RNA Phosphorylation: a Polynucleotide Kinase Function in Mouse L Cell Nuclei†

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ABSTRACT: Analysis of [γ-^{32}P]ATP in vitro labeled nuclear RNA shows transfer of γ-^{32}P from ATP to form the 5′-terminal monophosphate for large RNA molecules. This finding in an actively transcribing nuclear system capable of guanylation and methylation reactions indicates that polynucleotide kinase activity in the eukaryotic nucleus may be functional in kinase reactions involving RNA. It further suggests a participation in the posttranscriptional modification reactions involved in RNA processing. All four nucleosides were found to act as acceptors at the 5′ termini of RNA. It is also shown that both ATP and GTP can serve as donors in the nuclear polynucleotide kinase reaction.

Polynucleotide kinase was first observed in *Escherichia coli* extracts after infection with either T_4 or T_2 bacteriophage (Richardson, 1965; Novogrodsky and Hurwitz, 1966). This enzyme showed a lack of acceptor specificity in the transfer of γ phosphate from ATP to the 5′-OH of a polynucleotide (DNA or RNA) and on further inspection was also shown to use GTP, CTP, and UTP as phosphate donors.

Polynucleotide kinase activity has also been detected in nuclei of eukaryotic cells (Novogrodsky et al., 1966; Ichimura and Tsukada, 1971; Teraoka et al., 1975), using 5′-OH DNA as the acceptor molecule, with some indication (Teraoka et al., 1975) that the eukaryotic polynucleotide kinase also may be able to use 5′-OH of RNA as the acceptor species. A deoxyribonucleic acid kinase has also been purified from rat liver nuclei which is inactive on RNA (Levin and Zimmerman, 1976). The function of polynucleotide kinase in the eukaryotic nucleus is unknown despite the presumption that it plays a significant role in DNA repair together with polynucleotide ligase.

I wished to determine the role of polynucleotide kinase, if any, in the posttranscriptional modification of nuclear RNA. Monophosphorylated ends have been shown for both small nuclear RNA species (Marzluff et al., 1974) and nuclear RNA greater than 18 S (Schibler and Perry, 1976). Such monophosphorylated ends could arise from either cleavage activity on triphosphorylated 5′ termini of primary transcripts, or cleavage of internal phosphodiester bonds. Since nuclei contain ribonucleases which can produce both 5′ phosphate ends and 5′-OH ends (cf. Winicov and Perry, 1974, for review; Robertson and Mathews, 1973; Cordis et al., 1975), rephosphorylation of the 5′-OH termini may play an important part in subsequent disposition of the cleaved molecules.

Therefore, I looked for polynucleotide kinase activity in a nuclear system (Winicov and Perry, 1976), which had been shown in vitro to transcribe large RNA molecules, methylate and "cap" nuclear heterogeneous RNA, and thus might also be expected to carry out other nuclear modification reactions. This paper shows that polynucleotide kinase can be seen in such nuclei acting on nuclear RNA and the products of the reaction are found in both large and small nuclear RNA size classes. The phosphate donor for this reaction can be the γ phosphate of ATP as well as GTP.

Experimental Procedures

Isolation of in Vitro Labeled Nuclear RNA. L cell nuclear preparation was incubated in vitro at 1.5×10^8 nuclei/mL for 30 min at 37 °C as described previously (Winicov and Perry, 1976) in the presence of 2.6 μM *S*-[*methyl*-^{3}H]adenosylmethionine ([^{3}H]SAM) (New England Nuclear Corp., 8.82 Ci/mmol) and either 50 μM [γ-^{32}P]ATP (New England Nuclear Corp., 4 Ci/mmol) or 80 μM [$\beta\gamma$-^{32}P]GTP (ICN Pharmaceuticals, Inc., 4.5 or 2.25 Ci/mmol in γ-^{32}P).

RNA Extraction and Fractionation. RNA was extracted, separated from mononucleotides on a Sephadex G-50 column, denatured in 80% Me_2SO and separated on a sucrose–sodium dodecyl sulfate gradient (Winicov and Perry, 1976) in order to exclude contamination with any acid soluble nucleotides and separate RNA according to size class. The isolated RNA was digested with RNAse T_2 (50 units in 0.3 mL of 0.1 M NaOAc, pH 4.5, 5 h at 37 °C).

Analysis of T_2 Digests of in Vitro Labeled 5′ Termini. Mononucleotides, oligonucleotides, and 5′-phosphorylated termini were separated from capped termini on DBAE-cellulose (McCutchan et al., 1975) using elution with 1 M sorbitol in application buffer (Furuichi et al., 1975). The nonretained fraction containing mononucleotides, oligonucleotides, and 5′ termini was separated by DEAE-Sephadex column chromatography in 7 M urea (Perry et al., 1975a,b). Individual peaks of pXp or pX nucleotides were desalted and concentrated on small DEAE-Sephadex columns (carbonate form) and eluted with 30% triethylammonium carbonate, pH 7.8. The samples were concentrated by drying under vacuum.

The dried pXp nucleotides were dissolved in a small volume and spotted on polyethylenimine (PEI) thin layer plates (Merck) with appropriate markers or treated with S_1 nuclease (Schibler and Perry, 1976) and rechromatographed on DEAE-Sephadex. The pXp nucleotides were treated with bacterial alkaline phosphatase (50 μg/mL, 1 h, 37 °C) and spotted directly on PEI plates for chromatography.

Results

Isolation of 5′-Terminal Structures from in Vitro Labeled RNA. The labeling of 5′-terminal RNA structures with [γ-

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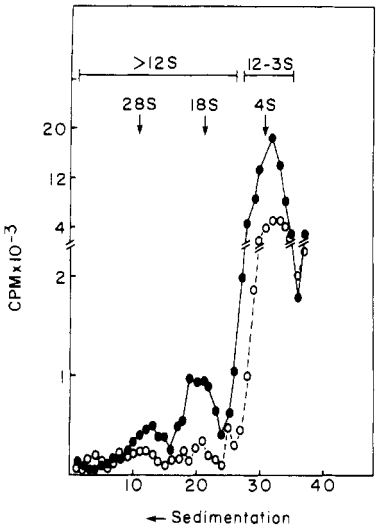

FIGURE 1: Sucrose gradient separation of in vitro labeled nuclear RNA. L cell nuclear preparation was labeled in vitro with [γ-^{32}P]ATP and [^{3}H]SAM as described in Experimental Procedures. The extracted RNA was separated from mononucleotides on a Sephadex G-50 column, denatured in 80% Me_2SO at 60 °C for 2 min, and sedimented through a 5–25% sucrose–sodium dodecyl sulfate gradient (with a 1.5 mL 45% sucrose cushion) for 16 h at 20K rpm in an SW27 rotor. Positions of absorbance markers are indicated with arrows; (○) ^{32}P cpm; (●) ^{3}H cpm. The bars designate fractions pooled as >12S and 3–12S RNA.

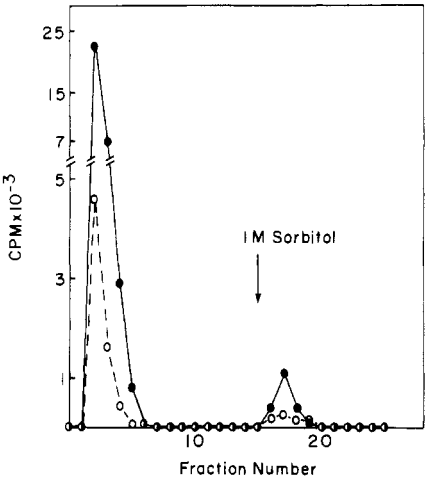

FIGURE 2: DBAE-cellulose separation of capped termini from 5′-phosphorylated termini and internal nucleotides. The >12 S RNA fraction shown in Figure 1 was extensively digested with T_2 RNase and diluted with 10 vol of DBAE application buffer, chromatographed on a 0.4 × 4 cm DBAE-cellulose column. The retained material was eluted with 1 M sorbitol in application buffer. (○) ^{32}P cpm; (●) ^{3}H cpm.

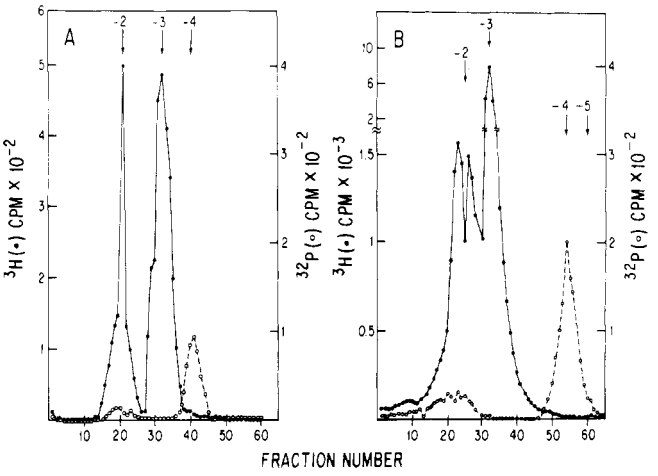

FIGURE 3: Separation of DBAE nonretained material on DEAE-Sephadex columns. The DBAE nonretained material (shown as fractions 2–5 in Figure 2) was diluted to 7 M urea–20 mM Tris (pH 7.4)–4 mM EDTA and separated on a DEAE-Sephadex column with a gradient of 0.1–0.5 M NaCl in 20 mM Tris-HCl (pH 7.4)–7 M urea. Arrows indicate positions of OD charge markers. (A) RNA > 12 S; (B) RNA was 3–12 S.

^{32}P]ATP in actively transcribing nuclei would be expected to yield both triphosphate termini of initial transcripts as well as any monophosphorylated termini if polynucleotide kinase activity was functional in such nuclei. It was also of interest to differentiate any possible polynucleotide kinase activity with regard to its nuclear substrate size classes. Because the nuclear system transcribes small molecular weight RNA efficiently (Winicov and Perry, 1976), the molar quantities of these 5′ termini would be expected to contribute significantly to the total number of termini analyzed. Therefore the in vitro labeled RNA was denatured by heating in 80% Me_2SO and separated according to size on a sucrose–sodium dodecyl sulfate gradient (Figure 1). Since little or no internal phosphate groups should be labeled under our experimental conditions with [γ-^{32}P]ATP labeling, the ^{32}P sedimentation profile resembles that of [^{3}H]methyl labeling from *S*-adenosylmethionine which was included as a control in the incubation assay. Thus most of the γ-^{32}P label is seen in the low molecular weight region of Figure 1. The RNA was separated in two size classes: >12S and 3–12S RNA for further analysis of the 5′-monophosphorylated termini.

The phosphorylated termini as well as internal mononucleotides were separated from capped termini on acetylated DBAE-cellulose after digestion of each RNA size class with RNase T_2. The elution profile of such a DBAE-cellulose column is shown in Figure 2 for a digest of in vitro labeled RNA > 12 S. Most of the [^{3}H]methyl and ^{32}P counts were not retained on the column and can be seen eluting with the void volume. The cap termini, as shown by the [^{3}H]methyl counts, were retained on the column and were eluted by 1 M sorbitol. The small amount of ^{32}P counts in this peak subsequently chromatographed as inorganic ^{32}P on a DEAE-Sephadex–urea column (data not shown).

The material in the nonretained fraction from the DBAE column was further separated on a DEAE-Sephadex–urea column as shown in Figure 3. As expected, little ^{32}P incorporation from [γ-^{32}P]ATP is found in the mononucleotide peak eluting at −2 charge, and none can be seen in the dinucleotide peak at −3 charge in RNA > 12 S (Figure 3A) or the 3–12 S fraction (Figure 3B). This observation is consistent with negligible equilibration of the γ-^{32}P from ATP with the nucleotide pools in the incubation system. The most prominent ^{32}P peak in Figure 3A,B is shown to elute with a −4 charge, which would be the expected position for a monophosphorylated 5′ terminus of the type pXp. The high level of radioactivity in the structure eluting at −4 charge in comparison with the ^{32}P counts recovered from internal monophosphates suggested that this might indeed be a pXp 5′-terminal mononucleotide in which the 5′-phosphate group had been donated by [γ-^{32}P]ATP in a polynucleotide kinase type reaction. As shown in Figure 3A,B this structure can be found in both >12S and 3–12S RNA. Identical column profiles were observed when the in vitro nuclear system was labeled with [$\beta\gamma$-^{32}P]GTP, which is consistent with the report that polynucleotide kinase can also use the γ-phosphate from GTP as a donor in the reaction (Novogrodsky et al., 1966).

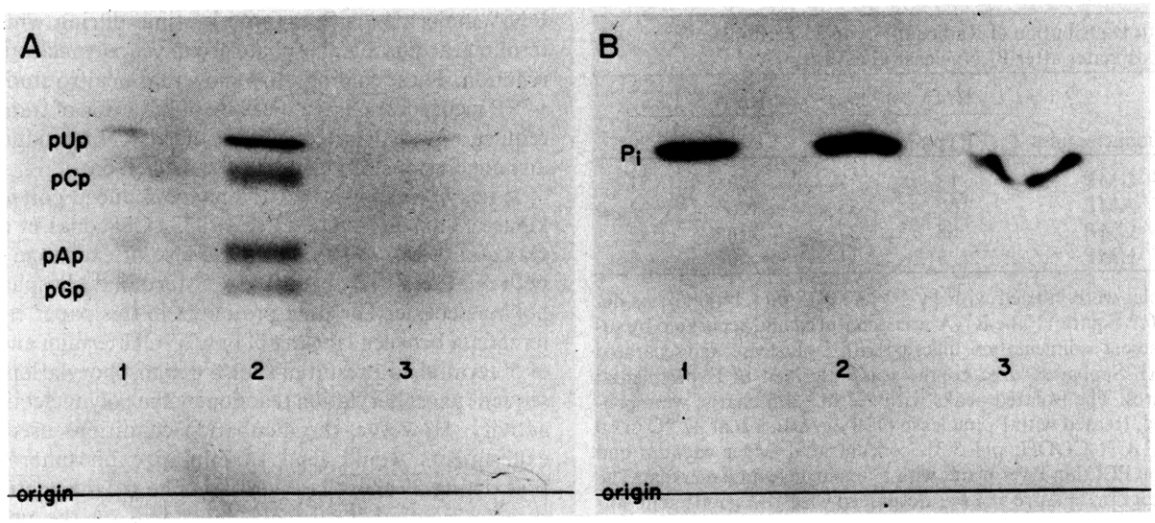

FIGURE 4: Distribution of radioactivity in 5′-terminal monophosphates and its sensitivity to bacterial alkaline phosphatase cleavage. The −4 peak T_2 digestion product of in vitro labeled >12S RNA and 3–12S RNA was desalted and concentrated as described in Experimental Procedures. The material was dissolved in a small amount of H_2O and either spotted directly on PEI-cellulose with the pXp markers (gift from Dr. U. Schibler) as indicated, or adjusted to 20 mM Tris-HCl (pH 7.4), incubated with bacterial alkaline phosphatase, and then spotted on the thin layer plates. The plates were developed with 0.75 M potassium phosphate buffer (pH 3.5). (Column 1) Peak −4 from >12S RNA (Figure 3A) labeled with [γ-^{32}P]ATP; (2) pXp markers; (3) peak −4 from 3–12S RNA labeled with [$\beta\gamma$-^{32}P]GTP. (A) Autoradiograph of pXp from >12S and 3–12S RNA. (B) Autoradiograph of same materials as in A, except treated with bacterial alkaline phosphatase.

TABLE I: Distribution of Radioactivity in 5′-Terminal Monophosphates from RNA > 12 S and 3–12 S.

	% radioactivity		
	>12 S[a]		3–12 S[b]
5′ nucleotide	I	II	I
pGp	33	49	31
pAp	29	23	34
pCp	27	17	13
pUp	11	11	24

[a] The −4 peak shown in Figure 3A was pooled, desalted, and concentrated as described in Experimental Procedures. [b] Nuclei were labeled with [$\beta\gamma$-^{32}P]GTP (sp act. 4.5 Ci/mmol; 80 μM) as described for [γ-^{32}P]ATP in Figure 1. The RNA was separated as shown in Figures 2 and 3. The peak fractions were pooled, desalted, and concentrated. The 5′ termini were analyzed by thin-layer chromatography as: (I) pXp in Figure 4A; (II) 5′-pX, after S_1 nuclease digestion of the same sample as shown in Figure 5a,b. The radioactive spots were identified by autoradiography (Figure 4A) or UV absorbance (Figure 5b), cut out, and counted on a liquid scintillation system. Recoveries from TLC plates were 75% and 74% for >12S RNA experiments I and II, respectively, and 51% for 3–12S RNA.

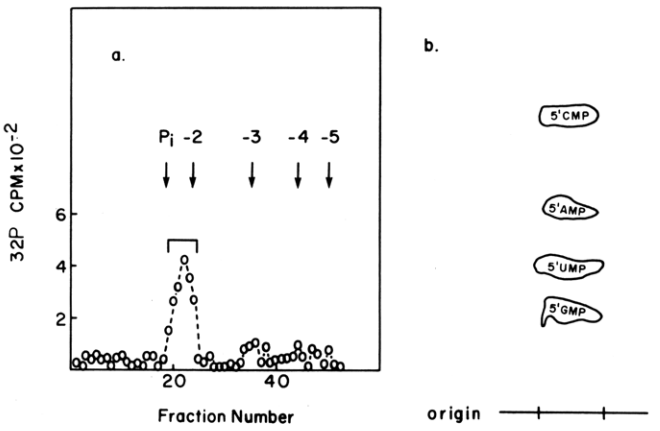

FIGURE 5: Analysis of S_1 nuclease treated pXp. The −4 peak T_2 digestion product of in vitro labeled >12S RNA was desalted, concentrated, and treated with S_1 nuclease as described in Experimental Procedures and rechromatographed on a DEAE-Sephadex column. The designated fractions were pooled, desalted, and concentrated as before and applied on a PEI plate with 5′ mononucleotide markers. The chromatogram was developed as described in Table II and 5′ mononucleotide markers identified by UV absorbance. (a) DEAE-Sephadex column profile. Arrows indicate position of charge markers. (b) Separation of 5′ mononucleotide markers on PEI thin-layer plate spotted with P_i or S_1 nuclease-treated −4 peak from DEAE-Sephadex–urea column (Figure 3). The plate was developed with 1 M acetic acid followed by 0.3 M LiCl (Randerath and Randerath, 1965).

Identification of 5′-Monophosphate Termini. Identity of the 5′-monophosphate termini was confirmed by several methods using thin layer and column chromatography. Figure 4A shows the separation of components of the isolated −4 charge peaks from the two RNA size classes on PEI-cellulose plates. Column 1 represents the separation of material from RNA > 12 S and column 3 depicts the material from 3–12S size class as compared with pXp markers in column 2. All four monophosphorylated ends appear to be present in both size classes of RNA as can be judged from the autoradiogram and a more detailed analysis as presented in Table I. All the ^{32}P can be removed from the structures by treatment with bacterial alkaline phosphatase as shown in Figure 4B, where it migrates with P_i at the ion front.

Further characterization of the presumed pXp structure was carried out by hydrolysis of the −4 peak with S_1 or P_1 nuclease, known to act specifically on mononucleotides as a 3′-phosphatase (Schmincke et al., 1976). Rechromatography of the S_1 nuclease treated −4 peak on DEAE-Sephadex showed (Figure 5a) a single peak eluting slightly before a −2 charge marker, a position consistent with 5′-XMP structure, and subsequently shown to consist of 5′-XMP (Table I). Distribution of radioactivity among the 5′-terminal monophosphate structures from a −4 peak treated with P_1 nuclease is shown in Figure 5b and Table II. All four nucleotides were found to be phosphorylated as in Table I, although the levels of 5′-UMP in >12S RNA are not significant and the distribution of counts in this experiment (Table II) is somewhat different from the one shown in Table I. Thus, there appears to be no marked

TABLE II: Distribution of Radioactivity in 5′-Terminal Monophosphates after P_1 Nuclease Cleavage.[a]

	RNA >12 S		RNA 3-12 S	
5′ mononucleotides	^{32}P cpm	%	^{32}P cpm	%
5′-GMP	13	7	502	51
5′-AMP	100	55	326	33
5′-CMP	68	37	116	12
5′-UMP	3	1	50	5

[a] Nuclei were labeled with [γ-^{32}P]ATP (7.09 Ci/mmol) as described for Figure 1. The RNA was denatured and separated by sucrose gradient sedimentation, digested with T_2 nuclease, and separated on DEAE-Sephadex-urea columns as described in Experimental Procedures. The isolated peaks with −4 to −4.5 charge were concentrated, treated with P_1 nuclease (150 μg/mL, 1 h at 37 °C in 10 mM NH_4CH_3COOH, pH 5.3), concentrated under vacuum and spotted on PEI thin-layer plates with 5′ mononucleotide markers. The chromatogram (Figure 5b) was developed with 1 M acetic acid, followed by development in 0.3 M LiCl (Randerath and Randerath, 1965). The mononucleotide spots were identified by UV absorbance, each spot was cut out and counted in a liquid scintillant to determine the radioactivity. Recovery of cpm from the TLC plates was 87% for >12S RNA and 85% for 3-12S RNA.

nucleoside specificity for polynucleotide kinase in the 5′-terminal position of the acceptor molecule in either of the nuclear RNA size classes.

Discussion

The concept of RNA processing in recent years has focused on two types of ribonucleases, one leaving a 5′-phosphate and the other a 5′-OH group. Analysis of functional RNA molecules in the cell has shown them to be mostly phosphorylated or "capped" at their 5′ termini and having a 3′-OH terminus. These findings together with the specificity of known processing RNases, such as those involved in tRNA processing (Altman, 1975) and RNase III (Crouch, 1974), seem to identify the processing ribonucleases with cleavage properties which would produce a 5′-phosphate and 3′-OH products. On the other hand, ribonucleases which produce a 5′-OH and 3′-phosphate product seem to be categorized with scavenging enzymes, directed toward rapid and complete breakdown of unnecessary RNA sequences.

The finding of polynucleotide kinase activity in L cells, which is functional in phosphorylation of 5′ termini of a variety of nuclear RNA size classes, seems to add another level of complexity to the RNA processing question. Certainly, a considerable number of RNA molecules, which had been cleaved, had been rephosphorylated by the polynucleotide kinase activity in a system which is capable of carrying out transcription as well as methylation and capping reactions, and thus may represent a fairly intact nuclear system. The finding of all four nucleosides represented in this monophosphorylated terminal class indicates that at least those molecules terminating with pUp and pCp had been substrates for the polynucleotide kinase activity after cleavage of a precursor molecule, since only purines have been shown to initate eukaryotic RNA polymerase reactions (Chambon, 1974; Schmincke et al., 1976). Unlike the present study, analysis of pXp termini isolated from in vivo transcribed nuclear RNA > 18 S (Schibler and Perry, 1976) shows the predominant 5′ terminus as pUp and pCp (about 70% of all pXp), but the in vivo experiments would have measured products of both cleavage reactions and kinase reactions, while only the kinase activity could be measured in these experiments. It would seem that at least some of the nuclear cleavages producing 5′-OH termini result in RNA molecules with a finite lifetime during which the 5′ terminus acquires a phosphate group via polynucleotide kinase reaction. These findings also show that in vitro studies, using γ-^{32}P incorporation as a measure of initiation of transcription, require a more detailed analysis of the RNA products before any conclusions can be drawn from such data.

It has been shown that the phage-induced polynucleotide kinase (Van de Sande, et al., 1973; Chaconas et al., 1975; Okazaki et al., 1975) is capable also of exchange reactions between the γ-^{32}P of ATP and 5′-terminal phosphate of the polynucleotide. The data presented in this paper cannot differentiate between labeling of only 5′-OH termini and labeling of 5′ termini as a result of such a dephosphorylation and subsequent phosphorylation reaction by the polynucleotide kinase activity. However, the incubation conditions used in these experiments would tend to minimize phosphorylation of preexisting 5′-phosphate termini. The polynucleotide kinase reverse reaction is strongly dependent on the presence of equimolar ADP concentrations in the reaction mixture (Chaconas et al., 1975) and the K_{IS} of ADP is approximately 100 μM (Lillehaug and Kleppe, 1975) with respect to ATP. Since the endogenous ADP concentration in vivo is about a factor of 5–10 lower than ATP concentration (Plagemann and Erbe, 1973) and the L cell system has been severely depleted of its soluble substrates as shown by its dependence on added substrates in vitro (Winicov and Perry, 1976), it is very likely that the kinase is mostly saturated with the added ATP (50 μM) during the time of incubation. It has also been shown that at pH 7.6 the rate of the reverse reaction is 2% of that of the forward reaction (Van de Sande et al., 1973). These considerations assume that this eukaryotic polynucleotide kinase has properties similar to those of the phage induced enzyme, which may not be the case. Until this enzymatic activity can be purified and further characterized, it cannot be ruled out that some of the 5′-terminal labeling is the result of the reversal of the polynucleotide kinase reaction.

The function and identity of the phosphorylated molecules remain unknown at this time. The apparent lack of 5′-terminal acceptor specificity as judged only by the nucleotide composition may be misleading if secondary structure contributes to the specificity of the reaction. The function of polynucleotide kinase in posttranscriptional modification of eukaryotic RNA is still unknown. It may act on certain classes of nuclear RNA molecules in its own right together with RNases producing 5′-OH termini, or as a correction or salvage factor in special circumstances. While these questions await further experiments, it is quite clear that the activity of polynucleotide kinase in the eukaryotic nucleus is not confined to DNA, but involves RNA as well and may possibly serve as a marker to differentiate between nuclear RNA species.

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Properties of an Altered RNA Polymerase II Activity from an α-Amanitin-Resistant Mouse Cell Line†

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ABSTRACT: α-Amanitin-resistant clones were selected in the mouse lymphoblastoid cell line L5178Y. One resistant clone, named A169b, was recloned and the properties of its DNA-dependent RNA polymerases were examined. The RNA polymerase II activity from A169b differs from the parental cell line in that approximately half the activity is resistant to 0.5 μg/mL α-amanitin, while the parental enzyme is 50% inhibited at 0.005 μg/mL. The enzymes from A169b and the parental line were purified free of polymerase III and their properties compared. The two preparations were identical in their apparent affinities for the four nucleoside triphosphates, in their salt and divalent cation preferences, and in their preference for denatured over native DNA. They differed in their response to α-amanitin. The apparent K_I for the parental enzyme was 3.5×10^{-9} M; plots of $1/V$ vs. α-amanitin concentration gave a biphasic curve with A169b enzyme. The two apparent K_I values were 4.1×10^{-9} and 2.1×10^{-6} M. In addition, the enzyme from A169b showed a twofold higher activity on poly[d(AT)] as template, compared to native DNA, than that of the parental enzyme. Other template preferences may be affected, but differences were marginal. These results indicate that mutation to α-amanitin resistance may alter other enzymatic parameters; such mutations may be helpful in elucidating structure-function relationships in these complex enzymes.

Eukaryotic cells contain three major forms of DNA-dependent RNA polymerase (EC 2.7.7.6) each of which appears to be responsible for the synthesis of a specific class of RNA (Weinmann and Roeder, 1974; Blatti et al., 1970; Reeder and Roeder, 1972). The various forms of RNA polymerase are composed of two large polypeptides in association with a series of smaller polypeptides (Gissinger and Chambon, 1972; Kedinger and Chambon, 1972; Sklar et al., 1975). Ascertaining how the structural components of RNA polymerase interact to determine functional properties is important for an understanding of the role played by RNA polymerase in the regulation of transcription.

One approach to the investigation of structure-function relationships is to correlate changes in functional properties with changes in structure. Huet et al. (1976) have presented evidence that removal of two polypeptides from RNA polymerase I (or A) in yeast decreases DNA binding but does not affect chain propagation. Bell et al. (1976) have also observed changes in function as a result of a structural alteration of yeast RNA polymerase I.

Another method of altering structure is through mutation. Mutations affecting RNA polymerase II (or B) in mammalian cells can be isolated on the basis of resistance to the mushroom toxin α-amanitin (Chan et al., 1972; Amati et al., 1975; Somers et al., 1975), which inhibits the elongation step in RNA syn-

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