

Modification interference approach to detect ribose moieties important for the optimal activity of a ribozyme

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ABSTRACT

A new approach for modification interference studies is presented. It involves the use of phosphorothioates as a handle to analyze any desired base or sugar modification. This method was applied to identify ribose and phosphate moieties which could be important in the pre-tRNA recognition of *E.coli* RNase P RNA (M1 RNA). The utility of this technique was confirmed by detecting the inhibitory effect of a deoxyribose in the 5'-flank (position -1). This site was already known to interfere with RNase P cleavage, if modified. We have analyzed pre-tRNA^{Tyr} and pre-tRNA^{Phe} and found different interference patterns for both tRNAs. Two unpaired regions were involved in both pre-tRNAs. Phosphorothioates interfered at the transition between acceptor- and D-arms. The results with deoxythymidines in the T-loop indicated that deoxyribose moieties or the extra methyl group in thymidine could interfere with RNase P cleavage. These data suggest that even in complete pre-tRNAs, only a few intact ribonucleotides are important in the substrate recognition by RNase P. We have demonstrated the potential of this new approach which offers many future applications in all fields involving nucleic acids, for example RNA processing, action of ribozymes, tRNA charging and studies related to DNA promoter recognition.

INTRODUCTION

Chemically synthesized RNAs which contain modified nucleotides at a few specific positions have been used to stabilize the RNAs and to elucidate the importance of these nucleotides in various studies. Prerequisites for such strategies are, first, the characterization of nuclease-resistant modifications, like phosphorothioates, or 2'-modified ribose moieties; second, the identification of the positions where modifications can be introduced into an oligonucleotide without loss of its activity. Unfortunately a serious drawback of this approach is the difficulty to predict all sites which can be modified without severely

effecting the catalytic activity of the ribozyme. This is a cumbersome analysis even for small RNAs, as experienced by others, and it will be difficult to draw a final conclusion, unless all positions are analyzed independently (1–6). Moreover, for large RNAs this approach will be more tedious or even impossible; already transfer RNAs are refractory. A powerful alternative could be the modification interference analysis (7–11). However, in most of the cases this approach requires methods for the detection of modified sites in the nucleic acid which is impossible for many modified nucleosides, like m⁵C.

We wish to report here a modification interference approach which can exploit phosphorothioates as a tool to analyze any kind of nucleoside modification in RNA and DNA. It is based on the presence of a low level (about 5-10%) of the modified nucleotide in enzymatically synthesized RNAs. The modification of interest is incorporated into an RNA transcript using an appropriate mixture of normal nucleoside triphosphates together with a modified NTP α S. As a result, each position with a modified base (or sugar) is next to a labile thio-substituted phosphodiester bond. In this fashion, any modification in sugar or base moieties can be combined with a scissile nucleic acid backbone; useful for both, RNA and DNA.

As an example we analyzed which ribose moieties in pre-tRNAs should remain intact for efficient cleavage by RNase P. RNase P is a ribonucleoprotein which makes a single endonucleolytic cut in the pre-tRNA forming the mature 5'-terminus of the tRNA molecule (12). Protein and RNA components of RNase P are essential *in vivo* (13), however, *in vitro* the eubacterial RNA component acts as a true enzyme (ribozyme) and it is able to cleave tRNA precursors at the correct site (14,15). In our study we used the *E. coli* RNase P RNA (M1 RNA).

Towards this end, we used dTTP α S and dATP α S to locate 2'-OH moieties which must remain intact for efficient pre-tRNA processing by RNase P. Obviously, one prerequisite of this analysis is the possibility to perform enzymatic RNA synthesis with the modified NTPs α S. However, only dATP α S and dTTP α S were efficient substrates for T7 RNA polymerase (more details about the use of T7 RNA polymerase and 2'-deoxy- or 2'-O-methyl NTPs α S will be published elsewhere).

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MATERIALS AND METHODS

Template preparations

The plasmid p67YFO containing the gene for mature *S. cerevisiae* tRNA^{Phe} (16) was a gift from Olke C. Uhlenbeck, Colorado, USA; plasmid pSU3 carrying the gene for *E. coli* pre-tRNA^{Tyr} (17) was a gift from Leif Kirsebom, Uppsala, Sweden. The plasmids p67YFO and pSU3 were digested with *Bst* NI (NE-Biolabs) and *Fok* I (NE-Biolabs) respectively.

Synthesis of pre-tRNA transcripts

RNAs containing 2'-deoxynucleoside phosphorothioates were obtained by transcription reactions in which one of the NTPs was replaced by 0.9 mM dNTP α S (Pharmacia, Uppsala) and 0.1 mM of the corresponding NTP. This resulted in 5–10% incorporation of the dNTP α S as evident from iodine-cleavage patterns. To obtain a similar modification level for ribonucleoside phosphorothioate containing pre-tRNAs, it was sufficient to use 0.1 mM NTP α S and 0.9 mM of the corresponding NTP. The concentration of the other NTPs was 0.5 mM. In all reactions a dinucleotide, adenylyl-guanosine (ApG, Sigma) as an initiator has been used (a 4:1 ratio of the dinucleotide to GTP) to facilitate the subsequent [5'-³²P]-end labeling of the pre-tRNAs, which otherwise would require a phosphatase treatment (20).

Preparation of M1 RNA

The plasmid pDW27 (19), containing a gene for M1 RNA behind the T7 promotor was a kind gift of Norman Pace, Indiana University, Bloomington, IN. The plasmid was cleaved with *Sna* BI (Boehringer Mannheim) and transcribed with 0.5 mM NTPs as described earlier (20).

Processing of pre-tRNAs

The processing of 5'-end labeled pre-tRNAs (about 200 fmoles) were carried out in 50 μ l reactions as described previously (20). A 1:1 molar ratio of M1 RNA to the substrate as the highest amount of enzyme and two serial tenfold dilutions were used. A control without M1 RNA was always included under identical conditions. After incubation for 1 h at 37°C the reaction products were applied on 8% denaturing polyacrylamide gels. The unprocessed substrates and products were visualized by autoradiography, excised and isolated by standard methods (18).

RNA analysis

(i) *Sequencing* The ³²P-labeled phosphorothioate transcripts were sequenced essentially according to the method reported by Schatz *et al* (21). The 5'-end labeled pre-tRNAs were dissolved in 10 μ l of 10 mM HEPES (pH 7.2), incubated at 70°C for 3 min, chilled in ice, spun briefly and treated with 1 μ l of 30 mM I₂/ethanol solution (w/v). The above reaction mixture was left for 1 min at room temperature, excess reagent was removed by ethanol precipitation and the pellet was washed with 70% aqueous ethanol. The dry samples were dissolved in 4 μ l water, 6 μ l urea/dye mixture (8 M urea, 0.03% xylene cyanol, bromophenol blue) and loaded directly on 8 or 20% denaturing polyacrylamide gels without prior heating.

(ii) *Densitometer tracing* An Ultra XL, enhanced Laser Densitometer (Pharmacia-LKB) was used for all densitometer analyses. The analyses of the extent of precursor tRNAs cleavage by RNase P and the autoradiograms of iodine cleavage patterns of uncleaved pre-tRNA were analyzed as described earlier (22).

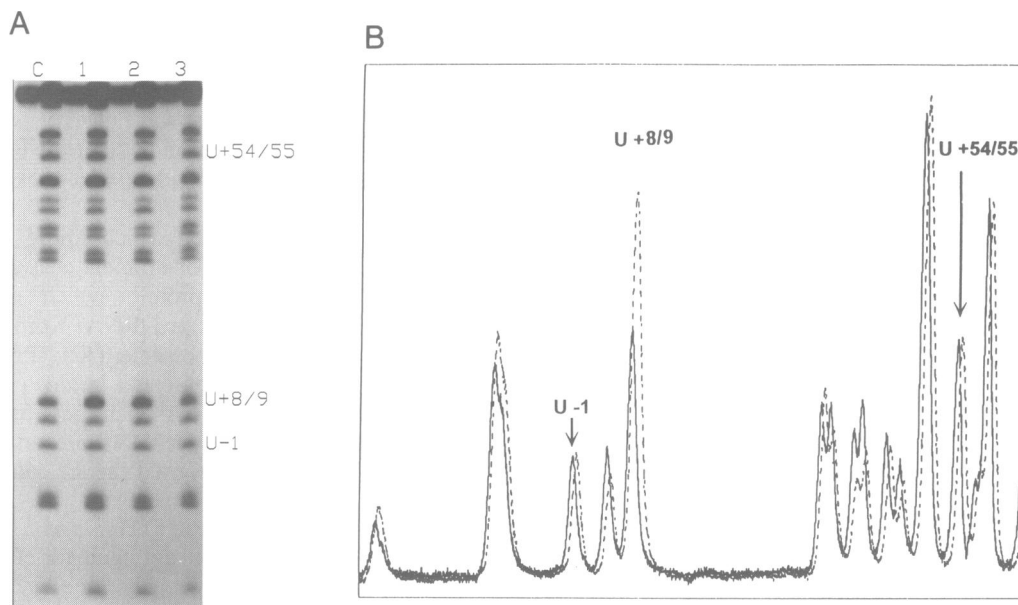


Figure 1. Modification interference with pre-tRNA^{Tyr} and uridine α S triphosphate. **Panel A** Sequencing gel with phosphorothioate cleavage patterns. The [5'-³²P]-labeled pre-tRNA^{Tyr} was incubated with *E. coli* RNase P RNA (M1 RNA). The uncleaved pre-tRNAs were gel-purified. We obtained cleavage of about 50% (1:1 molar ratio of M1 RNA to pre-tRNA; material analyzed in lane 1); 27% (10fold molar excess of pre-tRNA; analysis in lane 2) and 2% (100fold molar excess of pre-tRNA; analysis in lane 3). The RNAs were treated with iodine and analyzed on a 20% denaturing sequencing gel. Lane C, control without M1 RNA. The left lanes always contain RNAs not treated with iodine while the material in the right lanes was treated with iodine to cleave the thio-phosphodiester bonds. **Panel B** Densitometer tracings of lane C (solid line) and of lane 1 (broken line). In both panels, we indicated the relevant uridine positions. In addition to U+8/9 (interference), also U-1 and U+54/55 (no interference) are marked for more convenient comparison of the results with deoxythymidine (Fig. 2).

For each lane the average value was determined for four slightly shifted (800 μm steps), non-overlapping line tracings.

RESULTS

Interference sites in pre-tRNA^{Tyr}

Throughout this report we describe the use of [5'-³²P]-labeled phosphorothioate containing pre-tRNAs which were incubated without enzyme or with increasing amounts of M1 RNA (the catalytic *E. coli* RNase P RNA), isolation of the uncleaved substrates followed by their iodine treatment and analysis on sequencing gels. All products were treated identically to avoid possible gel artifacts (23). We have varied the enzyme to substrate ratio from 1/100 to equimolar, and routinely obtained a cleavage efficiency of pre-tRNA by RNase P ranging from 2 to 50%, as determined by densitometer tracing. The interference sites were identified by comparing the I₂/ethanol cleavage patterns of uncleaved pre-tRNAs (which were incubated with M1 RNA and isolated by gel electrophoresis) with the pre-tRNA incubated in the absence of M1 RNA. These comparisons were made by superimposing the densitometer traces of the gels obtained after iodine cleavage reactions of pre-tRNAs incubated in the presence or absence of M1 RNA.

We have analyzed *E. coli* pre-tRNA^{Tyr}-UMPS and the results are shown in Fig. 1. The structure of pre-tRNA^{Tyr} is shown in Fig. 5. It is evident from the densitometer tracings (Fig. 1B) that only the intensity of the band for U+8/9 was highly increased in the unprocessed substrates. This indicates that the corresponding thiophosphate interferes with RNase P cleavage; it was not possible to resolve the double band for both uridines, due to severe band compressions caused by base pairs formed between 5'-flank and acceptor stem.

For the results shown in Fig. 2 we used pre-tRNA^{Tyr}-dTMPS. In addition to thiophosphates, inhibitory effects of the 2'-deoxy groups were observed (possibly, also due to the extra methyl group in thymidines). These are evident by the accumulation of

dT+54/55 and dT-1. In agreement with our observations, it is already well documented that the 2'-hydroxyl at the cleavage site (position -1) is required for the efficient cleavage of pre-tRNAs by RNase P (24,25).

Mapping interfering sites in pre-tRNA^{Phe}

We have also analyzed a pre-tRNA with a short extra arm, the well characterized yeast tRNA^{Phe}. Its cloverleaf structure is shown in Fig. 6. Here, already the ribonucleoside phosphorothioates at U+54/55 and U+68/69 interfered. Essentially the same interference pattern was obtained with 2'-deoxy-thymidines (data not shown). This suggests that no additional uridine positions have important 2'-hydroxyl groups. The possibilities to compare both tRNAs were limited. The cytidines at +68/69 in tRNA^{Tyr} were not analyzed because dCTP α S was a poor substrate for T7 RNA polymerase (data not shown). In addition, position +9 is a uridine in tRNA^{Tyr} whereas it is an adenosine in tRNA^{Phe}. Therefore, we decided to analyze adenosines in tRNA^{Phe} also.

The interference analysis with pre-tRNA^{Phe}-AMPS is shown in Fig. 3. In addition to minor effects, a strong interference was observed at adenosine +9. Similarly, in the analysis with dATP α S, dA+9 interfered (Fig. 4). In contrast to the ribonucleoside phosphorothioates (Fig. 3) the analysis with pre-tRNA^{Phe}-dAMPS revealed a significant interference effect for dA+58 (Fig. 4). This suggests, the 2'-hydroxyl of the ribose at position +58 is important.

DISCUSSION

In general, most of the modification interference approaches are based on either chemical modifications of a normal transcript (22,23,26,27) or the RNA synthesis can be performed with a mixture of normal and modified NTPs (10,11,20). The modification interference approach has been applied successfully, for phosphorothioates inserted during transcription reaction

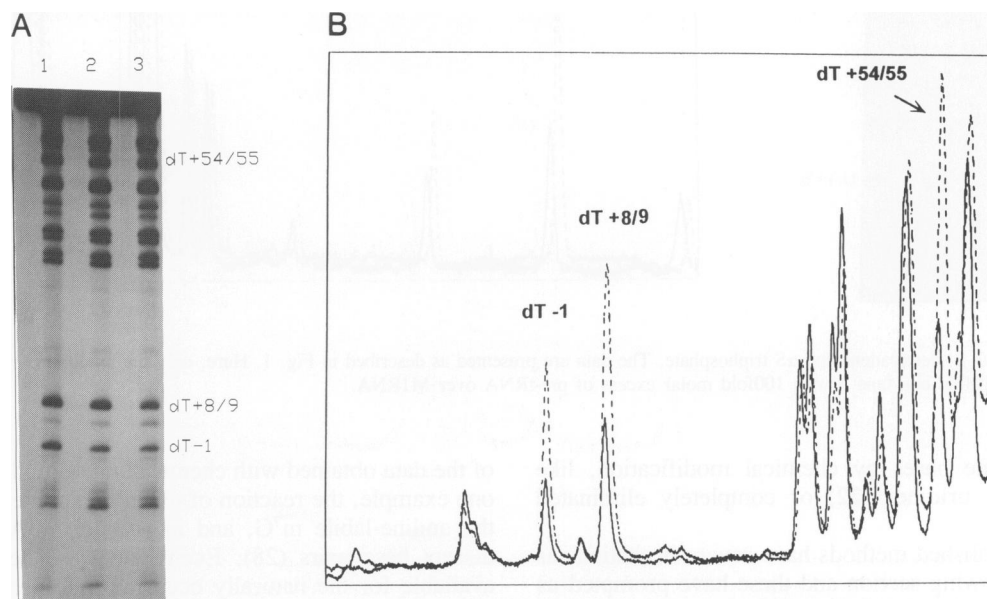


Figure 2 Pre-tRNA^{Tyr} with 2'-deoxy-thymidine α S triphosphate. The samples were analyzed and presented as described in Fig. 1. This example shows the effects of increasing amounts of M1 RNA, no control without RNase P RNA is shown. The densitometer tracings in panel B compare the products obtained with the lowest amount of M1 RNA (lane 3 in panel A; solid line in panel B) and the highest, 100fold higher amount (lane 1 in A; broken line in B).

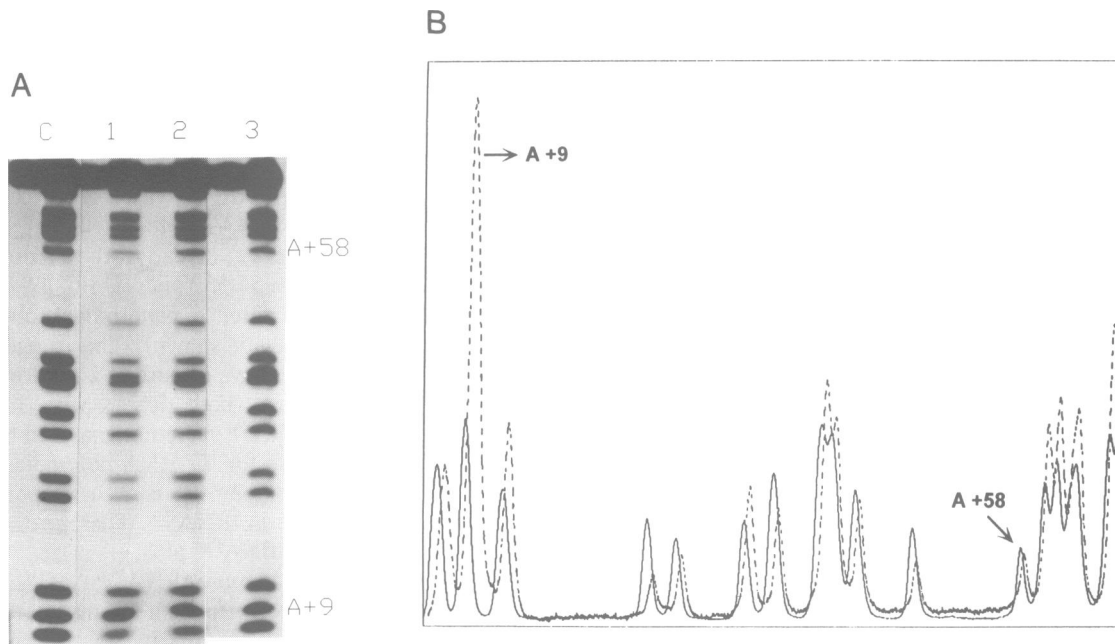


Figure 3 Pre-tRNA^{Phe} with adenosine α S triphosphate. The analysis was essentially performed as in Fig. 1, but here an 8% sequencing gel was used. In addition to A+9 (interference), also A+58 (no interference) is marked for more convenient comparison of the results with deoxyadenosine (Fig. 4).

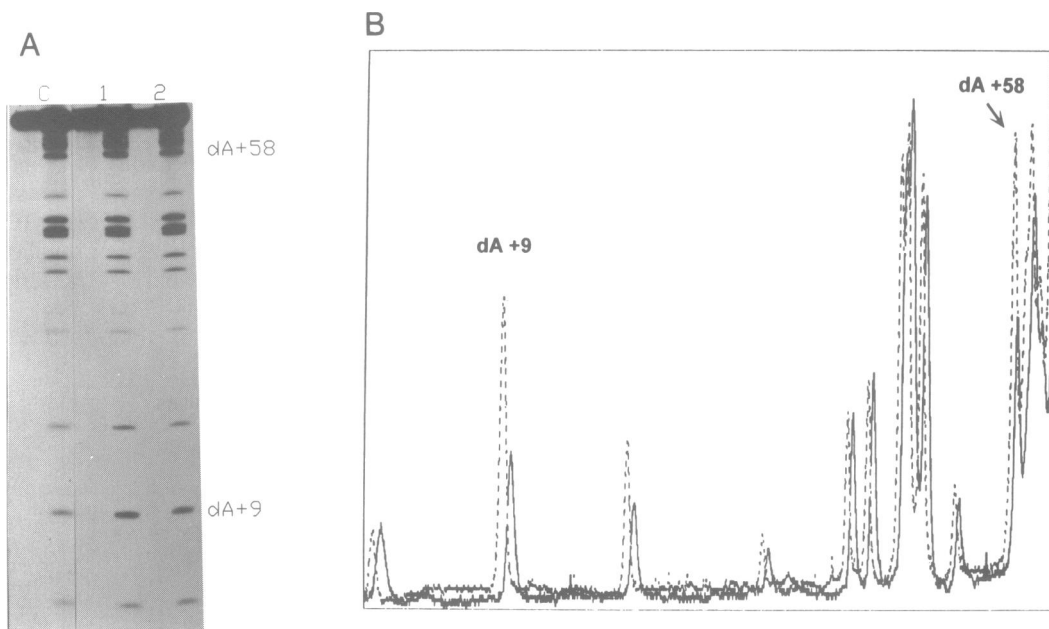


Figure 4 Pre-tRNA^{Phe} with 2'-deoxyadenosine α S triphosphate. The data are presented as described in Fig. 1. Here, only the products of two RNase P reactions are shown. Lane 1 with 10fold and lane 2 with 100fold molar excess of pre-tRNA over M1RNA.

(10,11) and for some bases by chemical modification, like alkylated purines or uridines (22) or completely eliminated pyrimidines (27).

However, the established methods have severe limitations as discussed in the following section and these have prompted us to develop a new approach. It is well established that in addition to the desired product, the chemical modification of nucleic acids results in the formation of other products which can even prevent Watson-Crick base pairing. This may obscure the interpretation

of the data obtained with chemically modified nucleic acids. As one example, the reaction of dimethyl sulfate with RNA yields the aniline-labile m⁷G, and in addition m¹A and m³C which disrupt base pairs (28). Even worse, no detection system is available for the naturally occurring modified bases like m⁵C, m²G, m⁶A, inosin, ribo-thymidine in RNA or 2'-deoxy-uridine in DNA.

In principle, the resistance against hydrolysis of the phosphodiester backbone next to 2'-modified nucleosides in RNA

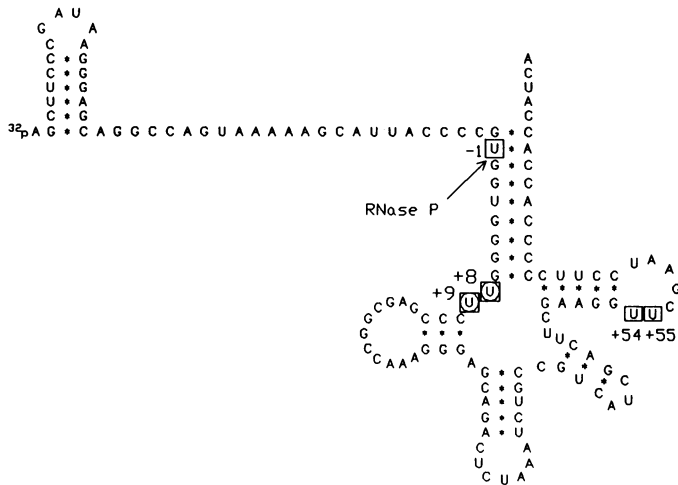


Figure 5 Cloverleaf model of pre-tRNA^{Tyr}. The [5'-³²P]-labeled pre-tRNA is shown, obtained by transcriptions in the presence of ApG dinucleotide. An arrow indicates the RNase P cleavage site and the modification interference sites are marked by circles (phosphorothioates) and squares (2'-deoxyribonucleotides).

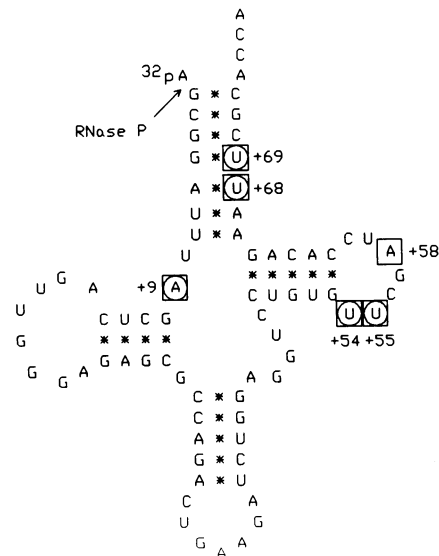


Figure 6 Cloverleaf model of pre-tRNA^{Phe}. The structure and results are shown as described for Fig. 5.

can be used as a detection method (5,29). However, it will be difficult to detect the desired low percentage of 5–10% modification in an RNA and this will be impossible in DNA. These considerations, together with the recently developed complex procedure to study 2'-deoxy-uridines in protein-DNA interactions (30) highlight the inherent limitations of the current approaches and demand for a better method of detection.

Obviously, all data obtained with the modification interference approach are only indirect. However, they are a useful first step in guiding future, more detailed and direct work with synthesized RNAs carrying the modification at defined positions only. We present an approach which is based on a phosphorothioate modification in the internucleotide phosphate backbone combined with the sugar or base modification of interest. This analysis is restricted to the S_P-diastereomer of the phosphorothioate since only the S_P diastereomer is a substrate for T7 RNA polymerase and is incorporated into the transcript with inversion of the configuration (31). We have used this method to identify the 2'-OH moieties in pre-tRNA^{Phe} and pre-tRNA^{Tyr} which could be important for efficient cleavage by RNase P. In order to distinguish between the thiophosphate interference and the interference due to sugar modification, we have compared the iodine cleavage patterns of pre-tRNA transcripts obtained with NTPαS with the iodine cleavage patterns of the corresponding pre-tRNA transcripts obtained with dNTPαS. The observed interfering sites are depicted in Figs. 5 and 6. In pre-tRNA^{Tyr}-UMPS, only thiophosphates at U+8/9 interfered with RNase P cleavage whereas with pre-tRNA^{Tyr}-dTmPS additional interference sites at -1 and +54/55 were identified. This suggests that 2'-hydroxyl at position -1, +54/55 are important for efficient RNase P cleavage; also the possible importance of the extra methyl group in thymidine should be considered. It was already known that a deoxyribose at the RNase P cleavage site (position -1) is inhibitory (24) and we have also detected it with our approach. In this study it was not possible to decide if the interference at position +8/9 was only due to the modified phosphate backbone or due to both, backbone as well as sugar modification. In pre-tRNA^{Phe}, thiophosphates at U+54/55,

U+68/69 and A+9 were inhibitory. These interference sites were also observed with 2'-deoxy-nucleosides, but with 2'-deoxy-adenosine an additional site was located at dA+58.

The two pre-tRNAs gave different results, however, position +9 was always inhibitory. Three other interference sites, U+54/55 and A+58 were located in the T-loop which is an important contact area between substrate and enzyme, evident from base modification interference studies (22) and from the observation that a distorted T-loop can abolish RNase P cleavage completely (32). We had reported previously that a pre-tRNA with a short extra arm (pre-tRNA^{Met}) has more contact points in the T-arm and base modifications were severely inhibitory, unlike a pre-tRNA with a long extra arm (pre-tRNA^{Ser}) (22). These differences agree with the data presented here. The inhibition in the RNase P cleavage of pre-tRNA^{Phe} (which has a short arm) was observed with both, thiophosphate and deoxyribose substitutions, whereas in pre-tRNA^{Tyr} (which has a long extra arm) only the deoxyribose substitutions were inhibitory.

In recent studies, additional important ribose moieties were identified near the cleavage site at positions -2, -1, +1 (25) and at the first cytidine (+74 in conventional tRNA numbering) in the 3'-terminal CCA-sequence (25). Small effects were also found in the 3'-terminal half of the acceptor stem, comparable to our interference data with U+68/69. In pre-tRNA^{Tyr}, we also detected the inhibition of a 2'-deoxyribose in position -1, whereas an analysis of the other sites was not possible in our system. In this comparison, it is important to note that Perreault and Altman (25) used small, chemically synthesized model substrates which lacked the complete D-, anticodon- and extra arms, as well as all connecting nucleotides. All these elements are not absolutely essential for RNase P cleavage, but they are clearly important, as evident from reduced cleavage efficiencies of minimal substrates. In the T-loop of the model substrates, no effect was observed after introducing 2'-deoxyriboses in the conserved sequence GUUC (the positions from +53 to +56,

referring to tRNA numbering); the position +58 was not analyzed by Perreault and Altman (25). In contrast to the findings with a minimal hairpin substrate, our data with full-size pre-tRNAs suggest that the 2'-hydroxyls at +54/55 and +58 may be important and this could be caused by the different RNA structures. The hairpin substrates contain the acceptor- and T-stems in one continuous helix with twelve base pairs, whereas the two helices are separated in tRNAs. Very likely, the structure of the D-arm region effects the stacking interactions of the helices, moreover, their geometries will be more easily effected by a distorted T-loop.

As a first step, the data reported here have pinpointed a number of positions where modified phosphates or ribose moieties can be expected to interfere with RNase P cleavage. Future studies with several chemically synthesized pre-tRNAs are needed to confirm these effects and they will reveal additional details.

As a more general basis for further studies, we have shown that phosphorothioates can serve as a handle to perform modification interference analyses with otherwise refractory modified sugar moieties. With an increasing number of available modified NTPs α S and dNTPs α S this approach can be useful in a variety of different fields.

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