ENHANCED ACTIVITY OF tRNA-PSEUDOURIDINE SYNTHETASE IN YOSHIDA ASCITES SARCOMA

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SUMMARY

Five days after transplantation of Yoshida ascites sarcoma cells into a rat, specific activity of tRNA-pseudouridine synthetase was extremely high in the supernatant of tumor cells and moderately high in the tumor-bearing rat liver compared with normal rat liver. Enzyme assay was performed at 37°C by determining the release of tritium from heterogeneous [^{3}H] tRNA extracted from E. coli B grown in the presence of [5,6-^{3}H]-uracil and resulting in the increased ratio of the amount of pseudouridine to uridine residues in [^{3}H] tRNA. Neither [5-^{3}H]-uridine, [5,6-^{3}H]-UTP, nor [5,6-^{3}H]-poly U released tritium in the present assay conditions.

INTRODUCTION

Increased urinary excretion of pseudouridine (ψ) in patients with neoplastic diseases (1) has been observed, and especially in chronic lymphatic leukemia subjects with selectively higher ratio of excretion of pseudouridine to uric acid (2). Pseudouridine, 5-β-D-ribofuranosyluracil, is found mainly in tRNA, and particularly in its TψC loop. Transfer RNA in tumor tissue shows quantitative and qualitative changes of methylated bases as well as methylase activity (3), and an elevated level of urinary excretion of methylated bases has been noticed (4). During chemical carcinogenesis, most carcinogens bind preferentially to tRNA among nucleic acids (5), and some of them modify tRNA to its particular sites (6).

Previous studies conducted in this laboratory revealed that an increased amount of ψ was excreted in the urine of rats after transplantation of Yoshida ascites sarcoma and that the urinary ψ was labeled by [6-^{3}H]-uridine

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via injection but not by either [5-^{3}H]-uridine or [^{14}C]-uracil and the labeling was diminished by treatment with actinomycin D (7).

As for the biosynthesis of ψ in mammals, two kinds of enzymes have been reported—one was ψMP synthetase found in calf thymus, which forms ψMP from ribose-P and uracil (8), and the other was tRNA-ψ synthetase observed in several cultured cell lines, an enzyme which catalyzes the conversion of tRNA-uridine to tRNA-ψ by an intramolecular rearrangement of the ribose from the N-1 position of uracil to the C-5 position. This activity is detected by the tritium-release from the C-5 position of uracil in heterogeneous [^{3}H] tRNA extracted from his T strain of Salmonella typhimurium deficient in the ψ-formation enzyme grown in the presence of [5-^{3}H]-uridine (9). Thus, we investigated tRNA-ψ synthetase activity in Yoshida ascites sarcoma cells and sarcoma-bearing rat liver by determining tritium-release from heterogeneous [^{3}H] tRNA extracted from E. coli B cultivated in the presence of [5,6-^{3}H]-uracil. This paper, to our best knowledge, is the first demonstration of enhancement of tRNA-ψ synthetase activity in tumor and liver of a tumor-bearing animal having increased urinary excretion of ψ.

MATERIALS AND METHODS

[5,6-^{3}H]-uracil (4.8 Ci/mmole), [5-^{3}H]-uridine (29 Ci/mmole), [5,6-^{3}H]-UTP (41.6 Ci/mmole) and [5,6-^{3}H]-poly U (4.65 Ci/mmole as UMP) were purchased from New England Nuclear, Boston, Mass. Snake venom phosphodiesterase and 5'nucleotidase were obtained from Worthington Biochemical Corp., Freefold, N. J. and Sigma Chemical Co., St Louis, Mo., respectively.

Yoshida ascites sarcoma was being maintained every 5 or 6 days by serial transplantation via intraperitoneal injection of 0.2 ml of ascites fluid to Donryu strain male rats, 5 weeks old weighing about 100 g.

Five days after tumor transplantation when rats usually showed the maximal excretion of ψ in the urine during the tumor growing process, harvested cells and removed tumor-bearing rat liver and control rat liver were homogenized with 4 volumes of 0.25M sucrose-0.01M β-mercaptoethanol-0.1M Tris-HCl (pH 8.0) and centrifuged at 105,000 x g for 60 min.

Enzyme assay was based on the method of Mullenbach et al. (9); assay mixture, 0.2 ml of total volume, contained 10 μmoles of Tris-HCl (pH 8.0), 0.25 μmole of magnesium acetate, 10 μmoles of NH_4Cl, 0.9 μmole of dithiothreitol, 100 μg of bovine serum albumin, 2.89 x 10^5 cpm of [^{3}H] tRNA (1.47 A_{260} unit) and an aliquot of the supernatant of the sarcoma cells, the sarcoma-bearing rat liver or normal rat liver. The reactions were carried out at 37°C for various lengths of time and terminated by adding 1.0 ml of 15% (w/v) Norit A in 0.1N HCl. These mixtures were warmed at 37°C for 30

min, then filtered through Whatman No.1 filter paper by centrifugation after addition of 0.5 ml of 0.1N HCl. Radioactivity in 1.0 ml of each filtrate was measured in Bray's solution by Beckman LS-250 scintillation counter. The 1.0 ml sample corresponded to about 62% of 0.2 ml reaction mixture.

[^{3}H] tRNA was prepared from E. coli B grown in the presence of [5,6-^{3}H]-uracil; cultivation was performed according to the procedure of Chirikjian and Davis (10) with 5.0 mCi of [5,6-^{3}H]-uracil and tRNA was extracted by the method of Zubay (11) and purified further by DEAE-cellulose column chromatography (12). Distribution of radioactivity in the obtained [^{3}H] tRNA was as follows: about 38% was in the UMP fraction including ψMP and rTMP and 62% in the CMP fraction, detected after separation of alkaline hydrolysate of [^{3}H] tRNA by Dowex 1x2 column chromatography (13); free tritium could not be found.

Hydrolysis of [^{3}H] tRNA was carried out by snake venom phosphodiesterase and 5'nucleotidase for 15 hr at 37°C. Uridine fraction which was isolated from the hydrolysate by Aminex-A7 column chromatography (14) was subjected to cellulose thin layer chromatography to separate ψ and rT from uridine with solvent system containing isobutyric acid, 0.5M NH_4OH and 0.5M EDTA (10/6/0.32, v/v/v).

For the detection of degradation of [^{3}H] tRNA during incubation with the tumor cell supernatant, assay medium was investigated by DEAE-Sephadex A-26 (6) and Dowex 1x2 (13) column chromatography.

RESULTS AND DISCUSSION

The tRNA-ψ synthetase activity in the supernatant of the tumor cells was 4 times higher and that of the tumor-bearing rat liver was 2 times higher than that of the control rat liver after incubation for 30 min (Fig. 1).

In order to prove the ψ-formation in [^{3}H] tRNA, radioactivities of ψ and uridine residues of [^{3}H] tRNA which had been treated for 60 min with the tumor cell supernatant used in the experiment shown in Fig. 2 were compared with those of the untreated intact [^{3}H] tRNA after hydrolysis by snake venom phosphodiesterase and 5'nucleotidase. The ratio of radioactivity of ψ to uridine in the treated tRNA was higher than that in the intact tRNA (0.14 and 0.04, respectively). Furthermore, when all radioactivity of released tritium and newly formed [6-^{3}H]-ψ residue was assumed to be converted from [5,6-^{3}H]-uridine residues and then all radioactivity of these three substances was defined as 100% (although cytidine and rT also were found to be labeled in [^{3}H] tRNA), distribution of radioactivity among released tritium, which was determined by the enzyme assay method separately, ψ and uridine was estimated at 8.7%, 11.7% and 79.6%, respectively, in the treated [^{3}H] tRNA, and 0%, 4.2% and 95.8% in intact [^{3}H] tRNA. Thus, changes of the distribution of

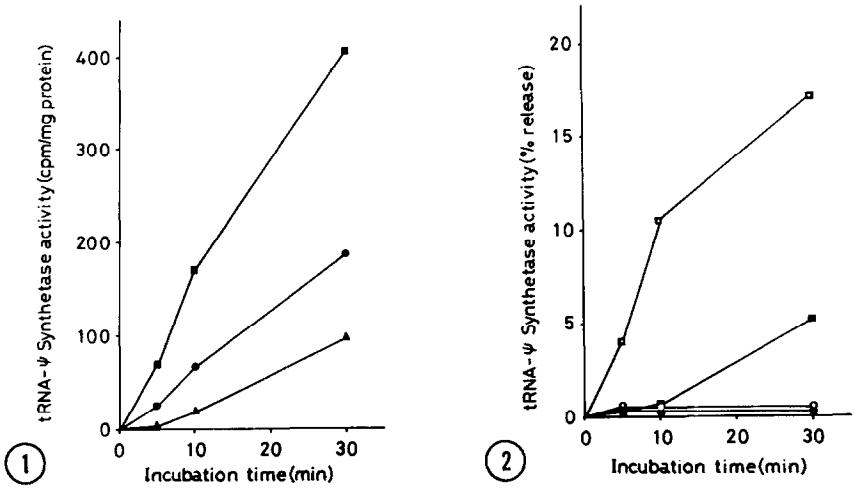

Fig. 1 Tritium-release from [^{3}H] labeled E. coli B tRNA as a function of time at 37°C. Enzyme assay was carried out as described in "Materials and Methods" using 1.38, 1.43 or 1.55 mg protein of the supernatant of Yoshida ascites sarcoma cells (■), the sarcoma-bearing rat liver (●) and normal rat liver (▲), respectively, and activity was expressed by radioactivity (cpm) of released tritium from [^{3}H] tRNA per mg protein.

Fig. 2 Release of tritium from [^{3}H] tRNA, [5,6-^{3}H]-poly U, [5,6-^{3}H]-UTP or [5-^{3}H]-uridine as a function of time by the supernatant of Yoshida ascites sarcoma cells. Extent of release was expressed as % release of tritium in the C-5 position of uracil moiety of each containing substrate. Total radioactivity of tritium located in the C-5 position was obtained by dividing total activity contained in the assay medium by two in the case of poly U and UTP and moreover multiplying it by 0.38 in the case of tRNA (see "Materials and Methods"). Assay details were as presented in "Materials and Methods". However, 5.59×10^5 cpm of [5,6-^{3}H]-poly U (0.32 mμmole as UMP), 5.59×10^5 cpm of [5,6-^{3}H]-UTP (0.036 mμmole) or 7.46×10^5 cpm of [5-^{3}H]-uridine (0.069 mμmole) took the place of [^{3}H] tRNA in 0.2 ml of the assay medium. Amount of enzyme protein was 0.94 mg. tRNA, (□); poly U, (●); UTP, (■); uridine, (○)

radioactivity in the treated [^{3}H] tRNA were increases of both released tritium and ψ of 8.7% and 7.5%, respectively, and a decrease of uridine of 16.2% of total radioactivity. Accordingly, as assumed above, the amount of increase in radioactivity of ψ was almost the same as either that of increase of released tritium or a half of the decrease in radioactivity of uridine. From this estimation, the conversion, on the average, of about 2 uridine residues to ψ residue in [^{3}H] tRNA can be calculated with the assumption of localization of 12 uridine residues in a tRNA molecule (15). Meanwhile, the

new formation of [^{3}H]-ribothymidine from [^{3}H] tRNA could not be detected in the present experimental conditions.

Specific activity of tRNA-ψ synthetase in the tumor cell supernatant measured above was higher than that shown in Fig. 1, probably owing to the process of tumor development, since in our previous work excretion of ψ was seen with a peak of increase in the urine of rats after tumor transplantation (7).

When [^{3}H] tRNA was investigated by DEAE-Sephadex A-26 (6) and Dowex 1x2 (13) column chromatography after incubation for 60 min with the tumor cell supernatant (shown in Fig. 2), about 65% of the total radioactivity of [^{3}H] tRNA was recovered as nucleosides, and about 20% as nucleotides and the remainder was eluted with 1M NaCl from DEAE-Sephadex A-26 column. Therefore, for the purpose of confirmation of detection of ψ-formation at the tRNA-level by this assay, the possible degraded forms of tRNA, [5-^{3}H]-uridine and [5,6-^{3}H]-UTP instead of UMP (which would probably be converted from UTP during the incubation) and other polymer, [5,6-^{3}H]-poly U were used as the substrate for this assay and compared with [^{3}H] tRNA. ψ-formation was firmly observed in tRNA, but not significantly in uridine, UTP and poly U (Fig. 2). In these assay conditions, equal level of radioactivity, hence, a lesser molar amount of each compound in comparison with [^{3}H] tRNA was used and the extent of ^{3}H-release was expressed as % of the total radioactivity of tritium in the C-5 position of uracil moiety of each compound; for example, the molar amount of UTP corresponded to about one thousandth of that of tRNA as uridine residue based on molar percentage of uridine residues in tRNA (15) as well as assumption that 1 A_{260} unit of tRNA is equal to 100 mμmoles of nucleotide residues (16). Therefore, if the tumor cell supernatant could utilize these compounds equally as tRNA, the value of released percentage of tritium from these compounds should be estimated to be much higher than that from [^{3}H] tRNA. In Fig. 2, a release of tritium was seen only in UTP but its released percentage was much lower than that of tRNA.

Although as already mentioned above, [^{14}C]-uracil did not label urinary ψ of Yoshida ascites sarcoma-bearing rats, the existence of ψ-formation from [5-^{3}H]-uridine via [5-^{3}H]-uracil by ψMP synthetase could not be excluded completely because the same assay conditions as those for tRNA-ψ synthetase were used in accordance with the purpose of the experiment. Besides this reason, since ψ-formation from UMP has been suggested (17), the significance of even a slight release of tritium from [5,6-^{3}H]-UTP in this study should not be neglected. Releasing curve of tritium from [^{3}H] tRNA as a function of time seemed to be sigmoid as shown in the figures. We have succeeded in performing further purification and characterization of the enzyme.

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