Base modification pattern at the wobble position of Xenopus selenocysteine tRNA<sub>Sec</sub>

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ABSTRACT

We examined the base modification pattern of Xenopus tRNA<sub>Sec</sub> using microinjection into Xenopus oocytes, with particular focus on the wobble base U34 at the first position of the anticodon. We found that U34 becomes modified to mcm<sup>5</sup>U34 (5-methylcarboxymethyluridine) in the oocyte cytoplasm in a rather complex manner. When the tRNA<sub>Sec</sub> gene is injected into Xenopus oocyte nuclei, 5<sup>5</sup>55 and m<sup>1</sup>A58 are readily obtained, but not mcm<sup>5</sup>U34. This will appear only upon cytoplasmic injection of the gene product arising from the first nuclear injection. In contrast, tRNA<sub>Sec</sub> produced by in vitro transcription with T7 RNA polymerase readily acquires 5<sup>6</sup>A37, 5<sup>5</sup>55, m<sup>1</sup>A58, and mcm<sup>5</sup>U34. The latter is obtained after direct nuclear or cytoplasmic injections. It has been reported by others that mcm<sup>5</sup>Um, a 2'-O-methylated derivative of mcm<sup>5</sup>U34, also exists in rat and bovine tRNA<sub>Sec</sub>. With both the gene product and the in vitro transcript, and using the sensitive RNase T2 assay, we were unable to detect under our conditions the presence of a dinucleotide carrying mcm<sup>5</sup>Um and that would be therefore refractory to hydrolysis. We showed that the unusual mcm<sup>5</sup>U acquisition pathway does not result from impairment of nucleocytoplasmic transport. Rather, these data can be interpreted to mean that the modification is performed by a tRNA<sub>Sec</sub> specific enzyme, limiting in the oocyte cytoplasm.

INTRODUCTION

Selenocysteine tRNA (tRNA<sub>Sec</sub>) mediates the incorporation of selenocysteine into the growing peptide chain in response to in frame UGA codons (1, 2). In prokaryotes, the tRNA<sub>Sec</sub> is delivered to the appropriate UGA codon owing to a stem-loop structure in the mRNA and SELB, a particular elongation factor used in lieu of EF-Tu (reviewed in ref. 1). Both the prokaryotic and eukaryotic tRNAs<sub>Sec</sub> exhibit unusual secondary and tertiary structures (3, 4). These are likely responsible, at least for prokaryotes, for the capacity of tRNA<sub>Sec</sub> to interact both with selenocysteine synthase, which converts serine to selenocysteine, and SELB (5).

In addition to being a particular tRNA at the level of its function, transcription of the tRNA<sub>Sec</sub> gene is initiated in eukaryotes from an atypic tRNA promoter located almost exclusively upstream of the coding region (6–8). This particular promoter, containing a TATA box, enables the start site of transcription by RNA polymerase III to coincide with the mature 5′ end of the gene product (7, 9). Therefore, there is no need for 5′ processing as in classical tRNAs.

It has previously been shown that eukaryotic pre-tRNA<sub>Sec</sub> undergoes in vitro a maturation step which consists in trimming a few nucleotides in the 3′ trailer sequence and adding the CCA end to produce the mature tRNA (9, 10). Modified nucleoside acquisition is another processing event followed by tRNAs to yield the mature, functional molecule. In particular, the importance of the modification at the wobble base (first position of the anticodon) is well-known. When the first position is occupied by a U, different types of modification can be encountered which either serve to promote flexibility in the codon—anticodon interaction or, in contrast, restrict this interaction to a perfect base complementarity in order to prohibit misrecognition of codons (reviewed in ref. 11). It has been reported that the wobble base of rat and bovine tRNA<sub>Sec</sub> is mcm<sup>5</sup>U (12, 13). In order to analyze the acquisition pathway of this and other modified bases in tRNA<sub>Sec</sub>, we employed the Xenopus oocyte which has been shown to be a powerful system to perform such an analysis (14, 15). Here, we show that the modification at the wobble position of Xenopus tRNA<sub>Sec</sub> is mcm<sup>5</sup>U and, in addition, that the base modification content of tRNA<sub>Sec</sub> is low, consisting in the four bases mcm<sup>5</sup>U34, 5<sup>6</sup>A37, 5<sup>5</sup>55, m<sup>1</sup>A58.

MATERIALS AND METHODS

DNA constructs

The <i>X.laevis</i> tRNA<sub>Sec</sub> gene carrying its external promoter elements is from (7). T7XlRNA<sub>Sec</sub>, the <i>X.laevis</i> tRNA<sub>Sec</sub> gene under the control of a T7 promoter, is from (4).
In vitro transcription of 32P-labeled tRNAs with T7 RNA polymerase

The procedure is described in detail in (16). Briefly, 8μg of linearized template is incubated in a 50 μl transcription buffer containing 125 μCi of the appropriate α-32P NTP (3000Ci/mmol, Amersham). The mixture is incubated for 3h at 37°C, phenol extracted and the tRNA transcripts fractionated on 12% denaturing polyacrylamide gels.

Oocyte injection

A 20 nl mixture containing 6 ng of DNA template carrying the X.laevis tRNA^ gene was injected into Xenopus oocyte nuclei in the presence of 300 nCi of the appropriate α-32P NTP (3000Ci/mmol, Amersham). Samples of 15 oocytes were incubated for 6 to 72h. Recovery of the tRNA material was performed as described in (7). In some experiments, the tRNA^ gene product obtained after 20h incubation was gel purified and either analyzed directly for its modified base content or injected into the oocyte cytoplasm at 2500 cpm per oocyte, in batches of 60 oocytes, and incubated for a further 72h.

Routinely, 20 nl containing 2–5×10^6 cpm (about 50 femtoles) of 32P-labeled in vitro transcribed tRNAs were injected into the nucleus or the cytoplasm of 60–90 oocytes. Incubation varied from 6 to 72 h. RNA extraction was done as above. tRNAs were fractionated on 10% denaturing polyacrylamide gels. Isolation of germinal vesicles was performed under mineral oil as described in (17) in order to avoid leakage of nuclear material.

Analysis of modified nucleosides

The modified nucleosides acquired by the 32P-labeled tRNAs arising from injection of the gene or in vitro transcription products were analyzed directly or following hydrolysis with 2 units of RNase T1 and fractionation of the digestion products on 20% denaturing polyacrylamide gels. Full-size tRNAs or tRNA fragments were degraded completely either to 5'-mononucleotides with 0.25 unit of nuclease P1 or to 3'-mononucleotides with 0.1 unit of RNase T2. The digestion products were separated by 2D thin layer chromatography on 10 x 10 cm or 20x20 cm cellulose plates using isobutyric acid-NH4OH-water, 66/1/33 (v/v/v) in the first dimension and isopropanol-HCl-water, (68/17,6/14,4 (v/v/v)) in the second dimension. Identification of the radioactive spots was done after autoradiography of the plates and comparison with published results (18).

RESULTS

The acquisition pattern of tRNA^ anticodon base modification in Xenopus oocytes is complex

The Xenopus tRNA^ gene (7) was injected into Xenopus oocyte nuclei in the presence of (α-32P)UTP. Oocytes were incubated for 6 to 72h. RNase T1 hydrolysis of the gel purified labeled tRNA^ yields, among other products, two characteristic 11-mer and 17-mer fragments after fractionation on 20% polyacrylamide gels. The 11-mer spans positions C32 to G42 which contain the anticodon, the 17-mer encompasses positions U54 to G69 containing the T stem and loop (Figure 1). The different 11-mer fragments arising from tRNA^ obtained after various incubation periods were digested totally by nuclease P1. Two-dimensional chromatography revealed that, except 32P-U residues, no labeled modified U derivatives appeared, even after the longest oocyte incubation (Figure 2a). This is not likely due to the inability of the oocyte to modify RNA molecules since the oocyte has been shown earlier to be able to fully process tRNA gene products to the mature, modified molecule (14, 15). Moreover, we observed that the tRNA-characteristic A58 to m' A58 (Figure 2b) or U55 to 3'55 modification (not shown) did occur in the 17-mer under our conditions.

We then asked whether the lack of U34 modification could result from impairment of nucleocytoplasmic transport of tRNA^ since it has been shown that in tRNAs, anticodon modifications are known to occur in the oocyte cytoplasm (15). As we observed that the pyrimidine biosynthesis pathway of the Xenopus oocyte converts part of the (α-32P)UTP input into 32P-labeled C and A (not visible in Figure 2a), we renounced using this label. Instead, (α-32P)CTP was used. To this end, injected oocyte nuclei were collected after 20h incubation, dissected under mineral oil and the RNAs extracted from the cytoplasmic and nuclear compartments separated on a polyacrylamide gel. Figure 3 shows that there is about twice as much tRNA^ in the...
Figure 3. Nucleocytoplasmic transport of Xenopus tRNA\textsuperscript{Sec}. The tRNA\textsuperscript{Sec} gene was injected into Xenopus oocyte nuclei with (\(\alpha\)-\(32\)P)CTP. After overnight incubation, germinal vesicles (small closed circle) and cytoplasm (large open circle) were manually dissected under mineral oil (17) and further examined for their tRNA\textsuperscript{Sec} content on a 10\% polyacrylamide gel.

Figure 4. Oocyte cytoplasmic injection of the tRNA\textsuperscript{Sec} gene product led to the appearance of spot X. The Xenopus tRNA\textsuperscript{Sec} gene was injected into Xenopus oocyte nuclei with (\(\alpha\)-\(32\)P)CTP. After 20 h, tRNA\textsuperscript{Sec} was purified, injected into the cytoplasm and incubated for 72 h. A 2D separation of a total RNase T2 digest of the tRNA\textsuperscript{Sec} obtained after these treatments is shown. Labeled spots are nearest neighbors to C residues. 5'-mono, di and triphosphate guanosines appear since initiation of tRNA\textsuperscript{Sec} transcription coincides with its mature 5' end (7, 9, 22).

The amount of tRNA\textsuperscript{Sec} molecules in this and the cytoplasmic injection experiment of the gene product is comparable. PI digestions of the 11-mer RNase T1 fragments obtained after various oocyte incubation periods are shown in Figure 5 (a, b, c). A spot with similar if not identical chromatographic mobility as spot X in Figure 4 is already detected 20 h after injection. To determine whether this base modification is nuclear or cytoplasmic, the same labeled transcript was injected into the cytoplasm. After overnight incubation, tRNA\textsuperscript{Sec} was digested to completion by nuclease P1. Figure 5d shows the presence of a spot equivalent to spot X. This experiment indicates that the anticodon base modification present in spot X is obtained in a cytoplasm-dependent manner. This finding corroborates the result obtained upon cytoplasmic injection of the gene product. In a complementary experiment, we showed that using a tRNA\textsuperscript{Sec} transcript labeled with (\(\alpha\)-\(32\)P)ATP, the base modification i6A37, occurring in the anticodon of a number of tRNAs, could be acquired 6 h after nuclear injection (Figure 5e and f).
To determine the identity of the modification, the following experiments were performed. If the modified base at U34 is known to contain mcm5U, a 2'-O-methylated version mcm5Um present at the same anticodon position in an isoacceptor species (12, 13). We wished to determine whether the 2'-O-methylation occurs in oocyte injected tRNAsec. A transcript labeled with (α-32P)ATP was injected into Xenopus oocyte nuclei. After various incubation periods, the 11-mer RNase T1 fragment containing the anticodon was submitted to RNase T2 digestion. If a 2'-O-methylation is to occur, then one should obtain an mcm5Um-Cp RNase T2-resistant dinucleotide carrying a 32P 3'-phosphate arising from A36. By comparison with the positions of known dinucleotides containing a 2'-O-methyl nucleoside (18), it can be deduced that mcm5Um-Cp cannot be superimposed with any of the four major nucleotides. Figure 7 indicates that even after 48h oocyte incubation no dinucleotide is visible on the plate. The C labeled residue is C35 since it is the only cytosine 5' to an A in the 11-mer fragment. This strongly suggests that 2'-O-methylation of mcm5U34 does not occur in Xenopus oocytes. The absence of RNase T2-resistant dinucleotide on the TLC plate shown in Figure 4 also argues that 2'-O-methylation is not obtained after cytoplasmic injection of the gene product.

**DISCUSSION**

We have shown that an X.laevis tRNAsec transcribed in vitro acquires four modified nucleosides upon injection into Xenopus oocytes. These are mcm5U34, iA37, $\Psi$55 and m1A58 which are also found in authentic mammalian tRNAsec (12, 13, 21, 22). These experiments show that an unmodified tRNAsec produced by in vitro transcription with T7 RNA polymerase can acquire in Xenopus oocytes the base modifications carried by authentic tRNAsec. No canonical dihydrouridines nor 5-methyluridine, usually found in tRNAs, could be detected. Therefore, the tRNAsec content in post-transcriptional base modification is rather low in comparison to other tRNAs.

In this work, we have focused our attention on the modification pattern of the wobble base, mcm5U34. We have shown that this modification takes place in the oocyte cytoplasm, an observation consistent with the general idea that anticodon modification enzymes are cytoplasmic (15). While a tRNAsec transcribed in vitro readily acquires this modification shortly after oocyte injection, this did not happen when the starting material was the tRNAsec gene itself. The U34 base modification appeared only when the gene product obtained after nuclear injection of the tRNAsec DNA was injected into the cytoplasm. Several possibilities can be raised to explain the lack of modification. Inhibition of nucleocytoplasmic transport was ruled out since we showed it to be fairly efficient. Another possibility resides in the fact that the modification enzyme is saturated and cannot cope with the amount of tRNAsec molecules transported to the cytoplasm. At first glance, this possibility would seem unlikely if one considers that using the same amount of injected DNA as in our experiments, it was previously shown by others that a tRNA gene product readily undergoes in Xenopus oocytes all the maturation pathway, including splicing (14, 15). However, an attractive explanation exists. Given the peculiar secondary and especially tertiary structures of the eukaryotic tRNAsec (4), one can invoke the existence of a modifying enzyme specific to tRNAsec, possessing as determinants some of these secondary
and/or tertiary motifs. This enzyme would therefore be limiting in the oocyte and thus could only modify the minor amounts contained in the cytoplasm when injecting the gene product or the T7 transcript.

The rat and bovine liver tRNAs5 have been described to contain two isoacceptor species. One carries mcm5U, the other mcm5Um (12, 13). In rat, accumulation of the latter modification is under the dependence of selenium concentration (12). We could not find the 2'-O-methylated version of mcm5U in Xenopus oocytes, even after long incubation periods of the oocytes in a medium containing sodium selenite (unpublished results). As a matter of fact, an enzymatic activity that 2'-O-methylates an otherwise unmodified wobble base in other tRNAs has been detected in Xenopus oocytes (15, 23). In this particular case, the extent of modification is significant after 48h and quantitative after 72h. Therefore, to explain the lack of Xenopus tRNA5 2'-O-methylation with regard to rat and bovine liver and to position 34 of other tRNAs in Xenopus, one must hypothesize that: i) the enzyme which methylates the ribose position of mcm5U in Xenopus differs from the position 34 sequence-unspecific activity described in (15, 23). ii) the 2'-O-methylation in tRNA5 is tissue specific or under developmental control. In this respect, it is worth mentioning that the mcm5Um version is limiting in rat brain and testis (12) and that developmental regulation of 2'-O-methylase activities has been already reported for Xenopus and mouse U1 snRNAs (24).

The importance of the modification at the wobble position in tRNAs is well-known (11). In this context, the occurrence of an unmodified U34 in E.coli tRNA5 is puzzling (25). Whether it reflects subtle differences in the decoding properties between prokaryotic and eukaryotic tRNAs is a matter of conjecture.

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