Vaccinia topoisomerase binds duplex DNA and forms a covalent DNA-(3'-phosphotyrosyl) protein adduct at the sequence 5'-CCCTT. The enzyme reacts readily with a 36-mer CCCTT strand (DNA-p-RNA) composed of DNA 5' and RNA 3' of the scissile bond. However, a 36-mer composed of RNA 5' and DNA 3' of the scissile phosphate (RNA-p-DNA) is a poor substrate for covalent adduct formation. Vaccinia topoisomerase efficiently transfers covalently held CCCTT-containing DNA to 5'-OH-terminated RNA acceptors; the topoisomerase can therefore be used to tag the 5' end of RNA in vitro.

Religation of the covalently bound CCCTT-containing DNA strand to a 5'-OH-terminated DNA acceptor is efficient and rapid (Kcat > 0.5 s⁻¹), provided that the acceptor DNA is capable of base pairing to the noncleaved DNA strand of the topoisomerase-DNA donor complex. The rate of strand transfer to DNA is not detectably affected by base mismatches at the 5' nucleotide of the acceptor strand. Nucleotide deletions and insertions at the 5' end of the acceptor slow the rate of religation; the observed hierarchy of reaction rates is as follows: 1 deletion > +2 insertion > -2 deletion. These findings underscore the importance of a properly positioned 5'-OH terminus in transesterification reaction chemistry, but they also raise the possibility that topoisomerase may generate mutations by sealing DNA molecules with mispaired or unpaired ends.

Vaccinia topoisomerase, a 314-amino acid eukaryotic type I enzyme, binds and cleaves duplex DNA at a specific target sequence, 5'-(T/C)CCCTT (1–3). Cleavage is a transesterification reaction in which the Tp N phosphodiester is attacked by Tyr-274 of the enzyme, resulting in the formation of a DNA-(3'-phosphotyrosyl) protein adduct (4). The covalently bound topoisomerase catalyzes a variety of DNA strand transfer reactions. It can religate the CCCTT-containing strand across the same bond that was originally cleaved (as occurs during the relaxation of supercoiled DNA) or it can ligate the strand to a heterologous acceptor DNA 5' end, thereby creating a recombinant molecule (5–7).

Duplex DNA substrates containing a single CCCTT target site have been used to dissect the cleavage and strand transfer steps. A cleavage-religation equilibrium is established when topoisomerase transesterifies to DNA ligands containing ≥18 bp of duplex DNA 3' of the cleavage site (8–11). The reaction is in equilibrium because the 5'-OH-terminated distal segment of the scissile strand remains poised near the active site by virtue of the fact that it is stably base paired with the nonscissile strand. About 20% of the CCCTT-containing strand is covalently bound at equilibrium (11). "Suicide" cleavage occurs when the CCCTT-containing substrate contains six or fewer base pairs 3' of the scissile bond, because the short leaving strand dissociates from the protein-DNA complex. In enzyme excess, >90% of the suicide substrate is cleaved (11).

The suicide intermediate can transfer the incised CCCTT strand to a DNA acceptor. Intramolecular strand transfer occurs when the 5'-OH end of the noncleaved strand of the suicide intermediate attacks the 3' phosphotyrosyl bond and expels Tyr-274 as the leaving group. This results in formation of a hairpin DNA loop (5). Intermolecular religation occurs when the suicide intermediate is provided with an exogenous 5'-OH-terminated acceptor strand, the sequence of which is complementary to the single strand tail of the noncleaved strand in the immediate vicinity of the scissile phosphate (5). In the absence of an acceptor strand, the topoisomerase can transfer the CCCTT strand to water, releasing a 3'-phosphate-terminated hydrolysis product, or to glycerol, releasing a 3'-phosphoglycerol derivative (12). Although the hydrolysis and glycerololysis reactions are much slower than religation to a DNA acceptor strand, the extent of strand transfer to non-DNA nucleophiles can be as high as 15–40%.

The specificity of vaccinia topoisomerase in DNA cleavage and its versatility in strand transfer have inspired topoisomerase-based strategies for polynucleotide synthesis in which DNA oligonucleotides containing CCCTT cleavage sites serve as activated linkers for the joining of other DNA molecules with compatible termini (13). In the present study, we examined the ability of the vaccinia topoisomerase to cleave and rejoin RNA-containing polynucleotides. It was shown previously that the enzyme did not bind covalently to CCCTT-containing molecules in which either the scissile strand or the complementary strand was composed entirely of RNA (9). To further explore the pentose sugar specificity of the enzyme, we have prepared synthetic CCCTT-containing substrates in which the scissile strand is composed of DNA- and RNA-containing halves. In this way, we show that the enzyme is indifferent to RNA downstream of the scissile phosphate, but it does not form the covalent complex when the region 5' of the scissile phosphate is in RNA form. Also, we assess the contribution of base pairing by the 5' end of the acceptor strand to the rate of the DNA strand transfer reaction.

**MATERIALS AND METHODS**

**Preparation of Tandem RNA-p-DNA and DNA-p-RNA Oligonucleotides**—CCCTT-containing 36-mer oligonucleotides containing a single internal 32P-label at the scissile phosphate were prepared by ligating two 18-mer strands (synthetic RNA or DNA oligonucleotides) that had been hybridized to a complementary 36-mer DNA strand. The sequence of the proximal CCCTT-containing 18-mer strand was 5'-CATATCCGT-CTGCCCTT as DNA or 5'-CAAUCCUGUGCUU as RNA. The sequence of the distal 18-mer strand was 5'-ATTCCGATATTGAC-TACA as DNA or 5'-AUUCCGAUGACUACA as RNA. The distal strand was made by ligating the proximal strand to a synthetic RNA oligonucleotide containing a CCCTT target site (14).
Topoisomerase-catalyzed DNA and RNA Strand Transfer Reactions

18-mer strand was 5'-labeled in the presence of [γ-32P]ATP and T4 polynucleotide kinase and then gel-purified. The sequence of the 36-mer strand was 5'-TGTAGTCACTAGGACAGCGATATTG. The strands were annealed 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 36-mer distal 18-mer to the proximal 18-mer and the 36-mer strand in the hybridization mixture was 1:4:4. The singly nicked product of the annealing reaction was sealed in vitro with purified recombinant vaccinia virus DNA ligase (14, 15). The ligation reaction mixtures (400 μl) contained 50 mM Tris-HCl (pH 8.0), 5 mM dithiothreitol, 10 mM MnCl₂, 1 mM ATP, 10 pmol of 5'-32P-labeled nicked substrate, and 180 pmol of ligase. After incubation for 4 h at 22°C, the reactions were terminated by the addition of EDTA to a final concentration of 25 mM. The samples were extracted with phenol-chloroform, and the labeled nucleic acid was recovered from the aqueous phase by ethanol precipitation. The 36-mer duplex products were dissolved in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Ligation of the labeled 18-mer distal strand to the unlabeled CCCCTT-containing 18-mer strand to form an internally labeled 36-mer product was confirmed by electrophoresis of the reaction products through a 17% denaturing polyacrylamide gel. The extents of ligation [36-mer/(36-mer + 18-mer)] were as follows: DNA-p-DNA, 88%; DNA-p-RNA, 67%; and RNA-p-DNA, 66%.

Covalent Binding of Topoisomerase to Internally Labeled 36-mer Duplexes—Recombinant vaccinia topoisomerase was expressed in bacteria and purified via phosphocellulose and SP5PW column chromatography as described (16, 17). Reaction mixtures for assay of covalent adenylate formation contained (per 20 μl) 50 mM Tris-HCl (pH 8.0), 25 pmol of 36-mer duplex, and 1 pmol of topoisomerase. The reactions were initiated by adding topoisomerase and halted by adding SDS to 1% final concentration. The samples were analyzed by SDS-polyacrylamide gel electrophoresis. Covalent complex formation was revealed by the transfer of radiolabeled polynucleotide to the topoisomerase polypeptide (3). The extent of adduct formation was quantitated by scanning the gel using a FUJIX BAS1000 phosphorimager and was expressed as the percentage of the input 5'-32P-labeled 36-mer substrate that was covalently transferred to protein.

DNA Strand Transfer to an RNA Acceptor—An 18-mer CCCCTT-containing DNA oligonucleotide (5'-CGTGGTCCTTTTTCG-3') was 5'-end-labeled in the presence of γ-32P-ATP and T4 polynucleotide kinase, then gel-purified and hybridized to a complementary 30-mer strand to form the 18-mer/30-mer suicide cleavage substrate. Covalent topoisomerase-DNA complexes were formed in a reaction mixture containing (per 20 μl) 50 mM Tris-HCl (pH 8.0), 0.5 pmol of 18-mer/30-mer DNA, and 2.5 pmol of topoisomerase. The mixture was incubated for 5 min at 37°C. The strand transfer reaction was initiated by the addition of an 18-mer acceptor strand, 5'-ATTCCGAGTAGTACAGTA (either DNA or RNA), to a concentration of 25 pmol/20 μl (i.e., a 50-fold molar excess over the input DNA substrate), while simultaneously the reaction mixtures were incubated at 37°C. The reactions were halted by addition of SDS and formamide to 0.2 and 50%, respectively. The samples were heat-denatured 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 strand transfer (expressed as the percentage of input labeled DNA converted to a 30-mer strand transfer product) was quantitated by scanning the wet gel with a phosphorimager.

Preparation of 32P-labeled 36-mer RNA—A 36-mer nucleotide run-off transcript was synthesized in vitro by T3 RNA polymerase from a Bluescript II-SK(--) plasmid template that had been linearized by digestion with endonuclease EagI. A transcription reaction mixture containing 100 μl (40 mM Tris-HCl (pH 8.0), 6 mM MgCl₂, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM UTP, 6.25 μM [γ-32P]GTP, 5 μg of template DNA, and 100 units of T3 RNA polymerase (Promega) was incubated for 90 min at 37°C. The reaction was halted by adjusting the mixture to 0.1% SDS, 10 mM EDTA, and 0.5 mM ammonium acetate. The samples were extracted with phenol-chloroform and ethanol-precipitated. The pellet was resuspended in formamide and electrophoresed through a 12% polyacrylamide gel containing 7 M urea in TBE. The radiolabeled 36-mer RNA was localized by autoradiography of the wet gel and eluted from the gel slice by soaking for 16 h at 4°C in 0.4 ml of buffer containing 1 mM ammonium acetate, 0.2% SDS, and 20 mM EDTA. The eluate was phenol-extracted and ethanol-precipitated. The RNA was resuspended in TE. Dephosphorylation of the RNA 5'-terminus was carried out in a reaction mixture (30 μl) containing 10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 10 mM MgCl₂, 1 mM dithiothreitol, 10 pmol of 36-mer RNA, and 30 units of calf intestine alkaline phosphatase (New England Biolabs). After a 1-h incubation at 37°C, the mixture was phenol-extracted and ethanol-precipitated. The phenosulfate-treated 36-mer transcript was repurified electrophoretically as described above.

RESULTS

Covalent Binding of Topoisomerase to a Duplex Substrate Containing RNA 3'-Oligonucleotide—Vaccinia topoisomerase does not bind covalently to CCCCTT-containing RNA duplexes, nor does it form a covalent complex on RNA-DNA hybrid duplexes in which one of the two strands is RNA (9). Control experiments showed that the failure to form a covalent adduct on a CCCUU-containing RNA strand was not caused by uracil substitution for the thymine bases in the CCCCTT sequence (9). To better understand why vaccinia topoisomerase does not form a covalent complex with all-RNA strands, we prepared 36-mer duplex substrates in which the scissile strand was a tandem RNA-DNA molecule and the noncleaved strand was all DNA (Fig. 1). These duplexes were uniquely labeled with 32P at the scissile phosphodiester. The substrate molecules were constructed by annealing two 18-mer oligonucleotides (one of which had been 5'-32P-labeled) to a complementary 36-mer DNA strand to form a singly nicked duplex. The 5'-labeled 18-mer strand was then joined to the unlabeled CCCCTT-strand (or CCCUU strand) in a reaction catalyzed by vaccinia virus DNA ligase. (The properties of vaccinia ligase in joining RNA and DNA strands will be described elsewhere.) The 36-mer duplex products were isolated and then used as substrates for vaccinia DNA topoisomerase. We will refer to these substrates as DNA-p-DNA, DNA-p-RNA, and RNA-p-DNA, with the labeled phosphate being denoted by p.

Transesterification by topoisomerase at the CCCCTT site will result in covalent binding of a 3'-32P-labeled 18-mer oligonucleotide to the enzyme. The extent of covalent complex formation on the DNA-p-RNA substrate in 10 min was proportional to input topoisomerase; 80–85% of the 36-mer strand was transferred to the topoisomerase at saturating enzyme (Fig. 1). The same level of topoisomerase covalently bound less than 1% of the RNA-p-DNA 36-mer strand. Hence, the topoisomerase tolerated RNA substitution downstream of the scissile phos-
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The RNA-p-DNA 36-mer was transferred to the topoisomerase, albeit very slowly. After 4 hours, 4% of the CCCTT-containing RNA strand was bound covalently to the enzyme (Fig. 2B). An end point was not established in this experiment. However, by comparing the initial rate of covalent adduct formation on RNA-p-DNA (0.04% of input substrate cleaved per min) to the amount adduct formed on DNA-p-DNA at the earliest time point (12% in 10 s), we estimate that RNA substitution of the CCCTT-portion of the substrate slowed the rate of covalent complex formation by about 3 orders of magnitude.

DNA Strand Transfer to an RNA Acceptor—Rejoining of the cleaved strand occurs by attack of a 5'-hydroxyl-terminated polynucleotide on the 3'-phosphodiester bond between Tyr-274 and the CCCTT site. This transesterification step can be studied independent of strand cleavage by assaying the ability of a preformed topoisomerase-DNA complex to religate the covalently held strand to a heterologous acceptor strand (5, 11). To form the covalent topoisomerase-DNA donor complex, the enzyme was initially incubated with a suicide substrate consisting of a 5'-32P-labeled 18-mer scissile strand (CTGGTCGCC-CCTTATTCCC) hybridized to a 30-mer strand. Cleavage of this DNA by topoisomerase is accompanied by dissociation of the 6-nucleotide leaving group, ATTCCTCC. With no readily available acceptor for religation, the enzyme is essentially trapped on the DNA as a suicide intermediate (Fig. 3). In a 5-min reaction in enzyme excess, >90% of the 5'-32P-labeled strand becomes covalently bound to protein. The strand transfer reaction was initiated by adding a 50-fold molar excess of an 18-mer acceptor strand (either DNA or RNA) complementary to the 5'-single-strand tail of the covalent donor complex (Fig. 3) while simultaneously increasing the ionic strength to 0.3 M NaCl. Addition of NaCl during the religation phase promotes dissociation of the topoisomerase after strand closure and prevents recleavage of the strand transfer product. Ligation of the covalently held 12-mer CGTGTCCGCCCTT to the 18-mer yields a 32P-labeled 30-mer (Fig. 4, lane 1). The suicide intermediate transferred 94% of the input CCCTT-containing strand to the 18-mer DNA strand (Fig. 3). The extent of religation at the earliest time point (5 s) was 90% of the end point value. We calculated from this datum a religation rate constant (k_{rel}) of >0.5 s^{-1}. We had determined previously (from experimental values for k_{cl} and K_{cl}) at 37 °C a k_{rel} value of -1.3 s^{-1}.

Topoisomerase readily ligated the covalently held 12-mer DNA to an 18-mer RNA acceptor to form a 30-mer product (Fig. 4, lane 5). 89% of the input CCCTT-strand was transferred to RNA, with 40% of the end point value attained in 5 s. We used this datum to estimate a rate constant of 0.1 s^{-1} for single-turnover strand transfer to RNA. Thus, religation to DNA was about 10 times faster than religation to RNA. The slowed rate of RNA religation is likely to account for the observed increase in the cleavage-religation equilibrium constant (K_{eq} = k_{cl}/k_{rel}) on the DNA-p-RNA 36-mer. Analysis of the Strand Transfer Reaction Product—The predicted product of strand transfer to RNA is a 30-mer tandem DNA-RNA strand (5'-CGTGTCCGCCCTTAAUCGGAGAU-GACUACA) uniquely 32P-labeled at the 5'-end. The structure of this molecule was confirmed by analysis of the susceptibility of this product to treatment with NaOH. The labeled 30-mer RNA ligation product was converted nearly quantitatively into a discrete species that migrated more rapidly than the input...
alkali-treated samples were neutralized by adding 1.2 pmol of 5'-labeled suicide DNA cleavage substrate, and 2.5 pmol of topoisomerase were incubated at 37 °C for 10 min. Strand transfer was then initiated by adding a 50-fold excess of the acceptor DNA (18-mer D; lanes 1 and 2) or acceptor RNA (18-mer R; lanes 5 and 6) while simultaneously adjusting the mixtures to 0.3 M NaCl. The religation reactions were quenched after a 10-min incubation by adding SDS to 0.2%. The samples were extracted with phenol-chloroform and ethanol-precipitated. The pellets were resuspended in either 12 m NaOH, 0.1 M NaOH, or 12 m of 10 m Tris- HCl, 0.1 mM EDTA (NaOH-EDTA). 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 topoisomerase were treated in parallel (lanes 1 and 2). The alkaline-treated samples were neutralized by adding 1.2 m of 1 M HCl. All samples were then ethanol-precipitated, resuspended in formamide, heated for 5 min at 95 °C, and electrophoresed through a 17% polyacrylamide gel containing 7 m urea in TBE. The structures of the covalent topoisomerase-DNA donor complex and the RNA acceptor are shown. The 5' single-strand tail of the suicide intermediate is complementary to the 18 nucleotides at the 5' end of the T3 transcript. Reaction mixtures contained (per 15 m) 50 m Tris-HCl (pH 8.0), 0.3 m NaCl, and 0.1 pmol of 32P-GMP-labeled T3 transcript. Religation was initiated by the addition of preformed topoisomerase-DNA donor (at a 10-fold molar excess over RNA acceptor). Incubation was at 37 °C. Aliquots (15 m) were removed at the times indicated and quenched immediately by adding SDS and EDTA. The samples were adjusted to 50% formamide, heated for 5 min at 95 °C, and electrophoresed through a 12% polyacrylamide gel containing 7 m area in TBE. Transfer of the 12-nucleotide DNA donor strand to the 5' end of the labeled 36-mer T3 transcript yielded a labeled 48-mer product. Conversion of input 36-mer to 48-mer was quantitated by scanning the gel with a phosphorimager.

18-mer CCCTT-containing DNA strand (Fig. 4, lane 6). The mobility of this product was consistent with a chain length of 13 nucleotides. The expected 32P-labeled alkaline hydrolysis product of the RNA strand transfer product is a 13-mer (5'-CCCTTCCCTTCCCTTAp). Control reactions showed that neither the 32P-labeled 18-mer scissile strand of the suicide substrate nor the 30-mer product of strand transfer to DNA was susceptible to alkaline (Fig. 4, lanes 4 and 2). We conclude that topoisomerase can be used to ligate RNA to DNA in vitro.

DNA Ligand Tagging of an RNA Transcript Synthesized in Vitro by T3 RNA Polymerase—Practical applications of topoisomerase-mediated strand transfer to RNA include the 5' tagging of RNA transcripts. Bacteriophage RNA polymerases have been used widely to synthesize RNA in vitro from plasmid DNA templates containing phage promoters. To test whether such transcripts were substrates for topoisomerase-catalyzed ligation, we constructed a CCCTT-containing suicide cleavage substrate that, when cleaved by topoisomerase, would contain a 5' single-strand tail complementary to the predicted 5' sequence of any RNA transcribed by T3 RNA polymerase from a pBlue-script vector (Fig. 5). A 36-nucleotide T3 transcript was synthesized in a transcription reaction containing [a-32P]GTP. The RNA was treated with alkaline phosphatase to dephosphorylate the 5' terminus. The topoisomerase-DNA covalent intermediate was formed on an unlabeled suicide substrate. Incubation of the radiolabeled T3 transcript with the suicide intermediate resulted in the conversion of the 36-mer RNA into a novel species that migrated more slowly during polyacrylamide gel electrophoresis (not shown). The apparent size of this product (48 nucleotides) was indicative of ligation to the 12-mer CCCTT DNA strand. The kinetics of DNA ligation to the T3 transcript are shown in Fig. 5. The reaction was virtually complete within 1 min; at its end point, 29% of the input RNA had been joined to DNA. No DNA-RNA ligation product was formed in reaction containing a T3 transcript that had not been treated with alkaline phosphatase (not shown).

Formulation of Insertions and Deletions: A Kinetic Analysis—The acceptor polynucleotides used in the experiments described above were capable of hybridizing perfectly with the 5' single-strand tail of the topoisomerase-DNA donor complex. It had been shown previously that the vaccinia virus topoisomerase is capable of joining the CCCTT-strand to an acceptor oligonucleotide that hybridizes so as to leave a single nucleotide gap between the covalently bound donor 3' end and the 5' terminus of the acceptor. Religation across this gap generated a 1-base deletion in the product compared with the input scissile strand (5). The enzyme also catalyzes strand transfer to an acceptor oligonucleotide that, when hybridized, introduces an extra nucleotide between the donor 3' end and the penultimate
Fig. 6. Kinetics of topoisomerase-catalyzed strand transfer reactions resulting in DNA deletions and insertions. The structure of the preformed donor complex is shown at the top of the figure. Religation reactions were performed under single-turnover conditions as described under "Materials and Methods." All DNA acceptors were included at a 50-fold molar excess over the input CCCTT-containing substrate. A, deletion formation. The structures of the completely base-paired 18-mer acceptor DNA oligonucleotide (open circle), a 17-mer oligonucleotide that anneals to the donor complex to leave a 1-nucleotide gap (filled square) and a 16-mer strand that anneals to leave a 2-nucleotide gap (open square) are shown. B, insertion formation. The structures of the completely base-paired 18-mer acceptor (open circle), a 19-mer oligonucleotide containing 1 extra 5' nucleotide (filled triangle), and a 20-mer acceptor containing 2 extra 5' nucleotides (open triangle) are shown. The extent of religation is plotted as a function of incubation time.

Base paired nucleotide of the acceptor. Religation in this case will produce a 1-base insertion (5). Deletion and insertion formation in vitro have also been documented for mammalian type I topoisomerase (19). However, there has been no report of the effects of acceptor strand gaps and insertions on the rate of strand joining by these enzymes.

We assessed the kinetics of strand transfer by the vaccinia topoisomerase covalent intermediate to acceptor oligonucleotides that base pair with the donor complex to form either a fully base-paired 3' duplex segment or 3' duplexes with a 1- or 2-nucleotide gap. 84% of the input DNA substrate was ligated to the fully paired acceptor in 10 s, the shortest time analyzed (Fig. 6A). The size of the strand transfer product was 30 nucleotides, as expected (Fig. 7, lane 5). No 30-mer product was formed in the absence of the added acceptor strand (Fig. 7, lane 2).

Religation across a 1-nucleotide gap was highly efficient, albeit slow. 85% of the input substrate was joined across a 1-nucleotide gap to yield the expected 29-nucleotide product (Fig. 6A and Fig. 7, lane 4). The kinetic data in Fig. 6 fit well to a single exponential with an apparent rate constant of 0.005 s⁻¹. Thus, single-turnover strand closure by topoisomerase across a 1-nucleotide gap was 2 orders of magnitude slower than the rate of joining across a fully paired nick. Vaccinia topoisomerase catalyzed strand transfer across a 2-nucleotide gap to form the anticipated 28-nucleotide product (Fig. 7, lane 5), but this reaction was feeble (Fig. 6A). We observed linear accumulation of the 2-nucleotide gap product over a 2-h incubation, at the end of which only 10% of the input DNA had been joined. We estimated, based on the initial rate, that religation across the 2-nucleotide gap was 2 orders of magnitude slower than joining across a 1-nucleotide gap (and hence 4 orders of magnitude slower than the rate of joining across a nick).

Similar experiments were performed using DNA acceptors that contained either 1 or 2 extra nucleotides at their 5' ends (Fig. 6B). Religation to these acceptors yielded labeled strand transfer products of 31 and 32 nucleotides, respectively (Fig. 7, lanes 6 and 7). 90% of the input DNA was religated to form the 1-nucleotide insertion product (Fig. 6B). We calculated a rate constant of 0.04 s⁻¹ for religation with 1-nucleotide insertion. A similar end point was achieved in the formation of a 2-nucleotide insertion product, but the strand transfer rate was considerably slower (Fig. 6B). The observed rate constant for 2-nucleotide insertion was 0.001 s⁻¹, i.e., 3 orders of magnitude lower than k₂rel at a nick.

Effect of 5' Acceptor Base Mismatch on Strand Transfer—We examined strand transfer by topoisomerase to a set of 18-mer acceptors that were capable of forming base pairs with the 5' tail of the donor complex from positions −2 to −18 (relative to the scissile +1 T:A base pair of the CCCTT element) but that have a base mismatch at the −1 position immediately 3' of the scissile bond. The control acceptor, which has a normal −1 A:T base pair, reacted to completion in 10 s; 89% of the end point was achieved in 5 s (Fig. 8). DNAs containing T:T, C:T, or G:T mispairs at the −1 position supported the same extent of strand transfer; 77% of the end point was attained in 5 s in each case (Fig. 8). Thus, within the limits of detection of this experiment, mismatch at the −1 position had little effect on the strand transfer reaction.
There are clear and instructive differences between the effects of base mismatches versus a single nucleotide deletion on the rate of the strand joining step.

**Kinetics of Intramolecular Hairpin Formation**—In the absence of an exogenous acceptor oligonucleotide, the 5’-OH terminus of the nonscissile strand of the 12-mer/30-mer covalent complex can flip back and act as the nucleophile in attacking the DNA-(3-phosphotyrosyl) bond (5). The reaction product is a hairpin molecule containing a 12-bp stem and an 18-nucleotide loop. The kinetics of this reaction were examined under single turnover conditions. In the experiment shown in Fig. 9A, 65% of the input CCCCTT strand was converted to hairpin product in 3 h. The observed rate constant was 5.7 × 10⁻⁴ sec⁻¹. In parallel, we analyzed the rate of hairpin formation by the covalent complex formed on an 18-bp cleavage substrate (Fig. 9A). In this case, an attack by the 5’-OH of the nonscissile strand yielded a hairpin molecule containing a 12-bp stem and a 6-nucleotide loop. 69% of the input CCCCTT strand was converted to hairpin product in 10 h. The observed rate constant was 8.2 × 10⁻⁵ sec⁻¹. Thus, the 18-nucleotide 5’ tail was ~7 times more effective than the 6-mer 5’ tail as the attacking nucleophile for strand transfer in cis. Note that hairpin formation by these covalent complexes occurs without any potential for base pairing by the single-strand tails.

To examine the contribution of base pairing to the rate of religation, we altered the 5’ terminal and penultimate bases of the bottom strand of the 18-mer/30-mer suicide substrate to 5’-ATT (Fig. 9A). Now, the 5’ terminal three bases of the bottom strand (5’-ATTCCC); hence, the single-strand tail is self-complementary and capable of forming three base pairs adjacent to the scissile phosphate. Intramolecular hairpin formation on this DNA was extremely fast; the reaction was complete in 5 sec. Thus, the topoisomerase-catalyzed DNA cleavage substrates were prepared by annealing the 5’-³²P-labeled 18-mer scissile strand to a 30-mer complementary strand (filled circle) or an 18-mer complementary strand (open circle); the structures of the substrates are shown, with the topoisomerase cleavage sites indicated by arrows. Reaction mixtures containing (per 20 μl) 50 mM Tris-HCl (pH 7.5), 0.5 pmol of DNA substrate, and 1 pmol of topoisomerase were incubated at 37 °C for 10 min. The mixtures were then adjusted to 0.3 M NaCl. Aliquots (20 μl) were withdrawn immediately prior to the addition of salt (time 0) and at various intervals after the addition of salt; the reactions were quenched immediately by adding an equal volume of stop solution (1% SDS, 95% formamide, 20 mM EDTA). The samples were heat-denatured and electrophoresed through a 17% polyacrylamide gel containing 7 M urea in TBE. The extent of intramolecular strand transfer (expressed as percentage of input labeled substrate converted to hairpin product) is plotted as a function of time after the addition of NaCl. B, hairpin formation with potential for base pairing. The structure of the 18-mer/30-mer cleavage substrate is shown, with the topoisomerase cleavage site indicated by the arrow. A reaction mixture containing (per 20 μl) 50 mM Tris-HCl (pH 7.5), 0.5 pmol of DNA substrate, and 1 pmol of topoisomerase was incubated at 37 °C for 2 min. The mixtures were then adjusted to 0.3 M NaCl. Aliquots (20 μl) were withdrawn immediately prior to the addition of salt (time 0) and at various intervals after the addition of salt. The extent of intramolecular strand transfer is plotted as a function of time after the addition of NaCl.
DISCUSSION

Vaccinia topoisomerase catalyzes a diverse repertoire of strand transfer reactions. Religation of the covalently bound DNA to a perfectly base-paired acceptor DNA oligonucleotide provides a model for the strand closure step of the DNA relaxation reaction. Here, we have analyzed the kinetics of strand transfer to alternative nucleic acid acceptors. Our findings provide new insights into the parameters that affect transfection rate, illuminate the potential for topoisomerase to generate mutations in vivo, and suggest practical applications of vaccinia topoisomerase as an RNA modifying enzyme.

Sugar Specificity for Covalent Adduct Formation Resides within the CCCTT Element—Vaccinia topoisomerase is apparently incapable of binding covalently to CCCUU-containing RNA strands. This is the case whether the CCCUU strand is part of an RNA-RNA or an RNA-DNA duplex (9). We have now shown that the sugar specificity of the enzyme is attributable to a stringent requirement for DNA on the 5′ shown that the sugar specificity of the enzyme is attributable to a stringent requirement for DNA on the 5′ side of the scissile phosphate, i.e. the CCCTT site must be DNA. Moreover, the CCCTT element must be a DNA-DNA duplex, because earlier experiments showed that a CCCTT strand is cleaved when it is annealed to a complementary RNA strand (9). The RNA-DNA hybrid results are informative, because they suggest that the CCCTT site must adopt a B-form helical conformation to be cleaved. RNA and DNA polynucleotide chains adopt different conformations within an RNA-DNA hybrid, with the RNA strand retaining the A-form helical conformation (as found in double-stranded RNA), whereas the DNA strand adopts a conformation that is neither strictly A nor B, but is instead intermediate in character between these two forms (20, 21). Vaccinia topoisomerase makes contacts with the nucleotide bases of the CCCTT site in the major groove (9, 22). It also makes contacts with specific phosphates of the CCCTT site (23). Adoption of the CCCTT site of a non-B conformation may weaken or preclude these contacts.

Our finding that vaccinia topoisomerase is relatively insensitive to the nucleotide sugar composition downstream of the scissile phosphate implies that the conformation of the helix in this portion of the ligand is not important for site recognition or reaction chemistry. Topoisomerase cleaves DNA-p-RNA strands in which the leaving strand is RNA. Indeed, the extent of cleavage at equilibrium is significantly higher than that achieved on a DNA-p-DNA strand.

Strand Transfer to RNA—The increase in the cleavage-religation equilibrium constant $K_{eq} = \frac{k_{rel}}{k_{cle}}$ on the DNA-p-RNA substrate can be explained by our finding that the rate of single-turnover RNA religation $k_{relRNA}$ is about one-tenth that of $k_{relDNA}$. Nonetheless, the extent of religation to RNA is quite high, i.e. ~90% of the input CCCUU strand is religated to an 18-mer RNA acceptor strand in a 2 min reaction. We have shown that a CCCTTT-containing DNA strand can be rapidly joined by topoisomerase to a transcript synthesized in vitro by bacteriophage RNA polymerase; ~30% of the RNA was transferred to the DNA strand in a 2-5 min reaction. This property can be exploited to 5′-tag any RNA for which the 5′ terminal RNA sequence is known, i.e. by designing a suicide DNA cleavage substrate for vaccinia topoisomerase in which the noncisile strand is complementary to the 5′ sequence of the intended RNA acceptor. Some practical applications include: (i) $^{32}P$-labeling of the 5′ end of RNA; and (ii) affinity labeling of the 5′ end of RNA, e.g. by using a biotinylated topoisomerase cleavage substrate. A potential advantage of topoisomerase-mediated RNA strand joining (compared with the standard T4 RNA ligase reaction) is that ligation by topoisomerase can be targeted by the investigator to RNAs of interest within a complex mixture of RNA molecules.

Frameshift and Missense Mutagenesis—It was reported earlier that vaccinia topoisomerase can religate to complementary DNA acceptors containing recessed ends or extra nucleotides, thereby generating the equivalent of frameshift mutations (5). Similar reactions have been described by Henningfeld and Hecht (19) for the cellular type I topoisomerase. A key question is whether these aberrant religation reactions are robust enough to implicate topoisomerase as a potential mutagen in vivo. Our kinetic analysis suggests that they are and provides the first clue as to what spectrum of frameshift reactions is most likely to occur (taking into account only the intrinsic properties of the topoisomerase). For the vaccinia enzyme, the hierarchy of frameshift generating religation reactions is as follows: +1 insertion > −1 deletion > +2 insertion > −2 deletion.

The slowest of these topoisomerase-catalyzed reactions is strand closure across a 2-nucleotide gap (initial rate = 0.002% of input DNA religated/sec). In this situation, the attacking nucleophile is held in place at some distance from the DNA-protein phosphodiester by base pairing with the noncisile strand. Moving the 5′-hydroxyl 1 base pair closer to the phosphodiester enhances reaction rate by a factor of 100. Extra nonpaired nucleotides appear to pose much less of an impediment to strand joining to form 1- or 2-nucleotide insertions. The active site of the topoisomerase may be able to accommodate extrahelical nucleotides; alternatively, these nucleotides may intercalate into the DNA helix at the topoisomerase-induced nick.

There are two potential pathways for topoisomerase to form minus frameshifts in vivo, which differ as to how the acceptor strand is generated: (i) the 5′ end of the leaving strand can be trimmed by a nuclease, after which ligation could occur across the resulting gap; or (ii) a homologous DNA single strand could attack the covalent intermediate. The second pathway presumably requires a helicase to form the invading strand (and perhaps also to displace the leaving strand). In the case of plus frameshifts, only the latter pathway would be available to the topoisomerase, i.e. because no mechanism exists to add nucleotides to the 5′ terminus of the original leaving strand. No matter which pathway is taken, it is reasonable to assume that the most rapidly catalyzed mutagenic strand-joining reactions are the ones most likely to make their mark in vivo. If the religation reaction is slow, as for −2 frameshifting, then the cell has greater opportunity to repair the mutagenic lesion, e.g. by removing the covalently bound topoisomerase. This could entail: (i) excision of a patch of the DNA strand to which the topoisomerase is bound; or (ii) hydrolysis of the topoisomerase-DNA adduct. An enzyme that catalyzes the latter reaction was discovered recently by Yang et al. (24).

Introducing a base mismatch at the −1 position immediately flanking the scissile phosphate has almost no effect on the rate of religation. This result is in stark contrast to the $10^{-2}$ rate effect of a 1-nucleotide gap. We infer that the −1 base mismatches do not significantly alter the proximity of the 5′-hydroxyl nucleophile of the terminal nucleotide to the scissile phosphate at enzyme’s active site. Our results indicate clearly that topoisomerase has the capacity to generate missense mutations in vitro. The single-strand invasion pathway invoked above for frameshift mutagenesis could, in principle, provide the opportunity for topoisomerase to create missense mutations in vivo. The kinetics of ligation in vitro suggest that topoisomerase-generated missense mutations would predominate over frameshifts.

Kinetic Contribution of Base Complementarity—Kinetic analysis of intramolecular hairpin formation by the vaccinia topoisomerase provides the first quantitative assessment of the
role of base complementarity in strand closure. The rate constant for attack on the DNA-(3′-phosphotyrosyl) bond by a nonpairing 18-nucleotide single strand linked in cis to the covalent complex was 5.7 × 10^{-4} \text{sec}^{-1}. Altering only the terminal bases of the single-strand tail to allow the formation of base pairs at the −1, −2, and −3 positions increased the rate constant for hairpin formation by 350-fold. The rate of religation in cis with 3 potential base pairs was nearly the same as the rate of religation to a non-covalently linked acceptor strand that forms 18 base pairs 3′ of the scissile bond. The ability of the covalently bound enzyme to take up and rapidly rejoin DNA strands with only three complementary nucleotides lends credence to the suggestion that vaccinia topoisomerase catalyzes the formation of recombination intermediates in vivo (25), either via strand invasion or by reciprocal strand transfer between two topoisomerase-DNA complexes. Efforts to model these reactions in vitro are under way.

REFERENCES
1. Shuman, S., and Prescott, J. (1990) J. Biol. Chem. 265, 17826–17836
2. Shuman, S. (1991) J. Biol. Chem. 266, 1796–1803
3. Shuman, S. (1991) J. Biol. Chem. 266, 11372–11379
4. Shuman, S., Kane, E. M., and Morham, S. G. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 9793–9797
5. Shuman, S. (1992) J. Biol. Chem. 267, 8629–8627
6. Shuman, S. (1992) J. Biol. Chem. 267, 16755–16758
7. Sekiguchi, J., Seeman, N. C., and Shuman, S. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 785–789
8. Stivers, J. T., Shuman, S., and Mildvan, A. S. (1994) Biochemistry 33, 327–339
9. Shuman, S., and Turner, J. (1993) J. Biol. Chem. 268, 18943–18950
10. Petersen, B. Ø., Witschieben, J., and Shuman, S. (1996) J. Mol. Biol. 263, 181–195
11. Petersen, B. Ø., and Shuman, S. (1997) J. Biol. Chem. 272, 3891–3896
12. Petersen, B. Ø., and Shuman, S. (1997) Nucleic Acids Res. 25, 2091–2097
13. Shuman, S. (1994) J. Biol. Chem. 269, 32878–32884
14. Shuman, S. (1995) Biochemistry 34, 16138–16147
15. Sekiguchi, J., and Shuman, S. (1997) Nucleic Acids Res. 25, 727–734
16. Shuman, S., Golder, M., and Mass, B. (1988) J. Biol. Chem. 263, 16401–16407
17. Morham, S. G., and Shuman, S. (1992) J. Biol. Chem. 267, 15984–15992
18. Cheng, C., Wang, L. K., Sekiguchi, J., and Shuman, S. (1997) J. Biol. Chem. 272, 8263–8269
19. Henningfeld, K. A., and Hecht, S. M. (1995) Biochemistry 34, 6120–6129
20. Salazar, M., Federoff, O. Y., Miller, J. M., Elbeto, N. S., and Reid, B. R. (1993) Biochemistry 32, 4207–4215
21. Arnott, S., Chandrasekaran, R., Millane, R. P., and Park, H. (1986) J. Mol. Biol. 188, 631–640
22. Sekiguchi, J., and Shuman, S. (1996) EMBO J. 15, 3448–3457
23. Sekiguchi, J., and Shuman, S. (1994) J. Biol. Chem. 269, 31731–31734
24. Yang, S. W., Burgin, A. B., Huizenga, B. N., Robertson, C. A., Yao, K. C., and Nash, H. A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 11534–11539
25. Shuman, S. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 10104–10108