Complexity of poly(A⁺) and poly(A⁻) polysomal RNA in mouse liver and cultured mouse fibroblasts

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ABSTRACT

RNA excess hybridization experiments were used to measure the complexity of nuclear RNA, poly(A⁺) mRNA, poly(A⁻) mRNA, and EDTA-released polysomal RNA sedimenting at <80 S in mouse liver and in cultured mouse cells. With both cell types, poly(A⁻) RNA was found to contain 30-40% of the sequence diversity of total mRNA. In the case of liver this represents 5,700 poly(A⁻) molecules and 8,600 poly(A⁺) molecules for a total of approximately 14,300 different mRNAs. Comparison of the complexity of mRNA with that of nuclear RNA revealed that in liver and in cultured cells, mRNA has only 10-20% of the sequence diversity present in nuclear RNA. This latter observation is consistent with existing data on mammalian cells from this and other laboratories.

INTRODUCTION

The discovery of a poly(A) tract of approximately 200 residues at the 3' terminus of mRNA in eukaryotic cells has proved interesting both in terms of its biological function and also because it provides a relatively simple means for the isolation and further characterization of cellular mRNA (for review, see ref. 1). At first it appeared that, except for histone mRNA, all eukaryotic mRNAs were polyadenylated.² It has since become clear, however, that in organisms as diverse as sea urchins,^{3,4} mouse L cells,⁵ and human HeLa cells⁶ a substantial fraction (30-50% by weight) of mRNA is not polyadenylated. In the case of the sea urchin and HeLa cells, hybridization studies have shown that the poly(A⁺) and poly(A⁻) mRNAs represent distinct populations which show little or no overlap in the sequences which they contain.^{3,6} On this basis it has been concluded that the poly(A⁻) mRNA is not derived from poly(A⁺) RNA which either has yet to be polyadenylated or from which the poly(A) tract was lost during isolation.

With few exceptions⁷ attempts to measure the number of different mRNA sequences present in mammalian cells have been based either on the use of cDNA copies of poly(A⁺) mRNA⁸⁻¹⁴ or the use of poly(A⁺) RNA as the driver in

RNA-excess hybridization experiments.¹⁵ How well these measurements represent the true complexity of cellular mRNA is principally determined by the extent to which the sequence diversity of the total mRNA is reflected in the population of poly(A⁺) mRNAs. To the best of our knowledge, no measurements on the complexity of poly(A⁻) mRNA have been published. Since information on the sequence diversity of poly(A⁻) mRNA is essential to a complete assessment of the overall complexity of mRNA in mammalian cells, a series of experiments were undertaken to make such measurements using RNA from mouse liver and from cultured mouse fibroblasts. In order to properly evaluate the data, the complexities of total mRNA and of poly(A⁺) mRNA were also determined under identical conditions. Since the primary concern was to determine the total complexity of the RNA from the various cellular fractions, it must be emphasized at the beginning that the experiments were intended to measure saturation values as opposed to resolving the number of frequency classes in the different RNA preparations.

A study of the complexity of poly(A⁻) mRNA can at present only be approached by using large quantities of RNA to drive reactions containing small amounts of radiolabeled DNA. Furthermore, since only a small fraction of the genome is represented in liver polysomal RNA^{13,14} such experiments require DNA labeled to high specific activity and partially purified transcribed sequences. The first criterion was fulfilled by labeling DNA in vitro in a manner similar to that applied by Galau et al.¹⁶ to sea urchin DNA. The second criterion was met by hybridizing total liver RNA with total nonrepetitive DNA and isolating the DNA from the hybrids for use in the experiments with poly(A⁺) and poly(A⁻) mRNA.

In the case of the particular line of cultured mouse cells used in this study, we showed previously⁷ that a surprisingly large proportion of the genome is transcribed. Therefore, measurements with poly(A⁺) and poly(A⁻) mRNA could be made using DNA labeled in vivo and without prior enrichment for the transcribed sequences. Thus the data obtained with the cultured cells provided a control for any bias that might result from the more complicated manipulations involved in the measurements on liver cells.

There were also several factors to be considered with regard to the preparation of RNA for this investigation. One of these was the necessity to minimize the possibility that the poly(A⁻) mRNA would be contaminated with poly(A⁺) mRNA from which the poly(A) had been lost. In contrast with this was the need to purify the mRNA sufficiently to remove any heterogeneous nuclear RNA (hnRNA) that leaked from the nuclei during the process

of cell fractionation. Since both of these goals could not be achieved with a single methodology, RNA was isolated in two ways: (1) Poly(A⁺) and poly(A⁻) mRNAs were obtained using a procedure that involved minimum handling prior to the separation of the two populations; (2) Total mRNA was prepared from polysomes by initially selecting those that sediment at >100 S, dissociating these polysomes with EDTA³³ and then selecting the ribonucleo-protein sedimenting at <80 S. It was reasoned that any difference in complexity between a mixture of poly(A⁺) and poly(A⁻) mRNA obtained by the first method and the <80 S mRNA by the second method would provide an estimate of the maximum contribution that would be made by hnRNA.

MATERIALS AND METHODS

Preparation of Mouse Liver. Ten-week-old male NYLAR mice were sacrificed by cervical dislocation. After removal of the gall bladder, the liver was excised and placed in 10 ml of ice-cold buffer A (0.14 M NaCl, 0.01 M Tris-HCl (pH 8.4), 0.001 M MgCl₂). The livers from 4 to 8 mice were pooled, minced with a sterile scalpel, the buffer poured off and 10 ml of fresh buffer A added. The minced liver was then forced through a 40-mesh tissue sieve (E. C. Apparatus Corp.) using 20 ml of buffer A. Triton X-100 was added to 0.05% and the suspension stirred for 5 min at 4°C. The suspension was filtered through sterile gauze and the gauze rinsed with 10 ml of buffer A containing 0.05% triton X-100. The nuclei were collected by centrifuging for 5 min at 500 g and 4°C. The nuclear pellet was washed twice with 10 ml of buffer A containing 0.05% triton X-100. The final nuclear pellet was used to prepare nuclear RNA. The supernatants from the lysis and washing steps were pooled and centrifuged for 25 min at 8,000 g and 4°C. The supernatant from this last centrifugation was layered over 3.5 ml of 2.0 M sucrose in 0.14 M NaCl, 0.01 M Tris-HCl (pH 7.4), 0.008 M MgCl₂ and centrifuged for 2 h at 55,000 rev/min and 4°C using the 60 Ti rotor in a Beckman L5-65 centrifuge. The polysome pellet was either used to isolate poly(A⁺) and poly(A⁻) RNA, or further processed to obtain EDTA-released RNA sedimenting at <80 S. The number of nuclei were counted in a hemacytometer at each step after the initial addition of triton X-100. No change in the number of nuclei was observed during subsequent washings with 0.05% triton X-100. A total of three detergent washings were required to obtain nuclei which appeared clean when observed with a phase-contrast microscope. It was found that either raising the triton X-100 concentration to 0.1%, or washing with other detergents (NP-40, sodium deoxycholate,

Tween-40) resulted in substantial lysis of the nuclei.

Isolation of Polysomes from Cultured Cells. PYAL/N cells are a line of mouse fibroblasts transformed by polyoma virus. The cells were grown in half-gallon roller bottles in a modified Eagle's medium (Grand Island Biological Co. #71035) plus 10% fetal bovine serum (Grand Island Biological Co.). The cells were harvested using 0.05% trypsin and 0.02% EDTA, washed twice in serum-free medium, and finally resuspended at 4°C in buffer A. The cell membrane was disrupted by adding triton X-100 to 0.05% and stirring for 5 min at 4°C. The nuclei were collected and washed exactly as in the case of mouse liver. Polysomes were harvested by pelleting through 2.0 M sucrose in the same manner as for mouse liver.

Isolation of Poly(A⁺) mRNA. The procedure for isolating poly(A⁺) mRNA closely followed that of Pemberton et al.¹⁷ The polysomal pellets obtained after centrifugation through 2.0 M sucrose were resuspended in 2.0 ml of 0.5 M NaCl, 0.01 M Tris-HCl (pH 7.8), 0.5% SLS and passed over a column of oligo(dT) cellulose (Type 3, Collaborative Research, Inc.) equilibrated with the same buffer. The column was washed with the starting buffer until the absorbance at 260 nm of the eluant reached background. The poly(A⁺) RNA bound to the column was then eluted with 0.01 M Tris-HCl (pH 7.5), 0.5% SLS. The poly(A⁺) fraction was made 0.5 M NaCl and again passed over oligo(dT) cellulose as above. Approximately 60% of the material in the first poly(A⁺) fraction was recovered as poly(A⁺) RNA from the second column. Further passage of the poly(A⁺) RNA over oligo(dT) cellulose did not change the amount of poly(A⁺) RNA recovered. The poly(A⁺) fraction was brought to 0.02 M MgCl₂ and 25 µg/ml of bentonite-treated α-amylase¹⁸ and 25 µg/ml of RNase-free DNase (kindly provided by Dr. T. H. Plummer, Jr. of this laboratory) were added and the mixture incubated for 1 h at 37°C. This was followed by the addition of pronase to 50 µg/ml and another 1 h incubation at 37°C. The mixture was then brought to 0.1 M with sodium acetate (pH 5.5) and the RNA extracted using an equal volume of phenol-chloroform (1:1 v/v) saturated with 0.01 M NaCl, 0.001 M EDTA, 0.1 M sodium acetate (pH 5.5),¹⁹ for 10 min at room temperature. The aqueous layer was removed and the RNA precipitated with 95% ethanol. After being held overnight at -20°C, the RNA precipitate was collected by centrifugation. The RNA was resuspended in 1.0 ml, 0.1 M sodium acetate (pH 5.5) and passed over a column of Sephadex G-100 (Pharmacia) equilibrated with the same buffer. The material eluting in the void volume was collected and precipitated with 95% ethanol. The precipitate was held overnight at -20°C and collected by centrifugation as

before. The final RNA pellet was resuspended in 0.05 M sodium acetate (pH 6.5). The purity of the poly (A⁺) mRNA was examined by electrophoresis through polyacrylamide-agarose gels. Approximately 15-20% of the preparation migrated as ribosomal RNA.

Isolation of the Poly(A⁻) RNA. The RNA that did not bind to oligo (dT) cellulose was purified as the poly(A⁻) RNA. The RNA was isolated by hot phenol extraction as previously described.²⁰ The only change in the purification procedure was the inclusion of a treatment with α -amylase concomitant with the DNase treatment. It is worth noting that the efficiency of the DNase treatment was monitored by prior addition of ³H-labeled DNA.²⁰ Less than 0.1% of the label remained in the final RNA preparations.

Isolation of Nuclear RNA. The nuclei were resuspended in 10 ml of 0.1 M NaCl, 0.01 M sodium acetate (pH 5.9), 0.001 M EDTA. Bentonite was added to 2.0 mg/ml and the nuclei lysed with 0.5% SLS. RNA was purified by hot phenol extraction exactly as described above for poly(A⁻) RNA.

Isolation of RNA Sedimenting at <80 S. The procedure for obtaining an RNA fraction sedimenting at <80 S was similar to that described by Galau et al.²¹ The polysome pellets obtained after centrifugation through 2.0 M sucrose were resuspended, sedimented through linear 15-30% sucrose gradients, and the polysomes sedimenting at >100 S were collected. These polysomes were dissociated by treatment with EDTA, then centrifuged through a second 15-30% sucrose gradient and the RNA sedimenting at <80 S was recovered and purified for use in the hybridization experiments. The exact details of the procedure have been published.⁷

Labeling DNA In Vivo. Secondary cultures of mouse embryo cells were grown in half-gallon roller bottles in a modified Eagle's medium containing 10% fetal bovine serum. When the cells were about 50% confluent, the medium was poured off and fresh medium containing 5 μ Ci/ml of thymidine methyl-³H (New England Nuclear Corp., sp. act. = 91 Ci/mM) was added. After 24 h the medium was changed again and fresh medium containing thymidine methyl-³H as above was added. Forty-eight hours after the first medium change the cells were harvested by trypsinization. Nuclei were prepared and DNA purified as described by McCarthy and Hoyer.²² Purified DNA was sheared and the nonrepetitive sequences isolated and characterized as previously described.²⁰ The final DNA preparation had a sp. act. = 5.6×10^5 cpm/ μ g.

Labeling of DNA In Vitro. The general procedure for the in vitro labeling of DNA was similar to that described by Galau et al.¹⁶ Secondary cultures of mouse embryo cells were grown to confluence in half-gallon roller bottles. The cells were harvested by trypsinization and the DNA isolated by the method of McCarthy and Hoyer.²² Purified DNA was suspended in 1.5 mM NaCl, 0.15 mM sodium citrate at a concentration of 600 µg/ml and sheared in a French press at 25,000 psi. The sheared DNA was adjusted to a concentration of 471 µg/ml in 0.093 M PB, 1.35 M NaCl. The DNA was then denatured in a boiling water bath for 5 min, followed by an incubation at 60°C to a C_0t equivalent²³ to 220. The sample was diluted to 0.06 M PB and passed over a column of hydroxylapatite (HAP) equilibrated with 0.06 M PB at 60°C. The single-stranded DNA was eluted by washing with 10 column volumes of 0.12 M PB and the double-stranded DNA was collected by washing with 5 column volumes of 0.4 M PB. Based on absorbance at 260 nm, 68% of the DNA was single-stranded. This single-stranded DNA was concentrated to 100 µg/ml and the PB concentration adjusted to 0.4 M. The DNA was again denatured, then incubated at 60°C to a C_0t equivalent to 220. The sample was diluted and passed over a column of HAP as described above. Slightly more than 80% of the DNA remained single-stranded after the second incubation to $C_0t = 220$. The single-stranded DNA was concentrated to 1.583 mg/ml and dialyzed into 0.1 M PB, 1.35 M NaCl. The DNA was then incubated at 60°C to a C_0t equivalent to 20,000, diluted into 0.12 M PB and passed over a column of HAP at 60°C. The column was washed as above to obtain single-stranded and double-stranded DNA. The double-stranded DNA was then used in the in vitro labeling reaction.

The in vitro labeling reaction contained, in 0.5 ml of 0.05 M PB, pH 7.6: 15 µg of C_0t 20,000 DNA, 2×10^{-5} M each of dATP, dGTP, dCTP (P-L Biochemicals), 0.01 M $MgCl_2$, 0.001 M B-mercaptoethanol, 0.5 mCi 3H TTP (New England Nuclear Corp., sp. act. = 46 Ci/mmol), 40 units E. coli DNA polymerase (Grand Island Biological Co.). The reaction was assembled at 0°C, then incubated at 12°C for 28 h. The reaction was terminated by the addition of 0.334 ml distilled water, 0.023 ml 5.0 M NaCl, and 0.017 ml 0.5 EDTA. The sample was mixed well, heated in a boiling water bath for 5 min, held at 50°C for 1 min, then passed over a 1 cc column of HAP equilibrated with 0.03 M PB, 0.135 M NaCl at 50°C until no further radioactivity washed from the column. Single-stranded DNA was eluted by washing with 1.0 ml portions of 0.12 M PB at 50°C until no further counts were recovered. Duplex DNA was washed from the column with 6, 1.0 ml volumes of 0.4 M PB.

The bulk of the single-stranded counts eluted in fractions 3-5 of the 0.12 M PB washes. Based on absorbance at 260 nm, these fractions contained 70% of the mass of the input DNA. The sp. act. of this DNA was 10^7 cpm/ μ g.

To examine the reassociation behavior of the in vitro labeled non-repetitive DNA, 0.06 μ g of this DNA was mixed with 2.3 mg of sheared, unlabeled and unfractionated mouse embryo DNA in 1.0 ml of 0.12 M PB. The sample was denatured in a boiling water bath for 5 min, then incubated at 60°C. At various times, 0.025 ml aliquots were removed, diluted into 3.0 ml 0.12 M PB + 0.4% SLS at 60°C and single-stranded and double-stranded DNA separated on a column of HAP.

Isolation of DNA Transcribed in Mouse Liver. Total liver RNA, prepared from liver homogenates by hot phenol extraction²⁰ was mixed with in vitro labeled nonrepetitive mouse DNA in 0.18 M PB and incubated at 60°C for 72 h. The RNA concentration was 12 mg/ml and the DNA concentration was 12 μ g/ml in a total volume of 0.3 ml. After incubation, the sample was diluted to 0.12 M PB containing 0.4% SLS at 60°C and passed over a column of HAP at 60°C. The unhybridized DNA was washed from the column with 8 column volumes of 0.12 M PB. The RNA-DNA hybrids were then collected by washing with 3 column volumes of 0.3 M PB. To remove the RNA from the hybrids, the 0.3 M PB fraction was brought to 0.3 N NaOH and held at room temperature for 90 min. The sample was neutralized with HCl, diluted to 0.05 M PB and passed over a column of HAP equilibrated with 0.05 M PB, then the single-stranded DNA was collected by washing with 8 column volumes of 0.12 M PB. The single-stranded DNA was concentrated to 1.0 ml and passed over a column (0.7 x 24 cm) of Bio-gel A (Bio-Rad Laboratories) equilibrated with 0.001 M PB. The radioactivity eluting in the void volume was used as expressed DNA (E-DNA) in the hybridization experiments.

RNA-DNA Hybridization. Hybridization reactions were carried out at 60°C in 0.1 ml of 0.18 M PB, 0.01 M EDTA. The reactions were run in polypropylene micro test tubes (Bio-Rad Laboratories) and were layered with paraffin oil to prevent evaporation. All of the reactions except those using poly(A⁺) RNA contained 12 mg/ml of RNA and 0.1 μ g/ml of liver E-DNA. In the case of poly(A⁺) RNA the concentration was 240 μ g/ml. In mixing experiments, poly(A⁺) RNA and poly(A⁻) RNA were used at concentrations of 240 μ g/ml and 12 mg/ml respectively. Control reactions containing DNA alone were run in parallel to each set of experimental reactions. At 24 h intervals, 20- μ l samples were removed, diluted into 2.0 ml of 0.12 M PB containing 0.4% SLS at 60°C and passed over a column of HAP at 60°C. The

procedure for determining the distribution of radioactivity between single-stranded and hybridized DNA has been published.²⁴ At zero-time, 0.5% of the DNA in both experimental and control samples bound to HAP. After 168 h of incubation at 60°C the DNA in the control sample had reached a $C_0t = 0.21$ and only 0.9% bound to HAP. Thus in no instance was the experimental data corrected for more than 0.4% due to DNA-DNA self reaction.

Experiments with RNA from PYAL/N mouse cells grown in culture were carried out in essentially the same manner as those described above for mouse liver. In this case, however, the reactions contained 12 µg/ml of total nonrepetitive DNA that had been labeled in vivo. Again, 0.5% of the DNA bound to HAP at zero-time. Following 168 h at 60°C, 1.5% of the DNA in the control sample bound to HAP.

RESULTS

In Vitro Labeled DNA. To determine whether the in vitro labeled DNA retained the ability to reassociate normally, a small amount of the labeled DNA was allowed to react with a large quantity of unlabeled and unfractionated mouse DNA. As illustrated in Figure 1, the labeled DNA was free of contaminating repetitive sequences and reassociated with the kinetics of single component DNA.

At the highest C_0t examined, slightly more than 70% of the in vitro DNA had reassociated without any indication of significant deviation from the ideal second-order curve. Identical results were obtained using two independently labeled DNA preparations. On this basis it was concluded

Figure 1. The reassociation kinetics of a small amount of nonrepetitive mouse DNA labeled in vitro in the presence of a large quantity of unlabeled and unfractionated mouse embryo DNA (●). The dashed line represents an ideal second-order reaction with a $C_0t \frac{1}{2} = 3,400$. Reassociation of unfractionated mouse DNA (○).

that valid measurements of transcription could be made with DNA labeled in vitro.

Distribution of RNA in Liver Cells. The typical distribution of RNA between the various cell fractions is shown in Table 1. The proportion of the total RNA recovered as poly(A⁺) mRNA is similar to that reported by Ryffel and McCarthy⁹ for mouse L cells.

Isolation and Characterization of DNA Expressed in Liver. In a reaction with total liver RNA, 3% of the in vitro labeled nonrepetitive DNA formed hybrids (Figure 2). This value agrees well with earlier reports using mouse DNA labeled in vivo which gave a range of 1.6-4% for the extent of nonrepetitive DNA expression in mouse liver²⁵⁻²⁷ and provides additional confidence in the use of DNA labeled in vitro.

When the DNA in the total RNA-DNA hybrids was isolated (this isolated DNA is hereafter referred to as E-DNA) and then incubated a second time with total RNA, 35% of the DNA reacted (Figure 2).

A similar improvement in hybridization was observed by Grouse et al.²⁷ when they isolated the DNA sequences expressed in mouse brain. It must be recognized, however, that the E-DNA contains sequences from DNA-DNA self-reaction as well as from RNA-DNA hybrids and this fact, combined with the probable loss of hybridizability after successive incubations, most likely accounts for the failure to observe more extensive hybridization. On the other hand, a 35% reaction represents a ten-fold purification of the transcribed sequences and for the present purpose was sufficient to permit a comparison of the relative complexities of the poly(A⁺) and poly(A⁻) mRNA populations.

TABLE 1

Amount of RNA Recovered in the Various Fractions of Mouse Liver Cells.^a

Cell Fraction	Amount of RNA (g)	Percent of Total RNA
Nuclei	283	3.3
Cytoplasmic poly(A ⁺)	134	1.5
Cytoplasmic poly(A ⁻)	8,352	95.3

^aLivers from 8 mice were processed as described under Materials and Methods.⁷ The total liver wet weight was 9.19 g. The number of nuclei/g was 4.1×10^7 .

Complexity of Nuclear RNA. Figure 2 shows that the sequence diversity of liver cell nuclear RNA is the same as that of total RNA. However, under identical conditions the nuclear RNA takes approximately twice as long as total RNA to reach one-half of the final saturation value. The reason for this is presently unclear and we are unaware of any published data from other laboratories with which to compare this observation. However, it has recently been reported in studies with rat liver that the poly(A⁺) mRNA sequences have a narrower frequency distribution in the nucleus than they do in the cytoplasm.²⁸ Since total RNA contains all of the sequences from both nucleus and cytoplasm, the higher concentration of some mRNAs in the cytoplasm could at least partly account for the difference between total and nuclear RNA. Other factors that may have contributed to this observation include differences in the efficiency of extraction of hybridizable RNA sequences and differences between preparations in RNA fragment size. Whatever the explanation, we obtained similar results with PY AL/N mouse cells grown in culture.

Mouse Liver Poly(A⁺) RNA. In Figure 3A it is shown that 4.3% of nonrepetitive E-DNA reacts with poly(A⁺) mRNA from mouse liver. Similar results were obtained using two separate poly(A⁺) mRNA preparations and two independently labeled and isolated E-DNA fractions. Since 35% of the E-DNA reacts with total RNA and is equivalent to 3% of total nonrepetitive DNA, the 4.3% of E-DNA which hybridized with poly(A⁺) mRNA represents 0.37% of mouse nonrepetitive DNA. Assuming that the number average molecular weight of mammalian cell mRNA is about 6×10^5 daltons,^{8,13} and that the complexity of the nonrepetitive portion of the mouse genome is 1.4×10^{12}

Figure 2. Total mouse liver RNA hybridized to in vitro labeled nonrepetitive DNA (Δ). The DNA from the hybrid (E-DNA) was isolated and reacted a second time with total liver RNA ($\bullet$). E-DNA was also hybridized to liver nuclear RNA ($\circ$).

daltons,¹³ then 0.37% of the nonrepetitive sequences codes for approximately 8,600 different mRNAs. In comparison, several laboratories using cDNA copies of poly(A⁺) mRNA have reported anywhere from 7,600 to 12,000 different mRNAs in mammalian cells.^{9,10,12-14} The good agreement between our results and those obtained with cDNA further supports the validity of both techniques for assessing the complexity of poly(A⁺) RNA.

Mouse Liver Poly(A⁻) RNA. The reaction between liver poly(A⁻) mRNA and mouse E-DNA is also illustrated in Figure 3A. Again, two different preparations of poly(A⁻) mRNA and E-DNA yielded essentially identical data. In this case, saturation occurs when 3.3% of the E-DNA, or 0.28% of total nonrepetitive DNA, has hybridized. This is equivalent to an additional 5,700 different mRNA molecules. Although the poly(A⁺) RNA consists almost entirely of mRNA, the poly(A⁻) preparation contains predominantly rRNA and tRNA. The combined complexity of these latter two species is only about 3×10^6 daltons²⁹ and thus their contribution to the measured complexity of poly(A⁻) RNA is negligible. Furthermore, there is no evidence that rRNA or tRNA cause nonspecific binding of DNA to HAP. Nemer et al.³ showed that the presence of rRNA did not alter the hybridization of DNA with poly(A⁺) RNA and along with other laboratories, we have found that large amounts of total RNA from nontransformed cells do not cause nonspecific binding of viral DNA.³⁰

To determine whether the poly(A⁻) RNA was a distinct population, an experiment employing a mixture of poly(A⁺) and poly(A⁻) RNA was carried out. Under these conditions, 7.7% of the E-DNA formed hybrids (Figure 3B).

Figure 3. (A) Hybridization of E-DNA with liver poly(A⁺) (●) and poly(A⁻) (○) mRNA. (B) The reaction of E-DNA with a mixture of liver poly(A⁺) and poly(A⁻) mRNA (Δ) and with liver < 80 S RNA (▲).

Thus it appears that in mouse liver as much as 43% of the total mRNA complexity may reside in poly(A⁻) RNA. The absence of overlap between poly(A⁺) and poly(A⁻) RNA has also been reported by Nemer et al.³ who found that no more than 8% of cDNA prepared from sea urchin poly(A⁺) RNA reacted with poly(A⁻) RNA.

Complexity of <80 S Liver RNA. The purpose in making measurements with the <80 S RNA was to provide data using a polysomal RNA preparation free of any contaminating hnRNA. When this RNA was reacted with E-DNA (Figure 3B), 6% of the DNA formed hybrids. This value is less than the 7.7% observed with a mixture of poly(A⁺) and poly(A⁻) RNA, but significantly greater than the saturation values obtained with either of these RNAs alone. Although the discrepancy between the <80 S RNA data and that from the mixture of poly(A⁺) and poly(A⁻) sequences raises the possibility that hnRNA is present in one or both of the latter RNA preparations, it is also conceivable that some RNA species were lost during the processing required to obtain <80 S RNA. Nevertheless, even if all of the difference between the RNA mixture and the <80 S RNA is attributed to contamination of the poly(A⁻) RNA, the poly(A⁺) RNA can only account for about 70% of the sequence diversity of the <80 S RNA. The conclusion is therefore reached that at least 30%, and perhaps as much as 40%, of the total mRNA complexity in mouse liver occurs in a distinct population of RNA molecules that are not polyadenylated.

Poly(A⁺) and Poly(A⁻) RNA in Cultured Cells. In earlier reports we showed that total RNA and nuclear RNA from PYAL/N mouse cells grown in culture were able to react with 15% of mouse nonrepetitive DNA.^{7,20} It was also shown that <80 S RNA from these cells saturated 1.5-2% of the nonrepetitive sequences. This represents about 36,000 different mRNA molecules. and is three times greater than the sequence diversity of mouse liver mRNA. Since such a large proportion of the genome is represented in the mRNA of these cells, it was possible to measure the complexity of the poly(A⁺) and poly(A⁻) RNAs without resorting either to in vitro labeling of DNA or the preliminary isolation of the transcribed sequences. The results of experiments with poly(A⁺) and poly(A⁻) mRNA from PYAL/N cells are presented in Figure 4A. The poly(A⁺) RNA hybridizes with 1% and poly(A⁻) RNA with 0.8% of the nonrepetitive DNA. When a mixture of poly(A⁺) and poly(A⁻) RNA was used, 1.6% of the DNA reacted, suggesting that in this instance there may be a small overlap between the poly(A⁺) and the poly(A⁻) RNA populations. Several independently isolated poly(A⁺) and poly(A⁻) mRNA preparations gave similar results. On the other hand, the saturation value obtained from the

Figure 4. (A) In vivo labeled mouse nonrepetitive DNA hybridized with poly(A⁺) (o) and poly(A⁻) (o) mRNA from PYAL/N mouse cells grown in culture. (B) A similar reaction using a mixture of poly(A⁺) and poly(A⁻) mRNA from PYAL/N cells (Δ).

mixing experiment compares favorably with our earlier measurements using <80 S RNA. Overall, the data on cultured cells also suggests that 30-40% of mRNA is not polyadenylated and thus supports the conclusions derived from the experiments with mouse liver.

DISCUSSION

The major goal of this study was to provide information on the contribution poly(A⁻) mRNA makes to the total sequence diversity of polysomal mRNA. Our results show that in both mouse liver and cultured mouse cells, 30-40% of the total mRNA complexity is derived from poly(A⁻) mRNA. The proportion of the mRNA complexity residing in poly(A⁺) and poly(A⁻) sequences was determined using the same E-DNA preparation and therefore is not dependent on the amount of the E-DNA that remains reactable. This is also true for comparison of these measurements with those using < 80 S RNA and for evaluating the relative complexities of nuclear and polysomal RNA. However, uncertainty as to the amount of E-DNA that remains reactable does complicate calculation of the number of different molecules present in each class of RNA. Minimum values are obtained if it is assumed, as was done in the results section, that all of the E-DNA can react and in this case one obtains approximately 8,600 different poly(A⁺) mRNAs and an additional 5,700 poly(A⁻) species for a total of 14,300 individual mRNA molecules. On the other hand, since it was shown that at least 70% of the in vitro labeled DNA could react, using this as a terminal value should give a reasonable estimate of the upper limit of the number of different RNA species in each category. Under these circumstances the number of distinct mRNAs are 12,200 poly(A⁺) and 8,100 poly(A⁻), or a total of 20,300 diverse mRNAs. In both cases, the estimated number of poly(A⁺)

molecules is within the range reported by other investigators using cDNA copies of poly(A⁺) mRNA.^{9,10,12-14} Regardless of the absolute number of distinct mRNAs, the overall results merit serious consideration since one area of current interest involves the differences which exist between the mRNA populations of either resting and growing cells, or between cells from different tissues. The data which have been presented suggest that attempts to study this problem using only poly(A⁺) mRNA, though useful, are limited because they do not account for a substantial portion of the total information present in mRNA.

It should probably be cautioned here that one cannot rule out with certainty the possible existence of a complex class of mRNAs which occur at such a low frequency that they would not be detected in these experiments. Should such a class exist, it is conceivable that its complexity could be distributed among poly(A⁺) and poly(A⁻) mRNA in such a way as to alter the distribution revealed by our experiments.

An unexpected feature of these experiments was that the percentage of total mRNA complexity contained in poly(A⁻) mRNA was similar to the proportion of poly(A⁻) mRNA which occurs on a weight basis.³⁻⁶ In this regard it is also interesting to note that in an investigation of the sequence diversity of nuclear RNA in mouse brain, Bantle and Hahn¹⁵ observed that the total complexity was distributed such that 64% was in poly(A⁺) hnRNA and 36% in poly(A⁻) hnRNA. Although obtained with different tissues, if the data of Bantle and Hahn are combined with the results of the present study there is a strong suggestion that the distribution of total complexity between poly(A⁺) and poly(A⁻) RNA is the same in the cytoplasm as in the nucleus.

In the mouse liver experiments total mRNA had only 22% of the sequence diversity of nuclear RNA based on the mixtures of poly(A⁺) and poly(A⁻) RNA, and 17% when <80 S RNA was used. This is similar to our report that the complexity of mRNA in the cultured PYAL/N cells was 10-20% of that contained in nuclear RNA.⁷ Both of these results compare favorably with those from other laboratories which found anywhere from a ten-fold³¹ to a five-fold^{11,15,32} difference in complexity between nuclear and mRNA in eukaryotic cells.

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33. Abbreviations used: EDTA, ethylenediaminetetraacetic acid; Tris, tris (hydroxymethyl) aminomethane; SLS, sodium lauryl sulfate; PB, phosphate buffer containing equimolar amounts of Na_2HPO_4 and NaH_2PO_4