Bases Methylated *in vitro* by Cell-Free Extracts of Adenovirus-18-Induced Tumours

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The tRNA methylase activity *in vitro* of adenovirus-18-induced tumour was studied. The activity expressed per mg of protein was the same for extracts of tumours induced by either adenovirus-12 or adenovirus-18. The methylated bases isolated after incubation with extract from adenovirus-18-induced tumour were *N*¹-methylguanine, 1-methyladenine, 3-methyladenine and *N*⁶-methyladenine.

After the discovery by Trentin *et al.* (1962) that adenovirus-12 was oncogenic in hamsters, Huebner *et al.* (1962) confirmed this observation and showed that adenovirus-18 was also oncogenic.

Since the early work with oncogenic adenovirus, various workers have shown that many, if not all, human adenoviruses are oncogenic in hamsters or can transform rat embryo cells *in vitro*. Green and co-workers have subdivided the human adenoviruses into four groups, based on DNA-hybridization experiments and on the incidence of tumour production (Green, 1969).

We have shown (McFarlane & Lee, 1970; McFarlane, 1969; McFarlane & Shaw, 1968) that, in comparison with normal hamster liver and muscle-connective tissue tRNA methylases (enzymes that transfer methyl groups from *S*-adenosylmethionine to specific positions in tRNA), the tRNA methylases from adenovirus-12-induced tumour cells are more metabolically active, possess different substrate specificities, different pH optima, different stabilities on storage and methylate different bases. In view of the genetic relationship between adenovirus-12 and adenovirus-18, a study was made to determine whether the tRNA methylases isolated from tumours produced after injections of adenovirus-12 or adenovirus-18 were the same or different.

Materials and Methods

Animals and tumour production

Pregnant random-bred Syrian hamsters (*Mesocricetus auratus*) were obtained from Lakeview Hamster Colony, Newfield, N.J., U.S.A., a week before parturition.

The adenovirus-12 used in these experiments was Huie strain and was supplied by Dr. J. J. Trentin, Baylor University, Houston, Texas, U.S.A. When received, the virus was in the eighth HeLa-cell passage and has been passed in our laboratory up to the

twenty-third HeLa-cell passage. The TCID₅₀ (tissue-culture infective dose for 50% of the cells) for HeLa cells was 10^{3.4}/1.0 ml.

Adenovirus-18 was purchased from the American Type Culture Collection and has been passed twice in HeLa cells. The TCID₅₀ for HeLa cells was 10^{4.2}/1.0 ml. Within 24 h of birth, the liver of the newborn hamster was inoculated with 0.1 ml of adenovirus-12 or adenovirus-18. The hamsters were weaned at 3 weeks of age. Uninoculated hamsters of the same age were used as the source of control tissue. Animals inoculated with adenovirus-12 and adenovirus-18 were housed in separate rooms, and were fed on mouse pellets and given water *ad lib*.

Preparation of tRNA methylases

The enzyme was prepared as described by McFarlane & Shaw (1968). Hamsters were anaesthetized with ether and were killed by heart puncture. The appropriate tissue was quickly removed and homogenized in 6 vol. of cold 0.25 M-sucrose–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 was centrifuged in an International model B-60 refrigerated centrifuge at 105000g for 1 h at 4°C. The clear supernatant from the 105000g centrifugation was used for enzyme studies.

Preparation of methylated tRNA in vitro

The enzyme mixture contained 500 μmol of tris-HCl buffer, pH 8.5, 200 μmol of dithiothreitol, 100 μmol of MgCl₂, 2 mg of *Escherichia coli* B tRNA (Schwarz BioResearch Inc., Orangeburg, N.Y., U.S.A.) and 0.5 μCi of *S*-adenosyl-L-[Me-¹⁴C]-methionine (specific radioactivity 40.0 μCi/μmol; New England Nuclear Corp., Boston, Mass., U.S.A.) in a final volume of 1.0 ml. Enzyme extract (1.0 ml,

containing 6mg of protein) was added to each assay, i.e. sufficient enzyme to cause maximum incorporation of ^{14}C -labelled methyl groups into 1mg of tRNA.

The mixture was incubated at 37°C for 1h, at the end of which time the reaction mixture was adjusted to pH 9.5 by adding 2M- Na_2CO_3 . For determination of the total activity of the enzyme preparation the reaction was stopped by the addition of 0.5vol. of 6M-HCl and 1 vol. of 10% (w/v) trichloroacetic acid. The precipitate was transferred to a Whatman glass-fibre filter (GF/A) and was washed with 15ml of 5% (w/v) trichloroacetic acid, 10ml of aq. 95% (v/v) ethanol and 10ml of ether.

If the tRNA was to be isolated, the incubation mixture was mixed with 1 vol. of water and 2 vol. of aq. 90% (w/v) phenol. The phenol mixture was stirred at 4°C for 30min and then centrifuged at 1000g for 20min 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 aq. 95% (v/v) ethanol. The RNA was col-

lected by centrifugation in an International model PR-2 refrigerated centrifuge at 1000g for 20min at 4°C and the RNA pellet was dissolved in water (Zubay, 1966).

Hydrolysis and chromatography of nucleic acids

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

Two-dimensional descending chromatography was carried out on Whatman 3MM paper. The solvent for the first dimension was butan-1-ol-aq. 0.6M- NH_3 soln. (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), or propan-2-ol-11.6M-HCl-water (680:170:144, by vol.). The first dimension was run for either 40 or 60h at 20°C , and the second dimension was run for 24h at 20°C . The bases were identified by co-chromatography with standard preparations 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

Table 1. R_F values of purines and pyrimidines

The R_F values were obtained by chromatography on Whatman no. 1 paper, run in a descending fashion. Solvents: A, butan-1-ol-aq. 0.6M- NH_3 soln. (6:1, v/v); B, butan-1-ol-formic acid-water (77:11:12, by vol.); C, propan-2-ol-11.6M-HCl-water (680:170:144, by vol.).

Solvent ...	A	B	C
Adenine	0.32	0.27	0.24
1-Methyladenine	0.25	0.17	0.15
3-Methyladenine	0.37	0.19	0.20
N^6 -Methyladenine	0.61	0.44	0.34
N^6 -Dimethyladenine	0.67	0.47	0.35
7-Methyladenine	0.28	0.37	0.26
9-Methyladenine	0.20	0.30	0.28
Guanine	0.03	0.14	0.10
1-Methylguanine	0.26	0.12	0.12
3-Methylguanine	0.16	0.15	0.17
7-Methylguanine	0.10	0.10	0.18
9-Methylguanine	0.17	0.21	0.22
N^6 -Methylguanine	0.19	0.25	0.24
3-Methylxanthine	0.06	0.08	—
Cytosine	0.24	0.19	0.39
1-Methylcytosine	0.38	0.29	0.38
3-Methylcytosine	0.46	0.31	0.38
5-Methylcytosine	0.30	0.28	0.40
Uracil	0.20	0.33	0.67
1-Methyluracil	0.36	0.50	0.75
3-Methyluracil	0.51	0.42	0.84
5-Methyluracil	0.39	0.52	0.72
1,3-Dimethyluracil	0.60	0.68	0.76

Table 2. tRNA methylase activity in cell-free extracts from normal hamster liver and adenovirus-induced tumour

The incubation mixture, incubated at 37°C for 60min, contained 500 μmol of tris-HCl buffer, pH 8.5, 200 μmol of dithiothreitol, 100 μmol of MgCl_2 , 2mg of *E. coli* B tRNA, 0.5 μCi of *S*-adenosyl-L-[$\text{Me-}^{14}\text{C}$]-methionine and 6mg of enzyme protein in a total volume of 2.0ml. After incubation, the protein-nucleic acid precipitate was transferred to and washed on a Whatman glass-fibre filter and counted for radioactivity with a Nuclear-Chicago scintillation counter.

Expt. no.	Radioactivity (c.p.m./mg of tRNA)	
	1	2
Source of extract		
Adenovirus-18-induced tumour	15000	13964
Adenovirus-12-induced tumour	13296	16950
Normal hamster liver	1930	1206

Table 3. *Specific methylated bases isolated after incubation of E. coli B tRNA as methyl acceptor with enzyme preparations derived from adenovirus-induced tumours and normal hamster liver*

The incubation mixture, incubated at 37°C for 60 min, contained 500 μ mol of tris-HCl buffer, pH 8.5, 200 μ mol of dithiothreitol, 100 μ mol of MgCl₂, 2 mg of *E. coli* B tRNA, 0.5 μ Ci of *S*-adenosyl-L-[Me-¹⁴C]methionine and 6 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 aq. 95% (v/v) ethanol and 0.1 vol. of aq. 20% (w/v) potassium acetate. Hydrolysis was for 1 h at 100°C and the bases were separated by two-dimensional paper chromatography.

Bases isolated	Source of extract ...	[¹⁴ C]Methylated bases (c.p.m./mg of tRNA)		
		Adenovirus-18-induced tumour	Adenovirus-12-induced tumour	Normal liver
N ¹ -Methylguanine		700	0	0
1-Methyladenine		3001	2684	386
3-Methyladenine		5960	6036	741
N ⁶ -Methyladenine		750	798	0

Table 4. *tRNA methylase activity of adenovirus-18-induced hamster tumours, measured by using E. coli B tRNA previously methylated by extracts from adenovirus-12-induced tumour or normal liver*

The incubation mixture, incubated at 37°C for 60 min, contained 500 μ mol of tris-HCl, pH 8.5, 200 μ mol of dithiothreitol, 100 μ mol of MgCl₂, 2.0 mg of *E. coli* B tRNA (previously methylated by cell-free extracts from adenovirus-12 or adenovirus-18-induced tumour or normal liver with unlabelled *S*-adenosyl-L-methionine and then reisolated), 0.5 μ Ci of *S*-adenosyl-L-[Me-¹⁴C]methionine and 6 mg of cell-free protein extract from adenovirus-18-induced tumour in a total volume of 2.0 ml. After incubation the tRNA precipitate was transferred to a Whatman glass-fibre filter and counted for radioactivity in a Nuclear-Chicago scintillation counter.

Expt. no. ...	Radioactivity (c.p.m./mg of tRNA)	
	1	2
<i>E. coli</i> B tRNA	16795	12654
<i>E. coli</i> B tRNA previously methylated by adenovirus-12-induced tumour extract	451	304
<i>E. coli</i> B tRNA previously methylated by hamster liver extract	14061	9875
<i>E. coli</i> B tRNA previously methylated by adenovirus-18-induced tumour extract	0	0

(5-*t*-butylbenzoxazol-2-yl)thiophen. Radioactivity of samples was counted in a Nuclear-Chicago mk. I scintillation counter.

Duplicate samples were chromatographed on Dowex 50 (H⁺ form; X8) (Craddock *et al.*, 1968). The column was eluted at room temperature with a gradient of 250 ml each of 1M-HCl to 4M-HCl. Every two fractions were combined, evaporated to dryness over phosphorus pentoxide and dissolved in 0.1 ml of water. Each sample was fractionated according to the two-dimensional descending chromatography as described above. The *R_F* values for the various purines and pyrimidines are given in Table 1.

Results and Discussion

Table 2 shows that a cell-free extract of adenovirus-18-induced tumour can transfer ¹⁴C-labelled methyl groups from *S*-adenosyl-L-[¹⁴C]methionine to *E. coli* B tRNA. The activity found in the cell-free extract of adenovirus-18-induced tumour is approximately the same as that found in adenovirus-12-induced tumour cell-free extract, and about eight times the activity found in normal hamster liver. The addition of 0.192M-ammonium acetate to the incubation mixture *in vitro* can double the enzyme activity observed with all cell-free extracts, i.e. from adenovirus-12-induced tumour, adenovirus-18-induced tumour and normal hamster liver. The optimum stimulation with all tissues occurred at 0.192M-ammonium acetate; at lower or higher ammonium acetate concentrations the enzyme activity was less than that observed in the enzyme extract without ammonium acetate.

Table 3 shows the bases isolated after incubation of *E. coli* B tRNA and various cell-free enzyme extracts. From adenovirus-18-induced tumour cell-free extract four methylated bases were produced: N¹-methylguanine, 1-methyladenine, 3-methyladenine and N⁶-methyladenine. These bases agree reasonably

Chemical Co., Los Angeles, Calif., U.S.A. After chromatography the paper was cut in pieces 3 cm wide and 1 cm in depth. All samples were placed in vials with 15 ml of toluene containing 0.4% 2,5-bis-

Table 5. *Specific methylated bases isolated after incubation of E. coli B tRNA (previously methylated with cell-free extract from adenovirus-12-induced tumour or hamster liver and reisolated) as methyl acceptor with enzyme preparations from adenovirus-18-induced tumours*

The incubation mixture, incubated at 37°C for 60 min, contained 500 μ mol of tris-HCl buffer, pH 8.5, 200 μ mol of dithiothreitol, 100 μ mol of MgCl₂, 2.0 mg of *E. coli* B tRNA (previously methylated by cell-free extracts from adenovirus-12-induced tumour or normal liver with unlabelled *S*-adenosyl-L-methionine and reisolated), 0.5 μ Ci of *S*-adenosyl-L-[Me-¹⁴C]methionine and 6 mg of cell-free protein extract from adenovirus-18-induced tumour in a total volume of 2.0 ml. After incubation the tRNA precipitate was transferred to a Whatman glass-fibre filter and counted for radioactivity in a Nuclear-Chicago scintillation counter.

tRNA previously methylated by ...	[¹⁴ C]Methylated bases (c.p.m./mg of tRNA)	
	Adenovirus-12-induced tumour extract	Hamster liver extract
N ¹ -Methylguanine	495	602
1-Methyladenine	0	2226
3-Methyladenine	0	4824
N ⁶ -Methyladenine	65	901

well with those (N¹-methyladenosine and N¹-methylguanosine) found by Mittelman *et al.* (1967b) in a preparation of yeast tRNA methylated *in vitro* with hamster liver extract. No methylated pyrimidines were found, either in the present work or in that of Mittelman *et al.* (1967b), after incubation with hamster liver extract. N¹-Methylguanine, the most common methylated base present in hamster liver (Mittelman *et al.*, 1967a), could not be detected after incubation with either adenovirus-12-induced tumour or liver cell-free extract. N⁶-Methyladenine has been isolated after incubation with adenovirus-12-induced tumour cell-free extracts (McFarlane & Lee, 1970). Although no standard preparation of N²-methylguanine or N²-dimethylguanine was available, these bases were eliminated by using published *R_f* values. It has also been shown (Iwanami & Brown, 1968) that all guanines elute from Dowex 50 in one general area; only one radioactive spot was found in the eluate from the guanine area and it corresponded to N¹-methylguanine.

To determine whether the same bases are methylated in the same position by enzymes from adenovirus-12-induced tumour and adenovirus-18-induced tumour, *E. coli* B tRNA was incubated with unlabelled *S*-adenosyl-L-methionine and enzyme of adenovirus-12-induced tumour, reisolated and then incubated with *S*-adenosyl-L-[Me-¹⁴C]methionine and adenovirus-18-induced tumour extract. Tables 4 and 5 show that the tRNA already methylated *in vitro* by enzyme of adenovirus-12-induced tumour can be further methylated to a limited amount. This further methylation appears to be limited to formation of N¹-methyladenine and a very small amount of N⁶-methyladenine. When the *E. coli* B tRNA was previously methylated with normal hamster liver, reisolated and then incubated with adenovirus-18-

induced tumour extract, a large amount of methylation occurred. All bases isolated were found to be present after this second step of methylation by adenovirus-18-induced tumour enzyme, as if methylation was carried out by adenovirus-18 tumour enzyme alone. It would appear that the extracts from adenovirus-12- and adenovirus-18-induced tumour cells *in vitro* methylate the same bases, and at the same positions in the tRNA, with the exception of N¹-methylguanine. Why N¹-methylguanine is not detected after incubation with extract of adenovirus-12-induced tumour or normal liver cell-free extract is not known. It may be that this enzyme, N¹-guanine methylase, is either more unstable or has a more potent inhibitor (Kerr, 1970) than the other methylases, as it is sometimes detected in very small amounts during the purification of normal hamster liver methylases (E.S. McFarlane, unpublished work). The presence of tRNA methylase inhibitors in the crude enzyme extract could influence the bases methylated *in vitro* and explain variations between laboratories.

It would appear that the methylases present in adenovirus-12- and adenovirus-18-induced tumours are very similar as far as base and site specificity are concerned; this is not surprising when one considers the degree of relationship between these two viruses (Green, 1969).

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