Overexpression, Purification, and Properties of Escherichia coli Ribonuclease II*

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Ribonuclease II (RNase II) is a major exonuclease in Escherichia coli that hydrolyzes single-stranded polyribonucleotides processively in the 3' to 5' direction. To understand the role of RNase II in the decay of messenger RNA, a strain overexpressing the rnb gene was constructed. Induction resulted in a 300-fold increase in RNase II activity in crude extracts prepared from the overexpressing strain compared to that of a non-overexpressing strain. The recombinant polypeptide (Rnb) was purified to apparent homogeneity in a rapid, simple procedure using conventional chromatographic techniques and/or fast protein liquid chromatography to a final specific activity of 4,100 units/mg. Additionally, a truncated Rnb polypeptide was purified, solubilized, and successfully renatured from inclusion bodies. The recombinant Rnb polypeptide was active against both [3H]poly(A) as well as a novel (synthetic partial duplex) RNA substrate. The data show that the Rnb polypeptide can disengage from its substrate upon stalling at a region of secondary structure and reassociate with a new free 3'-end. The stalled substrate formed by the dissociation event cannot compete for the Rnb polypeptide, demonstrating that duplexed RNAs lacking 10 protruding unpaired nucleotides are not substrates for RNase II. In addition, RNA that has been previously trimmed back to a region of secondary structure with purified Rnb polypeptide is not a substrate for polynucleotide phosphorylase-like activity in crude extracts. The implications for mRNA degradation and the proposed role for RNase II as a repressor of degradation are discussed.

Because the rate of synthesis of any given protein is directly proportional to the concentration of its message, regulating the balance between mRNA decay and its synthesis is an important aspect of gene expression. In Escherichia coli, it is widely accepted that mRNA decay is initiated by a series of endonucleolytic cleavages catalyzed by RNase E (1–3) or occasionally by RNase III (4, 5) followed by processive exonucleolytic degradation of the message to oligo- and mononucleotides (1–3). Two 3'-exonucleases have been implicated in this process: ribonuclease II (RNase II)† and polynucleotide phosphorylase (PNPase) (6). RNase II, which is responsible for the majority of the exonucleolytic activity in E. coli extracts (7), hydrolyzes RNA to release 5'-mononucleotides (8), while PNPase phosphorylates RNA to mononucleoside diphosphates (9). Although RNase II activity was first described over three decades ago (10, 11) and purified from whole cells several years later (12–14), details of its role in mRNA degradation are still poorly understood.

RNA structure, known to be an important determinant of mRNA stability, can protect upstream sequences from digestion by the 3'-exonucleases. The Rho-independent terminator sequence (trp t) of the tryptophan operon (15) and the intergenic (malE-malF) REP sequence of the maltose operon (16) are classic examples of secondary structures that protect upstream RNA from 3'-exonucleolytic degradation both in vitro and in vivo. These investigations implied that the observed protection by 3'-stem-loop structures was the result of an impediment to the processive activities of RNase II and, to a lesser extent, of PNPase (15–17). Recent observations of the decay of RNA-OUT, the antisense RNA that regulates Tn10/IS150 transposition, demonstrate that the higher the thermal stability of the RNA structure, the larger the barrier to degradation by RNase II (18). Degradation by PNPase is much less affected by the relative stability of the RNA-OUT structure (18). Interestingly, RNA-OUT appears to be stabilized approximately 3-fold against PNPase attack by RNase II (18). In addition, the rpsO mRNA is also stabilized significantly by the presence of RNase II (19). Although the mechanism by which RNase II shields upstream sequences from further exonucleolytic attack is not understood, the observed protection was attributed to the formation of a stable RNase II-RNA complex, which sequesters the 3'-end of the transcript (18, 19).

As part of the investigation of the functional and biophysical properties of RNase II and its role in the overall decay of mRNA, we have overexpressed RNase II and developed a rapid and simple purification of the enzyme free of other nucleases. The purified enzyme was used to investigate the mechanisms by which stem-loop structures impede exonucleases and the ability of RNase II to act as a repressor of PNPase activity.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—The E. coli strain 18-11 (rna-, rnb-, rmd-, rbn-, rnt-) (20) was obtained from Dr. M. P. Deutscher (University of Connecticut Health Center, Farmington), while the strain CF881 F Δlac argA trp recB1009 Δ(xth-pnc)-1 dna was obtained from Dr. M. Cashel (National Institutes of Health). The vector pET-11 and its host strain BL21(DE3) (21) were obtained from Novagen. The plasmid pRP400 (22) was obtained from Dr. N. Sonenberg (McGill University, Montreal). The following oligonucleotide primers were synthesized based on the previously published rnb sequence (23): fp1 (5'-GGCAGATCCACAGGTTGACATATGTTGCTTCCGAAAAC) and rp1 (5'-GGGAGATCTCCTTTTCTATCGGCGTTCTCGATTAT). An additional reverse primer p2 (5'-GGGAGATCTCCTTTTCTATCGGCGTTCTCGATTAT) was constructed based on the partial DNA sequences of pZ8A17 and pRZA18 obtained from Dr. C. M. Arreajano (Centro de Tecnologia Química e Biológica, University of Lisbon, Portugal) which contain the 3'-untranslated region of the rnb gene. The predicted coding sequence of the rnb gene of E. coli was amplified from genomic DNA of strain MV1190 by the polymerase chain reaction. The products were cleaved with BamHI and ligated into the unique BamHI site of pET-11. The orientation of the 2.4- (pP1-pP2) and 1.9-kilobase pair (pP1+pP1) BamHI

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‡ The abbreviations used are: RNase II, ribonuclease II; FPLC, fast protein liquid chromatography; PNPase, polynucleotide phosphorylase; DTT, dithiothreitol; nt, nucleotide(s); IPTG, isopropyl β-D-thiogalactopyranoside.
Purification of Recombinant RNase II

**RESULTS**

Exonucleolytic Activity in Crude Extracts From E. coli—A partially duplexed RNA substrate (Fig. 1a) was used to assay extracts generated from various E. coli strains for putative RNA cleavage activities. Instead of detecting an activity that could unwind the duplexed RNA to monomers, we observed the partial degradation of the synthetic substrate in extracts that are wild type for RNase II activity but not in extracts deficient for a number of exonucleases including RNase II (Fig. 1, compare b and c). Complete conversion of the 92-nt substrate to a relatively stable 77-nt degradative intermediate was observed in crude extracts prepared from strain CF881 over a 60-min time course (Fig. 1b). The exact size of the product was determined on a sequencing gel (data not shown). In contrast, crude extracts prepared from strain 18-11 were unable to digest the substrate (Fig. 1c). Several additional experiments were undertaken to confirm that the 77-nt degradation product (shown in Fig. 1a) corresponds to the product of RNase II-stalling 9 nucleotides 3' to the double-stranded region of the substrate. First, the denatured 77-nt product retains a 5'-end label (data not shown). Second, the partial duplex substrate is resistant to digestion by the purified Ams/Rne/Hmp-1 polypeptide, the catalytic subunit of RNase E (27), under conditions where authentic substrates would be processed to completion (data not shown). Third, incubation of the 92-nt substrate under conditions where PNPase, the other major exonuclease activity in E. coli, would be active also generates a 77-nt product but only...
in the presence of 10 mM sodium phosphate (Fig. 2a). In this case, however, the 77-nucleotide product can be degraded further in prolonged incubations (data not shown). Moreover, extracts prepared from a strain containing the mutant pnpA7 allele, which largely lacks PNPsase activity but does contain RNase II activity, also generate the 77-nucleotide product while retaining the PNPase activity of the wild-type strain. These observations strongly suggest that the 77-nucleotide product is formed as a result of PNPase activity.

Overexpression and Purification of RNase II (Rnb)—The predicted coding sequence of the rnb gene of E. coli was amplified by the polymerase chain reaction as described in “Experimental Procedures.” The digestion products were analyzed by electrophoresis through a 10% polyacrylamide gel denaturing under reducing conditions. The 92-nucleotide substrate (s), previously digested substrate (S), and the 77-nucleotide degradative intermediate (P) are indicated with arrows. NP denotes a control lane containing substrate incubated in the absence of protein for 30 min.

The initial step relies on the polymerase chain reaction since concentration, desalting, and significant purification of Rnb from most contaminants could be achieved by affinity chromatography on heparin-agarose or by ion exchange chromatography (Table I) may have been due to the inhibition by Ca2+ ions leached from the column at high ionic strength, as Ca2+ has been reported to inhibit RNase II activity (10, 11). Final purification of Rnb from most contaminants could be achieved by affinity chromatography on heparin-agarose or by ion exchange chromatography (FPLC). A sample of the purified Rnb polypeptide is shown in Fig. 4, lanes 7 and 8. Based upon Coomassie Blue or silver staining of overloaded polyacrylamide

Cibacron blue-agarose chromatography to remove the bulk of the nucleic acids and contaminating proteins while the majority (>90%) of the Rnb polypeptide remains bound to the column. Considerable efficiency was gained by loading the 3 M NaCl eluate from this column directly onto a hydroxylapatite column. This proved to be an invaluable step in the purification method since concentration, desalting, and significant purification of the Rnb polypeptide could take place in a single step. The apparent loss of activity after hydroxylapatite chromatography (Table I) may have been due to the inhibition by Ca2+ ions leached from the column at high ionic strength, as Ca2+ has been reported to inhibit RNase II activity (10, 11).
The Resource Q column. Although a significant amount of activity could be eluted from the column as a sharp peak at a NaCl concentration active polypeptides present in the preparation. RNase II activity reflecting the several different populations of misfolded and in- ization, reduction, and refolding of the truncated Rnb* polypeptide, resulted in the formation of insoluble inclusion bodies. Further purification of the recombinant Rnb polypeptide. Rnb polypeptide (0.5 \(\mu\)g/ml) was incubated in the absence of substrate for the indicated times (in minutes) prior to addition to a complete reaction mixture. Incubation was continued for an additional 15 min at a final concentration of Rnb polypeptide of 8.3 \(\mu\)g/ml. The digestion products were analyzed by gel electrophoresis. a, b, c, d, e, f, g, h, i, j, k, l, m, n, o, p, q, r, s, t, u, v, w, x, y, z.

**TABLE I** Purification of recombinant RNase II

| Fraction | Protein (mg) | Units | Specific activity (units/mg) |
|----------|--------------|-------|----------------------------|
| S-30     | 60.0         | 71,040| 1,184                      |
| Blue agarose* | 19.4      | 57,317| 2,955                      |
| Hydroxylapatite A | 6.3   | 16,265| 2,603                      |
| Hydroxylapatite B | 6.1   | 10,290| 1,695                      |
| Heparin-agarose* | 2.0   | 8,195 | 4,098                      |
| Resource Q* | 0.7   | 2,533 | 3,725                      |

* Based on loading 60 mg of the S-30 fraction onto the blue agarose column.

Based on loading 2.5 mg of the hydroxylapatite (pool A) fraction onto the heparin-agarose column.

Based on loading 0.8 mg of the hydroxylapatite (pool A) fraction onto the Resource Q column.

Digestion of the t40B transcript is complete after a 60-min incubation with 2.0 milliunits of RNase II activity (Fig. 5, lane 7). Approximately 20% of the substrate is resistant to degradation by the Rnb polypeptide even after addition of 200 milliunits of fresh enzyme (data not shown). A fraction of the substrate appears to form concatemers and as a result does not have free 3'-ends accessible to the enzyme. Interestingly, digestion of t40B for 60 min at 37°C with 4 units of enzyme resulted in a further shortened (73 nt) but stable degradation intermediate depicted by the arrowhead (Fig. 5a, lane 8). This experiment suggests that at high concentrations, the Rnb polypeptide can remove three to four additional unpaired residues in the t40B duplex remaining from a previous round of digestion.

Several previously published reports have suggested that RNase II, of varying degrees of purity, is readily inactivated by heat (10–13, 28). We have tested the purified Rnb polypeptide and found that the recombinant enzyme is also susceptible to thermal inactivation (Fig. 5b). A comparison of Fig. 5b, lanes 2 and 3, shows that less than 1% of the activity remains after a 5-min incubation of the Rnb polypeptide in the absence of substrate at 37°C. Interestingly, the enzyme is stabilized in the presence of substrate and can remain active up to 60 min at 37°C (Fig. 5a). Activity can also be stabilized by the addition of substrate to Rnb polypeptide, which has been partially inactivated by a brief incubation in buffer at 37°C. Once activity has been lost to thermal inactivation, however, it cannot be regained upon addition of substrate (data not shown). The 77-nt product also stabilized the enzyme against heating. Rnb polypeptide was incubated in the presence of 1.5 pmol of partially digested t40B for 5 min at 37°C prior to incubation with full-length t40B transcript. The 77-nt product not only protected the Rnb polypeptide against thermal inactivation but also appeared to stimulate the activity of the enzyme for the full-length substrate by approximately 2-fold (data not shown). The apparent stimulation may be attributable to a decreased rate of thermal inactivation. Taken together, the data demonstrate that the enzyme can be stabilized by both substrate and product. In contrast, both a single-stranded DNA oligonucleotide (33-mer) and double-stranded plasmid DNA inhibited the activity of RNase II but were unable to provide significant protection from heating (data not shown) unlike oligonucleotides of deoxy(C)\(_{27}\), which can reduce the rate of thermal inactivation (28).
implies that in the absence of any free 3'-single-stranded ends, the Rnb polypeptide can bind RNA even if it is not a substrate. To test this hypothesis, t40B was incubated briefly with a large excess of Rnb polypeptide, sufficient to digest it to 73 nt, and then subjected to UV photocross-linking. Fig. 6, lane 3 shows labeling of a band of 70 kDa, the size expected for the Rnb polypeptide. In addition, there is label associated with a band of 70 kDa, the size expected for the Rnb polypeptide in the absence of any other proteins or cofactors. A 70-kDa protein, corresponding to the molecular mass of RNase II, was also labeled in crude extracts prepared from strain CF881 (Fig. 6, lane 3). All bands were sensitive to proteinase K treatment (Fig. 6, lanes 2 and 4). A comparison of Fig. 6, lanes 1 and 3, also demonstrates that UV cross-linking can provide an important assessment of the purity of the enzyme preparation in light of the affinity chromatography techniques utilized in the purification. Since there are a large number of RNA binding proteins in crude extracts prepared from E. coli that have a significant affinity for the t40B transcript, the presence of even a small percentage these contaminants would be readily detected in the purified material (Fig. 6, compare lane 3 to lane 1).

We have also tested whether the 77-nt product would inhibit the activity of the Rnb polypeptide in subsequent rounds of digestion. In the first experiment, the Rnb polypeptide (3.3 milliunits) was incubated with 25 pmol of unlabeled t40B (2.5-fold molar excess over labeled t40B) for 2.5 min at 37 °C prior to addition of labeled t40B. The kinetics of digestion of labeled t40B over a 60-min time course were identical to those in an incubation in which the same amount of enzyme was incubated directly with labeled t40B (data not shown). In the second experiment, the t40B transcript, which had been previously digested with Rnb polypeptide, extracted with phenol/chloroform, and ethanol precipitated, was used in a competition experiment. Equimolar amounts of digested t40B did not alter the kinetics of disappearance of the 92-nt substrate and thus were unable to compete effectively for the Rnb polypeptide (Fig. 7). Although the 77-nt product can protect the enzyme from thermal inactivation, it cannot inhibit its activity.

Recent observations have suggested that RNase II can protect “upstream” RNA sequences from PNPase attack through the formation of a stable RNA-RNase II complex (18, 19). We have further investigated this hypothesis by incubating the 77-nt product, produced by the action of the Rnb polypeptide (see above), with crude extracts prepared from strain 18–11 in the presence of 10 mM sodium phosphate. The data demonstrate that the 77-nt product is resistant to digestion by a PNPase-like activity (Fig. 2b, lanes 1–4). As discussed above, the 92-nt substrate is rapidly shortened to approximately 77 nt in the presence of phosphate over a 30-min time course of digestion (Fig. 2a, lanes 5–8).

DISCUSSION

The Mechanism of Action of RNase II on a Novel Substrate—We envisage that the action of RNase II on t40B can be described by the following sequential steps: 1) binding to a free 3'-end on the 92-nt substrate, 2) processive hydrolysis of 15 phosphodiester bonds, 3) stalling of the enzyme approximately 9 unpaired nucleotides from the 10-bp G-C-rich stem, 4) dissociation of the enzyme from the substrate, and 5) thermal inactivation of a fraction of the dissociated enzyme. The duration of each such cycle at steady state can be calculated from the apparent turnover number, which we estimate as 9 nt·s⁻¹ based on a rate of 0.16 pmol of product formed per min at 43 °C from 10-fold excess of enzymes. This yields a cycle time of 1.67 s, the time to remove 15 nucleotides from each 3'-end (15 nt/9 nt·s⁻¹). The time actually required for hydrolysis of 15 phosphodiester bonds (step 2 in the cycle) is only 0.21 s, however, as the reported turnover number for RNase II acting on poly(A) is 70 nt·s⁻¹ (28). If we assume that this turnover number also applies to the 15 residues removed from t40B and that no enzyme is lost to thermal inactivation (step 5), then steps 1, 3, and 4 account for 1.46 s (1.67 − 0.21 s) of each cycle. As a consequence, RNase II cannot remain bound to a substrate once processive hydrolysis has ceased any longer than 1.46 s. The latter represents a maximum value for step 3 in the proposed cycle, as binding (step 1), dissociation (step 4), and thermal inactivation (step 5) are not negligible.

The linear kinetics observed for the reaction demonstrate that RNase II stalls at regions of secondary structure, however briefly, but can disengage from the “stalled” substrate and reassociate with a new free 3'-end. This is substantiated by the demonstration that the Rnb polypeptide can cycle from an unlabeled to a labeled substrate. Our finding of dissociation from a substrate with 9 unpaired protruding nucleotides at the 3'-end of the 77-nt product is in good agreement with the 10–15-nt digestion limit product obtained for RNase II acting...
purification of recombinant RNase II activity—This partially duplexed RNA is an effective substrate for investigating the properties of RNase II and offers at least four significant advantages over assays previously utilized for detecting RNase II activity. First, the t40B transcript resembles natural mRNA substrates more closely than the homopolymeric substrates utilized in traditional assays as it contains both 3′-unpaired extensions of essentially random composition and a stable duplex mimicking stem-loop structures found in natural mRNAs. Second, the pairing of the enzyme at the duplexed region reflects the known behavior of RNase II on RNAs containing regions of extensive secondary structure (15–18). Third, the formation of a stable degradative intermediate provides an internal control that distinguishes RNase II activity from single and double strand-specific endonucleases. Finally, the high specific activity of the synthetic transcript increases the sensitivity of the assay and allows for the detection of activity at low substrate concentrations (10^{-10}–10^{-11}) closer to the physiological range.

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Note Added in Proof—We have discovered that an A→G transition, resulting in a single amino acid change, Ser456→Gly456, was inadvertently incorporated into plasmid pGCI100.

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