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# Enzymatic RNA synthesis with deoxynucleoside 5′-*O*-(1-thiotriphosphates)

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We have investigated the incorporation of 2′-deoxynucleoside-5′-*O*-(1-thiotriphosphates) into RNA transcripts using T7 RNA polymerase. With the exception of [α-S]dGTP, we obtained full-length transcripts of pre-tRNA$^{Phe}$ and pre-tRNA$^{Tyr}$ using an appropriate mixture of 2′-deoxynucleoside 5′-*O*-(1-thiotriphosphate) and the corresponding normal nucleoside triphosphate. The yields of the transcripts were comparable to those obtained with unmodified NTPs. Both substrates, [α-S]dTTP and [α-S]dATP, were inserted specifically. However, [α-S]dCTP was excluded at specific sites. We could not obtain transcripts using the deoxyguanosine derivative.

T7 RNA polymerase; Transcription, in vitro; Modification interference; Pre-tRNA; Modified nucleotide

## 1. INTRODUCTION

The last few years have seen a growing interest among molecular biologists in understanding the mechanism of a number of reactions involving nucleic acids, e.g. RNA self-splicing [1], catalytic action of ribozymes [2], pre-tRNA cleavage by RNase P RNA [3] and the specificity of the charging of tRNA, microhelices and more recently RNA tetra loops by the cognate aminoacyl tRNA synthetases [4–6]. Considerable progress has been made in the identification of nucleotides which are crucial and therefore must remain intact in these processes.

Recently, 2′-deoxy-, 2′-*O*-methyl-, 2′-fluoro-2′-deoxy- and 2′-amino-2′-deoxyribonucleotides were incorporated into oligoribonucleotides by phosphoramidite based automated chemical synthesis to identify the positions where the 2′-hydroxyl group is essential for the catalytic activity of hammerhead ribozymes [7–10] and the cleavage of minimal substrate by RNase P RNA [11]. However, the chemical synthesis of oligoribonucleotides is limited to relatively short RNAs and for longer RNAs, the enzymatic RNA synthesis is still the method of choice.

In vitro synthesis of RNA using T7 or SP6 RNA polymerases has become a powerful method for the synthesis of virtually any RNA sequence, using normal and a few modified nucleoside triphosphates [12–16]. In this context, we [17] and others [18] have reported earlier the site-specific incorporation of modified nucleotides in RNAs by combining chemical and enzymatic techniques. However, the modification of interest could only be introduced at a specific site in the RNA transcripts.

These fully modified RNAs are useful in a variety of studies; however, for the modification interference approach it is desirable to have partially modified RNAs [19]. Here we wish to report a versatile method for the synthesis and analysis of such partially modified RNAs, i.e. transfer RNAs using [α-S]dNTP with T7 RNA polymerase. The $S_P$ diastereomers of nucleoside 5′-*O*-(1-thiotriphosphates) are substrates for T7 RNA polymerase and they are inserted into the RNA transcript with an inversion of configuration, thereby generating an $R_P$ phosphorothioate [20]. The specificity of the $I_2$/ethanol treatment to cleave the phosphorothioate bond selectively made it possible to identify the sites in the transcript where the [α-S]dNTPs were inserted or excluded.

## 2. MATERIALS AND METHODS

### 2.1. *Transcription reaction*

The plasmid p67YFO containing the gene for mature *S. cerevisiae* tRNA$^{Phe}$ behind a T7 promoter was a generous gift from Olke C. Uhlenbeck (Colorado, USA) [21] and the plasmid pSU3 encoding *E coli* pre-tRNA$^{Tyr}$ was a kind gift from Leif Kirsebom (Uppsala, Sweden) [22]. For run-off transcriptions, the plasmids p67YFO and pSU3 were cleaved with *Bst*NI (NE-Biolabs) and *Fok*I (NE-Biolabs), respectively.

A 50 μl transcription reaction consists of 40 mM Tris-HCl (pH 8.0), 20 mM $MgCl_2$, 2 mM spermidine, 10 mM DTT, 8% PEG 6000 (Serva), 0.01% Triton X-100 (Boehringer Mannheim), 2.5 μg template DNA, 100 units of T7 RNA polymerase (Pharmacia), 2 mM dinucleotide

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*Abbreviations:* [α-S]NTP, ribonucleoside 5′-*O*-(1-thiotriphosphate); [α-S]dNTP, 2′-deoxynucleoside-5′-*O*-(1-thiotriphosphate); NTPs, nucleoside triphosphates; dNTPs, deoxynucleoside triphosphates.


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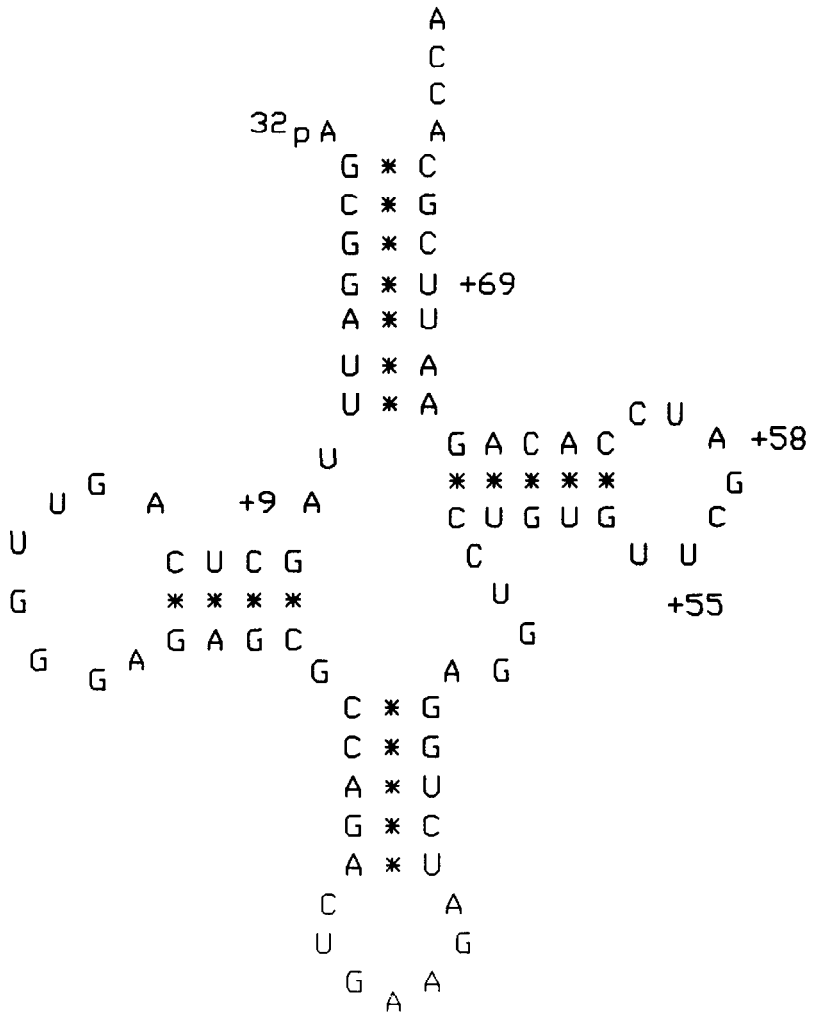


Fig. 1. Cloverleaf model of pre-tRNA$^{Phe}$. This transcript was obtained with ApG as initiator dinucleotide [17].

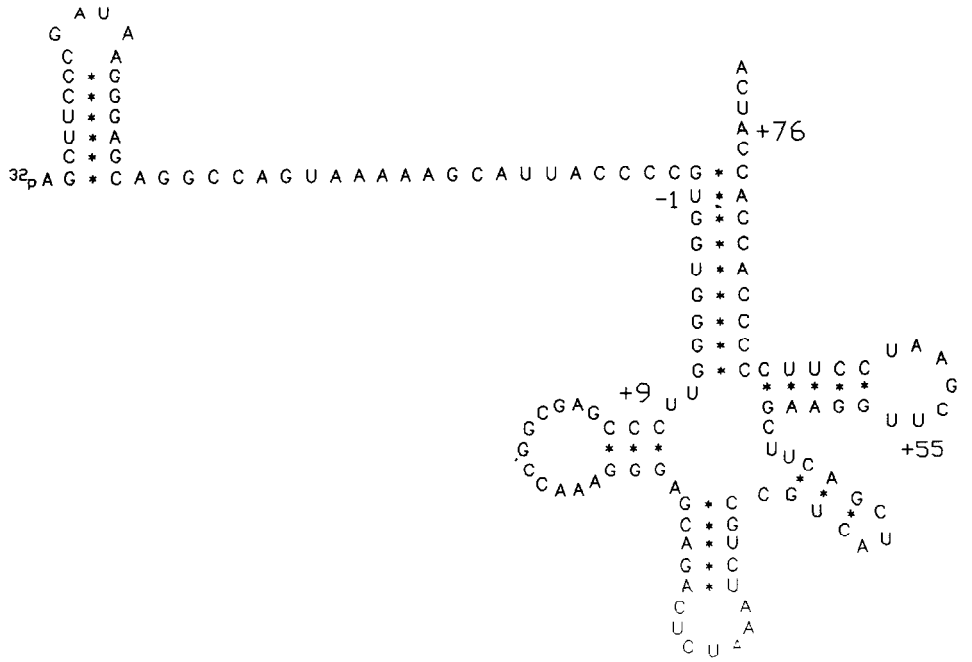


Fig. 2. Cloverleaf model of pre-tRNA$^{Tyr}$, as described for pre-tRNA$^{Phe}$ in Fig. 1.

ApG (from Sigma or USB), 0.5 mM NTPs; a small amount (about 1 mCi) of [α-$^{32}$P]UTP or [α-$^{32}$P]GTP (obtained from DuPont-NEN) was included. For the preparation of [α-S]dNTP containing transcripts, four separate transcription reactions were carried out in which one nucleoside triphosphate was replaced by 0.9 mM of [α-S]dNTP (Pharmacia) and 0.1 mM of the corresponding nucleoside triphosphate. As reference, a transcription reaction was performed using a mixture of 0.1 mM [α-S]NTP and 0.9 mM of the corresponding nucleoside triphosphate. The concentration of the other NTPs was 0.5 mM. After 2 h of incubation at 37°C, the reaction was terminated by the addition of 50 μl of 4 M ammonium acetate, 1 μl glycogen (5 μg/μl) and 250 μl of ethanol. After washing with 70% ethanol, the pellet was dissolved in 10 μl of urea/dye mixture (8 M urea, 0.03% xylene cyanol, Bromophenol blue) and analyzed on an 8% denaturing polyacrylamide gel. After autoradiography, the desired band containing the full-length product was excised and eluted [16]. The yields of the transcription reactions were estimated by Cerenkov counting or by densitometer scanning of the autoradiograms [19].

2.2. *End labeling*

To facilitate the 5′-end labeling of transcripts, all transcription reactions were initiated by ApG, using a fourfold excess of the dinucleotide over GTP. The normal template-encoded transcripts would start with pppG, but here, all RNA products start with the dinucleotide and therefore contain an extra adenosine and a free 5′-hydroxyl group. The gel-purified RNAs were end labeled with [γ-$^{32}$P]ATP (7000 Ci/mmol; ICN Biomedicals) and polynucleotide kinase (NE-Biolabs) as described [23].

2.3. *Sequencing*

The 5′-$^{32}$P-labeled phosphorothioate transcripts were sequenced according to the method described by Schatz et al. [24]. The iodine cleaved products were analyzed on 8% or 20% denaturing polyacrylamide gels, followed by autoradiography.

## 3. RESULTS

The cloverleaf structures of the two transcripts, pre-tRNA$^{Phe}$ and pre-tRNA$^{Tyr}$, used in this study are shown in Figs. 1 and 2, respectively. We could not obtain significant yields of the full-length transcripts of any of the two tRNAs, using 100% [α-S]dNTP or mixtures of [α-S]dGTP and GTP. In contrast, pre-tRNA transcripts were obtained with the other three [α-S]dNTPs by using a mixture of 0.9 mM [α-S]dNTP and 0.1 mM of the corresponding normal NTP. The yields of full-length transcripts were about 50% as compared to the transcription reactions with 100% normal NTPs.

According to the cleavage patterns shown in Fig. 3, [α-S]dATP and [α-S]ATP were incorporated specifically in the pre-tRNA$^{Phe}$ transcript at all expected positions, as predicted by the known sequence. As judged from the comparable cleavage efficiencies of the transcripts obtained with NTP mixtures containing either 10% [α-S]ATP or 90% [α-S]dATP, both modified RNAs contained about 10% of the thio-substituted phosphodiester bonds. It was confirmed in other experiments that the cleavage rates depend on the modification level (Kahle, unpublished data). Similar results were obtained with other tRNA transcripts containing [α-S]dATP or [α-S]dTTP. Unlike these data, the incorporation of [α-S]dCTP into the RNA transcript was less reliable. The analysis of 5′-end labeled tRNAs$^{Phe}$ obtained with [α-S]CTP or [α-S]dCTP is shown in Fig. 4. Evidently, the deoxycytidines in positions 27, 28 and

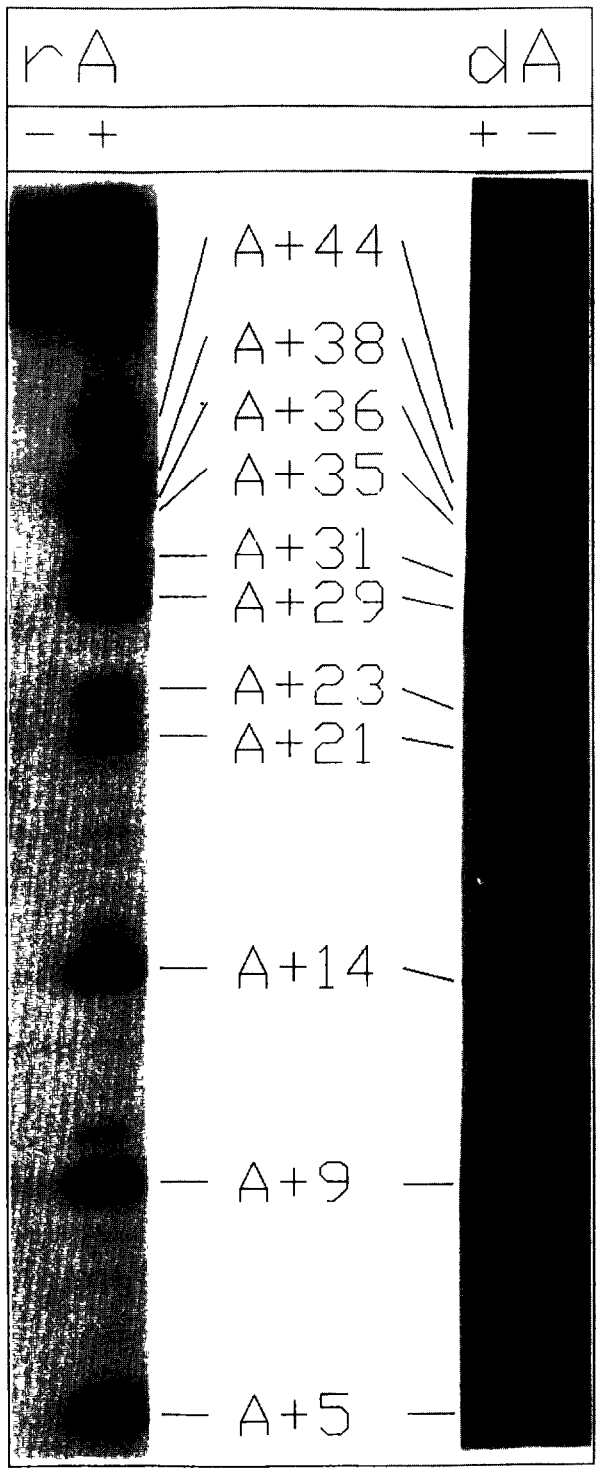


Fig. 3. Sequence analysis of [5′-$^{32}$P]-labeled pre-tRNA$^{Phe}$. The transcripts were obtained using [α-S]ATP (left panel) or with [α-S]dATP (right panel). The gel-purified RNAs cleaved with iodine/ethanol (lanes with +) or left untreated (lanes −) and analyzed on an 8% sequencing gel. Adenosine positions in the tRNA are assigned to the bands on the autoradiograms.

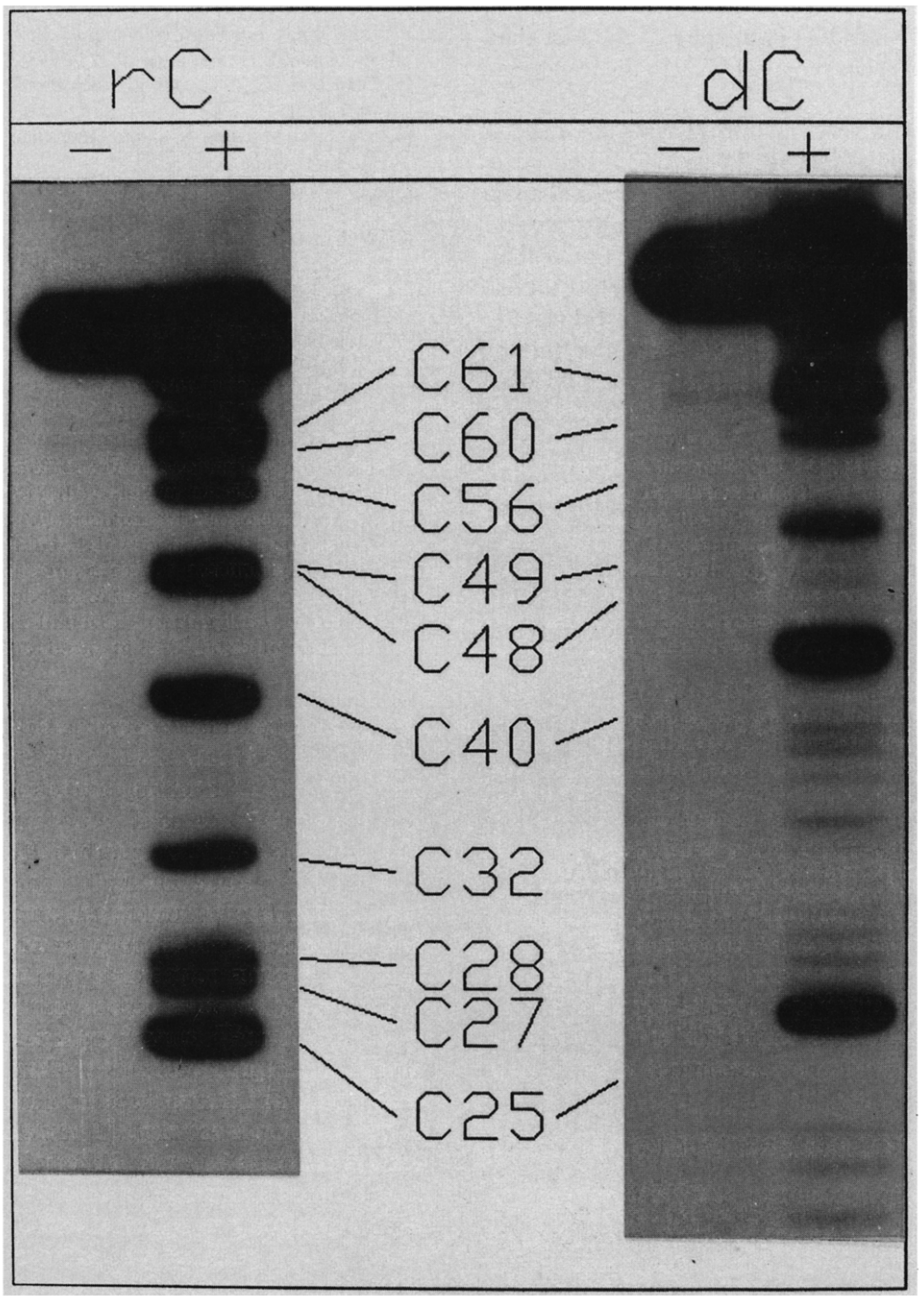


Fig. 4. Analysis of pre-tRNA$^{Phe}$. The analysis was performed as described in Fig. 1. Here, the RNAs were obtained with [α-S]CTP (left panel) and with [α-S]dCTP (right panel). The cytidine positions are assigned.

32 are missing, whereas all other deoxycytidines were incorporated specifically like the normal cytidines.

## 4. DISCUSSION

Our interest to study the role of 2′-OH groups in pre-tRNA recognition by RNase P and the recent preliminary results of Wyatt and Walker with dNTPs [25] have prompted us to investigate the efficiency of incorporation of [α-S]dNTPs into RNA transcripts in order to develop a new modification interference approach for identifying important 2′-OH groups in pre-tRNAs. We have shown that [α-S]dATP, [α-S]dCTP and [α-S]dTTP can be incorporated into RNA transcripts as large as pre-tRNA$^{Tyr}$. However, these deoxynucleoside triphosphates are poor substrates and have to be used in mixtures together with normal NTPs. Transcripts with only about 10% thio-substitions were obtained, if these mixtures contained 90% of dATP, dCTP or [α-S]dTTP supplemented by 10% of the corresponding NTPs. Only deoxyadenosines and deoxythymidines were incorporated reliably and specifically, whereas a few positions

were excluded with deoxycytidines. No transcripts could be obtained with dGTP, in spite of the fact that a dinucleotide (ApG) has replaced GTP in the transcription initiation.

Similar studies with very small transcripts (14 nucleotides) using 100% of dATP, dCTP or dTTP had been performed previously by Wyatt and Walker and they also found that dCTP is the least efficient substrate for T7 RNA polymerase [25]. Surprisingly, we found that dCTP is efficiently inserted at most cytidine positions. However, a very low level was observed for cytidines 27, 28 and 32. We have no explanations for these differences since neighbouring cytidines were not excluded at positions 48, 49 and 60, 61. Considering the different results with the four dNTPs, we conclude that T7 RNA polymerase is more selective for exocyclic bases in comparison to the sugar and phosphate moiety of the nucleoside triphosphate.

We have shown that rather large RNAs can be obtained with about 10% incorporation of [α-S]dNTP using T7 RNA polymerase. This level is ideal for the modification interference approach and it will find broad application, since it involves the convenient detection system based on the $I_2$/ethanol cleavage of thio-substituted phosphodiester bonds. Moreover, a similar approach will be possible with any modification in the exocyclic base or sugar.

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