Proc. Natl. Acad. Sci. USA
Vol. 78, No. 10, pp. 6096–6100, October 1981
Biochemistry

Plasmid ColE1 incompatibility determined by interaction of RNA I with primer transcript

(mutants/transcription/copy number/hybridization/secondary structure)

JUN-ICHI TOMIZAWA AND TATEO ITOH

Laboratory of Molecular Biology, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20205

Communicated by Terrell L. Hill, July 2, 1981

ABSTRACT **Mutants of plasmid pNT7 that can coexist with plasmid pMB9 in growing bacteria have been isolated. These mutants show altered incompatibility properties and increased copy numbers. Each mutant has a single base change at or near the center of one of the three palindromes in the region that specifies two RNA species: a larger one (primer transcripts) that provides a primer for DNA replication and a smaller one (RNA I) that is the incompatibility-specific inhibitor of primer formation. *In vitro* transcription studies show that the single base changes affect both the ability of RNA I to inhibit primer formation and the sensitivity of primer formation to inhibition by RNA I. RNA I hybridizes to the primer transcript, and the rate of hybridization is reduced by the single base changes. Based on analyses of inhibition of *in vitro* primer formation by RNA I and of *in vivo* properties of the mutant plasmids, we conclude that incompatibility between two plasmids can be attributed to inhibition of primer formation on one of the plasmids by the RNA I of the other. Inhibition of primer formation by RNA I appears to be the mechanism that determines the copy number of pNT7 and its derivatives.**

Transcription of ColE1 DNA by *Escherichia coli* RNA polymerase *in vitro* starts at two sites in the region required for maintenance of the plasmid. Transcription from one of these sites yields products (primer transcripts) that extend beyond the origin of DNA replication. Approximately one-third of these products form a stable hybrid with the template DNA during the course of transcription and can be cleaved by RNase H to give a 555-nucleotide-long primer for DNA replication (1). Transcription from the other site proceeds on the opposite strand and terminates close to the primer initiation site to yield an RNA (species I or RNA I) about 108 nucleotides long (2). In the presence of added RNA I, an increased fraction of transcripts that pass the origin do not form a hybrid with the template DNA and thus primer formation is suppressed. Primer formation is not inhibited by RNA I once the hybrid has been formed, nor is it affected by the species I RNAs of plasmids that belong to several other incompatibility groups (3).

Here we report that a change of a single base in the region that specifies both RNA I and the primer transcripts can affect the inhibitory activity of RNA I and the sensitivity of primer formation to the small RNA. Such a change can alter both the incompatibility and copy number.

MATERIALS AND METHODS

Bacterial and Plasmid Strains. *E. coli* K-12 strain N100 is identical to W3102 *rec*A3 (4). NT101 is a *thy*$^-$ derivative of N100. Plasmids pNT7 (1), pMB9 (5), and pBR322 (6) belong to the ColE1 incompatibility group; pST10 (3) is compatible with ColE1. pNT31 was constructed by inserting a 785-base-pair (bp) *Taq* I fragment that carries the chloramphenicol acetyltransferase gene of Tn9 (7) into the unique *Taq* I site in the β-lactamase gene of pNT7. The transferase gene is transcribed from the promoter of the β-lactamase gene (data not shown).

Isolation of Mutants. Plasmid pNT7 is readily lost from growing bacteria when the bacteria carry pMB9 (see below and also ref. 8). To isolate mutants of pNT7 that are insensitive to pMB9, a culture of N100 carrying pNT7 was grown to 3×10^8 cells per ml at 37°C in 5 ml of L broth; chloramphenicol (100 μg/ml) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (20 μg/ml) were added, and incubation was continued overnight. Plasmid DNA from these cells was used to transform N100 carrying pMB9 (about 6×10^8 cells) (9). L broth (5 ml) was added to the transformation mixture and incubated overnight at 37°C. The culture was diluted 1:25 into broth containing ampicillin (25 μg/ml) and tetracycline (10 μg/ml) and incubated overnight. About 30% of the cultures prepared in this way grew to saturation overnight; none did so when unmutagenized pNT7 was used (30 cultures tested). A culture showing full growth was diluted $1:10^4$ into broth containing both drugs and incubated overnight. Bacteria in each culture were purified by single-colony isolation on a plate containing both drugs. DNA preparations from the bacteria were used to transform N100 to ampicillin resistance. All plasmids thus isolated showed altered incompatibility properties (see Table 2). These include pNT51, pNT52, and pNT57 through pNT62. By using mutagenized pMB9 to transform N100 carrying pNT7, mutants of pMB9 that do not readily exclude pNT7 were also isolated. One of these is pNT91.

Measurement of Plasmid Copy Number. The copy number was determined essentially as described (10). NT101 bacteria carrying a plasmid (monomer) were exponentially grown in a medium containing 1% Tryptone, 0.3% NaCl, 0.1% glucose, and 2 μg of [^{3}H]thymine per ml. DNA was extracted and hybridized to denatured pNT7 DNA that had been fixed on membrane filters. The average size of a replicating *E. coli* chromosome was taken to be 3.5×10^9 daltons [1.4 times the size of the unreplicating chromosome (11)], and the plasmid molecules were assumed to be in the unreplicating form.

Transcription of Plasmid DNA and Cleavage of the Transcripts by RNase III. General conditions used for transcription studies (1) and preparation of RNase III (12) have been described.

RESULTS

Base Changes in Mutant DNAs. The locations of mutations in plasmids were determined by exchanging a restriction fragment of the parent plasmid DNA with the corresponding fragment of a mutant plasmid DNA. Mutant DNA was cleaved by *Pst* I and *Bst*EII, and the smaller fragment was ligated to the larger fragment of pNT31 DNA or *vice versa* (Fig. 1). In each

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviation: bp, base pair(s).

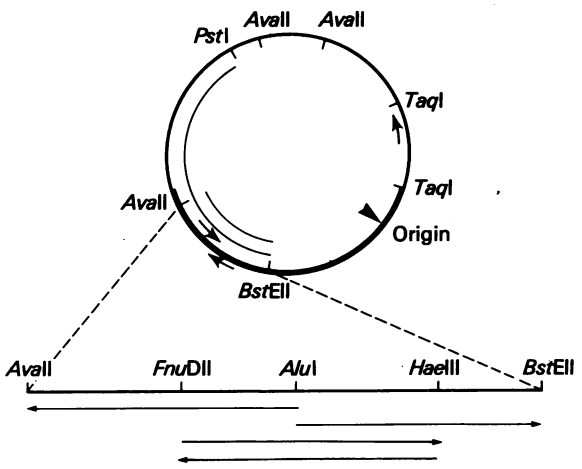

FIG. 1. Restriction map of pNT7. Heavy line, region derived from ColE1; thin line, portion derived from Tn3 transposon (1). Relevant restriction sites are indicated. In the upper part, arrows indicate the three major sites of transcription initiation and the direction of elongation, and an arrowhead indicates the origin of DNA replication. The arcs inside the circle indicate the two restriction fragments on which the mutations were found. Below is shown a portion of the genome with arrows indicating the 5′ → 3′ orientation of fragments used for nucleotide sequence determination.

case, the resultant plasmid containing the smaller *Pst* I–*Bst*EII segment of mutant DNA showed the mutant property (data not shown). To delimit the altered region further, *Ava* II–*Bst*EII fragments containing the primer promoter (Fig. 1) were isolated from the mutant DNAs and ligated to the appropriate fragment obtained from pNT31 DNA through partial *Ava* II digestion followed by complete *Bst*EII digestion (Fig. 1). Characterization of the resultant plasmids showed that the mutations were in the *Ava* II–*Bst*EII segment which contains the primer promoter and specifies RNA I (data not shown).

By determining the nucleotide sequences of the *Ava* II–*Bst*EII segments of eight mutant plasmids (Fig. 2), we identified four different point mutations. Each is present at or near the center of one of three palindromes in the region that specifies RNA I and the primer transcripts. The mutations in pNT59, pNT52, and pNT60 have been named *inc*1, *inc*3, and *inc*4, respectively. Plasmid pNT51 has two mutations, *inc*1 and *inc*2.

The nucleotide sequence of pMB9 in the region that specifies RNA I was found to be identical to that of pBR322 (15) and to differ from that of pNT7 at one position in palindrome I. We refer to the pNT7 sequence as *inc*$^+$ and call this difference *inc*9. An *inc*9 derivative (pNT73) of pNT7 was constructed by replacing a *Hpa* II segment of pNT7 by the corresponding segment of pBR322 that contains the *inc*9 allele. Plasmid pNT91 was found to be an *inc*2 mutant of pMB9.

Effect of Species I RNAs on Primer Formation. We examined the effect of the species I RNAs made by pNT7 and six representative mutants on primer formation on each plasmid. Experiments were performed under conditions that permitted initiation of transcription selectively from the primer promoter (Fig. 3), thus eliminating any effect due to RNA I synthesized *de novo*. Representative electrophoretograms are presented in Fig. 3. The results are summarized in Table 1. At a given RNA I concentration, the extent of inhibition of primer formation depended on the exact combination of template DNA and RNA I. Single base change can alter both the sensitivity of primer formation to RNA I (compare the values within a column of Table 1) and the activity of RNA I (compare the values across a row). Inhibition with homologous mutant pairs was always weaker than that with the wild-type pair (compare the values along a diagonal from the upper lefthand corner). When the template DNA and RNA I differed at two positions, the inhibition generally was poor; when they differed at three positions, the inhibitory effect was always small.

Interaction Between Primer Transcripts and RNA I. The finding that the region of plasmid DNA which specifies RNA I also determines sensitivity of primer formation to the RNA suggests that RNA I inhibits primer formation by interacting with the homologous region of the primer transcript or the template DNA. However, because RNA I is not degraded by RNase

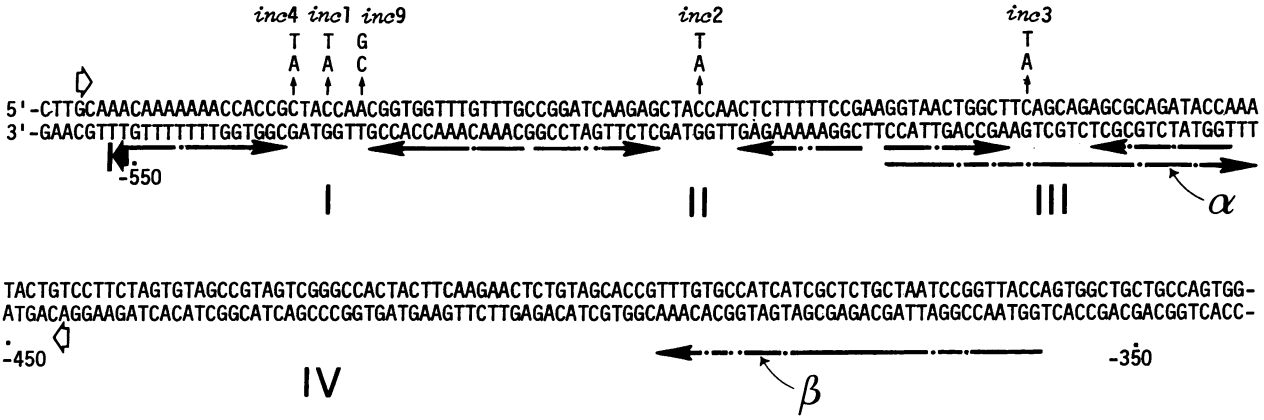

FIG. 2. Nucleotide sequences. The nucleotide sequences of the *Ava* II–*Bst*EII region (Fig. 1) of the mutants were determined as follows. The whole plasmid DNA was cleaved completely by *Alu* I. The resultant fragments were labeled with [γ-^{32}P]ATP and polynucleotide kinase and then cleaved by *Ava* II and *Bst*EII. A 142-bp *Alu* I–*Bst*EII fragment and a 157-bp *Ava* II–*Alu* I fragment were isolated and their nucleotide sequences were determined (13). Each mutant DNA was found to have a single base change in this region except for pNT51 which had two such changes. The change present in pNT52 recurred in pNT61 and pNT62; that in pNT57 was also found in pNT58 and pNT59. The alterations were confirmed by determination of nucleotide sequences of the complementary strands of four representative strains (pNT51, pNT52, pNT59, and pNT60) as follows. The DNAs of pNT51 and pNT52 were cleaved with *Hae* III and end-labeled. After cleavage with *Fnu*DII, a 160-bp fragment was isolated and its sequence was determined. The DNAs of pNT51, pNT59, and pNT60 were cleaved with *Fnu*DII, end-labeled, and then cleaved with *Hae* III to generate a 160-bp fragment whose sequence was determined. The sequences of the region that specifies RNA I of pMB9 and pNT91 were similarly determined. The locations of the mutations in these plasmids are indicated with the pNT7 sequence (14) of the region that specifies both RNA I and the first 220 nucleotides of the primer. Open arrows, major sites of transcription initiation and direction of elongation; solid arrow with bar, site at which most RNA I transcripts terminate; solid arrows, major palindromes in regions pertinent to the present report; dot in arrow, unpaired nucleotide within a palindrome. Palindromes I, II, III, and IV specify stem and loop structures I, II, III, and IV of the primer transcript and structures I′, II′, and III′ of RNA I. The sequences that specify the stem of structure IV are indicated by α and β.

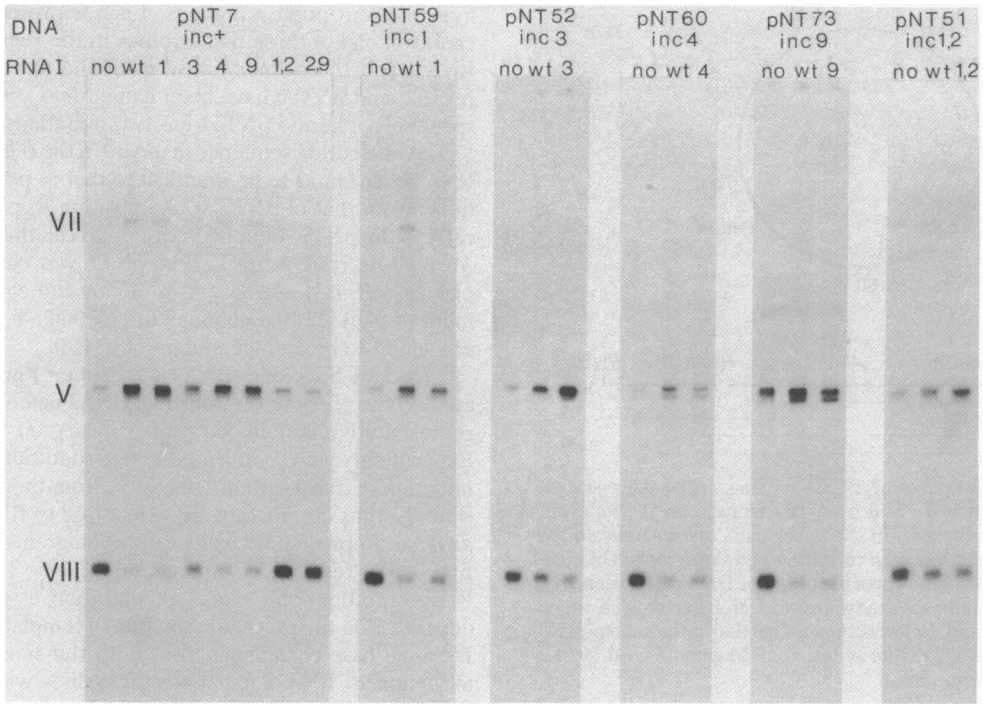

FIG. 3. Effect of RNA I on primer formation. RNA synthesis was initiated with RNA polymerase (3 units/ml), plasmid DNA (10 μg/ml), uridylyl(3',5')uridine (500 μM), GTP and CTP (10 μM each), and [α-^{32}P]ATP (2 μM, 400 Ci/mmol) in the presence or absence of RNA I (1 μg/ml). After 10 min at 30°C, rifampicin (10 μg/ml) was added to block new initiation, and incubation was continued for 10 min. Because of the absence of added UTP, synthesis of primer RNA was arrested after synthesis of about 30 nucleotides (3). After addition of UTP (40 μM) and RNase H (1.5 units/ml) and adjustment of concentrations of ATP, GTP, and CTP to 200, 40, and 40 μM, respectively, incubation was continued for 30 min. After termination of the reaction (1), an appropriate amount of [α-^{32}P]AMP-labeled runoff transcripts (342 nucleotides) from the β-lactamase promoter (1) made on *Rsa* I fragments of pNT7 was added. This was used to monitor the recovery of labeled RNAs. RNA from an equal volume of each reaction mixture was analyzed in a 3% polyacrylamide/urea gel (1). Synthesis of RNA I was not detected (data not shown). The *inc* allele of the RNA I present in each reaction is indicated by a number or by wt (i.e., *inc*$^+$). Thus 1, 1,2, and 2,9 indicate *inc*1 RNA I, *inc*1 *inc*2 RNA I, and *inc*2 *inc*9 RNA I, respectively. No addition of RNA I is indicated by "no." The Roman numeral VIII indicates the position of the primer RNA (species VIII) and V and VII indicate those of the read-through transcripts (species V and VII) (1).

H added to the transcription mixture (1), hybridization of RNA I to the template DNA is unlikely. Rather, the following results with RNase III, which cleaves double-stranded RNA, indicate formation of hybrids between RNA I and the primer. Although neither the pNT7 primer transcript nor RNA I is sensitive to RNase III when alone, both can be cleaved at several specific sites when mixed together (Fig. 4).

Table 1. Inhibition of primer formation by RNA I

	Template DNA					
RNA I	*inc*$^+$	*inc*1	*inc*3	*inc*4	*inc*9	*inc*1 *inc*2
inc$^+$	1.00	0.33	0.11	0.23	0.38	0.09
*inc*1	0.39	0.21	0.07	0.13	0.23	0.14
*inc*3	0.13	0.04	0.39	0.07	0.08	<0.02
*inc*4	0.33	0.14	0.07	0.20	0.29	0.06
*inc*9	0.34	0.13	0.11	0.18	0.29	0.07
*inc*1 *inc*2	<0.02	0.03	<0.02	<0.02	0.02	0.15
*inc*2 *inc*9	<0.02	<0.02	<0.02	<0.02	0.03	0.12

The relative strength of inhibition of primer formation by RNA I measured with various combinations of template DNAs and species I RNAs. This table is based on the results of about 300 reactions like those of Fig. 3. For each reaction, the radioactivity in a gel slice containing the primer RNA was measured and then normalized to that in a slice containing the reference (partial β-lactamase) RNA. The concentration of RNA I required to reduce the yield of primer by 50% was calculated from the extent of inhibition observed at three or four different concentrations of RNA I (0.05–4 μg/ml, depending on the activity). The values presented here are the inverse of the RNA I concentration at 50% inhibition. These are expressed relative to the value for pNT7 RNA I acting on pNT7 primer formation (0.2 μg/ml of RNA I for 50% inhibition). Means of triplicate determinations that varied within ±30% of each mean are presented.

To examine the rate of hybridization of pNT7 primer to various species I RNAs, RNA I and labeled primer were incubated together for various times and then any hybrids formed were cleaved by RNase III. Results (Fig. 4*B*) show that the wild-type RNA I hybridized rapidly with pNT7 primer whereas *inc*1 *inc*2 RNA I hybridized slowly. Hybridization of *inc*3 RNA I was slower than that of the wild-type RNA I but much faster than that of *inc*1 *inc*2 RNA I. An intermediate rate was also observed with the *inc*1 and *inc*4 species I RNAs (data not shown). These results indicate that a single base change can alter the rate of hybrid formation and that the rates observed with various species I RNAs correlate with their inhibitory activity.

Copy Number and Incompatibility of Mutant Plasmids. By use of DNA·DNA hybridization, we found that the numbers of monomeric molecules of pNT51 (*inc*1 *inc*2), pNT52 (*inc*3), pNT59 (*inc*1), pNT60 (*inc*4), and pNT73 (*inc*9) per chromosome in exponentially growing bacteria were about 220, 50, 60, 90, and 45, respectively, whereas the copy number of pNT7 was about 30 (data not shown). The correlation of increased copy number with decreased sensitivity of primer formation to homologous RNA I suggests that the copy number is determined by the inhibitory activity of RNA I.

The results described above suggest that *in vivo* replication of plasmid is susceptible to RNA I just as is *in vitro* primer formation. When two plasmids are present in a cell, any difference in susceptibility to the species I RNAs present in the cell ought to result in a difference between the rates of replication of the two. In the extreme, it is possible that one type of plasmid will

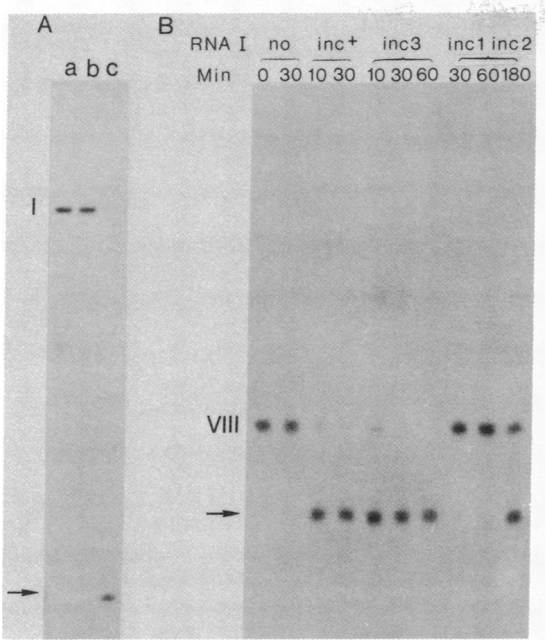

FIG. 4. Cleavage of RNA I (*A*) and primer (*B*) by RNase III. (*A*) pNT7 primer (unlabeled) was synthesized as in Fig. 3 except that the concentrations of ATP, GTP, and CTP were kept at 40 μM each throughout the reaction. After incubation to the completion of transcription (40 min), purified [γ-^{32}]ATP-labeled RNA I (0.01 μg/ml) was added and incubation was continued for 30 min. RNase III (60 units/ml) was then added and the mixture was incubated for 10 min at 30°C (lane c). Lane b shows labeled RNA I treated with RNase III in the absence of primer; lane a shows untreated RNA I. RNA was analyzed in a 10% polyacrylamide/urea gel. Arrow, cleavage product containing about 20 nucleotides. (*B*) Primer was synthesized as in *A* with [α-^{32}P]ATP (40 μM, 1 Ci/mmol) and then incubated with various species I RNAs (2 μg/ml) as indicated. RNase III was added at 10, 30, 60, or 180 min after addition of RNA I and incubation was continued for an additional 10 min. RNA was analyzed in a 3% polyacrylamide/urea gel. Species I RNA used are indicated in the figure. Arrow, cleavage product containing about 460 nucleotides. Weak spots above those of species VIII RNA were formed by species V RNA or its cleavage products. Although the hybrids were cleaved at several specific sites, only the shortest products of RNA I that retained the original 5′ end (in *A*) and the shortest products of the primer that had the original 3′ end (in *B*) are seen. When RNase III treatment was carried out at 250 mM KCl, the hybrid molecules were cleaved predominantly at approximately 70 bp from the 5′ end of the primer. Around that site there is a 5-bp sequence that is also found near the RNase III cleavage sites of some other RNAs (16–18).

be lost because its replication is suppressed by the RNA I of the other plasmid. To test such a possibility, we performed the incompatibility experiments described in Table 2. In the transformation tests, a reduction in the efficiency of transformation due to the presence of a resident plasmid indicates exclusion of the incoming plasmid by the resident. A greater reduction of the efficiency resulting from selection for both resident and incoming plasmids reflects exclusion of the resident. The segregation tests reveal the relative strengths of the two exclusion phenomena.

As bacteria which originally carry two plasmids grow, both plasmids continue to coexist, a particular plasmid is lost, or either plasmid is lost. All three situations are found in the results presented in Table 2. Both plasmids continued to coexist if only one of them was *inc*1 *inc*2 or *inc*3. In these cases, the RNA I of one plasmid inhibited primer formation on the other only slightly. The *inc*$^+$ plasmid was lost selectively when the *inc*1, *inc*4, or *inc*9 plasmid was present. In all these cases, both the *inc*$^+$ and *inc*$^-$ species I RNAs inhibited *inc*$^+$ primer formation more than *inc*$^-$ primer formation. Thus, when one copy of an

Table 2. Incompatibility tests

Plasmid A	Plasmid B	Relative efficiency of transformation, % For A only	Relative efficiency of transformation, % For A and B	Plasmid retained after 20 generations, % A only	Plasmid retained after 20 generations, % B only	Plasmid retained after 20 generations, % A and B
inc$^+$	pNT31	14	9*	16	24	60
*inc*1 *inc*2	*inc*$^+$	95	90	0	0	100
*inc*1		78	10*	100	0	0
*inc*3		98	83	2	0	98
*inc*4		75	20*	73	0	27
*inc*9		70	63*	97	0	3
inc$^+$	pNT36	95	92	0	0	100
*inc*1 *inc*2	*inc*1 *inc*2	12	14*	5	10	85
*inc*1		90	80	0	0	100
*inc*3		89	90	0	0	100
inc$^+$	pMB9	0.005	0.005	0	100	0
*inc*1 *inc*2	*inc*9	109	110	0	0	100
*inc*1		40	30	5	37	58
*inc*3		87	48	2	8	90
*inc*4		29	17*	10	30	60
*inc*9		10	10*	0	100	0
inc$^+$	pNT91	114	80	0	0	100
*inc*9	*inc*2 *inc*9	90	80	0	0	100

To determine the efficiency of transformation, about 6×10^8 cells of N100 carrying plasmid B were transformed with 0.01 μg of plasmid A (pNT7 or a derivative) except that 0.6 μg of pNT7 was used to transform N100 (pMB9). Plasmid DNA in bacteria and the purified DNA were monomeric. After the standard treatment for transformation, bacteria were spread on plates containing one or two appropriate drugs to select for the incoming plasmid only (A) or both incoming and resident plasmids (A and B). Tetracycline (10 μg/ml) was used to select for pMB9 or pNT91, chloramphenicol (20 μg/ml) for pNT31 or its *inc*1 *inc*2 derivative (pNT36), and ampicillin (25 μg/ml) for other plasmids. After incubation for 18 hr at 37°C, the visible colonies were counted. The number of transformants of the plasmid carrying bacteria was then divided by the number of transformants obtained in a similar experiment with N100 bacteria. The relative competence of each recipient culture was estimated by transformation with pST10, which is compatible with ColE1. The values shown have been corrected for the relative competence of the recipients. For the segregation test, a sample of the transformation mixture was diluted with L broth containing both drugs and incubated overnight. A sample that contained about 100 bacteria was then diluted into L broth without drugs and incubated. After about 20 generations of growth, bacteria were spread on a plate lacking drugs and the colonies that grew were replica-plated onto plates with either or both drugs. About 200 colonies per plate were examined. The fractional values for each class of resistant bacteria are calculated. In column B, blank indicates use of the same plasmid shown immediately above.

* Colony sizes were irregular.

inc$^-$ plasmid enters a cell carrying many copies of the *inc*$^+$ plasmid, the *inc*$^-$ plasmid can replicate more often than the *inc*$^+$ plasmid, and, finally, replication of the *inc*$^+$ plasmid will be blocked by the *inc*$^-$ RNA I. The third situation, in which either one or the other plasmid is eventually lost, occurs when both plasmids have an identical *inc* genotype. A remarkable case is the incompatibility of the plasmids with the *inc*1 *inc*2 genotype which are compatible with all other plasmids tested. Thus, these plasmids form a new incompatibility group of their own.

The results of incompatibility tests involving plasmids with heterologous genetic backgrounds are not easy to explain. Both the exclusion of pNT73 (*inc*9) by pMB9 (*inc*9) and the stronger exclusion of pNT7 (*inc*$^+$) by pMB9 than by pNT73 show that some factor in addition to RNA I influences incompatibility. The factor of pMB9 did not have a significant effect when the plas-

mid had the *inc*2 mutation or when pNT7 had the *inc*1 *inc*2 mutations. The factor could modify expression of the effect of inhibition of primer formation by RNA I.

DISCUSSION

Several mutants of pNT7 that have altered incompatibility and increased copy number have been isolated. In each plasmid, a mutation is present in the region that specifies RNA I and a portion of the primer transcripts. The results of *in vitro* experiments show that a single base change can simultaneously affect the inhibitory effect of RNA I on primer formation and the sensitivity of primer formation to inhibition by RNA I. The copy number of a given plasmid appears to be determined by inhibitory activity of RNA I on primer formation on the plasmid and the incompatibility between two plasmids can be attributed to inhibition of primer formation on one of the plasmids by RNA I of the other. Several recent reports have described mutations that affect the copy numbers of ColE1 (19), pMB8 (20), or CloDF13 (21) and are located in the region of each plasmid which specifies RNA I.

RNA I interacts with the primer transcripts to form an RNA·RNA hybrid. Because the rate of hybrid formation correlates well with the extent of inhibition of primer formation, we believe that this interaction is fundamental to the effect of RNA I on primer formation. The rate of hybridization can be reduced by a mutation at or near the center of any of three palindromes. This suggests that interaction of the loop portions of all three stem-and-loop structures of RNA I with the primer transcript is required for inhibition of primer formation and that the initial step of the interaction may involve the pairing of bases in the loop portions of the structures in RNA I with complementary sequences in the primer transcript. Thus, except for the *inc*9 transcripts (discussed below), changing of a possible G·C pairing between the primer transcript and RNA I to any other combination at the site of mutation greatly reduces the inhibition of primer formation. However, a possible A·U pairing is not always more effective than the G·U or A·C combinations at causing inhibition. For example, the homologous *inc*1/*inc*1 combination is slightly less inhibitory than the heterologous *inc*1/*inc*$^+$ combination. Some alterations of the structures of the transcripts by the mutation seem to modify the interaction. In contrast, there are cases in which structural changes of the transcripts seem to have a primary effect on the interaction. The *inc*9 mutation, located at a joint of the loop and the stem, may be such a mutation because the homologous *inc*9/*inc*9 combination, which can form a G·C pair at the *inc*9 site, is less inhibitory than the homologous *inc*$^+$/*inc*$^+$ combination which can form an A·U pair. We suspect that the previously described copy number mutations of ColE1 (19) and pMB8 (20) and the *cop*3 mutant of CloDF13 (21) exert their effect by preventing the proper formation of folded structures.

How does the interaction of RNA I with the primer transcript result in inhibition of primer formation? It seems likely that the interaction prevents formation of a structure necessary for efficient primer formation. Such a structure may involve a sequence in the region of the primer transcript that is complementary to RNA I and a second sequence of the transcript further downstream. For example, the primer transcript has a 30-nucleotide sequence in palindrome III (α region) that is highly complementary to a sequence 70 nucleotides downstream (β region) (Fig. 2). Base pairing between these sequences would result in formation of structure IV (Fig. 2), which is more stable than structure III. Obviously, hybridization of RNA I to the primer transcript will block formation of structure IV. This could explain the effect of RNA I if formation of structure IV is somehow involved in primer formation.

In fact, such a mechanism is suggested by the analysis of pNT7 mutants in which either a part or whole of the α or β region is deleted (data not shown). These mutants replicate poorly *in vivo*. The efficiency of *in vitro* primer formation on these mutants is low because of poor hybrid formation between primer transcripts and template DNA. Possibly, structure IV facilitates formation of a structure that is required for primer formation which involves sequences further downstream.

It may be worthwhile to comment on the possibility of involvement of RNase III in ColE1 DNA replication *in vivo* (22) or in initiation of ColE1 DNA synthesis *in vitro* (20). We have found that cell extracts (23) prepared from three RNaseIII-deficient strains can support normal formation of early replicative intermediates (data not shown). Recently, *in vivo* ColE1 DNA replication has been shown to be independent of the presence of active RNase III (ref. 24; G. Selzer, personal communication).

We thank G. Selzer and Y. Sakakibara for comments on the manuscript.

1. Itoh, T. & Tomizawa, J. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 2450–2454.
2. Morita, M. & Oka, A. (1979) *Eur. J. Biochem.* **97**, 435–443.
3. Tomizawa, J., Itoh, T., Selzer, G. & Som, T. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 1421–1425.
4. Gottesman, M. E. & Yarmolinsky, M. B. (1968) *J. Mol. Biol.* **31**, 487–505.
5. Bolivar, F., Rodriguez, R. L., Betlach, M. C. & Boyer, H. W. (1977) *Gene* **2**, 75–93.
6. Bolivar, F., Rodriguez, R. L., Greene, P. J., Betlach, M. C., Heyneker, H. L., Boyer, H. W., Crosa, J. H. & Falkow, S. (1977) *Gene* **2**, 95–113.
7. Alton, N. K. & Vapnek, D. (1979) *Nature (London)* **282**, 864–869.
8. Hashimoto-Gotoh, T. & Timmis, K. N. (1980) *Cell* **23**, 229–238.
9. Cohen, S. N., Chang, A. C. Y. & Hsu, L. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 2110–2114.
10. Yamaguchi, K. & Tomizawa, J. (1980) *Mol. Gen. Genet.* **178**, 525–533.
11. Cooper, S. & Helmstetter, C. E. (1968) *J. Mol. Biol.* **31**, 519–540.
12. Darlix, J.-L. (1975) *Eur. J. Biochem.* **51**, 369–376.
13. Maxam, A. M. & Gilbert, W. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 560–564.
14. Ohmori, H. & Tomizawa, J. (1979) *Mol. Gen. Genet.* **176**, 161–170.
15. Sutcliffe, J. G. (1978) *Cold Spring Harbor Symp. Quant. Biol.* **43**, 77–90.
16. Robertson, H., Dickson, E. & Dun, J. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 822–826.
17. Rosenberg, M. & Kramer, R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 984–988.
18. Bram, R. J., Young, R. A. & Steitz, J. A. (1980) *Cell* **19**, 393–401.
19. Muesing, M., Tamm, J., Shepard, H. M. & Polisky, B. (1981) *Cell* **24**, 235–242.
20. Conrad, S. E. & Campbell, J. L. (1979) *Cell* **18**, 61–71.
21. Stuitje, R. A., Spelt, C. E., Veltkamp, E. & Nijkamp, H. J. J. (1981) *Nature (London)* **290**, 264–267.
22. Kahn, M., Figurski, D., Ito, L. & Helinski, D. (1978) *Cold Spring Harbor Symp. Quant. Biol.* **43**, 99–104.
23. Sakakibara, Y. & Tomizawa, J. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 802–806.
24. Ely, S. & Staudenbauer, W. L. (1981) *Mol. Gen. Genet.* **181**, 29–35.