Synthesis, Methylation, and Capping of Nuclear RNA by a Subcellular System†

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ABSTRACT: A subcellular system is described which is capable of in vitro synthesis of large nuclear RNA and the formation of both cap I [$m^7G(5')pppX^mpYp$] and cap II [$m^7G(5')pppX^mpY^mpZp$] structures. This system, which consists of partially purified intact nuclei and residual cytoplasmic tags, carries out both guanosine addition, utilizing GTP, and the appropriate methylation reactions, utilizing *S*-adenosylmethionine as the methyl donor. The general structure of the caps was verified by analyses of methylated derivatives recovered after RNase T_2 hydrolysis and after digestion with P_1 nuclease, bacterial alkaline phosphatase, and nucleotide pyrophosphatase. Cap formation in large nuclear RNA species was found to be closely associated with transcription, as indicated by α-amanitin sensitivity and a requirement for the presence of all four nucleoside triphosphates. Recovery of a class of cap II structures, in which only the methyl group at position Y is labeled, as well as cap II structures in which all methylated constituents are labeled, indicates the presence of at least two independent methylation events in the in vitro system.

Recent findings in a number of laboratories (Wei et al., 1975; Perry et al., 1975a; Adams and Cory, 1975; Desrosiers et al., 1975; Furuichi et al., 1975) have shown that most eukaryotic mRNA molecules have cap structures of the form $m^7G(5')pppX^mp(Y^mp)Zp$ at their 5′ termini. The $m^7G(5')pppX^mpZp$ form of the cap structure is present in heterogeneous nuclear (hn[1]) RNA (Perry and Kelley, 1974; Perry et al., 1975b; Salditt-Georgieff et al., 1976), as well as in nuclear precursor molecules containing SV40 sequences (Lavi and Shatkin, 1975) and adenovirus sequences (Wold et al., 1976; Sommer et al., 1976).

These findings have raised several questions regarding the capping process in relation to the maturation of heterogeneous nuclear RNA. In particular, one would like to know the timing of the guanylation and methylation reactions, the mechanisms by which these reactions occur in eukaryotic cells, and their relevance to mRNA processing. To this end, we have developed a subcellular system consisting of partially purified L-cell nuclei capable of forming cap structures on hnRNA which are indistinguishable from those formed in intact cells. In this report, we describe the general characteristics of this in vitro system, including a dependence on continued RNA synthesis for cap formation and the formation of cap II structures by two sequential methylation events.

Groner and Hurwitz (1975) have also described cap formation by a subcellular system derived from HeLa cells, although, in this case, the species of capped RNA was not characterized. Moreover, these authors claimed that their system produced caps with diphosphate bridges in addition to the caps with triphosphate bridges normally observed in vivo. No such diphosphate bridges were produced by our in vitro system.

Experimental Procedures

Preparation and in Vitro Incubation of Partially Purified Nuclei. Mouse L cells were grown as described by Perry and Kelley (1968) and pretreated with actinomycin D (80 ng/ml) for 40 min to suppress rRNA synthesis (Perry and Kelley, 1970). About 1.5×10^8 cells were chilled, harvested, and washed with a balanced salt solution. All subsequent steps were carried out at 4 °C. Cells were then washed with 0.25 M sucrose, 5.5 mM $CaCl_2$, and suspended and lysed in 12 ml of 0.25 M sucrose, 5.5 mM $CaCl_2$ by Dounce homogenization, a modification of the method originally described by Lynch et al. (1970). The homogenization procedure was repeated with 10 ml of 0.25 M sucrose, 5.5 mM $CaCl_2$. The nuclei were pelleted by centrifugation at 1500 rpm for 3 min at 4 °C in an International centrifuge, and the pellet was washed with 6 ml of 0.25 M sucrose, 1.4 mM $CaCl_2$ by pipetting. At this point, the nuclei still showed considerable contamination with cytoplasmic tabs, as visualized by staining with methyl green pyronine (nna). Attempts were made in preliminary experiments to remove the residual cytoplasm by detergent washing of the nuclei, but the resultant preparations exhibited negligible incorporating activity for *S*-adenosylmethionine. The crude nuclear pellet was suspended by the addition of 0.2 ml of 0.25 M sucrose, 1.4 mM $CaCl_2$ and used in the incubation system at a concentration of 1.5×10^8 nuclei/ml.

The incubation system contained the following components at the indicated final concentration: 0.2 mM ATP, CTP, and UTP; 0.1 mM GTP; 100 μg/ml of yeast tRNA, as protection against degradation by nonspecific nucleases; 2 mM dithiothreitol; 50 mM Tris-HCl, pH 7.4; 2 mM $MgCl_2$; 1 mM $MnCl_2$; 40 mM KCl; 20 mM $(NH_4)_2SO_4$, to enhance the activity of RNA polymerase II; 2.6 μM *S*-[methyl-^{3}H]adenosylmethionine (^{3}H-SAM) (New England Nuclear Corp., 11.6 Ci/mmol)—neutralized at the time of addition; and [α-^{32}P]GTP (New England Nuclear Corp.) as indicated in individual experiments. The incubations were carried out for 30 min at 37 °C. In some experiments, nuclei were washed after incubation with 2 volumes of 10 mM Tris-HCl, pH 7.4, 10 mM

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[1] Abbreviations used: hn RNA, heterogeneous nuclear ribonucleic acid; [^{3}H]SAM, *S*-[*methyl*-^{3}H]adenosylmethionine; DOC, deoxycholate; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid.

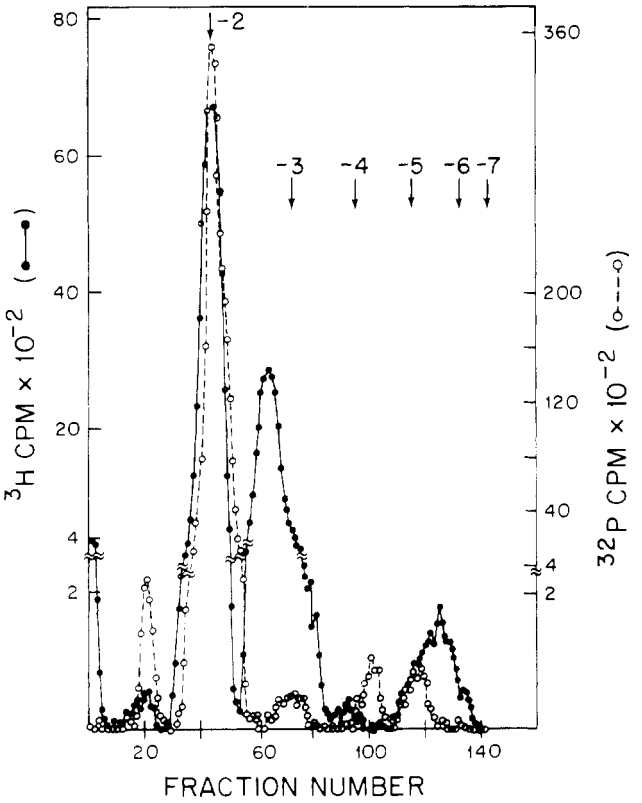

FIGURE 1: Separation of alkali digestion products of in vitro labeled nuclear RNA on DEAE-Sephadex A-25 column. Partially purified L cell nuclei were incubated with [^{3}H]SAM and [α-^{32}P]GTP (2 mCi/μmol) for 30 min. The RNA was extracted, purified on a Sephadex G-50 column, and hydrolyzed in 0.3 N KOH, 18 h, 37 °C. The hydrolysate was neutralized and separated on a DEAE-Sephadex A-25 column in 0.02 M Tris-HCl, pH 7.4, 7 M urea with a 0.1–0.5 M NaCl gradient in the same buffer. Arrows indicate charge positions of elution of [^{14}C]oligo(A markers. (●) ^{3}H cPM; (○) ^{32}P cPM.

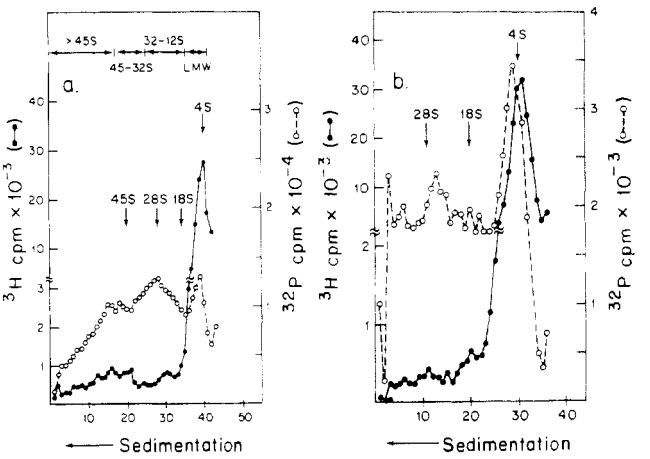

FIGURE 2: Sucrose gradient sedimentation of in vitro labeled RNA. L cell nuclear preparations were labeled with [^{3}H]SAM and [α-^{32}P]GTP (2 mCi/μmol for a, 0.2 mCi/μmol for b), and RNA was extracted and purified as in Figure 1. (a) Sedimentation in a 15–30% (w/w) sucrose gradient in sodium dodecyl sulfate buffer for 17 h at 14 000 rpm, 20 °C, in a Beckman SW27 rotor. (b) RNA was heated at 60 °C for 2 min in 80% Me_2SO, 1 mM EDTA, immediately diluted, and layered on a 5–25% (w/w) sucrose gradient in sodium dodecyl sulfate buffer with a 1 ml 40% (w/w) sucrose cushion. Centrifugation was for 16 h at 20 000 rpm, 20 °C, in a SW27 rotor. Fraction 23 is estimated as position for 12S RNA sedimentation.

NaCl, 1.5 mM $MgCl_2$, containing 0.5% deoxycholate (DOC) and 1% Tween 40 (Tween–DOC wash).

RNA Extraction and Fractionation. RNA was extracted from the Tween–DOC wash fraction as described by Perry et al. (1972). RNA was extracted from the nuclear preparation by a modification (Hames and Perry, submitted) of the procedure by Holmes and Bonner (1973), and separated from mononucleotides on a Sephadex G-50 column. The isolated RNA was separated by 5–25% (w/w) or 15–30% (w/w) sucrose gradient centrifugation in sodium dodecyl sulfate containing buffer (Perry and Kelley, 1974) after denaturation in 80% dimethyl sulfoxide at 60 °C for 2 min.

Mononucleotides and oligonucleotides were separated by DEAE-Sephadex column chromatography in 7 M urea after hydrolysis with KOH or various enzymes, as indicated in legends to figures and tables (Perry et al., 1975a,b; Perry and Kelley, 1976).

Results

Incorporation of [α-^{32}P]GTP and S-[methyl-^{3}H]Adenosylmethionine (SAM). Incubation of partially purified L cell nuclei with [α-^{32}P]GTP and [^{3}H]SAM resulted in both ^{32}P and ^{3}H incorporation into RNA that could be isolated in the void volume of a G-50 Sephadex column. The overall extent of incorporation of both ^{3}H and ^{32}P increases with time during a 30-min incubation. Alkaline hydrolysis of the in vitro labeled RNA followed by DEAE-Sephadex chromatography gave the radioactivity profile shown in Figure 1. In addition to the large peaks of mononucleotides with −2 charge and dinucleotides with −3 charge, a significant amount of material labeled with both ^{32}P and ^{3}H could be recovered in a peak migrating with a charge of −5 to −6. As will be demonstrated below, essentially all of the ^{3}H radioactivity and about 80% of the ^{32}P radioactivity in the −5 to −6 peak represent cap constituents (see below and Figure 5). From the specific activities of the [^{32}P]GTP and [^{3}H]SAM in the incubation mixture, we calculated an incorporation of 0.30 pmol of methyl group and 0.42 pmol of GMP per 10^8 nuclei in the −5 to −6 peak. A peak of material containing ^{32}P, but no ^{3}H label, was observed eluting in the region of the −4 marker. This, most likely, represents pGp derived from uncapped 5′ termini, or any pXp in which the 3′P comes from an adjacent nearest-neighbor G residue.

It was of interest to determine the extent to which cap formation is associated with continued RNA synthesis in this in vitro system. Therefore, the ability of the system to incorporate [^{3}H]methyl and [^{32}P]GMP was measured in the absence of two of the four nucleoside triphosphates. UTP and CTP were

TABLE I: In Vitro Incorporation of [^{3}H]Methyl and [^{32}P]GTP in the Absence of Two of the Nucleoside Triphosphates.

	^{3}H cpm		%
	Complete Mixture	minus UTP and CTP	Residual Incorp.
	^{32}P		
Total RNA (~100% mononucleotide)[a]	18 400	1 250	7
	^{3}H		
Total RNA	27 100	17 300	64
Oligonucleotide (−5 to −6)	1 500	<36[b]	<2
Mononucleotide (−2)	17 800	12 000	68
Dinucleotide (−3)	7 820	5 130	66

[a] In this experiment, the amount of [^{32}P]GTP used was not sufficient to detect incorporation into components other than mononucleotides. [b] Estimated maximum counts, within counting error, for the entire −5 to −6 region of the DEAE-Sephadex column.

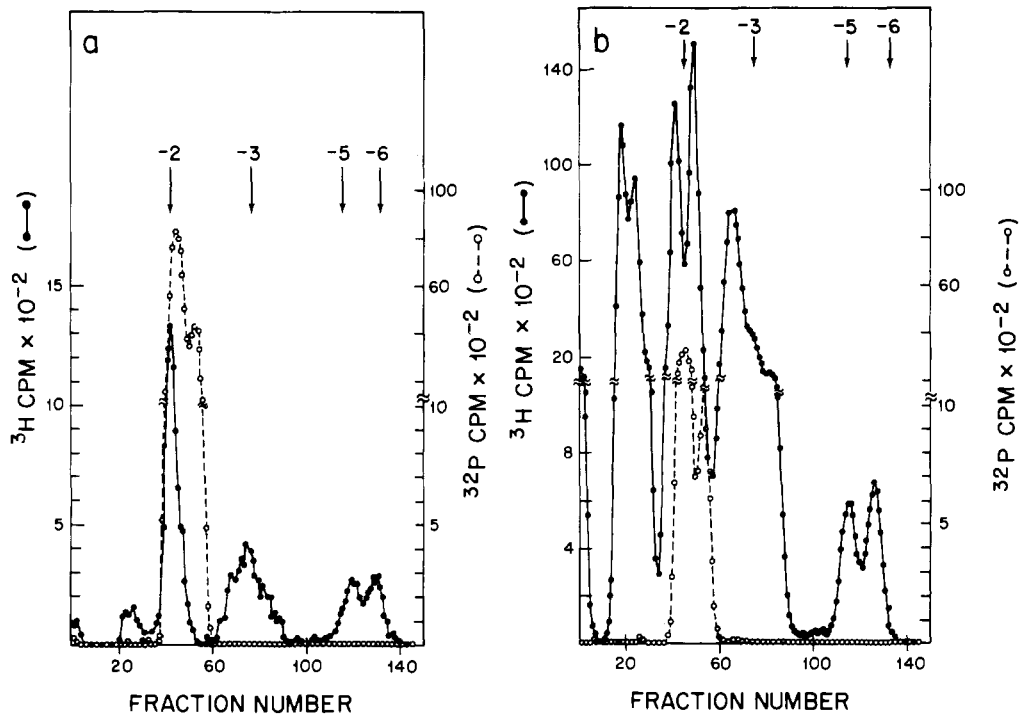

FIGURE 3: Analysis of the methylated derivatives of in vitro labeled RNA fractionated according to size. Sucrose gradient fractions >12 S (2–23, Figure 2b) and <12 S (24–34, Figure 2b) were pooled, the RNA was isolated and hydrolyzed with RNase T_2, and the products were submitted to DEAE-Sephadex chromatography, as described for Figure 1. (a) >12S RNA; (b) <12S RNA; (●) ^{3}H cpm; (○) ^{32}P cpm.

omitted from the incubation mixture, since these nucleotides, although essential for RNA synthesis, are presumably not directly involved in cap formation. As can be seen in Table I, omission of UTP and CTP led to an 85% reduction in ^{32}P incorporation and a reduction in overall ^{3}H incorporation of 36%. Analysis by DEAE-Sephadex chromatography indicated that the residual ^{3}H incorporation was confined exclusively to mononucleotides and dinucleotides, which, as shown below (Figures 2 and 3), are derived mainly from internal methylations of low-molecular-weight RNA species. No detectable incorporation of [^{3}H]methyl was observed in the −5 to −6 oligonucleotides. These results indicate that continued RNA synthesis is necessary for cap formation in this cell-free system. The relative insensitivity of methyl incorporation in the mononucleotide and dinucleotide fractions to the lack of a full complement of nucleoside triphosphates presumably reflects a significant pool of preformed molecules that are capable of accepting methyl groups at internal positions. This pool could include the added yeast tRNA, which is present in our system.

Characterization of the in Vitro Labeled RNA. The size distribution of the in vitro RNA products was examined by sedimentation analysis on sucrose gradients (Figure 2) with and without exposure to stringent denaturation conditions (80% Me_2SO, 2 min at 60 °C). It is seen that [^{32}P]GMP and [^{3}H]methyl groups are incorporated into all size classes of nuclear RNA, the labeled GMP being predominant in large RNA molecules and the [^{3}H]methyl groups in small (<12 S) molecules. However, even after denaturation, there is a significant amount of [^{3}H]methyl radioactivity which sediments ≥12 S (Figure 2b). Since the cells had been treated with a low dose of actinomycin D prior to preparation of the subcellular extract, the synthesis and methylation of ribosomal RNA are suppressed, and there are no discernible peaks of radioactivity corresponding to rRNA or rNA precursors. These results indicate that the in vitro system is capable of synthesizing and methylating RNAs of sizes similar to those isolated from nuclei labeled in vivo (Perry et al., 1975b).

The methylated components in RNA >12 S and in the low molecular weight (<12 S) were analyzed by DEAE-Sephadex chromatography after digestion with ribonuclease T_2 (Figure 3). The distribution of methylated components in RNA >12 S (Figure 3a) is very similar to that observed for in vivo methylated mRNA (Perry et al., 1975a,b). There are the mononucleotide and dinucleotide peaks representative of internal base and 2′-*O*-methylations, respectively, and the oligonucleotide peaks representative of cap I and II structures. The amount of [^{32}P]GTP used in this experiment was not sufficient to detect incorporation into components other than mononucleotides. The identity of the −3 peak as dinucleotide is consistent with the fact that this material loses two charges upon treatment with alkaline phosphatase (data not shown). The identities of the −5 and −6 peaks as cap I and II structures were established by a detailed analysis described below. In the in vivo labeled mRNA preparations, the internal base-methylated nucleotides consist exclusively of 6-methyladenylate, and the internal 2′-*O*-methylated nucleotides are attributable to small quantities of contaminating rRNA.

The chromatographic pattern of the low-molecular-weight RNA (Figure 3b) is more complex and less readily interpretable. The amount of labeled mono- and dinucleotide relative to the −5 to −6 oligonucleotide is vastly greater than in the case of the >12S RNA, and there is an additional peak eluting before the mononucleotides, which is not observed with in vivo labeled mRNA. Some of these methylated components are probably derived from the small nuclear RNA molecules (Reddy et al., 1974; Shibata et al., 1975), which contain capped termini and a substantial number of internal 2′-*O*-methylated residues. The small nuclear RNA molecules that have been analyzed to date (U_1, U_2, and U_3) contain a 5′-terminal cap

TABLE II: Synthesis and Capping in Relation to Size.

RNA Size	Incorp. [α-^{32}P]GTP (pmol) Total	Cap[a]	Total pmol of NTP Incorp[b]	pmol of Cap[c]	Total Nucleotides Incorp/Cap	No. Av Mol Wt[d] (No. of Nucleotides)
>32S	49.6	0.057	198	0.044	4500	13 345
32–10S	28.9	0.054	115	0.042	2740	3 360

[a] The incorporation into cap was calculated from radioactivity (−3 to −4) in oligonucleotides after KOH hydrolysis and bacterial alkaline phosphatase digestion of RNA fractionated as in Figure 2a. [b] Assuming 25% G in hnRNA. [c] Assuming 1 mol of m^7pG and 0.3 mol of pGm per mol of cap. [d] The number average molecular weight of each fraction calculated as described by Hames and Perry (submitted).

TABLE III: Effect of α-Amanitin on [^{3}H]SAM Incorporation.[a]

RNA Size	Methylated Derivatives	^{3}H cpm Control	^{3}H cpm +α-Amanitin	% of Control
>45S	Total cap	160	0	0
	Mononucleotide	602	101	17
10–32S	Cap I	972	368	38
	Cap II	1 260	412	33
	Mononucleotide	13 170	3910	30
<10S	Cap I	1 160	1486	128
	Cap II	1 685	1196	71
	Mononucleotide	10 600	9480	89

[a] Partially purified L cell nuclei were incubated in vitro for 30 min, as described for Figure 1 in the presence and absence of 0.4 μg/ml of α-amanitin. The extracted RNA was separated on a 15–30% (w/w) sucrose gradient in sodium dodecyl sulfate buffer after denaturation in 80% Me_2SO for 2 min at 60 °C. The indicated size classes of RNA were pooled, digested with RNase T_2 and bacterial alkaline phosphatase, and analyzed by DEAE-Sephadex chromatography in 7 M urea in the presence of appropriate ^{14}C-marker oligonucleotides.

structure with two adjacent 2′-*O*-methyl nucleotides, which would elute with a −6 charge. If these cap structures are formed by sequential 2′-*O*-methylation reactions, as is the case for mRNA caps (Perry and Kelley, 1976), an intermediate structure eluting at −5 might also be expected. Some of the other species of methylated small nuclear RNAs, e.g., U_{1b}, $4.5S_I$, and $5S_{III}$, may also contain either a cap I or cap II structure. Other methylated components may be derived from transfer RNA and from incompletely transcribed molecules of various types.

Since a substantial portion of the in vitro transcription might be expected to represent extension of in vivo initiated RNA molecules, it was of interest to determine the extents of synthesis and cap formation in relationship to the size of the hnRNA. RNA molecules were labeled in vitro with [α-^{32}P]GTP, separated on sucrose gradients, analyze in terms of number average molecular weight, and assayed for cap structures by DEAE-Sephadex chromatography. The data, summarized in Table II, indicate that there are roughly equal amounts of cap produced in the >32S and 32–10S RNA fractions. The smaller RNAs incorporate less total NTP per cap, but relative to their size the fractional incorporation is actually greater. For large (>32 S) RNA, the number of nucleotides incorporated per cap is substantially less than the number average molecular weight, suggesting that most large chains are only extended in vitro and that little, if any, initiation of transcription occurs for this size class of RNA. For the 10–32S RNA, the number of nucleotides incorporated per cap is about 0.8 of the number average molecular weight. For this size class, there may be initiation of RNA transcription, as well as chain extension.

α-Amanitin Inhibition of the in Vitro System. In order to further our understanding of the relationship between the transcription and capping reactions in this in vitro system, we studied the effects of α-amanitin on the incorporation of [^{3}H]SAM into the methylated components of the various size classes of RNA. At low concentrations, α-amanitin specifically and rapidly inhibits RNA polymerase II (Balatti et al., 1970; Lindell et al., 1970; Weinmann and Roeder, 1974), and would thus be expected to affect the methylation reactions which are specifically associated with hnRNA synthesis. As may be seen in Table III, both internal methylation and cap formation are severely inhibited in nuclear RNA >45 S. About a 65% inhibition in both reactions was observed in the 10–32S RNA fraction. Since some of the residual mononucleotide and cap in this fraction are probably derived from contamination by the low-molecular-weight region of the gradient, the true inhibition of the 10–32S hnRNA is likely to be even greater than 65%. In contrast, the methylation of <10S RNA is very little affected by the α-amanitin concentration used in these experiments. The foregoing observations suggest that RNA polymerase II activity is not necessary for the methylation of the low-molecular-weight RNA, but is essential for synthesis and cap formation in RNA >10 S.

Detailed Analysis of Caps. To substantiate the identification of the cap structures, to clarify further the relationship between the two types of caps, and to determine whether both guanylation and methylation reactions occurred in vitro, we analyzed the constituents of the −5 to −6 oligonucleotides derived from >12S RNA. Digestion of these oligonucleotides with nucleotide pyrophosphatase yields presumptive m^7pG, pX^mpZp and pX^mpY^mpZp, which migrate at −1 and −5 to −6, respectively (Figure 4a). In an experiment of this type, in which the oligonucleotides were doubly labeled with [α-^{32}P]GTP and [methyl-^{3}H]SAM, the ring opened m^7pG eluting with a −2 charge was estimated to contain 0.09 pmol of methyl and 0.05 pmol of phosphorus, and the pX^mpZp and pX^mpY^mpZp to contain 0.12 pmol of methyl and 0.11 pmol of phosphorus (Figure 5). In addition, we observed 0.04 pmol of pGp eluting with a −4 charge; this was presumably derived from some pppGp and/or ppGp that cochromatographed with the cap material. The presence of labeled phosphorus, as well as labeled methyl group in the m^7pG constituent (Figure 5), is evidence that cap formation in vitro involves guanosine addition, as well as methylation. The presence of ^{32}P label in the pY^mpZP constituent (−5 charge) demonstrates that some of these capped sequences were transcribed in vitro. Since a large part of the transcriptional activity represents chain extension, rather than chain initiation, we may presume that some of these 5′-

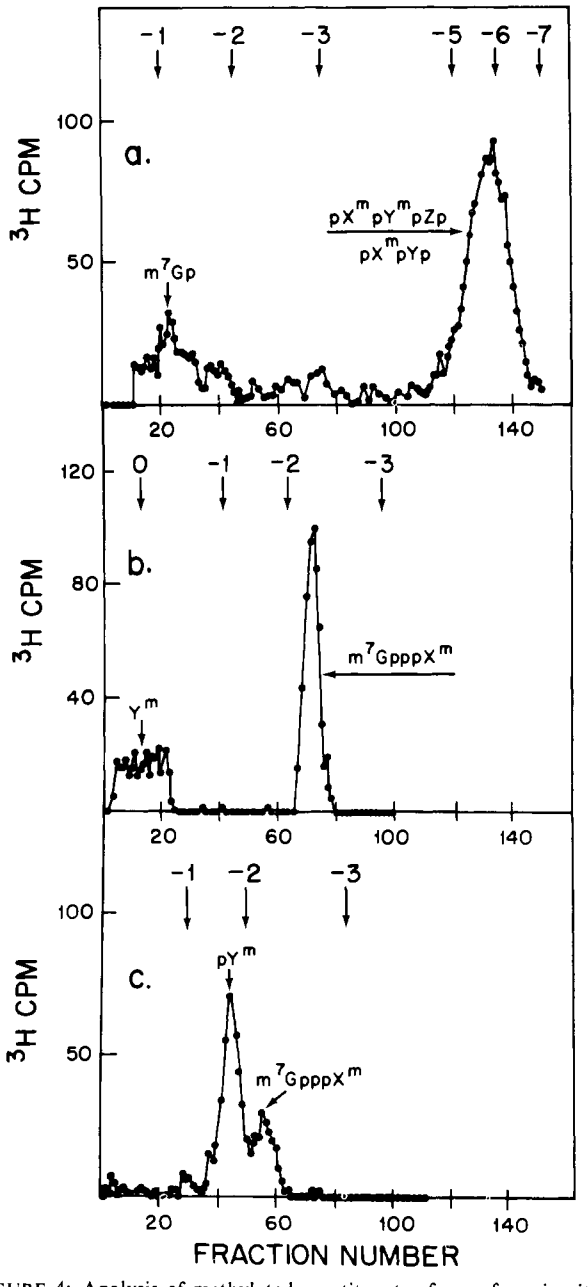

FIGURE 4: Analysis of methylated constituents of caps from in vitro labeled RNA >12 S. The oligonucleotides eluting between −5 and −6 charge units in Figure 3a were concentrated on DEAE-Sephadex. One portion (a) was treated with 40 μg of nucleotide pyrophosphatase/ml of 1.3 mM $MgCl_2$ for 40 min at 37 °C (this reaction went to 78% completion as estimated from data in Figures 4b and 3a), and the remainder (b) with 250 μg/ml of P_1 nuclease (*Penicillium* nuclease) in acetate buffer at pH 6.0 for 45 min at 37 °C, followed by 50 μg of bacterial alkaline phosphatase/ml at pH 7.2 for 2 h at 37 °C. (c) The cap II peak was isolated from a T_2 and alkaline phosphatase digest of >12S RNA labeled for 30 min in vitro with [^{3}H]SAM. The cap II material was concentrated and treated with P_1 nuclease as in panel b, but not with alkaline phosphatase. Products of these reactions were separated by DEAE-Sephadex chromatography.

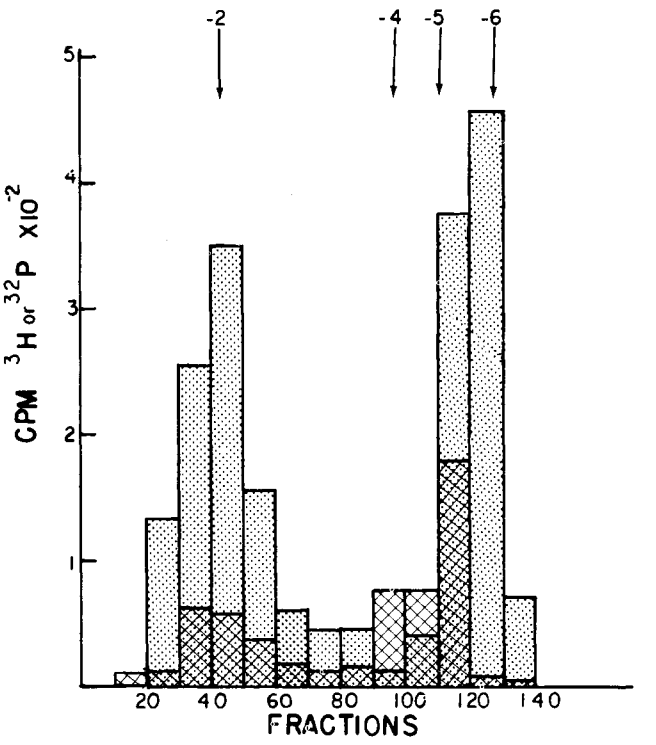

FIGURE 5: Analysis of nucleotide pyrophosphatase derivatives of caps labeled in vitro with [α-^{32}P]GTP and [^{3}H]SAM. The oligonucleotide peak eluting between −5 and −6 charge units in Figure 1 was concentrated on DEAE-Sephadex and treated with nucleotide pyrophosphatase, as described for Figure 4a. The digest was separated by DEAE-Sephadex chromatography. The stippled and cross-hatched bars, respectively, represent ^{3}H and ^{32}P radioactivity recovered from the column fractions.

terminal sequences arise by processing cleavages in the regions of the hnRNA which are transcribed in vitro.

Digestion of the −5 to −6 oligonucleotides with P_1 nuclease and alkaline phosphatase (Figure 4b) yields uncharged material (nucleosides), which is not absorbed to the DEAE-Sephadex column, and a peak of material corresponding to a charge of about −2.5, consistent with the structure m^7GpppX^m. This result indicates that the pyrophosphate linkage of the in vitro formed cap structure must be analogous to the triphosphate linkage observed in mRNA cap structures in vivo (Wei et al., 1975; Furuichi et al., 1975; Adams and Cory, 1975), and not a diphosphate linked cap structure (Groner and Hurwitz, 1975), the charge of which would be about −1.5. Another preparation of [^{3}H]SAM labeled −5 to −6 oligonucleotides was treated with P_1 nuclease and alkaline phosphatase and was separated by two-dimensional chromatography on cellulose plates (Figure 6). The [^{3}H]methyl-labeled derivatives comigrated with individual m^7GpppX^m (cap core) markers in this system. These results, as well as those cited above, confirm the identification of the in vitro labeled cap cores.

Cap II appears to be the predominant cap form after short labeling times in vivo, and, to a large extent, is labeled only in the Y^m moiety of the molecule (Perry and Kelley, 1976). It is, therefore, of interest to determine whether the cap II, recovered in the present experiments, was synthesized entirely in vitro or, also, was a product of a second ribose methylation of an in vivo preformed cap structure. The data in Figures 3a and 4b enable us to estimate the relative fractions of cap II structures which have been formed by a second O-methylation of previously formed cap I structures, and of cap II which had been formed entirely in vitro. The total cpm in caps I and II is represented by the sum of radioactivities in the Y^m (305 cpm) and m^7GpppX^m (617 cpm) peaks of Figure 4b, i.e., 922 cpm. The fraction of this total which is in cap I is given by the ratio of cap I to total cap counts in Figure 3a, i.e., 0.52. Thus, there are 0.52 × 922 = 480 cpm in cap I. Accordingly, the amount of label in the m^7GpppX^m (core) portion of cap II is 617 cpm less 480 cpm or 137 cpm. Since there are two methyl groups in the core and one in Y^m, the ratio of the moles of core to moles of Y^m is

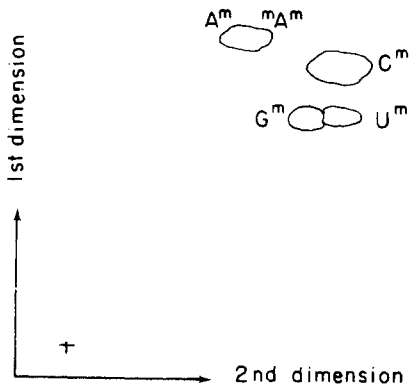

FIGURE 6: Cap core analysis by thin-layer chromatography on cellulose plates. The >12S RNA labeled with [^{3}H]SAM in vitro was digested with T_2 RNase and the cap material in the $-5/-6$ region was isolated from a DEAE-Sephadex (urea)column. The caps were desalted, concentrated, and further digested with P_1 nuclease and bacterial alkaline phosphatase, and the digest was separated by two-dimensional thin-layer chromatography on cellulose plates (Merck) with cap core (m^7GpppX^m) standards (PL Biochemicals). Development in first dimension was with isobutyric acid:0.5 M NH_4OH (5:3); development in second dimension was with saturated $(NH_4)_2SO_4$:1 M NaOAc:2-propanol (40:9:1). The cap core markers were located by uv fluorescence, cut out, and counted in liquid scintillant. Appropriate blank spots from the chromatogram were treated in a similar manner and corresponded to: 16, 15, and 16 cpm each. Spots on the plate, marked by the nucleotide in the X^m position of each "cap core", contained the following amounts of radioactivity (corrected for blanks):

cap core	^{3}H cpm	% cpm
$m^7GpppA^m/m^7Gpppm^6A^m$	380	82
m^7GpppG^m	34	4
m^7GpppC^m	71	12
m^7GpppU^m	22	1

$(137/2) \div 305 = 0.22$. This value is equivalent to the fraction of radioactive molecules which are labeled in both core and Y^m, i.e., the fraction formed entirely in vitro. The complementary fraction, 0.78, represents molecules labeled only in Y^m, i.e., those formed by a second methylation of preformed cap I structures.

An independent measurement of these ratios was obtained from cleavage of isolated cap II by P_1 nuclease, as shown in Figure 4c. The recovery of radioactivity in pY^m, eluting at about -2, and in m^7GpppX^m, eluting at about -2.5, was 532 and 200 cpm, respectively. Accounting for the difference in the number of methyl groups, as in the calculation above, we have for the ratio of moles of core to moles of Y^m: $(200/2) \div 532 = 0.19$. The values from the two independent measurements are in very good agreement, and indicate about 20% of the cap II molecules have been formed entirely in vitro and 80% are molecules which were brought to the cap I stage in vivo and then received their second 2′-O-methylation in vitro. This provides strong evidence for the occurrence of at least two independent methylation events in the formation of caps. Both of these events can apparently occur in the in vitro system.

Cellular Site of Second 2′-O-Methylation. Previous reports by Perry et al. (1975b) and Perry and Kelley (1976) indicated that cap II formation was probably a cytoplasmic event, since only cap I structures could be recovered from nuclear hnRNA and Y^m labeling of cap II structures was found on polyribosomal mRNA after very brief labeling times. Since both cap I and cap II structures were recovered after in vitro incubation in >12S RNA, an attempt was made to differentiate between the cytoplasmic and nuclear components of the partially purified nuclear preparation. A crude nuclear preparation was incubated with [^{3}H]SAM and afterwards the nuclei were washed with a detergent mixture (Tween–DOC), which is effective in removing residual cytoplasmic tabs (Penman, 1966). RNA extracted from detergent-cleaned nuclei and from the wash fraction was sedimented through a sucrose gradient and the components >12 S were analyzed for methylated derivatives after digestion with RNase T_2. The data, summarized in Table IV, indicate that a considerable portion of both mononucleotide and cap components are extractable by the detergent wash. Some of the extractable material may represent artifactual leakage from the in vitro incubated nuclei. Alternatively, it is possible that the in vitro system is also capable of processing reactions, which would result in the translocation of nuclear synthesized products to the (cytoplasmic) wash fraction. As indicated by the ratio of cap II/I, the wash fraction is considerably enriched in cap II, as compared to the nuclei. This would be expected on the basis of in vivo data (Perry et al., 1975b; Perry and Kelley, 1976) if the cytoplasmic remnants were contained in the detergent wash fraction.

TABLE IV: Removal by Detergent Wash of Methylated Components of >12S RNA Labeled During in Vitro Incubation of Partially Purified Nuclei.

	^{3}H cpm		% cpm
	Detergent Wash	Nuclear Residue	Removed by Wash
Total RNA >12 S	18 300	11 800	61
Cap II	2 890	634	82
Cap I	1 980	684	74
Mononucleotide	7 460	4 080	65
Ratio cap II/cap I	(1.5)	(0.9)	

[a] Partially purified L cell nuclei were incubated in vitro and washed with Tween-deoxycholate, as described under Experimental Procedures. RNA was extracted from the residual nuclei and the wash fraction and separated on a 5–25% (w/w) sucrose–sodium dodecyl sulfate gradient after denaturation in 80% Me_2SO for 2 min at 60 °C. The fractions containing RNA larger than 12 S were pooled, the RNA was hydrolyzed with T_2 RNase and bacterial alkaline phosphatase, and the labeled oligonucleotide components were analyzed by DEAE-Sephadex chromatography in 7 M urea.

Discussion

The subcellular system described herein is capable of in vitro synthesis and capping of large nuclear RNA. The fact that our system exhibits continued incorporation upon incubation at 37 °C for up to 30 min may reflect the presence of contaminating cytoplasm which helps stabilize the system. Much of the transcriptional activity observed in our system seems to involve the extension of RNA chains, rather than the initiation of new ones, at least insofar as the large RNA molecules are concerned.

Since we wish ultimately to study nuclear RNA synthesis and processing by following posttranscriptional capping and methylation reactions in vitro, it is of utmost importance to establish that the in vitro products resemble those produced in vivo. A significant portion of the in vitro RNA product is indeed >45 S. Under conditions where rRNA synthesis has been inhibited with actinomycin D, greater than 32% of the labeled GMP is found in molecules which are larger than 28 S after exposure to stringent denaturation conditions. The average size of the hnRNA molecules labeled in vitro is somewhat smaller than that observed for in vivo labeled hnRNA (Perry et al., 1975b), suggesting that some degradation and/or processing has occurred during the in vitro incu-

bation. Nevertheless, a considerable quantity of large molecules can still be recovered after a 30-min incubation.

The observed in vitro cap formation and internal methylation of nuclear RNA indicates that the system contains the enzymes and specificity determinants for base modification and cap formation. In the hnRNA >12 S, the predominant methylated components are internal base-methylated nucleotides and caps. A variable dinucleotide peak is observed, which is probably attributable to methylation of preformed ribosomal RNA precursors and of a small amount of pre-rRNA which is made in spite of the preincubation with actinomycin D. Analysis of the cap constituents indicated that the cap-forming reactions involve both guanylation, in which the α-phosphate of GTP is contributed, and methylations, in which the methyl group of *S*-adenosylmethionine is donated. It was also evident that at least some of the capped sequences had been transcribed in vitro. In view of the data suggesting little or no initiation in our system for large molecules, it is reasonable to suppose that some of these capped molecules are derived from the cleavage of molecules initiated in vivo and elongated in vitro in the region of the cleavage site.

The system is capable of forming both cap I and cap II structures. Some of the cap II structures are completely formed in vitro, whereas others are processed to the cap I stage in vivo and receive the second *O*-methylation at position Y^m in vitro. The second methylation is probably carried out in the peripheral cytoplasmic material, which surrounds the nuclei in our preparation, and which is mostly removable by a postincubation detergent wash. If this is the case, then the fact that cap II structures formed entirely in vitro are also found in the detergent wash suggests that our in vitro system may also be capable of reactions that mimic the transport of capped molecules from nucleus to cytoplasm. Moreover, since the system exhibits both the nuclear and cytoplasmic methylating activities, it has the potential to discriminate between the species of mRNA molecules which do and do not serve as substrates for the second 2′-*O*-methylation. Analysis of the constituents of both types of cap structures after treatment by nucleotide pyrophosphatase, P_1 nuclease, and P_1 nuclease plus alkaline phosphatase demonstrated that the in vitro formed caps are analogous to those obtained from mRNA and nuclear hnRNA in vivo, all caps containing m^7G in a triphosphate linkage to one or two *O*-methylated nucleosides. No evidence was obtained for a cap containing a diphosphate linkage, as postulated by Groner and Hurwitz (1975).

By the use of α-amanitin, which through patterns of inhibition in cell-free systems clearly differentiates between RNA polymerase I, II, and III (Balatti et al., 1970; Lindell et al., 1970; Weinmann and Roeder, 1974), it was possible to investigate whether the in vitro capping reactions depend on transcriptional activity of RNA polymerase II. Our results indicated that the production of caps in >12S RNA does indeed require the continued activity of RNA polymerase II. In contrast, the production of cap-like structures on low-molecular-weight RNA is considerably less sensitive to inhibition by α-amanitin. That the caps in the small RNA molecules are not derived from the breakdown of larger RNA is indicated by short-term labeling data. All of the cap structures labeled during a 5-min incubation are in small RNA molecules; none are detected in >12S RNA.

A more likely source of the cap structures in the <12S RNA are the low-molecular-weight stable nuclear RNA species (Weinberg and Penman, 1968; Ro-Choi et al., 1970; Hellung-Larsen and Fredriksen, 1972), most of which are methylated (see Weinberg, 1973, for a review). At least three of these RNAs have been shown to contain a $m_3^{2,2,7}Gp$ in the 5′-terminal oligonucleotide linked by a 5′,5′-pyrophosphate bond to adjacent 2′-*O*-methylated nucleotides (Ro-Choi et al., 1974). If these RNAs were synthesized by polymerase III, as are some of the other small RNA species (Weinmann and Roeder, 1974), then their synthesis would not likely be inhibited by the concentrations of α-amanitin used in our experiments.

The requirement for all four nucleoside triphosphates, together with the α-amanitin inhibition of cap formation, would seem to indicate that continued synthesis of nuclear RNA is necessary for cap formation to occur in the in vitro system. In this cell-free system, there does not seem to be a large pool of hnRNA with termini that may be potentially capped. The coupling with transcription that we observe for large hnRNA molecules may mean that, for these molecules, further elongation and separation from the DNA template are required for cap formation. Even the RNAs that are methylated in vitro only at position Y^m seem to require some additional transcription before the second *O*-methylation. If the methylations at position Y are indeed occurring in the peripheral cytoplasm, as we have postulated above, we would interpret this result to mean that some growing RNA chains are already capped at their 5′ termini and that those chains are sufficiently extended in vitro to allow their release from the DNA template, either by transcriptional termination or by a processing cleavage. It is possible that the pool of fully transcribed hnRNA that could serve as substrate for cap formation is very small, or, alternatively, that it is depleted during the preparation of our in vitro system. Preferential methylation of newly synthesized molecules has also been noted for preribosomal RNA in isolated rat liver nucleoli (Grummt et al., 1975).

Finally, it should be pointed out that, although our in vitro system does yield presumptive 5′ termini of the type ppGp and pGp, we have not been able to detect any uncapped termini that contain 2′-*O*-methylated derivatives, for example, pX^mpYp, etc. Since such structures have not been found in vivo either, the speculation that 2′-*O*-methylations could occur internally in hnRNA and mark the sites of future cleavage and capping reactions (Rottman et al., 1974; Salditt-Georgieff et al., 1976) seems very unlikely. It is more reasonable to suppose that the order of capping events in eukaryotic cells is similar to that described for viral systems (Ensinger et al., 1975; Furuichi et al., in press) in that the formation of the m^7G precedes the 2′-*O*-methylation at position X^m.

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Poly(adenylic acid) Synthesis in Isolated Rat Liver Mitochondria†

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ABSTRACT: Purified rat liver mitochondria were shown to synthesize poly(adenylic acid) (poly(A)) in vitro. Detection of the poly(A) synthesizing activity was facilitated by addition of NaF to the reaction mixture. The product of the reaction was shown to be poly(A) by its insensitivity to digestion with pancreatic RNase and RNase T_1, its degradation by venom phosphodiesterase and its retention on poly(uridylic acid) cellulose columns. The average chain length of the product was 20-23 AMP units and it was covalently attached to the endogenous RNA in the mitochondria. Poly(A) synthesis required ATP and a divalent ion and was maximally active in the pH range of 7-8. The reaction was inhibited by atractyloside, cordycepin triphosphate, Rose Bengal, rifamycin derivative AF/013, sodium pyrophosphate, and *N*-ethylmaleimide. These studies indicate that the mitochondrial poly(A) polymerase previously described in our laboratory (Jacob, S. T., Rose, K. M., and Morris, H. P. (1974), *Biochim. Biophys. Acta 361,* 312-320) is involved in the posttranscriptional addition of poly(A) sequence to mitochondrial RNA.

It is well recognized that many eukaryotic mRNAs contain a poly(A)[1] sequence at their 3′ termini (for review, see Brawerman, 1974). In addition to polysomal mRNA, mitochondrial mRNA also has been shown to contain a poly(A) tract, albeit of a shorter length (Perlman et al., 1973; Ojala and Attardi, 1974; Hirsch and Penman, 1974). Although the exact physiological function of poly(A) has not been established, there is mounting evidence to suggest that it confers stability to, and consequently enhances translational efficiency of, some mRNAs (Marbaix et al., 1975; Sheiness et al., 1975; Levy et al., 1975).

Poly(A) polymerase, the enzyme presumed to be involved in the posttranscriptional addition of poly(A) to mRNA, has been well characterized in a variety of prokaryotic and eukaryotic cells. This enzyme has been identified in the nucleus (Edmonds and Abrams, 1960; Hyatt, 1967; Niessing and Se-

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[1] Abbreviations used: poly(A), poly(adenylic acid); poly(U), poly(uridylic acid); Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.