[13] *Escherichia coli* Ribonuclease III: Affinity Purification of Hexahistidine-Tagged Enzyme and Assays for Substrate Binding and Cleavage

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Introduction

Ribonuclease III of *Escherichia coli* (EC 3.1.26.3) is a double-strand (ds)-specific, Mg^{2+}-dependent endoribonuclease. RNaseIII initially was detected as an activity in *E. coli* cell-free extracts that digests polymeric dsRNA to acid solubility,[1] and was shown subsequently to be involved in the maturation of the ribosomal RNA precursor, and bacteriophage T7 mRNA precursors. RNase III is now known to participate in the degradation as well as maturation of diverse cellular, phage, and plasmid RNAs (for reviews, see Refs. 2–4). Ongoing genome sequencing projects are revealing that RNase III is highly conserved in bacteria, with orthologs occurring (often in multiple copies) in plants, animals, fungi, and even a eukaryotic virus.[5] No RNase III ortholog has yet been noted in any sequenced archaeal genome. The reader is referred to the first article in this series for a bioinformatic analysis of the RNase III family.[5a]

The *E. coli* RNase III polypeptide (226 amino acids; molecular mass, 25.6 kDa) is encoded by the *rnc* gene, which is the first of three genes in the *rnc* (RNase III) operon, mapping at 55 min on the chromosome. Cells lacking RNase III are viable, but grow slowly and exhibit altered levels of specific proteins. RNase III-deficient strains also accumulate an ~5500-nucleotide (nt) species (30S RNA) that contains the rRNA sequences. As mentioned above, RNase III cleaves this transcript to provide the immediate precursors to the mature 16S, 23S, and 5S rRNAs. However, in the absence of RNase III, an alternative pathway provides functional rRNAs. Studies are indicating that pre-rRNA processing is a conserved function for RNase III family members.[6,7] *Escherichia coli* RNase III negatively regulates its expression by site-specific cleavage within the 5′ untranslated region of its mRNA, which

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destabilizes the transcript.[8,9] A full consideration of RNase III and its role in RNA maturation and decay, and gene regulation is beyond the scope of this article, and the reader is referred to other comprehensive reviews.[2–4,5a]

Escherichia coli RNase III is homodimeric in structure, and requires a divalent metal ion (preferably Mg^{2+}) for catalytic activity. Mn^{2+}, Ni^{2+}, and Co^{2+} can substitute for Mg^{2+}, whereas Ca^{2+}, Sr^{2+}, and Zn^{2+} are inactive. Zn^{2+} also inhibits the Mg^{2+}-dependent reaction.[10] The RNase III reaction employs a water molecule as nucleophile to cleave phosphodiesters, creating 5′-phosphate, 3′-hydroxyl product termini. Exhaustive cleavage of polymeric dsRNA provides duplex products averaging 12–15 bp in length, which corresponds to slightly greater than one turn of the A-helix (11 bp).[2,11] The dsRNA cleavage product termini exhibit two nucleotide, 3′ overhangs,[2–4,12] which places the scissile phosphodiesters on the same face of the helix, and across the minor groove.

Escherichia coli RNase III cleaves its cellular substrates in a site-specific manner. The cleavage sites are located within local secondary structure motifs, such as stem–loops, which have been termed processing signals.[12] The site of cleavage is determined by a combination of substrate sequence and structure. Substrates that exhibit strict double-helical structure undergo coordinate cleavage of both strands, while substrates with internal loops or bulges usually are cleaved at a single site, which is contained within or near the internal loop or bulge.[2–4,12] Specific Watson–Crick (WC) base pair sequences at defined positions relative to the cleavage site can inhibit cleavage, which have been termed "antideterminants,"[13] in analogy to the antideterminant sequences in tRNAs that control tRNA–protein transactions.[14] These substrate reactivity epitopes are not conserved among RNase III family members. For example, the *Saccharomyces cerevisiae* RNase III homolog, Rntlp, recognizes a specific hairpin tetraloop sequence, and cleaves within the stem at a defined distance from the tetraloop.[15,16]

The *in vivo* processing reactions of *E. coli* RNase III can be accurately reconstructed *in vitro* using small RNAs containing the critical reactivity epitopes, and using physiologically relevant ionic conditions (i.e., 1–10 m*M* Mg^{2+}, 150–250 m*M* salt).[17,18] However, lowering the salt concentration, or replacing Mg^{2+} by

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Mn^{2+}, promotes cleavage of additional ("secondary") sites, which are not normally recognized *in vivo*.[19,20]

The *E. coli* RNase III polypeptide contains two functional subdomains: one involved in substrate recognition, and the other important for catalysis. A conserved dsRNA-binding domain (dsRBD) is positioned near the C terminus and is required for productive binding of substrate. This domain is used by other dsRNA-binding proteins with diverse functions.[21] The N-terminal portion of the RNase III polypeptide contains the catalytic domain, which exhibits a number of highly conserved residues. One residue (Glu-117) has been shown to be important for phosphodiester hydrolysis.[22] The domain structure and conserved sequence elements indicate a common catalytic mechanism for RNase III family members, which has not yet been defined. The conserved structural organization of RNase III family members is discussed by Aravind and Koonin.[5a]

The existence of extensive biochemical and genetic data on *E. coli* RNase III function in cellular RNA metabolism, and the large set of characterized cellular, viral, and plasmid-encoded processing substrates, make this enzyme an excellent prototype for understanding the mechanism of action of other RNase III family members. Biochemical studies of RNase III and its specific mutants require (as with any enzyme) an efficient purification protocol. RNase III was first purified to near homogeneity from nonoverproducing *E. coli* cells.[19] Because RNase III is a low-abundance protein, large amounts of cells were required. The procedure also used dsRNA–agarose affinity chromatography, which exploited the ability of RNase III to bind dsRNA in the absence of Mg^{2+} (omission of divalent metal ion was necessary to avoid cleavage of the dsRNA affinity matrix). However, several *E. coli* RNase III mutants we are studying are defective in binding dsRNA, which necessitated a purification procedure that did not rely on dsRNA-binding ability. We describe here a protocol for the overproduction and affinity purification of *E. coli* RNase III carrying an N-terminal hexahistidine (His_6) sequence. We also describe the preparation of small processing substrates and their use in gel-based assays for RNase III binding and cleavage. The reader is referred to other protocols that describe the partial or complete purification of native *E. coli* RNase III from overproducing strains.[10,23–26]

Materials

Chemicals are molecular biology grade or reagent grade, and are generally obtained from Sigma (St. Louis, MO) or other major suppliers such as Fisher Scientific (Pittsburgh, PA) or VWR (San Francisco, CA). Restriction enzymes and

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modifying enzymes are purchased from New England BioLabs (Beverly, MA). T7 RNA polymerase is purified in-house from an overproducing bacterial strain as described.[27] More recently we have used His_6-tagged T7 RNA polymerase, purified as described by Ni^{2+} affinity chromatography.[28] The pET plasmid vectors and *E. coli* DE3 strains are obtained from Novagen (Madison, WI). Calf intestinal alkaline phosphatase is obtained from Roche Molecular Biochemicals (Indianapolis, IN). Ultrapure ribonucleoside 5′-triphosphates are purchased from Amersham-Pharmacia Biotech (Piscataway, NJ) or from Roche Molecular Biochemicals. *Escherichia coli* bulk stripped tRNA (Sigma) is used as a carrier for radiolabeled RNA purification. It has previously been shown that tRNA has no major inhibitory effect on *E. coli* RNase III cleavage of substrate.[1] The tRNA is purified further by repeated phenol extraction. The extracted tRNA is ethanol precipitated, and the pellet is collected by centrifugation and washed with cold 70% (v/v) ethanol. The tRNA is briefly dried *in vacuo*, resuspended in water at a concentration of ~50 mg/ml, and stored in small aliquots at −20°. The A_{260}/A_{280} ratio should be ~2.0. Radiolabeled nucleotides are obtained from Dupont-NEN (Boston, MA). Isopropyl-β-D-thiogalactopyranoside (IPTG) stocks are prepared in sterile water (1 *M* concentration) and stored at −20°. For Polymerase chain reaction (PCR) cloning of the *rnc* gene and its mutants we use Vent DNA polymerase (New England BioLabs), as it has proofreading capability. To date we have encountered few instances of adventitious, PCR-induced mutations.

Overproduction and Purification of Hexahistidine-Tagged RNase III

The pET plasmid protein expression vectors[29] provide a convenient route for high-level overproduction of RNase III. We had purified previously native RNase III from an *E. coli* DE3 strain carrying plasmid pET-11(*rnc*).[10] This plasmid carries the *rnc* gene under the control of an IPTG-inducible T7 promoter and T7 translation initiation signal. To create pET-11 (*rnc*) the *rnc* gene is amplified by PCR, using a chromosomal DNA preparation as template, and then cloned into the *Nde*I and *Bam*HI sites of plasmid pET-11a. RNase III is overproduced at high levels in *E. coli* BL21(DE3),[30] with a substantial fraction of the protein

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TABLE I
STEADY-STATE KINETIC PARAMETERS OF PURIFIED His_6–RNase III AND RNase III[a]

Enzyme	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1}/min^{-1})
RNase III	7.7	0.26	3×10^7
His_6–RNase III	3.8	0.34	1.1×10^7

[a]The substrate used for both enzymes in enzymatic cleavage assays was internally ^{32}P labeled R1.1 RNA.[18] Reactions were performed at 37° in buffer that included 250 m*M* potassium glutamate and 10 m*M* $MgCl_2$ (see text). Data for RNase III are from Li *et al.*[10]

accumulating in the soluble portion of sonicated cell extracts. RNase III is purified in several steps, which include poly(I)–poly(C)–agarose affinity chromatography and gel filtration.[10]

The protocol summarized above would not be useful in purifying RNase III mutants that are defective in dsRNA binding. We therefore have applied an alternative approach, using the hexahistidine (His_6) sequence tag and immobilized metal (Ni^{2+} ion) resin.[31] This approach affords several advantages. First, the His_6 tag allows purification independent of the particular biochemical properties of an RNase III mutant. Second, the small size of the affinity tag minimizes any deleterious effect on RNase III structure and activity. Indeed, the N-terminal His_6 tag has only a minor effect on RNase III binding and cleavage of substrate (see below). Third, the His_6 tag can bind to Ni^{2+} resin in the presence of denaturants (e.g., urea), thereby allowing purification of RNase III mutants with reduced solubility. Fourth, the His_6 tag can be removed if necessary by thrombin cleavage. To provide RNase III with an N-terminal His_6 tag, the *rnc* gene is transferred from pET-11a (*rnc*) (see above) into the *Nde*I and *Bam*HI sites of plasmid pET-15b. Briefly summarized, His_6-tagged RNase III can be efficiently overproduced in an *E. coli* DE3 strain [e.g., BL21(DE3) or HMS174(DE3)], and accumulates in the soluble portion of sonicated cell extracts. Purified His_6–RNase III has a slightly lower catalytic efficiency (k_{cat}/K_m) than native RNase III (Table I), and exhibits the same cleavage site specificity as native enzyme (data not shown).

The overexpression and purification of His_6–RNase III mutants present an additional challenge, in that the activity of endogenous (chromosomal) RNase III must be suppressed. Such contamination, albeit small, could complicate the analysis of RNase III mutants with low activities. Although immobilized Ni^{2+} columns do not bind native RNase III, we employ an *E. coli* DE3 strain in which the activity of the chromosomally encoded RNase III is suppressed by a point mutation.

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The *rnc105* mutation changes the highly conserved Gly-44 to aspartic acid in the catalytic domain and abolishes RNase III activity.[32,33] The bacterial strain BL21(DE3)*rnc105* has been prepared by Yamamoto and co-workers to overproduce a yeast RNase III ortholog.[34] To enhance the stability of the recombinant plasmid we have introduced the *recA*::Tn*9* allele into this strain by P1 transduction (the donor strain for the *recA*::Tn*9* allele is *E. coli* DB1318, provided by D. Court). BL21(DE3)*recA,rnc105* is slow growing, but allows overproduction of His_6–RNase III (see below). We have frequently observed that significant levels of His_6–RNase III are produced in the absence of IPTG induction. The reason for this is unclear, but may reflect the pleiotropic effects of the *rnc105* mutation on gene expression,[4] and the presence of a lambda (λ) lysogen, which uses RNase III in its gene regulatory circuitry.[4]

His_6–RNase III is prepared as follows. An overnight culture (in 5 ml of LB broth plus ampicillin at 100 μg/ml) (LB-Amp) is grown from a single colony of BL21(DE3)*rnc105,recA* cells freshly transformed with pET-15b(*rnc*). Although we have not noticed any significant instability of the pET-15b(*rnc*) plasmid in this strain, it is possible that plasmids carrying specific *rnc* gene mutants may be unstable. If necessary, the fraction of cells carrying plasmid can be determined by colony counts using LB-agar plates with or without ampicillin. An aliquot (1 ml) of the culture is added to 200–300 ml of LB-Amp at 37°. Alternatively, several fresh colonies can be collected from an LB-Amp agar plate and used as inoculant. Cultures are grown at 37° with vigorous aeration to an OD (600 nm) of 0.3–0.4. Immediately before IPTG addition an aliquot (1 ml) is removed for sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis (see below). IPTG is added (final concentration, 0.5–1 m*M*), and incubation continued at 37° with vigorous aeration. Additional 1-ml aliquots are removed at 1, 2, and 3 hr after induction for SDS–PAGE analysis. After 3–4 hr, the culture is cooled on ice, and the cells collected by centrifugation at 4°. The cell pellet is stored at −20° prior to protein purification (see below).

RNase III overproduction is checked by SDS–PAGE. The collected aliquots (see above) are briefly centrifuged, and the pellets are fully resuspended in Laemmli gel loading buffer (~100 μl).[35] The samples are heated at 100° for 3–5 min, and aliquots are analyzed by electrophoresis in an 12% (w/v) polyacrylamide gel containing SDS.[35] Prestained protein size markers (Life Technologies, Bethesda, MD) are included in a side lane. The gel is stained with Coomassie Brilliant Blue R, and then destained and dried by standard methods. The image is captured by scanning or photography. The His_6–RNase III polypeptide electrophoreses as

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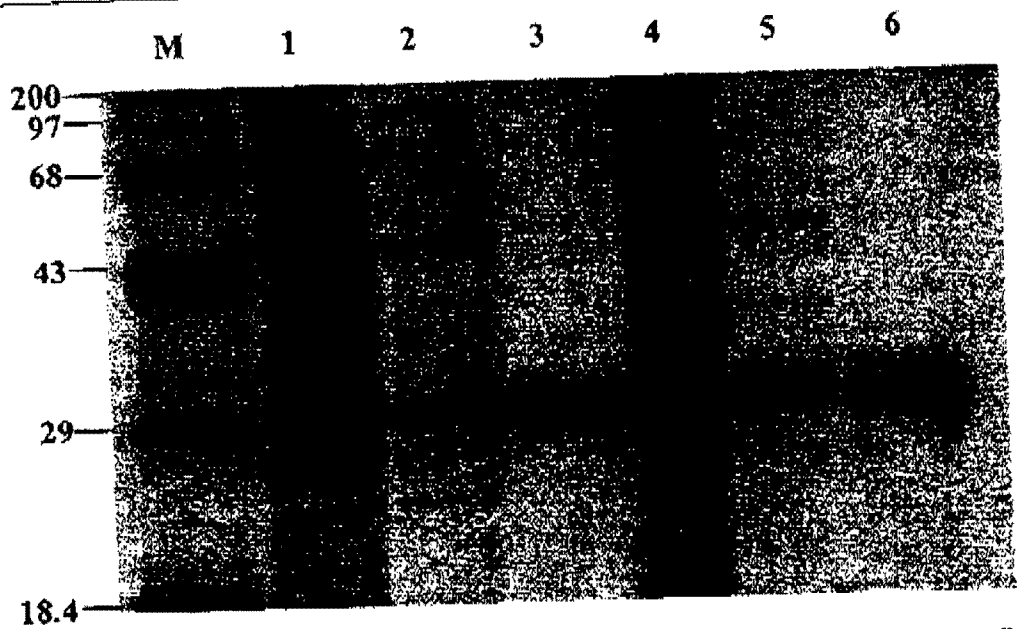

FIG. 1. Overproduction and purification of His_6-tagged RNase III. BL21(DE3)*rnc105recA* cells carrying pET-15b (*rnc*) were grown in LB broth containing ampicillin, and protein expression was induced with IPTG as described in text. Aliquots were taken and analyzed by SDS–PAGE [12% (w/v) polyacrylamide]. Proteins were visualized by Coomassie blue staining. Lane 1, total cell protein, 4 hr after IPTG addition. Lane 2, protein present in the soluble portion of sonicated cell lysates. Lane 3, protein in the soluble extract eluted from the Ni^{2+} affinity column. Lane 4, protein in the insoluble portion of sonicated cell lysates. Lane 5, protein present in the insoluble portion, solubilized with 6 *M* urea. Lane 6, protein present in the 6 *M* urea fraction, eluted from the Ni^{2+} affinity column. Lane M, prestained protein size markers (Life Technologies). The numbers on the left indicate the apparent molecular masses (kDa). The His_6–RNase III polypeptide comigrates with the 29-kDa marker.

a species of ~29 kDa molecular mass, and is the majority species in the cell by 4 hr after induction (Fig. 1, lane 1). As mentioned above, appreciable expression of the polypeptide is often obtained in the absence of IPTG.

Ni^{2+} Affinity Chromatography

Purification of His_6–RNase III is based on the protocol provided in the HisBind resin system manual (Novagen). We have incorporated a number of changes that have improved the yield and quality of enzyme. We have not found it necessary to include a protease inhibitor (e.g., phenylmethylsulfonyl fluoride) in the purification buffers, as long as the solutions are kept ice cold and affinity chromatography is performed as soon as possible after cell disruption. The following steps are performed at ~4°. The cell pellet is thawed and thoroughly resuspended in 30 ml of buffer A [500 m*M* NaCl, 20 m*M* Tris-HCl (pH 7.9)] containing 5 m*M* imidazole (Im). The cell suspension is transferred to a 30-ml Corex centrifuge tube, and subjected to repeated sonication bursts on ice, using an ultrasonic homogenizer (model

XL2007; 4-W power setting; Misonix, Farmingdale, NY). Sonication bursts are for 1 min, repeated four times with intermittent cooling on ice. Complete cell disruption is determined by visual examination, as indicated by the decrease in the initial viscosity to a final constant level. The sample is centrifuged at 7000 rpm for 20 min in a Sorvall SS34 rotor, and the supernatant is removed to a separate tube. If necessary, the centrifugation step is repeated to obtain complete clarification. His_6–RNase III is present in both the soluble and insoluble fractions of the sonicated cell mixture. Sufficient protein exists in the soluble fraction for purification. We have found that some RNase III mutants are obtained in largely insoluble form. If an RNase III mutant is being purified for the first time, aliquots are taken from the soluble and insoluble fractions for SDS–PAGE analysis to determine which fraction contains the protein. Purification of protein from the soluble fraction is described first, followed by the protocol for purification from the insoluble fraction.

The Ni^{2+} affinity column is prepared in a 10-ml glass pipette. The column can be operated in a cold room or in a cabinet refrigerator. Typically, 1–1.5 ml of HisBind resin is sufficient to purify protein from a 200- to 300-ml culture. A small plug of sterile glass wool is used as column support, and a short length of Tygon tubing is used to direct the eluent to the recipient tubes. An adjustable clamp is used to control the flow rate. The resin is washed with 10 column volumes of buffer A plus 5 m*M* Im, and then charged with a 50 m*M* $NiSO_4$ solution. The clarified protein solution (see above) is slowly applied to the column (approximately 1 hr of loading time for a 30-ml volume). The resin is washed with 10 column volumes of buffer A plus 5 m*M* Im, and then with 6 column volumes of buffer A plus 60 m*M* Im. The protein is eluted with three aliquots (1 ml each) of buffer consisting of 1 *M* NaCl, 400 m*M* Im, and 20 m*M* Tris-HCl (pH 7.9). The majority of His_6–RNase III elutes in the first three fractions, with the greatest amount in eluate fraction 2. The samples are combined, placed in dialysis tubing (SpectraP or membrane, 8000 MW cutoff; Spectrum, Laguna Hills, CA) and dialyzed against buffer (1 *M* NaCl, 400 m*M* Im, 60 m*M* Tris-HCl, pH 7.9) for ~2 hr, and then dialyzed for ~2 hr against the same buffer, but lacking Im. Dialysis is continued for 12–16 hr against buffer consisting of 1 *M* NaCl, 60 m*M* Tris-HCl (pH 7.9), 1 m*M* EDTA, 1 m*M* dithiothreitol (DTT). Purified enzyme is stored at −20° in 50% (v/v) glycerol, 0.5 *M* NaCl, 30 m*M* Tris-HCl (pH 7.9), 0.5 m*M* EDTA, 0.5 m*M* DTT at −20°. There is a negligible loss of activity over many months, and concentrations of ~1–2 mg/ml are stable. Attempts to store protein at higher concentrations led to some precipitation. Typically, ~2 mg of His_6–RNase III can be obtained from a 300-ml bacterial culture. The purity of His_6–RNase III is at least 90%, as estimated by gel electrophoresis (see Fig. 1, lane 3), and is free from contaminating nuclease activities.

We have found that proper execution of the dialysis procedure is critical for a successful purification. In a preliminary protocol we had originally used buffer A plus 1 *M* Im as elute buffer, followed by dialysis against buffer A without Im.

However, this caused protein precipitation. We suspected that the rapid drop in Im concentration was responsible for the precipitation. We therefore lowered the concentration of Im to 400 m*M* in the elute buffer and the first-step dialysis buffer. We also observed that inclusion of EDTA and DTT in the second dialysis step caused yellow–brown discoloration of the dialysate, and formation of similarly colored precipitates on the dialysis membrane with an accompanying loss of protein. We suspected that the combination of EDTA and DTT with Ni^{2+} ion (which coelutes with protein and is not efficiently removed by dialysis) promotes a chemical reaction that affects protein solubility. We therefore included 400 m*M* Im in the first-step dialysis buffer to help entrain the coeluting Ni^{2+}, and added EDTA and DTT only in the final dialysis step. Finally, the salt concentration influences RNase III solubility. We used 1 *M* NaCl in the dialysis buffer, because salt concentrations lower than ~500 m*M* caused progressively greater amount of protein precipitation, whereas concentrations greater than 1 *M* did not improve solubility. We did not observe any strong dependence of solubility on the type of metal ion (Na^+, K^+, NH_4^+).

As mentioned above, the purification of RNase III mutants presented additional challenges, because mutations can affect protein solubility. One approach to increase solubility is to overproduce the protein at lower temperatures (e.g., 25–30°). If this does not provide enough soluble protein in the sonicated cell lysate, His_6–RNase III and mutants can be purified from the insoluble fraction as follows. This protocol uses urea, rather than nonionic or ionic detergents, as we have found the latter reagents to be difficult to remove, and that at least for one detergent (Triton X-100), the secondary cleavage site activity of RNase III is enhanced (our unpublished observations, 2000). After sonication and centrifugation as described above, the supernatant is removed, and the inclusion body is washed several times with buffer A plus 5 m*M* Im. The pellet is then treated with buffer A plus 6 *M* urea on ice for 1–3 hr. The sample is centrifuged (10,000 rpm, 10–15 min) and the clarified supernatant is loaded on a Ni^{2+} column as described above. The column is washed with buffer A plus 5 m*M* Im, without urea. In this step the urea-solubilized protein undergoes renaturation on the Ni^{2+} column. The protein is eluted and dialyzed as described above. If necessary the solubility of the eluted protein (wild-type or mutant) can be maintained by including 10% (v/v) glycerol in the dialysis buffer. The purity of RNase III obtained by this route is also estimated to be >90% (see lane 6, Fig. 1).

RNase III Substrate Preparation

The original assays for *E. coli* RNase III took advantage of the ability of the enzyme to cleave polymeric dsRNA to acid solubility. The synthesis of dsRNA substrates can be accomplished by a number of routes, including transcription of a synthetic DNA copolymer [e.g., poly(dA-dT)] by *E. coli* RNA polymerase in

the presence of radiolabeled ribonucleoside triphosphates.[1,11] The product is purified by CF11 cellulose chromatography, which separates dsRNA from ssRNA in ethanol-containing buffers.[36] With the development of phage RNA polymerase-based transcription systems, dsRNAs of virtually any sequence can be obtained by transcription of both strands of a specific DNA template, followed by annealing of the complementary RNAs. In one method, a plasmid carrying a defined sequence between convergent phage promoters (e.g., T7 and T3) is linearized in separate reactions, using a restriction site on either side of the target sequence. Alternatively, a DNA template with the same promoter configuration can be generated by PCR using primers containing the appropriate promoter sequence. The purified templates are transcribed in separate reactions *in vitro*, using the phage RNA polymerase. The complementary ssRNA sequences are purified and annealed, and the dsRNA is purified by gel electrophoresis. The dsRNA can be radiolabeled on either strand, or both strands. For examples of this protocol, see Refs. 37–39.

Although synthetic dsRNAs retain their value in detecting RNase III-like activities—especially in the absence of knowledge of the specific cellular substrate sequence requirements—they are not convenient for detailed enzymological studies. We are interested in how *E. coli* RNase III recognizes and cleaves its cellular and viral processing signals, which typically occur within much larger RNA sequences. These substrates typically are small (~40–60 nt) stem–loop structures. These substrates are readily generated by transcription of single-stranded oligodeoxynucleotides, using the method developed by Uhlenbeck and coworkers.[40,41] Several earlier studies from our laboratory used this technology to prepare a wide variety of *E. coli* RNase III substrates.[10,14,17,22] Figure 2 shows the structure of R1.1 RNA, an RNase III substrate we have used in a number of investigations. R1.1 RNA is based on the R1.1 processing signal, which is encoded in the phage T7 genetic early region between genes 1.0 and 1.1.[10,17,18] The 60-nt RNA is cleaved *in vitro* at a single site within the internal loop (indicated by the arrow in Fig. 2). The RNA can be enzymatically synthesized with T7 RNA polymerase and a 77-nt DNA template, annealed to an 18-nt "promoter oligonucleotide" (Fig. 2). The RNA synthesis and purification protocol is provided below.

General Precautions

Several steps are taken to avoid ribonuclease and metal ion contamination. Water is deionized and distilled. Gloves are worn, and sterile plasticware (tips,

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[41] J. F. Milligan and O. C. Uhlenbeck, *Methods Enzymol.* **180**, 51 (1989).

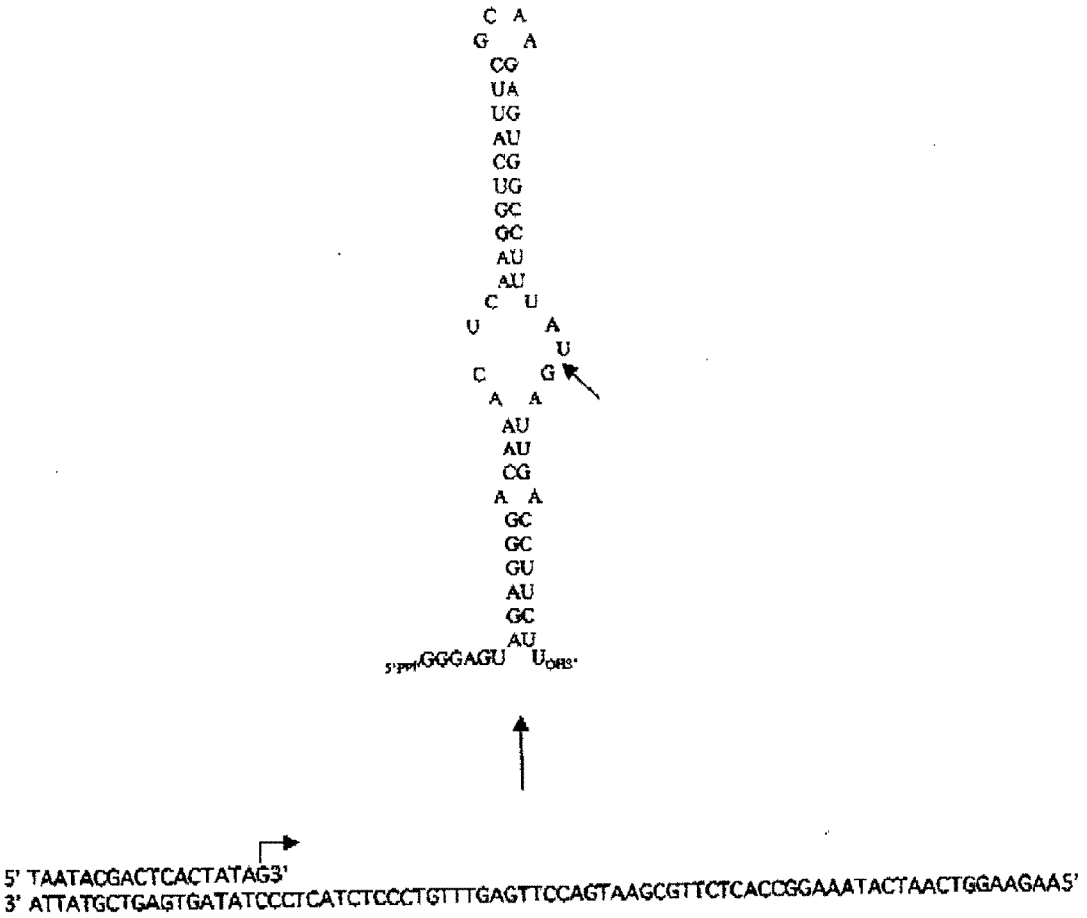

FIG. 2. Structure of R1.1 RNA, and the corresponding transcription template. The arrow indicates the site of RNase III cleavage in the internal loop. The 18-nt promoter oligonucleotide is shown annealed to the 77-nt transcription template. The bent arrow indicates the transcription start site.

tubes) is used. In this regard, radiolabeled RNAs generally can be recovered in higher yield by using siliconized microcentrifuge tubes. Liquid-handling devices (e.g., pipettors) are dedicated to RNA-containing samples. Glassware is thoroughly washed, rinsed thoroughly in deionized, distilled water, and baked before use. Glass plates for electrophoresis are treated with chromic acid, rinsed thoroughly in deionized, distilled water, and then dried (baking is optional). Using these procedures we have not found it necessary to include ribonuclease inhibitors (e.g., diethyl pyrocarbonate) in the buffer stocks and water.

Template Preparation

Synthetic oligodeoxynucleotides are obtained from a commercial source, and contain a T7 promoter sequence directly upstream of the sequence encoding

the RNase III substrate. The 18-nt promoter oligonucleotide is also procured, which is complementary to the T7 promoter sequence.[40,41] Because of promoter sequence constraints the transcripts usually carry a specific 5′-end sequence (5′-pppGGGAGA...3′) corresponding to the T7 promoter +1 to +6 sequence. We have not found this sequence to affect substrate cleavage and binding activities (see below). There is some latitude allowed in the 5′ sequence, although changes from the canonical sequence (see above) can lower transcription yields.[40,41]

The oligodeoxynucleotides are purified by denaturing polyacrylamide gel electrophoresis. The crude, deprotected DNA (~50 nmol) is resuspended in TE buffer (~300 μl), combined with a one-third volume of dye solution [89 m*M* Tris base, 89 m*M* boric acid, 22 m*M* EDTA, 7 *M* urea, 20% (w/v) sucrose, 0.04% (w/v) each xylene cyanol and bromphenol blue], and electrophoresed (~22 V/cm) at room temperature in a 20% (w/v) polyacrylamide gel containing 7 *M* urea in TBE buffer. The gel region containing the full-length DNA is located by UV shadowing[42] and excised with a clean scalpel. The gel slice is crushed and incubated in 0.5 *M* ammonium acetate (pH 7.8) and 10 m*M* EDTA (~400 μl) for 12–16 hr at room temperature. The sample is centrifuged (14,000 rpm, 10 min, 4°), and the supernatant is carefully transferred to a fresh tube. One-tenth volume of 3 *M* sodium acetate (pH 5.2) is added, followed by 2.5 volumes of ethanol and incubation at −70° (if necessary, the supernatant volume can be reduced before ethanol addition by repeated *n*-butanol extraction). The sample is centrifuged at 4° or room temperature (14,000 rpm, 30 min), and the DNA pellet is briefly dried *in vacuo* and then resuspended in 50 μl of TE buffer [10 m*M* Tris-HCl (pH 8.0), 1 m*M* EDTA]. The amount of DNA is determined by the absorbance at 260 nm, and the purity is assessed by the A_{260}/A_{280} ratio, which should be >1.7. The DNAs are stored at −20°, but −80° is preferable for long-term storage.

RNA Synthesis

In a typical transcription reaction, DNA template (~1.6 μM) and the promoter oligonucleotide (~1.8 μM) are annealed in 100 μl of 10 m*M* Tris-HCl (pH 8.2) by heating for 5 min at 65°, followed by quick cooling on ice. One-tenth volume of the annealed template mix is added to a transcription reaction (typically a 50- to 100-μl volume) containing 40 m*M* Tris-HCl (pH 8.2), 20 m*M* $MgCl_2$, 10 m*M* spermidine, 0.01% (v/v) Triton X-100, polyethylene glycol (PEG) 8000 (80 mg/ml), 5 m*M* DTT, the four rNTPs (1 m*M* each), ~5–15 μCi of [α-^{32}P]UTP (or CTP) (3000 Ci/mmol), and ~400 units of T7 RNA polymerase. The reaction is incubated at 37° for 3–4 hr, and then stopped by adding one-third volume of dye mix containing 20 m*M* EDTA (see above), and then immediately loaded on a 15-cm, 20% (w/v) polyacrylamide gel containing 7 *M* urea in TBE buffer. The sample is

[42] S. M. Hassur and H. W. Whitlock, *Anal. Biochem.* 59, 162 (1974).

electrophoresed at ~22 V/cm until the bromphenol blue runs off the bottom. The gel region containing the radiolabeled RNA is identified by autoradiography (nonradioactive RNAs are located by UV shadowing), excised, and soaked in 400 μl of extraction buffer (see above) containing ~1 μg of tRNA for 12–16 hr at room temperature. One-tenth volume of 3 *M* sodium acetate (pH 5.2) is added, followed by 2.5 volumes of ethanol and incubation at −70°. The sample is centrifuged for at least 30 min, and the RNA pellet is briefly dried and resuspended in TE buffer. For longer RNAs (>100 nt) efficient extraction can be achieved by soaking the gel slice instead in 400 μl of TE buffer (plus 1 μg of tRNA) and proceeding as described above.

For 5′-end radiolabeling, RNA (100 pmol) is dephosphorylated with ~1–2 units of calf intestine alkaline phosphatase in 50 m*M* Tris-HCl, 0.1 m*M* EDTA, pH 8.5 for 30 min at 37° (room temperature is also sufficient). The sample is phenol–chloroform extracted, and the RNA is recovered by ethanol precipitation as described above. The RNA is reacted in a small amount of TE buffer and stored at −20° until the next step. Approximately 25–50 pmol of the dephosphorylated RNA is treated with 2 units of T4 polynucleotide kinase and 5–10 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) in the buffer supplied with the enzyme. The reaction is electrophoresed in a denaturing polyacrylamide gel, and the RNA is isolated as described above. The RNA is resuspended in a small amount of TE buffer and stored at −20° before further use.

Substrate Cleavage Assay

The original assay for RNase III used a synthetic substrate [e.g., [^{3}H]poly (A-U)], which was added to partially purified cell extracts, and the time-dependent decrease in trichloroacetic acid-precipitable radioactivity was determined.[1] A unit of RNase III activity was defined as the amount of enzyme that solubilizes 1 nmol of acid-precipitable (dsRNA) polymer phosphorus in 60 min at 35°.[1,2] With the availability of near-homogeneous enzyme and small, specific substrates, evaluation of enzyme activity can be made by determination of initial cleavage rates and the steady state kinetic parameters (see Table I). For enzymatic analyses in the steady state (e.g., determination of initial cleavage rates, and measurement of K_m and k_{cat} values), the RNase III concentration is typically ~10 n*M* (dimer form), and internally ^{32}P-labeled substrate is used at concentrations greater than ~50 n*M*. For initial velocity measurements the concentrations of enzyme and substrate are both lower than the K_m, such that changes in velocity would reflect changes in either K_m or k_{cat}.[43] Reactions are initiated by adding substrate, or enzyme, or divalent metal ion ($MgCl_2$). We have obtained the most consistent results by using Mg^{2+} addition as the initiating step. The addition of dye mix

[43] A. Fersht, "Enzyme Structure and Mechanism." W. H. Freeman, New York, 1985.

containing excess EDTA is an effective reaction quench. Polypropylene (0.65 ml) microcentrifuge tubes are convenient reaction vessels that provide reproducible results.

Immediately before the assay, an appropriate amount of ^{32}P-labeled RNA (either internally or 5′ labeled) is briefly heated (100°, 30 sec) in TE buffer, and then cooled on ice. This step removes intermolecular complexes that can form during storage at −20°. Aliquots are combined with RNase III in a buffer containing 30 m*M* Tris-HCl (pH 8), 250 m*M* potassium glutamate (or 160 m*M* NaCl), 5 m*M* spermidine (optional), tRNA (0.01 μg/μl), 0.1 m*M* EDTA, and 0.1 m*M* DTT. Samples are preincubated at 37° for 5 min, and $MgCl_2$ (prewarmed at 37°) is added to initiate cleavage (10 m*M* final concentration). Reactions are stopped by addition of EDTA–dye mix. Samples are loaded on a denaturing 15% (w/v) polyacrylamide gel containing TBE buffer and 7 *M* urea, and electrophoresed for 1–2 hr at 350 V. The top gel plate is removed, the excess buffer is removed, and the gel is wrapped in plastic. The cleavage reaction is followed by the rate of appearance of a specific cleavage product, and is quantitated by phosphorimaging (Storm 860 Phosphorimager system; Molecular Dynamics, Sunnyvale, CA) or by radioanalytic imaging (Ambis, San Diego, CA). To ensure linear kinetics the reaction times are kept short (0.5–3 min), such that only a small fraction of substrate is converted to product (typically <30%). For determination of steady state kinetic parameters, the substrate concentration is varied at a fixed enzyme concentration (usually ~10 n*M*), and saturation of initial velocity is determined by graphic analysis. The K_m and V_{max} values are determined by nonlinear least-squares curve fitting (Kaleidagraph; Synergy Software, Reading, PA), and the k_{cat} is determined from the V_{max}. For the k_{cat} determination it is necessary to have accurate determination of the protein concentration, and of the amount of [^{32}P]RNA recovered in the gel lanes.

Gel Mobility Shift Assay for Substrate Binding

The application of the gel mobility shift assay to monitor *E. coli* RNase III binding to substrate has been described.[22,44] Since then additional changes have been instituted. In the absence of divalent metal ion, some RNase III–substrate complexes cannot be directly observed in a nondenaturing polyacrylamide gel.[22] We have shown that Ca^{2+} can stabilize RNase III–substrate complexes during electrophoresis, while disallowing cleavage.[22] The gel shift assay also can be performed in the presence of Mg^{2+}, and using a catalytically inactive mutant of RNase III (e.g., the Glu117Lys or Glu117Ala mutants).[22] The gel shift protocol described below may not necessarily be applicable to the analysis of RNase III

[44] B. S. Chelladurai, H.-L. Li, K. Zhang, and A. W. Nicholson, *Biochemistry* **32**, 7549 (1993).