Identification and sequencing of two isopentenyladenosine-modified transfer RNAs from Chinese hamster ovary cells

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ABSTRACT

To determine the presence and identity of isopentenyladenosine-containing transfer RNAs (tRNAs) in a mammalian cell line, we adopted a novel method to isolate, clone and sequence these RNAs. This method was based on 3' polyadenylation of the tRNA prior to cDNA synthesis, PCR amplification, cloning and DNA sequencing. Using this unique procedure, we report the cloning and sequencing of the selenocysteine-tRNA and mitochondrial tryptophan-tRNA from Chinese hamster ovary cells which contain this specific tRNA modification. This new method will be useful in the identification of other tRNAs and other small RNAs where the primary sequence is unknown.

Eukaryotic and bacterial transfer RNAs (tRNAs) reading codons starting with uridine (U) often have a large hydrophobic moiety attached to position 37, the site 3' of the anticodon (1). One common modification is isopentenyladenosine (i6A). Previous reports showing incorporation of [3H]mevalonic acid into RNA indicated that i6A-containing RNAs existed in cultured mammalian cells (2,3). However, the function of the i6A modification on tRNA in mammalian cells is unknown. In our pursuit to determine the function of this tRNA modification, we first set out to identify the tRNAs that contain i6A. Here, we document the presence of i6A-containing tRNAs in Chinese hamster ovary (CHO) cells. Using a unique procedure, we have identified three i6A-tRNAs and here report the cloning and sequencing of two of these tRNAs from CHO cells.

The sequences described in this manuscript can be obtained in GenBank with the following accession numbers: CHO selenocysteine tRNA (accession no. AF033379) and CHO mitochondrial tryptophan tRNA (accession no. AF033380).

To unequivocally determine if i6A-modified tRNAs are present in CHO cells, we utilized previously described antibodies against i6A (4) to specifically immunoprecipitate those tRNAs which carried this modification. We took advantage of the fact that the isopentenyl moiety is derived from mevalonic acid and utilized a CHO variant cell line, met-18b-2, with greatly enhanced uptake and metabolism of mevalonate compared to normal CHO cells (5). Met-18b-2 cells were radiolabeled for 48 h with [3H]mevalonic acid (60 Ci/mmol; American Radiolabeled Chemicals), after which either total RNA was isolated (6) or cell lysates were immunoprecipitated using anti-i6A serum in the presence or absence of competing i6A. Met-18b-2 cell pellets were solubilized in a buffer containing 20 mM Tris–HCl, 1% (v/v) Triton X-100, 0.5% (w/v) Na deoxycholate, 0.2% (w/v) SDS, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA and 0.02% (w/v) Na2S (pH 7.4) and centrifuged for 10 min at 13 000 g at 4°C. Supernatates were diluted 1:1 (v/v) with a buffer containing 20 mM Tris–HCl, 1% (v/v) Triton X-100, 0.5% (w/v) Na deoxycholate, 10 mg/ml ovalbumin, 10 mM methionine, 0.85 M NaCl, 1 mM EDTA, 1 mM EGTA and 0.02% (w/v) Na2S (pH 7.4). Anti-i6A serum was added and the mixture agitated for 4 h at room temperature. Protein A linked to Sepharose CL-4B was used to precipitate the immune complexes. For polyacrylamide gel electrophoresis, the precipitated material was released from the Sepharose CL-4B by incubation at 80°C for 5 min in a mixture containing 1% (w/v) SDS and gel-loading buffer [0.1 M Na acetate, 60% (w/v) sucrose, 8 M urea and 0.1% (w/v) xylene cyanol (pH 4.5)] was added. Immunoprecipitates were electrophoresed on 15% polyacrylamide gels containing 4 M urea.

As seen in Figure 1, gel electrophoresis of 3H-labeled total RNA (lane 1) yielded one major (Rf = 0.60) and two minor [3H]mevalonate-labeled species (Rf = 0.71 and 0.73, respectively) whose electrophoretic mobilities overlapped with the spectrum of purified yeast tRNA (Rf = 0.62–0.77) (data not shown). These same [3H]mevalonate-labeled species were observed when CHO lysates were immunoprecipitated with anti-i6A serum (lanes 2 and 4). However, these molecules were not detected when competing i6A was added during the immunoprecipitation (lane 3). Exogenous adenosine (added at 140 µM) did not affect the immunoprecipitation of the [3H]mevalonate-labeled species (data not shown). Additional experiments demonstrated that [3H]uridine, [3H]adenosine and [32P]phosphoric acid, but not [35S]methionine–cysteine or [3H]leucine, were incorporated into

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molecules with identical relative electrophoretic mobilities (data not shown). These results indicate that met-18b-2 cells have at least three \( i^6A \)-containing RNAs co-migrating with tRNAs.

Next, we set out to identify these \( i^6A \)-containing RNAs using a unique method outlined in Figure 2. Using anti-\( i^6A \) serum, we isolated nanogram quantities of \( i^6A \)-containing RNAs from CHO cells and incubated the material with yeast poly(A) polymerase to synthesize a site for primer attachment. The length of the poly(A) tail was controlled by including cordycepin \( 5' \)-triphosphate in the reaction mixture. Incorporation of this analogue of ATP terminates further tail elongation (7). Preliminary experiments indicated the optimal ATP:cordycepin molar ratio to be 8:1 (data not shown).

The \( i^6A \)-containing immunoprecipitated material was then polyadenylated for 6 h at 37°C using 1000 U of yeast poly(A) polymerase (USB), 10 mM MgCl\(_2\), 0.7 mM MnCl\(_2\), 50 mM KCl, 20 mM Tris–HCl (pH 7.0), 10% (v/v) glycerol, 100 µg/ml acetylated BSA, 62.5 µM cordycepin \( 5' \)-triphosphate and 0.5 mM ATP.

To synthesize cDNAs, the \( i^6A \)-polyadenylated \( i^6A \)-tRNAs were incubated for 3 h at 37°C with 200 U of Superscript II reverse transcriptase (Gibco BRL), 62 mM KCl, 2 mM MgCl\(_2\), 10 mM dithiothreitol, 3.3 µM primer (‘\( 5'\)-CCGGAAATTCGAGGCTTG-3’) and 0.3 mM dNTP in the presence of [\( \alpha \)-\( ^32P \)]dGTP (3000 Ci/mmol; NEN DuPont).

Individual cDNAs were separated on an 8% polyacrylamide gel containing 4 M urea and purified. Following RNAase H digestion to remove any DNA:RNA hybrids, second priming sites were created by \( 5' \)-polyoxyguanylation using 15 U of terminal deoxynucleotidyl transferase (Gibco BRL) and 0.1 mM \( \alpha \)-[\( ^32P \)]dCTP. PCR amplification was performed using 2.5 U Taq DNA polymerase (Fisher Scientific), 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl\(_2\) and 0.001% (w/v) gelatin with primers (‘\( 5'\)-CCGGATCTCTTTTTTTTTTTTTTGG-3’) and (‘\( 5'\)-CCGGATCCCCCCCCCCCCCCCCCCCC-3’) under the following conditions: 94°C denaturation, 55°C anneal and 72°C for extension. Reaction products were separated on 2% low-melt agarose and specific PCR products were ligated into pGEM-T (Promega). After bacterial transformation, several positive clones were isolated and DNA sequencing (8) was performed by the Tufts Physiology Core Facility.

DNA sequence analyses of four independent clones indicated that the largest cDNA is \( >94\% \) identical to bovine tRNA\[Ser\]Sec (9). Analyses of two independent clones revealed the second largest cDNA had \( >90\% \) identity to rat liver mitochondrial tRNA\[Trp\] (10).

To confirm the identity of the \( i^6A \)-tRNAs, we performed northern analyses on both total RNA and anti-\( i^6A \) immunoprecipitated material (Fig. 3). The northern blot was hybridized with a 193 bp \( AvaI\)-HindIII fragment of \( UCpST \) encoding the human tRNA\[Ser\]Sec gene (11) and the oligonucleotide probe (‘\( 5'\)-AGGGG-CCTTGAAGGCTTGACTG-3’) for the mitochondrial tRNA\[Trp\] (10). The blot shows that both the tRNA\[Ser\]Sec (Fig. 3A) and mitochondrial tRNA\[Trp\] (Fig. 3B) are immunoprecipitated by our anti-\( i^6A \) serum (lanes 1 and 3). For both tRNAs, the immunoprecipitated products show identical migration to the hybridizing bands in total CHO RNA (lane 4). The absence of hybridizable material from immunoprecipitations performed in the presence of \( i^6A \) (lane 2), further confirms the presence of this modification on these tRNAs. Total RNA from HEK-293 (human embryonic kidney) cells (lane 5) was used as a positive control for the tRNA\[Ser\]Sec hybridization since we utilized the human tRNA\[Ser\]Sec gene in preparing the probe. (Note: the migration
Isopentenyladenosine may be present on additional tRNAs (e.g., Leu, Ser, Tyr, Phe, Cys and Trp). However, with the exception of the major seryl-tRNA (data not shown), we have not detected these other i6A-tRNAs. Perhaps, a low relative abundance, poor antibody recognition due to folding of the tRNA, or inefficient cDNA synthesis may explain their lack of detection.

In this paper, we have described a unique method for the identification of tRNAs where the primary sequence is unknown. Starting with cell lysates or total RNA, one can clone and sequence DNA that is complementary to the RNA of interest. This protocol will be particularly helpful in the identification of other tRNAs and possibly other small RNAs especially when they can be enriched or partially purified.

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