The IncF plasmid protein TraI functions during bacterial conjugation as a site- and strand-specific DNA transerase and a highly processive 5' to 3' DNA helicase. The N-terminal DNA transerase domain of TraI localizes the protein to nic and cleaves this site within the plasmid transfer origin. In the cell the C-terminal DNA helicase domain of TraI is essential for driving the 5' to 3' unwinding of plasmid DNA from nic to provide the strand destined for transfer. In vitro, however, purified TraI protein cannot enter and unwind nicked plasmid DNA and instead requires a 5' tail of single-stranded DNA at the duplex junction. In this study we evaluate the extent of single-stranded DNA adjacent to the duplex that is required for efficient TraI-catalyzed DNA unwinding in vitro. A series of linear partial duplex DNA substrates containing a central stretch of single-stranded DNA of defined length was created and its structure verified. We found that substrates containing ≥27 nucleotides of single-stranded DNA 5' to the duplex were unwound efficiently by TraI, whereas substrates containing 20 or fewer nucleotides were not. These results imply that during conjugation localized unwinding of >20 nucleotides at nic is necessary to initiate unwind-}

**Extent of Single-stranded DNA Required for Efficient TraI Helicase Activity in Vitro**

Received for publication, September 9, 2003
Published, JBC Papers in Press, September 23, 2003, DOI 10.1074/jbc.M310025200

Vanessa C. Csitkovits and Ellen L. Zechner†

*From the Institut für Molekularbiologie, Biochemie und Mikrobiologie, Karl-Franzens Universität Graz, Universitätsplatz 2, 8010 Graz, Austria*

The conjugative helicases initiate duplex unwinding unidirectionally from the plasmid transfer origin after strand-specific cleavage at nic by their N-terminal relaxase domains. The

---

* This work was financed through a direct grant from the Austrian Bundesministerium für Bildung, Wissenschaft und Kultur, Fonds zur Förderung der Wissenschaftlichen Forschung Projects P13227GEN and P16722-B15, and European Union Grant QLK2-2000-01624. 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.

†To whom correspondence should be addressed. Tel.: 43-316-3805624; Fax: 43-316-3809898; E-mail: ellen.zechner@uni-graz.at.

---

2 The abbreviations used are: Rep, replication initiation proteins; nt, nucleotide(s); dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; MOPS, 3- N-morpholino)propanesulfonic acid; DTT, dithiothreitol.
molecular mechanisms of these early steps of plasmid strand transfer remain poorly understood. Genetic and biochemical approaches have characterized the role of auxiliary factors in assisting the ntc-specific cleavage reaction as well as the contribution of origin DNA sequence and topology (25–27, 42–44). Nonetheless, the product of that reaction, the open circular form, is not directly accessible to the duplex unwinding activity of the physically linked DNA helicase domain. Thus, although recruitment of the conjunctive helicase to oriT is achieved by site-specific recognition of the N-terminal relaxase domain and/or via interaction with additional oriT binding auxiliary proteins, the reaction as reconstituted thus far fails to effectively load the helicase at ntc (45). In the cell, then, the relaxosome probably acquires a higher order structure in a staged initiation process that triggers helicase activation, similar perhaps to the orchestrated unwinding of origin DNA during replication initiation in prokaryotic and eukaryotic cells (46).

**Purified TraI and TrvC proteins do not exhibit sequence specificity for binding and translocating (5 to 3) on ssDNA (29, 47, 48). Reconstitution of the duplex-unwinding activity in vitro requires a region of ssDNA adjacent to the duplex (49). In early work Hoffmann-Berling and co-workers (47) estimated that the length of ssDNA required by TraI on the duplex 5’–3’ requires a region of ssDNA adjacent to the duplex (49). In early work Hoffmann-Berling and co-workers (47) estimated that the length of ssDNA required by TraI on the duplex 5’–3’ requires a region of ssDNA adjacent to the duplex (49).**

**Experimental Procedures**

**Expression and Purification of R1 TraI**—The expression construction pH2 (50) carries the 5.3-kilobase pair traI gene of plasmid R1vd16 (GenBank accession number Y12454) under the control of the P promoter in vector pGZ119HE (51). Cultures (600 ml) of E. coli SCS1[pH2] were grown at 37 °C in 2X TY (16 g/liter Bacto-Tryptone, 10 g/liter Bacto yeast extract, 5 g/liter NaCl) medium (52) supplemented with 0.1% (v/v) glucose, 25 mM MOPS (pH 8.0), 25 mM thiamine/HCl, and 10 μg/ml chloramphenicol. At an A_{600} of 0.5, isopropyl-1-thio-d-3-

---

**Table I**

| Table I | Oligonucleotides used in this study |
|---------|----------------------------------|
| A for | 5’-CGGGCGCTTTCGCTATTAGG-3’ |
| A rev | 5’-CCTGCCATTACCTGGATTTCT-3’ |
| B | 5’-CAATTGCCCTATAGTATGGC-3’ |
| 12ss | 5’-CCCCTGAGGATGCAGGCGG-3’ |
| 16ss | 5’-TGGAGTCGACGATGATCGG-3’ |
| 20ss | 5’-GGACCGTACGTAGCTG-3’ |
| 27ss | 5’-GGATGATACTTACGATG-3’ |
| 30ss | 5’-ATCGTAAAGCTCTGATCAATTTCC-3’ |
| 40ss | 5’-TTGATATGGATCTCTGGAG-3’ |
| 67ss | 5’-GATCAGGTGTCTCTAGAAGGG-3’ |

---

**Table II**

| Table II | Purification of R1TraI |
|---------|-----------------------|
| ND, not determined. | |

**Verification of Partial Duplex Structure**—Typical nuclear protein based on Coomassie-stained polyacrylamide-denaturing gels. Protein concentration was determined using the Bradford protein assay (Bio-Rad) with bovine serum albumin as standard.

**ATPase Assay**—The γ-phosphohydrolase activity of partially purified protein fractions was determined in a 25-μl reaction mixture containing 25 fmol of circular single-stranded DNA donor, 25 mM Tris/HCl (pH 7.5), 20 mM NaCl, 3 mM MgCl₂, 5 mM β-mercaptoethanol, ~0.66 pmol of [γ-32P]ATP (1.85 × 10⁵ cpm/pmole), and 2 μl unlabeled ATP. Components were combined in a volume sufficient for 13 reactions and warmed to 30 °C. The reaction was started by the addition of ~60 ng of protein/25-μl reaction mixture (25 μl) were removed at 0, 0.5, 1, 2, 3, 4, 5, 6, 9, 11, 13, 15, and 20 min, and the reaction was stopped by the addition of ice-cold EDTA to 85 mM. 6-μl aliquots were spotted on polyethyleneimine-cellulose TLC plates and developed in 1 N LiCl to separate inorganic phosphate from the mono-, di-, and triphosphates. The extent of ATP hydrolysis was quantified using a PhosphorImager and ImageQuant software (Molecular Dynamics).

**Preparation of Helicase Substrates**—Linear partial duplex substrates containing a single-stranded gap were prepared in several steps. Three dsDNA fragments (A, B, C) were generated in independent PCR reactions performed without α,β,γ-ATP to the PCR reaction mixture. The conditions for amplification were 94 °C for 3 min then 30 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min followed by one step at 72 °C for 5 min. For the 30s and 40s substrates 55 °C annealing temperature was required. The 5’-phosphorylated strand of each PCR fragment was then selectively degraded by 5 units of T4 exonuclease III (New England Biolabs) followed by purification of 1 μg of fragment in a phenol/chloroform extraction followed by ethanol precipitation. The products were purified with Qiagen PCR purification kits. The yield and concentration of ssDNA were determined on 1.4% agarose Tris borate EDTA gels. To create the partial linear duplex DNA substrates, the three protected strands of fragments A, B, and C were combined at a molar ratio of 3:1 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 reiterating cycles of decreasing temperature (1 °C over four 25-° increments per cycle) to a final temperature of 16 °C. This hybridization mix was used directly in helicase assays or stored at –20 °C.
reaction mixtures (20 μl) contained 3–3.5 ng (375 pm) of intermediate products or hybridized helicase substrates and 2.5 units (S1 nuclease and HindIII), 4 units (XhoI and NotI), 5 units (ApaI), or 7.5 units (PstI, PvuII, and HindIII) of enzyme. All enzymes and buffers were supplied by Takara. Incubation was at 37 °C for 30 min. The products were resolved with 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-32P]ATP were used as standards for gel mobility. Radiolabeled DNA species were visualized by autoradiography of the dried gels.

Helicase 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 MgCl2, 1 mM DTT, 10% glycerol, 50 μg/ml bovine serum albumin, 1.8 mM ATP, ~3–3.5 ng of DNA (375 pm) substrates, and 0–52 nM TraI. For kinetic studies carried out in the presence of 52 nM enzyme a scale-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 0.2 volumes of loading buffer (50 mM EDTA, 50% glycerol, 1% SDS, and 0.1% bromphenol blue). 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 (Molecular Dynamics). The percent unwound helicase substrate was determined after background correction: % unwound = signal intensity in displaced fragment divided by total substrate signal.

RESULTS

Expression, Purification, and ATPase Activity of R1 TraI Protein—TraI was overexpressed in E. coli SCS1 and purified from crude cell extracts as described under “Experimental Procedures.” The final fraction contained a greater than 90% homogeneous solution of TraI as judged by SDS-PAGE and Coomassie Blue staining (data not shown). The purified protein exhibited an apparent molecular mass of 180 kDa. Typically, 20–25 mg of protein were obtained from 4.8 liters of cultured cells.

Extensive studies have characterized the NTPase activity of TraI from plasmid F (53, 54). In good agreement with these reports the ATPase activity of TraI from plasmid R1 was dependent on the presence of a ssDNA effector and activated by Mg2+. In the presence of 2 mM ATP maximal activity was observed at 2 mM MgCl2, and half-maximal values were obtained at 0.3 and 20 mM MgCl2 (not shown). One unit of enzymatic activity is defined as the amount of enzyme needed to hydrolyze 1 nM ATP in 20 min at 30 °C. The progress of the purification procedure was monitored by measuring the ATPase activity at the later stages, as summarized in Table II. A specific activity of 1970 kilounits/mg was determined for the final fraction containing TraI.

Preparation and Verification of the Helicase Substrate Structures—The TraI protein of plasmid F is known to require a ssDNA region centered on the single-stranded gap (47–49). To evaluate the requirement for a 5’ ssDNA tail, we chose to create a series of linear partial duplex substrates containing a centrally located single-stranded gap of variable length. Duplex arms were generated by hybridizing two distinct non-overlapping single-stranded fragments to one common longer fragment of complementary ssDNA as illustrated in Fig. 1. To be able to control the gap size precisely, the position of the 5’ end of the primer utilized to amplify fragment C was varied incrementally relative to the start of the left arm duplex (Fig. 1B). A series of partial duplex molecules were generated with this approach that differed only in the defined length of the single-stranded region present after hybridization.

The structure of the resulting products was verified based on nuclease sensitivity (Fig. 2). The oriT region of IncFII plasmid R1 is essentially devoid of commonly used restriction endonuclease cleavage sites. Utilization of this DNA for creating suitable helicase substrates would make verification of the gap size in each substrate prohibitively difficult. Therefore, pBluescript DNA was chosen to be able to exploit the dense arrangement of restriction endonuclease recognition sites in the multiple cloning site when these are positioned in and around the single-stranded gap of the partial duplex hybridization products. For each substrate preparation sensitivity of intermediate products and the product of their hybridization to restriction endonucleases and single strand-specific nuclelease S1 was analyzed. The results for three of the seven different substrates used in this study are presented in detail.
In Fig. 2A the structure of the hybridization product expected to contain a single-stranded gap 67 nt in length is demonstrated. Importantly, for every substrate just one of the component ssDNA fragments was radiolabeled. Thus, not all DNA species are visible in the autoradiogram. The different DNA species present after H9261-exonuclease III treatment of the pooled PCR products before their hybridization (lane 2) were also treated with several nucleases and resolved electrophoretically through non-denaturing agarose gels (lanes 3–6). The fastest and the slowest migrating labeled species were insensitive to restriction endonucleases (lanes 3–5) but were completely degraded by S1 nuclease (lane 6). In contrast, the band migrating somewhat less than the 405-bp double-stranded standard DNA fragment (lane 13) was resistant to S1 nuclease (lane 6). Treatment with PstI did not alter the apparent migration of this band (lane 3). Digestion with NotI, however, yielded a slightly smaller fragment (lane 4). The addition of PvuII caused this species to disappear altogether and two smaller fragments to emerge (lane 5). One exhibited an apparent migration intermediate between that of the 200- and 100-bp standard DNA fragments, and the second was slightly retarded compared with the 200-bp standard (lane 1). These results are
consistent with values expected for nuclelease-treated double-stranded PCR product C (see inset, Fig. 2A) before one strand was removed with λ-exonuclease III. We conclude, therefore, that this band represents a residual double-stranded 427-bp fragment C resulting from incomplete exodegradation. The slowest migrating band was not present in all preparations. Taken together, the resistance to endonucleases, sensitivity to S1, and the observations that this band was eliminated by heat denaturation of the samples (not shown) or incubation with TraI helicase (Fig. 3A), suggests that this is a partial duplex molecule with a central region of ssDNA flanked by two duplex arms. The single-stranded gap can be cleaved by S1 nuclease to release the right and left arms, but only the 427-bp labeled right arm is visible. Duplex character left of the central single-stranded gap was confirmed with PvuII. Two PvuII recognition sites are present in the fragment of pBluescript DNA common to all partial duplex substrates (fragment Δ). Treatment of the hybridization products would be expected to yield two radiolabeled fragments 448 and 170 bp long. Release of the 448-bp fragment requires the site left of the single-stranded gap is present in double-stranded form. PvuII digestion of this preparation yielded two bands exhibiting similar mobility. Correlation of expected and existing gap size is provided by sensitivity to NotI and resistance to ApaI and PstI endonuclease digestion in accordance with the sequence of the forward primer used to generate fragment C (Fig. 1B).

The structure of intermediate products and hybridized products in the preparation of all seven partial duplex substrates described in this study was demonstrated using the same general analysis (not shown). The selection of enzymes was varied as the expected gap size was narrowed from 67 to 12 nt. Verification of the structure of the substrate with the shortest

S1 treatment (lane 12). Incubation with NotI released a slightly more mobile band than that produced by S1 (lane 10). These results support the conclusion that the prominent labeled DNA in lane 7 is a partial duplex molecule with a central region of ssDNA flanked by two duplex arms. The single-stranded gap can be cleaved by S1 nuclease to release the right and left duplexes, but only the 427-bp labeled right arm is visible. Duplex character left of the central single-stranded gap was confirmed with PvuII. Two PvuII recognition sites are present in the fragment of pBluescript DNA common to all partial duplex substrates (fragment Δ). Treatment of the hybridization products would be expected to yield two radiolabeled fragments 448 and 170 bp long. Release of the 448-bp fragment requires that the site left of the single-stranded gap is present in double-stranded form. PvuII digestion of this preparation yielded two bands exhibiting similar mobility. Correlation of expected and existing gap size is provided by sensitivity to NotI and resistance to ApaI and PstI endonuclease digestion in accordance with the sequence of the forward primer used to generate fragment C (Fig. 1B).

The structure of intermediate products and hybridized products in the preparation of all seven partial duplex substrates described in this study was demonstrated using the same general analysis (not shown). The selection of enzymes was varied as the expected gap size was narrowed from 67 to 12 nt. Verification of the structure of the substrate with the shortest
region of ssDNA relied on digestion with XhoI, which cuts the right duplex arm only in this case, combined with continued sensitivity to S1 nuclease. For completeness, the results for two additional substrates containing single-stranded regions 27 and 16 nt in length are shown in Fig. 2. The preparation of the 27-nt gapped duplex (Fig. 2B, lane 8) contains some residual double-stranded fragment C. Thus, PvuII digestion of the substrate (lane 12) yields in addition to those products predicted for the partially duplex DNA, an ~300-bp fragment diagnostic for the fully duplex species. Members of this series of partial duplex substrates are designated according to the confirmed length of single-stranded gap in nt, for example, 67ss.

The 67ss DNA Substrate Supports Helicase Activity—To ascertain whether the length of ssDNA present in 67ss, which is an intermediate value in the earlier estimation of 12–200 nt (47), is sufficient for efficient TraI helicase activity, the dependence of the reaction on protein concentration and the time course of fragment displacement from the 67ss substrate at 52 nM TraI were determined (Fig. 3). The lowest concentration of protein that yielded detectable unwinding (16%) after 20 min at 37 °C (Fig. 3A) was 2.6 nM. This corresponds to a protein (monomer) to substrate ratio of 7:1. Given that a 4–6-fold excess of unlabeled ssDNA was also present in each labeled substrate preparation, we estimate that the protein to DNA ratio for this reaction mixture approaches 1:1. Performance of the enzyme under these conditions was, thus, similar to reported activities of F TraI at similar protein to DNA ratios on partially duplex circular DNA substrates (48). The comparison is important because in that case TraI was exposed to thousands of nt of ssDNA for binding before displacing the annealed complementary fragment. Complete unwinding of 67ss was observed at protein concentrations greater than 26 nM, again in good agreement with the performance of a >10-fold excess of F TraI on heteroduplex single-stranded circular substrates (48). The requirement for ATP and divalent cation cofactors was assessed by omission (Fig. 3A, lanes 11–13). As expected no helicase activity was detected in the absence of ATP. The addition of MgCl₂ was not essential, as has been observed previously (48). At the maximum protein concentration (Fig. 3B) 55% of the fragment was displaced after 90 s. Near quantitative unwinding of the substrate was observed after 7.5 min at 37 °C. We conclude that a single-stranded region 67 nt long 5’ to the duplex is, therefore, sufficient for efficient TraI helicase activity.

Maximal Helicase Activity on Substrates with Shortened Single-stranded Regions—Given that the 67-nt gap was sufficient to support highly efficient DNA unwinding activity by TraI, additional substrates were prepared, and their structures were characterized, as described above (Fig. 2 and data not shown), which limited the extent of single-stranded region adjacent to the duplex in a stepwise manner (67, 40, 30, 27, 20, 16, and 12 nt). The relative efficiency of DNA unwinding activity catalyzed by TraI on the series of substrates was compared using the standard helicase reaction and increasing amounts of purified protein (Fig. 4). The data are presented graphically in Fig. 5. The efficiency of the reaction was dependent on protein concentration and the length of ssDNA present on the substrate. At the highest protein concentrations nearly quantitative unwinding of the 67ss and the 40ss substrates was observed. In comparison, the maximum efficiency of unwinding was reduced to 80 and 60% when the ssDNA was shortened to 30 or 27 nt, respectively. Very limited unwinding of the partial duplex DNAs containing a single-stranded region ≤20 nt was observed. These experiments were repeated (n ≥ 5) using two independent preparations of each substrate (Fig. 5).

The kinetics of the unwinding reaction on the series of substrates at 52 nM TraI were also compared (Fig. 6). The rates of the overall reaction decreased as the length of ssDNA present in the substrates was limited. The extent of unwinding on substrates containing ≤40 nt of ssDNA did not continue to increase to 100% efficiency but reached a plateau after ~7.5 min. Although the assay employed here measures the combined efficiency of loading the helicase and duplex unwinding, we infer that the rate-limiting step in the overall reaction is the initiation of the unwinding process, which is the binding to the available ssDNA and entering the duplex, and not the rate of unwinding itself. We conclude that a single-stranded region at least 27 nt in length is sufficient to support efficient TraI-
catalyzed unwinding of the adjacent duplex. In contrast, single-stranded regions shorter than 20 nt are not sufficient to stimulate efficient helicase activity.

DISCUSSION

The initiation pathway for TraI-catalyzed unwinding of duplex DNA in isolation or as part of the conjugative relaxosome has not been described. Recruitment of replicative helicases to replication origins typically requires the activity of sequence-specific DNA-binding proteins that confer site specificity to the helicase activity as well as induce localized distortions in the duplex to provide regions of ssDNA for the helicase to bind (46). The IncF-IncW family of DNA/mobilizing systems achieve site specificity in oriT DNA unwinding by physical linkage of the conjugative helicase to an N-terminal DNA relaxase domain (34, 38, 55, 56), which acts to cleave the transfer origin with nt precision, and presumably via interactions with additional components of the relaxosome. In contrast to studies of the initiation process of replication at prokaryotic origins of replication, however, reconstitution of origin unwinding and the initiation of conjugal DNA synthesis in vitro has not been achieved. Although some helicases initiate the unwinding reaction from a nicked substrate, this activity has not been observed in vitro for TraI (54). The observations that (i) the intermediate (open) product of the TraI-catalyzed cleaving-joining reaction at nic failed to support helicase entry to the open circular form as it has been reconstituted in vitro thus far (45) and (ii) the helicase activity when studied in isolation required a single-stranded tail 5' to the duplex (49) imply that localized duplex melting at nic is necessary to load the TraI helicase. In vitro reconstitution of helicase activity for enzymes that require single-stranded ends to enter the duplex is typically achieved when a short fragment or oligonucleotide targeted for displacement is annealed to a comparatively large single-stranded circular or linear phage DNA. Substrates of this type offer an excess of entry sites as the helicase can bind anywhere on the available ssDNA and translocate to the duplex region. Early studies of TraI by Hoffmann-Berling and co-workers (47) sought to limit the extent of ssDNA at the single strand/double strand junction, resulting in the estimation that ~200 nt of ssDNA tail were required for this enzyme (47). With the aim of gaining insight to the initiation pathway utilized by TraI as a conjugal helicase, the present study required preparation of a series of linear DNA substrates where the length of single-stranded region adjacent to the target duplex could be defined. The linear substrates used contained a continuous DNA lattice with two terminally flush duplex arms separated by a central single-stranded region of variable length. The effect of varying the extent of ssDNA present at single strand/double strand junctions on the efficiency of TraI-catalyzed unwinding was observed. The end product of the assay measured the combined efficiency of loading at the single-stranded region and subsequent unwinding of a ~450-nt fragment from the downstream target duplex. We found that decreasing the available single-stranded region stepwise from 67 to 40 nt did not curtail the efficiency of unwinding, but a further reduction to 30 nt resulted in a loss of 20% of the maximal activity under standard assay conditions. Limiting the ssDNA to 27 nt decreased the maximal activity to only 60% that observed on the 67ss and 40ss substrates. When the gap size was decreased further to 20, 16, or 12 nt, efficient duplex unwinding was no longer observed.

This assay cannot distinguish the effect of gap size on individual steps presumed to occur in the initiation process such as binding affinity, enzyme orientation, translocation on the ssDNA lattice, duplex entry, etc. In view of the fact that during conjugation loading of TraI helicase is both site- and strand-specific, we expect that the nature of the region flanking nic in oriT is an important effector of the initiation pathway. Dissection of these steps, therefore, will be carried out on sequence-specific substrates. The necessity of conducting the current study on different DNA is due to the virtual absence of restriction recognition sites in the IncF oriT. In the absence of suitable sites, confirmation of the helicase substrate gap size would be quite difficult to prove. Moreover, all gel shift assays we have tested were unable to differentiate large fragments of fully duplex linear DNA from molecules containing 30 bp or less of melted duplex for a similar series of oriT-specific substrates (described below). Thus the rigorous evaluation of the extent of ssDNA present in the substrates that is crucial to the present analysis would be practically untenable.

The current findings have been integrated into the construction of oriT-specific heteroduplex DNA substrates where variable lengths of the sequence surrounding nic are present as open (noncomplementary) duplex and flanked by two double-stranded arms, one of which contains double-stranded oriT DNA in its native context relative to nic. We presently know that reconstitution of both the nic-cleaving and the DNA-unwinding activities of TraI on substrates of this type is possible. The effect of the extent of duplex melting around nic on the efficiency of TraI-catalyzed nic cleavage and DNA unwinding is under investigation. These studies will further assess the effects of the additional presence of auxiliary DNA-binding proteins of the IncF relaxosome as well as E. coli SSB protein on the activities of TraI at its origin-specific initiation site.

Acknowledgments—We thank Drs. C. Kratky, R. Zechner, and W. Keller for helpful discussions.

REFERENCES

1. Delagoutte, E., and von Hippel, P. H. (2002) Q. Rev. Biophys. 35, 431–478
2. Delagoutte, E., and von Hippel, P. H. (2003) Q. Rev. Biophys. 36, 1–49
3. Caruthers, J. M., and McKay, D. B. (2002) Curr. Opin. Struct. Biol. 12, 123–133
4. Marians, K. J. (2000) Structure Fold. Des. 8, 227–235
5. Geider, K., and Hoffmann-Berling, H. (1981) Annu. Rev. Biochem. 50, 233–260
6. Willette, N., and McIntire, S. (1979) Contrib. Microbiol. Immunol. 6, 137–145
7. Matson, S. W., Sampson, J. K., and Byrd, D. R. (2001) J. Biol. Chem. 276, 2372–2379
8. Zechner, E. L., de la Cruz, F., Eisenbrandt, R., Grahn, A. M., Koraimann, G., Lanka, E., Muth, G., Pansegrau, W., Thomas, C. M., Wilkins, B. M., and Zatko, M. (2000) in The Horizontal Gene Pool: Bacterial Plasmids and Gene Spread (Thomas, C. M., ed) pp. 87–174, Harwood Academic Publishers, Amsterdam
9. Birn, I., Ippen-Bik, K., and Skurrany, R. A. (1996) in Echerichia coli and Salmonella (Neidhardt, F. C., Curtiss, R. III, Ingraham, J. L., Lin, E. C. C., Low, K. B., Magasanik, B., Reznikoff, W. S., Riley, M., Schaefer, M., and Umbarger, H. E., eds Vol. 2, pp. 2377–2401, American Society for Microbiology, Washington, D. C.
10. Pansegrau, W., and Lanka, E. (1996) Prog. Nucleic Acid Res. Mol. Biol. 54, 197–251
11. Francis, V. M., Varsaki, A., Garciñí-Laria, M. P., Latorre, A., Drainas, C., and de la Cruz, F. (2003) FEMS Microbiol. Rev., in press
12. Kline, B. C., and Helinski, D. R. (1971) Biochemistry 10, 4975–4980
13. Moncali, G., Grandoso, G., Llosa, M., and de la Cruz, F. (1997) J. Mol. Biol. 270, 188–200
14. Moncali, G., Valles, M., Valpuesta, J., and de la Cruz, F. (1999) Mol. Microbiol. 34, 1641–1652
15. Fürste, J. P., Pansegrau, W., Ziegelin, G., Kroger, M., and Lanka, E. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 1771–1775
16. Pansegrau, W., Balzer, D., Krub, V., Lurz, R., and Lanka, E. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 6555–6559
17. Ziegelin, G., Pansegrau, W., Lurz, R., and Lanka, E. (1992) J. Biol. Chem. 267, 17279–17286
18. Brach, M. A., and Meyer, R. J. (1986) J. Bacteriol. 167, 703–710
19. Scherzinger, E., Lurz, R., Otto, S., and Dobrinski, B. (1992) Nucleic Acids Res. 20, 41–46
20. Perwez, T., and Meyer, R. (1996) J. Bacteriol. 178, 5762–5767
21. Zhang, S., and Meyer, R. J. (1997) Mol. Microbiol. 25, 509–516
22. Everett, R., and Willetts, N. (1980) J. Mol. Biol. 136, 129–150
23. Inamato, S., Fukuda, H., Abo, T., and Ohtsubo, E. (1994) J. Biochem. (Tokyo) 116, 838–844
24. Nelson, W. C., Howard, M. T., Sherrard, J. A., and Matson, S. W. (1995) J. Biol. Chem. 270, 28574–28580

V. C. Csiszovits and E. Zechner, unpublished information.
25. Howard, M. T., Nelson, W. C., and Matson, S. W. (1995) J. Biol. Chem. 270, 28381–28386
26. Kupelwieser, G., Schwah, M., Hogenauer, G., Koraimann, G., and Zechner, E. L. (1996) J. Mol. Biol. 275, 81–94
27. Karl, W., Bamberger, M., and Zechner, E. L. (2001) J. Bacteriol. 183, 909–914
28. Abdel-Monem, M., Taucher-Scholz, G., and Klinkert, M. Q. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 4659–4663
29. Karl, W., Bamberger, M., and Zechner, E. L. (2001) J. Bacteriol. 183, 909–914
30. Matson, S. W., and Morton, B. S. (1991) J. Biol. Chem. 266, 16232–16237
31. Reygers, U., Wessel, R., Muller, H., and Hoffmann Berling, H. (1991) EMBO J. 10, 2689–2694
32. Sherman, J. A., and Matson, S. W. (1994) J. Biol. Chem. 269, 26220–26226
33. Llosa, M., Grandoso, G., Hernando, M. A., and de la Cruz, F. (1994) Eur. J. Biochem. 226, 403–412
34. Traxler, B. A., and Minkley, E. G., Jr. (1988) J. Mol. Biol. 204, 205–209
35. Llosa, M., Grandoso, G., Hernando, M. A., and de la Cruz, F. (1996) J. Mol. Biol. 264, 56–67
36. Im, D. S., and Muzyczka, N. (1990) Cell 61, 447–457
37. Hickman, A. B., Ronning, D. R., Kotin, R. M., and Dyda, F. (2002) Mol. Cell 10, 327–337
38. Datta, S., Larkin, C., and Schildbach, J. F. (2003) Structure 11, in press
39. Owens, R. A., Weitzman, M. D., Kyostio, S. R., and Carter, B. J. (1993) J. Virol. 67, 997–1005
40. Linden, R. M., Ward, P., Giraud, C., Winocour, E., and Berns, K. I. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 11288–11294
41. Zhou, X., Zolotukhin, I., Im, D. S., and Muzyczka, N. (1999) J. Virol. 73, 1580–1590
42. Tsai, M. M., Fu, Y. H., and Deonier, R. C. (1990) J. Bacteriol. 172, 4603–4609
43. Luo, Y., Gao, Q., and Deonier, R. C. (1994) Mol. Microbiol. 11, 459–469
44. Rice, P. A., Yang, S., Mizuuchi, K., and Nash, H. A. (1996) Cell 87, 1295–1306
45. Byrd, D. R., and Matson, S. W. (1997) Mol. Microbiol. 25, 1011–1022
46. Konieczny, I. (2003) EMBO Rep. 4, 37–41
47. Kuhn, B., Abdel-Monem, M., Krell, H., and Hoffmann-Berling, H. (1979) J. Biol. Chem. 254, 11343–11350
48. Lahn, E. E., and Matson, S. W. (1988) J. Biol. Chem. 263, 3298–3315
49. Abdel-Monem, M., Lauppe, H. F., Kartenbeck, J., Durwald, H., and Hoffmann-Berling, H. (1977) J. Mol. Biol. 110, 667–685
50. Zechner, E. L., Pruger, H., Grommann, E., Espinosa, M., and Hogenauer, G. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 7435–7440
51. Lessl, M., Balzer, D., Lura, R., Waters, V. L., Guiney, D. G., and Lanka, E. (1992) J. Bacteriol. 174, 2493–2500
52. Miller, J. H. (1972) Experiments in Molecular Genetics, p. 433, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
53. Abdel-Monem, M., and Hoffmann-Berling, H. (1976) Eur. J. Biochem. 65, 431–440
54. Abdel-Monem, M., Durwald, H., and Hoffmann-Berling, H. (1976) Eur. J. Biochem. 65, 441–449
55. Byrd, D. R., Sampaio, J. K., Ragonese, H. M., and Matson, S. W. (2002) J. Biol. Chem. 277, 42645–42653
