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

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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-

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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.

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sulted in a higher yield of oligo(U+) RNA, this method of pretreatment was adopted for the hybridization experiments. However, formaldehyde modification of HeLa cytoplasmic poly(A+) RNA completely inactivated it as a template for translation in a rabbit reticulocyte lysate even after treatment to reverse the formaldehyde treatment. Therefore, removal of poly(A) using RNase H, which had little effect on poly(A+) mRNA translation, was employed for the translation studies.

Experimental Procedures

Materials. [^{3}H]dCTP (26 Ci/mmol) and [^{3}H]leucine (146.5 Ci/mmol) were purchased from New England Nuclear. Unlabeled dNTPs were from Sigma. S1 nuclease was a product of Boehringer Mannheim, and AMV reverse transcriptase was obtained from Life Sciences, Inc. Chelex-100 and all of the chemicals and molecular weight protein markers used for gel electrophoresis were from Bio-Rad. Nonidet P-40 and urea were from BRL as was the nuclease-treated rabbit reticulocyte translation kit (−leucine). DE-81 filter circles were obtained from Whatman. Formaldehyde (Baker reagent grade) and dimethyl sulfoxide (Eastman) were used without additional purification. LKB was the source of the ampholines (40% stock solutions), the slab gel apparatus, and the glass gel tubes used for the first-dimension separations, which were carried out in a Hoeffer tube gel unit. Sources for most of the other materials used have been previously described (Wood & Edmonds, 1981).

Cell Culture, RNA Extraction, and Oligo(dT)–Cellulose Chromatography. Maintenance of HeLa cells in suspension culture was previously described (Edmonds et al., 1971). Three liters of cells at 4×10^5 per milliliter was harvested and the RNA extracted from the postmitochondrial supernatant adjusted to 0.05 M Hepes buffer, pH 7.2, and 0.01 M EDTA by using SDS and hot phenol essentially as described previously (Korwek et al., 1976). Poly(A+) RNA was isolated by three successive bindings to ~1 g of oligo(dT)–cellulose (Nakazato & Edmonds, 1974).

Poly(A) Removal, Formaldehyde Modification, and Poly(A)–Agarose Chromatography. Poly(A) was removed as described in a previous report (Wood & Edmonds, 1981) using calf thymus RNase H (~250 units/50 μg of RNA) directed by $(dT)_{12-18}$ (~2 μg/50 μg of RNA). Formaldehyde treatment of RNA was essentially as described by Molloy (1980). RNA was dissolved in 10 mM sodium phosphate, pH 7.8, to a concentration not exceeding 380 μg/mL. One-tenth of a volume of 37% (v/v) formaldehyde was added and the sample heated to 65 °C for 10 min. After the sample was plunged in ice, it was diluted with 3 volumes of 0.2 M NETS before precipitation with ethanol at −20 °C. Poly(A)–agarose chromatography was carried out as outlined in Wood & Edmonds (1981). Bound fractions were eluted as described in the appropriate figure or table legend. To remove the formol adjuncts, RNA was dissolved in 0.1 M Tris-HCl, pH 7.4, and 0.01 M EDTA, heated to 80 °C for 10 min, and finally precipitated with ethanol at −20 °C. Recoveries at each step in oligo(U+) RNA isolation were monitored by inclusion of a trace amount of ^{32}P-labeled poly(A+) RNA added prior to formaldehyde treatment or poly(A) removal.

cDNA Synthesis. The method used for cDNA synthesis was adopted from that of Rosen & Monahan (1981); 250 μCi of [^{3}H]dCTP was dried down and resuspended in 50 mM Tris-HCl, pH 7.8, 9 mM $MgCl_2$, 20 mM dithiothreitol, 5 μg/mL $(dT)_{12-18}$, 0.4 mM each of dTTP, dATP, and dGTP, 4 mM sodium pyrophosphate, and 50 mM KCl. To this was added HeLa cytoplasmic poly(A+) RNA at 50 μg/mL. After the reaction was allowed to remain at 0 °C for 5 min, 30 units of AMV reverse transcriptase was added and incubation continued for 20 min at 46 °C with occasional mixing, before termination by addition of 50 mM EDTA and 100 μg/mL poly(A). Separation from unincorporated [^{3}H]dCTP was achieved by passage over a Sephadex G-50 column equilibrated with 0.1 M NaCl, 0.1 M Tris-HCl, pH 7.4, and 2 mM EDTA. After incubation with 0.1 M NaOH overnight, to digest RNA, the [^{3}H]cDNA was neutralized with glacial acetic acid and stored until needed at −20 °C under ethanol with 80 μg of poly(A) as carrier. Alkaline agarose gel electrophoresis with appropriate DNA size markers showed the weight-average size of the cDNA to be 1200 nucleotides (data not shown).

Hybridization Reactions. All solutions used were passed over a Chelex-100 column to remove heavy-metal ions. Three separate RNA concentrations were used to drive the hybridization of [^{3}H]cDNA (10^{-4}, 10^{-3}, and $10^{-2.5}$ mol of nucleotide/L). RNA pellets were rinsed with 70% ethanol and dried before being dissolved in 0.2% SDS containing the cDNA. RNA was always in at least 500-fold excess over cDNA. The hybridization reaction was started by addition of an equal volume of 2× hybridization buffer (1.2 M NaCl, 0.2 M Hepes, pH 7.0, and 2 mM EDTA). The samples were incubated at 68 °C for appropriate times. In order to prevent evaporation for incubation times greater than 100 s, aliquots were sealed in siliconized capillary tubes and immersed in a water bath at 68 °C. After incubation, samples were expelled into 55 μL of 2× S1 nuclease buffer (0.4 M sodium acetate, pH 4.5, 0.8 M NaCl, and 5 mM $ZnCl_2$) and stored temporarily at −20 °C. The extent of hybrid formation was assayed by removing two equal aliquots from each reaction, one of which was incubated at 37 °C for 90 min with 527 units of S1 nuclease and the other kept at 0 °C to determine total radioactivity. The surviving hybrids were captured by spotting the samples onto a 2.5-cm DE-81 filter and washing 3 times for 5–20 min with 0.5 M sodium phosphate, pH 6.8, with occasional agitation. Salt was removed with two water rinses followed by a final rinse with 95% ethanol. Dried filters were counted directly in toluene/Omnifluor.

In Vitro Translations, Gel Analysis, and Fluorography. RNA samples to be translated were ethanol precipitated twice from 0.2 M potassium acetate, dissolved in sterile water at 100 μg/mL, and stored at −85 °C until needed. Each translation reaction (30 μL) contained 0.5 μg of RNA and 25 μCi of [^{3}H]leucine and was incubated at 30 °C for 20 min (unless otherwise specified) in a rabbit reticulocyte lysate following the protocol supplied with the kit (see Materials) except that additional $MgCl_2$ was not required and the potassium acetate concentration was optimized for HeLa poly(A+) mRNA at 80 mM. Aliquots (2.5 μL) were removed, decolorized, and tRNA deacylated by incubation at 37 °C for 20 min in an alkaline peroxide solution before precipitating at 0 °C for 30 min with 10% Cl_3CCOOH in the presence of unlabeled leucine. Precipitates were collected on glass fiber filters, washed with 5 × 2 mL of cold 5% Cl_3CCOOH, solubilized, and counted in toluene/Omnifluor.

^{3}H-Labeled translation products for gel analysis were incubated with RNase A (~100 μg/mL) for 15 min at 30 °C. Aliquots to be applied to one-dimensional SDS slab gels were diluted to 26 μL with H_2O, and 13 μL of a solution containing 6% (w/v) SDS, 15% (v/v) mercaptoethanol, 30% (v/v) glycerol, and 0.015% (w/v) bromophenol blue was added. Prestained molecular weight protein markers were run in parallel lanes. All samples were boiled for 5 min and allowed to cool before application to the gel. The discontinuous SDS–PAGE method of Laemmli (1970) was used with the

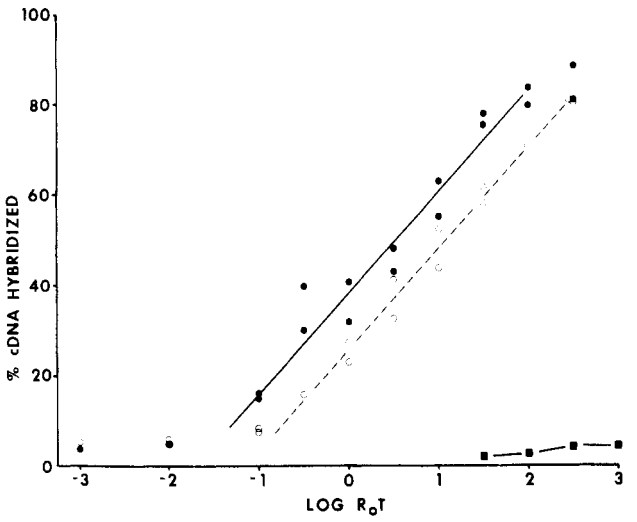

FIGURE 1: Hybridization of oligo(U+) and oligo(U−) RNA with cDNA reverse-transcribed from poly(A+) mRNA. Poly(A+) mRNA was treated with formaldehyde and bound to poly(A)–agarose (see Experimental Procedures) in 0.4 M NETS. After intermediate washes with 0.2 and 0.1 M NETS, the bound material was eluted with ETS. The ETS fraction was retreated with formaldehyde, rechromatographed on a second column of poly(A)–agarose, and eluted directly with ETS omitting the 0.2 and 0.1 M NETS washes (70% rebound). The twice-bound material oligo(U+) RNA and original unbound fraction oligo(U−) RNA were treated to remove formol adjuncts and hybridized to cDNA reverse-transcribed from poly(A+) mRNA (see Experimental Procedures). (●) Oligo(U−) RNA; (○) oligo(U+) RNA; (■) *Escherichia coli* tRNA. Duplicate points are either from identical incubations or from incubations of different concentrations hybridized to an equivalent R_0t. The relative concentration of the hybridizing sequence is calculated by computing the inverse log of the difference in the log (R_0t) value at any point on the linear portion of the two curves to be compared. For the curves shown above, at 50% hybrid formation, this gives a value of $1/10^{1.1-0.5} = 0.25$ (or 25%) for the amount of oligo(U+) RNA sequence present in oligo(U−) RNA.

following modifications. The resolving gel was a 10–20% gradient polyacrylamide gel with DATD as cross-linker and Tris-HCl, pH 8.8, at a concentration of 0.525 M. The stacking gel was 5% polyacrylamide with DATD and Tris-HCl, pH 6.8, at 0.125 M. Electrophoresis (in a chamber cooled to 10 °C) was carried out at 40 mA per gel (1.5-mm thickness) until the dye was 1 cm from the bottom. 2-D gel electrophoresis was performed as described with isoelectric focusing (IEF) (O'Farrell, 1975) or nonequilibrium pH gel electrophoresis (NEpHGE) (O'Farrell et al., 1977) in the first dimension. Aliquots were dried down, dissolved in 50 μL of sample buffer containing the appropriate ampholines, and applied to 3-mm-diameter tube gels. After first-dimension separation, gels were gently shaken for 1–2 h in equilibration buffer and frozen at −85 °C until needed. Electrophoresis in the second dimension with parallel molecular weight markers was exactly as described for one-dimensional gels. Gels were stained with Coomassie brilliant blue, destained, and prepared for fluorography as described by Bonner & Lasky (1974). Exposure was to prefogged Kodak XAR 5 film for the time indicated.

RESULTS

Hybridization Kinetics. To determine whether oligo(U+) RNA shared sequence information with oligo(U−) RNA, both were tested to see to what extent they could hybridize to a cDNA reverse-transcribed from total poly(A+) mRNA. The R_0t curves obtained are shown in Figure 1. Apparently, there was no difference in the percent of the cDNA (>80%) which both RNAs could ultimately protect; however, their kinetics of hybridization did vary. The sequence complementary to

Table I: Effect of Formaldehyde Treatment on Translatability of Poly(A+) mRNA from HeLa Cells[a]

input RNA	translational act.[b] total	translational act.[b] −endo	% of poly(A+) RNA
endo (−mRNA)	27.7		
poly(A+) mRNA	139.9	112.2	100
HCHO-treated poly(A+) mRNA[c]	25.8	0	0
mock-treated poly(A+) mRNA[d]	137.5	109.8	98
globin mRNA[e]	449.8	422.1	376

[a] Translations were carried out for 30 min using a BRL rabbit reticulocyte lysate translation kit as described under Experimental Procedures with 5 μCi of [^{3}H]leucine per reaction. [b] Cl_3CCOOH-insoluble [^{3}H]leucine incorporated (cpm × 10^{-3}). [c] Poly(A+) RNA was treated with formaldehyde and ethanol precipitated, and formol adjuncts were removed as described under Experimental Procedures. [d] Mock-treated RNA was carried through the same heating steps as for formaldehyde treatment except that water was substituted for formaldehyde. [e] Globin mRNA, which was supplied with the translation kit, was added at a level of 0.15 μg per reaction.

the cDNA was present at a lower concentration (approximately 25%) in the oligo(U+) RNA fraction than the oligo(U−) RNA, thereby requiring a higher R_0t value to protect the same amount of cDNA. The oligo(U−) RNA exhibited almost identical hybridization kinetics as poly(A+) mRNA, the template for the cDNA (data not shown). The simplest interpretation was that 25% of the oligo(U+) RNA molecules contained essentially all of the sequence information present in the reverse transcript of total poly(A+) RNA, but the remainder of the oligo(U+) RNA is, in fact, sequence which is represented at very low abundance in the total mRNA and contributes little to the cDNA template. Another possibility, that each oligo(U+) RNA molecule has part of its sequence content (averaging 25%) represented by oligo(U−) RNA sequence information, although unlikely, cannot be discounted.

In Vitro Translatability. As a second approach to examine the sequence content of oligo(U+) RNA, we decided to look at its translational properties as measured in a heterologous protein-synthesizing system from rabbit reticulocytes. Reaction with formaldehyde resulted in inactivation of poly(A+) mRNA as a template for translation (Table I) despite treatment presumed to remove formol adjuncts. A control [see mock-treated poly(A+) mRNA, Table I] demonstrated that the heating steps involved in the treatment were not responsible for the loss of activity. Treatment with RNase H in conjunction with oligo(dT) to remove poly(A) can also be used to facilitate isolation of oligo(U)-containing RNA although lower recoveries than with formaldehyde are usually encountered (Wood & Edmonds, 1981). Sippel et al. (1974) had previously shown that such treatment had little effect on the translatability of globin mRNA. Table II shows that depolyadenylation of poly(A+) mRNA resulted in no significant reduction in [^{3}H]leucine incorporation in a rabbit reticulocyte lysate. However, as is shown in Table II, the oligo(U+) RNA bound and eluted twice from poly(A)–agarose consistently incorporated 75% less [^{3}H]leucine than oligo(U−) RNA (on a microgram basis) which was not reduced relative to the unfractionated controls [poly(A+) RNA or its depolyadenylated derivative]. The material not retained after a second agarose column [oligo(U±) RNA] exhibited an intermediate translatability (38%), suggesting that it was not comprised solely of oligo(U−) RNA contamination. However, two bindings were deemed necessary to ensure the purity of oligo(U+) RNA. The reason for the observed impairment of oligo(U+) mRNA translatability is at present unknown, but the following trivial reasons could contribute to a lowered template efficiency. (1) Oligo(U+) RNA, when isolated from

Table II: Translation of RNA Subfractions Derived from Poly(A+) mRNA[a]

input RNA	translational act.[b] total	translational act.[b] −endo	% of poly(A+) mRNA
endo (−mRNA)	1.18		
poly(A+) mRNA	15.07	13.89	100
poly(A+) mRNA, −pA[c]	14.69	13.51	97
oligo(U−) RNA[d]	15.52	14.34	103
oligo(U±) RNA[d]	6.47	5.29	38
oligo(U+) RNA[d]	4.72	3.54	25
globin mRNA[e]	41.53	40.35	290

[a] Translations were carried out as described under Experimental Procedures. [b] Cl_3CCOOH-insoluble [^{3}H]leucine incorporation (cpm × 10^{-5}). [c] Poly(A+) mRNA was depolyadenylated by using RNase H and oligo(dT) (see Experimental Procedures). [d] Oligo(U−) RNA was the fraction of depolyadenylated poly(A+) mRNA unbound to poly(A)−agarose in 0.4 M NETS (see Experimental Procedures). Oligo(U±) RNA was the fraction bound and eluted from poly(A)−agarose with ETS that did not rebind to a second column. Oligo(U+) RNA was eluted with ETS initially and was eluted with ETS from a second column after an intermediate 0.2 M NETS wash. [e] Globin mRNA was added as in Table I.

depolyadenylated mRNA fractions, consistently exhibited a slightly higher sedimentation value on sucrose gradients than oligo(U−) RNA (Wood & Edmonds, 1981). The reduced number of 5′ ends per microgram of input RNA may result in a lowered initiation frequency of oligo(U+) RNA, although initiation is apparently not rate limiting in the reticulocyte system. (2) Nicking during the RNase H treatment would lead to loss of 5′ caps which are required for efficient translation (Shatkin, 1976). This is unlikely as the depolyadenylated and oligo(U−) RNA fractions which showed little, if any, inhibition of translation (Table II) presumably should have undergone a similar degree of nicking. (3) The presence of a diffusible inhibitor in oligo(U+) RNA preparations was ruled out by mixing experiments which showed no additional inhibition when oligo(U+) fractions were included in translations with fully functional templates (W. M. Wood, unpublished results). Other reasons for the reduced translatability of oligo(U+) RNA will be discussed later.

Characterization of the in Vitro Translation Products by Gel Electrophoresis. To determine further the extent of sequence homology between oligo(U+) and oligo(U−) RNA, the coding potentials of both RNAs were compared by gel electrophoresis of their in vitro synthesized proteins. Figure 2 is a fluorogram of ^{3}H-labeled products separated by SDS–PAGE specified by RNA subfractions derived from total poly(A+) mRNA. A striking similarity was seen in the patterns of lanes 1, 2, and 3 which represented the products encoded by poly(A+) mRNA [A(+)], the depolyadenylated derivative of poly(A+) mRNA (−pA), and the fraction not retained by poly(A)−agarose [U(−)], respectively. Clearly, poly(A) removal and subsequent depletion of the oligo(U+) material do not detectably alter the array of products specified by the abundant mRNAs as displayed by one-dimensional gel analysis. However, a quantitatively different pattern is revealed by the oligo(U+) RNA products [U(++); lane 4], with enrichment of bands corresponding to molecular weights of 65 000, 40 000, 25 000, and 15 000. The oligo(U±) RNA pattern (lane 6) exhibits similarities to both the oligo(U+) and oligo(U−) RNA lanes, indicating as before that it contains RNA templates common to both populations.

The more powerfully resolving technique of 2-D gel electrophoresis developed by O'Farrell was employed to determine if the above observed differences reflected enrichment of existing abundant products or whether oligo(U+) RNA is

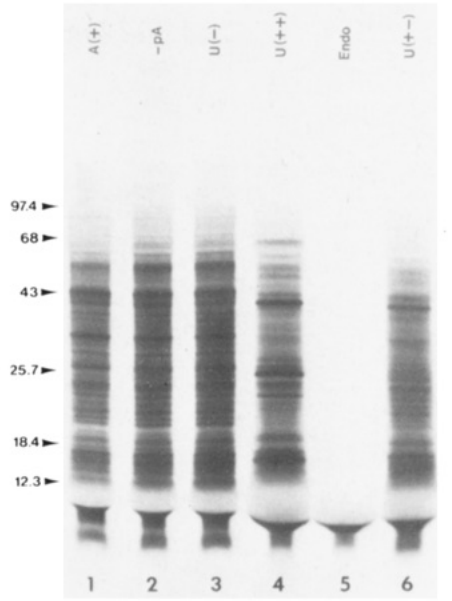

FIGURE 2: Denaturing SDS gel electrophoresis of in vitro translation products specified by subfractions derived from poly(A+) mRNA. Aliquots from the translation reactions (see Table II) were electrophoresed on a 10–20% gradient polyacrylamide gel as described under Experimental Procedures. Lanes are labeled with an abbreviation of the corresponding input RNA. Each lane contained 90 000 net (i.e., −endo) cpm except the endogenous lane which contained ~30 000 cpm [volume applied equal to the U(++) lane]. The dried gel was exposed for 7 days at −85 °C. Arrows denote positions of the prestained protein markers phosphorylase *b* (97.4), bovine serum albumin (68), ovalbumin (43), α-chymotrypsinogen (25.7), β-lactoglobulin (18.4), and cytochrome *c* (12.3) (values in parentheses are molecular weights × 10^{-3}) run in a parallel lane.

comprised of a distinct class of RNA templates not highly represented in poly(A+) mRNA. Fluorograms of these analyses are presented in Figure 3 using IEF in the first dimension and Figure 4 using NEpHGE in the first dimension, a technique which encompasses virtually the entire p*I* range of analyzable proteins. Figures 3 and 4 (upper panels), which displayed products specified by those RNAs lacking oligo(U), were virtually identical with the corresponding fluorograms derived by using total poly(A+) RNA as a template for in vitro translation (data not shown), demonstrating that both populations were comprised of the same abundant translation templates. Careful scrutiny of superimposed fluorograms of the IEF patterns revealed 26 spots on the pattern of oligo(U+) RNA products (numbered arrows, Figure 3, lower panel) which were absent or greatly diminished in the corresponding region of the oligo(U−) RNA derived fluorogram (Figure 3, upper panel). A similar examination of the NEpHGE patterns (Figure 4) revealed a further 7 spots specific to oligo(U+) RNA, bringing to 33 the total number of products enriched in RNAs selected for oligo(U)-rich regions. The total number of spots accountable by both analyses (IEF and NEpHGE) is approximately 300. Figure 5 strikingly contrasts the differences (numbered arrows) between the oligo(U+) and oligo(U−) RNA translation products displayed by both 2-D techniques. This was achieved by making a contact negative of the oligo(U+) fluorogram and rephotographing it with the corresponding oligo(U−) fluorogram superimposed; hence, oligo(U+) RNA specific products appear as white spots whereas oligo(U−) RNA derived spots are black. Spots of similar intensity common to both are identified as black with white halos (obvious samples being spots 1 and 2). Superimposition was possible only with fluorograms from gels whose first- and second-dimension separations were carried out in

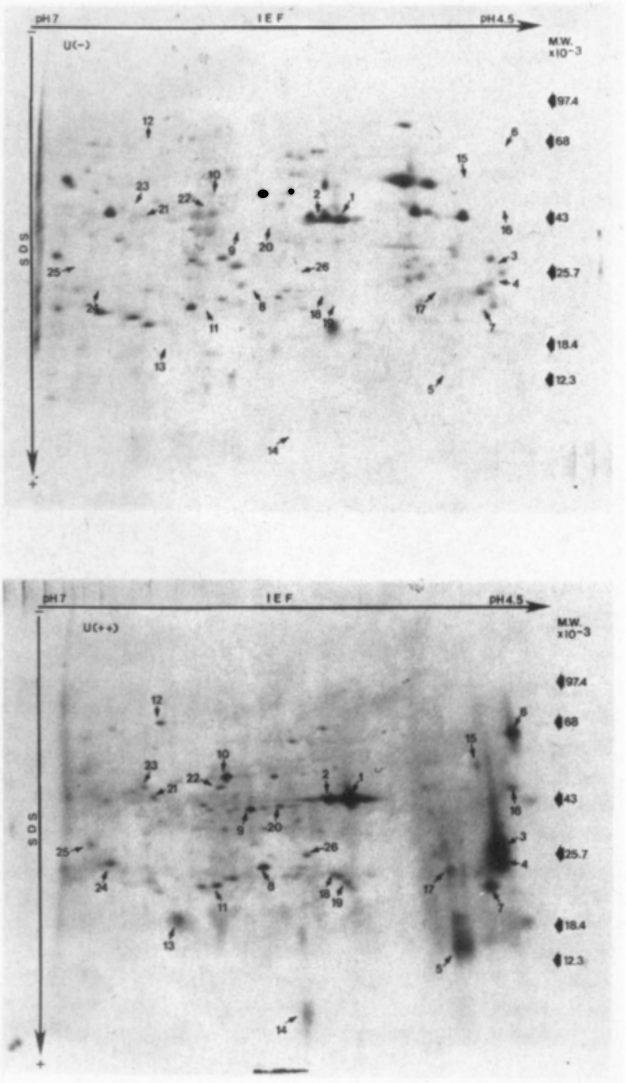

FIGURE 3: 2-D electrophoresis of in vitro translation products specified by subfractions derived from poly(A+) mRNA. IEF and SDS gel analysis. Fluorograms are labeled according to their designation in Figure 2. IEF and gel analysis were performed as described under Experimental Procedures. Sample volumes applied were identical with those for Figure 2. Exposure was for 36 days at −85 °C. Numbered arrows refer to spots which are present solely in the U(++) pattern or are highly enriched relative to those in the U(−) pattern. Prestained protein markers as in Figure 2 were electrophoresed in a side lane in each 2-D gel.

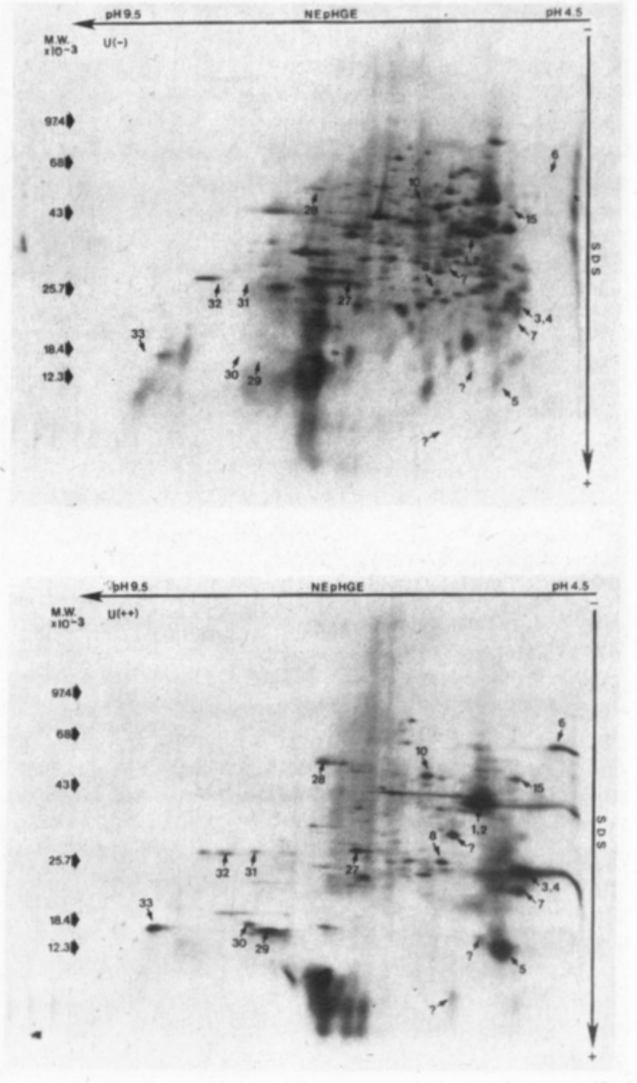

FIGURE 4: 2-D electrophoresis of in vitro translation products specified by subfractions derived from poly(A+) mRNA. NEpHGE and SDS gel analysis. Fluorograms are labeled as in Figure 3. NEpHGE and gel analysis were performed as described under Experimental Procedures. Sample volumes were identical with those in the legend to Figure 3. Arrows with question marks refer to U(++)-specific spots that have no identifiable counterpart in Figure 3.

parallel. Inspection of the relative intensities of the spots reveals that the major translatable oligo(U+) templates are comprised of only a few abundant mRNAs coding for proteins 1–7. It must be pointed out that spots 1 and 2, although somewhat enriched, are not unique to the oligo(U+) RNA pattern and are in fact fairly abundant oligo(U−) RNA products. Spot 1 has an isoelectric point and molecular weight (~42 000) characteristic of β-actin. Spot 2 probably represents the more basic unacetylated form of β-actin (δ-actin), a nonprocessed species which accumulates in in vitro systems (Toyama & Toyama, 1983). Why only a fraction of the RNAs coding for spots 1 and 2 appears to contain oligo(U) sequences is presently unknown, but experiments utilizing a cDNA clone for human β-actin are currently under way in an attempt to resolve this apparent discrepancy. Some of the less abundant oligo(U+) RNA directed products are also present in the pattern of oligo(U−) RNA products. That these are indicative of contamination of oligo(U+) RNA by the oligo(U−) fraction is unlikely as some very intense spots on Figure 3 (upper panel) are not visible at all in the corresponding region of Figure 3 (lower panel). This point will be discussed later with respect to the hybridization kinetics results. It appears, therefore, that the oligo(U+) RNA population consists of a small set of abundant mRNAs not highly represented in the total mRNA population (except spots 1 and 2) in addition to many less prevalent RNAs some of which code for the same proteins as oligo(U−) RNA.

Discussion

In this report, we have extended our studies of oligo(U)-containing mRNA from HeLa cells by examining to what extent its sequence content and relative sequence abundance differed from the total mRNA and from those molecules lacking oligo(U) sequences. It was immediately apparent from all of the analyses that oligo(U+) RNA differed considerably from oligo(U−) RNA in regard to sequence abundance, translatability, and coding potential as exhibited by in vitro translation products. Although it differed in sequence abundance, oligo(U+) RNA ultimately protected the same amount of cDNA as oligo(U−) RNA, implying that it contained virtually all of the abundant sequence information represented

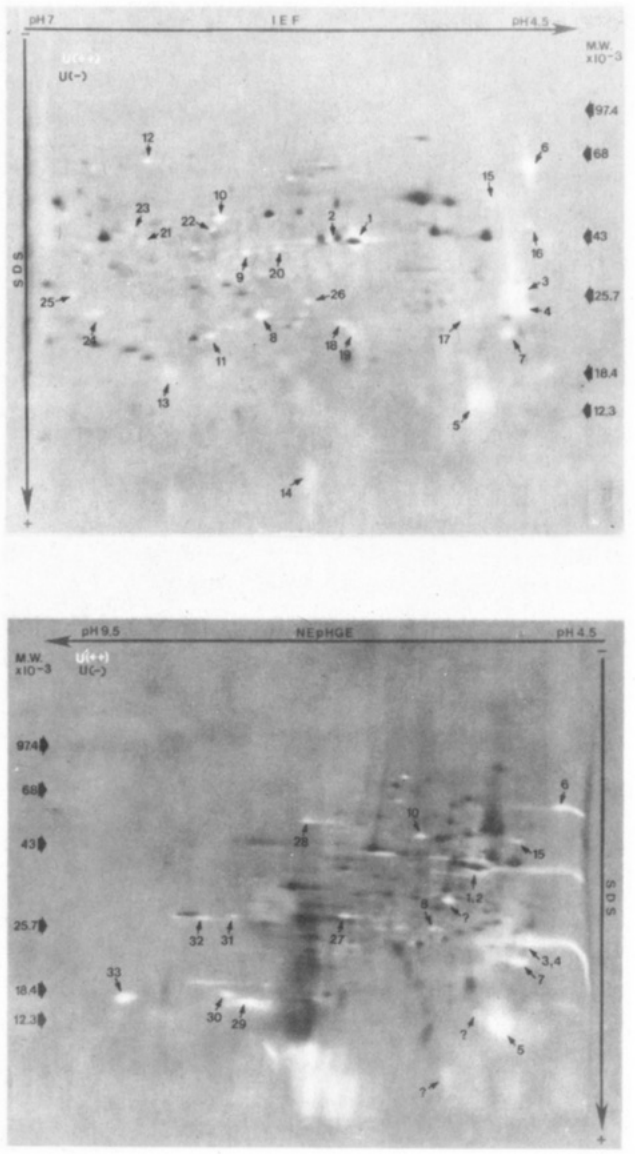

FIGURE 5: Superimposition of (upper panel) contact negative of Figure 3 [U(++)] with original fluorogram of Figure 3 [U(−)] and (bottom panel) contact negative of Figure 4 [U(++)] with original fluorogram of Figure 4 [U(−)].

in the cDNA. This appeared to be at variance with the 2-D in vitro translation product analyses which showed that several major products specified by oligo(U−) RNA were practically undetectable in the corresponding region of the fluorogram of oligo(U+) RNA products, whereas some minor spots common to both patterns were of similar intensity. This apparent discrepancy could be explained if the oligo(U+) templates which code for oligo(U−) RNA products were present at a different relative abundance within the oligo(U+) RNA population.

A somewhat surprising finding was the consistently lower translational capacity of oligo(U+) RNA. Several factors which could contribute to the reduced translatability were discussed under Results, but they are unlikely to account for the degree of impairment observed. Other possibilities related to the structure of the oligo(U+) RNA may be involved. The importance of the 5′-terminal cap structure in translation has been extensively reviewed by Shatkin (1976), and reduced cap content was proposed to account for the inefficient translation of postpolysomal mRNA from mouse kidney cells (Ouellette et al., 1982). However, in the preceding paper, we have shown that oligo(U+) RNA from HeLa cells is not inherently less capped than total mRNA (Kulkosky et al., 1985). Reduced 5′-cap content was not the reason for the lowered translational efficiency of postpolysomal RNA from developing sea urchin embryos (Rudensey & Infante, 1979). Instead, the authors suggest that there are intrinsic differences in the abilities of specific RNAs to be translated. In support of this, Kaempfer et al. (1978) proposed that initiation of translation is primarily regulated by an internal region of the mRNA and secondarily by the cap structure. Thus, it is feasible that oligo(U+) RNA from HeLa cells is merely comprised of templates less effective than those of total mRNA at directing protein synthesis in the reticulocyte system. Furthermore, when anchorage-dependent mouse fibroblast cells are detached from their substratum, the poly(A+) RNA extracted from them is modified in such a way as to render it poorly translated in both rabbit reticulocyte and wheat germ cell-free systems (Farmer et al., 1978). It is not degraded and retains both the sequence organization and level of 5′ caps as the mRNA before detachment. While most identifiable spots disappear from the two-dimensional gel pattern of protein products specified by suspended cell mRNA, a few polypeptides are still synthesized in relatively normal amounts although they were different products in the two systems. The authors argue that it is conceivable that the in vitro systems may handle the messages differently than the protein synthetic apparatus of an intact cell. The presence of several intense spots in the fluorogram of HeLa oligo(U+) RNA products (spots 1–7, Figure 3, lower panel) suggested that a few select templates may be efficiently translated in the reticulocyte lysate. This may be analogous to that for the suspended cell poly(A+) RNA where a heterologous translational apparatus may not be duplicating the protein synthetic system of another cell. A possible approach to solving this problem may be to develop an endogenous translation system from HeLa cells.

Whatever the reason for the inefficient translation of oligo(U+) RNA, it appears that its coding potential as expressed in the rabbit reticulocyte system is very different from that of oligo(U−) RNA with respect to abundant products. Except for those templates specifying spots 1 and 2 (Figure 3, upper panel), the RNAs coding for the major polypeptides of oligo(U+) RNA are not highly represented in oligo(U−) RNA and hence the total mRNA population. This is compatible with the results of the hybridization experiments which show that 75% of the sequences in oligo(U+) RNA are poorly represented in poly(A+) mRNA and these presumably code for the abundant oligo(U+) RNA specific translation products, the identity of which is presently unknown.

There are many reports describing the isolation, from a variety of sources, of a fraction of RNA with affinity for poly(A) columns [see Wood & Edmonds (1981) for references]. When analyzed, a number of these have been shown to contain an oligo(U)-rich sequence with a length and uridylate content similar to those found in HeLa cells (Wood & Edmonds, 1981; Molloy, 1980). In only a few of these studies do the authors examine the in vitro translational properties of such molecules. In all but one of these reports, the oligo(U+) RNA was detected only in the nonpolyadenylated RNA fraction from wheat embryos (Dobrzanski et al., 1980; Tomaszewski, 1982) and Friend leukemia cells (Katinakis & Burdon, 1981). Only in the case of rat liver was oligo(U+) RNA found in poly(A+) RNA (Nichols & Pryhitka, 1982), the other studies perhaps being hampered by the need to eliminate the interfering effect of poly(A) (see the introduction). In most cases, oligo(U+) RNA exhibited a similar template efficiency in cell-free systems as total mRNA except

in the later stages of wheat embryo germination where its translational activity relative to poly(A+) RNA was significantly reduced (Tomaszewski, 1982). In rat liver where 2-D gel electrophoresis was applied to the analysis of the in vitro translation products, the authors concluded that the oligo(U+) fraction represented a subset of the poly(A+) species although the proportions of the protein products were quite different and several proteins were found to be unique to the oligo(U+) RNA class. However, there was little similarity between the array of products directed by rat liver oligo(U+) RNA and those specified by HeLa oligo(U+) RNA described in this report. The qualitative differences between the in vitro products of oligo(U+) RNA and poly(A+) RNA were more obvious in the HeLa fluorograms than in those from rat liver where overexposure of several areas made interpretation difficult.

There are several examples of oligo(dT) sequences encoded in DNA, from which oligo(U) would be derived by transcription. The Alu family is a highly repetitive interspersed element within the human genome which is often flanked by long, uninterrupted stretches of dA residues (or dTs in the complementary strand) at its 3′ end (Deininger et al., 1981). Alu family members are transcribed into hnRNA where they can be isolated as readily renaturable double-stranded regions (Jelinek & Schmid, 1982). In several sequenced human genes, the Alu-associated oligo(dT) sequences are located in introns as in the adrenocorticotropic hormone–lipotropic hormone (ACTH-LPH) precursor (Takahashi et al., 1983), the proto-oncogene c-myc (Battey et al., 1983), and β-tubulin (Gwo-Shu Lee et al., 1984). However, sequence complementary to the Alu family has been reported in cytoplasmic poly(A+) RNA from cultured cells derived from human tumors using Northern blot hybridization (Elder et al., 1981) and electron microscopy (Calabretta et al., 1981).

Recent genomic and cDNA sequence data have revealed the presence of oligo(U) sequences within the 3′-noncoding region of several mRNAs. These are the chick ovalbumin-related Y gene mRNA (Heilig et al., 1982), β-actin mRNA of human fibroblasts (Ponti et al., 1984), an mRNA for a mouse histocompatibility (H-2) antigen (Lalanne et al., 1982), and the mRNA for the α-1 chain of chick type II procollagen (Sandell et al., 1984). Interesting among these is the β-actin cDNA which contains the sequence 5′... TTTGTTTTTTTTGTTTTGTTTTGGTTTTTTTTTTT-TTTTT...3′. As described under Results, a very prominent in vitro translation product specified by HeLa oligo(U+) RNA has the 2-D mobility characteristics of β-actin, strongly supporting the evidence that we are selecting molecules with uridylate-rich regions. None of the sequenced oligo(U+) mRNAs have recognizable Alu-type sequences adjacent to the oligo(U) region, although in two cases a small sequence immediately upstream from the oligo(U) region is repeated a few hundred bases downstream. Direct repeats of this type, 9 bases with 1 mismatch in the case of β-actin and 11 perfectly matched in procollagen, have been implicated as sites of mobile element insertion. Whether oligo(U) or as yet unidentified repetitive elements with which they may be associated are important to the function of RNA molecules which possess them will have to await further experimentation.

Added in Proof

Since submission of this report, we have obtained preliminary evidence that β-actin mRNA is quantitatively retained on poly(A)–agarose when the applied fraction of HeLa cytoplasmic poly(A+) RNA is pretreated with formaldehyde. This was demonstrated by Northern blot analysis using a nick-translated human β-actin-specific cDNA probe generously provided by Dr. Peter Gunning of Stanford University.

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Mechanism of the Hepatic Lipase Induced Accumulation of High-Density Lipoprotein Cholesterol by Cells in Culture†

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ABSTRACT: Hepatic lipase can enhance the delivery of high-density lipoprotein (HDL) cholesterol to cells by a process which does not involve apoprotein catabolism. The incorporation of HDL-free (unesterified) cholesterol, phospholipid, and cholesteryl ester by cells has been compared to establish the mechanism of this delivery process. Human HDL was reconstituted with ^{3}H-free cholesterol and [^{14}C]sphingomyelin, treated with hepatic lipase in the presence of albumin to remove the products of lipolysis, reisolated, and then incubated with cultured rat hepatoma cells. Relative to control HDL, modification of HDL with hepatic lipase stimulated both the amount of HDL-free cholesterol taken up by the cell and the esterification of HDL-free cholesterol but did not affect the delivery of sphingomyelin. Experiments utilizing HDL reconstituted with ^{14}C-free cholesterol and [^{3}H]cholesteryl oleoyl ether suggest that hepatic lipase enhances the incorporation of HDL-esterified cholesterol. However, the amount of free cholesterol delivered as a result of treatment with hepatic lipase was 4-fold that of esterified cholesterol. On the basis of HDL composition, the cellular incorporation of free cholesterol was about 10 times that which would occur by the uptake and degradation of intact particles. The preferential incorporation of HDL-free cholesterol did not require the presence of lysophosphatidylcholine. To correlate the events observed at the cellular level with alterations in lipoprotein structure, high-resolution, proton-decoupled ^{13}C nuclear magnetic resonance spectroscopy (90.55 MHz) was performed on HDL_3 in which the cholesterol molecules were replaced with [4-^{13}C]cholesterol by particle reconstitution. The loss of HDL phospholipid following incubation with hepatic lipase or phospholipase A_2 in the presence of albumin caused cholesterol molecules at the surface of the HDL particle to exhibit greater segmental motion as indicated by a decrease in line width of the 4-^{13}C resonance at a chemical shift of 41.70 ppm. Removal of phospholipid molecules was also associated with some redistribution of cholesterol molecules from the core to the surface of the HDL particle and an increased polar group segmental motion of the phospholipid molecules remaining in the surface. It follows that phospholipid and cholesterol molecules are apparently more widely spaced in the surface of modified HDL. These results support the hypothesis that hepatic lipase, via its phospholipase activity, shifts the equilibrium of free cholesterol between HDL and the plasma membrane, resulting in a net delivery of free cholesterol to the cell by a surface transfer process.

The role of high-density lipoproteins (HDL)[1] in lipid metabolism has not been clearly defined. The most popularly held concept is that HDL participates in the removal of cholesterol from peripheral cells to the liver for biliary excretion, a process often referred to as reverse cholesterol transport. Although this proposal is consonant with a number of reports implicating HDL in the removal of cholesterol from cells (Bates & Rothblat, 1974; Stein et al., 1976; Oram et al., 1981), the mechanism involved in the delivery of this cholesterol to the liver has not been established. Uptake may occur by a receptor-mediated endocytosis, with subsequent catabolism of the lipoprotein as a single unit (Roheim et al., 1972; Sigurdsson et al., 1979). Such a process would result in the

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[1] Abbreviations: apo, apolipoprotein; EC, esterified cholesterol; FC, free (unesterified) cholesterol; HDL, high-density lipoprotein(s); lyso-PC, lysophosphatidylcholine; MEM, Eagle's minimal essential medium; NMR, nuclear magnetic resonance; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PL, phospholipid; SM, sphingomyelin; TC, total (free plus esterified) cholesterol; δ, chemical shift; $\Delta\nu_{1/2}$, line width; TLC, thin-layer chromatography; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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