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QUANTITATIVE MEASUREMENT OF mRNA CAP 0 AND CAP 1 STRUCTURES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

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SUMMARY

Viral and eukaryotic mRNA molecules have a unique 5'-end. The 5'-terminus consists of $m^7G(5')ppp(5')N'(m)pN''(m)$, which is termed a "cap" structure. The study of these cap structures has led to the development of many methods of identification and analysis. Many of the methods have been time-consuming or have not been able to distinguish between the different caps, and they are quantifiable only by employing radiolabels. This paper presents the use of reversed-phase high-performance liquid chromatography as a rapid and efficient tool for the separation, identification and quantitation of caps. An ion-exchange enrichment procedure was also developed for the isolation of cap 0 and cap 1 structures from unfractionated RNAs. The recoveries of different caps ranged from 83 to 99%, with a relative standard deviation range of 1.3—4.4%. In this method, caps were released from

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commercially obtained rabbit globin mRNA by nuclease P1 digestion. The products of digestion were treated with alkaline phosphatase and separated on an octadecylsilyl column using stepwise or gradient elution. Cap structures and any internal modified nucleosides were identified by their retention times and UV spectra relative to reference compounds. The amount of each cap 0 or cap 1 structure was determined by its UV absorbance relative to a known quantity of reference compound. This method allows the quantitation of 0.2 nmol or more of cap 0 and cap 1 structures. Total UV spectra can be obtained for 0.5 nmol or more of cap. This methodology permits investigations on viral and eukaryotic mRNA cap biosynthesis and turnover during viral transformation, differentiation, cap synthesis in the cell cycle, etc.

INTRODUCTION

The 5′-termini of many viral and eukaryotic mRNAs contain unique structures known as "caps", which consist of inverted 7-methylguanosine (m^7Guo) linked to the penultimate nucleoside through a 5′–5′ triphosphate bridge [1, 2]. The 5′-end of these mRNAs takes the form of m^7G(5′)ppp(5′)N′(m)pN″(m). If the N′- and N″-nucleosides are not methylated, the dinucleotide with a 5′—5′ triphosphate bridge is termed a cap 0 structure. When the N′-nucleoside is methylated at the 2′–O position and the N″-nucleoside is not methylated, this dinucleotide with 2′—O methylation is termed a cap 1 structure. Additionally, in a cap structure the N′- and the N″-nucleosides are both methylated at the 2′—O position. It has been shown that the capping and the N′-methylation reactions occur in the nucleus, whereas the N″-methylation occurs in the cytoplasm [3, 4].

Cap structures appear to be involved with the longevity and functionality of mRNA. Owing to the inverted 5′—5′ linkage, capped mRNAs are resistant to 5′-exonuclease activity [5]. In addition, it has been shown that the presence of the intact cap structure on some mRNAs increases the efficiency of translation at these mRNAs [6, 7].

Various methods of analysing cap structures have included digestion of radiolabeled mRNA with nuclease P1, or RNase T_1 and T_2, followed by separation of the resulting products by electrophoretic fingerprinting [8] or chromatography on DEAE-Sephadex and pellinox WAX [3, 4, 9]. Another method of analysis involves a periodate oxidation and β-elimination procedure on the mRNA, then fingerprinting [10] or DEAE-cellulose chromatography [11]. More recent developments involve NaB^3H_4 labeling of the mRNA, followed by nuclease P1 digestion and analysis of the products by high-performance anion-exchange chromatography [12]. In addition, a technique has been developed involving selective hydrolysis of [^{3}H]methyl-labeled mRNA to cap structures and nucleosides, which are then separated by reversed-phase liquid chromatography (RPLC) [13].

This paper presents the development of a rapid, efficient, sensitive and quantitative procedure for analysing mRNA cap 0 and cap 1 structures without the need for radiolabels. The method is adaptable to cap 2 structures and to the $m_2^2m^7G$ cap structures of small nuclear RNAs. Our method uses RPLC for separating caps from nucleosides, with the use of either a stepwise buffer system or a gradient curve buffer system. Identification of cap structures is by retention time of known reference caps and UV spectra (190–400 nm) of the RPLC

peaks. Quantitation is accomplished using an internal standard method with our established relative molar response factors (RMRs) of all nine caps. Also, an anion-exchange technique has been developed to separate cap structures from other nucleosides that result from nuclease P1 and bacterial alkaline phosphatase (BAP) hydrolysis of total crude RNA. An accurate quantitative analysis of cap molecules will aid in the study of the presence and function of caps and help to provide an insight into the significance of the different types of cap structures in mRNA synthesis and function.

Potential uses of an accurate quantitative chromatographic method for separation and measurement of cap structures include: screening of mutants for cap production; study of cap production in the cell cycle; investigation of the presence of different caps before and after viral infection, transformation during differentiation and after hormonal changes; identification and quantitation of caps in single species of mRNA; and studies on the half-lives of mRNA and caps. This RPLC method will also find useful application for the determination and study of cap structures in biological samples and for their correlation to plant and animal diseases.

EXPERIMENTAL

Chromatography of cap structures and ribonucleosides

The chromatographic separation of a mixture of nine cap 0 and cap 1 standard dinucleoside 5′—5′ triphosphates (m^7GpppC, m^7GpppG, m^7GpppA, m^7GpppGm, m^7Gpppm^{6}Am, m^7GpppU, m^7GpppCm, m^7GpppUm and m^7GpppAm) (P.-L. Biochemicals, Milwaukee, WI, U.S.A.), and four major ribonucleosides and their corresponding 2′—0 methylated nucleosides was successfully accomplished using a Supelcosil LC-18 column (25 cm × 4.6 mm I.D., 5 μm particle size) (Supelco, Bellefonte, PA, U.S.A.). The RPLC system used for stepwise separations consisted of a Perkin-Elmer Series 2 solvent delivery pump and a Perkin-Elmer LC 75 variable-wavelength UV detector. The RPLC system used for gradient separations consisted of a Perkin-Elmer Series 4 solvent delivery pump and a Hewlett-Packard 1040A RPLC detection system (Hewlett-Packard, San Diego, CA, U.S.A.). A Perkin-Elmer Sigma 15 chromatography data station and two HP 3390A integrators were employed for peak integration. The column flow-rate was 1.0 ml/min and the column temperature was maintained at 23° C using a constant-temperature circulating water bath, Haake Model FJ (Saddle Brook, NJ, U.S.A.) connected to an aluminum column jacket. The buffers used for stepwise chromatography of the mixture were composed of 0.1 *M* KH_2PO_4 (Baker-analyzed, J.T. Baker, Phillipsburg, NJ, U.S.A.), pH 6.0, and the following methanol concentrations (EM Chemicals, Gibbstown, NJ, U.S.A.): buffer A, 1.0% methanol; buffer B, 4.0% methanol; and buffer C, 15.0% methanol.

Gradient elution of mRNA cap 0 and cap 1 structures was accomplished according to the binary elution conditions given in Table I. The gradient curve is as described in the Perkin-Elmer Series 4 solvent delivery system operations manual, section 4, p. 27.

TABLE I

TWO-BUFFER GRADIENT ELUTION FOR SEPARATION OF mRNA CAP 0 AND 1 STRUCTURES

All buffers: 0.10 *M* KH_2PO_4, pH 6.0, 23°C; column; Supelcosil LC-18 (25 cm × 4.6 mm I.D., 5 μm).

Step	Time (min)	Flow-rate (ml/min)	Buffer A (1.0% methanol)	Buffer B (15% methanol)	Buffer C (70% methanol)	Gradient curve
1	20*	1.0	100.0	0.0	0.0	
2	10	1.0	50.0	50.0	0.0	2
3	15	1.0	0.0	100.0	0.0	1
4	20	1.0	0.0	100.0	0.0	
5	10**	1.0	0.0	0.0	100.0	

*Equilibration.
**Wash.

Hydrolysis of intact cap structures and chromatography of hydrolysate

Intact cap 0 and cap 1 structures were enzymatically hydrolysed to ribonucleosides with snake venom nucleotide pyrophosphatase and BAP (type III) (Sigma, St. Louis, MO, U.S.A.). A stock solution of 5 A_{260}/ml (ca. 250 μg/ml) of each cap 0 and cap 1 structure was prepared using nanopure glass-distilled water. Aliquots (5—25 μl) of this stock solution were used in the following hydrolysis reaction mixture: Tris—HCl, pH 7.8 (final concentration 50 m*M*), magnesium acetate (final concentration 1 m*M*), pyrophosphatase (ca. 0.20 U), BAP (ca. 0.15 U) for a total reaction volume of 55—75 μl. This reaction mixture was incubated in a 37°C water bath for 2 h [13]. After incubation, 5 μl of 1.0 *M* KH_2PO_4 buffer solution (pH 4.0) were added to lower the pH in order to prevent degradation of m^7Guo to m^7Gua. The internal standard (Br^8Guo) (2 nmol Br^8Guo per nmol hydrolysed cap) was added to the solution prior to enzymatic hydrolysis. After hydrolysis, the reaction mixture was directly injected onto the RPLC column for quantitation.

Detailed procedures for the quantitative measurement of ribonucleosides resulting from hydrolysis of caps have been published [14—17].

Hydrolysis and chromatography of globin mRNA

Intact rabbit globin mRNA (BRL, Gaithersburg, MD, U.S.A.) was hydrolysed to cap structures and ribonucleosides using nuclease P1 (Boehringer Mannheim Biochemicals, Indianapolis, IN, U.S.A.) and BAP. The reaction mixture consisted of: 25 μl of mRNA (25 μg), 25 μl of nuclease P1 (25 μg) in sodium acetate buffer (pH 5.3) and 10 μl of 10 m*M* Zn^{2+}. Following a 60-min incubation at 37°C, 50 μl of BAP (1 U) and 50 μl of 10 m*M* Tris (pH 8.7) were added (final pH ca. 7.8). The total mixture was incubated for an additional 60 min at 37°C [17]. For quantitation, the internal standard Br^8Guo was added to the hydrolysate and then the sample was injected directly onto a Supelcosil LC-18 RPLC column. Independent mRNA hydrolysates were chromatographed, employing the stepwise elution and the gradient separation systems.

Isolation of cap structures using anion-exchange

Type 0 and 1 caps can be quantitatively obtained from mRNAs by complete nuclease P1 digestion. Type 2 caps are also effectively cleaved from mRNA, but yield a type 1 cap and an additional methylated nucleoside (N''m). The intact cap 2 structures can be obtained by using RNase T_2 [2].

The resin used for the ion-exchange isolation of cap structures was Ag 1 × 2 (100—200 mesh) (Bio-Rad, Richmond, CA, U.S.A.), which is a strong anion exchanger. The total resin volume of 50 μl was packed in a yellow Eppendorf pipet tip supported with a small glass-wool plug and attached to a three-way Luer-lock valve. This allows refilling of the syringe without detaching it from the yellow Eppendorf pipet tip anion-exchange column. The other end of the valve was attached to a 3.0 ml syringe. As the resin was supplied in the chloride form and was to be used in a formate form, an on-column regeneration process was performed. This consisted of an initial 5.0-ml wash with methanol to elute organic contaminants. Next, 3.0 ml of 1 *M* sodium hydroxide were applied, followed by a water wash (2 × 1 ml), 1.0 ml of 1 *M* formic acid, and a final rinse with 3.0 ml of nanopure glass-distilled water (as per Bio-Rad instructions). The flow-rate was ca. 0.5 ml/min and was controlled by depressing the syringe plunger.

The sample solution (pH 7—8) was applied to the anion-exchange column, and the major nucleosides were completely eluted with 5 ml of 40% methanol in water. The caps remained on the column. Cap structures were eluted with 1 ml of 2 *M* ammonium formate in water. The 2 *M* ammonium formate eluate was collected, lyophilized, and then dissolved in 200 μl of nanopure glass-distilled water. Nearly the entire sample (195 μl) was injected onto a Supelcosil LC-18 column and chromatographed according to the stepwise buffer system. Peaks were detected and their areas were measured by UV absorption at 254 nm. A Perkin-Elmer chromatography intelligent terminal (CIT) with a re-integration function was used to achieve the necessary precision and accuracy of the measurements. Measured areas were then converted to moles of each cap using an external molar response factor. Recoveries could then be calculated and are found in Table IV.

RESULTS

The reproducible identification and quantitation of the caps and nucleosides present in RNA hydrolysates is a problem requiring high-resolution separation of each of the caps from each of the nucleosides present in the digest (approximate ratio in pure mRNA is one cap per 1000 nucleosides). This paper addresses the problem by providing a method of separating caps from each other and the major nucleosides, and a method for separating all caps from all mononucleosides.

We chose RPLC for studies of the separation of caps due to its demonstrated excellent selectivity of the four major ribonucleosides and the four 2'—0 methylated nucleosides [15, 16]. The nine cap dinucleoside triphosphates possess structural differences important to achieving chromatographic separations in the N'-residues of the four cap 0 (A, C, G, U) and the five 2'—0 methylated cap 1 (Am, Cm, Gm, Um, m^6Am) nucleosides. Thus, we postulated

that reversed-phase separation of the caps should be achieved easily. Another consideration was selection of mobile phase parameters to minimize the positive charge on the m^7Guo nucleoside and to decrease the ionization of the phosphate groups by using a high-ionic-strength mobile phase. This, in effect, increases the hydrophobicity of the caps and reduces the adsorption interactions with the silanol residues on the support.

To effect the separation of all type 0 and 1 caps, the following column chromatographic parameters were studied with regard to the elution buffer: KH_2PO_4 concentration, methanol concentration and pH. In addition, column temperature was investigated. A Waters μBondapak C_{18} column was used initially owing to proven success in the separation of mononucleosides and dinucleoside monophosphates [17].

An increase in column temperature above ambient reduced the capacity factors (k') of all the cap structures and nucleosides and provided a higher column efficiency, but the desired selectivity was not achieved. Thus, a temperature of 23°C was used throughout the experiments to achieve the required selectivity. Initial studies with a mobile phase of low ionic strength (0.01 *M* KH_2PO_4), low pH (3—4) and a methanol gradient demonstrated that acidic conditions and low ionic strength caused poor peak shape in the elution of the cap structures. As the elution buffer had a pH > 6.0, this proved to be

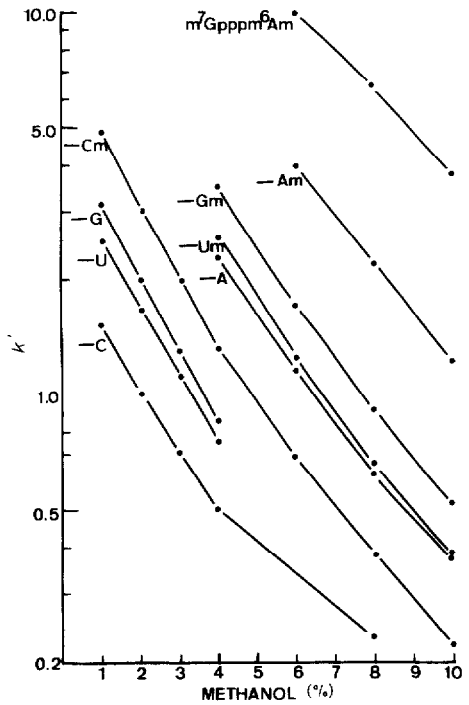

Fig. 1. Capacity factor of cap structures 0 and 1 versus methanol concentration in the elution buffer. The column capacity factor versus methanol was determined for each cap structure at a column temperature of 23°C and in the elution buffer of 0.1 *M* potassium biphosphate (pH 6.0). A Supelcosil LC-18 column was used. The RPLC system used is described in Experimental. Each line in the figure is labeled with the respective N'-nucleoside of the m^7GpppN' cap molecule.

detrimental to column longevity, and thus this pH range was not investigated.

Therefore, effects of KH_2PO_4 molarity and methanol concentrations on retention times were studied at 23° C at a pH of 6.0. Separation of the nine cap 0 and 1 structures was studied using elution buffers of increasing methanol concentrations with 0.1 *M*, 0.2 *M* and 0.3 *M* KH_2PO_4. Fig. 1 depicts the changes in the capacity factors versus methanol concentration at 0.1 *M* KH_2PO_4 for each cap. (Data for 0.2 and 0.3 *M* KH_2PO_4 are not shown.) The k' value of each cap increases proportionately as the KH_2PO_4 molarity in the mobile phase is increased. There is no obvious optimal concentration that will give a best separation of the nine caps and the four major nucleosides. A KH_2PO_4 molarity of 0.10 *M* was chosen to reduce peak-tailing and to give a low background absorption. A KH_2PO_4 molarity of > 0.1 *M* should be used to obtain good peak symmetry and reduced peak tailing. From this information, the composition of buffers A, B and C was determined (see Experimental).

Fig. 1. demonstrates that 1.0% methanol yields optimal separation of cap structures, which elute early (N′ = C, U, G and Cm). Also, this figure demonstrates that 4.0% methanol is optimal for the next eluting set of cap structures (N′ = A, Um and Gm). Since the last two cap structures (N′ = Am and m^6Am) were observed to elute slowly, a final buffer containing 15% methanol was chosen to elute these two molecules.

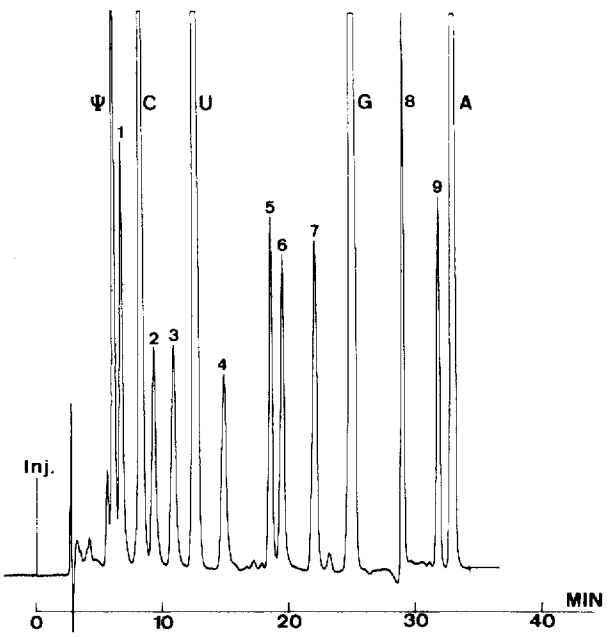

Fig. 2. Cap separation using stepwise gradient elution. The stepwise elution system for separation of the nine type 0 and 1 caps, ψ, and the four major ribonucleosides consisted of buffer A for 7 min (see Experimental) followed by buffer B for 13 min and ending with buffer C for 5 min, all at 1 ml/min. The Supelcosil LC-18 column temperature was maintained at 23° C. Absorbance at 254 nm was measured at a sensitivity of 0.04 a.u.f.s. Eluted peaks are designated as follows: 1 = m^7GpppC; 2 = m^7GpppU; 3 = m^7GpppG; 4 = m^7GpppCm; 5 = m^7GpppA; 6 = m^7GpppUm; 7 = m^7GpppGm; 8 = m^7GpppAm; 9 = m^7Gpppm^{6}Am.

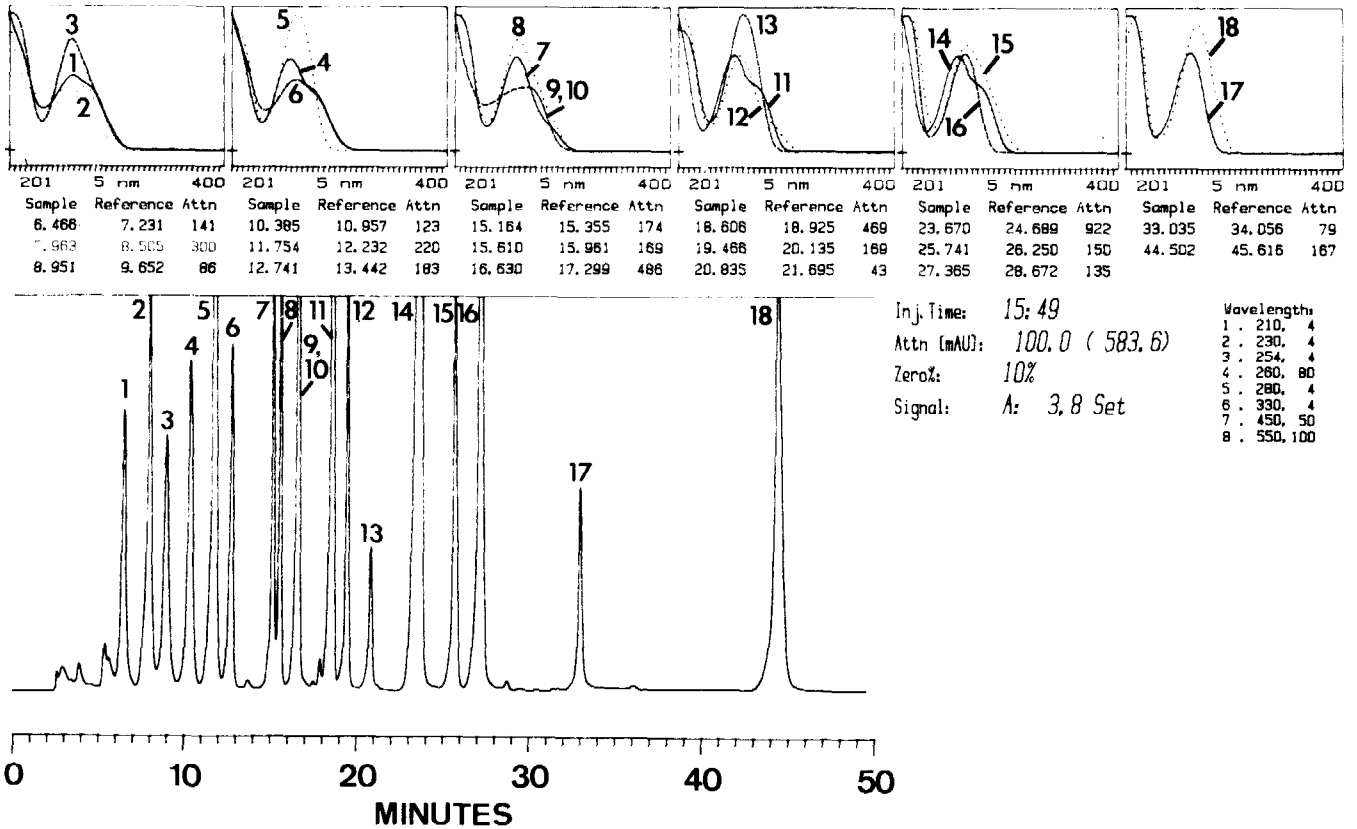

Fig. 3. Cap separation with gradient curve elution (Table I). Separation of caps by a gradient elution system incorporated first a gradient from 1.0 to 8.0% methanol in 10 min. The increase in methanol followed Perkin-Elmer gradient curve 2 (Perkin-Elmer Series 4 manual). The second gradient was composed of 8.0—15.0% metanol, required 15 min and followed a Perkin-Elmer curve 1. Elution ended with buffer B (containing 15% methanol) for 5 min, with a total analysis time of 50 min. Absorbance at 254 nm was measured at a sensitivity of 0.02 a.u.f.s. The absorption spectra were obtained using a Hewlett-Packard 1040 diode array detector. All of the spectra were obtained in real time of the eluted peak.

Chromatographic performance of a Supelcosil LC-18 column was evaluated in comparison to that of the Waters μBondapak C_{18} columns after the elution buffer composition had been established using the latter. This Supelcosil column gave a much higher efficiency than the Waters μBondapak C_{18} column. In addition to yielding sharper peaks and less peak tailing for all nine caps, it was capable of separating m^7GpppA and m^7GpppUm, which co-eluted on the Waters column. Therefore, the Supelcosil LC-18 column was employed in the remaining studies. It should be noted that, owing to the anionic nature of the cap molecules, a Supelcosil LC-18 DB column, shown to be excellent for mononucleoside separation [17], yields undesirable peak shape for the cap molecules and is unsuitable for separations involving cap structures.

The chromatographic separation of the cap 0 and cap 1 structures and the four major ribonucleosides was accomplished using both a stepwise buffer system (Fig. 2) and a gradient buffer system (Table I, Fig. 3). Chromatographic details are given in the figure legends. Separations were achieved in 35—45 min.

Determination of UV relative molar response factors

Once a fast, reproducible and efficient baseline separation of cap 0 structures, cap 1 structures and the four major ribonucleosides had been achieved, the RMR factor was determined for each cap with reference to Br^8Guo as the internal standard (I.S.). (RMR cap/I.S. is the ratio peak area per mole of each cap to peak area per mole of internal standard.)

To determine the RMR cap/I.S., first each cap structure was subjected to total enzymatic hydrolysis to m^7Guo and the respective N′-nucleoside. Two enzymes were used, snake venom nucleotide pyrophosphatase and BAP [13]. Pyrophosphatase cleaves the 5′—5′ triphosphate bridge between the two nucleotides, yielding nucleoside 5′-monophosphates and inorganic phosphate. BAP cleaves the phosphate from the nucleoside 5′-monophosphate, yielding nucleosides and inorganic phosphate. Experimental details of the enzymatic hydrolysis reaction procedure are given in the Experimental section. The enzymatic hydrolysis reaction was performed using several cap structures, with incubation times in the range 0—4 h. A 2-h incubation period was found sufficient for complete hydrolysis under the chosen conditions. At longer periods of incubation, there was no detectable increase in the amount of N′ or any decomposition of the N′-nucleoside. However, m^7Guo is unstable, and thus limiting the incubation time to 2 h avoids problems with decomposition of m^7Guo. We used a large excess of pyrophosphatase and BAP to achieve total hydrolysis of the cap structure in a minimum time.

After hydrolysis of each cap structure to m^7Guo and the respective N′-nucleoside, the hydrolysates were chromatographed according to procedures previously established for ribonucleosides [15, 17]. Fig. 4 presents the chromatographic separation of a cap 0 (m^7GpppA) hydrolysate product. This separation of the constituent nucleosides allowed accurate measurement of the molar concentration of m^7Guo and the N′-nucleoside in the hydrolysed cap solution through the use of previously established molar response factors for the individual nucleosides. The nanomoles of intact cap structure in the hydrolysed sample solution is equal to the nanomoles of either m^7Guo or N′. A small quantity (25.0 μl) of each solution containing intact cap and a known molar concentration of I.S. was chromatographed

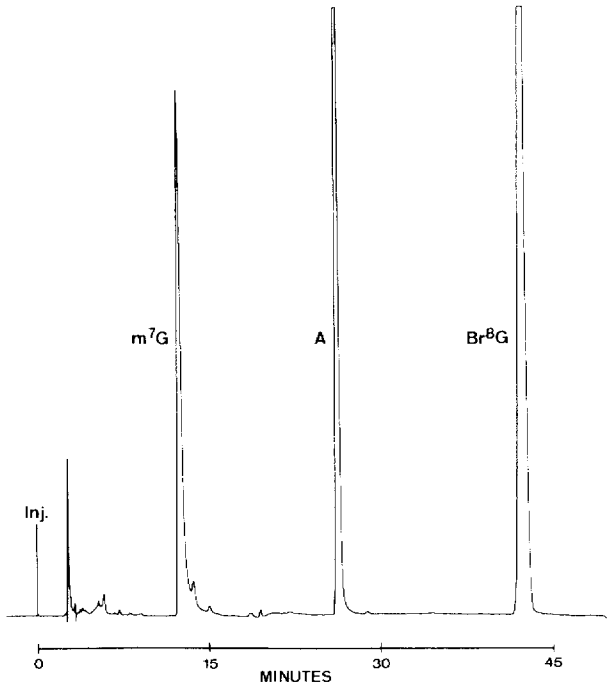

Fig. 4. Characteristic chromatogram of a cap hydrolysate. The cap 0 structure m^7GpppA was hydrolysed to nucleosides according to the enzymatic procedure given in the text. An aliquot of the hydrolysate was chromatographed according to conditions previously published [17]. The absorbance at 254 nm was measured at a sensitivity of 0.02 a.u.f.s.

TABLE II

RELATIVE MOLAR RESPONSE (RMR) VALUES FOR TYPE 0 AND TYPE 1 CAP STRUCTURES

Perkin-Elmer Series 4 solvent delivery system (Table I) used in determining RMR values; a Waters Model 440 detector was used, with Br^8Guo as internal standard.

	Cap	RMR ± R.S.D. (n = 3) (%)	
		254 nm	280 nm
Type 0	m^7GpppC	0.878 ± 0.6	0.967 ± 1.2
	m^7GpppU	0.817 ± 0.3	0.655 ± 2.3
	m^7GpppG	1.329 ± 0.5	1.008 ± 0.7
	m^7GpppA	1.292 ± 2.2	0.726 ± 2.7
Type 1	$m^7GpppCm$	0.771 ± 2.2	0.858 ± 2.3
	$m^7GpppUm$	1.103 ± 1.8	0.895 ± 4.3
	$m^7GpppGm$	1.369 ± 1.2	1.058 ± 1.8*
	$m^7GpppAm$	1.262 ± 1.7	0.743 ± 1.0
	m^7Gpppm^6Am	1.286 ± 1.8	1.285 ± 2.0

*Average of two determinations. Perkin-Elmer Series 4 solvent delivery system (Table I) used in determining RMR values; a Waters Model 440 detector was used, with Br^8Guo as internal standard.

according to the procedures in this paper to obtain the ratio of peak areas of each intact cap structure to the I.S. This measured area ratio of $A_{cap}/A_{I.S.}$, multiplied by the experimental values of molar concentration of I.S./molar concentration of cap, is equal to the RMR cap/I.S. The molar response relative to the I.S. could then be determined for each of the nine cap structures studied, and are presented in Table II. The use of an internal standard method led to excellent precision and quantitation, with repeated injections of ca. 1–3 nmol of each of the cap structures with relative standard deviations of 2.9—4.3%.

TABLE III

ULTRAVIOLET ABSORPTION ADDITIVITY OF CAP STRUCTURE COMPONENT NUCLEOSIDES

The cap and its component nucleosides and Br^8Guo were measured under identical chromatographic conditions: chromatographed with 15% methanol (pH 6.0) and 0.1 *M* $NH_4H_2PO_4$ at 23°C. Area ratio of nucleoside or cap structure/area of Br^8Guo. Br^8Guo (internal standard) was added to each cap solution before enzymatic hydrolysis. Then the appropriate aliquots were taken and measured by RPLC.
Each value in columns 2 and 3 is an average of two independent hydrolysates and two RPLC measurements, and for columns 6 and 7 of two independent measurements. m^6Am is not in close agreement, due to other cap structures present in the sample.

	Hydrolysed cap*		Calculated from hydrolysed cap		Measured from unhydrolysed cap	
	254 nm	280 nm	254 nm	280 nm	254 nm	280 nm
C	0.88	1.04	2.13	1.92	2.12	1.83
m^7G	1.25	0.88				
Cm	0.90	1.16	1.91	1.85	1.95	2.01
m^7G	1.01	0.68				
U	1.33	0.43	2.53	1.09	2.28	1.14
m^7G	1.20	0.66				
Um	1.02	0.61	2.25	1.84	2.43	1.81
m^7G	1.23	1.23				
G	1.60	0.75	2.71	1.35	2.71	1.30
m^7G	1.11	0.60				
Gm	1.11	1.48	2.16	2.57	2.02	2.60
m^7G	1.05	1.09				
A	1.38	0.38	2.41	1.38	2.23	1.42
m^7G	1.03	1.00				
Am	1.60	0.48	2.89	1.84	2.60	1.50
m^7G	1.29	1.36				
m^6Am	1.18	2.07	2.24	3.10	2.84	2.77
m^7G	1.06	1.03				

*Hydrolyzed with snake venom pyrophosphatase and bacterial alkaline phosphatase. See experimental.

Difficulty in commercial availability of pure cap structures makes routine direct determination of the RMRs for the cap structures unfeasible. Thus, we studied an attractive alternative approach and established a method for calculation of the cap response factors. We found that when the experiments were made under exactly the same chromatographic conditions, then the absolute molar response value for a cap structure is equal to the sum of the individual molar response values for each component nucleoside in the cap (Table III). Additional studies are in progress to measure the additivity of the three nucleosides in the cap 2 structures.

Application of method to single species in RNA

The RPLC method was applied to the determination of the type and quantity of cap in a single species of mRNA. The amount and type of cap structure present in a commercially obtained rabbit globin mRNA was investigated. Hydrolysis of the mRNA was accomplished using nuclease P1, which cleaves the phosphodiester bond between the nucleotides but leaves the cap 0 or cap 1 structures intact. Following the nuclease P1 treatment, BAP was added to cleave the phosphates from the other nucleotides, yielding ribonucleosides and the cap 0 or cap 1 structures (see Experimental).

Previous studies report that the cap structure present in rabbit globin mRNA is m^7Gpppm^6AmpCm, a type 2 cap [10]. Chromatography of the hydrolysed rabbit globin mRNA yielded one type of cap structure, m^7Gpppm^6Am (m^7GpppN'), and one type of modified ribonucleoside, Cm (N''), in addition to the four major ribonucleosides. Quantitative measurement of cap and Cm showed that the cap structure m^7Gpppm^6Am was present in ca. 60% of the amount expected in the commercial mRNA preparation, whereas the N''-nucleoside, Cm, was present in close to 100% of the theoretical cap value. One possible explanation for the lower amount of intact cap in the mRNA is that endonuclease cleaved m^7Gpppm^6Am from ca. 40% of the mRNA molecules during preparation of the mRNA.

Anion-exchange enrichment of cap structures from unfractionated RNAs

A procedure for isolation of cap structures from a nuclease P1 and BAP hydrolysate of bulk high-molecular-weight RNA had first to be developed in order to apply the RPLC measurement of caps to studies of cells and tissues. This technique would be used to separate cap 0 and cap 1 structures from the large quantity of nucleosides present in the hydrolysate and would ensure accurate quantitation of small amounts of cap 0 and cap 1 structures. Owing to the anionic nature of the triphosphate bridge between m^7Guo and the N'-nucleoside of the cap structure, the cap structures are retained on an anion-exchange column while the ribonucleosides elute under low ionic concentration of salt in 40% methanol. The second elution step consists of 2 *M* ammonium formate, which elutes all cap 0 and cap 1 structures (see Experimental).

A mixture of known quantities of the nine cap 0 and cap 1 structures (ca. 0.125 μg each) plus the four major ribonucleosides (ca. 20 μg each) were subjected to the anion-exchange procedure using three independent ion-exchange columns. The 2 *M* ammonium formate eluate was collected for quantitative

TABLE IV

RECOVERY OF NINE CAP STRUCTURES (m^7GpppN') USING ANION-EXCHANGE CHROMATOGRAPHY

m^7GpppN'	Recovery (%)*	R.S.D. (%)
Cyd	88	4.4
Urd	89	2.2
Guo	89	2.3
Ado	85	2.4
Cm	90	1.5
Um	83	3.4
Gm	98	3.6
Am	98	1.9
m^6Am	99	1.3

*Data based on three independent ion-exchange (IE) columns to isolate caps:

$$\text{Recovery} = \frac{\text{amount of cap after IE (by RPLC)}}{\text{amount of cap before IE (by RPLC)}} \times 100\%$$

RPLC analysis (see Experimental). Table IV shows the percentage recovery observed for each cap structure for each of the three anion-exchange columns. No detectable ribonucleoside peaks were found in the ammonium formate eluate using this procedure.

The overall anion-exchange recovery range was ca. 83—99%, with relative standard deviations ranging from 1.3 to 4.4% from the three independent analyses. Cap decomposition on the ion-exchange column has been discounted, since extended cap contact time with the column does not lead to decreased recovery. Also, the lyophilization of the eluate before the RPLC analysis does not destroy or change the cap structures. Fractions from the last washing step and from an extended 2 *M* ammonium formate elution step were analysed by RPLC, and no cap structures were observed in these eluates. Recoveries were increased from ca. 70 to 90% with the following adjustments: (a) use of a 2 *M* $HCOONH_4$ eluent in nanopure water instead of in 50% methanol; (b) a 100—200 mesh particle size resin; and (c) a very low flow-rate through the anion-exchange column.

This anion-exchange method for obtaining cap molecules can be adapted to the separation of cap structures from bulk nucleic acid after the nucleic acid has been digested with nuclease P1 and BAP. The hydrolysate would then contain cap 0 and cap 1 structures, any N''-nucleosides (from cap 2 structures) and the major nucleosides. The cap 0 and cap 1 structures could then be separated using the anion-exchange procedure, with an expanded first elution step and quantitation by RPLC.

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