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The *Escherichia coli pcnB* gene promotes adenylylation of antisense RNAI of ColE1-type plasmids *in vivo* and degradation of RNAI decay intermediates

(RNA decay/pBR322/antisense RNA/plasmid replication/polyadenylylation)

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ABSTRACT **Previous work has shown that RNase E-mediated cleavage of RNAI, an antisense repressor of the replication of ColE1-type plasmids, relieves repression *in vivo* by endonucleolytically converting RNAI to a rapidly decaying product. We report that mutations in the *Escherichia coli pcnB* gene result in a 10-fold prolongation of the half-life of RNAI decay intermediates and also of truncated RNAI primary transcripts lacking sites attacked by RNase E. Using Northern blotting, primer extension analysis, [^{32}P]GTP capping of 5′-triphosphate termini, and PCR amplification methods, we show that *pcnB*-mediated acceleration of RNAI degradation is associated with posttranscriptional 3′ addition of adenosine residues *in vivo* to native and processed forms of RNAI. Accumulation of antisense RNAI decay products in *pcnB* mutants potentially explains the reduced copy number of ColE1-type plasmids seen in the mutated bacteria.**

Replication of ColE1 and related *Escherichia coli* plasmids is initiated by a primer, RNAII, which forms a hybrid with its template DNA and then is cleaved by RNase H—producing a free 3′-OH end for the addition of deoxyribonucleotides. A second RNA, RNAI, is transcribed in an orientation opposite to that of RNAII from a segment of DNA that encodes the 5′ end of RNAII. Interaction of the antisense RNAI with RNAII causes a conformational change in the primer, inhibiting its binding to the template DNA and consequently preventing the initiation of leading-strand DNA synthesis (for reviews, see refs. 1 and 2). The rate of decay of RNAI recently has been found to be a key element in the *in vivo* control of plasmid pBR322 replication; pBR322 RNAI is cleaved near its 5′ end by the endonuclease RNase E, converting full-length antisense RNAI into relatively unstable pRNAI$_{-5}$ and consequently relieving repression of replication (3).

Mutations in various host genes implicated in DNA replication can affect plasmid copy number (4–6). Additionally, mutation of the *pcnB* gene (7–10), which is distinct from other *E. coli* chromosomal loci known to affect plasmid DNA synthesis, has been shown to reduce the copy number of ColE1-type plasmids in otherwise normal bacteria but to have no effect on plasmids whose replication is not controlled by antisense RNA. In the experiments reported here, we show that mutation of *pcnB* decreases the rate of decay of products generated by RNase E-mediated cleavage of RNAI, leading to intracellular accumulation of decay intermediates capable of repressing plasmid DNA replication. Moreover, we show that RNAI normally is polyadenylylated posttranscriptionally in *E. coli in vivo* and that mutation of *pcnB* prevents such polyadenylylation.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. *E. coli* strains Mri7 (F$^-$ *ΔlacU169 araD139 thiA rpsL relA Δrbs-7 pcnb*$^+$) (8) and Mri93 (Mri7 *zad*::Tn*10 pcnB80*) were obtained from J. Beckwith. Cells were grown in LB medium at 37°C containing appropriate antibiotics as required (ampicillin at 50 μg/ml and chloramphenicol at 25 μg/ml). Plasmid pJL89-Cm was constructed by replacing the 5.3-kb *Pvu* II–*Bgl* II fragment containing the gene encoding ampicillin resistance in pJL89 (11) with the 3.0-kb *Pvu* II–*Xmn* I gene fragment encoding chloramphenicol resistance from pACYC184. Other plasmids used were described in ref. 3.

DNA and RNA-Related Methods. The methods used were essentially those described (3) except that RNA samples were electrophoresed for 12 h at 40 W in 12% Hydrolink (AT Biochem, Malvern, PA) sequencing polyacrylamade gels containing 8 M urea; the electrophoresis buffer contained 50 mM Tris·HCl (pH 8.3) and 40 mM glycine. *In vitro* synthesis of RNAI transcripts of pBR322 and pSC-L101 was carried out as described (12) using 0.7-kb *Pst* I–*Alw*NI fragments of the plasmids as templates. M13 phage construct pM21 (13) was used for the *in vitro* production of 5′-GGG-RNAI using T7 RNA polymerase (Promega). *In vitro* polyadenylylation of 5′-GGG-RNAI used *E. coli* poly(A) polymerase (Life Technologies, Gaithersburg, MD). Purification of RNAI by hybrid selection used M13mp7.RII DNA (14) coupled to diazophenyl thioether-cellulose paper (Schleicher & Schuell) and was carried out according to the manufacturer's recommendations except that after hybridization filters were washed six times in 0.1% SDS/0.1× standard saline citrate at 42°C and the hybridized RNA was eluted by heating at 70°C in buffer containing 10 mM Tris·HCl (pH 7.5), 1 mM EDTA, 0.1% SDS, and 98% (vol/vol) formamide.

Primer extension analysis was carried out as described (11) using Superscript I reverse transcriptase (Life Technologies) except that the primer/RNA mixture was heated in water at 80°C for 4 min and then quickly chilled on ice prior to extensions. Labeling of 5′-triphosphate termini by [^{32}P]GTP used guanylyltransferase as recommended by the manufacturer (Life Technologies). PCR was performed using the GeneAmp RNA PCR kit and the Ampliwax PCR Gem 100 system (Perkin–Elmer/Cetus). The primers used are indicated in the figure legends. T4 RNA ligase (Bio-Rad) and restriction enzymes were used as recommended by the manufacturers. Sequencing of cloned cDNA complementary to RNAI was carried out by using modified T7 DNA polymerase (Sequenase, United States Biochemical).

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RESULTS

RNAI Is Heterogeneous in Length. Total RNA isolated from the pBR322-containing *E. coli* strains Mri7 and Mri93 and from Mri93 containing a plasmid (pJL89-Cm) carrying the cloned *pcnB* gene was separated by electrophoresis and hybridized with a ^{32}P-labeled RNA probe complementary to RNAI (Fig. 1*A*) (3). The sizes of the RNAI species were calibrated using unprocessed 108-nt RNAI ($RNAI_U$) and a mutationally truncated RNAI variant ($pppRNAI_{-5}$) synthesized *in vitro* (12) on pBR322 and pSC-L101 (3) templates, respectively, as standards (Fig. 1*B*). As shown in Fig. 1, three hybridizing RNAI species longer than the 108-nt pBR322 RNAI made *in vitro* were observed in cells containing a functional *pcnB* gene either on the chromosome or cloned on a plasmid; these had appeared as a single broad band in lower resolution analyses done previously (e.g., refs. 3, 13, and 15). In contrast, no RNAI species longer than $RNAI_U$ was detected by Northern blotting in the *pcnB* mutant, which instead showed massive accumulation of transcripts (Fig. 1 *A Center* and *B*, lane 1) that appeared to be 6–10 nt shorter than 108-nt pBR322 RNAI transcripts made *in vitro* and 1–4 nt shorter than 103-nt pSL-C101 RNAI transcripts made *in vitro*. Experiments using other (7) *pcnB* alleles (data not shown) produced similar results.

Analysis of the 5′ and 3′ Ends of RNAI Species Made in *pcnB*$^+$ Bacteria and *pcnB* Mutants. To understand the nature of the elongated RNAI species isolated from *pcnB*$^+$ strains and of the truncated RNAI derivatives accumulating in *pcnB* mutants, we analyzed their 5′ and 3′ ends. RNAI transcripts were purified by filter hybridization with an RNAII sequence cloned on bacteriophage M13 (13), and their 5′-triphosphate termini were labeled with [^{32}P]GTP by vaccinia virus guanylyltransferase; using identical DNA sequence ladders to calibrate the positions of bands, the lengths of capped transcripts (Fig. 2) were compared with the lengths of the calibrated transcripts detected by Northern blotting in an adjacent section of the same gel (Fig. 1*B*). The results indicate that the 108-nt RNAI species observed in Northern blots of RNA from both *pcnB*$^+$ and *pcnB* mutant strains (Fig. 1) contains a 5′-triphosphate terminus and therefore is a primary transcript. 5′-Triphosphate termini were also demonstrated by [^{32}P]GTP capping on the three RNAI species longer than 108 nt that were found in *pcnB*$^+$ but not in *pcnB* mutant bacteria, and on a 106-nt species detected in both strains (Fig. 2). However, the truncated RNAI transcripts that accumulated to a high concentration in the *pcnB* mutant were not labeled by the capping enzyme, suggesting that they are decay intermediates.

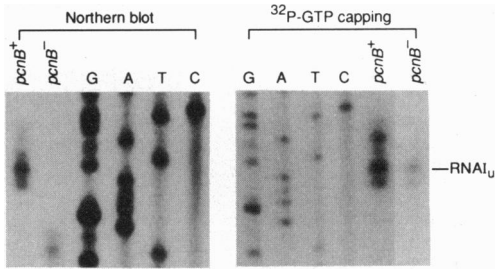

FIG. 2. Analysis of 5′ termini of RNAI species by Northern blotting and [^{32}P]GTP capping. RNA was separated in parallel for both experiments in gels containing the identical sequence ladder. The position of $RNAI_U$ in the Northern blot was used in conjunction with the sequence ladders to determine the lengths of the RNAI species capped in *pcnB*$^+$ and *pcnB*$^-$ strains.

Primer extension analysis of RNAI from the *pcnB*$^+$ strain using an oligonucleotide primer internal to RNAI revealed a 5′ end that corresponds to the 5′ terminus of the 108-nt primary transcript (Fig. 3, lane 1). In addition, a single band that appears to result from the initiation of some transcripts at an adventitious site (S.L.-C., T.-T. Wong, K. McDowall, and S.N.C., unpublished data) was observed for the several cap-labeled RNAI species longer than 108 nt isolated from *pcnB*$^+$ bacteria, implying that the differences in length observed for the capped transcripts are the result of heterogeneity at the 3′ end. Primer extension analysis of RNA isolated from the *pcnB*$^+$ strain also revealed 5′ ends that have been shown to result from cleavage of RNAI at positions 3 and 5 from the 5′ end of the 108-nt primary transcript.

A faint band representing the 5′ end of the 108-nt primary transcript was also detectable in the *pcnB* mutant (Fig. 3, lane 2). In addition, prominent 5′ ends that on the basis of our capping data are not triphosphate termini were identified by primer extension at positions 5, 6, and 7 nt downstream from the start site of the 108-nt transcript. Whether the bands seen at positions 6 and 7 result from multiple sites of primary cleavage of pBR322 RNAI in the *pcnB* mutant host or, alternatively, are produced by exonucleolytic removal of nucleotides from the 5′ terminus of the initial cleavage product is not known.

Mutation of *pcnB* Increases the Stability of RNAI Products Generated by RNase E Cleavage and also of Primary Transcripts Lacking an RNase E Cleavage Site. The multiple RNAI

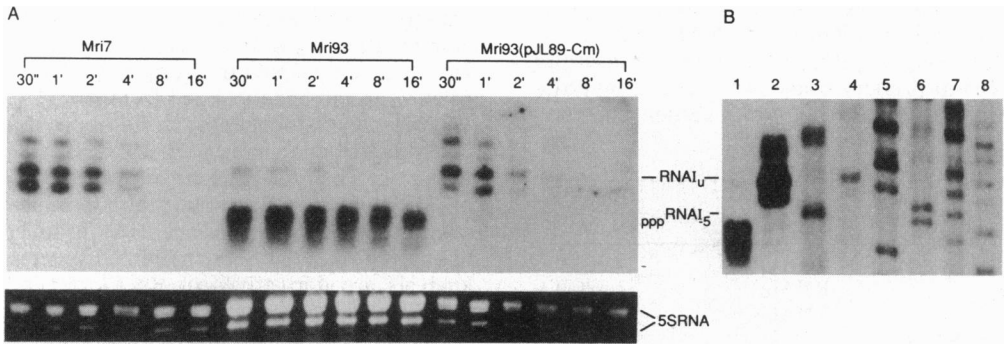

FIG. 1. (*A*) Processing of pBR322 RNAI in *E. coli pcnB*$^+$ and *pcnB* mutant strains. Total cellular RNA isolated at various times after rifampicin (0.5 mg/ml) was added to logarithmic-phase cultures (OD_{600} = 0.6) was subjected to Northern blotting analysis as indicated in *Materials and Methods*. 5S RNA was used for calibration of RNA loaded in different lanes; note the relative overloading of gels necessary to visualize RNAI bands in total RNA from the *pcnB* mutant, which contains the plasmid at low copy number. (*B*) RNAI synthesized *in vitro* from pBR322 (lane 4) and pSC-L101 (lane 3) was hybridized in Northern blots with an RNAII RNA probe (13) to calibrate the lengths of the RNAI species isolated from Mri7 (*pcnB*$^+$) (lane 2; also *A*) and Mri93 (*pcnB*$^-$) (lane 1; also *A*). $RNAI_U$ and $pppRNAI_{-5}$ indicate the position of unprocessed 108-nt pBR322 RNAI and 103-nt pSL-C101 RNAI, respectively. A larger transcript possibly resulting from initiation at an adventitious site *in vitro* is also seen for pSL-C101. Lanes 5–8 contain a sequence ladder.

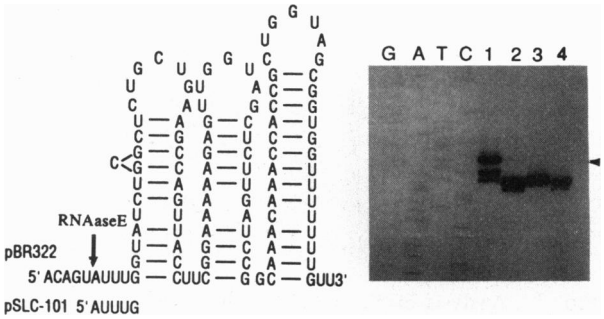

FIG. 3. (*Left*) Sequence of RNAI species encoded by pBR322 and pSL-C101. The RNase E cleavage site in pBR322 RNAI is indicated. (*Right*) Primer extension analysis. The oligonucleotide 5'-GCTA-CCAGCGGTGGTTTGTTTGCCGGATC-3' was used for both primer extension analysis and synthesis of pBR322 DNA sequence ladders. Lanes 1 and 2, pBR322-encoded RNAI from *pcnB*$^+$ and *pcnB* mutant strains, respectively; lanes 3 and 4, pSC-L101-encoded pppRNAI$_{-5}$ from the same strains. An arrowhead indicates the position of the pBR322 5' end that corresponds to RNAI$_U$.

primary transcripts 108 nt or longer synthesized in *pcnB*$^+$ strains and the 108-nt RNAI species (RNAI$_U$) found also in the *pcnB* mutant all had a 1.4-min half-life (Fig. 1 and Table 1), which is slightly shorter than the half-life determined previously for pBR322 RNAI under somewhat different growth and sampling conditions (3, 15). As reported previously, the 103-nt RNAI species (pRNAI$_{-5}$) generated by RNase E cleavage of primary RNAI transcripts *in vitro* and in *pcnB*$^+$ bacteria (3, 16) decayed rapidly (Fig. 1*A*); in contrast, degradation of the truncated RNAI species that mapped to a similar position in the mutated *pcnB* host was severely retarded ($t_{1/2}$ about 11 min) (Fig. 1 and Table 1). Analogous inhibition of degradation of pRNAI$_{-5}$ derivatives from the ColE1-related plasmid pACYC184 was also observed in *pcnB* mutant bacteria (data not shown).

Fig. 4 shows the result of Northern blot analysis of the decay of RNAI transcripts encoded by the mutated pBR322 derivative, pSL-C 101 (i.e., pppRNAI$_{-5}$, diagrammed in Fig. 3). It was previously found (3) that the half-life of pppRNAI$_{-5}$ transcripts, which lack a site cleaved by RNase E at their 5' ends, is significantly longer in wild-type bacteria than the half-life of pBR322-encoded RNAI. The rate of decay of pppRNAI$_{-5}$ was further decreased in the *pcnB* mutant ($t_{1/2}$ > 60 min; Fig. 4*A* and Table 1), leading to reduced plasmid copy number (data not shown). As the mutant transcript lacks 5 nt at the 5' end required for RNase E cleavage, the prolonged half-life of pppRNAI$_{-5}$ in the *pcnB* mutant indicates that the effects of the *pcnB* gene on RNAI decay do not require initial processing by RNase E. While transcripts shorter than the 103-nt primary transcript encoded by pSL-C101 were seen in both *pcnB*$^+$ and *pcnB* mutant bacteria,

Table 1. Half-lives of RNAI species

Strain and plasmid(s)	RNAI species	Half-life, min
Mri7 (pBR322)	RNAI$_U$	1.4 ± 0.1
Mri93 (pBR322)	RNAI$_U$	1.3 ± 0.1
	RNAI$_P$	11.0 ± 0.5
MRi93 (pJL89-Cm, pBR322)		
	RNAI$_U$	1.1 ± 0.2
MRi7 (pSL-C101)	pppRNAI$_{-5}$	7.8 ± 0.5
MRi93 (pSL-C101)	pppRNAI$_{-5}$	>60

Determination of RNAI half-life has been described (3). RNAI$_U$ in Mri7 and Mri93 (pJL89-Cm) represents the half-life of pBR322 RNAI transcripts of 106, 108, 109, 110, and 111 nt, all of which have similar decay rates. RNAI$_P$ indicates the half-life of nonseparated products of RNase E cleavage of pBR322 RNAI (see text and Fig. 1).

RNAI derivatives longer than the pppRNAI$_{-5}$ primary transcript were detected in only the *pcnB*$^+$ host (Fig. 4*A*). [^{32}P]GTP capping of pSL-C101-encoded transcripts purified from *pcnB*$^+$ bacteria by hybridization with DNA containing the RNAII sequence showed the presence of 5'-triphosphate termini on multiple transcripts longer than 103-nt pppRNAI$_{-5}$ (Fig. 4*A*); however, primer extension analyses using a primer internal to the transcript showed only a single 5' end mapping to position 5 (Fig. 3, lane 3), indicating that, as in the case of pBR322 RNAI, elongation of pSL-C101-encoded pppRNAI$_{-5}$ results from events that occur at the 3' end. pSL-C101 transcripts isolated from *pcnB* mutant bacteria showed three intense bands at the same positions (5, 6, and 7 nt downstream from the band corresponding to the 5' end of 108-nt pBR322 RNAI; Fig. 3, lane 4) as the 5' termini found for pBR322 RNAI in *pcnB* mutant bacteria.

***pcnB* Mediates 3' Adenylylation of RNAI.** In preliminary experiments, we had observed that RNAI isolated only from *pcnB*$^+$ strains could bind to an oligo(dT) column or oligo(dT) nylon paper, suggesting the presence of poly(A) tracts in this RNA; we also found that cDNA made on the oligo(dT)-binding RNAI template could be amplified by PCR using an RNAI-specific internal primer and a primer that consisted of an oligo(dT) sequence. Moreover, extracts of cells in which the *pcnB* gene was overexpressed from a controlled bacteriophage T7 promoter (17) had four times the adenylylating activity found in identically prepared extracts from cells that did not overexpress *pcnB*, as assayed *in vitro* (18) using yeast tRNA or synthetic pBR322 RNAI as substrate. Collectively, these data suggested that the RNAI transcripts extended at their 3' ends *in vivo* might result from *pcnB*-mediated addition of adenosine residues.

To determine whether RNAI is in fact adenylylated *in vivo* in *pcnB*$^+$ cells but not in a *pcnB* mutant, we carried out PCR using two pairs of RNAI-specific primers to amplify a single-stranded cDNA copy of RNAI. One pair, A and B (Fig. 5 *Upper*), was entirely internal to RNAI, whereas the other pair consisted of primer A plus a second primer, C, that contained a 5-nt sequence complementary to the 3' end of RNAI plus a distal oligo(dT) segment. Primer C was also used for synthesis of the cDNA strand that served as the template for PCR amplification. The targets of cDNA synthesis and amplification were a 5'-GGG-RNAI variant (13) made *in vitro* using T7 RNA polymerase, the same synthetic RNAI sample that had been polyadenylylated at its 3' end *in vitro* by *E. coli* poly(A) polymerase, and RNAI preparations purified from *pcnB*$^+$ and *pcnB* mutant cells by hybridization with a DNA segment containing the sequence of RNAII.

As shown in Fig. 5 *Lower*, DNA segments of the same size (95 bp) were generated from all four RNAI targets after PCR amplification using the internal pair of primers (A and B). However, only RNAI transcripts that had been adenylylated *in vitro* or had been isolated from *pcnB*$^+$ bacteria produced PCR-amplified fragments of the appropriate size (130 bp) when the dT-containing primer overlapping the 3' end (i.e., primer C) was used in place of primer B. Sequence analysis of the cloned PCR amplification products obtained from transcripts isolated from the *pcnB*$^+$ strain confirmed that the appropriately sized 3'-adenylylated species observed by gel analysis are derivatives of RNAI rather than nonspecific products of PCR amplification.

During analysis of cDNA sequences corresponding to RNAI by another procedure, we discovered that RNAI can undergo 3' adenylylation at sites other than its natural terminus. In these experiments, RNAI isolated from the *pcnB*$^+$ strain Mri7 and purified by filter hybridization was joined by RNA ligase to the *Bgl* II-generated 5' terminus of a pSP72 DNA fragment containing a polylinker sequence that had been cleaved by *Pvu* II and then treated with alkaline phosphatase prior to digestion with *Bgl* II (Fig. 6 *Left*). After

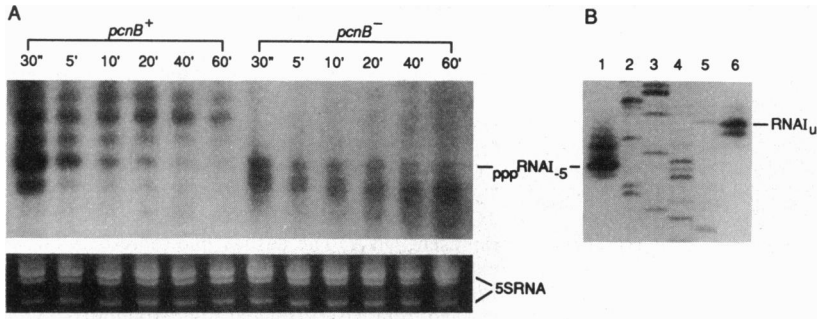

FIG. 4. Effect of the *pcnB* gene on decay of truncated pSL-C101-derived RNAI variant transcripts. RNA designations and procedures used for Northern blotting (*A*) and capping analysis (*B*) are indicated in Figs. 1 and 2. The position designated as pppRNAI$_{-5}$ in *A* was calibrated using the gel position of pppRNAI$_{-5}$ made *in vitro* (Fig. 1*B*, lane 3). The size of the [^{32}P]GTP-labeled pppRNAI$_{-5}$ (lane 1) was determined in relation to the size of RNAI$_U$ (lane 6) using a sequence ladder (lanes 2–5).

extension of the polylinker strand by reverse transcriptase using the ligated RNA as a template, part of the resulting cDNA segment was amplified by PCR using a primer (primer A) internal to RNAI together with a primer (primer D) complementary to the polylinker. Further PCR amplification of the RNAI/vector junction segment was then carried out by using an additional pair of nested primers (primers B and C) located at the positions shown in Fig. 6.

The amplified product was subjected to polyacrylamide gel electrophoresis, and DNA extracted from gel slices corresponding to fragments 82–118 bp in length was treated with *Eco*RI and cloned by ligation to *Eco*RI–*Sma* I-treated pUC19 DNA. Sequencing of 33 clones was then carried out using primers complementary to pUC19. RNAI-specific sequences were observed in all but one clone, which contained DNA

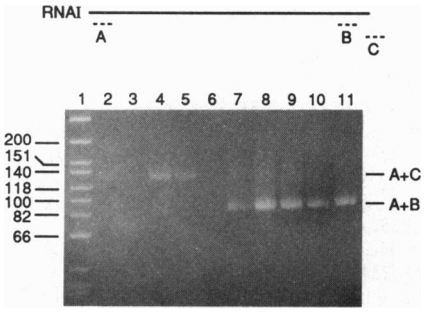

FIG. 5. Amplification of RNAI-specific cDNA sequences by PCR. The locations of primers A (5′-ATTTGGTATCTGCGCTCTGC), B (5′-AACCACCGCTACCAACGGTGGTTT), and C (5′-GACTCGAGTCGACAAGCTTTTTTTTTTTAACAA) are indicated. A segment containing multiple restriction site sequences (underlined) distal to the oligo(dT) tract on the primer facilitated the cloning and subsequent sequence analysis of PCR products amplified by primers A and C. RNAI samples synthesized *in vivo* were purified by hybridization with M13mp7 RNAII prior to synthesis of cDNA; 5′-GGG-RNAI synthesized *in vitro* by T7 RNA polymerase was either used directly for cDNA synthesis or was polyadenylylated *in vitro* prior to cDNA synthesis where indicated. Oligomers B and C were used to prime cDNA synthesis on all RNA templates, and the single-stranded cDNA products served as templates for separate PCR amplification in separate reactions that used primer pairs A and C or A and B. RNA samples used for cDNA synthesis are as follows: lanes 2 and 7, polyadenylylated GGG-RNAI at 0.165 ng/μl; lanes 3 and 8, nonpolyadenylylated GGG-RNAI at 31.3 ng/μl (present in excess to ensure that the failure of primers A and C to produce a detectable amplified product did not result from insufficient template); lanes 4 and 9, pBR322 RNAI from Mri7 at 3.77 ng/μl; lanes 5 and 10, same RNA as in lanes 4 and 9, except diluted 10-fold; lanes 6 and 11, pBR322 RNAI from Mri93 at 1.03 ng/μl. Oligonucleotide primers and cDNA were heated to 80°C for 4 min and cooled gradually to 39°C prior to cDNA synthesis. PCR amplification (50 cycles) was carried out as recommended by the manufacturer (Perkin–Elmer/Cetus) using the following parameters: denaturation, 1.5 min at 95°C; annealing, 3 min at 50°C; extension, 1 min at 72°C. A final extension step was done for 5 min at 72°C. Samples were loaded on 8% polyacrylamide gels, electrophoresed, and stained with ethidium bromide. Lane 1, ΦX174 DNA markers digested with *Hin*fI.

that presumably was amplified nonspecifically by PCR. In 30 of the 32 clones containing RNAI-derived cDNA, the RNAI segment was truncated at multiple locations within the third stem–loop structure rather than at the actual 3′ terminus of RNAI. In 24 of these, we detected processed RNAI derivatives that had their 3′ termini located at positions internal to RNAI and ended with the nucleotides CA or C(A)$_n$ (Fig. 6 *Upper Right*). While the presence of adenosine residues in the native RNAI sequence at positions that correspond to the adenosine residues observed in nearly all of these processed RNAI derivatives left uncertain whether adenosine residues were added to the truncated RNAI segments posttranscriptionally, in one instance (clone 64; Fig. 6 *Right*) the 3′ end of the RNAI remnant was followed by a run of adenosine residues that were not present in the native RNAI sequence and thus are presumed to have been added to the RNAI derivative after it had been processed. One clone (clone 11) showed three adenosine residues attached distally to the 3′ end of the third stem–loop, providing direct evidence of adenylylation of RNAI at this site *in vivo* in *pcnB*$^+$ bacteria.

DISCUSSION

In wild-type *E. coli*, decay of pBR322 RNAI is initiated by endonucleolytic cleavage of the antisense transcript by RNase E within a 10-nt single-strand segment near its 5′ end. This cleavage, which is dependent on a gene (*ams*; ref. 19) that also affects the activity of other characterized RNases as well as the chemical decay of bulk mRNA (19–21), ordinarily leads to formation of a decay intermediate, pRNAI$_{-5}$, which is rapidly degraded (3). The findings reported here indicate that a function of the *E. coli pcnB* gene facilitates the decay of pRNAI$_{-5}$. While the *pcnB* gene does not affect the rate of cleavage of pBR322 RNAI by RNase E, the prolonged half-life of the RNase E cleavage product(s) in *pcnB* mutants results in the intracellular accumulation of RNAI derivatives that, as has been shown (3), have antisense activity and consequently can inhibit initiation of pBR322 DNA replication. The observed effect of *pcnB* mutations on the degradation of RNAI decay intermediates potentially explains the phenotype that led to the initial identification of *pcnB* mutants—namely, decreased copy number of ColE1-type plasmids (7–10).

Our discovery that RNAI and its derivatives are extended in length at their 3′ ends in cells containing a functional *pcnB* gene, but that such extensions are not detected in *pcnB* mutants, led us to investigate the nature of the 3′ additions. Our consequent finding that the *pcnB* gene product can adenylylate RNAI *in vivo* and *in vitro* is consistent with recently published evidence that the *pcnB* locus encodes a poly(A) polymerase (22) and that specific mRNA species (e.g., *lpp* mRNA; ref. 22) can be polyadenylylated in *E. coli*. However, whereas adenylylated bacterial RNA has been reported to be relatively resistant to exonucleolytic degradation (22, 23), we find that the decay of RNAI is accelerated by *pcnB*-mediated adenylylation.

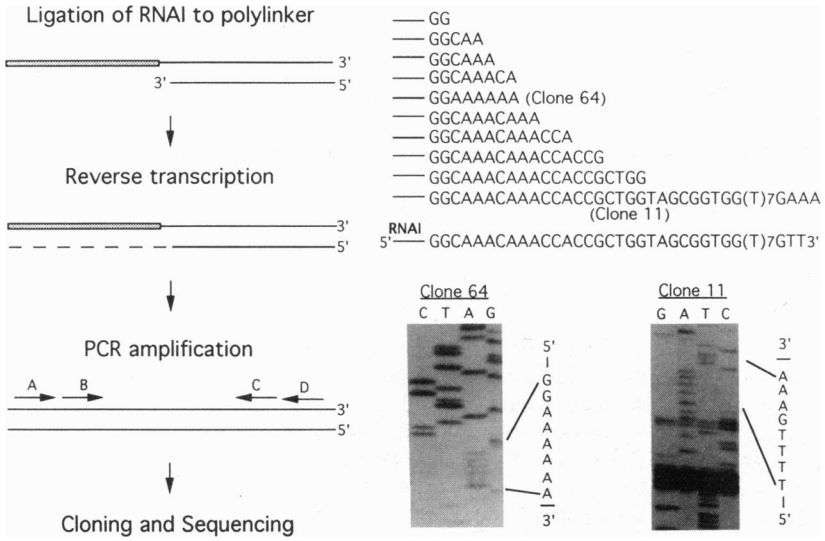

FIG. 6. Analysis of 3' termini of RNAI cDNA amplified using internal and external pairs of primers. (*Left*) The procedures followed are described in the text and Fig. 4. The sequences of the primers are as follows: A, 5'-ATTTGGTATCTGCGCTGAA; B, 5'-CCTTCGGAAAAGAGTTGGTAGCT; C, 5'-GGTACCGCTCGAATTCATCGAT; and D, 5'-CCTTCGGAAAAGAGTTGGTAGCT. The solid lines represent DNA. The dashed line represents cDNA extended on RNA template (stippled bar). (*Upper Right*) DNA sequences of cloned cDNA segments derived from RNAI isolated from strain Mri7. Solid lines represent the common sequence. The corresponding segment of the DNA sequence encoding unprocessed RNAI is indicated. (*Lower Right*) Sequencing gel of cDNA (primer, 5'-CCCAGTCACGACGTTGTAAAACG) showing adenylylation internal to RNAI (clone 64) and, for the complementary strand in clone 11, at the RNAI 3' end. Sequencing of the other strand resolved the band compressions shown but yelded compressions elsewhere in the gel.

During our Northern blot analysis of pBR322 RNAI isolated from *pcnB* mutant cells, we observed RNAI species 6–10 nt shorter than the 108-nt RNAI transcripts made *in vitro* on pBR322 DNA template (Fig. 1*B*, compare lanes 1 and 4). Primer extension analysis and capping studies indicated that these RNAI derivatives have lost nucleotides at their 3' ends in addition to having nucleotides removed from their 5' ends by the action of RNase E and possibly other RNases (3, 16). The generation of these species in *pcnB* mutants potentially could occur by removal of nucleotides 3' to the distal stem–loop structure by a 3' to 5' exonuclease or by premature termination of some transcripts. We speculate that the capped 106-nt RNAI-derived transcripts we observed in both *pcnB*$^+$ and *pcnB* mutant bacteria may have been formed by one of these mechanisms.

Our Northern blotting results suggest that the tails added to the native 3' ends of pBR322 RNAI and pSL-C 101-encoded pppRNAI$_{-5}$ *in vivo* by the action of the *pcnB* gene are shorter than the poly(A) tail identified on *lpp* mRNA made in *E. coli* (24). However, the *lpp* mRNA experiments were carried out in bacteria mutated in 3' to 5' exonucleases, while the strains we used contain no such mutations. Interestingly, the stem–loop structure present at the 3' end of *lpp* mRNA is similar to the one found at the 3' end of RNAI. While posttranscriptional addition of adenosine residues to 3' termini of RNAI molecules accounts for the processing of at least some RNAI transcripts in *pcnB*$^+$ bacteria, our data do not exclude the possibility of additional *pcnB*-mediated effects. It has been observed that the PcnB protein includes a domain resembling one in the *E. coli* enzyme tRNA nucleotidyltransferase, which generates CCA residues at the 3' termini of tRNA molecules (10, 25). Given the structural similarity between the two proteins, it is of interest that most of the processed RNAI derivatives we have detected in a *pcnB*$^+$ strain terminate with the sequence C(A)$_n$.

Although the rate of initial cleavage of RNAI by RNase E is not altered by *pcnB* mutations, our findings suggest that the subsequent steps in RNAI decay are influenced by the *pcnB* gene product. In *pcnB* mutant cells, degradation of a truncated variant of RNAI (pppRNAI$_{-5}$) that lacks an RNase E cleavage site near its 5' end is greatly impeded, indicating that the ability of *pcnB* to affect RNAI decay does not require initial RNase E-mediated processing of the transcript. Moreover, our identification by primer extension analysis of 5' termini 1 and 2 nt 3' to the 5' end of pppRNAI$_{-5}$ in *pcnB* mutant cells suggests that these transcripts may contain termini that have been generated by 5' to 3' exonucleolytic activity.

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