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   a. Electron microscopic studies on the growth characteristics of animal viruses
   b. Studies on the ultrastructure of virus and enzyme
   c. Purification and chemical composition of animal viruses
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   e. Interaction of nucleic acid with animal cells-viral interference induced by RNA
3. Department of Biochemistry
   a. Enzymatic studies on nucleotide metabolism
   b. Studies on the regulatory mechanism of DNA synthesis in mammalian tissues and virus-infected cells
   c. Studies on the control mechanism of cellular growth
   d. Biochemical studies on the regulatory mechanism of RNA synthesis in *E. coli* and in the related phages
   e. Studies on structure and function of DNA-dependent RNA polymerase
   f. Genetic regulatory mechanisms in the expression of the tryptophan operon in *E. coli*
4. Department of Serology and Immunology
   a. Studies on biochemical events in cells destined to lysogeny after phage infection
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   c. Chemical structures of somatic antigens and phage receptors in bacterial strains
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   a. Fundamental studies on live attenuated Japanese encephalitis vaccine and its application
   b. Studies on variation and oncogenicity of adenovirus
   c. Studies on immunity to adeno-tumor
   d. Protection mechanism against viral infection
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      (4) Cellular receptor for Japanese encephalitis virus
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   a. Studies on the mechanism of viral leukemogenesis
   b. In vitro control of growth and differentiation of blood cells

c. Studies on the biology of viral inclusion
d. Morphological studies of rabies virus multiplication

7. Department of Genetics
   a. Genetics of DNA-dependent RNA polymerase of *E. coli*
   b. Control mechanisms in bacteriophage growth
   c. Regulation of chromosomal and episomal replication
8. Virological Diagnosis Center
   a. Studies on viral agents of the upper respiratory diseases
   b. Studies on the antigenic differences between influenza type A group virus strains
   c. Virological and serological studies on the specimens collected in Burma
   d. Studies on the agent of hand, foot and mouth disease
   e. Studies on the epidemics of ECHO virus

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Effect of Modification of Guanine Residues Near Amino Acid Acceptor End of Transfer RNA on its Acceptor Function

Yoko YAMAMOTO, Makoto OHWAKI and Yoshimi KAWADE*

I. Introduction

The structural basis of the functions of tRNA molecules has been the subject of numerous studies but still remains largely unsolved. Various means of chemical modification of the bases have been applied to tRNA[1], but they are often of very limited value, because the reaction takes place more or less randomly along the molecule and it is usually difficult or impossible to correlate the structural modification of the bases at specified positions to the function of the molecule. In the present study, an experimental design was devised which used random photooxidation of guanine bases but still would allow us to decide whether intactness of the guanines nearest the acceptor end of a tRNA molecule is required for its aminoacylation function. The experiments on *Torulopsis utilis* $tRNA^{Ser}$, $tRNA^{Tyr}$ and $tRNA^{Phe}$ described below indicated that the first guanine from the acceptor end, and probably also more guanines if present up to about the ninth base from the end, may be modified without necessarily resulting in inactivation of the molecule.

The basic experimental design is as follows. Illumination of nucleic acid in solution by visible or near-ultraviolet light in the presence of such dyes as methylene blue[2] and lumichrome[3] has been shown to photooxidize guanine residues specifically. Inactivation of tRNA by such means has been reported[4,5]. KUWANO *et al.*[5] showed that the phosphate-sugar bond on the 3'side of guanine, which is the specific site of action of RNase T1, becomes resistant to the enzyme after photooxidation mediated by methylene blue or 4-nitroquinoline oxide. It is well known that when unmodified, unfractionated tRNA is charged with one kind of radioactive amino acid, digested completely with RNase T1 and chromatographed on DEAE Sephadex using salt gradient containing urea, the oligonucleotides are fractionated mainly according to their chain length, or number of negative charges[6], and the aminoacyl oligonucleotide cut from the first guanine (hereafter the first guanine means the one nearest to the acceptor end of the molecule) will readily be located on the chromatogram by its radioactivity[7,8]. If the first guanine of the aminoacyl tRNA has been photooxidized before RNase T1 digestion, the digest will contain

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longer aminoacyl oligonucleotide, readily distinguishable on the chromatogram from the "normal" aminoacyl oligonucleotide arising from tRNA with unoxidized first guanine. Now, suppose tRNA is photooxidized and inactivated to some extent, and then the surviving molecules are charged with radioactive amino acid. If the first guanine need not be intact for the tRNA molecule to accept the amino acid, then complete digestion of the photooxidized and charged tRNA will yield radioactive oligonucleotide longer than the normal one. If, on the other hand, the intactness of the first guanine is necessary for amino acid acceptance, no such oligonucleotides longer than the normal should appear on the chromatogram. Thus, one can in principle distinguish which is actually the case experimentally, without using purified tRNA specific for a single amino acid. Photooxidation of guanine residues will of course take place more or less at random along the molecule, but by this experimental design, the effect of modification of only the first guanines may be studied.

II. Materials and Methods

Transfer RNA. Crude tRNA of *Torulopsis utilis* extracted from whole cells by phenol, was generously supplied by the Toyo Spinning Co., Inuyama, Aichi. The tRNA was purified in a similar way to WADA *et al.*[9], namely by treatment with phenol, adsorption to and elution from DEAE Sephadex, and precipitation with 0.1 M HCl. It was treated with pH 10 carbonate buffer to remove attached amino acids, precipitated with ethanol, dissolved in and dialyzed against distilled water, and stored frozen at −20 °C until use.

Aminoacyl tRNA synthetase. An enzyme preparation from baker's yeast was used. Cells of *Saccharomyces cerevisiae* in late log phase were suspended in 2 mM phosphate buffer pH 7.7, 2 mM $MgSO_4$, 50 mM reduced glutathione (or 10 mM mercaptoethanol), and disrupted in a blendor with glass beads (Minnesota Mining Co., 3 M Type 100–5005)[10]. The extract was clarified and the supernatant from centrifugation at 105,000 xg for 150 min was treated with DEAE cellulose, as described previously[9].

Assay for amino acid acceptor activity of tRNA. The assay procedure was similar to the one described previously[9], expect that the acid-insoluble reaction product was dissolved in 0.5 ml 1 M NH_4OH, added to 16 ml scintillation cocktail (10 ml toluene, 6 ml methyl cellosolve, 4 mg 2,5–diphenyloxazole and 1 mg 2,2′–p-phenylene-bis(5–phenyloxazole)), and counted in a Nuclear Chicago scintillation counter. The [^{14}C] amino acids used were, L-serine, 12.3 μc/μmole (New England Nuclear Co.), L-tyrosine, 6.5 μc/μmole (Calbiochem.), and L-phenylalanine,

5.33 μc/μmole (New England Nuclear Co.). Their concentration in the reaction mixture was at the saturation level, 0.1, 0.025 and 0.05 μmole/ml, respectively.

Preparation of aminoacyl tRNA. The reaction mixture was similar in composition to the one for the aminoacylation assay, but radioactive amino acids of higher specific activity were used in slight undersaturation. Their specific activity (μc/μmole) and concentration (μmole/ml) were, respectively: L[^{14}C] serine, 123, 0.02; L[^{3}H] serine, 3740, 0.005; L[^{14}C] tyrosine, 368, 0.005; L[^{3}H] tyrosine, 7940 or 33700, 0.002; L[^{14}C] phenylalanine, 355, 0.01–0.02; L[^{3}H] phenylalanine, 2500, 0.02. These were purchased from New England Nuclear Corp. or Schwarz BioResearch Co. Non-radioactive 19 other amino acids were also added in 10 to 20-fold excess. After incubation at 37° for 20 min, the aminoacylated tRNA was purified by phenol treatment, ethanol precipitation and 0.1 N HCl precipitation.

Digestion of aminoacyl tRNA with RNase T1. Aminoacyl tRNA was digested by RNase T1 (Sankyo Co) to completion as described by ISHIDA and MIURA[7], namely with 500–1000 units of enzyme/mg RNA in 0.1 M potassium acetate buffer pH 5.4 containing 2 mM EDTA at 37° for 4 h. Completeness of digestion under these conditions was confirmed by measuring the increase with time of acid-soluble material produced from unmodified rRNA. The number of units of the enzyme preparation used was determined according to EGAMI *et al.*[11].

Column chromatography of RNase T1 digests. The digests were analyzed by DEAE Sephadex columns (A25, fine, Pharmacia, 4 mm × 40–50 cm) using NaCl gradient in 7 M urea. The eluent was bufferized by 0.01 M potassium acetate at pH 5.4, and a NaCl gradient was produced using a single 200 ml mixing chamber of the buffer alone, connected to a 0.6 M NaCl reservoir. Usually after the sixth absorbance peak was eluted, the remaining material was eluted with 1 M NaCl. The column was run at 4°. 2 ml fractions were collected, their absorbance was measured, and 0.5 ml aliquots were counted in a liquid scintillation counter as described before.

Photooxidation. Lumichrome was kindly donated by Dr. T. MASUDA of Takeda Chemical Ind., Ltd. Because of its limited solubility near neutrality, it was first dissolved in hot 0.001 M NaOH and then diluted in appropriate buffer solution.

The light source for irradiation was a 500-W Xenon lamp (Ushio Optics). The tRNA solution containing lumichrome was put in a quartz cell with 1 cm light path, and irradiated at a distance of about 25 cm, under constant stirring with a small magnetic stirrer. Two filters were placed immediately before the cell, one being a 1 cm quartz cell filled with distilled water to absorb infrared light, the other a UV-35 filter (Toshiba) to absorb ultraviolet light shorter than

350 nm. Effective removal of the ultraviolet components was confirmed since a uridylic acid solution did not show any spectral change upon irradiation, and also tRNA irradiated in the absence of lumichrome was not inactivated. The tRNA solution was in dilute salt, 5 mM potassium phosphate buffer pH 4.5 containing 0.1 mM EDTA, in order to labilize its intramolecular ordered structure. To eliminate it more effectively, the tRNA was irradiated either in the presence of 7 M urea, or at high temperature. For the latter purpose, the sample cuvette was placed in a 80° water bath for 2 min, taken out and immediately irradiated for 1 or 2 min. The temperature after 1 and 2 min was about 65° and 57°, respectively. If not irradiated, no inactivation of tRNA was observed under these conditions. In every case, the irradiated solution was left standing overnight at room temperature, in order to make the dark reaction complete its course. The photooxidized tRNA was precipitated twice with ethanol to remove lumichrome (and urea).

III. Results

1) Photooxidation of guanine by lumichrome.

BERENDS *et al.* have presented evidence for selective modification of guanine among the bases in nucleic acid by visible light irradiation in the presence of lumichrome[3]. They also showed that the photoreactions that guanine undergoes are complex, yielding a number of different photoproducts. The change we observed in ultraviolet absorption spectrum of a 5′ GMP solution upon irradiation for various periods is shown in Fig. 1. Since an after-effect was found, the spectra recorded are those obtained after standing the irradiated solution overnight at room temperature, when the spectrum ceased to change with time (the same end result may be obtained by heating the irradiated solution at 80° for 10 min). The

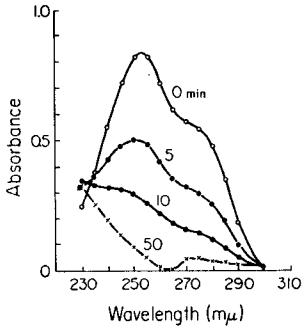

Fig. 1. Absorption spectra of 5′ GMP (0.1 mM) irradiated for various periods as indicated, in the presence of 0.01 mM lumichrome in 5 mM potassium acetate buffer pH 4.5 and 0.1 mM EDTA.

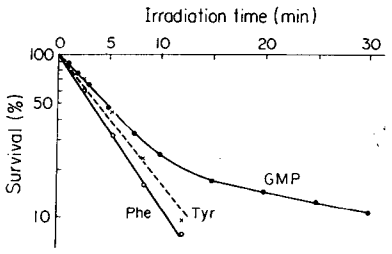

Fig. 2. Survival curves of 5' GMP expressed as the decrease of $A_{265\,nm}$, and of tyrosine and phenylalanine acceptor activities of tRNA. The GMP data are from the experiment of Fig. 1. tRNA, 0.04 mM in total nucleotide, was irradiated in the presence of 0.01 mM lumichrome under the same conditions at room temperature.

survival curve of guanine is represented in Fig. 2 as the decrease in $A_{265\,nm}$. Other nucleotides, AMP, CMP and UMP, showed little or no detectable change in absorption spectrum at least up to 20 min of irradiation. No spectral change was observed when GMP was irradiated without lumichrome.

2) Resistance of modified guanine site to RNase T1.

KUWANO *et al.*[5] have shown that the photooxidized guanine sites are resistant to RNase T1. To confirm this point in our system, poly G was photooxidized, digested completely by RNase T1, and fractionated chromatographically. If the modified guanine sites are resistant to RNase T1 the mononucleotide fraction must contain only intact GMP, and the oligonucleotides must consist of a sequence of N ($\geqq$1) modified guanines with an intact guanine at the 3′ end. (A possible exception: if some poly G chains are phosphorylated at the 3'end, and their guanine is photooxidized while the next guanine is not, the modified terminal GMP will appear in the mononucleotide fraction.) A poly G (Miles Laboratories, Inc.) solution was photooxidized in the usual way until its $A_{265\,nm}$ fell to 47%, and its RNase T1 digest was separated into 4 fractions by DEAE Sephadex column as shown in Fig. 3: (a) unadsorbed fraction, (b) mononucleotide fraction, (c) oligonucleotides eluted with 0.40 M NaCl, and (d) longer oligonucleotides eluted with 1 M NaCl. Their absorption spectra are shown in Fig. 4. As expected, fraction (b) closely resembles intact GMP in the spectrum and other fractions do not. Fraction (a) is most likely some split product from guanine ring structure. The materials in fractions (b) and (d) were recovered free of urea by adsorption to charcoal and elution by ammonia-ethanol (1 : 1 : 0.006, water : ethanol : 28% NH_4OH by volume), and their phosphorus content was determined according to TAKEMURA and MIYAZAKI[12]. The molar ratios of phosphorus to guanine, the latter deduced from $A_{260\,nm}$, were found to be 1.18 and 4.96, for (b) and (d), respectively.

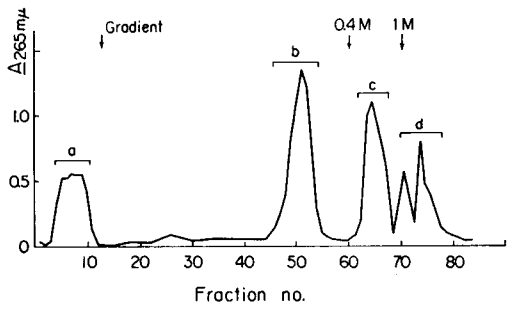

Fig. 3. Chromatographic analysis of RNase T1 digest of photooxidized poly G. Poly G, 0.4 mg/ml (about 1 mM) in the dilute acetate buffer was irradiated in the presence of 0.1 mM lumichrome for 7.5 h, at which time its $A_{265\,nm}$ was 47% of the original value. After eliminating lumichrome by ethanol precipitation, the photooxidized poly G (0.3 mg/ml) was digested completely by RNase T1 (200 units/ml) in 0.015 M Tris HCl buffer pH 7.5 containing 0.1 mM EDTA at 37° for 23 h. The digest was charged to a DEAE Sephadex column, and eluted at 4° using 7 M urea in 0.005 M Tris buffer pH 7.5. $A_{265\,nm}$ of the effluent was recorded. After an unadsorbed component, Fraction a, was eluted, a gentle NaCl gradient was applied to elute mononucleotide, Fraction b. Then the NaCl concentration was raised to 0.4 M to give an oligonucleotide Fraction c, then to 1 M to elute the remaining Fraction d. The recovery of $A_{265\,nm}$ in Fractions a, b, c, and d was 21, 28, 21 and 14%, respectively.

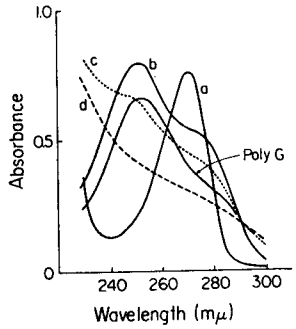

Fig. 4. Absorption spectra of Fractions a, b, c and d of Fig. 3, and of poly G before photooxidation.

Thus it appears that the mononucleotide fraction consists mostly or entirely of intact GMP, although a slight contamination with modified GMP may not be excluded, and that oligonucleotides contain modified guanine residues, in harmony with the expectation mentioned above. It may therefore be concluded that most, if not all, of the modified guanine sites are resistant to RNase T1.

To be exact, a slight degree of susceptibility to the enzyme may not be ruled out. For example, some forms of guanine photoreaction might leave the site