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# A uridine-rich sequence required for translation of prokaryotic mRNA

(Shine–Dalgarno sequence/ribosomal protein S1/protein synthesis)

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ABSTRACT **Binding of 30S ribosomal subunits to mRNA during the initiation of prokaryotic translation is known to be influenced by the initiation codon and the Shine–Dalgarno sequence. Site-directed mutagenesis of *rnd*, the *Escherichia coli* gene encoding RNase D, has now shown that a $U_8$ sequence upstream of the Shine–Dalgarno region is also essential for expression of this mRNA. Alteration of two to five uridine residues within this sequence has no effect on mRNA levels but decreases RNase D protein and activity by as much as 95%, indicating that the U-rich sequence acts as an enhancer of translation. Moreover, mutant transcripts bind to 30S ribosomes *in vitro* with lower affinity than their wild-type counterparts, suggesting that the role of the $U_8$ sequence is in the initial binding of ribosomes to the translation initiation region of the message. These data demonstrate that sequences other than those previously recognized can be essential for translation initiation.**

Selection of the correct initiation codon by 30S ribosomal subunits is a primary determinant of accurate translation of mRNA in prokaryotic cells. However, despite extensive study, it is still not understood how this sequence of three nucleotides is distinguished from other residues in a message (1, 2). Factors known to influence the efficiency of binding of ribosomes to the translation initiation region of a mRNA include the initiation codon itself, a run of three to seven nucleotides upstream of the initiation codon termed the Shine–Dalgarno sequence, the spacing between these two segments, and the secondary structure of the initiation region (3, 4). However, statistical analyses (5) and isolation of functional translation initiation regions (6) have shown that other sequences in these regions are nonrandom and may also play a role in the initiation process. Several possibilities for sequences upstream of the Shine–Dalgarno region that might function as translational enhancers have been proposed (7, 8), although their significance is unclear. In addition to the RNA–RNA interactions between mRNA and ribosomes, it is likely that ribosomal proteins also participate in the selection of translation start sites, and protein S1 of the 30S subunit, in particular, has been implicated in this process (9). Based on UV crosslinking of protein S1 to phage and bacterial messages *in vitro*, it was suggested recently that pyrimidine-rich regions upstream of the Shine–Dalgarno sequence might interact with protein S1 and serve as ribosome recognition sites (10).

During the course of studying the *Escherichia coli rnd* gene, which encodes the tRNA-processing enzyme RNase D, we identified (11, 12) a potential stem–loop structure followed by eight uridine residues located upstream of the initiator UUG codon and the Shine–Dalgarno sequence in the mRNA (Fig. 1). Although such a structure has features of a transcription terminator, when the stem–loop and uridine residues were deleted neither the *rnd* mRNA level nor the transcription start site was altered, but RNase D expression was eliminated almost completely (12). This result persisted even when the poor initiation codon UUG was converted to the 10-fold more efficient codon AUG (13). The effect of these alterations was shown not to be on mRNA processing or stability or on termination of an upstream gene. In addition, computer analysis using the FOLD program suggested that no inhibitory secondary structures that might block the Shine–Dalgarno or initiation codon were generated (12, 13). From these findings we concluded that all or part of the stem–loop plus $U_8$ region serves as a positive effector for translation of the *rnd* gene (12, 13).

To examine this region in more detail and to specifically determine whether the $U_8$ sequence participates in the translational enhancement observed, we have changed residues within this stretch by site-directed mutagenesis and examined the effect of these alterations on RNase D expression. Here we show directly that the sequence of uridine residues is essential for expression of the *rnd* gene *in vivo* and for binding of its mRNA to ribosomes *in vitro*. These findings demonstrate conclusively that regions other than the Shine–Dalgarno sequence can be important for translation of prokaryotic mRNA.

## MATERIALS AND METHODS

**Bacterial Strains, Plasmids, and Phages.** The *E. coli* strains UT481, 18-11, and CJ236 were described previously (12). They were used for transformation, RNase D assays, and site-directed mutagenesis, respectively. Plasmids pUC18 and pOU61 were used for multicopy and single-copy expression, as described (12). Phages M13mp18 and M13mp19 were used for DNA sequencing. pBluescript SK for transcript synthesis was obtained from Stratagene. Cells were routinely grown in YT medium with ampicillin (50 μg/ml), as needed. Growth was generally at 37°C except for cells harboring pOU61, which were grown at 30°C to maintain the plasmid in single copy.

**Materials.** [$\alpha$-$^{32}$P]dATP, [$\gamma$-$^{32}$P]ATP, and [$\alpha$-$^{32}$P]UTP were purchased from NEN. Restriction enzymes, polynucleotide kinase, and DNA ligase were obtained from New England Biolabs. Phage T7 DNA polymerase (Sequenase) was from United States Biochemical, and phage T3 RNA polymerase was from BRL. Avian myeloblastosis virus reverse transcriptase and $tRNA^{fMet}$ were purchased from Boehringer Mannheim. Oligodeoxyribonucleotides for probe labeling were products of Pharmacia LKB, and oligodeoxyribonucleotides for site-directed mutagenesis and primer extension were prepared on a Cyclone DNA synthesizer. Phosphodiesterase-treated [$^{32}$P]tRNA for RNase D assays was prepared as described (14). Total RNA from strain 18-11 transformed with pUC18 containing the wild-type or mutant *rnd* gene was isolated as described (12).

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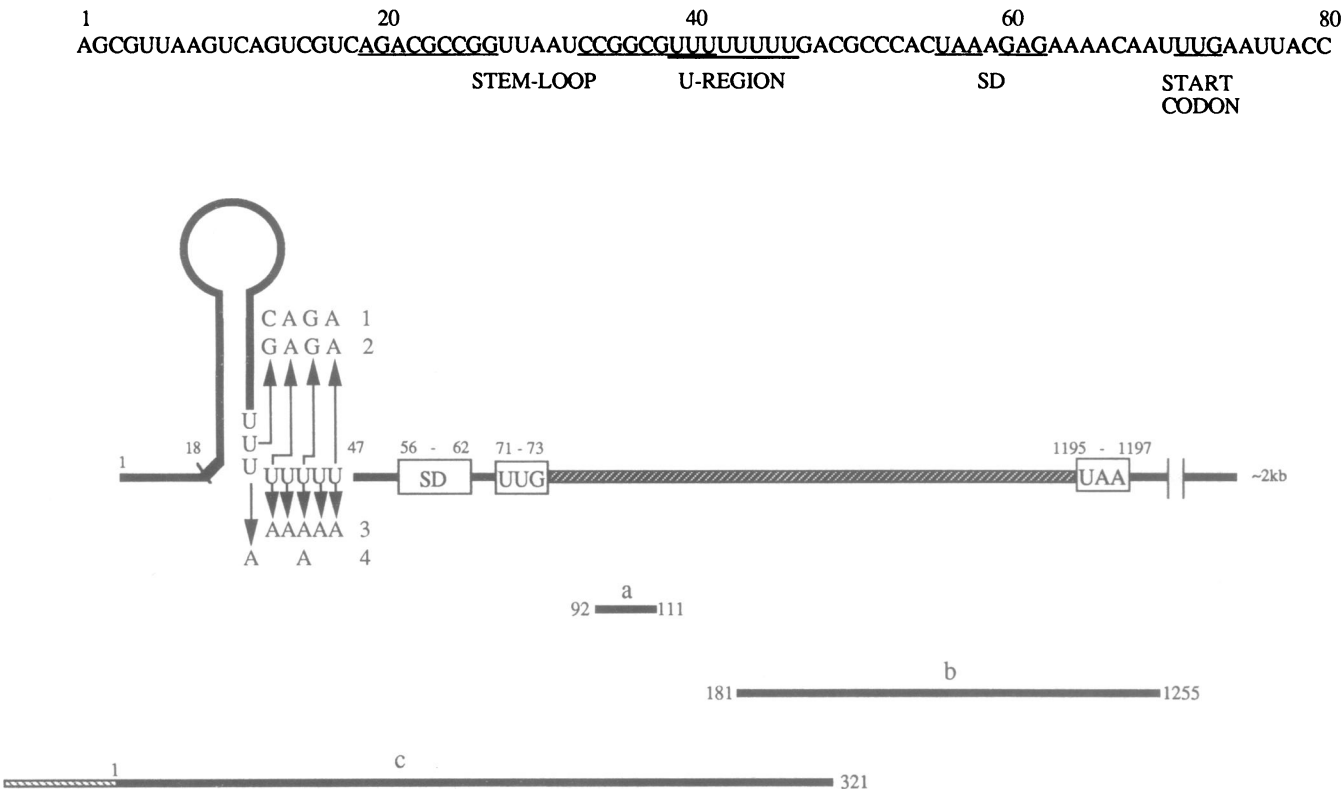


FIG. 1. Relevant sequence and proposed structure of *rnd* mRNA. The stem–loop is followed by the $U_8$ sequence, the Shine–Dalgarno region (SD), and the UUG initiation codon. The mutated residues in the $U_8$ sequence are indicated by arrows to their new identities, and the mutant number is shown to the right of the mutant sequence. Also noted are the locations of the DNA primer used for primer extension (a), the DNA probes used for Northern analysis and dot hybridization (b), and the *in vitro* transcripts used for ribosome binding (c). kb, Kilobases.

**Site-Directed Mutagenesis.** Mutations in the $U_8$ sequence were constructed by oligonucleotide-directed mutagenesis (15). Complementary primers of 36 or 37 nucleotides with two to five alterations, as shown in Fig. 1, were used for second-strand synthesis. All mutant sequences were confirmed by dideoxy sequencing.

**Ribosome Binding Assay.** Wild-type and mutant *rnd* genes on 1.4-kb fragments were inserted into pBluescript SK (Stratagene) and purified on CsCl/ethidium bromide gradients. After linearization with *Bgl* II, RNAs were synthesized with T3 RNA polymerase and [$\alpha$-$^{32}$P]UTP and purified by electrophoresis in 6% polyacrylamide/8 M urea gels. Small (30S) ribosomal subunits were isolated from strain 18-11 according to Spedding (16). Transcripts and ribosomes were quantitated by absorbance at 260 nm. The 30S ribosomes and $^{32}$P-labeled transcripts were incubated and assayed for binding as described in the legend to Fig. 3.

**Other Assays.** RNase D activity was measured by hydrolysis of phosphodiesterase-treated [$^{32}$P]tRNA (17). RNase D protein was determined by Western blotting (18). Quantitation of *rnd* mRNA by dot blot hybridization was as described (12), using DNA probe b (see Fig. 1); the amount of *bla* mRNA in each preparation was used as an internal control to normalize mRNA levels.

## RESULTS

**RNase D Activity of *rnd* Mutant Clones.** A series of *rnd* mutant genes was constructed by site-directed mutagenesis. The mutants included two in which alternate uridine residues were changed (mutants 1 and 2), one in which the last five uridine residues were converted to adenosine residues (mutant 3), and one in which the third and sixth uridine residues were changed to adenosine residues (mutant 4) (see Fig. 1 and Table 1). We believe that nucleotide changes are preferable to deletion of the U-rich sequence for these studies because they maintain the original spacing relationships present in the wild-type message. Moreover, computer analysis of the mutant sequences (19, 20) indicated little or no effect on the stability of the stem–loop structure.

As shown in Table 1, modification of the $U_8$ sequence in the various mutants had a profound effect on RNase D activity when expressed from either the multicopy plasmid, pUC18, or the single-copy plasmid, pOU61. Thus, in mutants 1–3, in which four or five of the eight U residues were altered, <20% of the wild-type RNase D activity was found in the multicopy situation, and <10% with the single-copy plasmid (after

Table 1. RNase D activity of wild-type and mutant clones

| *rnd* gene on plasmid | RNase D activity, % | |
|---|---|---|
| | Multicopy | Single copy |
| Wild type (UUUUUUUU) | 100 | 100 |
| Mutant 1 (UCUAUGUA) | 25 | 16 |
| Mutant 2 (UGUAUGUA) | 15 | 9 |
| Mutant 3 (UUUAAAAA) | 18 | 10 |
| Mutant 4 (UUAUUAUU) | 60 | 30 |
| Vector alone | 5 | 8 |

Residues within the $U_8$ sequence were changed by oligonucleotide-directed mutagenesis. The sequence of each mutant was verified by dideoxy sequencing and is shown in parentheses. Extracts for RNase D assay were prepared by sonication of transformed strain 18-11 cells (RNase I$^-$, II$^-$, D$^-$, BN$^-$, T$^-$) that had been grown in YT medium with ampicillin (50 $\mu$g/ml) to 1 $OD_{550}$ unit, as described (17). The substrate for assay was phosphodiesterase-treated [$^{32}$P]tRNA (14), and the assay measured release of acid-soluble radioactivity. Data are expressed as specific activities relative to the wild-type gene in each vector [pUC18 (multicopy) or pOU61 (single copy)], which has been set at 100. Earlier work (17) showed that too high an RNase D activity is deleterious to *E. coli*. Consequently, the single-copy value is a more accurate measure of RNase D activity, whereas the multicopy value is more sensitive for low levels of activity.

subtraction of activity with the vector alone). When only two of the eight uridine residues were altered (mutant 4), the level of RNase D activity was somewhat higher, but it was still decreased from that of wild-type with both the multicopy and the single-copy plasmid. These data demonstrate the importance of the $U_8$ sequence for the full expression of the *rnd* gene, and they indicate that even a small change from the wild-type sequence, such as from eight to six uridine residues, can have a major effect.

Since all of the mutants behaved similarly, one of them, mutant 1, was chosen to analyze the decreased RNase D expression in more detail. RNase D protein was barely detectable by Western blotting of extracts from a strain containing pUC18 carrying the mutant gene, whereas a strong signal was observed with the wild-type gene under the same conditions (data not shown). These results indicate that the decreased RNase D activity was a consequence of the synthesis of less RNase D protein.

**Analysis of *rnd* mRNA.** To determine whether the decreased RNase D activity was a consequence of a transcriptional or a translational defect, the amount of *rnd* mRNA in cells containing mutant 1 was measured. There was no change in the level of *rnd* mRNA in the mutant compared with wild type, as determined by quantitative dot blots (96–118% of wild-type level in two experiments). These findings are in complete agreement with those previously observed upon deletion of the stem–loop plus $U_8$ region (12), and they indicate that the U-rich sequence does not affect the level of transcription of the *rnd* gene, although it is essential for RNase D expression.

To analyze the mutant *rnd* mRNA in more detail and to eliminate the possibility that the decreased synthesis of RNase D might be due to some change in mRNA structure, we carried out both primer extension and Northern blot analysis of the mutant and wild-type mRNA preparations. The two mRNAs gave identical extension products (Fig. 2*A*) demonstrating that they have the same 5′ terminus, corresponding to the previously reported transcription start site at residue A99 (12). The similar intensities of the extension products confirm the data above indicating that the wild-type and mutant *rnd* mRNAs are present at nearly equal levels. The additional, shorter extension product seen with both mRNAs corresponds to the beginning of the proposed stem–loop structure and indicates that this structure does exist in mRNA under the conditions of the extension reaction.

Northern analysis showed that the wild-type and mutant *rnd* mRNAs were the same size, ≈2 kb (Fig. 2*B*). Inasmuch as the *rnd* insert in the plasmid is only about 1.4 kb, the transcription termination site for these mRNAs must reside in the vector. The various data on the mutant and wild-type *rnd* mRNAs (quantitative dot blots, primer extension, and Northern blotting) all indicate that the mutant mRNA is intact and present at normal levels. Thus, the low level of RNase D in the mutant clones is apparently due to the inability of these mutant mRNAs with alterations in their $U_8$ sequences to be translated efficiently.

**Binding of *rnd* mRNA to Ribosomes.** To examine further the translational defect of the mutant *rnd* mRNA, wild-type and mutant *rnd* transcripts were synthesized *in vitro* with phage T3 RNA polymerase. The transcripts encompassed the first 420 nucleotides of the wild-type and mutant *rnd* clones and were shown to have the correct 5′ terminus by primer extension analysis. Radioactive transcripts were prepared using [$^{32}$P]UTP in the *in vitro* transcription reactions. Gel electrophoresis showed that the two radioactive transcripts had the same size, ≈420 nucleotides, and were each at least 90% pure (data not shown). Thus, the only known difference between the two transcripts was the alteration of four of the eight uridine residues within the mutant $U_8$ sequence.

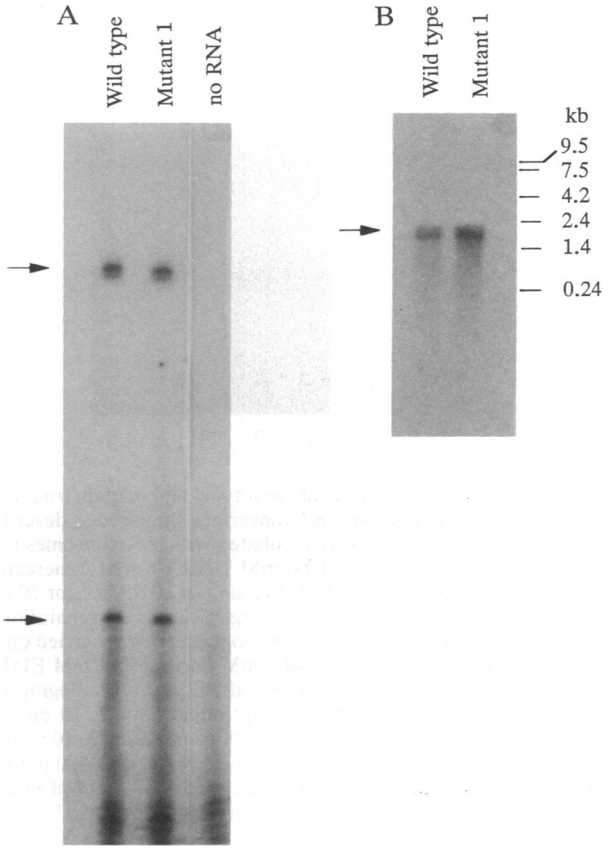


FIG. 2. Comparison of wild-type and mutant *rnd* mRNA by primer extension and Northern analysis. (*A*) Primer extension analysis was carried out (21) with 30 μg of total RNA isolated as described (12) and a $^{32}$P-labeled 20-mer DNA primer (see Fig. 1). (*B*) For Northern hybridization, 10 μg of RNA was denatured with glyoxal and dimethyl sulfoxide, fractionated according to size by agarose gel electrophoresis, transferred to nitrocellulose membranes, and hybridized with a $^{32}$P-labeled DNA probe (see Fig. 1) as described (22).

The functionality of the wild-type and mutant *rnd* transcripts was compared in an *in vitro* ribosome binding assay (Fig. 3). In the presence of *E. coli* 30S ribosomal subunits and tRNA$^{fMet}$, radioactive wild-type *rnd* transcript bound to the ribosome and consequently its migration was retarded on agarose gel electrophoresis (compare lane 2 with lane 1). This binding was completely abolished by a 50-fold excess of the unlabeled transcript (lane 3). In contrast to the binding of wild-type transcript, the mutant transcript was unable to bind to 30S subunits under these conditions (compare lane 5 with lane 2).

The relative binding affinity of the two transcripts was defined in more detail by varying the concentration of transcript added. Increasing the amount of wild-type transcript 4-fold in equal increments (Fig. 4, lanes 1–4) led to a corresponding linear increase in the amount of RNA in the retarded band. With the mutant transcript (lanes 5–8) a small amount of binding was observed only at the highest concentration of transcript (lane 8). We estimate from these data that the mutant transcript binds to 30S ribosomal subunits about 1/10th as strongly as the wild-type transcript. These data support the *in vivo* finding that the $U_8$ sequence plays an important role in translation of *rnd* mRNA and suggest that its function is in the initial binding of ribosomes to the translation initiation region.

## DISCUSSION

The data presented here provide direct evidence that regions other than the Shine–Dalgarno sequence can be important for

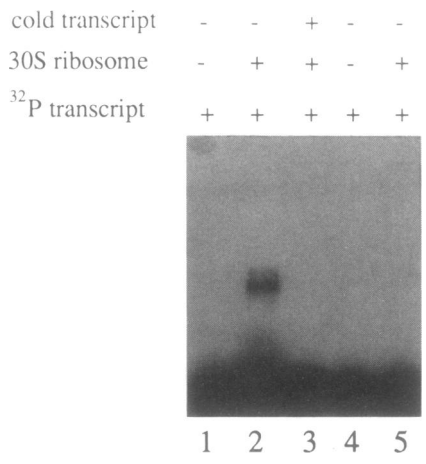


FIG. 3. Ribosome binding of wild-type and mutant *rnd* transcripts. Wild-type and mutant *rnd* transcripts, prepared as described in *Materials and Methods*, were incubated with 30S ribosomes in 10 μl of 10 mM Tris·OAc, pH 7.4/60 mM $NH_4Cl$/6 mM 2-mercaptoethanol/10 mM Mg $(OAc)_2$ with 1 $A_{260}$ unit of $tRNA^{fMet}$ for 20 min at 37°C. Samples were applied to 1.4% agarose gels for separation of free and bound RNA transcripts. Electrophoresis was carried out at 4°C in 0.5× TBE (44.5 mM Tris/44.5 mM boric acid/1 mM EDTA, pH 8). After electrophoresis, the gel was dried and autoradiographed. Lane 1, 10 pmol of wild-type transcript alone; lane 2, 10 pmol of wild-type transcript plus 15 pmol of 30S ribosome; lane 3, 10 pmol of wild-type transcript plus 15 pmol of 30S ribosome plus 500 pmol of unlabeled ("cold") wild-type transcript; lane 4, 10 pmol of mutant transcript alone; lane 5, 10 pmol of mutant transcript plus 15 pmol of 30S ribosome.

translation initiation in prokaryotes (1–8). In the situation described here, an intact sequence of uridine residues is essential for translation *in vivo* and for ribosome binding *in vitro* even though a Shine–Dalgarno sequence is present. Preliminary results indicate that for this mRNA the Shine–Dalgarno sequence is necessary as well (unpublished observations). Since we have previously shown that conversion of the UUG initiation codon to AUG stimulates RNase D synthesis >10-fold (12), these findings indicate that at least three distinct sequences in the translation initiation region of *rnd* mRNA are necessary for its efficient translation.

The fact that the essential region upstream of the Shine–Dalgarno sequence is U-rich melds these data with earlier work indicating that protein S1 favors interaction with pyrimidine-rich sequences (9, 10). Taken together, these findings suggest that the affinity of protein S1 for U-rich sequences plays an important role in the interaction of *rnd* mRNA with the 30S ribosome. What is most surprising is that alteration of as few as two of the eight uridine residues can have profound effects on translation. Further work will be necessary to determine exactly how protein S1 may interact with this sequence. It is not clear what function is served by the stem–loop structure. Earlier work showed that mutations that disrupted the stem also could decrease translation (13), but it remains to be determined whether the stem–loop has a direct role in interacting with ribosomes or functions to keep the U-rich sequence exposed.

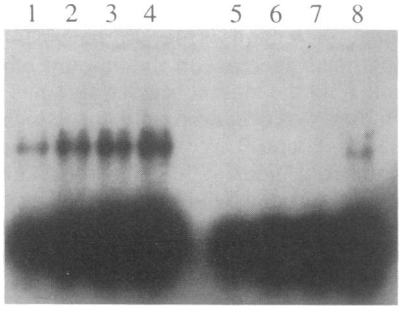


FIG. 4. Effect of transcript concentration on binding to 30S ribosomes. Ribosome binding was carried out as described in Fig. 3 except that the amounts of wild-type transcript (lanes 1–4) and mutant transcript (lanes 5–8) were varied (8, 16, 24, and 32 pmol).

It is likely that the importance of a U-rich region for translation is not restricted to the *rnd* gene. Boni *et al.* (10) have compiled a list of other *E. coli* genes that contain U-rich sequences in this region of their mRNA, and it will be interesting to see whether alteration of these sequences in other genes will also have major effects on their translation. On the other hand, it is also likely that a U-rich sequence is not an essential determinant for expression of all genes, inasmuch as many of them lack such a sequence. Rather, a U-rich region may only become necessary for mRNAs that contain weaker initiation codons or weaker Shine–Dalgarno sequences. In these cases, stable binding between ribosomes and the translation initiation region of mRNA may require additional sites for RNA–RNA or RNA–protein interaction.

One additional feature of this system that remains to be explored is the stability of the mutant *rnd* mRNAs. As shown here, and with other mutations in the upstream region of *rnd* mRNA (12, 13), the mutant messages are present at wild-type levels despite the fact that they are being translated very poorly. For many messages, interference with translation can have a destabilizing effect, although this is not a universal finding (23). Studies of the means by which mutant *rnd* mRNAs are stabilized may provide useful information about mechanisms of mRNA decay.

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