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# Differential degradation of the *Escherichia coli* polynucleotide phosphorylase mRNA

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## ABSTRACT

The transcript covering *pnp*, the gene encoding polynucleotide phosphorylase, is processed by RNaseIII at the 5'-upstream site of the *pnp* gene. In the RNaseIII-deficient strain, three species of the unprocessed transcript with different lengths could be detected. In this study, the stability of each transcript was analyzed by SI nuclease protection assay. The results show that the half-lives of the unprocessed transcripts are 8 min, whereas the half-life of the processed transcript is 1.5 min. It is also shown that the 5' segment of the unprocessed transcripts is more stable than the middle or the 3' segment.

## INTRODUCTION

In prokaryotes, the stability of the messenger RNA is one of the important factors which determine the level of gene expression. For example, the stability of different mRNA species in *E.coli* varies at least by a factor of 50(1) and the half-lives of some mRNA species are growth rate-dependent(2). The differential expression of genes within an operon encoding a polycistronic transcript can be accomplished by the difference in mRNA stability(3,4,5,6). The difference in mRNA stability in turn could be accounted for by the difference in its susceptibility to degradation by cellular endonucleases and 3'-exonucleases. Although the presence of the structural determinants for the mRNA stability, located near the 5' end or the 3' end of the transcripts, has been reported in several operons, the basic mechanism which determines the susceptibility of the mRNA to the degradative enzymes is still unclear(for a review, see 7).

The gene for polynucleotide phosphorylase, *pnp*, is located at 69 min on *E.coli* chromosome together with *rpsO*, the gene for ribosomal protein

 

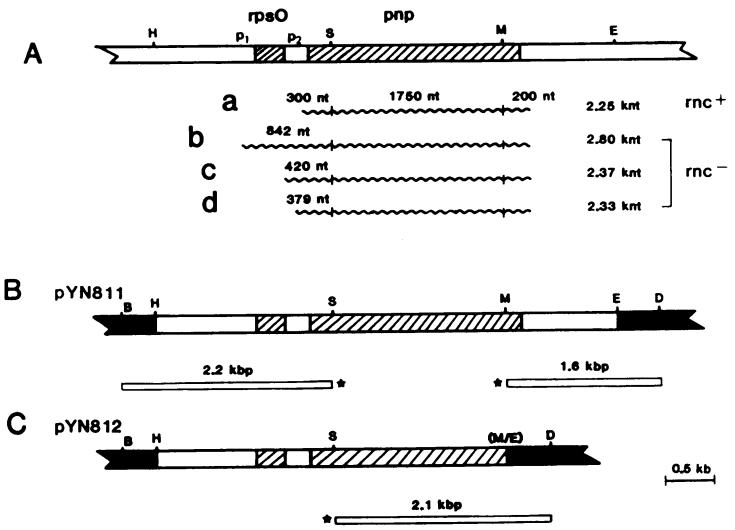


Figure 1. Structural organization of the *rpsO-pnp* operon and the DNA probes used in the S1 nuclease analysis. Panel A: The genes for *rpsO* and *pnp* are shown by the shaded line. Two promotors, $p_1$ and $p_2$, and the sites for the restriction enzymes are indicated: H, *Hind*III; S, *Sma*I; M, *Mlu*I; E, *Eco*RI; B, *Bam*HI; D, *Dra*I. Below the map, the transcripts covering *pnp* are presented; the transcript processed by RNaseIII(a), the transcript initiated at $p_1$(b), the transcript initiated at $p_1$ and cleaved by an unknown endonuclease other than RNaseIII(c) and the transcript initiated at $p_2$(d). The length of the transcripts and the segment of the transcript is shown in nucleotides(nt) or kilonucleotides(knt). The *rnc*$^+$ and the *rnc*$^-$ strains used are indicated on the right. These figures are due to the published results(10,11,12). Panel B: The structure of the plasmid pYN811 and the DNA probes derived from it. These probes are 5'-end-labeled *Bam*HI-*Sma*I fragment and 3'-end-labeled *Mlu*I-*Dra*I fragment. Labeled end is indicated by an asterisk. Panel C: The plasmid pYN812 and 3'-end-labeled *Sma*I-*Dra*I DNA fragment derived from it.

S15. Transcription of the *pnp* gene is initiated at two promoters, $p_1$ and $p_2$, located upstream of *rpsO* and between *rpsO* and *pnp*(8,9, Fig.1A). A portion of the transcripts initiated at $p_1$ is cleaved endonucleolytically downstream of *rpsO* by an unknown endonuclease. All such transcripts are processed by RNaseIII at the site upstream of *pnp* resulting in the synthesis of homogenous *pnp* mRNA.

In the RNaseIII-deficient(*rnc*$^-$) strain, three species of unprocessed transcripts with different 5'-end structure could be detected : one that covers *rpsO* and *pnp*, and two others that cover only *pnp*(10,11,12). A striking effect of the absence of RNaseIII is the significant increase in the amount of *pnp* mRNA up to 3-fold in the cells grown in M9 medium(11) and 11-fold in cells grown in LB medium(12). Analysis of the half-life of the processed and unprocessed transcripts shows that the increase in the amount of mRNA is mainly due to the stabilization of the transcript.

In the experiments mentioned above, the stability of the transcripts was analyzed with a limited segment of *pnp* mRNA and the results obtained show an average stability of the heterogenous transcripts. In order to get more information on the role of RNA structure in determining the stability of the *pnp* mRNA, a decision was made to investigate the decay rate of the entire region of each transcript by S1 nuclease analysis. The results show that the half-life of the unprocessed transcripts is increased 5-fold as compared to that of the processed transcript and there is a significant difference in the stability of the segments in the unprocessed transcripts.

## MATERIALS AND METHODS

### Bacterial strains and plasmids

*E.coli* RNaseIII-deficient strain N2077(*rnc105 thi argH nadB lacY gal malA* $\lambda^r$ $\lambda^-$ *xyl ara mtl* $str^r$ *tonA supE*) and its isogenic parent strain N2076(*rnc*$^+$) were gifts from Dr. D. Apirion(13). Plasmid pYN81($Ap^r$ *pnp*$^+$) was supplied by Dr. Y. Nakamura and it contains the *pnp* gene as a part of a 16 kilo-base pairs(kbp) *Eco*RI fragment of *E.coli* DNA cloned into *Eco*RI site of pBR322(14). *Eco*RI-*Hind*III fragment of 4.7 kbp, which includes *pnp*, was isolated from pYN81 and cloned into *Eco*RI-*Hind*III site of pBR322(pYN811, Fig.1B). Plasmid pYN811 was then digested with *Mlu*I and *Eco*RI and ligated as described(15, pYN812, Fig.1C). These two plasmids, pYN811 and pYN812, were used as a source of DNA fragment for preparation of the end-labeled probes.

### Preparation of the end-labeled probes

Plasmid DNA and DNA fragments were isolated as described(15). Restriction endonucleases and other enzymes were purchased from Takara Shuzo Co., Japan. DNA restriction fragments were 5'-end-labeled with polynucleotide kinase or 3'-end-labeled with Klenow fragment of *E.coli* DNA polymerase I as described(15). Then these $^{32}$p-labeled

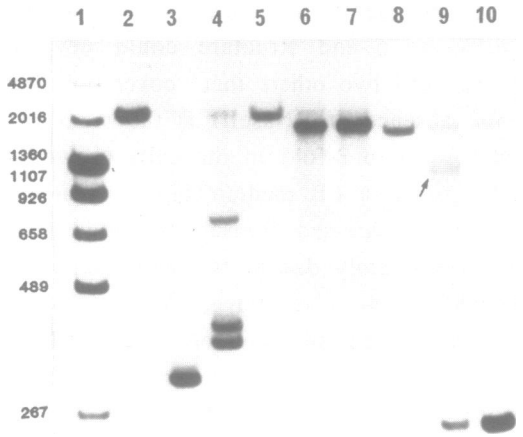


Figure 2. S1 nuclease analysis of the *pnp* transcripts in the *rnc*+ and the *rnc*- strains. The end-labeled DNA probes were hybridized with the total cellular RNA and subjected to S1 nuclease digestion. Lanes 2,3,4; experiments with the 5'-end-labeled *Bam*HI-*Sma*I probe. Lanes 5,6,7; experiments with the 3'-end-labeled *Sma*I-*Dra*I probe. Lanes 8,9,10; experiments with the 3'-end-labeled *Mlu*I-*Dra*I probe. Lanes 2,5,8; DNA probe only. Lanes 3,6,9; hybrid with *rnc*+ RNA. Lanes 4,7,10; hybrids with *rnc*- RNA. Lane 1; size marker($^{32}$p-labeled *Hind*III-*Hae*III fragments of pHYPLK purchased from Takara Shuzo Co.). The faint band is indicated by the arrow in lane 9.

fragments were digested with the second restriction nuclease to prepare the DNA probes which was labeled at one 5' or 3' end. These probes are 5'-end-labeled *Bam*HI-*Sma*I fragment of 2.2 kbp, 3'-end-labeled *Sma*I-*Dra*I fragment of 2.1 kbp and 3'-end-labeled *Mlu*I-*Dra*I fragment of 1.6 kbp(Fig.1B,C). The entire region of the *pnp* transcript is covered by these three probes.

S1 nuclease protection assay

S1 nuclease assays were performed essentially according to Maniatis et al.(15). Total *E.coli* RNA was prepared from mid-log cultute of bacteria in LB medium by the hot phenol method of Salser et al.(16). In RNA-DNA hybridization reaction, 40 μg of RNA was mixed with 0.05-0.2 μg of end-labeled DNA probe in 30 μl of the hybridization buffer. After denatured at 72 °C, the reaction mixture was cooled gradually to 37 °C. S1 nuclease digestion(300 units per ml) was carried out for 40 min at 37 °C. After nuclease treatment, the hybrids were electrophoretically

separated on 5 % polyacrylamide gels(15). Autoradiographs were quantitated by densitometric analysis as previously described(11). The assay of RNA degradation was carried out as described(11). Rifampicin(Sigma) was added(0.5 mg per ml) to the cell culture and samples were withdrawn at 2 min intervals for RNA extraction.

## RESULTS

### Identification of the *pnp* transcript in the *rnc*$^+$ and *rnc*$^-$ strains by S1 nuclease analysis

For identification of the *pnp* mRNA, three species of end-labeled DNA probes were prepared for S1 nuclease assay as described in Materials and Methods(Fig.1B,C). These probes cover the 5', the middle and the 3' segments of the transcript and they include, in all, the entire region of *pnp* mRNA. Probes were hybridized with RNA prepared from the *rnc*$^+$ or the *rnc*$^-$ strain(*rnc*$^+$ or *rnc*$^-$ RNA) and subjected to S1 nuclease digestion. Hybridization of the 5'-end-labeled *Sma*I-*Bam*HI probe with *rnc*$^+$ RNA resulted in the hybrid band of 310 base pairs(bp)(Fig.2, lane 3), which corresponded to the 5' segment upstream from *Sma*I site of the processed transcript. Three bands with the length of 800 bp, 400 bp and 360 bp could be observed when the same probe was hybridized with *rnc*$^-$ RNA(Fig.2, lane 4), which corresponded to 5' segments of the unprocessed transcripts. The relative amounts of these bands were estimated by densitometry as 20%, 35% and 45% respectively. When the 3'-end-labeled *Sma*I-*Dra*I fragment was used, the hybrid band of 1.8 kbp could be seen in hybridization with both *rnc*$^+$ and *rnc*$^-$ RNA(Fig.2, lanes 6,7). This band corresponded to the middle segment from *Sma*I to *Mlu*I site of the processed or unprocessed transcript. No other hybrid band could be observed on the autoradiograph suggesting that neither attenuation nor processing occured within this middle region of the transcript. Hybridization of the 3'-end-labeled *Mlu*I-*Dra*I fragment with *rnc*$^+$ and *rnc*$^-$ RNA gave 220 bp band(Fig.2, lanes 9,10), which corresponded to the 3' segment of the processed and unprocessd transcripts. In addition to the 220 bp band, a faint band with the length of 1.1 kbp could be seen(indicated by the arrow). This band would show the read-through product from the operon as reported before(17). The amount of the band was estimated as 30% of the total transcripts in the *rnc*$^+$ RNA. Considering that the lengths of the hybrid band observed in these experiments represented approximate values determined by comparison

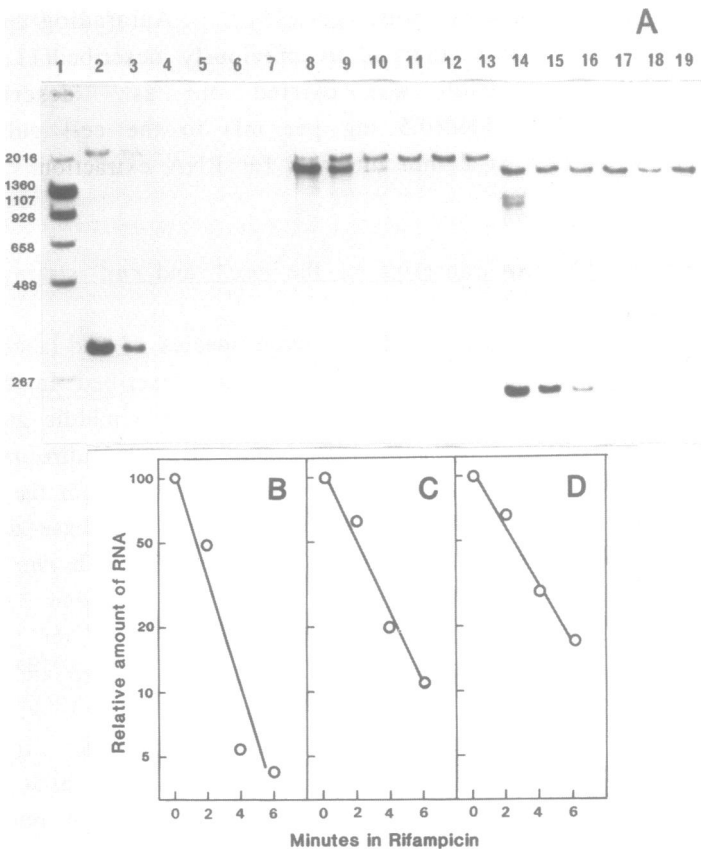


Figure 3. Decay of the *pnp* transcript in the *rnc*$^+$ cells. Panel A: S1 nuclease analysis of the *pnp* transcript. Lanes 2-7; analysis of RNA prepared from the *rnc*$^+$ cells incubated in Rifampicin for 0, 2, 4, 6, 8 and 10 min respectively with the 5'-end-labeled *Bam*HI-*Sma*I probe. Lanes 8-13; similar analysis with the 3'-end-labeled *Sma*I-*Dra*I probe. Lanes 14-19; similar analysis with the 3'-end-labeled *Mlu*I-*Dra*I probe. Lane 1; size marker as described in Fig.1. Panel B: Decay of the 5' segment hybridized with *Bam*HI-*Sma*I probe. The relative amount of RNA was obtained by densitometry of the hybrid bands and expressed as the percentage of RNA hybridized at zero time. Panel C: Decay of the middle segment hybridized with *Sma*I-*Dra*I probe. Panel D : Decay of the 3' segment hybridized with *Mlu*I-*Dra*I probe. In this experiment, as well as those described below, the RNA-DNA hyrbidization was performed under condition of DNA excess and the amount of the hybrid was within the range of linear relation to the amount of RNA added(data not shown).

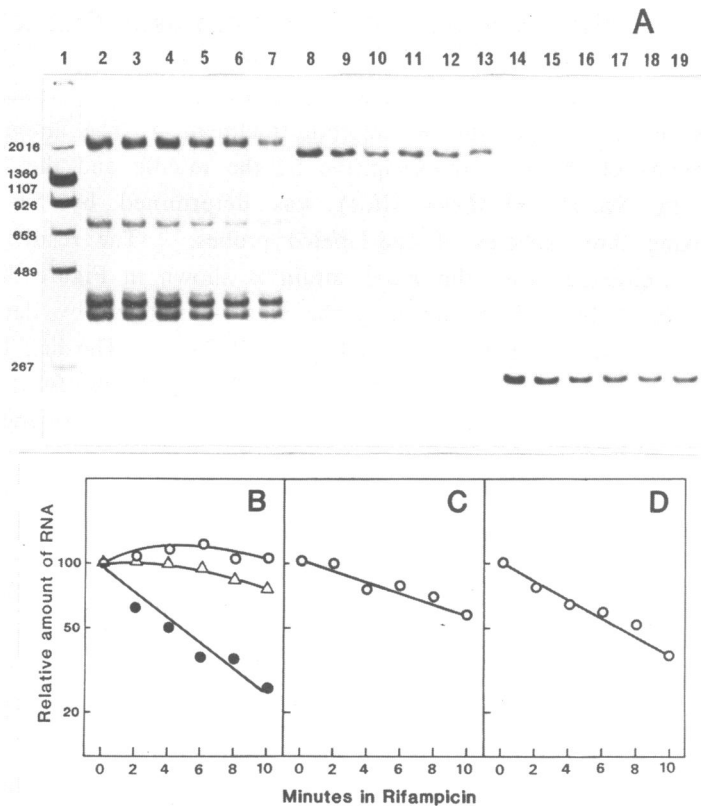


Figure 4. Decay of the *pnp* transcripts in the *rnc*⁻ cells. Panel A: S1 nuclease analysis of the *pnp* transcript. Lanes 2-7; analysis of RNA prepared from the *rnc*⁻ cells incubated in Rifampicin for 0, 2, 4, 6, 8 and 10 min respectively with the 5'-end-labeled *Bam*HI-*Sma*I probe. Lanes 8-13; similar analysis with the 3'-end-labeled *Sma*I-*Dra*I probe. Lanes 14-19; similar analysis with the 3'-end-labeled *Mlu*I-*Dra*I probe. Lane 1; size markers as described in Fig.2. Panel B: Decay of the 5' segments. Symbols; filled circle, 800 nt segment; open circle, 400 nt segment; triangle, 360 nt segment. Panel C: Decay of the middle segment. Panel D: Decay of the 3' segment.

with the reference markers, the size of each band correlated well with that predicted from the published sequence(9,10,17, see Fig.1A). Thus, S1 nuclease assay by using three species of the end-labeled DNA probes described made it possible to estimate the amount of the entire sequence of the *pnp* transcripts with different ends.

Stability of the three segments of the *pnp* transcripts in the *rnc*$^+$ and the *rnc*$^-$ cells

Stability of the *pnp* transcript was analyzed by estimating the amount of the transcript after Rifampicin treatment. The amount of the three segments of the *pnp* transcript(the 5', the middle and the 3' regions separated by *Sma*I and *Mlu*I sites) was determined by S1 nuclease analysis using three species of end-labeled probes. The results obtained with RNA extracted from the *rnc*$^+$ strain is shown in Figure 3A. The amount of RNA hybridized to the probe was quantitated by densitometry of the hybrid band and it is shown in Figure 3B,C,D. The half-life of the three segments was estimated as 1.5 min for the 5', 2 min for the middle and 2.3 min for the 3' segment. The half-life of the 3' segment in the read-through transcript was 1.5 min(data not shown). Thus, each segment of the processed transcript shows approximately the same stability. Assuming that the sequence within the transcript, which shows the most rapid decay rate, determine the stability of the full-length transcript, we conclude that the half-life of the processed *pnp* transcript with the length of 2.25 kilonucleotides(knt) is about 1.5 min.

The results of the S1 nuclease analysis with *rnc*$^-$ RNA are shown in Figure 4. The half-life of the 5' segment with the length of 800 nucleotides(nt) was estimated as 4.7 min, whereas half-lives of the other two 5' segments were more than 30 min(Fig.4B). Since the 2.8 knt transcript, from which the 800 nt segment was derived, was cleaved to generate the 2.37 knt transcript, the half-life of 4.7 min probably reflected the rate of cleavage at the position upstream of $p_2$(see Fig.1A). It should be noted that this cleavage was mediated by an unknown endonuclease other than RNaseIII(9). Such a speculation is in good agreement with the observation that the amount of the 400 nt segment showed a slight increase after Rifampicin treatment(Fig.4B). The half-lives of the middle and the 3' segment were estimated as 13 and 8 min respectively(Fig.4C,D). The result showed a greatly enhanced stability for the 5' segments of the unprocessed transcripts with the length of 2.37 knt and 2.33 knt as compared to the middle or the 3' segments. The half-lives of the unprocessed, full-length transcripts with the length of 2.37 knt and 2.33 knt is estimated as 8 min on the assumption described before. These results indicate that the stability of the unprocessed transcrpts increased 5-fold as compared to that of the processed transcript with regard to their half-lives. In the *rnc*$^-$ strain, the

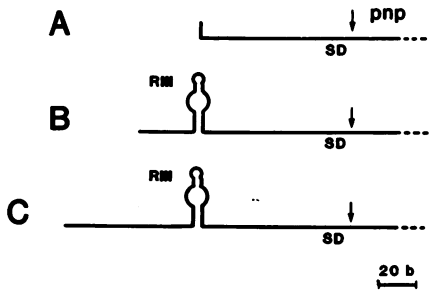


Figure 5. The 5'-end structure of the *pnp* mRNA. A, The transcript processed by RNaseIII with the length of 2.25 knt. B,C, the unprocessed transcripts with the length of 2.33 and 2.37 knt. RIII, RNaseIII processing site. SD, ribosome binding site. The arrow indicates the position of the initiation codon for *pnp*. This figure is based on the published sequence(9,10,11).

synthesis rate of the *pnp* mRNA increased 1.6-fold(12). The combination of these two effects, increased stability and increased synthesis rate, would be sufficient for the 10-fold increase in the amount of polynucleotide phosphorylase in the *rnc*$^-$ strain(9, also confirmed in this experiment).

## DISCUSSION

Two conclusions could be deduced from the present study. One is that the three segments of the processed *pnp* transcript have approximately the same stability, whereas those of the unprocessed transcripts show great difference in their stability. The other is that the stability of the unprocessed, full-length transcripts is greatly increased as compared to that of the processed, full-length transcript. The differential decay rate observed with the three segments of the unprocessed transcript could be most simply explained by a decay mechanism involving a 3'-exonuclease(e.g. RNaseII and/or polynucleotide phosphorylase) that initiates at the 3'-terminus of the intact transcripts. It implies that endonucleolytic cleavage of the unprocessed transcript is suppressed and the transcript is degraded preferentially by the 3'-exonuclease. The decay of the *ompA* transcript also shows a 3'-to-5' directionality(18). On the other hand, all segments of the processed transcript are degraded at approximately the same rate, which suggests

that not only the 3'-exonucleases but also the endonuclease(s) are involved in its degradation.

Three segments of the unprocessed transcripts show an enhanced stability as compared to their counterparts in the processed transcript. The difference in their stability should be explained by the altered sequence and/or structure in these transcripts. As shown in Figure 5, the two unprocessed transcripts have the additional 5'-end sequence of 79 nt and 120 nt, which include the RNaseIII processing site(the longest transcript is supposed to be cleaved to generate the 2.37 knt transcript. see Text). One possibility is that the additional sequence at the 5' end of the transcripts prevents the cleavage by the endonuclease(s). Endonucleolytic cleavage in the 5'- noncoding region in *ompA* is known to facilitates the degradation of the message(19). Absence or decreased rate of endonucleolytic cleavage in the 5' end of the unprocessed *pnp* transcript might lead to the preferential degradation from the 3' end of the intact transcript by 3'-exonucleases, which results, at the same time, in the decreased decay rate in all the segments of the transcript. Stem-loop structure, which is the RNaseIII processing site, might be important in this mechanism. Another possibility is that the 5'-end sequence of the processed transcript prevents ribosome binding to the mRNA. Seven nucleotides at the 5' end of the processed transcript are included in the stem of the RNaseIII processing site(Fig.5A). When processed, this sequence would be liberated and it might interfere with other regions of the transcript resulting in the formation of some stable secondary structure. Such a structure might in turn prevent ribosome binding to the transcript. Previous reports have shown that depriving a transcript of its ribosomes destabilizes the transcript(20,21). Therefore, the *pnp* transcript with decreased amount of loaded ribosome could be more susceptible to endo- or exonucleases. We could not find any sequence near the ribosome binding site that made a stable structure with the 7 nucleotides at the 5' end of the processed transcript, but it is still possible that the freed 5' end of the processed transcript does prevent ribosome binding by a yet unknown mechanism.

The 5'-end sequence of the mRNA is one of the main structural determinants for its stability. Susceptibility of the 5'-end sequence to the endonucleolytic attack determines the decay rate of the mRNA in several operons(for a review, see 7). It is still not proved that the 5' regions of the processed or unprocessed *pnp* transcripts accept

endonucleolytic cleavage, but it seems clear enough that the 5'-end sequence of the transcript plays a key role in determining the stability of the *pnp* mRNA.

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