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Minimal Essential Origin of Plasmid pSC101 Replication: Requirement of a Region Downstream of Iterons

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The minimal replication origin (*ori*) of the plasmid pSC101 was defined as an about 220-bp region under the condition that the Rep (or RepA) protein, a plasmid-encoded initiator protein, was supplied in *trans*. The DnaA box is located at one end of *ori*, as in other plasmids, like mini-F and P1. The other border is a strong binding site (IR-1) of Rep which is a palindromic sequence and lies in an about 50-bp region beyond the repeated sequences (iterons) in *ori*. This IR-1 is located just upstream of another strong Rep binding site (IR-2), the operator site of the structure gene of Rep (*rep*), but its function has not been determined. The present study shows that the IR-1 sequence capable of binding to Rep is essential for plasmid replication with a nearly normal copy number. Furthermore, a region between the third iteron and IR-1 is also required in a sequence-specific fashion, since some one-base substitutions in this region inactivate the origin function. It is likely that the region also is a recognition site of an unknown protein. Three copy number mutations of *rep* can suppress any one-base substitution mutation. On the other hand, the sequence of a spacer region between the second and the third iterons, which is similar to that of the downstream region of the third iteron, can be changed without loss of the origin function. The requirement of the region downstream of iterons in pSC101 seems to be unique among iteron-driven plasmid replicons.

An autonomously replicating DNA molecule has one or more specific replication origins. Approximately 20-bp repeats are found in a number of replication origins; they have been named iterons (31). Iterons are believed to be targets for replicon-specific initiator proteins. Plasmid pSC101 has three iterons in its replication origin (*ori*) region (3, 7, 40, 50), which can support replication initiation when a plasmid-encoded initiator protein, Rep (or RepA), is supplied either in *cis* or in *trans* (23, 49). Previous deletion analysis defined the pSC101 *ori* to span nucleotide positions 223 to 587 (50) in Fig. 1. Also, insertion of Tn*1000* between positions 422 and 423 inactivated the origin (Ori) function, but insertion between positions 473 and 474 did not (7, 23). The thus-defined *ori* region includes, in addition to iterons, an about 80-bp AT-rich region and binding sites of host proteins, DnaA (15, 35) and integration host factor (IHF) (35, 36), both of which are required for plasmid replication (8, 11, 16, 17). These structural features except for the IHF binding site are also found in other plasmid replicons, such as mini-F (29, 30), P1 (1, 2, 44), R6K (27, 34), and Rts1 (20, 21). In these replicons, the DnaA box is located at one end of the *ori* region, while iterons are at the other end and the AT-rich region is between these two elements. This fact suggests that a common mechanism exists in the initiation reactions of these replicons. However, unique features are also seen around the *ori* region of the individual replicons. In the case of pSC101, iterons are followed by two inverted repeats, IR-1 and IR-2, which overlap the promoter region of *rep* encoding the Rep protein and are the operator site for autoregulation of the *rep* gene expression (24, 42, 47). Half repeats of IR sequences are partially similar to the sequences of iterons. Purified Rep can bind preferentially to both IR-1 and IR-2 (35, 38, 41), but IR-2 alone seems to be sufficient for the operator function both in vivo (47) and in vitro (38). Only one pair of inverted repeats of the iteron-like sequence is found in promoter regions of initiator genes in mini-F (30) and R6K (10). Thus, the function of the first inverted repeat, IR-1, in pSC101 is so far not clear.

In the present study, we determined the minimal essential region of pSC101 *ori*, 220 bp in size, including from the DnaA box to IR-1; that is, IR-1 is indispensable for plasmid replication. Furthermore, a region between iterons and IR-1 is also required in a sequence-specific fashion. The requirement of the region downstream of iterons (or upstream of the *rep* gene) seems to be unique in the pSC101 replicon.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* K-12 strains used were JM109 *recA1* Δ(*lac proAB*) *endA1 gyrA96 thi hsdR17 supE44 relA1*/F' *traD36 proAB lacI*^{q}-ZΔ*M15* (51), WA802 F$^-$ *metB1 lac-3 lacY1 galK2 galT22 supE hsdR2* (37), and WA802 *polA*.

Plasmid pUC19 was described previously (51). Both pKMY292*dl*6 (formerly designated pKMY292*d*6 [50]) and pKMY213-A62 (49) are composites of pBR322 and pSC101 replication systems. The former retains *ori*$^+$ and *rep*$^+$ (coordinates 223 to 1886) of the pSC101 moiety, while the latter retains *ori*$^+$ but lacks *rep* (coordinates 1 to 585). A Rep protein supplier, pMIK6, is an R6K derivative containing the *rep* gene with its promoter (49).

General methods. Culture media, bacterial transformation with plasmid DNA, agarose gel electrophoresis, and DNA manipulations have been described elsewhere (47, 48). Restriction endonucleases, T4 DNA ligase, and T4 polynucleotide kinase were purchased, if not indicated otherwise, from Nippon Gene; BAL 31 exonuclease was from New England Biolabs; calf intestinal phosphatase was from Boehringer Mannheim; and T4 DNA polymerase was from Toyobo. Reactions with these enzymes were carried out as recommended by the suppliers.

Plasmid construction. Plasmid pKMY292*dl*6 was cleaved at a unique *Bam*HI site introduced at position 223 and

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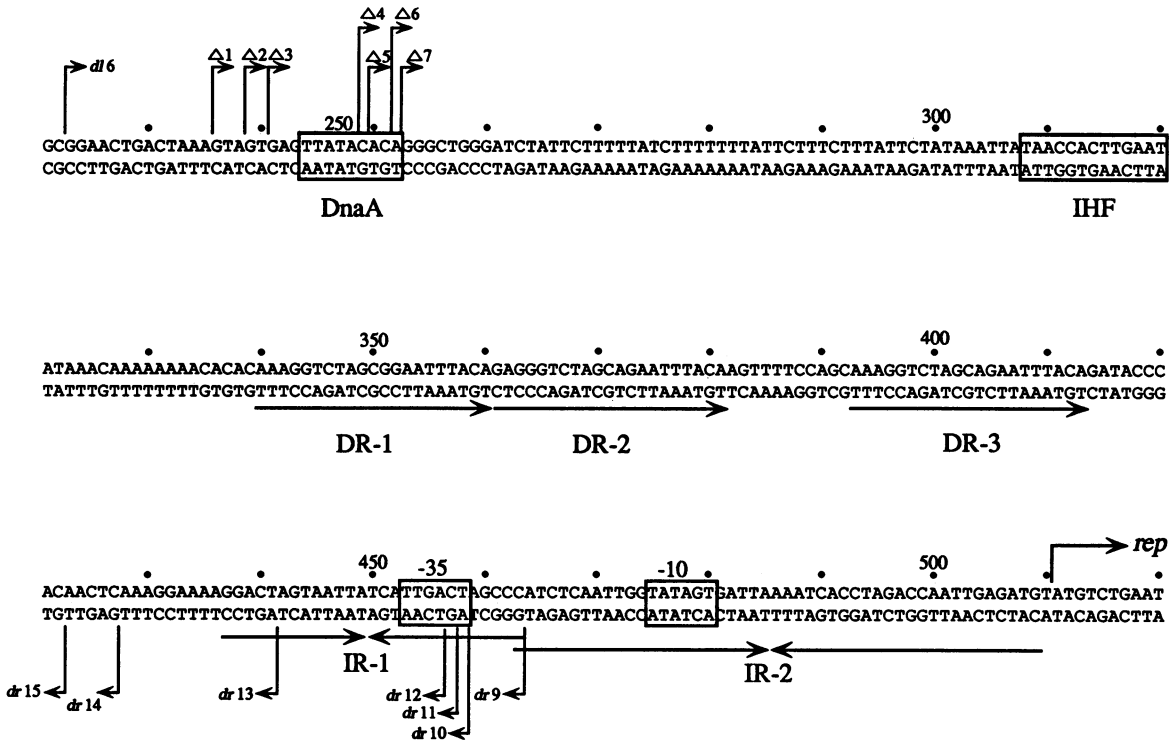

FIG. 1. Nucleotide sequence and structural features of the *ori* region of pSC101. Boxes show the DnaA protein binding sequence (DnaA), IHF binding sequence (IHF), and the *rep* promoter (−35 and −10). Arrows present three direct repeats (DR-1, DR-2, and DR-3) and inverted repeats (IR-1 and IR-2). Figures above the sequence are the coordinates of the sequence (50). The terminal nucleotide of the remaining fragment in each deletion mutant is indicated along the sequence by an arrow (➡ or ⬅) which means that the sequence is deleted from the left or right up to this site, respectively.

digested with BAL 31 nuclease. Trimmed DNA was circularized again with a *Bam*HI linker. The extent of the deleted regions was established by DNA sequencing by the dideoxy termination method (32). Resultant mutants were designated pKMY292*dl*6-Δ1 to pKMY292*dl*6-Δ7.

Plasmids pKMY292*dl*6t_{fd} and pKMY292*dl*6-Δ3t_{fd} were constructed by insertion of a synthetic sequence (t_{fd}) (18) mimicking a general transcriptional terminator of phage fd into pKMY292*dl*6 and pKMY292*dl*6-Δ3, respectively. A *Hin*dIII-*Sal*I fragment containing two tandemly repeated t_{fd} sequences was filled in by T4 DNA polymerase and ligated with pKMY292*dl*6 or pKMY292*dl*6-Δ3 cleaved with *Bam*HI and filled in. DNA sequencing confirmed that the direction of t_{fd} was correct to be functional for the rightward transcription from outside to *ori*.

Base substitution mutants within the downstream region of iterons were isolated as follows (Fig. 2). First, pUC-223/513 was constructed by tandemly inserting the *Bam*HI-*Sau*3AI fragment (positions 223 to 259) from pKMY292*dl*6 and the *Sau*3AI-*Bam*HI fragment (positions 260 to 513) from pUC-260/513 (38) into the *Bam*HI site of pUC19. Next, pUC-223/513 was digested with *Eco*RI and *Spe*I, and the resultant small fragment was separated from the large one retaining the vector moiety. Upon partial digestion of the small fragment with *Mae*I (Boehringer Mannheim), the second- and the third-largest fragments containing positions 223 to 399 and 223 to 367, respectively, were recovered and ligated with a synthetic DNA (A) in which positions A-413 and G-414 were replaced with G and T, respectively, to create a *Sna*BI site, which was then connected with the large *Eco*RI-*Spe*I vector fragment from pUC-223/513. In the resulting plasmid, pUC-223/513*Sna*BI carrying the second-largest *Eco*RI-*Mae*I segment, positions 413 to 439 can be removed by digestion with *Sna*BI (Takara Shuzo) and *Spe*I and replaced with synthetic DNA fragments carrying optional base substitutions. For example, in order to introduce mutations at position 420, the *Sna*BI-*Spe*I region of pUC-223/513*Sna*BI was replaced with a synthetic DNA fragment corresponding to positions 413 to 443,

AGATACCNACAACTCAAAGGAAAAGGA
TCTATGGNTGTTGAGTTTCCTTTTCCTGATC

On the other hand, a plasmid, pUC-223/513(Δ368/399)*Sna*BI, carrying the third-largest *Eco*RI-*Mae*I segment is in fact missing the sequence from positions 382 to 413 since *Mae*I sites are located within the repeated sequences. Thus, plasmid pUC-223/513(Δ382/413) was constructed by replacement of the *Sna*BI-*Spe*I segment of pUC-223/513(Δ368/399)*Sna*BI with the natural sequence from positions 413 to 443.

Copy number mutations, *rep1* (formerly designated *cop1* [19]), *rep21*, and *rep28*, increase the plasmid copy number five- to sixfold compared with that of the wild type. They have all single-base substitutions resulting in single amino acid changes, Glu-115→Lys, Glu-83→Lys, and Arg-46→Gln, respectively. (We previously reported that *rep1* [*cop1*] had three point mutations, Glu-115→Lys, Arg-257→His, and His-289→Tyr, based on the complete sequence of pSC101 [4]. However, as mentioned by Xia et al. [46], the conclusion of the last two amino acid changes was

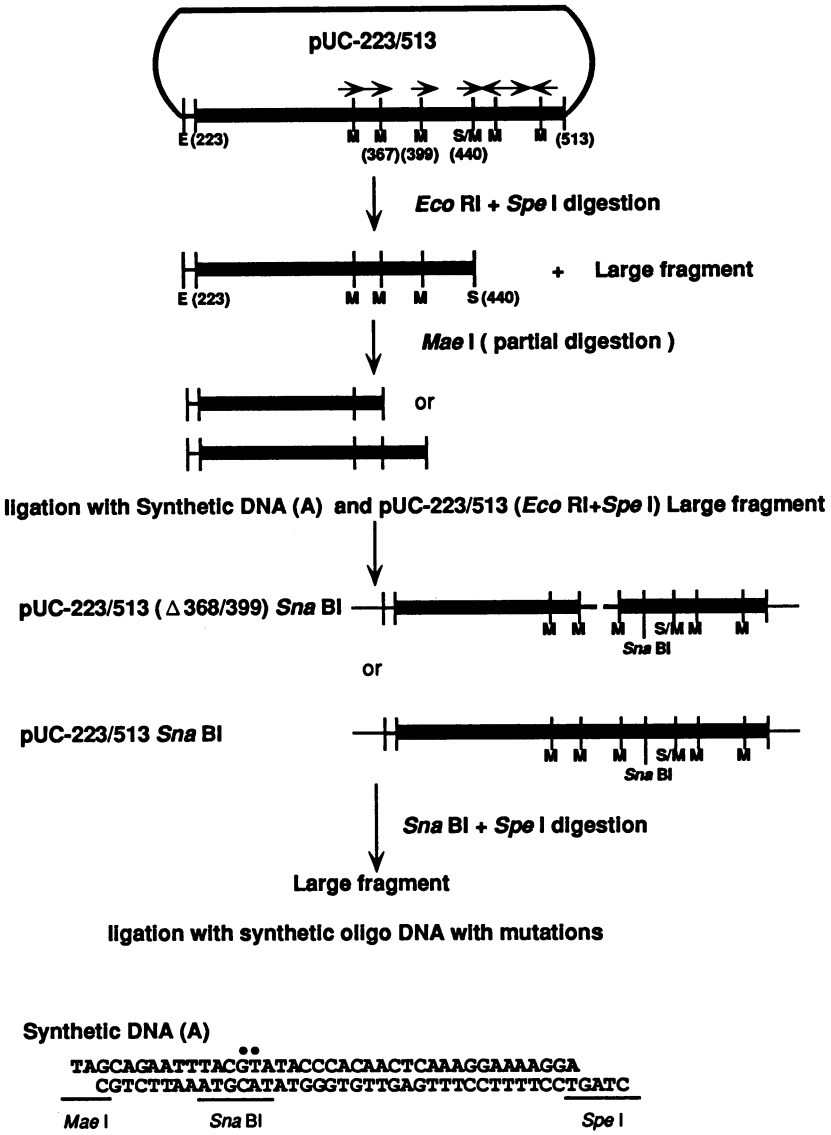

FIG. 2. Introduction of base substitution mutations into the downstream region of iterons. Boldface lines and thin lines show segments derived from pSC101 and pUC19, respectively. A dotted line indicates the deleted region. Figures in parentheses are the coordinates of the sequence. The sequence of synthetic DNA (A), in which the natural sequence was changed at dotted positions to create a *Sna*BI site, is shown at the bottom of the figure. E, *Eco*RI; M, *Mae*I; S, *Spe*I.

caused by errors in the original sequence. Our result of resequencing of the parental *rep* gene confirmed the sequence of Xia et al. Therefore, *rep1* has only one point mutation, resulting in one amino acid substitution at position 115.) Detailed characteristics of these mutations will be described elsewhere. DNA fragments carrying mutated *rep* genes (positions 443 to 1886) were cloned onto an R6K-based vector, pMI52, as pMIK6 carrying wild-type *rep* was constructed (49). Resultant plasmids were designated pMIK-*rep1*, pMIK-*rep21*, and pMIK-*rep28*.

DNA mobility shift assay. The sequence (positions 434 to 466) containing the natural IR-1 and the corresponding sequences of pKMY213*dr*10, pKMY213*dr*11, and pKMY 213*dr*12 were synthesized and cloned onto the *Sma*I site of pUC19 (designated pUC-434/466, pUC-434/466*dr*10, pUC-434/466*dr*11, and pUC-434/466*dr*12, respectively). Upon digestion with *Eco*RI and *Pst*I, 68-bp DNA fragments were purified by agarose gel electrophoresis. Probe DNA (1 μg) was dephosphorylated with calf intestinal phosphatase and then was 5′ end labeled with [γ-^{32}P]ATP (Amersham) as described elsewhere (38). The amount of DNA was estimated with 4′,6-diamidine-2-phenylindole (22).

End-labeled DNA probe (25 fmol), 2.5 μg of sonicated calf thymus DNA, and various amounts of competitor DNA were incubated with 0.5 pmol of Rep (38) in 30 μl of 10 mM Tris-HCl (pH 7.5)–10 mM $MgCl_2$–200 mM KCl–6 mM 2-mercaptoethanol for 10 min at 25°C. After addition of 1.5 μl of 0.5% bromophenol blue–80% glycerol, the reaction mixture was subjected to 2.5% agarose gel electrophoresis in 25 mM Tris–190 mM glycine–1 mM EDTA (pH 8.5) at 2.5 V/cm. Upon fixing and drying of the gel, radioactivities on the gel

TABLE 1. Transformation of *polA* cells with *dl* mutant plasmids

Plasmid	No. (10^5) of transformants/μg of DNA with AMP concn of (μg/ml)[a]:	
	15	30
pKMY292*dl*6	5.8	5.4
pKMY292*dl*6-Δ1	6.0	5.0
pKMY292*dl*6-Δ2	4.6	4.1
pKMY292*dl*6-Δ3	6.9	6.8
pKMY292*dl*6-Δ4	<0.002	NT
pKMY292*dl*6-Δ5	<0.001	NT
pKMY292*dl*6-Δ6	<0.001	NT
pKMY292*dl*6-Δ7	<0.001	NT
pKMY292*dl*6t_{fd}	3.1	3.1
pKMY292*dl*6-Δ3t_{fd}	2.0	1.7
pBR322	<0.001	NT

[a] WA802 *polA* competent cells were transformed with 50 ng of plasmid DNA. Cells were plated on duplicate agar plates supplemented with 15 or 30 μg of AMP per ml. The plates were incubated at 37°C for 18 to 24 h. When JM109 *polA*$^+$ cells were also transformed with the same DNA samples, the frequency of transformation ranged from 3.8×10^5 to 1.9×10^6 transformants per μg of donor DNA. NT, not tested.

TABLE 2. Transformation of *polA* cells with *dr* mutant plasmids

Plasmid	No. (10^5) of transformants/μg of DNA with AMP concn of (μg/ml)[a]:		
	15	30	75
pKMY213*dr*9	NT	11.0	8.6
pKMY213*dr*10	NT	7.8	4.3
pKMY213*dr*11	1.7[b]	0.068[b]	<0.006
pKMY213*dr*12	1.5[b]	0.034[b]	<0.003
pKMY213*dr*13	0.30[b]	0.014[b]	<0.003
pKMY213*dr*14	0.068[b]	0.008[b]	<0.003
pKMY213*dr*15	<0.002	<0.002	NT

[a] WA802*polA* cells were transformed with 40 ng of plasmid DNA and selected on agar plates containing AMP at the indicated concentrations as described in Table 1, footnote *a*. The number of colonies that appeared after incubation at 37°C for 24 h was counted. The frequency of transformation of JM109 *polA*$^+$ with the same DNA samples ranged from 4.7×10^5 to 1.3×10^6 transformants per μg of donor DNA. NT, not tested.

[b] The size of transformant colonies was very heterogeneous or small (less than 1 mm in diameter).

were determined with a Fuji Bio Image Analyzer BA100 (13).

RESULTS

The leftward limit of *ori*. Plasmid pKMY292 is a composite of pBR322 and pSC101 replication systems and requires DNA polymerase I for initiation of replication from the pBR322 origin. Only when the pSC101 replication system is active can the plasmid be maintained in DNA polymerase I-deficient (*polA*) cells. We have previously shown that its derivatives (pKMY292*dl*6 and the others) carrying the region rightward of position 223 retained the replication ability in *polA* cells, while rightward deletions beyond position 252 inactivated it (50). To define more precisely the limits of the DNA site required for pSC101 replication, rightward deletions from position 223 were carried out with BAL 31 nuclease. The mutants obtained were measured for their transformation efficiencies in a *polA* strain (WA802 *polA*). As shown in Table 1, deletions until position 240, which still preserve the DnaA box, had little effect on the replication function. Extension of the deletion until position 248 or further resulted in loss of the replication ability in *polA* cells. These results strongly suggest that the DnaA box is located in the leftmost portion of the region essential for the pSC101 replication system.

External transcription into the origin region affects the replication ability positively or negatively. In the case of pSC101, insertion of Tn*1000* between positions 213 and 214 had no effect on the replication function, but when the segment rightward of position 214 including the whole *rep* gene together with a small portion of Tn*1000* was cloned on pBR322, no plasmid could be maintained in *polA* cells (23). Transcription from a promoter within Tn*1000* was suggested to permit replication of the plasmid integrated with Tn*1000*. To test whether the replication ability in *polA* cells of plasmids used in the present study depended on rightward transcription into *ori*, such as a read-through of transcription for primer RNA of the pBR322 replication system, we inserted the strong transcription terminator, t_{fd}, upstream of position 223 (pKMY292*dl*6) or 241 (pKMY292*dl*6-Δ3) in the correct orientation so that rightward transcription could be efficiently repressed. As shown in Table 1, these plasmids also gave transformants nearly as well in *polA* cells. The discrepancy between our results and previous ones is so far unexplained, but a remaining part of Tn*1000* cloned together with the pSC101 segment into pBR322 may be unfavorable to the plasmid replication.

The rightward limit of *ori*. In order to trim leftward the *ori* region, we used pKMY213A62 as a parental plasmid (49). This plasmid contains the pBR322 replication origin and the sequence from positions 1 to 585 of the pSC101 moiety, resulting in the inability to replicate by itself in *polA* cells. Therefore, to test the origin function (Ori) of leftward-deletion mutants from this plasmid, they were introduced into *polA* cells carrying pMIK6, which supplies the wild-type Rep in *trans* (Table 2). A large number of ampicillin (AMP) (75 μg/ml)-resistant (Amp^r) transformants were obtained with pKMY213*dr*9 and pKMY213*dr*10 DNA. On the other hand, pKMY213*dr*11, which had only one more deleted base pair (position 458) than pKMY213*dr*10 no longer gave rise to transformants in the presence of 75 μg of AMP per ml. However, significant numbers of transformants were obtained at 15 μg of AMP per ml, suggesting that pKMY213*dr*11 is present at a lower copy number. This is supported by the level of single-cell resistance to AMP; that is, the concentration of AMP giving half-survival of *polA* cells carrying both pMIK6 and pKMY213*dr*11 was 16 μg/ml, while that of cells carrying pMIK6 and pKMY213*dr*9 or pKMY213*dr*10 was about 150 μg/ml, as was that of cells carrying pMIK6 and its parent, pKMY213-A62. Similarly, pKMY213*dr*12, pKMY213*dr*13, and pKMY213*dr*14 also gave transformants resistant to the low concentration of AMP at somewhat lower efficiencies than pKMY213*dr*11 did. When leftward deletions extended beyond position 423 (pKMY213*dr*15), transformants were no longer obtained even at 15 μg of AMP per ml. Considering the drastic difference between pKMY213*dr*10 and pKMY213*dr*11 with regard to their Ori activities, it is noteworthy that the IR-1 sequence is present in this region. Sequence comparison (Fig. 3) shows that, at the distal portion of the right half of IR-1, the inserted *Bam*HI linker recovers some correct base pairs in pKMY213*dr*10 but not in pKMY213*dr*11. Therefore, the rightward limit of *ori* is likely related to the intactness of IR-1, which is relevant to Rep binding. In order to test this possibility, the DNA fragments containing positions 434 to 466 of pKMY213*dr*9 (IR-1_{dr9}) and the corresponding posi-

		Ori activity	Rep-binding
wild (*dr*9)	GGACTAGTAATTATCATTGACTAGCCC	+	++
*dr*10	GGACTAGTAATTATCATTGACTgGCCg	+	+
*dr*11	GGACTAGTAATTATCATTGACggcCgg	±	–
*dr*12	GGACTAGTAATTATCATTGAggccgga	±	–

FIG. 3. Nucleotide sequence of deletion mutants at the right border. The capital letters represent the sequence from the wild type and identical bases. The Ori activities and Rep-binding activities are described in the text.

tions of pKMY213*dr*10, pKMY213*dr*11, and pKMY213*dr*12 (IR-1_{dr10}, IR-1_{dr11}, and IR-1_{dr12}, respectively) were prepared and in vitro binding of Rep to them was performed by the DNA mobility shift assay. Complex formation of Rep with natural IR-1 increased as the Rep concentration increased, and the equilibrium dissociation constant, K_d, was 3 to 4 nM as previously determined (data not shown). Relative binding affinities of these DNA fragments for Rep were compared precisely by using these DNAs as competitors for Rep binding to labeled natural IR-1 DNA. Figure 4 shows that formation of the labeled complex was most effectively inhibited by homologous DNA. Also, the IR-1_{dr10} sequence had an affinity for Rep although it was lower than that of IR-1. These data were replotted to linear presentation (5), and the relative K_d (K_d of IR-1_{dr10}/K_d of IR-1_{dr9}) was calculated to be 7.8. This was confirmed by evidence that Rep binding to labeled IR-1_{dr10} DNA was directly observed in the DNA mobility shift assay (data not shown). In contrast, neither the IR-1_{dr11} nor the IR-1_{dr12} sequence had detectable effect on Rep binding to IR-1. The above results suggest strongly that

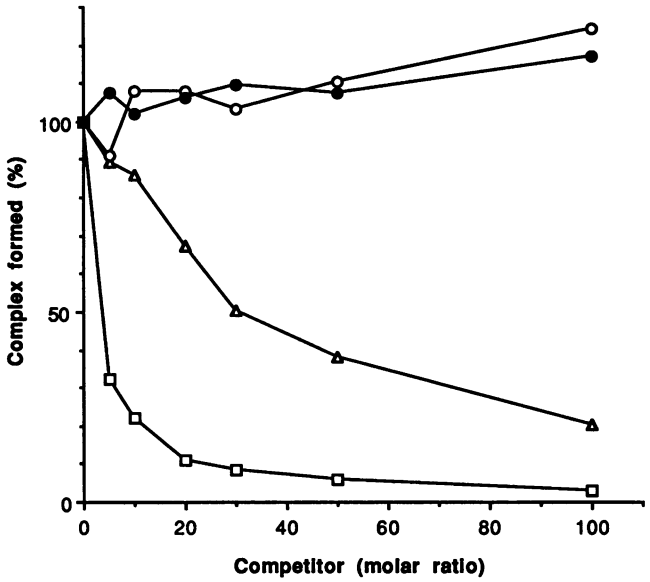

FIG. 4. Competition of Rep binding to IR-1 with various DNA fragments. End-labeled wild-type IR-1 (IR-1_{dr9}) (25 fmol) was incubated with 500 fmol of Rep in the presence of a 5- to 100-fold molar excess of unlabeled IR-1_{dr9} (□), IR-1_{dr10} (△), IR-1_{dr11} (○), or IR-1_{dr12} (●). Upon agarose gel electrophoresis, DNA-protein complex formation was determined as described in Materials and Methods. Under the condition without competitors, approximately 55% of labeled DNA was recovered from the retarded DNA bands.

the Ori$^+$ activity correlates to the Rep-binding affinity of the IR-1 sequence. It is most likely that the rightward limit of *ori* is functional IR-1.

Since we used plasmids lacking the *par* region (28, 39) in the present studies, these were gradually segregated from growing *polA* cells. Manen et al. (25) have shown that deletion of *par* caused a decrease in the plasmid copy number. Either pKMY213*dr*9 or pKMY213*dr*10 with or without *par* gave similar copy numbers, judging from the Amp^r of cells carrying these plasmids (AMP concentrations allowing 50% survival of cells carrying plasmids with and without *par* were 150 and 180 μg/ml, respectively) and Southern blotting analyses (data not shown). Loss of *par* might have different effects on DNA replication in different contexts around the *ori* region. Thus, the present data led us to conclude that the minimal essential region for maintenance of pSC101 at the nearly normal copy number spans from the DnaA box to the IR-1 sequence.

Ori$^-$ mutations within the region downstream of iterons. Deletion analyses indicate that an about 50-bp region downstream of the third iteron is essential for the Ori function. The distal IR-1 is a Rep binding site as described above. Is then the spacer region (positions 414 to 436) between the third iteron and IR-1 necessary for the Ori function? In order to answer this question, we induced single-base substitutions as well as a two-base substitution into the region proximal to the iteron, as described in Materials and Methods. The mutant DNAs were introduced into *polA*/pMIK6 cells for assay of their Ori activities. Transformants were selected at three different concentrations of AMP. Figure 5 shows that point mutations at positions 413 to 419 or 423 to 426 had little or no effect on the Ori function, while those at positions 420 to 422 severely inactivated the function only when the base was substituted by some other specific bases. Especially, mutations of C-420→G, A-421→G, and C-422→T gave rise to remarkably low efficiencies of transformation. When a lower concentration (15 μg/ml) of AMP was used for selection, transformants were obtained at efficiencies about 10^3-fold lower than that of transformation with the wild-type DNA (data not shown). Therefore, these mutants could retain some Ori function and be maintained at lower (one copy or fewer per host chromosome) copy numbers. On the other hand, a mutant, pUC-223/513(Δ382/413), completely missing the third iteron gave no transformant even when 15 μg of AMP per ml was used for selection, suggesting complete loss of the Ori function (see Fig. 6). Although we did not induce mutations into other positions in the spacer region (positions 427 to 436), at least positions 420 to 422, would be required in a sequence-specific fashion for the Ori function. One can notice that a sequence, CCAGCAA, similar to CCACAA (positions 419 to 424) is located in the region downstream of a direct repeat (DR-2) (positions 388 to 394). We introduced single-base changes into positions 389 to 391 (C-389→G or T, A-390→G or T, and G-391→A, C, or T) and found that no detectable change was observed in either transformation efficiencies of *polA*/pMIK6 cells or the level of Amp^r (data not shown). Therefore, the sequence itself of the downstream region of DR-2 would be changeable.

Sequence-specific requirements in the downstream region of DR-3 suggest that it is also a recognition site of some proteins. Recently, we found that purified Rep protein can bind efficiently in vitro to the DR sequence when the downstream region of DR-3 is single stranded, suggesting that a structural change of the downstream region is favorable to Rep binding to the DR sequence (14). It is likely that

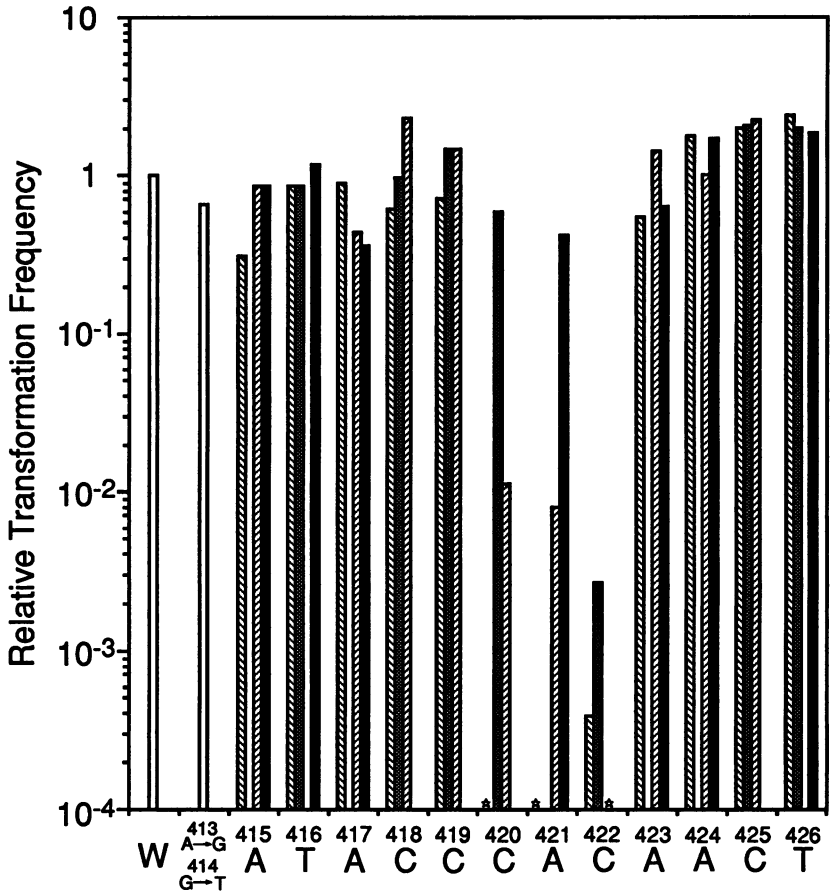

FIG. 5. Ori⁻ mutations induced by single-base substitutions within the downstream region of DR-3. Each nucleotide of positions 413 to 426 shown at bottom was substituted to G (▧), A (▩), T (▨), or C (■). W and $A \xrightarrow{413} G$ $G \xrightarrow{414} T$ mean plasmids pUC-223/513 and pUC-223/513*Sna*BI, respectively. WA802 *polA*/pMIK6 cells were transformed with 100 ng of plasmid DNA. The number of Amp^r (50 μg/ml) transformants was normalized with that of WA802 *polA*$^+$ cells obtained with the same DNA sample. Each bar represents the transformation frequency with each mutant DNA relative to that with pUC-223/513. Stars mean that no transformant was obtained.

the downstream region participates in Rep binding to DR in vivo. So we tested whether these Ori⁻ mutations are suppressed by some mutations within the *rep* gene. We used *rep* copy number mutants. Ori⁻ mutants were introduced into *polA* cells in which mutated Rep proteins were supplied by helper plasmids. Figure 6 shows that every Ori⁻ mutation was suppressed by all three copy number mutations tested since they were efficiently established in these cells. In other words, these suppressor mutations of *rep* are not allele specific for Ori⁻ mutations.

DISCUSSION

We have shown that the DnaA box in the pSC101 *ori* region is required for the origin function, as expected from evidence that functional *dnaA* is essential for its DNA replication (8, 11, 17) and the DnaA protein binds in vitro to the box (15). The DnaA box lies at the leftmost portion of *ori* followed by the A+T-rich region and three 21-bp repeats, iterons. A similar arrangement has been found in several other replicons, such as mini-P1 (1, 44), Rts1 (20), mini-F (29, 30), and R6K-γ origin (27, 34). In these replicons, except for pSC101 and R6K, multiple DnaA boxes are present in one terminal region and a single box match to the consensus sequence is sufficient for the origin function. In the case of Rts1, both DnaA boxes can be deleted with a twofold decrease in the copy number but the deleted mutants still require the DnaA protein for replication (21). These features of DnaA boxes, their number and position, in these plasmid replicons contrast with those in *oriC*, the replication origin of the *E. coli* chromosome, in which four boxes are scattered in the *ori* region and deletion of just one of them inactivates the activity of the origin (6). Therefore, the role of the DnaA protein in these plasmid replicons might be different from its role in *oriC* as previously suggested (44).

Despite similarities in the structural features of the left border of various *ori* regions carrying iterons, the right border of pSC101 *ori*, downstream of the iterons, is unique. Deletion analyses have shown that iterons are located at another end of *ori* in mini-P1 (44), Rts1 (20), and mini-F (30), and the external region of iterons can be removed without loss of the origin function. On the other hand, the pSC101 replicon requires clearly the about 50-bp sequence downstream of the third iteron to maintain the nearly normal copy number. This includes the IR-1 sequence, one of strong binding sites of Rep. The origin activities in mutants with a partial deletion of IR-1 (*dr*10, *dr*11, and *dr*12) correlated with the Rep-binding affinities of their IR-1-like sequences, suggesting that Rep binding to IR-1 is necessary for the origin function. If this is the case, since the mutant pKMY213*dr*10

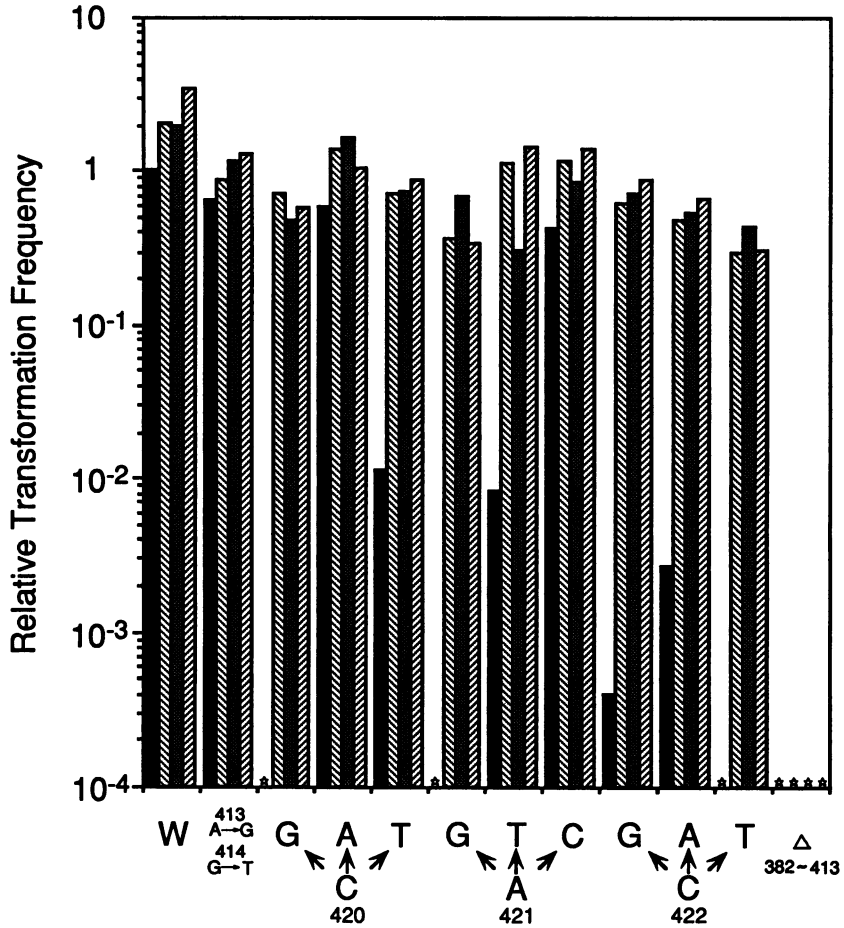

FIG. 6. Suppression of Ori⁻ phenotype with copy number mutations of Rep. Plasmids pUC-223/513 (W), pUC-223/513*Sna*BI ($A \xrightarrow{413} G$ $G \xrightarrow{414} T$), pUC-223/513(Δ382/413) (Δ382~413), and single-base-substituted mutants shown at the bottom were introduced into *polA* cells carrying pMIK6 (*rep*⁺) (■), pMIK-*rep1* (▧), pMIK-*rep21* (▩), or pMIK-*rep28* (▨). The number of Amp^r (50 μg/ml) transformants was normalized as for Fig. 5, and the relative transformation frequency with each plasmid DNA was calculated. Stars mean that no transformant was obtained.

is present at the normal copy number but its IR-1_{dr10} sequence has the significant but lower affinity for Rep than that of natural IR-1 (Fig. 4), Rep binding to IR-1 would not be a limiting factor of the initiation reaction. We have previously reported that the Rep protein binds in vitro strongly to the palindromic sequences IR-1 and IR-2 but a concentration of the protein an order of magnitude higher is required for binding to the iterons (38). When the region downstream, but not upstream, of iterons is single stranded, Rep binds to iterons at lower concentrations (14). This suggests that a conformational change of this region like unwinding of duplex DNA promotes Rep binding to iterons. Therefore, the mode of Rep binding to iterons is likely different from that of binding to IR-1. In the case of P1 replication protein RepA, DnaJ and DnaK proteins convert the dimer form of RepA to the monomer form, resulting in activation of the protein capable of efficient binding to iterons (45). Although purified Rep protein of pSC101 is almost entirely present in the dimer form (38), Manen et al. (26) have recently suggested that the Rep protein of pSC101 also binds to iterons in the monomer form. In these experiments iterons in the absence of IR-1 were tested for binding to Rep. It is plausible that some activation mechanisms of Rep and/or DNA structure of the downstream region are required for the most efficient binding of Rep to iterons. Rep binding to IR-1 might participate in this activation mechanism. Thus, the fact that IR-1 is included in the minimal essential region of the origin function suggests that the Rep protein has the third function in addition to two known functions, the replication initiator through binding to iterons and the autogenous repressor through binding to the *rep* operator (the IR-2 sequence). However, a possibility that IR-1 has another function independent of Rep binding for replication is not ruled out.

The *par* region was first identified as an element which confers stable maintenance of pSC101 in growing cells (28, 39). Thereafter, it was demonstrated that *par* is a preferential binding site for DNA gyrase (43). A recent study showed that deletions in the *par* region have a marked effect on plasmid copy number, that is, on plasmid replication (25). Although in the present study the *par* region had not so remarkable effect on the copy number of pKMY213*dr*9 or pKMY213*dr*10, lack of the *par* region might increase the defect in the Ori function of mutants tested. Even if this is the case, this means that both IR-1 and *par* are necessary for the complete system of the pSC101 replication and a significant role of IR-1 should still be noticed. With respect to this, it is noted that lack of IR-1 (pKMY213*dr*11 to pKMY213

*dr*14) in addition to *par* can still permit replicating at lower frequencies (Table 2). It is plausible that the region from the DnaA box to the downstream region of iterons (positions 241 to 427) comprises a core of *ori* and that both IR-1 and *par* function as enhancer elements for *ori*.

We showed furthermore that some single-base substitutions in the region downstream of iterons were deleterious for the origin function. Specific base changes such as C · G→G · C at 420, A · T→G · C at 421, and C · G→T · A or G · C at 422 have severe effects on replication. It is most likely that this downstream region also is a recognition site of an undetermined protein. Rep is one of the candidates for this binding protein, since copy number mutations of *rep* are able to suppress these Ori$^-$ mutations. These suppressions are, however, non-allele specific, since all three copy number mutations tested can suppress any mutations in CAC. One cannot rule out a possibility that copy number mutations independently recover detriments to replication by the base changes. It was reported that IHF promotes DnaA binding to two regions, positions 377 to 385 (TTACAAGTT) and 409 to 417 (TTACAGATA) (35), although they fit neither the consensus DnaA box TTAT(C/A)CA(C/A)A (15) nor a revised one (T/C)(T/C)(A/T/C)T(A/C)C(A/G)(A/C/T)(A/C) (33). However, one- or two-base substitutions within positions 413 to 417 have no detectable effect on the Ori activity (Fig. 5). This fact argues against functional participation of these weak DnaA boxes in pSC101 replication. With respect to mutations in the downstream region of iterons, McEachern et al. (27) have noted that a consensus sequence, CCACAGGNNNAA, is conserved in regions adjacent to the last iterons in plasmids R6K, R485, pSa, RK2, and Rts1, and the change of the fourth C to T (8 bp downstream of the last iteron) caused great reduction of the origin function of R6K. Thereafter, it was found that the sequence of R6K, CAA CAGGTTGAA, is a binding site of IHF (9). However, these sequences in other replicons are divergent from the consensus sequence of IHF binding, (C/T)AANNNNTTGAT(A/T) (12). A corresponding sequence in pSC101 is CCACAACT CAAA (positions 419 to 430), and CAC is indispensable for replication. No binding of IHF to this element was observed (35). Although whether these elements are essential for other plasmids remains to be solved, they may have a common function in DNA replication. Isolation of suppressors for single-base substitution mutations in this element is worthy for further understanding this element. An alternative explanation for the role of the element CAC is that it plays a structural role. As noted, Rep has a high binding affinity for iterons with the single-stranded downstream region including CAC. This element might affect local stability of DNA helices with or without unknown protein. Phenotypes attributed to point mutations in CAC might be affected by the presence of *par*, which is a DNA gyrase binding site (43). This line of experiments is in progress in our laboratory.

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