JOURNAL OF BACTERIOLOGY, Jan. 1992, p. 456–463
0021-9193/92/020456-08$02.00/0

Vol. 174, No. 2

Replication Origin Mutations Affecting Binding of pSC101 Plasmid-Encoded Rep Initiator Protein

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Received 19 April 1991/Accepted 7 November 1991

To investigate the role of binding sites for Rep initiation protein in the replication of pSC101, a series of plasmids was constructed which carried different combinations of mutations in three binding sites within the minimal origin of replication. Mutation of all three sites reduced the affinity of purified Rep protein for the origin by 100-fold, as measured by a competition binding assay. Mutations in individual binding sites prevented binding of Rep protein to the mutant site but not to adjacent wild-type sites. Transformation efficiency, copy number, and stability over 150 generations were measured for each of the mutant plasmids. Unlike other similar plasmids related to pSC101, the Rep binding sites were found not to be equivalent. A mutation in the site RS1, proximal to repeated sequences which serve as DnaB helicase entry sites in *oriC*, had a severe effect on replication activity. A similar mutation in the distal site RS3 caused a reduction in copy number, but the mutant plasmid was stably maintained despite a broadened distribution of copy number within the population. A mutation in the middle RS2 site had no significant effect on pSC101 replication.

The plasmid pSC101 confers resistance to tetracycline upon its bacterial host (9, 10); it is present at about 15 copies per cell during growth in rich medium (7), and its replication has been recently reviewed (26). Replication of pSC101 requires a plasmid-encoded protein, Rep (1, 24, 52, 58), as well as the host-encoded DnaA (13, 19) and integration host factor (IHF) (18, 46) proteins. These three proteins bind to specific sequences within the minimal origin of replication of pSC101, *ori*, and are somehow involved in the initiation of plasmid replication. In the host, DnaA protein is required for initiation of replication from *oriC*, the origin of replication of the *Escherichia coli* chromosome (16, 20). IHF, a small basic protein, is required for site-specific integration and excision of bacteriophage lambda DNA (33); it also regulates the expression of a variety of genes in *E. coli* (reviewed in reference 14).

The minimal origin of replication of pSC101 has been defined by deletion analysis and is 200 bp in length (see Fig. 1) (24, 52, 58). The most striking features of this region are an AT-rich tract, which contains a binding site for IHF, and three tandemly repeated sequences, denoted RS1 to RS3 (26), which bind Rep protein (8, 46, 50, 52, 53). Immediately adjacent to *ori* lies the promoter region of the *rep* gene (25). This region contains two inverted repeats denoted IR-1 and IR-2 (50). IR-1 and IR-2 are composed of pairs of overlapping, inverted sequences which are related but not identical to the three Rep binding sequences found in *ori* (53). These pairs of inverted sequences, RS4-RS4′ and RS5-RS5′, overlap the −35 and −10 regions of the *rep* promoter (25, 54, 57), respectively. Binding of Rep protein to the pairs of inverted sequences represses transcription of the *rep* gene, causing autoregulation of Rep synthesis (25, 54, 57). This arrangement of multiple binding sites involved in replication and autoregulation of a plasmid-encoded replication protein is typical of a number of plasmids, including P1, F, R6K, and Rts1 (for reviews, see references 42 and 34), but in pSC101 the autoregulatory Rep binding sites are not absolutely required for replication (24).

Structurally, the origin of replication of pSC101 is similar to *oriC*, the *E. coli* origin of replication (see Fig. 1) (31, 49). At *oriC*, in the presence of the small basic protein HU (12), 30 to 40 DnaA monomers bind to four GTTATACACA sequences to form a large complex (15). These sequences are conserved among bacterial origins of replication (61), and point mutations in a single sequence can abolish replication activity (35). An adjacent AT-rich region contains three repeats of a 13-mer GATCTnTTnTTTT sequence (6). DnaA protein also interacts specifically with these 13-mers (60) and catalyzes the denaturation of the AT-rich region (6). DnaB helicase then further unwinds the DNA and permits the priming of replication to occur (3).

The AT-rich region in the pSC101 origin is similar to that of *oriC* and contains two copies of the same 13-mer sequence (8, 52). However, although DnaA also binds to the pSC101 origin (45), the orientation of the strong DnaA binding site with respect to the 13-mer sequences is different from that found in *oriC* (45). As shown in Fig. 1, Rep protein binds to the pSC101 origin at positions analogous to those of DnaA at *oriC*. A second difference between pSC101 and *oriC* is the absolute requirement for IHF in the replication of pSC101 (18). IHF binds to a specific site very close to the 13-mers in the pSC101 origin and bends the DNA (46), enhancing binding of DnaA protein (45). Although IHF binds to a similar site in *oriC* and also bends *oriC* DNA (36), IHF is not required for replication to initiate at *oriC*. However, IHF can substitute for HU protein during in vitro replication of *oriC* (44), and it is not known if either IHF or HU protein is used preferentially in vivo.

To learn more about how replication of pSC101 occurs and may be controlled, we have analyzed the role of the three origin Rep binding sites, RS1 to RS3. A detailed examination of the interaction of purified Rep protein with *ori* DNA suggests that the three sites have markedly different affinities for Rep protein (50, 53), which could indicate that binding of Rep to individual sites plays different roles in the replication of the plasmid. To test this idea, we have constructed a series of plasmids carrying mutations in the different Rep

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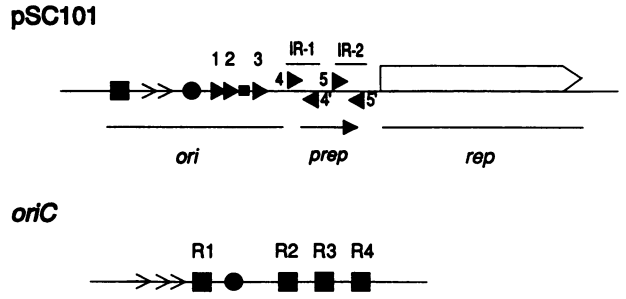

FIG. 1. Comparison of the replication region of pSC101 with *oriC*. The upper part of the figure shows important features of the minimal replication region of pSC101. The region denoted *ori* is 200 bp long. The *rep* gene is not drawn to scale. The arrow denoted *prep* shows the position of the *rep* promoter. IR-1 and IR-2 denote inverted repeats in the *rep* promoter region. Numbers refer to the individual Rep binding sites RS1 to RS5'. Symbols: ▶, Rep binding sites; ■, DnaA binding sites (the large square represents a strong binding site and the small square represents two weak binding sites which partially overlap Rep binding sites RS2 and RS3); ●, IHF binding site; >, 13-mer sequence. The lower part of the figure shows important features of *oriC*. R1 to R4 refer to DnaA binding sites. Other symbols are as described above for pSC101.

binding sites and shown that these mutations decrease binding of purified Rep protein. We have determined the effects of these mutations on the replication and maintenance of pSC101.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The following *E. coli* strains were used: LC707 F^+ *asn thi* (L. Caro), RY 2504 (F' *his* Tn9) *dut ung* (Ry Young), BW360 *polA* (43), K38 (λ) *met ura* (June Scott), BL21 (DE3) (47). Cultures were grown in LB medium (32), which was supplemented with 1.5% agar for solid media. Plasmids used were pLC712 (28), which consists of a 2.2-kb *Hin*cII-*Rsa*I fragment of pSC101 (nucleotides 4524 to 6737 [4]) cloned into the *Eco*RI site of pBR322 (5), pUC18 (59), Bluescript pKSM13$^+$ (Stratagene), and pSP73 (Promega).

Purification of Rep protein. (i) Method 1. The *rep* gene of pSC101 was cloned into a pKSM13$^+$ plasmid downstream of a bacteriophage T7 promoter. This cloning was accomplished by using oligonucleotide-directed mutagenesis to replace the entire autoregulatory region upstream of the initiation codon of *rep* with a *Bam*HI restriction endonuclease cleavage site and an improved ribosome binding site and then by cloning a *Bam*HI-*Eco*RI fragment containing the whole *rep* gene directly into pKSM13$^+$ to produce the plasmid pAASD12. The mutagenesis was carried out as described below by using the oligonucleotide 5'-AATTCAGACATTTT ATTTCCTCCTGGATCCGGCTAGTCAATG-3'. The entire Rep coding sequence of pAASD12 was verified by DNA sequencing. pAASD12 was then introduced into K38 containing the plasmid pGP1-2; this latter plasmid carries the gene encoding bacteriophage T7 RNA polymerase under the control of a thermosensitive bacteriophage λ cI repressor (51).

To purify Rep protein, 1-liter cultures of strain K38 (pAASD12/pGP1-2) were grown in LB medium at 31°C in 4-liter flasks. At an optical density at 600 nm of 0.5 to 0.6, each culture was placed in a shaking water bath at 42°C and incubated for 1 h. All subsequent steps were performed at 4°C. The cells were centrifuged at 6,000 rpm for 10 min in a Sorvall GSA rotor, and the cell pellets were suspended in a buffer containing 40 mM Tris-Cl (pH 7.6), 1 mM EDTA, 10 mM $MgCl_2$, 3 mM β-mercaptoethanol, and 250 mM NaCl. A 5-ml volume of buffer was used for each gram of bacterial pellet. The cells were lysed by being passed twice through a French pressure cell, and the lysates were centrifuged at 42,000 rpm for 3 h in a Beckman Ti45 rotor. The pellet was suspended and homogenized in R buffer (25 mM Tris-Cl [pH 7.5], 1 mM EDTA, 3 mM β-mercaptoethanol) containing 50 mM NaCl. The suspended material was recentrifuged at 42,000 rpm for 3 h in a Beckman Ti45 rotor. The supernatant was loaded onto a heparin-agarose column preequilibrated in R buffer with 50 mM NaCl, and the column was eluted with a 200 to 600 mM gradient of NaCl in R buffer. Rep-containing fractions were pooled and dialyzed against R buffer. The Rep protein precipitated during dialysis and was recovered by centrifugation and then by suspension in R buffer containing 500 mM NaCl.

(ii) Method 2. The *Bam*HI-*Eco*RI fragment of pAASD12 carrying the *rep* gene was recloned into pSP73 downstream of a bacteriophage T7 promoter to produce pCCU1001. This plasmid was introduced into strain BL21 (DE3) (47). This strain contains a λ prophage carrying the bacteriophage T7 RNA polymerase under the control of the *lac* repressor.

Two 2-liter cultures of strain BL21 (DE3)(pCCU1001) in LB medium were grown at 37°C to an optical density at 550 nm of 0.5. IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 0.4 mM, and the cultures were incubated for 90 min at 37°C. We experienced difficulties because of the toxic effects of Rep on the bacterial cells and employed the precautions outlined by Studier et al. (48) in storage and growth of the Rep-producing strain. Because Rep comigrates with an abundant bacterial protein in polyacrylamide gels, induction was best monitored by Western blotting (immunoblotting) and detection of Rep with polyclonal rabbit anti-Rep serum. After induction of Rep synthesis, cells were collected by centrifugation at 4,000 rpm for 10 min in a Sorvall GSA rotor at 4°C. All subsequent manipulations were done on ice or at 4°C unless indicated. The cells were suspended in 30 ml of buffer A (50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid] [pH 7.5], 1 mM EDTA, 10% glycerol, 1 mM β-mercaptoethanol) containing 250 mM NaCl. Spermidine (final concentration, 20 mM) and lysozyme (200 μg/ml) were then added. The suspension was incubated at 37°C for 4 min with gentle mixing every minute and then passed through a French pressure cell. The volume of the suspension was measured, and 5 M NaCl dissolved in buffer A was added to give a final concentration of 1 M NaCl. Debris was removed by centrifugation at 42,000 rpm for 60 min in a Beckman Ti70.1 rotor. The supernatant was dialyzed three times against 1 liter of buffer A containing 50 mM NaCl, and the last dialysis was for overnight. The dialyzed supernatant was centrifuged at 12,000 rpm for 20 min in a Sorvall SS34 rotor. The pellet containing most of the Rep protein was suspended in buffer A containing 250 mM NaCl. Approximately 9 mg of protein was loaded onto a 10-ml heparin-agarose column preequilibrated with buffer A containing 100 mM NaCl. The column was eluted with a 100 to 500 mM gradient of NaCl in buffer A. Fractions containing Rep were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, pooled, and dialyzed against buffer A containing 250 mM NaCl. The protein was stored at −80°C.

Construction of Rep binding site mutations. The pSC101-derived *Eco*RI fragment of pLC712 was cloned into bacteriophage M13mp19 (59), and the resulting phage was grown on RY2504 to produce uracil-containing single-stranded

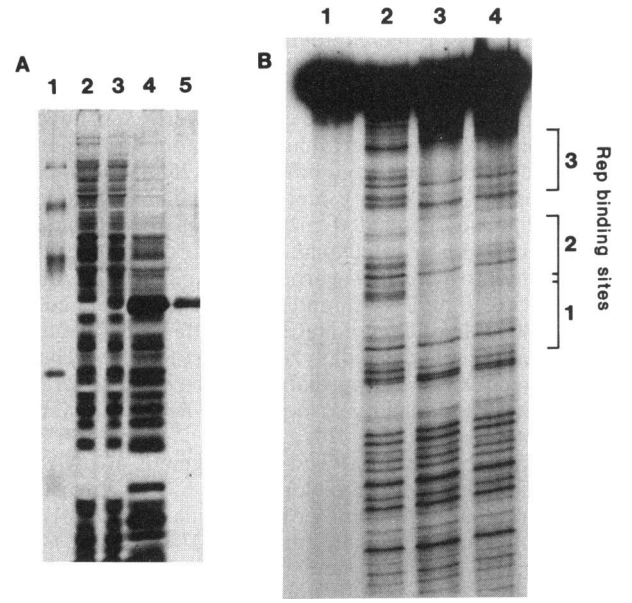

FIG. 2. Purification and DNA binding properties of Rep protein. (A) Silver-stained gel showing purification of Rep protein by using method 2. Lanes: 1, molecular weight markers; 2, lysate from uninduced cells; 3, lysate from cells induced with IPTG; 4, low-salt precipitate; 5, after chromatography on heparin-agarose. (B) Protection of a radiolabelled *Spe*I-*Ava*I fragment from digestion with DNase I by Rep protein. The positions of the Rep binding sites RS1 to RS3 are indicated. Lanes: 1, −DNase, +40 ng of Rep per μl; 2, +DNase, −Rep; 3, +DNase, +40 ng of Rep per μl; 4, +DNase, +20 ng of Rep per μl.

DNA (22). This DNA was annealed with the following phosphorylated synthetic oligonucleotides: 5′-GCTAGACCCTC TGTAAATTCCGCTGAGCCTTTGTGTGTTTTTTTT-3′ (site RS1), 5′-CTGGAAAACTTGTAAATTCTGCTGAGCCCTCT GTAAATTC-3′ (site RS2), 5′-GTTGTGGGTATCTGTAAA TTCTGCTGAGCCTTTGCTGGAAAACT-3′ (site RS3). Annealed primers were then elongated with the Klenow fragment of DNA polymerase, and, after treatment with DNA ligase, the resulting DNA was used to transfect LC707. Plaques were screened by preparing phage DNA and sequencing by using the dideoxynucleotide chain termination method (41). Once phage with mutations in site RS1, RS2, or RS3 were recovered, they were then used to produce template DNA for further rounds of mutagenesis to recover phage with mutations in more than one site. Mutations were transferred from phage to plasmids by substituting a restriction fragment. The same pSC101-derived *Eco*RI fragment used to construct the M13mp19 derivative used in the mutagenesis procedure was cloned into pUC18 to produce pCCU1. A *Hin*dIII-*Spe*I fragment isolated from phage carrying binding site mutations was then used to replace the analogous fragment of pCCU1. The structure of the resulting plasmids and the presence of the expected mutations was verified by DNA sequencing.

Binding of Rep to DNA. (i) DNase I footprinting (17). In the experiment shown in Fig. 2B, Rep protein, purified by method 1, was incubated at room temperature in 48 μl of binding buffer containing 50 mM Tris-Cl (pH 7.5), 1.1 mM EDTA, 7 mM $MgCl_2$, 3 mM $CaCl_2$, 100 mM KCl, 10% glycerol, 2 mM β-mercaptoethanol, and 400 μg of bovine serum albumin per ml. A 50-ng amount of poly(dI-dC) and then 35,000 cpm (0.5 to 1 ng) of ^{32}P-labelled restriction enzyme DNA fragment were added. The mixture was incubated for 1 h at room temperature, and then 2 ng of DNase I was added. After 30 s, the reaction was stopped by adding 12.5 μl of a solution containing 120 μg of yeast RNA per ml, 1.5 M ammonium acetate, and 0.25 M EDTA. The samples were then extracted with a mixture of phenol and chloroform and precipitated with ethanol before electrophoresis. The experiment shown in Fig. 4 was performed in a similar manner except that the binding buffer lacked $CaCl_2$ and glycerol and poly(dI-dC) was omitted.

(ii) Filter binding and competition assays (38). A 350-ng amount of Rep protein, purified by method 1, was incubated for 1 h at room temperature in the same binding buffer used in the footprinting assays. A 12-ng amount of poly(dI-dC), 10,000 cpm of ^{32}P-labelled restriction enzyme fragment, and increasing amounts of unlabelled competitor DNA were added, and the reaction mixtures were incubated for 1 h at room temperature. Competitor DNA was produced from the plasmids pCCU1 (wild type) and pCCU8 (mutant in sites RS1, RS2, and RS3) by polymerase chain reaction amplification (39) by using the oligonucleotides 5′-ACCACTTGAA TATAAAC-3′ and 5′-TAATTACTAGTCCTTTTC-3′ as primers. The samples were then slowly filtered through nitrocellulose filters which had been presoaked in washing buffer (10 mM Tris-Cl [pH 7.5], 1 mM EDTA, 7 mM $MgCl_2$, 100 mM KCl). The filters were washed with 1 ml of washing buffer and dried, and the radioactivity bound to the filters was measured by scintillation counting.

Plasmid copy number assays. Overnight cultures of strain BW360 containing mutant plasmids were diluted 1:1,000 in the presence of 25 μg of ampicillin per ml. After incubation at 37°C, aliquots were removed from exponential-phase cultures, diluted, and plated in 3 ml of soft agar on fresh plates containing increasing concentrations of ampicillin. For each ampicillin concentration, the soft agar contained the same concentration of ampicillin as the plates.

Measurement of plasmid stability. Overnight cultures of strain BW360 containing mutant plasmids were grown in LB medium containing 25 μg of ampicillin per ml. These cultures were diluted 1:1,000 in LB without ampicillin and allowed to grow overnight into stationary phase. They were then diluted 1:1,000 in LB medium without ampicillin and allowed to grow again. This procedure was repeated 14 times. An aliquot of each overnight culture was diluted and plated on nonselective plates as well as on plates containing 25 μg of ampicillin per ml, and the fraction of ampicillin-resistant colonies was measured.

Other techniques. All other techniques, including cloning procedures and gel electrophoresis, were performed by using standard methods (40).

RESULTS

Purification of Rep protein. The Rep protein used in the DNA binding experiments reported in Fig. 2B and 3 was purified by using method 1. This involved recovering Rep protein from the pellet fraction after centrifuging a cell lysate and then purifying the protein by chromatography on heparin-agarose and then by low-salt precipitation. We subsequently modified the purification of Rep as described in method 2. Inclusion of a higher salt concentration in the lysis buffer allowed the isolation of a substantial fraction of Rep in soluble form, which could then be recovered by low-salt precipitation and purified by chromatography on heparin agarose. The purification of Rep by method 2 is shown in Fig. 2A. Both methods produced protein of the same purity,

as judged by SDS-PAGE (23), but method 2 proved to be more reproducible and resulted in significantly higher yields of protein. Protein purified by either method bound specifically to DNA fragments containing either *ori* or the *rep* promoter region. That Rep protein protects the three Rep binding sites within *ori* from digestion by DNase I (17) is shown in Fig. 2B.

Construction of Rep binding site mutations within *ori*. Detailed studies of binding of a Rep–β-galactosidase fusion protein has shown that three adjacent purine residues, AGA, are present within each binding site and are in close contact with bound Rep protein (53). Methylation of the central G residue prevents Rep binding in vitro (53), and a mutation has been characterized where the G residue in one of the autoregulatory binding sites has been changed to an A (25). This mutation substantially reduces the efficiency of repression by Rep protein of transcription from the *rep* gene promoter (25). We therefore designed oligonucleotides which would enable us to change the AGA triplet to GAG in each of the origin Rep binding sites. Because the three Rep binding sites within *ori* have the same sequence, we used three different oligonucleotides, each with an extra 10 bases extending beyond the ends of the Rep binding site. These extra bases provided sufficient specificity so that we were able to recover mutations in a particular site by using a given oligonucleotide. We constructed a series of plasmids carrying mutations in each one of the three Rep binding sites, the three possible combinations of double mutations, and a plasmid with mutations in all three sites. These plasmids all carried the entire pSC101 replication region (Fig. 1), as well as the *par* locus (30) which is on the opposite side of *ori* from the *rep* gene, cloned in pUC18.

Rep binding to mutant DNA fragments. To confirm that the mutations did impair binding of Rep protein to the replication origin, we first compared the efficiency with which DNA fragments carrying either three intact or three mutant binding sites could compete for Rep binding with a radiolabelled wild-type fragment. The competing fragments were prepared from appropriate plasmid templates by polymerase chain reaction amplification (39) and mixed in increasing amounts with fixed amounts of radiolabelled *ori* DNA and Rep protein. After incubation, the mixtures were filtered through nitrocellulose, and the amount of radiolabelled DNA retained on the filters was determined (38). As shown in Fig. 3, the fragment carrying mutations in all three binding sites was 100 times less efficient as a competitor for Rep binding than a fragment with all three binding sites intact.

We then tested by footprinting with DNase I the effects of mutations in the individual sites RS1 to RS3 on Rep protein binding. The results of experiments using radiolabelled *Ava*I-*Spe*I DNA fragments (carrying the three binding sites RS1 to RS3), prepared from pCCU2 (mutant in site RS1), pCCU3 (mutant in site RS2), and pCCU4 (mutant in site RS3), are shown in Fig. 4. We found that each mutation abolished a prominent DNase I cleavage site which was present in wild-type DNA and which was not protected by Rep protein. A comparison of lane 1 with lanes 2 and 3 showed that, in the presence of Rep protein, DNA from pCCU2 (mutant in site RS1) was cleaved by DNase I in the region of site RS1, but sites RS2 and RS3 were protected from cleavage. Similarly, for plasmids pCCU3 and pCCU4 (mutant in sites RS2 and RS3, respectively) in the presence of Rep protein, mutant sites were cleaved by DNase I while other sites were protected (Fig. 4, lanes 4 to 6 and 7 to 9). We therefore concluded that the mutations abolished Rep binding to individual mutant sites and that any effects of the mutations on pSC101 replication were most likely due to impaired Rep binding to individual mutant binding sites rather than to other less direct effects of the mutations on global origin structure and function.

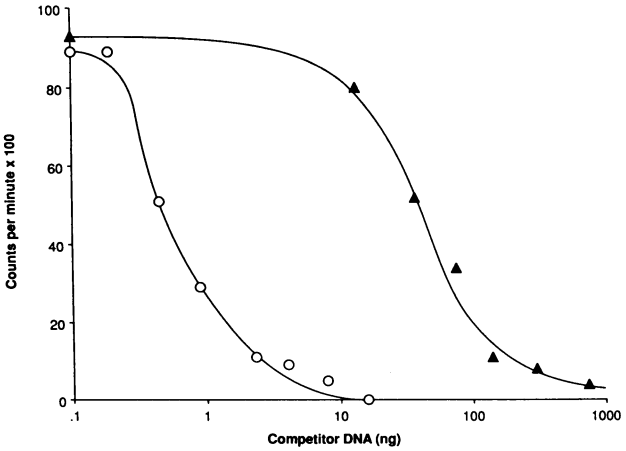

FIG. 3. Binding of Rep protein by DNA fragments containing wild-type or mutant binding sites RS1 to RS3. Results of a filter binding assay with radiolabelled *Spe*I-*Ava*I restriction fragment incubated with Rep protein and increasing amounts of competitor are shown. Symbols: ○, wild-type competitor; ▲, mutant competitor.

Transformation efficiency of the mutant plasmids. The pUC18 plasmids containing either wild-type or mutant pSC101 replication segments were tested for their ability to transform $CaCl_2$-treated (11) strain BW360 *polA*. The pUC18 origin of replication does not function in *polA* mutant strains, and therefore the pSC101 origin of replication must function in order to obtain transformants (7). The transformation efficiencies obtained with the different plasmids are presented in Table 1. The plasmid carrying mutations in all three

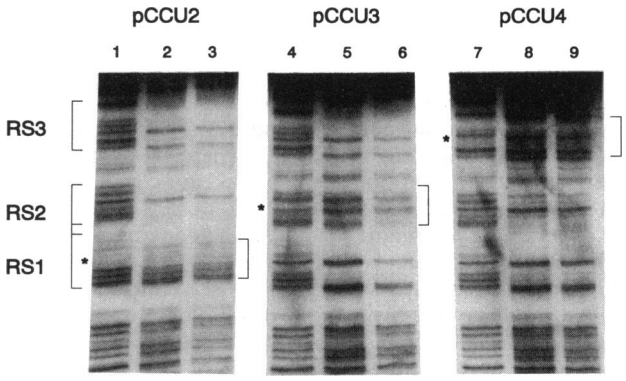

FIG. 4. Protection by binding of Rep protein of mutant DNA fragments from cleavage by DNase I. Lanes: 1 to 3, pCCU2 DNA (mutant in site RS1); 4 to 6, pCCU3 DNA (mutant in site RS2); 7 to 9, pCCU4 DNA (mutant in site RS3); 1, 4, and 7, −Rep protein; 2, 5, and 8, +40 ng of Rep protein per μl; 3, 6, and 9, +80 ng of Rep protein per μl. The brackets at the left side of the figure, labelled RS1, RS2, and RS3, show the position of sites RS1 to RS3 in the DNA sequence. The asterisk at the left of each panel marks the site of DNase I cleavage, referred to in the text, which was abolished by the mutation in each plasmid. The bracket at the right of each panel indicates DNase I cleavage sites within a mutant Rep binding site which are not protected by Rep binding.

TABLE 1. Transformation of strain BW360 *polA* by plasmids carrying Rep protein binding site mutations

Plasmid	Rep binding sites	Transformants per μg of DNA
pCCU1	$RS1^+$ $RS2^+$ $RS3^+$	8.2×10^5
pCCU2	$RS1^-$ $RS2^+$ $RS3^+$	1.7×10^4
pCCU3	$RS1^+$ $RS2^-$ $RS3^+$	2.0×10^5
pCCU4	$RS1^+$ $RS2^+$ $RS3^-$	8.3×10^4
pCCU5	$RS1^-$ $RS2^-$ $RS3^+$	80
pCCU6	$RS1^-$ $RS2^+$ $RS3^-$	1.2×10^5
pCCU7	$RS1^+$ $RS2^-$ $RS3^-$	1.4×10^5
pCCU8	$RS1^-$ $RS2^-$ $RS3^-$	

binding sites (pCCU8) failed to transform BW360 to ampicillin resistance, indicating a complete lack of pSC101 replication function. A comparison of the results for plasmids with mutations in single binding sites shows that the mutation in site RS1 (pCCU2) reduced the transformation efficiency by 50-fold, whereas the mutation in site RS2 (pCCU3) or RS3 (pCCU4) caused a 4- or 10-fold reduction, respectively. In addition, the transformant colonies obtained with the plasmid carrying a mutation in site RS1 (pCCU2) were very small after 24 h of incubation on selective media, indicating a substantial reduction in plasmid copy number in the transformants. Transformant colonies obtained with plasmids carrying a mutation in site RS2 or RS3 (pCCU3 and pCCU4) were of normal size. This behavior suggests that although all three sites are required for normal pSC101 replication activity, site RS1 is the most important.

The properties of the plasmids carrying double mutations are also presented in Table 1. The mutations in sites RS1 and RS2 together (pCCU5) almost completely abolished transformation. When DNA was prepared from these rare transformants and retested, it no longer displayed the mutant phenotype and so the few transformants obtained were presumably a result of contamination. The other double-mutant plasmids, carrying mutations in either sites RS1 and RS3 (pCCU6) or sites RS2 and RS3 (pCCU7), both showed a sixfold reduction in transformation activity, but there was a significant difference between the transformant colonies obtained with the two plasmids. As with the plasmid carrying a single mutation in site RS1 (pCCU2), the transformants obtained with the plasmid carrying mutations in sites RS1 and RS3 (pCCU6) were very small, whereas those from the plasmids with a mutation in sites RS2 and RS3 (pCCU7) were of normal size.

Copy number of mutant plasmids. The copy number of the different mutant plasmids was measured by plating exponentially growing cultures of BW360 *polA* bacteria containing each plasmid on increasing concentrations of ampicillin. In this strain, the level of resistance to ampicillin has been shown to be proportional to the number of plasmid copies (27).

As shown in Fig. 5, the plasmid copy number was reduced most markedly by mutations in binding site RS1 (plasmids pCCU2 and pCCU6). This is in agreement with the transformation efficiencies and small-colony phenotype described above for these plasmids. When a concentration of ampicillin at which 50% of the cells exhibited resistance was used, single mutations in binding sites RS2 or RS3 (pCCU3 and pCCU4, respectively) decreased copy number by 12 and 29%, respectively. A double mutation in binding sites RS2 and RS3 (pCCU7) decreased copy number by 65%. For both

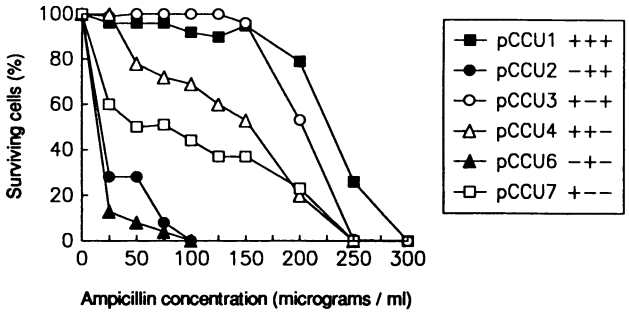

FIG. 5. Copy number of wild-type and mutant plasmids in strain BW360 *polA*. The key shows the symbol corresponding to each plasmid and the status (− indicates mutation) of binding sites RS1, RS2, and RS3 from left to right.

of the plasmids mutated in binding site RS3 (pCCU4 and pCCU7), the resistance to ampicillin declined over a broad range of ampicillin concentrations. This indicates a broader than normal range of plasmid copy numbers within the population, suggesting that a mutation in site RS3 affects the regulatory mechanism which corrects fluctuations in copy number.

Plasmid stability. The stability of the mutant plasmids during growth in the absence of selection was measured by repeated growth and dilution of strain BW360 *polA* containing the mutant plasmids. After each period of growth, aliquots were diluted and plated on media with and without ampicillin to determine the proportion of cells which remained resistant to ampicillin. As shown in Fig. 6, the wild-type plasmid (pCCU1) and plasmids with mutations in binding site RS2 or RS3 (pCCU3 and pCCU4) were stable over 15 subcultures or approximately 150 generations. The other mutant plasmids (pCCU2, pCCU6, and pCCU7) were unstable. The two plasmids mutant in site RS1 (pCCU2 and pCCU6) were lost most rapidly. In general, the stability of the plasmids correlated with plasmid copy number, the only surprising result being the difference between plasmids pCCU4, mutant in site RS3, which had a reduced copy number but was stable, and pCCU7, mutant in sites RS2 and RS3, which also had a reduced copy number but which was unstable.

DISCUSSION

Mutations in the Rep protein binding sites of the origins of replication of other plasmids similar to pSC101, including P1

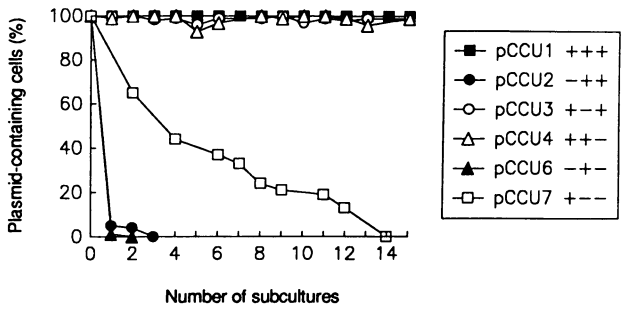

FIG. 6. Stability of wild-type and mutant plasmids in strain BW360 *polA*. The key shows the symbol corresponding to each plasmid and the status (− indicates mutation) of binding sites RS1, RS2, and RS3 from left to right.

and R6K, have been described, but a systematic study of the function of each repeat has not been reported. Deletion of either one or two repeats from the P1 origin of replication results in a replication-defective phenotype (2, 56). Mutations within single binding sites of the gamma origin of R6K also result in a defective replication origin, although precise deletion of the mutated repeat causes partial restoration of origin function (29).

Our results indicate that, although the three Rep binding sites within the origin of pSC101 are all required for normal pSC101 replication, they are not functionally identical. All plasmids bearing a mutation in binding site RS1 showed severe impairment of pSC101 replication function. Those plasmids, mutant in site RS1 and which productively transformed the *polA* strain BW360 (pCCU2 and pCCU6), were present at very low copy numbers, as indicated by the small colony size on selective media and the very low single-cell resistance to ampicillin in comparison to the wild-type plasmid (pCCU1). The replication defects shown by this group of mutant plasmids indicates that binding of Rep protein to site RS1 is crucial for pSC101 replication.

Binding site RS1 is adjacent to an AT-rich tract within the origin which contains 13-mer sequences identical to those sequences in *oriC* which are unwound by DnaA protein (6). Although the function of Rep in pSC101 replication is not known, Rep binding to site RS1 may facilitate the unwinding of the origin within the A-T tract. Replication of pSC101 is dependent upon IHF (18). Stenzel et al. have shown that IHF-induced bending promotes the interaction of DnaA protein with the replication origin and that Rep protein enhances DnaA protein binding (45). Such an interaction could be largely dependent on the presence of Rep protein bound to site RS1. Other evidence also suggests that binding site RS1 plays a major role in pSC101 origin function. By using a Rep-β-galactosidase fusion protein, Vocke and Bastia have shown that the inverted repeats overlapping the *rep* promoter are preferential Rep binding sites at a low protein concentration (53). More recent studies by Sugiura et al. with native Rep protein have shown a similar preferential binding of purified Rep protein to the autoregulatory sites (50). In both studies, there is evidence of binding of Rep protein to site RS1 at low protein concentrations before binding to sites RS2 and RS3 is observed.

Mutations in site RS2 caused only a slight reduction in plasmid copy number, and otherwise the mutant plasmids (pCCU3, mutant in site RS2, and pCCU7, mutant in sites RS2 and RS3) had the same properties as similar plasmids with a normal site RS2 (pCCU1, wild type, and pCCU4, mutant in site 3 alone, respectively). The one exception was that, while pCCU7 segregated during prolonged growth, pCCU4 was stably maintained. This difference is probably caused by different copy number distributions of the two plasmids and is discussed in more detail below. These results showing that mutation in site 2 has little effect on replication are contradicted by earlier studies (24). An insertion of Tn*1000* which destroys site RS2 completely abolishes *ori* function, although sites RS1 and RS3 remain intact (24). On the basis of our results reported here, one might have expected some residual function of the interrupted origin because of the presence of an intact binding site RS1. One explanation for these contradictory results could be that, in the case of the transposon insertion, the normal spacing between sites RS1 and RS3 was destroyed. An alternative explanation would be that transposase and IHF proteins bound at the end of Tn*1000* (55) prevent Rep binding to site RS1.

Mutations in site RS3 (pCCU4, mutant in site RS3, and pCCU7, mutant in sites RS2 and RS3) reduced the copy number and resulted in a broadening of the copy number distribution. This broadening is quite distinct from the effects of other mutations which reduce pSC101 copy number. Deletion of the *par* locus (27), as well as sequences between *par* and *ori* (37), reduces copy number, but the distribution of copy numbers, as measured by the slopes of ampicillin resistance curves, remains the same as that for the wild-type plasmid. This implies that, while the average number of plasmid copies is reduced, the mechanism which controls fluctuations in copy number is still working. In the case of site RS3 mutations, it appeared that both replication and the copy control mechanism were affected, resulting in a reduced average copy number and a broader copy number distribution. This broadening was more pronounced for pCCU7, mutant in sites RS2 and RS3, than for pCCU4, mutant in site RS3 alone. The copy number distributions for both of these plasmids are similar to those observed for *oriC* plasmids, which have no copy control mechanism (21).

The difference in copy number distribution between pCCU7, mutant in sites RS2 and RS3, and pCCU4, mutant in site RS3 alone, can explain the difference in stability observed for these plasmids. Resistance to 25 μg of ampicillin per ml corresponds to a plasmid copy number of approximately 1/10 the wild-type level (225 μg/ml) or 1 or 2 copies per cell. It can be seen in Fig. 4 that, in a culture containing pCCU7, approximately 40% of the cells were killed by 25 μg of ampicillin per ml, suggesting that they had either no or one plasmid. In contrast, for pCCU4, mutant in site RS3 alone, all cells survived at a concentration of 25 μg of ampicillin per ml, and therefore each one contained at least one plasmid.

Finally, we have reported a new method of Rep protein purification that is easier than previously published procedures (50) and yields Rep of similar purity and activity.

ACKNOWLEDGMENTS

This work was supported by Public Health Service Grant GM36437 from the National Institute of General Medical Sciences and BRSG S07 RR05364 from the Biomedical Research Support Grant Program, Division of Research Resources, National Institutes of Health.

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