Location of Oligo(uridylic acid) Sequences within Messenger Ribonucleic Acid Molecules of HeLa Cells†

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Received October 29, 1984

ABSTRACT: A significant fraction of the polyadenylated mRNAs of HeLa cells contain an oligo(uridylic acid) [oligo(U)] sequence of 15–30 nucleotides. Several different experimental approaches were used to determine if these oligo(U)'s occupied similar sites within all mRNAs. In one approach, poly(adenylic acid)-containing mRNAs [poly(A+) mRNAs] averaging 2800 nucleotides in length were reduced to an average size of 500 nucleotides by controlled alkaline hydrolysis. Over 20% of the oligo(U)-containing fragments isolated from the hydrolysate retained a poly(A) sequence, showing that oligo(U)'s were not exclusively located near 5′ ends of mRNA although 20% were apparently close to 3′ ends. To confirm these observations, oligo(U)-containing mRNA [oligo(U+) mRNA] was exposed to the 3′-exonucleolytic activity of polynucleotide phosphorylase to produce fragments containing the 5′ regions of mRNA. Each of a set of fragments of decreasing length generated by increased times of exposure of the mRNAs to the enzyme was found to have about the same oligo(U) content, including the shortest that averaged 550 nucleotides. These data not only eliminated an exclusive location for oligo(U) in either 3′ or 5′ ends of mRNA but also suggested that oligo(U)'s might be close to the 5′ ends of some mRNAs. To verify this last observation, periodate-oxidized poly(A+) mRNA was labeled at the 5′ caps and at 3′-adenosine residues by sodium [^{3}H]borohydride reduction before it was nicked 3–5 times with alkali to produce 5′ and 3′ end-labeled pieces that could be separated with oligo(thymidylic acid)–cellulose. Quantitation of radioactivity in the oligo(U+) fragments (averaging 450 nucleotides in length) isolated from each population showed that oligo(U) was associated with the 3′-polyadenylated fragments 3 times more often than with 5′-capped fragments of the same average length. However, at least half of the oligo(U)'s were not associated with these end-labeled fragments, confirming the experiments cited above that suggested that most oligo(U) sequences are not close to the ends of mRNA molecules. Thus, oligo(U) sequences occupy different sites in different mRNAs. Their distribution is not random, however, since 3′ regions are favored over 5′ regions. The localization of oligo(U) sequences in untranslated regions of mRNA is discussed in relation to the sequence data currently available for eukaryotic mRNAs.

It has been known for some time that nearly 20% of the messenger RNA molecules of HeLa cells contain an oligo(uridylic acid) [oligo(U)][1] sequence in addition to a 3′-terminal poly(A) sequence (Korwek et al., 1976). Intramolecular hybridization of this oligo(U) to poly(A) prevents the isolation of this subset of mRNAs unless poly(A) is either removed or masked. Removal has been achieved by specific endonucleolytic destruction of the poly(A) with RNase H while it is hybridized to added oligo(thymidylate) (Wood & Edmonds, 1981). Poly(A) can also be masked by formolation of exocyclic amino groups with formaldehyde from which only UMP residues are spared (Molloy, 1980). Messenger RNAs modified by either treatment that contain oligo(U) sequences can then be selected on poly(A) affinity columns. The oligo(U)-containing mRNA population is on the average larger than oligo(U)-lacking mRNA but remains heterogeneous in size (Molloy, 1980; Wood & Edmonds, 1981). The oligo(U) sequences prepared from this population range from 15 to 30 UMP residues and occur on average only once in each molecule (Molloy, 1980; Wood & Edmonds, 1981). A detailed analysis of the 5′ termini of oligo(U+) mRNAs has shown some molecules to have type 1 and others type 2 caps. The distribution and base composition of these two cap structures resemble those of the mRNA population as a whole (Wallace et al., 1981).

The location of the oligo(U) sequence within any mRNA molecule is important information that we expected could be quite easily obtained from analyses either of the entire population or of size-selected classes derived from it, if the following assumptions were valid. First, oligo(U) was at a similar, although not necessarily identical, locus in all mRNA molecules regardless of their size. Second, this locus would not be within a coding region since six to eight phenylalanyl residues are a highly unlikely occurrence in proteins. This latter assumption then relegates the oligo(U) sequence either to the 5′- or to the 3′-noncoding regions that characterize eukaryotic mRNAs.

Should oligo(U) sequences serve some regulatory function, it also seemed unlikely that oligo(U) would be in 3′-untranslated regions of some molecules and in 5′-untranslated regions of others. Given these assumptions, it was possible to envision several experimental approaches that might dis-

† This work was supported by Grants CA 18065 and GM 32585 from the National Institutes of Health.

[1] Abbreviations: poly(A), poly(adenylic acid); poly(A+) RNA, poly(A)-containing RNA; oligo(U), oligo(uridylic acid); oligo(U+) RNA, oligo(U)-containing RNA; oligo(U−) RNA, oligo(U)-lacking RNA; oligo(dT), oligo(thymidylic acid); UMP, uridine 5′-monophosphate; GMP, guanosine 5′-monophosphate; RNase, ribonuclease; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; HCHO, formaldehyde; ETS, 0.002 M EDTA, 0.01 M Tris-HCl (pH 7.5), and 0.1% SDS; NETS, same as ETS plus NaCl at the indicated concentration; ET, 0.1 M Tris-HCl (pH 7.4) and 0.01 M EDTA.

0006-2960/85/0424-3678$01.50/0

tinguish oligo(U)'s in 3′-untranslated regions from those in 5′-untranslated regions. We now report the results of experiments that rule out a unique location for oligo(U) with respect to either end of these mRNAs. However, our most refined data show that oligo(U)'s tend to be associated with 3′ ends 3 times more often than with 5′ ends. The significance of these results with respect to the localization of oligo(U) in untranslated regions is discussed in light of the currently available sequence data for eukaryotic mRNAs.

Experimental Procedures

Materials. Formaldehyde (reagent grade), supplied by Baker, was used without further purification. Polynucleotide phosphorylase from *Micrococcus luteus* (specific activity 342 units/mg) was obtained from Miles Research Products. Sodium metaperiodate, in crystalline form, was supplied by Sigma. Sodium [^{3}H]borohydride (>50 Ci/mmol) was obtained in lyophilized crystalline form from New England Nuclear. RNA was eluted from gels by using the ECU-40 elution apparatus supplied by CBS Scientific Co. Sources for most of the other materials used have been described previously (Wood & Edmonds, 1981).

In Vivo Labeling, Isolation, and Fractionation of RNA. Maintenance and ^{32}P labeling of HeLa cells have been described (Edmonds et al., 1971), as have the techniques for RNA extraction and oligo(dT)–cellulose chromatography (Nakazato & Edmonds, 1974) except that RNA was extracted from the cytoplasmic fraction at 60 °C with aqueous phenol buffered at pH 7 rather than pH 5.

In Vitro Labeling of RNA with Sodium [^{3}H]Borohydride. Cytoplasmic RNA, extracted from 3 L of HeLa cells (4×10^5 cells/mL), was subjected to two cycles of binding and elution from 1 g of oligo(dT)–cellulose. A final yield of 350–400 μg of polyadenylated RNA was usually obtained to which ^{32}P-labeled cytoplasmic polyadenylated RNA was added as tracer. Labeling of the *cis*-hydroxyls in the RNA was done essentially as reported (Breter et al., 1979). The ethanol-precipitated polyadenylated RNA was dissolved in 2.5 mM EDTA, pH 7.4, to a concentration of 1 mg/mL and heated to 65 °C for 1 min. After the solution was cooled on ice, 8 mM $NaIO_4$ was added to a final concentration of 2.5 mM. The reaction was kept on ice in the dark for 1.5 h. Ten volumes of 0.2 M sodium acetate, pH 5.5, were then added, and the RNA was precipitated with ethanol at −20 °C. The dried RNA pellet was dissolved in 0.1 M potassium phosphate, pH 6.8, to a concentration of 2 mg/mL. The sample was then added directly to the ampule of NaB^3H_4, and the reaction was kept in the dark for 1 h at room temperature. Five volumes of 0.2 M sodium acetate, pH 5.5, were then added, and the reaction was further incubated at room temperature for 15 min. The RNA was precipitated with ethanol at −20 °C and then passed over oligo(dT)–cellulose. Bound material was eluted as described and used for further experimentation.

Sucrose Density Gradient Fractionation of RNA. RNA was dissolved in 60 μL of ETS to which 140 μL of dimethyl sulfoxide was added. The solution was heated to 63 °C for 3 min. An equal volume of 0.1 M NETS was then added, and the solution was layered atop a 12-mL 15–30% sucrose gradient in 0.1 M NETS. The gradients were centrifuged for 16 h at 28 000 rpm in a Beckman SW-40 rotor. Fractions (0.44 mL) were collected from which aliquots were taken and counted for Cerenkov radiation. Appropriate fractions were pooled, and the RNA was precipitated with ethanol at −20 °C.

Alkali Treatment. ^{32}P-Labeled poly(A+) mRNA from a 4-h labeling of cells was precipitated from ethanol, suspended in 80 μL of sterile deionized H_2O, and placed on ice. The solution was then adjusted to 0.2 M NaOH with the addition of 20 μL of cold 1 M NaOH. Hydrolysis was allowed to proceed, on ice, for various times. The reaction was neutralized by the addition of 20 μL of 2 M HCl and then diluted with 300 μL of ET. NaB^3H_4-labeled RNA was treated in the same manner except that the volume of the reaction was increased 10-fold to maintain an approximately equivalent RNA concentration.

Formaldehyde Treatment and Poly(A)–Agarose Affinity Chromatography. Formaldehyde treatment of RNA was as described by Molloy (1980). RNA was dissolved in 10 mM sodium phosphate, pH 7.8, not exceeding an RNA concentration of 380 μg/mL. This solution was made 1.1 M HCHO with the addition of 0.1 volume of 37% (v/v) HCHO. The sample was heated to 65 °C for 10 min, cooled to 0 °C, and diluted with 3 volumes of 0.2 M NETS before precipitation with ethanol at −20 °C. Isolation of oligo(U)-containing RNA, using poly(A)–agarose, was performed essentially as described by Wood & Edmonds (1981), with the following modifications. Poly(A)–agarose (1 mL of packed gel) was loaded in the column as a suspension in ETS, washed with several column volumes of the same buffer, and then preequilibrated with 10 column volumes of 0.4 M NETS. Ten minutes after the RNA sample was loaded, the column was washed with 20 mL of 0.4 M NETS. Following an intermediate wash with 10 mL of 0.2 M NETS, the bound RNA was eluted with ETS at 37 °C. Formol groups were removed from the RNA by heating at 80 °C for 10 min in 0.1 M Tris-HCl, pH 7.4, and 0.01 M EDTA. After being adjusted to 0.1 M NaCl, the RNA was precipitated with ethanol at −20 °C.

Oligo(U) and Poly(A) Content Analysis. Formol groups were removed from oligo(U)-containing RNA as described above, and the RNA was depolyadenylated by digestion with RNase H directed by oligo(dT) (Wood & Edmonds, 1981). The reaction was diluted with 2.5 volumes of sterile H_2O, and 5 μg of poly(U) as carrier and 200 units of T1 RNase were then added. The sample was incubated for 30 min at 37 °C after which the reaction was adjusted to 0.4 M NaCl and oligo(U) fragments were then bound to poly(A)–agarose. Oligo(U) was eluted as described above for oligo(U+) mRNA. Base composition determinations of the isolated oligo(U) fragments were carried out by high-voltage electrophoretic separation (Salzman & Sebring, 1954) of the 3′-nucleotide monophosphates produced by incubation for 16 h at 37 °C with RNase T2 (2500 units/mL) in 20 μL of 10 mM Tris, pH 7.4, containing 25–50 μg of ribosomal RNA carrier. Poly(A) fragments generated by RNase A and RNase T1 were quantitated as described by Edmonds et al. (1971).

Exonuclease Digestion of RNA. ^{32}P-labeled oligo(U)-containing RNA was formolated and precipitated with ethanol at −20 °C in the presence of 2–4 μg of cold poly(A). Poly(A) was employed as carrier because it is rapidly removed by polynucleotide phosphorylase (Soreq et al., 1974) and thus should not impede the action of the enzyme on the desired templates. The RNA pellet was suspended in sterile H_2O and adjusted to 25 mM Tris-HCl, pH 8.5, 5 mM $MgCl_2$, and 10 mM potassium phosphate, pH 8.5. A 5-fold molar excess of polynucleotide phosphorylase compared with RNA was added at a concentration of 75 units/mL. Reactions incubated for increasing times at 37 °C were then passed over a Sephadex G-75 column (0.7 × 28 cm) which had been preequilibrated with ETS. Fractions (0.45 mL) were collected and counted for Cerenkov radiation. Radiolabeled material excluded from

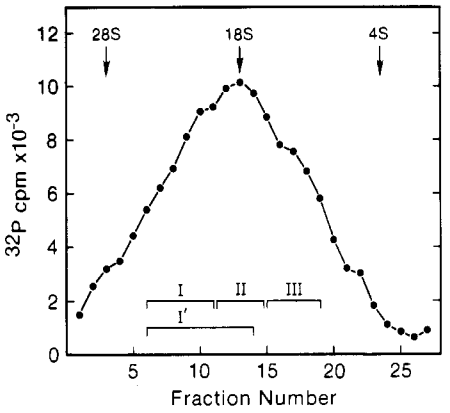

FIGURE 1: Sucrose density gradient sedimentation of ^{32}P-labeled HeLa cell cytoplasmic polyadenylated mRNA. Regions designated with Roman numerals indicate RNA fractions pooled. Arrows represent positions of HeLa cytoplasmic poly(A−) markers in a parallel gradient.

the column was precipitated with ethanol at −20 °C and then analyzed for oligo(U) content as described above.

Gel Electrophoresis and Electroelution of RNA. Electrophoresis of RNA in 98% formamide–4.2% polyacrylamide gels has been described (Nakazato et al., 1975). The ECU-40 elution concentrator apparatus was used to elute RNA fragments from gels. Electroelution blocks were sealed by using a sterile Spectropore 2 dialysis membrane (25-mm width). The chambers were partially filled with 10 mM Tris–acetate, pH 7.5, and 1 mM EDTA elution buffer. Gel portions, containing RNA of the desired size range, were cut into several pieces and loaded into the chambers which were then filled with buffer. To optimize recovery, 50 μg of HeLa rRNA was added per chamber. Gel fragments were electrophoresed for 5 h at 60 V, resulting in 85–88% recovery of the samples. A second round of elution with fresh buffer recovered an additional 10% of the RNA while 2–5% was retained in the gel.

Quantitation of Labeled Caps and Adenosine. The products of a complete RNase T2 digestion of ^{3}H-end-labeled mRNA were applied in about 20 μL to the center of a 1-cm lane on 20 × 40 cm Whatman 3MM paper wet with 0.05 M pyridinium acetate and 0.01 M Na_2EDTA adjusted to pH 3.5. Electrophoresis in this buffer at 75 V/cm was continued until the picrate marker was 15–18 cm from the origin. ^{3}H-Labeled material migrating toward the cathode was coincident with adenosine included in the sample. Material migrating toward the anode that was included in a region encompassing the GMP and UMP standards was taken as cap 1 plus cap 2, since cap 1 has previously been shown to display such a mobility in this system (Wallace et al., 1981). Additional evidence confirming that this ^{3}H material represented caps was obtained by chromatography of the digest on DEAE-Sephacel. Two components (cap 1 and cap 2) having charges of 5− and 6− together comprised the same percent of the total ^{3}H of the sample as did the material migrating on paper in the region of UMP and GMP (data not shown).

RESULTS

Location of Oligo(U) Sequences Relative to the 3′-Poly(A) Sequence of mRNA. The poly(A) sequence allows 3′ fragments detached from mRNA by nicking with alkali to be recovered on oligo(dT)–cellulose. If oligo(U) sequences were confined to the 3′ regions of mRNA, they would be concentrated in such fragments. Since the total mRNA population of HeLa cells ranges from 600 to 3500 nucleotides in length, smaller mRNAs would escape a limited amount of nicking. Such unnicked molecules included in the 3′ fragments bound

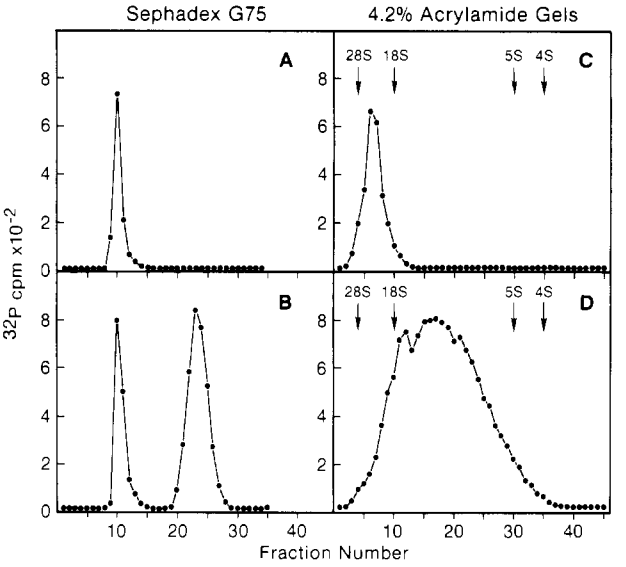

FIGURE 2: Effect of polynucleotide phosphorylase treatment of HeLa cell mRNA. (A) Sephadex G-75 profile of poly(A+) mRNA pool I from sucrose density gradient in Figure 1. (B) Sephadex G-75 profile of oligo(U+) mRNA [isolated from poly(A+) mRNA pool I] after 45-min treatment with polynucleotide phosphorylase as described under Experimental Procedures. (C) Polyacrylamide gel electrophoresis of poly(A+) mRNA pool I. (D) Polyacrylamide gel electrophoresis of material excluded from Sephadex G-75 (fractions 9–13) in (B).

Table I: Number of Oligo(U) Sequences per mRNA Molecule

oligo(U+) mRNA	oligo(U) content (%)	oligo(U) length[c] (nucleotides)	UMP/GMP ratio	RNA average length[d] (nucleotides)
pool I[a]	1.03 (1215)[b]	29.4	23/1	2855
pool II	1.25 (1247)	26.6	21/1	2130
pool III	1.70 (1685)	30.0	24/1	1760

[a] Pools from gradient of Figure 1. [b] Numbers in parentheses indicate cpm analyzed. [c] Determined from the ratio of ^{32}P in total nucleotides to that in GMP in the fragments bound to poly(A)–agarose. [d] Calculated by assuming one oligo(U) per molecule.

to oligo(dT)–cellulose could obscure the interpretation of these location experiments, particularly if oligo(U) was near the 5′ ends of such short molecules. To avoid this, a more homogeneous population lacking such small molecules was selected from the mRNA profile displayed in the sucrose gradient of Figure 1. This fraction designated as pool I migrated during electrophoresis in a denaturing gel as a population ranging from 2500 to 3500 nucleotides in length (see Figure 2C). The oligo(U)-containing RNA isolated from pool I was analyzed for oligo(U) content as shown in Table I. Base analysis of the oligo(U) fragments generated by RNase T1 yielded an average fragment length of 29.4 nucleotides with a UMP/GMP ratio of 23. If it is assumed that each molecule contains only one oligo(U) sequence, an average length for this mRNA pool of 2855 nucleotides was calculated. This value is in good agreement with the electrophoretic mobility of these molecules noted above. When oligo(U)-containing RNAs from intermediate (pool II, Figure 1) and small molecules (pool III) were analyzed in this same way, RNA lengths of 2130 and 1760 nucleotides, respectively, were calculated (Table I). These calculated lengths also are compatible with electrophoretic mobilities of these RNAs in denaturing gels (data not shown). From these results, we conclude it is unlikely that an mRNA molecule will contain more than a single oligo(U) sequence.

A timed exposure of an aliquot of the mRNA of pool I to alkali gradually reduced its binding to oligo(dT)–cellulose as shown in the second column of Table II. A direct analysis

Table II: Association of Oligo(U) Sequences with 3′-Polyadenylated mRNA Fragments

alkali exposure (min)	% mRNA fragments bound to $(dT)_n$–cellulose[a]	% mRNA fragments bound to poly(A)–agarose[b]	% oligo(U+) fragments bound to $(dT)_n$–cellulose[c]
0	83	14	83
0.5	72	12	67
1	66	9	65
2	55	7	56
3	39	5	44
8	21	2	24

[a] Obtained from an aliquot of each alkaline digest of the poly(A+) mRNA from pool I of Figure 1. [b] RNA fragments from each digest were treated with formaldehyde and bound to poly(A)–agarose as described under Experimental Procedures. [c] Oligo(U)-containing fragments eluted from poly(A)–agarose were treated to remove formol groups and then bound to oligo(dT)–cellulose.

Table III: Association of Oligo(U) Sequences with 5′ Ends of mRNA

enzyme treatment[a] (min)	5′-RNA fragments, % of original RNA	% oligo(U) content[c]	% of predicted oligo(U) content for 5′ location[d]
0	100[b]	0.83	0.83
10	80	0.92	1.04
15	76	0.75	1.09
30	48	1.10	1.74
40	34	0.93	2.45
45	31	0.93	2.68
50	18	0.93	4.62

[a] Formaldehyde-treated RNA of pool I of Figure 1 was digested with polynucleotide phosphorylase. [b] Each digest was passed over Sephadex G-75 (as under Experimental Procedures). The cpm in the excluded peak (see Figure 2) is given as a percent of the total radioactivity from the column. [c] The material excluded from Sephadex G-75 was analyzed for oligo(U) content as described under Experimental Procedures. [d] The oligo(U) content of truncated mRNA molecules assuming complete retention of oligo(U) sequences. Calculated as 100(0.83/% of original RNA).

of the resultant polyadenylated fragments for oligo(U) sequences was avoided since the formation of poly(A)–oligo(U) intermolecular hybrids favored by the 5-fold higher concentration and 5-fold greater lengths of the poly(A) sequences in the RNase T1 digests could limit recovery of oligo(U) on poly(A) affinity columns. Instead, the fragments produced by alkali cleavage were treated with formaldehyde to mask poly(A) which then allowed those containing oligo(U), which is unaffected by formaldehyde, to be selected directly on poly(A)–agarose. The fraction of the total mRNA fragments that contain an oligo(U) sequence is shown for each treatment period in column 3 of Table II. Following deformolation, each of the oligo(U+) fragments was analyzed for poly(A) on oligo(dT)–cellulose. In this case, oligo(U)'s are too short to interfere with the binding of poly(A)-containing fragments to oligo(dT)–cellulose. The possibility that oligo(U+) fragments that lack poly(A) might hybridize to the poly(A)-containing fragments was also ruled out when radiolabeled oligo(U) sequences added to the fragments before hybridization to oligo(dT)–cellulose remained unbound.

The last column of Table II lists the fraction of each set of oligo(U)-containing fragments that retains the 3′-poly(A) sequence. That these fragments contain one oligo(U) and one poly(A) sequence was confirmed by direct analysis of the smallest fragments (8 min in alkali) for both oligo(U) and poly(A) by techniques described under Experimental Procedures. Poly(A) accounted for 29% and oligo(U) 5% of the total nucleotides of the fragments. Using characteristic lengths of 150 nucleotides for poly(A) and of 25 nucleotides for oligo(U), an average fragment size of 517 nucleotides was calculated from the poly(A) content and 500 nucleotides from the oligo(U) content. These lengths are in close agreement with the average lengths calculated from the electrophoretic mobilities of these fragments (data not shown).

It is clear from Table II that as the size of the oligo(U)-containing fragments is decreased there is a parallel decrease in the number that contain poly(A), but there is no sudden loss of poly(A) from oligo(U+) RNA fragments as might be expected if most oligo(U) sequences were near 5′ ends of the original mRNA population. On the other hand, there is no marked retention of poly(A) in the shortest oligo(U)-containing fragments as might be expected if oligo(U)'s were close to 3′ ends. At the last point in the hydrolysis when 21% of the RNA nucleotides remain as 3′-polyadenylated fragments (column 2), 24% of the oligo(U+) fragments remain attached to a poly(A) sequence. This retention of oligo(U)'s in the shortest polyadenylated fragments shows that the oligo(U)'s in some of these mRNAs are 350 nucleotides or less away from the 150-nucleotide poly(A) sequence.

A similar set of experiments carried out on mRNA from pools II and III from the gradient of Figure 1 gave similar but somewhat less readily interpretable results because of the shortened distance between the ends of mRNAs in these populations.

Location of Oligo(U) Sequences Relative to the 5′ Ends of mRNA. The results just described would rule out an exclusive location for oligo(U) close to either the 5′ or the 3′ end of mRNA. The possibility that oligo(U) has no fixed locus or that it may be in a 3′-untranslated region remained. The latter had to be considered since recent sequence data have shown the length of this region to vary greatly in individual mRNAs. While in many molecules it comprises 30–40% of the total length, examples in which it exceeds 50% have been reported (see Discussion). An analysis of 5′ fragments similar to that carried out above on 3′-polyadenylated fragments would help to distinguish such possibilities. However, the lack of a dependable technique for selecting 5′-terminated pieces led us to use some different approaches for obtaining the oligo(U) content of 5′ fragments.

In one approach, the 3′-exonucleolytic activity of polynucleotide phosphorylase was used to remove nucleotides successively from the 3′ ends of mRNA. The resulting 5′ fragments were then separated by gel filtration from the nucleoside diphosphates released by this hydrolysis (Figure 2A,B). The oligo(U) content of fragments exposed to polynucleotide phosphorylase for increasing time periods was measured to assess the location of oligo(U) with respect to the 5′ ends of the intact mRNA. Table III lists a set of such 5′ fragments derived from a population of mRNA molecules similar to pool I of Figure 1 that were decreased in length by exposure to polynucleotide phosphorylase. Over time, the loss of nucleotides from the 3′ ends of these mRNAs roughly paralleled the loss of oligo(U) sequences since the oligo(U) contents of the fragments remained essentially unchanged. As would be predicted from the previous experiments with 3′-polyadenylated fragments, oligo(U) was removed from some molecules after only 20% of the mRNA nucleotides were removed, confirming a 3′ location for some oligo(U)'s. Oligo(U)'s are also not preferentially located in 5′ regions since the predicted increases in oligo(U) content for such a possibility are not seen in Table III. On the other hand, oligo(U) is still found in the smallest fragments generated that range from 400 to 1200 nucleotides in length (Figure 2D). This leaves open

the possibility that some oligo(U)'s could be close to 5′ ends.

Before we conclude from these analyses of both 3′ and 5′ fragments that oligo(U) may have no specified location with respect to either end of an mRNA molecule, certain assumptions involved in the interpretation of the data for 5′ fragments generated by polynucleotide phosphorylase need to be noted. First, in contrast to the 3′ fragments, it has been assumed, but not demonstrated, in the experiment of Table III that these fragments do contain the 5′ end of the original mRNA molecules and, second, that their lengths are roughly proportional to that fraction of the original radioactivity they now contain. This presumes that polynucleotide phosphorylase attacks all molecules simultaneously and that the overall rate of this processive removal of nucleotides is similar for each mRNA. An approximately 5-fold molar excess of polynucleotide phosphorylase over mRNA was used in these experiments to favor a simultaneous initiation of hydrolysis. Formolated mRNA was used to eliminate secondary structures that might cause variations in the rates of hydrolysis. Rates of hydrolysis of these formolated mRNAs were, in fact, similar to those reported for polynucleotide phosphorylase on native globin mRNA (Soreq et al., 1974).

Finally, endonucleolytic cleavages that may occur during the course of the reaction that would create new 5′ ends must also be considered, particularly in the experiments of longer duration. The most highly purified polynucleotide phosphorylase preparations from *M. luteus* retain low levels of nuclease that may be an intrinsic property of the enzyme (Craine & Klee, 1976; Barbehenn et al., 1982), although such activity is reported to be minimal at the pH of our experiments. It is apparent that the gel filtration elution profile of digestion products remaining after 50 min is that expected for an exonuclease. As seen in Figure 2B, most material is found either in the excluded volume or in the included mononucleotide fraction. The profile of the same digest after electrophoresis in denaturing gels does show an accumulation of intermediate-sized fragments as digestion proceeds (Figure 2D), but very few smaller fragments. Although this indicates that endonuclease activity was undoubtedly limited up to this point of the digestion, the heterogeneity of the initial substrate (2500–3500 nucleotides) as well as any tendency for such reactions to become asynchronous as they progress would make it difficult to assess the contribution a limited endonuclease activity might make to the reaction products.

To obtain more direct evidence for the association of oligo(U) sequences with 5′ fragments suggested by these results, we turned to an approach that avoids difficulties inherent in the 3′-exonuclease reaction. In this case, mRNA was fragmented with alkali, and the oligo(U)-containing fragments of different lengths were analyzed for their contents of 5′ caps and 3′-poly(A). Label was introduced into periodate-oxidized mRNA in vitro by the reduction of the oxidized 2′- and 3′-*cis*-glycols at each end of such RNA with ^{3}H-labeled sodium borohydride. The ^{3}H-labeled adenosine and ^{3}H-labeled caps released from the ends of the RNAs with RNase T2 were then separated by high-voltage electrophoresis. The top panel of the total RNA seen in Figure 3 shows the radioactivity profile of ^{3}H in adenosine and in caps. The ^{3}H remaining at the origin (~15%) is apparently not oligonucleotide in nature since it was completely resistant to nuclease P1 as well as to RNase T2 hydrolysis. We assume this represents some artifact associated with the chemical labeling procedure since it is not found in digests of the same RNA labeled in vivo with ^{32}P. The ratio of radioactivity in caps to that in adenosine shows that most RNAs are capped (Table IV). A similar analysis

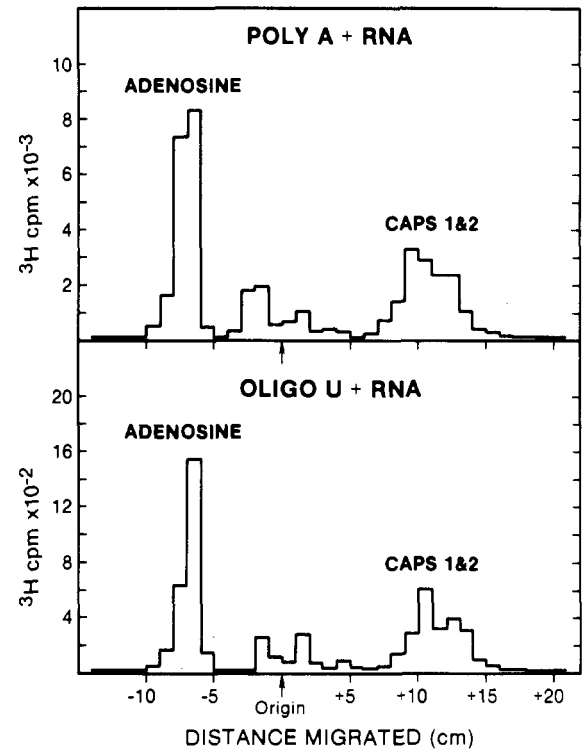

FIGURE 3: Paper electrophoretic analysis of the RNase T2 digestion products of ^{3}H end-labeled HeLa cell polyadenylated mRNA. (Top panel) Products from poly(A+) mRNA pool I′ from sucrose density gradient in Figure 1. (Bottom panel) Products from oligo(U+) mRNA isolated from poly(A+) mRNA pool I′.

Table IV: Labeled Ends of Polyadenylated Messenger RNA[a]

RNA	expt 1		expt 2	
	5′ caps	3′-adenosine	5′ caps	3′-adenosine
total poly(A+) RNA (^{3}H cpm)	46000	62000	136000	158000
oligo(U−) mRNA (^{3}H cpm)	44000	55500	127300	145000
oligo(U+) mRNA (^{3}H cpm)	4400	5700	12600	14700
oligo(U+) mRNA (%)	9.1	9.3	9.0	9.2
^{3}H-caps/[^{3}H]adenosine ratio				
total poly(A+) RNA	0.74		0.86	
oligo(U+) mRNA	0.77		0.86	

[a] An aliquot of ^{3}H end-labeled poly(A+) RNA, selected from pool I′ (see Figure 1), was treated with formaldehyde and bound to poly(A)–agarose as described under Experimental Procedures. Quantitation of the RNase T2 products (^{3}H-cap and [^{3}H]adenosine) of the RNA fractions from poly(A)–agarose is also described under Experimental Procedures. Values represent cpm normalized for the aliquots taken. The remaining RNA was treated with alkali to obtain the fragments analyzed in Table V.

of the ends of the oligo(U+) mRNAs obtained from this RNA shows them to be capped to a similar extent (Figure 3 and Table IV).

Table IV is a summary of data from two different experiments showing the distribution of ^{3}H in both 5′ and 3′ ends of periodate-oxidized mRNA reduced with NaB^3H_4. Seventy-four percent of the labeled polyadenylated mRNAs in experiment 1 and 86% in experiment 2 are capped. Similar values are found for the oligo(U)-containing mRNAs obtained from these RNAs which eliminates concern about losses of 5′ ends during the isolation and chemical labeling of this subset of the mRNA population. Table IV also shows that about 9% of the stable polyadenylated mRNAs of this pool of large

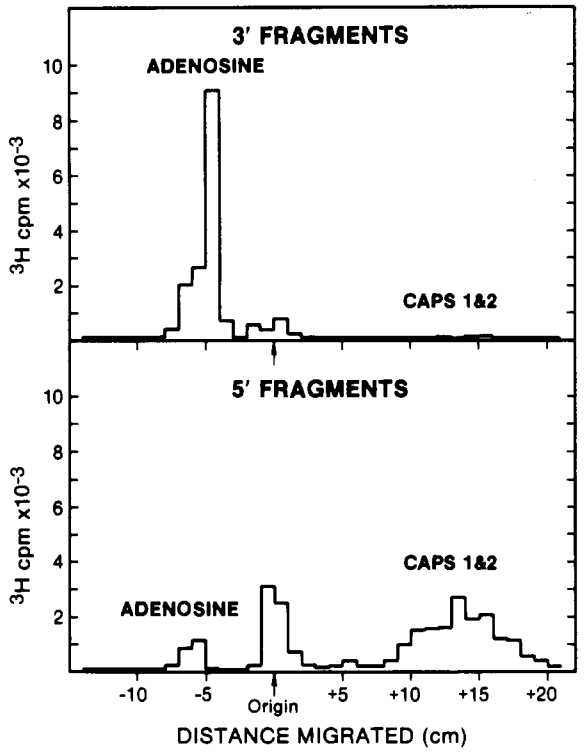

FIGURE 4: Paper electrophoretic analysis of the RNase T2 digestion products from alkali-treated ^{3}H end-labeled polyadenylated mRNA fractionated on oligo(dT)–cellulose. (Top panel) Products of 3′ fragments bound to oligo(dT)–cellulose. (Bottom panel) Products of 5′ fragments unbound to oligo(dT)–cellulose.

mRNAs contain an oligo(U) sequence.

This ability to label specifically both 3′ and 5′ ends of mRNA allowed us to address the question of the location of oligo(U) sequences with respect to the ends of mRNAs more directly than in previous experiments. The mRNA pools of the two experiments of Table IV were subjected to alkali cleavage so that about 25% of the fragments rebound to oligo(dT)–cellulose. Figure 4 shows that all mRNAs were cleaved at least once, since caps were not found in the 3′ fragments that rebound to oligo(dT)–cellulose. The small amount of label found in adenosine in the unbound fragments (Figure 4, bottom panel) was apparently the result of some pieces with poly(A)'s too short to bind to oligo(dT)–cellulose, since they were not seen in subsequent experiments when the NaCl level was raised from 100 to 200 mM before the extract was passed over oligo(dT)–cellulose. Also, caps did not appear in these bound 3′ fragments (data not shown).

The separated 5′ and 3′ fragments were each passed over poly(A)–agarose to obtain those end-labeled fragments that still contained an oligo(U) sequence. An analysis of the caps and the adenosine in each set of fragments is shown in Table V. It is obvious that many of the oligo(U) sequences have been detached from the ends of mRNA by this nicking since the recovery of ^{3}H in fragments of both experiments is much reduced when compared with that originally present in the intact oligo(U+) mRNA. In experiment 2, only about 13% of the capped ends (1670 cpm of the 12 600 cpm shown in Table IV) and 37% of the poly(A) sequences (5490 vs. 14 700 cpm) present in the intact oligo(U+) mRNA remain associated with oligo(U+) fragments of this length. This recovery of only 50% of the oligo(U) sequences attached to ends means that almost half are not close to either end of mRNA. It is clear, however, that those oligo(U)'s remaining attached to an end are more likely to be associated with a labeled adenosine than with a labeled cap.

Table V: Association of Oligo(U) Sequences with 3′ and 5′ Fragments[a] from mRNA

RNA	expt 1 5′ fragments	expt 1 3′ fragments	expt 2 5′ fragments	expt 2 3′ fragments
RNA (pool I′) (^{3}H cpm)	46000	62000	155000	177000
fragments oligo(U–) (^{3}H cpm)	39000	48000	141000	178000
oligo(U+) (^{3}H cpm)	750	1400	1670	5490
fragments recovered (%)	86.4	79.6	92	103
fragments containing oligo(U) (%)	1.87	2.85	1.12	2.99
oligo(U) sequences recovered (%)[b]	51		45	

[a] RNA, described in Table IV, was treated with alkali and bound to oligo(dT)–cellulose to obtain 5′ and 3′ end-labeled fragments. Each population of fragments was modified with formaldehyde and passed over poly(A)–agarose as described under Experimental Procedures. Values represent cpm normalized for aliquots taken for paper electrophoretic analysis. [b] Percent of oligo(U) sequences associated with end-labeled fragments after alkaline hydrolysis of intact RNA calculated as 100{[% 5′-oligo(U+) fragments + % 3′-oligo(U+) fragments]/[% oligo(U+) RNA of total poly(A+) RNA]} (see Table IV).

To interpret these data in terms of the location of the oligo(U) sequence more precisely, the size of these fragments was assessed from their electrophoretic mobilities in denaturing gels as seen for the more abundant oligo(U–) fragments shown in Figure 5. The distribution of ^{3}H in 5′ (top panel) and 3′ fragments (bottom panel) is compared, in each case, with the ^{32}P distribution in fragments produced from the ^{32}P-labeled mRNA added as marker. In each case, the difference between the ^{32}P and ^{3}H profiles is that expected for a weight-average vs. a number-average molecule distribution that results from the two different types of mRNA labeling. This is more clearly seen for the 3′ fragments bound to oligo(dT)–cellulose. The ^{3}H profile for the unbound 5′ fragments shows an anomalous migration of ^{3}H in faster moving components that is apparently due to the contaminant discussed earlier. As noted at that time, this material is well separated from the ^{3}H-labeled caps and adenosine during paper electrophoresis of the digested RNA. Figure 5 also shows a calculated number-average distribution of fragments obtained from the ^{32}P content and length of these fractions that agrees well with the distribution of the ^{3}H-end-labeled fragments (bottom panel, Figure 5).

We estimate from these gels that the fragments analyzed in Table V ranged in length from 150 to 1000 nucleotides with a number-average length of 450 nucleotides. These lengths are compatible with those predicted for this population of mRNA (1800–3500 nucleotides, pool I′, Figure 1) in which each molecule should have been nicked 3–5 times, depending on the original length, as judged by 23% rebinding of 3′ ends to oligo(dT)–cellulose. In any case, it is clear from Table V that many oligo(U)'s are not close to an end since they did not remain attached to end-labeled fragments that averaged 450 nucleotides in length. Since this population included a range of fragment sizes (Figure 5), it was also possible that even the oligo(U)'s that remained attached to ends were not close to an end but were concentrated in the longer fragments at a distance from the labeled end. To investigate this possibility, both oligo(U+) and oligo(U–) fragments of experiment 2 were divided into small and large fragments as designated for the oligo(U–) population in Figure 5. The large fragments encompassed a range of sizes of 600–1200 nucleotides and the small 150–600 nucleotides. It should be noted that all of the

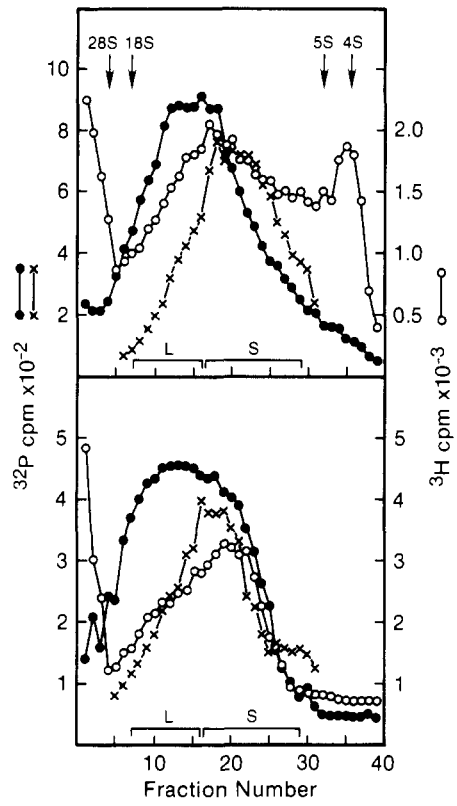

FIGURE 5: Polyacrylamide gel electrophoresis of 5′ and 3′ end-labeled fragments. 5′ and 3′ fragments, separated on oligo(dT)–cellulose after alkali treatment of ^{3}H end-labeled mRNA containing ^{32}P-labeled tracer mRNA, were passed over poly(A)–agarose. The unbound [oligo(U–) fragments] and bound [oligo(U+) fragments] fractions were subjected to polyacrylamide gel electrophoresis. L and S denote large and small fragment regions excised and electroeluted from gels for further analysis. Arrows indicate positions of ^{32}P-labeled cytoplasmic poly(A–) HeLa cell marker RNA in a parallel gel. (Top panel) Fragments unbound to oligo(dT)–cellulose and unbound to poly(A)–agarose. (Bottom panel) Fragments bound to oligo(dT)–cellulose and unbound to poly(A)–agarose. (○) ^{3}H-labeled mRNA fragments; (●) ^{32}P-labeled mRNA fragments; (×) number-average distribution of ^{32}P-labeled molecules, calculated by dividing the total ^{32}P in each fraction by the average length of molecules in the fraction.

3′ fragments contain a 150-nucleotide poly(A) sequence that is unlikely to have been reduced in length since poly(A) is resistant to the alkaline hydrolysis conditions used here (R. Almendinger and M. Edmonds, unpublished results). Thus, in terms of RNA nucleotides, 3′ fragments are approximately 150 nucleotides shorter than the corresponding 5′ fragments. When these populations were analyzed for 5′- and 3′- labeled ends, the results of Table VI were obtained. First, it should be noted that in both the large and small fragment populations, oligo(U) sequences were more likely to be associated with the 3′-polyadenylated than with the 5′-capped fragments. This difference could be expected to be even larger if the reduced length of the actual RNA sequence of the 3′ fragments caused by the poly(A) tail were taken into account. This bias for 3′ ends is consistent with the distribution of oligo(U) in the unseparated fragments displayed in Table V. It should be noted, however, that although there were 2.5 times more fragments in the pool of smaller pieces for both the 3′ and 5′ fragments not containing oligo(U), the fragments containing oligo(U) were almost evenly distributed between the two pools. The fact that oligo(U)'s tended to be concentrated in larger fragments shows again that many are not very close to either end of the mRNA. This result was not unexpected in view of the previous experiments that pointed to a rather random distribution of sites for oligo(U) in different mRNAs (Table II and III). This examination of end-labeled fragments, however, has revealed that this distribution is by no means random, since oligo(U)'s were found in 3′-polyadenylated fragments 3 times more often than in 5′-capped fragments of similar lengths. The failure of the 3′-exonuclease digestion experiments (Table III) to reveal this discrepancy must mean that the fragments analyzed, particularly in the later stages of the digestion, were not comprised exclusively of 5′ ends of the original population of mRNAs. One or more of the several pitfalls discussed earlier in connection with this approach were most probably encountered in our experiment.

Table VI: Retention of Oligo(U) Sequences Relative to Terminal Fragment Length[a]

	5′ fragments[b]		3′ fragments[c]	
	oligo(U–)	oligo(U+)	oligo(U–)	oligo(U+)
fragments				
expt 2 (pool I′) (^{3}H cpm)	141000	1670	178000	5490
large (^{3}H cpm)	33700	570	44300	2740
small (^{3}H cpm)	85000	660	115800	2390
recovery from gels (%)	84	74	90	93

[a] Fragments from experiment 2 (Table V) were fractionated by electrophoresis in gels as described in Figure 5. Recovery from gels and analysis for caps and adenosine are described under Experimental Procedures. [b] ^{3}H cpm in caps. [c] ^{3}H cpm in adenosine.

DISCUSSION

We began these experiments with the expectation that the single oligo(U) sequence present in many polyadenylated mRNAs of HeLa cells would be localized in one of the two untranslated regions found at the opposite ends of these molecules. The plausibility of this view (reviewed in the introduction) was enhanced by evidence emerging at the time that a hexanucleotide, AAUAAA, was localized within 15–25 nucleotides of the 3′-poly(A) sequence of most of the then-sequenced mRNAs (Jou & Fiers, 1978).

The experiments were designed to reveal which of the two ends contained the oligo(U) sequence. We have found, however, that oligo(U) is not confined to some defined region of each mRNA molecule but has a different location in different mRNAs. Although no site has been ruled out by these experiments, the study of end-labeled fragments shows that oligo(U) is more likely to be near a 3′ than a 5′ end of mRNA although a large number of oligo(U) sequences are apparently not close to either end of mRNA.

It is of interest to relate these results to more recent mRNA sequence data that have accumulated since these studies began. A recent compilation of sequence data for the 5′-untranslated region of more than 200 cellular and viral mRNAs (Kozak, 1983) shows that for more than 80% of these molecules the 5′-untranslated region is between 10 and 80 nucleotides, while for 15% it is somewhere between 80 and 190 nucleotides. The few remaining RNAs have 5′-untranslated regions longer than 200 nucleotides. That for the mRNA encoding the c-myc gene product is 572 nucleotides (Watt et al., 1983).

We have examined the 3′-untranslated regions of 86 fully sequenced mRNA species of humans and higher vertebrates obtained from the Protein and Nucleic Acid Sequence Data Base of the National Biomedical Research Foundation of Georgetown University Medical Center and found the length of this region to extend from 3 to 1152 nucleotides. In 60% of the molecules, the sequence fell within a range of 100–300 nucleotides while in 28% it was longer than 300 nucleotides. These data clearly point out the marked differences in length

of the two untranslated regions that largely reflect length differences already observed in individual sequenced mRNAs. As would be expected from the range of actual lengths noted here, the 3′-untranslated region is not some constant fraction of the total length of the mRNA. The value of this fraction was spread quite evenly over a range of 5–45% for 80 of these 86 species. In fact, it is not uncommon to find similar size mRNAs from the same species to differ markedly in the length of the 3′-untranslated region. Transferrin (Jeltsch & Chambon, 1982) and muscle pyruvate kinase (Lonberg & Gilbert, 1983) of the chick each contain approximately 2350 nucleotides, but the 3′-untranslated region of transferrin is 183 nucleotides while that of pyruvate kinase is 678 nucleotides.

When the results of the oligo(U) localization experiments for HeLa cell mRNA described here are viewed against this background of variability in lengths of the untranslated regions, it is clear that our data cannot consign oligo(U) sequences exclusively to noncoding regions in all mRNAs, even though we expect this to be the case for reasons noted earlier. The oligo(U)'s that our results have shown to be centrally located in many HeLa cell mRNAs are likely to be in noncoding regions since it is not uncommon for the 3′ region to comprise 45% or more of the total length of an mRNA.

We now know of four mRNA molecules that contain an oligo(U) sequence in the 3′-untranslated region that would be long enough to allow the mRNA to be recovered as an oligo(U+) species by our techniques. However, we are not aware of a sequenced mRNA with an oligo(U) in a 5′-untranslated region. The mRNAs for the chicken ovalbumin Y gene product (Heilig et al., 1982) and that for human β-actin (Ponte et al., 1984) have runs of 24 and 16 uninterrupted UMP residues that reside at distances 21% and 27%, respectively, of the total length of the RNA starting from the 3′-poly(A) addition site. Oligo(U)'s found in 3′-noncoding regions of two other mRNAs for which partial sequences were available include a U_{12} sequence in one member of the mouse H2 antigen multigene family (Lalanne et al., 1982) and a U_{18} sequence in type II procollagen of the chicken (Sandell et al., 1984). Although their 3′-noncoding regions do not differ greatly in length (488 nucleotides for H2 antigen vs. 525 nucleotides for type II procollagen), oligo(U) was only 43 nucleotides from the 3′-poly(A) addition site of the H2 antigen mRNA, but 482 nucleotides for procollagen. Although these four mRNAs comprise too small a sample to be compared with the much greater number of HeLa oligo(U+) mRNAs in the mixed populations we have studied, the fact that oligo(U) is in a 3′-noncoding region in each case would be expected from our data showing that oligo(U) is much more often associated with a 3′ than with a 5′ end-labeled fragment. However, at the same time, our data predict that some mRNAs will have oligo(U)'s near 5′ ends. The sequence data for these four mRNAs also confirm our conclusion for the HeLa cell mRNAs that oligo(U) is not positioned at some relatively fixed distance from the 3′-poly(A) tail as is the case for the AAUAAA sequence.

Adjacent to the 5′ end of the oligo(U) sequence in each of these four sequenced mRNAs is a UMP-rich region that is interrupted periodically by one or occasionally by two GMP residues. A single AMP or CMP occurs no more than once in any one of these UMP-rich regions that range from 17 nucleotides for the H2 antigen mRNA to 75 nucleotides for the ovalbumin Y gene product mRNA. This suggests that oligo(U) could be part of a larger sequence representing a repetitive element. Detection of oligo(U)'s in other sequenced mRNAs should allow the generality of this observation to be assessed. The availability of cloned cDNAs for human β-actin mRNA would also allow an experimental testing of the occurrence of oligo(U) and its adjacent sequences in the various oligo(U+) mRNA populations of HeLa cells. From what has been said thus far, it is apparent that β-actin mRNA should be present in the oligo(U+) mRNA population analyzed in our experiments. In a separate paper (Wood et al., 1985), we present in vitro translation data that strongly suggest this is indeed the case.

Certain speculations about the structure and function of oligo(U+) mRNAs can be ruled out by our localization studies and by the mRNA sequence data. For example, because of its variable distance from poly(A), it is no longer reasonable to expect that the hybridization of oligo(U) to the 3′-poly(A) tail will produce some looped-out or circular structure common to all such mRNAs; rather, a variety of such structures might be expected.

The precursor role once suggested for oligo(U+) mRNA based on the greater average length of this population and some evidence for sequences shared with oligo(U−) mRNAs (Wood & Edmonds, 1981; Wood et al., 1985) must be discarded in view of the occurrence of oligo(U) in mature mRNAs. Turnover studies have also failed to reveal any obvious differences in the metabolic stability of the oligo(U+) and oligo(U−) mRNAs (Wallace, 1983).

It is not obvious from the source, structure, or protein coded by each of the four mRNAs described above that oligo(U+) mRNAs comprise a class of mRNAs with some related function(s). Of course, the continued accumulation of sequence data for oligo(U+) mRNAs may yet reveal such relationships. At present, the effect that an oligo(U) sequence or some larger sequence containing it may have on the function of an mRNA molecule is unknown. If such an effect were shared by all oligo(U+) mRNAs, then our data would predict it to be independent of the precise location of oligo(U) within mRNA.

Registry No. Oligo(U), 27416-86-0.

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Sequence Content of Oligo(uridylic acid)-Containing Messenger Ribonucleic Acid from HeLa Cells†

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Received October 29, 1984

ABSTRACT: Oligo(uridylic acid)-containing [oligo(U+)] RNA was isolated from poly(adenylic acid)-containing [poly(A+)] mRNA from HeLa cells by using either formaldehyde pretreatment or poly(A) removal, both of which resulted in increased accessibility of oligo(U)-rich sequences to a poly(A)–agarose affinity column. In this report, we compared the sequence content of oligo(U+) RNA with that of molecules lacking oligo(U) [oligo(U–) RNA] by their relative hybridization to cDNA reverse-transcribed from poly(A+) mRNA and by comparison of their in vitro translation products synthesized in a rabbit reticulocyte lysate. Formaldehyde-modified poly(A+) RNA, treated to remove the formol adjuncts, was inactive as a template for in vitro protein synthesis; consequently, only depolyadenylated RNA, which retains its translatability, could be used in the translation studies. The hybridization kinetic experiments revealed that oligo(U+) RNA contained most of the sequence information present in oligo(U–) RNA but at a reduced level (ca. 25%), the majority of the oligo(U+) RNA sequences being poorly represented in the cDNA. This result was supported by one- and two-dimensional gel analysis of their in vitro translation products which showed that oligo(U+) RNA, although less effective as a template for translation than oligo(U–) RNA, coded for proteins, the most abundant of which were encoded by rare messages not highly represented in oligo(U–) RNA or the total poly(A+) RNA. Although some minor products were synthesized by both oligo(U+) and oligo(U–) RNA, at least 33 proteins were unique to or highly enriched in the pattern of products directed by oligo(U+) RNA. Of these, only two species, which have the mobility characteristics of β-actin and its unacetylated derivative, were also abundant oligo(U–) RNA products.

Previous reports from our laboratory (Wood & Edmonds, 1981) and another (Molloy, 1980) have shown that an appreciable fraction of HeLa cell poly(adenylic acid)-containing mRNA [poly(A+) mRNA][1] contains a short uridylate-rich region [oligo(U)]. Before such molecules can be selected on a poly(A) affinity column, they must undergo a pretreatment which renders the oligo(U) sequences accessible to the column. Two methods have been employed to this end. Molloy (1980) found that when poly(A+) RNA derived from polysomes was treated with formaldehyde, it exhibited enhanced binding to poly(A)–Sepharose. Enhanced binding to poly(A)-agarose of total cytoplasmic poly(A+) RNA was also achieved in our laboratory by first removing the 3′-poly(A) tails with RNase H directed by oligo(dT) (Wood & Edmonds, 1981). Molecules isolated by the latter procedure were shown to be on average slightly larger than the total mRNA and to bear both cap 1 and cap 2 at their 5′ termini (Wallace et al., 1981), corroborating earlier evidence that they are cytoplasmic in location. To gain further insight into the function of oligo(U)-containing RNA, we decided to compare its sequence content with that of molecules lacking oligo(U). This was approached in two ways. First, their relative ability to hybridize to a cDNA reverse-transcribed from total poly(A+) mRNA was investigated. Second, their in vitro translation products were compared. Because use of formaldehyde re-

† This work was supported by Grants CA 18065 and GM 32585 from the National Institutes of Health.
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[1] Abbreviations: poly(A), poly(adenylic acid); poly(A+) RNA, poly(A)-containing RNA; oligo(U), oligo(uridylic acid); oligo(U+) RNA, oligo(U)-containing RNA; oligo(U–) RNA, oligo(U)-lacking RNA; oligo(U±) RNA, fraction bound and eluted from poly(A)–agarose with ETS that did not rebind to a second column; –pA and U(++), used in figures to signify depolyadenylated poly(A+) RNA and oligo(U+) RNA, respectively; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; oligo(dT), oligo(thymidylic acid); RNase, ribonuclease; ETS, 0.002 M EDTA, 0.01 M Tris-HCl (pH 7.5), and 0.1% SDS; NETS, same as ETS plus NaCl at the indicated concentration; Cl_3CCOOH, trichloroacetic acid; PAGE, polyacrylamide gel electrophoresis; DATD, *N*,*N*′-diallyltartardiamide; 2-D, two-dimensional; IEF, isoelectric focusing; NEpHGE, nonequilibrium pH gel electrophoresis; R_0t, concentration of RNA in moles of nucleotide per liter multiplied by time in seconds; HCHO, formaldehyde; endo, endogenous incorporation without added RNA.

0006-2960/85/0424-3686$01.50/0