J. Biochem. Mol. Biol. Vol. 30, No. 2, pp. 162~165 March 31, 1997

5' Processing of RNA I in an *Escherichia coli* Strain Carrying the *rnpA49* Mutation

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(Received February 28, 1997)

Abstract : RNA I, a negative controller of ColE1-type plasmid replication, is metabolized by several RNases in *Escherichia coli*. Two small derivatives of RNA I are accumulated at nonpermissive temperatures in an *E. coli* strain carrying the *rnpA49* mutation, a thermosensitive mutation in the *rnpA* gene encoding the protein component of RNase P. A primer extension analysis was carried out to compare 5' processing of RNA I in the *E. coli rnpA49* cells at both permissive and nonpermissive temperatures. Derivatives of RNA I having different 5' ends were observed in the cells grown at permissive and nonpermissive temperatures. Some of the derivatives may be generated by the cleavage of RNase P.
Key words : ColE1-type plasmid, RNA metabolism, RNA I, *rnpA*.

Replication of the ColE1-type plasmid is initiated by a plasmid-specified RNA molecule, termed RNA II. RNA II serves as a primer for DNA synthesis by providing DNA polymerase I with a free 3' OH end (Cesareni *et al.*, 1991; Eguchi *et al.*, 1991). Formation of an active RNA II requires both transcription initiation at 555 base pairs upstream from the replication origin and cleavage of transcripts at the origin by RNase H. This replication is negatively controlled by a second RNA of 108 nucleotides, termed RNA I. Since RNA I is transcribed from a part of the DNA region encoding RNA II in an orientation opposite to that of RNA II transcription, RNA I is complementary to the 5' terminal region of RNA II and can act as an antisense RNA against RNA II. Interaction of RNA I with the RNA II transcript inhibits the generation of the functional 3' OH end of RNA II by cleavage of RNase H and consequently prevents initiation of plasmid DNA synthesis. Therefore, RNA I and RNA II are key elements in the replication control of the ColE1 type plasmid and their metabolism is closely related with the replication (Jung and Lee, 1996).

Several RNases such as RNase E and polynucleotide phosphorylase (PNPase) are involved in the metabolism of RNA I (Tomcsanyi and Apirion, 1978; Cohen, 1995). RNA I is cleaved endonucleotically by RNase E near its 5' end and digested exonucleotically by PNPase at the 3' end. We have previously shown the presence of intermediates of RNA I metabolism (Jung *et al.*, 1992). The intermediates, termed RNA X and RNA Y, accumulate at nonpermissive temperatures in an *E. coli* strain carrying the *rnpA49* mutation, which is responsible for the thermosensitivity of RNase P due to the mutation in the *rnpA* gene encoding the protein component of the enzyme, C5 protein (Kirsebom *et al.*, 1988). This result implies that RNase P, a tRNA processing enzyme which generates the mature 5' end of tRNA from precursor tRNAs, is involved in RNA I metabolism and that the defect of C5 protein is responsible for the accumulation of RNA X and RNA Y.

Since sequences at the 5' end of RNA I are implicated to play an important role in the interaction of RNA I with RNA II (Tomizawa, 1984; Tamm and Polisky, 1985), in this study, we compared 5' ends of intermediates of RNA I metabolism in the *E. coli rnpA49* cells at permissive and nonpermissive temperatures.

Materials and Methods

Bacterial strains and plasmids

The *E. coli* strain carrying the *rnpA49* mutation was A49 (Schedl and Primakoff, 1973). *E. coli* JM109 (Yanisch-Perron *et al.*, 1985) was used as a wild type strain for *rnpA*. Plasmid pLM1 is a derivative of pGEM3 containing a replicon of ColE1-type plasmid pMB1. This plasmid carries a functional *rnpB* gene (Lee *et al.*, 1989).

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Preparation of total cellular RNA

E. coli cells containing plasmid pLM1 were grown up at the permissive temperature (30°C) to an optical density of 0.3 at 600 nm in LB media supplemented with ampicillin of 50 μg/ml. The cells were divided into two cultures. One culture was shifted to a nonpermissive temperature (43°C), and further incubated for 3 h. Growth of the other culture was continued at 30°C. Total cellular RNAs were directly extracted from the cultures with a phenol mixture at 65°C, which contained one-tenth volume of 10× RNA extraction buffer (RNA extraction buffer: 0.02 M sodium acetate, pH 5.2, 0.5% SDS, 1 mM EDTA) and the same volume of phenol saturated with the RNA extraction buffer. The aqueous phase was then re-extracted with the same volume of the phenol at 65°C, ethanol-precipitated, and resuspended in water. The amount of RNA was estimated by determining the UV absorbance at 260 nm.

Primer extension analysis

Primer 5'-ACCACCGCTACCAGC-3', which is complementary to the 3' region of RNA I, was 5' end-labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase as described by Sambrook *et al.* (1989). Total cellular RNAs of 0.5 μg were mixed with 0.5 pmol of the labeled primer in 30 μl of hybridization buffer (80% formamide, 40 mM PIPES, pH 6.4, 1 mM EDTA, pH 8.0, 0.4 M NaCl), denatured at 90°C for 10 min, slowly cooled down to 30°C, and further incubated at 30°C for 12 h. The primer extension reactions were carried out in 25 μl of the reaction buffer (34 mM Tris-HCl, pH 8.3, 50 mM NaCl, 5 mM $MgCl_2$, 0.5 mM dNTP, 5 mM dithiothreitol) using 20 U of AMV reverse transcriptase. After incubating for 2 h at 37°C, the reaction products were precipitated with ethanol and analyzed on a 8% polyacrylamide sequencing gel. DNA sequencing ladders (Sanger *et al.*, 1977), which were obtained using the same primer on the plasmid template, were electrophoresed alongside the products of the primer extension assay.

Results and Discussion

Primer extension analysis (Fig. 1) was carried out to analyze the 5' ends of RNA I intermediates in an *E. coli* A49 mutant strain carrying the *rnpA49* mutation (Schedl and Primakoff, 1973). Total cellular RNA was prepared from the *rnpA49* cells containing pLM1 (Lee *et al.*, 1989), a ColE1 type plasmid which carried the cloned *rnpB* gene encoding M1 RNA, the RNA component of RNase P. This was because the growth defect of the *rnpA49* cells at nonpermissive temperatures can be complemented by a production of excess M1 RNA, the RNA component of RNase P (Jain *et al.*, 1982; Motamedi *et al.*, 1982), and the *rnpA49* cells containing pLM1 still accumulate RNA X and RNA Y at nonpermissive temperatures (Jung *et al.*, 1992). The cells were grown at permissive or nonpermissive temperatures. Three major extension products were observed in the cells grown at the nonpermissive temperature. These products were formed by extension to positions 6, 7, and 36 (Figs. 1 and 2), which corresponds to the 5' ends of RNA I_{-5} (an RNA derivative lacking 5 nucleotides at the 5' end of RNA I), RNA X, and RNA Y, respectively (Tomcsanyi and Apirion, 1985; Jung *et al.*, 1992). At the permissive temperature the cells generated other RNA I derivatives possessing different 5' ends. The 5' ends of the major derivatives were assigned to positions 11, 16, 42, 54, and 60. This result suggests that RNAs possessing the 5' ends of positions 11 and 16 are derived from RNA X and those possessing the 5' ends of positions 42, 54, and 60 are derived from RNA Y or possibly RNA X. Since they were not observed in the cells grown at the

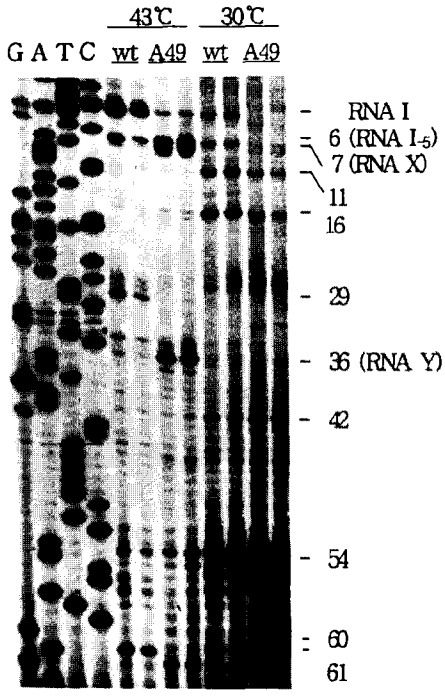

Fig. 1. Mapping of the 5' ends of RNA I derivatives. The 5' ends of the derivatives were determined by primer extension analysis. The total cellular RNA used for the primer extension was prepared from the *E. coli* A49 or JM109 cells containing pLM1 which were grown at 30°C and 43°C. The analysis was performed in duplicate using two different cultures. DNA sequencing ladders marked as G, A, T, and C were produced using the same primer and separated in parallel with the extension products. The major extension products are indicated by numbers corresponding to the positions of 5' ends. RNA I_{-5}, RNA X, and RNA Y possessing the specific 5' ends are given in parentheses at the corresponding positions.

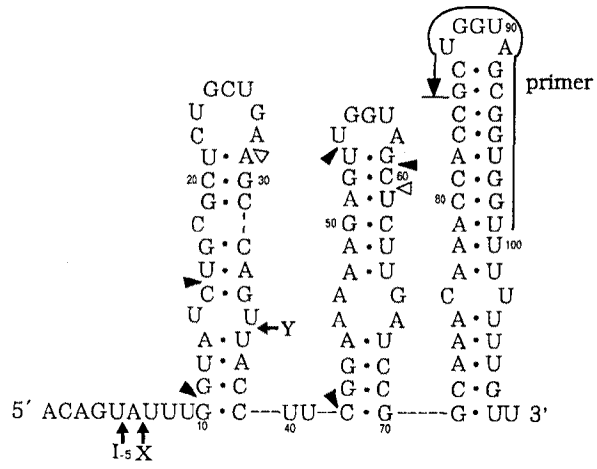

Fig. 2. *In vivo* derivatives of RNA I. The 5′ ends of RNA I derivatives present in *E. coli rnpA49* mutant or wild type cells were shown. The 5′ end termini of RNA I_{-5}, RNA X, and RNA Y, which accumulate in the *rnpA49* cells at a non-permissive temperature (43°C), are indicated. Solid arrowheads indicate 5′ ends of RNA I derivatives observed predominantly at a permissive temperature (30°C). The 5′ ends of RNA I derivatives observed in the wild type cells at 30°C are identical that in the *rnpA49* cells at 30°C. Open arrowheads indicate 5′ ends of RNA I derivatives observed predominantly in the wild type cells at 43°C. The location of the primer sequence for primer extension analysis is indicated.

nonpermissive temperature, it seems likely that some of them were generated by the cleavage of RNase P. If this is the case, RNA X or RNA Y would be a substrate for RNase P. As a control experiment, the same primer extension analysis was carried out using total cellular RNA from a wild type strain (Fig. 1). The 5′ ends of the RNA I derivatives from the wild type cells were the same as those from the *rnpA49* mutant cells at the permissive temperature (Figs. 1 and 2). However, they are different at the nonpermissive temperature (Figs. 1 and 2), implying that RNA X or RNA Y can have various conformations favorable at each temperature which may be differently processed.

Since M1 RNA alone can carry out the RNase P reaction under certain conditions such as in buffers containing more than 20 mM Mg^{2+}, the catalytic activity of the enzyme must lie on the RNA component (Guerrier-Takada *et al.*, 1983). *In vivo* and in buffers containing 10 mM Mg^{2+}, however, both M1 RNA and C5 protein are required for the RNase P function as a ribonucleoprotein (Schedl and Primakoff, 1973; Sakano *et al.*, 1974). C5 protein confers upon the RNase P holoenzyme a sensitivity not only to the rate of cleavage of a particular substrate but also to the precise site of cleavage within certain substrates (Gopalan *et al.*, 1994). Overexpression of M1 RNA can complement the growth defect of the *rnpA49* cells at nonpermissive temperatures (Jain *et al.*, 1982; Motamedi *et al.*, 1982). This complementaion can be explained by an enhancement of the interaction between C5 protein and M1 RNA (Lawrence and Altman, 1988). However, the complementation is not complete because the specific activity of RNase P is only partially restored and tRNA precursors are still accumulated (Jain *et al.*, 1982), suggesting that the mutant C5 protein resulting from the *rnpA49* mutation is also responsible for the defect of the RNase P function even in the presence of excess M1 RNA. Since RNA X and RNA Y accumulate in the *rnpA49* cells carrying a functional *rnpB* gene in high copy (Jung *et al.*, 1992), the accumulation is presumably related to the mutant C5 protein itself.

Although RNase P was initially characterized as an RNA processing enzyme that cleaves tRNA precursors to generate the mature 5′ termini (Robertson *et al.*, 1972), besides tRNA precursors several RNAs such as 4.5 S RNA precursors, derivatives of plant viral RNAs, and small model substrates, can be substrates for RNase P (Altman, 1993). Since RNA X and RNA Y are new candidates for substrates of RNase P, intermediates identified by this study can be used for elucidation of a relation of substrates to products for the RNase P reaction involved in RNA I metabolism.

Acknowledgement

This work was supported by grants from the Genetic Engineering Research Program of the Korean Ministry of Education (1995) and the Biotec 2000 program of the Korean Ministry of Science and Technology.

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