The methylation of one specific guanosine in a pre-tRNA prevents cleavage by RNase P and by the catalytic M1 RNA

Dietmar Kahle, Uta Wehmeyer, Shoba Char¹ and Guido Krupp

Institute for General Microbiology, Christian-Albrechts-Universität, Am Botanischen Garten. 1-9, D-2300 Kiel, FRG and ¹Department of Molecular Biophysics and Biochemistry, Yale University, PO Box 6666, New Haven, CT 06511, USA

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ABSTRACT

Several modified nucleosides were introduced during *in vitro* RNA synthesis into a pre-tRNA^{Ser}. The pre-tRNAs were used as substrates for RNase P enzymes. No effects were observed with biotin-8-ATP or [α -S]-GTP, whereas with m⁷GTP, the cleavage reaction was completely inhibited. Analysis of pre-tRNAs which contained m⁷G at various positions has revealed a single base at the 5'-end of the acceptor stem where this modification absolutely prevents cleavage by catalytic M1 RNA, eukaryotic and prokaryotic RNase P holoenzymes. These results suggest that a critical contact must be made between pre-tRNA substrate and enzyme/ribozyme or that the approach of the potential cleaving agent (a positive magnesium ion) is made impossible by the positive charge at N-7 of the guanosine. In addition, we have shown that a pre-tRNA containing only m⁷G's can still form a complex with M1 RNA in a gel retardation assay.

INTRODUCTION

The transcription products of tRNA genes undergo a series of enzymatic reactions before mature tRNAs are obtained (1,2). The 5'-termini of mature tRNAs are generated by the endonuclease RNase P (3). *In vivo*, the eubacterial, yeast and human RNases P consist of essential protein and RNA components (4-8). *In vitro*, the eubacterial RNAs alone are efficient and specific catalysts at high magnesium concentrations (9, 10).

RNase P from one organism can recognize all homologous and a variety of heterologous pre-tRNAs. The sequence and size of the 5'-flanks and even of the mature tRNA domains vary considerably, only the structure of the tRNA is conserved and the main recognition features must be in the mature domain (11-14). To obtain more direct information about the essential elements, minimal 'pre-tRNAs' were analyzed (15), including also the 3'-terminal tRNA-like domain of TYMV RNA (16, 17) which excluded conserved tRNA domains. These studies revealed that crucial contacts must be made between an intact (seven base pairs) acceptor stem and RNases P.

A different approach for the study of enzyme recognition mechanisms is based on the introduction of modified bases into

nucleic acids. Subsequently, the modified nucleic acid is incubated with the enzyme of interest. The modified bases at essential contact points will be excluded from the products and are revealed by an appropriate analysis. The chemical modification of bases in tRNAs was used recently for the identification of contact points between tRNA and tRNA nucleotidyltransferase (18). The difference in our approach was the introduction of the modified nucleosides during the *in vitro* RNA synthesis. This allows a control of the modification percentage and also the specific insertion at predetermined positions. Additional, undesired modifications are avoided. These occur with dimethyl sulphate treatment of RNA where, in addition to guanosines (m⁷G), adenosines (m¹A) and cytidines (m³C) become methylated (19). As another advantage, the specifically introduced m⁷G does not interfere with the formation of Watson-Crick base pairs (20). In the L-shaped tertiary tRNA structure, Hoogsteen base pairs are important and these would be destroyed by N-7 methylations (20). However, with our chosen tRNA^{Ser}, a class II tRNA, Hoogsteen pairs with guanosines were not observed (21).

For RNase P activities, we could show that the base at the 3'-side of the cleaved phosphodiester bond is intimately involved in the reaction. For the holoenzyme, this base is at the 5'-end of the acceptor stem; for the catalytic M1 RNA the position is shifted to the last, non base-paired nucleotide of the 5'-leader.

MATERIALS AND METHODS

Template DNAs

For the standard pre-tRNA, pSI, we used the plasmid pSSI, derived from pSP64, containing the *S. pombe supSI* gene. Transcripts were obtained after *TaqI* cleavage, as described previously (6). The templates for the short pre-tRNAs with pG and pA as 5'-flanks were constructed with a technique involving 'terminator oligos' in combination with a 'portable T7 promoter' for the synthesis of 'initiator oligos'. This method has been described in detail (22; see Figure 1). For pG as 5'-flank and CCA at the 3'-end, the complete transcript sequence was encoded by the DNA template 'I-24' (22). For pA as 5'-flank, the coding sequence encoded only G+1 of the mature tRNA and the extra nucleotide was inserted by the dinucleotide primer ApG (see below).

Synthesis of Pre-tRNAs

The standard pre-tRNA pSI was obtained with SP6 RNA polymerase (NE Biolabs) and α -[32 P]-UTP (from Amersham) with *Taq*I-cleaved pSSI, as described (6). Modified pSI(100% m⁷G) and pSI(50% m⁷G) were obtained with 0.5 mM ATP, CTP and UTP (Boehringer Mannheim) and for 100% with 0.5 mM m⁷GTP (Sigma) or for 50% with 0.25 mM each, GTP and m⁷GTP. Similarly, 100% biotin-8-ATP (Sigma) and [α -S]-GTP were introduced. [α - 35 S]-GTP was obtained from NEN, the unlabeled compound was a generous gift of Prof. F. Eckstein, Göttingen.

The short pre-tRNA with pm⁷G as 5'-flank was obtained with template DNA 'I-24' (described above), transcribed with T7 RNA polymerase (NE Biolabs) as described (22). Different from the standard protocol, the reactions contained 0.25 mM NTPs and 2 mM pm⁷G (Sigma) to promote initiation with the mononucleotide (23). For the short pre-tRNA with pA as 5'-flank, unmodified G+1 and 100% internal m⁷G's, the template 'IV-24' encoded G+1 as starting nucleotide. The extra 5'-terminal adenosine was added with the primer dinucleotide ApG as described (24). Transcriptions were performed as above, using 0.3 mM ApG (Sigma), 0.5 mM each ATP, CTP, UTP and m⁷GTP.

All transcript sequences were confirmed by direct RNA sequencing (22). The 5'-terminal sequences were exactly as given in Figure 1, whereas one or two extra nucleotides were present at the 3'-terminus (ref. 22 and unpublished data). Also, the gel-purified RNAs retained this 3'-heterogeneity at a reduced percentage. No severe interference with clear gel sequencing patterns of the 3'-labeled RNAs was observed, and here, a more rigorous purification was not attempted.

Preparation of RNases P

S. pombe and *E. coli* RNase P were prepared as previously described, using two consecutive DEAE-cellulose columns and a gel filtration with Sephacryl S-300 (6, 25). The active enzyme fractions were pooled, dialyzed against storage buffer [50 mM Tris-HCl (pH 8), 10 mM MgCl₂, 50 mM KCl (for *S. pombe*) or 100 mM KCl (for *E. coli*), 10 mM mercaptoethanol and 50% glycerol] and stored at -20°C.

Synthesis of M1 RNA

The plasmid pDW27, containing the gene for M1 RNA behind a T7 promoter, was a generous gift of Norman Pace, Indiana University, Bloomington, IN. It was cleaved with *Sna*BI, transcribed with 0.5 mM NTPs and the produced M1 RNA was gel-purified. Quantification was possible by the inclusion of a low level of α -[32 P]-UTP and silver staining of analytical 8% polyacrylamide gels (6).

Processing of Pre-tRNAs

For RNase P holoenzymes from *S. pombe* and *E. coli*, 10 μ l reactions contained 50 mM Tris-HCl (pH 8), 5 mM MgCl₂, 100 mM KCl and the specified amounts of pre-tRNAs and enzymes. Incubations with M1 RNA alone were performed in 50 mM Tris-HCl (pH 8), 100 mM MgCl₂, 100 mM NH₄Cl and 5% polyethylene glycol (26).

For the analysis on polyacrylamide gels, the sample was transferred onto 10 μ l urea/dye mix (8 M urea; 0.03% xylene cyanol, bromophenol blue), previously dried down. After boiling for 2 min and chilling on ice, it was applied on 20% or 8% denaturing polyacrylamide gels, as appropriate. For thin layer

chromatography, the samples were boiled for 2 min, dried down in a Speed-Vac (Savant Instruments) and dissolved in 2 μ l solution of the appropriate mixture of marker nucleotides (5 μ g each).

End-Labeling of RNAs

3'-Labeling. About 0.1 to 5 pmol RNA were denatured in 2 μ l 10 mM Hepes-KOH (pH 8.3), 33% deionized DMSO (dimethyl sulphoxide) by boiling for two minutes and chilling on ice. Then 2 μ l buffer were added, consisting of 120 mM Hepes-KOH (pH 8.3), 30% DMSO, 10 mM dithiothreitol, 30 mM MgCl₂, 80 μ M ATP and 30 μ g/ml autoclaved gelatine. The mixture was added to 200 μ Ci 5'-[32 P]-pCp, prepared and dried down as previously described (27). Then it was incubated with 2 μ l (20 U) T4 RNA ligase (NE Biolabs) for 16 h at 10°C. Excess label was removed by ethanol precipitation, adding 20 μ l 4 M ammonium acetate (pH 7), 14 μ l H₂O, 5 μ g glycogen (Boehringer) as carrier and 100 μ l ethanol. The pellet was dissolved in 10 μ l urea/dye mix (8 M urea; 0.03% xylene cyanol, bromophenol blue) and applied on an 8% polyacrylamide gel. After autoradiography, the RNA was excised and eluted (22).

5'-Labeling. RNAs with 5'-terminal phosphates (all RNAs used, except the short pre-tRNA with ApG as dinucleotide primer) were dephosphorylated first (27); RNA with 5'-OH could be labeled directly with 150 μ Ci gamma-[32 P]-ATP (7,000 Ci/mmol; ICN Biomedicals) and 5 U polynucleotide kinase (NE Biolabs) as described (27). Excess label was removed and the labeled RNAs were isolated as described above for 3'-labeled RNA.

RNA Analyses

Chemical sequencing reactions. The 3'-[32 P]-labeled RNAs containing m⁷G were directly subjected to aniline cleavage (28), since prior sodium borohydride reduction was not required (29). The alkali cleavage for a sequence ladder has been described (30).

Thin layer chromatography. The end group analyses have been described previously (30). Briefly, the 3'-labeled RNAs were digested completely with RNase T2 (BRL) to obtain 3'-[32 P]-labeled nucleoside-3'-monophosphates. The 5'-labeled RNAs were cleaved with nuclease P1 (Pharmacia) to obtain 5'-[32 P]-labeled nucleoside-5'-monophosphates. The products were analyzed by cochromatography with authentic marker nucleotides on cellulose plates (Merck), developed in isobutyric acid/ammonia.

For the analysis of RNase P cleavage products, the dried samples were analyzed as described above. However, it was essential to use only 5 μ l aliquots of the digests with M1 RNA and to rinse the plates extensively with ethanol (using a wash bottle) and to dry them with a hair dryer before the development. Otherwise, polyethylene glycol and glycerol will severely impair the resolution.

Densitometer analysis. A Hoefer GS 300 Scanning Densitometer was used. Three slightly shifted scans were recorded for each lane and the average of the integrated peak values were taken.

Gel Retardation Assays with M1 RNA

All assays contained in 10 μ l 50 mM Tris-HCl (pH 8), 100 mM MgCl₂, 100 mM NH₄Cl, about 10 ng (10,000 cpm) of tRNA, and specified amounts of M1 RNA. The mature tRNA^{Phe} from *Saccharomyces cerevisiae* (Sigma) was 3'-[32 P]-labeled and mixed with 10 ng of unlabeled tRNA^{Phe}. pSI and pSI(100%

m⁷G) were *in vitro* transcripts, labeled with α -[³²P]-UTP. The samples were mixed on ice and M1 RNA was added. After 10 min on ice, 5 μ l of 50% glycerol, 0.03% dyes (xylene cyanol, bromophenol blue) were added. Then, the samples were applied on a 4 % non-denaturing polyacrylamide gel (200 $\times$ 200 $\times$ 0.5 mm) containing 100 mM MgCl₂ in addition to 90 mM Tris-borate. The running buffer contained 100 mM MgSO₄ (to prevent the formation of chlorine gas). Gels were prerun for one hour in the cold (4°C) with 130 V and about 40 mA. Electrophoresis was performed under the same conditions for about 4 hours, until bromophenol blue had migrated about 17 cm. Free tRNAs comigrated approximately with xylene cyanol tracking dye. The gels were stained with silver as described (6), dried on Whatman 3MM paper and exposed for autoradiography.

RESULTS

Effects of modified bases on the 5'-processing of a pre-tRNA

We have synthesized a pre-tRNA^{Ser}, pSI, where unmodified nucleosides were replaced 100% by their modified counterparts by using biotin-8-ATP, [α -S]-GTP and m⁷GTP. The sequence of pSI is shown in Figure 1. This RNA was obtained with SP6 RNA polymerase. By comparing transcription reactions with appropriate, different templates (see below), we found for SP6 RNA polymerase that [α -S]-GTP and m⁷GTP were incorporated with the same efficiency as GTP, whereas with T7 RNA polymerase and m⁷GTP, the transcript yields were reduced about fourfold (not shown). Very low RNA yields were obtained with biotin-8-ATP and SP6 RNA polymerase (about 1% of the normal values). No inhibition of the 5'-processing reactions was observed after the insertion of biotin-8-adenosine or of [α -S]-GTP (not shown). The cleavage in this pre-tRNA occurs between two guanosines (Figure 1), and the insertion of [α -S]-GTP could be useful for the analysis of the stereochemistry of the reaction (31, 32). In Figure 2, processing reactions with the unmodified pre-tRNA, pSI, are compared with the modified pSI(100% m⁷G).

Figure 1. Cloverleaf Model of Synthetic Pre-tRNA^{Ser} and Variants. Nucleotides are numbered according to the yeast tRNA^{Phe} system. The standard substrate (pSI) has a 28 nucleotide 5'-flank and terminates with G+73. The two short pre-tRNAs contain the nucleotide pm⁷G or pA, respectively, as 5'-flank and they include the 3'-terminal CCA [in brackets]. The predominant cleavage sites of RNase P holoenzymes (G+1) and catalytic M1 RNA (G-1) are indicated by arrows.

The standard pSI was efficiently cleaved, whereas pSI(100% m⁷G) was completely resistant.

All internal m⁷G's are present in processed tRNAs

In the following experiments we compared only the two extreme examples, the eukaryotic *S. pombe* RNase P holoenzyme and prokaryotic catalytic M1 RNA. The use of 3'-[³²P]-labeled, partially modified pre-tRNA pSI(50% m⁷G), allows the isolation of end-labeled, uncleaved pre-tRNA and 5'-matured tRNA. Each modified guanosine in the pre-tRNA which interferes with RNase P cleavage should be absent in the processed tRNA. All m⁷G's can be easily detected by direct aniline cleavage, a reduction with sodium borohydride is not necessary (29). The comparison of

Figure 2. Processing of Unmodified Pre-tRNA and m⁷G-Containing Pre-tRNA. The left half panel shows the products with unmodified pre-tRNA, pSI, obtained by transcription with GTP. On the right half, the same reactions were performed with 100% modified pre-tRNA, pSI(100% m⁷G), resulting from transcription with m⁷GTP. About 100 fmol of α -[³²P]-UTP-labeled pre-tRNAs were incubated under standard conditions. Lane C, without enzyme as control; lanes S.p. (two neighbouring lanes), with two different amounts, 1 μ l undiluted and tenfold diluted *S. pombe* RNase P; lanes E.c., with 2 μ l undiluted and tenfold diluted *E. coli* RNase P; lanes M1, with 50 and 5 ng M1 RNA. Lane L, partial alkali digest of a 5'-[³²P]-labeled RNA as a sequence ladder. With *S. pombe* RNase P, the shorter products were observed only with excessive enzyme amounts and it is possible that they were caused by a nuclease contamination. The positions of pre-tRNA (pSI), 5'-matured tRNA (SI) and liberated 5'-flank (5') are indicated.

Figure 3. Identification of m^7G -Positions not Interfering with RNase P Cleavages

Panel A. Partially modified pSI(50% m^7G) was 3'-[^{32}P]-labeled and subjected to standard processing reactions. The gel-purified reaction products were cleaved with aniline and analyzed on sequencing gels. An 8% polyacrylamide gel is shown here as an example. Cleavages occurred at all guanines and the bands are numbered according to Figure 1. Lane C, unprocessed pre-tRNA from a control reaction without enzyme; lane S.p., 5'-matured tRNA with *S. pombe* RNase P; lane M1, processed RNA with M1 RNA. Lane L, an alkali digest as sequence ladder.

Panel B. End group analyses of pre-tRNA and processed RNAs. The three samples were obtained as described for panel A. After digestion with RNase T2, the nucleoside-3'-monophosphates were identified by thin layer cochromatography with authentic markers, visualized under UV-light. The densitometer tracings are shown below the autoradiogram and the percentages of the integrated values are given for the indicated peaks. m^7Gp is resolved only as a shoulder from the peak for Ap, and the separating line for the integration is marked.

Panel C. Processing of a short pre-tRNA with $^{32}Pm^7G$ as 5'-flank and without internal m^7G 's. As shown by Burkard et al. (34), this short pre-tRNA can be cleaved by M1 RNA. In pre-tRNAs with a long 5'-terminal flank, this is a secondary cleavage of the primary reaction product. Lane P1, liberation of the 5'-terminal nucleoside-5'-monophosphates with nuclease P1. Lane C, control incubation of the pre-tRNA (about 15 fmol) under standard conditions without enzyme; lane S.p., with 1 μ l *S. pombe* RNase P; lane M1, with 50 ng M1 RNA.

the obtained cleavage patterns with pre-tRNA and processed tRNAs did not reveal any difference; an example is shown in Figure 3A. With this analysis technique we could show that almost all m⁷G's in the mature tRNA domain do not interfere with RNase P cleavage. For several positions, however, no results were obtained at this time. These were the guanosines at the 3'-end (G+73) and at the 5'-end of the tRNA (G-1 and G+1).

For G+73, we determined the 3'-terminal nucleosides of the samples analyzed in Figure 3A. The nucleoside-3'-phosphates were liberated by treatment with RNase T2 and separated by thin layer chromatography (Figure 3B). In addition to the expected guanosines, also Cp and Ap were detected. This was due to the imprecise termination of RNA polymerases, adding one or two extra 3'-terminal nucleosides beyond the end of the template (22). The gel-purified material was still cross-contaminated with these longer transcripts. Due to the preference of T4 RNA ligase for adenosines and cytidines (33), these nucleotides are rather prominent in the 3'-end group analysis. The ratio of Gp and m⁷Gp was also far from the expected ratio of 1:1 (50% GTP and m⁷GTP were used in the transcription reaction). Again, this may be caused by different efficiencies in the ligase labeling reactions. However, it is evident that pre-tRNA (lane C in Figure 3B) and processed tRNAs (lanes S.p. and M1) show a similar ratio of about 27 ± 3 to 1 for Gp and m⁷Gp, as determined by densitometer analysis. This means, that also m⁷G+73 does not interfere with RNase P cleavage.

The m⁷G immediately preceding the cleavage site can be liberated by RNases P

For the analysis of G-1, a different, very short pre-tRNA was synthesized. The template DNA was constructed using the simplified approach for the synthesis of mutated RNAs. This method with 'initiator' and 'terminator oligos' was presented in detail recently, and here we used the described template 'I-24' (22). In this pre-tRNA, the 5'-flank was only one guanosine. A pre-tRNA with the same 5'-flank is the primary cleavage product with M1 RNA (Figure 2; ref.34). It was shown recently, that such short pre-tRNAs can be cleaved again by M1 RNA (34). However, this reaction is rather inefficient (Krupp et al., unpublished data) and an improved cleavage was desired. Here, the general observation was helpful, that an up to tenfold increased reaction rate with M1 RNA can be achieved, if the pre-tRNA contains the 3'-terminal CCA-sequence of mature tRNAs (35). Therefore, these short pre-tRNA constructs were made with a 3'-terminal CCA (see Figure 1). The introduction (at least partially) of m⁷G as the first nucleotide in the synthesized pre-tRNA was possible by including high concentrations of nucleoside-5'-monophosphate (pm⁷G) as initiating nucleotide (23). Prior to processing studies, the isolated transcript was dephosphorylated and 5'-[³²P]-labeled. The end group analysis with nuclease P1 is shown in Figure 3C (lane P1). The products of RNases P reactions were analyzed on the same thin layer plate. With densitometer tracing we found 64 to 67% ³²pm⁷G in all three samples. Consequently, also the modification of the guanosine at the 5'-side of the cleavage site does not inhibit RNase P.

These results were obtained with a short, one nucleotide long 5'-flank. In order to exclude the possibility that the resistance to RNase P had been caused by the now absent guanosines in the original 28 nt. long flank, two additional modified variants were analyzed. They had one guanosine as 5'-flank and were obtained by transcription with 100% m⁷GTP, using pG or pm⁷G as primer. Both pre-tRNAs were completely resistant to cleavage

by *S. pombe* RNase P or by M1 RNA (not shown), as already observed for the long pre-tRNA, pSI (Figure 2). From these results, we must conclude that one guanosine or perhaps the combination of several guanosines in the mature tRNA is responsible for the resistance.

The guanosine immediately following the cleavage site is crucial for the reaction

The last possibly important unique guanosine was the 5'-terminal nucleoside in the 5'-matured tRNA. This corresponds to G+1 for RNase P holoenzymes and predominantly G-1 for M1 RNA, see Figures 1 and 2, and ref. 34. For the analysis of this position, the processed tRNAs were purified. The corresponding pre-tRNA, pSI(50% m⁷G), had been previously internally labeled with α -[³²P]-UTP and additionally 3'-[³²P]-labeled with 5'-[³²P]-pCp. Thus, labeled processed tRNAs could be isolated. For the analysis of the 5'-terminal nucleotides, the RNAs were dephosphorylated and 5'-[³²P]-labeled. The products were again gel-purified and their 5'-end groups were determined after cleavage with nuclease P1 (Figure 4A). Quantitative data were obtained by densitometer tracing. The uncleaved pre-tRNA contains 62% pG and 38% pm⁷G (lane C, Figure 4A), reasonable values for pSI(50% m⁷G). The product with M1 RNA contained the unpaired G-1 and had been labeled very efficiently. Here, exclusively (above 97%) unmodified guanosine was obtained (lane M1, Figure 4A). With *S. pombe* RNase P, the 5'-terminal G+1 was in the stable acceptor stem and poorly labeled. Consequently, the previous history of the tRNA is revealed by the presence of ³²pU (10%) from labeling with α -[³²P]-UTP and ³²pC (39%) from the 3'-terminal ³²pCp. However, a clear result was obtained for the liberated 5'-flank; unmodified ³²pG was detected in high amounts (47%), whereas only a very low amount of ³²pm⁷G was found (about 4%) (lane S.p. in Figure 4A). It is unlikely, that the exclusion of m⁷G in these positions is due to the artifactual 'gel exclusion' (see below), because strong, G-specific cleavages were observed with the pre-tRNA (Figure 3A).

All results reported so far, suggest that the guanosine, 3'-proximal to the cleavage site, is the only detectable, crucial contact point between pre-tRNA and RNase P activities. However, apart from the clearer direct identification of m⁷G and G by thin layer chromatography, the results with aniline cleavage products are more indirect. As a potential problem, unspecific fragmentation may occur, especially evident at positions in the anticodon loop (Figure 3A). This may mask missing m⁷G's in the processed tRNAs. The variable band intensities of authentic m⁷G's are also not expected, because 50 % m⁷GTP should have been incorporated at every position (see also Discussion).

For a definite resolution of this problem, we synthesized a pre-tRNA which starts with ³²pAG... (pA is the 5'-flank) and contains 100% m⁷G at all other positions. The template DNA was again constructed with two oligos and the 'initiator oligo' encoded the mature 5'-terminus, starting at G+1. The use of the dinucleotide primer ApG permitted the introduction of an extra 5'-terminal adenosine in the transcript (24) and its simplified 5'-labeling. We used ApG as primer, because unprecise initiation had been observed with GpG (24). Again, the 3'-CCA was included for improved cleavage by M1 RNA (35). This short pre-tRNA was processed with *S. pombe* RNase P, albeit with a lower rate, and very efficiently by M1 RNA (Figure 4B). This demonstrated that all guanosines in this pre-tRNA, except the one following the cleavage site, can be modified to m⁷G,

Figure 4. Identification of m⁷G's in Pre-tRNAs which Inhibit 5'-Maturation

Panel A. Processing of partially modified pSI(50% m⁷G). Here, we used a pre-tRNA, internally labeled with α -[³²P]-UTP and previously also 3'-[³²P]-labeled with pCp for other analyses. The uncleaved pre-tRNA and the 5'-matured tRNAs were gel-purified after standard processing reactions. They were dephosphorylated, 5'-[³²P]-labeled and again gel-purified. The 5'-end groups were determined after cleavage with nuclease P1 by cochromatography with authentic markers. The 5'-labeling efficiency of *S. pombe* RNase P cleaved tRNA was very low (G+1 is base-paired), and consequently, the internal pU and 3'-terminal pC were detected in addition to the 5'-terminal guanosine. The densitometer tracings are shown below the autoradiogram and the percentages of the integrated values are given for the indicated peaks.

Panel B. Processing of a short pre-tRNA with ³²pA as 5'-flank, unmodified G+1 and 100% internal m⁷G's. About 5 fmol (2000 cpm) 5'-[³²P]-labeled pre-tRNA were incubated under standard conditions. Lanes C and CM, without catalyst under holoenzyme conditions and catalytic RNA conditions, respectively. Lanes 1-3, with 2 μ l undiluted, fourfold and twentyfold diluted *S. pombe* RNase P; lanes 4-6, with 25, 5 and 1 ng M1 RNA, respectively. The products were analyzed by thin layer chromatography. The material migrating in the middle of the panel is possibly inorganic phosphate, and already present in the control lanes. Cleavage with M1 RNA was very efficient and the right half was obtained after a shorter exposure time.

without preventing the cleavage by RNase P holoenzyme or catalytic M1 RNA.

Gel retardation assays with M1 RNA

The 100% substitution of G by m⁷G in a pre-tRNA prevented 5'-processing. However, in contrast to the pre-tRNAs with destroyed base pairs in the acceptor stem (13), no aberrant cleavages were observed here (Figure 2). They could result from incorrect binding of the substrate by RNase P. In our case, it is possible that pSI(100% m⁷G) is not bound at all by RNases P or that binding can occur, perhaps properly. Only after the substrate is bound, the specific and aberrant cleavages are inhibited by the presence of m⁷G. For the detection of possible complexes of catalyst and substrates, we have chosen M1 RNA. It is a very well defined system, and both components can be visualized by autoradiography (substrate) or silver staining (catalyst). We have mixed the tRNAs and M1 RNA on ice in normal M1 RNA reaction buffer, but omitted the enhancing polyethylene glycol (26). The samples were analyzed in the cold on native 4% polyacrylamide gels containing 100 mM magnesium (Figure 5). No retarded products were observed if magnesium was absent during the electrophoresis (not shown). Here, only with pSI(100% m⁷G) a possibly specific retarded product was observed, using 10 ng (25 nM) pre-tRNA and 50 ng (40 nM) M1 RNA. This complex migrates just above the bulk free M1 RNA, detected by silver staining (Figure 5). This can be expected for a complex between the 380 nucleotide long M1 RNA and a 120 nucleotide pre-tRNA. No similar complex was observed with mature yeast tRNA^{Phe} and with unmodified pSI. Mature tRNA has a lower affinity, and the bound pSI may become processed during the long electrophoresis time (at least four hours) with subsequent release of the products. Under standard conditions at 37°C, 5 ng M1 RNA would be sufficient for complete cleavage of the pre-tRNA present in the assay.

DISCUSSION

For the study of pre-tRNAs containing the modified nucleoside m⁷G, we have synthesized RNAs directly with m⁷GTP. It was efficiently incorporated by SP6 RNA polymerase and with a moderately reduced rate by T7 RNA polymerase. This approach permitted the convenient control of the m⁷G content, i.e., 100% or 50% at each position. The use of an initiating nucleoside monophosphate (23) permits the specific insertion or exclusion of m⁷G at the transcription start, dinucleotide primers extend this to the free choice of an extra nucleotide in position -1 (24). With dinucleotides, RNAs are obtained with free 5'-hydroxyls for convenient direct 5'-[³²P]-labeling. This should be possible also without the addition of an extra nucleotide by using the guanosine nucleoside as primer (36).

These techniques were used to reveal which guanosines in a pre-tRNA, if any, must not be methylated in substrates cleaved by RNase P holoenzyme or catalytic M1 RNA. The first approach was the search for specific positions, where m⁷G was present in pre-tRNA, but excluded from 5'-matured tRNA. The presence of m⁷G can be simply detected by aniline cleavage of 3'-[³²P]-labeled RNAs. With this method, we did not observe any excluded m⁷G's in 5'-matured tRNAs. This analysis seems problematic because unspecific degradation can obscure the results and some guanosines can be absent completely in this

Figure 5. Formation of Complexes with M1 RNA and tRNAs

All reactions contained about 10 ng tRNA (10,000 cpm) and the indicated nanogram amounts of M1 RNA in 10 μ l standard catalytic RNA conditions, but without polyethylene glycol. tRNA^{Phe} was 3'-labeled; pSI(100% m⁷G) and pSI were α -[³²P]-UTP-labeled transcripts. The products were analyzed on a 4% native polyacrylamide gel, containing 100 mM magnesium. Free RNAs form diffuse bands, retarded material is seen only with pSI(100% m⁷G) and 50 ng M1 RNA. In this lane, the position of bulk, unlabeled M1 RNA is indicated by brackets. It was visualized by silver staining, followed by gel drying and autoradiography.

Figure 6. Model of the Cleavage Site

The model of an RNA dinucleotide was drawn according to Saenger (20) and the approaching magnesium cation, bound by M1 RNA or RNase P, is shown as suggested by Guerrier-Takada et al. (26). The positive charges of Mg²⁺ and at the N-7 of the guanosine, 3'-proximal to the cleavage site, are marked. Two arrows indicate possible directions of the Mg approach from the front (No. 1) or the rear (No. 2).

partial sequence ladder. This effect was termed 'gel exclusion', because it occurred only after gel purification of the alkylated RNAs (18). This effect may be caused by strand breakage at alkylated purines in the alkaline polyacrylamide sequencing gels (8M urea raises the pH to 8.9) at elevated temperatures (up to 55°C). Also elution and other treatments at pH values above 7 should be avoided. This phenomenon was minimized by electrophoresis at 40°C and elution at pH 7.0. However, uneven band intensities for m⁷G cleavages and unspecific degradation were still observed (Figure 3A).

In combination with this more indirect technique, we performed end group analyses of specific pre-tRNA constructs. Finally, only one crucial guanosine remained, the one 3'-proximal to the RNase P or M1 RNA cleavage sites, i.e., G+1 and G-1, respectively. Both catalysts can cleave at G+1 in a short pre-tRNA with a single adenosine as 5'-flank. We have shown that all other guanosines in this pre-tRNA could be replaced by m⁷G's without preventing 5'-maturation, if G+1 remained unmodified. This experiment confirmed the results of the combined, more indirect analyses. M1 RNA can efficiently process this highly modified pre-tRNA, whereas it is a poorer substrate for *S. pombe* RNase P holoenzyme. It is possible that in the holoenzyme, several other nonessential guanosine contacts are important in addition to the crucial G+1. The exceptional importance of the nucleotides in position +1 and +2 for efficient RNase P cleavage had been shown earlier (13).

In this report, we considered only absolutely essential unmodified guanosines and positions with only partially reduced cleavage rates were not analyzed.

The inhibition by m⁷G may be caused by two, intimately related processes. Contacts between bases of the substrate and the enzyme RNA could be affected. Here, Watson-Crick base pairing is not involved, because there is no interference by the methylation at N-7 of guanosines. For the interaction between bases, other pairing schemes have to be considered also, e.g. in Hoogsteen base pairs the free N-7 of the guanosine is required as proton-acceptor. This does not mean, that Watson-Crick base pairs are not important at all, since they may be important for additional contact points between pre-tRNA and RNases P. It is also plausible that the positive charge in m⁷G interferes with the approach of the magnesium cation, which could be the actual cleaving agent. A model for this situation is shown in Figure 6, according to Guerrier-Takada et al. (26). Two routes are possible and the positive charge at N-7 would make route No. 2 rather difficult. The same inhibitory effect of one specific m⁷G was observed for a prokaryotic ribozyme and a eukaryotic RNase P holoenzyme. This indicates that the detailed reaction mechanism may be conserved in all RNase P activities.

A more complete picture of the contacts between pre-tRNAs and RNase P activities will require the study of other tRNAs with different nucleotide sequences and the analysis of other important nucleotides. The insertion of biotin-8-adenosine did not inhibit RNase P activities. Similar to m⁷G, Watson-Crick base pairs were not disrupted. It is possible, that in pre-tRNA^{Ser}, no adenosines are involved in crucial contacts between pre-tRNA and enzymes. The biotin modification does not lead to cleavage by aniline and a more detailed study is not possible. Unfortunately, the direct synthetic approach cannot be extended. Apart from m⁷GTP, no other properly modified nucleoside triphosphates are available, which permit subsequent aniline cleavages of the modified RNAs. On the other hand, the good agreement between the results from aniline cleavage patterns and more direct approaches will encourage future chemical nucleotide modification studies.

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