Methylated Nucleosides in Globin mRNA from Mouse Nucleated Erythroid Cells*

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Poly(A)-containing mRNAs labeled with \(\text{methyl-}^{3}\text{H}\)methionine were isolated from nucleated erythrocyte cells obtained from the spleens of anemic mice. The RNAs were further separated into non-globin poly(A)-containing RNAs and highly purified globin mRNA by globin cDNA-cellulose affinity chromatography. DEAE-Sephadex column chromatography of the T1 ribonuclease digestion products of the cDNA-purified globin mRNA fraction yielded methylated resistant fragments with charges of -4.7 (Cap 1) and -5.3 (Cap 2). Digestion of the non-globin RNA fraction revealed a similar pattern with the addition of a methylated mononucleotide identified as 6-methyladenosine at -2 charges. Alkaline phosphatase treatment of the T1 resistant fragments reduced their charges by approximately 2, which is consistent with the removal of one terminal phosphate. Treatment of the globin T2 and alkaline phosphatase-resistant fragments with penicillin P1, nuclease and alkaline phosphatase yielded a P1-resistant core structure in both fragments. In addition to the core, 2'-O-methylcytidine (Cm) was released from the more negatively charged globin fragment. The P1-resistant cores of the cap structures eluted from DEAE-Sephadex with the known standard \(\text{m'}\text{G}''\text{ppp}''\text{Am}\) and were found to be pyrophosphatase-sensitive establishing a 5'-5'-triphosphate linkage. The pyrophosphatase and alkaline phosphatase digestion products of the globin Cap 1 and Cap 2 core structures were analyzed by high voltage electrophoresis and paper chromatography and found to be 7-methylguanosine (\(\text{m'}\text{G}\)) and the dimethylated nucleoside 6-methyl-2'-O-methyladenosine (\(\text{N}''\text{mAm}\)). A small amount of the singularly methylated adenosine, 2'-O-methyladenosine (Am) was also observed. The predominant sequences of the methylated nucleosides in the globin cap structures are therefore \(\text{m'}\text{G}''\text{ppp}''\text{N}''\text{mAm}\) and \(\text{m'}\text{G}''\text{ppp}''\text{N}''\text{mAm}\).

EXPERIMENTAL PROCEDURES

Sources of Materials – Enzymes were purchased from Calbiochem (RNase T2), P-L Biochemicals (Escherichia coli alkaline phosphatase and P, nuclease), and Worthington Biochemical Corp. (snake venom phosphodiesterase). Methylated nucleoside markers were purchased from Sigma Chemical Corp. and P-L Biochemicals. The cap sequence \(\text{m'}\text{G}''\text{ppp}''\text{Am}\) was obtained from P-L Biochemicals. \(\text{m'}\text{H}\)Methionine was obtained from Research Products International Corp. and Amersham/Searle.

Isolation of Globin mRNA Purified from Nucleated Erythroid Cells – Nucleated erythroid cells from the spleens of anemic mice, obtained as previously described (22), were suspended in 5 x 10^7 cells/ml in methionine-free RPMI-1640 tissue culture media containing 20 mM formate, 20 mM adenosine, 20 mM guanine, 4 mM uridine, and 3.6 \(\mu\)M \(\text{m'}\text{H}\)Methionine (11 Ci/mmol). After incubation for 5 min at 37°C in 5% CO_2 in air, the media was made 18 \(\mu\)M in methionine by adding unlabeled methionine and incubated an additional 4 h. The cells were harvested, washed twice with phosphate-buffered saline, pH 7.5, and resuspended in 0.9% saline. Poly(A)+ RNA was isolated as described by Merk et al. (22) and separated on a 2.8 M CsCl gradient.

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† Postdoctoral fellow of the National Cancer Institute.
‡ The abbreviations used are: \(\text{m'}\text{G}\), 7-methylguanosine; \(\text{Am}\), 2'-O-methyladenosine; \(\text{N}''\text{mAm}\), 6-methyl-2'-O-methyladenosine; \(\text{Cm}\), 2'-O-methylcytidine; \(\text{Um}\), 2'-O-methyluridine; \(\text{Gm}\), 2'-O-methylguano-
sine; poly(A), polyadenylic acid; cDNA, complementary DNA; SDS, sodium dodecyl sulfate; RNase, heterogeneous nuclear RNA; P1, penicillin nuclease; \(\text{m'}\text{G}\), 2-aminoo-4-hydroxy-5-\(\text{N}\)-methylformamide-6-ribosylaminopyrimidine. N, N' and N" are used to designate unknown nucleosides one, two, and three bases removed from the 5'-terminal nucleoside (\(\text{m'}\text{G}\)).
into purified globin mRNA and non-globin RNA by globin cDNA-cellulose affinity chromatography. The cDNA-cellulose was prepared by a modification (23) of the procedure of Venetianer and Leder (24). Poly(A)+ RNA from mouse nucleated erythroid cells was hybridized to a 1 ml globin cDNA-cellulose column at 65° for 30 min in 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1 mM EDTA, and 0.2% sodium dodecyl sulfate. The unbound fraction was eluted in this buffer at 65° and the bound fraction was eluted with H2O at the same temperature. Recovery from the globin cDNA-cellulose column was >95% of the input radioactivity. The globin cDNA-cellulose purified globin mRNA ran as a single peak at 10 S on sucrose density gradients.

Enzymatic Treatment and Chromatography of Poly(A)+ RNA – Globin mRNA and non-globin poly(A)+ RNAs (10 to 20 μg) were digested with RNase T1 (50 units/ml) in 0.5 ml of NH4Ac buffer at pH 4.5 for 4 h at 37°. The reaction mixture was then diluted with 10 ml of triethylammonium bicarbonate at pH 8.0. The samples were lyophilized and then digested with yeast tRNA. Digestions with alkaline phosphatase (30 units/ml) or a combination of alkaline phosphatase and snake venom phosphodiesterase (60 units/ml) were done in 20 μl of 50 mM Tris-HCl (pH 8.0), 5 mM MgCl2, and incubated for 3 h at 37°. P. nuclea (500 μg/ml) digestion were in 50 μl of 10 mM sodium acetate (pH 6.0) for 1 h at 45°. The modified 2'-O-methyladenosine was depurinated by heating at 100° for 1 h in 1 N HCl and chromatographed as described by Moyer and Banerjee (10). Electrophoresis of digested samples was run on Whatman No. 3MM paper for 4 h at 3500 V in Buffer A (pyridine:acetate, pH 3.5). Paper chromatography solvents employed were as follows. (A) butanol:acetic acid:H2O (50:36:20), (B) methanol/ethyl acetate:H2O (2:3:1), (C) ethyl acetate:propanol:7.5 M ammonium hydroxide (2:3:1), (D) isobutyric acid:20% ammonium hydroxide (25:100:201), (E) butanol:ammonium hydroxide:H2O (68:170:144), (F) isopropyl alcohol:ammonium hydroxide (7:2:1), and (G) isopropyl alcohol:concentrated hydrochloric acid:H2O (680:170:144). Ribose was identified from the appearance of a reddish brown color after dipping the paper chromatogram into an aniline phosphate reagent (aniline:H2O:acetic acid:phosphoric acid, 2:20:18:1) mixed 2:3 with acetic acid, and heating for 5 min at 90° (25).

RESULTS

Purification of methyl-3H-labeled Globin mRNA – Analysis of the methylated constituents of a specific eukaryotic mRNA requires that the RNA be prepared free of other cellular RNAs and that a sensitive method for detecting methylated nucleosides be available. Although highly purified preparations of globin mRNA can be obtained from mammalian reticulocytes, detection of methylated nucleotides using radioactive precursors is not possible because these cells do not synthesize RNA. Radioactivity can be introduced into the 5' cap structure of mRNA by periodate oxidation followed by reduction with radioactive borohydride (17), however, internal methylated nucleosides are not labeled and their presence cannot be detected by this method.

We have recently shown that globin mRNA can be prepared from nucleated erythroid cells obtained from the spleens of anemic mice (22). These cells actively incorporate the methyl group of [methyl-3H]methionine into RNA when incubated in vitro, and therefore provide an ideal system for investigating the methylated constituents of a well characterized mRNA.

The nucleated erythroid cells were incubated for 4 h with [methyl-3H]methionine under labeling conditions previously shown to prevent incorporation of methionine methyl groups into the purine ring (26). Although we have shown that globin mRNA can be prepared from nucleated erythroid cells by a combination of oligo(dT)-cellulose affinity chromatography and sucrose density gradient centrifugation of RNA extracted by phenol:chloroform:isoamyl alcohol (22), there is contamination of this preparation with other 9 S poly(A)-containing RNAs. Because the purity of the RNA is critical for these studies, the poly(A)+ RNAs were further purified using a globin cDNA-cellulose column (23). The column was prepared by copying highly purified globin mRNA into cDNA with reverse transcriptase, while the mRNA was annealed to oligo(dT)-cellulose. The globin cDNA copy is attached to cellulose through the covalently bound oligo(dT) which serves as the primer. The properties of this column have been investigated and conditions determined where globin mRNA is retained by the column while non-globin RNAs are not (23). This column was used to further separate the poly(A)+ RNAs isolated from nucleated erythroid cells into globin mRNA and non-globin poly(A)+ RNAs. Forty-five per cent of the methyl-3H-labeled poly(A)-containing RNA from nucleated erythroid cells was retained by the globin cDNA-cellulose column, while 55% was not. R3 c2 analysis, performed in solution, of the cDNA-cellulose-bound RNA showed that it is as pure as the globin mRNA isolated from the polysomes of mouse reticulocytes by a combination of oligo(dT)-cellulose and sucrose density gradient centrifugation (23).

Enzymatic Digestion and Product Analysis – Globin mRNA

\[ R_3 c^2 \] is defined as the product of the concentration of RNA (in moles of nucleotides per liter) and time (seconds).
and non-globin poly(A)+ RNAs were digested with T1 ribonuclease and the methylated products analyzed by DEAE-Sephadex chromatography (Fig. 1). Pancreatic RNase digests of yeast RNA served as internal markers for the various sized oligonucleotides. Two resistant nucleotide fragments with charges of -4.7 (Cap 1) and -5.3 (Cap 2) were found in the T1 digestion products of globin mRNA (Fig. 1a). No other radioactivity eluted from the column. The absence of label at the dinucleotide position indicated that the mRNA preparation was free of ribosomal RNA. A similar elution pattern was observed for the non-globin fraction with the addition of a large peak eluting at the mononucleotide position (Fig. 1b). The ratio of Cap 1 to Cap 2 also differed between globin and non-globin mRNAs, the Cap 1/Cap 2 ratio in globin mRNA at 4 h incubation was 0.43, while this ratio was 2.13 in the non-globin poly(A)+ RNAs. The radioactive material eluting at the mononucleotide position from the non-globin fraction was identified as 6-methyladenosine by autoradiography following two-dimensional thin layer chromatography (Fig. 2). Although internal 6-methyladenosine has been observed in mixtures of cellular mRNAs, and occurs in the non-globin poly(A)+ RNAs of mouse nucleated erythroid cells, no internal 6-methyladenosine is present in purified globin mRNA.

The T1-resistant fragments from globin mRNA and non-globin poly(A)+ RNA were treated with alkaline phosphatase and chromatographed on DEAE-Sephadex A-25. The phosphatase-treated fragments lost 1.7 to 1.8 negative charges, indicating the loss of one terminal phosphate. This is compatible with the unique 5'-terminal cap structures m7Gppp5'NmpNP and m7Gppp5'NmpNmpNP found in mixtures of eukaryotic cellular mRNAs. Ten to twelve per cent of the radioactivity eluted in the wash after alkaline phosphatase treatment. This may result from a small amount of uncapped material which, after T1 digestion, has a similar charge to Cap 1 or Cap 2 and, therefore, elutes with these oligonucleotides. If the adenosine residue was 2'-O-methylated, then the T1 ribonuclease and alkaline phosphatase digestion products would result in a dinucleotide and would not elute in the 20 mM Tris HCl (pH 7.6) wash. This suggested a base methylation and because of this and the finding that the methylated materials electrophoresed with methyladenosine, it is proposed that some pppNmpAp is present in the globin mRNA preparation. There was, however, insufficient material to confirm this.

Following desalting, the two cap structures isolated from globin mRNA (Fig. 1a) were converted to nucleosides by digestion with a combination of snake venom phosphodiesterase and alkaline phosphatase. The resulting nucleosides were analyzed by high voltage electrophoresis and plotted in the form of a histogram (Figs. 3a and 6a). Three radioactive peaks were identified as methylated bases by autoradiography following two-dimensional thin layer chromatography of the internal 3H-methylated base from non-globin poly(A)+ RNAs. The radioactive material eluting at the mononucleotide position from the non-globin fraction was identified as 6-methyladenosine by autoradiography following two-dimensional thin layer chromatography (Fig. 2). Although internal 6-methyladenosine has been observed in mixtures of cellular mRNAs, and occurs in the non-globin poly(A)+ RNAs of mouse nucleated erythroid cells, no internal 6-methyladenosine is present in purified globin mRNA.

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obtained from the globin Cap 1 structure (Fig. 3a). Peak I migrated in the position of 2'-O-methyluridine, 2'-O-methylguanosine, and the open ring form of 7-methylguanosine while Peaks II and III migrated with 2'-O-methyladenosine and 7-methylguanosine, respectively. The nucleosides present in these three peaks were further analyzed by ascending paper chromatography (Fig. 3, b to d). Peak I was found to contain predominantly ring-opened 7-methylguanosine (Fig. 3b). 7-Methylguanosine undergoes ring scission to form 2-amino-4-hydroxy-5-(N-methyl)formamide-6-riboylaminopyrimidine (ring-opened m7G) when analyzed in the alkaline solvent employed for this chromatography (12). The 7-methylguanosine present in Peak I of Fig. 4a was in the ring-opened form prior to analysis by paper chromatography, as is evident from its electrophoretic migration. Ring-opened 7-methylguanosine is an uncharged compound and migrates at or near the origin after electrophoresis at pH 3.5, whereas authentic 7-methylguanosine is positively charged and migrates away from the origin toward the anode (Fig. 3c). The third peak resolved by electrophoresis (Fig. 3a) also co-chromatographed with ring-opened 7-methylguanosine in the alkaline solvent (Fig. 3d). It was not, however, in the ring-opened form prior to analysis by paper chromatography because it electrophoresed with authentic 7-methylguanosine and co-chromatographed with authentic 7-methylguanosine when analyzed by two-dimensional paper chromatography (Fig. 4). It is evident from this data that approximately 20% of the 7-methylguanosine in both Cap 1 and Cap 2 undergoes ring scission prior to electrophoretic analysis. Because 7-methylguanosine undergoes ring scission even at neutral pH values (27), it is probable that conversion to the open ring form occurs during isolation of the cap structures. The methylated material from Cap 1 which electrophoresed with 2'-O-methyladenosine was resolved into two components by paper chromatography. The majority of the radioactivity migrated faster than authentic 2'-O-methyladenosine (Fig. 3c). To determine if this component was a base-methylated derivative of 2'-O-methyladenosine it was depurinated and the products analyzed by descending paper chromatography in a solvent which separates ribose from methyladenine (10). Radioactivity migrated with both the ribose and the 6-methyladenine marker indicating that the nucleoside is methylated on both the ribose and the adenine base (Fig. 5a). Because 6-methyladenine and 6,6-dimethyladenine run similarly in this solvent and because more radioactivity appeared in the base than in the ribose, there was a possibility that the adenine was dimethylated. The base-methylated material was, therefore, chromatographed using a solvent which separates 6,6-dimethyladenine from 6-methyladenine (Fig. 5b). The radioactivity co-chromatographed with 6-methyladenine indicating that the methylated adenosine in the Cap 1 structure of globin mRNA is 6-methyl-2'-O-methyladenosine. This nucleoside has also been observed in vesicular stomatitis virus and BHK cells (10), HeLa cells (28), and L cells (29).

The methylated nucleosides of globin Cap 2 were identified using the procedures employed for globin Cap 1. Following the digestion of globin Cap 2 with snake venom phosphodiesterase and alkaline phosphatase, four peaks were resolved by high voltage paper electrophoresis (Fig. 6a). Three of the four peaks (I, II, and IV, Fig. 6a) had the same migration properties as

![Fig. 4. Two-dimensional thin layer chromatography of 3H-methylated m7G. The methyl-3H-labeled nucleoside from Peak III in Fig. 3a and Peak IV in Fig. 3a (558 cpm) were mixed with unlabeled marker and spotted on Whatman No. 3MM chromatography paper. The nucleosides were chromatographed by Solvent G in direction one and by Solvent F in direction two. The unlabeled nucleosides were located on the paper by ultraviolet absorbance. The ultraviolet absorbent spots were cut out, dried, and treated with 0.5 ml of a 10% solution of aniline phosphate reagent (25). The methylated adenine was eluted from the paper and reanalyzed on ascending paper chromatography in Solvent E.](http://www.jbc.org/)
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Fig. 6. Identification of methylated nucleosides in Cap 2 of globin mRNA. Cap 2 of globin mRNA was analyzed as described in Fig. 3 except that 0.25-ml aliquots from 2-ml fractions were counted. a, electrophoresis of alkaline phosphatase and snake venom phosphodiesterase digested globin Cap 2. b, paper chromatogram of Peak I. c, paper chromatogram of Peak II. d, paper chromatogram of Peak III. e, paper chromatogram of Peak IV.

Fig. 7. Identification of methylated nucleosides in Cap 1 of non-globin poly(A)+ RNA. Cap 1 of non-globin poly(A)+ RNA was analyzed as described in Fig. 3. a, electrophoresis of alkaline phosphatase and snake venom phosphodiesterase digested non-globin Cap 1. b, paper chromatogram of Peak I. c, paper chromatogram of Peak II. d, paper chromatogram of Peak III. e, paper chromatogram of Peak IV.

Table I

Fraction of methylated nucleosides in globin mRNA and non-globin poly(A)+ RNA cap structures

<table>
<thead>
<tr>
<th>Nucleoside</th>
<th>Globin</th>
<th>Non-globin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cap I</td>
<td>Cap II</td>
</tr>
<tr>
<td>Cm</td>
<td>0.04</td>
<td>0.59</td>
</tr>
<tr>
<td>Um + Gm</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Am</td>
<td>0.08</td>
<td>0.04</td>
</tr>
<tr>
<td>N'mAm</td>
<td>0.68</td>
<td>0.27</td>
</tr>
<tr>
<td>m'G</td>
<td>0.18</td>
<td>0.07</td>
</tr>
<tr>
<td>*m'(m'G)</td>
<td>0.05</td>
<td>0.02</td>
</tr>
<tr>
<td>m'G total</td>
<td>0.23</td>
<td>0.09</td>
</tr>
</tbody>
</table>

the three peaks resolved by electrophoresis of the digested nucleosides of globin Cap 1 (Fig. 3a). Chromatographic analysis of these three peaks revealed results identical to those observed for globin Cap 1 (Fig. 6b, c, and e). The additional peak (Peak III) observed after electrophoresis of the digested fragments of globin Cap 2, chromatographed with 2'-O-methylcytidine (Fig. 6d). The overall composition of globin Cap 1 and Cap 2 is given in Table I and is expressed as the ratio of radioactivity in each nucleoside to the total radioactivity in the given cap structure.

The composition of the Cap 1 and Cap 2 structures of the non-globin poly(A)+ RNAs was determined using procedures identical to those employed for Cap 1 and Cap 2 of globin mRNA. These non-globin RNAs were found to contain 7-methylguanosine and all four 2'-O-methylnucleosides in their capped structures (Figs. 7 and 8 and Table I). In addition, 6-methyl-2'-O-methyladenosine was also observed in both of the non-globin capped structures (Table I).

The sequences of the methylated Cap structures of globin mRNA were determined as follows. Globin mRNA was digested with T1 RNase and alkaline phosphatase and Cap 1 and Cap 2 purified by DEAE-Sephadex chromatography. The cap structures were then treated with P1 nuclease followed by alkaline phosphatase. P1 nuclease cleaves adjacent to 2'-O-methylated nucleosides, but will not cleave the 5'-5' triphosphate linkage found in cap structures. The reactions are:

- m'G'ppp'N,mNpN,pN2 ↓ P1 nuclease
- m'G'ppp'N,mN + pN2 ↓ Alkaline phosphatase
- m'G'ppp'N,m + N2 ↓ P1 nuclease
- m'G'ppp'N,mN + pN2 + pN3 ↓ Alkaline phosphatase
- m'G'ppp'N,mN + N2 + N3
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Fig. 8. Identification of methylated nucleosides in Cap 2 of the non-globin poly(A)+ RNA. Cap 2 of non-globin poly(A)+ RNA was analyzed as described in Fig. 3. a, electrophoresis of alkaline phosphatase- and snake venom phosphodiesterase-digested non-globin Cap 2. b, paper chromatogram of Peak I. c, paper chromatogram of Peak II. d, paper chromatogram of Peak III. e, paper chromatogram of Peak IV.

Fig. 9. Analysis of the P, digestion products of the Cap 1 and Cap 2 structures of globin mRNA. Purified Cap 1 and Cap 2 of globin mRNA were treated with P, nuclease followed by alkaline phosphatase, mixed with unlabeled nucleoside markers and analyzed by high voltage electrophoresis in Buffer A. The paper was dried and cut into 1-cm strips, and the components were eluted with 1 ml of H2O. Aliquots (0.2 ml) were counted. a, Cap 1; b, Cap 2.

Fig. 10. DEAE-Sephadex A-25 column chromatography of Cap 1 and Cap 2 P, resistant cores. The 3H-methylated Cap 1 and Cap 2 P, resistant cores were mixed with 1.5 A, units of the known standard m7G5'ppp5'Am in 7 M urea, 0.02 M Tris-HCl (pH 7.6), and layered on a DEAE-Sephadex A-25 column (40 x 0.9 cm). The compounds were eluted with a 0 to 0.5 M NaCl 400-ml gradient. Fractions (2 ml) were collected and counted. Unlabeled standard was located by ultraviolet absorbance (solid line).

Fig. 11. Identification of nucleosides in P, resistant cores of globin Cap 1 and Cap 2. The P, resistant cores from both Cap 1 (Peak I, Fig. 9a) and Cap 2 (Peak I, Fig. 9b) of globin mRNA were eluted from the paper digested with pyrophosphatase and alkaline phosphatase and analyzed by high voltage electrophoresis in Buffer A. The paper was dried, cut into 1-cm strips, and counted as described in Fig. 3. a, Cap 1 P, resistant core; b, Cap 2 P, resistant core.

The resulting products were analyzed by high voltage electrophoresis (Fig. 9). Because only the methyl groups are labeled, the nucleoside N, in Cap 1 and N, in Cap 2 will not be radioactive and will not be observed by our analysis. A single peak of radioactivity migrating with m7G5'ppp5'Am was observed after digestion of Cap 1 with P, nuclease and alkaline phosphatase (Fig. 9a). A peak with identical migration properties was observed after enzymatic treatment of Cap 2, however, an additional peak was found which migrates with 2'-O-methylcytidine (Fig. 9b). The P, resistant cores of both Cap 1 and Cap 2 were eluted from the paper and analyzed by DEAE-Sephadex column chromatography (Fig. 10). The labeled Cap 1 and Cap 2 P, resistant cores eluted at the same NaCl concentration as the known standard m7G5'ppp5'Am establishing a triphosphate linkage. The core structures were digested to nucleosides with pyrophosphatase and alkaline phosphatase and reanalyzed by high voltage electrophoresis (Fig. 11). The products migrated with 7-methylguanosine and 2'-O-methyladenosine in both cases. The majority (88 to 90%) of the nucleoside migrating with 2'-O methyladenosine is the dimethylated derivative 6-methyl-2'-O-methyladenosine (Table I). The following are, therefore, the predominant sequences of globin Cap 1 and Cap 2: m7G5'ppp5'N5nAm and m7G5'ppp5'NmAmCm.
Methylnucleosides in Globin mRNA from Mouse Erythroid Cells

DISCUSSION

We have identified the methylated nucleosides and determined their sequence in highly purified globin mRNA isolated from nucleated erythroid cells. All of the methylated nucleosides reside in one of two blocked structures at the 5' end of the mRNA referred to as Cap 1 and Cap 2. Both caps were found to contain P1-resistant, pyrophosphatase-sensitive cores characteristic of the blocked 5' structures m7G5'ppp'N^-m and m7G5'ppp'N^-mmpN, found in mRNAs. The sequences of globin Cap 1 and Cap 2 were found to be m7G5'ppp'N^-mAmp and m7G5'ppp'N^-mAmpCm. However, in both structures approximately 11% of the adenosine was not base-methylated. The different methyl groups in these sequences reside in one of two blocked structures at the 5' end of the mRNA referred to as Cap 1 and Cap 2. Both caps were found to have their sequence in highly purified globin mRNA isolated from different pools (nuclear and cytoplasmic) or that methylated in 2'-O-methylcytidine in Cap 2. Recent evidence indicates that differential nuclear cytoplasmic methylation may, however, explain the large amount of radioactivity resulting from turnover of HnRNA from mouse L cells have shown that the per cent of adenosine which is dimethylated is the same in the HnRNA as in the class of mRNAs derived from the HnRNA (29). This result implies that differential nuclear cytoplasmic methylation is not the cause of the unequal labeling of the methyl groups on N^-mAm. Differential nuclear cytoplasmic methylation may, however, explain the large amount of radioactivity in 2'-O-methylcytidine in Cap 2. Recent evidence indicates that Cap 2 is derived from Cap 1 by an additional ribose methylation on the base adjacent to Cap 2 (30). Investigation has also shown that while the methylations responsible for Cap 1 are nuclear events, the conversion of Cap 1 to Cap 2 occurs in the cytoplasm. During continuous labeling, an equilibrium between Cap 1 and Cap 2 is established at longer incubation times (30). The conversion of any Cap 1, present prior to incubating with [methyl-3H]methionine, to Cap 2 will result in Cap 2 with only the 2'-O-methylcytidine labeled. This could explain a greater amount of radioactivity in 2'-O-methylcytidine at short incubation times. It is not known why the residue radioactivity observed in 7-methylguanosine is low in both cap structures. Less than equal molar ratios of m7G in cap structures have also been observed in L cells and BHK cells (10, 29). These observations may result from turnover of the 7-methylguanosine, however, further investigation is necessary to establish this.

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Note Added in Proof—A similar 5'-terminal cap sequence for mouse globin mRNA has been found by Cheng and Kazazian (31).

REFERENCES

Methylated nucleosides in globin mRNA from mouse nucleated erythroid cells.
W L Heckle, Jr, R G Fenton, T G Wood, C G Merkel and J B Lingrel


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