The Postnatal Methylation of Transfer Ribonucleic Acid in Brain

EVIDENCE FOR THE METHYLATION OF PRECURSOR TRANSFER RIBONUCLEIC ACID

By ELAHE ELAHI* and OTTO Z. SELLINGER†
*Laboratory of Neurochemistry, Mental Health Research Institute,
University of Michigan Medical Center, Ann Arbor, MI 48109, U.S.A.*

(Received 17 October 1978)

Incubation of 3-day-old rat brain with L-[methyl-³H]methionine resulted in the rapid labelling of low-molecular-weight cytoplasmic RNA. Electrophoresis in 15% polyacrylamide gels provided evidence for the methylation of precursor tRNA molecules, and high-performance liquid chromatography demonstrated N²-methylguanine to be the predominant methylated base formed during the first 2 min of labelling.

In most studies of tRNA methylation *in vitro*, eukaryotic tRNA species have proved to be poor substrates for homologous tRNA methyltransferases (Nau, 1976). Hence prokaryotic tRNA species, which are less abundantly methylated than their eukaryotic counterparts (Randerath *et al.*, 1974), have been used as substrates, and, more recently, experimentally hypomethylated eukaryotic tRNA species have also served this purpose (Jank & Gross, 1974; Friedman, 1977). However, since we recently demonstrated the occurrence of homologous tRNA methylation in the brain of the immature rat (Cummins *et al.*, 1975; Salas *et al.*, 1976), it became necessary to investigate to what extent such methylation involves tRNA of mature size as against larger-precursor tRNA species (Munns & Sims, 1975; Majima *et al.*, 1977). The present report presents evidence that indicates that precursor tRNA serves as substrate for a significant portion of the tRNA-methylating reactions taking place in the 3-day-old rat brain. A preliminary report of some of the findings has appeared (Sellinger *et al.*, 1978).

Experimental

Labelling and isolation of low-molecular-weight cytoplasmic RNA

The cerebral cortices of 3-day-old Sprague–Dawley rats were dissected out rapidly and minced on ice into fragments of approx. 1 mm³. The mince from two cortices was transferred to a 50 ml Erlenmeyer flask containing 15 ml of incubation medium preincubated

at 37°C in an atmosphere of O₂/CO₂ (19:1). The incubation medium was similar to that used by Judes *et al.* (1973) and contained, in addition, 1.36 g of sodium formate/litre and 10 mCi of L-[methyl-³H]-methionine (11.25–15.0 Ci/mmol; New England Nuclear, Boston, MA, U.S.A.; or Research Products International, Elk Grove Village, IL, U.S.A.). The pH of the medium was adjusted to 7.4 with 7.5% (w/v) NaHCO₃. After incubation for 2, 5, 10 or 45 min, the flasks were placed on ice and 10 ml of ice-cold Hanks solution (Microbiological Associates, Walkerville, MD, U.S.A.), supplemented with 1 mM-L-methionine, was added. Tissue fragments were collected by centrifugation at 5900g for 7 min, the pellet was washed three times with the methionine-supplemented Hanks solution, and then homogenized in 3.5 vol. of 0.25 M-sucrose, containing 35 mM-Tris/HCl, pH 7.4, 25 mM-KCl and 5 mM-MgCl₂. The homogenate was centrifuged at 17300g for 10 min, and the supernatant was recentrifuged at 160000g for 90 min, yielding the high-speed supernatant, which was made 1% (w/v) in sodium dodecyl sulphate. An equal volume of phenol/*m*-cresol/water (7:1:2, by vol.) was added, and the RNA extracted by vigorous shaking for 10 min at 65°C and 15 min at room temperature. The phenol phase was re-extracted at room temperature with 0.5–1 vol. of 0.1 M-sodium acetate. The combined aqueous layers were re-extracted until no interface was detected, and the RNA was precipitated three times by addition of 0.1 vol. of 20% (w/v) potassium acetate, pH 5.5, followed by 2.5 vol. of 95% ethanol.

Electrophoresis

Electrophoresis was performed in 10 and 15% (w/v) polyacrylamide gels, which were stained with Methylene Blue (Peacock & Dingman, 1967).

* Present address: Department of Biochemistry and Biophysics, School of Medicine, University of California, San Francisco, CA 94143, U.S.A.

† To whom requests for reprints should be addressed.

Extraction of [methyl-³H]RNA from gel slices

Piperidine (0.5–1.0 ml; 10%, w/v, in 1 mM-EDTA) was added to vials containing one or two 1 mm-length gel slices, and the capped vials were incubated at 55°C for 24 h. After drying at 60°C water (0.5 ml) was added and the capped vials were vigorously mixed. Then 10 ml of 3a70B scintillation 'cocktail' (Research Products International) was added and the radioactivity determined.

Methylated-base analysis

Low-molecular-weight cytoplasmic RNA species were hydrolysed, and their methylated base constituents identified by h.p.l.c. (high-performance liquid chromatography) and quantified by liquid-scintillation counting. The procedures have been described in detail elsewhere (Salas *et al.*, 1976; Salas & Sellinger, 1977).

Fig. 1. Electrophoresis of RNA

This was performed by the method of Peacock & Dingman (1967) on 10% polyacrylamide gels. Gels were 8.5 cm long and direction of migration was from top to bottom (toward anode). (a) RNA from *E. coli*. Tube 1: 2.4 µg of *E. coli* tRNA and 2.4 µg of 5S ribosomal RNA (Miles Biochemicals, Elkhart, IN, U.S.A.); tube 2: 5.6 µg of tRNA^{Val} and 2 µg of tRNA^{Phe}. (b) 0.104 mg of low-molecular-weight cytoplasmic RNA from brain tissue, incubated for 5 min. The brain RNA was prepared as described in the Experimental section.

Results and Discussion

The extraction procedure resulted in a recovery of 16–24 µg of RNA per cortex. (One A_{260} unit corresponds to the amount of RNA in 1 ml, measured in a 10 mm cell. A conversion factor of 1 A_{260} unit/40 µg of RNA was used.) The A_{260}/A_{280} ratio of the extracted RNA was consistently greater than 2, and its specific radioactivity (d.p.m./µg) increased markedly over the time from 2 min (11) to 45 min (3977). The migration pattern of an RNA sample prepared from brain mince incubated for 5 min is

Fig. 2. Radioactivity profiles of the low-molecular-weight cytoplasmic RNA molecules on 10% polyacrylamide gels

The isolation, electrophoresis and extraction of the radioactive RNA molecules from the gel slices were as described in the Experimental section. RNA was extracted from one 1 mm-length gel slice after (a) 5, (b) 20 and (c) 45 min of incubation. The significance of zones I and II is explained in the text. Dashed lines demarcate zones I and II. The numerical I/II values on the Figure represent ratios of radioactivity with the value of zone I normalized to unity. Vials were counted to below an error factor of 7%. Abbreviation: BPB, Bromophenol Blue tracking dye.

shown in Fig. 1(b). Most of the RNA migrates as an intense band in the lower portion of the gel. However, particularly in this gel, which was intentionally overloaded to permit recovery of detectable radioactivity, several slower-moving bands are also visible. *Escherichia coli* 5S ribosomal RNA and *Escherichia coli* bulk and specific tRNA were electrophoresed on parallel gels (Fig. 1a). The position of 5S RNA, which migrated as a single band when electrophoresed alone (not shown), is indicated in tube 1, uppermost band. The additional bands in tube 1 are contributed by the components of bulk *E. coli* tRNA. As with the cortical RNA, although most of the *E. coli* tRNA migrated as a single intense and wide band, some species migrated more slowly

in the region ahead of the 5S marker and as thin, distinct bands. Tube 2 (Fig. 1a) illustrates the widely different migration, on co-electrophoresis, of two specific *E. coli* isoaccepting tRNA species, tRNA^{Val} and tRNA^{Phe}.

The radioactivity profiles of the brain RNA (Figs. 2 and 3) reveal a broad region of radioactivity in the lower halves of the gels. *E. coli* 5S ribosomal RNA run on parallel gels (not shown) migrated behind the region of radioactivity on both sets of gels. Given the broad region of migration of *E. coli* tRNA species (Fig. 1a), the widely dispersed radioactivity may in part reflect methylation of the entire gamut of mature-size cerebral tRNA species present. However, concurrent methylation of cerebral pre-

Fig. 3. Radioactivity profiles of low-molecular-weight cytoplasmic RNA molecules on 15% polyacrylamide gels. These profiles were obtained as described in the legend to Fig. 2. RNA was extracted from one 1 mm-length gel slice after (b) 5, (c) 20 and (d) 45 min of incubation, whereas two 1 mm slices were combined to extract the RNA labelled for 2 min (a). The error factor in counting was less than 7%.

Table 1. Distribution of [methyl-³H]bases in cortical low-molecular-weight cytoplasmic RNA

For the isolation and hydrolysis of the RNA and for its base analysis, see the Experimental section. Results are from two to three determinations for each incubation time. The samples contained approx. 2000, 5000, 15000 and 30000 d.p.m. at 2, 5, 20 and 45 min. The values represent percentages of the radioactivity after h.p.l.c. normalized to a recovery of 100%.

Incubation time (min)	N ² -Methyl-guanine	1-Methyl-adenine	5-Methyl-cytosine	1-Methyl-guanine	5-Methyl-uracil	1-Methyl-hypoxanthine	7-Methyl-guanine	N ² -Dimethyl-guanine	2-Methyl-adenine	N ⁶ -Methyl-adenine
2	67.4	4.1	4.1	1.3	0	13.6	5.2	4.2	0	0.1
5	44.1	6.7	7.8	8.2	3.0	12.6	9.2	7.7	0	0.7
20	32.6	11.9	11.7	13.1	1.8	11.3	9.1	7.8	0	0.7
45	31.9	17.7	12.6	10.6	1.5	9.5	7.6	7.3	0.2	1.1

cursor tRNA species is indicated by the shift in the shape of the radioactivity profile toward smaller species with increasing incubation time. A fairly distinct 'point of inflection' is evident in corresponding positions of the radioactivity profiles of RNA from 5, 20 and 45 min of incubation of 10% polyacrylamide gels (Fig. 2). This point was arbitrarily chosen to divide the radioactive region into zones I and II, corresponding to larger and smaller RNA species respectively. Calculation of the zone I/zone II radioactivity ratio in 10% gels showed a 2-fold decrease between 5 and 45 min (Fig. 2). A more pronounced shift in the profile was detected after electrophoresis in 15% gels (Fig. 3) and by analysis of a 2 min incubation sample (Fig. 3a). The zone I/zone II ratio decreased 37-fold between 2 and 45 min. This shift is interpreted as reflecting cleavage of previously methylated precursor tRNA molecules to mature size. An alternative explanation, that larger tRNA molecules as a class are methylated more rapidly than smaller ones, seems unlikely. To the best of our knowledge, there is no evidence for a rapidly methylated low-molecular-weight cytoplasmic RNA species not related to tRNA molecules.

The time course of the formation of the individual [methyl- ^3H]bases (Table 1) illustrates a temporal shift in their distribution, the most striking being that affecting formation of N^2 -[^3H]methylguanine, which decreased from 67.5 to about 33% between 2 and 20 min. Significant shifts also characterized the formation of N_2 -[^3H]dimethylguanine, which almost doubled between 2 and 5 min, and those of 1-[^3H]methyladenine, 5-[^3H]methylcytosine and 1-[^3H]methylguanine, which continued to increase over virtually the entire 45 min incubation period. The five minor bases also underwent characteristically individual shifts.

The present findings extend the occurrence of precursor tRNA species to immature rat brain. Blatt & Feldmann (1973) obtained evidence for a similarly rapid (2 min) labelling of precursor tRNA in yeast cells, and noted that its methylated bases also became labelled within 2 min. This finding suggests that methylation of precursor and mature-size tRNA can proceed simultaneously, although not necessarily in synchrony (Munns & Sims, 1975). A

similar conclusion may be drawn from the results shown in Table 1.

Munns & Sims (1975) showed that base methylation occurs at different stages of tRNA processing. Although the findings in Table 1 were not obtained under conditions identical with those used by Munns & Sims (1975), they provide evidence that suggests the following methylation sequence: N^2 -methylguanine forms early on precursor tRNA, 5-methyluracil forms later on both precursor and mature-size tRNA, and 1-methyladenine, 5-methylcytosine and 1-methylguanine form late, mainly on mature-size tRNA. It is important to note, however, that 'early', 'later' and 'late' mirror only an approximation of the methylation sequence of any individual tRNA species, be it precursor or mature size.

This work was supported in part by grant NINCDS 06294 from the U.S. Public Health Service. E. E. was a post-doctoral trainee on training grant MH 07417 from the U.S. Public Health Service.

References

- Blatt, B. & Feldmann, H. (1973) *FEBS Lett.* **37**, 129–133
- Cummins, C. J., Salas, C. E. & Sellinger, O. Z. (1975) *Brain Res.* **96**, 407–412
- Friedman, S. (1977) *Biochem. Biophys. Res. Commun.* **74**, 1505–1510
- Jank, P. & Gross, H. J. (1974) *Nucleic Acids Res.* **1**, 1259–1268
- Judes, C., Sensenbrenner, M., Jacob, M. & Mandel, P. (1973) *Brain Res.* **51**, 241–251
- Majima, R., Tsutsumi, K.-I., Suda, H. & Shimura, K. (1977) *J. Biochem. (Tokyo)* **82**, 1161–1166
- Munns, T. W. & Sims, H. F. (1975) *J. Biol. Chem.* **250**, 2143–2149
- Nau, F. (1976) *Biochimie* **58**, 629–645
- Peacock, A. C. & Dingman, C. W. (1967) *Biochemistry* **6**, 1818–1827
- Randerath, E., Chia, L. S. Y., Morris, H. P. & Randerath, K. (1974) *Biochim. Biophys. Acta* **366**, 159–167
- Salas, C. E. & Sellinger, O. Z. (1977) *J. Chromatogr.* **133**, 231–236
- Salas, C. E., Cummins, C. J. & Sellinger, O. Z. (1976) *Neurochem. Res.* **1**, 369–384
- Sellinger, O. Z., Dainat, J., Elahi, E. & Salas, C. E. (1978) *Trans. Am. Soc. Neurochem.* **9**, 144