JOURNAL OF BACTERIOLOGY, Nov. 2009, p. 6877–6887 Vol. 191, No. 22
0021-9193/09/$12.00 doi:10.1128/JB.00918-09

Plasmid R1 Conjugative DNA Processing Is Regulated at the Coupling Protein Interface▽

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Received 14 July 2009/Accepted 9 September 2009

Selective substrate uptake controls initiation of macromolecular secretion by type IV secretion systems in gram-negative bacteria. Type IV coupling proteins (T4CPs) are essential, but the molecular mechanisms governing substrate entry to the translocation pathway remain obscure. We report a biochemical approach to reconstitute a regulatory interface between the plasmid R1 T4CP and the nucleoprotein relaxosome dedicated to the initiation stage of plasmid DNA processing and substrate presentation. The predicted cytosolic domain of T4CP TraD was purified in a predominantly monomeric form, and potential regulatory effects of this protein on catalytic activities exhibited by the relaxosome during transfer initiation were analyzed in vitro. TraDΔN130 stimulated the TraI DNA transesterase activity apparently via interactions on both the protein and the DNA levels. TraM, a protein interaction partner of TraD, also increased DNA transesterase activity in vitro. The mechanism may involve altered DNA conformation as TraM induced underwinding of *oriT* plasmid DNA in vivo ($\Delta L_k = -4$). Permanganate mapping of the positions of duplex melting due to relaxosome assembly with TraDΔN130 on supercoiled DNA in vitro confirmed localized unwinding at *nic* but ruled out formation of an open complex compatible with initiation of the TraI helicase activity. These data link relaxosome regulation to the T4CP and support the model that a committed step in the initiation of DNA export requires activation of TraI helicase loading or catalysis.

Type IV secretion systems (T4SS) in gram-negative bacteria mediate translocation of macromolecules out of the bacterial cell (14). The transmission of effector proteins and DNA into plant cells or other bacteria via cell-cell contact is one example of their function, and conjugation systems as well as the transferred DNA (T-DNA) delivery system of the phytopathogen *Agrobacterium tumefaciens* are prototypical of the T4SS family. Macromolecular translocation is achieved by a membrane-spanning protein machinery comprised of 12 gene products, VirB1 to VirB11 and an associated factor known as the coupling protein (VirD4) (66). The T4SS-associated coupling protein (T4CP) performs a crucial function in recognition of appropriate secretion substrates and governing entry of those molecules to the translocation pathway (7, 8, 10, 30, 41). In conjugation systems substrate recognition is applied to the relaxosome, a nucleoprotein complex of DNA transfer initiator proteins assembled specifically at the plasmid origin of transfer (*oriT*). In current models, initiation of the reactions that provide the single strand of plasmid (T-strand) DNA for secretion to recipient bacteria is expected to resemble the initiation of chromosomal replication (for reviews, see references 18, 54, and 81). Controlled opening of the DNA duplex is required to permit entry of the DNA processing machinery. The task of remodeling the conjugative *oriT* is generally ascribed to two or three relaxosome auxiliary factors, of host and plasmid origin, which occupy specific DNA binding sites at this locus. Intrinsic to the relaxosome is also a site- and strand-specific DNA transesterase activity that breaks the phosphodiester backbone at *nic* (5). Upon cleavage, the transesterase enzyme (also called relaxase) forms a reversible phosphotyrosyl linkage to the 5′ end of the DNA. Duplex unwinding initiating from this site produces the single-stranded T strand to be exported. A wealth of information is available supporting the importance of DNA sequence recognition and binding by relaxosome components at *oriT* to the transesterase reaction in vitro and for effective conjugative transfer (for reviews, see references 18, 54, and 81). On the other hand, the mechanisms controlling release of the 3′-OH generated at *nic* and the subsequent DNA unwinding stage remain obscure.

Equally little is known about the process of nucleoprotein uptake by the transport channel. DNA-independent translocation of the relaxases TrwC (R388), MobA (RSF1010), and VirD2 (Ti plasmid) has been demonstrated; thus, current models propose that the relaxase component of the protein-DNA adduct is the substrate actively secreted by the transport system after interaction with the T4CP (42, 66). Cotransport of the covalently linked single-stranded T strand occurs concurrently (42). The mechanisms underlying relaxosome recognition by T4CPs are not understood. Direct interactions have been observed biochemically between the RP4 TraG protein and relaxase proteins of the cognate plasmid (65) and heterologous relaxosomes that it mobilizes (73, 76). TrwB of R388 interacts in vitro with relaxase TrwC and an auxiliary com-

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▽ Published ahead of print on 18 September 2009.

ponent, TrwA (44). TraD proteins of plasmid R1 and F are known to interact with the auxiliary relaxosome protein TraM (20) via a cluster of C-terminal amino acids (3, 62). Extensive mutagenic analyses (45) plus recent three-dimensional structural data for a complex of the TraM tetramerization domain and the C-terminal tail of TraD (46) have provided more detailed models for the intermolecular contacts involved in recognition.

Application of the Cre recombinase assay for translocation of conjugative relaxases as well as effector proteins to eukaryotic cells is currently the most promising approach to elucidate protein motifs recognized by T4CPs (56, 68, 78, 79). Despite that progress, the nature of the interactions between a T4CP and its target protein that initiate secretion and the mechanisms controlling this step remain obscure. In contrast to systems dedicated specifically to effector protein translocation, conjugation systems mobilize nucleoprotein complexes that additionally exhibit catalytic activities, which can be readily monitored. These models are therefore particularly well suited to investigate aspects of regulation occurring at the physical interface of a T4CP and its secretion substrate. For this purpose the MOB_F family of DNA-mobilizing systems is additionally advantageous, since DNA processing within this family features the fusion of a dedicated conjugative helicase to the DNA transesterase enzyme within a single bifunctional protein. The TraI protein of F-like plasmids, originally described as *Escherichia coli* DNA helicase I (1, 2, 23), and the related TrwC protein of plasmid R388 (25) are well characterized (reviewed in reference 18). Early work by Llosa et al. revealed a complex domain arrangement for TrwC (43). Similar analyses with TraI identified nonoverlapping transesterase and helicase domains (6, 77), while the remaining intermediate and C-terminal regions of the protein additionally provide functions essential to effective conjugative transfer (49, 71). The ability to physically separate the catalytic domains of TraI and TrwC has facilitated a detailed biochemical characterization of their DNA transesterase, ATPase, and DNA-unwinding reactions. Nonetheless, failure of the physically disjointed polypeptides to complement efficient conjugative transfer when coexpressed indicates a role(s) for these proteins in the strand transfer process that goes beyond the need for their dual catalytic activities (43, 50). The assignment of additional functional properties to regions within TraI is a focus of current investigation (16, 29, 49).

In all systems studied thus far, conditions used to reconstitute relaxosomes on a supercoiled *oriT* plasmid have not supported the initiation steps necessary to enable duplex unwinding by a conjugative helicase. The question remains open whether additional protein components are required and/or whether the pathway of initiation is subject to specific repression. In the present study, we applied the IncFII plasmid R1 paradigm to investigate the potential for interaction between purified components of the relaxosome and its cognate T4CP, TraD, to exert regulatory effects on relaxosome activities in vitro. In this and in the accompanying report (72), we present evidence for wide-ranging stimulatory effects of the cytoplasmic domain of TraD protein and its interaction partner TraM on multiple aspects of relaxosome function.

MATERIALS AND METHODS

Protein purification. Chromatography columns used for protein isolation were from Amersham Biosciences. The R1 plasmid proteins TraY and full-length TraI were purified as described previously (16) except that the final TraI fractions were pooled and applied to a Superdex 200 HR 10/30 column. *E. coli* integration host factor (IHF) was purified as described previously (22).

For overproduction of R1 TraDΔN130, a one-liter culture of *E. coli* C41(DE3) (51) carrying pSETraD was grown at 37°C in Luria-Bertani (LB) medium plus 100 μg/ml ampicillin. Isopropyl-1-thio-α-D-galactopyranoside (IPTG) was added to 1 mM at a culture density of an A_{600} of 0.5. After 6 h of shaking at 37°C, the cells were harvested by centrifugation and frozen at −80°C. Frozen cells were thawed overnight at 4°C and lysed as described previously (75). Solid ammonium sulfate (AS) was slowly added to the supernatant (fraction I) to 0.3 g/ml, and then the mixture was stirred for 30 min on ice. The precipitate was collected by centrifugation at 138,000 × *g* for 90 min at 4°C and then dissolved in a total volume of 15 ml buffer A (50 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)], pH 6.5, 0.1 mM EDTA, 5 mM magnesium acetate, 1 mM dithiothreitol [DTT], 0.1 mM phenylmethanesulfonyl fluoride [PMSF], 0.5% [vol/vol] Triton X-100) plus 0.02 M potassium acetate (KOAc). Fraction II was dialyzed against 2 liters of buffer A plus 0.02 M KOAc for 3 h. The dialyzed fraction was loaded on two 5-ml HiTrapQ columns connected in tandem. Adsorbed proteins were eluted with a 100-ml linear gradient of 0.02 to 0.5 M KOAc in buffer A. TraD eluted between 0.15 and 0.2 M KOAc. These fractions were pooled and dialyzed against buffer A plus 0.02 M KOAc. Fraction III was applied to a Resource Q 1-ml column, and proteins were eluted with a 30-ml gradient of 0.02 to 0.05 M KOAc in buffer A. Peak fractions were pooled, supplemented with glycerol to a final concentration of 20% (vol/vol), and stored at −80°C (fraction IV). This fraction was greater than 99% pure based on mass spectroscopy analysis. The oligomeric state was analyzed by blue native polyacrylamide gel electrophoresis as described previously (60). Final fractions of TraDΔN130 or TraI (as standard) were adjusted to equivalent final volumes with loading buffer containing 750 mM ε-aminocaproic acid and 5% Coomassie blue G250. Electrophoresis was performed through a linear 6 to 12% polyacrylamide blue native gel at 100 V at 4°C for 1 h. The buffer was exchanged before continuing for 2 h at 200 V. Proteins were stained with Coomassie brilliant blue. The molecular mass of the homo-oligomeric complexes was deduced by comparison with TraI monomer and the HW native protein marker kit (GE Healthcare).

TraM protein of plasmid R1 was overproduced in a 1-liter culture of *E. coli* C41(pET3a_TraM) grown at 37°C in LB medium containing 100 μg/ml ampicillin to an A_{600} of 0.5 before IPTG was added to 1 mM. Induced cultures were grown with shaking for 6 h at 37°C and then lysed in a French pressure cell. The cytoplasmic fraction was obtained by centrifugation at 126,000 × *g* for 30 min at 4°C. Solid AS (0.25 g/ml) was added to the supernatant, and the mixture was centrifuged at 126,000 × *g* for 1 h at 4°C. Solid AS (0.6 g/ml) was added to the supernatant, the precipitate was centrifuged, and the purification continued as described previously (59) with one additional chromatography step. Following size exclusion, the fractions containing TraM were loaded on a 1-ml Resource Q column equilibrated with buffer A (20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 1 mM DTT, 5% glycerol) plus 0.05 M NaCl. Proteins were eluted with a 30-ml linear gradient of 0.05 to 2 M NaCl in buffer A. Glycerol was added to TraM-containing fractions to a 40% final concentration, and the solution was stored at −80°C. The purified protein was >95% pure as determined by mass spectroscopy analysis. The expected oligomeric state (tetramer) of TraM was confirmed by gel filtration chromatography (Superdex 200 HR 10/30). Bovine serum albumin (BSA), β-lactoglobulin, cytochrome *c*, aprotinin, and vitamin B_{12} were used for calibration.

The C-terminally His_6-tagged transesterase domain, TraIN309, was overproduced in 500-ml cultures of *E. coli* BL21(DE3)(pET28aTraIrel) grown in LB medium containing 40 μg/ml kanamycin at 37°C to an A_{600} of 0.5 before IPTG was added to 1 mM. Cultures were incubated with shaking for 4 h at 37°C, harvested by centrifugation, and frozen at −80°C. Frozen cells were thawed overnight at 4°C and resuspended in 7 ml of a buffer containing 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 5% glycerol per gram of cell paste. The cells were lysed with 0.5 mg/ml lysozyme for 1 h at 0°C. This crude cell extract was centrifuged at 100,000 × *g* for 90 min at 4°C. The supernatant was applied to a 5-ml HiTrap chelating column equilibrated with buffer A (20 mM sodium phosphate, pH 7.4, 0.5 M NaCl) plus 10 mM imidazole. Bound protein was eluted with 5 column volumes of buffer A plus 500 mM imidazole. The TraIN309-containing fractions were pooled and dialyzed overnight against 4 liters of buffer B (25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl, 10% glycerol, 1 mM PMSF). Soluble AS was added to the final concentration of 1 M to the dialyzed fraction, and the fraction was loaded into a 5-ml phenyl Sepharose HP column equilibrated with

buffer B plus 1 M AS. The column was developed with a 50-ml decreasing gradient of 1 to 0 M AS. Peak fractions eluting at 270 mM AS were dialyzed against buffer B supplemented to 30% glycerol, concentrated with an Amicon filter device (Millipore), and then stored at −80°C.

All protein concentrations were determined using the Bradford protein assay (Bio-Rad) with BSA as a standard.

Mass spectrometry analysis. Selected protein bands were excised manually from the gel and subjected to in-gel digestion as described previously (70). Peptide extracts were dissolved in 0.1% formic acid, separated on a nano-high-performance liquid chromatography system (Ultimate 3000; LC Packings, Amsterdam, The Netherlands), and analyzed with a Thermo-Finnigan LTQ linear ion trap mass spectrometer (Thermo, San Jose, CA). The tandem mass spectrometry data were analyzed by searching the NCBI nonredundant public database with SpectrumMill Rev. 03.03.078 (Agilent, Darmstadt, Germany) software (4). Acceptance parameters were three or more identified distinct peptides as described previously (9).

DNA constructions. DNA-modifying reagents were used according to the manufacturers' recommendations. T4 DNA ligase and all restriction enzymes were provided by Fermentas. DNase I was obtained from Sigma. The Expand High Fidelity PCR system (Roche) was used for DNA amplification. The R1 *traM* allele from plasmid pGK111 (36) was modified to create appropriate ends, and a BamHI/NdeI fragment was introduced into pET3a (Novagen).

The first 924 bp of the R1 *drd19 traI* gene (GenBank accession no. AY423546) were amplified by PCR using primer pair 5′-AATTCCTCGAGAGCTGGCCC GGG-3′ and 5′-CCCTCTAGAAGGAGATATACAATGATGAGTATCGCGC AG-3′. The underlined XhoI and XbaI restriction sites, respectively, facilitated ligation to pET28a (Novagen).

Plasmid R1 *traD* was amplified from position 1458 to 3273 (GenBank accession no. AY684127) using 5′-GGAGATATACATATGAGCTGGATTCTGGG GCGTCAGG-3′ and 5′-CGCGGATCCTCAGAAATCATCTCCCGGCTC-3′, digested with NdeI and BamHI, and ligated to pET3a to obtain pSETraD.

Relaxase assay on supercoiled DNA. Reaction mixtures (20 μl) contained 40 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10% glycerol, 200 ng (4.4 nM) of pDE100 DNA (16), and either 75 nM TraI or 500 nM TraIN309. Various concentrations of single auxiliary proteins were additionally present within a series of repeated titrations ($n > 3$). The stimulatory effects of combinations of auxiliary proteins were then compared using final concentrations as indicated in the summary figures (see Fig. 1 and 3). Reactions were terminated after 20 min at 37°C by the addition of 1 μl of 20-mg/ml proteinase K and 5% sodium dodecyl sulfate (SDS). Samples were further incubated for 20 min at 37°C and then loaded onto 1.0% agarose gels containing 0.5 μg/ml ethidium bromide in Tris-borate-EDTA buffer (TBE). Products were resolved at 7 V/cm for 1 h in the presence of 0.1 volume of loading dye (1% SDS, 50% glycerol, 1× TBE, 0,05% bromophenol blue, 0,05% xylene cyanol). Gels were photographed under UV illumination, and the DNA was quantified using ImageQuant software (Molecular Dynamics). Statistical significance was determined by a one-sided Student *t* test. The data were fitted to a rectangular hyperbola using SigmaPlot 2008 software to generate curves in graphics shown.

Chloroquine agarose gel electrophoresis. Overnight cultures of *E. coli* AG1 (F^- *endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* λ^-) (Stratagene) harboring plasmid pGK111 (36) or pGK111M0 (57) were diluted to an optical density at 600 nm of 0.05 with 30 ml of fresh medium plus 100 μg/ml ampicillin and grown at 37°C with aeration for 10 h. The cultures were rapidly chilled in an ice-water bath, and the cells were collected by centrifugation at 8,000 × *g* for 5 min at 4°C. Supercoiled plasmid DNA was isolated by alkaline-SDS lysis and a Qiagen plasmid midikit according to the instructions of the manufacturer. The DNA was further purified with proteinase K treatment followed by phenol-chloroform extraction and then was recovered by ethanol. Electrophoresis was performed in horizontal slab gels containing 1% agarose and different concentrations of chloroquine (sodium diphosphate; Sigma), which was added to the agarose after boiling. For one-dimensional analyses, 0.5 μg of supercoiled plasmid DNA was separated by electrophoresis for 18 h at 2.6 V/cm in TBE buffer at room temperature. Chloroquine was removed from the gels prior to ethidium bromide staining by soaking the gels in distilled water for 6 h with hourly changes, followed by a wash in 1 mM $MgSO_4$ for 1 h. DNA was visualized after staining as described above. A test for the *cis* dependency of changed DNA topology required combining the same *oriT-traM* inserts in a relatively small vector backbone (pBR322) (34) with a second, larger replicon providing the *traM* gene (pMM-Mwt) or null allele (pMM-M0) in *trans* (39). *E. coli* AG1 transformants carrying each combination of two plasmids were cultivated, and the DNA was copurified as described above. Topoisomer resolution of 1 μg total DNA was performed in one dimension such that the two distinct plasmid populations in each sample were readily distinguished by size.

Two-dimensional gel electrophoresis was carried out by a procedure modified from that of Pruss (58). One microgram of supercoiled plasmid DNA was separated in the first dimension on a 1% agarose gel in Tris-phosphate-EDTA buffer (TPE), pH 7.2 (50 mM Tris base, 2.8 mM EDTA, 25 mM H_3PO_4), containing 25 μg/ml chloroquine for 18 h at 2.8 V/cm at 4°C. The running buffer was discarded, and the gel was soaked for 6 h at 4°C in fresh TPE buffer containing 100 μg/ml chloroquine. The gel was then rotated 90° in the electrophoresis chamber, and the separation was continued for 18 h at 1.7 V/cm in fresh TPE buffer containing 100 μg/ml chloroquine at 4°C. After electrophoresis, chloroquine was washed from the gel by soaking the gel in distilled water for several hours and finally in 1 mM $MgSO_4$ for 1 h. The gel was left overnight in distilled water and then photographed after being stained with ethidium bromide as described above.

Potassium permanganate assay. Supercoiled pGK111 (4.4 nM) was incubated at 37°C for 10 min with various concentrations of TraI, TraM, TraY, TraD, and IHF in 20 μl buffer containing 50 mM Tris-HCl, pH 7.5, 1 mM DTT, and 100 mM KCl. Potassium permanganate was added to a final concentration of 2 mM, and the incubation was continued for 2 min at room temperature. The reaction was stopped with 2.5 μl β-mercaptoethanol (14.7 M). Oligonucleotide primers were selected for both strands at flanking or internal positions relative to the protein binding sites: P1 (5′-TTTCCACCTCTGGTGAC), P2 (5′-GCACTTTC GCCATATGT), and P3 (5′-CACGAATTGAATCTAGAGTC). Primers were radiolabeled with T4 polynucleotide kinase (Fermentas) in the presence of [γ-^{32}P]ATP. DNA was ethanol precipitated and resuspended in 9 μl buffer containing 0.2 mM deoxynucleoside triphosphates, 1.5 mM $MgCl_2$, and 0.05 μM 5′-radiolabeled primers. Thermocycling was performed with 0.5 U DNA polymerase (Roche) for 25 cycles. Stop solution (95% formamide, 10 mM NaOH, 1 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol) was added (3 μl), and the reaction products were resolved electrophoretically on sequencing gels containing 8 M urea and 8% polyacrylamide (19:1) in TBE buffer at constant power (38 W) for 3 h. The gels were dried and visualized in a Typhoon 9400 system (Amersham Biosciences) using ImageQuant software.

RESULTS

Purification of TraD. T4CPs are inner membrane proteins, and their overproduction and purification have been hampered by problems of insolubility and a pronounced tendency of the proteins to form multimers or aggregates in vitro (65, 67). Deletion of the N-proximal transmembrane segments of the R388 T4CP, TrwB, resulted in a soluble form (52) suitable for biochemical and biophysical investigation (24, 74, 75). An experimental assessment of membrane topology of the F TraD protein (40) was consistent with hydropathy analyses (32), which predicted N-terminal membrane-spanning sequences extending to amino acid 130. Accordingly, for the current study we truncated the *traD* gene of plasmid R1 to overexpress solely the expected cytoplasmic domain of the protein. Protein TraDΔN130, lacking the 130-amino-acid N-terminal fragment, was abundantly overproduced in a soluble form that could be purified to homogeneity. Mass spectrometry confirmed that final fractions contained 99% TraD. The cytosolic form of TrwB, TrwBΔN70, was purified as a monomer, and yet under some in vitro conditions extensive multimers, which are thought to contain >20 monomers of native protein, form (52). The multimeric state of purified TraDΔN130 was evaluated using blue native polyacrylamide gel electrophoresis (63, 64). We estimate that 90% of the purified preparation of TraDΔN130 was monomeric, with minor amounts of trimeric and hexameric forms also present (not shown). In contrast to our previous work with full-length protein (65), larger multimers or aggregates were not detected.

TraD and TraM stimulate relaxosome activity in vitro. The availability of a pure, nonaggregating solution of TraD enabled investigation of its potential regulatory effects on the R1 relaxosome. The impact of TraD and its interaction partner

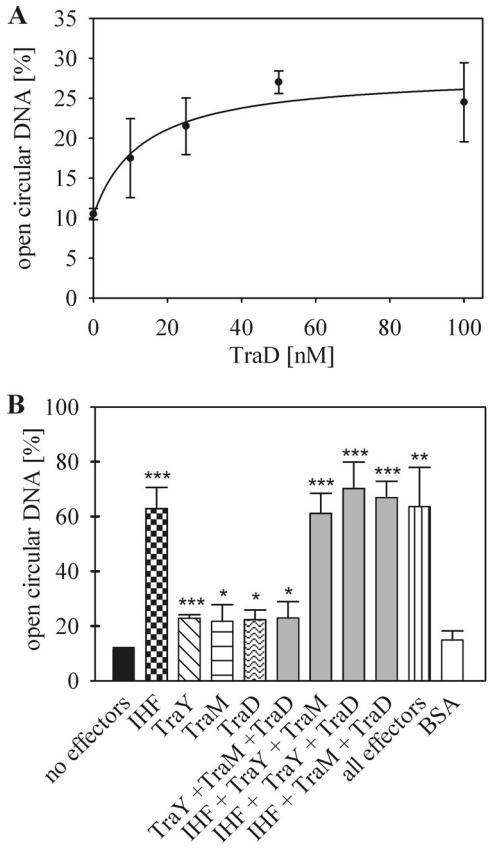

FIG. 1. Maximum stimulation of DNA transesterase activity. TraI-catalyzed conversion of supercoiled *oriT* DNA to the open circular form is expressed as percentage of total DNA substrate. *nic* cleavage was measured with 75 nM TraI alone or with increasing concentrations of individual auxiliary factors in independent experiments ($n = 3$), as illustrated for TraDΔN130 (A). The maximum stimulation of each effector (patterned bars) compared to TraI alone (black bar) is shown in summary (B). The final concentrations 75 nM TraI, 200 nM IHF, 200 nM TraY, 50 nM TraD, and 40 nM TraM (all proteins expressed as monomers, except for IHF, which was expressed as a heterodimer) were used to reconstitute the complete relaxosome (vertically striped bar) or the indicated subassemblies (gray bars). Statistical significance of the enhancement is shown (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

within the auxiliary components, TraM, on the TraI-catalyzed *nic* cleavage reaction on supercoiled DNA was compared with that of known effectors TraY and the *E. coli* IHF protein (16). Reaction mixtures contained a 17-to-1 ratio of TraI to substrate to mediate a low initial activity (12% open circle formation) in this assay. The effect of increasing concentrations of each protein was measured individually, as illustrated for TraDΔ130 (Fig. 1A). For each effector, the lowest concentration supporting maximum stimulation of the transesterase is shown (Fig. 1B). Significant enhancement was observed for each factor, with IHF supporting the highest (5.4-fold) level of stimulation. The positive effect of TraD and TraM on TraI was equivalent to that of protein TraY under these conditions. As we observed previously for TraY and IHF, the auxiliary activities of TraM and TraD were manifest independently and did not require the presence of other relaxosome components. This in vitro property distinguishes the R1 relaxosome from that of the related plasmid F (53, 59). The capacity of each effector to stimulate the reaction was observed at three independent TraI concentrations (not shown). To assess whether the stimulatory effect of each component may be additive, combinations of pairs (not shown) and triplets of effectors were surveyed. Synergistic effects were not observed under these conditions (Fig. 1), nor was stimulation observed in a control containing the maximum additional protein concentration (490 nM) as BSA. Notably, all reaction mixtures containing IHF showed a level of stimulation equivalent to that of the completely reconstituted relaxosome complex. We infer therefore that *nic* cleavage occurring during R1 conjugative transfer is predominantly enhanced by this host protein. A stimulatory role for the R1 TraM protein has not been shown previously in vitro but was expected based on our observations of TraM-mediated enhancement of *nic* cleavage in vivo (39). These data provide the first evidence of a contribution of the T4CP TraD to regulation of relaxosome function within the F-like systems. This capacity to stimulate its cognate relaxase is shared by the T4CP TrwB of plasmid R388 (52).

TraM modulates plasmid DNA topology. A hallmark feature of relaxosome auxiliary factors is that occupation of their specific DNA binding sites in the vicinity of *nic* introduces substantial topological distortion. Localized duplex melting facilitates sequence recognition by the cognate relaxase and cleavage at *nic* (5, 26, 81). In F-like systems, TraI stimulation within the relaxosome is thought to occur due to DNA topology changes, since the interaction of IHF with DNA induces strong distortions (61) and TraY binding to the F plasmid *oriT* induced bending of 50° (47). Paradigm MOB_P and MOB_F T4CPs investigated in vitro bind to single-stranded DNA (ssDNA) and double-stranded DNA without sequence specificity (52, 65). The efficiency of DNA strand passage catalyzed by eukaryotic type I topoisomerases is sensitive to deformations related to DNA writhing (11, 13, 48). The DNA relaxation reaction of the calf thymus enzyme has been used to monitor changes in plasmid supercoiling brought about by interactions with T4CP TrwB of R388 (52). A similar capacity to enhance plasmid supercoiling may therefore be predicted for the related TraD protein. In contrast, nothing was known about the potential for TraM protein to alter the topology of *oriT* DNA by interaction with its adjacent binding sites. To assess this potential activity, we checked for variation in the superhelical density of plasmid DNA due to the expression of TraM. Agarose gel resolution of plasmid topoisomers in the presence of the intercalating agent chloroquine is a very sensitive technique for measuring topological change. The two recombinant *oriT* plasmids compared were of identical size and carried the same fragment of plasmid R1 *oriT*, including the wild-type TraM binding sites. The adjacent *traM* gene of pGK111 is wild type, whereas in pGK111M0 a single T-for-G nucleotide substitution in the ATG start codon prevents *traM* translation. The null allele is defective for transfer and does not produce immunodetectable TraM protein (57). The plasmids were propagated in the same host background and then purified of protein. The populations of plasmid topoisomers were resolved in multiple agarose gels containing a broad range of chloroquine concentrations. Typical examples of the variation in the abundance and the distribution of topoisomers revealed by different conditions are shown in Fig. 2. Topoiso-

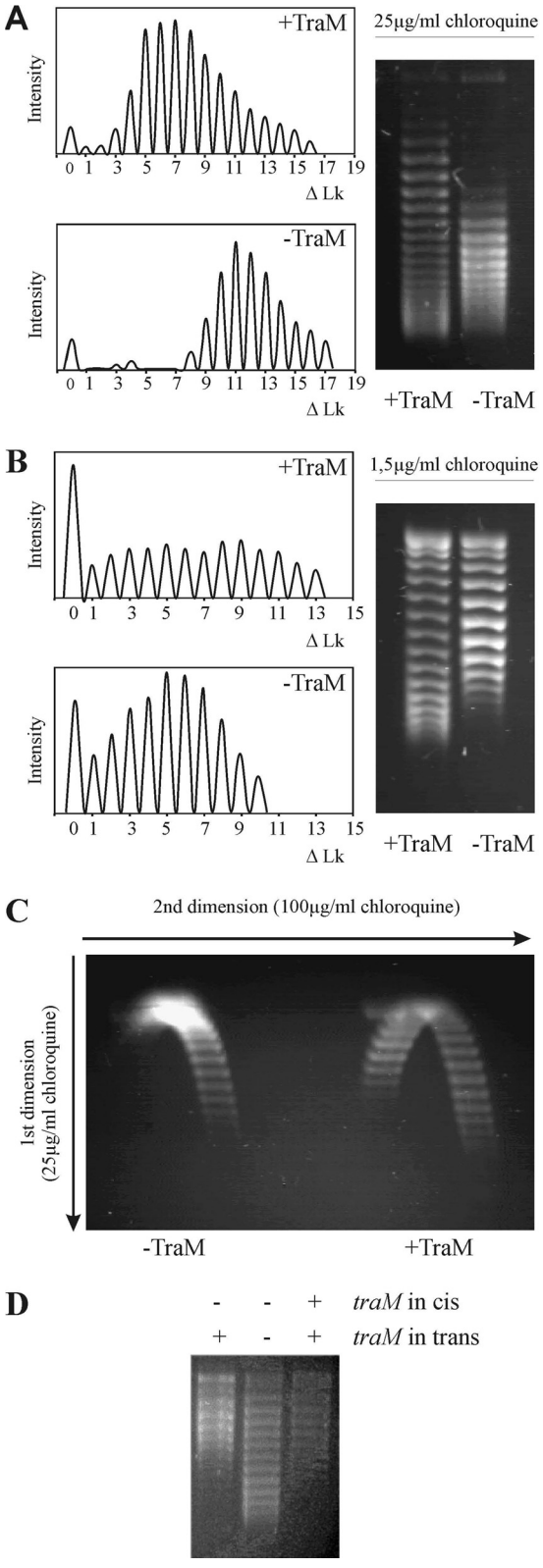

FIG. 2. *traM* expression in *cis* or in *trans* alters *oriT* plasmid supercoiling in vivo. Comparison of the supercoiling distributions of plasmid DNA propagated in bacterial cells in the presence of TraM (pGK111) and in the absence of TraM (pGK111M0). Populations of plasmid topoisomers were resolved by electrophoresis in the presence of the intercalating ligand chloroquine. (A and B) Plasmid DNA (500 ng) was resolved by one-dimensional electrophoresis in the presence of 25 μg/ml chloroquine (A) or 1.5 μg/ml chloroquine (B). The direction of migration was from top to bottom. Topoisomers were visualized subsequently by ethidium bromide staining as described in Materials and Methods. Quantification of the bands is illustrated graphically. The left side of the traces begins with the relaxed form (0), and the traces advance to the right in the direction of gel migration and depict the relative intensity of topoisomers with increasing relative linking numbers (ΔLk). (C) Electrophoretic resolution of 1 μg of pGK111 (+TraM) and pGK111M0 (−TraM) DNA through two dimensions. Chloroquine was present at concentrations of 25 μg/ml in the first dimension and 100 μg/ml in the second dimension. The directions of migration in each dimension are indicated. The distribution of negatively and positively supercoiled molecules is apparent along the left and right halves of the arcs, respectively. (D) Plasmid DNA isolated from cells carrying a substrate *oriT* plasmid and a second *traM* expression vector was resolved in agarose with 25 μg/ml chloroquine. The topoisomers of substrate plasmids only are shown. Sources of *traM* expression are indicated.

mers in adjacent bands differ by one in linking number (19). The supercoiling distribution exhibited by the TraM-producing plasmid was notably broader than that exhibited by the *traM* null plasmid. In the presence of 25 μg/ml chloroquine (Fig. 2A), the mobility of the DNA is impeded in direct proportion to the original level of negative supercoiling (19). Resolution of topoisomers of these plasmids in the presence of less chloroquine (1.5 μg/ml) confirmed the broader distribution of supercoiling and the increased superhelical density of plasmid exposed to TraM compared to those of the plasmid not exposed to TraM (Fig. 2B). At this concentration of chloroquine, more highly supercoiled topoisomers migrate more rapidly. Application of two-dimensional gel electrophoresis enables the resolution of negatively and positively supercoiled topoisomers with the same linking number, which comigrate in the first dimension. Under these conditions, the left side of each arc corresponds to negatively supercoiled DNA and the right portion corresponds to positively supercoiled topoisomers. These data demonstrate that plasmid pGK111 was more negatively supercoiled in vivo than was pGK111M0 DNA. This TraM-dependent variation in *oriT* plasmid topology was not limited to strain AG1 but was observed with a variety of *E. coli* K-12 strains (not shown). The effect was manifest in the absence of any other plasmid-encoded conjugation protein. To test whether the expression of *traM* had a general impact on cellular plasmid DNA topology, we analyzed the distributions of topoisomers of a second plasmid lacking the TraM binding sites when the source of TraM protein was provided in *trans*. An indirect mechanism of this nature seems unlikely, as the populations of pACYC184 topoisomers were indistinguishable in each case (not shown). The *oriT*-containing plasmids examined thus far also supported in *cis traM* transcription and translation (pGK111) or just transcription (pGK111M0 [57]). Given that both processes develop torsional stress, which may contribute to the observed variation, we examined the impact of TraM expressed in *trans* to an *oriT*-containing substrate plasmid. The test plasmid carried the *oriT* fragment including the *traM* null or wild-type allele. A much larger vector harboring the wild-type *traM* gene or the *traM* null allele in *trans* was simultaneously maintained in the same host cells. When analyzed in parallel, one-dimensional resolution of the pBR322-

based test plasmid revealed the same modulation of superhelical density in *oriT* DNA due to TraM expressed in *cis* as that observed when the source of TraM protein was provided in *trans* only (Fig. 2D). The shift in the degree of the underwinding observed was again typically equivalent to four linking numbers. Moreover, when mutant forms of TraM which are known to lack site-specific DNA binding capacity were tested, relative variation in superhelical density of the *oriT* plasmids was no longer observed (not shown). We conclude that the presence of TraM protein in vivo increases the negative supercoiling of plasmid DNA carrying the TraM sites of binding and that the observed modulation is a direct consequence of the interactions of the protein with these sites. This property may be sufficient to explain the enhancement of TraI-catalyzed *nic* cleavage during conjugation but does not rule out a functional contribution from interactions between the TraM and TraI proteins at this or later stages of conjugative DNA processing.

The auxiliary proteins but not TraD stimulate the isolated TraI transesterase domain in cleaving supercoiled DNA. To gain insight into whether interactions between proteins have a measurable impact on TraI catalysis in vitro, we performed comparative analyses of enzyme activities in a number of characterized assays. In the first approach, we replaced the full-length TraI protein (1,756 amino acids) with a truncated form, TraIN309 (residues 1 to 309), which manifests the N-terminal DNA transesterase domain of the protein, and asked whether the effector-mediated stimulation of *nic* cleavage was also exhibited by the much smaller transesterase domain. Significant enhancement of the reaction catalyzed by the isolated transesterase domain was observed for the individual effectors IHF, TraY, and TraM, but notably the stimulation mediated by TraD in combination with the full-length TraI (Fig. 1) was no longer exhibited by TraIN309 (Fig. 3). Reconstitution of the reaction with all effectors revealed a significant additive effect relative to the stimulation measured for each individual protein and additionally compared to the combined effect of IHF, TraY, and TraD. The capacity of each protein to stimulate a much smaller relaxase protein than the full-length TraI neither supports nor rules out a mechanism of stimulation that relies on protein-protein interaction. In contrast, the failure of TraD to stimulate the transesterase domain alone suggests that interactions between TraD and a TraI domain not present on the truncated TraIN309 protein are important to regulation. To establish whether TraDΔN130 stimulation of this reaction is indeed unique to the full-length protein, enzyme activities were analyzed in multiple parallel experiments. For comparison of the effects of TraDΔN130 on the distinct relaxases, averaged data from repetitions over the same concentration range of effectors were normalized by the initial relaxase activity exhibited without TraD. The activity of relaxase alone was set to 1, and the enhancement observed as a function of TraDΔN130 concentration was plotted as an increase in enzyme activity (Fig. 3A). These data confirmed that product formation with the full-length TraI was typically more than doubled in the presence of TraD, while the reaction catalyzed by TraIN309 was not affected.

Conjugative relaxases catalyze *nic* site cleavage on ssDNA and, when presented with an additional ssDNA substrate containing the *nic* recognition sequence, can perform a recombination via joining the cleaved reaction products of distinct

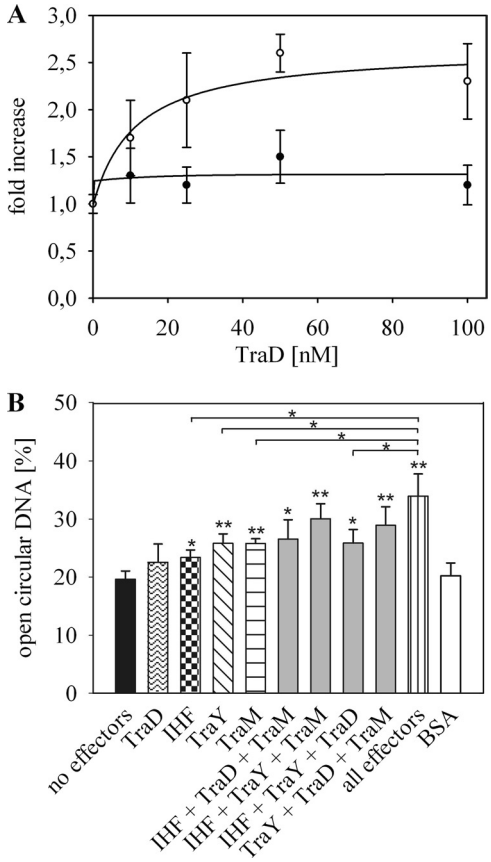

FIG. 3. TraD stimulation of DNA transesterase activity is lost with the N-terminal TraIN309 domain. (A) The relative effects of the presence of TraDΔN130 on the relaxation reactions catalyzed by full-length TraI (○) and by the isolated transesterase domain (TraIN309) (●) on supercoiled *oriT* DNA were compared. Variation in open circle formation as a function of TraD concentration is expressed as increase in activity over the activity exhibited by enzymes alone (value set to 1). (B) Conversion of supercoiled *oriT* DNA to the open circular form by the isolated transesterase domain (TraIN309) is expressed as percentage of total DNA substrate. *nic* cleavage was measured with 500 nM TraIN309 alone or with increasing concentrations of individual auxiliary factors in independent experiments ($n = 3$). The maximum stimulation of each effector (patterned bars) compared to that of TraI alone (black bar) is summarized. The final concentrations 500 nM TraIN309, 12.5 nM IHF, 200 nM TraY, 20 nM TraD, and 12 nM TraM (all proteins expressed as monomers, except for IHF, which was expressed as a heterodimer) were used to reconstitute the complete relaxosome (vertically striped bar) or the indicated subassemblies (gray bars). Statistical significance of the enhancement compared to TraIN309 alone is shown above the bars (*, $P < 0.05$; **, $P < 0.01$). Statistical significance of the additive enhancement mediated by all effectors compared to one or more effectors is indicated above the brackets.

substrates (55). We chose this assay as an alternative approach to screen for evidence of protein-induced effects on TraI catalysis. Since the oligonucleotide *nic* substrates lack double-stranded DNA recognition sequences for the auxiliary proteins, it was assumed that modulation of the cleavage and recombination assay would arise only due to interactions on the protein level. This strategy was successful in the R388 system for detecting TrwA-TrwC interactions, which alter TrwC activity (12). Systematic application of the R1 compo-

nents using two oligonucleotides containing *nic* and both the full-length TraI and TraIN309 failed to reveal an influence of either of the auxiliary components alone, or in combination, on the yield or relative proportion of cleaved or recombinant oligonucleotide products (not shown). Moreover, TraD had no impact on this reaction.

In a final screen, the DNA-dependent ATPase activity of TraI was analyzed in the coupled enzyme assay (27) performed according to the method of Tato et al. (75). The kinetic parameters of TraI activity were in good agreement with our previous data and published values (38). The presence of additional conjugation proteins over a broad concentration range had no observable impact on this reaction (not shown). A significant inhibition of the ATP hydrolyzing activity was detected for *E. coli* IHF at higher concentrations. We postulated that the finding reflected a nonspecific interaction of IHF with the required ssDNA effector of ATPase activity. Indeed, inhibiting ratios of IHF to M13 DNA in the ATPase assay produced a detectable mobility shift for that DNA in an agarose gel (not shown). In summary, these data reveal that the *nic* cleavage reaction is potentially regulated by interactions of TraD with regions of TraI outside the relaxase domain but generally support a model where control of TraI catalysis is largely independent of specific enzyme-effector interactions.

TraI binding induces duplex melting at *nic*. To better understand the mechanistic contributions of relaxosome proteins to the initiation stage of T-strand transfer, we sought to map the position and extent of relaxosome-induced duplex melting at *oriT*. In support of the transesterase reaction, binding of single proteins or relaxosome assembly would be predicted to distort duplex DNA at *nic*. Moreover, the subsequent process of introducing the TraI helicase onto the DNA for directional translocation and duplex unwinding requires a substantial single-stranded character. Previous work (16) demonstrated that efficient TraI helicase activity on heteroduplexes of the R1 *oriT* was attained only when substrate DNA harbored broad regions of open duplex (≥60 bp). In the current study, we applied potassium permanganate footprinting to determine whether and to what extent the binding of relaxosome components to supercoiled *oriT* DNA in vitro induced melting at *nic* and/or the surrounding sequences. Each effector protein was independently varied over a broad concentration range (5 to 800 nM). In the subsequent primer extension, both the cleaved T strand and the complementary strand were analyzed for hypersensitivity to permanganate. In all experiments, we detected strong polymerase termination sites in an AT-rich region that was present on the supercoiled plasmid independently of protein (Fig. 4). Notable protein-induced distortion was not detected for any of the individual effectors under these conditions (not shown). In contrast, TraI alone generated localized melting at the *nic* position (Fig. 4). The additional presence of the reconstituted relaxosome (with TraD) present in concentrations that supported the maximum of TraI transesterase activity on supercoiled DNA (Fig. 1) did not result in an extension of duplex melting at *nic* or elsewhere in the *oriT* region. Moreover, this combination of protein in the absence of TraI was not sufficient to unwind detectably at *nic*.

To our knowledge, this study combines the most extensive set of IncF conjugation proteins investigated to date and demonstrates that initiation of TraI helicase activity on supercoiled DNA in vitro requires reconstitution of a subsequent, undefined step of *oriT* activation. A minimum prerequisite for that activation induces sufficient duplex melting to support helicase loading. Moreover, these data establish that interaction with the T4CP contributes to DNA processing steps, which are essential to relaxosome function.

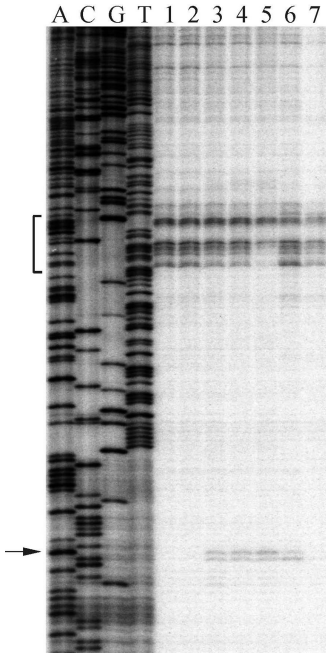

FIG. 4. Protein-induced duplex melting at *oriT*. Primer extension of the complementary (noncleaved strand) was performed in vitro with supercoiled pGK111 DNA in the absence of protein with no prior $KMnO_4$ treatment (lane 1) or after $KMnO_4$ incubation (lane 2). In all other samples permanganate footprinting followed a coincubation of DNA and protein as indicated. Lanes 3 to 5, increasing concentrations of TraI: 50 nM (lane 3), 85 nM (lane 4), and 200 nM (lane 5). Lanes 6 and 7, all effectors (200 nM IHF plus 200 nM TraY plus 40 nM TraM plus 50 nM TraD) with 85 nM TraI (lane 6) or without TraI (lane7). A, C, G, and T designate R1 *oriT* sequencing reactions created with the same primer (P1). The location of the hypersensitive region at *nic* is shown (arrow). The A/T-rich strong-stop region for polymerase is bracketed.

DISCUSSION

The molecular mechanisms of macromolecular secretion by T4SSs, including those governing the initiation of translocation, have not been elucidated. In the case of T4SSs dedicated to protein translocation only, secretion can be monitored with reporter assays indicative of recipient cell uptake on the basis of changed cellular morphology, physiology, or gene expression profiles. The assays have some limitations in that the outcome ultimately reflects a culmination of effects occurring over the entire process. The premise behind this study was that conjugation systems should provide a tractable model for investigating the mechanisms controlling secretion initiation since the substrate proteins are enzymes with measurable activities. Moreover, these enzymes are integral components of larger nucleoprotein complexes exhibiting well-defined biochemical properties. We further postulated that interactions

between proteins at the interface of a T4CP and a relaxosome displayed as a translocation substrate are likely to be crucial for delivery to the T4 transport channel and possibly T-strand production per se.

A corollary aim for the study was thus to advance our understanding of T-strand transfer initiation beyond the current impasse in reconstituted systems. In the in vitro approaches taken thus far, relaxosomes do not progress from a preinitiation complex (i.e., high-affinity interactions of the relaxase and auxiliary factors that support *nic* cleavage) to a true initiation complex competent to unwind the plasmid T strand. The challenge resembles that of reconstituting eukaryotic DNA replication initiation (15, 21, 33, 69). The conjugative helicase is recruited to *oriT* by virtue of the sequence recognition properties of the relaxase domain but remains inactive. The initiation of helicase motor activity and duplex entry may require an active step to induce melting at the transfer origin or perhaps the reversal of an intrinsic repression. It is further conceivable that this "activation" mechanism depends on physical contact between the relaxosome and components of the T4 transport channel, with the T4CP, or even additional unidentified factors.

In this and the accompanying work (72), we explore the potential for T4CP TraD of the conjugative paradigm plasmid R1 to modulate biochemical properties ascribed to the relaxosome. In this report, we describe functional effects of TraD on the topology of supercoiled *oriT* DNA and on in vitro properties of the relaxosome which are central to preinitiation. The degree of functional integration is apparently strong, since these experiments revealed properties of TraD that are conventionally used to define a relaxosome accessory factor. We show that the R1 TraI DNA transesterase activity was stimulated by TraD and TraM in addition to the known in vitro effectors TraY and *E. coli* IHF (16). Stimulation observed with IHF was the most pronounced; thus, enhancement of *nic* cleavage may be the primary role for IHF at transfer initiation. Cleavage at *nic* is a prerequisite for T-strand transfer, but additional high-affinity interactions of relaxosome components with sequences surrounding *nic* are likely to be important to the progression of DNA processing during conjugation. The number of alternative components in the R1 relaxosome that enhance the cleaving reaction in vitro and in vivo (34, 39) hints at a complex series of (yet-undefined) interactions that coordinate the production of T strand for uptake to the secretion pathway. As demonstrated in the accompanying report, TraD, TraM, and IHF indeed enhance the efficiency of TraI helicase activity at *oriT*, presumably by facilitating an early step in the multistep process of duplex unwinding (72).

The underlying mechanisms of positive regulation of *nic* cleavage observed here may rely on protein-induced DNA distortion, require interaction between proteins, or involve components of both. To differentiate between hypotheses, we compared TraI catalytic activities in different tests, which by design could reflect only changed enzyme activity due to protein-protein interaction. Neither the DNA-dependent ATPase activity nor oligonucleotide cleaving and recombination reactions of TraI were altered by the presence of the effector proteins. Recent evidence suggests that TraM and TraI of plasmid F interact physically via the TraI C terminus (59). Although a similar interaction between R1 components is likely and may be important to regulation of the transesterase, that contact did not alter other TraI catalytic activities under our test conditions. In contrast, TraD stimulation of the relaxase reaction on supercoiled DNA was lost when the full-length TraI protein was replaced by the isolated transesterase domain. This finding implies that interaction between TraD and a region of TraI outside the N terminus could contribute to regulation. This interpretation has been strengthened in an independent study aimed at mapping motifs in TraI required for TraD-mediated protein translocation (S. Lang and E. L. Zechner, unpublished data). We find that the portions of the TraI protein mediating uptake by the T4CP and the secretion pathway lie outside the relaxase domain. Beyond this observation, the experiments reported here support a model where strong protein interactions are not required to control the *nic* cleavage reaction.

A substantial impact on plasmid DNA topology was demonstrated for TraM that has not been reported previously. Isolation of populations of topoisomers of plasmids carrying the R1 TraM binding sites, *sbmA* and *sbmB*, revealed the extent of modulation in superhelical density induced in TraM-producing *E. coli* cells. Broader topoisomer distributions and a relative increase in negative supercoiling ($\Delta L_k = -4$) were generally observed when TraM—expressed either in *cis* or in *trans*—was able to occupy those sites. Detection of the shift in linking number for a plasmid population required both regions of TraM binding and was independent of additional conjugation proteins. Our earlier work revealed an enhancement of negative supercoiling by the TraD-related T4CP TrwB (52). The F TraY protein increases bending of F *oriT* (47) and is believed to wrap DNA into nucleosome-like structures when bound to simple repeats of $d(GA)_n/d(TC)_n$ on unrelated molecules (37). Extreme planar bending is a hallmark of the IHF interaction with DNA (61). Nonetheless, for topological distortion of plasmid DNA to be relevant to relaxosome function at the preinitiation stage, the effect should manifest in nick site unwinding. To support a transition to open complex formation and helicase activation, the point of duplex melting must spread to a sufficient extent to mediate loading of the TraI helicase domain. Potassium permanganate mapping of the protein-induced topological change on supercoiled *oriT* DNA in vitro revealed a highly specific opening of 2 to 3 bp at *nic* that was mediated by TraI alone or in the reconstituted relaxosome. In contrast single effectors and their combinations did not result in localized melting anywhere else in the entire *oriT* region that would be compatible with the ssDNA length requirements previously defined for detectable TraI helicase activity (16, 17). The simplest explanation for the lack of progression of the reconstituted preinitiation complex to a complex competent for T-strand unwinding would be that the torsional strain induced during assembly of this initial complex in vitro is distributed generally over the plasmid and does not force extended duplex melting at *nic*-adjacent sequences. In contrast to the in vitro situation, it can be speculated that closed topological domains may exist on the 100-kb conjugative R1 plasmid in vivo that would have the potential to constrain protein-induced torsional stress to the *oriT* region.

Results in this report focus on preinitiation functions of the relaxosome and advance our understanding of the regulation of this stage while the relaxosome is docked to the T4CP

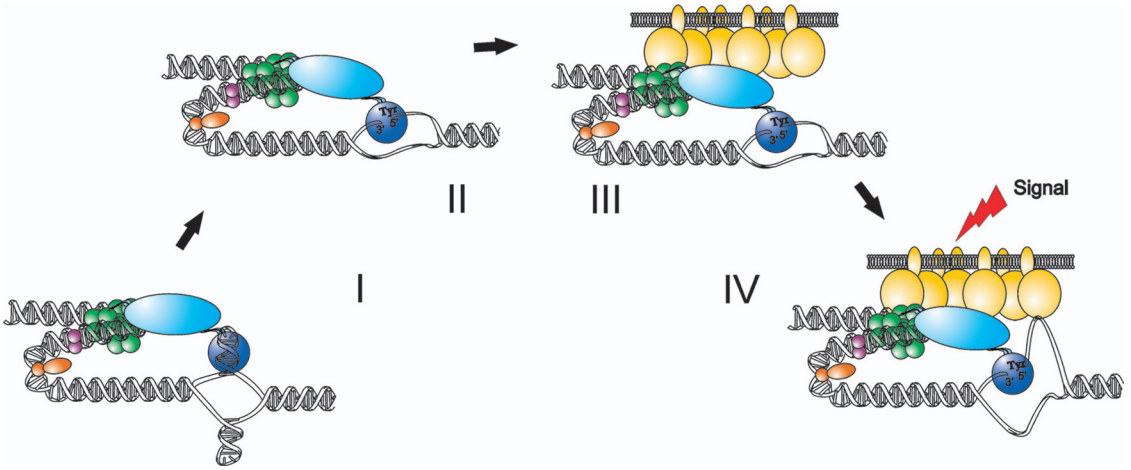

FIG. 5. Model for regulation of the preinitiation stage of relaxosome function. Early steps in the initiation pathway of MOB_F conjugative plasmid strand transfer (I to IV) are described in the text. Protein components are illustrated as follows: the bifunctional TraI transesterase (blue) with active site tyrosine (Tyr) and TraI helicase domains (light blue), IHF (orange), TraY (purple), TraM (green), and the T4CP TraD (yellow).

conjugative pore. Control of the TraI transesterase was affected by the T4CP and accessory protein components via DNA and protein interactions. Our findings are summarized in the following model (Fig. 5). (i) In vitro a relaxase can both bind and cleave supercoiled *oriT* DNA in isolation. Auxiliary components facilitate assembly of the relaxosome initiation complex, induce topological change, and enhance *nic*-cleaving activity. Although dispensable in vitro, these additional factors and their cognate DNA binding sites are essential for efficient conjugative DNA transfer. (ii) An equilibrium of the cleaving and joining reaction maintains the relaxosome at preinitiation readiness. (iii) Physical contact with the membrane-anchored T4CP contributes to preinitiation DNA processing in a manner analogous to that of traditional effectors IHF, TraM, and TraY. In vitro the reconstituted complex supports a focused duplex unwinding at *nic*, but in the absence of extended melting, TraI helicase cannot productively load onto an ssDNA template and initiate helical unwinding. (iv) Progression to a committed step of T-DNA production requires activation of origin melting and helicase initiation. Given the intimate association of the relaxosome with the T4CP at the pore of the membrane-spanning transport channel, induction of transfer initiation should be exerted at this interface. Monomers of T4CP TrwBΔ70 build hexamers in vitro in a manner stimulated by interactions with R388 relaxosome components and both DNA and nucleotide ligands (74). In the current study, we sought to stimulate multimer formation by TraDΔN130 in the presence of R1 relaxosome proteins, nucleotide triphosphates, and various DNA substrates. Although TrwBΔ70 performed as expected in these experiments, hexamer formation by R1 TraDΔN130 was not observed. It follows that this property of TrwB may not be general for T4CPs. Instead, transporter components or conditions particular to the membrane environment missing in our analysis may be additionally important to functional assembly of TraD and possibly other T4CPs. In support of the latter hypothesis, the presence of the membrane-spanning domain altered interactions of native TrwB with some ligands in vitro (31). Moreover, Traxler and colleagues have shown that associations between F plasmid TraD monomers in vivo require N-terminal transmembrane sequences and that formation of stable, higher-order TraD oligomers in the inner membrane also appeared to involve other F proteins (28). Accordingly, the model of Fig. 5 proposes that, in the absence of conjugation, the T4CP monomers are maintained in the inner membrane as a subassembly. Formation of functional hexamers and activation of the essential nucleotide triphosphate hydrolyzing activity would require positive regulation. In that case, conjugative transfer initiation would be controlled by external signals, possibly emerging through productive intercellular contacts conveyed over the conjugative pilus and further communicated from the T4CP to the DNA processing machinery. Early genetic experiments with the F plasmid fueled speculation about a similar induction pathway (80). Remarkably, N. Willetts' prognosis was made when only rudimentary mechanistic detail was known (35). Nonetheless, advances in the field over 30 years, including the current results, continue to support this very early hypothesis.

ACKNOWLEDGMENTS

We thank G. Rechberger for his assistance with mass spectroscopy and L. Frost, K. Zangger, R. Zechner, W. Keller, and B. M. Mayer for helpful discussions. S. Köstenbauer, M. Gauster, and H. Gerhold are acknowledged for their contributions to this study.

The work was supported by the Austrian Science Fund through FWF grants P18607 and W901-B05 DK: Molecular Enzymology, the Austrian ÖAD 23/2004, and the EU FP6 PL 019023; Spanish Ministry of Education grant BFU2008-00995/BMC; and the Spanish Ministry of Health RD06/0008/1012 (RETICS research network, Instituto de Salud Carlos III).

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