THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 269, No. 42, Issue of October 21, pp. 26220–26226, 1994
Printed in U.S.A.

Escherichia coli DNA Helicase I Catalyzes a Sequence-specific Cleavage/Ligation Reaction at the F Plasmid Origin of Transfer*

(Received for publication, May 18, 1994, and in revised form, August 17, 1994)

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Recent studies have shown that the *Escherichia coli* F plasmid-encoded *traI* gene product (TraIp), also known as DNA helicase I, catalyzes the formation of the site- and strand-specific nick that initiates F plasmid DNA transfer. Scission of the phosphodiester bond at the *nic* site within the origin of transfer (*oriT*) is accompanied by the covalent attachment of TraIp to the 5′-phosphate of the nicked DNA strand. This mechanism suggests that TraIp may also be capable of catalyzing a DNA ligation reaction using the energy stored in the protein-DNA intermediate. To test this possibility, an *in vitro* assay was designed that utilized short single-stranded DNA oligonucleotides of different lengths derived from the region within *oriT* that spanned the *nic* site. Purified TraIp was capable of efficiently cleaving single-stranded DNA that contained a *nic* site, and upon cleavage, the protein became covalently linked to the 5′-end of the *nic* site. When TraIp was incubated with two oligonucleotides of different length that contained the *nic* site, there was formation of novel recombinant products resulting from a TraIp-catalyzed cleavage/ligation reaction. Furthermore, the cleavage and ligation reactions were both sequence-specific. These data suggest that TraIp plays an important role in the initiation and termination of conjugative DNA transfer.

The transfer of DNA from one bacterial cell to another, mediated by transmissible plasmids, is an important mechanism for genetic exchange within a bacterial population. Classically, our understanding of the process of bacterial conjugation derives from genetic studies of transmissible plasmids such as F, R100, RP4, and others (for reviews see Refs. 1–3). In each case, a donor and a recipient cell establish cell-cell contact, and a single strand of DNA is transferred from the donor to the recipient cell with an overall 5′ to 3′ polarity. The transferred strand of DNA either circularizes to form an episome or is integrated, via recombination, into the chromosome of the recipient cell. Although well understood from a genetic perspective, the biochemical mechanism of this process remains poorly defined.

Molecular genetic studies of the *Escherichia coli* F plasmid have described 24 gene products of the *tra* family that are involved in conjugative DNA transfer from a donor F^+ cell to a recipient F^- cell (1, 4). Twenty of these genes are, to some extent, coordinately regulated in the large 35-kilobase pair *tra* operon (1, 4), and their functions can be divided into three categories: (i) proteins that form the pilus, (ii) proteins that recognize other F^+ cells and discourage mating, and (iii) proteins that function in the DNA metabolic events associated with strand transfer. Only four of the *tra* gene products appear to interact directly with the F plasmid DNA during strand transfer, specifically the gene products of *traY*, *traI*, *traM*, and *traD* (3).

Biochemical studies have established that F plasmid DNA strand transfer is initiated by the formation of a site- and strand-specific nick at the origin of transfer (*oriT*) (2). The nicked strand is then unwound, presumably by a helicase, and transferred to the recipient cell. The products of the *traD* and *traM* genes have both been shown to be necessary for DNA transfer during conjugation (5, 6). The *traM* gene product has been shown to bind sequences near the *nic* site within *oriT*, but is not required for nicking to occur (7, 8). No well defined biochemical role has been assigned to the *traD* gene product, although it has been identified as an inner membrane protein (9). The *traY* gene product (TraYp) has been overexpressed, purified, and shown to bind a region of *oriT* that is near, but not coincident with, the site that is nicked to initiate strand transfer (10). This finding, in conjunction with genetic evidence indicating that TraYp is necessary for nicking the transferred strand (8), suggests that TraYp may play a role in regulating the site- and strand-specific nicking event. While there is speculation regarding the specific roles of the *traM* gene product, the *traD* gene product, and the *traY* gene product, the specific biochemical functions of these proteins are still poorly understood.

On the other hand, the *traI* gene product (TraIp), also known as *E. coli* DNA helicase I, has been well studied biochemically. This protein was originally isolated as a DNA-dependent ATPase with helicase activity (11, 12) and has subsequently been shown to be encoded by the F plasmid *traI* gene (13). The purified protein catalyzes a processive 5′ to 3′ helicase reaction (12, 14), and it has been suggested that TraIp drives the transferred strand of DNA from the donor to the recipient cell during conjugation (3). More recently, TraIp has been shown to catalyze the site- and strand-specific nicking reaction at *oriT* that serves to initiate the transfer of F DNA (15, 16). It has also been shown that, subsequent to strand scission, TraIp remains covalently bound to the 5′-end of the nicked DNA strand (17).

In view of the covalent attachment of TraIp to the 5′-end of the nicked DNA strand, it seemed possible that TraIp might also mediate recircularization of the transferred strand via a ligation activity of the protein. The energy for this ligation event would come from the original phosphodiester bond energy preserved in the DNA-protein covalent intermediate. To test this idea, an intermolecular recombination assay was de-

* This work was supported in part by National Institutes of Health Grant GM 33476 and American Cancer Society Grant NP-833C (to S. W. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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TABLE I
Oligonucleotide sequences

The sequences for the oligonucleotides used in this study are shown. Each oligonucleotide was named based on its length in nucleotides. Only the 22-mer (23-mer) and the 30-mer (31-mer) contained the *nic* site. The sequences of the 3′-end labeled 23- and 31-mers were identical to the 22- and 30-mers, respectively, except a ^{32}P-labeled ddATP was added on the 3′-end as described under "Experimental Procedures." The 1-nucleotide addition is represented by "(A)."

22-mer (23-mer)	5′-TTTGCGTGGGGTGTGGTGCTTT(A)-3′
30-mer (31-mer)	5′-CTTGTTTTTGCGTGGGGTGTGGTGCTTTTG(A)-3′
41-mer	5′-GGTGCGTTTGGTGGTGAGAACCTTGTTTTTGCGTGGGGTGT-3′
25-mer	5′-TTGAATTCTCTACAATAAAAAGTTT-3′
17-mer	5′-GTAAAACGACGGCCAGT-3′

vised using single-stranded DNA (ssDNA)[1] oligonucleotides containing the *oriT nic* site. This assay was based on methods employed to investigate the possibility of a cleavage/ligation activity for the TraIp-like proteins of other transmissable plasmids (18, 19). The ssDNA oligonucleotides were efficiently cleaved by TraIp at the expected site, and when two oligonucleotides of different length were mixed, specific recombinant products of novel length were formed. Thus, in addition to its well documented helicase reaction, TraIp catalyzes a sequence-specific cleavage/ligation reaction, suggesting that TraIp likely catalyzes the ligation of the transferred strand in the process of conjugative DNA transfer.

EXPERIMENTAL PROCEDURES

Enzymes—TraIp (DNA helicase I) was purified as described previously (14) to >90% homogeneity as judged by polyacrylamide gel electrophoresis in the presence of SDS. Terminal deoxynucleotidyl transferase, phosphodiesterase I, and proteinase K were purchased from U. S. Biochemical Corp. T4 polynucleotide kinase was purchased from New England Biolabs.

Oligonucleotides and Nucleotides—The oligodeoxyribonucleotides used in this study were synthesized using standard phosphoramidite chemistry and purified on 20% polyacrylamide, 8 M urea denaturing gels as described (20). Their sequences are shown in Table I. Oligonucleotides were either labeled on the 5′-end using bacteriophage T4 polynucleotide kinase and [γ-^{32}P]ATP as described (20) or on the 3′-end using terminal deoxynucleotidyl transferase and [α-^{32}P]ddATP as described (20). Labeled oligonucleotides were separated from free nucleotides by gel filtration chromatography using a 1.5-ml Sephadex G-50 column (0.6 cm × 7.5 cm). [γ-^{32}P]ATP and [α-^{32}P]ddATP were from Amersham Corp. Oligonucleotide concentrations, expressed as moles of the oligonucleotide, were determined by spectrophotometric analysis of the purified, unlabeled oligonucleotide. Recovery from the G-50 column was estimated at 75%.

Oligonucleotide Cleavage Reactions—The complete reaction mixture (17 μl) contained 50 mM Tris-HCl (pH 7.5), 10 mM NaCl, 6 mM $MgCl_2$, 1 pmol of DNA (either 5′-end labeled 30- or 22-mer), and ~2 pmol of TraIp (400 ng),. Reactions were assembled at room temperature and incubated at 37 °C for 2 h. Reactions were stopped by the addition of SDS to 0.1%, and incubation was continued at 37 °C for 10 min. Ten μl of 85% formamide, 1 × TBE (0.089 M Tris, 0.089 M borate, 0.002 M EDTA), 0.1% dyes were added, and the samples were denatured at 100 °C for 3 min prior to loading on a 16% polyacrylamide, 8 M urea denaturing gel. Electrophoresis was at 5 watts constant power in 1 × TBE running buffer. The gels were then exposed to x-ray film or a PhosphorImage screen (Molecular Dynamics, Inc).

Markers were prepared by digesting 50 pmol of the 5′-end labeled 30-mer with 8×10^{-4} units of phosphodiesterase I in 40 mM Tris-HCl (pH 9) and 2 mM $MgCl_2$. The reaction (100 μl) was assembled on ice and incubated at 37 °C. Aliquots (20 μl) were removed at 2.5, 5, 10, 15, and 20 min and stopped by the addition of 80 μl of 100 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 100 μl of phenol/chloroform. After phenol/chloroform extraction, 4-μl samples were resolved on a 16% polyacrylamide, 8 M urea denaturing gel to determine the progress of the reaction at each time point. Typically, the 2.5 and 5 min time points were pooled for use as markers. The DNA was extracted 3 times with phenol/chloroform, precipitated with ethanol, and resuspended in 40 μl of 10 mM Tris-HCl (pH 9.0), 1 mM EDTA.

Label Transfer Reactions—The reaction mixtures were the same as for the cleavage reactions using 2 pmol of 3′-end labeled 30-mer and either 500 ng or 2 μg of TraIp. Incubation was for 20 min at 37 °C, and the reactions were stopped by the addition of an equal volume of 2 × gel loading buffer (100 mM Tris-Cl (pH 6.8), 200 mM dithiothreitol, 4% SDS, 0.2% bromphenol blue, and 20% glycerol). The samples were denatured by boiling for 3 min prior to loading on a 9% SDS-polyacrylamide gel. Electrophoresis was at 100 V (constant voltage), until the bromphenol blue reached the bottom of the gel. The gel was fixed and stained with Comassie Blue. After destaining, the gel was dried and exposed to x-ray film.

Oligonucleotide Recombination Reactions—Reaction mixtures were the same as for the cleavage reaction, except that 1 pmol of a second unlabeled oligonucleotide was added. The specific second oligonucleotide used is noted in appropriate figure legends. When 5′-end labeled oligonucleotides were used, the reactions were terminated as described above for the cleavage reactions. When 3′-end labeled oligonucleotides were used, the reactions were stopped by the addition of SDS to 0.1% and 0.2 mg/ml proteinase K. Incubation was continued for 20 min at 37 °C. The products were resolved on a 16% polyacrylamide, 8 M urea denaturing gel. Electrophoresis conditions were as described above for the cleavage reactions.

To test the effect of protein denaturants on the cleavage/ligation reaction catalyzed by TraIp, the recombination assay reaction mixtures (using 5′-end labeled DNA) were increased to 51 μl. Upon completion of the 2-h incubation, each reaction was divided into three aliquots. One set of reactions was treated as described above for the cleavage reactions. One set of reactions was heated at 65 °C for 20 min prior to loading on the gel, and one set of reactions was directly loaded on the gel. All of the samples were resolved on a 16% polyacrylamide, 25% glycerol native gel. Electrophoresis was at 2.5 W for 8 h in 0.5 × TBE running buffer.

RESULTS

Cleavage of ssDNA Oligonucleotides by TraIp—Previous studies have shown that TraIp catalyzes a site- and strand-specific nicking reaction *in vitro* at *oriT* (15, 16). This reaction depends on the presence of $MgCl_2$ and a supercoiled DNA substrate. To determine if TraIp could cleave a ssDNA substrate in a manner similar to the site- and strand-specific nicking of supercoiled DNA, two ssDNA oligonucleotides, selected from a region within *oriT* that contained the *nic* site, were constructed (Table I, 30- and 22-mer). The 30-mer oligonucleotide contained the *nic* site after the 20th nucleotide, and the 22-mer oligonucleotide contained the *nic* site after the 14th nucleotide. The 30- and 22-mer were each radioactively labeled at their 5′-ends and incubated with an equimolar amount of purified TraIp. The result of this experiment is shown in Fig. 1. After incubation with TraIp, cleavage products were observed comigrating with the marker at 20 nucleotides for the 5′-end labeled 30-mer (Fig. 1*A*, *lane 2*) and at 14 nucleotides for the 5′-end labeled 22-mer (Fig. 1*A*, *lane 5*). In both cases the primary cleavage product observed indicated that cleavage occurred at the *nic* site. In addition, the reaction was dependent on the presence of $MgCl_2$ (Fig. 1*A*, *lanes 3* and *6*).

In addition to the expected cleavage products, other ssDNA cleavage products were also apparent. In the case of the 30-mer, there was cleavage between the 18th and 19th nucleotide to produce a 5′-end labeled product 18 nucleotides in length and cleavage between the 12th and 13th nucleotides to produce a 12-nucleotide product (Fig. 1*A*, *lane 2*). In the case of the 22-mer, there was cleavage between the 12th and 13th nucleotide

[1] The abbreviation used is: ssDNA, single-stranded DNA.

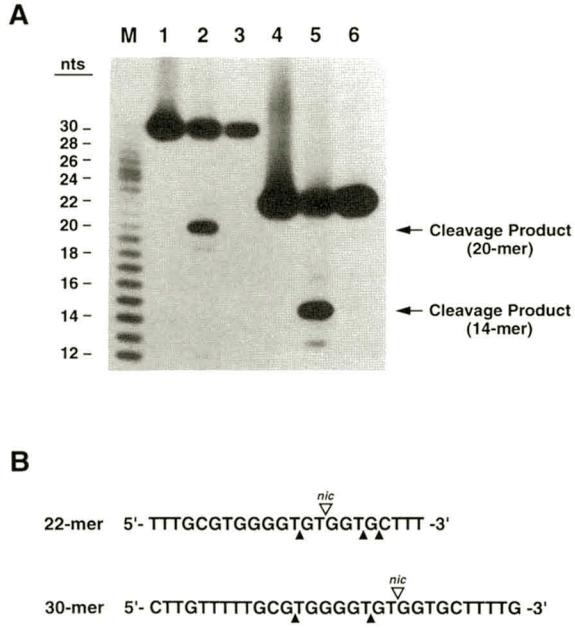

FIG. 1. **Cleavage of single-stranded oligonucleotides by TraIp.** *A*, cleavage reaction mixtures containing 5′-end labeled oligonucleotides (1 pmol) (30-mer, *lanes 1–3* and 22-mer, *lanes 4–6*) were as described under "Experimental Procedures." *Lanes 1* and *4*, no protein; *lanes 2* and *5*, 400 ng (2 pmol) of TraIp; *lanes 3* and *6*, 400 ng (2 pmol) of TraIp, no $MgCl_2$, and 5 mM EDTA. Markers were prepared as described under "Experimental Procedures." Cleavage products corresponding to phosphodiester bond scission at the *nic* site are indicated at the *right*. *B*, schematic of the oligonucleotide sequences showing the *nic* site (▽) and additional secondary sites (▲) cleaved by TraIp.

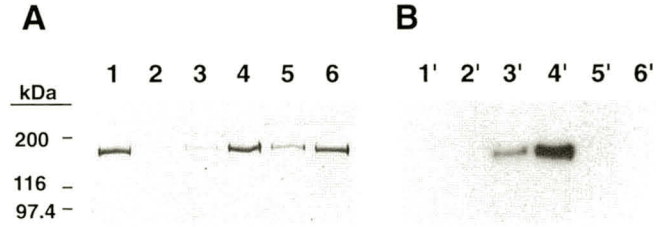

FIG. 2. **TraIp becomes covalently bound to the 5′-end of the cleaved oligonucleotide.** *A*, cleavage reaction mixtures contained either the 3′-end labeled 31-mer (2 pmol) (*lanes 2–4*) or the 5′-end labeled 30-mer (2 pmol) (*lanes 5–6*) and TraIp as described under "Experimental Procedures." *Lane 2*, no TraIp; *lane 3*, 0.5 μg of TraIp; *lane 4*, 2 μg of TraIp; *lane 5*, 0.5 μg of TraIp, *lane 6*, 2 μg of TraIp. All reactions were stopped with the addition of an equal volume of 2 × gel loading buffer. The samples were then boiled for 3 min prior to loading on a 9% SDS-polyacrylamide gel. *B*, autoradiograph of dried SDS-polyacrylamide gel from *panel A*. *Lanes 1′-6′* of *panel B* correspond to *lanes 1–6* of *panel A*.

to generate a 12-nucleotide product, cleavage between the 17th and 18th nucleotide to produce a labeled product 17 nucleotides in length, and cleavage between the 18th and 19th nucleotide to produce a fragment 18 nucleotides in length (Fig. 1*A*, *lane 5*). Cleavage by TraIp at these sites occurred at a much lower frequency than cleavage at the *nic* site. Inspection of the sequences of the oligonucleotides used in this experiment revealed that the 12- and 17-mers formed in the 22-mer cleavage reaction and the 18-mer formed in the 30-mer cleavage reaction lie just 3′ of a GT dinucleotide (Fig. 1*B*). The site cleaved in the 22-mer to generate the 18-mer lies just 3′ of a TG dinucleotide, and the site cleaved in the 30-mer to generate the labeled 12-mer secondary product lies just 3′ of a CG dinucleotide (Fig. 1*B*). The cleaved phosphodiester bond at the *nic* site is just 3′ of a GT dinucleotide. It seems likely that these secondary sites are cleaved under our *in vitro* conditions due to their similarity with the *nic* site and/or due to the relaxed interaction of TraIp with short single-stranded substrates. Experiments measuring reaction kinetics have shown that these sites are cleaved more slowly by TraIp than is the *nic* site (data not shown).

TraIp Is Covalently Attached to the 5′-End of the Cleavage Site—TraIp becomes covalently bound on the 5′-end of the nicked DNA strand upon scission of the phosphodiester bond at the *nic* site on a supercoiled DNA substrate (17). To determine if TraIp would function in a similar manner with regard to cleavage of ssDNA substrates, a label transfer experiment was performed. The 30-mer was labeled, either on the 5′-end using T4 polynucleotide kinase or on the 3′-end using terminal deoxynucleotidyl transferase and [α-^{32}P]ddATP. In the latter case, the length of the oligonucleotide has been extended by one base. The labeled oligonucleotides were incubated separately with TraIp, and the samples were analyzed by polyacrylamide gel electrophoresis in the presence of SDS. As expected, purified TraIp migrated as a single polypeptide with an apparent M_r = 190,000 (Fig. 2*A*, *lanes 1*, *3-6*). The polyacrylamide gel was subsequently exposed to x-ray film to visualize labeling of the protein by the covalently bound DNA (Fig. 2*B*). When TraIp was incubated with the 3′-end labeled 31-mer, transfer of the radioactive label to the protein was observed (Fig. 2*B*, *lanes* 3′ and *4′*). Incubation of TraIp with the 5′-end labeled DNA did not result in transfer of the radioactive label to the protein (Fig. 2*B*, *lanes 5′* and *6′*). This confirms the covalent attachment of TraIp to the DNA upon cleavage of the phosphodiester bond, as these samples were boiled in SDS prior to being resolved on the polyacrylamide gel. Moreover, the covalent linkage must be to the 5′-end of the cleavage site since 3′-end labeled DNA is covalently bound to TraIp while 5′-end labeled DNA is not. Thus, upon cleaving the 30-mer oligonucleotide, TraIp releases the 20-nucleotide fragment and remains covalently bound on the 5′-end of the 10-nucleotide fragment that lies 3′ to the *nic* site.

TraIp Catalyzes a Cleavage/Ligation Reaction—To investigate the possibility of a cleavage/ligation activity intrinsic to TraIp, an intermolecular oligonucleotide recombination assay was devised (Fig. 3*A*). TraIp was incubated with both the 30-mer and the 22-mer; one of the oligonucleotides was 5′-end labeled. If TraIp catalyzes a cleavage/ligation reaction, then specific recombinant molecules of novel size should be formed from intermolecular rearrangements between the two oligonucleotides cleaved by TraIp. When the labeled oligonucleotide is the 30-mer, four ligation products can be envisioned. The 10-mer cleavage product, with TraIp covalently bound on the 5′-end, could be ligated to the 20-mer cleavage product to regenerate the starting substrate. Alternatively, it could be ligated to the unlabeled 14-mer produced by cleavage of the 22-mer. The latter event would not be observed since the 22-mer was not 5′-end labeled. Similarly, the 8-mer cleavage product, with TraIp covalently bound on the 5′-end, could be ligated to the 14-mer to regenerate the 22-mer. As before, this event would not be observed. However, the 8-mer could be ligated to the 5′-end labeled 20-mer that results from TraIp-catalyzed cleavage of the labeled 30-mer. This would generate a 5′-end labeled 28-mer resulting from an intermolecular recombination event promoted by TraIp. When the 22-mer is 5′-end labeled and the 30-mer is unlabeled, the predicted intermolecular recombinant molecule should be 24 nucleotides in length.

Fig. 3*B* shows the results from such an experiment performed using a labeled 30-mer and an unlabeled 22-mer (*lanes 1-4*) or a labeled 22-mer and an unlabeled 30-mer (*lanes 5-8*). The labeled oligonucleotide was incubated with TraIp in the absence of the unlabeled oligonucleotide to demonstrate cleavage at the *nic* site. As expected, the 30-mer was cleaved to yield a 5′-end labeled 20-mer (*lane 2*), and the 22-mer was cleaved to produce a 5′-end labeled 14-mer (*lane 6*). When the second,

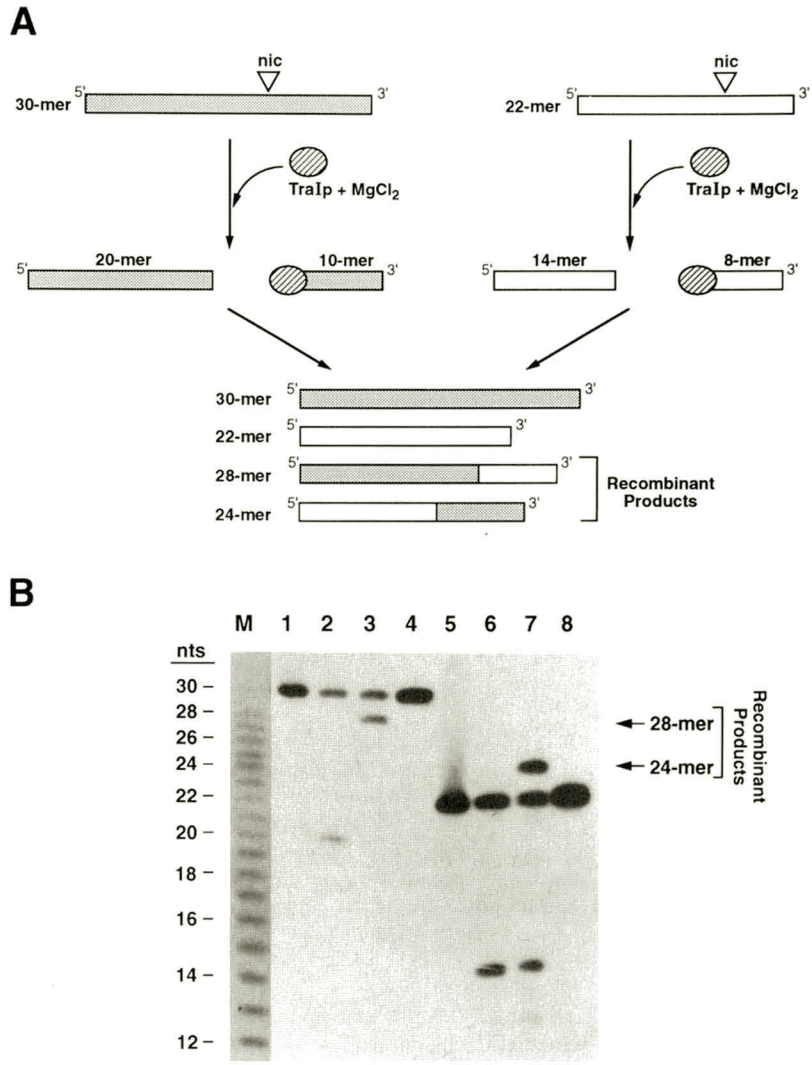

FIG. 3. **Site-specific oligonucleotide recombination catalyzed by TraIp.** *A*, schematic to illustrate the methodology employed to investigate the cleavage/ligation reaction catalyzed by TraIp. *B*, TraIp was incubated with 5′-end labeled oligonucleotides (1 pmol) (30-mer, *lanes 1–4* and 22-mer, *lanes 5–8*) as described under "Experimental Procedures." *Lanes 1* and *5*, no protein; *lanes 2* and *6*, 400 ng (2 pmol) of TraIp; *lane 3*, 400 ng (2 pmol) of TraIp and 1 pmol of unlabeled 22-mer; *lane 4*, 400 ng (2 pmol) of TraIp, no $MgCl_2$, 5 mM EDTA, and 1 pmol of unlabeled 22-mer; *lane 7*, 400 ng (2 pmol) of TraIp and 1 pmol of unlabeled 30-mer; *lane 8*, 400 ng (2 pmol) of TraIp, no $MgCl_2$, 5 mM EDTA, and 1 pmol of unlabeled 30-mer. Reactions were stopped with the addition of SDS to 0.1%, and incubation was continued at 37 °C for 10 min. The positions of the recombinant products are indicated at the *right*.

unlabeled oligonucleotide was added and incubated with TraIp, the predicted recombinant products were observed (28-mer, *lane 3* or 24-mer, *lane 7*). The formation of recombinant products was dependent on the presence of $MgCl_2$ in the reaction (Fig. 3*B*, *lanes 4* and *8*). In each case, the intermolecular recombinant products were formed at a high frequency, indicating that TraIp does indeed catalyze an efficient cleavage/ligation reaction.

To insure that the cleavage/ligation reaction observed was not a secondary effect due to the presence of denaturants, the following experiment was performed. Large scale oligonucleotide recombination reactions were incubated for 2 h at 37 °C using the 5′-end labeled 30-mer and the unlabeled 22-mer. Each reaction mixture was then divided into three aliquots. One set of reaction tubes was treated with 0.1% SDS as described previously, one set of reaction tubes received no further treatment, and one set of reaction tubes was incubated at 65 °C for 20 min. All of the samples were then resolved on a native polyacrylamide gel as described under "Experimental Procedures." In each case, the cleavage/ligation reaction proceeded as before (data not shown). Thus, the cleavage/ligation reaction proceeds in the absence of any denaturing treatment, and therefore cannot be a secondary effect of protein denaturation.

The Cleavage/Ligation Reaction Catalyzed by TraIp Is Sequence Specific—In an effort to begin to define the 3′-end sequence requirements for TraIp-mediated ligation, oligonucleotide recombination assays were performed using the 3′-end labeled 23-mer and unlabeled oligonucleotides that could not be cleaved by TraIp (see Table I). The 41-mer used in this experiment contains the sequence located immediately to the 5′ side of the site nicked by TraIp at *oriT*. The 17-mer contains a GT dinucleotide at the 3′-end, as does the 41-mer, but is otherwise unrelated to the *nic* site at *oriT*. The 25-mer contains a TT dinucleotide at its 3′-end and is also otherwise unrelated to the *nic* site at *oriT*.

We reasoned that if TraIp recognized a specific 3′-end sequence prior to catalyzing the ligation of the two DNA strands then after cleavage of the 23-mer, the protein covalently bound on the 5′-end of the 9-mer should be able to ligate to the 41-mer to form a 50-mer. On the other hand, the protein should not be able to ligate to the 17-mer to form a 26-mer or to the 25-mer to form a 34-mer since the sequences on the 3′-end of these oligonucleotides were unrelated to *oriT*. This was in fact the case, as shown in Fig. 4. TraIp accurately cleaved the 3′-end labeled 23-mer to produce an unlabeled 14-mer, and a labeled 9-mer covalently bound by TraIp (Fig. 4, *lane 2*). When the unlabeled 30-mer, containing a complete *nic* site, was added to the reaction, there was efficient intermolecular recombination to yield a labeled 29-mer as expected (Fig. 4, *lane 3*). When the unlabeled 41-mer with a 3′-end identical with that of a cleaved *nic* site was added to the reaction we, observed formation of the predicted 50-mer recombinant molecule (Fig. 4, *lane 4*). There was no intermolecular recombination observed when either the 17- or 25-mer were added to the reaction (Fig. 4, *lanes 5* and *6*, respectively). However, in each case there was efficient cleavage of the 23-mer as evidenced by the ladder of DNA fragments

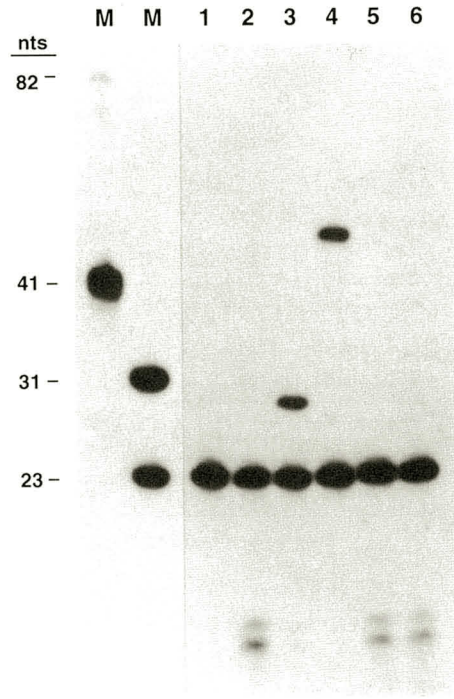

FIG. 4. **The ligation reaction catalyzed by TraIp is sequence specific.** TraIp was incubated with the 3′-end labeled 23-mer (1 pmol) as described under "Experimental Procedures." *Lane 1*, no protein; *lane 2*, 400 ng (2 pmol) of TraIp; *lanes 3–6*, 400 ng (2 pmol) of TraIp and 1 pmol of a second, unlabeled oligonucleotide (either the 30-mer (*lane 3*), the 41-mer (*lane 4*), the 25-mer (*lane 5*), or the 17-mer (*lane 6*). Reactions were stopped by the addition of SDS to 0.1% and 0.2 mg/ml proteinase K, and incubation was continued for 10 min at 37 °C. Markers (*lanes M*) were the 3′-end labeled 23-mer and 31-mer as well as the 5′-end labeled 41-mer and an 82-mer species formed from a ligation of two 5′-end labeled 41-mer oligonucleotides.

migrating at the position of a 16–17-mer corresponding to the 3′-end labeled 9-mer retarded in the gel by the covalent association of one or several amino acids resistant to the proteinase K digestion. We conclude that TraIp recognizes a specific sequence at the 3′-OH end prior to catalyzing a ligation reaction. Moreover, this recognition site must extend beyond the GT dinucleotide sequence present on two of the oligonucleotides used in this experiment.

DISCUSSION

Previous studies from this lab, and others, have demonstrated that the F plasmid-encoded TraIp, also known as DNA helicase I, catalyzes a site- and strand-specific nicking reaction at the *nic* site located within the F plasmid *oriT* (15, 16). Nicking requires a superhelical DNA substrate, and it has been suggested that the single-stranded character present in supercoiled DNA is important for recognition by TraIp (16). We have directly demonstrated that TraIp is able to catalyze the cleavage of 5′-end labeled ssDNA oligonucleotides containing the *nic* site from *oriT*. Cleavage of the ssDNA oligonucleotides occurred rapidly (data not shown) and required the presence of $MgCl_2$ in the reaction mixture. This is consistent with previous results obtained using superhelical DNA substrates containing *oriT* (15, 16). Two ssDNA oligonucleotides of different length were used for these studies and, in each case, the phosphodiester bond scission occurred at the same site that is nicked *in vivo* to initiate conjugative DNA strand transfer (8).

While cleavage occurred primarily at the *nic* site on each ssDNA oligonucleotide, there was detectable cleavage at other sites. Kinetic studies showed that formation of products from these sites occurred at a lower rate than did formation of products from cleavage at the *nic* site (data not shown). We have also been able to alter cleavage specificity by changing the salt concentration in the reaction. Higher, more physiological salt concentrations (150–200 mM) favor cleavage at the *nic* site as do extremely low concentrations of salt (10 mM), like those used in our assays. At intermediate salt concentrations, the *nic* site and secondary sites are cleaved with equal efficiency (data not shown). In addition, we have not been able to detect any recombinant products resulting from TraIp cleavage at the secondary sites. So, while these products are formed in the cleavage reaction, we suggest that they are a consequence of the conditions of the experiment and may not have any biological relevance. Inspection of the DNA sequence surrounding several of these secondary cleavage sites revealed that they contained a 5′-GT-3′ dinucleotide pair immediately 5′ to the phosphodiester bond that was cleaved. Since the *nic* site also contains a 5′-GT-3′ dinucleotide, it is likely that cleavage at the secondary sites is due to a relaxed interaction between TraIp and the ssDNA oligonucleotide under our *in vitro* conditions.

Cleavage reactions were also performed using ssDNA oligonucleotides labeled at the 3′-end (data not shown). In this case, the labeled cleavage product migrated as a series of bands with retarded mobility on a polyacrylamide gel (see Fig. 4, *lanes 2–6*). This result was expected since previous studies (17) have shown that TraIp covalently binds the 5′-end of the cleaved strand as it catalyzes phosphodiester bond scission. Thus the 3′-end labeled cleavage product, after proteinase K digestion, would be expected to have one or more amino acids covalently bound on the 5′-end of the DNA strand.

To directly demonstrate the covalent attachment of TraIp to the 5′-end of the cleavage site, a label transfer experiment was performed. The ^{32}P label on the DNA was efficiently transferred to TraIp in a linkage that is apparently covalent since it is resistant to boiling in the presence of SDS. Moreover, transfer of ^{32}P label from DNA to protein was observed only when a 3′-end labeled ssDNA oligonucleotide was used. Thus TraIp must covalently bind the 5′-side of the cleaved phosphodiester bond. This confirms previous results obtained using superhelical plasmid DNA (17).

The data from these experiments also further our understanding of the DNA structure that TraIp prefers. It has been suggested that TraIp acts on DNA with single-stranded character that is produced by the supercoiling of the F plasmid. The data presented here show that TraIp can cleave ssDNA and that this reaction has biochemical properties identical with the reaction observed when supercoiled dsDNA is used as the substrate.

In addition to the ssDNA cleavage activity intrinsic to TraIp, we have directly demonstrated that the protein-DNA intermediate is able to mediate a ligation reaction when provided with an appropriate 3′-OH end (see Fig. 3*A*). Thus, TraIp catalyzes both a site- and strand-specific cleavage reaction and a site-specific ligation reaction. This reaction is analogous to the cleavage/ligation reaction catalyzed by the ϕX174 CisA protein (21), and a similar reaction has been demonstrated for the nicking proteins encoded by other transmissible plasmids (18, 19). This mechanism for endonucleolytic cleavage of the DNA followed by ligation of the transferred strand by TraIp-like proteins seems to be highly conserved among transmissible plasmids. Indeed, this conservation extends to the mechanism of transfer used by the Ti plasmid to transfer from *Agrobacterium tumefaciens* to plant cells (22). The significant difference in the F plasmid system is the existence of the cleavage/ligation activity on the same polypeptide as the helicase activity, which is presumably responsible for driving strand transfer. The other nicking enzymes that have been characterized do not

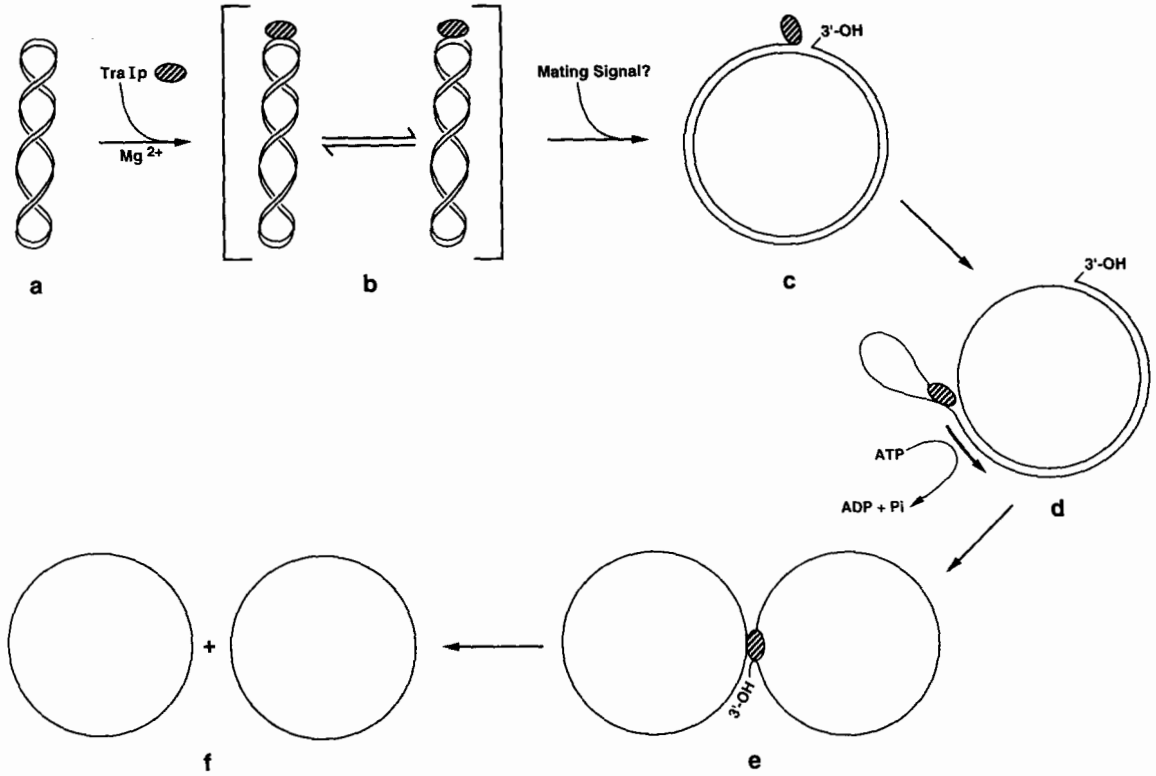

FIG. 5. **A model depicting the role of TraIp in the initiation and termination of conjugative DNA strand transfer.** See text for details.

contain a helicase activity. Moreover, the helicases involved in strand transfer in the RP4 system and the Ti plasmid system have yet to be identified.

The site specificity of the ligation reaction was investigated using three different acceptor oligonucleotides in the recombination assay. One of these oligonucleotides corresponded precisely to the sequence found on the 3′-side of the *nic* site. Recombination between this oligonucleotide and a TraIp-bound cleavage product proceeded to essentially the same extent as did recombination between oligonucleotides containing the *nic* site. Recombination between the TraIp-bound cleavage product and oligonucleotides that contained sequences that diverge from those found at the 3′ side of the *nic* site, was not detected. One of these potential acceptor molecules contained a GT dinucleotide on the 3′-end as does the 3′-side of the *nic* site. Since this oligonucleotide failed to recombine with the TraIp-bound cleavage fragment, we conclude that TraIp recognizes a specific 3′ acceptor sequence and the recognition site extends beyond the terminal dinucleotide. These findings are further supported by Gao *et al.* (23) who found that termination of strand transfer requires sequence determinants extending at least 9 nucleotides 5′ of the *nic* site. Experiments are in progress to further define the sequence requirements for TraIp catalyzed ligation. It will be interesting to determine if the site recognized by TraIp for cleavage is identical with the site recognized for ligation.

The studies described above have demonstrated yet another enzymatic activity associated with the F plasmid TraIp. It is now clear that TraIp is a multifunctional protein with multiple roles in the DNA metabolism associated with conjugative DNA strand transfer (Fig. 5). TraIp is a DNA-dependent NTPase and a helicase. Presumably these two coupled activities are involved in catalyzing the unwinding event required to generate the ssDNA that is transferred to the recipient cell (Fig. 5*d*). In addition, TraIp is an endonuclease/ligase capable of introducing a site- and strand-specific nick in the F plasmid *oriT* and then resealing that nick via a ligation reaction. We suggest that the ligation activity has two roles in the strand transfer process *in vivo*. Initially TraIp binds a specific site within *oriT* on a supercoiled plasmid in the presence of $MgCl_2$ (Fig. 5*a*). The bound protein catalyzes a site- and strand-specific nicking reaction within *oriT* that results in covalent attachment of the protein to the 5′-end of the cleaved strand (17). We suggest that the nicked (although not relaxed) species exists in equilibrium with the covalently closed form of the plasmid as a result of the endonuclease/ligation activity of TraIp (Fig. 5*b*). This idea is consistent with the observation that upon isolation of the F plasmid from cells, and subsequent to treatment with agents that disrupt protein structure, both supercoiled and nicked DNA molecules were recovered (Ref. 24; for reveiw, see Ref. 25). For clarity of presentation, the 3′-end of the cleaved strand has been depicted as not associated with the protein. In fact, the 3′-end of the cleaved strand is tightly held by TraIp such that the superhelical density of the nicked and covalently closed forms of the TraIp-bound plasmid are identical (17).[2] Some as yet unidentified protein-protein interaction presumably drives this equilibrium in the direction of the nicked DNA with subsequent relaxation of the plasmid when an appropriate mating signal is received (Fig. 5*c*). Then, the molecule of TraIp covalently bound on the 5′-end of the cleaved strand, acting as a helicase, unwinds the double-stranded DNA to generate ssDNA for transfer (Fig. 5*d*). This reaction requires the concomitant hydrolysis of ATP and could, in fact, be catalyzed by other molecules of helicase I. This remains to be determined. The 3′-OH on the cleaved strand could be utilized in donor-conjugal DNA synthesis, but this is not a requirement since strand transfer is not obligatorily coupled with donor-conjugal DNA synthesis (6). To simplify this model, synthesis from the 3′-OH has not been shown. Finally, the molecule of TraIp covalently bound on the 5′-end of the cleaved strand is poised to ligate the

[2] J. A. Sherman and S. W. Matson, unpublished observations.

two ends of the "transferred" strand as the unwinding reaction is completed (Fig. 5*e*). The result is two ssDNA circles that are complementary to one another (Fig. 5*f*). Thus, TraIp is involved in both the initiation and termination of strand transfer through the catalysis of the cleavage/ligation described here.

Acknowledgments—We thank Dr. Mike Howard, Bill Nelson and Dr. Dan Bean for stimulating discussions and Susan Whitfield for preparation of the artwork.

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