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In Vivo and In Vitro Studies of a Copy Number Mutation of the RepA Replication Protein of Plasmid pSC101

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The RepA replication protein of plasmid pSC101 binds as a monomer to three repeated sequences (RS1, RS2, and RS3) in the replication origin of the plasmid to initiate duplication and binds as a dimer to two inversely repeated sequences (IR1 and IR2) in its promoter region (D. Manen, L. C. Upegui-Gonzalez, and L. Caro, Proc. Natl. Acad. Sci. USA 89:8923–8927, 1992). The binding to IR2 autoregulates *repA* transcription (P. Linder, G. Churchward, G. X. Xia, Y. Y. Yu, and L. Caro, J. Mol. Biol. 181:383–393, 1985). A mutation in the protein RepA(cop) that affects a single amino acid increases the plasmid copy number fourfold. In vivo experiments show that, when provided in *trans* under a foreign promoter, the RepA(cop) protein increases the replication of a plasmid containing the origin of replication without *repA*, whereas it decreases the repression of its own promoter. In vitro experiments show that the purified RepA(cop) protein binds more efficiently to the repeated sequences within the origin than does RepA and that its binding to these sequences is more specific than that of RepA. Binding to an inversely repeated sequence within the *repA* promoter gives opposite results: the wild-type protein binds efficiently to that sequence, whereas the mutated protein binds less efficiently and less specifically. Footprint experiments confirmed these results and, in addition, showed a difference in the pattern of protection of the inversely repeated sequences by the mutant protein. Equilibrium binding experiments showed that the formation of protein-probe complexes at increasing concentrations of protein had a sigmoidal shape for binding to RS sequences and a hyperbolic shape for binding to IR sequences. The results, together with earlier work (G.-X. Xia, D. Manen, T. Goebel, P. Linder, G. Churchward, and L. Caro, Mol. Microbiol. 5:631–640, 1991), confirm that the binding of RepA to RS sequences plays a crucial role in the regulation of plasmid replication and that its binding to IR sequences plays a role in the autoregulation of RepA expression. They also demonstrate that the two separate functions of the protein are effected by two different forms of binding to the target sites.

The functions and sites required for the replication of plasmid pSC101 (11) in *Escherichia coli* lie within a 2.2-kb *Hin*cII-*Rsa*I fragment of the plasmid (Fig. 1) (2, 10, 23, 25, 26, 28, 37, 41). The region contains three directly repeated sequences, RS1, RS2, and RS3 and two inversely repeated sequences, IR1 and IR2, partially homologous to the direct repeats. It encodes a 37-kDa, 316-amino-acid-long initiator protein, RepA, which acts at at least two levels: positively, it initiates plasmid replication by binding to the repeated sequences RS1, RS2, and RS3 in the *ori* locus; negatively, it autoregulates its transcription by binding to the palindromic sequences IR1 and IR2 that overlap its promoter (24, 36, 38). In addition, pSC101 replication requires several *E. coli* proteins, in particular IHF (17) and DnaA (14, 19). Both bind to specific sites in the origin region (Fig. 1). Sequences rich in AT bracket the IHF site. The DNA of the *ori* segment bends around the IHF binding site, and the curvature is enhanced by the binding of IHF (34). Interactions between DnaA, IHF, and RepA in their binding reactions to the origin of pSC101 have been demonstrated (33).

Previous work in our laboratory has shown that a point mutation in the *repA* gene, changing amino acid 93 from glutamic acid to lysine in the protein, results in a four- to fivefold increase in plasmid copy number and a 1.7-fold increase in the amount of protein produced, pointing to a dual effect on replication of the plasmid and on autoregulation of the protein (40). Band shift assays performed with crude extracts indicated that the mutant protein RepA(cop) was bound more efficiently than the wild-type protein to repeated sequences in the origin. To verify that the changes were produced by the mutation in the protein rather than by a modification of the origin structure due to the mutation, we have studied its effects in vivo when provided in *trans*. To further elucidate the properties of the mutant and of the wild-type proteins, we have purified them and studied their binding to directly repeated sequences in the origin and to palindromic sequences in the promoter region. We have confirmed that the purified protein binds the repeated sequences more efficiently and the palindromic sequences less efficiently than does the wild-type protein.

MATERIALS AND METHODS

Bacterial strains and plasmids. The plasmids used are described in Table 1. The bacterial strains used were HB101 [*supE44 hsdS20* (r_B^- m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*] (4), XL1-Blue [*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac*/F′ (*proAB*$^+$ *lacI*^{q} *lacZ*ΔM15 Tn*10* (Tet^r)] (7), and BL21(DE3) [*hsdS gal* (μc*Its*857 *ind*1 *S*am7 *nin*5 *lac*UV5 T7 gene *1*)] (35).

Plasmid constructions. The coordinates used throughout the text are those of the origin region of pSC101, as defined in Churchward et al. (10). We obtained from Gordon Churchward a plasmid in which the *repA* gene had been separated from its promoter by the insertion of a *Bam*HI

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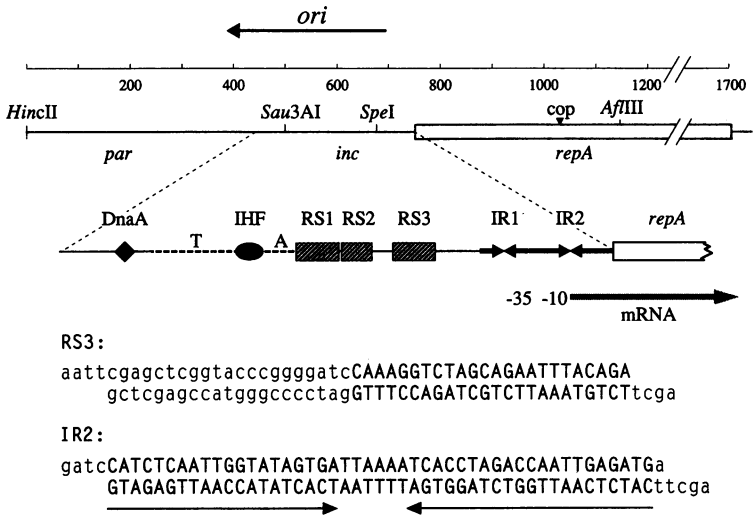

FIG. 1. pSC101 origin region and its relevant features. Coordinates are according to Churchward et al. (10). The sequence of the two major probes used in band shift experiments is shown underneath. Bold capital letters represent pSC101 sequences; lowercase letters represent foreign DNA sequences. The notations T and A on either side of the IHF site indicate that the region to the left of this site is rich in T in the 5'-3' strand (T), and rich in A on the right of the site in the same strand (A).

restriction site (1). From this plasmid, a fragment containing pSC101 sequences between coordinates 750 and 2215 (Fig. 1) was cloned under a T7 promoter in plasmid pET3c, yielding plasmid pET(wt). The site-directed mutagenesis method of Arini et al. (1) was also used to introduce the mutation *repA*(cop) into a similar construction [plasmid pET(cop)].

Both the *repA* and the *repA*(cop) fragments were also cloned under a T5 promoter controlled by *lac* operator sequences and inducible by IPTG (isopropyl-β-D-thiogalactopyranoside) in plasmid pUHE20, kindly provided by H. Bujard (6), yielding plasmids pUHE(wt) and pUHE(cop). In none of the experiments reported here was IPTG induction used for these strains. The residual expression of the genes produced sufficient protein, and it was found that overexpression of RepA was deleterious to the cell.

Plasmid pGB3 is a mini-pSC101 carrying the origin of replication, including the *repA* gene and the various protein binding sites, and resistance to spectinomycin (9). A pGB3(cop) plasmid was constructed by replacing the *Spe*I-*Eco*RI fragment of plasmid pGB3 with the homologous *Spe*I-*Rsa*I fragment of plasmid pLC716-UV45 carrying the *repA*(cop) mutation (40). In another construction, the *repA* gene of pGB3 was deleted by digestion with *Spe*I and *Eco*RI. After filling in of the ends with Klenow enzyme and treatment with ligase, the DNA was introduced by transformation into a strain producing RepA. Colonies containing the plasmid were selected by spectinomycin resistance. The resulting defective plasmid is called pGB3Δ*rep*.

The pXLΩKm plasmid was constructed by cloning the *Spe*I-*Hin*dIII fragment of plasmid 1983-36A (24), containing a fusion *repA-lacZ* gene, into the *Pvu*II site of plasmid pACYC184, to obtain a plasmid compatible with the RepA-producing plasmids. An omega-Km fragment (13, 31) cut from pHP45ΩKm by *Sma*I was inserted into the *Xmn*I site to

TABLE 1. Plasmids

Plasmid	Relevant markers		Replicon	Source or reference
	Resistance markers	Gene and promoter		
pGB3	Spc/Sm	P_{repA}-*repA*$^+$	pSC101	10
716UV45	Amp	P_{repA}-*repA*(cop)$^+$	pSC101	40
pGB3(cop)	Spc/Sm	P_{repA}-*repA*(cop)$^+$	pSC101	This work
pGB3Δ*rep*	Spc/Sm	*repA*	pSC101	This work
pMX3	Amp	P_{repA}-*repA*$^+$	pUC19	40
pMX131	Amp	P_{repA}-*repA*(cop)$^+$	pUC19	40
pLysS	Cm Lys		pACYC184	35
pET3c	Amp		pBR322	35
pET(wt)	Amp	P_{T7}-*repA*$^+$	pBR322	This work
pET(cop)	Amp	P_{T7}-*repA*(cop)$^+$	pBR322	This work
pUHE20	Amp Cm		pBR322	6
pUHE(wt)	Amp Cm	P_{T5}-*repA*$^+$	pBR322	This work
pUHE(cop)	Amp Cm	P_{T5}-*repA*(cop)$^+$	pBR322	This work
1983-36A	Amp	P_{repA}-β-galactosidase	pBR322	24
pXLΩKm	Tc Km	P_{repA}-β-galactosidase	pACYC184	This work
37Y	Amp		pBR322	26
pACYC184	Tc Cm		pACYC184	8

protect the pACYC184 origin region from transcription coming out of the *repA-lacZ* fusion.

Medium. Unless otherwise indicated, cultures were grown in L broth or plated on L agar plates supplemented as required with ampicillin (50 μg/ml), chloramphenicol (25 μg/ml), spectinomycin (100 μg/ml), kanamycin (20 μg/ml), or tetracycline (25 μg/ml).

DNA methods. Enzymes were purchased from commercial suppliers, and reactions were carried out under the recommended conditions. Cloning procedures, transformations into *E. coli*, DNA nick translation, Southern blots, and preparation of plasmid DNA were performed as described by Sambrook et al. (32).

DNA extraction. For each strain, an overnight culture was diluted 1:100 into 10 ml of LB medium supplemented with the appropriate antibiotic and grown to an optical density at 600 nm (OD_{600}) of 0.3. Cells were collected by centrifugation and washed with 10 ml of 0.14 M NaCl. After centrifugation, the cells were suspended in 1.25 ml of 10 mM Tris-HCl (pH 8)–1 mM EDTA–150 μl of RNase A (1 mg/ml)–100 μl of lysozyme (50 mg/ml). Incubation was for 20 min on ice. The sample was gently mixed with 1.4 ml of Sarkosyl mix (0.75% Sarkosyl, 10 mM Tris-HCl [pH 8.0], 10 mM EDTA) and incubated for 8 min at 65°C. RNase A (0.5 ml of a 1-mg/ml solution) was added for 1 h at 37°C, and then 40 μl of proteinase K (10 mg/ml) was added for another hour at 37°C. The samples were extracted twice with a mixture of phenol saturated with Tris-HCl (pH 8), chloroform, and isoamyl alcohol (24:24:1) and once with chloroform-isoamyl alcohol (24:1). After ethanol precipitation, the final pellet was suspended in 200 μl of TE buffer.

β-Galactosidase assay. The β-galactosidase assays were performed by the method of Miller (29). Samples (0.1 ml) of cultures were taken at an OD_{600} of 0.5 and assayed for enzyme activity at 28°C. The values shown are averages of those obtained from two cultures in each single experiment.

Copy number determination. Plasmid copy number was determined by comparing autoradiograms of Southern hybridizations. Total DNA was extracted from either strain HB101 harboring pMX and pGB3Δ*rep* plasmids or XL1-Blue harboring pUHE and pGB3Δ*rep* plasmids. About 4 μg of total DNA was digested with *Pst*I and transferred to nitrocellulose membranes from a 1% agarose gel. Two probes were prepared by nick translation, one for plasmid pGB3 and the other for the chromosomal *nusB* gene, used as an internal control to normalize the assay. Hybridization was carried out as described by Sambrook et al. (32). The autoradiograms were analyzed by densitometry with a Shimadzu dual-wavelength flying spot scanner (model CS-9000), and the relative copy numbers were determined.

Crude protein extracts. The proteins were extracted by sonication by the method of Bétermier et al. (3). The quantity of total protein was measured by the method of Bradford (5).

Protein purification. The *repA* gene (wild type and *cop*) was expressed under the control of a T7 promoter (see above) on plasmid pET3c in strain BL21(DE3) containing plasmid pLysS (35). Production of lysozyme in this strain inactivates residual T7 polymerase produced without induction, thus avoiding unwanted gene expression. When T7 polymerase is induced, this strain produces enough RepA to be used for protein purification. Cells were induced by adding 0.4 mM IPTG to a culture grown to an OD_{550} of 0.5 and then incubated at 37°C for 3 h. The procedure of Arini et al. (1) for RepA purification was followed with a few modifications. Cells were disrupted by sonication by the procedure used for making crude protein extracts (see above). The lysate was adjusted to 1 M NaCl. Cell debris was removed by centrifugation at 45,000 rpm in a Spinco Ti60 rotor for 1 h. The supernatant was dialyzed three times against 50 mM NaCl–buffer A (50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.5], 1 mM EDTA, 10% glycerol, 1 mM β-mercaptoethanol) and then centrifuged at 12,000 rpm for 20 min in a Sorvall SS34 rotor. The pellet was resuspended in 250 mM NaCl–buffer A and loaded on a heparin-agarose column. Protein elution was done with a 0.1 to 0.6 M linear NaCl gradient in buffer A. The fractions were assayed for specific DNA binding and on sodium dodecyl sulfate (SDS) gels. The RepA peak eluted at approximately 0.45 M NaCl. The fractions containing RepA were pooled and dialyzed against 50 mM NaCl; an aggregate was then visible. The protein was collected by centrifugation at 12,000 rpm for 20 min in a Sorvall SS34 rotor and resuspended in 0.5 M NaCl–buffer A. From 2 liters of culture, the yield of wild-type protein was about 1 mg, as determined by the method of Bradford (5).

RepA(cop) was purified similarly, but differences were observed: low-salt precipitation was not as efficient as with wild-type RepA (see Fig. 3B, lane 4), the elution gradient was from 0.1 to 0.7 M NaCl in buffer A, and the mutant protein ran out at about 0.55 M NaCl. After precipitation of the fractions containing the protein, an additional step was necessary to eliminate minor contaminants visible on Coomassie-stained SDS gels; the pellet was dissolved in 2 ml of 0.25 M NaCl–buffer A (the volume is important for correct aggregation of the protein), centrifuged again for 20 min at 12,000 rpm in a Sorvall SS34 rotor, and resuspended in 0.5 M NaCl–buffer A. The yield of RepA(cop) was about 0.25 mg from 2 liters of culture.

Band shift experiments. A 20-μl binding reaction mixture contained either 15 μg of total protein or the specified amount of purified protein, 0.5 μg of calf thymus DNA, and approximately 0.1 pmol of ^{32}P-labeled DNA fragment in buffer containing 10 mM Tris-HCl (pH 8), 11 mM EDTA, 50 μg of bovine serum albumin per ml, 0.1 mM dithiothreitol, 25 mM $MgCl_2$, and 0.2 M NaCl. Binding was carried out at 20°C for 20 min. The bound complex was analyzed by electrophoresis on a 7.5% acrylamide gel. After running at 150 V for 2 h, the gel was dried and subjected to autoradiography. The proportion of labeled DNA retained was measured by densitometry analysis of the bands in autoradiograms as described above.

Equilibrium binding studies. Increasing concentrations of RepA and RepA(cop) proteins were used to bind a fixed amount of labeled RS3 fragments, and the complexes formed were analyzed by the band shift assay under the conditions described above.

DNase I footprinting. The DNase I footprinting method was adapted from that of Galas and Schmitz (16). Approximately 10 ng of a *Sau*3AI-*Afl*III DNA fragment (Fig. 1) containing all the RepA-specific binding sites in the origin of pSC101 was labeled either at the 5′ end with [γ-^{32}P]ATP and polynucleotide kinase or at the 3′ end with [α-^{32}P]ATP and Klenow enzyme. After cleavage with the second enzyme, the end-labeled *Sau*3AI-*Afl*III fragment was purified from a low-melting-point agarose gel and incubated with various amounts of purified RepA protein under the conditions described above for the binding assay. One microliter of freshly diluted DNase I (50 μg/ml) was added to the reaction mix and incubated on ice for 3 min. The reaction was terminated by the addition of 200 μl of a solution containing 100 mM Tris (pH 7.5), 10 μM EDTA, 100 mM NaCl, and

0.1% SDS. Ten microliters of proteinase K (10 mg/ml) was added, and the samples were incubated at 37°C for 30 min and then extracted with phenol-chloroform. DNA was precipitated with ethanol in the presence of 1 μl of yeast tRNA (10 mg/ml). The DNase I cleavage products were separated on a 6% polyacrylamide–7 M urea wedged sequencing gel.

Measurement of dissociation kinetics. To start the dissociation assay at approximately the same concentrations of DNA-protein complexes, the following molar ratios of protein to DNA probe were used: RepA/RS3, 35; RepA(cop)/RS3, 17.5; RepA/IR2, 7.5; and RepA(cop)/IR2, 17.5. These ratios were determined by control experiments that had determined the relative activity of each preparation toward each probe at the concentrations used. After a 20-min binding period, under the conditions described for gel retardation, a 100-fold excess of unlabeled RS3 or IR2 DNA fragments was added to the mixture. Controls (not shown) showed that this amount was sufficient to reduce labeled-DNA binding to nearly zero. Samples were taken at intervals and applied immediately to a running polyacrylamide gel. The concentration of the complexes was measured by densitometry analysis of the retarded bands in the autoradiogram.

Competition experiments. The labeled probes were incubated in competition with increasing amounts, 200 to 1,200 ng, of purified *E. coli* DNA to 4 ng of probe. No calf thymus DNA was used. The experiments were run and analyzed under the conditions described for gel retardation except that the binding buffer contained 50 mM Tris (pH 7.5), 1 mM EDTA, 200 μg of bovine serum albumin per ml, 70 mM KCl, 7 mM $MgCl_2$, 3 mM $CaCl_2$, and 1 mM β-mercaptoethanol.

Computer searches. Homology searches between RepA and other plasmid replication proteins were conducted with the IDEAS sequence analysis package (21); sequence alignments were done with Michael Zuker's SEQALIGN (42).

RESULTS

Effects of RepA and RepA(cop) supplied in *trans* on the replication of a mini-pSC101Δ*repA* plasmid. A defective mini-pSC101 plasmid, pGB3Δ*rep*, was constructed by deletion of *repA* in pGB3 (see Materials and Methods). Since this plasmid is replication defective, it replicates only in cells that contain an independent RepA source. Cells carrying wild-type *repA* or mutant *repA*(cop), under the control of either their own promoter [pMX3(wt) and pMX131(cop)] or a foreign promoter [pUHE(wt) and pUHE(cop)] were used. The efficiency of transformation varied with the nature of the protein. With RepA, it was low with spectinomycin (25 μg/ml); the transformed cells grew poorly, and the colony size was markedly smaller than normal. No transformants were found on 50 or 100 μg of spectinomycin per ml (Table 2). In contrast, the efficiency of transformation of pGB3Δ*rep* in cells carrying a RepA(cop) source was normal; transformants were found at all three concentrations of spectinomycin, and the colony size was much larger than in the presence of RepA. Thus, RepA(cop) furnished in *trans* allows pGB3Δ*rep* to replicate more efficiently than does RepA.

We determined the relative copy number of pGB3Δ*rep* in the presence of RepA and of RepA(cop) by Southern blot analysis (Fig. 2). Two series of determinations were made, one with the pMX plasmids and the other with the pUHE plasmids to provide the proteins. The *nusB* gene of the *E. coli* chromosome served as an internal control. Densitometry of the autoradiograms showed that providing RepA(cop) in *trans* instead of RepA increased the copy number by a factor of 8.8 in the pUHE series and 8.1 in the pMX series (Table 2).

TABLE 2. Effect of RepA and RepA(cop) supplied in *trans* on replication of pGB3Δ*rep*[a]

Resident plasmid	Transformation by pGB3Δ*rep* at spectinomycin concn (μg/ml):			Copy number of pGB3Δ*rep*	
	25	50	100	Relative to pGB3	RepA(cop)/RepA
None	−	−	−		
pMX3(wt)	±	−	−	0.17	
pMX131(cop)	+	+	+	1.38	8.1
pUHE(wt)	±	−	−	0.12	
pUHE(cop)	+	+	+	1.06	8.8

[a] Plasmids pMX3 and pMX131 were used in strain HB101. The pUHE(wt) and pUHE(cop) plasmids were used in strain XL1-Blue, grown in the absence of IPTG. The ± symbol indicates a smaller-than-normal colony size. The relative copy numbers of pGB3Δrep were determined by densitometry of the autoradiograms shown in Fig. 2. For each plasmid, the copy number was estimated relative to the *nusB* chromosomal gene. Then, the ratio to the copy number of a pGB3 plasmid in a culture grown to the same OD was evaluated. The ratio of plasmid copy number obtained with RepA(cop) in *trans* relative to that obtained with RepA was calculated from these values.

Effects of RepA and RepA(cop) on the activity of the *repA* gene promoter. To examine the effect in vivo of the mutation on the activity of the *repA* promoter, a *repA-lacZ* fusion (plasmid pXLΩKm) was introduced into strain XL1-Blue harboring either pUHE(wt) or pUHE(cop). Transcription from the *repA* promoter was monitored by measuring β-galactosidase activity. The results showed that when RepA-

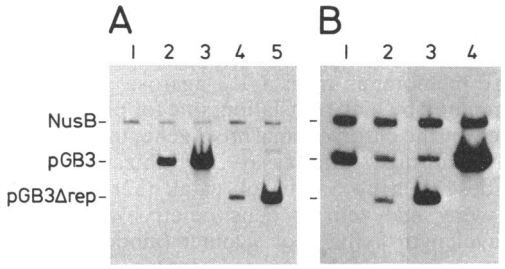

FIG. 2. Southern blot analysis. (A) DNA was extracted from XL1-Blue cells harboring (lane 1) no plasmid, (lane 2) pGB3, (lane 3) pGB3(cop), (lane 4) pGB3Δ*rep* plus pUHE(wt), or (lane 5) pGB3Δ*rep* plus pUHE(cop). The DNA was hybridized with two mixed probes: the Ω fragment (31), which hybridizes with the spectinomycin resistance gene of pGB3 and of pGB3Δ*rep*, and a fragment containing part of the *nusB* gene (22a), which hybridizes with the chromosomal *nusB* gene. Plasmid pUHE(wt) produces RepA, and plasmid pUHE(cop) produces RepA(cop), both under the control of a T5 promoter. (B) DNA was extracted from HB101 cells carrying (lane 1) pGB3, (lane 2) pGB3Δ*rep* plus pMX3, (lane 3) pGB3Δ*rep* plus pMX131, or (lane 4) pGB3(cop). The probes used for hybridization were the same as for panel A. Plasmid pMX3 produces RepA, and plasmid pMX131 produces RepA(cop), both under the control of the *repA* promoter. The middle bands in lanes 4 and 5 of panel A and lanes 2 and 3 of panel B result from an unspecific hybridization to sequences present in both the pMX and pUHE plasmids.

TABLE 3. Effect of RepA and RepA(cop) supplied in *trans* on activity of the *repA* promoter[a]

RepA source	β-Galactosidase activity (Miller units)	Activity (% of control)
None (control)	13,900	100
pUHE(wt)	187	1.3
pUHE(cop)	726	5.2

[a] XL1-Blue cells harboring pUHE(wt) or pUHE(cop) were transformed with pXLΩKm containing a fusion of the *repA* promoter and of the *lacZ* gene. The β-galactosidase activity was measured (29). Both proteins were produced in pUHE plasmids under the control of the T5 promoter without IPTG induction.

(cop) was provided in *trans*, β-galactosidase activity was four times higher than when RepA was supplied (Table 3), suggesting impaired autorepressor activity of the RepA(cop) protein.

Purification of RepA and of RepA(cop) proteins. A simple method for purifying RepA has been developed by Churchward's group (1). It is based on the self-aggregation properties of the protein and involves only two major steps: low-salt precipitation followed by chromatography on a heparin-agarose column. We have followed this procedure with only a few modifications (see Materials and Methods). Figure 3 shows the results of the procedure for the RepA and RepA(cop) proteins. Lane 5 in both panels of Fig. 3 shows, for each protein, the results of an SDS gel after purification. Although, under Coomassie stain, the protein appears to be completely pure, silver staining of the SDS gels showed a small amount of a ca. 16-kDa protein. In glycerol gradients and in gel filtration experiments, this protein appeared with the front of the RepA dimer band, giving rise to the speculation that it might be bound to them. We determined, however, that it is not required for binding to either RS3 or IR2 sequences (data not shown). As noted in Materials and Methods, the behavior of RepA and RepA(cop) during the purification procedure differed slightly.

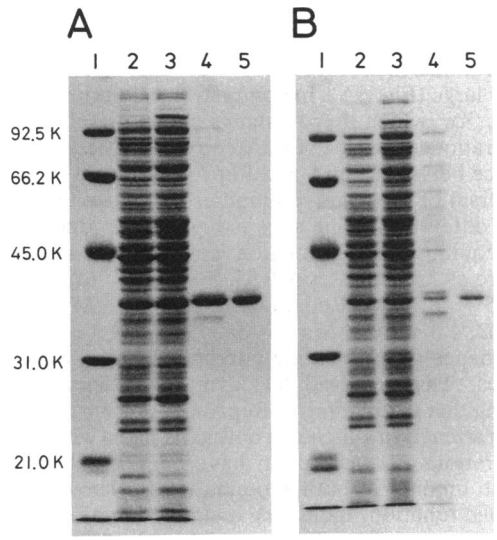

FIG. 3. Purification of proteins. Samples from various purification steps were run on a Coomassie-stained SDS–12.5% polyacrylamide gel. (A) RepA; (B) RepA(cop). Lane 1, molecular size markers; lane 2, noninduced cells; lane 3, induced cells; lane 4, fraction recovered after low-salt precipitation; lane 5, final preparation recovered from heparin-agarose pool. Sizes are shown in kilodaltons.

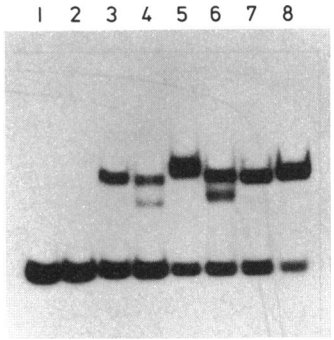

FIG. 4. Band shift assay of RepA proteins binding to the third repeated sequence (RS3). The cell extracts were from strain BL21(DE3)/pLysS with or without a RepA- or RepA(cop)-producing plasmid, as indicated. Lane 1, reaction without protein; lane 2, crude cell extract without RepA; lane 3, crude cell extract with RepA [plasmid pET(wt)]; lane 4, purified RepA protein (230 ng); lane 5, crude cell extract with RepA(cop) [plasmid pET(cop)]; lane 6, purified RepA(cop) (230 ng); lane 7, same as lane 4 plus 3 μl of crude cell extract without RepA; lane 8, same as lane 6 plus 3 μl of crude cell extract without RepA.

Binding of RepA and RepA(cop) to directly repeated sequences. We have demonstrated previously (40), using crude extracts, that the RepA(cop) mutant protein binds more efficiently to the directly repeated sequences than does the wild-type protein. To verify this result, we examined by a band shift assay the binding properties of the purified proteins (Fig. 4). The same concentrations and molar ratios of protein and probe were used in both preparations. The more efficient binding of RepA(cop) than of RepA to the RS3 repeated sequence is clearly seen.

While the interaction of an RS3 probe with a crude cell extract resulted in one retarded band (40), interaction with both purified proteins yielded two retarded bands. The upper band ran at the position found for the unique band observed with crude extracts containing RepA; the other band ran faster (Fig. 4, lanes 4 and 6; Fig. 5, lanes 2 and 4). The relative proportions of the two bands varied and seemed to depend critically on the conditions of the experiment (salt and protein concentrations and other unidentified factors).

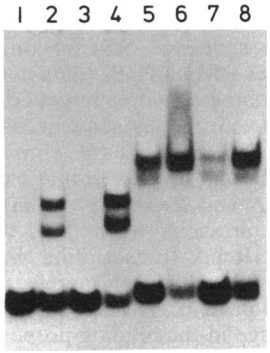

FIG. 5. Band shift assay with purified RepA and RepA(cop) proteins and RS3 (lanes 1 to 4) or IR2 (lanes 5 to 8) as the probe. Lanes 1 and 5, RepA, 23 ng; lanes 2 and 6, RepA, 230 ng; lanes 3 and 7, RepA(cop), 23 ng; lanes 4 and 8, RepA(cop), 230 ng.

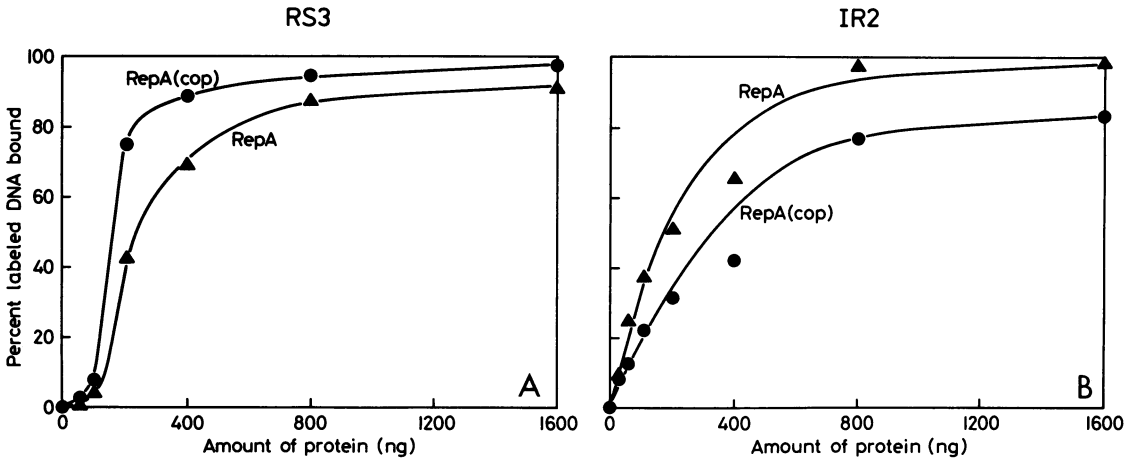

FIG. 6. Binding of RS3 (A) and IR2 (B) DNA fragments by RepA and RepA(cop) at various protein concentrations. Approximately 0.1 pmol of labeled RS3 or IR2 fragments was incubated with increasing amounts of either protein and subjected to a band shift assay. Autoradiograms of the gel were analyzed by densitometry.

Identical results were obtained with a probe containing only the 23 bases of the RS3 sequence without extraneous sequences. We postulated that the lower band resulted from the lack of a factor or a function that is present in crude extracts but limited or absent in the purified protein. Accordingly, we ran band shift assays with a mixture of purified RepA protein and a crude extract from cells without RepA; in this situation, the lower band all but disappeared and was chased to the upper position (Fig. 4, lanes 7 and 8). We also noticed a significant increase in the overall efficiency of binding, as shown by the amount of free probe remaining, as well as a slight shift in the position of the bands for both RepA and RepA(cop). Analysis on SDS gels of the complexes extracted from band shift experiments made with the purified protein alone (data not shown) revealed only the presence of the RepA protein in both bands. Possible factors involved include an undetected small peptide or a nonprotein component. Such a factor(s) could be present in the band and undetected, or it could act on the protein to yield two different configurations with different mobilities in the gel.

Both of the bands observed with the RepA(cop) complex were slightly slower than with the wild-type RepA complex. The reasons for this have not been established but could include a changed DNA-protein interaction between RepA(cop) and its target DNA, resulting in an altered conformation, or the charge difference (+2) caused by the mutation.

Similar results were obtained with a target DNA containing the repeated sequence RS1 or one containing both repeated sequences RS1 and RS2 (data not shown). Identical results were also obtained with a target consisting of the RS3 site only, without extraneous sequences. Footprint experiments (see later) indicate that the entire probe should be protected by the bound protein in that case.

Binding of RepA and RepA(cop) to an inversely repeated sequence. In our previous work (40), we found that the autoregulation of RepA transcription was reduced in the mutant, resulting in an in vivo increase of 1.7-fold in the production of the protein. We had assumed that this effect was due to a change in the binding properties of the protein to the palindromic sequences overlapping its promoter. To test our hypothesis, we used as the probe a synthetic DNA fragment identical with the inversely repeated sequence IR2 (coordinates 692 to 749; Fig. 1) to monitor the binding of RepA and RepA(cop). Comparison of lanes 7 and 5 and of lanes 8 and 6 of Fig. 5 shows that RepA(cop) binds less efficiently than RepA to IR2, confirming our hypothesis. Here also the RepA(cop)-DNA complex shows decreased mobility on the gel. At high protein concentrations, RepA binding to IR2 forms a slow-moving smear above the normal band. This is not seen with RepA(cop). As previously observed by others (36, 38) the wild-type RepA protein binds more strongly under the conditions used to the inverted repeated sequences than to the directly repeated sequences (Fig. 5). A double band is observed with IR2 as with RS3, but it is less pronounced under the conditions used than that formed with RS3.

Binding of the target DNA to increasing concentrations of protein. We studied by band shift assay the binding of a fixed amount of the RS3 or IR2 DNA fragments by increasing concentrations of purified RepA or RepA(cop) (Fig. 6). The results indicate that RepA(cop) binds the RS3 site at lower concentrations than does RepA, although the difference is not very large (Fig. 6A). In contrast, the situation is reversed for IR2, for which RepA shows more attachment at all concentrations than does RepA(cop) (Fig. 6B). Although the difference between RepA and RepA(cop) is not large either, the reversal of the relative affinities of the two proteins for the two different binding sites is highly reproducible.

The equilibrium dissociation constants estimated from Fig. 6 are as follows: RepA/RS3, 3.8×10^{-7} M; RepA(cop)/RS3, 2.5×10^{-7} M; RepA/IR2, 2.2×10^{-7} M; and RepA(cop)/IR2, 4.3×10^{-7} M.

The shapes of the binding curves differ strikingly for the two sites. That for RS3 has a marked sigmoidal shape, indicating that the binding of RepA and of RepA(cop) to RS3 relies on a reaction which can only take place when sufficient concentrations of the protein have been reached. In contrast, the binding of both proteins to IR2 shows a straight hyperbolic function, indicating a simple reaction.

DNase I footprinting. To better define the different binding behaviors of RepA(cop) and RepA, we performed comparative DNase I footprinting experiments (16). The 647-bp-long *Sau*3AI-*Afl*III fragment (see Materials and Methods) carrying all of the RepA binding sites in the origin of pSC101 was labeled at either the 5′ end of the upper strand or the 3′ end of the lower strand and bound to various amounts of

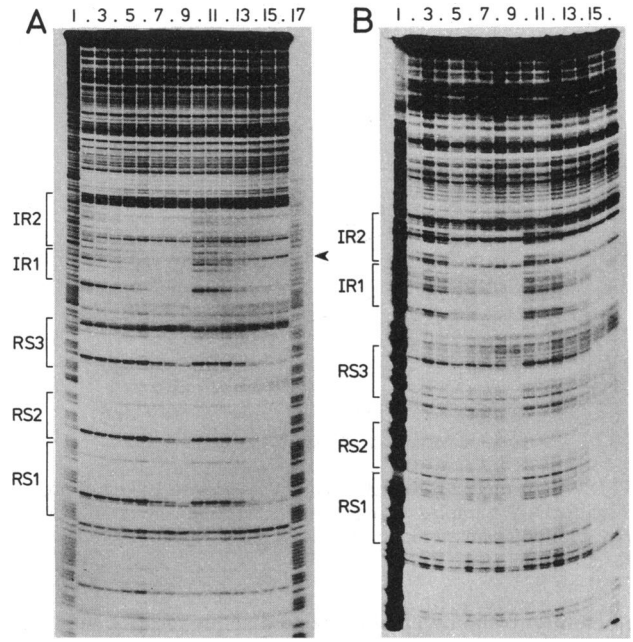

FIG. 7. DNase I footprinting experiments. (A) The DNA fragment was labeled at the 5′ end of its upper strand. (B) The DNA fragment was labeled at the 3′ end of its lower strand. Lanes 1 and 17, A+G cleavage reaction of the fragment; lane 2, reaction without protein; lanes 3 to 9, reaction with increasing concentrations of RepA from 5, 10, 25, 50, 100, 230, to 460 ng, respectively; lanes 10 to 16, reaction with increasing concentration of RepA(cop) from 5, 10, 25, 50, 100, 230, to 460 ng, respectively. The arrowhead shows the enhanced cleavage at coordinates 701 and 702.

purified RepA proteins. Figure 7A shows the footprint for the upper-strand DNA, and Fig. 7B shows the footprint for the lower strand. Although the protection patterns for the two proteins were similar, an increased affinity of RepA(cop) for the repeated sequences within the origin was observed; less than half as much protein was required to reach the level of protection obtained with RepA (Fig. 7A, lanes 9 and 14; Fig. 7B, lanes 9 and 15).

In addition, a conspicuous change in the protection pattern of RepA(cop) for the promoter sequences was found. At coordinates 701 and 702, the end of the right arm of IR1, at the junction between IR1 and IR2, a strongly enhanced cleavage site was observed on the upper strand when the DNA was protected by RepA(cop) but was not seen when it was protected by RepA (Fig. 7A, arrowhead).

Kinetics of RepA dissociation. To determine whether differences in the binding properties of the wild-type and mutant proteins could be detected independently of the relative concentrations of various forms of the protein, we measured the kinetics of RepA protein dissociation from its target DNA with the RS3 or IR2 DNA fragment as a probe. In these experiments, 4 ng of probe DNA was first reacted with the protein. Then, a 100-fold excess of cold probe DNA was added, and samples taken at various times after this chase were analyzed in a gel retardation assay (15). The experiments shown in Fig. 8 showed that RepA(cop) and RepA have nearly the same dissociation kinetics when bound to an RS3 target (Fig. 8A). Similar results were obtained for their binding to IR2 (Fig. 8B). In both cases the dissociation was rapid, the 50% level being reached in a little more than 1 min for RS3 and a little less than 1 min for IR2.

Competition with *E. coli* DNA. To establish the possible effect of the mutation on the specificity of binding, we performed competition experiments in which 4 ng of probe DNA and various amounts of *E. coli* DNA were reacted with a fixed amount of protein. The results appear in Fig. 9. The results indicate that RepA(cop) binds to RS3 with a higher specificity than does RepA and that it binds to IR2 with a lower specificity. The specificities of the binding of RepA to RS3 and to IR2 seem to be similar.

DISCUSSION

In a previous publication (40), we described the isolation and partial characterization of a copy number mutant of plasmid pSC101. The copy number phenotype was associated with a point mutation in the *repA* gene of the plasmid,

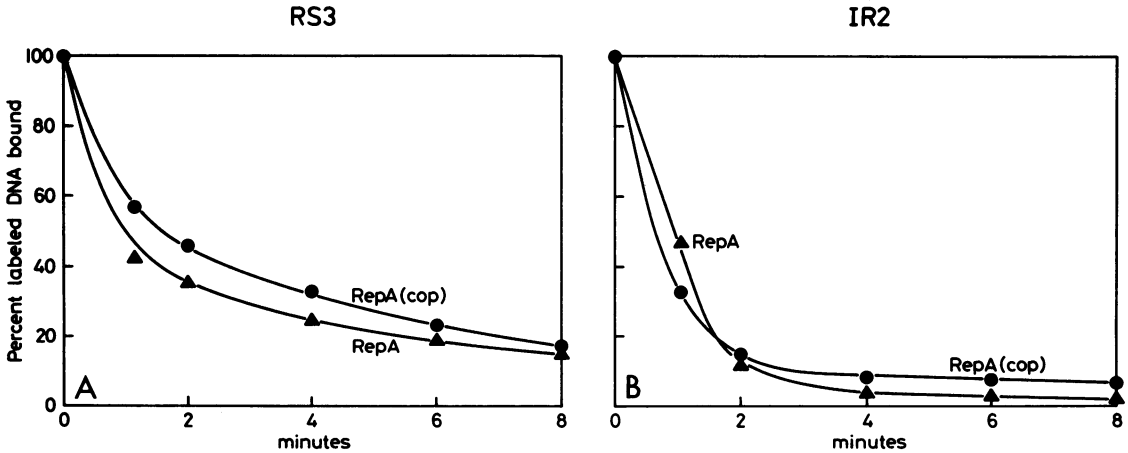

FIG. 8. Measurement of dissociation kinetics of RepA and RepA(cop). RepA-RS3 (A) and RepA-IR2 (B) complexes were formed as described in Materials and Methods, with either form of the protein. After a 20-min incubation at 20°C, an aliquot was withdrawn and loaded onto a running gel; a 100-fold excess of unlabeled RS3 fragment (A) or of unlabeled IR2 fragment (B) was then added. Samples were taken at 2, 4, 6, 8, and 10 min. The retarded bands in the autoradiograms were measured by densitometry, and the values were plotted against reaction time.

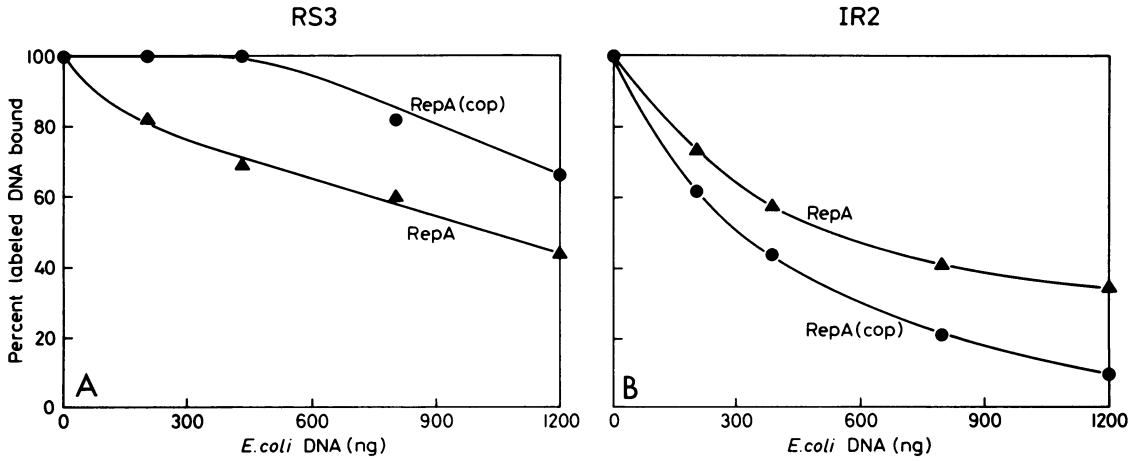

FIG. 9. Competition with *E. coli* DNA. Approximately 0.1 pmol of RS3 DNA (A) or IR2 DNA (B) was incubated with 100 ng of protein, either RepA or RepA(cop), in the presence of 0, 0.2, 0.4, 0.8, or 1.2 μg of *E. coli* DNA. Samples were taken after 20 min. The retarded bands in the autoradiograms were measured by densitometry, and the values were plotted against reaction time.

changing amino acid 93 of the protein from glutamic acid to lysine. We showed that the mutation produced two distinct effects: it reduced repression of the transcription of the gene, resulting in a 1.7-fold increase in mRNA and in protein production, and it led to an increase in the binding of protein from crude extracts to DNA fragments containing one or several of the three directly repeated sequences present in the origin of replication of the plasmid. The use of crude extracts in our binding assays and the multiplicity of interactions of the RepA protein with the origin of replication gave rise to several uncertainties, some of which have been resolved here.

Recent results in our laboratory have demonstrated that RepA proteins bind to the repeated sequences in the origin as monomers and to its promoter sequence as dimers, that the monomer form of RepA does not bind significantly to the promoter sequences, and that the dimer form does not bind to the directly repeated sequences in the origin (27). Gordon Churchward's group has also demonstrated the existence of two forms of the RepA protein with different specificities of binding (personal communication).

The in vivo studies presented in the Results section show that a plasmid containing a fragment of pSC101 deleted for *repA* and its promoter region replicates only poorly when the RepA protein is provided in *trans* under the control of either its own promoter or a foreign promoter, but that it replicates quite efficiently when the RepA(cop) protein is similarly provided. This confirms the high initiation potential of the RepA(cop) protein and rules out a *cis* effect of the mutation. The studies also show that the *repA* promoter is less strongly repressed by the mutant protein provided in *trans* than by the wild type. Here again, *cis* effects appear to be ruled out.

These seemingly paradoxical results, indicating a differential effect of the mutation on the interaction of the protein with origin sites that have sequence homologies with one another, led us to investigate these interactions in vitro with purified protein.

Using purified RepA and RepA(cop) protein, thus avoiding interference with other DNA-binding proteins found in crude extracts, and band shift assays involving probes constituted by a single binding site, we confirmed our previous conclusions. RepA(cop) binds more efficiently to RS sequences than does RepA (Fig. 4), and it binds less efficiently to the inversely repeated sequence IR2 than does the wild-type protein (Fig. 5). The paradox mentioned above is thus confirmed; a point mutation in the RepA protein results in its increased binding to one type of site and in its decreased binding to the other.

Studies in which various amounts of protein were bound, at equilibrium, to a fixed amount of probe DNA confirmed these results (Fig. 6). Although the differences in binding were not large, they were reproducible in several repeats of these experiments under various conditions. At all protein concentrations, the amount of RS3 bound by RepA(cop) was greater than that bound by RepA (Fig. 6A). The reverse was true for IR2 (Fig. 6B). The equilibrium constants calculated from these curves have only a relative value; because of the presence of a small but undetermined amount of contaminant, the protein concentration could not be evaluated very precisely, and it was not possible to ascertain in each case the proportion of dimers and monomers of RepA in the preparation (each form being specific for one target type).

The footprint experiments shown in Fig. 7 also confirmed our conclusions on the nature of the *cop* mutation. Quantitatively, they showed that RepA(cop) protected the RS sequences at a lower concentration than did RepA. The appearance in the RepA(cop) footprint of an enhanced DNase I cleavage site not seen in the RepA footprint is an additional indication of a difference in the interactions of the two proteins with their DNA target. The site of this singularity, between bp 701 and 702, is at the junction between the two sets of inverse repeats IR1 and IR2. It seems, thus, that the *cop* mutation produces an altered conformation of the protein-DNA complex in the IR1-IR2 region. Although we have found (data not shown) that the RS2 sequence alone does not bind RepA unless very high concentrations are used, the data in Fig. 7 and those of Vocke and Bastia (37) and of Sugiura et al. (36) show that it is protected at roughly the same protein concentrations as RS1 and RS3. This suggests a cooperative binding of the protein at the adjacent RS sites. Perri et al. (30) have noted such cooperative effects in the binding of the TrfA-33 protein of plasmid RK2 to a group of five repeated sequences in the origin of replication. They observed that binding to one single sequence was highly unstable but that stable binding was achieved when the target DNA contained two or five repeated sequences.

Finally, competition experiments (Fig. 9) in which a fixed amount of probe DNA and of protein were reacted in the presence of increasing amounts of *E. coli* DNA showed that the RepA(cop)-RS3 reaction was more specific than the RepA-RS3 reaction (i.e., less sensitive to competition), while the reverse was true for the IR2 target. A feature of both the equilibrium binding experiments and the competition experiments is the reversal of the effect of the mutation on the binding to the two types of targets.

The observed differences in the binding of wild-type and mutant proteins to their targets could be due to a variety of reasons: altered binding affinity, altered dimerization properties affecting the proportion between dimers and monomers in the purified protein preparation and in crude extracts, and altered aggregation properties releasing a higher proportion of the mutant in active form. In view of all the previous observations, the results in Fig. 8, in which the dissociation of protein previously bound to probe DNA was measured in the presence of an excess of cold probe DNA, are surprising because little difference was found for either probe in the behavior of RepA and RepA(cop). The differences in the binding constant might be due to different association rates. It is also possible that differences in the dissociation rates cannot be detected by the band shift assay, which is somewhat unreliable for studying effects during very short time intervals. In the absence of information on the relative proportions of dimers and monomers in the various preparations, we could only measure apparent binding constants. It is certainly possible that the mutation affects the dimerization properties of the protein and thus the relative quantities of dimers and monomers.

The shape of the equilibrium binding curves was surprising; the binding of RepA and RepA(cop) monomers to RS sequences had a distinct and reproducible sigmoidal shape, indicating that some concentration-dependent interaction must take place before binding is accomplished. The nature of that putative interaction is at present puzzling. The usual interpretation of such a shape is that binding requires the formation of a protein dimer or a higher multimer from monomeric forms. Yet earlier experiments (27) performed with crude cell extracts had given strong indications that the protein binding to the RS fragment was in the monomeric form. Band shift experiments with crude extracts and with purified protein showed an identical position for the major band of RS3-protein complexes in both cases (Fig. 4). This indicated that the protein was in the same state, presumably monomeric, for both. Another possibility to explain the sigmoidal shape of the curve is that RepA interacts with another protein present in the purified preparation. We have found (data not shown) that the only other protein component present in the purified RepA preparation, the 16-kDa protein, is not present in the binding complex. In fact, no protein other than RepA could be detected in the shifted DNA bands. It is still possible that the 16-kDa protein or a nonprotein component is involved or that formation of a RepA dimer or multimer is needed before attachment, although the final result seems to be the binding of a monomer to a single site. The possibility that the shape of the curve is due to a strong interaction with some site present on the carrier DNA seems to be ruled out by the fact that a similar curve was obtained with poly(dI-dC) as the carrier instead of calf thymus DNA. In contrast to the results for RS3, the curve for binding to the IR sequence, requiring dimers of the protein, is hyperbolic, indicating a direct reaction. This could be explained by the fact that the purified protein consists mainly of dimers, which therefore do not need to be assembled before binding.

Several authors have remarked that RepA binds at lower concentrations to its promoter region than to the repeated sequences (27, 36, 38), an observation confirmed by the footprint experiments of Fig. 7. Yet the equilibrium binding constants calculated from the data in Fig. 6 do not show a striking difference, and the dissociation experiments of Fig. 8 show, if anything, that binding to IR is more unstable than binding to RS. It would seem, in fact, that the major difference in the binding resides in the shapes of the binding curves. At low protein concentrations, because of the sigmoidal shape of the binding curve, binding to RS is strongly reduced relative to binding to IR. A complicating factor in interpreting footprint and other binding experiments is that two different forms of the protein bind to the two types of targets: monomers of RepA bind to RS, dimers bind to IR sequences. The dimer form is predominant in the purified protein and is quite stable (36; our unpublished observations). The balance between the two forms will affect differential binding to the two target types. This balance is, however, not stable and will vary with the age of the protein preparation and the state of dilution of the protein prior to the experiment. Hence, there is great variability in the results. The only valid results are those which present relative observations for the same lot of protein at the same time on the two different targets.

It appears likely that RepA(cop) undergoes a conformation change relative to RepA for the following reasons: (i) during the purification procedure, which depends on the aggregation of RepA protein under low-salt conditions, we found that RepA(cop) aggregates less well than RepA, resulting in a fourfold-lower yield; (ii) RepA(cop) eluted from the heparin-agarose column at an NaCl concentration of approximately 0.55 M rather than 0.45 M for RepA; and (iii) a slightly lower mobility of the RepA(cop)-DNA complexes than of the RepA complexes was observed in band shift assays; the smear seen in the band shifting of IR2 by RepA (Fig. 5, lane 6) is probably due to aggregation of free protein to the IR2-RepA dimer complex. No such smear is seen with RepA(cop), indicating again a possible modification of its aggregation properties. A double role of the same small region in binding and in overall conformation is certainly conceivable, as pointed out by Wickner et al. (39), who have proposed an overlapping region of binding and dimerizing in the RepA protein of plasmid P1.

In a different approach to studying the relations between RepA binding to the RS sites and plasmid replication, Arini et al. (1) isolated mutations within each of the three RS binding sites. They found that mutations that reduced the binding of purified RepA protein to the three sites also resulted in reduced copy number. The effect was particularly severe for a mutation within RS1 which also impaired plasmid stability. Similarly, a mutation at bp 705, in the right arm of IR2, increased the level of transcription from the *repA* promoter, showing the importance of that site in the autoregulation of *repA* (24).

Other copy number mutations have been isolated in plasmid pSC101 (2, 18, 20, 40). As shown in Fig. 10, they present the remarkable property of being grouped within a 24-bp region, seven of eight being within 5 bp of one another. The RepE replication protein of the mini-F plasmid presents important sequence homologies with pSC101 RepA. Many copy number mutations in that protein, compiled by Kawasaki et al. (22), map within a 40-bp region that is strongly homologous to the mutated region of RepA mentioned above

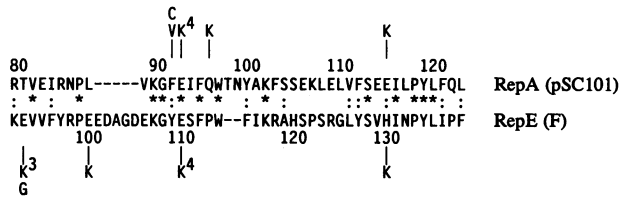

FIG. 10. Location of copy number mutations on the RepA protein of pSC101 and on the RepE protein of F. A partial sequence of each of the two proteins is shown. The amino acids changed by the mutations are indicated by a vertical bar, and the resulting amino acids are shown above or below. The exponents give the number of independent occurrences of a given mutation. Sequence homologies are marked: *, identity; :, conservative substitution. The figure shows only the relevant portion of an alignment between the entire sequences.

(Fig. 10). For both pSC101 RepA and mini-F RepE, the most common mutation is a change from glutamic acid to lysine. Because of the dual role of the protein in self-regulation and in replication, the constraints placed on possible copy number mutations are strong for both pSC101 and mini-F. These constraints may account for the grouping of the mutations. The TrfA replication protein of plasmid RK2 also presents some sequence homology with RepA. The homology, although weaker than that with the RepE protein, is nevertheless significant. Six copy number mutations have been mapped in a 24-amino-acid region of TrfA (12). This region is also aligned with the region of RepA shown in Fig. 10 (data not shown). In all these cases, the tightness of that grouping may well indicate that the region concerned interacts direct with the DNA sites. All the mutations included in this survey were selected as copy number mutations or as revertants of temperature-sensitive mutants of the protein. The method of selection might have affected the results, and it is conceivable that other types of replication mutations might yield different results.

The results, together with earlier work (40), show that the high copy number resulting from the *cop* mutation of RepA is correlated with an increased binding of the mutated protein to the directly repeated sequences in the origin, while the effect of the mutation in increasing the rate of production of RepA is correlated with decreased binding to the inverted sequences in the *repA* promoter. Part of the effect may be due to a differential effect of the mutation on monomer binding to RS sequences and on dimer binding to IR sequences. We have not, however, ruled out an effect on the balance between dimers and monomers in the mutated protein preparations. We think, in fact, that such an effect is probable.

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