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In vitro construction of yeast $tRNA^{Asp}$ variants: nucleotide substitutions and additions in T-stem and T-loop

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ABSTRACT

A procedure for the construction of 3'-end labelled yeast $tRNA^{Asp}$ harboring substitutions or additions of any desired nucleotide in T-stem and T-loop (position 57 to 61) has been developed. This was done by in vitro enzymatic manipulations of the yeast $tRNA^{Asp}$ involving specific hydrolysis with RNases, phosphorylation and dephosphorylation with T4 polynucleotide kinase and ligation with T4 RNA ligase. Using this procedure we have replaced conserved or semi-conserved nucleotides located in position 57 to 61 of yeast $tRNA^{Asp}$. We have also constructed different yeast $tRNA^{Asp}$ with eight bases instead of seven in T-loop. Further use of these $tRNA^{Asp}$ variants will be discussed with the help of the crystallographic three-dimensional structure.

INTRODUCTION

The three-dimensional structure of a tRNA molecule determines many of its biological activities. The crystallographic structure at high resolution of two elongator tRNAs from yeast, that of $tRNA^{Phe}$ (1-4) and that of $tRNA^{Asp}$ (5), are available at present. These tRNAs represent a model system for solution conformational compared studies. Chemical modifications of end-labelled RNAs followed by determination of the sites of modification by sequencing gel methodologies (6-7) has become a powerful approach for monitoring the solution structure of yeast $tRNA^{Phe}$ (8-9) and $tRNA^{Asp}$ (9). It is now well established that conserved or semi-conserved nucleotides play a central role in the three-dimensional conformation of the different tRNAs. Among the different domains in tRNAs, the T-loop and its extension in T-stem (the first base pair that closes the loop) is one of the most conserved region. For instance, it might be noticed the presence of the quasi-constant TψCR sequence in all elongator tRNAs. It has been proposed that this part of T-loop might be involved in interactions with the ribosome (10-12) although their exact nature remains controversial (13-14). The T-loop has also a structural

role in tRNA dynamic by participating in the stabilization of the D-loop (15). Conformational studies of variants from yeast $tRNA^{Asp}$ or $tRNA^{Phe}$ harboring altered T-stem and loop should allow us to define the relative importance of the conserved or semi-conserved nucleotides in maintaining the local or overall structure of tRNAs.

For a few years it has become possible to manipulate in vitro nucleotide sequence of RNAs by combinaison of several enzymes, such as RNases, T4 polynucleotide kinase and T4 RNA ligase. Several kinds of tRNA variants were prepared by this approach and used for studies on the structure-function relationship (16-21). tRNA variants were equally used for studies of various parameters involved in anticodon-loop base modifications (22-26, for a review see ref. 26). However, replacement of nucleotides into tRNAs were generally limited to the anticodon-loop and to the 3'-terminal region. Recently, the construction of T.utilis $tRNA^{Tyr}$ containing deletions or substitutions of nucleotides in D-loop has been reported (27). In vitro tRNA reconstruction is currently the only available technique allowing preparation of tRNA variants carrying naturally modified nucleotides.

In this paper we describe a procedure for the in vitro construction of 3'-end labelled yeast $tRNA^{Asp}$ with alteration of nucleotide sequence in T-stem and T-loop (position 57 to 61 according to the numbering system of nucleotides in tRNAs (28). Using this procedure we have created ten variants from yeast $tRNA^{Asp}$ harboring nucleotide substitutions or additions at position 57 to 61 in place of conserved or semi-conserved nucleotides. This report is the first describing a nucleotide substitution introduced in a stem using the T4-RNA ligase technology. Further solution structural mapping (using chemical or enzymatical probes) of these 3'-end labelled yeast $tRNA^{Asp}$ variants will be a useful step to better understand the role of conserved and semi-conserved nucleotides in the three-dimensional solution structure of yeast $tRNA^{Asp}$.

MATERIALS AND METHODS

tRNA, oligonucleotides and enzymes

$tRNA^{Asp}$ from brewer's yeast was purified as described by Keith et al. (29).

Oligonucleotide $_{HO}AUG_{OH}$ was from Sigma. The trinucleotide diphosphate $_{HO}AAU_{OH}$ was obtained as described by Haumont et al. (24). Oligonucleotide $_{HO}UUU_{p}$ was prepared by limited alkaline hydrolysis at

60°C of poly U (Sigma) as described by Mc Farland and Borer (30). $_{HO}UUU_{p}$ was separated from the mixture on a 1 X 60 cm DEAE-Sephadex A25 column (Pharmacia Fine Chemicals) eluted using a linear gradient of triethylammonium bicarbonate pH 8.0 from 10 mM to 750 mM. The sample was desalted by rotary evaporation aided by the addition of methanol. Oligomer was subsequently desalted on a Sephadex G10 column equilibrated in water. $_{HO}UUUp$ is then dephosphorylated by E.coli alkaline phosphatase (B.A.P) in the following conditions : 100 A_{260} $_{HO}UUUp$/ml in Tris HCl 50 mM pH 8.0 and 5 units of B.A.P/A_{260} $_{HO}UUUp$. The reaction was performed at 37°C for 4h and $_{OH}UUU_{OH}$ was isolated by chromatography on a 2 ml DEAE-Sephadex A 25 column. The trimer was then concentrated and desalted as described before.

E.coli alkaline phosphatase, nuclease P1, spleen phosphodiesterase, T4 polynucleotide kinase and T4 polynucleotide kinase lacking the 3'-phosphatase activity were purchased from Boehringer Mannheim. Pancreatic RNase (RNase A) was obtained from Worthington and RNase T1, RNase T2 and RNase U2 from Calbiochem. T4 RNA ligase was obtained from Pharmacia. [γ^{32}P]ATP (110 TBq/mmole) and [5'^{32}P]pCp(110 TBq/mmole) were purchased from Amersham.

Gel electrophoresis and recovery of RNA

After each enzymatic reaction the RNA fragments were usually purified by electrophoresis on denaturing polyacrylamide gel (P.A.G.E) containing 8.3 M urea ; 100 mM Tris-borate pH 8.3 and 3 mM EDTA. For preparative purpose, tRNA fragments were purified on thicker (2-4 mm polyacrylamide urea slab gels). The RNA bands were detected by ultraviolet shadowing (31), excised and eluted from the gel slices according to the crush and soak method (32). After a centrifugation step to remove acrylamide fragments, the RNA was ethanol precipitated. This step was repeated twice in order to remove SDS and impurities from the gel. Intermediates in variant tRNA construction and variant tRNAs were purified on thin (0.5 mm) polyacrylamide urea slab gels. In this case, the RNA bands were detected by autoradiography.

Preparation of yeast tRNA^{Asp} fragments

Preparation of 5'-half and 3'-half tRNA

5'-half ($pU\overset{}{\text{——}}Gp^{34}$) and 3'-half ($_{HO}U^{35}\text{——}Gp^{73}$)tRNA (Fig. 1, step 1) were prepared essentially as described by Carbon et al. (22).

Preparation of quarter tRNA

Quarter tRNA A : $_{HO}U^{35}\text{——}Cp^{56}$ was obtained by limited digestion of

3'-half tRNA by RNase A followed by preparative polyacrylamide gel electrophoresis (Fig.1, step 2). The standard reaction mixture contained 1 mg/ml (about 80 µM) of 3'-half tRNA ; 50 mM Tris-HCl pH 7.5 ; 10 mM $MgCl_2$; 100 mM NaCl and 0.5 µg RNase A/mg RNA. The reaction was performed at 4°C for 30 min, then stopped by addition of an equal volume of phenol saturated with the hydrolysis buffer containing 2.5% (w/v) SDS and 0.34% (w/v) sucrose. After extraction, the RNA was ethanol precipitated.

Quarter tRNA B : $HO^{35}U\text{———}A^{57}_p$, C : $HO^{35}U\text{———}A^{58}_p$, D : $HO^{59}U\text{——}G^{73}_p$ and E : $HO^{58}A\text{———}Gp^{73}$ were obtained by partial hydrolysis of 3'-half tRNA with RNase U2 (Fig. 1, step 3). Enzymatic digestion was performed at 4°C for 20 mn in 20 mM ammonium acetate pH 4.5 containing 1 mg/ml of 3'half tRNA and 2 units of enzyme/mg RNA. The reaction was stopped by phenol extraction followed by preparative P.A.G.E.

Fragment F : $HO^{60}U\text{——}Gp^{73}$ and fragment G : $HO^{61}C\text{———}Gp^{73}$ were obtained by sequential resection of fragment D (or E) using spleen phosphodiesterase (S.P.D.E) (Fig.1, step 4). Experimental conditions were the following : 40 µM fragment D ; 100 mM Imidazole-HCl pH 6.0 and 30 µg/ml of S.P.D.E. Incubation was performed at 37°C for 15 min followed by phenol-extraction and P.A.G.E.

Fragment H : $HO^{62}C\text{———}Gp^{73}$ was obtained by RNase A (Fig.1, step 5) partial hydrolysis of fragment D (40 µM) in 20 mM Tris-HCl pH 7.5 ; 100 mM NaCl ; 10 mM $MgCl_2$. Hydrolysis was carried out at an RNase/RNA ratio of 1.25 µg RNase A/µg RNA, for 30 min at 4°C. The reaction was stopped by phenol extraction and after ethanol precipitation, fragment H was purified by P.A.G.E.

The RNA fragments resulting from the above ribonuclease digestion were identified by their terminal nucleotides as described in (22) and by their nucleotide sequence using ^{32}P and post-labelling techniques. Sequence analysis was performed essentially as described by Donis-Keller et al. (33).

3'-end dephosphorylation of RNA fragments

Removal of the 3'-terminal phosphate from the 5'-half tRNA and from the quarters A,B,C,E,F,G,H was carried out by using the 3'-phosphatase activity of T4 polynucleotide kinase (34). The products at a concentration of 24 µM in 100 mM Imidazole-HCl pH 6.0 ; 10 mM $MgCl_2$; 3 mM DTE and 20 µg/ml of BSA were incubated for 2h30 at 37°C with 50 units/ml of T4 polynucleotide kinase. The reaction was terminated by heating for 3 min at 65°C and the RNA was ethanol precipitated.

3'-end labelling of RNA fragments

The 3'-end labelling of the dephosphorylated fragments E,F,G and H (the products of step 1 in Fig.2A) was done by addition of [5'-^{32}P] pCp to the 3'-hydroxyl group using T4 RNA ligase (step 2 in Fig.2A). Reaction mixture contained 30 µM fragment (300 pmoles) ; 10 µM [5'-^{32}P]pCp (110 T Bq/mmole) ; 100 µM ATP and 450 units/ml of T4 RNA ligase in 50 mM Hepes-KOH pH 7.5 ; 10 mM $MgCl_2$; 3.3 mM DTT ; 10 µg/ml BSA and 10% DMSO. Incubation was for 6h at 17°C and products were isolated by P.A.G.E and eluted from the gel. The 3'-end labelled fragments E,F,G,H were respectively denominated I,J,K,L.

Construction of 3'-end labelled yeast $tRNA^{Asp}$ variants

Preparation of 3'-end labelled quarter tRNA variants

Fragment J was joined (in the presence of T4 RNA ligase) with HO^{AUG}_{OH} or HO^{UUU}_{OH} ; fragment K was joined with HO^{AUG}_{OH} or HO^{AAU}_{OH} or HO^{UUU}_{OH} and fragment L was joined with HO^{UUU}_{OH} (Fig.2B). Prior to the ligation step, the 5'-end of fragments was phosphorylated (step 3 in Fig. 2B) in the following conditions : 1.5 µM of fragments J,K,L in 50 mM Tris-HCl pH 7.6 ; 10 mM $MgCl_2$; 5 mM DTT ; 10 µg/ml BSA ; 15 µM ATP were incubated with 500 units/ml of T4 polynucleotide kinase lacking the 3'-phosphatase activity. After 2h30 at 37°C the enzyme was inactivated by heating at 70°C for 3 min and the RNA was recovered by ethanol precipitation. The joining reaction (step 4 in Fig. 2B) of trinucleotides ($_{OH}^{UUU}_{OH}$, HO^{AAU}_{OH}, HO^{AUG}_{OH}) was performed in 50 mM Hepes-KOH pH 7.5 ; 10 mM $MgCl_2$; 3,3 mM DTT ; 10 µg/ml BSA and 20% DMSO. Concentrations of the acceptor (trinucleotide), of ATP, of donor (5'-end phosphorylated fragment J,K,L) and T4 RNA ligase were 1.5 mM ; 1.5 µM ; 1.5 µM and 360 units/ml respectively. The reaction was performed at 17°C for 8h and the joined products were purified by P.A.G.E and eluted from the gel. The ligation products of trinucleotides HO^{AUG}_{OH} and HO^{UUU}_{OH} with fragment J were denominated J_1 and J_2 respectively. Addition products of trinucleotides HO^{AUG}_{OH}, HO^{AAU}_{OH}, HO^{UUU}_{OH} to fragment K were denominated K_1, K_2, K_3 respectively and the ligation product of HO^{UUU}_{OH} to fragment L was denominated L_1.

Preparation of 3'-end labelled tRNA variants

5'-end phosphorylated fragments I, J_1, J_2, K_1, K_2, K_3 and L_1 were used as donors and 3'-end dephosphorylated fragments A, B, C as acceptors in different ligation reactions to form the various 3'-half tRNA variants (step 5 in Fig. 2C). The different combinations of donor

and acceptor are indicated in Fig. 2C. The reaction conditions were similar to those for the previous ligation step (step 4 in fig. 2B) except that the concentration of the donor, acceptor, ATP and T4 RNA ligase were 0.5 µM, 16 µM, 5 µM and 180 units/ml respectively. Before the addition of T4 RNA ligase the mixture was incubated at 60°C for 5 mn and then cooled slowly to room temperature. After incubation at 17°C for 4h, enzyme was inactivated by heating at 70°C for 3 mn and the RNA was ethanol precipitated. The 3'-half tRNA variant was then submitted to a 5'-end phosphorylation (as described above) and ligated with the 3'-end dephosphorylated 5'-half tRNA to form tRNA-sized molecules (step 6 in fig. 2C). The ligation conditions were similar to those described above for the preparation of 3'-half tRNA variants except that the concentration of T4 RNA ligase was 40 units/ml. After incubation for 1h at 37°C, the various tRNA variants were purified by P.A.G.E and the 3'-end labelled $tRNA^{Asp}$ variants were localized by autoradiography. Electrophoretic mobility of these variants was compared with that of an authentic unlabelled yeast $tRNA^{Asp}$, detected by U.V. shadowing.

Sequence analysis of 3'-end labelled $tRNA^{Asp}$ and of 3'-end labelled $tRNA^{Asp}$ variants

Sequence analysis was performed by the direct chemical method of Peattie (35). The unmodified yeast $tRNA^{Asp}$ was 3'-end labelled by addition of [5'-^{32}P]pCp (110 TBq/mmole) with T4 RNA ligase to the 3'-termini of yeast $tRNA^{Asp}$. Conditions of labelling were similar to those described above.

RESULTS AND DISCUSSION

Using a combination of different enzymes : RNase T1, RNase A, RNase U2 and spleen phosphodiesterase under appropriate conditions it was possible to prepare various fragments of yeast $tRNA^{Asp}$. These fragments and various trinucleotides were then used in conjunction with T4 polynucleotide kinase and T4 RNA ligase to reconstitute *in vitro* 3'-end labelled $tRNA^{Asp}$ variants having substitution or addition of nucleotides in T-stem (position 61) and in T-loop (position 57 to 60).

Experimental procedure

Preparation of yeast $tRNA^{Asp}$ fragments

Figure 1 shows the different hydrolyses used for preparing yeast $tRNA^{Asp}$ fragments. The products of each hydrolysis step were isolated by polyacrylamide gel electrophoresis. Due to the steric hindrance, it was not possible by enzymatic digestion of overall tRNA to obtain high yields

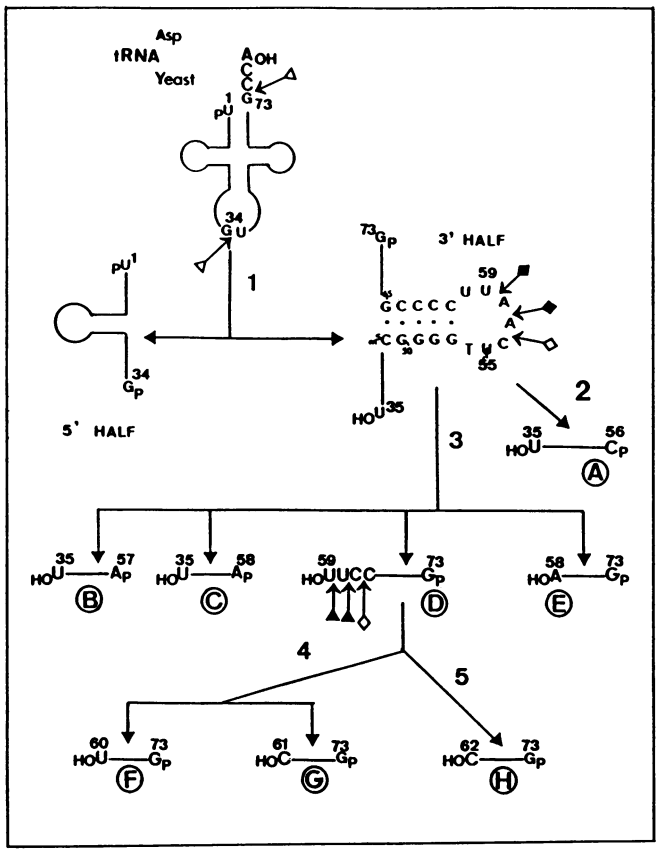

Figure 1 : Scheme for the preparation of different tRNA fragments. Step 1, 3, 4 : limited digestion with RNase T1, RNase U2 and spleen phosphodiesterase (SPDE) respectively ; steps 2 and 5 : RNase A partial digestion. Letters A to E denote the different fragments used in this study. ▷⟶ RNase T1 cleavage site ; ◆⟶ RNase U2 cleavage site ; ◇⟶ RNase A cleavage site ; ▶⟶ SPDE cleavage site.

of RNA fragments generated by enzymatic cuts in T-loop. This problem was overcome by working on the isolated 3'-half tRNA. Under mild conditions, RNase T1 cleaves (22-29) predominantly (Fig.1 step 1) in the anticodon loop of yeast $tRNA^{Asp}$ between residues G34 and U35 (up to 75% - 80%) and in the 3'-extremity between residues G73 and C74 (up to 50%) generating the 5'-half tRNA and the 3'-half tRNA (with or without 3'-terminal CCA). Due to the persistence of T-stem structure in isolated 3'-half tRNA, (P. Romby et al., unpublished results) it was possible, using RNase A or

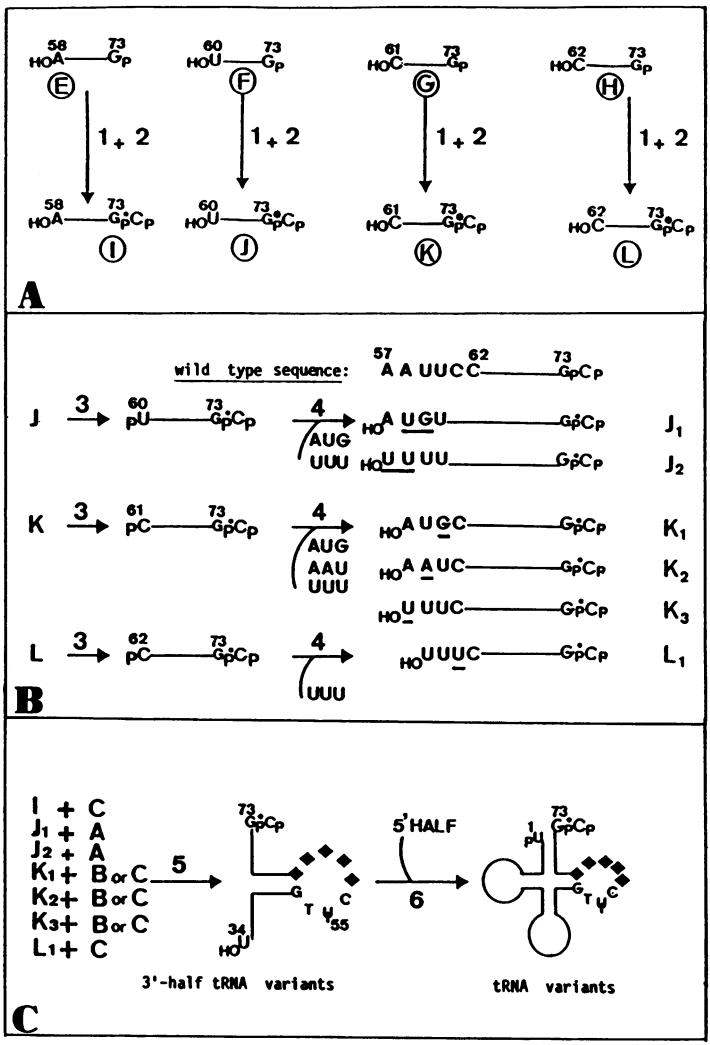

Figure 2 : Scheme for the construction of 3'-end labelled $tRNA^{Asp}$ variants.
A : 3'-end labelling of quarter $tRNA^{Asp}$. E to H correspond to quarter tRNA described in Fig. 1. Step 1 : 3'-end dephosphorylation of RNA fragments with T4 polynucleotide kinase. Step 2 : 3'-end labelling using [5'-^{32}P]pCp and T4 RNA ligase. The products of step 2 (fragments I to L) were isolated by P.A.G.E. * position of [^{32}P] label. B : Construction of 3'-end labelled quarter $tRNA^{Asp}$ variants. Prior to the ligation step 4, fragments J,K and L were 5'-end phosphorylated (step 3). The products of step 4 were isolated by P.A.G.E. Fragments J1,J2,K1,K2,K3,L1 correspond to the extended fragments J,K,L.Trinucleotides AUG, UUU and AAU have hydroxyl groups at their 5' and 3'-ends. Altered nucleotides are underlined.
C : Construction of 3'-end labelled 3'-half tRNA variants and 3'-end

labelled tRNA variants. Fragments A,B,C correspond to fragments described in Fig. 1. Prior to the ligation step 5, fragments A,B and C were 3'-end dephosphorylated and fragments I,J1,J2,K1 to K3 and L1 were 5'-end phosphorylated. The products of step 5 were not isolated by P.A.G.E. Prior to the ligation step 6,5'-half tRNA was 3'-end dephosphorylated and 3'-half tRNA variants were 5'-end phosphorylated. The products of step 6 were isolated by P.A.G.E. ◆ denotes the different altered nucleotides.

RNase U2 partial hydrolysis (Fig.1 step 2 and 3 respectively) of the 3'-half tRNA (without the 3'-terminal CCA) to obtain various quarter tRNAs (fragments A,B,C,D,E in Fig.1) by enzymatic cuts in T-loop. Partial hydrolysis with RNase A (Fig.1 step 2) introduces multiple cleavages in the 3'-half tRNA. However, among multiple fragments, we could recover $HO^{35}U---^{56}Cp$ as the major product (about 15% of recovery). Under mild conditions, RNase U2 (Fig.1 step 3) cleaves exclusively in the T-loop of the 3'-half tRNA between residues A_{57} and A_{58} and between residues A_{58} and U_{59} generating fragments B, C, D and E (see Fig.1).80% of the starting material was recovered in these four RNase U2 hydrolysis products. Limited hydrolysis of fragment D with spleen phosphodiesterase (Fig.1 step 4) or with RNase A (Fig.1 step 5) was used to generate RNA fragments F, G and H corresponding to fragment D differently truncated in its 5'-part. Partial digestion of fragment D with spleen phosphodiesterase causes important recurrent cleavage, between U_{59} and U_{60}, between U_{60} and C_{61} generating RNA fragments F : $HO^{60}U---^{73}Gp$ and G : $HO^{61}C---^{73}Gp$. Fragments F and G were obtained in good yields (about 20% of the starting material for each fragment). This yield is best explained if we consider the presence of a series of four cytosines that slow down dramatically the exonuclease activity of the enzyme (36). Limited digestion of fragment D with RNase A (Fig. 1 step 5) was used to generate fragment H corresponding to fragment D lacking the three 5'-terminal nucleotides. In early attempts, considerable difficulty was encountered in consistently obtaining a correct yield of fragment H. However the digestion described under Materials and Methods gave a final recovery of at least 25% of the starting fragment D. The reason for this RNase A preferential cleavage between residues C_{61} and C_{62} is not obvious. The absence of secondary structure in quarter tRNA D (15 nucleotide long) can explain the great difficulty in obtaining hydrolysis products of this fragment in high yields. Directed RNase H cleavage may be used as an alternative to possibly overcome this difficulty (37).

Construction of 3'-end labelled $tRNA^{Asp}$ variants

The RNA fragments resulting from the above ribonuclease diges-

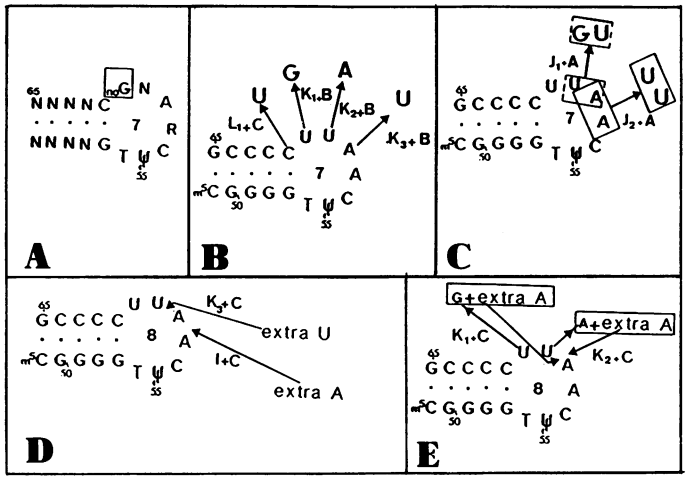

Figure 3 : A : T-stem and loop consensus sequence of eukaryotic elongator tRNAs (44). N corresponds to any nucleotide ; R corresponds to a purine nucleotide ; no G indicates that G is never found at position 60. B,C,D,E : T-stem and loop sequences of $tRNA^{Asp}$ variants. B and C : tRNA variants with seven nucleotide long T-loop and harboring one (B) or two (C) nucleotide substitutions. D and E : tRNA variants with eight nucleotide long T-loop resulting from one nucleotide addition (D) or one nucleotide addition and one nucleotide substitution (E). Substituted or (and) added nucleotides are indicated by an arrow and fragments used in the ligation step 5 (see Fig. 2C) are indicated adjacent to the arrow.

tions as well as various trinucleotides were used to restructure the T-stem and T-loop of yeast $tRNA^{Asp}$ using the T4 RNA ligase technology. Figures 2A, B and C outline the procedure used to construct 3'-end labelled $tRNA^{Asp}$ variants. Additions or substitutions of nucleotides occurring in the ten $tRNA^{Asp}$ variants were indicated in Figures 3B, C, D and E. Synthesis of almost all of the 3'-end labelled $tRNA^{Asp}$ variants was essentially carried out in four ligation steps (step 2 in Fig. 2A, step 4 in Fig. 2B, step 5 and 6 in Fig. 2C) catalysed by the T4 RNA ligase. This enzyme catalyses the ATP-dependant formation of a phosphodiester bond between a donor molecule bearing a 5'-phosphate and an acceptor molecule bearing a 3'-hydroxyl group (38). Correct donor and acceptor molecules were obtained by using the 3'-end phosphatase activity of T4 polynucleotide kinase (34) and the 5'-kinase activity of T4 polynucleotide kinase devoid of the 3'-phosphatase activity (39). The yield of the different dephosphorylation steps was almost 90%, that of the phosphorylation was about 70%. No degradation of RNA fragments occurred during the incubation

with T4 polynucleotide kinase and it was not necessary to purify the products after phosphorylation or dephosphorylation steps.

The overall yield of such tRNA variant construction is low, therefore it is necessary to work with highly labelled molecules as starting material. The first ligation step (step 2 in Fig. 2A) using [5'-^{32}P]pCp and T4 RNA ligase introduces a ^{32}P label at the 3'-end dephosphorylated quarters tRNAs E, F, G and H. Typical experiments yielded highly labelled fragments I, J, K and L (2.10^8 dpm/µg of starting material). After purification by gel electrophoresis, fragments J, K, L were 5'-end phosphorylated with T4 polynucleotide kinase devoid of 3'-phosphatase activity (step 3 in Fig. 2B). Sequence alterations of yeast $tRNA^{Asp}$ was essentially performed in the second ligation step (step 4 in Fig. 2B). In this ligation, 5'-end phosphorylated fragments J, K, L (donor molecules) were joined to the 3'OH of various acceptor trinucleotides ($_{HO}AUG_{OH}$, $_{HO}UUU_{OH}$, $_{HO}AAU_{OH}$). At this step, the presence of a 3'-phosphate blocking group on donor molecules was required in order to prevent cyclisation or self addition of these donors. The resulting fragments were purified by gel electrophoresis. Figure 4A shows an example of the addition of K and J fragments to trinucleotides $_{HO}UUU_{OH}$ or $_{HO}AUG_{OH}$. Yields in this step were 20-40% depending on acceptor nucleotide sequence. In addition $_{HO}UUU_{OH}$ is a poor acceptor as described by Romaniuk et al. (40). After the phosphorylation step 3 (Fig. 2B), the reaction products were not separated by P.A.G.E, explaining the presence of fragments K and J bearing no 5'-phosphates, as illustrated in Figure 4A. The third ligation step (step 5 in Fig. 2C) gave rise to the 3'-end labelled 3'-half tRNA variants. Before this ligation, the 3'-end labelled quarter tRNA variants J_1, J_2, K_1, K_2, K_3, L_1 (see Fig. 2B) were 5'-end phosphorylated using T4 polynucleotide kinase lacking the 3'-phosphatase activity in order to protect the 3'-phosphate. The RNA fragments were then annealed with 3'-end dephosphorylated fragments A, B or C (see Fig. 2C for the description of different combinations of donors and acceptors). The T-loop was subsequently sealed by incubation with T4 RNA ligase. The acceptor molecules (3'-end dephosphorylated fragments A, B or C) bear a hydroxyl group at their 5'-end in order to prevent their cyclisation or self addition. The mixture was then incubated with ATP and T4 polynucleotide kinase devoid of 3'-phosphatase activity. The 3'-end labelled 3'-half tRNA variants were annealed with the 3'-end dephosphorylated 5'-half tRNA and the anticodon loop was joined with a limited amount of T4 RNA ligase (Fig. 2C step 6). In this

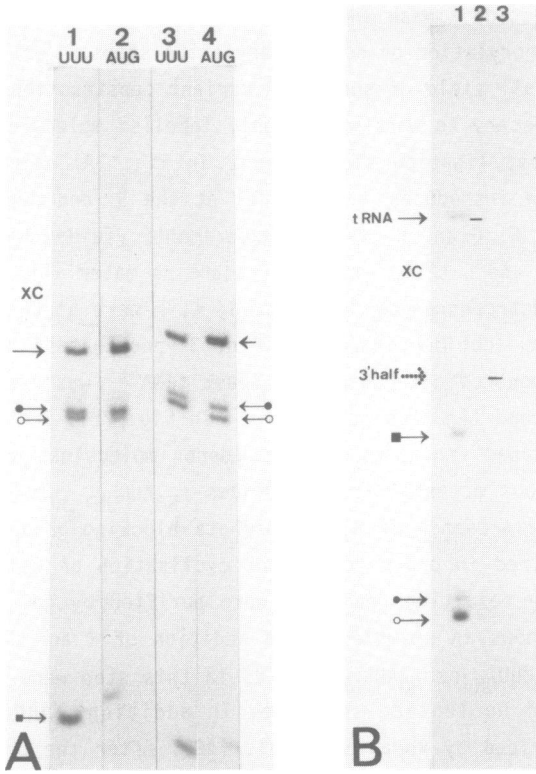

Figure 4 :
A : Elongation of 5'-end phosphorylated fragments K and J by addition of trinucleotides $_{HO}UUU_{OH}$ or $_{HO}AUG_{OH}$ with T4 RNA ligase. Autoradio graphy of a 20% polyacrylamide gel. Lane 1 : fragment K + $_{HO}UUU_{OH}$; lane 2 : fragment K + $_{HO}AUG_{OH}$; lane 3 : fragment J + $_{HO}UUU_{OH}$; lane 4 : fragment J + $_{HO}AUG_{OH}$. ⟶ indicates the position of elongated product ; ○⟶ indicates the position of residual 5'-end phosphorylated fragments K and J ; ●⟶ indicates the position of fragments K and J non phosphorylated at their 5'-end ; product indicated by ■⟶ corresponds probably to reversed action of T4 RNA ligase ; XC denotes the position of xylene cyanol marker dye.
B : Joining of the 3'-half containing altered T-stem (C_{61} U_{61}) and the 5'-half tRNA with T4 RNA ligase to obtain tRNA-sized molecule. Autoradiography of a 10% polyacrylamide gel. Lane 1 : ligation of 3'-end labelled 3'-half tRNA variant (product of L_1 + C ligation, step 5 in Fig. 2C) with 5'-half tRNA ; lane 2 : native $tRNA^{Asp}$, position (-) detected by U.V. shadowing ; lane 3 : unlabelled 3'-half tRNA (with 3'-terminal CCA), position (-) detected by U.V. shadowing ; ·⟶ indicates the position of residual 3'-end labelled 3'-half tRNA ; ●⟶ residual non 5'-end phosphorylated fragment L1 ; ○⟶ residual 5'- end phosphorylated fragment L_1 ; ■⟶ product of T4 RNA ligase reversal action ; XC denotes the position of xylene cyanol marker dye.

fourth and last ligation step the acceptor molecule (3'-end dephosphorylated 5'-half tRNA) bears a 5'-phosphate and a 3'-hydroxyl group. The annealing of the two tRNA halves prevents the circularisation of this acceptor (41). The final products were isolated by denaturing polyacrylamide gel electrophoresis. Example of final product purification is shown in Figure 4B. The presence of residual quarter tRNA is due to the fact that 3'half tRNA variants are not isolated by P.A.G.E. The yield of ligation steps 5 and 6 (Fig. 2C) was about 45% and 75% respectively. As in the ligation step 4 (Fig. 2B and Fig. 4A), undesired RNA fragments appear in ligation steps 5 and 6. They are probably due to the reverse action of T4 RNA ligase (42) and contribute to the decrease in the yield of the ligation steps.

All yeast $tRNA^{Asp}$ variants (except for the tRNA variant $C_{61} \longrightarrow U_{61}$) migrated in the expected position on denaturing gels. All the different preparations of the T-stem variant $C_{61} \longrightarrow U_{61}$ migrated more slowly than the products prepared by trinucleotide insertion in the T-loop. The $tRNA^{Asp}$ variants were obtained in radiochemical amounts and did not contain the 3'-terminal CpA_{OH}. Overall yields of the final products were usually 0.5%-1% of the 3'-end labelled starting material (quarter fragment J, K, L). The limiting step in constructing these tRNA variants is the ligation of the trinucleotides to the fragments J, K, L (step 4 in Fig. 2B, 20-40% yield). Besides, its products being purified by P.A.G.E, this additional step strongly contributes to decrease the overall yield of the construction. One out of the ten 3'-end labelled $tRNA^{Asp}$ variants (containing an extra adenosine between residues A_{57} and A_{58}) was obtained essentially in three ligation steps. The yield of the final product with an extra adenosine residue in T-loop was generally 10-15% of the starting 3'-end labelled quarter I (see Fig. 2A and C for the construction). The yield of this construction is 10-20 fold as high as that of others. The explanation is found in the omission of ligation step 4 and purification step that follows.

The ten 3'-end labelled $tRNA^{Asp}$ variants were sequenced by the chemical method (35). Autoradiograms from sequencing six 3'-end labelled $tRNA^{Asp}$ variants and 3'-end labelled authentic yeast $tRNA^{Asp}$ are shown in Figure 5. The sequencing gels confirm that the sequence of each synthetic tRNA variant corresponds to the predicted sequence. All the data indicate that the tRNA $C_{61} \longrightarrow U_{61}$ (Fig. 5B) has the same sequence as $tRNA^{Asp}$ except the cytosine to uridine base substitution in T-stem. The abnor-

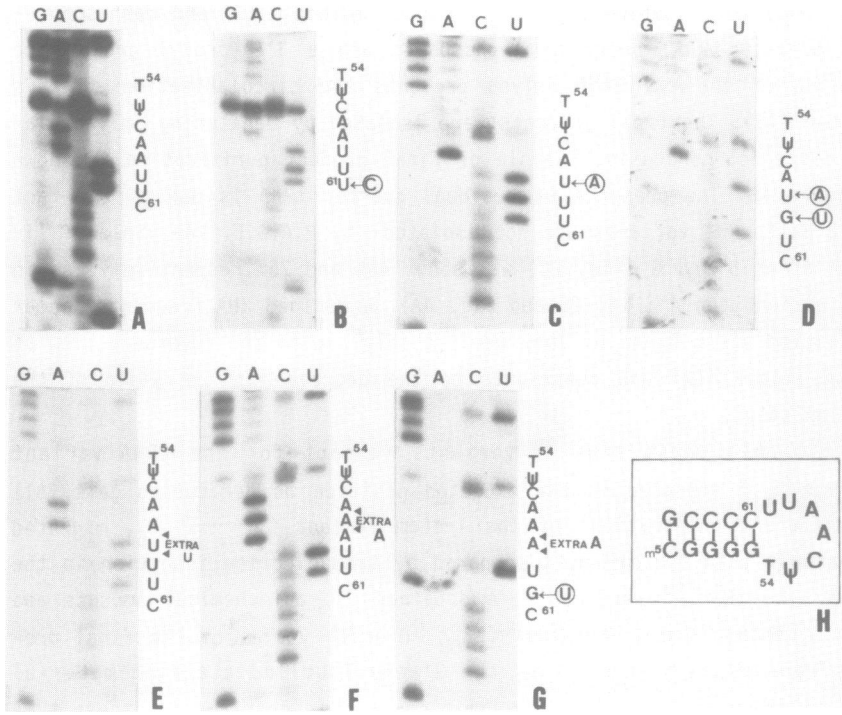

Figure 5 : Chemical RNA sequencing of 3'-end labelled $tRNA^{Asp}$ variants. Autoradiography of sequencing gels. G : guanosine reaction ; A : adenosine major and guanosine minor reaction ; C : cytidine major and uridine minor reaction ; U : uridine major reaction. Authentic $tRNA^{Asp}$: part A. tRNA variants : part B, $C_{61} \longrightarrow U_{61}$; part C, $A_{58} \longrightarrow U_{58}$; part D, $A_{58} \longrightarrow U_{58}$ and $U_{59} \longrightarrow G_{59}$; part E, extra U between residues 58 and 59 ; part E, extra A between residues 57 and 58 ; part G, $U_{60} \longrightarrow G_{60}$ and extra A between residues 58 and 59. T-stem and loop of authentic yeast $tRNA^{Asp}$: part H.

mal migration of this variant on polyacrylamide gel is probably due to an altered conformation of this molecule resulting from a G-U base pair at the end of the T-stem. An altered electrophoretic mobility of the same order has been reported for a species of E.coli 5S RNA where a cytidine to uridine transition transforms a G-C base pair to a G-U base pair (43).

Sequence characteristics of $tRNA^{Asp}$ variants

Nucleotide sequence of T-stem and T-loop of the ten $tRNA^{Asp}$ variants are shown in Figures 3B, C, D and E. $tRNA^{Asp}$ variants can be classified in four groups : tRNA with seven bases in T-loop harboring one (Fig. 3B) or two (Fig. 3C) nucleotide substitutions and $tRNA^{Asp}$ with

eight bases in T-loop resulting from one nucleotide addition (Fig. 3D) or from one nucleotide addition and one nucleotide substitution (Fig. 3E). The altered nucleotides were generally the conserved or semi-conserved nucleotides according to the consensus sequence of eukaryotic elongator tRNAs (44) (see Fig. 3A).

Four yeast $tRNA^{Asp}$ variants harbor each a single distinctive nucleotide substitution (Fig. 3B) : $A_{58} \longrightarrow U_{58}$, $U_{59} \longrightarrow A_{59}$, $U_{60} \longrightarrow G_{60}$, $C_{61} \longrightarrow U_{61}$. Two of these replace the conserved nucleotides A_{58} (located in T-loop) and C_{61} (located in T-stem) by uridines. Another replaces U_{60} by a guanosine residue. In all authentic eukaryotic elongator tRNAs the nucleotide in this position is never a guanosine residue. In the fourth, the residue U_{59} (any nucleotide in this position in eukaryotic elongator tRNA) is replaced by an adenosine.

Two $tRNA^{Asp}$ variants carry each two adjacent nucleotides substitutions (Fig. 3C). In one of them, residues A_{57} (always a purine in eukaryotic elongator tRNAs) and A_{58} (always an adenosine in eukaryotic elongator tRNAs) are replaced by a uridine. In the other one, residues A_{58} and U_{59} are replaced by a uridine and a guanine respectively.

Two $tRNA^{Asp}$ variants show an additional nucleotide at two different locations in T-loop (Fig. 3D). In one variant an extra adenosine is inserted between residues A_{57} and A_{58} ; in the other one, an extra uridine is inserted between residues A_{58} and U_{59}. These nucleotide insertions yield $tRNA^{Asp}$ variants with an eight base T-loop instead of seven like in all authentic tRNAs.

Two other $tRNA^{Asp}$ variants also have an 8 base T-loop. These two tRNAs arise from the addition of an extra adenosine between residues A_{58} and U_{59} and from a substitution at two different locations : residue U_{59} is replaced by an adenosine in one variant and residue U_{60} is replaced by a guanosine in the other one.

Implications for conformational studies of yeast $tRNA^{Asp}$

Nucleotide additions or substitutions in T-loop may alter some tertiary interactions involved in three-dimensional structure of yeast $tRNA^{Asp}$, especially at the level of the T-D loop contact. It is now possible to probe the solution structure of end-labelled RNAs using chemical or enzymatical probes. These approach need only limited amounts of [^{32}P] end-labelled material. The direct conformational analysis in solution of 3'-end labelled yeast $tRNA^{Asp}$ variants will allow to stress the importance of tertiary interactions, involving conserved or semi-conserved nu-

cleotides, in maintaining the local or overall structure of tRNAs. In one instance the importance of the constant reverse Hoogsten base pair T_{54}-A_{58} will be analysed by structural mapping of the $tRNA^{Asp}$ variant where the A_{58} is replaced by a uridine (see Fig. 3B). A seven base T-loop may be required to allow the correct formation of reserve Hoogsten T_{54}-A_{58} base pair. This issue and the role of the constant size of T-loop (always 7 nucleotides) might be elucidated by monitoring the solution structure of $tRNA^{Asp}$ variants with eight nucleotides in T-loop (see Figs. 3D and 3E). In the $tRNA^{Asp}$ variant where the constant C_{61} was replaced by a uridine, the hydrogen bond (p_{60}-C_{61}) that units phosphate 60 (phosphate located beetween C_{61} and C_{62}) with the N4 atome of the C_{61} (9) is impaired. Conformational studies of this variant will allow to determine the exact role of the p_{60}-C_{61} hydrogen bond. A guanosine residue is never found at position 60 in all eukaryotic elongator tRNAs (see Fig. 3A). Conformational analysis of variant tRNA where U_{60} is replaced by a G should help understand the reason for this absence.

Preparation of $tRNA^{Asp}$ variants according to the rationale described in this paper as well as structural analysis in solution (P. Romby et al. unpublished results) constitute an additional approach to conformational analysis of tRNAs.

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