ABSTRACT

To isolate cDNA clones of low-abundance mRNAs expressed in monkey cerebral cortex but absent from cerebellum, we developed an improved subtractive cDNA cloning procedure that requires only modest quantities of mRNA. Plasmid DNA from a monkey cerebellum cDNA library was hybridized in large excess to radiolabeled monkey cortex cDNA in a phenol emulsion-enhanced reaction. The unhybridized cortex cDNA was isolated by chromatography on hydroxyapatite and used to probe colonies from a monkey cortex cDNA library. Of 60,000 colonies screened, 163 clones were isolated and confirmed by colony hybridization or RNA blotting to represent mRNAs, ranging from 0.001% to 1.1% abundance, specific to or highly enriched in cerebral cortex relative to cerebellum. Clones of one medium-abundance mRNA were recovered almost quantitatively. Two of the lower-abundance mRNAs were expressed at levels reduced by a factor of 10 in Alzheimer disease relative to normal human cortex. One of these was identified as the monkey preprosomatostatin I mRNA.

In primates, the cerebral cortex has become regionally specialized to a high degree, as evidenced by observed cytoarchitectonic and functional differences between cortical areas. The cerebral cortex is also one of the major sites for the neuronal degeneration associated with Alzheimer disease in humans, a process that minimally involves the cerebellum (1). Preliminary studies have shown that relatively few brain mRNAs with regionally heterogeneous distributions are of sufficient abundance to permit detection of their corresponding cDNA clones by differential colony screening (2). To find cDNA clones representing low-abundance mRNAs present in cerebral cortex but absent from cerebellum, we developed an improved subtractive cloning strategy. This strategy has the advantage of requiring only modest amounts of starting RNA, an important concern when using precious tissues, and it permits the isolation of cDNA clones representing mRNAs with abundances as low as 0.001%. With this approach we have isolated several clones of low-abundance mRNAs that are highly enriched in neocortex. Two of these clones correspond to mRNAs whose concentrations are reduced by a factor of ≈10 in the cerebral cortex from Alzheimer disease patients. One of these is a clone of the mRNA encoding the somatostatin 1 precursor peptide.

MATERIALS AND METHODS

Preparation of RNA. Young adult male cynomolgus (Macaca fascicularis) monkeys were anesthetized with pentobarbital and their brains were perfused with ice-cold phosphate-buffered saline for 3 min. The chilled brains were removed and dissected into primary visual cortex (Brodmann area 17), motor cortex (area 4), dorsal prefrontal cortex (areas 9, 10, and 46), and cerebellum minus deep cerebellar nuclei. Liver was also dissected from monkey for RNA preparation. Cytoplasmic RNA was prepared by the procedure of Schibler et al. (3). Total cellular RNA was prepared from frozen tissues, including frontal cortex autopsy specimens from four patients with pathologically confirmed Alzheimer disease and from six adult humans with no known central nervous system pathology, using the procedure of Chirgwin et al. (4). Enrichment for polyadenylated [poly-(A)+] RNA was performed by the procedure of Aviv and Leder (5) with oligo(dT)-cellulose.

Construction of cDNA Libraries. Two cDNA libraries were constructed in the plasmid vector pGEM4 (Promega Biotec, Madison, WI) by using a vector-primed cloning procedure as described (6). One was prepared from 2 μg of poly(A)+ monkey cortex RNA (pooled from the three cortical areas) and the other was from 2 μg of poly(A)+ monkey cerebellum RNA. Both libraries were amplified by transforming Escherichia coli strain MC1061 with the final ligation mixtures and then growing the transformed cells in liquid media under ampicillin selection, following the procedure of Okayama and Berg (7). Based on the number of ampicillin-resistant colonies obtained from an aliquot of the transformation mixture taken prior to amplification, and the percentage of these shown in minipreps to harbor plasmids containing inserts, the number of recombinants in both libraries was estimated to be 105. Five micrograms of the monkey cortex plasmid DNA library was size-selected by electrophoresis on low melting temperature agarose, and supercoiled DNA containing inserts between 0.5 and 6 kilobases (kb) was used as a transformation stock for high-density colony hybridization plating.

Subtractive Probe Preparation and Screening. A [32P]-cDNA probe was prepared from 2 μg of poly(A)+ monkey cortex RNA by the procedure of Timberlake (8) using oligo(dT) as a primer and 1 mCi of [α-32P]dCTP (3000 Ci/mmol; 1 Ci = 37 GBq) resulting in a total incorporation of 106 cpm. After cDNA synthesis, the RNA template was removed by treating with 0.1 M NaOH for 20 min at 68°C. Cerebellum plasmid cDNA (120 μg) was prepared as a hybridization driver by restriction endonuclease digestion with Pst I and EcoRI to release insert fragments from the vector. Before hybridization, the restricted cerebellum cDNA driver was combined with the cortex cDNA probe and denatured by heating for 5 min at 100°C. Hybridization was performed with the phenol emulsion reassociation technique in a 400-μl solution containing 1.5 M NaSCN and 8% phenol at 25°C for 4 hr by the procedure of Kohn et al. (9). An emulsion of the phenol and aqueous phases was maintained throughout the hybridization by continuous agitation on a Vortex mixer.

After hybridization, the mixture was extracted with CHCl3, precipitated with 1 ml of ethanol, and dissolved in 1 ml of 50 mM sodium phosphate buffer (pH 6.5) (PB). The
mixture was loaded onto a jacketed column (Bio-Rad) maintained at 60°C containing a 3-ml bed vol of hydroxyapatite (DNA Grade Bio-Gel HTP, Bio-Rad) equilibrated with 50 mM PB. The column was washed with 9 ml of 50 mM PB to remove low molecular weight fragments. The single-stranded cDNA was eluted with 10 ml of 140 mM PB and the double-stranded cDNA was eluted with 10 ml of 400 mM PB.

Sixty thousand colonies from the size-selected monkey cortex library were plated, and Biotrans filter (ICN) colony lifts were probed by hybridization with the single-stranded hydroxyapatite fraction (140 mM PB eluate) in a high-density colony screen by the method of Grunstein and Wallis (10). All colonies giving a positive signal in this initial screen were transferred to a grid array in 96-well microtiter dishes after the method of Wallis et al. (11).

A low-density secondary screen on duplicate filters from this grid array was performed using the differential colony hybridization procedure of Dworkin and Dawid (12). Probes were constructed by first synthesizing unlabeled cDNA from 2 μg of poly(A)⁺ cortex and cerebellum RNA using an oligo(dT) primer. The RNA template was removed by treating for 20 min at 68°C with 0.1 M NaOH, and a separate labeling reaction was performed using random hexamer oligonucleotide priming (13) with 100 μCi of [α-³²P]dCTP.

Colonies that gave no hybridization signal with either the cortex or cerebellum probes on the differential colony screen were placed into a second grid array. These colonies were screened with a repeat cortex-minus-cerebellum subtracted cDNA probe prepared as described above.

RNA Abundance Determination. Quantitation of the pMC4G8 and the pMC5B3 mRNAs was performed by electrophoresis of 2 μg of monkey cortex poly(A)⁺ RNA in parallel with 100 pg, 10 pg, and 1 pg of heat-denatured restriction endonuclease-digested pGEM4 plasmid (added to 2 μg of yeast tRNA carrier) on an agarose formaldehyde gel in an RNA blotting-type experiment (see Fig. 2 legend for experimental details). These blotss were probed with nick-translated whole-plasmid DNA containing the pMC4G8 or pMC5B3 insert and washed in 0.25 x SSC at 68°C (1 x SSC = 0.15 M NaCl/0.015 M sodium citrate). The resulting autoradiographs were scanned with a laser densitometer (LKB UltrScan).

RESULTS

To isolate cDNA clones of mRNAs enriched in monkey cerebral cortex relative to cerebellum, we screened a monkey cortex cDNA library with a cortex cDNA probe from which sequences representing mRNAs also expressed in the cerebellum had been removed in a modified subtractive hybridization reaction. The initial [³²P]cDNA probe prepared from 2 μg of monkey cortex mRNA was hybridized in a phenol emulsion reaction mixture to 120 μg of denatured plasmid driver DNA prepared by amplifying a monkey cerebellum cDNA library. Assuming that cerebellum mRNA-derived sequences constituted 20% of the plasmid mass, the cerebellum cDNA hybridization driver was in 80-fold excess over the probe. Based on previously described calculations (9), a Cₛ value of between 20,000 and 80,000 M-sec was reached by the cerebellum driver. The hydroxyapatite single-stranded peak fraction contained 5 x 10⁶ cpm, which constituted 5% of the total counts.

Six hundred and sixty colonies (1%) of 60,000 from the cortex library gave a detectable hybridization signal with the subtracted probe. These were picked into a microtiter dish grid array and probed in duplicate with unsubtracted [³²P]cDNA prepared from cortex and cerebellum (Fig. 1). One hundred and sixty colonies (24%) gave a signal with the cortex probe but not the cerebellum probe. These represented candidate cDNA clones of "cortex-specific" mRNAs. In addition, 37 colonies (6%) gave no hybridization signal with either the cortex or cerebellum probes. The remainder of clones gave signals with both cortex and cerebellum probes, representing sequences incompletely subtracted during the hybridization reaction or human error in picking colonies from high-density plates. The low-density grid was probed with pMC1H8a, a cDNA clone representing a monkey cortex-specific mRNA with an estimated abundance of 0.05% isolated in a previous screen (2). Sequences homologous to pMC1H8a were detected in 59 of the 660 subtractive-selected colonies. When the original high-density filters from the cortex cDNA library were reprobed with pMC1H8a, it was found to hybridize to 60 of the 60,000 unselected colonies; thus near-quantitative recovery was realized for this sequence.
Ten of the 160 candidate cortex-specific cDNAs were selected at random and used to probe RNA blots containing RNA from monkey cortex, cerebellum, and liver, as well as Alzheimer disease cortex and normal human cortex. All 10 clones detected RNAs that were either specific to cortex or significantly enriched in cortex over cerebellum (e.g., Fig. 2 B and C).

The 37 colonies picked in the first screen that gave no signals with either the cortex or cerebellum unsubtracted probes were picked into a second microtiter dish grid and reprobed with another preparation of cortex-minus-cerebellum subtracted cDNA. Nine of these colonies gave a clear signal with the second subtracted probe (Fig. 3). The remaining 28 clones, negative with the second subtracted probe, probably arose from unavoidable errors in picking colonies from the high-density screen. The nine positive clones represented candidate low-abundance cortex-specific cDNAs and were used for RNA blot analysis. Three of these detected mRNAs that were cortex specific at the sensitivity of the assay (Fig. 4). One of these clones (pMC4G8) detected a low-abundance RNA that migrated as a doublet at \( \approx 700 \) bases and was reduced by a factor of \( \approx 10 \) in RNA samples from patients with Alzheimer disease relative to controls from normal human cortex. Clone pMC5B3 detected a low-abundance monkey cortex-specific RNA of \( \approx 2.7 \) kb that was also reduced by a factor of \( \approx 10 \) in Alzheimer disease cortex relative to normal human cortex. Clone pMC2C8 detected a cortex-specific RNA of \( \approx 1 \) kb, not reduced in Alzheimer disease cortex. Three of the remaining six clones gave no signal on the RNA blots (probably representing very-low-abundance mRNAs), and the other three gave weak signals in both the cortex and cerebellum lanes. When the original high-density filters were reprobed with pMC4G8, 2 of the 60,000 unselected colonies were detected.

The absolute abundances of the pMC4G8 and pMC5B3 mRNAs were determined by comparing their autoradiograph signal densities with the signal density from 1 pg of plasmid DNA on the adjacent lane in an RNA blotting experiment (data not shown). Assuming equal transfer of similar-sized RNA and DNA fragments, and equal stability of the DNA:DNA and DNA:RNA hybrids under our stringency-

**Fig. 3.** Subtractive colony autoradiograph of 37 clones previously negative with both unsubtracted cortex and cerebellum cDNAs. Nine colonies gave a clear hybridization signal in this experiment when reprobed with cortex-minus-cerebellum cDNA preparation. Clone pMC2C8 (a), pMC4G8 (b), and pMC5B3 (c). Clone pMC2D6 (d) gave a signal with the unsubtracted cortex probe and was included as a positive control.

wash conditions, an abundance estimate of 0.001% was obtained for both of these mRNA species in monkey cortex poly(A)⁺ RNA. For these calculations, we took into account the relative lengths of the DNA and RNA targets and that both strands of the DNA were available for hybridization. The RNA blot hybridization pattern of clone pMC4G8 resembled that obtained (G.H.T., unpublished observations) with a cDNA probe derived from the human preprosomatostatin I mRNA (15). Southern blot hybridization of insert DNA from this clone with the human cDNA probe confirmed that pMC4G8 was related to preprosomatostatin I. Nucleotide sequence analysis revealed that clone pMC4G8 contained a cDNA insert of 604 base pairs in addition to a poly(A) tract (Fig. 5). The nucleotide sequence of clone pMC4G8 was 97% similar to the human preprosomatostatin I mRNA and contained an insertion of 3 nucleotides in the 5'-untranslated region. On the amino acid level, the monkey preprosomatostatin I protein sequence was identical to the human sequence (15). Since pMC4G8 begins with the same nucleotide at the 5' end as does the human mRNA, it likely represents a full-length cDNA clone.

**Fig. 2.** RNA blot analysis of three clones selected as cortex (+)/cerebellum (-) with the differential colony screen. RNA samples [2 \( \mu g \) of poly(A)⁺] included normal human frontal cortex (lanes Hu), Alzheimer disease frontal cortex (lanes Al), monkey cerebral cortex (lanes Cx), monkey cerebellum (lanes Cbm), and monkey liver (lanes Li). Conditions for RNA blotting were as described (14). The specific activity of probe in the hybridization mix was 10⁷ cpm/ml and the autoradiographs were exposed for 16 hr using Kodak XRP film and a DuPont Cronex image intensifying screen. Blots were probed subsequently with a CDNA clone (p1B15) of rat cyclophilin (D), a ubiquitously expressed mRNA, as a control for gel loading and RNA integrity. RNA size standard positions (kb) are indicated.
DISCUSSION

mRNA species expressed in cells with anatomic distribution are likely to be present at low absolute abundance in a total brain RNA preparation. For this reason, the differential colony hybridization technique, which is only able to detect RNA species with an abundance of 0.05% or greater (12), is of limited use for cloning cDNAs representing brain mRNAs with regionally heterogeneous distributions (2). RNA-driven subtractive hybridization (8) has the theoretical advantage of permitting the detection of cDNA clones of rarer differentially expressed mRNAs, but it has important disadvantages. First, a large excess of RNA driver over probe (20- to 50-fold) is required for quantitative subtraction. To isolate cDNA clones representing rare mRNAs, at least one secondary screen is necessary. Thus, 80–200 µg of

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**FIG. 5.** Nucleotide sequence of cDNA pMC4G8 determined by “shotgun” sequencing strategy (16). The single long open reading frame is represented by the region of codon triplets with the predicted amino acid sequence of monkey preprosomatostatin I above. Differences between the monkey and human (15) preprosomatostatin I cDNA sequences are indicated as single base substitutions below the monkey sequence. A 3-nucleotide insertion (***)) in the monkey sequence is indicated.
driver poly(A)$^+$ RNA from the complementary tissue is required for cloning. Because oligo(dT)-primed cDNA has a 3'-end overhanging, the amount of driver RNA required is often underestimated. For many applications these mRNA quantities are of the order of a few hundred nanograms. Although R values of 1000–2000 M-sec are theoretically attainable in an RNA-driven hybridization reaction, the values achieved under experimental conditions are actually far lower. This is due to chemical degradation of the RNA during the extended hybridization time at 68°C, which occurs despite careful protection of the RNA from RNases (G.H.T., unpublished observations).

In the subtractive cloning approach presented here, amplified plasmid cDNA from monkey cerebellum in 80-fold excess was used as a driver in a competitive hybridization reaction with a monkey cortex cDNA probe. Because this reaction involved DNA-DNA hybridization, chemical degradation of the driver was less of a problem. Furthermore, the hybridization kinetics were significantly enhanced by performing the reaction in a phenol emulsion (9). A total investment of 6 μg of poly(A)$^+$ RNA was required for each tissue for cDNA library preparation and the subtractive probe screening.

Approximately 1% of 60,000 colonies in a monkey cortex library gave a hybridization signal with the cortex-minus-cerebellum subtracted probe. When these were probed in parallel with unsubtracted cortex and cerebellum cDNA, 160 clones (24%) gave a signal with the cortex but not with the cerebellum probe. Ten clones selected randomly from this group of 160 showed cortex-specific or cortex-enriched distributions when analyzed by RNA blotting. The recovery of clones of mRNAs in this group was nearly quantitative, as 59 of 60 pMC1H8a homologous clones were collected.

Thirty-seven (=6%) of the clones from the original subtractive screen gave no signals with either of the unsubtracted probes on the secondary screen. When these clones were probed with a second cortex-minus-cerebellum subtracted cDNA preparation, about one-fourth did give a clear hybridization signal. RNA blot analysis revealed that three of these clones actually represented low-abundance mRNAs present in cortex but not detectable in cerebellum. The absolute abundances for two of these mRNAs (pMC4G8 and pMC5B3) were both determined to be 0.001% of monkey cortex poly(A)$^+$ RNA.

One of the clones identified with the subtracted probe (pMC4G8) was found to represent the somatostatin I precursor mRNA. On the amino acid level, the cDNA-derived monkey preprosomatostatin I sequence was shown to be identical to the human protein, while 3% sequence divergence was observed on the nucleic acid level (15). When RNA blots containing similar quantities of frontal cortex RNA from normal human and Alzheimer disease brains were probed with pMC4G8, a reduction by a factor of 10 in hybridization to the Alzheimer RNA was observed. Reduction in the amount of somatostatin immunoreactivity in Alzheimer disease (17) as well as the colocalization of somatostatin immunoreactivity with neuritic plaques and neuronal tangles in Alzheimer disease brains (18, 19) has been reported. Reduction in the amount of the somatostatin mRNA in Alzheimer cortex supports the relationship between neurons expressing this neuropeptide and the Alzheimer disease process. This could result either from transcriptional down-regulation or reduced stability of the somatostatin I precursor mRNA in intact cells or from selective loss of somatostatin-containing neurons.

Clone pMC5B3 hybridized to an mRNA with a distribution and abundance similar to that of the pMC4G8 mRNA; it was expressed in neocortex but was not detectable in cerebellum, and it was reduced by a factor of $\approx10$ in Alzheimer disease cortex relative to normal human frontal cortex. Clone pMC5B3 may be useful, in conjunction with pMC4G8, for identifying cells that undergo degeneration in Alzheimer disease. Because it is present in cerebral cortex but not detectable in cerebellum, the pMC5B3 mRNA may be neuron specific.

The studies in this communication have led to the formulation of a method for isolation of cDNA clones corresponding to mRNA molecules present in one tissue sample but absent from another. The method requires modest amounts of input RNA, is technically straightforward, and can result in nearly quantitative recovery of clones representing medium-abundance tissue-specific mRNAs and the recovery of some clones representing low-abundance tissue-specific mRNAs. The present results demonstrate that although there are no high-abundance mRNAs present in cerebral cortex but undetectable in cerebellum of the cynomolgus monkey, several mRNAs with this distribution exist in the medium-abundance group, and presumably even more are present in the low-abundance group. Such mRNAs are likely to encode proteins that give the neocortex some of its specific functional properties.

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