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Mutations in the *trfA* Replication Gene of the Broad-Host-Range Plasmid RK2 Result in Elevated Plasmid Copy Numbers

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Mutated forms of *trfA*, the replication protein gene of plasmid RK2, that support a minimal RK2 origin plasmid in *Escherichia coli* at copy numbers up to 23-fold higher than normal have been isolated. Six such high-copy-number (copy-up) mutations were mapped and sequenced. In each case, a single base transition led to an amino acid substitution in the TrfA protein primary sequence. The six mutations affected different residues of the protein and were located within a 69-base-pair region encoding 24 amino acids. Dominance tests showed that each of the mutants can be suppressed by wild-type *trfA* in *trans*, but suppression is highly dependent on the amount of wild-type protein produced. Excess mutant TrfA protein provided in *trans* significantly increased the copy number of RK2 and other self-replicating derivatives of RK2 that contain a wild-type *trfA* gene. These observations suggest that the mutations affect a regulatory activity of the TrfA replication protein that is a key factor in the control of initiation of RK2 replication.

Control of the frequency of initiation of the broad-host-range plasmid RK2 is not well understood. Replication is known to be initiated at a single origin, termed *oriV* (11, 36, 39, 42, 43). The only other plasmid locus essential for replication is the *trans*-acting gene *trfA* (11, 39, 42). This gene encodes two proteins, termed TrfA-43 and TrfA-32, at least one of which is essential for replication in all hosts (9, 16, 26, 30, 33). It has been proposed that RK2 copy number is determined primarily by regulating the synthesis of the TrfA proteins such that these proteins are present in rate-limiting amounts. Several studies have provided evidence consistent with this hypothesis (25, 28, 41). However, it is clear that there is a mechanism capable of controlling RK2 replication that is independent of TrfA concentration. In the accompanying paper (10), raising TrfA protein levels to two or three times above the normal cell level in *Escherichia coli* was shown to increase RK2 copy number by only 30%. Further increases in TrfA concentration (up to 170-fold) had no additional effect on plasmid copy number, indicating that TrfA concentration is not the sole determinant of RK2 replication frequency. It was also found (10) that a plasmid containing only the RK2 origin of replication and two antibiotic resistance genes exhibited copy numbers similar to those of RK2 itself in response to various TrfA protein concentrations, suggesting that the TrfA-concentration-independent control mechanism is a function of the properties of the RK2 *oriV* region, the TrfA proteins, or both.

In order to study the role of TrfA proteins in replication and copy control, the minimal *trfA* coding sequence was cloned and mutagenized. Mutants were isolated that support *oriV* at substantially elevated copy numbers compared with those of the wild-type gene. The properties of these mutants suggest that they are altered in an activity of TrfA that is critical to copy control both in minimal replicons and in whole RK2. TrfA may, therefore, be a bifunctional protein that acts as both an initiator and a negative regulator of plasmid replication initiation.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains and plasmids used in this study are listed in Table 1. The *trfA* fragment in pRD110-34 (Fig. 1) was derived from pCT88Δ34. This plasmid was partially digested with *Hae*II and treated with T4 DNA polymerase to blunt the ends. *Pst*I linkers (CCTG CAGG; New England BioLabs) were added to the ends, and the resulting DNA was digested with *Pst*I and *Eco*RI. This mixture of fragments was added to *Eco*RI- and *Pst*I-digested pBR322 and treated with T4 DNA ligase. Transformants were selected for tetracycline resistance in *E. coli*, pooled, and retransformed with the *oriV* plasmid pCT45. Only those cells that contained a derivative of pBR322 carrying *trfA* in place of the *bla* gene were able to support pCT45. Individual transformants were analyzed to identify a plasmid containing the smallest functional *trfA* fragment, corresponding to nucleotides 408 to 1618 of the *trfA* sequence (32). This plasmid was designated pRD110-34. A similar scheme was used to construct pRD110-16 from pCT88Δ16 (Fig. 1). The 3′ end of the *trfA* gene is the same in both plasmids, but pRD110-16 lacks 265 base pairs (bp) of *trfA* sequence at the 5′ end of the gene. As a result, pRD110-34 encodes TrfA-32 and TrfA-43, while pRD110-16 encodes only TrfA-32. The native *trfA* promoter is absent in each of these constructs, but transcription from the pBR322 anti-*tet* promoter (37) appears to be sufficient to drive the synthesis of TrfA, as evidenced by the ability of both pRD110-16 and pRD110-34 to support replication of pCT45.

pRD119-34 and pRD119-16 (Fig. 1) were derived from pRD110-34 and pRD110-16 as follows. The 793-bp *Hin*dIII-to-*Bam*HI fragment containing the promoterless chloramphenicol resistance gene of pUC8-CAT⁻ was filled in at the *Hin*dIII site and inserted into the *Eco*RV and *Bam*HI sites of each of the two pRD110 plasmids. This inactivated the *tet* gene and placed *cat* under the transcriptional control of the *tet* promoter. The same procedure was used to convert pRD110*trfAcop* plasmids to pRD119*trfAcop* plasmids. Plas-

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TABLE 1. Strains and plasmids used

E. coli strain or plasmid	Remarks	Source or reference
E. coli strain		
HB101	F⁻ *leuB6 proA2 recA13 thi-1 ara-14 lacY1 galK2 xyl-5 mtl-1 rpsL20* λ⁻ *supE44 hsdS20* r_B^- m_B^-	6
TB1	JM83 r^- m^+	1
Plasmid		
pAL100	RSF1010 replicon containing the promoterless *trfA* gene; Tet^r	A. Greener (unpublished)
pAL102	pAL100 with R6K *pir** promoter inserted upstream of *trfA*; Tet^r	A. Greener (unpublished)
pBR322	Laboratory construct; Pen^r Tet^r	5
pCT45	RK2 replicon; 700-bp *Hae*II-*oriV*; Kan^r; requires *trfA* in *trans* for replication	43
pCT88Δ16	ColE1 replicon; deletion derivative of pCT88; Tet^r	9
pCT88Δ34	ColE1 replicon; deletion derivative of pCT88; Tet^r	9
pFF1	RK2 replicon; contains *trfA* from pRD110-34 inserted downstream of the Tn*5 neo* promoter; *oriV oriT*; Pen^r Cam^r	This work
pRD110-16	pBR322 with *trfA* from pCT88Δ16 inserted into the *Eco*RI-to-*Pst*I site; encodes only TrfA-32; Tet^r	This work
pRD110-34	pBR322 with *trfA* from pCT88Δ34 inserted into the *Eco*RI-to-*Pst*I site; encodes TrfA-32 and TrfA-43; Tet^r	This work
pRD119-16	pRD110-16 with *cat* inserted into *tet*; Cam^r	This work
pRD119-34	pRD110-34 with *cat* inserted into *tet*; Cam^r	This work
RK2	Naturally occurring plasmid; Tet^r Pen^r Kan^r	15
pSV16	RK2 replicon; 700-bp *Hae*II-*oriV*; Pen^r Kan^r; requires *trfA* in *trans*	S. Valla (unpublished)
pTJS65	pUC8 with 700-bp *Hae*II-*oriV* inserted as a blunt fragment into the *Sma*I site; Pen^r	26
pUC8-CAT⁻	pUC8 with a 773-bp *Taq*I fragment containing the *cat* gene inserted into the *Acc*I site in reverse orientation relative to P*lac*; Pen^r Cam^r	R. Durland (unpublished)

mid pSV16 (Fig. 2) contains the *Eco*RI-to-*Bam*HI *oriV* fragment of pTJS65 (26) coupled to a *Bam*HI fragment containing the *kan* gene of pUC5 (45) and an *Eco*RI-to-*Bam*HI fragment containing the *bla* gene derived from pBR322 (bp 3104 to 4363) (38). Plasmid pFF1 (Fig. 2) is a self-replicating RK2 derivative containing the *Bcl*I-to-*Eco*RI *oriV* fragment of pRK248 (42). The *trfA* gene in pFF1 is derived from pRD110-34. Transcription of the *trfA* gene is provided by a 354-bp *Eco*RI fragment containing the *neo* promoter of Tn*5* (bp 1196 to 1519) (4). A 270-bp fragment containing the minimal RK2 origin of transfer (13, 14) is also present. No other RK2 loci are present in pFF1. The *bla* and *cat* resistance markers were derived from pBR322 (5) and pUC9Cm, respectively, (K. Buckley, Ph.D. thesis, University of California, San Diego, La Jolla, Calif., 1986).

Mutagenesis of *trfA*. Mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) was carried out essentially by the method of Miller (19). Five-milliliter overnight cultures of *E. coli* TB1 containing pRD110-34 or pRD110-16 were grown at 37°C in LB containing 25 μg of tetracycline per ml. Each culture was diluted 1:20 into two separate flasks of the same medium and shaken at 37°C for 1.5 h. The cells were centrifuged, washed twice with 10 ml of citrate buffer (19), and suspended in 25 ml of the same buffer. A 1.25-ml portion of 1-mg/ml NTG in citrate buffer was added to each suspension, and the cells were incubated for 20 or 40 min at 37°C. After incubation with NTG, the cells were washed with 10 ml of phosphate buffer (19) and suspended in 10 ml of LB without antibiotic. The cultures were incubated at 37°C for 1.5 h, and then each was added to a 2.8-liter fluted flask containing 250 ml of LB with 25 μg of tetracycline per ml. These were shaken overnight at 37°C.

Plasmid DNA was isolated from an alkaline lysate of each 250-ml culture by CsCl density centrifugation. A 10-μg sample of each DNA was digested with *Eco*RI and *Pst*I, and the *trfA* fragment was agarose gel purified. Approximately 2 μg of each of the isolated fragments was mixed with 1 μg of pBR322 that had been previously digested with *Eco*RI, *Pst*I, and *Sca*I (to inactivate the pBR322 *bla* gene). After ligation, the subcloned plasmids were transformed into *E. coli* TB1, yielding 30,000 to 100,000 colonies for each of the four preparations. These colonies were pooled to form the four mutant banks (i.e., two time points of mutagenesis for each of the two pRD110 derivatives).

Mapping and DNA sequencing of *cop* mutations. Digestion of pRD110-16, or its copy-up mutant derivatives, with *Hin*cII yielded 3.6- and 0.95-kilobase (kb) fragments, while *Nde*I digestion yielded 2.95- and 1.6-kb fragments. *Hin*cII digestion of pRD110-34 or its derivatives yielded 3.6- and 1.2-kb fragments, while *Nde*I digestion yielded 3.2- and 1.6-kb fragments. These two enzymes were chosen because they cleave pRD110 once in the vector sequence and once in the *trfA* gene (Fig. 1). The fragments were separated by electrophoresis on a 0.7% agarose gel, and each was isolated separately by electroelution.

To map a particular mutation pairwise, combinations of purified fragments were ligated together such that a pRD110-type plasmid would be reconstructed, i.e., the 3.6-kb *Hin*cII fragment from the wild-type plasmid was ligated to the small *Hin*cII fragment from the mutant and vice versa, and similarly for the *Nde*I fragments. Ligation mixtures were then used to transform competent *E. coli* HB101 cells to Tc^r. Since one of the *Hin*cII sites is in the gene encoding Tc^r and one of the *Nde*I sites is in the vector *ori*, the only ligation products which should give transformants are those in which the two different *Hin*cII fragments or the two different *Nde*I fragments are present and oriented as they are in pRD110.

If the phenotype of each of the copy-up *trfA* genes was due to a single mutation, then for each enzyme one combination of fragments should give a plasmid with a wild-type phenotype, while the reverse combination would give a plasmid with a copy-up phenotype. The latter would localize the mutation to a particular restriction fragment. The mutations mapped in this way were *cop271C* and *cop254D* (using pRD110-16 as the wild-type plasmid) and *cop273C*, *cop267L*, *cop251M*, and *cop250V* (with pRD110-34 as the wild-type plasmid).

Typically, three HB101 transformants of a given reconstructed plasmid were tested for their abilities to support the origin plasmid, pSV16, at a wild-type or elevated copy number as detected by relative penicillin resistance levels or

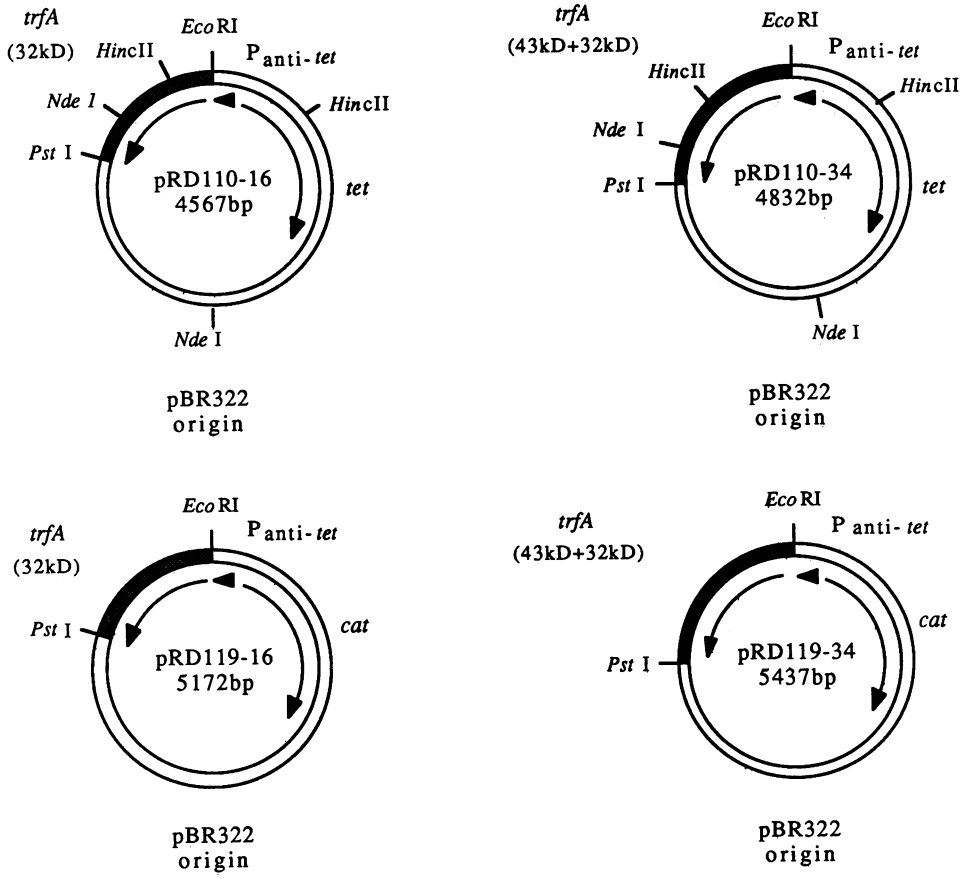

FIG. 1. Maps of plasmids containing wild-type and mutant *trfA* genes. All plasmids are derived from pBR322 and differ only in antibiotic resistance and whether or not they encode TrfA-43 in addition to TrfA-32, as indicated. The *trfA* gene (shaded) lacks its native promoter and is most likely transcribed by the promoter labeled $P_{anti\text{-}tet}$. This promoter has also been called P1 (37). Antibiotic resistance genes are *tet* (tetracycline) and *cat* (chloramphenicol). kD, Kilodalton.

as visualized on agarose gels. The results obtained were consistent with all six mutations mapping within the 379-bp *Hin*cII-*Nde*I fragment. This fragment from each of the mutants was then sequenced.

Plasmids for sequencing were constructed by isolating the 379-bp *Hin*cII-*Nde*I fragment from each mutant pRD110 plasmid, filling in the *Nde*I site by using Klenow polymerase as described previously (17), and then ligating the fragment into *Hin*cII-digested pBluescriptSK+ (Stratagene Cloning Systems, San Diego, Calif.). Sequencing plasmids with the insert in either orientation were obtained for each mutant. Single-stranded template DNA was prepared in accordance with instructions supplied with the vector. DNA sequencing was performed by using the Sequenase kit purchased from U.S. Biochemical Corp. The labeling reactions were separated on 8% denaturing polyacrylamide wedge gels.

Other methods. Estimations of TrfA levels by Western blots (immunoblots) were performed as described in the accompanying paper (10). Plasmid copy numbers were determined by the dot blot procedure of Shields et al. (29), modified as described in the accompanying paper (10). NTG was obtained from K&K Laboratories, Hollywood, Calif. Antibiotics were obtained from Sigma Chemical Co., except tetracycline, which was obtained from CalBiochem. Bacterial transformation (8), DNA isolation (2), and gel purification (17) were done as described previously.

RESULTS

Mutagenesis of the *trfA* gene. Figure 1 shows the structures of plasmids pRD110-16 and pRD110-34, which were used for the mutagenesis of the *trfA* gene. These plasmids are derivatives of pBR322 in which the *Eco*RI-to-*Pst*I fragment containing the 5′ end of the *bla* gene has been replaced with a fragment carrying *trfA*. The *trfA* fragment in pRD110-34 includes bp 408 to 1618, while the fragment in pRD110-16 includes bp 673 to 1618 (numbering in accordance to the system in reference 32). The *trfA* gene normally encodes two proteins, called TrfA-43 and TrfA-32, because of the presence of two in-frame initiation codons (16, 30, 33). pRD110-34 retains this property, but pRD110-16 lacks sequences necessary for synthesis of TrfA-43 and specifies only TrfA-32. Transcription of *trfA* is most likely directed by the anti-*tet* promoter (designated P1 in reference 37) that is located just clockwise of the *Hin*dIII site in the pBR322 vector sequence. An important feature of the pRD110 construct is that *trfA* expression and activity appear to have little or no effect on the replication of the pBR322 origin of the plasmid. This allowed the generation of mutant banks without imposing any initial selection for *trfA* phenotypes.

Mutagenesis was performed by treating *E. coli* TB1 containing either pRD110-16 or pRD110-34 with NTG for 20 and 40 min as described in Materials and Methods. After being

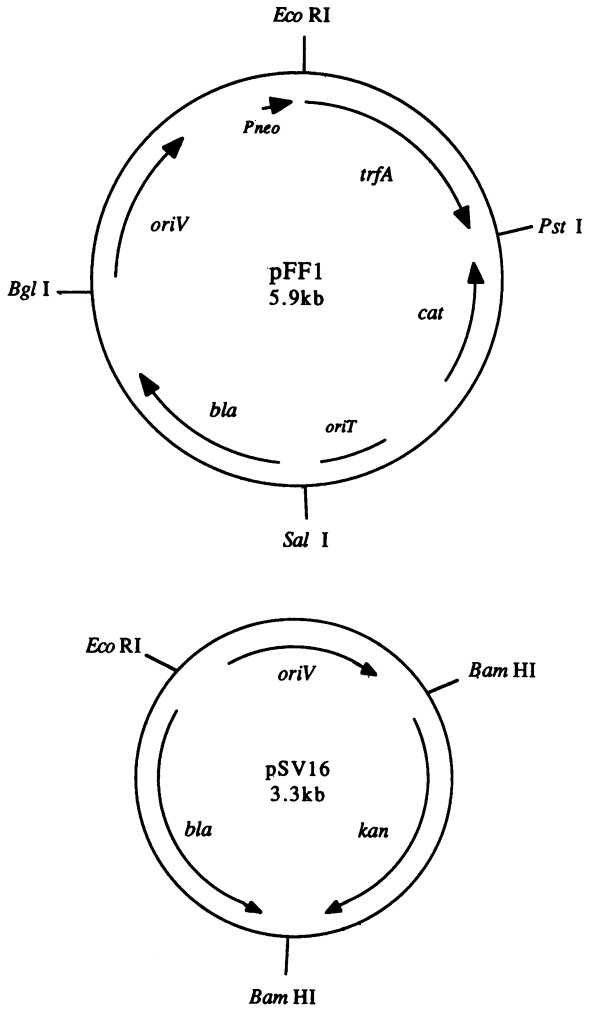

FIG. 2. Maps of autonomous mini-RK2 plasmids pFF1 and pSV16. RK2 loci are *oriV*, *oriT*, and *trfA*. The other loci are P*neo*, the neomycin phosphotransferase promoter from Tn*5* (4); *cat*, the Tn*9* chloramphenicol resistance gene from pUC9Cm (K. Buckley, Ph.D. thesis); *bla*, the penicillin resistance gene from pBR322; and *kan*, the kanamycin resistance gene from pUC5 (45).

washed, the surviving cells were diluted into fresh medium and grown overnight. Plasmid DNA was isolated, and the *Eco*RI-to-*Pst*I *trfA* fragment from each preparation was gel purified. The mutagenized *trfA* preparations were recloned into the *Eco*RI and *Pst*I sites of pBR322 to recreate pRD110-16 and pRD110-34, respectively. This step was included to reduce the frequency of mutations in the pBR322 vector sequences that might complicate subsequent analysis. These procedures yielded four independent mutant banks (two for each plasmid) in which the *trfA* gene had been treated with NTG for 20 or 40 min.

Selecting copy-up mutations in *trfA*. Advantage was taken of the fact that resistance to β-lactam antibiotics is generally proportional to gene dosage. Thus, an increase in the copy number of a plasmid carrying the *bla* gene leads to a proportional increase in penicillin resistance (44). This fact has been used previously to select copy-up mutations in other systems (20, 27). In this case, the RK2 origin plasmid pSV16 (*oriV bla kan*) was used to select for mutations in *trfA* leading to higher *oriV* copy numbers and increased penicillin resistance. *E. coli* TB1 carrying pRD110-16 or pRD110-34 (wild type) was transformed with pSV16 DNA and plated on LB containing different levels of penicillin G. In each case, transformants grew well at 250 μg of penicillin per ml, but frequencies of transformation were reduced 10- to 20-fold at 500 μg/ml, and no transformants were obtained at 1 mg/ml. *E. coli* TBI carrying each of the four *trfA* mutant banks was then transformed with pSV16 and selected on 750 μg of penicillin per ml. Samples of each transformation were also plated on 50 μg of kanamycin per ml to determine the number of total transformants capable of supporting pSV16. Of the total transformants, 0.4% from the pRD110-16 20-min bank, 1.1% from the pRD110-16 40-min bank, 4.0% from the pRD110-34 20-min bank, and 10.6% from the pRD110-34 40-min bank were resistant to 750 μg of penicillin per ml. A control transformation of cells containing unmutagenized pRD110-34 yielded no colonies on 750 μg of penicillin per ml (less than 0.1%).

Individual colonies growing on plates containing 750 μg of penicillin per ml were tested to confirm that they were in fact resistant to higher levels of penicillin than colonies containing pSV16 and unmutagenized pRD110-16 or pRD110-34. This was found to be the case for at least 90% of the colonies examined. Plasmid DNA was isolated from 44 such colonies. Agarose gel electrophoresis indicated that four colonies contained plasmids of an anomalous size, and these were discarded. The remaining 40 colonies contained two plasmids identical in size to pSV16 and either pRD110-16 or pRD110-34, as expected. In at least 70% of these colonies, the amount of pSV16 DNA relative to pRD110 DNA was clearly greater than in the unmutagenized situation, consistent with the interpretation that the high penicillin resistance was due to increased pSV16 copy number. The pRD110 plasmids from six such colonies were introduced into *E. coli* HB101 and retransformed with pSV16 to confirm that the mutant phenotype was associated with the pRD110 plasmid. Two of these plasmids, derived from the 20-min mutagenesis pool of pRD110-16, were designated pRD110-16*cop271C* and pRD110-16*cop254D*. Three were from the 20-min pool of pRD110-34 and were designated pRD110-34*cop273C*, pRD110-34*cop267L*, and pRD110-34*cop251M*. The final mutant, designated pRD110-34*cop250V*, was from the 40-min pool.

Properties of *trfA* copy-up mutants. To facilitate subsequent experiments, the *tet* gene of pRD110-16, pRD110-34, and the six mutant plasmids was in each case inactivated by the insertion of a promoterless *cat* gene, yielding the chloramphenicol-resistant derivatives pRD119-16, pRD119-34, pRD119-16*cop271C*, pRD119-16*cop254D*, pRD119-34*cop273C*, pRD119-34*cop267L*, pRD119-34*cop251M*, and pRD119-34*cop250V*, respectively (Fig. 1). The relative amounts of pSV16 in the presence of each of these eight plasmids in *E. coli* HB101 are shown in Fig. 3. The copy number of pSV16 relative to that of pRD119 was clearly elevated in the presence of each of the mutants compared to the wild-type situation. In the presence of pRD119-34*cop267L* or pRD119-34*cop250V*, the copy number of pSV16 was clearly well above that of the pBR322 replicon present in the pRD119 derivatives.

The absolute copy number of pSV16 in the presence of the two wild-type and the six mutant pRD119 derivatives was determined by using the dot blot assay as described previously (10). To avoid possible cointegration of pSV16 and pRD119 via shared DNA sequences, these and subsequent copy number determinations were performed in the *E. coli* *recA* strain HB101. The resulting autoradiogram is shown in Fig. 4. The intensity of each spot was quantified and com-

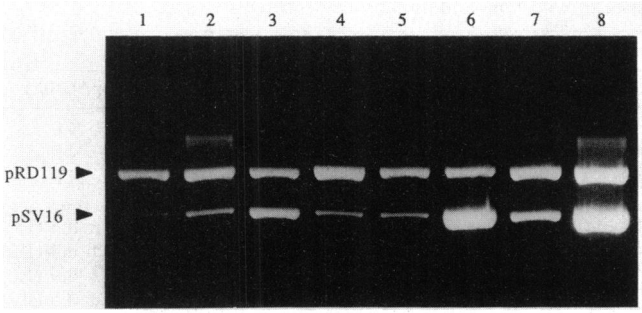

FIG. 3. Agarose gel analysis of pSV16 copy number in the presence of wild-type and mutant *trfA* genes. DNA was isolated from *E. coli* HB101 and digested with *Eco*RI to linearize each plasmid. Each sample contains pSV16 and the following plasmids: pRD119-16 (wild type) (lane 1), pRD119-16*cop271C* (lane 2), pRD119-16*cop254D* (lane 3), pRD119-34 (wild type) (lane 4), pRD119-34*cop273C* (lane 5), pRD119-34*cop267L* (lane 6), pRD119-34*cop251M* (lane 7), and pRD119-34*cop250V* (lane 8).

pared with the standard curve obtained from purified *oriV*-containing standard DNA to allow calculation of the total number of origins in each original culture. The results are presented in Table 2. During logarithmic growth, the mutant pRD110 derivatives supported replication of pSV16 at copy numbers as much as 23 times higher than the wild-type level.

Western blot analysis was performed on each mutant derivative to determine whether the relative size or amount of the TrfA proteins was affected. The results are presented in Fig. 5. The amounts of the TrfA proteins synthesized by

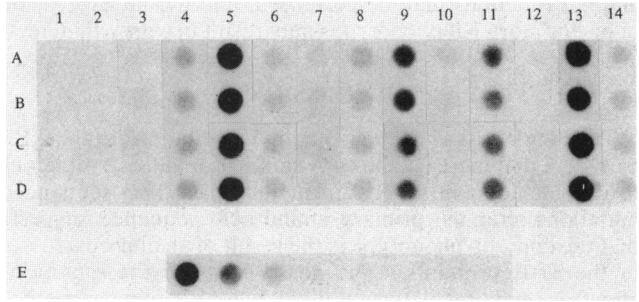

FIG. 4. Dot blot analysis of pSV16 copy number in the presence of wild-type and mutant TrfA proteins. Samples in rows A through D contain material from 0.02 OD_{600} units of *E. coli* HB101 with the following plasmids. Lanes: 1, none; 2, pRD119-34wt; 3, pRD119-16wt and pSV16; 4, pRD119-16*cop271C* and pSV16; 5, pRD119-16*cop254D* and pSV16; 6, 1:5 dilution of lane 5; 7, pRD119-34*wt* and pSV16; 8, pRD119-34*cop273C* and pSV16; 9, pRD119-34*cop267L* and pSV16; 10, 1:5 dilution of lane 9; 11, pRD119-34*cop251M* and pSV16; 12, 1:5 dilution of lane 11; 13, pRD119-34*cop250V* and pSV16; 14, 1:5 dilution of lane 13. The 1:5 dilutions in lanes 6, 10, 12, and 14 were prepared with plasmid-free HB101. In each lane, rows A and B are duplicates of one culture, and rows C and D are duplicates of an independent culture. More than 90% of the cells in cultures with pSV16 contained the plasmid (determined as described in reference 10), with two exceptions. About 70% of HB101 (pRD119-34*cop267L*) cells and about 30% of HB101 (pRD119-34*cop250V*) cells had lost pSV16. These values were taken into account in calculating plasmid copy number. Standards (row E) are twofold serial dilutions of purified *oriV*-containing pTJS65 DNA, with the highest amount being equivalent to 10^9 molecules of plasmid. Hybridization was with the nick translated *Dra*I-*Bam*HI *oriV* fragment of pTJS65, as previously described (10).

each of the mutant plasmids were similar to those of the wild-type plasmids, with some variation. However, the data presented in the accompanying paper (10), although dealing with the wild-type TrfA protein, indicate that the mutant phenotype is not a result of altered TrfA protein levels. Thus, a qualitative rather than quantitative alteration in TrfA is responsible for the copy number increase. It is interesting that the TrfA-32 protein is resolved into two distinct bands in this blot. This is true for both the wild-type and mutated plasmids. We have observed this on several occasions, primarily when the TrfA proteins are overproduced (relative to the levels synthesized by RK2). At present we do not know the significance of this observation, but it is worth noting that the relative amounts of the two TrfA-32 bands are altered in pRD119-16*cop271C* and that migration of the TrfA-32 bands appears to be anomalous in pRD119-34*cop273C* and pRD119-34*cop267L*.

The ability of different amounts of wild-type TrfA proteins to suppress the copy-up phenotype of these mutants in *trans* was determined by using two plasmids, pAL100 and pAL102. These plasmids are RSF1010 replicons that are compatible with the pBR322 and minimal RK2 origins present in pRD110 and pSV16, respectively. pAL100 carries the promoterless wild-type *trfA* gene downstream of a polylinker. In pAL102, a derivative of the R6K *pir* promoter (35) was inserted into the polylinker of pAL100 to increase the expression level of *trfA*.

Table 2 lists the copy numbers of pSV16 in the presence of wild-type or mutant pRD119-34 together with either pAL100 or pAL102. In general, wild-type *trfA* exhibited at least partial dominance over the mutants. Only pRD119-34*cop267L* and pRD119-34*cop273C* were fully recessive to pAL100. With the other copy-up derivatives, pAL100 only partially suppressed the copy number of pSV16. However, in the presence of pAL102, pSV16 copy number was reduced to near-normal levels in all cases. A possible explanation for these observations lies in the relative amounts of wild-type and mutant proteins in each situation. Western analysis indicated that pRD119-34 made about 240 ng of total TrfA per unit of optical density at 600 nm (OD_{600}) (data not shown). The mutant derivatives all made similar amounts (Fig. 5). Previous experiments estimated the total TrfA produced by pAL100 and pAL102 at 28 and 280 ng per OD_{600} unit, respectively (10). Thus, when mutant *trfA* is present in substantial excess over wild-type *trfA* (approximately ninefold), the copy number of pSV16 is generally intermediate between the wild-type and mutant levels. When wild-type and mutant *trfA* are present in equivalent amounts, the copy number of pSV16 approaches the wild-type level. Additional experiments with other copy-up *trfA* derivatives suggest that this is a general property of such mutants (R. Durland, unpublished observations). However, it is noteworthy that in some cases, such as pRD119-34*cop267L*, the mutant is recessive to even low levels of wild-type TrfA proteins provided in *trans* (i.e., from pAL100).

Mapping and sequencing of the mutations in *trfA*. The location of the mutation responsible for the copy-up phenotype was mapped in the six mutant plasmids by performing reciprocal exchanges of restriction fragments between individual mutant and wild-type plasmids (described in Materials and Methods). In all six cases, the mutant phenotype was found to be associated with the *Hinc*II-to-*Nde*I fragment of the *trfA* gene, corresponding to bp 967 to 1346. The sequence of this fragment was determined for each of the six mutated plasmids. Comparison with the wild-type sequence revealed a single G:C to A:T transition in each case (Fig. 6). Each

TABLE 2. Copy number of pSV16 in the presence of wild-type and mutant TrfA proteins

trfA plasmid	*trfA* allele	Copy no. with the following source of additional wild-type TrfA[a]:		
		None	pAL100	pAL102
pRD119-16	Wild type	5.2 ± 1 (1)	NT[b]	NT
	cop271C	18 ± 1 (3.5)	NT	NT
	cop254D	83 ± 6 (16)	NT	NT
pRD119-34	Wild type	5.5 ± 1 (1)	5.1 ± 0.3 (1)	5.2 ± 0.4 (1)
	cop273C	16 ± 1 (3)	6.8 ± 0.6 (1.3)	6.3 ± 0.4 (1.2)
	cop267L	120 ± 6 (23)	8.3 ± 0.5 (1.6)	6.4 ± 0.9 (1.2)
	cop251M	32 ± 1 (6)	14 ± 1 (2.7)	8.7 ± 0.5 (1.7)
	cop250V	120 ± 3 (23)	79 ± 2 (15)	13 ± 1 (2.5)

[a] Copy number was determined in logarithmically growing *E. coli* HB101 as described in the accompanying paper (10). Units are 10^9 molecules per OD_{600} unit (mean ± standard deviation). Numbers in parentheses are approximate relative copy numbers, with the average wild-type value (~5.2×10^9 per OD_{600} unit) taken to be 1. Total TrfA levels in cells containing the following plasmids were as follows: pRD119-34, ~235 ng per OD_{600} unit; pAL100, ~28 ng per OD_{600} unit; pAL102, ~280 ng per OD_{600} unit (determined as described in reference 10). One OD_{600} unit is equivalent to ~3.7×10^8 cells (determined with a Petroff-Hauser chamber).
[b] NT, Not tested.

mutation resulted in a single substitution in the TrfA amino acid sequence, indicating that the mutant phenotype is a result of a change in the TrfA protein(s). All six mutations occurred within a stretch of 69 bp, and with the exception of the *cop250V* mutation, all resulted in nonconservative amino acid substitutions.

Effect of copy-up *trfA* mutations on the replication of RK2 plasmids in *trans*. The properties of the replication mutants indicate that TrfA is more than a simple positive factor required for RK2 replication. The above results indicate that at least for a minimal replicon (pSV16), an activity of the TrfA protein(s) is intimately involved in determining replication frequency. Since RK2 carries various other loci (e.g., *kil* and *kor*) that are known or suspected to affect copy number or plasmid maintenance (3, 12, 25, 28, 30–32, 39, 41, 42, 46), we wished to determine how the *trfAcop* mutants interact with intact RK2 and other RK2 derivatives. pRD110-34 and pRD119-34 were used to test the ability of copy-up *trfA* mutants in *trans* to increase the copy number of RK2 and a small self-replicating derivative, pFF1 (Fig. 2). The latter plasmid contains *oriV* and the minimal *trfA* gene transcribed by the constitutive Tn*5* *neo* promoter (9) but lacks all known *kil* and *kor* genes.

The copy numbers of both pFF1 and RK2 were substantially increased by certain mutant *trfA* genes in *trans* (Table 3). In the presence of *trfAcop250V*, the copy number of each plasmid was increased approximately fourfold. Previous results indicate that this is not due to an increase in TrfA concentration per se, since a large excess of wild-type TrfA protein increases RK2 copy number by only 30% (10). These results demonstrate that certain mutant TrfA proteins are capable of interfering with or partially overcoming the normal replication controls present in RK2. Interestingly, we observed that cells containing RK2 and either pRD119-*cop251M* or pRD119-34*cop250V* grew slowly and formed small colonies on LB agar plates. This was not true for RK2 in the presence of pRD119-34 (wild type) or the other two mutants tested, nor was it true in any of the combinations with pFF1, suggesting that it is due to the higher RK2 copy number in the presence of the *cop251M* or *cop250V* mutations. It is possible that the growth inhibition is related to the RK2 *kil* genes, whose host lethality is normally suppressed by the RK2 *kor* genes (3, 12, 25, 28, 31, 47). At elevated RK2 copy numbers, the complex regulatory network involving *kil* and *kor* functions may be perturbed, and partial expression of one or more Kil phenotypes may result in a growth defect.

DISCUSSION

This work describes the isolation of mutations in the RK2 *trfA* gene that increase the copy number of all RK2 replicons tested, including intact RK2. The fact that all six sequenced mutations alter the primary amino acid sequence suggests that the copy-up phenotype is the result of an altered activity of the TrfA protein(s). The possibility that the mutations affect a small regulatory protein whose coding sequence overlaps the *trfA* gene is small. No single alternative reading frame encompasses the mutated region in such a way that all

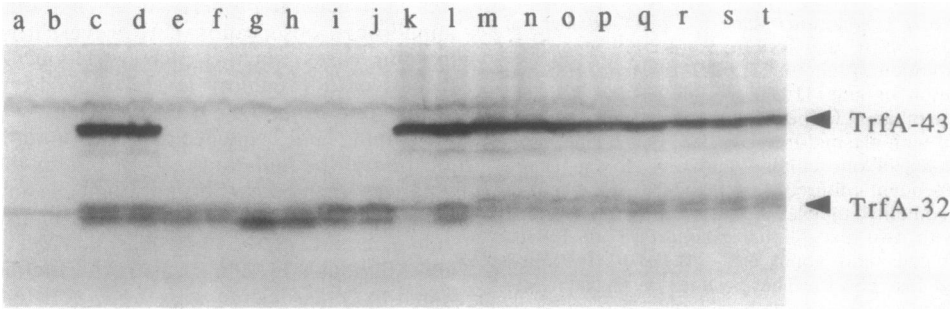

FIG. 5. Anti-TrfA Western blot of *E. coli* HB101 (0.5 OD_{600} units per lane) containing the following plasmids. Lanes: a and b, none; c and d, pRD119-34*wt*; e and f, pRD119-16*wt*; g and h, pRD119-16*cop271C*; i and j, pRD119-16*cop254D*; k and l, pRD119-34*wt*; m and n, pRD119-34*cop273C*; o and p, pRD119-34*cop267L*; q and r, pRD119-34*cop251M*; s and t, pRD119-34*cop250V*. Samples in lanes e through t also contained pSV16.

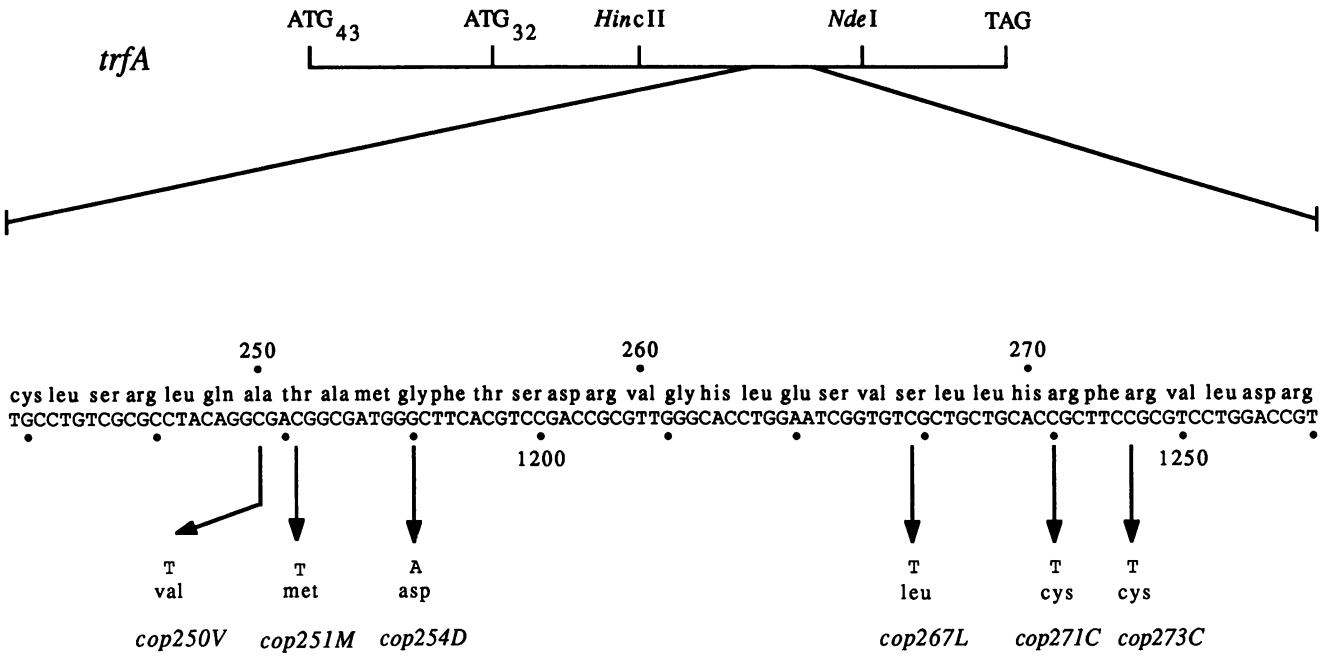

FIG. 6. Nucleotide sequence changes in six copy-up *trfA* mutations. The upper line represents the coding sequence for the *trfA* gene, indicating the two start codons and the stop codon. All copy-up mutations map to the region between the *Hin*cII and the *Nde*I sites. The region containing the sequenced mutations is expanded below. The sense strand of the DNA is numbered according to the system of Smith and Thomas (32). Amino acid residues are numbered from the first methionine of TrfA-43.

six sequence changes lead to amino acid substitutions (32). It is formally possible that the mutations affect a *trans*-acting regulatory RNA molecule. However, studies on the RK2 system to date have not suggested the existence of any such RNA molecule specified by the *trfA* gene. Thus, we consider that these mutations define a region of the TrfA protein(s) that is intimately involved in RK2 replication initiation and its control. The fact that mutations with similar phenotypes are found regardless of the presence or absence of TrfA-43 suggests that the function of the larger protein is not qualitatively different from that of the smaller one in this regard.

Although little is known about the activities of the TrfA proteins, it has been observed that one or both proteins bind to repeated sequences (iterons) present in the *oriV* region (24; R. Durland and S. Perri, unpublished observations). It is therefore worth noting that all six mutations are in or near a proposed helix-turn-helix DNA-binding domain of the TrfA protein (32). The amino acid substitutions in the *cop254D* and *cop250V* mutants reduce the similarity of this region to a consensus sequence derived from the DNA-binding domains of proteins such as λ repressor and λ Cro (21, 22). This raises the possibility that these mutations may affect the ability of TrfA to bind to *oriV*. At present, however, there is no experimental evidence to support an involvement of this region of TrfA in DNA binding. Further experiments will be necessary to determine whether the mutant TrfA proteins have altered DNA-binding activities.

TABLE 3. Copy number of pFF1 and RK2 in the presence of excess wild-type or mutant TrfA proteins

trfA allele in *trans*[a]	Plasmid copy number[b] with:	
	pFF1	RK2
Wild type	16 ± 0.8 (1)	7.1 ± 0.5 (1)
cop273C	21 ± 1 (1.3)	12 ± 1 (1.7)
cop267L	18 ± 1 (1)	7.8 ± 0.6 (1)
cop251M	50 ± 3 (3)	23 ± 4 (3.2)
cop250V	69 ± 5 (4.3)	27 ± 4 (3.8)

[a] Additional TrfA was provided by pRD110-34 (for experiments with pFF1) or pRD119-34 (for experiments with RK2), which differ only in antibiotic resistance genes.

[b] Copy number was determined in logarithmically growing *E. coli* HB101 as described in the accompanying paper (10). Units are 10^9 molecules per OD_{600} unit (mean ± standard deviation). Numbers in parentheses are relative copy number, with the wild-type situation for each plasmid (pFF1 or RK2) taken to be 1. Total TrfA levels in cells containing the following plasmids were as follows: pRD110/119, ~235 ng per OD_{600} unit; pFF1, ~23 ng per OD_{600} unit; RK2, ~6.4 ng per OD_{600} unit (determined as described in reference 10). One OD_{600} unit is equivalent to ~3.7×10^8 cells (determined with a Petroff-Hauser chamber).

All mutants examined thus far are recessive to the wild-type gene. In the case of pRD119-34*cop267L*, the copy-up phenotype is suppressed by wild-type TrfA protein even when the mutant protein is present in considerable excess. The properties of these mutants suggest that TrfA protein, in addition to being required for initiation, also participates in the regulation of replication, and that the mutants are altered in this regulatory activity. In most cases it appears that the relative amounts of wild-type and mutant proteins are an important factor in the degree to which the mutant phenotype is suppressed. The origin region of RK2 contains at least nine copies of a 17-bp sequence that is likely to be the TrfA-binding site (36, 40). Thus, it is possible that when present, both wild-type and mutant proteins bind to a single plasmid origin. If multiple binding events are required for the initiation or regulation of replication, binding of both wild-type and mutant proteins could lead to intermediate phenotypes. This interpretation could explain the observed dependence of the mutant phenotype on the amount of wild-type TrfA protein provided in *trans*. In addition, it is possible that the functional TrfA protein is in the form of a dimer or higher multimer and that mixed multimers of wild-type and mutant proteins may form, having intermediate phenotypes.

It is most significant that three of four mutants tested increase the copy number of intact RK2 in *E. coli* when the mutant proteins are provided in *trans*. In the presence of pRD119-34*cop251M* and pRD119-34*cop250V*, the RK2 copy number is increased approximately fourfold. This is well above the maximum RK2 copy number obtained by substantially overproducing the wild-type TrfA proteins (10). These observations support a multi-functional role for TrfA, with one or more domains of the protein acting in a positive sense and at least one involved in the negative control of plasmid RK2 replication. Similar positive and negative activities for a replication initiation protein have been proposed for plasmids R6K (18, 34) and P1 (7, 23). It is not yet clear why different plasmid derivatives of RK2 show different responses to individual copy-up TrfA proteins provided in *trans*. In particular, pRD119-34*cop250V trfA* increases the copy number of the origin plasmid pSV16 15-fold in the presence of moderate amounts of wild-type TrfA provided in *trans* by pAL100. In contrast, pFF1 and RK2 copy numbers increase only fourfold in the presence of pRD119-34*cop250V*. Since pFF1 and RK2 each produce wild-type TrfA at levels similar to or substantially less than that specified by pAL100, it seems likely that other elements present on pFF1 and RK2 are modulating the copy-up effect of the TrfAcop250V mutant protein.

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