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Specific replacement of Q base in the anticodon of tRNA by guanine catalyzed by a cell-free extract of rabbit reticulocytes

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ABSTRACT

Guanylation of tRNA by a lysate of rabbit reticulocytes was reported previously by Farkas and Singh. This reaction was investigated further using 18 purified *E. coli* tRNAs as acceptors. Results showed that only $tRNA^{Tyr}$, $tRNA^{His}$, $tRNA^{Asn}$ and $tRNA^{Asp}$ which contain the modified nucleoside Q in the anticodon acted as acceptors. Analysis of the nucleotide sequences in the guanylated tRNA showed that guanine specifically replaced Q base in these tRNAs.

INTRODUCTION

Farkas and his coworkers (1-3) previously reported guanylation of tRNA in rabbit reticulocytes. Guanylation takes place without transcription of tRNA, and the guanine is incorporated into a normal internal position. It seems to be incorporated into a specific location, because after digestion of the tRNA with RNase T_1, incorporated radioactive guanine was recovered as a single oligonucleotide. Farkas and coworkers suggested that the guanine is incorporated into an isoaccepting $tRNA^{His}$. However, this conclusion seems not unambiguous because their assignments of guanylated tRNA species were made only by comparison of the profile of guanylated unfractionated tRNA with that of amino acid acceptor activity on column chromatography.

We studied this unique guanylation reaction further to identify the exact position of guanylation, and the guanylated species of tRNA. Contrary to the report of Farkas and Singh (3), we found that *E. coli* tRNA as well as yeast tRNA are good acceptors for guanylation. Among 18 purified tRNAs tested, only four tRNAs, i.e. $tRNA^{Tyr}$, $tRNA^{His}$, $tRNA^{Asn}$ and $tRNA^{Asp}$ acted as acceptors for guanylation. These four tRNAs all contain the modified nucleotide Q in the anticodon, so our results suggested that guanine specifically replaces Q base in these tRNAs. This was confirmed by nucleotide sequence analysis of guanylated *E. coli* $tRNA^{Asp}$ and $tRNA^{His}$. Thus

guanylation of tRNA is a new type of post-transcriptional modification of nucleic acid, the replacement of a particular modified base in a specific location by normal guanine.

MATERIALS AND METHODS

Isolation of partially purified enzyme for the guanylation reaction. A lysate of rabbit reticulocytes was prepared as described by Farkas and Singh (3). It was dialyzed against 0.01 M Tris-HCl (pH 7.5)-0.01 M $MgCl_2$-0.001 M EDTA-0.006 M β-mercaptoethanol-10% glycerol [buffer A] for 10 hrs and then centrifuged. The supernatant (60 ml) was applied to a column (1 x 3 cm) of DEAE-cellulose (DE-52) previously equilibrated with buffer A, The colomn was washed with buffer A, until hemoglobin had been eluted completely, and then with buffer A containing 0.1 M NaCl to elute the guanylating enzyme. The enzyme was precipitated from the eluate by 85% saturation with ammonium sulfate. The precipitate was dissolved in a small volume of buffer A, and dialyzed against buffer A for 6 hrs.

Assay of guanylating enzyme. The procedure described by Farkas and Singh (3) was slightly modified as follows: The reaction mixture contained 1 ml of lysate, 75 μmoles of Tris-HCl (pH 7.5), 15 μmoles of $MgCl_2$, 3~17 A_{260} units of tRNA as specified and 1 μCi of 8-^{14}C-guanine (specific activity, 55 Ci/mole) or 2-^{14}C-guanine (specific activity, 20 Ci/mole) in a final volume of 1.5 ml. The mixture was incubated at 37° as specified and then 0.4 ml of the mixture was applied to a column (0.3 x 1 cm) of DEAE-cellulose (DE-52). The column was washed with 5 ml of 0.02 M Tris-HCl (pH 7.5)-0.01 M $MgCl_2$-0.2 M NaCl. Then tRNA was eluted with 1 ml of 0.02 M Tris-HCl (pH 7.5)-0.01 M $MgCl_2$-1 M NaCl. tRNA was precipitated by addition of 1/10 volume of 100% (w/v) trichloroacetic acid. The precipitated tRNA was collected on a glass fiber disc, and its radioactivity was counted in a liquid scintillation counter.

Separation and nucleotide sequence analysis of guanylated oligonucleotide. For guanylation, 3 A_{260} units of *E. coli* $tRNA^{His}$ or $tRNA^{Asp}$ were incubated with 1 mg of purified guanylating enzyme for 2 hrs at 37° in a total volume of 1.5 ml. In the case of ^{32}P-labeled *E. coli* $tRNA^{His}$, 5000 cpm of the tRNA were incubated with 10 mg of the enzyme in the presence of 0.5 A_{260} unit of cold *E. coli* $tRNA_1^{Ser}$. After incubation for 2 hrs, the tRNA was recovered by DEAE-cellulose column chromatography and digested with 2 units of RNase T_1 in 0.1 ml of 0.02 M Tris-HCl buffer (pH 7.5). The digest was fractionated

by two-dimensional PEI-cellulose chromatography as described by Mirzabekov and Griffin (4). The radioactive spot on the PEI-cellulose sheet was detected by radioautography. For sequence determination of oligonucleotide containing ^{14}C-guanine, the radioactive spot was eluted with 2 M triethylamine bicarbonate (pH 7.6), and eluate was evaporated to dryness. The radioactive oligonucleotide was mixed with 0.2 A_{260} unit of RNase T_1 digest of *E. coli* unfractionated cold tRNA, and digested with spleen phosphodiesterase (equivalent to hydrolyze 0.1 μmole of p-nitrophenol for 20 min) in 0.01 ml of 0.01 M ammonium acetate buffer. Samples taken at different times (0, 5, 10, 20, 30 and 40 min) after the start of incubation were applied to PEI-cellulose and fractionated by two-dimensional chromatography. The nucleotide sequence of the tetranucleotides containing ^{14}C-guanine at the 3'-end was deduced from the change in mobility of the shortened oligonucleotide according to the principle described by Barrell (5).

Materials. Unlabeled amino acid-specific tRNAs were isolated from cells of *E. coli* B, using combinations of several column chromatographies such as DEAE-Sephadex A-50, benzoylated DEAE-cellulose and RPC-5 column chromatography at different pH values. Details of the purifications for some of these tRNAs were described previously (6,7). The purities of the final preparations were between 70 and 95%. For isolation of ^{32}P-labeled *E. coli* $tRNA^{His}$, ^{32}P-labeled unfractionated tRNA (specific activity, 5 x 10^7 cpm/A_{260}) was first fractionated using acetylated DBAE-cellulose as described by McCutchan *et al.* (8). The tRNAs containing Q eluted in the last fraction were precipitated with ethanol, and further fractionated by two-dimensional polyacrylamide gel electrophoresis as reported by Ikemura and Dahlberg (9). In this way $tRNA^{His}$ was isolated as a pure species. Yeast unfractionated tRNA was prepared from baker's yeast as described previously (10). 8-^{14}C-Guanine (specific activity, 55 Ci/mole) and 2-^{14}C-guanine (specific activity, 20 Ci/mole) were obtained from the Radiochemical Centre, Amersham, England and the CEA-IRE-Sorin, France, respectively. Acetylated DBAE-cellulose was kindly provided by Dr. Söll. RNase T_1 and T_2 were products of Sankyo Co. Ltd., Tokyo. Spleen phosphodiesterase was obtained from Worthington Biochemicals, and purified further by DEAE-Sephadex A-50 column chromatography as described by Bernardi and Bernardi (11).

RESULTS

Table I showed the incorporation of 8-^{14}C-guanine or 2-^{14}C-guanine into

Table I. Incorporation of 8-^{14}C-guanine or 2-^{14}C-guanine into unfractionated *E. coli* and yeast tRNAs by the lysate of rabbit reticulocytes.

^{14}C-guanine added	tRNA added	^{14}C-guanine incorporated (pmole/0.5 ml of the reaction mixture)
8-^{14}C-guanine	yeast tRNA	8.6
" "	*E. coli* tRNA	8.0
" "	minus tRNA	1.4
2-^{14}C-guanine	yeast tRNA	11.0
" "	*E. coli* tRNA	10.0
" "	minus tRNA	1.8

17 A_{260} units of tRNA were added per 1.5 ml of reaction mixture. The incubation time was 2 hrs.

unfractionated *E. coli* and yeast tRNA, catalyzed by a lysate of rabbit reticulocytes. Contrary to the report of Farkas and Singh (3), unfractionated *E. coli* tRNA was good acceptor in guanylation as yeast tRNA. Both 8-^{14}C-guanine and 2-^{14}C-guanine were incorporated into tRNA with similar efficiency, indicating that total guanine moiety is involved in the reaction. Surprisingly, among the purified individual *E. coli* tRNAs tested as acceptors for guanylation, four tRNAs, i.e. tRNA^{Asp}, tRNA^{His}, tRNA$_2^{Tyr}$ and tRNA^{Asn}, were very active (Figure 1 and Table 2). *E. coli* tRNA^{Asp} was the most

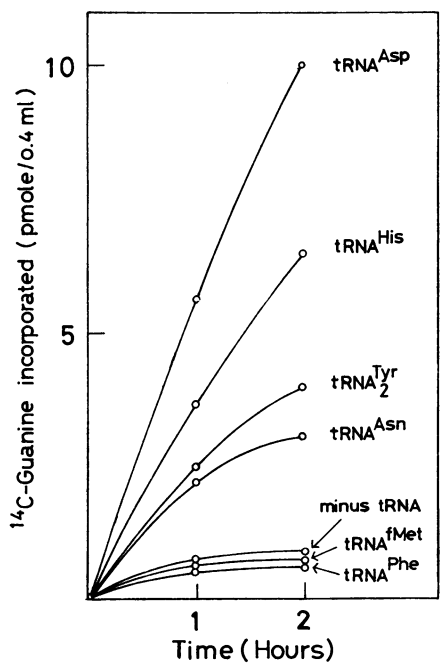

Figure 1. Acceptor abilities of the four E.coli tRNAs in guanylation. Conditions for guanylation were as described in Table I.

Table II. Incorporation of ^{14}C-guanine into purified amino acid specific *E. coli* tRNAs by the lysate of rabbit reticulocytes.

tRNA added	^{14}C-guanine incorporated (pmoles/0.4 ml of the reaction mixture)
$tRNA^{Asp}$	10.0
$tRNA^{His}$	6.5
$tRNA_2^{Tyr}$	4.0
$tRNA^{Asn}$	3.2
$tRNA_3^{Ser}$	2.5
$tRNA^{Lys}$	1.8
$tRNA_2^{Leu}$	1.6
$tRNA_1^{Val}$	1.4
$tRNA_2^{Glu}$	0.8
$tRNA^{Ala}$	0.2
$tRNA^{Trp}$	0.5
$tRNA^{fMet}$	1.0
$tRNA^{Phe}$	0.5
$tRNA_1^{Ser}$	0.6
$tRNA^{Cys}$	0.5
$tRNA^{Ile}_{major}$	0.5
$tRNA^{Met}$	0.4
$tRNA^{Arg}$	0.4
minus tRNA	0.9

3 A_{260} units of tRNA were added per 1.5 ml of reaction mixture. The incubation time was 2 hrs. 8-^{14}C-guanine was used.

active, and was 5 times better as a substrate than unfractionated yeast or *E. coli* tRNA (note that amounts of purified tRNAs added were one fifth as compared with unfractionated tRNA). Incorporation of guanine into these tRNAs was proportional to the incubation time for times of up to 2 hrs (Figure 1). Among the *E. coli* tRNAs tested only $tRNA_3^{Ser}$, $tRNA^{Lys}$, $tRNA_2^{Leu}$ and $tRNA_1^{Val}$ were slightly effective, and they were found to be appreciably contaminated with $tRNA^{Asp}$, $tRNA^{His}$ or $tRNA^{Tyr}$. Thus probably only $tRNA^{Tyr}$, $tRNA^{His}$, $tRNA^{Asn}$ and $tRNA^{Asp}$ act as acceptors. Farkas and his coworkers recovered the guanine incorporated into homologous tRNA as guanosine 2'(3')-phosphate after alkaline hydrolysis of the tRNA (1,3). We confirmed this using *E. coli* $tRNA^{Asp}$ and $tRNA^{His}$. ^{14}C-Guanosine 3'-phosphate was isolated by two-dimensional thin-layer chromatography after RNase T_2 digestion (12) (Data not shown).

The four tRNAs that are active in guanylation all recognize XA^U_C codons, and contain the modified nucleoside Q in the first position (13,14). These common characters suggest that incorporation of guanine must be associated with a specific anticodon structure, namely, either Q nucleoside in the first position or U in the second position of the anticodon. To identify the position of guanylation, *E. coli* $tRNA^{Asp}$ and $tRNA^{His}$ were labeled with ^{14}C-

guanine using purified guanylating enzyme and then digested with RNase T_1, and the digests were fractionated by two-dimensional PEI-cellulose chromatography. As shown in Figure 2, radioactivity was found in only one oligo-

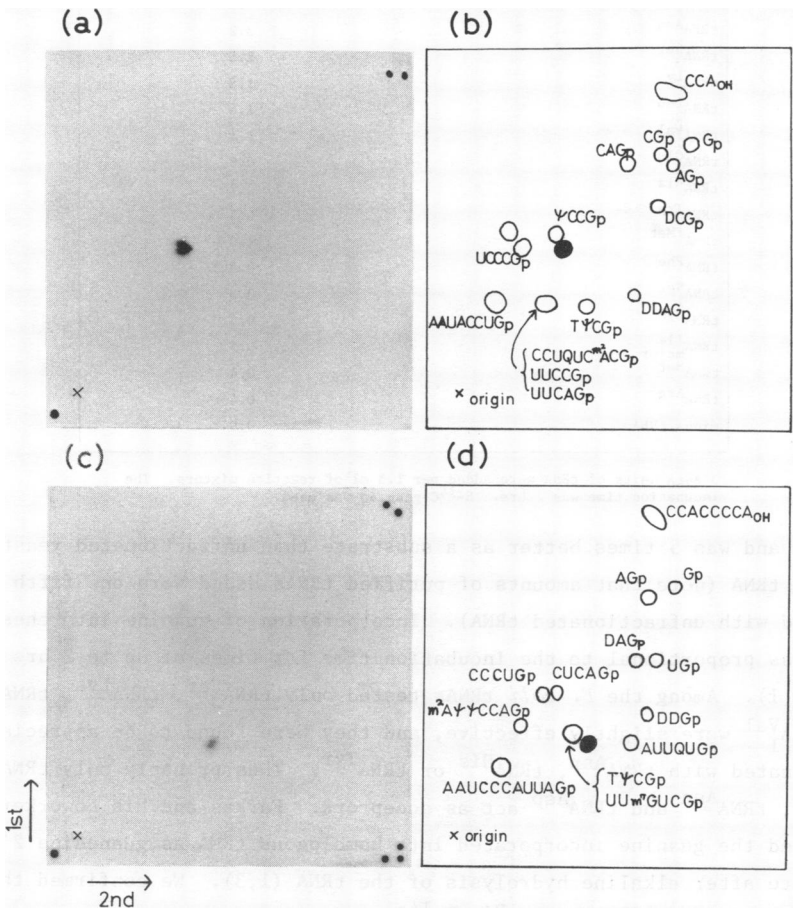

Figure 2. Two-dimensional PEI-cullulose chromatography of the RNase T_1 digest of ^{14}C-guanine labeled E.coli tRNA^{Asp} and tRNA^{His}. (a) auto-radiogram of the chromatogram of the digest of tRNA^{Asp}; (b) composite tracing of (a) in relation to the location of unlabeled oligonucleotides derived from tRNA^{Asp}; (c) auto-radiogram of the chromatogram of the digest of tRNA^{His} ; (d) composite tracing of (c) in relation to the location of unlabeled oligonucleotides derived from tRNA^{His} .

nucleotide on each chromatogram. The oligonucleotides labeled with ^{14}C-guanine seemed to be tetranucleotides judging from their relative mobilities. None of cold the oligonucleotides derived from untreated $tRNA^{Asp}$ or $tRNA^{His}$ were in the same position as the radioactive oligonucleotides. These results can be explained by supposing that Q base is exchanged with guanine, thus resulting in cleavage of the original oligonucleotide containing Q into two portions. [The phosphodiester linkage of Q nucleoside is resistant to RNase T_1 (13). Therefore, if Q is replaced by guanine, the original sequence C-C-U-Q-U-C-m^2A-C-Gp of $tRNA^{Asp}$ should give C-C-U-G(^{14}C)p and U-C-m^2A-C-Gp, while A-U-U-Q-U-Gp of $tRNA^{His}$ should give A-U-U-G(^{14}C)p and U-Gp (15,16)]. Another possibility is that $tRNA^{Asp}$ and $tRNA^{His}$ might be contaminated with some RNA which acted as acceptor, so that the radioactive oligonucleotides obtained did not correspond to any oligonucleotide derived from $tRNA^{Asp}$ and $tRNA^{His}$. To exclude this second possibility, uniformly ^{32}P-labeled $tRNA^{His}$ was extensively guanylated with cold guanine. The guanylated ^{32}P-labeled $tRNA^{His}$ was digested with RNase T_1 and again fractionated by PEI-cellulose chromatography. As shown in Figure 3, a new radioactive spot corresponding to ^{14}C-guanine labeled oligonucleotide [arrow in Figure 3a] appeared when $tRNA^{His}$ was incubated with, but not without guanine. The amount of A-U-U-Q-U-Gp only decreased when the tRNA was incubated with guanine (compare Figure 3a with 3b). The additional spots that appeared either in the presence or absence of guanine were presumably due to non-specific cleavage of the tRNA by nuclease present in the enzyme preparation. These results show that the oligonucleotide labeled with ^{14}C-guanine was in fact derived from $tRNA^{His}$.

The nucleotide sequence of ^{14}C-guanine labeled oligonucleotides were determined by partial digestion of the oligonucleotides with spleen phosphodiesterase as described in the Materials and Methods. The sequences of the oligonucleotides derived from $tRNA^{Asp}$ and $tRNA^{His}$ were found to be C-C-U-Gp (data not shown), and A-U-U-Gp, respectively (Figure 4). Oligonucleotides with these sequences can only be obtained when Q base in the tRNAs is replaced by guanine [see primary structures of *E. coli* $tRNA^{Asp}$ and $tRNA^{His}$ (15,16)].

DISCUSSION

The present work clearly indicated that Q base in the first position of the anticodon of *E. coli* tRNAs was replaced by guanine by the guanylating enzyme from rabbit reticulocytes. These results were obtained with a heterologous system using *E. coli* tRNAs, but the same type of reaction probably proceeds in homologous systems. Support for this idea is that we

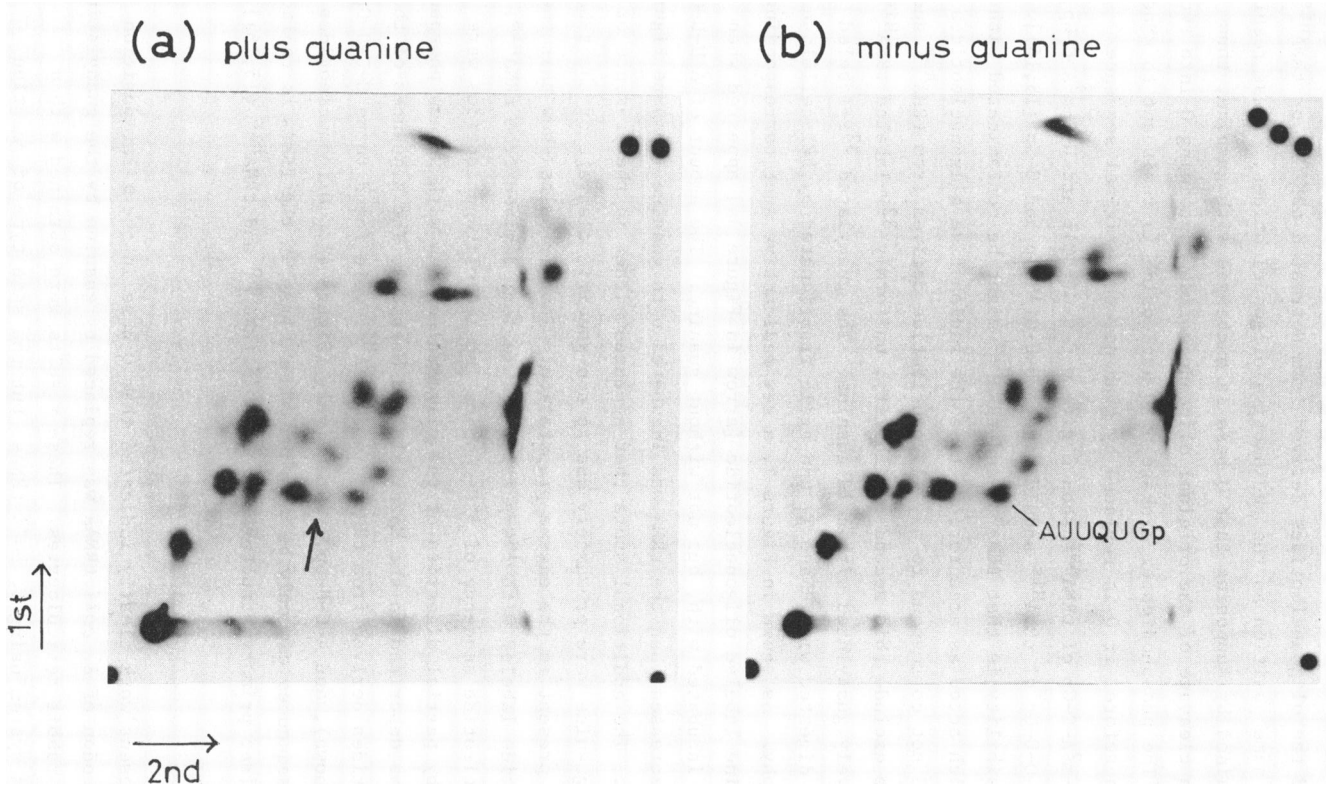

Figure 3. Two-dimensional PEI-cellulose chromatography of the RNase T_1 digest of ^{32}P-labeled $tRNA^{His}$ after incubation with the guanylating enzyme in the presence or absence of cold guanine. Incubation conditions were as for Figure 2, except that 10 times more enzyme preparation and 20 nmoles of cold guanine were added.

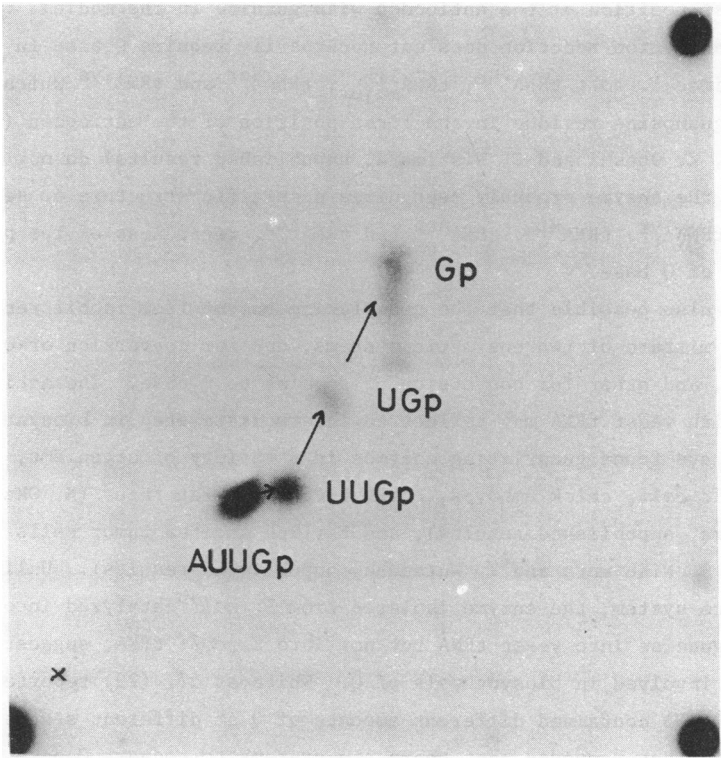

Figure 4. Two-dimensional PEI-cellulose chromatography of partial digests of the ^{14}C-guanine labeled oligonucleotides with spleen phosphodiesterase.

have found Q and Q* (a derivative of Q) in several mammalian tRNAs (17), and Q* in purified rabbit liver $tRNA^{Asp}$ (F. Harada, M. Yamada, S. Nishimura and H. Gross, unpublished results). The mechanism of replacement of Q base by guanine is unknown. Confirming the report of Farkas and Singh (3) we found that ATP was not required for guanylation. In biosynthesis of pseudouridine transglycosylation was also found not to require an energy source (18,19). The most likely mechanism of replacement of Q base by guanine is exchange of the whole Q base moiety with a guanine molecule, since 2-^{14}C-guanine as well as 8-^{14}C-guanine was incorporated into tRNA (Table I). If this occurs free Q base should be liberated. However, we have not yet been able to isolate Q base owing to technical difficulty of obtaining tRNA containing highly labeled Q.

The reticulocyte system also catalyzed the incorporation of guanine into yeast tRNA. Yeast tRNA does not contain modified nucleoside Q (17), so the enzyme in reticulocytes probably also catalyzes exchange of guanine

in the first position of the anticodon with guanine in the medium. That is, the guanylation reaction does not necessarily require Q base in tRNA. However, since *E. coli* $tRNA^{Phe}$, $tRNA^{Ile}_{major}$, $tRNA^{Ser}_{3}$ and $tRNA^{Cys}$ which contain a guanosine residue in the first position of the anticodon (20-22) (K. Tanaka, Z. Ohashi and S. Nishimura, unpublished results) do not act as acceptors, the enzyme probably recognizes a specific structure or sequence common to $tRNA^{Tyr}$, $tRNA^{His}$, $tRNA^{Asn}$ and $tRNA^{Asp}$, regardless of the presence or absence of Q base.

It is also possible that the guanylating enzyme from rabbit reticulocytes is a mixture of two enzymatic systems, one for conversion of Q base to guanine, and other for conversion of guanine to Q base. The activity detected with yeast tRNA may reflect an intermediate step in biosynthesis of Q. We have found guanylating enzymes in a variety of organisms, including *E. coli*, chick embryos, chick liver, and rat fetus (N. Okada and S. Nishimura, unpublished results), and Ehrlich ascites tumor cells (Y. Ito, F. Harada, S. Nishimura and Y. Watanabe, unpublished results). Unlike the reticulocyte system, the enzyme isolated from *E. coli* catalyzed incorporation of guanine into yeast tRNA but not into *E. coli* tRNA, suggesting that it is involved in biosynthesis of Q. White *et al.* (23) reported that *Drosophila* tRNA contained different amounts of Q at different stages in the life cycle. They proposed that there are systems to convert Q base to guanine and guanine to Q base without transcription. The guanylating reaction reported here is also probably involved in post-transcriptional modification of *Drosophila* tRNA, which may be important in regulating the function of this tRNA (24).

The guanylating reaction reported here is a new type of reaction for replacing modified base by normal guanine. Similar types of base exchange reactions without cleavage of the phosphodiester linkage in the polynucleotide chain are theoretically possible in DNA modification or DNA repair, since a base acts as donor. It will be interesting to examine the incorporations of other normal and modified bases into RNA and DNA.

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