Cloning of DNA Corresponding to Rare Transcripts of Rat Brain: Evidence of Transcriptional and Post-Transcriptional Control and of the Existence of Nonpolyadenylated Transcripts

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To examine the expression of genes encoding rare transcripts in the rat brain, we have characterized genomic DNA clones corresponding to this class. In brain cells, as in all cell types, rare transcripts constitute the majority of different sequences transcribed. Moreover, when compared with other tissues or cultured cells, brain tissue may be expected to have an even larger set of rare transcripts, some of which could be restricted to subpopulations of neural cells. We have identified seven clones whose transcripts are nonabundant, averaging less than three copies per cell. Clone rg13 (rat genomic 13) RNA was detected only in the brain, whereas RNA of a second clone, rg40, was also detected in the brain and in a melanoma. Transcripts of rg13 were found in cerebellum, cerebral cortex, and regions underlying the cortex, whereas rg40 transcripts were not detected in the cerebellum. Transcripts of both rg13 and rg40 were found in pelleted polysomal RNA. RNA of another clone, rg34, was found in the brain, liver, and kidney but was found in pelleted polysomal RNA only in the brain, suggesting that its expression may be post-transcriptionally controlled. The remaining four clones represent rare transcripts that are common to the brain, liver, and kidney; rg18 RNA is restricted to the nucleus, whereas rg3, rg26, and rg36 transcripts are found in the cytoplasm of all three tissues. Transcripts of the brain-specific clone, rg13, and the commonly expressed clone, rg3, are nonpolyadenylated, presumably belonging to the high-complexity, nonpolyadenylated class of transcripts in the mammalian brain.

The mammalian brain, the most complex organ in terms of function and structure, is also the most complex in terms of the diversity of its transcripts. The diversity or complexity of an RNA population can be measured by hybridizing RNA in excess to trace amounts of radioactive, nonrepetitive (unique-sequence) DNA, such that the amount of DNA driven into hybrids at saturation gives a direct measure of the percentage of nonrepetitive DNA transcribed. Complexity measurements have consistently shown that brain RNA in rodents displays a greater complexity than RNA from other tissues (mouse tissue [2, 6, 15, 16, 19, 26, 49]; rat tissue [8, 9, 13, 14, 17, 25, 28, 43, 47, 51]). For example, Chikarashi et al. (9) have found the nuclear RNA complexity of rat brain (31.2% of unique-sequence DNA coding capacity, assuming asymmetric transcription) to be higher than that of liver (21.8%) or kidney (10.6%).

A higher proportion of nuclear RNA complexity (nearly two-thirds) is conserved as cytoplasmic or polysomal RNA in the brain than in the liver or kidney (3, 8), although, presumably, not every brain cell expresses the entire repertoire of mRNAs found in whole brain. The polysomal mRNA complexity of brain is sufficient to account for 150,000 to 200,000 different mRNA molecules of 1,500 nucleotides each. Based on the kinetics of hybridization, these complex mRNAs are present at an average concentration of 0.1 copies of RNA or less per cell (2). Surprisingly, about one-half of these mRNAs are nonpolyadenylated [poly(A)⁻] in

rats and mice (8, 49). Chaudhari and Hahn (7) have reported that the high complexity, poly(A)⁻ class of mRNAs is restricted to the brain and appears after birth. That these poly(A)⁻ RNAs are genuine messenger molecules is supported by their presence on polysomes in a form that can be released by puromycin or EDTA and by their ability to be translated *in vitro* (8, 49); however, the translation assay is biased primarily toward the more abundant transcripts.

In brain, most of the nuclear and cytoplasmic transcripts (both poly(A)⁺ and poly(A)⁻) are present at an average of less than one copy per cell (2, 8, 9, 25). Although the functional significance of rare transcripts is unknown, the steady-state level of some housekeeping proteins could be maintained by rare transcripts. Galau et al. (12) have calculated that many metabolically important proteins could be translated from messenger molecules present at less than 10 copies per cell based on known protein and RNA turnover rates and protein steady-state concentrations. Alternatively, a transcript may be abundant in only a subset of cells, and so its rare occurrence might simply reflect the composite nature of cell types in a tissue such as brain. Evidence suggesting that only a subset of mRNAs is expressed in certain cell types was reported by Beckmann et al. (3), who observed that although cell lines of the central nervous system and brain sections (notably the cerebellum) had almost as high a nuclear RNA complexity as brain, the complexities of their cytoplasmic or polysomal RNA fractions were two- to threefold lower than those determined for brain as a whole. Therefore, a rare transcript may serve either a housekeeping function in every cell type or a unique function in a specialized cell.

Most methods (plaque, colony, northern, or dot hybridiza-

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tion) for screening DNA clones fail to detect those whose transcripts are present at less than one copy per cell. For example to be detected by a technique employing a cDNA intermediary prepared from the poly(A)⁺ fraction of RNA, a transcript must be present at 10 to 50 copies per brain cell or about 10⁻⁴% of the total RNA (4, 35). Consequently, most characterized clones do not represent the high-complexity, rare class of RNAs. Moreover, the cloning of brain-specific sequences via a cDNA intermediate poses an additional problem: one-half of the mRNAs in brain are poly(A)⁻ (8, 49) and would be absent from cDNA libraries prepared from poly(A)⁺ RNA. Therefore, we have developed a cloning and screening technique specifically designed to detect both poly(A)⁺ and poly(A)⁻ rare transcripts, which account for greater than 90% of the diverse transcribed sequences in the brain.

We have prepared a partial genomic library, enriched for rat unique-sequence DNA, in the single-stranded phage M13. A total of 50 random isolates were screened for expression in brain tissue by a modified transcription mapping procedure which was sensitive enough to detect transcripts present at ≥ 0.05 copy per cell. Of the 50 clones, 7 contained unique-sequence DNA homologous to rare brain transcripts, (at an average of three copies per cell [or fewer]), consistent with the percentage and occurrence predicted by earlier RNA complexity measurements. We have surveyed the tissue and subcellular distribution of these rare transcripts. Transcripts from two clones were restricted to certain tissues. RNA from one other clone was restricted to the nucleus, whereas transcripts of a fourth clone were present in the total RNA of all tissues studied but were in pelleted polysomal RNA only in brain tissue. Transcripts from two clones (which are present in pelleted polysomes) are poly(A)⁻.

MATERIALS AND METHODS

Materials, cDNA clones, and cell lines. Restriction enzymes were purchased from Bethesda Research Laboratories or New England Biolabs; T4 polynucleotide kinase was purchased from P-L Biochemicals, Inc. or New England Nuclear Corp.; and S1 nuclease was purchased from New England Nuclear Corp. or Bethesda Research Laboratories. [γ -³²P]ATP (9,000 Ci/mmol) was prepared by a modification of the technique of Johnson and Walseth (24). Oligodeoxythymidylate cellulose (type III) was purchased from Collaborative Research, Inc.

A cDNA clone (pc β 23) for rat β -casein was generously provided by Jeff Rosen. A *Sau*3A fragment of this cDNA clone (nucleotides 619 to 768 [39]) was subcloned into M13mp7 for use in transcription mapping (clone β). The cDNA subclone for rat tyrosine hydroxylase was prepared in M13 as described elsewhere (9a).

The isolation and culture of RT4-D and RT4-B8 cells were as described previously (23). The B27 and B103 lines were from Dave Schubert (42), and the rat melanoma Tr. CLXXXVIII^c (22) was provided as a transplantable tumor carried in AC rats, by E G & G Mason Research Institute, Worcester, Mass.

Preparation of RNAs. RNA was prepared from nuclei purified through a sucrose step gradient as described by Beckmann et al. (3), for use in the nuclear RNA complexity hybridizations. For these studies, the lower pair of mammary glands from 13-day-pregnant Sprague-Dawley rats was excised and predigested for 2 h at 37°C in medium 199 containing 2 mg of collagenase per ml (11). Total RNA from cells or tissues was extracted with guanidinium thiocyanate

and purified by centrifugation through 5.7 M CsCl as described by Chirgwin et al. (10). Polysomal RNA was prepared as pelleted polysomes (40). Kidney cytoplasmic RNA was prepared as described previously (3). Oligo(dT)-cellulose chromatography (two cycles) was as described previously (1).

Nuclear RNA hybridizations. Nuclear RNA at 20 mg/ml was hybridized to radio-iodinated, unique-sequence DNA (2×10^7 cpm/ μ g). The hybrids were assayed by hydroxylapatite (lot 17725; Bio-Rad Laboratories) chromatography as described previously (9).

Preparation and labeling of the unique-sequence rat DNA library in M13. Total rat DNA was digested to completion with the restriction endonuclease *Mbo*I. Unique-sequence DNA was isolated from the total digest by three cycles of reassociation to *C*₀t 200, followed by hydroxylapatite chromatography. The unique-sequence DNA (200 to 400 bases in length) was ligated into the *Bam*HI site of the replicative form of the bacteriophage M13mp7, and recombinant (clear) plaques were selected from indicator plates. Single-stranded DNA from individual recombinant M13mp7 clones was prepared as described previously (20) and digested with *Sau*3A, which restricts at the same site as *Mbo*I but is unaffected by DNA methylation that occurs in the *Escherichia coli* host JM103 cells. The rat insert is preferentially released because it is flanked by a 14-base-pair duplex formed by the symmetrical sequence between the *Bam*HI site and *Eco*RI site in M13mp7 (which includes the *Sau*3A site). The other *Sau*3A sites in the M13 part of the recombinant are also cut, albeit at lower efficiency, presumably because of transient, double-stranded conformations (50). The restricted DNA was end-labeled with carrier-free [γ -³²P]ATP and 1 to 2 U of polynucleotide kinase in a final volume of 20 μ l by either the forward reaction (32) or the exchange reaction (32), which we have found to be simpler and more reproducible. The labeling was performed without removal of the *Sau*3A, which did not affect the reaction. The reaction was terminated with the addition of 80 μ l of 2.5 M ammonium acetate–50 mM EDTA. Unincorporated [γ -³²P]ATP was removed by G-50 column chromatography, followed by extraction with water-saturated phenol and then by ethanol coprecipitation with 10 μ g of carrier *E. coli* tRNA. The labeled DNA was a mixture of M13 fragments and the rat insert fragment, the latter of which had an estimated specific activity of 1×10^7 to 5×10^7 cpm per μ g of DNA.

Transcription mapping on single-stranded DNA. Labeled single-stranded DNA (2 to 5 ng) and the indicated amount of cold RNA were first boiled together in microfuge tubes and then hybridized in 4 $\times$ SSC (1 $\times$ SSC = 0.15 M NaCl, 0.015 M sodium citrate [pH 7.0]) for 12 to 18 h at 65°C in a final volume of 50 μ l and overlaid with mineral oil. After hybridization, the mixture was diluted to 350 μ l with cold S1 incubation mixture (0.3 M NaCl, 0.03 M sodium acetate [pH 4.5], 3 mM ZnCl₂, 50 mM NaF [the NaF being added immediately before the reaction was begun to inhibit phosphatase activity present in most commercial S1 preparations]) containing 500 (Vogt) U of S1 nuclease per ml. The reaction mixtures were vortexed, centrifuged briefly, and transferred to a fresh tube. The digestion was carried out at 37°C for 1 h, terminated with the addition of 50 μ l of 2.5 M ammonium acetate–50 mM EDTA, and extracted twice with water-saturated phenol and once with chloroform-isoamyl alcohol. Carrier *E. coli* tRNA (20 μ g) was added, and the nucleic acids were precipitated with ethanol. The size of the pelleted, S1-resistant DNA was determined by electrophore-

sis on 8% polyacrylamide–8 M urea gels in 0.05 M Tris-borate (pH 8.3), as previously described (20). Autoradiography was for 5 to 10 days with XAR-5 film (Kodak) with a Lightning-Plus intensifying screen at -70°C .

Sensitivity of the assay. β -Casein mRNA is 1,154 nucleotides long and comprises 0.4% of total cellular RNA from mammary glands of 8-day-lactating rats (21). One nanogram of this RNA (designated LMG RNA) was mixed with 50 μg of tRNA. This corresponds to one copy of β -casein mRNA per 7.6 pg of RNA (the amount of RNA per average brain cell [31]). Therefore, although there are no β -casein transcripts in the brain, our reconstruction experiments are set up such that 1 ng of LMG RNA in a total of 50 μg of RNA corresponds to one copy of a transcript per brain cell equivalent.

Determination of copy numbers. For this experiment, a known amount of β -casein mRNA was hybridized to a homologous DNA sequence cloned into M13 (clone β) and assayed as above. The resulting autoradiographic signal was compared with that of two of our clones, rg3 and rg13. As a control, some of the hybridizations were mixtures of labeled DNA of clones β and rg3 or rg13. In these controls, the mixed, labeled DNA was hybridized to both brain and β -casein RNA.

Because all of our hybridizations are performed under conditions of DNA excess, the signal strength is proportional not only to the amount of RNA in our reactions but also to the specific activity of the DNA. Therefore, we measured the molar specific activities of the rat insert fragments of rg3, rg13, and β -casein, which were 5×10^5 , 6×10^5 , and 6×10^5 cpm/pmol, respectively.

The hybridizations were performed as described in the legend to Fig. 8A and were treated as above. The resulting autoradiograph was scanned with a Shimadzu CS-930 dual-wavelength thin-layer chromatography scanner. A linear relationship between the area under the plotted peaks (or peak heights) and the relative amounts of β -casein RNA in our standards was observed. The copy numbers of clones rg3 and rg13 were calculated with the aid of the plot in Fig. 8B.

RESULTS

Cloning of genomic DNA transcribed in the brain. Our strategy for cloning sequences encoding rare transcripts exploits the fact that a high percentage of unique-sequence DNA (31%) is transcribed in brain (9). Based on complexity measurements, nearly one-third of random clones of double-stranded, unique-sequence DNA should be transcribed in brain. However, since we found greater sensitivity by using single-stranded probes, we chose the single-stranded phage M13 as a cloning vector rather than a double-stranded vector. Thus, only about 15% of our clones should be expressed in brain, assuming an equal probability of cloning a coding versus a noncoding strand.

We prepared the genomic library as detailed above. Briefly, DNA from rat liver was completely digested with the restriction endonuclease *Mbo*I, yielding fragments averaging 200 to 300 nucleotides in length. Nonrepetitive DNA was isolated by C_{0t} reassociation, followed by hydroxylapatite chromatography, and ligated into the *Bam*HI site of the replicative (double-stranded) form of phage M13 strain mp7.

Phage DNA samples from 50 individual recombinants, selected at random, were screened for expression as brain RNA by transcription mapping (5), modified as diagrammed in Fig. 1. The phage DNA was digested with the restriction endonuclease *Sau*3A (an isoschizomer of *Mbo*I), which

FIG. 1. Transcription mapping of unique-sequence DNA cloned into M13. The cloning of rat DNA and details of this procedure are presented in the text. DNA from an individual clone of single-stranded phage M13, strain mp7, with a rat insert (indicated by the heavy line), is drawn schematically. The inverted repeat (14 nucleotides) between the *Eco*RI and the *Sau*3A sites (S) is shown as double stranded. The rat insert is preferentially excised by *Sau*3A, and the resulting fragments are end-labeled with [γ - ^{32}P]ATP. After hybridization to RNA, S1 nuclease digestion eliminates single-stranded nucleic acid not hybridized to RNA. The S1 nuclease-resistant fragments are separated by electrophoresis on polyacrylamide gels and visualized by autoradiography. Schematic examples of gel results are shown. The rat insert fragment indicated by the arrow is easily distinguished from the known *Sau*3A fragments generated by M13, both by size and specific activity. Lane 1, no bands are seen, illustrating complete digestion by S1 nuclease (no RNA hybridization which protects the labeled 5' end); lane 2, partial protection (the protected fragment is less than the full length of the insert); lane 3, full protection from S1 nuclease digestion; lane 4, pattern obtained without S1 nuclease treatment. The absence of labeled phage fragments after S1 nuclease digestion is an important internal control. An additional control in which a nonhomologous RNA (*E. coli* tRNA) gives no bands was also performed for each assay.

recognizes the internal four bases of the *Bam*HI site. The rat insert was preferentially cleaved out of this single-stranded molecule because it is flanked by a 14-base-pair duplex formed by the symmetrical sequence between the *Bam*HI site (*Sau*3A) and the *Eco*RI site in M13 mp7 (50). The *Sau*3A sites unique to M13 were also cut, but at reduced efficiency. Restricted recombinant phage DNA (2 ng, end-labeled to a high specific activity with [γ - ^{32}P]ATP and polynucleotide kinase) was hybridized to 50 μg of total cellular RNA from Sprague-Dawley rat brains under conditions in which the labeled DNA was in excess over its homologous RNA. Rat DNA fragments engaged in a hybrid with RNA were resistant to digestion by the single-strand-specific nuclease S1 and were resolved by gel electrophoresis. Of the 50 original isolates, 9 were judged to have inserts homologous to rat RNA by this screen.

FIG. 2. Hybridization of unique-sequence DNA to nuclear RNA. Unique-sequence [125 I]DNA (2×10^7 cpm/ μ g) at 1 μ g/ml was hybridized to nuclear RNA at 20 mg/ml. The curves are a best least-squares fit to a pseudo-first-order equation and are a composite of five separate analyses of at least three RNA preparations. R_0t (RNA concentration in moles of nucleotide per liter $\times$ time in seconds) on the abscissa is corrected to that in 0.12 M sodium phosphate (pH 6.8). Tissue preparation, nuclear RNA isolation, hybridizations, and analyses are as described in the text. Nuclear RNA from (A) mammary gland saturates 11.8% of a unique-sequence probe and (B) the melanoma saturates 13.3% of a unique-sequence probe, corresponding to 23.6% and 26.6%, respectively, of unique-sequencing coding capacity, assuming asymmetric transcription.

When DNA of each of the nine clones was used as a probe in Southern hybridizations with *Eco*RI-digested rat DNA, we found that two of the clones (rg21 and rg51) contained moderately repetitive elements (data not shown), and they were not further characterized.

Tissue distribution of transcripts. The seven unique-sequence clones whose transcripts appeared in the brain were further screened with total cellular RNA from the liver and kidney. Only two clones were brain-specific by these criteria, rg13 and rg40. Hybridization was strand-specific since the opposite DNA strand cloned into M13 did not hybridize to brain RNA (data not shown), as would be predicted for asymmetric transcription.

Our initial selection used brain RNA because of its high transcriptional complexity. However, two other ectodermal cell types also exhibit high nuclear RNA complexity, although not as high as that of the brain. Figure 2 shows the hybridization of nuclear RNA from normal, midpregnancy mammary gland and the melanoma, Tr. CLXXXVIII^c (22),

to unique-sequence DNA. RNA from mammary gland saturates 24% of the coding capacity of unique-sequence DNA, assuming asymmetric transcription; 26% complexity is observed for the rat melanoma tumor. Clones rg13 and rg40 were screened with these RNAs as well as RNA from three regions of the brain (cerebral cortex, regions underlying the cortex, and cerebellum), four neural cell lines, and newborn brain. Clone rg13 transcripts are detected in whole adult and newborn brain and in all adult brain sections, but not in mammary gland, melanoma, or any of the neural cell lines (Fig. 3A). Transcripts of clone rg40 are present in the whole brain, cortex, regions underlying the cortex, newborn brain, and the melanoma just within our limits of detection. No clear transcript signal was seen with RNA from the cerebellum, mammary gland or the neural cell lines (Fig. 3B). Clones rg40 and rg13 gave a positive signal with pelleted polysomal RNA from brain (Fig. 3C).

Clone rg34 contained rat DNA sequences transcribed in the brain, liver, and kidney. However, when hybridized to brain and liver pelleted polysomal RNA, or to kidney cytoplasmic RNA, the insert fragment was protected only by brain polysomal RNA (Fig. 4).

The rat DNA inserts of the four other clones, rg3, rg18, rg26, and rg36, were found to be protected by total cellular RNA from the brain, liver, and kidney. Transcripts homologous to rg3, rg26, and rg36 were in pelleted polysomes of both brain and liver; however, there was no protection of rg18 DNA by pelleted polysomal RNA from either the brain or the liver (Fig. 5).

Clones rg3 and rg13 encode poly(A)⁻ RNA. Brain poly(A)⁻ RNA (but not poly(A)⁺ RNA) protected the rat insert fragments of the commonly expressed clone rg3 and the brain-specific clone rg13, indicating that their transcripts were poly(A)⁻. The determination as to whether or not these two clones encode poly(A)⁺ or poly(A)⁻ RNA was possible because they gave the strongest signals in our assay and are thought to have the most abundant transcripts among our clones (see below). This is important in that we can unambiguously assign them to a poly(A)⁺ or poly(A)⁻ class, as we would be able to detect minor signals if such transcripts were bimorphic or if our RNA was improperly fractionated. As a control (with the same RNA preparation and concentrations) an M13 clone containing a partial cDNA sequence for tyrosine hydroxylase, known to be encoded by a poly(A)⁺ mRNA (30), showed protection in this assay by poly(A)⁺ RNA (but showed only slight protection by poly(A)⁻ RNA), indicating that our RNA was, for the most part, intact and that it had been fractionated properly (Fig. 6).

Sensitivity of the assay. To estimate the sensitivity of the transcription mapping technique, a known amount of a specific mRNA was annealed to its homologous cDNA and the resulting hybrids were assayed under our standard conditions. For this purpose, a *Sau*3A fragment of a cloned rat β -casein cDNA, pc β 23 (39), was subcloned into the *Bam*HI site of phage M13 (clone β). Known amounts of RNA from mammary glands of 8-day-lactating rats (LMG RNA), mixed with 50 μ g of *E. coli* tRNA, were hybridized to 2 ng of labeled single-stranded fragments of clone β . The results shown in Fig. 7 indicate that a signal was detected when as little as 0.1 ng of LMG RNA was used (lane 7), which is equivalent to 0.2 pg of β -casein mRNA. This reconstruction experiment is designed such that 1 ng of LMG RNA in 50 μ g of tRNA represents the equivalent of 1 copy of an RNA species per brain cell (although there are no β -casein transcripts in brain [see below]). Therefore, we can detect the equivalent of 0.1 to 0.05 copies per cell.

Estimation of copy number. The intensities of the labeled fragment of clone β protected from S1 nuclease digestion by known amounts of LMG RNA were measured by densitometry. By comparing the intensities of these standards with those generated by clones rg3 and rg13, an RNA copy number for these clones could be estimated (Fig. 8). To insure that the hybridization and digestion conditions for the β -casein probe were identical to those used for our clones, LMG RNA was mixed with brain RNA and hybridized in the same reaction mixture with both clone β and either rg3 probes or rg13 probes. Because the size of the clone β insert is different from that of either rg3 or rg13, both can be distinguished in the same gel lane (as indicated by the arrows in Fig. 8A). Under these conditions, there was no detectable cross-hybridization of clone β with brain RNA and no cross-hybridization of clone rg13 or clone rg3 with LMG RNA (lanes 1, 14, and 15, respectively). Because all probe inserts had the same molar specific activity, quantitation of the brain transcripts was directly determined by densitometric scanning of the resulting autoradiograph. As shown in Fig. 8B, clones rg3 and rg13 were present at levels of 1.3 and 2.7

copies per brain cell, respectively. Since expression of the concentration of these RNAs in terms of the percentage of the total RNA requires knowing the length of the complete transcripts, and because northern hybridizations were not sensitive enough to allow us to measure these lengths, we expressed our data in terms of copies per cell.

Based on comparisons with clones rg3 and rg13, transcripts from the other five clones are 1 copy or less per brain cell, although no direct determinations have been made. A summary of our results is presented in Table 1.

DISCUSSION

We have isolated and characterized genomic clones which code for rare transcripts (at an average of 0.05 to 3 copies per brain cell) in rat brain by a sensitive screening procedure. With this approach, investigation of the majority of the diverse transcribed sequences is now feasible. In the limited repertoire of clones we have so far studied, several interesting features have emerged.

Individual clones conform to sequence complexity data. We have found 14% of random single-stranded clones of unique-

FIG. 3. Transcription mapping of clones rg13 and rg40. Labeled *Sau3A* fragments of clones rg13 and rg40 were annealed to 50 and 100 μ g, respectively, of total cellular RNA from the indicated sources. The hybrids were digested with S1 nuclease, separated by electrophoresis on denaturing polyacrylamide-urea gels and visualized by autoradiography as described in the text. (A) The 75-nucleotide rat insert fragment of clone rg13 (indicated by arrow) is protected in this assay by RNA from whole adult brain (lane 1), newborn brain (lane 4), cerebellum (lane 5), regions underlying the cortex (lane 6), and cortex (lane 7) samples. Lane 13, pattern obtained without S1 digestion. The protected bands are all 75 nucleotides (the gel has "smiled"). (B) The 206-nucleotide rat insert fragment of clone rg40 (indicated by the arrow) is protected in this assay by RNA from adult brain (lane 1), melanoma (lane 3), newborn brain (lane 4), regions underlying the cortex (lane 6), and the cortex (lane 7). Lane 13, pattern obtained without S1 digestion. (C) Clones rg13 (lane 1) and rg40 (lane 2) show protection of rat insert bands (indicated by the arrows) in this assay by pelleted polysomal RNA from adult brain. Exposure of (A) and (B) was for 5 days; exposure for (C) was for 10 days.

FIG. 4. Transcription mapping of clone rg34. Labeled *Sau3A* fragments of clone rg34 were annealed to 50 μ g of RNA from the indicated sources and were treated as described in the legend to Fig. 3. The insert fragment of 181 nucleotides (indicated by the arrow) is protected in this assay by total cellular RNA from adult brain (lane 1), liver (lane 2), and kidney (lane 3) and polysomal RNA from adult brain (lane 4). No protection was observed with liver polysomal RNA (lane 5), kidney cytoplasmic RNA (lane 6), or tRNA (lane 7). Lane 8, pattern obtained without S1 nuclease digestion. Exposure of the gel was for 7 days.

sequence DNA to be transcribed in brain. This is in agreement with the earlier complexity measurements that showed about 15% of single-stranded unique-sequence DNA to be complementary to the nuclear RNA of rodent brain and suggests that those sequences detected by complexity measurements can be selected as individual clones. Both methods detect the complex class of rare transcripts, and the sensitivities of the methods are similar, detecting RNAs present at ≥ 0.05 copy per cell.

Chikaraishi et al. (9) have observed that after saturation hybridization of unique-sequence DNA with brain nuclear RNA there is no additional hybridization with nuclear RNAs from either liver or kidney cells. In contrast, unique-sequence DNA hybridized to saturation with either liver or kidney nuclear RNA supported additional hybridization when brain nuclear RNA was subsequently added, the new saturation value being equal to that of brain alone. These data predicted the existence of a class of sequences transcribed in the brain but not in the liver or kidney, and clones rg13 and rg40 may be representative of this class. Similar additivity experiments with kidney and liver nuclear RNAs generalized this phenomenon to a nested hierarchy of RNA complexity, such that sequences transcribed in a given tissue are also transcribed in any other tissue exhibiting higher

sequence complexity. This observation, based on nuclear RNA saturation hybridization results is not necessarily true for all transcribed sequences but, rather, for most of the rare, complex sequences detected by saturation hybridization. Because a difference of 1 to 2% in complexity is the experimental error in such studies, several thousand different transcripts may not fall into this generalization (including β -casein and albumin [see below]); however, the small random sample of unique-sequence DNA represented by our clones does follow this pattern.

Transcript distribution patterns implicate transcriptional and post-transcriptional control of rare transcripts. The transcription patterns of the seven unique-sequence clones investigated are summarized in Table 1. All but one of the seven clones were found in pelleted polysomal RNA of the brain, again in agreement with the earlier complexity data which show that a high proportion (nearly two-thirds) of brain nuclear transcripts are conserved as polysomal sequences. The nonpolysomal clone rg18 (found in the brain, liver and kidney) appeared to be restricted to the nucleus. Because our partial library was prepared from genomic DNA rather than cDNA, clone rg18 may encode an intron or 3' end that was removed by nuclear RNA processing events. Alternatively, it may be a polysomal RNA in a tissue which we have not yet examined.

Clones rg3, rg26, and rg36 transcripts are common to both nuclear and cytoplasmic RNA from the brain, liver, and kidneys and may be mRNAs involved in housekeeping functions. This pattern demonstrates that the rare transcripts of the brain are not necessarily brain-specific. The distribution of clone rg34 transcripts appears to be more complex; its transcripts appeared in all tissues but were polysomal only in the brain. Clone rg34 may, therefore, represent a gene controlled post-transcriptionally, similar to rare transcript clones described for sea urchins (29). The two remaining clones, rg40 and rg13, appear to be transcriptionally regulated. Clone rg40 RNA was detected only in the brain and the melanoma Tr. CLXXXVIII^c. Transcription of rg40 in this melanoma may be a function of the tumor properties of the tissue, or it may reflect the neural crest origin of melanocytes. Although it is likely that transformation is associated with the expression of new nuclear transcripts, the nuclear RNA complexities of the brain, the liver, and mammary gland are similar to those of their transformed counterparts (3, 45; Noboru Sueoka and Jim Beeson, personal communication).

We did not detect transcripts of clone rg13 or rg40 in any of the four neural cell lines tested. B103 and B27 (42) were derived from the central nervous system and exhibit neuronal and glial properties, respectively. Two others, RT4-B and RT4-D, are derived from a peripheral nerve tumor (23). RT4-B has neuronal attributes (48), whereas RT4-D has glial-like characteristics (23). All four lines were derived from nitrosoethylurea-induced tumors. These lines were chosen for study because previous work (3) has demonstrated high-nuclear-RNA complexity in B103 (29%) and B27 (27%) and lower complexity in RT4-D (22%), relative to adult brains (31%). The lack of rg13 and rg40 transcripts in these cell lines may reflect cell-type specificity. Additionally, although we detected transcripts of clone rg40 in other parts of the brain, we did not detect them in the cerebellum. Nonhomogeneous distribution of such transcripts implies that, although rare when averaged for the whole brain, these transcripts may be present at a higher copy number in a limited set of cells.

Poly(A)⁻ transcripts. Transcripts of brain-specific clone rg13 are poly(A)⁻. The poly(A)⁻ class may be important in

brain development as suggested by Chaudhari and Hahn (7), who reported an increase in brain nuclear-RNA complexity from birth to adulthood (2 months) in mice. In their study, the increase in nuclear-RNA complexity was mainly attributed to the appearance of poly(A)⁺ transcripts, although a small fraction of the adult poly(A)⁺ sequences were detected in newborn brains. These poly(A)⁺ sequences appeared first in the nucleus but were almost entirely expressed as polysomal poly(A)⁺ mRNA by 2 months of age (7). Since clone rg13 was detected in newborn brains, it may be among the earliest activated genes of this class.

Transcripts of clone rg3 were found on brain pelleted polysomes but in contrast to rg13 were also found in other tissues (see Table 1). In light of the data which suggest that most poly(A)⁺ transcripts are unique to adult brain (7), we were surprised to find that rg3 transcripts were poly(A)⁺. It may have been fortuitous that we found a clone correspond-

ing to a common poly(A)⁺ transcript in our limited screen. Poly(A)⁺ transcripts have been reported in non-neural cells: HeLa cells (34), mouse and rat liver (14-16, 36, 51), rat mammary gland (45), and mouse kidney (37). However, these poly(A)⁺ transcripts do not contribute significantly to the overall mRNA complexity. Taken together, these results suggest that although poly(A)⁺ transcripts are important in the brain, there is at least a small class of poly(A)⁺ RNAs which are neither brain-specific nor limited to postnatal expression.

What is the significance of rare transcripts? All of our clones represent rare transcripts that were present at three or fewer copies per cell in the total brain. Although it can be argued that some of these rare RNAs are products of leaky or read-through transcription, either this phenomenon must be sequence specific or certain leaky transcripts must be preferentially stabilized to allow their detection, since most

FIG. 5. Transcription mapping of clones rg3, rg18, rg26, and rg36. Labeled *Sau3A* fragments of clones rg3 (A), rg18 (B), rg26 (C), and rg36 (D) were annealed to 50 μ g of RNA from the indicated sources and were treated as described in the legend to Fig. 3. All four clones showed protection of insert fragments in this assay by total cellular RNA from adult brain (lane 5), kidney (lane 4), and liver (lane 3). The pattern obtained without S1 nuclease digestion is shown in lane 1 for each clone. (A) Clone rg3. In addition to protection by total cellular RNAs, protection of 75 nucleotides of the 80-nucleotide insert (indicated by arrows) is shown in this assay by polysomal RNA from adult brain (lane 6) and liver (lane 7). (B) Clone rg18. Although protected by total cellular RNAs, the 140-nucleotide insert (indicated by arrow) is not protected by polysomal RNA from adult brain (lane 6) or liver (lane 7). (C) Clone rg26. About 44 nucleotides of the 136-nucleotide insert (indicated by arrows) are protected by total cellular RNAs and polysomal RNAs of adult brain (lane 6) and liver (lane 7). (D) Clone 36. There are two *Sau3A* insert fragments of 98 and 255 nucleotides (indicated by arrows). Protection of the 98-nucleotide fragment by total cellular RNAs and polysomal RNAs of adult brain (lane 6) and liver (lane 7) is observed. Exposure of lanes 1 to 6 was for 5 days; exposure of lanes 6 and 7 was for 7 days.

FIG. 6. Transcription mapping using poly(A)⁺ and poly(A)⁻ RNA. Labeled *Sau*3A fragments of clones rg3 (A), rg13 (B), and an M13 clone containing a partial cDNA sequence to tyrosine hydroxylase (C) were annealed to RNA as indicated. The hybrids were treated as described in the legend to Fig. 3. The same total cellular RNA from adult brain was used as the source of both poly(A)⁺ and poly(A)⁻ RNA, which were fractionated twice on an oligo(dT)-cellulose column. In these assays, 50 μ g of the total cellular RNA (lane 1), 2 μ g of poly(A)⁺ RNA mixed with 48 μ g of *E. coli* tRNA (lane 2), 50 μ g of poly(A)⁻ RNA (lane 3), and 50 μ g of *E. coli* tRNA (lane 4) were used. The pattern obtained without S1 nuclease digestion is shown in lane 5. (A) Clone rg3: 75 nucleotides of the insert fragment of 80 nucleotides (indicated by arrow) are protected by total cellular RNA (lane 1) and by poly(A)⁻ RNA (lane 3). (B) Clone rg13: The insert fragment of 75 nucleotides (indicated by arrow) is protected by total cellular RNA (lane 1) and by poly(A)⁻ RNA (lane 3). (C) M13 containing cDNA of tyrosine hydroxylase: A *Sau*3A fragment of 175 nucleotides (indicated by arrow) is protected by total cellular RNA (lane 1) and by poly(A)⁺ RNA (lane 2). Exposure of (A) was for 5 days; exposure of (B) and (C) was for 10 days.

of our randomly selected clones (41/50) did not encode detectable transcripts. Since our limit of sensitivity was about 0.05 to 0.1 transcript per cell (Fig. 7), we cannot exclude that the negative clones are at present at below 0.05 copies per cell. However, our assay was not indiscriminate, as we had not been able to detect transcripts for β -casein in brain (Fig. 7). Also, we have subcloned cDNAs encoding albumin (41) and a prostate glycoprotein (38) into M13 and did not detect these cell-specific transcripts in any but the appropriate tissue (data not shown).

Transcripts from all but one of our seven clones (rg18) were found as pelleted polysomal RNA (at least in brain) suggesting a possible messenger function. However, there appear to be twofold fewer transcripts of rg13 and rg3 in polysomal RNA than in total RNA from brain (note exposure times in Fig. 3 and 5; also, unpublished data). Therefore, it is possible that these sequences do not function as

messenger RNAs and their reduced polysomal concentration merely reflects nuclear RNA contamination. However, since some transcribed clones were not found in polysomal RNA, such nuclear contamination must be nonrandom. In addition, a probe containing an intron sequence of tyrosine hydroxylase did not anneal to the same preparation of pelleted polysomal RNA, whereas a strong signal was obtained with an exon-specific tyrosine hydroxylase sequence (data not shown). If rg13 and rg3 do function as mRNAs, then their unusual nuclear versus polysomal distribution may reflect cell-specific post-transcriptional processing. Most brain cells may have these sequences in their nuclear RNA, but only a subpopulation of cells may utilize these sequences as mRNAs.

We have sequenced the 75-nucleotide insert of rg13, and

FIG. 7. Sensitivity of the transcription mapping assay. Labeled *Sau*3A fragments of clone β (an M13 recombinant containing a β -casein cDNA fragment) were annealed to various amounts of total cellular RNA from mammary glands of 8-day-lactating rats (LMG RNA) mixed with 50 μ g of tRNA. Although there are no β -casein transcripts in brain (see Fig. 8), in this reconstruction experiment, 1 ng of LMG RNA mixed with 50 μ g of tRNA is equivalent in concentration to one copy of a transcript per brain cell. The hybrids were digested with S1 nuclease, and resolved by electrophoresis on denaturing polyacrylamide-urea gels. The gels were exposed for 3.5 days. The derivation of clone β and the copy number calculations are detailed in the text. The β -casein insert fragment (149 nucleotides) is indicated by the arrow. Lane 1, simian virus 40 *Eco*RII markers; lane 2, pattern obtained without S1 nuclease digestion; lanes 3 to 11, LMG RNA as indicated.

FIG. 8. Copy number of clones rg3 and rg13. (A) Labeled *Sau3A* fragments of clone β and either clone rg13 or rg3 were annealed to the amounts of total cellular RNA from brain, LMG RNA, or a mixture of both brain and LMG RNAs, as indicated, and were treated as described in the legend to Fig. 7. The details of this experiment are described in the text. The β-casein insert fragment (149 nucleotides) is indicated by an arrow. The arrow at 80 nucleotides indicates the size of the rg3 insert. The arrow at 75 nucleotides indicates both the insert and S1 nuclease-resistant fragment of rg13, as well as the S1 nuclease-resistant fragment of rg3. Lane 1, no protection of the clone β insert by brain RNA; lanes 2 to 4, labeled rg13 and clone β annealed to 50 μg of brain RNA and 5, 10, and 15 ng of LMG RNA, respectively; lane 5, labeled rg13 and clone β show no nonspecific protection by 50 μg of tRNA; lane 6, pattern obtained without S1 nuclease digestion of mixed rg13 and clone β labeled fragments; lanes 7 to 9, labeled rg3 and clone β annealed to 50 μg of brain RNA and 5, 10, and 15 ng of LMG RNA, respectively; lane 10, labeled rg3 and clone β show no nonspecific protection by 50 μg of tRNA; lane 11, pattern obtained without S1 nuclease digestion of mixed rg3 and clone β labeled fragments; lane 12, labeled rg13 and clone β annealed to 25 μg of brain RNA and 2.5 ng of LMG RNA; lanes 14 and 15, no protection of rg13 or rg3, respectively, by 15 ng of LMG RNA. Exposure of the gel was for 3.5 days. (B) The density of the transcription map signals from (A) are plotted against the known equivalent copy number of β-casein (○). On the basis of the standard plot, the density of the rg3 transcript signal (+) indicates 1.3 copies per brain cell and that of rg13 (Δ) indicates 2.7 copies per brain cell.

although we do not know whether our clone corresponds to a translated region of mRNA, it does have one open reading frame extending the length of the insert (data not shown). However, until the putative protein is found, we cannot be certain that this sequence has a messenger function.

Rare transcripts may result from low-level transcription of uninduced genes or from constitutive transcription of housekeeping genes. Indeed, metallothionein I mRNA in uninduced liver or kidney cells has been measured at 13 to 14 copies per cell (18). The mRNA for the cellular homolog to the *src* gene of avian sarcoma virus is present at about four copies per cell in chicken heart, muscle, and brain (44). Additionally, the mRNA of transferrin is ≤ 10 copies per cell in chicken pancreas, kidney, spleen, heart, lung, or intestine (33). Presumably these low transcript levels are sufficient to maintain these housekeeping functions. Our commonly expressed clones may fall into this category.

The rare transcripts of brain may also result from differential expression of certain genes in specific cell types. We estimate that transcripts for tyrosine hydroxylase, the rate-limiting enzyme in catecholamine biosynthesis, are present at fewer than 10 copies per cell for whole-brain samples (based on the relative specific activities and signal intensities

in Fig. 6). However, tyrosine hydroxylase transcripts are much more abundant in certain areas of the brain (e.g., the substantia nigra and locus ceruleus) than in whole brain (9a). Although clone rg13 transcripts were present in the three brain sections studied, the cerebellum, the cortex, and the regions underlying the cortex, there may be more variation in terms of transcript abundance at fine levels of dissection. Clone rg40 transcripts do appear to be localized in brain, as we did not detect these transcripts in cerebellum.

The significance and function of rare transcripts as a class remain to be determined. Careful analysis of more clones is necessary, as well as in situ localization of these transcripts and identification of their protein products. Antisera directed against synthetic peptides of moderately abundant brain-specific transcripts have been used successfully to localize these products (27, 46), and this approach may be possible if a clone of the rare class is more abundantly expressed in a subset of brain cells.

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TABLE 1. Characteristics and transcriptional expression of unique-sequence clones

Parameter and RNA source	Result characteristic of clone:						
	rg3	rg13	rg18	rg26	rg34	rg36	rg40
Parameter							
Insert (nucleotides)	80	75	140	136	181	98, 255	206
Size protected (nucleotides) ^a	75	75	140	44 ^b	181	98 ^b	206
Transcripts per brain cell ^c	1.3	2.7	1	1	1	1	0.05–0.1
RNA source^d							
Brain	+	+	+	+	+	+	+
Liver	+	–	+	+	+	+	–
Kidney	+	–	+	+	+	+	–
Brain polysomal	+	+	–	+	+	+	+
Liver polysomal	+	–	–	+	–	+	–
Kidney cytoplasmic	–	–	–	–	–	–	–
Brain polyA ⁺	+	+	–	–	–	–	–
Brain polyA [–]	+	+	–	–	–	–	–
Newborn brain	+	+	+	+	+	+	+
Mammary gland	+	–	+	+	+	+	–
Melanoma	+	–	–	+	+	+	+
Cerebellum	–	+	–	–	–	–	–
Cortex	–	+	–	–	–	–	+
Regions underlying cortex	–	+	–	–	–	–	+
B103	–	–	–	–	–	–	–
B27	–	–	–	–	–	–	–
RT4-B	–	–	–	–	–	–	–
RT4-D	–	–	–	–	–	–	–

^a The size of the insert fragment protected against S1 nuclease digestion after hybridization with total cellular RNA from adult brain.

^b The multiple bands observed may result from sequence-dependent imprecision of S1 nuclease.

^c Transcript copy number per brain cell was determined for rg3 and rg13 as described in the legend to Fig. 8. The other copy numbers are estimates, based on comparisons with rg3 and rg13.

^d RNA is total cellular RNA unless otherwise indicated. RNA isolation from the tissues, tumor, and cell lines was as described in the text. Symbols: (+) and (–) indicate the presence or absence, respectively, of corresponding transcripts in the RNA source.

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