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Characterization and Complementation of pMB1 Copy Number Mutant: Effect of RNA I Gene Dosage on Plasmid Copy Number and Incompatability

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A 16-base-pair insertion has been identified as the mutation responsible for the high-copy-number phenotype of the plasmid copy number mutant pFH118. The mutation is located near the plasmid origin of replication in a region of the genome that encodes two overlapping RNA transcripts. One of these transcripts, RNA I, acts as a negative regulator of plasmid replication. The second transcript is the precursor to the primer for the initiation of DNA synthesis. We demonstrate through complementation that the pFH118 DNA overproduction phenotype is a consequence of the reduced effectiveness of the mutant RNA I at inhibiting plasmid replication and not a consequence of an altered target site on the primer precursor. In addition, a series of plasmids containing multiple RNA I-coding genes was constructed for investigating the effects of RNA I gene dosage on plasmid copy number and incompatibility. The results of this study strongly support the inhibitor dilution model of plasmid copy control with RNA I as the plasmid-specified inhibitor responsible for both copy number control and incompatibility.

The stable inheritance of multicopy bacterial plasmids at defined copy numbers implies a mechanism that strictly regulates the number of plasmid replication events within each cell division cycle. The inhibitor dilution model (19, 20) proposes that initiation of plasmid DNA replication is repressed by a plasmid-specified, *trans*-acting element, whose cellular concentration reflects the concentration of plasmids in the cell. Plasmid replication increases the number of plasmid molecules and the number of active inhibitor genes inside the cell. When a "critical" level of inhibitor is obtained, initiation of plasmid DNA synthesis is completely repressed. During growth and division of the cell, the inhibitor is diluted below the critical concentration, permitting new initiation of plasmid DNA synthesis.

For the amplifiable plasmids containing the replicons of ColE1, pMB1, NTP1, p15A, or C1oDF13, there is accumulating evidence that a small, replicon-specified transcript, RNA I, functions as the primary inhibitor of plasmid DNA replication (6, 18, 25, 26). We previously proposed the involvement of RNA I in plasmid copy number control from our observation that the insertion mutation responsible for the mutant high-copy phenotype of the pMB1-derived plasmid, pFH118, lies within the coding region for this small transcript (6). Spontaneous revertants of pFH118, displaying the wild-type copy number, were found to have deleted the insertion sequence and produced a normal RNA I. Tomizawa et al. (26) later demonstrated that purified RNA I inhibits initiation of plasmid DNA synthesis in vitro by preventing the processing of a second plasmid transcript to form an RNA primer for DNA polymerase I. The primer precursor is initiated about 550 nucleotides upstream from the origin of DNA synthesis and terminates downstream of the origin (13). Processing of this transcript involves RNase H, an endonuclease which preferentially degrades RNA in RNA-DNA hybrids. RNA I, through an undefined mechanism, interacts with the primer precursor during its transcription to prevent the transcript from forming a stable RNA-DNA hybrid with its template near the replication origin (14, 25).

RNA I and the primer precursor are both transcribed from the same region of the plasmid genome, but from opposite DNA strands (13). One consequence of the transcripts' overlap is that the entire RNA I nucleotide sequence is complementary to the 5′ end of the primer precursor. The RNA I molecule has a high degree of internal sequence symmetry (17) and may, under normal physiological conditions, adopt a tRNA-like structure with three partially double-stranded regions and three single-stranded loops (designated by Roman numerals I, II, and III in Fig. 1b). The active sites of the RNA I

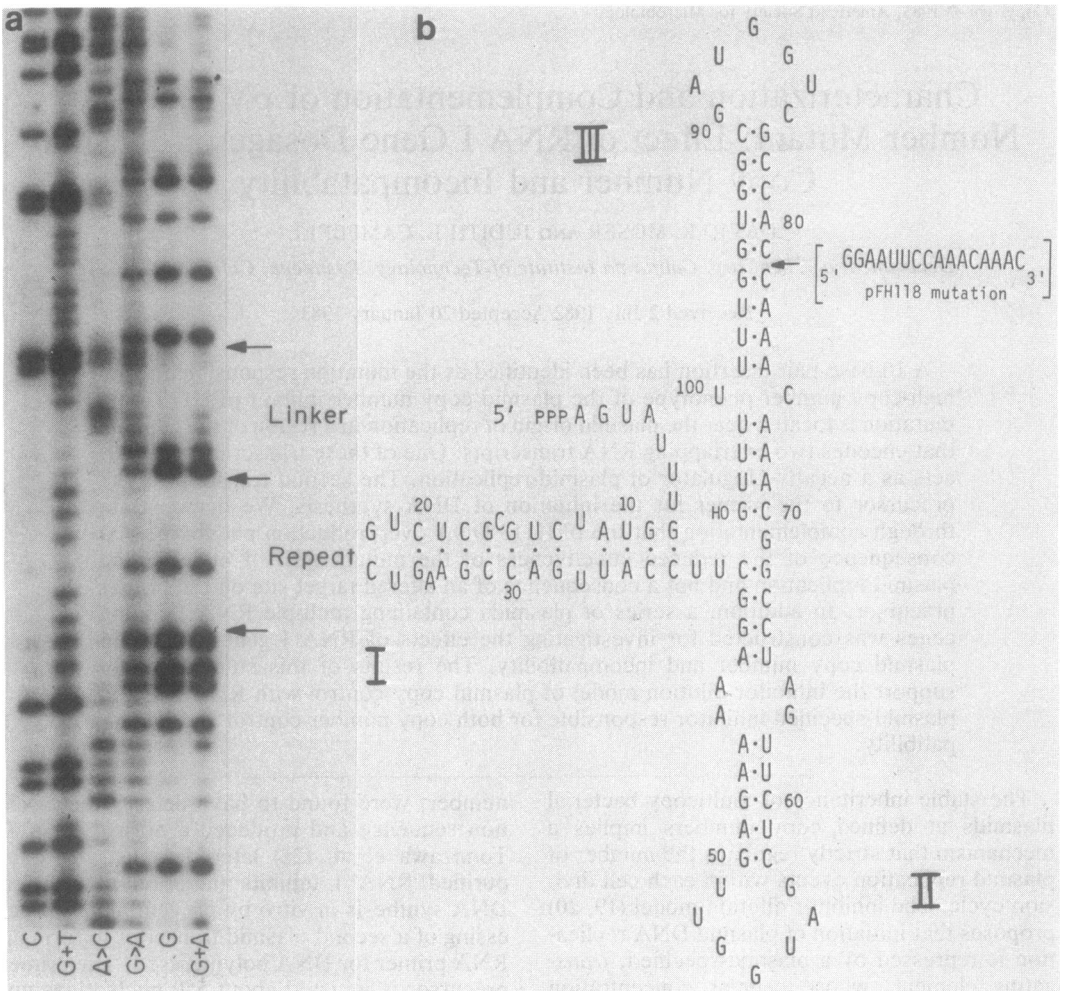

FIG. 1. (a) Representative sequencing gel of the RNA I-coding region of the copy number mutant pFH118. Sequencing was done by the method of Maxam and Gilbert (16). Details are provided in the text. The nucleotides bracketed by arrows are the additional nucleotides introduced into RSF1050 during mutagenesis. (b) Nucleotide sequence of RNA I from pFH118 as deduced from our DNA sequences. The secondary structure of the wild-type RNA shown is that proposed by Morita and Oka (17). The location of the insertion in pFH118 is indicated by the arrow, and the sequence of the additional nucleotides is presented in brackets. With the exception of this insertion, the nucleotide sequence of this region was found to be identical to that of pBR322 and pMB9, also derivatives of pMB1, and to differ from that of ColE1 at one position (24, 25).

molecule, like the active site of the tRNA, are considered to lie within one or more of the single-stranded loops (9, 14, 25). A similar stem-and-loop structure can also be predicted for the region of the primer precursor complementary to RNA I. From characterizations of inhibitor target mutants of pMB1, Lacatena and Cesareni (14) conclude that the secondary structure of this region of the primer precursor is required for successful inhibitory interaction. Sequence analysis of these mutants reveals the importance of the central loop (loop II) in the mechanism for plasmid copy number control.

In this paper we present results of complementation experiments with a high-copy-number mutant of pMB1 from which we conclude that loop structure III of RNA I is required for the transcript's inhibitory function. A 16-nucleotide insertion within the stem of this structure severely reduces the effectiveness of the transcript as a repressor of plasmid replication. The homologous insertion within the primer precursor does not appear to inactivate the target of inhibition. We also present evidence of a "critical" concentration of RNA I (as deduced from gene dosage) for complete repression of plasmid repli-

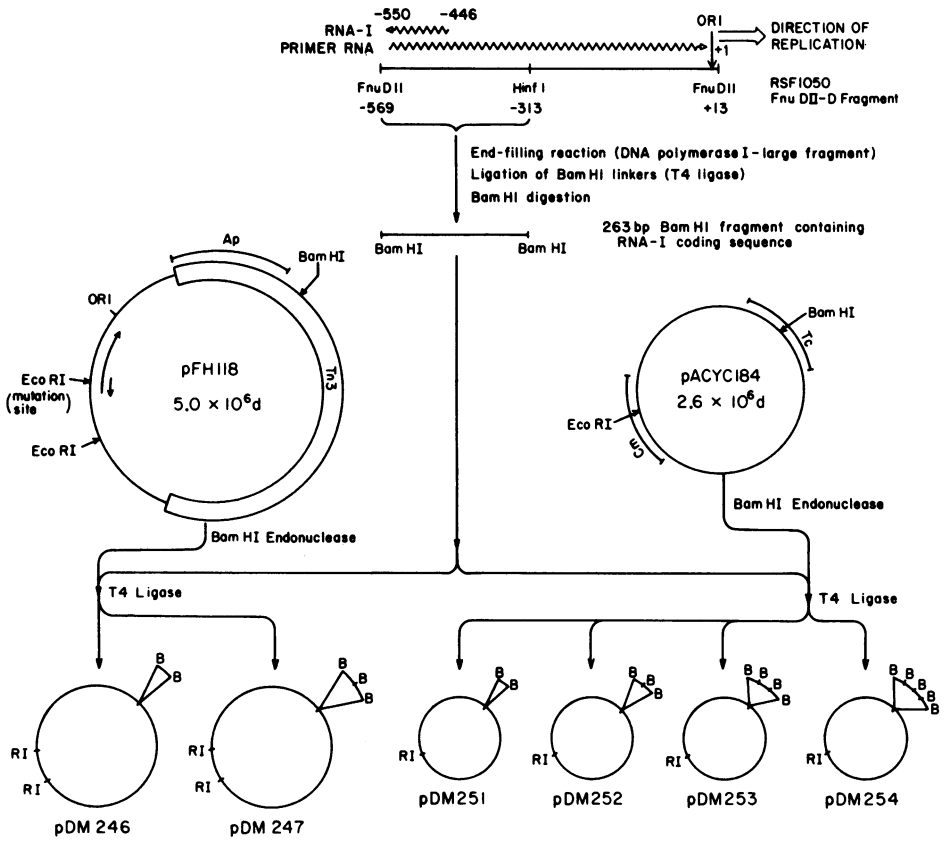

FIG. 2. Construction of plasmids. The 582-bp *Fnu*DII-D fragment of RSF1050 was cleaved with *Hin*fI to yield two fragments. The *Hin*fI ends of the fragments were made blunt ended by repair with DNA polymerase I. *Bam*HI ends were generated by attachment of *Bam*HI linkers followed by digestion with *Bam*HI endonuclease. The DNA fragments were separated from the unattached linkers by Bio-Gel A-5m (Bio-Rad Laboratories) chromatography and then ligated with *Bam*HI-digested pFH118. The ligation mixture was used to transform *E. coli* HB101. Ampicillin-resistant transformants were screened by restriction enzyme analysis of plasmid DNA from minilysates. Recombinant plasmids containing one and two 263-bp *Bam*HI inserts were isolated and designated pDM246 and pDM247, respectively. Purified inserts from *Bam*HI-digested pDM247 DNA were partially polymerized by allowing the inserts to self-ligate in the presence of T4 ligase. *Bam*HI-cut pACYC184 was then added, and ligation was continued. After transformation and screening of chloramphenicol-resistant, tetracycline-sensitive colonies, clones containing pACYC184 derivatives with one, two, three, and four *Bam*HI inserts were isolated. These plasmids were designated pDM251 through pDM254, respectively.

cation as postulated in the inhibitor dilution model. Finally, we present results which directly link plasmid copy-number control and incompatibility.

MATERIALS AND METHODS

Materials. *Bam*HI linkers (octamers) were obtained from Collaborative Research, Inc. Restriction enzymes were purchased from New England Biolabs. *Escherichia coli* DNA polymerase I (large fragment) was obtained from Boehringer Mannheim. Phage T4 polynucleotide ligase and kinase and *E. coli* RNA polymerase holoenzyme were gifts from Charles C. Richardson. [α-^{32}P]UTP (25 Ci/mmol) was from ICN Pharmaceuticals Inc.

Bacterial strains and plasmids. Experiments were carried out in *E. coli* HB101 *pro gal hsdR hsdM recA1*. Plasmid RSF1050 and its high-copy-number derivative, pFH118, have been described previously (12). Plasmid pACYC184 is a tetracycline- and chloramphenicol-resistant derivative of the cryptic miniplasmid p15A (3). Other plasmids used in these studies are described in Fig. 2 and Table 1.

Preparation of plasmid DNA. A modification of the method of Clewell and Helinski (4) was used to prepare plasmid DNAs and has been described previously in detail (5).

Agarose gel electrophoresis. Conditions for agarose gel electrophoresis have been previously described (7).

DNA sequencing. The 580-base-pair (bp) *Fnu*DII restriction fragment containing the origin region of RSF1050 (Fig. 2) was isolated from an acrylamide gel

TABLE 1. Plasmids used in this study

Plasmid	Replication origin	No. of copies[a]		Relative copy no.
		pMB1 RNA I-wt	pMB1 RNA I-mut	
RSF1050	RS1050 (wild type)	1		1
pFH118	pFH118 (mutant)		1	12
pDM246	pFH118	1	1	0.4
pDM247	pFH118	2	1	0.2
pDM248	pFH118		2	2.3
pACYC184	pACYC184			0.3
pDM251	pACYC184	1		0.3
pDM252	pACYC184	2		0.3
pDM253	pACYC184	3		0.3
pDM254	pACYC184	4		0.3

[a] The copy number of each plasmid was normalized to the amount of RSF1050, which was given a value of 1.

and labeled at the 5′ ends with T4 polynucleotide kinase and [γ^{32}P]ATP (21). This fragment was then cleaved with *Hae*III, generating a 430-bp fragment and a 150-bp fragment containing the RNA I-coding sequences. The nucleotide sequence of the 150-bp fragment was determined by the method of Maxam and Gilbert (16).

In vitro RNA synthesis. Covalently closed, circular plasmid DNA was transcribed by using purified *E. coli* RNA polymerase holoenzyme in a reaction mixture (0.1 ml) containing 50 mM Tris-hydrochloride (pH 7.9), 0.1 M KCl, 8 mM $MgCl_2$, 1 mM dithiothreitol, 10 μg of yeast tRNA per ml, 40 μM each of four ribonucleotide triphosphates (including [α-^{32}P]UTP), 5 U of RNA polymerase per ml, and 10 μg of plasmid DNA per ml. Incubation was at 37°C for 30 min. The reaction was terminated by addition of 5 μl of 250 mM EDTA. After precipitation with ethanol, transcription products were separated by electrophoresis in a denaturing polyacrylamide gel containing 7 M urea (15).

RESULTS

Nucleotide sequence of the copy number mutation in pFH118. The plasmid RSF1050 is a 5.0 × 10^6-dalton, ampicillin-resistant derivative of the plasmid pMB8 into which Tn*3* has been transposed (10). pMB8 is a miniderivative of the naturally occuring plasmid pBM1, which is closely related to, but not identical with, ColE1 (1, 22). The high-copy-number mutant plasmid pFH118 was generated from RSF1050 by a technique that employs synthetic oligonucleotide *Eco*RI linkers as mutagens (12). The mutant plasmid contains a new *Eco*RI site at the site of linker insertion. In pFH118 this site is located about 520 bp upstream (5′ to the direction of replication) from the origin of replication in the coding region for the RNA I transcript (6).

To determine the exact location of the linker insertion in pFH118, the nucleotide sequence of the RNA I-coding region was determined. A representative sequencing pattern and sequence of RNA I as deduced from the DNA sequence we have determined is shown in Fig. 1. The *Eco*RI octamer used to construct the mutant is inserted 57 bp from the RNA termination site. In addition, eight base pairs 5′ to this insertion are repeated on the 3′ side, making the total size of the insertion 16 bp. This repeat was probably generated from the in vitro mutagenesis procedure, in which linear plasmids with single-stranded ends are repaired with DNA polymerase I before the addition of *Eco*RI linkers. Similar structures have been reported among Tn*3* mutants prepared by this procedure (11). In vitro transcription of pFH118 in the presence of RNase H reveals that both RNA I and the primer RNA are 16 nucleotides longer than the corresponding transcripts from RSF1050 (data not shown). The 16 additional nucleotides fall within stem III of the proposed secondary structures of RNA I and its complementary region in the primer precursor.

Characterization of the pFH118 mutation: complementation by the wild-type RNA I. Since the mutation in pFH118 falls within the region of the plasmid that encodes the inhibitor RNA I and also the target site of RNA I inhibition on the primer precursor, the plasmid's high-copy-number phenotype could be attributed to either a defective inhibitor or a modified target site that is no longer recognized by the inhibitor or both. In the first case, the addition of wild-type RNA I should result in the complementation of the high-copy-number mutation. We decided to investigate this possibility by inserting the wild-type RNA I coding sequence into a nonessential region of the copy number mutant pFH118. To avoid ambiguities related to copy number effects mediated by plasmid regulatory elements other than RNA I, such as that identified by Twigg and Sherratt (27), we used a small restriction fragment which contains little more than the essential nucleotide information for transcription of the complete wild-type RNA I. A 256-bp *Fnu*DII-*Hin*fI restriction fragment from RSF1050 contains the complete wild-type RNA I-coding sequence plus 21 bp of DNA 3′ to the

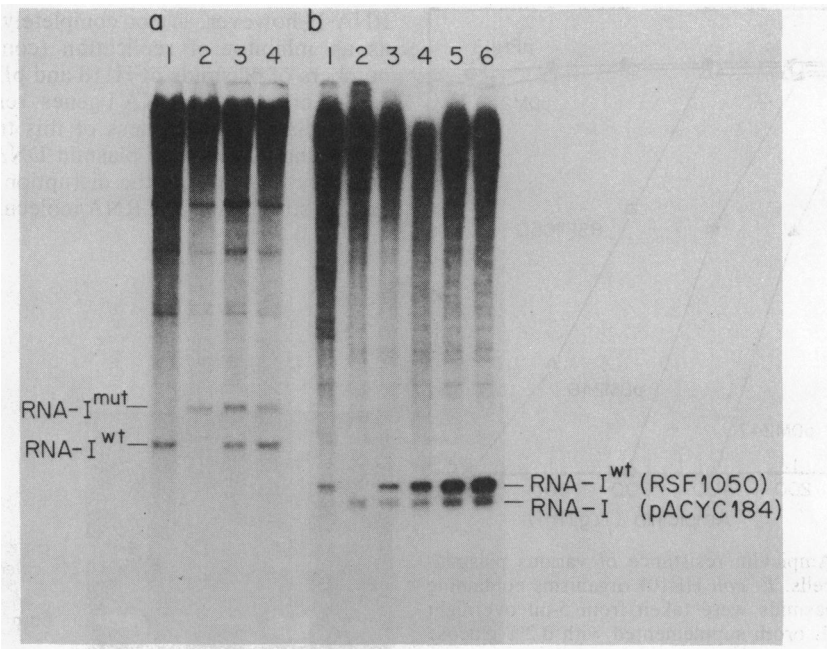

FIG. 3. In vitro synthesis of RNA I. Supercoiled plasmid DNA was transcribed in vitro as described in the text. ^{32}P-labeled transcripts were separated on either a 6% (a) or 8% (b) polyacrylamide gel (19:1 acrylamide/bisacrylamide ratio) containing 7 M urea. Gels were dried and autoradiographed as described previously (7). Plasmids transcribed were as follows: in a, (1) RSF1050, (2) pFH118, (3) pDM246, (4) pDM247; in b, (1) RSF1050, (2) pACYC184, (3) pDM251, (4) pDM252, (5) pDM253, (6) pDM254.

RNA I termination site and 130 bp of DNA 5′ to the transcript's initiation site, which includes the entire recognition sequence for RNA polymerase (Fig. 2 and Table 1). The fragment was inserted, by using *Bam*HI linkers, into the single *Bam*HI restriction site of pFH118, approximately 2.7 kb from the site of the copy number mutation. The recombinant plasmid, pDM246, when transcribed in vitro, produces two distinct RNA I transcript bands of approximately equal intensity which comigrate on a denaturing acrylamide gel with the RNA I transcripts of pFH118 (RNA I-mut) and RSF1050 (RNA I-wt) (Fig. 3a). A second recombinant which had picked up two *Bam*HI inserts was also isolated from cells transformed with the same ligation mixture. In vitro transcription of this plasmid, pDM247, yields both RNA I-wt and RNA I-mut transcripts at a ratio of approximately 2:1 (Fig. 3a). To test whether the wild-type RNA I transcribed from these plasmids complemented the high-copy-number mutation, we determined plasmid copy numbers.

Plasmid copy numbers were first examined in a general way from the relative resistances of plasmid-containing cells to ampicillin and then in a more quantitative way by direct analysis of plasmid DNA from cell lysates on agarose gels. Uhlin and Nordstrum (28) have shown for R1 plasmid derivatives that single cell resistance to ampicillin in agar plates is proportional to β-lactamase gene dosage. In Fig. 4, we compare ampicillin resistances of *E. coli* HB101 containing various plasmids. Bacterial cells which carry the high-copy-number mutant pFH118 are relatively insensitive to high levels of ampicillin up to 1,000 μg/ml, whereas bacteria carrying the homologous wild-type plasmid, RSF1050, are unable to grow at this drug concentration. Cells containing pDM246 are much more sensitive to lower ampicillin concentrations than is pFH118. This result suggests that the presence of the RNA I-wt gene complements the mutation in this plasmid, that is, that RNA I-wt binds better than RNA I mut to the pFH118 target. Cells containing plasmid pDM247 were the most sensitive to ampicillin, indicating that when two RNA I-wt genes are present, plasmid copy number is reduced even more. As a control, the RNA I-mut gene was isolated from pFH118 and inserted into the pFH118 *Bam*HI site. This plasmid, pDM248, is identical to pDM246, except that it carries the 16-bp insertion mutation within the cloned RNA I gene. Cells carrying pDM248, like cells carrying the high-copy number mutant pFH118, grow well on plates containing 1,000 μg of ampicillin per ml.

To confirm that plasmid copy number effects

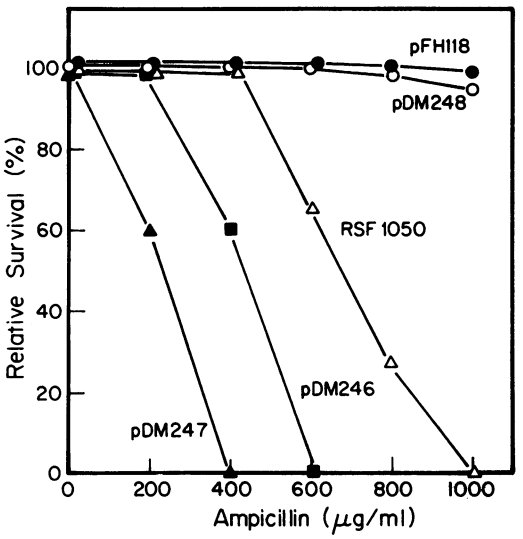

FIG. 4. Ampicillin resistance of various plasmid-containing cells. *E. coli* HB101 organisms containing indicated plasmids were taken from 5-ml overnight cultures in L broth supplemented with 0.2% glucose and spread onto L plates containing 0.2% glucose and various amounts of ampicillin. Visible colonies were counted after an 18-h incubation at 37°C. Relative survival is determined as a percentage of the colonies growing on plates without ampicillin.

were responsible for the observed differences in cellular resistance to ampicillin, quantitative determinations of plasmid copy number were made from electrophoretic analysis of plasmid DNA from cell lysates as described in the legend to Fig. 5. The plasmid copy numbers obtained with this procedure correlate well with the relative ampicillin resistances of cells containing these plasmids. These results are summarized in Table 1 and indicate that (i) the copy number of pFH118, as a result of the 16-bp insertion mutation within the RNA I coding region, is 12-fold higher than that of the homologous wild-type plasmid RSF1050; (ii) insertion of a 256-bp restriction fragment encoding the wild-type RNA I into pFH118 (pDM246) results in a 30-fold reduction of the plasmid copy number; (iii) insertion of a second RNA I-wt gene into this plasmid gives a further twofold copy number reduction (pDM247). In contrast, insertion of the mutant RNA I gene into the pFH118 *Bam*HI site gives only a fivefold reduction of copy number (pDM248). The copy number of plasmid pDM248 is still more than two times greater than that of the wild-type plasmid, RSF1050. From these observations we conclude that the plasmid overproduction phenotype of pFH118 results from the inability of RNA I-mut to effectively inhibit plasmid DNA replication. The mutant RNA I, however, is not completely ineffective as an inhibitor of replication (compare copy numbers of plasmids pFH118 and pDM248 containing one and two RNA I genes, respectively). The reduced effectiveness of this transcript in repressing initiation of plasmid DNA synthesis probably results from the disruption of the secondary structure of the RNA molecule by the 16

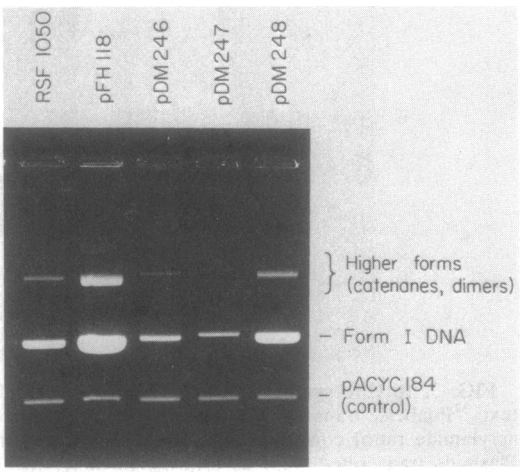

FIG. 5. Plasmid copy number comparison. *E. coli* HB101 cells containing plasmids RSF1050, pFH118, pDM246, pDM247, or pDM248 were grown in 150 ml of L broth containing 0.2% glucose and 25 μg of ampicillin per ml to an optical density at 550 nm between 0.6 and 0.9 and quickly chilled on ice. After dilution of the cultures to an optical density 0.6, 150 ml of each culture was mixed with an equal volume of a similarly diluted culture of HB101 cells containing the plasmid pACYC184. The addition of the pACYC184-containing cells was to provide an internal standard for monitoring the recovery of plasmid DNA after cell lysis. Cleared cell lysates were then prepared by the procedure of Clewell and Helinski (4), and supercoiled plasmid DNAs were isolated by equilibrium centrifugation in cesium chloride-ethidium bromide density gradients. The plasmid DNAs were then analyzed by electrophoresis through a 0.9% agarose gel. The lower band in each lane contains the supercoiled form of pACYC184. The upper bands contain the supercoiled forms of the pMB1-derived plasmids. The relative amounts of plasmid DNA were quantitated by densitometry. A photographic negative of another gel (not shown) containing different dilutions of these samples was scanned to ensure that comparative measurements of band densities were done within the linear range of the densitometer. Areas under the densitometry peaks were integrated with the aid of a Tektronix 4965 digitizing tablet interfaced to a Tektronix 4052 computer. By using the previously reported value of 18 for the minimal copy number of pACYC184 in HB101, the minimal copy numbers of the pMB1 plasmids were calculated to be as follows: RSF1050, 60; pFH118, 720; pDM246, 24; pDM247, 12; pDM248, 140.

additional nucleotides in stem III. Complementation of the pFH118 mutation by the wild-type RNA I indicates that the target of inhibition is not severely altered by the 16-nucleotide insertion within the primer precursor. This implies that stem structure III of the primer precursor is not of primary importance for this mechanism of copy number control.

RNA I and incompatibility. Two closely related plasmids are said to be incompatible if they are unable to stably coexist within the same bacterial cells growing under nonselective conditions. As cells which originally carry two incompatible plasmids divide, either one of the plasmids will be lost from the cell population or else the cells may segregate into two populations, each containing only one of the two plasmids. According to the inhibitor dilution model, incompatibility arises as a consequence of the inability of the plasmid-specified inhibitors to distinguish their respective replicons (20). Tomizawa and Itoh (25) have isolated several ColE1 mutants with altered incompatibility properties. These incompatibility mutants contain single-base-pair changes within the RNA I coding sequence. In vitro transcription experiments demonstrate that the single-base-pair changes affect both the ability of RNA I to inhibit primer formation and the sensitivity of primer formation to inhibition by RNA I. Each of the incompatibility mutants they examined was also a high-copy-number mutant, implicating RNA I as the plasmid-specified element involved in both copy number control and incompatibility.

We decided to further investigate incompatibility effects due to RNA I inhibition by inserting the pMB1 RNA I gene into a plasmid compatible with pMB1. The RNA I coding region from the pMB1-derived plasmid, RSF1050, was ligated into the single *Bam*HI restriction site of pACYC184 (Fig. 2). Plasmid pACYC184 contains the replicon from p15A and is compatible with ColE1-pMB1-derived plasmids (3). To compare the effects of RNA I gene dosage on incompatibility, four recombinant plasmids were constructed which contain from one to four identical pMB1-RNA I genes inserted into the pACYC184 *Bam*HI site. The chimeric plasmids, pDM251 through pDM254, each code for the transcription of two distinct species of RNA I (Fig. 3b). The slightly longer RNA I is identical to that transcribed from RSF1050. The smaller RNA I is transcribed from pACYC184.

We first examined the effects of the chimeric plasmids on the high-copy-number mutant pFH118. We attempted to prepare doubly transformed cells by introducing pFH118 by transformation into *E. coli* HB101 carrying pACYC184 or one of the chimeric plasmids, pDM251 through pDM254, and selecting on L agar plates containing chloramphenicol (25 μg/ml) and ampicillin (50 or 1,000 μg/ml). The results of this transformation are presented in Table 2. Cells containing pACYC184 were efficiently transformed to resistance to 50 μg of ampicillin per ml with pFH118. These double transformants also grew well on plates containing 1,000 μg of ampicillin per ml, indicating that pFH118 was present at high copy number. In contrast, transformation frequencies (from plates with 50 μg of ampicillin per ml) of cells containing any of the chimeric plasmids were appreciably lower and decreased with each additional pMB1 RNA I gene carried by the resident plasmid. With three or four of these genes present, no transformants with pFH118 could be obtained. If the resident plasmid contained only one or two of these genes, transformants with pFH118 could be isolated from plates with 50 μg of ampicillin per ml, but not from plates with 1,000 μg of ampicillin per ml, indicating that the copy number of pFH118 is reduced in these cells.

These results indicate that the pFH118 high-copy-number mutation is complemented by the pMB1 species of RNA I. In addition, we observe a correlation between the number of RNA I genes carried by the resident plasmid and the transformation frequency with pFH118 as the incoming plasmid. The most striking aspect of these results is the failure of pFH118 to become established in cells where there are more than two pMB1 RNA I genes on the resident plasmid. We interpret these results as indicating that the elevated level of RNA I in these cells at the time

TABLE 2. Transformation of various plasmid-carrying strains with pFH118 (Ap)[a]

Resident plasmid	No. of pMB1 RNA I coding regions	Transformants per μg of pFH118 DNA appearing on plates containing ampicillin	
		50 μg/ml	1,000 μg/ml
pACYC184 (Cm, Tc)	0	1.1×10^6	8.7×10^5
pDM251 (Cm)	1	6.0×10^5	$<10^3$
pDM252 (Cm)	2	2.0×10^5	$<10^3$
pDM253 (Cm)	3	$<10^3$	$<10^3$
pDM254 (Cm)	4	$<10^3$	$<10^3$

[a] HB101 cells carrying the indicated plasmids were transformed with 20 ng of pFH118 after a standard transformation procedure (8). Each transformation mixture was added to 2 ml of L broth and incubated at 37°C for 2 h before plating only L agar plates containing 0.2% glucose and the antibiotics chloramphenical (25 μg/ml) and ampicillin (50 μg/ml). After incubation for 18 h at 37°C the visible colonies were counted. A transformation frequency of $<10^3$/μg means that no transformants were observed.

TABLE 3. Exclusion of pFH118 and RSF1050 from cells after the establishment of a second plasmid encoding pMB1 RNA I[a]

Incoming plasmid	No. of pMB1 RNA I genes per plasmid	Fraction of total cells after 6 generations exhibiting ampicillin resistance	
		pFH118	RSF1050
pACYC184	0	1.0	1.0
pDM251	1	1.0	1.0
pDM252	2	0.8	1.0
pDM253	3	$<10^{-3}$	$<10^{-3}$
pDM254	4	$<10^{-3}$	$<10^{-3}$

[a] The plasmids indicated were used to transform *E. coli* HB101 carrying either pFH118 or RSF1050. Colonies which grew up on agar-plates containing both ampicillin (50 μg/ml) and chloramphenicol (25 μg/ml) were used to prepare cultures of cells growing in L broth supplemented with 0.2% glucose. After approximately six generations of growth at 37°C under nonselective conditions, samples of the cultures were spread onto L plates and L plates containing ampicillin (50 μg/ml). After overnight incubation of the plates at 37°C the colonies were counted, and the ratio of ampicillin-resistant cells to total cells was determined.

of pFH118 introduction completely inhibits replication of the incoming plasmid, thus preventing its establishment. In agreement with this interpretation we found that doubly transformed cells of pFH118 and each of the chimeras could be prepared by beginning with cells containing pFH118 and then introducing one of the chimeras by transformation. All of the doubly transformed cells prepared in this manner could be selected initially on plates containing chloramphenicol and 1,000 μg of ampicillin per ml.

Colonies from these plates were used to conduct a segregation test (Table 3). After six generations of growth in L broth in the absence of selective drugs, bacteria were spread onto L plates without drugs and L plates containing 50 μg of ampicillin per ml. We found that pFH118 was not excluded from the cells which also carried pACYC184 or the chimeras containing one or two pMB1 RNA I genes. However, pFH118 was rapidly lost from cells carrying the chimeras with three or four pMB1 RNA I genes. When segregaton tests were conducted with RSF1050 instead of pFH118, similar results were obtained, with RSF1050 being rapidly excluded only by pDM253 and pDM254.

These results can be explained according to the inhibitor dilution model. Since the p15A replicon of pACYC184 is not inhibited by the pMB1 RNA I species (26), the copy numbers of plasmids pDM251 through pDM254 are approximately equal to the copy number of pACYC184 (data not shown). We have observed that pACYC184 is maintained at a copy number that is approximately one-third that of the pMB1-derived plasmid, RSF1050 (Fig. 5). If the cellular concentration of RNA I is proportional to the number of RNA I genes, as postulated by the inhibitor dilution model, then the pMB1 RNA I concentration in cells containing the chimeric plasmids will be lower than the critical inhibitory concentration unless there are at least three copies of the gene transcribing this RNA per pACYC184 vector. The abrupt change in compatibility properties that we observe in going from two to three pMB1 RNA I genes per vector probably results from the increase in cellular RNA I concentration to a level exceeding the critical inhibitory concentration.

DISCUSSION

Perhaps the most powerful technique for investigating the molecular mechanisms responsible for the regulation of DNA synthesis in *E. coli* is the generation and characterization of plasmid copy number mutants. In this paper we extend our previous studies on the high-copy-number mutant pFH118 by identifying the mutation as a 16-bp insertion within the region of the plasmid genome which codes for the transcription of RNA I and the primer precursor. The mutation disrupts nucleotide pairing within the stem of one of three loop structures in the proposed secondary structure for both RNA I and the 5′ end of the primer precursor. We demonstrate through complementation studies that the mutation in pFH118 affects the ability of RNA I to inhibit plasmid replication without noticeably affecting the sensitivity of the plasmid to inhibition by wild-type RNA I. The pFH118 high-copy-number phenotype is suppressed when a 256-bp DNA fragment that codes for the transcription of the wild-type RNA I is cloned directly into pFH118 or into a second, unrelated plasmid within the same cell. The location of the mutation within the stem of the loop structure III (Fig. 1) suggests that this structure is critical for the inhibitory function of RNA I, but is not essential within the primer precursor for target site recognition. Lacatena and Cesareni (14) characterized dominant target mutations that were not complemented by wild-type RNA I. Their results demonstrated the importance of the central loop (loop II) in both RNA I and the primer precursor for successful inhibition. They have also found a dominant target mutation within the stem of loop I. Muesing et al. (18) have reported a copy number mutant of ColE1 that contains a single base-pair alteration within the stem of loop III. This mutation, like the mutation in pFH118, can be complemented in *trans* by the wild-type plasmid (23).

According to the inhibitor dilution model, plasmid copy number is regulated through a

mechanism of feedback inhibition. Plasmid replication increases the number of plasmids per cell, which in turn increases the concentration of the plasmid-specified inhibitor. Plasmid replication ceases when the level of the inhibitor reaches a "critical" level for the complete repression of initiation of plasmid DNA synthesis. One would predict from this model that if the number of inhibitor molecules produced from each plasmid molecule were doubled, the stable copy number of the plasmid would be reduced to half of its original value. In agreement with this prediction and with the evidence implicating RNA I as the plasmid-specified inhibitor of replication, we have observed that plasmid pDM247, which contains two identical wild-type RNA I genes, has a copy number which is one-half that of plasmid pDM246, which contains only a single wild-type RNA I gene.

We have also presented evidence directly linking RNA I inhibition to the phenomena of plasmid incompatibility, a finding previously reported by Tomizawa and Itoh (25). The plasmid pACYC184, which is normally compatible with the pMB1-derived plasmid, RSF1050, and its copy number mutant, pFH118, exhibits severe incompatibility toward these plasmids when more than two pMB1 RNA I genes are inserted into the plasmid's single *Bam*HI site. The rapid exclusion of RSF1050 and pFH118 from cells containing either pDM253 or pDM254 implies that the former pMB1-derived plasmids are unable to replicate in the presence of the latter plasmids containing three and four pMB1 RNA I genes. Transcription of RNA I from these plasmid genes is probably sufficient to maintain an RNA I level above the "critical" concentration for full inhibition of plasmid DNA synthesis from the pMB1 replicon.

Although it now seems clear from our work and the work of others that inhibition of plasmid DNA replication by RNA I is an important part of the mechanism for regulating plasmid copy number, there is evidence that other plasmid-encoded elements may be involved in copy control. Twigg and Sherratt (27) have shown that deletion of a nonessential region of ColE1 gives a derivative with an elevated copy number. The deletion mutation is complementable by a product encoded by the *Hae*II-C restriction fragment of ColE1. Cesareni et al. (2) have recently identified a gene within this fragment which encodes a 63-amino-acid polypeptide which they have designated rop (for repressor of primer). They proposed that the product of this gene regulates plasmid replication by repressing the transcription of the primer for DNA synthesis. Unlike RNA I, this product does not appear to be involved in determining plasmid incompatibility.

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