SELECTIVE MODIFICATION OF NUCLEOSIDES OF tRNA PRECURSORS ACCUMULATED IN A TEMPERATURE SENSITIVE MUTANT OF *ESCHERICHIA COLI*

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Received 12 September 1974

1. Introduction

It is generally accepted that tRNA is formed via a larger precursor molecule which is subsequently cleaved and modified in specific manners. However, the details of this 'maturation process' are still largely unclear. One of the interesting problems of these post-transcriptional events is how the cleavage and modification reactions are interrelated each other. Obviously, nucleotide analysis of various precursor molecules of tRNA's at a distinctive maturation stage might shed much light on this problem. We have shown that, in one of our thermosensitive mutants of *Escherichia coli* defective in tRNA synthesis, characteristic RNA molecules of larger molecular sizes are accumulated at high temperature [1]. In this mutant (TS241), at least three precursor molecules for T4 tRNA's are accumulated as the result of the endonuclease block [2]. In this paper, we report that, in the precursors for both cellular and T4 tRNA's accumulated in this mutant, specific modified nucleosides such as 2′-*O*-methyl guanosine (2′-*O*-mG) are missing, while others including ribothymidine (T), pseudouridine (ψ) and dihydrouridine (D) are present. These results indicate that modification of tRNA precursors is selective and possibly stage specific in the maturation process.

2. Materials and methods

TS241, a temerature sensitive mutant of *E. coli* defective in tRNA biosynthesis and its parental strain 4273 were described previously [1]. ^{32}P-labelling, extraction of RNA, and electrophoresis on polyacrylamide slab gel were also described earlier [1]. A dialyzed S 30 extract of *E. coli* Q13, used for in vitro cleavage of tRNA precursors, was prepared as described by Nathans [3]. RNase T2 digests of ^{32}P-labelled RNA were analyzed by two-dimensional thin-layer chromatography according to the method described by Nishimura [4]. Fingerprint analysis of RNase T1 or RNase A digests was performed as described by Barrell [5].

3. Results and discussion

As shown previously [2], the synthesis of T4 tRNA was drastically changed if TS241 was infected with phage T4 and subsequently grown at high temperature (fig. 1a). Appearance of three new bands (B, C and D) and an increase of band A were noted. These bands were characterized by the in vitro cleaving reactions using an S 30 cell-free extract from *E. coli* Q13 and by two-dimensional fingerprint analysis. Band D is a monomeric precursor for glycine tRNA. This precursor has an extra nucleotides at the 5′ terminus of glycine tRNA. Band A is found to be indistinguishable from the dimeric precursor for proline and serine tRNA's sequenced by Barrell et al. [6]. Band C, a mixture of several RNA species, seems to contain a monomeric precursor for ϵ-tRNA, a tRNA of unknown specificity. Band B is found as a precursor for band 1 that is not related to tRNA as characterized by Paddock et al. [7]. We have shown that all of these molecules accumulate in TS241 as the result of the impaired endonucleolytic function [2].

In the course of characterization of these tRNA precursors by fingerprint analysis, we found that a modified nucleoside 2′-*O*-methyl guanosine present in serine, glycine, and ϵ-tRNA's was missing in their precursor molecules. For instance, when RNase A digests of $tRNA^{Gly}$ was fractionated, a trinucleotide 2′-*O*-mGpGpDp was seen as shown in fig. 2. RNase T2 or alkaline digestion of this trinucleotide gave the dinucleotide 2′-*O*-mGpGp and Dp. In the RNase A fingerprint of band D, this trinucleotide was replaced by GpGpDp (or GpGpUp), which gave Gp (2 moles) and Dp (or Up) (1 mole) upon RNase T2 or alkaline digestion. This observation was further confirmed by two-dimensional chromatography of the RNase T2 digests of tRNA precursors on thin-layer plates. In fig. 3, chromatograms of the digests of band D and of the in vitro cleavage product of band A corresponding to $tRNA^{Ser}$ are compared to those of their mature tRNA counterparts. As seen in the figure, the dinucleotide 2′-*O*-mGpGp, present in $tRNA^{Ser}$ and $tRNA^{Gly}$, is totally absent in their precursor molecules. Similarly, band C which contains a precursor for ϵ-tRNA was analyzed after RNase T2 digestion. The dinucleotide 2′-*O*-mGpGp was detectable in ϵ-tRNA but not in band C (data not shown).

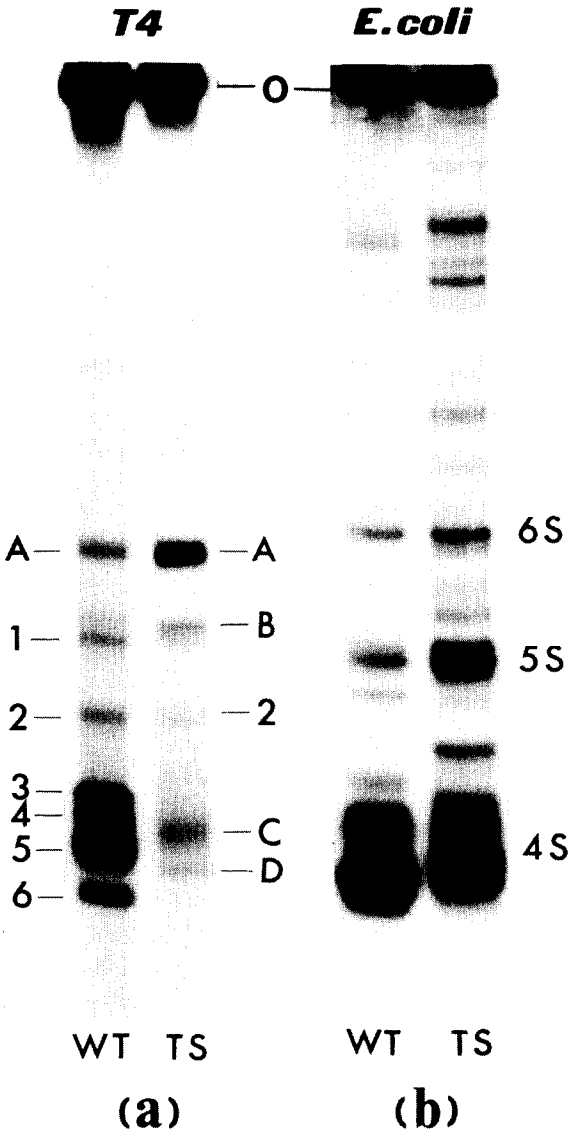

Fig. 1. Autoradioram of 10% polyacrylamide gel electrophoresis. T4 infected (a) or uninfected (b) cells were labelled with ^{32}P-orthophosphate at 42°C for 60 min. RNA's were prepared and electrophoresed as described earlier [1]. TS and WT in the figure represent TS241 and strain 4273, respectively.

According to Guthrie et al. [8], the dimeric precursors for proline–serine tRNA's and for α-δ tRNA's which they identified in wild type *E. coli* infected with T4 contained all nucleoside modifications with the exception of 2′-*O*-methyl guanosine. In our case, the in vitro cleavage product corresponding to $tRNA^{Ser}$ contains ψp, Tp, and Dp but the presence of 2′-methyl cytidine and N_2 (a derivative of U) is not clear. In addition, it appears that the A residue next to the 3′ terminal nucleotide of anticodon is barely modified. In band D, ψp and Tp are fully present but Dp seems to be only partial. Significance of these observations is not known. It is possible that nucleoside modification is generally incomplete for many nucleotides of these precursors. It is worth noting that pseudouridine in the anticodon stem of $tRNA^{Gly}$ is appreciably modified in its precursor, band D. This is rather contradictory to the previous observation of Schäfer et al. [9]. They showed that pseudouridine in the anticodon stem of *E. coli* $Su3^+$ $tRNA_1^{Tyr}$ was totally absent in its precursor molecule obtained after ϕ80pSu3 A25 infection, although pseudouridine in the TψC loop was present. According to Schäfer et al., pseudouridine in the anticodon stem of *E. coli* $tRNA_1^{Tyr}$ can only be formed after trimming of the precursor molecule by RNase *P*. It is possible that the mode of pseudouridylation of the anticodon stem of *E. coli* $tRNA_1^{Tyr}$ is different from that of T4 tRNA's. This point has to be clarified in the future.

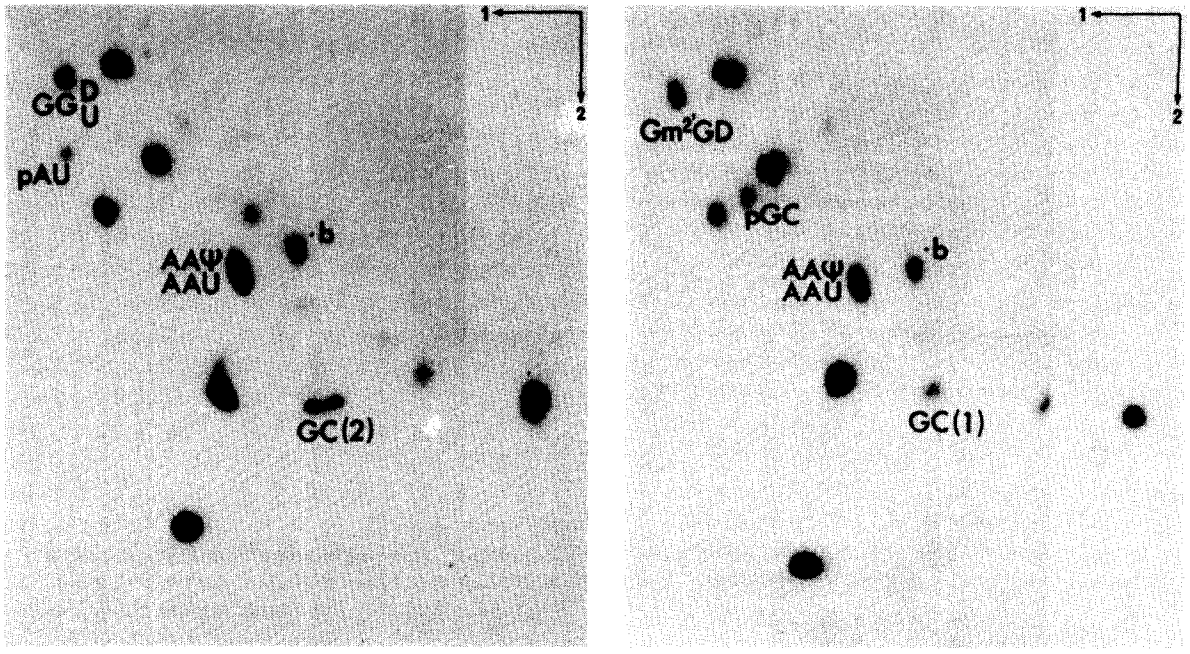

Fig. 2

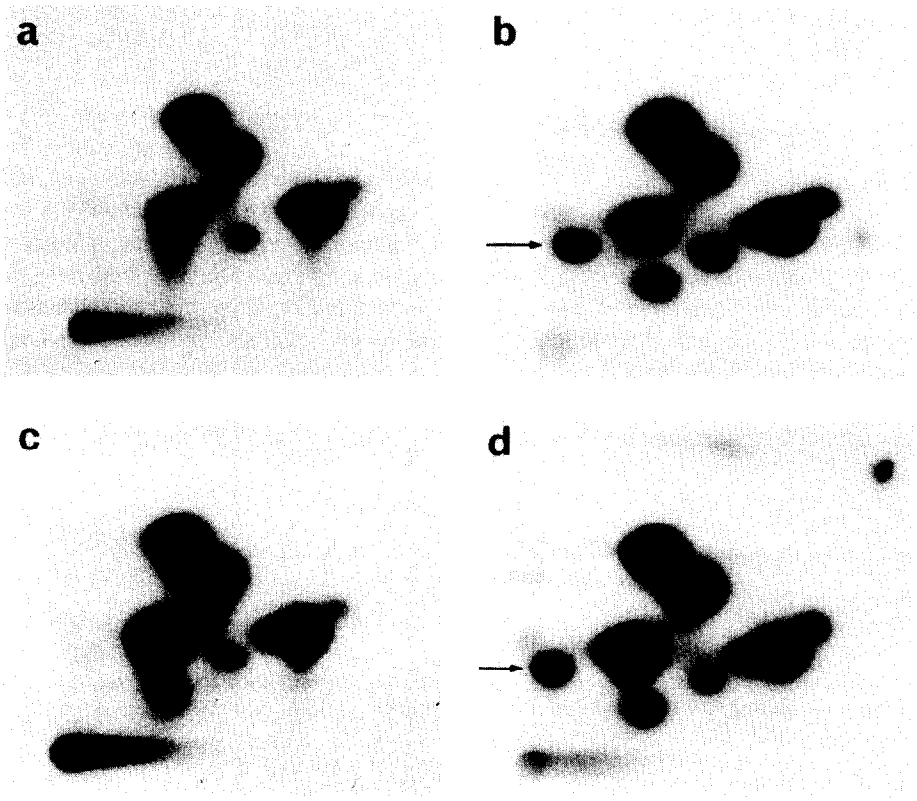

Fig. 3

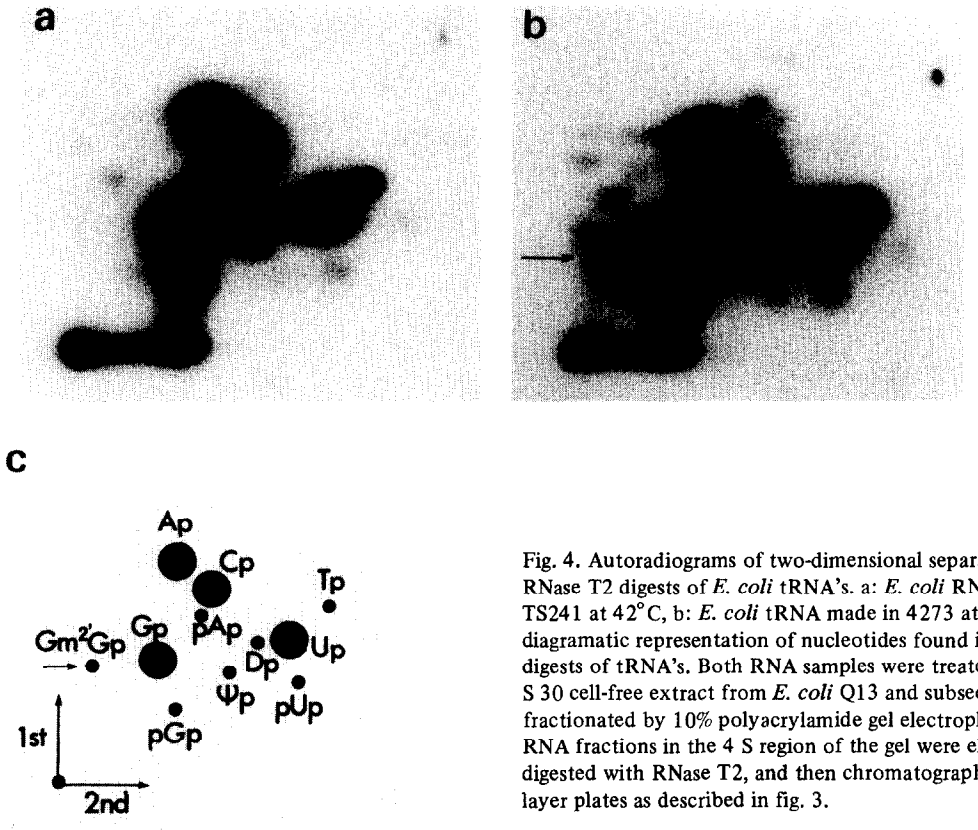

Fig. 4. Autoradiograms of two-dimensional separation of RNase T2 digests of *E. coli* tRNA's. a: *E. coli* RNA made in TS241 at 42°C, b: *E. coli* tRNA made in 4273 at 42°C, c: diagramatic representation of nucleotides found in RNase T2 digests of tRNA's. Both RNA samples were treated with an S 30 cell-free extract from *E. coli* Q13 and subsequently fractionated by 10% polyacrylamide gel electrophoresis. Total RNA fractions in the 4 S region of the gel were eluted and digested with RNase T2, and then chromatographed on thin-layer plates as described in fig. 3.

Although Guthrie et al. [8] were the first who pointed out the absence of 2'-*O*-methyl guanosine in the precursors of T4 tRNA's, their observation was confined to the dimeric precursors for proline-serine tRNA's and for α-δ tRNA's. We have demonstrated that at least 2'-*O*-methyl guanosine is consistently absent in both the dimeric and monomeric precursor molecules of T4 tRNA's detected in TS241. This observation was further generalized in precursors for *E. coli* tRNA's accumulated in TS241. When TS241 was grown at 42°C, slightly slower migration of 4 S RNA and appearance of new RNA bands of larger molecular weights were evident in a 10% polyacrylamide gel, as shown in fig. 1b. These abnormal bands disappeared and the gel pattern of mutant RNA's became indistinguishable from that of wild type, if the mutant RNA preparation had been treated with the Q13 cell-free extract prior to gel electrophoresis (data not

Fig. 2. Autoradiograms of two-dimensional fractionation of RNase A digests of T4 RNA's. (a): precursor for T4 $tRNA^{Gly}$ (band D of fig. 1a). (b): T4 $tRNA^{Gly}$ (band 6 of fig. 1a). The first dimension (right to left) is on cellulose acetate in pyridine acetate buffer (pH 3.5) with 7 M urea, and second dimension (top to bottom) on DEAE paper in 7% formic acid. The fingerprint analysis was performed according to the procedure described by Barrell [5].

Fig. 3. Two-dimensional separation of RNase T2 digests of ^{32}P-labelled RNA. RNA's were digested with RNase T2 and chromatographed on thin-layer plates according to the method described by Nishimura [4]; first dimension in isobutyric acid −0.5 M NH_4OH (5:3, v/v); second dimension in isopropanol–HCl–water (70:15:15, v/v/v). a: precursor for T4 $tRNA^{Gly}$, b: T4 $tRNA^{Gly}$, c: in vitro cleavage product of band A RNA ($tRNA^{Pro}$–$tRNA^{Ser}$ dimeric precursor) corresponding to T4 $tRNA^{Ser}$, d: T4 $tRNA^{Ser}$. The dinucleotide 2'-*O*-mGpGp is indicated by an arrow.

shown). We know that the majority of these abnormal RNA bands are converted to 4 S RNA as judged by electrophoretic mobility in a 10% polyacrylamide gel and thus probably represent precursors for *E. coli* tRNA's.

The converted 4 S RNA bands were extracted as a whole from the gel and digested with RNase T2. The digests were then chromatographed two-dimensionally on a thin-layer plate. As shown in fig. 4, 2′-*O*-mGpGp is not detectable in the mutant RNA, whereas the dinucleotide is definitely present in wild type RNA prepared in parallel with the mutant RNA. Also noted in the figure is that the number of modified nucleotide species detected in the mutant RNA is considerably much less than that of wild type RNA, although ψp, Tp, and Dp are clearly present as was the case of T4 tRNA precursors. This indicates that some other nucleosides are also missing in precursors for *E. coli* tRNA's. Thus, it appears that some nucleoside modifications such as pseudouridine, ribothymidine and dihydrouridine occur at relatively early stages of tRNA maturation process, at least before TS241 function cleaves tRNA precursors, while others including 2′-*O*-methyl guanosine occur after the precursors are processed by TS241 function. In this connection, it is interesting to note that some large tRNA precursors accumulated in TS241 contain more than two tRNA sequences, in which ψp, Tp and Dp are already present. It appears that these precursors represent a tRNA maturation stage earlier than the step of TS241 endonucleolytic cleavage (H. Sakano and Y. Shimura, unpublished).

At present we cannot totally exclude the possibility that TS241 is thermosensitive not only in the specific endonucleolytic function but also in modification reactions for certain nucleosides. On the basis of various biochemical and genetic experiments, however, it would be more tempting to assume that modification of specific nucleosides of tRNA molecules take place on their unique precursors at distinct stages of maturation process. Experiments are in progress in our laboratory to clarify these problems.

Acknowledgements

We thank Dr T. Ikemura of our laboratory for his help during the course of this work. This work was supported by a Scientific Research Grant from the Ministry of Education of Japan.

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