In Vivo Inhibition of Novikoff Cytoplasmic Messenger RNA Methylation by *S*-Tubercidinylhomocysteine†

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ABSTRACT: The analogue *S*-tubercidinylhomocysteine (STH) has been used to study the methylation of mRNA in vivo. Partial inhibition of cytoplasmic poly(A)-RNA methylation was observed using a level of inhibitor which still permitted cell growth. Characterization of the partially methylated mRNA indicated the presence of cap structures lacking 2′-*O*-methylnucleosides, m^7GpppN', which are normally not found in mammalian mRNA. Inhibition of additional methylated sites in mRNA at the second 2′-*O*-methylnucleoside, and at internal N^6-methyladenosine was also observed. Methylation of 7-methylguanosine was not affected under the conditions used in these experiments. The methylnucleoside composition of cap structures differed in STH-inhibited and uninhibited cells. These results indicate that a completely methylated cap is not required for transport of mRNA into the cytoplasm. Furthermore, it may now be possible to assess in vivo the sequential nature of mRNA methylation and its potential role in mRNA processing.

Most eukaryotic mRNA molecules are now known to contain blocked and methylated 5′-terminal structures called "caps" (cf. reviews: Shatkin, 1976; Rottman, 1976). These structures are commonly found in two forms, $m^7GpppN'mpN''$ (cap 1) and $m^7GpppN'mpN''mpN$ (cap 2).[1] In addition, some mRNA molecules contain internally located N^6-methyladenosine (m^6A) (Desrosiers et al., 1974; Adams and Cory, 1975; Perry et al., 1975a) and occasionally 5-methylcytosine (m^5C) (Dubin and Stollar, 1975).

Identification of the cap structures, together with evidence suggesting that these methylation events may occur at specific times and separate cellular locations (Perry and Kelley, 1976; Friderici et al., 1976), has intensified the need to address the question of mRNA methylation function in vivo. Previous studies on the function of mRNA methylation have focused on the role of the cap structure in vitro at the translational level, using cell-free protein-synthesizing systems (Muthukrishnan et al., 1975; Both et al., 1975a), ribosome binding studies (Dasgupta et al., 1975; Both et al., 1975b; Kozak and Shatkin, 1976), and cap analogues as specific inhibitors of binding and/or translational activity (Hickey et al., 1976; Weber et al., 1976). However, the possible relationship between methylation events and processing of mRNA remains to be established. Precedent for such a relationship exists in studies on rRNA maturation in HeLa cells grown under conditions of limiting methionine (Vaughn et al., 1967).

In preliminary studies performed in our laboratory, Novikoff hepatoma cells were deprived of methionine in an attempt to reduce methylation of mRNA, and thereby perturb mRNA processing. Under conditions which inhibited the appearance of cytoplasmic rRNA through reduced methylation of the nuclear precursor, mRNA methylation was maintained at the normal level. This result might reflect an "internal priority system" for the utilization of available methyl donors, the net result of which is to protect methylation of mRNA and ensure its complete complement of methyl groups. We concluded that methylation of mRNA could not be affected by depletion of the common amino acid methionine. If an evaluation of the interaction between methylation and processing of mRNA was to be feasible, a specific in vivo inhibitor of mRNA methylation would be necessary.

S-Tubercidinylhomocysteine (STH), the 7-deaza analogue of *S*-adenosylhomocysteine (SAH), has been synthesized in one of our laboratories (Coward et al., 1974) and has been shown to function as an effective in vivo inhibitor of tRNA methylation in cultured stimulated rat lymphocytes (Chang and Coward, 1975), and of dopamine methylation in murine neuroblastoma cells (Michelot et al., 1977) and human fibroblasts (X. O. Breakefield and J. K. Coward, unpublished results). The use of STH as a methylation inhibitor offers several advantages: it is able to permeate cell membranes, and it is not susceptible to the enzymes responsible for SAH metabolism in mammalian cells (Crooks et al., manuscript in preparation).

We have investigated the effect of STH on the methylation of Novikoff RNA and have presented below the characterization of the methyl distribution in the cytoplasmic poly(A)-RNA. The results of this study have indicated that STH inhibits mRNA methylation and that the use of this inhibitor may permit a qualitative and quantitative evaluation of the effects of methylation on mRNA processing. It may also enable determination of the relative time sequence of methylation in vivo at specific sites within mRNA.

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 (Desrosiers et al., 1974). Cells in midlogarithmic growth were harvested aseptically and resuspended at a concentration of 7.5×10^5/mL in fresh warm medium containing 2 mM methionine (one-fifth the normal concentration) for labeling experiments.

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[1] Abbreviations used are: HnRNA, heterogeneous nuclear RNA; m^7G, 7-methylguanosine; m^6A, N^6-methyladenosine; m^5C, 5-methylcytosine; m^6Am, N^6-2′-*O*-methyladenosine; m^7gua^*, ring-opened 7-methylguanine; HSLC, high-speed liquid chromatography; Nm, 2′-*O*-methylnucleoside; N′, nucleoside adjacent to pyrophosphates in 5′ caps; N″, penultimate nucleoside to pyrophosphates in 5′ caps; STH, *S*-tubercidinylhomocysteine; SAH, *S*-adenosylhomocysteine; DEAE, diethylaminoethyl.

The cells were equilibrated in the medium for 1 h; a portion of the cells was exposed to 250 μM STH for the last 40 min of the equilibration period. L-[*methyl*-^{3}H]Methionine (Amersham/Searle, 14 Ci/mmol) and [U-^{14}C]uridine (Amersham/Searle, 537 mCi/mmol) were added simultaneously at concentrations of 0.1 mCi/mL and 0.18 μCi/mL, respectively. Cells were incubated with radioisotope for 1 h.

S-Tubercidinyl-D,L-homocysteine (STH) was prepared by reaction of 5′-chloro-5′-deoxytubercidin (Coward et al., 1977) with D,L-homocysteine thiolactone, according to the general procedure of Legraverend and Michelot (1976). The crude product was purified by ion-exchange chromatography on Dowex 50 X-8 (H^+), the desired material being eluted with 1 N NH_4OH, following a water wash of the column to remove impurities. The peak tubes were pooled, and the contents lyophilized to give a white, fluffy powder in 83% yield. This material was identical by chromatographic and spectral comparisons with that prepared previously (Coward et al., 1974).

Isolation and Characterization of Poly(A)-RNA. Total cytoplasmic RNA fractions were isolated essentially as described (Desrosiers et al., 1974). Poly(A)-RNA from each fraction was isolated by oligo(dT)-cellulose chromatography, including a heat step prior to a second passage over the column to eliminate ribosomal RNA contamination (Desrosiers et al., 1975). RNA was analyzed by sedimentation through denaturing gradients (99% Me_2SO:5–20% sucrose) as described (Friderici et al., 1976) except that sedimentation was for 17 h.

Enzymatic and Acid Degradation of Poly(A)-RNA. The procedures for enzymatic degradation of poly(A)-RNA, and for high speed liquid chromatography (HSLC) methodology employed for analyses of cap structures, are detailed in previous publications (Friderici et al., 1976; Rottman et al., 1976) and will be briefly outlined below.

Cytoplasmic poly(A)-RNA was digested with RNase T2 (Sigma; Calbiochem) and alkaline phosphatase (Worthington) to produce nucleosides and intact 5′-terminal cap structures. After resolution of the reaction products on DEAE-Sephadex (7 M urea), intact cap 1 (m^7GpppN′mpN″) and cap 2 (m^7GpppN′mpN″mpN) structures were separately isolated and desalted on Bio-Gel P-2 (100–200 mesh); mononucleoside fractions were adsorbed on charcoal and eluted with 20% pyridine.

Further digestion of the desalted cap structures with nuclease P1 (Yamasa Shoyl Co. Ltd.) and alkaline phosphatase yielded methyl-labeled m^7GpppN′m from cap 1, and a mixture of m^7GpppN′m (core cap 2) plus released nucleosides (N″m) from cap 2. The nucleosides were resolved from the core cap on Bio-Gel P-2 (100–200 mesh).

Alternatively, cap 1 and core cap 2 structures were degraded to 2′-*O*-methylnucleosides and 7-methylguanosine with nucleotide pyrophosphatase and alkaline phosphatase. Analysis of these nucleosides on Aminex A-5 HSLC (see below) permitted an independent determination of methylnucleoside distribution. Acid hydrolysis of intact mRNA and of cap structures was performed in concentrated formic acid in sealed tubes at 100 °C for 1 h.

Distribution Analysis of Methylation. The distribution of base-methylated nucleosides was determined using Aminex A-5 chromatography in 0.4 M ammonium formate, pH 4.55. 2′-*O*-Methylnucleosides and 7-methylguanosine were separated using the same chromatographic system at pH 4.15 and in the presence of 40% ethylene glycol. Released purine bases in the acid hydrolysate were resolved on Aminex A-5 using 0.4 M ammonium formate at pH 5.30.

The distribution of cap species obtained from P1/alkaline phosphatase digested cap 1 and cap 2 was determined by Partisil-SAX HSLC (Whatman) using a gradient of 0.1–0.3 M KH_2PO_4, pH 3.45. Aliquots of the oligonucleotide eluting on DEAE-Sephadex with a −2.5 charge (m^7GpppN′) were injected directly and resolved by gradient elution from 0.1 M KH_2PO_4, pH 3.50, to 0.3 M KH_2PO_4, pH 3.90. Cap 1 and cap zero standards (m^7GpppNm and m^7GpppN, respectively; P-L Biochemical Co.) were monitored by A_{260}.

Results

Preliminary studies on Novikoff poly(A)-RNA, following exposure of the cells to STH, suggested that the compound effectively altered mRNA synthesis after relatively short time periods. No detectable decrease in cell viability, as monitored by vital staining, was observed up to 24 h of exposure to STH. These studies also indicated that STH concentration, exposure time to inhibitor, and labeling time influenced the extent of radioactivity incorporated. Conditions were chosen which resulted in partial inhibition of mRNA methylation. Presumably, higher concentrations of STH might further inhibit mRNA methylation, although cell viability may be decreased as well.

RNA was labeled for comparison of both RNA synthesis and methylation in the presence and absence of inhibitor. Since [^{14}C]uridine incorporation is indicative of total RNA synthesis and [^{3}H]methyl incorporation reflects the extent to which RNA was methylated, ^{3}H cpm/^{14}C cpm ratios for each RNA fraction can be employed for internal comparison of normal and inhibited samples. Table I summarizes the incorporation data obtained from parallel labeling of Novikoff cells in the presence (STH) or absence (normal) of inhibitor. Incorporation of both [^{14}C]uridine and [^{3}H]methyl groups into total cytoplasmic RNA was reduced to approximately the same extent in the presence of STH. However, incorporation of methyl groups into poly(A)-RNA was reduced (32% of the normal level) to a greater extent than incorporation of uridine (73% of the normal level). These results, coupled with the methyl distribution analysis presented below, indicated that *S*-tubercidinylhomocysteine functions as an in vivo inhibitor of mRNA methylation.

The size of poly(A)-RNA from STH-inhibited and normal cells was found to be nearly identical by denaturing sucrose gradient sedimentation (data not shown). No degradation was apparent in either sample and the size distribution was characteristic of cytoplasmic poly(A)-RNA obtained from these cells (Desrosiers et al., 1975). The poly(A)-RNA from normal and STH-treated cells was then further characterized to determine if the decreased incorporation of [^{3}H]methyl groups reflected site-specific or overall inhibition of mRNA methylation.

Cytoplasmic poly(A)-RNA was digested with RNase T2 and alkaline phosphatase, and the reaction products were separated on DEAE-Sephadex to resolve internal N^6-methyladenosine and the 5′-terminal cap structures. The radioactive profiles of both normal and STH-treated cytoplasmic-poly(A)-RNA digests are presented in Figure 1; the percent of total radioactivity eluting with each peak was as indicated in the figure. The most striking difference between the two samples was the appearance of a new oligonucleotide eluting as peak III in RNA isolated from STH-inhibited cells. This oligonucleotide, bearing a charge of approximately −2.5, was not a result of incomplete enzymatic digestion. Redigestion and rechromatography of a portion of the desalted peak fractions resulted in more than 92% of the material eluting identically with that shown in Figure 1. In addition, similar digestion and

TABLE I: Effect of STH on [^{14}C]Uridine and L-[*methyl*-^{3}H]Methionine Incorporation into Cytoplasmic RNA.

	[^{14}C]Uridine incorp		[^{3}H]Methyl incorp		^{3}H cpm/^{14}C cpm	
Fraction of RNA	Normal	STH	Normal	STH	Normal	STH
cpm in total RNA/10^7 cells	1.35×10^5 (100%)[a]	8.71×10^4 (65%)	3.69×10^6 (100%)	2.23×10^6 (60%)	27.5	25.6
% of cpm as						
Non-poly(A)-containing RNA	88.7	87.2	99.57	99.77		
Poly(A)-containing RNA	11.3	12.8	0.43	0.23	1.03	0.46
cpm in poly(A)-RNA/10^7 cells	15 200 (100%)	11 120 (73%)	15 730 (100%)	5110 (32%)		
A_{260} total RNA/10^7 cells	5.73 (100%)	5.88 (103%)				

[a] Figures in parentheses express cpm incorporated into STH cultures as a percentage of cpm incorporated into corresponding RNA fractions from normal cultures, the latter taken as 100% as indicated.

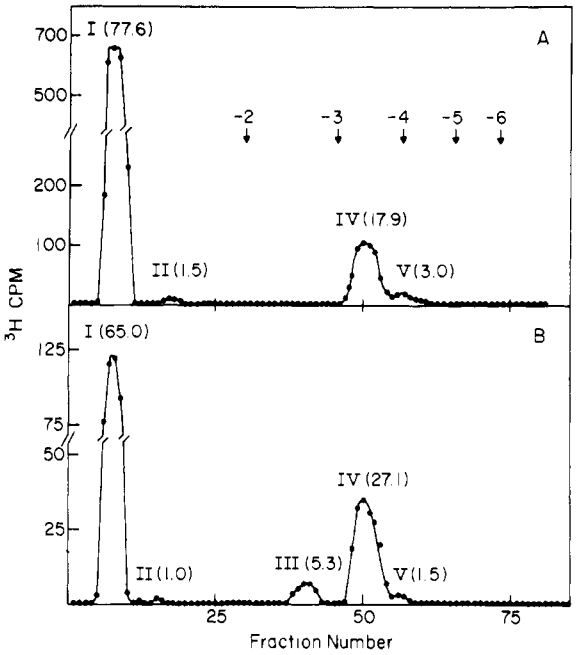

FIGURE 1: DEAE-Sephadex column separation of RNase T2 and alkaline phosphatase digestion products from whole mRNA. The reaction mixture was diluted with 4 volumes of 7 M urea, 0.02 M Tris-HCl, pH 7.0, and applied to a 0.9 × 25 cm DEAE-Sephadex column. The mononucleosides were eluted with 0.05 M NaCl, 7 M urea, 0.02 M Tris-HCl, pH 7.0. To resolve oligonucleotides, a 180-mL gradient of 0.1 M to 0.4 M NaCl in 7 M urea, 0.02 M Tris-HCl, pH 7.0, was used at a flow rate of ~8 mL/h; 2-mL fractions were collected, and 50-μL aliquots were removed for scintillation counting. Standard oligonucleotides $(pUm)_{1-5}$ were included to indicate approximate charge. (A) Digest of poly(A)-RNA isolated from normal cells; (B) digest of poly(A)-RNA isolated from STH-treated cells. Numbers in parentheses indicate the percent distribution among the labeled fractions: peak I, mononucleosides, Nm; peak II, dinucleotides, NmpN; peak III, oligonucleotide bearing −2.5 charge, identified as cap zero, m^7GpppN'; peak IV, cap 1, $m^7GpppN'mpN''$; and peak V, cap 2, $m^7GpppN'mpN''mpN$.

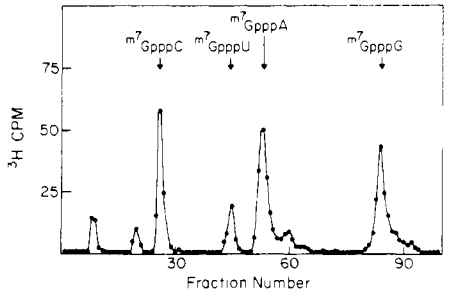

FIGURE 2: Distribution of ^{3}H radioactivity in cap zero species. Fractions eluted from a DEAE-Sephadex column at an approximate charge of −2.5 (peak III in Figure 1) were desalted on a 1.5 × 50 cm Bio-Gel P2 column by elution with 0.01 M NH_4HCO_3. Material in the void volume was made 20% with ethanol and evaporated. An aliquot was resuspended in 100 μL of 0.05 M KH_2PO_4, pH 3.50, and injected onto a Partisil PXS 1025-SAX column. The column was washed with 0.1 M KH_2PO_4, pH 3.50, until m^7GpppC began to elute; a 100-mL gradient from 0.1 M KH_2PO_4, pH 3.50, to 0.3 M KH_2PO_4, pH 3.90, was used to resolve the remainder of the cap species. Flow rate was approximately 1 mL/min (~1250 psi); 0.8-mL fractions were collected. Synthetic cap zero standards were injected with the sample for monitoring at 260 nm. The two peaks eluting in front of m^7GpppC are indicative of cap breakage and represent less than 10% of the total radioactivity applied.

chromatography of nuclear poly(A)-RNA fractions also indicated the presence of this oligonucleotide only in the RNA obtained from STH-treated cells (data not shown).

The −2.5 charge oligonucleotide, found only in the inhibited RNA sample, was further analyzed to determine its structure. An aliquot of the desalted peak fractions was acid hydrolyzed and the released purine bases separated by HSLC on Aminex A-5 resin. Greater than 95% of the base-methyl radioactivity migrated with 7-methylguanine and guanine, the latter representing 15% of the total cpm and arising from ring-label, which was not suppressed during the labeling period (Friderici et al., 1976). Less than 3% of the total cpm eluted in the solvent front with degradation products arising from 2′-*O*-methylnucleosides. These results suggested that this oligonucleotide (peak III in Figure 1) was in fact cap "zero", i.e., m^7GpppN', in which the 5′-5′ pyrophosphate linkage between 7-methylguanosine and the N′ nucleoside is intact, but the 2′-*O*-methylribose group is absent. Verification that the material eluting with a −2.5 charge from DEAE-Sephadex (Figure 1) was in fact cap zero was obtained by injection of an aliquot of the peak III fraction onto Partixil-SAX. The profile shown in Figure 2 demonstrates the resolution of peak III material into fractions coincident with authentic cap zero standards. Chromatography of a second aliquot of the −2.5 charged oligonucleotide under conditions employed for cap I separation resolved the sample into peaks which were not coincident with cap I standards (data not shown). All four cap zero products are present, with predominately purines in the N′ position. Since m^7Gpppm^6A was not available as a standard, its presence could not be directly verified. It is possible, however, that the small peak present after m^7GpppA (Figure 2) is in fact m^7Gpppm^6A.

The distributional analysis data for cap zero, and for both cap 1 and cap 2 from inhibited and normal cytoplasmic-poly(A)-RNA fractions, are summarized in Table II. In con-

TABLE II: Distribution of [^{3}H]Methyl cpm in Cap Structures of Cytoplasmic Poly(A)-RNA from Normal and STH-Treated Novikoff Cells.[a]

	STH RNA				
	$m^7GpppC(m)$	$m^7GpppU(m)$	$m^7GpppA(m)$	$m^7Gpppm^6A(m)$	$m^7GpppG(m)$
Cap zero (m^7GpppN')	22%	8%	38%	(0%)[b]	31%
Cap 1 ($m^7GpppN'm$)	20	6	27	16	31
Cap 2 core ($m^7GpppN'm$) (20% of cap 2 cpm)[c]	(Insufficient cpm for quantitative determination)				
	Normal RNA				
	$m^7GpppCm$	$m^7GpppUm$	$m^7GpppAm$	m^7Gpppm^6Am	$m^7GpppGm$
Cap 1 ($m^7GpppN'm$)	8	1	9	32	50
Cap 2 core ($m^7GpppN'm$) (38% of cap 2 cpm)[c]	9	1	8	46	36
	N″m nucleosides from cap 2 structures				
	Cm	Um	Am	m^6Am	Gm	m^7G
Cap 2 of normal RNA (62% of cap 2 cpm)[c]	24%	46%	16%	0%	13%	<2%
Cap 2 of STH RNA (80% of cap 2 cpm)[c]	+	+	+	ND[d]	+	−
	(Insufficient cpm for quantitative determination)					

[a] Distribution determined from Partisil-SAX HSLC; percentages normalized to 100% in caps only, without including breakdown products after P1/alkaline phosphatase digestion. [b] Appropriate standard unavailable; see text. [c] Percentage indicates separation of cap 2 core cpm from N″m cpm after P1/alkaline phosphatase digestion. [d] ND = not determined.

trast to cap zero analysis, caps 1 and 2 were subjected to nuclease P1/alkaline phosphatase digestion prior to analysis. Following this treatment, $m^7GpppN'm$ structures obtained from either cap 1 or cap 2 were analyzed directly on Partisil-SAX. If the base distributions at N′ of cap zero and cap 1 are compared, similar distributional data are observed. This indicates that inhibition of N′ 2′-O-methylation to produce cap zero is not base specific. A comparison of cap 1 distribution in mRNA from STH-inhibited and normal cells (Table II) indicates that less base methylation at N′ is occurring in the presence of STH. In addition, the data show that the composition of N′(m) in the cap structures from STH-treated and normal mRNA is considerably different. The low amount of radioactivity in cap 2 from mRNA of inhibited cells permitted only a limited qualitative analysis of N″m. It appears, however, that all four bases are present in N″m, and that Um is the predominant species, as is the case for normal cellular mRNA.

The mononucleoside fraction from DEAE-Sephadex (peak I in Figure 1), representing internal base-methylated nucleosides, was also analyzed. The nucleosides were desalted by charcoal adsorption and resolved on Aminex A-5 by HSLC as described. In both the normal and inhibited samples, more than 98% of the [^{3}H]methyl cpm was present as N^6-methyladenosine. If 5-methylcytosine was present in either sample, it consisted of less than 1.5% of the total radioactivity. Identical results were obtained by chromatography of the purine bases released by acid hydrolysis of the nucleoside fraction (data not shown).

The effect of STH on the level of internal base methylation can be assessed by comparison of the labeling of terminal 7-methylguanine with that of N^6-methyladenine. In order to avoid including the m^6A present in cap structures, the amount of m^6A was determined from acid hydrolysis data of the mononucleoside fraction eluting from DEAE-Sephadex. Total 7-methylguanine levels were determined by acid hydrolysis of intact poly(A)-RNA. In the normal sample, per 10^7 cells, 1038 ^{3}H cpm was incorporated as m^7G, and 10 822 ^{3}H cpm as m^6A in cytoplasmic poly(A)-RNA, resulting in an m^6A/m^7G ratio of 10.4. Corresponding incorporation in the STH-treated sample was 782 ^{3}H cpm as m^7G, and 2432 ^{3}H cpm in m^6A; the m^6A/m^7G ratio was 3.1. The ratio of m^6A to m^7G provides a measure of internal methylation in messenger RNA, relative to the amount of cap present.

Since partial ring opening of 7-methylguanine in cap structures can occur during the numerous analytical procedures performed over a period of several weeks, alternative analyses of methyl distribution in caps were determined from acid hydrolysis and nucleotide pyrophosphatase degradations, as outlined in Materials and Methods. For illustrative purposes, Figure 3 displays the profiles obtained from HSLC analyses of cap 1 from STH-inhibited cells. Resolutions of intact cap 1 species on Partisil-SAX (Figure 3A), of the nucleotide pyrophosphatase digest on Aminex A-5 (Figure 3B), and of the acid hydrolysate on Aminex A-5 (Figure 3C) are presented. The results for cap structures from the three types of analyses were internally consistent.

Discussion

Several in vitro systems, particularly those using viral sources (Rhodes et al., 1974; Shatkin, 1974; Martin and Moss, 1975, 1976), have provided information on the enzymatic generation of cap structures in mRNA. Analysis of isolated eukaryotic mRNA has produced a clearer understanding of the kinds of mRNA methylation which occur in eukaryotes, including 5′-terminal and internal methylation of both cytoplasmic mRNA and the corresponding nuclear precursors (Perry et al., 1975b; Salditt-Georgieff et al., 1976). Studies on the function(s) of mRNA methylation using these approaches, however, are not directed at the possible role of methylation in processing of mRNA. What is required for these studies is an in vivo system in which the role of methylation and its relationship to mRNA processing can be assessed. Since SAH is known to be a very effective inhibitor of mRNA methylases in vitro (Both et al., 1975a,b; Tonguzzo and Ghosh, 1976), and since STH, an analogue of SAH, has previously been shown to inhibit in vivo tRNA methylation (Chang and Coward, 1975), the effect of STH on mRNA methylation in vivo was studied.

Labeling of RNA with both [^{14}C]uridine and [^{3}H]-methylmethionine permits an evaluation of overall methylation relative to the total amount of RNA synthesized. The data

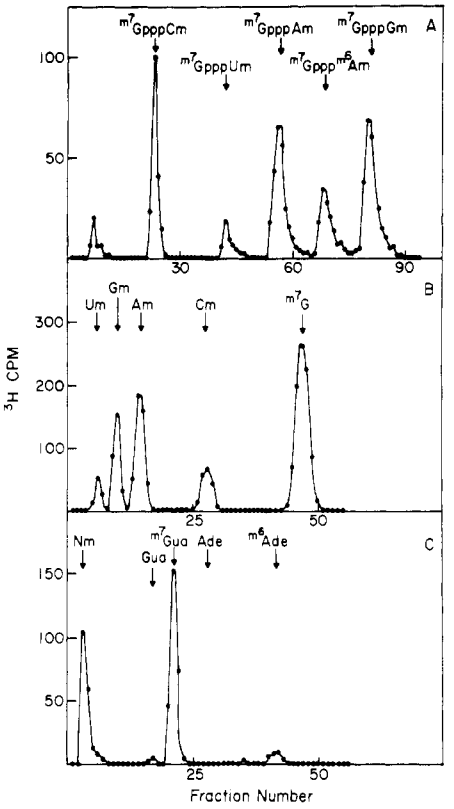

FIGURE 3: Analysis of cap 1 structures obtained from mRNA of STH-treated cells. (A) Resolution of cap 1 structures by HSLC on Partisil-SAX. Cap 1 fractions eluted from a DEAE-Sephadex column (peak IV in Figure 1) were desalted on Bio-Gel P-2 as described in Figure 2. The cap structures were digested with P1 nuclease and T2 RNase in the presence of 1 A_{260} unit carrier rRNA. The digest was injected onto a Partisil PXS 1025-SAX column and cap 1 species were resolved as described in Figure 2, except that the gradient elution was with 0.1 M KH_2PO_4, pH 3.45, to 0.3 M KH_2PO_4, pH 3.45. Synthetic cap 1 structures were used as standards. (B) Distribution of methylnucleosides in STH cap 1 structures. The desalted material was digested with nucleotide pyrophosphatase and alkaline phosphatase, dried with N_2, and resuspended in 125 μL of column buffer. HSLC on Aminex A-5 ($\frac{1}{8}$ in. × 90 cm) was performed in 0.4 M ammonium formate, pH 4.15, 40% ethylene glycol at 40 °C. Flow rate was ~10 mL/h (4000 psi); fraction size was ~0.8 mL until Cm had eluted, after which the volume was increased to ~1.2 mL/fraction. (C) Resolution of methylated bases released from cap 1 structures by acid hydrolysis. A portion of the sample was hydrolyzed in concentrated formic acid by heating at 100 °C for 1 h. The hydrolysate was dried with N_2 and dissolved in column buffer. The released bases were resolved by HSLC on Aminex A-5 ($\frac{1}{8}$ in. × 90 cm) in 0.4 M ammonium formate at pH 5.3, 40 °C.

presented in Table I provide a comparative overview of this relationship. The reduction of total radioactivity in RNA from STH-inhibited cells is nearly equivalent for both [^{3}H]methyl and [^{14}C]uridine incorporation. However, the methylation of poly(A)-RNA is significantly reduced, as reflected by the ^{3}H/^{14}C values for each poly(A)-RNA fraction.

Perhaps the most significant result of this study is the detection of cap zero structures in poly(A)-RNA isolated from the cytoplasm of cells exposed to STH. The appearance of peak III (Figure 1) led to extensive characterization of the −2.5 charged material. The oligonucleotide was not a result of incomplete digestion since redigestion with alkaline phosphatase and RNase T2 did not alter its retention on DEAE-Sephadex. Acid hydrolysis indicated that nearly all the radioactivity was present as m^{7}G, suggesting the structure was m^{7}GpppN′. Verification of the identity of this oligonucleotide as cap zero was obtained by HSLC on Partisil-SAX. As shown in Figure 2, the oligonucleotide fractions coeluted with actual cap zero standards.

The observation of a large amount of cap zero, which previously had not been identified as a component in any mammalian system, demonstrates that N′ methylation has been inhibited by the presence of STH. The similarity of base composition at N′ positions in cap zero and cap 1 structures (Table II) suggests that inhibition of methylation within this group of mRNAs is not selective for a particular base at N′. Instead an overall inhibition of N′ methylation appears to have occurred. It is possible, however, that base methylation at the 6-position of adenine may be inhibited to a greater extent than the ribose methylation at N′, since much less N^6-adenine is present in cap zero structures. The relative decrease observed in the amount of cap 2 from mRNA labeled in the presence of STH (Figure 1) indicates that 2′-O-methylation at N″ is also being inhibited.

The data presented in Table II indicate that the composition of N′(m) in cap structures from STH-treated samples is considerably different from the base distribution at N′m in normal RNA. The increased frequency of pyrimidines at N′(m) in STH-inhibited poly(A)-RNA is consistent with preferential inhibition of a subpopulation of mRNA. Inasmuch as RNA synthesis is initiated with a 5′-terminal purine triphosphate (Chambon, 1974; Schibler and Perry, 1976; Schmincke et al., 1976), and since cap 1 structures (m^{7}GpppN′mpN″) have been found in HnRNA (Perry et al., 1975a,b; Salditt-Georgieff et al., 1976), it has been postulated that 5′-terminal cap structures of mRNA might arise in two ways. Nascent 5′ termini of nuclear precursors would contribute to the mRNA species bearing caps of the type m^{7}GpppPu, and internal cleavage of HnRNA would generate RNA species terminated with either a purine or a pyrimidine, which would then be capped and processed (Furuichi et al., 1975; Ensinger et al., 1975; Schibler and Perry, 1976). The apparent enrichment of mRNAs containing a pyrimidine at the N′ position in cells exposed to STH raises the possibility that the presence of STH favors processing of mRNA bearing caps generated internally rather than from the 5′ terminus of a precursor molecule.

Although previous studies of nuclear poly(A)-RNA have suggested that methylation at the N′ site occurs in the nucleus (Perry et al., 1975a,b; Salditt-Georgieff et al., 1976), finding cap zero in cytoplasmic poly(A)-RNA indicated that methylation at the N′ position in mammalian mRNA is not a prerequisite for internal cleavage prior to capping. These results also suggest that transport of nuclear mRNA precursors does not require a fully methylated cap structure. Currently, investigations are in progress to determine if cap zero-containing mRNAs are located on polysomes, in an attempt to determine if N′-methylation is required for ribosomal binding of those mRNAs containing a capped 5′ terminus.

The data indicate that the presence of STH did alter the proportion of internal m^{6}A present in the mRNA fraction relative to the amount of m^{7}G observed. The ratio of m^{6}A/m^{7}G is much lower for mRNA derived from STH-treated cells, indicating that internal methylation is inhibited significantly in the presence of STH. This interpretation is supported by the fact that, when expressed as absolute cpm incorporated as m^{6}A, only 23% of the normal incorporation level is observed. It would be of interest to ascertain if this inhibition is expressed uniformly at all m^{6}A sites in mRNA molecules (Wei et al., 1976; Dimock and Stoltzfus, 1977).

In contrast, incorporation of [^{3}H]methyl as m^{7}G in mRNA from STH-treated cells was decreased to a lesser extent. The observed incorporation, 75% of the normal level, is comparable to the overall decrease in mRNA synthesis (73%, as measured by [^{14}C]uridine incorporation; cf. Table I). It appears that, under the conditions used in these experiments, in which partial

methylation is occurring, little or no inhibition of methylation at the 7-position of guanine is observed in the cytoplasmic poly(A)-RNA. These results may indicate a cellular response in terms of priority for methylation at the 7-methylguanosine site over internal base methylation, possibly reflecting the functional or sequential nature of methylation events during processing. Alternatively, RNA molecules which do not contain m^7G may not be transported into the cytoplasm and thus would not be observed. Experiments using ^{32}P-labeled RNA are currently being pursued in our laboratory in order to investigate further the possible inhibition of m^7G formation.

The data presented above indicate that STH inhibits methylation in vivo at several sites. Base methylation to generate m^6A is affected both at internal sites and at the N′ position of the caps. In addition, the presence of cap zero and the relative decrease in cap 2 structures in mRNA exposed to STH indicates inhibition of 2′-O-methylation at N′ and N″, respectively. This lack of complete selectivity for individual methylation sites by STH is in accord with previous data obtained using several isolated methylases (Coward et al., 1974; Borchardt, 1976). The fact that STH affects both 2′-O-methylation and base methylation indicates that this inhibitor may prove to be useful in examining the role of internal N^6-methyladenosine as well as the function of the cap structure.

In an earlier report describing the 5′-terminal cap structure (Rottman et al., 1974), a possible function of the cap in mRNA processing was proposed. The use of *S*-tubercidinylhomocysteine as an in vivo methylation inhibitor should provide a useful approach to this complex question. Although it can be stated that decreased and altered patterns of methylation are observed in the presence of STH, more specific questions can be asked if the system employed for this analysis permits evaluation of a single mRNA species. Studies of the effect of STH on methylation of a specific mRNA sequence are in progress. The analyses on poly(A)-RNA presented here indicate that STH does affect messenger RNA methylation in vivo, and that this in vivo approach may be useful in assessing the role of mRNA methylation and its relationship to mRNA processing.

While this manuscript was in preparation, Jacquemont and Huppert (1977) published evidence for inhibition of viral RNA methylation by another SAH analogue, *S*-isobutyladenosine. In contrast to STH, *S*-isobutyladenosine is a poor inhibitor of methylases in vitro (Legraverend et al., 1977) and thus the basis for its action in vivo is not clear.

Acknowledgment

We express our appreciation to Karen Friderici for her assistance in all phases of the experimental work, and her help in the preparation of this manuscript.

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