Ligation of RNA-Containing Duplexes by Vaccinia DNA Ligase

JoAnn Sekiguchi and Stewart Shuman*

Molecular Biology Program, Sloan-Kettering Institute, New York, New York 10021

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ABSTRACT: Vaccinia virus DNA ligase repairs nicked duplex DNA substrates consisting of a 5′-phosphate-terminated strand and a 3′-hydroxyl-terminated strand annealed to a bridging template strand. This study addresses the ability of vaccinia DNA ligase to seal nicked substrates containing one or more RNA strands. We found that the viral enzyme rapidly and efficiently joined a 3′-OH RNA to 5′-phosphate DNA when the reacting polynucleotides were annealed to a bridging DNA strand. In contrast, ligation of 3′-OH DNA to 5′-phosphate RNA was slow (0.2% of the rate of RNA-to-DNA ligation) and entailed the accumulation of high levels of RNA−adenylate intermediate. A native gel mobility shift assay showed that vaccinia DNA ligase discriminates at the substrate binding step between ligands containing 5′-phosphate DNA versus 5′-phosphate RNA at the nick. The enzyme displayed weak activity in RNA-to-RNA ligation on a bridging DNA template (0.01% of RNA-to-DNA activity). Vaccinia DNA ligase was incapable of joining two DNAs annealed on an RNA template. These results can be explained by a requirement for B-form helical conformation on the 5′-phosphate side of the nick. The robust RNA-to-DNA strand joining activity underscores the potential for vaccinia DNA ligase to catalyze RNA-based integration of host cell genetic information into the genome of cytoplasmic poxviruses.

The ATP-dependent DNA ligases catalyze the joining of 5′-phosphate-terminated strands to 3′-hydroxyl-terminated strands via three sequential nucleotidyl transfer reactions [reviewed in Lehman (1974), Engler and Richardson (1982), Lindahl and Barnes (1992), and Shuman (1996)]. In the first step, attack on the α-phosphate of ATP by DNA ligase results in displacement of pyrophosphate and formation of a covalent ligase−adenylate intermediate in which AMP is linked to the ϵ-amino group of a lysine. The AMP is then transferred to the 5′-monophosphate terminus of a nicked DNA duplex to form the DNA−adenylate intermediate, which consists of an inverted (5′)−(5′)-pyrophosphate bridge structure, AppN. Attack by the 3′-OH terminated strand of the nicked duplex on DNA−adenylate seals the nick and releases AMP.

Animal cells contain multiple ATP-dependent DNA ligase isozymes. Three different enzymes with distinctive substrate specificities were purified from animal cells by Tomkinson et al. (1991). DNA ligases I, II, and III are each capable of joining oligo(dT) molecules annealed to poly(dA), but they differ in their abilities to seal homopolymeric nucleic acid substrates containing RNA strands. DNA ligase I is unable to ligate oligo(dT) hybridized to poly(rA) (Tomkinson et al., 1991, 1992). In contrast, ligases II and III can ligate oligo(dT)−poly(rA) hybrids (Tomkinson et al., 1991). Ligation of oligo(rA) molecules annealed to a poly(dT) template is catalyzed by ligases I and III, but not by ligase II (Tomkinson et al., 1991).

Peptide sequencing of purified enzyme and the recent cloning of mammalian DNA ligase cDNAs indicate that the 70 kDa ligase II and 100 kDa ligase III proteins are encoded by a single cellular gene (Husain et al., 1995; Chen et al., 1995; Wei et al., 1995). The ligase II and ligase III polypeptides are probably generated by alternative RNA or protein processing events. Ligase III is itself subject to alternative RNA splicing to generate two isoforms, IIIα and III*β*, that differ at their carboxyl-termini (Mackey et al., 1997). DNA ligase I is a 102 kDa polypeptide encoded by a different cellular gene (Barnes et al., 1990). A previously unappreciated 96 kDa DNA ligase, now named ligase IV, was also identified by cDNA cloning (Wei et al., 1995). Ligase IV purified from human cells displays a substrate specificity reminiscent of ligase II; it joins oligo(dT) annealed to poly(rA), but it is unable to join oligo(rA) annealed to poly(dT) (Robins & Lindahl, 1996).

We are studying the structure and function of the eukaryotic DNA ligases using the vaccinia virus enzyme as a model. Vaccinia encodes a 552 amino acid DNA ligase (Smith et al., 1989; Colinas et al., 1990) that is strikingly similar in amino acid sequence to mammalian DNA ligases II and III (Wang et al., 1994; Husain et al., 1995; Wei et al., 1995; Chen et al., 1995). Because vaccinia DNA ligase and mammalian DNA ligases II and III are more similar to each other than to DNA ligases I and IV, these three enzymes can be regarded as a distinct subgroup within the eukaryotic DNA ligase family. The enzymatic properties of vaccinia DNA ligase have been studied using recombinant enzyme produced in bacteria (Shuman & Ru, 1995; Shuman, 1996; Odell et al., 1996; Sekiguchi & Shuman, 1997). The vaccinia enzyme requires high concentrations of ATP for strand joining. This is also the case for DNA ligase II, its cellular homologue.

Odell et al. (1996) reported that vaccinia DNA ligase seals oligo(dT) molecules annealed to a poly(dA) template. They also documented the ability of vaccinia DNA ligase to join oligo(dT) in the absence of a complementary nucleic acid. The single-strand joining activity of vaccinia DNA ligase is limited to oligo(dT); the enzyme will not join other single strand DNA homopolymers (Odell et al., 1996), nor will it join single-strand DNA oligonucleotides of mixed nucleotide sequence (Shuman & Ru, 1995; Odell et al., 1996). At this

* To whom correspondence should be addressed.

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point, the physiological significance of the homopolymer ligation reactions, and the specificity or lack thereof for DNA-versus RNA-containing substrates, remains unclear—for the vaccinia DNA ligase as well as the cellular DNA ligases.

Our studies of the nucleic acid specificity and fidelity of vaccinia DNA ligase have been performed using a presumptively "physiological" duplex DNA substrate containing a single nick (Shuman, 1996). The vaccinia ligase catalyzes efficient strand joining on nicked DNA, but is unable to seal strands across a 1-nucleotide or 2-nucleotide gap. Ligase action at a 1-nucleotide gap results in the accumulation of high levels of the normally undetectable DNA−adenylate reaction intermediate, whereas essentially no DNA−adenylate is formed at a 2-nucleotide gap. Vaccinia DNA ligase is capable of discriminating between nicked and gapped DNAs at the substrate binding step (Shuman, 1996). Properly positioned 5′-phosphate and 3′-hydroxyl termini are apparently required for substrate recognition. We presume that ligase interacts with the duplex at sites other than the 5′-phosphate and 3′-hydroxyl moieties, but the nature of such contacts has not been defined. One way to approach this issue is to alter the structure of the nucleic acid substrate without perturbing the 5′-phosphate and 3′-hydroxyl groups at the nick.

In the present study, we analyze the ability of vaccinia DNA ligase to seal nicked substrates containing one or more RNA strands. We find that the vaccinia enzyme catalyzes the efficient joining of 3′-OH-terminated RNA to 5′-phosphate-terminated DNA. Vaccinia DNA ligase is much less effective at joining 3′-OH-terminated DNA to 5′-phosphate-terminated RNA and is extremely weak at phosphodiester formation between two RNA strands. The substrate specificity is relaxed when manganese is substituted for magnesium in the ligase reaction. Thus, vaccinia DNA ligase can be used to synthesize defined polynucleotides containing tandem segments of RNA and DNA. The RNA strand joining activity of vaccinia DNA ligase suggests that this enzyme may play a role in the acquisition of cellular genes by the cytoplasmic poxviruses.

MATERIALS AND METHODS

Vaccinia DNA Ligase. Vaccinia DNA ligase was expressed in bacteria as an N-terminal His_{10}-tagged fusion protein and purified from bacterial lysates to near-homogeneity by Ni−agarose and phosphocellulose chromatography as described (Sekiguchi & Shuman, 1997). The protein concentration of the enzyme preparation was determined using the BioRad dye reagent with bovine serum albumin as a standard. Enzyme molarity was calculated from the protein concentration and the predicted molecular weight of the recombinant DNA ligase polypeptide.

Ligase Substrate. The standard substrate used in ligase assays was a 36-bp nucleic acid duplex containing a centrally placed nick. This DNA was formed by annealing two 18-mer oligonucleotides to a complementary 36-mer strand (Shuman, 1996). The 18-mer constituting the 5′-monophosphate-terminated strand (DNA oligonucleotide 5′-ATTCCGATAGTGACTACA or RNA oligonucleotide 5′-AUUCCGAUAGUGACUACA) was 5′-^{32}P-labeled and gel-purified as described (Shuman, 1996). The labeled 18-mer was annealed to the complementary 36-mer DNA (the template strand) in the presence of a 3′-OH 18-mer strand (either DNA oligonucleotide 5′-CATATCCGTGTCGCCCTT or RNA oligonucleotide 5′-CAUAUCCGUGUCGCCCUU) as described (Sekiguchi & Shuman, 1997).

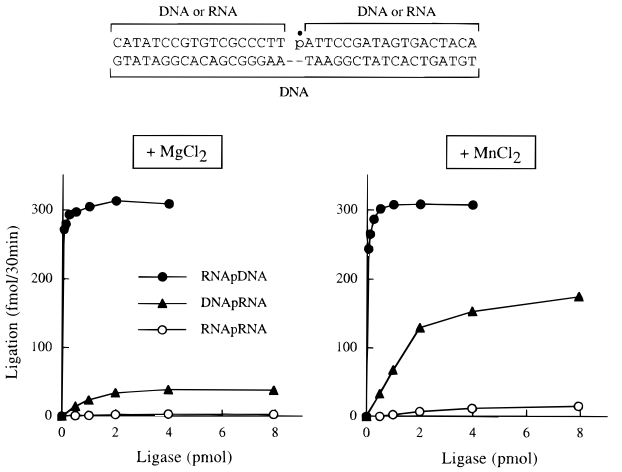

FIGURE 1: Ligation of RNA-containing substrates. The structure of the nicked duplex substrate used in the ligation reactions is shown. The ^{32}P-labeled 5′-phosphate at the nick is indicated by the dot. The 3′-OH and 5′-phosphate 18-mer strands were either DNA or RNA; the 36-mer bridging strand was all-DNA. Reaction mixtures (20 μL) containing 50 mM Tris-HCl (pH 8.0), 5 mM DTT, 500 fmol of substrate, 1 mM ATP, either 10 mM $MgCl_2$ (left panel) or 10 mM $MnCl_2$ (right panel), and ligase as indicated were incubated for 30 min at 22 °C. The yields of RNApDNA, DNApRNA, and RNApRNA ligation products are plotted as a function of input enzyme.

Ligation Assay. Reaction mixtures (20 μL) containing 50 mM Tris-HCl (pH 8.0), 5 mM DTT, 10 mM $MgCl_2$ or $MnCl_2$, 1 mM ATP, 500 fmol of 5′-^{32}P-labeled nicked duplex substrate, 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 EDTA and 5 μL of formamide. The samples were heated at 95 °C for 5 min and then electrophoresed through a 17% polyacrylamide gel containing 7 M urea in TBE (90 mM Tris−borate, 2.5 mM EDTA). The extent of ligation [36-mer/(18-mer + 36-mer)] was determined by scanning the gel using a FUJIX BAS1000 phosphorimager.

RESULTS

Ligation of Nicked Duplexes Containing RNA Strands. Studies of RNA strand joining by vaccinia DNA ligase were performed using a 36-mer duplex substrate containing a centrally positioned nick (Figure 1). The 3′-hydroxyl and 5′-phosphate termini at the nick are the only potentially reactive ends in the substrate molecule. Because the blunt ends of the duplex are not phosphorylated, it is not possible for the enzyme to ligate intermolecularly or even activate the blunt ends via formation of DNA−adenylate.

In the experiment shown in Figure 1, we tested the ability of vaccinia DNA ligase to catalyze phosphodiester bond formation on nicked substrates in which the 5′-phosphate-terminated strand, the 3′-hydroxyl-terminated strand, or both strands were composed of RNA. In every case, the 5′-phosphate and 3′-OH strands were annealed to a complementary DNA template strand. Ligation was assayed by conversion of the 5′-^{32}P-labeled 18-mer strand into an internally-labeled 36-mer product. The ligation reaction mixtures contained 25 nM (500 fmol/20 μL) nicked duplex,

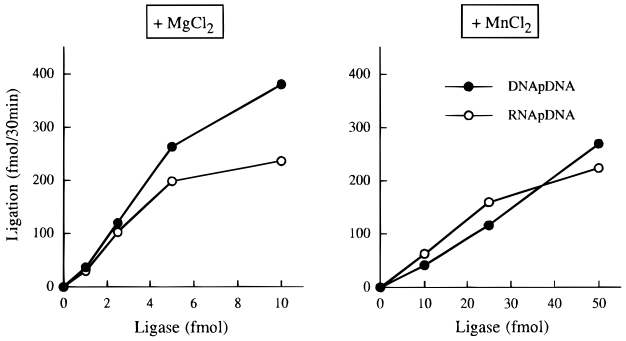

FIGURE 2: Efficiency of RNA-to-DNA versus DNA-to-DNA strand joining. Ligation reactins were performed as described in Figure 1. The yields of RNApDNA and DNApDNA ligation products are plotted as a function of input enzyme.

1 mM ATP, and 10 mM divalent cation, either $MgCl_2$ or $MnCl_2$.

Vaccinia DNA ligase readily joined a 3′-OH RNA to a 5′-phosphate DNA to yield a RNApDNA product; 310 fmol (62% of the input substrate) was ligated at saturating enzyme in the presence of either magnesium or manganese (Figure 1). The reaction was nearly complete at 62 fmol of input protein, the lowest level of enzyme tested in this experiment (Figure 1). The upper limit of the extent of ligation may reflect incomplete annealing of all three component strands to form the nicked substrate. Linear dependence of RNA-to-DNA strand joining on input enzyme was observed at lower protein concentrations (Figure 2). Specific RNA-to-DNA ligation activity in the presence of 10 mM $MgCl_2$ was about 7-fold higher than in 10 mM $MnCl_2$ (Figure 2). The activity of vaccinia ligase in RNA-to-DNA ligation was essentially equivalent to its activity in DNA-to-DNA strand joining, with either magnesium or manganese as the divalent cation cofactor (Figure 2).

Joining of a 3′-OH DNA to a 5′-phosphate RNA to produce a DNApRNA molecule was less efficient than was formation of RNApDNA or DNApDNA. Only 8% of the input substrate was ligated at saturating enzyme in the presence of magnesium (Figure 1). However, the extent of ligation was enhanced in the presence of manganese, in which case 35% of the substrate was sealed. The enzyme dependence of DNApRNA formation was shifted to the right compared to the titration profile in RNApDNA formation.

Vaccinia DNA ligase was quite poor at sealing a nicked duplex in which the 5′-phosphate and 3′-OH strands were both RNA; in reactions containing magnesium, only 0.6% of the substrate was joined (Figure 1). Manganese stimulated RNA-to-RNA ligation, but the reaction was still feeble compared to DNApDNA, RNApDNA, and DNApRNA production.

Kinetics of Ligation of RNA-Containing Strands. The kinetics of ligation were examined under conditions of enzyme excess (400 nM DNA ligase, 25 nM nicked duplex substrate) with either magnesium or manganese as the divalent cation cofactor. With either metal cofactor, the DNA ligase rapidly joined 3′-OH RNA to a 5′-phosphate DNA to form RNApDNA. The reactions proceeded to 80−83% of the end point values within 5 min (Figure 3). The initial rates calculated from the earliest time points (10, 20, and 30 s) were 7.5 fmol/s in magnesium and 4.5 fmol/s in manganese. We did not observed any accumulation of

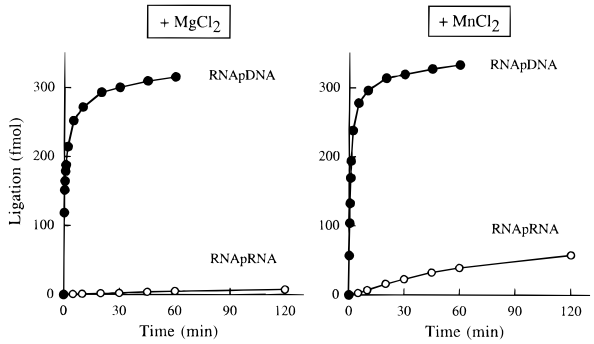

FIGURE 3: Kinetics of ligation of RNA-containing substrates. Reaction mixtures containing (per 20 μL) 50 mM Tris-HCl (pH 8.0), 5 mM DTT, 500 fmol of ^{32}P-labeled nicked substrates (with either 3′-OH RNA + 5′-phosphate DNA strands or 3′-OH RNA + 5′-phosphate RNA strands at the nick), 1 mM ATP, either 10 mM $MgCl_2$ (left panel) or 10 mM $MnCl_2$ (right panel), and 8 pmol of DNA ligase were incubated at 22 °C. The reactions were initiated by the addition of ligase. Aliquots (20 μL) were withdrawn at the indicated times and quenched immediately. The "time 0" points were taken prior to adding enzyme. The extents of formation of the 36-mer RNApDNA and RNApRNA ligation products are plotted as a function of incubation time.

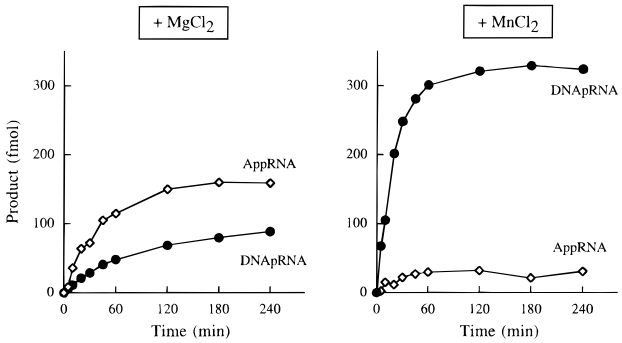

FIGURE 4: Kinetics of DNA-to-RNA ligation. Reaction mixtures containing (per 20 μL) 50 mM Tris-HCl (pH 8.0), 5 mM DTT, 500 fmol of ^{32}P-labeled substrate (with 3′-OH DNA + 5′-phosphate RNA strands at the nick), 1 mM ATP, either 10 mM $MgCl_2$ (left panel) or 10 mM $MnCl_2$ (right panel), and 8 pmol of DNA ligase were incubated at 22 °C. The extents of formation of the RNA−adenylate intermediate (AppRNA) and the 36-mer DNApRNA ligation product are plotted as a function of incubation time.

DNA−adenylate during the RNApDNA ligation reaction (not shown).

RNA-to-RNA ligation was extremely slow. The initial rate of RNApRNA formation in 10 mM magnesium was 0.01% of the rate of RNA-to-DNA ligation (Figure 3, left panel). The rate of RNA-to-RNA ligation was 10-fold higher in 10 mM manganese, but was still only 0.3% of the rate of RNA-to-DNA ligation (Figure 3, right panel). Accumulation of an RNA−adenylate intermediate was not observed during the RNA-to-RNA ligation reaction (not shown).

A strikingly different reaction profile was observed for the joining of 3′-OH DNA to 5′-phosphate RNA (Figure 4). A novel radiolabeled species was formed which migrated in a denaturing polyacrylamide gel at a position about 1 nucleotide longer than the 5′-labeled 18-mer RNA donor strand (not shown); this species corresponds to RNA−adenylate (AppRNA). When reactions were performed in 10 mM magnesium, the AppRNA intermediate accumulated steadily over 2 h, at which time 32% of the input RNA substrate had been adenylated (Figure 4, left panel). Accumulation of the DNApRNA ligation product lagged behind

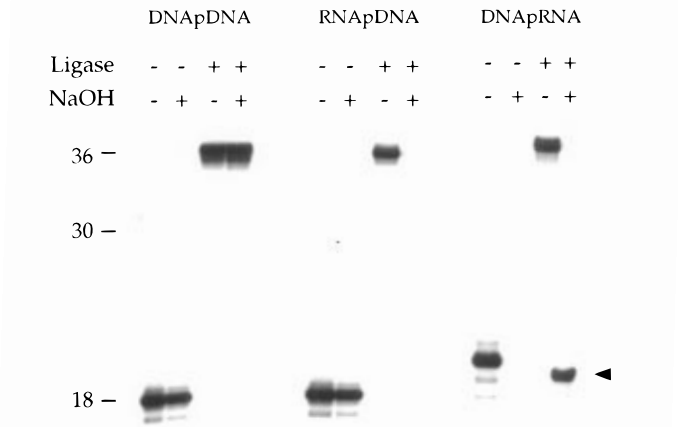

FIGURE 5: Analysis of the ligation products. Ligation reaction mixtures containing (per 20 μL) 50 mM Tris-HCl (pH 8.0), 5 mM DTT, 500 fmol of ^{32}P-labeled nicked substrates, 1 mM ATP, 10 mM $MnCl_2$, and 8 pmol of DNA ligase were incubated at 22 °C. The reactions were halted by the addition of EDTA to 25 mM. The reaction products were electrophoresed through a 17% polyacrylamide gel. The labeled 36-mer ligation products were localized by autoradiography and recovered by elution from gel slices. The eluates were ethanol-precipitated. The pellets were resuspended in either 12 μL of 0.1M NaOH, 1 mM EDTA (NaOH +) or 12 μL of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (NaOH −). These samples were incubated at 37 °C for 16 h. Control samples containing the input 18-mer DNA substrate that had not been exposed to ligase were treated in parallel (Ligase −). The alkali-treated samples were neutralized by adding 1.2 μL of 1 M HCl. All samples were then ethanol-precipitated, resuspended in formamide, heated for 5 min at 95 °C, and then electrophoresed through a 17% polyacrylamide gel containing 7 M urea in TBE. An autoradiograph of the gel is shown. The positions and chain lengths of 5′-^{32}P-labeled DNA oligonucleotide size markers are indicated at the left. Alkaline hydrolysis of the DNApRNA ligation product yielded a discrete species, which is denoted by an arrowhead at the right.

RNA−adenylate formation. It would appear that the rate-limiting step in DNA-to-RNA ligation is the attack by the 3′-OH moiety of the DNA strand on RNA−adenylate. The rate of DNA-to-RNA strand joining in magnesium was 12-fold higher than the rate of RNA-to-RNA ligation, but still only 0.2% of the rate of RNA-to-DNA ligation.

The reaction profile of DNA-to-RNA ligation was altered significantly by replacement of magnesium with manganese. In this case, AppRNA accumulated to much lower levels (6% at steady state), the rate of DNApRNA formation was increased by a factor of 10, and the reaction proceeded to completion in 1−2 h, at which time 65% of the input substrate was ligated (Figure 4, right panel). Manganese apparently accelerated the strand joining step (step 3) of the ligation pathway. The rate of DNA-to-RNA strand joining in manganese was 12-fold higher than the rate of RNA-to-RNA ligation, but only 4% the rate of RNA-to-DNA ligation under identical reaction conditions.

Analysis of the Ligation Reaction Products. We analyzed the susceptibility of the gel-purified RNApDNA ligation product to treatment with NaOH. The purified 36-mer product of 3′-OH DNA to 5′-^{32}P DNA ligation was analyzed in parallel. The input 5′-^{32}P-labeled 18-mer donor DNA strand was unaffected by alkali, as was the internally-labeled 36-mer DNApDNA ligation product (Figure 5, DNApDNA). In contrast, the 36-mer RNApDNA ligation product was quantitatively degraded after treatment with NaOH (Figure 5; RNApDNA). The predicted product of ligation of a 3′-OH RNA to a 5′-^{32}P DNA is a 36-mer tandem RNApDNA strand uniquely ^{32}P-labeled at the RNA−DNA junction. It

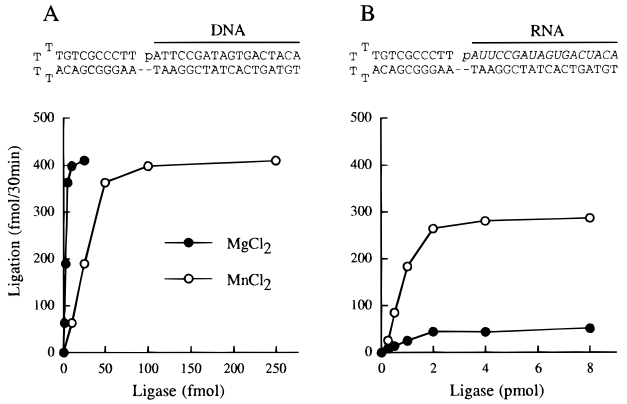

FIGURE 6: DNA-to-DNA versus DNA-to-RNA ligation. The structures of the nicked substrates used in the ligation reactions are shown. The labeled 5′-phosphate at the nick is denoted by "p". The ^{32}P-labeled 18-mer strand was either DNA (panel A) or RNA (panel B). Reaction mixtures (20 μL) containing 50 mM Tris-HCl (pH 8.0), 5 mM DTT, 1 mM ATP, 10 mM $MgCl_2$ or 10 mM $MnCl_2$, 500 fmol of nicked substrate, and DNA ligase as indicated were incubated for 30 min at 22 °C. The yield of ^{32}P-labeled 60-mer ligation product is plotted as a function of input enzyme. Note that the *x*-axis scales are different in panels A and B.

is expected that the ^{32}P-phosphate will be transferred to the upstream ribose sugar and converted into 2′- and 3′-UMP as the final product of alkaline hydrolysis.

The predicted product of ligation of a 3′-OH DNA to a 5′-^{32}P RNA is a 36-mer strand uniquely ^{32}P-labeled at the DNA−RNA junction. In this case, the input 5′-^{32}P 18-mer RNA strand was degraded completely by NaOH (Figure 5, DNApRNA). (Note that the 5′-^{32}P-labeled 18-mer RNA migrated slower than a 5′-^{32}P 18-mer DNA strand of identical sequence.) The gel-purified DNApRNA ligation product was converted nearly quantitatively into a discrete species that migrated at a position ~1 nucleotide longer than the 5′-phosphate-terminated 18-mer DNA donor (Figure 5, DNApRNA). This is consistent with the expected ^{32}P-labeled alkaline hydrolysis product, which is a 5′-hydroxyl and either 3′- or 2′-phosphate-terminated 19-mer 5′-CATATCCGTGTCGCCCTTp*A*p.

DNA-to-DNA versus DNA-to-RNA Strand Joining: Ligase Discriminates at the Substrate Binding Step. A direct comparison of DNA-to-DNA versus DNA-to RNA ligation was conducted using nicked substrates consisting of a self-complementary 3′-OH-terminated hairpin DNA (with a 10-bp stem and an 18-nucleotide 5′ single-strand tail) and a 5′-^{32}P-labeled 18-mer strand (either DNA or RNA) capable of base-pairing to the 5′ tail of the hairpin strand (Figure 6). Strand joining was measured in reaction mixtures containing 25 nM (500 fmol/20 μL) nicked duplex, 1 mM ATP, and 10 mM $MgCl_2$ or $MnCl_2$. At saturating enzyme, 82−85% of the 5′-^{32}P-labeled DNA strand was ligated in a 30 min reaction (Figure 6A). In the linear range of enzyme dependence, ligase sealed 74 fmol of nicks/fmol of enzyme in magnesium and 13 fmol of nicks/fmol of enzyme in manganese. The extent of DNA-to-RNA ligation on the hairpin-containing substrate at saturating enzyme was quite low in magnesium (10% of input 5′-^{32}P-labeled RNA strand ligated), but was increased in manganese (57% ligated) (Figure 6B). Note that the *x*-axis scales are different in Figure 6A,B and that the enzyme dependence of ligation of the 5′-phosphate RNA strand was shifted dramatically to the right compared to DNA strand joining. Vaccinia DNA ligase

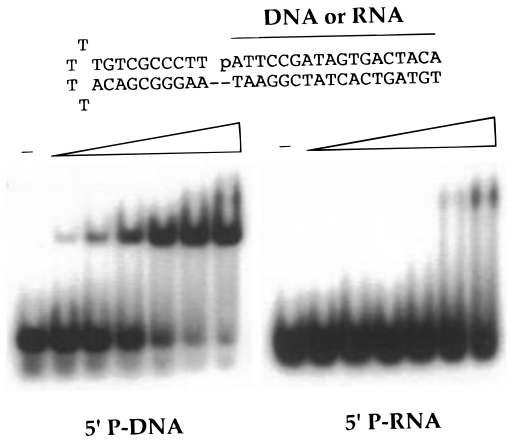

FIGURE 7: Native gel assay of the binding of vaccinia DNA ligase to nicked substrates with DNA versus RNA 5′-phosphate strands. The nicked duplex ligand used in the binding assays is shown. The ^{32}P-labeled 18-mer strand was either DNA or RNA. Binding reaction mixtures (20 μL) containing 50 mM Tris-HCl (pH 8.0), 5 mM DTT, 500 fmol of nicked ligand, and 0.1, 0.25, 0.5, 1, 2.5, and 5 pmol of vaccinia DNA ligase (proceeding left to right within each titration series) were incubated for 10 min at 22 °C. Control reactions (lane −) contained no ligase. Glycerol was added to 5%, and the samples were electrophoresed through a 6% polyacrylamide gel in TBE buffer (90 mM Tris−borate, 2.5 mM EDTA) at 60 V for 2 h. An autoradiograph of the dried gel is shown.

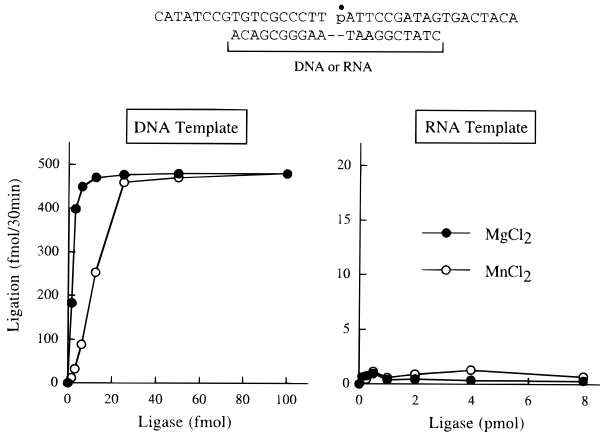

FIGURE 8: Ligation of DNA molecules annealed to an RNA template strand. The structure of the nicked duplex substrate used in the ligation reactions is shown; the ^{32}P-labeled 5′-phosphate at the nick is indicated by the dot. The 3′-OH and 5′-phosphate 18-mer strands were both DNA; the 20-mer template strand was either DNA or RNA. Reaction mixtures (20 μL) containing 50 mM Tris-HCl (pH 8.0), 5 mM DTT, 1 mM ATP, either 10 mM $MgCl_2$ or 10 mM $MnCl_2$, 500 fmol of nicked substrate containing a DNA template strand (left panel) or RNA template strand (right panel), and DNA ligase as indicated were incubated for 30 min at 22 °C. The extents of ligation are plotted as a function of input enzyme.

sealed only 0.03 fmol of 5′-^{32}P RNA strand/fmol of enzyme in magnesium and 0.15 fmol of RNA/fmol of enzyme in manganese (Figure 6B). We inferred from these data that vaccinia DNA ligase has reduced affinity for a molecule containing 5′-phosphate RNA at the nick.

A native gel mobility shift assay (Shuman, 1995) was used to directly examine the binding of vaccinia DNA ligase to the nicked hairpin ligands containing ^{32}P-labeled DNA or RNA strands. Binding reactions were performed in the absence of ATP or a divalent cation so as to preclude conversion of substrate to product during the incubation. Mixing the ligase with 0.5 pmol of nicked hairpin ^{32}P-DNA 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 7). The yield of this complex was proportional to input DNA ligase in the range of 0.1−1 pmol (Figure 7 and data not shown). Very little specific complex was detected when 0.1−1 pmol of DNA ligase was reacted with the nicked ^{32}P-RNA ligand (Figure 7). A smear of shifted material and trace amounts of a discrete band were detected at higher levels of input protein (Figure 7). This experiment shows that vaccinia DNA ligase is capable of discriminating at the substrate binding step between nicked ligands containing 5′-phosphate DNA versus 5′-phosphate RNA strands. By scanning the gel in Figure 7 and comparing the extents of binding at 1 pmol of input enzyme, we calculated that the enzyme's affinity for the RNA-containing nicked ligand was $^1/_{40}$th of that for the all-DNA ligand.

Ligation of DNA Strands on an RNA Template. We compared the ability of vaccinia DNA ligase to join two 18-mer DNA strands that were annealed to a 20-nucleotide template strand composed of either DNA or RNA. [The integrity of the template RNA and DNA 20-mer strands was confirmed in control experiments (not shown).] The annealed substrates contain a single nick flanked on both sides by 10 bp of duplex nucleic acid (Figure 8). The ligation reaction mixtures contained 25 nM (500 fmol/20 μL) radiolabeled substrate, 1 mM ATP, and 10 mM divalent cation, either $MgCl_2$ or $MnCl_2$. Vaccinia DNA ligase catalyzed efficient strand joining when the template strand was composed of DNA; 96% of the ^{32}P-labeled 18-mer DNA was ligated in 30 min at saturating enzyme in the presence of either magnesium or manganese. The reaction was nearly complete at 6 fmol of input enzyme (Figure 8). A kinetic analysis showed that ligation on a DNA bridging strand by 0.2 pmol of enzyme was complete in 2 min (not shown). In contrast, less than 0.1% of the ^{32}P-labeled 18-mer DNA was ligated in 30 min when the template strand was RNA, even in substantial enzyme excess (Figure 8). After a 4 h reaction with 8 pmol of DNA ligase, the extent of joining was 0.2% of input in magnesium and 0.4% in manganese (not shown).

DISCUSSION

We have shown that vaccinia DNA ligase is capable of joining RNA strands to DNA strands. The enzyme displays a strong preference for joining a 3′-OH RNA to a 5′-phosphate DNA compared to the joining of 3′-OH DNA to 5′-phosphate RNA. Vaccinia DNA ligase is feeble in RNA-to-RNA ligation and cannot ligate DNA molecules annealed on an RNA template. Kinetic analysis of these reactions provides instructive insights into the basis for substrate recognition. Our findings suggest practical applications of vaccinia DNA ligase as an RNA-modifying enzyme; in addition, they illuminate the potential for DNA ligase to catalyze RNA-based integration of host cell genetic information into the poxvirus genome.

RNA-to-RNA Strand Joining by DNA Ligases. It was shown previously that bacteriophage T4 DNA ligase could join two nonhomopolymeric RNA molecules annealed to a complementary DNA template strand to form a defined nicked duplex (Moore & Sharp, 1992). Of the labeled 5′-phosphate RNA strand, 45% was ligated to a 3′-OH strand in a 4 h reaction; the efficiency was estimated to be nearly 90% after taking into account the fact that half the ligation

substrate was not accurately bridged to form a nicked duplex (Moore & Sharp, 1992). Similar extents of RNA-to-RNA ligation by the T4 enzyme were observed when assays were performed using homopolymeric substrates (Fareed et al., 1971). The rate of RNA-to-RNA ligation on a DNA template by T4 DNA ligase was 1% of that of DNA-to-DNA strand joining (Fareed et al., 1971). Vaccinia DNA ligase appears to be even less active than the T4 protein in sealing two RNA strands on a DNA scaffold, as far as the yield of the RNApRNA ligation product is concerned. This is consistent with prior results indicating that the vaccinia ligase is more fastidious in DNA strand joining than the T4 enzyme; i.e., the vaccinia enzyme is less tolerant of gaps and base mismatches in the DNA substrate (Shuman, 1996). The RNA ligation activity of vaccinia DNA ligase could be enhanced by using manganese as a cofactor instead of magnesium. However, even in manganese, the rate of RNA-to-RNA ligation by the vaccinia enzyme is extremely slow compared to more favorable nicked duplex substrates.

The ability of eukaryotic DNA ligases to synthesize tandem 5′-RNA−RNA molecules on DNA templates has not been addressed previously with a physiological, i.e., non-homopolymeric, substrate. However, Tomkinson et al. (1991) showed that mammalian DNA ligases I and III joined oligo(rA) molecules annealed to poly(dT), whereas DNA ligase II did not catalyze this reaction. Robins and Lindahl (1996) showed that DNA ligase IV was unable to join oligo(rA)−poly(dT). These studies were conducted using magnesium as the divalent cation cofactor. Assuming that the activity of mammalian DNA ligases in sealing homopolymeric RNAs is reflective of their nucleic acid substrate specificities in general, we surmise that vaccinia virus DNA ligase resembles mammalian DNA ligases II and IV; i.e., these enzymes are very weak in RNA−RNA joining.

Specificity for DNA as the Bridging Template Strand. Vaccinia DNA ligase is essentially unable to join DNA molecules hybridized to a bridging RNA strand. The specific activity in ligating RNA-bridged DNA strands was less than 0.01% of the activity on DNA-bridged substrates. This stringent specificity for a DNA template is akin to that of DNA ligase I on homopolymer substrates; i.e., ligase I is incapable of sealing oligo(dT) on a poly(rA) template. Note that DNA ligases II, III, and IV are able to join oligo(dT)−poly(rA).

It is instructive that vaccinia DNA ligase cannot join nicked substrates that contain RNA on both sides of the nick, regardless of whether the chemically reactive "top strands" of the double helix (the 3′-OH and 5′-phosphate-terminated moieties) or the nonreactive "bottom" strand is composed of RNA. The suppressive effects of RNA−DNA hybrid formation on ligation suggest that the nicked substrate must adopt a B-form helical conformation in order to be sealed. The RNA strand of an RNA−DNA hybrid adopts the A-form helical conformation (as found in double-strand RNA) while the DNA strand adopts a conformation that is neither strictly A nor B, but is instead intermediate in character between these two forms (Arnott et al., 1986; Salazar et al., 1993). Vaccinia DNA ligase is nick-specific, but not sequence-specific. Hence, we assume that the protein makes nonspecific contacts with the phosphodiester backbone of the duplex in addition to specific contacts with the reactive 3′-OH and 5′-phosphate ends. Adoption of non-B conformation may weaken or preclude these backbone contacts.

RNA-to-DNA and DNA-to-RNA Ligation. Vaccinia DNA ligase rapidly and efficiently joins a 3′-OH RNA to 5′-phosphate DNA when the reacting polynucleotides are annealed to a bridging DNA strand. We surmise, therefore, that a B-form helical conformation on the upstream side of the nick is not essential for substrate recognition or reaction chemistry. In contrast, ligation of 3′-OH DNA to 5′-phosphate RNA is very slow compared to ligation involving a 5′-phosphate DNA. An implication of this result is that vaccinia DNA ligase requires a B-form helix on the downstream side of the nick for optimal activity. The enzyme dependence of DNA-to-RNA ligation suggests that the enzyme has low affinity for substrates containing a 5′-phosphate RNA strand at the nick. The native gel shift experiment confirms that vaccinia DNA ligase discriminates at the substrate binding step in favor of nicked ligands containing 5′-phosphate DNA versus 5′-phosphate RNA strands. DNA-to-RNA ligation is also limited at the level of reaction chemistry when ligation is assayed in enzyme excess. The accumulation of high levels of RNA−adenylate intermediate during the DNA-to-RNA joining reaction indicates that the strand closure step is rate-limiting. The stimulation of the overall DNA-to-RNA joining reaction, and step 3 in particular, by manganese may result from a cation-dependent change in the structure of the substrate (rendering it more like that of the all-DNA substrate) or a relaxation of the specificity of the ligase per se.

Early studies using homopolymeric substrates showed that bacteriophage T4 DNA ligase catalyzed phosphodiester bond formation between a 3′-OH RNA molecule and a 5′-phosphate DNA molecule (Nath & Hurwitz, 1974). Substrates that were ligated by the T4 enzyme included 3′-OH poly(rA) to 5′-^{32}P-poly(dA) on a poly(dT) template and 3′-OH poly(rU) to 5′-^{32}P-poly(dT) on a poly(dA) template. Remarkably, the T4 DNA ligase was not active in joining a 3′-OH DNA to a 5′-phosphate RNA molecule; i.e., 3′-OH poly(dA) was not ligated to 5′-^{32}P-poly(rA), and 3′-OH poly(dT) was not joined to 5′-^{32}P-poly(rU). Similarly, an early study of ligation of homopolymers by a DNA ligase from mouse L cells (this activity presumably corresponds to DNA ligase I) showed that the enzyme catalyzed the joining of 3′-OH RNA to 5′-phosphate DNA on a DNA template, but did not catalyze ligation of 3′-OH DNA to 5′-phosphate RNA (Bedows et al., 1977).

Practical Applications. The RNA-to-DNA and DNA-to-RNA joining properties of vaccinia DNA ligase with physiological substrates can be exploited to 5′ or 3′ tag any RNA for which the 5′- or 3′-terminal RNA sequence is known, i.e., by designing a bridging DNA template strand complementary to the terminal sequences of the intended RNA. Practical applications include: (i) ^{32}P-labeling of the 5′ or 3′ ends of RNA and (ii) affinity labeling the 5′ or 3′ ends of RNA, e.g., by using a biotinylated DNA oligonucleotide as the 5′-phosphate or 3′-OH strand. A potential advantage of DNA ligase-mediated RNA strand joining is that ligation can be targeted by the investigator to RNAs of interest within a complex mixture of RNA molecules.

Implications for Poxvirus Evolution. Poxviruses infect a diverse range of host species from insects to man. To accommodate their cytoplasmic lifestyle, the poxviruses encode a full complement of proteins required for DNA replication and mRNA synthesis. As a consequence, poxvirus genomes are among the largest of any viruses; they

range from 130 kbp to 300 kbp and consist almost entirely of protein-coding sequences [reviewed by Moss (1996)]. Poxvirus genes lack introns; this is not surprising, given that the host cell mRNA splicing machinery is exclusively nuclear. Diverse genera of poxviruses encode a conserved core of proteins that are essential for poxvirus replication in cultured cells. In addition, the poxviruses encode a large number of additional "viral defense proteins" that are dispensable for replication in culture, but which play critical roles in virus pathogenesis in the animal host. These include mitogens, soluble cytokine receptors, complement antagonists, interferon antagonists, and suppressors of apoptosis (Moss, 1996). The spectrum of such products can differ among the poxvirus genera. Because these defense factors are homologous to host proteins, it is assumed that the viral genes were acquired from the host. Such genes are clustered at the left and right termini of the vaccinia virus genome, whereas the core genes are situated centrally in the genome (Johnson et al., 1993).

The lack of introns suggests that cell-to-virus gene transfer occurs at the RNA level in the cytoplasm. The efficient ligation of RNA to DNA by vaccinia DNA ligase suggests a plausible pathway for initiation of RNA-based recombination. We speculate that the 3′ end of a cellular mRNA could be covalently joined to vaccinia virus genomic DNA by the viral DNA ligase. This would require limited complementarity between the mRNA 3′-OH end and viral template sequences. [Note that the vaccinia virus genome is very A-T rich and would provide ample opportunity for annealing of a 3′-oligo(A) tail of cellular mRNA to T-runs in the genome.] The mRNA strand is then copied into cDNA, that is contiguous with viral DNA, either by a host-derived reverse transcriptase or by the vaccinia DNA polymerase itself. The high levels of DNA recombination in vaccinia-infected cytoplasm would then permit the 3′ end of the integrated cDNA to be rejoined to viral genomic DNA. An alternative pathway, in which reverse transcription precedes uptake of the cellular coding information, is also conceivable and DNA ligase might participate in this pathway by joining either the RNA or the DNA strands to genomic DNA.

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