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Occurrence of Uridylate-Rich Oligonucleotide Regions in Heterogeneous Nuclear RNA of HeLa Cells

(hybridization/30-nucleotides long/messenger RNA)

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ABSTRACT **Heterogeneous nuclear RNA molecules from HeLa cells contain a specific segment of about 30 nucleotides length that is largely (about 80%) uridylic acid. This oligo(U) segment is located predominantly in the larger (70S–90S) heterogeneous nuclear RNA molecules, and is essentially absent in messenger RNA and 45S ribosomal precursor RNA molecules. The oligo(U) hybridizes rapidly to cellular DNA, suggesting that it is transcribed from the repeated regions of the DNA.**

Messenger RNA (mRNA) in mammalian cells appears to be derived from a class of high-molecular-weight nuclear precursor molecules of various sizes termed HnRNA (heterogeneous nuclear RNA) (1–5). Several post-transcriptional events are thought to be involved in the biogenesis of mRNA. (*i*) HnRNA molecules of all size classes have poly(adenylic acid) sequences about 200 nucleotides long appended to the 3′ end of the RNA chain (2, 6–8) (*ii*) After this addition, the HnRNA undergoes a reduction in size within the nucleus to yield mRNA, which also is terminated at the 3′ end by the same poly(A) sequence (6, 9–11). It seemed likely that such a highly ordered pathway might require recognition sites for enzyme actions that would be common to many of the HnRNA molecules, and that if such sites could be isolated they might be useful in helping to study further the structure and physiology of HnRNA. Two such potential classes of sequences have been already recognized in HnRNA: (*i*) regions that hybridize readily to the cellular DNA, implying that the DNA contains repeated similar complementary sites (12–15), and (*ii*) double-stranded, RNase-resistant regions within HnRNA molecules (16). Both of these portions of the HnRNA, however, probably contain many different members and do not represent single identical regions of HnRNA. With the discovery of poly(adenylic acid) regions in HnRNA molecules, it occurred to us that it might be possible to fractionate and purify the proposed common regions of HnRNA by first isolating HnRNA sequences rich in a particular nucleotide. This report describes the successful isolation of a common uridylic acid-rich sequence from HnRNA. This sequence arises from repeated rgions of the cell DNA.

METHODS AND MATERIALS

Growth, harvesting, and labeling of HeLa cells has been described, as have our techniques for cell fractionation and RNA isolation and analysis (17–21).

RESULTS

Isolation and characterization of the UMP-rich oligonucleotide

T1 ribonuclease, because it cleaves only the phosphodiester bond between guanylate and neighboring 3′ residues (5′—pXpGp |Xp—3′), offered the possibility of digestion of HnRNA leaving intact any pyrimidine-rich regions (22). Pancreatic RNase, on the other hand, would destroy such potential sequences.

A preparation of high molecular weight (50–100 S) HnRNA labeled with ^{32}P was divided; half was treated with pancreatic RNase and the other half was digested with T1 RNase. The ribonuclease-resistant RNA in each sample was applied to a column of poly(A)–Sepharose, which would be expected to adsorb uridylic acid-rich sequences (23); unadsorbed RNA was removed by washing with buffer (as in Fig. 1). The adsorbed RNA was eluted with 90% formamide–10% buffer (see Fig. 1) and subjected to polyacrylamide gel electrophoresis. The T1 RNase-resistant material (about 0.1% of the input) migrated as a relatively homogenous species that was not present in the sample digested by pancreatic RNase (Fig. 1). Base composition analysis showed the T1 RNase-resistant RNA to be 78.8% UMP, 8.8% CMP, 9.0% AMP, and 3.6% GMP (average of four determinations). Since the RNA fragment produced by T1 RNase should have only a single GMP residue located at its 3′ terminus, the 3.6% GMP content indicated that the UMP-rich oligonucleotide was about 30 nucleotides in length. This conclusion appeared correct, because treatment of the UMP-rich oligonucleotide [henceforth called oligo(U)] with alkaline phosphatase to eliminate the 3′-terminal phosphate of the T1 RNase-derived segment caused a 73% decrease in the ^{32}P content of the GMP in the oligo(U) (Fig. 2). The inability to remove all of the ^{32}P of GMP might have been due to incomplete phosphatase action or to the

TABLE 1.

RNA sample	Estimated length in nucleotides	Expected if there is one oligo(U) per molecule	% cpm as oligo-(U)
HnRNA, 70–90 S	30,000–40,000	0.1–0.075	0.13
50–60 S	15,000–20,000	0.2–0.15	0.06
28–30 S	5,000–6,000	0.6–0.5	0.04
mRNA	1,000–3,000	3–1	0.015
Ribosomal 45 S precursor RNA,	14,000	0.21	0.02

The sedimentation value of HnRNA prepared by sucrose-gradient sedimentation was estimated by comparison with 32S ribosomal precursor RNA; the chain length was estimated according to equations of Studier (25), which give good approximations to the size of ribosomal species.

presence of internal guanylate residues (possibly modified) in the oligonucleotide. In any event, most of the GMP appeared to be located at the 3′ terminus. In order to substantiate the size estimate, the electrophoretic mobility of the oligo(U) was compared with that of two oligonucleotides known to be 5 and 15 nucleotides long. The oligo(U) moved in comparison to these markers as if it had an average length of 31 nucleotides (for size calculation, see ref. 24) (Fig. 3). It was also demonstrated (Fig. 3) that pancreatic RNase digested the oligo(U) to units less than five nucleotides in length. Variation in the electrophoretic migration, and presumably the length, of the oligo(U) molecules [compare the width of the oligo(U) peak after electrophoresis with that of the Reovirus oligonucleotide marker] may be due to incomplete T1 RNase action, leaving an internal GMP residue in some molecules. It is also possible that a true heterogeneity exists in the length and sequence of the oligo(U) regions between the two nearest GMP residues.

Distribution of oligo(U) in HnRNA

As a first step in eventually learning the position of oligo(U) in HnRNA molecules and the role it might play in HnRNA metabolism, the oligo(U) content in nuclear-RNA molecules of different lengths (about 70–90 S, 50–60 S, and 28–30 S) was determined. As shown in Fig. 4*A*, the 70–90S RNA had the highest content of oligo(U), 0.13%, while the 50–60S and 28–30S RNA contained 0.06 and 0.04% oligo(U), respectively (average of three determinations). The oligo(U) in each of the HnRNA samples had essentially the same base composition and electrophoretic mobility. From the estimated molecular weight of each size class of HnRNA based on its sedimentation (25), and the percentage of oligo(U) in each sample, we calculated that every 100S HnRNA might contain an oligo(U) segment, but there was only one oligo(U) for every three 55S molecules and one for every twelve 28S molecules (Table 1). To test whether the oligo(U) was covalently attached to the HnRNA, the % of oligo(U) was measured in an HnRNA sample (80–100 S) that had been treated with 90% dimethylsulfoxide (Me_2SO) and reisolated by sucrose gradient centrifugation (16, 26). The Me_2SO-treated HnRNA sedimented similarly to an untreated sample, and contained the same amount of oligo(U), 0.13%, as untreated HnRNA. Thus, the oligo(U) is a part of the HnRNA chain.

To be certain that HnRNA was the source of the oligo(U), 45S ribosomal precursor RNA labeled with ^{32}P was prepared (5), digested with T1 RNase, and analyzed for the presence of oligo(U). Less than 0.02% of the 45S RNA behaved as oligo(U) (Table 1). Since the previous experiments were all done with nuclear RNA taken from cells treated with a low dose of actinomycin (27) to suppress ribosomal precursor RNA formation, the oligo(U) clearly resides in the HnRNA fraction of the nuclear RNA.

Absence of oligo(U) in mRNA

It has recently been demonstrated that poly(A) sequences that occur in HnRNA appear to be conserved when the mRNA is derived from HnRNA (1, 2). Furthermore, HnRNA molecules that contain poly(A), as determined by their adsorption to poly(U)–Sepharose columns, also contain the oligo(U) sequences (Molloy and Thomas, unpublished experiments). These findings suggested that mRNA should be examined for the presence of oligo(U). The length of most mRNA molecules falls between 1000 and 3000 nucleotides

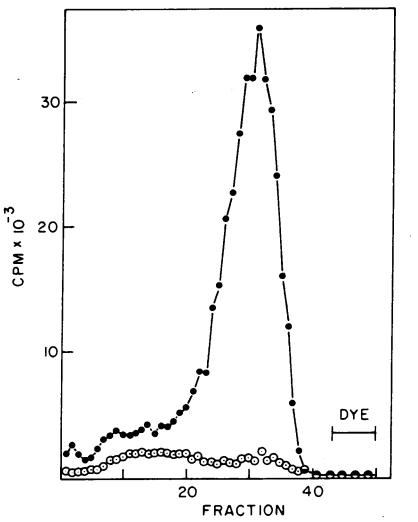


FIG. 1. Polyacrylamide-gel electrophoresis of the uridylate-rich oligonucleotide from HeLa-cell HnRNA. 3.7×10^8 HeLa cells in 150 ml of phosphate-free medium (17) supplemented with 5% dialyzed fetal-calf serum were exposed to 0.05 μg/ml of actinomycin D (27) for 30 min before labeling for 4 hr with 30–50 mCi of $H_3{}^{32}PO_4$ (carrier-free). Cells were lysed with 0.5% NP-40 (33) (Shell Chemical Co.) in 0.14 N NaCl–0.01 M Tris (pH 8.4)–1 mM $MgCl_2$. Nuclei were centrifuged at $700 \times g$ for 5 min, washed once in the same buffer containing 0.5% NP-40, and once in RSB buffer [10 mM Tris·HCl (pH 7.4)–1 mM $MgCl_2$–10 mM NaCl] containing 1% Tween 40 and 0.5% deoxycholate. The washed nuclei were lysed upon resuspension in high-salt buffer [0.5 M NaCl–50 mM $MgCl_2$–10 mM Tris·HCl (pH 7.4)] containing 100 μg/ml (20, 21) of DNase (Worthington, treated with iodoacetate) (34). The RNA was recovered after extraction with hot phenol and sucrose gradient sedimentation [15–30% sucrose (w/w)] in NETS "A" buffer [0.1 M NaCl–10 mM Tris·HCl (pH 7.4)–10 mM EDTA–0.2% sodium dodecyl sulfate] at 22,000 rpm for 9 hr in a Spinco SW27 rotor. Nuclear RNA sedimenting faster than 32 S was collected by ethanol precipitation and redissolved in 6 ml of 0.1 M NaCl–10 mM Tris·HCl (pH 7.4)–10 mM EDTA and divided into two 3-ml fractions. One fraction was treated with T1 RNase (13 units/ml, Sankyo) and the other with pancreatic RNase (2 μg/ml; Sigma Chemical Co.) for 60 min at 37°. The RNA solutions were then adjusted to 0.5% Na dodecyl SO_4, extracted twice with 2 volumes of phenol–chloroform 1:1, and precipitated with ethanol. It was necessary to repeat the RNase and extraction procedure a second time to obtain complete enzymatic hydrolysis of susceptible bonds. The digested RNA samples were placed on separate columns (0.6×3 cm) of poly(A)–Sepharose and chromatographed (2, 23); NETS "B" buffer (same as NETS "A," only 0.2 M NaCl) was used. Poly(A)–Sepharose was prepared as described (2, 23), with the substitution of poly(A) (Miles) for poly(U). The bound material was eluted from poly(A)–Sepharose with 3 ml of elution buffer [90% formamide–10% 10 mM Tris·HCl (pH 7.4)–10 mM EDTA–0.5% Na dodecylSO_4], precipitated with ethanol, resuspended on 0.35 ml of 20% glycerol–0.2% Na dodecylSO_4–8 M urea containing bromophenol blue, and incubated at 50° for 3 min immediately before it was layered on 15% acrylamide gels (0.075% *N,N′*-methylenebisacrylamide–8 M urea) (2, 23). Each gel fraction contained two 1-mm slices; ^{32}P-radioactivity was detected by Cerenkov radiation. ●——●, T1 RNase-resistant RNA; ⊙——⊙, pancreatic RNase-resistant RNA. The *bar* indicates the position of the bromophenol blue dye. Fractions 25–35 of the gel, containing the T1 resistant-RNA, were homogenized in NETS "A" buffer and centrifuged for 60 min at $17,000 \times g$. The RNA in the supernatant was precipitated with ethanol, and its base composition was determined.

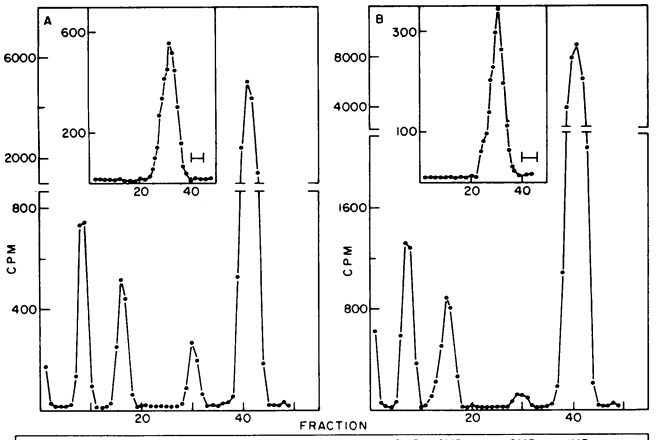
(10, 14, 19), so that if the oligo(U) were located adjacent to the mRNA sequence in the HnRNA molecules and not destroyed during nuclear processing, the mRNA should be about 1.5% oligo(U) (i.e., 30/2000). To minimize nuclear RNA contamination, ^{32}P-labeled mRNA was isolated from 30–70S structures released from polyribosomes by EDTA. This mRNA contained only 0.015% oligo(U), suggesting that it occurs in the HnRNA beyond the 5′ terminus of the mRNA and is destroyed during processing of the HnRNA molecules (Table 1).

Regions of DNA complementary to oligo(U)

Since the content of oligo(U) in the large HnRNA (70–90 S) is sufficient to allow for one oligo(U) per molecule, there might be many deoxyadenylate-rich regions in the DNA from which the oligo(U) was transcribed. When fragmented HnRNA molecules are exposed to relatively small amounts of DNA (e.g., 20–40 μg per filter; 18 hr), about 5–10% of the total RNA can be recovered as RNA–DNA hybrids (12–14). Therefore, we tested whether the oligo(U) might resemble this class of "rapidly hybridizing" RNA in its hybridization reactions with cell DNA. Fig. 5*A* shows that about 17% of the oligo(U) hybridized to HeLa DNA in 12 hr, a result that suggests that the oligo(U) probably is copied from the repeated regions of the DNA (28). In other experiments 30% of the oligo(U) hybridized in 12 hr. Fig. 5*A* also shows that about 67% of the hybridized oligo(U) was stable to pancreatic RNase; in other experiments, virtually all of the labeled oligo(U) was nuclease-resistant, a characteristic of specific hybrids. Another indicator of hybrid specificity, the temperature dissociation profile, was examined and found to be quite sharp (Fig. 5*B*), signifying a high degree of base-pairing. However, the T_m of the oligo(U) hybrid was 30°, which is 15° below the previously reported value for rU·dA (29). This lower melting temperature is probably due to the relatively short length of the oligo(U) as compared to the longer homopolymers previously studied, since the stability of rU·dA duplexes is sharply dependent on the length of the polymer involved (30).

DISCUSSION

This work demonstrates the occurrence in HnRNA molecules from HeLa cells of a specific segment of about 30 nucleotides length that is largely (about 80%) uridylic acid. The existence of cellular DNA that could give rise to such UMP-rich RNA sequences is suggested from other recent work. Although most evidence indicated that the 200-nucleotide segment of poly(A) is added to HnRNA after transcription (1, 2, 8, 31), we observed (31) that HeLa DNA would bind very small amounts of commercial poly(A) as a nuclease-resistant "hybrid," suggesting the presence of deoxythymidylate-rich regions. Such regions were absent from adenovirus DNA, although adenovirus-specific RNA contained the large poly(A) segment. If



	CMP	AMP	GMP	UMP	CMP	AMP	GMP	UMP
EXP 1	8.5	7.8	3.3	80.4	8.5	8.5	0.9	82.2
EXP 2	9.4	7.2	3.3	80.0	9.6	7.3	0.9	82.0

FIG. 2. Effect of alkaline phosphatase treatment on base composition of the UMP-rich oligonulceotide. An aliquot of the UMP-rich oligonucleotide (similar to fractions 25–35, Fig. 1*A*) was incubated at 37° for 60 min in 1 ml of 0.1 M NaCl–0.05 M Tris·HCl (pH 8.0) with about 10 units of alkaline phosphatase. An untreated control was included. Before use, alkaline phosphatase (Worthington, BAPF) was treated (37) with diethylpyrocarbonate to inactivate any contaminating endonuclease. After incubation, an aliquot was taken from each sample and analyzed by gel electrophoresis to ensure that the uridylate-rich fragment survived the phosphatase treatment. The remainder of the sample was extracted twice with 2 volumes of phenol–chloroform 1:1, precipitated with ethanol, and analyzed for base composition by paper electrophoresis. The electropherogram was cut into 1-cm fractions and counted. (*A*) Control not treated with alkaline phosphatase. (*B*) Sample treated with alkaline phosphatase. The *inserts* in both figures represent polyacrylamide gel analysis of the respective samples after incubation in the absence or presence of alkaline phosphatase.

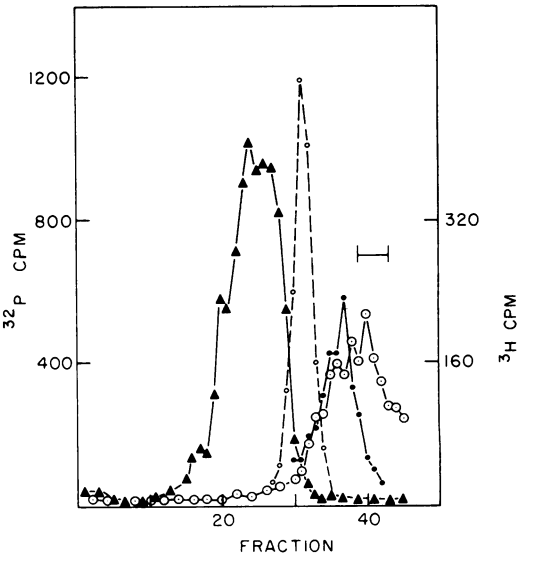


FIG. 3. Size determination of the UMP-rich oligonucleotide by polyacrylamide-gel electrophoresis. An aliquot of the HeLa cell oligo(U), similar to fractions 25–35 of Fig. 1, was analyzed on 20% acrylamide gels containing 8 M urea, along with two oligonucleotides of known molecular weight. ▲——▲, HeLa cell oligo(U); O– – –O, oligoadenylate [15 nucleotides long (38, 39)] from Reovirus labeled with ^{32}P (kindly provided by Dr. Martin Stokzfus); ●——●, pentanucleotide labeled with [^{3}H]uridine, characterized by elution from DEAE-cellulose; ⊙——⊙, [^{32}P]oligo(U) treated with pancreatic RNase (2 μg/ml) at 37° for 30 min before gel electrophoresis. The [^{32}P]oligo(U)—in the absence and presence of pancreatic RNase—and the [^{32}P]oligoadenylate were analyzed on three separate gels, each containing the [^{3}H]pentanucleotide and the bromophenol blue dye marker (|——|). In all gels the dye was in slices 39–42 and the pentanucleotide was in slice 37 or 38, so the results are plotted together.

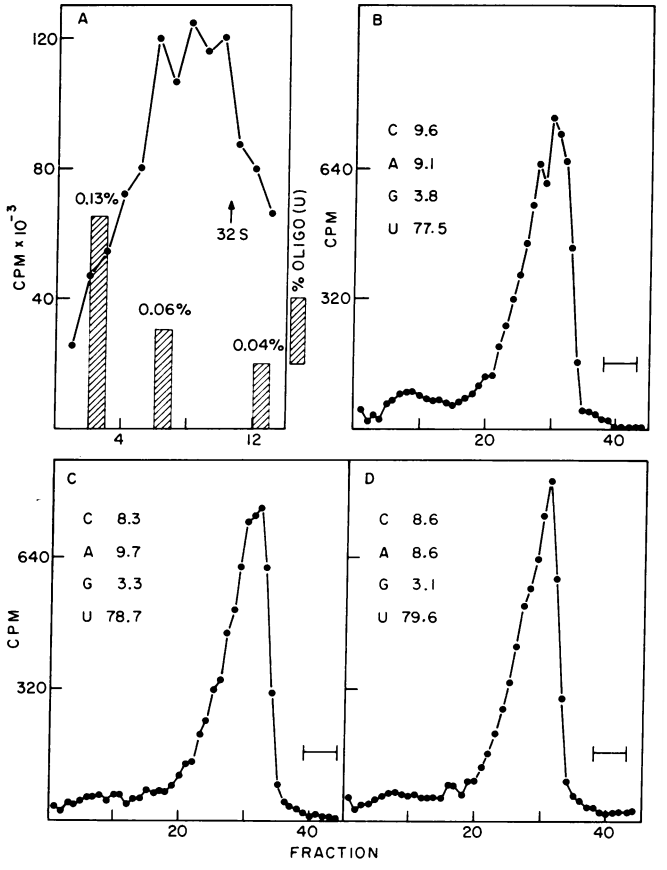


FIG. 4. Determination of the relative distribution of oligo(U) in different length HnRNA molecules. (*A*) Nuclear RNA was subjected to sucrose gradient centrifugation, as described in Fig. 1. Single fractions of RNA were selected from the 100S, 70–90S, 50–60S, and 28–30S regions of the gradient and separately analyzed for oligo(U) content. ●——●, Cl_3CCOOH-precipitable, alkali-labile cpm, determined on 0.3% of each fraction. *Histogram* indicates the content of oligo(U), expressed as the percent of RNA before T1 RNase treatment. *B*, *C*, and *D* show polyacrylamide-gel electrophoresis patterns (performed as in Fig. 1) and base compositions of the oligo(U) present in the 70–90S, 50–60S, and 28–30S samples, respectively. The content of oligo(U) was quantitated by inclusion of the RNA present in fractions 20–35 in *B*, *C*, and *D*.

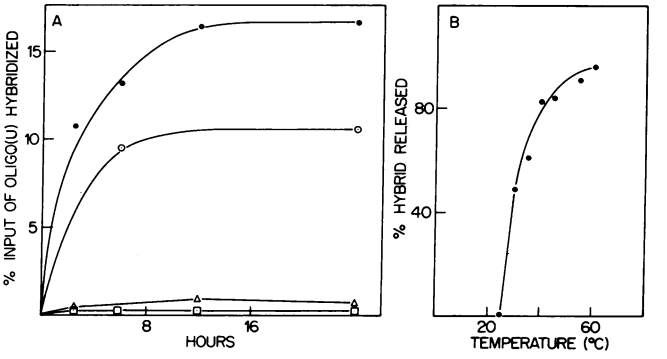


FIG. 5. (*A*) Oligo(U) was hybridized in 0.90 M NaCl–0.090 M Na citrate (35) at 25° to HeLa-cell DNA immobilized to nitrocellulose filters (36). At the indicated times, filters were removed and washed three times in 15 ml of NaCl–Na citrate for 15 min at 25°, and the amount of hybrid was determined. Hybridization of oligo(U) to: ●——●, 40 μg of HeLa cell DNA; △——△, 2 μg of adenovirus DNA; ⊡——⊡, filters lacking DNA; and ⊙——⊙, 40 μg of HeLa cell DNA after filters were washed, treated with pancreatic RNase (2 μg/ml) for 30 min at 25° in NaCl–Na citrate, and washed again. (*B*) oligo(U) was hybridized as described above at 25°; a filter was then removed and washed three times at the indicated temperature, and the amount of hybrid was determined.

sites for oligo(A) binding existed in one strand of cellular DNA, then oligo(U) should bind to the other strand. Indeed, Shenkin and Burdon have found recently that commercial poly(U) can bind to the DNA from several mammalian species (32). These DNA sites that bind poly(A) or poly(U) may, therefore, be the sites that when copied give rise to oligo(U).

The location of oligo(U) within the HnRNA molecules is a matter of great interest, but can not be determined from the present work. It is apparently not at the immediate 3′ end of the HnRNA molecules that give rise to mRNA. The distribution of oligo(U) within the various size classes of the HnRNA and its location within the HnRNA chain may give important clues as to the nature of recently completed molecules of HnRNA, as opposed to HnRNA molecules that have been modified after transcription. For example, the oligo(U) content of the largest HnRNA indicates that perhaps every HnRNA in its recently completed state contains one oligo(U). If this were true, and if the oligo(U) were at (or close to) the 5′ end of the HnRNA, then the smaller HnRNA—which has a low molar oligo(U) content—could be assumed to be mostly breakdown products in which the oligo(U) has been destroyed. If the oligo(U) were not close to the 5′ end of native HnRNA, then a substantial number of smaller HnRNA molecules might be nascent, but not yet far enough along in the course of their transcription to contain the oligo(U). The point of this discussion is not to reach a conclusion, but to point out that by study of the distribution and location of sequences such as poly(A), oligo(U), and double-stranded RNA in HnRNA much more about the physiology of HnRNA should become understood.

A final point deserves mention. Since the recognition of repeated sequences in eukaryotic DNA (28), and the recognition of HnRNA (3–5) and its content of rapidly hybridizing RNA (12, 13), it has been often suggested that HnRNA might be involved in regulation and that common repeated sequences might be the "signals" for such regulation. Except for the satellite DNA species (30) that do not participate in transcription, none of the repeated sequences—either in DNA or from transcribed RNA—has been purified. It would appear that the oligo(U) described here represents at least a part of such a sequence. The ultimate understanding of the role of a repeated sequence as a potential signal should be materially aided by its isolation as a discrete entity.

During the course of this work, Drs. R. H. Burdon and A. Shenkin informed us that they have detected uridylate-rich sequences in nuclear RNA of hamster cells by reaction of the RNA with poly(A) followed by isolation of the poly(A)·rU hybrid. 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). G. R. M. is a recipient of an American Cancer Society Postdoctoral Fellowship.

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