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Protein RepC is involved in copy number control of the broad host range plasmid RSF1010

(recombinant DNA/plasmid replication/replication proteins/DNA·DNA hybridization/replication control)

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ABSTRACT **Essential replication (*rep*) genes of the broad host range plasmid RSF1010 have been cloned onto controlled expression vectors and their protein products have been visualized, after induction, by $NaDodSO_4$/polyacrylamide gel electrophoresis of whole cell lysates. During this induction the replication of a coresident RSF1010 replicon, pKT210, was analyzed by quantitative DNA·DNA hybridization. The initiation of pKT210 replication was stimulated 6-fold by a simultaneous overproduction of the RepA and RepC proteins compared to cells in which only the RepA protein was overproduced. An enhanced synthesis of the RepB protein resulted in a 1.6-fold stimulation of pKT210 replication, whereas an overproduction of the RepA protein alone had no effect. Purified RepC protein has been shown to bind preferentially to DNA carrying the replication origin of RSF1010. Within this segment it was bound specifically to those DNA fragments that contained the 20-base-pair direct repeats of the origin region. These results suggest that RepC protein acts as a positive replication regulator, that its concentration is rate-limiting, and that the replication rate of RSF1010 is controlled, at least in part, at the level of RepC synthesis.**

RSF1010 (similar or identical to R300B and R1162) is a small [8.7-kilobase (kb)] nonconjugative plasmid of the *Escherichia coli* incompatibility group Q that specifies resistance to streptomycin and sulfonamides. Most remarkable is its ability to replicate in a very wide range of Gram-negative bacterial species (for review, see refs. 1 and 2). In *E. coli*, in which RSF1010 is maintained at 10–12 copies per chromosome (3), replication is initiated from a unique region (*oriV*) located at a distance of ≈2.6 kb from the single *Eco*RI site of the plasmid (4) (see Fig. 1). For R1162 it has been reported that both a mutation resulting in an increased plasmid copy number and a coding sequence responsible for expression of plasmid incompatibility are closely linked to *oriV* (5). More recently, the R1162 incompatibility determinant has been isolated as a 370-base-pair (bp) DNA segment and shown to contain three perfectly conserved 20-bp direct repeat units (6).

We have identified previously three essential replication genes in RSF1010: *repA*, *repB*, and *repC*. They are situated at considerable distance from *oriV* and separated from it by nonessential regions. It has been shown that a recircularized 2.1-kb DNA fragment containing *oriV* will replicate in *E. coli* if the three *rep* gene products are supplied in *trans*. Moreover, each of the three *rep* gene products of RSF1010 is also essential for initiating plasmid DNA replication *in vitro* (7, 8).

In the present work we cloned the *rep* genes of RSF1010 onto controlled expression vectors and examined, by labeling and DNA·DNA hybridization, the replication of a coresident RSF1010 replicon (pKT210) during amplification of individual Rep proteins. The results show that RepC protein binds specifically to *oriV* sequences of RSF1010 and is involved in the positive regulation of RSF1010 replication.

MATERIALS AND METHODS

Strains and Plasmids. The strains used were *E. coli* K-12 derivatives HB101 (9) and JD164, *leu thy ilv*::Mu λ*ind*⁻ Δ(srl-*recA*)306 *srl*R301::Tn*10*.

Plasmids RSF1010, pKT210 [a chloramphenicol resistance (Cm^r) derivative of RSF1010], and pMMB12 (a helper-dependent miniderivative of RSF1010) have been described (2, 7, 10). Plasmid pVH1 was constructed in the following way. The vector pKT101 (5.0 kb), a kanamycin resistance (Km^r) derivative of the multicopy plasmid ColD (7, 10), was linearized at its unique *Sst* I site and the single-stranded portions were rendered blunt-ended with the aid of T4 DNA polymerase. A 1.2-kb *Eco*RI fragment containing the $lacI^Q$ gene was excised from plasmid $placI^Q$ (obtained from J. Wang) and purified by agarose gel electrophoresis. After filling in the recessed ends, the $lacI^Q$ fragment was joined by blunt-end ligation to the linear pKT101 DNA preparation. This procedure created a plasmid of 6.2 kb, pVH1, that is compatible with and nonhomologous to pBR322.

The expression vectors used were the hybrid *trp–lac* promoter (*tac* promoter) containing pBR322 derivatives p*tac*12 (11) and pKK233-3; the latter plasmid contains unique *Eco*RI-, *Sma* I-, *Pst* I-, and *Hin*dIII-sensitive sites downstream of the *tac* promoter followed by the two transcription terminators of the *E. coli rrnB* operon (supplied by Pharmacia P-L Biochemicals).

Growth conditions, transformation of bacteria with plasmid DNA, and selection for antibiotic resistance were as described (7).

DNA Manipulation. Purification of plasmid DNA, restriction endonuclease analysis, and purification and ligation of DNA fragments were as described (7, 12). Hybridization of *in vivo* labeled DNA to DNA-charged nitrocellulose filters has been described in detail (13).

Nucleotide Sequence Analysis. Two strategies were used to determine the nucleotide sequence of the *oriV* region of RSF1010. In one of them, fragments obtained by sonic

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Abbreviations: Ap^r, ampicillin resistance; bp, base pair(s); Cm^r, chloramphenicol resistance; IPTG, isopropyl β-D-thiogalactopyranoside; kb, kilobase pair(s); Km^r, kanamycin resistance; *tac* promoter, hybrid *trp–lac* promoter.

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shearing of the *Hin*fI [1.9–3.0]* segment of RSF1010 were cloned into M13mp8 and M13mp9 vectors (14). In the second, the nonrandom deletion mutant library was generated from this fragment cloned into M13mp9 (15). Fragments obtained by either of these procedures were sequenced by the dideoxy chain-termination method (16).

Electrophoretic Analysis of Proteins. Centrifuged cells (equivalent to 1.0 ml at A_{590} = 1.0) were suspended in 20 μl of water, mixed with 20 μl of 2× concentrated sample buffer (17), heated 5 min at 50°C and then heated 2 min at 100°C, and centrifuged, and 25 μl of supernatant was loaded onto a $NaDodSO_4$/polyacrylamide gel (15% acrylamide) prepared and run according to Lugtenberg (17). Molecular mass standards were bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; chymotrypsinogen A, 25.7 kDa; and RNase A, 13.6 kDa.

RESULTS

Construction of Plasmids Allowing Controlled Expression of RSF1010 *repA*, *repB*, and *repC* Genes. In Fig. 1 a physical map of RSF1010, opened at its unique *Eco*RI site (origin of [kb] coordinates), is presented. It shows some of the restriction endonuclease-sensitive sites in RSF1010 DNA (1, 2) as well as the positions and the size of genes *repA*, *repB*, and *repC*, as determined by deletion mapping (7) and nucleotide sequence analysis (ref. 8; unpublished data). According to these analyses, the entire *repA* gene is contained within fragment *Bal* I [5.7–6.9] (1236 bp), there is no intergenic space between *repA* and *repC*, and the latter ends between the *Bst*EII [7.6] site and the *Pst* I [7.8] site. The entire *repB* gene is contained within fragment *Acc* I [3.6–5.5] (≈1900 bp).

To clone the *repA* gene under conditions of controlled expression, we inserted fragment *Bal* I [5.7–6.9], excised from RSF1010 and purified by agarose gel electrophoresis, into the *Pvu* II site of the vector p*tac*12 to yield plasmid pVH2. Plasmid pVH4, which contains both genes *repA* and *repC* under the control of the *tac* promoter, was constructed similarly by ligating the *Hin*fI [5.0]–*Pst* I [7.8] fragment of RSF1010 (2793 bp) to *Pvu* II-cleaved p*tac*12 DNA after the fragment has been made blunt-ended with the aid of T4 DNA polymerase. Plasmid pVH3, carrying the *repB* gene under *tac* control, was constructed by inserting fragment *Acc* I [3.6–5.5], excised from RSF1010 DNA and made blunt-ended with T4 DNA polymerase, into the unique *Sma* I site of vector pKK223-3.

Physical maps of these plasmids, indicating the orientation of the cloned RSF1010 fragments with respect to the *tac* promoter, are given in Fig. 1.

Each of the three recombinant plasmids was introduced into strain HB101 [pVH1] and the resulting strains were grown initially in the absence and subsequently in the presence of 1.0 mM isopropyl β-D-thiogalactopyranoside (IPTG) to induce expression of the *tac*-controlled *rep* genes. Whole cell lysates were prepared and subjected to $NaDodSO_4$/PAGE (Fig. 2). In each case the induction resulted in the appearance of at least one new prominent band in the gel pattern of Coomassie blue-stained proteins. Plasmid pVH2 directed the synthesis of a polypeptide of ≈29 kDa, the *repA* gene product (Fig. 2, lane 3). This polypeptide was not detectable in lysates of induced cells carrying a plasmid (pVH2′), in which the orientation of the *Bal* I fragment was opposite to that present in pVH2 (Fig. 2, lane 2). Moreover, using pVH2-carrying cells as a source, we purified the 29-kDa protein to near homogeneity, and the pure protein was found

*[ ] is used to indicate plasmid carrier state or plasmid kilobase coordinates.

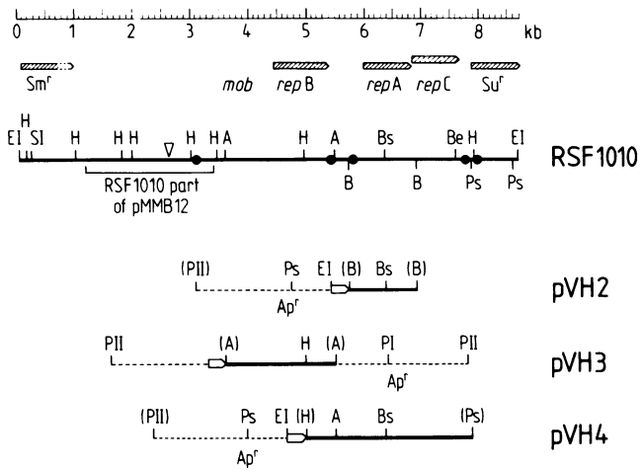

FIG. 1. (*Upper*) Physical and genetic map of RSF1010. Coordinates are in kilobases, starting at the unique *Eco*RI site. Restriction sites are shown as A, *Acc* I; B, *Bal* I; Be, *Bst*EII; Bs, *Bss*HII; EI, *Eco*RI; H, *Hin*fI; Ps, *Pst* I; and SI, *Sst* I. Of the numerous *Bal* I- and *Bss*HII-sensitive sites in RSF1010 DNA, only those relevant to plasmid constructions (see text) are indicated. The open triangle marks the primary origin of vegetative replication (*oriV*); it is contained within the mini-RSF1010 derivative, pMMB12 (7), used as a probe in DNA·DNA hybridization exeriments (see Table 1). The black circles represent *E. coli* RNA polymerase binding sites. Positions of the replication genes *repA*, *repB*, and *repC* are indicated by hatched boxes as are the positions of the genes conferring sulfonamide resistance (Su^r) and streptomycin resistance (Sm^r); the arrowheads of the boxes show the direction of transcription. Also indicated is a region (*mob*) encoding functions required for plasmid mobilization. The locations of genes *repA*, *repB*, and *repC* have been determined precisely by sequencing (ref. 8; unpublished data). Other data were taken from refs. 2 and 7. (*Lower*) Structures of the *rep* expression plasmids pVH2, pVH3, and pVH4. The thick solid lines represent RSF1010 sequences. Broken lines mark sequences of the vector plasmids; *Pvu* I and *Pvu* II restriction sites are abbreviated as PI and PII, respectively. Restriction sites shown in parentheses were lost during the constructions. The open boxes represent the *tac* promoter; its transcriptional orientation is indicated by the arrowheads. Ap^r, ampicillin resistance.

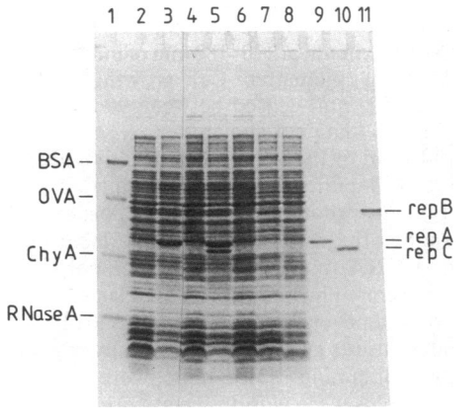

FIG. 2. Expression of RSF1010 *rep* genes inserted downstream of the *tac* promoter. To exponential cultures of HB101 [pVH1] harboring different *tac* promoter–*rep* fusions (Fig. 1), IPTG (1.0 mM) was added for 2 hr. Whole cell lysates were analyzed by $NaDodSO_4$/PAGE. Gels were stained with Coomassie blue. Lane 1, molecular mass standards (BSA, bovine serum albumin; OVA, ovalbumin; ChyA, chymotrypsinogen A; RNase A); lane 2, HB101 [pVH2′, pVH1]; lane 3, HB101 [pVH2, pVH1]; lane 4, HB101 [pVH4′, pVH1]; lane 5, HB101 [pVH4, pVH1]; lane 6, HB101 [pVH3′, pVH1]; lane 7, HB101 [pVH3, pVH1]; lane 8, HB101 [pVH1]; lanes 9, 10, and 11, purified RepA, RepC, and RepB proteins, respectively (≈2 μg each).

to be active in an *in vitro* system that specifically replicates plasmid DNA bearing the RSF1010 replication origin (ref. 7; unpublished data). Similarly, plasmid pVH3 directed, upon induction, the synthesis of active RepB protein, appearing as a 38-kDa protein band in the denaturing gel, whereas a control plasmid (pVH3′), containing the *Acc* I fragment inserted in the opposite orientation, did not express any visible RepB protein (compare lanes 6 and 7, Fig. 2). In the pVH4-carrying strain, as expected, two protein species were overproduced after IPTG induction: one corresponding to the 29-kDa RepA protein and a second of ≈27 kDa, the *repC* gene product (Fig. 2, lane 5). Both proteins were purified from this strain and both were found to be active for RSF1010 replication *in vitro* (unpublished data). These results establish unambiguously the direction of transcription of all three *rep* genes as being from left to right on the genetic map of RSF1010, as presented in Fig. 1.

For some undetermined reason, no RepC protein was overproduced with a plasmid (pVH5) containing the 1497-bp *Bss*HII [6.3]–*Pst* I [7.8] fragment inserted downstream of the *tac* promoter, although we have evidence from both DNA sequence and protein sequence analysis that the entire *repC* gene is contained within this RSF1010 fragment. We presume that the RepA and RepC proteins are normally synthesized from a polycistronic message and that *repA* translation is a prerequisite for optimal expression of *repC* (unpublished data).

Replication of RSF1010 Derivatives in Cells with Elevated Levels of Rep Proteins. The RepA, RepB, and RepC proteins have been shown previously to act in *trans* upon their target, the *oriV* of RSF1010 (7, 8). It was possible, therefore, to use the binary plasmid system described in the preceding section to vary the intracellular concentration of individual Rep proteins in the cell and in that way to determine their influence upon the copy number of an RSF1010 replicon.

To do this the following strains were constructed by transformation of JD164, a *recA thy*$^-$ strain that carries a single copy of prophage Mu inserted in the *ilv* locus: CB1130 = JD164 [pVH1, pVH2, pKT210], CB1131 = JD164 [pVH1, pVH4, pKT210], and CB1144 = JD164 [pVH1, pVH3, pKT210]. The inducibility by IPTG of the *tac*-controlled *rep* genes was verified for each strain by electrophoretic analysis of whole cell lysates (Fig. 3). For strain CB1131, we determined the time at which maximal RepA protein and RepC protein levels were attained after induction. It was found that the level of both proteins increased to a maximum at 120–180 min of induction at 37°C and decreased slightly thereafter. Cell growth, as measured by absorbance at 590 nm, ceased between 90 and 120 min after addition of IPTG (data not shown).

The replication of the RSF1010 derivative pKT210 during IPTG-induced amplification of individual Rep proteins was analyzed by a quantitative DNA·DNA hybridization method that determines the ratio of radioactive label incorporated into plasmid DNA to radioactive label incorporated into a marker of the host chromosome (13). Plasmid pVH1, replicating in the same cell, served as a control. In a typical experiment, cultures growing exponentially in supplemented M9 medium, containing [^{3}H]thymine, were exposed to IPTG (1.0 mM), samples were withdrawn at various times after the addition of the inducer, and the DNA in them was analyzed by hybridization, as described in the legend to Table 1 (for details, see also ref. 13).

Replication of the ColD-based plasmid pVH1 should not be influenced by the RSF1010-encoded Rep proteins and its copy number should not change during the exponential phase of cell growth. This was the case in all of the three strains, CB1130, CB1131, and CB1144: the ratio of DNA hybridized to ColD-charged filters over the DNA hybridized to Mu-charged filters did not change significantly during the 120 min of incubation with IPTG (Table 1, column 7).

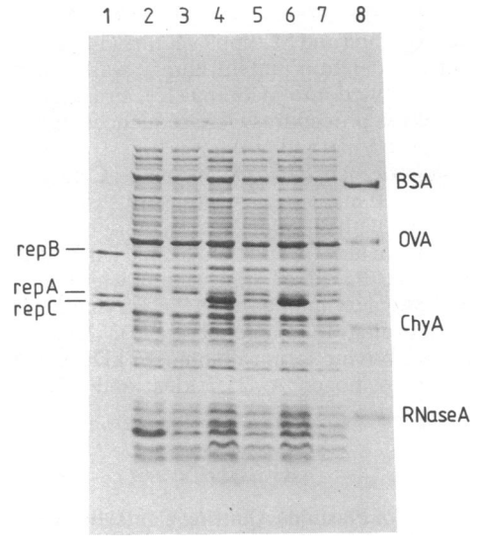

FIG. 3. Induction of Rep protein synthesis in strain JD164 harboring pVH1, pKT210, and pVH2, pVH3, or pVH4 (designated strains CB1130, CB1144, and CB1131, respectively). Cells were grown at 37°C in L broth, with appropriate antibiotics, to A_{590} = 0.5. Half of each culture was then exposed to 1.0 mM IPTG and all cultures were incubated for an additional 2 hr. Whole cell lysates were analyzed as in Fig. 2. Lane 1, mixture of purified RepA, RepB, and RepC proteins (≈2 μg each); lane 2, induced CB1144; lane 3, uninduced CB1144; lane 4, induced CB1131; lane 5, uninduced CB1131; lane 6, induced CB1130; lane 7, uninduced CB1130; lane 8, molecular mass markers (see legend to Fig. 2).

In contrast to pVH1, plasmid pKT210 showed a significantly different behavior upon induction of *rep* gene products, particularly in CB1131. In this strain, in which both RepA and RepC proteins are overproduced in the presence of IPTG, the ratio of DNA hybridized to pMMB12 filters over DNA that hybridized to Mu-charged filters increased progressively from 1.06 to 4.03 during the 120 min of the induction experiment. A small but significant increase in the ratio of DNA hybridized to pMMB12 over Mu DNA (1.6-fold) was also observed upon the amplification of the RepB protein (strain CB1144), whereas an overproduction of RepA protein alone (strain CB1130) had no effect (Table 1).

It must be noted that the concentration of pKT210 plasmid DNA in uninduced CB1131 cells was already 1.7-fold higher than that observed in uninduced cells of CB1130. This may be due to some leakiness of the *tac* promoter, even in the presence of a higher than normal level of *lac* repressor (supplied by the *lacI*^{Q} plasmid pVH1), or to *tac*-independent expression of the *repA–repC* gene cluster from its own promoter(s) present on the cloned *Hin*fI [5.0]–*Pst* I [7.8] fragment (see Fig. 1). Nevertheless, the induction experiments clearly indicate that an increased expression of the *repA–repC* gene cluster of RSF1010 leads to a prompt increase in the copy number of the plasmid and that this copy effect is not brought about by an increased expression of the *repA* gene alone.

Although the hybridization method used cannot distinguish between an increase in copy number of the plasmid and multiple initiations at the *oriV* region, we consider that our results reflect an increase in the number of the plasmid molecules since a net increase of the amount of pKT210 DNA per cell was always observed, upon induction, in strain CB1131 on agarose gel electrophoresis. The amount of ColD- or pBR322-derived DNA present in the same cell remained constant (results not shown).

Binding of RepC Protein to the *oriV* Region of RSF1010. The results described in the preceding section strongly predicted binding of RepC protein to the *oriV* region of RSF1010. To

Table 1. Effect of overproduction of RSF1010 Rep proteins on plasmid replication

Strain	Protein overproduced	Time, min*	[^{3}H]DNA retained on filter, cpm†			Ratio	
			ColD	pMMB12	Mu	ColD: Mu	pMMB12: Mu
CB1131	RepA + RepC	0	2587	3653	3457	0.75	1.06
		5	1519	2782	2170	0.70	1.28
		30	1470	4028	2078	0.71	1.94
		60	1473	4741	1983	0.74	2.39
		120	1318	7449	1849	0.71	4.03
CB1130	RepA	0	1635	1310	2239	0.73	0.58
		5	1651	1361	2310	0.71	0.59
		30	1462	1224	2321	0.62	0.53
		60	1465	1271	2424	0.60	0.52
		120	1822	1550	2636	0.69	0.59
CB1144	RepB	0	1876	1981	2681	0.70	0.73
		5	1754	1904	2506	0.70	0.76
		30	1770	2332	2425	0.73	0.96
		60	1631	2114	2146	0.76	0.99
		120	1815	2426	2368	0.77	1.02

Cultures were grown at 37°C in M9 medium supplemented with glucose (0.4 mg/ml), Difco Casamino acids (10 mg/ml), thiamine (0.5 μg/ml), and [*methyl*-^{3}H]thymine (4 μg/ml; 0.01 mCi/ml; 1 Ci = 37 GBq; Amersham–Buchler) to A_{450} = 0.5. IPTG was added (1.0 mM) and incubation was continued for 120 min. Samples equivalent to 1.0 ml of culture with A_{450} = 1.0 were withdrawn at the times indicated and poured into 2 vol of ethanol/phenol stop solution. After centrifugation, cells were resuspended in 1.0 ml of 0.5 M NaOH and heated for 10 min at 100°C. The resulting lysates were neutralized and incubated for hybridization with filters containing 2 μg of nonlabeled pMMB12, ColD, or Mu DNA (13).

*Time after induction.

†^{3}H cpm binding to each of the three probe filters were corrected for background binding determined on filters containing 2 μg of calf thymus DNA. The values shown were obtained in the same experiment, which is representative of two others.

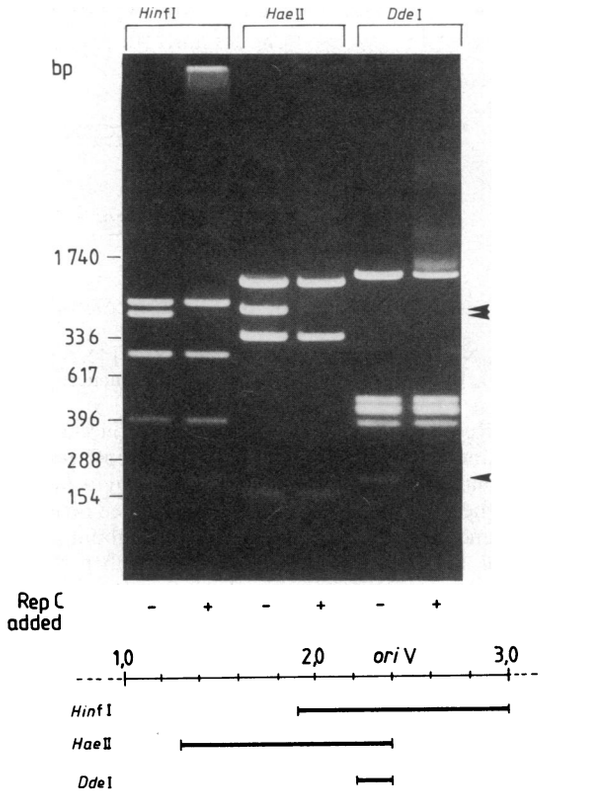

FIG. 4. Binding of RepC protein to the *oriV* region of RSF1010. Miniplasmid pMMB12 DNA was digested to completion with the indicated restriction endonucleases, and the mixture of DNA fragments was purified by phenol extraction, ether extraction, and ethanol precipitation. Approximately 0.5 μg of the cleaved DNA was incubated for 1 hr at 37°C with or without purified RepC protein (0.5 μg) in 20 μl (final volume) of binding buffer (40 mM Hepes/NaOH, pH 7.6/100 mM NaCl/10 mM $MgCl_2$/1 mM dithiothreitol/0.1 mg of bovine serum albumin per ml). Five microliters of 20% (wt/vol) Ficoll 400/0.1% bromophenol blue in electrophoresis buffer (40 mM Tris acetate/5 mM sodium acetate/1 mM EDTA, pH 7.9) was added to each sample and loaded into the wells of a 1.4% vertical agarose slab gel. Electrophoresis was run at a constant 5.5 V/cm for 3 hr at room temperature, and DNA was visualized by staining with ethidium bromide (0.5 μg/ml). Size markers were *Hpa* I fragments of T7 phage DNA and *Hin*fI fragments of pBR322 DNA. Restriction fragments that were specifically converted to RepC·DNA complexes, manifested by their greatly reduced electrophoretic mobilities, are indicated by arrowheads. The positions of these fragments on the RSF1010 map are shown in the bottom part of the figure. The numbers correspond to the coordinates in Fig. 1; *oriV*, origin of vegetative replication.

demonstrate this, the RepC protein was overproduced in strain HB101 [pVH4] and purified to near homogeneity (see Fig. 2, lane 10). Binding of the pure protein to DNA was assayed by an electrophoretic method that relies on the different mobilities of protein·DNA complexes and free DNA in agarose gels (18). Fig. 4 shows the results of such an experiment in which RepC protein was incubated at 37°C with pMMB12 miniplasmid DNA that had been cleaved with either *Hae* II, *Hin*fI, or *Dde* I. In each case, a single DNA fragment was retarded in moving through the gel. Restriction and nucleotide sequence analyses of the entire *Hin*fI [2.0–3.0] segment of RSF1010 have shown that each of the retarded fragments had in common the RSF1010 sequences located between map coordinates 2.2 and 2.4 (Fig. 4, map). In the nucleotide sequence of the 204-bp *Dde* I [2.2–2.4] fragment (Fig. 5), attention is immediately drawn to four 20-bp direct repeat units at positions 50–135. Three of these repeats, each separated from the other by 2 bp, are perfectly conserved and the remaining repeat differs from the others in only four positions. It is interesting to compare the sequence presented here with that of the 390-bp, incompatibility-exerting fragment of plasmid R1162 (6). The regions comprising the four direct repeats of these two plasmids (belonging to the same incompatibility group) are identical.

DISCUSSION

In this paper we provide direct evidence that protein RepC, encoded by RSF1010, binds specifically to the *oriV* region of the plasmid, presumably to the direct repeats located at the origin. Since RepC protein is essential for RSF1010 replication (7) we postulate that it is required for initiation and that intracellular concentration of RepC regulates the frequency with which RSF1010 replication is initiated (Table 1).

To monitor the changes in copy number of a given replicon in a cell carrying several different plasmids we have used the DNA·DNA hybridization method that determines the ratio of label in plasmid DNA to the label in a chromosomal marker (13). Although this ratio does not allow measurement of the absolute copy number of a plasmid, it is directly proportional to it and determines, therefore, quantitatively any changes of the plasmid copy number during an experiment and also among different experiments. In balanced growth, if plasmid replication is not disturbed, this ratio should be constant. This was the case for plasmid pVH1, a ColD-based replicon, regardless of the induction of RSF1010-specific replication proteins (Table 1). The replication of pKT210 (RSF1010 replicon), on the other hand, showed a marked dependence upon the concentration of Rep proteins. Of the three plasmid-

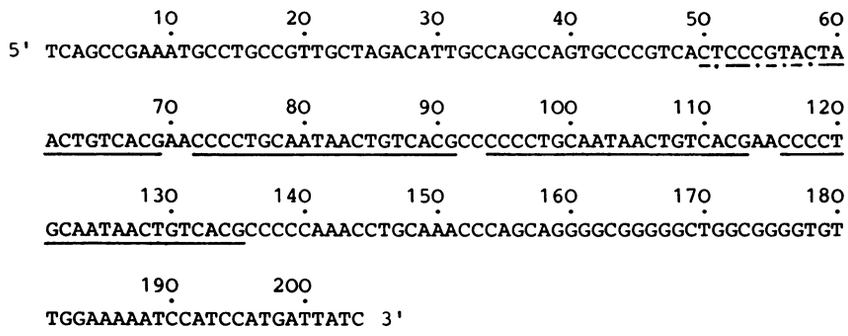

FIG. 5. Nucleotide sequence of fragment *Dde* I [2.2–2.4] of RSF1010. Numbering is from the *Dde* I cleavage site located at map coordinate 2.2 (Fig. 4). Underlines show 20-bp repeated sequences.

encoded Rep proteins, RepC had the most pronounced effect. A simultaneous induction of RepA and RepC proteins increased plasmid copy number of pKT210 by a factor of 6, whereas the induction of RepA alone produced no detectable increase and the induction of RepB brought about a small but significant increase (1.6-fold) in copy number of pKT210 (Table 1). At present it cannot be determined whether, for the observed copy number increase, it is required that both RepC and RepA proteins are overproduced simultaneously, since controlled expression of RepC protein alone could not be achieved to date.

The preparation of RepC protein used in this work was homogeneous on the $NaDodSO_4$/PAGE (Fig. 2). Specifically, it was free of contamination by RepA or RepB protein, as indicated by *in vitro* DNA replication studies (ref. 7; unpublished). Moreover, analysis of the amino acid sequence of our RepC preparation by the automatic Edman degradation procedure have shown that its purity was >95%. A mutant RepC protein that had lost 18 amino acids from its carboxyl terminus (purified from cells containing the *Hin*fI–*Bst*EII fragment of RSF1010) no longer exhibited origin-specific DNA binding (unpublished data). Purified RepA protein had no DNA-binding activity, it formed no physical complexes with RepC protein, even in the presence of *oriV* DNA, and it did not alter the binding properties of RepC (results not shown). We conclude, therefore, that binding to the *oriV* region of RSF1010 DNA is a specific property of the *repC* gene product.

We have shown that a minimal RSF1010 structure essential for RepA/RepB/RepC-dependent bidirectional replication *in vitro* resides in a segment of about 370 bp (located between map coordinates 2.3–2.7) that includes three of the four direct repeat units (unpublished data). On this basis we believe that these repeats are essential for binding of the RepC initiator protein and functionality of the replication origin. Consistent with this view is the observation that R1162 DNA containing the four *ori* repeats expresses incompatibility in *trans* (6).

The majority of plasmids studied to date contain direct repeats at their origin of replication. These include F (19), P1 (20), λdv (21), R6K (22), pSC101 (23), and RK2 (24). In each of these cases, the evidence indicates that repeated sequences are involved in the expression of incompatibility and hence in the copy number control. Thus, regulation of DNA replication by means of repeated DNA sequences appears to be a common mechanism.

The roles of RepA and RepB proteins in the replication of RSF1010 are not precisely known. However, a strong indication is at hand. Thus, *in vivo* (8) and *in vitro* (unpublished data) experiments have shown that several key components of the *E. coli* replication apparatus, including the primosomal proteins DnaB, DnaC, and DnaG, are not required for the replication of RSF1010. These observations strongly suggest that RSF1010 directs the formation of a plasmid-specific primosome composed of the plasmid-encoded Rep proteins. The control of DNA replication could therefore be exercised through the interplay of two, or all three, of the Rep proteins, as is the case for the interaction between the DnaA initiator protein and primosomal proteins involved in the replication of the *E. coli* chromosome (25). The slight increase in RSF1010 copy number after the induction of RepB protein synthesis is consistent with this hypothesis.

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