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

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ABSTRACT To characterize protein-DNA interactions involved in the initiation of conjugative transfer replication, we isolated and sequenced the transfer origin (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 trans 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 "nic 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 genes 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 Nx1 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-32P] (15) and 7-deaza-dGTP (16).

Quantitative Filter Matings. Appropriate donor (0.5 ml, A600 = 0.3) and HB101 NX+ recipient cells (4.5 ml, A600 = 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 NaDodSO₄ (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 recipient strains 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 se-

**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 BamHI site of pBR329, BamHI 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.

quences 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).

**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 A12, A1,
Fig. 3. Heterologous mobilization of deletion derivatives of the RP4 Tra1 core region. A physical map of deletions of the RP4 Not1 fragment F (pMS226) is shown. Deletions into the left end of the fragment were prepared from pMS26n (see Fig. 4), those into the right end were from pMS26u (the fragment orientation relative to the vector is designated by n or u). Plasmids pMS26n, -u were linearized at the unique SacI site in the vector portion and partially digested with BAL-31 to create successive deletions. BamHI linkers (10-mers) were attached to cleavage products and the larger portions of the fragments were isolated after PstI cleavage. These BamHI/ PstI 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 BamHI/PstI fragment isolated from pBR322, which contains the complete chloramphenicol-resistance gene (CmR). This strategy ensured that all deletion derivatives contained a vector portion of constant length. Clones were selected for CmR and tested for ampicillin resistance (ApR) 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 BamHI 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 AccI sites. The mobilization ability of the derivatives in the presence of K751 is given on the right. Mobilization efficiencies: + +, 2–3 x 10^6; (+), 10^5; −, ≤10^4. Solid bar represents oriT.

and Δ75. This indicates that the product(s) of the region deleted is to a certain extent interchangeable with K751 functions. The DNA sequence analysis of Tra1 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.

**Tra1 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 NaDODSO4 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 pMS26n was isolated and 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 AccI site. The assay was applied to the deletion derivatives to determine which part of Tra1 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 pMS26u 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 Tra1 core that is located to the left of oriT.

![Fig. 4. Assay for site- and strand-specific relaxation of RP4 Tra1 core plasmids. Relaxosomes were isolated from 30-ml cultures (late logarithmic phase) of HB101 (pMS26n) and treated with NaDODSO4. Open circular DNA (0.8 μg) was linearized with either BamHI, HindIII, or PstI; 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 a 1-kb ladder (Bethesda Research Laboratories). The physical structure of pMS26n and of the corresponding cleavage products with the position of the nick site (nic) are shown at the bottom.](image-url)

![Fig. 5. Relaxation assay of RP4 Tra1 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 PstI. pMS26n and pMS26u are designated n and u, respectively. The sizes of single-stranded DNA fragments are indicated.](image-url)
Identification of Tra1 Core Products by Expression Vector Cloning. The evidence that gene products of Tra1 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 NaDodSO4/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 (pJF161b) 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 Tra1 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 Tra1 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 Smal 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, BamHI linkers were attached, and the fragment was inserted into the unique BamHI 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, BssHII, HincII, 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.](image)

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 Tra1 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 Tra1 core region on both ends.

Specific Interaction of the RP4 TraJ Protein with the Transfer Origin. To gain insight into the physical interaction of Tra1 core products with the orIT site, it is necessary to study the in vitro reaction with purified components. Purification of the Tra1 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 non-denaturing 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
Figs. 8. Complex formation between oriT−DNA and the Rp4 TraJ protein. oriT fragments of Rp4 (pJF142; BamHI, 790 bp) and of R751 (pWP392; SaI 1/Nru I, 908 bp) were isolated and subsequently digested with Acc I and Acc I/HindI, 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 MgCl2/bovine serum albumin (10 µg/ml)] for 30 min at 37°C and electrophoresed on a 3% (wt/vol) non-denaturing 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 higher 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 traI gene. In addition, this ORF seems to be required for specific relaxation in vitro. However, the pMS226m 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, 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 phases. The cleavage reaction of the strand to be transferred is likely to be a more complex reaction than that seen with single-stranded DNA phases; 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 traI 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 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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