Volume 4 Number 6 June 1977 Nucleic Acids Research

Synthesis of messenger RNA-like molecules in isolated myeloma nuclei

Yves Y. Mory and Malcolm L. Gefter

Massachusetts Institute of Technology, Cambridge, MA 02139, USA

Received 21 January 1977

ABSTRACT

Nuclei isolated from mouse myeloma cells grown in tissue culture are capable of synthesizing RNA for prolonged periods of time. Addition of cytoplasmic extracts to the system stimulates slightly the rate and prolongs the time of synthesis.

As judged by sedimentation in SDS and in formamide gradients, the size of the RNA synthesized is heterogeneous from smaller than 10S to larger than 45S, thus resembling in vivo made RNA.

The characteristics of some of the RNA are in keeping with those expected to be for mRNA. Fifty percent of the RNA synthesis is sensitive to α-amanitin. After an incubation of two hours in the absence of α-amanitin about 10 percent of the newly synthesized RNA is found outside of the nuclei; it sediments with a broad distribution at 18S. A considerable fraction of the RNA that is released from nuclei in vitro can promote the formation of polyribosomes, and contains molecules that are polyadenylated and "capped".

INTRODUCTION

The understanding of transcription and maturation of RNA molecules in eukaryotic cells requires the availability of cell free systems in which RNA synthesis is faithfully performed.

Nuclei isolated from different cell lines have been observed to synthesize RNA. Nuclei isolated from HeLa cells are capable of synthesizing 45S ribosomal precursor RNA (1). Ribosomal genes are also faithfully transcribed in nuclei isolated from Xenopus laevis tissue culture cells (2). Nuclei isolated from adenovirus infected cells synthesize adenovirus specific RNA (3, 4, 5).

Jacobson et al. (6) have shown that nuclei isolated from the slime mold Dictyostelium discoideum can synthesize RNAs with properties almost identical to the cellular precursors to messenger and ribosomal RNAs.

Most of the eukaryotic messenger RNAs and some heterogeneous nuclear RNAs contain polyadenylic acid sequences (7, 8, 9).

Polyadenylic acid sequences are found also in RNA synthesized in vitro in nuclei isolated from mouse brain tissue (10) and from mouse myeloma cells

© Information Retrieval Limited 1 Falconberg Court London W1V 5FG England

(line 66-2) (11). Nuclei isolated from mouse myeloma lines are capable of synthesizing RNA for a long period of time and all molecular weight classes of RNA are synthesized.

In this article we report that heterogeneous RNA, comprising all molecular weight classes of RNA, is synthesized in nuclei prepared from the MOPC 315 myeloma cell line. In addition, a portion of the RNA synthesized in nuclei *in vitro*, is released from the nuclei and has some of the properties expected for mRNA.

MATERIALS AND METHODS

Materials. [^{3}H]GTP (8.1 Ci/mmole); [α-^{32}P]GTP (135 Ci/mmole); [^{3}H]uridine (45 Ci/mmole) and [^{3}H]thymidine (56 Ci/mmole) were purchased from New England Nuclear Corp.; ATP, CTP, GTP, UTP, amino acids, yeast tRNA and nucleotide pyrophosphatase from Sigma; creatine phosphate, creatine phosphokinase and ribonucleases T_1 and T_2 from Calbiochem; oligo (dT)-cellulose from Collaborative Research and α-amanitin from Boehringer Mannheim Corporation.

Cell Culture. Myeloma MOPC 315 cells, obtained from Dr. H. N. Eisen from this institution, were grown in suspension at 37°C in L-15 (Leibowitz) medium (GIBCO) supplemented with 0.58 mg/ml glutamine, 5 units/ml penicillin, 5 mg/ml streptomicin and 20% fetal calf serum.

Isolation of Nuclei. Nuclei were isolated by a slight modification of the procedure described by Marzluff et al. (11).

All procedures were carried out at 0°-4°C unless otherwise specified.

Cells (8-12 x 10^5/ml) were collected by sedimentation at 60g for 10 minutes and washed three times in 30 mM Tris HCl pH 7.5, 120 mM KCl, 5 mM Mg acetate, 7 mM 2-mercaptoethanol.

The packed cells were resuspended in 5 volumes of 0.3 M sucrose containing 2 mM Mg acetate, 3 mM $CaCl_2$, 10 mM Tris HCl pH 7.5, 0.1% Triton X-100 and 0.5 mM 2-mercaptoethanol, and homogenized in a B type Dounce homogenizer. The homogenate was mixed with an equal volume of 2M sucrose containing 5 mM Mg acetate, 10 mM Tris HCl pH 7.5 and 0.5 mM 2-mercaptoethanol. The mixture was layered over 2 ml of 2 M sucrose buffer and centrifuged for 45 minutes at 20,000 rpm and at 5°C in the Beckman SW50.1 rotor. The nuclei were resuspended in 25% glycerol containing 5 mM Mg acetate, 0.1 mM EDTA[1], 5 mM 2-mercaptoethanol and 50 mM Hepes NaOH pH 7.5 at a concentration of 1-2 x 10^8 nuclei/ml, and stored at -80°C.

Preparation of Cytoplasmic Extract. Preincubated extracts were prepared from myeloma cells according to the procedure described for Krebs II

ascites (12).

All steps were carried out at 0-4°C unless otherwise indicated.

Cells were collected by sedimentation at 60 g for 10 minutes and washed three times in 30 mM Tris HCl pH 7.5, 120 mM KCl, 5 mM Mg acetate, 7 mM 2-mercaptoethanol.

The packed cells were resuspended in two volumes of 15 mM KCl, 10 mM Hepes NaOH pH 7.5, 1.6 mM Mg acetate, 6 mM 2-mercaptoethanol, and kept on ice for 20 minutes. The cells were broken with 30 strokes in a B type Dounce homogenizer. 0.1 volume of concentrated buffer (200 mM Hepes NaOH pH 7.5, 1,200 mM KCl, 50 mM Mg acetate, 60 mM 2-mercaptoethanol) was added and the homogenate was centrifuged at 30,000 g for 20 minutes. After addition of ATP to a final concentration of 1 mM, GTP to 0.2 mM creatine phosphate to 8 mM and creatine phosphokinase to 0.2 mg/ml the supernatant was incubated for 30 minutes at 37°C. The extract was then passed through a Sephadex G-25 column (2.5 x 30 cm) which had been equilibrated with 20 mM Hepes NaOH pH 7.5, 120 mM KCl, 1.5 mM Mg acetate, 6 mM 2-mercaptoethanol. The column was eluted with the same buffer and the fractions containing the protein peak were pooled and frozen in small aliquots at -80°C.

Conditions for RNA synthesis. Nuclei (1-2 x 10^7 nuclei/ml) were incubated with or without cytoplasmic extract (1 mg of protein/ml) in 0.1 ml reaction mixtures having the following composition: 7.5% (w/v) glycerol; 19 mM Hepes NaOH pH 7.5; 3.8 mM Mg acetate; 84 mM KCl; 30 µM EDTA; 3 mM 2-mercapto-ethanol; 10 mM NH_4Cl; 130 µg/ml creatine phosphokinase; 10 mM creatine phosphate; 1 mM ATP; 250 µM CTP; 250 µM UTP; 50 µM GTP; 40 µM each of the 20 amino acids; 0.5 mM S-adenosyl-L-methoinine and [^{3}H] GTP or [^{32}P] GTP as indicated. Reaction mixtures were incubated at 25°C for the indicated times. At the end of the incubation the nuclei were pelleted by centrifugation for 10 minutes at 1,000 g and at 5°C. When only radioactivity had to be measured, the supernatant and the resuspended pellet were separately precipitated with 5% trichloroacetic acid containing 0.5% sodium pyrophosphate and the precipitate was collected on GF/C filters (Whatman). The filters were washed with 5% trichloroacetic acid and ethanol and dried. Radioactivity was measured by liquid scintillation.

When tritium had to be measured the filters were incubated overnight at 37°C - 45°C with 0.4 ml of NCS (Amersham) and 3 ml of toluene scintillation liquid. After addition of 3 ml of scintillation liquid and cooling the radioactivity was measured.

RNA extraction. RNA was extracted from nuclei and from supernatants by a

modification of the procedure described by Glišin et al. (13). The sample from which RNA had to be extracted was made up to 1% SDS, 10 mM EDTA and 50 mM Na acetate pH 5.1. The mixture was incubated at 37°C for 1 hour with occasional stirring. Thereafter, CsCl was added and the RNA was sedimented as described. Ribosomal RNA markers were prepared from whole cells which had been grown for 24-48 hours with [^{3}H]uridine. Diethyl pyrocarbonate was added to all the solutions as an inhibitor of RNAases at a concentration of 0.4% (v/v).

Sedimentation in Formamide Sucrose Gradients. The RNA prepared from nuclei or from supernatants was analyzed by velocity sedimentation under denaturing conditions through sucrose gradients in formamide. The RNA samples containing 0.5% sarkosyl, 3 mM EDTA, 3 mM Tris HCl pH 7.5 and 70% formamide were incubated at 37°C for 15 minutes. The samples (0.2ml) were layered over 4.4 ml linear sucrose gradients (5-20%) formed over a 0.6 ml cushion of 40% sucrose. The gradients contained 70% formamide, 3 mM EDTA, 3 mM Tris HCl pH 7.5 and 0.5% sarkosyl and were centrifuged for 15 hours at 43,000 and at 5°C in the Beckman SW50.1 rotor. After centrifugation fractions were collected from the bottom of the centrifuge tube and radioactivity was directly measured in a dioxane scintillation liquid.

Oligo (dT)-cellulose Chromatography. The RNA synthesized *in vitro* by isolated nuclei and purified as previously described was analyzed for poly(A) content by chromatography on an oligo (dT)-cellulose column. The sample in high ionic strength buffer (400 mM NaCl, 10 mM Tris HCl pH 7.5, 0.5% SDS and 1A_{260} unit/ml of yeast tRNA) (14), was passed 8 times through a 1 ml oligo (dT)-cellulose column which had been equilibrated with the same buffer. The column was washed with the high ionic strength buffer. The buffer was then changed to a low ionic strength buffer (10 mM Tris HCl pH 7.5, 0.05% SDS, 1A_{260} unit/ml of yeast tRNA). The buffers contained diethyl pyrocarbonate (0.4%, v/v). This substance, however, did not affect the quantitative results obtained with this method.

Polyribosome analysis. Cell free lysates prepared from rabbit reticulocyte were according to Villa-Kamaroff et al. (12). These were kindly supplied by Dr. H. F. Lodish of MIT. Details of the incubation conditions are given in the legend to Figure 7.

RESULTS

Conditions for RNA Synthesis. Myeloma nuclei were incubated under conditions that in other systems would also allow protein synthesis (15). The concentration of the salts, triphosphates, etc. were adjusted to give the maximal

rate of [^{3}H]GTP incorporation.

Under these conditions RNA synthesis is linear for at least 60 minutes; however, most preparations support synthesis at a linear rate for up to 3 hours of incubation (Figure 1). The rate of GTP incorporation is directly proportional to the number of nuclei added to the incubation mixture ($0.1 - 3.0 \times 10^7$).

When nuclei are incubated in the presence of cytoplasmic extract there is a two-fold enhancement in the rate of RNA synthesis as compared to the synthesis observed in the absence of cytoplasmic extract. Figure 2 shows also that about 10 to 15% of the RNA synthesized can be found outside of the nuclei. Such RNA is defined as that which does not sediment with the nuclei at 1000 g following termination of the reaction. The fractional release of RNA from the nuclei is not altered by the presence of cytoplasm.

Inhibition of RNA Synthesis by α-amanitin. Lindell et al. (16) have reported that α-amanitin inhibits the action of the RNA polymerase II enzyme. The drug does not affect nucleolar RNA synthesis in nuclei isolated from HeLa cells but it inhibits 50 percent of the nucleoplasmic activity when assayed at low ionic strength and 95 percent of it at high ionic strength (1). This drug therefore can be used in order to easily differentiate between ribosomal and nonribosomal RNA synthesis.

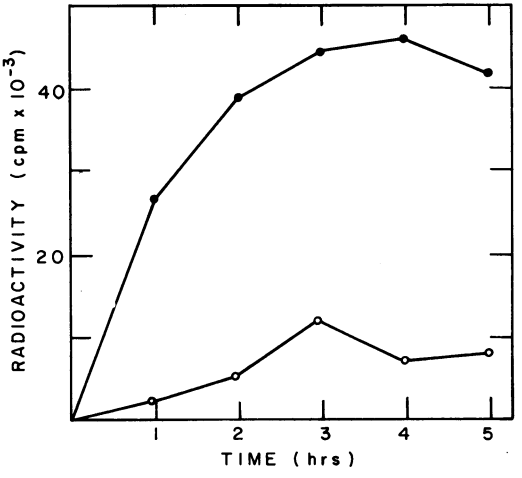

Figure 1. Kinetics of Synthesis and Release of RNA from Isolated Nuclei. Nuclei (2×10^6/reaction mixture) were incubated in standard reaction mixture containing 2 μCi [^{3}H] GTP and preincubated cytoplasmic extract. At the indicated times the reaction mixtures were divided into nuclear pellet and supernatant and each fraction was precipitated with TCA. The precipitates were collected on GF/C filters and radioactivity was measured.
●---● nuclear RNA 0---0 extranuclear RNA

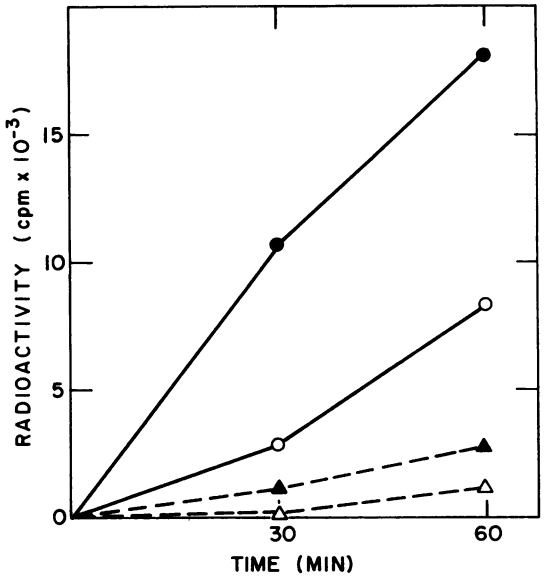

Figure 2. Effect of Cytoplasmic Extract on the Synthesis and Release of RNA from Isolated Nuclei. Nuclei (10^6/reaction mixture) were incubated in standard reaction mixtures containing 2 μCi of [^{3}H] GTP, with and without cytoplasmic extract. At the indicated times the reaction mixtures were divided into nuclear pellet and supernatant and each fraction was precipitated with TCA. The precipitates were collected on GF/C filters and radioactivity was measured. ●---● RNA synthesized in nuclei incubated with cytoplasm
0---0 RNA synthesized in nuclei incubated without cytoplasm
▲---▲ RNA released from nuclei incubated with cytoplasm
Δ---Δ RNA released from nuclei incubations without cytoplasm

RNA synthesis in nuclei isolated from mouse myeloma cells is partially inhibited by α-amanitin (11). Our experiments confirm these results. Sixty percent inhibition of total RNA synthesis is observed at 0.3 μg/ml. No further inhibition is seen on raising the α-amanitin concentration 5-fold. The incorporation of [^{3}H]GTP into the RNA that remains in the nuclei as well as incorporation into the RNA that is found outside the nuclei are equally inhibited by α-amanitin (0.3 μg/ml.) We conclude, therefore, that in the system we described at least 60 percent of total RNA synthesis is not ribosomal RNA synthesis.

Size Analysis of the RNA Synthesized in Isolated Nuclei. As previously reported (11), nuclei can synthesize RNA of a wide range in molecular weight. Analysis of the RNA synthesized *in vitro* by sedimentation velocity in the presence of SDS confirms the report that all molecular weight classes of RNA (4-80S) are synthesized (data not shown).

In the experiment described in Figure 3 the RNA synthesized *in vitro* was subjected to sedimentation analysis under denaturing conditions in the

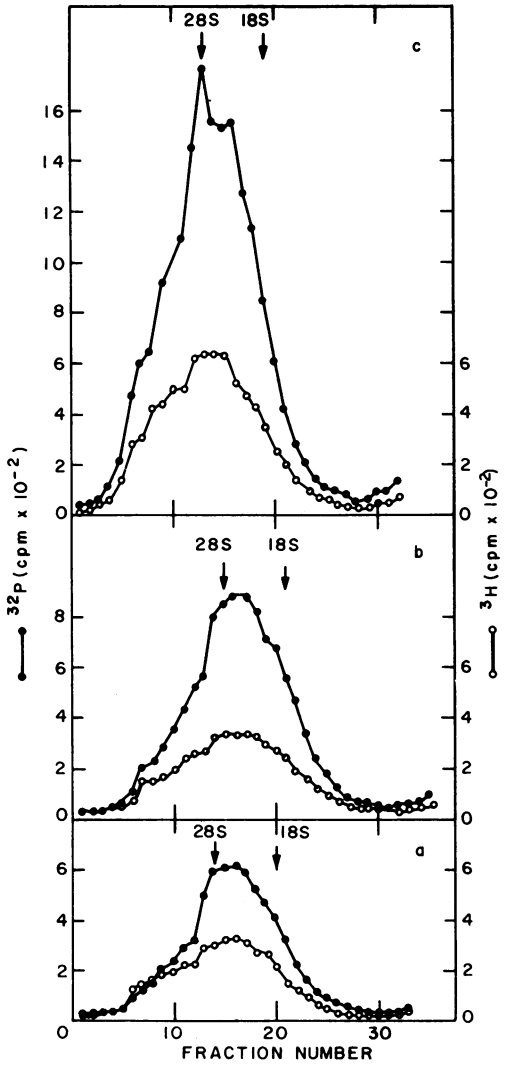

Figure 3. Sedimentation Analysis of RNA Synthesized in Isolated Nuclei. Cells were grown to a concentration of 10^6/ml in 300 ml of culture. The RNA was then pulsed-labelled with [^{3}H] uridine (7μCi/ml; 45 Ci/mmole) for 15 minutes. Nuclei were isolated and incubated for 30, 60 and 120 minutes in standard reaction mixtures including [α-^{32}P] GTP and cytoplasmic extract. At the indicated times the nuclei were pelleted and the RNA was extracted from the nuclear pellet as described under materials and methods. The RNA preparations were denatured and sedimented through 5-20% sucrose gradients prepared in 70% formamide.

Ribosomal RNA markers, isolated by the same procedure, were treated in the same way and sedimented in a parallel gradient.

a, b, and c show the pattern of RNA synthesized in isolated nuclei at 30, 60 and 120 minutes respectively.

●---● [^{32}P]RNA 0---0 [^{3}H]RNA

presence of 70% formamide. By this method of analysis the largest RNA molecules that can be found have a sedimentation coefficient of 40S. The difference between the results obtained by sedimentation analysis in SDS and the ones obtained in formamide is probably due to the tendency of heterogeneous RNA molecules to aggregate (17). Figure 3 shows that RNA molecules of all size classes are synthesized and that the sedimentation profile remains unchanged even after prolonged incubation. These results suggest that all size classes of RNA chains are elongated and are synthesized continuously. There is not a selective disappearance of molecules during incubation.

To investigate the possibility of RNA turnover during incubation the

cells were prelabelled with [^{3}H] uridine, the nuclei were then isolated and incubated under standard conditions for RNA synthesis. RNA synthesized *in vitro* was differentially labelled with [α-^{32}P] GTP. The sedimentation profile of the pre-labelled RNA (Figure 3, open circles) is the same as the one of the *in vitro* synthesized RNA. The relative stability of the pre-existing RNA indicates that there is not a vast amount of non-specific nuclease activity in the nuclear and cytoplasmic preparations.

The RNA that does not sediment with the nuclei has different sedimentation properties than RNA found in the nuclei. This extranuclear RNA (Figure 4) has a mean sedimentation rate of 18S and thus does not appear to arise simply by lysis of the nuclei *in vitro*. Furthermore, as seen in Figure 4, RNA labelled *in vivo* that does not sediment with nuclei after incubation *in vitro* also sediments at about 18S. These results suggest selectivity with respect to size, of the RNA that is released from the nuclei *in vitro*. These results were obtained by incubating the nuclei with cytoplasmic extract; the same qualitative results are obtained if the nuclei are incubated in the absence of cytoplasmic extract. The specific requirements for this selective release have not been investigated further.

The possibility was addressed that extranuclear RNA arises by lysis of nuclei followed by degradation of the released RNA. A monitor of nuclear

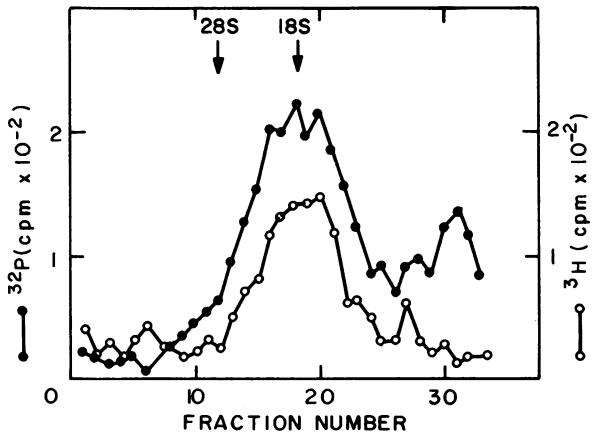

Figure 4. Sedimentation Analysis of RNA Released from Nuclei Incubated Under Conditions for RNA Synthesis. Cells were pre-labelled with [^{3}H] uridine as described under Figure 3 and nuclei were isolated and incubated for 60 minutes in standard reaction mixtures including [α-^{32}P] GTP and cytoplasmic extract in the reaction mixture. The nuclei were then removed and the RNA was extracted from the supernatant, denatured in 70% formamide and sedimented through a 5-20% sucrose gradient prepared in 70% formamide as described under Materials and Methods.

lysis was the release of DNA. Nuclei were prepared from cells grown for several generations in [^{3}H] thymidine. These nuclei were incubated under standard conditions for RNA synthesis. Although no time-dependent release of DNA could be observed, not more than 0.4% of the DNA could be recovered in the 1,000 x g supernatant even after one hour of incubation. In addition, there is no apparent lysis or clumping of nuclei during incubation as evidenced by phase contrast microscopy.

A monitor of non-specific degradation of free RNA in the cytoplasm was the stability of ribosomal RNA. A trace amount (3.5% of the weight of total endogenous RNA) of ^{14}C-labelled ribosomal RNA was added to a standard

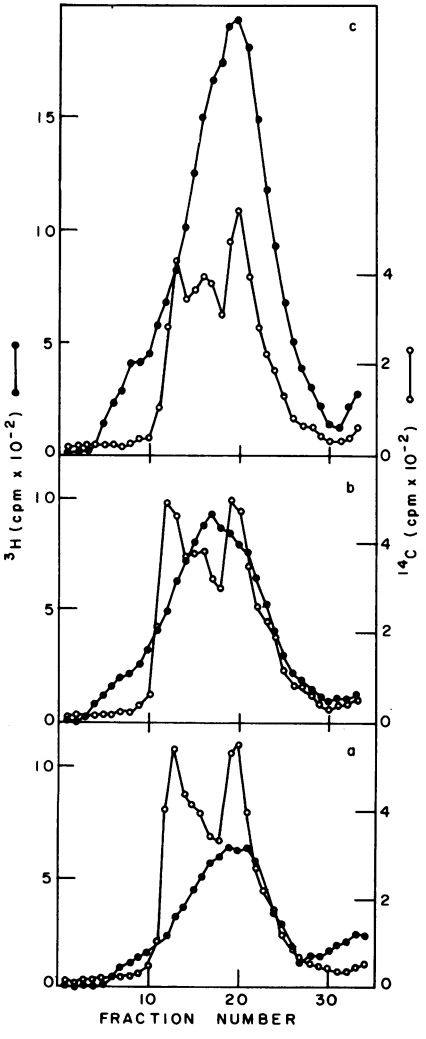

Figure 5. Analysis of Non-specific Ribonuclease Activity. Isolated nuclei were incubated with cytoplasmic extract in 0.1 ml standard reaction mixtures containing ^{14}C labelled ribosomal RNA (open circles) (16,000 cpm/reaction mixture, specific activity, 9,000 cpm/µg). After incubation the RNA was extracted from each reaction mixture and sedimented in 5-20% sucrose gradients in 70% formamide as described under Materials and Methods.

a, b, and c show the sedimentation pattern of the RNA after 15, 30 and 60 minutes of incubation. The RNA products synthesized are monitored by incorporation of [^{3}H] GTP (closed circles).

reaction mixture. Degradation was monitored by sucrose density gradient sedimentation analysis. From the profiles seen in Figure 5, only a slight amount of degradation of 28S RNA is seen even after one hour of incubation. Thus we can conclude that non-specific nucleases are not present in the system in sufficient amount such that they could convert the high molecular weight RNA to species sedimenting 15-18S in the times of incubations used. We cannot exclude the possibility of non-specific degradation of nuclear RNA followed by its selective release.

Association of in vitro Synthesized RNA with Ribosomal Particles. Messenger RNA has usually been identified employing the criterium that it is released from polysomes by EDTA treatment in the form of slowly sedimenting ribonucleoprotein particles (18) (19).

In order to further characterize the RNA synthesized and released from myeloma nuclei we attempted to see if this RNA, which has the sedimentation properties expected for mRNA after deproteinization (∿18S) could be found associated with polysomes. After incubation and removal of the nuclei from the cytoplasmic extract the reaction mixture was layered on a sucrose gradient under conditions that would preserve polyribosomes. The size of the molecules into which GTP has been incorporated was determined. After two hours of incubation (Figure 6) the radioactivity is separated into two species: one with sedimentation coefficient of about 15-18S and the second as material with sedimentation coefficient of 80S or higher. If the same preparation is first treated with EDTA and then sedimented in the same way as above, the 80S species cannot be observed and all the in vitro synthesized RNA is found in slower sedimenting material (40-70S). Thus a fraction of the RNA released from nuclei can be found to be associated with structures having the properties expected for monosomes or small polysomes.

As can also be seen in Figure 6, the association of in vitro synthesized RNA with ribosomes is a time-dependent phenomenon. Although by 30 minutes of incubation there is extranuclear RNA in measureable amounts, it is not associated with ribosomes. This also demonstrates that the association of RNA with ribosomes is not due to non-specific aggregation.

Further confirmation of the fact that RNA having the properties expected for mRNA was being synthesized in vitro was obtained using a protein synthesizing system from rabbit reticulocytes. This system has the advantage of being efficient in promoting polysome formation. In addition the specific conditions of polysome formations have been optimized as opposed to our in vitro nuclei system in which the conditions for RNA synthesis have been optimized.

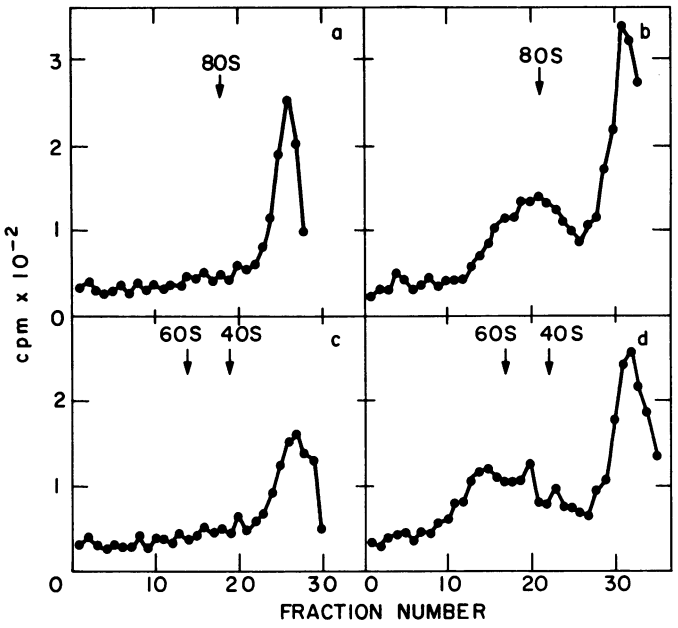

Figure 6. Sedimentation Analysis Under Non-denaturing Conditions of the RNA Synthesized and Released from Isolated Nuclei. Isolated nuclei (16 x 10^6 nuclei/reaction mixture) were incubated with cytoplasmic extract in 0.6 ml standard reaction mixtures containing 12 µCi [^{3}H] GTP.

After 30 and 120 minutes of incubation, the reaction mixtures were separated into nuclear pellet and supernatant.

In a and b 200 µl of supernatants prepared after 30 and 120 minutes of incubation respectively were directly layered on top of 4.4 ml sucrose linear gradients (7-47%) formed over a 0.6 ml cushion of 70% sucrose. The gradients contained 10 mM Tris HCl pH 7.5, 100 mM NaCl and 1.5 mM $MgCl_2$ and were centrifuged for 85 minutes at 45,000 rpm and at 4°C in the Beckman SW50.1 rotor. A polysomal marker was run in a parallel gradient.

In c and d 200 µl of the supernatants prepared after 30 and 120 minutes of incubation respectively were made 10 mM in EDTA and layered on 7-47% linear sucrose gradients prepared in the same buffer as in a and b but including 10 mM EDTA. The gradients were centrifuged for 120 minutes at 45,000 rpm and at 4°C in the Beckman SW50.1 rotor. A polysomal marker treated in the same way was run in a parallel gradient.

Fractions were collected from the bottom of the centrifuge tube on paper filters. The filters were washed with 5% TCA and ethanol, dried and the radioactivity content determined.

Extranuclear RNA was isolated following 30 minutes of incubation under standard conditions. The poly(A)-containing RNA (see below) was isolated and incubated in the reticulocyte system under conditions of protein synthesis. Although the isolated RNA sediments as the bulk extranuclear RNA does (15-18S), in the reticulocyte system it promotes the formation of rapidly sedimenting species; a portion of it sediments at 80S (monosomes) and a portion greater than 80S (polysomes) (Figure 7). The formation of "polysomes"

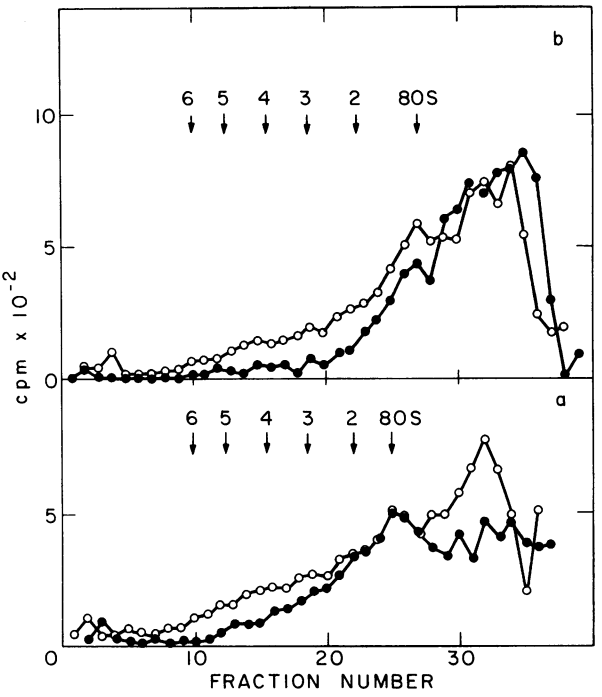

Figure 7. Formation of Polysomes in a Reticulocyte System. Nuclei were incubated for 30 minutes under standard conditions for RNA synthesis. Cytoplasmic extracts were included in the reaction mixture and [^{3}H]GTP was added as an RNA precursor.

After incubation, the nuclei were pelleted and the RNA was extracted from the supernatant as described under Materials and Methods. The RNA was dissolved in high ionic strength buffer and the poly(A) containing RNA was isolated. The poly(A)$^+$ RNA was washed four times by ethanol precipitation and resuspended in sterile H_2O.

This RNA (35,000 cpm reaction mixture) was incubated in reticulocyte cell-free reactions (230 µl) (12).

In (a) a standard reaction (0---0) and a reaction containing a specific inhibitor of polypeptide chain elongation (20) anisomycin (●---●) at a concentration of 1 mg/ml were incubated for four minutes at 30°C.

In (b) two standard reactions were incubated for 0, (●---●---●); and four minutes, (0---0---0).

After incubation the reaction mixtures were chilled and 1.2 ml of high salt buffer (0.5 m NaCl, 0.03 $Mg(OAC)_2$, 0.02 M Hepes pH 7.5) was added. The samples were layered on a 36 ml 15-30% linear sucrose gradient made up in high salt buffer and centrifuged in a Beckman SW 27 rotor at 4°C at 26,500 rpm for 3.5 hours. Samples were collected through a flow cell in a Gilford recording spectrophotometer directly into scintillation vials. The arrows represent optical density peaks of the reticulocyte polysomes. Each sample was bleached with alkaline hydrogen peroxide and counted in Aquasol 2 (New England Nuclear). Due to incomplete bleaching of hemoglobin, the counting of radioactivity in the upper part of each gradient is unreliable. The total radioactivity applied to each pair of gradients however is identical.

is time-dependent (Figure 7b) and reduced by anisomycin (Figure 7a), a specific inhibitor of polypeptide chain elongation (20).

These results demonstrate that at least some portion of the extranuclear RNA made in vitro has the functional characteristics expected for messenger RNA. Although the amount of RNA sedimenting in the polysome region is a small fraction of the total, its appearance is highly reproducible.

Polyadenylic Acid Content of the in vitro Synthesized RNA. Most of the eukaryotic messenger RNAs and some of the HnRNA contain poly(A) sequences at their 3' end (21) (7) (8) (9). Marzluff et al. (11) have shown that nuclei isolated from mouse myeloma cells are capable of synthesizing poly(A) sequences. Our experiments have confirmed these findings. The proportion of poly(A)-containing RNA is not altered by the presence of cytoplasmic extract. We analyzed the RNA released from the nuclei after an hour of incubation, by oligo dT-cellulose affinity chromatography. About fifty percent of this RNA binds to the column at high ionic strength and it is released as a sharp peak from it at low ionic strength. We, therefore, conclude that 50% of the in vitro synthesized RNA which is released from the nuclei contain poly(A) sequences (The amount of RNA binding to the oligo dT-cellulose column was determined by passing the RNA eight times over the column. 70% to 90% of the amount adhering was obtained on the first passage. Greater than 85% of the low salt-eluted material will re-adhere to the column on subsequent passage.).

The distribution of poly(A) with respect to size of the nuclear DNA molecules was determined. After incubation under standard conditions, nuclei were separated from the supernatant and the RNA was extracted. The RNA was separated into three fractions by sedimentation through 5-20% sucrose gradient in 70% formamide as in Figure 3: RNA sedimenting faster than 28S, RNA sedimenting between 28S and 18S and RNA sedimenting slower than 18S was pooled separately and the poly(A) content of each determined on an oligo (dT) celluslose column as described in Materials and Methods. The results of this experiment indicate that 77% of the RNA sedimenting faster than 28S, 40% of the RNA sedimenting between 28S and 18S and 58% of the RNA sedimenting slower than 18S contain polyadenlic acid sequences. Thus the poly(A) containing RNA in the nucleus is distributed over all size classes; this distribution is non-random. The extranuclear poly(A) containing RNA however, distributes itself with respect to size as the total extranuclear RNA does.

Isolation of in vitro Synthesized 5'-Terminal Structures. Messenger RNAs from many animal viruses and animal cells contain at the 5' end a structure

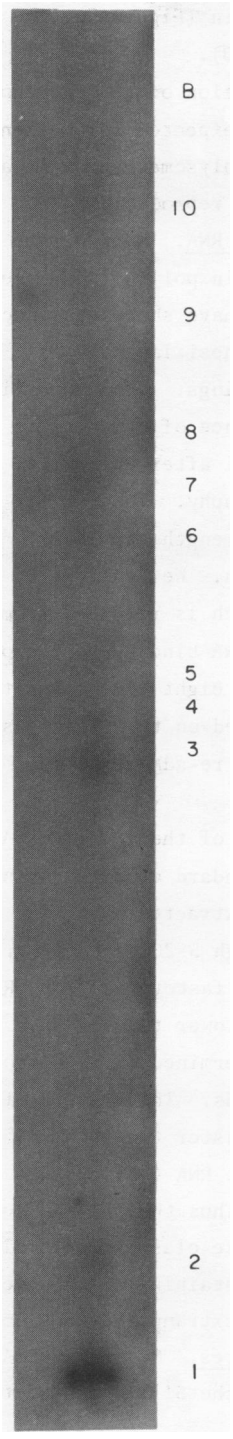

Figure 8. Electrophretic Mobility of T_2 Resistant Alkaline Phosphatase Resistant Oligonucleotides from in vitro Synthesized RNA. Isolated nuclei were incubated for thirty minutes in a 2 ml reaction mixture under standard conditions for RNA synthesis. Cytoplasmic extract was included in the reaction mixture and [α-^{32}P]GTP (1.5mCi; 130 Ci/mmol) was added as a precursor.

After incubation the RNA was extracted as described under Materials and Methods and washed four times by ethanol precipitation.

The washed RNA was dried under vacuum and dissolved into 200 µl of a mixture containing 600 units/ml ribonuclease T_1, 800 units/ml ribonuclease T_2, 0.24 mg/ml ribonuclease A, 40 mM ammonium acetate pH 5.5 and 20 mM EDTA (28). Incubation was for fifteen hours at 37°C. The T_2 digest was lysophilized and redissolved in 100 µl of a mixture containing 0.1 mg/ml alkaline phosphatase, 20 mM Tris HCl pH 7.5 and 2 mM $MgCl_2$. The reaction mixture was incubated for two hours at 37°C and the phosphorilated oligonucleotides were fractionated by electrophoresis on DEAE paper at pH 3.5 until the xylene cyanol marker dye (B) had migrated 40 cm.

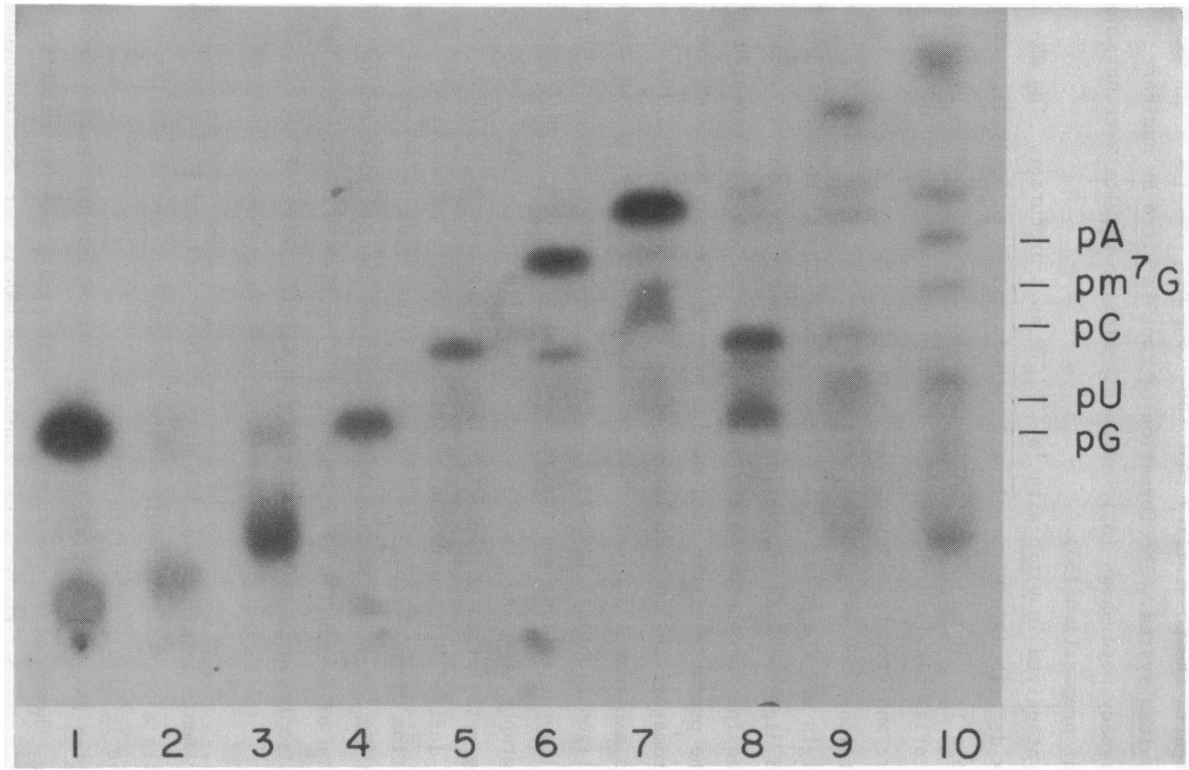

Figure 9. Identification of 7 methyguanosine monophosphate. The oligo- nucleotides (1 to 10) of the DEAE electrophoresis were eluted with 2M triethylamine bicarbonate pH 7 and digested with nucleotide pyrophosphatase in a 10 µl of a reaction containing 0.3 units/ml nucleotide pyrophosphatase, 20 mM Tris HCl pH 7.5, 20 mM $MgCl_2$ and 5 mM β-glycerophosphate. The digests were analyzed by chromatography on thin layers of cellulose using as solvent isobutyric acid /NH_3 /H_2O (66:1:33, V:V). Internal markers of pm^7G and the other mononucleotides were included in all samples and localized by ultraviolet light.

that is commonly called "cap" that can be generalized as $m^7G(5'ppp5')NmpN(m)p$ (22) (23) (24) (25) (26) (27) (28) (29). Structures of the same kind have been observed also in heterogeneous nuclear RNA (30) (31).

We investigated the possibility that the "capping" reaction could occur also in our in vitro system. The formation of caps in vitro by nuclear extracts has already been shown (32). Recently similar results were obtained in whole nuclear preparation (33).

RNA was synthesized in vitro with [α-^{32}P]GTP as a precursor. The RNA was isolated as described in Materials and Methods and extensively digested with ribonuclease T_2. The T_2 resistant oligonucleotides were dephosphorylated with bacterial alkaline phosphatase and the dephosphorylated oligonucleotides were analyzed by electrophoresis on DEAE paper at pH 3.5 (Figure 8). At least ten oligonucleotides could be identified which migrate between the origin and the xylen cyanol dye. Each of the oligonucleotides were eluted and digested with nucleotide pyrophosphatase in order to verify if 7 methylguanosine monophosphate could be released as would be expected for "caps". The products of the nucleotides pyrophosphatase reactions were analyzed by chromatography on thin layers of cellulose (Figure 9). The results of this experiment indicate that pm^7G is present in two of the ten T_2 resistant oligonucleotides (number 7 and 10). The presence of pm^7G in the T_2 resistant oligonucleotides was further verified by two dimensional thin layer chromotagraphy (data not shown). The other oligonucleotides may be 5' termini lacking methylation at the 7 position of the guanosine residue, or are internally-derived oligonucleotides containing ribose methylation.

The formation of pm^7G-containing "caps" in this system is dependent upon continued RNA synthesis in that "cap" formation can be inhibited by 70% by the inclusion of α-amanitin (1 µg/ml) in the reaction mixture. Similar results have also recently been obtained in an L cell system (33).

DISCUSSION

The experiments described in this article were designed to derive from eukaryotic cells a system that would synthesize RNA in vitro as similar as possible to the in vivo synthesized RNA species.

The in vitro RNA synthesizing system consists of isolated nuclei from myeloma cells. These nuclei are capable of synthesizing RNA for relatively long times. The size of the in vitro synthesized RNA is very heterogeneous with a distribution on sucrose gradients, that is almost identical to the in vivo HnRNA.

About ten percent of the in vitro synthesized RNA is found outside of

the nuclei after an incubation of two hours. Several properties of this RNA suggest but do not prove that it is selectively released from nuclei. It has been reported that the release of adenovirus specific RNA from isolated nuclei is an ATP-dependent reaction (34). We have not yet addressed the specific role of ATP in our system.

Several lines of evidence suggest that myeloma nuclei, when incubated under the conditions described here, synthesize RNA molecules with properties similar to mRNA.

(1) α-amanitin, an inhibitor of RNA polymerase II which is responsible for the synthesis of mRNA (1) causes an inhibition of about 50% of the rate of RNA synthesis in this system.

(2) About 50 percent of the in vitro synthesized RNA which is released from the nuclei contain poly(A) sequences.

(3) RNA released from nuclei contain 5' "caps". We have not yet determined the fraction of released RNA bearing 5' "caps", however. "Cap" structures have also been obtained from poly(A)-containing released RNA (data not shown).

(4) When nuclei are incubated with cytoplasmic extract some of the in vitro synthesized RNA is found associated with ribosomes. EDTA releases the RNA from the ribosomes in the slower sedimenting particles. This RNA is also capable of promoting polysome formation in a protein synthesizing system derived from reticulocytes.

RNA synthesis in isolated nuclei is compatible with the presence of cytoplasmic extracts. The conditions used for RNA synthesis are those described for the support of protein synthesis. The fact that cytoplasmic extracts have no apparent qualitative effect on RNA synthesis in isolated nuclei does not mean that cytoplasmic factors are not required for RNA synthesis observed here. Although our nuclei preparations are apparently free of cytoplasm as judged by electron microscopic examination, a small amount of cytoplasmic contamination cannot be excluded.

Although the system described herein appears to synthesize HnRNA similar to what is seen in vivo, we have not observed the synthesis of ribosomal precursor or mature RNA. The nature of the RNA synthesized in the presence of α-amanitin is yet to be determined.

Future work will be also directed towards obtaining definitive proof of the messenger activity of this RNA. The MOPC 315 myeloma cells used in this study synthesize large quantities of immunoglobulin (5-10% of the cell protein). We will examine the translation potential of the RNA in the homo-

logous (myeloma) and the heterologous translation systems and assess the level of de novo synthesized mRNA.

ACKNOWLEDGEMENTS

This work was supported by a grant from the American Cancer Society (#NP-6E).

ABBREVIATIONS

Abbreviations used are: EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; HnRNA, heterogeneous nuclear RNA; poly(A)$^+$RNA, RNA containing poly(adenylic acid); TCA, trichloroacetic acid.

REFERENCES

1 Zylber, E.A. and Penman, S. (1971) Proc. Nat. Acad. Sci. USA 68, 2861-2865
2 Reeder, R.H. and Roeder, R.G. (1972) J. Mol. Biol. 67, 433-441
3 Price, R. and Penman, S. (1972) J. Virol. 9, 621-626
4 Price, R. and Penman, S. (1972) J. Mol. Biol. 70, 435-450
5 Wallace, R.D. and Kates, J. (1972) J. Virol. 9, 627-635
6 Jacobson, A., Firtel, R.A. and Lodish, H.F. (1974) J. Mol. Biol. 82, 213-230
7 Edmonds, M., Vaughan, M.H., Jr. and Nakazato, H. (1971) Proc. Nat. Acad. Sci. USA 68, 1336-1340
8 Lee, S.Y., Mendecki, J. and Brawerman, G.B. (1971) Proc. Nat. Acad. Sci. USA 68, 1331-1335
9 Darnell, J.E., Wall, R. and Tushinski, R.J. (1971) Proc. Nat. Acad. Sci. USA 68, 1321-1325
10 Banks, S.P. and Johnson, T.C. (1973) Science 181, 1064-1065
11 Marzluff, W.F., Jr., Murphy, E.C., Jr. and Huang, R.C.C. (1973) Biochemistry 12, 3440-3446
12 Villa-Kamaroff, L., McDowell, M., Baltimore, D. and Lodish, H. (1974) Methods in Enzymology 30, 709-721
13 Glisin, V., Crkvenjakov, R. and Byus, C. (1974) Biochemistry 13, 2633-2637
14 Singer, R.H. and Penman, S. (1973) J. Mol. Biol. 78, 321-334
15 Aviv, H., Boime, I. and Leder, P. (1971) Proc. Nat. Acad. Sci. USA 68, 2303-2307
16 Lindell, T.J., Weinberg, F., Morris, P.W., Roeder, R.G. and Rutter, W.J. (1970) Science 170, 447-449
17 MacNaughton, M., Freeman, K.B. and Bishop, J.O. (1974) Cell 1, 117-125
18 Perry, R.P. and Kelley, D.E. (1968) J. Mol. Biol. 35, 37-59
19 Penman, S., Vesco, C. and Penman, M. (1968) J. Mol. Biol. 34, 49-69
20 Lodish, H.F. (1961) J. Biol. Chem. 246, 7131-7138
21 Edmonds, M. and Caramela, M.G. (1969) J. Biol. Chem. 244, 1314-1324
22 Wei, C.M. and Moss, B. (1974) Proc. Nat. Acad. Sci. USA 71, 3014-3018
23 Abraham, G., Rhodes, D.P., Barnejee, A.K. (1975) Cell 5, 51-58
24 Furuichi, Y., Morgan, M., Muthukrishnan, S. and Shatkin, A. (1975) Proc. Nat. Acad. Sci. USA 72, 362-366
25 Furuichi, Y., Morgan, M., Shatkin, A., Jelinek, W., Salditt-Georgieff, M. and Darnell, J.E. (1975) Proc. Nat. Acad. Sci. USA 72, 1904-1908
26 Rose, J.K. (1975) J. Biol. Chem. 250, 8098-8104
27 Wei, C.M., Gershowitz, A. and Moss, B. (1975) Cell 4, 379-386
28 Cory, S. and Adams, J. (1975) J. Mol. Biol. 99, 519-547
29 Perry, R.P., Kelley, D.E., Friderici, K.H. and Rottman, F.M. (1975) Cell 4, 387-394

30 Perry, R.P., Kelley, D.E., Friderici, K.H. and Rottman, F.M. (1975) Cell 6, 13-19
31 Salditt-Georgieff, M., Jelinek, W., Darnell, J.E., Furuichi, Y., Morgan, M. and Shatkin, A. (1976) Cell 7, 227-237
32 Groner, Y. and Hurwitz, J. (1975) Proc. Nat. Acad. Sci. USA 72, 2930-2934
33 Winicov, I. and Perry, R.P. (1976) Biochemistry 15, 5039-5046
34 Raskas, H. (1971) Nature, New Biol. 233, 134-136