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Mutagenesis of the Tra1 Core Region of RK2 by Using Tn5: Identification of Plasmid-Specific Transfer Genes

DONALD G. GUINEY,[1]* CORNELIA DEISS,[1] VIRGINIA SIMNAD,[1] LISA YEE,[1] WERNER PANSEGRAU,[2] AND ERICH LANKA[2]

Department of Medicine H811F, UCSD Medical Center, 225 Dickinson Street, San Diego, California 92103,[1] and Max-Planck-Institut fur Molekulare Genetik, Abteilung Schuster, D-1000 Berlin 33, Federal Republic of Germany[2]

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The conjugation system of the IncPα plasmid RK2/RP4 is encoded by transfer regions designated Tra1, Tra2, and Tra3. The Tra1 core region, cloned on plasmid pDG4Δ22, consists of the origin of transfer (*oriT*) and 2.6 kilobases of flanking DNA providing IncPα plasmid-specific functions that allow pDG4Δ22 to be mobilized by the heterologous IncPβ plasmid R751. Tn5 insertions in pDG4Δ22 define a minimal 2.2-kilobase region required for plasmid-specific transfer of *oriT*. The Tra1 core contains the *traJ* and *traK* genes as well as an 18-kilodalton open reading frame downstream of *traJ*. The *traJ* and *traK* genes were shown to be required for transfer by complementation of inserts within these genes. Genetic evidence for the role of the 18-kilodalton open reading frame in transfer was obtained, although this protein has not been detected in cell lysates. These studies indicate that at least three transfer proteins are involved in plasmid-specific interactions at *oriT*.

The broad-host-range conjugation systems of IncP plasmids are able to mediate DNA transfer into a wide variety of bacterial cells (9). The detailed molecular mechanisms of the transfer process are not understood. The general model of conjugative DNA transfer involves nicking of a single plasmid DNA strand at the origin of transfer (*oriT*) sequence and subsequent transfer of the nicked strand to the recipient (17). Conjugal DNA synthesis in the donor and recipient cells reconstitutes the daughter plasmid molecules. In support of this model, the site-specific, single-strand nick induced in vitro by the IncP plasmid RK2/RP4 relaxation complex maps within the minimal *oriT* sequence defined by genetic studies (5–7). In addition, IncP plasmids encode DNA primases that function in conjugation to ensure efficient priming of complementary-strand synthesis in the recipient, thus promoting the broad-host-range transfer properties of these plasmids (11, 13).

The protein-DNA interactions at *oriT* that initiate the transfer process are plasmid specific. Conjugation systems do not generally recognize *oriT* sequences from unrelated plasmids, and small nonconjugative plasmids such as ColE1 and R1162/RSF1010 encode specific mobilization proteins to allow their *oriT* sequence to be transferred (1, 3, 4, 16). Even within the IncP group of plasmids, the transfer systems of the α and β subgroups (2, 12, 18) have diverged such that R751 (IncPβ) cannot mobilize the RK2/RP4 *oriT* sequence in the absence of specific RK2/RP4 transfer gene products (5, 18). The RK2/RP4 and R751 *oriT* regions have overall structural similarities but significant sequence differences, which account for this specificity of *oriT* transfer (9, 14). We have used R751 mobilization of the RK2 *oriT* as an assay to identify RK2/RP4 transfer genes and products that interact specifically with *oriT*.

Previous results have shown that R751 can mobilize pDG4, which contains 12 kilobases (kb) of RK2 counterclockwise from the *Eco*RI site, but cannot transfer pDG5, which contains a 760-base-pair *Hae*II *oriT* fragment (18). Subsequently, we found that a *Hae*II-generated deletion derivative of pDG4, designated Δ22 (Fig. 1), could also be transferred efficiently by R751. pDG4Δ22 contains a 2.6-kb piece of RK2 DNA surrounding *oriT* and comprising four contiguous *Hae*II fragments (10). pDG4Δ22 encodes a functional RK2 DNA-protein relaxation complex, which can be induced in vitro to cleave a single DNA strand within the *oriT* sequence (7, 14).

To define the region of pDG4Δ22 required for R751 mobilization, we isolated a series of Tn5 inserts and tested these for transfer by both R751 and pRK231, an ampicillin-sensitive RK2 derivative (8). The Tn5 inserts were mapped by a combination of restriction digests (Fig. 1). Tn5 inserts with transfer frequencies reduced 10-fold or more compared with pDG4Δ22 were considered R751 transfer deficient. As a control, all these mutants were transferred normally by pRK231, indicating that the *oriT* sequence was fully functional. The locations of the transfer-deficient mutants indicate that almost all of the 2.6-kb contiguous *oriT* region is required for R751 mobilization. Recently, Fürste et al. (5) have found that a 3.77-kb *Not*I fragment from the *oriT* region of RP4 is also transferred by R751 and have defined 2.2 kb by deletion analysis as required for heterologous transfer. Since RK2 and RP4 are probably identical, we have designated this region the RK2/RP4 Tra1 core.

Sequence analysis of the Tra1 core indicates three open reading frames from the region defined by the transfer-deficient Tn5 inserts (Fig. 1) corresponding to 13-kilodalton (kDa) (*traJ*), 14-kDa (*traK*), and 18-kDa proteins (5, 9). The *traJ* and *traK* gene products have been detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of lysates from cells containing these cloned genes, but the 18-kDa protein has not been identified (5). Transcription of this region is known to arise from two divergent promoters within *oriT* (9). The locations of these open reading frames correlate well with the map positions of the transfer-deficient Tn5 inserts and predict that all three proteins are required for efficient mobilization.

The role of the *traJ* gene product has been investigated in detail. The protein has been identified and purified by expression vector cloning, and it migrates as an 11-kDa polypeptide on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (5). The purified TraJ protein binds to

* Corresponding author.

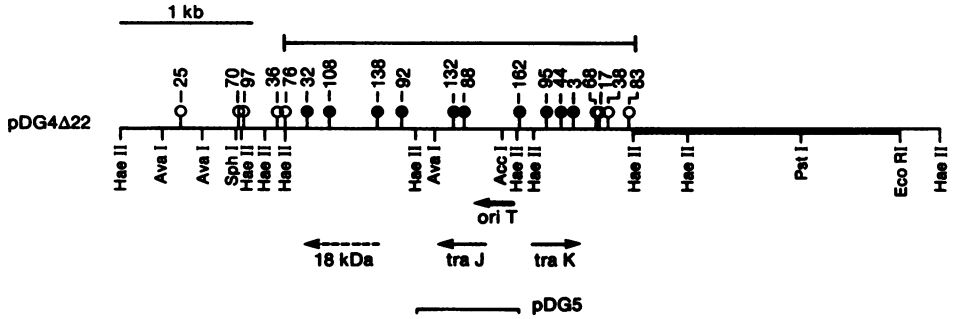

FIG. 1. Map of pDG4Δ22 showing the location of Tn*5* inserts. pBR322 DNA is indicated by the thick line. The bar indicates the four contiguous *Hae*II fragments surrounding *oriT* as found in the native RK2 plasmid. Tn*5* inserts, which are reduced in R751-mediated transfer 10-fold or more, are shown by the solid circles. The 250-base-pair minimal *oriT* is shown by the thick arrow indicating the direction of transfer. The reading frames for the *traJ*, *traK*, and 18-kDa protein are indicated by the arrows indicating direction of transcription. The *Hae*II fragment from the *oriT* region cloned in pDG5 is shown at the bottom (see reference 10 for a complete map of pDG5). Tn*5* inserts were isolated by transforming pDG4Δ22 into HB101::Tn*5* and selecting the resulting pDG4Δ22::Tn*5* by transfer into HB101 Nal^r by using the helper pRK2073 in triparental matings (10). Transfer by R751 was measured by transforming the pDG4Δ22::Tn*5* plasmids into HB101 (R751) and then mating these strains with HB101 Nal^r and using pDG4Δ22 and pDG5 as positive and negative controls, respectively (18).

the *oriT* sequence (5). Since Tn*5* inserts 88 and 132 map within the *traJ* coding sequence, we determined whether these inserts abolished synthesis of the TraJ protein, by using antiserum to the purified protein to detect specific bands on an immunoblot of cell lysates (Fig. 2). The results show that insert 88 eliminates production of the TraJ protein, while insert 132 leads to a slightly truncated gene product, confirming the map positions of these mutants. Insert 162 does not affect TraJ synthesis, a finding consistent with the location of the *traJ* promoter at the *Acc*I site (5). The immunoblot shows that pDG5, which contains the 760-base-pair *Hae*II *oriT* fragment (Fig. 1), also encodes the TraJ protein. Since pDG5 cannot be mobilized by R751 (18), these results confirm that *traJ* alone is not sufficient for heterologous mobilization.

The transfer-deficient phenotype of Tn*5* insert 88 does not prove that the *traJ* gene product is required for transfer, since this mutation could have a polar effect on a downstream gene, such as the 18-kDa open reading frame. To demonstrate directly that the TraJ protein is essential for transfer, we constructed plasmid pMS266-5 (Fig. 3) to provide the *traJ* gene in *trans* for a complementation experiment. pMS266-5 contains the coding sequence for the TraJ protein cloned as an *Eco*RI-*Bam*HI fragment in pWP201, a hybrid vector based on a ColD plasmid replicon. pMS266-5 was transformed into HB101 containing pDG4Δ22::Tn*5*-88, and mobilization by R751 was measured by a modification of the procedure described previously (10, 18). The transformants were grown by using double selection (penicillin [200 μg/ml] and sulfathiazole [400 μg/ml]), mixed 1:1 with HB101 (R751), and incubated on filters for 90 min. Cells were washed off the filters, mixed 1:1 with HB101 Nal^r, and incubated for another 90 min on filters. Following mating, the cells were washed off the filters, diluted, and plated on selective media containing penicillin (1 mg/ml) and nalidixic acid (20 μg/ml) for pDG4Δ22::Tn*5*-88 selection or trimethoprim (100 μg/ml) and nalidixic acid (20 μg/ml) for R751 selection. Penicillin-resistant transconjugants were shown to be sensitive to sulfathiazole but still resistant to kanamycin, indicating that recombination with the pMS266-5 had not occurred and the Tn*5* insert had not been lost. The results (Table 1) show that pMS266-5 increases the mobilization of pDG4Δ22::Tn*5*-88 by more than 100-fold. The control indicates that the parent plasmid, pWP201, has no effect on pDG4Δ22::Tn*5*-88 transfer. This complementation demonstrates that the TraJ protein is involved in the *oriT*-specific transfer process. The level of transfer of pDG4Δ22::Tn*5*-132 by R751 was higher than that of Tn*5*-88 (Table 1) and was not increased further by pMS266-5 (data not shown). This finding suggests that the slightly truncated TraJ protein encoded by pDG4Δ22::Tn*5*-132 (Fig. 2) is still functional.

The Tn*5* inserts 88 and 132 may also affect expression of the downstream 18-kDa open reading frame. In fact, pMS266-5 increased the transfer of pDG4Δ22::Tn*5*-88 only up to levels seen with insert 132 and other Tn*5* inserts, such as 92 and 138, that map downstream of *traJ*. Mutations in this region gave consistently higher transfer frequencies than insertions in either *traJ* or *traK* (Table 1). This finding is also consistent with results obtained by Fürste et al. (5), who

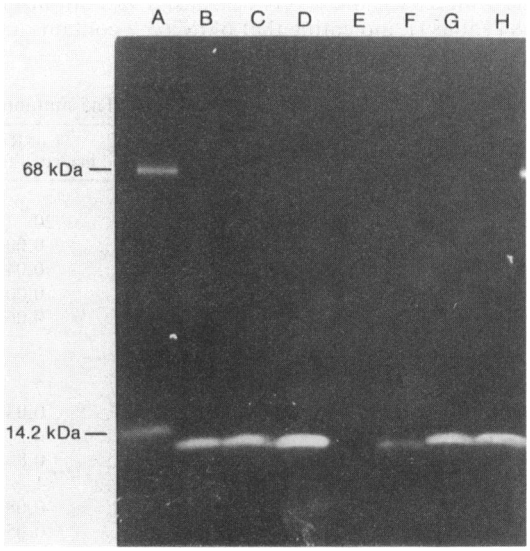

FIG. 2. Immunoblot using antisera to the TraJ protein to detect production in cells carrying various plasmids. The plasmids were transformed into HB101; cell lysates were prepared and run on a denaturing 17.5% polyacrylamide gel. The solid-phase immunoassay was done as described previously (12) by using antisera to the purified TraJ protein. Lanes: A, markers (bovine serum albumin [68 kDa] and lysozyme [14.2 kDa]); B, 25 ng of purified 11-kDa protein; C, pDG4Δ22; D, insert 162, E, insert 88; F, insert 132; G, insert 92; H, pDG5.

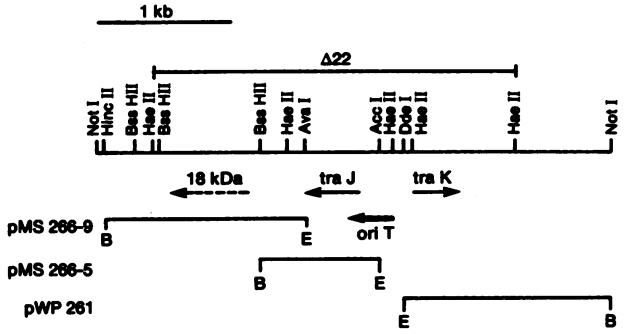

FIG. 3. Maps of plasmids used in the complementation experiments. The physical and genetic map of RP4 *Not*I fragment F (5) is drawn to the same scale as Fig. 1, with the addition of sites for *Hin*cII, *Bss*HII, and *Dde*I as determined by the nucleotide sequence (5, 14). The bar indicates the four contiguous *Hae*II fragments present in pDG4Δ22 (Fig. 1). The vector plasmid pWP201 (6.60 kb) used for the insertion of RP4 DNA fragments was derived from pMMB2 (15) by deletion of the smaller (3.48-kb) *Bst*EII fragment. A unique *Bam*HI site was created by insertion of a *Bam*HI linker (8′-mer) into the unique *Hpa*I site. The ColD-based replicon encodes kanamycin and sulfonamide resistance. To generate plasmids pMS266-9, pMS226-5, and pWP261, RP4 DNA fragments were isolated from pJF166uΔ7, pJF166uΔ5 and pJF161n, respectively (5). *Eco*RI-*Bam*HI fragments were inserted into pWP201 between the unique *Eco*RI (E) and *Bam*HI (B) sites so that transcription of the *tra* genes was under control of the promoter transcribing the sulfonamide resistance gene.

found that deletions in the TraI core distal to *traJ* give reduced but detectable transfer frequencies with R751. Presumably, the function abolished by inserts 92 and 138 can be partially provided by R751. We used plasmid pMS266-9, containing the region of TraI core from RP4 distal to the *traJ* gene (Fig. 3), to complement these insertion mutants. pMS266-9 increased the levels of transfer of both inserts 92 and 138 (Table 1), indicating that pMS266-9 contains a gene,

TABLE 1. Complementation of pDG4Δ22::Tn*5* mutants

Complementation plasmid and mutant	% of R751-mediated transfer
pMS266-5	
pDG4Δ22::Tn*5*-70	20
pDG4Δ22::Tn*5*-88	0.0001
pDG4Δ22::Tn*5*-88+pMS266-5	0.04
pDG4Δ22::Tn*5*-88+pWP201	0.0001
pDG4Δ22::Tn*5*-132	0.06
pMS266-9	
pDG4Δ22::Tn*5*-70	17
pDG4Δ22::Tn*5*-92	0.03
pDG4Δ22::Tn*5*-138	0.2
pDG4Δ22::Tn*5*-92+pMS266-9	0.8
pDG4Δ22::Tn*5*-138+pMS266-9	1
pDG4Δ22::Tn*5*-92+pWP201	0.08
pDG4Δ22::Tn*5*-138+pWP201	0.05
pWP261	
pDG4Δ22::Tn*5*-70	20
pDG4Δ22::Tn*5*-44	<0.0006
pDG4Δ22::Tn*5*-95	<0.0007
pDG4Δ22::Tn*5*-44+pWP261	0.02
pDG4Δ22::Tn*5*-95+pWP261	0.06
pDG4Δ22::Tn*5*-44+pWP201	<0.0005
pDG4Δ22::Tn*5*-95+pWP201	<0.0005

most likely the 18-kDa open reading frame, that is required for efficient transfer by R751. We also attempted to complement the activity of the *traK* gene abolished by inserts 44 and 95. pWP261, which contains the 14-kDa *traK* gene, increased the transfer of both pDG4Δ22::Tn*5*-44 and Tn*5*-95 by more than 100-fold (Table 1).

None of the complementing plasmids were able to restore the mobilization of pDG4Δ22::Tn*5* mutants to the wild-type level. With pMS266-5 complementation of insert 88, the transposon probably has a polar effect on expression of the downstream region including the 18-kDa open reading frame. This possibility is supported by the ability of pMS266-9 to increase the level of transfer of inserts 92 and 138 further toward wild-type levels. pWP261 is able to partially complement inserts in *traK*, but the transfer frequency remains low relative to that of the wild type. The reason for this inefficient complementation is not known but may involve poor expression and/or function of the *tra* gene products when the genes are not transcribed in their native configuration. Only small amounts of the TraK protein are found in cells even by using expression vectors, and the 18-kDa protein has not been detected. However, the complementation results indicate that the Tn*5* inserts are not acting to inhibit transfer of pDG4Δ22 by an alteration in structure or simply by proximity to *oriT*.

The transposon insertion mutants in pDG4Δ22 define the minimal RK2/RP4 region that can be mobilized by the heterologous conjugation system of R751. This functional unit, designated the TraI core, consists of the *oriT* sequence and three adjacent genes. The exact biochemical function of these gene products is still not known. Fürste et al. (5) showed that only the region to the left of *oriT* is needed for nicking, although the activity was lower than with the complete TraI core. The TraJ protein has been purified and shown to bind to the *oriT* region (5). Although a protein corresponding to the 18-kDa open reading frame has not yet been identified, the genetic experiments reported here suggest that this gene is a component of the TraI core and probably functions in the nicking reaction, since plasmids encoding only *traJ* (pDG5) do not form relaxation complexes (10). *traK* constitutes the third gene of the unit and is transcribed rightward from *oriT*. The role of this protein is not known, since it does not appear to be required for nicking. However, the absolute requirement for *traK* in mobilization of the RK2/RP4 *oriT* region by R751 suggests that *traK* functions in sequence-specific interactions at the transfer origin.

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