BIOSYNTHESIS OF PSEUDOURIDINE IN THE IN VITRO TRANSCRIBED $tRNA^{Tyr}$ PRECURSOR

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1. Introduction

The biosynthesis of a tRNA molecule in prokaryotes starts with the transcription of an RNA precursor which is converted to tRNA via a multistep process involving nucleolytic cleavages, which progressively reduce its length, and the modification of several nucleosides along the sequence [1].

Since the intracellular concentration of the maturation intermediates is generally very low, the study of tRNA biosynthesis has been greatly facilitated by the isolation of mutants altered in some specific steps of the biosynthetic pathway, with consequent accumulation of tRNA precursors [2–6].

In a previous study [3,7] it has been reported that *his*T mutants of *Salmonella typhimurium* lack an enzyme responsible for the biosynthesis of pseudouridine in the anticodon region of several tRNA species. tRNA Extracted from *his*T mutants has been used as substrate for in vitro biosynthesis of pseudouridine and an assay has been set up, which is based on the release of tritium from [5-^{3}H]uridine-labeled *his*T tRNA, upon conversion of uridine into pseudouridine [7].

On the basis of this study [7] it has been possible to postulate the existence of two pseudouridylate synthetases:

(i) Pseudouridylate synthetase I, coded by the *his*T gene and responsible for the biosynthesis of pseudouridine in the anticodon region of several tRNA species

(ii) Pseudouridylate synthetase II, responsible for the biosynthesis of pseudouridine in the TψCG loop of every tRNA molecule.

Since the pseudouridine of the anticodon region can be synthesized in vitro as the last step of tRNA maturation, the question is open as to at what step it is actually synthesized in vivo. Guthrie et al. [8] and Sakano et al. [9] have found that the pseudouridine of the anticodon region and of the TψCG-loop are both present in the T4 coded tRNA dimeric precursors. A different result was obtained by Shaefer et al. [10] who found that in the $tRNA^{Tyr}$ precursor accumulating in *Escherichia coli* cells infected by $\phi 80 psu_3^+$ A25 phage mutant strain only the pseudouridine of the TψCG-loop (ψ_{64}) was present, whereas the pseudouridine of the anticodon region (ψ_{40}) was not present and could be synthesized in vitro only after cleavage of $tRNA^{Tyr}$ precursor by RNAase P.

In this paper we report a study on the in vitro biosynthesis of pseudouridine using as substrate the $tRNA^{Tyr}$ precursor, transcribed in vitro from $\phi 80 psu_3^{+,-}$ phage DNA, carrying both suppressor and wild-type $tRNA_1^{Tyr}$ genes [11]. We have found that

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this molecule is substrate for both pseudouridylate synthetases and that the pseudouridine of the anticodon region can be synthesized at the level of uncleaved $tRNA^{Tyr}$ precursor transcribed in vitro.

2. Materials and methods

2.1. *Chemicals*

T1 Ribonuclease was purchased from Sigma Chemical Co. Ltd., and all other chemicals used were of reagent grade.

2.2. *Bacterial and bacteriophage strains*

Isogenic *his*T (TA253) and wild-type (TA265) *S. typhimurium* LT-2 have been described [7].

Bacteriophages $\phi 80$ and $\phi 80 psu_3^{+,-}$ have also been described [11,12].

2.3. *Preparation of S30 cell-extract*

Preparation of S30 cell-extract from TA265 and TA253 strains was performed as described in the procedure for partial purification of pseudouridylate synthetase I (see later).

2.4. *Partial purification of pseudouridylate synthetase I*

Salmonella typhimurium TA265 strain was grown in a medium containing 8% nutrient broth (Difco) and 1.5% NaCl at 37°C and harvested in late log-phase (A = 1.0 at 650 nm). The cells were resuspended in 2 vol. buffer A (10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 10% glycerol) and sonicated four times for 15 s each. The extract was centrifuged at 30 000 × g for 60 min and the resulting supernatant (S30) was centrifuged again at 150 000 × g for 90 min to obtain the S100 extract. The specific activity of pseudouridylate synthetase I in the S100 extract was 30 ± 10 units of enzyme/mg protein. Pseudouridylate synthetase I was precipitated from S100 extract by addition of 20 mg/ml streptomycin sulphate. The recovery of the enzyme activity was about 70% with a specific activity of 200 ± 50 units/mg protein. It has to be emphasized that the optimal concentration of streptomycin sulphate for the precipitation of the enzyme and for the recovery of the activity changed significantly from preparation to preparation and had to be determined each time by a pilot experiment. The streptomycin sulphate precipitate was resuspended in buffer A, 0.33 M NaCl, at a protein concentration of 50 mg/ml, then 450 mg/ml of ammonium sulphate were added. After centrifugation the precipitate was resuspended in buffer A and desalted on a Sephadex G-25 column. After this step there was a recovery of about 60% of the enzymatic activity with a specific activity of 400–600 units/mg of protein. The ammonium sulphate fraction was applied to a DEAE-cellulose column (100 mg protein/cm^2 resin). The flow-rate was half-column bed-volume/hour. After washing with two bed volumes of buffer A, a linear gradient of NaCl (0–0.185 M) was applied. The enzymatic activity eluted at about 50 mM NaCl. The fractions containing enzymatic activity were pooled and ammonium sulphate was added to reach a 70% saturation. After centrifugation the enzyme was resuspended in buffer A at a protein concentration of 10 mg/ml and stored at 4°C. The specific activity of this preparation was about 4000 units/mg of protein with a purification factor of 160.

2.5. *Preparation of labeled* his*T tRNA and $\phi 80$ and $\phi 80 psu_3^{+,-}$ RNA*

[5-^{3}H]Uridine-labeled *his*T tRNA was prepared according to Cortese et al. [7]. [5-^{3}H]Uridine-labeled $\phi 80$ RNA and [5-^{3}H]uridine-labeled RNA, [^{14}C] uridine-labeled RNA, [α-^{32}P]CMP-labeled RNA from $\phi 80 psu_3^{+,-}$ phage were prepared by transcribing in vitro $\phi 80$ phage DNA and $\phi 80 psu_3^{+,-}$ phage DNA respectively, according to Daniel et al. [11,12].

2.6. *Pseudouridylate synthetase assay*

Pseudouridylate synthetase assay was performed as described [7]. [5-^{3}H]Uridine-labeled $\phi 80 psu_3^{+,-}$ RNA was found to release tritium in the medium non-enzymatically at a rate much higher than [5-^{3}H] uridine-labeled *his*T tRNA. In order to minimize this background radioactivity in our assay, we stored $\phi 80 psu_3^{+,-}$ RNA in ethanol at −20°C and centrifuged and resuspended it every time it was needed. One unit of pseudouridylate synthetase is defined as the amount of enzyme able to synthesize 1 pmol pseudouridine in 10 min at 37°C using [5-^{3}H]uridine-labeled *his*T tRNA as substrate.

2.7. *Slab-gel electrophoresis*

Electrophoresis on polyacrylamide slab gel was

performed on a 30 X 15 X 0.15 cm 16% gel in Tris–borate buffer [13] at 200 V for 13–15 h.

2.8. *Fluorography*

Fluorographic detection of oligonucleotides on polyacrylamide gel was performed according to the procedure of Bonner and Laskey [14].

2.9. *Paper chromatography*

Nucleotides separation was observed by paper chromatography. The chromatogram was soaked in a 7% (w/v) PPO* solution in diethyl ether [15]. The chromatogram was then agitated until the ether had evaporated completely and fluorographed on an X-ray film (Kodak Regulix BB54).

3. Results and discussion

3.1. *$tRNA^{Tyr}$ precursor as pseudouridylate synthetase substrate*

In vitro transcribed $tRNA^{Tyr}$ precursor is substrate for both pseudouridylate synthetase I and II. The in vitro transcription of $tRNA^{Tyr}$ genes using purified RNA polymerase and template [11,12,16,17] has provided completely unmodified $tRNA^{Tyr}$ precursors which are suitable substrates for pseudouridylate synthetase I, which is lacking in *his*T mutants, and pseudouridylate synthetase II normally present in *his*T mutants.

Using the in vitro transcriptional product of $\phi 80psu_3^{+,-}$ DNA as substrate we have assayed the pseudouridylate synthetase activities present in the strain TA265 (a wild-type *S. typhimurium*) and in the strain TA253 (carrying the *his*T 1504 mutation). Figure 1 shows that the amount of tritium released in the medium from [5-^{3}H]uridine-labeled $\phi 80psu_3^{+,-}$ RNA, in the presence of TA265 S30 cell-extract, is almost twice the amount of tritium released in the presence of TA253 S30 cell-extract. This result is in agreement with the observation that in vivo TA265 is able to synthesize both ψ_{40} and ψ_{64} [18], whereas TA253 is able to synthesize only ψ_{64} [3].

The tritium released in the medium in the presence of TA253 S30 cell extract is a measure of the activity of pseudouridylate synthetase II, but any attempt to purify this enzyme failed since every fractionation procedure we tried caused the loss of the enzymatic activity. On the contrary, pseudouridylate synthetase I showed to be a less 'delicate' enzyme and it could be purified about 150 times (see Materials and methods). The enzymatic activity was monitored along the whole purification procedure by using either [5-^{3}H]uridine-labeled *his*T tRNA or [5-^{3}H]uridine-labeled $\phi 80psu_3^{+,-}$ RNA as substrate: identical results were obtained. The last purification step, a DEAE-cellulose column chromatography, is shown in fig.2. The elution profiles of pseudouridylate synthetase I, measured using *his*T tRNA and $\phi 80psu_3^{+,-}$ RNA as substrate, are practically coincident.

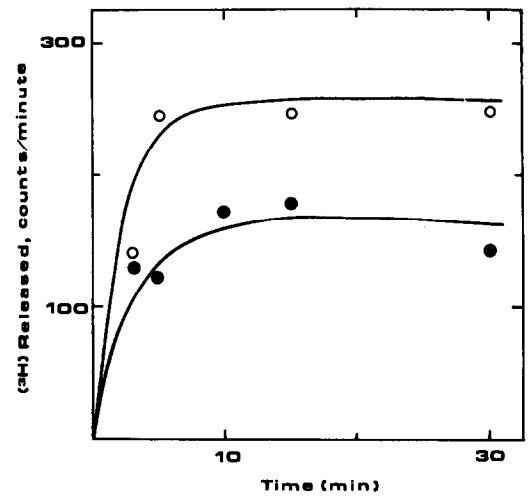

Fig.1. Tritium release as a function of time from [5-^{3}H] uridine-labeled in vitro synthesized $tRNA^{Tyr}$-precursor in the presence of TA265 (○——○) and TA253 (●——●) S30 cell-extract. Enzymatic assay was performed as described [7]. In 0.1 ml reaction mixture [7] 0.2 μg of labeled RNA (1.3×10^6 cpm/μg) and 1.2 mg protein were added.

3.2. *Specificity of ^{3}H-release from [5-^{3}H]uridine-labeled $tRNA^{Tyr}$ precursor*

When the tritium release assay for pseudouridylate synthetase I was developed, several evidences were provided for its specificity [7]. Here we show that tritium is specifically released from [5-^{3}H]uridine-labeled in vitro transcribed $\phi 80psu_3^{+,-}$ RNA incubated in the presence of partially purified pseudouridylate synthetase I. As shown in fig.3, upon incubation with

* *Abbreviation:* PPO, 2,5-Diphenyloxazole

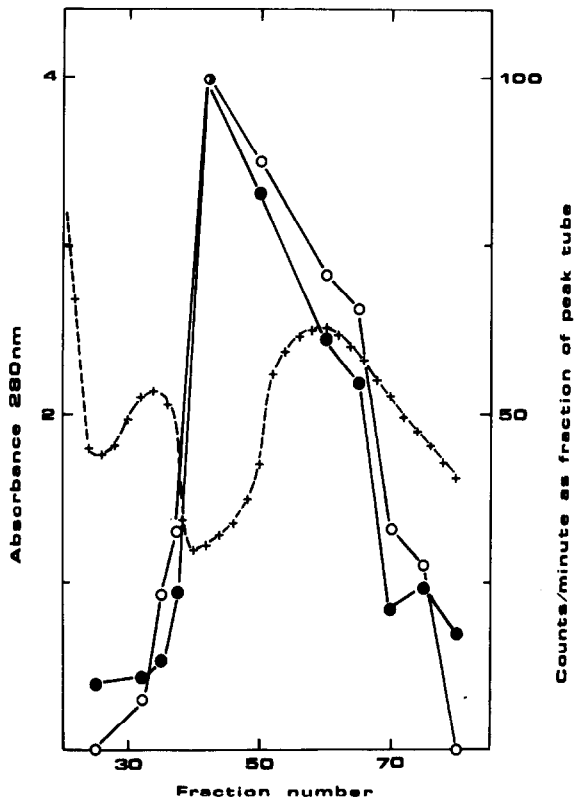

Fig.2. DEAE-Cellulose column chromatography of pseudouridylate synthetase I. The sample deriving from ammonium sulphate fractionation, containing 380 mg protein was applied to a 1.5 X 7 cm column. The column was developed with 200 ml of a linear NaCl-gradient (0–0.185 M) in buffer A, at a flow-rate of 5 ml/h and 1.5 ml fractions were collected. Enzymatic assay was performed on 30 μl aliquots of each fraction as described in the legend to fig.1 and in ref. [7]. The incubation time was 20 min; (+——+) absorbance at 280 nm; (○——○) activity measured with [5-^{3}H] uridine-labeled tRNA^{Tyr} precursor; (●——●) activity measured with [5-^{3}H]uridine-labeled *his*T tRNA.

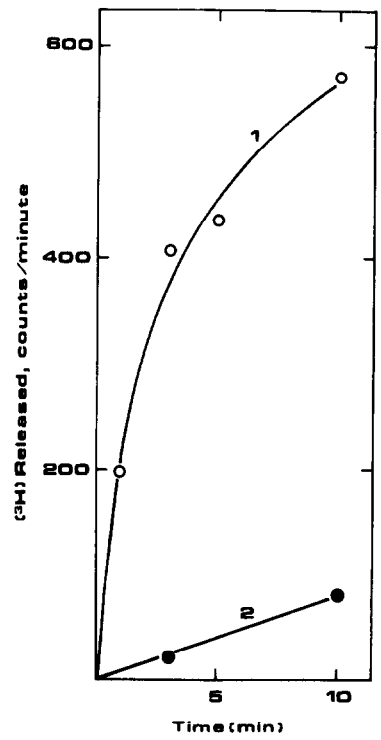

Fig.3. Tritium release as a function of time from [5-^{3}H] uridine-labeled $\phi 80 psu_3^{+,-}$ (○——○) and $\phi 80$ (●——●) RNA. Assay conditions were as described in the legend to fig.1. Partially purified pseudouridylate synthetase I was 7 μg/0.1 ml assay mixture. Labeled $\phi 80 psu_3^{+,-}$ and $\phi 80$ RNA had the same specific activity (2.7 X 10^6 cpm/μg) and were added in the same amount (0.2 μg/0.1 ml incubation mixture).

the enzyme, [5-^{3}H]uridine-labeled $\phi 80 psu_3^{+,-}$ RNA releases 15 times more tritium in the medium (Curve 1) than [5-^{3}H]uridine-labeled $\phi 80$ RNA, which does not contain tRNA^{Tyr} sequences (Curve 2). It is interesting to note that, in this experiment, $\phi 80$ RNA releases an amount of tritium in the medium which is about twice higher than the background of non-enzymatic release. Although no pseudouridine has been found until now in messenger RNA sequences, we cannot exclude that $\phi 80$ transcribed RNA contains sites which can be recognized by pseudouridylate synthetase enzyme.

Further evidence for the specificity of the tritium release reaction has been provided from an experiment in which we measured the inhibition exerted by cold tRNA on the tritium release from [5-^{3}H]uridine-labeled $\phi 80 psu_3^{+,-}$ RNA during incubation in the presence of partially purified pseudouridylate synthetase I (fig.4). A marked inhibition by cold wild-type tRNA was observed (Curve a). This inhibition is also observed when [5-^{3}H]uridine-labeled *his*T tRNA is used as substrate (Arena, F., Ciampi, M. S., Ciliberto, G. and Cortese, R., manuscript in preparation) and it is probably a general property of the enzyme. The inhibition exerted by cold *his*T tRNA

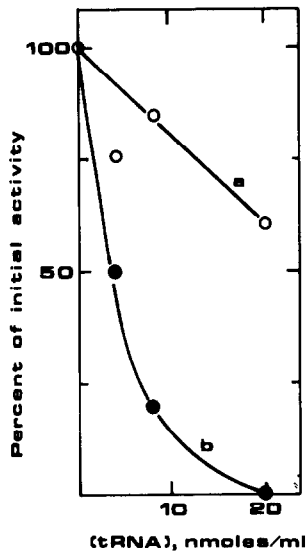

Fig.4. Effect of cold wild-type tRNA (○——○) and cold *his*T tRNA (●——●) on tritium release from [5-^{3}H]uridine-labeled $tRNA^{Tyr}$ precursor incubated with partially purified pseudouridylate synthetase I. Assay conditions were as described in the legend to fig.3. The incubation time was 10 min.

was however more severe and complete (Curve b) as one would expect if $tRNA^{Tyr}$ precursor and *his*T tRNA had to compete for the same site on the enzyme molecule.

The mechanism of pseudouridine synthesis is not known in detail and it is possible that it occurs by a multistep process involving, at a certain stage, the release of tritium from carbon 5 of the pyrimidine ring. Therefore, when the amount of tritium released in the medium is measured, it might be that only a partial reaction is measured, without any information on whether it goes to completion and pseudouridine residues are synthesized. With respect to this point it was shown that, using [5-^{3}H]uridine-labeled *his*T tRNA and [^{32}P]*his*T tRNA as substrate and wild-type S100 cell-extract, the tritium release was paralleled by synthesis of pseudouridine identified in the [^{32}P] tRNA hydrolysate by its characteristic electrophoretic and chromatographic behaviour [7]. Figure 5 shows that this is true also using partially purified pseudouridylate synthetase I instead of crude extract and [α-^{32}P]CMP-labeled $tRNA^{Tyr}$ precursor instead of *his*T tRNA as substrate.

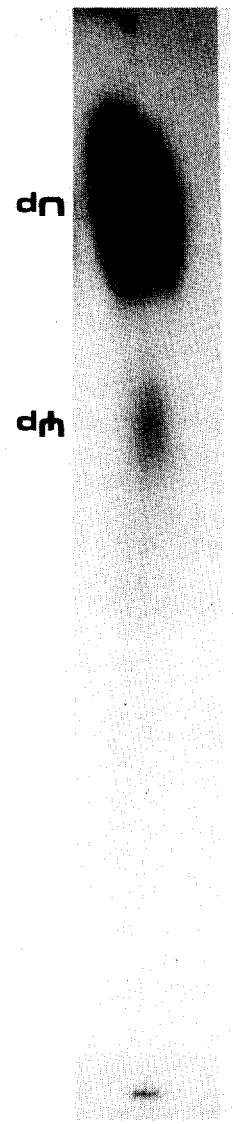

Fig.5. Paper chromatography of alkaline hydrolysate of in vitro pseudouridylated [α-^{32}P]CMP-labeled $tRNA^{Tyr}$ precursor. [^{32}P]CMP RNA 1.4 μg (1.5 × 10^7 cpm/μg) were incubated with 50 μg of partially purified pseudouridylate synthetase I for 15 min as described in the legend to fig.1. The reaction was stopped by adding 4 μg of cold carrier *S. typhimurium* tRNA and 100 μl of 0.1 M Tris–HCl saturated phenol pH 7.5. The RNA was extracted, alkali digested and the resulting nucleotides were fractionated by high-voltage electrophoresis on Whatman No. 52 paper in pyridine acetate buffer pH 3.5 [19]. The spot corresponding to UMP was eluted, UMP and ψMP were separated by paper chromatography in isopropanol–HCl–water (680 : 176 : 144, v/v/v) system [19] and evidenced by autoradiography.

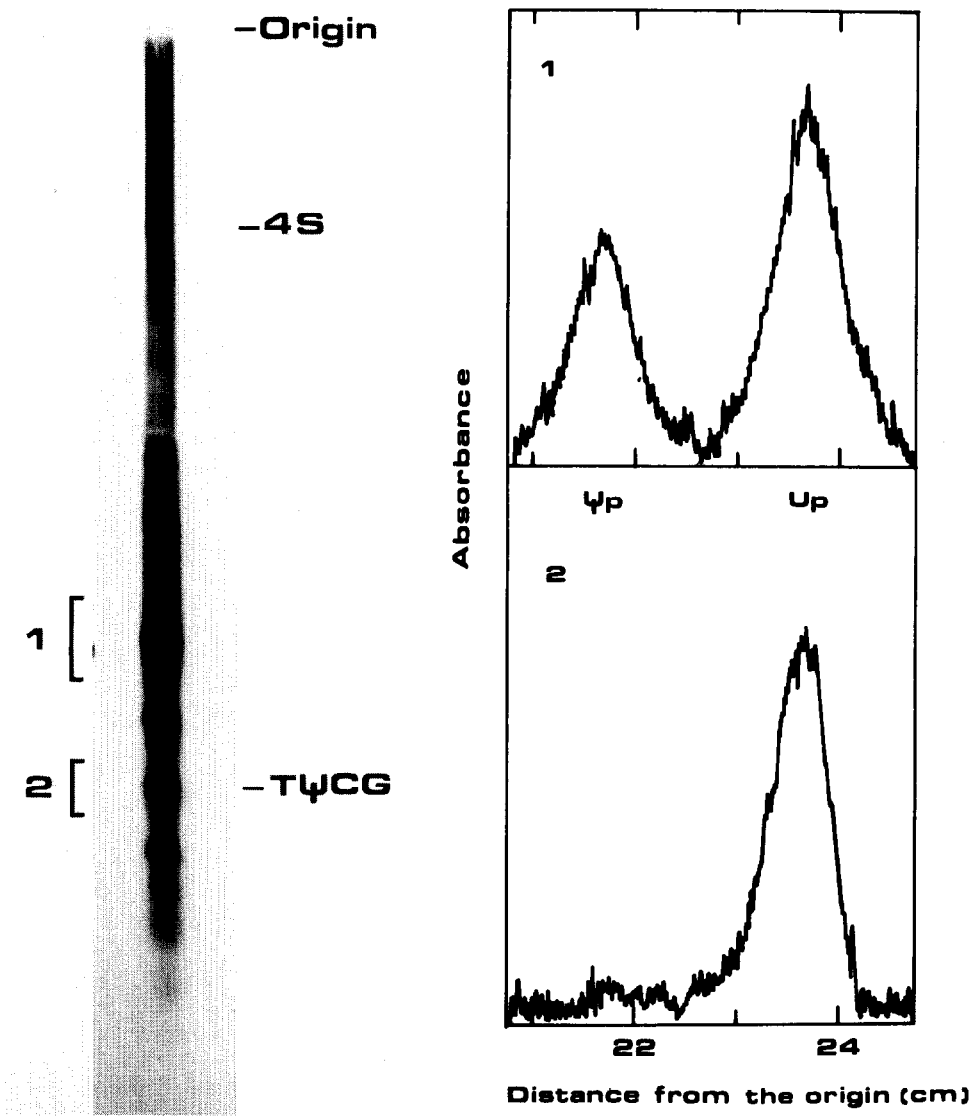

Fig.6. Left panel: fluorogram of 16% polyacrylamide gel electrophoretic separation of T1 digested [^{14}C]uridine-labeled tRNA^{Tyr} precursor. Labeled RNA precursor, 1 μg, was incubated with 70 μg pseudouridylate synthetase I for 20 min as described in the legend to fig.1. The reaction was stopped by addition of 10 μg of cold tRNA and 100 μl Tris-saturated phenol. RNA was extracted, digested by T1 RNAase [19] and the oligonucleotide fragments were fractionated by electrophoresis on 16% polyacrylamide gel [13]. As markers in parallel slots [5-^{3}H]uridine labeled 4 S RNA from *S. typhimurium* and TψCG tetranucleotide, purified from a T1 digest of [*methyl*-^{3}H]methionine-labeled tRNA of *S. typhimurium*, were run. After fluorography oligonucleotides were extracted from Regions 1 and 2 of the gel by a procedure which will be published elsewhere (Bossi, L. and Ciampi, M. S., manuscript in preparation) and digested by alkali. UMP and ψMP were separated by paper chromatography as described in the legend to fig.5 and evidenced by fluorography. The film was scanned by a Gilford spectrophotometer Mod 2400 at 550 nm. On the right panel are the film densitometric scanning profiles of ψMP and UMP contained in oligonucleotides extracted from Region 1 (Profile 1) and 2 (Profile 2) of the gel.

3.3. ψ_{40} *Synthesis at the uncleaved tRNA^{Tyr}-precursor level*

The two pseudouridine modifications contained in both tRNA^{Tyr} su_3^+ and tRNA^{Tyr} su_3^- sequences can be easily distinguished. After T1 RNAase digestion in tRNA^{Tyr} su_3^+ ψ_{40} is contained in a dodecanucleotide fragment and in tRNA^{Tyr} su_3^- it is contained in an octanucleotide, whereas ψ_{64} is contained only in the tetranucleotide TψCG in both cases. Our partially purified pseudouridylate synthetase I is able to synthesize only ψ_{40} and not ψ_{64}. After incubation with the enzyme preparation [^{14}C]uridine-labeled $\phi 80psu_3^{+,-}$ RNA was hydrolyzed with T1 RNAase and the resulting fragments were separated by electrophoresis on 16% polyacrylamide gel (fig.6, left panel). Oligonucleotides were extracted from two regions of the gel: Region 2 at the level of a tetranucleotide marker, run in a parallel slot of the gel, and Region 1 which, on the basis of the relationship between molecular weight and migration distance on polyacrylamide gel electrophoresis [20], should contain both the dodecanucleotide and the octanucleotide fragments. The oligonucleotides were subjected to alkaline hydrolysis and the resulting nucleotides were separated by paper chromatography. In fig.6 (right panel) scanning Profile 1 shows that a considerable amount of pseudouridine is found in the oligonucleotides extracted from gel Region 1 and scanning Profile 2 shows that only uridine is present in the tetranucleotides extracted from gel Region 2.

Since our partially purified pseudouridylate synthetase I is free of nucleases (the size of the in vitro transcribed tRNA^{Tyr} precursor, which migrates as an 85 RNA molecule on acrylamide gel electrophoresis [11], does not change after incubation with the enzyme, data not shown), the results described above constitute a good evidence that ψ_{40} can be synthesized at the level of uncleaved tRNA^{Tyr} su_3^+, su_3^- precursor. This is in agreement with the results reported by Guthrie et al. [8] and Sakano et al. [9], but not with those of Shaefer et al. [10] who found that ψ_{40} could be synthesized in vitro only after cleavage of tRNA^{Tyr} su_3^+ A25 precursor by RNAase P. A possible explanation for this discrepancy is that Shaefer et al. used a tRNA^{Tyr}-precursor, extracted from *E. coli* cells infected with $80psu_3^+$ A25 mutant phage, which reaches an abnormally high intracellular concentration because of its reduced sensitivity in vivo to RNAase P. It is therefore conceivable that this RNA precursor is altered in such a way as to be generally a bad substrate for many maturation enzymes, including pseudouridylate synthetase I. It is relevant to this point the finding that a single point mutation in tRNA genes can lead to transcriptional products which are not recognized by the majority of modifying enzymes [21].

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