Volume 11 Number 16 1983 Nucleic Acids Research

Gene expression in rat brain

Robert J.Milner and J.Gregor Sutcliffe

A.V. Davis Center for Behavioral Neurobiology, The Salk Institute, P.O. Box 85800, San Diego, CA 92138, and Department of Molecular Biology, Research Institute of Scripps Clinic, 10666 N. Torrey Pines Road, La Jolla, CA 92037, USA

Received 29 April 1983; Revised and Accepted 13 July 1983

ABSTRACT

191 randomly selected cDNA clones prepared from rat brain cytoplasmic poly $(A)^+$ RNA were screened by Northern blot hybridization to rat brain, liver and kidney RNA to determine the tissue distribution, abundance and size of the corresponding brain mRNA. 18% hybridized to mRNAs each present equally in the three tissues, 26% to mRNAs differentially expressed in the tissues, and 30% to mRNAs present only in the brain. An additional 26% of the clones failed to detect mRNA in the three tissues at an abundance level of about 0.01%, but did contain rat cDNA as demonstrated by Southern blotting; this class probably represents rare mRNAs expressed in only some brain cells. Therefore, most mRNA expressed in brain is either specific to brain or otherwise displays regulation. Rarer mRNA species tend to be larger than the more abundant species, and tend to be brain specific; the rarest, specific mRNAs average 5000 nucleotides in length. Ten percent of the clones hybridize to multiple mRNAs, some of which are expressed from small multigenic families. From these data we estimate that there are probably at most 30,000 distinct mRNA species expressed in the rat brain, the majority of which are uniquely expressed in the brain.

INTRODUCTION

Neural specificity at the cellular level is the consequence of a cell's anatomical location, its particular connections, and the set of proteins it expresses. The brain is a complex organ and its cells express an enormous number of specific proteins, yet very few brain specific proteins have thus far been characterized. In order to understand ultimately the basis of brain function, it will be necessary to know the proteins involved. Since so few have been enumerated, and so very many exist, a descriptive approach seems desperately needed. Although proteins which are shared with other tissues doubtlessly play important roles in brain functions, they are so outnumbered by proteins which are specific to the brain that it seems reasonable to assume that the shared proteins are important biochemically but that the informational nature of brain function results from the specific proteins.

In addition, brain offers several advantages for studying tissue specific gene expression. Estimates of brain mRNA complexity based on

solution hybridization are higher than those from other tissues: 0.5-1.5 x 10^5 distinct mRNAs are thought to be expressed in the rodent brain, over 50% of which are thought to be expressed only in brain (1-3). Furthermore, since the messenger RNA expressed by the brain encodes the proteins expressed there, by studying brain mRNA we should be able to learn about both brain specific RNA and brain-specific protein expression. The study of 10^5 mRNAs or proteins is indeed a formidable task; however, it seems probable that certain molecular themes have been frequently repeated such that by understanding how a reasonably small fraction of these molecules function, one might construct accurate generalizations.

We have begun a study of rat brain mRNA by generating a cDNA library from brain cytoplasmic poly $(A)^+$ RNA. Some evidence indicates that a substantial portion (perhaps 50%) of adult rodent brain mRNA complexity may be accounted for by poly $(A)^-$ RNA species (34), so the present study may not represent the entire content of brain mRNA. The rat is a suitable animal in which to study the brain because it is extensively used in behavioral, neurophysiological, biochemical, and neuroanatomical studies. We have characterized the steady state abundance, size and tissue distribution of mRNAs corresponding to each of many individual clones taken at random from the collection in order to get an overall description of brain gene expression. We have examined 172 brain mRNA molecules, representing by mass some 30% of the total poly $(A)^+$ RNA population of rat brain and including all or most of the very abundant RNAs. These studies thus provide a detailed description of the pattern of RNA expression in a highly differentiated tissue and establish a set of clones corresponding to brain specific mRNAs which are a starting point for investigating those proteins whose expression determines brain function.

MATERIALS AND METHODS

RNA Preparation

Cytoplasmic RNA was isolated from fresh brains, livers or kidneys or adult male Sprague-Dawley rats (Charles River) by phenol/chloroform/isoamyl alcohol (50:50:1) extraction (4) and enriched for poly$(A)^+$ RNA by passage over oligo(dT) cellulose (5).

cDNA Cloning

cDNA clones were prepared from brain cytoplasmic poly$(A)^+$ RNA by modification of the methods of Wickens *et al.* (6) and Gough *et al.* (7). Reverse transcription was carried out for 60 min at 42°C in a volume of 200 µl

under the following conditions: 50mM Tris-HCl (pH 8.3), 10mM $MgCl_2$, 30mM 2-mercaptoethanol, 70mM KCl, 1mM each dATP, dCTP, dGTP, TTP, 250 μCi/ml ^{32}p dCTP (800 Ci/mmol, NEN), 25 μg/ml oligo dT, 100 μg/ml poly(A)$^+$ RNA, and 1000 units/ml reverse transcriptase (from Dr. J.W. Beard, Life Sciences, Inc.). Two variations of this technique were used: for preparation 1, the RNA was pretreated with 2.5 mM CH_3HgOH for 5 min at room temperature and actinomycin D (30 μg/ml) was added to the reaction; for preparation 2, the RNA was not pretreated and sodium pyrophosphate (4mM) was added to the reaction. After reverse transcription, preparation 1 was extracted with phenol/chloroform then ether, and both preparations were precipitated several times with 0.3M sodium acetate and ethanol, dried and resuspended in water. The samples were heated at 100°C for 3 min and chilled rapidly on ice to dissociate mRNA-cDNA hybrids.

Second strand synthesis was carried out for 3 hrs at 15°C in a reaction volume of 200 μl containing 50 mM HEPES, pH 7.2, 70 mM KCl, 10mM $MgCl_2$, 10mM dithiothreitol, 0.5mM each dATP,dCTP,dGTP, TTP and 200 units DNA polymerase I, Klenow fragment (BRL). The reaction was stopped by the addition of EDTA to 10mM and the dscDNA was ethanol precipitated. S_1 nuclease digestion was carried out for 30 min at 37°C in a volume of 200 μl containing 50mM sodium acetate (pH 4.5), 0.3M NaCl, 1 mM $ZnSO_4$, 100 units S_1 nuclease (PL Biochemicals). After S_1 nuclease digestion, the dscDNA preparations were extracted with phenol/chloroform, ether and precipitated with 0.3M sodium acetate and ethanol. At this stage the dscDNA preparations were enriched for larger material on Biogel A-150m (BioRad) in 0.15M NaCl and 2mM EDTA, pH 8.0 (7): preparation 1 was divided into fractions 1A (approximately 1000-1800 bp) and 1B (500-1000 bp); one fraction, 2A (> 500 bp) was derived from preparation 2. Oligo dC tails (8-15 nucleotides) were added to each dscDNA preparation with terminal deoxynucleotide transferase (PL Biochemicals) 1,000 units/ml, in 25 μM dGTP, 100mM potassium cacodylate (pH 6.9), 2mM $CoCl_2$, 1mM EDTA for 5 min at 37°C (8). The tailed dscDNA preparations were extracted with phenol/chloroform, ether and precipitated with ethanol. Aliquots of each preparation (10 μg/ml) were annealed with oligo dG tailed (8-15 nucleotide tails, prepared as described for the insert) Pst I-cleaved pBR322 (10 μg/ml) in 100mM NaCl, 10mM Tris-HCl, pH 8, 0.5mM EDTA: the mixtures were heated at 67°C for 10 min, 50°C for 30 min and allowed to cool to room temperature overnight.

E. Coli C600 cells were transformed with the recombinant plasmids and transformants selected on tetracycline (10 μg/ml) plates. Individual

colonies were transferred to duplicate ampicillin (33 μg/ml) and tetracycline plates. $Amp^{s}Tet^{r}$ colonies were selected for further study. The colonies were numbered and identified as to the origin of the dscDNA insert used in each case, i.e. from dscDNA insert preparations 1A, 1B or 2A. Clones identified as "pO" originate from a pilot cloning experiment using the method of Wickens et al. (6).

$Amp^{s}tet^{r}$ clones were grown as 1 ml overnight cultures in 2YT medium and plasmid DNA was extracted by the method of Birnboim and Doly (9). One quarter of this material was digested with Pst I (N.E. Biolabs) and fractionated on a 1% agarose gel (50mM Tris-borate, pH 8.3, 1mM EDTA) in parallel with DNA size standards. Clones giving excisable insert bands of 500 bp or larger were selected for further study.

Northern Blot Analysis

$Poly(A)^{+}$ samples (usually 2 μg) were fractionated by electrophoresis on 1.5% agarose gels in the presence of 1M formaldehyde (10) and transferred to nitrocellulose (11). The blots were prehybridized overnight at 42°C in 50% formamide, 0.75M NaCl, 25mM PIPES (pH 6.8), 0.2% SDS, 25mM EDTA, 100 μg/ml salmon sperm DNA, 100 μg/ml yeast RNA and 5X Denhardt's solution (12) and hybridized with ^{32}P labeled probes overnight at 42°C in the same solution but with 1X Denhardt's solution. One quarter or one-eighth of each crude plasmid extract or (for followup screening) 100 ng of purified super-coiled plasmid were labeled with ^{32}P by nick translation (13) to specific activities of 2-4 x 10^{8} cpm/ μg. Blots were washed in two changes of 2X SSC, 0.2% SDS for 60 min each at 42°C and once in 0.1X SSC, 0.2% SDS for 15 min at 67°C and exposed to Kodak XRP-5 or XAR-1 X-ray film at -50°C using Cronex Lightening Plus intensification screens for 1 or 14 days. Size estimates were based on comparisons with pBR322 standards (14).

Southern Blot Analysis

DNA was extracted from rat liver nuclei after treatment with proteinase K and SDS, digested with Hind III or Eco RI restriction endonucleases, and fractionated on 0.6% agarose gels. The DNA was transferred to nitrocellulose (15) and hybridized with ^{32}P labeled probes as described for Northern blots.

mRNA Abundance Measurements

Recombinant plasmid DNA from 11 clones was prepared from chloramphenicol-treated bacterial cultures by the method of Tanaka and Weisblum (16) and 10 μg of purified DNA from each clone was denatured and immobilized on duplicate nitrocellulose filters (17). Brain cytoplasmic $poly(A)^{+}$ RNA (4 μg) was broken with alkali by incubation at 90°C for 15 min

in 50 µl 10mM Tris, pH 9.5, 1mM spermidine, 0.1mM EDTA and end-labeled by incubation at 37°C for 30 min with 6 ul 0.5M Tris (pH 9.5), 0.1M $MgCl_2$, 50mM DTT, 50% Glycerol containing 0.5 mCi γ-^{32}P-ATP (2000 Ci/mmol) and T4 polynucleotide kinase (4 units) to give a product RNA population of approximately 100 nucleotides. The ^{32}P-RNA was hybridized to the plasmid DNA, the filters washed and treated with pancreatic RNase and proteinase K under the same conditions used by Derman et al. (33). The filters were counted, and the percent of steady state poly(A)$^+$ RNA hybridizing to each clone was calculated using the following formula:

$$\frac{\text{number average mRNA size}}{\text{cDNA insert size}} \times \frac{\text{(hybrid-background) cpm}}{\text{total cpm}} \times 1.5 \times 100.$$

The number average size (1760 nucleotides) was used to obtain a number percent rather than a weight percent. The factor 1.5 was used because our poly (A)$^+$ RNA preparation was judged to be contaminated by about 30% ribosomal RNA. The calculated abundance was rounded off into one of our abundance classes; these are marked in Tables 1 and 3. The abundance of the other clones was estimated by comparison with these 11 standards, using probe specific activity, autoradiograph exposure times and band intensity.

RESULTS

Generation of a Random Collection of Brain cDNA Clones

Cytoplasmic poly(A)$^+$ RNA was prepared from the brains of 20 adult male Sprague-Dawley rats and converted into double-stranded cDNA (dscDNA) using standard procedures (see Methods). dscDNA molecules larger than 500 base pairs (bp) were tailed with oligo d(C) and annealed to dG-tailed, Pst I endonuclease cleaved pBR322, and these recombinant plasmids were used to transform E. coli. Tetracycline resistant, ampicillin sensitive bacterial colonies were judged likely to contain plasmids with cDNA inserts at the Pst I site since such inserts should interrupt and inactivate the pBR322 ampicillin resistance gene. In this study, 1629 tet^{r}amp^s colonies from three separate dscDNA preparations were selected for further screening.

Ideally, the cloning procedure generates two Pst I sites within the recombinant plasmid, one at each end of the cDNA insert. Thus Pst I digestion of the recombinant plasmid should produce a fragment corresponding to linear pBR322 and DNA fragments corresponding to the cDNA insert. We prepared plasmid DNA from 1 ml overnight cultures of each of the tet^{r}amp^s colonies (9) and digested a portion with Pst I. The products of the digestion were sized by electrophoresis on agarose gels. Many of the digests

contained a fragment which comigrated with linear pBR322 and one or more smaller fragments with mobilities between 300 and 2000 bp; other digests contained no excisable fragments, but in many cases these digests contained a single fragment with a gel mobility slower than pBR322, suggesting that one Pst I site was not properly generated during the cloning procedure. These and other clones exhibiting anomalous digestion patterns were not pursued. Using this procedure, we selected for further study 191 individual clones with cDNA inserts larger than 500 bp.

We imposed only three selection constraints on the collection: 1) the size of the dcsDNA molecules we tailed and cloned, 2) the size class of the cDNA insert (as judged by Pst I digestion of the plasmid) which we chose to study, and 3) the generation of Pst I sites. It is possible that some poly(A)$^+$ RNA molecules are converted to cDNA and successfully cloned in the bacteria more or less well than the average case. Other studies (18) have demonstrated, however, that at least the reverse transcription reaction generates a cDNA population which is a faithful representation of the original brain mRNA population. As cytoplasmic poly(A)$^+$RNA and polysomal mRNA are thought to be essentially equivalent sets (19), in general we believe our collection to be a small random sample of brain mRNA molecules larger than 500 nucleotides in length.

Tissue Distribution of mRNA Molecules Detected by Brain cDNA Clones

Plasmid DNA from each of the 191 clones having cDNA inserts larger than 500 bp was individually labeled with ^{32}P by nick translation (13). Each labeled plasmid was used to probe a "Northern blot" in which rat brain, liver and kidney poly (A)$^+$RNA had been separated by electrophoresis under denaturing conditions in three adjacent lanes of an agarose gel and transferred to nitrocellulose (11). In general, the cDNA clones hybridize only to one or a few discrete mRNA species. These hybridization patterns were used to estimate the size (by comparison to known standards), abundance (from the intensity of the autoradiographic signal and probe specific activity), and tissue distribution of the mRNA molecules corresponding to each clone. The abundance of the mRNA corresponding to several of the clones was independently assessed by hybridizing alkali broken, ^{32}P-end-labeled poly(A)$^+$ brain RNA to excess purified plasmid DNA. The clones have been classified by the tissue distribution patterns of the mRNAs they detected which fell into one of the five classes presented in Tables 1-4, summarized in Table 5, and discussed in turn below.

Class I clones (29 of the 191 clones) hybridized equally well to brain,

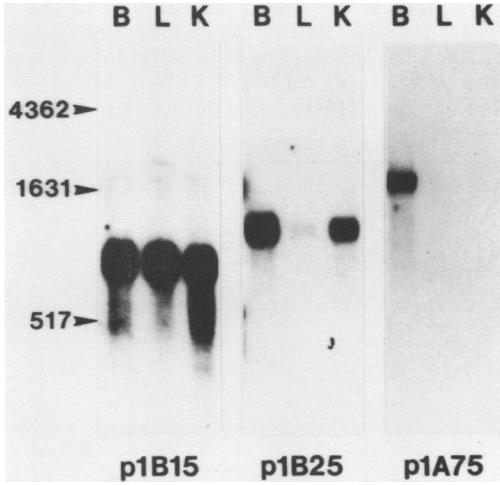

Figure 1: Typical Northern blot hybridization patterns of Class I, II and III clones to brain (B), liver (L), or kidney (K) cytoplasmic poly(A)$^+$ RNA. p1B15, a typical Class I clone; p1B25, a Class II clone and p1A75, a Class III clone. The positions and lengths (nucleotides) of DNA size standards, Pst I and Hinf I digests of pBR322 (14) are indicated at the left.

liver and kidney RNA; an example (p1B15) is shown in Figure 1. These clones were ranked according to the abundance of the corresponding mRNA (Table 1; clones hybridizing to more than one mRNA appear more than once in the list). These cDNA clones may represent the mRNAs of "housekeeping" proteins which are required in essentially equal amounts in many rat cell types, some of which could be encoded by the mitochondrial genome. One Class I clone, p1B15, has subsequently been used as a hybridization probe with many other tissues (adrenal, spleen, testes, heart, muscle, thymus, pituitary and intestine and various rodent cells in culture), and its corresponding mRNA is found in approximately the same concentration in each of these cell types (data not shown). Such clones are useful as controls for normalizing mRNA concentration and for detecting occasional RNA preparations which have suffered degradation.

Class II clones (41 of 191) hybridize to RNAs that are differentially represented in the RNA of brain, liver and kidney; an example (p1B25) is shown in Figure 1. These clones have been ranked by the estimated abundance of their mRNA in brain (Table 2) and the relative mRNA concentrations in liver and kidney are indicated. A few clones assigned to this class could in fact belong to Class I: they hybridize to large mRNA molecules which could

Table 1: The Class I clones which hybridize equally to the mRNA of brain, liver and kidney are identified by their laboratory codes and ranked first by estimated abundance, then by increasing mRNA size in nucleotides. Clones which hybridize to multiple mRNAs are marked M, those which detect precursor RNA species are marked P. Abundances determined by filter hybridization are indicated (*).

CLASS I

Clone	Abundance (%)	Size	Comments
1B211	3.0	600	P
1B447	2.0	1300	P
1B364	1.0	800	
1B15	1.0	1000	
1B258	1.0	1300	M,P
1B258	1.0	3000	M,P
2A559	0.5	600	
1B359	0.5	1500	P
1B372	0.5	2400	
1B203	0.2	400	
2A154	0.2	700	
2A111	0.1	1200	
1B268	0.1	1400	
1B334	0.1	1400	
1B374	0.1	1500	M
1A124	0.1	2000	
1A71	0.1	3200	
1B365	0.05	650	
1B368	0.05	1000	
1B435	0.05	1200	M
2A361	0.05	1500	M
1B446	0.05*	2000	M
1B446	0.05*	4000	M
1B374	0.02	1000	M
1B243a	0.02	2000	
1B871	0.02	2200	
0-35	0.02*	2500	
1B905	0.02	3000	
1B7	0.01*	1600	
2A344	0.01	2500	
2A361	0.01	2500	M
1B338	0.01	2800	
0-25	0.01	4000	

have been preferentially lost from one of our test tissue RNA preparations due to slight RNA degradation. However, most members of Class II clearly correspond to mRNA present in different amounts in the three tissues. They must, therefore, represent mRNAs which are required in higher levels in the cells of certain tissues. One such Class II clone (p2A290), for example, hybridizes to an mRNA with a tissue distribution pattern and relative

Table 2: The Class II clones which hybridize differentially to the mRNA of brain, liver and kidney are listed as described in Table 1. In addition, the amount of hybridization in liver (L) and kidney (K) relative to brain is indicated. (a) possible β-tubulin clone, (b) mRNA has length polymorphism, (c) also hybridizes to an unregulated RNA of about 150 nucleotides, (ND) no hybridization detected (possibly sometimes due to degradation).

Class II

Clone	Abundance%	Size	Comments	L	K
0-31	1.0	1400	P	.2	1
1B327	1.0	1400	P	.05	.1
1B868	1.0	1400	P	.2	.2
1B330	1.0	2500	M,P	.05	.05
2A558	0.5	800		3	3
1B350	0.5	1600		.05	.2
1B267	0.5	1700		4	.2
2A290	0.5	1900	P,a	.1	.05
1B360	0.5	2200	P	.1	.3
0-44	0.2	600	b	2	2
2A384	0.2	700		0	.05
1B366	0.2	1700		.1	5
1B328	0.2	2000		.02	.05
1B330	0.2	4000	M,P	.05	.05
2A278	0.1	1200	M	5	1
2A261	0.1	1300	M	.2	ND
1B25	0.1	1300		.01	.2
2A346	0.1	1600		.2	1
2A548	0.1	1600		.2	.2
0-38	0.1	1700	M	.5	.1
1A188	0.1	2000		2	.2
1B354	0.1	2000		.1	.5
0-26	0.1	2500		.05	.2
0-38	0.1	3000	M	.5	ND
1A76	0.1	3200		.2	.05
2A372	0.1	6000		.2	.5
0-29	0.05	1500	M	.5	.1
1B209	0.05	2000		2	1
1B376	0.05	2000	M	5	1
2A388	0.05	2500	M	.1	.1
2A261	0.05	2500	M	.5	ND
1B212	0.05	2500		.2	.2
0-42	0.05	2500	M	2	ND
0-42	0.05	3000	M	.5	ND
1B347	0.05	3000	M	2	.5
1B227	0.05	3500		5	ND
0-42	0.05	3500	M	.5	ND
0-42	0.05	4000	M	.5	ND
1B347	0.05	4000	M	2	.5
1B426a	0.05	4000		1	.2
2A345	0.05	5000	M	.05	.1
2A550	0.02	1400		ND	.1
2A366	0.02	3500		.1	.1
2A383	0.01	1200		20	1
2A388	0.01	1200	M	.5	.5
1A74	0.01	1700	c	.2	.2
1B867	0.01	2500		100	50
1B20	0.01	3000		.1	.1
1B260	0.01	4000		2	ND

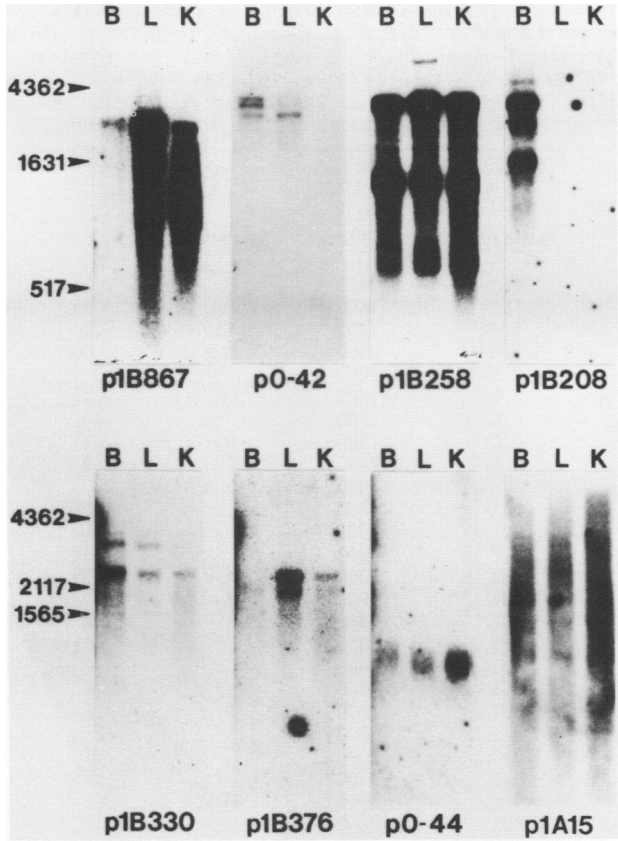

Figure 2: Northern blot patterns with brain (B), Liver (L) and kidney (K) poly(A)$^+$ RNA for various brain cDNA clones of interest. The positions and length (nucleotides) of DNA size standards (Pst I and Hinf I or Rsa I digests of pBR322) are shown at the left. In some cases the films were overexposed to bring out less intense details.

abundances similar to that reported for chicken tubulin mRNA (20), an mRNA which is expressed at a higher level in chicken brain than in other tissues. The differential tissue expression of the mRNAs which hybridize to some of these clones is not trivial. For 31 of the 49 Class II mRNAs, hybridization of the clone to brain RNA is significantly higher than to the other two tissues; in 11 of these cases (p1B327, p1B330 (2 species), p2A290, p2A384, p1B328, p2A388, p2A345, p2A550, p2A366, p1B20), the difference is tenfold or more. Other mRNAs are either up or down in liver or kidney, occasionally in the extreme as for the mRNA hybridizing to clone p1B867 which is one hundredfold less abundant in brain than in liver or kidney (Fig. 2). Class I

Table 3: The Class III clones which hybridize only to brain mRNA are listed as described in Table 1. (ID) the five clones which hybridize to the same small target (22), (a) ID clone which also hybridizes to a brain specific mRNA. Abundances determined by filter hybridization are indicated (*).

Class III

Clone	Abundance%	Size	Comments
0-27			ID
1B224			ID
2A120	2.0*	160	ID
1B308			ID
1B337			ID
1B208	2.0	3200	M,P
1B208	1.0	1600	M,P
1B431	1.0	2000	
1B373	0.5	2400	
1B426b	0.2*	1400	
2A278	0.1	1500	M
1B207	0.1	1600	P
1A75	0.1*	1700	
1B213	0.1	2000	
1B238	0.1	2500	
1B335	0.1	3200	
1B424	0.1	3500	
1A216	0.05	1700	P
0-29	0.05	2000	M
1A211	0.05	2500	
2A563	0.05	2500	
2A355	0.05	2500	M
0-17	0.05	3000	
1B319	0.05	3000	
1B361	0.05	3000	M
2A120	0.05	3200	ID,a
1B361	0.05	3300	M
1A168	0.05	4000	P
0-40	0.05	4000	
1A273	0.05	4500	
1A186	0.05	4500	
2A218	0.05	4500	
1B356	0.05	4500	
1B346	0.05	5000	
1B205	0.05*	5500	
1B243b	0.02*	3000	
0-30	0.02	3500	
2A543	0.02	5000	
1B236	0.01*	1700	
1B261	0.01*	2500	
1B369	0.01	3200	
1B435	0.01	3500	M
1B304	0.01	4000	
2A355	0.01*	4000	M
1B401	0.01	4000	
2A302	0.01	4000	
1B353	0.01	5500	
1B352	0.01	6000	
1B315	0.01	6000	
2A347	0.01	6000	
2A393	0.01	6000	
2A345	0.01	8000	M
2A345	0.01	10000	M

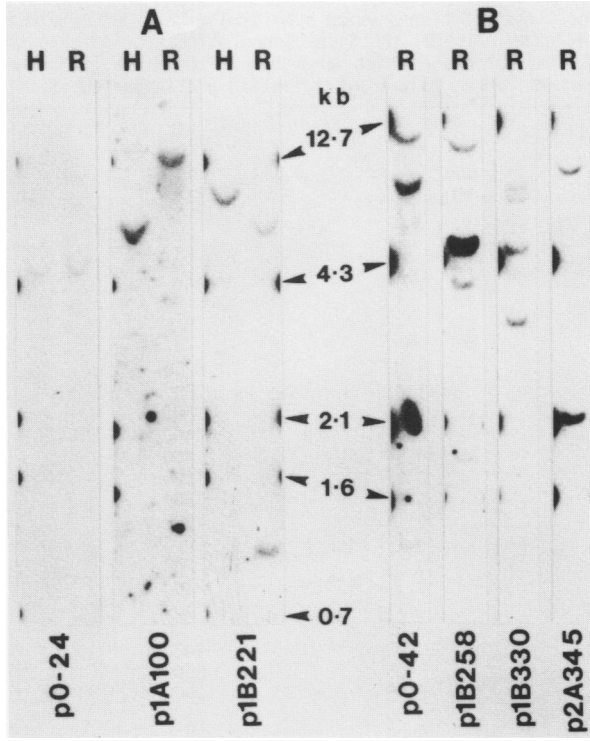

Figure 3: Southern blot hybridization analysis of rat brain cDNA clones to rat liver DNA digested with Hind III (H) or Eco RI (R) restriction enzymes. (A) Detection of genomic sequences by plasmids showing no detectable hybridization with brain mRNA in Northern blots. (B) Detection of multiple genomic sequences by plasmids hybridizing with multiple RNA species in Northern blots. The positions of DNA size standards (Pst I digests of plasmid pMLV-1 (32)) and pBR322 and a Rsa I digest of pBR322) are shown.

and Class II clones together number 70 clones that correspond to 82 mRNAs which are not restricted to expression in the tissue from which the cDNA clones were generated, brain.

Class III clones (43 of 191) hybridize to RNA species detected in brain but not liver or kidney, for example plA75 (Fig. 1; Table 3). These clones correspond to 48 mRNA species which presumably represent the mRNAs for proteins expressed in the neurons and/or glia of brain, but not required in liver or kidney. Furthermore, some of these Class III mRNAs produce proteins found only in a subset of brain cells, suggesting that the mRNA is expressed only in that subset (21). Additionally, five Class III clones hybridize to a 160 nucleotide brain specific, cytoplasmic poly(A)$^+$ RNA target, even though the cDNA insert in each clone is 500-1250 nucleotides long. These clones

Table 4: Class IV and V clones, which do not hybridize to any mRNA in a Northern blot experiment, are classified by their Southern blot pattern, actual or estimated (est.).

	Class IV	Class V
Southern positive	19	--
Southern negative	--	12
Untested--36 clones	22(est)	14(est)
Peculiar		11
Total	41	37

represent precursors of brain specific mRNAs which are a small (2-3%) part of our original poly(A)$^+$ cytoplasmic RNA preparation but contain a repetitive sequence element (ID sequence) which is also found in the small brain specific RNA molecules (22).

Sixty-seven of the 191 clones failed to detect an RNA target in brain, liver or kidney. These clones could either represent plasmids which do not contain cDNA copies of rat brain mRNA and are somehow artifactually present in our collection, or they could represent plasmids containing cDNA copies of relatively rare mRNAs. To distinguish between these possibilities, we tested for the presence of rat genetic information in the plasmids by using nick translated plasmid to probe Southern blots (15) of rat DNA. Of 31 clones selected at random from the 67 Northern negative clones, 19 (61%) hybridized to restriction fragments in Southern blots of rat liver DNA (examples in Fig. 3) suggesting that these 19 clones did correspond to rare mRNAs transcribed from rat genes. We therefore assume that 61%, or 22, of the remaining 36 untested clones would also fall into the same category, giving a total of 41 clones corresponding to rare brain mRNAs; we have called these Class IV clones (Table 4). Nucleotide sequencing of the cDNA insert of one such clone demonstrated that it contained a sequence ending with a poly(A) tail which is preceded by a poly(A) addition signal (AATAAA) 19 bases upstream (data not shown) suggesting that the clone was derived from a bona fide mRNA.

The remaining 26 Northern-negative clones are presumably artifacts of cloning, termed by us Class V (Table 4); this class also includes 11 clones which showed peculiar hybridization patterns which could not be classified into Classes I-IV. Usually these latter clones gave a smeared hybridization pattern to brain, liver and kidney RNAs, with no discrete bands. An example,

plA15, is shown in Figure 2. Nucleotide sequence analysis of clones plA15 has revealed that the insert at the pBR322 Pst I site consisted of a poly(A) tract of approximately 100 nucleotides with oligo dG-dC tails. An additional insert of the bacterial insertion sequence IS-1 which contains a Pst I site, was located in the ampicillin resistance gene and accounts for the increase in plasmid size and an apparent cDNA insert of greater than 500 bp as revealed by Pst I digestion. Presumably the smeared hybridization pattern is due to hybridization of the poly(T) sequence in the plasmid to the poly(A) tails present on the mRNAs of all size classes in all three tissue RNA preparations.

In sum, we have examined a total of 191 clones of which 154 correspond to 171 mRNAs (including the 41 presumed Class IV mRNAs). Together, these clones account for greater than 28% of the total cytoplasmic poly(A)$^+$ RNA (by mass) of the rat brain. An additional 2% of the poly(A)$^+$ RNA is accounted for by the brain specific 160 nucleotide RNA. The fraction of clones falling into each class is indicated in Table 5. The largest fraction is that of the brain specific Class III clones (30%). If one assumes that all or most of the Class IV clones also correspond to rare brain specific mRNAs (see discussion) then more than half of these clones, represented in our random sampling of brain mRNA, may correspond to mRNAs uniquely expressed in the brain.

Brain Specific mRNA is Considerably Larger than Non-Specific mRNA

The arithmetic and number average sizes of the mRNAs listed in Tables 1-3 were computed for each class and for overall brain mRNA (Table 5). The arithmetic average represents the average length of the mRNAs accessed by our probes. The number average reflects the average length of the population of mRNAs represented by our clones; it is weighted by the abundances of each particular mRNA and is the appropriate figure to compare with measurements of the average length of heterogeneous mixtures of RNA molecules such as those used in solution hybridization determinations of mRNA complexity. The overall number average length of the brain mRNAs detected by our clones (Classes I-III) is 1790 nucleotides, a figure compatible with the measurements of previous authors (1,2,23) which ranged from 1400-1900 nucleotides. The arithmetic average length of the set of Class I-III clones is considerably larger, 2690 nucleotides. This indicates that the most prevalent mRNAs in the brain tend to be smaller than the rarer mRNAs. The same conclusion holds when the two averages are computed for the mRNAs of each individual class, namely, the more prevalent mRNAs tend to be shorter

Table 5: The clones are summarized by Class, the percent of those actually classified as cDNA inserts (I-IV) are calculated, the number of mRNAs which hybridize to the clones and the sum of the abundances are indicated. The average size was computed arithmetically: the number average is the sum of the product of the size and abundance divided by the sum of the abundance. (a) These figures include the ID sequence, (b) these figures include the assumed but undetected Class IV mRNAs (c), this figure takes into account the four clones which fall into more than one class.

Summary of Clones by Classes

Class	Clones	mRNA	%total mRNA	size avg.	number avg.
I	29(18%)	33	11.95	1780	1250
II	41(26%)	49	9.55	2350	1870
III	47(30%)	48 (49)[a]	6.51 (8.51)[a]	3660	2640
IV	41(26%)	(41)[b]	--	--	--
V	37	--	--	--	--
TOTAL	191[c]	130 (171)[b]	28.01 (30.01)[a]	2690	1790

than the rarer mRNAs. Surprisingly, Class III mRNAs are, on average, more than twice as large as those expressed equally in other tissues.

Sequence Related mRNAs may be Coordinately Controlled

Eighteen of the 191 clones in this study hybridize with approximately comparable intensity to two or more mRNA species of different sizes (Table 6), including clones p2A345 and p0-42 which hybridize to 3 and 4 targets respectively; several examples (p0-42, p1B208, p1B330) appear in Figure 2. Such hybridization patterns are distinct from those observed with clones that, in addition to a major target mRNA species, also detected larger RNA species with much fainter relative hybridization intensities. We interpret the larger species in these latter patterns as precursor RNAs which are present as slight contaminants of our cytoplasmic RNA preparations (examples p1B258, p1B208, p1B330, p1B376 in Fig. 2). Clones with visible presumptive precursors tend to correspond to relatively abundant mRNAs (i.e., derived from precursors of sufficient abundance to be detected on long autoradiographic exposures).

The clones in Table 6 which hybridize to multiple targets are notable

Table 6: Clones which hybridize to more than one mRNA are presented, with the Class assignment, size, abundance, and, where appropriate, relative tissue abundance of each mRNA. The number of bands detected by Southern blotting are indicated for those clones which have been tested. (a) the amount in liver is 0.5%, (ND) no hybridization detected. Clones whose target mRNAs appear to be coordinately expressed are indicated (*).

Multiples

Clone	Classes	Sizes	Abundance in Brain	Class II Brain:Liver:Kidney	Southern Bands
1B258*	1	3000	1.0		6
	1	1300	1.0		
1B374*	1	1500	0.1		-
	1	1000	0.02		
1B435	3	3500	0.01		-
	1	1200	0.05		
2A361*	1	2500	0.01		2
	1	1500	0.05		
1B446*	1	4000	0.05		-
	1	2000	0.05		
1B330*	2	4000	1.0	1.0:0.05:0.05	5
	2	2500	0.2	1.0:0.05:0.05	
2A278	3	1500	0.1		-
	2	1200	0.1	1:5:1	
2A261	2	2500	0.05	1:0.5:ND	2
	2	1300	0.1	1:0.2:ND	
0-38 *	2	3000	0.1	1:0.5:ND	2
	2	1700	0.1	1:0.5:0.1	
0-29	3	2000	0.05		2
	2	1500	0.05	1:0.5:0.1	
1B376	2	2200	ND	0:10:1[a]	-
	2	2000	0.05	1:5:1	
2A388	2	2500	0.05	1:0.1:0.1	2
	2	1200	0.01	1:0.5:0.5	
0-42	2	4000	0.05	1:0.5:ND	6
	2	3500	0.05	1:0.5:ND	
	2	3000	0.05	1:0.5:ND	
	2	2500	0.05	1:2:ND	
1B347*	2	4000	0.05	1:2:0.5	2
	2	3000	0.05	1:2:0.5	
2A345	3	10,000	0.01		2
	3	8000	0.01		
	2	5000	0.05	1:0.05:0.1	
1B208*	3	3200	2.0		2
	3	1600	1.0		
2A355*	3	4000	0.01		1
	3	2500	0.05		
1B361*	3	3300	0.05		-
	3	3000	0.05		
1A87	4	--	--		9

for two reasons. First, they represent 10% of our total collection of clones and hybridize to 30% of the RNA molecules we have observed, including mRNAs which fall into all abundance ranges and tissue distribution patterns. Second, the mRNAs which hybridize to 10 of the 18 clones show absolute coordinate expression in the three tissues, while those which hybridize to another 4 clones are somewhat coordinately expressed. The multiple representation of the cloned sequences in different discrete target RNA species could result either from the transcription of several distinct genes containing related sequences, or from alternative modes of expression of a single gene producing two or more distinct RNA molecules. To investigate these different formal possibilities, we used Southern blots to determine the number of genes corresponding to twelve of the clones in Table 6. Ten of the twelve clones hybridized to two or more restriction fragments of the rat genome when the DNA was cleaved with Eco RI or Hind III (examples with Eco RI shown in Fig. 3). Three of these hybridize to 5 or 6 bands; therefore, the multiple RNAs hybridizing to these clones probably come from different genes. One Class IV clone, p1A87, hybridized to 9 genomic fragments in a Southern blot, suggesting that it may represent the mRNA from a multigene family of rare brain mRNAs. Eight clones hybridize to only two Southern fragments; these may represent two genes. Alternatively, these could be two fragments from a gene which contains a restriction cleavage site, either in coding or intron sequence. One clone, p2A355, hybridized to two Class III mRNAs but to only one genomic restriction fragment, when either Hind III or Eco RI was used, suggesting that the two mRNAs are encoded by a single gene or two very closely linked tandem genes. The two RNAs which hybridize to this clone (and perhaps some which correspond to clones that hybridize to two Southern fragments) could, therefore, be expressed by alternative initiation or termination signals for transcription (or polyadenylation), or by alternative choices for splicing the mRNA precursors.

Two other clones display unique hybridization patterns. Clone p1B376 hybridizes to a Class II RNA of 2000 nucleotides which is expressed five fold more in liver than brain or kidney (Fig. 2). It also hybridizes to a 2200 nucleotide species which is even more prevalent in liver, but not at all detected in brain. Our clone must be a copy of the smaller species, as we started with brain cDNA. The larger mRNA shares sequence homology with the smaller RNA; the two protein products may be functionally similar. Clone pO-44 hybridizes to a 600 nucleotide brain RNA species. Although we have defined this as a Class II mRNA, the species to which it hybridizes in liver

and kidney is estimated to be about 50 nucleotides shorter than the mRNA detected in the brain (Fig. 2), possibly indicating a polymorphism in polyadenylation or tissue specific RNA processing.

DISCUSSION

In order to eventually understand the proteins expressed by brain, we have begun to investigate a population of brain mRNA molecules by examining random individual clones from a rat brain cDNA library. Of the first 191 clones examined, 47 hybridize to mRNAs expressed in brain but not liver or kidney and are considered brain specific (Class III) mRNAs and 70 hybridize to mRNAs whose expression is not limited to brain (Classes I and II). Forty-one of the non-brain specific clones hybridize to mRNAs which are, nonetheless, regulated in their expression in the three tissues. There is a redundancy of four clones which hybridize to a Class III mRNA and also to a Class I or II species. An additional 41 (estimated) clones (Class IV) contain copies of mRNAs present in brain below the level of detection of our experiments. The tissue distribution, abundance, and size of the mRNAs corresponding to these 191 clones provide data which can be analyzed from a number of interesting aspects.

The Class IV clones must represent very rare mRNAs (less than a few copies per cell). However, given that the cellular heterogeneity of brain is much greater than that of either liver or kidney, many of these clones may represent brain specific mRNAs which are rare when the brain is considered as a whole, but which may be relatively abundant in one, or a few, cell types. If so, can we estimate the number and average size of Class IV mRNAs in spite of not being able to detect the mRNA in the Northern analysis? Furthermore, how many mRNAs are expressed overall in the rat brain, and what portion are specific to brain? The key to making these calculations is the abundance estimate we have made for each mRNA, and these numbers are admittedly soft. The abundance of the mRNA corresponding to 11 clones was measured directly. Other abundances are based on comparing hybridization probe specific activities, autoradiographic exposure times and relative band intensities to the directly measured cases. The resulting estimated abundances were rounded off so that each mRNA was placed into one of several discrete abundance classes, hence our abundance estimates are at best accurate to a factor of two. Furthermore, whenever our estimate fell below 0.01% (which was the case in several instances), we rounded up to 0.01%. It is our impression that in general, we may have slightly overestimated the abundances of these mRNAs.

Table 7: The mRNAs detected by the clones are ranked by estimated abundance. The sum in all classes and in each class was used to calculate the total of that abundance class in brain by assuming that random sampling produced a representative division. For example, 26/171 mRNAs are present at the 0.01% level; therefore 15.2% of brain mRNA is present at 0.01% requiring 1520 mRNA species. The arithmetic average is equivalent to the number average when the data are presented in this fashion.

	Actual				Calculated				Size Average			
Abundance(%)	Total	I	II	III	Total	I	II	III	Total	I	II	III
3	1	1	0	0	1	1	0	0	600	600	--	--
2	2	1	0	1	2	1	0	1	2250	1300	--	3200
1	10	4	4	2	10	4	4	2	1640	1530	1680	1800
0.5	9	3	5	1	11	4	6	1	1680	1500	1640	2400
0.2	8	2	5	1	23	6	14	3	1440	550	1800	1400
0.1	25	6	12	7	146	35	70	41	2160	1780	2280	2290
0.05	39	6	15	18	456	70	175	211	3050	1725	3170	3510
0.02	10	5	2	3	290	145	58	87	2710	2140	2450	3800
0.01	26	5	6	15	1520	292	351	877	3900	2680	2270	4960
TOTALS	130	33	49	48	2459	558	678	1223				

With these strong caveats, and bearing in mind that when we fragment this collection into classes we may introduce errors due to small sample size, we present our calculations in Table 7. The mRNAs are grouped by abundance, and the numbers of total mRNAs in each abundance class are estimated from the frequency that they occur in our collection, assuming that the cloning procedure randomly samples the original brain mRNA population. Our calculations predict that there are about 2460 mRNAs present at 0.01% or greater abundance in the rat brain, of which 50% are specifically expressed in the brain. Together, these mRNAs have a complexity of about 8×10^6 nucleotides. Of greatest interest is the trend that the rarer the mRNA species, the more likely it is to be brain specific. Conversely, more than 80% of the mRNAs present at 0.2% or greater are shared with other tissues. These trends are not sensitive to the possible errors in our original abundance estimates, assuming we dealt evenly with all clones.

A striking finding of this study is that mature, brain specific (Class III) mRNA molecules are on average longer, almost by a factor of 2, than mature, non-specific mRNAs. This is evident as a trend in Table 7. Also rarer mRNAs in all classes tend to be larger than the more abundant species. A similar relationship between mRNA length and abundance was also found by

Meyuhas and Perry (24) in mouse L cell mRNA populations using solution hybridization techniques. In our study, the rarest detectable brain specific mRNAs have an average size of about 4960 nucleotides--three times the number average size of brain mRNA. These data can be used to place upper and lower limits on the number of mRNA species expressed in brain--that is, including the Class IV mRNA. On the low side, if the 41 Class IV clones represent mRNAs that just barely escaped detection by us, then an additional 26% of the mass of brain mRNA is present at an abundance of just less than 0.01%--about 2600 Class IV mRNAs giving a total brain mRNA number of 5060. Because of the manner in which we estimated abundance, this figure is probably an underestimate.

Alternatively, if the highest published mRNA complexity measurements (1) are taken at full value (1.4×10^8 nucleotides), and it is assumed that Class IV mRNAs include all brain mRNAs except the 2460 Class I, II and III mRNAs (8×10^6 nucleotides) estimated in Table 7, Class IV mRNAs must have a total complexity of about 1.3×10^8 nucleotides. Previous workers have used the number average length of the total mRNA population and the total complexity of the RNA population in computing the number of mRNA species made by a tissue. As discussed by Meyuhas and Perry (24), if more abundant mRNAs tend to be shorter than less abundant mRNAs, then the overall number average size of the population may be an underestimate and hence may affect any estimate of the total number of mRNA species. Our studies suggest that indeed the more abundant mRNAs are shorter and hence such estimates may be greatly distorted. From the observed trends in the computed data in Table 7, it is reasonable to assume that the average Class IV mRNA length is greater than the average length of the least abundant, yet detectable mRNAs (3900 nucleotides) and perhaps exceeds the Class III 0.01% abundance value (4960 nucleotides), since we might expect many Class IV species to be very rare, brain specific mRNAs. This allows us to calculate the number of Class IV mRNAs (dividing 1.3×10^8 by 4960 or 3900) to be between 26,000 and 33,000 species, the lower value best reflecting the trend of rarer mRNAs to be larger than more prevalent, common species. Therefore, somewhere between 5,000 and 30,000 mRNA are expressed in the rat brain. Between 50 and 90% of these are specific to the brain, depending on whether the Class IV clones are, as a group, mostly like rare, detectable brain mRNAs or are very rare (0.01%) brain specific mRNAs. These estimates could be sharpened experimentally by using the clones in this collection in quantitative hybridization studies. As upper and lower boundaries, however, our estimates

are relatively firm, not being very sensitive to the soft abundance determinations.

We do not yet know if the larger size of brain specific mRNA is a consequence of tissue specificity per se or some brain specific property. Why might tissue specific mRNAs be longer than non-specific mRNAs? The extra sequences could be localized in noncoding regions of specific mRNAs, providing signals which direct processes unique to that tissue. For example, the recently characterized mRNA for the β-neo-endorphin/dynorphin precursor (25), which would be classified as a brain specific RNA by our criteria contains a 1500 nucleotide 3' non-coding region with a coding region of less than 800 nucleotides. Possible functions of such non-coding regions might be translation at specific cellular sites or interactions with particular proteins. Extraordinarily long non-coding regions could be non-functional, present merely because these transcripts represent genes whose function has begun late in evolution. As recent acquisitions, these genes may not yet have become as streamlined as their older cousins. Alternatively, the protein coding regions of the tissue specific mRNAs may contain the extra nucleotides, implying that proteins specific to a tissue may be larger, on average, than non-specific proteins. Perhaps tissue specific protein systems are more often synthesized as polyproteins, facilitating the coordinated expression of various subunits of the system. Since many differentiated tissues are relatively recent evolutionary arrivals compared to the proteins common to every cell and responsible for basic nonspecific cellular functions, these new tissue specific systems may not have had the evolutionary time required for the coding regions of their different subunits to disperse through the genome and establish coordinately regulated synthesis. Brain may be the extreme case of this situation, since it has been subjected to an enormous expansion in function rather recently in evolution. Certainly, several neuropeptide precursors, for example, proopiomelanocortin (26), the vasopressin-neurophysin precursor (27) and the β-neo-endorphin/dynorphin precursor (25) are each processed to give several proteins or peptides with different functions and are the only eukaryotic examples of polyproteins other than those produced by certain eukaryotic viruses.

Another interesting finding is that about 10% of our clones hybridize to multiple mRNAs, most of which seem to be coordinately controlled, many of which are transcribed from different genes. At least one of our clones hybridizes to multiple Class III mRNAs which may be transcribed from the same

gene by differential use of different initiation, termination (polyadenylation) or splicing signals. Alternate exon expression from the same gene has been observed for the immunoglobulins (28,29) and calcitonin (30) genes. Thus, the clones which hybridize to multiple mRNAs identify two separate genetic phenomena--multigenic families and single genes with multiple modes of expression. Surprisingly, both phenomena seem to be relatively frequent strategies for gene (and protein) expression.

One aspect of the collection of cDNA clones which this study has not directly addressed is the possibility of separate but identical independent isolates. Since 191 randomly selected clones have been examined, we ought to expect multiple representation of some mRNAs present at the 1% level. Some pairs of clones exhibit similar hybridization patterns, detecting relatively abundant mRNAs of approximately the same size and tissue distribution. These putative pairs could be unequivocally matched or shown to be distinct by a series of somewhat laborious experiments were it desirable to do so. Most of the independent clones examined here represent mRNAs of abundance considerably less than 1% and hence most clones in the collection are probably not multiply represented. Therefore, this concern should not significantly affect the conclusions of this paper.

The vast majority of clones in our collection hybridize to mRNAs whose expression is controlled. This is a surprising result--at least 80% of the different mRNAs expressed in the brain are regulated either in an on or off fashion (Class III) or in a relative fashion (Class II) in the three tissues examined. Furthermore, many of the Class III RNAs may be expressed in only some cell types within the brain as shown by Sutcliffe *et al*. (21). The Class IV clones represent very rare mRNAs, most of which are probably expressed in only a few cell types. Differential gene expression is the rule rather than the exception. This provides a biochemical complexity--5,000 to 30,000 mRNA species, most of which are regulated--which may be sufficient to account for the diversity of brain structure and function. Indeed, the observation that various Class II mRNAs are differently differentially controlled implies that a single system acting at one level of gene expression does not account for its entire pattern. Many different mechanisms operating at different levels of mRNA generation, such as those recently reviewed by Darnell (31), must contribute to the overall mRNA distribution. Of particular interest are the mechanisms controlling the genes that represent the coordinately controlled mRNAs with homologous sequence.

Because of the efforts of previous workers (1) we anticipated that the brain would be a rich source of tissue specific mRNA. Indeed, we now have lists of almost 200 brain mRNAs, many of which are brain specific, and estimates of their size and abundance. Our finding that rare brain specific mRNA species are much larger than abundant species, means that previous estimates of the number of mRNAs expressed in brain were probably too high, and that no more than 30,000 mRNAs are probably expressed in brain. It is foreseeable that in the not too distant future, the existence of a few thousand brain mRNAs may be catalogued, a substantial sample of the total. Such a list together with the corresponding cloned cDNAs provides an excellent starting point for finding out the location and function of the brain specific protein products of these mRNAs (21).

ACKNOWLEDGEMENTS

We acknowledge the technical expertise of Judy Ogata, Elizabeth Olinger, and Mark Lane, and thank Drs. R.A. Lerner, F.E. Bloom, T.M. Shinnick, A.-P. Chen, M.C. Wilson and R.T. Ogata for discussions and criticism of the manuscript. This work was supported, in part, by grants to J.G.S. from the American Cancer Society (NP-359) and McNeil Laboratories. This is publication no. 2923-IMM from the Research Institute of Scripps Clinic.

REFERENCES

1. Bantle, J.A. and Hahn, W.E. (1976) Cell 8, 139-150.
2. Hastie, N.D. and Bishop, J.O. (1976) Cell 9, 761-774.
3. Chikaraishi, D.M. (1979) Biochem. 18, 3249-3256.
4. Schibler, K., Tosei, M., Pittet, A.-C., Fabiasi, L. and Wellames, P. (1980) J. Mol. Biol. 142, 93-116.
5. Aviv, H. and Leder, P. (1972) Proc. Natl. Acad. Sci. USA 69, 1408-1412.
6. Wickens, M.P., Buell, G.N. and Schimke, R.T. (1978) J. Biol. Chem. 253, 2483-2495.
7. Gough, N.M., Webb, E.A., Cory, S. and Adams, J.M. (1980) Biochem. 19, 2702-2710.
8. Roychoudhury, T., Jay, E. and Wu, R. (1976) Nucleic Acids Res. 3, 865-877.
9. Birnboim, H.C. and Doly, J. (1979) Nucleic Acids Res. 7, 1513.
10. Rave, N., Ckvanjakou, R. and Boedtker, H. (1979) Nucleic Acids Res. 6, 3559-3567.
11. Thomas, P.S. (1980) Proc. Natl. Acad. Sci. USA 77, 5201-5205.
12. Denhardt, D. (1966) Biochem. Biophys. Res. Comm. 23, 641-646.
13. Rigby, P.W.J., Dieckmann, M., Rhodes, C. and Berg, P. (1977) J. Mol. Biol. 113, 237-251.
14. Sutcliffe, J.G. (1978) Nucleic Acids Res. 5, 2721-2728.
15. Southern, E. (1975) J. Mol. Biol. 98, 503-517.
16. Tanaka, T. and Weisblum, B. (1975) J. Bacteriol. 121, 354-362.
17. Melli, M., Ginelli, E., Corneo, G. and diGernia, R. (1975) J. Mol. Biol. 93, 23-28.
18. Van Ness, J. and Hahn, W.E. (1980) Nucleic Acids Res. 8, 4259-4269.

19. Campo, M.S. and Bishop, J.O. (1974) J. Mol. Biol. 90, 649-664.
20. Cleveland, D.W., Lopata, M.A., MacDonald, R.J., Cowan, N.J., Rutter, W.J. and Kirschner, M.W. (1980) Cell 20, 95-105.
21. Sutcliffe, J.G., Milner, R.J., Shinnick, T.M. and Bloom, F.E. (1983) Cell, 33,671-682.
22. Sutcliffe, J.G., Milner, R.J., Bloom, F.E. and Lerner, R.A. (1982) Proc. Natl. Acad. Sci. USA 79, 4942-4946.
23. Gros, F., Croizat, B., Portier, M.-M., Berthelot, F. and Felsani, A. (1982) In: Molecular Genetic Neuroscience. F.O. Schmitt, S.J. Bird and F.E. Bloom, eds. Raven Press, New York, pp. 335-347.
24. Meyuhas, O. and Perry, R.P. (1979) Cell 16, 139-148.
25. Kakidani, H., Furutani, Y., Takahashi, H., Noda, M., Morimoto, Y., Hirose, T., Asai, M., Inayama, S., Nakanishi, S. and Numa, S. (1982) Nature 298, 245-249.
26. Nakanishi, S., Inoue, A., Kita, T, Nakamura, M., Chang, A.C.Y., Cohen, S.N. and Numa, S. (1979) Nature 278, 423-427.
27. Land, M., Schultz, G., Schmale, M. and Richter, D. (1982) Nature 298, 299-303.
28. Alt, F.W., Bothwell, A.L.M., Knapp, M., Siden, E., Mather, E., Koshland, M., and Baltimore, D. (1980) Cell 20, 293-301.
29. Early, P., Rogers, J., Davis, M., Calame, K., Bond, M., Wall, R. and Hood, L. (1980) Cell 20, 313-319.
30. Amara, S.G., Jonas, V., Rosenfeld, M.G., Ong, E.S. and Evans, R.M. (1982) Nature 298, 240-244.
31. Darnell, J.E. (1982) Nature 297. 365-371.
32. Shinnick, T.M., Lerner, R.A. and Sutcliffe, J.G. (1981) Nature 293, 543-548.
33. Derman, E., Krauter, K., Walling, L., Weinberger, C., Ray, M. and Darnell, J.E. (1981) Cell 23, 731-739.
34. Chaudhari, N., and Hahn, W.E. (1983) Science 220, 924-928.