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Concomitant Reconstitution of TraI-catalyzed DNA Transesterase and DNA Helicase Activity *in Vitro**

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TraI protein of plasmid R1 possesses two activities, a DNA transesterase and a highly processive 5′-3′ DNA helicase, which are essential for bacterial conjugation. Regulation of the functional domains of the enzyme is poorly understood. TraI cleaves supercoiled *oriT* DNA with site and strand specificity *in vitro* but fails to initiate unwinding from this site (*nic*). The helicase requires an extended region of adjacent single-stranded DNA to enter the duplex, yet interaction of purified TraI with *oriT* DNA alone or as an integral part of the IncF relaxosome does not melt sufficient duplex to load the helicase. This study aims to gain insights into the controlled initiation of both TraI-catalyzed activities. Linear double-stranded DNA substrates with a central region of sequence heterogeneity were used to trap defined lengths of R1 *oriT* sequence in unwound conformation. Concomitant reconstitution of TraI DNA transesterase and helicase activities was observed. Efficient helicase activity was measured on substrates containing 60 bases of open duplex but not on substrates containing ≤30 bases in open conformation. The additional presence of auxiliary DNA-binding proteins TraY and *Escherichia coli* integration host factor did not stimulate TraI activities on these substrates. This model system offers a novel approach to investigate factors controlling helicase loading and the directionality of DNA unwinding from *nic*.

Conjugative DNA strand transfer is a complex process requiring highly specific protein-protein and protein-DNA interactions. Processing of the plasmid DNA is initiated when the relaxosome, a stable nucleoprotein complex, is formed at the transfer origin (*oriT*). This complex includes a DNA transesterase, also known as relaxase, which cleaves a specific phosphodiester bond, *nic*, within *oriT* and auxiliary DNA-binding proteins (1). After initial cleavage of the T-strand[1] (the DNA strand that is transferred to the recipient cell), the nascent terminus 3′ to *nic* remains covalently bound to a catalytic tyrosine of the protein by a 5′ phosphotyrosyl linkage, whereas the 3′ end is released. This reaction is reversible, and in the absence of transfer, an equilibrium of cleaving and resealing is established. If transfer is initiated, T-DNA is unwound from its complementary strand and transmitted to the recipient bacterial cell in a process requiring the conjugative Type IV secretion machinery. Our current understanding of relaxosome assembly and function is derived from extensive *in vitro* and *in vivo* investigation of several conjugation systems (for reviews, see Refs. 2–4). Comparatively little detail is available for steps in the initiation process after relaxase-catalyzed strand cleavage.

Duplex unwinding from *nic* releases the T-strand in single-stranded form and provides a substrate for translocation by the type IV secretion machinery. A recent report by Cascales and Christie (5) provides the first mechanistic insights into the initial engagement and subsequent progression of a T-DNA substrate via the *Agrobacterium* translocation machinery. T-strand unwinding is, thus, functionally linked to the preinitiation steps executed by the relaxosome and the substrate selection events that deliver the molecule to transporter proteins. Indeed, functional integration of helicases into macromolecular machines is common for helicases engaged in the physiological processes of nucleic acid manipulation and rearrangement *in vivo* (6, 7).

Although the majority of conjugative systems do not specify a DNA helicase activity dedicated to transfer, the IncF and functionally related IncW conjugation systems offer well studied exceptions (8–12). The C-terminal domain of the IncF TraI protein (also known as DNA helicase I of *Escherichia coli* (13)) catalyzes a 5′ to 3′ DNA helicase activity that is essential for conjugative transfer (14, 15). Similarly to other bacterial helicases, the interaction of TraI helicase domain with DNA lacks sequence specificity, and the capacity to initially enter duplex DNA is dependent on auxiliary factors. Assisted loading is commonly required by DNA helicases involved in DNA replication, gene expression, and recombination. Localization of the IncF conjugative helicase to the site of plasmid DNA strand transfer is achieved by its own independently folded N-terminal domain, which exhibits site- and strand-specific DNA binding and transesterase activity (15–17). Fusion of both activities in one polypeptide is expected to streamline regulation and efficiency of initiation of the transfer process (18). Yet in cells carrying derepressed IncF plasmids, relaxosomes are constitutively active for *nic* cleaving-joining in the absence of conjugation without concomitant activation of their intrinsic helicase. Furthermore, IncF relaxosomes reconstituted *in vitro* thus far fail to initiate DNA unwinding from *nic* (19). Thus events or processes that drive the opening of dsDNA around *nic* are still unknown, and mechanistic details about the coordination of TraI activities remain obscure.

To gain insights to regulation of the conjugative helicase

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[1] The abbreviations used are: T-strand, transferred strand; R-strand, retained strand; nt, nucleotides; dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; T-DNA, transferred DNA; DTT, dithiothreitol; hd, heteroduplex; IHF, integration host factor; IR, inverted repeat.

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TABLE I
DNA oligonucleotides used in this study

Homologous and non-homologous sequences are indicated. Numbers designate the length of *oriT* T-strand replaced with a primer pair. Complementary sequences of primer pairs are underlined. for, forward primer; rev, reverse primer.

Name	Non-homologous 5′ extension	Homology to template: *oriT*
60 for	5′-CACAGCCGGATTTTGATAATGTCGCGAACACGCTGATC	GTTGAGCCTTTTTGTGGAGT-3′
60 rev	5′-ACATTATCAAAATCCGGCTGTGTCAGGCACTCCTTCCACAAATC	TTTGCTATTTGAATCATTAACTTAT-3′
30 for	5′-GATAATGTCGCGAACACGCTGATC	GAGAACCACCAACCTGTTGA-3′
30 rev	5′-CAGCGTGTTCGCGACATTATCAAAATC	ACAAGTTTTTGCTGATTTGCTA-3′
21 for	5′-GATAATGTCGCGAACACGCT	GTGGTGAGAACCACCAACC-3′
21 rev	5′-GCGTGTTCGCGACATTATCA	CAAAAACAAGTTTTTGCTGAT-3′
oriT-ClaI	5′-ACCTGCAG	ATCGATGAATCGAATCGTG-3′
Name	Non-homologous 5′ extension	Homology to template: vector
067		5′-GCGGATAACAATTTCACACAGG-3′
pBST-std		5′-GCGGGCCTCTTCGCTATTACG-3′
pBSR-std		5′-CCTGCGTTATCCCCTGATTCTGTG-3′

TraI, we are studying how the enzyme is loaded at *oriT*. The requirement for ssDNA 5′ to the duplex junction for helicase activity was discovered early (20) but not further defined. Recently we reconstituted TraI ssDNA binding, 5′ to 3′ translocation, and duplex unwinding activity on linear duplex substrates containing a central gap of ssDNA and demonstrated that TraI requires ≥27 nt of ssDNA 5′ to the duplex junction to initiate unwinding efficiently (21). These data imply that localized melting of approximately three helical turns surrounding *nic* is required during conjugative transfer to load the helicase. In the present study we test this model with a subsequent series of substrates specific for *oriT*. Linear dsDNA molecules with a central region of sequence heterogeneity were used to trap defined lengths of R1 *oriT* sequence in unwound conformation. Use of these substrates models putative intermediates of the initiation process and enables us to reconstitute for the first time *in vitro* both *nic* cleavage and unwinding of the adjacent duplex.

EXPERIMENTAL PROCEDURES

Enzymes—Restriction endonucleases were provided by Takara, S1 nuclease, and T4 polynucleotide kinase from Roche Applied Science. The thermostable DNA polymerase used in all applications was Dynazyme II (Finnzymes). *E. coli* ssDNA-binding protein was purchased from Sigma. TraI protein of plasmid R1 was purified as described previously (21). *E. coli* integration host factor (IHF) was a gift of Dr. Peter Dröge (University of Cologne). The expression construction for TraY protein (kindly provided by Dr. Joel F. Schildbach, Johns Hopkins University) carried the 227-bp wild type *traY* gene of plasmid R1*drd*19 (GenBank™ accession number M19710) under the control of the P_{lac} promoter in vector pET21 (Novagen). Cultures (600 ml) of *E. coli* BL21 DE3[codon plus] [pET21cTraY] were grown at 37 °C in 2×TY medium (22) supplemented with 10 μg/ml tetracycline, 20 μg/ml chloramphenicol, and 100 mg/ml ampicillin. Isopropyl-1-thio-α-D-galactopyranoside was added to a concentration of 1 mM after the culture density reached A_{600} = 0.5. Shaking of cells was continued at 30 °C for 3 h. Cells were harvested by centrifugation and washed in 0.1 volumes 20 mM Tris/HCl (pH 7.5). After harvesting again by centrifugation, the cells were frozen at −20 °C. Frozen cells were thawed overnight at 4 °C and resuspended in 7 ml of buffer containing 50 mM Tris/HCl (pH 7.6), 50 mM NaCl, and 5% (v/v) glycerol per gram of cells. Cell lysis was achieved by adding 3.5 mg lysozyme per gram of harvested cells and 0.1 mM phenylmethylsulfonyl fluoride. After 1 h at 0 °C the lysis mixture was centrifuged at 100,000 × *g* for 90 min at 4 °C. This crude cell extract (Fraction I) was cleared through filtration and loaded on a Amersham Biosciences HiTrap™ heparin-Sepharose column. Proteins were eluted with a 0.05–1.2 M gradient of NaCl using buffer B (50 mM Tris/HCl (pH 7.6), 5% glycerol (v/v), 0.1 mM EDTA, 1 mM DTT, 1.2 M NaCl). TraY eluted at 220 mM NaCl (Fraction II). TraY-containing fractions were pooled, and the mixture was directly applied to a Amersham Biosciences HiTrap™ Blue HP column equilibrated with buffer C (50 mM Tris/HCl (pH 7.6), 5% glycerol (v/v), 0.1 mM EDTA, 1 mM DTT, and 220 mM NaCl). The column was developed with a gradient of 0.22–4 M NaCl in buffer D (50 mM Tris/HCl (pH 7.6), 5% glycerol (v/v), 0.1 mM EDTA, and 1 mM DTT). The TraY protein eluted at ~3 M NaCl (Fraction III). Peak fractions were pooled, dialyzed against buffer A (50 mM Tris/HCl (pH 7.6), 5% glycerol (v/v), 0.1 mM EDTA, 1 mM DTT, 50 mM NaCl), concentrated against PEG 20000, supplemented with glycerol to a final concentration of 40% (v/v), and stored at −20 °C (Fraction IV). This fraction was greater than 99% pure TraY protein based on Coomassie-stained polyacrylamide denaturing gels (data not shown). The purified protein exhibited an apparent molecular mass of 14 kDa. Typically, 9–10 mg of purified protein per liter of cultured cells were obtained. Protein concentration was estimated by absorbance at 280 nm using a theoretical extinction coefficient of 2560 M^{-1} cm^{-1}, calculated by the PEPTIDESORT program of the GCG software package.

Plasmid Constructions—DNA oligonucleotides are listed in Table I. The *oriT* region of plasmid R1 extending from the BglII site at position 1901 to the ClaI site at 2309 (numbering according to Graus *et al*. (23)) was amplified by PCR from pMM-wt (24) using primer 067, specific for vector sequence proximal to the BglII junction, and primer *oriT*-Cla, complementary to the R1 ClaI sequence plus a PstI 5′ extension. The amplified DNA was digested with EcoRI and PstI, and the resulting 426-bp fragment was ligated to EcoRI/PstI-prepared pBluescript II KS (Stratagene) to create pDE100. pDE110 carried a 388-bp insert in pBluescript II KS, including a 363-bp ClaI/PstI fragment of the R1 *traD* gene (positions 1766–2129, GenBank™ accession number AY684127) plus polylinker extensions on both ends. pDE100 served as substrate and pDE110 as the negative control in the supercoiled nicking assay and were used to create the prototype heteroduplex 426.

Templates for generating additional heteroduplex substrates (60, 30, and 21) were constructed where defined segments of both *oriT* strands were replaced with non-homologous spacer fragments of equivalent length. Replacement of *oriT* DNA was achieved in two steps. First one-half of *oriT* and the adjacent vector sequence was amplified by PCR from pDE100. In an independent reaction the alternate *oriT* half and vector arm was amplified. Both products were reisolated. The pairs of *oriT* primers in each case contained *oriT* sequence at their 3′ end and 5′ extensions of heterogeneous sequences of variable lengths (Table I). Each 5′ extension determined the length and content of the non-homologous spacer fragment. The identity of *oriT* sequence in each oligonucleotide determined the position where sequence replacement ended (and native *oriT* sequence continued) in the final constructions. For a given replacement the 5′ extensions of the cognate pair of *oriT* primers were additionally complementary. Therefore, when the two resulting left and right fragments of *oriT* were combined, heat-denatured, and allowed to reanneal, some hybrid molecules formed where the only complementary region was the overlapping terminal non-*oriT* sequence specified by the 5′ extensions of the original primers. One combination of hybrids (the complementary fragments to the original primers) contained 3′-OH termini suitable for extension by DNA polymerase. The "filled in" products were subsequently amplified using the original left and right vector-specific primers. The products were fully duplex fragments of *oriT*, where *nic* and surrounding sequences were replaced by heterogeneous DNA of defined lengths. The products were digested with EcoRI and BamHI, then ligated to prepared pBluescript II KS. DNA sequencing verified the correct recombinant DNAs: pVC21, pVC30, and pVC60.

Preparation of Heteroduplex Substrates—For each heteroduplex substrate the source of *oriT* T-strand was pDE100. The complementary R-strand originated from constructions where specific segments were replaced by heterologous sequences (pDE110, pVC21, pVC30, and pVC60). Each plasmid was digested with SspI, AlfIII, and PvuI over-

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night at 37 °C, and the *oriT*-containing 1059-bp AlfIII/PvuI fragment was gel-purified. Before hybridization, the desired strands were synthesized in independent asymmetric PCR reactions from 20 ng of template DNA with 0.2 μM primer that accumulates the desired single strand, 0.002 μM opposite primer (pBST-std and pBSR-std in Table I), 200 μM concentrations of each dNTP, and 1 unit of Dynazyme in the buffer provided. T-strand was common to every substrate and was radiolabeled with [α-^{32}P]dATP during synthesis. The conditions for its amplification were 94 °C for 3 min, 25 cycles at 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 3 min, and 1 step at 72 °C for 5 min. R-strand synthesis from all templates required an annealing temperature of 64 °C. To prepare heteroduplex substrates 60, 30, and 21 the ssDNA products of the asymmetric PCR reactions were resolved on 1.4% agarose-Tris borate EDTA gels and recovered using Nucleospin® extract columns. Yield and concentration of ssDNA was determined by agarose gel electrophoresis.

To create the heteroduplex substrates, labeled T-strand PCR products were combined with a 3-fold excess of a unique, unlabeled R-strand in 10 mM Tris/HCl (pH 8.5) and 200 mM NaCl and heat-denatured at 94 °C for 5 min. Annealing was achieved in reiterated cycles of decreasing temperature (1 °C over four 25-s increments per cycle) to a final temperature of 16 °C. This hybridization mix was used directly in the standard assay or stored at −20 °C.

Verification of Open Duplex Structure—Reaction mixtures (20 μl) contained 0.35–0.75 ng (54 pM) of intermediate products of the asymmetric PCR or hybridized DNA and 2.5 units (S1 nuclease), 4 units (XhoI), 5 units (NdeI), or 7.5 units (SalI, BamHI) of enzyme. Incubation was at 37 °C for 1 h except for S1 nuclease (30 min). The products were resolved through 1.4% agarose gels in Tris borate-EDTA buffer at 7 V/cm for 2.5 h. Mixtures of dsDNA fragments of known length radioactively end-labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP were used as standards for gel mobility. Radiolabeled DNA species were visualized by autoradiography of the dried gels.

Combined Cleavage/Unwinding Assay—Standard reactions (20 μl) were performed at 37 °C for 20 min in a solution containing 40 mM Tris/HCl (pH 7.5), 4 mM $MgCl_2$, 1 mM DTT, 10% glycerol, 50 μg/ml bovine serum albumin, 1.8 mM ATP, ~0.35–0.75 ng of DNA (54 pM) substrate, and 0–52 nM TraI. For kinetic studies carried out in the presence of 52 nM enzyme, a scaled up (14-fold) reaction mixture was assembled and warmed to 37 °C, and the reaction was started by the addition of protein. Portions (20 μl) were removed at 0.5, 1, 1.5, 2, 3, 4, 5, 7.5, 10, 12.5, 15, and 20 min, and the reactions were stopped by the addition of 0.1 volumes of stop mix (final concentrations of 22.5 mM EDTA, 0.11% SDS, and 0.9 mg/ml proteinase K). Incubation was continued for 20 min at 37 °C. 0.1 volumes of loading dye (40% glycerol, 0.05% bromphenol blue) was added, and the products were resolved on 1.4% agarose gels in Tris borate-EDTA buffer at 7 V/cm for 2.5 h. Radiolabeled DNA was visualized by autoradiography of the dried gels. Data were quantified using ImageQuant software (Amersham Biosciences). The percent unwound and unwound plus nicked product was determined after background correction: % unwound = signal intensity in displaced full-length fragment divided by total substrate signal. % unwound + nicked = signal intensity in nicked product bands divided by total substrate signal.

Cleavage Assay on Supercoiled DNA—Reaction mixtures (20 μl) contained 40 mM Tris/HCl (pH 7.5), 8 mM $MgCl_2$, 15% glycerol, 200 ng of plasmid DNA (5.5 nM), 85 nM TraI, IHF, and TraY as indicated. The reactions proceeded for 20 min at 37 °C and were terminated by the addition of 2 μl of 10 mg/ml proteinase K and 2.5% SDS and then incubated further for 20 min at 37 °C. The entire reaction mixture was loaded onto 1.0% agarose gels containing 0.5 μg/ml ethidium bromide in Tris borate-EDTA buffer, and samples were resolved at 5 V/cm for 1.5 h in the presence of 0.1 volumes of loading dye (40% glycerol, 0.05% bromphenol blue).

RESULTS

Preparation and Verification of Substrate Structure—Although *in vitro* the TraI N-terminal relaxase domain cleaves *nic* on supercoiled plasmid DNA, the resulting open circular form is not recognized as a suitable substrate by the C-terminal helicase domain (19, 25). Recently we determined that substrates containing ≥27 nt of ssDNA 5′ to the duplex are unwound efficiently by TraI *in vitro*, whereas substrates containing 20 or fewer nt are not (21). A plausible model for intermediate stages of initiation of transfer in this system, therefore, invokes localized melting of the sequences surrounding *nic* to allow the TraI C-terminal domain to enter the duplex. To reconstitute both nicking and unwinding activities *in vitro* we constructed *oriT*-specific heteroduplex DNA substrates where variable lengths of the sequence surrounding *nic* are present as open (non-complementary) duplexes flanked by two double-stranded arms. The extent of T-DNA present in heteroduplex form was determined by replacing varying lengths of the opposite (R) strand with heterologous sequence. Because the R-strand does not appear to interact with relaxase,[2] altering these sequences should not affect activity. The choice of which T-strand sequence to expose as ssDNA in the heteroduplex constructions took into consideration several prior observations. Studies by Stern and Schildbach (26) using the N-terminal 36-kDa DNA transesterase domain of TraI and a set of oligonucleotides containing overlapping 22-nt *oriT* sequences indicated that the highest affinity interactions between TraI and the target DNA require at least two bases 3′ to *nic* and at least 11 bases 5′ to *nic*. The latter, "core" sequence includes all nt between *nic* and the proximal end of the inverted repeat (IR). Interaction of the related IncW protein TrwC with supercoiled plasmid containing the R388 *oriT* target site induces localized melting at *nic* that begins on the *nic* proximal side of the IR and extends a few base pairs 3′ to *nic* (27). Similarly we verified through $KMnO_4$ footprinting of supercoiled R1 *oriT* DNA that the most distal sequence induced to $KMnO_4$ sensitivity upon binding of full-length TraI was the run of four thymidines immediately 3′ to *nic*.[3] Therefore, substrates were designed where the minimum length of T-DNA present in unwound form begins with the core 11 bases 5′ to *nic* and extends 10 bases beyond to include the T-rich region 3′ to *nic* (Fig. 1). For each subsequent substrate the junctions of open duplex with fully complementary *oriT* duplex were positioned at increasing distances from *nic* and symmetric to that point.

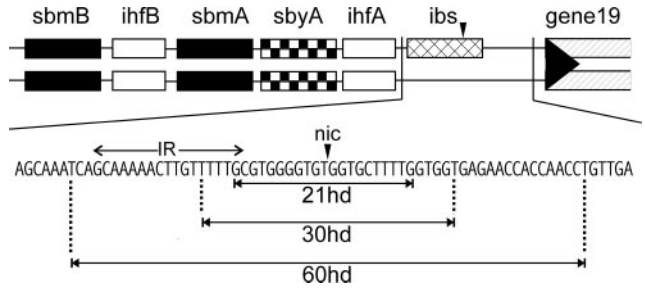

FIG. 1. **Preparation of heteroduplex substrates.** All substrates have the same overall length (1059 bp) and contain the same length of T-strand *oriT* DNA. Each substrate differs in the extent of T-DNA present in heteroduplex form (426–21) by varying the length of a non-complementary spacer DNA in the opposing strand. A general map of the R1 *oriT* sequence contained in every substrate including sites of binding for auxiliary factors IHF (*ihfA*, *ihfB*), TraY (*sbyA*), and TraM (*sbmA*, *sbmB*) is shown. The start of gene 19 (*right*) is outside of *oriT*. Detailed sequence of the T-strand with *nic* (*arrow*) and the IR is shown in the expanded view *below*. The complementary strand is present in every substrate except variable length stretches of heterogeneous sequences centered at *nic*. The nucleotide sequence and extent of T-DNA exposed as ssDNA in the hd substrates is indicated (*21hd*, *30hd*, *60hd*). The sequence for hd 426 is not shown.

The structure of each substrate preparation was verified based on sensitivity to double- and single strand-specific nucleases. To demonstrate the procedure in detail (Fig. 2), a prototype substrate containing an extended heteroduplex region (entire *oriT*) was chosen, since this heteroduplex can be readily resolved from fully duplex fragments by electrophoresis. Sensitivity of the intermediate products of asymmetric PCR (*lane 3*) and the product of their hybridization (*lane 8*) to restriction

[2] J. Schildbach, personal communication.
[3] S. Köstenbauer and E. L. Zechner, unpublished information.

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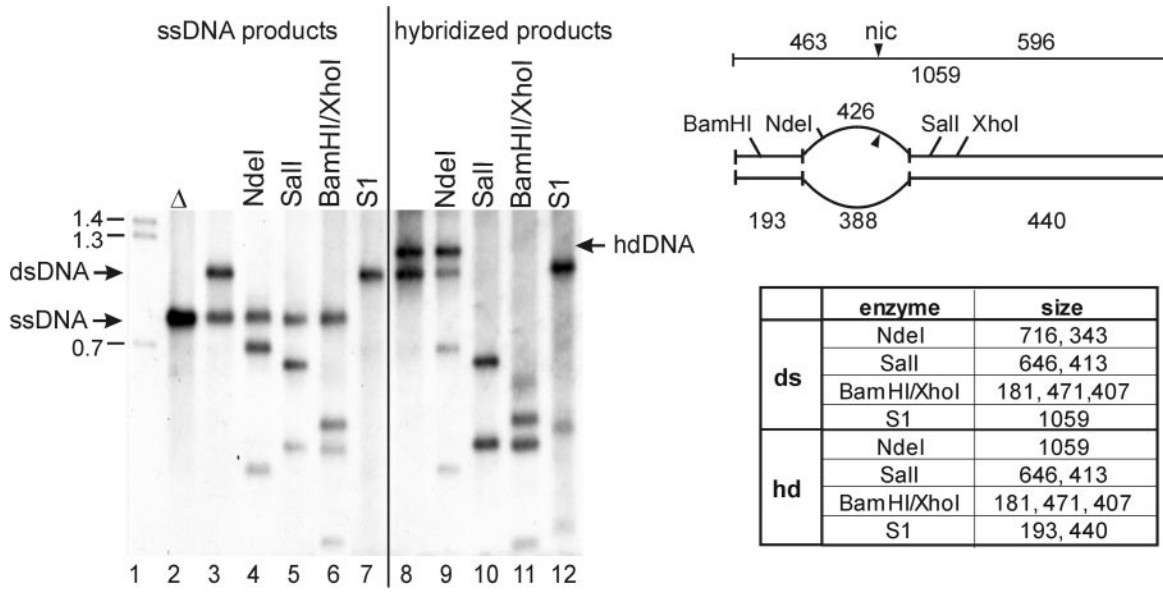

	enzyme	size
ds	NdeI	716, 343
	SalI	646, 413
	BamHI/XhoI	181, 471,407
	S1	1059
hd	NdeI	1059
	SalI	646, 413
	BamHI/XhoI	181, 471, 407
	S1	193, 440

FIG. 2. **Nuclease sensitivity verifies the heteroduplex substrate structure.** An autoradiograph revealing sensitivity of intermediate products (*left*) and the product of their hybridization (*right*) to restriction endonucleases and single strand-specific nuclease S1 is shown. Reaction products are visible as radiolabeled T-DNA in single-stranded (*left*, ssDNA), double-stranded (dsDNA), and heteroduplex form (*right*, hdDNA) as indicated. For comparison, labeled DNA fragments of known size (in kilobases) (*lane 1*) and heat-denatured products (*lane 2*) were applied. A sketch of the relative positions of nuclease sites in the heteroduplex molecule is shown (*upper right*). Sizes of the predicted products of nuclease treatment when the labeled DNA is present as a fully duplex intermediate product or heteroduplex hybridized product are indicated in the *upper* or *lower* portions, respectively, of the *insets* (*lower right*).

endonucleases as well as S1 nuclease was analyzed. After the PCR step a double-stranded byproduct and the desired single-stranded T-DNA species containing *nic* were observed. After hybridization of the single-stranded T-DNA species with unlabeled complementary strand, heteroduplex molecules were identified where *nic* is trapped in open confirmation by flanking duplex arms. Members of this series of heteroduplex (hd) substrates are designated according to the expected length of the T-strand in open conformation.

Nic Cleavage and DNA Unwinding Is Reconstituted on the 426-hd Substrate—The prototype substrate presents the entire *oriT* sequence of the T-strand in open conformation. In this model we predicted that interaction of the full-length TraI protein with the hd DNA would begin with high affinity binding of the N-terminal relaxase domain to the core sequence. *Nic* cleavage might then occur and/or 5′ to 3′ translocation of TraI along the single-stranded T-strand leading to unwinding of the duplex arm downstream of *nic*. Given the extended region of open duplex, TraI was also expected to interact nonspecifically with ssDNA of the opposite strand, translocate along this lattice, and enter and unwind the duplex 5′ to *nic*. Treatment of reaction products with SDS and proteinase K before loading on non-denaturing agarose gels was done to ensure that the products of both nicking and unwinding could be distinguished from unwinding alone.

The affect of increasing concentrations of TraI protein on the 426-hd substrate was investigated (Fig. 3*A*). Two bands were present in the substrate population (*lane 5*) as shown in the nuclease analysis described above (Fig. 2). The upper band represents the hd molecules, and the lower band contains labeled dsDNA present in the unhybridized starting material (Fig. 3*A*, compare *lanes 3*, *4*, and *5*). In the presence of increasing concentrations of TraI, the abundance of the dsDNA species remained constant, whereas the intensity of the hd band was reduced to background levels (*lanes 6–10*). A new band exhibiting a similar migration as the single-stranded T-DNA present in the unhybridized PCR products (*lane 3*) and heat-denatured substrate (*lane 2*) emerged (*lanes 6–10*). At the maximum concentration of TraI the abundance of this latter species appeared to decrease, and two faster migrating species were detected. A similar diminution of single-stranded T-DNA and emergence of two new species with identical migration were observed when 52 nM TraI was incubated with the unhybridized PCR products (*lane 4*). We conclude that TraI acts on the 426 hd to unwind the labeled T-strand, which subsequently migrates as a 1059-nt ssDNA. If the unwound T-strands are additionally cleaved at *nic*, two new single-stranded fragments emerge representing the 596-nt species 3′ to *nic* and the 463-nt species upstream of that site.

Detectable unwinding (~20%) was observed at 0.3 nM, the lowest concentration of TraI (Fig. 3*A*, *lane 6*). Reaction mixtures contain 0.054 nM substrate, thus corresponding to a protein to DNA ratio of roughly 5:1, assuming that all molecules of TraI were active and that the active species is a monomer. For complete unwinding of the T-strand, TraI must bind opposite strands and enter both duplex junctions. Given that there is additionally a 2–3-fold excess of unlabeled ssDNA in each substrate preparation, we estimate that the protein to hd substrate ratio approaches 1:1. These data are in good agreement with reported activities of TraI helicase at the same protein to DNA ratio (21, 28). *Nic* cleavage activity was readily detected (26%) only in the presence of 52 nM protein (*lane 10*). For comparison the cleaving activity of this preparation of purified TraI reached a maximum of ~50% on supercoiled *oriT* plasmids with equimolar amounts of protein and DNA (not shown). Moreover a single-stranded oligonucleotide, 5′-CTTGTTTTTGCGTGGGGTGTΔGGTGCTTTTGG-3′, including the inner arm of the inverted repeat, the core (underlined) and *nic* (Δ) was cleaved with 29% efficiency at protein to DNA ratios of 1:1 and reached a maximum efficiency of 48% with a 12-fold protein excess.

Dependence of the combined nicking/unwinding reactions on a divalent cation cofactor and ATP was assessed (Fig. 3*A*, *lanes 11–13*). Helicase activity required ATP (*lanes 11*, 13), but not Mg^{2+} (*lane 12*), consistent with prior observations (21, 28). Nicking activity required Mg^{2+} (*lane 12*). The DNA transesterase should not be dependent on ATP, but detection of cleaved T-strands on non-denaturing gels requires their prior release from

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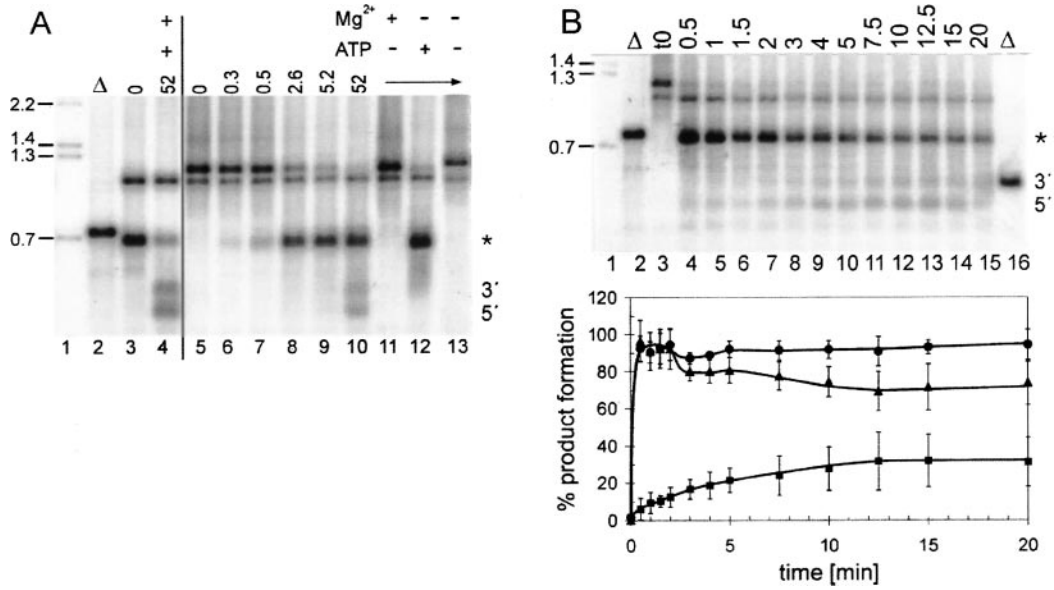

FIG. 3. **Heteroduplex substrates support the *nic* cleavage and DNA unwinding activities of TraI *in vitro*.** *A*, increasing amounts of TraI, as indicated *above* the *lanes* (nM), were incubated in the presence of Mg^{2+} and ATP with the hd substrate in the standard assay, and the products were resolved electrophoretically on native Tris borate-EDTA-agarose gels. Controls lacking cofactors are indicated (*lanes 11–13*). Heat-denatured substrate was applied to *lane 2*. The positions of migration of unwound T-DNA (*asterisk*) as well as unwound, nicked T-strands (5′, 3′) are indicated. The products of reaction mixtures containing non-hybridized PCR product without (*lane 3*) or with 52 nM TraI (*lane 4*) were also resolved (*left*). Labeled dsDNA fragments of known length (kilobases) were applied for reference (*lane 1*). *B*, the kinetics of nicking and unwinding from the 426-hd substrate were determined in the presence of 52 nM TraI under standard assay conditions. Portions of the reaction were removed at the times indicated *above* the *lanes* (min), and catalysis was terminated in ice-cold EDTA. Products of the reactions (*lanes 3–15*), heat-denatured samples of the substrate (Δ, *lane 2*) and a denatured 639-nt DNA standard (Δ, *lane 16*) were resolved electrophoretically. The bands containing unwound T-strand (*asterisk*) as well as 5′ and 3′ ends of unwound, nicked T-DNA (5′, 3′) are indicated. Labeled dsDNA fragments of known length (kilobases) were applied for reference (*lane 1*). Quantitation of TraI activities was depicted graphically as a function of time. The yield of unwound T-strand (▲), unwound and cleaved DNA (■), or the sum of both activities (●) was expressed as percent of total substrate signal converted to product. Values represent the mean of at least four independent experiments using three different preparations of substrate. S.D. are shown.

the hd via the ATP-dependent action of the helicase (*lane 11*).

A time course of these reactions was performed at the maximum TraI concentration (Fig. 3*B*). Quantitative T-strand displacement was observed after 30 s at 37 °C. The cleaving-joining reaction reached a plateau at 30% of the initial substrate cleaved after 7.5 min under the same conditions.

Maximal Helicase and Nicking Activities on Substrates with Shortened Heteroduplex Regions—To create hd DNA substrates that presumably more closely resemble intermediate structures generated at the initiation of transfer *in vivo*, the extent of *oriT* DNA present in open conformation was limited in a stepwise manner to 60, 30, and 21 bases by replacing shorter sequences of complementary R-strand *oriT* with heterologous sequence (Fig. 1). The extent of fully duplex *oriT* was increased as a result. Guasch and coworkers (27) report that the N-terminal relaxase domain of the related R388 TrwC protein forms a tight complex with oligonucleotides derived from *oriT* sequences only when the substrate contains sequences 5′ to *nic* including a six-base IR that is extruded as a cruciform. In our series of shortened hd substrates the inner and outer arms of the IR of plasmid R1 are present in every case either entirely in single-stranded form (hd 60), as a ssDNA with the potential to anneal partially to the complementary sequence (hd 30), or together with the entire complementary strand (hd 21). It is important to note that although we assume that the IR of hd 60 can fold into a hairpin, we do not know whether the T-strand IR of hd 21 and hd 30 are extruded as a hairpin under these conditions *in vitro*.

The relative efficiency of DNA unwinding and *nic* cleavage activities catalyzed by TraI on the series of substrates was compared (Fig. 4). The efficiency of both reactions was dependent on protein concentration and on the length of T-DNA trapped in unwound conformation in the substrates. Total activity, namely, unwinding plus unwinding and cleavage is expressed as a function of protein concentration (Fig. 4*B*). Unwinding of the T-strand from hd 426 was nearly quantitative at the highest protein concentrations. Cleavage was detected on 25% of the unwound strands. T-DNA unwinding was observed from a maximum of 50% of those substrates harboring 60 bases in open conformation at the highest protein concentration. 10% of these T-strands were detectably cleaved. Substrates containing only 30 or 21 bases of open duplex at *nic* supported extremely low levels, 7 and 5%, respectively, of T-strand unwinding and no detectable cleaving activity. These experiments were repeated ($n \geq 5$) using at least two independent substrate preparations.

Thus, although TraI could enter and unwind duplex DNA with moderate efficiency on templates containing 60 bases of open duplex, the protein was essentially inactive on substrates where helicase loading was required from an unwound region of 30 or fewer bases. In a recent study using simpler linear partial duplex substrates where the protein is loaded exclusively on one strand, we determined that 27 nt of ssDNA adjacent to the duplex was sufficient for efficient TraI helicase activity (21). Our current findings suggest, therefore, that one or several factors, *i.e.* a specific aspect of the *oriT* sequence, presence of the high affinity binding site for the relaxase domain, or the additional presence of the opposite strand, which enables bidirectional loading of the helicase, limits the efficiency of initiation and, thus, the overall reaction. The additional presence of *E. coli* ssDNA-binding protein over a concentration range of 0–500 nM did not affect either DNA helicase or transesterase activities of TraI on any of the four hd substrates (not shown).

Reconstitution of the R1 Relaxosome—Helicases typically function as integral parts of protein machineries. During bac-

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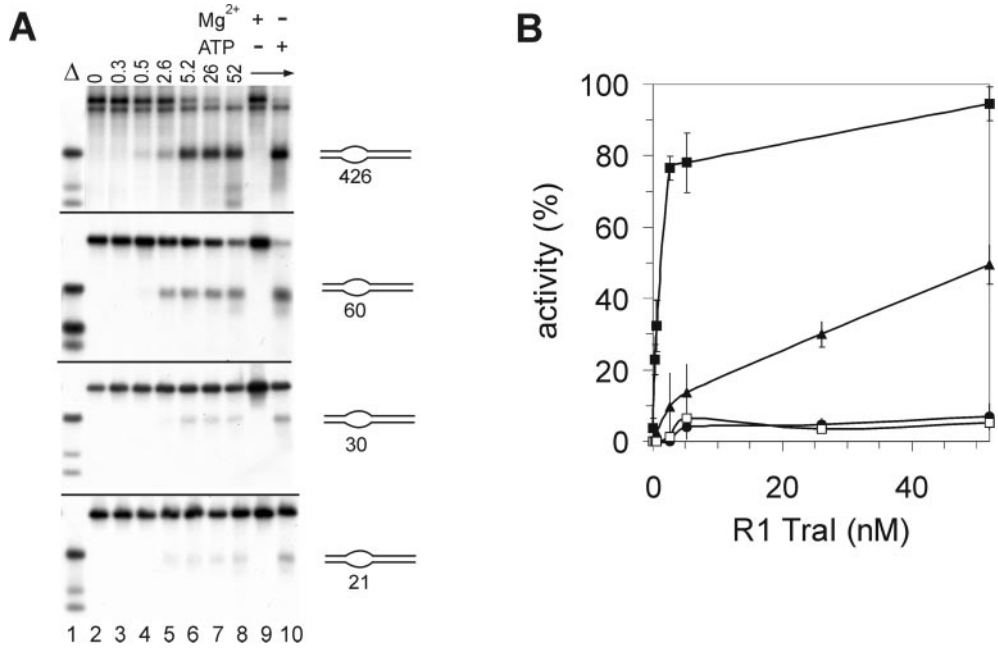

FIG. 4. **The efficiency of TraI *nic* cleavage and duplex unwinding is decreased as the extent of single-stranded heteroduplex DNA is limited.** *A*, the maximal relaxase and helicase activities were determined using four DNA substrates with defined regions of open duplex surrounding *nic*. Each panel shows an autoradiogram (*left*) of a representative gel for substrates containing heteroduplex regions ranging from 426 to 21 nt, as illustrated (*right*). Combined relaxase and helicase assays were performed with increasing amounts of purified TraI protein as indicated *above* the *lanes* (nM). Products of the reactions (*lanes 2–10*) were resolved on native Tris borate-EDTA-agarose gels. Controls lacking cofactors are indicated (*lanes 9* and *10*). Labeled heat-denatured DNA fragments of known length (1059, 639, and 475nt) were applied for reference (*lane 1*). *B*, the efficiency of the site- and strand-specific transesterase and helicase activities (expressed as total percent substrate unwound or unwound and cleaved) was compared on substrates containing heteroduplex regions of variable length 426 (■), 60 (▲), 30 (●), and 21 (□). Values represent the mean of at least five independent experiments using two different preparations of each substrate. S.D. are shown.

terial conjugation, the essential helicase activity of TraI is integrated in the multiprotein relaxosome complex and is present in the relaxosome-coupling protein interface, which is thought to mediate delivery of selected T-strands to the type IV secretion system. Therefore, the additional presence of IncF relaxosome components may be expected to affect TraI relaxase and helicase activities on the hd substrates. *E. coli* IHF and the plasmid-encoded factors TraY and TraM from IncF systems bind to DNA sequence proximal to *nic* and stimulate *nic* cleavage activity of TraI *in vivo* or on supercoiled or linear double-stranded *oriT* DNA *in vitro* (24, 29–33). To be able to investigate the effect of auxiliary relaxosome components on TraI with the hd system, we first purified R1 TraY protein to homogeneity, as described under "Experimental Procedures." We then sought to determine a minimal relaxosome reconstitution for the system defined as those purified proteins that exhibited a stimulatory affect on TraI *nic*-cleaving activity in the supercoiled to open circular assay. Proteins fulfilling those criteria were TraY and IHF. The purified fractions of TraM and TraD proteins tested do bind to DNA *in vitro* (34, 35) but did not stimulate TraI in this assay. A typical experiment showing the stimulatory effects of increasing concentrations of TraY protein, IHF, and the combination of both factors on TraI-catalyzed *nic* cleavage is shown (Fig. 5). Incubation of 85 nM TraI with 5.5 nM supercoiled *oriT*-containing plasmid pDE100 resulted in an ~50% conversion to open circular form, as expected (Fig. 5*A*). Stimulation of the reaction to a maximum of 80% through the addition of increasing concentrations of TraY was observed. Supercoiled plasmid pDE110 lacking *oriT* was not affected by incubation with TraI alone or both TraI and TraY. A similar stimulatory effect was observed when substrates were incubated with TraI and increasing concentrations of IHF (Fig. 5*B*). However, IHF in excess of 125 nM, *i.e.* a 1.5-fold higher concentration than TraI led to an inhibition of the reaction. Generally, in three protein reactions with 85 nM

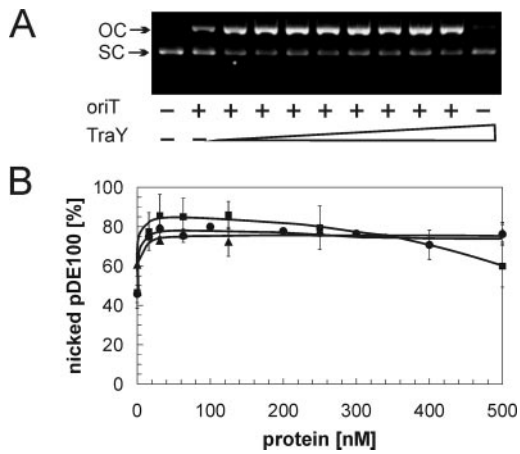

FIG. 5. **TraY and IHF stimulate TraI on supercoiled *oriT* DNA.** *A*, a representative gel-resolving supercoiled plasmid from the open circular form generated by TraI (85 nM) with no or increasing amounts of TraY. The position of migration of the supercoiled DNA substrate (*SC*) and the relaxed open circular DNA product (*OC*) are indicated. A supercoiled plasmid that lacks *oriT* was also incubated with these proteins to demonstrate reaction specificity. *B*, efficiency of site- and strand-specific transesterase activity of TraI as a function of accessory protein concentration is summarized. Reaction mixtures contained 85 nM TraI and increasing concentrations of IHF (■), TraY (●), or TraY (▲) with IHF held constant at 31 nM as indicated. Values represent the mean of at least three independent experiments. S.D. are shown.

TraI, a fixed concentration of one auxiliary factor and variable concentrations of the second enhancement of the reaction was comparable with that observed for a single auxiliary factor (Fig. 5*B*). Earlier work with the R100 system demonstrated a stimulatory function for IHF and the R100 TraY protein in the supercoiled cleavage assay (30). In contrast, a study involving F plasmid proteins did not detect an enhancement of the nick-

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ing reaction by the individual factors TraY and IHF (32). These authors did, however, describe the stimulatory effect of the combination of proteins. In agreement with their accompanying publication (31) we found the order of addition of the reaction components to be crucial. IHF and TraY must be present in the reaction mixture with supercoiled DNA before the addition of TraI to observe an enhancement. The order of subsequent addition of TraY and IHF was irrelevant (not shown).

Effect of IHF and TraY on the Combined nic Cleavage/DNA-unwinding Assay—Given that the R1 TraY protein and IHF increased cleavage activity on supercoiled DNA and that the F proteins are known to stimulate *nic*-cleavage activity also on linear double-stranded *oriT* DNA *in vitro* (32), we chose to assess the effect of these factors on TraI in the combined cleaving/unwinding assay. hd 60 was used, as this DNA supports both TraI-catalyzed activities with moderate efficiency. A series of experiments was performed where the concentration of the protein components was systematically varied, and the effect of their order of addition was compared (not shown). Each variation was repeated in two to four independent experiments. No enhancement of helicase or nicking activity catalyzed by TraI on hd 60 was detected in the additional presence of both auxiliary factors at all concentrations tested, although the same conditions were capable of stimulating TraI in the supercoiled assay (Fig. 5*B*). To be able to detect whether the efficiency of *nic* cleavage was enhanced independently of the DNA unwinding reaction, reaction products were divided in two portions, and half were heat-denatured before their resolution on non-denaturing gels. In all experiments a slight increase in the abundance of nicked product bands was observed when the samples were heat-treated before loading compared with the untreated portions (not shown). This indicates that some of the molecules in the reaction mixture were nicked but not fully unwound. Detection of the total yield of nicked product bands through this procedure revealed that under these conditions, for the combined assay on hd 60 the maximal cleaving activity approaches 15%. However, this slight increase was not altered significantly by the additional presence of the auxiliary factors. These results on hd 60 were consistent for all protein combinations tested, *i.e.* no stimulation of either the unwinding reaction or nicking activity was observed. Similar variations of IHF and TraY concentration in reaction mixtures containing the hd 30 and hd 21 substrates failed to show enhancement of the very low levels of DNA unwinding catalyzed by TraI alone (not shown). An increase in nicking activity was not detected. Thus, although TraY and IHF do stimulate the transesterase activity of TraI in a variety of assays, the additional presence of these auxiliary proteins did not stimulate either the transesterase or helicase activities of TraI in this system.

DISCUSSION

The mechanisms and regulation of the DNA-processing reactions that occur on plasmid molecules during conjugative DNA strand transfer are poorly understood. In this study the T-strand of R1 plasmid *oriT* DNA was trapped in open conformation to model intermediate structures predicted for the initiation stage of the strand transfer process. Use of the hd substrates enabled a concomitant reconstitution of both the TraI-catalyzed DNA transesterase and helicase activities *in vitro*, a precedent in the biochemical investigation of IncF conjugative DNA-processing reactions. To our knowledge this is also the first report of effective loading of the TraI helicase to *oriT*-specific sequences *in vitro*. The system, therefore, offers a promising approach to investigate the mechanisms involved in strand transfer initiation and the potential for coordinate regulation of TraI-catalyzed activities. In this report we describe the reconstituted reactions and evaluate properties of helicase loading on *oriT*-specific sequences partially unwound in defined, variable lengths.

We found that hd substrates with 60 bases of T-DNA in open conformation supported efficient TraI helicase activity. Unwinding from the loading region of sequence heterogeneity was bi-directional. The maximum *nic*-cleavage activity, 15% of the unwound strands, was comparatively poor on these substrates. The lack of significant unwinding on hd molecules possessing 30 or 21 bases of T-DNA in open conformation was not unexpected. We have previously analyzed the capacity of the full-length TraI protein to efficiently enter and unwind duplex DNA via interactions with various lengths of ssDNA 5′ to the duplex junction and found that a tail of ≥27 nt was required for a maximal activity of 60%. Yet substrates in the previous study did not contain *oriT* sequence; therefore, the TraI N-terminal relaxase domain would not mediate high affinity interactions with its specific recognition sequence. Moreover, the gapped linear substrates did not possess a second single strand of DNA accessible for loading the protein to the opposite duplex junction. In contrast the hd substrates employed in the current study present multiple targets for the full-length protein, (i) sequence-specific T-strand for the relaxase domain, (ii) single-stranded T-DNA for helicase loading, and (iii) an equivalent length of non-complementary ssDNA accessible for potential loading to the opposite strand. This complexity apparently diminishes the efficiency of loading compared with the simpler gapped linear duplexes. Little is known about the interactions of the distinct functional domains of TraI with DNA in a heterogeneous population of target- and nonspecific sequences of mixed single-stranded/double-stranded nature. Nevertheless, the diversity of potential DNA-protein interactions reconstituted in this system mirror the situation expected in the cell. It is conceivable that, in accordance with the present findings, TraI *in vivo* requires localized unwinding of over six helical turns to interact properly with the target sequence and enter the adjacent duplex. An alternative explanation may be that an improper orientation of the enzyme or a steric hindrance imposed by bi-directional loading of the protein disrupts the efficiency of initiation. The observed incongruity in *nic*-cleavage efficiency relative to helicase activity supports the latter hypothesis.

Yet, during conjugation TraI indeed differentiates between complementary T- and R-strands, translocates on just one ssDNA lattice, and initiates duplex unwinding from just the appropriate duplex junction. Moreover, in the cell TraI acts as an integral part of a multiprotein relaxosome. It is reasonable to assume that the additional presence of auxiliary DNA-binding proteins in this nucleoprotein complex has important consequences for the mechanisms of initiation and potentially for the coordinate regulation of the distinct domains of TraI. To evaluate the effect of additional relaxosome components on TraI activities in the combined assay we chose to reconstitute the complex with factors that exhibit a stimulatory affect on TraI transesterase activity in other *in vitro* assays including, importantly, *nic* cleavage on linear dsDNA substrates (32). The auxiliary proteins TraY and IHF interact with *oriT* DNA at specific binding sites immediately proximal to the outer arm of the IR (see Fig. 1) (36–38). We predicted that occupation of these sites or interactions between the proteins might affect the combined activity assay by improving nicking efficiency independently of the helicase activity or in a coordinated manner discernable as increased parity between the two reactions. It also seemed reasonable to postulate that the additional presence of TraY and IHF might stimulate unwinding from hd 60 or assist in loading of TraI to the inactive substrates hd 30 and hd

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21, thus facilitating the unwinding reaction on these targets to detectable levels. Despite the broad titration ranges tested for this combination of relaxosome components, their presence did not influence either TraI-catalyzed activity in the combined assay (although they are clearly capable of doing so in other assays). Thus, the architecture of the nucleoprotein complex formed on the hd substrates and/or the contacts between proteins within that reconstituted complex do not result in a detectable effect on TraI performance.

Perhaps the most intriguing question raised by this study is how helicase loading is limited to just one strand during transfer initiation. In the cell the result is unidirectional unwinding relative to *nic*. *In vitro* TraI loading on the opposing single-stranded R-strand and entry of the *nic* proximal duplex arm was clearly not limited by the sequence composition. Nor was duplex entry restricted by occupation of the TraY and IHF binding sites. *In vivo* the IncF relaxosome contains additionally the plasmid-encoded DNA binding protein TraM. When TraY is expressed, this factor may be dispensable for *nic* cleavage but remains essential for a later stage of the strand transfer process (24). Protein TraD establishes specific contact to IncF relaxosomes via interactions with TraM (39, 40). TraD belongs to the family of conjugation proteins that, in the case of R388, has been shown to be anchored in the cytoplasmic membrane (41) in physical interaction with the TrwE (VirB10-like) core component of the secretion system (42). In summary, these interactions are thought to physically couple the relaxosome to the membrane-spanning type IV secretion system. Conceivably this complex association of proteins assembled on the *nic*-proximal site of *oriT* prevent TraI translocation and duplex entry in this direction. Last, the process of conjugation involves replication of the T-DNA in the donor cell to ensure replacement of the exported plasmid strand. Complementary strand synthesis is believed to initiate at the 3′ hydroxyl of *nic* by DNA polymerase III (43, 44). Initiation of DNA replication from this site may additionally block helicase entry from the opposing direction. Reconstitution of TraI-catalyzed DNA unwinding from *oriT*-specific sequences *in vitro* offers a promising approach to address these questions and provides insights to the nature of this regulation.

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Concomitant Reconstitution of TraI-catalyzed DNA Transesterase and DNA Helicase Activity *in Vitro*

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