Control of the Position of RNase P-mediated Transfer RNA Precursor Processing*

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Two Bacillus subtilis tRNA\textsuperscript{His} precursors (Green, C. J., and Vold, B. S. (1988) \textit{J. Biol. Chem.} 263, 652-657) were processed by \textit{Escherichia coli} RNase P in the presence of varying \([\text{Mg}^2+]\). The wild type precursor was processed under all conditions to afford a single tRNA product containing 8 base pairs in the acceptor stem. In contrast, the position of processing of a mutant tRNA\textsuperscript{His} precursor (containing a \(G^\prime \rightarrow A^\prime\) alteration) was shown to be condition-dependent. Processing occurred at \(A^7\) under conditions consistent with formation of an \(A^7\cdot C^{100}\) base pair in the acceptor stem but at \(G^8\) under conditions that disfavored base pair formation. The ability to control the site of RNase P-mediated tRNA precursor processing is unprecedented and permits analysis of the chemical factors that promote processing.

Ribonuclease P (RNase P),\textsuperscript{1} an enzyme responsible for processing the 5'-end of tRNA precursors, was purified from \textit{Escherichia coli} by Robertson et al. (1). The holoenzyme has an RNA subunit (M1 RNA) 375 nucleotides in length and a protein subunit (C5 protein) of \(M_f \sim 14,000\) (2). Remarkably, the RNA is the catalytically competent subunit; M1 RNA processes tRNA precursor substrates in vitro in the absence of C5 protein, albeit at relatively high \([\text{Mg}^2+]\) (3).

RNase P's of bacterial and yeast origin process homologous and heterologous tRNA precursor substrates as well as a variety of mutant, synthetic, and abnormal substrates (4-13). The way in which the processing site is determined is not understood although substrate conformation must be important since oligonucleotides identical in sequence to authentic RNase P processing sites are not necessarily substrates (14). Suggested recognition elements have included the 3' terminal CCA sequence of the mature tRNA (15), the invariant 5'-GT\textsubscript{Cy-CpU}-3' sequence (16), the length (17), complementarity (5, 17, 18), or nucleotide composition (5, 6, 8, 9) of the acceptor stem, and the nature of the 5'-leader sequence (8, 9). Further complicating the analysis is the observation that while the processing site of a given substrate is generally independent of the source of the RNase P, there are examples of substrates processed at different sites by RNase P's from different organisms (6, 9).

To permit an analysis of the chemical factors that control the facility and site of tRNA precursor processing, we sought to obtain a substrate whose processing could be influenced by alteration of experimental conditions. The reports that the site of processing was influenced by the position of G-C base pairs in the acceptor stem (5, 6, 8, 9) and that mismatches in the acceptor stem greatly diminished the facility of processing (17, 18) prompted us to focus on a mutant \textit{Bacillus subtilis} tRNA\textsuperscript{His} precursor substrate (4) having 7 base pairs in the acceptor stem and a potential A-C base pair in position 1 (and 74).

Presently, we report that the mutant, but not wild type, tRNA\textsuperscript{His} underwent processing at two different positions and that the ratio of processing at these sites could be controlled as a function of \([\text{Mg}^2+]\). At low \([\text{Mg}^2+]\) processing occurred exclusively on the 3'-side of \(A^7\), the potential participant in an A-C base pair. With increasing \([\text{Mg}^2+]\), processing occurred with increasing facility and gave increased amounts of processing on the 5'-side of \(A^7\). The alteration of processing site is unprecedented; all previous reports of changes in RNase P processing sites have resulted from actual changes in nucleotide sequence of the tRNA precursor substrate (4-6, 9-11, 19) or M1 RNA molecule (20); none changed as a function of experimental conditions.

**EXPERIMENTAL PROCEDURES**

\textbf{Materials—SP6 RNA polymerase and RNasin ribonuclease inhibitor were from Promega. Calf intestinal phosphatase and EcoRI restriction endonuclease were obtained from Boehringer Mannheim; T4 polynucleotide kinase was from United States Biochemical Corp. \([\gamma-\text{P}]\text{ATP} (=7000 \text{ Ci/mmol}) was purchased from ICN K&K Laboratories Inc. Nensorb-20 nucleic acid purification cartridges were from Du Pont-New England Nuclear. pSP64 plasmids encoding either wild type or mutant tRNA\textsuperscript{His} (4) were isolated from \textit{E. coli} JM101 (21). RNase P, isolated by the method of Robertson et al. (1), was a gift from Dr. Robert Payne, University of Virginia.}
The reaction mixture was incubated at 37 °C for 90 min and then applied to a 20% polyacrylamide sequencing gel (350 x 180 x 0.5 mm) and run at 700-1000 V for 15-20 h. The labeled tRNAH* precursors were excised from the gel, isolated by the crush and soak method, and recovered by ethanol precipitation.

Processing of tRNAH* Precursors with RNase P—Reaction mixtures (5-µl total volume) contained 10 mM Tris-HCl, pH 8.0, 10 mM NH4Cl, 1 µl of RNase P preparation, ~75 nM base concentration (~20,000 cpm) of 5'-end-labeled precursor tRNAH*, and either 5, 50, or 100 mM MgCl2. Reactions were incubated at 37 °C for 1 h and then quenched by the addition of 3 µl of loading buffer (80% (v/v) formamide, 50 mM Tris borate, pH 8.3, 0.1 mM EDTA, 0.1% (w/v) xylene cyanol, and 0.1% (w/v) bromphenol blue). The reaction mixtures were then loaded directly onto 20% 8 M urea sequencing gels, run at 2,500 V for ~3 h, and analyzed by autoradiography.

Some reactions also contained 5 mM spermidine, 5% polyethylene glycol (M<sub>n</sub> ~8000), 5% glycerol, and 50 mM Tris instead of 10 mM Tris alone; or 100 mM Tris and 1.2 M NH4Cl in place of the normal buffer (vide supra); or 10, 100, or 1000 mM NaCl in addition to the normal buffer.

RESULTS

As shown in Fig. 1, a mutant tRNAH* precursor constructed by Green and Vold (4) differed from the wild type species in that G<sub>57</sub> was replaced by A<sub>57</sub>, thus eliminating the G·C base pair. Treatment of the 5'-27P-end-labeled wild type tRNAH* precursor with RNase P under buffer conditions used routinely for processing (10 mM Tris-HCl, pH 8.0, containing 10 mM NH4Cl and 5 mM Mg<sup>2+</sup>) afforded a single RNase P-dependent product (Fig. 2). RNA sequence analysis (23) in the presence of 5'-end-labeled precursor tRNA<sup>H*</sup>, and either 5, 50, or 100 mM Mg<sup>2+</sup> (Fig. 2, lanes 1-4) or mutant tRNA<sup>H*</sup> precursor (lanes 5-7) was treated with RNase P as described under "Experimental Procedures" in the presence of 5 (lanes 2 and 3), 50 (lanes 3 and 6), or 100 mM (lanes 4 and 7) Mg<sup>2+</sup>. A control experiment employed the wild type tRNA<sup>H*</sup> and RNase P but no Mg<sup>2+</sup> (lane 1). The observed Mg<sup>2+</sup> dependence of the site of processing of mutant tRNA<sup>H*</sup> precursor was verified in replicate experiments; the derived data were also used for densitometric analysis to establish the ratio of processing at the two sites. The results of a typical experiment are given in Fig. 3. As shown, while the wild type tRNAH* precursor still gave a single set of products (lane 3), the mutant tRNAH* precursor was processed at two sites within the 5'-leader (lane 6); these were shown to be 26 and 27 nucleotides in length (22) (cf. Fig. 1). The same phenomenon was also seen at 100 mM Mg<sup>2+</sup> (Fig. 2); in other experiments that provided a more detailed [Mg<sup>2+</sup>] profile, it was found that as little as 10 mM Mg<sup>2+</sup> could produce substantial processing at the second site (not shown).

The observed Mg<sup>2+</sup> dependence of the site of processing of mutant tRNA<sup>H*</sup> precursor was less efficient than that of the wild type precursor. In this experiment, processing of the mutant precursor was modest at 5 mM Mg<sup>2+</sup> but increased to ~25% of that obtained for the wild type precursor at 50 mM Mg<sup>2+</sup> and further to ~50% at 100 mM Mg<sup>2+</sup>. Since processing of the wild type tRNAH* precursor clearly increased in absolute terms with increasing Mg<sup>2+</sup> (cf. lanes 2-4, Fig. 2), proc-
processing at both sites in the mutant tRNA\textsuperscript{His} precursor must have increased substantially with increasing Mg\textsuperscript{2+} (Figs. 2 and 3).

To characterize further the effect of Mg\textsuperscript{2+} on the processing reaction, we studied the processing of the mutant tRNA\textsuperscript{His} precursor as a function of temperature at 5 and 25 mM Mg\textsuperscript{2+}. As shown (Fig. 4), total processing at 5 mM Mg\textsuperscript{2+} proceeded to a lesser extent than at 25 mM Mg\textsuperscript{2+}; processing was substantially more efficient at the higher temperatures tested in the presence of 25 mM Mg\textsuperscript{2+}, presumably reflecting better maintenance of some RNA conformation required for RNase P-mediated processing at the higher temperature.\textsuperscript{3}

Also investigated were the effects of other reagents known to alter or stabilize nucleic acid tertiary structure. When used in the presence of 5 mM Mg\textsuperscript{2+}, 5% polyethylene glycol (M\textsubscript{r} ~8,000) greatly enhanced overall processing and provided readily detectable quantities of the 26-nucleotide oligonucleotide product in addition to the predominant 27-nucleotide oligomer. However, admixture of this reagent to reaction mixtures containing 50 or 100 mM Mg\textsuperscript{2+} simply diminished processing overall. In contrast, 5 mM spermidine did not affect the extent or site of processing at any of the three tested Mg\textsuperscript{2+} concentrations. The addition of NaCl (10 mM–1 M) to reaction mixtures containing 5 mM Mg\textsuperscript{2+} simply suppressed processing.

\section*{Discussion}

Most tRNAs contain 7 bp in the acceptor stem, a circumstance that clearly reflects the nature of substrate processing by RNase P. In identifying a tRNA precursor whose site of processing might be amenable to experimental control, we sought to exploit the observation that the histidine-specific tRNAs from a number of species including spinach chloroplast, E. coli, B. subtilis, and Salmonella typhimurium, have 8 bp in the acceptor stem (4–6, 9). For prokaryotic and spinach chloroplast RNase P, tRNA\textsuperscript{His} precursor processing affords a tRNA with 8 bp in the acceptor stem (6). As it is known that the site of E. coli tRNA\textsuperscript{His} precursor processing can be altered by additional G-C base pairs within the acceptor stem, with processing occurring on the 5'-side of the first G involved in a base pair (5, 6, 9), it seemed possible that alternate site processing could be obtained as a function of experimental conditions for a tRNA\textsuperscript{His} precursor containing a potential base pair of marginal stability at the end of the acceptor stem. Accordingly, we compared the processing of two tRNA\textsuperscript{His} precursors, one having a G-C base pair as the last of 8 bp within the acceptor stem and the other in which G\textsuperscript{27} was replaced by A (Fig. 1).

As noted, the mutant tRNA\textsuperscript{His} precursor exhibited condition-dependent alternate site processing as predicted. At low [Mg\textsuperscript{2+}], processing occurred exclusively on the 3'-side of A\textsuperscript{27}, as [Mg\textsuperscript{2+}] was raised increasingly above 10 mM, the proportion of processing on the 5'-side of A\textsuperscript{27} increased steadily. The total extent of processing of both tRNA\textsuperscript{His} precursors also increased as a function of increasing Mg\textsuperscript{2+} up to ~75–100 mM Mg\textsuperscript{2+}, above which processing began to decrease. Processing also decreased with increasing temperature unless the [Mg\textsuperscript{2+}] was increased to compensate the effect of temperature. While the mutant tRNA precursor was processed predominantly on the 3'-side of A\textsuperscript{27} at all temperatures studied when [Mg\textsuperscript{2+}] was 5 mM, increasingly great amounts of Mg\textsuperscript{2+} were required to promote processing on the 5'-side of A\textsuperscript{27} at the higher temperatures studied. These effects were specific for Mg\textsuperscript{2+}; neither spermidine nor NaCl had any effect in enhancing the extent or influencing the site of processing.

The accumulated data suggest strongly that Mg\textsuperscript{2+} controls...
the processing site by directing conformational changes in the structure of the mutant tRNA^{His} precursor. Given that the effect on the processing site was specific for the mutant tRNA^{His} precursor, which differs from the wild type species only at nucleotide 27, it is logical to conclude that increased Mg^{2+} concentration induces the formation of a base pair between A^{27} and C^{100} and that the formed base pair promotes alteration of the site of processing.

In this context it may be noted that Mg^{2+} stabilizes the active form of tRNAs (24); for tRNA^{His}, the sites of Mg^{2+} binding have been characterized (25). In addition to these Mg^{2+} binding sites, it is speculated that high [Mg^{2+}] induces mispairing between the anticodon of tRNAs and the codon of mRNAs (26–29). Studies involving the misincorporation of leucine for phenylalanine at high [Mg^{2+}] in response to a poly(U) template indicated that the change from correct incorporation to misincorporation occurred at concentrations of Mg^{2+} between 10 and 20 mM (28, 29). Misincorporation was attributed to the direct effect of high [Mg^{2+}] on anticodon-codon base pair interactions. The concentrations of Mg^{2+} that were found to induce misincorporation of leucine are strikingly similar to those which we observed to induce alternate site processing of the mutant tRNA^{His} precursor by E. coli RNase P.

Interestingly, the mutant tRNA^{His} precursor was processed exclusively on the 3′-side of A^{27} by M1 RNA at all tested [Mg^{2+}] up to 200 mM. This is in agreement with the findings of Green and Vold (4) for the same tRNA^{His} precursor substrate and parallels those of Burkard et al. (5) for the E. coli RNase P and M1 RNA-mediated processing of E. coli tRNA^{His} precursors altered at the end of the acceptor stem. In agreement with Burkard et al. (6), we conclude that the C5 protein must play a role in providing the holoenzyme with a more generalized recognition for its substrates, compared with the rather strict specificity seen with the M1 RNA subunit, a process that would logically involve additional recognition sites in the substrate tRNAs. While RNase P may accommodate structural diversity in its tRNA substrates to a greater extent than M1 RNA, RNase P may also be more sensitive to structural alterations in the substrates and change its processing site accordingly.

Guerrier-Takada et al. (20) have recently described structural mutants of E. coli M1 RNA that process E. coli tRNA^{Tyr} precursor at altered positions. Interestingly, the site of processing could be controlled experimentally as a function of [Mg^{2+}] or by the addition of C5 protein.

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