A Study of Various Changes in Transfer Ribonucleic Acid Methylase Activity during Adenovirus-12 Transformation *in vitro*

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A number of parameters were used to correlate a change in control or expression of tRNA methylase activity and the transformation by adenovirus-12 *in vitro*. The earliest change observed corresponding to visible morphological changes was a decrease in the tRNA methylase inhibitor(s).

The possibility that changes in the cellular content of tRNA methylases might play a role in the development and in the growth of tumours has been postulated by Borek and his co-workers (Borek, 1968). An increase in the activity and/or change in the base specificity of the tRNA methylases in a number of cell-free extracts has been reported by numerous workers (Borek, 1971; Borek & Kerr, 1972). However, reports have recently begun to appear indicating that the contents of the assay medium *in vitro* could influence the expression of various methylated bases (Leboy, 1970, 1971; Kerr, 1972). No doubt similar conditions could exist *in vivo* resulting in the expression of one tRNA methylase in one tissue and not in another (Leboy, 1970).

A number of difficulties are encountered when tumour cells isolated after growth in a whole organism are compared with the normal tissue. The ionic concentration in the two tissues may not be the same and the cell type from which the tumour growth resulted cannot always be determined. Factors which appear to influence the expression of the tRNA methylases include the cell type from which the tumours arose, the absence of specific tRNA methylase inhibitors and the composition of the assay medium *in vitro*. To control as many of these variables as possible a study was carried out comparing tRNA methylase expression in normal gerbil lung cells and normal hamster kidney cells with the respective cell types after transformation *in vitro* by adenovirus-12.

A number of parameters were used to determine the expression of tRNA methylases and also to determine the time of adenovirus-12 transformation. The study was set up to relate (a) changes in tRNA methylase activity, (b) expression of a new methylated base and (c) the decrease in tRNA methylase inhibitor present in normal cells, to (a) the morphological changes which occur after transformation, (b) the appearance of a tumour-specific transplantation (T) antigen and (c) the production of tumours after

inoculation of transformed cells into newborn gerbils or newborn hamsters.

From these comparisons it appears that the earliest change observed corresponding to visible morphological changes *in vitro* was a decrease in the tRNA methylase inhibitor(s).

Materials and Methods

Virus

Adenovirus-12, strain HU1E, was obtained from Dr. J. J. Trenton, Baylor University, Houston, Texas, U.S.A., and was passed 22 times in HeLa cells in our laboratory. The TCID₅₀ (tissue culture infective dose) for HeLa-cell cultures was $10^{3.4}$ for 1.0 ml.

Cell cultures

Lungs were removed aseptically from young (3-months old or less) gerbils after killing by heart puncture. The cells were minced, washed, treated with 0.25% trypsin (Embil & Faulkner, 1964) and seeded at 10^4 cells per milk-dilution bottle in Eagle's basal medium (GIBCO, Grand Island, N.Y., U.S.A.) modified to contain four times the concentration of amino acids and vitamins and supplemented with 20% foetal calf serum (Embil & Faulkner, 1964). Cells to be transformed were exposed to 2.0 ml of adenovirus-12 suspension per milk-dilution bottle for 2 h. After exposure to the virus the cells were not treated with trypsin for 21 days, but the medium was changed twice a week (Shaw & McFarlane, 1969).

Hamster kidneys were removed from hamsters within 6 weeks of birth and were treated in the same manner as were the gerbil lung cells.

All cell cultures were monitored daily for morphological change. After the initial 21 days the cells were treated with trypsin as a routine at 8-day intervals and the medium was changed every 4 days.

Preparation of tRNA methylases and tRNA methylase inhibitors

The transformed and the normal cells were treated with trypsin, suspended in Eagle's basal medium, counted by exclusion-staining with Trypan Blue and pelleted by centrifugation for 10 min at 200g in an International refrigerated centrifuge model PR2. The cell pellet was suspended in 0.2M-sucrose containing 0.05M-Tris-HCl, pH 8.2, 0.1 mM-EDTA and 0.1 mM-dithiothreitol at a concentration of 1.0 ml for each 10^6 cells. The cell extract was sonicated for 30 s in a Raytheon sonicator at 10 kHz.

The sonicated cell-free extract was centrifuged for 15 min at 15000g in an International refrigerated centrifuge model B-20. After this the supernatant was centrifuged for 1 h at 105000g in an International refrigerated centrifuge model B-60. The 105000g supernatant was the source of the tRNA methylase enzymes and the tRNA methylase inhibitor(s).

The enzyme was separated from the inhibitor by the method of Kerr (1971). The pH of the 105000g supernatant was adjusted to 5.0 by the addition of 1M-acetic acid. The precipitate was collected by centrifugation for 10 min at 10000g in an International refrigerated centrifuge model B-20. The precipitate was dissolved in 0.25M-sucrose containing 0.05M-Tris-HCl, pH 8.2, 0.1 mM-EDTA and 0.1 mM-dithiothreitol. The pH of the 105000g supernatant was adjusted to pH 8.2 with 1M- Na_2CO_3 and this supernatant was used as the source of tRNA methylase inhibitor.

Test for tumour antigen

The complement-fixation test was used to investigate the transformed cells for the presence of tumour-specific transplantation antigens (Huebner *et al.*, 1963). Serum positive for T antigen was obtained from Flow Laboratories, Rockville, Md., U.S.A. Transformed and normal cells sonicated for 30 s and centrifuged at 15000g for 15 min were tested against 4 units of antibody (Sever, 1962).

Assay of tRNA methylases in vitro

The enzyme mixture contained 500 μmol of Tris-HCl buffer, pH 8.5, 200 μmol of dithiothreitol, 100 μmol of MgCl_2 , 1 mg of *Escherichia coli* B tRNA (Schwartz BioResearch Inc., Orangeburg, N.J., U.S.A.) and 0.5 μCi of *S*-adenosyl-L-[$\text{Me-}^{14}\text{C}$]-methionine (specific radioactivity 40 $\mu\text{Ci}/\mu\text{mol}$; New England Nuclear Corp., Boston, Mass., U.S.A.) in a final volume of 0.8 ml. Enzyme extract equivalent to 10^6 cells (transformed or normal) was added to each assay.

The mixture was incubated at 37°C for 1 h, at the end of which time the reaction mixture was adjusted to pH 9.0 with 1M- Na_2CO_3 and incubated for a further 30 min. For determination of the total activity of the enzyme preparation the reaction was stopped

by the addition of 0.5 vol. 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 washed with 15 ml of 5% (w/v) trichloroacetic acid, 10 ml of aq. 95% ethanol and 10 ml 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 30 min and then centrifuged at 1000g for 20 min at 4°C in an International refrigerated centrifuge model PR2. The aqueous layer was removed and the tRNA was precipitated by the addition of 0.1 vol. of 20% (w/v) potassium acetate and 2 vol. of aq. 95% ethanol. The tRNA was collected by centrifugation in an International refrigerated centrifuge model PR2 at 1000g for 20 min at 4°C and the tRNA 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 60 min, diluted fivefold with water and clarified by centrifugation. Hydrolysis with HClO_4 yields both free purine and pyrimidine bases (Bendich, 1957). Duplicate samples were hydrolysed in 1M-HCl at 100°C for 60 min. This gave free purine bases and pyrimidine nucleosides (Schmidt, 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 (6:1, v/v) and the solvent for the second dimension was butan-1-ol-formic acid-water (77:11:12, by vol.) or propan-2-ol-11.6M-HCl-water (340:85:72, by vol.) (Markham & Smith, 1949).

The conditions of separation of the bases and the standards co-chromatographed were as described previously (McFarlane, 1972).

Results and Discussion

For 21 days after exposure of either hamster or gerbil cells to adenovirus-12 the cells were not treated with trypsin. After this they were treated with trypsin as a routine at 8-day intervals and the medium was changed every 4 days. After trypsin treatment the virus-exposed cells grew very slowly at first, but by the fourth trypsin treatment they were growing faster than the control cells. By the 5th day after trypsin treatment several piles of cells were seen on the cell sheet. These piles of (transformed) cells were scraped off, treated with trypsin and placed in a milk-dilution bottle with Eagle's basal medium modified to contain 4 times the concentration of amino acids and vitamins and 20% calf serum. The cultures which arose from these piles of cells were treated with trypsin five more times (to increase the number of cells) and these cells were used in the various assays.

There was no significant difference in the time of

Table 1. *Properties of adenovirus-12-transformed and normal cell lines*

The transformed cells were isolated from visible piles of cells after the fourth trypsin treatment after exposure to the virus and were then treated with trypsin the number of times noted. The cells used in the various tests were sonicated for 30s in a Raytheon sonicator at 10kHz and centrifuged as described in the text. The T antigen was determined by complement fixation as described by Huebner *et al.* (1963). The incubation mixture for tRNA methylase activity contained 500 μ mol of Tris-HCl buffer, pH 8.5, 200 μ mol of dithiothreitol, 100 μ mol of MgCl₂, 1 mg of *E. coli* B tRNA, 0.5 μ Ci of *S*-adenosyl-L-[Me-¹⁴C]methionine and the pH 5-precipitated tRNA methylases from 10⁶ transformed or normal cells. To determine the tRNA methylase inhibitor activity from 10⁶ transformed or normal cells the pH 5 supernatant whose pH had been readjusted to pH 8.2 was added to the above methylase incubation mixture and the tRNA methylase activity was determined. After incubation the protein-nucleic acid precipitate was transferred to and washed on a Whatman glass-fibre filter and counted for radioactivity in a Nuclear-Chicago scintillation counter as described by McFarlane (1972).

Cell type	Number of times treated with trypsin after isolation as transformed cells	T antigen	tRNA methylase activity (c.p.m./mg of tRNA)	tRNA methylase inhibitor activity (c.p.m./mg of tRNA)
Gerbil lung	Not exposed to virus	Negative	3462	1043
Adenovirus-12-transformed gerbil lung	5	Negative	3608	3264
	10	1:4	5206	5094
	20	1:16	10054	9648
Hamster kidney	Not exposed to virus	Negative	5801	1808
Adenovirus-12-transformed hamster kidney	5	1:2	5908	5001
	10	1:8	8264	7614
	20	1:32	15824	13843

Table 2. *Specific methylated bases isolated after incubation of E. coli B tRNA as methyl acceptor with enzyme preparations derived from normal and adenovirus-12-transformed cells*

The transformed cells used were isolated from visible piles of cells after exposure to the adenovirus-12 and were then treated with trypsin the number of times noted. 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, 1 mg of *E. coli* B tRNA, 0.5 μ Ci of *S*-adenosyl-L-[Me-¹⁴C]-methionine and the tRNA methylases precipitated from 10⁶ cells. 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 on the sulphonic resin Dowex 50 (H⁺ form; X8) followed by two-dimensional paper chromatography (McFarlane, 1972).

Cell type	Number of times treated with trypsin after isolation as transformed cells	[¹⁴ C]Methylated bases (c.p.m./mg of tRNA)			
		1-Methyl-adenine	1-Methyl-guanine	N ⁶ -Methyl-adenine	Unknown pyrimidine
Gerbil lung	Not exposed to virus	0	1043	0	0
Adenovirus-12-transformed gerbil lung	5	0	2130	0	0
	10	502	3043	490	0
	20	784	5469	834	0
Hamster kidney	Not exposed to virus	0	2164	0	0
Adenovirus-12-transformed gerbil lung	5	0	2843	0	0
	10	364	4498	641	384
	20	498	7647	898	598

appearance of transformed cells in either the hamster or gerbil cell lines.

The various properties of the normal hamster or gerbil cell lines and the adenovirus-12-transformed hamster or gerbil cell lines are compared in Table 1, which shows that there was limited change in the production of T antigen and in the tRNA methylase activity after five passages after isolation of transformed cells. However, even at this time the activity

of tRNA methylase inhibitor present in the normal cells was decreased in the transformed cells. When the normal-cell inhibitor was added to normal-cell tRNA methylase extract a decrease in tRNA methylase activity was seen. This decrease in activity was not observed with transformed-cell inhibitor. If normal-cell inhibitor was added to transformed-cell tRNA methylase the transformed-cell tRNA methylase was inhibited (not shown in Table 1). No change in

either tRNA methylase activity or in tRNA methylase inhibitor activity could be seen after trypsin treatment of normal hamster or gerbil cells up to 20 treatments.

The presence of T antigen and increased tRNA methylase activity were observed at later passages.

After injection of 10^5 transformed cells at passage 5 into 30 newborn hamsters or 30 newborn gerbils tumour production was seen between 6 and 7 weeks. All animals developed tumours in 12 weeks. If transformed cells from passage 10 or more were used tumours developed within 4–5 weeks. All animals developed tumours in 8 weeks.

It appears that the first detectable biochemical change is a decrease in the tRNA methylase inhibitor activity, which correlates with the appearance of piles of cells in tissue cultures. These piles of cells can produce tumours *in vivo*.

Further studies were carried out to determine the time of appearance of expression of any new tRNA methylases. Table 2 shows that although several new bases were found in the transformed cells, their appearance was not detected until passage 10 or later. Similar results were noted (E. S. McFarlane, unpublished work) when the bases were methylated *in vivo*. No new bases were noted until at least passage 10. It would appear that for a new tRNA methylase to be expressed a change first has to occur in normal tRNA methylase inhibitors within the cell. This might mean that the change induced by the presence of the virus genetic material is in the inhibitors, which in

turn results in change of tRNA methylase expression. The new tRNA methylases may result from derepression of normal cellular tRNA methylases.

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