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Analysis of Mutations in *trfA*, the Replication Initiation Gene of the Broad-Host-Range Plasmid RK2

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Plasmids with mutations in *trfA*, the gene encoding the replication initiation protein of the broad-host-range plasmid RK2, were isolated and characterized. Mutants identified from a nitrosoguanidine bank were defective in supporting the replication of a wild-type RK2 origin in *Escherichia coli*. Most of the mutations were clustered in a region of *trfA* corresponding to the carboxy-terminal quarter of the TrfA protein. 5′ and 3′ deletion mutants of *trfA* were also constructed. A C-terminal deletion of three amino acids of the Tr A protein was completely nonfunctional for RK2 replication. However, a deletion of 25 amino acids from the start of the 33-kDa TrfA protein was still competent for replication. Further characterization of the point and deletion *trfA* mutants in vivo revealed that a subset was capable of supporting RK2 replication in other gram-negative bacteria, including *Pseudomonas putida*, *Agrobacterium tumefaciens*, and *Azotobacter vinelandii*. Selected mutant TrfA proteins were partially purified and characterized in vitro. Velocity sedimentation analysis of these partially purified TrfA proteins indicated that the wild-type protein and all mutant TrfA proteins examined exist as dimers in solution. Results from in vitro replication assays corroborated the experimental findings in vivo. Gel retardation results clearly indicated that the point mutant TrfA-33:151S, which was completely defective in replication of an RK2 origin in all of the bacterial hosts tested in vivo, and a carboxy-terminal deletion mutant, TrfA-33:CΔ305, were not able to bind iterons in vitro. In addition to the proteins from mutants that were totally defective in DNA binding, several other mutant proteins were either partially defective or could not be distinguished from the wild-type protein in binding to the origin region. The mutant proteins with apparently normal DNA-binding activity in vitro either were inactive in all four gram-negative bacteria tested or exhibited differences in functionality depending on the host organism. These mutant TrfA proteins may be altered in the ability to interact with the replication proteins of the specific host bacterium.

RK2 is a 60-kb self-transmissible plasmid that can be stably maintained in a wide variety of gram-negative hosts (9, 21, 27, 32, 33, 40). It is a member of the IncP1 incompatibility group, and the number of copies of RK2 in *Escherichia coli* has been estimated as four to seven per chromosome (17). Two plasmid elements that are essential for RK2 plasmid replication are the *cis*-acting origin of replication, *oriV*, and the *trans*-acting replication initiation protein, TrfA (16, 47, 50, 51). The origin of replication was originally defined as a 700-bp *Hae*II fragment. In addition to eight 17-bp direct repeats, or iterons, arranged in two clusters of five and three, the origin contains an A+T- and G+C-rich region and sequences homologous to DnaA boxes (43). In *E. coli*, a 393-bp *Hpa*II fragment containing the five-iteron cluster, three of the putative DnaA boxes, and the A+T- and G+C-rich region is also a functional origin. However, the deletion of the upstream cluster of three iterons results in an increase in the number of copies of RK2 derivatives in *E. coli* (39, 48, 51). The *trfA* gene encodes two polypeptides in the same reading frame, a 44-kDa protein (TrfA-44) and a 33-kDa protein (TrfA-33); TrfA-33 is the product of an internal translational start (24, 41, 42). Either TrfA-44 or TrfA-33 alone is sufficient for replication in many hosts, including *E. coli*. However, in *Pseudomonas aeruginosa*, there is a specific requirement for TrfA-44 in the stable maintenance of the RK2 replicon (11, 15). TrfA protein can be supplied in *cis* or in *trans* to an RK2 origin plasmid and initiates replication presumably by binding to the iterons (36, 37).

To better understand RK2 replication and control, different classes of TrfA mutants have been isolated and characterized. Genetic and biochemical studies on TrfA copy-up mutants, mutants of TrfA that increase the copy number of RK2 replicons in vivo, support a model for the replication control of RK2 involving the intermolecular coupling of TrfA-bound iterons (13, 23). This model was first proposed for the iteron-containing plasmid R6K (29, 34). In addition, mutant *trfA* genes that are temperature sensitive for RK2 replication have also been examined in vivo (49, 53). In this paper, we present the results of genetic and biochemical studies involving a number of TrfA mutants that are altered in their ability to support RK2 replication. Deletion analyses clearly indicate that the carboxy terminus of the 33-kDa TrfA protein is much more sensitive than the amino terminus to alteration. Furthermore, a *trfA* mutation that results in total loss of binding to the iterons at the RK2 origin also causes loss of functionality in vivo in three different gram-negative hosts in addition to *E. coli*. Finally, TrfA mutants that had no apparent loss of DNA binding activity but were either defective in all four bacterial hosts or showed an altered host range were isolated. The properties of these mutants confirm some of the proposed functions of the TrfA initiation protein and offer insight into the mechanism of TrfA function in the initiation of RK2 replication.

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Remarks	Source or reference
E. coli		
BB3	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA lac* (F′ *proAB lacI*^{q} *ZΔM15* Tc^r)	6
BL21	F$^-$ *hsdS*(r_B^- m_B^-) *gal*	44
C2110	*polA his rha*	22
C600	F$^-$ *thr leu thi lacY supE44 tonA*	
HB101	F$^-$ *leuB6 proAB recA13 thi-1 ara-14 lacY1 galK2 xyl-5 mtl-1 rpsL20* lambda$^-$ *supE44*	4
JM109	*recA1 endA1 gyrA96 thi-1 hsdR17*(r_K^- m_K^+) *supE44 relA1* Δ(*lac-proAB*) (F′ *traD36 proAB lacI*^{q}*ZΔM15*)	57
TB1	JM83 (r_K^- m_K^+)	1
Agrobacterium tumefaciens A136	Rif^r Nal^r Ti plasmid-cured derivative of wild-type C58	19
Azotobacter vinelandii uw	Wild type	3
Pseudomonas putida 2440	*hsdR* derivative of strain mt-2	M. Bagdasarian
Plasmids		
pAT388	In vivo DNA binding vector, NR1 replicon, Cm^r Sp^r	52
pBK3	Overexpression vector for TrfA-33, derivative of pKK233-3	22a
pBK11	393-bp *Hpa*II RK2 origin, Pn^r, RK2 *oriT*, requires TrfA in *trans*	22a
pEC1-33	TrfA-33 in *Eco*RI-*Pst*I of pUC18 (*Nde*I site of vector filled in)	7
pJL1-33	RK2/pBR322 replicon, TrfA-33, Tc^r	This work
pJL2-33	pJL1-33 with *Pst*I site replaced with *Nhe*I linker	This work
pJL4-33, pJL4-44/33	RK2/pBR322 replicon, TrfA-33 or TrfA-44/33, Pn^r	This work
pKK233-2	Overexpression vector for N-terminal deletions	Pharmacia
pMAB14	RK2/pBR322 replicon, TrfA* (Tn*5* insertion in *trfA*), Tc^r	3a, 40, 47
pRD110-34	pBR322 with *trfA* inserted into *Eco*RI-*Pst*I, TrfA-44/33, Tc^r	13
pRD110-16	Same as pRD110, but encodes only TrfA-33	13
pRK2013	ColE1 replicon, RK2 *tra*$^+$	16
pRR10	RK2 replicon, *oriT oriV trfA*, Pn^r	38
pSP6	393-bp *Hpa*II RK2 origin cloned into *Sma*I of pUC19	36
pSV16	700-bp *Hae*II RK2 origin, Pn^r Km^r, requires TrfA in *trans*	52
pTJS26	RK2 repicon, eight-iteron functional origin, Tc^r	39
pTJS42	RK2 replicon, five-iteron functional origin, Tc^r	39

MATERIALS AND METHODS

Materials. Restriction endonucleases, the Klenow fragment of *E. coli* DNA polymerase, T4 DNA polymerase, and T4 DNA ligase were obtained from Bethesda Research Laboratories (Gaithersburg, Md.), Boehringer Mannheim Biochemicals (Indianapolis, Ind.), New England BioLabs (Beverly, Mass.), and Stratagene Cloning Systems (La Jolla, Calif.) and were used according to the manufacturers' instructions. Antibiotics were supplied by Sigma Chemical Co. (St. Louis, Mo.). Components for solid and liquid growth media were supplied by Sigma or GIBCO Scientific (Grand Island, N.Y.).

Plasmids and strains. The bacterial strains and plasmids used in this study are listed in Table 1. Deletions Δ16 and Δ34 in plasmids pRD110-16 and pRD110-34 (13) designate specific 5′ deletions of the *trfA* operon flanked by *Eco*RI and *Pst*I linkers. These deletions produce the 33-kDa form of the TrfA protein only (Δ16) or both TrfA-44 and TrfA-33 (Δ34). All plasmids generated in this study that contain the *trfA* Δ16 *Eco*RI-*Pst*I fragment, such as pJL1, pJL2, and pJL4, are designated with the suffix -33. Accordingly, constructs bearing the Δ34 *trfA* are indicated with the suffix -44/33. Plasmid pJL1-33 was constructed by inserting a Klenow fragment-treated *Hin*dIII-*Sal*I fragment from pMAB14 (3a), which contains an eight-iteron RK2 *oriV* in which the *Bal*I site has been eliminated and replaced by a *Bam*HI linker, into the *Pvu*II site of pRD110-16. This places the RK2 origin directly next to and opposing the pBR322 origin. pJL2-33 is a modification of pJL1-33 in which the *Pst*I site downstream of the *trfA* gene has been eliminated by T4 DNA polymerase and replaced with an *Nhe*I linker d(CTAGCTAGCTAG) (New England BioLabs). Plasmids pJL4-44/33 and pJL4-33 were generated by ligating *Eco*RI-*Pvu*II restriction fragments (which contain *trfA* and the pBR322 origin) from pRD110-34 and pRD110-16, respectively, to an *Eco*RI-*Sma*I fragment (*bla oriT* of RK2 and the RK2 origin) isolated from pRR10 (38), creating double-origin plasmids in which the pBR322 and RK2 origins no longer oppose each other. Plasmid pEC1-33 was constructed by first eliminating the *Nde*I site in pUC18 by restricting with *Nde*I, filling in with the Klenow fragment, and ligating the vector closed. The *trfA* gene from pRD110-16 was then subcloned into the *Eco*RI and *Pst*I sites of the pUC18 polylinker (7).

Isolation of point mutants from a *trfA* mutant bank. Mutant *trfA* genes were obtained from the *trfA* mutant bank prepared in plasmid pRD110-34 (13). Mutant candidates were isolated by transforming individual members of the bank with the eight-iteron RK2 origin plasmid, pSV16. This plasmid requires a source of functional TrfA supplied in *trans* in order to replicate. Since many transformations were done simultaneously, a modified procedure was used in which individual members of the bank were made competent by diluting 100 μl of an overnight culture into 2 ml of LB broth (28) and allowed to grow at 37°C for 1 h. Cells were

recovered and resuspended in 1 ml of cold 100 mM $CaCl_2$ for 30 min on ice. Once again, cells were spun down and resuspended in 100 μl of 100 mM $CaCl_2$ containing pSV16 DNA. Samples were kept on ice for 1 h and placed at 42°C for 90 s before 400 μl of LB broth was added. After 75 min of incubation at 37°C, entire transformation mixes were plated, with selection for the replication of the RK2 origin plasmid on LB-penicillin (250 μg/ml) or LB-kanamycin (25 μg/ml) plates. Mutants were named for the codon changed and the amino acid change incurred, as determined by mapping and DNA sequence analysis.

Construction of deletion mutants. C-terminal deletions of *trfA* were generated by linearizing plasmid pJL1-33 with *Pst*I and incubating the DNA with *BAL* 31 exonuclease (New England BioLabs) and the buffer conditions described by Maniatis et al. (28) for 90 s at room temperature. The treated DNA was then extracted with phenol-chloroform and precipitated with ethanol. Uneven ends left by the exonuclease were filled in by adding nucleotides and the Klenow fragment. *Nhe*I linkers [d(CTAGCTAGCTAG)] that add termination codons in all three possible reading frames were added to the blunt ends by ligation. The extents of the deletions and the presence of the linker were visualized by restriction digestion and gel electrophoresis in 1.2% agarose. Appropriately sized deletions were restricted with *Nhe*I and then subcloned into pJL2-33. This cloning was facilitated by the presence of another *Nhe*I site in the *tet* gene of pBR322. Amino-terminal deletions were constructed in a similar manner. pJL1-33 was linearized at the *Eco*RI site, which precedes the start of the *trfA* coding sequence. Deletions were generated, filled in, and subcloned into the vector pKK233-2 (Pharmacia LKB Biotechnology, Pleasant Hill, Calif.). pKK233-2 contains an *Nco*I site that, when filled in, creates an artificial start codon optimally spaced in front of the inducible *trc* promoter. The deletions were then screened by Western immunoblot analysis and restriction digestion. An additional C-terminal deletion, TrfA-CΔ305, was made by adding an *Nhe*I linker at the unique *Nde*I site within the *trfA* gene in plasmid pEC1-33. TrfA-NΔ180 was created by cloning the *Hinc*II-*Pst*I segment of *trfA* from pEC1-33 into pKK233-2. Deletion mutants were named for the amino acid position at which the TrfA protein begins (N-terminal deletions) or ends (C-terminal deletions).

Characterization of *trfA* mutants in vivo. The *trfA* mutations were cloned into vectors pRD110-34, pRD110-16, and pJL4-44/33. Mutations mapping to the C-terminal portion of *trfA* (314S, 329S, 331L, 338C, 360DUP, 361K, 363TER, CΔ379) were cloned into these vectors via an *Nde*I fragment ligation, so that only the part of *trfA* containing the mutation was moved. N-terminal deletions (NΔ116, NΔ123) were cloned into the various vectors by using an *Eco*RI-*Pst*I fragment. This particular cloning scheme preserves the promoter and artificial start of the N-terminal deletions. One point mutant (151S) mapping to the N-terminal half of *trfA* was moved into otherwise wild-type vectors by exchanging an *Sfi*I-*Pst*I restriction fragment. To test RK2 replication in *trans*, competent *E. coli* HB101 cells carrying pRD110-34, pRD110-16, or one of their mutant derivatives were transformed with either an eight-iteron origin plasmid, pSV16 (53), or a five-iteron plasmid, pBK11 (22a). Transformation mixes were then spread on LB-agar plates with penicillin at 200 or 100 μg/ml. N-terminal deletion constructs containing the inducible *trc* promoter were tested with or without induction, respectively, by adding or not adding 250 μM isopropyl-β-D-thiogalactopyranoside to the plates. Mutants TrfA-CΔ305 and TrfA-NΔ180, generated on vectors conferring penicillin resistance, were tested with pSV16 on LB plates containing kanamycin (50 μg/ml). RK2 replication in *cis* was determined by using the vector pJL4-44/33. pJL4-44/33 and its mutant derivatives were transformed into *E. coli* C2110 (*polA*), forcing the vectors to rely on only the RK2 origin. Once again, transformants were selected on LB-agar plates containing penicillin (200 and 100 μg/ml).

Analysis of TrfA mutants in other gram-negative organisms. pJL4-44/33 and its mutant derivatives were moved into *Azotobacter vinelandii* and *Agrobacterium tumefaciens* by triparental mating with pRK2013 as the source of RK2 transfer genes as described by Schmidhauser and Helinski (40). Exconjugants were selected with nalidixic acid (20 μg/ml) and carbenicillin (1,000 μg/ml) in YEP medium (1% peptone, 1% yeast extract, 0.5% NaCl) for *A. tumefaciens* or carbenicillin (50 μg/ml) in Burk medium (31) for *A. vinelandii*. Plasmids were introduced into *Pseudomonas putida* 2440 via transformation by the procedure of Cohen et al. (8). Transformants were selected with carbenicillin (1,000 to 2,000 μg/ml) in LB containing 0.5% glucose. Transformants and transconjugants were tested for the presence of the plasmid by clone analysis. Transformation of isolated mutant plasmids back into *E. coli* and then clone analysis, restriction analysis, and transformation into *E. coli* C2110 were employed to confirm mutant phenotypes.

Analyses of mutants in vitro. Partial purification of TrfA mutants as 33-kDa proteins was performed essentially as described by Perri et al. (36). Plasmid pBK3, which was constructed to overproduce TrfA-33 protein (22a), contains a *trfA* gene encoding TrfA-33 cloned into the vector pKK223-3 in front of the inducible *tac* promoter. Mutant *trfA* genes were cloned into pBK3 via an *Sfi*I-*Pst*I fragment exchange. Mutant TrfA-33:151S and TrfA-33:329S proteins were purified from *E. coli* BB3, whereas the wild type, TrfA-33:wt, and mutants TrfA-33:338C, TrfA-33:361K, TrfA-33:CΔ305, TrfA-NΔ116, and TrfA-NΔ123 were purified from *E. coli* BL21 (44). Protein concentrations were determined with protein microassays (Bio-Rad, Richmond, Calif.) The protocols for in vitro replication experiments were as described by Kittell and Helinski (23) with the following modifications (22a). Ammonium sulfate cuts of the *E. coli* C600 extracts were performed with a solution of saturated ammonium sulfate instead of powdered ammonium sulfate. After the 40% cut, a 40 to 52% cut was also made. The amount of [*methyl*-^{3}H]TTP was reduced to 100 cpm/pmol. Conditions for replication of pTJS42 (five-iteron RK2 origin plasmid) and of pTJS26 (eight-iteron RK2 origin plasmid) were optimized for wild-type TrfA protein. Samples of 150 to 200 μg of 40% extract were used to replicate pTJS42, whereas mixtures of 150 to 200 μg of 40% extract, 50 μg of 40 to 52% extract, and 50 μg of DnaA-enriched extract were used to replicate pTJS26. Assay reaction conditions were as described previously (23). Gel mobility shift experiments were performed as described previously (36), except that the concentration of the nonspecific DNA competitor poly(dI-dC) in the binding buffer was increased to 50 μg/ml and EDTA was used at 0.1 mM. Mutants were also tested for their ability to bind RK2 iterons in vivo with the vector system of Elledge et al. (14) modified for the RK2 system (52).

Sucrose gradient analysis of TrfA proteins. Protein preparations containing 30 to 50 μg of TrfA were analyzed in 4.8-ml 5 to 15% (wt/wt) sucrose gradients. Sucrose solutions were made in a buffer containing 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 8.0), 50 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, and 50 μg of phenylmeth-

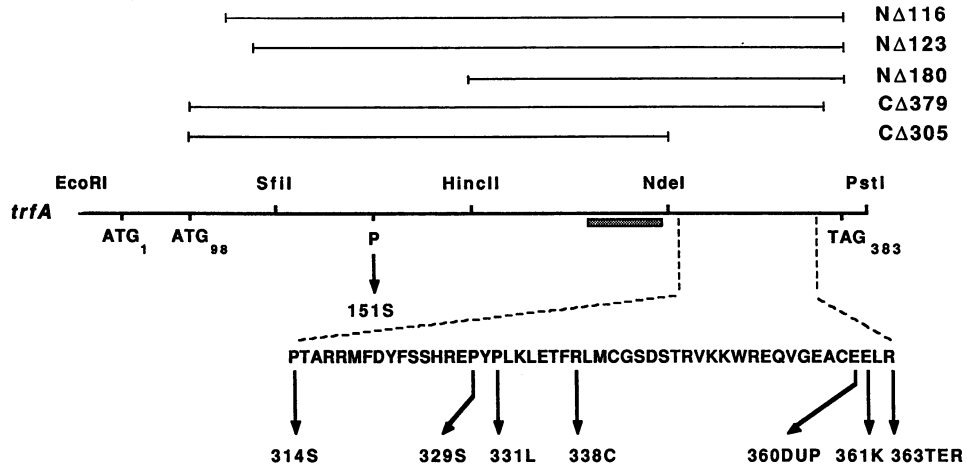

FIG. 1. Amino acid changes and approximate sizes of deletions in mutant TrfA proteins. Mutants are named for the codon changed and the change incurred, except in the cases of 360DUP, which contains an insertion of three amino acids (alanine, cysteine, and glutamic acid), and 363TER, in which an arginine is changed to a stop codon. The deletion mutants are named for the amino acid position at which the TrfA protein begins (N-terminal deletions) or ends (C-terminal deletions). Amino acid residues are numbered from the first methionine of TrfA-44. The shaded box represents the approximate location of six previously isolated copy-up *trfA* mutations (13) and the putative helix-turn-helix DNA-binding domain (42).

ylsulfonyl fluoride (Sigma) per ml. Centrifugation was carried out in a Beckman SW50.1 or SW55Ti rotor at 152,000 × *g* for 24 h under refrigeration (4°C). A set of marker proteins (β-amylase, bovine serum albumin, ovalbumin, carbonic anhydrase, and cytochrome *c*) was run under the same conditions in a separate tube. Fractions of approximately 110 μl were collected from the bottom of the tube. Marker proteins were visualized on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (12.5% polyacrylamide) stained with Coomassie blue. Aliquots of gradient fractions containing TrfA were filtered through a slot blot apparatus (Bio-Rad) onto nitrocellulose for detection of TrfA by Western analysis (12). Photographic reproductions of slot blots were scanned on a laser densitometer (LKB Ultroscan XL model 2222). Densitometric analyses were performed with software written by AMBIS (San Diego, Calif.)

Other methods. Basic cloning methods were performed as described by Maniatis et al. (28). Other techniques, including mapping and DNA sequencing of the mutations with Sequenase (U.S. Biochemical, Cleveland, Ohio) (13), clone analyses (2, 20), and SDS-PAGE (26), were performed as described previously. Western blotting with a semidry horizontal blotting apparatus was done as described by Kyhse-Andersen (25) with a polyvinylidene difluoride membrane (Immobilon-P (Millipore, Bedford, Mass.) instead of nitrocellulose. TrfA was detected with a polyclonal antibody preparation as described previously (12).

RESULTS

Isolation and mapping of defective TrfA mutants. Plasmids with defective mutations in *trfA* were from a nitrosoguanidine mutant bank prepared by Durland et al. (13). The *trfA* mutant genes were carried on a pBR322-derived plasmid, pRD110-34. *E. coli* cells carrying individual members of the bank were made competent, transformed with the RK2 origin plasmid pSV16, and plated on LB containing penicillin G or kanamycin. Members of the bank that gave no pSV16 transformants were provisionally considered to be defective in supporting RK2 replication. After an initial screening, 20 of 166 candidates from the 40-min nitrosoguanidine-treated bank were determined to be defective. After clone analyses to confirm that the *trfA* gene in these mutants was intact and Western blotting with a polyclonal anti-TrfA antibody preparation to determine the size of the mutant protein synthesized (data not shown), a group of mutations producing full-length or near-full-length TrfA proteins was selected for further study. This group included mutations designated 151S, 314S, 329S, 331L, 338C, 360DUP, 361K, and 363TER. All mutations, except 314S, were initially mapped within the *trfA* gene by performing reciprocal exchanges of restriction fragments between individual mutant and wild-type plasmids, as described by Durland et al. (13). In the process of mapping mutation 314S, a novel *Hin*cII site that was subsequently determined to be the result of the mutation was identified in the *trfA* gene. All of the point mutations were sequenced, and the nature of the changes and their locations are shown in Fig. 1. The *trfA* mutations are named by designating the number of the codon that was changed and the change incurred with the single-letter amino acid designation. Seven of these eight mutations cluster in the *Nde*I-*Pst*I segment of *trfA* corresponding to the 3′ end of the *trfA* gene. Only one mutation, 151S, was found to map upstream in the *Sfi*I-*Hin*cII region. The mutation 363TER results in a termination codon 20 amino acids before the natural translational stop of *trfA* and produces a truncated mutant TrfA protein. Additional deletion mutations were generated by using *BAL* 31 exonuclease (CΔ379, NΔ116, NΔ123), linker insertion (CΔ305), and simple cloning strategies (NΔ180) as described in Materials and Methods. These mutations were also sequenced, and the approximate sizes of the resulting protein products are indicated in Fig. 1. Mutant protein TrfA-CΔ379 is missing three amino acids from the C terminus of TrfA. Mutant protein TrfA-CΔ305 is truncated by 77 amino acids also at the carboxy terminus; however, the inserted *Nhe*I linker adds a leucine, an alanine, and a serine before the termination codon. In N-terminal deletion mutants TrfA-NΔ116 and TrfA-NΔ123, 18 and 25 amino acids, respectively, from the start of the 33-kDa TrfA protein are

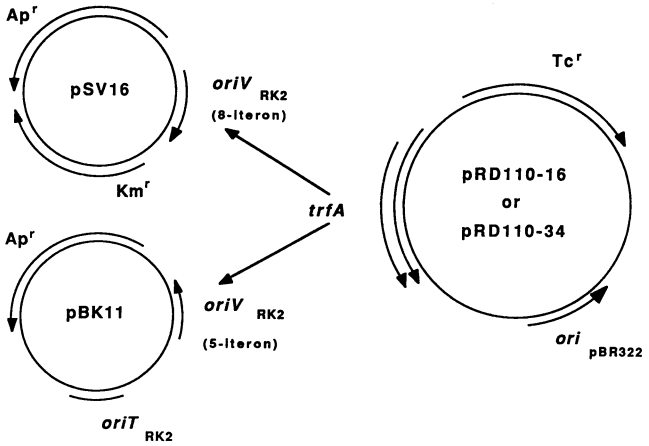

FIG. 2. Minimal RK2 replication in *trans*. To assay RK2 replication in *trans*, *E. coli* HB101 cells carrying pRD110-16 (33-kDa TrfA protein only) or pRD110-34 (44- and 33-kDa TrfA proteins) plasmids were transformed with RK2 origin vector pSV16 (eight-iteron RK2 origin) or pBK11 (five-iteron RK2 origin) as described in Materials and Methods.

deleted. In TrfA-NΔ180, 82 N-terminal amino acids are deleted.

Properties of *trfA* mutants in vivo. The functionality of these mutants in vivo was studied in more detail by subcloning them into a variety of plasmid vectors so they could be tested in *E. coli* in *trans* (Fig. 2) with respect to an eight-iteron (pSV16) or a five-iteron (pBK11) origin plasmid or in *cis* (Fig. 3) with respect to an eight-iteron RK2 origin (pJL4-44/33) and in other gram-negative organisms (Table 2). All mutations, except for the N-terminal deletions and CΔ305, were subcloned into plasmid constructs producing the mutant TrfA protein as the 33-kDa protein only or as a mix of TrfA-44 and TrfA-33. The results for the constructs expressing TrfA-33 and TrfA-44/33 in *E. coli* were very similar (data not shown); therefore only the observations for the constructs expressing TrfA-44/33 are shown (Table 2). When tested in *trans* for the ability to support an RK2 origin plasmid, mutants TrfA-44/33:151S, TrfA-44/33:329S, TrfA-44/33:331L, TrfA-44/33:360DUP, TrfA-44/33:363TER, TrfA-44/33:CΔ379, TrfA-44/33:CΔ305, and TrfA-NΔ180 were completely unable to support the replication of either an eight-iteron origin (pSV16) or a five-iteron origin (pBK11), whereas mutant TrfA-44/33:314S behaved like the wild type in both situations. Mutants TrfA-44/33:338C, TrfA-44/33:361K, and TrfA-NΔ123 were only partially defective in their ability to replicate pSV16 (eight-iteron origin plasmid) in *trans*. These mutants (in particular, TrfA-44/33:361K) showed better activity in vivo with the five-iteron origin plasmid pBK11. N-terminal deletion mutant TrfA-NΔ116 was as effective as wild-type TrfA with pSV16 but less effective at supporting the five-iteron origin plasmid pBK11.

The vector pJL4-44/33 was designed to test the ability of mutant TrfA proteins to replicate an eight-iteron RK2 origin present in *cis* with respect to the *trfA* gene in *E. coli* and other gram-negative bacteria. pJL4-44/33 contains both a functional RK2 origin and a pBR322 origin as well as the RK2 origin of transfer, *oriT* (Fig. 3). The pBR322 origin is present to allow for the maintenance of defective *trfA* mutations in a permissive (*polA*$^+$) *E. coli* host. When this construct is transformed into C2110, a *polA* strain, the ColE1 origin, which cannot replicate without *E. coli* DNA polymerase I, is nonfunctional. Replication of the vector is then entirely dependent on interactions between TrfA and the eight-iteron RK2 origin (Table 2). All mutants, with the exception of those with N-terminal deletions, were completely defective at replicating this eight-iteron RK2 minireplicon. The presence of the RK2 origin of transfer, *oriT*, in pJL4-44/33 allowed the conjugal transfer of wild-type and mutant derivatives into *A. tumefaciens* and *A. vinelandii*. Plasmids with the *trfA* mutations were introduced by transformation into *P. putida*. Although all of the TrfA mutants were nonfunctional in *E. coli* C2110, TrfA-44/33:314S and TrfA-44/33:338C were functional in all three other gram-negative bacteria. TrfA-44/33:361K, on the other hand, was unable to replicate in *A. tumefaciens* but functional in the other two gram-negative bacteria. The N-terminal deletions were not established in *A. vinelandii*; both plasmids carrying the deletions suffered rearrangement upon transfer to this organism, as determined by subsequent clone analysis.

Purification of defective TrfA proteins. A select group of TrfA mutants were overproduced in *E. coli* strains BB3 or BL21 from plasmid pBK3, which produces only the TrfA-33 form of the TrfA protein. Mutants TrfA-33:151S and TrfA-33:329S were purified from BB3. TrfA-33:314S, TrfA-33:338C, TrfA-33:361K, TrfA-33:CΔ305, TrfA-NΔ116, and TrfA-NΔ123 were purified with fewer contaminants and better yield from *E. coli* BL21 than from *E. coli* BB3 (data not shown). Wild-type TrfA-33 isolated from either strain showed similar activity in in vitro replication assays and DNA binding assays (data not shown). TrfA protein preparations purified from BB3 do have a previously observed (36) anomalous form of TrfA present at 36 kDa that was not seen with Western blotting of TrfA protein preparations purified from BL21. The effects, if any, of this minor form of TrfA are unknown. The TrfA yields and contaminants in the preparations from all of the mutants were not identical to those of the wild type with this procedure. Mutants such as TrfA-33:338C, TrfA-33:361K, TrfA-33:CΔ305, and the N-terminal deletion mutants were not substantially overproduced from plasmid pBK3. In addition, mutant TrfA-33:CΔ305 eluted from the heparin-agarose column at a lower KCl concentration than did the wild type TrfA-33, resulting in the presence of small amounts of additional *E. coli*

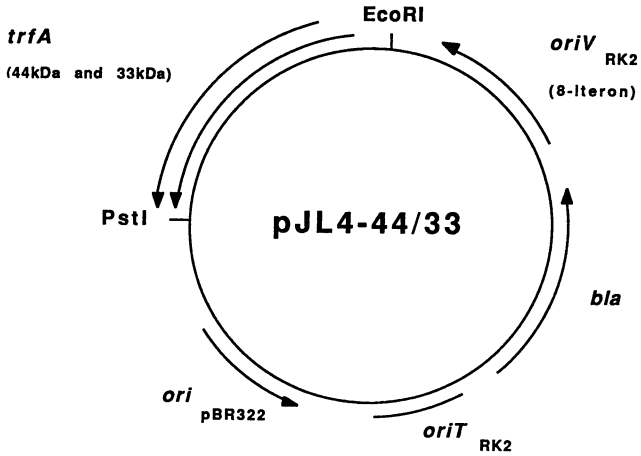

FIG. 3. Map of the double-origin plasmid pJL4-44/33. The pBR322 origin of replication allows for the maintenance of TrfA mutants in PolA$^+$ hosts. *oriT* from RK2 allows for the conjugal transfer of this plasmid into other gram-negative bacterial strains.

TABLE 2. In vivo replication properties of TrfA mutants

TrfA mutant	Replication[a] in:					
	HB101(pRD110-34, pSV16)	HB101(pRD110-34, pBK11)	C2110 (pJL4-44/33)	*A. vinelandii* (pJL4-44/33)	*P. putida* (pJL4-44/33)	*A. tumefaciens* (pJL4-44/33)
TrfA-44/33	+++	+++	+++	+++	+++	+++
TrfA-44/33:151S	−	−	−	−	−	−
TrfA-44/33:314S	+++	+++	−	+++	+++	+++
TrfA-44/33:329S	−	−	−	−	−	−
TrfA-44/33:331L	−	−	−	−	−	−
TrfA-44/33:338C	±	+	−	+++	+++	+++
TrfA-44/33:360DUP	−	−	−	−	−	−
TrfA-44/33:361K	±	+++	−	+++	+++	−
TrfA-44/33:363TER	−	−	−	−	−	−
TrfA-44/33:CΔ379	−	−	−	−	−	−
TrfA-44/33:CΔ305[c]	−	NT[b]	NT	NT	NT	NT
TrfA-NΔ116	+++	+	+++	−	+++	+++
TrfA-NΔ123	++	++	++	−	+++	−
TrfA-NΔ180[c]	−	NT	NT	NT	NT	NT

[a] Replication is described as the level of growth (+++ to ±) or no growth (−) of transformants.
[b] NT, not tested.
[c] CΔ305 and NΔ180 were tested in vivo in the construct pEC1-33.

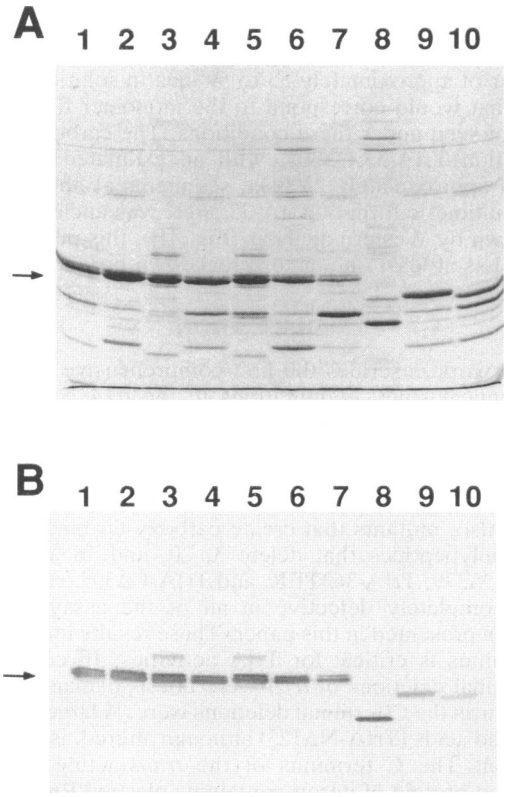

FIG. 4. Mutant TrfA protein preparations: (A) SDS-PAGE gel (10% polyacrylamide) stained with Coomassie blue (15 μg of total protein per lane); (B) corresponding Western blot (2 μg of total protein per lane) of TrfA protein preparations. Lanes: 1, TrfA-33; 2, TrfA-33; 3, TrfA-33:151S; 4, TrfA-33:314S; 5, TrfA-33:329S; 6, TrfA-33:338C; 7, TrfA-33:361K; 8, TrfA-33:CΔ305; 9, TrfA-NΔ116; 10, TrfA-NΔ123. The arrows indicate the positions of TrfA-33 protein and undeleted mutant derivatives. Protein preparations in lanes 1, 3, and 5 were prepared from *E. coli* BB3. All other purifications shown were prepared from *E. coli* BL21.

proteins. As determined from Coomassie blue-stained gels (Fig. 4A) and the corresponding Western blots (Fig. 4B) of the partially purified TrfA proteins, the relative purity of the protein preparations is estimated to be 20 to 50% of the total protein. Several attempts were made to purify TrfA-33:CΔ379, TrfA-33:363TER, and TrfA-NΔ180 by similar procedures. However, all such attempts resulted in minimal yields of these particular mutant TrfA proteins.

Activities of TrfA mutants in in vitro replication. The in vitro replication system described previously (23) is based on soluble *E. coli* C600 extracts, which replicate both the five-iteron RK2 plasmid pTJS42 and the eight-iteron RK2 plasmid pTJS26 when purified TrfA protein is added. This system, with the modifications described in the Materials and Methods, was employed to assay the replication activity of mutant TrfA protein preparations. Since some of the mutant preparations were not as pure as TrfA-33, the amount of protein preparation used in the replication assay was such that further addition of protein did not increase activity. The results for templates pTJS26 and pTJS42 (Table 3) show that mutants TrfA-33:151S, TrfA-33:329S, TrfA-33:361K, and TrfA-33:CΔ305 did not replicate the eight-iteron RK2 origin. The specific activities of TrfA-NΔ116 and TrfA-

TABLE 3. Replication of RK2 origin plasmids initiated by TrfA-33 or mutant TrfA-33 proteins

TrfA	DNA synthesized per reaction[a] (pmol)	
	pTJS26 (eight-iterons)	pTJS42 (five iterons)
TrfA-33	120	170
TrfA-33:151S	0	0
TrfA-33:314S	80	58
TrfA-33:329S	8	0
TrfA-33:338C	30	14
TrfA-33:361K	4	260
TrfA-33:CΔ305	0	0
TrfA-NΔ116	250	240
TrfA-NΔ123	130	200

[a] Results are averages of at least three experiments where 1.5 μg of each protein preparation was used. Background synthesis of DNA averaged 10 pmol. The numbers presented are corrected for this value.

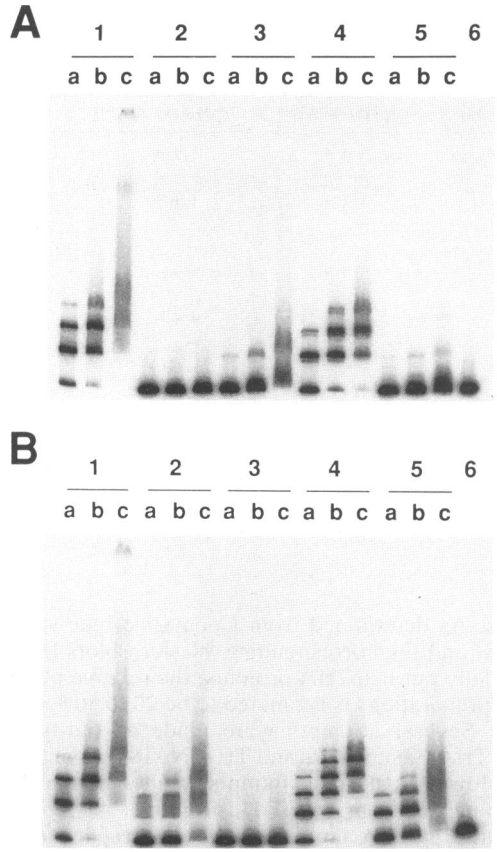

FIG. 5. Mutant TrfA binding to the RK2 minimal origin. The gel electrophoretic mobility shift generated by binding of TrfA-33 and mutant TrfA protein preparations to 400-bp *Eco*RI-*Hin*cII minimal RK2 *oriV* fragment from pSP6 was measured as described previously (36). Each set of three lanes contains the same amounts of total protein per binding reaction: a, 0.5 μg; b, 1.0 μg; c, 1.5 μg. (A) TrfA protein preparations used in each set of binding reactions: 1, TrfA-33; 2, TrfA-33:151S; 3, TrfA-33:314S; 4, TrfA-33:329S; 5, TrfA-33:338C; 6, no TrfA. (B) TrfA preparations used: 1, TrfA-33; 2, TrfA-33:361K; 3, TrfA-33:CΔ305; 4, TrfA-NΔ116; 5, TrfA-NΔ123; 6, no TrfA.

NΔ123 were comparable to or higher than that of wild-type TrfA-33. TrfA-33:314S and TrfA-33:338C demonstrated some activity. In vitro replication of the five-iteron plasmid pTJS42 is similar to that of the eight-iteron origin in almost all cases, except for mutant TrfA-33:361K, which is active with pTJS42 and completely inactive with the eight-iteron plasmid, pTJS26. In general, in vitro replication results correlated well with results obtained with the same mutants in vivo.

Iteron-binding properties of TrfA mutants. To study the interaction of mutant TrfA proteins with the RK2 origin of replication in vitro, the gel mobility shift procedure of Perri et al. (36) was used. A 400-bp fragment containing the functional five-iteron *Hpa*II RK2 origin was end labeled and served as the probe. Figures 5A and B show the iteron-binding activities of the mutant TrfA preparations. TrfA-33:329S, TrfA-NΔ123, TrfA-NΔ116, and TrfA-33:361K show strong binding activity with the origin-containing fragment. TrfA-33:329S gives a pattern of complex formation identical to that of the wild type. TrfA-NΔ116 appears to form complexes that are more distinct than those of TrfA-33, as opposed to TrfA-33:361K, which forms less distinct complexes with the probe. TrfA-33:314S, and TrfA-33:338C also demonstrate the ability to shift the origin fragment but not as well as the wild type, TrfA-33, as evidenced by weak complex formation. The binding of these particular mutant preparations to iterons was not improved by the addition of more protein (data not shown). Mutants TrfA-33:151S and TrfA-33:CΔ305 showed no ability to bind to the iteron-containing DNA fragment.

A vector system designed by Elledge et al. (14) was adapted for the RK2 system (52) to provide a means to identify TrfA proteins that will bind to two iterons from the RK2 origin in vivo. Binding of TrfA to the two iterons results in the transcriptional repression of a constitutive promoter and allows the expression of the convergent antibiotic resistance gene *aadA* (spectinomycin). Analyses of the TrfA mutants with this system correlate well with in vitro DNA binding results in that the mutant proteins that bind well in vitro are able to bind in vivo, whereas the mutant protein preparations that are weak in binding are not able to bind in vivo (Table 4).

Dimeric properties of TrfA mutant proteins in solution. Velocity sedimentation analyses with linear 5 to 15% (wt/wt) sucrose gradients revealed that wild-type TrfA-33 (Fig. 6), TrfA-33:151S, TrfA-33:314S, TrfA-33:329S, TrfA-33:338C, TrfA-NΔ116, TrfA-NΔ123, and TrfA-33:CΔ305 (data not shown) are dimers in solution. The position of TrfA protein in the gradient was detected by Western analysis. When compared with a set of marker proteins, TrfA appears to be a dimer of approximately 55 to 59 kDa in solution. A major peak that would correspond to the monomer form of TrfA was not seen under these conditions. The carboxy-terminal truncation TrfA-33:CΔ305, with an estimated monomeric size of approximately 25 kDa, sediments at about 45 kDa. The multimeric form of TrfA-33:361K was unclear because, as shown by Western analysis (Fig. 4B), this protein preparation has at least one major breakdown product.

DISCUSSION

This work describes the first comprehensive genetic and biochemical study of mutations in the *trfA* gene that are defective in supporting RK2 replication. With the TrfA mutants identified from a nitrosoguanidine bank (13), seven of eight mutations can be found in the carboxy-terminal quarter of *trfA* delineated by the *Nde*I and *Pst*I restriction sites. Also, mutants that create carboxy-terminal truncated TrfA polypeptides that delete 3, 20, and 78 amino acids (TrfA-CΔ379, TrfA-363TER, and TrfA-CΔ305, respectively) were completely defective in all of the assays for TrfA function presented in this paper. These results imply that the C terminus is critical for TrfA activities. In contrast, the N-terminal deletions of *trfA* were not as damaging to TrfA function as the C-terminal deletions were. A large deletion of 25 amino acids (TrfA-NΔ123) although altered, is still able to function. The C terminus of the *trans*-acting replication initiation protein of iteron-containing plasmid Rts1 has been shown to be essential as well. Substitutions in the carboxy-terminal six amino acids produce mutant RepA proteins that no longer activate the Rts1 origin (46).

Selected characteristics of all of the mutants are displayed in Table 4. For most of the mutants described in this study, the nature of the defect is not yet known and can only be inferred. A proposed model (10) for the positive activities of TrfA in RK2 replication initiation and control suggests that steps that might lead to the initiation of plasmid DNA

TABLE 4. Summary of selected characteristics of TrfA mutants

TrfA mutant	Replication			DNA binding		Dimerization
	In *E. coli*[a]	In *P. putida*	In vitro[b]	In vitro	In vivo	
TrfA-wt	+++	+++	+++	+++	+++	+
TrfA-151S	−−−	−−−	−−−	−−−	−−−	+
TrfA-314S	−−−	+++	−−+	−++	−−−	+
TrfA-329S	−−−	−−−	−−−	+++	+++	+
TrfA-331L	−−−	−−−	NT[c]	NT	+++	NT
TrfA-338C	−−−	+++	−−+	−−+	−−−	+
TrfA-360DUP	−−−	−−−	NT	NT	−−−	NT
TrfA-361K	−−−	+++	+++	+++	+++	?[d]
TrfA-363TER	−−−	−−−	NT	NT	−−−	NT
TrfA-CΔ379	−−−	−−−	NT	NT	−−−	NT
TrfA-CΔ305	−−−	NT	−−−	−−−	−−−	+
TrfA-NΔ116	+++	+++	+++	+++	+++	+
TrfA-NΔ123	−−+	−−+	+++	+++	+++	+
TrfA-NΔ180	−−−	NT	NT	NT	−−−	NT

[a] Results are reported for replication in *cis* with the vector pJL4-44/33, except in the cases of CΔ305 and NΔ180, where the observations of replication in *trans* is shown.
[b] Results of in vitro replication experiments with the five-iteron RK2 origin plasmid pTJS42 are shown.
[c] NT, not tested.
[d] Results from sucrose gradient analysis of this protein were uninterpretable.

synthesis include dimerization of TrfA, binding of dimers to the iterons at the origin, nucleation to form a preinitiation structure, and, finally, interaction of TrfA with specific host factors. The mutants described in this paper potentially are defective in any one of these proposed steps. Accordingly, some of the mutants characterized in this study serve to confirm some of these proposed activities and properties. For example, other studies have shown that replication initiation proteins from various systems such as π of R6K (18) and RepA of P1 (45, 55) exist as dimers in solution. The results from the sucrose gradients have shown that TrfA is also a dimer. Even TrfA-33:CΔ305, an extensive deletion protein, appeared to sediment as a dimer in these gradients. However, it remains to be proven whether the dimer form of TrfA is the active form of this protein in replication. It has been shown that in the P1 system the heat shock proteins DnaJ and DnaK convert RepA dimers to monomers in an ATP-dependent reaction. Monomerization of RepA renders the initiation protein more active for binding to the P1 origin of replication (54).

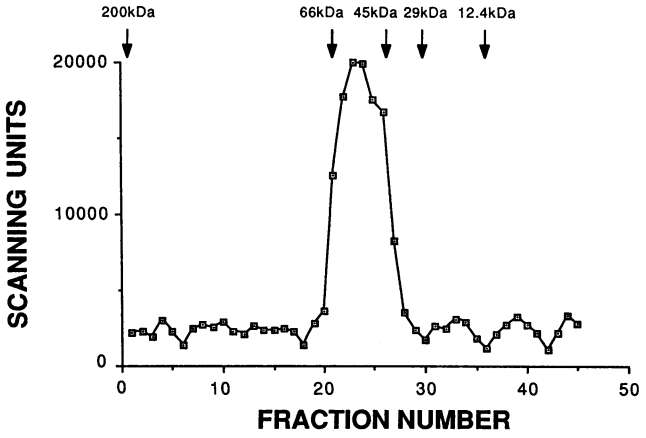

FIG. 6. Sedimentation analysis of wild-type TrfA-33 protein. Wild type TrfA-33 protein and five protein standards were analyzed on 5 to 15% (wt/wt) sucrose gradients. Photographic representations of Western blots used to detect TrfA protein were scanned on a laser densitometer as described in Materials and Methods. The peak positions of the five protein standards are indicated by the arrows. Approximate molecular sizes: β-amylase, 200 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa; cytochrome *c*, 12.4 kDa.

It is known that certain host proteins, such as DnaA, DnaB, DnaG, gyrase, and DNA polymerase III (37), are involved in RK2 replication. Although no specific interactions have been shown, some of the TrfA mutants presented here may be defective in host protein interactions. For example, mutant protein TrfA-33:329S is a dimer in solution and binds origin DNA as well as wild-type TrfA-33 protein, yet the same protein is inactive in RK2 replication in vitro and in vivo. Perhaps TrfA-33:329S is defective in the formation of a preinitiation structure, in the interaction with a particular host factor, or in other functions of TrfA that are independent of iteron binding and protein dimerization. TrfA-44/33:314S, TrfA-44/33:338C, and TrfA-44/33:361K are also interesting in that they show different replicative properties in gram-negative hosts other than *E. coli*. The ability of these three TrfA mutants to replicate in *P. putida*, *A. vinelandii*, and *A. tumefaciens* (TrfA-44/33:314S and TrfA-44/33:338C only) suggests variations in host-specific interactions for TrfA. The N-terminal deletion mutants TrfA-NΔ116 and TrfA-NΔ123 also show variable activity in the gram-negative hosts tested. This indicates that the N terminus of TrfA might play an important role in the host-range capabilities of TrfA. The specific requirement of the 44-kDa TrfA protein in *P. aeruginosa* supports this idea (11). However, host protein-TrfA interaction may not account fully for the differences in the behavior of these mutants in other gram-negative organisms. Protein conformation and modification, variations in host-specified proteins, differences in ionic environments, and variations in replication control among the different hosts may contribute to the contrasting phenotypes of these mutants outside of *E. coli* (49, 53). In addition, two (TrfA-314S and TrfA-361K) of the three mutants are functional in *E. coli* in specific situations. The amount of TrfA produced may play a role in vivo for mutants TrfA-33:314S. In *trans*, where it is produced at relatively high levels from a constitutive promoter (12), it mimics wild-type TrfA, yet it is defective in *cis*. The copy number of the vectors

used for the in *trans* assay is about three- to fivefold above that of the vectors used to test replication in *cis*. In vitro and in vivo DNA binding results demonstrate that TrfA-33:314S binds iterons poorly. An unstable interaction between TrfA-33:314S and the RK2 origin may be somehow overcome by the constitutive production of the mutant TrfA in vivo, a situation that cannot be reproduced in vitro. Accordingly, perhaps the amount of TrfA required for RK2 replication is somewhat dependent on the host. TrfA-33:361K is also interesting in that it is more active with the five-iteron origin than with the eight-iteron origin in vivo and in vitro. These results suggest that there are different requirements for the replication of each type of origin. Origin of replication requirements among various hosts may be different.

Binding of TrfA to RK2 iterons has been proposed to be a necessary step in the initiation of replication. TrfA-33:CΔ305 and TrfA-33:151S have been shown to be completely defective in sequence-specific DNA binding in vitro and in vivo. The fact that these mutants are also completely defective in RK2 replication indicates that binding of TrfA to the iterons is, indeed, necessary for replication to proceed. Mutants TrfA-33:314S, TrfA-33:338C, and TrfA-33:361K, which cannot support the replication of RK2 origins under certain circumstances in *E. coli*, show binding that is weakened or altered compared with that of wild type protein (Fig. 5, Table 4). Nevertheless, all three mutants are fully functional in several other gram-negative organisms. The reasons for these observations are unclear, but one possible explanation is that the interactions between TrfA and the RK2 origin are influenced by host cell factors that might stabilize or facilitate TrfA binding. These factors are likely to be somewhat different structurally from host to host. Since several of the mutations (314S, 331L, 338C, 360DUP, and 361K) found in the carboxy terminus of *trfA* result in proteins that are altered in their ability to bind iterons, regions of the C terminus and of the N terminus near 151S may be involved in DNA binding. For the O protein of lambda dv, a DNA-binding domain has been localized to the N-terminal 162 amino acids (56). In previous work, a helix-turn-helix putative DNA-binding domain for TrfA was proposed (42) for a region encompassing the previously identified RK2 "copy-up" region (Fig. 1) (13). However, according to Brennan and Matthews (5), the likelihood of the proposed region having the helix-turn-helix DNA-binding motif is low. Computer searches of TrfA with the programs PC/GENE and FASTA (30, 35) have not revealed any standard DNA binding motifs or relevant identities. Although it is necessary to carry out further mutational analyses of specific regions of *trfA* to more clearly define possible DNA binding and other domains of the replication initiation protein TrfA, the mutants isolated and characterized in this study should prove very useful in delineating the structural features of the RK2 replication initiation protein that account for the ability of TrfA to initiate plasmid replication in a wide range of gram-negative bacteria.

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