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Guide DNA technique reveals that the protein component of bacterial ribonuclease P is a modifier for substrate recognition

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Abstract We developed a guide DNA technique with which the cleavage efficiency of pre-tRNA substrate raised in the RNase P reaction. The 20-mer guide DNAs hybridizing to the upstream region of the cleaving site enhanced the cleavage reactions of RNA substrates by *Escherichia coli* RNase P. This guide DNA technique was also applicable to cleavage site selection by choosing the DNA-hybridizing site. Results showed that RNase P accepts DNA/RNA double-stranded 5′-leader region with high catalytic efficiency as well as single-stranded RNA region in pre-tRNAs as substrates, which suggests that the protein component of bacterial RNase P prefers bulky nucleotides. The protein component did not affect the normal 5′-processing reaction of pre-tRNAs, but enhanced the mis-cleaving (hyperprocessing) reactions of tRNA in non-cloverleaf folding. Our results suggested that the protein component of RNase P is a modifier for substrate recognition.

Key words: RNase P; Ribozyme; tRNA; Hyperprocessing; M1 RNA; C5 protein

1. Introduction

The relationship between the enzyme structure and the mechanism has long been a matter for argument. Ribonuclease P (RNase P) is one of tRNA-processing enzymes to produce mature tRNA molecules by cleaving the tRNA precursor at the 5′-end [1–3]. RNase P is one of ribonucleoprotein enzymes, consisting of one RNA subunit and one or more protein subunit(s). The RNA subunit of bacterial and of some archaeal RNase P is a ribozyme, being able to perform the RNase P activity without the protein component [1–4]. Reich et al. showed that the protein subunit of bacterial RNase P is a stabilizer for the RNA subunit, countervailing the surface charge of the nucleotides [5]. Kurz et al. showed that the bacterial protein subunit enhances the catalytic activity of the ribozyme by raising the affinity to the tRNA precursor substrate [6]. True et al. showed that the protein subunits of the eukaryotic RNase P contribute to the enzyme architecture [7]. The role of the protein subunit of the RNase P has been thought as additional one. Recently, the structural analysis of the protein subunit of the bacterial RNase P and the cross-linking experiments revealed that the single-stranded 5′-leader region of the tRNA precursor contacts to the protein subunit of the bacterial RNase P in the substrate recognition steps by the enzyme [8–11]. Park et al. and Loria et al. showed by using artificial tRNA substrates that the bacterial RNase P protein lowers substrate specificity in recognition of the 5′-leader region of the tRNA precursor [12,13]. These results suggest that the protein subunit of the RNase P is an acceptor for the 5′-leader region of the tRNA precursor. Although many studies have been done on the enzyme RNase P, especially on *Escherichia coli* and *Bacillus subtilis* enzymes [14–17], not all roles of the protein component of the RNase P are solved. It is true that the protein component of RNase P affects the substrate specificity of the enzyme, but many questions still remained. The RNase P protein is really an enhancer, or is substrate-discriminator?

In the contemporary biosynthesis systems, tRNA molecules are synthesized as single-stranded RNA molecules, and that tRNA molecule has single-stranded RNA as 5′-leader region. From this fact, we tend to expect that RNase P should not accept bulky nucleotides such as double-stranded 5′-leader region: most studies on RNase P are done with pre-tRNA substrates having short single-stranded RNA as 5′-leader region. Does RNase P have the strand-discrimination activity? To examine whether RNase P accepts pre-tRNA having double-stranded 5′-leader region as substrate or not, we prepared pre-tRNA and the 'guide DNA', of which the base sequence is complementary to the 5′-leader region of pre-tRNA.

In this paper, we show that the pre-tRNA substrates which contain double-stranded 5′-leader region with the guide DNA are more efficiently cleaved by the bacterial RNase P, and we also developed a guide DNA technique to control the cleavage site of tRNA substrates by the *E. coli* RNase P. By using the heterologous combination system, using eukaryotic tRNAs and bacterial RNase P, the hidden features of the protein component of the RNase P will be revealed. Here, we also developed the 'hyperprocessing' reaction as a tool to detect tRNA molecules in the non-cloverleaf folding [18–23]. The hyperprocessing reaction is defined as internal cleavage reaction of tRNA molecule by RNase P: the reaction can be observed when the cloverleaf shape of tRNA is melted to newly form the alternative hyperprocessible fold. Until now, three hyperprocessible tRNAs are known: *Drosophila* initiator methionine tRNA, alanine tRNA, and histidine tRNA. Our hyperprocessing strategy will give a useful tool for analysis of the relationships between tRNA and RNase P molecules.

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2. Materials and methods

2.1. Preparation of nucleotides and chemicals

The *E. coli* RNase P RNA and two eukaryotic tRNA precursors were prepared by in vitro transcription with T7 RNA polymerase (Toyobo) from pGEM-3Z-derived plasmids [20,23]. The pre-tRNAs were labeled at the 3′-end with [5′-^{32}P]pCp and T4 RNA ligase as described [23]. Three DNAs, gDNA-1 (19-mer), gDNA-2 (20-mer), and gDNA-3 (20-mer), were synthesized by Funakoshi Corporation (Japan) according to our specific order in the base sequences shown in Fig. 1. Other enzymes and chemicals were purchased from commercial available sources as described [20,23].

2.2. Preparation of E. coli RNase P protein subunit

The gene for *E. coli* protein was prepared from *E. coli* strain JM109. This gene was isolated from the genome DNA by PCR techniques and was cloned to *E. coli* strain BL21(DE3)pLysS using a protein expression vector pET15b (Novagen) with restriction sites, *Nde*I and *Bam*HI sites. The *Nde*I site was overlapped to the codon for methionine at the N-terminus of the protein, and the *Bam*HI site was added downstream of the termination codon. The *E. coli* gene encodes additional His-tag peptide sequence at the N-terminus of the protein, and the gene includes no artificial codon exchanges in the DNA sequences. The RNase P protein was purified to homogeneity from *E. coli* cells according to Niranjanakumari et al. and Rivera-Leon et al. [24,25]. The protein was purified by two steps of ion-exchanging column chromatography under denaturing and refolding conditions from the cell lysate. Sodium chloride at gradient concentration was used to elute the protein. Finally, about 9 mg *E. coli* protein was obtained from 1 l culture medium.

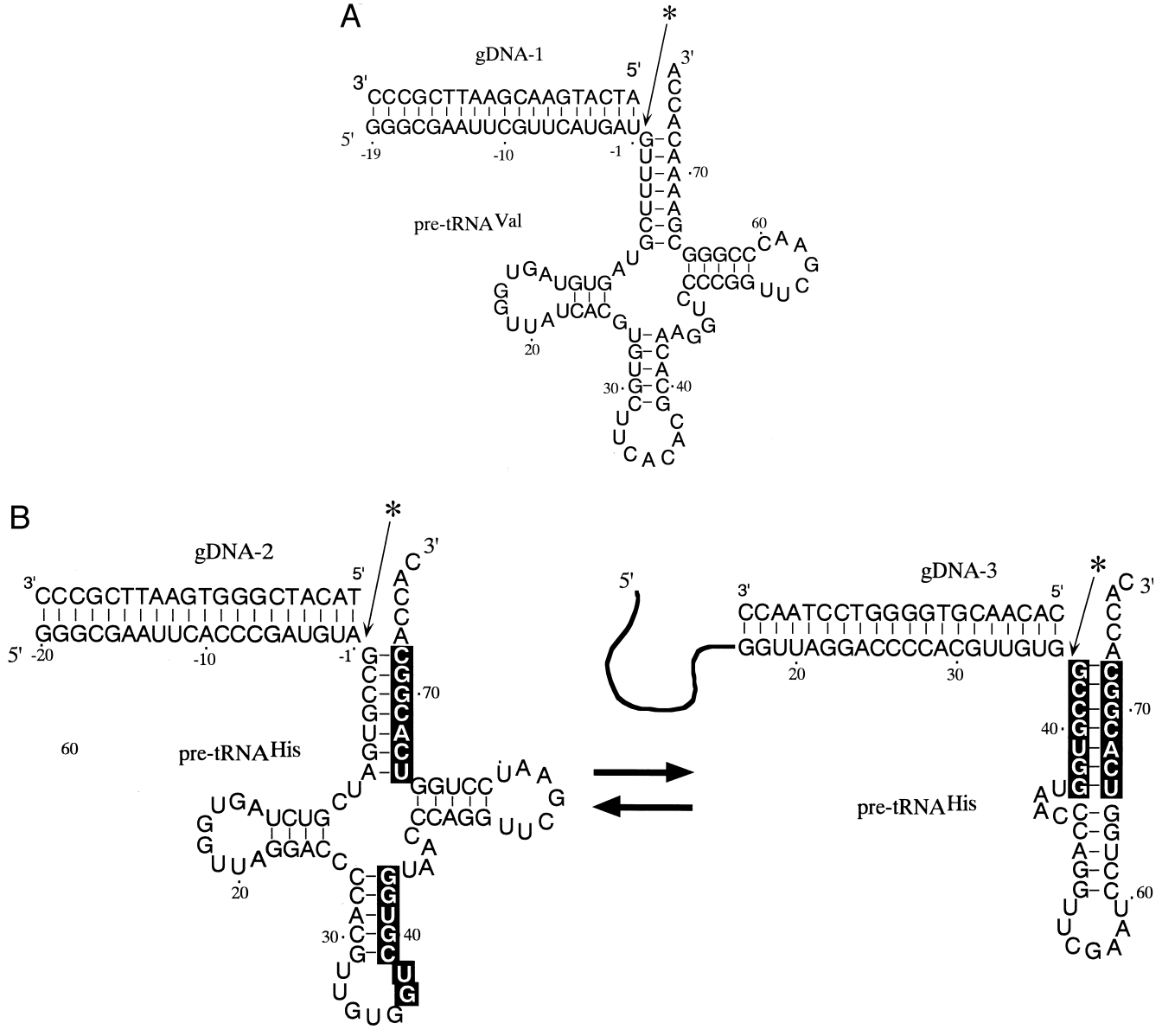

Fig. 1. Design for the cleavage reaction of pre-tRNAs by bacterial RNase P. (A) *Drosophila* pre-tRNA^{Val} is shown with the guide DNA (gDNA-1). The gDNA-1 can hybridize to the −19 to −1 region of the pre-tRNA molecule. The target cleavage site of the tRNA by the RNase P is shown by an arrow with asterisk. (B) *Drosophila* pre-tRNA^{His} is shown either in the cloverleaf structure (left) or in the double hairpin structure (right), with each guide DNA (gDNA-2 or gDNA-3, respectively). The gDNA-2 can hybridize to the −20 to −1 region of the pre-tRNA molecule, and the gDNA-3 can hybridize to the 17–36 region of the tRNA molecule, respectively. The target cleavage site of the tRNA by the RNase P is also shown by an arrow with asterisk. The complementary regions (G^{37}–G^{43} and U^{66}–C^{72}), contributing to form the alternative folding, within this tRNA are shown in boxes [23]. The numbers shown above the tRNA sequences are according to Sprinzl et al. [26].

2.3. Cleavage reactions

The standard reaction mixture contained 50 mM Tris–HCl (pH 7.6), 100 mM NH_4Cl, 60 mM $MgCl_2$, 5% (w/v) polyethylene glycol, 0.5 nM M1 RNA and 0.2 μM 3′-end-labeled pre-tRNA in a total volume of 10 μl reaction mixture. When C5 protein was added to the reaction mixture, the amount of it was equimolar with the M1 RNA. The RNase P holo-enzyme was reconstituted through the incubation at 70°C for 10 min. Each of guide DNA was added to the reaction mixture in a 25-fold amount of the pre-tRNA. The DNA/RNA annealing process was done through pre-incubation at 70°C for 15 min and additional incubation at 37°C for 4 h. The concentrations of M1 RNA and guide DNAs were measured spectrophotometrically by the absorbance at 260 nm, and the concentrations of 3′-labeled pre-tRNAs were measured by the radioactivities. The mixture was incubated at 37°C for 30 min, and products were developed on 10% polyacrylamide gel electrophoresis [20,23]. Quantitative analyses of the reactions were done by counting photo-stimulated luminescence of the product bands in a radiogram of the gel using PhosphorImager (Molecular Dynamics).

3. Results and discussion

As the RNA subunit of *E. coli* RNase P is a ribozyme (M1 RNA) under in vitro conditions, we used both apo-enzyme (M1 RNA alone) and holo-enzyme (M1 RNA with C5 protein) for the following experiments. The M1 RNA was prepared by in vitro transcription, and the recombinant C5 protein was purified from *E. coli* cells. The activities of the M1 RNA and the reconstituted holo-enzyme were examined and confirmed by other experiments (data not shown). Two tRNA precursors, pre-$tRNA^{Val}$ and pre-$tRNA^{His}$, were prepared to examine the enzyme activities (Fig. 1). The $tRNA^{His}$ is one of hyperprocessible tRNAs. The pre-$tRNA^{His}$ is also used to examine the cleavage site selection experiment. These tRNA precursors contain natural mature sequences and additional 19 or 20 nucleotides at the 5′-flanking region. These tRNAs were also prepared by in vitro transcription from plasmid DNAs. The sequences of the synthetic guide DNAs, gDNA-1 and gDNA-2, are complementary to the 5′-region of pre-$tRNA^{Val}$ and pre-$tRNA^{His}$, respectively. In the presence of these guide DNAs, the 5′-leader regions of tRNA precursors are designed to be double-stranded as DNA/RNA helices (see Fig. 1A,B, left). The sequence of gDNA-3 is complementary to the internal region of pre-$tRNA^{His}$, which is designed to induce the $tRNA^{His}$ to the internally cleavable, hyperprocessible folding (see Fig. 1B, right; [23]).

3.1. RNase P accepts double-stranded 5′-leader region of pre-tRNA as substrate

The results are shown in Fig. 2A. RNase P cleaved pre-$tRNA^{Val}$ both in the presence and absence of C5 protein (lanes 2 and 4). When the 5′-leader region of pre-tRNA was double-stranded by the addition of gDNA-1, the pre-tRNA was also cleaved by RNase P both in the presence and absence of C5 protein (lanes 6 and 8). These results show that the protein component of RNase P (C5 protein) has no effect to the cleavage efficiency. Interestingly, the presence of the guide DNA enhanced the RNase P activities. As the DNA/RNA helix formed by gDNA-1 and pre-tRNA is expected to be energetically stable, it is certain that RNase P recognized this gDNA-1-bound pre-tRNA as substrate, which means that RNase P accepts DNA/RNA double-stranded 5′-leader region in its substrate binding site. These results are consistent with the structural analysis of *Bacillus* RNase P protein [9]: there is a large cleft on the surface of the RNase P protein enough to accept such DNA/RNA helix as well as single-stranded RNA. The results that the reactions occurred equally both in the presence and absence of the C5 protein indicate that either M1 RNA or C5 protein has no strand selection activity. Both apo- and holo-enzymes preferred DNA/RNA double-strand to RNA single-strand as 5′-leader region in pre-tRNA. These results are consistent with the biological features. In most cases, tRNA molecules are synthesized as clustered form which contains two or more tRNAs. Each tRNA molecule is cleaved out from it. The spacer RNA length between two adjacent tRNAs is not so long, sometimes being less than 10 bases. The tRNA molecule at the upstream location should contact to the adjacent downstreamed tRNA, which means that RNase P contacts to the upstreamed tRNA in the recognition of the downstreamed tRNA as substrate. The cavity for the bulky RNA in the substrate binding site of RNase P as well as the cleavage enhancement effect for the double-stranded 5′-leader region of pre-tRNA are advantageous for the efficient RNase P activities.

Above results indicated that addition of the guide DNA is applicable to cleavage enhancement of RNase P reactions as described in Section 3.2.

3.2. Cleavage enhancement and cleavage site selection by guide DNAs

The results of the cleavage reactions are shown in Fig. 2A,B. When the guide DNA gDNA-1 was present with pre-$tRNA^{Val}$, the amount of the cleaved product (the mature $tRNA^{Val}$) was extremely raised to almost 100% (see Fig. 2A, the band '2' of lanes 6 and 8), being about 2-fold of lanes 2 and 4. The cleavage enhancement was also observed with the gDNA-2 (lane 10). This is because about half of the gDNA-2 is also hybridizable to pre-$tRNA^{Val}$ (see Fig. 3). These results indicated that the guide DNA technique is applicable to enhance the cleavage reactions of tRNA precursors by RNase P, and that partial hybridization of about 10 nucleotide length is enough for the cleavage enhancement effects. Same tendency was observed with pre-$tRNA^{His}$ (Fig. 2B). The cleaved products corresponding to the band '2' were also extremely raised to more than 95% (lanes 3, 5, and 15).

Above results also indicated that guide DNAs are applicable to cleavage site selection by choosing the DNA-hybridizing positions. In the presence of gDNA-2, the cleavage reaction of pre-$tRNA^{His}$ was mainly observed at the normal processing site, the A^{-1}–G^{1} bond (Fig. 2B, lanes 3 and 5). Interestingly, the cleavage product by hyperprocessing reaction was reduced in the presence of the gDNA-2 (compare lanes 3 and 5 with lanes 11 and 13; see Fig. 2D). Apparently, the cloverleaf structure of $tRNA^{His}$ was somewhat stabilized by the addition of the guide DNA gDNA-2. Considering that the hyperprocessing reaction of tRNA molecule should be disadvantageous to the host cells, the 5′-region of the pre-tRNAs may contribute to the stabilization of the tRNA precursor molecules in the RNase P reaction. On the other hand, the cloverleaf structure of this tRNA was greatly destabilized by the addition of the internal guide DNA gDNA-3. The product of normal processing reaction (the band '2' in Fig. 2B) was reduced to only 2%, and the product of hyperprocessing reaction was raised in the presence of gDNA-3 (Fig. 2B, lanes 7 and 9; Fig. 2D). These results indicate that the cloverleaf shape of tRNA can be easily destroyed with the complementary DNAs and that the guide

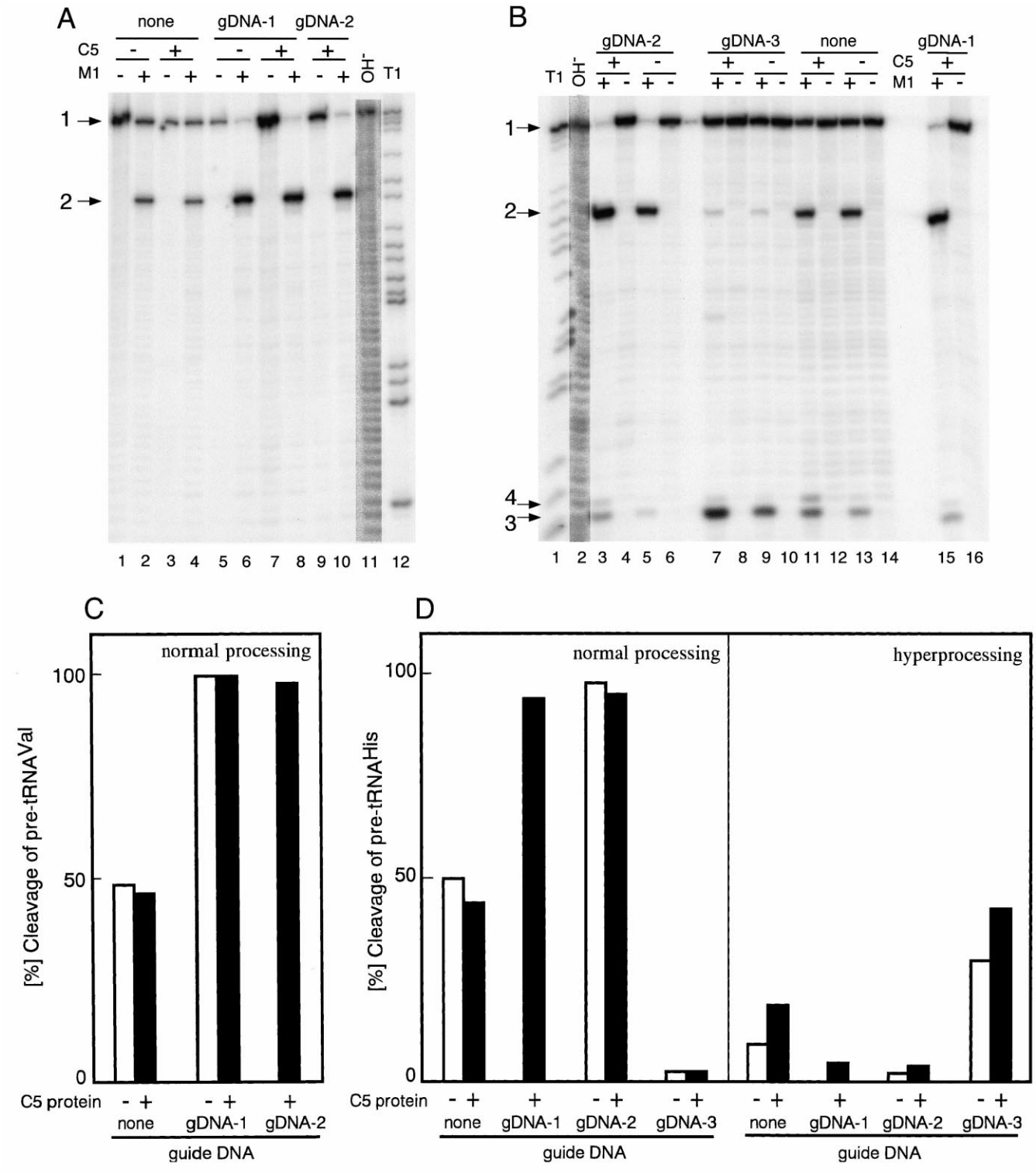

Fig. 2. Cleavage reaction of pre-tRNAs by bacterial RNase P. (A) Cleavage reaction of *Drosophila* pre-tRNA^{Val}. The bands '1' and '2' represent the pre-tRNA^{Val} and mature tRNA^{Val}, respectively. The digits 'C5' and 'M1' represent the protein subunit of the RNase P (C5 protein) and the RNA subunit of the RNase P (M1 RNA), respectively. Lanes 1–4, reactions in the absence of any guide DNA; lanes 5–8, reactions in the presence of gDNA-1; and lanes 9 and 10, reactions in the presence of gDNA-2. Lanes 3, 4, 7–10 represent the reactions in the presence of C5 protein. Lanes 11 and 12 represent the partially alkaline hydrolyzed ladder marker, and the RNase T1 cleaved marker, respectively. Reactions were done under 60 mM Mg^{2+}-containing conditions. (B) Cleavage reaction of *Drosophila* pre-tRNA^{His}. The bands '1'and '2' represent the pre-tRNA^{His} and mature tRNA^{His}, respectively. Both bands '3' and '4' represent the hyperprocessed products. The band '3' corresponds to the cleaved product shown in Fig. 1B, right. Lanes 1 and 2 represent the size markers. Lanes 3–6, reactions in the presence of the external guide DNA gDNA-2; lanes 7–10, reactions in the presence of the internal guide DNA gDNA-3; lanes 11–14, in the absence of any guide DNA; and lanes 15 and 16, in the presence of gDNA-1. Lanes 3 and 4, 7 and 8, 11 and 12, and 15 and 16 represent the reactions in the presence of C5 protein. Reactions were done under 60 mM Mg^{2+}-containing conditions. (C, D) Summary of quantitative analyses of the cleavage reactions. Percent cleavage ratio was shown in each case.

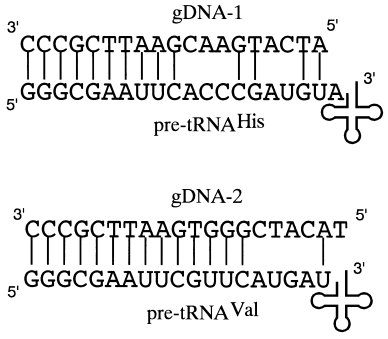

Fig. 3. Schematic representation of complementarities between the guide DNAs and tRNA molecules. The combination of gDNA-1 and pre-tRNA^{His} (above) and the combination of gDNA-2 and pre-tRNA^{Val} (below) are indicated. Possible base-pairings are shown with horizontal bars.

DNA technique is a useful tool for manipulation of cleavage site selection.

3.3. Effect of C5 protein

The results of cleavage efficiencies of pre-tRNA^{Val} and pre-tRNA^{His} by *E. coli* RNase P are summarized in Fig. 2C,D. In the absence of guide DNA, the amount of the cleaved product at normal processing reaction site was about 50% in every case (compare Fig. 2A, lanes 2 and 4; Fig. 2B, lanes 11 and 13). These results indicated that the protein component of RNase P performed no effect to normal processing reactions: no enhancement was observed with the cleavage reactions at the correct processing site. Even in the presence of the guide DNAs, no effect was observed with the normal processing reaction by the protein component of RNase P. These results, however, were apparently not consistent with the report on *Bacillus* enzyme by Kurz et al. [6]. Considering that our reaction mixture contains the enzyme at high concentration compared to normal Michaelis–Menten-type reactions, the effects of raising affinity for the substrate by the protein component were not observed. In contrast with the normal processing reactions, the hyperprocessing reactions were enhanced in the presence of RNase P protein in case of pre-tRNA^{His} (see Fig. 2B,D, right). Besides, the presence of the protein component enhanced 'mis-cleaving' reactions; an additional by-product appeared (see Fig. 2B, band '4'). The results for the hyperprocessing reaction are consistent with the previous reports on the role of the RNase P protein [6,12,13].

Our results suggest that RNase P protein is not itself a substrate specificity determinant, but a modifier for substrate recognition. RNase P protein has two faces: RNase P protein is silent when canonical cloverleaf tRNA is the substrate, but acts as an enhancer when unsuitable non-cloverleaf RNA is the substrate. RNase P protein lowers the substrate specificity of the enzyme, not by mis-recognition of the substrate, we suppose, but by enhancing the cleavage of mis-folded tRNAs.

Why does the RNase P protein have two faces in substrate recognition? Compared with the eukaryotic RNase P, the bacterial RNase P is to cleave not only cloverleaf-shaped pre-tRNA but also non-cloverleaf-shaped pre-rRNA. The RNase P RNA, we suppose, strongly prefers cloverleaf-shaped RNAs, therefore requires the additional subunit which lowers the substrate specificity to accept the non-cloverleaf-shaped RNAs. We think that the protein component of the RNase P is one of evolutionarily acquired molecular switches in substrate recognition.

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