Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 654–658, February 1984
Biochemistry

Replication of the broad host range plasmid RSF1010: Requirement for three plasmid-encoded proteins

(plasmid replication/origin/*rep* gene/replication factor)

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Communicated by Bruce N. Ames, September 9, 1983

ABSTRACT **Cloning of specific regions of plasmid RSF1010, in conjunction with *in vitro* replication studies, has revealed three novel genes: *repA*, *repB*, and *repC*. They are clustered in one region of the plasmid, separated from the origin of replication by regions that are not essential for plasmid viability in an *Escherichia coli* host. *In vivo*, a 2.1-kilobase segment of the plasmid, bearing the replication origin, can establish itself as an autonomous replicon if the DNA region carrying the three *rep* genes is present in the same cell on an independent plasmid. *In vitro*, RSF1010 DNA is efficiently replicated by an ammonium sulfate fraction from the *E. coli* extract, provided the extracts are prepared from cells that can supply the required *rep* gene products. Using cells containing the cloned *rep* gene region as a source of elevated levels of the rep proteins, we have partially purified these proteins in functional form. When added to an enzyme fraction derived from plasmid-free cells, they specifically promote the replication of plasmid DNA bearing the RSF1010 origin.**

Bacterial plasmids of the incompatibility group IncQ, represented by RSF1010 (1), R300B (2), and R1162 (3), are nonconjugative, multicopy replicons conferring resistance to streptomycin and sulfonamides. The most remarkable feature of this group of plasmids is their ability to replicate in a wide range of Gram-negative bacterial species. This property, shared only by certain members of the conjugative IncP group plasmids (4), has made them attractive for use as DNA cloning vehicles (5, 6) and has been the motivating factor in studies of their mode of replication.

For RSF1010 [8.9 kilobases (kb)], replication in *Escherichia coli* starts at a unique site and proceeds via θ-form intermediate structures either bi- or unidirectionally from this origin (7). Attempts at reducing the size of the RSF1010 replicon by *in vitro* manipulations have shown that genes essential for replication are located elsewhere on the plasmid (ref. 8; this work). Recently, two cell-free systems for the study of RSF1010 replication in *E. coli* and *Pseudomonas aeruginosa* have been developed (9). With these systems, it has also been shown that RSF1010 needs plasmid-encoded factors for its replication.

In the present study, we have used the technique of fractionation and reconstitution of cell extracts to resolve and identify the RSF1010-specified replication proteins. Chromatography on heparin-Sepharose of a protein fraction from plasmid-carrying *E. coli* resulted in the separation of three proteins that, when mixed together, complemented an extract of plasmid-free cells to permit the semiconservative replication of RSF1010 DNA. The genes specifying the three proteins have been identified and their positions on the physical map of RSF1010 have been determined.

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MATERIALS AND METHODS

Strains and Plasmids. The strains used were the *E. coli* K-12 derivatives SK1592 (10) and C600 (11). The plasmids used were RSF1010 (1), pKT228 (RSF1010::Tn*3*) (8), pHSG415 [pSC101 carrying the genes for chloramphenicol, kanamycin, and ampicillin resistance (Cm^r, Km^r, Ap^r)] (12), and pKT101 (a Km^r derivative of the multicopy plasmid ColD) (13). Transformation of *E. coli* with plasmid DNA was carried out by the method of Kushner (10). Drug resistance was tested with chloramphenicol at 50 μg/ml, kanamycin at 100 μg/ml, and streptomycin at 50 μg/ml.

Enzymes, DNA, and Biochemicals. T4 DNA ligase, BAL-31 nuclease, and restriction endonucleases were from New England BioLabs; ϕX174 replicative form DNA was from Bethesda Research Laboratories; nucleotides were from Boehringer Mannheim; [*methyl*-^{3}H]dTTP was from Amersham-Buchler (Braunschweig), and heparin-Sepharose Cl-6B was from Pharmacia.

DNA Procedures. The methods used for DNA purification, restriction enzyme digestion, and ligation were those described by Andres *et al.* (14). Plasmid deletions were generated by limited digestion of linear DNA with nuclease BAL-31, followed by blunt-end ligation of the product molecules (15). DNA·DNA heteroduplexes were formed and examined in the electron microscope as described (16).

Preparation of Extract. All steps were carried out at 0–4°C. Crude cell extract, prepared from exponentially growing *E. coli* as described (9), was adjusted to a final concentration of 2.7% streptomycin sulfate with a freshly prepared solution of 30% (wt/vol) streptomycin sulfate, stirred for 30 min, and centrifuged at 30,000 × *g* for 20 min. The supernatant was adjusted to 73% saturation with ammonium sulfate (0.47 g/ml), stirred for 30 min, and centrifuged as before. After removal of the supernatant, the precipitate was packed by recentrifugation for 10 min. The pellet obtained from 1 ml of crude extract was dissolved in 0.2 ml of 25 mM Hepes/KOH, pH 8.0/50 mM KCl/0.1 mM EDTA/1 mM dithiothreitol and dialyzed against the same buffer for 3.5 hr. The dialyzed material (fraction AS [0–73] or extract), containing 30–35 mg of protein/ml, was frozen and stored in liquid N_2.

Assay of DNA Synthesis. The standard reaction mixture (25 μl) contained Hepes/KOH (pH 8.0), 40 mM; KCl, 80 mM; $Mg(OAc)_2$, 11 mM; dithiothreitol, 800 μM; ATP, 2 mM; GTP, CTP, and UTP, each at 400 μM; dATP, dGTP, dCTP, and dTTP, each at 50 μM with [*methyl*-^{3}H]dTTP at 180 cpm/pmol of total deoxynucleotide; NAD, 50 μM; cAMP, 50 μM; creatine phosphate, 15 mM; creatine kinase, 100 μg/ml; and polyethylene glycol 6000, 2.5% (wt/vol). Supercoiled plasmid DNA and *E. coli* protein fractions were added to the assay mixture as indicated in the individual experiments. Components were assembled on ice, and reactions

Abbreviations: Cm^r, Km^r, and Su^r, chloramphenicol, kanamycin, and streptomycin resistance, respectively; kb, kilobase(s).

were initiated by incubation at 30°C. After 60 min, DNA synthesis was measured by determining incorporation of labeled deoxynucleotide into acid-insoluble material as described (17).

RESULTS

Identification of Essential RSF1010 Regions. The origin of vegetative replication (*ori*V), previously mapped by electron microscopy (7), is located on our physical map of RSF1010 in the area around coordinate 2.6 (Fig. 1). According to restriction analysis, this area should be contained within the stretch of DNA that is comprised of fragments *Hae* II [1.2–2.3], *Hae* II [2.3–2.4], and *Hae* II [2.4–3.3]. Attempts to generate from partial *Hae* II digests of RSF1010 DNA self-replicating miniplasmids repeatedly failed. This led us to consider the possibility that genes essential for replication or maintenance are located in regions of the plasmid remote from *ori*V. To identify these regions, we constructed hybrid plasmids carrying different segments of RSF1010 on an unrelated replicon and determined their ability to rescue *ori*V-containing *Hae* II fragments in the form of satellite plasmids. One such hybrid was constructed by inserting a 5.0-kb *Sst* I fragment from pKT228 (RSF1010 with transposon Tn*3* inserted at coordinate 4.2) into the single *Sst* I site of the ColD-based vector pKT101. The resulting plasmid, pMMB2, contains the RSF1010 region located between coordinates 4.2 and 8.9, plus a 0.3-kb segment of Tn*3* (Fig. 1). *E. coli* SK1592 (pMMB2) was then used as a recipient for recombinant DNA that was formed in a ligation mixture containing *Hae* II-digested RSF1010 DNA and a nonself-replicating 1.3-kb *Hae*

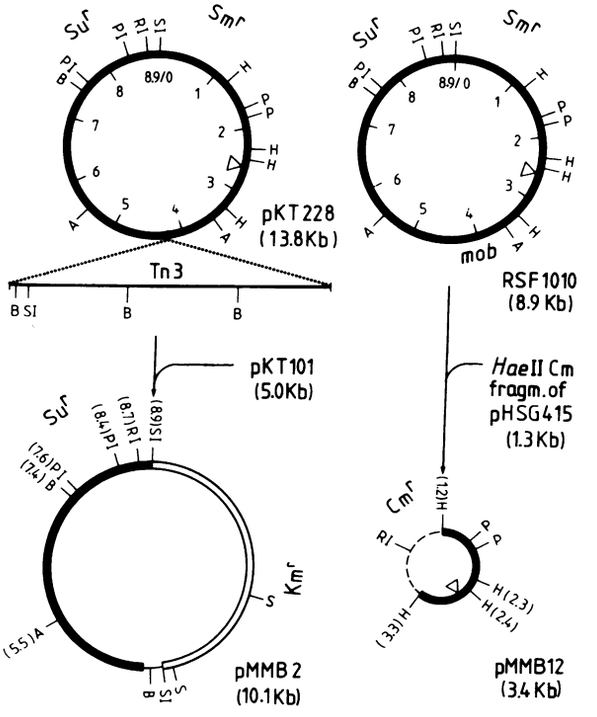

FIG. 1. Construction of pMMB2 and pMMB12. The thick solid line represents DNA derived from RSF1010. Restriction sites (refs. 6 and 8; this work) are indicated as follows: A, *Acc* I; B, *Bst*EII; RI, *Eco*RI; H, *Hae* II; PI, *Pst* I; P, *Pvu* II; S, *Sma* I; SI, *Sst* I. Of the numerous *Hae* II sites in RSF1010, only those relevant to the construction of pMMB12 are indicated. Numbers in parentheses refer to positions of restriction sites in the parental RSF1010 DNA (in kb from the unique *Sst* I site). The open triangle locates the origin of RSF1010 DNA replication (7). Also indicated are the positions of the genes conferring Su^r and Sm^r (1) and a region (*mob*) encoding mobilization functions (8).

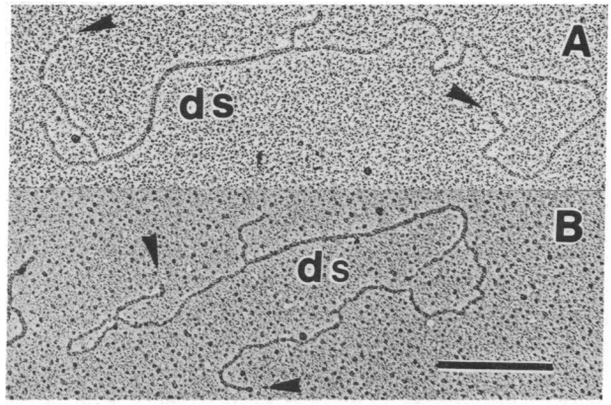

FIG. 2. Electron micrographs of heteroduplex DNA formed between *Eco*RI-cut pMMB12 and RSF1010 cut with either *Eco*RI (*A*) or *Bst*EII (*B*). The ends of RSF1010 DNA are marked by arrows. Each molecule shows a duplex segment (ds) that is 2.1 kb (± 0.06 kb, $n = 28$) long and whose termini are located at the RSF1010 coordinates 1.2 and 3.3 as defined in Fig. 1. (Bar = 1 kb.)

II Cm^r fragment derived from pHSG415 (12). Cm^r colonies were selected, and 25 were screened for plasmid content. All were found to carry, in addition to pMMB2, a second plasmid element. The smallest, pMMB12 (3.4 kb), was saved for further characterization. Restriction analysis showed it to consist of the *Hae* II fragments [1.2–2.3], [2.3–2.4], and [2.4–3.3] of RSF1010, as well as the *Hae* II Cm^r fragment of pHSG415. Its structure deduced from this analysis (Fig. 1) was confirmed by electron microscopic analysis of pMMB12·RSF1010 heteroduplex DNA (Fig. 2).

The ability of pMMB12 to be stably replicated in *E. coli* was tested by using its DNA, separated from pMMB2 DNA on a 1% agarose gel, to transform strains C600, C600(pKT101), C600(RSF1010), and C600(pMMB2) to Cm^r. The data in Table 1 show that the miniplasmid will establish itself only in the presence of RSF1010 or pMMB2. Thus, pMMB12 replication or maintenance is dependent on *trans*-acting functions encoded by the 4.2- to 8.9-kb region of RSF1010 that is present on pMMB2.

Requirement of pMMB2-Encoded Products for RSF1010 Replication *in Vitro*. To show that the 4.2- to 8.9-kb region of RSF1010 is actually required to provide replication functions, we turned to *in vitro* studies. An ammonium sulfate fraction, AS[0–73], was prepared from crude cell extracts of strains C600(RSF1010), C600(pMMB2), and C600(pKT101), and the resulting preparations were tested for their ability to support the replication of exogenous RSF1010 DNA. As shown in Fig. 3, extracts of both C600(RSF1010) and C600(pMMB2) catalyzed extensive DNA synthesis dependent on RSF1010 DNA but the extract made from the pKT101-carrying strain was totally inactive, showing that the replication capacity of the C600(pMMB2) extract was indeed due to products specified by RSF1010 sequences. Although the activity of the extracts varied somewhat from preparation to preparation, the level of DNA synthesis in extracts of C600(pMMB2) was always at least twice that ob-

Table 1. Transforming activity of miniplasmid pMMB12

Recipient strain	Transformants per μg of DNA
C600	<200
C600(pKT101)	<200
C600(pMMB2)	8.0×10^5
C600(RSF1010)	1.4×10^5

Approximately 10^9 cells were exposed to 5 ng of gel-purified pMMB12 DNA isolated from strain SK1592 (pMMB2, pMMB12). Transformants were selected for Cm^r.

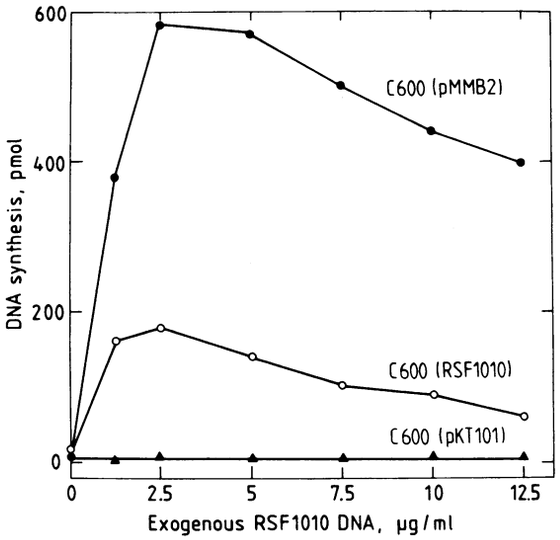

FIG. 3. RSF1010 replication in partially purified extracts of *E. coli*. Reactions were carried out with fraction AS[0–73] (280 μg of protein) prepared from the indicated strains.

tained with extracts of C600(RSF1010). In further experiments, therefore, strain C600(pMMB2) was used as a source of RSF1010 replication proteins.

Fractionation of the Replication System. The first step was to prepare an AS[0–73] fraction from strain C600 lacking a plasmid. As reported (9, 18), this enzyme system proved capable of supporting the replication of plasmid ColE1 but was inactive in RSF1010 replication. ColE1 DNA synthesis was generally optimal with 200–300 μg of protein in a 25-μl assay mixture. When such a quantity of C600 protein was combined with relatively small amounts of an AS[0–73] fraction from C600(pMMB2), RSF1010-dependent DNA synthesis occurred (Fig. 4*A*). The requirement for C600(pMMB2) proteins could not be satisfied by an AS[0–40] fraction, whereas an AS[40–73] fraction stimulated DNA synthesis to an extent similar to that of the AS[0–73] fraction. Unlike the latter fraction, the AS[40–73] fraction was by itself devoid of any replication activity (Fig. 4*B*) because of the absence of host enzymes such as DNA polymerase III (19).

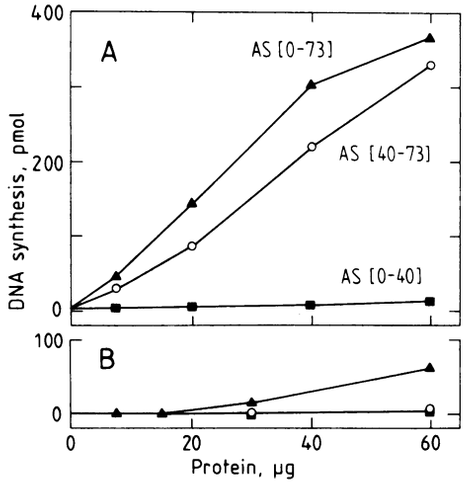

FIG. 4. Requirement of added protein fractions from strain C600(pMMB2) for RSF1010 replication in an extract of strain C600. (*A*) Reactions were in the presence of 64 ng of RSF1010 DNA, 280 μg of C600 As[0–73] protein, and various amounts of the indicated protein fractions from C600(pMMB2). AS[0–40] and AS[0–73], fractions obtained by adding to streptomycin sulfate-treated crude extract ammonium sulfate to 40% and 73% saturation, respectively; AS[40–73], fraction obtained by increasing the ammonium sulfate concentration of the AS[0–40] supernatant to 73% saturation. (*B*) Conditions were as in *A*, except that C600 extract was omitted from the reaction mixtures.

We next subjected an AS[40–73] fraction from C600 (pMMB2) to chromatography on heparin-Sepharose. In a preliminary experiment (Fig. 5, *Inset*), three fractions were collected: the flow-through (I), a 0.1–0.6 M NaCl eluate (II), and a 0.6–1.2 M NaCl eluate (III). Each subfraction was assayed singly and in various combinations for ability to effect synthesis of RSF1010 DNA in the presence of a saturating level of C600 extract. The results showed that combinations of all three fractions were most active (data not shown).

The fractionation procedure was then repeated starting from a new AS[40–73] preparation and now using a salt gradient to elute the proteins (Fig. 5). To find the several activities in individual column fractions, aliquots of each fraction were added to three assay mixtures, each one containing, in addition to the C600 extract, a different pair of the previously prepared fractions I, II, and III. Three well-separated activity peaks were detected by this assay procedure: one in the flow-through (A), a second in the 0.4–0.6 M NaCl region of the gradient (B), and the third in the 0.7–0.9 M NaCl region

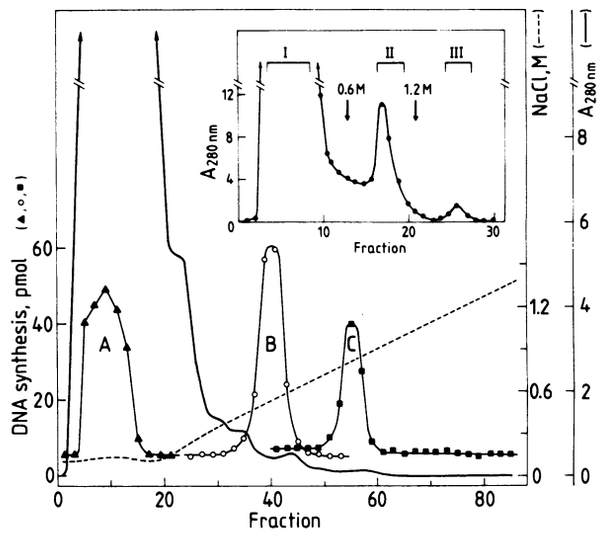

FIG. 5. Heparin-Sepharose chromatography of an AS[40–73] fraction from strain C600(pMMB2). A streptomycin sulfate supernatant, prepared from 360 g of cell paste as described in *Materials and Methods*, was adjusted to 40% saturation with ammonium sulfate (0.226 g/ml), stirred for 30 min, and centrifuged at 25,000 × *g* for 20 min. The supernatant was then brought to 73% saturation with ammonium sulfate (0.212 g/ml), stirred for 30 min, and centrifuged as before. The pellet was suspended in a minimal volume of buffer A (20 mM Tris·HCl, pH 8.0/1 mM dithiothreitol/0.1% Brij 58/5% ethylene glycol) and dialyzed against the same buffer until the conductivity of the solution was equal to that of buffer A/0.1 M NaCl. This fraction (185 ml, 6.8 g of protein) was applied to a column (5 × 9.2 cm) of heparin-Sepharose in buffer A/0.1 M NaCl. The column was washed with 180 ml of the same buffer and then eluted with a 1.8-liter gradient of NaCl (0.1–1.5 M) in buffer A. The flow rate was kept at 180 ml/hr; 24-ml fractions were collected, and aliquots (1 μl) of each fraction were assayed for ability to stimulate DNA synthesis in reaction mixtures containing 32 ng of RSF1010 DNA, 220 μg of C600 AS[0–73] protein, and 1 μl each of the various previously prepared fractions: ■, I + II; ○, I + III; ▲, II + III. (*Inset*) Heparin-Sepharose chromatography step used to prepare fractions I, II, and III. An AS[40–73] fraction prepared from 16 g of C600(pMMB2) cells (8.5 ml, 0.27 g of protein) was applied at a flow rate of 8 ml/hr to an 8-ml column equilibrated with buffer A/0.1 M NaCl. The column was washed with 16 ml of buffer A/0.1 M NaCl, and bound proteins were eluted with 16 ml of buffer A/0.6 M NaCl followed by 24 ml of buffer A/1.2 M NaCl. Individual column fractions were pooled as indicated by the bars.

Table 2. Requirements for RSF1010 DNA synthesis with partially purified replication proteins

Reaction components	DNA synthesis, pmol
Complete	65
+ novobiocin (10 μg/ml)	2.8
+ rifampicin (20 μg/ml)	56
− fraction A	1.3
− fraction B	4.1
− fraction C	1.1
− C600 extract	<1.0
− RSF1010 DNA	<1.0
RSF1010 DNA replaced by	
pMMB12 DNA (32 ng)	57
ColE1 DNA (64 ng)	61
pMMB2 DNA (64 ng)	2.9
φX174 replicative form DNA (64 ng)	1.8

The complete reaction mixture contained 32 ng of RSF1010 DNA, 220 μg of C600 AS[0–73] protein and 1 μl each of fractions A (25 μg of protein), B (0.5 μg), and C (0.15 μg). The antibiotics were incubated for 5 min at 0°C together with the C600 extract prior to assembling the reaction mixture.

(C). Tubes containing these peaks (5–14, 38–42, and 53–57) were pooled to give the respective fractions A, B, and C. Portions of these fractions were dialyzed against 25 mM Hepes, pH 8.0/0.5 M KCl/1 mM dithiothreitol/5% ethylene glycol and used in the experiments described below.

Properties of the Reconstituted System. Extract of C600 supplemented with fractions A, B, and C catalyzed DNA synthesis dependent on RSF1010 DNA (Table 2). In the presence of 1 μl of each fraction, synthesis to the extent of 65 pmol, equivalent to 65% of the input DNA, was observed. Larger amounts of the fractions were less effective, due to inhibitory effects exerted by stabilizing buffer ingredients (salt, Brij, ethylene glycol). DNA synthesis proceeded linearly for 30 min and leveled off after 40–60 min (data not shown). As expected for semiconservative replication, novobiocin, an inhibitor of DNA gyrase, blocked the reaction. Rifampicin, an inhibitor of RNA polymerase, on the other hand, had little if any inhibitory effect; the same result has been obtained with a crude system derived from RSF1010-harboring cells (9). No DNA synthesis was detected in the absence of fractions A and C, but in the absence of fraction B, DNA synthesis (up to 10% of the level of the complete system) occasionally occurred. This residual synthesis probably resulted from contamination of fractions A and C with fraction B activity because, on further purification of these fractions, the requirement for fraction B was absolute. Heating fractions A, B, or C at 100°C for 2 min destroyed their activity (data not shown).

In addition to RSF1010 DNA, pMMB12 DNA and ColE1 DNA were also active as templates in the reconstituted system (Table 2). However, the replication of ColE1, unlike that of pMMB12, was neither dependent on the presence of fractions A, B, and C nor rendered resistant to rifampicin by their presence (data not shown). Two other templates tested, phage φX174 replicative form DNA and pMMB2 DNA, were essentially inert in this system. Thus, the fractions A/B/C-dependent DNA synthesis appears to be specific for plasmid DNA containing the RSF1010 origin.

Ordering of the *rep* Genes. To locate the plasmid genes that specify proteins A, B, and C, we subjected pMMB2 to a deletion analysis; *Acc* I- or *Pst* I-cut DNA was partially digested with BAL-31 nuclease, religated, and used to transform strain C600 to Km^r. A third BAL-31 deletion series was generated from *Sst* I-cut DNA. In this case, the parent plasmid was a previously constructed derivative of pMMB2, Δ58, in which ≈1 kb around the single *Eco*RI site had been deleted to remove the adjacent *Sst* I site. Plasmids smaller than

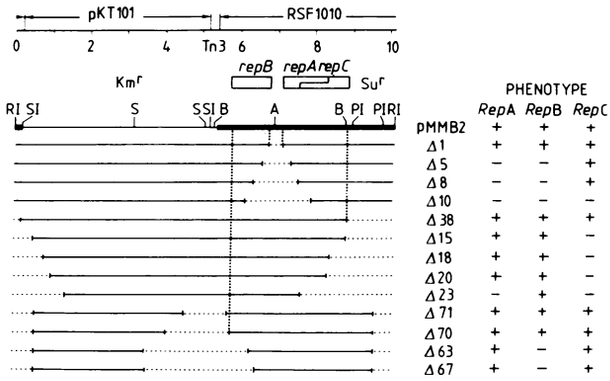

FIG. 6. (*Upper*) Physical and genetic map of pMMB2. Distances given are in kb from the unique *Eco*RI site. Abbreviations for restriction enzymes are those given in the legend to Fig. 1. The open boxes represent the maximum extent of the genetic elements as deduced from the data in the table and deletion mapping. (*Lower*) Diagrams of the pMMB2 derivatives used in this work. Broken lines indicate areas of deletion, as determined by heteroduplex mapping. (*Table*) Survey of individual rep activities specified by the various plamids shown. The activity tests were carried out as exemplified in Table 4. RepA$^-$, -B$^-$, or -C$^-$ was assigned if omission of the corresponding heparin-Sepharose fractions from the assay mixture reduced DNA synthesis by >90%; RepA$^+$, -B$^+$, or -C$^+$ was assigned if the reduction of DNA synthesis was <50%.

pMMB2 or Δ58 were isolated and their deletions mapped by heteroduplex analysis. Maps of 13 such derivatives are given in Fig. 6.

The ability of miniplasmid pMMB12 to be introduced by transformation into the various deletion-harboring strains was tested. The outcome was that pMMB12 could establish itself as a replicon as long as the pMMB2 derivatives had retained, from the original DNA molecule (Fig. 6), the RSF1010 sequences located between coordinates 5.8 and 6.8 (defined by the end points of Δ70 and Δ1) and between coordinates 7.1 and 8.9 (defined by the end points of Δ1 and Δ38) (Table 3).

Concurrent with the *in vivo* studies, we made extracts from the various strains and screened them for deficiencies in individual rep activities by assay of RSF1010 DNA synthesis in the presence of different combinations of fractions A, B, and C. As predicted by the pMMB12-rescue experiments, all deletions that affected the regions of pMMB2 between coordinates 5.8 and 6.8 or between coordinates 7.1 and 8.9 also affected the expression of at least one of the three rep activities. Typical results of these tests are presented in Table 4, and the results of tests performed on the complete set of 13 strains are summarized in Fig. 6. These results unambiguously establish the gene arrangement shown in the upper part of Fig. 6. We denote the genes that specify proteins A, B, and C as *repA*, *repB*, and *repC*, respectively.

Table 3. Ability of pMMB2 deletion derivatives to support replication of miniplasmid pMMB12

pMMB2 derivative	Site of deletion*	Cm^r transformants† per 1 μg of DNA
Δ1	*Acc* I	5.0×10^6
Δ5, Δ8, Δ10	*Acc* I	<200
Δ38	*Pst* I	4.5×10^6
Δ15, Δ18, Δ20, Δ23	*Pst* I	<200
Δ70, Δ71	*Sst* I	$2–4 \times 10^6$
Δ63, Δ67	*Sst* I	<200

*The extent of the deletions is shown in Fig. 6.
†Approximately 10^9 cells of *E. coli* C600 carrying one of the indicated pMMB2 derivatives were exposed to 5 ng of miniplasmid pMMB12 DNA.

Table 4. Detection of individual replication activities specified by deletion derivatives of pMMB2

Protein fractions present	DNA synthesized (relative amount)*				
	C600 (pMMB2)	C600 (Δ38)	C600 (Δ20)	C600 (Δ5)	C600 (Δ63)
A, B, and C	100	100	100	100	100
A and B	98	104	2	90	84
A and C	85	99	88	5	6
B and C	91	78	75	3	92
None	83	77	2	3	4

*100 = 223 (pMMB2), 208 (Δ38), 94 (Δ20), 59 (Δ5), or 61 (Δ63) pmol of total dNMP incorporated.

Alignment of the map of pMMB2 with that of RSF1010 (Fig. 1) shows that they extend from the *Pst* I site at coordinate 7.6 to a site near coordinate 4.5 in the order *repC-repA-repB*.

DISCUSSION

We have identified and mapped three essential genes in RSF1010, *repA*, *repB*, and *repC*, and have shown, by partial purification, that the protein products of these genes act positively in replication of the plasmid. All three genes are situated at a considerable distance from the origin of replication and are separated from it by regions that can be deleted without loss of plasmid viability in *E. coli* (8). That the essential components of the replicon of RSF1010 are not clustered clearly distinguishes it from the narrow host range plasmids studied to date (e.g., ColE1, R1, R6-5, R6K, F, and pSC101), which have their replication/maintenance determinants, including the origin, confined to a small (0.5- to 2.5-kb) segment of the plasmid genome (20–25). Its replicon organization resembles that of the much larger IncP-group plasmid RK2 (57 kb), in which the determinants for replication and maintenance are distributed over a 20-kb portion of the genome (26). The scattered arrangement of essential regions in RK2 has led to the speculation that possibly broad host range plasmids encode more functions for their replication than do the narrow host range plasmids, making them less dependent on host replication proteins and thus allowing a greater degree of promiscuity (26). Our finding that RSF1010, in spite of its small size, carries a set of at least three replication genes supports this view. We know of no example of a narrow host range plasmid having a comparable equipment with replication genes.

Among the host proteins that have been implicated in the initiation of replication of several narrow host range plasmids (including λdv), at least one, the rifampicin-sensitive RNA polymerase, is not required for *in vitro* RSF1010 replication (ref. 9; see also Table 2). In the replication of plasmid ColE1, the major role for RNA polymerase is to provide a primer for DNA synthesis (27). In phage λ, it appears that transcription by RNA polymerase activates the origin by altering its secondary structure, perhaps by exposing a primosome assembly site (28). Analogous functions could conceivably be carried out by the RSF1010-determined rep proteins. Differences in the specificities of RNA polymerases among Gram-negative bacteria may represent a barrier for those plasmids that rely on this particular enzyme for the initiation of their replication. Thus, the exclusion of RNA polymerase from the initiation process could well be part of the strategy used by the broad host range plasmids to survive in different bacterial hosts.

Subsequent to the heparin-Sepharose chromatography step (Fig. 5), each of the three RSF1010 rep proteins has been further purified through at least two steps of ion exchange chromatography and one step of gel filtration. One of the proteins, the *repC* gene product, has been obtained in electrophoretically pure form. Preliminary DNA binding studies with the purified protein (probably a dimer of 27,000-dalton protomers) indicate that it binds tightly and preferentially to the origin region within supercoiled RSF1010 DNA (unpublished results). By this criterion, it resembles the phage λ O protein (28) and the *E. coli* dnaA protein (29), two proteins that are known to bind to their autologous origins and to play a crucial role in the initiation of DNA replication.

We thank H. Schuster for support and continuous encouragement and C. C. Richardson for critical remarks on the manuscript. This work was partially supported by a grant from the Deutsche Forschungsgemeinschaft (to M.B.).

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