Efficient aminoacylation of a yeast tRNA^{Asp} transcript with a 5′ extension

Véronique Perret, Catherine Florentz and Richard Giegé

Laboratoire de Biochimie, Institut de Biologie Moléculaire et Cellulaire du CNRS, 15 rue René Descartes, 67084 Strasbourg Cedex, France

Received 30 May 1990; revised version received 12 July 1990

A yeast aspartic acid tRNA with a 5′ extension of 14 nucleotides was obtained by in vitro transcription with T7 DNA dependent RNA polymerase. This transcript, called extended tRNA^{Asp} transcript, retains its aspartylation capacity with the same K_m and only three times reduced k_{cat} values as compared to those measured for canonical tRNA^{Asp}. This result indicates that the 5′ extension of the amino acid acceptor stem of tRNA^{Asp} does not interfere with recognition by aspartyl-tRNA synthetase. However, in contrast to the wild-type tRNA^{Asp} transcript, the 5′ extended molecule presents a reduced capacity to be mischarged by arginyl-tRNA synthetase, suggesting the existence of different structural requirements in aspartyl- and arginyl-tRNA synthetases for tRNA^{Asp} recognition.

tRNA^{Asp} transcript; Precursor; Aminoacylation; RNA engineering; Mischarging

1. INTRODUCTION

The requirements that lead to specific recognition between tRNAs and aminoacyl-tRNA synthetases are still not known in detail and their knowledge represents one of the major prerequisites for a thorough understanding of the translation of the genetic message. Besides crystallography [1–3], one classical way to approach this problem is to induce changes in the tRNA molecules and to study their effects on their aminoacylation activities (e.g. [4]). The development of RNA engineering method and of in vitro transcriptional systems now gives the opportunity to create a variety of mutations in the wild-type molecules. This methodology yields tRNAs without modified nucleotides but which fully retain their aminoacylation properties as far as the wild-type molecules have been investigated (e.d. [5–7]), although they can acquire new specificities [7].

To better understand the relationship between the structure of tRNA^{Asp} near its amino acid accepting end and its aspartylation capacity, a variant tRNA^{Asp} molecule was created (called extended tRNA^{Asp} transcript) with a 14 nucleotide-long extension at the 5′ end of the molecule (Fig. 1). This was achieved by site directed mutagenesis of a modified pUC 18 plasmid containing the wild-type synthetic gene of tRNA^{Asp} starting with the T7 promoter. The aspartylation capacity and the mischarging ability of the in vitro transcripts of this gene were assayed in the presence of aspartyl- and arginyl-tRNA synthetases. The results will be discussed in relation with tRNA precursors [8], tRNA-like structures [9–11], and the structural requirements at the 5′ termini of tRNAs for recognition by aminoacyl-tRNA synthetases.

Correspondence address: V. Perret, Laboratoire de Biochimie, Institut de Biologie Moléculaire et Cellulaire du CNRS, 15 rue René Descartes, 67084 Strasbourg Cedex, France

2. MATERIALS AND METHODS

2.1. *tRNA, enzymes and chemicals*

Fully modified yeast tRNA^{Asp} was purified by chromatographies on BD-cellulose and Sepharose 4B columns after counter-current distribution of bulk brewer's yeast tRNA (Boehringer Mannheim, France SA, Meylan) [12,13]. Unmodified tRNA^{Asp} molecules were obtained by in vitro transcription of Bst N1 linearized plasmid pTA (variant pG1-C72, called transcript A) as described previously [7,14]. Pure yeast aspartyl-tRNA synthetase was prepared as described in [2] and pure arginyl-tRNA synthetase by a procedure derived from [15,16]. The T7 RNA polymerase was purified according to King et al. [17]. RNasin was from Promega (Madison WI, USA); pyrophosphatase from Sigma (St. Louis, MO, USA), L-[^{3}H]aspartic acid at 32 Ci/mole and L-[^{3}H]arginine at 57 Ci/mole from Amersham France (Les Ulis), and restriction enzymes from Boehringer Mannheim France SA (Meylan). For mutagenesis experiments, the Amersham kit (ref. RPN 1523) was used.

2.2. *Cloning and site directed mutagenesis of the synthetic tRNA^{Asp} gene*

Plasmid pTA containing the variant (G1-C72) gene of tRNA^{Asp} was constructed as described in [7,14]. To perform site-directed mutagenesis on single-stranded DNA, a new plasmid pTFMA was created by insertion of phage f1 intergenic region into plasmid pTA according to Vernet et al. [18]. The tRNA^{Asp} gene (G1-C72 variant) was then excised from this new plasmid and replaced by a synthetic gene corresponding to the wild-type sequence (U1, A72 [19]) of tRNA^{Asp}. After transformation of *E. coli* strain JM103 by this plasmid called pTFMWT, infection of the cells with filamentous helper phage M13K07, single-stranded DNA with the complementary

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

$tRNA^{Asp}$ sequence was prepared following standard procedures (Pharmacia Analects 14-1, 1986). For further experimental details see [20].

The insertion of a 14-nucleotide long extension between the T7 RNA polymerase promoter and the $tRNA^{Asp}$ sequence by site-directed mutagenesis of single stranded DNA from pTFMWT was done following the procedure of Eckstein and coworkers [21] as described in the Amersham mutagenesis kit. The following 35-nucleotide long oligonucleotide (ACTCACTATA**GGGCAAGCGTATTG**TCCGTGATAG) was used for the mutagenesis, with the 5′ extension indicated in bold characters. The sequence of the gene was verified using the T7 DNA polymerase methodology [22].

2.3. *In vitro transcription*

Transcription of the extended $tRNA^{Asp}$ gene follows essentially the conditions used for pTA [7,14]. Conditions were 40 mM Tris-HCl, pH 8.1, 22 mM $MgCl_2$, 1 mM spermidine, 5 mM dithioerythritol, 0.01% Triton X-100, 4 mM NTP (of each), 16 mM GMP, 40 units RNasin, 0.55 units pyrophosphatase (Sigma, USA), and 80 mg Bst N1 linearized DNA and 3000 units T7 RNA polymerase per ml of incubation mixture. Typical transcriptions were carried out in 150 μl. After 3 h of incubation at 37°C transcripts were phenol extracted, purified by electrophoresis on 10% polyacrylamide/8 M urea gels, and electroeluted. Due to the presence of GMP in the transcription media, 96% of the transcripts start with a 5′ monophosphate as verified by TLC analysis.

2.4. *Aminoacylation of tRNAs*

Aspartylation of tRNAs was performed in a buffer containing 100 mM Tris-HCl (pH 7.2), 5 mM ATP, 10 mM $MgCl_2$ and 30 mM KCl. Arginylation assays were conducted in a buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM ATP, 15 mM $MgCl_2$, 30 mM KCl, 2.5 mM glutathione and 50 μM radioactive amino acid. The plateau levels of aspartylation and arginylation were determined with concentrations of 200 nM of tRNA and 30 nM of the specific aminoacyl-tRNA synthetase. Initial velocities for aspartylation were obtained in the presence of pure aspartyl-tRNA synthetase at a final concentration of 2.5 nM and in a concentration range of tRNA from 2 to 20 nM. Initial velocities for arginylation were obtained in the presence of pure arginyl-tRNA synthetase at a final concentration of 30 or 3 nM and in a concentration range of transcripts from 75 to 330 nM or 300 to 1500 nM for the extended or wild-type transcripts, respectively. Aminoacylation results were determined as trichloroacetic acid precipitable radioactivity. Data from kinetic experiments were analyzed on Lineweaver-Burk plots.

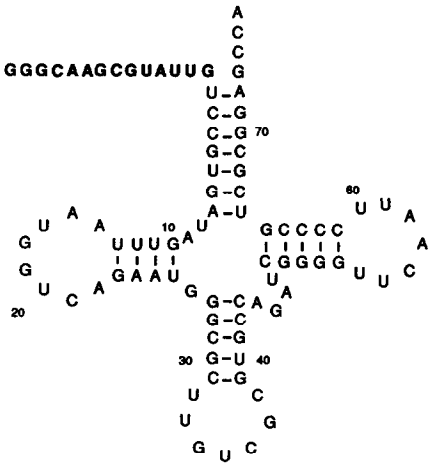

Fig. 1. Cloverleaf secondary structure of extended $tRNA^{Asp}$ transcript. The sequence of the tRNA (nucleotides 1–76) is according to [19]. The nucleotide sequence of the extension is given in bold characters.

3. RESULTS AND DISCUSSION

3.1. *Design and transcription of extended $tRNA^{Asp}$ gene*

The sequence of the extension introduced at the 5′ end of $tRNA^{Asp}$ was chosen according to three criteria: (i) to keep free the 4 nucleotides of the 3′ end of the tRNA (G73C74C75A76); (ii) to prevent internal secondary structure in the 14-nucleotide long extension and to prevent refolding of the transcript; (iii) to give an optimized start to transcription [23]. Thus the sequence (A-4U-3U-2G-1) has been chosen for the 3′ part of the extension and three contiguous G residues (G-14G-13G-12) have been introduced at its 5′ end. Further, the internal portion of the extension consists of a random sequence without possibilities of Watson-Crick base pairings. We note that the sequence of the extension differs from that of the spacer region, 10-nucleotide long, which was found upstream of $tRNA^{Asp}$ in the *Saccharomyces cerevisiae* gene containing the dimeric $tRNA^{Arg3}$-$tRNA^{Asp}$ precursor [23].

As expected the transcription level of the extended $tRNA^{Asp}$ is high and the yield of PAGE purified RNA is 50 μg for a typical 150 μl incubation. It is comparable to that of the (pG1-C72) variant and much better (about 100 fold) than that of the wild-type (pU1-A72) molecule [7].

3.2. Aminoacylation of extended $tRNA^{Asp}$ *transcript*

In a previous study, we have demonstrated that both (pU1-A72) and (pG1-C72) $tRNA^{Asp}$ variants can be aspartylated as efficiently as modified natural $tRNA^{Asp}$ [7]. Since the (pG1-G72) variant is active and can be easily transcribed, it was used rather than the (pU1-A72) molecule as a control in this work.

3.2.1. Aspartylation of extended $tRNA^{Asp}$ transcript

Fig. 2 displays comparative aspartylation assays conducted on extended $tRNA^{Asp}$ transcript (pG1-C72) $tRNA^{Asp}$ transcript, and natural modified $tRNA^{Asp}$. As could not be predicted *a priori*, the extended transcript is efficiently aspartylated as compared to the control

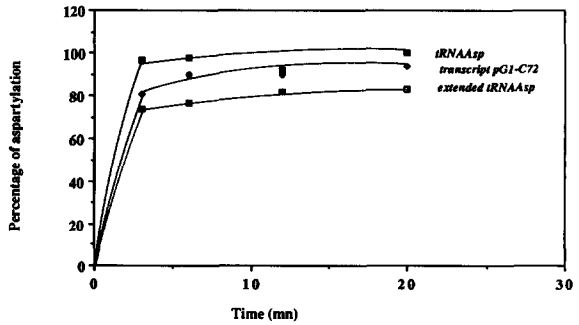

Fig. 2. Aspartylation plateaus of canonical modified yeast $tRNA^{Asp}$, and of the two $tRNA^{Asp}$ transcripts, variant (pG1-C72) and extended $tRNA^{Asp}$. The aminoacylation reactions were conducted on 100 μl samples and each experimental point corresponds to 20 μl aliquots.

tRNAs. Not only a 83% to 90% aspartylation level is observed for the extended molecule, but also its kinetic parameters are very similar to those of the controls (Table I). Indeed the Michaelis-Menten constant, K_m of $tRNA^{Asp}$ is 60 nM, whereas that of the extended molecule is 76 nM. A small difference is found for the k_{cat} value of the extended transcript, which is three times lower than that of native $tRNA^{Asp}$. Further the specificity constants k_{cat}/K_m are quasi-identical for the different kinds of tRNA. These results clearly show that the presence of 14 extra-bases at the 5′ end of $tRNA^{Asp}$ does neither prevent the recognition by aspartyl-tRNA synthetase, nor the formation of a catalytically active complex.

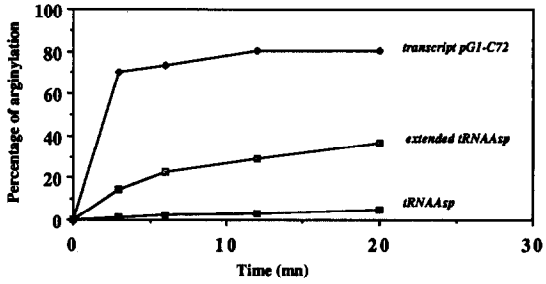

Fig. 3. Arginylation plateaus of canonical modified yeast $tRNA^{Asp}$, and of the two $tRNA^{Asp}$ transcripts, variant (pG1-C72) and extended $tRNA^{Asp}$. Aminoacylation reactions were conducted as described in Fig. 2.

3.2.2. Arginylation of extended $tRNA^{Asp}$ transcript

Beside being aspartylatable, $tRNA^{Asp}$ transcripts, either wild-type or the pG1-C72 variant, exhibit particular functional characteristics compared to the natural $tRNA^{Asp}$: they are efficiently mischarged by the non-cognate side arginyl-tRNA synthetase [7]. In the present work, we show that the extended $tRNA^{Asp}$ transcript is also recognized and aminoacylated by arginyl-tRNA synthetase. As presented in Fig. 3, the arginylation level of the extended transcript is significant (36%) and much higher than that of modified $tRNA^{Asp}$ (5%). However, this level is not as high as that of the pG1-C72 transcript which reaches 80%. Thus, recognition by arginyl-tRNA synthetase is influenced by the presence of the extension in the transcript. As seen in Table I, the influence of the extra-bases is essentially expressed at the level of the reaction rate as reflected by a ten fold lower k_{cat} for the mutant than for the wild-type transcript. The slightly better K_m for the arginylation of the extended molecule could reflect an enhanced affinity of the variant molecule for arginyl-tRNA synthetase due to nonspecific interactions of the extension with the synthetase. A similar effect was observed for the TYMV tRNA-like molecules interacting with valyl-tRNA synthetases [24]. As a consequence, the specificity constant k_{cat}/K_m for mischarging is only slightly decreased in the case of the extended molecule, the k_{cat} effect being compensated by the K_m effect.

3.3. *Role of the 5′ end of tRNA for aminoacylation*

Earlier footprinting experiments of $tRNA^{Asp}$ in contact with aspartyl-tRNA synthetase [13] as well as the low resolution crystal structure of the aspartic acid complex [1], have shown that the tRNA interacts with the protein through the face comprising the variable region. Since aspartylation remains efficient with extended $tRNA^{Asp}$, it implies that the 5′ terminus does not interfere with complex formation and/or aminoacylation activity. A likely structural explanation would be that the nucleotide sequence of the tRNA presents a kink between residues 1 and −1, allowing the extension to leave the enzyme surface. Consequently the region in tRNA around residue 1 seems not to be important for aspartylation, a conclusion also supported by other independent experimental evidences. Indeed the wild-type sequence of the tRNA could be mutated from U1-(A72) to G1-(C72) without a loss of the aminoacylation activity, contrarily to what could be expected from sequence comparison of yeast tRNAs that points to a strong correlation between the nature of this nucleotide and aspartic acid identity [25]. Further, the 5′ end of the tRNA, normally starting with a monophosphate group could be replaced by a triphosphate without affecting the aspartylation activity [7].

It can be questioned whether these properties and structural implications apply to other tRNA aminoacylation systems. As far as the phosphorylation

Table I

Kinetic parameters of aminoacylation of modified $tRNA^{Asp}$, (pG1-C72) and extended $tRNA^{Asp}$ transcripts by yeast aspartyl- and arginyl-tRNA synthetases

	Plateau	k_{cat} (s^{-1})	K_m (nM)	k_{cat}/K_m (relative)
Aspartyl-tRNA synthetase				
native $tRNA^{Asp}$	100	0.60	60	100
(pG1-C72) variant	96	0.69	60	115
extended transcript	85	0.22	76	29
Arginyl-tRNA synthetase				
(pG1-C72) variant	80	0.11	1300	100
extended transcript	36	0.012	430	33

of the 5′ termini is concerned, it is known for instance that triphosphorylated $tRNA^{Phe}$ transcript [5] or non-phosphorylated *E. coli* $tRNA^{fMet}$ [26] and yeast $tRNA^{Phe}$ [27] can also be charged. Larger structural changes are found at this location in tRNA-like ends of viral genomes. In that case the acceptor arm involves a pseudo-knotted folding in which nucleotides equivalent to residues −1 (or more upstream) of a 5′ extension of a canonical tRNA are found (see for instance the 4 nucleotides from loop L1 in the tRNA-like structure of TYMV [28]). Despite this structural peculiarity, viral tRNA-like molecules are efficiently aminoacylated. In contrast however, *E. coli* $tRNA^{His}$ which is known to have an extra G-1 residue at the 5′ end of its acceptor stem has its aminoacylation activity affected by introduction of a triphosphate at the 5′ terminus [29], but this behaviour may be explained by the fact that G-1 base-pairs with the discriminator residue C73 and thus brings the triphosphate group in closer vicinity to the catalytic site of the synthetase.

It is interesting to note that the arginylation ability of extended $tRNA^{Asp}$ is reduced as compared to that of the normal-length $tRNA^{Asp}$ transcript, while its aspartylation ability is similar. This means that the recognition process of this extended tRNA by arginyl- and aspartyl-tRNA synthetases is not exactly the same. Accordingly, the protein conformation around the binding sites of the tRNA 5′ termini is not the same for the two synthetases which also differ in molecular mass and oligomeric structure: α1, M_r 74 000 [15], and α2, M_r 130 000 [30], for arginyl- and aspartyl-tRNA synthetase, respectively. So, this observation is a further evidence that the structure of the synthetase governs the interaction of the tRNA [31].

3.4. *Outlooks*

To date and as far as we know, aminoacylation experiments on tRNA precursors have not been described. While recognition (or charging after addition of the 3′ terminal CCA) by aminoacyl-tRNA synthetases of precursor tRNAs containing introns appears unlikely, it may be a likely possibility for other precursors deprived of such structural adducts, in particular for the dimeric $tRNA^{Arg3}$-$tRNA^{Asp}$ precursor from yeast [32]. Could such a putative interaction of an aminoacyl-tRNA synthetase with precursor tRNA have a physiological significance? Although it is premature to answer this question it might be suggested that synthetases present in nuclei [33] could be involved in the regulation of the processing of tRNA precursors. From another point of view, the study of extended tRNA transcript could represent a new way for studying the type of interaction of tRNAs with synthetases of different oligomeric structures. Finally, from a practical point of view the $tRNA^{Asp}$ extended transcript could serve to produce large quantities of wild-type $tRNA^{Asp}$ transcripts starting with U1 (and thus not easily transcribed in vitro by T7 polymerase) after processing with M1 RNA from *Escherichia coli* RNase P [34].

Acknowledgements: This work was supported partly by grants from the Centre National de la Recherche Scientifique (CNRS), the Ministère de la Recherche et de l'Enseignement Supérieur (MRES), Université Louis Pasteur (Strasbourg, France) and NEDO (Japan). We thank F.W. Studier (Brookhaven, USA) for the clone of T7 RNA polymerase, M. Ruff for a gift of aspartyl-tRNA synthetase, and A. Hoeft for the synthesis of DNA fragments. We thank D. Moras for fruitful discussions.

REFERENCES

[1] Podjarny, A., Rees, B., Thierry, J.C., Cavarelli, J., Jésior, J.C., Roth, M., Lewit-Bentley, A., Kahn, R., Lorber, B., Ebel, J.P., Giegé, R. and Moras, D. (1987) J. Biomol. Struct. Dyn. 5, 187–198.
[2] Ruff, M., Cavarelli, J., Mikol, V., Lorber, B., Mitschler, A., Giegé, R., Thierry, J.C. and Moras, D. (1988) J. Mol. Biol. 201, 235–236.
[3] Rould, M.A., Perona, J.J., Söll, D. and Steitz, T.A. (1989) Science 246, 1135–1142.
[4] Normanly, J. and Abelson, J. (1989) Annu. Rev. Biochem. 58, 1029–1049.
[5] Sampson, J. and Uhlenbeck, O.C. (1988) Proc. Natl. Acad. Sci. USA 85, 1033–1037.
[6] Schulman, L.D. and Pelka, H. (1988) Science 242, 793–796.
[7] Perret, V., Garcia, A., Grosjean, H., Ebel, J.P., Florentz, C. and Giegé, R. (1990) Nature 344, 787–789.
[8] Sharp, S.J., Schaack, J., Cooley, L., Johnson-Burke, D. and Söll, D. (1985) CRC Crit. Rev. Biochem. 19, 107–144.
[9] Ebel, J.P., Giegé, R. and Florentz, C. (1989) in: Evolutionary Tinkering in Gene Expression (Grunberg-Manago, M., Clark, B.F.C. and Zachau, H.G. eds) Plenum, pp. 29–39.
[10] Guerrier-Takada, C., Van Belkum, A., Pleij, K. and Altman, S. (1988) Cell 53, 267–272.
[11] Green, C.J., Vold, B.S., Morch, M.D., Joshi, R.L. and Haenni, A.L. (1988) J. Biol. Chem. 263, 11617–11620.
[12] Dirheimer, G. and Ebel, J.P. (1967) Bull. Soc. Chim. Biol. 49, 1679–1687.
[13] Romby, P., Moras, D., Bergdoll, M., Dumas, P., Vlassov, V.V., Westhof, E., Ebel, J.P. and Giegé, R. (1985) J. Mol. Biol. 184, 455–471.
[14] Perret, V., Garcia, A., Puglisi, J., Grosjean, H., Ebel, J.P., Florentz, C. and Giegé, R. (1990) Biochimie (submitted).
[15] Gangloff, J., Schutz, A. and Dirheimer, G. (1976) Eur. J. Biochem. 65, 177–182.
[16] Kern, D., Dietrich, A., Fasiolo, F., Renaud, M., Giegé, R. and Ebel, J.P. (1977) Biochimie 59, 453–462.
[17] King, G.C., Martin, C.T., Pham, T.T. and Coleman, J.E. (1986) Biochemistry 25, 36–43.
[18] Vernet, T., Dignard, D. and Thomas, D.Y. (1987) Gene 52, 225–233.
[19] Gangloff, J., Keith, G., Ebel, J.P. and Dirheimer, G. (1971) Nature New Biol. 230, 125–127.
[20] Garcia, A. and Giegé, R. (in preparation).
[21] Taylor, J.W., Ott, J. and Eckstein, F. (1985) Nucleic Acids Res. 13, 8765–8784.
[22] Zaug, A.J., Kent, J.R. and Cech, T.R. (1984) Science 224, 574–578.
[23] Dunn J.J. and Studier, F.W. (1983) J. Mol. Biol. 166, 477–535.
[24] Dreher, T.W., Florentz, C. and Giegé, R. (1988) Biochimie 70, 1719–1727.
[25] Nicholas, Jr, H.B. and MacClain, W.H. (1987) Comp. Appl. Biosci. 3, 177–181.
[26] Schulman, L.H. and Pelka, H. (1977) Biochemistry 16, 4256–4265.

[27] Sprinzl, M. and Graeser, E. (1980) Nucleic Acids Res. 8, 4737–4744.
[28] Dumas, P., Moras, D., Florentz, C., Giegé, R., Verlaan, P., Van Belkum, A. and Pleij, C.W.A. (1987) J. Biomol. Struct. Dyn. 4, 707–728.
[29] Himeno, H., Hasegawa, T., Ueda, T., Watanabe, K., Miura, K.I. and Shimuzu, M. (1989) Nucleic Acids Res. 17, 7855–7863.
[30] Lorber, B., Kern, D., Dietrich, A., Gangloff, J., Ebel, J.P. and Giegé, R. (1983) Biochim. Biophys. Res. Commun. 117, 259–267.
[31] Moras, D., Lorber, B., Romby, P., Ebel, J.P., Giegé, R., Bentley, A.L. and Roth, M. (1983) J. Biomol. Struct. Dyn. 1, 209–223.
[32] Schmidt, O., Mao, J., Ogden, R., Beckmann, J., Sakano, H., Abelson, J. and Söll, D. (1980) Nature 287, 750–752.
[33] Mirande, M., Le Corre, D., Louvard, D., Reggio, H., Pailliez, J.P. and Waller, J.P. (1985) Exp. Cell Res. 156, 91–102.
[34] Guerrier-Takada, C., Gardiner, K., Marsh, T., Pace, N. and Altman, S. (1983) Cell 35, 849–857.