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Conjugative transfer of promiscuous IncP plasmids: Interaction of plasmid-encoded products with the transfer origin

(evolutionary conservation/protein–DNA interactions/"relaxosomes"/*oriT* binding protein/nick site)

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ABSTRACT **To characterize protein–DNA interactions involved in the initiation of conjugative transfer replication, we isolated and sequenced the transfer origins (*oriT*) of the promiscuous IncP plasmids RP4 and R751. The central initiating event at the transfer origin of a conjugative plasmid is the cleavage at a unique site (*nic*) of the strand to be transferred to a recipient cell. This process can be triggered after the assembly of "relaxosomes" (plasmid DNA–protein relaxation complexes), requiring plasmid-encoded gene products. We analyzed the nicking reaction for plasmid RP4 and demonstrated that one of the plasmid strands is specifically cleaved within *oriT*. The fully functional *oriT* of RP4 represents an intergenic DNA region of ≈350 base pairs. Dissection of *oriT* revealed that a portion carrying *nic* and symmetric sequence repeats determines *oriT* specificity. This part of *oriT* is contiguous to a region that is essential for efficient mobilization of *oriT* plasmids. In addition, *oriT* contains potential promoter sites allowing divergent transcription of two operons flanking *oriT*. We overproduced gene products and, from analyzing the products of defined deletion mutants, deduced the gene arrangements. Formation of RP4 relaxosomes is likely to depend on the presence of at least two plasmid-encoded components, which act in trans. Corresponding genes map on one side of *oriT*. Purification of the *traJ* product revealed it to be an 11-kDa polypeptide that binds to *oriT* DNA *in vitro*. The protein recognizes the part of *oriT* that is responsible for *oriT* specificity.**

Conjugation is the process that allows efficient gene transfer from one bacterial cell to another through plasmid-encoded functions. Conjugative plasmids of the IncP group are of particular interest because they are capable of mediating efficient DNA transfer between virtually any Gram-negative species (1). This promiscuity is important because of its role in the spread of antibiotic resistance and its application to gene manipulation in widely different bacteria.

Conjugative transfer requires both a cis-acting site, the origin of transfer (*oriT*), and a number of trans-acting functions that are necessary for mating pair formation, initiation and continuation of DNA transfer, and control of these processes. Despite increasing knowledge, derived primarily from studies of the fertility factor F, the molecular mechanisms of the initiation of transfer DNA replication are poorly understood (2). The general model for conjugative DNA synthesis proposes that one of the plasmid's strands is transferred to the recipient cell. The single strand is created by cleavage at the "nick site" within *oriT* and subsequent strand displacement through rolling circle type replication. The discovery of "relaxosomes" supports this hypothesis (3). Relaxosomes are thought to function as intermediates in the initiation reaction. Upon treatment of these complexes with protein-denaturing agents the superhelical plasmid DNA undergoes transition to the open circular form.

Plasmid RK2 and the probably identical plasmid RP4 have been shown to form relaxosomes (4, 5). We have investigated the composition and function of relaxosomes by using the IncPα plasmid RP4 [60 kilobases (kb)] and the related IncPβ plasmid R751 (53 kb). The nucleotide sequences of the two IncP transfer origins were compared in an effort to define common features. These data demonstrate that the structural organization of the IncP-type transfer origins is conserved. While most of the transfer functions, including the mating-pair formation system, can be utilized by both plasmids, the interaction at the transfer origin of *tra* gene products is plasmid specific (6). We used this observation to map the genes encoding *oriT*-specific functions within the regions immediately flanking *oriT*. Furthermore, an electrophoretic assay was developed to analyze rapidly the nicking reaction; the assay allowed us to locate genes required for relaxosome nicking within the region encoding *oriT*-specific functions. Expression-vector cloning of fragments carrying *oriT*-specific functions facilitated the analysis of gene organization and the overproduction of gene products. One of these proteins, the *traJ* gene product, specifically binds to the *oriT* region *in vitro*, thus suggesting an important role of the protein in triggering the initial events of transfer DNA replication.

MATERIALS AND METHODS

Strains, Phages, and Plasmids. *Escherichia coli* strain HB101 (7) was used as a host for plasmids and the nalidixic acid-resistant strain HB101 Nx^r as a recipient for filter matings. Phages M13mp18 and M13mp19 and recombinants derived from them were propagated in JM105 (8). Plasmids used were RP4 (9), R751 (10), pBR329 (11), and pJF118EH (12).

DNA Techniques. Standard molecular cloning techniques were performed as described (13). DNA was sequenced by the dideoxynucleotide chain-termination method (14) using dATP[α-^{35}S] (15) and 7-deaza-dGTP (16).

Quantitative Filter Matings. Appropriate donor (0.5 ml, A_{600} = 0.3) and HB101 Nx^r recipient cells (4.5 ml, A_{600} = 0.3) were mixed and filtered on a Millipore filter (0.45 μm, 25 mm in diameter). The filter was incubated for 1 hr at 30°C on a nutrient agar plate without selection. Cells were resuspended and plated on selective medium (10 μg of chloramphenicol per ml; 30 μg of sodium nalidixate per ml). The transfer frequency is the number of transconjugants per donor cell.

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Abbreviation: ORF, open reading frame.

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Relaxation Assay. Plasmid DNA was isolated by the gentle lysis technique as described (3). Relaxation was induced by $NaDodSO_4$ (2.5%). Plasmid DNA (open circular form) was isolated electrophoretically and concentrated by ethanol precipitation.

RESULTS

Structural Conservation of Inverted Repeats at the Transfer Origins of IncP Plasmids. Recombinant *oriT* plasmids were screened from libraries created by insertion of RP4 *Not* I fragments into the unique *Xma* III site of the small nonmobilizable vector plasmid pBR329 (Fig. 1). It was found that the RP4 *Not* I fragment F (3.77 kb) contains the fully functional *oriT* region because the corresponding plasmid (pMS226) was mobilized into recipients at the same frequency as the parental plasmid. The *Not* I fragment F is part of the TraI region of RP4 as judged by its map position (17). Subcloning of a 776-base-pair (bp) *Xma* III fragment (pJF142) helped to define with greater accuracy the limits of the functional *oriT*. Plasmid pJF145, which carries a 457-bp *Xma* III/*Dde* I fragment, was then generated to locate the minimal *oriT* region. Dissection of the *oriT*-containing *Xma* III fragment of plasmid pJF142 at the *Acc* I site allowed us to define two functional domains of the transfer origin. Plasmid pJF143 carrying the smaller *Xma* III/*Acc* I fragment is still mobilizable; however, transfer frequencies are reduced by a factor of 100–200 (Fig. 1). The plasmid containing the larger *Acc* I/*Xma* III fragment cannot be mobilized (data not shown). These results suggest that the region to the left of the *Acc* I site contains the proposed nick site, while the rightward region, responsible for efficient mobilization, is likely to include additional recognition site(s).

As a prelude to investigating the interaction of plasmid-encoded functions with the transfer origin, we compared the nucleotide sequence of the RP4 *oriT* with the sequence of the R751 *oriT* (Fig. 2). The sequences of the two transfer origins have 71% positional identity, indicating a close evolutionary relationship between the two transfer systems. Comparison to *E. coli* promoter consensus sequences identify, for both *oriT* regions, a pair of potential promoter sites that allow for divergent transcription. Promoter region(s) have been located in RP4 *oriT* by visualizing *E. coli* RNA polymerase binding sites using electron microscopy (20). Positions for the RNA polymerase binding sites are in good agreement with those of the promoter consensus sequences. The *oriT* sequences revealed no continuous open reading frame (ORF), implying that the IncP transfer origin is intergenic. Among the origin sequences, three groups of nucleotides are found to be arranged with dyad rotational symmetry (Fig. 2). The structural conservation of the repeats in both IncP transfer origins suggests that they are important for *oriT* function, probably in recognizing and interacting with transfer factors as has been suggested (21).

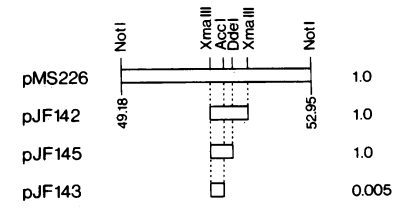

FIG. 1. Molecular cloning of RP4 transfer origin into pBR329. Coordinates according to the standard map of RP4 are indicated (17). For *oriT* subcloning into the unique *Bam*HI site of pBR329, *Bam*HI linkers (10-mer) were attached to the *Xma* III, *Xma* III/*Dde* I, and *Xma* III/*Acc* I fragments. The mobilization ability of the derivatives (numbers right) is given as the ratio of the mobilization frequency to the transfer frequency of RP4. On average, the transfer frequency of RP4 was 1.0 per donor cell.

TraI Core Is the Minimal Region Necessary for Heterologous Mobilization. Plasmid pMS226, carrying the *Not* I fragment F, can be mobilized by both RP4 and R751, although at frequencies reduced 1–2 orders of magnitude in the heterologous system (data not shown). In contrast, transfer of plasmid pJF142 can be mediated by RP4 only. This indicates that part of the transfer machinery is interchangeable, while homologous functions encoded by the RP4 portion of pMS226 are essential to properly recognize *oriT*.

To determine the minimal fragment size of the RP4 TraI region that is necessary for efficient heterologous mobilization, derivatives with deletions from either side of the *Not* I fragment F in pMS226 were generated (Fig. 3). The effect of these deletions on transfer efficiency demonstrates that the minimal region (TraI core) comprises ≈2.2 kb (1.3 kb to the left and 0.5 kb to the right of *oriT*). Plasmid pGZ226-1 was constructed to confirm the mapping of TraI core; it exerts the same effects as pMS226.

Two distinct regions of TraI core were defined by the differential behavior of the pMS226 deletion derivatives in R751-mediated mobilization. A significant drop in R751-mediated mobilization was measured with mutants Δ12, Δ1,

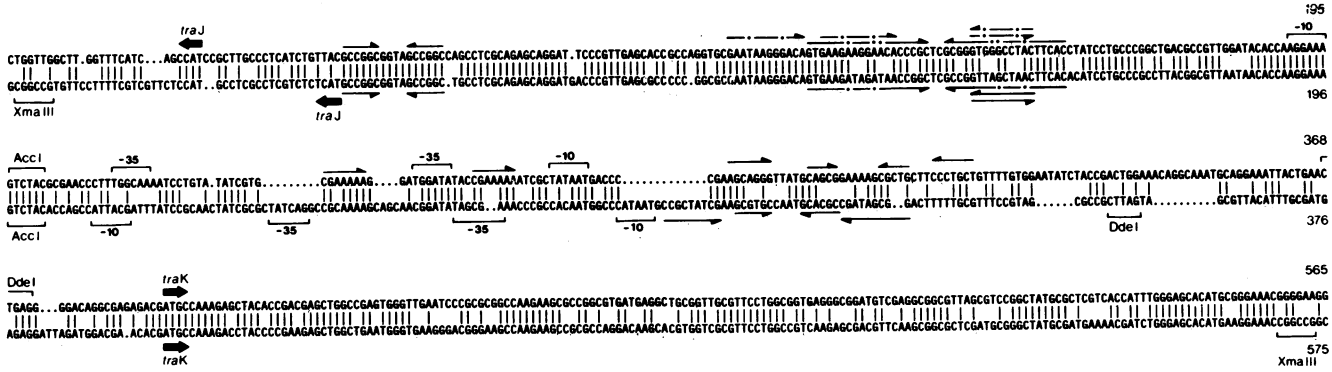

FIG. 2. Comparison of the nucleotide sequences of IncP transfer origins. The *oriT* sequence of RP4 (upper lines) was determined from the *Xma* III fragment (776 bp) present in pJF142; the nucleotide sequence of the R751 *oriT* (lower lines) was derived from the *oriT*-containing *Xma* III fragment (574 bp) of plasmid pWP392 (unpublished data). Both strands were sequenced. The total sequence of the R751 *Xma* III fragment and the corresponding part of the RP4 *Xma* III fragment are shown. The RP4 sequence for nucleotides 59–170 is in complete agreement with the published sequence of the RK2 *oriT*. Sequences were aligned by the BESTFIT program of the University of Wisconsin Genetics Computer Group software package (18). Vertical lines indicate identical nucleotides. The −10 and −35 regions of potential promoter sequences are marked by brackets. Promoter sequences were detected by a computer program based on data for 263 *E. coli* promoters (19). Restriction sites *Acc* I, *Dde* I, and *Xma* III correspond to those shown in Fig. 1. Boldface arrows represent the proposed 5′ ends of genes. Direct and inverted repeats are indicated by arrows; dots within arrows indicate mismatches.

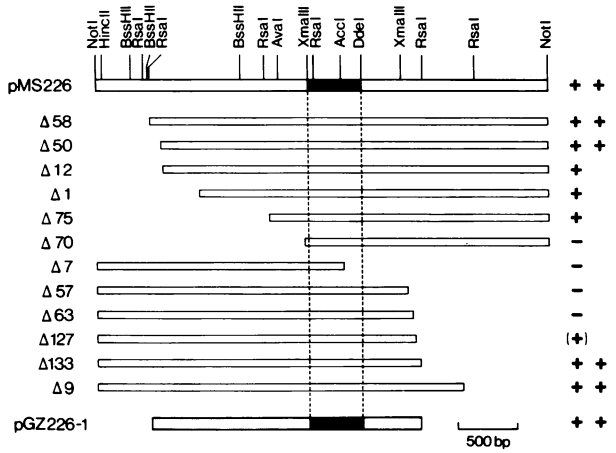

FIG. 3. Heterologous mobilization of deletion derivatives of the RP4 Tral core region. A physical map of deletions of the RP4 *Not* I fragment F (pMS226) is shown. Deletions into the left end of the fragment were prepared from pMS226n (see Fig. 4), those into the right end were from pMS226u (the fragment orientation relative to the vector is designated by n or u). Plasmids pMS226n, -u were linearized at the unique *Sal* I site in the vector portion and partially digested with BAL-31 to create successive deletions. *Bam*HI linkers (10-mers) were attached to cleavage products and the larger portions of the fragments were isolated after *Pst* I cleavage. These *Bam*HI/*Pst* I fragments (a population of differently sized fragments) contain the pBR origin but no functional antibiotic resistance gene. The fragments were ligated in the presence of the 2006-bp *Bam*HI/*Pst* I fragment isolated from pBR329, which contains the complete chloramphenicol-resistance gene (Cm^r). This strategy ensured that all deletion derivatives contained a vector portion of constant length. Clones were selected for Cm^r and tested for ampicillin resistance (Ap^r) to demonstrate the proposed phenotype. Sizes of the deletions were estimated by restriction endonuclease cleavage. The end points of all derivatives presented were determined by nucleotide sequencing of the plasmids. A sequencing primer was used that hybridizes near the *Bam*HI site in the vector portion of the plasmids. Plasmid pGZ226-1 has been constructed from Δ58 (left part) and Δ133 (right part) via the unique *Acc* I sites. The mobilization ability of the derivatives in the presence of R751 is given on the right. Mobilization frequencies: ++, 2–3 × 10^{-3} (not significantly different from the mobilization frequency of pMS226); +, 5–10 × 10^{-5}; (+), 10^{-5}; −, ≤10^{-7}. Solid bar represents *oriT*.

and Δ75. This indicates that the product(s) of the region deleted is to a certain extent interchangeable with R751 functions. The DNA sequence analysis of Tral core (unpublished data) identified an 18-kDa ORF that contains the termini of deletion derivatives Δ12, Δ1, and Δ75. This ORF is transcribed to the left with respect to the map shown (Fig. 3). The second region, defined by the termini of deletion derivatives Δ70, Δ7, Δ57, and Δ63, encodes functions active in the RP4 system only; the transfer frequencies of these plasmids in the heterologous system lie below detection limit (Fig. 3). Within this region, the DNA sequence allowed the assignment of a 13.3-kDa ORF (*traJ*) to the left and a 14.4-kDa ORF (*traK*) to the right of *oriT* that are transcribed divergently (Fig. 2).

We concluded that at least three gene products are likely to be required for efficient heterologous mobilization in addition to the transfer factors supplied by the helper plasmid. We next investigated the activity and identity of these products.

Tral Core Genes Are Required for Nicking Within *oriT*. Presumably, relaxosomes are composed of supercoiled plasmid DNA with transfer factors complexed at *oriT*. Treatment of these complexes with $NaDodSO_4$ induces site- and strand-specific relaxation. To analyze the relaxed forms, we developed a gel electrophoretic assay using denaturing agarose gels (Fig. 4). The relaxed DNA of pMS226n was isolated and

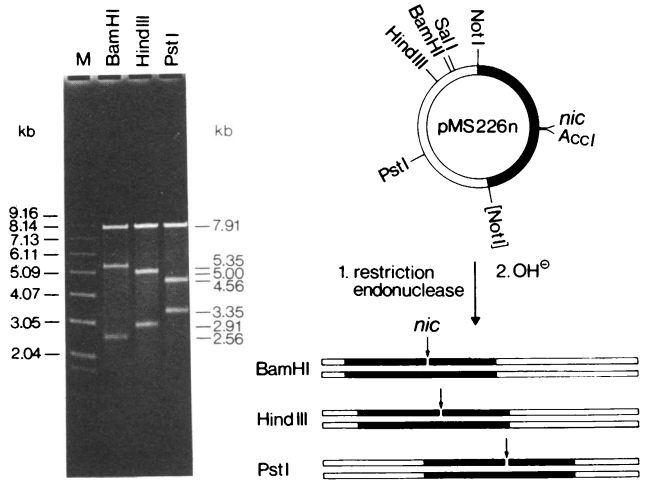

FIG. 4. Assay for site- and strand-specific relaxation of RP4 Tral core plasmids. Relaxosomes were isolated from 30-ml cultures (late logarithmic phase) of HB101 (pMS226n) and treated with $NaDodSO_4$. Open circular DNA (0.8 μg) was linearized with either *Bam*HI, *Hin*dIII, or *Pst* I; denatured in 0.1 M NaOH; and electrophoresed for 7.5 hr (3.5 V/cm) on a 0.9% alkaline agarose gel (22). Lane M contains 1-kb ladder (Bethesda Research Laboratories). The physical structure of pMS226n and of the corresponding cleavage products with the position of the nick site (*nic*) are shown at the bottom.

linearized with restriction endonucleases that cleave the plasmid DNA only once. After alkaline denaturation into single strands, the DNA separates into three distinct bands, one of which corresponds to the full-length linear of the plasmid. The existence of two additional bands with higher mobility indicates site-specific cleavage of one of the plasmid strands; the sum of these fragment sizes is one full-length linear. The cleavage was located from fragment sizes within *oriT* ± 50 bp to the left of the *Acc* I site. The assay was applied to the deletion derivatives to determine which part of Tral core is needed to cause relaxation (Figs. 3 and 5). While *oriT*-specific nicking of derivative Δ12 occurred, resulting in two closely migrating single-stranded fragments of 3.45 and 3.35 kb, only full-length single strands of the derivatives Δ1, Δ75 (data not shown), and Δ70 (data not shown) could be observed. In contrast, nicking occurred for all pMS226u deletion derivatives tested. Even for derivative Δ7, the two expected fragments (3.57 and 2.07 kb) could be observed, although nicking appeared to occur at a slightly reduced efficiency. Thus, the relaxation ability requires the region of Tral core that is located to the left of *oriT*.

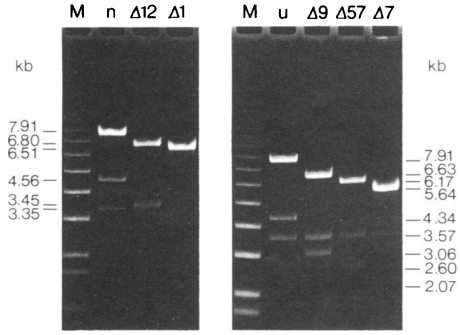

FIG. 5. Relaxation assay of RP4 Tral core deletion derivatives. Reaction conditions were as described in the legend to Fig. 4. The numbers refer to derivatives shown in Fig. 3. Open circular DNA was linearized with *Pst* I. pMS226n and pMS226u are designated n and u, respectively. The sizes of single-stranded DNA fragments are indicated.

Identification of Tral Core Products by Expression Vector Cloning. The evidence that gene products of Tral core interact specifically with the *oriT* site needed to be substantiated by biochemical data. Overproducing and identifying the proteins involved are critical for addressing the problem. The strategy for overproduction was guided by the observation that the *oriT* region is intergenic and contains putative promoter sites for divergent transcription. Thus, fragments from either side of *oriT* were inserted into the *tacP* expression vector pJF118EH (Fig. 6).

Upon induction of HB101 cells harboring the plasmid pJF161n, which contains the region to the right of *oriT*, two polypeptides of 26 (TraL) and 12 kDa (TraM) were readily detectable by electrophoresis on $NaDodSO_4$/polyacrylamide gels (Fig. 7). A third polypeptide of ≈15 kDa (TraK) was seen as a rather faint band on the gel. Two deletion derivatives—pJF161n Δ1 and Δ2—allowed us to determine the map location of the corresponding genes (Fig. 6). The *Dde* I/*Ava* I fragment inverted relative to the *tac* promoter (pJF161u) did not allow the production of inducible gene products, as judged by comparing extracts with those of HB101 (pJF118HE). Plasmid pJF166u containing the left-hand side of Tral core did not support any overproduction of proteins, regardless of the fragment orientation relative to the *tac* promoter (data not shown). Removal of the 150 bp to the right of the *Acc* I site resulted in pJF166u Δ1 (Fig. 6), lacking part of the proposed promoter region (Fig. 2). Extracts of induced HB101 cells carrying this plasmid contain two additional protein bands corresponding to molecular masses of 11 (TraJ) and 22 (TraH) kDa (Fig. 7). The map locations of the corresponding genes were determined by analyzing the expression pattern of several defined deletion derivatives (Fig. 6). Insertion of the RP4 *Not* I fragment F into the *tacP* expression vector system,

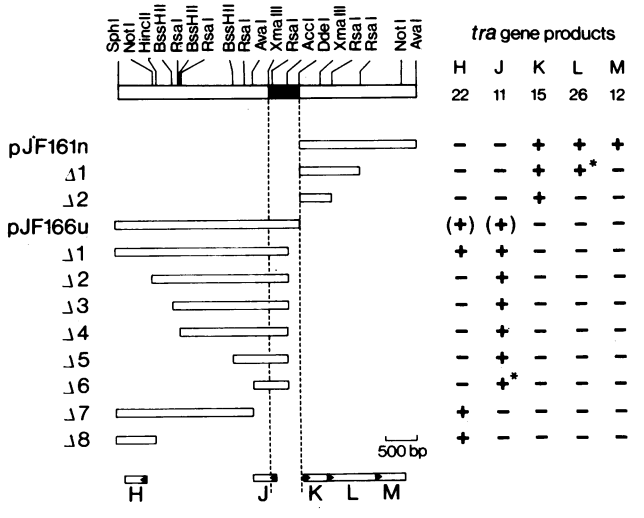

FIG. 6. Molecular cloning of RP4 Tral core fragments into a *tacP* expression vector. To construct pJF161n, the *Dde* I/*Ava* I fragment (1.7 kb) of pMS204 (23) was inserted into the unique *Sma* I site of the polylinker of pJF118EH. Two defined deletion derivatives (Δ1 and Δ2) were obtained by *Rsa* I partial digestion. To construct pJF166u, the *Sph* I/*Dde* I fragment (2.7 kb) of pMS204 was isolated, *Bam*HI linkers were attached, and the fragment was inserted into the unique *Bam*HI site of the polylinker of pJF118EH. pJF166u was used to generate defined deletion derivatives (Δ1–Δ8) by partial digestion of linearized plasmid with *Acc* I, *Ava* I, *Bss*HII, *Hin*cII, or *Not* I. Bars represent the RP4 DNA remaining in the derivatives. The pattern of protein expression in the induced state is shown on the right. (+), Protein is expressed in amounts detectable only immunologically; +*, truncated products; +, protein visible on stained gels (see Fig. 7); −, products not detectable. The extension of the genes is indicated by arrowheads. Numbers below the gene designations are the molecular masses of products in kDa. Solid bar represents *oriT*.

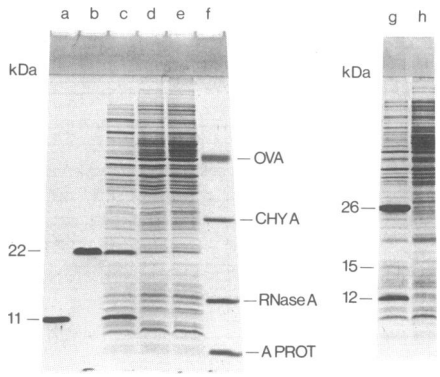

FIG. 7. Overproduction of RP4 Tral core products. Electrophoresis of cell extracts (2.5-μl aliquots) on 17.5% (wt/vol) polyacrylamide gels (12). Lanes: a, 0.75 μg of purified TraJ protein; b, 0.75 μg of purified TraH protein; c and d, HB101 (pJF166uΔ1); c, isopropyl β-D-thiogalactopyranoside (IPTG)-induced cells; d, noninduced cells; e, HB101 (pJF118EH) induced cells; g and h, HB101 (pJF161n); g, IPTG-induced cells; h, noninduced cells. Size markers in lane f are ovalbumin (OVA), 43.6 kDa; chymotrypsinogen A (CHYA), 25.7 kDa; RNase A, 13.6 kDa; aprotinin (APROT), 6.8 kDa, 1 μg each.

in both orientations relative to the *tac* promoter, did not lead to detectable overproduction of proteins.

In summary, these data demonstrate that the TraJ and the TraK proteins are transfer gene products encoded by Tral core. Their structural genes belong to two polycistronic operons arranged on either side of *oriT*. Divergent transcription is likely to begin at the proposed promoter sites within the *oriT* region (see Fig. 2). As indicated by the positions for the *traH*, *-L*, and *-M* genes (Fig. 6), the operons extend beyond the Tral core region on both ends.

Specific Interaction of the RP4 TraJ Protein with the Transfer Origin. To gain insight into the physical interaction of Tral core products with the *oriT* site, it is necessary to study the *in vitro* reaction with purified components. Purification of the TraJ protein—i.e., its binding to heparin-Sepharose (unpublished data)—suggested that it might bind to DNA (5). To prove this assumption, a DNA fragment retardation assay on nondenaturing polyacrylamide gels was performed (Fig. 8). Fragments were chosen that separate the two groups of sequence repeats (Figs. 2 and 8). The electrophoretic mobility of fragment B (RP4) decreased in the presence of TraJ protein and resulted in band B*, demonstrating stable complex formation between the *oriT* fragment and the protein (Fig. 8, lanes b–d). Since only one of the two DNA fragments (A or B) is affected, the binding is regarded to occur specifically. The protein/DNA ratio was estimated to be ≈4:1. The specificity of binding is also obvious from the experiment with R751 *oriT* fragments (lanes f–h). Restriction fragments of R751 *oriT*, of sizes similar to those of the RP4 fragments, were prepared. Binding of RP4 TraJ protein to the R751 fragments is suggested by the observation that increasing amounts of protein cause bands to become more diffuse. However, this kind of binding characterizes the general DNA binding ability of the protein. Thus, R751 *oriT* does not offer a recognition sequence for the RP4 TraJ protein.

DISCUSSION

Genetic and biochemical evidence demonstrate a specific interaction between the RP4 *traJ* product (11 kDa) and the transfer origin. The intact *traJ* gene is required for the mobilization of the RP4 *oriT* by the related helper plasmid R751. Since the *traJ* gene is flanked by the 18-kDa ORF and the *oriT*, a role of TraJ in relaxation cannot be concluded from the data gained by the deletion derivatives. However, the

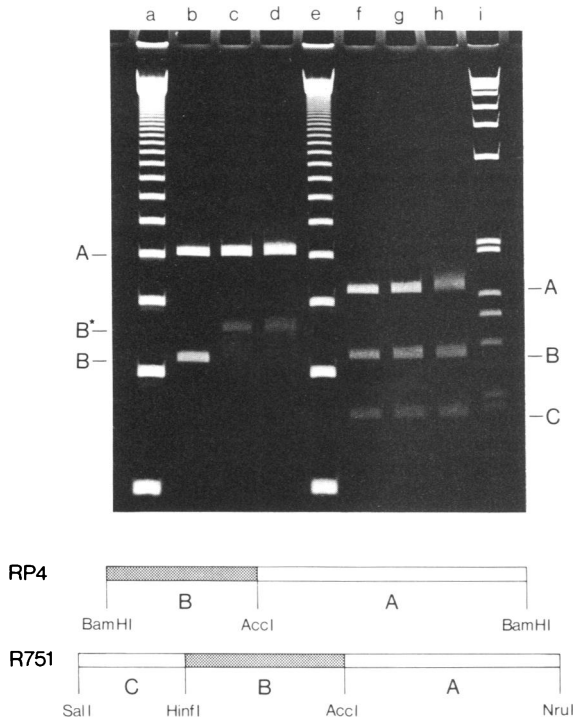

FIG. 8. Complex formation between *oriT*–DNA and the RP4 TraJ protein. *oriT* fragments of RP4 (pJF142; *Bam*HI, 790 bp) and of R751 (pWP392; *Sal* I/*Nru* I, 908 bp) were isolated and subsequently digested with *Acc* I and *Acc* I/*Hin*fI, respectively. Maps of the fragments visualized on the gel are at the bottom of the figure. The protein–DNA complex is designated B*. Mixtures of TraJ protein and 0.5 μg of DNA fragments were incubated in a total volume of 20 μl [20 mM Tris·HCl, pH 7.6/50 mM NaCl/5 mM $MgCl_2$/bovine serum albumin (10 μg/ml)] for 30 min at 37°C and electrophoresed on a 3.5% (wt/vol) nondenaturing polyacrylamide gel (8 V/cm). Lanes: b–d (RP4), b, protein omitted; c, 0.06 μg of protein; d, 0.3 μg of protein; f–h (R751), f, protein omitted; g, 0.06 μg of protein; h, 0.3 μg of protein. Lanes a, e, and i contain DNA fragment size markers; a and e, 123-bp ladder; i, 1-kb ladder.

TraJ protein binds specifically to the RP4 *oriT in vitro*, suggesting it to be part of the relaxosome.

Since the *Xma* III/*Acc* I fragment of pJF143 contains the proposed nick site (Fig. 1), TraJ was suggested to bind within this region. Alteration of the electrophoretic mobility of this fragment in the presence of the TraJ protein confirmed a specific interaction (Fig. 8). At first glance, it appears to be paradoxical that the domain with higher sequence conservation determines *oriT* specificity. This indicates, however, that the small sequence divergence must be responsible for high precision protein–DNA interaction. The possible targets for specific binding are the inverted sequence repeats in the RP4 fragment. The significance of these repeats is underscored by their structural conservation between IncP plasmids.

Efficient heterologous mobilization requires the intact 18-kDa ORF adjacent to the *traJ* gene. In addition, this ORF seems to be required for specific relaxation *in vitro*. However, the pMS226n derivative Δ12, which lacks seven of the 3′-terminal codons of the 18-kDa ORF, maintains its relaxation ability. A likely interpretation of this observation is that this deletion derivative specifies a fusion protein, which still can support *oriT*-specific nicking but is unable to assist in efficient *oriT*-specific initiation of transfer DNA replication. In assessing other components contributing to relaxation, the possible participation of host factors should be considered.

What other functions are encoded by TraI core? Although the *traK* gene product is essential for heterologous mobilization, in that it reacts specifically with *oriT*, a clearly defined function cannot yet be assigned. However, the slightly reduced yield of specifically relaxed DNA in the absence of TraK suggests that this gene product strengthens the interaction of the TraJ protein or the 18-kDa protein, or both, with *oriT* DNA.

Our data support the general model of the initiation reaction of the transfer replication, which is based on the mode of replicative form to single-strand replication of single-stranded DNA phages. The cleavage reaction of the strand to be transferred is likely to be a more complex reaction than that seen with single-stranded DNA phages; the data suggest that at least two gene products function in the initiation of the IncP transfer replication, cleaving one of the two plasmid strands at *oriT*. The specific contribution of the components—the TraJ and the 18-kDa protein—in mediating this event are still unknown. A possible role of the *traJ* gene product involves a sequential mechanism; the TraJ protein-mediated nucleoprotein structure at the transfer origin might serve as a recognition signal for the assembly of a multiprotein initiation complex. Thus, the binding of TraJ would be the initiating event for a complex series of reactions, culminating in *oriT*-specific initiation of transfer DNA replication. This and other questions concerning the assembly of relaxosomes should be addressed through the isolation of the other TraI core products in amounts sufficient for functional analysis.

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