JOURNAL OF BACTERIOLOGY, Aug. 1981, p. 509-516
0021-9193/81/080509-08$02.00/0

Vol. 147, No. 2

Replication Control and Switch-Off Function as Observed with a Mini-F Factor Plasmid

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Received 9 March 1981/Accepted 8 May 1981

Mini-F is a fragment of the F plasmid, consisting of 9,000 base pairs, which carries all of the genes and sites required for replicon maintenance and control. Its copy number is one to two per chromosome. This plasmid is joined to ColE1, whose copy number is 16 to 20. Under normal circumstances the composite plasmid replication exhibited ColE1 characteristics, maintaining a high copy number. However, when ColE1 replication was inhibited by deoxyribonucleic acid polymerase I inactivation, its replication exhibited mini-F characteristics, maintaining a low copy number. These observations are in complete agreement with those of Timmis et al. (Proc. Natl. Acad. Sci. U.S.A. **71**:4556-4560, 1974), who examined the behavior of a recombinant plasmid formed between pSC101 and ColE1. The transition from high to low copy number allowed us to examine the control system acting in cells carrying plasmids exhibiting intermediate copy numbers. The initiation of the mini-F replication system as represented by deoxyribonucleic acid synthesis of the composite plasmid was completely blocked when there were multiple copies of mini-F in a cell. It was not restored until the copy number was lowered to one to two, after which replication was first detected. ppF, a mini-F replicon packaged in a phage λ head behaved similarly: its replication was completely shut off when the resident mini-F genome copy number was high and was inhibited partially when the resident mini-F genome copy number was low. These experiments clearly demonstrate that there is a switch-off mechanism acting on deoxyribonucleic acid synthesis (initiation) in a cell carrying mini-F, and its intensity is related to the plasmid copy number. This result supports the "inhibitor dilution model" proposed by Pritchard et al. (Symp. Soc. Gen. Microbiol. **19**:263-297, 1969). The nature of the hypothetical inhibitor is discussed.

Replication control of plasmids has been studied intensively as a model system for understanding the control of more complex replicons, such as chromosomes. We have chosen the mini-F factor (hereafter referred to as mini-F) for these studies. The mini-F (8, 18, 21) is a plasmid containing about one-tenth of the *Escherichia coli* sex factor F (7) DNA. It replicates under a stringent replication control, with one or two copies of the plasmid maintained per chromosome. All of the genes and elements for replication and control of the F factor are clustered in the mini-F. Thus, the mini-F replicates in the same fashion as does the parental F factor. However, the genes and sites playing roles in replication control of this replicon have not yet been clarified.

Evidence has been accumulating in recent studies which show that a large part of replication control can be explained by a negative control model (15, 16). This model assumes that a negatively acting substance(s), such as a repressor, is accumulated in the cells, thereby switching off the initiation event most of the time, except when the copy number is lowered below a critical level. At a low copy number this switch-off function is relaxed, and the plasmid initiates a round(s) of replication. We were interested in examining the nature of the switch-off function and its mode of action in the replication control of mini-F. For this purpose, we chose a composite plasmid consisting of ColE1 and mini-F. This composite plasmid replicates under relaxed replication control of ColE1, with about 14 to 16 copies maintained per chromosome.

It was found that the composite plasmid exerts a strong switch-off function against other mini-F genomes carried in the same cell in an autonomously replicating state. This phenomenon seems to be similar to incompatibility in which a chromosomally integrated F factor (in an Hfr state) hinders stable maintenance of an autono-

mously replicating F factor (12; for further discussion of incompatibility between two autonomously replicating elements, see reference 6 and 13). Upon elimination of host *polA* function, the composite plasmid replicates by the mini-F replication system and the copy number is lowered to one or two. By using an appropriate *polA*(Ts) host strain and changing the temperature, intermediate composite plasmid copy numbers can be attained. Thus, we can artificially control the copy number of mini-F and examine the switch-off function.

Activity of the switch-off function was monitored with ppF, a mini-F joined to a region of phage λ along with a gene coding for ampicillin resistance (5). This genome has no system for replication other than mini-F. It is packaged into λ heads and can be injected into cells. The injected genome soon starts to replicate and becomes a plasmid if the cell does not carry an F-related plasmid showing FI incompatibility. As the DNA of ppF can be readily separated from mini-F, ColE1, or the composite plasmid by its difference in size, it is possible to monitor the replication of each of these genomes in cells.

Using this system we were able to show that a multicopy mini-F genome in a cell establishes a switch-off function. No replication of the mini-F genome is allowed under such conditions. The switch-off function disappears when the copy number of mini-F is lowered to about two.

MATERIALS AND METHODS

Bacterial strains. Km805 (*galT1 galK2 rpsL sup*^{0}) and km1213 [*polA*(Ts) *his argG metB leu rpsL xyl lacY thy*] are both derivatives of *E. coli* K-12 and were obtained from A. D. Kaiser as strain 594 and from A. Oka as strain JC411, respectively. The km1213 strain grows normally in PBBYT medium (see below) at temperatures below 40°C, but grows poorly at 42°C. At 30 to 37°C its *polA* function acts normally.

Plasmids. The plasmids used in this study are listed in Table 1.

Construction of the composite plasmids, ColAp-mini-F and ColKm-mini-F. ColAp-mini-F was constructed by insertion of mini-F (the f5 fragment of F′ *lac*) (8, 18, 21) into the *Eco*RI site of ColAp. ColKm-mini-F was constructed similarly, except that ColKm (8) was partially cleaved by *Eco*RI and used in place of ColAp.

Media. PBB and PB media have been described elsewhere (9). PBBYT medium is PBB containing 0.25% yeast extract and 20 μg of thymine per ml. PBCMM medium is PB supplemented with 10 μg of Casamino Acids per ml, 0.2% maltose, and 10 mM $MgSO_4$. Plates were made from the PBBYT with 1.5% agar. Ampicillin and kanamycin were purchased from Takeda Pharmacetucial Co. and Meiji Pharmaceutical Co., respectively. These drugs were used at concentrations of 20 μg/ml.

TABLE 1. *Plasmids used*

Plasmid	Source and properties
Mini-F-Ap (pSC138)	Described by Timmis et al. (21). It consists of the f5 fragment (a 9.0-kilobase *Eco*RI fragment of F′ *lac*) and a staphylococcal plasmid DNA fragment (6.3 kilobases) that carries the gene for β-lactamase. Obtained from S. N. Cohen.
Mini-F-Km (pML31)	The f5 fragment joined to an *Eco*RI-treated fragment (6.3 kilobases) from pML21 (8) carrying the gene for kanamycin resistance. It is the same as pML31 (8).
ColAp (ColE1-Tn*3*)	A ColE1 plasmid (6.4 kilobases) carrying an ampicillin-resistant transposon, Tn*3*, in or near the *mob* region of ColE1 (14). Obtained from J. Asaka.
ColKm	A recombinant plasmid consisting of the ColE1 plasmid and a DNA fragment carrying the gene for kanamycin resistance. It is the same as pML21 (8).
ColAp-mini-F	A recombinant plasmid consisting of the f5 fragment and ColAp joined together at the *Eco*RI sites. This work.
ColKm-mini-F	A recombinant plasmid consisting of the f5 fragment and ColKm cleaved at one site by *Eco*RI. This work.
ppF	A recombinant plasmid consisting of the mini-F-Ap and a region of phage λ from which the λ replication genes have been eliminated. This recombinant genome can be packaged in a λ phage coat and injected into cells, and can replicate as a plasmid (5).

Enzymes. The restriction enzyme *Eco*RI was a gift of T. Sakata. T4 DNA ligase was furnished by E. Akaboshi.

Determination of plasmid copy number. Plasmid carrier cells were labeled uniformly with [^{3}H]-thymidine as described previously (9), except that 7 ml of bacterial culture in PBBYT and 20 μCi of [^{3}H]thymidine were employed. They were then lysed with lysozyme and sodium sarkosynate, and the whole lysate was centrifuged in a CsCl-ethidium bromide density gradient to measure the amount of ^{3}H in the covalently closed DNA form (plasmid) and in the linear form (chromosome).

Sucrose gradient sedimentation. Sucrose gradient sedimentation of the DNA synthesized in bacteria infected with ppF was done as described previously (9).

RESULTS

Characteristics of composite plasmids; copy number and incompatibility. According to Timmis et al. (20) and Cabello et al. (1), a recombinant plasmid with two replication origins formed between two plasmids having different copy numbers has the characteristics

of the replicon with the higher copy number. When the replication of the higher-copy-number plasmid is impaired, then the composite plasmid has the characteristics of the lower-copy-number plasmid. We constructed two composite plasmids, ColAp-mini-F and ColKm-mini-F, both consisting of ColE1, a high-copy-number replicon, and of mini-F, a low-copy-number replicon. These plasmids were transformed into an *E. coli* K-12 *polA*(Ts) strain. *polA* function, which is necessary for autonomous ColE1 plasmid replication, is active at 30°C, but inactive at temperatures above 40°C, in this strain. Thus, copy numbers of the composite plasmids should be high at low temperatures and low at higher temperatures. The results in Table 2 demonstrate that the composite plasmids behaved as expected, making it highly likely that the ColE1 autonomous replication system was used at low temperatures, and the mini-F system was used at high temperatures.

To monitor the replication control system acting in cells carrying the mini-F or composite replicon, we infected cells with ppF, which carries an ampicillin-resistance marker. Cells perpetuating mini-F-Km or ColKm-mini-F were infected with ppF, and the numbers of kanamycin-resistant and ampicillin-resistant cells that carried the resident and incoming plasmids were measured. The results in Fig. 1 show that mini-K-Km and ppF coexisted transiently and gradually segregated. On the other hand, ColKm-mini-F and ppF did not show any sign of transient coexistence. In this case, it was always ppF that was excluded. Transformation of cells carrying mini-F-Ap or ColAp-mini-F by mini-F-Km DNA showed similar results (Table 3). Here again, establishment of plasmid state by incoming mini-F-Km was hindered by a resident mini-F-Ap and was completely blocked by the composite plasmid ColAp-mini-F. We concluded that incompatibility between the ColE1-mini-F plasmid and mini-F is much more extreme than that between two mini-F plasmids. These observations are in agreement with that of Cabello et al. (1) with ColE1-pSC101 and pSC101 and suggest that the mini-F genome in the high-copy-number composite plasmids is replicating by the ColE1 system. Since the normal copy number of mini-F is exceeded, its replication system is switched off.

TABLE 2. *Copy number of mini-F-Ap, ColAp, and composite plasmids in a polA(Ts) host cell at 30 and 40°C*[a]

Plasmid	Copy no. at:	
	30°C	40°C
Mini-F-Ap	2	2
ColAp	14	0
ColAp-mini-F	16	2
ColKm-mini-F	14	2

[a] Bacteria [km1213, *polA*(Ts)] carrying the indicated plasmids were grown overnight in PBBYT at the indicated temperature, diluted, and regrown at the same temperature in the presence of [^{3}H]thymidine. Cells were then lysed, and the DNA was banded in CsCl-ethidium bromide density gradients as described in the text. The copy number was measured from the ratio of DNA in covalently closed circular form to that of chromosome.

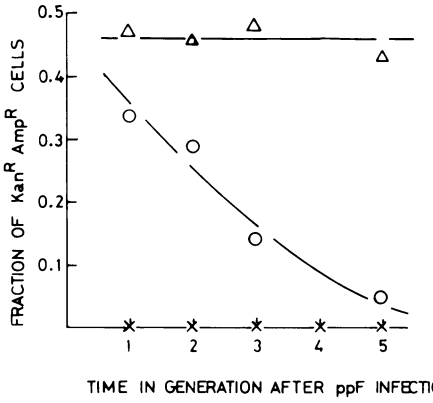

FIG. 1. *Incompatibility between resident mini-F-Km or ColKm-mini-F and incoming ppF. Bacteria (km805) carrying mini-F-Km* (○), *ColKm-mini-F* (×), *or ColKm* (△) *were grown in PBB to 2 × 10^8 cells per ml and then infected with ppF at a multiplicity of 0.5 (time zero). The cells were allowed to grow in the absence of drugs. At the times indicated samples were withdrawn, appropriately diluted, and spread over PBBYT agar with or without kanamycin plus ampicillin, to score for the fraction of double antibiotic-resistant cells that carry incoming ppF and resident plasmid.*

TABLE 3. *Efficiency of transformation with mini-F-Km DNA of cells carrying ColAp, mini-F-Ap, or ColAp-mini-F*[a]

Resident plasmid	No. of Kan^r transformants
None	177
ColAp	183
Mini-F-Ap	30
ColAp-mini-F	0

[a] Bacteria (km805) carrying the indicated plasmids were grown to 6 × 10^8 cells per ml, washed, and treated with $CaCl_2$. To samples (2 × 10^8 cells) of the $CaCl_2$-treated cells was added 0.03 μg of mini-F-Km DNA; cells were kept at 0°C for 30 min and then incubated at 37°C for 30 min. The cells were then spread over PBBYT agar containing kanamycin to score for the number of kanamycin-resistant transformants.

Temperature shift experiments to switch replication systems. If the passive replication of the mini-F replicon by the higher-copy-number ColE1 replicon is associated with a switch-off function, it follows that one can estimate the critical concentration of the mini-F genome at which it no longer exerts the switch-off function by following the reduction in copy number of the composite plasmid upon blocking the function of the ColE1 replication system. To monitor the change in copy number, we first measured the kinetics of loss of ColAp in *polA*(Ts) cells at 40°C (Fig. 2).

Plasmidless cells appeared four generations after the temperature shift, and the nonreplicating plasmids were lost exponentially from the cells. The profile is in perfect agreement with a model (6, 13) in which an average of 16 plasmids per cell are unable to replicate and are randomly assorted into dividing daughter cells. According to this model, at four generations, the average plasmid number per cells is reduced to 1. Composite plasmids will behave similarly after a temperature increase until the mini-F replication system becomes active. The temperature increase did not impair the growth of the host cells, as judged from the number of viable colony formers, growth rates, and rates of chromosomal DNA synthesis before and after the temperature increase (data not shown). The presence of plasmids, including ColAp, mini-F-Ap, or ColAp-mini-F, did not affect these results.

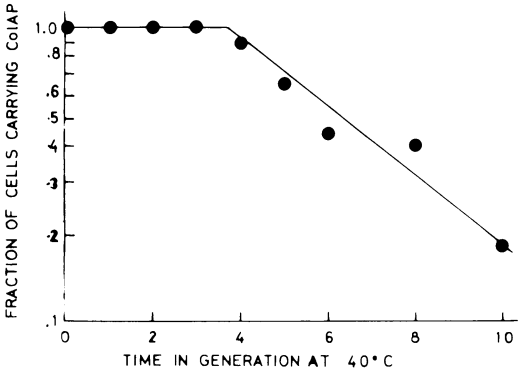

FIG. 2. *Kinetics of loss of ColAp from a polA(Ts) host at high temperature. PolA(Ts) bacteria (km1213) carrying ColAp were grown in PBBYT to 3 × 10⁸ cells per ml at 30°C. The temperature was shifted up to 40°C (time zero), and the cells were maintained at the high temperature with twofold dilutions with fresh PBBYT at every cell doubling (60 min). At the times indicated, samples were withdrawn, appropriately diluted, and plated on PBBYT agars with or without ampicillin to determine the number of cells carrying the ColAp or total viable cells, respectively.*

Figure 3A shows that ColAp plasmid DNA synthesis was abolished soon after the temperature increase. Figure 3B shows that replication of composite ColAp-mini-F plasmid did not occur for at least three generations after the temperature increases. Only after five generations did the synthesis of the composite plasmid DNA become detectable. These experiments were repeated three times with identical results. Under the same conditions the mini-F replication system was not impaired, as mini-F-Ap was maintained at a copy number of one to two per chromosome in the same *polA*(Ts) cell and exhibited a normal rate of DNA synthesis without lag after a temperature increase (data not shown). These results, in combination with those in Fig. 2, suggest that the mini-F replication system was switched off while there were multiple mini-F copies per cell and became active after three to five generations at 40°C when the copy number was lowered to one or two.

To examine the reverse process, viz., shift from mini-F-driven to ColE1-driven replication, *polA*(Ts) cells harboring one or two copies of the composite plasmid per chromosome as a result of prolonged growth at a high temperature were transferred to a low temperature. The rate of plasmid DNA synthesis indicated that it was occurring via the ColE1 replication system soon after the temperature decrease (Fig. 4). At zero, one, and two generations, 40, 60, and 80%, respectively, of the maximum amount of replication was restored. The gradual increase may reflect the gradual restoration of DNA polymerase level, though we do not have enough data to support this idea at present.

Replication of superinfecting ppF genomes. To further monitor the intensity of the switch-off function as a function of the mini-F copy number, we examined DNA synthesis of ppF that had recently infected cells carrying ColAp-mini-F or mini-F-Ap. If the strength of the switch-off activity depends on the copy number of the resident mini-F genome, such differences may be reflected in the extent of DNA synthesis of ppF that replicates by the mini-F replicational system. This was monitored by radiolabeling the infected cells with [^{3}H]thymidine, purifying the mixture of plasmid DNAs in the covalently closed circular form, and separating the resident plasmid and ppF DNAs in velocity sedimentations through sucrose gradients.

The results in Fig. 5 demonstrate clearly that DNA synthesis of incoming ppF is limited (≤10% of normal level) in cells carrying mini-F (one or two copies). It is completely switched off in cells carrying a ColAp-mini-F composite plasmid (16

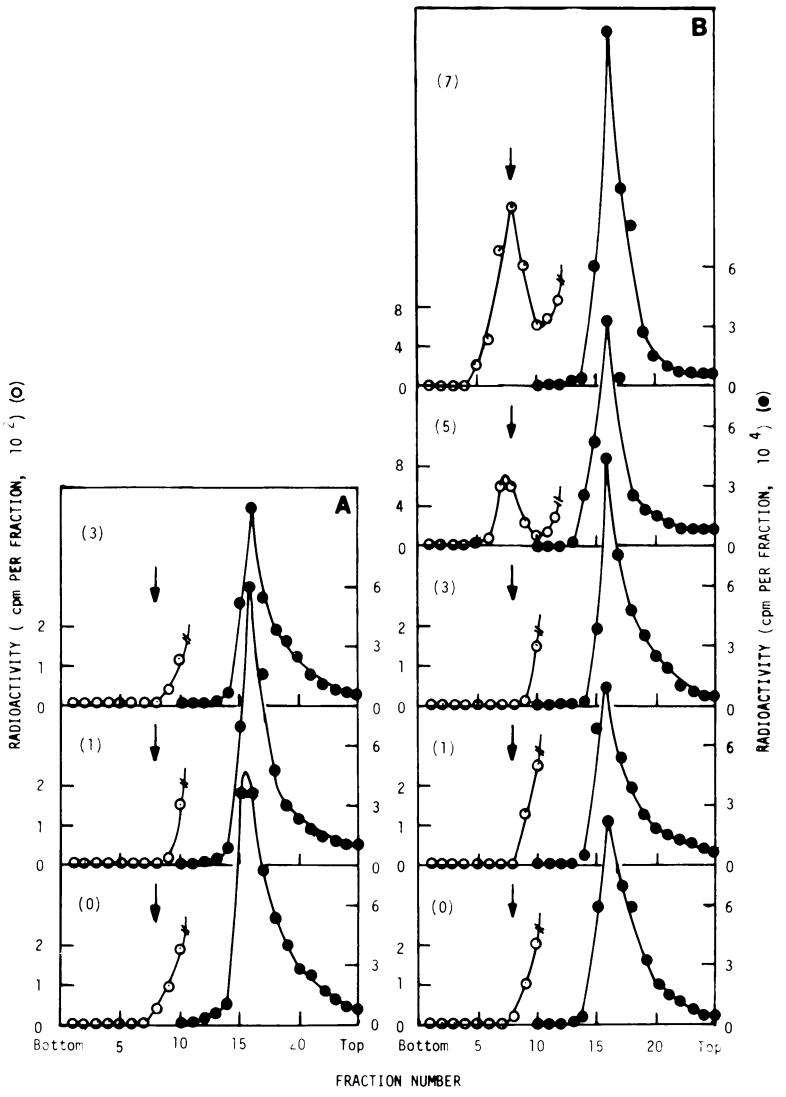

FIG. 3. *Absence of replication of ColAp or ColAp-mini-F in a polA(Ts) host cell upon elevating the temperature and resumption of replication upon prolonged incubation. Bacteria (km1213) carrying ColAp (A) or ColAp-mini-F (B) were grown at 30°C, and then the temperature was shifted to 40°C. The cells were allowed to grow at that temperature with periodic dilutions as described in the legend to Fig. 2. At the indicated (generation) times samples of the culture were taken and incubated for 60 min with [^{3}H]thymidine at the same temperature. The cells were lysed, and DNAs were banded in CsCl-ethidium bromide density gradients as described in the text. The location of plasmid DNAs in covalently closed circular form is indicated by the arrow. The major DNA band represents the chromosomal DNA. Note that neither ColAp nor composite plasmid covalently closed circular DNA had been synthesized for three generations after the temperature increase. As expected, ColAp DNA synthesis was not detected in five- and seven-generation samples. The data were omitted, however, for the sake of clarity. Synthesis of composite plasmid DNA was detected in five- and seven-generation samples. Numbers represent time in generations at 40°C when [^{3}H]thymidine was added. Note the differences in scale of the ordinates.*

copies). Thus, the results of both of our observations on the switching off of replication, one with the composite plasmid and the other with the incoming ppF, are in agreement.

DISCUSSION

Cabello et al. (1) constructed a composite plasmid consisting of ColE1 and pSC101 and observed that under normal circumstances only

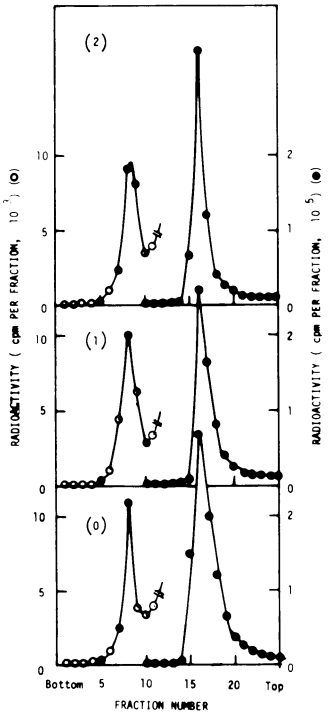

FIG. 4. *Resumption of ColAp-mini-F DNA synthesis in polA(Ts) cells upon decrease of temperature. Experiments were done as described in the legend to Fig. 3, except that cells carrying ColAp-mini-F were grown at 40°C, and the temperature was shifted to 30°C at time zero. Numbers indicate time (in generations) of incubation at 30°C before the addition of [³H]thymidine.*

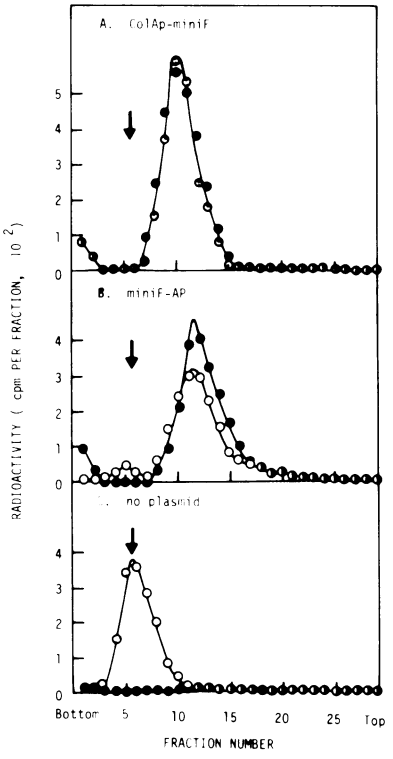

FIG. 5. *Synthesis of ppF DNA upon infection of cells carrying mini-F-Ap or ColAp-mini-F. Bacteria (km805) carrying the indicated plasmids were grown to log phase at 30°C and then infected with ppF (○) or mock infected (●) at a multiplicity of infection of 0.8. After 20 min, samples were taken and incubated for 50 min with [³H]thymidine. Cells were lysed, and covalently closed circular DNAs were prepared and sedimented through neutral sucrose gradients. Sedimentation was from right to left. Sedimentation profiles of these two samples are superimposed for the sake of clarity. Arrows indicate the position of ppF DNA in covalently closed circular form. The large peaks in A and B, respectively, represent ColAp-mini-F and mini-F-Ap, both in covalently closed circular form.*

the replication system of ColE1, the replicon with the higher copy number, is active. The replication system of pSC101, the replicon with the lower copy number is switched off. When the ColE1 replication system is blocked by denaturing the thermosensitive *polA* enzyme, the pSC101 replication system starts to function. Our observations on composite plasmid consisting of ColAp and mini-F are in complete agreement.

Kinetic studies demonstrated that the ColAp-mini-F composite replicon that was initially replicating by the ColE1 system stopped replicating when ColE1 replication was blocked by denaturing the thermosensitive *polA* function. The multicopy plasmid was diluted upon cell growth, indicating that the mini-F system remained switched off while the copy number was high. It was not until the copy number was lowered to near one or two that the mini-F replication system in the composite plasmid became active. The occurrence of random initiation, though reduced in frequency, was not observed while the cells carried extra mini-F copies. In other words, the mini-F replication system started to function only when the initiation mass became similar to that defined in the normal process of autonomous mini-F replication.

The switch-off function also blocked the superinfecting ppF replicon from synthesizing its DNA. The intensity of the blocking was strong when the cell carried the multicopy ColAp-mini-F and was moderate when the cell carried a low-copy-number, autonomously replicating mini-F. The switch-off of DNA synthesis of an incoming F plasmid by resident F has also been observed

by Saitoh and Hiraga (17). These observations fit well with the inhibitor dilution model of Pritchard et al. (15, 16), who assumed that cells harboring plasmids establish a switch-off mechanism to control plasmid copy number. They suggested that the switch-off of initiation by a repressor is followed by dilution of the repressor as a result of cellular mass increase to allow a switch-on of the next round of replication. This cycle of events is postulated to play a major role in the control of plasmid copy number. A similar switch-off effect has been observed with an incoming ColE1 inserted into a λ genome by a resident ColE1 plasmid (4).

Molin and Nordström (11) showed that the R1 replication system can be switched off by coexisting mini-R1 genomes. The switch-off function seemed to be more intense when they employed pBR322-derived multicopy pBR322-mini-R1 composite plasmid, rather than a single copy of R1. Timmis et al. (19) and Hashimoto-Gotoh and Inselburg (4) cloned a segment from R6-5 plasmid and ColE1, respectively, and saw similar effects. Thus, these replicons commonly exhibit copy number-dependent expression of incompatibility. Whether they share a similar mechanism for initiation control waits further elucidation.

The switch-off function, of necessity, involves a *trans*-acting control mechanism, such as a "diffusible inhibitor," that limits the frequency of initiation (2, 3, 16). Our study has demonstrated that the switch-off function acts quantitatively, its intensity being related to the copy number of the mini-F genome. Naturally, the replication system of these genomes must also have been subjected to the switch-off function. The nature of such *trans*-dominant diffusible inhibitors is not clear at present: Theoretically, they could be proteinaceous repressors as shown with R1 (2, 3, 22), or with λdv (10). RNA-like materials seen with ColE1 (Tomizawa, personal communication) could also be candidates. Alternatively, the diffusible inhibitors may not be the products of plasmid genes, but part or all of the plasmid DNA which, if present in excess in the cytoplasm, titrates some factor(s) indispensable for the initiation event. A model for such a system has been recently postulated by Tsurimoto (personal communication). He observed that a segment of the λdv genome carrying the replication origin that does not code for a unique protein or RNA interferes with the replication of λ phage λdv plasmid when carried in multiple copies in a cell.

Studies with nucleotide sequence in mini-F have revealed the presence of unique, tandemly repeating sequences (Murotsu et al., Gene, in press). The cloning of a region which is unlikely to code for a protein, but which carries these repeats, has been shown to express an incompatibility property when they are in a ColE1-type plasmid vector (Tsutsui and Matsubara, manuscript in preparation). These problems will be solved in the near future through obtaining and elucidating the plasmid region(s) responsible for the inhibitor production along with the site of its action.

ACKNOWLEDGMENT

This work was supported by grants from the Japanese Ministry of Education, Science and Culture.

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