Stabilization of Circular rpsT mRNA Demonstrates the 5'-End Dependence of RNase E Action in Vivo*

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RNase E is the major intracellular endonuclease in Escherichia coli. Its ability to cleave susceptible substrates in vitro depends on both the cleavage site itself and the availability of an unstructured 5' terminus. To test whether RNase E activity is 5'-end-dependent in vivo in the presence of all the components of the RNA degradative machinery, a known substrate, the rpsT mRNA, has been embedded in a permuted group I intron to permit its efficient, precise circularization in E. coli. Circular rpsT mRNAs are 4–6-fold more stable in vivo than their linear counterparts. Even partial inactivation of RNase E activity further enhances this stability 6-fold. However, the stabilization of circular rpsT mRNAs depends strongly on their efficient translation. These results show unambiguously the importance of an accessible 5'-end in controlling mRNA stability in vivo and support a two-step ("looping") model for RNase E action in which the first step is end recognition and the second is actual cleavage.

The degradation of mRNAs is an important, if incompletely understood, aspect of the regulation of gene expression. In Escherichia coli, the initiating step in the decay process is usually mediated by RNase E (1–3), a 5'-end-dependent endonuclease (4). In vitro, RNase E activity is conferred by a multi-enzyme complex, the degradosome (5, 6). In small RNAs, such as the ColE1 replication regulator RNA 1, and the rpsO or rpsT mRNAs encoding ribosomal proteins S15 and S20, respectively, a single RNase E cleavage is capable of inactivating the mRNA and rendering it susceptible to complete destruction to mononucleotides (reviewed in Ref. 3). In larger mRNAs this initiating endonucleolytic event can trigger a 5' → 3' "wave" of subsequent endonucleolytic cleavages, which rapidly inactivate the entire mRNA (7). The 3' termini generated by RNase E cleavages are scavenged by 3'→5'-exonucleases (1, 3).

Two features in the 5'-extremity of an mRNA, secondary structure and the triphosphate terminus, can control the susceptibility of the entire mRNA toward RNase E. The 5'-terminal stem-loop structure of the ompA mRNA is largely responsible for the atypical stability of this mRNA and can confer stability to heterologous mRNAs to which it is grafted (8, 9). Stabilization is abolished by as few as three single-stranded residues at the extreme 5'-end of an RNA (10). These effects of 5'-terminal secondary structure on mRNA stability are mediated directly through Rne (4, 11). Evidence for the critical role of the free 5'-end of RNA and its phosphorylation state in mRNA turnover arises from several experiments. Most notably, circular derivatives of the well-characterized RNase E substrates, rpsT mRNA or 9 S RNA, are highly resistant to cleavage in vitro by Rne or degradosomes (4). An RNase E cleavage at a site 5 residues from the 5'-end of RNA 1 destabilizes the 103-residue 3'-cleavage product in vivo. However, an artificial RNA identical to the initial cleavage product but containing a triphosphorylated terminus is significantly more stable (12). Likewise, oligonucleotide-directed cleavage of the rpsT mRNA by RNase H shows that the 5'-segment containing a triphosphorylated terminus is quite stable, even in relatively crude extracts. In contrast, the 3'-segment that would be monophosphorylated undergoes RNase E cleavage at an accelerated rate compared with the unmodified substrate (11, 13). Finally, either Rne protein alone or purified degradosomes preferentially cleave monophosphorylated substrates 20–30-fold more rapidly than their triphosphorylated counterparts (4). Together, these results imply that the vectorial nature of mRNA decay is a reflection of the inherent preference of Rne for 5'-monophosphorylated substrates.

To address whether Rne or degradosomes display 5'-end dependence in vivo where a circular RNA would be exposed to all the components of the degradative machinery, I have constructed chimeric RNAs in which a portion of the rpsT mRNA is embedded in a permuted group I intron, a construction that should permit its precise, autocatalytic circularization (14, 15). In this work I show that circular rpsT mRNAs form efficiently and are 4–6-fold more stable than their linear counterparts. Circular RNAs are, however, cleaved slowly by RNase E in a 5'-end-independent fashion, which is highly sensitive to ongoing translation.

EXPERIMENTAL PROCEDURES

Strains and Plasmids—Bacterial strains JM109 (F' traD36 lacZΔlacZ(M15) proA B' lacY1 tnaA Δ(lac-proAB) thi gyrA96 endA1 lsdR17 relA1 supE44 recA1) and GM323 (JM109 Δ(glpU) Ref. 16) are from our collection; MG1693 (thyA715 rph1) and SK5665 (thyA715 rph1 rne1) were obtained from Dr. S. R. Kushner, University of Georgia. The vector prR1 (17) was supplied by Dr. M. Ares, University of California, Santa Cruz. The rpsT sequences in pGM110 and pGM113 were amplified by PCR1 using oligonucleotides 982 (5'-GGAATTCCCCATGGAATTTCTTCAGCAAATTGGC) (3'-primer), 983 (5'-TT-GCAAGATCTTTCAGAAAACACATCTGGGAG) (5'-primer), and previously described DNA templates (13, 16). Amplified DNAs were purified electropheretically, cleaved with the appropriate enzymes, repurified, and ligated into pRR1 cleaved with NcoI and BglII (see Fig. 1b for a schematic). Following transformation into JM109, the recombinant plasmids were confirmed by restriction analysis and DNA sequencing. Extraction and Analysis of RNA— Cultures were grown to midexponential phase in LB medium (supplemented with ampicillin and 20 mg/liter thymidine as needed) at either 29 °C (MG1693 or SK5665)

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1 The abbreviations used are: PCR, polymerase chain reaction; IPTG, isopropyl-1-thio-β-D-galactopyranoside; RT, reverse transcription; Pipes, 1,4-piperazinediethanesulfonic acid; bp, base pair(s).
or 37 °C (JM109), shifted to 39.5 °C as required, induced with 0.5 mM IPTG for 20 min, and when necessary treated with rifampicin (>160 μg/ml) to inhibit transcription. Portions (2.0 ml) of the culture were harvested at intervals thereafter, and RNA was extracted using method II (18). Yields were quantified by A_{260} and the quality of the RNA was assessed by the intactness of rRNA. For RT-PCR, 2 μg of total RNA (in some cases pretreated with RNase H and oligonucleotide 481 (13)) was denatured at 90 °C and then mixed with a buffer containing 25 mM Tris-HCl, pH 8.3, 37.5 mM KCl, 1.5 mM MgCl₂, 0.5 mM dNTPs, 10 mM dithiothreitol, 2.5 μg oligonucleotide 992 (5'-CTGAAATGGCAAGCTTCATGGAC; complementary to residues 151–169 in the rpsT sequence (13)). Following addition of 100 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) and incubation for 30 min at 42 °C, a portion of the products corresponding to 0.25 μg of RNA template was amplified with 5 units of Taq DNA polymerase (Life Technologies, Inc.) for 30 cycles in 10 mM Tris-HCl, pH 6.4, 25 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM MgCl₂, 100 μM dNTPs, and 1 μg of primer 991 (5'-GCTAAAGTGCGATCCACAAA; complementary to residues 319–340 in the rpsT sequence (13)) and primer 992. For Northern blotting, samples of total RNA were dissolved in a buffer containing 8M urea. RNAs were transferred electrophoretically to Hybond-N (Amersham Pharmacia Biotech), fixed, and probed to detect the rpsT mRNA with either a complementary RNA or 5'-32P-labeled oligonucleotide 993 (5'-GTTAGACCTGGAATCTTC). Hybridization with oligonucleotides was performed at 37 °C in a buffer containing 0.75 M NaCl, 75 mM sodium citrate (pH 7), 0.1% polyvinylpyrrolidone, 0.1% Ficoll, 0.1% bovine serum albumin (fraction V), 20 mM Pipes (pH 6.4), 25 μg/ml salmon sperm DNA, 62.5 μg/ml yeast RNA, 0.1% SDS. Membranes were washed with 0.3 M NaCl, 0.03 M sodium citrate (pH 7), 0.1% SDS at 38–39 °C.

RESULTS AND DISCUSSION

Synthesis and Characterization of Circular rpsT RNAs—Based on the finding that the separate “halves” of a group I intron can self-assemble to form an active ribozyme (19) (Fig. 1a), permuted group I introns have been designed to drive the circularization of “passenger” sequences inserted between the halves of the intron (14, 15). Sequences from the E. coli rpsT gene (13) were inserted into the polylinker in pRR1 (17), flanked by the appropriate group I intron sequences (Fig. 1b) and verified by DNA sequence analysis. A set of constructions, whose prototype is pGM110, contains rpsT sequences from residue 93 to 413, including the entire 5’-untranslated region, the coding sequence, and part of the 3’-untranslated region but lacking the transcriptional terminator (Fig. 1b). The plasmids pGM113 and pGM116 (see below) differ from pGM110 only by point mutation. The insert in pGM110 extends from residue 132 adjacent to the initiation codon (altered from UUG to AUG) to residue 413 and lacks the 5’-untranslated region completely (Fig. 1b). All constructions contain a major internal RNase E cleavage site at rpsT residues 300–301 as well as other minor sites (20).

A Northern blot of total cellular RNA extracted from cultures of JM109/pGM110 or JM109/pGM111 with or without induction showed that expression of putative circular rpsT RNAs of 336 or 297 nucleotides, respectively, was induced by IPTG to appreciable levels. These RNAs migrated completely with those predicted from the sequence of pRR1 and pGM110 (data not shown). The amplification products of “inverse” RT-PCR were purified, cloned into pUC19, and sequenced. Both sequences agreed completely with those predicted from the sequence of pRR1 and the group I intron-rpsT boundaries in pGM110 and pGM111 (data not shown).

The amplification products of “inverse” RT-PCR were purified, cloned into pUC19, and sequenced. Both sequences agreed completely with those predicted from the sequence of pRR1 and the group I intron-rpsT boundaries in pGM110 and pGM111 (data not shown). Finally, a point mutation, G → C at position J7/8-5 (21), known to reduce the rate of self-splicing by group I introns by >10³, was introduced into the 3’-“half” of the group I intron sequence in pGM110 generating pGM116. This mutation abolishes formation of rpsT/circ-336 RNA (not shown). Taken together, the Northern blots, the permutation analyses, and the sequences of the RT-PCR products demonstrate unambiguously that expression of the rpsT sequences embedded in pGM110 or pGM111 yields circular rpsT RNAs, which accumulate to readily detectable levels, roughly 4–5-fold higher than those of the endogenous rpsT mRNAs (Fig. 2a, lanes 1–5).

Stability of Circular rpsT mRNAs—Measurements of RNA half-life were performed using standard methods (1) (see “Experimental Procedures”).
and the chromosomally encoded rpsT group I intron sequences. Triangles annealing to oligonucleotide 993 (rpsT extraction of RNA at various times thereafter shown above each panel (see "Experimental Procedures"). Circular oligonucleotide 993 complementary to lanes 1–5 XpGM110 (b–e, decay of circular or chimeric RNAs after rifampicin treatment. Cultures of JM109/pGM111 (m33 s (data not shown). A typical Northern blot of RNA extracted from strain JM109/pGM110 was measured initially (see "Experimental Procedures"). The rate of decay of rpsT mRNAs is considerably more stable than its linear precursors. Although significantly stabilized, the circular rpsT RNAs described above are, nonetheless, metabolically labile. To determine whether RNase E is responsible for initiating the degradation of circular rpsT RNAs in vivo, the stabilities of the rpsT/circ-336 RNAs in either MG1693/pGM110 (Fig. 2d) or SK5665 (rne1/pGM110 (Fig. 2e) were measured after cultures grown at the permissive temperature of 29 °C were induced with IPTG and shifted to 39.5 °C for 20 min to achieve partial inactivation of the rne1 gene product prior to RNA extraction. The half-life of the rpsT/circ-336 species in strain SK5665/pGM110 is 1800 ± 155 s (Fig. 2e). This represents a 6-fold increase relative to the same RNA in the wild type strain under identical conditions (296 ± 33 s (Fig. 2d).

Circular RNAs, including the rpsT/circ RNAs, are translated in vivo (Ref. 17 and additional data not shown). To assess a role for translation in the stability of circular RNAs, half-lives were determined for several circular rpsT RNAs containing mutations affecting translational efficiency (Table I). For comparison, half-lives are also given for a set of linear, chimeric rpsT mRNAs.

FIG. 2. Detection of circular RNAs by Northern blotting. a, total RNA from JM109 (10 μg; lanes 1 and 6) or from JM109 containing pGM110 (5 μg; lanes 2, 3, 7, and 8) or pGM111 (5 μg; lanes 4, 5, 9, and 10), extracted prior to (−) or after induction (+) was analyzed by Northern blotting (see "Experimental Procedures"). RNAs in lanes 1–5 were detected by a cRNA probe, and RNAs in lanes 6–10 were detected by annealing to oligonucleotide 993 complementary to rpsT mRNAs with a circular junction. Numbers in the left margin give the sizes (nucleotides [nt]) of markers and the chromosomally encoded rpsT mRNAs. The triangles show the circular RNAs; X denotes circular RNA nicked during isolation or handling. b–e, decay of circular or chimeric RNAs after rifampicin treatment. Cultures of JM109/pGM111 (b and c), MG1693/pGM110 (d), or SK5665/pGM110 (e) were induced with IPTG at 37 °C (b and c) or at 39.5 °C (d and e) for 20 min prior to inhibition of transcription by rifampicin and extraction of RNA at various times thereafter shown above each panel (see "Experimental Procedures"). Circular rpsT mRNAs were detected by annealing to oligonucleotide 993 (b, d, and e). RNAs in c were probed with oligonucleotide 995 (5′-GCTGCATATCTATCACC) complementary to group I intron sequences. Triangles in the right margin denote circular rpsT species. The triangles in the left margin of c refer to the RNAs mentioned in the text. X denotes nicked species.

FIG. 3. Inverse PCR detection of circular rpsT RNA. a, principle of the method. Reverse transcription is primed by oligonucleotide 992, and the product is amplified by oligonucleotides 991 and 992 (see "Experimental Procedures"). The position and orientation of the primers are such that circular RNA (left) but not linear RNA (right) can serve as an amplifiable template. b, analysis of inverse RT-PCR products. Template RNA was extracted from JM109/pGM110 (lanes 2, 3, 6, and 7; predicted product of 189 bp) or JM109/pGM111 (lanes 4 and 5; predicted product of 149 bp) and used for RT-PCR (see "Experimental Procedures"). In lanes 2 and 5, the template RNA was linearized outside the region of amplification with RNase H and oligonucleotide 481 (13) prior to RT-PCR. In lane 7, reverse transcriptase was omitted in the first step.
TABLE I

| Initiation feature | Half-life |
|--------------------|----------|
|                    | rps7614c | rps7614a |
| UUG                | 295 ± 30 | 56       |
| −3, −4             | 166 ± 19 | 24       |
| AUG                | 570 ± 20 | 142      |

The rps7614c RNAs (Fig. 1b) were detected with oligonucleotide 993.

The rps7614a mRNAs were detected with an rps7 cRNA. Half-life data are taken from Ref. 16.

(rps7614) containing similar or identical rpsT sequences embedded between 40 residues of lac operon sequence in their 5′-leader and a portion of the 3′-end of the rnb operon (16). Such transcripts also effectively mimic the linear precursors to the circular rpsT RNAs. The data in Table I show that a circular rps7 RNA is 4–6-fold more stable than a linear mRNA containing the same rpsT sequences regardless of translational efficiency. Circular rpsT/circ-336 (UUG) RNA, which spans residues 93–412 (Fig. 1b), exhibits a half-life 5-fold greater than rps7614 (UUG) mRNA spanning the same rpsT sequences (Table I, line 1). A UUG to AUG change at the initiation codon increases expression of the S20 protein up to 6-fold in vivo (16). The half-life of the rpsT/circ-297 (AUG) RNA is 4-fold greater than its linear counterpart, rps7614 (AUG) (Table I, line 3). A double mutation in the rpsT leader (16) (G129C, A130U; −3, −4), which reduces S20 expression substantially, was introduced into pGM110 to form pGM113. The half-life of the rpsT/circ-336 RNA encoded by pGM113 is almost 2-fold lower than the nearly identical circular RNA encoded by pGM110 (compare lines 1 and 2 in Table I) but is still much higher than the corresponding rps7614 mRNA containing the −3, −4 mutation (Table I, line 2). These results show that the stabilities of circular mRNAs are subject to almost the same degree of translational modulation as their linear counterparts and more interestingly that circularization protects the rpsT mRNA most effectively when translation is highly efficient (compare rpsT297-circ (AUG) to rpsT336-circ (−3, −4) in Table I).

The resistance of a circular RNA to exoribonucleases is obvious (14); it is thus surprising, if not counterintuitive, that a circular RNA is significantly more resistant to RNase E, a single-stranded specific endoribonuclease of limited sequence preference (2, 3, 22), when the same RNA in linear form is an excellent substrate (4, 13, 20). This finding is, however, fully consistent with both the in vitro (4) and more recently the in vivo (5) inaccessibility of the circular rpsT/circ-336 (UUG) mRNA when RNase E activity is reduced shows that other ribonuclease activities in E. coli (3), including RNase III, RNase G/CafA, and RNase H/M, are unable to compensate efficiently. These findings clearly demonstrate that RNase E is a 5′-end-dependent endoribonuclease in vivo as well as in vitro (4, 23, 24) and that the frequently observed 5′ → 3′ vectorial character of mRNA decay in vivo (1, 3, 4, 7) is due to the end dependence of RNase E (4, 23).

The initial attack of RNase E on circular RNAs in vivo is 5-fold less efficient than on linear RNAs of similar primary sequence. This can be rationalized by a two-site or looping model (3). In this model, RNase E in the degradosome would first contact linear substrate mRNAs at their extreme 5′-ends. This 5′-contact would stabilize the enzyme-substrate complex and would facilitate, possibly in a first order rearrangement (“looping”), recognition of internal cleavage sites that may be partially hindered by adjacent secondary structures. In circular RNAs, there would be no 5′-contact, and cleavage site recognition itself would become second order and much less efficient unless the cleavage site were particularly accessible or exposed. Thus the rate of attack of RNase E on a circular RNA, rather than on a triphosphorylated RNA as suggested elsewhere (23), defines its basal rate of activity. As shown here, linear RNA with an unobstructed 5′-triphosphate terminus is attacked 5-fold faster than a circular RNA, showing that a triphosphate terminus can assist substrate recognition. A 5′-monophosphate terminus provides a further 20–25-fold stimulation of the initial rate of RNA cleavage by RNase E in vitro (4, 23) and by a significant but undefined amount in vivo (12). One clear implication of these data for the two-step model is that translation affects the second (rearrangement) step rather than the initial recognition of the 5′-end of the substrate.

It is likely that the degradation of many mRNAs will follow the behavior of the rpsT mRNA and exhibit 5′-end dependence. Any exceptions that prove to be insensitive to the state of the 5′-terminus will likely be explained by the presence of readily accessible RNase E sites in the body of the mRNA (permitting an increase in the basal rate of RNase E cleavage) or by inefficient translation.

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