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Plasmid RP4 Specifies a Deoxyribonucleic Acid Primase Involved in Its Conjugal Transfer and Maintenance

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We surveyed plasmids representative of most incompatibility groups for their conferred deoxyribonucleic acid (DNA) primase activity. RP4 (IncP) was one of the few with such activity although, unlike the derepressed IncIα plasmids (which also specify a primase), it did not suppress the *dnaG* mutation. Using deletion and Tn7 derivatives of RP4, we located the presumed primase structural gene (*pri*) in the 37- to 42-kilobase region. Tn7 insertions in the adjacent TraI region also reduced or caused overproduction of primase. We purified the RP4 primase to a single polypeptide of molecular weight 118,000. It is an anisometric molecule and functions as a monomer, initiating complementary strand synthesis on φX174 DNA in *Escherichia coli dnaG* cell extracts in the presence of ribonucleotide triphosphates and rifampin. It is immunologically unrelated to either the *E. coli dnaG* or the IncIα plasmid-specified DNA primases. RP4 *pri* mutants conjugated with a lower efficiency into some bacterial species, including *Salmonella typhimurium*. Back-transfer experiments showed that this effect was recipient specific. There was also a comparable reduction in mobilization efficiency of R300B by RP4 *pri* into such recipients. Loss of RP4 primase led to detectable plasmid instability. The RP4-specified primase therefore seems to serve two functions: the single DNA strand transferred during conjugation is primed by it in the recipient cell, and it appears to be necessary for the efficient priming of discontinuous plasmid DNA replication despite the presence of the chromosomal priming system.

It has been shown that plasmids of incompatibility group (Inc) Iα that are derepressed for transfer (*drd* mutants) partially suppress the temperature-sensitive phenotype of *dnaG* mutants of *Escherichia coli* (8, 27). This phenomenon has also been reported for IncIγ plasmids (19). IncIα plasmids specify the synthesis of a DNA primase which seems likely to be the protein responsible for this suppression (14). Recently it has been demonstrated that during the cloning of a primase specified by ColI*drd*9, a deletion that considerably reduced the molecular weight of the primase also improved the suppression of the *dnaG* phenotype by a factor of about 100 (28). This almost certainly indicates that the plasmid-coded primase is able to substitute for the function of the *dnaG* primase in such temperature-sensitive mutants, that is, for the priming of Okazaki fragments during chromosome replication. It is, however, not clear what function the plasmid-coded primase serves for the plasmid, although the fact that its synthesis is derepressed together with plasmid transfer functions suggests a role in the latter.

During a survey of primase activity specified by plasmids representative of most of the known incompatibility groups (see Table 2), we discovered that few of these plasmids specify this activity. IncP plasmid RP4 is one of those that does confer such a DNA primase activity. The availability of previously mapped Tn7 mutations of RP4 (4, 6, 7) enabled us to locate the coding region of this primase. We also describe in this paper the purification of this enzyme as a single polypeptide of molecular weight 118,000. It seems to be necessary for efficient plasmid transfer to certain bacterial species and for plasmid stability.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used are listed in Tables 1 and 2, respectively. The Tn7 derivatives of RP4 have been previously described (4, 6, 7).

Media. Minimal medium was M9 (17) with glucose (2 mg/ml) and suitable additions for auxotrophs (7). Rich medium was Luria broth (17). *Methylophilus methylotrophus* was grown on M9 plus $FeCl_3$ (10 μg/ml) plus 1% methanol. Agar plates were made by the addition of 15 g of agar per liter to these media. Strains

TABLE 1. *Bacterial strains used*

Strain	Genetic markers[a]	Reference or source
E. coli		
K-12 J53	*proA metF*(λ)	2
C600	*thr leu thi lacY*	2
W3110 T^-	*thy deoC*	2
BC1304	*dnaB1304 dnaC201 arg thy polA1 end rpsL*	21
HfrH252	*dnaB252 pro thy thi*	15, 31
BT1011	*dnaG thy polA1 endI rpsL tsx*	25
MSI	*dnaB266*(Am) *supF trp*(Am) *thy thi rpsL nal lac*(Am)	14
NY731	*dnaG3 leu thy rpsL rpoB*	16
BW86	*dnaG3 leu thy deoB rpsL cir* Δ(*chlA-uvrB*)	B. M. Wilkins (8)
A. tumefaciens C58C1	—[b]	J. Draper
A. eutrophus 17698	—	ATCC
K. aerogenes K16	—	N. Datta
M. methylotrophus AS1	—	29
P. mirabilis 13	—	N. Datta
Providencia sp. strain 164	—	R. W. Hedges
P. aeruginosa 280	*met*	G. Jacoby
P. putida A312	—	M. Worsey
S. typhimurium Ut3692	—	N. Datta
S. typhimurium LT2 SL329	*trp*	N. Datta
S. marcescens	—	G. O. Humphreys

[a] Genetic symbols are as given by Bachmann and Low (3).
[b] —, Not done.

were grown at 37°C except for *Agrobacterium tumefaciens*, *Alcaligenes eutrophus*, and *Pseudomonas putida*, for which 30°C was used. The swarming of *Proteus mirabilis* was inhibited by the addition of 0.1% chloral hydrate or by the use of fivefold normal agar concentration. Antibiotics were used at the following concentrations unless otherwise indicated: ampicillin (Ap), 100 μg/ml; tetracycline-HCl (Tc), 10 μg/ml; kanamycin-sulfate (Km), 25 μg/ml; trimethoprim lactate (Tp), 100 μg/ml; streptomycin-sulfate (Sm), 15 μg/ml; and sulfathiazole (Su), 500 μg/ml. Strain antibiotic resistances were tested on Isosensitest plates (Oxoid Ltd.) with Multodiscs (code no. 6903E; Oxoid Ltd.).

Plasmid conjugal transfer. To test the effect of plasmid-coded primase on the conjugal transfer of RP4, we used as donors the various RP4::Tn*7* derivatives in the *thy E. coli* strain W3110T^-. Colonies of these were cross-streaked into the various bacterial species listed in Table 1, spread across half an Isosensitest plate containing a suitable drug for selection. After incubation (generally at 37°C but 30°C for *A. tumefaciens*, *A. eutrophus*, and *P. putida*), the efficiency of conjugation could be estimated from the relative number of transconjugants in each of the cross-streaks. Isosensitest medium, devoid of thymine or its deoxynucleotides, but otherwise a rich medium, permits efficient mating and counterselection of the *thy* donor in all of these crosses. All conjugations into *M. methylotrophus* utilized the methanol minimal medium instead. Mobilization of the nonconjugative broad-host-range plasmid R300B (4, 5) by each of the RP4::Tn*7* derivatives was carried out similarly, using streptomycin (50 μg/ml) or sulfathiazole selection.

For crosses with *Salmonella typhimurium* as donor, we used M9 glucose medium containing Casamino Acids (5 mg/ml) plus a selective drug, but otherwise the same technique. This medium selects against *trp* donors.

Plasmid stability testing. Overnight cultures of the various strains were grown in broth with kanamycin selection. These were subinoculated into prewarmed nonselective broth three times successively with 10^2-fold dilutions and allowed thereby to grow through about 20 generations at 37°C. Serial dilutions were spread onto nonselective plates. About 100 colonies from these (for each strain) were then patched onto kanamycin-selective and nonselective plates. After incubation the Km^s patches were counted.

Isolation of deletions from RP4::Tn*7* plasmids. Derivatives of RP4 with specific deletions were isolated from suitable Tn*7* insertion mutants by excising the DNA between the single *Hin*dIII site on RP4 and the three *Hin*dIII sites on the transposon as previously described (4). Ampicillin-resistant transformants were tested for kanamycin sensitivity. Plasmid DNA from suitable transformants was analyzed by restriction enzyme mapping with horizontal agarose gels (4).

Primase assay. Cells grown on tryptone-yeast extract plates containing an appropriate antibiotic were used to prepare crude cell extracts, which were assayed for primase activity. Two colonies of each strain were suspended in 50 μl of a lysis mixture containing 50 mM Tris-hydrochloride (pH 7.6), 10 mM spermidine-3HCl, 100 mM NaCl, 1 mM EDTA, and 2 mg of lysozyme per ml. After freezing and thawing of the cell suspension three times, Brij 58 was added to a final concentration of 0.25%. After further incubation for 1 h at 0°C and subsequent centrifugation (80,000 × *g*, 30

TABLE 2. *Survey of plasmids specifying DNA primase activity*

Plasmid[a]	Incompatibility group[b]	Pilus synthesis[c]	Primase activity[d] (mU/mg of CCE)	Plasmid[a]	Incompatibility group[b]	Pilus synthesis[c]	Primase activity[d] (mU/mg of CCE)
R16	B	Repr	0	R621a	Iγ	Repr	122.0
R16*drd*4	B	Const	2.7	RM413	Iδ	Repr	0
RA1	C	Repr	0	R721	Iδ	Repr	0
R40a	C	Repr	0	R821a	Iδ	Repr	0
R55	C	Repr	0	R821a*drd*1	Iδ	Const[e]	0
R711b	D	Const	0	R805a	Iζ	Repr	0
ColV2	F I	Const	0	TP114	I2	Repr	0
ColVBtrp	F I	Const	0	TP114*drp*1	I2	Const	0.15
R1*drd*16	F II	Const	0	R391	J	Repr	0
R1*drd*19	F II	Const	0	R387	K	Repr	0.1
R100*drd*1	F II	Const	0	R446b	M	Const	1.5
R538-1	F II	Repr	0	R831b	M	Const	6.4
ColB4	F III	Repr	0	N3	N	Const	0
R124	F IV	Repr	0	RP4	P	Const	10.3
R27	H1	Repr	0	R300B	Q	—[f]	0
R478	H2	Repr	0	Rts1	T	Const	0
R64	Iα	Repr	2.9	R905	V	Repr	0
R64*drd*11	Iα	Const	35.0	S-a	W	Const	0
R112	Iα	Repr	1.8	R7K	W	Const	0
R144	Iα	Repr	0.7	R7K::Tn7	W	Const	0
R144*drd*3	Iα	Const	4.6	R6K	X	Const	0
ColI	Iα	Repr	1.3	P1	Y	—	0
ColI*drd*1	Iα	Const	47.7				

[a] Most plasmids were kindly provided by N. Datta. They were resident in J53 except for the IncIα plasmids, which were in MSI. The TP114 plasmids were from H. R. Smith, and R831b was from D. E. Taylor.

[b] Incompatibility group designations were according to Datta (10; personal communication that IncE = IncC and that IncI2 probably = incIδ). R831b has been reclassified as IncM by Bradley et al. (9).

[c] Information on pilus synthesis (Repr = repressed, Const = constitutive) is taken from Bradley et al. (9) and personal communication.

[d] Primase activity was measured by using crude cell extracts (CCE) of plasmid-containing strains as described in the text. 0, No detectable activity.

[e] This plasmid may have become spontaneously repressed for pilus synthesis (David Bradley, personal communication).

[f] —, No pilus synthesis.

min), primase activity and protein concentration were measured as described previously (14). The primase assay is based on the ability of crude cell extracts from plasmid-harboring cells to restore phage fd DNA complementary-strand synthesis in an extract of the *dnaB dnaC* mutant BC1304 in the presence of rifampin. One unit of primase converts 1 μmol of dTMP to an acid-insoluble product in 30 min at 30°C.

SDS-PAGE of crude cell extracts. Cell extracts were prepared from 1 ml of stationary-phase bacteria at an absorbance at 450 nm of 1. Cells were harvested by centrifugation, washed with 1 ml of 20 mM Tris-hydrochloride (pH 6.8) containing 50 mM NaCl, and lysed in 30 μl of cracking buffer (10% [wt/vol] glycerol, 100 mM Tris-hydrochloride [pH 6.8], 5% [wt/vol] sodium dodecyl sulfate (SDS), 1 M β-mercaptoethanol). The suspension was incubated 5 min at 60°C followed by 5 min at 100°C. The lysate was centrifuged (80,000 × *g*, 30 min) at 25°C and totally applied to the slot of the SDS-polyacrylamide gel (13).

Agarose gel electrophoresis of φX174 DNA (RF II) synthesized in vitro. Complementary-strand synthesis on φX174 *am*3 DNA in a primase assay mixture (50 μl) was terminated by the addition of 100 μl of 80% (wt/wt) phenol (in 0.1 M Tris base), and the reaction mixture was extracted. DNA was precipitated from the aqueous phase in the presence of 50 mM sodium acetate with 0.4 ml of 96% ethanol at −70°C for 10 min. The pellet was recovered by centrifugation, washed once with 70% ethanol at 0°C, dried in a desiccator under vacuum for 5 min, and dissolved in 20 μl of 10 mM Tris-hydrochloride (pH 7.6) containing 0.1 mM EDTA. A sample was digested with the restriction enzymes *Pst*I or *Hpa*I, as described by Godson and Roberts (11), and electrophoresed on vertical 1.5% agarose (Bio-Rad) slab gels (0.4 by 15 by 22 cm) after addition of 6 μl of sample buffer (20% [wt/vol] Ficoll, 1% SDS, 0.1% bromophenol blue in 40 mM Tris-hydrochloride [pH 7.9], 5 mM sodium acetate, and 1 mM EDTA). Agarose gels were formed and run in 40 mM Tris-acetic acid (pH 7.9) containing 5 mM sodium acetate for 6 h at 4.5 V/cm.

Purification of RP4 DNA primase. Bacteria of strain J53(pRP92) were grown at 37°C in LB medium (17) supplemented with tetracycline. Fermentation was performed at a constant pH of 7.5 in a 100-liter

Bioengineering fermentor to 5×10^8 cells per ml. The cells were harvested at 25°C (317 g of wet cell paste), suspended in 1 liter of 50 mM Tris-hydrochloride (pH 7.6) containing 100 mM NaCl, and centrifuged. After suspension to a total volume of 1 liter with 5% (wt/vol) sucrose, 50 mM Tris-hydrochloride (pH 7.6), 10 mM spermidine-3HCl, and 1 mM EDTA, the cell suspension was quickly frozen with liquid nitrogen and stored at −20°C. All further operations were carried out at about 2°C. After thawing, the bacteria were lysed with lysozyme (0.04%) and Brij 58 (0.25%) for 1 h. The highly viscous lysate was centrifuged for 90 min at 70,000 × *g* at 2°C. An 820-ml amount of supernatant (fraction I) was recovered, diluted with 10% glycerol-20 mM Tris-hydrochloride (pH 7.6)-1 mM dithiothreitol to an ionic strength equal to 50 mM NaCl, and directly applied to a heparin-Sepharose CL-6B column (5.3 cm^2 by 24 cm) equilibrated with 10% glycerol-20 mM Tris-hydrochloride (pH 7.6)-50 mM NaCl-1 mM dithiothreitol-0.1 mM EDTA (buffer A). The column was washed with 500 ml of the same buffer before elution of the proteins with a 2-liter linear gradient from 0.05 M to 0.8 M NaCl in buffer A. The primase activity eluted in a single peak at a sodium chloride concentration of 450 mM. Fractions containing enzyme activity were pooled (fraction II, 290 ml) and directly applied to a hydroxyapatite column (5.3 cm^2 by 6 cm) equilibrated with 10% glycerol-20 mM potassium phosphate (pH 6.8)-0.1 mM EDTA (buffer B). The column was washed with 100 ml of buffer B. Proteins were eluted with a 440-ml linear gradient, 20 to 300 mM potassium phosphate (pH 6.8). The fractions containing the bulk of the enzyme activity which eluted at ~150 mM potassium phosphate were pooled (fraction IV, 110 ml), diluted with 330 ml of 10% glycerol-1 mM dithiothreitol-0.1 mM EDTA, and directly applied to a phosphocellulose column (2 cm^2 by 12 cm) equilibrated with 10% glycerol-50 mM Tris·H_3PO_4 (pH 7.0)-1 mM dithiothreitol-0.1 mM EDTA (buffer C). After the column was washed with 100 ml of buffer C, proteins were eluted with a 450-ml linear gradient, 0 to 0.6 M NaCl in buffer C. Primase activity eluted at 200 mM NaCl, and fractions containing activity were pooled and dialyzed against buffer A with two 250-ml changes (fraction VI, 42 ml). Fraction VI was applied to a DEAE-Sephacel column (2 cm^2 by 10 cm) equilibrated with buffer A. The column was washed with 50 ml of buffer A, and the enzyme was eluted at 200 mM with a 300-ml, 50 to 400 mM NaCl linear gradient in buffer A. Fractions containing primase activity were pooled, dialyzed against buffer A (pH 7.5) with two changes of 250 ml each (fraction V, 55 ml), and applied to a second DEAE-Sephacel column (0.64 cm^2 by 8 cm) which was equilibrated with buffer A (pH 6.5). Primase activity was eluted with a 100-ml, 50 to 400 mM linear NaCl gradient. The enzyme eluted at about 200 mM NaCl. Concentration was achieved by dialysis against buffer A containing 50% (wt/vol) glycerol (fraction VI, 4.3 ml). Fraction VI has been stored at −20°C and remained stable for more than 6 months.

Glycerol gradient centrifugation. A 35-μl sample of fraction VI (25 μg of protein) was layered onto a 3.7-ml linear 18 to 43% (wt/vol) glycerol gradient in buffer A at a concentration of 200 mM NaCl. Sedimentation was at 60,000 rpm for 23.5 h in a Beckman SW60 Ti rotor at 2°C. Fractions of 8 drops (177 μl) were collected from the bottom of the tube and assayed for primase. Reference proteins—alcohol dehydrogenase from yeast (M_r = 141,000), *E. coli* alkaline phosphatase (M_r = 80,000), ovalbumin (M_r = 43,000), and lysozyme (M_r = 13,970)—were run in a parallel tube.

RESULTS

Survey of plasmid-coded primase activity. We made cell-free extracts from strains of *E. coli* carrying plasmids representative of most of the described incompatibility groups (10). These were then tested for DNA primase activity as described above. The results in Table 2 show that primase is specified by few plasmid groups, i.e., IncB, IncIα (as already published [14]), IncIγ (as expected from the *dnaG* suppression data [19]), IncI2, IncK, IncM, and IncP. Among the IncI groups it is noteworthy that derepression of plasmid transfer functions was accompanied by an increase in primase activity of about 10- to 50-fold. It is important to note for what follows that Tn*7* did not specify primase activity (see R7K::Tn*7*, Table 2).

Deletion of the three transfer regions of RP4. The relatively well-understood IncP plasmid RP4 provided an attractive subject for further investigation of primase activity. Because of the close relationship between transfer functions and primase activity in the plasmids noted above, we first constructed deletion derivatives of RP4 that removed the three transfer regions that have been mapped so far (4, 7). These were made from *Hin*dIII restrictions of suitable Tn*7* derivatives of RP4 as described previously (4). The deletions used are shown in Fig. 1. We confirmed their structures from agarose gel electrophoresis analysis of intact and suitably restricted plasmid DNA samples (data not shown). Primase activity was measured in extracts of cells containing each of these plasmid derivatives. pTB72 and pTB75 specified primase activities of 12.9 and 12.0 mU/mg of protein, respectively, but both pTB73 and pRP261 gave undetectable levels. These results suggest that the primase gene is located on the RP4 genome in the region defined by the pRP261 deletion.

Primase specified by RP4::Tn*7* derivatives. We assayed primase in cell extracts from strains containing RP4 plasmids with Tn*7* insertions that have been mapped in the 36- to 50-kilobase (kb) region of RP4. The results shown in Fig. 2 suggest that the structural gene for primase may be located in the 37- to 42-kb region of the map. However, there was also a marked dip in primase activity from Tn*7* insertions in the 42.5- to 44-kb region. In contrast, some of

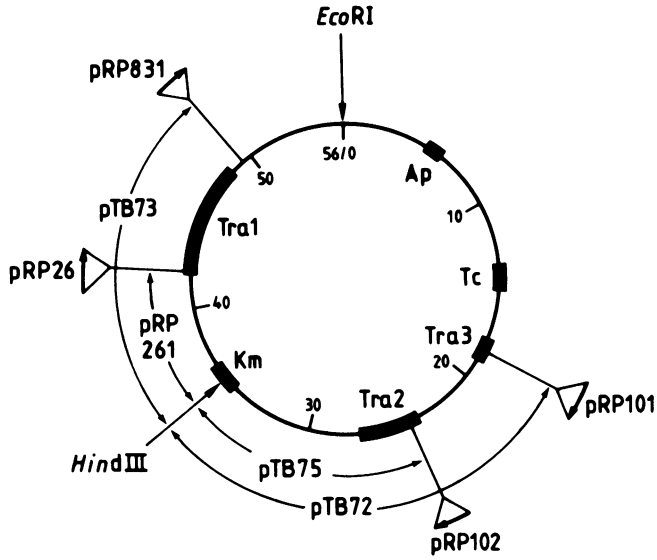

FIG. 1. *Map of RP4 deletions used for this work. The triangles represent Tn7 insertions into RP4 that have been previously mapped as shown (4, 7). The arrowheads on the Tn7 symbols represent the lefthand end of Tn7, i.e., close to the genes conferring trimethoprim and streptomycin resistance (7). The isolation of pRP261 was previously described (6). The other deletions were similarly isolated by HindIII excision as described in the text, and the structure of all four was confirmed. Map coordinates are in kilobase units from the single EcoRI site.*

the Tn7 insertions caused overproduction of primase. We designate the structural gene for the RP4-specified DNA primase as *pri*.

Purification of the RP4 primase. To characterize the RP4 DNA primase, it was purified as described above, with the aid of an in vitro assay (14). The results of a purification are summarized in Table 3. We used the advantage of a primase-overproducing strain [J53(pRP92); Fig. 2], which greatly aided in the purification of the protein to near homogeneity. Heparin-Sepharose chromatography was remarkably useful in a first step starting with a crude cell extract. In addition to the chromatographic absorbents used during the purification, RP4 primase also bound to single-stranded DNA-agarose and could be eluted from the column with 0.3 M NaCl (data not shown). Conventional column chromatography on hydroxyapatite, phosphocellulose and DEAE-Sephacel resulted in fraction VI, which was composed of three major polypeptides (Fig. 3, track a). The largest protein, with an apparent molecular weight of 118,000, cosedimented in a glycerol gradient with the enzyme activity as revealed by SDS-polyacrylamide gel electrophoresis (PAGE) of samples of the fractions (Fig. 3). The next smaller polypeptide (80 kilodaltons [kdal]) may also have had priming activity (Fig. 3), but the smallest appeared to have none. The primase sedimented with 5.5S, which led us to the conclusion that the enzyme has an anisometric shape and functions as a monomer.

The RP4 primase was readily detectable as a stained band after SDS-PAGE of crude cell extracts. Extracts of cells of primase-overproducing strain J53(pRP26) and the strain harboring wild-type plasmid J53(RP4) both contained a band corresponding to a molecular weight of 118,000 (Fig. 4). In the case of strain J53(pRP1), which did not produce detectable amounts of primase, the latter band was absent. From a series of similar gels, we analyzed all the RP4::Tn7 plasmid-containing strains used in this study for their production of the 118-kdal protein band. The results in Table 4 show that there was a close correlation between the primase activity specified by each plasmid (Fig. 2) and the presence or absence of the 118-kdal protein in crude cell extracts. Scanning of the stained protein bands in Fig. 4 with a microdensitometer was used to estimate the amount of primase relative to the two subunits of *E. coli* RNA polymerase $\beta' + \beta$. *E. coli* RNA polymerase subunits $\beta' + \beta$ were about 1% of the total soluble protein of the cell as revealed by the scanning procedure. Strain J53(pRP26) contained primase and $\beta' + \beta$ in equal amounts, which means that about 1% of the soluble protein was primase, whereas for strain J53(RP4) the value for primase was 2.5-fold lower.

RP4 DNA primase initiation of comple-

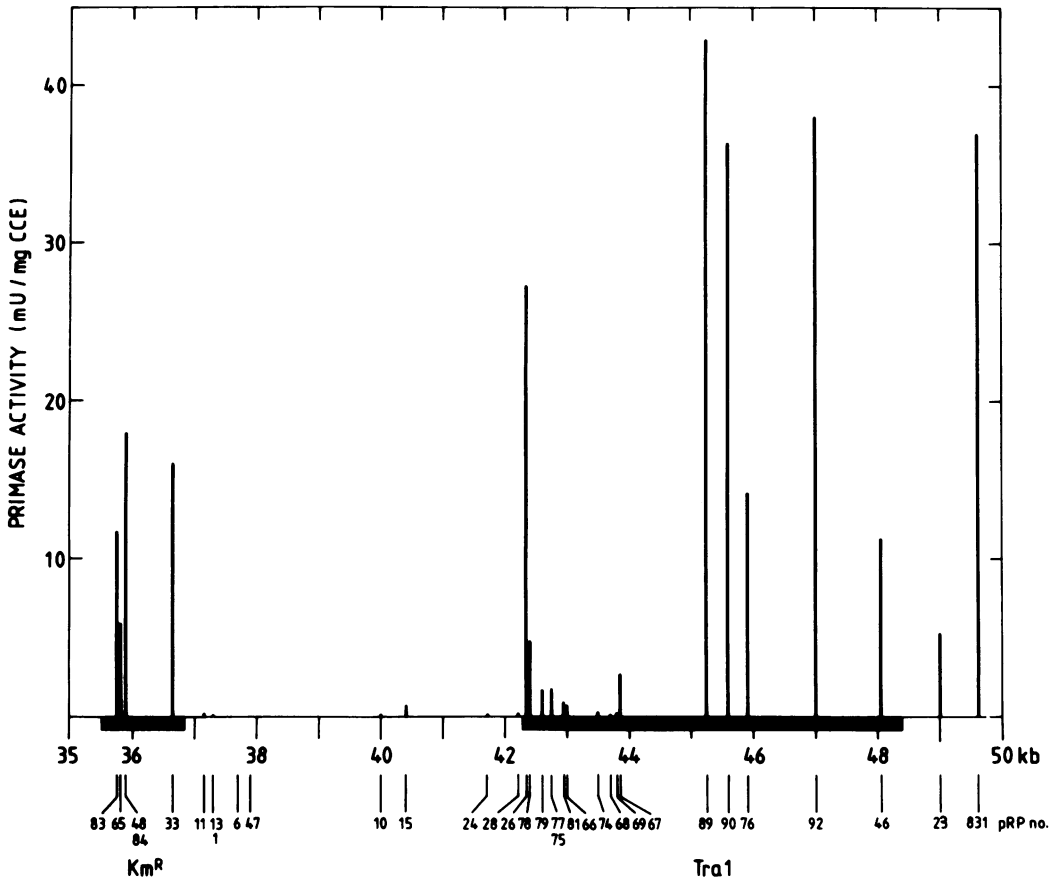

FIG. 2. *Map of Tn7 insertions in the 36- to 50-kb region of RP4. The level of primase activity specified by each plasmid derivative (in J53) is expressed by a line. The kanamycin resistance and Tra1 regions previously defined are marked as blocks. RP4 gave a primase activity of 10.3 mU/mg of crude cell extracts (CCE) (Table 2).*

TABLE 3. *Purification of RP4 DNA primase*

Fraction	Activity (total U)	Protein (total mg)	Sp act (U/mg)	% Recovery
I. Crude cell extract	375	13,960	0.027	100
II. Heparin-Sepharose CL-6B	132	257	0.51	35
III. Hydroxyapatite	108	48	2.25	29
IV. Phosphocellulose	77	9.2	8.4	21
V. DEAE-Sephacel I	41	3.5	11.7	11
VI. DEAE-Sephacel II	43	3.3	13.0	12

mentary-strand synthesis on φX174 DNA in *E. coli dnaG* mutant extracts. *E. coli* proteins precipitated by 43% saturated ammonium sulfate from crude cell extracts (ammonium sulfate fraction) contained a sufficient level of replicative proteins to mediate (-) strand synthesis on single-stranded phage DNA. Conversion of φX174 single-stranded to duplex DNA was rifampin resistant and required, in addition to other proteins, the *dnaG* gene product (20, 26). DNA synthesis in a *dnaG* mutant extract was restored after addition of purified *dnaG* protein isolated from *dnaG*$^+$ cells. Addition of purified RP4 or R64*drd*11 DNA primase instead of *dnaG* protein had the same effect (Table 5). The priming reaction on circular φX174 DNA with the *E. coli* primase is dependent on, among other proteins, the presence of the *dnaB* gene product (30). Plasmid DNA primases encoded by plasmids RP4 and R64*drd*11 initiated (-) strand synthesis on φX174 DNA in the absence of an active *dnaB* protein (Table 5) or *dnaC* protein (data not shown). These reactions required ribonucleotide triphosphates and were resistant to rifampin. The necessity of ribonucleotide triphosphates reflects their need for RNA primer synthesis. The purified enzyme of RP4 catalyzed the synthesis of short oligoribonucleotides only on single-stranded DNA templates (to be published elsewhere).

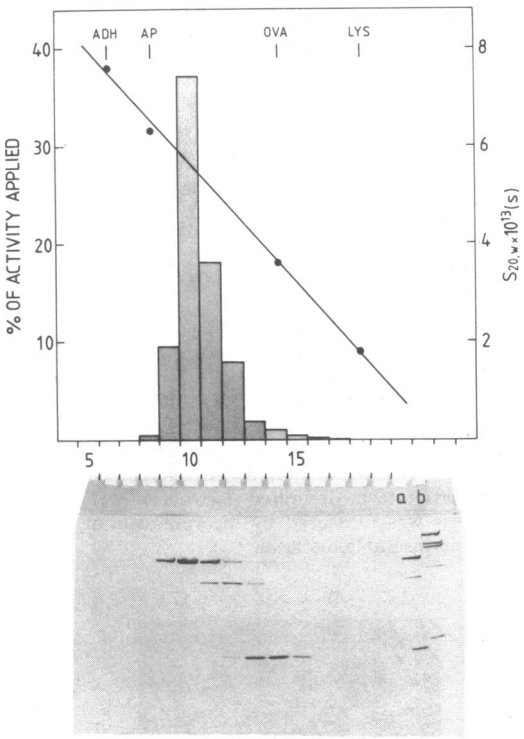

FIG. 3. *Glycerol gradient centrifugation of RP4 DNA primase. Conditions are described in the text. Sedimentation was from right to left. Fractions were assayed for primase, and 90-μl samples were subjected to SDS-PAGE (13). Track a, material applied to the glycerol gradient (7.7 μg of protein, fraction VI); track b, molecular weight standards myosin (212,000), E. coli RNA polymerase β' (165,000), β (155,000), σ (89,000), and α (39,000). ADH, Alcohol dehydrogenase; AP, alkaline phosphatase; OVA, ovalbumin; LYS, lysozyme.*

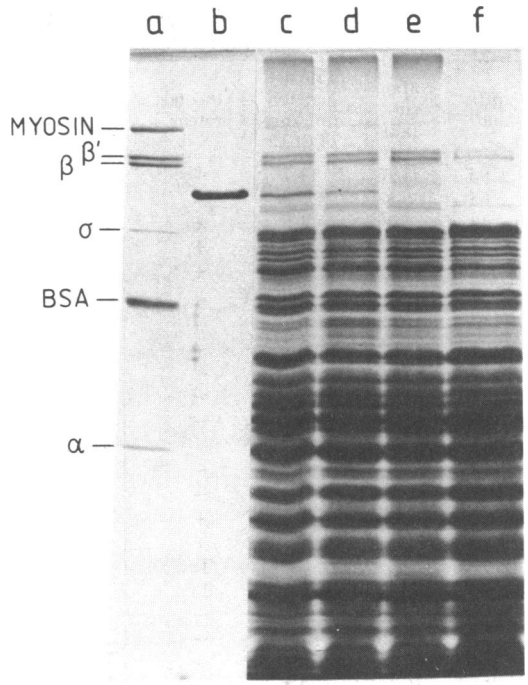

FIG. 4. *SDS-PAGE of purified RP4 DNA primase and of crude cell extracts. Track a, molecular weight standards—1 μg of myosin (212,000); 3 μg of E. coli RNA polymerase β' (165,000), β (155,000), σ (89,000), and α (39,000); and 1 μg of bovine serum albumin (BSA; 68,000). Track b, 40 μl of glycerol gradient fraction no. 10 (Fig. 3). Tracks c, d, e, and f, crude cell extracts of J53(pRP26), J53(RP4), J53(pRP1), and J53, respectively.*

The major product of DNA synthesis on circular φX174 DNA in initiation-defective extracts supplemented with RP4 primase was replicative form II (RF II), and there were traces of supercoiled DNA (RF I), as revealed by agarose gel electrophoresis and restriction enzyme analysis (Fig. 5).

Immunological unrelatedness of RP4 DNA primase to the *E. coli dnaG* protein and IncIα DNA primase. The primases specified by the plasmids RP4 and R64*drd*11 behave enzymatically rather similarly with regard to their ability to initiate (-) strand synthesis on a variety of different single-stranded phage DNAs, i.e., φX174, G4, fd, or If1, without the aid of accessory replication proteins like the *dnaB* or *dnaC* gene product, which are components of the primosome (1). The enzymatic similarity between the two plasmid primases raised the question of whether they are antigenically related to each other. We could not detect an immunological relationship between RP4, R64*drd*11, and *E. coli* primase with an IncIα primase-specific antiserum (28) and antiserum directed against *dnaG* protein. Anti-*dnaG* did not significantly affect the plasmid-mediated process, nor did the anti-IncIα primase show significant inhibition of DNA synthesis dependent on RP4 primase or *dnaG* protein (Table 6). Immunological cross-reaction could also not be demonstrated between the three primases and the appropriate antisera by the method of Towbin et al. (22), which involves electrophoretic protein transfer from SDS-polyacrylamide gels to nitrocellulose sheets and their subsequent immunochemical reactions (data not shown).

Biological role of the RP4-coded primase. (i) Conjugation. The observation that in plasmids which specify primase activity, derepression of their transfer functions led to a significant increase in this activity, (8, 19, 27; Table 2) suggests that primase may play a role in plasmid conjugation. However, in our analysis of RP4 with Tn*7* insertions (7), we found no effect on

TABLE 4. *Detection of the 118-kdal protein specified by RP4::Tn7 plasmids*

pRP no[a]	Map site[b] (kb)	Primase activity[c] (mU/mg of CCE)	118-kdal protein[d]	Transfer frequency[e]
83	35.75	11.7	+	Normal
65	35.8	5.9	+	
48	35.9	11.8	+	
84	35.9	18.0	+	
33	36.65	16.0	+	
11	37.15	0.2	−	Normal
13	37.3	0.1	−	
1	37.3	0	−	
6	37.7	0	−	
47	37.9	0	−	
10	40.0	0.2	−	
15	40.4	0.6	−	
24	41.7	0.1	−	
28	42.2	0.2	−	
26	42.35	27.3	+	10^{-5}
78	42.4	4.8	+	$<10^{-9}$
79	42.6	1.7	Tr	
77	42.75	1.8	Tr	
75	42.75	0.3	−	
81	42.95	1.0	−	
66	43.0	0.8	−	
74	43.5	0.3	Tr	
68	43.7	0.1	−	
69	43.8	0.2	−	
67	43.85	2.7	Tr	
89	45.25	56.0	+	10^{-5}
90	45.6	36.4	+	10^{-6}
76	45.9	14.1	+	10^{-6}
92	47.0	38.1	+	10^{-4}
46	48.05	11.3	+	10^{-4}
23	49.0	5.3	+	Normal
831	49.6	36.8	+	

[a] The pRP plasmids (RP4::Tn7 derivatives) were resident in J53 and were previously described (7).

[b] The Tn7 insertion map sites were taken from the previous data (7) and recalculated from megadaltons into kilobase units, taking into account that the previous estimate of RP4 as 36 megadaltons is now generally agreed to be closer to 56 kb.

[c] Primase activity was measured as before (Table 2). CCE, Crude cell extracts.

[d] The 118-kdal protein was detected by SDS-PAGE of crude cells extracts (+, presence; −, absence). One such gel is shown in Fig. 4.

[e] The transfer frequency is expressed as transconjugants per donor per hour in *E. coli* to *E. coli* crosses taken from our previous data (7) where "normal" had a value of about 10^{-3}.

conjugation by those insertions that we have now identified as probably being in the structural gene for primase (Fig. 2). These conjugation tests were, however, only done with strains of *E. coli*. As primase may have a role in the broad-host-range conjugal ability of RP4, we extended the tests to other bacterial species.

We used the solid-medium mating technique described above to transfer a number of *pri*$^+$ and *pri* RP4::Tn7 derivatives from *E. coli* W3110 T$^-$ into *E. coli* J53 and the 10 other

TABLE 5. *φX174 DNA complementary-strand synthesis in E. coli dna mutant extracts[a] in the presence of different DNA primases*

DNA primase added	pmol of dTMP incorporated: *dnaG* extract	pmol of dTMP incorporated: *dnaB* extract
None	7.2	2.1
RP4	57.3	52.1
R64*drd*11	86.0	87.9
E. coli dnaG protein	69.1	0.7

[a] Ammonium sulfate fractions of cell extracts of strains BT1011 and HfrH252 were prepared as described (21). The assay mixtures (50 μl) contained 132 and 120 μg of protein from BT1011 and HfrH252, respectively. Purified primases were added: 77 ng of RP4 enzyme (fraction VI), 0.7 ng of R64*drd*11 enzyme (fraction VII, reference 14), and 78 ng of *dnaG* protein (purification to be described elsewhere). φX174 DNA synthesis was measured by incorporation of dTMP into acid-insoluble material.

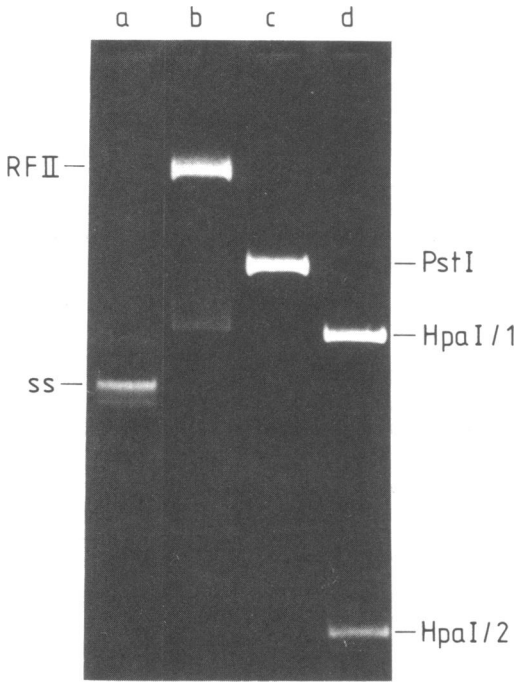

FIG. 5. *Product analysis by agarose gel electrophoresis of φX174 DNA synthesized in a BC1304 mutant extract in the presence of RP4 DNA primase. Experimental conditions were as described in the text. Track a, 0.15 μg of φX174 DNA; track b, DNA synthesized in the presence of RP4 primase and isolated by phenol extraction. Digestion of a sample of the material applied to slot b with restriction endonuclease PstI (c) and HpaI (d).*

bacterial species described in Table 1. The results in Table 7 show that for 5 of the 11 species used as recipients, there was a correlation between the loss of primase activity and a significant reduction in plasmid transfer frequency. For *Klebsiella aerogenes*, *Providencia* sp., and *Serratia marcescens*, this reduction was only about 5-fold, whereas for *P. mirabilis* and *S. typhimurium* it was about 25-fold.

The transconjugants of these five species were purified and then tested for the plasmid-conferred drug resistance phenotypes with Multodiscs. Both the *pri*$^+$ and *pri* RP4::Tn*7* transconjugants had all the expected resistances (ampicillin, tetracycline, kanamycin, and not just trimethoprim, and streptomycin) the selected one. They all appear therefore to have received the appropriate donor RP4::Tn*7* plasmid. Thus, the lack of primase reduced, but did not abolish, plasmid transfer into these five strains.

TABLE 6. *Effect of antisera directed against DNA primases on φX174 DNA complementary-strand synthesis in a dnaG extract*[a]

Serum added	pmol of dTMP incorporated when synthesis mediated by:		
	RP4 protein	R64*drd*11 protein	*dnaG* protein
Preimmune	45.3	70.4	60.7
Anti-IncIα	41.3	<1	60.4
Anti-*dnaG*	39.6	59.4	11.2

[a] Conditions were as described in Table 5, footnote *a*, using the ammonium sulfate fraction of strain BT1011, except that the proteins were incubated 5 min at 30°C with the appropriate serum as indicated. Enzymatic reactions were then started by the addition of the substrates to complete the assay mixture.

We next measured the transfer frequency of the *pri*$^+$ and *pri* RP4::Tn*7* plasmids from *S. typhimurium* back into *E. coli* and also into some of the other bacterial species. We checked that RP4 expressed primase activity in *S. typhimurium*. It also did so in *P. aeruginosa*. The results in Table 8 show that the primase-correlated effect on conjugation frequency depended only on the recipient and not on the donor species. Thus *E. coli* and *M. methylotrophus* showed no effect, but the other species showed a correlation with plasmid primase level approximately as seen in Table 7 for the *E. coli* donor.

(ii) Mobilization. R300B is a broad-host-range, nonconjugative plasmid that did not specify primase activity (Table 2) and is efficiently mobilized by RP4 (4). We tested whether this mobilization was affected by the RP4-coded primase. RP4 and each of the RP4::Tn*7* derivatives listed in Table 7 were transferred into W3110 T$^-$ (R300B). The double-plasmid strains were purified, checked for their resistance phenotypes, and then plate-mated with each of the strains listed in Table 7, using streptomycin or sulfonamide selection. In the majority of these matings, there was no discernible difference between the mobilization efficiency of the *pri*$^+$ and *pri* RP4 derivatives (data not shown). However, it can be seen in Table 9 that the recipients *P. mirabilis* and *S. typhimurium* did show a correlation between mobilization frequency and the RP4-coded primase level. It should be noted that the mobilizations given by *pri* derivatives pRP1, -11, and -13 into *P. mirabilis* and by pRP28 into *S. typhimurium* were scarcely reduced below the *pri*$^+$ plasmid controls. It may be significant that these mapped at the extremities of the region that we suggest contains the

TABLE 7. *Relative transfer frequency of RP4 pri*$^+$ *and pri derivatives from E. coli*

Recipient strain	Drug[b]	Relative transfer frequency[a] from donor strain W3110 T$^-$ of plasmid (pRP no.):													
		1 (−)	2 (+)	3 (+)	6 (−)	10 (−)	11 (−)	13 (−)	14 (+)	15 (−)	23 (+)	24 (−)	27 (+)	28 (−)	RP4 (+)
A. tumefaciens	Km	1	1	1	1	1	1	1	1	1	1	1	1	1	1
A. eutrophus	Tc	1	1	1	1	1	1	1	1	1	1	1	1	1	1
E. coli J53	Km	1	1	1	1	1	1	1	1	1	1	1	1	1	1
K. aerogenes	Km	0.03	0.7	0.9	0.2	0.1	0.1	0.25	1.3	0.3	1.6	0.25	1.0	0.2	1.0
M. methylotrophus	Km	1.0	1.0	1.0	1.0	0.75	1.0	1.0	1.0	0.5	2.0	1.0	1.0	1.0	1.0
P. mirabilis	Km	0.04	1.0	1.0	0.05	0.06	0.04	0.05	1.0	0.02	1.0	0.03	1.0	0.01	1.0
Providencia sp.	Tp	0.2	1.0	1.0	0.2	0.2	0.2	0.2	1.0	0.4	1.0	0.15	1.0	0.1	0
P. aeruginosa	Km	1	1	1	1	1	1	1	1	1	1	1	1	1	1
P. putida	Km	1	1	1	1	1	1	1	1	1	1	1	1	1	1
S. typhimurium[c]	Km	0.03	1.0	1.0	0.04	0.05	0.05	0.04	1.0	0.07	1.0	0.03	1.0	0.03	1.0
S. marcescens	Tp	0.04	0.7	0.8	0.14	0.3	0.08	0.2	1.2	0.3	1.0	0.15	1.0	0.1	0

[a] Transfer frequencies are expressed relative to that given by RP4, except when trimethoprim was the selective drug (RP4 is Tp^s, Tn*7* confers trimethoprim resistance), then pRP27 was used as the yardstick instead. Matings were repeated several times on solid media as described in the text; the averages are presented in the table. + and −, Presence and absence of DNA primase activity.

[b] Matings were done using various levels of the selective drugs. Ampicillin was always found to be unsatisfactory, presumably because of secreted β-lactamase. The most satisfactory selection data are presented. For *K. aerogenes* and *P. mirabilis*, the kanamycin level was 10 μg/ml. The other levels are as given in the text.

[c] Results for both strains of *S. typhimurium* given in Table 1 were essentially the same.

TABLE 8. *Relative transfer frequency of pri⁺ and pri RP4 plasmids from S. typhimurium LT2*

Recipient strain	Drug	Relative transfer frequency[a] from donor strain *S. typhimurium* LT2 of plasmid (pRP no.):													
		1 (−)	2 (+)	3 (+)	6 (−)	10 (−)	11 (−)	13 (−)	14 (+)	15 (−)	23 (+)	24 (−)	27 (+)	28 (−)	RP4 (−)
E. coli J53	Km	1	1	1	1	1	1	1	1	1	1	1	1	1	1
K. aerogenes	Km	0.04	1	1	0.03	0.06	0.03	0.05	1	0.07	1	0.04	1	0.15	1
M. methylotrophus	Km	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Providencia sp.	Tp	0.03	1	1	0.05	0.04	0.03	0.03	1	0.15	1	0.1	1	0.08	0
S. typhimurium 3692	Km	0.05	1	1	0.05	0.05	0.02	0.05	1	0.1	1	0.1	1	0.1	1

[a] Conditions were as described in Table 7, footnote *a*.

TABLE 9. *Relative mobilization frequency of R300B by the RP4 pri⁺ and pri derivatives*

Recipient strain	Drug	Relative mobilization frequency[a] from donor strain W3110 T⁻ of plasmid (pRP no./R300B):													
		1 (−)	2 (+)	3 (+)	6 (−)	10 (−)	11 (−)	13 (−)	14 (+)	15 (−)	23 (+)	24 (−)	27 (+)	28 (−)	RP4 (+)
P. mirabilis	Sm	0.75	1.0	1.0	0.08	0.05	0.4	0.4	0.7	0.05	1.0	0.07	1.0	0.08	1.0
S. typhimurium	Sm	0.2	0.6	1.0	0.2	0.15	0.3	0.35	1.0	0.1	1.0	0.1	1.0	0.6	1.0

[a] R300B was mobilized into all 11 recipients listed in Table 7 as described in the text, but only for the two species shown was there any RP4 *pri*-correlated effect. (No data were obtained for *A. tumefaciens* because the selections were inadequate.) The high (50 μg/ml) streptomycin selection was used to differentiate between the high streptomycin resistance conferred by R300B and the much lower level conferred by Tn7. The sulfathiazole selections gave similar results to those shown in the table. + and −, Presence and absence of DNA primase activity.

primase structural gene (Fig. 2).

(iii) Plasmid stability. We tested whether the RP4-coded primase had any influence on plasmid stability in *E. coli* or *S. typhimurium*. After 20 generations of unselected growth in broth, both species containing each of the five *pri* plasmids suffered a small percentage of loss of plasmid, whereas none of the *pri⁺* plasmids was lost (Table 10). Thus the primase also seems to be involved in RP4 maintenance.

Suppression of *dnaG*. We discussed above the evidence that the suppression of the *dnaG* mutation in *E. coli* by IncIα or Iγ *drd* plasmids is due to their specifying a DNA primase. We therefore tested whether RP4 and a number of the RP4::Tn7 plasmids could suppress the thermosensitivity of NY731 or BW86 (Table 1). We made transconjugants of these strains at 30°C and then looked for colony-forming ability at 40°C. None of the plasmids tested was able to suppress the *dnaG*(Ts) mutation.

TABLE 10. *Stability of RP4::Tn7 pri⁺ and pri derivatives*

Plasmid pRP no.	DNA primase activity	% Loss of plasmid[a] with host bacterium:	
		E. coli	*S. typhimurium*
1	−	4	1
2	+	0	0
3	+	0	0
6	−	3	3
10	−	3	2
14	+	0	0
15	−	2	1
23	+	0	0
28	−	6	4
RP4	+	0	0

[a] Plasmid loss from *E. coli* J53 or *S. typhimurium* LT2 was measured after 20 generations of unselected growth as described in the text.

DISCUSSION

We discovered that RP4 specifies a DNA primase and that this activity is found only rarely among the various plasmid incompatibility groups that have been described. Using in vitro-generated deletions and Tn7 insertion mutations, we located the probable structural gene for this enzyme in the 37- to 42-kb region of RP4. The primase was purified to near homogeneity (Fig. 4) and shown to consist of a single polypeptide of 118 kdal. Using antiserum raised against this polypeptide, we identified a second cross-reacting polypeptide of about 80 kdal (to be published elsewhere). This may be a processed product of the larger protein or may be generated by an in-phase second translational start within the *pri* gene. A similar situation has been found to obtain for the primase specified by the plasmid Col*Ird*1 (28), although the polypeptide sizes are different from the above.

The conversion of single-stranded circular phage DNA to the duplex forms in vitro requires,

in the presence of RP4 DNA primase, a DNA polymerase III fraction (14) and single-stranded DNA binding protein. The *E. coli* primase action, however, on φX174 DNA is strictly dependent on accessory replication proteins organized in the primosome (1). The RP4 DNA primase is enzymatically similar to the primases encoded by IncIα plasmids (14, 28) with regard to the rifampin-resistant priming reaction, its dependence on ribonucleotide triphosphates, and its ability to initiate complementary-strand synthesis on all single-stranded circular phage DNAs which have been tested so far. I-type DNA primases and the RP4 enzyme, however, are antigenically unrelated. The wide host range of RP4 suggests that the RP4 DNA primase must be able to cooperate with a variety of different DNA elongation systems within the spectrum of gram-negative bacteria.

There is a discrepancy between the coding requirements of a protein of 118 kdal (about 3.5 kb) and the region of RP4 that appears to code for it (about 5 kb). This may be resolved by the presence of a positive control region in the latter. Certainly, the variations in primase activity given by Tn7 insertions throughout the Tra1 region (Fig. 2) suggest that there are complex controls on the expression of the *pri* gene. These observations, together with the *pri* gene mapping site and our finding that primase plays a role in the conjugation of RP4 with some bacterial species (Tables 7 and 8), suggest that the *pri* gene is functionally part of the Tra1 region. This is represented in Fig. 6 which summarizes the genes on RP4 involved in conjugation.

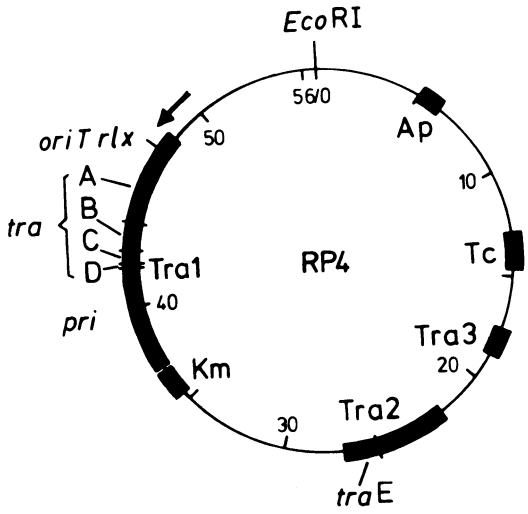

FIG. 6. *Map of RP4 transfer genes. This presents a summary of the regions of RP4 involved in conjugation: the five tra complementation groups (7), named traA through -E; the extension to Tra2 and Tra3 involved in surface exclusion (4); and the site of the transfer origin (oriT) and relaxation site (rlx) (12). The orientation of transfer has been determined (N. J. Grinter, Plasmid, in press; and P. T. Barth, unpublished data). The pri gene has been put into the context of the Tra1 region as discussed in the text.*

The RP4 DNA primase represents the first function discovered on a broad-host-range plasmid that is responsible in part for its promiscuity. It does this in two ways, first, by increasing the efficiency of conjugal transfer to some bacterial species and second, by being necessary for plasmid stability in at least two species (Table 10). (The effect on conjugal transfer cannot be explained in terms of the stability effect alone because [i] the instability is only a few percent over 20 generations of growth and [ii] *pri* mutants are unstable in *E. coli* but show no measurable effect on conjugal transfer to this species.)

With respect to conjugal transfer, Vapnek and co-workers (23, 24) have shown that IncFI, FII, and Iα plasmids transfer only a single specific strand to the recipient. Assuming that this is also true for RP4 transfer, we conclude that the RP4 primase is involved in the conversion of this single strand to double-stranded DNA. It follows that for recipient bacteria which happen to produce a DNA primase that does not efficiently recognize the priming sites on RP4, the RP4-coded primase will have the greatest effect on the establishment of transconjugants (such as *P. mirabilis* and *S. typhimurium*, Tables 7 and 8). The recipient-specific effect of the RP4 primase is very intriguing and shows, in at least the majority of matings, that the transferred strand cannot be primed in the donor. This means that the RP4 primase is either expressed in the recipient from the transferred single DNA strand (for which there is no known mechanism) or that the primase molecules are transferred from donor to recipient during conjugation, perhaps by becoming attached to priming sites on the transferred single strand of plasmid DNA. In none of the strains studied did we find that the RP4 *pri* mutation completely blocked transconjugant formation. Therefore, in such crosses a minority of the transferred RP4 strands must be primed either before transfer by the donor's primase or after transfer by the recipient's primase.

With respect to plasmid mobilization, our results in Table 9 show that R300B mobilization was also affected by the *pri* gene of RP4 (although to a less marked degree than RP4 self-transfer). As R300B did not code for a DNA primase (Table 2), this is perhaps the expected result. However, Nordheim et al. (18) have recently proposed a model for the conjugal transfer

of several plasmids (including RSF1010, which is indistinguishable from R300B [4]) whereby the recipient cell receives a single plasmid DNA strand which has been primed at its proximal end by a short stretch of complementary DNA. This mechanism would make the transfer of such a plasmid independent of the requirement for a plasmid- or recipient-specified DNA primase. Our results clearly do not support this model although a minority of transferred R300B DNA strands may have this proposed structure.

Our observation that *pri* RP4 mutants are also somewhat unstable (Table 10) presumably means that the RP4 primase is involved in priming not only a single-stranded plasmid DNA form transferred during conjugation, but also the double-stranded form during normal Okazaki discontinuous plasmid DNA synthesis. This surprising finding suggests that the chromosomal priming system cannot altogether substitute for the plasmid one. Coupled with the observation that RP4 (and R68·45 [16]) cannot complement the *dnaG* mutation, this implies a considerable functional distinction between the chromosomal and plasmid priming systems. However, it is possible to isolate mutants of RP4 (like those reported for R68·45 [16]) that are capable of suppressing the *dnaG* mutation. We are at present investigating what change has taken place in such mutants.

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