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Mutational Analysis of Essential IncPα Plasmid Transfer Genes *traF* and *traG* and Involvement of *traF* in Phage Sensitivity

VIRGINIA L. WATERS,[1]* BETTINA STRACK,[2] WERNER PANSEGRAU,[2] ERICH LANKA,[2] AND DONALD G. GUINEY[1]

Division of Infectious Diseases, Department of Medicine, University of California, San Diego Medical Center, 225 Dickinson Street, San Diego, California 92103-8416,[1] and Max-Planck-Institut für Molekulare Genetik, Abteilung Schuster, D-1000 Berlin 33, Germany[2]

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Although the broad-host-range IncP plasmids can vegetatively replicate in diverse gram-negative bacteria, the development of shuttle vector systems has established that the host range for IncP plasmid conjugative transfer is greater than the range of bacteria that sustain IncP replicons. Towards understanding IncP plasmid conjugation and the connection between IncP conjugation and *Agrobacterium tumefaciens* T-DNA transfer to plants, two sets of mutants were generated in the larger transfer region (Tra1) of the IncPα plasmid RK2. Mutagenesis strategies were chosen to minimize transcriptional polar effects. Mutant Tra1 clones were mapped, sequenced, and processed to reconstruct 49.5-kb Tra2-containing plasmid derivatives in order to assay for transfer activity and IncP plasmid-specific phage sensitivity. Focusing on the activities of the gene products of *traF* and *traG* in *Escherichia coli*, we found that mutations in *traF* abolished transfer activity and rendered the host cells phage resistant and mutations in *traG* abolished transfer activity but had no effect on phage sensitivity. Complementation of these mutant derivatives with corresponding *trans*-acting clones carrying *traF* or *traG* restored transfer activity and, in the case of the *traF* mutant, the phage sensitivity of the host cell. We conclude that in *E. coli*, both TraF and TraG are essential for IncP plasmid transfer and that TraF is necessary (but not sufficient) for donor-specific phage sensitivity, and sequencing data suggest that both TraF and TraG are membrane spanning.

The remarkable broad-host-range transfer properties of the IncP plasmids have stimulated investigation of the basic mechanism of conjugation as well as the use of these plasmids as tools for genetic manipulation of a wide variety of bacteria. The IncP plasmids can replicate in diverse gram-negative organisms, but the host range defined by conjugative transfer ability is considerably greater than the range of vegetative replication proficiency (15, 19, 50). With the construction of shuttle vector systems consisting of more than one host-specific replication system and the IncP transfer system, plasmid derivatives have been shown to transfer from gram-negative bacteria to highly divergent gram-negative organisms (17, 30, 52), to acid-fast bacteria (26), to gram-positive bacteria (47), and to yeast (22). In addition, significant similarities have recently been described between the IncP conjugation system and the T-DNA and *vir* loci of *Agrobacterium tumefaciens* tumor-inducing (Ti) plasmids that promote DNA transfer to plants (49, 56). These similarities are underscored by the recent demonstration that the *A. tumefaciens* Vir region can mobilize the small non-self-transmissible IncQ plasmids (2) among agrobacteria in a manner similar to the established IncP mobilization of these IncQ plasmids (11).

Unlike the F plasmid, in which the conjugative transfer genes are organized into a single contiguous operon (51), the IncP plasmids have a more complicated arrangement of their transfer systems. In the IncPα plasmid RP4 (apparently identical to RK2), these genes are located in two regions 10 kb apart, designated Tra1 and Tra2. We have recently described the molecular cloning of the Tra1 and Tra2 regions on separate vectors, with reconstitution of a functional transfer system by *trans* complementation (27). The Tra2 region (formerly Tra2/Tra3 [1]) consists of an 11-kb fragment and appears to be transcribed from promoters located adjacent to the essential replication gene *trfA* (27). A surface exclusion locus consisting of two genes (*eex1* and *eex2*) has been mapped in Tra2 (28), as well as other loci affecting sensitivity to donor-specific phage (27).

The genetic organization of Tra1 is complex, and the limits of the essential transfer region have not yet been determined. Fifteen putative genes spanning about 15 kb have been identified by DNA sequence analysis and are arranged in at least four transcriptional units (20, 23a, 55, 56). Of these loci, essential roles in *Escherichia coli* to *E. coli* transfer have been assigned to the products of *traI*, *traJ*, and *traK* (13, 39, 49, and 54). TraI and TraJ are required for relaxosome formation and site-specific nicking at *oriT* (39), and TraI becomes covalently associated with the 5′ end of the DNA strand destined for transfer to the recipient cell (42). TraK is involved in the DNA-protein complex at *oriT* and binds in the leading strand region (55). Within *traI* is a small open reading frame, *traH*, which encodes a protein proposed to stabilize the relaxosome (39), but the in vivo requirement of *traH* for transfer has not been shown.

We report the isolation and characterization of specific mutations in the two genes located downstream from *traIH*, the *traF* and *traG* loci. The DNA sequences of *traG* for RP4 and R751 have been reported previously, and homology of the TraG proteins to the VirD4 proteins of the *A. tumefaciens* Ti plasmids was noted (56). We find that both *traF* and *traG* are essential for conjugative transfer in *E. coli*. The

* Corresponding author.

TABLE 1. Bacterial plasmids

Plasmid	Description	Marker(s)	Reference
pRK231	49.5-kb *Pst*I deletion derivative of RK2	Km^r Tc^r	32
pRK231B	pRK231 with *Bam*HI linker at unique *Eco*RI site	Km^r Tc^r	27
pVWDG23110	22.1-kb Tral-containing *Pst*I-*Bam*HI fragment cloned into pBR329 (9)	Km^r	27
pBS140	2.2-kb *Hin*cII-*Sph*I *traG*-containing fragment of RP4 cloned into pJF119EH (12)	Ap^r	This work
pWP471	0.7-kb *Nsp*HI-*Hae*II *traF*-containing fragment of RP4 cloned into pJF119EH (12)	Ap^r	This work
pBS730Δ1	5.0-kb *Sst*II-*Sph*I RP4 fragment containing *traE*, *traF*, and *traG*[a]	Ap^r	This work
pBS730Δ3	4.0-kb *Sst*II-*Sph*I fragment containing *traE* and *traF*[a]	Ap^r	This work
pBS701	3.0-kb *Sst*II-*Not*I fragment containing *traE* and *traF*[a]	Ap^r	This work
pBS702	2.3-kb *Sst*II-*Eco*RI fragment containing *traE*[a]	Ap^r	This work
pBS703Δ1	3.5-kb *Not*I-*Sph*I fragment containing *traF* and *traG*[a]	Ap^r	This work

[a] Cloned into pT7-6 (45).

traF sequences for RP4 and R751 are presented and each displays a signal sequence motif. *traF* is necessary (but not sufficient) for donor-specific phage sensitivity, while mutations in *traG* have no effect on such phage sensitivity.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* HB101 (6), *E. coli* DH5 (21), *E. coli* RU4404 (48), and *E. coli* K38 (45) were grown in Luria-Bertani liquid or solid media (33). Plasmids were maintained in *E. coli* DH5 and are listed with relevant characteristics in Table 1. Although the IncPα plasmids RK2 and RP4 remain molecularly indistinguishable, it should be noted that genetic analyses described herein were performed with RK2, protein expression was done with RP4, and sequencing results were obtained with RK2 and RP4.

Mutagenesis of the RK2 Tral region. One set of Tral mutants was generated by transposon mutagenesis via Tn*1725*, which carries a chloramphenicol resistance marker (48). The Tral clone pVWDG23110 (27), retaining the kanamycin resistance marker from RK2, was transformed into *E. coli* RU4404, which harbors a chromosomal copy of Tn*1725*, and the culture was passed for 30 generations. Plasmid DNA was prepared and the presumed mutant bank was transformed into *E. coli* DH5 and plated with selection for chloramphenicol and kanamycin resistance. Colonies were screened by DNA clonal analysis (5), and unique clones with full-size inserts were selected for large-scale plasmid DNA preparation. Digestion with *Eco*RI and religation deleted all but 35 bp of the transposon (48). The location of each 35-bp insert was mapped with *Eco*RI in double digestion with *Not*I, *Hin*dIII, *Bam*HI, or *Pst*I. A second set of Tral mutants was obtained by controlled digestion of pVWDG23110 with DNase I in the presence of Mn^{2+} (43). Randomly linearized plasmid molecules were ligated to *Xba*I site-containing 14-mer linkers, -CTAGTCTAGACTAG- (New England Biolabs, Beverly, Mass.), containing translation termination codons in all three reading frames. After transformation, plasmid DNA was prepared and digested with *Xba*I. Full-size linearized molecules were electroeluted, ligated, and retransformed into *E. coli* DH5. The resulting mutant bank was screened by mapping the location of the new *Xba*I site.

Sequence analysis of RP4, RK2, and R751 *traF* genes. Sequence analysis was performed by using the dideoxy chain-termination method (44) with modifications to obtain high-quality autoradiographs (4, 14). Single-stranded DNAs of phage M13 subclones containing Tral fragments served as templates for DNA sequencing reactions, which were run with DNA polymerase (large fragment) or *Taq* DNA polymerase. To reduce the formation of secondary structures during electrophoresis, 7-deaza-dGTP was used instead of dGTP (34) and 6% (wt/vol) polyacrylamide gels were run at 70°C. Nucleotide sequences were analyzed by using version 7 of the University of Wisconsin, Madison, Genetics Computer Group (10) software package.

Expression of the *traF* and *traG* gene products. Tral gene products were specifically labeled by using the T7 RNA polymerase-T7 ϕ10 promoter system. Appropriate Tral fragments were inserted into the multiple cloning site of the T7 promoter plasmid pT7-6 (45). The resulting recombinant plasmids were introduced by transformation into *E. coli* K38 cells containing the T7 RNA polymerase plasmid pGP1-2 (29). Cells were grown in M9 medium supplemented with thiamine (20 μg/ml) and 18 amino acids (200 μg/ml each, without cysteine and methionine) at 30°C until an A_{600} of 1.5 was reached. The temperature was shifted to 42°C, rifampin was added, and after an additional 10 min at 42°C, the cells were grown for 20 min at 30°C. After pulse-labeling with [^{35}S]methionine (10 μCi, 800 Ci/mmol) for 5 min at 30°C, the cells were harvested, resuspended in 60 mM Tris-HCl (pH 6.8)–1% (wt/vol) 2-mercaptoethanol–10% (wt/vol) glycerol–0.01% (wt/vol) bromophenol blue, and heated to 95°C for 3 min. Aliquots were analyzed on sodium dodecyl sulfate (SDS)–17.5% (wt/vol) polyacrylamide gels.

Plasmids used for complementation of mutations in *traF* and *traG* were obtained by cloning the genes into the multiple cloning site of the autoregulated P*tac*/*lacI*^{q} vector pJF119EH (12). Plasmid pWP471 contains a 668-bp *Nsp*HI-*Hae*II RP4 fragment. This DNA fragment does not contain the original *traF* translational initiation signal and lacks also the first 6 codons of the *traF* structural gene. The 5′ end of the gene was reconstructed by inserting a double-stranded synthetic oligodeoxyribonucleotide (TGGAAGCGGCTCA, TATGAGCCGCTTCCAGCGC), connecting *traF* to a 38-bp *Xba*I-*Nde*I fragment of pT7-7 that contains the efficient translational initiation signal of the phage T7 gene *10*. The clone pBS140 was obtained by insertion of a 2,214-bp *Hin*cII-*Sph*I RP4 fragment (RP4 coordinates 46.4 to 48.6) containing gene *traG* into pJF119EH. Both *traF* and *traG* genes as contained in the 3.5-kb *Not*I-*Sph*I insert of pBS703Δ1 (see Fig. 3A) were also used to complement pRK231B mutants (Table 2).

Determination of transfer frequency. Conjugation experiments were performed on solid media as previously described (20) with *E. coli* HB101 (streptomycin resistant) as the recipient and *E. coli* DH5 carrying the RK2 plasmid or derivative as the donor. With a donor-to-recipient cell ratio of 1:4, the mixed culture was filtered, the filters were incubated on plates for 45 min (to minimize the occurrence

TABLE 2. Transfer proficiency and phage sensitivity of *traE*, *traF*, and *traG* mutants

Plasmid(s) in donor	Transfer frequency[a]	GU5 and PRD1 phage sensitivity[b]
RK2	2×10^{-1}	S
pRK231 or pRK231B	2×10^{-1}	S
pBS140 (*traG*)		R
pWP471 (*traF*)		R
pBS703Δ1 (*traFG*)		R
pRK231B*traF*168	5×10^{-7}	R
pRK231B*traF*168 + pBS140	6×10^{-7}	R
pRK231B*traF*168 + pWP471	2×10^{-6}	S
pRK231B*traF*168 + pBS703Δ1	1×10^{-1}	S
pRK231B*traF*32	1×10^{-7}	R
pRK231B*traF*32 + pBS140	4×10^{-7}	R
pRK231B*traF*32 + pWP471	5×10^{-2}	S
pRK231B*traF*32 + pBS703Δ1	2×10^{-1}	S
pRK231B*traG*64	1×10^{-7}	S (turbid plaques)
pRK231B*traG*64 + pBS140	2×10^{-2}	S (turbid plaques)
pRK231B*traG*64 + pWP471	2×10^{-7}	S (clear plaques)
pRK231B*traG*64 + pBS703Δ1	3×10^{-2}	S (clear plaques)
pRK231B*traG*18	1×10^{-7}	S (turbid plaques)
pRK231B*traG*18 + pBS140	7×10^{-2}	S (turbid plaques)
pRK231B*traG*18 + pWP471	1×10^{-6}	S (clear plaques)
pRK231B*traG*18 + pBS703Δ1	8×10^{-2}	S (clear plaques)
pRK231B*traG*16	6×10^{-7}	S
pRK231B*traG*16 + pBS140	1×10^{-1}	S
pRK231B*traG*16 + pWP471	6×10^{-8}	S
pRK231B*traG*16 + pBS703Δ1	1×10^{-1}	S
pRK231B*traG*110	1×10^{-8}	S
pRK231B*traG*110 + pBS140	2×10^{-2}	S
pRK231B*traG*110 + pWP471	9×10^{-8}	S
pRK231B*traG*110 + pBS703Δ1	1×10^{-2}	S

[a] Transfer frequencies, representative of three or more conjugation experiments, are expressed as the ratio of the number of exconjugants per recipient.
[b] Phage sensitivity patterns for phage GU5 and PRD1 were always identical. S, sensitive; R, resistant.

of multiple transfer events) and washed into 150 mM NaCl, and serial dilutions were plated onto selective media. Transfer frequencies were expressed as the ratio of the number of exconjugants per recipient cell, to keep frequency values less than 1.0. To obtain transfer data with RK2 mutant derivatives of pVWDG23110, large 49.5-kb recombinant clones were constructed. DNA of each derivative was digested with *Hin*dIII and *Bam*HI, and the 21-kb Tral-containing fragment was electroeluted and ligated to the 28-kb *Hin*dIII-*Bam*HI fragment of pRK231B. Recombinants were then transformed into *E. coli* DH5 for transfer into *E. coli* HB101. In selected experiments, the *E. coli* DH5 transformants were further transformed with potentially complementing *trans*-acting clones.

Determination of phage sensitivity. Phage stocks of PRD1 (38) and GU5 (1, 23) were prepared (38), and sensitivities were determined by performing standard plaque assays. *E. coli* DH5 cells carrying the relevant plasmid derivative were grown overnight with selection in Luria-Bertani broth. An aliquot of the culture was mixed with an equal volume of diluted phage and plated. Results were reported as S for phage sensitive, for which the phage titers were about 10^{10}/ml for GU5 and 10^{9}/ml for PRD1, and R for phage resistant, for which no plaques were obtained with the most concentrated phage solutions.

Nucleotide sequence accession number. The nucleotide sequences reported in this paper have been submitted to GenBank with accession numbers M94366 (*traF* RP4) and M94367 (*traF* R751).

RESULTS

Derivation of the RK2 Tral clone. The native 60-kb plasmid RK2 encodes resistance to the antibiotics ampicillin, tetracycline, and kanamycin, as well as one replication origin and the two large transfer-related regions Tral and Tra2 (15). We employed the 49.5-kb deletion derivative pRK231B, which lacked the ampicillin resistance marker but retained full self-transmissibility (Table 2). From this 49.5-kb derivative we constructed the 24-kb Tral-containing clone pVWDG23110. As expected, pVWDG23110 alone did not transfer (data not shown) for lack of the gene products from the Tra2 region, but it was more amenable to genetic manipulation and mutagenesis than pRK231B. Derivatives of pRK231B were reconstituted by employing the mutant derivatives of Tral made from pVWDG23110 and wild-type Tra2-containing DNA from pRK231B.

Generation of RK2 Tral mutants. The Tral clone pVWDG23110 was mutagenized by transposon insertion with Tn*1725* (48) and random insertion of translation termination linkers. Upon examination of plasmid DNA preparations from the former collection of transposon mutants, we found that a significant number had undergone deletions of various sizes at the site of Tn*1725* insertion. Such deletion derivatives were disregarded, and only unique and full-size insertion derivatives were selected. The number of distinct transposon mutants was consequently reduced, but two were processed for this study (Fig. 1). Most of the transposon was excised from each mutant by digestion with *Eco*RI and religation, leaving a 35-bp insert which would cause a frameshift in the reading of the mutated gene (48). The location of a new *Eco*RI site remaining at the site of excision was determined by restriction mapping and DNA sequencing. Mutagenesis by controlled DNase I cleavage gave randomly linearized molecules which were ligated to *Xba*I-containing translation-termination linkers. Mutant preparations were screened by *Xba*I-*Not*I digestion, and selected mutants were precisely mapped and processed for phenotypic assays (Fig. 1). Both of these strategies were chosen in order to minimize the severity of transcriptional polarity that is often observed with conventional transposon mutagenesis (3).

Transfer phenotypes of mutations in *traF* and *traG*. Two transposon mutants of pVWDG23110 were chosen for analysis. Restriction enzyme map positions confirmed by sequencing showed that one 35-bp insert, pVW16, was in the *traG* gene, and the other, pVW32, was in the *traF* gene (Fig. 1, 2A, and 3A). *Xba*I-linker mutants of *traF* and *traG* were also selected for analysis. In order to supply Tra2 transfer and phage-related functions in *cis*, reconstituted large plasmid derivatives were constructed for each mutation. The 28.5-kb *Hin*dIII-*Bam*HI fragment of pRK231B, containing the *oriV*, *trfA*, and Tra2 regions, was electroeluted and ligated to the similarly electroeluted 21.0-kb Tral-containing *Hin*dIII-*Bam*HI fragment of each mutant derivative. The resulting recombinants, selected by the restoration of the kanamycin and tetracycline markers, have the native RK2 replication and Tra2 regions with the desired mutation in the Tral region. *E. coli* conjugation frequencies for each mutant

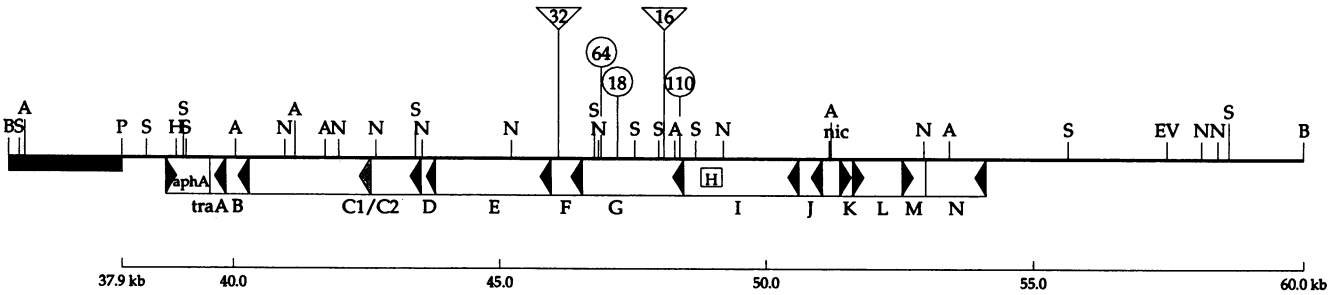

FIG. 1. Restriction map of TraI-containing clone pVWDG23110, spanning 22.1 kb from coordinates 37.9 to 60.0 kb on the standard RP4 map. Indicated are transfer genes *traA* through *traN*, including *traH*, which is an overlapping reading frame within *traI*, and the kanamycin resistance gene *aphA*. Transposon Tn*1725* insertion mutants are numbered within triangles, and translation-terminator linker mutants are numbered within circles. The darkened line represents DNA from pBR329, and restriction enzyme sites are abbreviated as follows: A, *Acc*I; B, *Bam*HI; EV, *Eco*RV; H, *Hin*dIII; N, *Not*I; P, *Pst*I; and S, *Sph*I.

were calculated as the number of exconjugates per recipient cell. In a 45-min mating experiment with a donor-to-recipient cell ratio of 1:4, the positive controls RK2 and pRK231B transferred at a frequency of 10^{-1}, while transfer of the pRK231B*traF*32 and pRK231B*traG*16 transposon mutants occurred at frequencies of less than 10^{-6} (Table 2). Recombinants similarly constructed, but starting with DNA preparations of the *Xba*I-linker mutants pVW64, pVW18, and pVW110 (Fig. 1), also transferred with severely reduced transfer frequencies (pRK231B*traG*64, pRK231B*traG*18, and pRK231B*traG*110 [Table 2]). *trans* complementation of the TraF- or TraG-deficient phenotype was achieved with the corresponding gene cloned in ColEI-type plasmids: pBS140 carrying the *traG* gene, pWP471 carrying *traF*, and pBS703Δ1 carrying *traF* and *traG*. (Table 2). The restoration of conjugative transfer confirms the specificity of each mutation and rules out severe polar effects on downstream loci. These results demonstrate the essential role of TraF and TraG in matings between *E. coli* strains and also show that these proteins are not required in *cis*. In the case of TraG, the complete functional loss with translation terminator mutations mapping in the latter part of the gene, i.e., with pVW18 and pVW64 (Fig. 1), indicates that the N-terminal and central domains of the protein are not sufficient for TraG transfer activity. TraF is a much smaller protein, but the same can be said for the necessity of its C terminus, according to the mapped position of pVW32 (Fig. 1).

trans complementation of both *traF* and *traG* pRK231B mutants was also attempted with the described *traF*- and/or *traG*-containing ColEI clones in the recipient (*E. coli* HB101). Transfer proficiency was never restored in these experiments (data not shown).

Donor-specific phage sensitivity of *E. coli* harboring RK2 mutants. Plaque assays were performed by employing IncP plasmid-specific phages PRD1 and GU5 to infect *E. coli* DH5 carrying the *traF* and *traG* mutants with and without the complementing cloned gene. Results indicate that cells harboring an RK2 derivative with a mutation in the *traF* gene are completely resistant to the donor-specific phages PRD1 and GU5 (Table 2). Phage sensitivity, as determined by high-titer plaquing of each phage, was restored when the TraF phenotype was returned via the expression of either of the complementing *traF*-carrying clones pWP471 or pBS703Δ1. That TraF by itself is insufficient for productive phage infection was demonstrated by phage resistance seen with *E. coli* DH5 carrying only pWP471 or pBS703Δ1 (Table 2). No effect on the ability of these phages to produce high titers was observed with the *traG* N terminus mutants, pRK231B*traG*16 and pRK231B*traG*110 (Table 2). However, there was a definitive and reproducible effect on phage infection, resulting in plaques that appeared turbid, with *traG* C terminus mutants pRK231B*traG*18 and pRK231B*traG*64. In complementation experiments employing these mutants with *traF* clones, plaque clarity was restored (Table 2).

Sequence analysis of the *traF* genes of RP4 and R751. The DNA and deduced amino acid sequences of the *traF* genes from RP4 (IncPα) and R751 (IncPβ) are shown in Fig. 2. The DNA sequences are 80% identical, and the proteins share 77% identity. This result is consistent with the degree of homology seen between other loci of the two plasmids (40, 56). With the exception of *traJ* and *traK*, which we call specificity determinants, all components of the RP4 Tra system are interchangeable with those of R751 (23a). The high degree of similarity between the two *traF* loci is therefore not surprising and suggests that the *traF* gene products perform the same transfer function in these two plasmids. Both TraF proteins contain excellent signal sequences and are probably membrane spanning or translocated to the periplasm or cell surface.

Expression of the *traF* and *traG* gene products. To establish that the protein products of the *traF* and *traG* genes are produced, this region was cloned downstream from the T7 promoter as a 4.8-kb *Sst*II to *Sph*I fragment (pBS730Δ1 [Fig. 3A]). Deletions of this fragment were generated to progressively eliminate the *traG* and *traF* coding regions (Fig. 3A). Expression from the T7 promoter of these clones was induced in *E. coli* K38, and the resulting labeled proteins were resolved by SDS-polyacrylamide gel electrophoresis (Fig. 3B). Parent clone pBS730Δ1 produces all four predicted proteins, TraG, TraF, TraE, and the TraD* fusion product. As expected, intact TraG is eliminated in pBS730Δ3 and pBS701 expression. Notably, the amount of TraF is decreased in these two clones as well, suggesting translational coupling of the *traF* and *traG* loci. This hypothesis is consistent with sequence analysis which suggests that TraI genes are organized in a few major operons, with long messages transcribed from promoters for *traG*, *traJ*, and *traK*. The reading frames for *traF* and *traG* contain overlapping start/stop codons (23a, 56). In cells carrying the smallest derivative, pBS702, which lacks *traF* and *traG*, only TraE and the TraD* fusion product are expressed. Overall, these results demonstrate that both TraF and TraG are produced as predicted from the DNA sequence analysis.

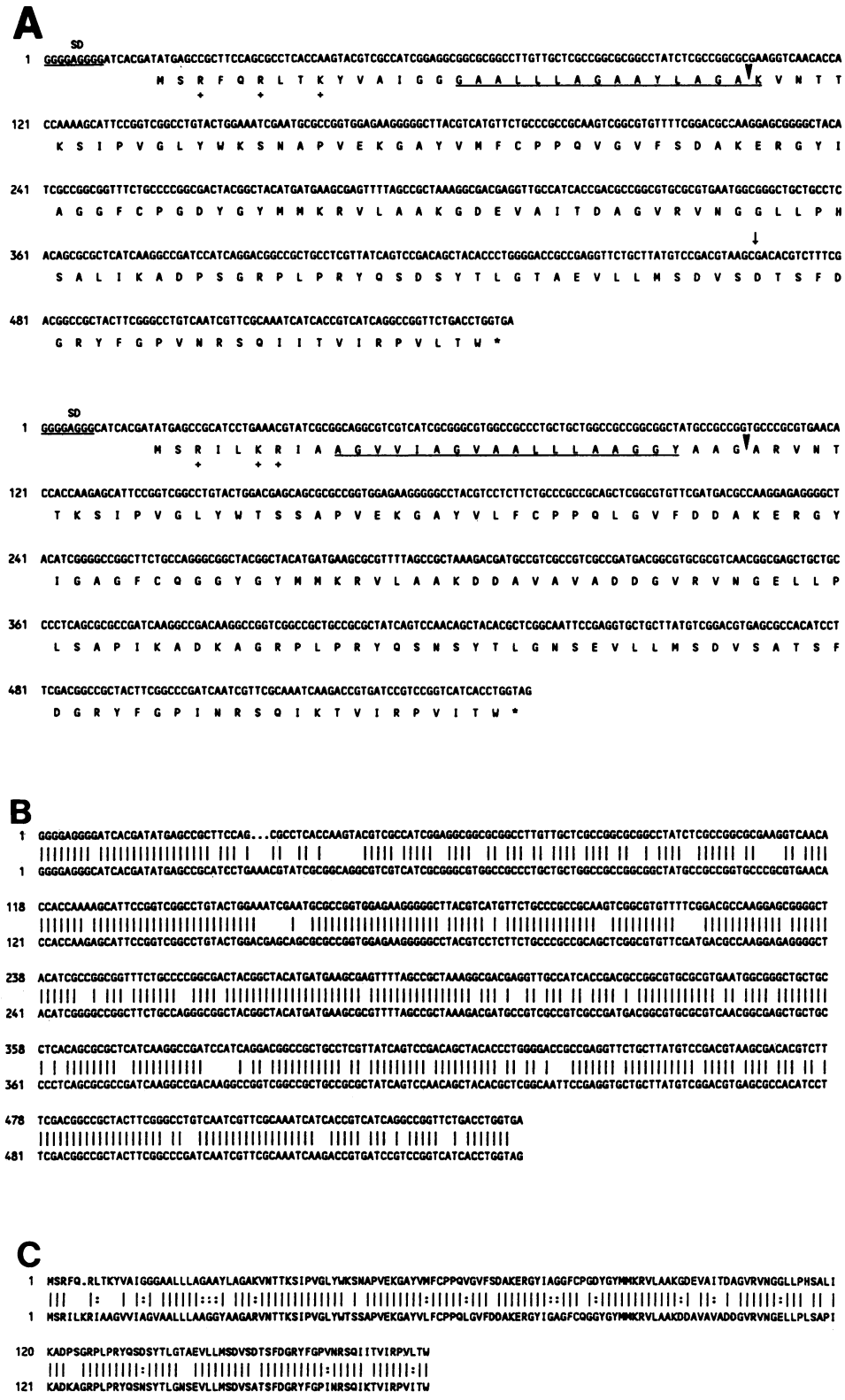

FIG. 2. Sequence analyses of transfer gene *traF* from the IncPα plasmid RP4 (upper) and the IncPβ plasmid R751 (lower). (A) DNA, encoded amino acids, upstream Shine-Dalgarno sequences (SD), proposed leader sequence and cleavage point, basic amino acids (+), and position of insert mutation (downward arrow); (B) DNA sequence comparison showing 80% identical nucleotides as vertical lines and one gap indicated as a dotted line; (C) protein comparison showing 87% amino acid similarity and 77% identity indicated, respectively, as double dots and vertical lines, with a single gap indicated as a dot in the RP4 sequence.

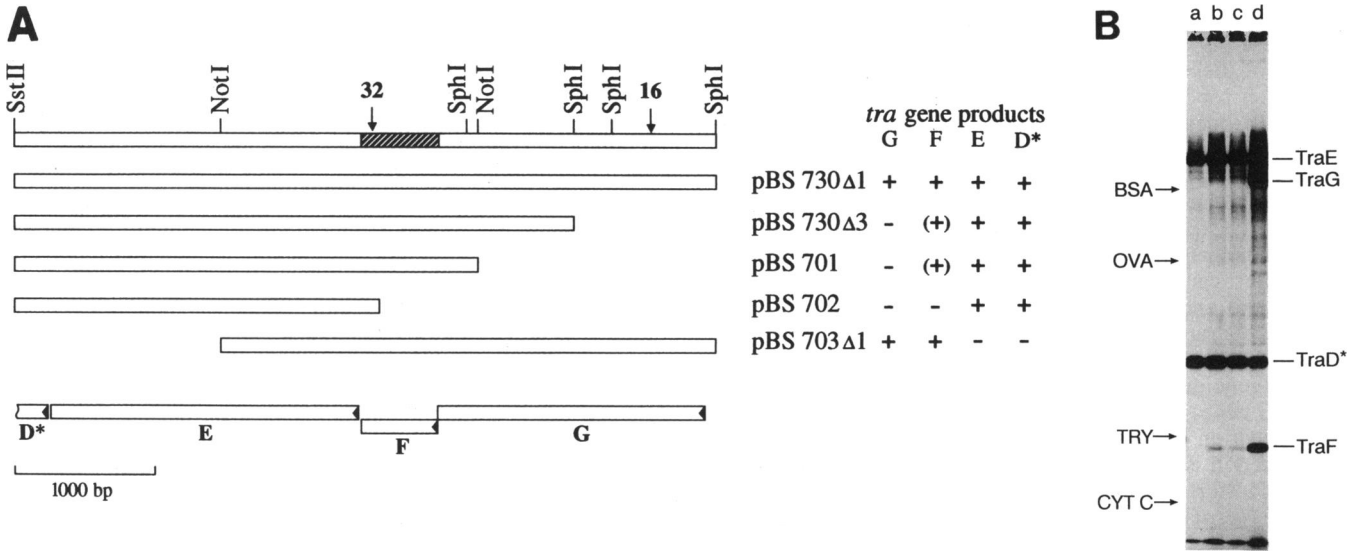

FIG. 3. Specific labeling of RP4 Tra proteins with the T7 RNA polymerase/promoter system described in Materials and Methods. (A) Subclones of RP4 defined by DNA segment cloned and by the gene products expressed, with map sites of the transposon insertions 16 and 32 indicated (also in Fig. 1); (B) polyacrylamide gel electrophoresis of expressed proteins: lane a, pBS702; lane b, pBS701; lane c, pBS730Δ3; lane d, pBS730Δ1. The positions of the reference proteins, bovine serum albumin (BSA, 68 kDa), ovalbumin (OVA, 43 kDa), trypsin inhibitor (TRY, 25.7 kDa), and cytochrome *c* (CYT C, 11.7 kDa), are marked.

DISCUSSION

The conjugation systems of the IncPα plasmids are arranged in two separate regions called Tra1 and Tra2. The Tra2 region has been defined by deletion analysis to be located within coordinates 18.03 to 29.26 kb on the standard RP4 map (24, 40). Functions mapping within Tra2 include phage sensitivity, entry exclusion, and the locus *kilB*, also designated *trbB* (28, 35). The *kilB* gene, which has homology with *virB11* of *A. tumefaciens* (35), has been shown by mutation and complementation analysis to be required for transfer (27). The extent of the Tra1 region that is essential for conjugation between *E. coli* strains is currently under study. Within Tra1 is the transfer origin and nick site, surrounded by the divergently transcribed genes *traJ*, *traK*, and *traI*, which are essential for mobilization of IncP plasmids (16, 18). DNA sequence analyses, combined with expression and purification of the protein products, have identified 12 other genes within the Tra1 region (19, 56), and the question of which of these other proteins are essential for plasmid transfer is addressed herein regarding TraF and TraG. The RP4 DNA primase genes, designated *traC* and *traC'*, are located toward the end of Tra1 (Fig. 1) (25, 31). Mutations in *traC* were reported to have an effect on the host range of transfer but did not affect matings between *E. coli* strains or transfer to *Pseudomonas aeruginosa* (36).

The transfer-deficient phenotypes of the *traF* and *traG* mutations define them as essential Tra1 transfer genes. *traF* and *traG* are located immediately downstream from *traI*, encoding the site- and strand-specific endonuclease which cleaves at the nick site (39, 42). Both *traF* and *traG* appear to have good export signal sequences. *traG* sequence data and promoter analysis from RP4 and R751 indicate that *traG* is transcribed as the first gene in a long operon that may extend for seven genes to *traA* (23a, 31, 56). The function of TraG in transfer is unknown, but the recently described amino acid homology of TraG and VirD4 of an *A. tumefaciens* Ti plasmid provides some clues (56). Like TraG, VirD4 is required for DNA transfer and is encoded downstream from the genes (*virD1* and *virD2*) encoding unwinding and site- and strand-specific nicking activities for the T-DNA borders of Ti plasmids (53, 56). These right and left directly repeated borders, delimiting the T-DNA segment that is transferred and integrated into the plant genome, not only share a functional similarity with the *oriT* of RP4 but also display sequence identity (41, 49). TraG and VirD4 are of similar size, are thought to operate at the membrane level, and may share an analogous function(s) in the DNA transfer process. VirD4 may operate at the inner membrane of *A. tumefaciens*, protruding its C-terminal end into the periplasmic space (37). Although the TraG protein has not been localized in *E. coli*, our mutation analysis indicates that both the C-terminal and N-terminal ends of the protein are required for activity, and we suspect that each of the domains of TraG (and VirD4) have specific roles. To address the possibility that TraG or TraF could be functioning in some capacity in the recipient cell, *trans* complementation of mutants was attempted with the complementing clone resident in the recipient. The complete lack of such complementation supports a model of IncP plasmid transfer in which these proteins must operate at the surface of the donor cell.

traF is the first locus in Tra1 shown to be required for donor-specific phage sensitivity. This result is consistent with the early mutation analysis of Barth et al. (1), who reported that certain Tn*7* insertions in the Tra1 region affected phage sensitivity. Whether these Tn*7* insertions were located in *traF* or exerted polar effects on *traF* expression is unclear, since the precise map position of these mutations within the presently known Tra1 sequence is unknown. The *traF* genes of RP4 and R751 contain excellent signal sequences, suggesting that the proteins are membrane spanning or exported. It has often been assumed that plasmid mutations resulting in plasmid-specific phage resistance are indicative of lesions in the genes encoding pilus biosynthesis. Although certain of the IncP donor-specific phages

appear to adsorb to pili (7, 8), it has not been demonstrated that these pili are sex pili or that all phage resistance is due to loss of these pili. In fact, adsorption to the cell wall has also been reported (38), and resistance to PRD1 has been shown to occur with IncN plasmid mutants which indeed produce transfer pili and are transfer proficient (46). Electron microscopic studies are presently addressing the question of whether TraF is involved in IncP pilus biosynthesis. Since *traF* expression appears to be coupled to that of *traG* at the level of translation, an attractive hypothesis is that TraF interacts with TraG at the cell membrane in an equimolar ratio and that TraF is an essential component of the mating apparatus (and phage production) at the cell surface.

Translational coupling of TraF and TraG was suggested by in vitro gene expression; in clones carrying *traF* but lacking the *traG* gene, *traF* expression is severely reduced (Fig. 3). An indication of the in vivo biological relevance of this phenomenon is the change in phage plaquing obtained with translation-terminator *traG* mutants pRK231B*traG*64 and pRK2231B*traG*18. Unless complemented with a clone carrying *traF*, phage PRD1 or GU5 produces very turbid plaques when plated on *E. coli* carrying these mutants. We presume that expression of *traF* is reduced in these *traG* mutants by translational polarity, which results in inefficient phage plaquing but has no effect on transfer activity. The differential effect could be because efficient phage production requires more TraF molecules per cell than does transfer. The absence of apparent polarity in pRK231B*traG*110, in which the insert is further upstream from the *traF* gene, is not likely due to secondary translational start codons, since only one TraG protein species has been shown by anti-TraG solid-phase immunoassay (56).

The mobilization of DNA in transfer, either from bacteria to bacteria in conjugation or from bacteria to plants in *Agrobacterium* tumor induction, requires two categories of (nonregulatory) proteins: (i) those that interact directly with the DNA and form a relaxosome or protein-DNA complex and (ii) those that mediate the passage of this complex from the donor to the recipient cell. We believe that the TraI, TraJ, and TraK IncP proteins and the VirD1, VirD2, and VirE Ti proteins are in the first category; the TraF and TraG IncP proteins and the VirD4 and VirB Ti proteins would then be in the second category.

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