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Regulatory Interactions between RepA, an Essential Replication Protein, and the DNA Repeats of RepFIB from Plasmid P307

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The control of RepFIB replication appears to rely on the interaction between an initiator protein (RepA) and two sets of DNA repeat elements located on either side of the *repA* gene (BCDD'D" and EFGHIJ). In vivo genetic tests demonstrate that the BCDD'D" repeats form part of the origin of replication, while some of the downstream repeat elements (HIJ) are involved in the sensing and setting of plasmid copy number. RepA DNA binding to these groups of repeats has been investigated in vivo by utilizing the fact that the replicon contains three active promoters (ori_p, $repA_p$, and EF_p), one of which has previously been shown to control the expression of *repA* ($repA_p$). All three promoters are closely associated with the repeat elements flanking *repA*, and an investigation using *lacZ* or *cml* gene fusions has demonstrated that RepA expressed in *trans* is able to repress each promoter. However, these assays suggest that the transcriptional responses of ori_p and $repA_p$ to RepA repression are significantly different, despite the fact that both promoters are embedded within the BCDD'D" repeat elements. Extra copies of the BCDD'D" or EFG repeats in *trans* have no effect on RepA repression of $repA_p$ embedded in a second copy of the BCDD'D" repeats, but copies of the HIJ or EFGHIJ repeats are able to derepress $repA_p$, suggesting that there is a fundamental difference between RepA-BCDD'D" or -HIJ complexes and RepA-EFG or -EFGHIJ complexes.

The RepFIB replicon is a step function replicon (30) expressing *incE* incompatibility with a tightly regulated copy number of 1 to 2 copies per chromosome (5, 23, 30, 35). The term "step function" refers to the very rapid and stringent manner in which a replicon (such as mini-F or mini-P1) responds to an artificially high copy number by preventing further replication until the copy number is sufficiently reduced by cell division. The replicons which show such a response share a similar genetic organization, and copy number control appears to be achieved through the interaction between an initiator protein and sets of DNA repeat elements located on either side of the initiator gene. RepFIB replicons from the enterotoxinogenic plasmid P307 (35, 38) and the colicin-producing plasmid pColV3-K30 (32, 33) have been examined at the genetic level, and a sequence comparison has previously shown that there is remarkably little variation among RepFIB replicons, despite the diverse nature of the parental plasmids (17).

Like all step function replicons (12), RepFIB contains a single initiator gene (*repA*) which is flanked by a series of repeat elements (upstream, BCDD'D"; downstream, EF GHIJ; see Fig. 1). The *repA* promoter ($repA_p$) is located within the BCDD'D" repeat elements, and RepA translation is initiated from a CTG start codon to express a 39-kDa protein (38). The expression of the gene is autoregulated by RepA binding to the BCDD'D" repeats (15, 38), which presumably prevents RNA polymerase access to $repA_p$. Repression can also be achieved when only the D" repeat is present (38), and RepA DNA binding to the repeat elements may be cooperative (15). RepA DNA binding to the BCDD'D" repeats has been demonstrated in vitro by filter-binding studies (15) and by Western-DNA analysis in which RepA bound to a nitrocellulose membrane was incubated with probe DNA (38).

The organizational homology between RepFIB and the step function replicons strongly suggests that the BCDD'D" repeat elements and ≈200 bp of DNA to the left of repeat B represent the origin of replication. The homology analysis also predicts that the downstream repeat elements of RepFIB are involved in the sensing and setting of plasmid copy number, although it is known that the HIJ repeats are not essential for replication (36). Step function replicons appear to control replication and limit plasmid copy number by autoregulation of the initiator gene and titration of the initiator against the downstream repeat elements. However, the concurrent autoregulation and titration have presented a paradox for control models which has only recently been circumvented by the proposal of several DNA-looping models (2, 31). While the finer details of these models differ, they suggest that copy number control might be achieved through the formation of DNA-initiator protein complexes producing DNA loops (intermolecular linkages) in which the initiator protein binds to both the origin and titration repeat elements or intramolecular linkages in which the initiator protein joins two copies of the replicon in a parallel or antiparallel manner. For mini-P1 and R6K, inter- and intramolecular linkages caused by the DNA binding activity of the initiator protein have been visualized by electron microscopy, and genetic evidence supporting such linkages in mini-P1 and R6K has been reported previously (24, 26, 27, 31).

Our current focus is on determining whether RepFIB replication involves the same kind of control elements found in other step function replicons. In this work, we present in vivo and in vitro experimental results which demonstrate RepA DNA binding to the BCDD'D" and EFGHIJ repeats. We have examined the functional significance of RepA DNA binding, and in doing so we have located the origin of replication and titration elements of the replicon. Our observations of the behavior of RepA and the protein's interaction with the repeat elements suggest that the organizational homology seen between RepFIB and step function replicons

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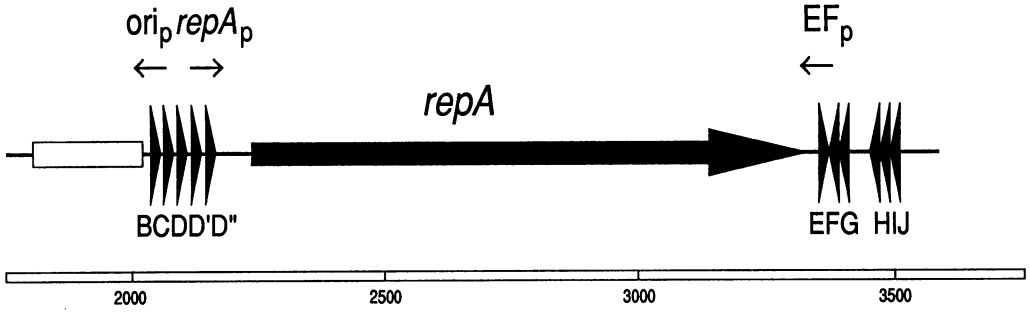

FIG. 1. RepFIB replicon. The RepFIB replicon of P307 contains a single initiator gene (*repA*) flanked on either side by a number of repeat elements (BCDD'D" and EFGHIJ; the A and K repeat elements lie outside the replicon region). The BCDD'D" repeat elements are associated with a number of origin-like sequences (boxed) and contain two active promoter elements, *ori*$_p$ and *repA*$_p$. The EF repeats contain a third promoter, EF$_p$. The promoter sequences are given in Results. The scale below is in base pairs. The coordinates are from Saul et al. (35).

can be extended to functional similarity, despite the fact that the replicons share little DNA sequence homology.

MATERIALS AND METHODS

Bacterial strains and transformation. *Escherichia coli* DH5α [F$^-$ φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR17*(r_K^- m_K^+) *supE44* λ$^-$ *thi-1 gyrA relA1*; Bethesda Research Laboratories] and PB1905 [F$^-$ *ara* Δ(*lac-pro*) *thi*, streptomycin resistant; a gift from B. Kline] were used for general cloning purposes and in vivo repression assays, while PB1849 [F$^-$ *leu thi thy* Δ(*proB-lac*) *dra*/*drm dnaA46*(Ts), streptomycin resistant; D. Lane], PB2989 [*thi-1 dam-3 lacU169* λ(*tyrP-lac*); a gift from A. J. Pittard], DK249 (*sdrA244 dnaA850*::Tn*10 ilv metB his-29 trpA9605 thyA deoB* [or *C*] *rpoB*) (20), AQ699 (*sdrA224 metD88 ilv metB his-29 trpA9605 thyA deoB* [or *C*] *rpoB*) (20), and the isogenic pairs PB1965 (Tn*10*, tetracycline resistant) and PB1966 (*polA1*::Tn*10*, tetracycline resistant; P. L. Bergquist) were used for replication assays. Competent cells were prepared and transformed by the method of Lederberg and Cohen (21). AQ699 and DK249 were grown in minimal (defined) medium with 56/2 salts (4) with appropriate supplements, while the other strains were grown in L medium (22).

Plasmid DNA. All RepFIB DNA used to construct new plasmids was isolated from pNZ945, Xho31 (35), or pNZ965 (this laboratory) or pAS1, pAS38, or pAS40 (39). pHP45 was used as a source of the omega (Ω) resistance fragment (34). pBS$^+$ (Stratagene) was used for general cloning purposes. Gene fusions were formed with pKK232-8 (8) and pMU575 (43). pKK232-8 carrying an active promoter expresses chloramphenicol acetyltransferase, while a fusion in pMU575 will express a hybrid β-galactosidase. pHSG576 (40) and pUC19 (44) have been previously used as controls in some assays. In some assays, RepA was expressed in *trans* from pAS60 (38). This plasmid also carries a copy of *lacI*^{q} which fully represses the expression of RepA from p_{tcr} unless induced with IPTG (isopropyl-β-D-thiogalactopyranoside).

pNZ945, pNZ955, *Sna*BI-*Bal*I Δ, pSS3928, pMA4322, and pWM114 are all RepFIB miniplasmids. In pNZ945, the E11 fragment from P307 which carries RepFIB has been ligated to pBS$^+$ (35); in pWM114, it was ligated to pBR325 (a gift from W. Maas), while in pSS3928 it was ligated to the Ω fragment (a gift from S. Saadi). pUC19 was inserted into the Ω portion of pSS3928 to give pNZ955 (37). *Sna*BI-*Bal*I Δ is a deletion derivative of pNZ945 and lacks the HIJ repeat elements (35). pMA4322 (a gift from W. Maas) is a copy mutant of pSS3928 which has a single-residue alteration in RepA (35). pNZ965 carries the RepFIB *Pst*I 1.2-kb fragment in pCGN565 (39).

Plasmid construction. The 200-bp *Bam*HI-*Pst*I, 480-bp *Hin*dIII-*Bam*HI, and 210-bp *Hin*dIII-*Sna*BI fragments from pNZ945 were ligated into pBS$^+$ to give pAS4, pAS15, and pAS16, respectively. pAS15 was digested with *Sna*BI and *Sal*I, treated with Klenow fragment to produce blunt ends, and then self-ligated to give pAS16. The 270-bp *Sna*BI-*Bam*HI fragment from pNZ945 was ligated to *Sma*I–*Bam*HI-cut pHSG576 to give pAS18. pAS19 and pAS20 are *Pst*I and *Bam*HI deletions of Xho31. pAS21 and pAS22 contain the 200-bp *Bam*HI fragment from pAS19 in pKK232-8 in both orientations. In pAS21, the plasmid *cml* gene is controlled by *repA*$_p$, while in pAS22 the gene is controlled by *ori*$_p$. The Ω fragment was inserted at the *Bam*HI site of the RepFIB portion of pAS19 to give pAS28. The 480-bp *Hin*dIII-*Bam*HI fragment from pNZ945 was ligated into pMU575 to give pAS31. In this plasmid, the hybrid β-galactosidase gene is controlled by EF$_p$. The 200-bp *Bam*HI fragment from pAS19 was ligated into pMU575 in both orientations to produce pAS32 and pAS33. In pAS32, *ori*$_p$ controls the expression of the hybrid β-galactosidase gene, while in pAS33 the gene is controlled by *repA*$_p$. pAS19 was digested with *Bam*HI, and the DNA was religated. A recombinant plasmid containing a *Bam*HI fragment inversion was isolated to give pAS36. The ≈0.8-kb *Pst*I fragments from pAS38 and pAS40 were ligated to pBS$^+$ to give pAS58 and pAS59, respectively. pAS60 and pACYC184 (9) were digested with *Sal*I and *Bam*HI, respectively, and then treated with Klenow fragment. The DNAs were then ligated to produce pAS64. The Ω fragment was inserted at the *Sna*BI site of pAS15 to give pAS65. pNZ965 was digested with *Pfl*MI and then treated with Klenow fragment before being digested with *Eco*RI to release a 1.14-kb fragment. This fragment was then ligated to the 6.26-kb *Eco*RI-*Bam*HI fragment of pAS1 (in which the *Bam*HI 3′ overhang had been filled in with the Klenow fragment) to give pAS69. The *Eco*RI-*Bam*HI fragment of pAS1 is identical to *Eco*RI–*Bam*HI-cut pMLB1034 (36).

MIC assays. Mid-log-phase test cultures were diluted into L broth (1/200) and then aliquoted into tubes with final concentrations of 0 to 250 μg of chloramphenicol per ml. These cultures were incubated overnight at 37°C before bacterial growth was measured by determining the culture's optical density at 600 nm. pAS21 and pAS22 were maintained in DH5α with 100 μg of ampicillin per ml.

Repression assays. The repression of various promoter-probe plasmids carrying *repA*$_p$, *ori*$_p$, or EF$_p$ promoters was tested on agar plates and in liquid cultures. The repression of

pAS21 and pAS22 by pMA4322 was demonstrated with agar plates which had been overlaid with strips of paper containing chloramphenicol. Strips of Whatman filter paper were soaked in 20 mg of chloramphenicol and then placed onto dry L-agar plates which had been spread with an early-log-phase culture. The plates (with the strips) were incubated overnight at 37°C before the zone of inhibition was measured. pAS21 and pAS22 were maintained in DH5α with 100 μg of ampicillin per ml, and pMA4322 was maintained with 50 μg of spectinomycin per ml. The repression of pAS9, pAS31, pAS32, and pAS33 by pAS60 and pAS64 induced with IPTG was examined by assays of cells taken from liquid cultures. Test cultures were inoculated from overnight cultures (1/100 dilution) and grown at 37°C for 90 min in L broth to reach early to mid-log phase before portions were added to tubes containing various concentrations of IPTG. These cultures were incubated for 2 h further before β-galactosidase activities were determined (25). pAS60 was maintained in PB1905 with 100 μg of ampicillin per ml, while pAS31, pAS32, and pAS33 were maintained with 10 μg of trimethoprim per ml. pAS64 was maintained in DH5α with 25 μg of chloramphenicol per ml, pAS9 was maintained with 40 μg of kanamycin per ml, and pAS4, pAS15, pAS16, pAS17, pAS58, pAS59, and pAS65 were maintained with 50 μg of ampicillin per ml.

Copy number determination. Plasmid copy numbers were determined in PB1966 (Pol^-) by preparing total cellular DNA, dot blotting it onto a GeneScreen membrane (New England Nuclear), and hybridizing it with a suitable RepFIB probe according to a previously established procedure (23).

Western-DNA analysis. We have used a modification of the standard Western transfer technique to demonstrate RepA DNA binding activity in vitro (38). Overexpressed RepA protein has been previously isolated in a crude extract (≈40 to 50% RepA), and by following the protocol described below, we have been able to recover RepFIB DNA-specific RepA DNA binding activity (38).

The complex mixture of proteins was first separated by sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis. After electrophoresis, the gel was washed in transfer buffer before being electrotransferred to a Hybond-ECL (Amersham) nitrocellulose membrane. The membrane was washed three times in Tris-buffered saline (TBS) (20 mM Tris, 137 mM NaCl [pH 7.6])–0.1% Tween 20 for 5 min each time. The membrane was then incubated in a blocking solution (TBS, 0.1% Tween 20, 3% nonfat milk powder, 5 μg of sonicated calf thymus DNA per ml) for 2 h before being incubated in TBS–0.1% Tween 20–0.6% nonfat milk powder–5 μg of sonicated calf thymus DNA–200 to 500 ng of ^{32}P-end-labelled probe DNA for 2 h further at room temperature. Unbound probe DNA was removed by three washes in TBS–0.1% Tween 20 for 5 min each before autoradiography.

RESULTS

Identification of the origin of replication. The origin of replication (≈1,900- to 2,200-bp coordinates) was initially defined by a sequence analysis of the RepFIB replicon which identified a number of origin sequence features associated with other plasmid replicons (these features are shown in Fig. 2) and by a comparison of RepFIB and mini-P1. The most characteristic features of the putative origin of replication are the BCDD'D" repeat elements, a single *dnaA* box, and several small repeat elements which include the Dam methylase recognition sequence, GATC. The replicative ability of this region was tested genetically by a DNA polymerase I (PolI) rescue assay which relied on the fact that RepFIB replication is independent of PolI (16) and the assumption that RepFIB consists of a functionally divisible origin of replication and a *trans*-activating initiator protein (this assumption was supported by the similarities between RepFIB and other step function replicons). In such a system, a PolI-dependent plasmid such as pBS^+ containing the RepFIB origin should be able to replicate in a Pol^- host if RepA was supplied in *trans*.

We have investigated the ability of the putative RepFIB origin to rescue pBS^+ by forming a number of pBS^+-origin plasmids and testing their replicative abilities in a Pol^- host (PB1966) in which RepA was supplied in *trans* from a copy mutant RepFIB miniplasmid (pMA4322). From the results given in Table 1, it is clear that pAS19 containing the complete putative origin is capable of replication. However, if either of the BCDD'D" repeats (pAS19) or the *dnaA* box section of the putative origin is removed (pAS4), the pBS^+-origin plasmid is unable to replicate in the Pol^- host carrying pMA4322. Replication is still possible if the BCDD'D" repeats are inverted with respect to the *dnaA* box (pAS36), but origin activity is completely lost if the repeats and the *dnaA* box are separated by the insertion of 2 kb of DNA (pAS28).

In our initial trials of this experiment, we attempted to compare the rescue of pBS^+-origin plasmids in Pol^+ and Pol^- strains which contained the wild-type RepFIB miniplasmid pSS3928 with the rescue in strains containing the copy mutant pMA4322. However, we were unable to rescue any plasmids when RepA was supplied in *trans* by pSS3928. These results may be due to either a higher level of expression of the mutant RepA by pMA4322 than by pSS3928 (35) or perhaps a greater tolerance to incompatibility elements present in *trans* resulting from the expression of an altered RepA from pMA4322 (there is a 1-residue difference in the RepA proteins expressed by pSS3928 and pMA4322 [35]).

Replication requirement for Dam and DnaA. The putative origin of replication contains a single perfect *dnaA* box and a number of small repeats which include the sequence GATC. The appearance of *dnaA* boxes and GATC repeats in the origins of other low-copy-number replicons (such as mini-P1) has usually correlated well with a replication requirement for host-encoded DnaA and Dam methylase (for examples, see references 1, 3, 18, 19, and 42). RepFIB replication dependence on host Dam and DnaA was tested by comparing the relative transformation frequencies of RepFIB plasmids into *dam*/*dam*$^+$ and *dnaA*/*dnaA*$^+$ strains (Table 2). The use of chimeric RepFIB miniplasmids containing Dam or DnaA replication-independent replicons in conjunction with Dam or DnaA replication-independent plasmids provided positive controls for these assays. RepFIB miniplasmids are unable to transform *dam* and *dnaA* strains, indicating that RepFIB replication is dependent on host Dam and DnaA functions. The DnaA-dependent replication of RepFIB was also tested with a temperature-sensitive DnaA strain (PB1849). PB1849 containing a chimeric RepFIB miniplasmid (pNZ945) which includes a DnaA-independent replicon was able to grow at the nonpermissive temperature of 42°C in the presence of antibiotic selection. However, PB1849 transformed with a miniplasmid containing only the RepFIB replicon (pSS3928) was unable to grow at 42°C, confirming that RepFIB replication is DnaA dependent.

Copy numbers of RepFIB miniplasmids. The copy number of the RepFIB miniplasmid pSS3928 is 1 to 2 copies per

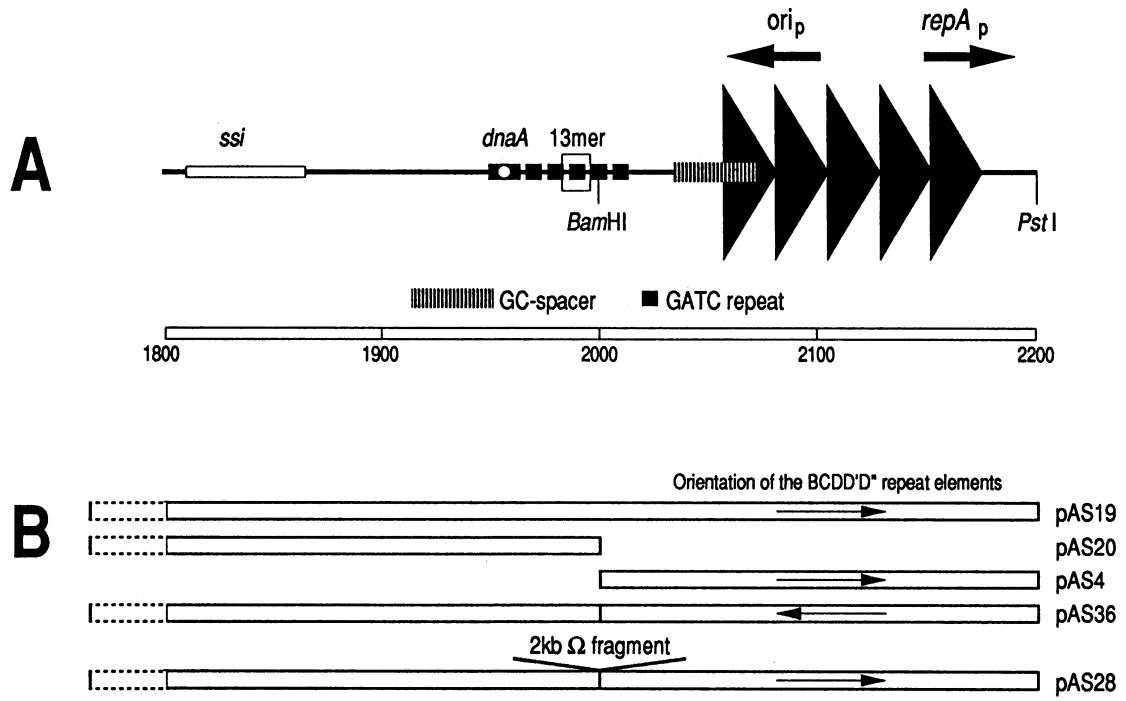

FIG. 2. Sequence features of RepFIB origin region. (A) The region between the 1,800- to 2,200-bp region of RepFIB contains a number of features identified by sequence homology, which suggests that it represents the origin of replication. The region includes five direct repeat elements, BCDD'D" (large triangles) (35, 38), seven small repeats associated with the sequence GATC recognized by Dam methylase (■), a single *dnaA* box (○) (13), and a single-strand initiation sequence (*ssi*) (29). The best of four potential 13-mer sequences (6) is located next to the *dnaA* box, and a GC spacer (broken box) homologous to the mini-P1 spacer (7) is located to the right of the GATC repeats and overlaps the B repeat element. The region also contains two transcriptionally active promoters located within the BCDD'D" repeats, ori_p (transcription to the left) and $repA_p$ (transcription to the right). Promoter sequences are given in Results. (B) The regions of the origin used to produce pBS$^+$-origin plasmids, shown schematically. pAS19 contains the entire origin, pAS20 contains the *dnaA* box section, and pAS4 contains the BCDD'D" repeats. The *Bam*HI fragment containing the BCDD'D" repeats in pAS19 has been inverted to give pAS36, while the Ω fragment has been inserted between the *dnaA* box and the repeats in pAS28. The scale between panels A and B is in base pairs according to Saul et al. (35).

chromosome in a Pol$^-$ host (23, 35). We have determined that the copy number of a RepFIB plasmid with the HIJ repeat elements removed (*Sna*BI-*Bal*I Δ) increases to 2 to 4 copies relative to pSS3928. If the HIJ repeats were present in *trans* (carried by pAS18), the copy number of *Sna*BI-*Bal*I Δ increases to ≈10 copies per chromosome. From these results, we conclude that the EFGHIJ repeats are involved in the sensing and setting of RepFIB copy number. Since the copy number of RepFIB replicons increases when the HIJ repeats either are deleted from the miniplasmid or are added in *trans*, we conclude that the repeats are binding or titrating an inhibitor of replication. In the context of this analysis, the inhibitor is presumed to be RepA (the paradox generated by the initiator and inhibitor qualities of RepA is a common feature of step function replicons; for a review, see reference 41).

TABLE 1. Origin rescue assay[a]

Plasmid	Characteristic feature(s)	Efficiency (%)[b]
pHSG576	PolI-independent replication	100
pBS$^+$	PolI-dependent replication	<0.3
pAS20	*dnaA* box section only	<4
pAS4	BCDD'D" repeat section only	<1
pAS19	*dnaA* box and BCDD'D" sections	34
pAS36	*dnaA* box and inverted BCDD'D" section	27
pAS28	Ω inserted between the *dnaA* box section and the BCDD'D" repeats	<0.6

[a] pMA4322 was maintained with 50 μg of spectinomycin per ml. Transformant colonies containing pHSG576 were selected with 25 μg of chloramphenicol per ml, and pBS$^+$, pAS4, pAS19, pAS20, pAS28, and pAS36 were selected with 50 μg of ampicillin per ml.

[b] Efficiency is the ratio of transformation frequencies of PB1966 plus pMA4322 and PB1965 plus pMA4322 (Pol$^-$/Pol$^+$) for which the relative competencies of the two strains have been corrected so that transformation with pHSG576 gives a value of 100%.

Identification of the ori_p and EF_p promoters and comparison with $repA_p$. The ori_p and EF_p promoter sequences were initially identified by an analysis of RepFIB sequence with TargSearch (28) and have been named on the basis of their locations in the RepFIB replicon (ori_p: −35, AGTTTATC CGTAACAT, 2,092 to 2,077 bp; −10, AGCTTATGTTAT CG, 2,071 to 2,058 bp, with a TargSearch score of 49%; and EF_p: −35, AGTGCTCTTCACTGAC, 3,326 to 3,311 bp; −10, AGCGGGATTTGAAG, 3,296 to 3,283 bp, 44%; coordinates from Saul et al. [36]). ori_p is embedded within the BC repeat elements and is in the opposite orientation to the *repA* promoter (−35, ACATAAACTATGGTCA, 2,149 to 2,164 bp; −10, AGTTGTTAAATACA, 2,178 to 2,191 bp, 47% [38]), which is located in the D" element ≈40 bp away. EF_p is embedded within the EF repeat elements located downstream of *repA*.

TABLE 2. Dam methylase and DnaA transformation assays

Assay and plasmid[a]	Characteristic	Replication efficiency (%)[b]
Dam methylase		
pBS$^+$	Dam-independent replicon	100
pSS3928	RepFIB miniplasmid	<6.8
pMA4322	RepFIB miniplasmid copy mutant	<9.5
pNZ955	RepFIB-pUC19 hybrid	61.3
DnaA		
pUC19	DnaA-independent replicon	100
pHSG576	DnaA-dependent replicon	<0.05
pMA4322	RepFIB miniplasmid copy mutant	<0.05
pWM114	RepFIB-pBR325 hybrid	100

[a] Transformant colonies containing pBS$^+$, pUC19, pNZ955, and pWM114 were selected with 50 μg of ampicillin per ml, pSS3928 and pMA4322 were selected with 50 μg of spectinomycin per ml, and pHSG576 was selected with 25 μg of chloramphenicol per ml.

[b] The replication efficiency is the ratio of transformation frequencies of PB2989 and PB2946 (Dam$^-$/Dam$^+$) calculated so that transformation with pBS$^+$ gives a value of 100% or that with pUC19 with AQ699 and DK249 (DnaA$^-$/DnaA$^+$) gives a value of 100%.

The ori_p and EF_p promoter sequences were used to produce gene fusions in pMU575, which is a low-copy-number promoter-probe plasmid (43). Cells containing the fusion plasmids (pAS31 and pAS32) expressed β-galactosidase, indicating that the promoters were transcriptionally active. ori_p is not positioned to express an open reading frame (ORF) in RepFIB. The ability of EF_p to express a protein from ORF4 (35) was examined with an ORF4–β-galactosidase fusion plasmid (pAS69). However, cells containing pAS69 did not express significantly more β-galactosidase activity than the appropriate control strain, suggesting either that EF_p transcripts did not extend the necessary ≈700 nucleotides to reach ORF4 or that although the transcripts reached the ORF, the reading frame is not translated.

The 200-bp *Bam*HI-*Pst*I fragment containing ori_p also contains $repA_p$. For comparative purposes, a $repA_p$ gene fusion was produced in pMU575 (pAS33), while ori_p and $repA_p$ gene fusions were also produced in pKK232-8 (pAS21 and pAS22). β-Galactosidase assays of cells containing pAS31, pAS32, and pAS33 indicated that the transcriptional strengths of ori_p and $repA_p$ were comparable, while EF_p was ≈10× weaker. Examination of pAS21 and pAS22 in DH5α by MIC liquid culture assays and the measurement of growth inhibition caused by the diffusion of chloramphenicol from strips of filter paper placed on agar plates confirmed that ori_p and $repA_p$ are of similar strengths (data not shown).

Repression of EF_p, ori_p, and $repA_p$ by RepA in *trans*. In previous work, we have demonstrated that *repA* is autoregulated by RepA binding to the BCDD′D″ repeat elements (38). Since ori_p and EF_p were also embedded within copies of the repeat elements, we have tested whether RepA expressed in *trans* can also repress the transcriptional activity of these promoters. From the results presented in Fig. 3 it is clear that all three promoters are repressed by RepA. The repression characteristics of ori_p and EF_p appear to be similar, and the reduction of transcriptional activity appears to begin with levels of RepA expression corresponding to induction with 1 μM IPTG. In contrast, $repA_p$ repression is more gradual than that of the other two promoters and appears to be more sensitive to levels of RepA. The repression of $repA_p$ begins at 0.125 μM IPTG, one-eighth of the level required to begin the repression of ori_p and EF_p.

Demonstration of RepA DNA binding to the BCDD′D″, EFG, and HIJ repeat elements in vitro. We have used the Western-DNA procedure to demonstrate that RepA is capable of binding specifically to RepFIB DNA containing copies of the BCDD′D″ repeat elements (38). We have extended our analysis to show that RepA will bind plasmid DNA carrying copies of the EFG and HIJ repeats as well (Fig. 4). Although a small amount of pBS$^+$ plasmid DNA is bound nonspecifically by RepA, increased washing of the Western-DNA membrane after probe incubation reduces the level of binding compared with that by a pAS4 probe containing the BCDD′D″ repeats. pAS20 contains the A repeat element found outside the replicon region of the E11 fragment. Since pAS20 probe DNA is bound as weakly as pBS$^+$ DNA, we assume that the A repeat element does not bind RepA. This conclusion is supported by the observation that the A repeat

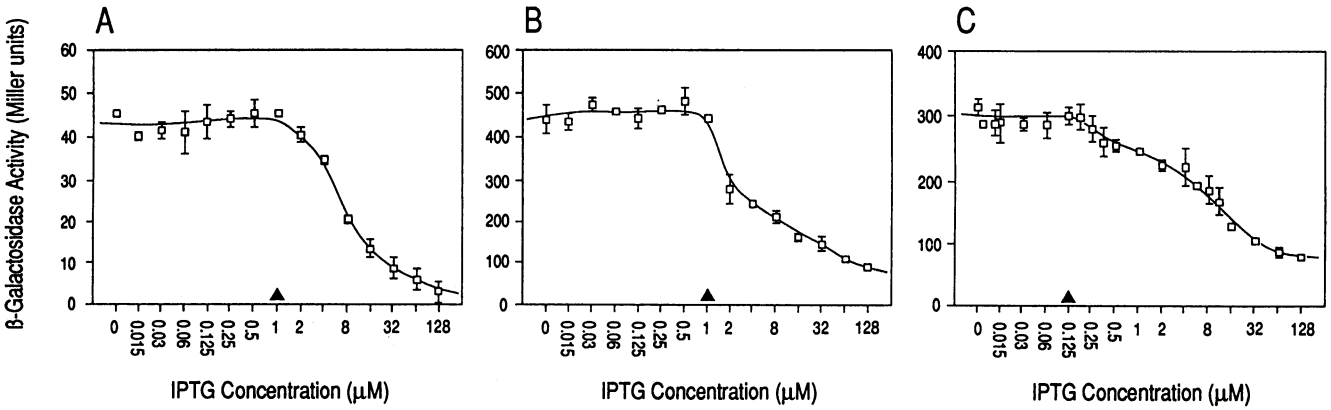

FIG. 3. Repression of EF_p, ori_p, and $repA_p$ by RepA in *trans*. RepA expressed from pAS60 represses three different promoters controlling the expression of a hybrid β-galactosidase gene in pAS31, pAS32, and pAS33. The repression of EF_p in pAS31 (A), ori_p in pAS32 (B), and $repA_p$ in pAS33 (C) is shown. Arrowheads mark the IPTG concentrations at which sufficient RepA is produced to begin to repress each promoter. Mean Miller units (± standard error) and the lines of best fit are shown. The assays were performed in PB1905 with mid-log phase cultures. pAS60 was maintained in PB1905 with 100 μg of ampicillin per ml, and pAS31, pAS32, and pAS33 were maintained with 10 μg of trimethoprim per ml.

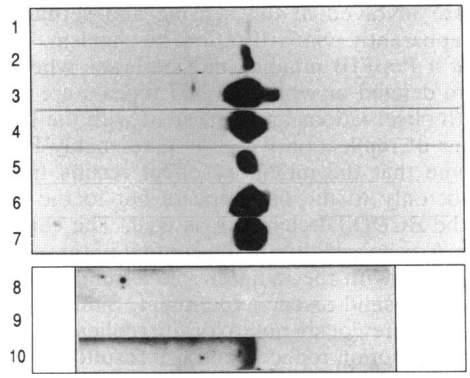

FIG. 4. In vitro RepA DNA binding. RepA DNA binding was investigated by Western-DNA analysis as described in Materials and Methods. Membrane strips were incubated with various plasmid probes. Each strip has been positioned so that the origin is at the left border and the dye front is at the right border of the box. Lanes: 1, pBS$^+$ (control); 2, pAS20 (containing the *dnaA* box section of the origin and repeat A); 3, pAS19 (*dnaA* box section and repeats A and BCDD'D"); 4, pAS4 (BCDD'D"); 5, pAS16 (EFG); 6, pAS15 (EF GHIJ); 7, pAS17 (HIJ); 8, 9, and 10, same as lanes 1, 2, and 3, except that the membrane strips were more thoroughly washed after incubation with probe DNA. Approximately 2 μg of total protein (40 to 50% RepA) was loaded into each lane. DNA probes were produced by end labelling *Xba*I-digested plasmid DNA with Klenow fragment and [^{32}P]dCTP.

element shows one of the lowest degrees of homology with the A-to-K repeat element consensus sequence (35).

Competitive RepA binding to the BCDD'D", EFG, and HIJ repeats. In vivo and in vitro experiments have shown that RepA binds to the repeat elements which flank *repA*. The sequence, orientation, spacing, and numbers of repeat elements differ between the upstream (BCDD'D") and the downstream (EFGHIJ) groups of repeat elements, and these differences might result in a variation in RepA DNA binding. Such variation was investigated by a competition assay in which RepA supplied in *trans* (from pAS64) bound either to a copy of the BCDD'D" repeats in a RepA–β-galactosidase fusion plasmid (pAS9) to repress $repA_p$ or to the repeat elements carried by a third compatible plasmid.

From earlier work, we know that induction of pAS64 with 64 μM IPTG is sufficient to bring about the partial repression of a high-copy-number *repA-lacZ* fusion plasmid similar to the lower-copy-number plasmid pAS9 (38). On the basis of a comparison of RepA expression from a RepFIB miniplasmid and that from an expression plasmid similar to pAS64 (37), we expect that pAS64 is capable of expressing levels of RepA 100× or more than wild-type levels when induced with 64 μM IPTG. This high level of expression is partially offset by the high copy number of pAS9 (≈10× that of RepFIB) and the even greater copy numbers of the pBS$^+$-based third plasmids. The results of the β-galactosidase assays for this experiment (shown in Table 3) indicate that in most cases, RepA is able to repress $repA_p$ despite the presence of copies of identical or different repeat elements on the high-copy-number third plasmid. However, when the HIJ or EFGHIJ repeats were carried by the third plasmid (pAS17 or pAS15), $repA_p$ was not repressed when pAS64 was induced to express RepA. Repression occurred when the EFG and HIJ repeats were separated by 2 kb of DNA in pAS65. These results might be explained if the copy numbers of pAS15 and pAS17 were significantly different from those of pAS4, pA16, pA58, pA59, and pA65. If this were the case, the HIJ repeats present in pAS15 and pAS17 may bind more RepA than the other plasmids. Although the copy numbers of the third plasmids have not been determined, we feel that they are not likely to differ significantly, since each plasmid was derived from pBS$^+$. An insufficient amount or excess of RepA with respect to the copy numbers of the plasmids used might provide an alternative explanation for these results. If too little RepA was expressed by pAS64, we would expect to see the partial repression of pAS9 when no third plasmid was present. However, when RepA is expressed, pAS9 is repressed to 4% of its former level. If too much RepA was expressed, we would expect to see pAS9 repressed to the same level, regardless of which third plasmid was present. The fact that pAS15 and pAS16 (with three and six repeats, respectively) give results substantially different from those for pAS16 (three repeats) and pAS65 (six repeats) suggests that an excess of RepA cannot explain the observations that we have made. We conclude from these results that RepA bound to the BCDD'D" or EFG repeats is capable of binding to a second copy of the BCDD'D" repeats to repress $repA_p$. We are currently unable to offer an explanation for why $repA_p$ is derepressed when copies of the HIJ repeats are present in *trans*. However, it is possible that a RepA-HIJ complex is unable to bind other copies of repeat elements because of an altered physical structure. This alternate structure is also formed when the EFG repeats are immediately adjacent to the HIJ repeats, but not when the two sets of repeats are sufficiently separated. In this case, two

TABLE 3. RepA binding to repeat elements

Plasmids[a]	Repeats	β-Galactosidase activity		% Activity
		No IPTG	64 μM IPTG	
pAS9, pAS64		560.8 ± 11.7	23.3 ± 3.7	4.2
pAS9, pAS64, pAS4	BCDD'D"	556.7 ± 14.4	35.5 ± 2.5	6.3
pAS9, pAS64, pAS59	CDD'D"	438.7 ± 26.0	11.9 ± 9.4	2.7
pAS9, pAS64, pAS58	D"	425.8 ± 41.7	0.0 ± 8.5	<0.2
pAS9, pAS64, pAS16	EFG	510.7 ± 7.7	29.1 ± 5.0	5.9
pAS9, pAS64, pAS17	HIJ	729.6 ± 6.3	734.7 ± 10.2	100.7
pAS9, pAS64, pAS15	EFGHIJ	728.7 ± 5.6	691.5 ± 11.2	94.9
pAS9, pAS64, pAS65	EFGΩHIJ	417.7 ± 44.4	14.7 ± 8.8	2.1

[a] pAS9 carries a *repA-lacZ* fusion under the control of $repA_p$ as well as the BCDD'D" repeats. pAS64 is a RepA expression plasmid induced with IPTG, pAS4, pAS15, pAS16, pAS17, pAS58, pAS59, and pAS65 are pBS$^+$ plasmids carrying the indicated various sets of repeat elements. The assays were carried out in DH5α with mid-log-phase cultures, pAS9 was maintained in DH5α with 40 μg of kanamycin per ml, pAS64 was maintained with 25 μg of chloramphenicol per ml, and the other plasmids were maintained with 50 μg of ampicillin per ml. In pAS65, Ω has been inserted between the EFG and HIJ repeat elements.

complexes would form on the same piece of DNA, one of which was capable of binding additional copies of the repeat elements and one which was not.

DISCUSSION

The RepFIB replicon is a low-copy-number replicon which has significant organizational homology to step function replicons. Although these replicons may not share high levels of DNA sequence similarity, step function replicons all appear to control plasmid copy number by the interaction of a single initiator protein with a number of repeat elements flanking the initiator gene (for examples, see references 2, 11, 12, and 41). For RepFIB, the initiator protein is RepA, and 5 copies of a 21-bp repeat sequence form a direct repeat upstream of *repA* (the BCDD'D" repeats) and 6 additional copies are located downstream in a more complicated arrangement (EFGHIJ repeats). The similarities between RepFIB and the mini-P1 replicon are more extensive than those between RepFIB and other step function replicons, since the RepA proteins share a significant level of sequence similarity which is especially well conserved in the potential DNA binding domains found in each (17), despite the fact that the repeat elements which bind the proteins are quite different.

The organizational similarities between RepFIB and other step function replicons have allowed us to propose three clear predictions about the control elements involved in RepFIB replication. The first prediction is that RepA should be autoregulated by RepA binding to the BCDD'D" repeat elements, a prediction which has been confirmed in vivo and in vitro (15, 38). The second prediction is that the BCDD'D" repeat elements form part of the origin of replication, while the EFGHIJ repeats downstream of *repA* are involved in the sensing and setting of plasmid copy number (via titration). The final prediction is that although concurrent autoregulation and titration clearly result in a control paradox, autoregulation, titration, and origin activation by RepA DNA binding to the appropriate repeat elements should be experimentally demonstrable.

We have examined the functional significance of the BCDD'D" and EFGHIJ repeat elements and have found that the BCDD'D" repeats form an essential part of the origin of replication. The origin covers ≈400 bp and appears to be divided into two sections, one of which appears to be RepFIB specific and contains the BCDD'D" repeats and the other which appears to be host specific and includes a *dnaA* box and Dam methylation sites. Our designation of the origin is in agreement with that of Gammie and Crosa (14), who have defined the origin for the pColV3-K30 RepFIB replicon by a similar assay. RepFIB replication is dependent on host DnaA and Dam, which is characteristic of step function replicons (for examples, see references 1, 18, and 42), although DnaA binding sites are not always necessary for replication (3).

For the mini-P1 origin, a GC spacer is required to maintain the correct distance between the DnaA and RepA binding sites (7). Although the RepFIB origin has an analogous sequence, it is displaced ≈20 bp toward the BCDD'D" repeats and overlaps the B repeat element. Activation of the RepFIB origin is possible when the BCDD'D" repeat elements are inverted with respect to the *dnaA* box. This result suggests that if a spacer is required for origin activity, then the BCD repeats are more important in initiation than D'D", since the *dnaA* box-to-repeat C distance is the same in both the normal and inverted origins.

We have shown that the HIJ repeats located downstream of *repA* are involved in the sensing and setting of copy number, apparently via a titration mechanism. The copy number of a RepFIB miniplasmid increases when the HIJ repeats are deleted or when the HIJ repeats are present in *trans*. Both observations are consistent with the titration of an inhibitor of replication which is presumably RepA, and both assume that the inhibitory effect results from RepA binding not only to the HIJ repeats but to the EFG and possibly the BCDD'D" elements as well. The characterization of RepA as an inhibitor in the context of this experiment is incompatible with the initiator role assumed in the Pol$^-$ pBS$^+$-origin plasmid rescue experiment. Similar incompatibility has been previously noted for the replication protein of another step function replicon and has resulted in an apparent control paradox (see reference 41).

In previous incompatibility analyses, we have shown that the BCDD'D", EFG, and HIJ repeats carried by high-copy-number vectors are incompatible with a RepFIB miniplasmid with a copy number of 1 (38). Superficially, the origin and copy number experiments reported here appear to contradict our earlier claims. However, the origin experiment differs from the incompatibility analysis in three important respects. First, a mutant RepFIB miniplasmid (pMA4322) with a higher copy number (35) and a more relaxed *inc* function (37) than pSS3928 was used to supply RepA to activate the origin carried by the incoming test plasmids. Second, the copy number of the test plasmids in the Pol$^-$ host is significantly lower than that of the incompatibility analysis test plasmids, since replication was from the RepFIB origin rather than from the vector plasmid (pBS$^+$, ColE1-derived) origin. Third, both plasmids were selected with the appropriate antibiotics. In contrast to our earlier work, the expected incompatibility reactions between pMA4322 and the test plasmids were sufficiently reduced by mutation and relative copy numbers to allow the stable maintenance of both plasmids under selection. For the copy number experiment, relative copy numbers (a higher-copy-number miniplasmid and a low-copy-number plasmid carrying the HIJ repeats) and dual selection overcame the inherent incompatibility reactions between the two plasmids and allowed us to grow the necessary test cultures.

The BCDD'D" repeat elements contain two active promoters, $repA_p$, which expresses *repA* and is autoregulated (15, 38), and ori_p, a promoter described here which produces a transcript which has the potential to cross the origin of replication and which does not appear to express a protein. ori_p has transcriptional strength similar to that of $repA_p$, but the ori_p homolog in mini-P1 (pI [11]) is far weaker (only 5% of $repA_p$). The EFG repeats also contain a functional promoter (EF_p). Although EF_p transcripts have the potential to express ORF4, which is analogous to the 9-kDa genes of mini-F (41), they do not appear to express a protein.

Locating ori_p, $repA_p$, and EF_p within the repeat elements has enabled us to examine RepA DNA binding by a simple genetic repression assay. Gene fusions in pMU575 in which the promoters control the expression of a hybrid β-galactosidase protein have been formed. A comparison of the repression behavior of each fusion plasmid indicates that although all three are repressed when RepA is supplied in *trans*, $repA_p$ appears to be more sensitive to RepA than either ori_p or EF_p. In addition, $repA_p$ is repressed over a greater range of RepA levels (corresponding to a greater range of IPTG concentrations used in the induction of RepA) than the other two promoters.

Gammie and Crosa (15) have suggested that RepA binding to the BCDD'D" repeat elements is cooperative. This sug-

gestion was based on in vitro RepA binding to DNA containing the D″, D′D″, BDD′D″, or BCDD′D″ repeat elements. From their results, it is clear that BCDD′D″ DNA binds more RepA than would be expected if a linear relationship existed between RepA binding and the numbers of repeats present in the probe DNA. However, our observations of the repression of ori_P and $repA_P$ demonstrate that RepA binding to the individual repeats within the BCDD′D″ repeat group varies, and in particular, RepA appears to have a higher affinity for element D″ (which covers the −35 sequence of $repA_P$) than for element B (which covers the −10 sequence of ori_P). For this reason, we feel that the claim of cooperative RepA DNA binding is premature and requires further examination. Given the wide variation in the sequences of the BCDD′D″ repeats, we feel that a claim of cooperative RepA binding cannot be substantiated unless Gammie and Crosa's filter-binding experiment is repeated with 1 to 6 copies of exactly the same repeat sequence (for example, repeat B, which shows the highest similarity to the A-to-K consensus sequence). The modification that we have suggested represents an important test, since differential binding affinities to different groups of repeats have been proposed as a control mechanism in some replicons (such as mini-F [41]).

We have demonstrated previously that RepA is able to specifically bind DNA containing the BCDD′D″ repeat elements in vitro by the Western-DNA technique (38), and we have now extended that analysis to demonstrate that RepA binds to the EFG, HIJ, and EFGHIJ repeat elements, confirming our in vivo demonstration of RepA DNA binding. We have also investigated RepA binding affinity for each of the BCDD′D″, EFG, and HIJ groups of repeat elements by a competition assay in which RepA expressed in *trans* can bind either to a copy of the BCDD′D″ repeats in a *repA-lacZ* fusion plasmid and thus repress $repA_P$ or to a third plasmid carrying various groups of repeat elements. The results from this experiment were unexpected, since $repA_P$ repression occurred even when the third plasmid carried either the BCDD′D″, CDD′D″, or D″ repeats. We had expected that the extent of $repA_P$ repression would be reduced when a second copy of the BCDD′D″ repeats was present in *trans* because of the simple titration of RepA by both plasmids. However, our results strongly suggest that RepA bound to repeat elements on one plasmid is able to repress $repA_P$ located on a second plasmid. The ability of RepA to bind to two pieces of DNA is a necessary requirement in the new DNA-looping models proposed for mini-P1 (2, 31). In such models, RepA might link the origin and titration repeats of the replicon to form a loop or it might link the origin and titration repeats of two copies of the replicon in a parallel or antiparallel manner. Since RepFIB and mini-P1 appear to be related, we feel that DNA-looping mini-P1 models will probably apply equally well to RepFIB.

The competition assay has also indicated that the HIJ repeat elements are functionally different from either the BCDD′D″ or EFG repeats, since the HIJ repeats in *trans* can derepress $repA_P$. The derepressive action of the HIJ repeats is dominant and distance dependent, since a third plasmid containing the EFGHIJ repeats can derepress $repA_P$, but the EFG repeats alone have no effect, and the effect is lost when the EFG and HIJ repeats are separated by 2 kb.

The different repression characteristics of ori_P and $repA_P$ and the action of the HIJ repeats being different from those of the BCDD′D″ and EFG repeats suggest that RepA recognizes and reacts in different ways to individual repeat elements and to different repeat groups. Such differential interaction is reminiscent of the hierarchical binding model proposed for mini-F (10) in which the E protein bound first to repeats to autoregulate *E* gene expression, then to the downstream repeats to titrate E, and finally to the upstream repeats to activate the origin of replication. For RepFIB, a cladistic analysis of the repeat element sequences has shown that individual elements are not grouped together in a manner which reflects their spatial relationships (37), which implies that the groupings of repeats do not reflect strict functional divisions. However, autoregulation and origin activation may be controlled by the levels of RepA bound to the BCDD′D″ repeats, with autoregulation occurring first and origin activation at higher levels of RepA occurring second.

RepFIB is proving to be an intriguing replicon. The genetic organization of the replicon and the response to artificial changes in copy number (23) clearly classify RepFIB as a member of the step function group of replicons. The more extensive homology between RepFIB and mini-P1, especially in the sequence of RepA and in the origin regions, indicates that mini-P1 and RepFIB are closely related homologs (17). Although RepFIB replication seems to involve the same kind of control elements as that of mini-P1, our observations suggest that the control system may in fact be best described by a hybrid constructed from the early hierarchical binding model (11) and the current DNA-looping models (2, 24, 31).

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REFERENCES

1. **Abeles, A. L., and S. J. Austin.** 1987. P1 plasmid replication requires methylated DNA. EMBO J. **6:**3185–3189.
2. **Abeles, A. L., and S. J. Austin.** 1991. Antiparallel plasmid-plasmid pairing may control P1 plasmid replication. Proc. Natl. Acad. Sci. USA **88:**9011–9015.
3. **Abeles, A. L., L. D. Reaves, and S. J. Austin.** 1990. A single DnaA box is sufficient for initiation from the P1 plasmid origin. J. Bacteriol. **172:**4386–4391.
4. **Adelberg, E. A., and S. N. Burns.** 1960. Genetic variation in the sex factor of *Escherichia coli*. J. Bacteriol. **79:**321–330.
5. **Bergquist, P. L., H. E. D. Lane, L. Malcolm, and R. A. Downard.** 1982. Molecular homology and incompatibility in the IncFI plasmid group. J. Gen. Microbiol. **128:**223–238.
6. **Bramhill, D., and A. Kornberg.** 1988. Duplex opening by dnaA protein at novel sequences in initiation of replication at the origin of the *E. coli* chromosome. Cell **52:**743–755.
7. **Brendler, T., A. Abeles, and S. Austin.** 1991. Critical sequences in the core of the P1 plasmid replication origin. J. Bacteriol. **173:**3935–3942.
8. **Brosius, J.** 1984. Plasmid vectors for the selection of promoters. Gene **27:**151–160.
9. **Chang, A. Y. C., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. **134:** 1141–1156.
10. **Chattoraj, D., K. Cordes, and A. Abeles.** 1984. Plasmid P1 replication: negative control by repeated DNA sequences. Proc. Natl. Acad. Sci. USA **81:**6456–6460.
11. **Chattoraj, D. K., A. L. Abeles, and M. Yarmolinsky.** 1985. P1 plasmid maintenance: a paradigm of precise control, p. 355–381. *In* D. R. Helinski, S. N. Cohen, D. B. Clewell, D. A. Jackson, and A. Hollander (ed.), Plasmids in bacteria. Plenum Press, New York.
12. **Filutowicz, M., M. McEachern, A. Greener, P. Mukhopadhyay, E. Uhlenhopp, R. Durland, and D. Helinski.** 1985. Role of the π

initiation protein and direct nucleotide sequence repeats in the regulation of plasmid R6K replication, p. 125–140. *In* D. R. Helinski, S. N. Cohen, D. B. Clewell, D. A. Jackson, and A. Hollander (ed.), Plasmids in bacteria. Plenum Press, New York.
13. **Fuller, R. S., and A. Kornberg.** 1983. Purified dnaA protein in initiation of replication at the *Escherichia coli* chromosomal origin of replication. Proc. Natl. Acad. Sci. USA **80:**5817–5821.
14. **Gammie, A. E., and J. H. Crosa.** 1991. Roles of DNA adenine methylation in controlling replication of the REPI replicon of plasmid pColV-K30. Mol. Microbiol. **5:**495–503.
15. **Gammie, A. E., and J. H. Crosa.** 1991. Co-operative autoregulation of a replication protein gene. Mol. Microbiol. **5:**3015–3023.
16. **Gardner, R., J. McAnulty, E. Feher, and D. Lane.** 1985. Location of *rep* and *inc* sequences in the F secondary replicon. Plasmid **13:**145–148.
17. **Gibbs, M. D., A. J. Spiers, and P. L. Bergquist.** RepFIB: a basic replicon of large plasmids. Plasmid, in press.
18. **Hansen, E. B., and M. B. Yarmolinsky.** 1986. Host participation in plasmid maintenance: dependence upon *dnaA* of replicons derived from P1 and F. Proc. Natl. Acad. Sci. USA **83:**4423–4427.
19. **Itoh, Y., and Y. Terawaki.** 1989. Replication properties of mini-Rts1 derivatives deleted for DnaA boxes in the replication origin. Plasmid **21:**242–246.
20. **Kogoma, T., and K. von Meyenburg.** 1983. The origin of replication, *oriC*, and the *dnaA* protein are dispensable in stable DNA replication (*sdrA*) mutants of *Escherichia coli* K-12. EMBO J. **2:**463–468.
21. **Lederberg, E. M., and S. N. Cohen.** 1974. Transformation of *Salmonella typhimurium* by plasmid deoxyribonucleic acid. J. Bacteriol. **119:**1072–1074.
22. **Luria, S. E., and J. N. Burrous.** 1957. Hybridization between *Escherichia coli* and *Shigella*. J. Bacteriol. **74:**461–476.
23. **Maas, R., S. Saadi, and W. K. Maas.** 1989. Properties and incompatibility behaviour of miniplasmids derived from the bireplicon plasmid pCG86. Mol. Gen. Genet. **218:**190–198.
24. **McEachern, M. J., M. A. Bott, P. A. Tooker, and D. R. Helinski.** 1989. Negative control of plasmid R6K replication: possible role of intermolecular coupling of replication origins. Proc. Natl. Acad. Sci. USA **86:**7942–7946.
25. **Miller, J.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Mukherjee, S., H. Erickson, and D. Bastia.** 1988. Enhancer-origin interaction in plasmid R6K involves a DNA loop mediated by initiator protein. Cell **52:**375–383.
27. **Mukherjee, S., H. Erickson, and D. Bastia.** 1988. Detection of DNA looping due to simultaneous interaction of a DNA-binding protein with two spatially separated binding sites on DNA. Proc. Natl. Acad. Sci. USA **85:**6287–6291.
28. **Mulligan, M. E., D. K. Hawley, R. Entriken, and W. R. McClure.** 1984. *Escherichia coli* promoter sequences predict *in vitro* RNA polymerase selectivity. Nucleic Acids Res. **12:**789–800.
29. **Nomura, N., H. Masai, M. Inuzuka, C. Miyazaki, E. Ohtsubo, T. Itoh, S. Sasamoto, M. Matsui, R. Ishizaki, and K.-I. Arai.** 1991. Identification of eleven single-stranded initiation sequences (*ssi*) for priming of DNA replication in the F, R6K, R100, and ColE2 plasmids. Gene **108:**15–22.
30. **Nordström, K.** 1985. Control of plasmid replication: theoretical considerations and practical solutions, p. 184–214. *In* D. R. Helinski, S. N. Cohen, D. B. Clewell, D. A. Jackson, and A. Hollander (ed.), Plasmids in bacteria. Plenum Press, New York.
31. **Pal, S. K., and D. K. Chattoraj.** 1988. P1 plasmid replication: initiator sequestration is inadequate to explain control by initiator-binding sites. J. Bacteriol. **170:**3554–3560.
32. **Perez-Casal, J. F., and J. H. Crosa.** 1987. Novel incompatibility and partition loci for the REPI replication region of plasmid ColV-K30. J. Bacteriol. **169:**5078–5086.
33. **Perez-Casal, J. F., A. E. Gammie, and J. H. Crosa.** 1989. Nucleotide sequence analysis and expression of the minimum REPI replicon region and incompatibility determinants of pColV-K30. J. Bacteriol. **171:**2195–2201. (Erratum, **173:**2409.)
34. **Prentki, P., and H. M. Krisch.** 1984. *In vitro* insertional mutagenesis with a selectable DNA fragment. Gene **29:**303–313.
35. **Saul, D., A. J. Spiers, J. McAnulty, M. D. Gibbs, P. L. Bergquist, and D. F. Hill.** 1989. Nucleotide sequence and replication characteristics of RepFIB, a basic replicon of IncF plasmids. J. Bacteriol. **171:**2697–2707.
36. **Shultz, J., T. J. Silhavy, M. L. Berman, N. Fiil, and S. D. Emr.** 1982. A previously unidentified gene in the *spc* operon of *Escherichia coli* K12 specifies a component of the protein export machinery. Cell **31:**227–235.
37. **Spiers, A.** Unpublished results.
38. **Spiers, A. J., and P. L. Bergquist.** 1992. Expression and regulation of the RepA protein of the RepFIB replicon from plasmid P307. J. Bacteriol. **174:**7533–7541.
39. **Stalker, D. M., K. E. McBride, and L. D. Malyj.** 1988. Herbicide resistance in transgenic plants expressing a bacterial detoxification gene. Science **242:**419–423.
40. **Takeshita, S., M. Sato, M. Toba, W. Masahashi, and T. Hashimoto-Gotoh.** 1987. High-copy-number and low-copy-number plasmid vectors for *lacZ*α complementation and chloramphenicol- or kanamycin-resistance selection. Gene **61:**63–74.
41. **Trawick, J. D., and B. C. Kline.** 1985. A two-stage molecular model for control of mini-F replication. Plasmid **13:**59–69.
42. **Wickner, S. H., and D. K. Chattoraj.** 1987. Replication of mini-P1 plasmid DNA *in vitro* requires two initiation proteins, encoded by the *repA* gene of phage P1 and the *dnaA* gene of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **84:**3668–3672.
43. **Yang, J., and J. Pittard.** 1987. Molecular analysis of the regulatory region of the *Escherichia coli* K-12 *tyrB* gene. J. Bacteriol. **169:**4710–4715.
44. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 vectors. Gene **33:**103–119.