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Methylation and Capping of RNA Polymerase II Primary Transcripts by HeLa Nuclear Homogenates†

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ABSTRACT: HeLa nuclear homogenates incubated in vitro incorporate [β-^{32}P]ATP and *S*-[*methyl*-^{3}H]adenosylmethionine ([^{3}H]SAM) into blocked methylated 5′ termini of newly synthesized RNA. Approximately 10% of the RNA chains initiated in vitro with [β-^{32}P]ATP are subsequently blocked by condensation of GMP to di- or triphosphate terminated RNA. The blocked termini can then be methylated by transfer of methyl groups from [^{3}H]SAM to the 7 position of the guanosine and 2′-O position of the adenosine to form m^{7}Gpp*pAm- capped terminus. In addition to conventional triphosphate caps, HeLa nuclear homogenates produce capping structures containing two phosphate residues in the pyrophosphate bridge. The two distinct cap forms were separated by DEAE-cellulose chromatography and analyzed. In contrast to triphosphate caps (m^{7}GpppXm) in which X can be any one of the four nucleosides (G, A, C, or U), in diphosphate caps (m^{7}GppXm), more than 95% of the penultimate nucleoside Xm is G. Incorporation of both [β-^{32}P]ATP and [^{3}H]SAM into caps was markedly reduced by low concentrations of α-amanitin. However, an ammonium sulfate fraction of the nuclear homogenate can cap β-^{32}P-labeled RNA (pp*pA-RNA) to form m^{7}Gpp*pA-RNA, in the presence of 0.5 μg/mL of α-amanitin. Therefore, the nuclear capping enzyme is resistant to this drug. Our results indicate that RNA polymerase II primary transcripts are the substrate for the cellular capping enzyme and that the β phosphate in the pyrophosphate bridge (m^{7}G$^{\gamma}$p$^{\beta}$p$^{\alpha}$pXm) is derived from the 5′ ends of the RNA chains.

A wide variety of eukaryotic and viral mRNAs have been found to contain capping structures of the type m^{7}G(5′)ppp(5′)Xm at their 5′ termini (7-methylguanosine linked by 5′-5′ pyrophosphate bridge to the adjacent nucleotide) (for review, see Shatkin, 1976a). Capping structures are important for ribosome binding and mRNA translation (Muthukrishnan et al., 1975, 1976; Both et al., 1975a, 1976b) and cap analogues can cause inhibition of capped mRNA translation (Hickey et al., 1976; Canaani et al., 1976; Groner et al., 1976; Roman et al., 1976) as well as impairment of IF-M$_3$ mRNA interaction (Shafritz et al., 1976). It has also been suggested that capping and methylation may play a role during biogenesis of mammalian mRNAs and virus replicating in the nucleus (Rothman et al., 1974; Cory & Adams, 1975b; Salditt-Georgieff et al., 1976). The reactions involved in the formation of caps at the 5′ termini of viral mRNAs have been worked out for several viruses. It has been shown that vaccinia virus (Wei & Moss, 1975; Urishibara et al., 1975), reovirus (Furuichi et al., 1975), cytoplasmic polyhydrosis virus (cpv) (Furuichi & Miura, 1975), and vesicular stomatitis virus (VSV) (Abraham et al., 1975) particles contain virion-associated activities that are capable of methylating and capping these viral mRNAs. Three distinct enzymatic activities were implied (Furuichi et al., 1976; Moss et al., 1976) and subsequently isolated from vaccinia virus (Ensinger et al., 1975; Martin et al., 1975; Martin & Moss, 1975). In vaccinia virus, reovirus, and cpv, the capping reaction involves the transfer of guanosine monophosphate from GTP to the diphosphate terminated mRNA (ppX-) followed by methylation at the 7 position of the guanosine to form a m^{7}GpppX- terminus. However, a different mechanism that involves capping of a 5′-monophosphate end (pX-) was found for vesicular stomatitis virus (VSV) (Abraham et al., 1975).

Little is known about the biochemical steps and enzymes involved in the synthesis of capping structures in eukaryotic nuclear and messenger RNAs. It was previously reported that isolated HeLa nuclei (Groner & Hurwitz, 1975) and L cells nuclei (Winicov & Perry, 1976) incubated in vitro are able to form blocked methylated 5′ termini under conditions of RNA synthesis. α-Amanitin, a specific inhibitor of RNA polymerase II, markedly reduced formation of blocked termini. Thus, there appears to be some relationship between RNA synthesis catalyzed by HeLa and L cells nuclei and incorporation of [α-^{32}P]GTP (Groner & Hurwitz, 1975) or [^{3}H]SAM (Winicov & Perry, 1976) into 5′ caps. Recently, an enzyme that specifically methylates the guanosine residue of capping structures (RNA guanine-7-methyltransferase) was isolated from HeLa cells (Ensinger & Moss, 1976).

In order to gain further information about the mechanism of capping in eukaryotic cells, experiments were performed to

† From the Department of Virology, Weizmann Institute of Science, Rehovot, Israel. *Received August 19, 1977.* This research was supported by a grant from the United States-Israel Binational Science Foundation (BSF), Jerusalem, Israel, and in part by Contract NOI CP33220 from the National Cancer Institute.

0006-2960/78/0417-0977$01.00/0

determine whether initiation of RNA chains in vitro is coupled to capping. Cap formation in HeLa nuclear homogenates was studied using [β-^{32}P]ATP and [^{3}H]SAM as radioactive precursors. Our results indicate that, in HeLa nuclei, caps are formed at the 5′ termini of initial transcripts. Capping takes place preferentially at the 5′ end of RNA polymerase II transcripts by condensation of pG with either di- or triphosphate ends.

Experimental Procedures

In Vitro Incubation of Nuclear Homogenates. HeLa cell nuclei were prepared as described previously (Groner & Hurwitz, 1975) and stored in small aliquots in liquid nitrogen. Frozen nuclei were disrupted immediately when thawed. Nuclei were suspended in incubation medium, at a concentration of 10^8 nuclei/mL. Reaction mixtures (1–5 mL) contained: 40 mM Hepes[1]-KOH (pH 8.0); 1 mM dithiothreitol; 4 mM $MgCl_2$; 1 mM $MnCl_2$; 0.5 mM each of CTP, UTP, and GTP; 0.05–0.1 mM [β-^{32}P]ATP (50–72 Ci/mmol), 1.8 μM *S*-[*methyl*-^{3}H]adenosylmethionine ([^{3}H]SAM) (67 Ci/mmol). Incubation was for 60 min at 37 °C. Reactions were stopped by addition of 2 volumes of buffer containing 20 mM Tris-HCl (pH 8.0), 1% sodium dodecyl sulfate, 3 M urea, 300 mM NaCl, 0.5 mM EDTA, and RNA was extracted and separated from nucleotides as described by Groner & Hurwitz (1975) and Gilboa et al. (1977).

Synthesis of [β-^{32}*P*]*ATP.* Preparation of [β-^{32}P]ATP was as described in detail by Gilboa et al. (1977). The method consisted of two steps: (1) an exchange reaction using ADP and ^{32}P in the presence of highly purified *E. coli* polynucleotide phosphorylase (Soreq & Littauer, 1978); and (2) phosphorylation of the β-^{32}P-labeled ADP by pyruvate kinase to generate ATP. Specific activity values of 50 Ci/mmol or more were obtained.

Ammonium Sulfate Fractionation of Nuclear Homogenate. To 1 mL of nuclei suspension (1.8×10^8 nuclei), 476 mg of ammonium sulfate was slowly added. After 20 min in the cold, the suspension was homogenized with a Dounce homogenizer; the precipitate was collected by centrifugation at 18 000*g* of 15 min and resuspended in 0.2 mL of 4 mM Hepes–KOH (pH 8.0), 50 mM $(NH_4)_2SO_4$, 1 mM dithiothreitol, and 5% glycerol (buffer A). The ammonium sulfate extract was filtered through a Sephadex G-50 column as follows: A 4-mL Sephadex G-50 bed was prepared in a 5-mL syringe and was equilibrated with buffer A. The syringe was spun at 1800*g* for 5 min to drain excess of buffer. The ammonium sulfate fraction was applied to the top of the packed Sephadex bed and respun at 1800*g* for 20 min. The effluent (approximately 200 μL) was kept in liquid nitrogen.

Chromatography and Electrophoresis. DEAE-cellulose chromatography in 7 M urea (pH 8.0) was carried out on a column (0.5 × 35 cm) equilibrated with 50 mM Tris-HCl (pH 8.0), containing 7 M urea and 50 mM NaCl. Samples (0.5–1 mL) were applied to the column together with tRNA hydrolyzates and eluted with a linear gradient (100 mL) of NaCl (0.05–0.4 M). Absorbance at 260 nm was monitored and the radioactive content of each fraction measured in a Triton X-100 based scintillation fluid. Descending paper chromatography on Whatman 52 paper was performed with 1-butanol–NH_4OH–H_2O (86:5:14). Paper electrophoresis on Whatman No. 3MM was carried out at 1.85 V/cm^2 for 2–3 h in pyridine–acetate buffer (pH 3.5). The dried paper was cut into 1-cm strips and radioactivity was determined either in toluene-based scintillation fluid or, after extracting the radioactivity with water, in Triton X-100 based scintillation fluid.

Enzymes Treatment. For RNase T_2 digestion, RNA was dissolved in 100 μL of 0.05 M sodium acetate buffer (pH 4.5) and incubated overnight at 37 °C with 50 U/mL of T_2 ribonuclease. For combined digestion with Penicillium nuclease (P_1) and bacterial alkaline phosphatase, RNA was dissolved in 50–100 μL of 10 mM sodium acetate buffer (pH 6.0) and incubated with nuclease P_1 (1 mg/mL) for 60 min at 37 °C. The pH of the mixture was then adjusted to 8.0 with 1 M Tris base and incubation continued for 60 min with bacterial alkaline phosphatase. Two units of enzyme was added four times at 15-min intervals. Digestion with nucleotide pyrophosphatase (0.3 units/mL) was carried out for 60 min at 37 °C in 50 mM Tris-HCl buffer (pH 7.4), containing 10 mM $MgCl_2$ and 5 mM β-glycerophosphate to compete for any phosphatase activity in the enzyme preparation (Duck et al., 1973). Under these conditions no phosphatase activity was detected as judged by control incubation with both α-^{32}P- and ^{3}H-labeled mononucleotides. Incubation with spleen phosphodiesterase (5.5 U/mL) in 20 mM Tris-HCl (pH 7.2) was for 60 min at 37 °C.

Enzymes and Chemicals. RNase T_2, Penicillium nuclease, and nucleotide pyrophosphatase were purchased from Calbiochem, Yamasa Shoyu Co., and Sigma Chemical Co., respectively. Bacterial alkaline phosphatase and spleen phosphodiesterase were from Worthington Biochemical Corp. *S*-Adenosyl[*methyl*-^{3}H]methionine (67 Ci/mmol) was purchased from New England Nuclear. [^{32}P]Orthophosphate was obtained from Radiochemical Center, Amersham, England. Nucleotides were obtained from P-L Biochem. Co. *E. coli* polynucleotide phosphorylase was a kind gift of Dr. H. Soreq.

Results

Incorporation of [β-^{32}*P*]*ATP and* [^{3}*H*]CH_3 *into in Vitro Synthesized Capping Structure.* RNA was synthesized in vitro in nuclear homogenates with [β-^{32}P]ATP and *S*-[*methyl*-^{3}H]adenosylmethionine (SAM) as radioactive precursors. Under the conditions applied (see Experimental Procedures) both ^{32}P and ^{3}H were incorporated into RNA. To determine which groups in the RNA were labeled, RNA was isolated, extensively digested with RNase T_2, and analyzed by DEAE-cellulose chromatography. As seen in Figure 1, most of the ^{3}H-labeled material, which consisted of nucleosides and mononucleotides, eluted before or at −2 charge. Nevertheless, a significant amount of *methyl*-^{3}H-labeled material eluted with a net negative charge of 2.5 to 3.5 and another peak of both ^{32}P and ^{3}H was eluted between charge of −4 to −5. RNase T_2 resistant capping structures of the general type m^{7}GpppXmpYp and noncapped diphosphate termini (pp*Ap–) are both expected to elute in this region. In order to distinguish between the two and isolate the caps, fractions 52 to 64 were pooled, desalted, and digested with nuclease P_1 followed by treatment with alkaline phosphatase and the products analyzed by paper electrophoresis (Figure 2A). About 70% of the ^{3}H radioactivity was P_1 nuclease and alkaline phosphatase resistant cap structures and migrated in two negatively charged spots (peaks I and II). The remainder of the *methyl*-^{3}H-labeled material was positively charged and comigrated with all four nucleosides (since the electrophoretic mobility at pH 3.5 is unaltered by 2′-*O*-methylation, unmethylated nucleosides, and nucleotides were used as markers).

[1] Abbreviations used: Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; m^{7}G, 7-methylguanosine; Nm, 2′-*O*-methyl ribonucleoside; P_i, inorganic phosphate; [^{3}H]SAM, *S*-[*methyl*-^{3}H]adenosylmethionine; HnRNA, heterogeneous nuclear RNA.

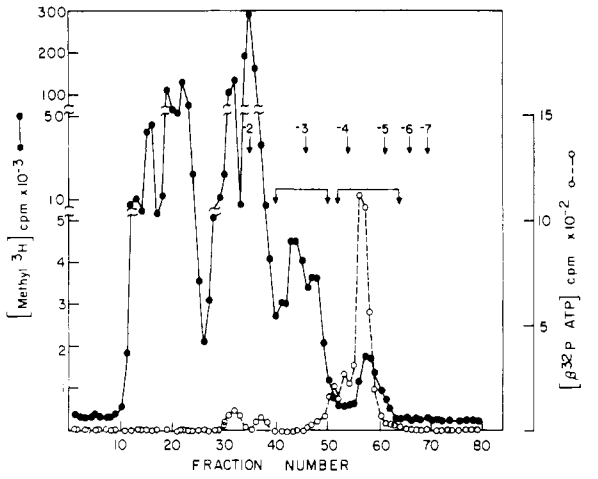

FIGURE 1: Column chromatography of ^{32}P- and ^{3}H-labeled nucleotides released by RNase T_2 digestion. RNA synthesized in vitro in the presence of [β-^{32}P]ATP and [^{3}H]SAM was digested with RNase T_2 and analyzed by DEAE-cellulose chromatography as described under Experimental Procedures. The positions of oligonucleotide markers of net charge −2 to −5 determined by absorbance at 260 nm are indicated by arrows. Regions comprising fractions 52 to 66 and fractions 40 to 50 were pooled separately as indicated for subsequent analysis described in Figures 2 and 4, respectively. (●—●) *methyl*-^{3}H; (○ - - - ○) ^{32}P.

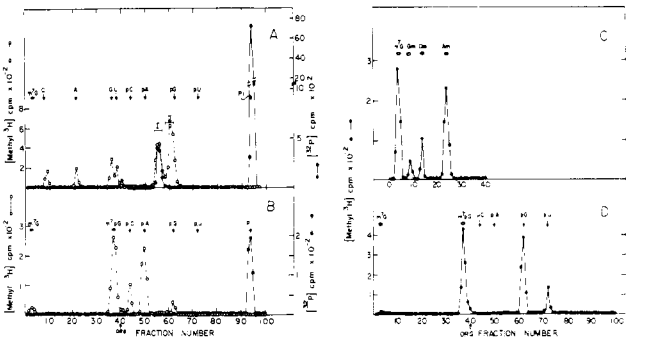

FIGURE 2: Analysis of capping structures isolated from DEAE-cellulose column described in Figure 1. (A) Fractions 52 to 66, eluted from the DEAE-cellulose, were pooled, desalted, digested with P_1 nuclease followed by treatment with alkaline phosphatase, and analyzed by paper electrophoresis. (B) Peak I in panel A was eluted; an aliquot was treated with nucleotide pyrophosphatase in the presence of 5 mM β-glycerophosphate and analyzed by paper electrophoresis. (C) Another aliquot of material eluted from peak I in panel A was treated with nucleotide pyrophosphatase plus alkaline phosphatase and analyzed by descending paper chromatography in 1-butanol-NH_4OH-H_2O (86:5:14). (D) Analysis of peak II material as in B. The arrows indicate the position of radioactive markers. Methylated nucleosides m^7G, Gm, Cm, and Am were included with the sample and located by ultraviolet light.

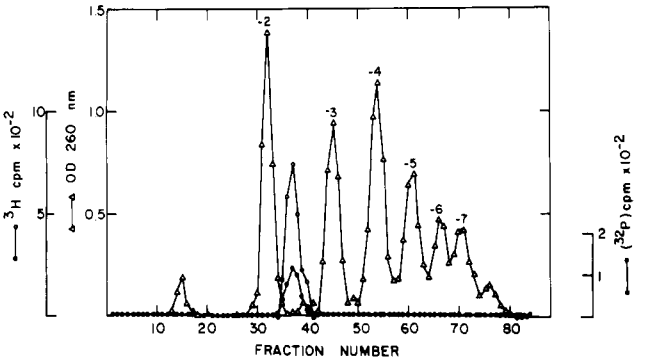

FIGURE 3: DEAE-cellulose chromatography of ^{32}P- and ^{3}H-labeled capping structures. P_1 nuclease and alkaline phosphatase resistant ^{32}P- and ^{3}H-labeled material in peak I from experiments identical with that described in Figure 2A was eluted from the paper mixed with tRNA hydrolysate and analyzed by DEAE-cellulose chromatography as described under Experimental Procedures. (△—△) Elution profile of marker oligonucleotides monitored at 260 nm and denoted by increasing net negative charge; (○—○) elution profile of ^{3}H- and (●—●) ^{32}P-labeled material.

It can also be seen (Figure 2A) that ^{32}P radioactivity pooled from the DEAE-cellulose column yielded two spots after the treatment with P_1 nuclease and alkaline phosphatase. 90% of the ^{32}P (originally derived from [β-^{32}P]ATP was converted to orthosphosphate (P_i) but a significant amount (10%) comigrated with peak I in the paper electrophoresis (Figure 2A). It should be noted that the same amount of ^{32}P radioactivity (90%) was converted to P_i when the pooled material was treated with alkaline phosphatase alone (not shown). These results imply that about 10% of the β-^{32}P incorporated into the 5′ ends of newly synthesized RNA became subsequently engaged in capping structure and therefore alkaline phosphatase resistant. The rest of the P_i which was sensitive to alkaline phosphatase is derived from unblocked termini.

To further analyze the labeled caps, material from peaks I and II was eluted separately, treated with nucleotide pyrophosphatase (in the presence of β-glycerophosphate as described under Experimental Procedures), and analyzed by paper electrophoresis. All the ^{32}P from peak I was liberated as P_i (Figure 2B), indicating that the ^{32}P was indeed at the β position in caps with the structure m^7Gpp*pAp. On the other hand, ^{3}H radioactivity from peak I yielded four *methyl*-^{3}H-labeled components (Figure 2B). About 50% migrated as m^7pG and the remainder as AMP (37%), CMP (10%), and GMP (3%). Liberation of *methyl*-^{3}H-labeled nucleosides monophosphate by the nucleotide pyrophosphatase demonstrated the absence of phosphatase activity during this reaction. These methylated nucleotides were quantitatively converted to nucleosides by incubation with alkaline phosphatase and then further identified by paper chromatography (Figure 2C). The sum of the 2′-*O*-methylated nucleotides was approximately equal to that of the m^7G, in agreement with the cap structure m^7GpppNm, where Nm is (in peak I) Am, Cm, and Gm.

Capping structures in peak II (Figure 2A) were similarly analyzed (Figure 2D). After nucleotide pyrophosphatase digestion and paper electrophoresis, radioactivity was present in material that migrated in the position of m^7pG (47%), GMP (42%), and UMP (10%). The identities of the 2′-*O*-methylguanosine and 2′-*O*-methyluridine separated by the electrophoresis were confirmed by elution and rechromatography with markers in both isobutyric acid:NH_3 and in isopropyl alcohol:NH_3 (data not shown). As in peak I, the amount of 2′-*O*-methylated nucleotides approximated that of m^7pG.

The conclusions from these experiments are that peak I contains caps of the structure m^7Gp[^{32}P]pAm and m^7GpppCm, whereas peak II contains m^7GpppGm and m^7GpppUm. The small amount of Gm found in peak I is probably due to incomplete separation of peaks I and II in Figure 2A. ^{32}P radioactivity derived from [β-^{32}P]ATP was present only in capping structures migrating in paper electrophoresis in peak I, consistent with the fact that A caps (m^7GpppAm) are present only in this peak.

Peak I from Figure 2A was further analyzed by chromatography on DEAE-cellulose column (Figure 3). All the radioactivity eluted in one peak containing both ^{32}P and ^{3}H between marker nucleotides of charge −2 and −3. This is characteristic of P_1 nuclease and alkaline phosphatase resistant cap having m^7G with ½ a positive charge and 3 phosphate residues

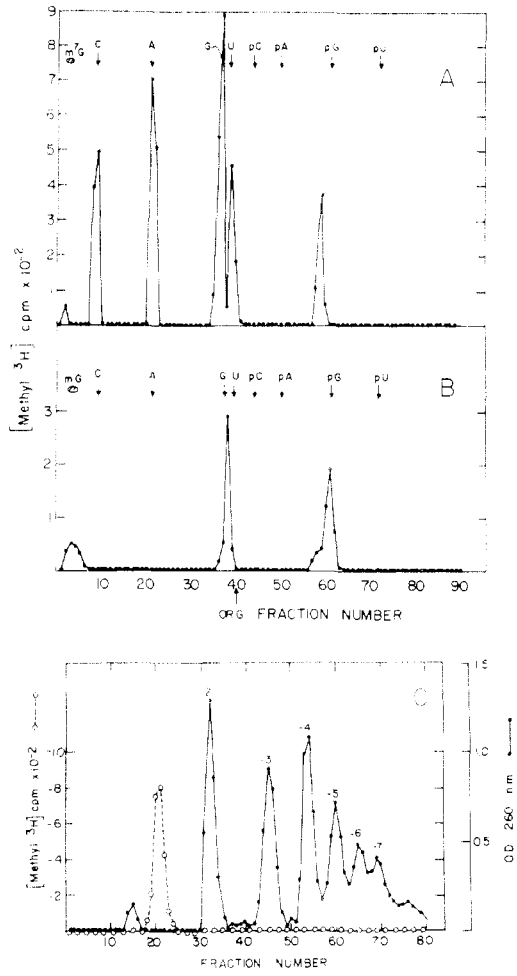

FIGURE 4: Analysis of diphosphate capping structures. (A) Electrophoretic analysis of ^{3}H-labeled oligomer from DEAE-cellulose described in Figure 1. Fractions 40–50 of the DEAE-cellulose were pooled, digested with P_1 nuclease followed by alkaline phosphatase, and analyzed by paper electrophoresis as described under Experimental Procedures. The arrows indicate the position of radioactive markers. m^7G is a spot detected under ultraviolet light. (B) P_1 nuclease and alkaline phosphatase resistant material migrating in fractions 58–60 in panel A was eluted and digested with nucleotide pyrophosphatase in the presence of 5 mM β-glycerophosphate, and the products were analyzed by paper electrophoresis as above. (C) Determination of the number of phosphate residues in the pyrophosphate bridge. P_1 nuclease resistant material eluted from fractions 58–60 in panel A was mixed with tRNA hydrolysates and analyzed by DEAE-cellulose chromatography as described under Experimental Procedures. (●—●) Elution profile of marker oligonucleotides monitored at 260 nm. (○ - - - ○) Elution profile of ^{3}H-labeled material eluted from fractions 58–60 in panel A.

each contributing one negative charge. Similar results were obtained when material from peak II was chromatographed on DEAE-cellulose column (not shown). The amount of ^{32}P in Figure 3 is about 16% of that of the ^3H radioactivity. Taking into account the specific activities of the two isotopes and the efficiency of counting, it can be calculated that about 72% of the *methyl*-^3H caps in peak I (Figure 2) contain ^{32}P. Since one ^{32}P and two ^3H-labeled methyl groups are present in $m^7Gpp*pAm$, this value is in good agreement with the presence of 37% 2′-*O*-methyladenosine in the nucleotide pyrophosphatase and alkaline phosphatase digest of peak I (Figure 2C). Thus, it can be concluded that about 10% of the RNA chains initiated in vitro with [β-^{32}P]ATP were also capped by transfer of GMP residue from GTP to form $m^7Gp[^{32}P]pAmp$-capped termini.

Capping Structures Containing Two Phosphate Residues in the Pyrophosphate Bridge. It has been reported previously (Groner & Hurwitz, 1975) that HeLa nuclear homogenates are capable of synthesizing caps with two phosphate residues in the pyrophosphate bridge. As presented above, only triphosphate caps were isolated from the −4 to −5 fraction of the DEAE-cellulose column in Figure 1. Nevertheless, P_1 nuclease and alkaline phosphatase resistant capping structures were also present in the −3 charge fraction of the DEAE-cellulose column and as will be shown below these caps contain two phosphate bridges. To isolate the capping structure in this region, fractions 40–50 in Figure 1 were pooled, digested with P_1 nuclease followed by treatment with alkaline phosphatase, and analyzed by paper electrophoresis. As can be seen in Figure 4A, only one *methyl*-^3H-labeled negatively charged spot was resolved which contained about 10% of the total radioactivity in this fraction. From the specificity of P_1 nuclease and alkaline phosphatase, the negatively charged material was presumed to be a dinucleotide connected through a pyrophosphate linkage. To further analyze this capping structure, *methyl*-^3H-labeled material in fractions 58–60 (Figure 4A) was eluted and incubated with (a) spleen phosphodiesterase, (b) 0.3 M NaOH, and (c) nucleotide pyrophosphatase (in the presence of β-glycerophosphate as described under Experimental Procedures). Treatments a and b did not degrade the radioactive material, again indicating the lack of both 5′-OH and conventional 5′ 3′-phosphodiester linkage. In contrast, after treatment with nucleotide pyrophosphatase, the diphosphate caps yielded two *methyl*-^3H-labeled nucleotides (Figure 4B) in the positions of m^7pG and pG. The fact that methylated nucleotides, but not methylated nucleosides, were liberated by the nucleotide pyrophosphatase treatment is consistent with a pyrophosphate linkage in the caps. m^7G and pGm were always present in the diphosphate caps. However, in some experiments low amounts (>5%) of pCm and pUm were also found, but pAm was never detected in this fraction. To determine the number of phosphates in the pyrophosphate linkage, radioactive material from fractions 58–60 in Figure 4A was mixed with oligonucleotide markers derived from pancreatic RNase digestion of tRNA and analyzed by DEAE-cellulose chromatography under conditions where nucleotides are eluted according to their charge. As shown in Figure 4C, radioactivity eluted ahead of the mononucleotide marker with a net negative charge of approximately −1. A dinucleotide which is resistant to P_1 nuclease plus alkaline phosphatase, having m^7G and a net charge of −1, is most likely to contain two phosphate residues in the pyrophosphate bridge.

These results indicate that diphosphate caps having the structures m^7GppXm (where X is primarily G) are formed in HeLa nuclear homogenates by a mechanism still unknown. Nevertheless, as will be shown below, formation of diphosphate caps was less sensitive to α-amanitin than the triphosphate species.

Inhibition of in Vitro Cap Formation by α-Amanitin. RNA polymerase II is specifically inhibited by a low concentration of α-amanitin (Stirpe & Fiume, 1967; Chambon, 1970; Balotti et al., 1970). We have previously observed (Groner & Hurwitz, 1975) that α-amanitin produces a marked reduction in the in vitro incorporation of [α-^{32}P]GTP into 5′ caps. It was also reported (Winicov & Perry, 1976) that α-amanitin severely inhibited the in vitro [^3H]SAM incorporation into both internal sites and caps of nuclear RNA larger than 45 S.

Therefore, it was of interest to study the effect of α-amanitin on the incorporation of ^{32}P derived from [β-^{32}P]ATP into capping structures. For this purpose, RNA was synthesized and capped in nuclear homogenates as described above. [β-^{32}P]ATP and [^3H]SAM were radioactive precursors and α-amanitin (0.5 μg/mL) was added at the beginning of the in-

TABLE I: Effect of α-Amanitin on [^{32}P]- and [^{3}H]SAM Incorporation into Capping Structures.[a]

Radioactive peaks on electropherogram	No additions (cpm)		+ α-amanitin (cpm)		Inhibition (%)	
	^{3}H	^{32}P	^{3}H	^{32}P	^{3}H	^{32}P
Peak I, Figure 2A	1320	930	260	42	80	96.5
Peak II, Figure 2A	1690		338		83	
P_i, Figure 2A		8830		9400		0
Diphosphate caps, Figure 4	550		248		65	

[a] Capping structures synthesized in vitro with and without α-amanitin (0.5 μg/mL) were isolated and analyzed as outlined under Experimental Procedures and in Figures 1, 2, and 4. The values presented (mean value of three experiments) represent the radioactivity found on the paper after electrophoresis as described in Figures 2A and 4.

cubation. Labeled RNA was isolated and digested with RNase T_2, and oligonucleotides were separated by DEAE-cellulose chromatography as in Figure 1. Material eluting between −4 and −5 was pooled and digested with nuclease P_1 and alkaline phosphatase, and the products were analyzed by paper electrophoresis as in Figure 2A. Radioactivity in capping structures (peaks I and II) as well as in P_i was determined and the results are summarized in Table I. Although there is no inhibition of total ^{32}P incorporation into 5′ termini of nuclear RNA, β-^{32}P incorporation into capping structures was inhibited more than 95% by a low concentration of α-amanitin. [^{3}H]SAM incorporation into triphosphate caps was also severely reduced (80–83%). These results signify that, at least under the in vitro conditions we used, capping as well as methylation occurs primarily at the 5′ ends of newly initiated RNA polymerase II transcripts, rather than at the 5′ termini of processed products which would presumably be insensitive to α-amanitin. In this context it is interesting to note (Table I) that formation of methylated caps, containing two phosphates in the pyrophosphate bridge, is significantly less sensitive to α-amanitin as compared with the triphosphate caps.

To exclude the possibility that, in addition to RNA polymerase II, the capping enzyme itself is sensitive to α-amanitin, the following experiment was performed: β-^{32}P-labeled synthetic RNA, synthesized by *E. coli* RNA polymerase using [β-^{32}P]ATP, according to Maitra & Hurwitz (1973), was incubated in vitro in our standard reaction mixture. The ammonium sulfate fraction (0–75%) of HeLa nuclear homogenates (see Experimental Procedures) was used as a source for capping enzyme. After incubation, RNA was isolated and analyzed for cap formation by paper electrophoresis following treatment by P_1 nuclease and alkaline phosphates, as described in Figure 2A. Although the capping activity was low (from 10^6 cpm input an average of 20 000 cpm (2%) were found in capping structures m^7Gpp*pA), it was totally resistant to 0.5 μg/mL of α-amanitin. From these results we concluded that the impairment of cap formation by the drug was indeed due to inhibition of RNA polymerase II.

Discussion

It was previously shown (Gilboa et al., 1977) that [β-^{32}P]-ATP incubated with isolated nuclei is incorporated exclusively into the 5′ ends of newly synthesized RNA chains. It has also been reported that capping structures are formed in nuclei incubated in vitro under conditions of RNA synthesis (Groner & Hurwitz, 1975; Winicov & Perry, 1976). In this report we have used both [β-^{32}P]ATP (as nucleoside triphosphate donor) and [^{3}H]SAM (as a methyl donor) to investigate the steps leading to the formation of caps at the 5′ termini of cellular RNA. The incorporation of ^{32}P derived from [β-^{32}P]ATP into capping structures as described below signify that a mechanism for cellular cap synthesis exists which is very similar to that of vaccinia and reovirus (Moss et al., 1976; Furuichi & Shatkin, 1977). Namely, diphosphate 5′ termini of initial RNA transcripts (ppX) are capped by a condensation of pG residue derived from GTP to form $G^{5'}ppp^{5'}X$ termini. These blocked termini are subsequently methylated by transfer of methyl groups from SAM. While this manuscript was in preparation, we learned that Wei and Moss have also found that capping enzyme isolated from HeLa cells can cap diphosphate terminated RNA (Wei & Moss, 1977).

Consistent with our results that most of the RNA chains initiated in vitro did not enter capping structures are the observation of Gilboa et al. (1977) that the predominant RNA species initiated in vitro in nuclei is 4–5S RNA synthesized by RNA polymerase III. Only 10% of the total [β-^{32}P]ATP incorporated in our system was found in caps. Interestingly, only those RNA termini that were initiated by RNA polymerase II were subsequently capped since [β-^{32}P]ATP incorporation into caps was severely inhibited by a low concentration of α-amanitin. These results indicate that the specificity of the capping reaction is maintained in the nuclear homogenates and only a selective group of RNA chains is capped. The ability to select for RNA polymerase II transcripts is lost when the nuclear homogenate is fractionated by ammonium sulfate precipitation since, as we have shown, the ammonium sulfate fraction is capable of capping 5′-triphosphate termini of *synthetic* RNA. A plausible explanation for the latter result is that capping is coupled to HnRNA transcription and takes place on nascent RNA chains which are still bound to chromatin. This is consistant with the presence of capping structures in HeLa and L cell HnRNA (Perry et al., 1975; Perry & Kelly, 1976; Salditt-Georgieff et al., 1976). Moreover, the fact that both capping and methylation are inhibited by low concentrations of α-amanitin implies that the majority of the capping occurs shortly after the initiation of transcription. Consequently, there is no accumulation of preformed noncapped RNA termini that can serve as a substrate for the cellular capping enzyme. Hence, the majority of caps are added to RNA polymerase II primary transcripts which have undergone no previous processing.

We have previously observed (Groner & Hurwitz, 1975) that, in addition to caps with triphosphate residues in the pyrophosphate bridge (m^7GpppXm), diphosphate caps m^7GppXm are formed in vitro in HeLa nuclear homogenates. Since at that time the two types of capping structures were not separated, we could not assess the distribution of 2′-*O*-methylated nucleosides in each type of bridge compound. As described below, the two caps species were isolated by DEAE-cellulose chromatography and analyzed. In contrast to the triphosphate caps, where the penultimate nucleotide is any one of the four 2′-*O*-methylated derivatives, the diphosphate species contain almost exclusively Gm. We cannot rule out the possibility that formation of diphosphate caps is an artifact of the in vitro incubation condition. However, it should be pointed out that the HeLa cells methyl transferase (which has a

specificity for 5′ terminal caps) can methylate the diphosphate cap GppG, although less efficiently than the triphosphate type (GpppG) (Ensinger & Moss, 1976). It is not yet clear whether capping structures found in some of the low molecular weight nuclear RNAs contain di- or triphosphates (Reddy et al., 1974; Ro-Choi et al., 1975; Cory & Adams, 1975a). Nevertheless, since it has been recently shown that U_1 RNA inhibits translation of mRNA in cell-free systems (Rao et al., 1978), it will be of interest to compare the effect of triphosphate cap analogues on in vitro protein synthesis (Canaani et al. 1976; Groner et al., 1975), with that of the diphosphate structures.

Acknowledgment

We are grateful to Dr. Michel Revel for his encouragement and support.

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