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Conditional high copy number ColE1 mutants: resistance to RNA1 inhibition *in vivo* and *in vitro*

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We describe three independently isolated copy number mutants of a plasmid ColE1 derivative which undergo temperature- and growth-phase-dependent DNA amplification in *Escherichia coli*. These mutants have single base-pair alterations in a highly localized region of the plasmid genome encoding the replication primer RNA. The mutations map immediately upstream of the RNA1 transcript, altering the sequence between conserved elements of the RNA1 promoter. These mutants have 2- to 4-fold increased copy number relative to wild-type plasmids in exponential growth at 37°C but undergo 20-fold amplification of copy number relative to wild-type when cells enter stationary phase. Cells containing these plasmids grow with normal kinetics at 37°C but grow poorly at 42°C. The poor growth is associated with high-level plasmid amplification. Both the temperature and growth phase plasmid DNA amplification are suppressed if the ColE1 *rop* gene product is provided *in trans* from a compatible plasmid. Analysis of steady-state RNA1 levels indicates that DNA amplification occurs in the presence of RNA1 made by the mutant plasmid. Thus, the DNA amplification of the copy mutant is not due to an inability to synthesize RNA1. Using an *in vitro* transcription system containing RNase H, we show that mutant primer processing by RNase H is resistant to levels of the replication initiation inhibitor RNA1 that inhibit wild-type primer processing. The defect in inhibition appears not to be at the level of association of RNA1 with nascent primer. These results indicate that mutant plasmid amplification is due to the ability of its primer precursor transcripts to serve as substrates for RNase H despite the presence of RNA1.

Key words: DNA replication/plasmid/RNA conformation/copy number control

Introduction

The plasmid ColE1 has proved to be a useful model for the study of the control of DNA replication (reviewed in Polisky, 1986). Investigation of this multi-copy plasmid by genetic and biochemical approaches has revealed an unusual scheme for the control of leading DNA strand synthesis. Crucial to this control is the interaction of two complementary RNA species encoded by the plasmid (Tomizawa and Itoh, 1981). One RNA is the replication primer which is capable of forming a localized RNA–DNA hybrid in the vicinity of the replication origin (Itoh and Tomizawa, 1980). The hybrid serves as a substrate for the host enzyme ribonuclease H (RNase H), which cleaves the RNA to create a specific 3′ terminus. This terminus is recognized by DNA polymerase I, which adds deoxyribonucleotides to generate the leading DNA strand. The ability of RNase H to process the hybridized primer precursor can be inhibited by the addition of a 108-nt RNA, called RNA1, encoded by the plasmid (Tomizawa *et al.*, 1981). RNA1 is complementary to the 5′-terminal region of the primer, and is transcribed from the opposite DNA strand, terminating near the initiation site of primer RNA transcription. The complementary RNAs can be shown to interact *in vitro* to form an RNA–RNA duplex (Tomizawa, 1984; Tamm and Polisky, 1985). From studies of plasmid mutants altered in incompatibility properties, it appeared likely that these RNAs interact in highly folded conformations (Lacatena and Cesareni, 1981; Tomizawa and Itoh, 1981). Biochemical studies of these RNAs *in vitro* have confirmed that they possess stable secondary and tertiary structural characteristics (Morita and Oka, 1979; Tomizawa and Itoh, 1982; Tamm and Polisky, 1983; Wong and Polisky, 1985; Masukata and Tomizawa, 1986). The association of RNA1 with the nascent primer transcript alters primer conformation and thereby prevents it from forming a stable hybrid at the origin (Masukata and Tomizawa, 1986).

In addition to RNA1, ColE1-type plasmids encode a small polypeptide, known as Rop or Rom, which also plays a negative role in copy number control (Twigg and Sherratt, 1980; Cesareni *et al.*, 1982). This polypeptide has been shown to catalyze the rate of association of the two RNAs *in vitro* (Som and Tomizawa, 1983; Cesareni *et al.*, 1984; Lacatena *et al.*, 1984; Tomizawa and Som, 1984). The *rop* gene is an auxiliary control element; deletions of this gene cause relatively minor increases in plasmid copy number compared to changes in the RNA1-primer overlap region (Twigg and Sherratt, 1980).

A variety of mutants of ColE1 have been described that have altered copy number (Muesing *et al.*, 1981). We have previously described two mutants whose copy number increased after a temperature shift from 30°C to 42°C (Wong *et al.*, 1982). The mutants, designated pEW2705 and pMM1, contained sequence changes in the primer RNA, but in a region of the plasmid genome immediately upstream of the first nucleotide of RNA1. Here we describe two additional mutants with similar features which map in the same region of the plasmid genome. To gain information on the molecular events responsible for the conditional DNA amplification displayed by this class of mutations, we have carried out experiments to define more precisely the roles of RNA1, primer RNA and the *rop* gene in this process. Our results indicate that the replication primer encoded by the mutant plasmids is resistant to the inhibitory effects of RNA1.

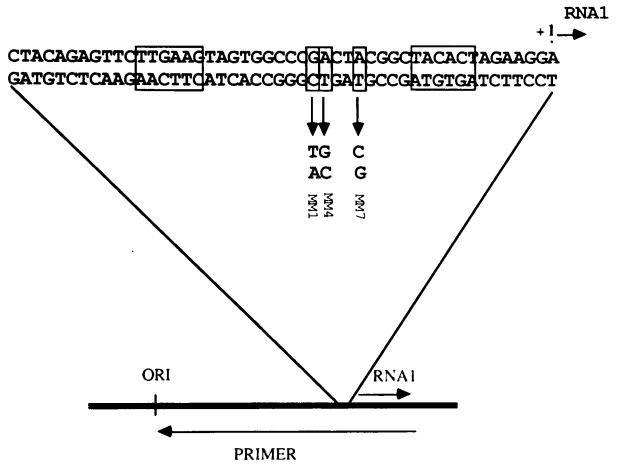

Fig. 1. Sequence changes associated with conditional high-copy-number mutants. The lower bold line represents part of the sequence of the 4.2-kb ColE1 derivative plasmid pNOP42. The horizontal arrows represent the overlapping transcriptional arrangement of RNA1 and primer RNA. The sequence shown is a portion of the DNA sequence upstream of the initiation of transcription of RNA1. The horizontal rectangles refer to the conserved sequence elements of the RNA1 promoter at positions −13 and −37 upstream of the initiation nucleotide of RNA1. The single boxed bases are the locations of the changes responsible for the phenotypes of the mutants in this study.

Results

Copy number behavior of wild-type and mutant plasmids

We isolated a number of ColE1 plasmid mutants which had elevated copy numbers when cells were grown to saturation. These plasmids were derivatives of the *cop*$^+$ plasmid, pNOP42, a 4.2-kb ColE1 plasmid that contains RNA1, the replication primer, and the ColE1 origin, but lacks the *rop* gene. pNOP42 contains the *bla* gene and confers ampicillin resistance. The mutants were selected on the basis of the elevated antibiotic resistance to both methicillin and ampicillin which they conferred on cells harboring them (Wong and Polisky, 1985). One mutant, called pMM1, was selected to have a thermal-regulated copy number, i.e. low copy number at 30°C, and high copy number after a shift in growth temperature to 42°C. The other two mutants, pMM7 and pMM4, were selected as high copy number mutants at 37°C. Sequence analysis of the group of plasmids described here indicated that each mutant contained a single base alteration in a highly localized region of the plasmid genome encoding the primer RNA (Figure 1). Because of the complementary relationship between the primer RNA and RNA1, the changes also alter the sequence of the RNA1 promoter.

In preliminary experiments, we were surprised to observe that pMM7 actually had a rather low copy number in exponentially growing cells compared to that which we had seen in cells grown to stationary phase. Accordingly, we determined plasmid copy levels of the wild-type parent plasmid, pNOP42, and the mutants at different times during the growth of bacterial cultures at 37°C. Representative samples of plasmid DNA present in cleared lysates were electrophoresed on agarose gels (Figure 2). These results indicate that a large increase in mutant plasmid copy number occurs when cells enter stationary phase.

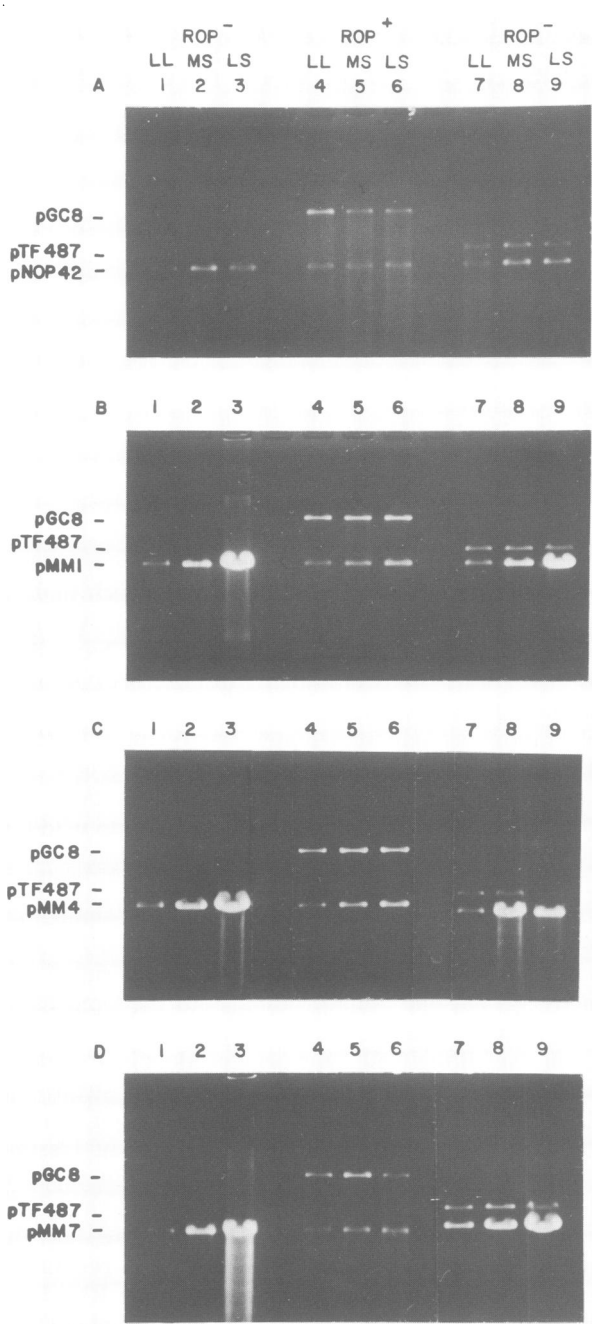

Fig. 2. Copy number changes of wild-type and mutant plasmids at different stages of growth, with and without *rop* gene. Bacterial cultures containing various plasmids were grown at 37°C. At different times during growth, cleared lysates from identical numbers of cells were prepared as described in Materials and methods. The plasmid DNAs in the lysates were cleaved with appropriate restriction enzymes to linearize them. DNA was electrophoresed on 0.8% agarose gels, stained with ethidium bromide, destained and photographed. LL is late logarithmic, MS is mid-stationary, LS is late stationary. These correspond to OD_{650} values of 1.3, 3.9 and 5.6 respectively. *Rop* gene function is provided *in trans* by the compatible R6K derivative pGC8; pTF487 is a *rop*$^-$ deletion derivative of pGC8. (**A**) pNOP42 copy number. Lanes 1−3, pNOP42 alone; lanes 4−6, pNOP42 and pGC8. lanes 7−9, pNOP42 and pTF487. (**B**) pMM1 copy number. Lanes 1−3, pMM1 alone; lanes 4−6, pMM1 and pGC8; lanes 7−9, pMM1 and pTF487. (**C**) pMM4 copy number. Lanes 1−3, pMM4 alone; lanes 4−6, pMM4 and pGC8; lanes 7−9, pMM4 and pTF487. (**D**) pMM7 copy number. Lanes 1−3, pMM7 alone; lanes 4−6, pMM7 and pGC8; lanes 7−9, pMM7 and pTF487.

Table I. Quantitation of RNA1 and plasmid DNA levels

	Early log	Late log	Late log/stationary	Stationary	Late stationary
RNA1					
1a pNOP42	172	152	265	1130	1197
b pMM7	563	594	2351	8494	30590
5S RNA					
2a pNOP42	1493	1674	1475	1809	1688
b pMM7	1784	1854	2344	2082	2370
RNA1/5S					
3a pNOP42	0.12	0.09	0.18	0.63	0.70
b pMM7	0.32	0.32	1.00	4.08	12.91
RNA1/5S*					
4a pNOP42	1.0	0.8	1.5	5.3	5.8
b pMM7	1.0	1.0	3.1	12.7	40.4
Plasmid DNA (copies/cell)					
5a pNOP42	40	41	47	67	69
b pMM7	119	178	241	688	2465
Plasmid DNA* (copies/cell)					
6a pNOP42	1.0	1.0	1.2	1.7	1.7
b pMM7	1.0	1.5	2.0	5.8	21.0
RNA1/DNA					
7a pNOP42	56	41	72	176	190
b pMM7	50	34	78	109	303
RNA1/DNA*					
8a pNOP42	1.0	0.8	1.2	3.1	3.4
b pMM7	1.0	0.8	1.6	2.2	1.9

Quantitation of RNA1 and plasmid DNA levels in cells containing pNOP42 or pMM7. The data were obtained at different stages of growth of cultures grown in rich media at 37°C. The correspondence of stage of growth to OD_{650} is as follows: early log, 0.5; late log, 1.3; late log/stationary, 1.8; stationary, 4.2; late stationary, 5.6. The data in lines 1 and 2 are c.p.m. determined by scintillation counting of bands corresponding to either RNA1−RNA1* or the 5S species of *E.coli* excised from acrylamide gels. The data shown are averages of two separate determinations. Plasmid DNA copies per cell (lines 6a and b) were determined as described in Materials and methods. Data in lines 4a, 4b, 6a, 6b, 8a and 8b are normalized to the value in early log phase. To obtain the data in lines 7a and 7b, the value in line 3 was multiplied by 18 700 (the reported number of 5S molecules per cell) and the product divided by the value in line 5. This result gives the number of RNA1 molecules per monomeric plasmid DNA. We did not take into consideration changes in 5S copy number with cell growth in these estimates. We estimate the error in the calculation to be ~20%.

We determined copy number more quantitatively by slot-blot hybridization; these data are shown in Table I, lines 6a and b. The results indicate that the wild-type plasmid, pNOP42, has an approximately constant copy number per cell at different growth phases. Copy number of pNOP42 is ~40 copies/cell in early and late log phase, increasing to 69 copies in stationary phase, corresponding to a 1.7-fold increase per cell over the entire range of growth. pMM7 copy number is 119 copies/cell in early log phase and increases ~6-fold as cells enter stationary phase. In stationary phase pMM7 copy number continues to increase, reaching a copy number corresponding to a 21-fold increase relative to that in early log phase. These results suggest that under these conditions control of pMM7 copy number is altered in a growth-phase-dependent manner.

We observed that cells containing pMM7 also had a temperature-dependent growth phenotype. When shifted from growth at 23°C to 42°C, cells grew with normal doubling time for a few generations, then stopped (data not shown). In contrast, cells containing pNOP42 grew normally following the temperature shift. When plasmid copy number was examined, it was observed that pMM7 underwent extensive amplification within 1−2 h after the shift to 42°C (data not shown). The amplification correlated temporally with the growth inhibition. Behavior qualitatively similar to pMM7 was displayed by pMM4 and pMM1. The extensive amplification of pMM7 in stationary phase observed after growth at 37°C was not observed when cells were grown at 30°C. Under these conditions, pMM7 amplified ~2-fold and was essentially indistinguishable from the wild-type plasmid. Taken together, the experiments on temperature and plasmid amplification indicate that at higher temperatures amplification occurs earlier in the growth phase.

Synthesis of RNA1 by wild-type and mutant plasmids in vivo

From the location of these mutations, it was not evident whether their primary effect was on RNA1 or the replication primer or both. Although they map outside the RNA1 structural gene, the mutations lie in the RNA1 promoter. It was conceivable that they might alter the expression of RNA1 in a growth-dependent or temperature-dependent manner such that RNA1 levels were reduced leading to elevated replication initiation and high copy number. We approached this possibility in two ways. The first was to determine directly RNA1 levels in cells containing wild-type or mutant plasmids at different growth phases. Since steady-state RNA1 levels *in vivo* have not been previously quantitated for these plasmids, we thought such data would also prove useful in understanding overall replication control.

To assess directly RNA1 levels *in vivo*, total RNA from cells carrying either pNOP42 or pMM7 was isolated,

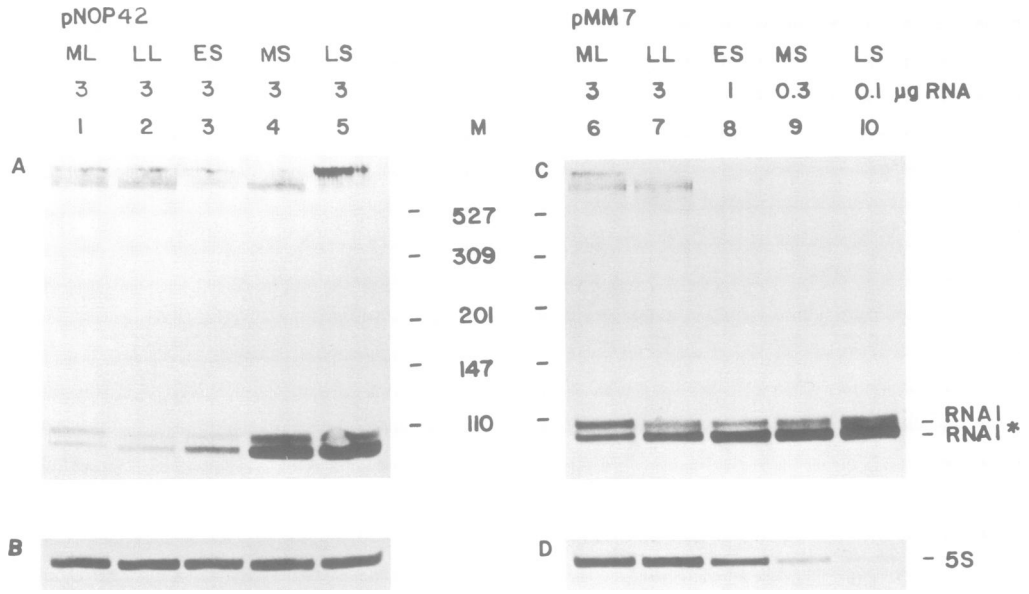

Fig. 3. RNA blot analysis of RNA1 transcribed from wild-type or mutant plasmids. Total cell RNA was isolated from DG75 cells carrying either pNOP42 or pMM7. RNA was prepared at different stages of culture growth: ML, mid logarithmic; LL, late logarithmic; ES, early stationary; MS, mid-stationary; LS, late stationary (see Table I for definitions of these periods). RNA was electrophoresed on a 8% acrylamide gel containing 8 M urea, then electrophoretically transferred to a nylon support. The amount (µg) of RNA loaded is shown at the top of the lane. Duplicate blots were probed with ^{32}P-labeled RNA complementary to RNA1 or to *E.coli* 5S RNA made as described in Materials and methods. (**A**,**C**) Autoradiographs using the RNA1 probe hybridized with RNA from cells carrying pNOP42 or pMM7 respectively. (**B**,**D**) Relevant sections of the autoradiographs using the 5S probe. The RNA1 probe did not hybridize to low mol. wt RNA from cells lacking plasmids (data not shown). The variable signal at the top of some lanes is due to interaction of the probe nonspecifically with rRNA. **Lane M** shows mobility of labeled denatured DNA marker fragments.

electrophoresed on an acrylamide gel, transferred to a nylon support and hybridized with a labeled probe specific for RNA1. To control for the amount of RNA loaded we also hybridized the blot with a probe for the host 5S RNA species. RNA samples were prepared at different times in the growth of the culture. Our hybridization data for 5S RNA indicate that the steady-state level of this species varies somewhat per cell at different growth phases; late stationary cells generally were observed to contain 20–30% more 5S RNA than cells at earlier stages (Table I). The results of the RNA blot analysis are shown in Figure 3. In cells containing the ColE1 plasmids, but not the host cell, the RNA1 probe hybridizes to two small RNA species. The larger of these species migrates at ~108–110 bases, and has identical mobility to RNA1 synthesized *in vitro* (data not shown). The smaller band is a processing product, termed RNA1* (Elble *et al.*, 1983), which results from removal of the 5′-terminal 5–6 nt of mature RNA1 by the host nuclease, RNase E (Tomcsanyi and Apirion, 1985). In log-phase and stationary-phase RNA isolated from cells containing either plasmid, it is apparent that both RNA1 and RNA1* are present (Figure 3). Quantitation of the ratio of the two forms indicated little difference between them at either stage of growth. RNA1* is 2- to 3-fold more prevalent than RNA1 in these samples.

In Table I we quantitate RNA1 levels derived from the blot analysis and correlate the data with plasmid copy number. In early and late log phase cells containing pNOP42, the steady-state levels of RNA1 are 9–12% that of 5S RNA. In stationary phase, this level increases to 63–70% of 5S RNA. Considering the 1.7-fold increase in plasmid copy number that occurs during this time, the data indicate a 3- to 3.4-fold increase in steady-state RNA1 levels in stationary phase relative to exponential phase. The number of 5S RNA molecules in exponentially growing *Escherichia coli* has been estimated to be 18 700 (Neidhardt, 1987). Taking into account the specific radioactivities of the probes for the 5S species and for RNA1, we estimated the number of RNA1–RNA1* molecules per plasmid DNA. As shown in Table I, this number ranges from ~56 molecules of RNA1–RNA1* per monomeric plasmid in early log phase to 190 in late stationary phase. These values are substantially higher than those reported by Chao and Bremer (1987), who detected about two RNA1 molecules per plasmid DNA. In cells containing the copy number mutant pMM7, RNA1–RNA1* levels per plasmid are similar to those of wild-type, ranging from 50 in early stationary phase to 303 in late stationary phase. The high copy number of pMM7 in stationary phase results in the RNA1–RNA1* species being far more prevalent than host 5S RNA; bands corresponding to RNA1–RNA1* are readily visible in ethidium-bromide-stained acrylamide gels of total RNA (data not shown).

From the perspective of copy number control, the most important conclusion from these measurements is that the RNA1 promoter activity in the mutant plasmid does not seem to be significantly altered relative to the wild-type promoter. Thus, pMM7 DNA amplifies in the presence of wild-type RNA1, and the levels of RNA1 present during amplification increase in an approximate gene-dosage-dependent manner.

Effect of the rop gene on mutant plasmid DNA amplification

Since RNA1 was present but apparently inactive during amplification of the mutant plasmid DNA, we investigated whether the growth-phase- and temperature-dependent amplification phenotypes were suppressed by the *rop* gene

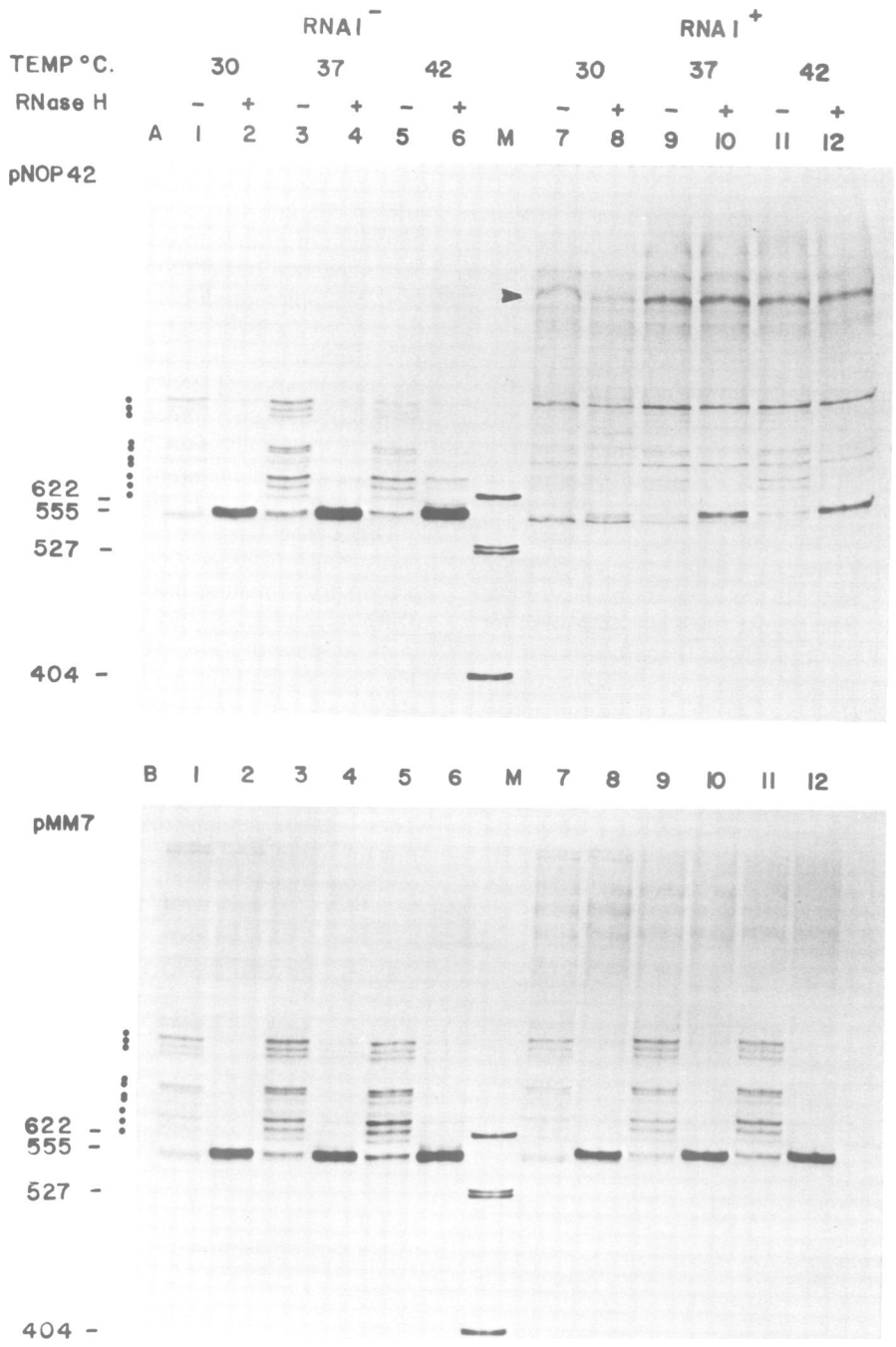

Fig. 4. Effect of RNA1 on wild-type and mutant plasmid primer RNA processing *in vitro*. Transcription of ^{32}P-labeled primer RNA was specifically initiated using supercoiled plasmid DNAs as templates *in vitro* as described in Materials and methods. Following initial synthesis of a short, labeled 5′-terminal primer RNA transcript, elongation of primer was carried out at three different temperatures, 30, 37 and 42°C. Each reaction was carried out in the presence and absence of RNase H and RNA1. The labeled primer RNA species were electrophoresed on a 8% acrylamide gel containing 8 M urea and autoradiographed. Because the samples were heated prior to loading, any RNA1–primer complexes were melted. **Lane M** shows denatured DNA markers whose sizes are on the left.

product. The Rop protein catalyzes the reversible, initial phase of the RNA1–primer interaction *in vitro* (Tomizawa, 1985). In addition, Rop protein is capable of suppressing certain defective RNA1 mutants *in vivo*, restoring their ability to mediate ColE1-type incompatibility (Dooley and Polisky, 1987).

We investigated pMM7 amplification in cells containing a compatible plasmid, pGC8, which is capable of providing Rop protein *in trans* (Cesareni *et al.*, 1984). As a control, cells carrying pMM7 and a *rop*$^-$ deletion derivative of pGC8, pTF487, were also tested. The results, shown in Figure 2, show that the presence of pGC8 reduces pNOP42 copy number as expected, and totally suppresses the amplification of pMM7 in stationary phase. The timing and extent of the amplification are unaffected by the presence of the control plasmid, pTF487. Similar results were seen with pMM1 and pMM4. We also observed that pGC8 eliminated the lethal effects of growth at 42°C for cells

containing pMM7 (data not shown). These results suggest that the defect in pMM7 is not at the level of RNA1 expression, but rather at the level of RNA1 function.

Effect of RNA1 on wild-type and mutant primer production in vitro

To gain further insight into the possible defect in RNA1 inhibition of pMM7 replication, we investigated the ability of RNA1 to inhibit primer formation *in vitro*, using an assay developed by Tomizawa *et al.* (1981). A single round of primer transcription from supercoiled plasmid DNA is specifically initiated at the primer promoter using a dinucleotide; subsequent rounds of transcription are inhibited by rifampicin. Elongation of the primer transcript is carried out in the presence of RNA1. Formation of the mature 555-nt primer is dependent on specific RNase H cleavage of transcripts that extend beyond the origin region. We carried out primer transcription reactions of pNOP42 or pMM7 DNAs at different temperatures in the presence and absence of RNA1, with and without RNase H addition. The results are shown in Figure 4. The primer transcription pattern for both templates in the absence of RNase H addition is similar. A series of discrete transcripts extending beyond the origin is apparent (vertical dots, Figure 4). Each transcript apparently terminates at a specific position in a rho factor-independent manner (Figure 4A, lanes 1, 3 and 5). This collection of transcripts comprises precursors to mature primer since subsequent addition of RNase H to the transcription reaction eliminates them and a new band of 555 nt is generated (Figure 4A, lanes 2, 4 and 6). In the absence of RNase H, the addition of purified RNA1 (final concentration of 2×10^{-8}M) has a clear effect on the primer transcription pattern from pNOP42 DNA; the discrete set of read-through transcripts is largely eliminated and several longer transcripts are apparent (Figure 4A, lanes 8, 9 and 11, see arrow). The addition of RNase H to these transcripts does not lead to the extensive generation of mature 555-nt primer (Figure 4A, lanes 8, 10 and 12). The extent of inhibition of primer formation by RNA1 can be estimated by comparison of densitometric scans of the 555-nt region of the samples produced with and without added RNA1 (Figure 4A, compare lanes 2, 4 and 6 with lanes 8, 10 and 12 respectively). These scans (data not shown) indicate that the concentration of RNA1 used inhibits RNase H cleavage of pNOP42 primer precursors by ~90%. No effect of different temperatures on the amount of primer transcription, or the extent of maturation of RNase H or inhibition by RNA1 are apparent *in vitro*.

The cognate reactions carried out with pMM7 DNA show two striking differences compared to pNOP42 DNA. First, precursors to mature primer are apparent in the presence of RNA1, and are identical in size and amount to those made in the absence of RNA1 (Figure 4B, lanes 1, 3, 5, 7, 9 and 11). Second, the addition of RNA1 has virtually no inhibitory effect on subsequent primer formation by RNase H (Figure 4B, lanes 8, 10 and 12). Furthermore, under these conditions *in vitro*, the resistance of pMM7 primer formation to RNA1 inhibition did not show appreciable temperature dependence. These results demonstrate that primer formation by the mutant template shows greatly reduced sensitivity to inhibition by RNA1.

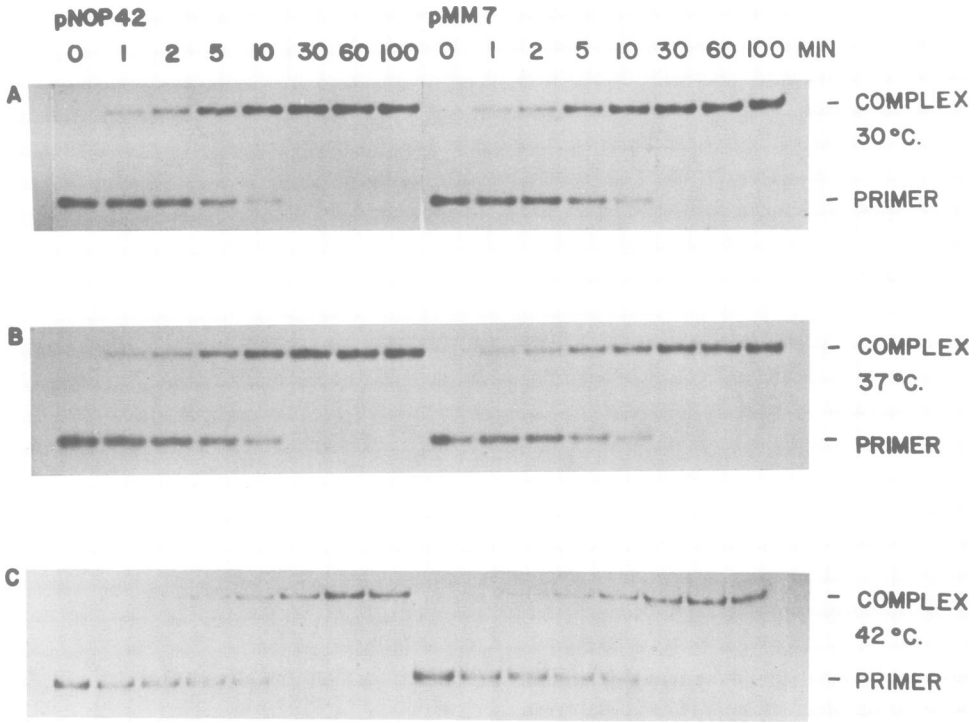

Fig. 5. Kinetics of interaction of wild-type and mutant primer RNA species with RNA1 *in vitro*. ^{32}P-Labeled wild-type or pMM7 primer RNA and wild-type sequence RNA1 were prepared as described in Materials and methods. Interaction between these complementary species was carried out in transcription buffer at various temperatures. The input molar ratio of RNA1:primer was 600:1 in these reactions. At various times samples were removed from the reaction, frozen and subsequently electrophoresed on a 4% acrylamide gel. The autoradiograph of the gel is shown. Numbers at the top of each panel are minutes after the two RNA species are mixed. **Panel A**, 30°C; **panel B**, 37°C; **panel C**, 42°C. Reactions on the left side of the panels use pNOP42 primer; those on the right use pMM7 primer. Complex refers to the RNA–RNA hybrid formed by the complementary RNAs under these conditions. Primer is the position of uncomplexed input primer.

Kinetics of RNA1–primer interaction in vitro

The basis for high-level replication of pMM7 might be due to reduced interaction kinetics between RNA1 and mutant primer RNA. We tested this possibility by measuring *in vitro* the kinetics of interaction between RNA1 and either wild-type primer or primer encoded by pMM7 (Tomizawa, 1984; Tamm and Polisky, 1985). In this assay, the complementary RNAs are allowed to interact at low salt and temperature to form an RNA–RNA duplex. The duplex can be electrophoretically distinguished from the input RNAs on a non-denaturing acrylamide gel. The kinetics of duplex formation reflect higher-order structural features of the RNAs since mutations in the RNAs that do not affect complementarity but do affect plasmid incompatibility properties *in vivo* can be shown to have reduced rate constants for association in this type of assay (Tomizawa, 1984).

We purified two different length subspecies of the primer precursor by run-off transcription *in vitro* at 30°C of either pNOP42 or pMM7 DNA. One primer precursor was 241 nt long (PR241); the other was 555 nt (PR555). We have previously examined the conformation of PR241 and shown that it contains stem–loops I, II and IV, but is lacking III (Wong and Polisky, 1985; see Figure 6). PR555 consists of mature primer after RNase H cleavage. These purified transcripts were separately mixed with excess RNA1 under conditions where they interact with pseudo-first-order kinetics. Since we have observed that pMM7 DNA undergoes amplification at 42°C, we performed the *in vitro* interactions at either 30, 37 or 42°C to examine whether the association of the RNAs might be temperature sensitive. The results are shown in Figure 5 for PR241 from pMM7 or pNOP42. No detectable difference was observed between wild-type and mutant primers at any of the three temperatures. Identical results were obtained for PR555 from these plasmids (data not shown). We repeated these experiments with identical length primer transcripts synthesized *in vitro* at 42°C rather than 30°C, because of concern that the RNAs might assume an altered conformation during synthesis which could not be attained simply by incubation at 42°C after synthesis at 30°C. The results of the interactions were completely identical to those obtained with transcripts made at 30°C (data not shown).

We conclude that the ability of the pMM7 primer transcript to escape from RNA1 inhibition is not due to a defect in its ability to associate with nascent primer.

Discussion

We have described several mutants of the ColE1 replicon which have unusual phenotypes. These mutants only display their high-copy-number phenotype in *E.coli* under certain physiological and genetic conditions. An interplay between temperature and growth phase on plasmid amplification is also evident since amplification in stationary phase after growth at 30°C is much less extensive than after growth at 37°C. Also, stationary phase entry is not required for amplification during growth at 42°C; under these conditions cell growth stops and amplification occurs within several generations.

While the behavior of these mutants is complex *in vivo*, we have shown a clear difference between them and wild-type plasmids *in vitro*. Mutant primer RNA processing by RNase H is essentially totally resistant to levels of RNA1 which greatly inhibit processing of wild-type primer. We have not observed a temperature effect on processing of the mutant primer *in vitro* consistent with their copy number behavior *in vivo*, i.e. sensitivity to RNA1 at low temperatures (30°C) and resistant to RNA1 inhibition at high temperature (42°C). Rather, we observe resistance at either temperature. There are many possible reasons for this behavior difference, including differences in the conformations of the RNAs *in vivo* and *in vitro*, the absence of accessory factors *in vitro* that play a role in the interaction, or inappropriate ionic environment *in vitro*. However, because the resistance to RNA1 inhibition *in vitro* correlates with the high copy number behavior of the mutant *in vivo*, we believe that this step in plasmid replication control is responsible for its phenotype.

In stationary phase, pMM1 and pMM7 DNA comprise >50% of total cell DNA. The observation of limited plasmid amplification in stationary phase without addition of protein synthesis inhibitors has been made previously with certain plasmids such as pBR322 (Stueber and Bujard, 1982) and the ColD plasmid (Frey and Timmis, 1985). However, it is important to point out that the parental *rop*$^-$ *cop*$^+$

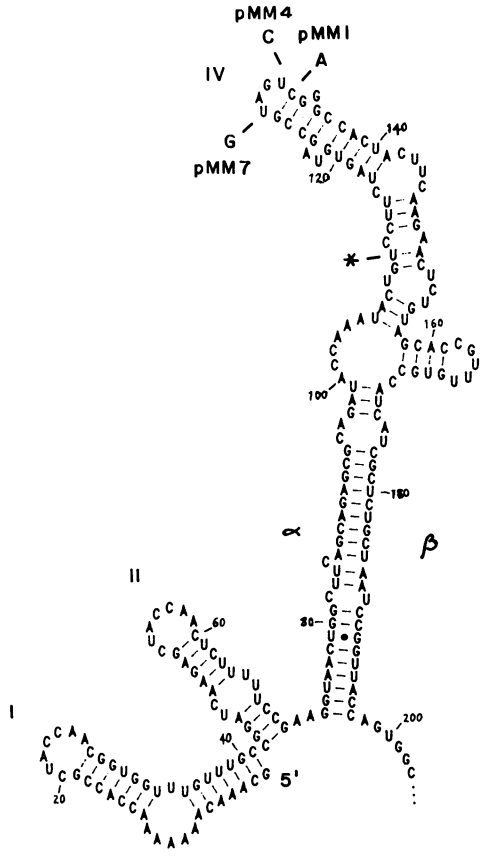

Fig. 6. Schematic representation of the 5′-terminal 203 nt of primer RNA folded using the Zuker program (Zuker and Steigler, 1981) without folding constraints. Stem–loop domains marked I, II and IV have been detected by biochemical analysis of RNAs made *in vitro* and subjected to partial digestion with enzymatic probes (Wong and Polisky, 1985; Masukata and Tomizawa, 1986). The location of the mutations pMM7, pMM1 and pMM4 in the primer transcript are shown. α and β refer to the prominent stem domain of stem–loop IV. The loop region consists of the four-base sequence 5′UAGU3′. The region of complementarity of primer RNA with RNA1 begins with the U at position 111 (marked with asterisk).

derivative used in our work, pNOP42, does not amplify appreciably in stationary phase. Thus, for the ColE1 family, stationary phase amplification under the growth conditions described here is not a property of either the wild-type plasmid, or a *rop*$^-$ derivative which contains a wild-type primer and RNA1 region. The behavior of pNOP42 indicates that ColE1 copy number is actively regulated in stationary phase. It seems reasonable to presume that prevention of plasmid amplification in cells that are not dividing would be a major function of copy number control elements. The observation reported here that the steady-state level of RNA1 increases several-fold upon entry into stationary phase may be relevant to maintenance of low copy number under these conditions.

From the location of the sequence changes responsible for these phenotypes, we have investigated several possibilities to explain their behavior. Although the sequence changes lie in the RNA1 promoter, for several reasons we do not believe that the mutations have a primary effect on the rate, timing or extent of RNA1 synthesis. First, RNA1 levels *in vivo* are not significantly reduced in cells containing the mutant plasmids, and during amplification of plasmid DNA in stationary phase, RNA1 levels increase in a roughly gene-dosage dependent manner. Second, the phenotype is suppressed by the Rop protein *in vivo*, which is known to require RNA1 for its function (Som and Tomizawa, 1983; Cesareni *et al.*, 1984). Consequently, the unusual replication behavior of these mutants is not due to a defect in their ability to produce RNA1, or to a defect in the structure of RNA1, but rather to the ability of the RNA1 to function as a negative regulator.

We have shown that RNA1 levels that inhibit wild-type primer formation *in vitro* have negligible effects on primer formation of the copy number mutant pMM7. The resistance of the mutant primer to RNA1 inhibition seems not to be due to an inability to associate with RNA1. Under a variety of temperatures, with different length primer transcripts, we have never observed a difference in association kinetics between wild-type and mutant RNAs with RNA1. This was surprising to us because a defect in association seemed the most plausible explanation of suppression of the mutant phenotype by the *rop* gene product. Although we cannot rule out the possibility that the association rates *in vivo* are different from those seen *in vitro*, there has been generally good correlation between *in vitro* rates of interaction and *in vivo* phenotypic behavior for a number of ColE1 incompatibility and copy number mutants (Tomizawa, 1985). The ColE1 primer is a structurally complicated RNA which contains a number of distinct domains (Masukata and Tomizawa, 1984, 1986). Furthermore, it undergoes at least one major rearrangement during its synthesis during which a stem−loop domain (called stem−loop III) is melted and later appears as part of a downstream stem structure (stem−loop IV; see Figure 6) as transcription proceeds (Wong and Polisky, 1985). Formation of stem−loop IV has been shown to be correlated with functional primer formation (Masukata and Tomizawa, 1986). Interaction of RNA1 with the nascent primer precludes stem−loop IV formation, and permits potential formation of another alternative structure involving further downstream regions of the primer (Masukata and Tomizawa, 1986). A mutation potentially destabilizing the stem region of stem−loop IV has been described which is replication defective and is reverted to function by a second site change that restores the potential to form a stem (Masukata and Tomizawa, 1986). Another mutation that potentially lengthens the stem region of stem−loop IV and has elevated copy number has been isolated (Castagnoli *et al.*, 1985). These considerations indicate that the rate of formation, structure and stability of stem−loop IV are critical for proper primer folding and regulation of initiation of replication by RNA1.

Two of the mutations described here, pMM7 and pMM4, map in the loop region of stem−loop IV, and therefore do not obviously alter the structure of the region (Figure 6). The third mutation, pMM1, alters a potentially based-paired C residue adjacent to the 3′ side loop IV. Each of the changes creates potential new base-pairs in the region which would have the effect of reducing the size of loop IV. It is possible that the loop region interacts with a second region of the primer located downstream; no direct evidence for this possibility exists. However, certain mutations that map near the loop are known to be suppressed by second-site changes that occur far downstream in the primer (Moser *et al.*, 1983; E.Wong, unpublished). It is possible to explain the suppression by a long-range interaction between distant domains of the primer.

A second possibility is that the mutations affect the rate of formation of stem−loop IV by affecting pausing of RNA polymerase during transcription of the primer, specifically accelerating the rate of formation of stem−loop IV. This model postulates a pause site in wild-type primer transcription at or near the loop which is eliminated or reduced by the mutations.

Regardless of the explanation, the conditional DNA amplification displayed by the mutants *in vivo* indicates that plasmid replication is tied to host physiology in a complicated manner. Whether the basis for this behavior is alterations of levels of primer promoter activity, primer stability or host factors that potentially play a role in the RNA−RNA interaction will require further investigation.

Materials and methods

Strains and plasmids

The host strain for these experiments was the *E.coli* K12 derivative DG75 (*hsdS1 leu-6 proA2 ara-14 galK2 xyl-5 mtl-1 rpsL20 thi-1 supE44* λ*F*$^-$). The parent plasmid used here is pNOP42, a 4.2-kb *cop*$^+$ derivative which has been described previously (Muesing *et al.*, 1981). The copy number mutants described here were spontaneously generated and selected by growth on plates containing methicillin and ampicillin as described previously (Wong *et al.*, 1982). The mutations were initially mapped to specific restriction fragments by construction of plasmids containing only one fragment derived from the mutant. Sequence determinations were done by the methods of Maxam and Gilbert (1977).

The plasmid used to provide the *rop* gene product *in trans* was pGC8, an R6K derivative (Cesareni *et al.*, 1984). This plasmid contains the *rop* gene from pBR322 present on a 2258-bp *Eco*RI fragment. A derivative of pGC8 which is deleted for the *rop* gene was constructed by removing the *Eco*RI fragment from pGC8. This plasmid was designated pTF487.

Cell growth and media

Cells were grown in 2XYT medium (Miller, 1972). Ampicillin was used at a concentration of 100 μg/ml. Prior to experiments in which plasmid copy number was measured at different points in the growth of cultures, cells containing the mutants pMM1, pMM4 and pMM7 were grown overnight at room temperature with no shaking. The slow growth rates ensured that cells had not entered stationary phase before the start of the experiment. Cells entering stationary phase undergo plasmid amplification and their subsequent plating efficiency has been observed to be reduced by several orders of magnitude. These overnight cultures were subsequently diluted 1:100-fold into fresh medium at the appropriate temperature. The OD_{650}

was monitored and samples were withdrawn at different points for RNA preparation and for plasmid copy number determination.

RNA preparation and blotting

Total RNA was prepared from plasmid-free or plasmid-containing DG75 cells by hot phenol extraction followed by DNase treatment and ethanol precipitation. All buffers used in RNA purification were treated with 0.1% (v/v) diethylpyrocarbonate. RNA concentrations were determined by OD_{260}. RNA samples were boiled for 3 min prior to loading onto an 8% polyacrylamide gel containing 8 M urea. The gel was electrophoresed in 1 × TBE buffer (TBE is 100 mM boric acid, 100 mM Tris base, 2 mM EDTA). After electrophoresis, RNA was transferred electrophoretically to Nytran (Schleicher and Schuell, 0.1-μm pore size) in 1 × TAE buffer (10 mM Tris−HCl, pH 7.8, 5 mM sodium acetate, 0.5 mM EDTA).

Radiolabeled probe for RNA1 was prepared by *in vitro* transcription of *Hin*dIII-cleaved plasmid pTF474 by T7 RNA polymerase using α-labeled [^{32}P]UTP (410 Ci/mmol, Amersham). pTF474 contains a 913-bp *Fnu*dII fragment of pNOP42 that contains the replication origin of ColE1 but lacks the −35 region of the primer promoter. Synthetic *Eco*RI linkers were added and the fragment was inserted into the cloning vector pT7/T3-18 (Bethesda Research Laboratories, BRL). Procedures for *in vitro* transcription were those described (BRL). The labeled probe contains the entire primer sequence and is complementary to RNA1. Probe for 5S RNA was prepared by nick translation of a *Hin*dIII−*Sca*I fragment of plasmid pKK223-3 (Pharmacia). This fragment contains the entire 5S coding region and was isolated by acrylamide gel electrophoresis and electroelution. Prehybridization for 3 h was at 42°C in 50% formamide containing 5 × Denhardt's solution, 5 × SSPE (1 × SSPE is 150 mM Nacl, 10 mM NaH_2PO_4, 1 mM EDTA), 200 μg/ml yeast RNA, 0.5% SDS. Hybridization was at 55°C for 15 h in the same solution except that 5% dextran sulfate was added. After hybridization, blots were washed successively with 2 × SSPE, 1 × SSPE and 0.1 × SSPE at 68°C for 30 min each. These buffers contained 0.1% SDS. Following autoradiography, relevant regions of the blot were excised and counted in a scintillation counter.

Plasmid copy number determination

Total bacterial DNA was prepared by the method of Schleif and Wensink (1981) from cells grown to different stages of growth. To determine copy number per cell, slot blots were carried out using a Schleicher and Schuell Minifold apparatus as recommended by the manufacturer. A series of diluted DNA samples were loaded into slots. Known amounts of CsCl-purified pMM7 DNA were also loaded as standards. The probe was [^{32}P]UTP-labeled primer RNA prepared by T7 RNA polymerase transcription of pTF474 DNA as described above. Conditions for prehybridization and hybridization were the same as described for RNA blot analysis. Cell numbers were determined by plate counts at the time of harvest. After autoradiography, slots were excised and counted in a scintillation counter. Plasmid copy number was calculated as monomeric plasmid equivalents per cell. During exponential growth, multimeric plasmid forms never constituted more than ~20% of total plasmid DNA.

Preparation of RNA1 and primer RNA

RNA1 was purified from total cell RNA as described (Dooley and Polisky, 1987), or by acrylamide gel electrophoresis after transcription of pMM7 DNA with *E.coli* RNA polymerase *in vitro*. Primer transcripts were synthesized *in vitro* basically by the selective initiation method previously described (Masukata and Tomizawa, 1984), except that the transcription buffer contained 100 mM potassium glutamate rather than 100 mM KCl. A primer species of 241 nt was produced by cleavage of the template with *Hin*fI as described previously (Wong and Polisky, 1985). Labeled primer RNA species were electroeluted after acrylamide gel electrophoresis.

RNA − RNA interaction kinetics

Conditions for the interactions have been described (Tamm and Polisky, 1985). RNA1 (1.8×10^{-8} M) was incubated with purified ^{32}P-labeled primer RNA (1.0×10^{-11} M) at various temperatures in a volume of 37 μl of transcription buffer (50 mM Tris−HCl, pH 7.5, 100 mM potassium glutamate, 10 mM $MgCl_2$, 1 mM dithiothreitol). At various times, 5-μl aliquots were transferred to an equal volume of ice-cold formamide loading buffer and frozen at −70°C prior to loading on a 4% acrylamide gel. The gel was then exposed to X-ray film.

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