Alterations in the Transfer Ribonucleic Acid Methylases after Bacteriophage Infection or Induction*

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ABSTRACT: The relative levels of base-specific transfer ribonucleic acid (t-RNA) methylases are constant in a given species. In *E. coli* B, grown on different nutrient media or after ultraviolet irradiation, the relative activities of enzymes which methylate adenine, guanine, and uracil in t-RNA remain constant within experimental error. On the other hand, after T2 infection major shifts in the relative levels of base-specific t-RNA methylases occur. For example, the methylations of uracil and adenine were enhanced 100% relative to the methylation of guanine. The methods employed detect only changes in the total number of methyl groups introduced, not changes in sites. With these methods no changes were detected upon infection with T1 bacteriophage.

However, after induction of λ in *E. coli* K_{12}, extensive changes were found both in the absolute and relative levels of the t-RNA methylases. The shifts in enzyme activities are not simply the sequels of ultraviolet irradiation but appear to be a result of the induction since irradiation of nonlysogenic organisms produced no measurable changes in the enzymes.

It has been found by Cohen that as a consequence of infection by T2 bacteriophage an enzyme capacity for the synthesis of 5-hydroxymethylcytosine appears *de novo* in the host cell. Subsequently, other profound metabolic alterations as a result of infection have been demonstrated in several laboratories. Inhibition of normal cellular synthesis as well as the production of new enzyme activities have been observed (Cohen, 1948, 1949; Franklin and Baltimore, 1962; Flaks and Cohen, 1957; Kornberg *et al.*, 1959; Keck *et al.*, 1960; Weissman *et al.*, 1963; Baltimore and Franklin, 1963; Bello *et al.*, 1961).

Since virus-induced metabolic changes are intimately associated with the modification of host nucleic acids, and since the enzymes which methylate nucleic acids have been found to be species specific, we speculated on the possibility that viral infection might also alter the activities of the transfer ribonucleic acid (t-RNA) methylases in the host cell.

The t-RNA methylases can be characterized by three different parameters: rates of methylation, total number of methyl groups introduced, and the sites of methylation. Since the various methylated bases are present in very low levels compared to the major components of RNA, a method of detecting changes in the levels of the methylated bases relative to each other has been employed in the present investigation with [*methyl*-^{14}C]-adenosylmethionine as the methyl donor. Inasmuch as all the methyl groups of t-RNA originate from the methyl group of methionine, the radioactivity of each methylated base is a reflection of its total amount.

The use of methyl-deficient t-RNA from *E. coli* K_{12}W6 as a substrate for the assay of relative levels of base-specific t-RNA methylases was the method of choice. Homologous enzymes introduce methyl groups into the methyl-deficient RNA in the same ratios and to the same extent as they do *in vivo* (Svensson *et al.*, 1963). The pattern of methylation of this substrate by heterologous enzymes is characteristic of each source of the enzymes. However, enzymes from a given source, ranging from microorganisms to animal organs, catalyze the transfer of the same number of methyl groups to methyl-deficient t-RNA, and the pattern of methylation in the product is constant (Srinivasan and Borek, 1963).

We report here changes in the activity patterns of t-RNA methylases in cells infected by T2 phage and in lysogenic cells induced by ultraviolet irradiation.

Materials and Methods

Preparation of Enzyme Extracts. The cells were disrupted by grinding in the cold with twice their weight of wet alumina and extracted with a buffer containing Tris (0.01 M, pH 8.0), $MgCl_2$ (0.01 M), mercaptoethanol (0.005 M), and DNAase (5 μg/ml) (Fleissner and Borek, 1963). After removal of cell debris and alumina by low speed centrifugation, the suspension was centrifuged at 105,000 × *g* for 75 min. The RNA methylase activity of the supernatant was stable in the frozen state for several months.

T2 Infection. A culture of *E. coli* B grown in broth to logarithmic growth phase was infected at a population of 4×10^8 cells per ml with T2 phage at a multiplicity of 8–10. The infected cells were vigorously

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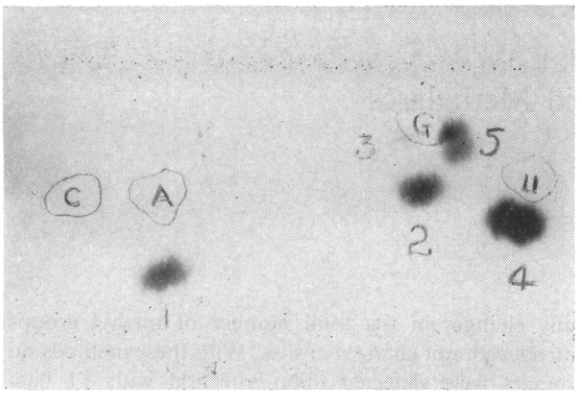

FIGURE 1: Radioautogram of an alkaline hydrolysate of methyl-deficient t-RNA isolated after exposure to RNA methylases in presence of [*methyl*-^{14}C] S-adenosylmethionine. The nonradioactive major nucleotides are outlined and labeled A, C, G, and U for adenylic, cytidylic, guanylic, and uridylic acids, respectively. Radioactive spot 1 is 6-methyladenylic acid, spot 2 is methylguanylic acid, spot 3 is dimethylguanylic acid, and spot 4 is methyluridylic acid. Spot 5 is an as yet uncharacterized methylated nucleotide.

aerated at 37° for 10 min. The culture was then poured over crushed ice and harvested by centrifugation in the cold.

T1 Infection. A logarithmic phase culture of *E. coli* B in mineral–glucose medium (Gray and Tatum, 1944) was supplemented with $MgCl_2$ to a final concentration of 7.5×10^{-3} M. T1 phages (10 per cell) were added and incubation at 26° was allowed to proceed for 10 min, at which time the culture was poured over cracked ice and harvested. Lysis time under these growth conditions is 22 min.

Ultraviolet Irradiation. E. coli K_{12}W6 (λ) was induced by ultraviolet irradiation (Lwoff *et al.*, 1950) using slight modifications of procedures previously described (Wainfan *et al.*, 1963). Cells were grown at 37° in mineral–glucose medium (Gray and Tatum, 1944) supplemented with 30 mg/l. of DL-methionine and 12.5 μg/l. of biotin. Cells in logarithmic growth phase were irradiated with a G.E. 15-w germicidal lamp at doses sufficient to induce 90% or more of the population. For induction of small batches of cells 250 ml of cell suspension in a 10.5 × 6.5 in. glass tray received a dose of 900 ergs/mm^2 while being agitated on a shaker. For larger quantities of cells 1 l. of a cell suspension containing 6×10^8 cells per ml was subjected to a dose of 4900 ergs/mm^2 in a 10.5 × 16.5 in. glass tray. Ultraviolet dose was determined with a Latarjet meter. The irradiated cells were immediately transferred, in the dark, to flasks and agitated at 37° for the stated periods. *E. coli* B cells in logarithmic growth phase were irradiated in the same way. Survival at the doses employed was less than 0.1%.

Enzyme Incubations. In vitro methylation of RNA was carried out with methyl-deficient t-RNA as substrate and [*methyl*-^{14}C]*S*-adenosylmethionine as methyl donor (Fleissner and Borek, 1963). With each extract a pilot experiment was carried out to determine the saturation levels of enzyme extract (*i.e.*, further addition of enzyme did not result in any significant change in total ^{14}C incorporated per mg of RNA). In a typical experiment 0.6 mg of methyl-deficient t-RNA accepted methyl groups to the following extent: 0.05 ml of enzyme, 4600 cpm; 0.1 ml of enzyme, 7100 cpm; 0.2 ml of enzyme, 12,100 cpm; 0.5 ml of enzyme, 12,100 cpm. The incubation mixture for the isolation of $^{14}CH_3$-t-RNA contained the following per ml: 0.6 mg of methyl-deficient t-RNA, 0.5 μcurie of [*methyl*-^{14}C]*S*-adenosylmethionine (specific activity 26–29.9 mcuries/mM), Tris buffer (0.01 M, pH 8), $MgCl_2$ (0.01 M), mercaptoethanol (0.005 M), and sufficient enzyme extract to yield saturation level. After 45 min of incubation at 37° 0.3 ml of 2 M hydroxylamine, pH 8, was added and the mixture was incubated for an additional 10 min. The mixture was cooled to room temperature and extracted with an equal volume of 88% phenol (Mallinckrodt, Liquefied Analytical Reagent). Residual phenol was removed from the aqueous extract with ether and the RNA was then precipitated with alcohol. The RNA precipitate was then dissolved in 0.2 M Tris, pH 9.5, held for 10 min at 50°, and then reprecipitated in the cold with trichloroacetic acid. This extraction procedure was repeated once more. The precipitated RNA was agitated with ether to remove the residual trichloroacetic acid. Approximately 0.5–0.8 mg of this RNA was used for the determination of the methylation pattern. Methyl-deficient t-RNA was prepared from methionine-starved cells of *E. coli* K_{12}W6 by methods described by Fleissner and Borek (1963).

Pattern of Methylation. The isolated RNA was hydrolyzed to nucleotides with 0.3 N NaOH at 37° for 18 hr. The RNA hydrolysate was deionized, subjected to paper electrophoresis in ammonium acetate buffer (pH 2.7), and followed by chromatography in 2-propanol–H_2O (70:30) and NH_3 in the vapor phase by the methods described by Ingram and Pierce (1962).

The position of each of the major base nucleotides was detected by its ultraviolet absorption and the radioactive, methylated bases present on the chromatogram were located by radioautography. A typical chromatogram is shown in Figure 1. The radioactive areas were cut into thin strips and extracted with 0.05 N HCl at 37°. The eluates were dried in stainless steel planchets and counted in a Nuclear Chicago gas flow end-window counter.

Results and Discussion

The methylating capacities of t-RNA by extracts derived from normal, irradiated, and T2 infected *E. coli* B cells are given in Table I. The specific activities of the methylated t-RNA isolated after exposure to enzyme extracts of *E. coli* B cells grown on minimal medium, broth medium, and after irradiation showed no significant differences. However, extracts from *E. coli* B cells prepared 10 min after infection with T2 catalyze

TABLE I: Total t-RNA Methylating Capacities of Enzyme Extracts from Normal, Irradiated, and T2 Infected *E. coli* B.

Experimental Conditions	Specific Activity of t-RNA[a] (cpm/mg)
Cells in logarithmic growth phase	
Grown on minimal medium	
1. Expt 1	35,000
2. Expt 2	38,500
Grown on broth medium	41,000
Ultraviolet-irradiated cells	
10-min postirradiation	36,000
20-min postirradiation	35,000
Cells infected by T2	
Expt 1	58,000
Expt 2	61,000

[a] Counts per minute per mg of isolated t-RNA after methylation under conditions of enzyme saturation. Refer to text for experimental details.

TABLE II: Relative Methylation *in Vitro* of Various Bases in t-RNA by Enzymes of *E. coli* B under a Variety of Conditions.[a]

Experimental Conditions	Ratio of[b] MeA/MeG	Ratio of[b] MeU/MeG
Controls		
Grown on minimal medium	0.4	2.6
Grown on minimal medium	0.6	2.4
Grown on broth medium	0.5	2.8
Irradiated with ultraviolet and harvested 20 min later	0.4	2.4
T2 infected cells		
Expt 1	0.9	5.9
Expt 2	0.9	5.2
T1 infected cells	0.4	2.2

[a] Values were obtained at saturation levels of methylation. For details see text. All ratios represent averages obtained from duplicate chromatograms. [b] The following abbreviations have been used in the table: MeA: methyladenylic acid; MeG: monomethylguanylic acid plus dimethylguanylic acid; MeU: methyluridylic acid. The ratios were calculated as follows: for example, in the experiment in line 1 the radioactivities of MeA, MeG, and MeU were 1310, 3050, and 7760 cpm, respectively. Therefore, MeA/MeG = 1310/3050 = 0.4 and MeU/MeG = 7760/3050 = 2.6.

the incorporation of at least 50% more methyl groups into methyl-deficient t-RNA than normal *E. coli* B extracts.

In Table II the pattern of methylation achieved by the extracts of uninfected cells, *i.e.*, controls, is presented. The enzymes were extracted from organisms which had been subjected to different environmental conditions, yet the base-specific relative methylating potencies are essentially constant. This is all the more remarkable when the complexity of the methylating systems is considered. The substrate consists of a mixture of all the t-RNA's and the enzyme extract contains at least six RNA methylases. The constancy of the relative base-specific methylating activities indicates a high degree of predisposition of particular nucleotide chains to specific methylations under a variety of metabolic conditions and enhances the biological significance of possible alterations in these normal patterns of methylation.

In Table II a summary of studies of the RNA methylases in *E. coli* B after infection by T2 and T1 bacteriophages is also presented. Major shifts in the relative activities of the base-specific methylases occur after T2 infection. The methylation of both adenine and of uracil relative to the methylation of the guanines is enhanced nearly twofold. That the changes in the relative amounts of the methylated bases stem from the action of some nuclease must be considered. Should some nuclease arise within the host cell as a result of the phage infection with an action specificity rooted around some methylated base, a preferential cleavage and release of some bases might alter the residual relative levels in the macromolecule. Assay for nuclease activity against t-RNA in the enzyme extracts from T2 infected cells proved to be negative. After 45 min of incubation, which is the duration of exposure for the methylation reaction, there was no measurable loss in radioactivity from t-RNA previously labeled with [^{14}C]methyl groups (initially the total radioactivity of an aliquot was 4300 cpm; after 45 min of incubation with T2 infected cell extract the total radioactivity was 4800 cpm).

After T1 infection no changes could be detected in the relative methylating activities. However, with the methodology used, the occurrence of other alterations cannot be ruled out entirely. The methylated base ratio changes represent only gross changes in RNA structure. Subtler modifications such as methylation of the same base in another position on the polymer chain cannot be detected by these methods. More searching analytical procedures are necessary to determine whether such changes occur universally after virus infection.

In Table III a summary of the findings on the levels of RNA methylase activities in *E. coli* K_{12} after the induction of λ phage is presented. The values reported were obtained at saturation levels of enzyme activity. Therefore the reduced incorporation of methyl groups by enzymes extracted up to 20 min after irradiation indicates that the enzymes search out fewer sites for methylation as a result of induction. The reduction of

TABLE III: RNA Methylase Activities of *E. coli* K_{12} (λ) before and after Ultraviolet Induction.[a]

Conditions	Specific Activity of t-RNA (cpm/mg)
No ultraviolet	31,000
10 min after ultraviolet	<5,000[b]
20 min after ultraviolet	8,500
30 min after ultraviolet	26,000

[a] Values were obtained at saturation levels of methylation. For experimental details see text. [b] This value varies among different experiments depending upon the sampling time.

enzyme activity is apparently caused by the induction and not related directly to the irradiation. This is substantiated by the lack of effect of ultraviolet irradiation on the RNA methylases of a nonlysogenic *E. coli* B (Tables I and II).

The activities of all of the base-specific RNA methylases are not uniformly reduced as a result of induction. For example, the reduction in the uracil-t-RNA methylase activity is the most pronounced (Wainfan *et al.*, 1965). Details of quantitative studies of the relative levels of the base-specific enzyme activity before and after the return to maximal activity will be published later.

Whether the changes observed in the methylating enzymes of T2 infected or induced cells represent the cumulative effects of inhibition, alteration of specificity, or, in the case of increased activity, *de novo* synthesis of enzymes is the subject of current study.

Phage infection or induction is known to produce profound disorganization in the metabolic processes of the host. What role, if any, the changes in the patterns of methylation of t-RNA described here play in the disruption of normal synthetic pathways can only be conjectured. Ames and Hartman (1963) have suggested the possibility of control of protein synthesis mediated by alterations of the t-RNA. Methylation is a plausible method of modification of t-RNA for such a modulator role. Two different lines of evidence indicate that methylation alters the physical characteristics of t-RNA. Methylation of methyl-deficient t-RNA causes a reduction in the absorbancy of the product at 260 mμ (Borek and Christman, 1965). Moreover, it has been shown by Lazzarini and Peterkofsky (1965) that in methyl-deficient t-RNA a new leucine acceptor activity appears, which behaves uniquely on a methylated albumin column.

On the other hand, Sueoka and Kano-Sueoka (1964) reported that after T2 phage infection leucine acceptor t-RNA's appear in bacterial cells, which also behave aberrantly on a methylated albumin column. Whether there is any relation between the formation of t-RNA's with new physical attributes as a result of phage infection and the new patterns of methylating activities remains to be elucidated.

At present it is not clear whether the alteration of patterns of methylation of the t-RNA's is the initiating mechanism for the disruption of normal controls by phage infection or induction or just an unrelated coincidence. If it should prove to be the former, the schemes for regulatory mechanisms proposed up to now, most of which assign DNA for the site of regulation, will have to be re-evaluated.

Added in Proof

Radioactive spot 5 in Figure 1 has now been tentatively identified as methylated products derived from 7-methylguanylic acid.

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