Uraguchi, K. (1962), *Nippon Yakurigaku Zasshi 58*, 45.
Uraguchi, K., Sakai, F., Tsukioka, M., Noguchi, Y., Tatsuno, T., Saito, N., Enomoto, M., Ishiko, T., Shikata, T., and Miyake, M. (1961), *Jap. J. Exp. Med. 31*, 435.
Von der Helm, K., and Krakow, J. S. (1972), *Nature (London) 235*, 82.
Walter, G., Zillig, W., Palm, P., and Fuchs, E. (1967), *Eur. J. Biochem. 3*, 194.
Wehrli, W., Knüsel, F., Schmid, K., and Staehelin, M. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 667.

Characterization of the Poly(adenylic acid) Regions and the Adjacent Nucleotides in Heterogeneous Nuclear Ribonucleic Acid and Messenger Ribonucleic Acid from HeLa Cells†

George R. Molloy‡,* and J. E. Darnell

ABSTRACT: Poly(A) segments derived from either Hn-RNA or mRNA molecules with T1 RNase consisted of two uridylate and one–two cytidylate residues per 195 adenylate residues and were devoid of any GMP residues. Pancreatic RNase produced poly(A) segments approximately 195 nucleotides long which consisted exclusively of adenylic acid. These experiments indicated that at least 80–90% of the poly(A) in Hn-RNA and mRNA is located at the 3′-OH termini and that the nucleotides adjacent to the poly(A) in Hn-RNA and mRNA are the same. This result further supports the hypothesis that some Hn-RNA molecules are precursors to mRNA molecules.

The nucleus of mammalian cells contains very high molecular weight RNA molecules varying in size from 6,000 to at least 30,000 nucleotides in length (Hn-RNA, heterogeneous nuclear RNA) which is similar in base composition to the messenger RNA fraction (mRNA) recovered from the polyribosomes (Scherrer *et al.*, 1963; Scherrer and Marcaud, 1965; Houssais and Attardi, 1966; Soeiro *et al.*, 1966) in the cell cytoplasm. The speculation was advanced a number of years ago that the mRNA was a product of posttranscriptional modification of the higher molecular weight Hn-RNA (Darnell, 1968). Recent experiments have shown that Hn-RNA and mRNA contain adenylic acid rich regions, poly(A), about 200 nucleotides long which are located at the 3′-OH terminus of the RNA molecules (Kates, 1970; Edmonds *et al.*, 1971; Lee *et al.*, 1971; Darnell *et al.*, 1971b; Mendecki *et al.*, 1972; Molloy *et al.*, 1972a; and Sheldon *et al.*, 1972). The poly(A) appears to be initially added to Hn-RNA molecules following transcription and is thereafter transferred to the cytoplasm as part of mRNA molecules (Darnell *et al.*, 1971a; Jelinek *et al.*, 1973). It appears therefore that mRNA is derived from a high molecular weight nuclear precursor and that the nucleotide sequences involved in protein synthesis are located near the 3′ terminus of the Hn-RNA precursor molecules.

In the experiments reported here an examination was made of poly(A) segments derived by the separate or combined use of T1 RNase (specific for guanylate residues; Arina *et al.*, 1968) and pancreatic RNase (specific for pyrimidine residues; Markham and Smith, 1952). From these studies it was possible to determine that the poly(A) segments in Hn-RNA and mRNA molecules consisted entirely of adenylic acid residues and that the nucleotides adjacent to the nuclear poly(A) were the same as those adjacent to the mRNA poly(A), giving further support to the hypothesis that some Hn-RNA molecules are precursors to mRNA molecules. In addition, as explained in detail below, base composition analysis of the poly(A) segments labeled and derived in various ways indicated that the great majority, if not all, of the poly(A) was located immediately at the 3′-OH end of the Hn-RNA and mRNA chains.

Experimental Procedures

Hela cells were grown in suspension culture as described (Eagle, 1959) and 3×10^8 cells was labeled in 150 ml of medium with either 5 mCi of [^{3}H]adenosine (18.3 Ci/mmol), 20 mCi of [^{3}H]uridine (24.3 Ci/mmol), or 50 mCi of $^{32}PO_4$ (carrier free, Schwarz-Mann). ^{32}P labeling was carried out by washing cells twice and resuspending in PO_4 free Eagle's medium supplemented with 5% dialyzed fetal calf serum. Unless otherwise stated, cells were treated for 30 min with 0.05 μg/ml of actinomycin D to stop ribosomal RNA synthesis prior to labeling with isotope for 4 hr (Perry, 1963).

Labeled cells were fractionated as previously described (Molloy *et al.*, 1972b) and phenol-extracted mRNA and Hn-RNA isolated after sucrose gradient sedimentation (Soeiro and Darnell, 1969). Nuclease digestion of purified RNA samples was carried out at 37° for 60 min in a buffer designated NET (0.1 M NaCl–0.01 M EDTA–0.01 M Tris, at pH 7.4) with pancreatic RNase (Sigma recrystallized 5×) 2 μg/ml or T1 RNase (Sankyo) at 10 units/ml. Nuclear samples were digested and extracted twice to ensure cleavage at all susceptible bonds. After digestion, samples were phenol extracted and RNase-resistant material precipitated with ethanol and redissolved in buffer designated as NETS (the same as NET with 0.2% sodium dodecyl sulfate added).

† From the Department of Biological Sciences, Columbia University in the City of New York, New York, New York 10027. *Received January 12, 1973.* This work was supported by grants from the National Institutes of Health (CA 11159-04), the National Science Foundation (GB 27691X), and the American Cancer Society (NP-5A).

‡ George R. Molloy is the recipient of an American Cancer Society Postdoctoral Fellowship.

TABLE I

	Possible Sequence	Enzyme Treatment	Product	Diagnostic Property
Poly(A) at 3′-OH	5′---PypGpA_{200}	T1	A_{200}	Entirely Ap
		P	GpA_{200}	Gp possible at 5′ end
	5′---GpPyppA_{200}	T1	PypA_{200}	Pyp possible at 5′ end
		P	A_{200}	Entirely Ap
Poly(A) internal to 3′-OH	5′---GpPyppA_{200}pPypGp---3′	T1	PypA_{200}pPypGp	Gp present at 3′ end
		P	A_{200}pPyp	Pyp present at 3′ end
	5′---PypGppA_{200}pGpPyp---3′	T1	A_{200}pGp	Gp present at 3′ end
		P	GpA_{200}pGpPyp	Pyp present at 3′ end

Adsorption of poly(A) from nuclease digested samples was carried out on poly(U)–Sepharose columns (0.5 × 3 cm) as previously described for samples from both Hn-RNA and mRNA (Jelinek *et al.*, 1973; Adesnik *et al.*, 1972). For samples of nuclear RNA digested with T1 alone, elution was performed with a formamide gradient, 0–90% formamide in ETS buffer (0.01 M EDTA–0.2% sodium dodecyl sulfate–0.01 M Tris (pH 7.4), while the mRNA samples and nuclear RNA samples digested with pancreatic RNase were eluted with 90% formamide–10% ETS immediately after adsorption and washing of the column. The poly(A) eluted at about 30% formamide while the smaller oligo(A) fragment, to be described elsewhere, eluted between 0 and 15% formamide.

Gel electrophoresis of column-adsorbed samples was carried out in 15% polyacrylamide gels containing 8 M urea as previously described (Jelinek *et al.*, 1973). Elution of poly(A) from gels was carried out by shaking gel slices in 10 ml of NETS buffer at 45°.

Alkaline phosphatase (Worthington) was rendered endonuclease-free by treatment with diethyl pyrocarbonate according to Wimmer (1972). The poly(A) segments were treated with 2 units of alkaline phosphatase in 0.015 M Tris-HCl (pH 8.0) for 50 min at 37°. Gel electrophoresis of an aliquot of the solution showed the complete absence of endonucleolytic degradation of the poly(A) as previously demonstrated (Molloy *et al.*, 1972b). The remainder of the poly(A) was extracted twice with phenol–$CHCl_3$ (1:1), precipitated with ethanol, and submitted to base composition analysis by electrophoresis at pH 3.5 according to Salzman and Sebring (1964).

Results

Isolation and Characterization of Poly(A) Containing RNA Segments from Hn-RNA and mRNA Molecules. T1 RNase hydrolyzes the phosphodiester bond between a guanylic acid residue and the adjacent 3′ nucleotide (5′---pGp|Xp----3′) while pancreatic RNase hydrolyzes the phosphodiester bond between pyrimidine nucleotides and the adjacent 3′-nucleotide (5′---pPyp|Xp---3′). Beers (1960) showed that pancreatic RNase can hydrolyze commercial poly(A) to small oligonucleotides in solutions of low ionic strength; 0.1 M NaCl renders poly(A) resistant to hydrolysis. Therefore, the hydrolysis of RNA molecules with T1 RNase should produce poly(A) segments containing any adjacent pyrimidine nucleotides and potentially a guanylic acid residue at the 3′ terminus (henceforth known as poly(A)–T1 segments). (See possibilities diagrammed in Table I.) However, if all of the poly(A) was located at the 3′-OH termini of the RNA chains, the alkaline hydrolysate of a poly(A)–T1 segment should be completely devoid of guanylic acid (Table I.) Digestion with pancreatic RNase in 0.1 M NaCl should produce poly(A) segments containing any adjacent guanylate residues and a potential pyrimidine nucleotide at the 3′ terminus (designated poly(A)–P segments). If the poly(A) were exclusively located at the 3′-OH terminus, an alkaline hydrolysate of the poly(A)–P segments should not contain any pyrimidine nucleotides.

It was reported earlier that the poly(A) obtained after digestion with both T1 and pancreatic RNase followed by affinity chromatography on poly(dT)-cellulose was 95–99% adenylic acid (Edmonds *et al.*, 1971). However, the content of other nucleotides was not given. It was also previously reported from this laboratory that poly(A) from HeLa RNA which had not been purified beyond gel electrophoresis of the digests consisted of 85–92% AMP residues (Darnell *et al.*, 1971a) with small amounts of the other three nucleotides. For the present experiments it was necessary to isolate intact poly(A) segments completely devoid of oligonucleotide contamination. ^{32}P-labeled Hn-RNA as well as mRNA were prepared and divided into equal fractions, one of which was treated with T1 RNase and the other with pancreatic RNase. The poly(A)-containing RNA segments were isolated from the remainder of the digested RNA by affinity chromatography on poly(U)–Sepharose followed by polyacrylamide gel electrophoresis.

When nuclear RNA was digested with T1 RNase, it was necessary to employ an exponential formamide gradient during poly(U)–Sepharose chromatography to separate the large *poly(A)* from smaller adenylate-rich RNA segments (henceforth, known as oligo(A); see Experimental Procedures). This oligo(A) had a base composition of 57% AMP, 15% CMP, 23% UMP, and 5% GMP and was observed to migrate on acrylamide gels as though its average length was 20 nucleotides, although a small amount of this material always migrated in the large poly(A) region. A more detailed investigation of the oligo(A) will be published separately. In the experiments where Hn-RNA was digested with pancreatic RNase, it was not necessary to employ the formamide gradient to isolate highly purified large poly(A) segments, probably due to the fact that any contaminating adenylate-rich regions were more thoroughly digested. Therefore, the poly(A)–P segments were eluted in one step from poly(U)-Sepharose with 3 ml of formamide elution buffer. Both the poly(A)–T1 and poly(A)–

[1] Abbreviations used are: Hn-RNA, heterogeneous nuclear ribonucleic acid; NET, 0.1 M NaCl–0.01 M EDTA–0.01 M Tris, pH 7.4; NETS, NET plus 0.2% sodium dodecyl sulfate; ETS, 0.01 M EDTA–0.2% sodium dodecyl sulfate–0.01 M Tris.

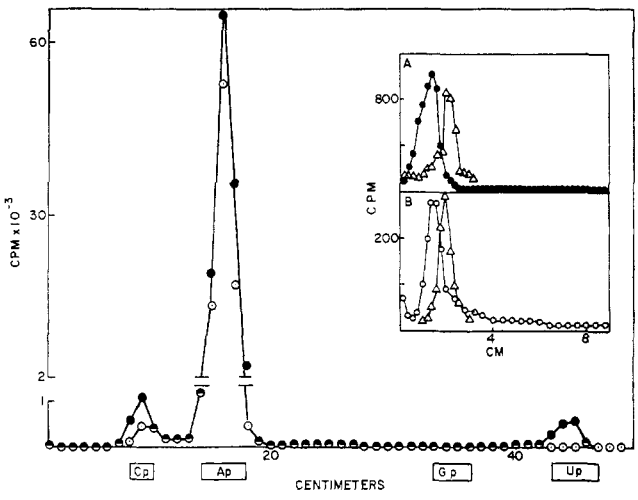

FIGURE 1: Isolation and base composition of the poly(A) segments derived from high molecular weight nuclear RNA. ^{32}P-labeled Hn-RNA greater than 32 S was prepared as previously described (Molloy *et al.*, 1972b) from cells that were not pretreated with a low dose of actinomycin D. The RNA was dissolved in NET buffer and portions were treated either with T1 RNase or pancreatic RNase (see Methods). The digested RNA samples were placed on separate columns (0.3 × 3 cm) of poly(U)-Sepharose and affinity chromatography was performed as described under Methods. Both poly(A)–T1 and –P segments were then precipitated with ethanol, resuspended in 0.35 ml of 20% glycerol–0.2% sodium dodecyl sulfate–8 M urea containing Bromophenol Blue, and incubated at 50° for 3 min immediately prior to layering on 8 M urea–15% polyacrylamide gels (0.075% *N,N'*-methylenebisacrylamide). Electrophoresis was allowed to proceed until the Bromophenol Blue dye had migrated 9 cm and the poly(A) segments approximately 1.5 cm, as determined by Cerenkov radiation. The poly(A) segments were eluted from the gels, precipitated in ethanol, and again chromatographed on separate poly(U)-Sepharose columns employing elution with 3 ml of formamide elution buffer. The poly(A) was precipitated with ethanol and hydrolyzed in 0.10–0.15 ml of 0.3 N KOH at 37° for 18–24 hr prior to base composition analysis (Salzman and Sebring, 1964). Alkaline-hydrolyzed nuclear RNA was subjected to electrophoresis along with the hydrolysate of the poly(A) segments to localize the four nucleotides. The figure shows the electropherogram of the pancreatic (○—○) and T1 (●—●) nuclear poly(A) segments after alkaline hydrolysis. The inset shows the migration on 15% gels of an aliquot of the nuclear poly(A) segments in relation to the mRNA poly(A)–T1 immediately prior to alkaline hydrolysis: (A) ●—●, nuclear poly(A)–T1; (B) ○—○, nuclear poly(A)–P; △—△, mRNA poly(A)–T1.

P segments derived from polysomal RNA could be eluted in this fashion because any contaminating adenylate-rich fraction was present only in trace amounts, if at all.

Figures 1 and 2 and Table II indicate that the nucleotide composition of the nuclear poly(A)–P and T1 segments were similar to the corresponding mRNA poly(A) segments. The poly(A)–T1 segment from Hn-RNA contained 1.5–1.6% CMP, 1.1% UMP, and >97% AMP, suggesting the structure $C_8U_2A_{195}$. The mRNA T1–poly(A) segment contained 1.7% CMP, 1.1–1.5% UMP, and >96% AMP which was consistent with the structure $C_8U_2A_{180-195}$. The slightly higher percentage of pyrimidines in the mRNA fragments is consistent with the slightly faster mobility of the mRNA poly(A) (inset, Figure 1). Several groups have noticed this decreased mobility of poly(A) segments that come from the mRNA of cells which have incorporated label for several hours (Mendecki *et al.*, 1972; Edmonds *et al.*, 1972;[2] Sheiness and Darnell, 1972). The shorter poly(A) in mRNA reflects a progressive shortening that occurs in the cytoplasm.

The complete absence of GMP in the poly(A)–T1 segments from both the Hn-RNA and the mRNA demonstrated that there was no guanylate residue between the poly(A) and the 3′-terminal nucleotide. Since as much as 130,000 cpm of the poly(A)–T1 segment was analyzed at one time, the presence of one GMP per poly(A) segment would have produced 650 cpm above background in the 4 cm of the electropherogram containing GMP. The radioactivity (excluding machine background) in the region of GMP was less than 0.2–0.1 this amount. Therefore, over 90% of the poly(A) from the Hn-RNA as well as the mRNA must be closer to the 3′-OH terminus than any region in the RNA chain containing a single GMP. This result does not exclude the possibility that a guanosine or other nucleoside might be the 3′-OH terminal residue. It could have been possible, of course, that some of the pyrimidine nucleotides present in the poly(A)–T1 were located between the 3′-OH terminal nucleotide and the poly(A). We expected that this latter possibility could be tested by analyzing the nucleotide composition of the poly(A)–P segments because only one CMP or UMP residue adjacent to the 3′ terminus of the poly(A) should remain after pancreatic RNase digestion. However, the poly(A)–P segments from both Hn-RNA and mRNA contained on first analysis what appeared to be 1% CMP and 99% AMP. Succeeding experiments will demonstrate, however, that there was no true CMP in poly(A)–P segments. Thus the results with both T1 and pancreatic RNase taken together show that no GMP residue and no pyrimidine residues exist on the 3′ side of the poly(A), *i.e.*, the poly(A) segment is at the 3′-OH terminus.

To return to the proof of the nature of the apparent CMP residues in poly(A)–P fragments, it was first noted that the CMP-like material frequently migrated 0.5–1 cm faster than the marker CMP, suggesting that it might not be truly CMP. In addition, the poly(A)–P fragment should contain only one CMP residue and the analysis showed 1% apparent CMP or two residues per poly(A) segment.

If there were two CMP's in the poly(A)–P fragment one should be at the 3′ terminus and its phosphate group should be removable with alkaline phosphatase. Subsequent alkaline hydrolysis and nucleotide analysis should show a disappearance of half the CMP in the poly(A)–P segment. Table II shows that alkaline phosphatase treatment of nuclear or mRNA poly(A)–P segments did not decrease the apparent CMP content of either. In other experiments, performed with the same reagents in this laboratory, alkaline phosphatase did remove the ^{32}P from GMP residues located at the 3′ terminus of a uridylic acid rich oligonucleotide (Molloy *et al.*, 1972b) and oligo(A) (Molloy *et al.*, 1973[3]). Thus, it appeared that no CMP-like residue was at the 3′ terminus of the poly(A)–P segment. It remained possible, however, that either the CMP was located internally in the poly(A) but was not susceptible to pancreatic RNase because of the conformation of poly(A) in 0.1 M NaCl (Beers, 1960) or indeed it was not truly CMP.

To test the possibility of a chemical modification of AMP which produced a nucleotide with electrophoretic properties similar to CMP, the following experiments were carried out. (1) mRNA was prepared from cells labeled with [^{3}H]adenosine and the poly(A)–P segment was isolated and subjected to base composition analysis. Figure 3A shows that indeed 1.5% of the material comigrates with or just slightly ahead of CMP

[2] Edmonds, M., *et al.* (1972), personal communication.

[3] Molloy, G. R., *et al.* (1973), unpublished information.

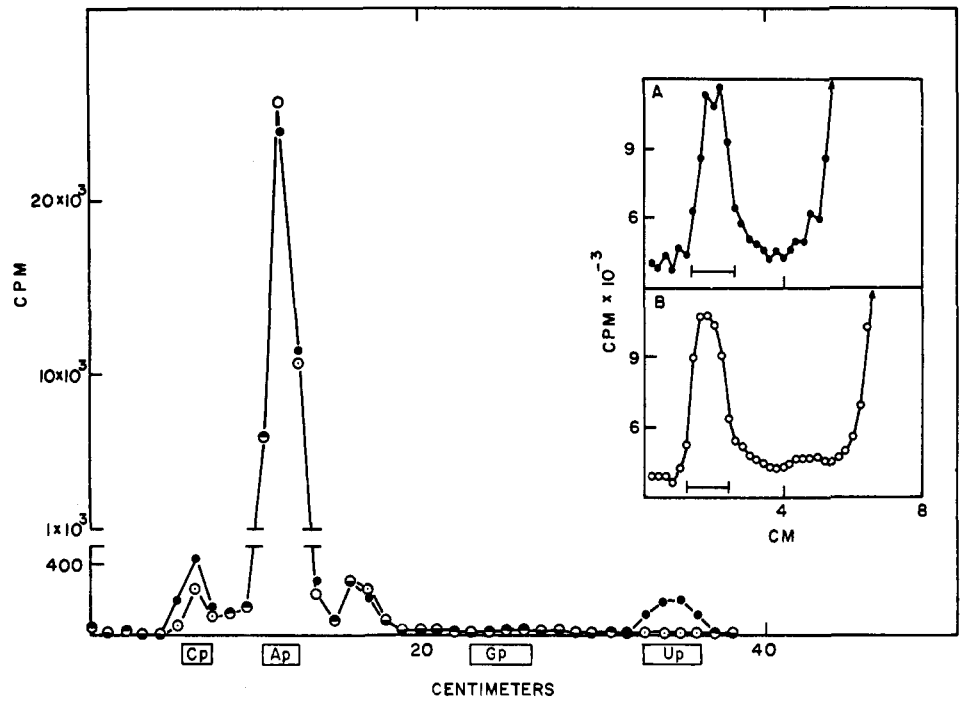

FIGURE 2: Isolation and base composition of the poly(A)-containing RNA segments derived from mRNA. ^{32}P-labeled polysomes were prepared as previously described (Penman *et al.*, 1963) and adjusted to 0.5% sodium dodecyl sulfate. The RNA was precipitated in ethanol, redissolved in NETS buffer, and extracted three times in 2 vol of phenol–chloroform (1:1). RNA between 9 and 28 S was recovered after sucrose gradient sedimentation (Molloy *et al.*, 1972a), divided into two equal fractions in RNase buffer, and treated once with either T1 or pancreatic RNase as described in Figure 1. After phenol extraction the RNase-digested samples were subjected to electrophoresis as described in Figure 1. The inset shows the electrophoretic pattern of the nuclease resistant fraction of the total T1 (A) and pancreatic (B) RNase digests. The poly(A) was eluted from the gel (the included fractions are indicated by the bars) and chromatographed on separate poly(U)–Sepharose columns employing elution with 3 ml of formamide elution buffer, and its base composition determined. The figure shows the electropherogram of the mRNA poly(A)–T1 (●—●) and poly(A)–P (○—○) segments after alkaline hydrolysis. The label which appears in fractions 16 and 17 was seen in many preparations with ^{32}P or adenosine label. It is in the position known to be ApAp and when this material was eluted and subjected to further alkaline hydrolysis it all migrated as AMP. Neighboring adenylate residues are known to be the most resistant to KOH hydrolysis (Lane and Cutler, 1959).

while the remainder of the material migrates similarly to AMP. This CMP-like material which is labeled by adenosine was isolated from ^{32}P labeled poly(A)–P and shown to be a mononucleotide by quantitative removal of the phosphate as inorganic phosphate by alkaline phosphatase. (2) Commercial [^{3}H]adenine labeled poly(A) was hydrolyzed and subjected to electrophoresis. Again approximately 1% of the radioactivity was recovered in the CMP region (Figure 3B). (3) When isolated AMP was subjected to alkaline hydrolysis and electrophoresis approximately 0.9% of the radioactivity migrated similarly to CMP (Figure 3C). Thus there appear to be no nucleotides in the poly(A)–P fragment except adenylic acid. The CMP-like material found associated with the poly(A)–P segments was the result of a chemical modification of 1% of the AMP during the standard alkaline hydrolysis (24 hr, 37°, 0.3 N NaOH).

The artifact complicated the estimation of the number of authentic CMP residues present in the poly(A)–T1 segments. For example, the total CMP-like material indicated 3 CMP residues per 200 AMP residues and correction due to the artifact indicated that 1 CMP residue was probably the correct number. Because of this uncertainty a second procedure was employed to determine the UMP and CMP content of the poly(A)–T1. Since both the UMP and CMP residues can be labeled by incubating cells in the presence of [^{3}H]uridine (Salzman and Sebring, 1959), poly(A)–T1 segments from such cells should also allow a determination of UMP and true CMP content. In addition, complete removal of pyrimidine label by pancreatic RNase without destruction of the poly(A) fragment would strongly imply an external location of all the true pyrimidine residues in the poly(A) segment and confirm that poly(A)–P fragments contained only AMP. [^{3}H]Uridine was, in fact, incorporated into both the CMP and UMP residues in the total mRNA; however, 70% of the radioactivity was in UMP while the base composition of the mRNA has previously shown that UMP and CMP are about equal. Thus, it appeared that during the formation of the labeled mRNA in this experiment the UMP pool was more radioactive than the CMP pool by a factor of 70:30. Figure 4 shows that it was possible to obtain a [^{3}H]uridine-labeled poly(A)–T1 segment from the labeled mRNA. Therefore, if the number of UMP and CMP residues in the poly(A)–T1 segment were about equal, as indicated in Table II, about 30% of the ^{3}H radioactivity should have been present in CMP. In fact, about 25% of the radioactivity was present in CMP and 75% in UMP, indicating approximately equal numbers of UMP and CMP residues in the poly(A)–T1. In addition all the pyrimidine residues in the poly(A)–T1 segments were accessible to pancreatic RNase.

Figure 4 shows that a second treatment of the [^{3}H]uridine-labeled poly(A)–T1 segment and a ^{32}P poly(A)–P internal marker with T1 RNase had, as expected, no effect on their electrophoretic migration. However, treatment with pancreatic RNase removed essentially all of the ^{3}H label (96%) from the poly(A)–T1 segment indicating that there were no true CMP residues present in a poly(A)–P segment (Figure 4). The fact that the structure of the internal marker, [^{32}P]poly(A)–P, was not affected by pancreatic RNase demonstrated that the

TABLE II: Base Composition of the Pancreatic and T1–Poly(A) Segment in Hn-RNA and mRNA.

	Expt	CMP[a] (%)	AMP (%)	GMP[b] (%)	UMP[b] (%)
T1 RNase–Poly(A) Segments					
Hn-RNA	1	1.6 (2064)	97.3 (125,867)	0 (33)	1.1 (1450)
	2	1.5 (1160)	97.4 (72,996)	0 (32)	1.1 (800)
mRNA	1	1.8 (805)	96.6 (42,070)	0.1 (44)	1.4 (633)
	2	1.3 (1330)	97.6 (99,961)	0 (27)	1.1 (1172)
	3	2.0 (1731)	96.4 (80,022)	0 (20)	1.5 (1216)
	4	1.3 (1514)	97.1 (109,588)	0 (21)	1.5 (1690)
Pancreatic RNase–Poly(A) Segments					
Hn-RNA	1	1.1 (980)	98.8 (87,400)	0 (20)	0 (28)
	2	1.0 (528)	98.9 (48,924)	0 (17)	0 (43)
+ Alkaline phosphatase[c]	3	0.9 (301)	99.1 (33,159)	0 (17)	0
mRNA	1	1.0 (425)	98.8 (42,390)	0.1 (40)	0
	2	1.2 (557)	98.7 (45,052)	0 (9)	0
+ Alkaline phosphatase[c]	3	0.9 (419)	98.1 (46,706)	0 (7)	0 (8)

[a] Figures in this column include radioactivity migrating similarly to CMP which was shown later not to be truly CMP. [b] Zero indicates less than 0.1% and the numbers in parentheses are the detectable counts per minutes above machine background, which was 25 cpm per sample. [c] The procedure for the alkaline phosphatase treatment of the poly(A)–P segments is described under Experimental Procedures.

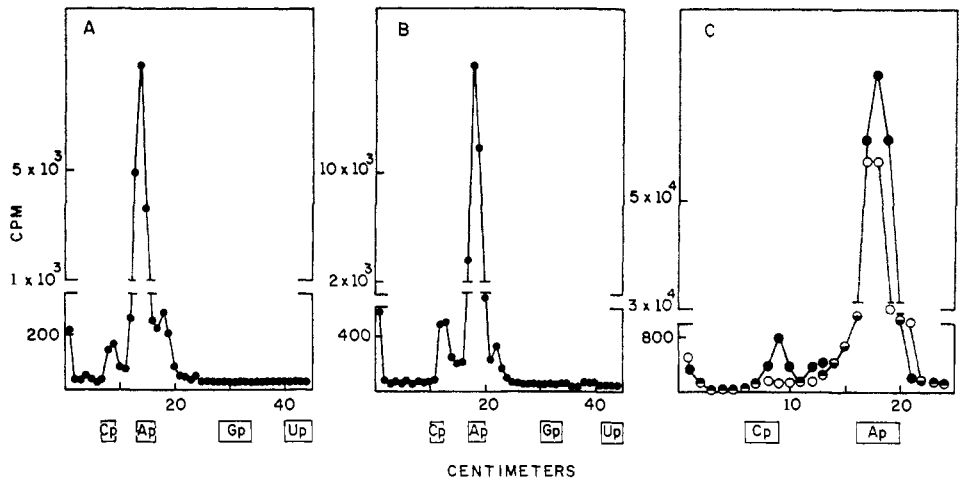

FIGURE 3: (A) Nucleotide analysis of the adenosine-labeled poly(A)–P segment. Cells were labeled with [^{3}H]adenosine and large molecular weight cytoplasmic RNA was prepared as in Figure 4A, treated with pancreatic RNase, and subjected to poly(U)–Sepharose chromatography followed by gel electrophoresis as described in Figure 2. After extraction from the gel, the poly(A) was again chromatographed on poly(U)–Sepharose prior to base composition analysis. (B) Nucleotide analysis of ^{3}H-labeled commercial poly(A) (Miles) which was subjected to the standard conditions of alkaline hydrolysis and high voltage electrophoresis at pH 3.5. (C) ^{32}P-Labeled nuclear RNA was subjected to alkaline hydrolysis and electrophoresis. The adenylic acid was eluted from the electropherogram, divided, and incubated either in 0.3 N KOH or sterile H_2O for 24 hr at 37° prior to electrophoresis: ●—●, AMP inclubated in 0.3 N KOH (total cpm, 2×10^5): ○—○, AMP inclubated in H_2O (total cpm, 1.5×10^5).

removal of [^{3}H]uridine-derived radioactivity from the poly(A) segment was not due to endonucleolytic degradation of the adenylate portion of the ^{3}H-labeled poly(A)–T1. In support of these results, it was found that mRNA prepared from cells labeled with [^{3}H]uridine as in Figure 4 and digested with pancreatic RNase did not give rise to a labeled RNA segment that would bind to poly(U)–Sepharose and migrate like poly(A) in 15% acrylamide gels, again indciating that CMP is not present in a poly(A)–P segment.

Discussion

This paper provides evidence concerning two problems: (1) the nucleotides next to poly(A) in mRNA and Hn-RNA and (2) the position of poly(A) within mRNA and Hn-RNA molecules and its mode of addition.

(1) When T1 RNase was used to prepare poly(A) segments from ^{32}P-labeled Hn-RNA or mRNA molecules approximately 2 UMP and 3 apparent CMP residues were found per 195 adenylate residues. About two-thirds of the CMP proved to be derived from chemical modification of the adenylate during alkaline hydrolysis. [^{3}H]Uridine-labeled poly(A)–T1 segments, synthesized when the cell pool contributed label to UMP and CMP in total mRNA in a ratio of 70:30, contained label in UMP and CMP in a ratio of 75:25. Since mRNA contains approximately equal amounts of UMP and CMP (Darnell *et al*, 1971b) it appears that on the average each ^{3}H-labeled poly(A)–T1 segment contains two residues of

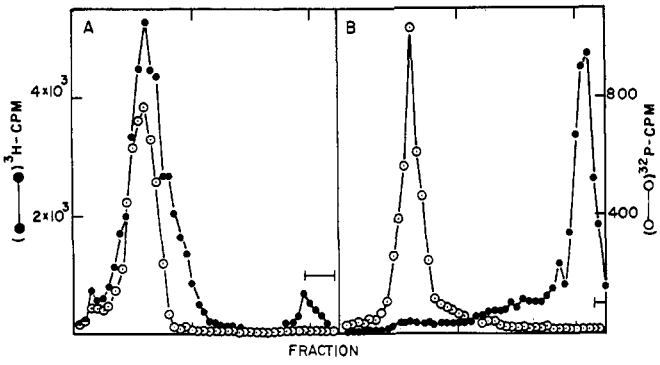

FIGURE 4: Uridine labeling of the poly(A)–T1 segment. The total cytoplasmic fraction from cells labeled with [^{3}H]uridine was adjusted to 0.5% Brij-58, 0.5% deoxycholate, and 0.5% sodium dodecyl sulfate and the high molecular weight RNA was precipitated with 2 M LiCl and extracted with phenol–cholorform. The relative amount of radioactivity in UMP was found to be 70% of the total and that present in CMP was 30%. The RNA was treated with T1 RNase, and phenol–chloroform was extracted and chromatographed on poly(U)–Sepharose. The poly(A)–T1 segments were eluted with 3 ml of 90% formamide elution buffer, subjected to polyacrylamide gel electrophoresis as in Figure 1, and subsequently eluted from the gel. The ^{3}H-labeled poly(A)–T1 segment (38×10^3 cpm) plus an internal standard of ^{32}P-labeled poly(A)–P (4×10^3 cpm) isolated from polysomal RNA was incubated in 0.2 ml of RNase buffer at 37° for 60 min with 10 units/ml of T1 RNase (A) and separate samples with 2 μg/ml of pancreatic RNase (B). After incubation each sample was adjusted to 0.5% sodium dodecyl sulfate–8 M urea and Bromophenol Blue dye was added immediately prior to gel electrophoresis as in Figure 1. The bar indicates the position of the Bromophenol Blue. Each fraction contains two 1-mm slices: ●—●, [^{3}H]poly(A)–T1; ○—○, [^{32}P]poly(A)–P.

UMP and two of CMP. Therefore, nucleotide analysis of poly(A)–T1 segments from Hn-RNA and mRNA indicates the presence of two UMP residues and one–two CMP residues. These data suggest that each poly(A)–T1 segment might contain the same sequence adjacent to the poly(A) and, since the nucleotide composition of the poly(A)–T1 segments was the same in the Hn-RNA and mRNA, such a similar sequence might be part of a recognition site for enzyme(s) that add poly(A) to Hn-RNA molecules. To further investigate the constancy of the poly(A)–T1 segment, attempts are now under way to define the sequence of the nucleotides adjacent to the poly(A) in both mRNA and Hn-RNA. It should be noted that although there are, on the average, three–four pyrimidine residues adjacent to the poly(A), it is not presently known if there are any AMP residues not contained within the strictly polyadenylate regions of the poly(A)–T1. These would not be detected as being different from the majority of the AMP after alkaline hydrolysis. Finally, it should be pointed out that if the scheme of poly(A) addition to Hn-RNA followed by transfer to the cytoplasm is correct, it is necessary that the adjacent nucleotides in both Hn-RNA and mRNA be the same; they are.

(2) During the completion of this work a number of reports have appeared which also indicated that poly(A) exists mainly at or close to the 3′-OH end of mRNA and Hn-RNA. Mendecki *et al.* (1972) reported the recovery of about one adenosine residue per 200 AMP residues from the poly(A) in mRNA and Hn-RNA molecules in mouse sarcoma ascites cells, although relatively small amounts of radioactivity were available for the determinations. Nakazato *et al.* (1973) have shown through end group analyses that a large fraction of the poly(A) was located at the 3′-OH termini of the mRNA and Hn-RNA molecules in HeLa cells. In addition, experiments performed with a highly purified 3′-OH specific exonuclease indicated that 90%, if not all, of the poly(A) in mRNA (Molloy *et al.*, 1972b) and Hn-RNA (Sheldon *et al.*, 1972) was located at the 3′-OH terminus. In the present experiments poly(A) segments prepared by T1 RNase contained sufficient radioactivity to allow the firm conclusion that at least 80–90% of the poly(A) is close enough to the 3′ terminus so as not to include a GMP. Furthermore, poly(A) segments prepared by pancreatic RNase or pancreatic RNase treatment of poly(A)–T1 segments showed the complete absence of any nucleotides other than AMP. Thus, from these results only a nucleoside could exist between the poly(A) and the 3′-OH terminus of the RNA chains and the results of Mendecki *et al.* (1972) suggest that an adenosine residue is, in fact, present at the 3′-OH terminus. In addition the present results show the poly(A) to consist exclusively of adenylic acid. These two properties accord well with the suggestion that the synthesis of poly(A) is probably not DNA dependent (Darnell *et al.*, 1971a) but is a post-transcriptional event. A final point should be made about a chemical artifact uncovered in this work which will plague others working on the chemistry of poly(A) segments. The normal conditions of alkaline hydrolysis of RNA lead to a chemical change in 1% of the AMP to yield something which migrates like CMP. The presence of this derivative was probably responsible for leading one group to conclude that the poly(A) in poliovirus had a CMP at its 3′ terminus (Armstrong *et al.*, 1972). This was apparently an erroneous result because Yogo and Wimmer (1972), employing experimental procedures not involving alkaline hydrolysis, have found that the poly(A) consisted entirely of AMP residues and was located at the 3′-OH terminus of poliovirus RNA.

Acknowledgments

We thank Dr. Warren Jelinek for critical reading of the manuscript and Mr. William Thomas for excellent technical assistance.

References

Adesnik, M., and Darnell, J. E. (1972), *J. Mol. Biol. 67*, 397.
Adesnik, M., Salditt, M., Thomas, W., and Darnell, J. E. (1972), *J. Mol. Biol. 71*, 21.
Arina, T., Uchida, T., and Egami, F. (1968), *Biochem. J. 106*, 609.
Armstrong, J. A., Edmonds, M., Nakazato, H., Phillips, B. A., and Vaughan, M. H. (1972), *Science 176*, 526.
Beers, R. F. (1960), *J. Biol. Chem. 235*, 2393.
Darnell, J. E. (1968), *Bacteriol. Rev. 32*, 262.
Darnell, J. E., Philipson, L., Wall, R., and Adesnik, M. (1971a), *Science 174*, 507.
Darnell, J. E., Wall, R., and Tushinski, R. (1971b), *Proc. Nat. Acad. Sci. U. S. 68*, 1321.
Eagle, H. (1959), *Science 130*, 432.
Edmonds, M., Vaughan, M. H., and Nakazato, N. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 1336.
Houssais, J. F., and Attardi, G. (1966), *Proc. Nat. Acad. Sci. U. S. 56*, 616.
Jelinek, W., Adesnik, M., Salditt, M., Sheiness, D., Wall, R., Molloy, G., Philipson, L., and Darnell, J. E. (1973), *J. Mol. Biol. 75*, 515.
Kates, J. (1970), *Cold Spring Harbor Symp. Quant. Biol. 35*, 743.
Lane, B. G., and Cutler, G. C. (1959), *Can. J. Biochem. Physiol. 37*, 1329.

Lee, Y., Mendecki, J., and Brawerman, G. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 1331.
Markham, R., and Smith, J. D. (1952), *Biochem. J. 52*, 552, 558, 565.
Mendecki, J., Leey, Y., and Brawerman, G. (1972), *Biochemistry 11*, 792.
Molloy, G. R., Sporn, M. G., Kelley, D. E., and Perry, R. P. (1972a), *Biochemistry 11*, 3256.
Molloy, G., Thomas, W., and Darnell, J. E. (1972b), *Proc. Nat. Acad. Sci. U. S. 69*, 3684.
Nakazato, H., Kopp, D., and Edmonds, M. (1973), *J. Biol. Chem. 248*, 1472.
Penman, S., Becker, Y., Scherrer, K., and Darnell, J. E. (1963), *Proc. Nat. Acad. Sci. U. S. 49*, 654.
Perry, R. P. (1963), *Exp. Cell. Res. 29*, 400.
Salzman, N. P., and Sebring, E. D. (1959), *Arch. Biochem. Biophys. 84*, 143.
Salzman, N. P., and Sebring, E. D. (1964), *Anal. Biochem. 8*, 126.
Scherrer, K., Latham, H., and Darnell, J. E. (1963), *Proc. Nat. Acad. Sci. U. S. 49*, 240.
Scherrer, K., and Marcaud, L. (1965), *Bull. Soc. Chim. Biol. 47*, 1697.
Sheiness, D., and Darnell, J. E. (1972), *Nature (London) 241*, 265.
Sheldon, R., Kates, J., Kelley, D., and Perry, R. P. (1972), *Biochemistry 11*, 3829.
Soeiro, R., Birnboim, H. C., and Darnell, J. E. (1966), *J. Mol. Biol. 19*, 362.
Soeiro, R., and Darnell, J. E. (1969), *J. Mol. Biol. 44*, 551.
Wimmer, E. (1972), *J. Mol. Biol. 68*, 537.
Yogo, Y., and Wimmer, E. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 1877.

Preparation, Molecular Weight, Base Composition, and Secondary Structure of Giant Nuclear Ribonucleic Acid†

David S. Holmes* and James Bonner

ABSTRACT: Previous methods of heterogeneous ribonucleic acid (HnRNA) extraction yield material which "disaggregates" into small molecules. This could be the fault of either ribonuclease knicks in the polymers sustained during the extraction procedure or disaggregation into real subunits. The present communication distinguishes between these possibilities by describing an RNA extraction procedure which does not yield subunits when HnRNA is denatured. By the criteria of sedimentation through sucrose, formaldehyde, and dimethyl sulfoxide, it is estimated that the majority of the radioactivity of giant HnRNA after a 30-min pulse of [^{3}H]uridine is associated with molecules in the range 5–10 $\times$ 10^6 daltons. In the electron microscope, under denaturing conditions, 84% (mass %) of giant HnRNA has a contour length of 4–9 μ corresponding to a molecular weight of about 5–10 $\times$ 10^6. Giant HnRNA has a "DNA-like" base composition (G + C = 46–54%) and has considerable secondary structure (*ca.* 60% helix conformation) as judged by its melting profile and reactivity with formaldehyde.

The nuclei of mammals contain a class of heterogeneous RNA (HnRNA) that is rapidly labeled, sediments heterogeneously in sucrose gradients, and has a DNA-like base composition (Scherrer *et al.*, 1963; Attardi *et al.*, 1966; Soeiro *et al.*, 1966; Schutz *et al.*, 1968; Soeiro and Darnell, 1970). A portion of HnRNA has a sedimentation coefficient greater than 45 S as judged by sedimentation through sucrose. This has been equated with a molecular weight in excess of 4 $\times$ 10^6 daltons, using the available equations to relate molecular weight and sedimentation velocity (Gierer, 1950; Spirin, 1961). However the dangers in using such equations have been pointed out by Gesteland and Boedtker (1964) and Strauss and Sinsheimer (1967). Estimates of the molecular weight of a variety of cellular and viral RNAs have been obtained by other methods such as sedimentation or electrophoresis in denaturing solvents (Boedtker, 1968; Fenwick, 1968; Strauss *et al.*, 1968; Staynov *et al.*, 1972), light scattering (Gesteland and Boedtker, 1964), viscosity (Mitra *et al.*, 1963), and electron microscopy (Gransboulan and Scherrer, 1969; Robberson *et al.*, 1971). However there are few reports in the literature in which such techniques have been applied to HnRNA. Granboulan and Scherrer (1969) describe a class of HnRNA molecules with a molecular weight in the range 5–10 $\times$ 10^6 daltons as judged by visualization in the electron microscope under partially denaturing conditions. On the other hand, Mayo and de Kloet (1971) using formaldehyde sucrose gradients, and Scott and Kuhns (1972) using electrophoresis in the presence of Me_2SO present evidence that giant HnRNA "disaggregates" into smaller molecules presumably because denaturation reveals hidden nicks in the RNA.

This paper describes a method for the isolation of giant HnRNA from rat ascites cells which does not disaggregate under denaturing conditions. Such RNA has low G + C content and is heterogeneous in size with a molecular weight in the range 5–10 $\times$ 10^6 daltons as judged by sedimentation and electron microscopy under denaturing conditions. The

† From the Division of Biology, California Institute of Technology, Pasadena, California 91109. *Received December 18, 1972.* Report of work supported by U. S. Public Health Service Grant No. GM-13762 and by the Lucy Mason Clark Fund of the California Institute of Technology.