Requirements for and Regulation of Origin Opening of Plasmid P1*

(Received for publication, April 2, 1998, and in revised form, June 28, 1998)

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Origin opening is essential for the initiation of DNA replication in the theta mode and requires binding of initiator proteins. Using reactivity to KMnO₄ in vivo as an assay, we find that, like initiation, origin opening of the Escherichia coli plasmid P1 requires the host initiators DnaA and HU and the plasmid-encoded initiator RepA. The ability to detect opening at the P1ori in vivo allowed us to study this activity at various copy numbers in chimeric replicons. The opening was prevented when the P1ori was cloned in high copy vectors or when excess RepA binding sites (iterons) were provided in trans. However, when RepA supply was also increased, the opening was efficient. A further increase in RepA prevented opening. Replication of an incoming P1 under these conditions correlated with opening. These results demonstrate that initiation is possible even at abnormally high origin concentrations and that oversupply of RepA, relative to iterons, can prevent replication by blocking origin opening. It appears that plasmid overreplication can be prevented either by limiting RepA or by accumulating RepA at a rate higher than that of the origin.

Initiation of DNA replication has been staged into discrete steps primarily from the work in vitro on plasmids carrying the Escherichia coli origin, oriC. Binding of initiators first opens the origin, and the opening provides the stage for the DnaC-mediated loading of the DnaB helicase (1). In the absence of the DnaB-DnaC complex, a stable open state of the origin could be obtained in vitro. Using a dnaC(ts) mutant host at the nonpermissive temperature, a stable open structure could also be obtained in vivo as assayed by reactivity to KMnO₄ (2). Reactivity was lost rapidly when the culture was shifted down to permissive temperature. It appears that once DnaB is loaded, the subsequent steps of priming and elongation can proceed rapidly and are unlikely to be the steps for controlling initiation frequency.

We have examined whether origin opening of plasmid P1 can be used to follow regulation of initiation in vitro. Plasmid P1 belongs to a family of plasmids characterized by the presence of short repeating sequences, called iterons (3). The iterons are binding sites for the plasmid-encoded initiator, RepA. Iterons cover about half of the minimal origin (ori) of P1 and also constitute the control locus, incC (see Fig. 1). The incC locus can be deleted, and such plasmids are maintained at an 8-fold higher copy number. The locus therefore plays only a negative regulatory role in plasmid replication. The origin iterons (called incC; see Fig. 1) are essential for initiation and are believed to be important for the control of copy number as well. The incC locus also includes the promoter of the initiator gene. Consequently, RepA binding to incC results in efficient repression of the RepA promoter. Autoregulated initiator synthesis is generally a conserved feature of iteron-carrying plasmids, implying that maintenance of initiator concentration is critical to the copy number control process.

We find that the requirements for the appearance of KMnO₄-reactive bases in P1ori are well correlated with the genetic determinants of P1 plasmid replication. Two host proteins, DnaA and HU (4, 5), and P1 RepA (6) are essential for initiation of P1 replication, and excess iterons or excess RepA inhibit P1 replication (7–9). These characteristics of plasmid replication were found to be also true for the opening reaction, validating the use of the opening assay to study initiation control.

We show that normally inhibitory concentrations of cloned origins or iterons were tolerated both for opening as well as replication, provided RepA concentration was correspondingly high. Further increases of RepA can be inhibitory. Thus, under the conditions of our experiments, the inhibitory activities of iterons and RepA depend upon their relative concentrations.

Experimental Procedures

Bacteria, Plasmids, and Phages—Bacteria and their relevant genotypes were DH5α, recA (10); PC2, dnaC2 (11); E3H827, dnaA (4); BR4587, hupA16 (12); BR4586, hupB11 (12); and BR4588, hupA16hupB11 (this study).

Plasmids supplying various amounts of RepA were pALA197 (3), pALA198, pALA646 (40), and pALA196 (40). Initially, repA was cloned in pBR322 under the control of a constitutive promoter bla-p2 (13). The resultant plasmid, pALA162, produced 40× more protein than the wild type P1 plasmid as determined by Western blotting. RepA production was reduced to 7× and 3× by interposing a transcription terminator (T1) in two orientations between the gene and the promoter. Finally, fragments containing the repA region from T1bla-p2 region were cloned into a pBR322 compatible vector, pST52 (14) to generate plasmids pALA169, pALA198, and pALA197.

Another 7× RepA source was pKP116, constructed by cloning a RepA-EcoRI fragment of pALA176 (15) into the HincII site of pGB2, a pSC101-derived vector with a spectinomycin resistance gene (16). The source of AP protein was pRML75 (pBR322 + λI577p15P) (17).

Plasmids carrying various cis elements were: pALA18 (pBR322 + incA), P1 coordinates 1505–1811 (15), pALA646 (pUC19 + a FlincC carrying fragment, P1 coordinates 405–610) (18), pALAB58 (pUC19 + P1ori, P1 coordinates 386–1000) (18), pISP102 (miniP1 containing P1ori + repA, P1 coordinates 406–1565) (19), and a vector control, pPF155 (pUC19 + a 30-base pair insert at the EcoRI site; the presence of the insert was incidental). An additional set of plasmids were constructed: pKP104 (pACYC184 + P1ori, the P1 fragment (coordinates 280–1000) was from pKLM370 (20) and was cloned at the EcoRI site of pACYC184), pKP124 (pACYC184 + incC, the P1 fragment (coordinates 406–610) was from pALA646 (18) and was cloned at the EcoRI site of pACYC184), and pKP126 (pACYC184 with the EcoRI site destroyed by end filling and used as a vector control).

Plagues were AΔDKC234-AP145R857Kan”, carrying the entire P1rep + par region, and AΔDKC274-AP1oriS857Kan” (21). The control plagues, AΔDKC275, was isogenic to AΔDKC274 except that the P1ori was replaced with incC.

The bacterial cultures were grown in M9 medium (22) supplemented with 0.2% Casamino acids, 0.002% thymine, and 0.001% vitamin B1. When desired, 100 μg/ml ampicillin, 100 μg/ml tetracycline, 5 μg/ml chloramphenicol, and 50 μg/ml kanamycin were added as necessary.

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20 μg/ml chloramphenicol, 30 μg/ml kanamycin, 15 μg/ml tetracyclin, and 40 μg/ml (400 μg/ml for PC2) spectinomycin were added to the medium in various combinations. Fresh overnight cultures were diluted 100-fold in the same medium and grown at 30 °C to an OD of 0.3. The culture was distributed into 10-ml aliquots. One set was maintained at 30 °C as controls, while the other set was shifted to 42 °C for inactivation of DnaC or induction of the AP protein. The induction was at 38 °C for 5 h, because its growth reduces above 37 °C. Incubations were for 1 h. When desired, rifampicin (Rif) was added to a final concentration of 0.1 mg/ml. 5 min prior to KmO4 treatment. KmO4 was diluted to a final concentration of 3 mm and incubated for 1 min at 42 °C for all cells. A 0.37 M KmO4 stock solution was made in water by boiling for 5 min and used up to a month.) The reaction was terminated by mixing the culture with an equal volume (10 ml) of ice-cold STE buffer (100 mM NaCl, 10 mM Tris, pH 8.0, and 1 mM EDTA) with 5 mM dithiothreitol and chilling the mixture on ice. The cells were pelleted at 3000 rpm in a GLC-4 centrifuge (Sorval) at 4 °C for 15 min, and the pellet was washed with 1 ml of 50 mM Tris buffer, pH 8.0, and resuspended in 100 μl of TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA). Plasmid DNA was isolated using a INSTA-MINI-PREP kit (5 Prime ~ 3 Prime, Inc., Boulder, CO) and used for polymerase chain reaction without further purification.

**Primer Extension**—The primer for the bottom strand, KP2 (5'-GG-GCGATGAGCTTAAATGC-3'), corresponded to the P1 coordinates 301–319. For the top strand, KP4 5' -CGCTGGAATGATCAGGG-3'), corresponded to P1 coordinates 710–692. They were end-labeled with [γ-32P]ATP. Primer extension was done in a thermocycler using sequencing grade Taq DNA polymerase (Promega). Reaction mixtures were consisted of 50 mM Tris, pH 9.0, 2 mM MgCl2, and 50 μM each of all four dNTPs. Reactions were terminated by adding 0.5 volume of a stop solution (10 mM NaOH, 95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanole) and heating to 95 °C for 5 min. Primer extension products were analyzed by electrophoresis in a 6% polyacrylamide gel containing 8 X urea followed by autoradiography.

**Lysogeny**—Freshly saturated cultures grown as described for KmO4 footprinting were simultaneously titered for viable counts and used for lysogeny. Typically, 100 μl of cells were mixed with phage at a multiplicity of infection of ≈ 10. After 30 min of incubation at room temperature, 0.5 ml of L broth was added to each tube. The mixtures were incubated at 30 °C for 2 h for expression of drug resistance and, after appropriate dilution, plated on media selective for resident plasmids and infecting phage (plasmid prophage).

**RESULTS**

**Stabilization of P1 Origin Opening by Blocking of DnaB Loading**—In exponentially growing cells, KmO4 reactivity of P1ori was found to be minimal, probably because only a small fraction of the plasmids were undergoing replication initiation at the time of KmO4 probing. To accumulate plasmids at the initiation step, an E. coli host with a dnaC(ts) mutation was used at the nonpermissive temperature. Inactivation of DnaC initiated from another DnaA-independent vector, pST52 (14), at 30 °C, allowed KmO4 to catch opening in other hosts. An alternative method of blocking DnaB loading was devised. The method utilized the KmO4 reaction to validate the KmO4 reactive patch. The signal was made (4). These experiments also revealed that the KmO4 reactive sites are shown by vertical arrows. Their heights represent the strength of the reaction. The KmO4 activity of P1ori is also present in E. coli origin, oriC. The positions of KmO4 reactive sites are shown by vertical arrows. Their heights represent the strength of the reaction. The KmO4 data are from Ref. 2. The P1 coordinates are from GenBank™ (R02380).

To assay P1ori opening in other hosts, an alternative method of blocking DnaB loading was devised. The method utilized the strong DnaB binding property of the bacteriophage λ protein P (17). The AP protein was supplied from a plasmid (pRLM75) under the control of a heat-inducible promoter (17). After 30 min of induction, a KmO4 reactive patch appeared similar to that seen in the dnaC(ts) host (Fig. 3A, lane 3). The signal was stable at least up to 70 min (Fig. 3A, lane 6). We conclude that P1ori opening can be conveniently assayed also in dnaC+ strains by blocking the loading of DnaB with the AP protein.

**Requirements of DnaA, HU, and RepA Initiators for P1ori Opening**—Host initiator proteins DnaA and HU, and the P1 RepA being essential for P1 plasmid replication, their role in the opening of P1ori was tested to validate the KmO4 reactivity assay as a reporter for initiation. Because miniP1 cannot replicate in a ΔdnaA host, P1ori was cloned in a DnaA-independent vector, pACYC184 (23). RepA was provided in trans from another DnaA-independent vector, pST52 (14), at 3X, 7X, and 40X where 1X is the amount that a wild type P1 plasmid produces normally.

In a ΔdnaA host, the KmO4 reactive patch was absent (Fig. 3B, lanes 1–4). When the dnaA gene was re-introduced by lysogenizing the ΔdnaA host with a dnaA transducing phage λDKCS65 (a imm derivative of λKO32, (24), the reactive patch could be seen (Fig. 3B, lanes 6–8). The intensity of the signal was weaker than that seen in dnaC+ cells. Although the basis for the quantitative difference remains to be determined, a correlation of the requirement of DnaA for the KmO4 reactivity of P1ori with the requirement of DnaA for P1 plasmid replication could be made (4). These experiments also revealed that the KmO4 reactivity is sensitive to RepA concentration. The 7X source was optimal, and reactivity decreased at both higher and lower RepA (Fig. 3B, lanes 5–8). As will be discussed, the requirement for RepA at higher than physiological concentrations is most likely due to the presence of the P1ori in a high copy vector. Otherwise, the dependence of the opening signal

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1 The abbreviation used is: Rif, rifampicin.
on DnaA and RepA and decrease of the signal with increase of RepA concentration are consistent with earlier replication studies (4, 21).

We next determined the requirement for HU protein on the appearance of the KMnO4 reactive patch. E. coli HU protein consists of two subunits, α and β, encoded by hupA and hupB genes, respectively. The protein normally exists as a heterodimer, but homodimers of either subunit can also be functional (12). For the KMnO4 reactivity of P1 ori, either of the homodimers was sufficient (Fig. 3C, lanes 2 and 4), but there was no apparent reactivity in the absence of both subunits (Fig. 3C, lanes 2 and 4 versus lane 6). These results provide further correlation of KMnO4 reactivity with initiation and encouraged the use of the assay to study initiation control.

P1 ori Opening in the Presence of Excess Iterons—As stated earlier, in miniP1 plasmids devoid of the incA locus (e.g. pSP102), although the mean copy number increases, it is still controlled. This is believed to be due to the origin (incC) iterons. When their concentration reaches some threshold, further increases in copy number are not allowed. The plasmid used in experiments of Fig. 3 (B and C), pKP104, a clone of P1 ori in pACYC184 vector, had a copy number very similar to that of pSP102. Because the concentration of incC iterons was comparable in the two cases (pSP102 and pKP104), the appearance of the opening signal in pKP104 was not surprising (Fig. 4A, lane 2). However, when the iteron concentration was significantly increased by providing incA in trans from pALA18 (pBR322+incA), our expectation from replication studies was that the opening would be totally inhibited. Such was the observation (Fig. 4A, lanes 7 and 8). However, increased RepA concentration did allow opening (Fig. 4A, lanes 7 and 8). Thus, under the conditions of the present experiment, it appears that incA inhibition of opening can be overcome by excess RepA.

A similar finding was made in a different experiment. In this experiment, P1 ori was cloned in pUC19, and the copy number of the resultant plasmid, pALA68, was 2.5-fold higher than that of pSP102 or pACYC184. However, opening was efficient in the presence of the 40× source (Fig. 4B, lane 3). We conclude
that at high concentrations of iterons, present either in cis as in the pUC19 + P1ori plasmid or in trans as in the pBR322 + incA plasmid, need not be inhibitory, provided sufficient RepA is also present. From these results it appears that the replication of pSP102 could be limited by the availability of RepA.

P1 Plasmid Replication in the Presence of Excess Iterons—If excess RepA could allow simultaneous opening of an abnormally high concentration of P1 ori (as in pUC19 + P1ori plasmid) and if the open-complexes were intermediates of initiation, as suggested by their rapid disappearance when the block to DnaB loading was removed (Fig. 4B, lanes 4-6), we argue that excess RepA would also allow replication of miniP1 plasmids in the presence of excess origins in trans. Functioning of the P1 ori was tested by lysogeny of AP1 chimeric phages, which are defective in recombination and depend on a functional P1 ori to maintain themselves as plasmid prophages. In a rcwA host, the lysogeny of an AP1 ori phage increased 4 orders of magnitude in the presence of RepA but was inhibited in the presence of the 40× source of RepA, as expected (Table I, column 1, rows 1-4). In the presence of excess iterons provided by a pACYC184 + incC plasmid, only the 7× but not the 3× source of RepA allowed lysogeny (Table I, column 1, rows 6-7). Results were similar when pACYC184 + incC was replaced with pACYC184 + P1ori plasmid (Table I, column 1, rows 9-12). This is evidence that incC suffices for control in the absence of incA. When the incoming phage was armiP1, which in addition to P1ori had the autoregulatory repA gene and the incA locus, lysogeny became dependent on additional (trans source) RepA only when excess iterons were present (Table I, column 2). The results of lysogeny with armiP1 were otherwise similar to P1ori. These experiments showed that an excess of origin iterons (incC) in trans can inhibit miniP1 replication and that this inhibition can be overcome by providing excess RepA.

Similar experiments were also done in the dnaC(ts) host used in some of our origin opening studies (Table II). In this case, extra iterons were provided at a higher concentration using a pUC19 + incC plasmid, whose copy number was 2.5 times higher than the pACYC184 + incC plasmid used in the experiments of Table I. The 7× RepA source was not enough; efficient lysogeny required the 40× source (Table I, column 2, row 8). We note that RepA concentrations are only relative and need not be compared between experiments of Tables I and II, because the copy number of the plasmids depended on the strain background. The copy numbers were lower in the dnaC(ts) host, which may explain why the so-called 40× source was not inhibitory (Table II, row 4). We conclude that replica-

**Fig. 3.** KMnO₄ reactivity of P1ori in dnaC⁻ hosts. In these hosts, block to DnaB loading was accomplished by thermal induction of AP protein synthesis from pRLM75 (pBR322 + λI857p.OP). A, kinetics of reactivity of pSP102 in DH5αlac. The reactivity was optimal at 30 min of induction at 42 °C. B, requirement of DnaA in a ΔdnaA host. The AP induction was done at 38 °C to avoid temperature sensitivity of the strain. P1ori was present in pKP104 (pACYC184-derived), and RepA was provided in trans from constitutive sources at 3×, 7×, and 40× physiological concentrations from pALA197, pALA198, and pALA169, respectively. Note that the reactivity is apparent only when the host had an integrated λdhaA phage (lanes 6-8). Note also that the opening was optimal at 7× RepA (lane 7), the concentration at which opening at the −10 region of prepA (horizontal arrow) was optimally repressed. Promoter repression is a reliable indicator of RepA binding to origin iterons, as evidenced by footprinting in vivo (10). C, requirement of HU protein. The origin was present in pKP104, as in Fig. 3B, and RepA protein was provided at 7× concentration from pKP116. The host was deleted either for one of two genes of the heterodimeric HU protein, hupA and hupB, or for both the genes. Upon temperature shift to induce λP to block DnaB, reactivity became more pronounced in ΔhupA and in ΔhupB (lanes 2 and 4) and remained unchanged in Δ(hupAΔhupB) (lane 6).

**Fig. 4.** Requirement of higher RepA for KMnO₄ reactivity in the presence of incA (A) or at increased P1ori copy number (B). The host was dnaCts. RepA sources were same as in Fig. 3. A, P1ori plasmid was pKP104. In the absence of incA, opening was optimal at 3× RepA (lane 2), whereas in the presence of incA, provided from a pBR322 vector, opening was best at 40× RepA (lane 8). B, P1ori plasmid was pALA658 (pUC19-derived). The cells also contained a source of LacI to repress the lac promoter present in pUC19. The reactivity was optimal at 40× RepA (lane 3) and was lost within 5 min of returning to 30 °C (lane 5) as seen in Fig. 2.
Control of DNA Replication of Plasmid P1

TABLE I
Relief of incC-mediated inhibition of replication by additional RepA in DH5Δinc

| Source of additional inc (plasmid name) | Relative [RepA] | Lysozymy of cells infected by | A·P Lori | AminiP1 |
|----------------------------------------|----------------|--------------------------------|----------|---------|
| None: vector alone control (pKP126)    | None           | 0.7 × 10^{-4}                  | 3.7      |         |
|                                        | 3×              | 0.4                            | 2.0      |         |
|                                        | 7×              | 0.6                            | 4.3      |         |
|                                        | 40×             | 1.5 × 10^{-4}                  | 6.0 × 10^{-4} |         |
| pACYC184+incC (pKP124)                 | None           | 0.9 × 10^{-4}                  | 2.2 × 10^{-4} |         |
|                                        | 3×              | 1.2 × 10^{-4}                  | 1.2 × 10^{-4} |         |
|                                        | 7×              | 0.5                            | 4.6      |         |
|                                        | 40×             | 1.4 × 10^{-4}                  | 2.2 × 10^{-4} |         |
| pACYC+P1ori (pKP104)                   | None           | 0.4 × 10^{-4}                  | 3.7 × 10^{-4} |         |
|                                        | 3×              | 3.6 × 10^{-4}                  | 1.9 × 10^{-4} |         |
|                                        | 7×              | 0.5                            | 4.8      |         |
|                                        | 40×             | 0.7 × 10^{-4}                  | 2.0 × 10^{-4} |         |

* The background lysogeny was nearly identical in all rows (0.011 ± 0.003) when the infecting phage, ΔDKC275, had incC and not complete P1ori.

TABLE II
Relief of incC-mediated inhibition of replication by additional RepA in E. coli PC2

| Source of additional inc (plasmid name) | Relative [RepA] | Lysozymy of cells infected by | A·P Lori | AminiP1 |
|----------------------------------------|----------------|--------------------------------|----------|---------|
| None: vector alone control (pPP155)    | None           | 0.01*                          | 3.1      |         |
|                                        | 3×              | 0.01                           | 1.6      |         |
|                                        | 7×              | 0.02                           | 1.1      |         |
|                                        | 40×             | 0.04                           | 1.7      |         |
| pUC19+incC (pALA646)                   | None           | 0.01                           | 0.1      |         |
|                                        | 3×              | 0.01                           | 0.1      |         |
|                                        | 7×              | 0.02                           | 0.5      |         |
|                                        | 40×             | 0.08                           | 2.3      |         |
| pUC19+P1ori (pALA658)                  | None           | 0.01                           | 0.04     |         |
|                                        | 3×              | 0.01                           | 0.05     |         |
|                                        | 7×              | 0.02                           | 0.1      |         |
|                                        | 40×             | 0.02                           | 0.05     |         |

* The background lysogeny was nearly identical in all rows (0.011 ± 0.003) when the infecting phage, ΔDKC275, had incC and not complete P1ori.

tion inhibition by excess iteron can be overcome by controlled overproduction of RepA.

DISCUSSION

Here we show that opening of the P1 plasmid origin in vivo can be detected efficiently by probing with KMnO₄, provided the DnaB helicase loading to the origin is blocked. This has been achieved either by inactivating DnaC (2) or by sequestering DnaB with AP protein (17). The opening was taken to represent initiation of DNA replication for the following reasons. It 1) required the presence of all three proteins that are essential for plasmid replication: host initiators DnaA and HU and plasmid-encoded initiator RepA, 2) was site-specific, localized to an A-T-rich region of the origin, 3) was transient, because it reversed when the block to DnaB loading was lifted, and 4) was regulated by iteron and RepA, the known regulators of P1 plasmid replication.

Opening of plasmid origins has been studied primarily in vitro. For P1, some opening was observed with DnaA alone (25). RepA alone was not effective, but it greatly stimulated opening when present together with DnaA. Results were similar with miniF plasmids except that these experiments also included HU (26). Although individually ineffective, a combination of RepE (the P1 RepA equivalent) and HU allowed opening, which was further stimulated by DnaA. Results were different for plasmid RK2 in one respect (27). DnaA alone, which was effective in opening the P1 and F origins, was ineffective for the RK2 origin. From our present results in vivo, it is seen that any of the pairwise combinations of DnaA, HU, and RepA were insufficient. A reproducible signal required cooperation of all three proteins, indicating that one reason the three are essential in replication is for opening the origin.

The ability to detect opening allowed us to study P1ori activity at various copy numbers in chimeric replicons and test the applicability of models that have been proposed for the negative feedback control of plasmid copy number by iteron. In the initiator titration model, the concentration of plasmid-encoded initiator protein that binds the iteron is assumed to be limiting (8, 15, 19, 28, 29). With increase of copy number, the limiting amount of initiator is believed to distribute to different origins, preventing saturation of any one origin. Thus, the increase of origin (or, more precisely, iteron) concentration relative to initiator provides the negative feedback signal. The model was questioned when extra initiators did not increase copy number significantly in many iteron-carrying plasmids: R6K (30), P1 (21), RK2 (31), pSC101 (32), and Rs1 (33), although not R1162 (34).

A second model, the handcuffing model, is based on the finding that the initiators can pair iterns in trans (21, 35). It is assumed that the pairing causes a steric hindrance to origin activity, increases with increased origin (iteron) concentration and cannot be relieved by increasing the concentration of RepA. As discussed above, this model was invoked when the titration model was found inadequate to explain insensitivity of copy number to increases of initiator concentration.

A third mode of control is conspicuous in some members of iteron-carrying plasmids: R6K (30), P1 (21), pSC101 (32), and Rs1 (33). In these plasmids, a modest increase of initiator concentration was shown to lead to a decrease in copy number. The mechanism of this mode of control is not clear, but in principle such a mechanism can be an alternative to handcuffing in restraining copy number when RepA concentration becomes excessive.

The results of the present paper are most easily explained by the initiator titration model. When the P1ori was cloned into the pUC19 vector with a copy number about 2.5-fold higher than that normally achieved by miniP1 plasmids (in the absence of the incC locus), the origins could still be opened but required higher concentrations of RepA (Fig. 4B). Such a high concentration of P1ori also did not inhibit replication of an incoming AminiP1 plasmid prophage when extra RepA was provided (Table II). These results are consistent with the conclusion from a different set of experiments that origins are poor inhibitors of each other when RepA supply is adequate (8, 36).

The inhibitory activity of iteron is clearly seen when they are not part of intact origins (8, 15) or when the origin is not functional as in high copy plasmid chimeras used here (Tables I and II). In some of these experiments, RepA was supplied from an autoregulatory source. One might expect that an autoregulatory source of RepA could promptly compensate for RepA titration. Were that true, the observed inhibition might be attributed to handcuffing. Experiments designed to determine the extent to which titration of RepA by iteron results in additional RepA synthesis showed that the induction of RepA was inefficient (20). This was also observed in the present studies (Tables I and II). The autoregulated source of RepA present in AminiP1 did not supply enough RepA to overcome inhibition by extra incC. The inhibition was overcome when extra RepA was provided from a constitutive promoter. One
reason for inadequate RepA supply from the autoregulated source could be due to the capacity of iteron-bound RepA to repress prepA by RepA-mediated handcuffing (20).

In experiments where the source of RepA was not autoregulatory and iteron-mediated inhibition prevails irrespective of the quantity of RepA supply, handcuffing remains the most satisfactory explanation (8, 21, 36). Even in experiments where the inhibition was overcome by oversupply of RepA (e.g. pBR322 + incA, Fig. 4A; pACYC184 + incC, Table I; and pUC19 + incC, Table II) and the results are adequately explained by titration, it is possible that handcuffing could have operated at steps after origin opening in experiments where replication was blocked (Fig. 4A) or replication of vector could have interfered with handcuffing (Tables I and II). Similar ambiguity remains in some of our previous experiments where initiator titration appeared to be the simplest explanation. In one, incA inhibition of integrative suppression by AminiP1 was overcome by extra RepA (15). In the other, a block to lysogeny by APlori was overcome by extra RepA (21), as in the present experiments (Tables I and II). In both of these experiments, incA was chromosomally located, and chromosomal replication could have interfered with handcuffing. Thus it is possible to accommodate most of our results as not contradicting the handcuffing model, if the conditions under which handcuffing operates are sufficiently constrained.

How is the copy number of incA-deleted miniP1 plasmids controlled? If origins per se are not inhibitory to each other at high concentrations, the copy number could be limited by the availability of RepA or by some essential host factor(s) (8). We already discussed that RepA supply from an autoregulated source could be limiting. Because RepA requires chaperones for activity (37), their availability could be limiting. Another possibility could be that the supply of too much RepA limits copy number because of an accumulated inhibitor synthesized concomitantly with RepA.

That the inhibitor is not RepA itself is suggested by two lines of evidence. Mutating the translation start codon of repA reduces RepA production drastically but not the synthesis of a replication inhibitor (9). Also excess of purified RepA does not cause replication inhibition in vitro (36, 38). When we exceeded the normal copy number of incA-deleted miniP1 in chimeric replicons, the normally inhibitory level of RepA synthesis was not inhibitory, most likely because the inhibitor distributed to different origins. Thus the relative rather than absolute levels of inhibitors and origins seem to be more important, and accumulating RepA at a rate higher than the increase of copy number can be a potential means to prevent runaway replication.

In summary, it appears that in addition to relative concentrations of RepA and iterons, there are probably other players in copy number control. We are interested to know whether the inhibition of replication that is seen when RepA is artificially overproduced happens under normal circumstances. An understanding of the nature and mode of action of the inhibitor(s) may help also to determine the conditions that favor handcuffing and those that favor titration.

Acknowledgments—We thank Stuart Austin and Roger McMacken for plasmids, Stuart Austin, Don Helinski, and Michael Yarmolinsky for thoughtful criticisms, and Michael Lichten and Michael Yarmolinsky for critical reading of the manuscript.

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