Role of the sequence of the rne-dependent site in 3′ processing of M1 RNA, the catalytic component of *Escherichia coli* RNase P

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Received 2 August 2001; accepted 13 August 2001

First published online 28 August 2001

Edited by Lev Kisselev

Abstract The 3′ processing of M1 RNA, the catalytic component of *Escherichia coli* RNase P, occurs by two pathways involving multiple steps. The precursor of M1 RNA has an rne-dependent site downstream of the processing site, whose sequence variation affects the processing efficiency. In this study, we showed that the sequence itself of the rne-dependent site possessed the ability to determine the processing pathways and that it also affected the cleavage specificity with the generation of the processing products at one nucleotide upstream or downstream of the normal cleavage sites. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: RNase P; M1 RNA; 3′ Processing; Rne-dependent site; *Escherichia coli*

1. Introduction

M1 RNA is the RNA component of *Escherichia coli* RNase P that endonucleolytically removes 5′ leader sequences from precursors of tRNAs, generating the mature 5′-ends of tRNAs [1,2]. M1 RNA of 377 nucleotides forms by 3′ processing of a major primary transcript of the rnpB gene, precursor M1 RNA (pM1 RNA) of 413 nucleotides [3–8]. Although RNase E is involved in the 3′ processing of M1 RNA [6–8], other enzymes seem to be required for the complete maturation of M1 RNA at the 3′-end [9]. An in vitro analysis of the processing also showed the presence of the +385/+386 RNA intermediates which are further processed to +378/+379 RNA by another processing activity [10]. Since the +378/+379 RNA can be generated in vitro with an endonucleolytic cleavage that is believed to be carried out by RNase E, it was suggested that there are at least two pathways for the 3′ processing of M1 RNA. Finally, the mature 3′-end of +377 should be generated from +378/+379 RNA products in vivo through cleavage of the extra nucleotides by exoribonucleases [9,10].

pM1 RNA has a pentanucleotide of GAUUU, immediately 3′ to the processing site [8]. This pentanucleotide accords with the consensus sequence, [A/G]AUU[A/U], of the the rne-dependent site [11]. The sequence of the rne-dependent site is thought to be recognized and cleaved by RNase E [11], although the primary sequence alone of the rne-dependent site was insufficient to account for the cleavage specificity by RNase E in some cases [12,13]. The rne-dependent site has been reported to be located in a single-stranded region that is rich in U and A nucleotides [14–16]. Although the rne-dependent site of pM1 RNA is the major factor for determining the processing efficiency [8], essentially nothing is known about the mechanism by which its sequence would affect the processing efficiency. This is because 3′ processing of M1 RNA is not a simple process, but a complex one that occurs through two pathways with multiple steps that require many enzymes [10].

In this study, we examined the effect of the sequence of the rne-dependent site on the pathways of 3′ processing of M1 RNA. We showed that the primary sequence itself of the rne-dependent site possessed the ability to determine the processing pathways. Therefore, the sequence of the rne-dependent site seems not only to affect the processing efficiency, but also to guide the RNA metabolic pathway. Our results may provide a mechanistic basis for the role of the rne-dependent site in control of biosynthesis of M1 RNA in *E. coli*.

2. Materials and methods

2.1. Bacterial strains and plasmids

E. coli JM109 [17] was used for the construction of plasmids and the preparation of cell extract. The plasmid vectors used were pGEM3 and pALTER-1 from Promega. Plasmid pMTd23 [8] is a derivative of pGEM3, which contains the rnpB transcription unit with an internal deletion of the sequence between +57 and +330. Plasmid pSPd23 [8] is a derivative of pMTd23, but it carries the SP6 promoter linked to the 5′-end of the M1 RNA-encoding sequence, and a *Dra*I site at position +415. pSP–RNE series [8] are derivatives of pSPd23 carrying mutations at the rne-dependent site; pMT–RNE series [8] are derivatives of pMTd23 carrying the same mutations at the rne-dependent site as the corresponding pSP–RNE series.

2.2. Site-directed mutagenesis

Site-directed mutagenesis was carried out according to the Promega-Altered System protocol to generate five more mutations at the rne-dependent site. The mutations were introduced to generate DNA templates for in vitro transcriptions of RNA having changed sequences between positions +378 and +382 (Fig. 1). Plasmid pSPd23 was used for mutagenesis. Oligonucleotides used for constructing the following mutant plasmids were as follows: pSP-RNE (GCCCU): 5′-GCGGGTTTTTACGTA**GGG**CAGGTGAAACTGACC-3′; pSP-RNE (GCCUU): 5′-CGGGTTTTTACGTAA**GG**CAGGTGAAACTGACC-3′; pSP-RNE (GCUUU): 5′-TTTACGTAAA**G**CAGGTGAAAC-3′; pSP-RNE (GUUUU):5′-TTTACGTAAAA**A**CAGGTGAAAC-3′; pSP-RNE (UUUUU): 5′-GGGTTTTTACGTAAAAA**A**AGGTGAAACTGACCG-3′. The mutated bases are shown in bold. All mutations generated by the mutagenesis were verified by DNA sequencing.

The 129-bp *Eco*RI–*Bam*HI DNA fragments of these pSP-RNE

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PII: S0014-5793(01)02822-8

plasmids were moved to plasmid pMTd23/*Eco*RI–*Bam*HI to yield the corresponding pMT–RNE series which had the rnpB transcription unit.

The DNA templates for transcripts having the 3′-end of +386 were constructed by polymerase chain reaction (PCR) from pSP-RNE plasmids with a pair of primers SP and RSA; SP and RSA were previously described [10]. The PCR products were cloned into the pGEM-Teasy vector (Promega).

2.3. In vitro transcription of substrates

For in vitro transcription of RNA substrates to be used for the in vitro processing assay, plasmid DNAs were cleaved with *Dra*I for the RNA end of +415 of *Rsa*I for that of +386, and used as templates for run-off transcription by SP6 RNA polymerase. In vitro transcription products were purified by gel elution, as described [18].

In vitro transcripts were labeled at the 3′-end with [^{32}P]pCp and T4 RNA ligase, or internally with [α-^{32}P]CTP, as described [18].

2.4. Northern hybridization

Total cellular RNAs from *E. coli* cells containing pMT–RNE series were prepared, as previously described [8]. RNAs were electrophoresed in a 5% polyacrylamide gel containing 7 M urea, electrotransferred to a nylon membrane (Hybond N+; Amersham) with a Hoefer Semi-Phor Semi-dry transfer unit, and then they were hybridized, as described [8]. The probe used was ^{32}P-labeled antisense M1 RNA prepared, as described [8].

2.5. In vitro processing assay

The 40% ammonium sulfate precipitation (ASP-40) was prepared from *E. coli* JM109 and used in vitro processing assay, as described [8]. The yeast total RNA of 0.7 mg/ml was added into the reaction mixture when required. RNA substrates were incubated at 37°C with ASP-40. The reaction products were analyzed by electrophoresis on a polyacrylamide sequencing gel containing 8 M urea.

3. Results and discussion

3.1. Changes of the processing pathway by the sequence variation at the rne-dependent site

Since 3′ processing of M1 RNA occurs through two pathways (Fig. 1; [10]), we determined whether the rne-dependent site would govern the pathway choice or not. We used p23 RNA (Fig. 1B) as a model substrate, a truncated M1 RNA which was generated from the rnpB gene with a deletion in the M1 RNA-structural gene [8,10]. The rne-dependent site mutants were previously constructed with random nucleotides

Table 1
Effects of the sequence variations at the rne-dependent site on the processing of p23 RNA

Sequence	Relative processing efficiency[a]
GAUUU (WT)	1.00
CGCCC	0.13[b]
CCCCA	0.09[b]
GCCAG	0.13[b]
GCCAC	0.14[b]
AUCAU	0.80[b]
UCACU	0.36[b]
UUCGG	0.47[b]
UUCAU	0.54[b]
GCCCU	0.14
GCCUU	0.39
GCUUU	0.79
GUUUU	0.94
UUUUU	1.00

[a]The processing efficiency was calculated as a ratio of the amount of the processed RNA (23 RNA) to that of the total transcripts (p23 RNA+23 RNA). The relative processing efficiency represents the processing efficiency of a derivative relation to that of the wild-type (WT).
[b]The values from Kim et al. [8]

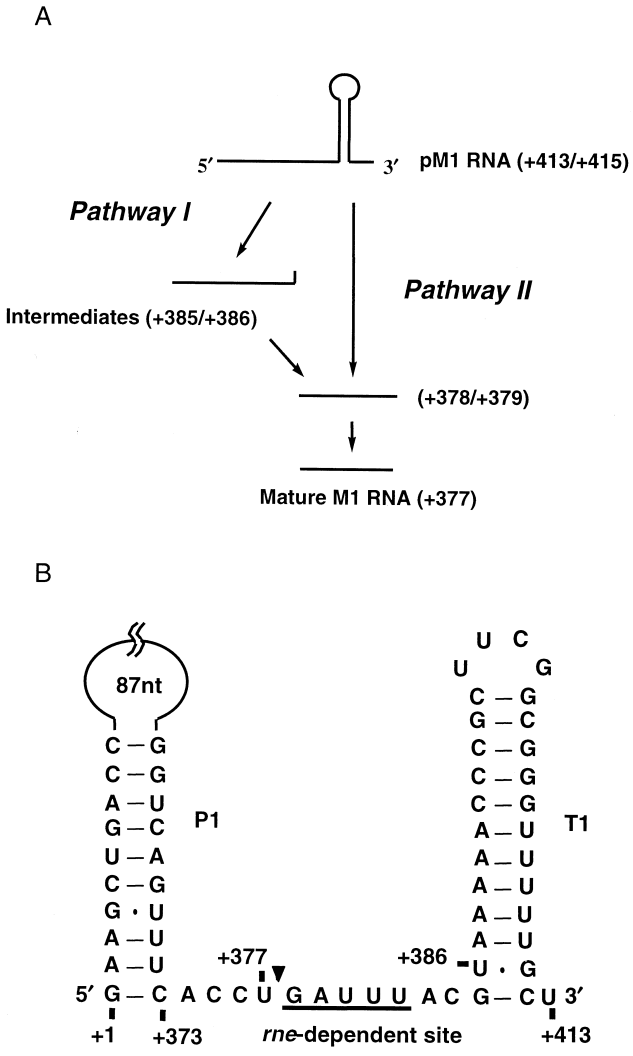

Fig. 1. A: Proposed model of 3′ processing pathways of M1 RNA [10]. Pathway I is involving the +385/+386 RNA intermediates, and pathway II is not. The final trimming step of one or two nucleotides from +378/+379 RNA to generate the mature M1 RNA with the 3′-end of +377 is also represented. B: A possible secondary structure of p23 RNA, a truncated pM1 RNA transcribed from the internally deleted rnpB gene is shown. The arrowhead indicates the 3′-end of the mature M1 RNA. The rne-dependent site is underlined.

differing from the consensus sequence of the rne-dependent site, and it was shown that all of the random mutants were processed less efficiently than the wild-type in vivo ([8], see also Table 1). The choice of the pathways was examined in the in vitro processing reaction using the ASP-40 as a partially purified cell extract in the absence or presence of excess yeast total RNA. Since the ASP-40 fraction lacks a processing activity responsible for the trimming of one or two nucleotides at the 3′-end of +378/+379 RNA intermediates, the final in vitro processing products are +378/+379 RNA. When excess yeast total RNA in included in the in vitro reactions, +385/+386 intermediates are accumulated because excess yeast total RNA blocks the second step of pathway I (Fig. 1; [10]). The time-course experiment has shown that these intermediates are processed to the final products [10]. Therefore, the appearance of the +385/+386 RNA intermediates in the in vitro reaction with excess yeast total RNA represents that the processing

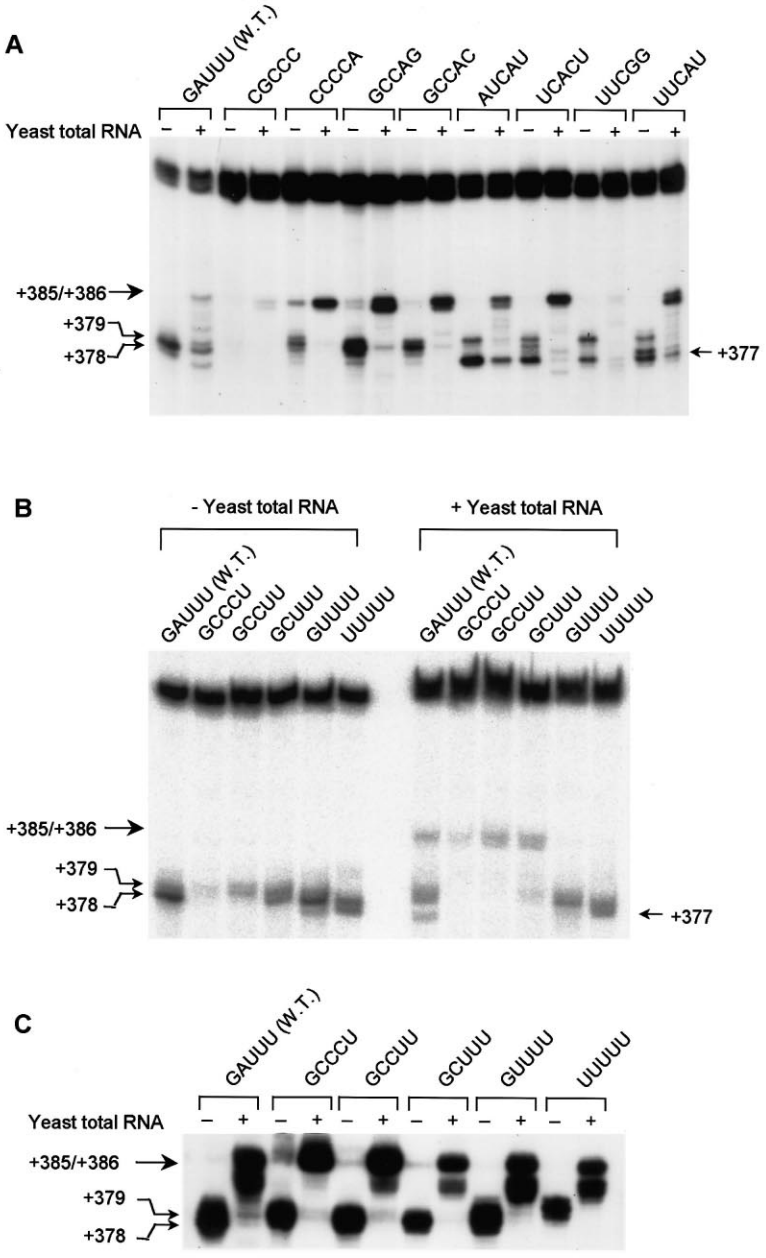

Fig. 2. In vitro processing of mutant p23 RNAs having changes at the rne-dependent site. Substrate RNAs were transcribed in vitro with [α-^{32}P]CTP. Synthetic transcripts were incubated at 37°C for 10 min with a partially purified cell extract (ASP-40) from JM109. The reaction products were resolved on a 5% polyacrylamide sequencing gel containing 8 M urea. The following substrates were used for the analysis: (A) p23 RNA variants of random mutations at the rne-dependent site, (B) p23 RNA variants of U-mutations, (C) +386 intermediates of the U-variants. The mutated sequences at the rne-dependent site are indicated above each lane. The presence and absence of yeast total RNA are indicated with a + or −. W.T. indicates the wild-type.

occurs by pathway I. On the other hand, the appearance of the +378/+379 RNA in the presence of excess yeast total RNA reveals that the processing occurs by pathway II.

DNA templates for generating p23 RNA substrates were originally designed in such a way that in vitro transcription by SP6 RNA polymerase was initiated at the first nucleotide of M1 RNA, and this, in turn, generated run-off transcripts having the 3′-end of +415 when cleaved with *Dra*I. However, SP6 RNA polymerase also terminates at the rnpB T1 terminator to generate a shorter transcript having the 3′-end of +413 ([10]; Sim and Lee, unpublished results). Since the 3′-end of pM1 RNA is +413, p23 RNA having the 3′-end of +413 was purified from the gel and used as a substrate.

Fig. 2A showed that the sequence of the rne-dependent site affected the formation of +385/+386 RNA intermediates, indicating that the pathways are determined by the sequence of the rne-dependent site. The rne-dependent site variants can be categorized according to their preference for the processing pathway (Fig. 3). Group I variants are processed only through pathway I. Group II variants are processed only through

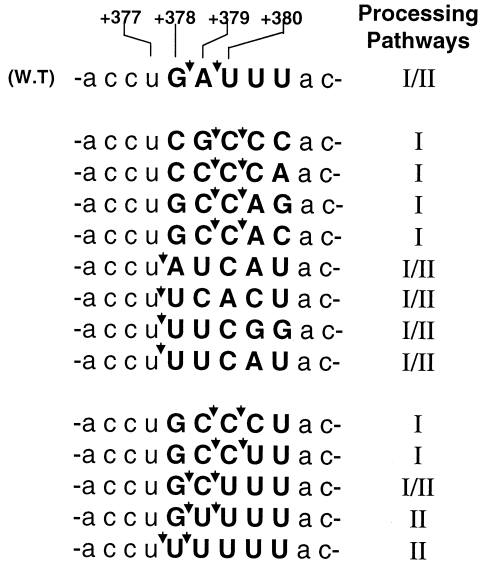

Fig. 3. Processing pathways deployed by the rne-dependent site variants and their cleavage sites. Group I, II, and III variants are processed only through pathway I, only through pathway II, and through both pathways, respectively. Arrows indicate the cleavage sites corresponding to the data in Figs. 2 and 5. The rne-dependent sites are shown in bold capital letters.

pathway II. Both pathways operate for the processing of group III variants. As well as the wild-type, p23 RNA variants bearing AUCAU, UCACU, UUCGG, and UUCAU belong to group III. On the other hand, p23 RNA variants of GCCAG, GCCAC, CGCCC, and CCCCA belong to group I. The G/C contents in the pentanucleotide sequence of the rne-dependent site seemed to be responsible for the pathway preference because p23 RNA variants rich in G/C nucleotides preferred to be processed by pathway I.

For the more systematic study of the influence of G/C contents, we constructed five U-variants by changing the U content from GCCCU, to GCCUU, GCUUU, GUUUU, and UUUUU. The more U nucleotides that were present in the rne-dependent site, the better the processing efficiency in vivo (Fig. 4 and Table 1), which is in accord with the previous result of the U preference for the efficient processing [8]. The in vitro processing of the five U-variants was assayed (Fig. 2B). The processing patterns of the five U-variants (Figs. 2 and 3) showed that the variants carrying high G/C

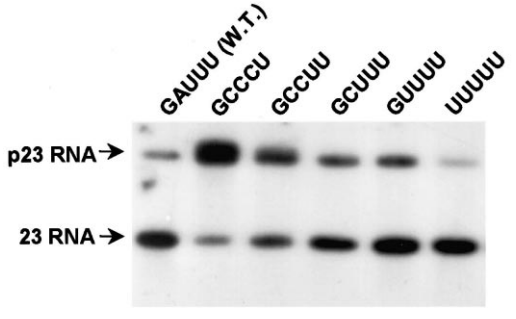

Fig. 4. In vivo analysis of U-variants of the rne-dependent site. Total cellular RNAs were prepared from 2×10^7 cells containing the mutant plasmids of pMT–RNE series. The RNAs were separated on a 5% polyacrylamide gel containing 7 M urea, and analyzed by Northern blotting. The mutated sequences of the rne-dependent site are indicated above each lane.

content in the rne-dependent site preferred the processing by pathway I to that by pathway II. One extreme was the GCCCU variant which was processed only by pathway I. The opposite extreme was the UUUUU variant which was processed only by pathway II. Compared with the U-variants, the wild-type of GAUUU had high efficiencies and was processed by both pathways (Table 1 and Fig. 3). Therefore, it is likely that the wild-type sequence provides a greater advantage because it can provide one more regulatory site, which may be needed for a finer tuning of the maturation of M1 RNA. These findings imply that the sequence of the rne-dependent site guides the RNA-metabolic pathway for regulation of RNA-processing in *E. coli*.

One may argue that excess yeast total RNA might not inhibit further processing of +385/+386 RNA intermediates of some variants, especially those of group II. However, Fig. 2C shows that excess yeast total RNA inhibited further processing of the +386 intermediates derived from all of the U-variants. The products, which were generated from the +386 RNA of the UUUUU variant by ASP-40 in the absence of excess yeast total RNA, were larger than the products from the corresponding p23 RNA. Since the UUUUU variant of p23 RNA did not generate the +385/+386 RNA intermediates (Fig. 2B), the RNA products from the +386 RNA in Fig. 2C could differ from the genuine processing products in the case of the UUUUU variant.

It has not been clear why 3′ processing of M1 RNA occurs by two pathways. The two pathways may be independently involved in regulation for producing the mature M1 RNA within the cell, which consequently should influence biosynthesis of RNase P. The fact that the wild-type rne-dependent site is the most appropriate sequence for efficient processing by each pathway supports this possibility. The synthesis rate of RNase P is expected to vary with growth conditions [19], as do the translational apparati such as ribosomes and tRNA [20,21]. The M1 RNA processing may play an important role in growth-dependent regulation for the synthesis of RNase P because there is some evidence that RNA processing or degradation activities, including RNase E, are also regulated by growth conditions [19,22–24]. Then each pathway of 3′ processing of M1 RNA would differentially contribute to this regulation under different growth conditions.

3.2. Changes of the cleavage sites by the sequence variation at the rne-dependent site

The sequence alterations at the rne-dependent site caused the variations of product sizes in vitro (Fig. 2). Since the RNA substrates were internally labeled, we examined whether or not this size variation was due to different 3′-ends of the products. The products were eluted from the gel and subjected to primer extension analysis. The primer extension analysis showed that all the products had the same 5′-ends (data not shown). This result confirmed that the different size resulted from the variation of the 3′-end. Although the in vivo 3′-end of 23 RNA, the processing product of the wild-type p23 RNA, is +377, ASP-40 generates the +378/+379 products having one or two nucleotides more than 23 RNA [10]. The variants of GCUUU and GUUUU produced +378/+379 products that had the same 3′-ends with that of the wild-type GAUUU. The variants of GCCCU and GCCUU, as well as GCCAG, GCCAC, CGCCC, and CCCCA, generated the processing products with the 3′-ends of +379/+380. On the

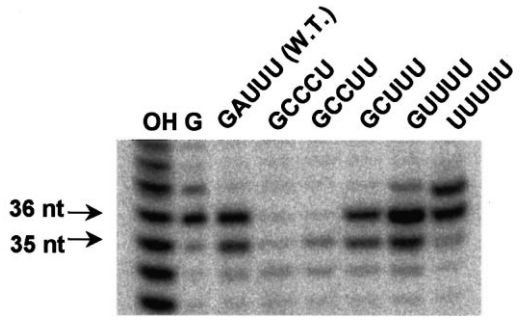

Fig. 5. Identification of downstream processing products. RNA substrates labeled at 3′-end were incubated with ASP-40 and analyzed on a 10% polyacrylamide sequencing gel. OH: Indicates alkaline hydrolysis ladders. G: Stands for G-specific (RNase T1) cleavage products.

other hand, the UUUUU variant, as well as the variants of AUCAU, UCACU, UUCGG, and UUCAU, produced the 3′-ends of +377/+378.

When the rne-dependent site variants were listed according to their cleavage sites (Fig. 3), the variants that produced the +379/+380 ends had all G/C at the first three nucleotides of the rne-dependent site, while the variants generating +377/+378 products had A/U at the first nucleotide of the rne-dependent site. Although we have not tested all possible variations, our results indicate that the position of specific bases at the rne-dependent site can determine in vitro cleavage sites by either pathway I or II. This cleavage specificity may explain a complicated relationship of sequence changes of the rne-dependent site to the cleavage specificity, which was observed in other RNA substrates [14,15].

To see whether different 3′-ends could be generated by an endoribonucleolytic cleavage involved in pathway II, the 3′-end-labeled substrates were used for the processing assay (Fig. 5). The variants of GCUUU, GUUUU, and UUUUU produced the downstream products corresponding to the upstream products formed by the endoribonucleolytic cleavage. Therefore, the sequence at the rne-dependent site determined the endonucleolytic cleavage sites of pathway II. In the case of the variants of GCCCU and GCCUU, the corresponding downstream cleavage products were not produced because it was processed only by pathway I. Interestingly, all the variants involving both pathways I and II for the processing generated the same 3′-ends, suggesting that the 3′-end formation by pathway I is also affected by the sequence at the rne-dependent site. RNase E is responsible for the endoribonucleolytic cleavage of pathway II by recognizing the rne-dependent site [10]. The pathway I-dependent cleavage specificity may also be related somehow to RNase E recognition.

Acknowledgements: This work was supported by a Korea Research Foundation grant (1998).

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