A new method for identifying the amino acid attached to a particular RNA in the cell

Tsutomu Suzuki[ab], Takuya Ueda[a], Kimitsuna Watanabe[a,*]

Received [a]Department of Chemistry and Biotechnology, School for Engineering, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan
[b]Department of Biological Sciences, Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology, Nagatsuta, Midori-ku, Yokohama 227, Japan

Received 4 January 1996; revised version received 23 January 1996

Abstract To investigate the function of tRNAs or any other aminoacylable RNAs in vivo, it is important to be able to estimate the amounts and species of aminoacylated RNAs in living cells. We have developed a method of analyzing amino acids attached to particular tRNAs obtained from cells. After the ester bond between the amino acid and the 3′-adenosine moiety of a specific aminoacyl-tRNA is stabilized by acetylation of the amino acid with [^{14}C]acetic anhydride, the aminoacyl-tRNA can be fished out with a solid-phase-attached DNA probe. The ^{14}C-labeled acetylamino acid is then released from the thus purified acetyl-aminoacyl-tRNAs by alkaline treatment and detected by TLC analysis.

Key words: Aminoacyl-tRNA (in vivo); Amino acid; Hybridization; *N*-Acetylation; *Candida*

1. Introduction

The aminoacylation level of tRNA is known to reflect the growth conditions of cells [1,2], and small RNAs other than tRNAs [3,4] and several plant viral RNAs [5] are able to be charged with specific amino acids in vitro, suggesting the possible involvement of these charging activities in unknown functions of RNAs in vivo. It is thus of great importance to analyze the amounts and species of aminoacylated RNAs in various cells at different stages.

Aminoacyl-tRNAs participating in the translational machinery in cells have been reported to be protected from deacylation by continuous association with several factors – aminoacyl-tRNA synthetase, elongation factors and ribosomes [6,7]. Since they are unstable if they have no interaction with other factors unless kept under acidic conditions, the quantitative analysis of aminoacyl-tRNA in cells has proved rather difficult.

It is possible to separate aminoacyl-tRNAs from uncharged tRNAs in cells by acid urea polyacrylamide gel electrophoresis (PAGE) and to determine the ratio of the charged and uncharged tRNAs by Northern hybridization [8,9], but this procedure does not detect what kind of amino acid is attached to the tRNA. Direct determination of an amino acid attached to a specific RNA would be a useful method for characterizing tRNAs or aminoacylable RNAs in cells.

One-step purification of a desired RNA using a solid-phase-attached DNA probe is one of the most powerful purification methods, especially of minor RNAs such as suppressor tRNAs or tRNAs in organelles [10,11]. However, this method cannot be applicated to the purification of a specific aminoacyl-tRNA because incubation at high temperature is necessary in a certain part of the process, which would in turn lead to deacylation of the aminoacyl-tRNA.

It is known that modification of the primary amino group of the amino acid attached to a tRNA prevents deacylation [12–14]. In order to stabilize the aminoacyl bond and specifically label the amino acid, we acetylated the amino acid attached to a tRNA obtained from cells with [^{14}C]acetic anhydride. Acetylated aminoacyl-tRNA can be successfully purified by a solid-phase-attached DNA probe, the acetylamino acid being retained at the 3′-end of the tRNA. The ^{14}C-labeled acetylamino acid deacylated from the purified tRNA can then be detected by TLC analysis. In order to demonstrate the validity of this method, we selected a serine tRNA having the anticodon CAG (tRNA^{Ser}CAG), corresponding to the universal leucine codon CUG, from *Candida cylindracea* [15,16]. We have already reported that the universal leucine codon CUG is read as serine in several *Candida* species by a specific serine tRNA corresponding to the codon CUG (tRNA^{Ser}CAG) [17,18], following an experiment showing that the codon CUG can in fact be translated as serine in an in vitro translation with this serine tRNA using synthetic mRNA containing the in-frame CUG codons [15,16]. Here, we attempted to analyze the aminoacylated form of this serine tRNA in the cell to ensure that the tRNA is also charged with serine even in vivo. As a control, a leucine tRNA with the anticodon ncm^{5}UmAA (tRNA^{Leu}U*AA) from *C. zeylanoides* was also analyzed.

2. Materials and methods

2.1. Materials

[1-^{14}C]Acetic anhydride (185 MBq/mmol) was purchased from American Radiolabeled Chemicals. Uniformly labeled [^{14}C]serine (5.99 MBq/mmol) or leucine (11.5 MBq/mmol) were from Amersham. Yeast extract and bactopeptone were purchased from Difco. DEAE Sepharose fast flow was from Pharmacia. Streptavidin agarose was from Gibco BRL. 3′-Biotinylated DNA probes, 5′-AGCAAGCTCAATGGATTCTGCGTCC-3′ for the purification of *C. cylindracea* tRNA^{Ser}CAG and 5′-TGAAGGATGCGAGGTTCGAACTCGCGC-3′ for *C. zeylanoides* tRNA^{Leu}U*AA, were synthesized by Sci. Media, Japan. Glycogen was from Boeringer. Other chemicals were obtained from Wako Chemical Industries.

2.2. Preparation of aminoacyl-tRNAs from growing Candida cells

About 10 mg of aminoacyl-tRNAs was prepared from 10 g of *Candida* culture, which was harvested aerobically with 4 l of YPD medium (1% yeast extract, 2% bactopeptone and 2% dextrose) in the early log-phase, by phenol extraction at 4°C under acidic conditions conferred by 0.3 M NaOAc (pH 5.0), according to the literature [8]. To remove the cell wall components as well as the phenol, the

*Corresponding author. Fax: (81) (3) 5800–6950.
E-mail: kw@kwl.t.u-tokyo.ac.jp

SSDI S0014-5793(96)00107-X

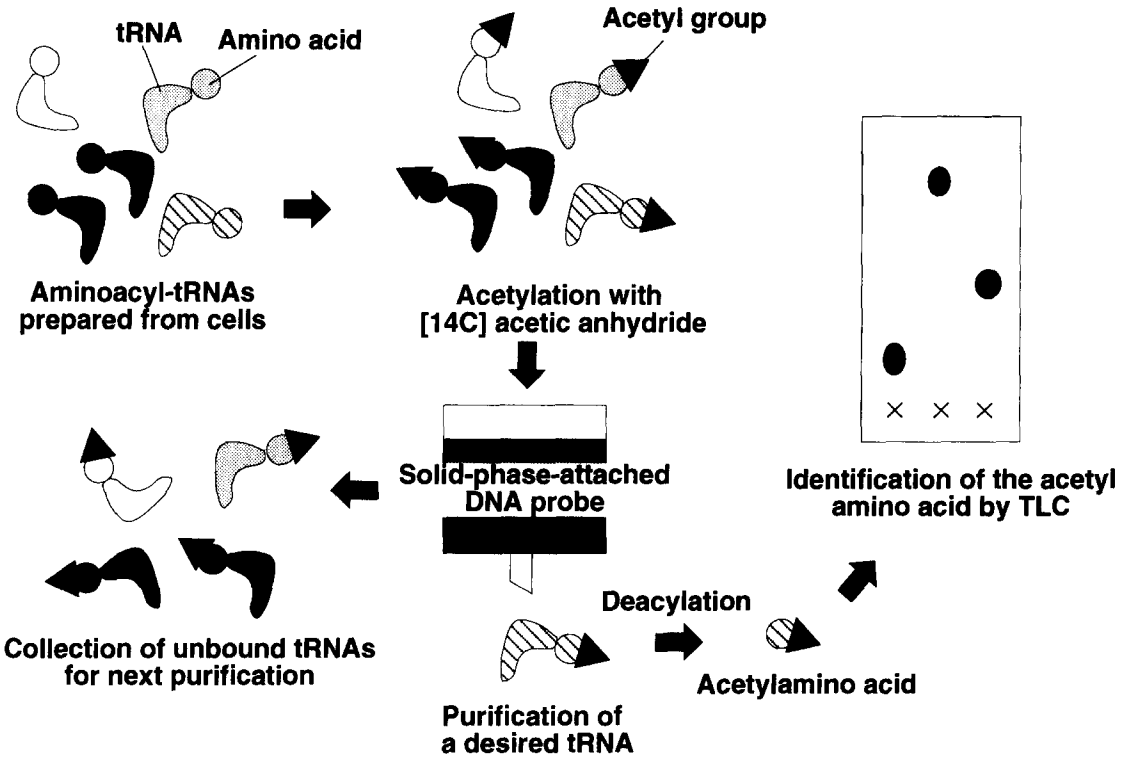

Fig. 1. Outline of experimental procedures for identification and quantitative analysis of aminoacyl-tRNA.

aminoacyl-tRNAs were applied onto a DEAE Sepharose fast-flow column which was initialized with a buffer consisting of 0.2 M NaCl and 50 mM NaOAc (pH 5.0) at 4°C. After washing out the unbound component with the initial buffer, aminoacyl-tRNAs were eluted with a buffer consisting of 1 M NaCl and 50 mM NaOAc (pH 5.0). Almost all the tRNAs thus prepared were aminoacylated as judged by an analysis of the results of Northern hybridization followed by 10% acid urea PAGE (data not shown).

2.3. Acetylation of amino acids attached to aminoacyl-tRNAs

Acetylation was performed according to Haenni and Chapeville [13]. 5 mg of aminoacyl-tRNAs was acetylated at 0°C in 250 μl of 0.2 M KOAc (pH 5.6) by the addition of 5-μl aliquots of [1-^{14}C]acetic anhydride (185 MBq/mmol) 4× at 15-min intervals. Care must be taken in handling this reaction because [1-^{14}C]acetic anhydride is a volatile material. We used pipet tips filled with activated charcoal filter (ART Solvent Safe, Molecular Bio-Products, San Diego, CA) to handle [1-^{14}C]acetic anhydride in a draft chamber. After being allowed to stand for 2 h on ice, the tRNAs were precipitated with ethanol 3× to completely remove unreacted acetic anhydride.

2.4. Purification of acetylated aminoacyl-tRNAs using solid-phase-attached DNA probes

The 3′-biotinylated DNA probes were immobilized with Streptavidin agarose as reported previously [11]. Acetyl-aminoacyl-tRNAs precipitated with ethanol were dissolved in 2 ml of 6×NHE buffer [1.2 M NaCl, 30 mM Hepes-KOH (pH 7.0) and 15 mM EDTA] and mixed with a solid-phase-attached DNA probe in a disposable filtration tube (Ultrafree CL, 0.45 μm pore size, Millipore). The desired tRNA was annealed with the corresponding DNA probe by incubation at 65°C for 10 min and cooling at room temperature for 5 min. Unbound tRNAs were thoroughly washed away from the probe-containing resin by vortexing followed by centrifugation in 2.5 ml of 3×NHE buffer [0.6 M NaCl, 15 mM Hepes-KOH (pH 7.0) and 7.5 mM EDTA] until the absorbance at 260 nm became less than 0.01 (the voltexing/centrifugation procedure needed to be repeated 7–10×). The purified acetyl-aminoacyl-tRNA was eluted from the probe-attached resin by warming at 65°C for 5 min with 2.5 ml of 0.1×NHE buffer [20 mM NaCl, 0.5 mM Hepes-KOH (pH 7.0) and 0.25 mM EDTA] 4× and precipitated with 20 μg/ml glycogen and ethanol.

2.5. TLC-plate analysis of acetylamino acids deacylated from purified acetyl-aminoacyl-tRNAs

The purified acetyl-aminoacyl-tRNAs were deacylated in 0.1 N NaOH at 37°C for 3 h and spotted on silica gel plates (0.036 A260 unit/plate) for TLC, which was developed by *n*-butanol/acetic acid/dH_2O (4 : 0.9 : 1, v/v/v) with ^{14}C-labeled markers of acetylserine and acetylleucine. These markers were prepared by deacylation of the acetyl-aminoacyl-tRNAs which were aminoacylated with [^{14}C]serine or leucine in vitro and acetylated with non-radioactive acetic anhydride. The radioactivities were detected with an imaging plate (Fuji Photo Systems) and visualized by an imaging analyzer (BAS-1000, Fuji Photo Systems).

3. Results and discussion

The experimental procedures are outlined in Fig. 1. Aminoacylated tRNAs are obtained from the cells under as mild conditions as possible. Amino groups of amino acids attached to the tRNAs are then acetylated with [^{14}C]acetic anhydride so as to label the amino acids and stabilize the ester bonds between amino acids and the 3′-terminal adenosine moieties in the aminoacyl-tRNAs. A desired acetyl-aminoacyl-tRNA is then fished out by a solid-phase-attached DNA probe, the sequence of which corresponds to a certain region of the desired tRNA. Flow-through fractions of acetyl-aminoacyl-tRNAs from which the desired tRNA has been squeezed can be re-used for further purification of other tRNAs. The acetylamino acid which was deacylated from the purified acetyl-aminoacyl-tRNA by alkaline treatment is analyzed by TLC.

Because acetylation with acetic anhydride proceeds efficiently, irrespective of the species of amino acid, all the aminoacyl-tRNAs except for prolyl-tRNA and N-blocked aminoacyl-tRNAs such as formylmethionyl-tRNA can be acetylated by this procedure. Before purification by DEAE-

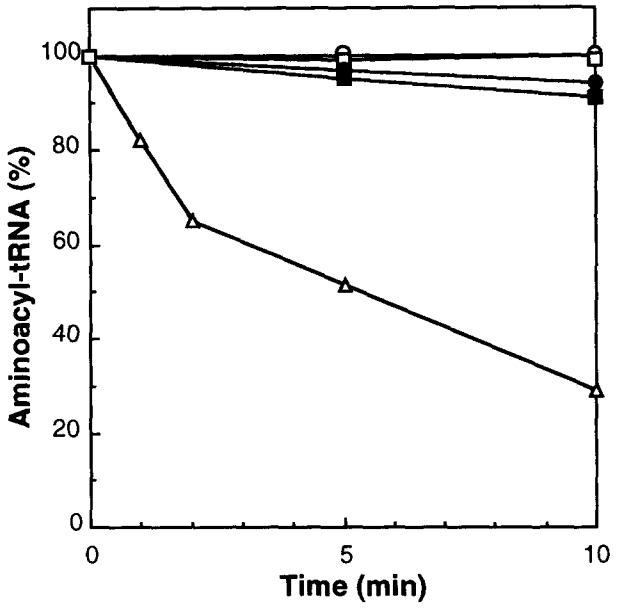

Fig. 2. Stabilization of acetylaminoacyl-tRNA in different concentrations of NHE buffer. Acetylseryl-tRNA^{Ser} in 6×NHE buffer (●) and 0.1×NHE buffer (○), acetylleucyl-tRNA^{Leu} in 6×NHE buffer (■) and 0.1×NHE buffer (□) and seryl-tRNA^{Ser} in 6×NHE (△).

Sepharose fast-flow column chromatography, aminoacyl-tRNA mixtures were acetylated only about 60% with [^{14}C]acetic anhydride as judged from the radioactivity of the acetyl group incorporated into the aminoacyl-tRNAs (data not shown), which was probably due to contamination by a large amount of cell wall components of the tRNA preparations arising from direct phenol extraction. Thus, it was necessary to remove the cell wall components from the aminoacyl-tRNA preparations for efficient acetylation. Increased acetylation was observed only after the aminoacyl-tRNAs were passed through the DEAE-Sepharose fast-flow column. Judging from the radioactivity, the extent of acetylation of the aminoacyl-tRNAs thus purified exceeded 100%, probably because partial acetylation of nucleic acid occured in addition to that of amino acid.

The procedure for purifying a specific tRNA using a solid-phase-attached DNA probe was performed according to the literature [11] with slight modifications. In order to avoid deacylation of the acetylamino acid moiety from the acetyl-aminoacyl-tRNAs during purification, a buffer consisting of Hepes-KOH (pH 7.0), NaCl and EDTA (NHE buffer) was used. Fig. 2 shows the stabilization of acetylseryl or acetylleucyl-tRNAs at 65°C in 6×NHE [1.2 M NaCl, 30 mM Hepes-KOH (pH 7.0) and 15 mM EDTA] and 0.1×NHE buffers [20 mM NaCl, 0.5 mM Hepes-KOH (pH 7.0) and 0.25 mM EDTA], respectively. The reaction was started by the addition of 5 pmol (5 μl) of ^{14}C-labeled seryl-, acetylseryl- or acetylleucyl-tRNA to the preincubated NHE buffer (55 μl). An appropriate volume of the mixture was spotted onto filteration paper (Wattman 3 MM) presoaked with 5% trichloroacetic acid (TCA) at the proper time. After washing out deacylated amino acid with 5% TCA, the radioactivities were measured by a liquid scintillation counter. About 70% of the control seryl-tRNA was deacylated by incubation at 65°C for 10 min. Although slight deacylation of both acetyl-aminoacyl-tRNAs was observed in the 6×NHE buffer, 90% of the tRNAs was retained. No deacylation was observed in the 0.1×NHE buffer.

From 5 mg of unfractionated aminoacyl-tRNAs, 0.576 A260 unit of *C. cylindracea* tRNA^{Ser}CAG and 0.036 A260 unit of *C. zeylanoides* tRNA^{Leu}U*AA were, respectively, obtained. The large difference in the yields of these two tRNAs is probably due mainly to the different amounts of each tRNA contained in the cells and slightly to the hybridizing efficiency of the DNA probes [19].

Spots of acetylserine and acetylleucine were actually detected from samples of *C. cylindracea* tRNA^{Ser}CAG and *C. zeylanoides* tRNA^{Leu}U*AA, respectively, as shown in Fig. 3, so it is evident that *C. cylindracea* tRNA^{Ser}CAG is in fact aminoacylated with serine not only in vitro but also in vivo. The intensities of the two spots quantified by an imaging analyzer were nearly the same, indicating that the charging activity of a certain tRNA in vivo may be quantifiable by this method.

The radioactivities remaining at the origin on the TLC plate may be due to a direct acetylation of nucleic acid of tRNAs, since the deacylation conditions used (described in section 2) are known to be sufficient for complete discharging of acetyl-aminoacyl-tRNAs.

Although it is possible to label aminoacyl-tRNAs in vivo by growing cells with isotopic amino acids, this is not practical because of the very high cost of large-scale cultivation, dilution of the labeled amino acid due to its incorporation into proteins or their proteolysis in the metabolic pathway [20], and sometimes difficulty in the cultivation of certain organisms. The labeling of amino acids attached to tRNAs using acetic anhydride is a useful method for all living organisms if aminoacyl-tRNAs can be obtained from them. Although this method requires a larger amount of aminoacyl-tRNA mixture, especially for analyzing a minor RNA, as compared to using an acid urea PAGE followed by a Northern hybridization, it is superior because amino acid identification is possible.

A solid-phase-attached DNA probe can be used to purify a specific aminoacyl-tRNA only if its sequence is known; otherwise, a series of column chromatographies or electrophoresis could be used for the purification. If unidentified amino acids

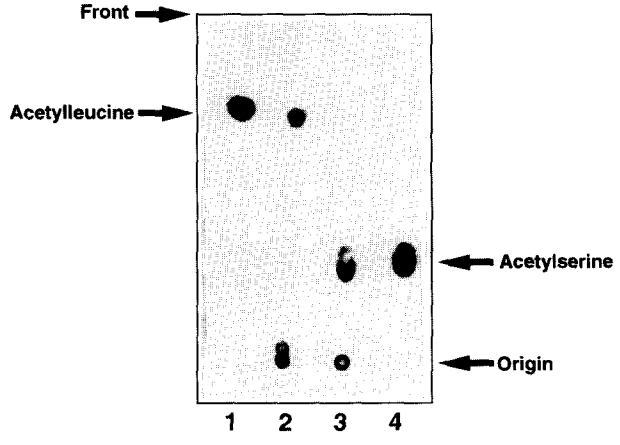

Fig. 3. Analysis of acetylserine and acetylleucine attached to specific tRNAs on TLC plates. Deacylated acetylamino acids from 0.036 A260 unit of *C. zeylanoides* tRNA^{Leu}U*AA (lane 2), 0.036 A260 unit of *C. cylindracea* tRNA^{Ser}CAG (lane 3), 1000 cpm of acetylserine (lane 1) and acetylleucine (lane 4).

were detected on the TLC plate, it would be necessary to obtain a larger amount of the RNAs to identify the amino acids by using a LC-Mass spectrometry or other appropriate techniques. And if a certain amino acid-attached RNA in which the linkage between the RNA and an amino acid is not an acyl bond was found, it would be another important problem to characterize the property of the linkage.

Limitation in the detection of an acetylamino acid depends on the specific activity of [^{14}C]acetic anhydride. About 0.005 A260 unit of aminoacyl-tRNA may be necessary for detection in using [^{14}C]acetic anhydride, whose specific activity is 185 MBq/mmol. It is effective to improve the method of labeling the amino group to achieve high intensity. For instance, uniformly labeled anhydride of a fatty acid having a longer side chain than the acetic acid will increase the specific activity, and tritium labeling instead of ^{14}C or fluorescence labeling with *o*-phthalaldehyde would be other effective means of labeling amino acids. This method proposed here will also be applicable to direct analysis of the conversion of amino acids attached to tRNAs, e.g. selenocystein tRNA [22] and glutamate tRNA for the formation of ∂-aminolevulinate [23], and tRNA having mischarging activity [21] in vivo, because two or more amino acids attached to the tRNA would be easily detected on the TLC plate. We expect that this method will be useful for research into tRNA identity in vivo and for the analysis of presently unknown functions of RNAs in vivo.

Acknowledgements: This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science, Culture and Sport of Japan and by a JSPS Fellowship for Japanese Junior Scientists (T.S.).

References

[1] Marr, A.G. (1991) Microbiol. Rev. 55, 316–333.
[2] Ofverstedt, L.G., Zhang, K., Tapio, S., Skoglund, U. and Isaksson, L.A. (1994) Cell 79, 629–638.
[3] Komine, Y., Kitabatake, M., Yokogawa, T., Nishikawa, K. and Inokuchi, H. (1994) Proc. Natl. Acad. Sci. USA 91, 9223–9227.
[4] Ushida, C., Himeno, H., Watanabe, T. and Muto, A. (1994) Nucleic Acids Res. 22, 3392–3396.
[5] Florentz, C. and Giege, R. (1995) in tRNA: Structure, Biosynthesis, and Function (D. Söll, D. and Raj Bhandary, U.L., Eds.), American Society for Microbiology, Washington, DC, 1995, pp. 141–163.
[6] Sivaram, P. and Deutscher, M.P. (1990) Proc. Natl. Acad. Sci. USA 87, 3665–3669.
[7] Stapulionis, R. and Deutscher, M.P. (1995) Proc. Natl. Acad. Sci. USA 92, 7158–7161.
[8] Varshney, U., Lee, C.-P. and Raj Bhandary, U.L. (1991) J. Biol. Chem. 244, 1104–1113.
[9] Pak, M., Willis, I.M. and Schulman, L.H. (1994) J. Biol. Chem. 269, 2277–2282.
[10] Tsurui, H., Kumazawa, Y., Sanokawa, R., Watanabe, Y., Kuroda, T., Wata, A., Watanabe, K. and Shirai, T. (1994) Anal. Biochem. 221, 166–172.
[11] Wakita, K., Watanabe, Y., Yokogawa, T., Kumazawa, Y., Nakamura, S., Ueda, T., Watanabe, K. and Nishikawa, K. (1994) Nucleic Acids Res. 22, 345–353.
[12] Herve, G. and Chapeville, F. (1965) J. Mol. Biol. 13, 757–766.
[13] Haenni, A.-L. and Chapeville, F. (1966) Biochim. Biophys. Acta 114, 135.
[14] Gillam, I., Blew, D., Warrington, R.C., Tigerstorm, M. and Tener, G.M. (1968) Biochemistry 7, 3459.
[15] Yokogawa, T., Suzuki, T., Ueda, T., Mori, M., Ohama, T., Kuchino, Y., Yoshinari, S., Motoki, I., Nishikawa, K., Osawa, S. and Watanabe, K. (1992) Proc. Natl. Acad. Sci. USA 89, 7408–7411.
[16] Suzuki, T., Ueda, T., Yokogawa, T., Nishikawa, K. and Watanabe, K. (1994) Nucleic Acids Res. 22, 115–123.
[17] Ohama, T., Suzuki, T., Mori, M., Osawa, S., Ueda, T., Watanabe, K. and Nakase, T. (1993) Nucleic Acids Res. 21, 4039–4045.
[18] Ueda, T., Suzuki, T., Yokogawa, T., Nishikawa, K. and Watanabe, K. (1994) Biochimie 76, 1217–1222.
[19] Kumazawa, Y., Yokogawa, T., Tsurui, H., Miura, K. and Watanabe, K. (1992) Nucleic Acids Res. 20, 2223–2232.
[20] Watt, P.W., Lindsay, Y., Scrimgeour, C.M., Chien, P.A., Gibson, J.N., Taylor, D.J. and Rennie, M.J. (1991) Proc. Natl. Acad. Sci. USA 88, 5892–5896.
[21] Schön, A., Kannangara, C.G., Gough, S. and Söll, D. (1988) Nature (London) 331, 187–190.
[22] Leinfelder, W., Forchhammer, K., Veprek, B., Zehelein, E. and Böck, A. (1990) Proc. Natl. Acad. Sci. USA 87, 543–547.
[23] Schön, A., Krupp, G., Gough, S., Berry-Lowe, S., Kannangara, C.G. and Söll, D. (1986) Nature (London) 322, 281–284.