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Separation and characterization of the main methylated nucleobases from nuclear, cytoplasmic and poly $(A)^+$ RNA by high-performance liquid chromatography and mass spectrometry

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Abstract

We were able to detect nine methylated nucleobases (3-methyluracil, 1-, 2-, 3- and 7-methylguanine, 1-, 2-, 3- and 6-methyladenine) in RNA from rat and calf liver, baker's yeast, *Torula* and *Euglena* cells by using reversed-phase high-performance liquid chromatography and thermospray mass spectrometry. Total cellular, nuclear, cytoplasmic and poly $(A)^+$ RNA from rat liver showed marked methylation, mainly of 1- and 3-methylguanine, and 3- and 2-methyladenine. These bases were especially abundant in nuclear RNA and, to a lesser extent, in poly $(A)^+$ RNA. In contrast, 7-methylguanine and 6-methyladenine were poorly represented in poly $(A)^+$ RNA.

1. Introduction

The natural methylated nucleobases in eukaryotic DNA are limited to thymine (Thy) and 5-methylcytosine (5-mCyt). Thymine is one of the main four DNA constituents, while 5-mCyt represents only 1–3% of the DNA nucleobases in animal tissues [1,2]. Nonetheless, 5-mCyt is important given its presumed role in regulating gene expression (see Refs. [3,4] for review). Another minor methylated nucleobase, 6-methyladenine (6-mAde), is known to intervene in the defense of prokaryotic DNA against nucleases [5,6]. It was recently demonstrated that 6-mAde is involved in the replication of *Escherichia coli* DNA [7], and it is also found in the DNA of some unicellular eukaryotes (*Chlamydomonas reinhardi*, *Tetrahymena pyriformis*) [8].

The methylated bases in RNA are far more diverse, but their distribution, amounts and

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function in the different RNA species are poorly documented. Numerous rare nucleobases have been identified in tRNA. Modifications of tRNA involve methylation not only on the base moieties but also on the ribose moiety. Thus, among the 29 modified nucleosides of *S. typhimurium* and *E. coli* tRNA, Buck et al. [9] have identified four that are methylated on the ribose ring and eight that are methylated on the base moiety, including the 5-methyluridine (ribothymidine). Furthermore, among the 67 identified nucleosides, Gehrke and Kuo [10] have identified 33 (10 A, 5 G, 5 C and 13 U) that are methylated on the base and/or sugar moiety.

Methylation of ribosomal RNA is known as a maturation modification like that occurring on cytosine after DNA replication [3]. In rRNA, the modification proceeds in 2 stages: first, methylation of ribose in position 2′, which protects the only free hydroxyl of the ribose ring, then methylation of the base moieties [11]. In DNA the same hydroxyl in the 2′ position supports the reduction converting ribose to deoxyribose and protects DNA molecules against nucleases. In bacteria, resistance to antibiotics has been correlated with the degree of methylation of rRNA subunits (see Ref. [12] for review). More recently, six nucleosides methylated on the base moieties (N6, N6-mA, 7-mG, N4- and 5-mC, 3- and 5-mU) have been identified [13]. In addition, Gehrke and Kuo [10] have detected 10 methylated nucleosides in the rRNA of *E. coli* (2-, 6- and 2,6-mAde; 3- and 5-mUra; 1-, 2- and 7-mGua; 4- and 5-mCyt). However, these studies did not indicate the relative proportion of the methylated compounds.

Poly $(A)^+$ messenger RNAs carry genetic information from the nucleus into the cytoplasm where they are translated to proteins. Before mRNA is exported to the cytoplasm, it supports a maturation process involving "capping" and other methylations as well as intron splicing and addition of a polyadenine tail. In 1974 and 1975, the methylated molecules composing the "capped" 5′-end structure of messenger RNA were identified as 7-mGua and N6-mAde (see Refs. [14,15] for review). The stability of the "cap" structure was conferred by complementary methylation on the 2′ hydroxyl of the ribose ring from guanine and the two following adenines. Less precise are data on methylation of the "internal" part of mRNA, and the nature of the methylated bases. Cell labelling with [^{3}H](methyl)methionine resulted in incorporation of about two methyl groups per 1000 nucleotides, mainly attributed to 6-methyladenine [16–21]. Therefore, identification of the various methylated compounds present in poly $(A)^+$ RNA was important owing to their probable role in regulating the function of messenger RNA.

In this study we used high-performance liquid chromatography (HPLC) and mass spectrometry to analyse the nucleobase composition of RNA from three unicellular organisms and two mammalian livers. Special attention was paid to RNA from nuclei and cytoplasm, and to poly $(A)^+$ messenger RNA from rat liver.

2. Experimental

The chromatographic separation of nucleobases was carried out as previously described, with minor modifications [22,23] and nucleoside were separated as described in [10].

2.1. Chemicals and solvents

Standard nucleobase and ribonucleoside components were from the following sources: adenine (Ade), guanine (Gua), cytosine (Cyt), uracil (Ura), 1- and 2-methyladenine (1-, 2-mAde), 2- and 7-methylguanine (2-, 7-mGua), 5-methylcytosine (5-mCyt), 3-methyluracil (3-mUra), adenosine (A), guanosine (G), cytidine (C), uridine (U), 5-methylcytidine (5-mC), 6-methyladenosine (6-mA), 1- and 7-methylguanosine (1-, 7-mG) were from Sigma (St. Quentin, Fallavier, France) and 1- and 3-methylguanine (1-, 3-mGua); 3- and 6-methyladenine (3-, 6-mAde) were from Fluka (Buchs, Switzerland).

Ammonium dihydrogen phosphate (AdHP) was from Sigma. Ammonium acetate (AA), potassium dihydrogen phosphate (PdHP), formic acid (98–100%) of analytical grade and H_3PO_4 were from Merck (Nogent/Marne, France).

Methanol Normapur grade, NH_4OH and KOH were from Prolabo (Paris, France). Water for HPLC was purified using the Milli Q system (Millipore, St. Quentin-Yvelines, France). Nuclease P1 (No. 8630), bacterial alkaline phosphatase (BAP) from *E. coli* type III (No. P-4252), baker's yeast and *Torula* RNAs, calf liver and ribosomal bovine liver RNAs were from Sigma.

2.2. Preparation of standards

Stock solutions of standard nucleobases were prepared at concentrations in the range 0.4–2.0 mg/ml (ca. 3–15 m*M*), for minor and major bases, respectively. Excepting for guanine, which was dissolved in 10 m*M* HCl, all nucleobase solutions were made up in HPLC water and sterilized by filtration through Millipore membranes (pore size 0.22 μm). These nucleobase solutions were stable for several weeks at −20°C. Working standard nucleobase solutions (0.5–10 μg/ml) were applied to the column in a 10–20 μl injection volume containing 5–200 ng of each base.

Stock solutions of 0.5 *M* AdHP (or AA for mass spectrometry) were also sterilized by filtration and stored at 4°C. Elution buffers were prepared daily by diluting an aliquot of the stock solutions with HPLC water and the appropriate amount of methanol previously filtered through HV filters (pore size 0.45 μm, Millipore).

Stock solutions of standard nucleosides, exclusively dissolved in water, were in the concentration range 0.2–2.8 mg/ml (0.7–10 m*M*) for minor and major nucleosides respectively. The addition of diluted HCl is not recommended to facilitate dissolution of the nucleosides, because of possible degradation of ribosides. Working standard nucleoside solutions (2–30 μg/ml) were applied to the column in a 50-μl injection volume containing 0.1–1.5 μg of each nucleoside.

2.3. Elution buffers

A binary system was used to elute nucleobases and nucleosides from the columns. For nucleobases, buffer A consisted of 4% methanol in 6 m*M* AdHP (pH 5.3), and buffer B of 6% methanol in 12 m*M* AdHP (pH 5.3). The pH of the nucleobase elution buffer was adjusted with a few drops of either 5% NH_4OH or 5% H_3PO_4.

For nucleosides, buffer C consisted of 2.5% methanol in 50 m*M* PdHP (pH 4.5), and buffer D of 20% methanol in 50 m*M* PdHP (pH 4.0). The pH of the nucleoside elution buffers was adjusted with 5% KOH or 5% H_3PO_4. The 0.2 *M* AdHP and 0.5 *M* PdHP stock solutions were filtered through 0.45-μm Millipore membranes (Type HA).

2.4. Isolation of RNA

Total cellular RNA was extracted from frozen pieces of rat liver or from frozen *Euglena* cells by the guanidine isothiocyanate–cesium chloride method using a Beckman SW 55 rotor for 16 h at 64 000 *g*, according to the procedure described by Maniatis et al. [24]. A 500-mg quantity of either rat liver specimens or *Euglena* cell pellets was frozen immediately after harvesting in 15-ml sterile tubes maintained for a few minutes in a dry-ice–ethanol bath, and stored at −80°C until extraction of RNA.

Cytoplasmic and nuclear RNA were prepared from ca. 5 g of fresh rat liver. Nuclei were purified according to the procedure first described by Blobel and Potter [25] and modified as follows: the whole liver was quickly washed with ice-cold phosphate-buffered saline (0.2 g KCl/l, 0.2 g KH_2PO_4/l, 8 g NaCl/l, 2.16 g $Na_2HPO_4 \cdot 7H_2O$/l), blotted and weighed; a 5-g specimen was then minced and homogenized in 10 ml of ice-cold RNase-free 0.3 *M* sucrose buffer containing 10 m*M* Tris-HCl (pH 7.4), 10 m*M* NaCl, 3 m*M* $MgCl_2$ and 7.5 m*M* dithiothreitol, using a 45-ml sterilized Kontes glass homogenizer (Poly Labo, Strasbourg, France) with a motor-driven Teflon pestle with 10–15 strokes at 0°C and 1700 rpm. The homogenate was filtered through four layers of cheese-cloth and then centrifuged at 15 000 *g* for 15 min at 4°C. Cytoplasmic RNA was extracted from the 15 000 *g* supernatant while nuclei were purified from the crude nuclear pellet.

Cytoplasmic RNA was extracted from a 6-ml aliquot of the 15 000 *g* supernatant of the homogenate to which 1.111 ml of the ten-fold concentrated RNA extraction buffer (0.25 *M* trisodium citrate (pH 7.0), 5% N-lauroyl sarcosine (SLS), and 20 m*M* EDTA), 111 μl of β-mercaptoethanol and 5.5 g of guanidine isothiocyanate powder were added. After complete dissolution of the guanidium salt, cytoplasmic RNAs were purified by the same procedure as for total cellular RNA [24].

To obtain purified nuclei, the crude nuclear pellet was carefully rehomogenized in 9 ml of the 0.3 *M* RNase-free sucrose buffer using a loose-fitting Dounce homogenizer (pestle A). Two volumes of 2.3 *M* sucrose, made up in the same buffer, were added to the suspension, which was thoroughly mixed by multiple inversions. The suspension was then layered onto 0.8-ml cushions of 2.3 *M* sucrose, made up in the same buffer but without dithiothreitol, in six Beckman SW 55 centrifuge tubes. After 30 min centrifugation at 4°C and 80 000 *g*, the translucid pellets were stored at −20°C in the centrifuge tubes until extraction of nuclear RNA using the RNA-Zol kit from Bioprobe Systems (Ref. Zol 1501, Montreuil-sous-Bois, France). Each of the six nuclear pellets was dissolved in 2 × 500 μl RNA-Zol, and the final nuclear solution (6 ml) was carefully homogenized using a Dounce homogenizer (pestle A), until the solution became clear. Nuclear RNA was then extracted for 1 min by shaking with 700 μl of chloroform. The emulsion was transferred to 2-ml Eppendorf tubes and centrifuged for 15 min at 12 000 *g*. Nuclear RNA was precipitated from the upper aqueous phase (3 ml) with one volume of isopropanol at −20°C. The RNA pellets were further washed in ethanol, rehydrated, precipitated again, and stored at −20°C.

Large quantities of poly $(A)^+$ messenger RNA were selected through two cycles of oligo d(T)-cellulose chromatography, as described in Ref. [24], but using lithium chloride instead of sodium chloride in the loading and washing buffers. Smaller quantities of poly $(A)^+$ messenger RNA were also obtained using the QuickPrep mRNA purification kit Ref. 27-9254-01 from Pharmacia (St. Quentin-Yvelines, France).

2.5. Hydrolysis of RNA samples

For nucleobase analyses, RNA samples stored at −20°C as ethanol precipitates were washed twice in 75% and once in 100% ethanol, and moderately dried under nitrogen before rehydration in HPLC water at a concentration of 0.5 μg/μl. A 5–10 μg quantity of RNA was then evaporated to dryness, 200 μl of formic acid were added, and RNA hydrolysis was carried out in sealed tubes at 175°C for 75 min. After cooling, formic acid was evaporated under a nitrogen stream, and the released nucleobases were solubilized in HPLC water.

For nucleoside analyses, 10–30 μg of ethanol-washed RNA was rehydrated in 100 μl of HPLC water, heated for 2 min in a boiling water bath and rapidly cooled on ice. Then, 10 μl of 10 m*M* $ZnSO_4$ and 100 μl of nuclease P1 (200 units/ml) in 30 m*M* sodium acetate (pH 5.4), were added. Enzymatic hydrolysis was carried out for 16 h at 37°C before the addition of 10 μl of 0.5 *M* Tris buffer (pH 8.3), and 10 μl of bacterial alkaline phosphatase [BAP: 100 units/ml in 2.5 *M* $(NH_4)_2SO_4$, Boehringer Mannheim, Meylan, France] for the dephosphorylation of nucleotides for 2 h at 37°C.

2.6. HPLC instrumentation and columns

A liquid chromatographic (LC) system (SP 8700, Spectra-Physics, San Jose, CA, USA) was used for all the HPLC profiles presented in this paper. The LC system consisted of a control unit, a solvent tray and helium degassing manifold, a pump and an organizer module. The LC work station is composed of an HPLC monitor (IBM/LB 506 C-12 Berthold), supported by operation software (Epson PC-AX 12 MHZ) running MS-Dos 3.3. A variable-wavelength monitor (Knauer 87-00) and an LX 800 Epson printer for hard copy data presentation were used.

Reversed-phase columns for nucleobase analysis (300 × 3.9 mm I.D.) were prepacked with μBondapak phenyl (P/N 27198) by Waters (Millipore, Molsheim, France). A guard precolumn, with the same solid phase, was repacked in the

laboratory every fortnight, or more often if necessary. A Supelco prepacked column NC 18 S for nucleoside analysis (150 × 4.6 mm I.D., particle size 5 μm) was obtained from Supelco (Bellefonte, PA, USA). A 2-cm guard column was also periodically repacked in order to increase the lifetime of the column.

2.7. Thermospray mass spectrometry equipment

Analyses were carried out on an HPLC–MS system consisting of a Waters Model 600 MS solvent delivery system, a Waters Model U6K injector (Millipore Waters division) equipped with a 50- μl loop, a Vestec thermospray interface (Vestec, Houston, TX, USA) and a Nermag R 10-10 L quadripolar mass spectrometer (Delsi-Nermag, Argenteuil, France).

A six-port Rheodyne valve, used as an injector shunt, allowed the chromatographic silica column (250 × 0.25 mm I.D. containing a μC_{18} stationary phase) to be bypassed in order to calibrate the mass spectrometer and to optimize the working conditions of the thermospray interface. The second solvent delivery system, originally used after the chromatographic column to introduce the ionization reagent ammonium acetate, was removed to maintain the efficiency of the chromatographic separation, to avoid dilution of the analyte, and to increase sensitivity.

2.8. Chromatographic conditions for mass spectrometry

Analyses were carried out according to the procedure described in Ref. [26]. The mobile phase (methanol–2 m*M* ammonium acetate, 6:94, v/v), degassed prior to use and kept under a stream of helium, was adjusted to a flow-rate of 1.0 ml/min through a stainless-steel column (150 × 3.9 mm I.D.) packed with NovaPak C_{18} (60Å, 4 μm), maintained at 25°C. Elution was isocratic. Ammonium acetate was only used as an ionization reagent for the thermospray interface and did not affect the chromatographic separation. The conditions used for the thermospray interface, optimized by a direct injection through the injector shunt, were as follows: control temperature 150°C; vaporized tip temperature 229°C; tension of repeller 355 V and source temperature 256°C. Mass spectra were scanned from *m/z* 110 to *m/z* 350 a.m.u. (atomic mass unit).

3. Results

To study the relative amount of methylated nucleobases in RNA samples, we selected ten standard methylated bases: four adenine derivatives (1-, 2-, 3- and 6-mAde), four guanine derivatives (1-, 2-, 3- and 7-mGua), and two methylated pyrimidines (3-mUra and 5-mCyt). The latter was barely detected in RNA samples. The choice of the standard compounds also depended on their commercial availability.

In a preliminary step, the standard nucleobases were used to set the optimum elution conditions for separation and identification of minor methylated nucleobases in RNA. Fig. 1 shows the ionized currents in mass spectrometry of eleven nucleobases according to their molecular mass, amount and retention time; they are shown as individual ions, with the exception of cytosine, which appears as a dimer.

In HPLC, standard nucleobases were eluted with a binary gradient system. As shown in Fig. 2, the methylated bases were regularly interspersed between the four major bases, while 2-mAde partially coeluted with the peak of adenine. To shorten the retention time of 3- and 6-mAde, the flow-rate was increased to 2.0 ml/min just after 2-mAde elution. Thus, total elution time varied between 25 and 29 min according to column age.

The nucleobase composition of RNA from two yeasts, *Saccharomyces cerevisiae* and *Torula* sp., and from the phytoflagellate *Euglena gracillis* are shown in Fig. 3. *S. cerevisiae* RNA showed the largest amount of methylated minor bases, namely 1-mAde, 3- and 1-mGua, and 2-mAde. The three latter bases were also abundant in *Torula* and *Euglena*. In contrast, 3-mAde and 6-mAde were less abundant than in rat liver RNA (see Figs. 5 and 6). They were usually separated by another minor peak, which has

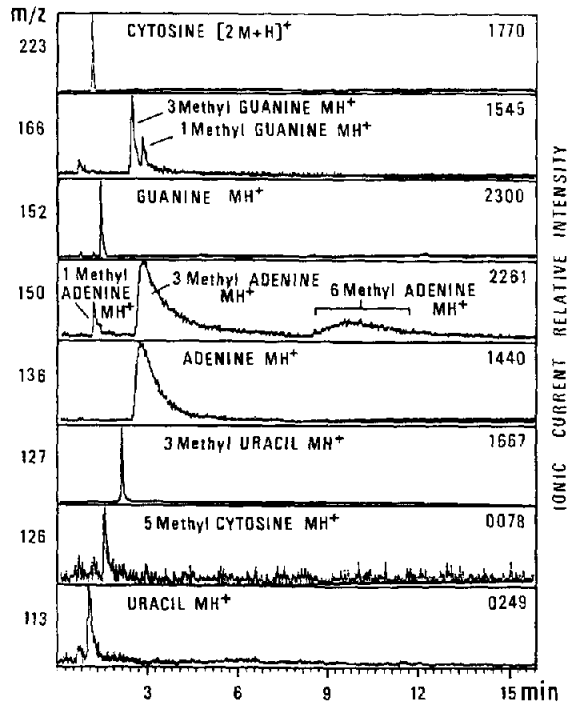

Fig. 1. Reconstitution of the ion currents of 11 standard nucleobases. Each ion mass is increased by one proton mass (MH^+). 3- and 1-Methylguanines have the same ion mass (166), just as the three methyladenine derivatives (ion mass 150), while cytosine is ionized as a dimer (ion mass 223). The numbers at the upper right of each *m/z* compartment correspond to the relative signal intensity.

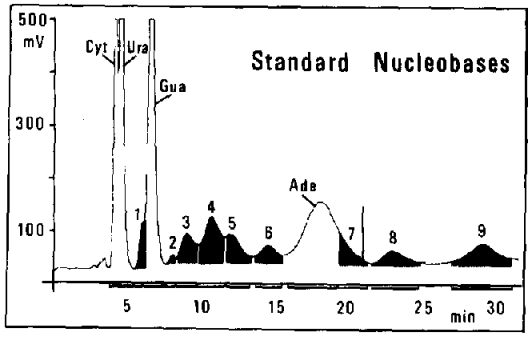

Fig. 2. Elution profile of 13 standard nucleobases using a binary gradient system. Elution buffer A was isocratic until 3 min, then buffer B was supplied in a linear gradient up to the elution of 6-mAde (peak 9). The flow-rate of 1.0 ml/min was increased to 2.0 ml/min at 22 min. Only the areas of the 9 methylated minor bases are shadowed. A 10-μl mix of the 4 major and 9 methylated minor bases was injected onto the column and nucleobases were eluted in the following order : Cyt (100 ng), Ura (70 ng), 1 = 1-mAde (22 ng), Gua (94 ng), 2 = 3-mUra (6 ng), 3 = 3-mGua (22 ng), 4 = 1-mGua (10 ng), 5 = 2-mGua (11 ng), 6 = 7-mGua (26 ng), Ade (78 ng), 7 = 2-mAde (54 ng), 8 = 3-mAde (54 ng), 9 = 6-mAde (54 ng).

been identified as a dimethylated derivative of guanine (1-, 7-mGua).

Fig. 4 shows the results for two commercial RNAs from bovine liver. Total cellular RNA nucleobases from calf liver showed minor peaks like those found in microorganisms. The main significant minor bases were 3-mGua, 1-mGua and 2-mAde. In bovine liver ribosomal RNA, a marked peak of 3-mAde was observed.

Fig. 5 shows the elution profiles of the major and methylated nucleobases from total RNA of rat liver, and from the purified nuclear and cytoplasmic RNAs. Each RNA sample was analyzed in triplicate with similar results. With nuclear RNA, two major peaks of 1-mGua and 3-mAde were observed. The amount of 2-mAde accounted for a large proportion of the total adenine peak. This characteristic composition of methylated nucleobases might correspond to methylation during the maturation process occurring in the nucleus after transcription of RNA precursors. Cytoplasmic RNAs contained far fewer methylated nucleobases than nuclear RNAs. It should be noted that in the case of the twin 3-mGua and 1-mGua peaks, the amount of 3-mGua tended to be larger than that of 1-mGua. The elution profiles of total cellular RNAs were similar to those of cytoplasmic RNA. This is not surprising given the small proportion of nuclear RNA in the total cellular RNA. Even the shoulder corresponding to 2-mAde (peak 7, Fig. 5) was quantitatively similar in cytoplasmic and total RNA.

The proportions of methylated nucleobases from poly $(A)^+$ messenger RNA and poly $(A)^-$ RNA of rat liver were also investigated. Particular attention was paid to 6-mAde, because of the number of adenine residues in the poly (A) tails of most messenger RNAs.

Fig. 6b shows the elution profile of poly $(A)^+$ RNA, with a marked peak of 3-mAde and a slight increase in 6-mAde relative to total liver and poly $(A)^-$ RNA (Figs. 6a and 6c). The

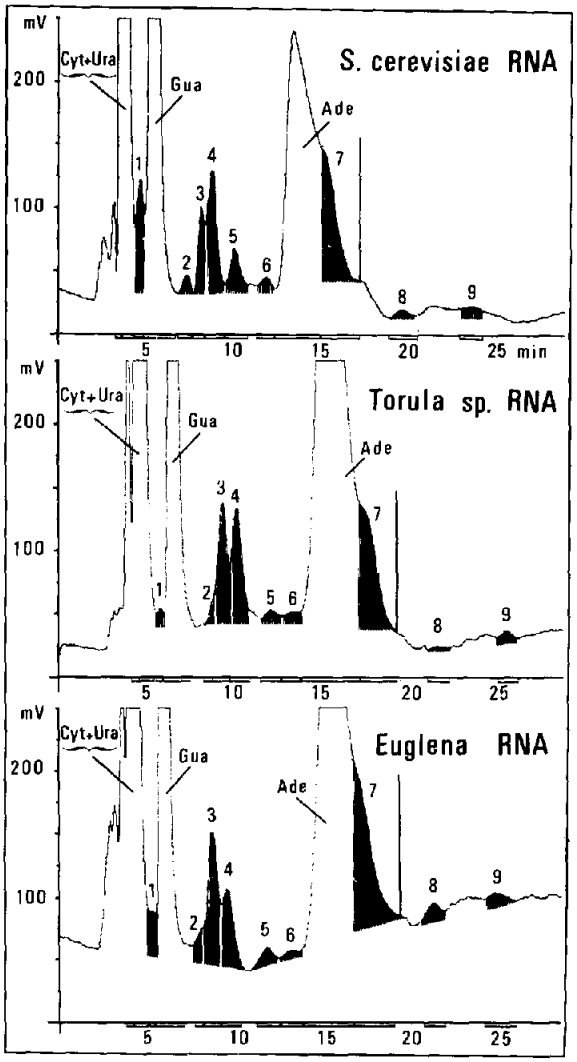

Fig. 3. Nucleobase elution profile of total cellular RNA of *Saccharomyces cerevisiae*, *Torula* sp., and *Euglena gracilis*. Identification of peaks: 1 = 1-mAde, 2 = 3-mUra, 3 = 3-mGua, 4 = 1-mGua, 5 = 2-mGua, 6 = 7-mGua, 7 = 2-mAde, 8 = 3-mAde, 9 = 6-mAde. Elution conditions as in Fig. 2.

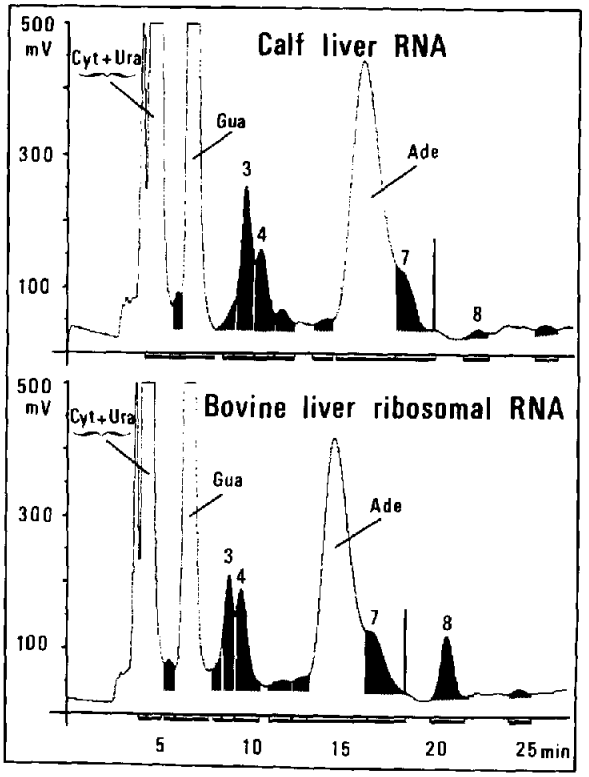

Fig. 4. Nucleobase elution profiles of two commercial RNAs of bovine liver : total RNA from calf liver, and ribosomal RNA from bovine liver. Identification of peaks : 3 = 3-mGua, 4 = 1-mGua, 7 = 2-mAde, 8 = 3-mAde. Elution conditions as in Fig. 2.

proportion of adenine was far higher in poly $(A)^+$ RNA, and the shoulder corresponding to 2-mAde was more pronounced. The proportion of 3-mGua was high in the three elution profiles but only in poly $(A)^+$ RNA was the 1-mGua peak consistent. Messenger RNA thus possesses high levels of 3-mGua, 3-mAde and 1-mGua and moreover a small increase in 6-mAde.

m/z-150 Fragments (Fig. 7) showed a much higher amount of 3- and 6-mAde in poly $(A)^+$ RNA (Fig. 7b) than in the equivalent amount of poly $(A)^-$ RNA (Fig. 7c). Moreover, the amount of 3-mAde was higher than that of 6-mAde, as shown by HPLC analysis (Fig. 6).

A complementary analysis of the nucleoside composition of RNA was performed to confirm the main results obtained for the nucleobase composition of nuclear and poly $(A)^+$ messenger RNAs. It was important to corroborate these results by using milder enzymatic hydrolysis. However, some standard ribonucleoside compounds are not commercially available, and are thus missing from the standard elution profile (upper part of Fig. 8). This is the case for 3-methylguanosine (3-mG), 3-methyladenosine (3-mA), and 1-methyladenosine (1-mA). These missing compounds enlarged the range of re-

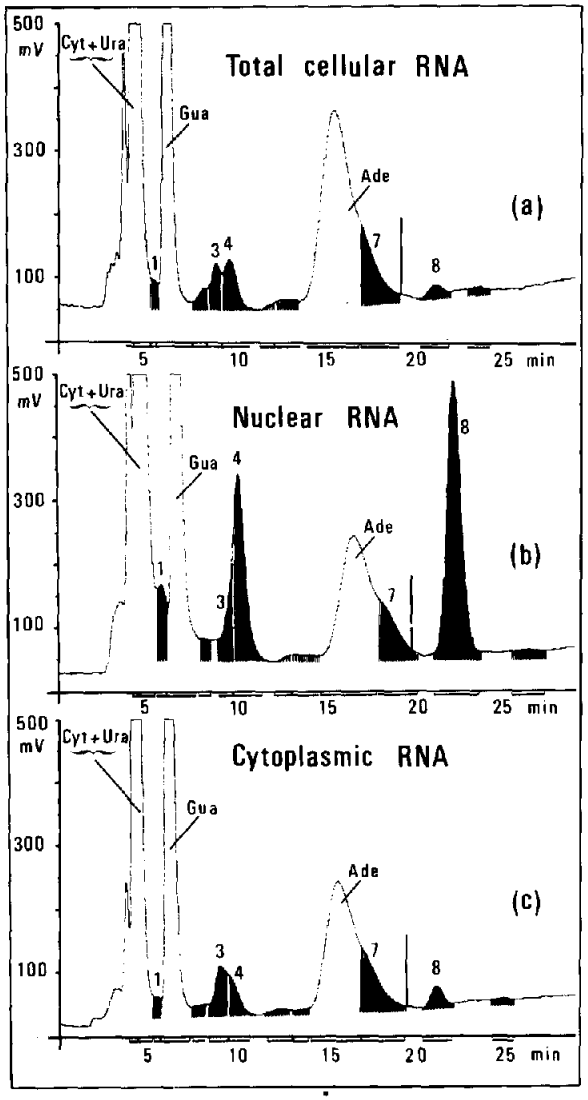

Fig. 5. Nucleobase elution profiles of three RNA samples of rat liver: (a) total cellular RNA, (b) nuclear RNA, and (c) cytoplasmic RNA. Identification of peaks : 1 = 1-mAde, 3 = 3-mGua, 4 = 1-mGua, 7 = 2-mAde, 8 = 3-mAde. Note that in nuclear RNA the proportion of the 3-mAde peak was large, and that of 1-mGua was far greater than that of 3-mGua. Elution conditions as in Fig. 2.

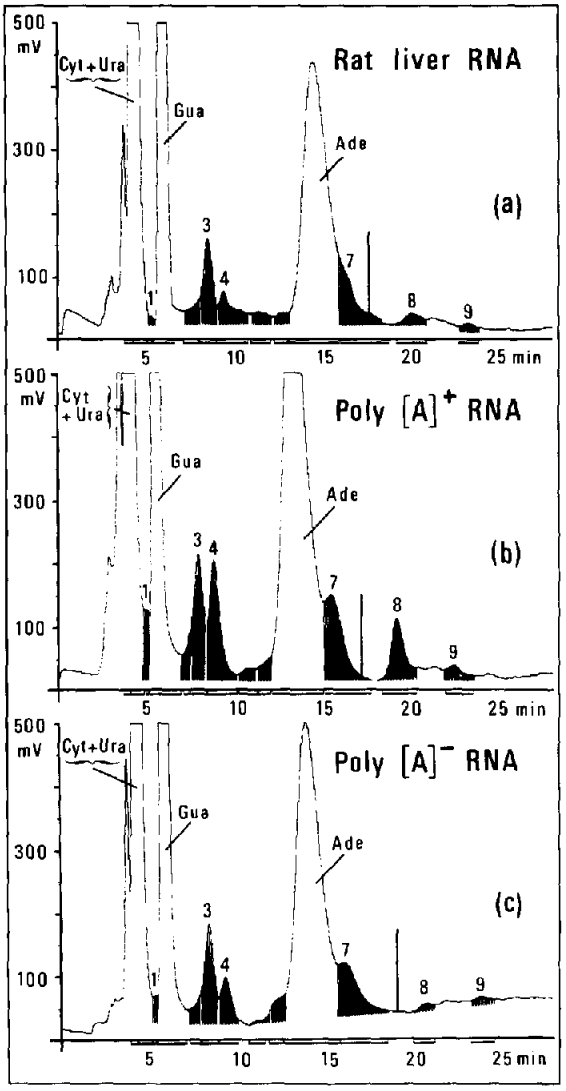

Fig. 6. Nucleobase elution profiles of poly $(A)^+$ messenger RNA and poly $(A)^-$ RNA from rat liver, compared with total liver RNA: (a) Total liver RNA, (b) poly $(A)^+$ messenger RNA, and (c) poly $(A)^-$ RNA. Identification of peaks : 1 = 1-mAde, 3 = 3-mGua, 4 = 1-mGua, 7 = 2-mAde, 8 = 3-mAde, 9 = 6-mAde. Note that in poly $(A)^+$ messenger RNA the proportion of 3-mAde is greater than that of 6-mAde, and the proportion of 3-mGua remains higher than that of 1-mGua. Elution conditions as in Fig. 2.

tention times in the elution profile of the standard compounds relative to nuclear RNA nucleosides (lower part of Fig. 8). In spite of these difficulties, analogous methylated nucleosides were identified in the RNA of rat liver nuclei.

7-mG was the only compound eluting earlier than the homologous nucleobases (Fig. 5b). This is in agreement with the retention time previous-

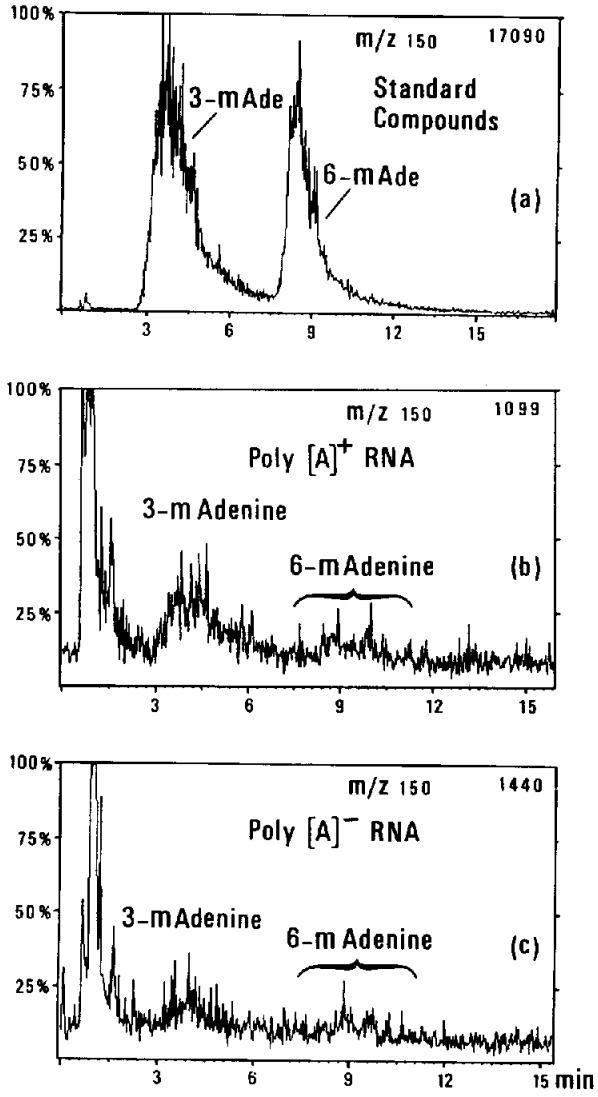

Fig. 7. Mass spectrometric detection of the fragments m/z 150 in RNA samples. 3- and 6-mAde standard compounds and equivalent amounts of poly $(A)^+$ and poly $(A)^-$ RNA hydrolysates were analyzed by mass spectrometry for the fragment m/z 150. According to the HPLC results (Fig. 6), the proportions of 3- and 6-mAde are larger in poly $(A)^+$ messenger RNA than in poly $(A)^-$ RNA. The numbers at the upper right of Figs. 7a, b and c correspond to the relative signal intensity.

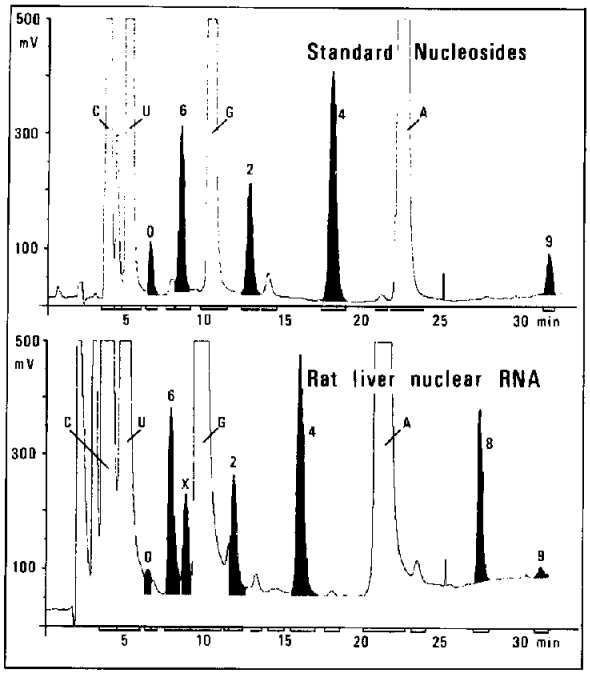

Fig. 8. Elution profiles of rat liver nuclear RNA (enzymatic hydrolysate) and standard commercial nucleosides. Since three standard compounds are not commercially available (3-mG, 3-mA and 1-mA), the retention times of the other standard nucleosides are somewhat delayed (upper part of Fig. 8), compared to the enzymatically digested nuclear RNA (lower part of Fig. 8). The large peak numbered "8" likely corresponds to 3-mA, as the large homolgous 3-mAde peak in the nucleobase elution profile of Fig. 5b. 3-mG possibly coelutes with 1-mG (peak 4, see also Fig. 9), while the peak noted "X" might correspond to 1-mA. Identification of peaks: 0 = 5-mC, 6 = 7-mG, X = possibly 1-mA, 2 = 3-mU, 4 = 1-mG which possibly coelutes with 3-mG, 8 = very likely 3-mA, and 9 = 6-mA.

ly observed for 7-mG by Gehrke and Kuo [10]. In the elution profile shown for nuclear RNA in the lower part of Fig. 8, peak "X" was presumed to correspond to 1-mA, and 3-mG probably coeluted with 1-mG (peak 4). 3-mA was easily identified by its abundance in nuclear RNA, as was the case for the nuclear RNA nucleobase elution profile (Fig. 5b), and by its equidistant position between adenosine and 6-methyladenosine (6-mA).

Finally, the existence of a larger proportion of 1- and 3-methylguanosines and 3- and 6-methyladenosines in poly $(A)^+$ RNA from rat liver, in comparison with total liver RNA, was confirmed by the ion-current reconstitution of nucleosides in mass spectrometry (Fig. 9).

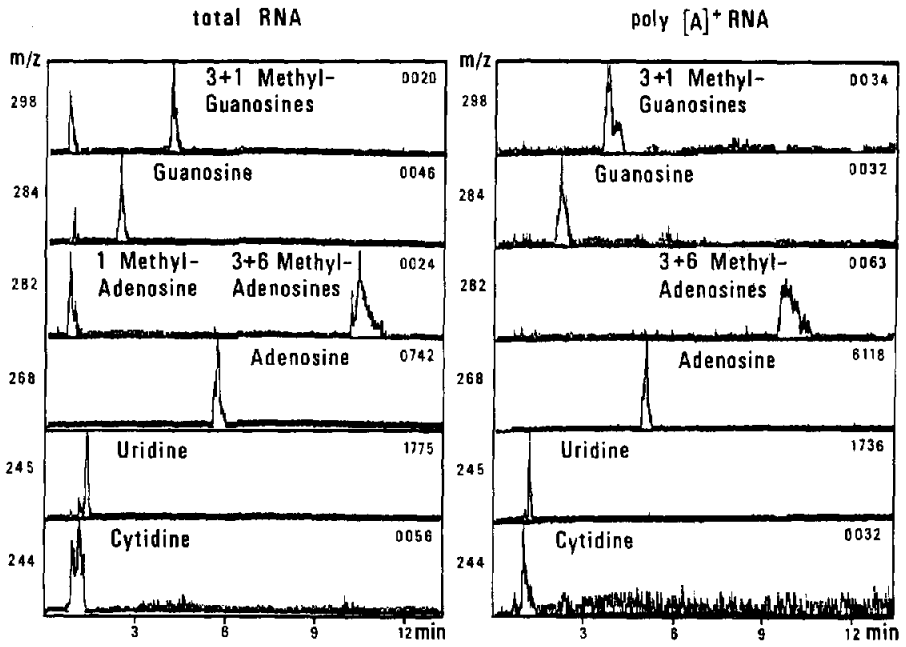

Fig. 9. Reconstitution in mass spectrometry of the ion currents of nucleosides from total liver RNA (left part) and poly $(A)^+$ messenger RNA from rat liver (right part). Three methylated guanosines (m/z 298) and three methylated adenosines (m/z 282) were detected. When comparing the relative intensity of the signals (numbers at the upper right of each m/z compartment), mass spectrometry confirmed the results obtained by HPLC analyses, i.e. poly $(A)^+$ RNA contains the highest proportion of methyladenosines and methylguanosines. Note the intensity of the adenosine signal in poly $(A)^+$ RNA (6118), on account of the number of adenosine residues present in the poly (A) tail, compared to the adenosine signal for total liver RNA (742).

3.1. Estimation of the proportions of methylated purines in nuclear and poly $(A)^+$ messenger RNA

An estimation of methylguanines and methyladenines in nuclear RNA and poly $(A)^+$ messenger RNA was made using the Stat view 512+ system (Brain Power, Calabasas, CA, USA). The calculated mean values represent an average of the peak areas obtained with four successive injections of each RNA hydrolysate.

In nuclear RNA, methylated guanines represented ca. 29% of the total guanine residues (22% for 1-mGua and 6% for 3-mGua), while methylated adenines represented 43% of the adenine residues (37% for 3-mAde).

In poly $(A)^+$ messenger RNA, methylated guanines represented ca. 26% of the total guanine residues (14% for 3-mGua and 12% for 1-mGua). In the case of methylated adenines, the proportion was far lower than in nuclear RNA (8.0%), with respectively 3.1% for 3-mAde and 4.2% for 2-mAde. Finally, 7-mGua and 6-mAde, essential minor bases in the "cap" structure of messenger RNAs, represented only 0.1% and 0.2% of the total guanine and adenine residues in messenger RNA.

4. Discussion

The reversed-phase HPLC method used here to separate RNA nucleobases is based on the procedure already described for the separation of the major and modified minor bases in DNA [22]. Nine methylated minor bases, together with the four major bases, were detected in RNA from unicellular organisms and mammalian tissues, in poly $(A)^+$ messenger RNA, and in RNA extracted from the different compartments of rat liver cells. To avoid nucleobase degradation during RNA hydrolysis in pure formic acid, all

traces of water must be eliminated. Under these conditions, the stability of nucleobases was verified for each individually hydrolyzed nucleobase, and also on standard nucleobase mixtures.

The binary gradient elution system can separate 13 minor and major compounds in less than 30 min. Baseline resolution is not obtained for all compounds, but the resolution is sufficient for quantitation. By increasing the flow-rate to 2.0 ml/min after elution of 2-methyladenine, 3- and 6-methyladenines can be quantified. In short, this procedure for the analysis of RNA base composition proved to be simple, rapid and inexpensive. In addition, all the standard compounds are commercially available, and 2 μg of RNA is sufficient.

For the analysis of nucleosides a larger quantity of RNA and advanced equipment are needed to resolve and identify the numerous nucleoside derivatives modified on both the base and sugar moieties [10]. For example, with our analytical equipment, a 10-fold larger quantity of RNA is needed to analyze a nucleoside hydrolysate. This is a disadvantage when working on limited quantities of RNA such as poly $(A)^+$ messenger RNA or nuclear RNA. Also, a number of standard nucleosides are not commercially available, such as 3-methylguanosine, and 1- and 3-methyladenosines. Nevertheless, analyses of nucleoside hydrolysates from nuclear RNA of rat liver yielded roughly equivalent results as for nucleobase analyses.

Recently, an ultrasonic nebulizer has been developed to generate the fine dispersion of the mobile-phase required for electrospray ionization mass spectrometry [27]. Undoubtedly this new equipment will enlarge the restricted range of mobile-phase compositions amenable to the electrospray process, including the use of mobile-phase gradients. However, the lack of commercially available modified nucleosides compounds remains the most severe limitation for routine analysis of biological samples. Indeed, in the absence of the 3-methyladenosine standard compound, the main shaded peak eluting between adenosine and 6-methyladenosine in Fig. 8 can be considered to correspond to 3-methyladenosine, as there was an equivalent peak in the nucleobase elution profile. Data obtained by mass spectrometry also confirmed the nucleobase analyses, i.e. a significant increase in 3- and 6-methyladenine in poly $(A)^+$ messenger RNA.

We found significant differences in the amount of methylated nucleobases, for example between cytoplasmic and nuclear RNA of rat liver. Cytoplasmic RNA contained the bulk of mature ribosomal RNA, while nuclear RNA was composed of various precursors of ribosomal RNA and heterogeneous nuclear RNAs, including premessenger RNAs. The results show that nuclear RNA possesses a large amount of 1-methylguanine and 3-methyladenine, and that the amount of 1- and 3-methylguanine, and 2- and 3-methyladenine is higher in poly $(A)^+$ messenger RNA than in poly $(A)^-$ RNA. 7-Methylguanine and 6-methyladenine, which are the modified minor bases present in the "cap" structure of poly $(A)^+$ messenger RNA, represented only a small proportion of the modified minor bases detected in total messenger RNA.

To interpret the abundance of methylated bases found in nuclear RNA and poly $(A)^+$ messenger RNA, it must be noted that they are preferentially methylated purines (1- and 3-methylguanine, 2- and 3-methyladenine), with the exception of one pyrimidine, 3-methyluracil, present in a moderate amount. In contrast, the only natural methylated bases occurring in DNA from higher eucaryotes are two pyrimidines, thymine and 5-methylcytosine. As far as we know, no analysis of the base composition of nuclear RNA has previously been undertaken. In the case of messenger RNAs, the study of methylated bases at the "internal" sites dates back 20 years [16,17]. It thus appears that the large field of the methylated bases present in the RNA precursors of nuclear and messenger RNA is now accessible to investigation with more refined techniques.

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