A central step in the transfer of genetic information during bacterial conjugation of the Escherichia coli F plasmid involves the formation of a protein-DNA complex, called the relaxosome, at the origin of transfer. During conjugation, the relaxosome introduces a site- and strand-specific nick from which the physical transfer of a single strand of DNA is initiated. At least two F-encoded proteins, Tralp (traY gene product) and TraYp (traY gene product), and one host-encoded protein, integration host factor, are involved in this process. In this report, we use DNase I protection and electron microscopic techniques to investigate the mechanism of relaxosome formation. Our results show that TraYp and integration host factor form a protein-DNA complex that facilitates the binding of TraIp to assemble a relaxosome capable of introducing a site- and strand-specific nick at the origin of transfer. This nick is identical to that observed during conjugation.

Bacterial conjugation, mediated by the Escherichia coli F factor, leads to the transfer of genetic material from one bacterial cell to another. Conjugation begins with the formation of a pilus bridge between a donor (F +) and recipient (F –) bacterium. In response to an as yet unidentified signal, DNA transfer is initiated from a site- and strand-specific nick within a region of F defined as the origin of transfer (oriT). From this site, a single strand of DNA is driven through the mating junction into the recipient cell in a 5' to 3' direction. DNA synthesis replaces the transferred strand in the donor and converts the transferred strand to double-stranded DNA in the recipient (for reviews, see Willetts and Wilkins (1984), Ippen-Ihler and Minkley (1986), Willetts and Skurray (1987), Wilkins and Lanka (1993), and Frost et al. (1994)).

A key step in this process is the formation of a protein complex at oriT called the relaxosome. Plasmids containing oriT can be isolated from F – bacteria as relaxosomes, so called because treatment of these plasmid protein-DNA complexes with protein denaturants results in the release of DNA superhelicity (Wilkins and Lanka, 1993; Thompson et al., 1989). Loss of superhelicity is due to the formation of a site-specific nick located on the strand destined for transfer during conjugation. The location of this site- and strand-specific nick has been identified by sequence analysis of relaxed DNA, and it is from this site that DNA transfer is initiated (Thompson et al., 1984; Thompson et al., 1989).

Genetic studies have revealed that mutations in the F plasmid tral and traY genes affect relaxosome formation in vivo, implicating these proteins in formation of the relaxosome at oriT (Everett and Willetts (1980), Traxler and Minkley (1988), also see Traxler and Minkley (1987)). Although biochemical and genetic studies have since determined a role for the traR gene product (TraIp in relaxosome formation (Traxler and Minkley, 1988; Matson and Morton, 1993; Reygers et al., 1991), until recently, the role of the traY gene product remained unknown (see below). Various TraI mutants have been studied, and the results suggest that the protein contains two distinct functional domains (Traxler and Minkley, 1988). The amino-terminal domain appears to be required for relaxosome formation, whereas the carboxyl-terminal domain is required for steps subsequent to formation of the relaxosome. Purified TraIp (also known as DNA helicase I) has long been known to contain DNA helicase activity (Abdel-Monem et al., 1983; Lahue and Matson, 1988), and it has been shown that the carboxyl-terminal half of the protein is a DNA-dependent ATPase (Traxler and Minkley, 1988; Reygers et al., 1991). Therefore, it appears that the traI gene encodes a bifunctional protein containing an amino domain with DNA strand scission activity and a carboxyl domain with DNA helicase activity.

Under defined conditions in vitro, TraIp alone has been shown to form relaxosomes with plasmids containing oriT (Matson and Morton, 1991; Reygers et al., 1991). Treatment of these relaxosomes with protein denaturants such as SDS results in the conversion of supercoiled plasmids to the relaxed open circular form via formation of a nick within oriT. The location of this nick is identical to that observed in plasmids isolated as relaxosomes in vivo. It is believed that the DNA is nicked prior to the addition of protein denaturants with the continuity of the DNA strand being maintained by a TraIp bridge. The addition of protein denaturants disrupts the protein bridge, exposing the nick and allowing DNA supercoils to be released. The nature of the reaction catalyzed by the TraIp to form the site- and strand-specific nick at oriT has been studied biochemically (Matson and Morton, 1991; Reygers et al., 1991; Matson et al., 1993; Sherman and Matson, 1994; Nelson et al., 1995). It occurs by a transesterification reaction that requires Mg2+, low salt conditions, and a supercoiled DNA substrate. Following DNA relaxation, the 5' phosphate at the nick site remains covalently associated with the TraIp, whereas the 3' hydroxyl is free and available for extension synthesis by DNA polymerase I. Together with the available genetic data, these results indicate that TraIp is the enzyme that introduces the site- and strand-specific nick within oriT prior to DNA strand transfer.

Genetic and biochemical analysis of oriT reveals the region...
adjacent to the nick site to be complex (see Fig. 8). It contains two binding sites for integration host factor (IHF) 1 (Tsai et al., 1990), one TraYp binding site (Nelson et al., 1993), one TraMp binding site (Laurenzo et al., 1992), two intrinsic DNA bends (Tsai et al., 1990), and several inverted sequence repeats (Frost et al., 1994). The occurrence of IHF, TraYp, and TraMp binding sites within oriT suggests these proteins may play a role in DNA strand transfer. A combination of linker-scanning and deletion mutagenesis has been employed to determine which of these cis-acting sequence elements are involved in the DNA strand transfer reaction (Fu et al., 1991; Nelson et al., 1995). Based on these studies, oriT can be divided into two regions. The first region, consisting of the IHF B and TraMp binding sites, is required for efficient DNA transfer but not relaxosome formation. The second region, containing the IHF A and TraYp binding sites, is required for both relaxosome formation and DNA transfer. These results suggest that, in addition to TraIp, IHF and TraYp are involved in steps leading to relaxosome formation and formation of a site- and strand-specific nick at oriT. TraMp is most likely required for subsequent steps during bacterial conjugation.

Nelson et al. (1995) have determined a biochemical role for TraYp and IHF in the formation of a relaxosome at the F plasmid oriT. They demonstrated that these proteins can stimulate the TraMp-catalyzed nicking of supercoiled DNA in vitro, relax the topological requirement for a supercoiled DNA substrate, and relieve NaCl inhibition. Although the oriT region also contains several binding sites for TraMp, the addition of purified TraMp had no detectable effect on relaxosome formation. In this report, we have investigated the mechanism by which IHF, TraYp, and TraIp coordinate with oriT DNA to form the relaxosome. The complex is formed in a stepwise manner in which IHF and TraYp assemble onto DNA prior to the TraIp. IHF and TraYp facilitate TraIp binding at oriT under conditions that preclude TraIp from binding alone. In addition, we have applied a combination of DNase I protection and electron microscopic techniques to probe the structure of the F plasmid relaxosome.

**EXPERIMENTAL PROCEDURES**

**Materials**

TraIp, TraYp, and IHF were purified as described in the accompanying paper (Nelson et al., 1995). Restriction enzymes, calf intestinal phosphatase, and T4 polynucleotide kinase were purchased from New England Biolabs and used according to manufacturers specifications. Poly(dI-dC)poly(dI-dC), used as competitor DNA in DNase I reactions, was obtained from Pharmacia Biotech Inc. Radiolabeled nucleotides were used in DNA-labeling and sequencing reactions were performed using the alkaline lysis method described in Maniatis et al. (1982) and twice banded on CsCl gradients to obtain plasmids that were >95% supercoiled.

**Methods**

Relaxosome Formation and DNase I Experiments—Relaxosomes were prepared by incubating 6.7 mM pBSoriT (plasmid pBS containing the F plasmid oriT, see Matson and Morton (1991)) with TraIP, IHF, and TraMp as described in appropriate figure legends in a reaction buffer that contained 40 mM Tris-HCl (pH 7.5), 6 mM MgCl2, 75 mM NaCl, 10% glycerol, 5 mM dithiothreitol, and 25 mM bovine serum albumin. The protein-DNA complexes established in this reaction mixture were then exposed to 1% SDS and 500 μg/ml proteinase K for 15 min at 37°C. Products were then resolved by electrophoresis on a 0.8% agarose gel and visualized by EtBr staining. Exposure of the gel to UV light (254 nm) for 1 h converted all species to relaxed open circular DNA. The gels were then restained. This process ensures that all DNA species within the gel bind an equal amount of EtBr. The relative quantities of DNA within the bands corresponding to supercoiled and nicked DNA were then determined by laser densitometry of photographic negatives taken of each gel.

Relaxosomes for DNase I protection experiments were prepared as described above except that 5 μg/ml poly(dI-dC)poly(dI-dC) was included and the substrate, pBSoriT, was digested with XbaI, treated with calf intestinal phosphatase, and 5' end-labeled with [γ-32P]ATP using T4 polynucleotide kinase according to manufacturer's specifications. The labeled DNA was then digested with SaeI to generate two fragments 20 and 3471 bp in size (the larger fragment contains oriT), each uniquely labeled at the XbaI end prior to incubation with TraIp, IHF, and TraIp. The protein-DNA complexes were then exposed to DNase I (1 μg/ml) for 2 min at 25°C. The reactions were stopped by the addition of EDTA to 50 mM. Products were resolved by electrophoresis through 8% acrylamide gels alongside DNA sequencing markers. Sequencing markers were generated using the dyeoxy sequencing method (Sanger et al., 1977) and the primer whose 5' end initiated at the XbaI site of the large DNA fragment and extended toward the oriT nick site. Products were visualized by film autoradiography.

Electron Microscopy—Relaxosomes were fixed by exposure to glutaraldehyde (0.6%) and formaldehyde (1.0%) for 5 min at 25°C. The samples were chromatographed on a 2-nl Biogel A5M (Bio-Rad) column. The samples were then applied in a buffer containing 2.5 mM spermidine, 75 mM NaCl, 50 mM KCl, 10 mM HEPES-OAc (pH 7.5), 1 mM MgCl2, 0.25 mM CaCl2 directly to carbon-coated copper grid, which had been exposed to UV light (254 nm) for 30 min. Grids were exposed to a graded EtOH series: 0, 25, 50, 100% for 3 min each. Grids were then rotary shadowcast at 10−6 torr and examined in a Zeiss transmission electron microscope. A summagraphics digitizing board was used to measure DNA molecules captured on electron micrographs.

**RESULTS AND DISCUSSION**

Plasmid pBSoriT contains the cis-acting F plasmid oriT sequence cloned into the pBS phagemid (Matson and Morton, 1991). Active relaxosomes can be formed by incubating purified proteins involved in relaxosome formation with supercoiled pBSoriT DNA (see accompanying paper). These protein-DNA complexes are detected by exposing the complexes to protein denaturants and following the conversion of the plasmid from a supercoiled to a relaxed form by agarose gel electrophoresis. The criteria which were applied to discriminate true relaxosome formation from nonspecific relaxation events were as follows: 1) the reaction must be dependent on the cis-acting oriT element and 2) the relaxed plasmid must contain a site- and strand-specific nick identical to that observed in DNAs isolated as relaxosomes in vivo. Using this assay it has been shown that, under defined conditions, TraIp alone can form a relaxosome at oriT (Matson and Morton, 1991; Reygers et al., 1993). Nelson et al. (1995) have furthered these studies by demonstrating that IHF and TraYp can act in conjunction with TraIp to form relaxosomes under conditions in which TraIp alone fails to form relaxosomes.

The Ability of IHF and TraYp to Facilitate Relaxosome Formation with the TraIp Protein Is Dependent Upon the Order of Addition of Each Component—In a reaction buffer containing 75 mM NaCl, which precludes TraIp alone from forming a relaxosome (Fig. 1, column a), pBSoriT was incubated with IHF, TraYp, and TraIp. The order of addition of each protein was varied, and the resulting protein-DNA complexes were treated with SDS and proteinase K and examined by agarose gel electrophoresis. The results shown in Fig. 1 demonstrate that IHF and TraYp must be present in the reaction with pBSoriT prior to the addition of TraIp in order to observe maximal relaxosome formation (Fig. 1, compare columns d and e to f–h). These three order of addition effects suggest that IHF and TraYp form a complex with DNA prior to TraIp binding and that this complex facilitates the assembly of an active relaxosome containing TraIp. The functional relaxosome could contain all three proteins, or IHF and TraIp may simply serve to promote binding of TraIp to the nick site at oriT, at which point one or both of these accessory protein could be released from the DNA.
To investigate this question further, assembly of the relaxosome was examined using DNase I protection methodologies. pBSoriT DNA was linearized and uniquely labeled at the 5′ end some was examined using DNase I protection methodologies. 168 and 194 and positions 205 and 240, respectively (numbered system of Frost 1994), from DNase I in a protein concentration-dependent manner (data not shown). In contrast, Tralp alone did not provide any protection from DNase I cleavage at any concentration tested up to a 20-fold molar excess of Tralp over DNA (Fig. 2). It appears that the Tralp alone is unable to bind linear double-stranded DNA in a stable manner at these concentrations and thus is unable to form a relaxosome on a linear DNA substrate under these conditions.

The addition of TraYp (Fig. 3) or IHF (Fig. 4) to the DNase I protection experiments containing Tralp did not produce any additional protection other than that expected for IHF or TraYp alone. However, the addition of all three proteins in the following order, IHF, TraYp, and then Tralp revealed a region of DNase I protection that included the IHF and TraYp binding sites plus an additional region of protection that extended across the nick site to position 135 (Fig. 5, also see Fig. 8). In addition, a site of DNase I hypersensitivity appears near the distal end of the IHF binding site at position 192 only when all three proteins are included in these experiments. These results indicate that neither IHF nor TraYp alone can facilitate the binding of Tralp, but together these two proteins act to promote the formation of a relaxosome that includes Tralp. These data are consistent with the observation that all three proteins are involved in the introduction of a site- and strand-specific nick at oriT (see Fig. 1 and Nelson et al. 1995).

Another characteristic of protein complexes that wrap DNA is a shortening of the curvilinear length of the DNA. The curvilinear length can be directly measured from electron micrographs of DNA containing a relaxosome and compared with protein-free DNA. To further examine this question, we prepared relaxosomes containing IHF, TraYp, and Tralp for examination in the electron microscope. Fig. 6A shows electron micrographs of a 1622-bp SspI/AflIII restriction fragment of pBSoriT in which the nick site is centrally located. 36 such molecules were measured, and the distance of the protein complex was determined from one end and plotted as a percentage
of the total distance (Fig. 6B). 34 protein complexes out of those examined were located at or within 10% of the nick site.

To examine the curvilinear lengths of relaxosomes, a smaller 684-bp HaeII restriction fragment of pBS oriT was assembled into relaxosomes and prepared for electron microscopy. The lengths of 33 DNA molecules with protein complexes at the expected location was compared with the lengths of 37 protein-free DNA molecules (Fig. 7). Wrapping or looping of 100 bp of DNA (the relaxosome covers approximately 110 bp) would result in a linear foreshortening of the DNA by approximately 0.025 μm. The measured length of 3 relaxosomes was 0.025 μm shorter than the mean. However, the mean length of all measured relaxosomes compared with free DNA molecules was identical. Although we cannot rule out the possibility that these three relaxosomes represent wrapped complexes, the majority of relaxosomes do not appear to be complexed in such a way as to result in a significant foreshortening of the DNA.

In conclusion, we have demonstrated that the relaxosome at the F plasmid origin of transfer is formed in a stepwise manner. IHF and TraYp assemble onto DNA prior to TraIp, and this complex facilitates TraIp binding at oriT under conditions that preclude TraIp from binding alone (Fig. 8). In addition, information about the superstructure of the relaxosome can be gleaned from DNase I protection experiments and electron microscopy. These results suggest that the DNA is not wrapped significantly around the surface or within the relaxosome complex as might be expected from the highly bent nature of oriT DNA and the presence of IHF and TraYp within the relaxosome, both of which are known to bend DNA. The presence of a DNase I hypersensitive site present only when all three proteins are bound to DNA, which is located at the distal end of the IHF binding site, suggests that the DNA at this site is exposed at the surface of the relaxosome and distorted to expose the minor groove to DNase I cleavage.

One model, consistent with the data presented in this paper, that can be advanced to explain the ability of TraYp and IHF to facilitate the formation of the F plasmid relaxosome invokes a DNA structural change induced by the binding of these proteins. An alteration in DNA structure could allow TraIp to interact with oriT DNA, which is normally refractory to TraIp

![Fig. 3. TraIp does not bind to linear pBS oriT DNA in the presence of TraYp.](image)

![Fig. 4. TraIp does not bind to linear pBS oriT DNA in the presence of IHF.](image)
binding. In support of this model, the ability of TraIp to nick supercoiled DNA in the absence of other proteins suggests that superhelical energy can mimic the affect of TraYp and IHF to favor TraIp binding at the nick site. It is important to note that negatively supercoiled or underwound DNA can promote the formation of non-B form DNA such as Z-DNA, cruciforms, and triplex structures depending on the primary sequence of the DNA (Palecek, 1991). Negative supercoiling can also drive transient melting of duplex DNA, especially within A-T-rich regions. The oriT region of F contains several inverted repeats, which could form cruciforms when exposed to either superhelical stress or localized stress induced by TraYp and IHF binding. These same factors could also induce transient DNA melting within the highly A-T-rich DNA region found just 5' of the nick site. In fact, recent evidence indicates that TraIp alone can efficiently cleave single-stranded oligonucleotides and heat-denatured DNA containing the oriT nick site (Sherman and

**FIG. 5.** TraIp binds linear pBSoriT DNA in the presence of IHF and TraYp. Uniquely end-labeled [\(^{32}\)P]DNA (6.7 nM), prepared as described under “Experimental Procedures,” was incubated at 23 °C for 5 min with varying amounts of IHF, followed by the addition of varying amounts of TraYp and an additional 5-min incubation. Next, varying amounts of TraIp were added and incubation continued at 37 °C for 15 min (lanes 2-5). Protein-DNA complexes were then exposed to DNAse I, and the products were resolved on a 8% polyacrylamide denaturing gel alongside DNA sequencing markers (C, T, A, G). The amount of IHF, TraYp, and TraIp in each reaction, corresponding to lanes 2-5 is indicated as follows: lane 2, 320 nM, 2 μM, and 100 nM; lane 7, 160 nM, 0.52 μM, and 40 nM; lane 8, 80 nM, 0.26 μM, and 20 nM; lane 9, 40 nM, 0.13 μM, and 10 nM. The locations of the IHF (IHF), TraYp (TraYp), and TraIp (TraIp) DNase I protection sites and the nick site (nic) are indicated to the left of the autoradiograph. The asterisk to the left of the autoradiograph shows the position of a DNase I hypersensitive site seen in lanes 2-5. Numbers to the right represent the distance in nucleotides from the BglII site located within oriT (see Frost et al. (1994)).

**FIG. 6.** Visualization of the relaxosome complex by electron microscopy. A, a 1622-base pair SspI/AflIII restriction fragment containing the oriT nick site located in the center of the molecule was incubated with IHF (320 nM), TraYp (2 μM), and TraIp (300 nM). The protein-DNA complexes were then fixed with formaldehyde/glutaraldehyde and prepared for electron microscopy as described under “Experimental Procedures.” The arrows indicate the location of relaxosome complexes. B, the distance from one end of the DNA to each relaxosome was measured, and that distance was plotted as a percentage of the total DNA length versus the number of molecules at each position.

**FIG. 7.** Absence of linear foreshortening of DNA within the relaxosome complex. A 684-bp HaeIII restriction fragment containing the F plasmid oriT was assembled into relaxosomes and prepared for electron microscopy as described in Fig. 6. The curvilinear lengths of 33 DNA molecules containing a relaxosome were measured from electron micrographs using a Summagraphics digitizing board, and the data were represented as a histogram (upper panel). The curvilinear lengths of 37 protein-free DNA molecules were measured from electron micrographs using a summagraphics digitizing board, and the data were represented as a histogram (lower panel).
Matson, 1994). These observations suggest that TraIp can either recognize single-stranded DNA as a substrate or that single-stranded DNA can adopt a conformation for the nicking reaction, which can also be formed by supercoiled DNA or DNA bound by TraYp and IHF.

The in vitro formation of a relaxosome at the F plasmid origin of transfer provides a substrate for examining the next step in DNA transfer; the conversion of DNA within the relaxosome from the supercoiled to relaxed form in response to a cellular mating signal. Extracts from mating bacteria or purified prospective mating signals can be added to relaxosomes in vitro, and the conversion of supercoiled relaxosomes to relaxed complexes can be easily assayed. Continued work along these lines will surely lead to the in vitro reconstitution and a more complete understanding of each event, which occurs during DNA strand transfer directed by the F plasmid.

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2 M. T. Howard, unpublished results.
