Mode of Degradation of Precursor-Specific Ribonucleic Acid Fragments by Bacillus subtilis

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A precursor of 5S ribosomal ribonucleic acid (rRNA) from Bacillus subtilis was cleaved by ribonuclease (RNase) M5 in cell-free extracts from B. subtilis to yield the mature 5S rRNA plus RNA fragments derived from both termini of the precursor. The released, mature 5S rRNA was stable in these extracts; however, as occurred in vivo, the precursor-specific fragments were rapidly and completely destroyed. Such destruction was not observed in the presence of partially purified RNase M5, so fragment scavenging was not effected by the maturation nuclease itself. The selective destruction of the precursor-specific fragments was shown to occur through a 3'-exonucleolytic process with the release of nucleoside 5'-monophosphates; the responsible activity therefore had the character of RNase II. Consideration of the primary and probable secondary structures of the precursor-specific fragments and mature 5S rRNA suggested that involvement of 3' termini in tight secondary structure may confer protection against the scavenging activity.

Little information is available regarding the detailed pathways by which cells degrade ribonucleic acid (RNA) rendered useless to the metabolic machinery. In prokaryotes, such unstable RNA includes most or all of the messenger RNA and precursor-specific segments trimmed from ribosomal RNA (rRNA) and transfer RNA (tRNA) molecules during their maturation. In eukaryotes, probably all cellular RNA is eventually degraded. Apirion (1) has provided considerable insight into RNA degradation in Escherichia coli. By analyzing the rates of degradation of total unstable RNA in various mutants deficient in enzymes capable of participation, he provided evidence that the major responsible activity probably is ribonuclease (RNase) II, a 3'-exonuclease yielding nucleoside 5'-monophosphates (14, 17). Polynucleotide phosphorylase (PNPase), also a 3'-exonuclease but releasing nucleoside diphosphates (13), may offer minor assistance in the process, whereas RNase I, an endonuclease producing nucleoside 3'-monophosphates (18), probably is not included in this scavenging process. These findings support earlier, in vitro analyses of RNA degradation by extracts from E. coli (12, 19).

Although genetic analysis may yield the mode of degradation of unstable RNA components, the approach probably cannot provide information bearing on the selectivity of the process. Since in prokaryotes only about half of the RNA output of cells undergoes eventual destruction (10), mechanisms must exist for distinguishing stable from unstable molecules. The stability of the rRNA molecules might be rendered by their close association with proteins, but the tRNA species enjoy no such protection. These molecules must contain structural features that protect them from cellular degradative activities, or perhaps lack information that specific degrading enzymes utilize in their selection of substrates.

Exploration of the various facets of selective RNA degradation requires identification and isolation of the responsible enzymes and the availability for comparative purposes of defined RNA molecules that are stable or unstable in growing cells. We have described (9) in some detail precursors of 5S rRNA (p5A, 179 nucleotides, and p5B, about 150 nucleotides) from Bacillus subtilis which, in the presence of an appropriate maturation endonuclease, undergo conversion in vitro to mature 5S (m5, 116 nucleotides) rRNA with the concomitant release of precursor-specific fragments from both the 5' and 3' termini of the precursors (15). Mature 5S rRNA is of course stable in growing cells, but the released, precursor-specific segments are rapidly destroyed. We here report that the selective degradation of the precursor-specific segments is demonstrable in cell-free extracts.
of B. subtilis and define the degradative activity as a 3'-exonuclease that yields 5'-nucleotide monophosphates. This degradative activity therefore is analogous to the RNase II of E. coli.

MATERIALS AND METHODS

Growth of cells. B. subtilis 168 (trp +) was grown in a New Brunswick Micro Ferm fermentor in medium 3XD (5), modified to contain tryptone (Difco) instead of Casamino Acids, with continuous air sparging to a turbidity corresponding to 500 Klett units. Cultures were chilled rapidly by addition of crushed ice, harvested in a Lourdes continuous-flow refrigerated centrifuge, washed once with 0.01 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 7.4/0.01 M MgCl₂/15% (wt/vol) glycerol/0.001 M diithiothreitol/0.0001 M disodium ethylenediaminetetraacetate (Tris/Mg/glycerol/DTT/EDTA) containing 0.05 M NH₄Cl, and stored as a frozen cell paste at −70°C.

Preparation of cell extracts. A 20-g amount of frozen cell paste was thawed and suspended in 20 ml of twofold-concentrated Tris/Mg/glycerol/DTT/EDTA containing 0.12 M NH₄Cl and 20 μg of electrophoretically purified deoxyribonuclease (Worthington Biochemicals Corp.). Cells were ruptured by two passes at 20,000 lb/in through an Amino French pressure cell, and then the lysate was diluted with 1 volume of Tris/Mg/glycerol/DTT/EDTA containing 0.06 M NH₄Cl. The diluted lysate was centrifuged at 20,000 rpm in a Sorvall SS34 rotor for 30 min, and the supernatant (Sup 0) was removed, taking care to avoid collecting a membranous pellicle at the surface, which is rich in nonspecific nucleases. Sup 0, the "crude extract" used in these experiments, is rich in RNase M5 activity and the precursor fragment scavenging RNase. However, a portion of the RNase M5 activity adheres to ribosomes (15) and could readily be freed from the scavenging nuclease as follows. A 20-Ml amount of Sup 0 was centrifuged at 30,000 rpm in a Beckman type 40 rotor for 2 h at 4°C. The resulting ribosomal pellet was rinsed with Tris/Mg/glycerol/DTT/EDTA containing 0.06 M NH₄Cl and then resuspended by stirring with a glass rod in 4 ml of the same buffer. The volume of the suspension was measured, and then sufficient 3M NH₄Cl was added to adjust the NH₄Cl concentration to 0.2 M. This salt concentration was sufficient to elute resident RNase M5 from the ribosomes, which then were pelleted at 40,000 rpm in a Beckman type 40 rotor for 2 h at 4°C. The upper two-thirds of the supernatant (Sup 2) was collected as a source of relatively nuclease-free RNase M5. More rigorous purification and characterization of this RNA maturation enzyme is described elsewhere (M. L. Sogin, B. Pace, and N. R. Pace, J. Biol. Chem., in press).

Preparation and analysis of RNA substrates. The isotopic labeling of concentrated, chloramphenicol-treated cultures of B. subtilis and the purification by phenol extraction and polyacrylamide gel electrophoresis of ³²P-labeled precursors of SS rRNA have been detailed previously (9, 16). In the isolation of the RNase M5 reaction products, ³²P-labeled p₁₅ or p₅₅ was incubated for 20 min in 0.2 ml of standard reaction buffer (0.01 M Tris-hydrochloride, pH 7.3/0.01 M MgCl₂/0.05 M NH₄Cl) containing about 0.2 mg of Sup 2 protein, and then the reaction was halted by addition of sodium dodecyl sulfate (SDS) to 0.2% and EDTA to 0.02 M. Reaction products were resolved preparatively by electrophoresis through 12% polyacrylamide gels, eluted from appropriate gel slices, and freed of soluble polyacrylamide by methylated albumin-kieselguhr chromatography, all as detailed previously (9, 16). When applicable, the isolated RNase M5 reaction products were digested completely with RNase T1 and the products were resolved by two-dimensional electrophoresis, first at pH 3.5 on cellulose acetate strips followed by transfer to diethylaminoethyl (DEAE) paper and electrophoresis at pH 1.7, all as described by Sanger and Brownlee (11). We recently have detailed the derivation of the complete nucleotide sequences of the precursor (16) and mature (7) segments of p₁₅ and m5 RNA; therefore T1 RNase oligonucleotides from the reaction products could be identified by their positions in the two-dimensional fingerprints. Novel oligonucleotides arising from T1 RNase digests of fragments generated in crude extracts could be identified unambiguously by quantitative analysis of their RNase A digestion products, with consideration to their behavior during two-dimensional electrophoresis.

Kinetic analysis of fragment release and degradation. Approximately 2 × 10⁶ cpm of ³²P-labeled p₁₅ was added to 0.4 ml of standard reaction mixture containing approximately 5 mg of Sup 0 protein or approximately 1 mg of Sup 2 protein, and incubation was continued at 37°C. At time intervals, 0.04-ml aliquots were withdrawn and mixed with 1 ml of ice-cold 0.003 M EDTA containing SDS to 1%. Identical amounts (approximately 10⁶ cpm) of purified ³²H-labeled m5 RNA, which served as recovery marker for the various samples, were added to each aliquot. RNA from all samples was purified by phenol extraction, methylated albumin-kieselguhr chromatography, and precipitation from ethanol as detailed previously (9, 16), and then reaction products were resolved by electrophoresis through 12% polyacrylamide gels. Gels were cut into 1-mm slices, which were dispersed by overnight incubation in 30% H₂O₂ and then evaluated for ³²H and ³²P contents by scintillation counting.

In determining the rate of degradation of the precursor-specific fragment F2 by Sup 0 in the presence or absence of added orthophosphate, ³²P-labeled F2 isolated from a RNase M₅ p₁₅ reaction as described above was added to 0.5 ml of standard reaction mixture with or without 0.01 M sodium phosphate buffer, pH 7.3. At time intervals, 0.04-ml aliquots were withdrawn and mixed with 0.01 ml of 1% SDS containing 0.1 M EDTA, and then reaction products were resolved by electrophoresis through a 15% polyacrylamide slab gel (1% bisacrylamide cross-linking). After drying and radioautography, regions of the slab gel corresponding to intact F2, nucleoside monophosphates, and all materials interconverting in size were excised for counting of resident radioactivity.
Analysis of acid-soluble components by Dowex-1-formate chromatography. Approximately 2 \times 10^6 cpm of 32P-labeled p5A was added to a 0.4-ml standard reaction mixture containing approximately 5 mg of Sup 0 protein, which then was incubated at 37°C for 40 min. The reaction mixture was then chilled to 0°C, 5 ml of 10% (wt/vol) trichloroacetic acid was added, and the mixture was held at 0°C for 10 min. The precipitate was removed by passing the mixture through a 47-mm membrane filter (HAWP, Millipore Corp., Bedford, Mass.). Then trichloroacetic acid was removed from the filtrate by four extractions with two volumes of diethyl ether, and the residual ether was removed from the final aqueous phase by bubbling at 37°C with a stream of air. The acid-soluble materials were then mixed with 1 mg of E. coli bulk RNA that had been hydrolyzed by overnight incubation in 0.2 N NaOH and were applied to a column (0.5 by 5 cm) of Dowex-1 (X 8)-formate. The column was eluted as described by Hayashi and Spiegelman (6), and aliquots of samples were monitored for optical absorbance at 260 nm and content of 32P radioactivity.

RESULTS

Specific degradation of fragments in vitro. The differential stabilities of the various products of the RNase M5 reaction were examined by incubating purified, 32P-labeled p5A precursor rRNA with either partially purified RNase M5 (Sup 2) or a crude cell extract (Sup 0) from B. subtilis and scoring the relative yields of the various reaction products with time. As detailed in Materials and Methods, aliquots from respective reaction tubes were withdrawn and mixed with known quantities of 3H-labeled m5 rRNA to provide a recovery measure, and then RNA components were resolved by electrophoresis through 12% polyacrylamide gel cylinders. The distributions of 32P and 3H in representative gels of Sup 0 reaction products are shown in Fig. 1; the various reaction products are identified in the figures. The amounts of radioactivity associated with regions of interest in the gels were summed and are expressed as a function of time in Fig. 2. It should be noted that the accumulation of m5 RNA is shown, but the mass ratios of the precursor-specific fragments, relative to the m5 product, are plotted. Selective loss of the released fragments therefore is readily manifest. Incubation of 32P-labeled p5, rRNA with partially purified (Sup 2) RNase M5 from B. subtilis resulted in the release of equimolar quantities of mature 5S rRNA (m5) and the precursor-specific fragments F1 (the 42-nucleotide segment derived from the 3' end of p5,) and F2 (the 21-nucleotide 5' terminus of p5,) (Fig. 2B). All of these reaction products were indefinitely stable in the presence of RNase M5, which had been freed of extraneous nuclease activities. The maturation nuclease itself was not the fragment-scavenging activity. However, in crude extracts from B. subtilis (Fig. 2A), the released, precursor-specific fragments were rapidly destroyed, whereas the m5 rRNA generated was stable to further degradation. There must exist within the crude extract an enzyme activity or activities capable of the selective destruction of the released precursor-specific segments.

Mode of fragment degradation. The three possible modes by which the released precursor-specific fragments may be degraded include: (i) endonucleolytic, (ii) 5'-exonucleolytic, and (iii) 3'-exonucleolytic degradation. We have previously reported the nucleotide sequence of the p5A rRNA precursor and defined the exact points of cleavage in the molecule by partially purified RNase M5 (15, 16). We noted during the course of the present experiments that the precursor-specific fragment F2 (the 5' end of the precursor) derived from crude extracts presented a broader band on polyacrylamide gels than did F2 released by more purified preparations of RNase M5. This suggested that heterogeneity in the length of F2 released by crude extracts is presumably due to the initiation of fragment degradation. We therefore analyzed the nature of the heterogeneity in F2 length; the positions of missing nucleotides should define the nature of the degradative process.

We previously have shown that F2, the 5'-terminal precursor-specific segment released by RNase M5 from p5A, has the 21-nucleotide sequence pU-G-A-G-A-G-A-A-C-A-C-U-C-U-C-A-A-U-U-U-G0H. Digestion of F2 with RNase T1 yields, per mol of F2, exactly 1 mol of pU-G-, 2 mol of A-G-, and 1 mol of A-A-C-A-C-U-C-A-A-U-U-U-G0H. In defining the structure of F2 recovered from cleavage by crude extracts as detailed in Materials and Methods, 32P-labeled p5A was incubated with an extract for 20 min and then treated with SDS and EDTA, and the products were resolved by gel electrophoresis. The radioactive band corresponding to F2 and slightly smaller materials was eluted from appropriate gel slices, recovered by precipitation from ethanol, and then completely digested with RNase T1. The resulting oligonucleotides were resolved by electrophoresis at pH 3.5 on cellulose acetate followed by transfer to DEAE paper and electrophoresis at pH 1.7, all as described by Sanger and Brownlee (11). The radioautogram of the resulting “fingerprint” is shown in Fig. 3. Of particular note is that the 3'-terminal T1 RNase oligonucleotide, A-A-C-A-C-U-C-U-C-A-A-U-U-U-G0H (fragment A in Fig. 3), is recovered in substantially lower
Fig. 1. Selective degradation of precursor-specific RNA fragments in vitro: polyacrylamide gels. As detailed in Materials and Methods, 32P-labeled p5A precursor rRNA was incubated with Sup 0 or Sup 2 (data not shown) extracts from B. subtilis. Samples withdrawn at time intervals were mixed with identical amounts of 3H-labeled m5 rRNA to provide a quantitative recovery marker, and products were resolved by electrophoresis through 12% polyacrylamide gels. Gels were sliced and monitored for 3H (x) and 32P (o) content; selected profiles are illustrated.
yields (about 0.3) than expected from the quantities of resident 5'-terminal pU-G- or internal A-G. Novel fragments, as indicated in Fig. 3, proved upon subsequent analysis, with consideration given to their positions in the two-dimensional fingerprint, to have the structures A-A-C-A-C-U-U-C-A-A-U-U-OH (fragment B), A-A-C-A-C-U-U-C-A-A-U-U-OH (fragment C), and A-A-C-A-C-U-U-C-A-A-U-U-OH (fragment D). The fragments, upon secondary digestion with RNase A and electrophoresis on DEAE paper at pH 3.5, were identical to fragment A with regard to relative contents of A-A-C-, A-C, and C-. However, fragment B lacked 1 mol of U-, fragment C 2 mol of U-, and fragment D 2 mol of U- and A-A-U-. RNase A digestion of fragment D yielded one novel product with the electrophoretic mobility expected of A-A-U-OH. The observed heterogeneity in F2 released by the cell extracts therefore was due to 3'-exonuclease trimming. We have not recovered significant amounts of fragment shorter than those noted, so presumably the subsequent exonucleolytic digestion is progressive in character; the substrate is not released but rather is rapidly and completely degraded.

Nature of the degradation products. Two types of 3'-exonucleases have been isolated from *E. coli*; we presume that equivalent degradative activities exist in *B. subtilis*. These enzymes, RNase II and PNPase, are distinguishable by the characters of their reaction products. RNase II is a hydrolytic enzyme that generates 5'-mononucleotides (14, 17), whereas PNPase effects the phosphorolytic release of 5'-dinucleotides (13). Moreover, degradation of polynucleotides by PNPase, in contrast to RNase II, requires rather high levels of orthophosphate for optimal degradative activity. We therefore tested these aspects of precursor-specific fragment degradation in extracts of *B. subtilis*.

As detailed in Materials and Methods, the terminal degradation products of the precursor-specific fragments were recovered as trichloroacetic acid-soluble components after incubation of 32P-labeled p5A with a *B. subtilis* Sup 0 extract. The character of these products then was defined by Dowex-1-formate column chromatography as described by Hayashi and Spiegelman (6). Figure 4 illustrates the resulting elution profile; the optical absorbance markers were supplied by an alkaline hydrolysate of *E. coli* total RNA (yielding 2'- and 3'-mononucleotides). More than 90% of the materials applied to the column eluted as the nucleoside 5'-monophosphates; nucleoside diphosphates would not elute from this column during the elution regimen used. The distribution of radioactivity among the four mononucleotides was approximately that expected from the base composition of the precursor-specific fragments.

Although the yield of nucleoside 5'-monophosphates suggests that the degradative activity is RNase II-like, it is conceivable that the reaction is effected by PNPase and that the resulting nucleoside 5'-diphosphates are reduced to mononucleotides by pyrophosphatase activity in the cell extracts. We therefore tested the capacity of added orthophosphate to stimulate the degradation. For clarity of the kinetic analysis, purified, precursor-specific fragment F2 was utilized as substrate. 32P-labeled fragment F2, isolated from RNase M5-p5B reactions as described in Materials and Methods, was
Fig. 3. T1 RNase fingerprint analysis of F2 released by Sup 0. Precursor-specific fragment F2 was isolated as detailed in Materials and Methods by gel electrophoresis from among the reaction products of Sup 0 acting on 32P-labeled p5A. The purified 32P-labeled F2 was completely digested with RNase T1, and the products were resolved by two-dimensional electrophoresis as detailed by Sanger and Brownlee (11); a radioautogram of the resulting finger-print is shown. All radioactive regions were excised from the DEAE paper and monitored for their 32P content; this permitted calculation of molar yields for known oligonucleotides. The oligonucleotides pUG-, AG-, and A (A-A-C-A-C-U-C-U-C-A-A-U-U-U-G) each have previously been characterized (15).
supplied to cell extracts in the presence or absence of added 0.01 M orthophosphate. Aliquots were withdrawn from the reaction mixtures at time intervals, and the products were resolved by polyacrylamide slab gel electrophoresis. Radioactive zones in the dried gel were located by radioautography and then excised and monitored for $^{32}\text{P}$ content by scintillation counting. The kinetics of the decay of F2 are illustrated in Fig. 5. It is evident that added orthophosphate had no effect on the initial attack of the fragment (Fig. 5A), although at later times it was inhibitory. However, the orthophosphate inhibited the accumulation of materials in the region of the gel containing nucleoside mono- or diphosphates. We conclude that the enzyme responsible for scavenging the precursor-specific fragments is RNase II-like in that it is a 3'-exonuclease that yields the 5'-mononucleotides, probably by a hydrolytic mechanism.

**DISCUSSION**

The coupled, in vitro RNA maturation-scavenging system described here mimics the substrate selectivity of the scavenging process that is observed in cells; mature 5S rRNA is conserved, whereas the released, precursor-specific fragments are concomitantly destroyed. Such selectivity offers some assurance that the implicated enzyme activity is responsible for the degradation of RNA fragments in the cell. The availability of RNA substrates, which in the cell are susceptible (precursor specific) or non-susceptible (mature) to scavenging, should be useful in defining the basis of selectivity by the degradative enzyme. Such analysis will require the use of highly purified enzyme preparations.

We here have determined that the responsible enzyme is a 3'-exonuclease that produces 5'-mononucleotide products and now can proceed with its purification, using these properties to distinguish it from other, irrelevant nucleases. From the character of the reaction products and the mode of degradation, we suggest that RNA fragment degradation in *B. subtilis* is effected by an enzyme corresponding in action to the RNase II of *E. coli*. Analysis of certain mutants by Apirion and his colleagues suggests that RNase II is responsible for destroying metabolically unstable RNA in *E. coli*, but direct demonstration of the involvement of this enzyme has not yet been possible. A similar conclusion...
was drawn by Sekiguchi and Cohen (12) and Spahr and Schlessinger (19), but they could not explore the specificity of the process. In contrast, Duffy et al. (4), from examination of the kinetics of $H_2^{18}O$ incorporation by B. subtilis, proposed that RNA turnover is effected by phosphorolysis. However, this conclusion probably is incorrect because of contamination of ostensible RNA with non-polynucleotide, phosphate-containing materials (9). Using the same methodology, Chaney and Boyer (2) provided evidence that RNA turnover in E. coli is by hydrolytic means.

We have determined the nucleotide sequence of the p5$_4$ rRNA precursor and evaluated the exact points of scission by RNase M5 (15, 16). The structures of the cleavage products (Fig. 6) suggest that the presence or absence of secondary structure at the 3' termini of potential substrates may predicate their susceptibility to the scavenging process. The precursor-specific segments (Fig. 6A and B) both have 3' termini (the site of initiation of degradation) that are not involved in obvious secondary structure, whereas the resistant, mature 5S termini (Fig. 6C) can be juxtaposed as anti-parallel, complementary strands. All 5S rRNA nucleotide sequences thus far determined possess complementary 5' and 3' termini (8), so it seems likely that the secondary structure drawn for the mature 5S rRNA in Fig. 6 in fact exists. If the 3'-exonuclease responsible for RNA scavenging cannot bind to and initiate degradation of double-helical polynucleotides, then this feature might in part explain the specificity of the scavenging process. It in fact has been shown (14, 17) that duplex polynucleotides are not good substrates for the RNase II of E. coli. In the case of the precursor-specific fragments from B. subtilis p5$_4$, however, after degradation is initiated, the scavenging enzyme probably ignores or at most is only somewhat retarded by duplex substrates; the susceptible precursor-specific segments both contain more or less extensive, self-complementary regions that likely collapse at physiological ionic strengths into double-helical structures as drawn. In the case of mature 5S rRNA, in addition to structural features possibly implicit in the RNA structure, protection to degradation presumably would be conferred by the intimate association of the molecule with ribosomal proteins. Such protection would not be available to the metabolically stable tRNA molecules, but their 3' termini of course are tightly associated with secondary structure. The 3'-terminal -C-C-A$_{OH}$ segment may be somewhat labile, but the enzyme nucleotidyl transferase (3) presumably is capable of effecting the repair of any tRNA that might be damaged in this manner.

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FIG. 6. Structures of the mature and precursor-specific products of the RNase M5 reaction. The nucleotide sequences of F2, F1, and m5 rRNA, as previously determined (16), are drawn in their most probable (minimum energy) secondary structures.

LITERATURE CITED


