Extended RNA Synthesis in Isolated Nuclei from Rat Pituitary Tumor Cells†

Debajit K. Biswas, Thomas F. J. Martin, and Armen H. Tashjian, Jr.*

ABSTRACT: Nuclei of GH_3 cells, isolated by detergent lysis, synthesized RNA for an extended period at 29 °C in the presence of rat liver ribonuclease inhibitor (RI). Extended RNA synthesis was dependent upon the presence of RI. Sucrose gradient sedimentation analysis of the cell-free reaction products showed that RNAs ranging from 4 S to greater than 28 S were synthesized. Further characterization of the RNA products was made by examining the sensitivity of synthesis to α-amanitin and actinomycin D as well as by oligo(dT)-cellulose binding properties. Evidence was obtained that RNA polymerases I, II, and III were functioning in isolated GH_3 nuclei. Newly synthesized RNAs were found in both the nuclear pellet and postnuclear supernatant fractions. RNA polymerase I products remained associated with the nuclear pellet throughout a 60-min incubation period whereas RNAs synthesized by RNA polymerase III emerged rapidly into the supernatant fraction. RNA polymerase II products were distributed in both fractions and were found to contain poly(A). De novo poly(A) synthesis was demonstrated and found to be inhibited by cordycepin triphosphate (3′-dATP). Supernatant RNAs synthesized by polymerase II contained a poly(A) segment of about 150 adenine residues; these transcripts sedimented heterogeneously with an apparent size distribution (under denaturing conditions) which was smaller than that of nuclear RNA polymerase II products and which resembled that of cellular mRNA. Qualitative differences in the nuclear and supernatant RNAs, the kinetics of appearance of the latter, and the differential effect of 3′-dATP on the extranuclear appearance of supernatant RNAs suggest that a process resembling nuclear–cytoplasmic RNA transport occurred in this cell-free nuclear system.

Understanding nuclear events involved in regulation of the synthesis of specific RNAs in differentiated cells would be greatly facilitated by the development of a cell-free system that performs these processes with fidelity. A system of isolated nuclei which initiated RNA synthesis and extensively transcribed the genes for rRNA,[1] mRNA, and low molecular weight RNAs (tRNA and 5S RNA) would partially fulfill such a need. To be especially useful, such a system should also perform RNA processing (e.g., poly(A) addition, methylation, and cleavage of precursor RNAs) as well as transport of RNA from the nucleus. Ideally, all these events should occur in an ionic milieu suitable for studies of concomitant cytoplasmic functions (e.g., translation of mRNA).

Transcription of the genes for rRNA, tRNA, and 5S RNA proceeds in isolated nuclei from a variety of cell types with fidelity similar to that observed in intact cells (Weinmann and Roeder, 1974). HnRNA (Zylber and Penman, 1971; Jacobson et al., 1974) and mRNA (Shutt and Kedes, 1974; Jacobson et al., 1974) biogeneses have also been reported. However, RNA synthesis in most of these nuclear cell-free systems is short-lived (Zylber and Penman, 1971; Tsai and Green, 1973; Reeder and Roeder, 1972; Widnell and Tata, 1966) and probably consists largely of elongation of RNA chains initiated in the intact cell. Two reports have appeared describing isolated nuclear systems capable of rather prolonged RNA synthesis.[2] Wu and Zubay (1974) described RNA synthesis for periods of up to 120 min that occurred only in the presence of added cytoplasm. Marzluff et al. (1973) described RNA synthesis which continued for 60 min but required ionic conditions unsuitable for concomitant protein synthesis.

Posttranscriptional processing of RNAs synthesized by isolated nuclei has received limited attention. Jelinek (1974) and Jacobson et al. (1974) reported that isolated nuclei are capable of poly(A) synthesis. Another report attempts to document posttranscriptional cleavage of precursors for adenovirus mRNA using prelabeled nuclei from virus-infected cells (Brunner and Raskas, 1972). Experiments designed to study the transport of RNA from the nucleus in cell-free systems have generally used prelabeled nuclei which release uncharacterized RNA into the extranuclear supernatant phase. Cytosol-modulated, ATP-dependent RNA release from prelabeled liver nuclei has been described (Schumm et al., 1973). The possible artifactual nature of RNA release in these systems has been noted (Chatterjee and Weissbach, 1973).

This report describes the development and partial characterization of a cell-free nuclear system derived from the GH_3 strain of rat pituitary tumor cells (Tashjian et al., 1968). This system exhibits extended transcription in the absence of cytoplasm. Evidence has been obtained for RNA synthesis directed by RNA polymerases I, II, and III. RNA synthesis continues for at least 120 min under ionic conditions suitable

† From the Laboratory of Pharmacology, Harvard School of Dental Medicine, and the Department of Pharmacology, Harvard Medical School, Boston, Massachusetts 02115. *Received January 22, 1976.* This investigation was supported in part by a research grant from the National Science Foundation (BMS 74-12703) and the National Institute of Arthritis, Metabolism and Digestive Diseases (AM 11011). T.F.J.M. was a Research Fellow of the American Cancer Society, Massachusetts, and subsequently a recipient of a U.S. Public Health Service Research Fellowship (F22, AM 00979). Part of the work was carried out during the period when D.K.B. was a Special Fellow of the Leukemia Society of America.

[1] Abbreviations used are: HnRNA, heterogeneous nuclear RNA; mRNA, messenger RNA; rRNA, ribosomal RNA; tRNA, transfer RNA; RI, rat liver ribonuclease inhibitor; Cl_3CCOOH, trichloroacetic acid; 3′-dATP, 3′-deoxyadenosine 5′-triphosphate, cordycepin triphosphate; TBS buffer, 0.02 M Tris-HCl, pH 7.5, 0.146 M NaCl, 0.011 M sucrose; TKM buffer, 0.03 M Tris-HCl, pH 7.6, 0.10 M KCl, 0.005 M $MgCl_2$, 0.005 M 2-mercaptoethanol.

[2] Following submission of this manuscript, extended RNA synthesis in isolated oviduct nuclei has been reported by Ernest et al. (1976).

for studies of concomitant protein synthesis and is dependent upon the presence of an inhibitor of ribonuclease activity (Gribnau et al., 1969). The data presented also indicate that processing (e.g., poly(A) addition) of newly synthesized RNA and transport from the nucleus are occurring in this experimental system. A preliminary report of this work has been presented (Martin et al., 1975).

Experimental Procedure

Materials. Materials used in these experiments were obtained as follows: α-amanitin (Calbiochem); Sephadex G-25 (Pharmacia); Millipore filters, 0.45 μm pore, 24 mm (Millipore Corp.); ribonuclease-free sucrose, Sigmacell cellulose (type 38), crystalline pancreatic ribonuclease (type III-A), and actinomycin D (Sigma Chemical Co.); Nonidet P-40 (Shell Oil Co.); [8-^{3}H]GTP (9 Ci/mmol), [5,6-^{3}H]UTP (36 Ci/mmol), [5-^{3}H]CTP (26 Ci/mmol), [2,8-^{3}H]ATP (32 Ci/mmol), [5,6-^{3}H]uridine (45 Ci/mmol), [^{3}H]poly(U) (24 Ci/mol of UMP), [2,8-^{3}H]adenosine (40 Ci/mmol), [α-^{32}P]GTP (10.9 Ci/mmol), and Omnifluor (New England Nuclear); glass fiber filters, GF/C (Whatman); and oligo(dT)-cellulose (dT_{2-10}) and rat liver ribonuclease inhibitor (specific activity 200–400 units/mg of protein) (G. D. Searle and Co.). One unit of rat liver ribonuclease inhibitor is defined as the amount of material producing 50% inhibition of 5 ng of crystalline pancreatic ribonuclease A. Cordycepin triphosphate (3′-dATP) was generously provided by Dr. R. J. Suhadolnik of Temple University School of Medicine, Philadelphia, Pa. Poly(A) fractions of defined chain length were obtained from Dr. R. G. Sommer of the Molecular Biology Department of Miles Laboratories. GH_3 cells used in these studies are a clonal strain of prolactin- and growth hormone producing rat pituitary tumor cells which have been propagated in culture since 1965 (Tashjian et al., 1968; Martin and Tashjian, 1976). They have been examined by Dr. Iolanda Low and found to be free of Mycoplasma at the time of these experiments.

Preparation of Nuclei. GH_3 cells, grown in suspension culture, were harvested and washed with ice-cold TBS buffer (0.02 M Tris-HCl, pH 7.5, 0.146 M NaCl, 0.011 M sucrose) as previously described (Biswas and Tashjian, 1974). All subsequent operations were carried out at 0–4 °C. Washed cells were suspended in two packed-cell volumes of TKM buffer (0.03 M Tris-HCl, pH 7.6, 0.10 M KCl, 0.005 M $MgCl_2$, 0.005 M 2-mercaptoethanol). Rat liver ribonuclease inhibitor (2 units/ml) was present throughout the isolation procedure unless otherwise noted. Cells were lysed in the presence of 0.2% Nonidet P-40 (10 min at 0 °C). Nuclei were sedimented at 500*g* for 5 min and the supernatant was withdrawn for the preparation of cytoplasm. Incubation with 0.2% Nonidet P-40 was repeated with the resuspended nuclear pellet. Nuclei obtained by sedimentation were washed three times with TKM buffer containing rat liver ribonuclease inhibitor (RI) and finally resuspended in the same buffer at a concentration of 2×10^7 nuclei/ml. Pipetting transfers were minimized by replicate preparation of nuclei in separate incubation tubes to which the components of the cell-free incorporation system were subsequently added. Isolated nuclei appeared uncontaminated by whole cells as assessed by phase contrast microscopy.

Preparation of Cytoplasm. Supernatant fractions (500*g*) from the nuclear preparation were filtered at 4 °C on a Sephadex G-25 column equilibrated with TKM buffer to remove endogenous nucleotides. Material appearing in the void volume was used as the cytoplasmic preparation and is referred to as Sephadex-treated cytoplasm.

Conditions for Cell-Free RNA Synthesis. Incubations with isolated nuclei were performed at 29 °C for the indicated times in reaction mixtures containing per ml: 30 μmol of Tris-HCl (pH 7.6); 100 μmol of KCl; 5 μmol of $MgCl_2$; 6 μmol of dithiothreitol; 1.5 μmol of ATP; 0.25 μmol of GTP; 0.2 μmol of CTP; 0.2 μmol of UTP; 10 μmol of creatine phosphate; 100 μg of creatine phosphokinase; 5×10^6 to 10^7 nuclei (90 to 180 μg of DNA); 2 units of RI (or as indicated); and 10 μCi of [^{3}H]UTP, [^{3}H]GTP, [^{3}H]CTP, or [^{3}H]ATP replacing the unlabeled nucleoside triphosphate unless otherwise indicated. Reactions were terminated by the addition of 100 μl of 0.2% sodium dodecyl sulfate in TKM buffer followed by the addition of 100 μg of yeast RNA as carrier and 2 ml of 10% trichloroacetic acid. Insoluble radioactivity was quantitated by filtration on GF/C filters which were counted at 30% efficiency in Omnifluor.

Isolation of RNA. Reaction mixtures were chilled on ice following incubation and separated into a nuclear pellet and supernatant fraction by centrifugation at 500*g* for 10 min at 4 °C. The nuclear pellet was washed four times with TKM buffer; the first wash was added back to the supernatant fraction. The nuclear pellet was lysed and digested with DNase (Worthington Biochemical Corp.) as described by Zylber and Penman (1971). RNA from the lysed nuclear pellet and supernatant fractions was extracted by the sodium dodecyl sulfate–phenol–pH 9 method of Lee et al. (1971). Alternatively, nuclear RNA was also extracted by the hot phenol–sodium dodecyl sulfate methods of Scherrer (1969) or Penman (1969). Rabbit reticulocyte 28S and 18S rRNAs, 45S rRNA from GH_3 cells, and *Escherichia coli* 4S RNA were prepared by conventional methods and added to the RNA samples before ethanol precipitation. Sodium dodecyl sulfate–sucrose gradient centrifugation was performed in an SW 41 rotor at 25 000 rpm for 15–24 h at 23 °C. Sucrose gradients consisted of 12.5 ml of a 10–70% linear sucrose gradient in 0.01 M Tris-HCl, pH 7.6, 0.1 M NaCl, 0.001 M EDTA, 0.2% sodium dodecyl sulfate. In the gradient shown in Figure 5A a 12-ml gradient was formed over a 0.5-ml cushion of 70% sucrose. Dimethyl sulfoxide–sucrose gradient centrifugation was performed by a modification of the method of J. C. Scott, Massachusetts General Hospital (personal communication) in an SW 50.1 rotor at 50 000 rpm for 21 h at 23 °C. Gradients consisted of 5.4 ml of 0–20% (w/w) sucrose in 85% (v/v) dimethyl sulfoxide, 0.05 M Tris-HCl, pH 7.5, 0.005 M EDTA. RNA was dissolved in water–85% dimethyl sulfoxide in buffer (1:1), heated to 65 °C for 3 min, cooled, and layered onto the gradient. Gradient fractions were precipitated with 10% trichloroacetic acid and collected on GF/C filters; 45S, 28S, 18S, and 4S RNA markers were used to align gradients for comparison.

Oligo(dT)-Cellulose, Sigmacell, and Millipore Filter Binding. Chromatography of RNA samples on oligo(dT)-cellulose was performed as described by Aviv and Leder (1972) using 0.01 M Tris-HCl, pH 7.5, 0.5 M KCl; 0.01 M Tris-HCl, pH 7.5, 0.1 M KCl; and 0.01 M Tris-HCl, pH 7.5, as application, wash, and elution buffers, respectively. Chromatography of RNA samples on Sigmacell was by the method of Schutz et al. (1972) employing 0.01 M Tris-HCl, pH 7.5, 0.5 M KCl, 0.0002 M $Mg(CH_3COO)_2$ and neutralized water as application and elution buffers, respectively. Millipore filter binding was performed as described by Lee et al. (1971) using 0.01 M Tris-HCl, pH 7.5, 0.5 M KCl, 0.001 M $Mg(CH_3COO)_2$ and 0.1 M Tris-HCl, pH 9.0, 0.5% sodium dodecyl sulfate as application and elution buffers, respectively.

Analysis of RNA for Poly(A) Content. RNA from cell-free postnuclear supernatant fractions or from the cytoplasm of

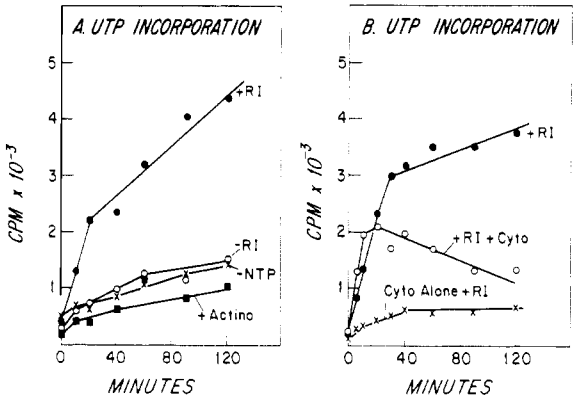

FIGURE 1: Characteristics of RNA synthesis by isolated GH_3 nuclei. Each point gives the incorporation by 10^6 nuclei taken from a common reaction mixture at the indicated times. Nuclei were prepared in the absence of RI. (A) [^{3}H]UTP incorporation in the complete system without (−RI) or with RI (+RI), 1 unit/ml; without GTP, CTP, and ATP (−NTP) plus RI; and with actinomycin D (2 μg/ml) plus RI (+Actino). (B) [^{3}H]UTP incorporation in the complete system with RI (+RI); with Sephadex-treated cytoplasm (200 μg of protein) plus RI (+RI + Cyto); and [^{3}H]UTP incorporation in the absence of nuclei but with cytoplasm plus RI (Cyto Alone + RI).

intact cells was analyzed for poly(A) content by digestion with pancreatic and T1 RNases under the conditions described by Jelinek (1974), followed by oligo(dT)-cellulose chromatography, and finally by polyacrylamide gel electrophoresis of the lyophilized low salt eluate from the oligo(dT)-cellulose column. Electrophoresis of [^{3}H]poly(A) was performed in cylindrical (3 mm × 60 mm) 10% polyacrylamide gels in buffer (0.04 M Tris-HCl, pH 7.5, 0.02 M sodium acetate, 0.002 M EDTA) containing 0.2% sodium dodecyl sulfate. Gels were preelectrophoresed for 30 min before the RNA samples were applied. Electrophoresis (6 mA/gel, constant current) was performed at room temperature. About 0.5 A_{260} unit of each of the poly(A) standards of defined nucleotide length and about 1–2 × 10^3 cpm of [^{3}H]poly(A) isolated from the cell-free supernatant and cytoplasmic fractions were applied to the gels. After completion of the electrophoresis, absorbance of the standard poly(A) gels was measured at 260 nm in a Gilford recording spectrophotometer. The relative mobility of poly(A) tracts of different nucleotide lengths was measured with reference to the bromophenol blue dye front. Each gel containing [^{3}H]-poly(A) was cut into 2-mm slices and incubated with H_2O_2 at 60 °C for 48 h, and radioactivity was determined in Bray's modified scintillation fluid at 30% efficiency.

Ribonuclease Activity Determinations. Ribonuclease activity in cellular fractions or of pancreatic ribonuclease was determined by measuring the hydrolysis of [^{3}H]poly(U). Incubations were performed at 37 °C for 60 min in 0.014 M Tris-HCl, pH 7.8, 0.1 M KCl, and 0.002 M $Mg(CH_3COO)_2$.

Results

Extended RNA Synthesis. Isolated nuclei from GH_3 cells incorporated [^{3}H]UTP into Cl_3CCOOH-insoluble material for at least 120 min at 29 °C in the presence of RI (Figure 1A). Incorporation in the absence of RI proceeded at a much slower rate. Incorporation of [^{3}H]UTP was dependent on the presence of the other three nucleoside triphosphates and was inhibited markedly by actinomycin D. Treatment of the reaction mixture following incubation with pancreatic RNase (10 μg/ml), 0.1 N NaOH, or hot 10% Cl_3CCOOH rendered the radioactivity acid soluble. From these results, we conclude that DNA-dependent RNA synthesis occurs in this cell-free system. Whole cells did not contribute substantially to the RNA synthesis observed because the inclusion of pancreatic RNase during incubation reduced [^{3}H]UTP incorporation by 95%, and furthermore no intact cells were seen by phase contrast microscopy.

Extended incorporation of [^{3}H]CTP, [^{3}H]GTP, and [^{3}H]ATP by isolated nuclei was also dependent upon the presence of RI (data not shown). Incorporation of ^{3}H-labeled nucleoside triphosphates was markedly dependent on the final concentration of the labeled precursor. For example, the incorporation of [^{3}H]GTP during 120-min incubation periods was 0.20, 2.8, and 10.2 pmol/μg of DNA at 0.3 μM, 12.8 μM, and 125 μM GTP, respectively. The time course of [^{3}H]GTP incorporation at each of the above concentrations was prolonged, suggesting that the extended duration of RNA synthesis was not a consequence of reduced incorporation rates caused by limiting nucleotide concentrations. Similar results were observed for low (0.6 μM) and high (200 μM) [^{3}H]ATP concentrations (see Figure 7B, below).

When Sephadex-treated cytoplasm from GH_3 cells was added to the incubation mixture, [^{3}H]UTP incorporation was substantially inhibited (Figure 1B). This inhibition was not a unique feature of cytoplasm or nuclei prepared by detergent treatment of GH_3 cells, for it was also observed with nuclei and cytoplasm prepared by Dounce homogenization. In addition, cytosol fractions prepared by centrifugation (S-30, S-100) were also found to be inhibitory. The mechanism of inhibition by cytoplasm is not known although the inhibition is apparently not caused by RNases sensitive to RI, since it could not be reversed or prevented by addition of larger amounts of RI than shown in Figure 1B (1 unit/ml).

Role of Ribonuclease Inhibitor. The presence of RI was essential for extended nucleoside triphosphate incorporation. Incorporation of [^{3}H]UTP was dependent on the RI concentration used, reaching a maximum at about 2 units/ml of RI. Because the RI preparation used was not homogeneous (2 units corresponds to about 8 μg of protein), it was possible that a component other than a ribonuclease inhibitor was responsible for the observed enhancement of [^{3}H]UTP incorporation. However, a thermal denaturation profile of the RI preparation showed that the inhibition of crystalline pancreatic RNase and the enhancement of nuclear [^{3}H]UTP incorporation by RI were inactivated in parallel (Figure 2). These findings are consistent with the conclusion that stimulation of [^{3}H]UTP incorporation by RI was due to its RNase inhibitory activity. In addition, direct measurement demonstrated RNase activity in GH_3 nuclear preparations which was substantially inhibited by RI. Thus, it appears that the slow rate and early termination of ^{3}H-labeled nucleoside triphosphate incorporation in this cell-free system in the absence of RI are due at least in part to the presence of RNase in the nuclear preparation.

Criteria for Characterization of Cell-Free RNA Products. Nucleolar RNA polymerase I directed rRNA synthesis in a variety of cell types is preferentially inhibited by low concentrations of actinomycin D. Nucleoplasmic RNA synthesis is inhibited at 20- to 50-fold higher concentrations of the drug (Perry et al., 1970; Penman et al., 1970). As shown in Figure 3, synthesis of nucleolar RNA in isolated GH_3 nuclei was inhibited by concentrations of actinomycin D between 0.1 and 10 μg/ml. In contrast, synthesis of nucleoplasmic RNA was only partially inhibited by concentrations of the drug as high as 25 μg/ml. Although the preferential inhibition of the synthesis of nucleolar RNA by actinomycin D is preserved in this cell-free system, concentrations of the drug much higher than those reported to inhibit RNA synthesis in intact cells were

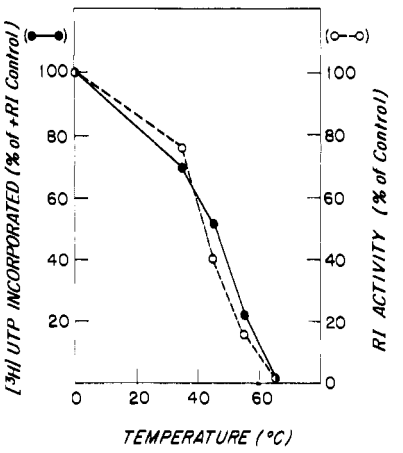

FIGURE 2: Effects of heat treatment on the activities of RI. Nuclei were isolated in the absence of RI and incubated in the complete system for 120 min. RI (1 unit/ml) was heated at the indicated temperatures for 10 min, chilled to 0 °C, and assayed for stimulation of [^{3}H]UTP incorporation (●-●) or inhibition of RNase activity (O- - -O) using crystalline pancreatic RNase as described in Experimental Procedure. Each point (●) gives the incorporation by 10^6 nuclei.

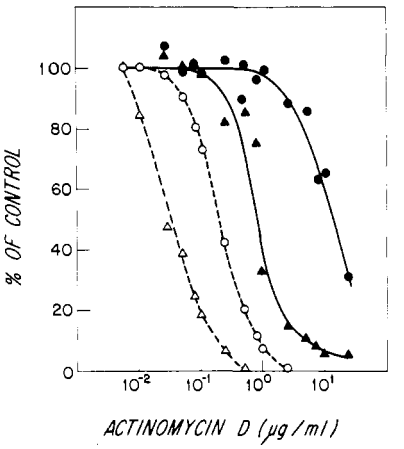

FIGURE 3: Actinomycin D inhibition of RNA synthesis in isolated GH_3 nuclei and intact GH_3 cells. Nuclei (10^6/ml) were incubated with [^{3}H]-GTP in a complete reaction mixture as described in Experimental Procedure with the indicated concentrations of actinomycin D. Following incubation at 29 °C for 15 min, nuclei were chilled, pelleted, washed three times with cold TKM buffer, and separated into nucleoplasmic and nucleolar fractions by the method of Penman (1969). Nucleoplasmic (●-●) and nucleolar (▲-▲) Cl_3CCOOH-insoluble radioactivity was assayed as described in Experimental Procedure. For comparison, intact GH_3 cells at 10^6/ml were incubated for 30 min at 37 °C with the indicated concentrations of actinomycin D and then for 15 min with 2 μCi/ml [^{3}H]uridine. Incubations were terminated by rapid chilling, and nuclei were prepared as described in Experimental Procedure. Radioactivity in nucleoplasmic (O- - -O) and nucleolar (Δ- - -Δ) fractions was determined as described for the cell-free incubations.

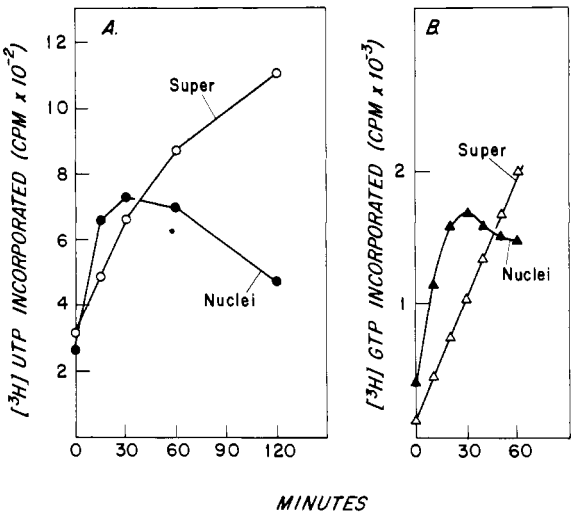

FIGURE 4: Time course of the incorporation of [^{3}H]UTP or [^{3}H]GTP into nuclear and supernatant RNAs. RNA was synthesized in a complete reaction mixture including RI (1 unit/ml) with [^{3}H]UTP (A) or [^{3}H]GTP (B). Each point represents the incorporation in a 100-μl aliquot withdrawn from a common reaction mixture at the indicated times and separated into a nuclear pellet (Nuclei) and supernatant fraction (Super) as described in Experimental Procedure.

required. The data in Figure 3 also show that RNA synthesis in intact GH_3 cells exhibited a sensitivity to actinomycin D comparable to that observed in other cell types, namely inhibition of nucleolar RNA synthesis between 0.01 and 0.1 μg/ml actinomycin D. Although the reason for the difference between the sensitivities of intact cells and isolated nuclei to inhibition of RNA synthesis by actinomycin D is not known, we have utilized actinomycin D to inhibit preferentially cell-free nucleolar RNA synthesis in subsequent experiments. Cell-free nucleolar RNA synthesis was inhibited in the experiments reported here either by inclusion of 3 μg/ml actinomycin D in the cell-free incubation or by pretreatment of intact cells with 0.1 μg/ml actinomycin D followed by cell-free incubation of isolated nuclei in the absence of the drug.

The toxin α-amanitin has been used to distinguish the activities of the multiple forms of eukaryotic DNA-dependent RNA polymerase (Blatti et al., 1970). Nucleolar RNA polymerase I is resistant to the action of α-amanitin, whereas nucleoplasmic RNA polymerases II and III are sensitive to the toxin. Furthermore, the activities of RNA polymerases II and III can be distinguished by their relative sensitivities to α-amanitin (Weinmann and Roeder, 1974). RNA polymerase II activity is inhibited by low concentrations (0.001 to 0.1 μg/ml) of α-amanitin whereas inhibition of RNA polymerase III requires much higher concentrations (1 to 500 μg/ml). In a 60-min incubation, low concentrations of α-amanitin (<0.1 μg/ml) inhibited incorporation of [^{3}H]GTP in isolated GH_3 nuclei by 23% and higher α-amanitin concentrations (300 μg/ml) inhibited incorporation an additional 30%. The residual 47% α-amanitin-resistant incorporation in isolated nuclei was inhibited by concentrations of actinomycin D of 3 μg/ml. From these findings, we conclude that about 23, 30, and 47% of the RNA synthesized by isolated GH_3 nuclei in a 60-min incubation in TKM buffer at 29 °C were directed by RNA polymerases II, III, and I, respectively.

Fate of [^{3}*H*]*RNA.* Release of newly synthesized RNA from nuclei was investigated by measurement of labeled Cl_3CCOOH-insoluble material associated with nuclear pellet and postnuclear supernatant fractions at different incubation times (Figure 4). The initial rate of appearance of [^{3}H]RNA in the reaction supernatant was slower than the rate of appearance of [^{3}H]RNA in the nuclear pellet. This finding is consistent with nuclear synthesis followed by subsequent release into the supernatant. RNAs in the supernatant fraction are qualitatively different from those present in the nuclear pellet as assessed by several methods described below.

RNA Associated with the Nuclear Pellet. RNA products synthesized by isolated GH_3 nuclei were characterized by sodium dodecyl sulfate–sucrose gradient centrifugation analysis. Panel A of Figure 5 shows the profile of [^{3}H]RNA which remained associated with the nuclear pellet during a 60-min incubation at 29 °C with [^{3}H]GTP. Also shown is nuclear

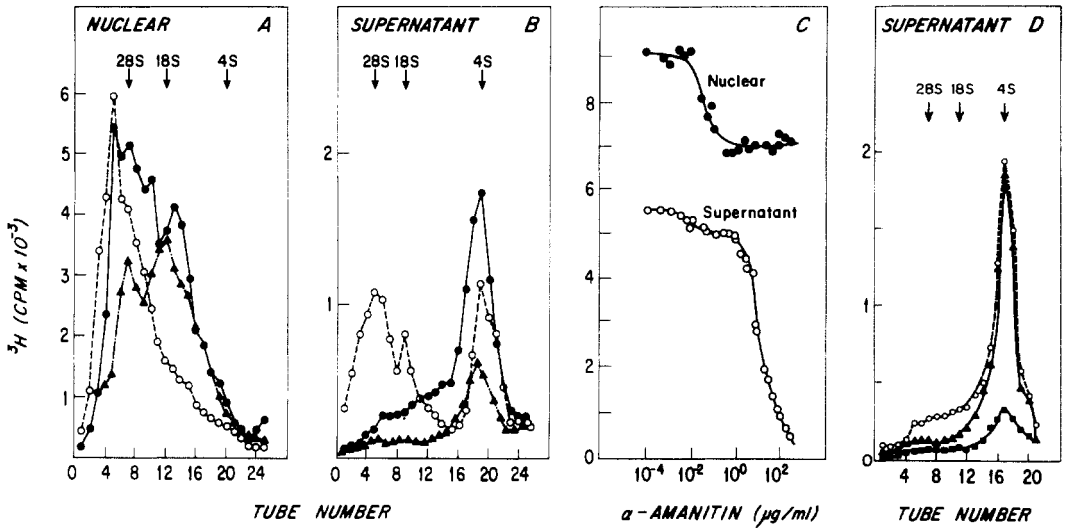

FIGURE 5: (A) Sodium dodecyl sulfate–sucrose gradient analysis of nuclear RNA synthesized by isolated GH_3 nuclei and by intact GH_3 cells. [^{3}H]RNA was synthesized during a 60-min incubation at 29 °C with isolated nuclei in a complete reaction mixture containing [^{3}H]GTP either without (●–●) or with (▲–▲) α-amanitin (5 μg/ml). [^{3}H]RNA was prepared from the nuclear pellet as described in Experimental Procedure. Nuclear [^{3}H]RNA was prepared from intact GH_3 cells incubated at 37 °C for 60 min with 5 μCi/ml [^{3}H]uridine (○– –○). Centrifugation was performed in sodium dodecyl sulfate–sucrose gradients for 20 h as described in Experimental Procedure. (B) Sodium dodecyl sulfate–sucrose gradient analysis of supernatant RNA synthesized by isolated GH_3 nuclei and of cytoplasmic RNA synthesized by intact GH_3 cells. [^{3}H]RNA was prepared from the postnuclear supernatant fractions of the incubations described in A. Supernatant RNA was prepared from incubations in which α-amanitin (5 μg/ml) was absent (●–●) or present (▲–▲). Cytoplasmic RNA was isolated from cells incubated with [^{3}H]uridine for 60 min as described in A (○– –○). Centrifugation was performed for 25 h. (C) Effects of α-amanitin on the synthesis of nuclear (●–●) and supernatant (○–○) RNA. Reaction mixtures containing 5 × 10^5 nuclei, [^{3}H]GTP, and the indicated concentrations of α-amanitin were incubated for 60 min at 29 °C. Nuclei were pelleted and washed three times with cold TKM buffer. Radioactivity in the nuclear pellet and supernatant fractions was analyzed as described in Experimental Procedure. (D) Sodium dodecyl sulfate–sucrose gradient analysis of supernatant RNA synthesized in the presence of selected α-amanitin concentrations. Supernatant [^{3}H]RNA (labeled with [^{3}H]GTP) was isolated from reaction mixtures containing no α-amanitin (○– –○), 0.5 μg/ml (▲–▲), and 100 μg/ml (■–■) α-amanitin. Analysis was performed as described in B.

RNA isolated from intact GH_3 cells incubated with [^{3}H]uridine for 60 min at 37 °C. The cell-free RNA was heterogeneous, sedimenting broadly between about 12 S and >28 S. An upper estimate of apparent size cannot be made from this gradient because of the presence of a 70% sucrose cushion which does not allow fractionation of RNAs larger than about 30 S.

The dose–response characteristics of the α-amanitin inhibition of RNA synthesis by GH_3 nuclei are shown in Figure 5C. Low concentrations of α-amanitin inhibited the synthesis of about 22% of the RNA associated with nuclei (Figure 5C, upper curve). Concentrations of α-amanitin higher than 0.3 μg/ml did not inhibit this synthesis further, suggesting that RNAs synthesized by RNA polymerase III were not present in the nuclear pellet following a 60-min incubation.

The synthesis of nuclear RNAs of all size classes was inhibited by α-amanitin (Figure 5A). The nuclear RNAs whose synthesis is sensitive to α-amanitin presumably are the products of RNA polymerase II and are evident as the difference between RNA from control and α-amanitin-containing incubations in Figure 5A. The heterogeneous sedimentation behavior of these RNA polymerase II products resembles that of HnRNA described for a variety of cell types (Darnell et al., 1973). RNA polymerase II directed HnRNA synthesis in isolated nuclei has been previously reported (Zylber and Penman, 1971; Jacobson et al., 1974). Most of the nuclear RNA synthesized in the presence of α-amanitin cosedimented with the 18S and 28S rRNA markers (Figure 5A). This cell-free RNA synthesis which was resistant to α-amanitin was inhibited by 3 μg/ml of actinomycin D or by pretreatment of intact cells with 0.1 μg/ml of the drug (data not shown). α-Amanitin-resistant, actinomycin D sensitive RNA synthesis probably represents RNA polymerase I directed rRNA synthesis by isolated GH_3 nuclei.

RNA in the Postnuclear Supernatant Fraction. The sedimentation profile of [^{3}H]RNA released into the supernatant fraction during a 60-min incubation at 29 °C is shown in Figure 5B. Also shown in Figure 5B are cytoplasmic RNAs isolated from intact GH_3 cells following a 60-min incubation with [^{3}H]uridine at 37 °C. Cell-free supernatant RNA was heterogeneous in size consisting of a peak at the position of the 4S marker and a broad shoulder ranging to about 28 S. The appearance of both the heterogeneous 5–28S component and the 4–5S peak in the supernatant was inhibited by α-amanitin (5 μg/ml). We presume that the action of α-amanitin is at the level of synthesis of supernatant RNAs rather than at the level of release from the nuclei.

The concentration dependence of the α-amanitin inhibition of the synthesis of supernatant RNAs is shown in the lower curve of Figure 5C. About 10% of the total supernatant RNA synthesized was inhibited by concentrations of α-amanitin in the range of 0.001 to 0.1 μg/ml; synthesis of the remaining 90% was inhibited by higher concentrations of the drug (1–300 μg/ml). The biphasic nature of the α-amanitin inhibition is consistent with the involvement of two polymerase systems (RNA polymerases II and III) in the synthesis of the supernatant RNA (see Weinmann and Roeder, 1974).

As shown by the lower curve of Figure 5C, α-amanitin at the highest concentrations used (300 μg/ml) inhibited completely the synthesis of supernatant RNAs. This result indicates that the products of RNA polymerase I, whose synthesis was resistant to the drug and which were present in the nuclear pellet (Figure 5A), do not appear in the supernatant fraction in a 60-min incubation period.

The sedimentation properties of supernatant RNAs accumulating at selected α-amanitin concentrations are shown in Figure 5D. RNAs whose synthesis was reduced by a low concentration of α-amanitin (0.5 μg/ml) sedimented broadly

TABLE I: Binding of Supernatant and Nuclear [^{3}H]RNA to Oligo(dT)-Cellulose, Millipore Filters, and Sigmacell.[a]

Expt. No.	Adsorbant	α-Amanitin[b]	cpm (×10^{-3}) Applied	cpm (×10^{-3}) Eluted with KCl			% of Applied cpm Eluted with 0.0 M KCl
				0.5 M	0.1 M	0.0 M	
			Supernatant RNA				
1A	Oligo(dT)-cellulose	−	15	13.6	0.6	1.1	7.3
1B	Oligo(dT)-cellulose	+	12	10	0.5	0.2	1.7
2	Millipore filters	−	5			0.7	14
3	Sigmacell	−	5			0.4	8
			Nuclear RNA				
4	Oligo(dT)-cellulose	−	104	73	5.7	16	15

[a] Samples of [^{3}H]RNA were prepared by incubation of a complete reaction mixture containing [^{3}H]GTP for 60 min at 29 °C followed by phenol extraction of the supernatant and nuclear fractions as described in Experimental Procedure. The columns and filters were washed with the buffers shown and the retained [^{3}H]RNA was eluted with low salt buffer (0.0 M KCl). [b] α-Amanitin (3 μg/ml) was present (+) or absent (−) during the incubation from which the RNA was isolated.

between about 5 and 28 S and are presumably the products of RNA polymerase II. RNAs whose synthesis was inhibited by 100 μg/ml, but not by 0.5 μg/ml, α-amanitin consisted of low molecular weight 4–5S RNAs which are presumably the products of RNA polymerase III.

Poly(A)-Containing Supernatant RNA. The cell-free supernatant RNA was examined for the presence of poly(A)[3] tracts. As shown in Table I, 7–14% of the supernatant RNA was adsorbed to and eluted from oligo(dT)-cellulose, Sigmacell, and Millipore filters under conditions reported to detect the presence of poly(A). Oligo(dT)-cellulose binding of supernatant RNA synthesized in the presence of low concentrations of α-amanitin was substantially reduced (Table I, experiment 1B) indicating that poly(A) was associated with RNAs synthesized by RNA polymerase II.

The sedimentation characteristics of cell-free supernatant RNA eluted from oligo(dT)-cellulose at low ionic strength are shown in Figure 6B. The poly(A)-containing RNA, which represented 7.5% of the total supernatant [^{3}H]RNA applied to the oligo(dT)-cellulose column, sedimented heterogeneously between about 5 and 28 S. The peak at approximately 5 S was of variable height in several similar experiments (see, e.g., Figure 9) and probably represents a small (1–4%) contamination from the 4–5S peak of the supernatant RNA applied to the column. Sedimentation analysis of total supernatant RNA and oligo(dT)-cellulose binding and nonbinding fractions in the same experiment showed that 75% of the heterogeneous 5–28S RNA component contained poly(A). The sedimentation properties of poly(A)-containing RNA extracted from polyribosomes of intact GH_3 cells and fractionated by oligo(dT)-cellulose chromatography are shown in Figure 6A. Cellular cytoplasmic (Figure 6A) and cell-free supernatant (Figure 6B) poly(A)-containing RNAs sediment over a similar broad range on sodium dodecyl sulfate–sucrose gradients.

Poly(A) Synthesis by Isolated Nuclei. The results of experiments shown in Table I and Figure 6B suggest that RNA polymerase II directed transcripts which appear in the postnuclear supernatant fraction of this cell-free system contain poly(A). In order to determine whether de novo synthesis of poly(A) occurred in isolated GH_3 nuclei, studies on the incorporation of [^{3}H]ATP into RNase-resistant poly(A) tracts

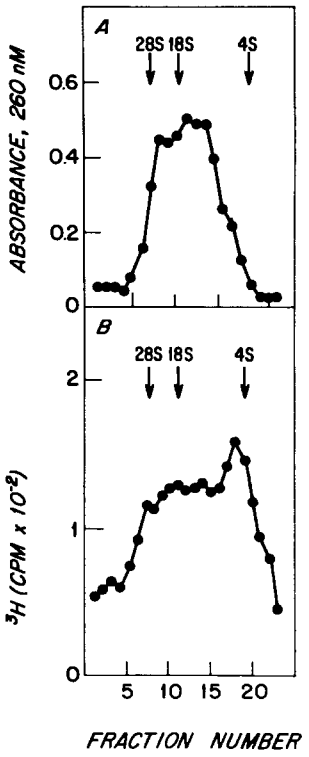

FIGURE 6: A comparison of sodium dodecyl sulfate–sucrose gradient profiles of poly(A)-containing GH_3 polysomal RNA and poly(A)-containing cell-free supernatant RNA. (A) Poly(A)-containing GH_3 polysomal RNA: polysomes were prepared from GH_3 cells by lysis with 0.2% Nonidet P-40 followed by fractionation of a postmitochondrial supernatant on a sucrose block gradient as described by Schimke et al. (1974). Polysomal RNA was extracted by the phenol–sodium dodecyl sulfate method and fractionated on oligo(dT)-cellulose as described in Experimental Procedure. (B) Poly(A)-containing cell-free supernatant RNA: [^{3}H]RNA was prepared from supernatant obtained from an incubation of isolated nuclei with [^{3}H]GTP at 29 °C for 60 min and purified by oligo(dT)-cellulose as described in Experimental Procedure. The 0.01 M Tris eluate which contained 7.5% of the total supernatant RNA was applied to the gradient. Sedimentation was performed in 10–70% sodium dodecyl sulfate–sucrose gradients for 20 h.

were performed. Figure 7 shows the extended time course of [^{3}H]ATP incorporation into total (Figure 7B) and RNase-resistant material (Figure 7A) at high and low concentrations of ATP. At 200 μM ATP, [^{3}H]ATP incorporation into RNase-resistant, Cl_3CCOOH-insoluble material in a 60-min incubation period was 240-fold higher than observed at 0.6 μM

[3] The term poly(A) is used to refer to adenine-rich tracts irrespective of their sizes. No distinction is made between large poly(A) tracts and smaller oligo(A) tracts both of which bind to oligo(dT)-cellulose and Millipore filters.

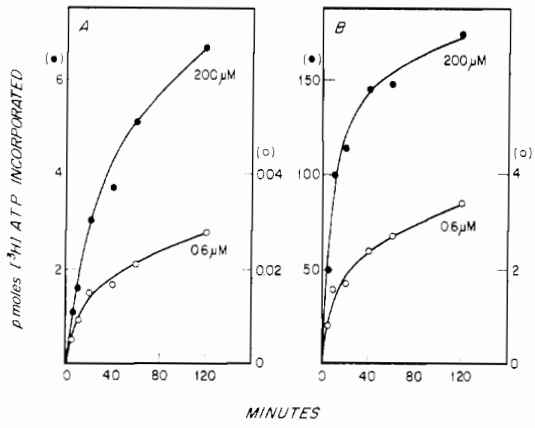

FIGURE 7: Effect of ATP concentration on poly(A) and total RNA synthesis. [^{3}H]RNA was extracted from the supernatant fraction of a cell-free reaction mixture in which 10^7 nuclei/ml were incubated with [^{3}H]ATP (50 μCi) at the indicated concentrations of nonradioactive ATP. Cl_3CCOOH-insoluble radioactivity was determined in an aliquot of the [^{3}H]RNA (panel B). Another aliquot was digested with pancreatic and T1 RNases followed by determination of Cl_3CCOOH-insoluble radioactivity (panel A). Note that incorporation at 0.6 μM ATP is read from the right-hand ordinates and incorporation of 200 μM ATP is read from the left-hand ordinates.

ATP (Figure 7A). The incorporation of [^{3}H]ATP into total Cl_3CCOOH-insoluble material at 200 μM ATP was 50-fold greater than observed at 0.6 μM ATP (Figure 7B). Incorporation of [^{3}H]ATP into poly(A) represented 0.8 and 3.5% of the total incorporation of [^{3}H]ATP in a 60-min incubation at 0.6 and 200 μM ATP, respectively (Figure 7). The requirement for a high concentration of ATP for poly(A) synthesis in this cell-free system is in agreement with the findings reported by Jelinek (1974) using isolated nuclei from HeLa cells. In subsequent experiments on poly(A) synthesis, 100–200 μM ATP was included in the reaction mixture. RNase-resistant poly(A) tracts prepared from the supernatant RNA fraction of a 60-min incubation were fractionated by oligo(dT)-cellulose chromatography and analyzed by polyacrylamide gel electrophoresis as shown in Figure 8. Of the RNase-resistant, Cl_3CCOOH-insoluble radioactivity 90–95% was recovered following oligo(dT)-cellulose binding and elution. The cell-free products were compared with RNase-resistant poly(A) tracts prepared by similar methods from cytoplasmic RNA of GH_3 cells incubated with [^{3}H]adenosine. The major peak of radioactive poly(A) of the cytoplasmic RNA corresponds to a tract of about 150 adenine residues (Figure 8, shown by arrow) based on a calibration of the gel system with standard oligo(A) preparations (see inset in Figure 8A). Poly(A) tracts isolated from cell-free supernatant RNA migrated in the same region of the gel (Figure 8B). The inclusion of α-amanitin (2 μg/ml) in the cell-free incubation inhibited completely the appearance of supernatant RNase-resistant poly(A) tracts (Figure 8B).

Sedimentation Properties of Nuclear and Supernatant Poly(A)-Containing RNA under Denaturing Conditions. Cell-free nuclear RNA also contained poly(A) by the criterion of oligo(dT)-cellulose binding (Table I, experiment 4). In addition, α-amanitin (3 μg/ml) reduced the synthesis of nuclear RNA which bound oligo(dT)-cellulose (not shown). Since both nuclear and supernatant RNA polymerase II products contained poly(A) and sedimented heterogeneously, these RNAs synthesized in the cell-free system were examined on dimethyl sulfoxide containing sucrose gradients. Under these conditions the secondary structure of RNA is destroyed and aggregates are dissociated (Strauss et al., 1968). Figure

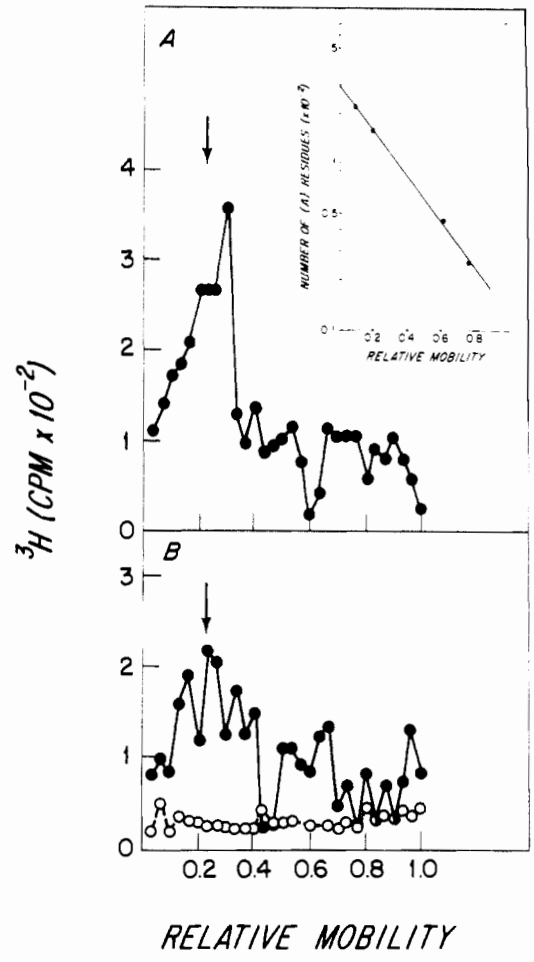

FIGURE 8: Electrophoretic analysis of [^{3}H]poly(A) in cytoplasmic and postnuclear supernatant RNA. GH_3 cells (10^6/ml) were incubated with 1 μCi/ml [^{3}H]adenosine for 6 h. [^{3}H]RNA was extracted from the cytoplasm as described by Jelinek (1974). Similarly, [^{3}H]RNA was isolated from the postnuclear supernatant fraction of a cell-free reaction mixture containing 5×10^7 nuclei, [^{3}H]ATP (0.2 mM), incubated for 60 min at 29 °C in the presence or absence of α-amanitin (5 μg/ml). Analysis of these RNAs for poly(A) content was performed as described in Experimental Procedure: (A) relative mobility of [^{3}H]poly(A) tracts isolated from cytoplasmic RNA; (B) relative mobility of [^{3}H]poly(A) tracts isolated from the postnuclear supernatant RNA from cell-free incubations performed in the presence (O- - -O) and absence (●–●) of α-amanitin. Inset in panel A shows the linear relationship between the logarithm of the number of adenine residues and relative mobility of the poly(A) tracts. The average length of these poly(A) fractions was determined by Dr. Ronald G. Sommer of Miles Laboratories, Inc. using a sedimentation velocity technique (Eisenberg and Felsenfeld, 1967).

9 shows the gradient profiles of oligo(dT)-cellulose fractionated RNA from the nuclear pellet and supernatant fractions of cell-free incubations. Cell-free nuclear RNA was obtained by incubation of nuclei from cells pretreated with 0.1 μg/ml actinomycin D such that 75% of the total nuclear RNAs synthesized in subsequent cell-free incubations in the absence of the drug were products of RNA polymerases II and III (i.e. sensitive to inhibition by 100 μg/ml α-amanitin). RNA eluting with low ionic strength buffer from oligo(dT)-cellulose represented 21% of the total nuclear RNA applied. Supernatant RNA was prepared from a 60-min cell-free incubation of nuclei isolated from cells not pretreated with actinomycin D; 7.7% of the total supernatant RNA applied to the oligo(dT)-cellulose column was recovered in the low salt fraction. As shown in Figure 9, poly(A)-containing nuclear and supernatant RNAs both sediment heterogeneously in dimethyl sulfoxide.

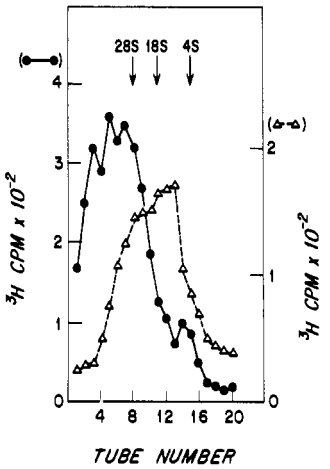

FIGURE 9: Sedimentation of cell-free poly(A)-containing nuclear and supernatant RNA in dimethyl sulfoxide–sucrose gradients. Intact GH_3 cells were incubated with 0.1 μg/ml actinomycin D for 30 min at 37 °C. Nuclei were prepared as described in Experimental Procedure and incubated for 15 min at 29 °C with [^{3}H]GTP in the absence of actinomycin D. [^{3}H]RNA was extracted from the nuclear pellet by the hot phenol–sodium dodecyl sulfate method. Supernatant [^{3}H]RNA was prepared from an independent 60-min incubation at 29 °C with [^{3}H]GTP using nuclei prepared from cells which had not been exposed to actinomycin D. Nuclear and supernatant RNAs synthesized in cell-free incubations were fractionated by oligo(dT)-cellulose chromatography. The 0.01 M Tris wash fractions were precipitated with ethanol and the RNA resuspended and sedimented in dimethyl sulfoxide–sucrose gradients as described in Experimental Procedure. The symbols are: nuclear poly(A)-containing RNA (●–●) and supernatant poly(A)-containing RNA (△- - -△).

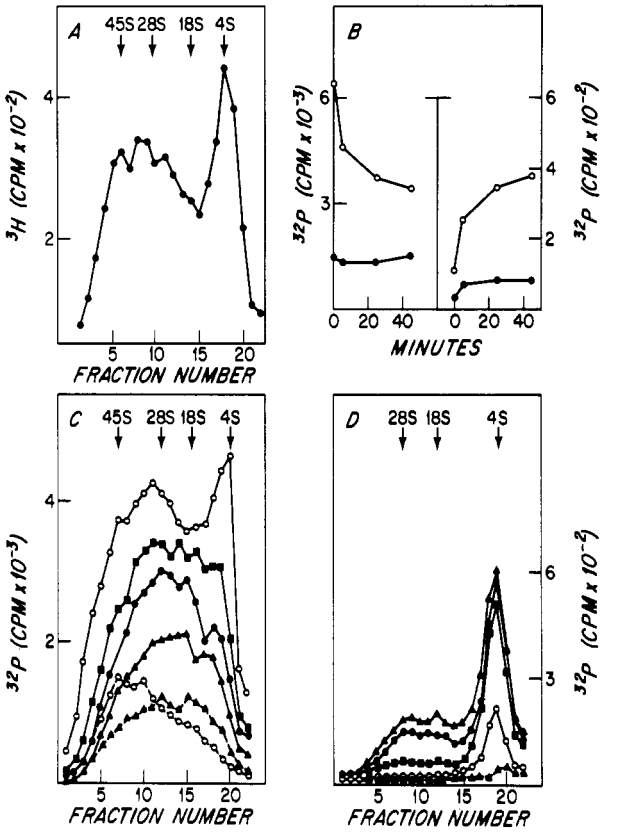

FIGURE 10: Fate of cell-free nuclear and supernatant RNAs in chase incubations following a 5-min pulse labeling period with [α-^{32}P]GTP and comparison with pulse-labeled nuclear RNA from intact cells. (A) Pulse-labeled nucleoplasmic RNA from intact GH_3 cells. Cells were incubated at 3×10^6/ml with 2 μCi/ml [^{3}H]uridine for 5 min at 37 °C. Cells were chilled and washed and nuclei were prepared with Nonidet P-40 as described in Experimental Procedure. [^{3}H]RNA was extracted from the nucleoplasmic fraction prepared by the high-salt DNase method of Penman (1969) utilizing hot phenol–sodium dodecyl sulfate. Sodium dodecyl sulfate–sucrose gradient centrifugation was performed on 10–70% sucrose gradients for 12 h. (B) Radioactivity in nuclear pellet (left side) and supernatant fraction (right side) during chase period. GH_3 nuclei (3×10^6/ml) were incubated in a complete reaction mixture for 5 min at 29 °C with 1 μCi/ml [α-^{32}P]GTP and 3 μg/ml actinomycin D in the presence (●–●) or absence (○- - -○) of 100 μg/ml α-amanitin. The reaction was terminated by chilling and nuclei were washed three times in TKM buffer at 4 °C. Nuclei were resuspended in TKM buffer and added to a reaction mixture identical with that of the 5-min incubation except that 0.25 mM GTP replaced the [α-^{32}P]GTP. The reaction mixture was incubated at 29 °C and aliquots were withdrawn at the indicated times, chilled to 0 °C, and separated into nuclear pellet and postnuclear supernatant fractions. Ten percent of each nuclear pellet and supernatant fraction was analyzed for Cl_3CCOOH-insoluble radioactivity. The abscissa gives the time following resuspension of pulse-labeled nuclei in the unlabeled reaction mixture. (C) Sodium dodecyl sulfate–sucrose gradient profiles of cell-free nuclear [^{32}P]RNA following chase incubations. RNA was extracted from aliquots representing 90% of the nuclear pellet fraction as prepared in B by the hot phenol–sodium dodecyl sulfate method and sedimented on sodium dodecyl sulfate–sucrose gradients for 12 h. RNA from the nuclear pellet fractions was isolated from nuclei pulse labeled in the presence of 100 μg/ml α-amanitin following 0 min (○- - -○) and 45 min (▲- - -▲) of chase incubation. RNA from nuclei pulse labeled in the absence of α-amanitin following 0 min (○–○), 5 min (■–■), 25 min (●–●), and 45 min (▲–▲) chase periods are shown. (D) Sodium dodecyl sulfate–sucrose gradient profiles of supernatant [^{32}P]RNA following chase incubations. RNA was isolated from aliquots of the postnuclear supernatant fractions from the chase incubations described in B, loaded on sodium dodecyl sulfate–sucrose gradients, and centrifuged for 20 h. Symbols used in this panel are the same as in C.

Although there was overlap in size, most of the poly(A)-containing nuclear RNA sedimented faster than the bulk of the poly(A)-containing supernatant RNA.

Rate of Appearance of Supernatant RNA following Pulse Labeling of Isolated Nuclei. Pulse-chase experiments were performed to examine the time course of appearance of supernatant RNA and the fate of nuclear RNA. These experiments with isolated nuclei were performed with actinomycin D (3 μg/ml) present during the pulse labeling period in order to inhibit by about 75% polymerase I directed RNA synthesis. As shown in Figure 10C, cell-free nuclear RNA synthesized during the 5-min pulse incubation was heterogeneous in size sedimenting from 4 S to greater than 45 S in sodium dodecyl sulfate–sucrose gradients. The synthesis of a large fraction of this RNA was inhibited by α-amanitin (100 μg/ml). The sedimentation profile of the cell-free nuclear RNA resembled that of nuclear RNA isolated from intact GH_3 cells pulse labeled with [^{3}H]uridine for 5 min (Figure 10A). Using a variety of RNA extraction procedures (see Experimental Procedure) we consistently observed low molecular weight (4–12 S) RNAs in nuclei following brief cell-free incubations as shown in Figure 10C. These RNAs were absent in nuclei incubated for longer periods (e.g., 60 min, Figure 5A). Most of the 4–12S nuclear RNA did not contain poly(A) as was shown in Figure 9. The synthesis of about 50% of the 4–12S nuclear RNA was inhibited by 100 μg/ml α-amanitin, but not by 0.5 μg/ml (not shown), indicating that 50% of this RNA was the product of RNA polymerase III. The remaining 50% may represent partially completed transcripts directed by RNA polymerase II, although we cannot rule out the possibility of degradation of larger RNA polymerase II products.

As shown in Figure 10B, with increasing periods of chase incubation following the 5-min pulse labeling, radioactivity associated with nuclei decreased (left) and there was a concomitant appearance of radioactivity in the supernatant fraction (right). Note the tenfold difference in ordinate scales between the nuclear (left) and supernatant (right) sides in

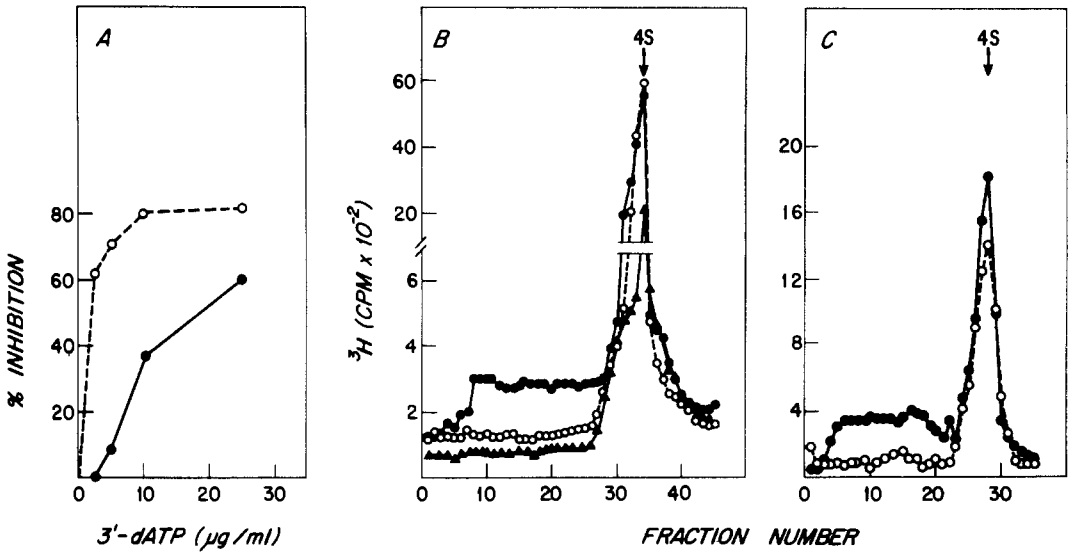

FIGURE 11: (A) Effects of cordycepin triphosphate (3′-dATP) on RNA synthesis in the cell-free system. Reaction mixtures (100 μl) containing 5 × 10^6 nuclei, [^{3}H]ATP (0.1 mM), and the indicated concentrations of 3′-dATP were incubated for 60 min at 29 °C. RNA was extracted from the reaction mixture and Cl_3CCOOH-insoluble radioactivity was measured in an aliquot (●-●). Another aliquot was subjected to pancreatic and T1 RNase treatment followed by determination of Cl_3CCOOH-insoluble radioactivity (○- - -○). (B) Sodium dodecyl sulfate-sucrose gradient sedimentation of postnuclear supernatant RNA synthesized in the presence of 5 μg/ml 3′-dATP (○- - -○), 5 μg/ml α-amanitin (▲-▲), or in the absence of either drug (●-●). Incubation was for 60 min at 29 °C with [^{3}H]GTP. (C) Sodium dodecyl sulfate-sucrose gradient sedimentation of supernatant RNA synthesized in the presence of [^{3}H]GTP for 10 min and chased for 45 min in the presence or absence of 3′-dATP (5 μg/ml). Following the 10-min pulse incubation with [^{3}H]GTP in the absence of 3′-dATP, nuclei were washed and reincubated in an unlabeled reaction mixture as described in the legend of Figure 9B. Supernatant RNA from a chase incubation performed in the absence (●-●) and presence (○- - -○) of 5 μg/ml 3′-dATP is shown.

Figure 10B. It is apparent that label associated with nuclear RNA during the pulse was not conserved during the subsequent chase. As shown in Figure 10C, the loss of radioactivity from nuclear RNA during the chase occurs for all size classes of nuclear RNA. However, as shown in Figure 10D, low molecular weight 4–5S RNAs appeared in the supernatant fraction more rapidly than did the heterogeneous 5 to 28S RNA; most of the 4–5S RNA emerged in a 5-min chase period in contrast to the heterogeneous 5 to 28S RNA which emerged gradually throughout the 45-min chase period. The rapid exit of 4–5S RNA from nuclei can also be seen by a comparison of Figure 10D with Figure 5D. The relative amount of supernatant RNA in the 4–5S peak is reduced in a pulse-chase experiment as compared with a continuous labeling experiment. This difference is accounted for by the fact that the postnuclear supernatant fraction from a 5-min pulse incubation (which is routinely discarded as the first wash) contains nine times the amount of 4–5S RNA than is found in the supernatant of the subsequent 45-min chase (Figure 10D).

Effect of Cordycepin Triphosphate on the Appearance of Supernatant RNA. Cordycepin (3′-deoxyadenosine) has been reported to inhibit preferentially addition of poly(A) to the 3′ terminus of heterogeneous nuclear RNA without affecting the synthesis of HnRNA in intact cells (Mendecki et al., 1972; Darnell et al., 1971). Since we had demonstrated that de novo poly(A) synthesis occurred in isolated GH_3 nuclei, we examined the effect of cordycepin triphosphate in this system. The phosphorylation of cordycepin to 3′-dATP by intact cells has been shown (Klenow, 1963), and it is presumed that this is the active form of the drug (Maale et al., 1975). As shown in Figure 11A, 3′-dATP inhibited preferentially the incorporation of [^{3}H]ATP into RNase-resistant Cl_3CCOOH-insoluble radioactivity in comparison with incorporation of [^{3}H]ATP into total Cl_3CCOOH-insoluble material. At 2.5 μg/ml 3′-dATP, poly(A) synthesis was inhibited by 62% without affecting total RNA synthesis. These results appear to conflict with those of Maale et al. (1975) who reported nonselective inhibition by 3′-dATP of isolated DNA-dependent RNA polymerase as well as of ATP-polynucleotidyl exotransferase. However, direct evidence that the ATP-polynucleotidyl exotransferase actually catalyzed 3′-end addition of AMP to HnRNA in vivo is lacking.

Cordycepin has also been reported to inhibit the appearance of pulse-labeled RNA in HeLa cell polyribosomes (Adesnik et al., 1972; Penman et al., 1970). The effect of 3′-dATP on the appearance of supernatant RNAs was examined in the GH_3 cell-free system. In order to inhibit substantially poly(A) synthesis without affecting markedly total RNA synthesis, 5 μg/ml 3′-dATP was used. As shown in Figure 11B, this concentration of 3′-dATP inhibited the appearance of the heterogeneous 5–28S supernatant RNA without affecting the appearance of 4–5S RNA. In order to eliminate a possible effect of 3′-dATP on RNA synthesis (as opposed to poly(A) synthesis), the appearance in the supernatant of RNAs synthesized in the absence of 3′-dATP was examined during a chase incubation in the presence of the drug. As shown in Figure 11C, the appearance of the heterogeneous 5–28S RNA in the supernatant fraction was again preferentially inhibited.

Discussion

The results presented in this report demonstrate that RNA synthesis in isolated nuclei from GH_3 cells continues for at least 120 min in the presence of rat liver ribonuclease inhibitor. The dependence of synthesis on RI suggests that the early termination of nucleoside triphosphate incorporation into RNA in the absence of RI is due to ribonuclease activity associated with the nuclei. The commonly observed short-lived nature of transcription in other cell-free nuclear systems may also result in part from such nuclease activity. An extended time course of transcription in a cell-free system is probably a necessary prerequisite for the de novo synthesis of complete RNA molecules, since the synthesis time for the largest RNAs (e.g.,

HnRNA of 50 000 nucleotides) may be as long as 10 min at 37 °C in intact cells (Derman and Darnell, 1974).

The results presented also demonstrate that RNA polymerases I, II, and III are functioning in isolated GH_3 nuclei incubated in TKM buffer. This buffer was chosen because it can support cell-free protein synthesis in lysates of GH_3 cells. RNA polymerase I directed products synthesized in the cell-free system remained associated with the nuclei (Figure 5A) and did not appear in the postnuclear supernatant fraction during a 60-min incubation at 29 °C (Figure 5B). In contrast, cellular rRNAs appeared in the cytoplasm of GH_3 cells during a 60-min incubation at 37 °C (Figure 5B). The nature of this difference has not been studied; however, it may be due to the presumed role of cytoplasmic factors in the nuclear processing and nuclear–cytoplasmic transport of rRNAs as ribosomal particles in intact cells (Maden, 1971). However, alternative explanations such as effects of temperature and ionic strength in the cell-free system cannot be excluded at the present time (Stevens and Amos, 1972).

RNAs synthesized by RNA polymerase III in this cell-free system sedimented as a prominent peak in the region of 4–5 S (Figures 5B and 5D). This RNA probably consists of pre-4S and 5S RNA species as has been reported for several nuclear transcription systems (McReynolds and Penman, 1974; Mauck and Green, 1974; Marzluff et al., 1974). RNA polymerase III products do not accumulate within the nuclei during a 60-min cell-free incubation (Figures 5A and 5C) and appear rapidly in the postnuclear supernatant fraction following synthesis (Figure 10D), a finding that resembles the immediate appearance of pre-4S and 5S RNA in the cytoplasm of intact cells (Weinberg, 1973). It is known that these RNAs do not undergo prolonged nuclear processing after transcription, and in fact cytoplasmic processing of 4.5S pre-tRNA to 4S tRNA has been shown (Marzluff et al., 1974; McReynolds and Penman, 1974).

Addition of poly(A) to HnRNA is believed to be a post-transcriptional event perhaps essential for the biogenesis of cellular mRNAs (Darnell et al., 1973). Poly(A) synthesis occurs primarily in the nucleus although cytoplasmic addition and removal of poly(A) have also been reported (Diez and Brawerman, 1974). Both nuclear and supernatant RNAs synthesized by isolated GH_3 nuclei contain poly(A). That these poly(A) tracts are associated with RNAs synthesized by RNA polymerase II is supported by the following findings. First, poly(A) tracts were associated with RNA molecules of high apparent molecular weight as shown by sedimentation in denaturing sucrose gradients (Figure 9). Secondly, concentrations of α-amanitin, which inhibited RNA polymerase II, inhibited the appearance of poly(A)-containing RNA in the supernatant fraction (Table I).

De novo synthesis of poly(A) by GH_3 nuclei has been demonstrated (Figure 8). The poly(A) tracts isolated were comparable in size (about 150 adenine residues) to those reported for eukaryotic mRNAs (Mendecki et al., 1972; Nakazoto et al., 1973). The appearance of newly synthesized poly(A) tracts in the supernatant fraction was prevented completely by α-amanitin (Figure 8B). Assuming the α-amanitin did not inhibit poly(A) synthesis itself, these results imply that at least some of the poly(A) associated with RNA polymerase II products was synthesized de novo.

Nucleoplasmic RNA polymerase II directs the synthesis of heterogeneous nuclear RNA (DNA-like RNA) and presumably also that of mRNA (Blatti et al., 1970). In the isolated GH_3 nuclear system, RNA polymerase II directed transcripts were present in both the nuclear pellet and postnuclear supernatant fractions. Both classes of RNA sedimented heterogeneously and contained poly(A). However, the appearance of the supernatant RNA was not merely a consequence of simple diffusion or leakage of nuclear RNA polymerase II products from damaged nuclei for several reasons. (1) There was a size difference between the nuclear and supernatant RNA. Much of the poly(A)-containing nuclear RNA sedimented faster than supernatant poly(A)-containing RNA under denaturing conditions (Figure 9). (2) There was enrichment for poly(A)-containing RNA in the supernatant heterogeneous RNA in comparison with nuclear heterogeneous RNA. Approximately 75% of the heterogeneous 5–28S supernatant RNA bound to oligo(dT)-cellulose. In contrast, nuclear RNA smaller than 18S did not contain poly(A) (Figure 9), and only about 30% of the high molecular weight (greater than 18S) nuclear RNAs contained poly(A) (not shown). (3) Only a small fraction of the radioactivity of nuclear heterogeneous RNA emerges in the supernatant in a pulse-chase experiment (Figure 10D). This finding of nonconservation was reproduced in five experiments and resembles that observed for HnRNA and mRNA in intact cells. (4) There was selective blockade by 3′-dATP of the emergence from nuclei of RNA polymerase II but not RNA polymerase III directed products (Figures 11B and 11C). (5) RNAs synthesized by RNA polymerase III emerged from nuclei substantially faster than RNA polymerase II directed products in a pulse-chase experiment (Figure 10D). The different nuclear retention times for the different classes of RNA were not due to a nonspecific mechanism based on the size of the molecules, for 18S and 28S RNA polymerase I directed products were retained in the nuclear pellet (Figure 5A) while RNA polymerase II directed transcripts as large as 18 and 28 S were found in the supernatant fraction. These findings, collectively, would not be expected if the appearance of RNAs in the supernatant were solely the result of nonspecific nuclear permeability; they are consistent with the occurrence in this cell-free system of a process resembling nuclear–cytoplasmic transport of RNA in intact cells.

The 5–28S supernatant RNA synthesized in the GH_3 nuclear system resembles cellular messenger RNA in several ways. (1) It contains poly(A) tracts comparable in size to those of GH_3 cytoplasmic RNA (Figure 8) and similar to those observed for a number of mRNAs from other cells (Mendecki et al., 1972; Nakazoto et al., 1973). (2) Cordycepin, a drug believed to inhibit the cytoplasmic appearance of mRNA (Adesnik et al., 1972; Penman et al., 1970), when used as the phosphorylated derivative, inhibited the extranuclear appearance of this class of supernatant RNAs (Figures 11B and 11C). (3) This RNA sedimented heterogeneously over the same broad range as poly(A)-containing polyribosomal RNA from intact cells (Figure 6). By these criteria, we infer that synthesis of mRNA-like RNA is occurring in this cell-free system. In order to confirm this conclusion, however, additional criteria are essential. These, of course, include the translation of this RNA into specific GH_3 cell proteins.

In summary, we conclude that several nuclear functions are preserved in nuclei isolated from GH_3 cells. These cells are a clonal strain of rat pituitary cells that synthesize and secrete two protein hormones, prolactin and growth hormone. Hormone synthesis by these cells can be modulated by hydrocortisone, estradiol (Tashjian and Hoyt, 1972), and the hypothalamic tripeptide thyrotropin-releasing hormone (Martin and Tashjian, 1976). The application of recently developed assay procedures for specific mRNAs (Roberts and Paterson, 1973; Sullivan et al., 1973) to this cell-free nuclear system

should permit an analysis of nuclear events involved in the regulation of prolactin and growth hormone biosynthesis.

Acknowledgment

The authors thank Miss Lenore Rothman for assistance, Dr. J. Scott, Massachusetts General Hospital, for help in the analysis of poly(A) content of the supernatant RNA, Dr. R. J. Suhdolnik, Temple University, for cordycepin triphosphate, Dr. R. G. Sommer, Miles Research Laboratories, for poly(A) samples of specific chain length, and Dr. Iolanda Low for examining GH_3 cells for the presence of Mycoplasma.

References

Adesnik, M., Salditt, M., Thomas, W., and Darnell, J. E. (1972), *J. Mol. Biol. 71,* 21.

Aviv, H., and Leder, P. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 1408.

Biswas, D. K., and Tashjian, A. H., Jr. (1974), *Biochem. Biophys. Res. Commun. 60,* 241.

Blatti, S. P., Ingles, C. J., Lindell, T. J., Morris, P. W., Weaver, R. F., Weinberg, F., and Rutter, W. J. (1970), *Cold Spring Harbor Symp. Quant. Biol. 35,* 649.

Brunner, M., and Raskas, H. J. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 3101.

Chatterjee, N. K., and Weissbach, H. (1973), *Arch. Biochem. Biophys. 157,* 160.

Darnell, J. E., Jelineck, W. R., and Molloy, G. R. (1973), *Science 181,* 1215.

Darnell, J. E., Philipson, L., Wall, R., and Adesnik, M. (1971), *Science 174,* 507.

Derman, E., and Darnell, J. E. (1974), *Cell 3,* 255.

Diez, J., and Brawerman, G. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 4091.

Eisenberg, H., and Felsenfeld, G. (1967), *J. Mol. Biol. 30,* 17.

Ernest, M. J., Schutz, A., and Feigelson, P. (1976), *Biochemistry 15,* 824.

Greenberg, J. R. (1975), *J. Cell Biol. 64,* 269.

Gribnau, A. A. M., Schoenmakers, J. G. G., and Blomendal, H. (1969), *Arch. Biochem. Biophys. 130,* 48.

Jacobson, A., Firtel, R. A., and Lodish, H. F. (1974), *J. Mol. Biol. 82,* 213.

Jelinek, W. R. (1974), *Cell 2,* 197.

Klenow, H. (1963), *Biochim. Biophys. Acta 76,* 347.

Lee, S. Y., Mendecki, J., and Brawerman, G. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 1331.

Maale, G., Stein, G., and Mans, R. (1975), *Nature (London) 255,* 80.

Maden, B. E. H. (1971), *Prog. Biophys. Mol. Biol.,* 129.

Martin, T. F. J., Biswas, D. K., and Tashjian, A. H., Jr. (1975), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 34,* 629.

Martin, T. F. J., and Tashjian, A. H., Jr. (1976), in Biochemical Actions of Hormones, Litwack, G., Ed., New York, N.Y., Academic Press (in press).

Marzluff, W. F., Jr., Murphy, E. C., Jr., and Huang, R. C. C. (1973), *Biochemistry 12,* 3440.

Marzluff, W. F., Jr., Murphy, E. C., Jr., and Huang, R. C. C. (1974), *Biochemistry 13,* 3689.

Mauck, J. C., and Green, H. (1974), *Cell 3,* 171.

McReynolds, L., and Penman, S. (1974), *Cell 1,* 139.

Mendecki, J., Lee, S. Y., and Brawerman, G. (1972), *Biochemistry 11,* 637.

Nakazoto, H., Kopp, D. W., and Edmonds, M. (1973), *J. Biol. Chem. 248,* 1472.

Penman, S. (1969), *Fundam. Tech. Virol.,* 35.

Penman, S., Fan, H., Perlman, S., Rosbash, M., Weinberg, R., and Zylber, E. (1970), *Cold Spring Harbor Symp. Quant. Biol. 35,* 561.

Penman, S., Vesco, C., and Penman, M. (1968), *J. Mol. Biol. 34,* 49.

Perry, R. P., Greenberg, J. R., and Tartof, K. D. (1970), *Cold Spring Harbor Symp. Quant. Biol. 35,* 577.

Reeder, R. H., and Roeder, R. G. (1972), *J. Mol. Biol. 67,* 433.

Roberts, B. E., and Paterson, B. M. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 2330.

Scherrer, K. (1969), *Fundam. Tech. Virol.,* 413.

Schimke, R. T., Palacios, R., Sullivan, D., Kiely, M. L., Gonzales, C., and Taylor, J. M. (1974), *Methods Enzymol.,* 631.

Schumm, D. E., Morris, H. P., and Webb, T. E. (1973), *Cancer Res. 33,* 1821.

Schutz, G., Beato, M., and Feigelson, P. (1972), *Biochem. Biophys. Res. Commun. 49,* 680.

Shutt, R. H., and Kedes, H. (1974), *Cell 3,* 283.

Stevens, R. H., and Amos, H. (1972), *J. Cell Biol. 54,* 1.

Strauss, J. H., Jr., Kelly, R. B., and Sinsheimer, R. L. (1968), *Biopolymers 6,* 793.

Sullivan, D., Palacios, R., Stavnezer, J., Tylor, J. M., Faras, A. J., Kiely, M. L., Summers, M. N., Bishop, M. J., and Schimke, R. T. (1973), *J. Biol. Chem. 248,* 7530.

Tashjian, A. H., Jr., and Hoyt, R. F., Jr. (1972), in Molecular Genetics and Developmental Biology, Sussman, M., Ed., Englewood-Cliffs, N.J., Prentice-Hall, p 353.

Tashjian, A. H., Jr., Yasumura, Y., Levine, L., Sato, G. H., and Parker, M. L. (1968), *Endocrinology 82,* 342.

Tsai, R. L., and Green, H. (1973), *Nature (London), New Biol. 243,* 168.

Weinberg, R. A. (1973), *Annu. Rev. Biochem. 73,* 329.

Weinmann, R., and Roeder, R. G. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 1790.

Widnell, C. C., and Tata, J. R. (1966), *Biochim. Biophys. Acta 123,* 478.

Wu, G-J., and Zubay, G. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 1803.

Zylber, E. A., and Penman, S. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 2861.