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Analysis of Functional Domains of Rts1 RepA by means of a Series of Hybrid Proteins with P1 RepA

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The RepA protein of the plasmid Rts1, consisting of 288 amino acids, is a *trans*-acting protein essential for initiation of plasmid replication. To study the functional domains of RepA, hybrid proteins of Rts1 RepA with the RepA initiator protein of plasmid P1 were constructed such that the N-terminal portion was from Rts1 RepA and the C-terminal portion was from P1 RepA. Six hybrid proteins were examined for function. The N-terminal region of Rts1 RepA between amino acid residues 113 and 129 was found to be important for Rts1 *ori* binding in vitro. For activation of the origin in vivo, an Rts1 RepA subregion between residues 177 and 206 as well as the DNA binding domain was required. None of the hybrid initiator proteins activated the P1 origin. Both in vivo and in vitro studies showed, in addition, that a C-terminal portion of Rts1 RepA was required along with the DNA binding and *ori* activating domains to achieve autorepression, suggesting that the C-terminal region of Rts1 RepA is involved in dimer formation. A hybrid protein consisting of the N-terminal 145 amino acids of Rts1 and the C-terminal 142 amino acids from P1 showed strong interference with both Rts1 and P1 replication, whereas other hybrid proteins showed no or little effect on P1 replication.

The minimal replication region of the plasmid Rts1, mini-Rts1, consists of 1,855 bp and contains two important components. They are the replication origin *ori* and a gene, *repA*, encoding the initiator protein RepA (29). Another component important for the negative control of Rts1 replication is *incI*, a group of direct repeated sequences (iterons) located downstream of *repA* (29). The minimal replicon structure is similar to those of F (40), P1(5), R6K (47), and pSC101 (54). The amino acid sequence of Rts1 RepA, consisting of 288 amino acids, shares 60% homology with that of the 286-amino-acid P1 RepA (5, 29). Nevertheless, Rts1 and P1 belong to different incompatibility groups (5, 14). Rts1 RepA exhibits dual functions for plasmid replication. It initiates the replication positively by binding to the *ori* sequence and also negatively regulates initiator synthesis by binding to the *repA* promoter region (P*repA*) (28, 29). The inhibitory function is observed when an excess amount of RepA is supplied in *trans* to the mini-Rts1 plasmid (51). In addition to the autorepression of RepA synthesis, pairing of RepA-DNA (*ori* or *inc* iterons) complexes may be involved in the replication inhibition of Rts1, as proposed for R6K and P1 replication (2, 12, 38, 39, 42).

Recently, we reported that the N-terminal half of RepA could activate Rts1 *ori* when a truncated or hybrid *repA* was positioned in *cis* with the *ori* in its natural configuration (48). To gain more insight into the structure and function of the Rts1 RepA molecule, in this study we constructed a series of hybrid *repA* genes consisting of the 5′ end portion from Rts1 *repA* and the 3′ end portion from P1 *repA*. In the constructs, the transcription was under the control of Rts1 P*repA*. The hybrid RepA proteins obtained were examined for Rts1 *ori* activation, for repression of Rts1 P*repA*, and for interference with mini-Rts1 and mini-P1 replication. Also, in vitro binding of the proteins to the Rts1 and P1 *ori* sequences, *incI* iterons, and to P*repA* was studied.

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MATERIALS AND METHODS

Bacterial strains and phages. *Escherichia coli* HB101 (F⁻ *leuB6 supE44 thi-1 hsdS20 recA13 ara-14 proAB lacY1 galK2 rpsL20 xyl-5 mtl-1*) (8) was used for plasmid-DNA construction. Strain JG112 (*polA lacY thy str*) (36) was used for the Rts1 *ori* activation assay, and N100 (*galK2 recA13*) (23) was used for the incompatibility test. Strain SE4006 (*araD relA thi rpsL recA56* Δ*lac169 rsl*::Tn*10*) (20) was used for the β-galactosidase assay. Strain JM109 [*recA1 supE22 endA1 hsdR17 gyrA96 relA1 thi* Δ(*lac-proAB*) F′(*traD36 proAB lacI*^{q} *lacZ*ΔM15)] (60) was the host for M13 phage. M13mp18 and M13mp19 phages (60) were used as cloning vectors for PCR fragments.

Media and chemicals. Luria-Bertani medium (Difco Laboratories, Detroit, Mich.) was used for transformation, and Penassay broth (Difco) was used for selection of transformants. 2YT medium (37) was used for isolation of plasmid DNA, and B broth (37) was used for the β-galactosidase assay. The following antibiotics were included in the medium when needed: ampicillin (50 μg/ml), chloramphenicol (10 μg/ml), spectinomycin (30 μg/ml), kanamycin (30 μg/ml), and tetracycline (10 μg/ml). T4 DNA ligase, Klenow fragment of DNA polymerase I, bacterial alkaline phosphatase, and linkers were obtained from Takara Shuzo, Kyoto, Japan. Restriction enzymes were from Takara Shuzo, New England BioLabs, Inc. (Beverly, Mass.), or Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). All of the enzymes were used as recommended by the suppliers. [γ-^{32}P]ATP used for end labeling DNA fragments was purchased from Amersham (Buckinghamshire, England).

DNA isolation and gene manipulation. Plasmid DNA was isolated by the method of Humphreys et al. (24). Transformation was performed with calcium-treated cells as described by Cohen et al. (16) or was done by electroporation (2,450 V/cm) with a Cellject Basic electroporation system (Equi Bio., Angleur, Belgium). Restriction endonuclease fragments were purified from agarose gels with a Prep-A-Gene DNA purification kit (Bio-Rad Laboratories, Richmond, Calif.).

Recovery of Rts1 *ori* plasmid from *polA* host. Lysate-containing plasmid DNA was prepared from JG112 transformants with pTW22-*ori* and pGB:Xn plasmids as described by Humphreys et al. (24). The lysate was used to transform N100(*recA*) cells, which were selected for pTW22-*ori* with 20 μg of ampicillin per ml and for pGB:Xn with 30 μg of spectinomycin per ml. Plasmid DNA was isolated from the ampicillin-resistant N100 cells, and its restriction profile was compared with that of pTW22-*ori* DNA.

PCR. The PCR was carried out with a DNA Thermal Cycler (model IJ1000; Takara Shuzo) by use of a GeneAmp PCR reagent kit with Ampli-Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.). Various oligonucleotides used as the primer were synthesized by the phosphoramidite methods as described previously (62) or obtained from Bio-Synthesis Inc., Lewisville, Texas.

DNA sequence analysis. The nucleotide sequences of PCR products inserted in M13 phages were determined by the dideoxy chain termination method (44) with a DNA sequencer (model 370A; Perkin-Elmer). DNA sequencing reagents and enzymes were obtained as a kit from Perkin-Elmer.

Construction of plasmids. The plasmids used in this study are listed in Table 1. To construct plasmid pTW22, the *Aat*II, *Eco*RV, and *Bam*HI sites of pBR322 were first replaced by insertion of *Bam*HI, *Bgl*II, and *Kpn*I linkers, respectively.

TABLE 1. Plasmids used in this study

Plasmid(s)	Description	Reference or source
pBR322	Ap^r Cp^r	7
pACYC184	Tc^r Cp^r	11
pSC101	Tc^r	15
pKK223-3	Ap^r	6
pUC18, pUC19	Ap^r	60
pGB2	pSC101 derivative; Sp^r	13
pFZY1	Mini-F-derived transcriptional *lacZ* fusion cloning vector; Ap^r	33
pALA109	Mini-P1; Km^r	3
pALA619	pGB2 + P1 *repA*; Ap^r	4
pALA630	pUC19 + P1 *ori*; Ap^r	10
pTW20	Deletion derivative of Rts1; Km^r	49
pTW100	pFD51 + Rts1 *ori* (coordinates 1141–1194) and *repA* (coordinates 1191–216); Ap^r	52
pTW541	pBR322 + Rts1 *repA* (coordinates 1213–216); Ap^r	27
pTW601	Mini-Rts1; Sp^r	26
pTW22	pBR322 + *rrnB* transcriptional terminator; Ap^r	This study
pTW22-*ori*	pTW22 + Rts1 *ori* (coordinates 1441–1191); Ap^r	This study
pTWV01	pTW22-*ori* + Rts1 *repA* (coordinates 1191–216); Ap^r	This study
pTWX01	pTW22-*ori* + Rts1 *repA* (coordinates 1191–260) without *incI* region; Ap^r	This study
pTWXn[a]	pTW22-*ori* + Rts1-P1 hybrid *repA*; Ap^r	This study
pTW11:X01	pACYC184 + Rts1 *repA* (coordinates 1191–260) without *incI* region; Cp^r	This study
pTW11:Xn[a]	pACYC184 + Rts1-P1 hybrid *repA* from pTWXn; Cp^r	This study
pSC:X01	pSC101 + Rts1 repA (coordinates 1191–260) without *incI* region; Tc^r	This study
pGB:X01	pGB2 + Rts1 *repA* (coordinates 1191–260) without *incI* region; Sp^r	This study
pGB:Xn[a]	pGB2 + Rts1-P1 hybrid *repA* from pTWXn; Sp^r	This study
pTWZ01	pFZY1 + promoter of Rts1 *repA* (coordinates 1213–1020); Ap^r	This study

[a] n, numbers 12 to 17 indicate the Rts1-P1 hybrid *repA*.

The modified 0.95-kb *Sal*I-*Bam*HI fragment of pBR322 was then ligated with the 4.0-kb *Sal*I-*Bam*HI fragment of pKK223-3 containing *rrnB* (the rRNA transcriptional terminator) (6), *bla*, and *ori* region, in which the *Hin*dIII site of the polylinker was deleted beforehand by digestion with exonuclease. The resulting plasmid pTW22 contained the unique restriction sites *Bgl*II, *Hin*dIII, *Eco*RI, and *Bam*HI, in that order.

Plasmid pTW100 (52) is a plasmid containing Rts1 *ori* in a 0.25-kb fragment flanked by *Hin*dIII and *Bam*HI sites (mini-Rts1 coordinates 1441 to 1194) (29) and the *repA* gene in a 1.0-kb *Hin*dIII-*Hin*dIII fragment (mini-Rts1 coordinates 1191 to 216). To construct plasmid pTW22-*ori*, *Bgl*II and *Hin*dIII linkers were inserted at the *Hin*dIII site (coordinate 1441) and the *Bam*HI site (coordinates 1191) of pTW100, and the resulting 0.25-kb *Bgl*II-*Hin*dIII fragment containing the Rts1 *ori* region was then ligated into the *Bgl*II-*Hin*dIII sites of pTW22.

The 1.0-kb *Hin*dIII fragment (mini-Rts1 coordinates 1191 to 216) of pTW100 containing Rts1 *repA* gene was ligated into the *Hin*dIII site of pTW22 such that the *Eco*RI site of pTW22 was located downstream of the *repA* gene to construct pTW22-*repA*. To eliminate one *Hin*dIII site at coordinate 216 downstream of *repA* in pTW22-*repA*, the *Xba*I-*Eco*RI fragment of pTW22-*repA* was replaced by the *Xba*I-*Eco*RI fragment (mini-Rts1 coordinates 516 to 1) of pTW601 (26). The *Hin*dIII-*Eco*RI fragment containing the reconstructed *repA* gene (mini-Rts1 coordinates 1191 to 1) was then inserted into the *Hin*dIII-*Eco*RI sites of pTW22-*ori*, giving rise to plasmid pTWV01 containing Rts1 *ori*, *repA*, and *incI* oriented as in the wild type (Fig. 1).

To construct plasmids carrying various Rts1-P1 hybrid *repA*, a series of fragments was synthesized by PCRs. The synthesized fragments had restriction endonuclease sites at both ends, which were used to construct each hybrid *repA* without insertion of any additional amino acids in the hybrid RepA (Fig. 1). The synthesized fragments were cloned into the polylinker sites of M13mp18 or M13mp19, and their DNA sequences were confirmed by sequencing.

pTWX01 was constructed by replacing the *Eco*RV-*Eco*RI fragment (mini-Rts1 coordinates 362 to 1) of pTWV01 with the synthesized *Xba*I-*Bam*HI fragment carrying the C-terminal sequence of Rts1 *repA* (mini-Rts1 coordinates 516 to 261) (Fig. 1). Plasmid pTWX01 contains Rts1 *ori* through wild-type *repA* but lacks the *incI* region. In the same way, plasmid pTWX12 was constructed by replacing the *Eco*RV-*Eco*RI fragment of pTWV01 with the synthesized *Eco*RV-*Bam*HI fragment of the C-terminal part of P1 *repA* (mini-P1 coordinates 1432 to 1527) (5). Plasmid pTWX13 was constructed by replacing the *Xba*I-*Eco*RI fragment of pTWV01 with the synthesized *Xba*I-*Bam*HI fragment (mini-P1 coordinates 1278 to 1527). The *Cla*I-*Eco*RI fragment of pTWV01 was replaced by the synthesized *Cla*I-*Bam*HI fragment (mini-P1 coordinates 1069 to 1527) to construct pTWX15 (Fig. 1). To construct pTWX14, the 0.59-kb *Hin*dIII-*Eco*RI fragment including the synthesized *Sac*I site (mini-Rts1 coordinate 605), which contained the N-terminal sequence of Rts1 *repA* (mini-Rts1 coordinates 1191 to 605), was ligated into the *Hin*dIII-*Eco*RI sites of pTW22-*ori*. Subsequently, the synthesized *Sac*I-*Bam*HI fragment containing the C-terminal sequence of P1 *repA* (mini-P1 coordinates 1186 to 1527) was inserted into the *Sac*I-*Bam*HI sites. In the same way, to construct pTWX16, the synthesized *Hin*dIII-*Bsp*MI fragment of Rts1 *repA* (mini-Rts1 coordinates 1191 to 744) and the synthesized *Bsp*MI-*Bam*HI fragment of P1 *repA* (mini-P1 coordinates 1060 to 1527) were inserted into the *Hin*dIII-*Bam*HI sites of pTW22-*ori*. To construct pTWX17, the synthesized *Hin*dIII-*Eco*RI fragment of Rts1 *repA* (mini-Rts1 coordinates 1191 to 790) and the synthesized *Eco*RI-*Bam*HI fragment of P1 *repA* (mini-P1 coordinates 1000 to 1527) were ligated in the *Hin*dIII-*Bam*HI sites of pTW22-*ori* (Fig. 1).

A series of pTW11:Xn plasmids and pGB:Xn plasmids were constructed by inserting the *Hin*dIII-*Bam*HI fragments from pTWXn plasmids, which contained hybrid *repA* genes, into the *Hin*dIII-*Bam*HI sites of pACYC184 (11) and pGB2 (13), respectively.

To construct pTWZ01 in which *lacZ* is transcribed from P*repA*, the *Eco*RI-*Bam*HI fragment (mini-Rts1 coordinates 1213 to 1020) containing the operator-promoter of Rts1 *repA* was inserted into the *Eco*RI-*Bam*HI sites of pFZY1 (33) after a *Bam*HI linker was inserted into the *Eco*T14I site (mini-Rts1 coordinate 1020) of pTW541 (27).

Rts1 *ori* activation. Rts1 *ori* activation by hybrid RepA in *trans* was examined by transforming plasmid pTW22-*ori* into JG112 harboring hybrid RepA producing plasmids pGB:Xn. Maintenance of the *ori* plasmid indicates an Rts1 *ori* activating function of the hybrid proteins.

Interference of hybrid RepA with the replication of mini-Rts1 and mini-P1. The inhibitory effects of hybrid RepA on mini-Rts1 or mini-P1 plasmid replication were examined by transforming pTW11:Xn plasmids into the *recA* strain N100 harboring either pTW601 or pALA109 (3). The transformants grown on plates containing chloramphenicol were transferred onto plates containing the resident marker drug (spectinomycin for pTW601 and kanamycin for pALA109) to check for the presence of the resident plasmid. Loss of the resident plasmid indicates an inhibitory function of the hybrid proteins on parent plasmid replication.

β-Galactosidase assay. The β-galactosidase activities of cells harboring the P*repA-lacZ* fusion and the hybrid RepA producing plasmids pTW11:Xn were assayed by a modification of the method of Miller (37) as described by Easton and Rownd (19).

Preparation of cell extracts of hybrid proteins and immunoblot analysis. Cell extracts of hybrid RepA proteins were prepared by ammonium sulfate fractionation of cell lysate by the method for preparation of fraction II reported by Fuller et al. (21) except that the dialysis buffer contained 50 mM Tris (pH 7.5), 1 M NaCl, 1 mM EDTA, 2 mM mercaptoethanol, and 10% (vol/vol) glycerol. The composition of the dialysis buffer was basically like that described by Abeles for P1 RepA preparation (1). Protein concentrations were determined with the Bio-Rad protein assay kit with bovine serum albumin as a standard.

Gel shift assay for hybrid protein-DNA binding. The binding of hybrid protein to DNA fragments was assayed as follows. The 10-μl binding mixture contained 20 mM Tris (pH 7.5), 40 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM

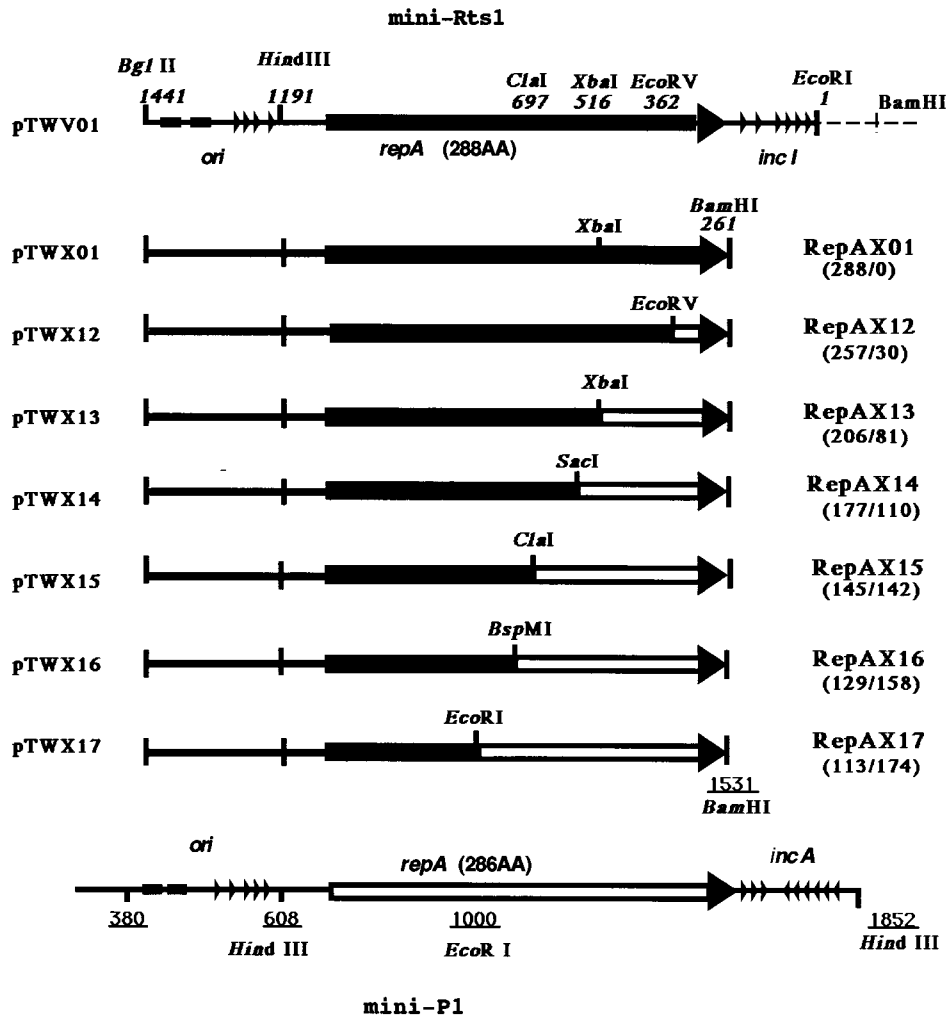

FIG. 1. Structure of pTWXn plasmids carrying the hybrid *repA* gene. Maps of mini-Rts1 (top) and mini-P1 (bottom) are shown. Numbers without and with underlines are mini-Rts1 (29) and mini-P1 (5) coordinates, respectively. Tandem closed rectangles are DnaA boxes. Bold arrows indicate the *repA* genes of Rts1 (closed) and P1 (open), encoding 288 amino acids (AA) and 286 amino acids, respectively. Hybrid RepA proteins (287 amino acids) consist of the N-terminal portion from Rts1 RepA and the C-terminal portion from P1 RepA. The restriction endonuclease cutting sites where the recombination took place are shown. The numbers of amino acids from each RepA which constitute the hybrid proteins are indicated in parentheses (number from Rts1 RepA/number from P1 RepA).

ATP, 5 mM EDTA, 50 μg of bovine serum albumin per ml, 50 μg of calf thymus DNA per ml, 0.5 pmol of ^{32}P-end-labeled DNA fragment, and various amounts of fraction II extracts containing hybrid proteins. The mixture was kept at 20°C for 20 min. Immediately after incubation, it was mixed with 5 μl of dye buffer (10% Ficoll-400, 0.1% bromophenol blue, 10 mM Tris [pH 7.7], 1 mM EDTA), loaded onto a 5% polyacrylamide gel, and subjected to electrophoresis at 5 V/cm. The gel was dried and autoradiographed with Fuji Medical X-ray film at room temperature for 12 to 20 h.

RESULTS

Interaction between the constituents of mini-Rts1 and mini-P1. Rts1 and P1 plasmids belong to different incompatibility groups, *incT* and *incY*, respectively. The mini-Rts1 plasmid pTW601 can coexist stably with the mini-P1 plasmid pALA109 (Table 2). However, we noticed recently that the Rts1 *ori* sequence cloned in pBR322 excluded pALA109. In contrast, the P1 *ori* sequence cloned in either pBR322 or pUC19 showed no effect on the replication of pTW601 (Table 2). Since pTW601 has a higher copy number (about three per host chromosome) than that of pALA109, a unit copy derivative plasmid of Rts1, pTW20, was also used. pTW20 contains the complete set of *incI* iterons, consisting of nine repeating units

TABLE 2. Incompatibility between mini-Rts1 and mini-P1 components

Plasmid		% Transformants with resident plasmid[a]
Donor	Resident	
pTW601	pALA109	100
pTW22-*ori*	pALA109	<1
pBR322:P1 *ori*	pTW601	100
pALA630[b]	pTW601	100
pALA630[b]	pTW20	100
pALA619[c]	pTW20	27[d]

[a] Each transformant colony selected for donor plasmid was picked and stabbed onto plates containing kanamycin (for pALA109 and pTW20, 30 μg/ml) or spectinomycin (for pTW601, 30 μg/ml). At least 100 colonies were examined.
[b] pUC19 recombinant plasmid containing P1 *ori*.
[c] pGB2 recombinant plasmid containing P1 *repA*.
[d] Poor growth.

TABLE 3. Rts1 *ori* and P1 *ori* activation by hybrid proteins

Resident plasmid	Hybrid RepA protein	No. of transformants/μg of DNA[a]	
		pTW22-*ori*[b]	pALA630[c]
pSC101	No	<1	<1
pSC:X01	RepAwt(Rts1)	1.0×10^3	<1
pGB2	No	<1	<1
pGB:X01	RepAwt(Rts1)	1.0×10^1	<1
pGB:X12	RepAX12	0.8×10^1	<1
pGB:X13	RepAX13	1.0×10^3	<1
pGB:X14	RepAX14	<1	<1
pGB:X15	RepAX15	<1	<1
pGB:X16	RepAX16	<1	<1
pGB:X17	RepAX17	<1	<1
pALA619	RepAwt(P1)	<1	6.5×10^3

[a] *E. coli* JG112 (*polA*) was used as the host. Results are averages of multiple experiments.
[b] pBR322 recombinant plasmid containing Rts1 *ori*.
[c] pUC19 recombinant plasmid containing P1 *ori*.

(30, 41, 50). pTW601 was generated from pTW20 by deleting four repeating units of *incI* iterons (26). As shown in Table 2, the cloned P1 *ori* also did not destabilize pTW20. The results suggest that P1 RepA protein can interact with Rts1 *ori*, whereas Rts1 RepA does not interact with P1 *ori*. If this notion is correct, P1 RepA protein supplied in *trans* in an excess amount would inhibit the replication of mini-Rts1 plasmid, and this result was obtained (Table 2).

Construction of a series of hybrid proteins. To obtain hybrid proteins consisting of the N-terminal peptide of Rts1 RepA and the C-terminal peptide of P1 RepA, a series of pTWXn plasmids was constructed (Fig. 1). Each of the hybrid *repA* genes was cloned into pACYC184 and pGB2 (or pSC101) to form pTW11:Xn and pGB:Xn (or pSC:Xn) plasmids, respectively, as described in Materials and Methods.

All of the hybrid RepA proteins contained 287 amino acids, and the residues at the junctions were the same as the native ones irrespective of base pair changes introduced to produce the new restriction sites.

Rts1 *ori* activation by the hybrid RepA. To examine whether these hybrid RepA proteins activate an Rts1 *ori* positioned in *trans*, pTW22-*ori* plasmid was transformed into the *polA* strain JG112 harboring pGB2 recombinant plasmids containing the hybrid *repA* genes. The results in Table 3 show that pGB:X12 and pGB:X13 supported the replication of pTW22-*ori*. However, since the transformation frequency of pTW22-*ori* into JG112 harboring either pGB:X12 or pGB:X01 was quite low, we tested whether these transformants really contained the *ori* plasmid (see Materials and Methods). The results showed that pTW22-*ori* was harbored in and could be recovered from cells transformed with pGB:X12, pGB:X13, or pGB:X01. We also examined the stability of pTW22-*ori* in these JG112 transformants by growing the cells at 37 and 42°C in Penassay broth for 3.5 h. The ampicillin-sensitive (pTW22-*ori* cured) cells appeared at 42°C with a frequency of 12 to 19%, but no spectinomycin-sensitive (pGB:Xn-cured) cells developed. At 37°C, no drug-sensitive cells appeared. These results also indicate the presence of pTW22-*ori* as plasmid in JG112 transformants.

We then tested whether the P1 *ori* sequence was activated by the hybrid proteins. The pUC19 recombinant plasmid containing P1 *ori*, pALA630 (10), was unable to transform JG112 harboring any of pGB:Xn plasmids. In contrast, the P1 *ori* plasmid was introduced quite efficiently into JG112 with pALA619 encoding P1 RepA wild-type protein. Thus, no hybrid RepA activated P1 *ori*.

TABLE 4. Inhibition of parent plasmid replication by hybrid proteins

Plasmid		% Transformants with resident plasmid[a]	% Cells with resident plasmid[b]
Donor	Resident		
pTW11:X01	pTW601	<1	ND[c]
pTW11:X12	pTW601	<1	ND
pTW11:X13	pTW601	100[d]	31
pTW11:X14	pTW601	<1	ND
pTW11:X15	pTW601	<1	ND
pTW11:X16	pTW601	100[d]	12
pTW11:X17	pTW601	100	99
pTW11:X01	pALA109	99	100
pTW11:X12	pALA109	100	100
pTW11:X13	pALA109	100	100
pTW11:X14	pALA109	98	98
pTW11:X15	pALA109	4	6
pTW11:X16	pALA109	98	100
pTW11:X17	pALA109	99	65

[a] Each transformant colony from chloramphenicol-containing plates was picked and stabbed onto plates containing spectinomycin (for pTW601) or kanamycin (for pALA109). At least 100 colonies were examined.
[b] A single transformant colony that was confirmed to be spectinomycin or kanamycin resistant by the data in the preceding column was suspended and streaked onto plates without drug. The colonies that developed were examined for the presence of the resident plasmid. At least four single colonies were examined.
[c] ND, not determined.
[d] Poor growth at the stabbed region.

Interaction of the hybrid RepA with mini-Rts1 and mini-P1 replication. An excess amount of Rts1 RepAwt protein supplied in *trans* inhibits replication of the mini-Rts1 plasmid (48, 51, 52). To examine the effect of the hybrid proteins on their parent plasmid replication, pACYC184 recombinant plasmids containing hybrid *repA* genes, pTW11:Xn, were transformed into N100 cells harboring pTW601 or pALA109. All transformant colonies were selected for pTW11:X12, pTW11:X14, and pTW11:X15 as well as pTW11:X01 and were shown to lose pTW601 (Table 4). Although the colonies with pTW11:X13 and pTW11:X16 appeared to retain pTW601 in the initial selection, each colony of the transformants contained a large proportion of pTW601-cured cells, 69% with pTW11:X13 and 88% with pTW11:X16, respectively (Table 4). Only pTW11:X17 coexisted stably with pTW601. In contrast to TW601, the mini-P1 plasmid pALA109 coexisted more stably with pTW11:Xn plasmids. The only notable exception was that pTW11:X15 inhibited strongly the pALA109 replication.

Autorepressor function of hybrid proteins. The autoregulation of Rts1 RepA synthesis is suggested from our in vitro binding of purified RepA to the region just upstream of P*repA* (28) and from the in vivo study using the *galK* expression system (62). To examine the autorepressor activity of the hybrid proteins, a transcriptional fusion was constructed such that the P*repA* fragment (mini-Rts1 coordinates 1213 to 1020) was inserted upstream of the promoterless *lacZ* gene in pFZY1, giving rise to pTWZ01. The hybrid RepA-producing plasmids, pTW11:Xn, were introduced into SE4006 carrying pTWZ01, and the level of β-galactosidase activity was measured (Table 5). A significant repression (more than 80%) of the β-galactosidase activity was observed only in SE4001(pTWZ01) transformed with pTW11:X12, which gave a repression similar to that of the cells with pTW11:X01. Thus, only RepAX12 is able to function as an autorepressor like the Rts1 RepAwt protein.

Interaction of the hybrid proteins in vitro with Rts1 *ori*, Rts1 P*repA*, *incI* region, and P1 *ori*. It was expected that the char-

TABLE 5. Autorepressor function of hybrid protein in cells[a] harboring the P*rep-lacZ* fusion plasmid pTWZ01

Coresident plasmid	Hybrid protein	β-Galactosidase activity (Miller units)[b]	Relative activity
pACYC184		680	100
pTW11:X01	RepAwt	160	24
pTW11:X12	RepAX12	120	18
pTW11:X13	RepAX13	590	87
pTW11:X14	RepAX14	700	103
pTW11:X15	RepAX15	748	110
pTW11:X16	RepAX16	700	103
pTW11:X17	RepAX17	650	96

[a] Host strain SE4006 contained pTWZ01 and effector plasmids pTW11:Xn which supplied hybrid proteins in *trans*.
[b] Results are averages of multiple experiments.

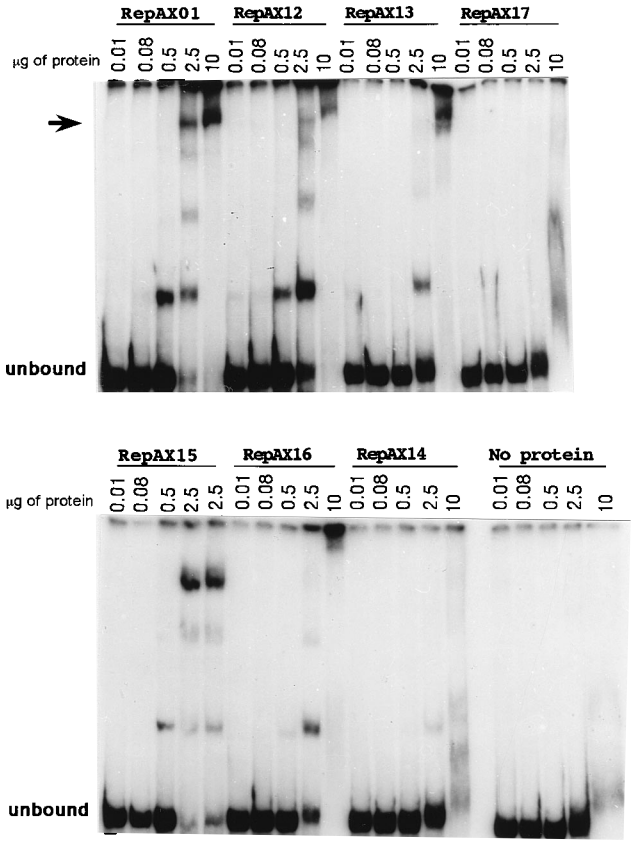

FIG. 2. Binding of hybrid proteins to Rts1 *ori*. The 0.42-kb *Bgl*II-*Eco*T14I fragment end labeled with ^{32}P (0.1 pmol of DNA per 10^5 cpm) was mixed with the fraction II extracts containing the hybrid protein and allowed to bind for 20 min at 20°C as described in Materials and Methods. The amount of total protein is shown at the top of each lane. The arrow indicates the position of the fourth retarded band which is missing for the RepAX15 mixture.

acteristics of the hybrid proteins shown in the experiments described above could be ascribed to their interaction with the Rts1 *ori*, *repA* promoter, and *incI* regions. Fraction II containing each hybrid protein (see Materials and Methods) was used for gel shift assays. The four DNA fragments used were as follows: (i) a 0.42-kb *Bgl*II-*Eco*T14I fragment containing the Rts1 *ori* region; (ii) a 0.19-kb *Hin*dIII-*Eco*T14I fragment containing the P*repA* region; (iii) a 0.36-kb *Eco*RV-*Eco*RI fragment containing the *incI* region; and (iv) a 0.3-kb *Rsa*I-*Hin*dIII fragment containing the P1 *ori* region.

The results of the gel shift assay with these fragments are shown in Fig. 2 to 4. Rts1 RepAwt, RepAX12, and RepAX15 bound strongly to Rts1 *ori*, whereas RepAX13, RepAX14, and RepAX16 bound weakly (Fig. 2). No binding by RepAX17 was detected in this assay. It should be mentioned that although the binding of RepAX13 was weak, it gave four low-mobility bands, as was seen with RepAwt and RepAX12, and one band was missing in the case of RepAX15 or RepAX16 (Fig. 2, arrow). Only RepAX12 bound as strongly to the P*repA* fragment as Rts1 RepAwt, and no retarded band appeared with the other hybrid proteins (Fig. 3). The result is consistent with the in vivo study where only RepAX12 showed evidence of autorepressor activity (Table 5). It appeared that P*repA* DNA was degraded when incubated with a large amount (2.5 μg) of RepAX14, RepAX16, and RepAX17 (Fig. 3). This might be due to some DNase activity remaining in the protein preparation, which degraded nonprotected DNA. Figure 4 shows the interaction of the hybrid proteins with the *incI* fragment. RepAX12, RepAX13, RepAX15, and RepAX16 appeared to interact as strongly as RepAwt did. RepAX14 bound weakly, and RepAX17 showed no binding with *incI* iterons. Thus, RepAX12 and RepAX15 bound strongly to both Rts1 *ori* and *incI* iterons, but the latter lost binding to Rts1 P*repA*.

As described, RepAX15 strongly inhibited the replication of both pALA109 and pTW601. Accordingly, we tested the in vitro binding of RepAX15 to the P1 *ori* sequence. Only one retarded band was detected when a large amount of the protein was used (data not shown), which was quite different from the binding of RepAX15 to Rts1 *ori*. A very weak binding to P1 *ori* was also detected with Rts1 RepAwt. Taking these findings together with the in vivo result that Rts1 RepAwt and P1 *ori* showed no effect on each other (Table 2), it appears that RepAX15 and Rts1 RepAwt do not interact efficiently with P1 *ori*.

DISCUSSION

Initiator proteins (Rep) of many plasmids have been analyzed in detail for their structure and function. In *Staphylococcus aureus*, the replication initiator proteins of the pT181 plasmid family are known to have an endonuclease activity, and this function is located in the N-terminal portion of the proteins (18, 43, 55). However, none of the Rep proteins from the plasmids of members of the family *Enterobacteriaceae* have shown specific enzymatic activity. This makes it difficult to determine the functional domains of the proteins. The dimeric form of Rep proteins from F, R6K, and pSC101 behaves as an autoregulatory protein, whereas the monomer form functions as the initiator (25, 35, 61). In addition to the autoregulatory mechanism, the Rep protein-dimer serves in R6K and P1 replication to form a regulatory complex consisting of a pair of Rep protein-DNA (*ori* and iteron) complexes, which inhibit the replication fork (2, 12, 34, 38, 42). Thus, the dimerization of Rep protein is thought to play a key role in the negative control of plasmid replication. An Rts1 RepA mutant, RepAz279 (Arg-279 to Gly), unable to activate Rts1 *ori*, showed increased interference with mini-Rts1 replication (62). Although we have not confirmed the dimer form of Rts1 RepA, this increased interference might be due to increased dimer formation by RepAz279 with itself or with the wild-type RepA. Since the amino acid change of RepAz279 is near the end of RepA, it is possible that the C-terminal region of Rts1 RepA is involved in dimerization.

Binding of RepA protein to the origin is essential for initiation of replication. As shown in the in vitro binding study,

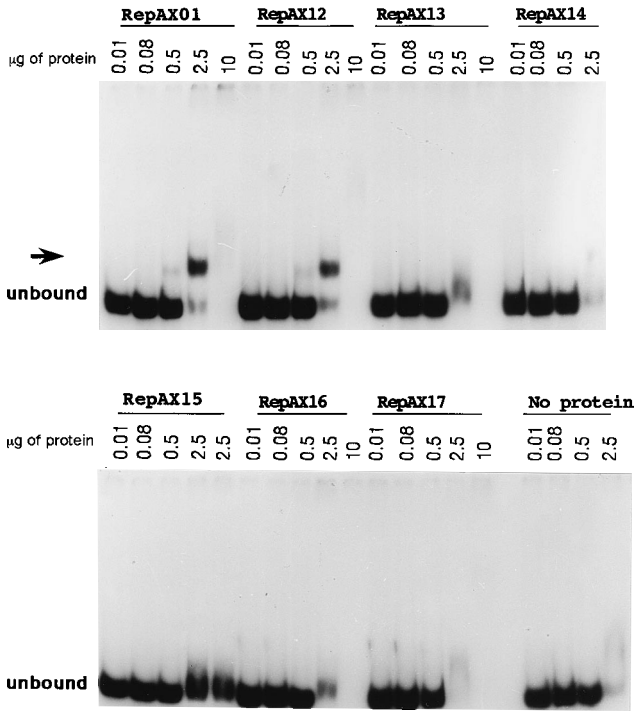

FIG. 3. Binding of hybrid proteins to P*repA*. The 0.19-kb *Hin*dIII-*Eco*T14I fragment end labeled with ^{32}P (0.1 pmol of DNA per 10^5 cpm) was mixed with the fraction II extracts containing the hybrid protein. Only one shifted band appeared with RepAwt and RepAX12 (indicated by the arrow).

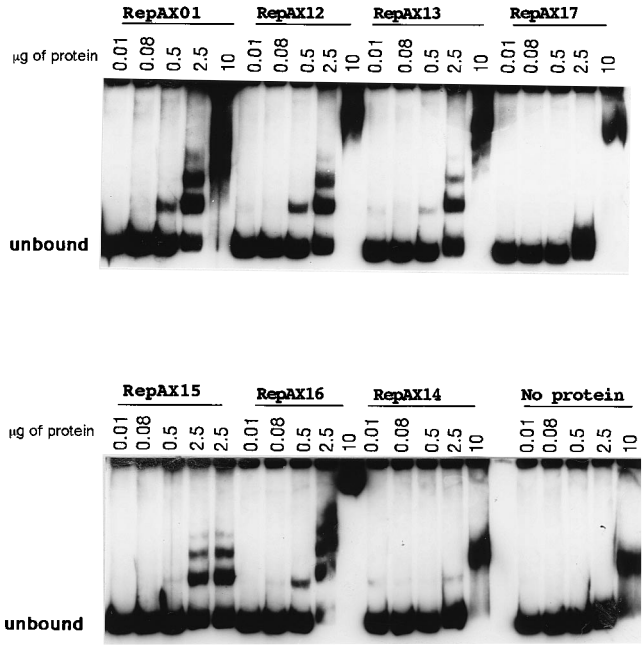

FIG. 4. Binding of hybrid proteins to *incI*. The 0.36-kb *Eco*RV-*Eco*RI fragment end labeled with ^{32}P (0.1 pmol of DNA per 10^5 cpm) was used. The experimental procedure was the same as that described for Fig. 2 and 3.

RepAX12 through RepAX16 bound to Rts1 *ori*-DNA, but RepAX17 did not. This suggests that the N-terminal region of RepA between amino acid residues 113 and 129 is important for the origin binding. Interestingly, the region is included in domain II (Rts1 RepA residues between 111 and 130), which is proposed by Gibbs et al. (22) as the DNA binding domain in Rts1 RepA. Other regions suggested as binding DNA are domain I (amino acids 63 to 87) and domain III (amino acids 205 to 224) in Rts1 RepA (22).

In the *ori* activation study in vivo, RepAX12 and RepAX13 but not RepAX14 induced replication from Rts1 *ori* in *trans*. This finding implies that a RepA subregion between residues 177 and 206 is required for *ori* activation in addition to the DNA binding region described above. This 30-residue segment of RepA might be necessary for proper folding of the protein to induce a duplex DNA opening at the origin as mediated by DnaA protein of the active form at *oriC* in the *E. coli* chromosome (9).

Previously, we found that the N-terminal 145-amino-acid portion of RepA activated Rts1 *ori* in *cis*, as demonstrated in pTW547, which is a pBR322 recombinant plasmid (48). This study, however, revealed that a hybrid RepA with the N-terminal 145-amino-acid portion was not sufficient but the 206-amino-acid region was required to activate the origin in *trans*. The result suggests that the C-terminal P1 RepA portion of the hybrid may have an inhibitory function because of its unproper folding or interaction with some abnormal host proteins. Alternatively, the constituents in pTW547 may support the replication, which has started at Rts1 *ori*, to proceed by transcription activation of the *bla* gene through the pBR322 replication origin. An observation that when a universal transcription terminator (6) was inserted between the *repA* and *bla* genes in pTW547, the recombinant plasmid was converted to a nonreplicative one in JG112 (our unpublished data), favors the latter possibility. However, the mechanism remains unclear.

All hybrid proteins except RepAX12 showed neither autorepression in vivo nor binding in vitro to the promoter region of Rts1 *repA*. This suggests that for P*repA* binding, Rts1 RepA may take a different form from that for origin binding. It might be a dimer form as reported with F, pSC101, and R6K (25, 35, 61). Recently, York and Filutowicz suggested that a short palindrome structure (8 to 9 bp) existing in the promoter-operator region of the *rep* gene could be important for the autoregulation of R6K, F, and Rts1 (61). Possibly, the dimer form of the hybrid proteins other than RepAX12 does not recognize this specific structure because of conformational distortions from the native Rts1 RepA. Alternatively, failure to autoregulate can be due to insufficient amounts of these hybrid proteins, if the proteins were unstable and degraded. However, the hybrid RepA proteins appear to be as stable as Rts1 RepAwt (our unpublished data).

The most interesting finding obtained in this study is that RepAX15, containing the N-terminal 145 amino acids from Rts1 RepA and the C-terminal 142 amino acids from P1 RepA, exhibited significant interference with the replication of both mini-Rts1 and mini-P1. In addition, although RepAX15 could not activate the Rts1 *ori*, it showed strong binding to Rts1 *ori* in vitro but no binding to Rts1 P*repA*. The replication inhibition by a mutant RepA such as RepAX15 could be caused by one or more of the following mechanisms when it is supplied in *trans* in excess: (i) competition for origin binding with RepAwt produced from the parent plasmid; (ii) repression of the RepAwt synthesis by binding to the promoter of the wild-type *repA* gene; (iii) inhibition of the replication fork by forming a RepA protein-DNA complex; or (iv) sequestration by the mutant RepA of an essential component for origin activation, such as RepAwt, or some host factors, such as DnaJ, DnaK, and GrpE, which are known to be required for initiation of replication of P1 (17, 39, 45, 46, 53, 56–59) and F

(31, 32). This interaction could be mediated by protein-protein contacts with or without DNA binding.

Interference of mini-Rts1 replication by RepAX15 would be caused by competitive binding to the Rts1 *ori* with Rts1 RepAwt. However, the inhibition of mini-P1 replication by RepAX15 is not simply explained. The N-terminal portion of the hybrid protein may not be involved in the inhibitory function, since RepAX15 as well as RepAwt did not bind efficiently in vitro to P1 *ori*. The in vivo study that showed no interaction of Rts1 RepA with P1 *ori* (Table 2) also supports this notion. The interference is, therefore, probably mediated by its C terminus to form a heterodimer with P1 RepAwt, resulting in sequestration of the wild-type protein. Another series of hybrid proteins consisting of the N-terminal region from P1 RepA and the C terminus from Rts1 RepA would be useful in gaining more precise knowledge of the functional domains of RepA.

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