Poly(uridylic acid) Sequences in Messenger Ribonucleic Acid of HeLa Cells†

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ABSTRACT: The poly(uridylic acid) [poly(U)] sequences of 30 to 40 nucleotides found in the heterogeneous nuclear ribonucleic acid (RNA) of HeLa cells are also present in the poly(adenylic acid) [poly(A)] containing messenger RNA (mRNA) of these cells. Twenty-five percent of the total poly(U) sequences in the cell are found in the cytoplasm after HeLa cells have been labeled for 2.5 h with ^{32}P. This value is close to 40% if only poly(A) containing RNA molecules of the cell are considered. The distribution of poly(U) sequences parallels that of poly(A) sequences in different size classes of cytoplasmic messenger RNA. From the concentrations and lengths of the two sequences it can be estimated that about 20% of the poly(A) containing mRNA molecules could contain one poly(U) sequence. Several lines of evidence have been developed to rule out poly(U) containing heterogeneous nuclear RNA (HnRNA) as the source of the poly(U) sequences in cytoplasm. Poly(U) sequences are also found in RNA molecules of the nucleus and cytoplasm which do not contain poly(A). Although poly(U) sequences are most abundant in nuclear RNA species lacking poly(A), they are well represented in a class of RNA molecules in cytoplasm which lack poly(A), but which otherwise resemble the polysomal mRNA of HeLa cells.

The massive heterogeneity of the rapidly metabolized RNA of the cell nucleus (HnRNA[1]), the putative precursor of the mRNA sequences in the cytoplasm of animal cells, has been a serious handicap in studies of its structure. The precursor role envisaged for large HnRNA molecules would predict that common sequences exist for the recognition of processing sites by the enzymes that release mature mRNA molecules. The poly(A) sequences at the 3′ ends of many HnRNAs provided the first experimental evidence for such common sequences although their role, if any, in processing is unclear. Following this, two shorter single base sequences were detected in the HnRNA of HeLa cells. One is an internal sequence of about 25 AMPs which is transcribed rather than added posttranscriptionally as is the large poly(A) at the 3′ end (Nakazato et al., 1973, 1974). The other is a poly(U) sequence of 30 to 40 nucleotides which is concentrated in the largest HnRNA molecules in regions distant from the poly(A) terminus (Molloy et al., 1972, 1974). Evidence that this UMP-rich sequence is uninterrupted has also been presented (Korwek et al., 1974).

We have repeatedly found as much as 20–25% of the total poly(U) sequences of HeLa cells in cytoplasm. Since we thought it unlikely that cytoplasm would invariably be contaminated with this large quantity of poly(U)-containing nuclear RNA, we made more detailed studies of the localization and properties of the RNA molecules containing these sequences. We now report that some mRNA molecules contain poly(U) sequences. Some characteristics of these sequences and the RNA molecules of both the nucleus and cytoplasm which contain them are described.

Experimental Procedures

Materials. Carrier-free [^{32}P]phosphoric acid was from New England Nuclear Corporation; Me_2SO (silylation grade) from Pierce Chemical Company, RNase A from Sigma Biochemicals, RNase T_1 from Sankyo Inc., and pancreatic DNase from Worthington Biochemical Corp. Poly(U) and poly(A) were from Schwarz/Mann.

Cell Culture, Labeling, Fractionation, and RNA Extraction. HeLa cells maintained as previously reported (Edmonds et al., 1971) were washed once with phosphate-free Eagle's minimal essential medium buffered with 10 mM Hepes and supplemented with 5% normal calf serum. The cells suspended at a concentration of 2×10^6 cells/ml were labeled with carrier-free $^{32}PO_4$ for the time indicated. Cells were fractionated as described (Nakazato et al., 1974). Polysomes were analyzed by sucrose density gradient centrifugation, but without the addition of Brij-58 and sodium deoxycholate to the postmitochondrial cytoplasm (Edmonds et al., 1971). RNA was extracted with hot phenol and sodium dodecyl sulfate (Girard, 1967).

Poly(U) Isolation and Quantitation. Poly(U) was isolated by a method described in detail elsewhere (Venkatesan et al., 1976). RNA was digested with 400 units of RNase T_1 in 1.7 ml of 0.03 M Tris-HCl, pH 7.4. After 15 min at 37 °C, it was cooled to 0 °C before adding 0.1 ml of 0.5 M Tris, pH 7.4, 0.16 ml of 0.1 M $MgCl_2$, and 0.02 ml of 0.2 M $CaCl_2$ and 10 μg of pancreatic DNase in 0.01 ml. After 15 minutes at 37 °C, sodium dodecyl sulfate, EDTA and unlabeled poly(U) followed by NaCl were added to give final concentrations of 0.5%, 0.05 M, 10 μg/ml, and 0.1 M, respectively. A methylenedianiline

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[1] Abbreviations used: poly(U), poly(uridylic acid); poly(A), poly(adenylic acid); mRNA, messenger ribonucleic acid; rRNA, ribosomal RNA; HnRNA, heterogeneous nuclear RNA; tcRNA, translational control RNA; AMP, adenosine 5′-monophosphate; UMP, uridine 5′-monophosphate; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; NETS, 0.1 M NaCl, 0.05 M EDTA, 0.01 M Tris (pH 7.5), 0.5% sodium dodecyl sulfate; EtS, same as NETS, except without NaCl.

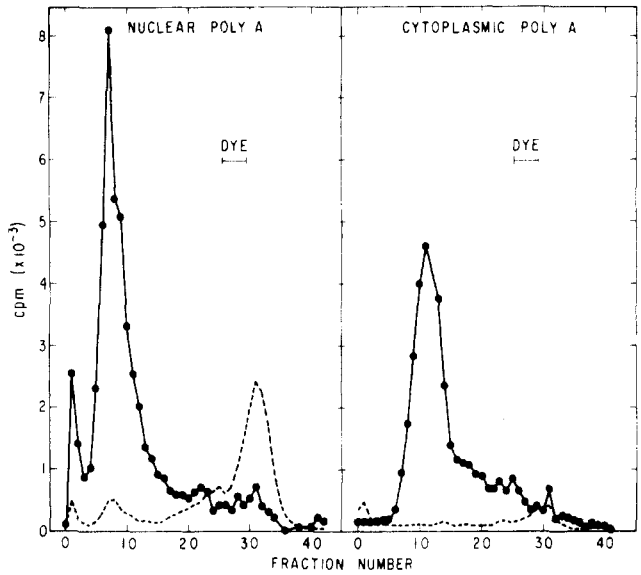

FIGURE 1: Separation of poly(A$^+$) RNA from poly(A$^-$) RNA. 1.5×10^8 HeLa cells were labeled for 2.5 h with 80 mCi of carrier-free $^{32}PO_4$. Cell fractionation and RNA extraction were as described in Experimental Section. Separation of poly(A$^+$) and poly(A$^-$) RNA was as reported (Nakazato and Edmonds, 1974). RNA was heated at 60 °C without addition of unlabeled poly(A) before binding to oligo(dT)-cellulose, poly(A) was isolated from an RNase digest of an aliquot of each RNA preparation and it was electrophoresed on polyacrylamide gel. Poly(A) fractions from poly(A$^+$) RNA (●-●) and from poly(A$^-$) RNA(- - -) electrophoresed in parallel gels were plotted in the same figures.

derivative of starch to which poly(A) was covalently linked by azo coupling was used to isolate poly(U) sequences. The preparation and use of this poly(A) resin are described in a separate publication (Venkatesan et al., 1976). Ten milligrams of poly(A) resin suspended in 1 ml of NETS (0.1 M NaCl, 0.05 M EDTA, 0.01 M Tris (pH 7.5), 0.5% sodium dodecyl sulfate) was added to 3 ml of the nuclease digest. The mixture was shaken for 30 min at room temperature before removal of the aqueous phase from the resin on a temperature-controlled filter. After washing the resin with NETS, poly(U) was removed with ETS (NETS without NaCl) at 60 °C. Poly(U) recovered from ETS by ethanol precipitation was analyzed by gel electrophoresis and quantitated as described in Figure 3.

Poly(A) Isolation. Two RNase digestion procedures were used. When poly(U) was not added, poly(A) was isolated by procedures previously described (Nakazato et al., 1974). When unlabeled poly(U) was added, the RNA was digested for 15 min at 50 °C in 1.7 ml of 0.005 M Tris buffer at pH 8.5 containing 0.01 M NaCl, 45 μg of unlabeled poly(A), 0.1 μg of RNase A, and 100 units of RNase T_1. The DNase treatment that immediately followed was as described in the previous section for poly(U) isolation.

Results

Separation of Poly(A$^+$) RNA from Poly(A$^-$) RNA. To answer many of the questions raised by the presence of poly(U) sequences in both nuclear and cytoplasmic RNAs, such as their number and distribution in different size classes and in poly(A$^+$) and poly(A$^-$) RNAs, a quantitative method for separating the latter two classes of RNA was needed. Such a method had already been described (Nakazato and Edmonds, 1974), and Figure 1 presents confirming evidence that it can effectively separate poly(A) containing from poly(A) lacking RNAs of both cytoplasm and nucleus. Separations such as these have been difficult to achieve in the case of HnRNA

TABLE I: Displacement of Oligo(U) Sequences from Polyribosomes by EDTA.[a]

Treatment of Cytoplasm	Polyribosomal RNA ($s > 74$ S) RNA (cpm × 10^{-6})	Oligo-(U) (%)	Oligo-(U) (cpm)	Postpolyribosomal RNA ($s < 74$ S) RNA (cpm × 10^{-6})	Oligo-(U) (%)	Oligo-(U) (cpm)
			Experiment 1			
None	3.5	0.07	2450	9.75	0.01	975
+EDTA	0.74	0.10	740	12.8	0.02	2560
			Experiment 2			
None	5.4	0.07	3780	6.2	0.05	3100
+EDTA	0.573	0.10	570	10.5	0.07	7350

[a] One-half of the postmitochondrial supernatant of HeLa cells labeled for 1 h with $^{32}PO_4$ was treated with 0.01 M EDTA. Each half was then sedimented through a sucrose gradient as described (Edmonds et al., 1971). After pooling and allowing the reaction mixture to stand in 1% sodium dodecyl sulfate and 0.2 M NaCl for 15 min at 23 °C, polysomal and postpolysomal fractions were precipitated with ethanol. RNA was extracted and assayed for poly(U).

particularly the larger species. A method based on an affinity for poly(U)-Sepharose, for example, bound only 50% of those poly(A) containing HnRNAs which sedimented faster than 45S RNA (Derman and Darnell, 1974). It is apparent from Figure 1 that essentially all poly(A) sequences of cytoplasm and more than 95% of those of the nuclease are recovered in RNA molecules that bind to oligo(dT)-cellulose. The sedimentation profiles of these two classes of RNA denatured by Me_2SO treatment show that little, if any, degradation results from this binding to oligo(dT)-cellulose (Figure 2).

A significant observation evident in these data, which has been noted and discussed elsewhere (Nakazato and Edmonds, 1974), is that HnRNA molecules containing the large poly(A) sequences have been separated from those containing a small transcribed poly(A). The very low levels of this small poly(A) relative to large poly(A) found in the mRNA also confirm a previous observation that small poly(A) sequences of this size are present in very few, if any, mRNA molecules (Nakazato et al., 1974).

Poly(U) Sequences in mRNA. A large fraction of the poly(U) sequences in the cytoplasm of HeLa cells is found in the polysome region of cytoplasm sedimented in a density gradient (Table I). The fact that a large portion of these sequences were shifted to regions containing more slowly sedimenting components when EDTA was included in the gradient suggested that poly(U) was in mRNA and not in an undefined polydisperse class of RNA which sediments in the polysome region of these gradients (Penman et al., 1968). Ribosomal RNA had previously been ruled out as a source of poly(U) sequences when the latter were not found in the 45S pre-rRNA of the nucleolus of HeLa cells (Korwek, 1974).

The detection of poly(U) sequences in polysomal RNA indicated that a detailed quantitative study of the distribution of poly(U) sequences in all classes of cellular RNA was needed to assess the significance of this observation. Figure 2 and Table II contain the results of analyses carried out on both poly(A$^+$) and poly(A$^-$) RNA of the nucleus and cytoplasm of cells labeled for 2.5 h with [^{32}P]phosphate. Each of these four RNA fractions was denatured by heating in the presence of an excess of unlabeled poly(U) for 3 min at 64 °C in 70% dimethyl sulfoxide before it was applied to the sucrose gradi-

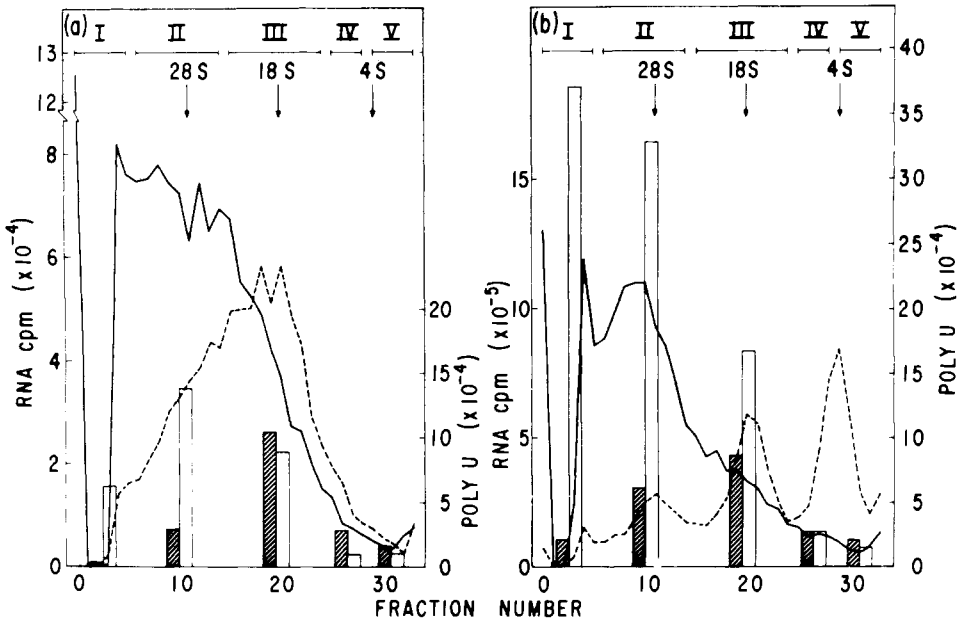

FIGURE 2: Poly(U) content of mRNA and HnRNA. The RNAs isolated and separated into poly(A^+) and poly(A^-) RNAs as described in legend to Figure 1 were each mixed with 25 μg of unlabeled commercial poly(U) in the presence of 0.1 M NaCl and were precipitated with ethanol. The precipitates collected by centrifugation were dissolved in 0.12 ml of ETS. After adding 0.28 ml of Me_2SO, the solutions were heated for 3 min at 64 °C before adding 0.40 ml of NETS and rapidly cooled in a water bath at 23 °C. This RNA solution was layered over 12 ml of a 15 to 30% linear sucrose gradient in NETS and centrifuged for 15 h at 29 000 rpm in an IEC SB-283 rotor at 21 °C. After fractionation, 5-μl aliquots were taken for acid-precipitable radioactivity, and the remainder of the fractions were pooled as designated by Roman numerals. That RNA which had precipitated (fraction = 0) was recovered by dissolving in NETS. It was added to pool I. After ethanol precipitation in the presence of 200 μg of yeast RNA, each pool was assayed for poly(U) and poly(A) content as described in the Experimental Section. Radioactivities in 5-μl aliquots (about $^1/_{100}$ of a fraction volume) of nuclear RNA (—) and cytoplasmic RNA (- - -), run in the same rotor are plotted. (a) Poly(A^+); (b) poly(A^-) RNA. Open bars are poly(U) content of nuclear RNA. Filled bars are poly(U) in cytoplasmic RNA.

ents described in Figure 2. The characteristic distribution of radioactivity in each of the four RNA fractions, sedimented in the same rotor, is seen in Figures 2a and 2b. Each gradient was divided into five identical pools and the poly(U) content of the cytoplasmic RNA of each pool is shown in the filled bars and that of nuclear RNA in the open bars. A numerical summary is included in Table II.

It is evident from Figure 2 and Table II that poly(U) sequences are found in all size classes of poly(A) containing mRNA and that a striking correlation is found between the distribution of the poly(A) sequence and the poly(U) sequences in each of the five fractions. A calculation of the average number of poly(U) sequences per mRNA molecule can be obtained from these data since both the poly(A) and poly(U) sequences are rather homogeneous (see gel patterns of Figures 1 and 3), and each mRNA contains only one poly(A) sequence. If estimates of 120 and 30 are used as the average lengths of the labeled poly(A) and poly(U) sequences, approximately 0.20 mol of poly(U) is present per mol of poly(A) in all size classes of mRNA. The deviations found in pools I and V can be ignored for this calculation since they constitute only 7% of the total poly(A^+) mRNA. It can be concluded that about 20% of the poly(A^+) mRNAs contain one poly(U) sequence, although more than one poly(U) per mRNA cannot be excluded. This is quite different from nuclear RNA where the largest HnRNA molecules have been shown to contain as many as three or four poly(U) sequences per molecule, although the smallest poly(A) containing HnRNAs of the size of mRNA contained less than 0.20 sequence per RNA molecule (Molloy et al., 1974). A similar, although less striking, result is seen in these experiments. The lower concentrations of poly(U) found in large HnRNA molecules in our experiments may be related to the fact that our poly(A) containing RNA preparations were denatured by heating in Me_2SO prior to sedimentation in these gradients. In the case of HnRNA, but not mRNA, a considerable overall reduction of sedimentation velocity is always observed (Figure 2 and Nakazato, unpublished observations).

Poly(U) Sequences in Poly(A^-) RNA. Many of the poly(U) sequences in the cytoplasm and especially those in the nucleus are found in RNA molecules lacking poly(A). Those of the cytoplasmic RNA are of particular interest since many are in an RNA fraction associated with polysomes which has properties similar to mRNA (Korwek, 1974). This poly(A^-) RNA in HeLa cytoplasm has recently been characterized as a class of mRNA (Milcarek et al., 1974) that is apparently unrelated structurally to poly(A^+) mRNA since it failed to hybridize to cDNA transcribed from the poly(A^+) mRNA by reverse transcriptase. The poly(U) sequences in this fraction are distributed in RNA size classes similar to, but somewhat larger than those in the poly(A^+) mRNA (Figure 2b). The latter may reflect the somewhat higher sedimentation velocities reported for poly(A^-) as compared with poly(A^+) mRNA (Milcarek et al., 1974).

More than 50% of the poly(U) sequences of cytoplasm are usually found in poly(A^-) RNA, although poly(A^-) mRNA constitutes only 30% of the total mRNA of HeLa cells regardless of the length of the labeling period (Milcarek et al., 1974). Although poly(U) sequences may be present in higher concentrations in poly(A^-) than in poly(A^+) mRNA, the possibility of contamination with poly(U) containing nuclear RNA is also greater for poly(A^-) RNA because of the excess of poly(U) sequences in poly(A^-) HnRNA (Table II).

Contamination of mRNA with Poly(U) Sequences from the Nucleus. Since the concentration of poly(U) containing RNA of the nucleus always exceeds that in cytoplasm, the

TABLE II: Distribution of Poly(U) Sequences in the RNA of Nucleus and Cytoplasm.[a]

	RNA $\times 10^{-6}$	Poly(A) ($\times 10^{-3}$ cpm)	Poly(U) $\times 10^{-3}$	Poly(U)[b]/ Poly(A)[c]
		Cytoplasm		
Poly(A^+) RNA				
I	3.39	55	3.8	0.27
II	27.2	686	29.6	0.17
III	48.4	2360	105.0	0.18
IV	5.64	505	29.3	0.23
V	2.95	171	16.7	0.39
Total	87.6	3777	184.0	0.19
Poly(A^-) RNA				
I	35.3		21.7	
II	157.0		61.9	
III	296.0		85.1	
IV	195.0		26.2	
V	228.0		21.3	
Total	211.0		216.0	
		Nuclei		
Poly(A^+) RNA				
I	27.8	261	62.7	1.7
II	65.5	1640	139.0	0.59
III	41.3	2370	89.5	0.26
IV	3.52	337	10.2	0.21
V	2.39	109	10.6	0.68
Total	140.5	4717	312.0	0.46
Poly(A^-) RNA				
I	414.0		371.0	
II	747.0		329.0	
III	343.0		167.0	
IV	59.4		27.7	
V	44.0		15.2	
Total	1607.0		910.0	

[a] RNA preparation and poly(U) isolation are described in the legend to Figure 2. Poly(A) was isolated by the method used when unlabeled poly(U) was added to the RNA preparation (in Experimental Section). [b] 30 UMPs in length. [c] 120 AMPs in length in cytoplasm and 210 in nucleus.

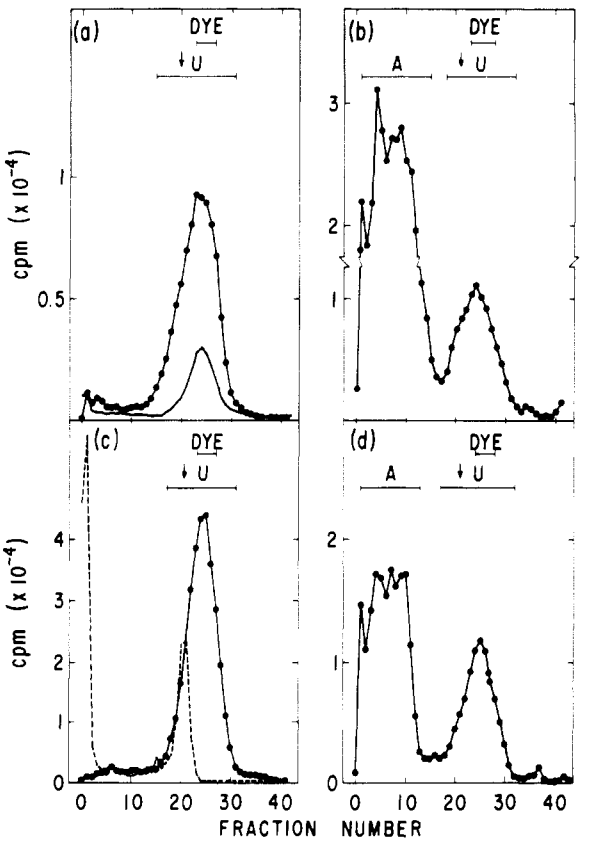

FIGURE 3: Electrophoretic mobilities of poly(U) fractions from the nucleus and cytoplasm. Poly(U) isolated from one pool of each of the nuclear and cytoplasmic gradients shown in Figure 2 was electrophoresed with [^{3}H]adenosine labeled poly(A^-) RNA from the cytoplasm of HeLa cells to provide a 4S RNA marker. (●—●) Poly(U) from: (a) cytoplasmic poly(A^-) RNA III; (b) cytoplasmic poly(A^+) RNA III; (c) nuclear poly(A^-) RNA II; (d) nuclear poly(A^+) III. [^{3}H]Adenosine-labeled marker for 4S RNA is shown by dotted line in c and by arrows in a, b, and d. Poly(U) from cytoplasmic poly(A^-) RNA V electrophoresed on a parallel gel is plotted as a solid line in a. Fractions were pooled as indicated by bars and were analyzed for base composition as shown in Table III.

question of nuclear contamination as the source of the cytoplasmic poly(U) must be considered. It would be reasonable to expect as much as 5% of the RNA of the nucleus to be recovered in cytoplasmic fractions prepared by Dounce homogenization of hypotonically swollen HeLa cells since 2 to 4% of the nuclear DNA is usually found in cytoplasm under these conditions (Borun et al., 1967). The experiment summarized in Figure 2 and Table II shows not only that the concentration of poly(U) in the cytoplasm relative to that in the nucleus exceeds this amount by an order of magnitude, but also that the poly(U) sequences are concentrated in different size classes of RNA in cytoplasm from those in the nucleus.

A comparison of poly(U) sequences in poly(A^+) RNA from the two sites provides compelling evidence for localization of poly(U) sequences in cytoplasmic mRNA. In this experiment the fraction of the total poly(U) sequences in the poly(A^+) RNA which is present in cytoplasm is similar to the fraction of the total cellular poly(A) found in cytoplasm (i.e., 37 vs. 44%). The poly(U) sequences are also distributed in a population of RNA molecules sedimenting after Me_2SO denaturation with velocities characteristic of mRNA rather than of poly(A^+) HnRNA (Figure 2a).

The question of nuclear contamination of the poly(A^-) RNA of cytoplasm is more serious because of the very high levels of poly(U) in poly(A^-) HnRNA (Figure 2b). However, in this case also, the distribution of the poly(U) in various size classes of RNA in the nucleus in no way resembles their distribution in cytoplasm. The RNA of pool I of the nucleus, for example, contains 25 times more poly(U) than pool I of cytoplasm suggesting that, for RNA of this size class, nuclear contamination did not exceed 4% even if all RNA in this cytoplasmic pool resulted from nuclear contamination.

These data would exclude any simple form of nuclear contamination as the source of most of the poly(U) containing RNA of cytoplasm. This is particularly clear in the case of the poly(U) sequences in poly(A^+) mRNA in our experiments where labeled poly(U) sequences are present in quantities similar to those in the nuclear poly(A^+) HnRNA molecules of similar size.

Properties of Poly(U) Sequences. Poly(U) sequences from poly(A^+) and poly(A^-) RNA of the nucleus and cytoplasm display similar electrophoretic mobilities (Figure 3), although those from cytoplasmic RNAs migrate somewhat more heterogeneously. The sequences electrophoresed in Figure 3 were obtained from the pool of each RNA containing a large amount of poly(U) (Figure 2). Poly(U) always migrated in the bromphenol blue dye marker slightly ahead of transfer RNA. A poly(A) fraction from pulse-labeled yeast mRNA that was measured by end-group analysis as 50 nucleotides comigrated with transfer RNA in similar gels (McLaughlin et al., 1973).

TABLE III: Composition of Sequences from RNase T_1 Digests Separated on Gels.

	Component A					Component U				
	Total Radioactivity (cpm)	%				Total Radioactivity (cpm)	%			
		C	A	G	U		C	A	G	U
Cytoplasm										
I[b]	NA[a]					126	9.5	10.3	5.6	74.6
II	823	2.1	93.3	0.7	3.9	1272	8.3	8.3	3.3	80.0
III	4840	2.4	95.6	0.2	1.9	2235	5.6	11.2	3.0	80.3
IV + V	1355	2.4	95.3	0.15	2.2	1363	3.5	7.2	1.2	88.0
Nuclei										
I	NA[a]					4160	8.2	10.2	3.7	77.9
II	2938	1.9	93.9	0.9	3.3	6606	5.9	8.2	2.8	83.2
III	4495	2.5	94.9	0.2	2.4	3947	7.0	10.2	3.3	79.5
IV + V	598	2.5	95.3	0.3	1.8	NA[a]				

[a] Not analyzed. [b] Fractions are as in Figure 2a, except that in this experiment, Fractions IV and V were pooled. Poly(U) was isolated from the RNA fractions in an experiment similar to that shown in Figure 2 and Table II. After gel electrophoresis, AMP-rich and UMP-rich fractions as shown in Figure 3 were assayed for base composition by paper electrophoresis (Salzman and Sebring, 1964).

The minimum size of these sequences can be estimated from the composition data for poly(U) fractions obtained from poly(A^+) RNAs (Table III) since each sequence in a complete RNase T_1 digest should be terminated by a single GMP. Sequences of 25 to 30 nucleotides would be calculated from the ratio of ^{32}P in UMP to that in GMP for the poly(U) obtained from each size class. This is in close agreement with the length obtained previously for the poly(U) sequences in HeLa nuclear RNA (Molloy et al., 1972). This value increases to 35 or 40 if this poly(U) fraction is further purified on DEAE-Sephadex (Korwek, 1974).

It is apparent from Figure 3 that the poly(U) fractions from poly(A^+) RNAs which are bound to the poly(A) resin contain components not found in RNA lacking poly(A). The composition analysis of Table III shows that this slowly migrating heterogeneous material is poly(A). In these experiments it represents about 10% of the poly(A) sequences in the RNA sample. Its separation from poly(U) during electrophoresis shows it is not covalently linked to poly(U), but may be hybridized to the unlabeled poly(U) added to the RNase T_1 digest to ensure a maximal reproducible binding of the poly(U) to the poly(A) resin (Venkatesan et al., 1976). Because of the large excess of poly(U) in the mixture, it is reasonable to expect that many single stranded regions remain which hybridize to the poly(A) resin and carry along segments of labeled poly(A) annealed to other regions of the same poly(U) chains.

Short UMP sequences have been detected in certain small cytoplasmic RNAs purified from specific protein initiation factors from embryonic chick muscle extracts (Bester et al., 1975) and also from the salt washes of reticulocyte ribosomes (Bogdanovsky et al., 1973). These RNAs are able to alter the translation of specific mRNAs in cell-free systems and have been named translational control RNAs (tcRNA). Although these RNAs are not well-characterized, they appear to be no larger than 40 nucleotides of which less than 20 are in a UMP-rich sequence (Bester et al., 1975). It is obvious from data presented in Figure 2 that almost all of the poly(U) containing RNAs of HeLa cells are far larger than this. These RNAs should be found in pool 5 of the RNA gradients of Figure 2. The poly(U) sequences recovered from the cytoplasmic poly(A^-) RNA of this pool migrated during electrophoresis with the same velocity as did the poly(U) from all other size classes of RNA (shown in the solid line of Figure 3a). A poly(U) sequence of 15 or 20 nucleotides as has been estimated for such RNA would migrate well ahead of the dye in this system. This survev of poly(U) sequences in cytoplasmic RNAs (Figure 2) shows not only that very few are in molecules as small as tcRNAs but that the poly(U) sequences in the smallest RNAs of HeLa cytoplasm are actually larger than have been estimated for the UMP-rich regions of such RNAs obtained from chick muscle (Bester et al., 1975) and from rabbit reticulocytes (Bogdandovsky et al., 1973). It should be noted that recent investigations of the factor(s) prepared from reticulocyte polysomes that were reported to stimulate the translation of globin mRNA in vitro do not support the earlier conclusion that the factor is a polynucleotide (Salden and Bloemendal, 1976).

Discussion

These experiments document the presence of poly(U) containing RNA in the cytoplasm of HeLa cells with properties characteristic of mRNA. Most of these RNAs are bound to polysomes from which they can be released as more slowly sedimenting components if EDTA is included in the sucrose gradient. About one-half of the poly(U) sequences are in poly(A) containing mRNA and the rest are in RNAs which lack poly(A), but have the other characteristics of mRNA. The distribution of poly(U) sequences in RNA of different size classes closely parallels that of poly(A) in mRNA (Table II). If it is assumed that only one poly(U) is present in each poly(A^+) mRNA, then about 20% of these mRNAs contain a poly(U) sequence.

Although we believe it to be quite unlikely, we have considered the possibility that poly(U) sequences which appear to be in poly(A) containing RNA molecules, might in fact be in poly(A^-) RNAs which have formed intermolecular hybrids by complexing with the poly(A) sequences of mRNA. If this were the case, however, it is hard to explain why such a large fraction of poly(U) containing RNAs do not form such hybrids, but are recovered in poly(A^-) RNA since the concentration of poly(A) sequences always exceeds that of poly(U) sequences in cytoplasm. It is also unlikely that poly(U) sequences would distribute in exactly the same size classes as do the poly(A) sequences of RNA denatured with Me_2SO in the presence of cold poly(U) (Table II) had they been generated during the isolation of poly(A) containing mRNA in inter-

molecular complexes. A different distribution might in fact be expected on the basis of the fact that the poly(U) sequences in poly(A^-) RNA tend to be concentrated in larger RNA molecules (Figure 2a vs. Figure 2b). This observation could be related to the tendency for poly(A^-) mRNA population of HeLa cells to contain a larger proportion of more rapidly sedimenting molecules than does the poly(A^+) mRNA (Milcarek et al., 1974).

The combination of the data and arguments presented here inevitably lead to the conclusion that some poly(A^+) mRNA molecules contain a poly(U) sequence. It is reasonable to assume that these poly(U) sequences, as well as the poly(A) sequences, are synthesized in the nucleus as HnRNA and are transported to cytoplasm as mRNA.

It has been generally assumed that poly(U) sequences are not present in mRNA since levels far less than those found in our experiments were reported in the polysomal RNA of HeLa cells several years ago (Molloy et al., 1972). We believe this discrepancy may have resulted either from the fact that the polysome pellet examined for poly(U) sequences in their experiment was from cells labeled in the presence of low levels of actinomycin D for a longer time (4 vs. 2.5 h) or from certain technical problems inherent in quantitative recovery of poly(U) sequences from cellular RNAs which could have been overlooked in these experiments since precautions to avoid them were not reported. The most significant of these stems from the coexistence in cytoplasm of the poly(U) sequences with higher concentrations of much longer poly(A) sequences. Conditions that normally are satisfactory for the quantitative recovery of poly(A) sequences from RNase digests, may in the case of poly(U) sequences, lead to greatly reduced recoveries since much of the poly(U) can hybridize to the longer poly(A) of the 3′-terminal fragments in the digest and subsequently fail to bind to the poly(A) affinity column. Five- to tenfold increases in poly(U) binding are obtained when an excess of unlabeled poly(U) is added after RNase T_1 digestion, but before addition of the salt needed to bind poly(U) to the poly(A) resin (Venkatesan et al., 1976). It is the addition of this poly(U) carrier that is largely responsible, however, for the concomitant binding of a small fraction of poly(A) sequences to the poly(A) resin (see Figure 3 and Results section).

The poly(U) sequences found in mRNA in these experiments are likely to be related to other UMP-rich sequences that have been found recently in polysomes and messenger RNA–protein particles. It is probable that the sequences associated with poly(A) fragments that are released either from polysomes directly or from polysomal RNA by a mild RNase A treatment are held together by these poly(U) sequences (Jeffrey and Brawerman, 1975). Although not actually identified as poly(U) in these experiments, it was suggested when neither heat nor formamide released these fragments from poly(A), but their reassociation was prevented by added poly(U) (Jeffrey and Brawerman, 1975).

A quite different set of observations have implicated UMP-rich sequences in the regulation of the translation of mRNA by the formation of complexes with the poly(A) region. These experiments concern small RNAs purified from extracts of muscle and reticulocytes which appear to inhibit the translation of specific mRNAs by blocking their binding to ribosomes during the initiation of protein synthesis. Although both the so-called translational control RNAs and their poly(U) stretches are much shorter than the RNAs and poly(U) sequences found in our experiments, they could be derived from them since the procedures used in their isolation would not have afforded the usual protection from RNases. It is also possible that these small UMP-rich RNAs may be present in concentrations that would have escaped detection in our analyses.

What is known of the location of poly(U) sequences in the nuclear RNA has been readily accommodated by models for the biogenesis of mRNA in which the mRNA sequences are derived from the 3′ ends of larger completed HnRNA molecules which have been polyadenylated posttranscriptionally (Darnell et al., 1973). Such observations include the much higher concentrations of poly(U) sequences in the larger HnRNA molecules and the fact that limited fragmentation of large poly(A^+) HnRNA resulted in a 90% decrease in the poly(U) content of the poly(A) containing fragments of sizes similar to mRNA (Molloy et al., 1974). These data are, of course, also compatible with the original report of the very low levels of poly(U) in polysomal bound mRNA (Molloy et al., 1972). While our detection of poly(U) sequences in some mRNA species is not inconsistent with this model, since some poly(U) sequences are found in poly(A) containing HnRNA molecules of the size of mRNA (Figure 2; Molloy et al., 1974), the finding would also be consistent with mRNA originating from the 5′ regions of HnRNA. Support for the presence of mRNA at the 5′ end of HnRNA has recently been provided by the detection of methylated blocked 5′ terminal sequences (so-called "caps") in very large (>32 S) HnRNA molecules of mouse L-cells (Perry et al., 1975).

The large amounts of poly(U) sequences found in HnRNA molecules lacking poly(A) (see Figure 2) would be accounted for in the usual models either as nascent RNA which has not yet been polyadenylated and/or RNA fragments released during processing reactions that generate mature mRNA. However, in view of the growing evidence for a distinct class of mRNA molecules that lack poly(A), the possibility that poly(A^-) HnRNAs may be precursors of such mRNAs should also be considered. The poly(U) sequences present in the poly(A^-) mRNA may be derived from such a class of HnRNA molecules.

Techniques which would allow the selection of poly(U) containing RNA molecules from mixed RNA populations would be especially helpful in answering the many questions raised in these experiments about the structure and function of poly(U) containing RNA. The relatively short length of the poly(U) sequence, coexisting with an excess of much longer poly(A) sequences, has made this a more difficult problem to solve than was the case for poly(A) containing RNA molecules.

Studies of the function and metabolism of poly(U) containing mRNA molecules will depend on the development of this capability.

References

Bester, A. J., Kennedy, D. S., and Heywood, S. M. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 1523–1527.

Bogdanovsky, D., Hermann, W., and Schapira, G. (1973), *Biochem. Biophys. Res. Commun. 54,* 25.

Borun, T., Robbins, E., and Scharf, M. D. (1967), *Biophys. Biochim. Acta 149,* 302–304.

Darnell, J. E., Jelinek, W. R., and Molloy, G. R. (1973), *Science 181,* 1215–1221.

Derman, E., and Darnell, J. E. (1974), *Cell 3,* 255–264.

Edmonds, M., Vaughan, M. H., and Nakazato, H. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 1331–1335.

Girard, M. (1967), *Methods Enzymol. 12,* 581–588.

Jeffrey, W. R., and Brawerman, G. (1975), *Biochemistry 14,* 3445-3450.
Korwek, E. L. (1974), Localization and Characterization of Oligouridylic Acid Sequences in the RNA of HeLa Cells, Ph.D. Thesis, University of Pittsburgh.
Korwek, E. L., Nakazato, H., and Edmonds, M. (1974), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 33,* 1486.
McLaughlin, C. S., Warner, J. R., Nakazato, H., Edmonds, M., and Vaughan, M. (1973), *J. Biol. Chem. 248,* 1466-1471.
Milcarek, C., Price, R., and Penman, S. (1974), *Cell 3,* 1-10.
Molloy, G. R., Jelinek, W., Salditt, M., and Darnell, J. E. (1974), *Cell 1,* 43-53.
Molloy, G. R., Thomas, W. L., and Darnell, J. E. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 3684-3688.
Nakazato, H., and Edmonds, M. (1974), *Methods Enzymol. 29,* 431-443.
Nakazato, H., Edmonds, M., and Kopp, D. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 200-204.
Nakazato, H., Kopp, D. W., and Edmonds, M. (1973), *J. Biol. Chem. 284,* 1472-1476.
Penman, S., Vesco, C., and Penman, M. (1968), *J. Mol. Biol. 34,* 49-69.
Perry, R. P., Kelley, D. E., Friderici, K., and Rottman, F. (1975), *Cell 4,* 387.
Salzman, N. P., and Sebring, E. D. (1964), *Anal. Biochem. 8,* 126.
Salden, M., and Bloemendal, H. (1976), *Biochem. Biophys. Res. Commun. 68,* 157.
Venkatesan, S., Nakazato, H., and Edmonds, M. (1976), *Nucleic Acids Res.* (in press).

Chromatographic and Functional Comparison of Human Placenta and HeLa Cell Tyrosine Transfer Ribonucleic Acids†

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ABSTRACT: Chromatographic and functional properties of tyrosine isoaccepting transfer ribonucleic acids (tRNAs) from placenta and HeLa cells were analyzed and compared. RPC-5 chromatography separated four major isoacceptors from each source, with those from HeLa cells eluting generally later than those from placenta. There was some overlap: HeLa $tRNA_1^{Tyr}$ eluted in a position between placenta $tRNA_3^{Tyr}$ and $tRNA_4^{Tyr}$; and HeLa $tRNA_2^{Tyr}$ and placenta $tRNA_4^{Tyr}$ eluted in similar positions with the HeLa isoacceptor eluting slightly later than the placental isoacceptor. Thus there are no isoacceptors common to both sources. The function of the individual isoacceptors was compared in a rabbit-reticulocyte, cell-free, protein-synthesizing system for both the rate of incorporation of tyrosine into the polypeptide chain and the site of incorporation in α-globin. Two isoacceptors were compared simultaneously in the same reaction, and overlapping comparisons were made to relate each isoacceptor to all the others. There were no significant differences in the rates of incorporation among the isoacceptors, nor were there any differences in the sites of incorporation. All eight isoacceptors donated tyrosine equally well into the three tyrosine containing tryptic peptides of α-globin. Whatever the structural differences among the tyrosine isoacceptors are, they do not affect the function of the tRNA in this protein-synthesizing system.

Isoaccepting tRNAs isolated from genetically related sources often exhibit different chromatographic properties. Transfer RNAs from many neoplastic cells differ from those of normal cells in this respect (Taylor et al., 1968; Baliga et al., 1969; Gallagher et al., 1972; Mushinski and Potter, 1969). The precise nature of the changes which occur upon transformation varies with cell type and with specific isoacceptors. Taylor et al. (1968), for example, showed that some transformed cells contained tyrosyl-tRNA species not found in normal cells, and that other transformed cells contained different ratios of tyrosyl isoacceptors than normal cells. New species of histidine, tyrosine, and asparagine tRNAs were seen in Novikoff hepatoma when compared with normal rat liver (Baliga et al., 1969) and transformation of rat embryo or mouse cells by SV40 resulted in the production of a new aspartyl-tRNA (Gallagher et al., 1972). Because transfer RNAs occupy a central position in the flow of genetic information and are known to be involved in genetic regulation, analysis of functional differences which might result from the structural changes seems desirable.

A few studies of this type have been performed. These functional studies have been carried out in several different systems. Sharma et al. (1975) used chick oviduct magnum explants and showed that bulk Novikoff hepatoma tRNA added to the medium specifically inhibited ovalbumin synthesis, while normal rat liver tRNA had no effect. Several groups have compared incorporation of amino acids from unfractionated tRNAs into specific proteins. In one such study, Mushinski et al. (1970) showed that there was no difference between unfractionated tRNA from normal mouse liver and plasma cell tumors in the transfer of leucine into rabbit hemoglobin in a cell-free system, even though the leucine tRNAs differed chromatographically. Bridges and Jones (1973) found a slight quantitative difference in incorporation of serine into the tryptic peptides of protein produced by a cell-free system from one plasmacytoma when the unfractionated tRNAs of a different plasmacytoma were used. There were significant

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