# Vaccinia Virus DNA Ligase: Specificity, Fidelity, and Inhibition†

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*Received August 18, 1995; Revised Manuscript Received October 10, 1995*⊗

ABSTRACT: Vaccinia DNA ligase and mammalian DNA ligases II and III comprise a distinct subgroup of structurally homologous enzymes within the eukaryotic DNA ligase family. The specificity and fidelity of the viral enzyme were investigated using purified recombinant ligase and synthetic duplex DNA substrates containing a single strand discontinuity. Vaccinia ligase catalyzed efficient strand joining on nicked DNAs in the presence of magnesium and ATP ($K_m$ = 95 $\mu$M). dATP, ITP, AMPPCP, 3′dATP, and ATPαS could not substitute for ATP; of these, 3′dATP and ATPαS were inhibitors of ligation. The vaccinia enzyme was unable to seal strands across a 1 nt (nucleotide) or 2 nt gap. Ligase action at a 1 nt gap resulted in accumulation of high levels of the normally undetectable DNA−adenylate reaction intermediate. In contrast, no DNA−adenylate was formed at a 2 nt gap. A native gel mobility shift assay showed that vaccinia DNA ligase was capable of discriminating between nicked and gapped DNAs at the substrate binding step. The ligase was fairly tolerant of mismatches at a nick involving the 5′ phosphate donor terminus but was inhibited strongly by mismatches at the 3′ OH acceptor terminus, especially by purine•-purine mispairs. These findings underscore the importance of a proper 3′ OH terminus in substrate recognition and reaction chemistry but also raise the possibility that ligase may generate mutations during DNA repair by sealing DNA molecules with mispaired ends. Ligase was inhibited by several DNA binding drugs, including, in order of decreasing potency, distamycin, ethidium bromide, and actinomycin. Strand joining by purified ligase was not affected by etoposide, a drug that inhibits vaccinia virus replication in vivo and which depends on the presence of vaccinia ligase for its antiviral action.

Eukaryotic DNA ligases are ATP-dependent strand-joining enzymes that participate in DNA replication, repair, and recombination (Lindahl & Barnes, 1992). Whereas a single essential DNA ligase has been identified in yeasts (Barker et al., 1985, 1987) mammalian cells contain four different DNA ligases encoded by separate genes (Barnes et al., 1990; Wang et al., 1994; Wei et al., 1995; Husain et al., 1995). Structural and biochemical similarity between the *Saccharomyces cerevisiae* DNA ligase (CDC9) and mammalian DNA ligase I suggests that these proteins have similar functions in vivo (Tomkinson et al., 1992). Conditional *cdc9* mutants of yeast exhibit defects in DNA replication and repair and are nonviable at restrictive temperature. Similarly, human cells harboring a DNA ligase I mutation have enhanced sensitivity to DNA damage (Barnes et al., 1992; Pringent et al., 1994), and gene-targeting experiments indicate that DNA ligase I is essential for viability of mammalian cells (Petrini et al., 1995). Apparently, ligases II−IV do not fully complement a null mutation in ligase I.

ATP-dependent DNA ligases are also encoded by two classes of eukaryotic DNA viruses: poxviruses (Kerr & Smith, 1989; Colinas et al., 1990; Parks et al., 1994; Skinner et al., 1994) and African swine fever virus (Hammond et al., 1992). Vaccinia DNA ligase, a 552-amino acid polypeptide, has been studied most extensively and can be considered the prototypal poxvirus DNA ligase. Vaccinia ligase is expressed early during virus infection and becomes localized within discrete cytoplasmic sites of viral DNA synthesis (Smith et al., 1989; Colinas et al., 1990; Kerr et al., 1991). Although the ligase gene can be deleted without consequence for virus replication in cultured cells (Colinas et al., 1990; Kerr & Smith, 1991), the ligase-negative strain is more sensitive to UV or bleomycin, suggesting a role in viral DNA repair. Vaccinia ligase is capable of complementing the growth defect of a thermosensitive *cdc9* mutant of *S. cerevisiae* (Kerr et al., 1991). Functional redundancy with cellular ligases may explain why vaccinia ligase is dispensable for viral replication.

Vaccinia ligase is strikingly similar at the amino acid sequence level to mammalian DNA ligases II and III (Wang et al., 1994; Wei et al., 1995; Husain et al., 1995). Ligase II, a 70 kDa polypeptide, has been purified from bovine liver (Wang et al., 1994). The amino acid sequences of 15 different peptides of ligase II exhibit 65% overall identity with the vaccinia DNA ligase; indeed, ligase II is more closely related to the vaccinia enzyme than it is to ligase I. cDNAs encoding human DNA ligases III and IV have been isolated recently (Wei et al., 1995). The amino acid sequence of the 103 kDa human ligase III is nearly identical to that determined by peptide sequencing of the 70 kDa bovine DNA ligase II (Wang et al., 1994; Wei et al., 1995). Ligase III, like ligase II, is more similar to vaccinia DNA ligase than it is to cellular ligases I or IV. The vaccinia ligase shows 55% homology over its entire length with a segment of ligase III from residues 173 to 744 (Wei et al., 1995).

It is likely that vaccinia ligase and mammalian ligases II and III constitute a distinct subgroup within the polynucleotide ligase family. Whether functional similarity accompanies sequence conservation remains to be determined by comparative biochemical studies of the three enzymes. Such analysis would be best performed using recombinant enzymes expressed from ligase genes or cDNAs. An initial study of the properties of vaccinia ligase was conducted using

† This work was supported by NIH Grant GM42498 and ACS Grant FRA-432.

⊗ Abstract published in *Advance ACS Abstracts,* November 15, 1995.

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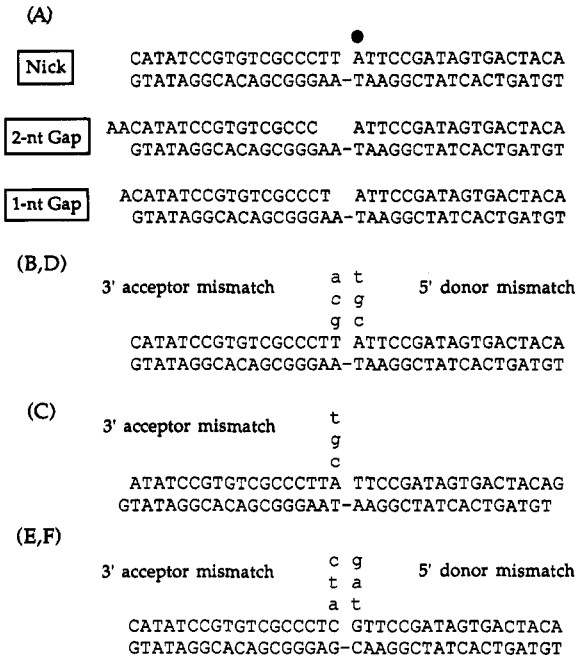


FIGURE 1: Ligase substrates. Duplex substrates for vaccinia ligase were prepared by annealing a 5′-labeled 18-mer donor strand to a 36-mer complementary strand and an 18-mer acceptor strand. The structure of the standard nicked duplex substrate is shown in A; the position of the 5′-labeled nucleotide is indicated by the closed circle. The structures of gapped duplexes and duplexes with mismatches at the 3′ OH or 5′ phosphate side of the nick are shown in A−F. Mismatch nucleotides are indicated in lowercase letters.

recombinant enzyme produced in bacteria (Shuman & Ru, 1995). The purified recombinant ligase resembled other eukaryotic enzymes in catalyzing the ATP-dependent joining of 5′ phosphate-terminated strands to 3′ hydroxyl-terminated acceptor strands via a covalent ligase−AMP intermediate. Mutational analysis implicated Lys-231 as the active site (Cong & Shuman, 1993; Shuman & Ru, 1995). The vaccinia enzyme readily sealed DNAs with cohesive ends but was poorly active on blunt-ended molecules. The vaccinia enzyme required high concentrations of the ATP for strand joining (Shuman & Ru, 1995); this is also the case for DNA ligase II (Lindahl & Barnes, 1992).

A more extensive characterization of the strand-joining reaction that focuses on the following issues is presented herein: (i) the NTP cofactor specificity, (ii) the ability of the ligase to discriminate nicked, gapped, and mismatched ends, (iii) the identification of the DNA−adenylate reaction intermediate, (iv) the requirements for initial binding of ligase to the DNA substrate, and (v) identification of compounds that inhibit strand ligation.

## MATERIALS AND METHODS

*Ligase Substrate.* The standard substrate used in ligase assays was a 36 bp (base pair) DNA duplex containing a centrally placed nick (Figure 1A). This DNA was formed by annealing two 18-mer oligonucleotides to a complementary 36-mer strand. The 18-mer constituting the donor strand was 5′ $^{32}$P-labeled and gel-purified as described (Shuman & Ru, 1995). The labeled donor was annealed to the complementary 36-mer in the presence of a 3′ OH-terminated acceptor strand in 0.2 M NaCl by heating at 65 °C for 2 min followed by slow cooling to room temperature. The molar ratio of the 18-mer donor to 36-mer complement to 18-mer acceptor strands in the hybridization mixture was typically 1:3:6.

*DNA Ligation.* Vaccinia DNA ligase was expressed in bacteria and purified from bacterial lysates by nickel agarose and phosphocellulose chromatography steps as described (Shuman & Ru, 1995). Standard ligation reaction mixtures (20 μL) containing 50 mM Tris-HCl (pH 8.0), 5 mM dithiothreitol (DTT), 10 mM $MgCl_2$, 1 mM ATP, 5′ $^{32}$P-labeled DNA substrate as indicated, and enzyme were incubated at 22 °C. Reactions were initiated by addition of enzyme and halted by the addition of 1 μL of 0.5 M ethylenediaminetetraaetic acid (EDTA) and 5 μL of formamide. The samples were heated at 95 °C for 5 min and then electrophoresed through a 12% polyacrylamide gel containing 7 M urea in TBE (90 mM Tris-borate and 2.5 mM EDTA) at 15 W constant power. The labeled 36-mer ligation product was well-resolved from the 5′-labeled 18-mer donor strand. The extent of ligation [36-mer/(18-mer + 36-mer)] was determined by scanning the gel using a FUJIX BAS1000 phosphorimager.

*Materials.* Novobiocin, purchased from Sigma, was stored as a 100 mM stock solution in water and diluted in water immediately prior to each use. Actinomycin D (Sigma) was stored as a 0.5 mM stock solution in water and diluted in water immediately prior to use; the concentration of actinomycin was determined spectrophotometrically ($\epsilon_{260}$ = 24 600). Distamycin A (Sigma) was prepared as a 7.8 mM stock solution in water; the concentration of distamycin was determined spectrophotometrically ($\epsilon_{303}$ = 34 000). The distamycin solution was not stored but rather diluted and tested immediately for inhibition of ligation.

## RESULTS

*Defined DNA Substrate for Strand Ligation.* The present characterization of the vaccinia ligase was performed using a 36-mer DNA duplex containing a centrally positioned nick (Figure 1A). This substrate offers several advantages over the 12 bp duplexes with 12 nt (nucleotide) complementary tails used previously. For example, ligation of the cohesive end substrate yielded a "ladder" of concatamers of the component strands (Shuman & Ru, 1995), most probably because the substrate had formed such concatamers during the annealing step of substrate preparation. Consequently, the ligase could catalyze multiple joining events on either strand of a substrate molecule without dissociating between catalytic cycles. The nicked duplex substrate has no potential to form multimers. The 3′ hydroxyl and 5′ phosphate termini at the nick are the only potentially reactive ends in the substrate molecule. Because the blunt ends are not phosphorylated, there is no possibility that the enzyme can ligate intermolecularly or even activate the blunt ends via formation of DNA−adenylate. Ligation could therefore be assayed simply by conversion of the 5′ $^{32}$P-labeled 18-mer donor strand to the unlabeled acceptor strand to yield a 36-mer reaction product. The use of the nicked substrate also made possible the analysis of ligase−DNA interaction via native gel electrophoresis (described in subsequent sections).

The extent of ligation of the nicked duplex (added at a 50 nM concentration with respect to the 5′-labeled donor strand) during a 10 min incubation in the presence of 1 mM ATP increased linearly with the concentration of vaccinia ligase from 0.1 to 1 nM enzyme (Figure 2A). In the linear range of enzyme dependence in this experiment, the recombinant

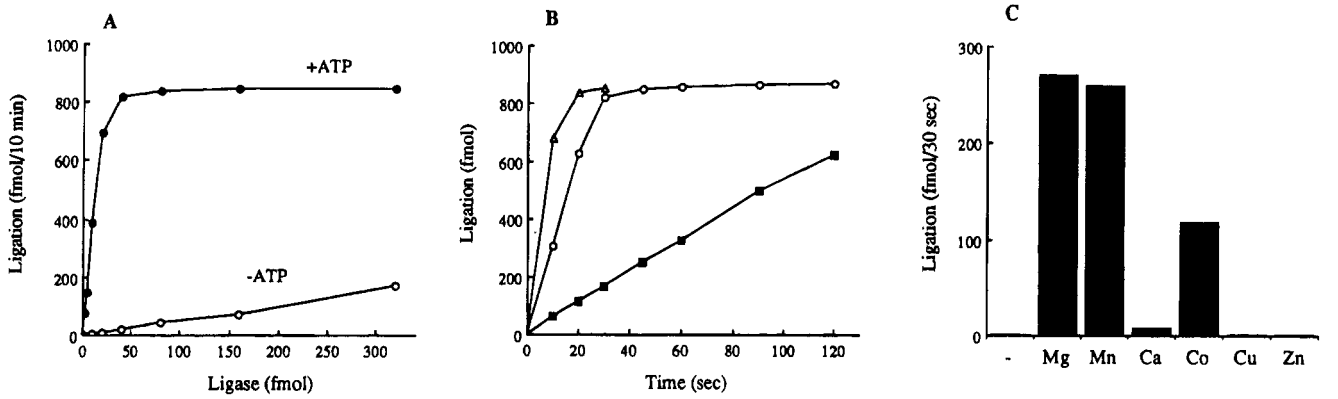


FIGURE 2: Ligation of duplex DNA containing a single nick. (A) Enzyme titration. Reaction mixtures (20 $\mu$L) containing 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM DTT, 1 mM ATP (where indicated), 1 pmol of nicked duplex substrate, and the indicated amounts of purified ligase (femtomoles) were incubated for 10 min at 22 °C. Ligation in the presence or absence of ATP is plotted as a function of input enzyme. (B) Time course. Reaction mixtures contained 1 mM ATP, 50 nM nicked DNA substrate, and either 4 nM (squares), 16 nM (circles), or 40 nM enzyme (triangles). Aliquots were withdrawn at the indicated times and the reactions quenched with EDTA and formamide. Ligation is plotted as a function of incubation time. (C) Divalent cation dependence of ATP-independent strand joining. Reaction mixtures (20 $\mu$L) containing 1 pmol of nicked duplex substrate, 640 fmol of ligase, and divalent cations as indicated (10 mM concentration) were incubated for 30 s at 22 °C. Mg, Mn, Ca, and Co were added as the chloride salt; Cu and Zn were added as the sulfate salt. Divalent cations were omitted from a control reaction (−).

ligase joined about 35−40 fmol of DNA ends per femtomole of enzyme. For the purpose of estimating the ratio of product to enzyme, the enzyme molarity was calculated on the basis of total protein concentration, assuming enzyme homogeneity (the enzyme preparation was composed predominantly of the 63 kDa ligase polypeptide). It was assumed that all enzyme molecules in the preparation were catalytically active. The reaction was saturated at 2 nM enzyme, with 85% of the labeled donor strand converted to 36-mer in 10 min. The extent of ligation at saturation was the same when the reactions were performed using bacteriophage T4 DNA ligase (data not shown). This upper limit of ligation probably reflected incomplete annealing of all three component strands in the formation of the nicked substrate.

The kinetics of ligation were examined at three concentrations of enzyme (Figure 2B). The reaction was linear for at least 2 min at 4 nM enzyme. At 16 nM enzyme, ligation was linear for 20 s and complete after 30 s. At 40 nM enzyme, the reaction was complete within 10−20 s. The initial rates were proportional to enzyme concentration.

*Adenosine Nucleotide Dependence.* The standard ligation assay included 1 mM ATP. The rate of ligation of the nicked duplex substrate increased with ATP concentration from 15 to 500 $\mu$M ATP and leveled off at higher concentrations. A $K_m$ of 95 $\mu$M ATP was calculated from a double reciprocal plot of the data (not shown). Ligation could be detected in the absence of added ATP, but only at relatively high levels of input enzyme. ATP-independent ligation was attributed to preadenylylated ligase in the enzyme preparation. The linear dependence of ATP-independent strand joining on enzyme indicated that about 0.5 mol of ends was sealed per mole of ligase (Figure 2A), implying that about half of the enzyme molecules had AMP bound at the active site. Kinetic analysis of ATP-independent ligation indicated that the reaction was virtually complete within 10 s (data not shown), as expected of a single turnover event. The abundance of preadenylated enzyme (EpA) permitted analysis of the cofactor requirement for reactions downstream of EpA formation. ATP-independent ligation at high enzyme concentrations depended completely on a divalent cation; this requirement could be fulfilled by 10 mM Mg or Mn and to a lesser extent by Co, but not by Ca, Cu, or Zn (Figure 2C).

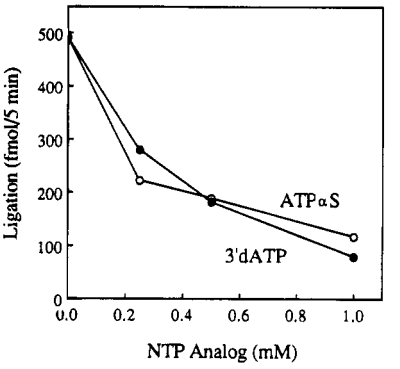


FIGURE 3: Inhibition by adenosine nucleotide analogs. Reaction mixtures (20 $\mu$L) containing 1 pmol of nicked duplex substrate, 20 fmol of ligase, 10 mM $MgCl_2$, 0.5 mM ATP, and either 3'dATP (cordycepin triphosphate) or ATPαS as indicated were incubated for 5 min at 22 °C. Ligation is plotted as a function of nucleotide analog concentration.

*Adenosine Nucleotide Specificity.* The NTP cofactor requirement for ligation by the vaccinia enzyme could not be satisfied by GTP, CTP, or UTP (Shuman & Ru, 1995). The nucleotide specificity was examined in greater detail using structural analogs of ATP, each at 1 mM concentrations. dATP and 3'dATP (cordycepin triphosphate) could not substitute for ATP, implying strict discrimination of the nucleotide sugar moiety. AMPPCP, which contains a methylene group in place of the bridging oxygen between the $\beta$- and $\gamma$-phosphates of ATP, did not substitute for ATP. Thiosubstitution of the α-phosphate of ATP also eliminated activity (ATPαS; Sp isomer). ITP did not support ligation, indicating that the 6 amino moiety of the purine ring was essential (data not shown). A mixing experiment was performed in which each ATP analog was added at 1 mM concentrations along with 1 mM ATP. It was found that dATP, AMPPCP, and ITP were not inhibitors of strand ligation (data not shown). 3'dATP and ATPαS were inhibitory in this screening assay (not shown); further experiments showed that the extent of inhibition by 3'dATP and ATPαS was concentration-dependent (Figure 3).

*DNA Substrate Specificity: Nicks vs Gaps.* The structure of the ligation substrate was altered such that the 3' hydroxyl-terminated acceptor strand was separated from the 5'

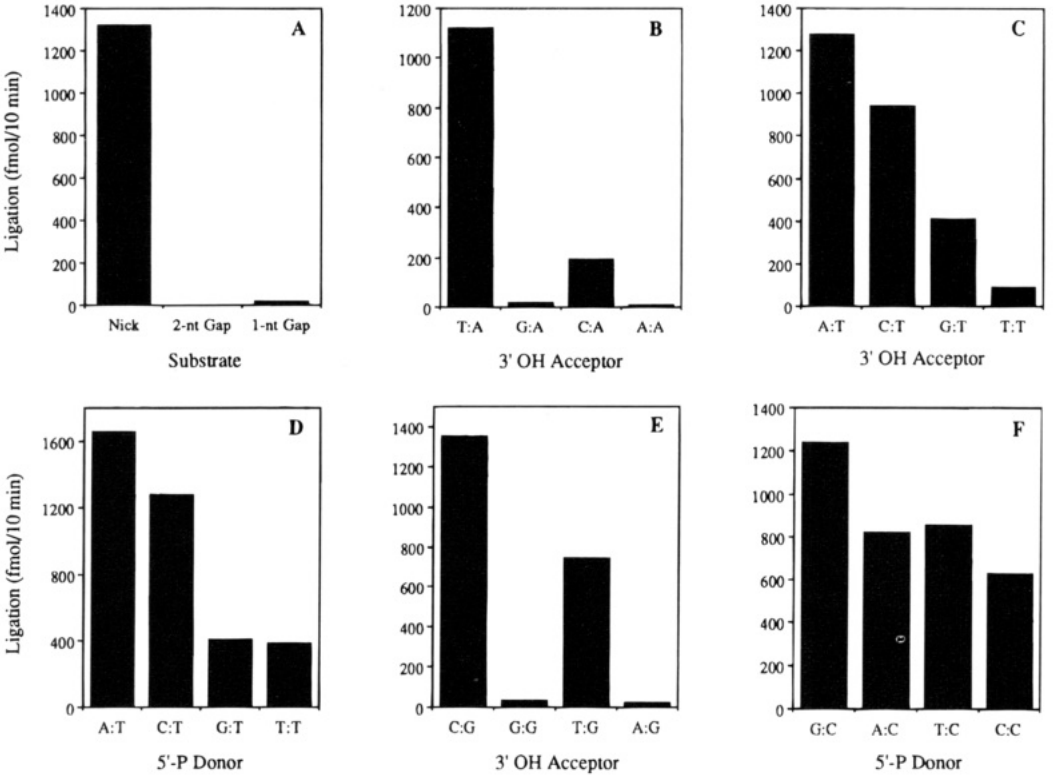


FIGURE 4: DNA substrate specificity. (A) Ligation reaction mixtures contained 320 fmol of enzyme and 1.5 pmol of DNA substrate with either a nick, a 2 nt gap, or a 1 nt gap. The structures of the substrates are shown in Figure 1A. (B) Reaction mixtures contained 40 fmol of enzyme and 1.5 pmol of DNA substrate with the indicated base pair configuration at the 3′ OH acceptor terminus. The structures of the substrates are shown in Figure 1B. (C) Reaction mixtures contained 20 fmol of enzyme and 2 pmol of DNA substrate with the indicated base pair configuration at the 3′ OH acceptor terminus. The structures of the substrates are shown in Figure 1C. (D) Reaction mixtures contained 20 fmol of enzyme and 2 pmol of DNA substrate with the indicated base pair configuration at the 5′ phosphate donor terminus. The structures of the substrates are shown in Figure 1D. (E) Reaction mixtures contained 80 fmol of enzyme and 2 pmol of DNA substrate with the indicated base pair configuration at the 3′ OH acceptor terminus. The structures of the substrates are shown in Figure 1E. (F) Reaction mixtures contained 80 fmol of enzyme and 2 pmol of DNA substrate with the indicated base pair configuration at the 5′ phosphate donor terminus. The structures of the substrates are shown in Figure 1F. All ligation reactions were carried out for 10 min at 22 °C.

phosphate donor terminus by a 2 or 1 nt gap (Figure 1A). The vaccinia ligase was completely incapable of joining strands across the 2 nt gap and catalyzed minimal ligation of the 1 nt gapped duplex (Figure 4A).

*DNA−Adenylate Formation on 1 nt Gap Duplex.* The DNA ligase reaction mechanism entails activation of the 5′ phosphate donor terminus by transfer of AMP from the enzyme to form the DNA−adenylate intermediate AppDNA. Demonstration of the DNA−adenylate typically requires extraordinary reaction conditions (Harvey et al., 1971). In the case of the vaccinia ligase, I have never detected accumulation of DNA−adenylate on the nicked duplex substrate under standard reaction conditions, even when enzyme was present in stoichiometric amounts relative to DNA. This was in sharp contrast to the situation with the 1 nt gap substrate. When this DNA was incubated with excess ligase, a novel radiolabeled species was formed which migrated in a denaturing polyacrylamide gel at a position about 1 nt longer than the 5′-labeled 18-mer donor strand and which corresponded to DNA−adenylate (Figure 5). A kinetic analysis showed that the AppDNA intermediate accumulated steadily over 10 min, at which time nearly half the input substrate had been adenylated (Figure 6); an apparent rate constant of 0.08 $min^{-1}$ was calculated from a semilog plot of the data (not shown). Strand joining by ligase was negligible with the 1 nt gap DNA. Clearly, the predominant impediment to ligation across a 1 nt gap was the inability of the 3′ OH moiety of the acceptor strand to engage in nucleophilic attack on DNA−adenylate. The

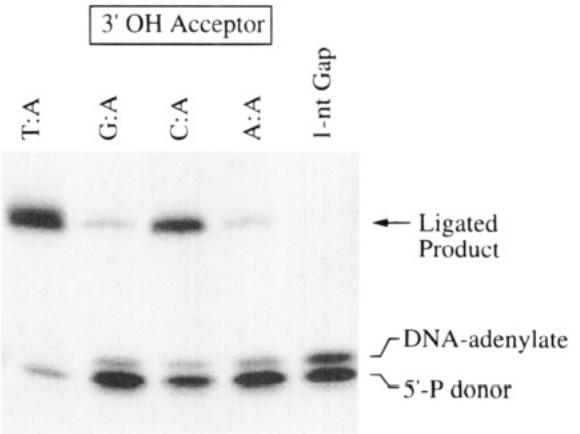


FIGURE 5: Reaction products formed with DNA substrates containing a 1 nt gap or 3′ OH mismatches. Reaction mixtures containing 480 fmol of enzyme and 400 fmol of DNA substrate with the indicated configuration at the 3′ OH acceptor position were incubated for 10 min at 22 °C. Reaction products were heat-denatured in formamide and electrophoresed through a 17% polyacrylamide gel containing 7 M urea in TBE. An autoradiogram of the gel is shown. The 3′ acceptor configuration is indicated above each lane. The structures of the 3′ mismatch substrates are shown in Figure 1B.

implication is that the 3′ OH must be positioned fairly precisely relative to the activated donor terminus for ligation to occur. Although the extent of DNA−adenylate formation was high on the 1 nt gap duplex, the rate of AMP transfer to DNA at a gap was still far less than that of AMP transfer to nicked DNA, which, although not measurable directly,

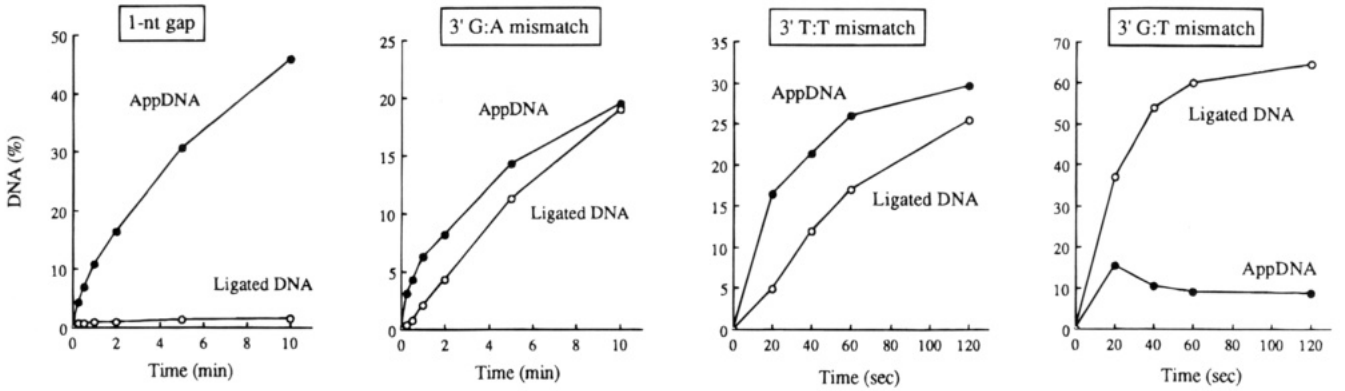


FIGURE 6: Kinetics of DNA−adenylate formation and strand joining on DNA containing a 1 nt gap or 3′ OH mismatches. Reaction mixtures contained 1 mM ATP, 10 mM $MgCl_2$, 27 nM ligase, and substrates as follows: 19 nM 1 nt gap DNA, 19 nM 3′ G·A mismatch DNA, 18 nM 3′ T·T mismatch DNA, and 18 nM 3′ G·T mismatch DNA. Aliquots were withdrawn at the indicated times and the reactions quenched with EDTA and formamide. Products were resolved by electrophoresis through a 17% polyacrylamide gel in 7 M urea. The amounts of DNA−adenylate (closed circles) and ligated product (open circles) were quantitated using a phosphorimager and were expressed as the percent of the total labeled DNA; these values are plotted in each panel as a function of incubation time.

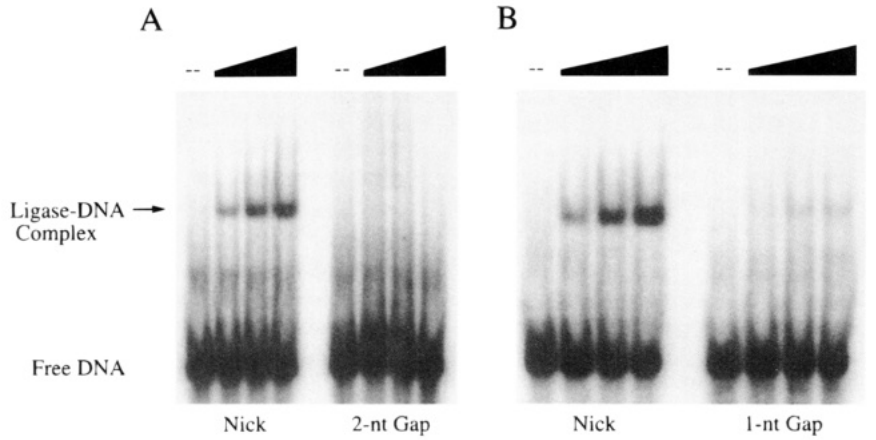


FIGURE 7: Binding of ligase to nicked DNA. (A) Reaction mixtures (20 μL) contained 50 mM Tris-HCl (pH 8.0), 5 mM DTT, 1.5 pmol of $^{32}$P-labeled DNA with either a nick or a 2 nt gap, and either 320, 640, or 960 fmol of ligase (proceeding from left to right within each titration series). Enzyme was omitted from a control reaction (lane --). (B) Reaction mixtures (20 μL) contained 50 mM Tris-HCl (pH 8.0), 5 mM DTT, 750 fmol of $^{32}$P-labeled DNA with either a nick or a 1 nt gap, and either 320, 640, or 960 fmol of ligase (proceeding from left to right within each titration series). Enzyme was omitted from a control reaction (lane --). The samples were incubated for 5 min at 22 °C, adjusted to 6% glycerol, and then electrophoresed at 150 V through a native 6% polyacrylamide gel in 0.25× TBE (22.5 mM Tris-borate and 0.6 mM EDTA). Autoradiograms of the gels are shown. Labeled species corresponding to free DNA and the ligase−DNA complex are indicated at the left.

was at least as fast as the overall rate of strand joining on nicked DNA (compare rates in Figures 6A and 2B). When similar kinetic analysis was performed for the 2 nt gap substrate in enzyme excess, there was no detectable accumulation of either DNA−adenylate or ligated product (not shown). Thus, the ligase actually requires duplex structure upstream of the 5′ phosphate terminus in order to transfer AMP to the donor strand. This would account for why single-stranded and blunt-ended duplex molecules are not effective substrates for ligation by the vaccinia enzyme.

*Specificity of Ligase Binding to DNA: Nick vs Gap.* A native gel mobility shift assay was employed to examine the binding of vaccinia ligase to the $^{32}$P-labeled nicked duplex DNA. Binding reactions were performed in the absence of magnesium and ATP so as to preclude conversion of substrate to product during the incubation. Mixing the ligase with nicked substrate resulted in the formation of a discrete protein−DNA complex that migrated more slowly than the free DNA during electrophoresis through a 6% native polyacrylamide gel (Figure 7A). The abundance of this complex increased in proportion to the amount of input ligase. No such complex was detected when ligase was reacted in parallel with the 2 nt gap duplex DNA (Figure 7A). This experiment shows that vaccinia DNA ligase binds specifically at a DNA nick and is capable of discriminating between nicked and 2 nt-gapped DNA at the substrate binding step.

A second experiment compared the binding of ligase to nicked duplex DNA vs the 1 nt gap duplex. A discrete complex was formed between ligase and the 1 nt gap DNA (Figure 7B). The mobility of this species resembled that of ligase bound to the nicked DNA; however, the yield of gap complex was <10% of that of the nick complex (Figure 7B). That ligase can bind a 1 nt gap is consistent with the finding that this DNA molecule is reactive in DNA−adenylate formation. Apparently, vaccinia ligase binds with higher affinity at a nick than it does at a 1 nt-gapped end. This affirms the importance of the 3′ OH acceptor strand in substrate recognition.

*Fidelity of Ligation: Effects of Base Mismatch at the 3′ OH Acceptor Site.* A series of nicked duplex ligation substrates was prepared such that the acceptor strand contained a mispaired base at the 3′ OH terminus (Figure 1B,C,E). Mismatch effects on ligation were tested initially in groups of four substrates under conditions of DNA excess. Changing a 3′ T·A base pair to a 3′ C:A mismatch (Figure

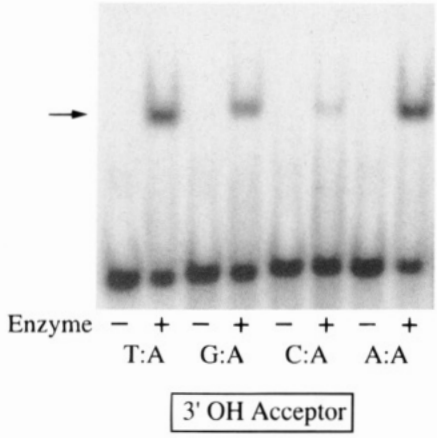


FIGURE 8: Binding of ligase to nicked DNA containing 3′ OH mismatches. Reaction mixtures containing 960 fmol of ligase (Enzyme +) and 750 fmol of DNA substrate with the indicated configuration at the 3′ OH acceptor position were incubated for 5 min at 22 °C. Control reactions lacking enzyme were run in parallel (Enzyme −). The samples were analyzed by native gel electrophoresis as described in the legend to Figure 7. An autoradiogram of the gel is shown. The ligase−DNA complex is indicated by an arrow. The structures of the 3′ mismatch substrates are shown in Figure 1B.

1B) partially inhibited strand joining (Figure 4B). In contrast, 3′ G·A and 3′ A·A mismatches were nearly as refractory to ligation as the 1 nt-gapped duplex (Figure 4B). The spectrum of reaction products at near-stoichiometric levels of ligase was remarkable for the presence of DNA−adenylate on the 5′-labeled donor strand of the 3′ C·A, G·A, and A·A substrates (Figure 5). Kinetic analysis of the 3′ G·A mismatch substrate under conditions of enzyme excess showed a steady accumulation of DNA−adenylate over the 10 min reaction (Figure 6). Ligated product was absent at the earliest time points but accumulated in near-linear fashion after a lag. The kinetic data were consistent with a precursor−product relationship between DNA−adenylate and ligated DNA. When the levels of AppDNA and ligated DNA were summed at each time point for the 3′ G·A mismatch DNA, it was apparent that the reactivity of the 3′ G·A mismatch substrate was comparable to that of the 1 nt gap substrate (see Figure 6; calculations not shown). Thus, the 3′ G·A mismatch elicited an effect on the rate of DNA adenylation that was quantitatively similar to the 1 nt gap, but the reaction was able to go forward on the 3′ G·A mismatch, albeit very slowly, with an attack by the 3′ OH of the mispaired dG nucleotide on the activated 5′ donor strand.

These 3′ mismatch duplexes were tested for binding to the ligase using the mobility shift assay (Figure 8). The 3′ A·A mismatch duplex, which was the worst substrate in strand joining, was bound by the ligase just as well as the 3′ T·A nicked duplex. The extent of complex formation on the 3′ G·A mismatch DNA was half that of the control DNA (Figure 8). Thus, the slow rate of DNA−adenylate formation and ligation of the 3′ purine·purine mismatch DNAs was most likely caused by mismatch effects on the chemical steps rather than by effects on substrate binding. Ligase binding to the 3′ C·A mismatch DNA was about 20% of that of the 3′ T·A control (Figure 8). Note that ligation of the 3′ C·A mismatch under multiple turnover conditions was affected to a similar degree (Figure 4B). The results indicate that different mismatches can have distinctive effects on individual steps in the ligation reaction pathway.

Effects of other 3′ mismatches on strand joining were surveyed and are summarized below. Ligation to an acceptor containing a 3′ T·G mispair was affected only modestly, whereas 3′ G·G and 3′ A·G pairs were ligated about 40 times less efficiently that an otherwise identical control substrate with a 3′ C·G base pair (Figure 4E). The results in Figure 4B,E make clear that purine·purine mismatches were far more detrimental than pyrimidine·purine mismatches. Mispairing of the 3′ acceptor nucleotide to a T residue in the complementary strand also affected ligation efficiency (Figure 4C). A 3′ C·T pair was well-tolerated; whereas the 3′ G·T pair was less active, and the 3′ T·T mismatch seriously impeded ligation. A kinetic analysis was performed for the 3′ T·T mismatch over a 2 min time course in enzyme excess (Figure 6). DNA−adenylate accumulated to significant levels within 20 s and leveled off between 1 and 2 min, while the ligated DNA level increased after an initial lag, again consistent with DNA−adenylate being a catalytic intermediate. The effect of the 3′ T·T mismatch on the rate of DNA adenylation was less profound than that observed for the 1 nt gap and the 3′ G·A mismatch (Figure 6). Still, the very fact that AppDNA accumulated to such an extent again underscored the message that the most kinetically significant impact of the 3′ mismatch was on the attack on AppDNA by the 3′ OH. This was reiterated in the case of the 3′ G·T mismatch, except that this mismatch had a less severe effect on the rate of the strand closure step, such that there was more rapid flux through DNA−adenylate to ligated product (Figure 6).

*Effects of Base Mismatches at the 5′ Phosphate Donor Site.* Oligonucleotides substituted at the 5′ end of the donor strand were 5′ $^{32}$P-labeled and annealed to various 18-mer acceptor and 36-mer complementary strands to form nicked duplexes with 5′ mismatches. A 5′ C·T mismatch was well-tolerated in the ligation reaction, whereas 5′ G·T and 5′ T·T mispairs were about one-fourth as effective as substrates compared to a 5′ A·T control DNA (Figure 4D). The 5′ A·C, 5′ T·C, and 5′ C·C mismatch substrates were all ligated fairly well compared to a 5′ G·C control (Figure 4F). Remarkably, 5′ mismatches to a G on the complementary strand were also ligated well (Figure 9), including the G·G and A·G mispairs that were so strongly inhibitory when present on the 3′ OH side of the nick. Apparently, the vaccinia ligase is more tolerant of base mismatches on the 5′ side of the nick than it is of mispairing at the 3′ OH acceptor terminus.

*Inhibitors of Vaccinia DNA Ligase.* Reports showing that T4 DNA ligase is inhibited by DNA-binding drugs, including antitumor agents (Montecucco et al., 1988, 1990a), prompted an evaluation of several DNA-binding compounds for their effect on the vaccinia DNA ligase (Figure 10). Ethidium bromide (EtBr) inhibited the vaccinia enzyme in a concentration-dependent manner. Strand joining at 10 μg/mL EtBr (25 μM) was <2% of the control value; 50% inhibition occurred at about 3.5 μg/mL (Figure 10A). Distamycin, which binds DNA in the minor groove, was even more potent, producing 97% inhibition of ligation at 5 μM and 50% inhibition at 1 μM (Figure 10B). Actinomycin D was less active than either distamycin or EtBr; actinomycin inhibited ligation by 90% at 50 μM and by 50% at about 18 μM (Figure 10C).

It was reported recently that vaccinia virus replication is blocked in vivo by etoposide (also called VP-16), a known inhibitor of eukaryotic DNA topoisomerase II (DeLange et

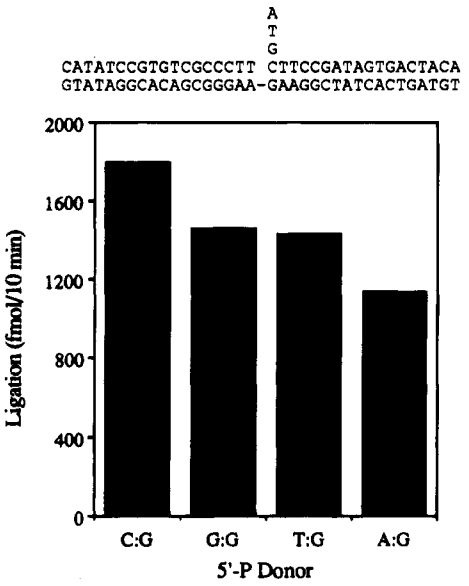


FIGURE 9: Ligation of nicked DNA containing a 5′ phosphate mismatch. Standard ligase reaction mixtures containing 20 fmol of enzyme and 2 pmol of DNA substrate with the indicated base pair configuration at the 5′ phosphate donor terminus were incubated for 10 min at 22 °C. The structures of the substrates are shown above the graph.

al., 1995). After isolating etoposide-resistant vaccinia viruses, these investigators mapped the resistance-conferring mutation to the gene encoding the vaccinia DNA ligase. Indeed, deletion of the vaccinia ligase gene also conferred etoposide resistance (DeLange et al., 1995). It was therefore of interest to examine what effects etoposide might have on the strand-joining activity of the purified vaccinia ligase. I found that a clinical preparation of etoposide [20 mg/mL in 3% benzyl alcohol, 8% polysorbate 80, 65% poly(ethylene glycol 300), and 30.5% ethanol], which blocked replication *in vivo* at 20−80 μg/mL (DeLange et al., 1995), did not affect DNA ligation at 2−20 μg/mL. Strand joining at 200 μg/mL was 85% of the control value, whereas activity at 2 mg/mL was 28% of the control. Although I suspect that inhibition by the highest concentration of etoposide was caused by the solvent, it was nevertheless apparent that etoposide did not inhibit strand joining by vaccinia DNA ligase in vitro at concentrations of the drug preparation that blocked virus replication in vivo by >99% (DeLange et al., 1995).

Several other topoisomerase inhibitors were tested against the DNA ligase. Camptothecin, a drug that targets eukaryotic DNA topoisomerase I, did not inhibit DNA ligase at drug concentrations up to 250 μM (not shown); this level is several fold higher than the concentration of camptothecin sufficient to induce maximal DNA breakage by topoisomerase I in vitro (Hsiang et al., 1985). The coumarin drugs novobiocin and coumermycin target the bacterial type II topoisomerases (Maxwell, 1993). These drugs also inhibit eukaryotic topoisomerase II and vaccinia topoisomerase I, as well as several other enzymes, albeit at much higher drug concentrations than are necessary to affect DNA gyrase. Vaccinia DNA ligase was inhibited completely by 2 mM novobiocin, with 50% inhibition at 0.7−0.8 mM drug (Figure 10D). Coumermycin was a more potent inhibitor of ligation than novobiocin; 80% inhibition occurred at 0.25 mM coumermycin (not shown).

## DISCUSSION

Vaccinia virus DNA ligase provides a useful model for mechanistic studies of the eukaryotic ligase family. Like other ligases, the vaccinia enzyme joins DNA strands via three partial reactions. First, the enzyme condenses with ATP to form a covalent enzyme−adenylate intermediate. AMP is then transferred to the 5′ phosphate terminus of the donor DNA strand to form DNA−adenylate. DNA−adenylate was not detected during ligation of nicked duplex DNA but did accumulate to substantial levels when the vaccinia ligase reacted with DNA substrates containing a 1 nt gap or certain base mismatches at the 3′ OH side of a nick. These alterations of the substrate drastically slowed the third step of the ligation reaction, the attack of the 3′ OH acceptor strand on DNA−adenylate, and thereby caused accumulation of the activated DNA intermediate. It is also the case for other DNA ligases that special maneuvers are required to detect DNA−adenylate. These include performing the ligation reactions at low temperatures and low pH or manipulating the substrate in ways similar to those described above (Harvey et al., 1971; Goffin et al., 1987; Wu & Wallace, 1989; Tomkinson et al., 1992; Husain et al., 1995). Thus, it appears for the vaccinia ligase, and perhaps for all ligases, that the normally rate-limiting step in the reaction pathway is DNA−adenylate formation.

Studies of the NTP cofactor requirement of vaccinia ligase revealed a stringent specificity for ATP. Structural analogs dATP, ITP, and AMPPCP did not substitute for ATP, nor did they inhibit enzyme activity in the presence of ATP. The simplest interpretation is that these analogs do not bind effectively to vaccinia ligase. 3′dATP and ATPαS were not substrates for strand joining, but they did inhibit the reaction, implying that these compounds do bind to enzyme, at least noncovalently. The results presented above do not reveal which step or steps are inhibited by the 3′dATP and ATPαS, e.g., nucleotide binding, EpA formation, etc. ATPαS has different effects on ligases from various sources. For example, ATPαS supports strand-joining by T4 DNA ligase at about one-third the reaction rate observed with ATP (Montecucco et al., 1990b). In contrast, ATPαS does not support the strand joining reaction by yeast ligase, although ATPαS does serve as a substrate for enzyme−thioadenylate formation (Tomkinson et al., 1992). The yield of yeast enzyme−adenylate was actually higher with ATPαS than with ATP, yet the sulfur substitution inhibited the two subsequent reaction steps: transfer of AMP-S from enzyme to DNA and closure of the thioadenylated DNA intermediate (Tomkinson et al., 1992). Another report described two ligases purified from rat liver that differed in their response to ATPαS. An enzyme designated ligase I did not carry out strand joining using ATPαS, yet did form the enzyme−thioadenylate, whereas a 100 kDa enzyme designated ligase II was able to catalyze overall strand joining with ATPαS, albeit less efficiently than with ATP (Elder & Rossignol, 1992). It is likely that the vaccinia and yeast enzymes are affected similarly by ATPαS, whereas the T4 and the 100 kDa liver enzymes share the ability to use this analog.

The high efficiency of vaccinia ligase in strand joining across a nick in duplex DNA contrasted sharply with the inability of the enzyme to seal strands across a 1 or 2 nt gap. This essentially rules out any role for vaccinia DNA ligase in generating frame-shift type deletion mutations. Yeast CDC9 is also unable to ligate across a 1 nt gap, and

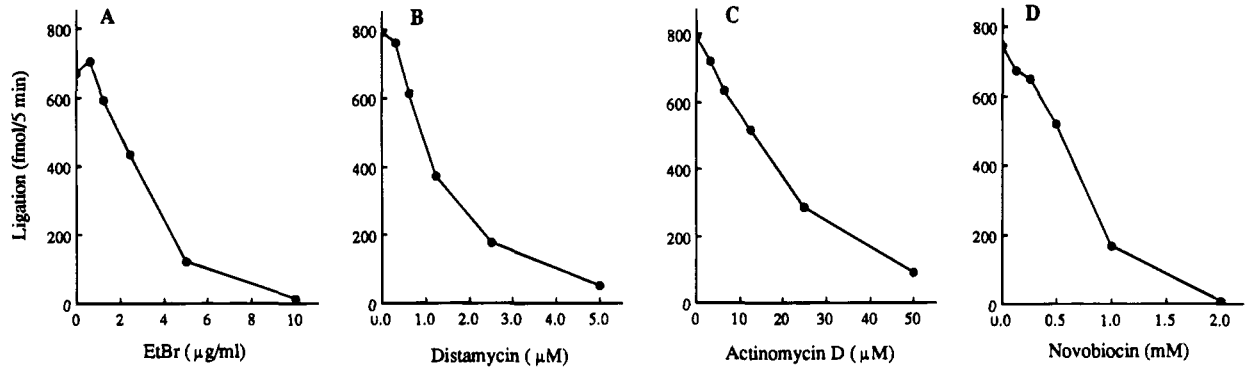


FIGURE 10: Inhibition of DNA ligation. Reaction mixtures containing 1 pmol of nicked duplex DNA substrate (Figure 1A), 20 fmol of enzyme, and inhibitors as indicated were incubated for 5 min at 22 °C. In each case, the inhibitor was added to the mixture containing Tris, magnesium, ATP, and DNA, and the ligation reactions were initiated by the addition of enzyme. Ligation is plotted as a function of inhibitor concentration: (A) ethidium bromide, (B) distamycin, (C) actinomycin, and (D) novobiocin.

like the vaccinia enzyme, CDC9 generates appreciable levels of DNA−adenylate under such circumstances (Tomkinson et al., 1992). These investigators indicated that CDC9 also formed DNA−adenylate on a 2 nt gap substrate, albeit 10-fold less than on the 1 nt gap DNA. In contrast, I was unable to detect any DNA−adenylate formed by the vaccinia ligase at a 2 nt gap, even when enzyme was in excess. T4 DNA ligase may be a bit less fastidious than the eukaryotic enzymes insofar as it can ligate to some extent across a 1 nt gap, albeit with difficulty, and with accumulation of DNA−adenylate (Goffin et al., 1987).

A native gel mobility shift assay was used to demonstrate that formation of a stable complex between vaccinia ligase and DNA depends on a DNA nick. The vaccinia enzyme discriminates clearly at the DNA binding step between nicked DNA molecules that can be sealed vs 2 nt-gapped molecules that cannot. Even a 1 nt gap significantly reduces the affinity of ligase for the DNA. This implies that the protein initially contacts both the donor and acceptor DNA strands on either side of the nick prior to any covalent modification of the DNA substrate. Facile electrophoretic resolution of the ligase−DNA complex from unbound DNA will permit the use of chemical and enzymatic footprinting techniques for delineation of the interface between ligase and the DNA substrate, a subject about which almost nothing is known for any DNA ligase. Of particular interest will be the identification of the region or regions of the ligase polypeptide that mediate nick recognition.

Fidelity of strand joining refers to the extent to which the enzyme can ligate substrates containing mismatched bases on either side of the nick. Vaccinia ligase was generally less tolerant of mismatches involving the 3′ OH acceptor strand than those involving the 5′ phosphate donor strand. Where mismatch inhibition of ligation occurred, it was accompanied by accumulation of DNA−adenylate to an extent that paralleled the magnitude of the inhibition. Hence, the inhibitory mismatches exerted their greatest impact on the strand closure step of the reaction pathway. Similar findings have been made for T4 ligase, yeast CDC9, and mammalian ligases I−III (Goffin et al., 1987; Wu & Wallace, 1989; Tomkinson et al., 1992; Husain et al., 1995). It is useful to compare the findings for vaccinia ligase on 3′ mismatch substrates with those reported for the cellular ligases. Bovine ligase III, like vaccinia ligase, was fairly effective at sealing nicked substrates with a 3′ C·T or 3′ T·G mismatch, was less active with a 3′ G·T mismatch, and was inactive at a 3′ A·G mismatch (Husain et al., 1995). Bovine ligase I differed in being more sensitive to 3′ T·G and 3′ C·T mismatches than either ligase II or vaccinia ligase (Husain et al., 1995). Yet, the CDC9 ligase, the yeast equivalent of ligase I, differed from the bovine ligase I in that CDC9 had no apparent difficulty joining the identical substrates containing 3′ G·T and 3′ C·T mismatches (Tomkinson et al., 1992). Vaccinia DNA ligase was inhibited by a 3′ T·T mismatch; the effects of this substrate configuration on the cellular ligases have not, to my knowledge, been reported.

The present findings raise the possibility that vaccinia DNA ligase may contribute to the generation of certain missense mutations by ligating strands containing mispairs on either side of a nick. This has been discussed previously for yeast DNA ligase (Tomkinson et al., 1992). Note that vaccinia virus encodes several other enzymes with potential roles in DNA repair, including an essential uracil DNA glycosylase and an essential DNA helicase related to human ERCC3 (Stuart et al., 1993; Millns et al., 1994; Bayliss & Condit, 1995). These proteins may constitute part of a repairosome that includes the viral DNA ligase. Increased sensitivity of ligase-negative vaccinia mutants to certain DNA-damaging agents is consistent with a role for ligase in DNA repair. Whether ligase fidelity is relevant to repair-induced mutagenesis in vivo is not at all clear, but such a role is at least biochemically feasible.

It has been surmised that bovine ligase I is a more faithful enzyme than bovine ligases II and III (Husain et al., 1995), and perhaps it is also more faithful than the vaccinia ligase. However, because none of the published studies of eukaryotic ligases tested all 16 base-pairing configurations at the 5′ phosphate and the 3′ OH sides of the nick (the present study tests 12 of 16 3′ pairs and 12 of 16 5′ pairs), it may be premature to draw conclusions about comparative fidelity of these enzymes. Conceivably, even homologous enzymes from different sources might vary in their ability to ligate particular mismatch structures, as in the case of CDC9 vs mammalian ligase I.

Several compounds that inhibit the vaccinia ligase were identified. Distamycin was the most potent of these. Distamycin binds DNA in the minor groove at A+T-rich sequences; the crystal structure of the distamycin−DNA complex shows that the drug covers five A·T base pairs (Coll et al., 1987). The standard nicked DNA substrate for strand joining by vaccinia ligase contains a 5 bp A+T sequence spanning the site of the nick; hence, it is likely that distamycin binding across the reactive 3′ OH and 5′

phosphate termini is responsible for the observed inhibition. Actinomycin was much less potent in blocking strand joining. Actinomycin binds preferentially to G+C-rich sequences and thus may have less impact on ligation of this particular DNA substrate. It would be of interest to see whether alterations in the base composition in the vicinity of the nick would reverse the potency of these two drugs as ligase inhibitors. The intercalator ethidium bromide also inhibited strand joining by the vaccinia enzyme, presumably via effects on DNA conformation. Earlier studies had shown that several DNA-binding drugs inhibit strand joining by T4 DNA ligase (Montecucco et al., 1988, 1990a).

Vaccinia DNA ligase is required for etoposide inhibition of vaccinia virus replication in vivo. Virus sensitivity to etoposide is enhanced by increasing ligase gene copy number, while deletion of the nonessential ligase gene confers etoposide resistance (DeLange et al., 1995). This situation is reminiscent of how the cytotoxicity of camptothecin is proportional to the level of topoisomerase I in the cell and how deletion of the nonessential *TOP1* gene of yeast confers resistance to camptothecin in vivo (Nitiss & Wang, 1988). Camptothecin stabilizes the covalent complex of topoisomerase bound at a DNA nick (Hsiang et al., 1985), and it is these lesions that lead to toxicity. A plausible model for the etoposide effect on vaccinia replication is that the drug induces vaccinia ligase to form lesions in viral DNA (for example, the accumulation of DNA adenylate) that block either DNA replication or the processing of replication intermediates. Yet, etoposide did not affect strand joining by the purified ligase and did not cause detectable accumulation of DNA−adenylate in vitro. Hence, the in vivo block to virus replication is probably not caused by a direct effect of drug on the ligation reaction. The present findings support the alternative model discussed by DeLange et al., whereby etoposide induces DNA breaks through its effects on cellular topoisomerase II, and these DNA breaks are then acted upon by vaccinia ligase in such a way as to block virus replication. If etoposide-induced breaks are somehow converted into 1 nt gaps, it becomes quite plausible on the basis of the results presented herein to suggest that vaccinia ligase would then bind to these ends and convert them into "dead-end" DNA−adenylates. Further studies are needed to clarify this interesting ligase-dependent antiviral mechanism.

## REFERENCES


Barker, D. G., White, J., & Johnston, L. H. (1985) The nucleotide sequence of the DNA ligase gene (*CDC9*) from *Saccharomyces cerevisiae*: a gene which is cell cycle regulated and induced in response to DNA damage, *Nucleic Acids Res. 13*, 8323−8337.

Barker, D. G., White, J., & Johnston, L. H. (1987) Molecular characterization of the DNA ligase gene, *CDC17*, from the fission yeast *Schizosaccharomyces pombe*, *Eur. J. Biochem. 162*, 659−667.

Barnes, D. E., Johnston, L. H., Kodama, K., Tomkinson, A. E., Lasko, D. D., & Lindahl, T. (1990) Human DNA ligase I cDNA: cloning and functional expression in *Saccharomyces cerevisiae*, *Proc. Natl. Acad. Sci. U.S.A. 87*, 6679−6683.

Barnes, D. E., Tomkinson, A. E., Lehmann, A. R., Webster, A. D. B., & Lindahl, T. (1992) Mutations in the DNA ligase I gene of an individual with immunodeficiencies and cellular hypersensitivity to DNA-damaging agents, *Cell 69*, 495−503.

Bayliss, C. D., & Condit, R. C. (1995) The vaccinia virus A18R gene product is a DNA-dependent ATPase, *J. Biol. Chem. 270*, 1550−1556.

Colinas, R. J., Goebel, S. J., Davis, S. W., Johnson, G. P., Norton, E. K., & Paoletti, E. (1990) A DNA ligase gene in the Copenhagen strain of vaccinia virus is nonessential for viral replication and recombination, *Virology 179*, 267−275.

Coll, M., Frederick, C. A., Wang, A. H. J., & Rich, A. (1987) A bifurcated hydrogen-bonded conformation in the d(A:T) base pairs of the DNA dodecamer d(CGCAAATTTGCG) and its complex with distamycin, *Proc. Natl. Acad. Sci. U.S.A. 84*, 8385−8389.

Cong, P., & Shuman, S. (1993) Covalent catalysis in nucleotidyl transfer: a KTDG motif essential for enzyme-GMP complex formation by mRNA capping enzyme is conserved at the active sites of RNA and DNA ligases, *J. Biol. Chem. 268*, 7256−7260.

DeLange, A. M., Carpenter, M. S., Choy, J., & Newsway, V. E. (1995) An etoposide-induced block in vaccinia virus telomere resolution is dependent on the virus-encoded DNA ligase, *J. Virol. 69*, 2082−2091.

Elder, R. H., & Rossignol, J. (1990) DNA Ligases from Rat Liver. Purification and Partial Characterization of Two Molecular Forms, *Biochemistry 29*, 6009−6017.

Goffin, C., Bailly, V., & Verly, W. G. (1987) Nicks 3′ or 5′ to AP sites or to mispaired bases, and one-nucleotide gaps can be sealed by T4 DNA ligase, *Nucleic Acids Res. 15*, 8755−8771.

Hammond, J. M., Kerr, S. M., Smith, G. L., & Dixon, L. K. (1992) An African swine fever virus gene with homology to DNA ligases, *Nucleic Acids Res. 20*, 2667−2671.

Harvey, C. L., Gabriel, T. F., Wilt, E. M., & Richardson, C. C. (1971) Enzymatic breakage and joining of DNA: synthesis and properties of the DNA adenylate in the phage T4 ligase reaction, *J. Biol. Chem. 246*, 4523−4530.

Hsiang, Y., Hertzberg, R., Hecht, S., & Liu, L. F. (1985) Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerase I, *J. Biol. Chem. 260*, 14873−14878.

Husain, I., Tomkinson, A. E., Burkhart, W. A., Moyer, M. B., Ramos, W., Mackey, Z. B., Besterman, J. M., & Chen, J. (1995) Purification and characterization of DNA ligase III from bovine testes: homology with DNA ligase II and vaccinia DNA ligase, *J. Biol. Chem. 270*, 9683−9690.

Kerr, S. M., & Smith, G. L. (1989) Vaccinia virus encodes a polypeptide with DNA ligase activity, *Nucleic Acids Res. 17*, 9039−9050.

Kerr, S. M., & Smith, G. L. (1991) Vaccinia virus DNA ligase is nonessential for virus replication: recovery of plasmids from virus-infected cells, *Virology 180*, 625−632.

Kerr, S. M., Johnston, L. H., Odell, M., Duncan, S. A., Law, K. M., & Smith, G. L. (1991) Vaccinia DNA ligase complements *Saccharomyces cerevisiae cdc9*, localizes in cytoplasmic factories and affects virulence and virus sensitivity to DNA damaging agents, *EMBO J. 10*, 4343−4350.

Lindahl, T., & Barnes, D. E. (1992) Mammalian DNA ligases, *Annu. Rev. Biochem. 61*, 251−281.

Maxwell, A. (1993) The interaction between coumarin drugs and DNA gyrase, *Mol. Microbiol. 9*, 681−686.

Millns, A. K., Carpenter, M. S., & DeLange, A. M. (1994) The vaccinia virus-ecoded uracil DNA glycosylase has an essential role in viral DNA replication, *Virology 198*, 504−513.

Montecucco, A., Pedrali-Noy, G., Spadari, S., Zanolin, E., & Ciarocchi, G. (1988) DNA unwinding and inhibition of T4 DNA ligase by anthracyclines, *Nucleic Acids Res. 16*, 3907−3918.

Montecucco, A., Pedrali-Noy, G., Spadari, S., Lestingi, M., & Ciarocchi, G. (1990a) Effects of DNA-binding drugs on T4 DNA ligase, *Biochem. J. 266*, 379−384.

Montecucco, A., Lestingi, M., Pedrali-Noy, G., Spadari, S., & Ciarocchi, G. (1990b) Use of ATP, dATP and their α-thio derivatives to study ligase adenylation, *Biochem. J. 271*, 265−268.

Nitiss, J., & Wang, J. C. (1988) DNA topoisomerase-targeting antitumor drugs can be studied in yeast, *Proc. Natl. Acad. Sci. U.S.A. 85*, 7501−7505.

Parks, R. J., Lichty, B. D., Karakis, C., & Evans, D. H. (1994) Characterization of the Shope fibroma virus DNA ligase gene, *Virology 202*, 642−650.

Petrini, J., Xiao, Y., & Weaver, D. T. (1995) DNA ligase I mediates essential functions in mammalian cells, *Mol. Cell. Biol. 15*, 4303−4308.

Pringent, C., Satoh, M. S., Daly, G., Barnes, D. E., & Lindahl, T. (1994) Aberrant DNA repair and DNA replication due to an inherited enzymatic defect in human DNA ligase I, *Mol. Cell. Biol. 14*, 310−317.

Shuman, S., & Ru, X. (1995) Mutational analysis of vaccinia DNA ligase defines residues essential for covalent catalysis, *Virology 211*, 73−83.

Skinner, M. A., Moore, J. B., Binns, M. M., Smith, G. L., & Boursnell, M. E. G. (1994) Deletion of fowlpox virus homologues of vaccinia genes between the 3$\beta$-hydroxysteroid dehydrogenase (A44L) and DNA ligase (A50R) genes, *J. Gen. Virol. 75*, 2495−2498.

Smith, G. L., Chan, Y. S., & Kerr, S. M. (1989) Transcriptional mapping and nucleotide sequence of a vaccinia gene encoding a polypeptide with extensive homology to DNA ligases, *Nucleic Acids Res. 17*, 9051−9062.

Stuart, D. T., Upton, C., Higman, M. A., Niles, E. G., & McFadden, G. (1993) A poxvirus-encoded uracil DNA glycosylase is essential for virus viability, *J. Virol. 67*, 2503−2512.

Tomkinson, A. E., Tappe, N. J., & Friedberg, E. C. (1992) DNA Ligase I from *Saccharomyces cerevisiae*: Physical and Biochemical Characterization of the *CDC9* Gene Product, *Biochemistry 31*, 11762−11771.

Wang, Y. J., Burkhart, W. A., Mackey, Z. B., Moyer, M. B., Ramos, W., Husain, I., Chen, J., Besterman, J: M., & Tomkinson, A. E. (1994) Mammalian DNA ligase II is highly homologous with vaccinia DNA ligase, *J. Biol. Chem. 269*, 31923−31928.

Wei, Y., Robins, P., Carter, K., Caldecott, K., Pappin, D., Yu, G., Wang, R., Shell, B., Nash, R., Schar, P., Barnes, D., Haseltine, W., & Lindahl, T. (1995) Molecular cloning and expression of human cDNAs encoding a novel DNA ligase IV and DNA ligase III, an enzyme active in DNA repair and recombination, *Mol. Cell. Biol. 15*, 3206−3216.

Wu, D. Y., & Wallace, R. B. (1989) Specificity of the nick-closing activity of bacteriophage T4 DNA ligase, *Gene 76*, 245−254.

BI9519664