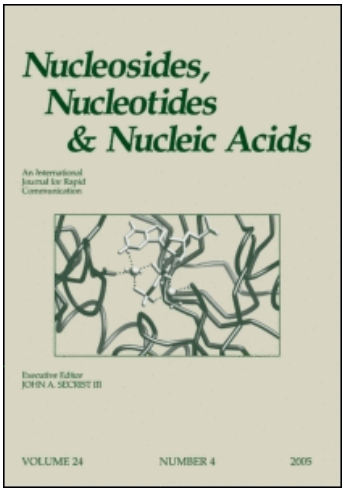

Nucleosides, Nucleotides and Nucleic Acids

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Incorporation of 2'-Modified Nucleotides into tRNA to Test Importance for Charging

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To cite this Article Persson, Tina , Vörtler, Claus Stefan , Fedorova, Olga and Eckstein, Fritz(1997) 'Incorporation of 2'-Modified Nucleotides into tRNA to Test Importance for Charging', Nucleosides, Nucleotides and Nucleic Acids, 16: 5, 797 — 800

To link to this Article: DOI: 10.1080/07328319708002954

URL: http://dx.doi.org/10.1080/07328319708002954

NUCLEOSIDES & NUCLEOTIDES, 16(5&6), 797–800 (1997)

INCORPORATION OF 2′-MODIFIED NUCLEOTIDES INTO tRNA TO TEST IMPORTANCE FOR CHARGING

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Abstract

Incorporation of 2′-deoxy nucleotides into tRNA is described using two complementary strategies: (a) in vitro transcription with a mutant T7 RNA polymerase and (b) enzymatic ligation of chemically synthesised tRNA fragments. The former permits incorporation at a low to a moderate substitution level for all nucleotides while the latter allows a complete exchange of ribonucleotides to deoxynucleotides as well as incorporation of point mutations. Both methods can be used for studying the influence of 2′-hydroxy groups at specific positions important for tRNA aminoacyl synthetase recognition.

Introduction

The role of 2′-hydroxyl groups in RNA is not always well defined. In many cases it is not certain if their presence at all positions is required for the particular function of an RNA. For example, it is known that in ribozymes modifications at some 2′-positions from a hydroxyl to a deoxy derivative are tolerated without great loss of biological activity[1,2]. This has also been shown for the recognition of $tRNA^{Ala}$ by its cognate synthetase[3]. The aim of our investigation is to study the influence of 2′-hydroxyl groups important for the interaction of $tRNA^{Asp}$ with its aminoacyl synthetase.

It is well known that a tDNA has considerably reduced rate in the charging reaction[4], indicating that at least certain hydroxyl groups might be of significance. One strategy to probe this position is to introduce 2′-deoxy nucleotides randomly into tRNA. This can be achieved by using the recently reported mutant T7 RNA polymerase[5]. Using this enzyme

for tRNA transcription in the presence of a dNTPαS/NTP mixture results in random 2′-deoxy substitution at various position depending on the nucleotide used. Such a pool of partially modified tRNA is the basis for an modification interference experiment, which consist of three steps:

1. Aminoacylation employing this pool.

2. Separation of charged from uncharged tRNA by acidic PAGE[6] or by $NaIO_4$[7].

3. Identification of the different 2′-deoxy modified positions in the tRNAs by phosphorothioate sequencing[8].

Finally, enzymatic ligation of chemically synthesised oligonucleotides to full length tRNA will be carried out as an complementary strategy to verify identified positions. In contrast to transcription, enzymatic ligation of two tRNA halves with T4 RNA Ligase allows the preparation of fully 2′-modified tRNA, without phosphorothioates, and also of tRNAs modified only at specific positions.

Result and discussion

Incorporation of dNTPαS by transcription using a mutant T7 RNA polymerase

The recently described mutant T7 RNA polymerase[5] allows replacement of ribonucleotides by deoxynucleotides in transcription reactions. We have used the mutant enzyme to check: (a) incorporation of dNTPαS into transcripts and (b) to determine the lowest incorporation level of dNTPαS/NTP in the transcription mixture still supporting incorporation. This is important since the modification interference procedure requires a pool of tRNA molecules containing the lowest level possible of 2′-deoxynucleotides per molecule. The incorporation level is monitored by phosphorothioate sequencing. As an example we here describe the dUTPαS/UTP system.

Incorporation of dUTPαS was only found when dUTPαS concentration was 20% of the total dUTP concentration, which support the result of Sousa[5] that dNTPs are not as good substrates for the mutant enzyme as rNTPs. Similar results have been obtained for the other nucleotides. At a dUTPαS/UTP ratio concentration of 80% a drastic decrease of full length product was observed. By using 100% dUTPαS in the transcription mixture internal labelling suggest formation of a premature termination transcript as a major product[9]. Similar results have been found for the other dNTPαS except for dGTPαS

which showed no product formation above 80% dGTPαS. One explanation could be that guanosine is the first nucleotide to be incorporated and in addition that for our particular template four additional Gs follow up to position seven which probably results in elongation problems. The efficiency for the formation of full length transcripts seems to be determined by the secondary structure of the transcripts[9].

In conclusion, besides the inability to obtain transcripts with 100% dNTPαS modification, the mutant enzyme can still be used to create a pool of modified tRNAs important for the first step in the modification interference procedure.

Enzymatic ligation of chemically synthesised oligonucleotides to full length tRNA

In contrast to transcription, enzymatic ligation of two tRNA halves with T4 RNA ligase[10] allows the preparation of fully 2´-modified tRNA, without phosphorothioate, and also of tRNAs modified only at specific positions[11-13]. We describe here the ligation of two all ribo tRNA halves to obtain unmodified tRNA as a control and as a first example of incorporation of 2´-deoxyuridine, the ligation of a 37mer with all uridines replaced by 2´-deoxyuridine as acceptor and a 39mer all ribo as donor in the ligation reaction.

Commonly used reaction conditions resulted in about 70% yield of $tRNA^{Asp}$ for the all ribo oligonucleotides. However, using the same conditions with the 2´-deoxy containing acceptor lowered the yield to about 30%. To improve the ligation reaction, various conditions were screened and using 10% DMSO resulted in a yield of 40%. Exchanging Mn^{2+} for Mg^{2+} decreased the yield to about 15%, other tested conditions had no effect. No self ligation of the donor molecule was observed eliminating the need of 3´-endprotection used by Ohtsuki et al[13].

tRNA prepared in this way will be used for kinetic analysis of single or multiple 2´-deoxy modified positions. The replacement of the 2´-deoxy modification in the ligation procedure will be directed by the outcome of the modification interference experiments with the transcribed pools of modified tRNAs.

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