NOVIKOFF MESSENGER RNA METHYLATION:

IMPLICATIONS OF METHYLATION IN PROCESSING

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1978

ABSTRACT

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The specific function(s) of the 5'-terminal cap structure (cap 1, m^7GpppN'mpN"p; and cap 2, m^7GpppN'mpN"mpNp) in viral and eukaryotic messenger RNA are unknown. Ribosome binding studies and cell-free translational assays have shown that cap structures are involved in the recognition and translation of mRNA. However, the data suggests that caps function as facilitators rather than requisites for these processes. Cap structures have also been observed in nuclear poly (A)-containing RNA, suggesting an involvement of capping and methylation in processing. We have investigated this possibility via two approaches: 1) kinetic analysis of the formation of cap structures; and 2) in vivo perturbation of mRNA methylation by S-tubercidinylhomocysteine.

Novikoff hepatoma cells were maintained in culture for these experiments. Methylation was monitored by incorporation of L[^{3}H-methyl]methionine; synthesis was measured by [U-^{14}C]uridine or [^{32}P]-orthophosphate incorporation. RNA was isolated by standard procedures, including proteinase K digestion prior to extraction with phenol-chloroform. Poly (A)-containing RNA was isolated by oligo(dT)-cellulose affinity chromatography. Sucrose gradient sedimentation analysis was employed to determine the size of the mRNA.

Analysis of the methylation patterns of mRNA involved specific nuclease digestions followed by various chromatographic procedures.

RNase T2 effectively degraded mRNA to mononucleotides plus the RNase-resistant cap structures. These digestion products were separated by DEAE-Sephadex (7 M urea) chromatography prior to further analysis. Cap structures were analyzed either as intact caps by Partisil-SAX high speed liquid chromatography (HSLC) or by further degradation to mononucleosides, which were resolved by Aminex A-5 HSLC.

The kinetics of cap formation were studied by monitoring incorporation of L-[^{3}H-methyl]methionine into specific sites of mRNA as a function of labeling time. After a short labeling period of 20 min, approximately 80% of the label incorporated into caps was located at the penultimate nucleoside to the pyrophosphate, i.e. in the (N"m) position of cap 2 structures. The ratio of cap 2/cap 1 was observed to change with time, and the amount of internal N^6-methyladenosine decreased, relative to cap, with longer labeling times. These results are consistent with a model in which methylation at three sites - generating 7-methylguanosine, the first 2'-O-methylnucleoside (N'm), and internal N^6-methyladenosine - occur in the nucleus. The second 2'-O-methylation (N"m) appears to be a cytoplasmic event.

Perturbation of methylation in Novikoff cells occurred *in vivo* in the presence of S-tubercidinylhomocysteine (STH). Characterization of the partially methylated mRNA indicated that all sites of methylation, except the 7-position of guanine, were inhibited. STH diminished the levels of internal N^6-methyladenosine and of 2'-O-methylnucleosides in cap structures. The cytoplasmic presence of cap structures devoid of 2'-O-methylation, i.e. cap zero (m^7GpppN'), suggested that ribose

methylation is not required for processing and transport of mRNA. The base composition of cap 1 structures from normal and STH-exposed mRNAs were considerably different. The composition of caps zero and 1 from inhibited samples were comparable, however, indicating that inhibition by STH at the N' position was not base specific.

In order to assess the functional nature of cap zero-bearing mRNA molecules, monosomal and polysomal poly (A)-containing RNA was isolated from both normal and inhibited cells. Cap zero structures were the predominant cap species from both monosomal and polysomal mRNAs. These results suggest that 2'-O-methylation is not requisite for ribosome binding and subsequent translation.

Nuclear poly (A)-containing RNA was also analyzed for its methylation patterns. The base composition of nuclear cap 1 structures was comparable to the corresponding cytoplasmic cap 1 structures. Cap zero sturctures were present in nuclear RNA isolated only from STH-inhibited cells. No accumulation of totally unmethylated cap structures was observed.

Dedicated to Dick for his love and tolerance during my graduate training.

ACKNOWLEDGEMENTS

I would like to express my appreciation to the Biochemistry Department personnel for the constructive and congenial atmosphere maintained in the department during my graduate work. The people with whom I have interacted have been very cooperative and helpful. In particular I thank the members of my guidance committee, Drs. Pam Fraker, Debbie Delmer, Ron Patterson, John Boezi and Arnold Revzin, for their insight and advice.

A special thanks is extended to my coworkers in Dr. Fritz Rottman's laboratory - Karen Friderici, Dr. John Nilson, Bruce Coffin, Drs. Sarah Stuart, Ron Desrosiers and Arlen Thomason, Nancy Sasavage and Don Bodeau. Their friendships are very valuable to me. I especially thank Karen for her continual assistance and helpful advice throughout my graduate career. Part II of this thesis was a collaborative effort with Karen, who was the principal investigator in that study. Parts III and IV of the dissertation describe the research our laboratory performed in collaboration with Dr. James Coward of Yale University. He has been most helpful and communicative during our collaboration.

I am especially grateful to Fritz for his role in the development of my career. He has been an excellent mentor and friend. His insight and expertise has been invaluable in many helpful discussions. In addition, his sensitivity to others has resulted in very positive interactions with people within and outside the laboratory. I have been fortunate to work with Fritz and hope to continue our friendship in the future.

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DBAE	dihydroxyborylaminoethyl
DEAE	diethylaminoethyl
DNase	deoxyribonuclease
EDTA	ethylenediaminetetraacetic acid
GDP	guanosine-5'-diphosphate
GMP	guanosine-5'-monophosphate
GTP	guanosine-5'-triphosphate
hnRNA	heterogeneous nuclear RNA
HSLC	high speed liquid chromatography
m^6A	N^6-methyladenosine
m^6Am	N^6,2'-O-dimethyladenosine
m^5C	5-methylcytosine
Me_2SO,DMSO	dimethylsulfoxide
m^7G	7-methylguanosine
m^7gua^*	ring-opened 7-methylguanine
mN	base methylated nucleosides
mRNA	messenger RNA
N	nucleosides
N'	nucleoside adjacent to pyrophosphates in 5'-caps
N"	penultimate nucleoside to pyrophosphates in 5'caps

Nm	2'-O-methylnucleoside
RNase	ribonuclease
rRNA	ribosomal RNA
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SDS	sodium dodecyl sulfate
STH	S-tubercidinylhomocysteine
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
UV	ultraviolet
VSV	vesicular stomatitis virus

LITERATURE SURVEY

The presence of methylnucleotides in eukaryotic messenger RNA (mRNA) was first discovered in 1974. Since that time several investigators have been actively studying mRNA methylation patterns with respect to the structural and functional properties of this posttranscriptional modification. This review focuses primarily on those investigations which have attempted to elucidate the function of methyl groups on viral and eukaryotic messenger RNA molecules. Reviews by Shatkin (1976) and Rottman (1976;1978) provide comprehensive discussions concerning the discovery and initial characterizations of messenger RNA methylation.

I. Methylation of Viral and Eukaryotic Messenger RNA

mRNA Methylation: An Historical Perspective

Perry and Kelley (1974) first published evidence that eukaryotic mRNA was methylated. Poly (A)-containing RNA was isolated from mouse L cells and found to contain 2.2 methyl groups per 1000 nucleotides, or approximately one sixth of the level of ribosomal RNA methylation. Upon alkaline hydrolysis of the mRNA, a significant proportion of the methylated material chromatographed as a highly charged oligonucleotide. In a simultaneous and independent study, Desrosiers, et al. (1974) reported that Novikoff hepatoma polysomal mRNA was also methylated. The methylated components included the four common 2'-O-methylnucleosides, N^6-methyladenosine (m^6A) and

an unidentified nucleoside (later shown to be 7-methylguanosine, Desrosiers, et al., 1975). This methyl distribution was distinct from, and much simpler than, the methylation patterns of either ribosomal or transfer RNA from Novikoff cells.

In addition, studies using cytoplasmic polyhedrosis virus (Muira, et al., 1974a; Furuichi, 1974), reovirus (Muira, et al., 1974b; Shatkin, 1974) and vaccinia virus (Wei and Moss, 1974) indicated that methylation was also occurring in viral systems, and that the methylating activity was present within the virions. Localization of the methylase activity in viruses suggested that the function of methyl groups was sufficiently important to be retained in such limited genomes, and provided a source from which to isolate the enzymes responsible for methylation.

Analysis of the methylated components in both eukaryotic and viral mRNAs generated seemingly contradictory results. The alkali-resistant oligonucleotide fraction was also resistant to RNase T2 digestion, but the low level of methyl groups present ruled out its identification as a series of 2'-O-methylnucleotides. Alkaline phosphatase digestion reduced the charge of the material by two, indicating a single phosphate was released. Attempts to phosphorylate the oligonucleotide with polynucleotide kinase were unsuccessful, suggesting that the 5'-hydroxyl group was inaccessible. These data were consistent, however, with the general structure proposed by Rottman, et al. (1974), in which 7-methylguanosine is linked via an inverted 5'-5' pyrophosphate bond to one or two 2'-O-methylated nucleotides (Figure 1). These atypical nucleotide structures were termed "caps", and were structurally similar to the 5'-termini of

Figure 1. The 5'-terminal end of eukaryotic mRNA. The nucleoside 7-methylguanosine is attached by an unusual 5'-5'-pyrophosphate linkage containing three phosphates to a 2'-O-methylnucleoside with the base indicated as N'. This terminus is called cap 1. An additional adjacent 2'-O-methylnucleoside with the base indicated as N", is present in cap 2 structures. (Taken from Rottman, 1978).

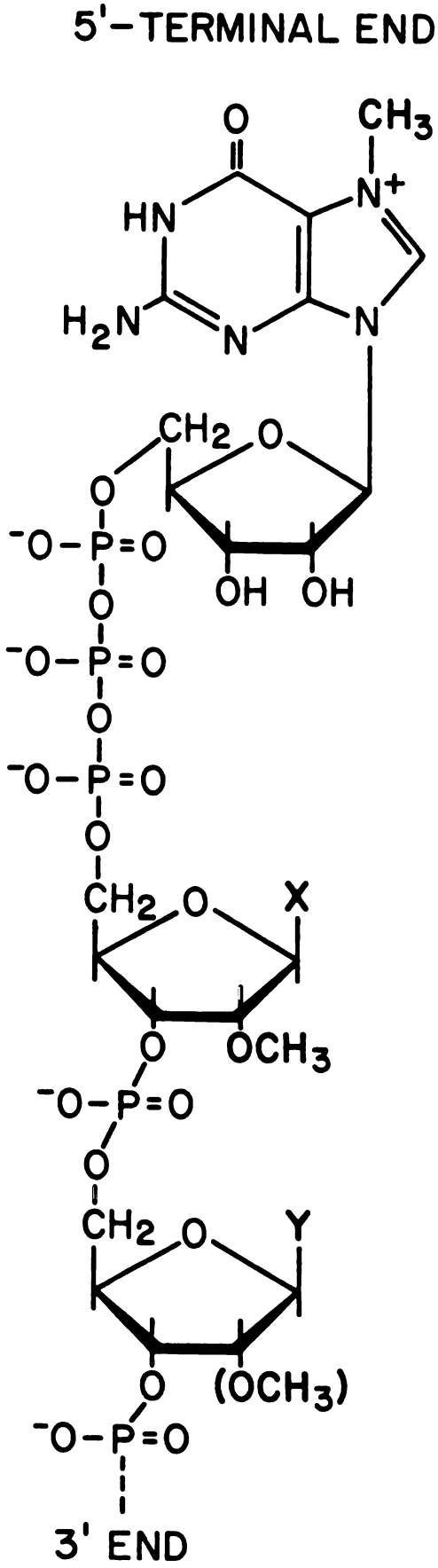
5'-TERMINAL END
O
CH3
N+
HN
H2N
N
N
CH2
O
O
-O-P=O
OH
OH
O
-O-P=O
O
-O-P=O
O
CH2
O
X
O
OCH3
-O-P=O
O
CH2
O
Y
O
(OCH3)
-O-P=O
3' END

Figure 1

low molecular weight nuclear RNAs (Reddy, et al, 1974; Ro-Choi, et al. 1974).

Distribution of Cap Structures

Several eukaryotic and viral messenger RNAs and virion RNAs have been shown to possess capped 5'-termini (refer to Table I). Three types of cap structures have been observed: cap zero, which contains no 2'-O-methylation (m^7GpppN'); cap 1, in which the penultimate nucleoside is ribose methylated (m^7GpppN'mpN); and cap 2, which contains two adjacent 2'-O-methylnucleosides (m^7GpppN'mpN"mpN). All of these structures are characterized by the 5'-5' pyrophosphate linkage of 7-methylguanosine to the penultimate nucleoside (N') of the ribopolymer. Tables I and II summarize the distribution of methylation in RNA from both eukaryotic and viral sources, respectively.

Cap zero structures are present only in a few viruses and in some lower eukaryotes. All mammalian and most viral systems studied to date contain cap 1 and/or cap 2 structures. The penultimate nucleoside in all viral mRNAs, and in some lower eukaryotic mRNAs, is always a purine, suggesting that these molecules are derived from primary transcription products. In contrast, mammalian mRNA contains both purines and pyrimidines at the N' position. The presence of pyrimidines requires that some 5'-termini must be generated from sites internal to the 5'-termini of the primary transcripts. In addition to the four common 2'-O-methylnucleosides, the dimethylated structure N^6,2'-O-dimethyladenosine (m^6Am) is also present at N' of caps 1 and 2. This doubly methylated nucleoside has not been found at the adjacent position N", however.

Table I

Distribution of Cap Structures and Internal Methylation in Eukaryotic mRNA[a]

Organism	Cap Zero (m^7GpppN')	Cap 1 ($m^7GpppN'm$)	Cap 2 ($m^7GpppN'mpN"m$)	Internal Methylation	References
Slime mold	N = A, G	N'm = Am	Absent	Absent	Dottin, et al. (1976)
Yeast	N = A, G	Absent	Absent	Absent	Sripati, et al. (1976); DeKloet and Andrean (1976)
Sea urchin embryos	Absent	N'm = Am,Gm,Cm,Um	Absent	m^6A	Faust, et al. (1976)
Brine shrimp	Absent	N'm = Am,Gm	Absent	m^6A	Groner, et al. (1976)
Insect					
Silkworm-(silk fibroin mRNA)	Absent	Absent	$m^7GpppAmUm$	N.D.[b]	Yang, et al. (1976)
Tobacco hornworm oocytes	GpppN[c]	Absent	Absent	N.D.	Kastern and Berry (1976)
Duck-globin mRNA	Absent	Present	Present	Absent	Perry and Sherrer (1975)
Rabbit-globin mRNA	Absent	N'm = m^6Am	N"m = Cm	Absent	Lockhard and RajBhandary (1976)

Table I (contd.)

Mouse					
L cells	Absent	N'm = Am,Gm,Cm,Um,m^6Am	N"m = Am,Gm,Cm,Um	m^6A	Perry, et al. (1975)
Myeloma(MPC-11-662)	Absent	N'm = Am,Gm,Cm,Um,m^6Am	N"m = Am,Gm,Cm,Um	m^6A	Cory and Adams (1975)
Kidney	Absent	Present	Present	Present	Ouellette, et al. (1975)
globin mRNA	Absent	N'm = Am,m^6Am	N"m = Cm	Absent	Cheng and Kazazian (1977)
Hamster-kidney (BHK-21)	Absent	N'm = Am,Gm,Cm,Um,m^6Am	N"m = Am,Gm,Cm,Um	m^6A,m^5C	Dubin and Taylor (1975)
Rat-Hepatoma (N1S1)	Absent	N'm = Am,Gm,Cm,Um,m^6Am	N"m = Am,Gm,Cm,Um	m^6A	Desrosiers, et al. (1975)
Erlich Ascites cells	Absent	Present	Present	N.D.	Bajszar, et al. (1976)
Monkey-kidney (BSC-1	Absent	N'm = Am,Gm,Cm,Um,m^6Am	N.D.	m^6A	Lavi and Shatkin (1975)
Human					
HeLa	Absent	N'm = Am,Gm,Cm,Um,m^6Am	N"m = Am,Gm,Cm,Um	m^6A	Wei, et al. (1976)
histone mRNA	Absent	N'm = Am,Gm,m^6Am	N"m = Am,Gm,Cm,Um	Absent	Moss, et al. (1977) Stein, et al. (1977)

[a]Adapted in revised form from Rottman, et al. (1978), Table 1-A.
[b]N.D. = not determined
[c]Methylation at 7-position of guanine is absent.

Table II

Distribution of Cap Structures and Internal Methylation in Viral RNAs[a]

Type of virus	Genome	Virion RNA 5'-Terminus	Viral mRNA 5'-Terminus	Internal Methylation	References
Plant:					
Brome Mosaic	ssRNA(+)	m^7GpppG	N.D.[b]	N.D.	Dasgupta, et al. (1976)
Tobacco Mosaic	ssRNA(+)	m^7GpppG	N.D.	N.D.	Keith and Fraenkel-Conrat (1975a); Zimmern (1975)
Alfalfa Mosaic	ssRNA(+)	m^7GpppG	N.D.	N.D.	Pinck (1975)
Cucumber Mosaic	ssRNA(+)	m^7GpppN	N.D.	N.D.	Symons (1975)
Cowpea Mosaic	ssRNA(+)	cap absent	N.D.	Absent	Klootwijk, et al. (1977)
Satellite Tobacco Necrosis	ssRNA(+)	$_7$ ppA	N.D.	N.D.	Wimmer, et al. (1968)
Wound Tumor	dsRNA	m^7GpppAm	N.D.	N.D.	Rhodes, et al. (1977)
Rice Dwarf	dsRNA	m^7GpppN	N.D.	N.D.	Muira, et al. (1975)
Avian:					
Rous sarcoma (Prague B)	ssRNA(+)	m^7GpppGm	N.D.	m^6A	Keith and Fraenkel-Conrat (1975b)
Sarcoma-B77	ssRNA(+)	m^7GpppGm	N.D.	m^6A	Furuichi, et al. (1975); Stoltzfus and Dimock (1976)
Newcastle disease	ssRNA(-)	N.D.	m^7GpppG(m)[c]	Absent	Colonno and Stone (1975)
Insect:					
Cytoplasmic Polyhedrosis	dsRNA	m^7GpppAm	N.D.	Absent	Furuichi and Muira (1975)
Mammalian:					
Vaccinia[d]	DNA	-	m^7Gppp(A,G,m^6A)mN"m	Absent	Wei and Moss (1975); Boone and Moss (1977)
Simian Virus 40[e]					
(in BCS-1 cells)	DNA	-	m^7Gppp(A,G)m		Lavi and Shatkin (1975);
(in CV_1 cells)	DNA	-	m^7Gpppm^{6}AmpU(m)		Haegeman and Fiers (1978)

Table II (contd.)

Adenovirus[e]	DNA	-	$m^7Gppp(m^6A,A)mpN"(m)$	m^6A,m^5C	Sommer, et al. (1976); Moss and Koczot (1976)
Herpes Simplex[e]	DNA	-	$m^7Gppp(A,G,m^6A)mN"(m)$	m^6A	Bartkosky and Roizman (1976); Moss, et al. (1977)
Reovirus[d]	dsRNA	$m^7GpppGm$	$m^7GpppGmpC(m)$	Absent	Shatkin (1974); Desrosiers et al. (1976)
Vesicular stomatitis[d]	ssRNA(-)	ppA	m^7Gpppm^6Am, $m^7Gpppm^6Amp(m^6A,A)m$	Absent	Moyer, et al. (1975); Rose (1975); Hefti and Bishop (1975)
Influenza (A and B)[e]	ssRNA(-)	ppA	$m^7Gppp(A,G,m^6A)m$	m^6A	Krug, et al. (1976); Moss, et al. (1978)
Sendai	ssRNA(-)	pppA	N.D.	N.D.	Leppert and Kolakofsky (1978)
Maloney murine leukemia	ssRNA(+)	$m^7GpppGm$	N.D.	m^6A	Bondurant, et al. (1976); Rose, et al. (1976)
Sindbis	ssRNA(+)	m^7GpppA	m^7GpppN	m^5C	Dubin and Stollar (1975)
Feline leukemia	ssRNA(+)	Cap absent	$m^7GpppGm(A,G)$	m^6A	Hefti, et al. (1976) Thomason, et al. (1976, 1978)
Poliovirus	ssRNA(+)	pUp,pAp	pUp	N.D.	Hewlett, et al. (1976); Nomoto, et al. (1976)
Encephalomyocarditis	ssRNA(+)	Cap absent	N.D.	Absent	Frisby, et al. (1976)
Foot-and-mouth disease	ssRNA(+)	Cap absent	N.D.	N.D.	Sangar, et al. (1977)
Mengo	ssRNA(+)	Cap absent	N.D.	N.D.	Fellner, et al. (1975)

[a] Adapted in revised form from Rottman (1978), Table I-B.
[b] N.D. = Not determined.
[c] mRNA synthesized in vitro by virion core.
[d] Replicates in cytoplasm of host cell.
[e] Replicates in nucleus of host cell.

In some cases, a distinction must be maintained between virion and viral messenger RNA termini (cf. Table II). The presence of cap 2 structures in viral mRNA, but not in virion RNA, of reovirus (Desrosiers, et al., 1976) suggests that host methylases may be involved in modifying the N" nucleotides. Some virion RNAs contain no cap structures at all, but the viral mRNAs are fully capped. Such is the case for RNA viruses whose genome consists of single-stranded, minus RNA strands (vesicular stomatitis virus (VSV), Newcastle disease virus and influenza virus), and for feline leukemia virus. The latter may be due to the defective nature of this virion (Thomason, et al., 1976; Rottman, 1976). In addition, the picorno-viruses (poliovirus, foot-and-mouth-disease virus, and encephalomyo-carditis virus) thus far appear to not be capped either in the virion or as messenger RNA. The significance of this is unknown.

Cap structures have also been identified on the 5'-termini of eukaryotic and viral-specific heterogeneous nuclear RNA (hnRNA). hnRNA of mouse L cells was shown to contain only cap 1 structures (Perry, et al., 1975). Similarly, cap 1 structures are found in hnRNA of HeLa cells (Salditt-Georgieff, et al., 1976) and Erlich ascites carcinoma cells (Bajszár, et al., 1976). Nuclear sequences specific for SV40 (Lavi and Shatkin, 1975) and adenovirus (Sommers, et al., 1976; McGuire, et al., 1976) are also capped. The composition of cap 1 structures in the mRNA and hnRNA of both mouse L cells (Schibler, et al., 1977; Perry, et al., 1975) and adenovirus-specific RNA (Sommer, et al., 1976) are very similar, consistent with a precursor-product relationship between these two RNA populations. No cap 2 structures have been identified in nuclear RNA.

Internal Methylation in mRNA and in hnRNA

In addition to methylated cap structures, several mRNAs contain base-methylated nucleosides. These modified nucleosides are not resistant to either RNase T2 digestion or alkaline hydrolysis, and can therefore be resolved from cap structures by chromatography on DEAE-Sephadex (7 M urea). In nearly all mRNAs and hnRNAs containing this modification, the base-methylated nucleoside has been identified as exclusively N-6-methyladenosine (m^6A). The exceptions - cultured hamster kidney cells (Dubin and Tayler, 1975), adenovirus (Sommer, et al., 1976) and Sindbus virus (Dubin and Stollar, 1975) have been reported to contain 5-methylcytosine (m^5C) in addition to m^6A.

Most higher eukaryotic mRNA and hnRNA molecules contain base-modified methylnucleosides. These modified bases have been located internal to the 3'-terminal poly (A) segment and to the 5'-terminal cap structure (Sommer, et al., 1976; Desrosiers, et al., 1975; Perry, et al., 1975). It is not known yet whether the methylated bases are within the coding regions of mRNA. However, the location of m^6A appears to be sequence specific in HeLa cells: Wei and Moss (1977) examined the nucleotides adjacent to m^6A and found only two sequences, Gpm^6ApC and Apm^6ApC. Of particular significance is the fact that these same two sequences were also detected in mouse L cells (Schibler, et al., 1977) and in avian sarcoma virus (Dimock and Stoltzfus, 1977), suggesting conservation of this sequence. The specificity of the location of m^6A may be related to its function, which is unknown. Internal methylation sites in Rous sarcoma virus have been located, however, in the 3'-terminal portion of the genome, in the region containing the sarc gene (Beeman and Keith, 1977).

The amount of internal m^6A in both mRNA and hnRNA has been correlated with molecular size (Lavi, et al., 1977; Sommer, et al., 1976; Perry and Kelley, 1976; Friderici, et al., 1976). Longer polynucleotides appear to contain more base-methylated residues than do shorter molecules. Some relatively small mammalian mRNAs, such as globin mRNA (Perry and Sherrer, 1975; Adams, et al., 1978) and histone mRNA (Stein, et al., 1977; Moss, et al., 1977) lack internal base modifications. Whether this reflects a simple size correlation, or is significant for some other reason, is unknown. This question has been complicated by the recent demonstration that the apparent levels of labeled m^6A in HeLa cells decreased with labeling time, an observation which may be due to demethylase activity, processing, and/or variation in turnover rates of mRNA subpopulations (Sommer, et al., 1978).

The presence of m^6A in viral RNA sequences appears to depend on whether these molecules are synthesized in the nucleus of host cells (Rottman, 1978). This distinction is consistent with the hypothesis that N^6-methylation of adenosine is a nuclear event, and that m^6A may be significant in processing and/or transport. Lower eukaryotic mRNA also lacks internal base methylation: no modified nucleosides except cap structures have been identified in the slime mold Dictostelium (Dotten, et al., 1976) or yeast (Sripati, et al., 1976; DeKloet and Andrean, 1976).

Enzymatic Mechanisms for Capping and Methylation

The sequential events of capping and methylation have been studied primarily in viruses. Elucidation of enzymatic mechanisms

for cap formation has been facilitated by two aspects of viral systems; 1) the virions themselves contain all the enzymes which are necessary to generate capped and methylated mRNA, thus providing a source for isolation and purification of the activities; and 2) unmethylated viral RNA, used as a substrate in these studies, can be synthesized in vitro in the presence of S-adenosylhomocysteine (SAH), an analog of the in vivo methyl donor S-adenosylmethionine (SAM).

Two basic mechanisms for capping and methylation have been postulated to function in viral systems (for details, cf reviews: Shatkin, 1976; Rottman, 1976, 1978). The first has been observed to function in vaccinia virus (Martin and Moss, 1975b; Moss, et al., 1976) and in reovirus (Furuichi, et al., 1976). It involves the transfer of a GMP residue from GTP to an RNA molecule terminated by a 5'-diphosphate, and may be depicted as follows:

1) Cleavage by polynucleotide 5'-triphosphatase activity to generate 5'-diphosphate termini:

$$pppN'pN''... \rightarrow\!\!\rightarrow ppN'pN''... + Pi$$

2) Capping by mRNA guanylyltransferase:

$$ppN'pN''... + GTP\ (\overset{***}{p}ppG) \rightarrow\!\!\rightarrow \overset{*}{G}pppN'pN''... + \overset{**}{P}Pi$$

3) Methylation by mRNA (guanine-7-)methyltransferase:

$$\overset{*}{G}pppN'pN''... + SAM \rightarrow\!\!\rightarrow m^7\overset{*}{G}pppN'pN''... + SAH$$

4) Ribose methylation at N' by mRNA (nucleoside-2'-)methyltransferase:

$$m^7\overset{*}{G}pppN'pN''... + SAM \rightarrow\!\!\rightarrow m^7\overset{*}{G}pppN'mpN''... + SAH$$

Polynucleotide 5'-triphosphatase has been purified and characterized (Tutas and Paoletti, 1977). Subsequently these investigators have demonstrated that this enzyme activity is induced in

HeLa cells within one hour after infection with vaccinia virus. Its induction was shown to be dependent upon de novo RNA and protein synthesis, but independent of DNA synthesis, suggesting this enzyme is a prereplicative or "early" viral product (Tutas and Paoletti, 1978).

Moss and his colleagues have identified activities in vaccinia virus which correspond to each of the other enzymes involved in the capping and methylation sequence depicted above (Ensinger, et al., 1975). mRNA guanylyltransferase and mRNA (guanine-7-)methyltransferase activities co-purify from vaccinia virions as a single enzyme of molecular weight 127,000 (Martin et al., 1975a). This enzyme contains molar amounts of two subunits whose molecular weights are 95,000 and 31,400 (Martin et al., 1975a). Subsequent characterization of these enzymatic activities (Martin and Moss, 1975, 1976; Moss, et al., 1976a) permitted determination of the above reaction sequence. Boone, et al. (1977) have also isolated the co-purifying activities from infected HeLa cells; their subsequent studies provided evidence that guanylyltransferase and the methyltransferases are prereplicative viral gene products. Moss (1977) has also demonstrated that the viral enzymes can be used to modify heterologous mRNAs, a technique which should be useful both for identification of 5'-terminal mRNA structures and for investigation of cap function(s).

Initial studies with reovirus by Furuichi and Shatkin (1976) indicated that short (<15 nucleotides) nascent reoviral RNAs, synthesized in vitro, were capped and methylated. These investigators initially concluded that RNA polymerase activity in reovirus was

coupled to capping and methylation. More recent studies, however, have indicated that RNA polymerase functions independently of guanylyltransferase and the methyltransferases, and vice versa (Furuichi and Shatkin, 1977). Carter (1977) has reported that reoviral methyltransferases are also capable of modifying the 5'-termini of single-stranded oligonucleotides which possess GpppG at their 5'- ends. These oligomers are present in the virion, and their function is unknown.

A second distinct mechanism for capping has been identified in the formation of capped VSV RNA (Abraham et al., 1975; Colonno and Banerjee, 1976; Testa and Banerjee, 1977). This mechanism differs from that described for vaccinia virus and reovirus in two important features: 1) both the α- and β-phosphates of GTP are transferred during capping; and 2) 2'-O-methylation of the penultimate nucleoside N' occurs prior to methylation of the 7-position of guanine. The mechanism is outlined below:

1) Cleavage reaction to generate monophosphorylated 5'-termini:

$$pppN'pN''... \rightarrow\!\!\rightarrow pN'pN''... + PPi$$

(or $pppNpN...NpN'pN'' \rightarrow\!\!\rightarrow pppNpNp... + pN'pN''...$

2) Capping by transfer of guanosine diphosphate to the RNA chain:

$$pN'pN''pN... + \overset{***}{p}ppG \rightarrow\!\!\rightarrow \overset{**}{G}pppN'pN''pN...$$

3) Ribose methylation at N' by mRNA (nucleoside-2'-)methyltransferase:

$$\overset{**}{G}pppN'pN''pN... + SAM \rightarrow\!\!\rightarrow \overset{**}{G}pppN'mpN''pN... + SAH$$

4) Methylation by mRNA (guanine-7-)methyltransferase:

$$GpppN'mpN''pN... + SAM \rightarrow\!\!\rightarrow m^7GpppN'mpN''pN... + SAH$$

The enzymes involved in this reaction scheme have not been purified. The capping and methylase activities are known to be

associated with the virion ribonucleoprotein core (Rhodes et al., 1974) and appear to be transcription-dependent (Abraham and Banerjee, 1976). Hefti and Bishop (1976) have suggested that the guanylyl-transferase from VSV possesses sequence specificity for the donor substrate mRNA.

The two methyltransferase activities involved in the reaction scheme indicated above have recently been identified in purified virions of VSV (Testa and Banerjee, 1977). The concentration of SAM, the methyl donor, appeared to determine the number and location of the methyl groups transferred to cap structures. Limiting SAM concentrations resulted in only 2'-O-methylation of the penultimate nucleoside, whereas saturating SAM concentrations permitted base methylation at the 7-position of guanine (Testa and Banerjee, 1977). These methyltransferase activities have been identified in virions purified from hamster, mouse and human host cells (Testa and Banerjee, 1977).

Since vesticular stomatitis virus replicates in the nucleus of its host cells, and since its mRNA appears to be generated from large nuclear precursors (Colonno, et al., 1976), this second mechanism was presumed most likely to be occurring in eukaryotes. Formation of cap structures onto a monophosphorylated terminus allowed for capping at sites located internally from the 5'-terminus of an hnRNA molecule. Such a mechanism is necessary owing to the presence of pyrimidines at the N' position of cap structures, since all transcription is believed to initiate with purines (Chambon, 1974; Schmincke, et al., 1976; Schibler and Perry, 1976). Investigation of the 5'-termini of hnRNA in mouse L cells, however, indicated that approximately

20% of the hnRNA population was terminated by diphosphates (Schibler and Perry, 1976; Schibler, et al., 1977). Furthermore, the base distribution of the diphosphorylated termini was very similar to the composition of N' nucleosides in cap structures from mRNA. These findings suggested that the capping mechanism described for vaccinia viral RNAs might be functional in eukaryotes as well.

Eukaryotic methylase activities have been observed in nuclear homogenates of HeLa cells (Groner and Hurowitz, 1975), mouse L cells (Winicov and Perry, 1976) and mouse myeloma (MOPC-21) cells (S. Stuart, unpublished observations). Groner, et al. (1978) recently identified RNA polymerase II transcripts as the substrates for the capping and methylating activities present in HeLa nuclear homogenates. These investigators further demonstrated that the β-phosphate of the pyrophosphate bridge originates from the RNA chain (Groner, et al., 1978). These results are consistent with the capping mechanism which occurs in vaccinia virus and reovirus.

An RNA (guanine-7-)methyltransferase had been partially purified from the cytoplasm of HeLa cells (Ensinger and Moss, 1976). The presence of this activity in the postnuclear supernatant was inconsistent with the presumed nuclear location of capping and 7-methylation of guanine, but the authors acknowledged the possibility of nuclear leakage during cell lysis. The enzyme was capable of modifying vaccinia viral mRNA, synthetic 5'-diphosphate-terminated ribopolymers, and GpppG, indicating its substrate specificity is comparable to the mRNA (guanine-7-)methyltransferase isolated from vaccinia virions.

II. Possible Function(s) of Capping and Methylation

A. Involvement of Methylated Cap Structures in mRNA Translation

Translation Studies.

Capping and methylation of both viral and eukaryotic mRNA suggested that these posttranscriptional modifications might function at the level of translation. The influence of methylation on translational efficiency of mRNA was first demonstrated by Both, et al. (1975). Reoviral and vesicular stomatitis viral mRNAs were synthesized in vitro in the absence and presence of SAM, in order to generate unmethylated and methylated viral mRNA. The unmethylated mRNA was translated with lesser efficiency in wheat germ extracts than was methylated mRNA. Addition of S-adenosylhomocysteine (SAH) to the cell-free extract further reduced the translational efficiency of unmethylated mRNAs, suggesting that SAM-mediated methylation of this mRNA might have occurred in the wheat germ extract. Positive identification of the endogenous methyltransferase activity was made by analysis of unmethylated mRNA after incubation in the wheat germ system containing [^{3}H-methyl]-labeled SAM (Both, et al., 1975). These results showed that the ability of mRNA molecules to direct protein synthesis was dependent upon the methylated state of the mRNA. Muthukrishnan and colleagues (Muthukrishnan, et al., 1975a) subsequently showed that the endogenous methylation produced cap zero structures, m^7GpppN', at the 5'-terminus of unmethylated mRNA, suggesting that translation of viral mRNA was specifically dependent upon the presence of 7-methylguanosine in capped mRNA.

Comparable translational studies using capped and fully-methylated mRNAs were performed after removal of the 5'-terminal m^7G by periodate

oxidation and β-elimination (Muthukrishnan, et al., 1975a). Decreased translation efficiency in cell-free protein synthesizing extracts was observed following β-elimination of rabbit reticulocyte mRNA (Muthukrishnan, et al., 1975a; Rose and Lodish, 1976), brine shrimp mRNA (Muthukrishnan, et al., 1975b), bovine parathyroid mRNA (Kemper, 1976), brome mosaic virus RNA-4 (Shih, et al., 1976) and reoviral mRNA (Muthukrishnan, et al., 1975a; Samuel and Lewin, 1976). In contrast, no difference in translational ability was observed after β-oxidizing satellite tobacco necrosis viral RNA (Kemper, 1976; Roman, et al., 1976), an uncapped RNA.

The harsh conditions required for β-elimination of m^7G has been criticized with respect to the nonspecific alterations of mRNA structure which may occur during the procedure (Rose and Lodish, 1976). Alternative methods of m^7G removal involving enzymes have been reported to be more specific and less damaging to the mRNA chain (Zan-Kowalczewska, et al., 1977; Abraham and Pihl, 1977). Enzymatic removal of pm^7G from the 5'-terminus of reovirus, rabbit globin and brine shrimp mRNAs, using purified potato nucleotide pyrophosphatase, resulted in a greater than 80% decrease in each mRNA's template activity (Zan-Kowatczewska, et al., 1977). In contrast, Abraham and Pihl (1977) have reported that "decapping" of rabbit globin and mouse immunoglobulin light chain mRNAs by polynucleotide kinase does not affect translation of these messengers.

The results of translational efficiency studies also appear to be dependent upon the degree of homogeneity in the assay systems. The use of wheat germ extracts with animal virus and cellular mRNAs represents a heterologous system, which may not adequately reflect

the regulatory functions and signals of the in vivo environment. Rose and Lodish (1976) reported that the translation of β-eliminated vesicular stomatitis viral mRNA was one-tenth as efficient as untreated mRNA in wheat germ systems, but was reduced to only one-fourth the control level in reticulocyte lysates. Similar results have been published by Samuel, et al (1977) for the translational efficiencies of methylated vs. unmethylated reovirus mRNA. In this study, unmethylated mRNA directed translation at less than 10% the rate of methylated mRNA in wheat germ extracts, but the two mRNA preparations were equally efficient in the homologous mouse ascites system (Samuel, et al., 1977). Toneguzzo and Ghosh (1976) obtained comparable results using vesicular stomatitis viral mRNA.

Held, et al (1977) also cautions against interpreting the results of translation studies as a structure-function analysis. These investigators demonstrated that uncapped reovirus and globin mRNAs can be translated up to 70% as efficiently as methylated-capped mRNAs in the presence of optimal concentrations of reticulocyte initiation factors. In contrast, optimal concentrations of ascites initiation factors or suboptimal levels of reticulocyte initiation factors resulted in a translational efficiency for unmethylated mRNA at 5 to 10% of the methylated-capped mRNA efficiency (Held, et al., 1977).

Ribosome Binding Studies.

More precise evaluation of the effect of methylation on mRNA translational efficiency has been obtained by ribosome binding assays. Both, et al. (1975b) showed that wheat germ ribosomes selectively bound reovirus mRNA molecules which contained 7-methylguanine in

the 5'-terminal cap. This selectively occurred during or prior to the formation of 40S-mRNA-containing complex, suggesting that methylation functions at initiation (Both, et al., 1975b). A majority of cap structures was sensitive to partial RNase digestion of the 80S-mRNA complexes, however, indicating that some caps were not physically protected by the ribosomes at the 80S level. Terminal oligonucleotides, containing 7-10 nucleotides and cap structures, did not rebind to ribosomes, suggesting that the presence of caps is insufficient for recognition by ribosomal subunits (Both, et al., 1975b).

The relative importance of the 5'-terminus and the base composition of the polynucleotide chain was evaluated by binding studies of synthetic ribopolymers (Both, et al., 1976; Muthukrishnan, et al., 1976a,b). The ribopolymers were synthesized with polynucleotide phosphorylase, and the reactions were primed with m^7GpppGmpC, its ring-opened derivative m^7G*pppGmpC, m^7GpppGpC, GpppGpC or ppGpC. Preferential binding was observed for m^7G-containing polymers and for those sequences rich in (A,U) (Both, et al., 1976; Muthukrishnan, et al., 1976a). Eleven to twenty per cent of the (A,U)-rich ribopolymers were complexed to ribosomes without regard to the 5'-terminus, indicating that 7-methylguanosine is not an absolute requisite for ribosomal recognition. The presence of 2'-O-methylation at the penultimate residue (N' position) enhanced ribosome binding of capped (A,U)-polymers five fold in reticulocyte lysates. Similar enhancement of binding in wheat germ extracts was observed only if 60S subunits were depleted by high speed centrifugation (Muthukrishnan, et al., 1976b).

The importance of cap structure in ribosomal binding affinity also seems to depend on the nature of the systems used. Lodish and Rose (1977) studied binding and translational efficiencies of vesicular stomatitis virus mRNAs in the heterologous plant cell-free extract and in the more homologous reticulocyte lysate. The presence of 7-methylguanosine appeared to be far more important for mRNA function in the wheat germ system (Lodish and Rose, 1977). Similar observations were made for reovirus mRNA (Muthukrishnan, et al., 1976b) and vaccinia virus mRNA (Muthukrishnan, et al., 1978).

Because these ribosomal studies implicated methylation at the initiation level, several investigators sought to identify a ribosomal protein or initiation factor which bound to cap structures. Fillipowicz, et al. (1976) reported that a ribosome-associated protein in brine shrimp extracts was capable of binding m^7GpppGpC, but could not identify this protein with any known initiation factor. Shafritz and colleagues, however, demonstrated that IF-M3, an initiation factor in reticulocyte lysates, specifically recognized cap structures and that its binding to capped mRNA was inhibited by analogues of the cap (Shafritz, et al., 1976). This factor is also necessary for the translation of uncapped picornovirus mRNAs, though, and therefore presumably possesses additional interactions with mRNA. Studies by Kaempfer, et al. (1978), however, argue that initiation factor EF-2 is involved in recognition of cap structures. A model involving primary recognition of an internal mRNA sequence, and secondary recognition of cap structures and methionyl-$tRNA_f^{Met}$, is proposed which provides a molecular basis for differential translation of mRNA species (Kaempfer, et al., 1978).

Further information about the involvement of 5'-terminal cap structures in ribosome attachment has been obtained by analysis of viral RNA fragments which are protected by ribosomes. Dasgupta, et al. (1976) demonstrated that the AUG codon is located just 10 nucleotides from the 5'-terminus of brome mosaic virus RNA-4. Eight of the 10 nucleotides are adenosine and uridine (the 5'-terminal nucleosides comprise the cap m^7GpppG). Kozak and Shatkin (1976) have similarly characterized the ribosome-protected fragments of reovirus mRNAs. The sequence of fragments from six of these mRNAs has been determined (Kozak and Shatkin, 1977a,b; Kozak 1977).

Comparison of the sequence and re-binding ability of these ribosome-protected fragments permitted identification of the following common features: a) in all cases the 40S-initiation complex protected a significantly larger segment of the mRNA (including the cap) than did the 80S complex; b) each 80S-complex protected a subset of the 40S-protected sequence and contained an AUG codon; c) ribosomes re-bound to the mRNA fragments at the same initiation site irrespective of the methylated state of the cap; d) only those partial digestion products which retained the AUG codon could form initiation complexes, although the efficiency of binding was reduced if either the 5'-terminal region (including the cap structure) or the 3'-terminal region to AUG was removed (Kozak and Shatkin, 1978). These results strongly suggest that a variety of parameters are involved in ribosomal recognition of mRNA, and that the presence of cap structures serves to facilitate binding rather than to determine it.

Analog Inhibition Studies.

An alternative approach to investigation into the role of cap structures in ribosome binding and translation was introduced by Hickey, et al. (1976a). These investigators demonstrated that 7-methylguanosine-5'-monophosphate (pm^7G) inhibited cell-free translation of rabbit globin mRNA, tobacco mosaic virus RNA, HeLa cell poly (A)-containing RNA and reovirus RNA. Translation of poly (A) and uncapped satellite tobacco necrosis virus RNA was unaffected by pm^7G, indicating that this inhibition was specific for translation of capped RNAs (Hickey, et al., 1976a). These findings have been confirmed and expanded by several independent investigations (Canaani, et. al., 1976; Levin and Samuel, 1976; Roman, et al., 1976; Suzuki, 1976; Groner, et al., 1976).

The inhibitory effect of pm^7G seems to be dependent upon both the methyl group in position 7 and the 5'-phosphate, since 7-methylguanosine-2',3'-monophosphate, 7-methylguanosine and guanosine-5'-monophosphate did not affect translation (Hickey, et al., 1976a,b; Canaani, et al., 1976). 7-Methylinosine-5'-monophosphate was also a poor inhibitor, suggesting that the amino group at position 2 of guanine may be important (Hickey, et al., 1977). However, addition of 5'-phosphate groups to generate ppm^7G and $pppm^7G$ resulted in an enhancement of inhibition relative to the monophosphorylated nucleotide (Hickey, et al., 1977).

Cap structures and their analogs have also been shown to inhibit protein synthesis, and are much more effective than the mononucleotide derivatives (Hickey, et al., 1977; Suzuki, et al., 1977; Canaani,

et al., 1976). The presence of 7-methylguanosine appears to be most important, since GpppN and GpppNm were not inhibitory, and m^7GpppNm and m^7GpppN were comparable to each other and to $pppm^7G$ and ppm^7G in inhibitory effect (Hickey, et al., 1977). Results from our laboratory have indicated that methylated tetraphosphate cap analogs are also potent inhibitors of protein synthesis (N. Sasavage, K. Friderici and F. Rottman, unpublished observations). The doubly-methylated analog, $m^7Gppppm^7G$, was more inhibitory than the singly-methylated structure m^7GpppG; no inhibition was observed in the presence of the unmethylated tetraphosphate cap (GppppG).

Inhibition by pm^7G has been determined to occur at the conversion from the 40S-initiation complex to the 80S-initiation complex, a transition which is mRNA-dependent (Roman, et al., 1976). The mono-phosphorylated derivative has been shown to equally inhibit the translation of all species present in HeLa cell poly (A)-containing RNA (Weber et al., 1976), and to be noninhibitory for the translation of unmethylated reoviral mRNAs (Levin and Samuel, 1977).

The use of these cap analogs in both translation and ribosome binding studies has indicated that the m^7G analogs compete with capped mRNA molecules, as predicted. However, the results of these studies must also be subject to the considerations described above, i.e. the use of heterologous vs. homologous systems. In addition, the observed effect of pm^7G on mRNA translation in both wheat germ and reticulocyte systems has also been shown to be dependent upon the potassium ion concentration used (Kemper and Stolarsky, 1977; Weber, et al., 1977, 1978). The effective inhibition by pm^7G increased with increased potassium concentrations up to the optimum K^+ level

for protein synthesis. The counterion of the potassium salt (Kemper and Stolarsky, 1977) and the incubation temperature (Weber, et al., 1978) used in these assays also influenced the apparent inhibition of protein synthesis by 7-methylguanosine-5'-monophosphate.

NMR studies by Hickey, et al. (1977) have generated an hypothesis which correlates inhibitory strength with the structure of a given analog. 7-Methylguanosine possesses a flexible conformation which becomes increasingly rigid by the introduction of one or two 5'-phosphate groups. This is due to an electrostatic interaction between the positively charged N-7 position of 7-methylguanine and the negatively charged phosphates, which results in a stable "W-shaped" conformation of the m^7G backbone. Use of this structural criterion has enabled accurate predictions of the inhibitory effect of analogs on mRNA translation. This hypothesis is also consistent with the similar inhibtory effects of ppm^7G, $pppm^7G$, m^7GpppN' and $m^7GpppN'm$: the conformational stability is maximized in the diphosphate mononucleotide (Hickey, et al., 1977).

Adams, et al (1978) also concluded that the positively charged imidazole moiety of m^7G and negatively charged phosphate groups were the important structural features of cap analogs. These investigators synthesized a variety of substituted 7-methylguanosine-5'-diphosphate compounds and determined the effect of these derivatives on reovirus mRNA binding to wheat germ ribosomes. Alkylation at the 7-position of guanine with methyl, ethyl and benzyl groups generated active cap analogs, but loss of inhibitory activity was observed if the positive charge on the imidazole ring was eliminated. The 2-amino group of guanine also influenced the inhibitory effect of

the analog, whereas the 2',3'-cis-diol moiety was not critical for ribosomal binding.

It should be noted that two enzymes have been detected which are capable of hydrolyzing cap structures. HeLa cell extracts contain a pyrophosphatase activity which cleaves m^7GpppN' to pm^7G and ppN', and possesses substrate specificity for m^7G-terminated oligonucleotides up to 10 residues in length (Nuss, et al., 1975). A distinct enzymatic activity has been identified in tobacco cells which is capable of hydrolyzing various phosphodiester and pyrophosphate bonds without degrading polynucleotides (Shinshi, et al., 1976). These enzymes may function to protect cells from the inhibitory effects of residual caps generated by degradation of mRNAs. Removal of the 5'-terminal pm^7G from tobacco mosaic virus by the tobacco enzyme has been shown to virtually destroy viral infectivity (Ohno, et al., 1976).

Regulatory Function at Translational Levels.

Differential translation of methylated and unmethylated mRNAs prompted investigations of the possible role of methylation in development. Nontranslated, stable "maternal mRNA" is known to be present in unfertilized oocytes of sea urchins and in brine shrimp cysts and embryos. Capping and/or methylation represented potential signals for activation of this mRNA population. However, embryonic brine shrimp mRNA (Muthukrishnan, et al., 1975b) and sea urchin oocyte and embryonic mRNAs (Hickey, et al., 1976b; Faust, et al., 1976; Sconzo, et al., 1977) were shown to already possess methylated cap structures. The stored maternal mRNA in tobacco hornworm oocytes, though, is capped but not methylated (Kastern and Berry, 1976); whether the cap becomes methylated after fertilization is unknown.

The appearance of 7-methylguanosine-5'-phosphate in RNA of mouse one-cell embryos has been reported (Young, 1977).

A regulatory function of methylation has been suggested by the impairment of reovirus mRNA methylation in interferon-treated Ehrlich ascites tumor cells (Sen, et al., 1975,1977). The relevance of these in vitro studies remains to be established.

The above experimental approaches used to assess the significance of cap structures at the level of translation have been facilitated by several characteristics of viral systems. These features include 1) the relatively simple, and generally well-characterized, viral genome, which permits monitoring of distinct RNA species and protein products; 2) the ability to synthesize viral mRNAs in vitro, which not only provides access to virtually pure RNAs, but also permits generation of unmethylated mRNAs to serve as substrate for enzyme assays and to enable comparative studies on the function(s) of methylation; and 3) the presence of the capping and methyltransferase activities within the virion, affording substantial purification (relative to a typical cellular enzyme) prior to the onset of isolation procedures. As a result, most experiments thus far performed have used viral mRNAs and enzyme preparations. A notable exception is globin mRNA, which is unusually simple to isolate relative to most eukaryotic mRNAs.

A massive amount of data has been generated as a result of these efforts to identify a role for cap structures in mRNA translation. An overall assessment of these results indicates that cap structures do seem to function in translation at the level of initiation, but its role is quantitative rather than qualitative. The

conformation afforded by electrostatic interaction of 7-methylguanosine with the adjacent phosphate groups appears to stabilize the ribosomal binding of caps, which would account for preferential translation of capped mRNAs.

Perhaps the most serious criticism of these experimental approaches, taken as a whole, is the heterologous nature of the in vitro assays. Fidelity of regulatory mechanisms is essential for structure-function studies, and an in vitro, heterologous system may not meet this criterion. A corollary to this criticism is that the factors important for recognition of viral-specific mRNA may be different than those involved in eukaryotic mRNA translational control. Future experimental designs must be developed to enable assessment of these more subtle aspects of translational control.

B. Role of Methylation in mRNA Processing

The concept of mRNA processing - that large, heterogeneous nuclear RNA molecules (hnRNA) are precursors of cytoplasmic mRNAs - was first proposed by Sherrer and Marcaud (1968). Processing encompasses posttranscriptional polyadenylation of the 3'-terminus, capping and methylation at the 5'-terminus, internal base methylation, and cleavage (or degradation) of the precursor in order to reduce its size (cf reviews: Darnell, et al., 1973; Greenberg, 1975; Lewin, 1975; Rottman, 1978). Only a few per cent of the transcribed sequences present in the nucleus reach the cytoplasm. Thus processing appears to control genetic expression at the posttranscriptional level by regulating cytoplasmic entry of mRNA sequences. Considerable efforts have been directed at elucidating the nature of processing events, but the control mechanisms involved remain ambiguous.

Theoretical and Technical Considerations.

Identification of a relationship between methylation and other posttranscriptional events is difficult for both theoretical and technical reasons. The apparent contradiction that both 3'-poly (A) and 5'-cap structures of hnRNA seemed to be conserved during processing, has most likely been resolved by the recent discovery of intragenic sequences in unique genes (Jeffreys and Flavell, 1977; Tilghman, et al., 1977; Breathnach, et al., 1977; Brack and Tonegawa, 1977). The demonstration that the intragenic sequences in β-globin genes are transcribed into a β-globin precursor RNA molecule, but are not present in β-globin mRNA (Tilghman, et al., 1978), requires that cleavage occur from within the hnRNA and thus permits conservation of both termini. Although these data have clarified the overall mechanism of processing, the precise sequential and regulatory interrelationships between specific posttranscriptional events remains vague.

Characterization of hnRNA has proven to be a difficult and often controversial task. As indicated by its name, hnRNA consists of a heterogenous population of RNA molecules both with respect to size and sequence. The sequence complexity of hnRNA is extremely high, and certain sequences appear to be restricted to the nucleus. Less than ten per cent of the hnRNA synthesized is believed to reach the cytoplasm, with the remainder being rapidly degraded and/or performing unknown functions. The halflife of most hnRNA molecules is believed to be extremely short - 3 to 23 minutes - and thus processing intermediates are also presumably short-lived. These characteristics make kinetic studies extremely complex, particularly if the design is toward elucidation of a precursor-product relationship.

Isolation of hnRNA is a technical problem in itself. Massive amounts of DNA and rRNA precursors must be separated from hnRNA. Eukayrotic nuclei contain a variety of ribonucleases and the large size of hnRNA renders it particularly susceptible to nicks and degradation during rigorous purification procedures. Isolation of specific hnRNA sequences requires additional sensitivity for its detection. Also, considerable controversy still exists as to the actual size distribution of hnRNA. In addition to being sensitive to degradation, this RNA class appears to form aggregates on gels and sucrose gradients. The recent and rapid development of cloning procedures, coupled with the sensitivity of complementary DNA hybridization techniques, should facilitate studies of hnRNA and mRNA processing.

Evidence Implicating Methylation in Processing.

The possibility that methylated cap structures are involved in processing was suggested by Rottman, et al. (1974) in the first communication which identified the 5'-terminal structure. Involvement of methylation in processing has precedent in rRNA processing of 45S precursor molecules. Although the 18S and 28S cytoplasmic rRNA species represent less than half the nucleotide sequences of the 45S precursor, all methylated sites of the precursor are retained in mature rRNA (cf. review: Perry, 1976).

The identification of cap structures in hnRNA molecules isolated from mouse L cells (Perry, et al., 1975b), HeLa cells (Salditt-Georgieff, et al., 1976), adenovirus-infected cells (Sommer, et al., 1976) and SV40-infected cells (Lavi and Shatkin, 1975) has been described above. In all cases, only cap 1 structures were observed. The presence of cap 1 in hnRNA, coupled with the similarity

in methylnucleotide composition of caps 1 from mouse L cell hnRNA and mRNA (Perry, et al., 1975b; Schibler and Perry, 1976; Schibler, et al., 1977) raised the possibility that caps might be conserved in processing. Perry and Kelley (1976) performed pulse-chase experiments with mouse L cells to determine the kinetics of cap formation. The data indicated that minimum cap turnover occurred in the nucleus and that virtually all the labeled hnRNA caps were chased into cytoplasmic mRNA after 3 h. The 2'-O-methylation at the N" nucleoside appeared to occur in the cytoplasm (Perry and Kelley, 1976; Perry, et al., 1976).

Similar results were observed by Friderici, et al. (1976; reproduced as part two of this thesis) for Novikoff mRNA methylation. These investigators analyzed the methylation patterns of mRNA as a function of labeling time. After a 20 min pulse, 80% of the radioactivity in mRNA was in N"m positions of cap 2. With longer labeling periods, a greater proportion of the mRNA radioactivity was observed in m^7G, N'm, and m^6A, i.e. in those sites which appear to be methylated in the nucleus. The kinetics of cap formation in both Novikoff and mouse L cells suggests that capping and methylation of terminal guanosine, N' nucleosides, and internal bases occur in the nucleus. The methylated hnRNA molecules are processed and transported into the cytoplasm prior to additional methylation at N" to generate cap 2 structures. The sequential methylation pattern of globin mRNA in mice, as determined by Cheng and Kazazian (1978), is consistent with this model.

The presence of internal base methylation in both hnRNA and mRNA (see above) is also suggestive of an involvement of methylation in processing. In this regard it is of interest to note that only those viruses which replicate in the nucleus contain internal base methyl groups. The fact that m^6A is present in the same specific sequence (G,A)pm^6ApC in mouse L cell mRNA and hnRNA (Schibler, et al., 1977) implies that m^6A-containing hnRNA sequences are conserved in mRNA. As mentioned above, the m^6A content in both mRNA and hnRNA has been correlated with molecular size (Perry and Kelley, 1976; Friderici, et al., 1976; Sommer, et al., 1977). In contrast, Lavi and Shatkin (1977) found that the m^6A residues per nucleotide number in HeLa cell mRNA is three to four times greater than that of HeLa hnRNA, suggesting that all internal base methylations in hnRNA are conserved during processing. Sommers, et al.,(1978) however, have shown that the apparent levels of m^6A in RNA molecules may reflect labeling periods, and, as pointed out by Schibler, et al. (1977), the current data on m^6A labeling kinetics may be interpreted in a variety of ways. Further studies are thus necessary to determine the fate of internal base methylnucleosides during processing.

Two other studies have implicated methylation in processing. McGuire, et al. (1976) analyzed adenovirus-specific RNA and found two types of 5'-termini. Both cytoplasmic and nuclear viral-associated RNA, believed to not function as viral mRNA, were terminated by guanosine tetraphosphate (pppGp). Viral nuclear precursor RNA, however, contained both pppGp and cap structures, whereas polysome-associated viral mRNA was terminated exclusively by cap structures. These findings suggest that capping and methylation of virus-specific

transcripts only occurs on those molecules which will perform mRNA functions (McGuire et al., 1976). Rose, et al.(1977) synthesized in vitro VSV mRNA and analyzed the products by gel electrophoresis. Analysis of the polyadenylated (and methylated) RNA produced in the presence of SAM showed discrete VSV mRNA species, while unmethylated mRNA, synthesized in the presence of SAH, electrophoresed as large, heterogeneous RNA species. Further analyses demonstrated that in the presence of SAH, very large heterogeneous poly (A) was present on the VSV mRNA species. These results implicate that a relationship does exist between the posttranscriptional events of methylation and polyadenylation, and that pertubation of methylation has disrupted regulation of polyadenylation. Further studies concerning the relationship between methylation, polyadenylation and cleavage of hnRNA will be of considerable interest with regard to posttranscriptional control of genetic expression.

Inhibition of mRNA Methylation In Vivo

Since the discovery of S-adenosylmethionine (Cantoni, 1952), a variety of SAM-dependent biological transmethylation reactions have been identified (cf. review: Borchardt, 1977). Most of these SAM-dependent methyltransferases are inhibited by the demethylated product S-adenosylhomocysteine (SAH), including catechol O-methyltransferase, phenolethanolamine N-methyltransferase, histamine N-methyltransferase, glycine N-methyltransferase, homocysteine S-methyltransferase, indoleethylamine N-methyltransferase and tRNA methyltransferases (Borchardt, 1977). Due to the biological importance of these SAM-dependent reactions, several laboratories have, in recent years, systematically synthesized and evaluated the inhibitory

properties of numerous SAH analogs in order to study SAM and SAH binding characteristics (Coward *et al.*, 1974; Borchardt, 1977; Pugh *et al.*, 1977). These studies have indicated that inhibitory activity requires the ribose and amino acid moieties remain virtually intact. Modifications of adenine, however, can result in potent inhibitory analogs if the planarity and aromaticity of the purine is retained (Coward, 1974; Borchardt, 1977).

The use of SAH analogs to inhibit mRNA methylation represented an alternative approach to the study of the function(s) of methyl groups. The generation of undermethylated eukaryotic mRNA was not feasible via *in vitro* RNA synthetic reactions, as has been described above for viral methylation studies. In addition, SAH cannot be used to inhibit methylation of cells in culture, since it is virtually impermeable to cellular membranes and is also a naturally-occurring metabolite subject to *in vivo* degradation. Our laboratory and others have thus initiated methylation studies which employ SAH analogs to inhibit eukaryotic mRNA methylation in cell cultures, in order to assess the functional nature of this posttranscriptional modification.

S-tubercidinylhomocysteine (STH), the 7-deaza analog of SAH, is structurally depicted in Figure 2. This analog exhibited equivalent or greater potency of inhibition than SAH when used to inhibit catechol *O*-methyltransferase, indoleethylamine *N*-methyltransferase, and tRNA methyltransferases *in vitro* (Coward *et al.*, 1974). Of greater significance was the demonstration of the *in vivo* inhibition by STH on tRNA methylation in phytohemagglutinin-stimulated lymphocytes (Chang and Coward, 1974) and on catecholamine methylation in neuroblastoma

Figure 2. The structure of S-tubercidenylhomocysteine, the 7-deaza analog of S-adenosylhomocysteine.

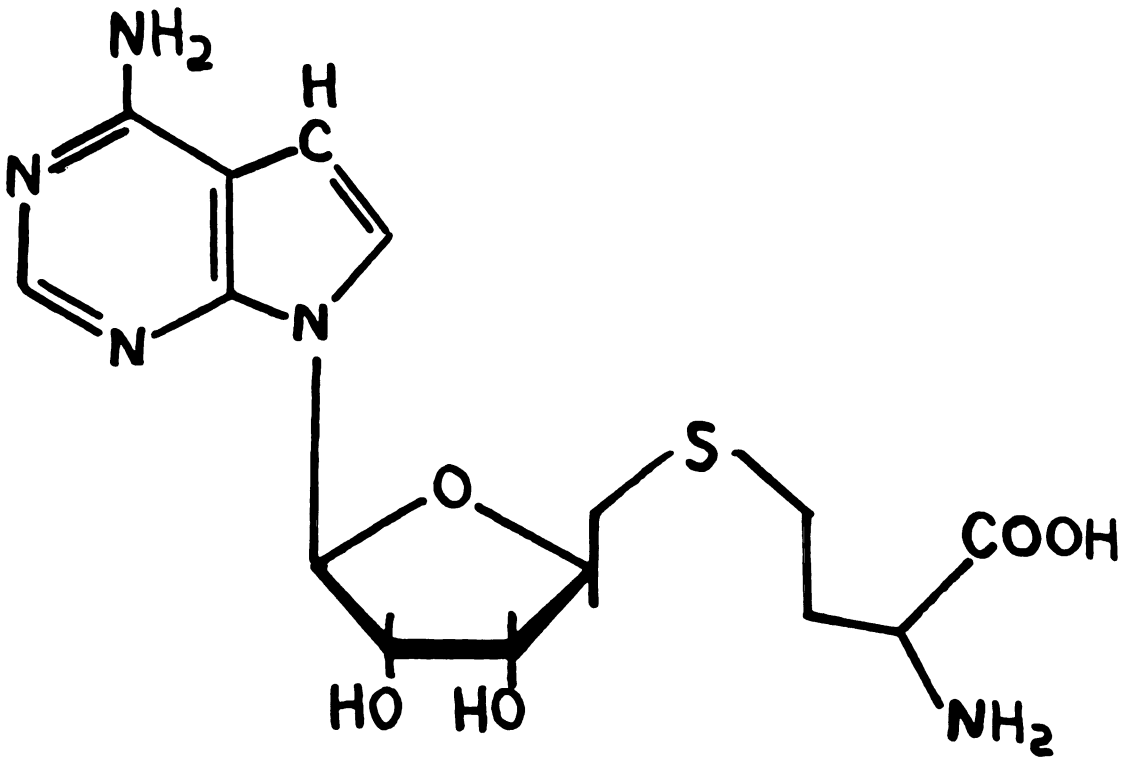

Figure 2

cells (Michelot, et al., 1977). The capability of STH to be inhibitory in vivo indicated both permeability and metabolic stability of the compound.

Our laboratory has employed STH as a means of perturbing Novikoff mRNA methylation. Preliminary studies suggested that the presence of STH in culture media was nontoxic to Novikoff cells, and that by 24 h after incubation with the drug, the cells had overcome the inhibitory effects of STH (M. Kaehler, J. Coward and F. Rottman, unpublished observations). At 250 μM, STH inhibited messenger RNA synthesis to 32% the level of control cells. Analysis of ^{3}H-methyl mRNA indicated that methylation at N',N" and internal base sites were affected by STH. Inhibition of 2'-O-methylation at N' positions generated cap zero structures (m^{7}GpppN') in both cytoplasmic and nuclear poly (A)-containing RNA, indicating that ribose methylation of the penultimate nucleoside is not necessary for processing and transport of Novikoff mRNA (Kaehler et al., 1977 (reproduced as part III of this thesis)). Subsequent location of cap zero-bearing mRNA molecules on both monosomes and polysomes of STH-inhibited Novikoff cells suggests that this 2'-O-methylation is not requisite for ribosomal binding or for translation (Kaehler, M., Coward, J. and Rottman, F., manuscript submitted and reproduced as Part IV of this dissertation). Non-poly (A)-containing RNA from normal and STH-treated cells was also analyzed and shown to contain virtually identical methylation patterns (M. Kaehler, J. Coward and F. Rottman, unpublished results). Analysis of tRNA methylation is in progress (P.Crooks, personal communication). Pugh et al. (1977) have recently found STH to be the most potent inhibitor of all SAH analogs tested

in vitro against Newcastle disease virus mRNA (guanine-7-)methyltransferase. Studies in our laboratory indicate that if 7-methylguanosine formation is blocked *in vivo* by STH, the resultant GpppN' structures are not stable (Kaehler, M., Coward, J. and Rottman, F., manuscript submitted).

Another SAH analog, S-isobutyladenosine (SIBA) has been reported by Jacquemont and Huppert (1977) to inhibit viral mRNA methylation *in vivo*. 1mM SIBA reversibly blocked Herpex simplex type 1 virus multiplication, viral protein synthesis, and viral mRNA methylation; the data is interpreted to establish a correlation of capping and methylation inhibition to decreased protein synthesis (Jacquemont and Huppert, 1977). It should be noted, however, that this interpretation does not explain the reversible inhibition of production observed for encephalomyocarditis virus, which does not contain capped mRNA (Jacquemont and Huppert, 1977). Cantoni (1977) has suggested that SIBA is an inhibitor of SAH hydrolase, rather than a competitive inhibitor for SAM binding sites. Robert-Gero *et al.* (1975) and Michelot *et al.* (1976) have demonstrated that SIBA, in contrast to both SAH and STH, is a very weak inhibitor of tRNA transmethylases *in vitro* in both normal and transformed cells.

Other compounds than SAH analogs have been shown to perturb mRNA methylation. Cycloleucine is an *in vitro* competitive inhibitor of methionine for SAM-synthetase (Lombardini, *et al.*,1970), and its mode of action *in vivo* presumably is to reduce intracellular concentrations of SAM (Caboche, 1977). Cycloleucine has been shown to quantitatively affect rRNA maturation processes (Caboche and

Bachellerie, 1977). More recently, Dimock and Stoltzfus (K. Dimock and C. Stoltzfus, personal communication) have demonstrated that the presence of 40 mM cycloleucine in low methionine medium results in greater than 90% inhibition of internal and N'methylations in avian sarcoma virus B-77. No inhibition of 7-methylation of guanine was detected (K. Dimock and C. Stoltzfus, personal communication). It should be noted that although similar inhibition patterns are observed for STH and cycloleucine, the two compounds are believed to function differently at the macromolecular level.

Glazer and Peale (1978) have recently reported that cordycepin (3'-deoxyadenosine) and xylosyladenine (9-β-D-xylofuranosyladenosine) inhibit methylation of nuclear RNA to a greater extent than RNA synthesis in L1210 cells. Cordycepin has been shown to be an effective inhibitor of nuclear RNA synthesis (Kann and Kohn, 1972; Darnell, et al., 1971), and xylosyladenine appears to effect similar results but with greater potency. Inhibition was also observed for both base-methylated mononucleotides and 2'-O-methylated dinucleotides, the latter presumably of rRNA origin. An oligonucleotide containing 7-methylguanosine and 2'-O-methylnucleotides was also present at decreased levels in the presence of either drug. These results are of considerable interest in light of the anticancer usage of cordycepin and xylosyladenine, but must be considered as preliminary since the entire nuclear RNA population was analyzed together (Glazer and Peale, 1978).

Kredich and Martin (1977) have recently studied adenosine-mediated toxicity in cultured mouse T lymphoma cells, and have concluded that SAH accumulation is the direct effect of adenosine

cytotoxicity. High levels of adenosine inhibit the enzyme SAH-hydrolase, and thus increase intracellular levels of SAH. In vivo DNA methylation was inhibited in correlation with increased SAH concentrations, suggesting the primary toxic effect is due to inhibition of SAM-mediated methyltransferases (Kredich and Martin, 1977). RNA methylation in human myeloma cells also appears to be inhibited by high adenosine concentrations (J.Bynum, personal communication). It would be of interest to determine what, if any, qualitative changes adenosine exposure might effect on mRNA methylation.

Finally, N^6-methyladenosine has been found to be a substrate for S-adenosylhomocysteine hydrolase both in vitro and in vivo, resulting in the synthesis of S-N^6-methyladenosylhomocysteine (Sm^6AH) (J. Hoffman, personal communication). This analog has been shown to be a potent inhibitor of both tRNA methyltransferases (Trewyn and Kerr, 1976) and mRNA (guanine-7-) methyltransferase (Pugh et al. 1977). Thus the potential usefulness of SAH analogs continues to expand.

Increased Resistance to Degradation

A straightforward yet nontrivial function of mRNA capping and methylation might be to provide additional resistance of the RNA molecules to ribonuclease attack. Jervis and DeBusk (1975) have shown that undermethylated tRNAs are more susceptible to nuclease attack than are normal tRNA samples. Methylation has been demonstrated to enhance the stability of both rRNA (Liau, et al., 1976) and synthetic ribopolymers (Stuart and Rottman, 1973).

III. An Argument for Control of Genetic Expression at the Posttranscriptional Level

The control of genetic expression is a major problem in cellular biology. The processes of development, tissue specialization, regeneration, and normal and abnormal cell growth must reflect those DNA sequences which are permitted to be expressed via eukaryotic mRNA species. Control mechanisms have been established at the transcriptional level, and reannealing experiments have indicated that most mRNA sequences are transcribed from unique DNA genes (cf. reviews: Lewin, 1975a,b). However, the sequence complexity of hnRNA, the primary transcript of DNA, is several fold higher than the complexity of cytoplasmic mRNA, suggesting a second level of control. This brief (and certainly incomplete) review will delineate evidence for posttranscriptional control of genetic expression.

One of the most dramatic demonstrations implicating posttranscriptional control of gene expression involved the characterization of two clones of the Friend cell line. The Friend erythroleukaemic cell line was isolated in 1966 (Friend, et al., 1966), and the addition of dimethylsulfoxide (DMSO) to cultures of virus-transformed Friend cells has been shown to induce erythroid differentiation and hemoglobin accumulation (Friend, et al., 1974). Paul and colleagues (Gilmore, et al., 1974; Harrison, et al., 1974) analyzed the basal and induced levels of globin cytoplasmic and nuclear globin-specific RNA sequences in two clones: M2 and 707. In clone M2, the globin mRNA on polysomes increased 50 to 100 times during induction, whereas the hnRNA sequences complimentary to globin mRNA increased only 5- to 6-fold (Gilmore,

et al., 1974). In addition, no detectable globin mRNA was synthesized from chromatin templates of uninduced M2 cells, but significant levels of globin mRNA were synthesized from induced cell chromatin. These data pointed to primary control of gene expression at the transcriptional level, with some secondary control mechanisms functioning posttranscriptionally. In contrast, although clone 707 cells were inducible by DMSO exposure, and induced globin mRNA levels in the cytoplasm increased to levels comparable to that of induced M2 clones, the basal level of cytoplasmic globin mRNA in uninduced 707 cells was much higher than that observed in M2 cells. No differences were observed in the induced and uninduced 707 cells for globin-specific nuclear RNA sequences or the capacity of the chromatin template to synthesize globin sequences. These results suggested that transcriptional control in clone 707 has been relaxed, and posttranscriptional mechanisms were responsible for the observed induction (Harrison, et al., 1974).

In subsequent studies, annealing experiments were performed to determine the complexity of Friend cell hnRNA and mRNA. At least five times more unique DNA gene sequences were represented in nuclear poly (A)-containing RNA than in the polysomal poly (A)-containing RNAs. Furthermore, some gene transcripts were enriched in the polysomal RNA relative to its concentration in hnRNA (Birnie, et al., 1974; Getz, et al., 1975). These results suggested that posttranscriptional mechanisms alter the relative concentration of some gene transcripts between nucleus and cytoplasm. These results have been verified by Kleiman, et al. (1977) using more sensitive hybridization

techniques. Similar conclusions, i.e. that there is a considerably greater number of unique DNA sequences represented in hnRNA than in mRNA, have been made for sea urchin embryos (Smith, et al., 1974), Xenopus liver cells (Ryffel, 1976), HeLa cells (Herman, et al., 1976), and mouse brain (Bantle and Hahn, 1976).

Evidence is also accumulating which indicates that cell phenotype may be determined more by the relative abundance of mRNA species on polysomes than by the absence or presence of specific RNA sequences. A high level of homology between the poly (A)-containing polysomal RNA of mouse brain, embryo, and liver tissue was demonstrated by Young, et al. (1976). Getz, et al. (1976) has studied the relationship between cell proliferation and the amount and diversity of mRNA sequences in mouse embryo cells. Within limits of detection, all species of poly (A)-containing mRNA present in growing cells are also present in resting cells. Humphries, et al. (1976) detected mouse globin RNA sequences present in hnRNA from nonerythroid tissues (including adult brain and liver, and lymophoma, untransformed, and transformed fiborblast cell cultures) as well as in erythroid tissues (reticulocytes and fetal liver). The proportion of the globin RNA sequences in hnRNA containing poly (A) was similar for all species, but in erythroid cells the cytoplasm contained a much greater percentage of the total globin sequences (Humphries, et al., 1976). The association of poly (A) with nuclear globin sequences indicated that poly (A) does not play a major role in selecting sequences for transport into the cytoplasm. This is consistent with the demonstration by Perry, et al. (1974) that nuclear poly (A) is not quantitatively converted to cytoplasmic poly (A), even

though these investigators have demonstrated the potential exists for most nuclear hnRNA molecules to function as mRNA precursors (Hames and Perry, 1977).

A second perspective which may be of importance then with regard to the presence of posttranscriptional control mechanisms is the rate of selection, processing, and transport of mRNA molecules into the cytoplasm. Studies which compare resting and induced fibroblasts (3T6 cells) are useful for this hypothesis. As summarized by Green (1974), a characteristic of growing fibroblasts and other cell types is a high messenger RNA to ribosome ratio. The increased mRNA levels in the cytoplasm has not been correlated with stabilization of the RNA, since the halflife of mRNA molecules is not significantly altered by induction (Abelson, et al., 1974). Nor can the higher mRNA levels be attributed to increased transcription rates (Mauch and Green, 1973), since the rate of transcription of hnRNA per unit of DNA does not change during induction or if DNA synthesis is blocked. However, the increased mRNA levels in the cytoplasm of serum-stimulated fibroblasts has been correlated with an increased rate of processing (Johnson, et al., 1974, 1975). These data taken together are consistently supportive of posttranscriptional control of gene expression, both with respect to qualitative and quantitative perspectives. It should be emphasized, however, that transcriptional control of genetic expression is certainly not excluded or minimized by the additional control mechanisms which may function posttranscriptionally. In this regard, Williams and Penman (1975) reported that approximately 3% of the mRNA in resting 3T6 cells will not crosshydridize with cDNA of mRNA from growing cells. Similar results were obtained

by crosshybridization of growing cell mRNA to cDNA from restng cell mRNA.

Egyhazi (1976) has quantitatively studied the intranuclear metabolism and transport of hnRNA transcribed in the Balbiani rings of Chironomus tentans salivary gland cells. Of the total amount of the 75S RNA synthesized at the ring loci, only 14-17% can be recovered in the nuclear sap whereas 4-7% is present in the cytoplasm. The remainder is presumably degraded, and experiments using inhibitors of transcription indicate very little of the 75S RNA can be chased into the cytoplasm. Since no size reduction of the 75S RNA occurs prior to export of this RNA molecule, the nucleotide sequences of 75S hnRNA and 75S mRNA are presumably the same. These results demonstrate for the first time that a specific, protein-coding sequence in hnRNA is degraded within the nucleus, and implicate posttranscriptional regulatory mechanisms. This system provides a unique opportunity for studying what role, if any, methylation and/or polyadenylation plays in determining the metabolic fate of the three pools of 75S RNA.

Regulation of virus gene expression at transcriptional and posttranscriptional levels has been demonstrated as well. The appearance and quantity of 47 viral RNA species and 35 viral proteins present in frog virus 3 was monitored by Willis, et al. (1977) as a function of time post-infection. Although proteins could be classified as early, intermediate or late polypeptides, viral mRNAs could not be classified according to time of maximum synthesis. Once the RNA synthesis began, most RNA species continued to be made at the same or elevated rates, implying that posttranscriptional

mechanisms controlled the shut-off of early and intermediate protein synthesis.

The realization that more than 90% of those RNA sequences transcribed in the nucleus are not permitted to enter the cytoplasm for ultimate gene expression has been historically puzzling. The inefficiency of such a method seemed incredible in view of the efficiency of the enzymatic metabolism and catabolism occurring elsewhere within cells. Relatively recent investigations such as those described above, however, make such a "wasteful" synthetic process plausible. A rationale for the necessity of posttranscriptional as well as transcriptional regulation of gene expression is presented below.

A Speculative "Control Hierarchy".

The eukaryotic genome contains a high complexity of gene sequences which must be conserved in each and every cell of the organism. At any given time in the life of the cell, a very small percentage of these genes are required for normal cell function. Both gross and subtle alterations in the cellular environment, however, require different combinations of gene products for adequate cellular response. The overall "off-on" control of genes occurs at the transcriptional level. The sensitivity of these control mechanisms is determined largely by the developmental or specialized state of the cell, and by gross environmental changes. Its response occurs throughout the genome.

Transcriptional control mechanisms may be envisioned to grossly monitor "sets" of genes which, due to the very bulk of the genome, include several gene sequences which are either undesirable or unnecessary in a given cell state. However, all genes of the set

must be transcribed in order to express those sequences which are needed. Thus posttranscriptional control mechanisms have evolved to provide finer, more sensitive regulation of those sequences which enter the cytoplasm. The events of processing - methylation at internal sites, capping and methylation of 5'-termini, polyadenylation of 3'-termini, cleavage of intragenic sequences, and other unidentified modifications of nuclear RNA - provide a molecule-by-molecule screening process of transcribed gene sequences. Such screening control may be sequential in nature, or the selection responsibility at a given time may rest with one specific processing event, which in turn is determined by the transcriptional regulation in effect at the time.

The appearance of wasteful and inefficient RNA transcription by eukaryotic cells may actually reflect an extremely complicated and sophisticated set of control mechanisms which permit both retention of the entire genomic information in the nucleus and extreme sensitivity for response to the cells needs. The adaptability and extent of control afforded by posttranscriptional regulation of gene expression makes such systems very attractive.

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PART II

THE KINETICS OF NOVIKOFF CYTOPLASMIC MESSENGER RNA METHYLATION

ABSTRACT

Methylation patterns of Novikoff cytoplasmic mRNA were determined as a function of labeling time with L-[methyl-^{3}H]methionine. The 5'-terminal $m^{7}G$ could be released from whole mRNA by treatment with nucleotide pyrophosphatase. Subsequent alkaline phosphatase treatment of this mRNA followed by KOH digestion yielded N'mpNp and N'mpN"mpNp from cap 1 ($m^{7}GpppN'mpN$) and cap 2 ($m^{7}GpppN'mpN"mpN$) respectively.

Our results indicate that the relative amounts of labeled cap structures do change with time and that the amount of internal N^{6}-methyladenosine decreases, relative to 5'-cap structures, as the cytoplasmic mRNAs age and the average size decreases. The formation of cap 2 structures by the addition of the second 2'-O-methyl group at position N"m appears to be a cytoplasmic event. Thus, after very short labeling times greater than 80% of the labeled methyl groups in cap 2 are found in this position. These results, along with earlier data obtained on L-cell heterogeneous nuclear RNA methylation, are consistent with a model in which the nucleus is the cellular site of three mRNA methylation events producing 5'-terminal $m^{7}G$, the first 2'-O-methylnucleoside (N'm) found in cap 1 structures and internal N^{6}-methyladenosine. Subsequently these nuclear methylations are followed by the cytoplasmic methylation at N"m.

Analysis of the methylnucleoside composition of cap 1 structures, along with comparable "core" structures ($m^{7}GpppN'm$) generated from cap 2 by removal of N"m, indicates that at any single labeling time the methylnucleoside composition of a given cap 1 and the cap 2

"core" structure is remarkably similar. On the other hand, comparisons of the methylnucleoside composition of the cap structures at different labeling times indicate an increase in Cm in the first 2'-O-methyl-nucleoside (N'm) with time.

INTRODUCTION

There is an increasing body of evidence to suggest that the majority of eukaryotic mRNA molecules from both cells (Rottman et al., 1974; Adams and Corey, 1975; Desrosiers et al., 1975; Furuichi et al., 1975a; Perry et al., 1975a; Wei et al., 1975) and viruses (Furuichi and Miura, 1975; Furuichi et al., 1975b; Furuichi et al., 1975c; Keith and Fraenkel-Conrat, 1975; Moyer et al., 1975; Wei and Moss, 1975) contain a unique methylated cap structure, m^7GpppN'mp(N"mp)Np on their 5'-termini. Furthermore, recent reports indicate that similar structures are also present on hnRNA (Perry et al., 1975b; Salditt-Georgieff et al., 1976), a class of molecules that contains mRNA precursors. In addition to a 5'-terminal cap structure, both cytoplasmic mRNA and hnRNA also contain N^6-methyl-adenylic acid located internally between the cap and 3'-poly(A) segment. Although this mononucleotide is apparently absent in many viral RNAs (Furuichi and Miura, 1975; Moyer et al., 1975; Wei and Moss, 1975), globin mRNA (Perry and Scherrer, 1975) and yeast mRNA (Sripati et al., 1976; Dekloet and Andrean, 1976), it can account for nearly 50% of the labeled methylnucleotides in cellular mRNA (Desrosiers et al., 1974).

In a recent characterization of Novikoff cytoplasmic mRNA we noted the presence of two types of cap structures: m^7GpppN'mpNp (cap 1) and m^7GpppN'mpN"mpNp (cap 2) (Desrosiers et al., 1975).

Similar results have been reported for mouse myeloma cells (Adams and Cory, 1975), L-cells (Perry et al., 1975a) and HeLa cells (Wei et al., 1975; Furuichi et al., 1975a). In an earlier publication (Desrosiers et al., 1975) it was suggested that the two cap structures might in fact represent separate classes of mRNA with different degrees of cytoplasmic stability. Previous studies on cap structures in cytoplasmic mRNA have utilized cells labeled for a single time period. Therefore, it was reasoned that the relative abundance of cap 1 and cap 2 structures might depend upon the length of labeling time, possibly reflecting different kinetics of labeling or turnover. The presence of such time-dependent changes might, however, also reflect other factors such as additional cytoplasmic methylation of partially methylated precursors.

To study the possibility that differential cytoplasmic mRNA stability affects the distribution of cap structures, and at the same time to permit continual cytoplasmic methylation to occur, we employed continuous labeling of Novikoff cells with L-[methyl-^{3}H]methionine for periods up to 24 h. The methylation patterns of mRNA were then analyzed as a function of time.

MATERIALS AND METHODS

Cell Culture and Labeling Conditions

Novikoff hepatoma cells (N1S1 strain) were grown in Swim's S-77 medium (GIBCO) containing 10% calf serum essentially as described (Desrosiers et al., 1974). For labeling with L-[methyl-^{3}H]methionine (Amersham/Searle, 5 Ci/mole), cells in midlogarithmic growth phase were harvested aseptically and resuspended in fresh warm medium

at a concentration of approximately 7.5 x 10^5 cells/ml. Labeling for the time course study was performed in the presence of 20 mM sodium formate and 40 μM each adenosine and guanosine to supress nonmethyl purine ring labeling; normal methionine levels were present for all labeling times except for 20 min, in which medium without methionine was used.

Labeling conditions for the distributional analysis experiments were altered to increase the specific activity of the methylated nucleotides in mRNA. Adenosine, guanosine and formate were omitted and the 5 and 24 h samples were labeled in 50 μM methionine, one half the normal concentration. Under these conditions there was no discernable change either in cell doubling time or in cell appearance when examined by phase microscopy.

In most experiments 15-20 μCi/ml of L-[methyl-^{3}H]methionine was used. At each time point an aliquot of the cells was harvested aseptically and the radioactive medium returned to the growing culture. Final cell concentrations at the time of harvest never exceeded 1.3 x 10^6/ml.

Isolation and Characterization of Poly (A)-Containing Cytoplasmic mRNA

Total cytoplasmic RNA was isolated as previously described (Desrosiers et al., 1974). Poly (A)-containing mRNA was isolated by oligo (dT)-cellulose chromatography, including a heat step prior to a second passage over the column (Desrosiers et al., 1975). This step was necessary to eliminate traces of rRNA that otherwise interfere with methylation analysis.

Sedimentation analysis of poly (A)-containing mRNA was performed using 4.8 ml gradients of 5-20% sucrose in 99% Me_2SO, 10 mM LiCl, 1 mM EDTA. The mRNA was made 91% Me_2SO, 10 mM LiCl, 1 mM EDTA in a total volume of 100 µl and heated at 60°C for 2 min prior to layering onto the gradient. Centrifugation was for 14.5 h at 25°C and 45,000 rpm in a Beckman SW 50.1 rotor.

Nucleotide Pyrophosphatase Treatment of Whole mRNA

Poly (A)-containing cytoplasmic mRNA, essentially free of tRNA and rRNA contamination, was digested with nucleotide pyrophosphatase from Crotalus atrox (Sigma). A 200 µl reaction contained 0.25 units enzyme, 9 A_{260} units RNA, 20 µmoles Tris HCl, pH 7.8 and 0.2 µmoles of magnesium acetate. After incubation at 37°C for 35 min the reaction was stopped by heating in a boiling water bath for 5 min. The high level of carrier RNA was added to suppress nonspecific diesterase activity which contaminates this enzyme. The RNA was separated from the released pm^7G by chromatography on Biogel P2 (100-200 mesh; 1.5 x 22 cm column) and treated with 0.25 units bacterial alkaline phosphatase (PL Biochemicals, electrophoretically pure) in 0.05 M Tris HCl, pH 7.8, 0.001 M magnesium acetate for 45 min at 37°C to remove the newly exposed 5'-terminal phosphates. The dephosphorylated RNA was then digested for 18 h at 37°C with 0.4 N KOH to obtain N'mpNp from cap 1 and N'mpN"mpNp from cap 2 structures. These oligonucleotides were resolved from each other and m^6Ap by chromatography on Pellionex WAX in the presence of 7 M urea (Desrosiers et al., 1975).

Preparation of mRNA for Methyl Nucleoside Distributional Analysis

Internal methylnucleosides and intact 5'-terminal caps were produced by enzymatic digestion of poly (A)-containing cytoplasmic mRNA with RNase T2 (Sigma) at 2 units/A_{260} unit of RNA in 0.9 M NaCl, 0.15 M sodium acetate pH 4.5, 0.01 M EDTA, for 2 h at 37°C. The reaction mixture was then adjusted to pH 8 with 1 M NaOH and made 0.017 M in magnesium acetate. Alkaline phosphatase that had been dialyzed against 0.05 M NH_4HCO_3 was added (0.25 units/A_{260} unit of RNA) and the reaction continued for 30 min at 37°C. The products of this reaction were resolved on DEAE-Sephadex (7 M urea). Intact cap structures to be used for subsequent analysis were desalted on Biogel P-2 (100-200 mesh). Nucleosides were adsorbed to charcoal and eluted with 20% pyridine.

Cap 2 structures (m^7GpppN'mpN"mpN) were purified on Pellionex-WAX to remove remaining traces of urea which were found to inhibit subsequent digestion with nuclease P1. Cap 2 oligonucleotides contained in a volume of 500 µl were applied to Pellionex-WAX (1/7" x 30 cm) and urea was removed by eluting with 10 ml 0.1 M ammonium acetate. The buffer was changed to 6 M ammonium acetate and the cap structure eluted in 2 ml total volume. Ammonium acetate was removed by lyophilization. Cap 2 structures were digested with 160 µg/ml nuclease P 1 (Yamasa Shoyl Co., Ltd.) in 0.01 M sodium acetate, pH 6.1. After 45 min at 37°C the sample was made 0.05 M Tris HC1, pH 7.8, 0.001 M in magnesium acetate and 0.3 units alkaline phosphatase/100 µl were added. Incubation was continued for 30 min at 37°C. The reaction mixture was diluted to 500 µl with water and reapplied to the Pellionex-WAX column described above. N"m

was eluted with 0.1 M ammonium acetate (2 ml total volume) and "core" oligonucleotide, $m^7GpppN'm$, eluted with 6 M ammonium acetate.

Both cap structures and "core" oligonucleotides generated from cap 2 were completely digested to nucleosides by incubation for 45 min at 37°C with 0.25 units nucleotide pyrophosphatase and 0.4 units alkaline phosphatase in 100 µl reactions containing 0.1 M Tris HCl, pH 7.8, 0.1 mM magnesium acetate.

Acid Hydrolysis

Acid hydrolysis of whole mRNA, 5'-terminal oligonucleotides and mononucleotides can be used to cleave the N-glycosidic bond of purine-containing nucleotides, thereby releasing free purine bases. Generally 1.5 A_{260} unit of RNA was dissolved in 0.5 ml concentrated formic acid, the tube sealed and the hydrolysis carried out at 100°C for 2 h, similar to the procedure of Munns et al.,(1974). The released bases were resolved by high speed liquid chromatography (HSLC) on Aminex A-5 (Desrosiers et al., 1975). [^{14}C]Adenosine was added as an internal standard to the [^{3}H]-labeled RNA before hydrolysis to permit determination of $^{14}C/^3H$ ratios after digestion and thus provide a measure of the methanol lost from 2'-O-methyl groups in the presence of strong acid.

RESULTS

Cap 1 and cap 2 structures differ from each other by containing one and two 2'-O-methylnucleosides respectively. These structures can be resolved in the presence of 7 M urea on DEAE-Sephadex columns or on Pellionex-WAX. Frequently the resolution obtained on intact whole cap structures on Pellionex-WAX is not satisfactory, even

in the presence of 7 M urea which is added to suppress base composition effects. Removal of terminal m^7G with nucleotide pyrophosphatase followed by treatment with bacterial alkaline phosphatase leaves the mRNA with a 5'-terminal end of N'mpNp... or N'mpN"mpNp....., corresponding to cap 1 and cap 2 structures, respectively. Subsequent alkaline hydrolysis of the remaining portion of mRNA produces N'mpNp and N'mpN"mpNp, which are easily and quickly separated by HSLC on Pellionex-WAX.

Novikoff mRNA was labeled for varying times with L-[methyl-^{3}H]-methionine and rigorously purified to eliminate rRNA as described in MATERIALS AND METHODS. Whole mRNA treated with nucleotide pyrophosphatase yielded a mononucleotide which was hydrolyzed with formic acid and the hydrolysate chromatographed on Aminex A-5. Greater than 85% of the released material eluted as 7-methylguanine (data not shown). The mRNA remaining after nucleotide pyrophosphatase treatment was further hydrolyzed with alkaline phosphatase and KOH. The internal base-methylated mononucleotide m^6Ap and the 5'-terminal oligonucleotides N'mpNp and N'mpN"mpNp were separated on Pellionex-WAX (Figure 1).

One of the main objectives of these studies was to examine the relative distribution of cap 1 and cap 2 structures as a function of time of continuous labeling with L-[methyl-^{3}H]methionine. A pronounced change in labeling of these cap structures was observed with different labeling times (Figure 1). After a short exposure of only 20 minutes, most of the label in cytoplasmic mRNA was contained in cap 2 while further labeling yielded an increase in cap 1. Similar determinations were made at later time points and the ratios of

Figure 1. HSLC resolution on Pellionex-WAX of KOH digestion products from mRNA which had previously been treated with nucleotide pyrophosphatase and alkaline phosphatase. A 1/8" x 40 cm column was developed at room temperature with a 100 ml gradient of 0-0.2 M $(NH_4)_2SO_4$ in 7 M urea, 0.005 sodium phosphate, pH 7.7 at a flow rate of 25 ml/h. The position of the oligo(Up) standards added as UV markers and carrier rRNA digestion products are shown. Poly (A)-containing RNA was isolated at the times indicated.

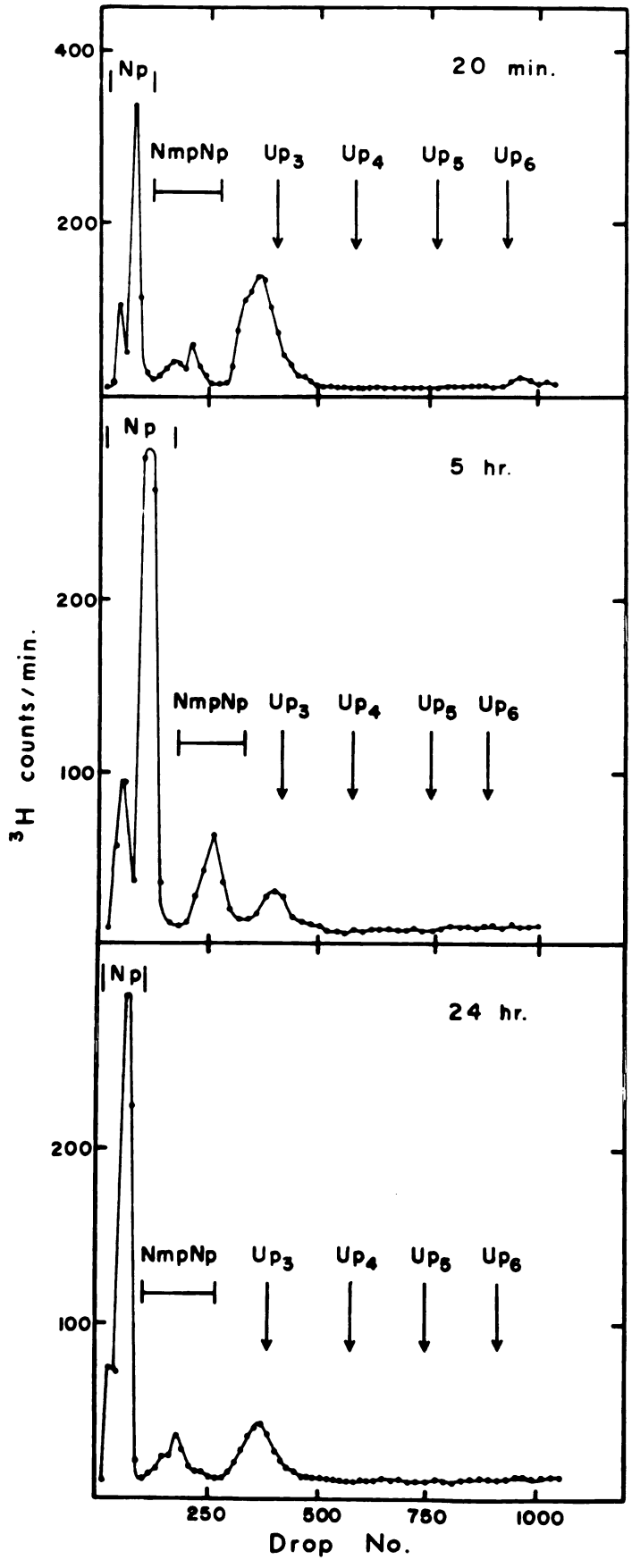
400
200
Np
20 min.
NmpNp
Up3
Up4
Up5
Up6
200
100
Np
5 hr.
NmpNp
Up3
Up4
Up5
Up6
3H counts/min.
200
100
Np
24 hr.
NmpNp
Up3
Up4
Up5
Up6
250
500
750
1000
Drop No.

Figure 1

radioactivity between cap 1 and cap 2 were observed to change (Figure 2).

To further identify and study the methylnucleoside distribution in internal and cap positions in poly (A)-containing mRNA, three periods of labeling were chosen: 20 min, 5 h and 24 h. Although Pellionex-WAX efficiently resolves N'mpNp and N'mpN"mpNp derived from cap 1 and cap 2, respectively, DEAE-Sephadex (7 M urea) is better suited for obtaining intact cap structures. Whole mRNA was digested with RNase T2 and alkaline phosphatase to produce nucleosides from internal base methylations plus the 5'-terminal caps 1 and 2. These products were resolved on DEAE-Sephadex developed in the presence of 7 M urea (Figure 3). It is again apparent that the distribution of ^{3}H-radioactivity between nucleoside, cap 1 and cap 2 varied significantly with time of labeling.

The nucleosides recovered from urea buffer by charcoal adsorption were analyzed on Aminex A-5 under conditions that resolve base-modified nucleosides (Desrosiers et al., 1974). N6-Methyladenosine comprised greater than 95% of the base methylated nucleosides at all times of labeling, but small amounts of radioactivity eluting with 5-methylcytidine were observed at later times (data not shown).

To correlate the number of internal methylations per messenger RNA molecule with labeling time, the amount of internal m^6A was compared to the amount of m^7G in whole poly (A)-containing mRNA which had been labeled for the times indicated (Table I). The amount of m^7G in mRNA should reflect the absolute number of modified 5'-termini without being complicated by the number of ribose methylations in each message (i.e., cap 1 vs. cap 2).

Figure 2. Change in ratio of N'mpNp to N'mpN"mNp in mRNA with time. The data was obtained from mRNA that had been labeled with L-[methyl-^{3}H]methionine for the times indicated and treated as described in the legend to Figure 1. Dinucleotide contamination from rRNA was determined by KOH hydrolysis of intact mRNA and chromatography on Pellionex-WAX as in Figure 1. Appropriate corrections were made. Ratio of radioactivity in N'mpN"mpNp, eluting with the $(Up)_3$ marker, vs N'mpNp was calculated.

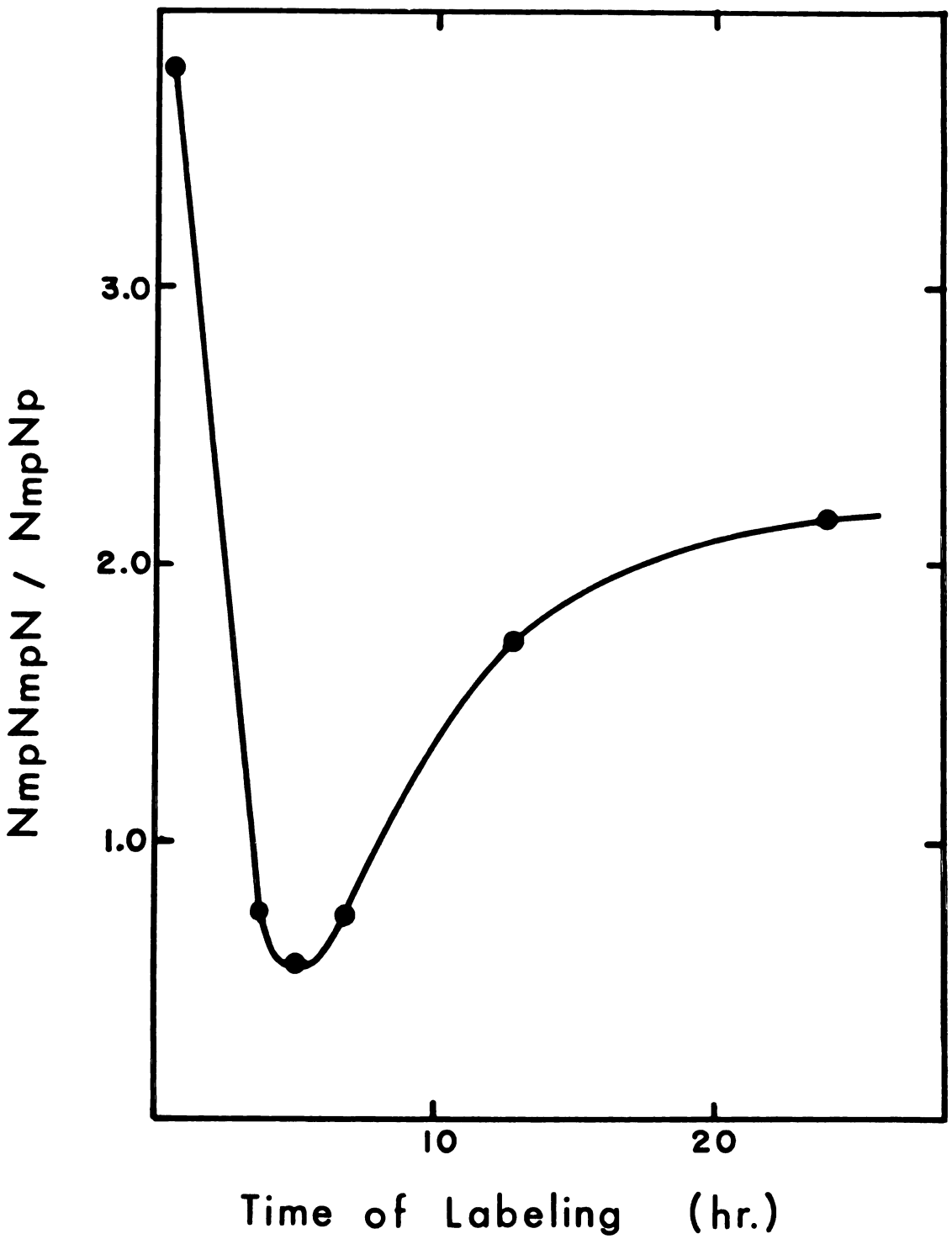

Figure 2

Figure 3. DEAE-Sephadex column separation of RNase T2 and alkaline phosphatase digestion products from whole mRNA. Poly (A)-containing mRNA which had been incubated with L-[methyl-^{3}H]-methionine for various time periods was digested with RNase T2 and alkaline phosphatase as described in MATERIALS AND METHODS. Following digestion the reaction mixture was diluted with 9 volumes of 7 M urea, 0.02 M Tris HCl, pH 7.4 and applied to a 0.9 x 25 cm DEAE-Sephadex column. The mononucleosides were eluted with 0.1 M NaCl, 7 M urea, 0.02 M Tris HCl, pH 7.4. To resolve oligonucleotides a 200-ml gradient of 0.1 M to 0.4 M NaCl in 0.02 M Tris HCl, pH 7.4, 7 M urea was used at a flow rate of 12 ml/h; 2 ml fractions were collected. Standard oligonucleotides $(pUm)_3$ and $(pUm)_4$ were included as markers to indicate approximate charge.

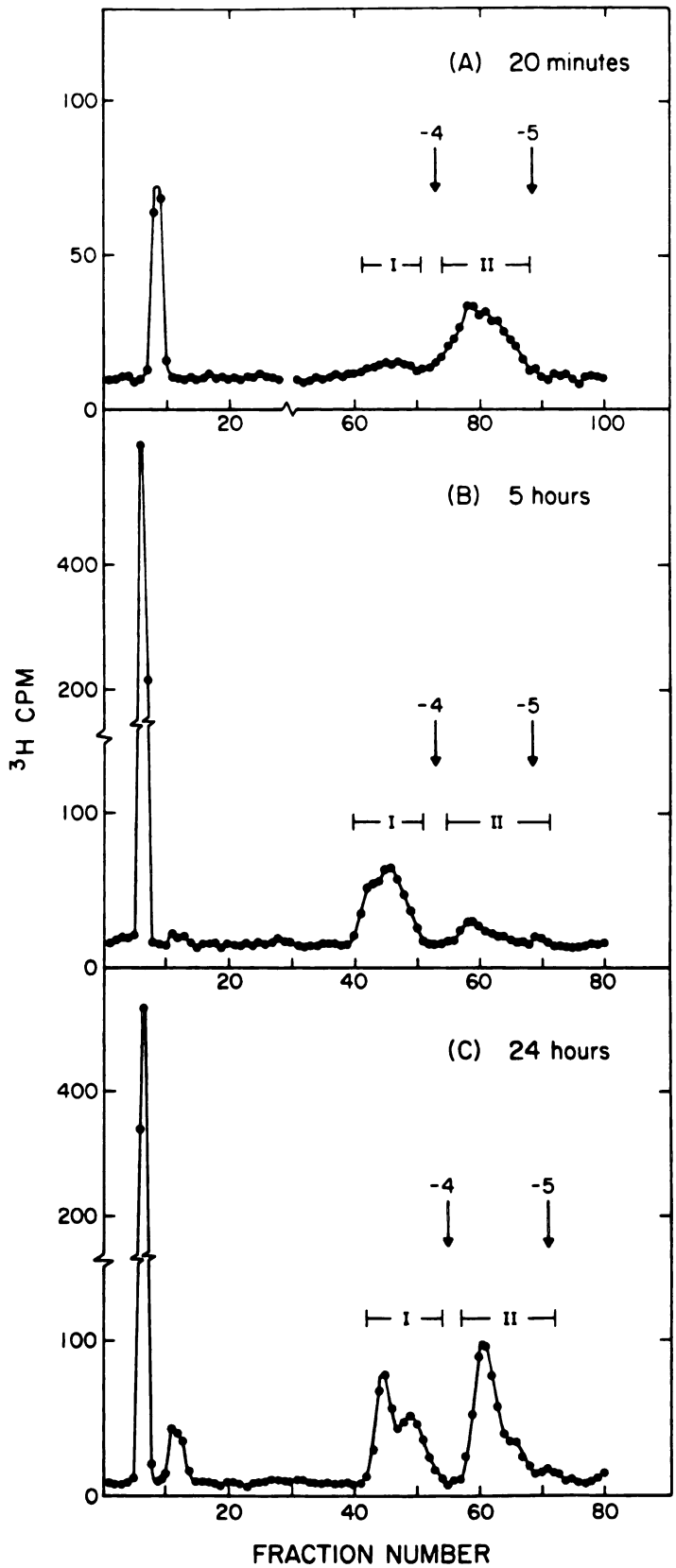
(A) 20 minutes
-4
-5
I
II
100
50
0
20
60
80
100
(B) 5 hours
400
200
100
0
20
40
60
80
(C) 24 hours
400
200
100
0
20
40
60
80
^{3}H CPM
FRACTION NUMBER

Figure 3

Table I

Amount of Internal m^6A Per Average mRNA: Variation with Time

	% of Label in mRNA as:		
	m^7G[a]	m^6A[b]	Ratio m^6A/m^7G
20 min	5.5	28	5.1
5 h	17	58	3.4
13 h	19	53	2.8
24 h	27	35	1.3

[a]Determinations were made by acid hydrolysis of whole mRNA and analyzed on Aminex A5 HSLC.

[b]Determination of m^6A was the amount of mononucleotide from the DEAE-Sephadex (7 M urea) column of T2 and alkaline phosphatase digest (cf. Figure 3) corrected for ring labeling when necessary.

As indicated in Table I the ratio of internal m^6A to terminal m^7G decreases with time, indicating that the average number of internal methylations in poly (A)-containing mRNA is reduced in longer labeling periods.

Since other reports have shown that larger hnRNA (Salditt-Georgieff et al., 1976) and mRNA (Perry and Kelley, 1976) have a higher average number of internal m^6A residues, it was of interest to examine the size of [^{3}H-methyl]-labeled mRNA as a function of time. Poly (A)-containing mRNA from three labeling periods was analyzed by Me_2SO-sucrose gradient centrifugation (Figure 4). Messenger RNA labeled for shorter times was substantially larger in size than mRNA labeled for longer periods, i.e., with increased labeling time the per cent of the molecules sedimenting at less than 18S changes from 32% to 54%.

Since there is as time dependent change in m^6A content, size of message, and cap 1 to cap 2 ratio, the methylnucleoside distribution within the caps was also studied. Cap 1 and cap 2 structures produced by RNase T2 and alkaline phosphatase were resolved on DEAE-Sephadex (7 M urea) (Figure 3) and desalted on Biogel as described in MATERIALS AND METHODS.

Cap 1 structures were digested with a mixture of nucleotide pyrophosphatase and alkaline phosphatase to produce 2'-O-methyl-nucleosides and m^7G. The separation of methylnucleosides found in cap 1 structures is presented in Figure 5. Only results from mRNA obtained at 5 and 24 h are included since the amount of radio-activity in cap 1 at 20 min is too small to analyze (Figure 3). Two important aspects of this data should be mentioned: first, the major change in methylnucleoside composition as a function of time

Figure 4. Me_2SO-sucrose gradient profiles of poly (A)-containing cytoplasmic RNA. Preparation of mRNA and gradient centrifugation was as described in MATERIALS AND METHODS. The percentage of the total radioactivity present in fractions smaller than 18S, between 18S and 28S, and greater than 28S was calculated for each RNA sample. Messenger RNA was isolated after labeling with L-[methyl-^3H]methionine for A) 20 min B) 5 h and C) 24 h, as described in MATERIALS AND METHODS.

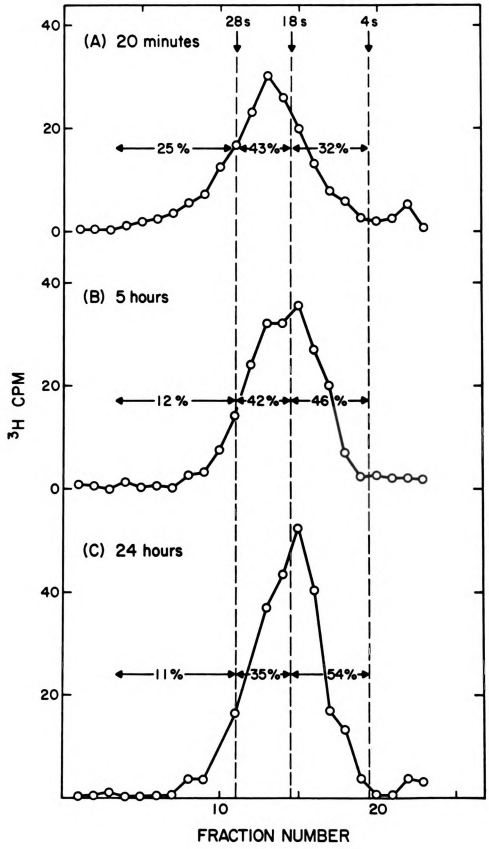
28s
18s
4s
(A) 20 minutes
25%
43%
32%
(B) 5 hours
12%
42%
46%
(C) 24 hours
11%
35%
54%
40
20
0
^{3}H CPM
10
20
FRACTION NUMBER

Figure 4

Figure 5. The distribution of methylnucleosides in cap 1 structures. Cap 1 structures produced by RNase T2 and alkaline phosphatase treatment were eluted from a DEAE-Sephadex (7 M urea) column in a volume of 10-20 ml and desalted on a 1.9 x 42 cm Biogel P2 column by elution with 0.02 M NH_4HCO_3. Material in the void volume was made 20% with ethanol and evaporated. Cap 1 structures were then digested with nucleotide pyrophosphatase and alkaline phosphatase as described in MATERIALS AND METHODS. The reaction mixture was dried with N_2 and dissolved in 125 µl column buffer. HSLC on Aminex A-5 (1/8" x 90 cm) was in 0.4 M ammonium formate, pH 4.25, 40% ethylene glycol at 40°C. Flow rate was 7 ml/h (4750 psi) for remainder of the run. Fraction size was 10 drop (0.4 ml) until Cm was eluted; fraction size was then doubled. A) Cap 1 from mRNA labeled for 5 h. Inset is the acid hydrolysis of the same 5 h cap 1 structure analyzed on Aminex A-5 in 0.4 M ammonium formate at pH 5.3. B) Cap 1 from mRNA labeled for 24 h. 2'-O-methyl-nucleosides and m^7guanosine were added as markers and detected at 260 nm.

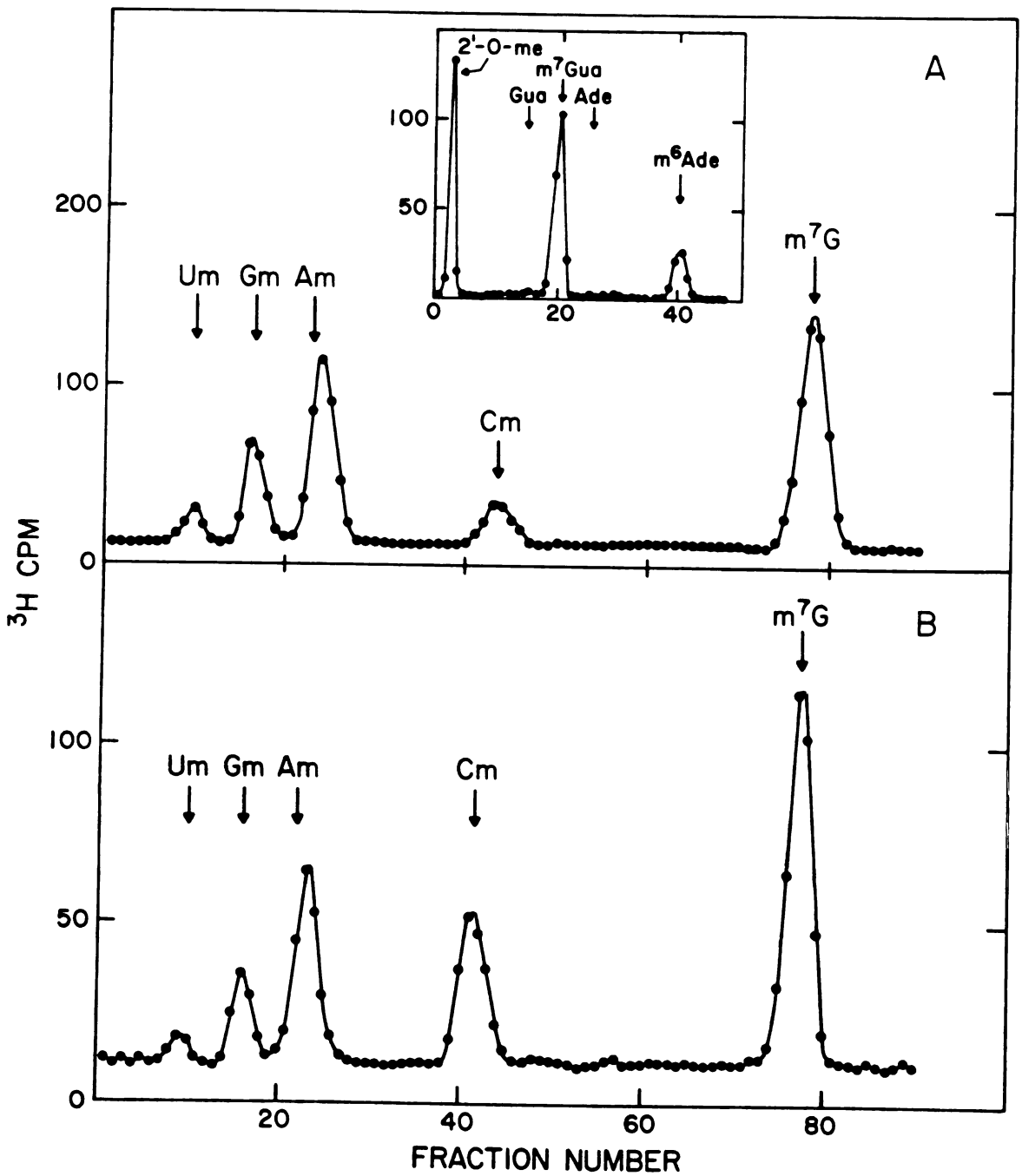
2'-O-me
m7Gua
Gua
Ade
m6Ade
A
B
Um Gm Am
Cm
m7G
3H CPM
FRACTION NUMBER
0
50
100
200
20
40
60
80

Figure 5

is the increase in Cm content and second, it appears that most of the Am is present as a doubly-methylated derivative, N^6, 2'-O-dimethyl-adenosine (m^6Am). Verification of the m^6A content in this dimethylated nucleoside was obtained by subjecting an aliquot of isolated cap 1 to acid hydrolysis and subsequently analyzing the free bases produced, as described earlier. After 5 h labeling, 50% of the label in m^6Am was converted to m^6Ade (cf. Figure 5, inset). Also the percentage of label in m^7Gua equals the percentage of label in 2'-O-methyl products. Similar results were obtained with acid hydrolysis of cap 1 derived from 24 h mRNA, i.e., 87% of the Am is found in the form of m^6Am.

The methylnucleoside distribution in the N"m position of cap 2 was determined by digesting cap 2 structures with nuclease P1 which produces $m^7GpppN'm$. The released methylnucleoside N"m can be separated from the remainder of the cap structure by HSLC on Pellionex-WAX in ammonium acetate and subsequently analyzed on Aminex A-5. Of the label in cap 2 after 20 min, 80% was released as N"m. The N"m position of 24 h labeled cap 2 appears to be particularly rich in Um, and contains a significant amount of Am (Figure 6). Acid hydrolysis of this N"m nucleoside produced no m^6Ade (data not shown).

The overall distribution of methylnucleosides at each specific site of Novikoff mRNA methylation after 20 min, 5 h and 24 h of continuous labeling with L-[methyl-^3H]methionine is shown in Table II.

Figure 6. Methylnucleoside distribtuion analysis of N"m nucleoside of mRNA labeled for 24 h by Aminex A-5 HSLC. The N"m nucleoside obtained from nuclease P1 digestion of cap 2 was eluted from Pellionex-WAX (ammonium acetate), lyophilized, dissolved in 0.4 M ammonium formate, pH 4.25 in 40% ethylene glycol and chromatographed as in Figure 5.

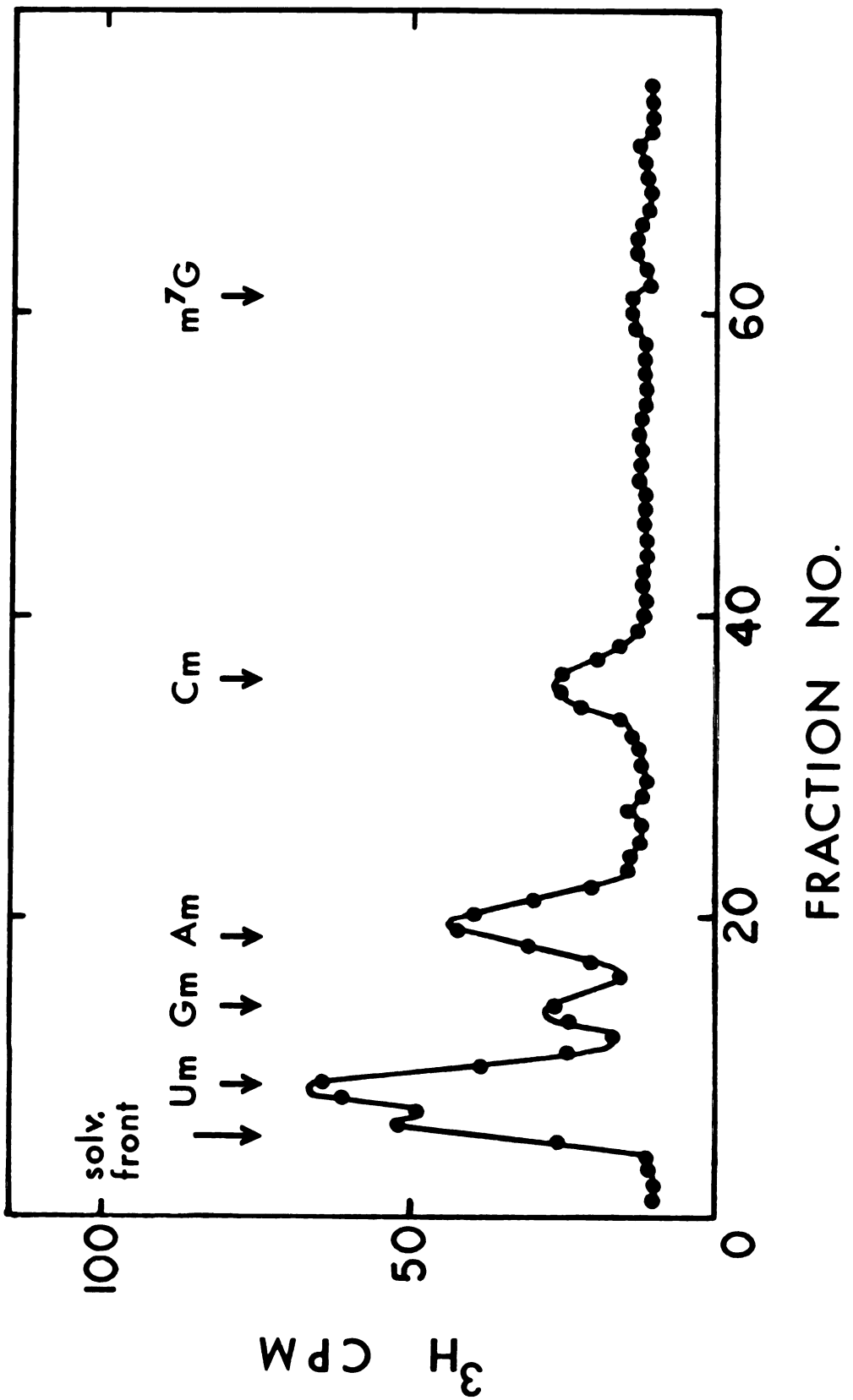

Figure 6

Table II. Whole poly (A)-containing mRNA was digested with RNase T2 and alkaline phosphatase; cap 1 and cap 2 structures were separated on DEAE-Sephadex (7 M urea). Following digestion of cap structures with penicillium nuclease, the "core" oligonucleotide and N"m nucleoside were resolved on a Pellionex-WAX column (NH_4 acetate). The distribution of nucleosides in N"m was determined as in Figure 6. Core oligonucleotide and cap 1 structures were digested with nucleotide pyrophosphatase and analyzed as in Figure 5. The data is presented as a percentage of the total radioactivity present in the structural position indicated.

Table II

Distribution of [^{3}H]methylnucleosides in Various Positions of Cap 1 and Cap 2 Structures as a Function of Labeling Time

	Labeling time	% of total label in:						
		Um	Gm	Am	$m^{6}Am$	Cm	$m^{7}G$	
Cap 1 (^{7}mGpppN'mpN)	5 hr	4.6	14.9	0	28.8	8.7	42.2	
	24 hr	2.4	9.4	3.5	19.6	21.1	44.0	
Cap 2 "core" (m^{7}GpppN'm)	5 hr	4.7	11.0	4.1	25.2	13.0	42.0	
	24 hr	1.8	8.0	9.3	20.4	18.4	42.1	
								% of Cap 2 as N"m
Cap 2 (N"m)	20 min	44	11	28	-	15	2	80
	5 hr	35	21	26	-	18	0	35
	24 hr	36	18	23	0	23	0	26

DISCUSSION

In earlier studies (Desrosiers et al., 1975) we examined the distribution of methylnucleosides in Novikoff mRNA after continuously labeling the cells for 13 h with L-[methyl-^{3}H]methionine. Analysis of mRNA methylnucleoside composition indicated that an average Novikoff mRNA contained 5.7 methyl groups, 3 of which were present as internal $m^{6}A$, 1 terminal $m^{7}G$ and 1.7 Nm's. The non-integral number of Nm's could result from an unequal distribution of cap 1 and cap 2 structures as well as $m^{6}Am$ in the N'm position. An explanation for the necessity of two different types of cap structure in mRNA was not readily apparent. However, the stabilizing effect that 2'-O-methyl groups have on RNA molecules in the presence of specific nucleases (Stuart and Rottman, 1973) led us to suggest that the ratio of these two cap structures might reflect multiple classes or fractions of mRNA with different cellular stabilities (Desrosiers et al., 1975). If this were true, one might expect the relative levels of these two structures to change as a function of labeling time.

Previous determinations of cap 1 to cap 2 ratios utilized mRNA that had been treated by periodate oxidation and β-elimination to remove terminal $m^{7}G$ prior to alkaline digestion. Since the elimination of $m^{7}G$ by periodate oxidation of mRNA was not reproducible in our laboratory, we explored the possibility of using the enzyme nucleotide pyrophosphatase on intact mRNA molecules and found this method of $m^{7}G$ removal to be superior. It should perhaps be noted that the susceptability of the 5'-terminal $m^{7}G$-containing cap to nucleotide pyrophosphatase implies that it must be open and accessible

to the enzyme and not buried within the folded structure of the mRNA molecules. Following removal of terminal phosphate and subsequent alkaline digestion, the N'mpNp and N'mpN"mpNp obtained from cap 1 and cap 2 structures, respectively, were separated by HSLC on Pellionex-WAX.

Poly (A)-containing mRNA was isolated from Novikoff cells that had been labeled with L-[methyl-^{3}H]methionine for different periods of time. Analysis of cap structures obtained from these mRNA samples indeed indicated that the relative labeling of cap 1 and cap 2 changed with time (Figure 2). At 20 min there was much more L-[methyl-^{3}H] label in cap 2, reflecting enhanced labeling at the N"m position of cap structures that were earlier methylated in the nucleus at m^7G and N'm from cold methyl precursors. After 5 h the ratios were reversed (Figures 1 and 3), and after longer periods (24 h) the ratio approaches a "steady-state" level of cap 2 to cap 1 of approximately two. These studies employed continuous labeling with L-[methyl-^{3}H]methionine to permit continued formation of newly methylated mRNA sequences, since the ratio of cap structures present at a particular time reflects both synthetic and degradative events.

The time-dependent changes observed in the relative labeling of each cap structure prompted us to further characterize the methylation occurring at each specific site within mRNA as a function of time. Such an approach necessitated the prior separation and isolation of 5'-terminal cap 1 and cap 2 structures as well as the m^6A located within the mRNA. DEAE-Sephadex (7 M urea) columns provided excellent resolution of the material obtained from 20 min, 5 h and 24 h-labeled mRNA (Figure 3). Analysis of the material present

in the mononucleotide fractions by subsequent HSLC on Aminex A-5 showed it to be mainly N^6-methyladenosine (data not shown). Since there have been several reports of 5-methylcytidine (m^5C) in viral and cellular mRNA (Dubin and Stoller, 1975; Salditt-Georgieff et al., 1976) the mononucleotide fraction was further analyzed. HSLC on Aminex A-5 provided separation of m^6A and m^5C and indicated a small peak of radioactive material eluting with m^5C at later labeling times (data not shown). After 20 min very little m^5C was detected. The maximum levels at 5 h and 24 h were 2% and 4% of the total internal methylnucleoside, respectively, indicating a low but possibly significant amount of this methylnucleoside that accumulates with time.

Another interesting time-dependent comparison involves the internal methylnucleoside m^6A and 5'-terminal m^7G. With longer labeling times the amount of m^6A relative to m^7G decreases (Table I). This could be due to a selective time-dependent loss of mRNAs rich in m^6A or an increase in the number of 5'-terminal m^7G residues on mRNA. Determination of the average size of Novikoff mRNA sequences on denaturing sucrose gradients indicates a distinct reduction in mRNA size as a function of labeling time (Figure 4). At 20 min most of the mRNA sediments in Me_2SO gradients at approximately 20S. At 5 h a biomodal distribtuion is obtained with components sedimenting at 20S and 15S while after 24 h most of the mRNA sediments at 15S. This indicates a reduction in the average size of mRNA sequences with time accompanied by a loss of m^6A which may be distributed at approximately equal intervals throughout the mRNA. Similar results have been obtained in duplicate experiments in which cells were grown in the presence or absence of adenosine, guanosine and sodium

formate, which were added to suppress purine ring labeling. Also it should be noted that these results on mRNA are in essential agreement with recent studies on HeLa hnRNA (Salditt-Georgieff et al., 1976) and L-cell mRNA (Perry and Kelly, 1976). However, the HeLa and L-cell studies concentrated on the relative reduction in the number of internal m^6A residues with reduction in size of RNA at a fixed labeling time. Thus in Novikoff cells it can be stated that with increased labeling time the average size of methyl-labeled mRNA gets shorter and the content of internal m^6A is reduced.

The isolation of labeled cap 1 and cap 2 permits a systematic analysis of specific methylated positions within these oligonucleotides as a function of labeling time. Comparisons can be made between cap 1 from different labeling times as well as between cap 1 and the analogous "core" structure, $m^7GpppN'm$, from cap 2. Earlier analyses were performed on either mixtures of cap 1 and cap 2 (Desrosiers et al., 1975) or on cap structures obtained at a single fixed time of labeling (Perry et al., 1975b). The methylnucleoside composition of cap 1, particularly at position N'm, appears to change with time. Although the relative labeling of Um, Gm and Am is nearly equivalent at 5 and 24 h (Figure 5 and Table II) there is a significant increase in Cm with time. This increase probably reflects a time-dependent enrichment of a sub-class of mRNA sequences with enhanced cytoplasmic stability. Also, as shown in Figure 5, virtually all of the Am present in the N'm position is found as the doubly methylated nucleoside, N^6, 2'-O-methyladenosine. At 5 h the amount of radioactivity recovered as N^6, 2'-O-methyladenine is consistent with the presence of only m^6Am and no Am, while at 24 h the distribution

is 87% m^6Am, 13% Am. This result differs from comparable analyses on L-cell mRNA which contained larger amounts of singly methylated Am in the N'm position (Perry and Kelley, 1976).

In an attempt to determine if the flux in cap 1 and cap 2 labeling was primarily due to labeling of a specific site within the cap structure or, alternatively, general labeling at all positions, the N"m position was selectively removed and separately analyzed. After 20 min, greater than 80% of the methyl label is in the N"m position (Table II). The distribution of methylnucleosides in the N"m position at 24 h labeling (Figure 6) indicates a high level of both Um and Am. None of the Am appears as the doubly methylated nucleoside m^6Am.

Removal of N"m from cap 2 results in the production of "core" structures that can be easily separated from N"m and recovered on Pellionex-WAX in the presence of ammonium acetate. Following complete degradation with nucleotide pyrophosphatase and alkaline phosphatase the methylnucleosides in the N'm position and m^7G can be analyzed on Aminex A-5. These data enable one to make an interesting comparison between the methylnucleoside composition of cap 1 and that of the analogous "core" structure derived from cap 2. The results summarized in Table II indicate that at a given labeling time the distribution between these two structures is remarkably similar. This correspondence in methylnucleoside distribution even extends to the relative increase in Cm observed at later labeling times. Data obtained with L-cell cap 1 and cap 2 "core" structures showed differences in composition at the N'm position (Perry and Kelley, 1976). Aside from different cell types used in both experiments

it should be noted that studies with L-cells were performed under pulse-chase labeling conditions while for Novikoff cells continuous labeling was employed. The data obtained with Novikoff mRNA do not suggest a selective methylation of certain mRNAs containing a unique cap 1 Nm content to which a N"m is added to form cap 2 structures. The presence of altered methylnucleoside compositions in mRNA's with different cytoplasmic half lives could produce the differences observed in pulse chase vs. continuous labeling.

Earlier data on hnRNA methylation patterns indicated the presence of 5'-terminal cap 1 structures and internal m^6A but no cap 2 (Perry et al., 1975b). These data, in combination with the present study, are consistent with a model in which m^7G, N'm and internal m^6A are products of nuclear methylation events. After exit of mRNA sequences containing these modifications into the cytoplasm, there is a cytoplasmic methylation event at the N"m position, forming cap 2. The selective enrichment, with time, of mRNA sequences containing a relatively high Cm composition suggests an enhances stability of a subclass of mRNAs that happen to have this altered methylnucleoside composition.

The complexities of this system in which thousands of mRNA sequences are being simultaneously synthesized and degraded are indeed enormous. Examination of methylnucleoside composition in mRNAs collectively grouped by cap 1 and cap 2 content is only a first step in following these mRNA modifications as a function of time. Of real interest will be those studies dealing with a specific homogeneous eukaryotic mRNA sequence.

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PART III

IN VIVO INHIBITION OF NOVIKOFF CYTOPLASMIC MESSENGER RNA METHYLATION BY S-TUBERCIDINYLHOMOCYSTEINE

ABSTRACT

The analogue S-tubercidinylhomocysteine (STH) has been used to study the methylation of mRNA *in vivo*. Partial inhibition of cytoplasmic poly (A)-containing 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 present 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.

INTRODUCTION

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). 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 structures 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., 1977).

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 poly (A)-containing cytoplasmic 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 asceptically

and resuspended at a concentration of 1.5 x 10^6/ml in fresh warm medium containing 20 μM methionine (one-fifth the normal concentration) for labeling experiments. 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 pre-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 alkylation 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 1N 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)-Containing RNA

Total cytoplasmic RNA fractions were isolated essentially as described (Desrosiers, et al., 1974). Poly (A)-containing 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., 1975) except that sedimentation was for 17 h.

Enzymatic and Acid Degradation of Poly (A)-Containing RNA

The procedures for enzymatic degradation of poly (A)-containing RNA, and for high speed liquid chromatography (HSLC) methodology employed for analyses of cap structures, are detailed in previous publications (Friderici, et al., 1975; Rottman, et al., 1976) and will be briefly outlined below.

Cytoplasmic poly (A)-containing 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 Biogel 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 Biogel 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 chromatogrpahy 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 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; PL Biochemical Co.) were monitored by A_{260} absorbance.

RESULTS

Preliminary studies on Novikoff poly (A)-containing 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)-containing 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)-containing 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)-containing RNA obtained from these cells (Desrosiers, *et al.*, 1974). The poly (A)-containing 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.

Table I

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

Fraction of RNA	^{14}C-Uridine incorporation		^{3}H-Methyl incorporation		^{3}H cpm/^{14}C cpm	
	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 poly (A)-containing RNA	11.3	12.8	0.43	0.23	1.03	0.46
non-poly (A)-containing RNA	88.7	87.2	99.57	99.77		
CPM in poly (A)-containing RNA 10^7 cells	15,200 (100%)	11,120 (73%)	15,730 (100%)	5,110 (32%)		
A_{260} total RNA/10^7 cells	5.73 (100%)	5.88 (103%)				

[a]Figures in parenthesis 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.

Cytoplasmic poly (A)-containing 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)-containing RNA digests are presented in Figure 1; the percentage 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. Re-digestion and re-chromatography of a portion of the desalted peak fractions resulted in more than 92% of the material eluting identically to that shown in Figure 1. In addition, similar digestion and chromatography of nuclear poly (A)-containing 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., 1975). 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

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 X 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 from scintillation counting. Standard oligonucleotides $(pUm)_{1-5}$ were included to indicate approximate charge. (A) Digest of poly (A)-containing RNA isolated from normal cells; (B) Digest of poly (A)-containing RNA isolated from STH-treated cells. Numbers in parentheses indicate the percentage distribution among the labeled fractions: Peak I - mononucleosides ^{m}N; Peak II - dinucleotides, NmpN: Peak III - oligonucleotide bearing -2.5 charge identified as cap zero, m^7GpppN'; Peak IV - cap 1, $m^7GpppN'pmpN$; and Peak V - cap 2, $m^7GpppN'mpN"mpN$.

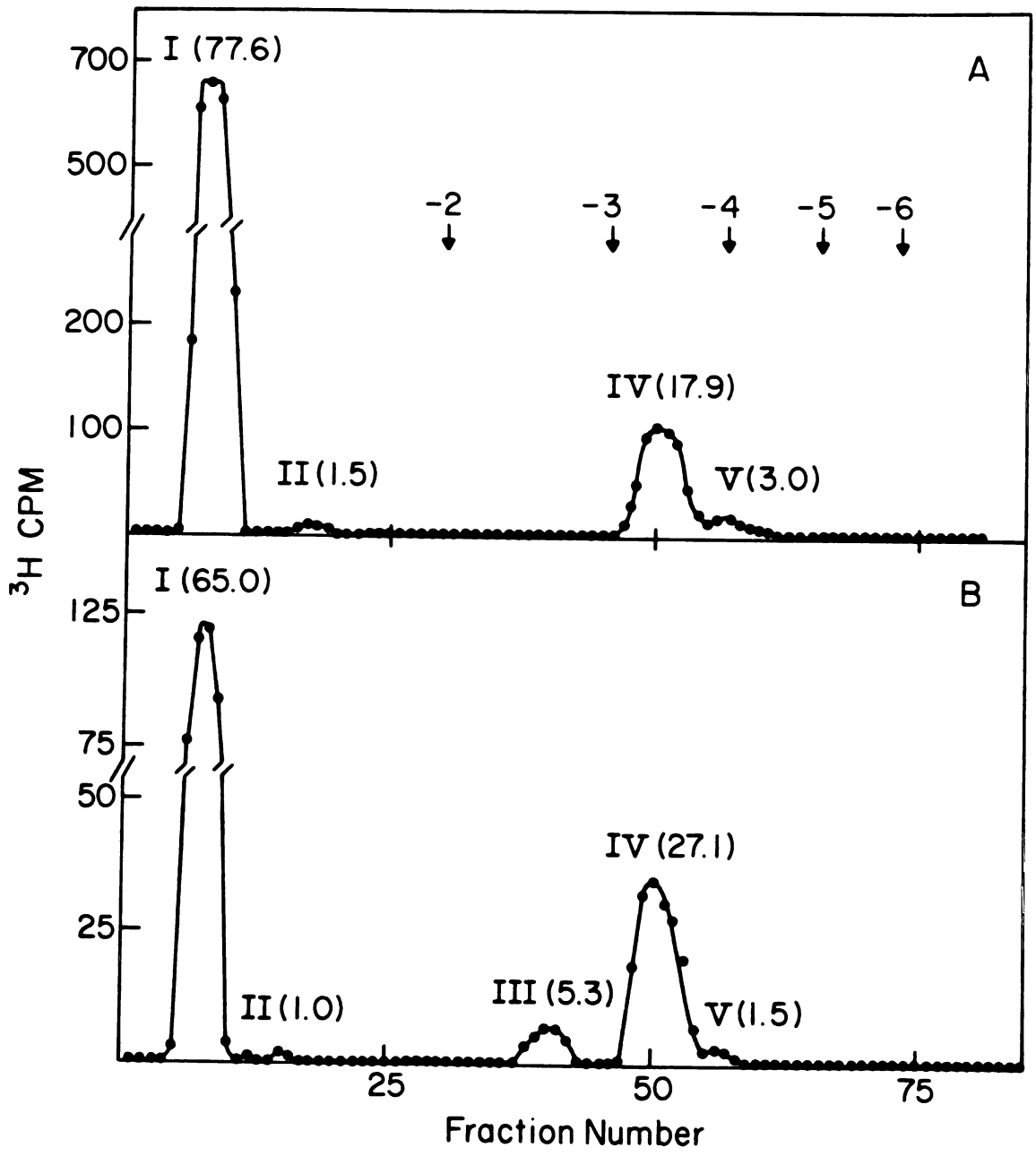
700
500
200
100
I (77.6)
A
-2
-3
-4
-5
-6
IV(17.9)
II(1.5)
V(3.0)
^{3}H CPM
125
75
50
25
I (65.0)
B
IV (27.1)
III (5.3)
II(1.0)
V(1.5)
25
50
75
Fraction Number

Figure 1

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-methyl ribose 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 Partisil-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 1 separation resolved the sample into peaks which were not coincident with cap 1 standards (data not shown). All four cap zero structures are present, with predominately purines in the N' position. Since m^7Gpppm^{6}A 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^{6}A.

The distributional analysis data for cap zero, and for both cap 1 and cap 2 from inhibited and normal cytoplasmic poly (A)-containing RNA fractions, are summarized in Table II. In contrast 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 obtained. This indicates that inhibition of N' 2'-O-methylation to produce cap zero is not base specific. A comparison of cap 2 distribution

Table II

Distribution of ^{3}H-methyl CPM in Cap Structures of Cytoplasmic Poly (A)-containing 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)	(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)	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]	+	+	+	N.D.[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] N.D. = not determined.

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 Fig. 1) were desalted on a 1.5 X 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 1 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^7GpppU 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.

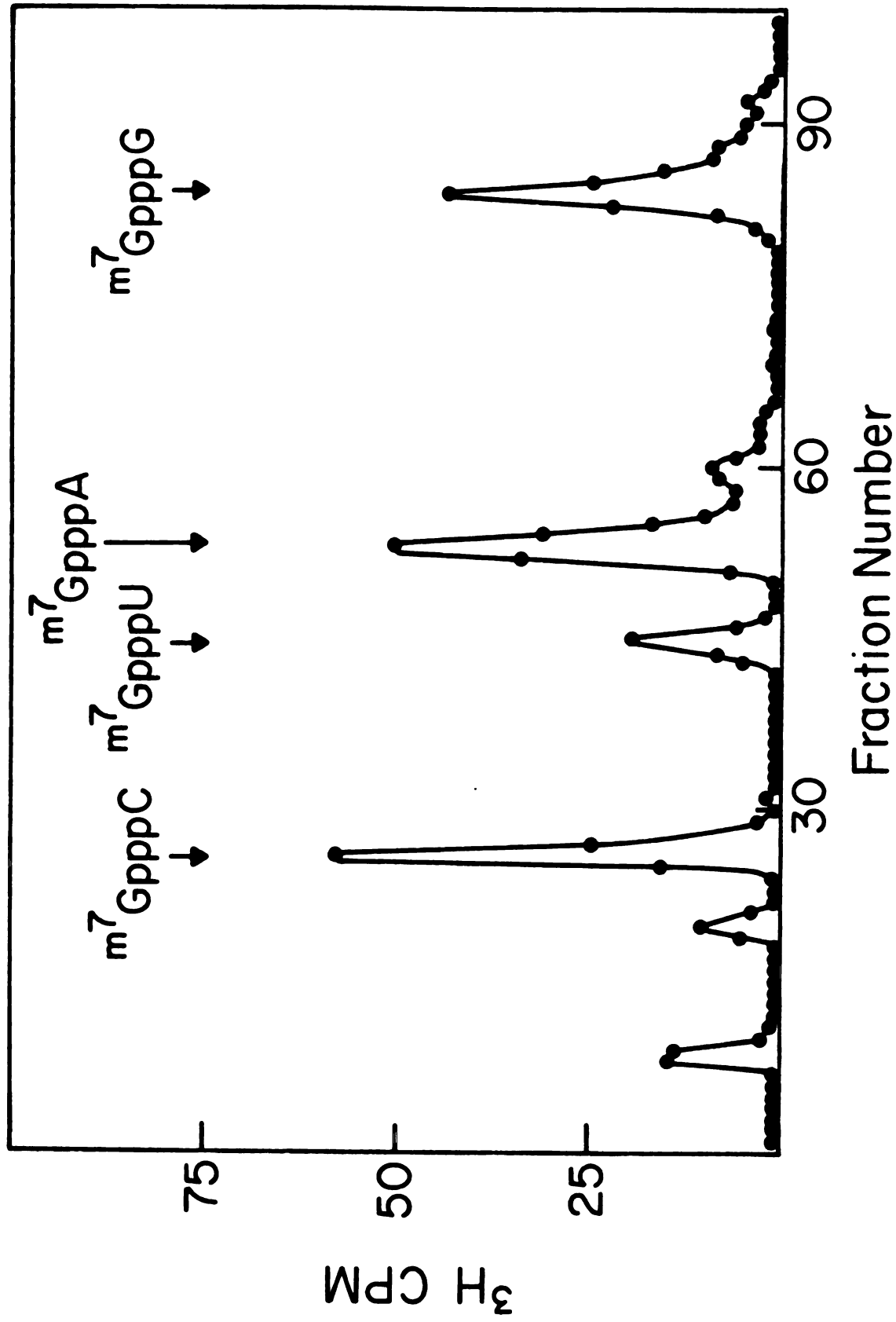
m7GpppG
m7GpppA
m7GpppU
m7GpppC
75
50
25
3H CPM
30
60
90
Fraction Number

Figure 2

in mRNAs 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 shows that the composition of N'(m) in the cap structures from STH-treated and normal RNA 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 mononucleoside 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 $m^{6}A$ present in cap structures, the amount of $m^{6}A$ 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 RNA. In the normal sample, per 10^{7} cells, 1038 ^{3}H-cpm was incorporated as $m^{7}G$, and 10,822 ^{3}H-cpm as

m^6A in poly (A)-containing cytoplasmic RNA, resulting in an m^6A/m^7G ratio of 10.4. Corresponding incorporation in the STH-treated sample was 782 ^3H-cpm as m^7G, and 2,432 ^3H-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 analysis of methyl distribution in caps was 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 anslysis of cap 1 from STH-inhibited cells. Resolution 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

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 Fig. 1) were desalted on Bio-Gel P-2 as described in Fig. 2. The cap structures were digested with P1 nuclease and T2 RNase in the presence of $1A_{260}$ unit carrier rRNA. The digest was injected onto a Partisil PXS 1025-SAX column and cap 1 species were resolved as described in Fig. 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 cap 1 structures isolated from STH-treated cells. The desalted material was digested with nucleotide pyrophosphatase and alkaline phosphatase, dried with N_2 and resuspended in 125 µl column buffer. HSLC on Aminex A-5 (1/8 in X 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° 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 (1/8 in. X 90 cm) in 0.4 M ammonium formate at pH 5.3, 40°C.

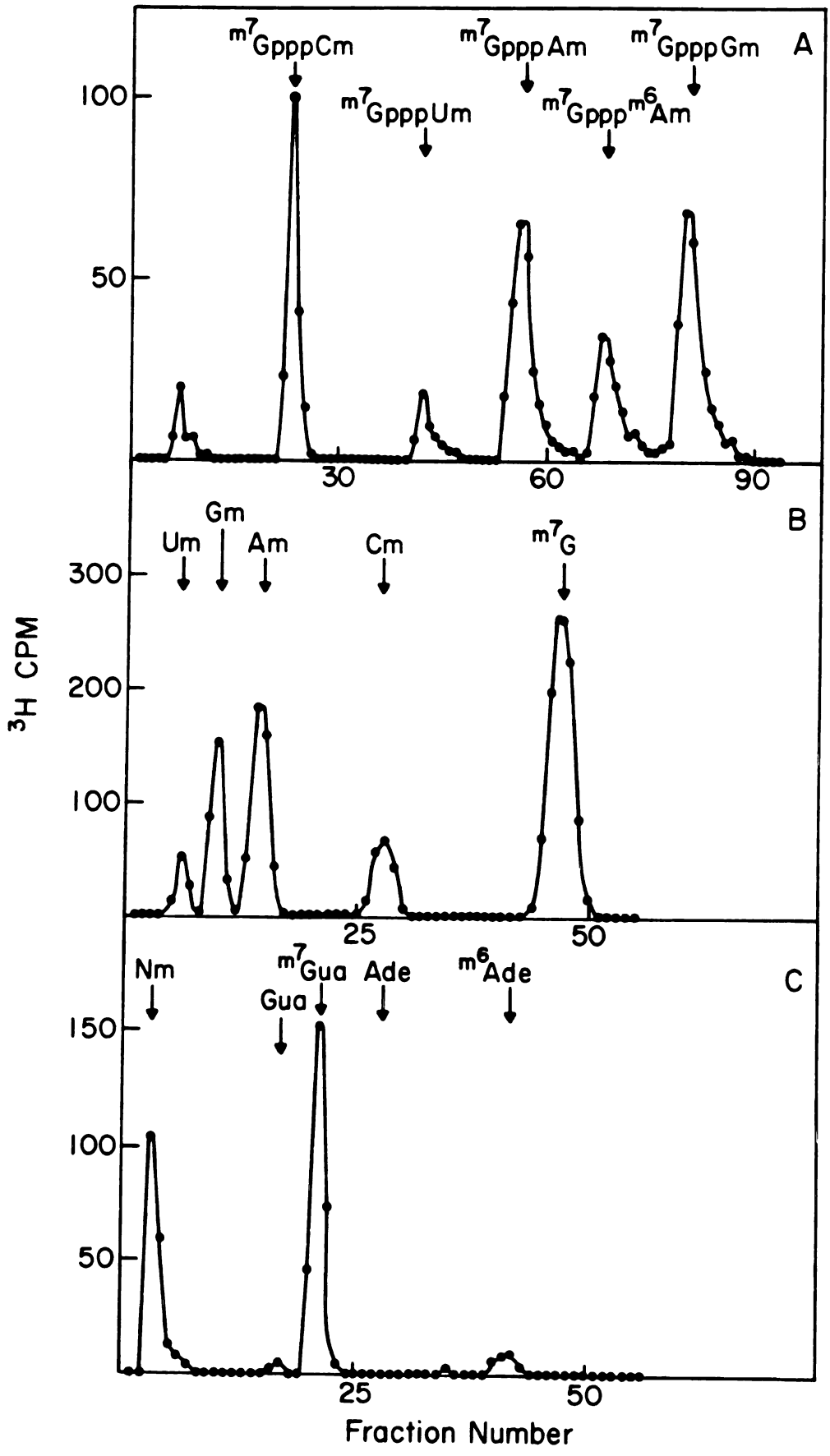
m7GpppCm
m7GpppUm
m7GpppAm
m7Gppp m6Am
m7GpppGm
A
100
50
30
60
90
Gm
Um
Am
Cm
m7G
B
300
200
100
25
50
Nm
m7Gua
Gua
Ade
m6Ade
C
150
100
50
25
50
3H CPM
Fraction Number

Figure 3

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, 1975b; Tonguzzo and Ghosh, 1976), and since STH, an analogue of SAH, has previously been shown to inhibit RNA methylation (Chang and Coward, 1975; Michelot, *et al.*, 1977), the effect of STH on mRNA methylation *in vivo* was studied.

Labeling of RNA with both [^{14}C]uridine and [^{3}H-methyl]methionine permits an evaluation of overall methylation relative to the total amount of RNA synthesized. The data presented in Table I provides 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)-containing RNA is significantly reduced, as reflected by the ^{3}H/^{14}C values for each poly (A)-containing fraction.

Perhaps the most significant result of this study is the detection of cap zero structures in poly (A)-containing RNA isolated from the cytoplasm of cells exposed to STH. The appearance of peak III (Fig. 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^7GpppN. 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 co-eluted 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 the 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 that 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 RNA labeled in the presence of STH (Fig. 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)-containing RNA is consistent with preferential inhibition of a subpopulation of mRNA. Inasmuch as RNA synthesis is initiated with a 5'-terminal purine triphoisphate (Chambon, 1974; Schibler and Perry, 1976; Schmincke, et al., 1976) and since cap 1 structures (m^7GpppN'mpN) have been found in hnRNA (Perry, et al., 1975; 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 $m^7GpppPu$, or 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)-containing RNA have suggested that methylation at the N' site occurs in the nucleus, (Perry, et al., 1975; Salditt-Georgieff, et al., 1976), finding cap zero in poly (A)-containing cytoplasmic 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 indicates that the presence of STH did alter the proportion of internal m^6A present in the RNA fraction relative to the amount of m^7G observed. The ratio of m^6A/m^7G 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^6A, 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^6A sites in mRNA molecules (Wei, et al., 1976; Dimock and Stoltzfus, 1977).

In contrast, incorporation of ^3H-methyl as m^7G 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 thus appears that under the conditions used in these experiments, in which partial methylation is occurring, little or no inhibtion of methylation at the 7-position of guanine is observed in the cytoplasmic poly (A)-containing RNA. These results may indicate a cellular response in terms of priority for methylation at the 7-methylguanosine site over base methylation, possibly reflecting the functional or sequential nature of methyaltion 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 indicates 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, et al., 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)-containing 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.

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PART IV

CYTOPLASMIC LOCATION OF UNDERMETHYLATED MESSENGER RNA FROM NOVIKOFF CELLS

ABSTRACT

Novikoff cells in culture were simultaneously labeled with L-[methyl-^{3}H]methionine and [^{32}P]orthophosphate in the presence or absence of S-tubercidinylhomocysteine, an inhibitor of RNA methylation. Total cytoplasmic, polysomal and monosomal poly (A)-containing RNAs from both normal and inhibited cells were analyzed to ascertain the subcellular location of undermethylated mRNA. Both monosomal and polysomal mRNA fractions from S-tubercidinylhomocysteine-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 either for ribosomal binding or for translation. These partially methylated cap structures are also present in nuclear poly (A)-containing RNA isolated from cells labeled in the presence of S-tubercidinylhomocysteine. Totally unmethylated cap structures, if present at all, are not accumulating in the cells.

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 (cf. reviews: Shatkin, 1976; Rottman, 1976, 1978). This posttranscriptional modification of mRNA has been the subject of a number of investigations which have attempted to elucidate cap function(s) within the cell. These efforts have generally focused on the role of methylated cap structures during translation (Muthukrishnan, et al. 1975; Kozak and Shatkin, 1977; Hickey, et al., 1977) and have indicated that cap structures

may facilitate translation, but are not absolutely required. However, the presence of cap structures on hnRNA molecules (Perry, et al., 1975; Salditt-Georgieff, et al., 1976) and the kinetics of methylation at specific sites within cap structures (Perry and Kelley, 1976; Friderici, et al., 1976; Rottman, et al., 1976), 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 (Jeffreys and Flavell, 1977; Tilghman, et al., 1977; Breathnach, et al., 1977; Brack and Tonegawa, 1977), and the demonstration that transcription of the intervening sequences into primary RNA transcripts occurs during expression of β-globin genes (Tilghman, et al., 1978), suggests a model for mRNA processing in which both 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 have generated undermethylated mRNA (Kaehler, et al., 1977). These studies demonstrated that S-tubercidinylhomocysteine (STH), the 7-deaza analogue of S-adenosylhomocysteine (SAH), inhibited mRNA methylation in viable Novikoff cells. The cytoplasmic presence of "cap zero" structures (m^7GpppN'), in which 2'-O-methylation was absent, indicated that ribose methylation of the N' nucleoside was not required for nuclear processing and transport of mRNA in viable Novikoff cells.

We 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 cells with STH present.

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 and resuspended at 1.5 x 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 STH for the final 50 min of the equilibration period. L-[methyl-^{3}H]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 labeled for 2 h. STH was synthesized and characterized as previously described (Coward, et al., 1976).

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 (Desrosiers, et al., 1974).

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 (Eschenfeldt and Patterson, 1975) 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, and aliquots equivalent to 5% of each fraction were removed and analyzed for radioactivity. 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 in ethanol.

Washed nuclei were resuspended in hypotonic buffer and vortexed for 3 sec in the presence of 1% sodium deoxycholate and 2% Tween40 prior to re-centrifugation 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; (Kwan, et al., 1977)), 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 (Desrosiers, et al., 1975). RNA was dissolved in high salt buffer (0.12 M NaCl, 1 mM EDTA, 0.2% SDS, 10 mM Tris, pH 7.0) and applied to a 2.5 ml oligo(dT)-cellulose column. Non-poly (A)-containing RNA fractions were pooled and precipitated by the addition of two volumes of ethanol. RNA which bound to the column was eluted with low salt buffer (1 mM EDTA, 0.2% SDS, 10 mM Tris, pH 7.0), made 0.2 M in NaCl, and precipitated in ethanol. The second chromatographic passage of RNA on oligo(dT)-cellulose was preceded by heat denaturation of the RNA in the presence of 90% DMSO, as described by Bantle, et al. (1976). Poly (A)-containing RNA was precipitated in the presence of unlabeled Novikoff rRNA by addition of two volumes of ethanol.

Enzymatic Digestion and Analysis of Poly (A)-Containing RNA.

Poly (A)-containing RNA was digested with approximately 2 units RNase T2 per A_{260} unit of RNA in 10 mM ammonium acetate, pH 4.5, for 2 h at 37°C. The digest was then diluted with four volumes of 7 M urea, 10 mM Tris, pH 7.0, and the digestion products were resolved on DEAE-Sephadex (7 M urea). A 200 ml gradient from 0.1 M to 0.4 M NaCl in 7 M urea, 10 mM Tris, pH 7.0 was used to elute the digestion products; $(pUm)_{1-5}$ were employed as absorbance markers.

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; (Thomason, et al., 1978)) 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 (Thomason, et al., 1978), 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 in NaCl, and passed over a 1 ml DEAE-cellulose column to remove urea and salt. The column was washed with 0.02 M ammonium acetate, and cap structures were eluted with 0.5 M ammonium acetate. The ammonium acetate was subsequently removed by repeated evaporation and lyophilization.

Cap structures were analyzed by Partisil-SAX (Whatman) high speed liquid chromatography (HSLC). Cap 1 structures were first digested with P1 nuclease in 10 mM ammonium acetate, pH 5.3 for 1 h at 37°C. The mixture was adjusted to 10 mM Tris, pH 8.3 and 10 mM magnesium acetate prior to digestion with alkaline phosphatase at 37°C for 45 min. The digestion mixture was evaporated under a gentle stream of nitrogen and resuspended in 100 µl 0.01 M KH_2PO_4, pH 3.33 for injection onto Partisil-SAX. 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.

Due to low levels of radioactivity in cap 2 species, the P1 nuclease-digested caps were chromatographed on DBAE-cellulose as described above, and fractions were analyzed for radioactivity. The ^{3}H-methyl distribution between core cap structures (m^{7}GpppN'mp) and N"mp was determined by the radioactivity in bound and unbound fractions, respectively.

Acid hydrolysis in concentrated formic acid, followed by chromatography of the hydrolysate on an Aminex A-5 column, was performed as previously described (Rottman, et al., 1976). 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 (Munns et al., 1974).

RESULTS

Previous studies in our laboratory have shown that STH is an effective inhibitor of mRNA methylation in vivo in Novikoff hepatoma cells (Kaehler, et al., 1977). In order to further establish the specificity of STH as a methylation inhibitor, the following experiment was performed. Cultured Novikoff cells 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 STH, (b) 250 μM SAH, (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 (Kaehler, et al., 1977). Analysis of the methyl distribution in poly (A)-containing RNA from cultures treated with SAH and homocysteine gave virtually identical results when compared to the

control (Table I). As predicted, tubercidin was toxic to the cells and both RNA synthesis and methylation sharply decreased. Only in cultures labeled in the presence of STH were cap zero structures observed to represent a significant portion of [^{3}H]methyl radioactivity. These results are summarized in Table I.

To determine if cap zero-bearing mRNA was functional in these cells we studied the cytoplasmic location of undermethylated mRNA molecules. In these experiments, simultaneous labeling with L-[^{3}H-methyl]methionine and [^{32}P]orthophosphate permitted evaluation of the effect of 500 μM STH on mRNA synthesis, methylation and transport during the labeling period. In agreement with our earlier observations (Kaehler, et al., 1977), ^{3}H-methyl incorporation was inhibited to a greater extent than ^{32}P-incorporation, i.e. to 18% and 70%, respectively, of the corresponding incorporation levels observed in the control cultures. This inhibition of methylation appears to depend upon the concentration of the inhibitor, since doubling the concentration of STH (from 250 μM to 500 μM) decreased the ratio of ^{3}H-methyl incorporation in STH-treated vs. normal cultures from .32 to .18. In contrast, mRNA synthesis (monitored by ^{14}C-uridine or ^{32}P-orthophosphate incorporation) was decreased to approximately 70% of normal levels at either STH concentration.

In order to establish the cytoplasmic location of cap zero-bearing mRNA molecules, monosomal and polysomal RNAs were separated by sedimentation of the postnuclear supernatant through sucrose gradients. Figure 1 shows the absorbance profiles obtained from both normal and STH-treated cells, and indicates the regions pooled

Table I

Effect of STH, SAH, Homocysteine and Tubercidin on Methylation of Poly (A)-containing RNA.[a]

	Control	STH	SAH	Homocysteine	Tubercidin
^{3}H-methyl cpm incorporated into total RNA/10^{7} cells	3.69×10^{6}	2.23×10^{6}	3.17×10^{6}	3.07×10^{6}	1.54×10^{5}
^{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[b]:					
% 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 (Kaehler, et al., 1977). 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]^{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.

Figure 1. Absorbance profiles of postnuclear supernatant 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) STH-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.

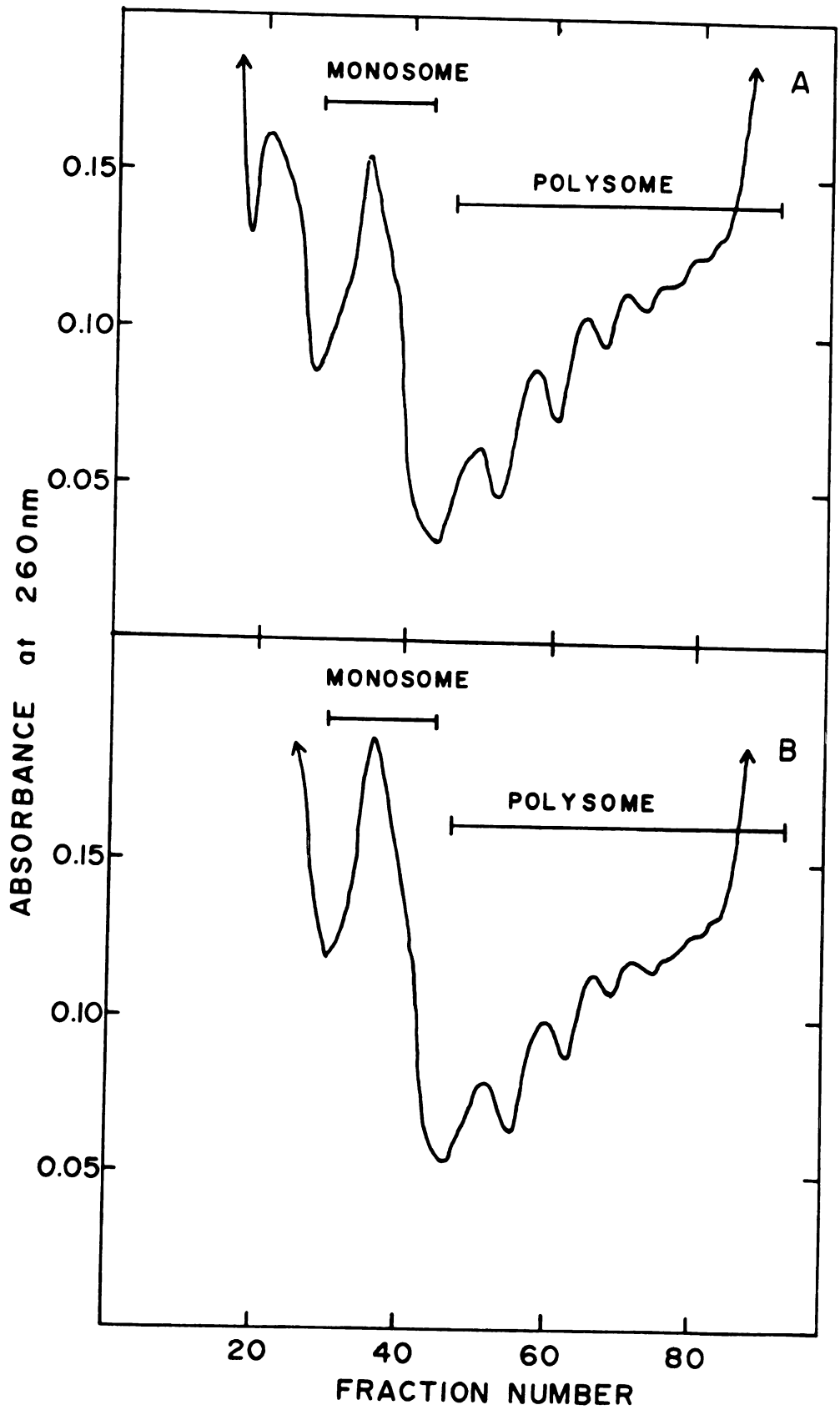
MONOSOME
A
POLYSOME
0.15
0.10
0.05
ABSORBANCE at 260nm
MONOSOME
B
POLYSOME
0.15
0.10
0.05
20
40
60
80
FRACTION NUMBER

Figure 1

for monosomal and polysomal RNA extraction. The monosomes to polysomes ratio in each sample was nearly identical, indicating that the protein-synthesizing machinery had not been significantly perturbed by the presence of STH.

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 STH-treated total cytoplasmic, polysomal, and monosomal mRNAs (Figures 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). Cap zero (peak II, Figure 2B,C,D) was the predominant cap structure in each of the RNA fractions isolated from STH-inhibited cells, indicating that cap zero-containing molecules were located on 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 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 (Friderici, et al., 1976; Perry and Kelley, 1976).

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 (Figure 2). However, greater sensitivity could be achieved if the RNase T2 digest was first passed over an acetylated DBAE-cellulose column. This step removed most of

Figure 2. DEAE-Sephadex (7 M urea) elution profiles of RNase T2 digestion products from poly (A)-containing RNAs. The RNAs were digested and the products were chromatographed as described in MATERIALS AND METHODS. 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 STH. 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 27% of the total cpm in peak I from normal and inhibited cytoplasmic samples, respectively (Figure 2A and B)). For clarity, ^{32}P-radioactivity profiles have been omitted.

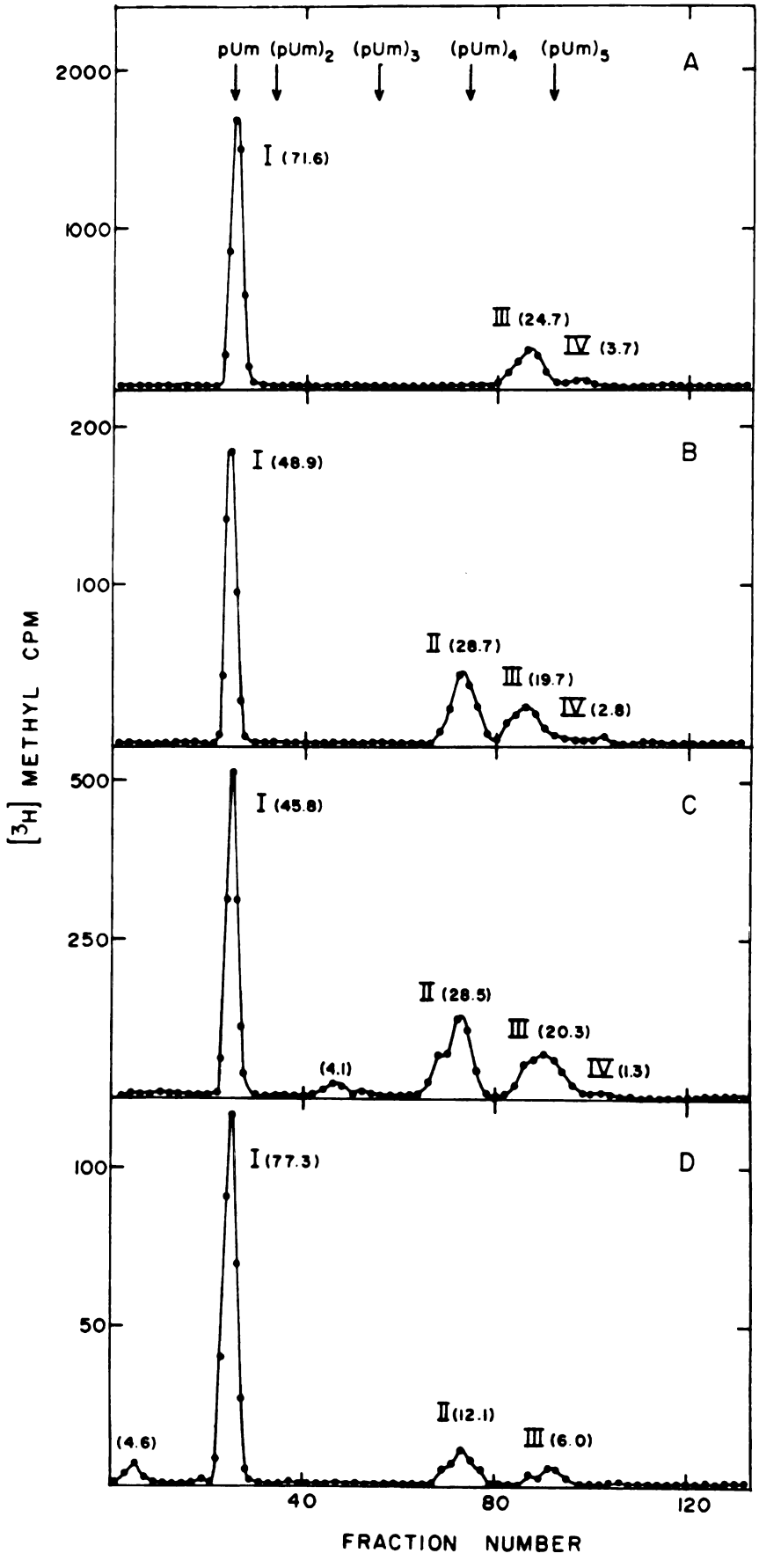
pUm
(pUm)2
(pUm)3
(pUm)4
(pUm)5
A
2000
1000
I (71.6)
III (24.7)
IV (3.7)
200
100
B
I (48.9)
II (28.7)
III (19.7)
IV (2.8)
500
250
C
I (45.8)
II (28.5)
III (20.3)
(4.1)
IV (1.3)
100
50
D
I (77.3)
II (12.1)
III (6.0)
(4.6)
40
80
120
[3H] METHYL CPM
FRACTION NUMBER

Figure 2

the ^{32}P-radioactivity from the sample prior to resolution of the cap structures by DEAE-Sephadex (7 M urea). As shown in Figure 3, more than 99% of the ^{32}P-radioactivity in total cytoplasmic mRNA from STH-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 4A). The distribution of ^{3}H-methyl cpm in caps zero, 1, and 2 (Figure 4A, 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 2B (peaks II, III and IV, respectively). Most of the ^{32}P-cpm which bound to DBAE-cellulose eluted as mononucleotides (Figure 4A, 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 STH-treated cytoplasmic mRNA, was distributed as follows: 0.10% in cap zero (Figure 4A, peak II), 0.04% in cap 1 structures (Figure 4A, peak III), and 0.03% eluting as peak V in Figure 4A.

Unmethylated cap structures would be expected to elute from DEAE-Sephadex (7 M urea) columns between caps zero and 1, since the unmethylated caps would not carry the partial positive charge of 7-methylguanosine. There was no detectable radioactivity eluting in this position, but rather peak V in Figure 4A was observed, at a position indicating an approximate charge of -8. The fractions containing this radioactive material were pooled and desalted for further analysis.

Figure 3. Acetylated DBAE-cellulose chromatography of RNase T2 digestion products from STH-inhibited cytoplasmic poly (A)-containing RNA. The digest was diluted with application buffer and chromatographed on a 1 ml acetylated DBAE-cellulose column as described in MATERIALS AND METHODS. Thirty drop fractions were collected, corresponding to a fraction size of 0.95 ml during application, and 1.6 ml during elution. Radioactivity present in each fraction was determined from 25 µl aliquots. Circles (—●—●—) indicate ^{3}H-methyl cpm; triangles (—▲—▲—) denote ^{32}P-cpm.

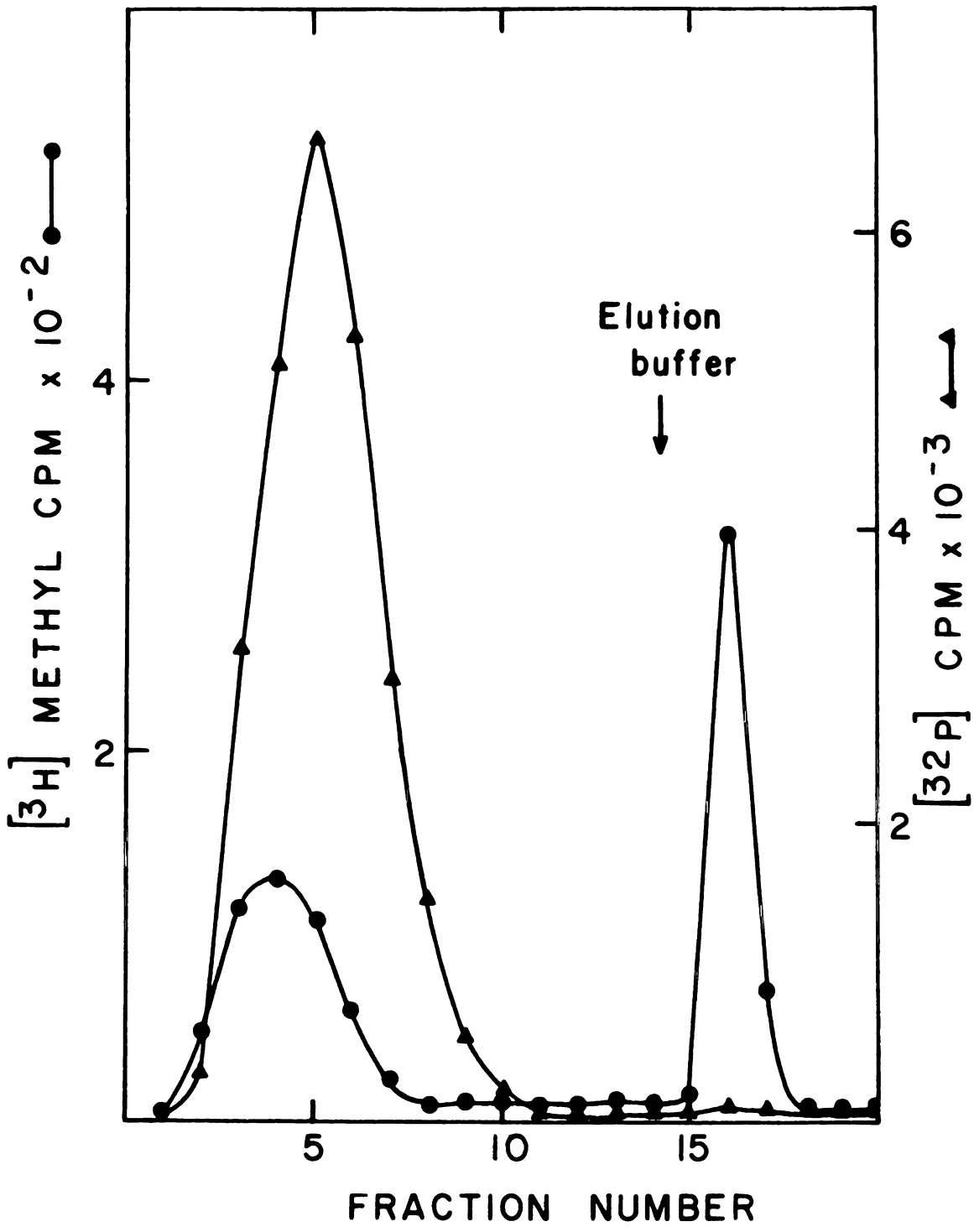
[3H] METHYL CPM x 10-2
[32P] CPM x 10-3
Elution buffer
FRACTION NUMBER
2
4
6
5
10
15

Figure 3

Figure 4. 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 (cf. Figure 3) 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 STH-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.

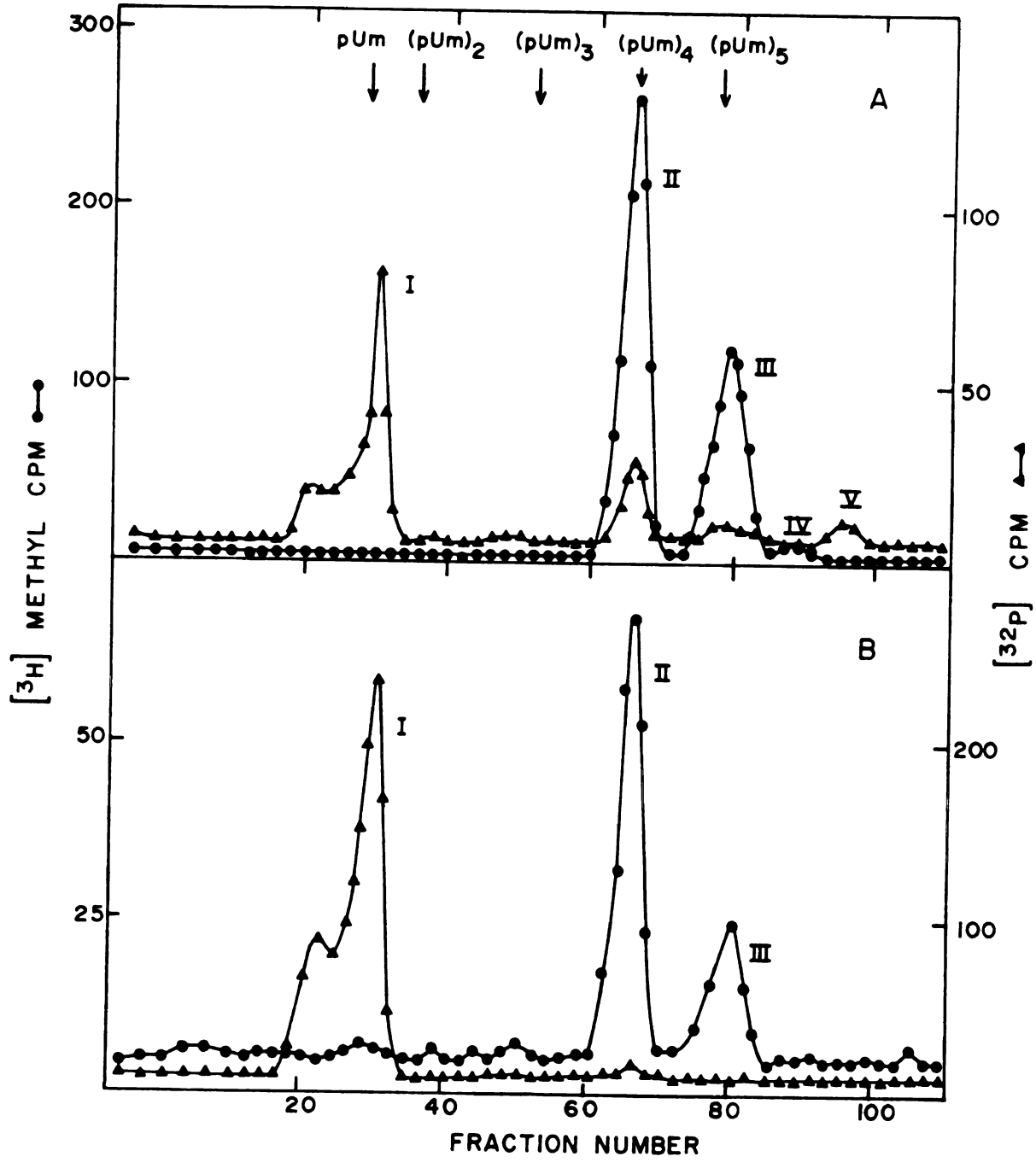
pUm
(pUm)2
(pUm)3
(pUm)4
(pUm)5
A
B
I
II
III
IV
V
300
200
100
50
25
100
50
200
100
[3H] METHYL CPM
[32P] CPM
20
40
60
80
100
FRACTION NUMBER

Figure 4

Although no absorbance standards were available for chromatography, it was possible that the anomalous elution behavior of the material contained in peak V is consistent with a GpppN'p structure. The desalted oligonucleotide eluting as peak V in Figure 4A was therefore treated with alkaline phosphatase and rechromatographed on DBAE-cellulose. Sixty per cent of the radioactive material bound to the column (Figure 5). This result is consistent with the predicted behavior of unmethylated cap structures only if 20% of the pyrophosphate linkages had been broken during desalting and/or phosphatase digestion. The low level of radioactivity in this highly negatively charged compound prevented further analysis.

Nuclear poly (A)-containing RNA isolated from STH-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 chromatographed on DBAE-cellulose. The DBAE-bound fraction was eluted on DEAE-Sephadex (7 M urea) and the resultant profile is shown in Figure 4B. ^{32}P-Radioactivity was detected only in the mononucleotide and possibly in cap zero fractions (Figure 4B, 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).

Figure 5. DBAE-cellulose profile of the highly charged material isolated from STH-treated cytoplasmic mRNA. The highly charged oligonucleotide fractions (peak V in Figure 4A) were desalted and digested with alkaline phosphatase as described in MATERIALS AND METHODS. The digest was chromatographed on an acetylated DBAE-cellulose column. Fractions were collected and measured directly for ^{32}P-radioactivity.

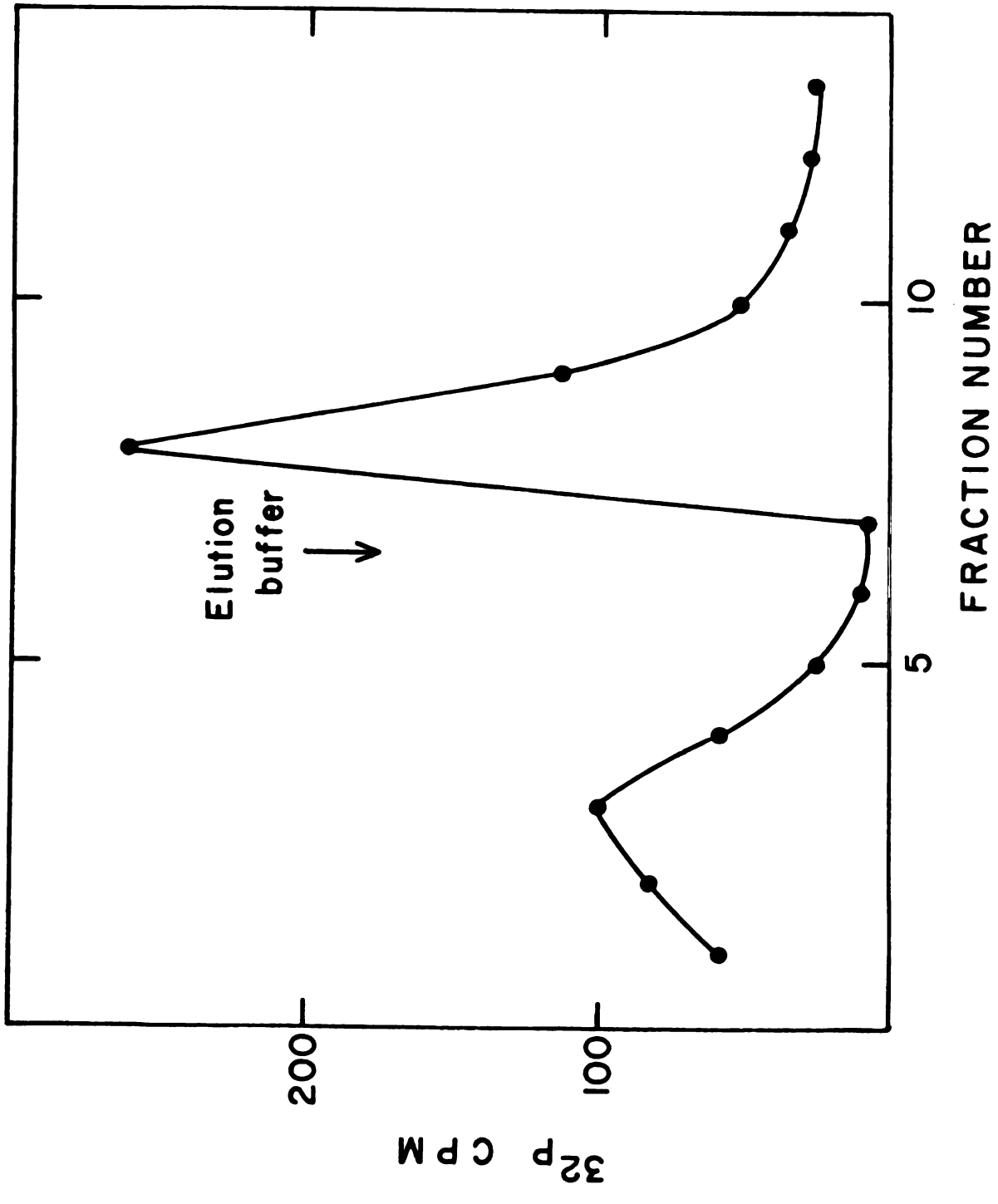

Figure 5

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 one and zero from both normal and STH-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 STH-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 STH-inhibited cells were similar and appeared to contain only the four common bases at N'. In addition, no m^{6}A was observed in this position, as detected by Partisil-SAX chromatography and, in the case of cytoplasmic cap zero, by Aminex A-5 HSLC of the acid-hydrolyzed structures (cf. Table II).

Since cap 2 structures contained low amounts of radioactivity, these samples were digested with P1 nuclease and chromatographed on DBAE-cellulose. The amount of labeled material present as N"mp was determined by the percentage of the total ^{3}H-methyl radioactivity which did not bind to the column; the bound fraction represented label localized in the "core cap" (m^{7}GpppN'm). Labeled N"mp accounted for 46% and 34% of the total ^{3}H-methyl radioactivity in cap 2 structures derived from normal and STH-inhibited mRNA, respectively. This is indicative of inhibition of ribose methylation at the N" position by STH.

Table II

Distribution of ^{3}H-Methyl Radioactivity From Cap Structures Determined by Partisil-SAX Chromatography

	% of ^{3}H-methyl cpm eluting as:				
	$m^7GpppC(m)$	$m^7GpppU(m)$	$m^7GpppA(m)$	$m^7Gpppm^6A(m)$	$m^7GpppG(m)$
Normal cultures:					
Cap 1 - cytoplasmic	17	13	24	31	15
nuclear	19	9	32	28	12
STH - 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^7Gpppm^6A 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 A5 HSLC to verify that m^6A was not present.

DISCUSSION

Determination of the role of mRNA methylation in eukaryotes is complicated 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 in the presence of S-tubercidinylhomocysteine. Cap zero was the predominant cap structure of both polysomal and monosomal mRNAs (Figure 2C and D, respectively). The location of these mRNAs on 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 indicate 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. It should be noted that these conclusions have been drawn from experiments using viable cell cultures. Both et al. (1975) and Muthrikrishnan, et al. (1975) have previously reported that 2'-O-methylation at N' was not necessary for ribosome binding and cell-free translation of capped reovirus mRNA in wheat germ extracts. Subsequent studies, however, indicated that ribosomes preferentially bound viral RNA and synthetic ribopolymers terminated by cap structures which contained ribose-methylated nucleosides at the N' position (Both, et al., 1976; Muthukrishnan, et al., 1976). Ribose methylation at N' does not appear to

significantly affect ribosomal binding of vaccinia mRNA (Muthukrishnan, et al., 1978), unless high concentrations of mRNA are used to enhance competition for binding.

Sedimentation analysis of the postnuclear supernatant of cells labeled in the absence and presence of STH (Figure 1A and B, respectively) also permitted evaluation of the inhibitor's effect on polysome distribution. Polysomal profiles have been recognized as a criterion of the specificity of various metabolic inhibitors of RNA synthesis (Craig, N., 1973). The similar monosome/polysome ratio observed for both control and STH-inhibited cells indicated that the inhibitor had not significantly altered the polysome levels, implying that protein synthesis has remained relatively unperturbed by the presence of STH. This nontoxicity of STH is highly desirable for studies involving RNA processing, since cell viability must remain high in order to maintain normal regulatory functions in the cells.

[^{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 were found in inhibited mRNA digests that chromatographed at the expected elution position of unmethylated caps (Figure 2B, C and D; Figure 4A and B). 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 STH-inhibited cytoplasmic mRNA eluted from DEAE-Sephadex in three distinct peaks (Figure 4A): 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 4A. 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 4A represents approximately one-third the level of incorporation observed into cap zero structures. Analysis of the material was limited by the low amount of radioactivity present. The structure possessed the following chracteristics: 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 phosphatase 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 STH-inhibited nuclear mRNA digests (although its detection would require this structure to be present in levels comparable to cap zero levels); and 5) it elutes from DEAE-Sephadex (7 M urea) columns as if it 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. (1978) have demonstrated that STH is a very 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 is consistent with the moderate decrease in RNA synthesis observed in the presence of STH.

The ^{3}H-methyl distribution among cap structures from total cytoplasmic mRNA of both control and STH-inhibited cultures are remarkably similar to the corresponding nuclear cap distributions (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 STH-exposed cells. The absence of $m^{6}A$ 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 (Cheng and Kazazian, 1978).

The predominance of uridine at N' in both nuclear and cytoplasmic cap one structures derived from inhibited cells was somewhat surprising (Table II), since m^{7}GpppU(m) is generally observed as a minor component of cap structures from Novikoff cells (Friderici et al., 1976; Kaehler, et al., 1977). The presence of 500 M STH 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, STH may selectively inhibit certain methylases to a greater extent than others. Such selectivity could account for the observation that cap zero structures from STH-treated cultures contain low levels of uridine at N'.

In an earlier report, the presence of cap zero-bearing mRNAs in the cytoplasm of cells labeled in the presence of STH had indicated that 2'-O-methylation was not required for processing and transport of mature mRNA (Kaehler, et al., 1977). The present study indicates that cap zero-containing mRNAs are present on both monosomes and polysomes of cells which remained viable in the presence of 500 μM STH. We conclude that ribose methylation at N' does not appear to be requisite for ribosomal binding and subsequent translation of the undermethylated mRNA.

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