Transfer Ribonucleic Acid Guanine Transglycosylase Isolated from Rat Liver†

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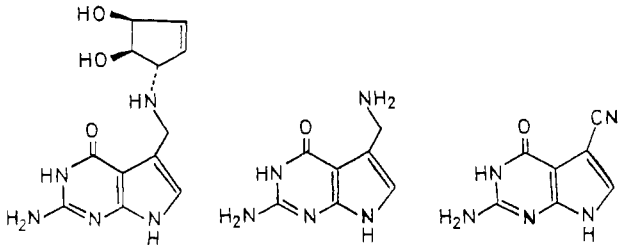
ABSTRACT: Transfer ribonucleic acid (tRNA) guanine transglycosylase (guanine insertion enzyme) was isolated from rat liver and extensively purified. The enzyme catalyzes an exchange of queuine (the base of queuosine, Q) as well as its precursors and guanine for guanine originally located in the first position of the anticodon of "undermodified" $tRNA^{Tyr}$, $tRNA^{His}$, $tRNA^{Asn}$, and $tRNA^{Asp}$ from an *Escherichia coli* mutant or rat ascites hepatoma cells. This is in contrast to the previous observation that *E. coli* tRNA-guanine transglycosylase catalyzes the exchange of queuine precursors, such as 7-(aminoethyl)-7-deazaguanine and 7-cyano-7-deazaguanine, but not of queuine itself [Okada, N., Noguchi, S., Kasai, H., Shindo-Okada, N., Ohgi, T., Goto, T., & Nishimura, S. (1979) *J. Biol. Chem. 254*, 3067–3073]. The K_m value for queuine of the rat liver enzyme is 9.2×10^{-7} M, much lower than the values for the bases of queuosine precursors or guanine. Thus, the actual substrate for tRNA-guanine transglycosylase in queuosine biosynthesis in vivo in rat liver may not be 7-(aminomethyl)-7-deazaguanine, which is thought to be an actual substrate guanine. the *E. coli* system. Queuine or some queuine derivative may be the actual substrate for the tRNA-guanine transglycosylase reaction in the biosynthesis of Q in tRNA of mammalian cells. 6-Thioguanine and 8-azaguanine are also found to be good substrates.

Exchange of free guanine in tRNA by a lysate of rabbit reticulocytes was previously reported by Farkas & Singh (1973), who originally conceived it to be an "insertion" reaction. We demonstrated that this reaction is a specific exchange of guanine for queuine[1] (structure **1**) or for guanine



Structure 1 Structure 2 Structure 3

located in the first position of the anticodon of $tRNA^{Tyr}$, $tRNA^{His}$, $tRNA^{Asn}$, and $tRNA^{Asp}$ (Okada et al., 1976). Namely, the enzyme is a kind of transferase, catalyzing base exchange without cleavage of the phosphodiester linkage in the polynucleotide chain. Although the enzyme from rabbit reticulocytes or Ehrlich ascites tumor cells catalyzes the exchange of queuine of *Escherichia coli* tRNA with guanine (Okada et al., 1976; Itoh et al., 1977), it did not seem to catalyze this exchange in a homologous tRNA–enzyme system (Farkas & Chernoff, 1976; Okada et al., 1978b; McKinnon et al., 1978). It was thought that the enzyme is involved in the biosynthesis of Q in tRNA, rather than in the conversion of Q-containing tRNA to G-containing tRNA. The actual substrate for the guanine insertion enzyme may not be guanine but queuine or its precursor. In the biosynthesis of Q, queuine or its precursor is synthesized without participation of tRNA and then exchanged for guanine in tRNA by the transglycosylase. In fact, we recently demonstrated that the *E. coli* enzyme (tRNA-guanine transglycosylase, previously named tRNA transglycosylase) catalyzes the incorporation of queuine precursors, such as 7-cyano-7-deazaguanine (structure **3**) and 7-(aminomethyl)-7-deazaguanine (structure **2**), into undermodified *E. coli* $tRNA^{Tyr}$ and $tRNA^{Asn}$ that contain normal guanine instead of quenine in the first position of the anticodon (Okada et al., 1979). Queuine was not incorporated into these undermodified tRNAs by *E. coli* tRNA-guanine transglycosylase, and free 7-(aminomethyl)-7-deazaguanine was found in the acid-soluble fraction of *E. coli* cells (Okada et al., 1979). These results indicated that 7-(aminomethyl)-7-deazaguanine is the actual substrate incorporated into tRNA in vivo in *E. coli* and that 7-(aminomethyl)-7-deazaguanine thus incorporated into the polynucleotide chain is successively converted to queuine in the polynucleotide chain.

We have shown that undermodified tRNAs containing guanine instead of queuine specifically appear in a variety of tumor cells (Okada et al., 1978b). These isoaccepting tRNA species can easily be detected by measuring the guanine acceptance of tRNA by using *E. coli* tRNA-guanine transglycosylase (Okada et al., 1978b). To understand how such undermodified tRNA species are formed in tumor tissues or how queuosine is synthesized in mammalian cells, it is necessary to investigate the properties of the tRNA-guanine transglycosylase isolated from these cells. For large-scale isolation of the enzyme from mammalian cells, rat liver was chosen as a source of the enzyme instead of rabbit reticulocytes or Ehrlich ascites tumor cells. In fact, the enzyme was found in rat liver and could be purified extensively by procedures similar to those used for purification of *E. coli* enzyme. The most notable finding on rat liver tRNA-guanine trans-

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[1] Abbreviations used: queuosine or Q, 2-amino-5-[(3*S*,4*R*,5*S*)-4,5-dihydroxycyclopent-1-en-3-ylaminomethyl]-7-(β-D-ribofuranosyl)-pyrrolo[2,3-*d*]pyrimidin-4-one (Ohgi et al., 1979), queuine is the corresponding base; $preQ_0$, 7-cyano-7-deazaguanosine; $preQ_1$, 7-(aminomethyl)-7-deazaguanosine; G-$tRNA^{Tyr}$, $preQ_0$-$tRNA^{Tyr}$, and $preQ_1$-$tRNA^{Tyr}$, *E. coli* "undermodified" $tRNA^{Tyr}$ containing guanosine (G), $preQ_0$, and $preQ_1$ in the first position of the anticodon, respectively; A_{260} unit, that amount of material that has an absorbance of 1.0 at 260 nm when dissolved in 1 mL of water and measured with a 1-cm light path.

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glycosylase is that, unlike the *E. coli* enzyme, it catalyzes the incorporation of queuine much more efficiently than of its insertion of bases of Q precursors. Thus, the actual substrate for tRNA-guanine transglycosylase in the mammalian system in vivo may not be 7-(aminomethyl)-7-deazaguanine but queuine or some queuine derivative.

Materials and Methods

Isolation of tRNA-guanine Transglycosylase from Rat Liver. The following buffer solutions were used for purification of the enzyme (Okada & Nishimura, 1979): buffer A, 10 mM Tris-HCl (pH 7.5)–10 mM $MgCl_2$–1 mM EDTA–0.5 mM dithiothreitol–10% glycerol; buffer B, 10 mM sodium phosphate buffer (pH 6.6)–0.5 mM dithiothreitol–10% glycerol; buffer C, 10 mM Tris-HCl (pH 7.5)–10 mM $MgCl_2$–1 mM EDTA–0.5 mM dithiothreitol–50% glycerol. A mixture of protease inhibitors (pepstatin, chymostatin, and leupeptin, 10 mg of each per L) was added to buffers A and B.

Male albino rats of the Donryu strain (250–300-g body weight) were killed by decapitation. The livers were quickly excised, and 520 g of liver was homogenized with 1300 mL of buffer A. The homogenate was centrifuged at 10000*g* for 30 min, and the supernatant was recentrifuged at 100000*g* for 4 h. The supernatant thus obtained was diluted threefold with buffer A and loaded on a column (4 × 17 cm) of DEAE-cellulose (DE52). The column was washed with 500 mL of buffer A and then eluted with 2 L of a linear gradient of 0–0.4 M NaCl in the same buffer at a flow rate of 100 mL/h. tRNA-guanine transglycosylase was eluted from the column as a single peak at the concentration of 0.04–0.06 M NaCl (fraction II). The fractions containing the transglycosylase were combined and dialyzed against buffer B for 2 h. Then, 0.01 volume of 1 M phosphate buffer (pH 6.6) was added and the preparation (180 mL) was applied to a phosphocellulose (P11) column (2.6 × 12 cm) equilibrated with buffer B. The column was washed with 60 mL of buffer B, and material was eluted with 800 mL of a linear gradient of 0–1 M NaCl in buffer B. Fractions of 10 mL of effluent were collected, and those with activity (fraction no. 21–27, eluted at a concentration of 0.15–0.21 M NaCl) were combined and dialyzed against buffer C for 16 h (fraction III). The purified enzyme was stored at −20 °C.

Determination of Molecular Weight of tRNA-guanine Transglycosylase by Sephadex G-200 Gel Filtration. A column (2 × 83 cm) of Sephadex G-200 was equilibrated with buffer A containing 0.1 M NaCl. The purified enzyme (0.55 mL of fraction III, 9.2 units, 0.17 mg) from the phosphocellulose column was loaded on the column and eluted with buffer A containing 0.1 M NaCl at a flow rate of 15 mL/h. Bovine serum albumin, γ-globulin, and myoglobin were used as marker proteins.

E. coli tRNA^{Tyr} Containing Queuosine, 7-Cyano-7-deazaguanosine, 7-(Aminomethyl)-7-deazaguanosine, or Guanosine in the First Position of the Anticodon. Undermodified *E. coli* tRNA^{Tyr}s containing 7-cyano-7-deazaguanosine and guanosine (preQ$_0$-tRNA^{Tyr} and G-tRNA^{Tyr}) were isolated from cells of a revertant derived from a temperature-sensitive mutant (Noguchi et al., 1978). The tRNA^{Tyr}s were purified by column chromatography on DEAE-Sephadex A-50 and then on BD-cellulose as described previously (Okada & Nishimura, 1979). Isolation of undermodified tRNA^{Tyr} containing 7-(aminomethyl)-7-deazaguanosine (preQ$_1$-tRNA^{Tyr}) from a mutant of *E. coli* (JE10651) was also carried out as described previously (Okada et al., 1979). Normal *E. coli* tRNA$_1^{Tyr}$ containing queuosine was isolated from unfractionated tRNA of *E. coli* B cells by successive column chromatographies on DEAE-Sephadex A-50 and RPC-1 (Nishimura, 1971).

Rat Liver and Rat Ascites Hepatoma tRNAs. Unfractionated tRNAs from rat liver and rat ascites hepatoma AH7974 cells were prepared from the respective tissues by phenol extraction, followed by isopropyl alcohol fractionation (Nishimura, 1971). A minor isoaccepting species of tRNA^{Asp} from rat ascites hepatoma AH7974 cells (rat ascites hepatoma tRNA$_2^{Asp}$) that contains guanosine in place of mannose-containing Q was prepared by RPC-5 column chromatographies at pH 7.5 and 4.5 as described previously (Okada et al., 1978b).

Preparation of [^{14}C]Guanine-Labeled Rat Ascites Hepatoma tRNA. One hundred A_{260} units of rat ascites hepatoma AH7974 tRNA was incubated with 40 μg of *E. coli* tRNA-guanine transglycosylase (fraction IV) and 2 μCi of [^{14}C]-guanine (specific activity 55 Ci/mol) in 70 mM Tris-HCl (pH 7.5)–20 mM $MgCl_2$ at 37 °C for 1 h in a total volume of 6 mL. Under these conditions, undermodified G-containing tRNA was completely labeled with [^{14}C]guanine. After incubation, the reaction mixture was loaded on a small column (0.4 × 4 cm) of DEAE-cellulose (DE52). The column was washed with 0.02 M Tris-HCl (pH 7.5)–0.01 M $MgCl_2$–0.2 M NaCl, and tRNA was eluted with 0.02 M Tris-HCl (pH 7.5)–0.01 M $MgCl_2$–1 M NaCl. The [^{14}C]guanine-labeled tRNA was precipitated by addition of 2.5 volumes of ethanol and dissolved in a small volume of water. One A_{260} unit of tRNA contained 1800 cpm of [^{14}C]guanine.

Identification of Queuine and 7-(Aminomethyl)-7-deazaguanine Incorporated into Rat Ascites Hepatoma tRNA$_2^{Asp}$ by tRNA-guanine Transglycosylase. The tRNA$_2^{Asp}$-enriched fraction prepared as described above (0.5 A_{260} unit, ~25% pure) was incubated with 6 units of rat liver tRNA-guanine transglycosylase (0.35 mL of fraction III, containing 0.1 mg of protein) and 0.8 A_{260} unit of queuine or 7-(aminomethyl)-7-deazaguanine at 37 °C for 2 h in a total volume of 1 mL of 70 mM Tris-HCl buffer (pH 7.5) containing 20 mM $MgCl_2$. The reaction mixture was treated with an equal volume of phenol, and the aqueous layer was loaded on a column (0.5 × 4 cm) of DEAE-cellulose (DE52). The column was washed with 0.02 M Tris-HCl (pH 7.5)–0.01 M $MgCl_2$–0.2 M NaCl, and the tRNA was eluted from the column with 0.02 M Tris-HCl (pH 7.5)–0.01 M $MgCl_2$–1 M NaCl. The tRNA eluted was precipitated by the addition of 2.5 volumes of ethanol and dissolved in a small volume of water. Modified nucleosides in the tRNA were analyzed by postlabeling using T_4 polynucleotide kinase and [γ-^{32}P]ATP as described by Silberklang et al. (1977) with a slight modification as reported previously (Okada et al., 1979).

Queuine, 7-Cyano-7-deazaguanine, 7-(Aminomethyl)-7-deazaguanine, and Other 7-Deazaguanine Derivatives. Since queuine and its precursors could not be obtained from naturally occurring nucleosides owing to the resistance of the N–C glycosyl bond to acid treatment, they were synthesized chemically. The procedure used for the synthesis of queuine and 7-(aminomethyl)-7-deazaguanine was described previously (Okada et al., 1979). The synthesis of 7-cyano-7-deazaguanine was described previously (Ohgi et al., 1978). The improved procedure will be published in detail elsewhere. Other 7-deazaguanine derivatives, such as 7-methyl-7-deazaguanine, 2-thio-7-methyl-7-deazaguanine, and 2-(methylthio)-7-methyl-7-deazaguanine, were also synthesized chemically (Ohgi et al., 1977). Authentic 7-deazaguanine was a gift from Dr. R. E. Bowman of Parke-Davis & Co., Pontypool, MT.

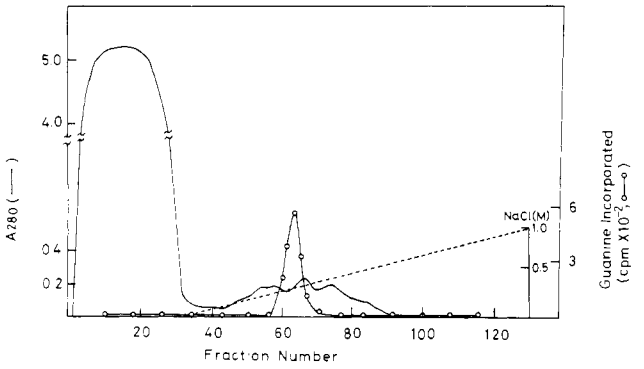

FIGURE 1: Purification of rat liver tRNA-guanine transglycosylase by phosphocellulose column chromatography. The experimental procedure is described under Materials and Methods. For assay of tRNA-guanine transglycosylase activity, 0.4 mL of each fraction was incubated with 5 A_{260} units of yeast unfractionated tRNA and 0.25 μCi of [8-^{14}C]guanine (specific activity 55 Ci/mol) in a total volume of 1 mL of 70 mM Tris-HCl (pH 7.5)–20 mM $MgCl_2$ at 37 °C for 1.5 h. After incubation, tRNA was precipitated by addition of an equal volume of cold 30% (w/v) trichloroacetic acid. The precipitated tRNA was collected on a glass fiber disk, and its radioactivity was counted in a liquid scintillation counter. One unit of enzyme was defined as the amount catalyzing the incorporation of 20 pmol of guanine into yeast tRNA under the conditions used. The enzymatic reaction proceeded linearly up to 7 units of the enzyme.

Other Materials. Pepstatin, chymostatin, and leupeptin were supplied under the Research Resources Program for Cancer Research of the Ministry of Education, Science and Culture, Japan. Unfractionated yeast tRNA and T_4 polynucleotide kinase were obtained from Boehringer. Pure *E. coli* $tRNA^{His}$, $tRNA^{Asn}$, $tRNA^{Asp}$, $tRNA^{fMet}$, and $tRNA^{Phe}$ were prepared by successive column chromatographies on DEAE-Sephadex A-50 at pH 7.5 and 4.0 and on BD-cellulose and RPC-5 as described previously (Nishimura, 1971). Unfractionated rabbit liver tRNA was kindly provided from Dr. H. J. Gross of Max-Planck-Institut für Biochemie, München. *E. coli* tRNA-guanine transglycosylase (fraction IV) used for the preparation of [^{14}C]guanine-labeled rat ascites hepatoma tRNA was prepared as described by Okada & Nishimura (1979). Myoglobin and γ-globulin were purchased from Schwarz/Mann, and bovine serum albumin was from Armour Pharmaceutical Co. [γ-^{32}P]ATP was prepared as described by Glynn & Chappell (1964). [8-^{14}C]Guanine (specific activity 55 Ci/mol) was obtained from the Radiochemical Centre, Amersham. RNase T_2 was a product of Sankyo Co., Tokyo, Japan. Nuclease P_1 was a gift from Dr. A. Kuninaka of the Research Laboratory of Yamasa Shoyu Co., Ltd., Choshi, Japan. Sephadex G-200 and blue dextran were obtained from Pharmacia Fine Chemicals, and phosphocellulose (P11) and DEAE-cellulose (DE52) were from Whatman. Thin-layer glass plates coated with Avicel SF cellulose were purchased from Funakoshi Pharmaceutical Co., Tokyo. Guanine, hypoxanthine, xanthine, 8-azaguanine, 6-mercaptopurine, and 7-methylguanine were obtained from Sigma Chemical Co. 2-Thiohypoxanthine was kindly provided by Dr. M. Ikehara of Osaka University, Osaka, Japan. 6-Thioguanine was a gift from Dr. M. Maeda of this institute.

Results

Purification of Rat Liver tRNA-guanine Transglycosylase. On DEAE-cellulose (DE52) column chromatography, rat liver tRNA transglycosylase was eluted as a single peak at a concentration of ~0.05 M NaCl. The dialzyed eluate (fraction II) was then fractionated by phosphocellulose (P11) column chromatography. On elution with a linear gradient of NaCl, most of the protein was not retained on the phosphocellulose column and the enzyme was then eluted with ~0.18 M NaCl, as shown in Figure 1. Both purification procedures were very effective, achieving 250-fold purification of the enzyme from the 100000*g* supernatant. Table I shows the yield, specific activity, and extent of purification of the enzyme. The rat liver tRNA-guanine transglycosylase thus obtained was stored in 50% glycerol at −20 °C. No loss of activity was observed during storage for 10 months. Addition of protease inhibitor to the buffers used for purification was essential for isolation of the enzyme. In the absence of protease inhibitors, little enzymatic activity was detectable after DEAE-cellulose column chromatography.

Table I: Summary of Purification of Rat Liver tRNA-guanine Transglycosylase

fraction	total protein (mg)	total units (units)	sp act. (unit/mg)	vol (mL)
(I) 100000*g* supernatant	7500	1650	0.22	900
(II) DEAE-cellulose column chromatography	460	1100	2.4	180
(III) phosphocellulose column chromatography	17	930	54.7	56

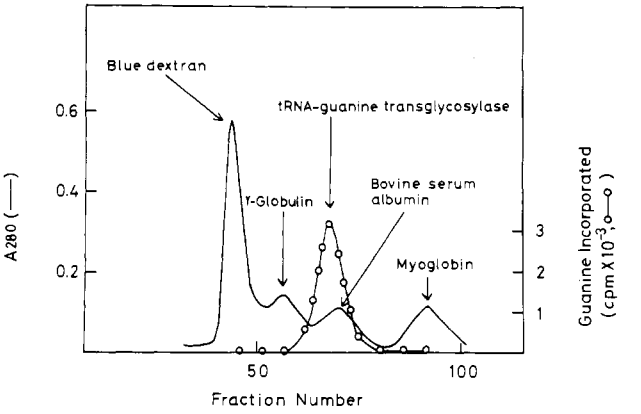

FIGURE 2: Determination of molecular weight of rat liver tRNA-guanine transglycosylase by Sephadex G-200 gel filtration. The experimental procedure is described under Materials and Methods. Enzymatic activity was assayed as described in Figure 1, except that 1 mL of each fraction in 3 mL of total reaction mixture was used.

Properties of Rat Liver tRNA-guanine Transglycosylase. For determination of the molecular weight of the tRNA-guanine transglycosylase, the enzyme and reference proteins were chromatographed on a Sephadex G-200 column as shown in Figure 2. From the results, the molecular weight of the enzyme was calculated to be 8.0×10^4. The pH optimum of the enzyme was found to be 7.3. The pH optimum curve was broad; at pH 7.0 and 7.5 the enzyme showed more than 80% of the activity observed at pH 7.3. Unlike *E. coli* tRNA-guanine transglycosylase, the rat liver enzyme was not stimulated by magnesium ion.

Acceptor Abilities of Various tRNAs for Guanine Inserted by Rat Liver tRNA-guanine Transglycosylase. We have previously shown that rabbit reticulocyte tRNA-guanine transglycosylase catalyzes the incorporation of guanine into *E. coli* tRNA, expelling the Q base (queuine) originally located in the first position of the anticodon (Okada et al., 1976). To find out whether rat liver tRNA transglycosylase has similar specificity to the rabbit reticulocyte enzyme, we tested the abilities of various purified *E. coli* tRNAs and of unfractionated yeast, rat liver, and rat ascites hepatoma tRNAs to accept guanine. As shown in Figure 3, as in the case of rabbit reticulocyte enzyme (Okada et al., 1976), $tRNA^{Asp}$ was found to be the best acceptor among the normal *E. coli* tRNAs

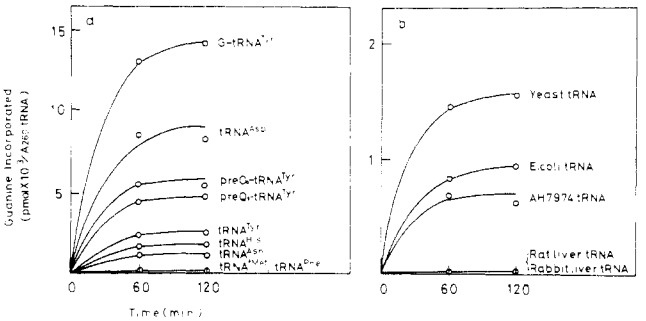

FIGURE 3: Incorporation of guanine into various tRNAs by rat liver tRNA-guanine transglycosylase. 3 A_{260} units each of *E. coli* $tRNA_1^{Tyr}$, $tRNA^{His}$, $tRNA^{Asn}$, $tRNA^{Asp}$, $tRNA^{fMet}$, $tRNA^{Phe}$, and undermodified $tRNA^{Tyr}$s containing guanosine, 7-cyano-7-deazaguanosine, or 7-(aminomethyl)-7-deazaguanosine in place of queuosine (G-$tRNA^{Tyr}$, $preQ_0$-$tRNA^{Tyr}$, and $preQ_1$-$tRNA^{Tyr}$) were separately incubated with 11.3 units of the enzyme (fraction III, 0.7 mL) and 0.5 μCi of [^{14}C]guanine (specific activity 55 Ci/mol) in 7 mL of 57 mM Tris-HCl (pH 7.5)–17 mM $MgCl_2$ at 37 °C. After various times of incubation, 2.2 mL of the reaction mixture was taken for measuring acid-insoluble radioactivity. In case of unfractionated tRNA from *E. coli*, yeast, rat liver, and rat ascites hepatoma AH7974, 15 A_{260} units of tRNA was added to the reaction mixture.

containing queuine tested ($tRNA^{Tyr}$, $tRNA^{His}$, $tRNA^{Asn}$, and $tRNA^{Asp}$). It is noteworthy that *E. coli* undermodified $tRNA^{Tyr}$s containing guanine, 7-cyano-7-deazaguanine, or 7-(aminomethyl)-7-deazaguanine instead of queuine (G-$tRNA^{Tyr}$, $preQ_0$-$tRNA^{Tyr}$, and $preQ_1$-$tRNA^{Tyr}$) were much better acceptors than normal $tRNA^{Tyr}$. G-$tRNA^{Tyr}$ showed the highest acceptor activity, while normal *E. coli* $tRNA^{fMet}$ and $tRNA^{Phe}$ were completely ineffective. As predictable from the previous results on rabbit reticulocytes (Farkas & Chernoff, 1976) and on the Ehrlich ascites tumor enzyme (Okada et al., 1978b), the rat liver enzyme was unable to insert guanine into queuine-containing tRNA from a homologous source; unfractionated rat liver tRNA was not utilized at all as an acceptor. Rat ascites hepatoma AH7974 tRNA was a fairly good acceptor of guanine. This is because tumor tRNA contains partially undermodified tRNA containing guanine instead of queuine or its hexose-containing derivatives (Okada et al., 1978b).

Guanine and Queuine Derivatives As Acceptors in the tRNA-guanine Transglycosylase Reaction. Since radioactive queuine derivatives are not available, the following experiments were carried out to examine whether these analogues can be utilized in place of guanine in the tRNA-guanine transglycosylase reaction. The tRNA-guanine transglycosylase reaction was performed with [^{14}C]guanine by using unfractionated yeast tRNA as acceptor. After incorporation of [^{14}C]guanine into tRNA had reached a plateau, a 50-fold excess of base analogue was added to each incubation mixture. Radioactive guanine incorporated into tRNA should be released if the analogue is incorporated into tRNA during later incubation. As shown in Figure 4, queuine caused a considerable decrease of radioactivity in tRNA, indicating that it is efficiently incorporated into tRNA. 7-(Aminomethyl)-7-deazaguanine, which is the natural precursor of queuine in *E. coli* (Okada et al., 1979), was also incorporated into tRNA but less efficiently than guanine. Other guanine or queuine analogues, such as 7-deazaguanine, 7-methyl-7-deazaguanine, and 7-methylguanine, were not utilized at all as donors. Table II also shows the amount of [^{14}C]guanine released from [^{14}C]guanine-labeled ascites hepatoma AH7974 tRNA by guanine and queuine analogues. In this experiment, rat ascites hepatoma tRNA labeled with [^{14}C]guanine by using *E. coli* tRNA-guanine transglycosylase and the labeled tRNA was

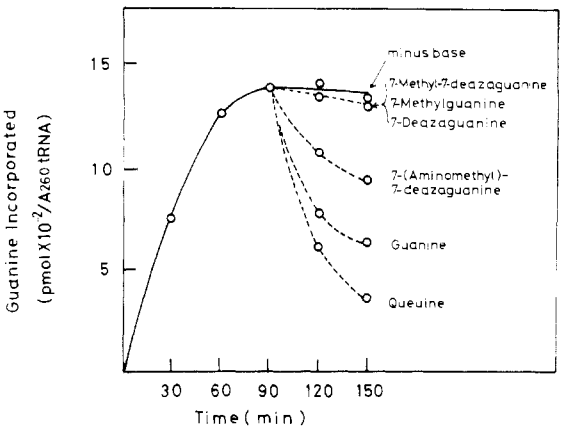

FIGURE 4: Release of [^{14}C]guanine from [^{14}C]guanine-labeled yeast tRNA by queuine and its analogues. Unfractionated yeast tRNA (3 A_{260} units) was preincubated with 0.5 μCi of [^{14}C]guanine (3 nmol) and 45 units of rat liver tRNA-guanine transglycosylase (2.6 mL of fraction III) in the presence of 10 A_{260} units of carrier rabbit liver tRNA in a final volume of 12 mL of 52 mM Tris-HCl buffer (pH 7.5) containing 10 mM $MgCl_2$. After incubation for 90 min, 11 nmol of the specified bases was added to 0.8 mL of reaction mixture and the mixture was incubated for 60 min. Acid-insoluble radioactivity was measured as described for Figure 3.

Table II: Release of [^{14}C]Guanine from [^{14}C]Guanine-Labeled Rat Ascites Hepatoma AH7974 tRNA by Rat Liver tRNA-guanine Transglycosylase in the Presence of Guanine, Queuine, and Their Analogues[a]

guanine derivatives	guanine released (pmol × 10^{-3})
nonradioactive guanine	4.95
queuine	5.83
7-cyano-7-deazaguanine	5.47
7-(aminomethyl)-7-deazaguanine	2.66
8-azaguanine	5.67
6-thioguanine	4.02
2-thiohypoxanthine	1.81
7-deazaguanine	0.65
7-methyl-7-deazaguanine	0.28
2-thio-7-methyl-7-deazahypoxanthine	0.69
2-(methylthio)-7-methyl-7-deazahypoxanthine	0.79
6-mercaptopurine	0.56
7-methylguanine	0.10
xanthine	0.10
hypoxanthine	0.20
minus base	0.10

[a] The reaction mixture contained 3.5 A_{260} units of [^{14}C]-guanine-labeled tRNA (950 cpm, 1.1×10^{-2} pmol of [^{14}C]-guanine), 7.3 units of rat liver tRNA-guanine transglycosylase (0.2 mL of fraction III), 4 A_{260} units of carrier rabbit liver tRNA, and 13 nmol of the specified base in a total volume of 1 mL of 40 mM Tris-HCl (pH 7.5)–10 mM $MgCl_2$. After incubation for 2 h at 37 °C, acid-insoluble radioactivity in the reaction mixture was measured.

isolated by phenol extraction and DEAE-cellulose column chromatography. This [^{14}C]guanine-labeled ascites hepatoma tRNA was then incubated with the bases and rat liver tRNA-guanine transglycosylase, and the release of [^{14}C]-guanine from the tRNA was measured as described in the legend to Table II. Table II shows that addition of 7-cyano-7-deazaguanine, which seems to be another precursor of queuine in the *E. coli* system, caused a marked reduction of the radioactivity in acid-precipitable tRNA, indicating that it was also incorporated into undermodified tRNA, with concomitant release of [^{14}C]guanine from the tRNA. It should be noted that artificial guanine analogues, such as 8-aza-

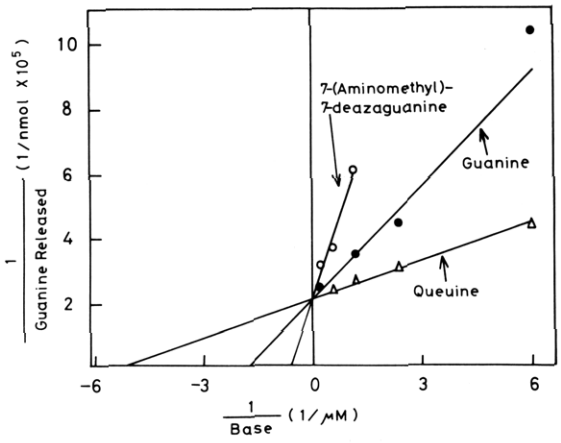

FIGURE 5: Double-reciprocal plot of initial velocity of release of [^{14}C]guanine from [^{14}C]guanine-labeled rat ascites hepatoma AH7974 tRNA by guanine, gueuine, and 7-(aminomethyl)-7-deazaguanine vs. their concentration. Incubation conditions were as described for Table II except that the specified amounts of bases were added and the incubation was carried out for 25 min.

guanine and 6-thioguanine, were also incorporated into tRNA. 2-Thio-7-methyl-7-deazahypoxanthine, 2-(methylthio)-7-methyl-7-deazahypoxanthine, 6-mercaptopurine, hypoxanthine, and xanthine were inactive, although 2-thiohypoxanthine was slightly active.

Kinetics of Incorporation of Guanine, Queuine, and 7-(Aminomethyl)-7-deazaguanine. The kinetic parameters of the rat liver tRNA-guanine transglycosylase reaction with guanine, queuine, or queuine analogues were determined by measuring the release of [^{14}C]guanine from prelabeled rat ascites hepatoma tRNA, as shown in Figure 5. From these data, the K_m values of queuine, 7-(aminomethyl)-7-deazaguanine, and guanine were determined to be 2.9×10^{-7}, 2.1×10^{-6}, and 8.3×10^{-7} M, respectively, while the V_{max} values for these bases are the same. These data clearly show that the affinity of the rat liver tRNA-guanine transglycosylase for queuine is much greater than that for 7-(aminomethyl)-7-deazaguanine and guanine.

Detection of Queuine and 7-(Aminomethyl)-7-deazaguanine Incorporated into tRNA As the Corresponding Nucleoside 5′-Monophosphate. To show that queuine and 7-(aminomethyl)-7-deazaguanine are in fact incorporated into tRNA, we incubated undermodified tRNA^{Asp} isolated from rat ascites hepatoma AH7974 tRNA with either queuine or 7-(aminomethyl)-7-deazaguanine in the presence of rat liver tRNA-guanine transglycosylase and then reisolated it by phenol extraction, followed by DEAE-cellulose column chromatography. The tRNA thus isolated was hydrolyzed completely by RNase T_2, and the mixture of nucleoside 3′-monophosphates formed was phosphorylated to obtain ^{32}P-labeled nucleoside 3′,5′-diphosphates by the postlabeling technique using [γ-^{32}P]ATP and T_4 polynucleotide kinase. The nucleoside 3′,5′-diphosphates were converted to nucleoside 5′-monophosphates by nuclease P_1 digestion and analyzed by two-dimensional thin-layer chromatography. As shown in Figure 6, queuosine 5′-monophosphate and 7-(aminomethyl)-7-deazaguanosine 5′-monophosphate were clearly detected in a digest of undermodified tRNA^{Asp} that had been incubated with queuine or 7-(aminomethyl)-7-deazaguanine in the presence of the enzyme but not in a digest of undermodified tRNA^{Asp} that had been incubated without the bases. This experiment clearly demonstrates that these bases are actually incorporated into the polynucleotide chain of the tRNA, because T_4 polynucleotide kinase can phosphorylate nucleoside 3′-monophosphate but not bases.

tRNA-guanine Transglycosylase from Rat Morris Hepatoma 7316A Cells. A tRNA-guanine transglycosylase was also isolated from rat Morris hepatoma 7316A cells (5.2 g of the wet cells) by small-scale fractionation on smaller columns of DEAE-cellulose (0.7 × 17 cm) and phosphocellulose (0.3 × 17 cm). A parallel experiment was carried out to isolate the enzyme from rat liver on the same scale to compare the specific activities of the enzymes from rat liver and rat ascites hepatoma. About the same amounts of enzyme (total units per total protein in the 100000*g* supernatant) were obtained from the ascites hepatoma cells and from rat liver by phosphocellulose column chromatography. The specificity of the tRNA-guanine transglycosylase isolated from rat ascites hepatoma cells was the same as that of the rat liver enzyme with respect to utilization of tRNA. Namely, the ascites hepatoma enzyme was able to insert [^{14}C]guanine into ascites hepatoma tRNA but not into normal rat liver tRNA. The enzyme was also able to insert queuine, 7-(aminomethyl)-7-deazaguanine, and 7-cyano-7-deazaguanine into rat ascites hepatoma tRNA with the same efficiency as the rat liver enzyme (data not shown).

Discussion

There are several unique differences in the properties of tRNA-guanine transglycosylases of *E. coli* and rat liver. The molecular weight of rat liver tRNA-guanine transglycosylase was 8.0×10^4, whereas that of the *E. coli* enzyme was much less, being 4.6×10^4. The most notable difference between the two enzymes is their specificity for substrates. Queuine was the best substrate for the rat liver enzyme, whereas it was not used by the *E. coli* enzyme (Okada et al., 1979). 7-(Aminomethyl)-7-deazaguanine was inserted into undermodified tRNA most efficiently by the *E. coli* enzyme, whereas queuine was inserted most efficiently by the rat liver enzyme

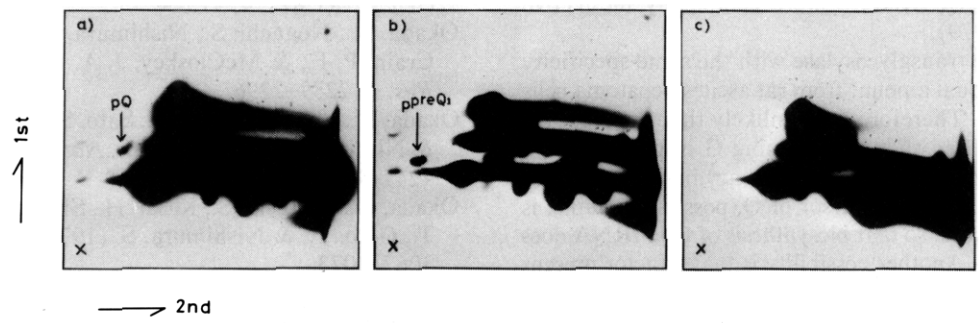

FIGURE 6: Detection of queuosine 5′-monophosphate (pQ) and 7-(aminomethyl)-7-deazaguanine 5′-monophosphate (p-preQ$_1$) in a digest of rat ascites hepatoma AH7974 tRNA$_2^{Asp}$ that had been incubated with queuine or 7-(aminomethyl)-7-deazagaunine by the postlabeling technique. Radioautograms of the digest of (a) ascites hepatoma AH7974 tRNA$_2^{Asp}$ incubated with queuine and rat liver tRNA-guanine transglycosylase, (b) ascites hepatoma AH7974 tRNA$_2^{Asp}$ incubated with 7-(aminomethyl)-7-deazaguanine and the enzyme, and (c) ascites hepatoma tRNA$_2^{Asp}$ incubated with the enzyme in the absence of the base.

(Okada et al., 1979). In *E. coli*, 7-(aminomethyl)-7-deazaguanine seems to be the actual substrate for biosynthesis of queuosine in tRNA in vivo, because (1) it was found in methyl-deficient tRNA or tRNA from a mutant selected for its deficiency of modified nucleosides (Okada et al., 1978a), (2) it was found as a free base in an acid extract of *E. coli* cells (Okada et al., 1979), and (3) a mutant of *E. coli* that lacks tRNA-guanine transglycosylase accumulated it in large quantity in the cells (S. Noguchi, H. Kasai, and S. Nishimura, unpublished experiments). So far attempts to isolate either free queuine or 7-(aminomethyl)-7-deazaguanine from an acid extract of rat liver cells have been unsuccessful. From the values for the kinetic parameters for queuine and 7-(aminomethyl)-7-deazaguanine in the rat liver tRNA-guanine transglycosylase reaction, it seems that the biosynthetic pathway of queuosine in tRNA of rat liver is very different from that in *E. coli*. Namely, 7-(aminomethyl)-7-deazaguanine is not a substrate in the in vivo reaction in mammalian cells. Further studies are needed to clarify whether queuine is the actual substrate of rat liver tRNA-guanine transglycosylase in vivo in the biosynthesis of queuosine in tRNA.

It was clearly shown that the purified tRNA-guanine transglycosylase from rat liver is able to expel queuine from Q-containing *E. coli* tRNAs by inserting guanine but not from Q-containing homologous tRNA, such as normal rat liver tRNA. Similar observations have been made on the enzymes from other eucaryotic cells, such as rabbit reticulocytes (Okada et al., 1976; Farkas & Chernoff, 1976), Ehrlich ascites tumor (Itoh et al., 1977; Okada et al., 1978b), and *Drosophila* cells (McKinnon et al., 1978). The enzyme seems to recognize the U-G(Q)-U structure of the anticodon that is common to tRNAs for tyrosine, histidine, asparagine, and aspartic acid. However, it is evident that some other region of the tRNA must also be involved in the tRNA–enzyme interaction, because heterologous tRNA is only utilized for expulsion of queuine, although heterologous and homologous tRNAs have the same anticodon structure. It is also possible that the attachment of mannose or galactose in queuosine of tRNA (Kasai et al., 1976; Okada et al., 1977) prevents the release of queuine from tRNA by the tRNA-guanine transglycosylase reaction.

The efficiency of incorporation of guanine is greatly influenced by the base located in the first position of the anticodon of tRNA. Of the various *E. coli* $tRNA^{Tyr}$s tested, G-$tRNA^{Tyr}$ was the best acceptor molecule, followed by $preQ_0$-$tRNA^{Tyr}$ or $preQ_1$-$tRNA^{Tyr}$, while $tRNA^{Tyr}$ that contains normal queuosine was the poorest acceptor (Figure 3). On the other hand, with *E. coli* tRNA-guanine transglycosylase, G-$tRNA^{Tyr}$ and $preQ_0$-$tRNA^{Tyr}$ were almost equally good acceptors, while $tRNA^{Tyr}$ and $preQ_1$-$tRNA^{Tyr}$ were completely ineffective (Okada et al., 1979).

tRNA-guanine transglycosylase with the same specificity was obtained in equal amount from rat ascites hepatoma cells as from rat liver. Therefore, it is unlikely that the presence of the undermodified tRNA containing G in place of Q in tumor tissues is due to reduction of the enzyme in tumor cells. But it is possible that a precursor of Q, possibly queuine, is limited in tumor cells, so that biosynthesis of Q in tRNA does not proceed fully. Another possibility is that a factor present in tumor cells inhibits the transglycosylase reaction. These possibilities require investigation.

It is interesting that the artificial guanine analogues 8-azaguanine and 6-thioguanine that act as anticancer reagents were efficiently incorporated into rat ascites hepatoma tRNA (Table II). Farkas & Singh (1973) previously reported a slight incorporation of 8-azaguanine into a rabbit reticulocyte system. It would be interesting to find out whether these bases are also inserted into the anticodon of tRNA in vivo.

After completion of this work, Howes & Farkas (1978) reported the isolation and purification of tRNA-guanine transglycosylase from rabbit erythrocytes. The molecular weight of the rabbit erythrocyte enzyme was 104 000, which is more than that of the rat liver enzyme reported here. Other properties of the rabbit erythrocyte enzyme, namely, its behavior on phosphocellulose column chromatography and its K_m value for guanine, are similar to those of the rat liver enzyme.

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