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RNAII transcribed by IPTG-induced T7 RNA polymerase is non-functional as a replication primer for ColE1-type plasmids in *Escherichia coli*

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ABSTRACT

RNAII, an RNA species encoded by ColE1-type plasmids, serves as a primer for plasmid DNA replication. Previous work has shown that overproduction of RNAII transcribed by *Escherichia coli* RNA polymerase results in elevated plasmid copy number. To produce a plasmid in which the elevation of its copy number is inducible, we placed transcription of RNAII under the control of a bacteriophage T7 late promoter regulated by IPTG-inducible T7 RNA polymerase. During induction of T7 RNA polymerase by IPTG, we found that RNAII was overexpressed, but that, surprisingly, this increase in RNAII did not result in elevation of plasmid copy number. These results suggest that RNAII transcribed by T7 RNA polymerase does not function as a primer for plasmid DNA replication. Since RNAII function requires folding of its multiple stem–loop structures in a precise conformation and folding of RNAII can be influenced by its rate of transcription, the extremely rapid rate of travel of the T7 RNA polymerase may preclude proper folding of RNAII during its elongation.

INTRODUCTION

ColE1-type plasmids are commonly used as vectors for the cloning and expression of foreign genes, because they are small and they replicate to a high copy number using only enzymes encoded by the *Escherichia coli* host chromosome. Replication of the plasmid is initiated by a 555 nt RNA transcript, RNAII, which forms a persistent hybrid with its template DNA in a segment 13–20 bp upstream of the origin (1). The RNAII–DNA hybrid is then cleaved by RNaseH at the replication origin yielding a 3′ hydroxyl terminus that serves as primer and allows addition of deoxynucleotides by DNA polymerase I (2). This priming of DNA synthesis is negatively controlled by RNAI, a cloverleaf-structured tRNA-like RNA molecule (3) which is complementary to the 5′-end of RNAII and thus can bind to RNAII to prevent formation of the persistent hybrid between elongating RNAII and the template DNA (4–6).

High copy number plasmids offer many advantages as vectors for the cloning and expression of foreign genes. Even more advantageous for the expression of certain gene products would be a cloning vector that has an inducible high copy number. Several R1-derived plasmids that show temperature-dependent regulation of copy number have been described (7); these plasmids replicate at a low copy number at 30°C and exhibit 'runaway replication' at 37°C, resulting in an exponential increase in the concentration of plasmid DNA. However, the host cells carrying these plasmids lyse under 'runaway' conditions.

Recent work by Bacharov *et al.* (8) has shown that IPTG-induced overproduction of RNAII transcribed from a strong synthetic promoter by *E.coli* RNA polymerase results in a 4-fold increase in plasmid copy number (8). In an attempt to further increase plasmid production, we constructed a plasmid that overexpresses RNAII using IPTG-inducible bacteriophage T7 RNA polymerase, which generates transcripts processively and efficiently from the bacteriophage T7 late promoters both *in vitro* and *in vivo* (9,10). We anticipated that this plasmid, when carrying an adventitious gene expressed from the T7 promoter, would replicate at an induced high copy number at the time when a high level of expression from the cloned gene of interest is induced. Here we report the unexpected finding that plasmid copy number does not increase during overexpression of RNAII by T7 RNA polymerase. These results suggest that the RNAII transcribed from bacteriophage T7 RNA polymerase is non-functional as a replication primer. A possible mechanism for this effect is discussed.

MATERIALS AND METHODS

Host strains and plasmids

The *E.coli* strain BL21(DE3) (10), which is isogenic to BL21 and includes a T7 RNA polymerase gene under the control of the *lac*UV5 promoter inserted into its genome, was used to study the copy number control of the chimeric replicon pCMRNAII-1. pCMRNAII-1 contains a 0.6 kb DNA fragment encoding RNAII under the regulation of the T7 promoter (Fig. 1). The 0.6 kb fragment was derived from plasmid pSL-C101, a pBR322 derivative that produces an RNAI lacking 5 nt at the 5′-end (11), and was prepared by PCR amplification using primers T7–RNAII and NSP1. Primer T7–RNAII contains the DNA sequence 5′-GCGGAATTCTAATACGACTCACTATAGG**GCAAACAAAAAAACCACC**-3′, which includes the DNA sequence of the

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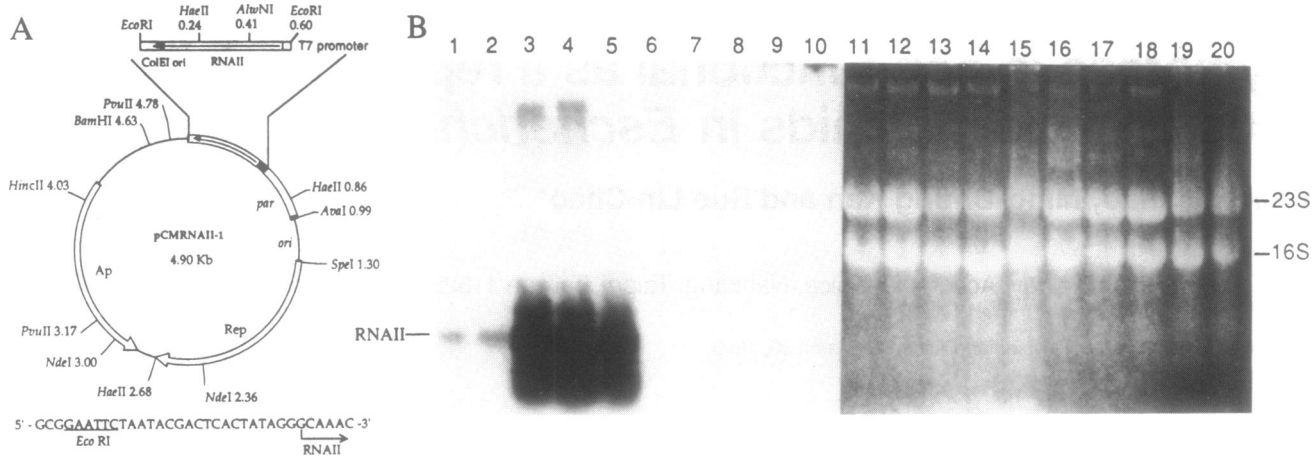

Figure 1. Map of plasmid pCMRNAII-1 (A) and its RNAII production induced by IPTG (B). The procedures used for plasmid construction are described in Materials and Methods. (**A**) A 0.6 kb fragment containing the RNAII gene under regulation of the T7 late promoter, the ColE1-replication origin, the *Eco*RI site used for the construction and the final size of plasmid pCMRNAII-1 are shown. The DNA sequence of the T7 promoter and its junction region with the segment encoding the RNAII gene are also shown. The replication region of the pSC101 derivative (*par*, *ori* and *Rep* segment) and directions of transcripts are indicated. The coordinates of the indicated restriction enzymes were deduced from the published DNA sequences and confirmed by restriction enzyme digestion analysis. (**B**) Northern blotting analysis of overproduction of RNAII from the T7 promoter by IPTG-induced T7 RNA polymerase in *E.coli* BL21(DE3). Lanes 1–10, autoradiograph of hybridizable RNAII molecules which were electrophoretically separated on a 1.5% agarose–formaldehyde gel. Lanes 11–20, the pre-blotted gel was stained with ethidium bromide for calibration of cellular RNA used in each lane; the position of 23S and 16S rRNA are indicated. Lanes 1–5 were prepared from cultures without IPTG treatment or 0, 30, 60 or 120 min after IPTG treatment respectively. The strain used for these cultures was *E.coli* BL21(DE3) containing pCMRNAII-1. Lanes 6–10, samples loaded in the same order as lanes 1–5, except that the strain used was *E.coli* N3433. Lanes 11–20 correspond to lanes 1–10.

T7 promoter plus the DNA sequence encoding the 5′-end of RNAII (bold nucleotides are complementary to nucleotides 3071–3088 of pBR322). Primer NSP1 contains the sequence 5′-CCCGGGAATTC**GACGCAGGAAAGAACATGT**-3′ (bold nucleotides correspond to nucleotides 2462–2480 of pBR322). Each primer contains a recognition sequence for *Eco*RI (underlined region), which allows the PCR-amplified DNA fragment to be inserted into the *Eco*RI site of pCM128 (12). The resulting plasmid, pCMRNAII-1, was then introduced by transformation into *E.coli* host N3433 (13). To ensure that the PCR-amplified DNA fragment contains a functional replication origin at the 3′-end of RNAII, an analogous PCR-amplified DNA fragment that carries the native RNAII promoter but lacks the T7 promoter was prepared and ligated to the *aph* (kanamycin resistance) gene. The resulting construct replicated as an autonomous plasmid in *E.coli* cells (data not shown).

Culture conditions

A single colony was picked and inoculated into 3 ml LB medium containing 50 μg/ml ampicillin (Ap) and cultured overnight at 37°C. The cells were then diluted 100-fold into LB medium and monitored for growth by measuring the optical density (OD) at 600 nm. At $OD_{600} \approx 0.4$–0.5, IPTG was added to a final concentration of 0.5 mM and the culture was diluted with an equal amount of LB medium containing 0.5 mM IPTG to maintain the culture in log phase growth. All samples taken for RNA isolation and plasmid copy number determinations were at equal cell mass.

Nucleic acid technology

RNA isolation, Northern blotting and copy number determination were as described previously (14,15), except that the ethidium bromide stained gel was photographed on a 254 nm wavelength transilluminator. DNA quantitation was performed using rectangular integration on a 300S Molecular Dynamics (MD) computing densitometer; film negatives exposed for various times were scanned to ensure a linear signal for accurate quantitation. Plasmid copy number was determined as amount of plasmid DNA per chromosomal DNA equivalent. Relative copy number was calculated as the copy number of pCMRNAII-1 over the copy number of pCM128, which was electrophoresed on the same agarose gel. For copy number comparisons, the size difference between these two plasmids was also normalized. Northern blot transfer was done using BioRad Zeta-Probe membranes and hybridization was done according to the manufacturer's instructions. A ^{32}P-labeled RNA probe complementary to RNAII was synthesized *in vitro* using T3 RNA polymerase from plasmid pUC18T7/T3 (11) as described (16,17).

RESULTS

pCMRNAII-1 (Fig. 1A) was constructed as described in Materials and Methods; the resulting plasmid includes two replication origins, the pSC101-derived origin of the pCM128 vector (18) and the ColE1-type origin under control of the T7 promoter. The fidelity of the PCR reaction was confirmed by DNA sequence analysis (data not shown); the junction region is shown in Figure 1. As shown by Northern blot analysis (Fig. 1B), in *E.coli* strain BL21(DE3) RNAII encoded by pCMRNAII-1 was transcribed at a high rate 30 min after IPTG induction of T7 RNA polymerase (lanes 2–5); otherwise only a basal level of expression of RNAII was observed (lanes 1–2). In both instances, the size of the major band of hybridizable RNAII molecules was determined to be ~500 nt by plotting a migration curve using 23S and 16S rRNA as size markers in ethidium bromide stained pre-blotting agarose gels (Fig. 1B, lanes 11–20). The size

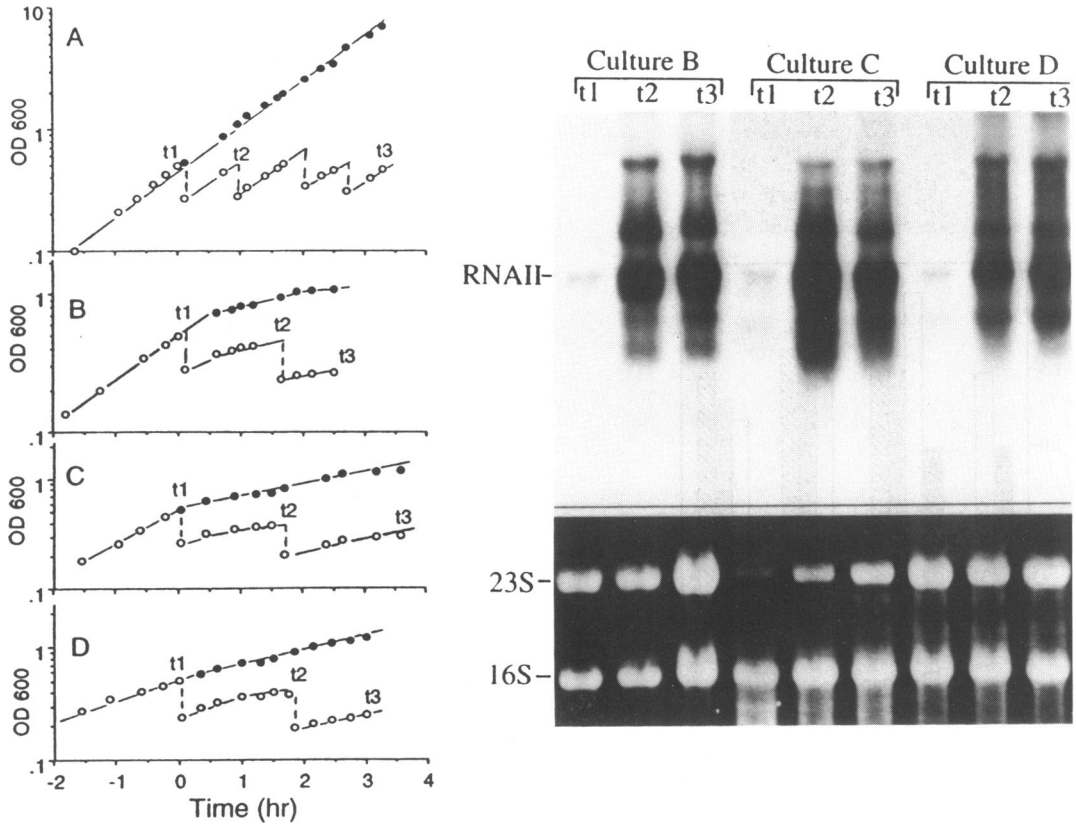

Figure 2. Growth characteristics and RNAII production of bacterial cultures after induction of T7 RNA polymerase by IPTG. (Left) IPTG was added to cultures at time zero (i.e. the first OD_{600} = 0.5); the cultures were then diluted with an equal volume of pre-warmed LB medium containing IPTG. To maintain the cultures in mid-log phase during subsequent growth, the cultures were diluted again whenever the OD_{600} had reached a value of ~0.5 (as indicated by the vertical lines). Multiplying the observed OD_{600} (open circles) by the accumulated dilution factors gave the upper, calculated OD curves (closed circles). (**A**) *Escherichia coli* BL21(DE3) cells lacking any plasmid grown at 37°C in LB medium. (**B** and **C**) BL21(DE3) cells containing plasmid pCMRNAII-1 cultured at 37°C in the absence (B) or presence (C) of 50 µg/ml ampicillin. (**D**) Culture medium as (C) except that cells were grown at 23°C. At the indicated times, t1, t2 and t3, constant OD units of culture mass were taken for determination of plasmid copy number (see Fig. 3) and cell viability. (Right) The RNA isolated from each corresponding culture from the left panel is indicated. The top and bottom panels show the autoradiograph of hybridizable RNAII molecules and the ethidium bromide stained gel, as described in Figure 1, respectively.

calculated for this band is about the same as the previously reported size of RNAII (555 nt) (1). In addition to the major band, several larger species appeared after 30 min induction. These bands are most likely RNAII species that terminate downstream of the replication origin. However, in host strain N3433, which lacks a gene encoding T7 RNA polymerase, accumulation of hybridizable RNAII molecules was not found (lanes 6–10) under the same growth conditions. These results indicate that over-expression of RNAII is dependent on the bacteriophage T7 RNA polymerase induced by IPTG and suggest that the small amount of RNAII produced in the uninduced condition in *E.coli* strain BL21(DE3) is due to T7 promoter-mediated transcription resulting from leaky expression of the T7 RNA polymerase gene.

To investigate whether overproduction of RNAII by induction of the T7 promoter increases pCMRNAII-1 copy number, we measured the copy number before and after IPTG treatment, as shown in Figure 2. In addition, for each time point the overproduced RNAII was analyzed from a duplicate culture sample. Surprisingly, we found that the plasmid copy number was unchanged (Fig. 3), even though RNAII was being overexpressed (Fig. 2). Thus overproduction of RNAII from the T7 promoter fails to stimulate replication from the ColE1-type replication origin, indicating that the RNAII transcribed from the T7 promoter by induced T7 RNA polymerase is not functional for priming of plasmid DNA replication.

During the above experiment we observed that cultures of *E.coli* BL21(DE3) containing pCMRNAII-1, but not the host strain which did not carry plasmid pCMRNAII-1, showed a decreased growth rate after IPTG induction of T7 RNA polymerase (Fig. 2). This decrease in growth rate was also observed when IPTG induction was at a lower temperature (culture D), at which the level of RNAII produced was reduced but was still increased ~10-fold over basal expression. To learn whether the growth effect was due to the addition of ampicillin, which might eliminate cells that had lost their plasmids during cell growth, we carried out a similar experiment in the absence of ampicillin. As shown in culture B, an identical result was observed. Plating of the cells from cultures B–D onto LB plates showed that colony formation on LB plates was sharply reduced (data not shown). From these results we conclude that slowed growth and decreased viability result from the overproduction of RNAII molecules, rather than from IPTG induction of T7 RNA polymerase. However, in contrast to the increase in copy number of ColE1-type plasmids that occurs when cell growth is slowed

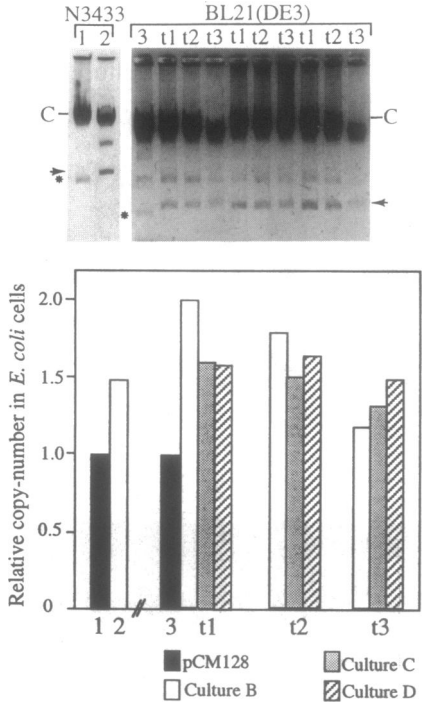

Figure 3. Analysis of plasmid copy number in *E.coli* N3433 or BL21(DE3) cells. The host cells used and the time points when culture samples were taken are indicated. Lanes 1 and 3, culture samples prepared from cells carrying plasmid pCM128. Lanes 2, t1, t2 and t3, culture samples prepared from cells containing plasmid pCMRNAII-1. The cell lysates were prepared as described in Materials and Methods. (Top) Electrophoresis patterns of chromosomal DNA (C) and plasmid DNA (pCM128 and pCMRNAII-1 are indicated with an asterisk and an arrow respectively; the upper band is the dimer form of each plasmid). From left to right, each set t1, t2 and t3 corresponds to samples taken from cultures C, D and B in Figure 2 respectively. (Bottom) Quantitative analysis of the copy number in the top panel. The relative copy number, as described in Material and Methods, is shown.

down by either reaching stationary phase (19) or by culturing in a poor medium (15), the slowed cell growth resulting from RNAII overproduction by T7 RNA polymerase was associated with an unchanged plasmid copy number, indicating that plasmid replication is correspondingly deceased.

DISCUSSION

In contrast to the elevated plasmid copy number observed when RNAII is transcribed from a strong *lac* promoter by *E.coli* RNA polymerase (8), we unexpectedly found that during overproduction of RNAII by bacteriophage T7 RNA polymerase, increased replication of pCMRNAII-1 does not occur. These results indicate that RNAII transcribed from the T7 promoter by T7 RNA polymerase is non-functional for the priming of plasmid DNA replication. Although RNAII molecules are barely detectable by Northern blotting in *E.coli* N3433, which does not contain the T7 RNA polymerase gene (Fig. 1, lanes 6–10), pCMRNAII-1 replicates at a higher copy number than pCM128 (Fig. 3, lanes 1 and 2). A similar elevation of pCMRNAII-1 copy number was also observed in the absence of IPTG treatment in the *E.coli* host BL21(DE3) (Fig. 3, lane 3 versus lane t1), where some RNAII synthesized by T7 RNA polymerase was observed due to leaky expression of this enzyme. As ColE1 replicons exert a dominant phenotype over pSC101-derived replicons (20), these results indicate that pCMRNAII-1 contains a functional ColE1-type replicon and suggest that there exists a putative promoter upstream of the RNAII gene capable of functioning together with the host RNA polymerase to synthesize a functional primer. The conclusion that pCMRNAII-1 contains a functional ColE1 replicon is also supported by the observation that a similar PCR-amplified DNA fragment that carries the native RNAII promoter, but lacks the T7 promoter, can replicate in *E.coli* cells when it is ligated to a kanamycin resistance gene (data not shown). Collectively, therefore, RNAII transcribed by *E.coli* RNA polymerase is functional as a replication primer, whereas RNAII transcribed by T7 RNA polymerase is not.

Why is RNAII transcribed by T7 RNA polymerase non-functional for priming of DNA replication? T7 RNA polymerase initiates RNA chains preferentially at G residues (21,22); one possibility is that the additional two G residues (the first residue of wild-type RNAII is G) added in our construct to ensure initiation of transcription by T7 RNA polymerase interferes with folding of RNAII. However, computer analysis of the secondary structure of wild-type RNAII and that of RNAII molecules containing the two additional G residues (Fig. 1) showed only minor differences at the 5′-end and essentially no differences in the region near the ColE1 origin (data not shown). Thus the difference in the 5′-end sequence is not likely to be the cause of the observed non-functionality of RNAII transcribed by T7 RNA polymerase. Moreover, earlier work has shown that base substitutions and deletions in the 5′-end region of RNAII do not abolish RNAII function (11,14).

Recent observations indicate that RNA transcripts made by *E.coli* RNA polymerase also cannot be functionally replaced by transcripts transcribed by bacteriophage T7 RNA polymerase (23); rRNA transcribed by T7 RNA polymerase was inactive in ribosomal protein synthesis both *in vivo* and *in vitro* (23). It was proposed that ribosome assembly was coupled to rRNA chain elongation speed and that rRNA synthesized by T7 RNA polymerase, which transcribes five times faster than *E.coli* RNA polymerase (9), cannot function in the formation of active ribosomal subunits *in vivo* (23). Another report by Lopez *et al.* (24) demonstrated that, while transcriptional efficiency is increased in the T7 expression system *in vivo*, translational efficiency surprisingly does not increase concomitantly (24). These authors suggested that, due to the high speed of transcription, T7 RNA polymerase actually outruns bacterial ribosomes, leaving the mRNA segment 5′ to T7 RNA polymerase essentially naked of ribosomes, thus enabling the naked mRNA to be attacked by endoribonucleases. Alternatively, they proposed that a rapidly elongating transcript devoid of ribosomes may be free to fold into complex structures that block access to the Shine–Dalgarno sequence (24).

For the pCMRNAII-1 plasmid we suggest that the rapid rate of synthesis of RNAII transcribed by T7 RNA polymerase may similarly interfere with proper folding of RNAII, thus interfering with its ability to function as a primer for plasmid DNA replication. Folding of RNAII is crucial to the formation of persistent hybrids between elongating RNA and its DNA template (1) and factors that affect this process interfere with the ability of RNAII to function as a replication primer. It has been proposed that control of transcriptional speed is crucial for facilitating proper folding of RNAII and subsequent interaction between its –265 region and the –20 'switch' site on the DNA

template (25). Consistent with this view is evidence that slowing of RNAII transcription by limiting dNTP concentration decreased the efficiency of hybrid formation *in vitro* (1). It has also been proposed that the secondary structure of RNAII regulates the transcriptional speed of RNAII made by *E.coli* RNA polymerase (25). T7 RNA polymerase, which is insensitive to transcription terminators (10) and probably also to other regions of localized secondary structure, might read through structure X of RNAII *in vivo* without pausing, causing improper folding of the secondary structure and inhibiting formation of the crucial persistent hybrid and, consequently, plasmid DNA replication. Our observation that bacteriophage T7 RNA polymerase cannot replace *E.coli* RNA polymerase to produce an active RNAII primer, together with those of others (24,26), provides further evidence that speed of transcription and its effects on folding of RNA may play a role in the control of replication of ColE1-type plasmids.

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