J. Gen. Appl. Microbiol., **43**, 309–316 (1997)

Characterization of copy number mutants of plasmid pSC101

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(Received September 19, 1997; Accepted November 28, 1997)

We have characterized three copy number mutants of the plasmid pSC101. These mutations caused single amino acid substitutions at the 46th, 83rd and 115th codons in the *rep* gene and an increase in the copy number by 4- to 8-fold. Although the in vivo and in vitro repressor activities of these mutated Rep proteins were quite different from each other, the intracellular concentrations of the proteins were maintained at higher levels than the wild-type protein. It has been reported that excess amounts of Rep inhibit pSC101 replication (Ingmer and Cohen, 1993). This inhibitory activity of Rep was markedly decreased in all three mutants. When both the wild-type and one of the mutated *rep* genes were retained in the same plasmids, the copy number of these plasmids was decreased compared with plasmids retaining a single mutated *rep* gene. These results support the theory that the inhibitory activity of Rep for its own replication plays an important role in copy number regulation.

Key Words——copy number; DNA replication; dominance test; plasmid; pSC101

Plasmid pSC101 (Cohen and Chang, 1977), 9.2 kilobase pairs (kb) in size (Yamaguchi and Yamaguchi, 1984b), is present at approximately 5 copies per *Escherichia coli* chromosome (Hasunuma and Sekiguchi, 1977), which are intermediate between the unit copy number (i.e., mini-F) and the high copy number (15–20 copies, i.e., R6K). A 1.3 kb segment of pSC101 responsible for autonomous replication consists of a particular gene, designated *rep* (or *repA*), encoding a replication protein (Rep or RepA) essential for its replication (Linder et al., 1983; Yamaguchi and Yamaguchi,1984a), and an origin region (*ori*) sufficient for the initiation of replication in the presence of Rep (Fig. 1). It has been proposed that Rep binds to the three direct repeats (DR) present in the *ori* region and thereby initiates replication, as in other DR-driven plasmids (Nordström, 1990). The expression of the *rep* gene is regulated autogenously by the binding of its own product to an inverted repeat (IR) element (IR-2) as an autorepressor (Linder et al., 1985; Sugiura et al., 1990; Vocke and Bastia, 1985; Yamaguchi and Masamune, 1985). In addition to IR-2, Rep binds to IR-1 and has a role in the enhancement of replication initiation (Manen et al., 1994; Ohkubo and Yamaguchi, 1995). Furthermore, overproduction of the Rep protein can inhibit the replication of plasmid pSC101 (Ingmer and Cohen, 1993).

Rep is thus a multifunctional protein. A mutation defective in a particular function would be useful for the analysis of the functions of Rep. Several mutants with an elevated copy number were isolated from pSC101 and mapped in the *rep* gene (Armstrong et al., 1984; Ingmer and Cohen, 1993; Ishige et al., 1987; Sugiura et al., 1993; Xia et al.,1991). A single amino acid substitution at the 93rd codon caused a decrease in autorepressor activity, resulting in high protein concentrations and an increase in the affinity of the protein for the DR sequences (Xia et al., 1991, 1993). When another mutated Rep at the 46th amino acid was overproduced, pSC101 replication inhibition activity decreased markedly (Ingmer and Cohen, 1993). We have also isolated three Rep mutants with elevated copy numbers which have single base changes resulting in the substitution of their corresponding single amino acids located at the 46th, 83rd and 115th codons in the *rep* gene (Ishige et al., 1987; Sugiura et al., 1993).

In this study, we characterized some properties of the mutant Rep proteins, and found that higher concentrations of the Rep proteins were maintained in cells carrying these mutant plasmids although their autorepressor activities were quite different. In addition, all of these point mutations showed markedly di-

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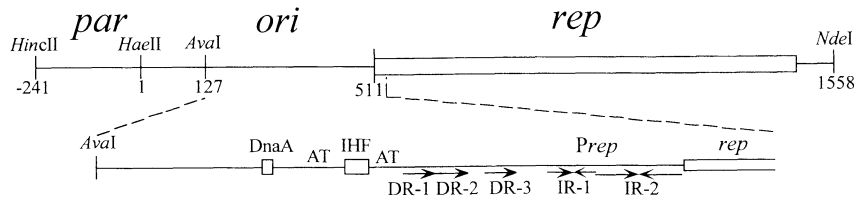

Fig. 1. The basic replicon of plasmid pSC101.

Boxes show the DnaA protein binding sequence (DnaA), the integration host factor binding sequence (IHF) and the structure gene (*rep*) of Rep. *par* and *ori* mean the partition region and replication origin, respectively. AT and P*rep* indicate an AT-rich stretch and the promoter of *rep*, respectively. The numbers under the map are the coordinates of the sequence (Yamaguchi and Yamaguchi, 1984b).

Table 1. Plasmids used.

Plasmids	Relevant properties[a]	Sources
pYUK101	Amp^r, *par*, *ori*, P*rep-rep*	Fueki and Yamaguchi (1991)
pYUK101-Kan	pYUK101, Kan^r	This work
pMIK4	Kan^r, R6K	This work
pMIK5	Kan^r, R6K	Yamaguchi and Yamaguchi (1984b)
pMIK6	Kan^r, R6K, P*rep-rep*	Yamaguchi and Yamaguchi (1984b)
pMIK-*rep1*	Kan^r, R6K, P*rep-rep1*	Sugiura et al. (1993)
pMIK-*rep21*	Kan^r, R6K, P*rep-rep21*	Sugiura et al. (1993)
pMIK-*rep28*	Kan^r, R6K, P*rep-rep28*	Sugiura et al. (1993)
pNR11	Amp^r, pBR322, P*lac-rep*	This work
pNC1	Amp^r, pBR322, P*rep-rep1*	This work
pNC21	Amp^r, pBR322, P*rep-rep21*	This work
pNC28	Amp^r, pBR322, P*rep-rep28*	This work
pSAW1	Amp^r, *par*, *ori*, P*rep-rep1*	This work
pSAW21	Amp^r, *par*, *ori*, P*rep-rep21*	This work
pSAW28	Amp^r, *par*, *ori*, P*rep-rep28*	This work
pSAZ-WT	Amp^r, *par*, *ori*, P*rep-rep*/*rep*	This work
pSAZ1	Amp^r, *par*, *ori*, P*rep-rep*/*rep1*	This work
pSAZ21	Amp^r, *par*, *ori*, P*rep-rep*/*rep21*	This work
pSAZ28	Amp^r, *par*, *ori*, P*rep-rep*/*rep28*	This work
pKMY213*dr1-lac*	Amp^r, pBR322, *ori*, P*rep-lacZ*	Yamaguchi and Masamune (1985)

[a] Amp^r, ampicillin (50 μg/ml) resistant; Kan^r, kanamycin (20 μg/ml) resistant; R6K, replication system of R6K; pBR322, replication system of pBR322. Definitions of other symbols are described in the legend of Fig. 1.

minished inhibition activities of the Rep proteins for plasmid replication.

Materials and Methods

Bacterial strains and plasmids. The strains of *E. coli* used were HI1006 F$^-$ *araD139 Δ*(*ara leu*) *7697 ΔlacX74 galU galk strA trpA38 recA1*, a derivative of MC 1000 (Casadaban and Cohen, 1980; provided by M. Imai) and JM109 *recA1 Δ*(*lac proAB*) *endA1 gyrA96 thi hsdR17 supE44 relA1*/F′ *traD36 proAB lacI^q-ZΔM15* (Yanisch-Perron et al., 1985). The plasmids used are listed in Table 1.

General methods. The culture media, methods for bacterial transformation with plasmid DNA, agarose gel electrophoresis and DNA manipulations have been described previously (Yamaguchi and Masamune, 1985; Yamaguchi and Tomizawa, 1980). The restriction endonucleases, T4 DNA ligase, Klenow fragment, T4 DNA polymerase, T4 polynucleotide kinase and bacterial alkaline phosphatase were purchased from Nippon Gene (Toyama, Japan), Toyobo (Osaka, Japan) and Takara Shuzo (Osaka, Japan). The reactions using these enzymes were carried out as recommended by the suppliers.

Construction of plasmids. Plasmid pYUK101 (Fueki and Yamaguchi, 1991) is a derivative of plasmid pSC101 and contains the *ori* region (*ori*), wild-type *rep* gene and *par* [coordinates 239–1885 (Yamaguchi and Yamaguchi, 1984b)]. We have constructed three plasmids (pSAW1, pSAW21 and pSAW28) which are identical to the pYUK101 plasmid except for *rep1*, *rep21*, and *rep28* mutations, respectively. The *rep* genes with mutations were amplified by polymerase chain reaction (PCR) (Saiki et al., 1988) and replaced the wild-type *rep* of pYUK101. The *rep1*, *rep21* and *rep28* genes have single base changes in nucleotide positions 853, 757 and 647 with substitu-

tions of the corresponding 115th, 83rd and 46th amino acids from Glu to Lys, Glu to Lys and Arg to Gln, respectively.

pYUK101-Kan was constructed by the insertion of the Kan^r fragment from pUC4K (Pharmacia Biotech, Tokyo, Japan) into the *Pst*I site in the *bla* gene of pYUK101.

Plasmids pSAZ-WT, pSAZ1, pSAZ21 and pSAZ28 are identical to pYUK101 except that they contain one additional wild-type *rep*, *rep1*, *rep21* and *rep28*, respectively. They were constructed by insertion of the DNA fragments (coordinates 443 to 1561) carrying the *rep* genes with their promoters into the *Nde*I site of pYUK101 with an *Nde*I linker. Both the *rep* genes and *rep* (in pSAZ-WT) or a mutated *rep* with its own promoters were connected to each other in the same direction of transcription.

Plasmids pNR11, pNC1, pNC21 and pNC28 have the *rep* or one of the mutated *rep* genes directed by the *lac* promoter (P*lac*), and were constructed as follows. First, the *lacI*^{q} fragment from pMJR1560 (Pharmacia) was inserted into the *Eco*RI site of p344, which is a pBR322 derivative containing P*lac* (Tsurimoto and Matsubara, 1981). The resultant plasmid was designated p344-*lacI*^{q}. Next, the *rep* gene (coordinates 481 to 1886) with or without mutation was amplified by PCR. The PCR products were inserted into the *Bam*HI/*Sph*I sites downstream of P*lac* of p344-*lacI*^{q} in the correct direction. The plasmids retaining *rep*, *rep1*, *rep21* and *rep28* were designated pNR11, pNC1, pNC21 and pNC28, respectively.

Determination of copy number. We determined the relative copy number conferred by the wild-type and mutant plasmids by the agarose gel method. Cells were grown in LB medium supplemented with 50 μg/ml of ampicillin and 20 μg/ml of kanamycin to an optical density of 0.4–0.5 at 600 nm. Cells from 1.5 ml cultures were suspended in 50 μl of 20 mM Tris HCl (pH 7.5), 100 mM NaCl and 10 mM EDTA, and mixed with 50 μl of phenol/chloroform. The supernatant was incubated with 1 μg of RNase A at 37°C for 15 min, then subjected to 1% agarose gel electrophoresis. We used here pMIK4 or pMIK5, a derivative of the plasmid R6K, as the internal control. Different amounts of DNA samples were applied in agarose gel to confirm the linear relationship of the amounts of DNA to the fluorescence of DNA bands after staining by SYBR-Green I (Molecular Probes, Eugene, OR, U.S.A.). The intensity of fluorescence was determined with a FluorImager-SI (Molecular Dynamics, Sunnyvale, CA, U.S.A.). The copy number of mutant plasmids was finally calculated by comparing with the value conferred by the wild-type plasmid, considering the unit value.

Assay of β-galactosidase activity. Cells to be assayed were grown in minimal-A medium (Miller, 1972) or LB medium. The β-galactosidase activities were determined as described previously (Miller, 1972).

Purification of the Rep proteins. The Rep, Rep1 and Rep21 proteins were purified as described previously (Sugiura et al., 1990). These proteins were greater than 95% pure as judged by SDS-polyacrylamide gel electrophoresis followed by staining with Coomassie Blue (Laemmli, 1970).

DNA mobility shift assay. A pUC19 derivative containing the IR-2 sequence was digested with *Bam*HI and *Eco*RI. The shorter fragment (68 bp) was isolated by electrophoresis and was 5′-end labeled with [γ-^{32}P] ATP (Amersham, Tokyo, Japan) and polynucleotide kinase. The end-labeled DNA probe (1 nM) was incubated with various amounts of Rep in 10 mM Tris HCl (pH 7.5), 10 mM $MgCl_2$, 200 mM KCl and 6 mM 2-mercaptoethanol for 10 min at 25°C. Electrophoresis was carried out as described previously (Sugiura and Yamaguchi, 1993).

Assay of inhibitory activity of Rep for plasmid replication. To overproduce the Rep proteins, *rep* genes were directed by the *lac* promoter. When strain HI1006 cells carrying both pYUK101-Kan and an overproducer of Rep were cultured in LB medium and grown to 0.1–0.2 at A_{600}, 1 mM IPTG was added. Samples were taken at every several generations of cell growth and spread on duplicate LB agar plates supplemented with both ampicillin and kanamycin or only ampicillin. The number of colonies that appeared after incubation overnight at 37°C was counted. The percentage of pYUK101-Kan containing cells with the number of generations reflects the inhibitory activity of Reps for the replication of pYUK101-Kan.

Immunodetection of the Rep proteins. Strain HI1006 cells were cultured in LB medium supplemented with ampicillin and grown to 0.4–0.5 at A_{600}, suspended in 50 mM Tris HCl (pH 8.0), 15 mM EDTA, frozen-thawed with lysozyme (final 0.65 μg/μl), and then treated with sonication. The total protein concentration was determined by Bio-Rad Protein Assay (Nippon Bio Rad Labs, Tokyo, Japan) using bovine serum albumin (BSA) as the standard. Less than 100 μg total protein was applied on a 12.5% acrylamide gel, and SDS-PAGE was carried out as described previously (Laemmli, 1970). After electrophoresis, Rep was electro-blotted to an Immobilon PVDF membrane (Nippon Millipore, Tokyo, Japan) in 25 mM Tris-base, 192 mM glycine and 15% methanol. The immunodetection of Rep was performed as described previously (Fueki et al., 1996) with partial modifications as follows. The membrane was blocked by incubation with 10% (v/v) Block Ace (Dainippon Seiyaku, Osaka, Japan) in TBS [20 mM Tris HCl (pH 7.5), 0.9% NaCl] and incubated with anti-Rep antiserum (rabbit) diluted 10,000-fold with 1% BSA and 0.05% (v/v) Tween-20 in

TBS at room temperature for 2 h. The protein was immunostained with biotinylated goat anti-rabbit IgG antibody (Peroxidase Vectastain ABC; Vector Labs, Burlingame, CA, U.S.A.) and Immunostaining HRP-1000 (Konika, Tokyo, Japan), and analyzed by densitometry with a Shimadzu dual-wavelength flying spot scanner (CS-9000; Shimadzu, Kyoto, Japan). The immunostaining was performed as recommended by the suppliers. We calculated the relative concentrations of Reps for each mutated Rep by considering that of the wild-type Rep as the unit value.

Results

Determination of copy number

The copy number of the pSC101 replicon is approximately 5 copies per host chromosome in exponentially grown cells (Hasunuma and Sekiguchi, 1977). To determine the relative copy numbers of mini-pSC101 (pYUK101) and its copy number mutants, we used the agarose gel analysis method with a second plasmid (pMIK5) as the internal control. The resulting value for the wild-type plasmid was considered the unit value, and the relative copy numbers of the three mutants were calculated by comparing their normalized values with that of the wild-type plasmid (Table 2). The copy number of the *rep1* mutant was maintained at a level about 5.5-fold, *rep21* about 4-fold and *rep28* about 8-fold higher than that of the wild-type plasmid.

Repressor activity of the Rep proteins

The Rep protein functions as an autorepressor for its own structural gene, *rep*. Its repressor activity is thought to affect the intracellular concentration level of Rep. We assessed the repressor activities of the mutated Rep proteins by β-galactosidase synthesis directed by the *rep* promoter (Yamaguchi and Masamune, 1985). The copy number (gene dosage) of *rep* may affect the concentration of Rep, which is directly related to the repressor activity. With this in mind, we cloned the *rep* genes onto an unrelated plasmid vector, a derivative of R6K. Thus, the copy number of *rep* genes would be expected to be similar among different strains. As shown in Fig. 2, in the presence of the wild-type Rep (lane 2), β-galactosidase activity was repressed to about 10% of that in the absence of the protein (lane 1). The Rep21 protein seems to have almost the same repressor activity as that of the wild-type protein, while those of Rep1 and Rep28 apparently decreased. For the synthesis of β-galactosidase, we grew the cells in LB medium as well as in minimal-A medium. In both cases, similar results for the three copy number mutants were obtained (data not shown).

Table 2. Relative copy numbers of mutant plasmids.

Plasmids	Relative copy numbers[a]	
	Expt. 1	Expt. 2
pYUK101 (*rep*$^+$)	1.00±0.19	1.00±0.05
pSAW1 (*rep1*)	5.80±0.19	5.53±0.21
pSAW21 (*rep21*)	4.64±0.12	3.85±0.13
pSAW28 (*rep28*)	8.22±0.14	7.21±0.27
pYUK101 (*rep*$^+$)	1.20±0.07	0.96±0.09
pSAZ-WT (*rep*$^+$/*rep*$^+$)	1.00±0.15	1.00±0.15
pSAZ1 (*rep*$^+$/*rep1*)	2.10±0.09	2.28±0.08
pSAZ21 (*rep*$^+$/*rep21*)	1.65±0.17	1.74±0.04
pSAZ28 (*rep*$^+$/*rep28*)	2.43±0.09	2.67±0.13

[a] The standard deviations were calculated from 4 determinations.

The intracellular concentration of Rep proteins

To test whether the differential repressor activities of Rep proteins result in differential levels of Rep concentrations in the cells, we immunologically determined the amounts of Rep proteins. The same amounts of total proteins in crude cell extracts were subjected to SDS-PAGE, and the Rep protein was detected by anti-Rep antibody. In this experiment, we used the same plasmids as those described in Fig. 2. Figure 3 shows that the intracellular concentration of Rep depended on the gene dosage, since the Rep level of pMIK6 (wild-type *rep* on the R6K derivatives; about 20 copies per host chromosome) was about 2-fold higher than that of mini-pSC101 (pYUK101) (lanes 1 and 2). In the case of mutants *rep1*, *rep21* and *rep28*, the Rep proteins were about 1.6-, 1.8- and 1.7-fold more abundant than that of the wild-type *rep* on the same R6K vector, respectively (Fig. 3, lanes

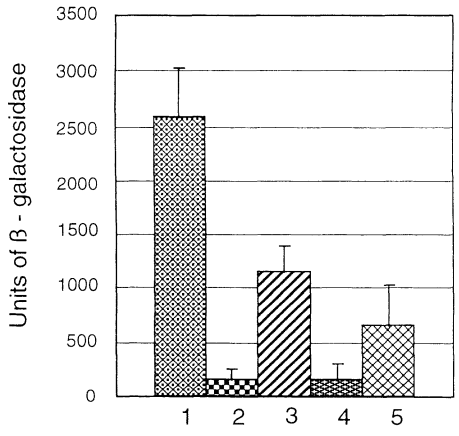

Fig. 2. In vivo repressor activities of the Rep and mutated Rep proteins.

Columns show the β-galactosidase activities of cells carrying pKMY213*dr1-lac* (P*rep-lacZ*) and pMIK5 (vector alone) (lane 1), pMIK6 (*rep*$^+$) (lane 2), pMIK-*rep1* (lane 3), pMIK-*rep21* (lane 4) or pMIK-*rep28* (lane 5). Bars at the top of the columns indicate the standard deviations from 8 determinations.

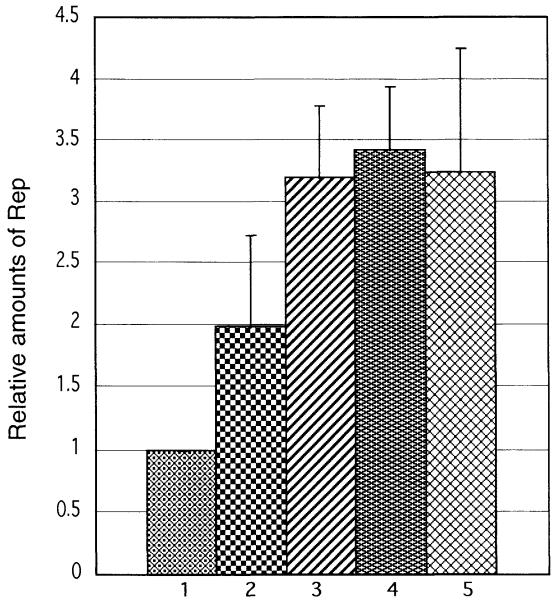

Fig. 3. Intracellular concentrations of the Rep proteins.
The columns represent the relative amounts of the Rep proteins in total proteins extracted from cells carrying pYUK101 (pSC101, *rep*$^+$) (lane 1), pMIK6 (R6K, *rep*$^+$) (lane 2), pMIK-*rep1* (lane 3), pMIK-*rep21* (lane 4) or pMIK-*rep28* (lane 5). Bars at the top of the columns indicate the standard deviations from 4 determinations.

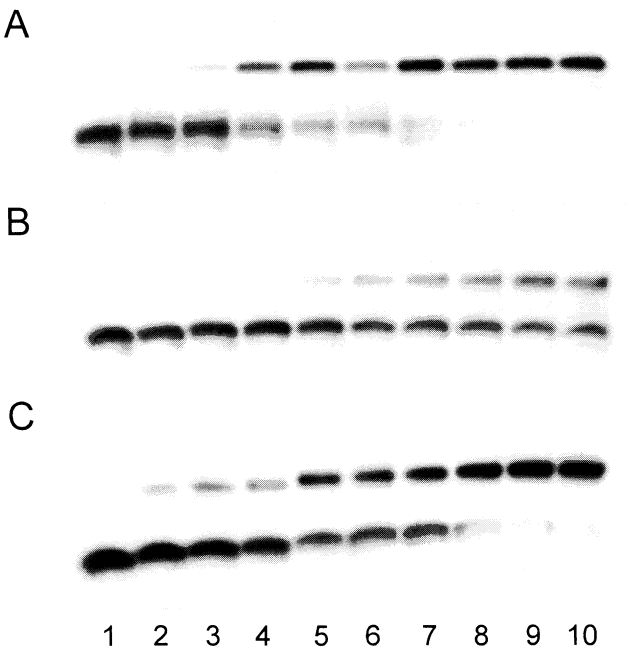

Fig. 4. Binding of purified Rep proteins to the IR-2 sequence.
In each reaction, 1 nM end-labeled IR-2 DNA was used. (A) The amounts of wild-type Rep added were 0, 0.39, 0.78, 1.6, 2.3, 3.1, 4.7, 6.3, 12.5 and 25 nM in lanes 1 to 10. (B) The amounts of Rep1 added were 0, 0.67, 1.0, 1.3, 2.0, 2.7, 4.0, 5.4, 8.0 and 10.7 nM in lanes 1 to 10. (C) The amounts of Rep21 added were 0, 0.78, 1.6, 3.1, 4.7, 6.2, 9.3, 12.5, 18.7 and 24.9 nM in lanes 1 to 10.

3–5). An estimation of the level of Rep protein was also performed using minimal-A medium side by side with LB medium. The results were similar in both media (data not shown).

DNA binding activities of purified Rep proteins

The in vivo repressor activity of Rep seemed not to be reflected in the intracellular concentration of Rep, as described above. Therefore, we purified Rep proteins except for Rep28 and tested their binding activity to the operator sequence (IR-2) of *rep*. Figure 4 shows the results of the DNA mobility shift assay of the Rep proteins using the IR-2 sequence as the DNA probe. The complex formation of wild-type Rep with IR-2 increased as the Rep concentration increased, and the equilibrium dissociation constant (K_d) was calculated to be 2 nM. The binding affinity of Rep21 to IR-2 was similar to that of Rep (K_d=3 nM), while that of Rep1 was clearly low (K_d=10 nM). Thus, the in vitro binding affinities of Rep proteins to the operator sequence support the in vivo repressor activity findings.

Inhibitory activity of excess amounts of Rep for plasmid replication

The Rep protein has another function, in that overproduction of the protein inhibits pSC101 replication specifically. However, excess amounts of a mutated Rep protein, which increased in the plasmid copy number, did not have this inhibitory activity (Ingmer and Cohen, 1993). We therefore determined the inhibitory activities of the wild-type and mutated Rep proteins. In this experiment, we used the *lac* promoter (P*lac*) to promote the production of the Rep proteins instead of the original *rep* promoter. In the presence of more than 0.15 mM IPTG, the intracellular concentration level of Rep in cells carrying pNR11 (P*lac-rep*) was 5- to 6-fold higher than that in cells carrying pYUK101-Kan (P*rep-rep*) (data not shown). At 1 h after the addition of 0.25 mM IPTG, a decrease in the copy number of pYUK101-Kan was observed in cells carrying both pNR11 and pYUK101-Kan (data not shown). As shown in Fig. 5, the induction of wild-type Rep synthesis with 1 mM IPTG caused the elimination of the mini-pSC101 plasmid. In contrast, the plasmid was stably maintained in cells even after the induction of any mutated Rep protein synthesis.

When Rep protein synthesis was directed by the stronger promoter, *tac*, the level of Rep concentration increased to 25- to 30-fold that of the standard level in pSC101-carrying cells. Such a high overproduction of mutated Reps generated pYUK101-Kan-free cells (data not shown). This result indicates that the inhibitory activities of mutated Reps were not completely diminished.

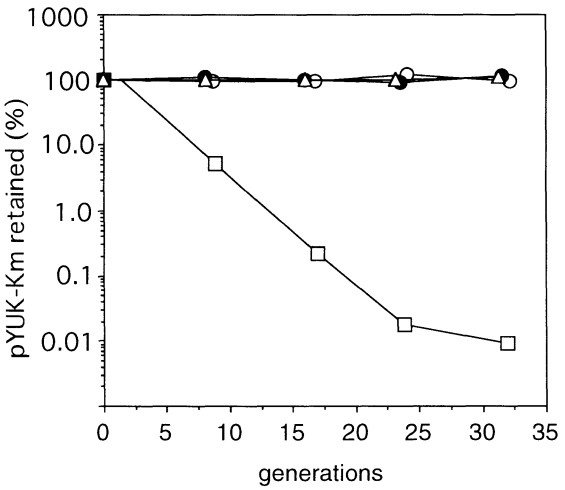

Fig. 5. Segregation of mini-pSC101 (pYUK101-Kan) after the induction of Rep synthesis.

To induce Rep synthesis directed by the *lac* promoter, 1 mM IPTG was added to a culture of cells carrying both pYUK101-Kan and pNR11 (*rep*$^+$) (□), pNC1 (*rep1*) (○), pNC21 (*rep21*) (●) or pNC28 (*rep28*) (△) at 0 generation. The fractions of cells retaining pYUK101-Kan are presented.

Effects of the coexistence of wild-type and mutant Rep proteins

To determine the effect of the coexistence of Rep wild-type and mutant Rep proteins on the plasmid copy number (i.e., to determine whether either one is dominant or recessive over the other), an additional *rep* gene fragment was inserted into mini-pSC101 (i.e., pYUK101) together with their own promoters. Thus, four plasmids containing either double *rep* gene were constructed; *rep*/*rep* (pSAZ-WT), *rep*/*rep1* (pSAZ-1), *rep*/*rep21* (pSAZ-21) and *rep*/*rep28* (pSAZ-28). We used another plasmid (pMIK4), a derivative of R6K, as the internal control since pMIK5 used as the control in the previous experiment had a molecular size similar to those of the pSAZ plasmids. The copy number of pYUK101 relative to pMIK4 was almost the same as that for pMIK5 (data not shown). When the relative copy number of pSAZ-WT was taken as 1, the relative copy numbers of pYUK101 and pSAZ-WT were almost the same (Table 2). This suggests that increasing the gene dosage of *rep* by 2-fold has no effect on the plasmid copy number. The plasmids carrying *rep* and any one of the mutated *rep*s were present in higher copy numbers (lanes 3–5), but their copy numbers were clearly less than those of the plasmids retaining a single mutated *rep* gene (Table 2). It was also noted that the order of the copy number among pSAZ-1, pSAZ-21 and pSAZ-28 corresponded to the order of the copy number among the single mutated *rep* plasmids. These results indicate that coexistence of the wild-type *rep* gene leads to a decrease in the high copy numbers directed by single mutated *rep* genes.

Discussion

We have isolated three independent mutants of Rep as a result of single nucleotide changes which substitute the corresponding amino acid in each case (Ishige et al., 1987; Sugiura et al., 1993). Previously reported mutants with the high copy number phenotype were mapped at the 46th, 92nd, 93rd and 96th codons of *rep* (Armstrong et al., 1984; Ingmer and Cohen, 1993; Xia et al.,1991). Therefore, *rep1* (115th codon) and *rep21* (83rd codon) are new types of copy number mutations. The mutation site of *rep28* is the 46th codon, but the amino acid substitution is from Arg to Gln, while that of the previous mutation was from Arg to Trp (Ingmer and Cohen, 1993).

The three *rep* mutations mentioned here caused an increase in the plasmid copy number by 5- to 8-fold compared with that of the wild-type plasmid (Table 2). The difference of copy numbers among the mutants was reproducible, and *rep28* showed the highest copy number. In addition, the repressor activities of the mutated Rep proteins were also different among the mutants (Fig. 2). The in vivo repressor activity of Rep21 was almost the same as that of the wild-type Rep, but that of Rep1 was decreased. This was confirmed by the binding activity of the purified Rep1 and Rep21 proteins to the operator sequence of the *rep* gene (Fig. 4). However, the repressor activities of these proteins were not reflected in the intracellular concentration of Rep proteins, since all of the mutated Rep proteins showed similar intracellular concentrations (Fig. 3). The reason for this is not yet known, but the stability of Reps inside the cells might be changed by the mutations. However, we noted that the level of every mutant Rep was about 3-fold higher than the standard level of mini-pSC101. The copy number of R6K used as a vector in these experiments is similar to those of the mutants. In fact, the Rep levels of pSC101 derivatives driven by these mutated Reps, such as pSAW1, were 3- to 4-fold higher than the wild-type Rep level (unpublished data).

A remarkable common feature of our copy number mutants is the decrease in their inhibitory activities for pSC101 replication, as shown in Fig. 5. This inhibitory activity of Rep may be explained by the handcuffing model proposed for the regulation of other plasmid replications in which, in addition to the binding of the initiator (Rep) to iterons (DR sequences in *ori*), two initiator-iteron complexes can pair up, resulting in the obstruction of the initiation of replication (Chattoraj et al., 1988; McEachern et al., 1989; Pal and Chattoraj, 1988). The overproduction of Rep from an adventitious promoter should lead to an increase in the

chances of origin-pairing. From this model, it is likely that mutated Rep1, Rep21 and Rep28 would be partially defective in the pairing of two initiator-iteron complexes. This pairing should be different from the dimer formation of Rep, because Rep21 is almost normal with respect to its repressor activity (Figs. 2 and 4), to which the dimer form of Rep is responsible (Manen et al., 1992; Sugiura et al., 1990). However, a higher level of Rep led to a complete loss of the plasmid from cells in this study (Fig. 5). This is not expected from the handcuffing model. This discrepancy might be explained by the evidence that an excess intracellular concentration of Rep interferes with plasmid DNA partitioning in addition to replication (Ingmer and Cohen, 1993). When wild-type Rep coexisted with a mutated Rep in the same cell, the copy number decreased (Table 2). A simple explanation is that the wild-type Rep protein functions as a negative regulator for DNA replication. Since the inhibitory activity of the wild-type Rep is active, the wild-type Rep would be expected to overcome the mutated Rep. If our mutant Reps increase their binding affinity to the DR sequences, resulting in elevation of the copy number as suggested previously (Xia et al., 1993), coexistence of the mutant Reps is thought to be dominant over the wild-type Rep. We have also isolated a Rep mutant (Ser→Arg at the 113th codon) deficient in inhibitory activity, and found that the mutation gave rise to an elevated copy number (Watanabe and Yamaguchi, unpublished observation). This also supports the theory that the inhibitory activity of Rep for its own DNA replication plays an important role in copy number regulation.

Purified Rep protein consists almost entirely of a dimer with very low binding affinity for the DR sequences (iterons) in *ori* (Sugiura et al., 1990), and the monomer form of Rep has a low affinity for DR [K_d=0.38 μM (Xia et al., 1993) or 1 μM (Ingmer et al., 1995)]. The isolation of the active initiator form (probably monomer form) of Rep should be performed to help clarify the molecular mechanism of copy number regulation.

We acknowledge colleagues in our laboratory for stimulating discussion and K. Arakawa and Y. Watanabe for constructing plasmids. This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science, sports, and Culture of Japan.

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