THE METHYLATED CONSTITUENTS OF GLOBIN mRNA

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1. Introduction

Recent findings indicate that messenger RNA from a wide variety of cellular and viral sources contains methylated constituents [1–5]. Studies of complex mixtures of mammalian mRNA's have indicated that the methyl groups occur in internal residues of N^6 methyl adenylate and in unusual sequences at the 5′-termini [5–11]. The 5′-terminal sequences appear to be of the form $X^{5'}$ ppp$^{5'}$ $(N^mp)_{1-2}Np$ . . . in which a 'capping nucleoside' X (generally m^7G), is joined by a 5′–5′ triphosphate linkage to sequences containing either one or two 2′-*O*-ribose methylated nucleotides (N^mp).

In order to determine whether such components are also characteristic of a well-defined species of eukaryotic mRNA, we investigated the methylated derivatives of mRNA in immature duck erythrocytes. These cells synthesize readily detectable quantities of 9 S globin mRNA in addition to a variety of other mRNA's [12]. Our findings indicate that 9 S globin mRNA also contains capped 5′-terminal sequences, but very little, if any, internal base methylated derivatives. Moreover, compared to a mixture of other mRNA species synthesized by these cells, the 9 S mRNA is relatively enriched in the sequences that contain two 2′-*O*-methyl nucleotides.

2. Materials and methods

Immature erythrocytes (approximately 8 ml packed volume) were isolated from 120 ml of blood of anemic ducks, and suspended in plasma as previously described [13]. The plasma (70 ml) was supplemented with 2.5 mC: [^{3}H]methionine (7.3 Ci/m mol), 20 mM sodium formate, 20 μM each of guanosine and adenosine, 0.1 mM hemin and 10 mM HEPES buffer. The pH was adjusted to 7.4, and the cells incubated for 4 hr at 37 °C with a gentle rocking motion. After incubation the cells were washed in a balanced salt medium, lysed by hypertonic shock and polyribosomal RNA isolated [13]. The RNA was fractionated by passage through an oligo dT-cellulose column [14] and by zonal sedimentation on sucrose gradients [13]. One portion of each mRNA fraction was analysed by electrophoresis on exponential acrylamide gels [15], and the remainder digested in 0.3 N KOH for 24 hr at 37 °C. After neutralization, portions of the alkaline digests were further digested for 2 hr at 37 °C with 200–400 μg (5–10 units) of bacterial alkaline phosphatase (Worthington Biochem., BAP) per ml. The nucleotide derivatives were analysed on DEAE-Sephadex (urea) columns as described previously [1,7].

3. Results

In preliminary experiments, in which the total polyribosomal RNA of methyl-labeled erythroid cells was isolated and analysed by sucrose gradient sedimentation, we observed an intense labeling of 4 S components and a lower level of radioactivity distributed more or less uniformly throughout the remainder of the gradient. When the RNA in the 9 S regions of such gradients was concentrated and analysed by gel electrophoresis, the absorbance profile and methyl-labeling pattern closely resembled those previously found for 9 S polyribosomal RNA labeled with [^{3}H]uridine [12]: a prominent peak of radioactivity (MW ~ 230 000) migrating slightly

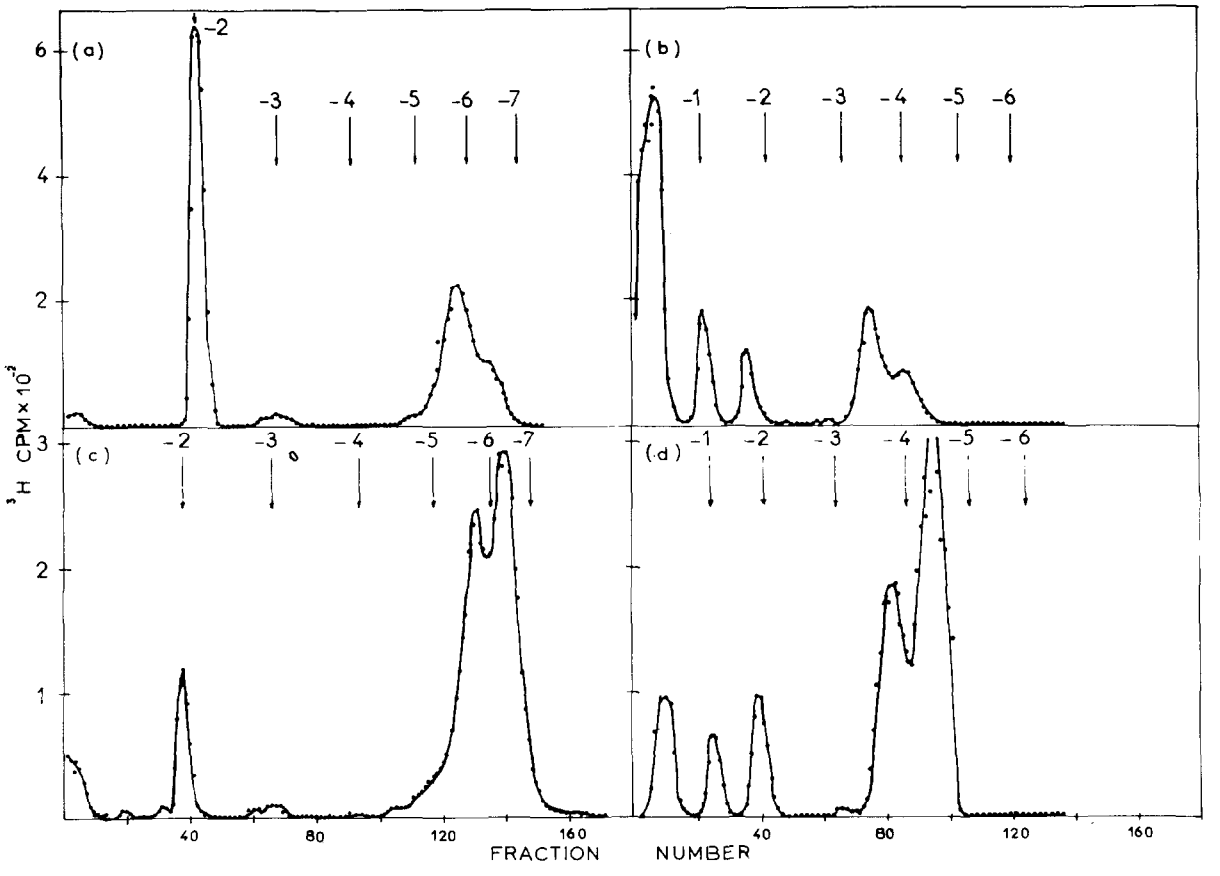

Fig.1. Sedimentation and electrophoretic mobility of 9 S and '21 S' methyl-labeled mRNA. (*a*) Sedimentation profile of polyadenylated mRNA isolated by dT cellulose chromatography from polyribosomal RNA of immature duck erythrocytes labeled for 4 hr with [^{3}H]methyl methionine. Sedimentation through isokinetic 5–19% sucrose gradient in 25 mM ionic strength buffer [13], 25 hr, 27,000 rpm. Horizontal arrows indicate fractions pooled as 9 S and '21 S' mRNA. (*b*) Electropherogram of 9 S RNA on 4–16% exponential acrylamide gel [15], run 12 hr at 10 V/cm. Shaded area indicates the radioactivity not considered to be associated with the 9 S globin mRNA. (*c*) Electropherogram of '21 S' fraction of mRNA, run as in panel b) on 1.8–20% exponential gel.

behind the absorbance peak (MW ~ 200 000) of globin mRNA (see below) and a second radioactive peak (MW ~ 180 000) migrating with a faster moving unidentified minor RNA component.

One cycle of chromatography on oligo dT cellulose, and selection for polyadenylated (*polyA*$^+$) RNA components eliminated most of the 4 S RNA, allowing us to discern a distinct peak of radioactivity in the 9 S region of the gradient as well as other more rapidly sedimenting labeled components (fig.1a). Gel-electrophoretic analysis showed approximately 84% of the radioactive RNA in the 9 S region to be in a band migrating slightly behind the bulk of the globin mRNA, with the rest in a trailing of slower migrating components (fig.1b). The more rapidly migrating component (MW ~ 180 000) that was found in preparations of total polyribosomal RNA was absent in the *polyA*$^+$ RNA, but could be recovered in 9 S fractions of *polyA*$^-$RNA. This component, which might represent the mRNA of histones F1 or F2c [16,17], has not yet been characterized further.

As a comparison for the 9 S mRNA, *polyA*$^+$ mRNA components sedimenting between 14 and 24 S (mode 21 S) were pooled (fig.1a) and also analysed.

When submitted to gel electrophoresis (fig.1c) these components migrated between the 28 S and 18 S ribosomal RNA markers. The fractions of 9 S and '21 S' *polyA*$^+$ mRNa had specific activities of 356 and 316 cpm/μg, respectively.

The mRNA's were hydrolysed with alkali, and the products chromatographed on DEAE-Sephadex (urea) columns at pH 7.5. The methylated components of the mRNA species sedimenting around 21 S (fig.2a) were found to have the same chromatographic properties as those previously observed for complex mixtures of mRNA's from mammalian sources [6,7,9–11]. About half of the methyl groups are in mononucleotides (charge −2) representing internal base-methylated derivatives. In mammalian mRNA these base methylated components have been found to consist exclusively of N^6 methyl adenylate [5–7]. The remainder of the methyl groups are in oligonucleotide derivatives carrying net charges of about −5.5 to −6.5. In other types of mRNA methylated

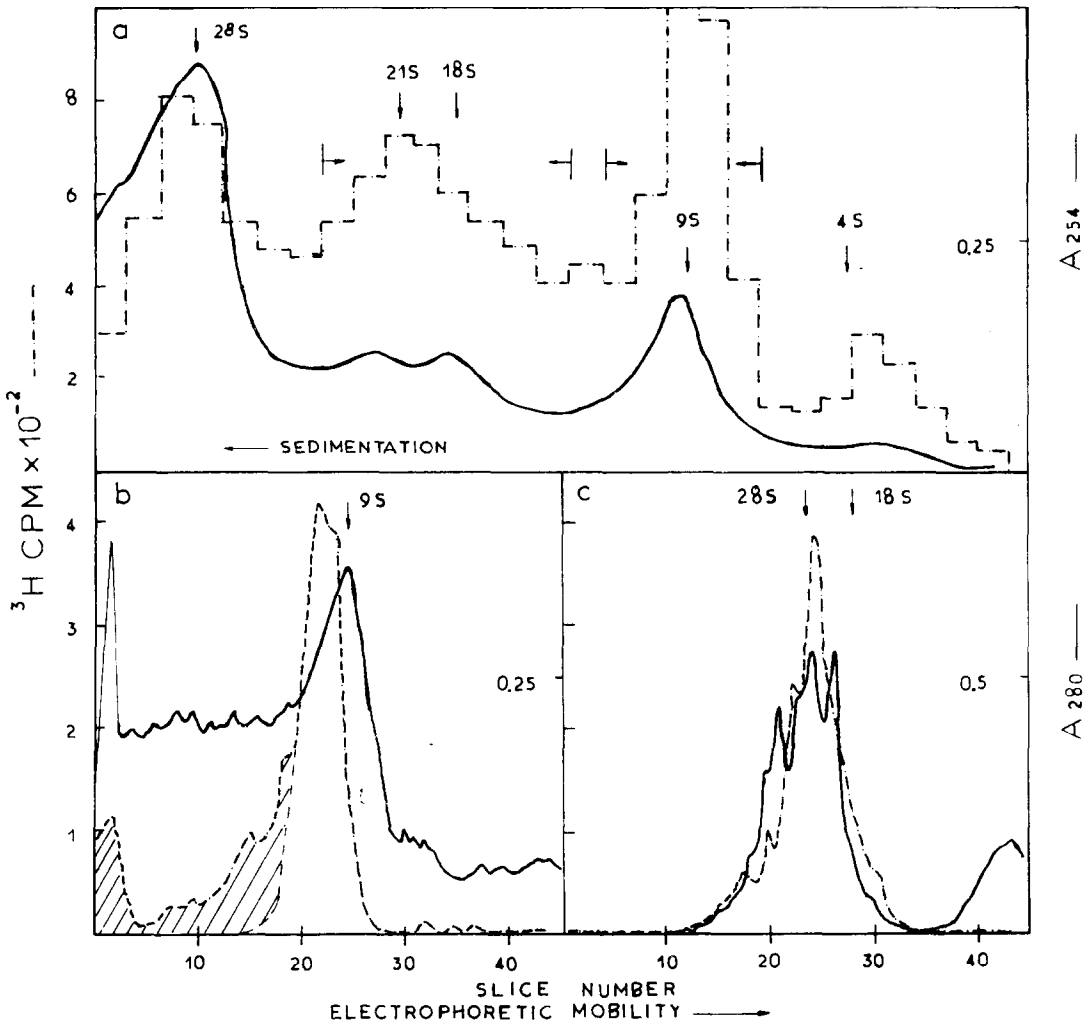

Fig.2. Analysis by chromatography on DEAE-Sephadex (urea) of the methylated derivatives of '21 S' (a,b) and 9 S (c,d) mRNA. The mRNA fractions shown in fig.1 were hydrolysed with alkali and the derivatives chromatographed without (a,c) or with (b,d) prior treatment with alkaline phosphatase. Elution by 400 ml gradient of 0.1–0.5 M NaCl in 7 M urea. Markers obtained from A_{260} profile of a ribonuclease A digest of yeast RNA.

derivatives with such chromatographic properties have been identified as $X^{5'}$ ppp$^{5'}$ N^mpNp and $X^{5'}$ ppp$^{5'}$ $(N^m p)_2$Np where the capping group, X is m^7G [6–10,18–20].

When the nucleotide derivatives of '21 S' mRNA were treated with alkaline phosphatase, the chromatographic profile shown in fig.2b was observed. The uncharged material, not absorbed to the column and recovered mainly in the wash prior the elution with the salt gradient (fractions 1 through 10), represents the internal base methylated derivates which were converted to nucleosides by the alkaline phosphatase treatment. The other methylated components, which are derived from the oligonucleotides, consisted of two species with net charges of approximately −3.5 and −4.5, presumably XpppN^{m}pN and Xppp $(N^m p)_2$N, and lesser amounts of two other species with charges of −1 and −2. The possible origin of these latter species, which we interpret to be N^mpN and $(N^m p)_2$N, is discussed below.

The profiles of the methylated derivatives of 9 S mRNA are shown in fig.2c and 2d. Two marked differences are apparent. First, the 9 S mRNA derivatives contain only a very minor amount (9%) of methylated mononucleotide. This small amount could be entirely attributable to the other mRNA components which contaminate the 9 S mRNA preparation (fig.1b) assuming that such contaminants are like '21 S' mRNA in that half of the methyl groups are internal base methylations. The other significant difference is in the relative proportions of the derivatives containing one and two 2′-*O*-methyl nucleotides. This is seen as reversed proportions of the -5.5 and -6.5 elution peaks (fig.2a and 2c), and after alkaline phosphatase treatment, in the relative proportions of components carrying charges of −1 and −3.5 vs. those carrying charges of −2 and −4.5 (figs. 2b and 2d). From these data we estimated the molar ratios of terminal sequences with one and two 2′-*O*-methyl nucleotides to be about 3 and 1 for '21 S' and 9 S mRNA, respectively (table 1). In a similar experiment with a different batch of labeled cells, a lower overall abundance of sequences containing two 2′-*O*-methyl nucleotides was observed for both '21 S' and 9 S mRNA; nevertheless the relative proportion of such sequences in the 9 S mRNA was again markedly greater than in the other mRNA species.

4. Discussion

The results presented here demonstrate clearly that the 9 S globin mRNA produced in duck erythrocytes is methylated. The newly made globin mRNA, which is synthesized by the erythroblast portion of the cell population [21], is identified as a radioactive component sedimenting coincidentally with the mature 9 S globin mRNA, and migrating slightly behind it on acrylamide gels [12]. The slower migration is presumably due at least in part to the fact that the *polyA* segment of newly made globin mRNA is three to four times longer than that of mature globin mRNA [22,23].

In view of the widespread occurrence of internal residues of N^6 methyl adenine in other types of eukaryotic mRNA [1,5–7,9–11], it was suprising not to find internal base methylations in globin mRNA. Since, during the 4-hr incubation, radioactive methyl groups were indeed incorporated into other methylated constituents of globin mRNA, and into internal nucleotides of other species of mRNA, it seems clear that this result is not due to ineffective labeling of the methyl donor pool, but rather to the distinctive methylation composition of globin mRNA. In this respect the globin mRNA seems to resemble several types of viral mRNA in which methylation occurs only at the 5′-termini.

The existence in globin mRNA of the derivatives characterestic of capped 5′-terminal methylated sequences is consistent with the findings that 5′-terminus of globin mRNA is not reactive with polynucleotide kinase [24], and that the template activity of the mRNA from rabbit reticulocytes is abolished by the β-elimination reaction which removes the m^7G moiety and 'uncaps' such 5′-terminal sequences [25].

The 9 S mRNA appears to contain the two types of capped 5′-terminal sequence characteristic of other eukaryotic mRNA's, that is sequences containing either one or two 2′-*O*-methylated residues adjacent to the cap [6–10]. Compositional and kinetic analyses of these sequences in complex mixtures of mRNA do not suggest a precursor–product relationship between the sequences containing one and two 2′-*O*-methyl residues, but rather suggest two separate classes of 5′-termini [9,26]. Thus, one might suppose that the 9 S mRNA consists of two

Table 1
Estimated proportions of various methylated constituents

RNA type	Internal base methylation (%)	5′-terminal nucleotides				
		$XpppN^mpN$ (%)	$Xppp(N^mp)_2N$ (%)	N^mpN (%)	$(N^mp)_2N$ (%)	Molar ratio $\frac{N^mp}{(N^mp)_2}$
'21 S' mRNA	47	23	9.8	12	8.2	3.1
9 S mRNA	0	32.5	52	5.9	9.6	1.0

Internal base methylation calculated from the fraction of radioactivity in mononucleotides (charge −2) in figs.2a and 2c. The value for 9 S mRNA was corrected on the assumption that the 16% of contaminating mRNA (shaded area in fig.1b) is of the '21S' type i.e. that approximately half of the methyl constituents comprise internal base methylation.

Proportions of different 5′-terminal derivaties calculated from the fractions of radioactivity in the various peaks of figs.2b and 2d with the following assignments: N^mpN (charge −1); $(N^mp)_2N$ (charge −2); $XpppN^mpN$ (charge <−4); $Xppp(N^mp)_2N$(charge >−4).

Molar ratios calculated on the assumption that X contains a methyl group, e.g. m^7G, and that each 2′-*O*-methylated nucleotide, N^mp, contains one methyl group. The molar ratio is thus given by:

$$[(XpppN^mpN)/2 + N^mpN]/[(Xppp(N^mp)_2N)/3 + (N^mp)_2/2]$$

populations of molecules, each with a distinctive 5′-terminus, as would be the case, for example, if the mRNA's for α and β globin had different 5′-terminal sequences.

One final point concerns the fraction of the methylated derivatives of both 9 S and '21 S' mRNA that lose four units of charge upon treatment with alkaline phosphatase. When the capping group, X, carries some positive charge, as in the case of m^7G [19], the derivatives Xppp $(N^mp)_{1-2}Np$ and $p(N^mp)_{1-2}Np$ would possess similar net charges and would thus be distinguishable by DEAE chromatography only after removal of the monoesterified phosphates. The uncapped derivatives, which have also been observed as minor components of the mRNA of mouse L cells [7], might be the result of a certain turnover of caps which could be regenerated by the reaction

$$p(N^mP)_{1-2}Np \ldots + Xppp \longrightarrow Xppp(N^mp)_{1-2}Np \ldots + p$$ [20,27].

Alternatively, the uncapped derivatives might be artifactually generated by cleavage of pyrophosphate bonds during alkaline hydrolysis or alkaline phosphatase treatment. However, in this regard, we did not observe a significant difference in their proportion when the concentration of alkaline phosphatase and the duration of incubation were both reduced concomitantly by factors of four.

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