The RNase Z Homologue Encoded by *Escherichia coli* elaC Gene Is RNase BN*

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In eukaryotes, archaea, and in some eubacteria, removal of 3′ precursor sequences during maturation of tRNA is catalyzed by an endoribonuclease, termed RNase Z. In contrast, in *Escherichia coli*, a variety of exoribonucleases carry out final 3′ maturation. Yet, *E. coli* retains an RNase Z homologue, ElaC, whose function is under active study. We have overexpressed and purified to homogeneity His-tagged ElaC and show here that it is, in fact, the previously described enzyme, RNase BN. Thus, purified ElaC displays structural and catalytic properties identical to those ascribed to RNase BN. In addition, an elaC mutant strain behaves identically to a known RNase BN− strain, CAN. Finally, we show that wild type elaC can complement the mutation in strain CAN and that the elaC gene in strain CAN carries a nonsense mutation that results in loss of RNase BN activity. These data correct a previous misassignment for the gene encoding RNase BN. Based on the fact that the original RNase BN mutation has now been identified, we propose that the elaC gene be renamed *rbn*.

In all organisms examined, tRNA genes are transcribed as precursor forms that contain extra 5′ and 3′ sequences that must be removed to generate mature, functional species (1). The 5′ processing event carried out by the endoribonuclease RNase P is quite similar among different organisms (2). However, 3′-end maturation varies considerably and may utilize an exonucleolytic or an endonucleolytic mechanism depending largely on whether or not the universal 3′-terminal CCA sequence is encoded (1–6).

In *Escherichia coli*, an organism in which all tRNA genes have the CCA encoded (5), 3′ maturation generally begins with an endonucleolytic cleavage by RNase E downstream of the mature 3′ terminus (7), followed by exonucleolytic trimming to expose the CCA sequence (1–5). Any one of five exoribonucleases, RNase II, RNase D, RNase BN, RNase T, or RNase PH, can carry out the trimming reaction (8), although RNases T and PH are most effective (8). In eukaryotic cells, in which the CCA sequence is not encoded (1–6), 3′ maturation is carried out by an endonuclease, termed RNase Z or 3′-tRNAse (6, 9, 10). Cleavage by this enzyme occurs after the discriminator base (6, 9, 10), followed by CCA addition by tRNA nucleotidyltransferase to generate mature tRNA (5).

Genes encoding RNase Z homologues are found in eukaryotes, archaea, and about half of the sequenced eubacterial genomes (11, 12). In *E. coli*, the RNase Z homologue is encoded by the *elaC* gene located at 51.3 min on the *E. coli* chromosome (13). The protein encoded by *elaC* was originally found to be a zinc phosphodiesterase (ZiPD) (14) and was subsequently shown to have RNAse activity on certain tRNA precursors (12, 15). However, the absence of a phenotype in an *elaC* knock-out mutant (16), and the presence of CCA in all *E. coli* tRNA genes (5), has made it difficult to understand what might be the role of ElaC in this organism.

Interestingly, the substrate specificity of RNase Z, i.e. removal of incorrect residues in the position of the CCA sequence, was reminiscent of a previously identified *E. coli* enzyme, termed RNase BN (17). This RNase was shown to be essential for maturation of certain bacteriophage T4 tRNAs that do not encode the CCA sequence (17, 18); thus, two *E. coli* mutant strains, BN (a B derivative) and CAN (a K-12 derivative) (19, 20), deficient in this RNase, could not support the growth of a phage T4 mutant that required a suppressor function of phage T4 tRNA<sub>ser</sub>, a tRNA for which the CCA is not encoded (18). RNase BN also can take over 3′ maturation of host tRNA precursors in a strain lacking the other tRNA maturation exoribonucleases, although it does so extremely poorly (3, 8).

Earlier, our laboratory reported that RNase BN is encoded by an orf (*yihY*) at 87.8 min on the *E. coli* genetic map, subsequently named *rbn* (21). Surprisingly, however, the *rbn* coding regions of strains BN and CAN were identical to wild type (21). Moreover, sequence analysis of *rbn* indicated that RNase BN was unlike other RNases, and in fact, was part of a hydrophobic protein family (22). These considerations led us to re-examine the identity of the gene that encodes RNase BN. We show here, using biochemical and genetic approaches, that RNase BN is actually encoded by the *elaC* gene and, therefore, that RNase BN is the RNase Z homologue of *E. coli*.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs Inc. The *Pfu* DNA polymerase was obtained from Stratagene. Calf intestine alkaline phosphatase was from Promega. The QIAEX II gel extraction kit was purchased from Qiagen Inc. [γ-<sup>32</sup>P]ATP (3,000 Ci/mmol) and [α-<sup>32</sup>P]UTP (3000 Ci/mmol) were obtained from PerkinElmer Life Sciences. The His-Trap HP column was obtained from Amersham Biosciences. Ultragel AcA44 was from Amersham Biosciences. Protein size markers were obtained from Bio-Rad. SequaGel, used to make urea-polyacrylamide gels, was purchased from National Diagnostics, Inc. DNA oligonucleotides were synthesized by Sigma. All other chemicals were reagent grade.

**Bacterial and Bacteriophage Strains**—Wild type *E. coli* K-12 strain CA265 and its RNase BN<sup>−</sup> derivative, strain CAN, have been described (17–19). Strain BL21II− (DE3)<sup>+</sup>LYS was also described previously (23). Wild type bacteriophage T4, the amber-suppressor T4 phage, B33, and phage P1vir were from our laboratory stock. An *elaC* deletion-insertion strain was constructed by replacing most of the *elaC* gene (all but four N-terminal amino acids of ElaC are removed) with a chloramphenicol resistance cassette using the method of Datsenko and Wanner (24). The pKD3 chloramphenicol resistance cassette was amplified by PCR using the following reverse and forward primers, 5′-GGGTGCGATTATAGGGGAAATGCTGTTTATTTAATAATGAAGCAGGATATGAAATATGCTCTTA-3′ and 5′-ATACATGCGGAATATTTTTTGTCGATTATAGGGGAAATGCTGTTTATTTAATAATGAAGCAGGATATGAAATATGCTCTTA-3′ and 5′-ATACATGCGGAATATTTTTTGTCGATTATAGGGGAAATGCTGTTTATTTAATAATGAAGCAGGATATGAAATATGCTCTTA-3′.
TTGGAAAATGATGCACTGAATTAATATTTTTTTTCTTC-3', respectively (residues homologous to elaC are underlined). The linear PCR product was first transformed into the electrocompetent strain BW25113/pKD46, and the elaC mutation was subsequently transferred into other strains by transduction with phage P1vir. Disruption of the elaC gene was verified by PCR using a variety of primers within and flanking the insertion cassette (data not shown).

**Plasmid Construction**—DNA fragments containing the elaC gene were amplified from chromosomal DNA of strains CA265 and CAN by PCR using the oligonucleotides ElaS732 (5'-CGGAATTCCGGCCAC-CTTGCTTTTTTCCAAAACGGAG-3') and ElaS738 (5'-CGGCTTC- GAGACGCGGGGTGTGATACAGGGACTTT-3') as primers. PCR products were inserted into the EcoRI and SalI restriction sites of plasmid pH797 to yield plasmids pElaC(CA265) (for the CA265 elaC gene) and pElaC(CAN) (for the CAN elaC gene). Construction of the overexpression plasmid, pET-ElaC, for production of ElaC, was carried out as follows: the elaC DNA sequence was generated by PCR from genomic DNA of *E. coli* strain CA244 using the forward primer (F3) 5'-GAACCCATTTGAATTTTTTTTAGTTACT-3', which introduced an NcoI restriction site (bold) and the reverse primer (P2) 5'-TAATACTCGAGTAAACGTTAACACGGT-3', which introduced an XhoI restriction site (bold). The PCR product was then cloned into the NcoI and XhoI restriction sites of the pET15b expression plasmid (Novagen). Since NcoI cuts the elaC gene internally into two fragments, insertion of the downstream fragment was done in two steps. Construction of the overexpression plasmid pET-ElaC(His) for production of His-tagged ElaC was performed as follows: the elaC DNA sequence was generated by PCR from genomic DNA of *E. coli* strain CA244 using the forward primer (P1) 5'-GAACCATTTGAATTTTTTTTAGTTACT-3', which introduced an NdeI restriction site (bold) and the reverse primer (P2). The PCR product was cloned into the NdeI and XhoI sites of pET15b.

**Expression and Purification of ElaC**—Strain BL21II (DE3)/pLys carrying the pET-ElaC(His) plasmid was grown at 37 °C in 1 liter of LB medium supplemented with 100 μg/ml ampicillin plus 34 μg/ml chloramphenicol to maintain the pLys plasmid. At an A600 of 0.6, 1 mM isopropyl β-D-thiogalactopyranoside was added, and growth was continued for another 2 h. Cells were harvested by centrifugation, and washed with 0.9% NaCl. The cell pellet was frozen at −80 °C until use.

All steps of the purification procedure were carried out at 4 °C. The frozen pellet was resuspended in 8 ml of buffer M (20 mM Tris-Cl, pH 7.5, 10 mM KCl, 10% (v/v) glycerol). Cells were ruptured by two passes through an Aminco French press at 12,000 p.s.i. The resulting crude extract was centrifuged at 30,000 × g for 20 min at 4 °C. The supernatant was added. After 2 h, cells were harvested by centrifugation and resuspended for another 2 h. Cells were harvested by centrifugation, and washed with 0.9% NaCl. The cell pellet was frozen at −80 °C until use.

**Gel Filtration**—Extracts containing ElaC(His) were applied to a Ultragel AcA44 gel filtration column (1.0 × 56.5 cm) equilibrated with 20 mM Tris, pH 7.5, 1 mM DTT, and equilibrated with buffer M. Proteins were eluted by a gradient from 0 to 600 mM imidazole, and the fractions were analyzed by SDS-PAGE. ElaC-containing fractions were pooled and concentrated by ultrafiltration using Ultrafree Biomax-10K.

**Preparation of Extracts**— Cultures (20 ml) of BL21II (DE3)/pLys cells carrying either pET-Ela or pET-Ela(CHis) were grown at 37 °C in LB medium. At an A600 of 0.6, 1 mM isopropyl β-D-thiogalactopyranoside was added, and cells were harvested by centrifugation and resuspended in 0.4 ml of buffer A (25 mM Tris, pH 7.5, 1 mM dithiothreitol). Resuspended cells were broken by sonication using two 20-s pulses.

**Substrates**—tRNA-C-[3H]A, tRNA-C-[32P]U, and tRNA-CA-[32P]A were used as substrates of RNase BN.

**Protein Determination**—Protein was determined by the method of Bradford (26).

**Enzyme Assays**—Standard assays for RNase BN were carried out in 100 μl reaction mixtures containing: 20 mM HEPES, pH 6.5, 0.2 mM CoCl2, 200 mM KCl, 10 or 20 μg of tRNA-C-[3H]A and the indicated amount of crude extract or purified protein.

**Gel Filtration**—Extracts containing ElaC(His) were applied to an Ultragel AcA44 gel filtration column (1.0 × 56.5 cm) equilibrated with 20 mM Tris, pH 7.5, 200 mM KCl, 1 mM dithiothreitol. Blue dextran 2000, bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsin (25 kDa), and vitamin B12 were used for calibration. Fractions of 1 ml were collected, and 50 μl of each was assayed for RNase BN.

**Phage Assays**—Bacteriophage T4 or T4 mutant strain BU33 (18) were incubated for 10 min at room temperature with 0.4 ml of *E. coli* at an A600 = 1. The suspension was then overlaid onto LB plates with 3 ml of top agar. Plates were incubated overnight at 37 °C prior to counting plaques.

**RESULTS**

**Purification of ElaC**—To examine its catalytic properties, we attempted to purify the ElaC protein both in its native and in a His-tagged form (see “Experimental Procedures”). RNases I and II were eliminated from the strain to reduce background RNase activity. Unexpectedly, we found that the active, native form of ElaC could not be overexpressed, whereas no such difficulty was found with the N-terminal His-tagged protein. Thus, as shown in Fig. 1A, induction of pET-ElaC essentially results in cessation of cell growth, whereas slower growth continues upon induction of pET-ElaC(His). Moreover, while the His-tagged form of ElaC protein could be readily overexpressed and led to increased RNase activity in extracts, results with the native form were quite variable (Fig. 1B). In some experiments, no overexpression was observed, whereas in others, such as shown in Fig. 1B, the protein was overexpressed, but it was inactive (data not shown). These data indicate that overexpression of ElaC is deleterious to *E. coli*. Second, it is likely that His-tagged ElaC is considerably less active than the native protein. However, direct confirmation of this conclusion must await purification of native ElaC.

To confirm that the elevated RNase activity observed in extracts was due to the overexpressed His-tagged ElaC, the extract was fractionated on an Ultragel AcA44 gel filtration column. Fractions were analyzed by SDS-PAGE and assayed for RNase activity (data not shown). His-tagged ElaC protein peaked in fractions 22 to 24, and the peak of RNase activity was found in the same position, supporting the conclusion that His-tagged ElaC is an active RNase.

His-tagged ElaC was purified to homogeneity using a HisTrap HP column. The purified protein had a specific activity with tRNA-CA as substrate of ~2,000 nmol/h/mg of protein. This compares with a specific activity of 55 nmol/h/mg of protein in the crude extract. On SDS-PAGE (Fig. 1C), the purified
protein migrated with an apparent molecular mass of 36 kDa. Taking into account the His-tag and additional amino acids introduced from the cloning, this value is in excellent agreement with the molecular mass calculated from the sequence of the \textit{elaC} gene (32.9 kDa). Based on an apparent molecular mass of \textasciitilde75 kDa, determined by gel filtration for the active, His-tagged enzyme, these data confirm that ElaC is a homodimer (14) and also show that it is similar in size to that reported earlier for RNase BN (17).

**Catalytic Properties of ElaC**—The reported preference of RNase Z for tRNA substrates lacking the CCA sequence appeared to be the same as that previously ascribed to \textit{E. coli} RNase BN (17, 18). Consequently, for the assays of the RNase Z homologue, ElaC, we used RNase BN assay conditions and tRNA-CA, a known substrate of RNase BN (17). Further examination of the specificity of ElaC for various tRNA substrates revealed that it acted on tRNA-CU, tRNA-CA, and tRNA-CCA essentially as previously found for RNase BN (5, 17). Purified ElaC also was active against bis(p-nitrophenyl) phosphate and thymidine-5'-p-nitrophenyl phosphate, as reported (14).

RNase BN is unusual among \textit{E. coli} RNases in that it is more active with \textit{Co}^{2+} than with \textit{Mg}^{2+} as the required divalent cation, and it is most active at pH values between 6 and 7 (17). As these are essentially unique signatures for RNase BN activity, we examined purified ElaC to determine whether these conditions were also optimal for its RNase activity. The data in Table I show that homogeneous ElaC acts on tRNA-CA much more efficiently with \textit{Co}^{2+} than with \textit{Mg}^{2+} and that the enzyme is more active at pH 6.5 than pH 7.5. These data support the idea that ElaC might be RNase BN.

As a further test of the relation between ElaC and RNase BN, we determined the product of ElaC action on tRNA-CU, a known substrate of RNase BN (17). Further examination of the specificity of ElaC for various tRNA substrates revealed that it acted on tRNA-CU, tRNA-CA, and tRNA-CCA essentially as previously found for RNase BN (5, 17). Purified ElaC also was active against bis(p-nitrophenyl) phosphate and thymidine-5'-p-nitrophenyl phosphate, as reported (14).

**Generation and Properties of an \textit{elaC} Mutant**—To further examine the relationship between ElaC and RNase BN, a deletion-insertion mutant of \textit{elaC} was constructed as described under "Experimental Procedures." Our ability to recover the mutant strain indicates that \textit{elaC} is not essential for \textit{E. coli} growth, confirming the conclusion of Schilling et al. (16). Growth of the \textit{elaC} mutant strain was indistinguishable from wild type on rich medium (LB) or minimal medium (M9/glucose) plates at 24, 37, and 42 °C. Thus, the absence of ElaC has no obvious effect on \textit{E. coli} growth. In earlier studies, it was shown that strain CAN, lacking RNase BN, also grows as well as wild type (17, 20, 27). Thus, these data show that both ElaC- and RNase BN- mutant strains are unaffected in growth.

The \textit{elaC} mutation was also transferred to a strain lacking RNase I, II, and D activity. In this background, the \textit{elaC} mutation reduced RNase activity against tRNA-C-[^3H]UA in extracts to \textasciitilde10% of that in a strain containing ElaC (data not shown). This finding indicates that elimination of ElaC dramatically reduces RNase activity, supporting the conclusion that ElaC is RNase BN. It was necessary to carry out this experiment in the RNase I II D background to eliminate other RNases that also act on the RNase BN substrate (17).

**Growth of T4 Phage in Wild Type and Mutant Strains**—\textit{E. coli} strains deficient in RNase BN are known to be unable to plate the mutant T4 phage strain BU33 (19) due to their inability to process the 3'-end of a phage-encoded suppressor tRNA\textsuperscript{+}

To obtain additional evidence for the identity of RNase BN and ElaC, wild type T4 and BU33 were plated on a wild type \textit{E. coli} strain (CA265), on a known RNase BN mutant strain (CAN) and on an ElaC mutant strain (BEZ33). The data in Table II show that wild type T4 is able to form plaques with equal efficiency on all three strains. In contrast, phage T4 BU33 could only form plaques on the wild type strain; the other strains could not support BU33 growth. These data indicate that ElaC- and RNase BN- mutant strains behave identically with respect to growth of T4 phage BU33 and consequently to the maturation of \textit{psu} \textsuperscript{+} tRNA\textsuperscript{+}.

The \textit{elaC} genes from wild type CA265 and mutant CAN also were compared for their ability to complement the T4 phage BU33 growth defect in strain CAN. Each \textit{elaC} gene was cloned into the low copy number plasmid pH7C79 and transformed into strain CAN (Table IV). The \textit{elaC} gene from strain CA265 can complement to allow phage BU33 growth, whereas that from strain CAN cannot. These data indicate that \textit{elaC} genes from strains CA265 and CAN are different and that the wild type \textit{elaC} gene can overcome the absence of RNase BN in strain CAN. These observations imply that the RNase BN defect in strain CAN is due to a mutation in its \textit{elaC} gene.

**Sequences of the \textit{elaC} Genes from Wild Type and RNase BN Mutant Strains**—To directly confirm the presence of a mutation in the \textit{elaC} gene of strain CAN, the cloned gene was sequenced and compared with the cloned \textit{elaC} gene from wild type strain CA265. This analysis revealed that nucleotide 259 in the \textit{elaC} gene of strain CAN was changed from C to T, converting the CAA codon of Gln-87 to a UAA stop codon, and resulting in synthesis of a protein fragment lacking RNase BN activity.

**DISCUSSION**

The information presented here clearly shows that the \textit{E. coli} ElaC protein is, in fact, the previously described enzyme, RNase BN. Thus, purified ElaC displays biochemical proper-

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**Table I**

| pH   | Co^{2+} | Mg^{2+} |
|------|---------|---------|
| 6.5  | 100     | 28      |
| 7.5  | 61      | 17      |

*Activity at pH 6.5 with CoCl₂ was set at 100.

**Table II**

| Nucleoside | Nucleotide | %          |
|------------|------------|------------|
| -Phosphatase | 1         | 99         |
| +Phosphatase | 93        | 7          |
Table III

| E. coli strain | Relevant phenotype | T4 plaques | BU33 plaques |
|---------------|-------------------|------------|--------------|
| CA265         | Wild type         | 35         | 108          |
| CAN           | RNase BN           | 25         | 0            |
| BEZ33         | ElaC              | 30         | 0            |

Table IV

| E. coli strain | Phasmid present | T4 plaques | BU33 plaques |
|----------------|-----------------|------------|--------------|
| CAN            | pH7C9 vector    | 498        | 0            |
| CAN            | pElaC (CA265)   | 410        | 234          |
| CAN            | pElaC (CAN)     | 460        | 0            |

ties identical to those previously described for RNase BN (17), including its homodimeric structure, its unusual optimal assay conditions, and its substrate specificity. Second, an elaC mutant strain behaves identically to the known RNase BN – strain, CAN (17), with respect to growth under a variety of conditions and to its inability to support plaque formation by mutant T4 phage BU33. This latter property is a direct biological assay for the presence of RNase BN activity (18). Third, whereas a cloned elaC gene from a wild type strain can complement the RNase BN defect in strain CAN, that from an RNase BN – strain cannot. Finally, direct sequencing showed that the elaC gene in strain CAN contains a nonsense mutation that would result in an inactivating protein.

Based on all of this information, it is apparent that the previous assignment (21) for the gene encoding RNase BN must have been incorrect. The new assignment of elaC as the relevant gene is much more consistent with that expected for the sequence of a ribonuclease. Moreover, the finding of a mutation in the elaC gene of strain CAN provides a clear explanation for the absence of RNase BN activity in this strain, in contrast to the earlier results in which no mutation was found (21). We have also observed that overexpression of the gene previously designated as rbn does not increase RNase BN activity and that deletion of this gene still allows plaing of T4 phage BU33 (data not shown). Consequently, we propose that the rbn designation be removed from the orf (yihY) at 87.8 min and that elaC be renamed rbn, especially since there is no particular significance to the elaC name (14).

One interesting question that remains is whether RNase BN is an exo or endoribonuclease or both. When examined, RNase Zs from other organisms are endoribonucleases, and even the E. coli homologue has been shown to cleave tRNA precursors in an endonucleolytic manner (12, 15). We have confirmed that RNase BN cuts a B. subtilis tRNAThr precursor (10) to generate a tRNA-sized product (data not shown), implying that it is an endonuclease. However, it is also true that RNase BN cuts tRNA-CA, tRNA-CU and tRNA-CCA to release the 3′-terminal nucleotide. It is this activity that led to its original designation as an exoribonuclease. We suspect that with these latter substrates, containing very short single-stranded 3′-ends or an inhibitory CCA sequence, endoribonucleolytic cleavage is constrained to the terminal residue, which may be the only residue able to enter the cleavage channel (11).

The finding that it was not possible to overexpress normal ElaC, whereas no such difficulty was observed with the His-tagged version, emphasizes a serious issue to be considered when working with tagged proteins. In this particular instance, the presence of the His-tag apparently results in a major effect on RNase BN activity that eliminates the deleterious effect of overexpressing the native protein. This is not so surprising considering that the His-tag would lead to a new, highly positively charged region in a protein that must interact with a negatively charged RNA substrate. This may result in incorrect binding and a consequent reduction in RNase activity.

Although the data presented here clarify the relation between RNase BN and ElaC, the question still remains as to its role in E. coli. The essentiality of RNase BN for maturation of the 3′-end of certain phage T4 tRNA precursors which do not encode the CCA sequence (18) fits exactly with the role of the homologous RNase Z in other organisms (4, 10). However, the E. coli host has no apparent need for such an activity as the CCA sequence is encoded in all its tRNA genes (5). Furthermore, RNase BN mutants show no growth phenotype (16, 27), suggesting that this RNase could not be required for maturation of any essential RNA. Thus, given the absence of an obvious function, we can expect continued examination into the possible role of this interesting RNase in E. coli.

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