Characterization of a chemically synthesized RNA having the sequence of the yeast initiator $tRNA^{Met}$

John Bratty[1], Taifeng Wu[2], Krikor Nicoghosian[1], Kelvin K. Ogilvie[2], Jean-Pierre Perreault[1], Gérard Keith[3] and Robert Cedergren[1]

[1]*Département de Biochimie, Université de Montréal, Montréal, Qué, H3C 3J7, Canada,* [2]*Department of Chemistry, McGill University, Montréal, Qué, H3A 2K6, Canada* and [3]*Institut de Biologie Moléculaire et Cellulaire du CNRS, F-67084 Strasbourg, France*

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A 75-unit long oligoribonucleotide corresponding to the sequence of the *Saccharomyces cerevisiae* initiator tRNA was synthesized chemically. The crude RNA was purified, and the sequence was verified by RNA sequencing techniques. A particularly useful purification step involved hydrophobic chromatography on BND-cellulose. The purified RNA could be aminoacylated to 28% of a bona fide initiator $tRNA^{Met}$ sample and threonylated to 76% of the level observed with native $tRNA^{fMet}$ from *E. coli*.

RNA; Chemical synthesis; Purification; Aminoacylation

1. INTRODUCTION

Until recently structure/function studies of RNA have been severely hampered by the lack of efficient methods for the synthesis of discrete RNA sequences. However, the situation has dramatically changed: first the in vitro RNA transcription system developed particularly by Melton et al. and Milligan et al. [1,2] and later the chemical synthesis procedure introduced by our group [3] have made it possible to obtain large amounts of specific RNAs for these studies. To illustrate the utility of the chemical synthesis protocol, a synthetic RNA having the sequence of the $tRNA^{fMet}$ of *E. coli* was prepared and characterized [4]. In another study, the synthesis of the same unmodified $tRNA^{fMet}$ was accomplished by ligation of chemically synthesized half molecules [5]. In both cases the fully synthetic tRNA had a modest but reproducible aminoacylation activity (approx. 10%). We now report the synthesis and characterization of an oligoribonucleotide having the sequence of the initiator $tRNA^{Met}$ of *Saccharomyces cerevisiae* [6] (see Fig. 1) together with an improved method for the purification of deprotected oligoribonucleotides.

Correspondence address: R. Cedergren, Département de biochimie, Université de Montréal, Montréal, Qué. H3C 3J7, Canada

Abbreviations: PAGE, polyacrylamide gel electrophoresis; BND-cellulose, benzoylated naphthoylated diethylaminoethylcellulose; EDTA, ethylenediaminetetraacetate; pCp, 3′,5′-cytidine diphosphate; Phy M and *B.c.*, RNases from Physarum and *Bacillus cereus*, respectively; TBAF, tetrabutylammonium fluoride. One OD_{260} is the quantity of material that, when dissolved in 1 ml, gives an A_{260} of one with a 1 cm pathlength

2. MATERIALS AND METHODS

[γ-^{32}P]ATP and [^{35}S]methionine were purchased from New England Nuclear; L-[^{3}H]threonine was obtained from Amersham. The sequencing enzymes (RNases U2, Phy M. *B.c.* and T1), T4 polynucleotide kinase, T4 RNA ligase and RNA guard were purchased from Pharmacia. RNase T2, BND-cellulose and a yeast extract enriched in methionyl-tRNA synthetase were obtained from Sigma Chemical Co. Natural *Saccharomyces cerevisiae* initiator tRNA was prepared as previously described [7].

2.1. *Synthesis of tRNA*

A 75-unit long oligoribonucleotide corresponding to the sequence of the yeast initiator tRNA [6] was chemically synthesized using the step-wise solid-phase method [3,8]. N-Protected (benzoyl for A,C; phenoxyacetyl for G) 5′-monomethoxytritylated 2′-silylated (*t*-butyldimethylsilyl for A, C. U; triisopropyl for G) ribonucleoside phosphoramidites were prepared according to standard methods [3,8]. All other reagents were prepared freshly. The automated ribonucleotide synthesis was performed on an Applied Biosystems DNA synthesizer using the cycle described previously [3,8]. Overall yield was 24% based upon quantification of the trityl group. At the end of the synthesis, the product was treated sequentially with thiophenoxide and methanolic ammonia to remove the phosphate- and base-protecting groups [3,8]. This stable (2′-protected) product was stored at −20°C. Portions were deprotected by treatment with 1 M TBAF for 16 h and passed through a Sephadex G-25 column to give the crude product.

2.2. *Purification by PAGE and sequencing of ^{32}P-labelled tRNA*

One preparation (16 OD_{260} units) of crude oligonucleotide was desalted on a 1 × 15 cm Sephadex G-50 column. The fractions containing the A_{260} peak were lyophilized and the resulting material was purified by 20% PAGE in 45 mM Tris/boric acid, pH 8.3, containing 1 mM EDTA and 7 M urea. After electrophoresis the position of the oligoribonucleotide was detected by UV shadowing and the slowest migrating band was cut from the gel, extracted with water for 24 h at room temperature and ethanol-precipitated. The pellet was resuspended in water and desalted on a spun column of Sephadex G-50. The eluent was lyophilized and a 1 μg fraction was 3′-labelled with [^{32}P]pCp using T4 RNA ligase [9]. A similar fraction was labell-

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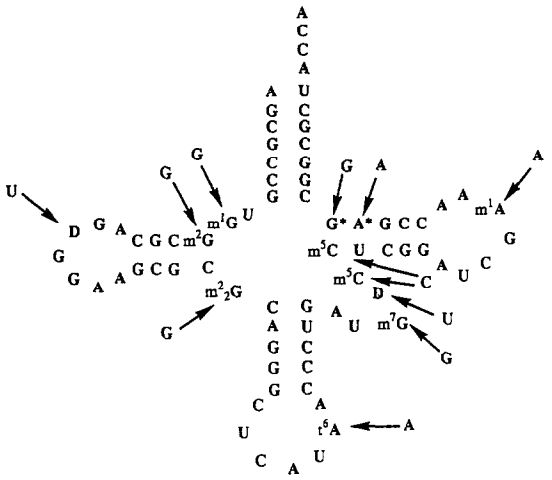

Fig. 1. The cloverleaf structure of yeast $tRNA^{iMet}$ [6]. Modified nucleotides are: m^1A, 1-methyladenosine; m^5C, 5-methylcytidine; D, dihydrouridine; m^1G, 1-methylguanosine; m^2G, N^2-methylguanosine; $m^2{}_2G$, N^2,N^2-dimethylguanosine; m^7G, 7-methylguanosine; t^6A, *N*-((9-β-D-ribofuranosylpurine-6-yl)-carbamoyl)-threonine; A*, modified A; G*, modified G. The modified bases occurring in the native tRNA were replaced by their respective parent nucleotides in the synthetic tRNA as indicated.

ed at the 5′ terminus with [γ-^{32}P]ATP and polynucleotide kinase [10]. The radioactive materials were repurified as above using 20% PAGE and the resulting 3′- and 5′-labelled oligoribonucleotides were subjected to enzymatic sequencing as described previously [11], as well as end group analysis by complete digestion with RNase T2 followed by two-dimensional TLC on 5×5 cm cellulose plates [3].

2.3. *Purification on BND-cellulose and sequencing*

Another RNA preparation was purified by BND-cellulose chromatography prior to PAGE as follows: a BND-cellulose column (1×16 cm) was prepared by loading the resin suspended in 1.5 M NaCl, 0.01 M $MgCl_2$, 0.01 M NaOAc, pH 4.5, followed by equilibration in 0.3 M NaCl with 0.01 M $MgCl_2$ and 0.01 M NaOAc at pH 4.5. Approximately 13 OD_{260} units of crude oligonucleotide was dissolved in 0.2 ml of 0.03 M NaCl, 0.01 M $MgCl_2$, 0.01 M NaOAc buffer and loaded on the column. The column was eluted sequentially with 3.5 ml of the 0.3 M NaCl buffer, 12 ml of the 1.5 M NaCl buffer and finally 12 ml of the 1.5 M buffer containing 20% ethanol. Fractions of 0.5 ml were collected and the absorbency at 260 nm determined. The material eluting in the 1.5 M buffer (fractions 22–25) was concentrated to 1.0 ml and desalted through a Sephadex G-25 column. RNA-containing fractions identified by their absorbency at 260 nm were pooled and dried. One μg of the resulting material was 5′-labelled using T4 polynucleotide kinase. This product and the remaining unlabelled tRNA were subjected to PAGE as described above. Full-length tRNA and two major faster migrating bands were extracted and sequenced as above.

2.4. *Aminoacylation assay*

RNA (0.3 μg) was dissolved in 100 μl of buffer with the following composition: 20 mM imidazole-HCl, pH 7.5, 0.1 mM EDTA, 2 mM ATP, 4 mM $MgCl_2$, 150 mM NH_4Cl, 10 μg/ml BSA, 17 μM [^{35}S]methionine and a yeast extract enriched in methionyl-tRNA synthetase. Tubes were incubated at 25°C; 20 μl aliquots were withdrawn and spotted onto Whatmann 3MM filter papers which were plunged immediately into an ice-cold 10% solution of trichloroacetic acid (TCA) containing 1 mM methionine. At the completion of the longest incubation, all filter papers were washed for a further 10 min in the same solution, then sequentially 4 times in cold 5% TCA for 10 min each and once in 95% ethanol for 10 min. Filter papers were air-dried and counted in a scintillation cocktail. The measurements of radioactivity incorporated into TCA-precipitable material were corrected for background calculated from control experiments which contained no tRNA or no yeast extract.

2.5. *Threonylation of tRNA*

Threonylation at the adenosine 3′ adjacent to the anticodon was assayed as described previously [5] using 1 μg of natural *E. coli* $tRNA^{fMet}$ and the synthetic yeast tRNA purified by PAGE. Control incubations in the presence of natural yeast initiator $tRNA^{Met}$ and in the absence of RNA and of protein were performed.

3. RESULTS AND DISCUSSION

The synthesis of the 75-unit oligonucleotide corresponding to the initiator $tRNA^{Met}$ of yeast was accomplished by a fully automatic synthesizer as described above. After deprotection and precipitation, the sample was submitted to denaturing polyacrylamide

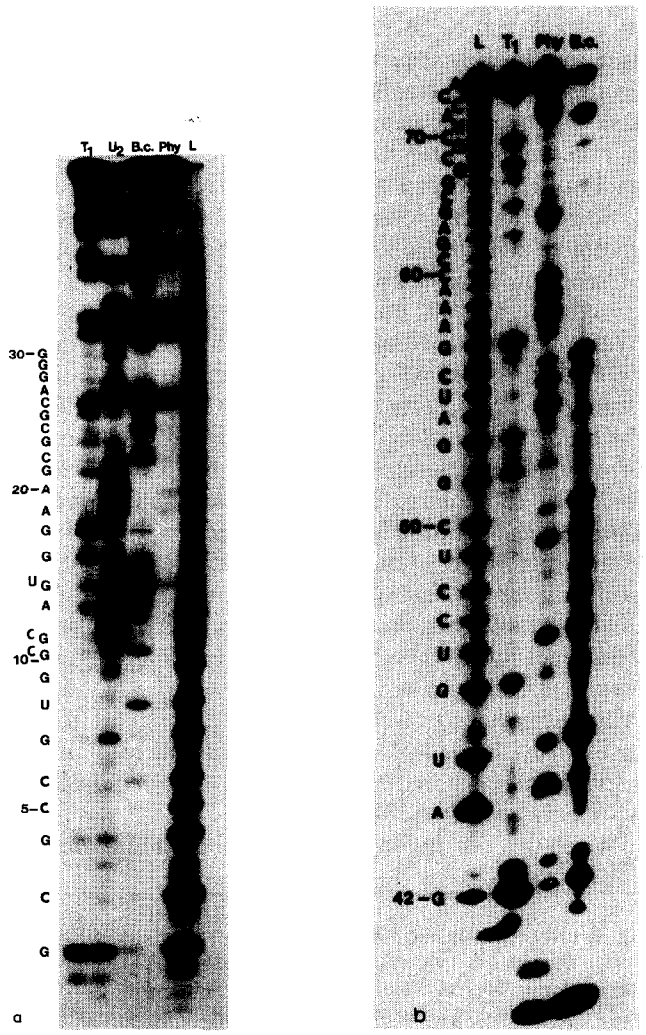

Fig. 2. Enzymatic sequencing gels of the synthetic tRNA, 5′-labelled with ^{32}P. L, the formamide cleavage ladder; T_1, RNase T_1; B.c., RNase from *Bacillus cereus*; Phy, RNase from *Physarum*; U_2, RNase U_2. Numbers refer to corresponding positions in the complete tRNA molecule. (a) Full length molecule. (b) Band F of Fig. 3c, corresponding to the 3′ half of the molecule.

gel electrophoresis and analysis thereof proved the sample to be a complex mixture of oligonucleotides. The crude 5′-[^{32}P]-labelled product gave the expected bands, but with a heavy background covering most of the gel (see below). Neither ethanol precipitation nor additional cycles of Sephadex G-25 or G-50 chromatography brought about acceptable purification of the fully deprotected species. The region of the gel corresponding to the full-length molecule was excised, labelled at the 3′ and 5′ termini and repurified by PAGE. Enzymatic sequence analysis showed that the oligonucleotide had the expected tRNA^{iMet} sequence. Representative gels showing the sequence of positions 1–30 and 42–75 are presented in Fig. 2.

Another, unlabelled sample prepared by two successive preparative gels served for preliminary aminoacylation experiments. Determinations of amino acid acceptance with this sample showed low and variable levels of aminoacylation. It is important here to underline the difference between chemical integrity as determined by sequencing and biological integrity of synthetic oligonucleotides determined by enzymatic assays. On the one hand, sequence analysis showed the sample to be correct, but on the other, functional studies demonstrated only marginal activities.

Some chemical heterogeneity could result from incomplete deprotection of the synthetic oligonucleotide. Since protecting groups, although having different structures, are all hydrophobic, we decided to use hydrophobic chromatography on benzoylated, naphthoylated DEAE-cellulose in order to eliminate incompletely deprotected species. The elution profile of the crude oligonucleotide is shown in Fig. 3. Recovery of oligonucleotides eluting in 1.5 M NaCl buffer from the BND cellulose column represented approximately 10% of the starting material suggesting that if all bound material contained protecting groups, deprotection was approximately 97% efficient at each position in the sample. Sequence analysis as presented above would not detect this low level of impurity. The problem has been noted in the case of oligodeoxyribonucleotides as well [12], but is more acute in the case of functional RNA, since the molecule must adopt a specific tertiary structure, not generally a requirement when using synthetic DNA.

When this purified product was labelled and analyz-

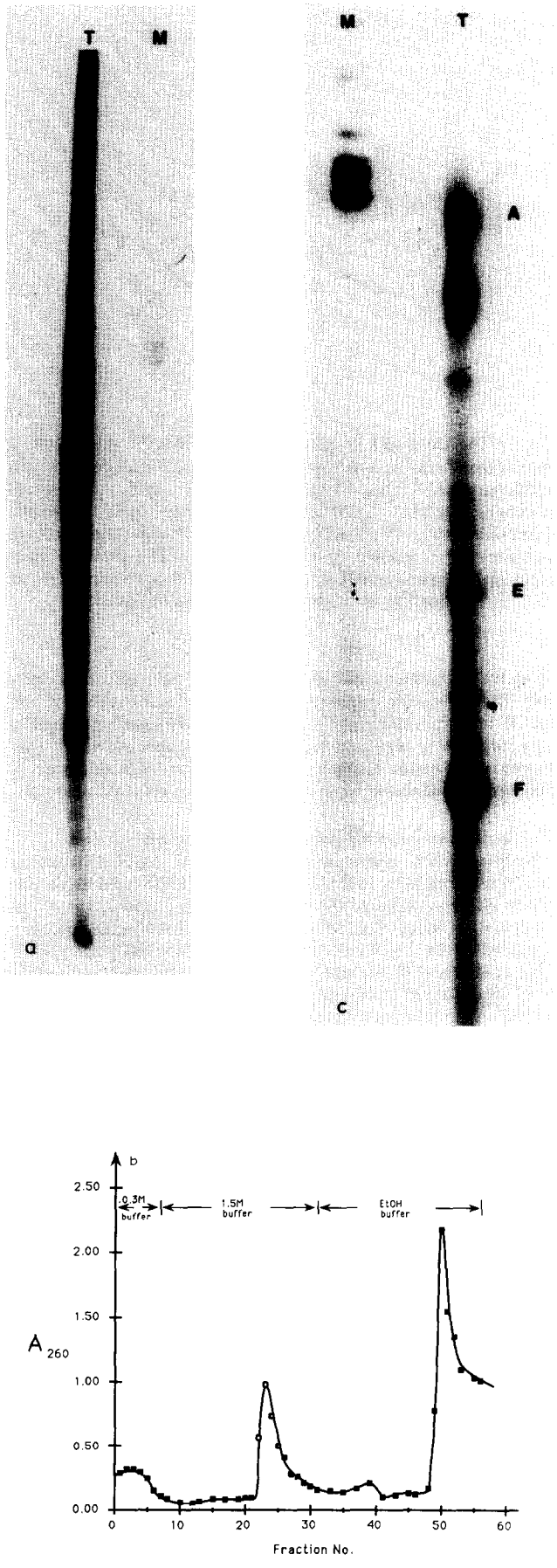

Fig. 3. BND-cellulose and PAGE purification of synthetic RNA. (a) Autoradiogram of a typical gel prior to BND-cellulose chromatography. Lanes: M, crude yeast tRNA extract; T, the synthetic tRNA. (b) Elution profile of crude oligonucleotide applied to BND-cellulose column. Fractions indicated by open circles are those used in subsequent analyses. (c) Polyacrylamide gel after purification by BND-cellulose chromatography. A, the full length synthetic tRNA used in the aminoacylation experiments. E and F refer to truncated products which were sequenced. Both A and F were initially cut as pairs of bands, but subsequent analysis indicated this distinction to be superfluous.

ed by denaturing PAGE (Fig. 3c), a great improvement in the definition of bands was observed; distinct bands were resolved, and the background was substantially reduced (cf. Fig. 3a). The full-length product and two major faster moving bands (E and F, Fig. 3c) were extracted and sequenced. The truncated fragments proved to represent molecules differing in the number of nucleotides missing from their 5′ termini (Fig. 2b). Since no molecules derived from the 5′ end were observed in this or similar analyses, the truncated molecules presumably represent points at which the chemical synthesis was arrested rather than degradation products. The 5′ termini of the truncated molecules were at positions G_{42} and C_{33} corresponding to the anticodon stem and loop of the native tRNA. It is not clear at this stage why the synthesis should have stopped at these points, although we note that in each case, the termination immediately preceded the addition of a U residue to the growing chain.

The synthetic RNA purified by this BND-cellulose method was aminoacylated to approximately 28% of the level obtained for the natural tRNA even though it lacks a 5′-phosphate and modified bases. This represents a substantial improvement over the 11% charging rate previously obtained with a synthetic *E. coli* $tRNA^{fMet}$ [4]. In other studies an *E. coli* $tRNA^{fMet}$ assembled by ligation of short, synthetic oligomers was charged to 4–6% [13], while a T7 RNA polymerase-transcribed $tRNA^{Gly}$ of *Mycoplasma mycoides* was charged by an *E. coli* synthetase extract with approximately 80% efficiency relative to the native $tRNA^{Gly}$ [14]. Similar results have been obtained with T7 RNA polymerase-transcribed *E. coli* $tRNA^{eMet}$ and yeast $tRNA^{Phe}$ [15,16].

Native yeast initiator tRNA is modified in vivo by threonylation of the 6-amino group of the adenosine 3′-adjacent to the anticodon (Fig. 1). This modification is believed to be important for the codon-anticodon interactions [17]. Although the *E. coli* $tRNA^{fMet}$ does not naturally have this modification [18], we have previously shown that it may be introduced in vitro using a protein extract from yeast [5]. In those experiments, synthetic $tRNA^{fMet}$ was threonylated to approximately 3% of the level of native *E. coli* $tRNA^{fMet}$. Here, the threonylation of the synthetic $tRNA^{iMet}$ attained 76% of the value of the native *E. coli* $tRNA^{fMet}$ (Table I). Since native yeast initiator tRNA already contains the t^6A modification, it cannot be used as the standard. Thus, the value of 76% threonylation may not be directly compared to the 3% obtained for the synthetic $tRNA^{fMet}$. However, the threonylation level is very significant, particularly in view of the fact that this sample of the synthetic RNA had not been subjected to the BND-cellulose purification step. This suggests that threonylation is much less sensitive to the global tRNA conformation than is aminoacylation.

Table I

Aminoacylation and threonylation of synthetic RNA

		Aminoacylation (pmol	% of OD_{260} control)	Threonylation (pmol	% of OD_{260} control)
$tRNA^{iMet}$	yeast	595	100	(16)	n/a
$tRNA^{fMet}$	*E. coli*	–	–	186	100
$tRNA^{iMet}$	synthetic	168	28	141	76

Levels are expressed as the absolute yield of radioactive product and as percent of control tRNA ($tRNA^{iMet}$ for aminoacylation and $tRNA^{fMet}$ for threonylation). Natural yeast $tRNA^{iMet}$ cannot be quantitatively threonylated, because the t^6A modification is already present.

The automated solid-phase synthesis of RNA [3–5] is very similar to DNA synthesis using phosphoramide chemistry. However, although chemical synthesis of small and medium-sized oligoribonucleotides is now a routine procedure, the synthesis of long oligoribonucleotides, such as the tRNA presented here, has not yet become widely used. These results clearly illustrate that full chemical synthesis can be used to produce large, biologically active RNA species. They also demonstrate that, at least in the case of aminoacylation, such activity can be highly sensitive to the residual presence of protecting groups. The BND-cellulose chromatography of synthetic RNA described herein should be a valuable procedure in the purification of biologically important, chemically synthesized RNA molecules.

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