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RIBONUCLEIC ACID SYNTHESIS IN

ISOLATED MOUSE MYELOMA NUCLEI

presented by

William Herman Eschenfeldt

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Ronald J Patterson
Major professor

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RIBONUCLEIC ACID SYNTHESIS IN ISOLATED MOUSE MYELOMA NUCLEI

By

William Herman Eschenfeldt

A DISSERTATION

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ABSTRACT

RIBONUCLEIC ACID SYNTHESIS IN ISOLATED MOUSE MYELOMA NUCLEI

By

William Herman Eschenfeldt

Ribonucleic acid (RNA) synthesis in nuclei isolated from the mouse myeloma cell line P3 (MOPC-21) was studied. Under the conditions used, the nuclei remained intact and ^{3}H-GTP was incorporated at a relatively linear rate for at least 30 minutes at 25^{o}C. At 2^{o}C no significant synthesis of RNA was observed. Synthesis of RNA was dependent upon the addition of the four ribonucleoside triphosphates. If CTP, GTP, or UTP were omitted from the reaction, synthesis did not occur. In the absence of exogneous ATP, synthesis was reduced to about 17% of control levels.

The RNA synthesized exhibited a heterogeneous size distribution on sucrose gradients, with a broad peak in the range of 18 to 28S. This size range was similar to that of nuclear RNA isolated from cells labeled in culture with ^{3}H-uridine. When nuclei labeled in culture with ^{3}H-uridine were incubated *in vitro* for 30 minutes, a reduction in the size of the RNA was noted. This suggests that some degradation and/or processing is occurring in the isolated nuclei during the *in vitro* incubation.

The synthesis was sensitive to α-amanitin and aurintricarboxylic acid (ATA). α-amanitin at 1 μg/ml reduced synthesis to 25 to 35% of

control levels, suggesting that 65 to 75% of the synthesis in this system is due to RNA polymerases II and III. ATA, an inhibitor of nucleic acid-binding proteins, reduced synthesis to 30 to 50% of control levels at a concentration of 0.1 mM. The combination of α-amanitin (1 ug/ml) and ATA (0.1 mM) reduced synthesis to levels lower than with either inhibitor alone.

5-mercuriuridine triphosphate (Hg-UTP), when substituted for UTP, supported RNA synthesis at about 35 to 45% of control levels. Synthesis also occurred when the ribonucleoside 5'-γ -thiotriphosphates, ATP-γ-S and GTP-γ -S were substituted for ATP and GTP, respectively. ATP-γ -S stimulated RNA synthesis by about 50%. Studies with α-amanitin indicated that RNA polymerase I was not affected by ATP-γ -S. Thus it appeared that RNA polymerase II and possibly RNA polymerase III were preferentially stimulated by ATP-γ -S. GTP-γ-S did not appear to stimulate RNA synthesis. When both analogs were added to the reaction, synthesis was again stimulated. Attempts to prepare mercury-Sepharose and to isolate thiol-containing nucleotides on mercury-Sepharose were unsuccessful. The use of γ-S ribonucleotides for the study of initiation of RNA synthesis and the preferential stimulation of RNA synthesis by ATP-γ -S are discussed.

to Chris

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The study of eukaryotic gene expression at the level of transcription has been an area of intense activity for a number of years. The use of eukaryotic chromatin and prokaryotic RNA polymerases to study eukaryotic transcription has been reported widely. In recent years, however, the use of isolated nuclear systems relying upon endogenous RNA polymerases has been proposed as an alternative (Bellard *et al.*, 1977). It has been suggested that transcription in intact nuclei more closely resembles the *in vivo* situation. In addition, the study of post-transcriptional processing may be possible in an isolated nuclear system. The first report of RNA synthesis in isolated nuclei was over 20 years ago (Allfrey *et al.*, 1957) but, in recent years, the literature has expanded rapidly. Reports have appeared in which RNA synthesis has been examined in nuclei from fungi and higher plants, amphibians and mammals.

Many workers believe that the isolated nuclear systems merely complete nascent chains which were initiated *in vivo*, and that no initiation of synthesis occurs *in vitro* (Bitter and Roeder, 1978; Tata and Baker, 1978). Others feel that at least some species of RNA are initiated *in vitro* (Tamm, 1977; Busiello and Di Girolamo, 1975). The use of ribonucleoside 5'-γ -thiotriphosphates has been proposed as a sensitive probe for *in vitro* initiation of RNA synthesis (Reeve *et al.*, 1977). If the gamma-thiol-containing nucleotides were incorporated as the initial nucleotide of a RNA chain, the thiol group would remain

intact and the completed RNA chain could be isolated by chromatography over a matrix coupled with mercury. Several studies using this technique with eukaryotic systems are reportedly in progress (Huang *et al.*, 1977; Smith *et al.*, 1978).

In the study reported here, we have examined the synthesis of RNA in nuclei isolated from mouse myeloma cells (MOPC-21). Conditions for optimum synthesis were determined and the effects of two inhibitors--α-amanitin and aurintricarboxylic acid--were investigated. The size and poly(A) content of the synthesized RNA was also examined. The effect of the ribonucleoside 5'-γ -thiotriphosphates, ATP-γ -S and GTP-γ -S, on RNA synthesis was studied. Some of the implications of these results on RNA initiation studies are discussed.

REVIEW OF THE LITERATURE

RNA Synthesis in Isolated Nuclei

Introduction

In an effort to understand the mechanisms controlling eukaryotic gene expression it has been necessary to examine both protein synthesis and RNA transcription. Since transcription is normally restricted to the nucleus, most studies have attempted to remove the nuclear transcription system from the influence of cytoplasmic factors. One method which accomplishes this involves isolating chromatin from cell nuclei and transcribing it by adding back exogenous polymerases along with the proper combination of salts and necessary substrates. A second method uses intact nuclei purified free of the cell cytoplasm. It was felt that this system might reflect more accurately the *in vivo* conditions of RNA transcription.

Basic Systems

The first report of nucleic acid synthesis in isolated nuclei was in 1957 by Vincent Allfrey and his co-workers (Allfrey *et al.*, 1957; Osawa *et al.*, 1957). At the time, these workers were primarily interested in protein synthesis in isolated nuclei. They reported that calf thymus nuclei actively incorporated labeled amino acids into protein and that DNA was necessary to this process. They also noted that glycine was incorporated into nucleic acid purines and orotic acid was incorporated into pyrimidines. DNA was also necessary for this RNA synthesis.

They reported that calf thymus nuclei contained the four ribonucleosides (adenosine, cytosine, guanosine and uridine) and that these could be phosphorylated to their triphosphate counterparts with the aid of cytoplasmic components. It was also demonstrated that the benzimidazole riboside, 5,5-dichloro-β-D ribofuranosyl-benzimidazole (DRB) at levels of 50 ug/ml inhibited RNA synthesis. Later that year, Allfrey and Mirsky (1957) reported a method for the fractionation of nuclear RNAs. In this report, they expanded upon their studies of RNA synthesis in isolated nuclei.

In 1959, Breitman and Webster published a report dealing with the effect of monovalent cations on protein and nucleic acid synthesis in isolated nuclei. They stated that the utilization of amino acids for protein and nucleic acid synthesis required sodium ions. However, the incorporation of nucleotides into RNA was not affected by the substitution of potassium for sodium.

In 1966, Mittermayer *et al.* reported a system using nuclei isolated from the myxomycete *Physarum polycephalum* which supported RNA synthesis. Synthesis was dependent upon the presence of magnesium ions, all four ribonucleoside triphosphates and intact DNA. They concluded that their nuclei contained a functional DNA-dependent RNA polymerase.

In recent years, *in vitro* RNA synthesis has been reported in nuclei isolated from a number of different sources. These include nuclei from rat liver and human leukemic cells (Tryfiates and Polutanovich, 1972), sea urchin embryos (Shutt and Kedes, 1974), mouse myeloma cells (Marzluff *et al.*, 1973), Krebs II ascites cells (Wu and Zubay, 1974), hen oviduct cells (Ernest *et al.*, 1976), *Xenopus laevis* embryos (Yasuda *et al.*, 1977) and soybean leaves (Rizzo *et al.*, 1978). All of these systems

have in common the requirements for divalent and monovalent cations and the four ribonucleoside triphosphates. Synthesis is due to the presence of endogenous DNA-dependent RNA polymerase. The systems vary with respect to nuclear isolation procedure, concentrations of the various substrates, and the length and temperature of incubation. The isolation and synthesis conditions most commonly used in recent years (regardless of the source of nuclei) are modifications of the system reported by Marzluff *et al.* (1973). These workers separated the nuclei from cytoplasmic components by pelleting through 2.0 M sucrose. The reaction buffer in which the nuclei were resuspended contained magnesium, manganese, Tris buffer (pH 8.0), EDTA, dithiothreitol (or 2-mercaptoethanol), potassium chloride (or ammonium sulfate), the four ribonucleoside triphosphates, and 12.5% glycerol to stabilize the nuclei. Incubation was at 25°C. They demonstrated that, under these conditions, RNA synthesis was linear for at least one hour, that it was sensitive to α-amantin and actinomycin D, and that the amount of RNA synthesized was directly related to the number of nuclei added to the reaction. The RNA synthesized was heterogeneous in size, ranging from 4S to 45S, and a portion of the RNA was polyadenylated.

Synthesis of Ribosomal RNA

As ribosomal RNA has proven to be the easiest specific RNA species to isolate in pure form, it is not surprising that the first reports of the synthesis of a specific RNA in isolated nuclei involved ribosomal RNA. Reeder and Roeder (1972) utilized nuclei isolated from *Xenopus laevis* tissue culture cells to demonstrate RNA synthesis *in vitro*. By hybridizing purified ribosonal DNA to the synthesized RNA they were able

to show that a major fraction of that RNA was ribosomal. Since the synthesis of this ribosomal RNA was totally insensitive to α-amanitin, an inhibitor of RNA polymerase II, they concluded that ribosomal RNA must be synthesized by form I of the enzyme. They could not, however, completely rule out the involvement of RNA polymerase III. At this same time, Caston and Jones (1972) reported the synthesis of high molecular weight RNA in nuclei from embryos of *Rana pipiens*. Their system was optimized for maximum methylation of RNA, and they found that the entire ribosomal gene was transcribed, although the large ribosomal RNA was apparently under-methylated, yielding a sedimentation value of 25S rather than the expected 28S.

Marzluff *et al.* (1974) identified two distinct RNA products of their mouse myeloma nuclear system as 5S ribosomal RNA and a 4.5S precursor to transfer RNA. The 4.5S RNA could be converted *in vitro* to 4S RNA. As the synthesis of these RNAs was not sensitive to α-amanitin, they concluded that they were not synthesized by polymerase II. Experiments using γ-^{32}P-GTP indicated that the synthesis of these two RNAs was initiated *in vitro*.

A number of studies have been reported which attempt to elucidate the control mechanisms for ribosomal RNA synthesis. Bolla *et al.* (1977) reported that ribosomal proteins stimulated the synthesis of 45, 18, and 28S RNAs in isolated nuclei. The ribosomal proteins were found associated with newly synthesized 45S RNA, forming 80S particles in the nucleolus. Lindell *et al.* (1978) have proposed that messenger RNA has a role in ribosomal RNA synthesis. They reported that low concentrations of actinomycin D administered *in vivo* caused a partial inhibition of nucleoplasmic RNA polymerase II *in vitro* as assayed in isolated rat liver nuclei.

The synthesis of rapidly labeled nuclear proteins was also inhibited at this concentration. They suggested that ribosomal RNA synthesis might be under the control of the mRNAs whose transcription is blocked by the low levels of actinomycin D.

Baserga *et al.* (1977) and Ide *et al.* (1977) have recently reported that preparations of SV40 T antigen stimulate ribosomal RNA synthesis in isolate rat liver nuclei. They suggested that this phenomenom may be related to the mechanism of viral transformation.

In studies of the synthesis of 5S rRNA and 4.5S tRNA precursor, Roeder and co-workers have demonstrated in mouse myeloma nuclei that these RNAs are synthesized by RNA polymerase III (Sklar and Roeder, 1977; Parker *et al.*, 1977). They used exogenous RNA polymerase III added to the nuclei as well as the endogenous polymerases. It was suggested that they might be observing transcription of chromatin fragments leaked out of the nuclei (Hagopian and Ingram, 1978). However, Roeder *et al.* (1978) felt that the controls in their system and the fact that they were using eukaryotic polymerases (as opposed to the *E. coli* polymerase used by Hagopian and Ingram (1978)) effectively ruled out this possibility.

Synthesis of Poly(A)-containing RNA

The demonstration that many eukaryotic messenger RNA molecules have a poly-adenylate (poly(A)) sequence at their 3' terminus (Edmonds *et al.*, 1971; Darnell *et al.*, 1971) made it feasible to identify and isolate mRNA in a state relatively free of contaminating non-messenger RNA. Studies of messenger RNA synthesis in isolated nuclei would be greatly enhanced if polyadenylation occurred *in vitro*. In the initial description of their synthesis system, Marzluff *et al.* (1973) reported that a

significant fraction of the newly synthesized RNA was retained on oligo(dT) cellulose, indicating that it contained poly(A) sequences. That same year, Banks and Johnson (1973) reported that nuclei isolated from mouse brain synthesized poly(A)-containing RNA. Jelinek (1974) reported poly(A) synthesis in isolated HeLa cell nuclei and demonstrated that poly(A) synthesis required ATP concentrations 75-200 times higher than that necessary for total RNA synthesis. Cooper and Marzluff (1978) reported that polyadenlyation in isolated mouse myeloma nuclei was dependent upon the addition of an extract of crude nuclei. The poly(A) was synthesized *in vitro* and was the same size as that found *in vivo* on heterogeneous nuclear RNA. They found that only polymerase II transcripts were polyadenylated. Nakanishi *et al.* (1978), on the other hand, reported that in nuclei isolated from Ehrlich ascites tumor cells a significant amount of poly(A) was found associated with polymerase I and III products (from 1 to 4% of the total RNA). They speculated that a small amount of hnRNA may be synthesized by polymerase I and/or polymerase III. DePomeral and Butterworth (1975) also reported that poly(A) could be added post-transcriptionally to processed fragments of the products of α-amanitin resistant polymerases. They suggested that the poly(A) segment is added by polymerase II which is bound to the DNA template. Biswas *et al.* (1976), using nuclei isolated from rat pituitary tumor cells, demonstrated *de novo* poly(A) synthesis and found that cordycepin triphosphate (3'-dATP) inhibited this synthesis. The effect of cordycepin triphosphate on poly(A) synthesis was also investigated in rat liver nuclei by Rose *et al.* (1977). They incubated nuclei with or without exogenous primer to distinguish between chromatin-bound poly(A) polymerase and free enzyme. They found that 80-times more inhibitor was needed to

achieve 50% inhibition of the free enzyme than for the bound enzyme. Similar high levels of inhibition were necessary for inhibition of DNA-dependent RNA synthesis.

In a recent study Kieras *et al.* (1978) have carefully examined a number of parameters affecting the synthesis of poly(A) sequences in mouse myeloma nuclei. They found that 5 mM KCl stimulated synthesis 10 to 20-fold over levels synthesized at 120 mM KCl. The poly(A) sequences were of similar size at the low and high KCl concentrations, but the RNA to which the poly(A) was attached was shorter at the lower concentration. They also found that manganese ions in the medium led to a heterogeneous population of poly(A) sequences. They reported optimum conditions which allowed synthesis of poly(A)-containing RNA resembling those found *in vivo*. They also suggested that some processing and turnover was taking place.

Synthesis of Viral RNA

Isolated nuclei have proven to be an excellent system for the study of viral replication in eukaryotic cells. Rymo *et al.* (1974) used nuclei isolated from chick embryo fibroblast cells to look at the synthesis of Rous Sarcoma virus RNA. They found that manganese, magnesium, ammonium sulfate, and the four ribonucleoside triphosphates were necessary for total RNA synthesis as well as viral RNA synthesis. Both were suppressed by pre-incubation with DNase or actinomycin D. Virus-specific RNA was sensitive to α-amanitin, suggesting that it was synthesized by polymerase II. In nuclei from infected cells, virus-specific RNA comprised 0.5% of the total RNA synthesized as opposed to 0.005 to 0.03% in control nuclei. They concluded that the tumor virus-specific RNA was synthesized

on a DNA template, probably by RNA polymerase II. At about the same time, Roeder and co-workers reported similar results for adenovirus 2 (Weinmann *et al.*, 1974). They found that late in infection polymerase I was not involved in transcription of viral RNA. Based on a differential sensitivity to α-amanitin, they suggested that both polymerase II and polymerase III were involved in viral RNA synthesis. A large fraction of the viral RNA was synthesized by polymerase II, but they concluded that the 5.5S viral RNA, as well as 5S cellular RNA, was synthesized by polymerase III. This group reported further work on this system using nuclei from a cloned cell line containing only the left 14% of the virus genome (Bitter and Roeder, 1978). Their characterization of this system was similar to previous work. They concluded that the viral RNA in this system was synthesized only by polymerase II. Also, they reported that, while only one strand of virus RNA is found in the cytoplasm of infected cells, both strands were transcribed. They have also reported further studies on the low molecular weight viral RNA synthesized by RNA polymerase III (Harris and Roeder, 1978). By hybridization and partial sequencing, they have shown that the small RNAs synthesized by polymerase III in adenovirus infected nuclei are indeed the authentic viral products. They also found that one of the products might be a larger precursor to the viral RNA, although they could not rule out the possibility that the polymerase III occasionally "reads through" the viral gene termination signal.

Gefter and co-workers have reported development of an *in vitro* system in which synthesis of adenovirus 2 RNA is initiated *de novo* in isolated nuclei (Manley *et al.*, 1978). They found that a substantial fraction of the viral RNA was greater than 10,000 nucleotides in length and

contained a unique 5' end which mapped between 15 and 17% on the adenovirus 2 map. They stated that this portion corresponds to the previously demonstrated 5' terminus of adenovirus 2 hnRNA as well as many adenovirus 2 late mRNAs. Weinmann and Aiello (1978) have also reported mapping of adenovirus 2 late genes. They showed that the 5' ends of the RNAs for late genes mapped at different locations from the early genes, suggesting that the transition from early to late functions is controlled at the transcriptional level.

Yamamoto and co-workers have reported studies on the hormone sensitivity of a mouse mammary tumor virus (Yamamoto *et al.*, 1977; Stallcup *et al.*, 1978). They used nuclei isolated from a mouse mammary tumor cell line. By hybridization with unlabeled viral RNA, they demonstrated that nuclei from cells pre-treated with glucocorticoid hormone synthesized mammary tumor virus (MTV) RNA at a level of about 0.2 - 0.4% of the total RNA. In control nuclei (untreated or dexamethsaone treated), only 0.01 - 0.03% of the total RNA was MTV-specific. Glucococorticoid treatment had no effect on total RNA synthesis by the nuclei.

Synthesis of Specific Messenger RNAs

Advances in the last several years have made it possible to obtain a number of individual eukaryotic messenger RNAs in a homogeneous, translationally active form. Through the use of specific complementary DNA (cDNA), the synthesis of several of these messenger RNAs has been studied in isolated nuclear systems. Fodor and Doty (1977) examined globin mRNA synthesis in chicken reticulocyte nuclei. They reported that 0.24% of the total RNA synthesized was globin specific. Orkin has studied globin synthesis in nuclei isolated from inducible murine

erythroleukemic cells (Orkin and Swerdlow, 1977; Orkin, 1978a; Orkin, 1978b). The murine erythroleukemic cells are arrested at the pre-erythroblast stage of differentiation. After induction by a varity of agents (dimethylsulfoxide is the most commonly used), the cells resume differentiation and accumulate globin. Orkin optimized a nuclear system from these cells for total RNA synthesis and then examined the products for the presence of globin mRNA with specific cDNA. He found that globin mRNA synthesis was markedly increased in nuclei from induced cells, concluding that globin accumulation was the result of transcriptional activation rather than post-transcriptional stabilization of the mRNA. He also demonstrated that only one strand of the globin gene was transcribed. Similar results were reported by Schütz and co-workers for ovalbumin synthesis in chicken oviduct nuclei (Schütz *et al.*, 1977; Nguyen-Huu *et al.*, 1978). Production of ovalbumin, conalbumin, ovomucoid, and lysozyme in chick oviduct is inducible by estrogen or progesterone. By examining the RNA synthesized in isolated nuclei from induced and uninduced chick oviduct, Schütz *et al.* (1977) found a significant increase in ovalbumin mRNA synthesis in induced nuclei. They reported that ovlabumin mRNA is preferentially transcribed in induced nuclei 1000-fold over random transcription of the genome by polymerase II. Approximately 0.1% of the total RNA synthesized was found to be ovalbumin-specific. Although the half-life of ovalbumin mRNA has been shown to be increased in induced cells (Palmiter and Carey, 1974), the results of Schütz *et al.* indicate that transcriptional controls are also involved.

Bellard *et al.* (1977) have also examined ovalbumin mRNA synthesis in nuclei from estrogen induced chick oviduct as well as from oviduct of mature laying hens. Their synthesis conditions included high salt and heparin in an attempt to obviate any *in vitro* initiation of RNA

synthesis as well as post-translational modifications of the RNA. They found that polymerase II synthesized the ovalbumin mRNA and only one strand of the DNA was transcribed. They estimated that there were approximately 2 to 3 transcribing polymerase molecules per gene in the chick and 5 per gene in the laying hen.

Isolated nuclei have also been used to examine the synthesis of histone mRNA at various stages of the cell cycle. Detke *et al.* (1978) used nuclei isolated from synchronized HeLa cells to examine transcription of histone mRNA. They reported that transcription of this mRNA was sensitive to α-amanitin and occurred during the S phase, but not during the G_1 phase, of the cell cycle.

Another hormonally controlled protein which has been studied is α2a-globulin, a male rat liver protein. Chan *et al.* (1978) examined RNA synthesized in isolated rat liver nuclei for the presence of α2a-globulin sequences. They found that in nuclei from male rat liver the specific mRNA sequences comprised 0.005% of the total RNA synthesized and was completely sensitive to α-amanitin. They were unable to detect α2a-globulin mRNA in female rat liver nuclei. They concluded that the absence of this protein in female rats is due to the lack of transcription of the gene.

Huang and co-workers have examined the synthesis of immunoglobulin kappa light chain mRNA in mouse myeloma nuclei (Smith and Huang, 1976; Huang *et al.*, 1977). They reported that the light chain mRNA synthesis is sensitive to α-amanitin as expected and is enriched about 300-fold over the haploid genome. They also reported some preliminary evidence that at least a portion of the mRNA synthesis is initiated *in vitro* (Huang *et al.*, 1977).

Processing and Transport of Synthesized RNA

One of the reasons for choosing an isolated nuclear system over *in vitro* RNA synthesis with isolated chromatin is the assumption that the former more closely resembles *in vivo* conditions. A number of studies have been done examining the fate of newly synthesized RNA in isolated nuclei to determine if it is processed or metabolized in a manner similar to the *in vivo* situation. Caston and Jones (1972) found, that in an isolated nuclear system from embryos of *Rana pipiens*, ribosomal RNA was synthesized that was indistinguishable from purified cytoplasmic rRNA. Under their conditions, the RNA was also methylated *in vitro*, although at least one of the species was apparently methylated to a lesser extent than its native counterpart. Busiello and Di Girolarmo (1975) reported similar results using nuclei isolated from HeLa cells. They found that there was initiation of RNA synthesis *in vitro* as well as completion of nascent chains initiated *in vivo* and that RNA synthesis started predominantly with a purine base. They also reported that nucleolar RNA was methylated *in vitro* with S-adenosyl-L-methionine serving as the methyl donor. Bolla *et al.* (1977) reported that ribosomal protein stimulated synthesis of 45S rRNA in isolated rat liver nuclei and that these proteins associated with 18 and 28S RNA to form ribonucleoprotein particles. Kozlov *et al.* (1978) examined the effect of low molecular weight nuclear RNA (lnRNA) on ribosomal RNA synthesis in isolated rat liver nuclei. They found that lnRNA had no effect on RNA polymerase I activity. Marzluff *et al.* (1974) looked at the synthesis of 5S rRNA and 4.5S precursor tRNA in mouse myeloma nuclei. They found that although both of the genes for these RNAs are nucleoplasmic, they were not transcribed by the major nucleoplasmic polymerase

(polymerase II). They also reported that the 4.5S tRNA precursor molecule could be converted *in vitro* to 4S tRNA. Cooper and Marzluff (1978) reported recently that their cell-free system of RNA synthesis using mouse myeloma nuclei synthesized poly(A) and added it to completed RNA molecules *in vitro*. Cap structures were added to completed RNA molecules and large hnRNA-like molecules were processed to mRNA-like molecules and transported out of the nucleus. *In vitro* synthesis and capping of RNA has also been reported by Wincov and Perry (1976). They found that nuclei from mouse L cells were capable of synthesizing large hnRNA-like molecules and forming both cap I and cap II structures. Wincov (1977) subsequently demonstrated a polynucleotide kinase function in this same system. Using γ-^{32}P-ATP, she demonstrated transfer of the gamma phosphate to the 5' terminus of large RNA molecules. It was also reported that GTP could serve as a donor.

Using nuclei isolated from slow and fast skeletal muscles, Held (1977) reported differential RNA synthesis. She found that polymerase II activity was increased in nuclei from slow-twitch soleus as compared to nuclei from fast-twitch soleus.

Biswas and co-workers used a rat pituitary tumor cell line (GH_3) to study synthesis and processing of RNA (Biswas *et al.*, 1976; Biswas, 1978). They found extended synthesis of RNA in isolated nuclei with polyadenylated polymerase II products resembling mRNA. The polymerase II products associated with proteins in the nucleus forming ribonucleoprotein particles (RNPs), some of which were transported out of the nucleus. Polymerase III products were released from the nucleus as free RNA.

Using isolated mouse brain nuclei, Johnson and co-workers have

also examined RNA metabolism *in vitro*. Banks-Schlegel and Johnson (1975) reported that RNA synthesized in brain nuclei from 12 and 30 day old mice was of much smaller molecular weight than that from neonatal mice. They also found that age effect varied with different cell populations. Glial cell nuclei were found to be most active in RNA synthesis and metabolism at birth, decreasing rapidly with age. Neuronal cell nuclei, however, increased in activity until 14 days of age, remaining essentially constant thereafter. Weck and Johnson (1978a) reported conditions under which newly synthesized RNA was released from isolated nuclei. Addition of cytosol to the system inhibited this release. Partial fractionation of the cytosol revealed fractions which stimulated release as well as fractions which inhibited release. It was suggested that the fractions which facilitated release were associated with cellular proteins. McNamara *et al.* (1975) have also reported release of newly synthesized RNA from isolated nuclei. They used rat liver nuclei and found prolonged synthesis and transport of both mRNA and rRNA in the presence of cytosol.

Sarma *et al.* (1976), using HeLa cell nuclei, have reported that although high molecular weight RNA is synthesized, only low molecular weight RNA is released from the nucleus and normal RNA processing does not appear to occur inside the nucleus. Using aurintricarboxylic acid (ATA), which they proposed as an inhibitor of chain initiation, they claimed that 80% of the RNA synthesized in their system was initiated *in vitro*.

Mory and Gefter (1977) have reported synthesis of *in vivo*-like RNA in nuclei isolated from mouse myeloma cells. A significant fraction of the RNA was polyadenylated and capped. A portion of the α-amanatin

sensitive RNA was released from the nucleus and could be incorporated into polyribosomes.

Effect of Cytosol on RNA Synthesis and Processing

One advantage of studying RNA synthesis in isolated nuclei is that the system is removed from the possibly complicating effects of the cytoplasmic components of the cell. However, it is of interest to know what effects the cytoplasm (cytosol) might have upon RNA synthesis and processing. A number of workers have reported studies on this subject.

McNamara *et al.* (1975) reported that in isolated rat liver nuclei, cytosol stimulated rRNA synthesis and, to a lesser extent, non-ribosomal RNA synthesis. Mory and Gefter (1977) reported similar results in myeloma nuclei. Addition of cytosol stimualted slightly the overall synthesis of RNA and prolonged the time of synthesis. In normal rat liver nuclei and rat hepatoma nuclei, Bastian (1977) found that RNA synthesis was stimulated when the nuclei were incubated in their homologous cytosol. The stimulation could be increased if cytosol from regenerating liver was used. She also found that although the size range of RNA synthesized in the presence of normal or regenerating liver cytosol was similar, hybridization analysis revealed that regenerating liver cytosol stimulated the synthesis of RNA from unique and slightly repetitive genes that were transcribed to a much lesser extent in the presence of normal liver cytosol. (Bastian, 1978).

Dvorkin *et al.* (1974) reported differences in the RNA synthesized in rat liver nuclei incubated in the presence of either homologous cytosol or cytosol from rat hepatoma cells. When homologous cytosol was used, analysis of the synthesized RNA by hybridization indicated that

the RNA was similar to that found in whole cells. Use of the hepatoma cytosol suppressed total RNA synthesis somewhat and yielded a more limited population of RNA. The authors reported that this RNA was similar to that found in intact rat hepatoma cells.

Johnson and co-workers have studied the effect of cytosol on RNA synthesis and release in mouse brain nuclei. They initially reported that cytosol from both young and adult mouse brain tissue stimulated RNA synthesis (Banks *et al.*, 1974). This stimulation was 3-fold greater in nuclei from newborn mice than in nuclei from adult mice. They subsequently reported that dialyzed cytosol from various mouse tissues as well as from guinea pig brain and neuroblastoma cells also stimulated RNA synthesis in mouse brain nuclei (Weck and Johnson, 1976). In addition, the size of the synthesized RNA was increased by the cytosol. Dialyzed mouse serum, on the other hand, had no effect on the rate of incorporation or the size of the products. The size increase of RNA caused by cytosol was found in 10 day old and adult brain nuclei but not in 2 day old nuclei (Weck and Johnson, 1978b). RNA from 2 day old mice was larger in size without cytosol and was unaffected by its addition. Cytosol did cause an increase in the amount of poly(A)-containing RNA in nuclei from 2 day old and adult animals, but no increase was observed with nuclei from 10 day old animals. It was also found that cytosol contained fractions which could either stimulate or inhibit release of RNA from isolated mouse brain nuclei (Weck and Johnson, 1978a).

Factors Which Enhance RNA Synthesis

In a number of cell lines of tissues which are inducible by various substances it has been shown that this induction results in the

preferential increase in synthesis of a specific RNA (or RNAs). Yamamoto *et al.* (1977) and Stallcup *et al.* (1978) demonstrated that glucocorticoid treatment of GR cells--a mouse mammary tumor cell line--resulted in the preferential transcription of mammary tumor virus RNA, while leaving overall RNA synthesis unaffected. A synthetic glucocorticoid, dexamethasone, had no effect. Mizuno *et al.* (1978) have reported that in nuclei from chick oviduct RNA synthesis decreased by 50% within 48 hours of estrogen withdrawal. They demonstrated that more than 90% of the RNA was complementary to unique sequence DNA. They have not yet examined the RNA for the presence of specific mRNA sequences. The fact that estrogen withdrawal leads to a decrease in overall RNA synthesis would seem to contradict the results reported by Yamamoto's group. However, since the four proteins induced by estrogen in the chick oviduct (ovalbumin, conalbumin, ovomucoid and lysozyme) comprise the bulk of the protein synthesis in the cell, the removal of estrogen might be expected to reduce the overall levels of RNA synthesis.

Zerwekh *et al.* (1974) reported that in nuclei from rachitic chick intestinal cells, 1-α, 25-dihydroxyvitamin D_3 stimulated RNA polymerase II activity two-fold. RNA polymerase I activity was not affected.

Biswas *et al.* (1976) found that rat liver nuclease inhibitor (RI) was necessary for extended RNA synthesis in their GH_3 nuclei. They did not examine the exact mechanism of action of the RI, but it is probable that the extended synthesis is simply the result of the functional removal of endogenous nucleases in the system. Baserga and co-workers have reported that addition of SV40 T antigen to nuclei from rat liver or quiescent hamster cells stimulated RNA synthesis by as much as 150%. This increase was shown to be completely α-amanitin resistant, indicating

that RNA polymerase I products are stimulated. Gershon *et al.* (1978) have reported that, in rat liver nuclei, RNA polymerase I activity was stimulated by the addition of the synthetic double-stranded polynucleotide poly(dAT). This stimulation was age dependent, as nuclei from 3 month old rats were stimulated by 33% whereas nuclei from 24 month old rats were stimulated by about 100%. No stimulation was observed with poly(dA), poly(dC), poly(dGdC), poly(U), or RNA from various sources. They suggested that poly(dAT) removes a specific regulating factor (or factors) from the chromatin, thus stimulating polymerase I activity.

Using isolated cardiac nuclei, Schreiber *et al.* (1978) demonstrated that RNA polymerase II activity was stimulated by hydrostatic pressure. The stimulation was about 30 to 40% and was completely sensitive to α-amanitin. They suggested that this may be the stimulus that triggers the augmented protein synthesis seen in pressure overload of cardiac cells.

Weck and Johnson (1976) reported that heparin stimulated RNA synthesis in mouse brain nuclei, although it had no effect on the size of the RNA. Coupar and Chesterton (1977) reported similar results in rat liver nuclei. They found that heparin had no effect on the levels of the various polymerases in the nucleus. The rate of chain elongation by polymerase I was not affected, but the rate of polymerase II was increased 2 to 4-fold. Neither of these reports, however, considered the physical effects which heparin has upon nuclei (such as condensation of chromatin) and how this would affect the interpretation of their results.

Factors Which Inhibit RNA Synthesis

The inhibitory effect of actinomycin D and α-amanitin on eukaryotic RNA polymerases has been studied in great detail (see review by Jacob, 1973). Their effect on isolated nuclei will not be considered here, with one exception. Lindell and co-workers, using both rat liver cells and isolated nuclei, presented evidence suggesting that actinomycin D might have an extra-nucleolar mechanism of action (Lindell, 1976; Lindell *et al.*, 1978). They found that low doses of actinomycin D partially inhibited RNA polymerase II in isolated nuclei. In whole cells, there was a time dependent inhibition of polymerase I activity. The synthesis of rapidly labeled nuclear proteins was also inhibited in whole cells. They suggested that polymerase I (rRNA transcription) might be under the control of the mRNA which codes for these rapidly labeled proteins.

Rose *et al.* (1977) have reported on the effects of cordycepin 5'-triphosphate on poly(A) synthesis in isolated nuclei. They found that low levels of the drug inhibited the chromatin-bound poly(A) polymerase enzyme but had no effect on nucleoplasmic poly(A) polymerases or on the DNA dependent RNA polymerases. Much higher levels of the drug were required to inhibit the latter two groups of enzymes.

The effect of the dye aurintricarboxylic acid (ATA) on RNA synthesis in isolated nuclei from HeLa cells was reported by Sarma *et al.* (1976). ATA is an inhibitor of nucleic acid binding proteins. It is also an inhibitor of protein synthesis (Huang and Grollman, 1972) and a general nuclease inhibitor (Hallick *et al.*, 1977; Tsutsui *et al.*, 1978). Sarma *et al.* (1976) found that in their system ATA inhibited RNA synthesis by 80%, the same level of inhibition as α-amanitin. They suggested that ATA was an inhibitor of initiation of RNA synthesis (although they sited

no evidence to this effect) and thus concluded that 80% of the RNA synthesis in their system was initiated *in vitro*.

The nucleoside analog 5,6-dichloro-β-ribofuranosyl benzimidazole (DRB) has been reported to be an inhibitor of hnRNA initiation in eukaryotic cells (Sehgal *et al.*, 1976). As early as 1957, Allfrey *et al.* (1957) noted that DRB inhibited RNA synthesis in isolated calf thymus nuclei. More recently, Tamm (1977) has reported that RNA synthesis in isolated HeLa cell nuclei is inhibited if the cells are pre-treated with DRB. About one-third of the hnRNA was resistant to the DRB pre-treatment, and the size of this resistant RNA was distributed over the entire range of hnRNA sizes. By using α-amanitin and heparin *in vitro* in conjunction with the DRB pre-treatment, Tamm found that the DRB-resistant RNA could be divided into two fractions, one 140 to 330 residues in length and the other 330 to 740 residues in length. The smaller size range was reinitiated *in vitro* while the larger size was not.

Initiation of RNA Synthesis

There is some disagreement in the literature as to the level of reinitiation of RNA synthesis which occurs *in vitro*. Some state that there is no reinitiation taking place (Bitter and Roeder, 1978a; Tata and Baker, 1978), while others claim that at least some species of RNA are initiated *in vitro* (Tamm, 1977; Busiello and Di Girolamo, 1975).

Huang and co-workers reported that in mouse myeloma nuclei 5S ribosomal RNA and 4.5S precursor tRNA were initiated *in vitro* (Marzluff *et al.*, 1974). They determined this by labeling with γ -^{32}P-GTP and detecting the presence of labeled guanosine tetraphosphate. They realized, however, that this method would probably not allow enough label

incorporation to detect RNA transcribed from single-copy genes (Huang *et al.*, 1977). Therefore, they investigated the use of nucleoside triphosphates with a sulfur group substituted for an oxygen on the gamma phosphate (Reeve *et al.*, 1978; Smith *et al.*, 1978). (See Yount, 1975, for a review of this and other nucleotide analogs). Using both synthetic DNA templates and bacteriophage λ DNA with *E. coli* RNA polymerase, they demonstrated that purine nucleoside 5'-(γ - S) triphosphates were incorporated into RNA which could be isolated by passage over affinity columns of mercury-agarose. By identifying γ - S purine tetraphosphates, they demonstrated that the sulfur-containing nucleotides were at the 5'-end of the RNA chains. No sulfur-containing nucleotides were found in internal positions in the RNA. Thus, this method allows the specific isolation of RNA molecules which were initiated *in vitro* and should allow the detection of single-gene transcripts. Huang *et al.* (1977) also stated that the γ - S nucleoside triphosphates are resistant to phosphatase cleavage, although they sited no evidence to this effect. A resistance to phosphatase cleavage would prevent capping of *in vitro* initiated mRNA molecules and allow their isolation on mercury-agarose. Huang *et al.* (1977) have reported preliminary results stating that about 15% of the RNA synthesized in isolated nuclei from mouse myeloma is initiated *in vitro*.

RNA Synthesis in Nuclei from Synchronized Cells

Grant (1972) reported differences in RNA polymerase I and RNA polymerase II activity in nuclei isolated from *Physarum polycephalum* at various stages of the cell cycle. He found a peak of polymerase I activity (presumably rRNA synthesis) during G_2, about 2.5 to 3 hours before

mitosis. RNA polymerase II activity peaked about 2.5 to 3 hours after mitosis. More recently, Davies and Walker (1978) have reported that polymerase II activity peaked 1 to 3 hours after mitosis, falling to a constant level for the remainder of the cell cycle. RNA polymerase I activity, however, was reported to be constant throughout the cell cycle, with the exception of mitosis, during which time all RNA synthesis ceases. They did not discuss the differences between their data and that of Grant (1972).

Rossini and Baserga (1978) examined RNA synthesis using a temperature sensitive mutant of a hamster cell line. At the non-permissive temperature, the cells are arrested at a point in mid-G_1. They reported that at the permissive temperature both polymerase I and polymerase II activity increased until the cells were well into S phase, peaking between 20 and 24 hours. At the non-permissive temperature, total RNA synthesis increased for about 16 hours. At this point, polymerase II activity declined rapidly, while polymerase I maintained its elevated level. The cells were now blocked in G_1. If maintained at the non-permissive temperature, polymerase II activity decreased to 50% at 24 hours and was not detectable at 48 hours. They suggested that polymerase II activity was necessary for entry of this mutant cell line into S phase.

The relationship of histone mRNA synthesis to the stage of the cell cycle was examined by Detke *et al.* (1978). They reported that HeLa cell nuclei isolated during S phase were capable of synthesizing histone mRNA while nuclei isolated during G_1 phase were not. Sensitivity to α-amanitin indicated that the histone mRNA was synthesized by RNA polymerase II.

Summary

It is evident that isolated nuclear systems have proven to be very useful in the study of eukaryotic gene expression. Isolated nuclei can serve as an assay system for the various RNA polymerases. Transcription of a number of individual RNA molecules has been reported. Processing of primary transcripts which resembles the processing seen *in vivo* has been demonstrated in isolated nuclei. Finally, some evidence suggests that isolated nuclei may exhibit many of the same transcriptional controls which are present *in vivo*. It is likely that isolated nuclear systems will continue in the future to be an important tool for the study of gene expression and regulation in eukaryotic cells.

MATERIALS AND METHODS

Cells

The mouse myeloma cell line MOPC-21 (P.3) (kindly provided by Dr. M.D. Scharff, Albert Einstein College of Medicine) was maintained in suspension culture in Dulbecco's Modified Eagle Medium (K-C Biologicals) supplemented with 10% fetal calf serum (K-C Biologicals). The cultures also contained 100 U/ml penicillin G, 75 μg/ml streptomycin, and 40 U/ml mycostatin. The cultures were incubated at 37°C in a moist atmosphere of 95% air and 5% CO_2. The cells were allowed to grow to 8 to 10 x 10^5 cells per ml and then diluted to 1 to 2 x 10^5 cells per ml with fresh medium. For the experiments reported here the cells were harvested during exponential growth, at levels from 4 to 8 x 10^5 cells per ml.

Nuclear Isolation

Nuclei were isolated essentially as described by Marzluff *et al.* (1973). Cells were pelleted at 500 x g for 8 to 10 minutes in an International refrigerated centrifuge. They were resuspended in lysis buffer (0.3 M sucrose, 2 mM $MgCl_2$, 3 mM $CaCl_2$, 10 mM Tris (pH = 8.0), 0.1% (v/v) Triton X-100, and 2 mM 2-mercaptoethanol). The cells were then lysed in a Dounce homogenizer with 2 to 3 strokes of the tight-fitting glass pestle. The homogenate was then mixed with 1 to 2 volumes of 2 M sucrose containing 5 mM $MgCl_2$, 10 mM Tris (pH = 8.0) and 2 mM 2-mercaptoethanol. This was layered over 2 mls of the 2 M sucrose in a polyallomer

centrifuge tube. The mixture was then centrifuged at 20,000 RPM for 45 minutes at 4°C in a Beckman SW 50.1 rotor. The nuclear pellet was drained and the insides of the tube wiped dry. The nuclei were resuspended gently in a buffer containing 25% glycerol, 10 mM $MgCl_2$, 50 mM Tris (pH = 8.0), 24 mM 2-mercaptoethanol, and 0.2 mM EDTA. To break up any aggregates of nuclei, the suspension was placed in the glass Dounce homogenizer and resuspended with one stroke of the tight-fitting pestle. The yield of nuclei by this procedure was 40 to 60%.

RNA Synthesis

RNA synthesis was generally done in either 800 ul or 200 ul reactions. For the 800 ul reaction, 200 ul of a solution of ribonucleoside triphosphates (containing 1.6 mM ATP, CTP, and UTP and 0.96 mM GTP (all from Calbiochem)) was added to 400 ul of the nuclear suspension. ^{3}H-GTP (Amersham (2 Ci/mmole) or ICN (16.7 Ci/mmole)) was dried under a continuous flow of compressed air at 4°C and then dissolved in 0.6 M KCl. A 200 ul aliquot of this solution was added to the reaction. The final reaction conditions were 12.5% glycerol, 5 mM $MgCl_2$, 25 mM Tris (pH = 8.0), 0.1 mM EDTA, 12 mM 2-mercaptoethanol, 150 mM KCl, 0.024 mM unlabeled GTP, and 0.4 mM each of ATP, CTP, and UTP. The reactions were incubated at 25°C in a gyratory water bath. Aliquots were removed at zero time and at various times during the reaction and spotted onto Whatmann 3MM filter paper discs (2.3 cm). The discs were air dried and then washed in three changes of ice cold 5% (w/v) trichloroacetic acid (TCA). The filters were individually rinsed under vacuum with approximately 10 mls of 5% TCA followed by 3 to 5 mls of 95% ethanol. The filters were then dried and counted by liquid scintillation in toluene containing 4 g/l

Omnifluor (New England Nuclear) in a Searle model Delta 300 liquid scintillation counter. Tritium efficiency on the filters was approximately 7 to 8%. The specific activity of the ^{3}H-GTP in the reaction mixture was normally 50 to 60 CPM per picomole of GTP.

RNA Isolation

RNA was isolated by a modification of the procedure described by Kwan *et al.* (1977). After 30 to 45 minutes of incubation at 25°C, a 800 *u*l reaction mixture was added to 5 ml of ice cold buffer containing 10 mM sodium acetate (pH = 5.2), 3 mM $MgCl_2$, 200 *u*g/ml heparin, 30 *u*g/ml polyvinyl sulfate, 200 *u*g/ml dextran sulfate, 3 mM each of 2'-3' AMP, CMP, and UMP and 40 *u*g/ml DNase (RNase-free, Worthington). This mixture was incubated at 25°C for 20 minutes. Sodium dodecyl sulfate (SDS) and EDTA were then added to final concentrations of 2% (w/v) and 5 mM respectively. Proteinase K (Beckman; pre-incubated in the isolation buffer for 15 minutes at 25°C) was added to a final concentration of 300 to 400 *u*g/ml and the mixture incubated at 25°C for 15 minutes. Sodium acetate (1.0 M, pH = 5.2) was then added to a final concentration of 50 mM. An equal volume of water-saturated phenol was added and the mixture vortexed for 30 to 60 seconds. An equal volume of chloroform: isoamyl alcohol (49:1) was added and the mixture again vortexed. The mixture was then centrifuged at 1500 x g for 10 minutes at 20°C. The lower phase was removed and an equal volume of chloroform:isoamyl alcohol added. The mixture was centrifuged again. The upper phase was removed, two volumes of 100% ethanol added to it, and the mixture was incubated overnight at -20°C. The precipitated RNA was centrifuged at 12,000 x g in a Sorvall RC-2B at 4°C, and the pellet was dried and

dissolved in sterile distilled water.

Sucrose Gradient Analysis of RNA

Isolated RNA was analyzed on 15 to 30% (w/v) sucrose gradients with a 45% (w/v) sucrose cushion. The sucrose was dissolved in a buffer containing 30 mM Tris (pH = 7.4), 100 mM NaCl, 5 mM EDTA, and 0.5% (w/v) SDS. The gradients were run in Beckman SW 50.1 tubes (0.5 x 2.0 inches). The 45% sucrose cushion was 0.5 ml. RNA samples were added to gradient buffer (2% SDS) and heated to 65^{o}C for 5 minutes before being rapidly cooled in ice and layered on top of the gradients. The gradients were centrifuged at 50,000 RPM for 2.75 hours at 25^{o}C. Samples of 200 ul were collected, precipitated with 5% TCA, collected on Whatmann GF/C filter discs and counted in toluene-Omnifluor.

Oligo(dT)-cellulose Chromatography

Oligo(dT)-cellulose, prepared according to Gilham (1964) was the kind gift of Dr. Fritz Rottman, Michigan State University. Column chromatography of RNA was done essentially as described by Aviv and Leder (1972). Columns were poured in pasteur pipettes and RNA was applied in a buffer containing 10 mM Tris (pH = 7.4), 0.2 mM $MgCl_2$, 500 mM NaCl, and 0.1% (w/v) SDS (application buffer). Bound material was eluted by washing the column with distilled water. Aliquots were TCA precipitated and counted on GF/C filters as described above.

Preparation of Mercury-Sepharose

Initially, mercury-Sepharose was prepared as described by Reeve *et al.* (1977) with the exception that Sepharose 2B (Pharmacia) was

employed instead of Bio-Gel A-15M. It was noted that the low levels of cyanogen bromide used for activation did not affect the pH or temperature of the reaction. It was also found that the parachloromercuribenzoate had to be dissolved in 0.2 to 0.25 M KOH before N,N-dimethylformamide could be added to a final concentration of 40% (w/v). At the pH of the final coupling reaction (4.8), the organomercury compound precipitated. This is apparently normal (R.C.C. Huang, personal communication).

The organomercurial content of the Sepharose was determined as described by Sluyterman and Wijdenes (1970) and Ellman (1959). Since this coupling procedure consistently yielded Sepharose with no detectable organomercury content, a second method was tried. AH Sepharose 4B (Pharmacia) was reconstituted by the addition of distilled water. This product is Sepharose 4B which has had a six-carbon spacer (diaminohexane) attached by cyanogen bromide activation. The AH Sepharose was washed and parachloromercuribenzoate coupled by reaction with carbodimide as described by Reeve *et al.* (1977).

Isolation of γ-S-containing RNA

RNA was fractionated over mercury-Sepharose columns essentially as described by Reeve *et al.* (1977). Columns were poured in disposable 5 ml plastic syringes plugged with glass wool. Total volume of packed Sepharose was approximately 2 ml.

RESULTS

Conditions of Synthesis

The conditions for RNA synthsis in isolated myeloma nuclei originally reported by Marzluff *et al.* (1973) included 1 mM manganese chloride in addition to those components listed in Materials and Methods. The initial experiments done in this study also contained manganese chloride in the reaction buffer. It was found subsequently that removal of manganese did not alter the overall RNA synthesizing capabilities of the system (W.F. Marzluff, personal communication). Kieras *et al.* (1978) have reported that poly(A) sequences synthesized in isolated mouse myeloma nuclei are more heterogeneous in size when manganese is present. They also found no effect on total RNA synthesis in the absence of manganese (M.L. Edmonds, personal communication). In view of these findings, it was decided to delete manganese from our synthesis system. As expected, RNA synthesis was not affected noticeably (data not shown).

Synthesis reactions from which multiple time points were to be taken were done in sterile plastic tubes (12 x 75 mm). The volume of the reaction was 0.8 ml. At time zero and all indicated time points thereafter, triplicate 30 ul aliquots were taken and spotted onto Whatmann 3MM filter discs. The filters were then processed as described in Materials and Methods. The incorporated radioactivity at each time point represents the average of the three aliquots. Unless otherwise noted, the values at time zero were subtracted from the values at the other time points. The specific activity of the GTP (determined

separately for each experiment) and the concentration of nuclei in the reaction were used to calculate picomoles of GTP incorporated per 10^6 nuclei for each time point.

Figure 1 illustrates a representative plot of RNA synthesis versus time. The curves shown are the averages of several different experiments. The control reactions contained ATP, CTP, UTP, and GTP and were incubated at either 25°C or 2°C. Also shown is a reaction in which the UTP had been replaced by 5-mercuriuridine triphosphate (Hg-UTP). In the control reaction at 25°C, synthesis is relatively linear for at least 30 minutes. The same reaction at 2°C shows virtually no synthesis. The reaction containing Hg-UTP and incubated at 25°C also shows relatively linear incorporation, but at a reduced rate compared to the control. After 30 minutes, the incorporation in the presence of Hg-UTP was about 40% of the control value. In subsequent experiments using Hg-UTP, incorporation was usually about 35 to 45% of the control values. This level of synthesis is somewhat lower than the 60 to 65% originally reported by Smith and Huang (1976) but is similar to values found by other workers (Orkin and Swerdlow, 1977; W.F. Marzluff, personal communication).

Actual levels of incorporation of GTP varied somewhat throughout the course of these studies. Initially, incorporation in control reactions was in the range of 150 to 200 picomoles of GTP per 10^6 nuclei. More recently, the values have averaged 80 to 150 picomoles per 10^6 nuclei. The shape of the incorporation curve also varied somewhat. Although always relatively linear for at least 30 minutes at 25°C, quite often the incorporation would plateau or even decrease slightly after 45 to 60 minutes. This is thought to be the result of endogenous nucleases in the system.

Figure 1. Incorporation of ^{3}H-GTP in isolated nuclei. The units of the ordinate are pico moles per 10^{6} nuclei. a. unmodified ribonucleotides at 25°C (– – –); b. Hg-UTP at 25°C (·····); c. unmodified ribonucleotides at 2°C (———).

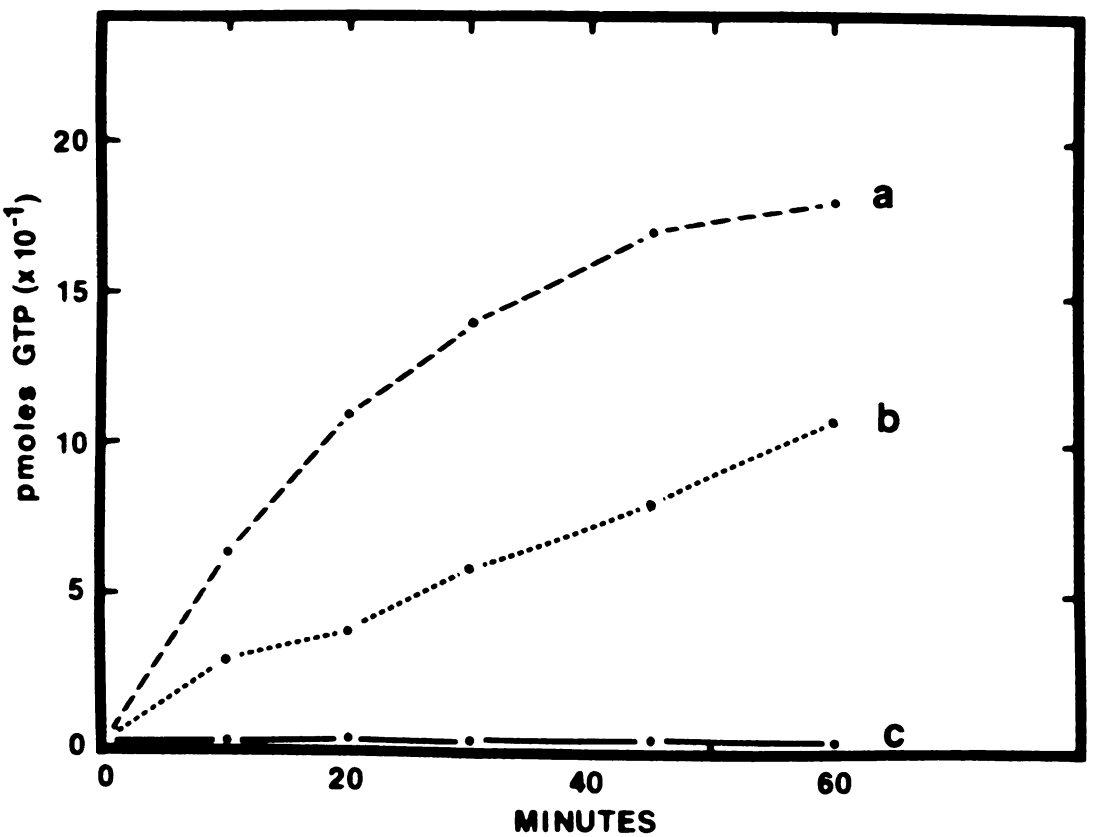


Figure 1

Dependence upon Exogenous Ribonucleoside Triphosphates

RNA synthesis in this system is dependent upon the addition of the four ribonucleoside triphosphates. This is shown in Figure 2. Reactions of 0.8 ml containing all four ribonucleotides or without ATP, CTP, or UTP were incubated at 25°C for one hour. The control curve shown in Figure 2 is the same as that in Figure 1. The deletion of either CTP or UTP from the reaction mixture resulted in virtually no RNA synthesis. After 30 minutes, the levels of incorporation were only 2.5% and 4.6% of control values in the absence of CTP and UTP, respectively. As the label used was ^{3}H-GTP, GTP could not be completely eliminated from the reaction. However, if unlabeled GTP was omitted from the reaction, the remaining ^{3}H-GTP concentration was insufficient for RNA synthesis (see Figure 4). The results were similar to the absence of either CTP or UTP. After 30 minutes, incorporation was only 3.0% of the control value (Figure 4). In contrast, in the absence of exogenous ATP, synthesis was reduced but not completely eliminated. After 30 minutes, incorporation was about 17% of the control value. This suggests that the nuclei contain low levels of endogenous ATP.

Effect of 2-Mercaptoethanol Concentration

Dale *et al.* (1973) noted that mercaptan concentration was very important for RNA or DNA synthesis by *E. coli* polymerases in the presence of mercurated nucleotides. Orkin and Swerdlow (1977) reported similar findings with eukaryotic RNA polymerases in isolated nuclei. Smith and Huang (1976), in their report of RNA synthesis using Hg-UTP in mouse myeloma nuclei, changed the reducing agent from 2.5 mM dithiothreitol (Marzluff *et al.*, 1973) to 12 mM 2-mercaptoethanol. We examined the

Figure 2. Dependence of RNA synthesis upon exogenous ribonucleoside triphosphates. The units of the ordinate are pico moles per 10^6 nuclei. All incubations at 25°C. a. all four ribonucleoside triphosphates added (– – –); b. without ATP (— – —); c. without CTP (·····); d. without UTP (————).

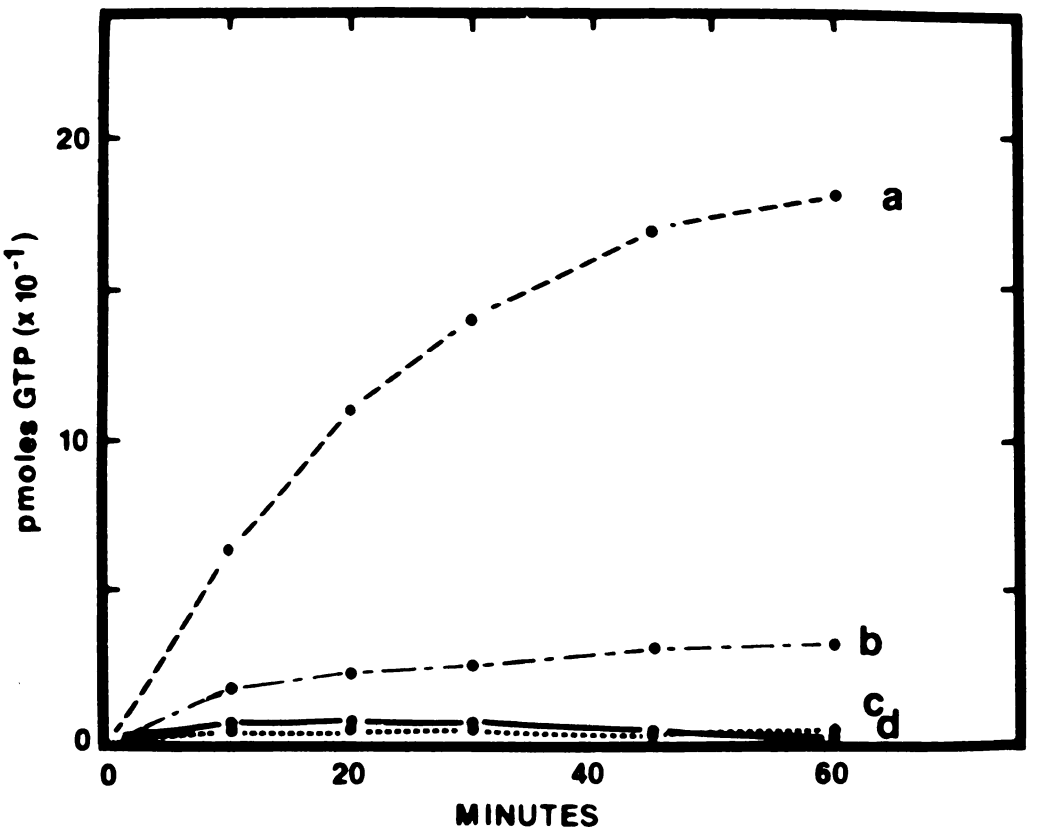


Figure 2

effect of the 2-mercaptoethanol concentration on RNA synthesis in our system. The results are shown in Figure 3. The reactions were 0.8 ml and Hg-UTP was substituted for UTP. It can be seen that there was little effect on synthesis over the range of 4 to 20 mM 2-mercaptoethanol. After 30 minutes of synthesis, incorporation of ^{3}H-GTP was decreased slightly at 4 mM 2-mercaptoethanol and increased slightly at 20 mM. For all subsequent reactions, 12 mM 2-mercaptoethanol was used, regardless of whether or not mercurated nucleotides were used.

Effect of GTP Concentration

Since the absolute levels of radioactivity incorporated into RNA in a given reaction are directly related to the specific activity of the precursor, it would be advantageous to use the highest specific activity precursor available. The two lots of ^{3}H-GTP which we used had specific activities of 2 Ci per millimole and 16.7 Ci per millimole. The reaction mixtures normally contained 10 uCi/ml ^{3}H-GTP which corresponds to a concentration of 5 uM GTP for the lower specific activity batch and 0.6 uM for the higher specific activity batch. These are both well below the 0.05 mM level reported by Marzluff *et al.* (1973). Thus it was necessary to add unlabeled GTP to the reactions and advantageous to determine the minimum concentration of GTP necessary for RNA synthesis. Reactions of 0.8 ml containing Hg-UTP and various levels of unlabeled GTP were incubated for 60 minutes at 25°C. The results are shown in Figure 4. When no unlabeled GTP was added, RNA synthesis was negligible. This is similar to the reaction in which CTP of UTP was omitted (see Figure 2). Increasing levels of unlabeled GTP yielded increasing levels of incorporation. At a concentration of 0.024 mM GTP, synthesis was only

Figure 3. Effect of 2-mercaptoethanol concentration on RNA synthesis. The units of the ordinate are pico moles GTP per 10^6 nuclei. Incubations at 25°C; Hg-UTP substituted for UTP. Final concentration of 2-mercaptoethanol: a. 20 mM (—··—); b. 16 mM (— — —); c. 12 mM (————); d. 8 mM (··········); e. 4 mM (—·—).

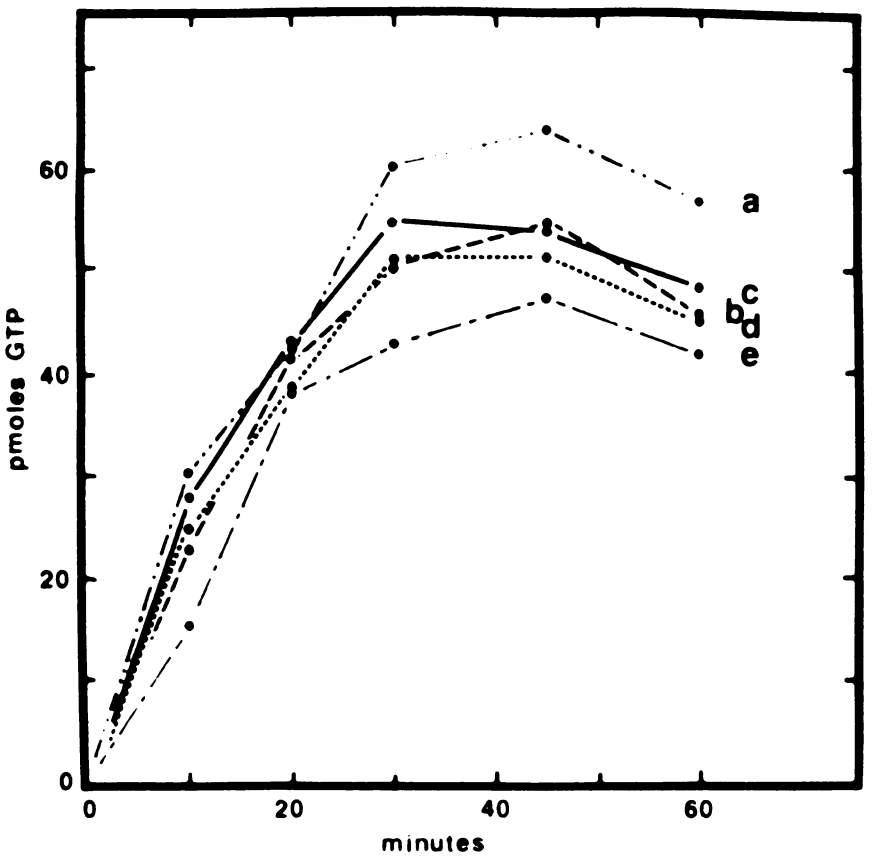


Figure 3

Figure 4. Effect of unlabeled GTP concentration. The units of the ordinate are pico moles GTP per 10^6 nuclei. Incubations at $25^{\circ}C$. Hg-UTP substituted for UTP. Final concentration of unlabeled GTP: a. 0.04 mM (— — —); b. 0.024 mM (————); c. 0.008 mM (·····); d. 0.004 mM (— — —); e. 0 mM (— ·· —).

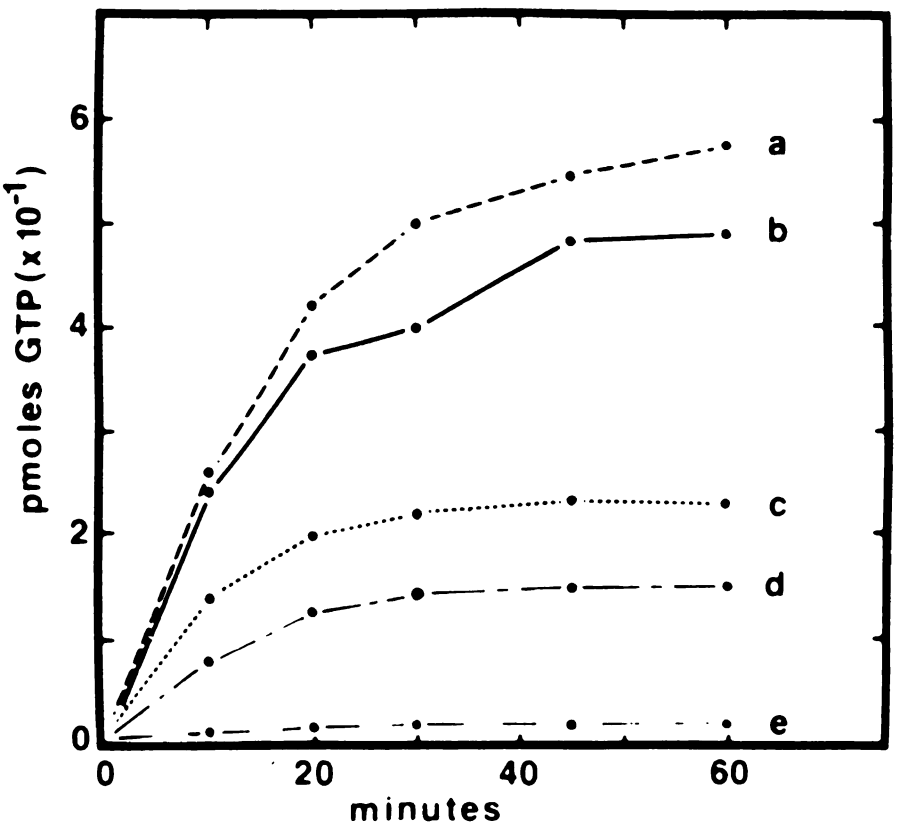


Figure 4

slightly decreased from the control levels (0.04 mM). The specific activity of the GTP was nearly doubled at this concentration: 50 to 60 CPM per picomole GTP compared to about 30 CPM per picomole or GTP at 0.04 mM. For all subsequent reactions, unlabeled GTP was added to a final concentration of 0.024 mM.

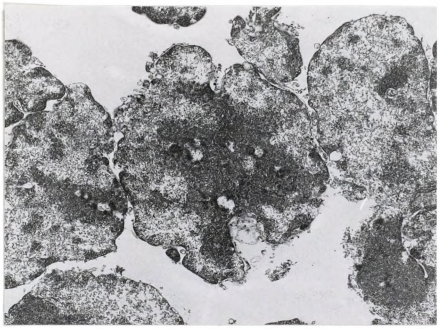
Condition of the Nuclei

Based on visual observation of the reaction tubes and the fact that the suspensions of nuclei could be pipetted, it was concluded that the nuclei did not aggregate significantly during the 30 to 60 minutes of incubation at 25°C. Observation by light microscopy before and after incubation at 25°C revealed that the nuclei were intact. The nuclei were also examined by transmission electron microscopy. These studies were performed by Mr. Stuart Pankratz of the Department of Microbiology and Public Health, Michigan State University. Figure 5 shows electron micrographs of nuclei incubated for 30 minutes at 2°C (Figure 5A) and at 25°C (Figure 5B). It can be seen that the nuclei are intact and the chromatin is not condensed. A nuclear membrane is not readily apparent. However, as the cells were lysed in the presence of a nonionic detergent (Triton X-100, 0.1%), the absence of a membrane was not unexpected (Stuart *et al.*, 1977).

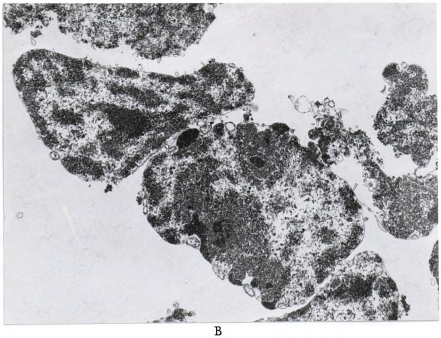
Effect of α-Amanitin

The effects of the toxin α-amanitin on eukaryotic RNA polymerases both *in vitro* and *in vivo* have been known for some time (see Jacob, 1973). Its effect on RNA polymerase activity in isolated nuclei was first reported by Stirpe and Fiume (1967). Using nuclei from mouse liver

Figure 5. Transmission electron microscopy of isolated nuclei. A. Nuclei incubated under RNA synthesis conditions for 30 minutes at 2°C. B. Nuclei incubated under RNA synthesis conditions for 30 minutes at 25°C. Magnification x 20,250.



A



B

Figure 5

cells, they demonstrated that the manganese-dependent polymerase (polymerase II) was inhibited by almost 80% by α-amanitin at levels as low as 0.02 *u*g/ml. α-amanitin has since become a standard method of differentiating between RNA polymerase I and RNA polymerase II. The minor RNA polymerase III activity is also inhibited by α-amanitin, but it is not quite as sensitive to the inhibitor as RNA polymerase II.

We have examined our synthesis system for sensitivity to α-amanitin. The results are shown in Figure 6A. Levels of synthesis are plotted as percent of control (without α-amanitin). The reactions were 0.8 ml, containing UTP, CTP, GTP, and adenosine 5'- γ -thiotriphosphate (ATP- γ-S; see below). Aliquots were taken at six different time points. The synthesis levels at 30 minutes are representative and are the only ones shown. Total RNA synthesis is inhibited to about 25% of control levels at 0.05 *u*g α-amanitin per ml. At concentrations of 1.22 *u*g/ml, synthesis is about 30%. Among a number of different experiments, the synthesis levels in the presence of α-amanitin ranged from 25 to 35% Thus, 65 to 75% of the RNA polymerase activity in our system is due to RNA polymerases II and III.

Effect of Aurintricarboxylic Acid

The dye aurintricarboxylic acid (ATA) has been shown to inhibit initiation and elongation of protein synthesis (Huang and Grollman, 1972), RNA and DNA synthesis (Tstutsui *et al.*, 1978; Sarma *et al.*, 1976) and has been reported to be a general nuclease inhibitor (Hallick *et al.*, 1977). Sarma *et al.* (1976) suggested that ATA was an inhibitor of initiation of RNA synthesis and thus could be used to determine the amount of RNA synthesis initiated in isolated nuclei. We have examined the

Figure 6. Effect of α-amanitin and aurintricarboxylic acid on RNA synthesis. Synthesis was for 30 minutes at 25°C. ATP-γ -S and GTP-γ -S were substituted for ATP and GTP, respectively. Data are plotted as fraction of control synthesis (without inhibitors). A. Effect of α-amanitin. B. Effect of aurintricarboxylic acid.

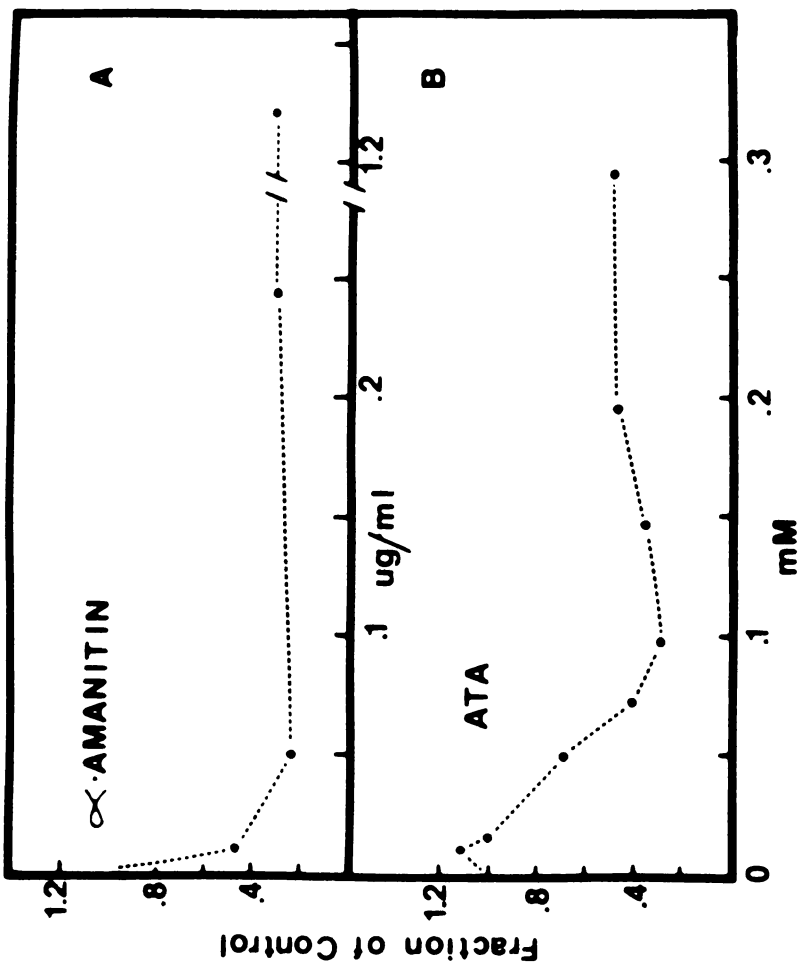


Figure 6

effect of ATA on RNA synthesis in our system. The results are shown in Figure 6B. As in Figure 6A, the data are plotted as percent of control values at 30 minutes versus inhibitor concentration. Maximum inhibition occurred at 100 uM ATA with synthesis levels about 30% of control values. Synthesis levels increased slightly at higher levels of ATA.

Effect of Ribonucleoside 5'-γ-thioptriphosphates

The use of purine nucleoside 5'-γ-thiotriphosphates as a probe for the initiation of RNA synthesis *in vitro* has been reported by Huang and co-workers (Reeve *et al.*, 1977; Smith *et al.*, 1978). ATP-γ-S has been shown to serve as a substrate for *E. coli* DNA-dependent RNA polymerase (Goody *et al.*, 1972) and some yeast tRNA synthetases (Yount, 1973). It is an inhibitor of *E. coli* alkaline phosphatase (Goody *et al.*, 1972). Using bacteriophage λ DNA, Smith *et al.* (1978) demonstrated that ATP-γ-S and GTP-γ-S were incorporated into RNA with only the 5'-terminal nucleotide retaining a thiol group. Thus, by chromatographing the newly synthesized RNA over a column of agarose coupled with an organomercury compound they were able to specifically isolate those RNA molecules which were initiated *in vitro*. They did not report, however, the effects of the γ-S-ribonucleotides on RNA synthesis in a eukaryotic system. Figure 7 shows the results of synthesis of RNA in our system in the presence of ATP-γ-S, GTP-γ-S, and a combination of the two. ATP-γ-S and GTP-γ-S were substituted for ATP and GTP, respectively, at concentrations equal to the controls (0.4 mM ATP and 0.024 mM GTP). When ATP-γ-S was substituted for ATP, RNA synthesis was stimulated by about 50% over control reactions containing ATP and GTP. GTP-γ-S, on the other hand, did not stimulate synthesis. Incorporation of ^{3}H-GTP

Figure 7. Effect of ATP-γ -S and GTP-γ -S on RNA synthesis. Nuclei were incubated at 25°C for 30 minutes with ATP-γ -S or GTP-γ -S substituted for ATP and GTP, respectively. Data are plotted as fraction of control (unmodified ribonucleotides). Striped bars are with α-amanitin (1μg/ml) and open bars are without α-amanitin.

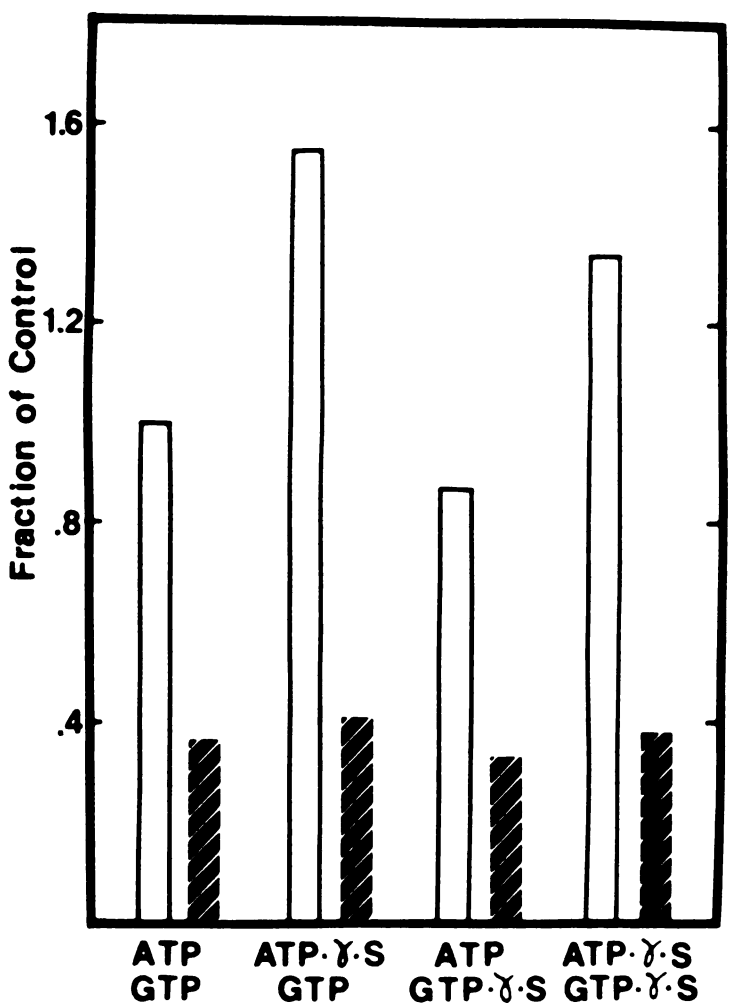

Fraction of Control
1.6
1.2
.8
.4
ATP
GTP
ATP·γ·S
GTP
ATP
GTP·γ·S
ATP·γ·S
GTP·γ·S

Figure 7

was 86% of the control levels. When both ATP-γ-S and GTP-γ-S were added to the reactions, a stimulation of synthesis was seen again. Incorporation was increased by about 35% over control levels. Interestingly, all of the reactions were inhibited to the same extent by α-amanitin (1 μg/ml). This suggests that the α-amanitin insensitive RNA polymerase (polymerase I) was not affected by the ATP-γ-S or the GTP-γ-S.

Combined Effect of ATA and α-amanitin on RNA Synthesis

The combined effects of ATA and α-amanitin on RNA synthesis were examined in control reactions (using unmodified ribonucleotides) and reactions using ATP-γ-S and GTP-γ-S. The results are shown in Figure 8. Synthesis was done in 0.2 ml volumes and aliquots were taken at 0 and 30 minutes. The net synthesis of RNA was determined as described in Materials and Methods. The data are plotted as percent of the control reaction (unmodified ribonucleotides without inhibitors). As expected, ATP-γ-S and GTP-γ-S stimulated synthesis by about 60% over the control values. α-amanitin alone inhibited both reactions to about 36 to 40% of the control values. ATA inhibited the reaction containing unmodified ribonucleotides to about 43% of the control values. The γ-S reaction was inhibited to about 60% of the control reaction. The combination of ATA and α-amanitin inhibited synthesis in both reactions to a greater extent than either inhibitor alone (23% in the reaction with unmodified ribonucleotides and 33% in the reaction with γ-S ribonucleotides). Thus, the inhibitory properties of these two compounds appear to be additive.

Figure 8. Effect of α-amanitin and aurintricarboxylic acid in combination on RNA synthesis. Nuclei were incubated at 25°C for 30 minutes. Aurintricarboxylic was added at 0.1 mM; α-amanitin was added at 1 μg/ml. Open bars represent reactions using unmodified ribonucleotides; striped bars represent reactions using ATP-γ -S and GTP-γ -S. Data are plotted as fraction of control (unmodified ribonucleotides without inhibitors).

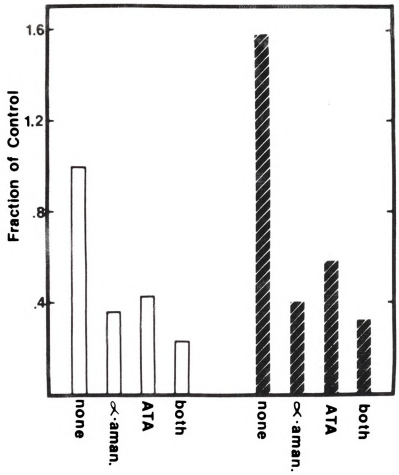

Fraction of Control
1.6
1.2
.8
.4
none
α-aman.
ATA
both
none
α-aman.
ATA
both

Figure 8

Sucrose Gradient Profiles of Synthesized RNA

The size of the synthesized RNA was examined by sedimentation through sucrose gradients. Nuclei were incubated in 0.8 ml reactions containing either unmodified ribonucleotides or γ-S ribonucleotides and either α-amanitin (1 ug/ml), ATA (100 uM), or no inhibitors. The RNA was extracted as described in Materials and Methods and separated from free nucleotides by gel chromatography on Sephadex G-25. Gradient fractions of 0.2 ml were precipitated with 10% trichloroacetic acid and collected on Whatmann GF/C filters. ^{14}C-ribosomal RNA was included on each gradient as a size marker (arrows indicate positions of 4S, 18S, and 28S RNAs). The results are shown in Figures 9 to 12.

The profiles of the control RNA (unmodified ribonucleotides) with and without ATA are shown in Figure 9. In the absence of ATA, the RNA shows a broad peak across the 18 to 28S region of the gradient with some material larger than 28S. In the presence of ATA, the profile of the RNA is shifted toward the top of the gradient. The peak of material is smaller than 18S with less material of larger size.

The effect of ATA on RNA synthesized with ATP-γ-S and GTP-γ-S (γ-S RNA) is shown in Figure 10. Without ATA, the profile is similar to the control in Figure 9. Most of the material is between 18 and 28S in size with some larger RNA. In the presence of ATA, smaller material is observed, although the shift is not as dramatic as in Figure 9 (see also Table I).

The effect of α-amanitin on the size of the RNA is illustrated in Figures 11 and 12. RNA synthesized with unmodified ribonucleotides is shown in Figure 11. The control profile is similar to the control shown in Figure 9. In the presence of α-amanitin, the shape of the

Figure 9. Sucrose gradient profiles of synthesized RNA: effect of aurintricarboxylic acid. Reactions were incubated at 25°C for 30 minutes with unmodified ribonucleotides. RNA was extracted and centrifuged on gradients as described in Materials and Methods. The direction of sedimentation is from left to right. Arrows indicate the position of 4S, 18S and 28S RNA. No inhibitors (·····); 0.1 mM ATA (——).

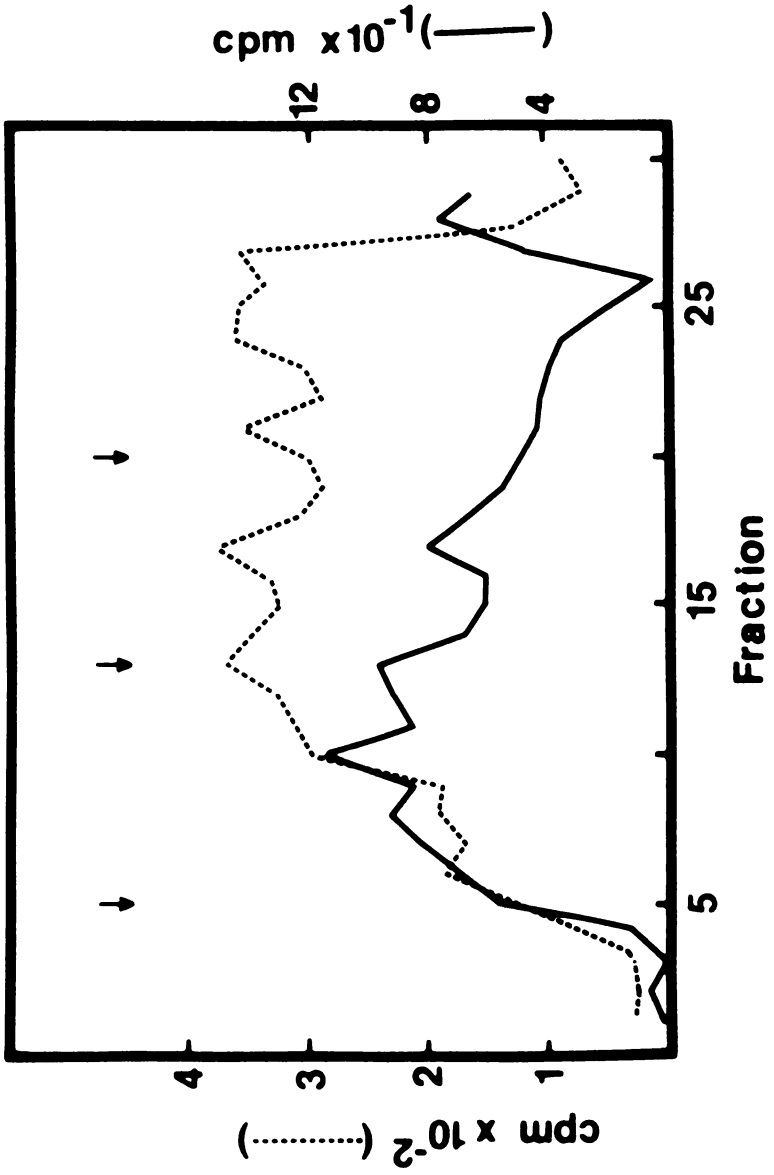


Figure 9

Figure 10. Sucrose gradient profiles of synthesized RNA: effect of γ -S ribonucleotides and aurintricarboxylic acid. Reactions were incubated at 25°C for 30 minutes with ATP-γ -S and GTP-γ -S. RNA was extracted and centrifuged on gradients as described in Materials and Methods. The direction of sedimentation is from left to right. Arrows indicate the position of 4S, 18S and 28S RNA. No inhibitors (••••••••••); 0.1 mM ATA (——————).

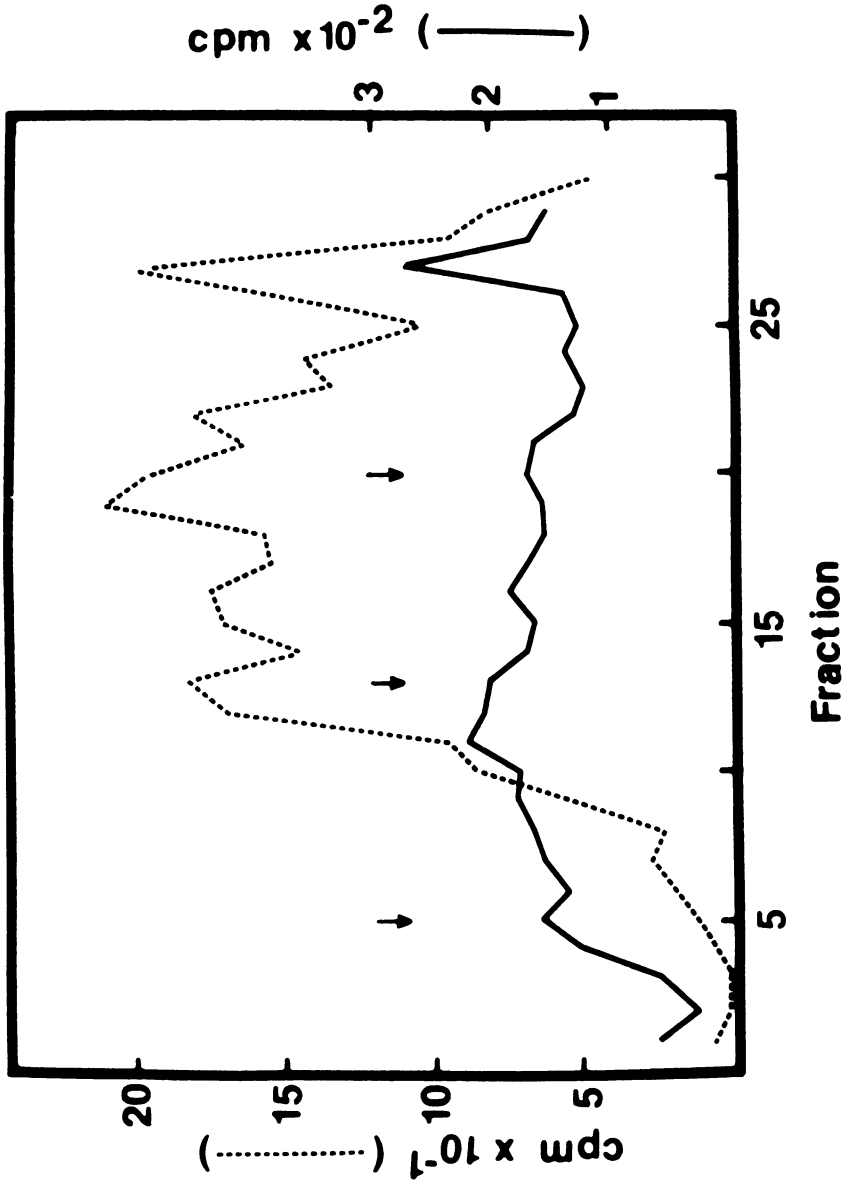


Figure 10

Figure 11. Sucrose gradient profiles of synthesized RNA: effect of α-amanitin. Reactions were incubated at 25°C for 30 minutes with unmodified ribonucleotides. RNA was extracted and centrifuged on gradients as described in Materials and Methods. The direction of sedimentation is from left to right. Arrows indicate the position of 4S, 18S and 28S RNA. No inhibitors (•••••••••); 1 µg/ml α-amanitin (——————).

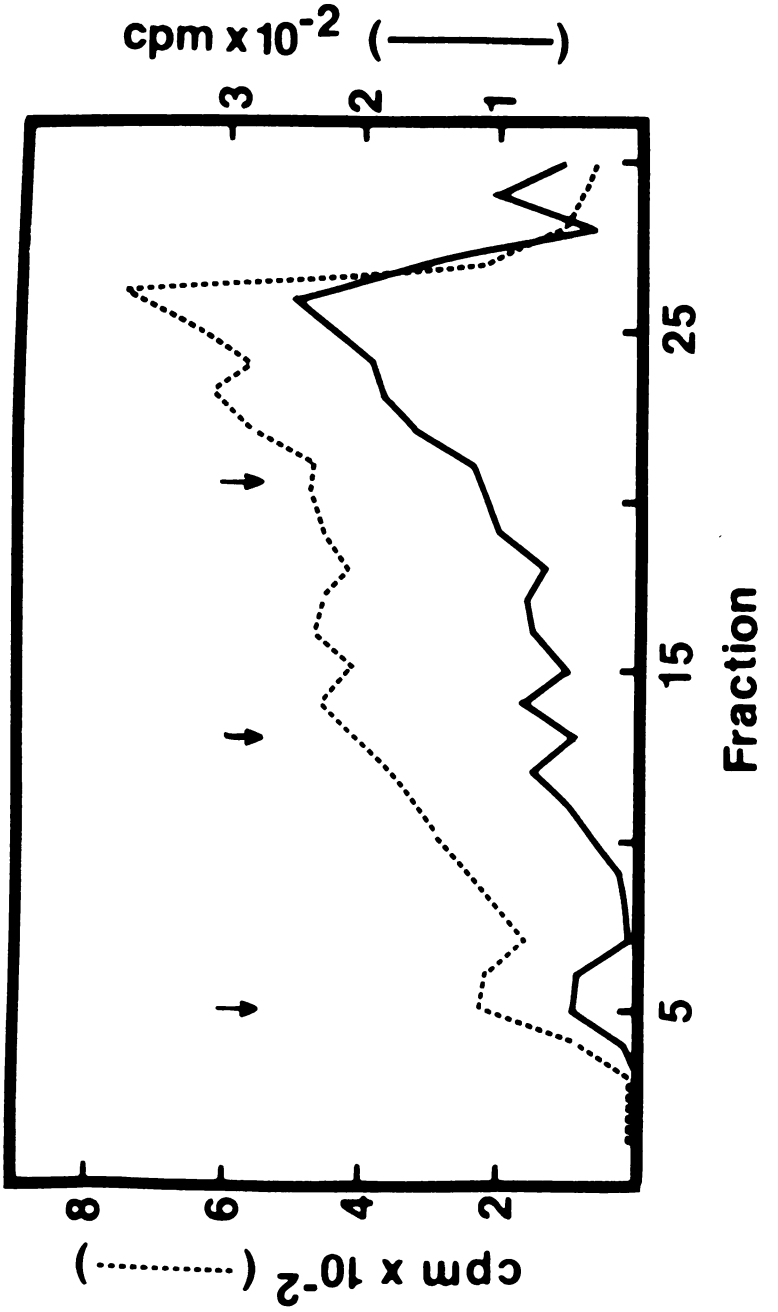


Figure 11

profile is similar but there is a decrease in the amount of RNA smaller than 28S. Figure 12 shows the profiles of γ-S RNA with and without α-amanitin. Again, a shift to larger size RNA is evident in the presence of α-amanitin.

Figure 13 shows profiles of nuclear RNA isolated from cells labeled in culture for 30 minutes with ^{3}H-uridine. In one experiment, the nuclei were isolated and the RNA immediately extracted as described in Materials and Methods. In another experiment, the isolated nuclei were incubated under *in vitro* RNA synthesis conditions at 25^{o}C for 30 minutes (in the absence of CTP and ^{3}H-GTP). The RNA was then extracted as described. Both profiles show a heterogeneous population of RNA similar to the profiles of the RNA synthesized *in vitro* (in the absence of inhibitors). The 30 minute incubation *in vitro* has caused an increase in smaller RNA with a corresponding decrease in the amount of large RNA.

The data from these figures are summarized in Table I. The size of the RNA is expressed as percent of material smaller than 18S, from 18 to 28S, and greater than 28S in size. It can be seen that with RNA synthesized with unmodified ribonucleotides, ATA caused an increase of smaller material (16% more RNA smaller than 18S) and α-amanitin caused an increase in larger RNA (approximately 20% more RNA larger than 28S). The effects were similar with γ-S RNA although the changes were not as large. ATA increased small material (less than 18S) by about 12% and α-amanitin increased larger material (greater than 28S) by about 9%. The *in vitro* incubation of *in vivo*-labeled nuclei caused an increase of smaller RNA (smaller than 18S) of about 16% with a corresponding decrease in RNA larger than 28S.

Figure 12. Sucrose gradient profiles of synthesized RNA: effect of γ -S ribonucleotides and α-amanitin. Reactions were incubated at 25°C for 30 minutes with ATP-γ -S and GTP-γ -S. RNA was extracted and centrifuged on gradients as described in Materials and Methods. The direction of sedimentation is from left to right. Arrows indicate the position of 4S, 18S and 28S RNA. No inhibitors (---------); 1 µg/ml α-amanitin (——————).

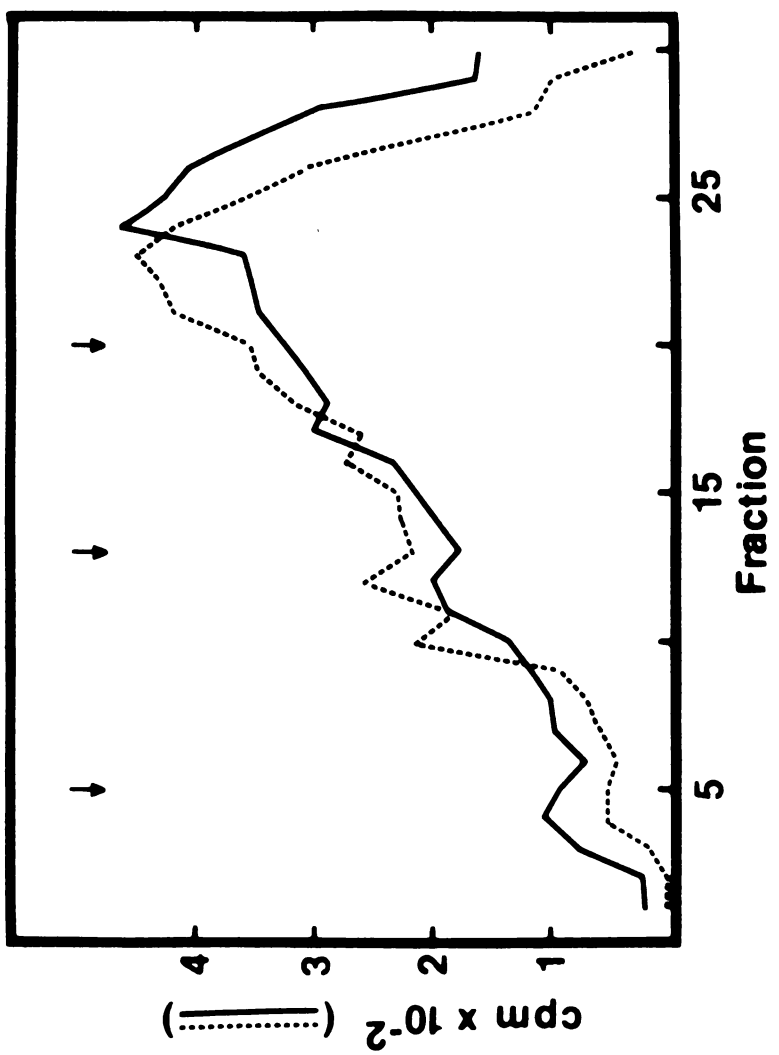


Figure 12

Figure 13. Sucrose gradient profiles of RNA labeled *in vivo*. RNA was labeled for 30 minutes in whole cells with ^{3}H-uridine. Nuclei were isolated and the RNA was either extracted immediately (••••••••••) or the nuclei were incubated under *in vitro* synthesis conditions (without CTP or ^{3}H-GTP) at 25°C for 30 minutes (——————). RNA was extracted and centrifuged on gradients as described in Materials and Methods. The direction of sedimentation is from left to right. Arrows indicate the position of 4S, 18S and 28S RNA.

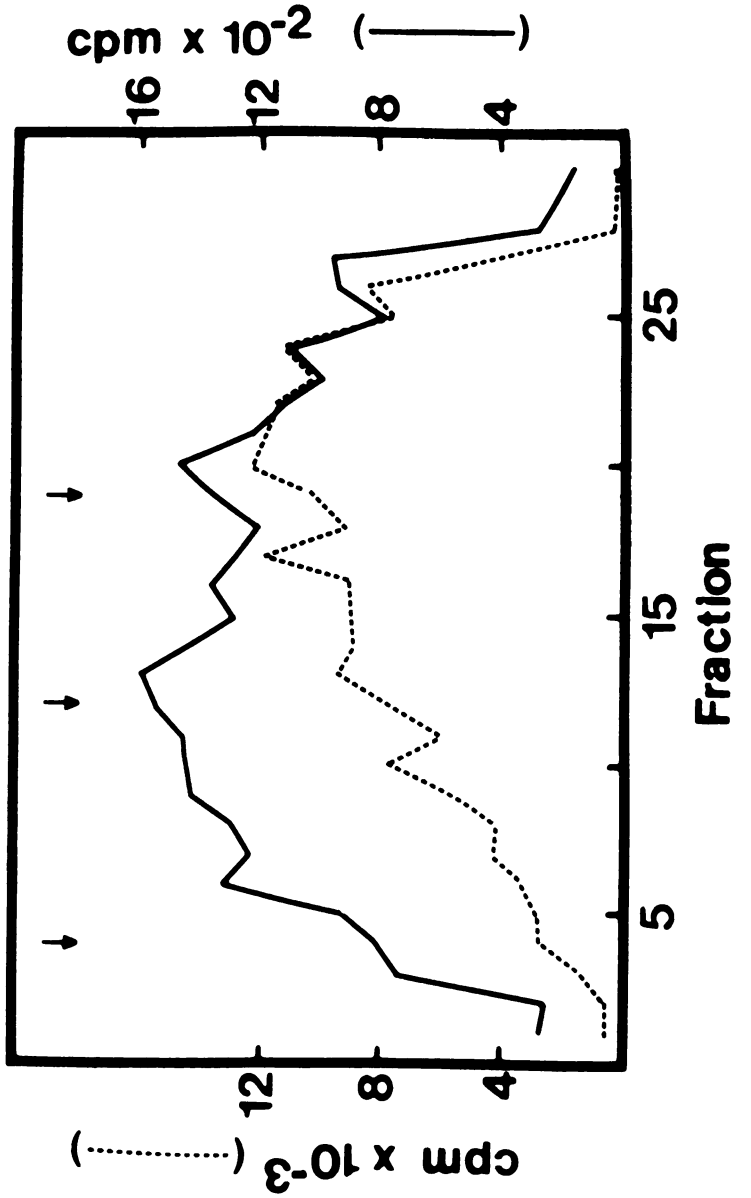


Figure 13

Table I. Size of synthesized RNA

	RNA	Inhibitor	<18S[a]	18-28S[a]	>28S[a]
1.	^{3}H-UDR[b]	none	19.6	39.4	41.0
2.	^{3}H-UDR[c]	none	35.7	35.4	28.9
3.	NTPs[d]	none	27.0	36.6	36.4
4.	NTPs[d]	none	21.5	41.6	36.9
5.	NTPs[d]	ATA	43.3	33.2	23.5
6.	NTPs[d]	α-aman.	11.4	31.2	57.6
7.	γ-S[e]	none	19.7	41.6	36.9
8.	γ-S[e]	none	12.8	40.6	46.6
9.	γ-S[e]	ATA	31.9	30.7	37.4
10.	γ-S[e]	α-aman.	15.7	29.0	55.4

[a]Percent of total RNA on gradient.

[b]Labeled for 30 minutes in whole cells with ^{3}H-uridine. The nuclei were isolated and RNA extracted immediately as described in Materials and Methods.

[c]Labeled for 30 minutes in whole cells with ^{3}H-uridine. The nuclei were isolated and incubated under *in vitro* RNA synthesis conditions (without CTP or ^{3}H-GTP). After 30 minutes the RNA was extracted as described in Materials and Methods.

[d]RNA synthesized with unmodified ribonucleotides.

[e]RNA synthesized with ATP-γ-S and GTP-γ-S.

Poly(A) Content of Synthesized RNA

The poly(A) content of *in vitro*-synthesized RNA was determined by affinity chromatography using oligo(dT) cellulose. Columns of approximately 1 ml packed volume were poured in pasteur pipettes and equilibrated with application buffer (see Materials and Methods). The samples were applied in 0.4 ml of application buffer and the columns were then washed with 4.6 ml of application buffer. The entire effluent was collected in a single fraction. The columns were then washed with 5.0 ml of distilled water to remove the bound material. This was also collected as a single fraction. The fractions were then precipitated with 10% trichloroacetic acid and collected on Whatmann GF/c filters. The results are shown in Table II. ^{3}H-poly(A) was used as a positive control for the oligo(dT) columns. Under the conditions used, greater than 99% of the ^{3}H-poly(A) was retained by the column. RNA synthesized with γ-S ribonucleotides had a slightly higher poly(A) content than did the RNA synthesized with the unmodified ribonucleotides (an average of 10.6% as compared to an average of 8.5%). ATA and α-amanitin each caused a decrease in the proportion of poly(A)-containing RNA in both reactions. In the presence of ATA, 6.3% of the synthesized RNA contained poly(A) sequences in both RNA samples. In the presence of α-amanitin, 6.4% of the RNA synthesized with unmodified ribonucleotides contained poly(A) while 8.2% of the RNA synthesized with γ-S ribonucleotides contained poly(A).

Mercury-Sepharose Chromatography of Synthesized RNA

We attempted initially to link parachloromercuribenzoate to Sepharose 2B with an ethylenediamine spacer as described by Reeve *et al.*

Table II. Poly(A) content of synthesized RNA

	RNA	Inhibitor	CPM Unbound	CPM Bound	% A^+
1.	^{3}H-poly(A)	none	116	32,476	99.6
2.	NTPs[a]	none	12,143	1,043	7.9
3.	NTPs[a]	none	14,623	1,448	9.0
4.	NTPs[a]	ATA	6,301	421	6.3
5.	NTPs[a]	α-aman.	9,311	637	6.4
6.	γ-S[b]	none	14,868	1,784	10.7
7.	γ-S[b]	none	16,176	1,908	10.6
8.	γ-S[b]	ATA	8,035	536	6.3
9.	γ-S[b]	α-aman.	14,806	1,323	8.2

[a]RNA synthesized with unmodified ribonucleotides.

[b]RNA synthesized with ATP-γ -S and GTP-γ -S.

(1977). The final product was washed as described (Reeve *et al.*, 1977) and the mercury content determined as described by Sluyterman and Wijdenes (1970) and Ellman (1959). This procedure consistently yielded no detectable mercury coupled to the Sepharose. Therefore, we tried a slightly modified procedure in which AH-Sepharose was used. AH-Sepharose is Sepharose 4B to which a six-carbon spacer (diamino hexane) has been coupled by cyanogen bromide activation. One end of the spacer remains available for reaction with the ligand (in this case, the organomercury compound). The AH-Sepharose should be similar to the ethylenediamine-coupled agarose which is the result of the first step of the procedure reported by Reeve *et al.* (1977). The organomercury compound was reacted with the AH-Sepharose using the procedure described by Reeve *et al.* (1977). The final material was washed under vacuum filtration and the organomercury content determined. This procedure yielded Sepharose which contained 0.36 *u* moles organomercury per ml packed Sepharose. A 2 ml column of the Hg-Sepharose was poured in a 5 ml plastic syringe and washed with a buffer containing 10 mM Tris (pH = 7.9), 1 mM EDTA, 100 mM NaCl, and 0.1% SDS (TNES; Smith *et al.*, 1978). The effluent of the column was found to contain a large amount of material which absorbed at 260 nm (greater than 1 A_{260}unit per ml). It was necessary to wash the 2 ml column with 40 to 50 ml of TNES to remove this material. Since the organomercury compound was precipitated during the coupling procedure, it was suspected that there was mercury trapped in the column material. A 1 mM solution of $HgCl_2$ was found to have an absorbance of approximately 1.0 at 260 nm. After the column had been washed free of the absorbing material, the organomercurial content was again determined, yielding a figure of 0.074 *u* moles mercury per ml

packed Sepharose.

The column was then tested for binding of RNA which had been synthesized with unmodified ribonucleotides. ^{3}H-poly(A) and ^{3}H-RNA (sample 4, Table II) were used. The samples were applied in 0.4 ml of TNES. A 0.1 ml aliquot was taken to determine the total amount of material applied to the column. The column was washed with five 2 ml aliquots of TNES followed by five 2 ml aliquots of TNES plus 50 mM 2-mercaptoethanol which should elute any material bound through thiol groups (Smith *et al.*, 1978). The results are shown in Table III. With both RNA samples about 99% of the RNA eluted in the "unbound" fractions. The recovery of approximately 100% indicates that there was no material left on the column. Thus, the column did not bind RNA which contained no thiol groups.

The free nucleotide ATP-γ-S was used as a positive control for the column. ATP-γ-S was dissolved in TNES. 3.8 A_{260} units of this material (2.54 u moles) was applied to the 2 ml column. The column was washed with five 2 ml aliquots of TNES and the absorbance at 260 nm was determined for each fraction. (As 2-mercaptoethanol absorbs strongly at 260 nm, it was not possible to elute with TNES plus 2-mercaptoethanol and determine absorbance.) The results of two such experiments are shown in Table IV. In one experiment 92% of the material was recovered in the unbound fractions while in the other experiment 98% of the material was recovered in the unbound fractions. The material unaccounted for--possibly "bound"--amounted to 0.32 A_{260} in the first experiment and 0.04 A_{260} in the second experiment. This corresponds to 0.02 and 0.006 u moles of ATP-γ-S, respectively. The expected capacity of a 2 ml column would be 0.148 u moles, based on the calculated mercury content

Table III. Binding of control RNA to Hg-Sepharose

RNA	Total Applied[a]	Unbound	Bound	% Bound	% Recovered
^{3}H-poly(A)	30,708	31,609	338	1.06	104.0
^{3}H-RNA[b]	20,648	20,776	248	1.18	101.8

[a]Sample applied to column in a volume of 0.4 ml. Total radioactivity applied determined from 0.1 ml aliquot of sample.

[b]RNA synthesized *in vitro* with unmodified ribonucleotides.

Table IV. Binding of ATP-γ-S to Hg-Sepharose

Sample	Total A_{260} Applied[a]	A_{260} Unbound	% Unbound
1	3.80	3.48	91.6
2	3.80	3.71	97.6

[a]Applied 20 μl of a stock solution of ATP-γ-S (190 A_{260}/ml).

of the washed column. Thus, it appears that little or no ATP-γ-S was bound by the column. Identical experiments using GTP-γ-S gave similar results (data not shown).

DISCUSSION

In recent years the use of isolated nuclear systems for the study of eukaryotic transcription has been reported widely. It is felt that this type of system not only resembles more closely transcription *in vivo* but also allows the study of post-transcriptional processing events which take place in the nucleus (Bellard *et al.*, 1977). Nuclei from a wide variety of sources have been used, including systems derived from fungi and higher plants, amphibians, and mammals. As a part of our effort to study the controls of immunoglobulin synthesis, we have examined RNA synthesis in nuclei isolated from the mouse myeloma tissue culture line P3 (MOPC-21).

Using as a basis the nuclear transcription system reported by Marluff *et al.* (1973) for another mouse myeloma line (MPC-11), it was demonstrated that ^{3}H-GTP was incorporated into acid insoluble material at a relatively linear rate for at least 30 minutes (Figure 1). (All of the synthesis reactions reported were incubated at 25^{o}C unless otherwise noted. As the reaction mixtures were prepared they were kept on ice. To determine what levels of synthesis were occurring on ice, a control reaction was incubated at 2^{o}C. The results indicated that synthesis at 2^{o}C was negligible.)

Incorporation of ^{3}H-GTP was dependent upon the addition of the four ribonucleoside triphosphates (Figures 2 and 4). If either CTP, UTP, or GTP were omitted from the reaction virtually no incorporation was detectable. If ATP was omitted from the reaction, synthesis was

reduced but remained at about 17% of control values after 30 minutes of incubation (Figure 2). This is not surprising since it might be expected that endogenous levels of ATP would be higher than the levels of the other ribonucleoside triphosphates. It has been reported that ATP is synthesized in isolated calf thymus nuclei (McEwen *et al.*, 1963).

An examination of the effect of the 2-mercaptoethanol (2-ME) concentration on the synthesis of RNA in our system revealed little differences over a concentration range of 4 to 20 mM (Figure 3) even in the presence of mercurated nucleotides which are known to be sensitive to mercaptan concentration (Dale *et al.*, 1973). The reported value of 12 mM 2-ME (Smith and Huang, 1976) was found to be suitable in our system for reactions which contained mercurated UTP as well as for reactions which contained unmodified ribonucleotides.

The label used for all of the synthesis reactions reported here was ^{3}H-GTP. As was stated above, in the absence of any unlabeled GTP, synthesis did not occur in the system. As it was not feasible to maintain the minimum concentration of GTP required for synthesis with only ^{3}H-GTP, it was necessary to add unlabeled GTP. An unlabeled GTP concentration of 0.024 mM was found to be the minimum which could support normal levels of synthesis (Figure 4).

Examination of the nuclei by both light microscopy and transmission electron microscopy (Figure 5) indicated that the nuclei were intact after 30 minutes of incubation at 25^{o}C or 2^{o}C. The absence of an intact nuclear membrane was expected since the cells were lysed in the presence of 0.1% Triton X-100. The component of the reaction which stabilized the nuclei apparently was glycerol, as even a small reduction in its concentration (from 12.5% to 10%) caused the nuclei to form

large aggregates within one minute of incubation at 25°C (data not shown).

The RNA synthesized under optimal conditions was shown to be heterogeneous in size when sedimented on sucrose gradients. It ranged in size from about 4S to larger than 28S with a broad peak in the 18 to 28S region (Figure 9). This profile was similar to that seen for nuclear RNA labeled for 30 minutes with ^{3}H-uridine in whole cells (Figure 13). It was noted that *in vitro* incubation of nuclei labeled *in vivo* caused a downward shift in the average size of the RNA. It is likely that some degredation and/or processing occurred during the 30 minute incubation *in vitro*.

The fungal toxin α-amanitin has been shown to be a potent inhibitor of eukaryotic RNA polymerases II and III (Jacob, 1973). In our system, RNA synthesis was inhibited by about 65 to 75% at α-amanitin levels as low as 0.05 *u*g/ml (Figures 6A and 8). Thus, about 65 to 75% of the RNA synthesis in our system must be due to RNA polymerase II and III. This is in agreement with results reported by other workers (Marzluff *et al.*, 1973; Cooper and Marzluff, 1978). The sucrose gradient profile of the RNA synthesized in the presence of 1 *u*g/ml of α-amanitin indicated that it was heterogeneous in size (Figure 11) with a higher proportion of larger material (greater than 28S) when compared to RNA synthesized in the absence of α-amanitin. The RNA synthesizing activity which remains in the presence of α-amanitin should be due to RNA polymerase I. Since polymerase I synthesizes primarily ribosomal RNA, the shift toward larger RNA in the presence of α-amanitin would seem reasonable.

The dye aurintricarboxylic acid (ATA) has been shown to be an inhibitor of protein synthesis initiation and elongation (Huang and

Grollman, 1972), nucleic acid synthesis (Sarma *et al.*, 1976; Tsutsui *et al.*, 1978), and ribonucleases and deoxyribonucleases (Hallick *et al.*, 1977). It has been suggested that ATA will inhibit most proteins which bind nucleic acids (Blumenthal and Landers, 1973). Sarma *et al.* (1976) reported the effect of ATA on RNA synthesis in isolated HeLa cell nuclei. They pre-treated their cells with low levels of actinomycin D to reduce the levels of RNA polymerase I activity. They found that α-amanitin inhibited 80% of the RNA synthesis in isolated nuclei. ATA at 0.1 mM or higher also inhibited synthesis by 80%. When α-amanitin and ATA were combined, they found no additive effect. RNA synthesis was still reduced by 80%. They suggested that ATA was inhibiting initiation of RNA synthesis and concluded that 80% of the synthesis in their system was initiated *in vitro*. In our system, we found that ATA at 100 *u*M inhibited to a lesser extent than did α-amanitin and, contrary to the results of Sarma *et al.* (1976), the effects of α-amanitin and ATA were additive (Figure 8). The combination of ATA (100 *u*M) and α-amanitin (1 *u*g/ml) consistently inhibited to a greater extent than either inhibitor alone. The combination, however, did not inhibit synthesis 100%. Our results suggest that a large portion of the RNA synthesis activity is sensitive to both α-amanitin and ATA and that a portion of the α-amanitin insensitive activity (polymerase I) is sensitive to ATA. It also appears that some of the polymerase II and/or III activity is insensitive to ATA. These results are consistent with thesis that ATA is inhibiting only initiation but they certainly do not prove it. Of the reports of *in vitro* initiation of RNA synthesis in the literature, none have suggested initiated RNA levels higher than about 15 to 20% (Busiello and Di Girolamo, 1975; Tamm, 1977; Huang *et al.*, 1977). Sucrose gradient

profiles of RNA synthesized in the presence of ATA showed a shift toward smaller material (Figures 9 and 10; Table I). This result would not seem to be consistent with continued elongation after the inhibition of initiation.

The poly(A) content of the synthesized RNA was examined by chromatography on oligo(dT) cellulose. About 8 to 9% of the RNA synthesized in the presence of unmodified ribonucleotides contained poly(A) sequences (Table II). Synthesis in the presence of ATA decreased the percentage of poly(A)-containing RNA by about 2%. This is consistent with the data indicating that ATA partially inhibits α-amanitin-sensitive polymerases. Synthesis in the presence of α-amanitin decreased the proportion of poly(A)-containing RNA to 6.4%. It would seem that if α-amanitin inhibits RNA polymerase II, no new (i.e. labeled) poly(A)-containing RNA should be synthesized in its presence. Thus the proportion of poly(A)-containing RNA should be at or near 0. It is not entirely clear why this is not the case. The presence of poly(A) sequences on the products of α-amanitin resistant polymerases has been reported in rat liver nuclei (De Pomeral and Butterworth, 1975) and in nuclei from Ehrlich ascites tumor cells (Nakanishi *et al.*, 1978). De Pomeral and Butterworth (1975) suggested that the polyadenylation of α-amanitin-resistant polymerase products might be an artifact. They stated that processing or random degradation of RNA in the nucleus might result in a loss of specificity of polyadenylation. Nakanishi *et al.* (1978) reported that the amount of poly(A)-containing RNA synthesized by α-amanitin -resistant polymerase ranged from 1 to 4% of the total RNA. Since RNA polymerase III is a minor component, they suggested that most of this RNA was probably a product of RNA polymerase I. They suggested

that perhaps polymerase I is transcribing some nucleolar genes other than ribosomal genes and that the products of these other genes are being polyadenylated. Since α-amanitin inhibits RNA synthesis in our system by 65 to 75%, the 6.4% poly(A)-containing RNA synthesized in the presence of α-amanitin represents about 1.5 to 2.5% of the total RNA synthesized in the absence of any inhibitors. This is similar to the values reported by Nakanishi *et al.* (1978). The possibility always remains that a small amount of non-polyadenylated RNA is being retained by the oligo(dT) column.

The use of ribonucleoside 5'-γ-S triphosphates as substrates for RNA synthesis has been reported (Reeve *et al.*, 1977; Huang *et al.*, 1977; Smith *et al.*, 1978). Both synthetic DNA templates and bacteriophage λ DNA were transcribed with *E. coli* RNA polymerase. The γ-S ribonucleotides were reported to serve as substrates and, when incorporated as the initial nucleotide, the γ-S group was retained, which allowed the specific isolation of the *in vitro*-initiated RNA molecules by affinity chromatography on mercury-agarose. Some discussion of the use of γ-S ribonucleotides for the study of eukaryotic transcription was included (Huang *et al.*, 1977; Smith *et al.*, 1978) but that work as yet is unpublished.

We examined the effect of ATP-γ-S and GTP-γ-S on transcription in MOPC-21 nuclei. To our surprise the substitution of ATP-γ-S for ATP stimulated the synthesis of RNA by 50 to 60% (Figure 6). The substitution of GTP-γ-S for GTP gave no stimulation of RNA synthesis. When both γ-S ribonucleotides were used, synthesis was again stimulated by about 50%. The reason for the stimulation by ATP-γ-S is not clear. GTP-γ-S may show no stimulation because of its lower concentration--

0.024 mM compared to 0.4 mM ATP-γ -S. However, it is possible that some stimulation is occurring with GTP-γ -S but it is not observable due to the fact that the RNA is labeled with ^{3}H-GTP. If GTP-γ -S stimulates RNA synthesis it might be expected that it would be incorporated preferentially over GTP, thus lowering the effective specific activity of the ^{3}H-GTP, obscuring any stimulation of incorporation. This could be tested simply by labeling with another ribonucleotide. During the course of these studies another labeled ribonucleotide was not readily available.

The levels of γ -S RNA synthesized in the presence of α-amanitin indicated that RNA polymerase I activity was not stimulated by ATP-γ -S (Figure 6). Thus it would appear that ATP-γ -S is stimulating primarily RNA polymerase II and possibly RNA polymerase III. (Opinions in the literature vary as to whether or not 1 μg/ml α-amanitin will inhibit polymerase III. In any event, since polymerase III is a minor component, it probably does not account for the major portion of the stimulation seen with ATP-γ -S.) The poly(A) content of the γ-S RNA was slightly higher (10.6% compared to 8.5%) than that of the RNA synthesized with unmodified ribonucleotides. The inhibitor ATA decreased the proportion of poly(A)-containing RNA to about 6.4%, a level similar to that seen with the RNA synthesized with unmodified nucleotides. α-amanitin decreased the proportion of poly(A)-containing RNA but did not eliminate it. About 8.2% of the RNA synthesized with γ -S ribonucleotides in the presence of α-amanitin was retained on oligo(dT) cellulose. This corresponds to about 2% of the total RNA synthesized in the absence of inhibitors.

Sucrose gradient profiles of the γ -S RNA revealed a size distribution similar to that of the RNA synthesized with unmodified ribonucleotides (Figures 10 and 12). The effects of ATA and α-amanitin on the size of the RNA was also similar to the RNA synthesized with unmodified ribonucleotides. ATA caused a decrease in large RNA while α-amanitin caused a decrease in smaller RNA.

Our attempts to prepare and use mercury-Sepharose columns were completely unsuccessful. Preparation of Hg-Sepharose as described by Reeve *et al.* (1977) yielded a product which contained no detectable organomercury groups when tested as described (Reeve *et al.*, 1977; Sluyterman and Wijdenes, 1970; Ellman, 1959). When Sepharose with a six-carbon spacer was used (AH Sepharose), the final product had an apparent organomercury content of 0.36 *u* moles per ml Sepharose, a number consistent with the report of Reeve *et al.* (1977). However, this product was found to contain a large amount of material which absorbed strongly at 260 nm and eluted with buffer. Extensive washing was necessary to remove this material. A subsequent determination revealed that the apparent organomercury content of the Sepharose had been reduced to 0.074 *u* moles per ml Sepharose.

^{3}H-poly(A) and ^{3}H-RNA (synthesized without γ -S ribonucleotides) were used to test the columns for non-specific binding. As expected, virtually none of this material was retained (Table III). The free nucleotide ATP-γ -S was used as a positive control to test the binding capacity of the columns. Very little of this material was retained (Table IV). In one of the experiments, as much as 8 to 9% of the ATP-γ -S may have been retained. This corresponds to about 0.32 A_{260} or 0.021 *u* moles ATP-γ -S. Based on the calculated mercury content,

the capacity of a 2 ml column should be about 0.15 u moles. Thus it appears that very little, if any, of the ATP-γ-S was specifically retained by the column.

Our results indicate that nuclei from MOPC-21 mouse myeloma cells remain intact and are capable of synthesizing RNA under *in vitro* conditions. RNA synthesis is dependent upon the addition of the four ribonucleoside triphosphates. The RNA synthesized exhibited a size distribution and poly(A) content similar to that of nuclear RNA isolated from cells labeled *in vivo*. RNA synthesis *in vitro* is sensitive to both α-amanitin and aurintricarboxylic acid. In addition, the ribonucleoside triphosphate analogs ATP-γ-S and GTP-γ-S can serve as substrates for RNA synthesis in this system. ATP-γ-S was shown to stimulate RNA synthesis by 50 to 60%. RNA polymerase II (and possibly RNA polymerase III) was stimulated preferentially. This raises some questions about the suitability of ATP-γ-S (and possibly GTP-γ-S) for the study of initiation of RNA synthesis in isolated nuclei. The preferential stimulation of RNA synthesis by ATP-γ-S would make RNA chain initiation studies using this analog difficult to interpret, at the very least. In addition, although it has been reported that transfer of the γ-thiophosphate to other nucleotides or nucleotide precursors does not occur in mouse myeloma cells (Smith *et al.*, 1978), it is not clear if transfer of the γ-thiophosphate to existing RNA molecules can occur. Winicov (1977) has reported a polynucleotide kinase activity in nuclei from mouse L cells which transfers the γ-phosphate of ATP or GTP to the 5' end of existing RNA molecules. She reported that any of the four ribonucleoside triphosphates could serve as an acceptor. γ-S ribonucleotides have been

reported to act as substrates for nucleoside diphosphate kinase (Goody *et al.*, 1972), but their reaction with polynucleotide kinase has not been reported to date. To maintain the validity of RNA initiation studies with γ-S ribonucleotides it is obviously important to establish that transfer of the γ-thiophosphate to existing RNA molecules does not occur.

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