A site- and strand-specific nick, introduced in the F plasmid origin of transfer, initiates conjugal DNA transfer during bacterial conjugation. Recently, molecular genetic studies have suggested that DNA helicase I, which is known to be encoded on the F plasmid, may be involved in this nicking reaction (Traxler, B. A., and Minkley, E. G., Jr. (1988) J. Mol. Biol. 204, 205-209). We have demonstrated this site- and strand-specific nicking event using purified helicase I in an in vitro reaction. The nicking reaction requires a superhelical DNA substrate containing the F plasmid origin of transfer,Msp" and helicase I. The reaction is protein concentration-dependent but, under the conditions used, only 50-70% of the input DNA substrate is converted to the nicked species. Genetic data (Everett, R., and Willetts, N. (1980) J. Mol. Biol. 136, 129-150) have also suggested the involvement of a second F-encoded protein, the TraY protein, in the oriT nicking reaction. Unexpectedly, the in vitro nicking reaction does not require the product of the F plasmid traY gene. The implications of this result are discussed.

The phosphodiester bond interrupted by helicase I has been shown to correspond exactly to the site nicked in vivo suggesting that helicase I is the site- and strand-specific nicking enzyme that initiates conjugal DNA transfer. Thus, helicase I is a bifunctional protein which catalyzes site- and strand-strand specific nicking of the F plasmid in addition to the previously characterized duplex DNA unwinding (helicase) reaction.

*Escherichia coli* DNA helicase I is the product of the F plasmid tral gene (1) and is required for conjugal DNA transfer from a donor to a recipient bacterium (2). This enzyme was the first *E. coli* DNA helicase to be identified and characterized on the basis of its ability to separate the two strands of duplex DNA in an energy-requiring reaction (3-5). The enzyme, which has been extensively characterized both as a DNA-dependent ATPase (3, 6) and as a helicase (4-8) catalyzes the processive unwinding of duplex DNA migrating in the 5' to 3' direction with respect to the strand of DNA on which it is bound. This reaction requires the concomitant hydrolysis of a NTP; there is no apparent preference as to which NTP (dNTP) is utilized in this reaction (6). Unwinding of regions of duplex DNA in excess of 100 kb¹ has been observed (9) suggesting that the enzyme is capable of unwinding long regions of duplex DNA in a processive reaction.

The tral gene encoding helicase I lies near the distal end of the 33-kb F plasmid traYZ operon; its complete DNA sequence and precise map position are now known (10, 11). Genetic studies indicate that the tral gene product is required for both physical transfer of the F plasmid from the donor to the recipient cell during bacterial conjugation and for donor conjugal DNA synthesis (12, for reviews see Refs. 13-15). Since the discovery that helicase I is encoded on the F plasmid (11), it has been assumed that helicase I utilizes its DNA unwinding activity to play a role in conjugal DNA transfer. The processivity and the polarity of the unwinding reaction make helicase I an attractive candidate for the unwinding of the F plasmid to generate single-stranded DNA (ssDNA) for transfer to a recipient bacterium. It has been suggested that the unwinding reaction catalyzed by helicase I may act as the motive force that drives strand transfer (13-15).

The initial DNA processing event in conjugal DNA transfer is the site- and strand-specific nicking of the F plasmid at the origin of transfer (oriT). This is followed by the unwinding of the F plasmid, presumably by helicase I, with the 5' end of the nicked strand entering the recipient bacterium first (reviewed in Refs. 13-15). The site- and strand-specific nick is believed to be introduced in oriT by the action of a two subunit endonuclease composed of the products of the traY and traZ genes (16). The traY gene has been cloned, its protein product overproduced and purified, and it has been shown to bind specifically to a region on oriT that lies 60 bp upstream of the nick site (17). The TraY protein alone does not catalyze a nicking reaction within the oriT region (17). The traZ gene was never precisely mapped, due to a lack of point mutations, although it was localized at the distal end of the F plasmid tra operon (16, 18). Minicell experiments suggest the existence of a coding region, distal to traZ, responsible for a protein product called 2b with a molecular mass of 78-79 kDa (18). This was thought to be the traZ gene (16, 18). Recent mapping and sequencing of the distal end of the tra operon indicates that there is insufficient room to encode a 79-kDa protein between the end of the traZ gene and the IS3 sequence which defines the end of the traYZ operon (11). The 79-kDa 2b protein observed in minicells appears, in fact, to be a restart product of the traZ gene (11). Additional studies have suggested that the nicking function originally assigned to traZ may be dependent on traZ sequences. Traxler and Minkley (19) have shown that insertions into and deletions of the traZ sequence abolish nicking in an in vivo nicking assay, suggesting that helicase I may be involved in the nicking reaction. In

¹ The abbreviations used are: kb, kilobase pairs; ssDNA, single-stranded DNA; oriT, origin of transfer; SDS, sodium dodecyl sulfate; bp, base pairs; nt, nucleotide(s).

² E. E. Lahue and S. W. Matson, unpublished observations.
this view, helicase I would have a dual function in conjugation; site- and strand-specific nicking at oriT, presumably in conjunction with the TrfY protein, leading to the F- plasmid. Although the helicase activity of TraI protein has been clearly demonstrated, a biochemical demonstration of an oriT nicking activity has never been accomplished.

In this report we demonstrate the oriT site- and strand-specific nicking reaction catalyzed by purified helicase I. The nicking reaction occurs within the oriT region on the F plasmid at the site shown to be nicked in vivo. The reaction requires a supercoiled DNA substrate and MgCl₂ but does not require the addition of ATP. Interestingly, the in vitro reaction does not require the product of the traY gene. Based on these results we suggest that helicase I is, in fact, the site- and strand-specific endonuclease that initiates strand transfer during bacterial conjugation.

**EXPERIMENTAL PROCEDURES**

**Materials**

*Enzymes—*DNA helicase I was purified as previously described (6) from an overproducing strain of *E. coli* containing pMPS 19 (18) which was kindly provided by Dr. T. Lohman (Washington University, St. Louis, MO). The purified enzyme (M̄ = 180,000) was greater than 95% homogeneous as determined on polyacrylamide gels run in the presence of sodium dodecyl sulfate (SDS). Restriction endonucleases were purchased from New England Biolabs, U. S. Biochemicals, or Life Technologies, Inc. and used according to the suppliers’ instructions. Proteinase K was from Boehringer Mannheim and euukaryotic topoisomerase I was from Life Technologies, Inc.

*DNAs and Nucleotides—*The Bluescript vector (Stratagene) (pBS) was grown in *E. coli* HB 101 and purified by alkaline/SDS lysis (20) followed by banding in CsCl/ethidium bromide gradients. The plasmid pBSoriT is a derivative of pBS containing the F plasmid oriT region. It was constructed by cloning the 529-bp BstYI/SalI fragment from pED 806 (21) into pBS cleaved with BamHI and SalI. The construction was confirmed by DNA sequencing. This 529-bp DNA fragment contains the F oriT region from the EcoRI site at position 1 on the recent map of the tra operon to the SalI site at position 529 (22).

The oligonucleotides used as primers in DNA sequencing reactions were synthesized on an Applied Biosystems oligonucleotide synthesizer and have the following sequence: primer 1, 5’dAATACGACTCACTATAG3’; primer 2, 5’dAACCACCCTAAAGGG3’. Nucleoside 5’-triphosphates were purchased from P-L Biochemicals, Inc., and concentrations were determined by directly reading absorbance at the appropriate wavelength. [α-32P]dTTP and [α-32P]dATP were from Amersham.

**Methods**

*Nicking Assay—*Reaction mixtures (16 µl) contained 50 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, 50 mM NaCl, 1 mM dithiothreitol, 30 µg/ml bovine serum albumin, 13–16% glycerol, 330 ng of plasmid DNA (Form I) and helicase I (TraI protein) as indicated. Incubation was at 37 °C for 30 min (unless otherwise indicated) followed by the addition of 4 µl containing 50 mM EDTA, 0.5% SDS, 500 µg/ml proteinase K. Incubation was continued for 30 min at 37 °C. The entire reaction mixture was loaded onto 1.0% agarose gels containing 0.5 µg/ml ethidium bromide. Electrophoresis was at 2 V/cm at 4 °C for 12–16 h. The running buffer was 40 mM Tris, 30 mM borate, 2 mM EDTA (TBE).

*Alkaline Agarose Gel Electrophoresis—*pBSoriT DNA nicked by helicase I in a standard reaction mixture (increased to 80 µl) was isolated on 0.8% agarose gels run in Tris/acetate/EDTA buffer and purified free of agarose using GeneClean (Bio-101, Inc.). Nicked DNA was incubated with the indicated restriction enzyme for 30–60 min at 37 °C, phenol/CHCl₃-extracted, precipitated with ethanol, and resuspended in 25 µl of 100 mM NaOH, 1 mM EDTA, 15% glycerol, 0.5% loading dyes. Alkaline agarose gels (1.0%) were cast in 50 mM NaCl, 1 mM EDTA, 100 mM NaOH as described (20); the gel running buffer was 100 mM NaOH, 1 mM EDTA. Electrophoresis was at 2 V/cm for 14–18 h at 4 °C. Gels were neutralized in 0.5 M Tris-HCl (pH 8) followed by soaking in TBE. DNA was visualized by staining with ethidium bromide (1 µg/ml) in TBE. For Southern blotting the gel was subsequently soaked in 1.5 M Tris-HCl (pH 7.4)/30 M NaCl for 30 min, and the DNA was transferred to nylon membranes. The probe was a 540-bp DNA fragment containing the oriT region isolated from pBSoriT and radioactively labeled using a random oligonucleotide labeling kit (U. S. Biochemicals).

**Other Methods—**Protein concentrations were determined using the Bio-Rad protein assay; bovine serum albumin was the standard. DNA concentrations were determined by directly reading the absorbance at 260 nm and are expressed in nucleotide equivalents. DNA sequencing was by the dideoxynucleoside 5’-triphosphate chain termination method (23) using Sequenase (U. S. Biochemicals) as described by the supplier.

**RESULTS**

*Helicase I Nicks a Plasmid Containing oriT—*Recently, insertion and deletion mutagenesis studies have suggested that helicase I might be one component of the enzyme responsible for site- and strand-specific nicking at F oriT (19). In an effort to directly demonstrate a site- and strand-specific nicking activity associated with this enzyme we tested purified helicase I, in the presence and absence of purified TraY protein, in a nicking assay using a plasmid which contained a cloned copy of F oriT (pBSoriT). Both enzymes were purified to greater than 95% homogeneity from F⁻ overproducing strains of *E. coli* and should, therefore, be free of any other F-encoded polypeptide.

Incubation of purified helicase I with pBSoriT resulted in the formation of a DNA species that migrated with nicked circular DNA on an agarose gel (Fig. 1, lanes 1–3). Analysis of the product of this reaction on agarose gels in the presence or absence of ethidium bromide indicated that this was a nicked molecule and not covalently closed relaxed circular DNA (data not shown). It should be noted that the formation of this nicked DNA species does not require the addition of TraY protein.

To determine whether the reaction was specific for plasmid DNA containing the F oriT region, helicase I was incubated with an identical plasmid (pBS) lacking the F oriT region (Fig. 1, lanes 7–9). In this case no nicked DNA was formed suggesting that the nick introduced into pBSoriT by helicase I was located within the oriT region. In addition, incubations of helicase I with pBSoriT were carried out in the absence of MgCl₂ (data not shown) and in the presence of EDTA (Fig. 1, lanes 4–6). Under these conditions there was no nicking of the plasmid DNA indicating that the reaction required the presence of Mg²⁺.

The experimental protocol utilized in the experiments de-
scribed above included an incubation in the presence of SDS and proteinase K after incubation with helicase I. This step was incorporated to trap nicked complexes generated by helicase I that might be analogous to the relaxosomes observed at the plasmid RP4 orfT (24, 25). To determine whether this step was necessary to generate nicked DNA, reactions were stopped using EDTA, EDTA and SDS, or EDTA, SDS, and proteinase K (data not shown). The results of these experiments suggested that proteinase K and SDS were not required for the conversion of supercoiled DNA to nicked DNA. However, in the absence of protein denaturants a large fraction of the nicked DNA migrated as a smear, more slowly than nicked molecules, suggesting the presence of helicase I bound to the nicked DNA. The addition of SDS alone reduced the intensity of the nicked DNA smear but did not eliminate the complex. Based on these results we are not able to conclusively determine whether a covalent DNA-protein complex exists.

The nicking reaction catalyzed by helicase I was shown to be protein concentration-dependent (Fig. 2). At a ratio of DNA molecules: helicase I monomers of 1:1 approximately 10% of the plasmid DNA was nicked by the enzyme in a 30-min incubation (Fig. 2, lane 2). At the highest concentration of helicase I tested over 50% of the DNA substrate was specifically nicked by the enzyme. Increasing the length of the incubation did not dramatically increase the fraction of substrate converted to nicked molecules (data not shown). Moreover, when residual supercoiled DNA was isolated subsequent to the nicking reaction it could be converted to a nicked DNA species by helicase I in a second incubation (data not shown). This rules out the possibility of an inhibitory structure present in some fraction of the substrate DNA. Thus, at a 32-fold molar excess of helicase I monomers over the DNA substrate the reaction failed to go to completion. At present we do not have an explanation for this observation. It may be due to a low affinity of helicase I for its binding site on pBSoriT. We are not able to demonstrate a stable protein-DNA interaction using linear DNA fragments containing orfT and helicase I in a gel-retardation assay (17, data not shown). However, this latter result may be due to the use of linear DNA molecules instead of a supercoiled substrate (see below). Alternatively, the nicked DNA species may compete with the supercoiled substrate for binding of helicase I. These two possibilities are not mutually exclusive and both may contribute to prevent quantitative conversion of the supercoiled substrate to a nicked DNA molecule.

The ability of helicase I to nick a covalently closed relaxed circular plasmid was tested using pBSoriT DNA that had been relaxed with the eukaryotic topoisomerase I (Fig. 3). As expected, helicase I converted supercoiled pBSoriT to a nicked species in the presence of MgCl$_2$ (Fig. 3, lanes 1–4). The enzyme failed to nick pBSoriT DNA that had been previously relaxed using calf thymus topoisomerase I (Fig. 3, lanes 5–8). The relaxed molecule migrates slightly faster than supercoiled plasmid on this agarose gel run in the presence of ethidium bromide due to intercalation of the dye. We were unable to detect any conversion of the relaxed plasmid to a nicked form suggesting that the nicking reaction catalyzed by helicase I required a superhelical DNA substrate. When supercoiled and relaxed plasmids were mixed and incubated with helicase I there was conversion of the supercoiled substrate to nicked DNA (Fig. 3, lane 9), although with somewhat lower efficiency than in the absence of the relaxed plasmid. Apparently the presence of a relaxed plasmid containing the oriT region does not markedly inhibit the interaction between helicase I and its supercoiled substrate. Moreover, preparation of the relaxed plasmid has not introduced any inhibitors of the nicking reaction into the reaction mixture.

Location of the Nick Site within the F orfT Region.—The experiment presented in Fig. 1 strongly suggests that the nick introduced into pBSoriT DNA by helicase I is located within the F orfT region. To further define the site of the nick, and to demonstrate the existence of a single, unique nick, the experiment diagrammed in Fig. 4A was performed. pBSoriT DNA that had been nicked by helicase I was isolated on an agarose gel, cut with a restriction endonuclease to produce a nicked, linear molecule, and the individual DNA strands resolved on an alkaline agarose gel. If the nick introduced by helicase I is both site- and strand-specific this strategy is expected to resolve three bands of ssDNA; a linear full length strand and two shorter molecules resulting from the nicked strand. The expected products after restriction with XmnI are DNA fragments of about 3490 nucleotides (nt), 2360 and 1130 nt in length if the nick site in orfT is at the position found in vivo (22). As can be seen in the alkaline agarose gel stained with ethidium bromide (Fig. 4B, lane 4), the products observed correspond to those expected suggesting that the

![Fig. 2. Protein concentration dependence of the nicking reaction.](image)

![Fig. 3. Helicase I does not nick relaxed covalently closed circular DNA.](image)
The position of the nick introduced in vitro is at or near the position of the nick site identified in vivo. These data also indicate the existence of a single, unique nick site. When a different restriction endonuclease was used to produce the nicked, linear DNA the sizes of the two DNA fragments resulting from the nicked strand were altered as expected (Fig. 4C, lane 3). In this case, SalI was used to produce the nicked, linear DNA molecule and, due to the small size of one of the predicted DNA strands, the DNA was transferred to a nylon membrane and probed with oriT DNA sequences to locate the three DNA fragments. The expected products after restriction with SalI are DNA fragments of 3490, 3100, and 390 nt in length. The autoradiograph shown in Fig. 4C (lane 3) demonstrates three DNA fragments of the expected sizes. As a control, nicked DNA cut with XmnI (Fig. 4C, lane 2) was included in this experiment and, as expected, DNA fragments of 3490, 2360, and 1130 nt in length were observed. Together these data suggest that the nick introduced into pBSoriT is unique and at, or very near, the site that is nicked in vivo to initiate DNA strand transfer during bacterial conjugation.

To define the site nicked by helicase I at single nucleotide resolution, nicked DNA was isolated and subjected to DNA sequencing reactions using the dideoxynucleotide chain termination method (Fig. 5). When a primer that hybridizes to the strand that has been nicked is utilized in these reactions a strong stop is expected in all four lanes at the site of the nick. This is due to the fact that the template strand is interrupted at this site forcing the DNA polymerase to cease synthesis. A strong stop was seen in all four lanes (Fig. 5, lanes 13–16) when primer 1 was utilized in reactions containing nicked pBSoriT DNA as the template. The site of the strand interruption was on the same strand and in precisely the same position as the nick site located by a similar method on plasmids isolated from the cell (22). A control experiment was done using primer 2 to direct synthesis on the opposite strand through the region expected to contain the nick site. In this case there was no strong stop (Fig. 5, lanes 5–8) indicating no interruption of the DNA strand. We conclude that purified helicase I makes a site- and strand-specific nick in the F oriT region under our in vitro conditions, and in the absence of any additional proteins, at the same site nicked in vivo.

**DISCUSSION**

Purified helicase I catalyzes the site- and strand-specific nicking of the F plasmid at oriT, a reaction required prior to unwinding, to initiate DNA strand transfer during bacterial conjugation. Thus this enzyme is likely to have two roles, as previously suggested (19), in conjugal DNA transfer. First, the enzyme nicks the F plasmid (or the chromosome in Hfr strains) in a site- and strand-specific manner at F oriT.
Participation of helicase I in this reaction has been inferred (19) but not previously demonstrated biochemically. Second, helicase I is thought to unwind the F plasmid from the nick site to generate the ssDNA transferred into the recipient bacterium. Helicase I has been characterized as a processive helicase I (4–6) that is likely to be capable of catalyzing this unwinding reaction. However, it has been suggested that other helicases found in E. coli may also be capable of catalyzing this unwinding reaction in tral mutants that lack helicase activity but retain nicking activity (19).

The F oriT nicking reaction catalyzed by helicase I requires a superhelical DNA substrate and Mg2++. Presumably the Mg2+ interacts with the DNA substrate to make the relevant phosphodiester bond in the DNA backbone more susceptible to scission by helicase I. The requirement for superhelical DNA is consistent with our previous observation that helicase I fails to interact with linear DNA fragments as determined using gel-retardation assays (17). Thus when the supercoiled substrate is replaced with covalently closed relaxed DNA there is no conversion to a nicked DNA species. In view of the fact that helicase I fails to retard DNA fragments containing oriT in a gel-shift assay, we suggest that helicase I does not recognize its binding site in oriT except when present in a supercoiled conformation. This result may suggest a reason for the apparent requirement for E. coli gyrase in bacterial conjugation (26, 27). Additional studies will be required to fully appreciate the role of supercoiled DNA in the nicking reaction catalyzed by helicase I.

The helicase I nicking reaction has been shown to be protein concentration dependent, but the reaction fails to go to completion under the conditions we have used. At a 32-fold molar excess of helicase I monomers over DNA molecules 50–70% of the plasmid DNA was nicked by helicase I (Fig. 2). Longer incubations or the inclusion of TraY protein in the reaction mixture did not significantly alter this result. It is possible that helicase I is active as a multimer and that a fraction of the purified enzyme is not active. Together these two factors could account for the incomplete reaction. Helicase I has been reported to be active as an aggregate of monomers in duplex DNA unwinding reactions (4). To determine whether this is true of the nicking activity will require further investigation.

Alternatively, helicase I may have a low affinity for its binding site on oriT or it may have a high affinity for the nicked DNA species. Nicked DNA-helicase I complexes have been observed as smears migrating more slowly than nicked DNA on agarose gels when SDS/proteinase K treatment is omitted (data not shown). This suggests that the enzyme has some affinity for the nicked DNA species and this molecule may compete with the supercoiled substrate for initial binding of excess protein. A critical component may also still be missing in the in vitro reaction. Binding sites for integration host factor have been defined within the oriT region on F (8). Perhaps binding of integration host factor to oriT is required for quantitative conversion to the nicked species by helicase I. At present we are unable to distinguish among these possibilities. It should be noted that reconstitution of the relaxosome and subsequent nicking at oriT in the plasmid RP4 system results in approximately 30% of the substrate converted to a nicked species (25). Thus a low nicking efficiency may be an inherent property of the in vitro reaction kinetics.

We have demonstrated that purified helicase I nicks oriT at precisely the same site shown to be nicked in vivo. Cis-dominant Tra− mutations in oriT have been isolated (28, 29) and involve nucleotide changes at positions 4 bases and 9 bases upstream of the nick site we have determined. These may be part of a recognition site for helicase I binding to oriT. Interestingly, no Tra− mutants in oriT were isolated which directly involve the nucleotides on either side of the phosphodiester bond cleaved by helicase I. This may be due to the particular methods used to generate or isolate oriT mutants. There have been no efforts as yet to saturate this region in oriT with mutations, and it is likely that additional mutants will be found which affect either binding of helicase I, nicking by helicase I, or both.

The most surprising result is the fact that this reaction does not depend on TraY protein. It has long been assumed that TraY protein, in conjunction with a second protein, would be responsible for site- and strand-specific nicking at oriT. Indeed, purified TraY protein has been shown to bind specifically to oriT (17) at a site just upstream of, but not including, the nick site. The notion of the TraYZI endonuclease is based largely on early work suggesting that the product of the trazl gene was responsible for oriT nicking (16). These experiments utilized a λ phage containing oriT and Flac plasmids which provided the Tra functions in trans. A fraction of the packaged λ phage contained nicked molecules which correspond to a nick in oriT. The formation of these nicked molecules was dependent on specific tra gene products. The traY mutants utilized in these studies were all deletion mutants which contained deletions of more than just the traY gene. In fact, no point mutants in traY have ever been isolated. In these studies deletion of tral also abolished the nicking reaction. Since the TraJ protein is thought to be a positive regulator of the traYZ operon, these data were interpreted as an effect on transcription of the traYZ operon resulting in little or no production of TraY protein. Perhaps the traY deletion mutants used in this study have a polar effect on the transcription of tral. Although this seems unlikely in view of the discovery of a promoter associated with the tral gene (30), it remains a formal possibility. Nevertheless, the in vitro nicking of F oriT does not require TraY protein. Additional roles for TraY protein in the metabolism of F DNA during bacterial conjugation can be envisaged and are likely. However, at this time the role of TraY protein remains unclear.

The F oriT nicking reaction seems remarkably simple compared to the in vitro nicking reaction demonstrated using the RP4 plasmid (24, 25). Plasmid RP4 requires the products of two genes in order to form a relaxosome capable of introducing a specific nick in the RP4 oriT (25). In addition, it has been shown that one of these proteins, the product of the RP4 tral gene, remains covalently bound to the 5' end of the nicked DNA strand (24, 25). At this point the nature of the nick introduced by helicase I in F oriT is not clear. The data presented here do not allow us to conclude whether or not helicase I remains covalently bound to either the 3' end or the 5' end of the nicked DNA strand. Previous data (31) suggest the formation of a relaxosome, similar to that seen in the RP4 system, on the F plasmid. This suggests the possibility of a protein-DNA complex at the nick site in F oriT. Additional work will be required to determine whether or not such a complex exists, and if so, where helicase I is bound.

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