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Cytoplasmic location of undermethylated messenger RNA in Novikoff cells

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ABSTRACT

Novikoff cells in culture were labeled with L-[methyl-^{3}H]methionine and [U-^{14}C]uridine in the presence of (a) TubHcy[2], (b) AdoHcy, (c) homocysteine, (d) tubercidin, or (e) without any additions. Only in cultures labeled in the presence of TubHcy were undermethylated cap structures observed to represent a significant portion of [^{3}H]methyl radioactivity.

Novikoff cells in culture were then simultaneously labeled with L-[methyl-^{3}H]methionine and [^{32}P]orthophosphate in the presence or absence of TubHcy. Total cytoplasmic, polysomal and monosomal poly(A)-containing RNAs were analyzed. Both monosomal and polysomal mRNA fractions from TubHcy-treated cells contain partially methylated cap structures, suggesting that 2'-O-methylation of the nucleoside adjacent to the pyrophosphate linkage in caps is not required for transport, ribosomal binding or translation. Comparison of nuclear and cytoplasmic cap structures from normal and inhibited cultures indicate that an altered mRNA population is generated in the presence of TubHcy.

INTRODUCTION

Methylated cap structures (cap 1, m^{7}GpppN'mpN and cap 2, m^{7}GpppN'mpN"mpN) have been identified at the 5'-terminus of a variety of viral and eukaryotic messenger RNAs [3,4,5]. The presence of cap structures on hnRNA molecules[6,7] and the kinetics of methylation at specific sites within cap structures[8,9,10] suggest that capping and methylation may be important processing events in the generation of mature mRNA. The discovery of intervening sequences within the coding region of unique genes[11-14] and the demonstration that transcription of the intervening sequences into primary RNA transcripts occurs during expression of β-globin genes[15], suggests a model for mRNA processing in which both the 5'- and 3'-termini of an hnRNA molecule are conserved during mRNA biogenesis.

In order to assess the possible role of methylation in mRNA processing, we have perturbed methylation *in vivo* and thereby generated undermethylated

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mRNA[16]. These studies demonstrated that S-tubercidinylhomocysteine (TubHcy), the 7-deaza analogue of S-adenosylhomocysteine (AdoHcy), inhibited mRNA methylation in viable Novikoff cells. The cytoplasmic presence of "cap zero" structures (m^7GpppN'), indicated that ribose methylation of N' nucleoside was not required for nuclear processing and transport of mRNA in Novikoff cells.

Data are presented below which indicate that the intact TubHcy molecule is required to generate partially methylated cap structures. We also report here the results of experiments designed to establish whether cap zero-bearing mRNA was associated with polysomes in Novikoff cells. Such an association would imply that these undermethylated mRNA molecules can be translated *in vivo*. In addition, we have investigated the possibility that totally unmethylated cap structures were generated in TubHcy-treated cells.

MATERIALS AND METHODS

Cell Culture and Labeling Conditions. Novikoff hepatoma cells (N1S1 strain) were grown in Swim's S-77 medium (GIBCO) supplemented with 10% calf serum[17]. For most experiments, cells in midlogarithmic growth were harvested and resuspended at 1.5×10^6/ml for labeling in fresh medium containing no phosphate and 20 μM methionine (one-fifth normal concentration). Cells were equilibrated for 3 h; a portion of the culture was exposed to 500 μM TubHcy for the final 50 min of the equilibration period. L-[methyl-^3H] methionine (Amersham, 8.8 Ci/mmole) and [^{32}P]orthophosphate (Amersham, 127 Ci/mg P) were added simultaneously at concentrations of 0.1 mCi/ml and 0.15 mCi/ml, respectively. Cells were exposed to isotopes for 2 h. In the experiments presented in Table 1, the cultures were treated as reported earlier[16]. TubHcy was synthesized and characterized as previously described[16].

Isolation of Poly (A)-Containing RNA. Cells were poured over frozen crushed saline and harvested by centrifugation at 1500 xg for 5 min. The washed cells were resuspended and allowed to swell in hypotonic buffer (10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris, pH 7.0). Cells were lysed by dounce homogenization in the presence of 1.5 mg/ml cycloheximide. Nuclei were pelleted by centrifugation at 1000 xg for 5 min. Total cytoplasmic RNA was isolated from the postnuclear supernatant as previously described[17].

Polysomal and monosomal fractions were separated by sucrose gradient sedimentation. A portion of the postnuclear supernatant was made 0.5% in

both sodium deoxycholate and Triton X-100[18] and layered onto 11 ml gradients of 10-40% sucrose in 100 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris, pH 7.0. The gradients were overlaid on a 0.5 ml cushion of 60% sucrose in the same buffer. Sedimentation was for 75 min at 40,000 rpm in a Beckman SW 41 rotor. Gradients were analyzed using a Gilford model 2480 gradient scanner. Appropriate fractions were pooled, brought to 0.2 M NaCl, 10 mM EDTA, 0.2% SDS and 20 mM Tris, pH 7.0, and digested with 200 μg/ml proteinase K (EM Biochemicals) for 10 min at 37°C. RNA was precipitated in 67% ethanol. The RNA was pelleted by centrifugation at 27,000 xg for 15 min, dried under nitrogen and dissolved in 0.1 M NaCl, 10 mM EDTA, 0.2% SDS, 10 mM Tris, pH 7.0 for redigestion with proteinase K. The RNA was extracted with phenol: chloroform:isoamyl alcohol (50:49:1) and reprecipitated with ethanol.

Washed nuclei were resuspended in hypotonic buffer and vortexed for 3 sec in the presence of 1% sodium deoxycholate and 2% Tween 40 prior to recentrifugation at 1500 xg for 5 min. Following resuspension of the nuclear pellet into anti-RNase buffer (3 mM $MgCl_2$, 3 mM each 2',3'AMP, 2',3'UMP and 2',3'CMP, 30 μg/ml polyvinyl sulfate, 200 μg/ml heparin, 10 mM sodium acetate, pH 5.2[19]) 40 μg/ml of DNase I (Worthington) was added and the solution was incubated at room temperature for approximately 10 min. Proteinase K digestion and extraction of nuclear RNA were performed as described above for cytoplasmic RNA.

Poly (A)-containing RNA was isolated from each RNA fraction by repeated binding to oligo(dT)-cellulose[20]. The second chromatographic passage of RNA on oligo(dT)-cellulose was preceded by heat denaturation of the RNA in the presence of 90% dimethylsulfoxide[21].

Enzymatic Digestion and Analysis of Poly (A)-Containing RNA. Poly (A)-containing RNA was digested with RNase T2 and the digestion products were resolved on DEAE-Sephadex (7 M urea) as previously described[10], except that a 200 ml gradient from 0.1 M to 0.4 M NaCl was used to elute the digestion products.

Alternatively, when cap structures were to be isolated for further analysis, an acetylated DBAE-cellulose column was used to separate mononucleotides from cap structures. The RNase T2 digest was diluted with six volumes application buffer (0.6 M KCl, 10 mM $MgCl_2$, 50 mM Tris, pH 7.7, 20% ethanol)[22] and applied to a 1 ml DBAE-cellulose column. The column was washed with application buffer until no further radioactivity was detectable. The cap structures were eluted with 0.2 M NaCl, 1 M sorbitol, 50 mM sodium acetate, pH 5.0[22], diluted to approximately 0.05 M NaCl with 7 M urea, 10

mM Tris, pH 7.0 and chromatographed on DEAE-Sephadex (7 M urea) as described above. Fractions containing cap structures were pooled, diluted to approximately 0.1 M NaCl, and passed over a 3 ml DEAE-cellulose column to remove urea and salt.

Cap structures were analyzed by Partisil-SAX (Whatman) high speed liquid chromatography (HSLC)[16]. Cap 1 structures were first serially digested with P1 nuclease and alkaline phosphatase[9,10]. Cap zero structures were digested with alkaline phosphatase prior to injection. Resolution of cap 1 species was achieved using a 100 ml gradient of 0.1 M to 0.3 M KH_2PO_4, pH 3.55; cap zero species were resolved with a similar gradient using 0.1 M KH_2PO_4, pH 3.64 to 0.3 M KH_2PO_4, pH 3.90. Cap standards (P-L Biochemicals) were monitored at 254 nm.

Acid hydrolysis in concentrated formic acid, followed by chromatography of the hydrolysate on an Aminex A-5 column, was performed as previously described[10]. Both mononucleotides and cap structures were analyzed. This method permits determination of [^{3}H]methyl groups present in methylated purine bases and of incorporation into purine ring structures through de novo synthesis[23].

RESULTS

Earlier studies in our laboratories have indicated that TubHcy inhibits methylation of both base and ribose moieties in Novikoff mRNA. The capability of TubHcy to be inhibitory in viable cells implies that the compound is both permeable to cellular membranes and metabolically stable within the cell. To demonstrate that TubHcy was specifically required for the observed inhibition, the following experiment was performed. Novikoff cells in culture were subdivided into five separate cultures and labeled with L-[methyl-^{3}H]methionine and [U-^{14}C]uridine in the presence of (a) 250 μM TubHcy, (b) 250 μM AdoHcy, (c) 250 μM homocysteine, (d) 250 μM tuberdicin, or (e) without any additions (control culture). The labeling conditions and analytical methods were identical with those previously described[16]. Tubercidin and homocysteine represent the breakdown products of TubHcy cleavage by AdoHcy hydrolase, the enzyme which presumably would mediate *in vivo* degradation of TubHcy if it were metabolically unstable. AdoHcy, a powerful *in vitro* inhibitor of mRNA methylation[3], is similarly broken down[24] and may be impermeable to cellular membranes[25].

The results of this experiment are summarized in Table I. Analysis of

Table I. Effect of TubHcy, AdoHcy, Homocysteine and Tubercidin on Methylation of Poly (A)-Containing RNA[a]

	Control	TubHcy	AdoHcy	Homo-cysteine	Tuber-cidin
[^{3}H]methyl cpm incorporated into total RNA/10^{7} cells[b]	3.69×10^{6} (100%)	2.23×10^{6} (60%)	3.17×10^{6} (86%)	3.07×10^{6} (83%)	1.54×10^{5} (4%)
[^{3}H]methyl/[^{14}C]uridine ratio in total RNA	27.5	25.6	28.6	25.2	28.6
% of total [^{3}H]methyl cpm in poly (A)-containing RNA	0.43	0.23	0.38	0.41	0.31
[^{3}H]methyl/[^{14}C]uridine ratio in poly (A)-containing RNA	1.03	0.46	1.07	1.29	3.02
[^{3}H]methyl distribution[c]:					
% as mononucleoside	78	65	78	79	43
dinucleoside	1	1	<1	<1	2
Cap zero	0	5	<1	<1	0
Cap 1	18	27	17	16	15
Cap 2	3	2	4	4	40

[a] Labeling conditions and analytical methods were as previously described[16]. Labeling with L-[^{3}H-methyl]methionine and [^{14}C]uridine was for 1 h in the presence of 250 μM inhibitor or in the absence of any addition (control).
[b] Numbers in parentheses indicate [^{3}H]methyl cpm incorporation as a percentage of control values.
[c] [^{3}H]methyl distribution was determined by DEAE-Sephadex (7 M urea) chromatography of the RNase T2/alkaline phosphatase digest of poly (A)-containing RNA.

the methyl distribution in poly (A)-containing RNA from cultures treated with AdoHcy and homocysteine gave virtually identical results when compared to the control (Table I). The presence of tubercidin in Novikoff cell cultures sharply reduced both mRNA synthesis and methylation (less than 5% of control levels, cf. Table I), reflecting the toxicity of this compound. The high level of radioactivity in mRNA cap 2 structures obtained from tubercidin-exposed cells presumably reflects the late cytoplasmic methylation of synthesized mRNAs before addition of the inhibitor[8,9]. Only in TubHcy-treated cultures was inhibition of mRNA methylation observed. This is reflected both in the decreased [^{3}H]methyl/[^{14}C]uridine ratio of isolated mRNA, and in

the appearance of cap zero structures in the elution profile of mRNA digests (Table I). These data indicate that TubHcy is the actual inhibitory compound. Similar studies in appropriate cell culture systems have led to the same conclusion for TubHcy inhibition of tRNA[26] and dopamine[27] methylation.

Novikoff cell cultures were then simultaneously labeled with L-[^{3}H-methyl]methionine and [^{32}P]orthophosphate to evaluate the effect of 500 μM TubHcy on mRNA synthesis, methylation and transport during the labeling period. [^{3}H]Methyl incorporation in TubHcy-containing cultures was inhibited to 18% of the level observed in the control cultures whereas [^{32}P]incorporation was 70% of the normal level. As expected, this inhibition of methylation appears to depend upon the concentration of the methylase inhibitor, since doubling the concentration of TubHcy (from 250 μM to 500 μM) decreased the ratio of [^{3}H]methyl incorporation in TubHcy-treated vs. normal cultures from 0.32 to 0.18. In contrast, mRNA synthesis, monitored by [^{14}C]uridine or [^{32}P]orthophosphate incorporation, was decreased to the same extent (60-70% of normal levels) at either TubHcy concentration.

In order to establish the cytoplasmic location of undermethylated mRNA molecules, monosomal and polysomal RNAs were separated by sedimentation of the detergent-treated postnuclear supernatant through sucrose gradients. Figure 1 shows the absorbance profiles obtained from both normal and TubHcy-treated cells, and indicates the regions pooled for monosomal and polysomal RNA extraction. The monosome to polysome ratio in each sample was nearly identical, indicating that the protein-synthesizing machinery had not been significantly perturbed by the presence of TubHcy.

The presence of cap zero structures was ascertained by DEAE-Sephadex (7 M urea) chromatography of RNase T2-digested mRNA. Figure 2 shows the profiles obtained from normal total cytoplasmic mRNA (Figure 2A), and from TubHcy-treated total cytoplasmic, polysomal, and monosomal mRNAs (Figure 2B, C and D, respectively). No cap zero structures were detected in normal cytoplasmic mRNA (Figure 2A). Similar profiles were observed for polysomal and monosomal mRNAs from normal cultures (data not shown). In contrast, cap zero (peak II, Figure 2B, C and D) was the predominant cap structure in each of the RNA fractions isolated from TubHcy-inhibited cells, indicating that cap zero-containing molecules were associated with both monosomes and polysomes. The actual molar ratio of cap zero to cap 1 structures (peak III, Figure 2) is higher than indicated by the radioactive distribution, since cap zero contains a single methyl group whereas cap 1 structures contain two

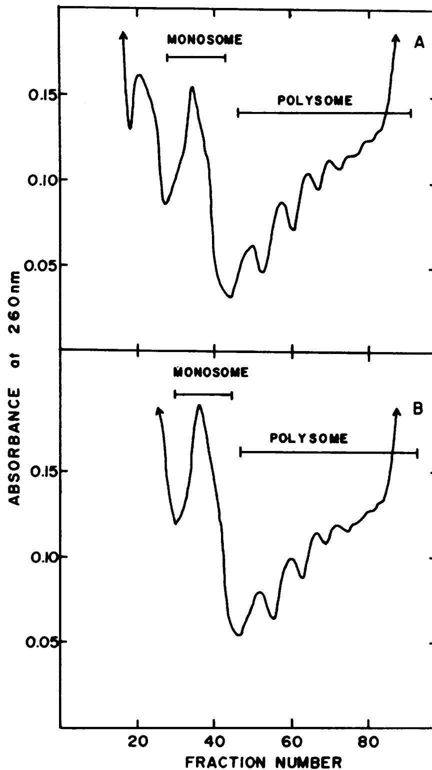

Figure 1. Absorbance profiles of postnuclear supernatants sedimented through 10-40% sucrose gradients, and scanned using a Gilford Model 2480 gradient analyzer. Polysomal profiles were obtained for postnuclear supernatants from (A) control cells; and (B) TubHcy-inhibited cells. Bars indicate fractions pooled for subsequent isolation of monosomal and polysomal RNA. In each case, the polysome region extended into the interface of the 60% sucrose pad at the bottom of the gradient.

methyl groups. The relative amount of cap 2 structures (Peak IV, Figure 2) in these samples is difficult to estimate, due to the low levels of labeling and its complex labeling kinetics[8,9].

The presence of totally unmethylated cap structures, GpppN'p was not detected in DEAE-Sephadex (7 M urea) profiles of poly (A)-containing RNA digests. However, greater sensitivity could be achieved if the RNase T2 digest was first passed over an acetylated DBAE-cellulose column to remove most of the [^{32}P]radioactivity from the sample prior to resolution of the cap structures by DEAE-Sephadex (7 M urea). More than 99% of the [^{32}P]radioactivity in total cytoplasmic mRNA from TubHcy-treated Novikoff cells did not bind to DBAE-cellulose after RNase T2 digestion. The bound fraction, containing 55% of the [^{3}H]methyl radioactivity and 0.9% of the total [^{32}P]cpm, was chromatographed on DEAE-Sephadex (7 M urea)(Figure 3A). The distribution of [^{3}H]methyl cpm in caps zero, 1, and 2 (Figure 3A, peaks II, III and IV, respectively) was virtually the same as the distribution observed in the DEAE-Sephadex (7 M urea) profile shown in Figure

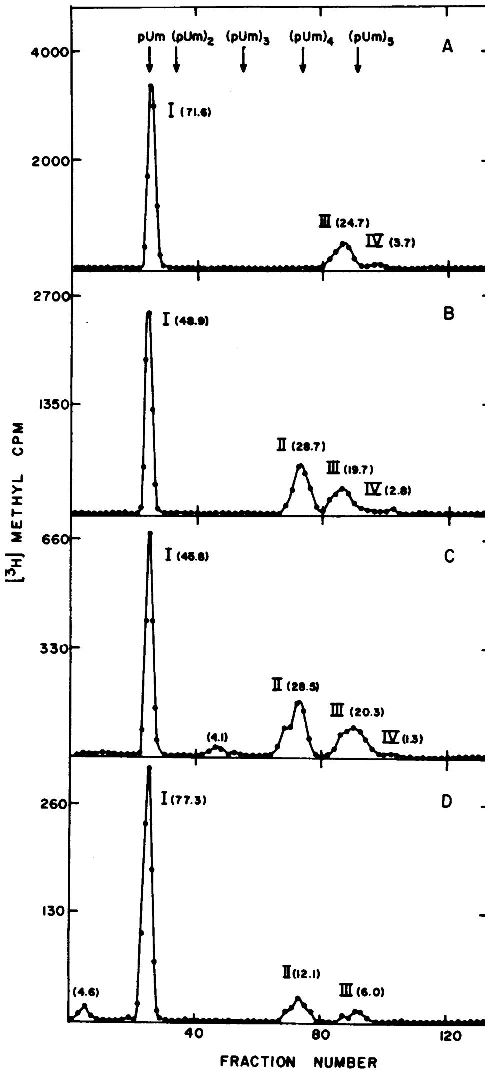

Figure 2. DEAE-Sephadex (7 M urea) elution profiles of RNase T2 digestion products from poly(A)-containing RNAs. Oligonucleotides $(pUm)_{1-5}$ added as internal standards were monitored by absorbance at 260 nm to determine the approximate charge of eluted digestion products. Poly(A)-containing RNA was derived from (A) total cytoplasm of normal cells; and from (B) total cytoplasm, (C) polysomes, and (D) monosomes of cultures labeled in the presence of TubHcy. Numbers in parentheses indicate the percentage of [^{3}H]-methyl radioactivity eluting among the labeled fractions: peak I, mononucleotides, mNp; peak II, cap zero; peak III, cap 1 and peak IV, cap 2. (Peak I contains some ring-labeled purines as well as base-methylated mononucleotides. Ring-labeling was measured by Aminex A-5 HSLC of acid-hydrolyzed peak I fractions and accounted for 16% and 26% of the total cpm in peak I from normal and inhibited cytoplasmic samples, respectively (Fig. 2A and B)). For clarity, [^{32}P]radioactivity profiles have been omitted. Ordinate values have been normalized such that each sample represents 10^7 cells.

2B (peaks II, III and IV, respectively). Most of the [^{32}P]cpm which bound to DBAE-cellulose eluted as mononucleotides (Figure 3A, peak I), presumably due to trailing of the unbound fraction. The remainder of the [^{32}P]cpm, representing 0.17% of the total radioactivity incorporated into TubHcy- treated cytoplasmic mRNA, was distributed as follows: 0.10% in cap zero (Figure 3A, peak II) 0.04% in cap 1 structures (Figure 3A, peak III), and 0.03% eluting at a position indicating an approximate charge of -8

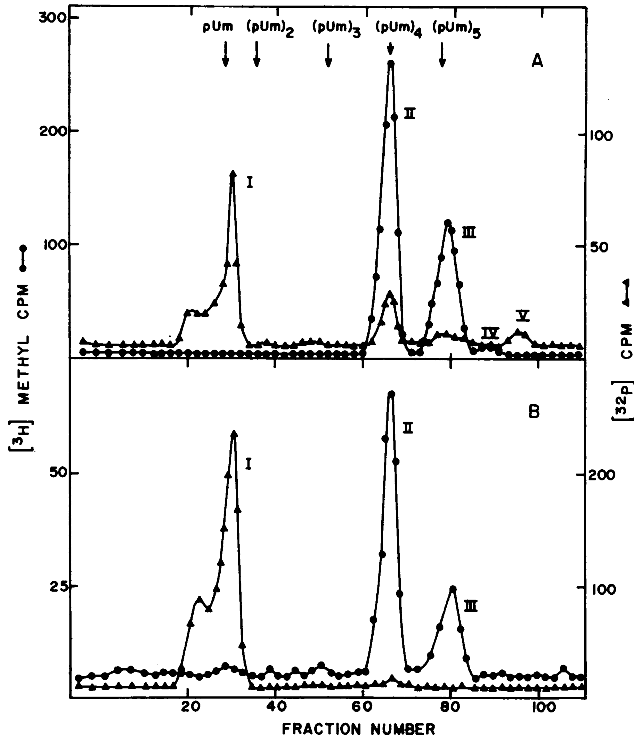

Figure 3. DEAE-Sephadex (7 M urea) elution profile of the DBAE-cellulose-bound fraction from RNase T2 digestion of poly (A)-containing RNA. The radioactive material released from acetylated DBAE-cellulose was chromatographed on DEAE-Sephadex (7 M urea) as described in Materials and Methods. Aliquots from each fraction were analyzed for radioactivity levels. Profiles are shown for (A) total cytoplasmic poly (A)-containing RNA, and (B) nuclear poly (A)-containing RNA, both from TubHcy-inhibited cultures. Radioactive material was present in peak I, mononucleotides, (Np and mNp); peak II, cap zero, peak III, cap 1; peak IV, cap 2 and peak V. Circles denote [^{3}H]-methyl cpm, and triangles indicate [^{32}P]radioactivity.

(Figure 3A, peak V).

The oligonucleotide eluting as peak V in Figure 3A was desalted, treated with alkaline phosphatase and rechromatographed on DBAE-cellulose. Sixty percent of the radioactive material bound to the column.

Nuclear poly (A)-containing RNA isolated from TubHcy-inhibited cultures was analyzed to determine if unmethylated cap structures were present in the

nucleus. The RNA was digested with RNase T2, and the digestion products were chromatographed on DBAE-cellulose. The DBAE-bound fraction was eluted on DEAE-Sephadex (7 M urea) and the resultant profile is shown in Figure 3B. [^{32}P]Radioactivity was detected only in the mononucleotide and possibly in cap zero fractions(Figure 3B, peaks I and II, respectively). It should be noted, however, that only 0.02% of the total [^{32}P]cpm incorporated into poly (A)-containing nuclear RNA eluted with cap zero structures. This level approached the limits of detection, and thus unmethylated cap structures would not be detected if present at a level below that of cap zero. Parallel analysis of normal nuclear poly (A)-containing RNA revealed no cap zero structures (data not shown).

The distribution of [^{3}H]methyl radioactivity in caps from nuclear and total cytoplasmic poly (A)-containing RNAs was analyzed by Partisil-SAX HSLC. The data are summarized in Table II, and permit comparison of caps 1 and zero from both normal and TubHcy-treated mRNAs. The methyl distribution of a given cytoplasmic cap structure is remarkably similar to its nuclear counterpart. The methyl distribution in cap 1 structures isolated from control cultures, however, was markedly different than that observed for cap 1 structures from TubHcy-exposed cultures. The structure m^{7}GpppUm was surprisingly predominant in the latter population. The [^{3}H]methyl distribution in cap zero structures from both cytoplasmic and nuclear fractions of TubHcy-

Table II. Distribution of [^{3}H]Methyl Radioactivity Within Cap Structures As Determined by Partisil-SAX Chromatography

	% of [^{3}H]methyl cpm eluting as cap structures (m^{7}GpppN'(m)) where N'(m) is:				
	C(m)	U(m)	A(m)	m^{6}A(m)	G(m)
Normal cultures:					
Cap 1 - cytoplasmic	17	13	24	31	15
nuclear	19	9	32	28	12
TubHcy-inhibited cultures					
Cap Zero - cytoplasmic	14	4	39	(0)[a]	42
nuclear	14	3	42	(0)	42
Cap 1 - cytoplasmic	19	39	14	14	14
nuclear	18	31	13	26	12

[a]The absence of m^{7}Gpppm^{6}A was inferred since no radioactivity migrated differently from the four cap zero structures for which absorbance standards were available. Cytoplasmic cap zero structures were acid hydrolyzed and chromatographed by Aminex A-5 HSLC to verify that m^{6}A was not present.

inhibited cells were similar and appeared to contain only the four common bases at N' - no m^6A was observed in this position.

DISCUSSION

The data summarized in Table I suggest that TubHcy is an effective inhibitor of mRNA methylation which is functioning as an intact molecule rather than through potential degradation products. The use of TubHcy to generate undermethylated mRNA molecules in viable cells thus provides an alternate approach for studying the function of methyl groups in mRNA. Fidelity of regulatory mechanisms is essential for structure-function studies, and the use of an *in vivo* inhibitor may approximate this condition more closely than *in vitro* cell-free systems.

Determination of the role of mRNA methylation in eukaryotes is complicated, however, by the inability to isolate a subclass of undermethylated mRNA molecules from cell cultures. We approached this problem indirectly by studying the intracellular location of cap zero-bearing mRNAs, which were formed only in the presence of S-tubercidinylhomocysteine. The cytoplasmic supernatant was treated with detergent prior to layering the gradients, to minimize nonspecific binding of RNAs to sedimenting material[28]. Sedimentation analysis of the postnuclear supernatant of cells labeled in the absence and presence of TubHcy (Figure 1A and B, respectively) permitted evaluation of the inhibitor's effect on polysome distribution. Polysomal profiles are sensitive indicators of the metabolic state of cells[29]. In this regard, it was interesting to note that the polysome to monosome ratio for control cells and those treated with TubHcy for approximately 3 h were similar, implying that protein synthesis has remained relatively unperturbed by the presence of TubHcy. Based on these results, TubHcy appears to be a useful inhibitor for studies involving RNA processing.

Cap zero was the predominant cap structure of both polysomal and monosomal mRNAs (Figure 2C and D, respectively). The association of these mRNAs with monosomes implies that ribosomal recognition of the undermethylated mRNAs was not prevented by lack of 2'-*O*-methylation at the N' position. Furthermore, identification of cap zero structures in the polysomes suggests that these undermethylated structures are translated *in vivo*. Although the results presented here imply that ribose methylation at N' was not requisite for either ribosomal binding or subsequent translation, these data do not exclude the possibility that 2'-*O*-methylation facilitates these processes *in*

vivo. The role of 2'-O-methylation in ribosomal recognition and translation is not clear. Yeast mRNA, however, has been shown to contain exclusively cap zero structures[30], supporting the hypothesis that ribose methylation may not be required for this mRNA function.

[^{32}P]Orthophosphate was used as a radioactive precursor in these studies to detect the possible presence of completely unmethylated cap structures. No detectable levels of [^{32}P]radioactivity in inhibited mRNA digests were observed to chromatograph at the expected elution position of unmethylated caps (corresponding to a charge of -5; cf. Figure 3A). Total [^{32}P]incorporation into cap structures of both normal and inhibited cytoplasmic mRNAs was slightly lower than predicted. Assuming an average mRNA length of 2000 nucleotides, cap structures should have contained approximately 0.20-0.25% of the total [^{32}P]cpm (depending on whether cap zero or cap 1 structures are used for calculation). Cap 1 structures derived from normal cytoplasmic mRNA contained 0.20% of the [^{32}P]cpm incorporated into the sample. Approximately 0.17% of the total [^{32}P]radioactivity in TubHcy-inhibited cytoplasmic mRNA eluted from DEAE-Sephadex in three distinct peaks (Figure 3A): 0.10% was in cap zero structures (peak II), 0.04% in cap 1 structures (peak III) and 0.03% in the late eluting fractions of peak V in Figure 3A. We estimate that the lower limit of detection of unmethylated caps would have enabled us to see one-fifth of the cap zero level in this sample, or 0.02% of the total [^{32}P]cpm incorporated into mRNA.

The small peak of [^{32}P]labeled material referred to as peak V in Figure 3A represents approximately one-third the level of incorporation observed in cap zero structures. Analysis of the material was limited by the low amount of radioactivity present. The structure possessed the following characteristics: 1) it contains no radioactive methyl groups, but is resistant to RNase T2 digestion; 2) it binds to acetylated DBAE-cellulose, and therefore must contain cis-hydroxyl groups; 3) alkaline phosphate digestion does not eliminate its ability to bind the substituted borate column, although 40% of the [^{32}P]cpm is released into the unbound fraction; 4) it does not appear in the DEAE-Sephadex (7 M urea) profiles of TubHcy-inhibited nuclear mRNA digests (although its detection would require this structure to be present in levels comparable to the low cap zero levels); and 5) it elutes from DEAE-Sephadex (7 M urea) columns as if it had contained a charge of approximately -8. With the exception of its apparent excessive charge, this material exhibited behavior consistent with unmethylated cap structures. The absence of this material in nuclear RNA, however, indicated that it is not

stable and therefore does not accumulate in the nucleus. Pugh *et al.*[31] have demonstrated that TubHcy is a potent inhibitor of mRNA (guanine-7-)-methyltransferase *in vitro*. The data presented here do not exclude the possibility that inhibition of methylation at the 7-position of guanine is occurring *in vivo*, but the resultant cap structures are rapidly degraded. This situation would be consistent with the moderate decrease in RNA synthesis observed in the presence of TubHcy.

The [^{3}H]methyl distribution among cap structures from total cytoplasmic mRNA of both control and TubHcy-inhibited cultures is remarkably similar to the corresponding nuclear cap distribution (Table II). This implies that transport of cap-bearing mRNA molecules is not selective relative to the presence or absence of a 2'-O-methyl group in the N' nucleotide of the cap. Cap zero structures were observed in the nuclear and cytoplasmic fractions of only TubHcy-exposed cells. The absence of m^6A at N' of cap zero structures sharply contrasts the relative predominance of this methylated base in cap 1 structures, and suggests that 2'-O-methylation of adenosine at N' may be necessary for subsequent base methylation. This sequence of methylation events has been observed in the formation of mouse globin mRNA cap structures[32].

The predominance of uridine at N' in both nuclear and cytoplasmic cap 1 structures derived from inhibited cells is somewhat surprising (Table II), since m^7GpppU(m) is generally observed as a minor component of cap structures from Novikoff cells[9,16]. The presence of 500 μM TubHcy in these cultures, however, may have altered the relative stability or processing efficiency of certain RNA species which happen to be enriched in uridine at the N' position of caps. An increase in the amount of pyrimidines at N' might reflect an increase in the processing and/or transport of RNA molecules whose 5'-termini are generated internally from primary RNA transcripts. Alternatively, TubHcy may selectively inhibit certain methylases. The observed differences in cap distributions suggest that *in vivo* perturbation of mRNA methylation has resulted in the maintenance of an altered mRNA population. The precise nature and significance of this observation is unknown at present.

This study has demonstrated that cap zero-containing mRNAs are associated with both monosomes and polysomes of cells which remained viable in the presence of 500 μM TubHcy. We therefore conclude that ribose methylation at N' does not appear to be requisite for transport to the cytoplasm, ribosomal binding and subsequent translation of the undermethylated mRNA. The altered

base distribution at the N' position of cap structures in the presence of TubHcy may reflect a more subtle influence of methylation on processing events. This possibility poses questions which may be assessed more directly using a specific mRNA sequence. Such studies are currently being undertaken in our laboratory.

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2. Abbreviations used: TubHcy, S-tubercidinylhomocysteine; AdoHcy, S-adenosylhomocysteine; N', nucleoside adjacent to the 5'-pyrophosphate linkage in cap structures; N", penultimate nucleoside to the 5'-pyrophosphate linkage in cap structures; Nm, 2'-O-methylated nucleoside; mN, base methylated nucleoside; DEAE, diethylaminoethyl; DBAE, dihydroxyborylaminoethyl; hnRNA, heterogeneous nuclear RNA; HSLC, high speed liquid chromatography.
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