Processing of the rne Transcript by an RNase E-independent Amino Acid-dependent Mechanism*

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RNase E is encoded by the rne (also known as amS or hmp) gene and is the principal enzyme that controls the chemical decay of bulk mRNA in Escherichia coli. Earlier work has shown that RNase E degrades its own mRNA, autoregulating production of the RNase E protein. Here we show that in cells lacking RNase E activity, the 3.6-kilobase rne transcript is cleaved site specifically at two locations near its center by a novel endonuclease whose activity is modulated by the presence or absence of amino acids in the culture medium. These cleavages produce a 2-kilobase RNase E-sensitive RNA fragment corresponding to the 3’ half of the transcript. Using primer extension and RNase protection analysis, we mapped RNase E-independent cleavages to sites 1558 and 1576 nucleotides from the 5’ end of the rne transcript (coordinates 1738 and 1747 of the rne gene). Our results indicate the existence of a previously unknown RNase E-independent mechanism for degradation of rne transcripts and further demonstrate that this mechanism responds to changes in cell growth conditions.

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1 The abbreviations used are: kb, kilobase; MOPS, 4-morpholinepropanesulfonic acid; CAA, casamino acid(s).

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions—The E. coli strains used for studying the rne mRNA process were N3433 and N3431 (rne-3071; Ref. 16). Plasmid pUC13 (17), which contains a full-length rne gene, was used to isolate DNA fragments as indicated in Fig. 1B (5’ probe a (da) and 3’ probe b (ds)) for making random priming DNA probes and for construction of other plasmids. For plasmid constructions, the XmnI DNA fragment (Fig. 1B) was ligated to a filled-in HindIII site of plasmid pT7/T3a19 (Life Technologies, Inc.); we then screened recombinant plasmids for the expression of riboprobe c from the T7 promoter (Fig. 1B). From this plasmid, the DNA fragment, which contains the 5’ end portion of the rne gene to the Mu1 site (Fig. 1B), was further deleted and self-ligated to generate a second plasmid in which the expression of the riboprobe d was under the control of the T3 promoter as shown (Fig. 1B).

Luria-Bertani broth (18) was used to grow E. coli for plasmid DNA preparation. The medium used to grow cultures for RNA studies was MOPS minimal medium (19) supplemented with 0.4% glucose, 1.32 mM K2HPO4, 0.02 mM (equal to 6.75 mg/ml) thiamine, and 0.04 mg/ml of the appropriate amino acids. 1% casamino acids (Difco) or a mixture of 20 individual amino acids (final concentration of 50 μg/ml, Sigma) was added in certain experiments as indicated.

RNA Isolation, Northern Blot, RNase Protection, and Primer Extension Analyses—Fresh overnight cultures grown at 33 °C in MOPS/glucose were diluted 1:100 with fresh medium as described in each experiment and cultured at 33 °C, or alternatively shifted to 43 °C, for different lengths of time prior to RNA isolation. Cell growth was monitored by taking the optical density reading at 460 nm using a Beckman DU-62 spectrophotometer. Total cellular RNA was isolated as described (20) at various times after rifampicin (250 μg/ml) treatment or after temperature upshift of logarithmic phase cultures (Δt = 0.4), except that after RNA precipitation at −20 °C, cellular chromosomal analysis and in vitro cleavage assay, no rne-dependent decay intermediate was observed by Northern blotting (14, 15), suggesting that the RNase E-generated products are rapidly degraded.

During the course of investigation of the effect of cell growth rate on the stability of rne mRNA, we observed a 2-kb1 RNA species corresponding to the 3’ half of the rne mRNA in the E. coli rne-3071 mutant strain grown in minimal medium containing casamino acids. However, accumulation of this rne transcript fragment at a nonpermissive temperature indicated that it was not produced by RNase E cleavage of full-length rne mRNA. Our subsequent investigations aimed at understanding the mechanism of production of this rne-derived transcript species have shown that inactivation of RNase E in cells growing in minimal medium leads to accumulation of full-length rne mRNA, which is processed in culture medium containing casamino acids to the 2-kb fragment. Our results imply that in the absence of RNase E activity, an rne-independent, physiologically regulated endoribonuclease is activated, and that this previously unidentified endonuclease cleaves full-length rne mRNA to produce an RNase E-degraded decay intermediate.

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DNA that forms strands in the solution was removed by spooling on a micropipette tip, and the precipitated RNA was then centrifuged at 4 °C for 15 min. For RNA sample preparation for gel electrophoresis analysis, the RNA pellet was washed with 70% ethanol and was then vacuum-dried and resuspended in 15 μl of 1× formamide gel-loading dye (21) containing 40 μg/ml ethidium bromide. RNA samples were denatured at 65 °C for 15 min and separated on 1% agarose, 6% formamide-gel loads (20 x 24 x 0.5 cm) in 1× formamide-MOPS buffer at 75 V for 10 h (bromphenol blue migrated approximately 16 cm from the well). A photograph of the individual gel showing ribosomal RNAs in each RNA sample was taken using Type 665 Polaroid film (Polaroid Corp., Cambridge, MA) and was used to control the amount of RNA loaded in each well. The RNA gel was then soaked in 10 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M trisodium citrate) for 12 h. RNA on membranes was then cross-linked to the membrane using a UV-Stratalinker 1800 (0.12 J, 2 min, Stratagene).

Hybridization procedures using either 32P-labeled random-priming double-stranded DNA probe (22) or riboprobe were performed according to the vendor’s instructions (Bio-Rad). The radioactively labeled bands were analyzed using either a Molecular Dynamics PhosphorImager or an x-ray autograph. Films showing ribosomal RNAs in each RNA sample were scanned and quantitated by the 3008 MD computing densitometer.

The riboprobes used for the RNase protection assay are indicated in Fig. 4A, and RNase protection was performed as described by the vendor’s instruction (Ambion). Primer extension analysis was done as described previously (23); the primers used are primer A1912 (5′-CA-GACATTTTTCATCAGCAGTGAAG-3′, rne gene coordinates 1912 to 1886) and primer AMS (5′-CCGCTAATCGTCCGAAAGATC-3′, rne gene coordinates 294 to 274).

Restriction endonucleases, T7 and T3 RNA polymerases, placentar ribonuclease inhibitor, and RNase-free DNase I were purchased from Amersham Corp., Life Technologies Inc., Promega, Boehringer Mannheim, or New England Biolabs and were used according to the vendor’s instructions. Plasmid DNA was isolated using an alkaline-lysis procedure (21). [α-32P]dCTP and [α-32P]UTP were used for random priming from the double-stranded DNA probe. [γ-32P]ATP was used for end labeling of oligoprimers. Agarose and polyacrylamide gel electrophoresis and DNA fragment isolations were performed as described by Sambrook et al. (21).

RESULTS

The 3′ Half of an rne mRNA Intermediate Accumulates in E. coli rne-3071 Strain Grown on Nonpermissive Temperature in Minimal Medium Containing Casamino Acids—Northern blot hybridization to a 32P-labeled double-stranded DNA probe that contains the rne coding region (325–3589 nucleotides; see Refs. 17 and 24) was used to detect the rne message and its decay intermediates. As previously observed (14, 15), we found that a transcript approximately the size of full-length rne mRNA accumulates in N3431 at a temperature nonpermissive for cell growth (43 °C), but not at a permissive temperature (33 °C) (Fig. 1A). However, in N3431 cultured at 43 °C in the same medium supplemented with casamino acids (CAA), but not in media lacking CAA, in addition to the full-length rne mRNA, an rne transcript species about 2 kb in length was observed (Fig. 1).

Using Northern blot analyses and double-stranded DNA probes (indicated as ds in Fig. 1, B and C) corresponding to either the 5′-end region (5′ probe a (ds)) or 3′-end region (3′ probe b (ds)) to further identify the origin of the 2-kb transcript species, we found that the 3′ probe but not the 5′ probe hybridized to the 2-kb species (Fig. 1C). Additional studies (Fig. 1C) using single-strand-specific riboprobes confirmed that this species is transcribed in the same direction as the full-length rne transcript rather than in the orientation of a transcript known to extend in an antisense direction into the 3′ end of the rne gene coding region from a downstream promoter (17) and thus implies that the 2-kb transcript species is a decay intermediate produced from full-length rne transcripts. As RNase E produced in strain N3431 is inactivated at 43 °C (16), the results show that the production of the 2-kb transcript species occurs by an RNase E-independent mechanism and furthermore that this mechanism is dependent on the presence of CAA in the culture medium. Analysis of the kinetics of decay of full-length rne mRNA and formation of the 2-kb transcript species at various times following rifampicin treatment of a culture that was temperature-upshifted from 33 to 43 °C for 15 (T15) or 30 (T30) min before the rifampicin was added, which inhibits new RNA synthesis, showed that the 2-kb transcript species was formed continuously (Fig. 2, T15 and T30, respectively) and that the intracellular concentration of full-length mRNA declined. Thus, production of the 2-kb species occurs despite inhibition of new RNA synthesis and therefore is in fact an rne decay intermediate. The 2-kb RNA intermediate observed at T30 is more abundant (Fig. 2) because the rifampicin was added 15 min later than T15 culture thus allowing the production of the very stable (see Figs. 2 and 3) decay intermediate to continue for another 15 min. Although E. coli N3433 whose RNase E is active under both 33 and 43 °C growing temperatures was studied, we found no detectable 2-kb transcript (either full-length or decay intermediate) in cells cultured in minimal medium supplemented with or without CAA (data not shown). These results are consistent with a previous report (14) showing that in the presence of RNase E activity the rne message is extremely unstable.

Production of the 2-kb Decay Intermediate Requires Amino Acids in the Culture Medium Prior to Temperature Upshift to 43 °C—To investigate the apparent dependence of production

**FIG. 1.** Northern blot analysis of rne mRNA showing an accumulation of its 3′-RNA transcript in N3431 (rne-3071) grown in CAA at 43 °C. Panel A, comparison of rne mRNA isolated from MOPS/glucose and MOPS/glucose/1% casamino acids. Bacteria were grown in MOPS/glucose (−CAA) or MOPS/glucose/1% casamino acids (+CAA) at 33 °C, and then an aliquot was shifted to 43 °C when A600 reached 0.4. Total RNA was isolated from the 33 and 43 °C cultures 30 min after temperature upshift. A film of ethidium bromide-stained 16 S and 23 S rRNAs (bottom panel) on the preblotted gel was used as the internal RNA loading standard. The probe used in panel A was a full-length rne (325–3589) probe synthesized by random priming, FL, full-length rne mRNA; I, the 3′ transcript (later identified as the rne mRNA decay intermediate). The sizes of the RNAs are indicated in parentheses. Panel B, regions of the double-stranded probes and riboprobes used for mapping the decay intermediate are shown. T7 and T3 represent the promoters used in synthesizing riboprobe c and d, respectively. Panel C, Northern blot analysis of the rne 3′ transcript using the probes shown in panel A. RNA was isolated from N3431 (rne-3071) grown in MOPS/glucose/1% casamino acids at 33 or 43 °C as indicated. The same amount of total RNA was loaded in each well, separated in the same gel, and transferred to the same blotting membrane. The membrane was then cut into strips for Northern blotting analysis using different hybridization probes as indicated. Coordinates shown are based on the DNA sequences of X67470 and L23942 (GenBankTM). The hybridizable RNA bands observed above and below the 3′ transcript result from cross-hybridization by 23 S and 16 S rRNA, respectively.
Northern blotting.

After temperature upshift to 43 °C. (Fig. 3) decrease in the level of full-length mRNA and significant accumulation of the decay intermediate started about 20 min after temperature upshift to 43 °C. (Fig. 3A, lanes 1–6, and B, bottom panel). However, accumulation of the 2-kb decay intermediate did not occur when CAA was added to minimal medium 10 min after the culture was shifted to 43 °C (Fig. 3A, lanes 7–11) or just prior to the temperature upshift (data not shown). When CAA was added only one-generation time (i.e. about 40 min) before the temperature upshift, accumulation of the decay intermediate RNA occurred about 30 min after the upshift and was delayed for roughly 10 min compared to the culture grown continuously in CAA (Fig. 3C, lanes 1–6 versus lanes 7–12). With further shortening of the interval between addition of CAA and temperature upshift, the delay in production of the 2-kb species was further prolonged (data not shown). However, in all cases accumulation of the intermediate RNA was associated with a decrease in the level of the full-length rne transcript (Figs. 2 and 3). Similar kinetics of production of the decay intermediate were observed in minimal medium containing a mixture of 20 individual amino acids (Fig. 4). Collectively, these results indicate that formation of the decay intermediate in the temperature-sensitive rne307I mutant requires the presence of amino acids prior to RNase E inactivation.

The 5’ Terminus of the Intermediate RNA Maps to the Middle of the rne Gene—RNase protection and primer extension analyses were used to map the 5’ terminus of the decay intermediate. Using two 32P-labeled RNA riboprobes, which are indicated in Fig. 5A (top panel) as probe a and b, respectively, and known RNA size markers in an RNase protection assay (see “Materials and Methods”), we detected two RNA fragments whose sizes are about 270 and 280 nucleotides in length (Fig. 5A (bottom panel), lanes 5 and 10). Using RNA size markers on the same gel, the 5’ termini of these species were mapped to the vicinity of rne coordinate 1750 (indicated with arrows in Fig. 5A). Based on these results, we designed an oligonucleotide complementary to a sequence downstream from the 5’ termini located towards RNase protection (i.e. primer A1912, coordinates 1912 to 1886) and used primer extension to map the precise locations of the 5’ termini; DNA sequence reactions

FIG. 2. Accumulation of the rne 3’ transcript species occurs continuously after inhibition of RNA synthesis by rifampicin treatment of N3431 grown in CAA at 43 °C. Two 60-ml aliquots of N3431 cultured in MOPS/glucose/1% casamino acids at 37 °C were shifted to 43 °C for 15 and 30 min (T15 and T30, respectively) at A660 0.3 and treated with rifampicin (250 μg/ml culture). RNA samples were isolated at different time points, as shown above the wells, after rifampicin treatment. Time points –1, 1, 2, 3, 5, 9, 13 (or 15), 17 are 1 min before (−1) and times (1–17 min) after rifampicin treatment. Top panel, Northern blot; bottom panel, ethidium bromide-stained gel prior to Northern blotting. FL and I represent full-length and rne mRNA decay intermediate, respectively.

FIG. 3. Production of rne decay intermediate requires casamino acids in minimal medium prior to temperature upshift. Panel A, Northern blot analysis of rne decay intermediate isolated from N3431. Lanes 1–6, N3431 was cultured in MOPS/glucose/1% casamino acids and then shifted to 43 °C as described in Fig. 1. Lanes 7–11, N3431 was first grown in MOPS/glucose without 1% casamino acids, and then casamino acids were added after shifting the culture to 43 °C. RNA samples prepared from cells harvested at various time points after the temperature upshift are shown (min). Panel B, line plots of full-length (FL) and the decay intermediate (I) of panel A. The top of panel B shows the amount of full-length rne RNA in minimal medium with CAA (+CAA, ○) and without CAA (−CAA, ▲) after various time points following upshift to 43 °C. The bottom of panel B shows the amount of decay intermediate (I, ●) and full-length (FL, ○) rne mRNA of lanes 1–6 of panel A. Full-length RNA at time 0 (lane 1) was set at 100%. Quantitation was done with a PhosphorImager and densitometer as described under “Materials and Methods,” in which 23 S rRNA in each sample was used as internal control. Panel C, lanes 1–6, 1% casamino acids was present continuously. Lanes 7–12, 1% casamino acids was added for 1 doubling time (about 40 min) prior to temperature upshift to 43 °C. RNA samples were isolated at various time points as indicated after temperature upshift. Films of ethidium bromide-stained 16 S and 23 S rRNAs on the preblotted gels are shown (bottom of panels A and C). FL, full-length rne mRNA; I, intermediate rne mRNA.

using the same primer (A1912) were used as the size standard. As shown in Fig. 5B, the 5’ termini of the intermediate RNA were detected in N3431 grown at 43 °C but not at 33 °C and were mapped at rne coordinates 1738 and 1747. The intensity of the terminus at position 1738 is stronger than that at 1747, consistent with our RNase protection data showing that the larger species is more abundant (Fig. 5A, bottom panel, indicated by arrows). Additional primer extension analysis demonstrated that the 2-kb decay intermediate is generated from intact rne mRNA rather than from the previously reported (14) RNA species produced by RNase E cleavage 48 nucleotides from the 5’ end of the full-length rne transcript. A 5’ terminus at rne gene coordinate 180 that is identical to the rne initiation site mapped by Jain and Belasco (14) was detected at both temperatures (Fig. 5C) as was a second site at coordinate 146 in N3431 grown at 43 °C (Fig. 5C, 43 °C). The primer extension signal at the nucleotide position 180 was much greater at 43 °C than at
33 °C, and the second 5’ end at nucleotide position 146 was detected only at 43 °C. These results, which are consistent with our results showing that the CAA-dependent cleavage that produces the 2-kb decay intermediate becomes evident when RNase E is inactivated, indicate that the 2-kb decay intermediate is the cleavage product of the intact full-length rne transcript.

**DISCUSSION**

Previous work has shown that RNase E autoregulates its production by degrading its own transcript (14, 15). We have now found that in the absence of RNase E activity a default mechanism involving cleavage by an endoribonuclease whose activity is regulated by the presence of amino acids in minimal medium initiates degradation of rne transcripts. In addition, our finding that amino acids in minimal medium can regulate the endoribonuclease activity in vivo has not been reported previously. This kind of default mechanism of regulating RNA decay pathway may also exist for other transcripts to compensate for deficiency in RNase E activity or to respond to different cell growth conditions, including oxygen concentration, which have been shown to modulate RNase E activity (25).2

The cleavages we observed at coordinates 1738 and 1747 in the rne message do not result from RNase III, which also has exoribonucleases have been identified in *E. coli* (26) to be involved in mRNA degradation. Whether the observed endoribonuclease whose activity regulated by amino acids in minimal medium is RNase P is unknown. However, there is no reported evidence that RNase P activity is affected by the presence or absence of amino acids. Therefore, production of the 2-kb decay intermediate appears to result from an unidentified endonuclease whose activity requires specific amino acids in the culture medium. Whether there was an initial cleavage at an upstream site coupled with subsequent processing to these termini cannot be ruled out. However, no 5’–3’ exoribonucleases have been identified in *E. coli*, and other decay intermediates resulting from endonuclease digestion would have been detected by the experiment shown in Fig. 1. The 2-kb decay intermediate observed here was not observed in previous studies (14, 15) where LB medium rather than CAA minimal medium was used.

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2 Recent data suggest that cell growth stage and oxygen can regulate the level of the rne transcript (A. von Gabain, manuscript in preparation).
It is worth noting that an essential glycolytic enzyme, enolase, that catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate has been found in the RNase E complex or degradosome independently by us and others (27, 28). Bacteria mutated in the eno gene cannot be cultured in glycerol minimal medium, but it can grow if either CAA, succinate, or malate is provided (29). As tight control of RNase E expression is essential for cell growth (13, 14), it is possible that this CAA or medium composition-rescued phenotype is related to the activation of the observed endoribonuclease activity that hydrolyzes rne mRNA.

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