JOURNAL OF BACTERIOLOGY, Nov. 1992, p. 6904–6910
0021-9193/92/216904-07$02.00/0

Vol. 174, No. 21

Function of the N-Terminal Half of RepA in Activation of Rts1 *ori*

YOSHIRO TERAWAKI,* YOSHIFUMI ITOH,† HONG ZENG,‡
TETSUYA HAYASHI, AND AKIRA TABUCHI

Department of Bacteriology, Shinshu University School of Medicine, Asahi 3-1-1, Matsumoto 390, Japan

Received 13 May 1992/Accepted 25 August 1992

The RepA protein of the Rts1 plasmid, consisting of 288 amino acids, is a *trans*-acting protein essential for replication. A mutant *repA* gene, *repA*ΔC143, carrying a deletion that removed the 143 C-terminal amino acids of RepA, could transform, but at a low frequency, an *Escherichia coli polA* strain, JG112, when *repA*ΔC143 was cloned into pBR322 with Rts1 *ori* in the natural configuration. The transformation was less efficient without the dyad DnaA box in the *ori* region, and no transformation occurred at 42°C, characteristic of Rts1 replication. A fusion of the 3′-terminal half of *repA* of the P1 plasmid to *repA*ΔC143 yielded a pBR322 chimeric plasmid that contained Rts1 *ori* through hybrid (Rts1-P1) *repA*. This plasmid was maintained much more stably in JG112 at 37°C. At 42°C, however, it was quite unstable. The overproduced hybrid RepA protein showed interference with mini-Rts1 replication in *trans* and also exhibited an autorepressor function, although both activities were decreased. These findings suggest that the N-terminal half of the RepA molecule of Rts1 is involved in the activation of the replication origin.

The essential replication regions of plasmids usually contain two important components: an origin sequence (*ori*) and a gene, *rep*, encoding a Rep protein (23). The Rep protein of various plasmids binds to *ori*, leading to the initiation of plasmid replication. It also binds to its promoter region, resulting in the autoregulation of Rep protein synthesis (2, 15, 21, 24, 30). P1 and Rts1 especially resemble each other in their basic replicon structures (2, 16). In addition, their Rep protein, RepA, shows high homology in amino acid sequence. P1 and Rts1, however, belong to different incompatibility groups.

To gain insight into the structure and function of the 288-amino-acid Rts1 RepA molecule, we constructed various *repA* mutants and examined their phenotypes with regard to Rts1 *ori* activation, autorepression, and inhibitory effect on mini-Rts1 replication (26, 27). Recently, we introduced site-directed mutations near the 3′ terminus of *repA* and obtained mutant RepA proteins that had lost the *ori* activation function. One of these, $RepA_{Z279}$ (Arg-279 to Gly), exhibited increased interference with mini-Rts1 replication (33). In this study, we determined that the RepA molecule of Rts1 with a deletion of the 143 C-terminal amino acids still retained the functions of Rts1 *ori* activation and autorepression, although at decreased levels.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* K-12 strains JC1569 (*recA1 gal leu his arg met str*) (5), JG112 (*polA lac thy str*) (20), JM109 [*recA1 supE endA1 hsdR17 gyrA96 relA1 thi* Δ(*lac-proAB*) F′ (*traD36 proAB*$^+$ *lacI*^{q} M15)] (32), and AB1157 (*galK2 leu thr pro his thi lacY ara xyl supE44 str*) (10) were used as host cells. The plasmids used are listed in Table 1. A mini-P1 plasmid, pALA109, containing the *rep* and *par* regions of plasmid P1 (2) with the kanamycin resistance gene from Tn*5* (18) and harbored in *E. coli* N100 (8), was kindly donated by A. Abeles.

Media and chemicals. Penassay broth (Difco Laboratories, Detroit, Mich.) was used for the cultivation of bacteria unless otherwise noted. L broth without glucose was used for transformation. MacConkey-Gal plates were prepared by adding galactose to a final concentration of 0.6% to MacConkey agar base (Difco) and used to examine the *galK* expression of AB1157 harboring the pFD51 chimeric plasmid. Restriction endonucleases, DNA polymerase I (Klenow fragment), *Eco*RI and *Sma*I linkers, and T4 DNA ligase were purchased from Takara Shuzo, Kyoto, Japan.

Plasmid DNA preparation and transformation. Plasmid DNA was prepared by the method of Humphreys et al. (11), and transformation was carried out as described by Cohen et al. (6).

Construction of pTW547 derivatives and other recombinant plasmids. pTW547, described previously (13), is a pBR322 recombinant plasmid in which a mini-Rts1 subregion (coordinates 1441 to 697) is inserted as a *Hin*dIII-*Cla*I fragment (Fig. 1). In the fragment, Rts1 *ori* and *repA*, with a deletion of the 3′-terminal half, are present in the natural configuration. Hence, pTW547 encodes a mutant RepA protein, RepAΔC143, that lacks the 143 C-terminal amino acids. At the C terminus of RepAΔC143, six amino acids were added by read-through into the pBR322 sequence. A *repA* subregion corresponding to that in pTW547 was isolated from pTW100 (26) (Fig. 1) as a 0.5-kb *Hin*dIII-*Cla*I fragment and cloned between the *Hin*dIII and *Cla*I sites of pBR322, giving rise to pTWb:*repA*ΔC143. The Rts1 *ori* sequence (coordinates 1441 to 1194) was isolated as a 0.25-kb *Hin*dIII-*Eco*RI fragment from pTW100. This 0.25-kb fragment was cloned between the *Hin*dIII and *Eco*RI sites of pBR322, giving rise to pTWb:Rts1 *ori*. The *lac* promoter, P*lac*, was isolated from pUC19 as a 181-bp *Pvu*II-*Hin*dIII fragment or as a 232-bp *Pvu*II-*Eco*RI fragment and cloned, respectively, into pTWb:*repA*ΔC143 at the *Eco*RV and *Hin*dIII sites or into pBR322 at the *Eco*RV and *Eco*RI sites, giving rise to pTWb:

* Corresponding author.
† Present address: Genetic Engineering Laboratory, Division of Applied Microbiology, National Food Research Institute, Ministry of Agriculture, Forestry and Fisheries, Tsukuba 305, Japan.
‡ Present address: Institute of Tokyo Immunological Pharmacology, Takada 3-41-8, Toshima-ku, Tokyo 171, Japan.

TABLE 1. Plasmids used

Plasmid	Mini-Rts1 composition or character[a]	Reference or source
pBR322	Ap^r Tc^r	3
pACYC184	Cp^r Tc^r	4
pFD51	Ap^r; promoterless *galK*	22
pUC19	Ap^r	32
pMY1123	Ap^r Tc^r	H. Danbara
pALA109	Mini-P1:Km^r	A. Abeles
pTW601	Mini-Rts1:Sp^r	12
pTW547	pBR322 (1441–697)	13
pTW547-P1A	pBR322 (1441–697 and 1000–1852)	This study
pTW547-P1AΔ1	pBR322 (1191–697 and 1000–1852)	This study
pTW550	pBR322 (1381–697)	13
pTWb:*repA*ΔC143	pBR322 (1191–697)	This study
pTWb:*repA*ΔC143:P*lac*	pBR322 (1191–697):Plac	This study
pTWb:Rts1 *ori*	pBR322 (1441–1194)	This study
pBR322:P*lac*	pBR322:Plac	This study
pTW11:*repA*-A	pACYC184 (1191–216)	33 and this study
pTW11:Rts1-P1 *repA*	pACYC184 (1191–697 and 1000–1852)	This study
pTW100	pFD51 (1441–1194 and 1191–216)	26
pFD51:*repA*	pFD51 (1191–216)	26
pTW1213-S	pFD51 (1213–1020)	This study

[a] Numbers in parentheses are mini-Rts1 coordinates (16) and mini-P1 coordinates (2) (two sets) and mini-Rts1 coordinates (one set). Ap, ampicillin; Tc, tetracycline; Cp, chloramphenicol; Km, kanamycin; Sp, spectinomycin.

*repA*ΔC143:P*lac* or pBR322:P*lac*, respectively. pTW1213-S, which was used as a target plasmid in the *galK* expression study, was constructed as follows. A mini-Rts1 subregion (coordinates 1213 to 510) was isolated from pTW1213-Xa (26) as a *Hin*dIII fragment and cloned into the *Hin*dIII site of pBR322 in the same orientation as the *tet* gene. The plasmid obtained, pBR322:1213-Xa, was digested with *Sty*I, and *Sma*I linkers were ligated to the restricted ends after the ends were made blunt with the Klenow fragment. Then, the mini-Rts1 subregion (coordinates 1213 to 1010) in which the operator-promoter sequence of *repA* is accompanied by a very short stretch of the N-terminal portion of *repA* was isolated as a *Hin*dIII-*Sma*I fragment and cloned in front of the promoterless *galK* gene of pFD51 (22), giving rise to pTW1213-S.

Construction of *repA* fusion gene. To construct a fusion of the Rts1 *repA* gene with the P1 *repA* gene, we converted the *Cla*I site of pTW547 to an *Eco*RI site by adding a 12-mer *Eco*RI linker after filling in the *Cla*I site with the Klenow fragment; this process yielded pTW547^E. First, the 3′ half of P1 *repA* (mini-P1 coordinates 1000 to 1852, encompassed by *Eco*RI and *Hin*dIII sites) was isolated from pALA109 as a 2.1-kb *Eco*RI-*Sma*I fragment conferring kanamycin resistance. Then, this 2.1-kb fragment, in which the *incA* locus of P1 is also contained, was inserted between the *Eco*RI and *Sca*I sites of pTW547^E, giving rise to pTW547-P1K. Thus, a hybrid (Rts1-P1) *repA* gene was generated. From this recombinant plasmid, Rts1 *ori* through Rts1-P1 *repA*, along with P1 *incA*, was isolated as a 1.6-kb *Hin*dIII fragment. This 1.6-kb fragment was cloned into the *Hin*dIII site of pBR322, giving rise to pTW547-P1A and pTW547-P1A′ (Fig. 2). In the former, the cloned fragment is in an orientation such that the hybrid *repA* gene is transcribed towards the *bla* gene of pBR322, and in the latter the fragment is in the reverse orientation. pTW547-P1AΔ1, which retains Rts1-P1 *repA* but lacks Rts1 *ori*, was constructed as follows. The smaller *Sty*I fragment of pTWb:*repA*ΔC143, which contains the 5′-terminal region of Rts1 *repA* (coordinates 1191 to 1020), was ligated to the larger *Sty*I fragment of pTW547-P1A, which contains the mini-Rts1 and mini-P1 regions (coordinates 1020 to 697 and 1000 to 1852), resulting in the generation of a 1.25-kb *Hin*dIII fragment (coordinates 1191 to 697 and 1000 to 1852) located in the *Hin*dIII site of pBR322. The hybrid *repA* gene carried on the 1.25-kb fragment also was cloned into the *Hin*dIII site of pACYC184, giving rise to pTW11:

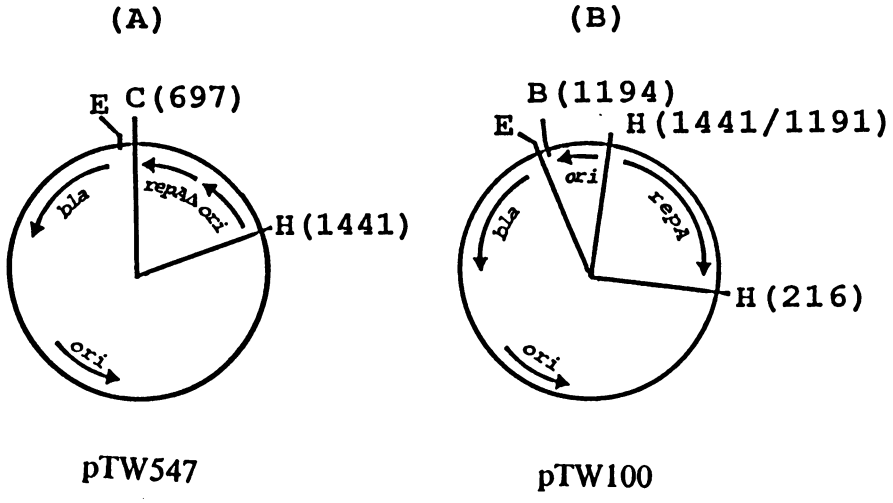

FIG. 1. Maps of mini-Rts1 derivatives pTW547 and pTW100. (A) A mini-Rts1 subregion (coordinates 1441 to 697; see Fig. 2) that contains Rts1 *ori* and a *repA* deletion, *repA*ΔC143, lacking the 3′-terminal half, was cloned into pBR322 between the *Hin*dIII and *Cla*I sites. Note that Rts1 *ori* and *repA*ΔC143 are present in the natural configuration. repAΔ, repAΔC143. (B) Wild-type *repA* with its native promoter (coordinates 1191 to 216) is positioned upstream of Rts1 *ori* (coordinates 1441 to 1194), which is inserted separately into pFD51. The pTW100-type plasmid encoding *repA* (wild type) could replicate in JG112, but that encoding $repA_{z279}$ could not (33). Numbers in parentheses are mini-Rts1 coordinates. Restriction sites: H, *Hin*dIII; E, *Eco*RI; C, *Cla*I; B, *Bam*HI.

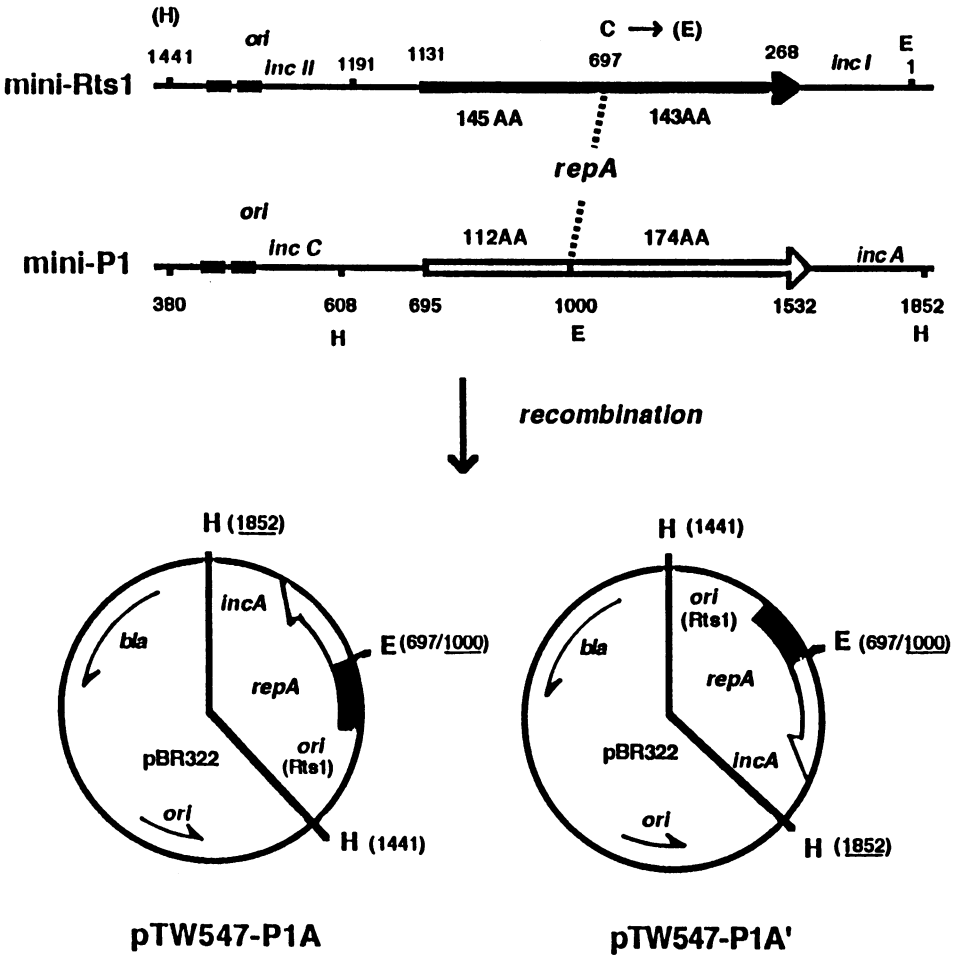

FIG. 2. Construction of pTW547 derivatives containing a hybrid (Rts1-P1) *repA* gene. Maps of mini-Rts1 and mini-P1 are shown in the upper portion. Numbers are mini-Rts1 (16) and mini-P1 (2) coordinates. Tandem closed squares are DnaA boxes. Bold arrows indicate the *repA* genes of Rts1 (closed) and P1 (open), encoding 288 amino acids (AA) and 286 AA, respectively. The *Cla*I (C) site in Rts1 *repA* was converted to an *Eco*RI (E) site by 12-mer linker ligation and then ligated to the *Eco*RI site in P1 *repA*. The resulting *Hin*dIII (H) fragment, which contains Rts1 *ori* and the hybrid *repA* gene, was finally cloned into pBR322 in both directions (see Materials and Methods). Numbers in parentheses in pTW547-P1A and pTW547-P1A' indicate mini-Rts1 coordinates (shown without underlining) and mini-P1 coordinates (shown with underlining).

Rts1-P1 *repA*, which was used as an effector plasmid in the *galK* expression study. The orientation of the insertion was the same as that of *repA* (wild type) in pTW11:*repA*-A i.e., opposite to the *tet* gene of pACYC184.

Immunoblot analysis of RepA and its derivatives. JC1569 cells harboring pFD51 or pBR322 recombinant plasmids containing wild-type or mutant *repA* genes were grown in 1.5 ml of L broth at 37°C. The cell lysates, which were prepared as described previously (33), were adjusted to contain the same amount of total protein in each well and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis by the method of Laemmli (17). Polypeptides in the gel were transferred to a nitrocellulose filter (pore size, 0.45 μm) by the method of Towbin et al. (29). The filter was blocked with bovine serum albumin, treated with anti-RepA antibody, and treated with alkaline phosphatase-conjugated anti-rabbit immunoglobulin G antibody (Promega Biotec, Madison, Wis.); color development was then done as recommended by the supplier.

Plasmid stability in single colonies. JG112 (*polA*) cells transformed with pTW547 or its derivatives formed small colonies on ampicillin (20 μg/ml)-containing plates after 30 h of incubation at 37°C. A single colony picked from each plate was suspended in Penassay broth and streaked onto an ampicillin-containing plate to yield a number of single colonies. Several of the single colonies were picked individually, suspended in Penassay broth, and then appropriately diluted. Twenty microliters of each suspension was spotted onto a plate with or without ampicillin (20 μg/ml). The number of colonies that developed on the plates was scored, and the ratio of plasmid-cured cells to total cells was determined.

Sensitivity of mini-Rts1 to overproduced RepA. The inhibitory effect of overproduced RepA on replication of a coexisting mini-Rts1 plasmid (pTW601) in *trans* was examined by introducing pBR322 recombinant plasmids carrying *repA* (wild type or hybrid) by transformation into JC1569 harboring pTW601. The transformants that developed on plates containing 20 μg of ampicillin per ml were picked individually and stabbed onto plates containing 30 μg of spectino-

mycin per ml to screen for the presence of resident plasmid pTW601. To examine the inhibitory function in detail, we examined the plasmid stability in single colonies as described above; i.e., each of the single colonies developed on plates containing ampicillin was individually suspended in Penassay broth and streaked onto a plate without the drug. The resulting colonies were examined for spectinomycin resistance.

***galK* expression study.** The autorepressor activities of the wild-type and hybrid RepA proteins were examined with a *galK* expression system as described previously (26) but with slight modifications. The target plasmid used in this study was pTW1213-S, in which the operator-promoter sequence of *repA* accompanied by a very short stretch of the *repA* N-terminal portion was inserted in front of the promoterless *galK* structural gene of pFD51 as described above. pACYC184 recombinant effector plasmid pTW11:*repA*-A (wild type) or pTW11:Rts1-P1 *repA* (hybrid) was introduced into AB1157 (*galK*). Then, the target plasmid was introduced by transformation into AB1157 harboring pTW11:*repA*-A or pTW11:Rts1-P1 *repA*. When the *repA* promoter of the target plasmid was strongly repressed, by RepA supplied in *trans*, the doubly infected cells formed white colonies on MacConkey-Gal plates containing both chloramphenicol and ampicillin. When no repression occurred, the colonies on the plate were red after 16 h of incubation at 37°C.

RESULTS

Rts1 *ori* activation by the N-terminal half of RepA. pTW547 (13) is a pBR322 chimeric plasmid that contains Rts1 *ori* and a deletion derivative of the Rts1 *repA* gene in the natural configuration, i.e., spanning mini-Rts1 coordinates 1441 through 697 (unique *Cla*I site). The *repA* derivative encodes RepAΔC143, which has a deletion of the 143 C-terminal amino acids of the wild-type RepA protein. Its synthesis in JC1569 was demonstrated in the immunoblotting profile (Fig. 3, lane 2). pTW547 could transform *E. coli polA* strain JG112. Although the transformants developed slowly on plates containing 20 μg of ampicillin per ml, they formed solid and isolated colonies after 16 h of incubation at 37°C. The transformants could subsequently form small colonies on serial purification. Single colonies purified on ampicillin-containing plates were shown to contain 0.1% or fewer drug-resistant cells, suggesting that pTW547 replicates inefficiently in the *polA* host. Since the percentages of single colonies harboring pTW547 did not increase on serial purification, it is unlikely that *polA*$^+$ revertants of JG112 were selected by the procedure. It is noteworthy that pTW550 (13), which lacks the dyad DnaA boxes of pTW547, could also transform JG112. In this case, however, the number and the size of the transformants on the drug-containing plates were smaller than those with pTW547. Furthermore, no transformants of JG112 developed on plates incubated at 42°C with either pTW547 or pTW550. The temperature sensitivity of replication and the less efficient replication without the DnaA boxes are characteristic of Rts1 replication (14, 28).

We then tested the individual effects of *repA*ΔC143 and Rts1 *ori* on the replication of pBR322 chimeric plasmids in JG112. *repA*ΔC143 with its native promoter (coordinates 1191 to 697) or the *ori* region (coordinates 1441 to 1194) was cloned into pBR322 at the site corresponding to that in pTW547; i.e., *repA*ΔC143 was inserted between the *Hin*dIII and *Cla*I sites, and Rts1 *ori* was inserted between the *Hin*dIII and *Eco*RI sites, giving rise to pTWb:*repA*ΔC143 and pTWb:Rts1 *ori*, respectively (see Materials and Methods). Unlike pTW547 and pTW550, neither of the pBR322 chimeric plasmids transformed JG112 (Table 2), indicating that both *repA*ΔC143 and Rts1 *ori* are required for transforming the *polA* host. Therefore, we concluded that the replication of pTW547 in JG112 was initiated at Rts1 *ori* and that *ori* activation was mediated by RepAΔC143. The initiation, however, was very inefficient, as shown by the plasmid stability in single colonies.

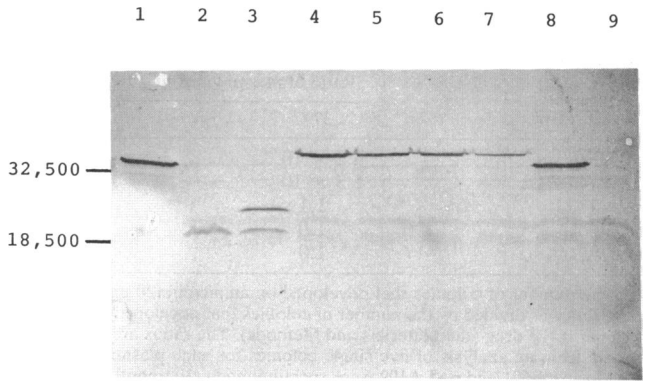

FIG. 3. Immunoblotting of the wild-type and mutant RepA proteins. Proteins were prepared from *E. coli* JC1569 harboring plasmids, except for lane 3, in which JM109 was used as the host. Lanes: 1, pFD51:*repA* (wild type); 2, pTW547; 3, pTWb:*repA*ΔC143:*Plac* (induced with IPTG); 4, pTW547-P1A; 5, pTW547-P1A′; 6, pTW547-P1AΔ1; 7, pTW11:Rts1-P1 *repA*; 8, pFD51:*repA* (wild type); 9, no plasmid. In pTW547, pTW547-P1A, pTW547-P1AΔ1, and pTWb:*repA*ΔC143:*Plac*, *repA* or Rts1 *ori-repA* was inserted in the orientation necessary to transcribe the *bla* gene of pBR322, and in pTW547-P1A′, the orientation of the insert was opposite. In pFD51:*repA*, wild-type *repA* was inserted in front of the promoterless *galK* structural gene of pFD51 to transcribe the *galK* gene. In pTW11:Rts1-P1 *repA*, the hybrid *repA* gene was inserted at the *Hin*dIII site of pACYC184 in an orientation opposite to that of the *tet* gene. Note that in lane 3, a product larger than RepAΔC143 is shown. It should be a fused protein consisting of the N-terminal oligopeptide from β-galactosidase and the N terminus of RepAΔC143. The numbers (32,500 and 18,500) are the molecular weights of carbonic anhydrase and lysozyme, respectively, purchased from Bio-Rad Laboratories, Richmond, Calif.

Insertion of the *lac* promoter upstream of *repA*ΔC143. It may be argued that the pTW547 replication observed in

TABLE 2. Transformation of a *polA* strain with pBR322:mini-Rts1 plasmids[a]

Plasmid	Mini-Rts1 component	Ratio of transformation (JG112 *polA*/JC1569 *polA*$^+$) at:	
		37°C	42°C
pTW547	*ori-repA*ΔC143	5.2×10^{-2}	$<1 \times 10^{-4}$
pTW550	*ori*(ΔD)-*repA*ΔC143	1.7×10^{-2}	$<1 \times 10^{-4}$
pTWb:*repA*ΔC143	*repA*ΔC143	$<1 \times 10^{-4}$	NT
pTWb:Rts1 *ori*	*ori*	$<1 \times 10^{-4}$	NT
pTWb:*repA*ΔC143:*Plac*	*repA*ΔC143	$<1 \times 10^{-4}$	NT
pBR322:*Plac*		$<1 \times 10^{-4}$	NT

[a] JG112 and JC1569 were transformed with 0.5 and 0.2 μg of plasmid DNA, respectively, and transformants were selected on plates containing 20 μg of ampicillin per ml by incubation at either 37 or 42°C for more than 30 h. *ori* (ΔD), Rts1 *ori* with a deletion of the DnaA boxes. NT, not tested.

TABLE 3. Stability of pTW547 and other plasmids in a *polA* host[a]

Plasmid	Ratio of plasmid-carrying JG112 cells at:	
	37°C	42°C
pTW547	6.2×10^{-4}	8.3×10^{-6}
pTW547-P1A	1.3×10^{-2}	6.5×10^{-5}
pMY1123	1.1	1.0
pTW601	1.0	8.8×10^{-5}
pALA109	1.0	1.2

[a] The number of colonies that developed on ampicillin (20 μg/ml)-containing plates was divided by the number of colonies that developed on plates not containing the drug (see Materials and Methods). The ratios are the averages of data from an analysis of five single colonies for each plasmid. The drugs used for pTW601 and pALA109 were spectinomycin (30 μg/ml) and kanamycin (10 μg/ml), respectively, instead of ampicillin. The temperatures indicated are for plating of cultures.

TABLE 4. Effect of overproduced RepA on mini-Rts1 plasmid replication

Plasmid		% of transformants with resident plasmid[b]	% of cells with resident plasmid[c]
Donor	Resident[a]		
pFD51:*repA*[d]	pTW601	37[e]	9[e]
pTW547-P1AΔ1[f]	pTW601	99	58
pBR322	pTW601	100	99
pFD51:*repA*	pALA109	100	100
pTW601	pALA109	100	100

[a] pTW601, mini-Rts1 plasmid; pALA109, mini-P1 plasmid.
[b] Each of the transformant colonies from ampicillin-containing plates (except for the pTW601 donor plates) was picked and stabbed onto spectinomycin (for pTW601)- or kanamycin (for pALA109)-containing plates. At least 100 transformant colonies were examined.
[c] A single transformant colony that was confirmed to be spectinomycin (or kanamycin) resistant by the data listed in the column to the left was suspended and streaked onto plates without drug. The colonies that developed were examined for the presence of the resident plasmid.
[d] At least six single transformant colonies were examined for each transformation. Overproduces wild-type RepA.
[e] Poor growth on spectinomycin-containing plates despite a large inoculum.
[f] Overproduces hybrid RepA.

JG112 was not due to the RepAΔC143-Rts1 *ori* interaction but that the promoter activity in the inserted mini-Rts1 subregion activated the pBR322 replication origin, leading to replication of pTW547 and pTW550. To test this possibility, we inserted the *lac* promoter from pUC19 upstream of *repA*ΔC143 in pTWb:*repA*ΔC143 or at the *Eco*RI site of pBR322 in an orientation such that transcription occurred in the direction of the *bla* gene (and hence towards the pBR322 origin), giving rise to pTWb:*repA*ΔC143:P*lac* or pBR322:P*lac*, respectively. As expected, a large amount of RepAΔC143 was visualized in the immunoblot profile of JM109 (*lac*I^q) (with isopropyl-β-D-thiogalactopyranoside [IPTG]) cells containing pTWb:*repA*ΔC143:P*lac* (Fig. 3, lane 3). The P*lac* plasmids, however, did not transform JG112 under any culture conditions, even when IPTG was added to the L broth culture for transformation (Table 2). Thus, the possibility that some promoter activity occurring upstream of the *bla* gene may have activated *ori* in pBR322 was ruled out.

Fusion of RepA of Rts1 with RepA of P1. The inefficiency of pTW547 replication in the *polA* host may have been due to the unnatural C-terminal sequence of RepAΔC143. Accordingly, we fused the 3′-terminal half of P1 *repA* (mini-P1 coordinates 1000 to 1852, contained in an *Eco*RI-*Hin*dIII fragment) (2) to the 5′-terminal half of Rts1 *repA* in pTW547 (see Materials and Methods). The recombinant plasmid obtained, pTW547-P1A, contains Rts1 *ori* through the hybrid *repA* gene, which is transcribed towards *bla* of pBR322. The hybrid protein, Rts1-P1 RepA, consists of 145 N-terminal amino acids from Rts1 RepA and 174 C-terminal amino acids from P1 RepA, and at the junction a proline residue is added by *Eco*RI linker insertion. Thus, Rts1-P1 RepA is composed of 320 amino acids. Its presence in JC1569 harboring pTW547-P1A was confirmed in the immunoblot analysis as a polypeptide slightly larger than wild-type Rts1 RepA (Fig. 3, lane 4).

pTW547-P1A transformed JG112 at 37°C but not at 42°C, as was the case with pTW547. The plasmid stability in single colonies of JG112 cells growing on ampicillin-containing plates revealed that pTW547-P1A was more stably maintained in the *polA* host than pTW547 at 37°C, while both plasmids were quite unstable at 42°C (Table 3). pMY1123, a mini-R100 plasmid with a low copy number, was used to examine the effect of temperature on *bla* gene expression. As shown in Table 3, JG112 carrying pMY1123 formed a large number of colonies at 42°C as well as at 37°C on ampicillin-containing plates, indicating that the expression of *bla* is not temperature sensitive. Since the *bla* genes in pTW547 and pTW547-P1A are derived from Tn*3*, as is that in pMY1123, we concluded that the temperature-sensitive growth of JG112 with pTW547 or pTW547-P1A on ampicillin-containing plates was due to the temperature sensitivity of plasmid replication. A mini-P1 plasmid, pALA109, was shown to be quite stable at both 37 and 42°C (Table 3). In contrast, mini-Rts1 plasmid pTW601 showed marked temperature sensitivity. Thus, Rts1 *ori* activation mediated by the hybrid RepA protein also exhibited the temperature sensitivity property of Rts1 replication, as was shown with RepAΔC143. It should be mentioned, however, that pTW547-P1A′, in which Rts1 *ori* through Rts1-P1 *repA* is inserted at the *Hin*dIII site of pBR322 in an orientation opposite to that of the *bla* gene, did not transform JG112 at any temperature. The amount of Rts1-P1 RepA synthesized in JC1569 with pTW547-P1A was approximately 1.6 times that synthesized in JC1569 with pTW547-P1A′ (Fig. 3, lanes 4 and 5), a result that was consistently observed. Because Rts1-P1 *repA* in pTW547-P1A is positioned downstream of the *P1* promoter of *bla* in pBR322 (25), transcription of the hybrid *repA* gene may have been enhanced by this *P1* promoter. However, we are uncertain as to whether the smaller amount of hybrid RepA in the pTW547-P1A′-carrying cells caused deficient activation of Rts1 *ori*.

Effect of RepA overproduction and autorepressor function. Since the Rts1-P1 RepA hybrid protein activated Rts1 *ori*, it was expected that the hybrid RepA protein might interact in *trans* with a mini-Rts1 plasmid (pTW601) and have an inhibitory effect on the replication of pTW601. To study this possibility, we constructed pTW547-P1AΔ1, which contains the hybrid *repA* gene but has a deletion of the Rts1 *ori* sequence, by using pTW547-P1A (see Materials and Methods). Incompatibility between pTW601 and mini-P1 plasmid pALA109 was also examined. In the initial study, each of the transformant colonies growing on plates containing the donor marker drug (ampicillin) was examined for resistance to the resident marker drug (spectinomycin). Almost all colonies selected for pTW547-P1AΔ1 also were shown to be spectinomycin resistant (Table 4). However, when individual cells that composed each of the single ampicillin-resistant

transformant colonies were examined for spectinomycin resistance, only 58% were shown to retain pTW601 (Table 4, last column). Thus, the hybrid RepA protein, when overproduced, showed a decreased interference with mini-Rts1 plasmid replication in *trans*. Wild-type Rts1 RepA supplied in an excess amount had no inhibitory effect on mini-P1 plasmid replication (Table 4), indicating that Rts1 and P1 are functionally independent replicons. Indeed, pTW601 and pALA109 coexisted quite stably (Table 4, last row). Therefore, the ability of Rts1-P1 RepA to interfere with pTW601 replication could be ascribed to the possibly overproduced N-terminal portion of the molecule, which was derived from Rts1 RepA.

The autorepressor activity of the hybrid RepA protein was investigated with a *galK* expression system as described in Materials and Methods. AB1157 (*galK*) cells harboring pTW11:Rts1-P1 *repA*, when transformed with pTW1213-S, formed red colonies (having red centers with white margins) on MacConkey-Gal plates containing both chloramphenicol and ampicillin after 16 h of incubation at 37°C. In contrast, AB1157 (pTW11:*repA*-A) transformed with pTW1213-S formed white colonies on the plates, and cells harboring pACYC184 (without *repA*) and transformed with pTW1213-S formed red colonies. These findings suggest that the hybrid RepA protein retains autorepressor activity but at a decreased level.

DISCUSSION

Many plasmids encode their own initiator Rep proteins (23). Frequently, these proteins exist as dimers. One would expect that these proteins would contain regions specific for forming dimers as well as domains involved in DNA binding. Besides these, Rep proteins may have a sequence for interacting with host factors to activate the replication origin. Recently, it was clearly demonstrated that the binding of the P1 RepA protein to the origin sequence was greatly facilitated by the host factors DnaJ and DnaK, which serve to keep the protein in the monomeric form (31). Thus, the Rep protein displays various functions. However, studies of the functional domains of Rep molecules are scarce, except for the π protein of R6K (9).

A most remarkable finding obtained in this study was that the N-terminal half of the Rts1 RepA molecule was able to activate Rts1 *ori*, although inefficiently. Hoping to obtain more efficient replication of a pBR322 recombinant plasmid in JG112 (*polA*), we fused the 174 C-terminal amino acids of P1 RepA to the N-terminal half of Rts1 RepA. We used P1 RepA because, although Rts1 is quite compatible with P1, their RepA proteins show a high homology (about 60%) in amino acid sequence (2, 16). As demonstrated by the plasmid stability in single colonies, pTW547-P1A, which encodes Rts1-P1 RepA, was more stably maintained in JG112 at 37°C than pTW547, which encodes RepAΔC143. The Rts1 *ori* activation mediated by Rts1-P1 RepA was temperature sensitive, as was the activation mediated by RepAΔC143, a result that is characteristic of Rts1 replication (28). The hybrid RepA protein, when supplied in *trans* in an excess amount, was inhibitory for mini-Rts1 replication and, in addition, showed an autorepressor function, although both activities were decreased in comparison with those of wild-type RepA protein. The low efficiency of *ori* activation by Rts1-P1 RepA and RepAΔC143 might be ascribed to lower DNA binding affinity of mutant RepA proteins, as suspected from the decreased autorepressor function of hybrid RepA protein. If we could measure the inhibition by RepA(Rts1-P1) on mini-P1 replication, the specificity of the hybrid protein could be analyzed easily. However, such an analysis is at present impossible, because pTW547-P1AΔ1, which encodes Rts1-P1 RepA, also contains a complete set of *incA* direct repeats of P1 at the 3′ terminus of the cloned P1 *repA* fragment.

Our previous studies had suggested the importance of the C-terminal region of RepA for Rts1 replication and incompatibility functions (26, 33). One of the C-terminal mutant proteins, $RepA_{z279}$ (Arg279 to Gly), showed increased interference with pTW601 replication in *trans* but could not induce replication from Rts1 *ori* (33), even when $repA_{z279}$ was positioned in the wild-type configuration, as in pTW547 and pTW547-P1A (unpublished data). Since $RepA_{z279}$ has an intact N-terminal portion, the deficiency in Rts1 *ori* activation in *cis* is apparently inconsistent with the findings obtained in this study. One possible explanation is that the C-terminal region of Rts1 RepA is involved in the association of RepA molecules, which mediates the pairing of RepA DNA (*ori* or *inc* direct repeats) complexes and results in the inhibition of initiation, as proposed in the regulation of R6K (19), RK2 (7), and P1 (1) replication. $RepA_{z279}$ might have an increased ability to form dimers and enhance the negative regulation of initiation. The increased inhibitory effect of $RepA_{z279}$ on mini-Rts1 replication in *trans* could also be explained by enhanced heterodimer formation with wild-type RepA molecules. If this is the case, the C-terminal region of Rts1 RepA should mediate strong incompatibility, as observed with wild-type RepA in this study.

It was recently reported that the π protein of R6K, lacking the 141 C-terminal amino acids, retained the negative control function of initiation (corresponding to the incompatibility function) but lost the initiator function along with DNA binding affinity (9). It appears, therefore, that the functional domains for *ori* activation and incompatibility are oppositely located in Rts1 RepA. For determination of the functional domains of Rts1 RepA in more detail, construction of hybrid protein molecules with various combinations of RepA from Rts1 and P1 accompanied by Rts1 *ori* or P1 *ori* would be useful, since Rts1 and P1 have different specificities with regard to replication and its regulation.

ACKNOWLEDGMENTS

We are very grateful to A. L. Abeles for mini-P1 plasmid pALA109.

This study was supported by a Grant-in Aid for Scientific Research (02454177) from the Ministry of Education, Science and Culture of Japan and by grants from the Yakult Foundation and the Aikō Foundation.

REFERENCES

1. **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.
2. **Abeles, A. L., K. M. Snyder, and D. K. Chattoraj.** 1984. P1 plasmid replication: replicon structure. J. Mol. Biol. **173:**307–324.
3. **Bolivar, F., R. L. Rodriguez, P. J. Green, M. C. Betlach, H. L. Heynecker, H. W. Boyer, and S. Falkow.** 1977. Construction and characterization of new cloning vehicles. II. A multiple cloning system. Gene **2:**95–113.
4. **Chang, A. C. Y., 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.
5. **Clark, A. J., M. Chamberlin, and R. P. Boyce.** 1966. Abnormal metabolic response to UV light of a recombinant deficient mutant of *E. coli* K-12. J. Mol. Biol. **19:**442–453.

6. **Cohen, S. N., A. C. Y. Chang, and L. Hsu.** 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R factor DNA. Proc. Natl. Acad. Sci. USA **69:**2110–2114.
7. **Durland, R. H., and D. R. Helinski.** 1990. Replication of the broad-host-range plasmid RK2: direct measurement of intracellular concentrations of the essential TrfA replication proteins and their effect on plasmid copy number. J. Bacteriol. **172:**3849–3858.
8. **Gottesman, M. E., and M. B. Yarmolinsky.** 1968. Integration negative mutants of bacteriophage lambda. J. Mol. Biol. **31:**487–505.
9. **Greener, A., M. S. Filutowicz, M. J. McEachern, and D. R. Helinski.** 1990. N-terminal truncated forms of the bifunctional initiation protein express negative activity on plasmid R6K replication. Mol. Gen. Genet. **224:**24–32.
10. **Howard-Flanders, P., E. Simson, and L. Theriot.** 1964. A locus that controls filament formation and sensitivity to radiation in *Escherichia coli* K-12. Genetics **49:**237–246.
11. **Humphreys, G. O., G. A. Willshaw, and E. S. Anderson.** 1975. A simple method for the preparation of large quantities of pure plasmid DNA. Biochim. Biophys. Acta **383:**457–463.
12. **Itoh, Y., Y. Kamio, Y. Furuta, and Y. Terawaki.** 1982. Cloning of the replication and incompatibility regions of a plasmid derived from Rtsl. Plasmid **8:**232–243.
13. **Itoh, Y., Y. Kamio, and Y. Terawaki.** 1987. Essential DNA sequence for the replication of Rts1. J. Bacteriol. **169:**1153–1160.
14. **Itoh, Y., and Y. Terawaki.** 1989. Replication properties of mini-Rts1 derivatives deleted for DnaA boxes in the replication origin. Plasmid **21:**242–246.
15. **Kamio, Y., Y. Itoh, and Y. Terawaki.** 1988. Purification of Rts1 RepA protein and binding of the protein to mini-Rts1 DNA. J. Bacteriol. **170:**4411–4414.
16. **Kamio, Y., A. Tabuchi, Y. Itoh, H. Katagiri, and Y. Terawaki.** 1984. Complete nucleotide sequence of mini-Rts1 and its copy mutant. J. Bacteriol. **155:**307–312.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
18. **Mazodier, P., P. Cossart, E. Giraud, and F. Gasser.** 1985. Completion of the nucleotide sequence of the central region of Tn5 confirms the presence of three resistance genes. Nucleic Acids Res. **13:**195–205.
19. **McEachern, M., 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.
20. **Miller, J., J. Manis, B. Kline, and A. Bishop.** 1978. Nonintegrated plasmid-folded chromosome complexes. Plasmid **1:**273–283.
21. **Murotsu, T., K. Matsubara, H. Sugisaki, and M. Takanami.** 1981. Nine unique repeating sequences in a region essential for replication and incompatibility of the mini-F plasmid. Gene **15:**257–271.
22. **Rak, B., and M. von Reutern.** 1978. Insertion element IS4 contains a third gene. EMBO J. **3:**807–811.
23. **Scott, J. R.** 1984. Regulation of plasmid replication. Microbiol. Rev. **48:**1–23.
24. **Stalker, D. M., R. Kolter, and D. R. Helinski.** 1979. Nucleotide sequence of the region of an origin of replication of the antibiotic resistance plasmid R6K. Proc. Natl. Acad. Sci. USA **76:**1150–1154.
25. **Stüber, D., and H. Bujard.** 1981. Organization of transcriptional signals in plasmids pBR322 and pACYC184. Proc. Natl. Acad. Sci. USA **78:**167–171.
26. **Terawaki, Y., Z. Hong, Y. Itoh, and Y. Kamio.** 1988. Importance of the C terminus of plasmid Rts1 RepA protein for replication and incompatibility of the plasmid. J. Bacteriol. **170:**1261–1267.
27. **Terawaki, Y., H. Nozue, H. Zeng, T. Hayashi, Y. Kamio, and Y. Itoh.** 1990. Effects of mutations in the *repA* gene of plasmid Rts1 on plasmid replication and autorepressor function. J. Bacteriol. **172:**786–792.
28. **Terawaki, Y., and R. Rownd.** 1972. Replication of the R factor Rts1 in *Proteus mirabilis*. J. Bacteriol. **109:**492–498.
29. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
30. **Vocke, C., and D. Bastia.** 1983. Primary structure of the essential replicon of the plasmid pSC101. Proc. Natl. Acad. Sci. USA **80:**6557–6561.
31. **Wickner, S., J. Hoskins, and K. McKenney.** 1991. Function of DnaJ and DnaK as chaperones in origin-specific DNA binding by RepA. Nature (London) **350:**165–167.
32. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.
33. **Zeng, H., T. Hayashi, and Y. Terawaki.** 1990. Site-directed mutations in the *repA* C-terminal region of plasmid Rts1: pleiotropic effects on the replication and autorepressor functions. J. Bacteriol. **172:**2535–2540.