Properties of the Transfer Ribonucleic Acid Methylase Activity *in vitro* of Hamster Tumours Induced by Adenovirus-12

BASE ANALYSIS

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The tRNA methylase activity *in vitro* of adenovirus-12-induced tumour and normal hamster tissues was studied. Enzyme extracts from adenovirus-12-induced tumour, hamster muscle-connective tissue and hamster liver methylated both *Escherichia coli* tRNA and yeast tRNA. The source of the enzyme and the pH of the incubation mixture influenced the bases methylated. However, the methylated bases that were isolated were not affected by the presence of NH_4^+ ions in the incubation mixture.

tRNA methylases are enzymes that transfer methyl groups from *S*-adenosylmethionine to specific positions in the tRNA. Such enzymes have been found in *Escherichia coli* (Hurwitz, Gold & Anders, 1964), yeast (Baguley & Staehelin, 1969; Bjork & Sorensson, 1969), bacteriophage-infected bacteria (Wainfan, Srinivasan & Borek, 1966a), mammalian tissues and various tumour tissues (Mittelman, Hall, Yohn & Grace, 1967b; Baguley & Staehelin, 1968; McFarlane & Shaw, 1968; Kerr, 1969; Rogg & Staehelin, 1969; Tsutsui, Srinivasan & Borek, 1969). Nucleic acid methylation has been implicated in cellular control mechanisms (Wainfan, Srinivasan & Borek, 1966b) and in the developmental process (Comb, 1965; Pillinger & Borek, 1969). However, the questions still arise as to how the tRNA methylases recognize specific sites in the tRNA and what the function of the methyl group on the tRNA is. A number of groups of workers (Kaye & Leboy, 1968; Kerr, 1969; Pillinger & Borek, 1969) have reported that the presence of ammonium acetate in the incubation medium *in vitro* greatly stimulates the tRNA methylase activity.

In view of previous work (McFarlane & Shaw, 1968; McFarlane, 1969) on normal hamster and adenovirus-12-induced tumour-cell enzymes, we have further studied these enzymes with respect to the bases methylated, the effect of ammonium acetate and the effect of pH on the pattern of methylation.

METHODS AND MATERIALS

Animals and tumour production. Pregnant random-bred Syrian hamsters (*Mesocricetus auratus*) were ob-

tained from High Oak Ranch Ltd., Richmond Hill, Ont., Canada, 2–4 days before expected parturition.

The adenovirus-12 used in these experiments was Huie strain and was supplied by Dr J. J. Trentin, Baylor University, Houston, Tex., U.S.A. When received, the virus was in the eighth HeLa-cell passage and was passed in our laboratory up to the twenty-third HeLa-cell passage. The TCID_{50} (tissue culture infective dose for 50% of the cells) for HeLa cells was $10^{3.4}/1.0\text{ ml}$.

Within 24 h of birth 0.1 ml of adenovirus-12 was inoculated into the dorsal subcutaneous tissue of the newborn hamster. The hamsters were weaned at 3 weeks of age. Uninoculated hamsters of the same age were used as the source of control tissue. Both inoculated and control hamsters were housed in the same room and were fed on carrots *ad libitum*.

Preparation of tRNA methylases. The enzyme was prepared essentially as described by McFarlane & Shaw (1968). Hamsters were anaesthetized with ether and killed by heart puncture. The tissue, i.e., liver, dorsal muscle-connective tissue or adenovirus-12-induced tumour, was quickly removed and homogenized in 6 vol. of ice-cold 0.25 M-sucrose containing 10 mM-MgCl₂. The homogenate was centrifuged in an International model B-20 refrigerated centrifuge at 15000g for 15 min at 4°C to remove nuclei, whole cells and mitochondria. The supernatant fluid was centrifuged in an International model B-60 refrigerated centrifuge at 105000g for 1 h at 4°C. The clear supernatant fluid from the 105000g centrifugation was used for enzyme studies.

RNA preparations. *E. coli* B tRNA and brewer's yeast tRNA were purchased from Schwartz BioResearch Inc., Orangeburg, N.Y., U.S.A.

Preparation of methylated tRNA in vitro. The enzyme assay mixture contained 500 μmol of tris-HCl buffer, pH 7.0 or 8.5 as indicated in the experiments, 200 μmol of dithiothreitol, 100 μmol of MgCl₂, 400 μmol of ATP, 2 mg of tRNA and 0.5 μCi of *S*-adenosyl-L-[Me-¹⁴C]-methionine (specific radioactivity 40.0 $\mu\text{Ci}/\mu\text{mol}$;

obtained from New England Nuclear Corp., Boston, Mass., U.S.A.) in a total volume of 2.0 ml. Then 1.0 ml of enzyme extract containing 10–12 mg of protein was added to each assay, i.e., sufficient enzyme to cause maximum incorporation of [^{14}C]methyl groups into 2 mg of tRNA.

The mixture was incubated at 37°C for 1 h, at the end of which time the reaction mixture was adjusted to pH 9.5 by adding 2 M- Na_2CO_3 and incubated for a further 30 min. The incubation mixture was then mixed with an equal volume of water and 2 vol. of 90% (w/v) phenol. The phenol mixture was stirred at 4°C for 30 min and then centrifuged at 1000g for 20 min at 4°C in an International model PR-2 refrigerated centrifuge. The aqueous layer was removed and the RNA was precipitated by the addition of 0.1 vol. of 20% (w/v) potassium acetate and 2 vol. of 95% (v/v) ethanol. The RNA was collected by centrifugation in an International model PR-2 refrigerated centrifuge at 1000g for 20 min at 4°C and the RNA pellet was dissolved in water (Zubay, 1966).

Hydrolysis of nucleic acids. RNA was hydrolysed in 11.7 M- HClO_4 at 100°C for 60 min, diluted fivefold with water and clarified by centrifugation. Hydrolysis with HClO_4 yielded both free purine and pyrimidine bases (Bendick, 1957).

Duplicate samples were hydrolysed in 1 M-HCl at 100°C for 60 min. This gave free purine bases and pyrimidine nucleosides (Schmidt, 1957).

Chromatography of hydrolysed nucleic acid. Two-dimensional descending chromatography was carried out on Whatman 3MM paper. The solvent for the first dimension was butan-1-ol-0.6 M- NH_3 (6:1, v/v) and the solvent for the second dimension was butan-1-ol-formic acid-water (77:11:12, by vol.) (Markham & Smith, 1949). The first dimension was run for 40 h at 20°C and the second dimension was run for 24 h at 20°C. The bases were identified by co-chromatography with standard prepara-

tions of methylated bases. Standard preparations of 1-methyladenine, 3-methyladenine, 7-methyladenine, 9-methyladenine, N^6 -methyladenine, N^6 -dimethyladenine, 1-methylguanine, 3-methylguanine, 7-methylguanine, 9-methylguanine, N^6 -methylguanine, 3-methylxanthine, 1-methylcytosine, 3-methylcytosine, 5-methylcytosine, 1-methyluracil, 3-methyluracil, 5-methyluracil and 1,3-dimethyluracil were purchased from Cyclo Chemical Co., Los Angeles, Calif., U.S.A.

After chromatography the paper was cut into 3 cm $\times$ 3 cm squares which were placed in vials containing 15 ml of 0.4% 2,5-bis-(5-*tert*-butylbenzoxazol-2-yl)thiophen. The radioactivities of samples were counted in a Packard scintillation counter.

RESULTS

Table 1 shows the methylated bases isolated after incubation of enzyme preparations from adenovirus-12-induced hamster tumours, normal hamster liver and normal hamster muscle-connective tissue, with tRNA from either *E. coli* B or brewer's yeast as substrate. The same bases are methylated with either substrate, although the extent of methylation appears greater with the *E. coli* B tRNA. The total activity recovered is about ten times greater with adenovirus-12-induced hamster tumour extracts than with normal hamster liver or normal hamster muscle-connective tissue extracts. One of the bases found only in muscle-connective tissue is yet to be identified. The unidentified base Y is a pyrimidine but is not 1-methylcytosine, 3-methylcytosine, 5-methylcytosine, 1-methyluracil, 3-methyluracil, 5-methyluracil or 1,3-dimethyluracil.

Table 1. *Specific methylated bases isolated after incubation of E. coli B tRNA or brewer's yeast tRNA as methyl acceptor with enzyme preparations derived from adenovirus-12-induced tumours and normal hamster tissues*

		[^{14}C]Methylated base recovered (c.p.m./mg of tRNA)					
Substrate	Base	Adenovirus-12-induced tumour enzyme		Muscle-connective tissue enzyme		Liver enzyme	
		HClO_4 hydrolysis	HCl hydrolysis	HClO_4 hydrolysis	HCl hydrolysis	HClO_4 hydrolysis	HCl hydrolysis
<i>E. coli</i> B tRNA	1-Methyladenine	2100	2100	0	0	280	200
	3-Methyladenine	6090	9000	750	900	560	720
	N^6 -Methyladenine	510	525	0	0	0	0
	Unidentified base Y	0	0	250	0	0	0
Brewer's-yeast tRNA	1-Methyladenine	750	680	0	0	125	150
	3-Methyladenine	500	550	210	220	240	300
	N^6 -Methyladenine	200	230	0	0	0	0
	Unidentified base Y	0	0	150	0	0	0

Table 2. *Effect of pH on the methylated base isolated after incubation of E. coli B tRNA or brewer's yeast tRNA with hamster muscle-connective tissue enzyme preparation*

The incubation mixture, incubated at 37°C for 60 min, contained 500 μ mol of tris-HCl buffer, pH 7.0 or pH 8.5, 200 μ mol of dithiothreitol, 100 μ mol of MgCl₂, 400 μ mol of ATP, 2 mg of tRNA, 0.5 μ Ci of *S*-adenosyl-L-[Me-¹⁴C]methionine and 10–12 mg of enzyme protein in a total volume of 2.0 ml. The tRNA was extracted with phenol and precipitated from the aqueous phase by the addition of 2 vol. of 95% (v/v) ethanol and 0.1 vol. of 20% (w/v) potassium acetate. Hydrolysis with HClO₄ was for 1 h at 100°C and the bases were separated by two-dimensional paper chromatography.

Base	[¹⁴ C]Methylated base recovered (c.p.m./mg of tRNA)			
	<i>E. coli</i> B tRNA		Brewer's-yeast tRNA	
	pH 7.0	pH 8.5	pH 7.0	pH 8.5
3-Methyladenine	1000	845	210	180
Unidentified base Y	300	0	150	0

Table 3. *Effect of the presence of ammonium acetate on the methylated bases isolated after the incubation of E. coli B tRNA or brewer's-yeast tRNA and the enzyme preparation derived from adenovirus-12-induced tumours and normal hamster tissues*

The incubation mixture, incubated at 37°C for 60 min, contained 500 μ mol of tris-HCl buffer (for tumour and liver, pH 8.5, and for muscle-connective tissue, pH 7.0), 200 μ mol of dithiothreitol, 100 μ mol of MgCl₂, 400 μ mol of ATP, 2 mg of tRNA, 0.5 μ Ci of *S*-adenosyl-L-[Me-¹⁴C]methionine, 10–12 mg of enzyme protein and, where indicated, 480 μ mol of ammonium acetate, in a total volume of 2 ml. The tRNA was extracted with phenol and precipitated from the aqueous phase by the addition of 2 vol. of 95% (v/v) ethanol and 0.1 vol. of 20% (w/v) potassium acetate. Hydrolysis with HClO₄ was for 1 h at 100°C and the bases were separated by two-dimensional paper chromatography.

Source of enzyme	Base	[¹⁴ C]Methylated base recovered (c.p.m./mg of tRNA)			
		<i>E. coli</i> B tRNA		Brewer's-yeast tRNA	
		Ammonium acetate present	Ammonium acetate absent	Ammonium acetate present	Ammonium acetate absent
Adenovirus-12-induced tumour	1-Methyladenine	15000	6350	2180	850
	3-Methyladenine	18500	8400	1215	585
	N ⁶ -Methyladenine	5000	1380	505	220
Muscle-connective tissue	3-Methyladenine	900	640	310	200
	Unidentified base Y	630	430	275	195
Liver	1-Methyladenine	400	240	300	140
	3-Methyladenine	1200	600	510	300

In view of previous work (McFarlane, 1969) indicating that the hamster muscle-connective tissue enzyme extract has two pH optima, bases were isolated after incubation at pH 7.0 and at pH 8.5 with either *E. coli* B tRNA or brewer's yeast tRNA as substrate. Table 2 shows that with either substrate base Y is present only after incubation at pH 7.0, whereas 3-methyladenine is found after incubation at either pH 7.0 or pH 8.5. Base Y was not found after incubation of cell-free extracts from either hamster liver or adenovirus-12-induced tumour with either *E. coli* B tRNA or yeast tRNA substrate.

In view of the reports of Kaye & Leboy (1968), Kerr (1969) and Pillinger & Borek (1969) that the presence of ammonium acetate can overcome the natural inhibitors present in various methylase extracts, we studied the effect of ammonium acetate in the assay medium on the pattern of base methylation. E. S. McFarlane & J. A. LeBlanc (unpublished work) have shown that 480 μ mol of ammonium acetate/2.0 ml of assay medium causes maximum stimulation of hamster liver, muscle-connective tissue and adenovirus-12-induced tumour enzyme extracts. Table 3 shows that the methylated bases isolated after incubation in the

presence of ammonium acetate are the same as those in its absence, although the total activity recovered is higher.

DISCUSSION

We have examined the tRNA methylase activities in enzyme extracts of adenovirus-12-induced hamster tumour, normal hamster liver and normal hamster muscle-connective tissue. We found that incubation of adenovirus-12-induced hamster tumour enzyme extract *in vitro* with tRNA from *E. coli* B or brewer's yeast resulted in the formation of three methylated bases, 1-methyladenine, 3-methyladenine and *N*⁶-methyladenine. Incubation of normal hamster muscle-connective tissue enzyme extract (the tissue from the area in which the adenovirus-12-induced tumour developed) *in vitro* with *E. coli* B tRNA or brewer's yeast tRNA resulted in the formation of two methylated bases, 3-methyladenine and an unidentified pyrimidine base Y. Incubation of normal hamster liver enzyme extract *in vitro* with either *E. coli* B tRNA or brewer's yeast tRNA resulted in the formation of two methylated bases, 1-methyladenine and 3-methyladenine. The extent of methylation was greater with adenovirus-12-induced tumour enzyme extract than with normal hamster liver or muscle-connective tissue enzyme extract. It is possible that the unknown base Y could correspond to the unknown nucleotide observed by Rogg & Staehelin (1969), but this has not been established.

Comparison of the tRNA methylase patterns found for normal hamster muscle-connective tissue and adenovirus-12-induced hamster tumour shows that the latter has two new enzyme activities and has lost one enzyme activity. These differences may mean that the part of the adenovirus-12 viral genome incorporated into the host's genome carried the information for the new enzymes. However, in view of Green's (1969) work, which indicates that the part of the viral genome transcribed codes for no more than five proteins, it may be that all hamster cells are predetermined to synthesize the same tRNA methylase enzymes and the differences found are due to cellular differentiation (Brown & Dawid, 1969). Both the hamster muscle-connective tissue and the adenovirus-12-induced tumour tissue may be predetermined to synthesize several tRNA methylase enzymes, and as a change in differentiation occurs a change may also occur in the tRNA methylase activities. These changes in tRNA methylase activities may be the result of changes in the natural inhibitors present within the cell (Kaye & Leboy, 1968).

Since the presence of ammonium acetate increases the extent of methylation *in vitro* by hamster liver, muscle-connective tissue and adeno-

virus-12-induced tumour enzyme extracts, but not the number of methylated bases isolated (Table 3), experiments are being carried out to determine the base sequences needed for methylation to occur (Baguley & Staehelin, 1969). Preliminary results indicate that the presence of ammonium acetate changes the base sequences in which methylation occurs, but not, as stated above, the number of methylated bases found (H. Wierzbicka & E. S. McFarlane, unpublished work). The effect of ammonium acetate might be explained in a number of ways: (a) it might modify the enzyme specificity, making it able to methylate sequences not normally methylated; (b) it might change the conformation of the tRNA, making more sites available; (c) it might remove an inhibitor, thus causing the activation of a number of enzymes that methylate the same substrate (Hurwitz *et al.* 1964). The work of Baguley & Staehelin (1969) suggests that the ammonium acetate changes the tertiary structure of the tRNA, making more sites available for methylation *in vitro*.

The changes observed on methylation at different pH values with muscle-connective tissue enzyme extract and not observed with either liver or adenovirus-12-induced tumour enzyme extracts are of note. It appears that the enzyme resulting in the methylation of the pyrimidine base Y is only active at pH 7.0 and only in muscle-connective tissue. The results for the formation of 3-methyladenine may indicate a wider pH optimum for this enzyme or it may indicate the presence of two enzymes that can methylate adenine at the 3-position (Hurwitz *et al.* 1964).

It is difficult to explain the absence of methylated guanine from our base analysis, especially in the light of the findings of Mittelman *et al.* (1967*b*) of methylated guanines in both normal hamster tissues and in SV40-induced hamster tumours with yeast tRNA as well as with hamster liver tRNA. Further experiments (G. J. Shaw & E. S. McFarlane, unpublished work) indicate that one of the bases methylated *in vivo* by hamster liver is a methylated guanine, probably *N*¹-methylguanine (Mittelman, Bonney, Hall & Grace, 1967*a*).

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