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Expression and Regulation of the RepA Protein of the RepFIB Replicon 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. Limited N-terminal sequence information obtained from a RepA:β-galactosidase fusion protein indicates that although the first residue of RepA is methionine, the initiation of translation of RepA occurs from a CTG codon rather than from the predicted GTG codon located further downstream. Overexpressed RepA in *trans* is capable of repressing a *repA:lacZ* fusion plasmid in which the expression of the fusion protein is under the control of the *repA* promoter. The *repA* promoter has been located functionally by testing a series of *repA:lacZ* fusion plasmids. Both in vivo genetic tests and in vitro DNA-binding studies indicate that *repA* autoregulation can be achieved by RepA binding to one or more repeat elements which overlap the *repA* promoter sequence.

The RepFIB replicon is characterized as a Step function replicon (26) expressing *incE* incompatibility with a tightly regulated copy number of one to two per chromosome. RepFIB is present in many IncF plasmids and was first isolated from the F plasmid, for which the minimal replicon and *incE* determinants were mapped (3, 4, 11, 12, 22). Since then, RepFIB replicons from the enterotoxin plasmid P307 (33) and the colicin-producing plasmid pColV3-K30 (28, 29) have been examined in some detail. The sequence analysis of RepFIB from P307 and ColV3-K30 RepFIB, plus seven additional RepFIB replicons, has shown RepFIB to be very highly conserved, despite the fact that the parental plasmids appear to be otherwise unrelated (12).

The RepFIB replicon contains an initiator gene (*repA*) sequence flanked on either side by a series of DNA repeat elements (29, 30, 33) (Fig. 1). Our initial analysis of the repeat elements identified 11 elements (A through K [33]). However, the A and K elements show less homology to the A–K repeat consensus sequence than do the other nine elements, and at least two additional repeat elements (D′ and D″ [28]) can be identified if the consensus sequence is allowed to diverge further. The genetic organization of RepFIB with respect to the placement of the repeat elements on either side of *repA* is very similar to a number of Step function replicons, such as mini-P1, mini-F, and R6K, although there is no DNA sequence similarity with mini-F and R6K and limited similarity with the mini-P1 *repA* coding region (8, 33). However, the predicted amino acid sequence of RepA shows a higher degree of similarity with the initiator protein (RepA) of mini-P1 (1) and the highly related replicons from P7, Rts1, and R401 (9, 20, 21, 27, 38). Approximately 1.6 kb of DNA is required for replication and includes the BCDD′D″ upstream repeat elements, *repA*, and three of the six downstream repeats (EFG). Deletion of *repA* or interruption by a transposon insertion has demonstrated that the gene is essential for replication, and a potential translation product has been observed by maxicell analysis (33).

The close organizational similarity between RepFIB and mini-P1 leads to a number of predictions about the mechanism and nature of the replication control system of RepFIB. In particular, the similarity predicts that RepFIB *repA* is autoregulated and that the RepA product is a DNA-binding protein which specifically binds to the DNA repeat elements located on either side of *repA*. A test of these two predictions requires a knowledge of the location of the *repA* promoter ($repA_p$) and ability to produce RepA protein under controlled conditions. Although a specific $repA_p$ and start codon have been predicted by ourselves and others on the basis of DNA sequence analysis (29, 33), the upstream region of *repA* contains at least five potential promoter sequences sharing a significant degree of homology with the *Escherichia coli* promoter consensus sequence (17) plus four possible translational start codons, which would result in a protein in agreement with that determined by maxicell analysis for RepA.

We decided to resolve the *repA* start codon used in translation by limited N-terminal sequencing in order to avoid future experimental problems that might arise if an elongated or truncated RepA was inadvertently used instead of the natural protein. In addition, we present the location of $repA_p$ determined by genetic analysis in this work, as well as evidence for *repA* autoregulation and RepA DNA binding.

MATERIALS AND METHODS

Bacterial strains. *E. coli* PB1905 [F⁻ ara Δ(*lac-pro*) *thi* streptomycin resistant] and 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) were used as host strains for plasmid construction, in vivo assays, and protein expression.

Plasmid DNA. All RepFIB DNA used to construct new plasmids was isolated from pSS3928 (a gift of S. Saadi), pMA4322, or pNZ945 (33). pNZ945 was also used as template DNA for polymerase chain reaction (PCR) amplification of selected regions of the RepFIB replicon. pMLB1034 (36) and pNZ338 (R. de Feyter, this laboratory) were used to produce RepA:β-galactosidase fusion proteins. pNZ338 is a low-copy-number plasmid containing the pSC101 replicon and the *lacZ* fusion cassette from pMLB1034. RepA overexpression plasmids were produced by using pJLA602 (34)

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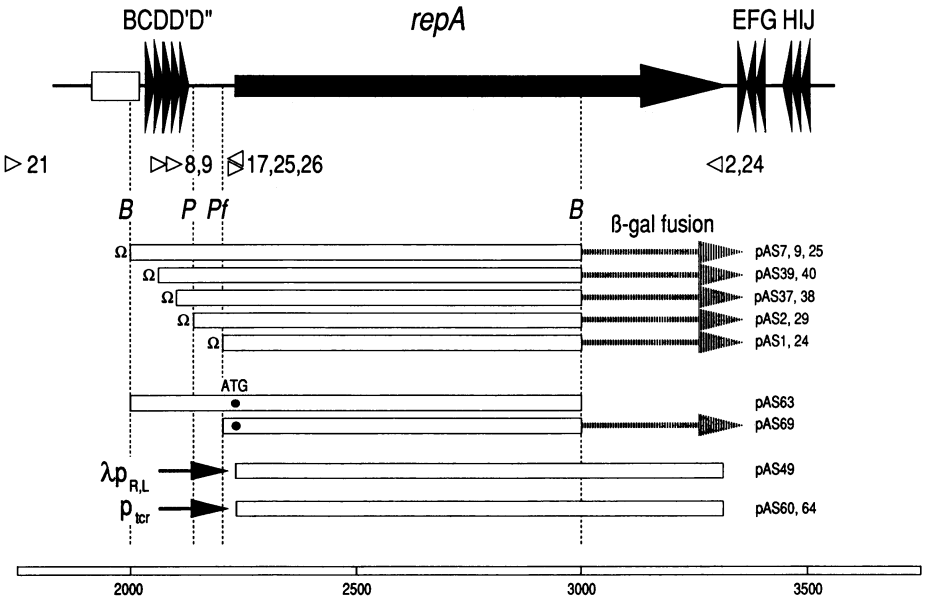

FIG. 1. RepFIB replicon. The RepFIB replicon of P307 consists of a single initiator gene (*repA*) flanked on either side by a number of repeat elements (BCD and EFGHIJ). The BCD repeat elements are associated with a number of origin-like sequences (boxed), and the organization of the replicon is very similar to the organization of the mini-P1 and mini-Rts1 replicons. The plasmids constructed for this work are shown below. pAS1, pAS2, pAS7, pAS9, pAS24, pAS25, pAS29, pAS38, pAS40, and pAS69 all contain RepA:β-galactosidase fusion proteins (striped arrows). pAS63 contains DNA obtained by mutagenic PCR whereby the *repA* translation initiation codon has been altered to ATG. pAS49, pAS60, and pAS64 are RepA expression plasmids. ▷, oligonucleotide PCR primer; B, *Bam*HI; P, *Pst*I; Pf, *Pfl*MI; ●, PCR-altered sequence; Ω, the omega resistance fragment was placed upstream of the *repA:lacZ* protein fusion in pAS24, pAS25, pAS29, pAS38, pAS40, and pAS64; p_{tcr}, promoter in pAS60 and pAS64; $\lambda p_{R,L}$, promoter in pAS49. Scale is in base pairs, using coordinates from Saul et al. (33).

and pKK233.2 (2). pJLA602 contains a hybrid λ promoter constructed by placing λp_R and λp_L in tandem (induced by incubation at 42°C), whereas pKK233.2 contains the hybrid p_{tcr} promoter (induced with IPTG). pACYC184 (7), pBS+ (Stratagene), and pCGN565 (37) were used for general cloning. The omega (Ω) resistance fragment was isolated from pHP45 (31), and the *lacI*^{q} gene was isolated from pMC9 (6). pACYC184 contains a replicon derived from p15A and is compatible with both pMLB1034 and pKK233.2, which contain replicons derived from pMB1.

Oligonucleotide primers. The oligonucleotide primers used for PCR amplification of RepFIB DNA are listed in Table 1. In some cases, specific changes were made to the RepFIB sequence to introduce restriction enzyme recognition sites.

Plasmid construction. The 0.70-kb *Pfl*MI (treated with Klenow fragment)-*Bam*HI fragment from pNZ945 was ligated into pMLB1034 digested with *Sma*I and *Bam*HI to give pAS1. The 0.77-kb *Pst*I (treated with Klenow fragment)-*Bam*HI fragment from pNZ945 was ligated into pMLB1034 digested with *Sma*I and *Bam*HI to give pAS2. The 0.97-kb *Bam*HI fragment from pNZ945 was ligated into pMLB1034 digested with *Bam*HI to give pAS7. The 0.97-kb *Bam*HI fragment from pMA4322 was ligated into pNZ338 digested with *Bam*HI to give pAS9. *Eco*RI-cut Ω was ligated into pAS1 digested with *Eco*RI to give pAS24. *Eco*RI-cut Ω was ligated into pAS7 digested with *Eco*RI to give pAS25. *Eco*RI-cut Ω was ligated into pAS2 digested with *Eco*RI to give pAS29.

A 1.17-kb fragment was generated by PCR from pNZ945, using primers 2 and 9. PCR involved 25 cycles of 1 min at 95°C, 2 min at 55°C, and 2 min at 72°C. The PCR DNA was digested with *Bam*HI, and the 0.83-kb fragment was ligated into pMLB1034 digested with *Sma*I and *Bam*HI to give pAS37. *Eco*RI-cut Ω was ligated into pAS37 digested with *Eco*RI to give pAS38.

A 1.2-kb fragment was generated by PCR from pNZ945 by using primers 2 and 8. PCR involved 25 cycles of 1 min at 95°C, 2 min at 55°C, and 2 min at 72°C. The PCR DNA was digested with *Bam*HI, and the 0.88-kb fragment was ligated into pMLB1034 digested with *Sma*I and *Bam*HI to give pAS39. *Eco*RI-cut Ω was ligated into pAS39 digested with *Eco*RI to give pAS40. The *Eco*RI fragment from pMC9 which carries the *lacI*^{q} gene was ligated into pKK233.2 digested with *Eco*RI to give pAS48.

A 1.02-kb fragment was generated by PCR from pNZ945 by using primers 2 and 17. PCR involved 25 cycles of 1 min at 95°C, 2 min at 55°C, and 2 min at 72°C. The PCR DNA was

TABLE 1. Oligonucleotide PCR primers

Primer	Sequence (5′→3′)[a]	Location[b]
2	G**GAATTC**ATCAGCGGGATTTGAAGA	3306–3282
8	CTGTAACATGCTAATGATAAGCTG	2091–2114
9	GTTAATGACATAAACTATGGTCAG	2142–2165
17	GGAGGCT**CCATGG**AAAACGAAAATTCAGACATC	2270–2302
21	TATAT**GAATTC**TACTTCGGATATGAGCGTACG	1721–1750
24	CTGACTAC**CCATGGAATTC**AGCGGGATTTGAAG	3315–3283
25	GGAGGCTTTATGGAAAACGAAAATTC	2270–2295
26	TTCGTTTTCCATAAAGCCTCCAGCCT	2290–2265

[a] Changes from the RepFIB sequence (33) are indicated by bold type, and restriction enzyme recognition sites formed by the changes are underlined (e.g., GAATTC [*Eco*RI] and CCATGG [*Nco*I]).
[b] P307 E11 base pair coordinates (33).

digested with *Nco*I and *Eco*RI and ligated into *Nco*I-*Eco*RI-cut pJLA602 to give pAS49.

A 1.02-kb fragment was generated by PCR from pNZ945, using primers 17 and 24. PCR involved 25 cycles of 1 min at 95°C, 2 min at 55°C, and 2 min at 72°C. The PCR product was digested with *Nco*I and ligated into pAS48 digested with *Nco*I to give pAS60.

A 0.6-kb fragment was generated by PCR from pNZ945 by using primers 21 and 26, and a second 1-kb fragment was generated by PCR from pNZ945 by using primers 2 and 25. Both reactions involved 25 cycles of 1 min at 95°C, 1.5 min at 55°C, and 1.5 min at 72°C. The two fragments were gel purified and mixed together to provide the template for a second round of PCR synthesis using primers 2 and 21. This PCR involved 10 cycles of 1 min at 95°C, 1.5 min at 35°C, and 1.5 min at 72°C followed by 10 cycles of 1 min at 95°C, 1.5 min at 55°C, and 1.5 min at 72°C. The 1.6-kb PCR product was digested with *Bam*HI, and the resulting 0.97-kb fragment was ligated into pCGN565 digested with *Bam*HI to give pAS63. The ≈5.5-kb *Sal*I (treated with Klenow fragment)-*Sca*I fragment from pAS62 was ligated into pACYC184 digested with *Bam*HI (treated with Klenow fragment) to give pAS64. The 0.7-kb *Pfl*MI (treated with Klenow fragment)-*Bam*HI fragment from pAS63 was ligated into pAS1 digested with *Sma*I and *Bam*HI (equivalent to *Sma*I-*Bam*HI-cut pMLB1034) to give pAS69.

Plasmid maps are shown in Fig. 1.

Isolation of the RepA:β-galactosidase fusion protein. Cells were harvested from overnight cultures by centrifugation, and the cell pellet was resuspended in 10 ml of buffer A (0.25 mM NaCl, 10 mM magnesium acetate, 20 mM Tris, 10 mM EDTA, 10 mM β-mercaptoethanol, 5% glycerol, 1 mM phenylmethylsulfonyl fluoride, 2 mM iodoacetamide [pH adjusted to 7.6]); 20 mg of lysozyme per ml was added to the suspension, which was left to stir gently at 4°C for 30 min. Cells were ruptured by five 30-s periods of sonication while on ice. The solution was cleared by centrifugation at 12,000 × *g* for 30 min. The fusion protein was concentrated from the supernatant by precipitation with ammonium sulfate to 40% of saturation. The pellet was collected and resuspended in TEP buffer (100 mM Tris-Cl [pH 7.4], 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) and dialyzed against TEP buffer for several hours. Portions of the dialyzed extract were passed through a 1-ml anti-β-galactosidase immunoaffinity column (Promega). The column was washed with 25 ml of TN buffer (50 mM Tris-Cl [pH 7.3], 0.2% Nonidet P-40) before the fusion protein was eluted with three 1-ml portions of 0.1 M sodium carbonate [pH 10.8] followed by 1 ml of Tris-buffered saline (TBS; 20 mM Tris, 137 mM NaCl [pH 7.6]). The resulting 4 ml of eluate was pooled, dialyzed for 1 h against 5 mM Tris-Cl (pH 7.5), and lyophilized in several fractions for storage.

Extraction of overexpressed RepA. L broth (1.2 liters) was inoculated with an overnight culture of DH5α (1/30 dilution) and grown for 2 h at 32°C. The culture was transferred to 42°C, and incubation continued for a further 3 h before the cells were harvested by centrifugation and resuspended in 10 ml of lysis buffer (50 mM Tris-Cl [pH 8.0], 1 mM EDTA, 100 mM NaCl); 20 mg of lysozyme per ml was added to the suspension, which was left on ice for 20 min. The mixture was centrifuged at 12,000 × *g* for 10 min. The pellet was resuspended in 10 ml of lysis buffer with 0.5% Triton X-100 and shaken for 5 min at room temperature. The mixture was again centrifuged, and the pellet was resuspended in 10 ml of 8 M urea–50 mM Tris-Cl (pH 8.0)–1 mM EDTA–100 mM NaCl. The mixture was shaken for 5 min at room temperature and then centrifuged at 12,000 × *g* for 10 min. The supernatant was kept at −70°C until required.

In vivo ^{14}C labelling. A 20-ml early-log-phase culture of *E. coli* DH5α containing pAS49 was shifted from 30 to 42°C, and 50 μCi of ^{14}C-labelled amino acids (Amersham) was added to the culture. Samples were removed at various times for analysis by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). After electrophoresis, the acrylamide gels were washed with Amplify (Amersham) before autoradiography.

N-terminal sequence analysis. Limited N-terminal protein sequence was obtained by using an Applied Biosystems 470A gas phase analyzer from protein bound to an Immobilon (Millipore) membrane (24).

Western immunoblot analysis. Proteins separated by SDS-PAGE were electrotransferred to a Hybond-ECL (Amersham) membrane, using a 20% methanol–192 mM Tris–89 mM glycine buffer at 50 V for 2 h. Membrane-bound proteins were visualized by using amido black staining solution (Sigma). Immunodetection of proteins was achieved by using either Promega anti-β-galactosidase monoclonal rabbit antibodies and Promega anti-rabbit horseradish peroxidase conjugate or an Amersham Western-ECL kit according to the supplier's instructions. Anti-RepA antiserum was obtained after immunizing a chinchilla rabbit with RepA protein isolated by SDS-PAGE.

Western-DNA analysis. Proteins separated by SDS-PAGE (12.5% polyacrylamide gel) were electrotransferred to a Hybond-ECL (Amersham) membrane by using a 10% methanol–192 mM Tris–89 mM glycine buffer at 50 V for 2 h. After transfer, the membrane was washed three times in TBS–0.1% Tween 20 for 5 min each time. The membrane was then incubated in TBS–0.1% Tween 20–3% nonfat milk powder–5 μg of sonicated calf thymus DNA for 2 h at room temperature. The membrane was transferred to 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 and incubated for a further 2 h at room temperature. The membrane was washed three times in TBS–0.1% Tween 20 for 5 min each time before autoradiography.

β-Galactosidase assays. Cultures used in β-galactosidase assays were inoculated from overnight cultures (1/100 dilution) and grown at 37°C for 2 h in L broth. β-Galactosidase activities were determined from early- to mid-log-phase cultures (25), and mean Miller units of activity with standard errors were calculated.

RESULTS

N-terminal sequence of RepA. An N-terminal fusion between RepA and β-galactosidase was formed by inserting the 0.97-kb *Bam*HI fragment of pNZ945 into pMLB1034 to form pAS7. The *Bam*HI fragment also contains $repA_p$, and as a result, colonies containing pAS7 are dark blue when plated with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). Although the fusion protein can be detected by enzymatic assay or by using anti-β-galactosidase antibodies on Western membranes, it is not readily detected by Coomassie blue staining after SDS-PAGE. Sufficient fusion protein was isolated from ammonium sulfate precipitates by using an anti-β-galactosidase immunoaffinity column. The column eluate was separated by SDS-PAGE and transferred to an Immobilon membrane. Membrane-bound fusion protein was identified by using anti-β-galactosidase antibodies and Coomassie blue staining of portions of the membrane. The

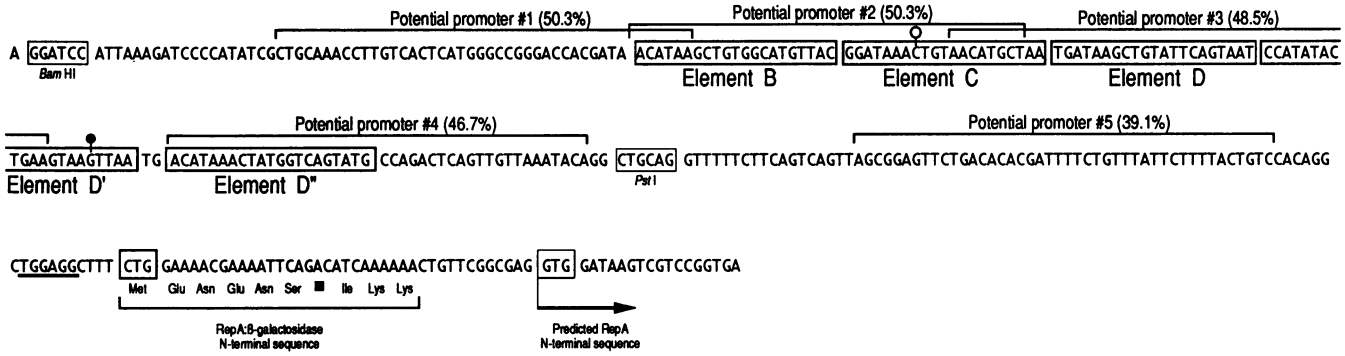

FIG. 2. RepFIB sequence upstream of *repA*. The region of RepFIB (33) from bp 2000 to 2340 contains the BCDD'D" repeat elements and the start of the *repA* coding sequence. This portion of DNA includes five potential promoter sequences (1 to 5) which may express *repA*. Brackets cover the potential −35 and −10 sequences, and the Targsearch value based on the degree of homology with the Hawley and McClure promoter consensus sequence (17) for each is given (compare these values with the promoter activities determined in Table 2). Arrows, predicted and actual N-terminal regions of RepA (the limited N-terminal sequence is given under the DNA sequence); ■, unresolved residue; ●, 5' nucleotide of oligonucleotide primer 8 used to produce pAS37; ○, 5' nucleotide of oligonucleotide primer 9 used to produce pAS39. A potential Shine-Dalgarno sequence is underlined. The *Bam*HI site is at bp 2001; the *Pst*I site is at bp 2194; the CTG start codon is at bp 2279.

band corresponding to the fusion protein was removed, and limited N-terminal protein sequence was obtained.

The band removed for sequencing contained two RepA:β-galactosidase fusion polypeptides that were distinguished from one another on the basis of signal intensity, reflecting a difference in the abundance of the two polypeptides. The sequence of the major polypeptide was compared with the translated sequence of the *repA* coding region (in all three frames), but no alignment was found. However, a perfect alignment occurs between the N-terminal sequence and RepA if the initiation of translation of RepA begins 14 codons upstream of the GTG start site predicted by Saul et al. (33) and 22 codons upstream of the ATG start site predicted by Perez-Casal et al. (29). The first residue of the RepA:β-galactosidase fusion protein is a methionine, and the alignment between the N-terminal sequence and the *repA* coding region indicates that the initiation of translation is from a CTG codon at bp 2279 (Fig. 2).

The minor polypeptide sequence (unresolved residue-Asn-Thr-Leu-Ser-Arg) corresponds to residues 42 to 47 in the central region of RepA (bp 2444 to 2461). The unresolved first residue does not map to an internal *repA* translation initiation codon but maps to a lysine codon (AAA). This codon is preceded by another lysine codon, which suggests that the minor polypeptide is a proteolytic cleavage product of the RepA:β-galactosidase fusion protein rather than a truncated fusion protein initiated from a secondary start codon.

Overexpression of RepA. PCR was used to amplify the coding region of *repA*, which was then inserted into the overexpression plasmid pJLA602. The resultant plasmid, pAS49, expresses large amounts of RepA when induced by incubation at 42°C. A DH5α strain carrying pAS49 was induced in the presence of a mixture of ^{14}C-labelled amino acids, and the labelled proteins were then separated by SDS-PAGE and detected by autoradiography. Limited N-terminal sequence of the 39-kDa ^{14}C-labelled protein presumed to be RepA demonstrated that it was RepA and that the protein was expressed from pAS49 by using the correct start codon. RepA expressed from pAS49 has been used to prepare rabbit antiserum which recognizes RepA expressed by a number of other plasmids, including a copy number mutant RepFIB miniplasmid (pMA4322) and pAS7, which expresses the RepA:β-galactosidase fusion protein used to obtain the N-terminal sequence of RepA. However, the titer of the antiserum was not great enough to allow detection of RepA expressed by a wild-type RepFIB miniplasmid (pSS3928), even when a concentrated cell lysate was used instead of cells lysed directly in SDS-PAGE sample buffer.

DNA binding by RepA in vitro. We have chosen to demonstrate the DNA-binding activity of RepA by using a Western-DNA technique developed from protein blotting (5, 18, 23). The technique is particularly suitable for an analysis of DNA binding by RepA and allows us to make use of denatured nonpurified RepA protein extracts. Protein samples were first separated by SDS-PAGE and then transferred to a membrane in a manner similar to that used for Western analysis. The membrane was washed several times to remove as much SDS and methanol from the bound proteins as possible. Protein renaturation may also occur in the blocking solution, which presumably removes more SDS by competition and blocks nonspecific DNA-binding sites. The membrane is incubated with end-labelled probe DNA in the presence of excess, nonhomologous DNA (calf thymus DNA). We have tried to enhance the degree of RepA DNA binding by washing the membrane in TBS, TBS–Triton X-100, TBS–8 M urea followed by TBS-Tween 20, or TBS–8 M urea serially diluted to 0.25 M followed by TBS-Tween 20. However, none of these treatments produces an increase in RepA DNA binding.

Two examples of the Western analysis are given in Fig. 3. In the first panel, various protein extracts have been used to demonstrate that only RepA binds to probe DNA containing the BCDD'D" repeat elements and that host proteins of similar electrophoretic mobilities are not responsible for probe binding. As the age of the 8 M urea extract containing RepA increases, the appearance of a RepA degradation product that also binds the probe DNA becomes more apparent. RepA binds probe DNA containing only the D" repeat element more strongly than probe DNA which lacks copies of the repeat elements (Fig. 3B). Although we have not tried to measure RepA binding affinities for multiple repeat elements or repeat elements of different sequences

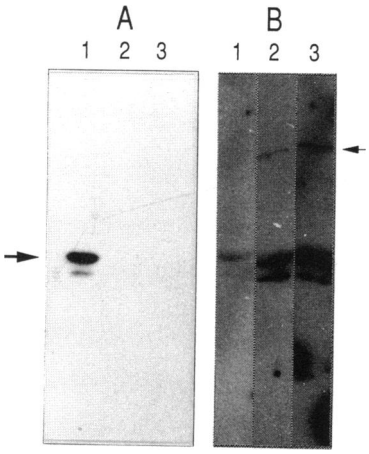

FIG. 3. In vitro RepA DNA binding. RepA specifically binds RepFIB DNA containing copies of the BCDD'D" repeat elements, and binding can be demonstrated by using the Western-DNA protocol described in Materials and Methods. The electrophoretic migration position of RepA is indicated by the arrow on the left of panel A, and the autoradiograph has been presented so that the SDS-PAGE origin and dye front correspond to the top and bottom edges, respectively, of the print. A PCR probe containing the BCDD'D" repeat elements binds to RepA but not to DH5α host proteins. (A) Lanes: 1, RepA extract; 2, DH5α extract; 3, DH5α whole-cell extract. The three strips of membrane were incubated with a PCR probe containing the BCDD'D" repeat elements. The faint band below RepA is the result of weak DNA binding by a RepA degradation product. Probe DNA containing the D" or the BCDD'D" repeat elements is bound by RepA with higher affinity than is probe DNA which lacks repeat elements. (B) Each lane was loaded with RepA extract. Lanes: 1, membrane incubated with pAS2 probe DNA (no repeat elements); 2, membrane incubated with pAS37 probe DNA (D" repeat element); 3, membrane incubated with pAS7 probe DNA (BCDD'D" repeat elements). The probe DNA is also bound by a high-molecular-weight host protein in lanes 2 and 3 (indicated by the arrow at the right) as well as by the RepA degradation product. Extracts (8 M urea) were prepared from DH5α-pAS49 (RepA extract) and DH5α (DH5α extract) cultures as described in Materials and Methods. The DH5α whole-cell extract was obtained by lysing cells from a mid-log-phase culture grown at 42°C directly in 8 M urea–50 mM Tris-Cl (pH 8.0)–1 mM EDTA–100 mM NaCl. The extracts were adjusted so that each sample loading (2 to 3 μg) contained the same amount of host protein (the RepA extract contained 40 to 50% RepA). Labelled DNA probes were produced by end labelling *Bam*HI-digested PCR-amplified DNA (using primers 2 and 8) and pAS2, pAS7, and pAS37 DNA with Klenow fragment and [^{32}P]dCTP.

quantitatively, we believe that they could be determined by using this technique.

Effect of CTG on expression. The CTG start codon may limit the expression of the RepA product. To determine whether the *repA* translational start codon was inefficient in the initiation of translation, we compared the expression of a RepA:β-galactosidase protein fusion containing the wild-type CTG *repA* start codon (pAS1) with that of a fusion protein in which the start codon had been altered to ATG (pAS69). The sequence alteration was achieved through site-specific mutagenic PCR whereby two overlapping complementary primers with single base mismatches (corresponding to the C in the CTG start codon), in conjunction with two flanking primers, were used to synthesize two fragments of DNA, each of which contained the CTG-to-ATG change (described by Higuchi [19]). The DNA was used to form a *repA:lacZ* fusion (pAS69) equivalent to pAS1, differing only in the translational start codon of the fusion protein. In both plasmids, the fusion protein mRNA transcripts are initiated from an upstream pMLB1034 sequence rather than from a RepFIB promoter sequence. The β-galactosidase activities of mid-log-phase DH5α cultures containing these plasmids show that pAS69 expresses almost four times more fusion protein than does pAS1 (114 ± 5 versus 30 ± 2 Miller units), and hence it appears that translation of *repA* is limited by the initiation CTG codon.

Location of $repA_p$. Sequence analysis of the region upstream of *repA* has identified five potential promoter sequences which may be involved in the expression of *repA*. Four of these sequences (sequences 1 to 4) are in addition to the promoter sequence (sequence 5) previously identified and assumed to be responsible for *repA* expression (29, 33). Sequences 1 to 4 are located around the BCDD'D" repeat elements and are some 100 to 300 bp upstream of the original promoter sequence (Fig. 2).

A number of *repA:lacZ* fusion plasmids were made to determine which of the five potential promoter sequences was $repA_p$. The fragments used to make the fusions all had the same 3' terminus (forming the *repA:lacZ* fusion) but had variable 5'-terminal regions that included one or more of the potential promoter sequences. Analysis of the levels of β-galactosidase activity expressed in PB1905 strains carrying each of these plasmids (Table 2) suggests that the major contribution to fusion protein expression is the result of transcriptional activity from promoter sequence 4. On this basis, we have concluded that promoter sequence 4 is $repA_p$. The −35 sequence of $repA_p$ (5'-ACATAAACTATGGTCA-3') covers the D" repeat element, whereas the −10 sequence (5'-AGTTGTTAAATACA-3') is located between the D"

TABLE 2. Relative expression of the *repA:lacZ* fusion protein[a]

Potential promoter(s) present in the fusion	β-Galactosidase activity				Relative *repA:lacZ* expression (%)
	Without Ω		With Ω-derivative plasmid		
	Plasmid	Miller units (mean ± SE)	Plasmid	Miller units (mean ± SE)	
- - - - -	pAS1	4.9 ± 0.3	pAS24	−0.7 ± 0.5	
- - - - 5	pAS2	28.9 ± 0.9	pAS29	0.4 ± 0.7	0.0005
- - - 4 5	pAS37	808.63 ± 43.9	pAS38	658.1 ± 38.4	86
- - 3 4 5	pAS39	675.1 ± 16.3	pAS40	606.8 ± 15.6	79
1 2 3 4 5	pAS7	904.3 ± 16.3	pAS25	768.7 ± 17.6	100

[a] A number of *repA:lacZ* fusions were formed by using the promoter-probe plasmid pMLB1034. The plasmids contain one to five potential promoter sequences located upstream of *repA*. The level of fusion protein expression from these plasmids in PB1905 was determined from mid-log-phase cultures according to Miller (25). The background expression of β-galactosidase activity by PB1905 is 1.7 ± 0.6 U, and pMLB1034 (without a fusion insert) expresses 0.4 ± 0.6 U.

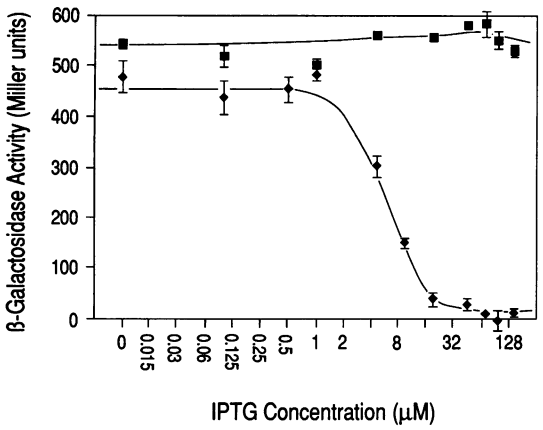

FIG. 4. In vivo demonstration of *repA* autoregulation. Autoregulation of *repA* is demonstrated by the effect of RepA expression from pAS60 on the expression of a RepA:β-galactosidase fusion protein from pAS9 in DH5α. RepA expression from pAS60 is induced by IPTG, and the level of fusion protein expression from the promoter sequence in pAS9 is determined by β-galactosidase assay. ■, pAS9 only; ◆, pAS9 and pAS60. A 1/100 dilution of an overnight culture was used to inoculate an L-broth culture grown for 90 min before portions were removed and added to tubes containing various concentrations of IPTG. The cultures were grown for a further 2 h before β-galactosidase activities were assayed (mean Miller units ± standard errors are shown).

repeat and the *Pst*I site at bp 2194 (Fig. 2). Although the fusion protein is weakly expressed from pAS1 and pAS2, the insertion of Ω upstream of the RepFIB DNA abolishes all β-galactosidase activity. Transcription and translation into or out of Ω is prevented by termination sequences located on either side of the resistance gene carried by Ω (31). Since pAS24 and pAS29 (Ω derivatives of pAS1 and pAS2) do not express the fusion protein, we conclude that the low-level expression from pAS1 and pAS2 is not the result of RepFIB promoter activity but rather is the result of pMLB1034 sequence-initiated transcription.

Autoregulation of *repA*. pAS9 and pAS60 were used to demonstrate *repA* autoregulation. Competent DH5α was transformed with both pAS9 and pAS60, and cotransformant colonies were selected on plates containing ampicillin and kanamycin. β-Galactosidase activities of pAS9-pAS60 cultures induced with various concentrations of isopropylthio-β-D-galactoside (IPTG) were determined 2 h after the induction of pAS60 with IPTG. Figure 4 shows the dramatic decrease of β-galactosidase activity as the IPTG concentration is increased, indicating that RepA from pAS60 is able to repress the synthesis of the fusion protein from pAS9 which is under the control of $repA_p$.

Repeat requirement for autoregulation. The number of repeat elements required for RepA repression of $repA_p$ was investigated by using the *repA:lacZ* fusion plasmids pAS7, pAS37, and pAS39 and a compatible RepA expression plasmid (pAS64). Repression assays of pAS7, pAS37, and pAS39 with use of pAS64 demonstrated that all three *repA:lacZ* fusion plasmids could be repressed when RepA was supplied in *trans* (Fig. 5). However, unlike the repression of pAS9 by pAS60 induced with 0.5 to 8 μM IPTG, $repA_p$ in pAS7, pAS37, and pAS39 is only partially (~50%) repressed by RepA expressed from pAS64 with use of 32 to 64 μM IPTG. Although the repression of $repA_p$ in pAS9 is easily achieved by the partial induction of pAS60, the repression of

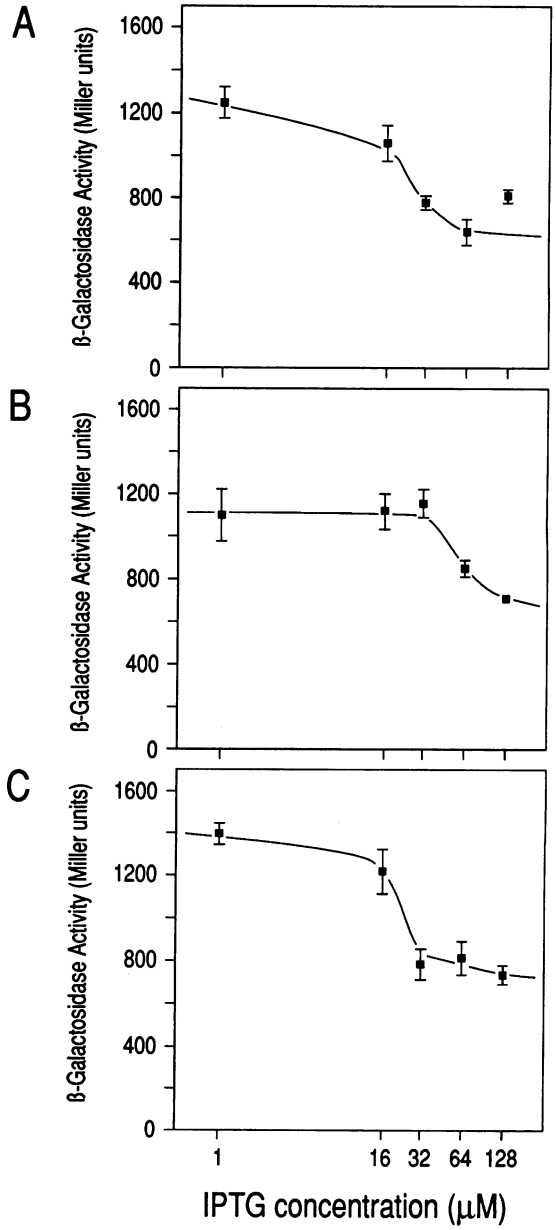

FIG. 5. Comparison of RepA binding in vivo to pAS7, pAS37, and pAS39. RepA expressed from pAS64 can repress $repA_p$ in pAS7, pAS37, and pAS39 to similar extents, despite a difference in the number of protein-binding sites (repeat elements) present in each plasmid. RepA expression from pAS64 is induced by IPTG, and the level of fusion protein expression in DH5α cultures was determined by β-galactosidase assay. (A) pAS7 (containing the BCDD'D" repeat elements) and pAS64; (B) pAS39 (containing half of C and the DD'D" repeat elements) and pAS64; (C) pAS37 (containing the D" repeat element) and pAS64. A 1/100 dilution of an overnight culture was used to inoculate an L-broth culture grown for 90 min before portions were removed and added to tubes containing various concentrations of IPTG. The cultures were grown for a further 2 hours before β-galactosidase activities were assayed (mean Miller units ± standard errors are shown).

repA$_p$ in the higher-copy-number plasmids pAS7, pAS37, and pAS39 cannot be achieved even with the complete induction of pAS64.

DISCUSSION

The replicon structure of RepFIB is characteristic of the Step function class of replicons (26), which employ flanking sets of DNA repeat elements to regulate the expression and action of a single initiator gene located between the two sets of repeat elements. The Step function class includes replicons such as mini-F, mini-P1, and R6K. In particular, RepFIB shows a striking similarity with the mini-P1 replicon (1, 33). Although DNA sequence is not well conserved between the two replicons, the amino acid sequences of the initiator proteins are similar (67% identical), and they share that same potential DNA-binding motifs (12, 33). Phylogenetic analysis of nine different examples of RepFIB indicate that it is a very highly conserved replicon (12), and comparison with the mini-P1 group of replicons, including P7, Rts1, and R401 (9, 20, 21, 27, 38), suggests that they represent two related, highly conserved groups of replicons. At the level of DNA sequence features, RepFIB (from P307) and mini-P1 appear to share the same genetic organization, and we are interested in determining whether RepFIB and mini-P1 are functionally similar.

In our analysis of the sequence of the P307 RepFIB, and on the basis of maxicell analysis of P307 RepFIB translation products (33), we predicted that the initiation of translation of *repA* would begin at a GTG codon (bp 2321 [E11 coordinate]) and produce a protein of ≈37 kDa. This prediction was not in agreement with that of Perez-Casal et al. (29), who have used the ATG codon at bp 2393 (E11 coordinate) to produced RepA for in vitro experimentation (10). We formed a RepA:β-galactosidase fusion protein which was identified and sufficiently purified by using antibodies directed against β-galactosidase to obtain N-terminal sequence information. Alignment of the N-terminal sequence with the *repA* coding region indicated that the initiation of translation was from a CTG codon (bp 2279) upstream of the predicted GTG and ATG start codons (29, 33). Initiation from the CTG codon results in the expression of a protein of 39 kDa, which is in agreement with the calculated molecular mass of 38,870 Da.

Although the majority of prokaryotic initiation of translation is from an ATG or GTG codon, seven of the possible nine triplets with a single variant nucleotide from the universal ATG codon have been reported as functional start codons (for references, see reference 32). Initiation factors are used by *E. coli* to select initiator tRNAs over elongator tRNAs during the initiation phase of translation. Initiation factor 3 (IF3) catalyzes the formation of 30S initiation complexes (39) and selects the initiator tRNA complex used to initiate translation (16), apparently on the basis of unique sequences located in the anticodon stem and loop of the tRNA^{fMet} (15). IF3 appears to "inspect" the anticodon end of the initiator tRNA^{fMet}, probably along with the initiation codon on the mRNA, and allows translation initiation to occur if the anticodon stem and loop sequences are correct. The 5′ nucleotide of the mRNA codon has little influence on IF3 selection, and a GUG or UUG codon can be used quite efficiently as a translation start site in vivo (13). In vivo translation initiation from CTG has not been reported widely, but Gold (14) has suggested that initiation is quite possible from this codon with an efficiency approaching that of initiation from a TTG codon. In contrast, the 3′ nucleotide of the mRNA codon appears to be inspected by the action of IF3; if the nucleotide is not a guanosine, IF3 destabilizes the tRNA:mRNA complex, and translation initiation does not occur in vitro (15) and occurs very poorly in vivo (35).

Therefore, the translation of RepA from a CTG start codon is expected to be lower than translation from an ATG codon. To test this prediction, we altered the *repA* start codon by mutagenic PCR from CTG to ATG and compared the expression of a RepA:β-galactosidase fusion protein in pMLB1034. Our early attempts to produce an ATG fusion protein under the control of *repA*$_p$ were unsuccessful, and we were able to produce only a fusion that was under the control of a pMLB1034 promoter located upstream of the point of insertion. However, this promoter was sufficiently strong to express the fusion protein, and β-galactosidase assays demonstrated that the ATG form expressed about four times more fusion protein than did the wild-type CTG form. We assume that the increased expression of the fusion resulting from the CTG-to-ATG change led to fatal levels of expression in cells carrying recombinant plasmids in which the fusion protein was under the control of *repA*$_p$. To survive, recombination events resulted in deletions that were seen in the electrophoretic examination of transformant DNA.

repA$_p$ has been located by in vivo genetic analyses using a set of promoter probe plasmids in which a number of potential *repA*$_p$ sequences were separated by restriction enzyme digestion or PCR amplification. An analysis of the RepA:β-galactosidase fusion protein expression from these plasmids indicates that one promoter sequence is responsible for at least 86% of the expression of the gene. This promoter sequence partially overlies the D″ repeat element and has been designated *repA*$_p$.

Gammie and Crosa (10) have used primer extension of RepA transcripts to locate the transcription initiation point (+1 nucleotide) and have then used this information to define the promoter sequence of *repA*$_p$. The ColV-K30 RepFIB *repA* +1 nucleotide corresponds to the cytosine residue at bp 2163 in the P307 RepFIB replicon. Since the sequence of a number of RepFIB replicons is highly conserved in this region (12), we presume that P307 RepFIB *repA* transcripts originate at this position. However, this assumption would place the +1 nucleotide within the −35 sequence of the promoter that we have determined on functional grounds to be *repA*$_p$. The only way to resolve the question of which promoter sequence is responsible for *repA* expression would be to clone the two potential promoter sequences (−35 and −10 sequences only) into a promoter probe vector to form gene fusions. Either the sequence determined here or the P307 RepFIB equivalent to the promoter defined by Gammie and Crosa (10) would be capable of expression, but not both.

The *repA* gene is autoregulated, and repression of *repA*$_p$ has been demonstrated by using pAS60 and pAS9. This combination of plasmids results in an almost complete repression of *repA*$_p$ activity. RepA may repress *repA*$_p$ by binding to the BCD elements to such an extent that RepA overhangs the promoter sequence and prevents RNA polymerase recognition of *repA*$_p$. Alternatively, RepA may recognize and bind to less well-conserved copies of the repeat elements A through K located closer to the −35 and −10 sequences of *repA*$_p$ and directly interfere with RNA polymerase access to the promoter. To determine which model best describes RepA repression of *repA*$_p$, we have tested the ability of RepA to repress *repA*$_p$ present in pAS7, pAS39, and pAS37 (containing BCDD′D″, half of C and DD′D″, and D″ repeat elements, respectively). RepA from pAS64 is

capable of repressing $repA_p$ in these three plasmids to similar degrees, and we have interpreted these results to mean that the D″ element is capable of RepA binding and is therefore functionally important. The inclusion of D′D″ results in a near-perfect direct-repeat group with similar spacing between elements. The D″ element covers the −35 sequence of $repA_p$, which, when coupled with the repression of pAS37 by pAS64, suggests that a single repeat element bound by RepA is able to prevent RNA polymerase access to the promoter sequence.

It was our intention to purify RepA expressed from pAS49 to allow a variety of in vitro experiments with which to test RepA-DNA binding characteristics. However, we have found that on induction, RepA expressed from pAS49 forms aggregates that are not readily separated from insoluble cellular material after cell lysis. We have prepared RepA protein extracts from the insoluble material by using 8 M urea and developed a DNA-binding assay which has allowed denatured RepA to refold into a functional protein (Western-DNA assay). The method that we have used is similar to protein blotting (5, 18, 23) whereby proteins are transferred to a membrane by capillary transfer with use of a urea buffer. However, unlike Herlt et al. (18), we have found that RepA renaturation can occur after SDS-PAGE separation, and the recovery of RepA DNA-binding activity does not require incubation and transfer in a renaturing urea buffer. We have demonstrated that membrane-bound RepA can specifically recognize and bind to DNA containing the BCDD′D″ repeat elements of RepFIB. The Western-DNA assay has also shown that RepA is able to bind probe DNA that contains a single copy of the D″ repeat element and provides complementary in vitro evidence supporting the repression of pAS37 by pAS64. By combining isolation (SDS-PAGE separation) and renaturation into a single assay, the in vitro proof of DNA binding has been far easier to demonstrate than if we had isolated, purified, and renatured RepA protein for use in gel retardation or filter retention assays.

Gammie and Crosa (10) have also shown that RepA is a DNA-binding protein by using a filter-binding assay. However, the semipurified RepA used by them appears to be a truncated form of RepA lacking the first 22 residues of the protein. An analysis of nine examples of RepFIB (12) show that there is no sequence variation in the region between the true *repA* start codon and the ATG start codon used by Gammie and Crosa, and we feel this degree of sequence conservation precludes the possibility that *repA* of pColV-K30 uses a different translation initiation start codon than does P307 RepFIB *repA*. Gammie and Crosa's observations suggest that DNA binding is not influenced by the N-terminal region of RepA, and such an interpretation is supported by the fact that the first of three potential DNA-binding motifs in RepA begins at residue 70 (12).

We have described in this work a number of in vivo and in vitro experiments which indicate that RepA is a DNA-binding protein, capable of binding to the BCDD′D″ repeat elements and autoregulating its own expression by repressing the *repA* promoter embedded within the D″ repeat element. Our experimental results are consistent with the classification of RepFIB as a Step function replicon, which was initially suggested after an analysis of the genetic organization of the replicon. The work presented here establishes the necessary basis for future research aimed at presenting a model for the control of RepFIB replication. In addition, the strong organizational homology between RepFIB and other Step function replicons suggests that a comparison of the RepFIB RepA control functions and the analogous functions in other replicons, especially mini-P1, might prove valuable in the development of a general control model for all Step function replicons.

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