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P1 Plasmid Replication: Initiator Sequestration Is Inadequate To Explain Control by Initiator-Binding Sites

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The unit-copy plasmid replicon mini-P1 consists of an origin, a gene for an initiator protein, RepA, and a control locus, *incA*. Both the origin and the *incA* locus contain repeat sequences that bind RepA. It has been proposed that the *incA* repeats control replication by sequestering the rate-limiting RepA initiator protein. Here we show that when the concentration of RepA was increased about fourfold beyond its normal physiological level from an inducible source in *trans*, the copy number of a plasmid carrying the P1 origin increased about eightfold. However, when the origin and a single copy of *incA* were present in the same plasmid, the copy number did not even double. The failure of an increased supply of RepA to overcome the inhibitory activity of *incA* is inconsistent with the hypothesis that *incA* inhibits replications solely by sequestering RepA. We propose that *incA*, in addition to sequestration, can also restrain replication by causing steric hindrance to the origin function. Our proposal is based on the observation that *incA* can bind to a RepA-origin complex in vitro.

The prophage P1 is faithfully maintained as a plasmid with a copy number of one to two per bacterial chromosome (17). The elements that are responsible for this controlled replication have been identified and found to be located within a 1.5-kilobase (kb) segment of the plasmid: a replication origin within 245 base pairs (bp), a 959-bp region that encodes a 32-kilodalton (kDa) initiator protein, RepA, and a control locus, *incA* (7). Nearly identical copies of a 19-bp sequence are present five times within the origin and nine times in *incA* (3). These repeat sequences bind the RepA protein (1, 16). Whereas repeats at the origin are essential for replication, the repeats of the *incA* locus are dispensable (16). Complete deletion of *incA* raises the copy number about eightfold. Similar observations have been made with mini-F, another low-copy-number plasmid, whose origin and control locus also contain repeat sequences (23, 26). In both cases, the observations led to the proposal that the control locus restrains replication by sequestering the initiator, which would otherwise be available to the origin (5, 26). The existence of about 20 RepA dimers per mini-P1 replicon is consistent with the idea that the initiator is normally rate limiting for replication (22). This low initiator level is maintained by efficient autoregulation of the *repA* gene: the promoter of the gene maps within the origin repeats (7), and it would appear that RepA binding to the origin excludes RNA polymerase from the promoter (22). The observation that *repA* is autoregulated might appear to contradict the notion of sequestration, since the protein lost to the control locus should be replenished by new synthesis. However, we have recently shown by electron microscopy that purified RepA can contact the origin and *incA* repeats simultaneously by DNA looping when the sites are in *cis* (6). The RepA can also pair the sites when they are in *trans* (6). In this way, promoter repression can be maintained even by the RepA bound to *incA*. As long as the *incA*-bound RepA does not participate in initiation, autoregulation need not be considered inconsistent with sequestration. In the case of the mini-F plasmid, autoregulation and sequestration have been reconciled by assuming that there are two different forms of the Rep protein (24, 29).

In order to test the model that the rate of replication is determined by the initiator concentration, we cloned the *repA* gene under the control of the inducible *lac* promoter, enabling us to vary RepA concentration in the cell. As expected, the copy number of a plasmid carrying the mini-P1 origin increased about eightfold when the RepA concentration was raised about fourfold beyond the physiological (autoregulated) level. However, the increase in copy number was nominal (less than double) when the mini-P1 origin and *incA* were present in the same plasmid. These results confirm that RepA alone can affect the rate of replication but also suggest that limiting RepA, by autoregulation and sequestration, cannot be the only mechanism by which copy number is controlled. In view of our election microscopic demonstration that *incA* can interact directly with the origin repeats through RepA (6), we propose that such interactions can cause steric hindrance to the process of replication initiation.

(A preliminary report of some of this work has already been presented [15].)

MATERIALS AND METHODS

Construction of *repA*-deleted mini-P1 plasmids pRJM345 and pRJM384. pRJM345 was derived from the mini-P1 plasmid pALA318 (Table 1) by deleting a 1-kb *Hin*dIII-*Bam*HI fragment containing the *repA* gene and the adjoining *incA* repeat 9 and inserting at the same sites a 3.4-kb fragment containing the partition locus (Par) of P1 (Fig. 1). The Par locus was obtained from the plasmid pALA270 (2). pRJM345 therefore lacks *incA* and *repA* and is dependent on a foreign source of RepA for replication. RepA was supplied from pALA69 (5). An *incA* derivative of pRJM345, pRJM384, was constructed by cloning the 306-bp *Bam*HI fragment carrying *incA* from pALA18 (5) into the *Bam*HI site of pRJM345. Both pRJM345 and pRJM384 were stable (<1% loss in 20 generations of growth without selection).

Construction of an inducible *repA*::*lacZ* operon fusion. A *Hin*dIII-*Bam*HI fragment (P1 coordinates 606 to 1529) of

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TABLE 1. Description of plasmids

Plasmid	Replicon	Relevant content (P1 coordinates)[a]	Source or reference
pALA18	pBR322	Repeats 1–9 of *incA* (1506–1812)	5
pALA69	pBR322	*repA* (606–1569); provides nearly physiological amount of RepA	5
pALA176	pBR322	*repA* (606–1569); provides 7 times the physiological amount	7
pALA177	pBR322	Same as pAlA176 but provides 3 times the physiological amount	7
pALA270	pBR322	P1 Par (1851–5241)	2
pALA318	Mini-P1	*ori* + *repA* + repeat 9 and 8′ of *incA* (−228–1569)	16
pBH20	pBR322	*lacpo*	11
pRJM345	Mini-P1	*ori* (−228–611) + P1 Par (1851–5241)	This work
pRJM384	Mini-P1	Same as pRJM345 but also carries *incA* (1506–1812)	This work
pSP102	Mini-P1	*ori* + *repA* (−228–1529)	16
pSP120	pBR322	*lacpo* + *repA* (606–1529)	This work
pSP206	pBR322	*lacpo* + *repA* + *lacZ*	This work
pVLRR10	pACYC184	*lacI*^{q}	28
pWS60	pBR322	λ *c*II-*lacZ* fusion gene	20
F′ *lacI*^{q}*ZYA*::Tn*5*	F	*lac* operon; Tn*5* is located somewhere outside the operon	R. Young

[a] The sequences between −228 and 2088 (3) and 1851 and 5683 (2) have been published.

plasmid pSP102 (16) carrying the *repA* gene without its own promoter was cloned between the *Hin*dIII and *Bam*HI sites of pBH20 (11). pBH20 is identical to pBR322 except for a 200-bp insert containing the *lac* promoter (p_{lac}) at the *Eco*RI site of pBR322. In the resultant plasmid, pSP120, the *repA* gene is downstream of p_{lac} and is separated from it by 29 bp of pBR322 sequences between its *Eco*RI and *Hin*dIII sites. Next, a 3.2-kb fragment carrying a λ *c*II-*lacZ* fusion gene, without a promoter but with a ribosome-binding site from plasmid pWS60(20), was cloned downstream of *repA* of pSP120 at the *Bam*HI site to give the plasmid pSP206. In pSP206, the *repA* and *lacZ* genes are cotranscribed from p_{lac}, so that the β-galactosidase activity should reflect relative amounts of RepA in the cell. The p_{lac}-*repA-lacZ* fusion was transferred in vivo to a *lacYZ′ ′bla* vector phage, λRZ5, by using *lac* and *bla* homologies as described previously (22). The phage was deleted of *lacY* by crossing with λp*lac5* (25) so that suboptimal induction of the *lac* promoter could be achieved without population heterogeneity (14). The final phage, λp_{lac}-*repA-lacZY$^-$bla*, also called λSP300 or λp_{lac}-*repA-lacZ*, was integrated into the chromosome of the *lac*-deleted strain MC4100 *recA56* (19). An F′ *lacI*^{q}*ZYA*::Tn*5* plasmid from strain PLH90 (gift from R. Young) was mated into the above lysogen to repress the *lac* promoter (Table 1). The basal level of β-galactosidase activity from individual lysogens could be grouped into discrete classes. Apparently the lysogens contained either one, two, or three prophages. A trilysogen was selected for further study (see Results). When absence of *lacY* was desired, pVLRR10 (28) was used, which contains *lacI*^{q} and the NH_2-terminal end of *lacZ* only (see Fig. 3c). When higher levels of RepA were needed, the multicopy plasmid pSP206 was used instead of the λ fusion phage λSP300.

Measurement of RepA protein and plasmid copy number. The measurement of RepA has been described (22). The copy number of plasmids was determined by fluorescence densitometry of ethidium bromide-stained agarose gels or by blot hybridization of cell lysates with a ^{32}P-labeled 826-bp *Hin*dIII fragment (P1 coordinates −228 to 598) carrying the mini-P1 origin as the probe (16). Throughout the text, copy numbers are expressed relative to that of our wild-type mini-P1 plasmid, λ-P1:5R, grown under identical conditions but in the absence of any RepA source in *trans* (see legend to Fig. 3). Under our growth conditions, a relative copy number of one corresponds to about four λ-P1:5R plasmids per cell (22).

Electron microscopy. In order to show DNA looping between *incC* and *incA* repeat 9, pALA318 was digested with *Pst*I so that one 2.5-kb fragment had the two repeat regions separated from each other by 0.9 kb of *repA* sequence. The other 1.8-kb fragment contained the *cat* gene and no P1 sequences (16). As a control, *Pst*I-digested pSP102 DNA was used that lacked the *incA* repeat but otherwise was identical to pALA318 (16). About 50 ng of DNA was mixed with 40 ng of purified RepA (27) in a 20-μl volume essentially as described elsewhere (6). The mixture was diluted about 10-fold, and 10 μl was sampled for electron microscopy (9).

RESULTS

Construction of an inducible source of RepA. In order to study the effect of increasing RepA concentration on replication of mini-P1 plasmids, we constructed a λ phage carrying the operon fusion p_{lac}-*repA-lacZ* (Materials and Methods). The phage was integrated into the chromosome of the *lac*-deleted strain MC4100 *recA56*. The same cell also contained a *lacI*^{q} gene (Fig. 1). Induction of the operon to different levels with various amounts of an inducer, isopropyl thiogalactopyranoside (IPTG), provided a convenient means of varying the RepA concentration. Furthermore, since both *repA* and *lacZ* genes are transcribed from the same promoter, the specific activity of β-galactosidase in steady-state cultures, which is easily measured (13), is expected to reflect the concentration of RepA in the cells.

Induction of β-galactosidase and RepA was measured after the cells were exposed to different amounts of IPTG for 12 generations. The time was sufficient for the β-galactosidase specific activity to reach a steady state in the range of inducer concentrations used. Without any IPTG the basal level of β-galactosidase activity was about 40 Miller units (M.U.) (13). The addition of up to 100 μM inducer increased β-galactosidase activity by about sixfold. In order to obtain higher levels of RepA, a pBR322 derivative plasmid, pSP206, which contains the same fusion, was used instead of the λ fusion phage. In this case, the basal β-galactosidase level without IPTG was about 150 M.U. and the induced level with IPTG was over 1,000 M.U.

Inducibility of RepA with IPTG was confirmed by measuring RepA directly with an immunological assay (Western blot) (Fig. 2). When the p_{lac}-*repA-lacZ* fusion was present in the chromosome, the basal level of RepA was about 70% of

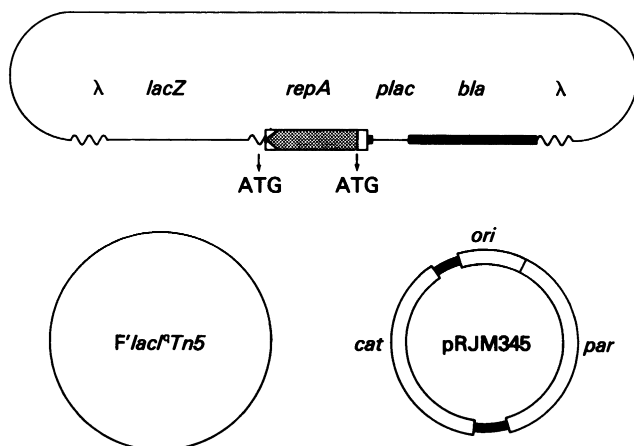

FIG. 1. Schematic representation of the tripartite system used here to determine the relationship of RepA concentration to copy number. A mini-P1 plasmid, pRJM345, was used to measure copy number. The chromosomally integrated prophage λp_{lac}-*repA-lacZ-bla* was used to supply RepA. An F' *lacI*^{q}::Tn*5* plasmid was used to repress the *lac* promoter. Thick black lines are sequences from pBR322, open boxes are sequences from P1cm, the stippled box represents the *repA* open reading frame, and wavy lines are phage λ sequences.

the physiological level (defined as the concentration of RepA in cells containing the unit-copy λ-P1 chimera λ-P1:5R [3]). With IPTG, RepA could be increased to about five times the physiological level. As expected, the RepA concentration was roughly proportional to β-galactosidase activity (Fig. 2C). This was also true in the case of cells carrying pSP206 (Fig. 2B and D).

Stimulatory effect of RepA on replication of mini-P1. In order to study the effect of RepA on replication, we used a plasmid (pRJM345) that has the mini-P1 origin but not the *repA* gene, so it can replicate only when RepA is supplied in *trans*. The plasmid was able to transform uninduced MC4100 *recA56* cells carrying three copies of the prophage λp_{lac}-*repA-lacZ* at λ^{att} of the chromosome and an F' *lacI*^{q}. Evidently, the basal level of RepA in the cell was sufficient to support the replication of pRJM345. The relative copy number of the plasmid under such conditions was about two relative to λ-P1:5R grown under identical condition. The transformed cells were grown in the presence of different concentrations of the inducer. In each culture, both β-galactosidase and the copy number of pRJM345 were measured. The copy number of pRJM345 increased with induction (Fig. 3a). At a β-galactosidase value of about 250 M.U., the relative copy number approached eight, which appears to be the maximum value pRJM345 can attain (see below). The results show that it is possible to increase the copy number of a mini-P1 plasmid by raising the initiator concen-

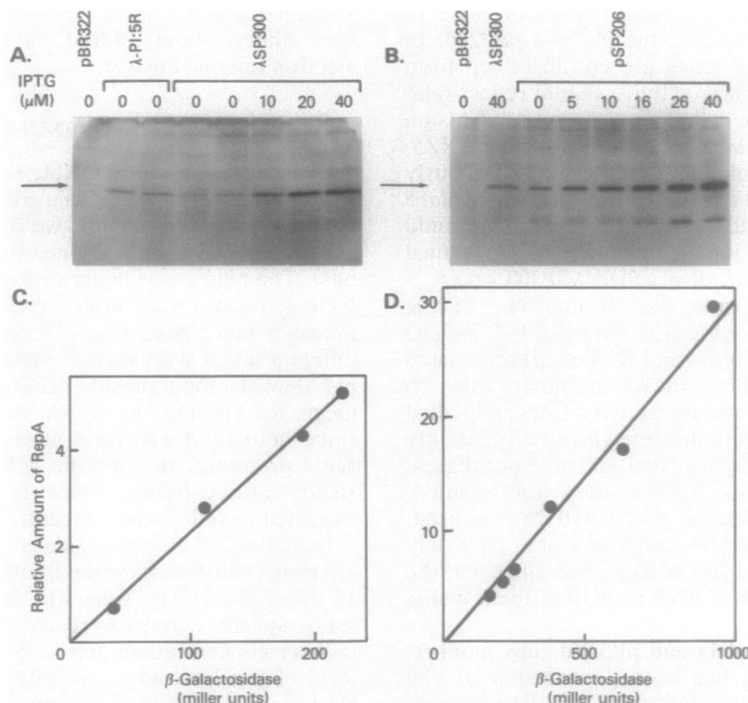

FIG. 2. Correlation between the amounts of RepA and β-galactosidase produced from the operon fusion p_{lac}-*repA-lacZ*. Cultures of MC4100 *recA56* (λp_{lac}-*repA-lacZ-bla*)/F' *lacI*^{q}::Tn*5* harboring plasmid pRJM345 (see Fig. 1) were grown overnight in LB medium containing ampicillin (25 μg/ml), chloramphenicol (20 μg/ml), and kanamycin (10 μg/ml). Overnight cultures were diluted 200-fold into the same fresh medium and grown to an A_{600} of 0.5. This was again diluted 5,000-fold into fresh medium containing various amounts of IPTG (0 to 40 μm). Cells were grown for 12 generations to an A_{600} of 0.4. Portions were withdrawn to assay β-galactosidase (13) and RepA (see Materials and Methods). (A) RepA produced from the chromosomal fusion prophage at the IPTG concentrations shown. A 300-μg amount of total cell protein extract was added to each lane. In the first lane is an extract from MC4100 *recA56*(pBR322) cells that do not carry a source of RepA. The next two lanes show the amount of RepA produced from our wild-type mini-P1 plasmid, λ-P1:5R. This amount is defined as unit level in panel C and is used to normalize RepA levels from the remaining lanes of panel A. Panels B and D are identical to A and C, respectively, except that the fusion was present on the multicopy plasmid pSP206. Arrow shows the 32-kDa RepA band as deduced from M_r markers. The identity of the lower-M_r band also induced by IPTG has not been examined.

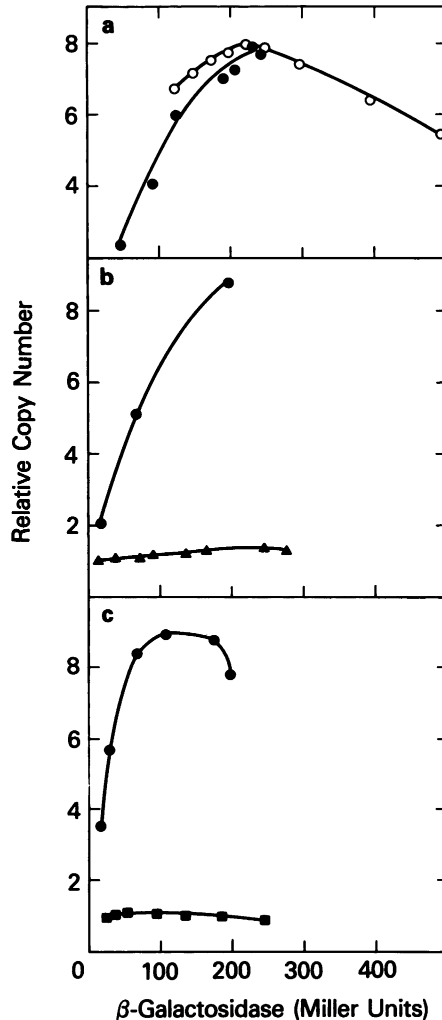

FIG. 3. (a) Effect of RepA on copy number of pRJM345. Cultures were grown as described in the legend to Fig. 2. Copy number was measured as described in Materials and Methods and plotted against corresponding amounts of β-galactosidase. Copy number measurement in lysogens of λp_{lac}-*repA-lacZ* fusion phage is represented by solid circles and in cells carrying the fusion in the multicopy plasmid pSP206 by open circles. The copy number was expressed relative to λ-P1:5R grown in MC4100 *recA56*/F′ *lacI*^{q} cells in otherwise identical conditions. Each curve represents the mean of three separate sets of measurements. (b) Effect of RepA on copy number in presence of *incA*. Cells were grown as for panel a except that the cell extracts were blotted to nitrocellulose paper and hybridized with a ^{32}P-labeled P1-specific probe. Copy number was determined by densitometrically tracing the autoradiogram bands (see Materials and Methods). A monolysogen of λ-P1:5R at the *attP1* site of the *E. coli* chromosome was used here as a standard for unit-copy plasmid. Symbols: ●, pRJM345; ▲, λ*cat*-P1:5R. (c) Experiments were done as in panel b except that λ-P1:5R was replaced with pRJM384 (■), which is isogenic to pRJM345 (●) except for *incA*. Also pVLRR10 was used to supply *lacI*^{q} instead of F′ *lacI*^{q}::Tn*5*, which carried the entire *lac* operon (see Materials and Methods). Unlike the previous cases, there is no *lac* permease present in these experiments, and β-galactosidase values represent activity from the chromosomal fusion prophage only. Mean results of two independent sets of measurements are shown here. The limits of error in copy number measurement were within 10%, and for β-galactosidase they were within 20%.

tration. Since the protein concentration had to be increased about fourfold above the physiological level in order to reach a relative copy number of eight, these results are consistent with the idea that RepA is normally rate limiting for replication.

Inhibitory effect of relatively high rates of *repA* expression. In order to study the effect of *repA* expression higher than that produced by the prophage λp_{lac}-*repA-lacZ*, the multicopy plasmid pSP206, carrying the same p_{lac}-*repA-lacZ* fusion, was used as a source of RepA. The basal level of RepA in this case allowed pRJM345 to replicate with a relative copy number of about seven (Fig. 3a). With increasing concentrations of IPTG, the copy number rose to about eight and then decreased with further induction. These results show that when *repA* expression exceeds the physiological level by about fourfold, the rate of replication begins to decrease. With higher amounts of IPTG, the copy number of mini-P1 under selection can be reduced to unity (15; unpublished results). The reason for this behavior is not understood.

Effect of *incA* on copy number. The effect of *incA* on copy number does not require the integrity of *incA* but appears to depend only on the number of individual repeating units of which it is composed (5, 16). Since these units are capable of binding RepA separately (1) but incapable of affecting *repA* transcription or translation (16) or of influencing the stability of RepA protein as measured by immunoblotting (6), it appears that *incA* exerts its effect only through its property of binding RepA. If the inhibitory activity of *incA* is mediated by RepA sequestration, then the copy number of a mini-P1 that includes *incA*, such as λ-P1:5R, should also increase in the presence of sufficient RepA. Whereas the relative copy number of pRJM345 reached eight, that of λ-P1:5R did not even reach two in parallel experiments (Fig. 3b). At higher concentrations of RepA, replication of λ-P1:5R was inhibited, as in the case of pRJM345 (data not shown). Essentially identical results were obtained with a second *incA*-carrying plasmid, pRJM384 (Fig. 3c). Plasmids pRJM345 and pRJM384 are isogenic except for a 306-bp *incA* fragment in the latter (see Materials and Methods). These results were not expected for RepA sequestration alone and suggest that *incA* is inhibitory under conditions in which RepA is apparently not limiting.

Effect of *incA* on lysogenization frequency. An alternative measure of the capacity of *incA* to inhibit replication is the frequency with which a λ-mini-P1, such as λ-P1:5R, will lysogenize *Escherichia coli*. We found that a mini-P1 plasmid could not be established in the presence of an integrated copy of λ-P1:5R, even under selection. The frequency of lysogeny of incoming λkan-P1:5R phage (λDKC235) was reduced by five orders of magnitude (Table 2). The rare lysogens apparently contained mutant λkan-P1:5R plasmids. The phages produced following induction of these mutant prophages could subsequently establish themselves in λ-P1-5R lysogens as easily as in nonlysogens. Somewhat surprisingly, even when RepA in sevenfold excess over the physiological concentration was supplied in *trans* from plasmid pALA176, there was little increase in the number of lysogens. Only when the incoming phage (λDKC274) lacked *incA* in *cis* was there efficient lysogeny in the presence of pALA176 (Table 2). A comparable impediment to lysogeny can be achieved with *incA* alone, as shown when instead of a host carrying λ-P1:5R, a λ *incA* polylysogen was used (Table 2). Although a λ *incA* monolysogen can destabilize mini-P1, at least two λ *incA* prophages at λ^{att} were required to prevent colony formation under selection for the incoming

TABLE 2. Effect of *incA* on lysogenization frequency

Plasmid and/or prophage in host MC4100 *recA56*	Relative amount of RepA[a]	No. of Kan[r] lysogens with infecting phage[b]:	
		λkan-P1:5R (λDKC235)	λkan-P1:*ori* (λDKC274)
pBR322		2×10^8	1×10^3
pALA177	3	2×10^8	2×10^7
pALA176	7	2×10^8	3×10^7
λ-P1:5R/pBR322	1	1×10^3	1×10^3
λ-P1:5R/pALA177	(3)	1×10^3	1×10^3
λ-P1:5R/pALA176	(7)	5×10^3	5×10^8[c]
λ-P1:*incA*[d]/pBR322		2×10^3	1×10^3
λ-P1:*incA*/pALA177	(3)	2×10^3	2×10^4
λ-P1:*incA*/pALA176	(7)	6×10^3	5×10^8[c]

[a] Numbers shown in parentheses are expected amounts that were not verified experimentally.

[b] λDKC235 was constructed by cloning a 1-kb kanamycin resistance gene cartridge between the two *Sal*I sites of λ-P1:5R (gift from S. Friedman). λDKC274 was constructed from λDKC231 (22), which has essentially the mini-P1 *ori* in the b2 region of phage λ. A kanamycin cartridge (Pharmacia) was incorporated in the middle of the *int* gene of λDKC231, and a Spi deletion (Spi-8 [21]) was crossed in so that the final phage was completely recombination deficient.

[c] About 20-fold-increased lysogenization versus that with pALA176 alone was apparently due to homoimmune infection.

[d] Polylysogens (22).

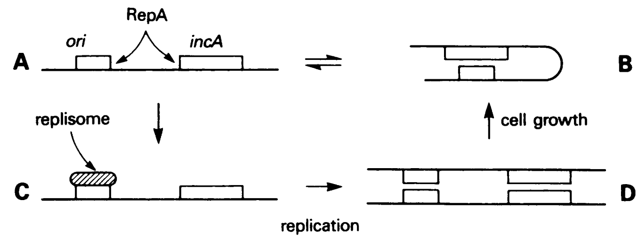

FIG. 4. Control of replication by steric hindrance at high RepA concentration. We imagine that the RepA-binding sites of *ori* and *incA* are mostly occupied when RepA dimers are not limiting (saturation binding of RepA is shown as open rectangles). At a low DNA concentration, mostly species A and B exist in equilibrium; replisome assembly at *ori* (species C) prevents pairing in *cis*. The increased DNA concentration created upon replication favors pairing in *trans* (species D); cell growth lowers the DNA concentration, favoring species B (and A). At low RepA concentrations, the primary rate-limiting step is the filling up of the binding sites, i.e., the formation of species A. The sequence of events otherwise stays the same except that upon replication, the pairing in *trans* could be weaker because there are fewer RepA dimer contacts. As long as RepA is limiting, however, the role of species D is less important. In this model, diagonal arrows between the species are also possible but have been omitted because their contribution to the control circuit is considered minor. Finally, species D must be thought of as only one of several possible pairs.

mini-P1. These results show independently that an inhibitory effect of *incA* can be seen on mini-P1 replication even when RepA is apparently not limiting. Also, the efficient lysogeny obtained with λDKC274 versus that by λDKC235 (Table 2) is consistent with our previous results, which indicated that *incA* is more inhibitory in *cis* to the P1 origin than it is in *trans* (16) (see below).

***incA* and origin can directly interact through RepA.** In this section, we propose that aside from RepA sequestration, *incA* can directly control initiation by steric hindrance to the origin activity. Recently we demonstrated by electron microscopy that the repeat sequences of the origin and the *incA* locus can interact with each other in the presence of purified RepA (6). When the two sites are in *cis*, the intervening DNA forms a loop. RepA also pairs the sites when they are in *trans*. It is conceivable that when the RepA dimers bound to the origin contact RepA dimers bound to a second locus, the origin is precluded from participating in the initiation of replication (Fig. 4). The failure of excess RepA to promote initiation is consistent with this steric hindrance by *incA* (Fig. 3b and c). The model is also in accord with the preferential *cis* action of *incA* (Table 2) (16). We have previously reported that the relative copy number of mini-P1 plasmids decreases from eight to about four in the presence of a single 19-bp *incA* repeat in *cis* (16). It is difficult to imagine how a single repeat can sequester so much RepA as to reduce the rate of replication twofold. From Fig. 2 and 3 it would appear that in order to reduce the copy number from eight to four, RepA concentration has to decrease about twofold as well. On the other hand, it is easy to imagine how a single site can be effective in interfering with the origin function by DNA looping. We verified that a single *incA* repeat can participate in loop formation (Fig. 5). When a 2.5-kb DNA fragment from pALA318 was reacted with purified RepA, DNA-protein complexes could be easily seen, accounting for about 10% of the input DNA. The protein appeared to be present at the origin in about 70% of the complexes (Fig. 5A), at the *incA* site in 7% (Fig. 5B), and in loops in the remaining 23% (Fig. 5C and D). Among the looped structures, more than 80% were of the expected size, 0.9 kb; the rest were heterogeneous. Only the heterogeneous loops were seen in the control DNA, pSP102, that lacked the *incA* repeat. In this case looped molecules accounted for only 5% of the DNA-protein complexes. We conclude from these preliminary studies that even a single repeat can stably interact with repeats at the origin in the presence of RepA alone. It should be noted that when all nine repeats were present at the *incA* locus, looped molecules accounted for 80% of the DNA-protein complexes, while RepA bound to either *incA* or *ori* accounted for the other 20% (6). In other words, loops formed more efficiently with intact *incA*. These results are consistent with the notion that *incA* can control replication by steric hindrance.

DISCUSSION

In this paper we have studied the relationship between the initiator protein RepA and the replication of mini-P1 plasmids both with and without the copy number control locus, *incA*. As we increased the RepA concentration above that normally produced by a unit-copy mini-P1 (the physiological level), the copy number of a mini-P1 plasmid lacking *incA* also increased. This is direct evidence that the initiator protein itself can control the rate of replication in the absence of *incA*. Similar experiments suggest that initiator concentration can also limit replication of plasmids pT181 (12), RSF1010 (10), and mini-F (18) and probably of *oriC* (4). There is at least one well-studied case, plasmid R6K, where this is not so (8).

It was originally proposed, under the assumption that RepA is rate limiting, that *incA* sequesters sufficient RepA to limit the rate of replication (26). The results of this paper argue that RepA sequestration is not the only role for *incA*, since the copy number barely changed when RepA concentration was increased well above physiological levels in the presence of *incA*. We expected that although more RepA would be needed to cause a given increase in copy number in the presence of *incA* than in its absence, copy number would nonetheless increase. For example, at a RepA concentration

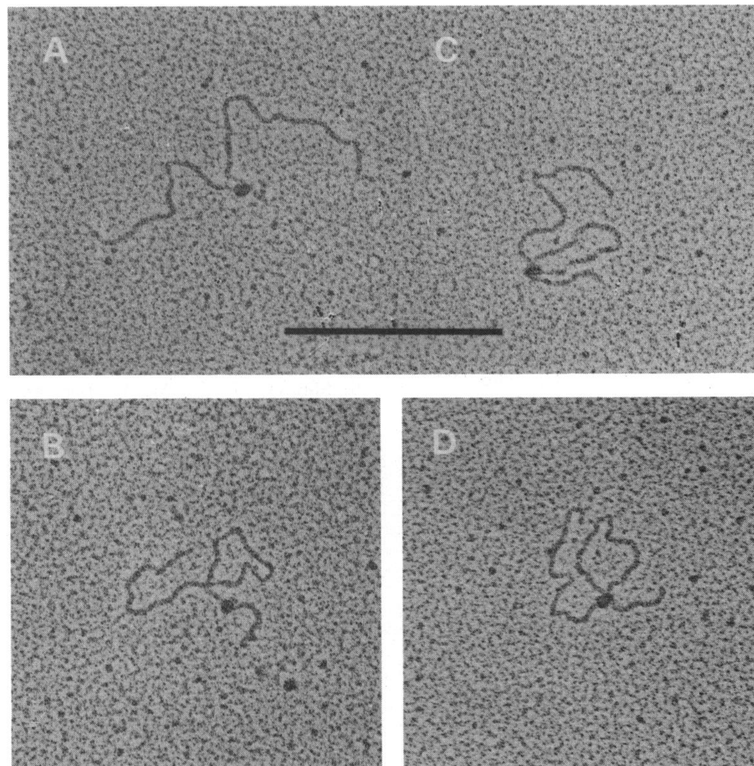

FIG. 5. Examples of DNA-protein complexes between a 2.5-kb DNA fragment from pALA318 and purified RepA. The protein is apparently present at the *ori* site (A), at the *incA* site (B), and in the two sites simultaneously, causing the intervening DNA to loop (C and D). Bar, 1 kb.

that raises the copy number of *incA*-deleted plasmids to eight, the copy number of *incA*-carrying plasmids should be about three to give the same final repeat concentration; clearly, it did not even reach two (Fig. 3). From in vitro gel retardation (1) and in vivo titration assays (6), the repeats of the *incA* and *incC* loci appear to bind RepA about equally well. These considerations lead us to believe that a stoichiometric binding to RepA cannot be the only mode of action of *incA*.

A second observation inconsistent with the sequestration hypothesis was that extra RepA could not relieve replication inhibition of one mini-P1 plasmid in the presence of another (Table 2). This result is, however, consistent with the finding that at no RepA concentration did the copy number of an *incA*-carrying plasmid reach even two (Fig. 3). In view of the ability of RepA to pair repeat sequences both in *cis* and in *trans*, we propose that extra repeats can cause steric hindrance to the origin function.

The control of copy number of mini-P1 replicons can be viewed as follows. Under physiological conditions, in which RepA is limiting, pairing in *cis* (DNA looping) is the primary interaction that slows down RepA synthesis by maintaining autorepression and causes steric hindrance to initiation at the same time. Eventually, when the sites are saturated with RepA dimers, initiation can occur, the probability of which depends on the reversibility of *cis* pairing. Following replication, the concentration of repeat sequences increases and so does the probability of pairing. The added opportunity to pair in *trans* could provide the negative feedback part of the control circuit. This negative feedback would presumably be in addition to the negative feedback elements of the sequestration model (the extra binding sites created after replication). RepA ceases to be rate limiting when its concentration is high enough to keep the binding sites occupied essentially all the time (Fig. 4). At such RepA concentrations, the replication rate is controlled solely by steric hindrance, the *trans* component of which increases following replication and decreases due to cell growth and consequent dilution of the DNA concentration. In order for the steric hindrance model to explain RepA-insensitive replication at relatively high RepA concentrations, the pairing ought to occur between two dimers, each bound to a repeat sequence, rather than between the two monomers of a RepA dimer. In the latter case, species B and D (Fig. 4) would not form, and so there could not be any control by steric hindrance. Efforts are under way to test this prediction.

A complication in understanding the relationship of RepA concentration to copy number is the finding that when *repA* expression was increased about fivefold over the physiological level, the copy number began to decrease (7) (Fig. 3a). The basis of this overexpression-inhibition in vivo is not yet understood. In contrast, in vitro replication of a mini-P1 plasmid was not appreciably inhibited even when the concentration of purified RepA was increased 100-fold over that required for maximal replication (27). When *repA* ATG was mutated to ATA (coordinate 666) in a plasmid that normally overproduces RepA in *trans*, the RepA protein band could not be seen in Western blots, as expected, but surprisingly, lysogen formation by our wild-type mini-P1, λ-P1:5R, was still inhibited (K. Muraiso and D. K. Chattoraj, unpublished results). These results indicate that the inhibitor may not be RepA itself but either *repA* mRNA or some other product from the same region of the DNA. Our current efforts are directed towards clarifying the nature of this inhibition.

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