Complexity of Cytoplasmic Polyadenylated and Nonpolyadenylated Rat Brain Ribonucleic Acids†

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ABSTRACT: Measured by saturation hybridization to radioiodinated single-copy DNA, rat brain cytoplasmic RNA annealed to 10.0% of the rat unique sequence DNA. By assuming asymmetric transcription, this complexity is equivalent to 20% of the single-copy genome or 3.6×10^8 nucleotides, enough information for 240 000 different 50 000-dalton proteins. Half the complexity resides in polyadenylated RNA and half in nonpolyadenylated RNA. Sequential additivity experiments show that poly(A)$^+$ and poly(A)$^-$ cytoplasmic RNAs contain nonoverlapping sets of sequences. Both RNA populations are composed of at least two abundance frequencies; the most abundant class has an average copy number of 5–7 copies per cell, while the rare frequency class is present at an average of 0.014 copy per cell for poly(A)$^+$ RNA and 0.18 copy per cell for poly(A)$^-$ RNA. Since saturation hybridization measures primarily high-complexity, low-abundance RNA, comparison of cell-free translation products directed by poly(A)$^+$ and poly(A)$^-$ RNAs was performed to adjudge sequence similarities between highly abundant RNAs. In contrast to the hybridization results, many of the major translation products synthesized in a rabbit reticulocyte translation system directed by poly(A)$^-$ RNA are also found among the poly(A)$^+$ translation products, a major common protein being β-actin. Although the most abundant proteins are held in common, the majority of the detectable poly(A)$^-$ products are unique to the poly(A)$^-$ fraction as analyzed by two-dimensional gel electrophoresis. To eliminate possible contamination of cytoplasmic RNA by high-complexity nuclear RNA, puromycin-released brain polysomal RNA was prepared. This RNA saturated 8% of probe DNA. Assuming asymmetric transcription, this is equivalent to 2.9×10^8 nucleotides or 192 000 different 1500-nucleotide-long mRNAs. By comparison, cytoplasmic liver and kidney RNAs have complexities of 8.6×10^7 and 5.8×10^7 nucleotides, respectively, equal to about 57 000 active liver genes and 39 000 active kidney genes.

In a previous communication (Chikaraishi et al., 1978), nuclear RNA complexities of various adult rat tissues were measured by RNA-driven hybridization to single copy DNA. Thirty-one percent of the unique sequence genome was expressed in brain RNA, 22% in liver RNA, and about 10% in kidney, thymus, and spleen RNAs, by assuming asymmetric transcription. A surprising result was that the vast majority of nuclear RNAs from brain, liver, and kidney represented a nested set of mutually inclusive sequences. Others have also reported high transcriptional diversity in rodent brain. Using saturation hybridization, Bantle & Hahn (1976) showed that mouse brain nuclear RNA annealed to 42% of single copy mouse DNA, and Grouse et al. (1978) saturated 33.6% of unique sequence DNA with rat brain hnRNA,[1] assuming asymmetric transcription. Using poly(A)$^+$ nuclear RNA, rather than total RNA, Bantle & Hahn (1976) saturated 26.6% of nonrepetitive mouse DNA, while Kaplan et al. (1978) saturated 24.6% of single copy rat DNA with total poly(A)$^+$ RNA. Since these clearly demonstrate high transcriptional diversity in rodent brain hnRNA, we were interested if brain cytoplasmic and polysomal RNA also possessed high complexity. In this report, we estimate the complexity of brain cytoplasmic and polysomal RNA. For comparison with nonneural tissues, the RNA complexity of liver and kidney cytoplasmic RNA is also measured.

Hybridization of poly(A)$^-$ RNA to cDNA prepared by reverse transcription from poly(A)$^+$ RNA has demonstrated that the large majority of poly(A)$^+$ sequences are not found in the poly(A)$^-$ RNA population in HeLa cells (Milcarek et al., 1974; Kaufmann et al., 1977) and in sea urchin embryo RNA (Nemer et al., 1974). Since cDNA experiments primarily measure abundant RNAs, we examine here the sequence similarity between poly(A)$^+$ and poly(A)$^-$ RNAs by saturation hybridization which is better able to compare high complexity, rare abundance RNAs.

Because of its high sequence diversity, brain cytoplasmic RNA is well suited for determining what proportion of the RNA diversity resides in complex polyadenylated vs. nonpolyadenylated transcripts. Recently, Hahn and his colleagues have demonstrated a complex class of nonpolyadenylated mouse brain mRNAs which are distinct from the polyadenylated species (W. E. Hahn, personal communication). Grady et al. (1978) have recently shown that complex poly(A)$^+$ and poly(A)$^-$ RNAs from mouse liver and cultured polyoma transformed mouse cells contain nonoverlapping sets of sequences.

In contrast to the above experiments that show high complexity, rare abundance RNAs fall either into the polyadenylated or nonpolyadenylated class, Kaufmann et al. (1977) demonstrated by in vitro translation that many of the proteins programmed by poly(A)$^-$ cytoplasmic RNA from HeLa cells were also programmed by poly(A)$^+$ RNA. In particular, β-actin was translated from both types of RNA. By preselecting cDNA homologous to the most abundant 20% of the poly(A)$^+$ RNAs, these authors further showed that about 10% of this preselected cDNA annealed to poly(A)$^-$ RNA. Since our complexity measurements are very insensitive to differences or similarities between small numbers of sequences, even though those sequences might be very abundant, we compared the in vitro translation products directed by brain cytoplasmic

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[1] Abbreviations used: C_0t, DNA concentration (mol of nucleotide/L) at $t_0 \times$ time (s); R_0t, RNA concentration at $t_0 \times$ time (s); [^{125}I]DNA, ^{125}I-iodinated DNA; HAP, hydroxylapatite; poly(A)$^+$, polyadenylated; poly(A)$^-$, nonpolyadenylated; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate; OD_{260}, optical density units at 260 nm; cDNA, complementary DNA; hnRNA, heterogeneous nuclear RNA; RNP, ribonucleoprotein.

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poly(A)$^+$ and poly(A)$^-$ RNAs in a messenger-dependent rabbit reticulocyte system.

Experimental Procedures

Preparation of [^{125}I]DNA. The preparation and characterization of the unlabeled and iodinated unique sequence DNA were exactly as previously described (Chikaraishi et al., 1978) except that β-mercaptoethanol was added to a final concentration of 0.14 M to the iodination reaction after the initial incubation of 20 min at 60 °C. The addition of β-mercaptoethanol improved the stability of the iodinated DNA. However, about 10–15% of the ^{125}I radioactivity was no longer bound to acid-precipitable DNA after 3 months of storage at 4 °C. Therefore, a new probe was prepared every 2 months. During hybridization, about 8–10% of the ^{125}I radioactivity was released from acid-precipitable DNA after 200 h at 68 °C. This radioactivity eluted from hydroxylapatite in the 0.03 M PB, 0.2 M NaCl, 0.06% $NaDodSO_4$ fraction and was not included in the calculation of the percent hybridization (Chikaraishi et al., 1978).

Preparation of Cytoplasmic RNAs. Between 6 and 9 g of fresh adult rat brain, liver, or kidney was homogenized (5 strokes) in 23 mL of 0.14 M NaCl, 0.01 M Tris (pH 8.4), 1.5 mM $MgCl_2$ (Lindberg & Darnell, 1970), 0.5% Nonidet P40, 0.5 mM sodium iodoacetate on ice. The homogenate was centrifuged at 12000*g* (av) for 10 min at 0 °C and the upper three-quarters of the supernatant put directly into $NaDodSO_4$–Pronase, as described previously (Chikaraishi et al., 1978). After at least 2 h at 37 °C, the RNA was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (24:24:1) containing 0.1% 8-hydroxyquinoline and saturated with 0.1 M Tris (pH 7.5). The RNA was ethanol precipitated, dissolved in 0.1 M NaCl, 0.02 M Tris (pH 7.5), 5 mM EDTA, 0.1% $NaDodSO_4$, and deproteinized as above until there was no further interphase. The RNA was stored as an ethanol precipitate.

Preparation of Poly(A)$^+$ and Poly(A)$^-$ RNAs. Cytoplasmic RNA was dissolved in 0.02 M Tris (pH 7.5), 0.1% $NaDodSO_4$, at 25–50 OD_{260}/mL, heated for 5 min at 68 °C, cooled to room temperature, and brought to 0.5 M NaCl. The RNA was fractionated on an oligo(dT)-cellulose (T3) column (Collaborative Research), as described by Aviv & Leder (1972). Poly(A)$^-$ RNA was eluted in 0.5 M NaCl, 0.02 M Tris (pH 7.5), 0.05% $NaDodSO_4$, until the effluent was less than 0.005 OD_{260}/mL. Poly(A)$^+$ RNA was then eluted with 0.02 M Tris (pH 7.5), 0.1% $NaDodSO_4$. In most cases, both the poly(A)$^-$ and poly(A)$^+$ RNAs were fractionated again on oligo(dT)-cellulose. The poly(A)$^+$ RNA sedimented on 85% formamide–sucrose gradients (Macnaughton et al., 1974) as a broad peak around 18 S.

Preparation of Polysomal RNA. Adult (2 months or older) rat brain was removed within 1 min of cervical dislocation, washed in ice-cold phosphate-buffered saline, and homogenized in 7–9-g batches in 0.2 M sucrose (Mann, RNase-free; pretreated with activated charcoal to remove absorbance at 260 nm and diethyl pyrocarbonate to inactivate ribonucleases), 0.1 M NH_4Cl, 10 mM $MgCl_2$, 20 mM Tris (pH 7.5), 1 mM dithiothreitol (Falvey & Staehlin, 1970), 0.5 mg/mL sodium heparin. The homogenate was centrifuged at 12000*g* for 10 min at 0 °C. The postmitochondrial supernatant was brought to 0.3% sodium deoxycholate and 0.3% Triton X-100, and batches of 23 mL of supernatant were layered over discontinuous step sucrose gradients consisting of 7.0 mL of 1.5 M sucrose and 7.0 mL of 0.7 M sucrose in 0.1 M NH_4Cl, 5 mM magnesium acetate, 20 mM Tris (pH 7.5). The interfaces were mixed and centrifuged for 17 h at 24000 rpm in an SW27 rotor at 3 °C. Polysomal pellets were resuspended at 100–200 OD_{260}/mL in 20 mM *N*-2-hydroxylethylpiperazine-*N'*-ethanesulfonic acid (pH 7.0), homogenized, layered on 0.48–1.8 M sucrose gradients in 0.02 M Tris (pH 7.5), 0.2 M KCl, 2 mM $MgCl_2$, and centrifuged for 4 h at 23000 rpm at 3 °C in an SW27 rotor. Polysomes sedimenting between 120 S and 250 S were pooled, diluted with an equal volume of H_2O, and collected by centrifugation at 24000 rpm, 3 °C, 18 h in an SW27 rotor over a 5.0-mL cushion of 1 M sucrose, 0.2 M KCl, 0.02 M Tris (pH 7.5), 2 mM $MgCl_2$. At this stage, between 2 and 3 OD_{260} units of polysomes was recovered per g of tissue.

The polysomal pellet was dissolved at 100–150 OD_{260}/mL in 0.5 M KCl, 0.02 M Tris (pH 7.5), 2 mM $MgCl_2$, 0.02 M puromycin, incubated at 0 °C for 15 min, 37 °C for 10 min, then layered at 100 OD_{260}/gradient on 0.48–1.8 M sucrose gradients in 0.5 M KCl, 0.02 M Tris (pH 7.5), 1 mM $MgCl_2$, and centrifuged for 13 h, 25000 rpm at 3 °C in an SW27 rotor. Fractions sedimenting between 5 S and 60 S were pooled and extracted with phenol–chloroform as above, and the RNA was stored as an ethanol precipitate.

^3H-Labeled mengovirus marker was a gift of Dr. Raymond Erikson.

Hybridization of Unique Sequence [^{125}I]DNA to RNAs. Single copy [^{125}I]DNA at 0.2–0.5 μg/mL (specific activity 1–2.5 × 10^7 cpm/μg) was annealed to RNA (concentration given in each figure). The hybridization and quantitation of hybrids by S_1 assay or HAP chromatography were performed as previously described (Chikaraishi et al., 1978), except that the loading buffer for HAP chromatography was 0.2 M NaCl, 0.03 M phosphate buffer (1 M phosphate buffer (1 M PO_4, pH 6.8) = equimolar mixture of monobasic and dibasic sodium phosphate), 0.06% $NaDodSO_4$. Zero-time background was always less than 1.5% and has been subtracted before plotting. We have also found highly significant variation between various batches of commercially available HAP for retention of RNA–DNA hybrids. Hydroxylapatite (HTP) was obtained from Bio-Rad. Lots 16346 and 17725 were used in the experiments reported here.

Hybridization of [^3H]Poly(uridylic acid) to Polysomal RNA. [^3H]Poly(uridylic acid) (New England Nuclear) at 84 nCi/μg was heated for 5 min at 80 °C at 2.7 μg/mL in 0.5 M NaCl, 0.02 M Tris (pH 7.5), 0.05% $NaDodSO_4$ and cooled; 50 μL of [^3H]poly(uridylic acid) was added to 50-μL fractions from the puromycin-released polysomal RNA gradient. After incubating for 30 min at room temperature, 1.0 mL of 20 μg/mL RNase A in 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0) was added and incubation continued for 30 min; the fractions were then precipitated with 5% trichloroacetic acid and counted by liquid scintillation.

Translation and Protein Gel Analysis. The cell-free translations were essentially performed as described by Pelham & Jackson (1976), as modified by Purchio et al. (1977). The concentration of poly(A)$^+$ RNA in the translation reaction was 35 μg/mL, that of poly(A)$^-$ RNA, 130 μg/mL. These concentrations gave the maximal stimulation of [^{35}S]-methionine incorporation. The products of translation were analyzed on two-dimensional gels as described by O'Farrell (1975), by using a 10% polyacrylamide separation gel in the second dimension. Purified chicken gizzard actin was a generous gift of Dr. Jon Izant.

Results

Cytoplasmic and Polysomal RNA Complexities. RNA complexities were measured by RNA-driven hybridization to radioiodinated unique sequence DNA. The preparation and

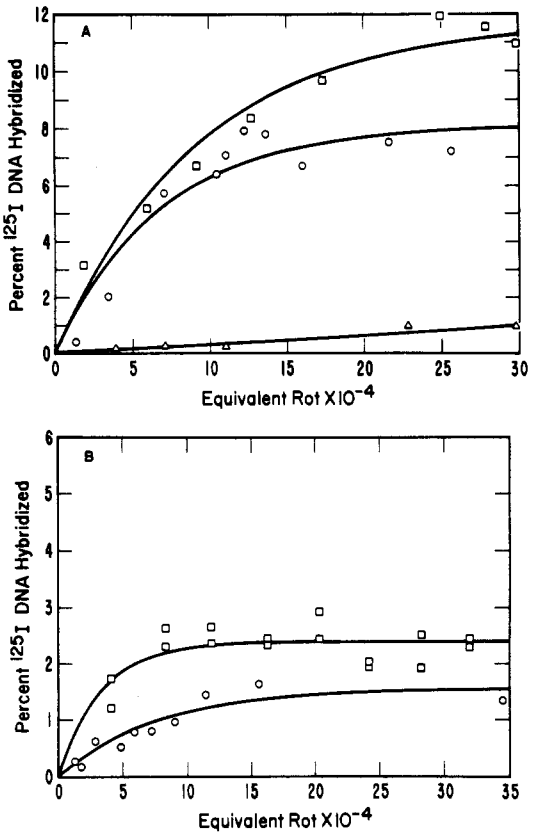

FIGURE 1: (A) Hybridization of brain cytoplasmic and polysomal RNA to single copy [^{125}I]DNA. Total cytoplasmic RNA at 16.8 mg/mL was annealed to single copy [^{125}I]DNA, and hybrids were assayed by S_1 digestion (□). RNA pretreated with RNase A at 20 μg/mL in H_2O and annealed at 20 mg/mL (△); puromycin-released polysomal RNA annealed at 15 mg/mL (○) assayed by HAP chromatography. Hybridization of liver and kidney cytoplasmic RNAs. Liver cytoplasmic RNA at 20 mg/mL (□) and kidney cytoplasmic RNA at 20 mg/mL (○) were annealed and assayed by digestion S_1. The curves in both figures are drawn as a best fit to a first-order reaction by using least-squares analysis. The observed rate constant (K_{obsd}) for the brain cytoplasmic RNA is 1.1×10^{-5} mol s^{-1} L; for liver cytoplasmic RNA, 3×10^{-5} mol s^{-1} L; for kidney cytoplasmic RNA, 1.3×10^{-5} mol s^{-1} L.

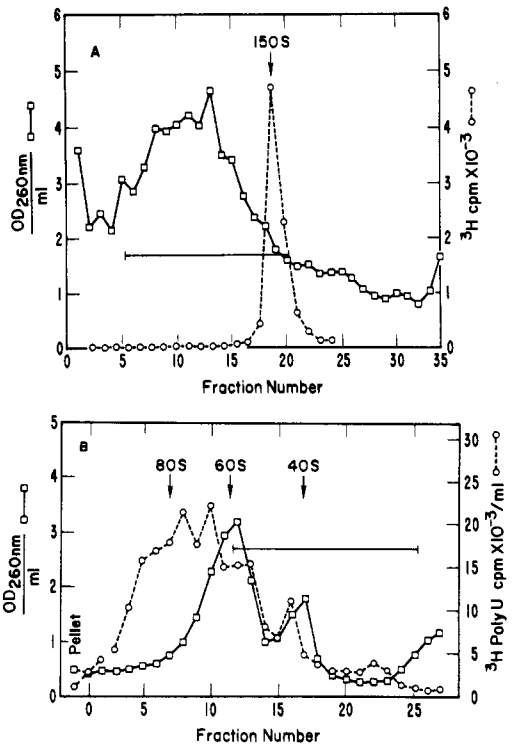

FIGURE 2: Preparative isolation of polysomal RNA by sucrose gradient centrifugation. (A) Brain polysomes (91 OD_{260} units/gradient) collected after step sucrose were sedimented through a 38-mL 0.48–1.8 M sucrose gradient in 0.2 M KCl, 0.02 M Tris (pH 7.5), 2 mM $MgCl_2$, as detailed under Experimental Procedures (□). [^{3}H]Mengo virus (150S) marker was sedimented in a parallel gradient (○). Sedimentation is from right to left. The fractions indicated by the bracket were pooled for puromycin treatment. (B) Sedimentation of polysomes after puromycin treatment in a 38-mL 0.48–1.8 M sucrose gradient in 0.5 M KCl, 0.02 M Tris (pH 7.5), 1 mM $MgCl_2$, as described under Experimental Procedures (□). Aliquots from each fraction were assayed for the presence of poly(A) by hybridization to [^{3}H]poly-(uridylic acid) (○), as described under Experimental Procedures. Fractions indicated by the bracket were pooled and RNA was extracted.

characterization of the probe DNA have been previously described (Chikaraishi et al., 1978); in brief, the iodinated DNA has an average length of 475 nucleotides and has a specific activity of 1.0 to 2.5×10^7 cpm/μg. When driven with excess unlabeled total genomic DNA, the probe anneals to greater than 93% with an observed $C_0t_{1/2}$ of 2.1×10^3, as assayed by hydroxylapatite (HAP) chromatography, and appears to have less than 3% contamination with repetitive sequence DNA. The percentage of the probe DNA in hybrids was analyzed either by digestion with S_1 single-strand-specific nuclease or by HAP chromatography.

Total brain cytoplasmic RNA anneals to 10% of unique sequence iodinated DNA, when the probe self-annealing of 1% is subtracted (Figure 1A). Assuming that RNA is asymmetrically transcribed fron one DNA strand, approximately 20% of the unique sequence coding capacity is expressed in brain cytoplasmic RNA, equivalent to 3.6×10^8 nucleotides or 240 000 messages, each 1500 nucleotides long. When the cytoplasmic RNA was predigested with ribonuclease A before annealing, the hybridization was reduced to about 1% at the termination of the experiments (Figure 1A). This background level is due to the self-annealing of the probe DNA, which was 1×10^7 cpm/μg in this experiment. With probes at greater than 1.5×10^7 cpm/μg, we were able to use less probe DNA in the annealing mixture and reduce self-annealing to less than 0.5% after 150 h of hybridization.

Since total cytoplasmic RNA had such high complexity, we were concerned that this might be due to nuclear RNA contamination. To eliminate contaminating hnRNA from cytoplasmic preparations, puromycin-released polysomal RNA was isolated. This was accomplished by first separating cytoplasm from nuclei, pelleting the postmitochondrial cytoplasmic supernatant over step sucrose gradients (Falvey & Staehlin, 1970), and sedimenting the polysomal pellet in a preparative sucrose gradient to display polysomes. Those polysomes that were greater than 120 S in size were pooled, concentrated, and treated with puromycin, and the released polysomes displayed again on a sucrose gradient. RNA was extracted and pooled from fractions between 5 S and 60 S. If contaminating nuclear ribonucleoproteins should cosediment with >120S polysomes in the first display gradient, they would have to be dissociated to less than 60 S by puromycin in order to cosediment with the released polysomes. Figure 2 shows a typical preparative fractionation. In order to determine where poly(A)$^+$ mRNAs sediment after puromycin release, [^{3}H]poly(uridylic acid) was hybridized across the gradient (Bishop et al., 1974). Since the majority of puromycin-released mRNPs from chick brain sediment at less than 80 S (Bryan & Hayashi, 1973), we were surprised to find most of the poly(A)$^+$ mRNPs sedimenting in a broad peak around 80 S. Therefore, the puromycin release may not have been complete

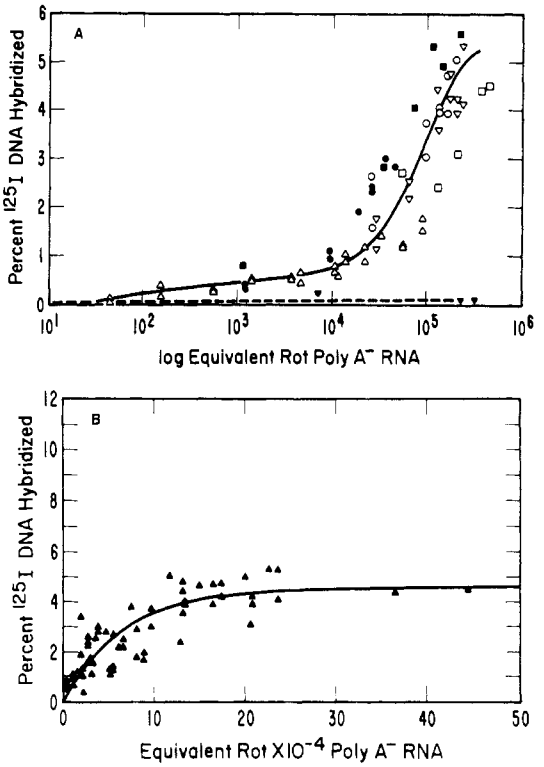

FIGURE 3: Hybridization of brain cytoplasmic poly(A)$^-$ RNA to single copy [^{125}I]DNA. (A) Hybridization of five different preparations of poly(A)$^-$ RNA to single copy [^{125}I]DNA. Preparation 1 was annealed at 5.8 mg/mL (○); preparation 2 at 18 mg/mL (▽); preparation 3 at 18 mg/mL (●). Preparations 1, 2, and 3 were assayed by digestion S_1. Preparation 4 was annealed at 6 mg/mL (△) and 20.8 mg/mL (■). Preparation 5 was annealed at 45 mg/mL (□); half of the preparation was treated for 18 h in 0.2 N NaOH, neutralized, annealed as above (▼). Preparations 4 and 5 were assayed by HAP chromatography. (B) Data in A plotted with a linear abscissa. The curve is a computer best fit to a first-order reaction with a K_{obsd} = 1.5 × 10^{-5} mol s^{-1} L.

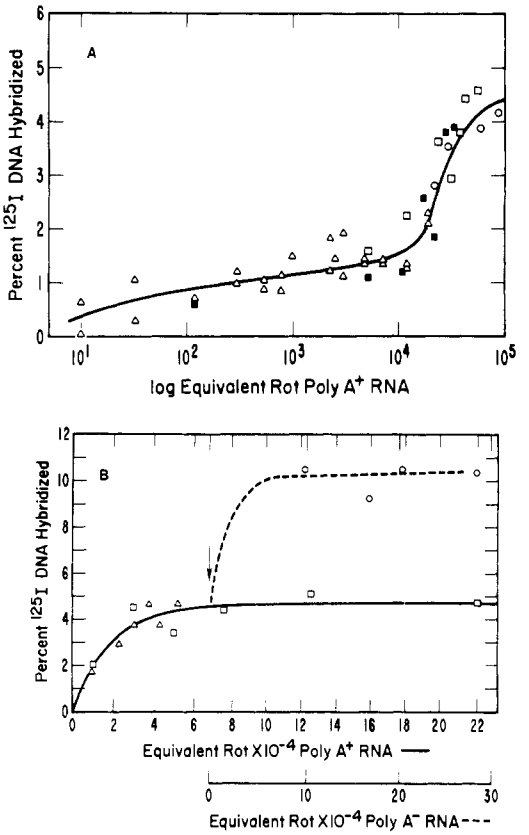

FIGURE 4: Hybridization of brain poly(A)$^+$ cytoplasmic RNA to single copy [^{125}I]DNA and additivity of poly(A)$^+$ and poly(A)$^-$ RNAs. (A) Hybridization of four different preparations of cytoplasmic poly(A)$^+$ RNA at 1.26 mg/mL (△); 3 mg/mL (■) assayed by HAP; 3.2 mg/mL (□) and 5.8 mg/mL (○) assayed by S_1. (B) Brain poly(A)$^+$ RNA was annealed at 7.8 mg/mL to [^{125}I]DNA (□). In another reaction, poly(A)$^+$ RNA was annealed at 5.9 mg/mL (△) and at the R_0t indicated by the arrow, poly(A)$^-$ RNA was added to final concentration of 20 mg/mL with respect to the poly(A)$^-$ RNA; hybrids were assayed by S_1. The abscissa is a linear scale.

or mammalian mRNPs may be larger than those found in chick brain, as suggested by the data of Auerbach & Pederson (1975). Changing the puromycin or salt (KCl and Mg^{2+}) concentration did not change the mRNP profile, although reducing the concentration of polysomes 10–20-fold did shift some of the mRNPs to the <40S region of the gradient.

Puromycin-released polysomal RNA saturates 8% of unique sequence DNA (Figure 1A). By assuming asymmetric transcription, this is equivalent to a complexity of 2.9 × 10^8 nucleotides, enough information for 192 000 different genes. We have considerable difficulty in obtaining enough high complexity polysomal RNA for hybridization, perhaps because much of the complex RNA is greater than 60 S after puromycin release. The 2% difference in saturation plateaus between total cytoplasmic RNA (10%) and polysomal RNA (8%) could be due either to nuclear RNAs contaminating the cytoplasmic RNA preparations or to cytoplasmic nonpolysomal RNAs. Although we have tried to eliminate hnRNA (or RNPs containing hnRNA), it is clear that any contamination of the polysomal RNA with hnRNA sequences would greatly increase the observed polysomal RNA complexity.

By assuming polysomal hybridization is an accurate measure of gene activity, the rat brain expresses an extremely large number of different genes. We were therefore interested in comparing brain with other adult tissues. Figure 1B shows that the total liver cytoplasmic RNA anneals to 2.4% of the unique sequence DNA probe, total cytoplasmic kidney RNA to about 1.6%. These are equivalent to transcription of 8.6 × 10^7 nucleotides and 5.8 × 10^7 nucleotides, respectively (by assuming asymmetric transcription), or about 57 000 liver and 38 000 kidney genes, each 1500 nucleotides in length. Therefore, four to five times fewer genes appear to be active in nonneural tissue. Since all cells probably require common housekeeping gene products, which are less than or at most equal to the number expressed in the tissue having the lowest complexity (in this case, kidney), this suggests that the high diversity of products in brain may not be used for general maintenance functions.

Brain Cytoplasmic Poly(A)$^+$ and Poly(A)$^-$ Complexities. Because brain cytoplasmic RNA exhibits high diversity, it is well suited for asking what proportion of the complexity resides in polyadenylated vs. nonpolyadenylated RNAs. We separated poly(A)$^+$ RNA from poly(A)$^-$ RNA by chromatography on oligo(dT)-cellulose (Aviv & Leder, 1972). Both fractions were usually purified by two cycles of oligo(dT) chromatography. After two passages through oligo(dT)-cellulose, 2% of the total cytoplasmic RNA fractionated as poly(A)$^+$. The size of the poly(A)$^+$ RNA was estimated to be about 18 S or 1500 nucleotides by sedimentation on 85% formamide–sucrose gradients (data not shown). By annealing with [^{3}H]poly(uridylic acid), we estimate that between 0.5 and 5% of the poly(A) or oligo(A) is in poly(A)$^-$ RNA. Figures 3 and 4 show hybridizations of poly(A)$^-$ and poly(A)$^+$ RNA to unique sequence DNA. At saturation, both poly(A)$^+$ and poly(A)$^-$ RNA anneal to 4.8%.

Since total cytoplasmic RNA saturates 10%, it appears that

the two populations contain nonoverlapping sets of sequences. A further demonstration of this is shown in Figure 4B. Poly(A)$^+$ RNA is hybridized to saturation; at that point, excess poly(A)$^-$ RNA is added to the same tube and the annealing continued. As shown, addition of poly(A)$^-$ RNA increases the hybridization plateau from about 4.5% to about 10%. This indicates the vast majority of high complexity poly(A)$^-$ RNAs contain different sequences than the high complexity poly(A)$^+$ RNAs. If poly(A)$^-$ RNA is pretreated for 18 h in 0.2 N NaOH, neutralized, and used for hybridization, all annealing is eliminated, suggesting that there is no appreciable self-annealing of the probe in this experiment and that there is no detectable DNA contamination contributing to the hybridization (Figure 3A).

Plotted on a logarithmic scale, the hybridizations of poly(A)$^+$ (Figure 4A) and poly(A)$^-$ RNA (Figure 3A) clearly show several abundance components. Although there may be additional components, the data were analyzed as a two-component population. As shown in Table I, by computer least-squares best-fit to a first-order reaction, both the poly(A)$^+$ and poly(A)$^-$ populations have a high abundance component annealing at low R_0t values. In the poly(A)$^+$ RNA, this component has a $R_0t_{1/2}$ of 40 and accounts for essentially all the mass of the poly(A)$^+$ RNA, or about 2% of the total cytoplasmic RNA. This component has a complexity of 4.9×10^7 nucleotides, enough for 32 600 mRNAs, present at an average of 5.1 copies per cell. In contrast, the rare poly(A)$^+$ RNAs have a complexity of 1.3×10^8 nucleotides, enough to encode 86 600 genes and have an average copy number of 0.014 copy per cell. The abundant class of nonribosomal poly(A)$^-$ RNA represents about 1.6% of the cyotplasmic RNA and by this rough measurement comprises about the same mass as poly(A)$^+$ RNA. The abundant poly(A)$^-$ RNAs represent about 19 000 different transcripts (2.9×10^7 nucleotides) present at 7.2 copies per cell, while the rare poly(A)$^-$ transcripts exist at about 0.19 copy per cell and contain enough information to encode 100 000 different polypeptides. The copy number estimates are very approximate, owing to assumptions made for their calculations.

Owing to lack of material, we have not performed additivity experiments with polysomal RNA, and therefore it is not known whether the reduced complexity of polysomal RNA compared with cytoplasmic RNA comes preferentially from the adenylated or nonadenylated class.

Translation of Poly(A)$^+$ and Poly(A)$^-$ RNAs. RNA complexity measured by RNA-driven saturation hybridization assays almost exclusively high complexity, rare abundance sequences. In order to assess sequence similarities between abundant poly(A)$^+$ and poly(A)$^-$ RNAs, we translated both RNAs in a messenger-dependent rabbit reticulocyte system described by Pelham & Jackson (1976) and modified by Purchio et al. (1977). Figure 5 compares the products of poly(A)$^+$ and poly(A)$^-$ RNA directed translations on two-dimensional gels (O'Farrell, 1975), in which the first dimension is isoelectric focusing and the second, $NaDodSO_4$ gel electrophoresis. With no RNA added to the translation system, two spots (no. 6 and 7) appear, which have the same molecular weight, 46 000, but different isoelectric points (Figure 5A). [^{35}S]Methionine-labeled products translated from poly(A)$^-$ RNA and poly(A)$^+$ RNAs show the same two endogenous spots, with a marked increase in the more acidic protein (no. 7). At least 18 other new spots could be detected in the poly(A)$^-$ RNA programmed products, 6 of which are also found among the poly(A)$^+$ translation products (spots no. 3, 9, 10, 11, 12, 17). Twelve minor proteins, however, are found only among the poly(A)$^-$ products. Four of the six common proteins were among the major products of translation. Most of the unique poly(A)$^-$ proteins were minor spots. Unfortunately, histones which are known to be translated from poly(A)$^-$ RNA do not isoelectric focus in this gel system.

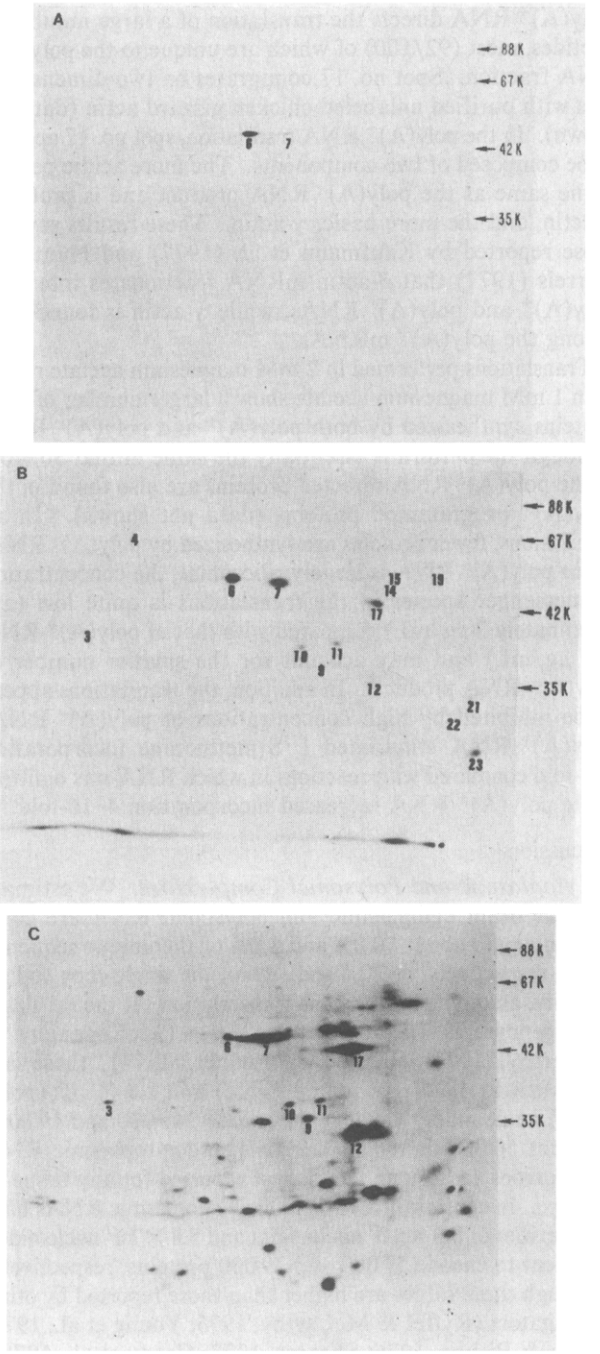

FIGURE 5: Fluorographs of two-dimensional gel electrophoresis of [^{35}S]methionine-labeled in vitro translation products programmed by brain cytoplasmic poly(A)$^+$ and poly(A)$^-$ RNAs. Translation of poly(A)$^+$ and poly(A)$^-$ RNA was performed as described under Experimental Procedures, and a 2-μL aliquot of the translation mixture was analyzed by the procedure of O'Farrell (1975). A 2-μL aliquot of the translation reaction programmed without RNA contained 6800 cpm (A); with poly(A)$^-$ RNA, 27 700 cpm (B); and with poly(A)$^+$ RNA, 27 200 cpm (C). The gels were stained, fluorographed (Bonner & Laskey, 1974; Laskey & Mills, 1975), dried, and exposed to Kodak X O-mat R film for 25 days at −70 °C. The first dimension is isoelectric focusing in which the acidic pole is at the right and the basic pole at the left. The second dimension is a 10% $NaDodSO_4$–polyacrylamide slab gel, with molecular weight markers (K = 1000) given on the right.

Poly(A)$^+$ RNA directs the translation of a large number of peptides, most (92/100) of which are unique to the poly(A)$^+$ RNA fraction. Spot no. 17 comigrates on two-dimensional gels with purified unlabeled chicken gizzard actin (data not shown). In the poly(A)$^+$ RNA translation, spot no. 17 appears to be composed of two components. The more acidic peptide is the same as the poly(A)$^-$ RNA product and is probably β-actin, and the more basic, γ-actin. These results support those reported by Kaufmann et al. (1977) and Hunter & Garrels (1977) that β-actin mRNA fractionates into both poly(A)$^+$ and poly(A)$^-$ RNAs, while γ-actin is found only among the poly(A)$^+$ mRNAs.

Translations performed in 2 mM magnesium acetate rather than 1 mM magnesium acetate show a larger number of total proteins synthesized by both poly(A)$^+$ and poly(A)$^-$ RNA, although the pattern is essentially the same in that 30–40% of the poly(A)$^-$ RNA directed proteins are also found in the poly(A)$^+$ programmed proteins (data not shown). In all translations, fewer proteins are synthesized by poly(A)$^-$ RNA. Since poly(A)$^-$ RNA is largely ribosomal, the concentration of messenger species in the translations is quite low (approximately 2 μg/mL) compared with that of poly(A)$^+$ RNA (35 μg/mL) and may account for the smaller number of poly(A)$^-$ RNA products. In addition, the translations appear to be inhibited by high concentrations of poly(A)$^-$ RNA. Poly(A)$^-$ RNA stimulated [^{35}S]methionine incorporation 3–5-fold compared with reactions in which RNA was omitted, while poly(A)$^+$ RNA increased incorporation 4–16-fold.

Discussion

Cytoplasmic and Polysomal Complexities. We estimate that rat brain cytoplasmic and polysomal RNA are complementary to about 10.0% and 8.0% of the unique sequence DNA, respectively, or 20% and 16% of the single copy coding capacity, assuming asymmetric transcription. If the rat single copy genome is 1.8×10^9 base pairs (McConaughy & McCarthy, 1970; Holmes & Bonner, 1974), these are equivalent to 3.6×10^8 (cytoplasmic) and 2.9×10^8 (polysomal) nucleotides, which could encode 240 000 and 192 000 different 50 000-dalton proteins. These cytoplasmic RNA complexities are among the highest reported for any tissue or cell type. In contrast, liver and kidney cytoplasmic RNAs have complexities of 8.6×10^7 nucleotides and 5.8×10^7 nucleotides, sufficient to encode 57 000 and 39 000 proteins, respectively. Although these values are higher than those reported by other investigators (Ryffel & McCarthy, 1975; Young et al., 1976; Hastie & Bishop, 1976; Shearer, 1977; Grady et al., 1978), the level of gene expression in the nonneural tissues is still four to six times lower than in brain.

Because of the high diversity of brain cytoplasmic and polysomal RNAs, one must be suspicious of possible hnRNA contamination contributing to this high complexity. After puromycin treatment of polysomes, which initially sedimented between 120 S and 250 S, we hoped to reduce hnRNA contamination by collecting material that resedimented between 5 S and 60 S after release. If, however, significant nicking of hnRNA occurred during puromycin treatment, nuclear RNA sequences could still contaminate polysomal RNA. Since the polysomal RNA complexity is only half that of the nuclear RNA (Chikaraishi et al., 1978), not all nuclear sequences would be suspected of leaking into the cytoplasm. However, leakage of a distinct subset of sequences is a possibility, especially when dealing with a tissue in which various cell types may exhibit varying nuclear fragilities.

There have been a number of measurements of the complexity of poly(A)$^+$ mRNA from rodent brain. Our data indicate that about 4.8% of the unique sequence DNA is transcribed into poly(A)$^+$ RNA, which is equivalent to about 1.7×10^8 nucleotides or about 120 000 different proteins, by assuming asymmetric transcription. Using saturation hybridization, Bantle & Hahn (1976) estimated a complexity of 1.4×10^8 nucleotides for mouse brain poly(A)$^+$ mRNA, and Grouse et al. (1978), a complexity of 1.3×10^8 nucleotides for rat brain poly(A)$^+$ mRNA, equivalent to about 90 000 average peptides. In contrast, three groups have reported much lower diversities in mouse brain RNA using cDNA hybridization to poly(A)$^+$ mRNA: Ryffel & McCarthy (1975), 3.5×10^7 nucleotides; Young et al. (1976), 4.5×10^7 nucleotides ($R_0t_{1/2} = 300$); and Hastie & Bishop (1976), 2.1×10^7 nucleotides ($R_0t_{1/2} = 21$). By assuming that the vast majority of mRNAs are transcribed from single copy DNA (Klein et al., 1974), the complexities estimated by cDNA kinetics range from 0.6% to 1.3% of mouse single copy DNA. These values are consistent with the annealing of the more abundant poly(A)$^+$ cytoplasmic RNAs comprising about 1.3% complexity and having a $R_0t_{1/2}$ of 40 (Table I). We estimate that there are on the average about five copies per cell of these relatively abundant RNAs, and, by comparing the expected and the observed rate constants for this annealing, these RNAs compose essentially all of the mass of the brain poly(A)$^+$ cytoplasmic RNA. It is likely that the RNAs that encode maintenance or housekeeping functions fall into this abundance class since, by cross-hybridization cDNA experiments, the majority of these sequences were shared among all tissues studied (Ryffel & McCarthy, 1975; Young et al., 1976; Hastie & Bishop, 1976).

The hybridization of poly(A)$^-$ cytoplasmic RNA to single copy DNA (Table I) also appears to be composed of several kinetic classes. The more abundant poly(A)$^-$ RNAs are present at about seven copies per cell. These poly(A)$^-$ RNAs may be analogous to the relatively abundant poly(A)$^+$ RNAs and may be common to many kinds of tissues.

Although about a quarter of the cytoplasmic hybridization can be accounted for by relatively abundant (5–10 copies per cell) poly(A)$^+$ and poly(A)$^-$ RNAs, the remaining complexity appears to reside in RNAs that are much rarer in abundance. We estimate that the rare poly(A)$^+$ RNAs are present at an average of about 0.014 copies per cell and the rare poly(A)$^-$ RNAs are at 0.19 copy per cell. Both Bantle & Hahn (1976) and Grouse et al. (1978) have shown that the least abundant class of poly(A)$^+$ mRNA in rodent brain anneals with an equivalent $R_0t_{1/2}$ of greater than 5000 and, therefore, is composed of RNAs present, on the average, at less than 0.05 copy per cell. These rare RNA sequences are probably absent entirely from most cells, but may be present at physiologically significant levels in a small proportion of cells; this, in fact, may reflect the diversity of cell types in the mammalian brain. The brain is certainly composed of many morphologically distinct cell types, both glial and neuronal, and, perhaps, even within a given neuronal cell type, there may be heterogeneity reflecting the specificity of neural connections. By assaying the RNA complexity of brain sections and clonal neural cell lines and tumors, our preliminary data suggest that the high cytoplasmic transcriptional diversity reported here may indeed result from the composite nature of the brain. In contrast, we have been unable to detect differences in the nuclear RNA complexity of various brain sections or clonal neural cells (D. M. Chikaraishi et al., unpublished results; S. L. Beckmann et al., unpublished results). In a recent study, Kaplan et al. (1978) have shown that regions of the rat brain (cerebral cortex, cerebellum, hypothalamus, and hippocampus) have

Table I: Analysis of Poly(A)$^+$ and Poly(A)$^-$ Brain Cytoplasmic RNAs

RNA	abundance class	complexity: % of unique sequence genome ± SD[a]	complexity: nucleotides[b]	$R_0t_{1/2}$[c]	K_{obsd}[c]	K_{exptd}[d]	fraction of RNA driving hybridization (K_{obsd}/K_{exptd})	no. of different 1500-nucleotide-long sequences	av copy no./cell[e]
poly(A)$^+$	high	1.35 ± 0.35	4.9×10^7	40	1.7×10^{-2}	1.7×10^{-2}	1.0	32 600	5.1
	low	3.45 ± 0.46	1.3×10^8	13000	5×10^{-5}	6.6×10^{-3}	0.0076	86 600	0.014
	high + low	4.80	1.7×10^8					119 200	
poly(A)$^-$	high	0.8 ± 0.19	2.9×10^7	1390	5×10^{-4}	2.9×10^{-2}	0.017	19 300	7.2
	low	4.0 ± 0.86	1.5×10^8	53000	1.3×10^{-5}	5.7×10^{-3}	0.0023	100 000	0.19
	high + low	4.8	1.7×10^8					119 300	

[a] SD = root mean standard deviation calculated from least-squares analysis from a best-fit first-order equation. Each abundance class was treated separately to estimate SD for each component. [b] Assumes that rat genome is 2.8×10^9 nucleotides and that 65% is unique sequence (McConaughy & McCarthy, 1970; Holmes & Bonner, 1974); assumes asymmetric transcription of DNA. [c] $K_{obsd} = K_{observed}$; calculated by least squares, best-fit to first-order equation from data in Figures 3 and 4A; calculated by using a 9821A Hewlett-Packard calculator. [d] $K_{exptd} = K_{expected}$; calculated on standard of a pure 2000-nucleotide sequence having a K_{exptd} of 8.6×10^2 mol s^{-1} L (Hastie & Bishop, 1976); corrected by $f = 2$ for the rate reduction due to length difference between tracer (475 nucleotides) and driver (1500 nucleotides) (Chamberlin et al., 1978). [e] Assumes that mass of total RNA per brain cell is 7.6 pg (Mandel et al. 1964); 90% of total RNA is cytoplasmic; 2% of cytoplasmic RNA is poly(A)$^+$; molecular weight of an RNA base is 330. Copy number per cell = [(total amount of RNA (g)/cell)(fraction of RNA in cytoplasm)(fraction of cytoplasmic RNA in poly(A)$^+$ or poly(A)$^-$ RNA)(fraction of RNA driving hybridization)]/[complexity of RNA (nucleotides) × 330/(6.02×10^{23})].

total poly(A)$^+$ RNA complexities similar to that of the total brain, although their data suggest that cultured neural cells may contain lower poly(A)$^+$ RNA complexity. In addition, there have been reports suggesting that neural activity and experience increase brain nuclear RNA diversity in rats (Uphouse & Bonner, 1975; Grouse et al., 1978).

Even though any given brain cell may express only a small percentage of the total possible brain polysomal RNAs, the fact that their summed complexity is so large suggests that there is an enormous number of active genes in the rat brain. If polysomal RNA complexity accurately reflects diversity of proteins, 192 000 different structural genes are expressed in neural tissues, which exceeds by about fivefold the limiting number of structural genes estimated by genetic arguments (Ohta & Kimura, 1971), although the assumptions upon which these calculations are based are somewhat controversial (O'Brien, 1973). Our estimates of nonneural tissues are equal to or greater than the 30 000 to 50 000 gene limit set by mutational considerations. Since the mutational load calculation counts only those genes in which a mutation would be deleterious to the organism, perhaps there is sufficient flexibility in redundancy of function among neural-specific genes, such that mutations in these genes are not selected against. Selection against neural mutations may be more complex and escape the classic genetic load arguments (N. Sueoka, personal communication).

Additivity of Cytoplasmic Poly(A)$^-$ and Poly(A)$^+$ RNAs. In sea urchins (Nemer et al., 1974; Fromson & Verma, 1976), pea seedling leaves (Gray & Cashmore, 1976), L cells (Greenberg, 1976), and HeLa cells (Milcarek et al., 1974), poly(A)$^-$ RNAs comprise between 30 and 65% of the mass of polysomal messenger RNA. By cross-hybridization of cDNA synthesized from poly(A)$^+$ mRNA to poly(A)$^-$ RNA, Nemer et al. (1974) and Milcarek et al. (1974) have shown that there is little or no homology between poly(A)$^+$ and poly(A)$^-$ RNAs in sea urchins and HeLa cells. By saturation hybridization, Grady et al. (1978) showed that poly(A)$^+$ and poly(A)$^-$ mRNAs from mouse liver and cultured Py AL/N cells contained nonoverlapping sets of sequences and that poly(A)$^-$ mRNA contained 30–40% of the sequence diversity of total mRNA.

Recently, W. E. Hahn and his colleagues (personal communication) have measured the complexity of poly(A)$^+$ and poly(A)$^-$ polysomal RNA from mouse brain. Their results show that 7.8% of the single copy DNA is transcribed into mouse brain polysomal RNA, with approximately half the complexity in poly(A)$^+$ mRNA and half in poly(A)$^-$ mRNA. Their results clearly support the notion that the complex polysomal adenylated and nonadenylated RNAs contain different sets of sequences.

Within the sensitivity of the technique, the complexities of cytoplasmic poly(A)$^+$ and poly(A)$^-$ RNAs are essentially additive. Addition of excess poly(A)$^-$ RNA to poly(A)$^+$ hybridization, which had already reached saturation with respect to poly(A)$^+$ RNA, increased the saturation plateau to about 10%. Experiments in which poly(A)$^+$ RNA was added to a hybridization in which poly(A)$^-$ RNA had reached saturation also showed additivity of poly(A)$^+$ and poly(A)$^-$ RNAs (data not shown). These results strongly suggest that the vast majority of complex poly(A)$^+$ RNAs have different sequences from the complex poly(A)$^-$ RNAs; i.e., the two classes of RNA represent nonoverlapping sets of genes. In addition, the additivity experiment suggests that extensive RNA degradation did not occur during preparation of the RNA. Random endonucleolytic breakage of poly(A)$^+$ RNA would generate RNAs of varying lengths which were no longer covalently attached to a 3′ poly(A)$^+$ tract. These nicked molecules would be expected to fractionate as poly(A)$^-$ on oligo d(T)-cellulose and contribute poly(A)$^+$ sequence complexity to the poly(A)$^-$ RNAs. Since the poly(A)$^-$ RNA does not contain appreciable poly(A)$^+$ sequences, nicking of poly(A)$^+$ RNA does not appear to be significant at the level of detection reported here.

Given that poly(A)$^-$ mRNAs represent a unique set of sequences which contain substantial complexity, measurements relying solely on poly(A)$^+$ RNAs could seriously underestimate the *total* complexity of a RNA population. Although it may be a more serious problem with cytoplasmic or polysomal RNAs, Bantle & Hahn (1976) showed that about 37% of the hnRNA complexity in mouse brain resided in nonpolyadenylated nuclear RNA. However, Ordahl & Caplan (1978) estimated that only 10% of the total RNA complexity resided in poly(A)$^-$ RNA in embryonic myoblasts.

Because saturation hybridization primarily measures the

diversity of high complexity, rare abundance RNAs, we translated poly(A)$^+$ and poly(A)$^-$ RNAs to compare abundant poly(A)$^+$ and poly(A)$^-$ RNAs, assuming that the major translation products reflect the most abundant RNAs in the population. In contrast to the hybridization additivity results, about one-third (6/18) of the proteins translated from poly(A)$^-$ RNA were also found among the poly(A)$^+$ RNA products. Similar results have been reported by others who translated poly(A)$^-$ and poly(A)$^+$ RNA from HeLa cells (Kaufmann et al., 1977), sea urchin eggs and embryos and amphibian oocyte and ovaries (Ruderman & Pardue, 1977), and pea seedling leaves (Gray & Cashmore, 1976). In fact, in these previous studies, a majority of the poly(A)$^-$ RNA directed products comigrated on NaDodSO$_4$ gels with poly(A)$^+$ RNA directed products. In an attempt to resolve this ambiguity, Kaufmann et al. (1977) selected cDNA homologous to the most abundant 20% of the poly(A)$^+$ mRNAs and showed that 10% of this preselected cDNA, which represented 2% of the total mass of HeLa cell polyadenylated mRNAs, annealed at relatively low R_0t's to poly(A)$^-$ RNAs. This shows that an abundant class of messages may exist whose members are both polyadenylated and nonpolyadenylated (or short polyadenylation) and encode common poly(A)$^-$ and poly(A)$^+$ mRNA products. β-Actin mRNA may fall into this class since β-actin appears as a major product in both poly(A)$^-$ and poly(A)$^+$ translations (Figure 5; Kaufmann et al., 1977; Hunter & Garrels, 1977) and its mRNA appears to have a continuous size spectrum of poly(A) tracts, some too short to fractionate as polyadenylated on oligo(dT)-cellulose (Hunter & Garrels, 1977).

A further explanation for the difference between our hybridization and translation results could be that some of the common poly(A)$^+$ and poly(A)$^-$ products are translated from repetitive sequence DNA transcripts. In mouse kidney and liver, high and middle abundance RNAs contain sequences homologous to DNA repeated approximately 100-fold in the genome (Hastie & Bishop, 1976). Since our hybridization probe contains only single copy DNA, overlaps in repetitive sequence transcripts would not be detected.

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References

Auerbach, S., & Pederson, T. (1975) *Biochem. Biophys. Res. Commun. 63*, 149–156.

Aviv, H., & Leder, P. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1408–1412.

Bantle, J. A., & Hahn, W. E. (1976) *Cell 8*, 139–150.

Bishop, J. D., Rosbash, M., & Evans, D. (1974) *J. Mol. Biol. 85*, 75–86.

Bonner, W. M., & Laskey, R. A. (1974) *Eur. J. Biochem. 46*, 83–88.

Bryan, R. N., & Hayashi, M. (1973) *Nature (London), New Biol. 244*, 271–724.

Chamberlin, M. E., Galau, G. A., Britten, R. J., & Davidson, E. H. (1978) *Nucleic Acids Res. 5*, 2073–2094.

Chikaraishi, D. M., Deeb, S., & Sueoka, N. (1978) *Cell 13*, 111–120.

Costantini, F. D., Scheller, R. H., Britten, R. J., & Davidson, E. H. (1978) *Cell 15*, 173–187.

Falvey, A. K., & Staehlin, T. (1970) *J. Mol. Biol. 53*, 1–19.

Fromson, D., & Verma, D. P. S. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 148–151.

Grady, L. J., North, A. B., & Campbell, W. D. (1978) *Nucleic Acids Res. 5*, 697–712.

Gray, R. E., & Cashmore, A. R. (1976) *J. Mol. Biol. 108*, 595–608.

Greenberg, J. R. (1976) *Biochemistry 15*, 3516–3522.

Grouse, L. D., Schrier, B. K., Bennett, E. L., Rosenzweig, M. R., & Nelson, P. G. (1978) *J. Neurochem. 30*, 191–203.

Hahn, W. E., Pettijohn, D. E., & Van Ness, J. (1977) *Science 197*, 582–585.

Hastie, N. D., & Bishop, J. O. (1976) *Cell 9*, 761–774.

Holmes, D. S., & Bonner, J. (1974) *Biochemistry 13*, 841–848.

Hough, B. R., Smith, M. J., Britten, R. J., & Davidson, E. H. (1975) *Cell 5*, 291–299.

Hunter, T., & Garrels, J. I. (1977) *Cell 12*, 767–781.

Kaplan, B. B., Schachter, B. S., Osterburg, H. H., DeVellis, J. S., & Finch, C. E. (1978) *Biochemistry 17*, 5516–5524.

Kaufmann, Y., Milcarek, C., Berissi, H., & Penman, S. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4801–4805.

Kleiman, L., Birnie, G. D., Young, B. D., & Paul, J. (1977) *Biochemistry 16*, 1218–1223.

Klein, W. H., Murphy, W., Attardi, G., Britten, R. J., & Davidson, E. H. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 1785–1789.

Laskey, R. A., & Mills, A. D. (1975) *Eur. J. Biochem. 56*, 335–341.

Lindberg, U., & Darnell, J. E. (1970) *Proc. Natl. Acad. Sci. U.S.A. 65*, 1089–1096.

Macnaughton, M., Freeman, K. B., & Bishop, J. O. (1974) *Cell 1*, 117–125.

Mandel, P., Rein, H., Harth-Edel, S., & Mardell, R. (1964) in *Comparative Neurochemistry* (Richter, D., Ed.) pp 149–163, Pergamon Press, New York.

McConaughy, B. L., & McCarthy, B. J. (1970) *Biochem. Genet. 4*, 425–446.

Milcarek, C., Price, R., & Penman, S. (1974) *Cell 3*, 1–10.

Nemer, M., Graham, M., & Dubroff, L. M. (1974) *J. Mol. Biol. 89*, 435–454.

O'Brien, S. J. (1973) *Nature (London), New Biol. 242*, 52–54.

O'Farrell, P. H. (1975) *J. Biol. Chem. 250*, 4007–4021.

Ohta, T., & Kimura, M. (1971) *Nature (London) 233*, 118–119.

Ordahl, C. P., & Caplan, A. I. (1978) *J. Biol. Chem. 253*, 7683–7691.

Pelham, H. R. B., & Jackson, R. J. (1976) *Eur. J. Biochem. 67*, 247–256.

Purchio, A., Erikson, E., & Erikson, R. L. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4661–4665.

Ruderman, J. V., & Pardue, M. L. (1978) *Dev. Biol. 60*, 48–68.

Ryffel, G. U., & McCarthy, B. J. (1975) *Biochemistry 14*, 1379–1384.

Shearer, R. W. (1977) in *Prevention and Detection of Cancer*, Part I, Vol. I, pp 53–68, Marcel Dekker, New York.

Uphouse, L. L., & Bonner, J. (1975) *Dev. Psychobiol. 8*, 171–178.

Young, B. D., Birnie, G. D., & Paul, J. (1976) *Biochemistry 15*, 2823–2829.