Characterization of the Poly(adenylic acid) Sequences in RNA Synthesized in Vitro by Mouse Myeloma Nuclei†

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ABSTRACT: The size and quantity of poly(A) sequences made by mouse myeloma nuclei in vitro are dependent on the concentration of KCl, ATP, other ribonucleoside triphosphates, as well as the nature of the divalent cation in the reaction medium. Reduction of the KCl concentration from 120 mM to 5 mM, for example, stimulates poly(A) synthesis 10- to 20-fold. These poly(A) sequences are similar in size to cellular nuclear poly(A), but the RNA molecules to which they are attached are much shorter than poly(A) containing RNA molecules made at 120 mM KCl. Presence of Mn^{2+} in the medium led to a much more heterogeneous population of poly(A) sequences. From such observations we have found reaction conditions in which nuclei synthesize molecules that resemble native nuclear poly(A) + RNA. Not only are the lengths and amounts of the poly(A) sequences similar, but they also undergo a terminal turnover like that of the poly(A) in hnRNA. An oligo(A) sequence that resembles the oligo(A) found in non-poly(A) containing hnRNA of mouse myeloma and HeLa cells is also synthesized in vitro. These observations suggest that some processing functions are retained during the in vitro incubation of these nuclei.

Poly(A)[1] and oligo(A) sequences are found in heterogeneous nuclear RNA (hnRNA) of animal cells. The first to be described was the poly(A) sequence of about 200 nucleotides that is added posttranscriptionally to the 3′ ends of hnRNA (Edmonds et al., 1971; Darnell et al., 1971). A transcribed internal small poly(A) sequence of about 25 nucleotides was subsequently isolated from the hnRNA of HeLa cells (Nakazato et al., 1973, 1974; Edmonds et al., 1976). The role of these sequences is not known, but it seems likely that either or both play some part in the processing of hnRNA to messenger RNA (mRNA). Isolated nuclei could be useful for studying such roles for these poly(A) sequences if natural transcription and processing reactions are retained in vitro. A nuclear system in which specific RNA transcripts could be identified would be especially valuable for such studies. Certain mouse myeloma cell lines that synthesize specific immunoglobulin light chains were selected as the source of our nuclear preparations since the specific mRNA coding for these abundant immunoglobin polypeptides synthesized by a specific cell type is an abundant species of mRNA (Stavnezer & Huang, 1971; Swan et al., 1972). A more practical consideration for this choice was the low level of ribonuclease reported for these nuclei that allows the recovery of large RNA transcripts characteristic of the native hnRNA of these cells (Marzluff et al., 1973). The synthesis of poly(A)-containing RNA has also been reported for myeloma nuclei (Marzluff et al., 1973; Mory & Gefter, 1977), as it has for nuclei from chick oviduct (Ernest et al., 1976), HeLa cells (Jelinek, 1974), and rat liver (De Pomerai & Butterworth, 1975). The poly(A) sequences recovered in the last two systems were, however, much shorter than the poly(A) found in the nuclear RNA of these same cells.

In this paper we describe conditions that allow the synthesis by isolated mouse myeloma nuclei of both large and small poly(A) sequences covalently bound to nuclear RNA that resemble native poly(A) sequences.

Methods and Materials

Cells. A subclone (66-2) of a light chain producing myeloma cell line, MPC-11, isolated by Coffino & Scharf (1972) was supplied through the courtesy of R. C. Huang of Johns Hopkins University. Cells were maintained as described (Marzluff et al., 1973) except the cell suspensions were grown in roller rather than stationary flasks.

Preparation of Nuclei. Nuclei were prepared as described by Marzluff et al. (1973). The nuclear pellets were carefully suspended (0.4–1.2 mg of DNA/mL) either in 25% glycerol containing 50 mM Tris (pH 8), 5 mM DTT (dithiothreitol), and 0.1 mM EDTA (suspension buffer A) or in 10% glycerol containing 120 mM KCl, 10 mM $MgCl_2$, and 1 mM DTT (suspension buffer B). Nuclei were freshly prepared for these experiments. Nuclei stored at −70 °C tended to have lower RNA synthetic capacity. We are indebted to Dr. William Marzluff for providing us with the composition of both the suspension and incubation buffers designated as "B" in these experiments.

RNA Synthesis. RNA synthesis in nuclei suspended in medium A was carried out for 30 min at 25 °C in a reaction that was 12.5% glycerol, either 5 or 120 mM KCl, 25 mM Tris (pH 8), 4.6 mM $MgCl_2$, 1 mM $MnCl_2$, 2.5 mM DTT, 0.05 mM EDTA, CTP, UTP, and GTP at 0.4 mM each and 2 mM ATP unless otherwise noted (incubation medium A). Nuclei suspended in buffer B were incubated as above but in 7.5% glycerol, 130 mM KCl, 5 mM $MgCl_2$, 7.5 mM Tris-HCl (pH 8), 0.75 mM DTT and 0.4 mM each of UTP, GTP, and CTP, and 1 mM ATP (incubation medium B). [2-^{3}H]ATP (20–30 Ci/mmol from Amersham/Searle) provided the label. After a 5-min lag, RNA synthesis occurs at a constant rate for 30 to 45 min and then declines rapidly although label continues to accumulate in the total RNA for about 60 min.

RNA synthesis was measured in some cases by the filter paper technique (Bollum, 1966) but more often by collecting Cl_3CCOOH-insoluble material from the reaction on GF/A glass fiber filters (Whatman). After suitable acid washing of papers or glass fiber filters, RNA was solubilized with NCS

† From the Department of Biological Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania 15261. *Received February 28, 1978.* This investigation was supported by Grant No. 18065 awarded by the National Cancer Institute, Department of Health, Education and Welfare.

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[1] Abbreviation used: poly(A), poly(adenylic acid).

0006-2960/78/0417-3221$01.00/0

TABLE I: Effect of KCl and ATP Concentrations on RNA and Poly(A) Synthesis.

[ATP] (mM)	nmol of AMP × 10^8 incorp per nucleus: total RNA 120[a]	total RNA 5[a]	RNA portion 120[a]	RNA portion 5[a]	poly(A) portion 120[a]	poly(A) portion 5[a]	% of incorp AMP in poly(A) 120[a]	% of incorp AMP in poly(A) 5[a]
0.05	1.1	0.37	1.034	0.32	0.066	0.05	6	13
0.20	1.56	2.7	1.34	1.78	0.220	0.92	14	34
2.00	9.8	15.3	8.92	6.10	0.88	9.20	9	60

[a] [KCl], mM.

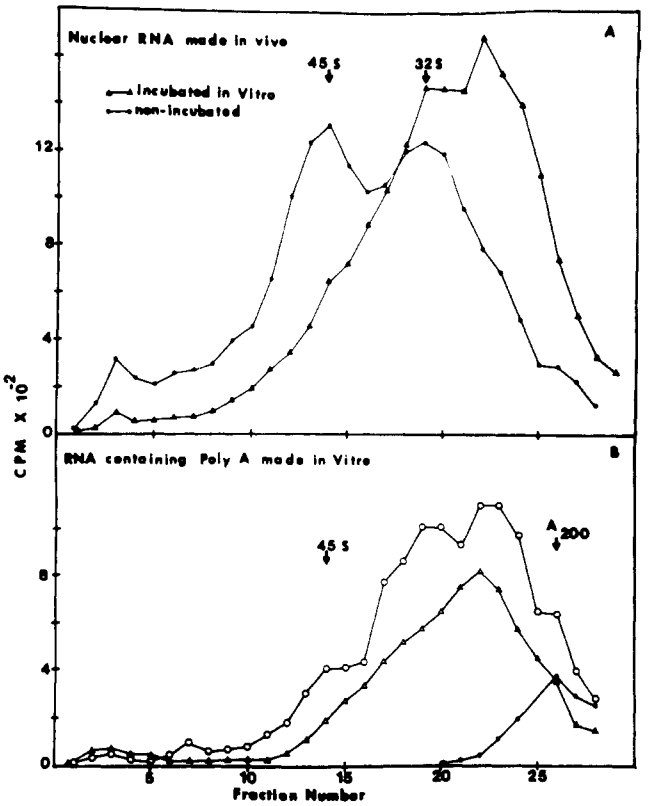

FIGURE 1: Sedimentation analysis of myeloma nuclear RNA made in vivo and incubated in vitro (A) and poly(A) + RNA made in vitro (B). Myeloma cells (2×10^6 cells/mL) were labeled during a 20-min incubation with [^{14}C]adenosine at 0.05 μCi/mL in Dulbecco's medium containing 10% horse serum. (A) Nuclei were isolated as described in Materials and Methods and were incubated in medium A for 30 min (Δ) or were not incubated (○). Total RNA was extracted from each nuclear preparation as described in Materials and Methods. Each RNA sample was centrifuged in a 15–30% sucrose gradient for 16 h at 18 000 rpm in a Beckman SW 40 rotor. (B) Nuclei were isolated and incubated in medium A containing 120 mM KCl and either 0.2 mM (Δ) or 2 mM (○) [^{3}H]ATP. ^{32}P-labeled poly(A) (●) from HeLa cell nuclear RNA was included as a marker in the gradient.

reagent. Radioactivity was determined in a Packard liquid scintillation spectrometer after the addition to the samples of an omnifluor toluene scintillator.

RNA Isolation. RNA was isolated by diluting the nuclear incubation mixture into 10 volumes of sodium acetate (pH 5.3) containing 0.5% sodium dodecyl sulfate. After vigorous vortexing, an equal volume of water saturated phenol at 60 °C was added and RNA was recovered as described (Girard, 1967). Poly(A) containing RNA was separated from the non-poly(A)-containing RNA by methods described previously for HeLa cells (Nakazato & Edmonds, 1974). Sedimentation analysis and Me_2SO denaturation of nuclear RNA were carried out as previously described for HeLa nuclear RNA (Korwek et al., 1976).

Isolation of Poly(A) Sequences. Both large and small poly(A) sequences released from RNA were purified on oligo(dT)-cellulose as described (Nakazato & Edmonds, 1974; Korwek et al., 1976). Poly(A) was heated at 60 °C in 8 M urea for 3 min to eliminate any noncovalently bound radioactivity prior to electrophoresis.

Analysis of Poly(A) Sequences. End-group analysis was carried out by measuring the ^{3}H in the AMP and adenosine recovered from alkaline hydrolysates of poly(A) sequences purified on polyacrylamide gels (Nakazato et al., 1973). Following electrophoresis and fractionation of the gel in a Maizel fractionator, the poly(A)-containing fractions were pooled and gel fragments were removed by filtration through a sintered glass funnel. Poly(A) was then rebound to oligo(dT)-cellulose. After washing with high salt buffer (0.5 M NaCl, 0.01 M Tris (pH 7.4), 0.2% sodium dodecyl sulfate), the poly(A) was eluted with seven successive 1-mL washes with 0.01 M Tris (pH 7.4), 0.2% sodium dodecyl sulfate at 60 °C. Poly(A) was precipitated with 2.5 volumes of ethanol after adding 150 μg of RNase-free soluble yeast RNA. The precipitated poly(A) was hydrolyzed in 20 μL of 0.33 M KOH at 37 °C for 16–18 h after which it was mixed with enough 1 M perchloric acid to bring to pH 4.0. After chilling, $KClO_4$ was removed. The remaining $KClO_4$ pellet was washed with 10 μL of a mixture containing 50 μg each of adenosine and AMP to serve as markers. The wash and original supernatant were pooled and applied as a 1-cm band to the center of a Whatman 3MM paper strip 40-cm long.

Electrophoresis (Camag high voltage electrophoresis apparatus) was carried out in pyridine acetate buffer (pH 3.5) at 3000 V as described (Salzman & Sebring, 1964). The markers were visualized in ultraviolet light. Each lane was cut out and the entire paper was cut in 50-mm strips that were eluted by shaking in 1 mL of 0.01 M HCl for 48 h at 23 °C. Ten milliliters of PCS (Amersham/Searle) was added to each sample to determine radioactive tritium as described above.

Results

RNA Synthesis. The data of Figure 1A indicate that nuclear RNA made in vivo undergoes some size reduction when incubated under conditions that support RNA synthesis in vitro. The extent of this reduction indicates that ribonuclease activity must be low as has already been reported for these nuclei (Marzluff et al., 1973) and suggests that processing may actually occur, although processing cannot be distinguished in this case from nonspecific degradation until pathways for the processing of specific RNA molecules are identified.

Figure 1B shows that as reported for these nuclei large RNA transcripts become labeled during the in vitro incubation (Marzluff et al., 1973). In this figure, only poly(A)-containing RNA molecules are displayed to emphasize that poly(A) can be added to RNA molecules similar in size to those synthesized in the cell. These RNA molecules sediment far more rapidly

TABLE II: Effect of Nucleoside Triphosphate Concentrations on RNA and Poly(A) Synthesis.

[ATP] (mM)	nmol of AMP × 10^8 incorp per nucleus in 5 mM KCl: total RNA	RNA portion	poly(A) portion	% of incorp AMP in poly(A)	[NTP] (mM)
0.05	0.544	0.154	0.39	72	0
0.05	0.475	0.455	0.02	4	0.5
0.20	1.72	1.01	0.71	41	0.1
0.20	1.41	1.21	0.20	14	0.5
0.20	1.29	1.21	0.08	6	1.0
2.0	6.06	1.70	4.36	72	0.1
2.0	3.26	2.32	0.94	29	0.5

than the HeLa nuclear poly(A) marker of 200 nucleotides cosedimented in this gradient.

Poly(A) Synthesis in Vitro. The effect of KCl and ATP concentration on both RNA and poly(A) synthesis is summarized in Table I. The quantity of AMP from [^{3}H]ATP incorporated in the RNA portion of the total RNA has been obtained by subtracting the amount of label recovered in the poly(A) portion from the total label in the RNA extracted from the incubated nuclei. Table I shows that increased labeling of both RNA and poly(A) occurred as the ATP concentration was raised. A major reduction in salt concentration (from 120 to 5 mM) had relatively small effects on AMP incorporation into RNA except at very low ATP levels. This was not the case for poly(A) synthesis which is markedly increased by reducing the salt concentration. In fact, it is apparent that in high salt the synthesis of poly(A) and RNA increased almost proportionally as ATP concentration was raised as indicated by the fact that poly(A) synthesis accounted for about 10% of the AMP incorporated at each ATP concentration. In low salt, however, there is an increase in percent of total label in poly(A) at high levels of ATP that results from an increase of poly(A) synthesis that greatly exceeds the increase in RNA synthesis. Poly(A) synthesis became predominant in nuclei incubated at low salt and high ATP concentrations. Similar results were reported for rat liver nuclei incubated at low ionic strength (De Pomerai & Butterworth, 1975).

A more detailed examination of the effects of various concentrations of nucleoside triphosphates on RNA and poly(A) synthesis in the low salt incubation is shown in Table II. At all levels of ATP, the presence of UTP, CTP, and GTP reduced poly(A) synthesis, but the effect on RNA synthesis at nucleotide levels above 0.1 mM was not particularly significant. The levels of nucleotides that strongly inhibit poly(A) synthesis are significantly higher, however, than those needed to attain close to maximal levels of RNA synthesis in these nuclei.

Characteristics of Poly(A) Synthesized in Vitro. In data presented thus far, poly(A) synthesis has been defined as radioactivity bound to oligo(dT)-cellulose from ribonuclease digests of total RNA. The fact that in addition to the larger 3′-terminal poly(A) sequence a small transcribed poly(A) sequence can also be recovered from ribonuclease digests of HeLa cell nuclear RNA (Nakazato et al., 1974) made it necessary to establish the size and intramolecular location of the poly(A) synthesized in isolated myeloma nuclei. Figure 2 displays conditions that are among those described in Tables I and II. Poly(A) made in high salt (Figures 2C and 2D) was highly heterogeneous, covering all sizes ranging from 20 to 200 nucleotides. The amount of poly(A) made was greatly increased at high ATP concentrations (Figure 2C). In low salt large amounts of large poly(A) were made when the ATP concentration was high (Figure 2A). At low ATP concentrations not only was much less poly(A) made, but most of it was much shorter (Figure 2B).

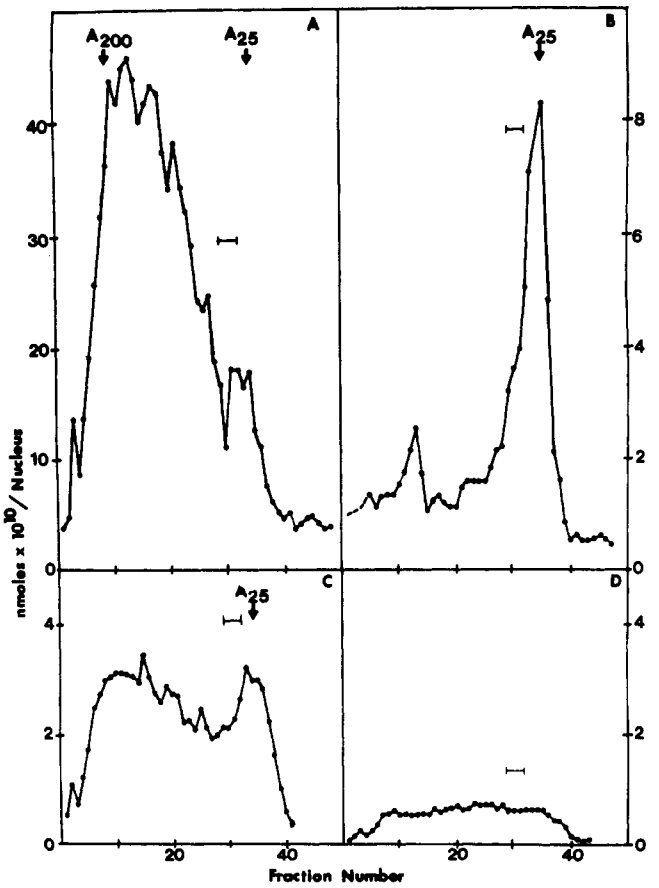

FIGURE 2: Polyacrylamide gel electrophoresis of poly(A) made in vitro. Nuclei were incubated in medium A but NTPs were lowered to 0.1 mM: (A) 2 mM ATP, 5 mM KCl; (B) 0.2 mM ATP, 5 mM KCl; (C) 2 mM ATP, 120 mM KCl; (D) 0.2 mM ATP, 120 mM KCl. Isolation of RNA and poly(A) and electrophoresis of poly(A) are described in Materials and Methods. Poly(A) radioactivity was normalized to nmol of AMP incorporated per nucleus from the specific activity of [^{3}H]ATP and from the number of nuclei in the reaction, as were counted in a hemocytometer.

Figure 3 (left panel) shows the electrophoretic mobility of those poly(A) sequences synthesized under conditions optimal for poly(A) synthesis in vitro, i.e., low salt and high ATP in the absence of other nucleoside triphosphates. Not only did most of these sequences have electrophoretic mobilities similar to natural nuclear poly(A), but an end-group analysis obtained from measuring the ratio of label in AMP to that in terminal adenosine showed the large sequences near the top of the gel to be labeled to about the same average length as the nuclear poly(A) from HeLa cells. When the other nucleoside tri-

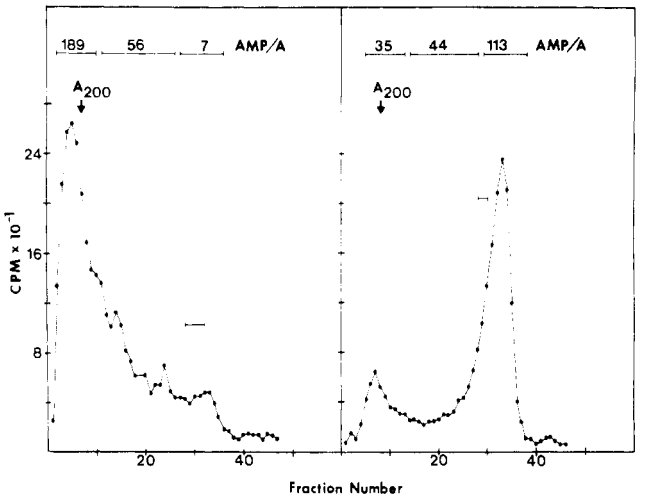

FIGURE 3: Effect of ATP and other nucleoside triphosphates on the electrophoretic mobility and length of poly(A). Poly(A) was isolated and electrophoresed as described in Figure 2. Gel fractions were pooled as indicated and the ratio of ^3H in AMP to ^3H in adenosine was determined by paper electrophoresis as described in Materials and Methods. Nuclei were incubated in medium A containing 5 mM KCl, 2 mM ATP, and no CTP, GTP, or UTP (left) or in 5 mM KCl, 0.2 mM ATP, and 0.1 mM each of GTP, UTP, and CTP (right).

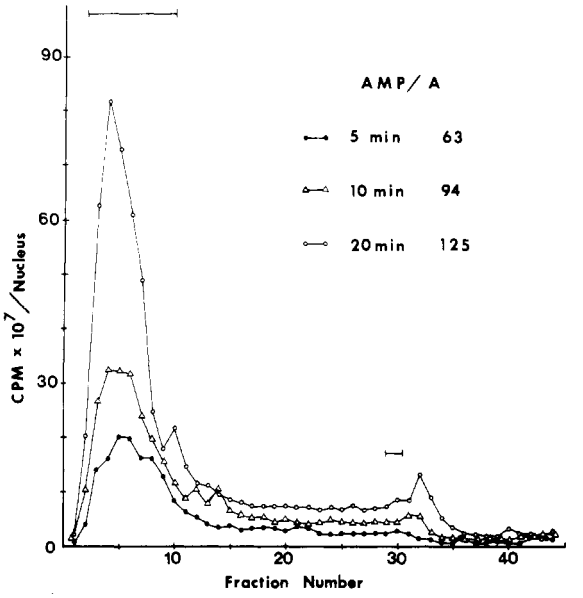

FIGURE 4: Increase in length of poly(A) chains labeled in vitro. Poly(A) was synthesized in nuclei incubated in medium A containing 5 mM KCl, 2 mM ATP, and no CTP, UTP, or GTP for (●) 5 min; (Δ) 10 min and (○) 20 min. Poly(A) was isolated and electrophoresed as in Figure 2. Chain lengths were measured as in Figure 3.

phosphates and reduced levels of ATP (0.2 mM) were present at these low salt concentrations (Figure 3, right) not only were most poly(A) sequences short, but end-group analysis indicated that the majority of the sequences in the rapidly migrating peak did not have adenosine with free hydroxyl groups at the 3′ end since the calculated average length of 113 exceeded the 20 or 30 nucleotide length expected for the observed electrophoretic mobilities. It is likely that most of these short poly(A) sequences are transcribed as part of larger RNA molecules, since in contrast to the experiment of Figure 3 (left panel) other nucleoside triphosphates were added here to allow RNA synthesis.

It is apparent from the experiment of Figure 3 (left panel) that large amounts of poly(A) similar in size to cellular nuclear poly(A) can be synthesized in these nuclei in vitro. An experiment examining the rate of this synthesis is shown in Figure 4. A glance at the electrophoretic mobilities of poly(A) synthesized 5, 10, and 20 min after the initiation of in vitro synthesis showed all labeled poly(A) to be similar in size. In this respect, it appeared to resemble poly(A) synthesis in HeLa cells where nascent chains are not observed even after the briefest labeling periods since the rate of poly(A) chain elongation is too rapid to observe the existence of intermediates (Jelinek et al., 1973). However, end-group analysis of the poly(A) synthesized during this time period clearly showed a progressive increase in the average length of poly(A) labeled. A relatively slow rate of completion of preexisting incomplete poly(A) sequences and/or a slow rate of synthesis de novo could account for this progressive increase in length of the poly(A) sequences labeled in vitro. However, either of these mechanisms should produce poly(A) sequences of increasing lengths as time elapsed, rather than the similar lengths seen at all times in Figure 4. Since a similar picture is seen in modified high salt incubation system about to be described, these data will be discussed in more detail below.

Poly(A)$^+$ RNA Synthesis in Vitro. Although large amounts of poly(A) comparable in size to natural poly(A) were made in the low salt system (Figures 2A and 3, left), patterns of poly(A) synthesis in high salt resembled more closely those of the cell, although the poly(A) sequences were more heterogeneous than native poly(A). Also, a significant reduction in sedimentation velocity was always observed in RNA preparations recovered from low salt incubations relative to RNA made in high salt (data not shown). These findings led us to concentrate on developing a medium that would permit the synthesis of poly(A) containing RNA molecules in isolated nuclei that resembled native molecules. Use of a modified high salt medium (medium B), in which the main difference from the high salt medium A is the absence of manganous ion, has allowed us to synthesize a much more homogeneous collection of poly(A) sequences (Figure 5, top) attached to large RNA molecules (Figure 6), some of which are transcribed in vitro (Table III). This medium has been used in all subsequent experiments.

It has previously been shown that RNA molecules containing the large terminal poly(A) can be separated from molecules containing small internal transcribed oligo(A) sequences by heating the RNA before applying it to oligo(dT)-cellulose (Nakazato & Edmonds, 1974; Edmonds et al., 1976). Only the molecules containing large poly(A) (poly(A) + RNA) bind to the oligo(dT)-cellulose. We performed the same kind of treatment to see if this separation occurred with RNA made in vitro. Table III summarizes five different experiments in which the quantity of poly(A) containing RNA molecules and their poly(A) content was measured. In most cases, poly(A) + RNA accounted for about 10–20% of the labeled RNA. This was also true when CTP rather than ATP was used to label RNA (experiment 5, Table III). These values are within the range of those reported for the number of polyadenylated RNA molecules in the hnRNA of HeLa (Nakazato & Edmonds, 1974) and mouse L cells (Perry et al., 1974).

Of special significance is the finding that the poly(A) sequence itself accounts for only 10–20% of the labeled AMP in the poly(A)-containing RNA (experiment 2 is an obvious exception). If the polyadenylation occurring in these experiments is the expected posttranscriptional addition of AMP to the 3′ ends of RNA molecules, it must be concluded that poly(A) has been attached to RNA molecules transcribed in vitro. If, for example, a poly(A) of 200 labeled AMPs is attached to a RNA

TABLE III: Properties of Poly(A)-Containing RNA Synthesized by Myeloma Nuclei in Vitro.[a]

labeled product	experiments[b] 1	2	3	4	5[c]
total RNA	1790	950	86	138	183
poly(A) + RNA	200	86	9.9	13.6	17
poly(A) − RNA	1600	550	—	—	166
poly(A)	24	35	1.7	2.6	—
oligo(A)	1.4	2.3	—	—	—
[^{3}H]AMP/[^{3}H]adenosine of poly(A)	29.1	32	—	—	—

[a] Four separate standard nuclear incubations each containing 6 × 10^6 nuclei in medium B (see Materials and Methods) and 0.6 mCi of [^{3}H]ATP were pooled to prepare the RNA for experiments 1 and 2, while a single nuclear incubation containing 0.05 mCi of [^{3}H]ATP was used for both experiments 3 and 4. In experiments 5, two standard nuclear incubations each containing 0.066 mCi of [^{3}H]CTP at 0.4 mM were pooled to prepare RNA. [b] In units of ^{3}H cpm × 10^{-3}. [c] [^{3}H]CTP replaced [^{3}H]ATP as the labeled nucleotide.

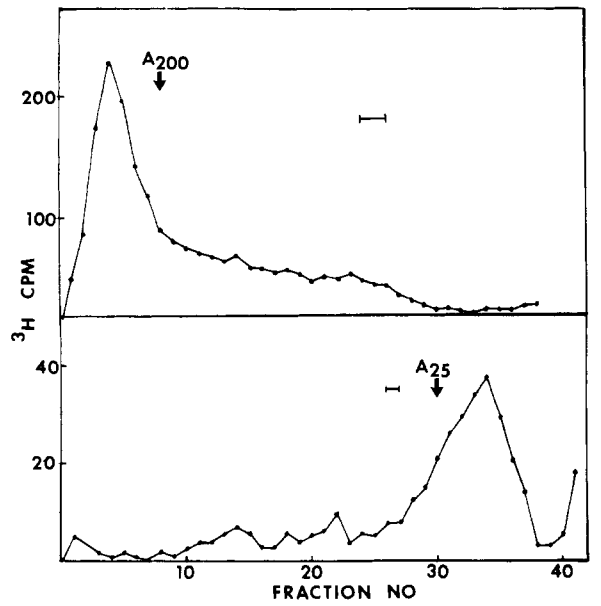

FIGURE 5: Separation of poly(A) + RNA from poly(A) − RNA labeled in nuclei incubated in medium B (see Materials and Methods). Poly(A) + RNA was separated from poly(A) − RNA as described for HeLa cell hnRNA by Nakazato & Edmonds (1974). The poly(A) sequences were isolated from RNase digests on oligo(dT)-cellulose. Fractions 2–9 were pooled for end-group analysis of experiment 1 in Table III. Poly(A) from RNA bound (top) and not bound (bottom) to oligo(dT)-cellulose was electrophoresed on polyacrylamide gels as described in Materials and Methods. ^{32}P-labeled poly(A) and oligo(A) sequences purified from hnRNA of HeLa cells were electrophoresed as markers on parallel gels.

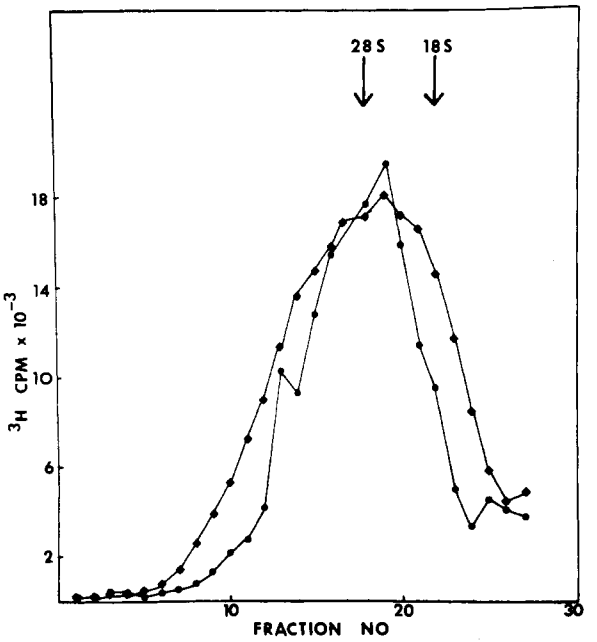

FIGURE 6: Sedimentation profile of poly(A) + RNA synthesized in myeloma nuclei incubated in medium B. Sedimentation was at 19 500 rpm for 17.5 h in an SW 40 Beckman rotor. Poly(A) + RNA without (■) and with (●) dimethyl sulfoxide treatment as described by Korwek et al. (1976). ^{32}P-labeled HeLa cell ribosomal RNA was cosedimented as marker in each gradient.

molecule labeled for a length of 5000 nucleotides of which 1400 are in AMP (the hnRNA of HeLa cells is 28% AMP), then poly(A) would account for 14.3% of the AMP in such RNA, which is within the observed range for most of the poly(A) + RNA of these experiments. These values for the fraction of incorporated AMP that is in the poly(A) portion indicate that poly(A) is added to RNA molecules in which an average of 4000–6000 nucleotides have been transcribed in vitro.

Properties of Poly(A) Synthesized in Medium B. The size of the poly(A) sequences in poly(A) + RNA synthesized in medium B (Figure 5, top) is large and covers a rather narrow range of sizes characteristic of native nuclear poly(A). The labeled RNA that did not bind to oligo(dT)-cellulose contained little large poly(A), but contained some ribonuclease resistant radioactivity that bound to oligo(dT)-cellulose when ATP, but not CTP (data not shown), provided the label. During polyacrylamide gel electrophoresis (Figure 5, bottom), much of this material migrated at the same rate as a small poly(A) sequence in the hnRNA of HeLa cells (Nakazato et al., 1974). Again as has been shown for HeLa cell hnRNA little of this material is found in the poly(A)-containing RNA (Figure 5, top). The oligo(A)s of HeLa cells are nonterminal transcribed sequences of about 25 uninterrupted AMP residues (Nakazato et al., 1974; Edmonds et al., 1976). Although we designate this material synthesized in isolated nuclei as oligo(A) because it resembles the oligo(A) sequences found in HeLa cells, it must be purified and analyzed more carefully to establish whether these are short terminal sequences added posttranscriptionally or transcribed internal sequences of the type found in the hnRNA of the cell or a mixture of both. The chain length data of Figure 3 (right) obtained in medium A in low salt indicated many of the latter were present. Preliminary chain length data on the oligo(A) from poly(A) − RNA (Figure 5, bottom) suggest most do not have adenosine at the 3′ end (Almendinger, unpublished experiments).

The poly(A)-containing RNA molecules synthesized in high salt in medium A have already been shown to sediment as large molecules (Figure 1B). Poly(A)-containing RNA molecules made in the modified high salt medium B are shown in Figure

6. In this experiment denaturation of the RNA in Me_2SO prior to sedimentation resulted in somewhat fewer molecules sedimenting more rapidly than 28S RNA than were found in nondenatured samples.

Although large poly(A) sequences recovered from this poly(A) + RNA migrated as lengths of about 200 nucleotides during electrophoresis (Figure 5), end-group analyses on two different preparations showed the average length of poly(A) labeled in these experiments to be only about 30 (Table III). A similar discrepancy has already been discussed in relation to the end-group analyses in the experiments of Figure 4. The experiment differs from that of Figure 4, where new transcripts were not made, in that here some of these poly(A) sequences are attached to RNA molecules that have been transcribed in vitro, at least in part. This can be deduced from the data in Table III showing that most of the AMP has been incorporated into the RNA portion of the poly(A) + RNA molecules. Given the fact that polyadenylation is posttranscriptional, we would expect the poly(A) portion of these RNAs to be completely labeled and show average lengths of about 200. The average labeled lengths of 30 AMP residues found on poly(A) migrating as sequences of about 200 nucleotides suggest that terminally labeled poly(A) sequences on endogenous unlabeled RNA have been mixed with poly(A) synthesized de novo. To interpret these data quantitatively in terms of the number of poly(A) sequences labeled only terminally would require information about the actual number of AMP residues added to preexisting poly(A) sequences. However, a simple calculation can show that more terminally labeled poly(A) sequences must be present in this mixture than poly(A) sequences synthesized de novo in order for 3.3% of the radioactivity in this approximately 200 nucleotide poly(A) sequence to be found at the 3′ end rather than the 0.50% expected. If it is assumed for sake of argument that only one labeled AMP has been added to the 3′ end of an endogenous poly(A) sequence of 200 nucleotides, the following calculation would show that five such labeled poly(A) sequences must be present for each poly(A) synthesized de novo, for example: 1 poly(A) with 200 labeled Ap's and 1 labeled A_{OH} and 5 poly(A)'s with no labeled Ap's, but 5 labeled A_{OH} would yield an Ap/A_{OH} ratio of 33.3.

If the terminal labeling involved more than the single AMP residue assumed in the above calculation, as seems likely, then an even larger number of such terminally labeled molecules would be present to account for the observed average chain lengths. We have attempted to separate those polyadenylated RNA molecules that are synthesized in vitro from endogenous RNA molecules that have been terminally adenylated in vitro by incorporating mercury from Hg UTP into RNA made in vitro. This should permit its isolation on sulfhydryl sepharose (Dale & Ward, 1975). A significant reduction in both amount and size of the mercurated poly(A) + RNA compared with RNA made with UTP only has not yet allowed us to make extensive use of this ingenious approach (Edmonds & Almendinger, unpublished experiments).

At first glance then it would appear that this preponderance of terminally labeled poly(A) sequences synthesized in isolated nuclei differs from poly(A) synthesis in the cell nucleus where polyadenylation is such a rapid reaction that nascent sequences are not seen in labeling periods as short as 1 min (Jelinek et al., 1973). HeLa cells labeled for longer periods do in fact contain poly(A) with chain lengths of about 200 as determined by end-group analysis (Nakazato et al., 1973). More recent analyses on poly(A) made in short time periods have shown, however, a relatively slow elongation of label in poly(A) from nuclear RNA of all size classes of hnRNA of mouse sarcoma and Chinese hamster cells (Brawerman & Diez, 1975). This apparent slow elongation was attributed to a terminal AMP addition reaction in the nucleus that occurs independently of the de novo poly(A) synthesis in these cells. These observations have been confirmed and extended to HeLa cells recently in experiments in which the lengths of labeled poly(A) sequences have been examined after brief labeling periods (Sawicki et al., 1977). In these experiments an estimate of the average number of AMP residues added to the 3′ ends of preexisting nuclear poly(A) was obtained by comparing the amount of label released by polynucleotide phosphorylase from the 3′ ends of briefly labeled (2 min) with completely labeled poly(A) (4 h). The data indicated that, within 2 min of labeling, about 5 to 10 AMPs were added to 5 to 10 pre-existing nuclear poly(A) sequences for each 200 nucleotide poly(A) made de novo. The poly(A) obtained from the mixing of these terminally labeled products with those synthesized de novo would account for the average chain length of 30 obtained in the 2-min labeling experiment. It is significant that similar labeling patterns are observed in the poly(A) made in vitro, suggesting that isolated nuclei retain activities characteristic of the cell nucleus and thus provide a system for examining these reactions in detail.

Discussion

These data demonstrating that mouse myeloma nuclei synthesize poly(A) sequences in vitro that are essentially indistinguishable from those found in the cell nucleus indicate that at least one well-established RNA processing reaction occurs in isolated nuclei. Not only are the poly(A) sequences similar in length, but they are covalently attached to an essentially equivalent number of nuclear RNA molecules that span size ranges comparable to native nuclear RNA molecules. Some earlier studies with different nuclear preparations had shown polyadenylation of RNA synthesized in vitro (Jelinek, 1974; De Pomerai & Butterworth, 1975), but most all of the poly(A) was much shorter than natural poly(A). The data presented here emphasizing the sensitivity of poly(A) synthesis to the concentration of components of the reaction medium such as salts, ATP, other nucleoside triphosphates, and divalent cations would readily account for such discrepancies. Poly(A), but not RNA synthesis in these nuclei, is especially sensitive to changes in ionic strength. Poly(A) synthesis was increased 10- to 20-fold by reducing the KCl in the incubation medium from 120 to 5 mM without greatly altering RNA synthesis except at the lowest ATP concentrations (Table I). Since we have observed a marked reduction in the size of the RNA synthesized in vitro in this same low salt system, it is possible that the increase in poly(A) synthesis results from an increase in the number of RNA pieces that can serve as primers for poly(A) synthesis. An activation of poly(A) polymerase in low salt could also be involved. The fact that conditions most favorable for poly(A) synthesis were not those leading to the synthesis of RNA molecules resembling native nuclear RNA either in size or poly(A) content led us to examine other media in which nuclei synthesize poly(A)-containing RNA molecules with sequences similar to native nuclear poly(A) (Table III; Figures 5 and 6). A modified high salt medium (B) gave poly(A) sequences more homogeneous in size than could be isolated from medium A (compare Figure 5 (top) with Figure 2C). We would attribute this increased homogeneity of poly(A) sequences to the omission of manganous ions from medium B, were it not for some changes in the concentration of other components in medium B. Without a systematic study of the effects of these changes we cannot be certain that the heterogeneity of poly(A) synthesized in medium A is due to the presence of Mn^{2+} ions. However, there are examples of

losses of specificity of polymerases (Berg et al., 1963) and very recently of restriction endonucleases (Hsu & Berg, 1978) when manganous ions replace magnesium ions in a reaction medium. It is possible that Mn^{2+} may activate or alter the specificity of an endogenous nuclease.

In addition to the large poly(A) sequence, a much shorter poly(A) sequence is synthesized in these nuclei that corresponds in amount and size to an oligo(A) sequence that is transcribed as part of hnRNA in HeLa cells (Nakazato et al., 1974) and is also present in myeloma hnRNA (Kieras, unpublished experiments). As reported for the hnRNA molecules from the HeLa cells, the RNA molecules made in these nuclei that contain oligo(A) sequences can be separated from those that contain poly(A) by a brief heat treatment before reacting with oligo(dT)-cellulose (Nakazato & Edmonds, 1974). This sequence similarity supports earlier evidence for the fidelity of in vitro transcription in nuclei obtained with specific DNA hybridization probes for ribosomal genes of Xenopus (Reeder & Roeder, 1972) and for immunoglobulin light chains of mouse myeloma (Smith & Huang, 1976).

The recent detection of a reaction in the nucleus that adds terminal AMP residues to the 3′ ends of poly(A) that is apparently distinct from the reaction that synthesizes poly(A) de novo (see discussion in Results section) suggests that nuclear poly(A) metabolism is more complicated than is indicated by the usual models in which nuclear polyadenylation is depicted as a stage in hnRNA processing that prepares nuclear transcripts for their eventual transport to the cytoplasm. Although the significance of this end addition reaction in cells is obscure, it is of considerable interest to the present study, since it provides additional evidence for the retention of specific nuclear functions in vitro. Studies with purified poly(A) polymerases have indicated that more than one polymerase may be present in the nucleus. A manganese-dependent activity was lost during the purification of a magnesium-dependent poly(A) polymerase from calf thymus nuclei (Edmonds & Abrams, 1965; Winters & Edmonds, 1973) while nuclear extracts from rat liver (Niessing & Sekeris, 1974) and from hamster embryo fibroblasts (Pellicer et al., 1975) have been separated during ion-exchange chromatography into two or more fractions with poly(A) polymerase activity. In no case, however, have the separate activities been shown to be due to different enzyme proteins. A more detailed discussion of multiple poly(A) polymerases is found in a recent review (Edmonds & Winters, 1976). In any case the similarity in the characteristics of total poly(A) synthesis in these myeloma nuclei incubated in vitro to those of a poly(A) polymerase purified from calf thymus nuclei makes it likely that an enzyme similar to the calf thymus nuclear poly(A) polymerase catalyzes poly(A) synthesis in mouse myeloma nuclei. Both reactions require high ATP concentrations for maximal activity, both are inhibited by other ribonucleoside triphosphates and by moderate ionic strengths (Tables I and II and Winters & Edmonds, 1973).

The two apparently distinct polyadenylation reactions observed in nuclei both in vivo and in vitro could also be catalyzed by different forms of the same enzyme rather than by different enzymes. A chromatin bound form of rat liver nuclear poly(A) polymerase has recently been described that differs in divalent cation and primer requirements, as well as sensitivity to cordycepin triphosphate, from an unbound or soluble activity extracted from the same nuclei (Rose et al., 1977a,b). However, the small fraction of the bound activity that could be released from the chromatin had properties indistinguishable from the so-called "free" enzyme. This included a greatly reduced sensitivity to cordycepin triphosphate that led these investigators to propose that the chromatin bound activity is responsible for the de novo synthesis of poly(A) attached to hnRNA while the "free" poly(A) polymerase may catalyze the addition of AMP residues to the 3′ end of poly(A) (Rose et al., 1977b). Although such a correlation will be difficult to establish, it is worth noting that the addition of AMP residues to the 3′ ends of nuclear poly(A) in HeLa cells has also been shown to be insensitive to inhibition by cordycepin (Sawicki et al., 1977). It would appear that these nuclei may provide a more useful system for understanding cellular poly(A) synthesis since, unlike the purified enzyme, the endogenous nuclear RNA molecules are serving as primers. Even more important may be the evidence presented here that the RNA transcribed in vitro can also be polyadenylated to lengths characteristic of nuclear poly(A). Thus far our attempts to separate these RNA molecules from endogenous RNAs that have been labeled only at 3′ ends by the addition of an undetermined number of [^{3}H]AMP residues have not been completely successful. The mercury UMP containing polyadenylated RNAs synthesized by these nuclei have not resembled nonmercury-containing RNA either in size or quantity. Similar results have been reported recently for poly(A)-containing RNAs made from HgUTP in a nuclear system from mouse L cells (Schäfer, 1977). However, although technical problems such as this one remain, mouse myeloma nuclei provide a system for examining molecular details of RNA processing reactions since, at least in the case of polyadenylation, nuclei retain many of their natural biosynthetic characteristics in vitro. Myeloma nuclei may also permit studies of the polyadenylation of specific gene transcripts, i.e., those for immunoglobulin light chains, since RNA transcribed in vitro by these nuclei has been hybridized to DNA sequences transcribed from messenger RNA specific for the kappa light chains synthesized by these cells (Smith & Huang, 1976).

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Small Nuclear RNA Molecules in Nuclear Ribonucleoprotein Complexes from Mouse Erythroleukemia Cells

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ABSTRACT: Ribonucleoprotein (RNP) complexes were prepared by sonic disruption of mouse erythroleukemia (MEL) cell nuclei. After removal of nucleoli and insoluble chromatin by centrifugation, the RNP sedimented on sucrose gradients as two populations: one at 10 S and the other at 30–60 S. Each RNP fraction contained both rapidly labeled and stable nuclear RNA molecules. The bulk of the stable RNA was recovered in the 10 S RNP. No ribosomal or preribosomal RNAs were recovered from the RNP. The labeling of the fast turnover RNA was not suppressed by low concentrations of actinomycin D. Most of the rapidly labeled RNA in both RNA fractions sedimented on sucrose gradients at 8–12 S. The buoyant density of formaldehyde-fixed RNP in CsCl was ρ = 1.38 g/cm^3 (10 S) and ρ = 1.436 g/cm^3 (30–60 S). These are characteristics exhibited by nuclear RNP complexes which contain degraded hnRNP. Both the 10S and 30–60S hnRNP contained several small, stable, monodisperse RNA molecules which belong to a class of cellular RNA called snRNA. The 10S hnRNP contained snRNA species A–E. The 30–60S hnRNP contained snRNA species, A, B, D, and E. No nucleolar snRNA species were recovered in the hnRNP. The snRNAs cosedimented with the hnRNP on sucrose gradients. With the exception of snRNA species A, nearly all of the snRNAs remained associated with that portion of the 10S and 30–60S hnRNP which bound to oligo(dT)-cellulose. The recovery of snRNAs from the bound hnRNP fraction was not due to nonspecific binding of deproteinated snRNAs to the column or an artificial association of snRNAs and nuclear protein. It is proposed that several snRNAs are integral components of MEL cell hnRNP complexes.

Eucaryotic cells contain a unique class of small, monodisperse, metabolically stable RNA molecules which are referred to collectively as small nuclear RNAs (snRNAs), since they appear to be confined to the nucleus, at least during the majority of their lifetime in the cell (Busch et al., 1971; Weinberg, 1973; Ro-choi and Busch, 1974). The cellular function of most snRNAs is unknown. It has been suggested that snRNAs may be involved in the regulation of gene expression in eucaryotic cells (Kanehisha et al., 1974; Goldstein, 1976). Some of the regulation by snRNAs could occur during the posttranscriptional processing of other species of RNA in the nucleus. The observation that three species of snRNA are hydrogen-bonded to 28S rRNA in the nucleolus is consistent with the hypothesis that these nucleolar snRNAs are involved in some unknown manner in the maturation of 28S rRNA from larger precursors and in one case (5.8S snRNA) with the function of 28S rRNA in the ribosome (Prestayko et al., 1970). Recently, it has been reported that individual snRNA species can be recovered from nuclear ribonucleoprotein preparations which contain heterogeneous nuclear RNA (hnRNP; Deimel et al., 1977). This raises the interesting possibility that the snRNAs in hnRNP are involved in the processing of hnRNA and perhaps also in the maturation and transport of mRNA to the cytoplasm. Before this possibility can be considered seriously it must be demonstrated that snRNAs are native components and not contaminating elements of hnRNP. In this report, we show that several nonnucleolar snRNA species copurify on sucrose gradients with hnRNP obtained by the sonic disruption of MEL cell nuclei under conditions where the artificial associ-

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0006-2960/78/0417-3228$01.00/0