The traY Gene Product and Integration Host Factor Stimulate Escherichia coli DNA Helicase I-catalyzed Nicking at the F Plasmid oriT*

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The F plasmid conjugative transfer is initiated by the introduction of a site- and strand-specific nick within the plasmid origin of transfer (oriT). Genetic studies have shown nick formation to be dependent on both the tra and traY genes. However, highly purified TraLP, the tra gene product, nicks oriT in a site- and strand-specific manner in the absence of the traY gene product (TraYP) in vitro (Matson, S. W., and Morton, B. S. (1991) J. Biol. Chem. 266, 16232-16237). Analysis of the oriT region has revealed binding sites for TraYP and the host protein integration host factor (IHF). To explore possible interactions occurring at oriT, highly purified TraLP, TraYP, and IHF were incubated with a supercoiled oriT-containing DNA substrate. A marked enhancement of the nicking reaction catalyzed by TraLP was observed in a reaction that required both TraYP and IHF. In addition, TraLP was able to nick a linear oriT-containing double-stranded DNA substrate when IHF and TraYP were present in the reaction; such a substrate is not nicked by TraLP alone. Individual protein concentration requirements for the supercoiled and linear nicking reactions were similar, and the reactions occurred at equal velocity, suggesting that they are biochemically identical. Concentrations of TraYP and IHF that yield half-maximal activity in the nicking assays compare well with the reported $K_d$ values for the IHF and TraYP binding sites in oriT. These data, coupled with data presented in the accompanying report, suggest that TraYP and IHF bind independent of one another, forming a nucleo-protein complex with oriT that can be recognized and nicked by TraLP.

The F plasmid is a 100-kilobase pair self-transmissible plasmid that inhabits many Escherichia coli strains. During a mating event, a single strand of the F plasmid is transferred, with the 5'-end leading, from the donor bacterium to the recipient bacterium. The transferred genetic material is stabilized in the recipient either by recombination into the chromosome or complementary strand synthesis (for reviews, see Refs. 1–3). Myriad other transmissible plasmids have been described that inhabit various species of bacteria, allowing this type of horizontal gene transfer to occur in an intra- or extraspecies-specific manner.

The tra region (for transfer) on the F plasmid encodes essentially all of the plasmid genes necessary to support bacterial conjugation (2). Of the 36 known genes encoded in this region, only four, traM, traY, traD, and traL, have been shown to be directly involved in DNA mobilization (1). Two of these genes, traY and traL, are required for formation of a site- and strand-specific nick at the origin of transfer (oriT) (4). The formation of the site- and strand-specific nick in oriT is generally considered the first step in DNA mobilization. TraMP and TraDP have been shown not to be involved in nick formation in vivo and are proposed to play a role in subsequent steps of mobilization. As such, they were not considered in this study.

The tra gene encodes DNA helicase I (5), an enzyme that has been well characterized in terms of both its DNA unwinding activity and its DNA-dependent ATPase activity (6–9). More recently, we and others have shown that TraLP also contains the catalytic site responsible for site- and strand-specific cleavage at oriT (10, 11). The large size of TraLP, in comparison with other known bacterial helicases, suggests the possibility of separate nicking and helicase domains. This notion is further supported by mutational analysis that localized nicking activity to the amino-terminal half of the protein and helicase activity in the carboxyl-terminal half (5, 9, 12). The oriT-specific nicking reaction catalyzed by TraLP requires magnesium and an oriT-containing DNA substrate that is either supercoiled or single-stranded (10, 11, 13). Cleavage occurs at exactly the same phosphodiester bond that is nicked in vivo. As the phosphodiester bond is cleaved, a covalent linkage forms between TraLP and the 5-phosphate of the nicked strand. This bond scission is the result of transesterification and not hydrolysis (14). The nicked product observed is actually a stable reaction intermediate. Consistent with the notion of a reversible transesterification, TraLP has been shown to release the break formed in the phosphodiester backbone (13). TraLP, therefore, likely plays a role in both the initiation and termination of strand transfer.

The product of the traY gene (TraYP) is a site-specific DNA-binding protein with three known binding sites (15–17). Two of these binding sites are located within oriT within 100 bp of each site and is nicked in vivo to initiate strand transfer. The position of these sites suggests the possibility of protein-protein contacts between TraLP and TraYP during the initiation of strand transfer. However, no such interactions have been demonstrated, and the addition of TraYP has no impact on the oriT-specific nicking reaction catalyzed by TraLP (10). The third TraYP binding site is coincident with the mRNA start site of the traYI operon and is proposed to be involved in...
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Transcriptional regulation (15).

Integration host factor (IHF) binds to two specific sites within oriT (18). IHF is a heterodimer encoded by the chromosomal genes himA and hip (19). It is involved in a wide variety of cellular processes including replication, transcription, and recombination. Moreover, IHF has been shown to play a role in expression of F tra genes. The identification of binding sites in oriT was the first indication that IHF might play a role in DNA metabolism during conjugative transfer (20).

Genetic studies have suggested that TraYp is involved in nick formation in vivo, and it is known that the protein binds oriT near the nic locus. Therefore, a role for TraYp in the in vitro reaction catalyzed by TraIp, which has been elusive, might be uncovered by exploring the roles of other proteins known to bind oriT. To this end, combinations of IHF, TraYp, and TraIp were incubated with supercoiled and linear oriT-containing DNA substrates. TraYp and IHF together stimulate the TraIp-catalyzed reaction on a supercoiled DNA substrate and confer recognition of a linear DNA as a substrate.

**EXPERIMENTAL PROCEDURES**

**Materials**

Enzymes—DNA polymerase I large fragment, T4 polynucleotide kinase, and restriction enzymes were purchased from Amersham Corp., New England Biolabs, or Boehringer Mannheim and used according to the supplier's specifications.

TraIp was purified using a modification of the protocol described previously (21). Fractions I–III were prepared as described previously (21). Fraction III was dialyzed against buffer A (50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 1 mM mercaptoethanol, 20% glycerol) containing 100 mM NaCl, and loaded onto a 30-ml (1.5 cm x 45 cm) heparin-agarose (Sigma) column equilibrated with buffer A containing 50 mM NaCl. The column was washed with 5 column volumes of buffer A and the protein was eluted with a 10-column volume linear gradient from 100 to 800 mM NaCl in buffer A. A gel mobility shift assay and a nuclease assay were performed on fractions from this column. The nuclease assay was identical to the gel mobility shift assay, except the reactions contained 10 mM MgCl2 and no EDTA. Nuclease activity, indicated by appearance of free labeled nucleotide on the binding gel, was undetectable under the conditions used. The final pool was concentrated using a Centriprep10 concentrator (Centricon), and adjusted to 50% glycerol. The protein was judged to be >90% homogeneous by SDS-PAGE (Fig. 1B).

The concentration of protein in the purified fraction was determined by the method of Lowry (23). The theoretical extinction coefficient of TraYp was calculated to be 10.870 M−1 cm−1 by the PEPTIDESORT program of the GCG software package. Comparing the absorption of equal amounts of TraYp under denaturing and nondenaturing conditions showed the extinction coefficient of the native protein to be equal to the calculated extinction coefficient of TraYp.

The active fraction of TraYp was determined by a modification of the method of Rigg et al. (24). Briefly, gel mobility shift assays were performed (as described above) in which the 32P-labeled DNA substrate was titrated (1.7–171 mM) against a fixed concentration of TraYp (85 nM). The fraction of substrate that was bound was determined for each reaction by Phosphorimage analysis. A double reciprocal plot of substrate concentration versus bound complex concentration yields the reciprocal of the concentration of active protein as the y intercept and the negative reciprocal of the Ks of TraYp for the substrate as the x intercept. In this manner, the active fraction of this TraYp preparation was determined to be 19.1%. The Ks of TraYp for sbYa was 36.6 nM.

**DNAs and Nucleotides**—The plasmid pBSoriT has been described previously (10). The substrate used in the linear nicking assays was a pBSoriT restriction fragment prepared as follows: pBSoriT DNA was cleaved to completion with XbaI and labeled at the 3′-end using [α-32P]dCTP and DNA polymerase I large fragment under reaction conditions suggested by the supplier. This linear DNA fragment was then cleaved with SacI and Sall, resulting in the formation of three DNA fragments: a 545-bp 32P-labeled DNA fragment containing the oriT region, a 13-bp 32P-labeled DNA fragment, and a 2887-bp unlabeled fragment.

Plasmids containing oriT deletions were kindly provided by Dr. Richard Deenon (University of Southern California). Plasmid pXRD620a 87 contains bp 1–285 of oriT (using the numbering system of Frost et al. (25)) inserted into the BamHI site of pUC8; pXRD620a 103 contains bp 1–237; pXRD620a 129 contains bp 1–285. The active fraction of TraYp was determined by a modification of the method of Rigg et al. (24). Briefly, gel mobility shift assays were performed (as described above) in which the 32P-labeled DNA substrate was titrated (1.7–171 mM) against a fixed concentration of TraYp (85 nM). The fraction of substrate that was bound was determined for each reaction by Phosphorimage analysis. A double reciprocal plot of substrate concentration versus bound complex concentration yields the reciprocal of the concentration of active protein as the y intercept and the negative reciprocal of the Ks of TraYp for the substrate as the x intercept. In this manner, the active fraction of this TraYp preparation was determined to be 19.1%. The Ks of TraYp for sbYa was 36.6 nM.

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RESULTS

TraYp and IHF Stimulate the Nicking Reaction Catalyzed by TraIp—An in vitro reaction has been described previously in which purified TraIp catalyzes a site- and strand-specific trans-esterification reaction at the nic locus in oriT (10, 11). The reaction requires MgCl₂ and a supercoiled DNA substrate containing oriT; relaxed and linear DNA molecules are not substrates for this reaction. Surprisingly, the addition of TraYp, which genetic studies indicate is required for site- and strand-specific nicking in vivo, is not required for the in vitro reaction catalyzed by TraIp (10). Further characterization of this reaction has shown it to be very sensitive to the addition of increasing concentrations of NaCl (Fig. 2) or potassium glutamate (data not shown). The nicking reaction was reduced by more than 70% in the presence of 75 mM NaCl and was essentially undetectable when the NaCl concentration was increased above 100 mM. These properties seem to be inconsistent with the notion that TraIp, by itself, catalyzes the site- and strand-specific nicking reaction that initiates bacterial conjugation in vivo.

Both TraYp and IHF binding sites have been located within the F plasmid oriT (15, 17, 18). These sites lie near, but not coincident with, the nic locus, and it was reasoned that these two proteins in combination might stimulate the transesterification reaction performed by TraIp. To test this possibility, various combinations of TraIp, IHF, and TraIp were incubated with a plasmid DNA substrate containing oriT in the presence of 75 mM NaCl. The increased concentration of NaCl was included in the reaction to reduce the site- and strand-specific nicking catalyzed by TraIp alone. The results of this experiment are presented in Fig. 3. Incubation of the plasmid DNA with TraIp resulted in the production of a minimal amount of nicked DNA (Fig. 3, lane 4) as expected under these conditions. TraYp and IHF also failed to nick the supercoiled plasmid (Fig. 3, lanes 2 and 3). Moreover, the addition of either TraYp or IHF to reaction mixtures containing TraIp had no effect on the amount of nicked DNA product formed (Fig. 3, lanes 6 and 7). However, when TraYp and IHF were incubated together with TraIp, there was a dramatic increase in the amount of nicked DNA produced (Fig. 3, lane 8). The plasmid was nicked at the same site and on the same strand that is nicked in vivo (data not shown). Quantitation of this data indicated that nicked molecule formation in the three-protein reaction was approximately 7-fold greater than in the reaction catalyzed by TraIp alone. The nicking reaction catalyzed by TraIp in the presence of both TraYp and IHF was also more resistant to increased NaCl concentrations than the reaction catalyzed by TraIp alone (see Fig. 2). Significant nicking of the plasmid DNA was still detectable at NaCl concentrations exceeding 150 mM. Thus, under these conditions, stimulation of the transesterification reaction catalyzed by TraIp is absolutely dependent on the addition of both TraYp and IHF.

To determine the optimal concentrations of TraIp, TraYp, and IHF required for nicking the supercoiled DNA substrate, a series of titrations was performed. In each case, two of the protein components were held at constant concentrations,
while the third was added in increasing concentrations. Production of nicked DNA was half-maximal, with TraIp present at a concentration of 7 nM, which is stoichiometric to the substrate (Fig. 4B, closed circles). Production of nicked DNA was half-maximal at a TraYp concentration of ~150 nM and an IHF concentration of ~20 nM (Fig. 4, A and C, closed circles). The IHF value is in reasonably close agreement with the reported apparent $K_D$ for binding to the IHF A site in oriT, whereas the value for TraYp is approximately 4-fold higher than the apparent $K_D$ for binding to sbyA (see "Experimental Procedures").

TraIp, TraYp, and IHF Nick a Linear DNA Substrate—TraIp, in the absence of other proteins, fails to catalyze a site- and strand-specific transesterification reaction at an oriT sequence that is on either a relaxed plasmid or linear duplex DNA (10, 11). To determine whether this DNA substrate could be specifically nicked when IHF and TraYp were present, various combinations of TraIp, TraYp, and IHF were incubated with a 545-bp linear oriT-containing DNA substrate (Fig. 5). TraIp alone did not nick the linear DNA substrate (Fig. 5, lane 4). The addition of either IHF (Fig. 5, lane 6) or TraYp (Fig. 5, lane 7) had no effect on this reaction. When all three proteins were incubated with this substrate, a portion of the DNA substrate was nicked at a specific site on a specific strand (Fig. 5, lane 8). If the site nicked in this reaction is identical to the site nicked in vivo, the nicked DNA product is expected to be 155 nucleotides in length. Using a 5' end-labeled linear substrate, the cleavage site was mapped and found to occur at the exact location nicked in vivo (data not shown). Thus TraYp, IHF, and TraIp together were able to catalyze oriT-specific nicking of a linear DNA substrate.

To determine the optimal concentration of each protein required to nick the linear DNA substrate, a series of titration experiments was performed. This analysis was similar to that described above using the supercoiled plasmid substrate. The concentration of each protein required for optimal nicking using the linear nicking assay was similar to that determined in the supercoiled nicking assay, although the extent of the reactions differed (see below). Production of nicked DNA was half-
maximal at a TraIp concentration of 4.5 nM, which is a 10-fold molar excess over substrate molecules (Fig. 4B, open circles). Production of nicked DNA was half-maximal at a TraYp concentration of 25 nM and an IHF concentration of 6 nM (Fig. 4A and C, open circles). In this case, the values for both IHF and TraYp compare well with reported Kₜ values for the binding sites in oriT. High concentrations of any of the three proteins inhibited nicking of the linear substrate. The results from this series of experiments are in reasonable agreement with those obtained from the titrations performed using the supercoiled DNA substrate.

Kinetics of the Transesterification Reaction—The kinetics of the transesterification reaction were determined in the presence of TraIp and IHF using both the linear and supercoiled DNA substrates. The reactions were initiated by the addition of TraIp to an equilibrated reaction mixture containing substrate DNA, TraYp, and IHF. Presumably, both IHF and TraYp bind their respective binding sites in oriT during the preincubation. The reactions proceeded linearly for approximately the first 5 min and were >90% complete in 15 min (data not shown). These kinetics are similar to those observed when TraIp alone degrades a single-stranded DNA substrate. The steady-state amount of nicked DNA observed in each case was below 100%. The reaction using the linear substrate achieved a plateau at 70% nicked, while the reaction using the supercoiled substrate only reached a level of 30% nicked. This likely reflects an equilibrium between nicked and ligated species expected in transesterification reactions (see "Discussion").

Nicking oriT Deletion Mutants—The oriT region is operationally defined as the −300 bp of cis-acting DNA sufficient to direct mobilization of a plasmid by F transfer factors (27). The region includes the nic site, two IHF binding sites (IHF A and IHF B) (18), a TraMp binding site (sbyC) (28), and two TraYp binding sites (sbyA and sbyC) (15, 17) (see Fig. 6). The site sbyC is coincident with IHF A, and presumably both sites cannot be bound by their respective proteins simultaneously. In addition, there are two intrinsic bends present in oriT, each bending the DNA by about 50° (18). Fu et al. have shown in vivo that elimination of IHF B, the second intrinsic bend, sbyC, and half of sbyA resulted in reduction of the transfer efficiency, with little effect on nicking at oriT (26). To determine which of these sequence features were required for our in vitro reaction, oriT deletion mutants were used as substrates in the supercoiled nicking assay. An advantage of the in vitro assay is the ability to quantify the nicking efficiency of the mutants.

Plasmid pXRD620Δ87 contains bp 1–285 of oriT (using the numbering system of Frost et al. (25)), including nic, IHF A, sbyA, and sbyC, but eliminating IHF B (Fig. 6). Plasmid pXRD620Δ104 contains bp 1–237, eliminating sbyC and one of the intrinsic bend sequences in addition to IHF B. Plasmid pXRD620Δ79 contains bp 1–222, eliminating approximately one-half of sbyA in addition to the deletions described for pXRD620Δ104. When either pXRD620Δ 87 or pXRD620Δ104 was incubated in the three-protein nicking reaction, the relative amount of nicked DNA formed was comparable with the amount of nicked DNA formed when the substrate was a fully intact oriT sequence. When plasmid pXRD620Δ79 was used, the amount of nicked DNA formed was about 75% of that observed when a plasmid containing the wild-type oriT was used. Elimination of sbyC and the second intrinsic bend sequence had no effect on the in vitro nicking assay reconstituted with three proteins. Also, this experiment shows that the IHF B site is not required for the reaction. The TraYp binding site sbyA can apparently be partially eliminated without loss of nicking competence. However, this plasmid was nicked with a somewhat lower efficiency. Importantly, control experiments indicate that nicking of the pXRD620Δ79 plasmid was IHF- and TraYp-dependent (data not shown). In addition TraIp is still able to bind the truncated oriT region of this plasmid, although with reduced affinity (data not shown).

DISCUSSION

We, and others, have previously reported that TraIp is able to catalyze the site- and strand-specific nicking reaction required to initiate DNA transfer during F plasmid-mediated bacterial conjugation (10, 11). The in vitro reaction catalyzed by TraIp is, in fact, a transesterification reaction that requires a supercoiled DNA substrate containing oriT, MgCl₂, and a molar excess of TraIp (14). A linear DNA substrate or a relaxed, circular DNA substrate cannot be nicked by TraIp. In this communication, we note that this reaction also requires a relatively low ionic strength. In the presence of greater than 75 mM NaCl, the reaction catalyzed by TraIp in the absence of additional proteins is almost undetectable. However, under these conditions, TraIp-catalyzed nicking is greatly stimulated.
when TraYp and IHF are both present in the in vitro reaction. This result is significant for two reasons. First, it establishes a biochemical role for TraYp in the initiation of conjugative DNA transfer. Previous genetic studies have suggested that TraYp is required for the formation of the site- and strand-specific nick that initiates DNA strand transfer (4). However, a biochemical role for this protein had not been elucidated. The data presented here suggest that TraYp plays an integral role in helping to recruit TraIp to the nic locus. Secondly, the results presented here reveal a critical role for the host-encoded IHF in the process of initiating conjugative DNA strand transfer. Thus a biochemical role for the previously described IHF binding sites in oriT (18) is revealed. It is important to note that both IHF and TraYp are required to stimulate the reaction catalyzed by TraIp; neither protein alone is sufficient. Moreover, extended titrations of both IHF and TraYp suggest that an increased concentration of one protein cannot compensate for the absence of the other protein. This is consistent with the data obtained in the deletion studies (see below).

The direct demonstration that TraYp, in conjunction with IHF, is required for site- and strand-specific nicking catalyzed by TraIp confirms a biochemical role for TraYp in generating IHF, is required for site- and strand-specific nicking catalyzed by TraIp. Furthermore, the concentration of TraYp required to observe half-maximal nicking of the linear DNA substrate (approximately 25 nM), in the presence of saturating concentrations of IHF and TraIp, is consistent with binding to the site previously identified as sbyA (15). Half-maximal nicking of the supercoiled substrate is observed at a TraYp concentration of approximately 150 nM. Apparently, alterations of the helical structure in supercoiled DNA reduce the affinity of TraYp for sbyA. These data suggest that TraYp binds independently to this site (as no binding cooperativity is observed between IHF and TraYp) and that sbyA must be occupied by TraIp in order for TraIp to bind and nick at the nic locus. Experiments using deletion mutants that encroach upon oriT from the right further underscore the importance of TraYp binding at sbyA for efficient nicking at oriT (26). In the in vitro experiments presented here, removal of the IHF B binding site resulted in a reaction that was still dependent on both TraYp and IHF. This indicates that IHF is bound at IHF A, presumably occluding sbyC, and therefore TraYp must be bound at sbyA. Also, a deletion that removed approximately one-half of the sbyA binding site reduced the efficiency of the nicking reaction. The affinity of TraYp for this truncated site was also slightly reduced, further supporting the notion that TraYp must be bound to sbyA to help recruit TraIp to the nic locus.

The biochemical role played by TraYp in the nicking reaction was previously unrecognized due to the absence of IHF in reconstituted nicking reaction mixtures. Tsai et al. (18) demonstrated the presence of two binding sites for IHF within the oriT region using direct footprinting studies. The IHF A site lies between the nic locus and the TraYp binding site. This IHF binding site has a higher affinity for IHF than the IHF B binding site, which is 50 bp distal to the TraYp binding site. Protein titration experiments and deletion studies support the idea that binding of IHF to the IHF A binding site, and not IHF B, is critical for recruiting TraIp to the nic locus. The concentration of IHF required for half-maximal nicking (~20 nM) in the presence of saturating concentrations of TraYp and TraIp is consistent with the occupation of the IHF A site by IHF. Moreover, deletion of the IHF B binding site has no effect on the in vitro nicking reaction as demonstrated here and had no effect on nicking observed in an in vivo assay (26). We suggest that IHF and TraYp bind independently to their respective sites in oriT and, under conditions of increased ionic strength, help recruit TraIp to the nic locus.

We envision two mechanisms by which IHF and TraYp might act to stimulate the site- and strand-specific nicking reaction catalyzed by TraIp. In one case, the two proteins might act to distort the DNA helix, perhaps to create a single-stranded DNA binding site for TraIp, at the nic locus. In support of this view, we have shown that TraIp is able to catalyze site-specific nicking of single-stranded DNA (13) while the protein does not specifically bind double-stranded DNA (7). Furthermore, TraIp can specifically nick the oriT region at low ionic strength, a condition that would favor the existence of single-stranded DNA character in a supercoiled DNA substrate. This mechanism is similar to that proposed for the action of DnaA protein at the origin of DNA replication (29). The second mechanism envisions protein-protein interactions that help to assemble a competent relaxosome at oriT. Both IHF and TraYp are known to bend the DNA when bound to their respective binding sites (18, 30). In addition, two sequence-directed bends have been localized in oriT (18). Together these factors might alter the overall conformation at oriT to bring the TraIp binding site into juxtaposition with the putative TraIp binding site. This could help to load TraIp at the nic locus and would be consistent with the notion of an interaction between TraYp and TraIp at the nic locus.

In summary, site- and strand-specific nicking at the oriT locus requires binding of both TraYp and IHF to their respective binding sites within oriT. Importantly, the IHF B binding site and the TraIp binding site do not seem to be important for nicking at oriT. This latter conclusion is now well supported by both in vivo and in vitro data. Presumably the binding of both IHF and TraYp help to direct the binding of TraIp to the nic locus. Precisely how this is accomplished remains to be determined. We also note that the results presented here are consistent with those recently reported by Inamoto et al. (31).

These authors performed a similar series of experiments using the traI and traY gene products from the related plasmid R100 and reached similar conclusions.

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The \textit{traY} Gene Product and Integration Host Factor Stimulate \textit{Escherichia coli} DNA Helicase I-catalyzed Nicking at the F Plasmid \textit{oriT}

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