Published in final edited form as:

Methods. 2008 February ; 44(2): 129–138.

The many applications of acid urea polyacrylamide gel electrophoresis to studies of tRNAs.

Caroline Köhrer and Uttam L. RajBhandary*

Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA.

Abstract

Here we describe the many applications of acid urea polyacrylamide gel electrophoresis (acid urea PAGE) followed by Northern blot analysis to studies of tRNAs. Acid urea PAGE allows the electrophoretic separation of different forms of a tRNA, discriminated by changes in bulk, charge, and/or conformation that are brought about by aminoacylation, formylation, or modification of a tRNA. Among the examples described are (i) analysis of the effect of mutations in the *Escherichia coli* initiator tRNA on its aminoacylation and formylation; (ii) evidence of orthogonality of suppressor tRNAs in mammalian cells and yeast; (iii) analysis of aminoacylation specificity of an archaeal prolyl-tRNA synthetase that can aminoacylate archaeal tRNA^{Pro} with cysteine, but does not aminoacylate archaeal tRNA^{Cys} with cysteine; (iv) identification and characterization of the AUA-decoding minor tRNA^{Ile} in archaea; and (v) evidence that the archaeal minor tRNA^{Ile} contains a modified base in the wobble position different from lysidine found in the corresponding eubacterial tRNA.

Keywords

acid urea polyacrylamide gel electrophoresis; Northern hybridization; aminoacylation; deacylation kinetics

1. Introduction

As adapter molecules between codons in a mRNA and amino acids in proteins, transfer RNAs (tRNAs) play a critical role in protein synthesis [1]. From their biosynthesis to their function on the ribosome, tRNAs interact with various proteins that include processing enzymes (such as tRNA processing RNases), modifying enzymes, aminoacyl-tRNA synthetases, translation factors (initiation and elongation factors), and ultimately the ribosome and mRNA. Analysis of tRNAs, therefore, constitutes an integral part of studies of protein synthesis. Methods for tRNA purification and sequence analysis, including the determination of base modifications, are essential for structure-function studies and have been described elsewhere [2,3]. Methods for separation of tRNAs on polyacrylamide gels under native or denaturing conditions followed by identification of individual tRNAs by Northern hybridization have also proven to be extremely useful. Here, we describe the analysis of tRNAs by acid urea polyacrylamide gel electrophoresis (acid urea PAGE).

*Corresponding author: Department of Biology, Massachusetts Institute of Technology 77 Massachusetts Ave., Room 68–671 Cambridge, MA 02139, USA Tel.: 617–253–4702 FAX: 617–252–1556 E-mail: bhandary@mit.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Acid urea PAGE provides the means for separating tRNAs at acidic pH under semi-denaturing conditions. Originally described by Ho and Kan [4] for analyzing the aminoacylation of eukaryotic suppressor tRNAs *in vivo*, Varshney *et al.* [5] adapted the approach for the separation of uncharged tRNA from aminoacylated tRNA (aa~tRNA) and formylaminoacylated tRNA (faa~tRNA), both of which show a clear electrophoretic mobility shift caused by a change in bulk, charge, and/or conformation of the aa~tRNA and faa~tRNA. In combination with the isolation of tRNAs under acidic conditions that preserve the labile ester linkage between tRNA and amino acid and thus allow the isolation of aa~tRNAs and faa~tRNAs, acid urea PAGE was firmly established as the method of choice to determine the *in vivo* state of aminoacylation or formylation of tRNAs. Initially used extensively for the *in vivo* analysis of mutants of the *E. coli* initiator tRNA^{fMet} ([5], and reviewed in [6-8]), the technique quickly found its way into the standard repertoire of tRNA analysis from all kingdoms including organelles, such as mitochondria [9,10].

In this review, we describe the basic experimental procedures for acid urea PAGE and present examples of applications (case studies 1–5) that include the *in vivo* analysis of steady state levels of aminoacylation and formylation of tRNAs; characterization of aminoacylation specificity of tRNAs and aminoacyl-tRNA synthetases; and identification of base modifications.

2. Experimental Procedures

Methods described below include the (1) isolation of total RNA under acidic conditions to maintain the amino acid ester-linkage between tRNA and amino acid; (2) deacylation of aminoacylated tRNAs; (3) separation of tRNAs by acid urea polyacrylamide gel electrophoresis; and (4) Northern hybridization.

2.1. Preparation of total RNA under acidic conditions

2.1.1. Isolation of RNA from *E. coli*. The protocol is based on the original RNA extraction procedure described by Varshney *et al.* [5]

- Cells (5 ml) are grown to mid-to-late log phase, chilled on ice for 5–15 minutes and pelleted at 4 °C. All subsequent steps are carried out in the cold.
- The cell pellet is resuspended in 0.5 ml sodium acetate buffer (0.3 M sodium acetate pH 4.5–5.0; 10 mM Na₂EDTA).
- One volume of phenol equilibrated with sodium acetate buffer is added, and the sample is mixed by vortexing (10 second-pulse); the cell suspension is kept on ice for 15 min with repeated vortexing. After centrifugation at 12,000 g (10–15 minutes), the aqueous phase is transferred to a fresh tube and the phenol phase is re-extracted with 0.25 ml of sodium acetate buffer.
- Aqueous phases from both centrifugation steps are combined and mixed with 2.5 volumes of ice-cold ethanol to precipitate nucleic acids (20 minutes, dry ice; or 2–3 hours at –20 °C).
- RNA is recovered by centrifugation at 12,000 g (15–30 minutes); the pellet is washed twice with 70% ethanol, left to dry on ice for several minutes (with the lid open), and finally dissolved in 20–50 µl of 10 mM sodium acetate pH 4.5–5.0, quick-frozen and stored at –80 °C. Typically, 1–2 A₂₆₀ units of total RNA are obtained from a 5 ml culture.

2.1.2. Isolation of total RNA from archaea, yeast, and mammalian cells

- The protocol described above can also be applied to the isolation of total RNA from archaea (e.g. various *Halobacterial* species [11]; *Methanocaldococcus jannaschii*; *Methanococcus maripaludis* [12]), yeast (*Saccharomyces cerevisiae* [13,14]), and mammalian cells (e.g. COS1, HEK293, HeLa cells [15-18]). We recommend the addition of acid-washed glass beads during phenol extraction (1/4 of suspension volume; 425–600 μm ; Sigma) for RNA preparations from methanogenic archaea and yeast to improve lysis efficiency.

2.1.3. Isolation of total RNA using Trizol

- Alternatively, total RNA can be isolated under acidic conditions using Trizol reagent (Invitrogen) following the instructions provided by the manufacturer. This extraction procedure is adapted from a protocol described by Chomczynski and Sacchi [19] for isolation of total RNA with unbuffered phenol in the presence of guanidinium hydrochloride.

2.2. Deacylation of aminoacylated tRNA and measurement of deacylation rates

Total RNA is isolated under acidic conditions as described above and resuspended in ice-cold sterile water.

- Preparation of bulk deacylated tRNA: A portion of the material is subjected to deacylation by addition of Tris-HCl pH 9.5 to a final concentration of 0.2 M. The deacylation reaction is usually performed at 37 °C for 30–120 min (most tRNAs will be deacylated after 30 min; however, certain tRNAs such as valine and isoleucine tRNAs require longer deacylation treatments [20]); deacylated tRNAs are re-precipitated with ethanol and stored in 10 mM sodium acetate pH 5.0; 10 mM Hepes-NaOH pH 7.4; or 10 mM Tris pH 7.5 at –80 °C.
- Measurement of deacylation rates: Deacylation is performed in 0.2 M Tris-HCl pH 9.2 at 37 °C. Aliquots corresponding to 0.1 A_{260} are removed at appropriate time intervals, mixed with an equal volume of acid urea sample buffer (see section 2.3.1), and quick-frozen on dry-ice. Reaction products are subsequently analyzed by acid urea PAGE/Northern hybridization.

2.3. Acid urea polyacrylamide gel electrophoresis (acid urea PAGE)

tRNAs are separated by acid urea PAGE followed by identification of individual tRNAs using Northern hybridization [5].

2.3.1. Preparation of acid urea polyacrylamide gel

- Gel dimension:
0.4 mm $\times$ 20 cm $\times$ 45 cm
- Gel composition:
6.5% polyacrylamide (19:1 acrylamide/bisacrylamide)
0.1 M sodium acetate pH 5.0
8 M urea
dissolve the above ingredients under stirring (without heating the solution); adjust volume to 50 ml and degas for 5–10 min
add TEMED (0.15% v/v) and ammonium persulfate (0.7% w/v)

cast gel and allow to polymerize for approximately 2 hours

- Acid urea sample buffer:
0.1 M sodium acetate pH 5.0
8 M urea
0.05% bromophenol blue
0.05% xylene cyanol FF

2.3.2. Gel electrophoresis

- It is recommended that a short pre-electrophoresis (~30 minutes) be performed prior to loading the samples and that the sample wells be cleaned carefully. The electrophoresis buffer is 0.1 M sodium acetate pH 5.0.
- Typically, 0.01–0.5 OD of tRNA (< 5 μ l) is mixed with one volume of sample buffer and loaded onto the gel. Electrophoresis is carried out in the coldroom at 500 V (~12 V/cm) for 18–24 hours until the bromophenol blue dye reaches the bottom of the gel.

2.4. Northern hybridization

2.4.1. Transfer of tRNAs to nylon membranes

- The portion of the gel containing the tRNAs (between xylene cyanol and bromophenol blue dyes) is transferred to Hybond-N+ membrane (Amersham GE) or Nytran Supercharge membrane (Millipore) using a Hoefer Electroblot apparatus (TE Series Tank Transphor Unit; Amersham GE). After electrotransfer, the membranes are baked for 2 hours at 70–80 °C.
- Transfer buffer:
40 mM Tris-HCl pH 8.0
2 mM Na₂EDTA
- Transfer conditions:
10 min at 20 V
2 hours at 40 V
coldroom

2.4.2. Northern hybridization

- tRNAs are visualized by hybridization using [³²P]-labeled DNA oligonucleotides following standard procedures [21]. Oligonucleotides are 5'-end labeled with γ -[³²P]-ATP (3000 Ci/mmol; Perkin Elmer) using T4-polynucleotide kinase (New England Biolabs). Excess γ -[³²P]-ATP is removed by Sephadex G-25 chromatography (e.g. MicroSpin G-25 columns; GE Healthcare).
- Prehybridization:
6 $\times$ SSC
10 $\times$ Denhardt's solution
0.5% SDS
6–12 hours at 42 °C
- Hybridization:

$6 \times \text{SSC}$

0.1% SDS

$[^{32}\text{P}]$ -labeled probe ($1\text{--}2 \times 10^6$ cpm/ml)

pass hybridization solution through 0.2 μm filter prior to addition to membrane

12–24 hours at 42 °C

- Membranes are washed at room temperature with $6 \times \text{SSC}$ (2×10 minutes; low stringency), followed by washes at $4 \times$ and $2 \times \text{SSC}$ (medium and high stringency). Northern blots are analyzed by autoradiography followed by PhosphorImaging.

2.4.3. Design of hybridization probes

- In most cases, we have used synthetic DNA oligonucleotides with a length of 17–25 nucleotides complementary to the desired tRNA target; if necessary, longer probes (up to 45 nucleotides) can be considered. For highest specificity, we recommend the design of hybridization probes targeting the anticodon stem and loop domain. Due to base modifications present in the anticodon loop of tRNAs interfering with efficient hybridization between tRNA and DNA probe, hybridization signals might sometimes not be strong enough. In such a case, alternative hybridization targets on the tRNA (e.g. 5' or 3' terminal region, variable loop) are advised. Alternatively, hybridization signals can be enhanced using either 'combinatorial oligonucleotide hybridization' [22], which uses a combination of $[^{32}\text{P}]$ -labeled DNA oligonucleotide and a molar excess of a non-labeled DNA oligonucleotide acting as an 'unfolders', or $[^{32}\text{P}]$ -labeled RNA oligonucleotides, providing for a more stable RNA:RNA hybrid. In both cases, specificity of hybridization has to be carefully monitored on a case-by-case basis.

3. Case Studies

The following examples highlight the many applications of acid urea PAGE/Northern hybridization to studies of tRNA in *E. coli*, mammalian cells, yeast and archaea.

3.1. Role of unique features of *E. coli* initiator tRNA^{fMet} in initiation of protein synthesis: Formylation

Initiator tRNAs are used exclusively for initiation of protein synthesis, whereas elongator tRNAs are used for the insertion of amino acids into internal peptide linkages. Translation initiation occurs universally with the amino acid methionine (Met) or its derivative formylmethionine (fMet). To fulfill their unique function in protein synthesis, initiator tRNAs possess a number of highly specific properties that direct them to the initiation pathway of translation [6–8]. These include (i) direct binding to the ribosomal P-site and (ii) exclusion from binding to elongation factor necessary for binding to the ribosomal A-site. In contrast to the protein synthesis systems of eukaryotes (cytoplasmic) and archaea, initiator tRNAs in eubacteria and eukaryotic organelles such as mitochondria and chloroplasts are unique in that the methionine attached to the initiator tRNA is formylated to formylmethionine (fMet~tRNA^{fMet}), by methionyl-tRNA transformylase (MTF) (Figure 1A). The formyl group acts as a positive determinant for initiation factor IF-2 to select fMet~tRNA^{fMet} from the pool of all cellular tRNAs and acts as a negative determinant for elongation factor EF-Tu to discriminate between fMet~tRNA^{fMet} and elongator tRNAs [23–25,8]. In addition, the eubacterial fMet~tRNA^{fMet} is insensitive to hydrolysis by peptidyl-tRNA hydrolase (PTH) [26], which cleaves the ester linkages between peptide and tRNAs dissociated from the ribosome and thereby allows tRNAs to recycle instead of accumulating as peptidyl~tRNAs.

For *in vivo* studies of structure-function relationships of the *E. coli* initiator tRNA^{fMet} and to establish the critical role of formylation, various mutant tRNAs were generated and examined for their activity in initiation *in vivo*. Acid urea PAGE followed by Northern hybridization analysis was used to determine the extent of *in vivo* aminoacylation and formylation of these mutant tRNAs. Control experiments using markers generated by *in vitro* aminoacylation and formylation of tRNA^{fMet} show that the different forms of initiator tRNA – uncharged tRNA^{fMet}, aminoacylated Met~tRNA^{fMet}, and formylaminoacylated fMet~tRNA^{fMet} – can be separated clearly (Figure 1B). Results in Figure 1C show that formylation is strongly affected by mutations in the acceptor stem, in particular mutations at the first, second and third base pair. Studies like these (reviewed in [6-8]) proved to be critical for the elucidation of how tRNA^{fMet} is recognized by methionyl-tRNA synthetase (MetRS) and MTF, and how fMet~tRNA^{fMet} is utilized in initiation in eubacteria.

As mentioned above, a well-established difference in translation initiation between *E. coli* and cytoplasmic protein synthesis of eukaryotes is the use of non-formylated Met ~ tRNA_i^{Met} in the latter [27,28]. Ramesh *et al.* [14] showed that expression of *E. coli* MTF in *S. cerevisiae* led to formylation of the cytoplasmic initiator Met ~ tRNA_i^{Met} (up to 70%) and possibly to initiation of protein synthesis with formylmethionine. The slow growth phenotype of yeast caused by expression of *E. coli* MTF could be rescued by co-expressing *E. coli* polypeptide deformylase (DEF), an enzyme that removes formyl groups from the N-terminus of proteins. Again, the *in vivo* state of formylation of the cytoplasmic yeast initiator tRNA_i^{Met} was monitored by acid urea PAGE/Northern hybridization linking the slow growth phenotype of yeast cells expressing MTF in the cytoplasm directly to formylation of the eukaryotic initiator tRNA_i^{Met}.

A striking example of the use of acid urea PAGE of tRNAs comes from studies of a mutant derived from the *E. coli* elongator tRNA (Mi:2 tRNA), which is active in initiation of protein synthesis in *E. coli* [29]. Activity of the mutant tRNA in initiation required the overproduction of *E. coli* MetRS. Acid urea PAGE/Northern hybridization explained the need for overproduction of MetRS. It was found that Mi:2 tRNA is normally aminoacylated in *E. coli* with lysine [30] and that Mi:2 tRNA aminoacylated with lysine is not a substrate for MTF [29] (Figure 2). Overproduction of MetRS leads to aminoacylation of a portion of the Mi:2 tRNA with methionine and to its consequent formylation by MTF (Figure 2B, lane 5). Besides highlighting the importance of the amino acid attached to the tRNA for its activity as a substrate for MTF, Figure 2B also shows quite clearly the separation by acid urea PAGE of four different forms of the same tRNA; tRNA, Lys~tRNA, Met~tRNA, and fMet~tRNA.

3.2. Orthogonal suppressor tRNAs for site-specific insertion of unnatural amino acids

An important advance in protein engineering has been the site-specific insertion of unnatural amino acids into proteins in prokaryotes or in eukaryotes. The most common approach is based on the suppression of an amber termination codon (UAG) at a predetermined site in the mRNA by an amber suppressor tRNA aminoacylated with the desired unnatural amino acid [31]. The key requirement for this approach is that the suppressor tRNA is 'orthogonal' (Figure 3A), i.e. not recognized by any of the aminoacyl-tRNA synthetases present in the respective expression systems. Otherwise, once the aminoacylated suppressor tRNA has inserted the unnatural amino acid at the designated site in the target protein, it will be re-aminoacylated with a natural amino acid and insert the natural amino acid instead of the analogue, thus generating a heterogeneous pool of target protein molecules. Many such orthogonal suppressor tRNAs have been established over the past years, including suppressor tRNAs derived from the eubacterial tRNA^{Tyr} [31-36], archaeal tRNA^{Tyr} [37], yeast tRNA^{Phe} [38], bacterial tRNA^{Trp} [39], *E. coli* tRNA^{Gln} [15,18], and even human and *E. coli* initiator tRNAs [40,16,13]. The next requirement is the use of an orthogonal aminoacyl-tRNA synthetase that specifically

recognizes the suppressor tRNA but no other tRNA in the cell (Figure 3A) (reviewed in [41-44]). The ultimate step is generation of mutants of the aminoacyl-tRNA synthetase, which use a specific unnatural amino acid instead of the natural amino acid and attach it to the orthogonal suppressor tRNA [37,35,45].

The first example of orthogonal suppressor tRNA/aminoacyl-tRNA synthetase pairs for use in mammalian cells consisted of an amber suppressor tRNA derived from *E. coli* tRNA^{Gln} and *E. coli* glutamyl-tRNA synthetase (GlnRS) [15]. The suppressor tRNA was expressed in mammalian cells, and its activity as a suppressor tRNA in readthrough of amber codons in a reporter gene was shown to be strictly dependent upon co-expression of *E. coli* GlnRS. Based on this orthogonal suppressor tRNA/aminoacyl-tRNA synthetase pair, a complete set of orthogonal amber, ochre, and opal suppressor tRNA/*E. coli* GlnRS pairs was generated [18]. Acid urea PAGE/Northern hybridization was used to confirm their orthogonality in mammalian cells (Figure 3B). Introduction of additional mutations in the anticodon loop of the amber, ochre, and opal suppressor tRNAs led to much enhanced suppression efficiencies (observed increases up to 36, 156, and 200-fold, respectively) allowing the concomitant suppression of two or three termination codons in a mRNA [18].

Another amber suppressor tRNA, orthogonal in mammalian cells and yeast, was derived from the human initiator tRNA^{Met}_i [16,13]. Mutations in the anticodon (U35A36) and T ψ C-arm (U50G51:C63A64/U54/C60) of the initiator tRNA abolished its recognition by MetRS and by initiation factor eIF-2. The tRNA mutant containing a CUA anticodon (U35A36 mutation) was now a substrate for *E. coli* GlnRS, and acted as an amber suppressor in yeast cells expressing *E. coli* GlnRS [13] suggesting that the tRNA could now bind the elongation factor EF-1. *In vivo* aminoacylation with glutamine, estimated to be ~50% (based on acid urea PAGE/Northern hybridization), allowed suppression of amber alleles in a yeast tester strain, *S. cerevisiae* HEY301-129 (*met8-1am*, *trp1-1am*, *his4-580am*) [32], indicated by growth on minimal media lacking methionine, tryptophan, or histidine (Figure 3C).

3.3. Formation of Cys~tRNA^{Cys} in archaea

In most organisms, the synthesis of Cys~tRNA^{Cys} is catalyzed by a canonical cysteinyl-tRNA synthetase (CysRS) that attaches cysteine to the corresponding tRNA^{Cys}. Surprisingly, certain methanogenic archaea (e.g. *Methanocaldococcus jannaschii*, *Methanothermobacter thermoautotrophicus*, and *Methanopyrus kandleri*) lack the gene encoding a canonical CysRS, yet genes encoding tRNA^{Cys} are present. This raised the intriguing question of how Cys~tRNA^{Cys}, an essential component of protein synthesis, is synthesized in these organisms. A number of studies, using incorporation of radiolabeled amino acids into total tRNA, showed that in some cases prolyl-tRNA synthetase (ProRS) from methanogenic archaea could aminoacylate total tRNA with cysteine [46,47], leading to the hypothesis that ProRS attaches proline to tRNA^{Pro} and cysteine to tRNA^{Cys}. In other words, ProRS – hence called ProCysRS – could be responsible for the formation of Cys~tRNA^{Cys}.

This intriguing hypothesis was put to rest (Figure 4A) [48,12] by aminoacylation of total *M. jannaschii* tRNA with nonradioactive proline or cysteine, followed by acid urea PAGE and Northern hybridization, using probes specific for tRNA^{Pro} and tRNA^{Cys}, to identify the tRNA species that had been aminoacylated [12]. The results showed clearly that purified recombinant ProRS – in addition to its canonical function to generate Pro~tRNA^{Pro} – could also aminoacylate tRNA^{Pro} with cysteine, but was unable to aminoacylate tRNA^{Cys} with cysteine (Figure 4B). In addition, acid urea PAGE revealed a clear difference in mobility of Pro~tRNA^{Pro} and Cys~tRNA^{Pro}. These data showed that ProRS cannot be responsible for generation of Cys~tRNA^{Cys} *in vivo*.

A subsequent study solved the mystery of how archaea lacking a canonical CysRS synthesize Cys~tRNA^{Cys} [49]. A two-step pathway was discovered: first, tRNA^{Cys} is aminoacylated with *O*-phosphoserine (Sep) by *O*-phosphoseryl-tRNA synthetase (SepRS) forming the intermediate Sep~tRNA^{Cys}; in a second step, Sep~tRNA^{Cys} is converted to the final product Cys~tRNA^{Cys} by Sep-tRNA:Cys-tRNA synthetase (SepCysS) (Figure 5A). Acid urea PAGE/Northern hybridization analysis of tRNA samples aminoacylated with a filtered *M. jannaschii* S-100 total cell extract (in the absence and presence of appropriate amino acid mixtures) was used to detect the formation of both Sep~tRNA^{Cys} and Cys~tRNA^{Cys}. Sep~tRNA^{Cys} formation was further confirmed using purified recombinant *M. jannaschii* SepRS (Figure 5B). In requiring more than one step for the synthesis of Cys~tRNA^{Cys}, the newly identified SepRS/SepCysS pathway in archaea is similar to the biosynthesis of Asn~tRNA^{Asn} and Gln~tRNA^{Gln} (in certain eubacteria and archaea), and of Sec~tRNA^{Sec} [50].

3.4. Identification of the AUA-reading minor isoleucine tRNA in archaea

Annotation of the complete genome of the extreme halophilic archaeon *Haloarcula marismortui* does not include a tRNA for translation of AUA, the rare codon for isoleucine. This is a situation typical for most archaeal genomes sequenced to date. One hypothesis stated that the AUA-reading isoleucine tRNA is derived through alternative splicing of a transcript to produce both a tryptophan tRNA and the isoleucine tRNA [51]. However, using acid urea PAGE, we have shown that the minor AUA-decoding isoleucine tRNA in *H. marismortui* and other archaeal species is derived from a CAU anticodon-containing tRNA, currently annotated as methionine tRNA, in which C34 in the anticodon is post-transcriptionally modified (Figure 6A) [11]. In addition, we show that the post-transcriptional modification of C at position 34 in the anticodon of this tRNA, responsible for the switch in amino acid and decoding specificity, is different from those present at position 34 of isoleucine tRNA species in eubacteria and in eukaryotes [52,53].

To investigate whether either of the two CAU anticodon-containing elongator tRNAs, annotated as tRNA₁₂ and tRNA₃₄ (The Genomic tRNA Database at <http://lowelab.ucsc.edu/GtRNAdb>) in *H. marismortui*, is possibly aminoacylated with isoleucine *in vivo*, we measured the kinetics of chemical deacylation of aminoacyl-tRNAs corresponding to tRNA₁₂ and tRNA₃₄ (Figure 6A), exploiting the well-known fact that the stability of the ester link between tRNA and amino acid is determined by the nature of the amino acid attached to the tRNA [20]; with isoleucine and valine being the most stable and methionine being much less stable. Aminoacyl-tRNAs present in total RNA were isolated from *H. marismortui* under acidic conditions to retain the aminoacyl-ester linkage and were subjected to base-catalyzed deacylation. Samples were subsequently analyzed by acid urea PAGE and kinetics of deacylation followed by Northern hybridization using probes specific for tRNA₁₂ and tRNA₃₄, respectively (Figure 6B). Aminoacyl~tRNA₁₂ is almost completely deacylated within 15 min, whereas deacylation of aminoacyl~tRNA₃₄ is incomplete even after 120 min. The half-lives of deacylation of aminoacyl~tRNA₁₂ is ~5 min which is the same as that of Met~tRNA_i^{Met}; whereas that of aminoacyl~tRNA₃₄ is ~35 min, which is the same as that of Ile~tRNA_{GAU}^{Ile} [11]. These data, in combination with *in vitro* re-aminoacylation using purified recombinant *H. marismortui* MetRS and isoleucyl-tRNA synthetase (IleRS), show that tRNA₁₂ is the elongator methionine tRNA_{CAU}^{Met}, and that tRNA₃₄ is the minor tRNA^{Ile} (renamed tRNA_{XAU}^{Ile}, X representing an unknown modified base).

The determination of deacylation kinetics has been used earlier to characterize the nature of the amino acid attached *in vivo* to a mutant human initiator tRNA initiating protein synthesis

from non-AUG codons [17], and to establish the importance of the anticodon sequence for recognition of tRNAs by MetRS and valyl-tRNA synthetase in archaea [54].

3.5. Base modifications

Some modified nucleosides play critical roles in the structure and function of tRNAs; in particular, base modifications in the anticodon loop are thought to be important for recognition of tRNAs by their cognate aminoacyl-tRNA synthetases and/or for codon/anticodon interactions. Post-transcriptional modifications at the wobble position 34 and the purine 37, 3' adjacent to the anticodon, are known to be involved in stabilizing the geometry of the anticodon loop and its dynamics for optimum codon/anticodon interaction [55,56]. Early work on tRNA analysis used combinations of column, paper, and thin layer chromatography of partial and complete nuclease digests of tRNAs to identify and characterize modified nucleosides [2,3]. Recent analyses include separation of tRNA fragments or nucleoside mixtures by liquid chromatography followed by mass spectrometric analysis of individual peak fractions (LC-MS), pioneered by the laboratories of Nishimura, McCloskey and Crain [57,58].

One modified base of particular interest is lysidine, a modified cytosine with a lysine side-chain attached to the O² of cytidine. Found in the wobble position of eubacterial minor isoleucine tRNAs ($\text{tRNA}_{\text{LAU}}^{\text{Ile}}$ or $\text{tRNA}_2^{\text{Ile}}$), lysidine has a dual role (reviewed in [53]): (i) the presence of lysidine in the anticodon allows $\text{tRNA}_{\text{LAU}}^{\text{Ile}}$ to translate specifically the minor isoleucine codon AUA; and (ii) modification of cytidine to lysidine is necessary for recognition of $\text{tRNA}_{\text{LAU}}^{\text{Ile}}$ by IleRS and, at the same time, for blocking its aminoacylation by MetRS. The enzyme, tRNA^{Ile}-lysine synthetase (TilS), responsible for modifying the minor isoleucine tRNA has been identified and characterized [59] (Figure 7A), and the crystal structure of the enzyme from *Aquifex aeolicus* has been solved [60]. For a recent study on archaeal minor isoleucine tRNA ([11]; see section 3.4), we have devised a simple method, involving the use of acid urea PAGE, to confirm the presence of lysidine in tRNAs. Total *E. coli* tRNA or a T7 transcript corresponding to *E. coli* $\text{tRNA}_{\text{LAU}}^{\text{Ile}}$ is incubated with sulfo-NHS-LC-biotin [N-hydroxysuccinimide (NHS) ester of biotin], which reacts with the free $\alpha\text{-NH}_2$ group of lysidine. Both, *in vitro* lysidinylation of the control T7 transcript using TilS and subsequent biotinylation of tRNAs can be monitored by acid urea PAGE followed by Northern blot analysis using a $\text{tRNA}_{\text{LAU}}^{\text{Ile}}$ -specific probe (Figure 7B). Introduction of lysidine into the T7 transcript causes a clear mobility shift (Figure 7B, compare lanes 1 and 3). This is expected since the $\alpha\text{-NH}_2$ group of lysidine carries a positive charge under the acidic conditions of the gel electrophoresis. Biotinylation of the modified transcript causes an additional supershift (Figure 7B, lane 4), most likely caused by the added bulk from the biotin; as expected, a T7 transcript lacking lysidine does not show a shift (Figure 7B, lane 2). Reaction of total tRNA from *E. coli* with the NHS-activated biotin, also gave rise to a clear shift of $\text{tRNA}_{\text{LAU}}^{\text{Ile}}$ confirming the presence of lysidine (Figure 7B, compare lanes 5 and 6); several minor shifts upon biotinylation were also observed, probably corresponding to partial reaction with other base modifications such as 3-(3-amino-3-carboxypropyl) uridine present in *E. coli* $\text{tRNA}_{\text{LAU}}^{\text{Ile}}$. In the course of the same study, we found that minor isoleucine tRNAs ($\text{tRNA}_{\text{XAU}}^{\text{Ile}}$) from *H. marismortui* (see section 3.4) and other archaea could not be biotinylated [11]. Thus, the base modification at C34 in the archaeal tRNA is not lysidine, which is consistent with the fact that, so far, no protein with significant homology to TilS could be identified in archaea. These results are intriguing from an evolutionary point of view; the overall pathway for supplying the cell with a minor isoleucine tRNA appears to be similar in eubacteria and most archaea and requires the post-transcriptional modification of a cytidine. Nevertheless, the base modification and the enzyme(s) responsible for this switch appear to be different between eubacteria and archaea.

4. Concluding Remarks

The separation of tRNAs by acid urea PAGE followed by identification of individual tRNAs by Northern hybridization provides a unique approach to analyze the state of tRNAs *in vivo* and enables us to gain insight into various aspects of protein synthesis; the determination of levels of aminoacylation and formylation, the characterization of aminoacylation specificity and detection of certain base modifications. One of the most important features of acid urea PAGE/Northern hybridization is the analysis of individual tRNA species present in total tRNAs isolated from cells or in total tRNAs subjected to *in vitro* manipulation. The combination of acid urea PAGE/Northern hybridization with other RNA techniques (native and denaturing PAGE, *in vitro* aminoacylation, reverse transcription, derivatization of functional groups etc.) allows one to experimentally verify the presence of any tRNA in a cell and, thereby, make possible experimental validation of predictions of tRNA genes based on *in silico* studies.

Acknowledgement

We would like to thank Dr. Jae-Ho Yoo for comments and suggestions on the manuscript. This work was supported by grants from the National Institutes of Health (GM17151 and GM67741) and the U.S. Army Research Office (W911NF-04-1-0353).

References

1. Crick FHC. Symp. Soc. Exp. Biol 1958;12:138–163. [PubMed: 13580867]
2. RajBhandary UL, Köhrer C. J. Biosci 2006;31:439–451. [PubMed: 17206064]
3. Grosjean H, Droogmans L, Roovers M, Keith G. Methods Enzymol 2007;425:57–101.
4. Ho YS, Kan YW. Proc. Natl. Acad. Sci. U S A 1987;84:2185–2188. [PubMed: 3031670]
5. Varshney U, Lee CP, RajBhandary UL. J. Biol. Chem 1991;266:24712–24718. [PubMed: 1761566]
6. RajBhandary UL. J. Bacteriol 1994;176:547–552. [PubMed: 7507918]
7. RajBhandary, UL.; Chow, CM. tRNA: Structure, Biosynthesis, and Function, American Society for Microbiology. Söll, D.; RajBhandary, UL., editors. Washington, DC: 1995. p. 511-528.
8. Mayer C, Stortchevoi A, Köhrer C, Varshney U, RajBhandary UL. Cold Spring Harb. Symp. Quant. Biol 2001;66:195–206. [PubMed: 12762022]
9. Enriquez JA, Attardi G. Proc. Natl. Acad. Sci. U S A 1996;93:8300–8305. [PubMed: 8710865]
10. Li Y, Holmes WB, Appling DR, RajBhandary UL. J. Bacteriol 2000;182:2886–2892. [PubMed: 10781559]
11. Köhrer C, Srinivasan G, Mandal D, Mallick B, Ghosh Z, Chakrabarti J, RajBhandary UL. RNA. 2007in press
12. Ambrogelly A, Ahel I, Polycarpo C, Bunjun-Srihari S, Krett B, Jacquin-Becker C, Ruan B, Köhrer C, Stathopoulos C, RajBhandary UL, Söll D. J. Biol. Chem 2002;277:34749–34754. [PubMed: 12130658]
13. Kowal AK, Köhrer C, RajBhandary UL. Proc. Natl. Acad. Sci. U S A 2001;98:2268–2273. [PubMed: 11226228]
14. Ramesh V, Köhrer C, RajBhandary UL. Mol. Cell. Biol 2002;22:5434–5442. [PubMed: 12101237]
15. Drabkin HJ, Park HJ, RajBhandary UL. Mol. Cell. Biol 1996;16:907–913. [PubMed: 8622693]
16. Drabkin HJ, Estrella M, RajBhandary UL. Mol. Cell. Biol 1998;18:1459–1466. [PubMed: 9488462]
17. Drabkin HJ, RajBhandary UL. Mol. Cell. Biol 1998;18:5140–5147. [PubMed: 9710598]
18. Köhrer C, Sullivan EL, RajBhandary UL. Nucleic Acids Res 2004;32:6200–6211. [PubMed: 15576346]
19. Chomczynski P, Sacchi N. Anal. Biochem 1987;162:156–159. [PubMed: 2440339]
20. Matthaei JH, Voigt HP, Heller G, Neth R, Schöch G, Kübler H, Amelunxen F, Sander G, Parmeggiani A. Cold Spring Harb. Symp. Quant. Biol 1966;31:25–38. [PubMed: 4966070]
21. Sambrook, J.; Fritsch, EF.; Maniatis, T. Molecular Cloning - A Laboratory Manual. Second edition. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, New York: 1989.

22. Buvoli A, Buvoli M, Leinwand LA. RNA 2000;6:912–918. [PubMed: 10864048]
23. Ono Y, Skoultschi A, Klein A, Lengyel P. Nature 1968;220:1304–1307. [PubMed: 4882917]
24. Sundari RM, Stringer EA, Schulman LH, Maitra U. J. Biol. Chem 1976;251:3338–3345. [PubMed: 776966]
25. Wakao H, Romby P, Westhof E, Laalami S, Grunberg-Manago M, Ebel JP, Ehresmann C, Ehresmann B. J. Biol. Chem 1989;264:20363–20371. [PubMed: 2684975]
26. Kössel H, RajBhandary UL. J. Mol. Biol 1968;35:539–560. [PubMed: 4877004]
27. Housman D, Jacobs-Lorena M, RajBhandary UL, Lodish HF. Nature 1970;227:913–918. [PubMed: 4915406]
28. Smith AE, Marcker KA. Nature 1970;226:607–610. [PubMed: 4315548]
29. Varshney U, Lee CP, RajBhandary UL. Proc. Natl. Acad. Sci. U S A 1993;90:2305–2309. [PubMed: 8460138]
30. Li S, Kumar NV, Varshney U, RajBhandary UL. J. Biol. Chem 1996;271:1022–1028. [PubMed: 8557626]
31. Noren CJ, Anthony-Cahill SJ, Griffith MC, Schultz PG. Science 1989;244:182–188. [PubMed: 2649980]
32. Edwards H, Schimmel P. Mol. Cell. Biol 1990;10:1633–1641. [PubMed: 1690848]
33. Köhler C, Xie L, Kellerer S, Varshney U, RajBhandary UL. Proc. Natl. Acad. Sci. U S A 2001;98:14310–14315. [PubMed: 11717406]
34. Kiga D, Sakamoto K, Kodama K, Kigawa T, Matsuda T, Yabuki T, Shirouzu M, Harada Y, Nakayama H, Takio K, Hasegawa Y, Endo Y, Hirao I, Yokoyama S. Proc. Natl. Acad. Sci. U S A 2002;99:9715–9720. [PubMed: 12097643]
35. Sakamoto K, Hayashi A, Sakamoto A, Kiga D, Nakayama H, Soma A, Kobayashi T, Kitabatake M, Takio K, Saito K, Shirouzu M, Hirao I, Yokoyama S. Nucleic Acids Res 2002;30:4692–4699. [PubMed: 12409460]
36. Köhler C, Yoo J, Bennett M, Schaack J, RajBhandary UL. Chem. Biol 2003;10:1095–1102. [PubMed: 14652077]
37. Wang L, Brock A, Herberich B, Schultz PG. Science 2001;292:498–500. [PubMed: 11313494]
38. Mamaev SV, Laikhter AL, Arslan T, Hecht SM. J. Am. Chem. Soc 1996;118:7243–7244.
39. Zhang Z, Alfonta L, Tian F, Bursulaya B, Uryu S, King DS, Schultz PG. Proc. Natl. Acad. Sci. U S A 2004;101:8882–8887. [PubMed: 15187228]
40. Lee CP, RajBhandary UL. Proc. Natl. Acad. Sci. U S A 1991;88:11378–11382. [PubMed: 1763051]
41. Hendrickson TL, de Crécy-Lagard V, Schimmel P. Annu. Rev. Biochem 2004;73:147–176. [PubMed: 15189139]
42. Köhler C, RajBhandary UL, Ibba M, Francklyn C, Cusack S. Aminoacyl-tRNA Synthetases, Landes Bioscience 2005:353–363.
43. Wang L, Xie J, Schultz PG. Annu. Rev. Biophys. Biomol. Struct 2006;35:225–249. [PubMed: 16689635]
44. Xie J, Schultz PG. Nat. Rev. Mol. Cell Biol 2006;7:775–782. [PubMed: 16926858]
45. Chin JW, Cropp TA, Anderson JC, Mukherji M, Zhang Z, Schultz PG. Science 2003;301:964–967. [PubMed: 12920298]
46. Lipman RS, Sowers KR, Hou YM. Biochemistry 2000;39:7792–7798. [PubMed: 10869184]
47. Stathopoulos C, Li T, Longman R, Vothknecht UC, Becker HD, Ibba M, Soll D. Science 2000;287:479–482. [PubMed: 10642548]
48. Ahel I, Stathopoulos C, Ambrogelly A, Sauerwald A, Toogood H, Hartsch T, Söll D. J. Biol. Chem 2002;277:34743–34748. [PubMed: 12130657]
49. Sauerwald A, Zhu W, Major TA, Roy H, Palioura S, Jahn D, Whitman WB, Yates Jr. Ibba M, Söll D. Science 2005;307:1969–1972. [PubMed: 15790858]
50. Feng L, Sheppard K, Namgoong S, Ambrogelly A, Polycarpo C, Randau L, Tumbula-Hansen D, Söll D. RNA Biol 2004;1:16–20. [PubMed: 17194933]
51. Ghosh Z, Chakrabarti J, Mallick B, Das S, Sahoo S, Sethi HS. Biochem. Biophys. Res. Commun 2006;339:37–40. [PubMed: 16307727]

52. Senger B, Auxilien S, Englisch U, Cramer F, Fasiolo F. *Biochemistry* 1997;36:8269–8275. [PubMed: 9204872]
53. Grosjean H, Björk GR. *Trends in Biochem. Sci* 2004;29:165–168. [PubMed: 15124629]
54. Ramesh V, RajBhandary UL. *J. Biol. Chem* 2001;276:3660–3665. [PubMed: 11058596]
55. Agris PF. *Nucleic Acids Res* 2004;32:223–238. [PubMed: 14715921]
56. Agris PF, Vendeix FAP, Graham WD. *J. Mol. Biol* 2007;366:1–13. [PubMed: 17187822]
57. Yokoyama, S.; Nishimura, S. *tRNA: Structure, Biosynthesis, and Function*, American Society for Microbiology. Söll, D.; RajBhandary, UL., editors. Washington, DC: 1995. p. 207-223.
58. Crain PF, McCloskey JA. *Curr. Opin. Biotechnol* 1998;9:25–34. [PubMed: 9503584]
59. Soma A, Ikeuchi Y, Kanemasa S, Kobayashi K, Ogasawara N, Ote T, Kato J, Watanabe K, Sekine Y, Suzuki T. *Mol. Cell* 2003;12:689–698. [PubMed: 14527414]
60. Nakanishi K, Fukai S, Ikeuchi Y, Soma A, Sekine Y, Suzuki T, Nureki O. *Proc. Natl. Acad. Sci. U S A* 2005;102:7487–7492. [PubMed: 15894617]

Figure 1. Role of unique features of *E. coli* initiator tRNA^{fMet} in initiation of protein synthesis. **(A)** Recognition of tRNA^{fMet} by methionyl-tRNA transformylase (MTF). **(B)** Separation of the three forms of *E. coli* initiator tRNA^{fMet}: tRNA, aminoacyl~tRNA (aa~tRNA), and formylaminoacyl~tRNA (faa~tRNA) by polyacrylamide gel electrophoresis under acidic conditions (acid urea PAGE) followed by detection of tRNA by Northern hybridization using a [³²P]-labeled DNA oligonucleotide. The markers for aa~tRNA and faa~tRNA were generated by *in vitro* aminoacylation and formylation of tRNA^{fMet} using purified MetRS and MTF. **(C)** Acid urea PAGE/Northern analysis of the effect of mutations on aminoacylation and formylation of the mutant tRNAs *in vivo*. Analysis of tRNA^{fMet} from *E. coli* transformants carrying the wild type (W.T.) gene or various mutant tRNA genes as indicated. Control, tRNA isolated from transformants carrying the plasmid vector without any tRNA gene (modified from [6]).

Figure 2.

Important role of the amino acid attached to tRNA in formylation and in initiation of protein synthesis in *E. coli*. **(A)** *E. coli* methionyl-tRNA transformylase (MTF) does not recognize Lys~tRNA^{Mi:2} as a substrate [29,30]. **(B)** Acid urea PAGE/Northern analysis of total RNA isolated from *E. coli* expressing the Mi:2 mutant elongator tRNA. Lanes 1 and 2, marker tRNA isolated from TG1 cells using a high-copy vector for expression of Mi:2 tRNA; tRNA isolated under acidic conditions before (lane 1) and after deacylation by base-treatment (lane 2). Lanes 3–5, tRNA isolated from CA274 transformants containing an additional plasmid that had no aminoacyl-tRNA synthetase gene (lane 3), the *E. coli* GlnRS gene (lane 4), or the MetRS gene (lane 5) (modified from [29]).

Figure 3.

Orthogonal suppressor tRNAs for site-specific insertion of unnatural amino acids. (A) The orthogonal suppressor tRNA is not recognized by any of the endogenous aminoacyl-tRNA synthetases (grey), but is aminoacylated by the orthogonal aminoacyl-tRNA synthetase (black), which recognizes no other tRNA in the cell and has been modified to use unnatural amino acids. aaRS, aminoacyl-tRNA synthetase; the star indicates the unnatural amino acid of interest. (B) A complete set of orthogonal amber, ochre, and opal suppressor tRNAs for use in mammalian cells [18]. The suppressor tRNAs (*hsup2am*, *hsup2oc* and *hsup2op*) are derived from the *E. coli* glutamine tRNA. Acid urea PAGE/Northern analysis of total RNA isolated from HEK293T cells, transfected with plasmids carrying genes for the respective suppressor

tRNA and *E. coli* GlnRS. The suppressor tRNAs were visualized using a [³²P]-labeled oligonucleotide complementary to nucleotides 57–72; a [³²P]-labeled oligonucleotide complementary to the human tRNA^{Ser} was used as internal standard. (C) Orthogonal amber suppressor tRNA/aminoacyl-tRNA synthetase pair for use in yeast [13]. A suppressor tRNA (*hM2am*) derived from the human initiator tRNA is co-expressed alongside *E. coli* GlnRS in *S. cerevisiae*. *hM2am* is aminoacylated in yeast only in the presence of *E. coli* GlnRS (top panel; acid urea PAGE/Northern analysis) and is active in suppression (bottom panel). The test for suppression of the *met8–1* amber allele was performed in *S. cerevisiae* HEY301–129 on a selective plate lacking methionine (modified from [13]).

Figure 4.

Characterization of *M. jannaschii* ProRS (ProCysRS). (A) *M. jannaschii* ProRS (ProCysRS) catalyzes the formation of Cys~tRNA^{Pro} but not Cys~tRNA^{Cys} [12]. (B) Acid urea PAGE/Northern analysis of the total RNA isolated from *M. jannaschii* cells and aminoacylated *in vitro* with proline and cysteine by *M. jannaschii* ProRS and *M. maripaludis* CysRS. The blots were probed with [³²P]-labeled oligonucleotides complementary to *M. jannaschii* tRNA^{Pro} and tRNA^{Cys}, as indicated. Bands indicated by 1, 2, and 3 correspond to uncharged tRNA^{Pro}, Cys~tRNA^{Pro}, and Pro~tRNA^{Pro}, respectively. Bands indicated by 4 and 5 correspond to uncharged tRNA^{Cys} and Cys~tRNA^{Cys}, respectively. OH⁻, tRNA after deacylation by base-treatment (modified from [12]).

Figure 5. Formation of Cys~tRNA^{Cys} in some methanogenic archaea. **(A)** Two-step conversion of tRNA^{Cys} to (i) Sep~tRNA^{Cys}, catalyzed by *O*-phosphoseryl-tRNA synthetase (SepRS), and (ii) Cys~tRNA^{Cys}, catalyzed by Sep-tRNA:Cys-tRNA synthetase (SepCysS) [49]. **(B)** Acid urea PAGE/Northern analysis of total *M. maripaludis* RNA charged with dialyzed *M. jannaschii* S-100, *M. maripaludis* CysRS, and *M. jannaschii* SepRS (Mja 1660/SepRS) in the presence of 20 amino acids (20 aa), phosphoserine (Sep), or a *M. jannaschii* S-100 cell extract filtrate (Y3). A portion of the sample was deacylated by base-treatment (OH⁻). The blots were probed with [³²P]-labeled oligonucleotides complementary to *M. maripaludis* tRNA^{Cys} (modified from [49]).

Figure 6.

Identification of the minor isoleucine tRNA in archaea. (A) *H. marismortui* contains two different CAU anticodon-containing tRNAs, tRNA₁₂ and tRNA₃₄, currently annotated as methionine tRNAs. tRNA₁₂ represents the elongator methionine tRNA (tRNA^{Met}_{CAU}); the minor isoleucine tRNA (tRNA^{Ile}_{XAU}) of *H. marismortui* is derived from tRNA₃₄, and carries an unknown modified base (X) in the anticodon responsible for its aminoacylation and decoding specificity [11]. (B) Determination of deacylation rates of tRNA₁₂ (tRNA^{Met}_{CAU}) and tRNA₃₄ (tRNA^{Ile}_{XAU}) from *H. marismortui*. Total RNA was isolated under acidic conditions and subjected

to deacylation by base-treatment as described under Experimental Procedures followed by acid urea PAGE/Northern analysis using probes specific for tRNA₁₂ (top panel) and tRNA₃₄ (bottom panel), respectively. The calculated half-lives of deacylation are indicated. aa~tRNA, aminoacyl-tRNA (modified from [11]).

Figure 7.

Detection of base modifications. (A) Formation of lysidine in eubacteria. A CAU anticodon-containing tRNA ($\text{tRNA}_{\text{CAU}}^{\text{Ile}}$) is modified by tRNA^{Ile}-lysine synthetase (TlsS) generating the minor isoleucine tRNA ($\text{tRNA}_{\text{LAU}}^{\text{Ile}}$), which carries lysidine at position 34 in the anticodon. (B) *In vitro* biotinylation of the free $\alpha\text{-NH}_2$ group present in lysidine of $\text{tRNA}_{\text{LAU}}^{\text{Ile}}$ from *E. coli* followed by acid urea PAGE/Northern analysis. A T7 transcript corresponding to $\text{tRNA}_{\text{LAU}}^{\text{Ile}}$ was generated as a marker. Lanes 1 and 2, unmodified T7 transcript; lanes 3 and 4, T7 transcript after *in vitro* modification with lysidine using TlsS; lanes 5 and 6, analysis of total RNA from

E. coli. A comparison of tRNAs before (lanes 1, 3, 5) and after (lanes 2, 4, 6) *in vitro* biotinylation is shown (modified from [11]).