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Importance of the C Terminus of Plasmid Rts1 RepA Protein for Replication and Incompatibility of the Plasmid

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RepA protein, essential for replication of plasmid Rts1, was found to bind in vivo immediately upstream of the *repA* promoter in studies with mini-Rts1 derivatives with deletions in the upstream region of *repA*. We constructed another series of *repA* mutants that would encode RepA derivatives containing oligopeptide substitutions in place of the carboxyl-terminal six amino acids. These modified RepA proteins could not activate *ori* (Rts1) at all and showed various degrees of incompatibility, or no incompatibility, toward a mini-Rts1 plasmid. These results suggest that the carboxyl-terminal six (or fewer) amino acids of RepA are important for exerting replication and incompatibility functions. One of the RepA derivatives, which showed an evident incompatibility without initiating replication, was examined for its ability to repress the *repA* gene.

The minimal replicon of plasmids, except for ColE1-type plasmids (21), generally contains a *rep* gene encoding a protein essential for plasmid replication (Rep protein) and the origin sequence for replication (*ori*) (17, 28). Besides these two components, some plasmids carry direct repeat sequences (iterons) located either upstream or on both sides of *rep*. R6K (18) and pSC101 (24) belong to the former group, and F (13), P1 (1), and Rts1 (8) are the latter-type plasmids that contain two sets of iterons. These iterons are known to regulate replication negatively by interacting with Rep protein, thereby expressing incompatibility. In addition, in these plasmids Rep protein functions as a repressor in one way by binding to the promoter region of its own gene and as an initiator to induce replication by binding to *ori* (3, 10, 15, 20, 25, 27), together with host factors. Recently, a two-stage model was proposed by Trawick and Kline (22) and Womble and Rownd (26) to explain the complex activities of the Rep molecule. They propose that the protein functions first as a repressor and then converts irreversibly to the initiator form. The molecule that is titrated by the iterons is considered to be the initiator form.

Mini-Rts1 encodes the 33,000-dalton RepA protein, consisting of 288 amino acids (8). The promoter sequence of *repA* was recently defined by both in vivo and in vitro studies (7). Mini-Rts1*cop1*, a high-copy-number mutant of mini-Rts1, contains a single base substitution in *repA* which replaces arginine (at amino acid position 142) with lysine (8). From in vivo studies (19) the RepA*cop1* protein is suspected to have a lowered binding affinity for the iterons. In the present study, we obtained a series of RepA derivatives with modified carboxyl-terminal regions and examined their functions relative to incompatibility and replication. In addition, the operator region to which RepA binds was determined.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* K-12 strains JC1569 (*recA1 gal leu his arg met str*) (4), JG112 (*polA lac thy str*) (12), and AB1157 (*galK lacY ara mtl xyl thr leu pro his arg thi str*) (6) were used as host cells. The plasmids used are listed in Table 1.

Media. Penassay broth (Difco Laboratories, Detroit, Mich.) and L broth were used for cultivation of bacteria. MacConkey-Gal plates were prepared by adding galactose to a final concentration of 1% to MacConkey agar base (Difco Laboratories) and used to examine *galK* expression of AB1157 harboring the pFD51 chimeric plasmid.

Chemicals. All restriction endonucleases used except *Nru*I (Nippon Gene, Toyama, Japan) were purchased from Takara Shuzo, Kyoto, Japan. *Eco*RI, *Hin*dIII, and *Bam*HI linkers were also from Takara Shuzo. [γ-^{32}P]ATP (7,000 Ci/mmol; New England Nuclear Corp., Boston, Mass.) and T4 polynucleotide kinase (Takara Shuzo) were used for determining nucleotide sequences.

Nucleotide sequence determination. Nucleotide sequences were determined by the method of Maxam and Gilbert (11). The procedures were as described previously (9).

Construction of deletions in *repA*. Mini-Rts1 derivatives pTW541 and pTW119-A, both of which have deletions in the upstream region of the RepA-coding frame (mini-Rts1 coordinates, 1131 to 268), were used as starting materials to introduce deletions within *repA*. The mini-Rts1 portion in pTW541 (coordinates 1213 to 1) is bounded by *Hin*dIII (linker) and *Eco*RI restriction sites, and that in pTW119-A (coordinates 1191 to 286) is bounded by *Hin*dIII (linker) and *Bam*HI (linker) sites (Fig. 1). It should be mentioned that during the construction of pTW119-A (i.e., at ligation of the 10-base-pair [bp] *Bam*HI linker to the *Alu*I site of mini-Rts1), a *Sma*I restriction site was unexpectedly generated beside the *Bam*HI site. The *repA* subregions excised from these plasmids were cloned into the polylinker region of a promoterless *galK* plasmid pFD51 (Fig. 1). Thus, we prepared two series of mini-Rts1 deletions; the upstream ends of mini-Rts1 deletions were coordinates 1213 and 1191, respectively. All of the cloned subregions, except in case of pTW1213-Xb, were aligned to read *repA* and *galK* in the same orientation starting from the promoter of *repA*. Among the pFD51 chimeric plasmids obtained, pTW1191, which contained *repA* starting at coordinate 1191 but had a deletion of the terminal 18 bp at the 3′ end of the noncoding strand, was a key plasmid in this study, since various derivatives were constructed from this plasmid. The derivatives pTW1191-S, pTW1191-BB, and pTW1191-SB (named pFD51:*repA*Δ*C* plasmids), were constructed by cutting and recircularizing pTW1191 DNA by using its appropriate restriction sites located downstream of *repA*Δ*C* (see Fig. 4). This allowed

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TABLE 1. Plasmids used[a]

Plasmid	Composition or character	Reference or source
Vectors		
pFD51	Promotorless *galK* Ap^r	14
pDR720	Ap^r	16
pACYC184	Cp^r Tc^r	2
pUC8	Ap^r	23
Mini-Rts1 derivatives		
pTW601	Mini-Rts1:Sp^r	9
pTW541	(1213–1):pBR322[b]	7
pTW119	(1191–216):pBR322	This laboratory
pTW119-A	(1191–286):pUC8	This laboratory
pTW1213-Xa	(1213–516):pFD51	This study
pTW1191-X	(1191–516):pFD51	This study
pTW1191	(1191–286):pFD51	This study
pTW1191-S	(1191–286 + α):pFD51[c]	This study
pTW11-S:AC	(1191–286 + α):pACYC184	This study
pTW11-HII:AC	(1191–216):pACYC184	This study

[a] The mini-Rts1 derivatives listed are the ones mainly used in this study.
[b] Numbers in parentheses are the mini-Rts1 coordinates (Fig. 3).
[c] α, Region added to the C terminal by readthrough.

readthrough in the pFD51:*repAΔC* plasmids from the RepA-coding frame into the vector for various lengths.

Cloning of *repAΔC* into pACYC184. To clone the entire RepAΔC protein-encoding region of pTW1191-S, a unique *Nru*I restriction site located 19 bp upstream of the *galK* start codon of the plasmid was used (see Fig. 4). A *Hin*dIII linker was added to the blunt end generated with *Nru*I digestion of pTW1191-S, and the *repAΔC* was isolated as a 1.1-kilobase (kb) *Hin*dIII fragment. The fragment was cloned into the *Hin*dIII site of pACYC184, giving rise to pTW11-S:AC. Also the entire genome of the *repA* wild type was isolated from pTW119, in which the coordinates 1191 to 216 are bounded by *Hin*dIII (linker) sites, as a 0.98-kb fragment and cloned into the *Hin*dIII site of pACYC184, giving rise to pTW11-HII:AC.

Reconstruction of *repAΔC:ori* (Rts1) plasmid. To investigate whether the RepA proteins deleted for the six C-terminal amino acids (designated RepAΔC6) can induce replication from *ori* of Rts1, an *ori* (Rts1) fragment was combined with the pFD51:*repAΔC* plasmids. The *ori* (Rts1) consists of a 248-bp fragment (coordinates 1441 to 1194) bounded by *Hin*dIII and *Bam*HI linkers in pTW554 (Fig. 2). This 0.25-kb *ori* fragment was isolated as a 0.5-kb *Eco*RI fragment after cloning the 0.25-kb fragment into the *Hin*dIII-*Bam*HI sites of pDR720. The 0.5-kb *ori* (Rts1) fragment was then cloned into the unique *Eco*RI site of pTW1191 and its derivatives, giving rise to pTW1191:*ori* (Rts1), pTW1191-S:*ori* (Rts1), pTW1191-BB:*ori* (Rts1), and pTW1191-SB:*ori* (Rts1). To construct a pFD51 chimeric plasmid containing the complete genome of *repA* and *ori* (Rts1), the 0.98-kb *Hin*dIII fragment (coordinates 1191 to 216) from pTW119 was inserted into the *Hin*dIII sites of pTW1191:*ori* (Rts1) in place of the *repAΔC* of the latter plasmid. The resulting plasmid was named pTW1191-HII:*ori* (Rts1).

pFD51 chimeric plasmid and *galK* expression. pFD51 is a promoterless *galK* plasmid (14). Accordingly, when a DNA fragment with promoter activity is inserted in front of the *galK* structural gene in the correct orientation, the chimeric plasmid confers a *galK*$^+$ phenotype to its host, AB1157 (*galK*). If the inserted promoter activity is weak, colonies developed on MacConkey-Gal plate are white with a pinpoint red center, whereas in the case of a strong promoter the colonies are fully red and larger than the former case.

Transformation and incompatibility. Transformation was carried out by the method of Cohen et al. (5). Incompatibility of the cloned *repA* subregions toward the mini-Rts1 plasmid was examined by transforming pTW601 with any of the pFD51 chimeric plasmids. Transformants were selected with a combination of 50 μg of ampicillin and 30 μg of spectinomycin per ml. When transformants were obtained on the plate, a single colony was picked and seeded onto the plate containing both drugs. From the doubly infected cells thus obtained, several single colonies were selected and streaked separately onto a plate without drugs. One hundred colonies from each plate without drugs were examined for their resistance to ampicillin and spectinomycin.

RESULTS

Promoter of *repA* and incompatibility. In mini-Rts1, the reading frame of *repA* is assigned from the coordinates 1131 to 268 (8), and its promoter, P*repA*, was recently determined to be located between coordinates 1191 and 1165 (7). To determine whether RepA protein can bind to P*repA*, mini-Rts1 subregions from a midpoint in *repA*, the *Xba*I site at coordinate 516, to bp 1213 and 1191 were cloned, respectively, in front of *galK* in pFD51 (Fig. 1), and their incompatibility toward pTW601 was tested in JC1569 (*recA*). Both pTW1213-Xa and pTW1213-Xb excluded pTW601, whereas pTW1191-X coexisted stably with pTW601. This suggests that RepA protein (from pTW601) does not bind to P*repA* but binds to the immediately upstream of P*repA* at coordinates 1213 to 1191, which contain a sequence similar to that of one of the iterons. Accordingly, we call this region (coordinates 1213 to 1191) an operator of *repA*, which is substantiated in the experiment described below.

It should be mentioned that AB1157 (*galK*) harboring pTW1191-X as well as pTW1213-Xa formed fully red colonies on MacConkey-Gal plates, indicating the presence of a promoter in the cloned fragment with the direction from the coordinate 1191 toward *repA*, as demonstrated previously (7). In contrast, pTW1213-Xb, in which the fragment (coordinates 1213 to 516) was inserted in the reverse orientation compared with that in pTW1213-Xa, did not confer the GalK$^+$ phenotype to AB1157.

***repA* and incompatibility.** In the incompatibility test, pTW1191, which contained coordinates 1191 to 286, unexpectedly showed a weak but apparent incompatibility toward pTW601; i.e., about 10% of JC1569(pTW601)(pTW1191) cells lost pTW601 preferentially during colony formation on a plate containing both ampicillin and spectinomycin. Accordingly, the mini-Rts1 subregion 1191 to 286 was divided, the parts were cloned separately into pFD51, and their incompatibility was examined. Neither of the divided subregions at coordinates 1191 to 361 and 516 to 286 in pFD51 expressed incompatibility to pTW601 (Fig. 3). It should be noted that the region at coordinates 1191 to 286 does not contain any part of *incI* and *incII* iterons (8). This suggests that the incompatibility mediated by pTW1191 is due not to the cloned DNA fragment but to a product from the fragment at coordinates 1191 to 286. Nucleotides 1131 to 286 in this fragment would encode a RepA protein deleted for its carboxyl-terminal six amino acid residues, RepAΔC6 protein, deduced from the nucleotide sequence of mini-Rts1 (8).

To make clear the extent of a readthrough from the *repAΔC* into the vector in pTW1191, the nucleotide sequence of the border region was determined (Fig. 4). The

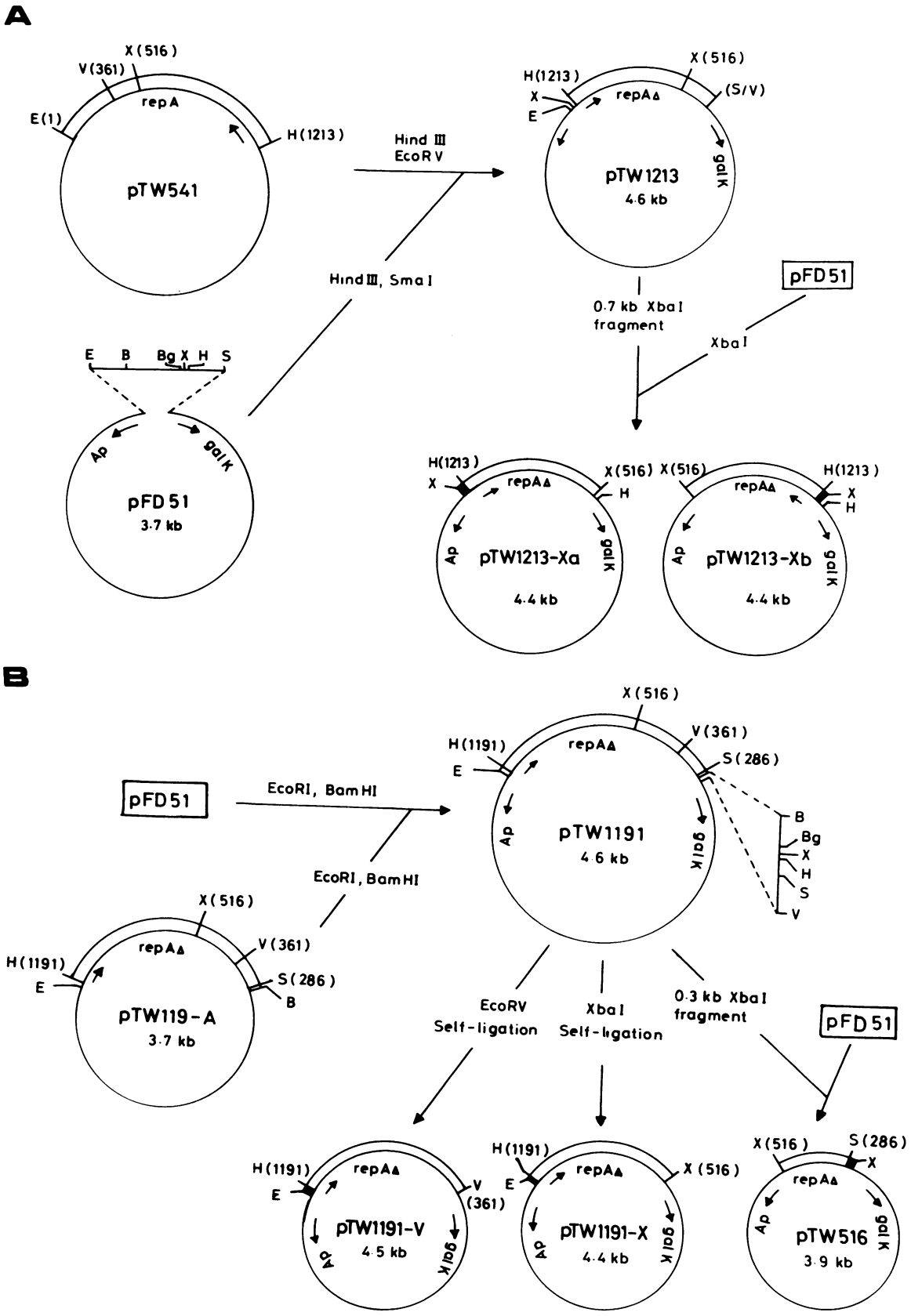

FIG. 1. Cloning of *repA* subregions. The 5′ ends of the cloned *repA* genes were the coordinates 1213 in (A) and 1191 in (B). pFD51 is a promoterless *galK* plasmid containing the ampicillin resistance gene. The numbers in parentheses are the coordinates of mini-Rts1 (see Fig. 3). Restriction endonucleases: E, *Eco*RI; V, *Eco*RV; X, *Xba*I; H, *Hin*dIII; B, *Bam*HI; Bg, *Bgl*II; S, *Sma*I.

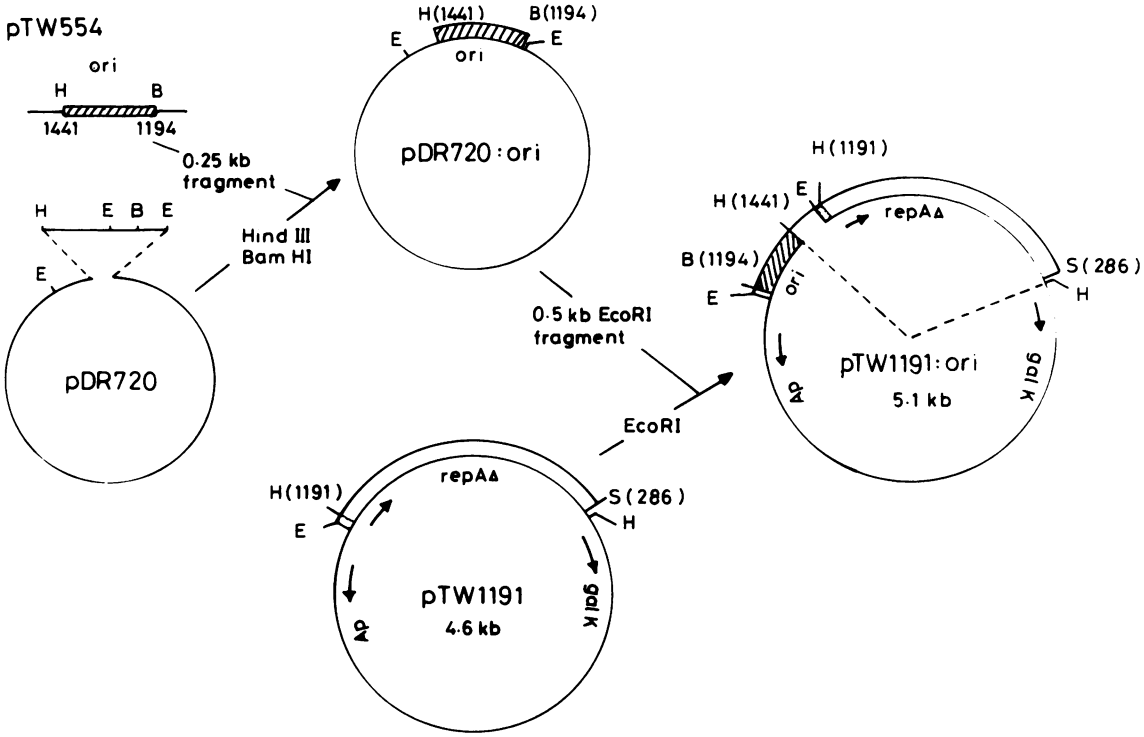

FIG. 2. Reconstruction of *repAΔC:ori* (Rts1) plasmid. A minimal origin sequences for Rts1 replication (coordinates 1441 to 1194) isolated as a 0.25-kb *Hin*dIII-*Bam*HI fragment from pTW554 was inserted into pDR720, from which *ori* (Rts1) was isolated as a 0.5-kb *Eco*RI fragment. The hatched bar represents *ori* (Rts1).

sequence analysis combined with the pFD51 nucleotide sequence (kindly supplied by S. Iida) revealed that 22 amino acids would be added to the C terminal of RepAΔC6 in pTW1191.

RepAΔC6 and incompatibility. In pTW1191, several restriction sites are located between the 3′ terminal of *repAΔC* and the front of *galK*, where six stop codons are placed in three phases (Fig. 4). These restriction sites and the *Sma*I and *Bam*HI sites at the 3′ end of *repAΔC* were used to construct pTW1191 derivatives. The numbers of amino acids added to the C terminal in place of the six amino acids (wild type) would be 3, 4, and 39 residues in pTW1191-S, pTW1191-BB, and pTW1191-SB, respectively (Fig. 4).

Among these, pTW1191-S was most interesting since it

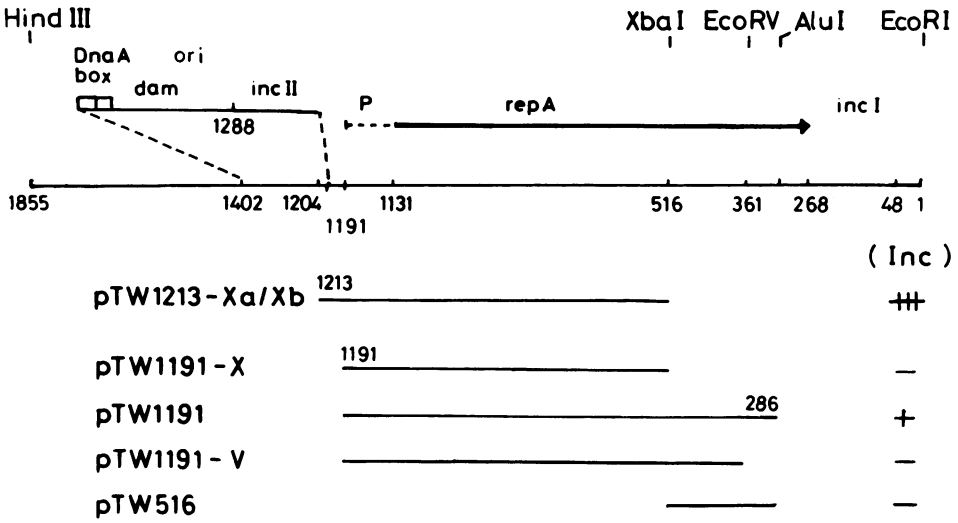

FIG. 3. Mini-Rts1 map and *repA* deletions. The *repA* subregions were cloned in front of the *galK* gene of pFD51. The 5′ ends of the cloned *repA* are the coordinates 1213 in pTW1213 and 1191 in pTW1191 series. Other features: DnaA box, tandem repeat of TTATCCACA sequences; P, promoter of *repA*; *repA*, reading frame of *repA* (coordinates 1131 to 268); *incI* and *incII*, five and three iterons, respectively; (Inc), incompatibility function of the cloned *repA* subregions toward the mini-Rts1 plasmid; (+++), no transformants obtained.

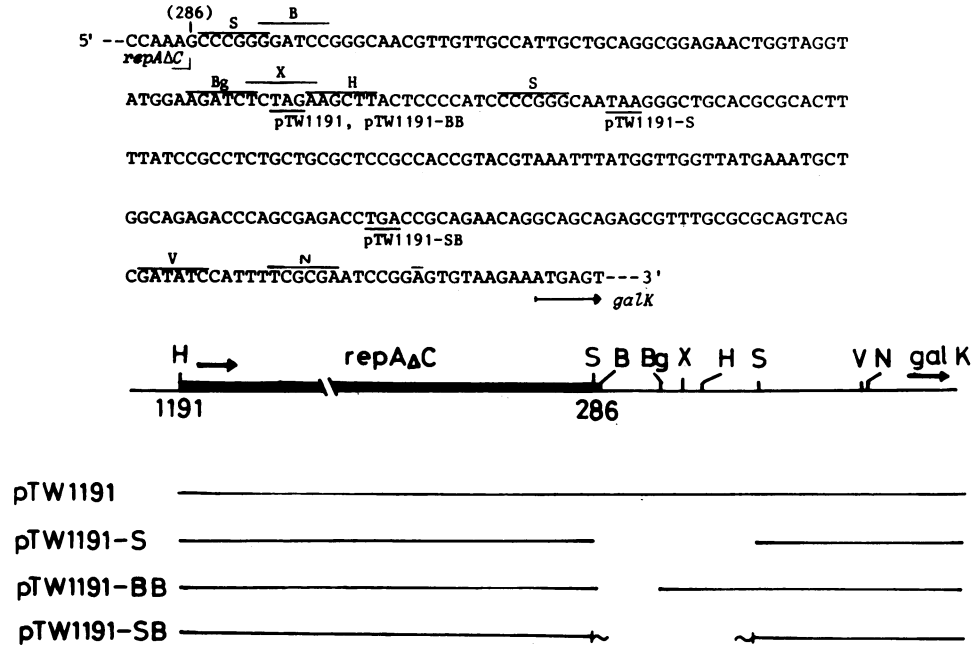

FIG. 4. Nucleotide sequence of the border region of pTW1191 between the carboxyl-terminal region of *repA* and the polylinker region of pFD51 (upper section) and deletion map of pTW1191 derivatives (lower section). The nucleotide sequence actually determined was from the *Eco*RV (mini-Rts1 coordinate 361) to *Bgl*II sites and from the *Bam*HI to the *Bgl*II sites. The coordinate 286 in parentheses is the *Alu*I site of *repA*, where a 10-bp *Bam*HI linker had been added during the construction of pTW119-A (Fig. 1). Stop codons used for the RepAΔC6 proteins in pTW1191 and its derivatives are underlined. Letters above the sequence indicate restriction sites: S, *Sma*I; B, *Bam*HI; Bg, *Bgl*II; X, *Xba*I; H, *Hin*dIII; V, *Eco*RV; N, *Nru*I. To construct pTW1191 derivatives, pTW1191 DNA was manipulated as follows: with *Sma*I digestion and ligation for pTW1191-S, with *Bam*HI-*Bgl*II digestion and ligation for pTW1191-BB, and with *Sma*I digestion and ligation after 8-bp *Bam*HI linkers were added to the blunt ends for pTW1191-SB.

showed clear incompatibility to pTW601. Transformation of JC1569(pTW601) with pTW1191-S DNA yielded much smaller colonies than transformation with pTW1191. Colonies were selected on plates containing both ampicillin and spectinomycin. In addition, only about 10% of the cells in a single colony retained pTW601 after restreaking on plates containing both drugs. Thus, RepAΔC6 produced from pTW1191-S (hereafter we call the protein RepAΔC6-S) appeared to mediate incompatibility toward mini-Rts1. pTW1191-BB showed an intermediate level of incompatibility between pTW1191-S and pTW1191, but pTW1191-SB showed no incompatibility with pTW601 (Table 2).

RepAΔC6 and replication functions. To determine whether the RepAΔC6 proteins can promote initiation of mini-Rts1 replication, an *ori* (Rts1) fragment was ligated to pTW1191 and its derivatives as described in Materials and Methods. None of the *repAΔC*:*ori* (Rts1) recombinant plasmids could replicate in the *polA* host JG112, suggesting that the RepAΔC6 is not capable of initiating replication (Table 2). That *ori* (Rts1) was actually present in these reconstituted plasmids was confirmed functionally by transforming JG112(pTW601) with these plasmids, where the *ori* (Rts1) in the incoming plasmid (the *repAΔC*:*ori* derivatives) was activated by RepA supplied in *trans* from the resident plasmid pTW601. Under these conditions transformants developed on plates containing both ampicillin and spectinomycin. Furthermore, when the *repAΔC* region of pTW1191 was replaced with the complete genome of *repA* (coordinates 1191 to 216) as described in Materials and Methods, the recombinant plasmid pTW1191-HII:*ori* (Rts1) could replicate autonomously and was maintained stably in JG112.

Effect of RepAΔC6-S on P*repA*. To investigate further the function of RepAΔC6-S, its entire encoding region was cloned into pACYC184 as described in Materials and Methods. This plasmid, pTW11-S:AC, was introduced into strain AB1157. AB1157(pTW11-S:AC) was then transformed with either pTW1213-Xa or pTW1191-X to determine whether RepAΔC6-S from the resident plasmid would affect the *galK* expression by the incoming plasmid. To compare the effect of RepAΔC6-S with normal RepA, pTW11-HII:AC encoding wild-type RepA was also used (see Materials and Methods).

AB1157(pTW11-S:AC)(pTW1213-Xa) formed two types colonies on MacConkey-Gal plates containing both ampicillin and chloramphenicol (10 μg/ml, used for selecting pACYC184 chimeras) (Table 3). The majority, about 70% of the colonies, had red centers with broad white margins, and the remaining colonies were mostly red. In contrast, all colonies from AB1157(pTW11-HII:AC)(pTW1213-Xa) were white (with a pinpoint red center in each colony). In addition, the size of the colonies of the latter was smaller than

TABLE 2. Incompatibility and replication functions conferred by RepAΔC6

Plasmid	Composition of RepA[a]	Incompatibility to pTW601[b]	Provocation of replication[c]
pTW1191-HII	Wild type	++	+
pTW1191	−6 + 22	+	−
pTW1191-S	−6 + 3	++	−
pTW1191-BB	−6 + 4	+	−
pTW1191-SB	−6 + 39	−	−

[a] −6, Deletion of C-terminal 6 amino acids of RepA; +, amino acids newly added to the C terminal.

[b] ++, 10% or less of cells in a single colony harbored pTW601 after development on a plate containing both Ap and Sp; +, 70 to 90% of cells retained pTW601; −, no loss of pTW601.

[c] The ability of the plasmid to replicate in a *polA* host.

TABLE 3. Effect of RepA and a deletion derivative on the promoter of *repA*

Plasmid				Inhibitory effect[a] on *galK* expression
Donor	(O P)[b]	Resident	Protein[c]	
pTW1191-X	(O^- P^+)	pTW11-S:AC	RepAΔC6-S	−
pTW1191-X	(O^- P^+)	pTW11-HII:AC	RepA	−
pTW1213-Xa	(O^+ P^+)	pTW11-S:AC	RepAΔC6-S	+
pTW1213-Xa	(O^+ P^+)	pTW11-HII:AC	RepA	++

[a] Effect of the resident plasmid on the *galK* expression by the incoming plasmid (see Materials and Methods). The host was AB1157 (*gal*K), and incubation of MacConkey-Gal plate was for 15 h at 37°C.
[b] Presence of operator (O) and promoter (P) of *repA* in pFD51 chimeric plasmid.
[c] Encoded protein on the resident plasmid.

that in the former. When pTW1191-X, which contains no operator of *repA*, was used as a donor plasmid, the transformants produced fully red colonies irrespective of the presence of either pTW11-HII:AC or pTW11-S:AC. These suggest that RepA binds to the operator just upstream of P*repA*, thereby inhibiting the expression of its own gene. RepAΔC6-S does bind (but weakly) to the operator.

DISCUSSION

Recently, we determined the promoter sequence of mini-Rts1 *repA*, P*repA*, and the 5′ end of the −35 region was defined at around the coordinate 1191 in the mini-Rts1 map (7). Indeed, a mini-Rts1 subregion, bp 1191 to a midpoint of *repA*, which was cloned in front of the promoterless *galK* structural gene of pFD51, conferred a $GalK^+$ phenotype to the host AB1157 as shown in this study.

In the plasmids that contain iterons vicinial to the *rep* gene, it has been established that the Rep protein binds to the promoter region of its own gene, by which Rep protein synthesis is negatively regulated (3, 10, 15, 25, 27). Therefore, the cloned promoter-operator region should manifest incompatibility toward its parent plasmid by titration of Rep protein molecules derived from the parent plasmid. On this assumption, we cloned P*repA* with its neighboring portions, and found that the upstream region (coordinates 1213 to 1191, where a similar sequence to *inc* iterons exists) of P*repA* was involved in the incompatibility to the mini-Rts1 plasmid, but P*repA* itself (bp 1191 to 1165) did not cause incompatibility. This finding was substantiated by the fact that normal RepA repressed the *galK* expression of coexisting pTW1213-Xa, which contains the *repA* operator region. In addition, the purified RepA was found to bind to the region (coordinates 1213 to 1191) by DNase I footprinting experiments (Y. Kamio and Y. Terawaki, manuscript in preparation). These indicate that RepA binds to the operator, located just upstream of P*repA*, and negatively autoregulates RepA synthesis. Thus, RepA of Rts1 is also an autoregulatory protein.

Comparing the Rep protein-binding sites in the promoter region of several plasmids, the E protein of F binds to the region from between positions −35 and −10 to downstream (20), and the π protein of R6K binds both to the portion between the −35 and −10 regions and to the upstream region (10). In the P1 replicon, −35 and −10 regions are completely included within *incC* iterons (3), indicating that RepA protein of P1 binds to the region encompassing the −35 and −10 sequences. In this connection, mini-Rts1 is similar to R6K in its promoter-operator location. This might be related in part to the not-so-restricted copy number of mini-Rts1 in comparison with mini-F and mini-P1 (the copy number of mini-Rts1 is estimated to be threefold as high as that in mini-F; our unpublished data).

A most interesting finding in this study is that the RepAΔC6-S protein, a derivative of RepA deleted for its C-terminal six amino acids with the addition of three different amino acids, showed an evident incompatibility to pTW601, whereas the protein could not induce replication from *ori* (Rts1). The presence of a 33-kilodalton protein corresponding to RepAΔC6-S was actually confirmed in pTW1191-S^+ cells by using the maxicell method (Zeng Hong, personal communication). The following possibilities are considered as a cause of the incompatibility mediated by RepAΔC6-S. First, the protein could inhibit the normal RepA synthesis by binding to the operator of *repA* of pTW601. Second, the overproduced RepAΔC6-S protein could suppress the replication of pTW601, as was seen with the overproduced P1 RepA on P1 replication (3). Third, the protein could compete with normal RepA at the replication origin of pTW601; that is, competition with normal RepA for *ori* binding or for making a complex with host-encoded factors required for the plasmid replication. The last possibility seems unlikely, since RepAΔC6-S and the other RepAΔC6 proteins could not activate *ori* (Rts1) at all, suggesting that the RepA derivative can not bind to *ori*, nor can it form a complex with the host factors. In an in vivo study, RepAΔC6-S could bind (but with a less efficiency) to the operator. Therefore, the first possibility may be likely. At present, however, the second possibility is not excluded, and both might contribute to the expression of the incompatibility. An in vitro binding experiment of the RepAΔC6 protein to the mini-Rts1 subregions including the operator would be helpful to solve the problem.

The two-stage model predicts that the DNA-binding domain of Rep would be common for both repressor and initiator forms (22, 26). If the model is applied to RepAΔC6-S, it is difficult to explain the dissociation of RepAΔC6-S functions; i.e., a complete loss of initiator function and a partial impairment of repressor activity. However, this does not contradict the model, since the C terminal of RepA might not be the DNA binding region but may be required to stabilize the RepA-DNA complex. Our mini-Rts1*cop1* contains a single base substitution at a midpoint in *repA* and results in a threefold increase in the plasmid copy number (19). This suggests that the midportion of RepA molecule is important for regulating the frequency of initiation of the plasmid replication.

Finally, we must mention the behavior of RepAΔC6, encoded on pTW1191-SB, which shows neither replication nor incompatibility functions. This derivative has a longer peptide substitution than the other RepAΔC6 proteins at the C terminal in place of the six amino acids. Thus, it appears

that an extensive modification of the C terminus of Rts1 RepA destroys both replication and incompatibility functions simultaneously.

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