Reconstruction of $tRNA^{Phe}$ molecules from the fragments by linkage with T-4 RNA ligase in double-stranded regions

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Phenylalanine-specific tRNA from yeast was hydrolysed with cobra venom ribonuclease in the double-stranded regions and the fragments isolated. The 'dissected' molecules with nicks in positions 28 and 41 were reconstructed from supplementary fragments and treated with T-4 RNA ligase. A phosphodiester bond between two fragments was formed when the fragment combination (1–28) + (29–76) was used. A strong discrimination in the ligation yield between different nick positions in the same helix is shown.

Cobra venom nuclease *RNA ligase* *$tRNA^{Phe}$* *tRNA fragments*

1. INTRODUCTION

The method of 'dissecting' molecules is widely used for structural and functional investigations of ribonucleic acids [1–3]. In particular, the self-assembly of biologically active tRNAs from fragments has been studied [2–4]. In order to compare the properties of the dissected molecule with those of the native one it would be necessary to link the fragments by a covalent bond into one molecule. Especially interesting are complexes containing fragments from different sources.

There exists an enzyme which catalyzes phosphodiester bond formation between ribonucleotide components – RNA ligase from the bacteriophage T-4-infected *E. coli* cells [5–7]. This enzyme has been widely used for different purposes [5–12].

However, RNA ligase-catalyzed phosphodiester bond formation between fragments of natural RNAs has not yet been described for substrates containing nicks in double-stranded regions. In order to cleave RNA molecules in these regions we have used cobra venom nuclease [13]. We have also developed a procedure for RNA ligase purification and obtained a preparation of the enzyme without contaminating endo- or exonuclease activity [14].

This work deals with RNA ligase-catalyzed linkage of natural fragments of yeast phenylalanine-specific RNA in the double-stranded region with the formation of native tRNA molecules.

2. MATERIALS AND METHODS

Phenylalanine-specific tRNA, terminal tRNA nucleotidyl transferase and phenylalanyl-tRNA synthetase were isolated from yeast and kindly provided by Drs P. Remy and M. Renaud from Professor J.-P. Ebel's laboratory. Radioactive [γ-^{32}P]ATP and [α-^{32}P]ATP of specific activity 2000–3000 Ci/mmol were purchased from Amersham (England). Cobra venom nuclease and phosphodiesterase were isolated as in [15]. The specific activity of the nuclease was 2400 units/ml [16]. Polynucleotide kinase was isolated as in [17], and alkaline phosphatase was kindly provided by Dr V.G. Korobko. Acrylamide was from Serva, *N,N'*-methylenebisacrylamide (Eastman Kodak). X-ray films RM-1 from Svema (Shostka, USSR) were used for autoradiography.

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2.1. *Isolation and testing of T-4 RNA ligase*

RNA ligase was isolated from the bacteriophage T-4amN82-infected *E. coli* B cells. The isolation procedure included disintegration of the cells, ammonium sulphate precipitation of the proteins, fractionation on phosphocellulose and DEAE-cellulose, DEAE-cellulose chromatography, isoelectric precipitation, gel filtration on Sephadex G-100, chromatography on hydroxyapatite and aminohexyl-Sepharose. A detailed description of the isolation procedure will be published elsewhere [14]. Specific activity of the isolated enzyme was 28 000 units/ml [18].

The absence of endonuclease contamination was shown by incubating 30–60 units RNA ligase with 10–20 μg bacteriophase MS-2 RNA at room temperature for 4–6 h or at 4°C for 24 h. Integrity of MS-2 RNA molecules was then analysed by electrophoresis in 4% polyacrylamide gel under denaturating conditions. For exonuclease contamination testing 200–250 units RNA ligase were incubated with 35 μmol of the synthetic hexanucleotide $(pA)_6$ at 37°C for 5 h. Chromatography of the mixture [19] on Aminochrom C3 was then carried out.

2.2. *Labeling of tRNA*

For 5'-labeling, $tRNA^{Phe}$ was dephosphorylated by alkaline phosphatase and then phosphorylated with [γ-^{32}P]ATP in the presence of polynucleotide kinase. For 3'-labeling, the CCA-end of tRNA was removed by snake venom phosphodiesterase and joined again by terminal tRNA-nucleotidyl transferase in the presence of CTP and [α-^{32}P]ATP. All the labeled tRNAs were purified by electrophoresis in 15% polyacrylamide gel under denaturating conditions. These procedures were described in our previous papers [20,21].

2.3. *Preparation of the tRNA fragments*

Hydrolysis of end-labeled $tRNA^{Phe}$ by cobra venom nuclease, separation of the fragments by electrophoresis in 20% polyacrylamide gel under denaturing conditions and isolation of the radioactive fragments from the gel were carried out as in [20,21].

2.4. *Reconstruction of tRNA molecules from the fragments and RNA ligase-catalyzed linkage*

Labeled supplementary fragments (2–10 μg) of $tRNA^{Phe}$ (1–28) + (29–76) or (1–41) + (42–76) or (1–67,68,69) + (68,69,70–76) were mixed in equimolar concentrations in 0.05 M Tris-HCl (pH 7.4), 0.01 M $MgCl_2$, heated to 75°C, and cooled to 4°C during 3 h. Ligation of the fragments was carried out in 0.05 M Tris-HCl (pH 7.4), 0.01 M $MgCl_2$, 0.01 M dithiotreitol, 0.5 mM ATP with 20–30 units RNA ligase at 4°C for 20 h. Analysis of the reaction mixture was made by electrophoresis in 20% polyacrylamide gel under denaturing conditions [20,21] with subsequent autoradiography. 5'-Fragments of tRNA were labeled at the 5'-termini and 3'-fragments at the 3'-termini, respectively.

3. RESULTS

We have studied RNA ligase-catalyzed reaction on the model of $tRNA^{Phe}$ from yeast. This molecule is convenient for such kinds of investigation because its primary, secondary and tertiary structures are well known [22], methods of RNA fragmentation and isolation of the fragments are well developed [20,21] and self-assembly of the initial structure of the tRNA molecule from its fragments has been shown for the molecules cleaved in both single-stranded [1–4] and double-stranded regions (in preparation).

We have used reconstructed tRNA molecules which have nicks in double-helical regions. In order to cleave the molecules in such a way a nuclease from cobra venom was used. This enzyme cleaves phosphodiester bonds in double-helical regions of RNA [13]. In the case of $tRNA^{Phe}$ from yeast the main cleaved phosphodiester bonds are 28 and 41 in the anticodon stem and 67–73 in the acceptor stem [20,21] (fig.1). The other advantage of this enzyme is the formation of 5'-phosphorylated hydrolysis products with non-phosphorylated 3'-termini. These products may be substrates for RNA ligase [5–7].

In order to eliminate contamination by native tRNA, minor fragments and products of subsequent hydrolysis, individual fragments of tRNA were isolated and the tRNA molecule was reconstructed from supplementary pairs of fragments. In our experiments the following pairs were used (the numbers indicate the first and the last nucleotide of the fragment): (1–28) + (29–76); (1–41) + (42–76) and (1–67,68,69) + (68,69,70–76).

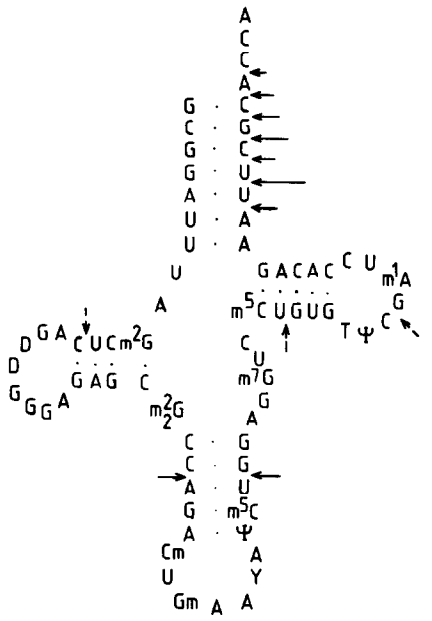

Fig. 1. 'Cloverleaf' structure of $tRNA^{Phe}$ with the main cleavage sites by the nuclease from cobra venom (indicated by the arrows) [20–22].

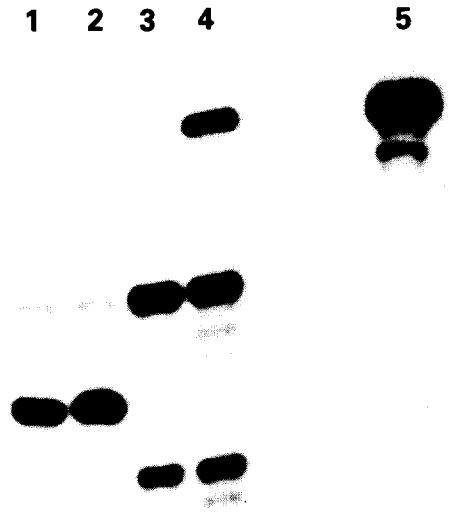

Fig. 2. Electrophoresis of RNA ligase-treated ^{32}P-labeled fragments of $tRNA^{Phe}$, (1) 5′-^{32}P-labeled fragment (1–41) and 3′-^{32}P-labeled fragment (42–76). (2) The same as 1 after RNA ligase treatment. (3) 5′-^{32}P-labeled fragment (1–28) and 3′-^{32}P-labeled fragment (29–76). (4) The same as 3 after RNA ligase treatment. (5) Intact 3′-^{32}P-labeled $tRNA^{Phe}$

Experiments with natural RNA require pure preparations of RNA ligase in order to avoid degradation of the material. The developed isolation procedure allowed us to isolate RNA ligase without endonuclease or exonuclease contamination [14]. The enzyme preparation did not degrade bacteriophage MS-2 RNA (a test system for endonuclease activity) and did not remove the terminal nucleotides from hexanucleotide $(pA)_6$ (a test system for exonuclease activity) even after prolonged incubation of these substrates with excess enzyme.

The results of RNA ligase treatment of the fragments corresponding in composition to tRNA molecules with one nick in the anticodon stem are shown in fig.2. It is seen that phosphodiester bond formation proceeds only when the reconstructed molecule has a nick in position 28. Formation of the phosphodiester bond in position 41 is negligible and can be seen only after prolonged autoradiography. In some experiments the ligase reaction in position 28 proceeded until one of the components was completely exhausted while bond formation in position 41 never exceeded 10%.

We were not successful in covalently binding the 3′-terminal octa- and nonanucleotides to the rest of the molecule (not shown).

In some experiments we examined the ability of the dissected tRNA molecules to be aminoacylated. In all cases the aminoacylation capacity of the reconstructed molecules was the same as that of native ones. We consider this as evidence of the correct assembly of the molecules from the fragments. We have not found RNA ligase-catalyzed formation of the cyclic tRNA in accordance with the data in [23].

4. DISCUSSION

In our experiments the possibility of enzymatic formation of phosphodiester bonds between rather long fragments of natural RNA in the double-stranded region has been demonstrated. These results should be considered from the point of view of steric conditions for RNA ligase-catalyzed linkage. Ligation of short oligonucleotides occurs

because they do not form stable complexes and the reaction takes place without any steric hindrance after the corresponding ends are combined in the active site of the enzyme. Quite a different picture is seen when the ends of long natural polynucleotides have to be combined. The fragments might form a complicated tertiary structure which could prevent coincidence of corresponding ends. Even when the fragments are isolated from one source, incorrect assembly may occur. In the case of $tRNA^{Phe}$ cleaved in position 41 the absence of the linkage can be explained by the strained structure of the 3′-half of the anticodon stem in the intact molecule. Despite correct assembly of the dissected molecule there is no coincidence of the 3′-OH group of ribose and the 5′-phosphate group of the supplementary fragment leading to phosphodiester bond formation. An alternative explanation is the steric hindrance for intermediate pyrophosphate derivative formation [17].

In the case of 3′-terminal oligonucleotides the same explanation may be given but the situation becomes more complicated by the rapid dissociation of these oligonucleotides which form very weak complexes with the rest of the molecule (in preparation).

From these data it can be concluded that RNA ligase does not play an active part in the reaction or, in another words, it does not bring together the ends which have to be linked when there is some steric hindrance to their coincidence.

Hence, our results show the possibility of using RNA ligase for construction of RNA molecules from fragments. But the use of RNA ligase is restricted by the steric factors and ligation occurs only when the corresponding ends of the fragments are brought into coincidence in a way favourable for the reaction.

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