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Genetics

Plasmid P1 replication: Negative control by repeated DNA sequences*

(incompatibility/DNA–protein interactions)

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ABSTRACT **The incompatibility locus, *incA*, of the unit-copy plasmid P1 is contained within a fragment that is essentially a set of nine 19-base-pair repeats. One or more copies of the fragment destabilizes the plasmid when present in *trans*. Here we show that extra copies of *incA* interfere with plasmid DNA replication and that a deletion of most of *incA* increases plasmid copy number. Thus, *incA* is not essential for replication but is required for its control. When cloned in a high-copy-number vector, pieces of the *incA* fragment that each contain only three repeats destabilize P1 plasmids efficiently. This result makes it unlikely that *incA* specifies a regulatory product. Our *in vivo* results suggest that the repeating DNA sequence itself negatively controls replication by titrating a P1-determined protein, RepA, that is essential for replication. Consistent with this hypothesis is the observation that the RepA protein binds to the *incA* fragment *in vitro*.**

Bacteriophage P1 lysogenizes *Escherichia coli* as a unit-copy plasmid (1). Despite this low copy number, the spontaneous segregation of plasmid-free cells is extremely rare (10^{-4}–10^{-5} per generation) (2). To understand the mechanisms responsible for this precise control, we identified the plasmid-determined plasmid maintenance elements in the λΔ(*att int*)-P1 hybrid phage, λ-P1:5R (3). This phage lysogenizes as a unit-copy plasmid much like P1 itself and includes a region necessary for unit-copy replication (Rep) and an adjacent region (Par) that assures plasmid equipartition (4) (Fig. 1).

The Rep region has previously been shown to include no more than ≈2.1 kilobase pairs (kb) (3). This region contains two incompatibility loci, *incA* and *incC*, that flank a gene for a protein, RepA, that is essential for replication (Fig. 1). *incA* and *incC* each include a series of highly conserved 19-base-pair (bp) sequences. In the region of the *incA* locus, there are nine of these repeated sequences spaced three to four turns of the helix apart in a stretch of 285 nucleotides (Fig. 1). The *incC* region has five repeated sequences separated from each other by only two turns of the helix. The two regions of repeated sequences were defined as *incA* and *incC* because, when each region was cloned into pBR322, the composite plasmid destabilized λ-P1:5R as efficiently as other derivations containing larger flanking P1 sequences (3). The *repA* gene and the two *inc* loci occupy ≈1.4 kb. The remaining 0.7-kb segment contains part of the origin and will be discussed in a separate paper. Here we show that the *incA* sequences are required only for negative regulation of replication. In the unit-copy sex-factor plasmid F, whose plasmid-maintenance region is organized similarly to that of P1 (3), replication is also controlled by dispensable repeated sequences that exert incompatibility (5). It was proposed that the repeating DNA sequence itself can limit replication by titrating a factor essential for replication.

A prediction of the model is that overproduction of the essential factor should overcome the inhibitory effect of the repeating sequences. Here we show that the essential protein, RepA, could be such a factor since its overproduction overcomes the inhibitory effect of *incA*. A more direct prediction that the RepA protein should bind to the *incA* fragment has also been verified *in vitro*. These results support the "titration" model of replication control by repeating DNA sequences (5).

MATERIALS AND METHODS

Construction of BR1622. The mini-P1 phage λ-P1:5R was integrated into the chromosome of a *recA56 dnaA46(ts) E. coli* strain as follows. First a λ*imm*21-P1:*Eco*RI-7 phage was integrated at *loxB* by site-specific recombination using the *lox-cre* system encoded by P1:*Eco*RI-7 (6). This strain is called NS1502. In BR1622, the *imm*21 prophage of NS1502 is replaced by the *loxP*-bearing λ-mini-P1, λ-P1:5R *Bam*HI-9, at the same chromosomal site, by the process described in ref. 6. The phage λcI857-P1:5R *Bam*HI-9 is described in ref. 7. The cI^+ version of it used here was obtained from a cross between the cI857 phage and λ*bio spi*B *nin-5* (8). BR1622, unlike its parent NS1502, is viable at 42°C (Table 1). That this viability is due to integrative suppression, rather than reversion of the *dnaA*46(ts) mutation, was confirmed by displacing the prophage with λ*imm*21-P1:*Eco*RI-7 to regenerate a strain that is temperature-sensitive.

Construction of λ *incA* Phages and *incA* Plasmids. In this work, *incA* or *incA* locus refers to the region of P1 DNA containing nine repeats. The term *incA* fragment refers to the DNA between two *Alu* I sites at coordinates 1505 and 1811 (Fig. 2). This 306-bp fragment includes 4 bp beyond repeat 1 and 17 bp beyond repeat 9 (Fig. 2). After the addition of *Bam*HI linkers, the fragment was inserted, in both orientations, into the single *Bam*HI site within the *int* gene of λD69 (10) to yield phages DKC177 and DKC181, and into pBR322 to yield pALA18 and pALA61. The *incA* fragment was equally active in either orientation; results will be discussed for only one (DKC177 or pALA18) of each pair of isolates.

Other details of materials and methods are described elsewhere (3, 11).

RESULTS

Integrative Suppression: An *in Vivo* Assay for Initiation of DNA Replication in P1 Miniplasmids. Previous work implicated *incA* in the control of DNA replication of plasmid prophage P1 and showed *incB*, which maps in the Par region, to be a centromere analog required for equipartition of the plasmids to daughter cells (4). We show here that *incA*, but not *incB*, interferes with the initiation of DNA replication.

The tester plasmid, λ-P1:5R (Fig. 1), was suitably integrated at *loxB* in the chromosome of a *dnaA*ts mutant of *E. coli* such that the replication initiated by the plasmid would permit replication and survival at 42°C of the otherwise nonvia-

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Abbreviations: bp, base pair(s); kb, kilobase pair(s).
*This paper is no. 2 in a series. Paper no. 1 is ref. 3.

Table 1. *incA* but not *incB* blocks integrative suppression mediated by λ-P1:5R

Plasmid	*inc* present	Prophage at *loxB*	Relative survival (42°C/32°C)	Strain
None	—	λ-P1:7	3×10^{-6}	NS1502
None	—	λ-P1:5R	1	BR1622
pBR322	—	λ-P1:5R	1	BR1627
pALA6	*incB*	λ-P1:5R	1	BR1624
pALA18	*incA*	λ-P1:5R	4×10^{-6}	BR1712
pALA8	*incA+B*	λ-P1:5R	9×10^{-7}	BR1625

Plasmids are pBR322 derivatives and were described previously (3, 9). pALA6 has the Par region from a *Xho I* site to the end of P1:*Eco*RI-5 and pALA8 has the entire P1:*Eco*RI-5 (Fig. 1). pALA18 is described in *Materials and Methods*.

*Initial titer at 32°C was 2–4 × 10^7 colony-forming units per ml. The differences between relative survivals in lines 1, 5, and 6 are probably not significant.

ble bacteria. Interference with bacterial survival at 42°C by an introduced *inc* fragment is taken as a measure of interference with the replication initiated by the mini-P1 replicon.

The survival of the integratively suppressed bacteria was unaffected by the presence of a pBR322 plasmid or of a derivative carrying *incB*; when pBR322 carried *incA*, the survival of bacteria at 42°C was reduced to approximately the frequency of *dnaA*$^+$ revertants (Table 1). These results indicate that the incompatibility exerted by *incA*, in contrast to that exerted by *incB*, is attributable to its inhibitory effect on P1-initiated DNA replication. All following experiments are consistent with this concept.

The λ-P1:5R prophage present in BR1622 contains both the Rep and Par regions of P1. To confirm that the Par region is irrelevant to the *incA*-mediated nonviability observed at 42°C, we repeated the above experiments with a λ-P1:5R derivative, λP1-5RΔ1005, from which the entire Par region had been deleted (9). As expected, λ-P1:5RΔ1005 was competent for integrative suppression and was as sensitive to *incA* (and insensitive to *incB*) as λ-P1:5R (data not shown). To simplify interpretation of data, most of our experiments were, therefore, done in the absence of the Par region.

Repeats Alone Constitute the *incA* Locus. The *incA* locus was previously mapped to within an 800-bp fragment. It destabilized P1 plasmids when present as a single cloned copy in *trans* (11). To see whether the 306-bp *incA* fragment containing the nine 19-bp repeats was active, we cloned the fragment in one copy into a λ vector, λD69, to yield the phage DKC177 and repeated the experiment done previously with the 800-bp insert (9). The λ*incA* phage was as effective in destabilizing P1 plasmids as various deletion derivatives of λ-P1:5R that carried *incA* and additional flanking P1 DNA (ref. 9; data not shown). It appears that repeats alone constitute the *incA* locus of P1.

Subsets of Repeats from *incA* Exert Incompatibility. The existence of repeats in the *incA* fragment prompted us to determine whether a subset of the repeats could exert incompatibility. To this end, various portions of the *incA* fragment were cloned separately in one copy into the *Bam*HI site of pBR322, and their effects on integrative suppression by λ-P1:5R prophage and on stability of maintenance of λ-P1:5R plasmid prophage were analyzed (Fig. 2). The stability of λ-P1:5R was not affected by the presence of pBR322 alone. However, except for pALA96, which contains less than two repeats, all other subclones of the *incA* fragment caused ≥97% loss of λ-P1:5R in about 25 generations. Repeats 1–6 (pALA63) and repeats 4–9 (pALA66) were as active as the intact *incA* of pALA18 in blocking integrative suppression. However, three repeats were not enough to block integrative suppression. We conclude that the repeats themselves are responsible for *incA* activity and that although the number of repeats present may determine the magnitude of the response, no single site in the *incA* fragment needs to be retained. The activity of discrete portions of *incA* suggests that a protein product is not involved in *incA* activity.

Deletion of *incA* Releases Copy-Number Control. We next tested whether *incA* is essential for replication. If *incA* is solely a negative regulator of replication, then it might be possible to delete the *incA* sequences and obtain a high-copy-number mutant plasmid. pALA96, which has less than two *incA* repeats and fails to express incompatibility toward λ-P1:5R (Fig. 2), was used to construct the *incA* deletion plasmids used in this work. Loss of *incA* from the minireplicon pALA33 yielded pALA97 (Fig. 3) which, like its parent, was found to be replication-proficient in a *polA* host (CM5649) (3), but its copy number, as determined by a gel assay, was about 6-fold higher (data not shown). Subsequently, ≈4.2 kb of DNA including the origin of replication of pBR322 was deleted from pALA97 (Fig. 3, legend) to yield the minimal plasmid pALA139. This plasmid also repli-

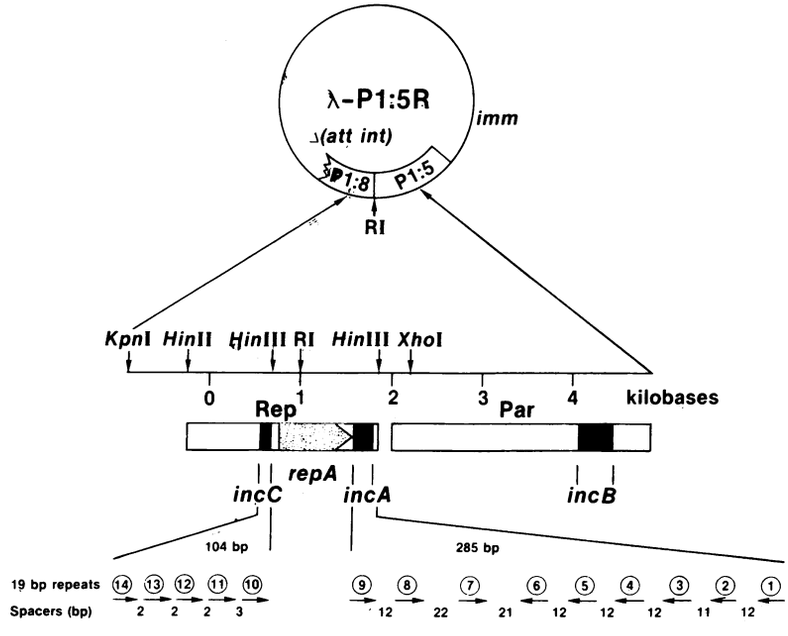

FIG. 1. Organization of the P1 plasmid maintenance region in λ-P1:5R phage. The 5′ guanosine of the *Eco*RI site is arbitrarily assigned the coordinate +1 kb (3). A 2.1-kb *Hin* II-*Hin* III fragment, marked Rep, is sufficient to confer the capacity for unit-copy replication on a plasmid. The adjacent region, marked Par, is involved in equipartition and includes *incB* (shaded rectangle). The Rep region contains sets of five and nine repeated sequences of 104 and 285 bp designated *incC* and *incA* (shaded rectangles), respectively. They flank the gene for the RepA protein (stippled area). The small arrows at the bottom represent the 19-bp repeats present in *incA* and *incC* sequences and indicate their orientations. Individual repeats are identified by circled numbers 1 to 14. The numbers below the gaps between the arrows indicate the number of base pairs in the spacer sequences between the repeats.

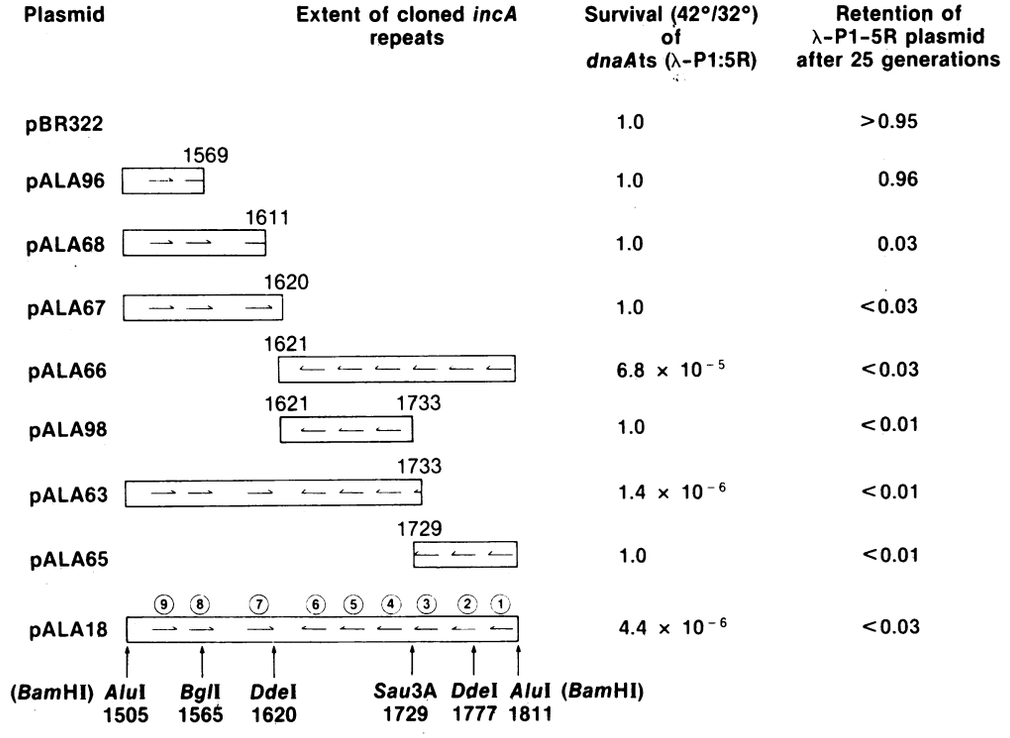

FIG. 2. Incompatibility expression from subsets of *incA* repeats. Various parts of *incA* were cloned into the *Bam*HI site of pBR322 by taking advantage of the restriction enzyme sites in *incA* as shown in the bottom line. P1 coordinates of the first base at the 5′ end of the restriction enzyme sites are shown below their names. Coordinates of the actual end points of the cloned fragments, as determined by DNA sequencing, are shown above the rectangles. Construction of pALA96 is described in the legend to Fig. 3. It contains repeat 9 and also has 15 of the 19 bp of repeat 8. pALA66, pALA67, and pALA68 were constructed by partially digesting the *Alu* I fragment with *Dde* I, filling in the ends of the resulting pieces using the Klenow fragment of DNA polymerase I and dNTPs, ligating the resultant mixture of fragments to *Bam*HI linkers, and cloning the sequences into pBR322. pALA68 has repeats 9 and 8 intact and has 16 of the 19 bp of repeat 7. pALA67 and pALA66 have repeats 9–7 and 1–6, respectively. pALA98 has *incA* repeats 4–6; this fragment was purified from pALA66 following *Sau*3A digestion. pALA63 and pALA65 were obtained by *Sau*3A digestion of the *Bam*HI fragment from pALA18 (bottom line) and cloning of the two *Bam*HI/*Sau*3A fragments. Measurements of integrative suppression and of λ-P1:5R stability are described in Table 1 and in ref. 9, respectively.

cated with a copy number of ≈6. These results directly implicate *incA* as a negative control element for mini-P1 plasmid DNA replication, a concept derived from earlier indirect studies. It also appears that at least most of *incA* is not essential for replication. It should be stressed that the *incA*-deleted plasmids still retain 46 bp of *incA* (coordinates 1524–1569, Fig. 2; ref. 3). Although this DNA is not enough to exert significant incompatibility, whether it is essential for replication has not been determined.

Relief of *incA*-Mediated Blockage of Integrative Suppression by P1 Subclones of the *repA* Region. To reveal the mechanism of action of *incA*, we tested whether any cloned P1 fragment could alleviate the inhibitory effect of *incA*. We found that clones containing the *repA* gene, when properly expressed, could counter the inhibitory effect of *incA*. These studies were done using blockage of integrative suppression as an assay for incompatibility and *incA* prophages as the source of the incompatibility-exerting element. Being integrated, the incompatibility-exerting and incompatibility-sensitive elements were stable. Also, *incA* could be provided in minimal gene dosage from integrated prophages so that their inhibitory effect could be overcome more easily.

One λ*incA* prophage integrated at the *attB* site failed to block integrative suppression (14). However, when the *dnaA*ts (λP1:5R) strain BR1622 was lysogenized with two λ*incA* phages, λ*imm*21*incA* (DKC177) and λ*imm*434-P1:*Eco*RI:5 (14), about one-third of the lysogens were nonviable at 42°C. One such isolate, BR2655, was used for subsequent studies (Table 2, line 1). It is likely that dilysogens of λ*incA* prophages were still viable at 42°C whereas those that had more than two prophages were not.

The effects of various levels of RepA protein on survival of BR2655 at 42°C are shown in Table 2. Variation in the level of the protein was achieved by controlling transcription. Maximal transcription was achieved from a pBR322 promoter in pALA162; the effectiveness of the promoter was reduced in pALA176 by interposing a partially active transcription terminator sequence between the promoter and the gene (Fig. 4). Transcription was even more reduced in pALA69 because the *repA* gene was oriented differently. The relative levels of *repA* transcription were determined by fusion of *repA* to the galactokinase gene (16) and found to be 24-, 5.5-, and 1.5-fold above background in pALA162, pALA176, and pALA69, respectively. The level of RepA, as judged from maxicell experiments (3), reflected the level of transcription; this level is described in qualitative terms in Table 2 because of the variable and unphysiological conditions of the maxicell experiments.

The results clearly show that the improvement of survival of BR2655 at 42°C is dependent upon the presence of the intact *repA* open reading frame and its proper expression. When intermediate levels of RepA were produced from pALA176, the survival of BR2655 was enhanced to the point where *incA* seemed to be entirely ineffective (Table 2, line 3). Both higher and lower amounts of the protein were marginally effective (Table 2, lines 2 and 4). Also, none of the *repA* plasmids could overcome the nonviability caused by a multicopy *incA* plasmid (data not shown), confirming the continued presence of the *dnaA*(ts) mutation. Although the effects of the relative levels of the RepA protein and the *incA* sequences are not yet fully understood in quantitative terms, these experiments are most simply interpreted in terms of a

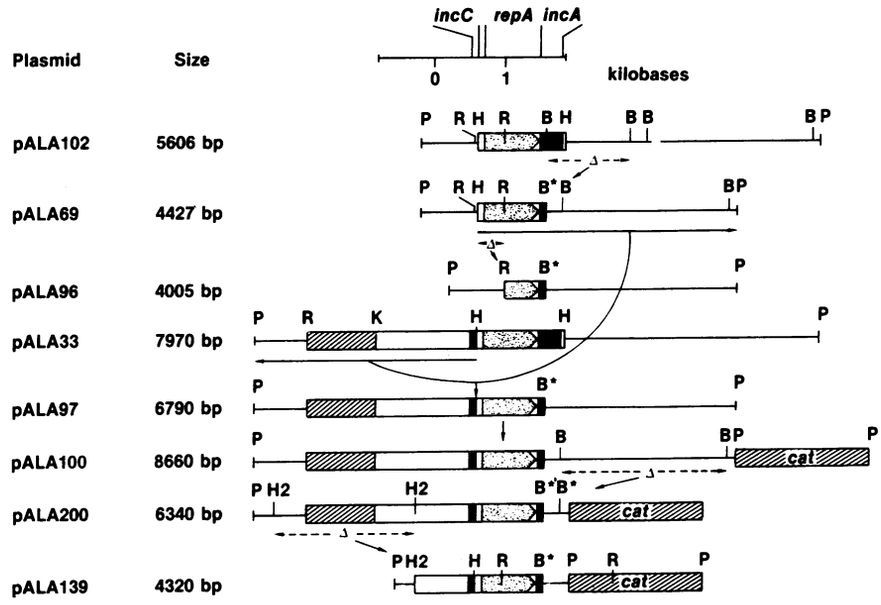

FIG. 3. Maps of pBR322 derivatives bearing fragments from the P1 Rep region. These fragments are represented by bars in which the *repA* open-reading frame is stippled and the *inc* loci are shaded. Circular plasmid maps are broken at the *Pst* I site at coordinate 3608 of the pBR322 map (11) and drawn as linear maps progressing from left to right instead of clockwise. Some of the restriction enzyme sites, used here as landmarks, are abbreviated as follows: *Pst* I (P), *Hin*dIII (H), *Eco*RI (R), *Bgl* I (B), *Kpn* I (K), and *Hin*cII (H2). The maps are aligned with respect to the *Eco*RI site at coordinate 1 kb of the P1 map (Fig. 1). The striped bars at the left represent DNA of the vector pKB111 (12), whose presence is incidental to this study, and the striped bars at the right represent the 1870-bp *Pst* I fragment from Tn*9* (13), which encodes the gene for chloramphenicol acetyltransferase (*cat*). The starting plasmid pALA102 carries the P1 *Hin*dIII fragment inserted at the *Hin*dIII site of pBR322. pALA69 was derived from pALA102 by deletion of the *Bgl* I fragment (←---Δ---→) from the *incA* repeat 8 (see Fig. 1) to the neighboring *Bgl* I site in pBR322 DNA. The protruding noncomplementary single strands at the *Bgl* I sites were removed with nuclease S1. After ligation, the hybrid site (B*) is resistant to *Bgl* I digestion. pALA96 was derived by deletion of the *Eco*RI fragment from pALA69. pALA33 has the entire Rep region as described previously (3). The *incA* region deleted from pALA69 was transferred to pALA33 by joining the appropriate *Pst* I-*Hin*dIII fragments (→). To the resultant plasmid pALA97 the *cat* gene was added, giving the chloramphenicol-resistance plasmid pALA100; pBR322 origin sequences contained in a 2.32-kb *Bgl* I fragment were deleted to yield pALA200. A 1.88-kb *Hin*cII fragment was deleted to yield pALA139, which has 1842 bp of P1 DNA and 659 bp of pBR322 DNA in addition to the *cat* gene.

direct interaction between the two components.

***In Vitro* Binding of the RepA Protein to the *incA* Fragment.** The results of our *in vivo* experiments strongly predicted binding of the RepA protein to the *incA* sequences. To test this prediction, the RepA protein was overproduced and purified to ≈90% homogeneity, as estimated by $NaDodSO_4$/PAGE (unpublished data). Binding of the protein to DNA was assayed as described (17, 18). This assay relies on the different electrophoretic mobilities of protein–DNA complexes and free DNA in a polyacrylamide gel. Fig. 5 shows results of an experiment in which a mixture of a ^{32}P-labeled *incA* fragment and a control restriction fragment from pBR322 were incubated together with different concentrations of the RepA protein. The mobility of only the *incA* fragment was retarded and several discrete new bands appeared. This result confirms the results obtained *in vivo* and shows directly that the RepA protein binds specifically to *incA*.

Table 2. Relief by *repA* of *incA*-mediated blockage of integrative suppression by λ-P1:5R

Plasmid	RepA level*	Relative survival (42°/32°)
pBR322	—	5×10^{-4}
pALA162	high	3×10^{-3}
pALA176	intermediate	1
pALA69	low	1×10^{-3}
pALA96	none	1×10^{-4}

Relative survival was measured as described in Table 1 and in the text. Plasmids are described in Figs. 3 and 4 and their legends. All except pBR322 contain the *incA* repeat 9 and part of repeat 8 (Fig. 3). pALA162, pALA176, and pALA69 contain *repA*; pALA96 lacks part of *repA* so that the RepA protein is not expressed.
*Indicates intensities of RepA protein band in maxicell experiments.

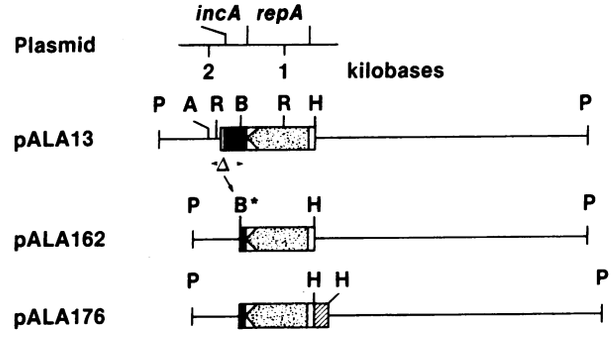

FIG. 4. Maps of plasmids that produce the RepA protein. Symbols are described in Fig. 3. pALA13 is the same as pALA102 (Fig. 3) except for the orientation of P1 DNA with respect to the pBR322 sequences. In pALA13, the *repA* open reading frame is transcribed from a pBR322 promoter, *bla-p2*, located immediately to the right of the *Hin*dIII site (H). This results in overproduction of the protein as described (3). A 394-bp DNA fragment between the pBR322 *Aat* II site (A) and the *Bgl* I site (B) in *incA* was deleted from pALA13 to yield pALA162. (B* in pALA162 is no longer a *Bgl* I site and is indicated for position only.) The levels of RepA protein derived from pALA13 and pALA162 are identical, but the levels from pALA176 are reduced because of the interposition of a 190-bp transcriptional terminator, T1 (hatched box), at the *Hin*dIII site (H). T1 terminator is described in ref. 15.

FIG. 5. Binding of RepA protein to *incA* fragment. The *incA* fragment with *Bam*HI linkers (320 bp) and the *Bam*HI/*Sal* I control fragment from pBR322 (281 bp) were obtained simultaneously by *Bam*HI and *Sal* I digestion of pALA18 (pBR322 + *incA*, see *Materials and Methods*). The fragments were end-labeled, using the Klenow fragment of DNA polymerase I and [α-^{32}P]dNTPs, and mixed with various amounts of partially purified RepA protein. The relative amounts are shown at the top of the lanes. The mixture was electrophoresed on a 5% polyacrylamide gel. The autoradiogram shows that the *incA* fragment formed several slow moving bands, apparently by complexing with the protein. The mobility of the control pBR322 restriction fragment (lower band) was not affected.

DISCUSSION

In this paper, we provide direct evidence that the repeating sequences of *incA* are involved in the stringent copy-number control of mini-P1. A single extra copy of all nine repeats or various three-repeat pieces cloned in high-copy-number vectors can effectively inhibit DNA synthesis originating in a mini-P1 plasmid. Conversely, deletion of *incA* repeats from a mini-P1 plasmid increases its copy number. In the stringently controlled sex-factor plasmid F, whose plasmid maintenance region is organized similarly to that of P1 (3), replication is also controlled by dispensable repeated sequences that exert incompatibility (5). It has been postulated that the repeated DNA sequences of the *inc* loci control replication by titrating out a factor essential for the initiation event (5). Our observations show that this model is applicable to P1. Moreover, we provide direct confirmation of the model with evidence that overproduction of RepA can overcome the inhibitory activity of the repeated sequences and that RepA binds specifically to these sequences. The findings that mutations that result in increased plasmid copy-number are located in the *repA* locus of P1 (19) and also in the analogous locus of F (20, 21) are also consistent with the hypothesis.

We recently mapped three promoter activities associated with repeat sequences: two in *incC* and one in *incA*. The RepA protein could repress all three promoters but not a fourth mini-P1 promoter that mapped outside of the repeats (unpublished data). These results are consistent with the notion of RepA–*incA* interaction *in vivo*.

The surprising result that the greater amount of RepA produced in pALA162 was less effective in countering the effect of *incA* than the smaller amount of the protein produced in pALA176 led us to conclude that, at higher concentration, the protein itself inhibits replication. Indeed, pALA162, but not pALA176, destabilized λ-P1:5R or pALA139 strongly (unpublished results). Thus, although RepA is required for replication, it also appears able to act as a negative control element in addition to *incA*.

Plasmids other than P1 and F have repeated DNA sequences associated with their origin of replication and/or incompatibility loci. These include λdv (22), RK2 (23), R6K (24), Rts1 (25), and pSC101 (26). In the cases of λdv (22), R6K (27), and pSC101 (28), plasmid-encoded initiator proteins have been shown to have sequence-specific DNA-binding activity. We report here evidence of RepA binding to *incA* sequences *in vitro* in the case of a stringently controlled replicon P1. Thus, control of DNA replication by means of repeated DNA sequences appears to be a common mechanism.

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