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Isolation of cDNAs of scrapie-modulated RNAs by subtractive hybridization of a cDNA library

(spongiform encephalopathy/avidin–biotin affinity chromatography/glial fibrillary acidic protein/metallothionein/α-crystallin)

JOHN R. DUGUID*†‡, ROBERT G. ROHWER§, AND BRIAN SEED¶||

*GRECC, Edith Nourse Rogers Memorial Veterans Administration Hospital, 200 Springs Road, Bedford, MA 01730; †Department of Biochemistry, Boston University School of Medicine, Boston, MA; §Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599; ¶Department of Molecular Biology, Massachusetts General Hospital, Boston, MA 02114; and ||Department of Genetics, Harvard Medical School, Boston, MA 02115

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ABSTRACT **We have developed a subtractive cloning procedure based on the hybridization of single-stranded cDNA libraries constructed in πH3M, a vector containing the phage M13 origin of replication. We have used this strategy to isolate three transcripts whose abundance is increased in scrapie-infected brain. DNA sequence analysis showed that they represent glial fibrillary acidic protein, metallothionein II, and the B chain of α-crystallin; the latter two may represent a response to stress.**

Scrapie is the best studied example of the subacute spongiform virus encephalopathies (1), a group of slow, transmissible, neurodegenerative diseases characterized by a long latent period, an insidious onset, and a progressive course that in scrapie results in ataxia, wasting, prostration, and death (for reviews, see refs. 1–3). Established human diseases in this class include Creutzfeld–Jakob disease and kuru (1). Scrapie, a disease of sheep and goats, has been passaged in hamsters (4), the source of tissue used in this study.

In this work, a subtractive cloning procedure was developed and used to isolate several cDNAs whose transcripts are modulated in scrapie-infected hamster brain. Nine recombinants were sequenced and shown to represent three different genes, encoding glial fibrillary acidic protein (GFAP), metallothionein, and the B chain of α-crystallin.** GFAP (5, 6) and its mRNA (7, 8) are known to be differentially expressed in scrapie-infected brain as a result of the prominent gliosis caused by the disease. Metallothionein and α-crystallin B-chain messages are an order of magnitude less abundant than GFAP mRNA and have not previously been shown to be modulated by scrapie infection.

Our cloning procedure is based on the subtractive hybridization of cDNA libraries constructed from normal and scrapie-infected brain RNA in the phage M13-origin vector πH3M (9). As shown in Fig. 1, single-stranded phage DNA derived from the normal library was biotinylated and hybridized with single-stranded DNA from the scrapie library. The random orientation of recombinant inserts in the single-stranded vector permits their hybridization, whereas the vector sequences are in a single orientation and therefore cannot hybridize. The biotinylated normal library DNA and the scrapie recombinants hybridized to it were removed with an avidin affinity resin. The remaining scrapie recombinants, enriched for those with an increased abundance in scrapie-infected brain, were used to transform *Escherichia coli*, yielding a "subtracted" library. Differentially expressed recombinants were identified by colony hybridization.

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MATERIALS AND METHODS

Tissue. Outbred weanling female golden Syrian hamsters (Charles River Breeding Laboratories) were inoculated intracerebrally with 50 μl of low-speed supernatant from a 10% brain homogenate (wt/vol in 6.7 mM sodium phosphate/145 mM NaCl, pH 7.4) prepared from hamsters in late clinical disease with hamster scrapie strain 263K [passage 3 of a stock from R. H. Kimberlin, (4)]. Animals in late clinical disease and age-matched controls were killed by CO_2 asphyxiation, and the brains were harvested, frozen on dry ice, and stored at −70°C.

RNA Preparation. Brains were shattered on dry ice in a sealed plastic bag, suspended in 5 M guanidinium thiocyanate/8% 2-mercaptoethanol/50 mM Hepes/5 mM EDTA, pH 7.5 (5 ml per brain), at 20°C (10), and immediately homogenized by using an STD Tissuemizer with either a 182EN (two brains) or a 100EN (one brain) generator (Tekmar, Cincinnati, OH) at full speed for 10 sec. Four volumes of 6 M LiCl was added and the mixture was kept overnight at 0°C, after which it was either used directly or stored at −70°C before centrifugation at 40,000 × *g* for 1 hr at 4°C. The pellet was resuspended in 4 M LiCl at 0°C and centrifuged as above. The washed pellet was dissolved in 10 ml of 0.5% $NaDodSO_4$/20 mM Hepes/10 mM EDTA, pH 7.5, and incubated with proteinase K (Bethesda Research Laboratories) at 100 μg/ml for 1 hr at 37°C. The solution was then extracted twice with phenol/chloroform (1:1, wt/vol) and once with chloroform and the RNA was ethanol-precipitated. mRNA was purified from total RNA by benzoylcellulose chromatography as described by Roberts (11). Benzoylcellulose was the gift of N. Chaudhari and W. Hahn (University of Colorado School of Medicine, Denver).

cDNA Synthesis and Library Construction. mRNA (2 μg) from two brains was dissolved in 5 μl of HE (10 mM Hepes/1 mM EDTA, pH 7.5) and 15 μl of water, heated to 65°C for 1 min, and cooled on ice. cDNA was synthesized by using Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) as recommended by the vendor, with random hexanucleotide (Pharmacia) at 0.5 mg/ml as primer. The second strand was synthesized as described by Okayama and Berg (12). Non-self-complementary *Bst*XI adapters were ligated to the double-stranded cDNA. Molecules longer than 150 base pairs (bp) were selected by potassium acetate gradient centrifugation and ligated into the *Bst*XI site of the vector πH3M as described by Aruffo and Seed (9). The ligation mixture was used to transform 2 ml of

Abbreviation: GFAP, glial fibrillary acidic protein.
‡To whom reprint requests should be addressed.
**The sequences reported in this paper are being deposited in the EMBL/GenBank data base (IntelliGenetics, Mountain View, CA, and Eur. Mol. Biol. Lab., Heidelberg) (accession nos. J03847, J03848, and J03849).

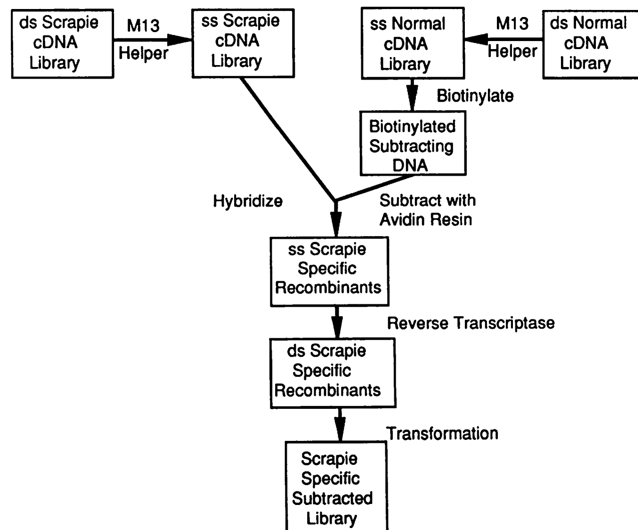

FIG. 1. Schematic diagram of the library subtraction procedure. ss, Single-stranded; ds, double-stranded.

competent host, *E. coli* XS127 (13) or FG2 (a kind gift of C. F. Menck, Centre National de la Recherche Scientifique, Villejuif, France), which were grown to saturation in 100 ml of selection medium [LB (14) containing ampicillin at 25 μg/ml and tetracycline at 6.25 μg/ml] and then stored at -70°C in 30% (vol/vol) glycerol.

Recombinant Propagation and DNA Isolation. Frozen cells (5 ml) representing either the scrapie or the control library were thawed, diluted with 20 ml of selection medium, and incubated with agitation at 37°C for 1 hr. The cultures were inoculated with 10^{12} plaque-forming units of the interference-defective M13 helper phage RV1 (13). After a further incubation for 30 min, the culture was diluted to 1 liter with selection medium and incubated with agitation at 37°C for 16 hr. Phage, phage DNA, and plasmid DNA were isolated as described (13).

Biotinylation. Single-stranded control library DNA (50 μg) was dissolved in 1 ml of HE and sonicated with a Branson sonicator operating at maximum power with the microprobe for 150 sec to attain a mean length of 500 bp. The DNA was ethanol-precipitated, dissolved in 50 μl of HE, and, under safelight, mixed with 50 μl of a 1-mg/ml solution of Photoprobe biotin (Vector Laboratories, Burlingame, CA) and processed as described (15).

Hybridization. Biotinylated control library DNA (25 μg), intact scrapie library DNA (2.5 μg), poly(A) (2 μg), and poly(C) (2 μg) were ethanol-precipitated, dissolved in 5 μl of water, and added to 5 μl of 1.5 M NaCl/50 mM Hepes/10 mM EDTA/0.2% $NaDodSO_4$, pH 7.5. The mixture was heat-sealed in a 20-μl capillary and incubated at 100°C for 1 min and then at 68°C for 20 hr. The mixture was then diluted with 90 μl of HE and incubated at 55°C for 5 min.

Affinity Resin. Sephacryl S-1000 (1 ml packed volume; Sigma) was activated with carbonyl diimidazole (Sigma) (16). All resin incubations were at room temperature with rotary agitation (Labquake, Labindustries, Berkeley, CA). The activated resin was washed once with 10 ml of dioxane and four times with 10 ml of 0.2 M sodium borate (pH 8.5) and then taken up in 1 ml of borate buffer containing 25 mg of biocytin (Sigma) and allowed to react overnight. After addition of ethanolamine to 1 M, the incubation was continued an additional hour. The resin was then washed four times with 10 ml of borate buffer and taken up in 1 ml of resin buffer (RB: 1 M NaCl/20 mM Hepes, pH 7.5). Packed resin (200 μl) was mixed with 200 μl of a 1-mg/ml solution of AvidinDN (Vector Laboratories), incubated 30 min, and then washed four times with 5 ml of RB.

Subtraction. The diluted hybridization mixture was made 1 M NaCl/20 mM Hepes, pH 7.5, and incubated with 100 μl of packed resin in a total volume of 200 μl for 30 min. The mixture was centrifuged 30 sec at 3000 $\times$ g and the supernatant was collected. The resin was washed three times with 200 μl of RB; the supernatants were combined and incubated with 50 μl of packed resin as before. The supernatant and three 100-μl RB washes were combined and ethanol-precipitated. At this point, the subtracted single-stranded scrapie library DNA could be either subjected to a second round of subtraction or used to transform competent cells to generate a subtracted library.

Transformation. The subtracted DNA was dissolved in 100 μl of reverse transcriptase reaction mixture (see above), excluding the random primers and actinomycin D but including 0.1 μM hybridization primer (17). Competent cells (1 ml) were transformed with the reaction mixture and plated on ten 9-cm selection plates. Control experiments showed that the transformation efficiency of double-stranded converted library DNA was 0.5–1.3 $\times$ 10^6 colonies per μg of starting DNA. About 75% of the colony-forming activity of the library was preserved through a mock hybridization and subtraction cycle.

cDNA Probes. cDNA probes were synthesized from scrapie-infected or control brain RNA by using random primers as described above except that [α-^{32}P]dCTP (3000 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear) at 2 Ci/ml replaced the unlabeled dCTP. After incubation at 37°C for 1 hr, the reaction was stopped by the addition of 20 μl of 0.2 M EDTA, and the RNA was hydrolyzed by the addition of 25 μl of 1 M NaOH and incubation for 30 min at 70°C. After neutralization, the unincorporated label was removed by centrifugal Sephadex G-50 chromatography (14).

Colony Hybridization. Replicas were made on nitrocellulose filters (HAWP, Millipore) and prepared for hybridization as described (18). The filters were hybridized with poly(A) at 10 μg/ml and normal hamster brain cDNA probe at 5 $\times$ 10^6 cpm/ml in a volume of 10 ml. After a moderate-stringency wash (four changes of 15 mM NaCl/1.5 mM trisodium citrate/0.1% $NaDodSO_4$, pH 7.0, for 15 min at 50°C), the filters were autoradiographed. The filters then were stripped of bound probe by incubation at 100°C for 5 min in 200 ml of 0.1$\times$ HE/0.1% $NaDodSO_4$ and then hybridized with the scrapie brain cDNA probe as above. Differentially expressed recombinants were identified by overlaying autoradiograms obtained with the two probes.

RNA Blot Analysis. Probe was generated from a selected recombinant by using insert DNA, liberated by *Xho* I digestion (New England Biolabs) and purified by electrophoresis in SeaPlaque agarose (FMC, Rockland, ME). A gel slice containing insert DNA was used to generate probe (19).

Electrophoresis and Hybridization. Total RNA (10 μg) from scrapie or control brain was denatured with formaldehyde and electrophoresed in a 1.5% agarose gel (14). After transfer (20) to GeneScreen*Plus* (New England Nuclear), the filter was baked, blocked, and hybridized with the insert-specific probe as recommended by the vendor. Molecular size standards were obtained from Bethesda Research Laboratories.

Sequence Analysis. Plasmid DNA, prepared as described above, was sequenced (21) by the dideoxynucleotide strategy (22) with avian myeloblastosis virus reverse transcriptase (Life Sciences, St. Petersburg, FL) or modified (23) phage T7 DNA polymerase (Sequenase, United States Biochemical, Cleveland). Oligonucleotide sequencing primers were the gift of John Smith (Massachusetts General Hospital, Boston). Sequence data were analyzed by using the National Biomedical Research Foundation nucleic acid data base[††] and the UWGCG sequence-analysis software (version 5.0).

[††]Protein Identification Resource (1987) Nucleic Acid Sequence Database (Natl. Biomed. Res. Found., Washington, DC), Release 31.0.

RESULTS

The cDNA libraries were constructed from RNA purified by benzoylcellulose chromatography and therefore included both poly(A)$^+$ and poly(A)$^-$ brain mRNA (24). High concentrations of random primers were used to maximize the representation of small RNAs, at the expense of insert length. The scrapie library contained 2.3×10^6 recombinants, with insert lengths of 0.15–1 kilobase (kb); the normal hamster brain library contained 10^6 recombinants.

In an initial experiment the scrapie library was subjected to a single cycle of hybridization and subtraction with the normal library to generate a subtracted library (Fig. 1). Colony hybridization of 10,000 of the resulting recombinants identified 5 that were differentially expressed to a high degree. All 5 of these recombinants cross-hybridized at high stringency (data not shown). The partial sequence of a 0.9-kb recombinant is aligned with that of murine GFAP (the hamster GFAP sequence is not shown) in Fig. 2*A*. Although the nucleic acid sequences differ in 10% of the positions, the translated protein sequences differ by a single conservative substitution. RNA blot analysis identified a 3.0-kb message in the scrapie-infected brain RNA that is considerably more prominent than in the control RNA (Fig. 3*A*).

A second subtracted library was constructed from which GFAP sequences were selectively removed. Insert DNA

A) GFAP

```
      E G Q S L K E E M A R H L Q E Y Q D L L N V K L A L D I E I A T Y R K L L E G E
   1  GAGGGCCAAAGCCTCAAGGAGGAGATGGCTCGCCACCTGCAGGAGTATCAAGATCTACTCAATGTCAAGCTAGCCCTGGACATCGAGATTGCCACCTATAGGAAATTGCTAGAAGGCGAG
 910  GAGGGCCAAAGCCTCAAGGAGGAGATGGCCCGCCACCTGCAGGAGTACCAGGATCTACTCAACGTTAAGCTAGCCCTGGACATCGAGATCGCCACCTACAGGAAATTGCTGGAGGGCGAA
                                A           Y Q     N V                   I     Y         L E E

      E N R I T I P V Q T F S N L Q I R E T S L D T K S V S E G H L K R N I V V K T V
 121  GAAAACCGCATCACCATCCCTGTACAAACTTTCTCCAACCTGCAAATCCGAGAAACCAGCCTGGACACCAAGTCCGTGTCAGAAGGACACCTCAAGAGGAACATCGTGGTAAAGACAGTG
1030  GAAAACCGCATCACCATTCCTGTACAGACTTTCTCCAACCTCCAGATCCGAGAAACCAGCCTGGACACCAAATCCGTGTCAGAAGGCCACCTCAAGAGGAACATCGTGGTAAAGACTGTG
                I     Q           L Q                         K         G                       T

      E M R D G E V I K E S K Q E
 241  GAGATGAGGGATGGTGAGGTCATTAAGGAGTCCAAGCAGGAG
1150  GAGATGCGGGATGGTGAGGTCATTAAGGACTCGAAGCAGGAG
          R               D S
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B) Metallothionein

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                                                                                M D P N C S C A T D G S C S C S G S C K
   1  CACTCAAGTTTCGACTTTTCCTCGGTCCTCAGCCGGTCTTCAACCGCCGCCTTCACTCGCCATGGACCCCAACTGCTCCTGTGCCACAGATGGATCCTGCTCCTGCTCTGGGTCTTGCA
   1                                TCAGCCGGTCTCCAACCGTCGTCTTCACTCGCCATGGACCCCAACTGCTCCTGTGCTACAGATGGATCCTGCTCCTGCGCTGGGTCTTGCA
                                                                                      A                   A

      C K E C K C T T C K K S C C S C C P V G C A K C S Q G C V C K E A S E K C S C C
 121  ATGCAAAGAGTGCAAATGCACCACGTGCAAGAAAAGCTGCTGCTCCTGCTGCCCGGTGGGCTGTGCGAAGTGCTCCCAGGGCTGCGTCTGCAAAGAGGCTTCGGAGAAGTGCAGCTGCT
  93  ATGCAAAGAGTGCAAATGCACCACCTGCAAGAAAAGCTGCTGCTCCTGCTGCCCGGTGGGCTGTGCGAAGTGCTCCCAGGGCTGCGTCTGCAAAGAGGCTTCGGACAAGTGCAGCTGCT
                              T                                                                   D

      A *
 241  CGCCTGAAGCGG.ATTCCCCTCAGCTGTCTGTAAATAGAGCAATGTGTAGAAACGTATTGGTTTTTTTACAACCCCGTCCTATTCTCC
 213  CGCCTGAAGTGGGATTCCCCTCAGCTGCCTGTAAATAGAGCAATGTGTAGAAACGTATT..TTTTTTACAACCCCGGCCTATTCGCC
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C) α-Crystallin B Chain

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                        M D I A I H H P W I R R P F F P F H S P S R L F D Q F F G E H L L
   1  CATACATTCACCTAGCCACCATGGACATCGCCATCCACCACCCCTGGATCCGCCGTCCCTTTTTCCCTTTCCACTCCCCCAGCCGCCTCTTTGACCAGTTCTTCGGAGAGCACCTGTTG
   3  CCTACATTCACCTAGCCACCATGGACATCGCCATCCACCACCCCTGGATCCGCCGTCCCTTTTTCCCTTTCCACTCCCCCAGCCGCCTCTTTGACCAGTTCTTCGGAGAGCACCTGTTG

      S D L F S T A T S L S P F Y L R P P S F L R A P S W I D T G L S E M R M E K D
 121  AGTCTGACCTCTTCTCAACTGCCACTTCTCTGAGTCCCTTCTACCTGCGGCCACCTTCCTTCCTTCGGGCACCCAGCTGGATTGACACTGGACTCTCAGAGATGCGGATGGAGAAGGAC
 123  AGTCTGACCTCTTCTCAACTGCCACTTCTCTGAGTCCCTTCTACCTGCGGCCACCTTCCTTCCTTCGGGCACCCAGCTGGATTGACACTGGACTCTCAGAGATGCGGATGGAGAAGGAC

      F S V N L D V K H F S P E E L K V K V L G D V V E V H G K H E E R Q D E H G F
 241  GATTCTCCGTCAACCTGGATGTGAAGCACTTCTCCCCGGAAGAGCTGAAAGTCAAGGTGCTGGGGGACGTGGTTGAAGTGCATGGCAAGCACGAAGAGCGCCAGGACGAACACGGCTTC
 243  GATTCTCCGTCAACCTGGATGTGAAGCACTTCTCCCCGGAAGAGCTGAAAGTCAAGGTGCTGGGGGACGTGGTTGAAGTGCATGGCAAGCACGAAGAGCGCCAGGACGAACACGGCTTC

      S R E F H R K Y R I P A D V D P L T I T S S L S S D G V L T V N G P R K Q A S
 361  TCTCTAGGGAGTTCCATAGGAAGTACCGGATCCCAGCTGATGTGGATCCTCTGACCATTACTTCATCCCTGTCATCTGACGGCGTCCTCACTGTGAATGGACCAAGGAAACAGGCCTCT
 363  TCTCTAGGGAGTTCCATAGGAAGTACCGGATCCCAGCTGATGTGGATCCTCTGACCATTACTTCATCCCTGTCATCTGACGGCGTCCTCACTGTGAATGGACCAAGGAAACAGGCCTCT

      P E R T I P I T R E E K P A V T A A
 481  GCCCCGAGCGTACCATTCCCATCACCCGTGAAGAGAAGCCTGCTGTCACTGCAGCCC
 483  GCCCCGAGCGTACCATTCCCATCACCCGTGAAGAGAAGCCTGCTGTCACTGCAGCCC
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FIG. 2. DNA sequence analysis of the isolated recombinants. The sequences of the isolated recombinants and their translation products are presented above the reference sequences. (*A*) Sequence of the first recombinant aligned with that of murine GFAP (25). (*B*) Sequence of the second recombinant aligned with that of hamster metallothionein II (26). (*C*) Sequence of the third recombinant aligned with that of the B chain of hamster α-crystallin (27). Amino acid sequences are given in standard one-letter symbols.

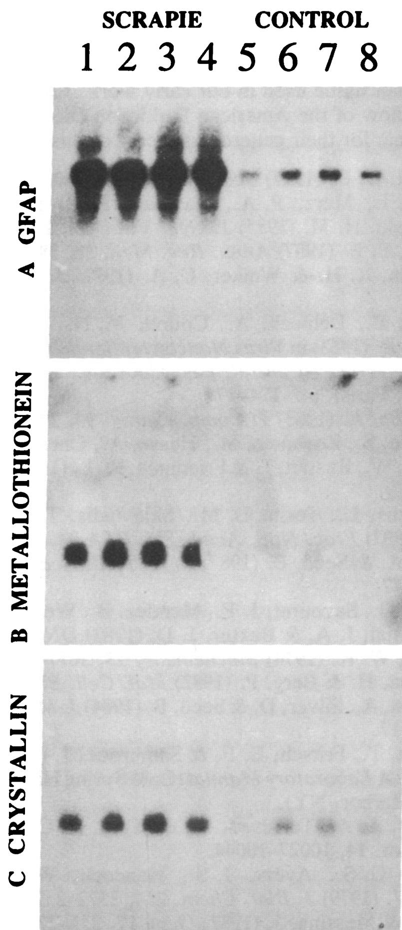

FIG. 3. RNA blot analysis of isolated recombinants. Total RNA (10 μg per lane) from four separate scrapie-infected brains (lanes 1–4) and four control brains (lanes 5–8) was fractionated by agarose gel electrophoresis and hybridized with the GFAP recombinant (*A*), the metallothionein recombinant (*B*), and the α-crystallin recombinant (*C*). Autoradiograms were exposed for 2.5, 24, and 24 hr, respectively.

from two GFAP recombinants was excised by *Xho* I digestion, purified by potassium acetate gradient centrifugation, biotinylated, and included in the hybridizations. Single-stranded DNA (15 μg) from the original scrapie library was subjected to two consecutive cycles of hybridization and subtraction with biotinylated control library DNA (50 μg) and two GFAP inserts (2 μg each) prior to double-strand conversion and transformation. Ten thousand independent recombinants were obtained, with an estimated 100-fold enrichment of scrapie-specific sequences, based on a 75% recovery of biological activity per subtraction cycle.

The colonies were transferred to nitrocellulose filters, hybridized with control cDNA probe, autoradiographed, stripped of the control probe, and then hybridized with scrapie cDNA probe. Autoradiograms of a pair of replicas, along with that of a filter of the unsubtracted scrapie library (Fig. 4), show that subtraction removes abundant sequences present in the scrapie library. Although the vast majority of recombinants in the subtracted library represent RNAs with an abundance below the limit of detection by the cDNA probe, differentially expressed sequences are easily identified (compare Fig. 4 *A* and *B*). Recombinants representing these transcripts were picked, and small-scale DNA preparations (14) were electrophoresed in agarose gels and transferred to nitrocellulose (28). The five most highly modulated sequences, identified by sequential hybridization with normal and scrapie cDNA probes, were selected for sequence analysis.

One of these recombinants contained GFAP sequences 300 bp downstream from the sequence presented in Fig. 2. This clone was spared subtraction because it did not overlap the GFAP sequences included in the construction of the second subtracted library.

A second recombinant, 330 bp long, was closely related to hamster metallothionein II (see Fig. 2*B*). The four differences in the coding sequence resulted in no amino acid change in two instances and conservative changes in the other two. RNA blot analysis using this cDNA identified a 550-base message in the scrapie-infected brain RNA that was considerably more prominent than in the control RNA (Fig. 3*B*). Differences in exposure time required to produce similar signal intensities suggested that the GFAP RNA is approximately an order of magnitude more abundant than the metallothionein RNA in scrapie-infected brain. The sequence of a second cDNA was identical with the first metallothionein isolate.

The sequence of the third message, isolated as a 550-bp recombinant, is presented in Fig. 2*C*. The coding region is identical to that of the B chain of hamster α-crystallin, though there is one discrepancy in the 5′ untranslated region. RNA blot analysis identified a 900-bp message whose abundance was moderately increased in scrapie-infected brain (Fig. 3*C*); the level of this message in scrapie-infected brain is similar to that of metallothionein.

The last sequence determined was that of 18S rRNA. It is not overexpressed in scrapie brain, and its selection here was presumably due to contamination of the scrapie mRNA preparation with rRNA.

DISCUSSION

We have developed a cloning strategy based on the subtractive hybridization of cDNA libraries. We used this procedure

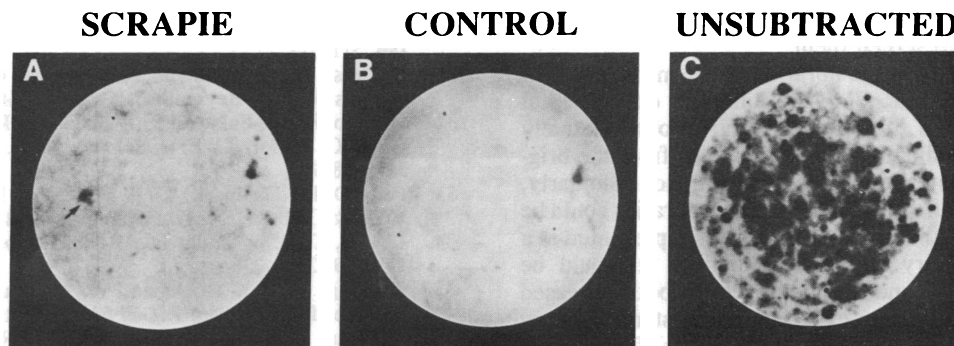

FIG. 4. Colony hybridization of the subtracted and unsubtracted scrapie libraries. One thousand colonies of the twice-subtracted library were hybridized with scrapie cDNA probe (*A*) or with control cDNA probe (*B*). The colony subsequently shown to represent a GFAP recombinant is indicated by an arrow. A filter containing 1000 colonies of the unsubtracted library hybridized with the scrapie cDNA probe is presented in *C*. All autoradiograms were exposed for 24 hr.

to isolate five recombinants representing GFAP mRNA, a moderately abundant message synthesized by fibrous astrocytes; it has been reported to be increased ≈20-fold in scrapie-infected brain (7, 8), which is consistent with our results. A second subtracted library was prepared from which these sequences were specifically removed, permitting the detection of two other modulated sequences.

One of these cDNAs, isolated in two independent recombinants, is closely related to metallothionein II and may represent an allelic polymorphism or a nonallelic form of metallothionein. Although it is an order of magnitude less abundant than GFAP, its degree of modulation is comparable. Metallothionein is induced by exposure to heavy metals, but it is also a stress protein, induced by glucocorticoids, interleukin 1, and bacterial lipopolysaccharide (29). Although there have been no reports of heavy-metal abnormalities in scrapie-infected brain, abnormal brain depositions of aluminum salts occur in Alzheimer disease (30, 31) and in the Guamanian forms of amyotophic lateral sclerosis and Parkinsonian dementia (32, 33). It could be either the nonspecific physiological stress resulting from the clinical disease or some intrinsic feature of the infectious process itself that results in the increased abundance of metallothionein mRNA in scrapie-infected brain.

The third cDNA identified encodes the B chain of α-crystallin. The RNA is moderately more abundant in scrapie-infected brain than in control brain. The B chain of α-crystallin, a major component of the lens, has a strong sequence similarity to the small heat shock proteins (34). Although α-crystallin is not induced in lens organ culture after heat shock (35), it may be induced in response to stress in other tissues.

The reason we did not detect a scrapie-specific cDNA may be that such messages have an abundance below the limit of detection by the cDNA probe used, about 0.01% (36). Alternatively, a message of less than 100 bp, or one with a large amount of secondary structure (11), would not have been represented in our library.

The subtraction system described here is based on the use of avidin affinity resins to bind biotinylated nucleic acids, as pioneered by Ward and colleagues (15). Library subtraction has several advantages over the use of subtracted probe or the cloning of subtracted cDNA (refs. 37 and 38; for review, see ref. 36). First, since large amounts of mRNA are not required for subtractive hybridizations, small nonrenewable tissue specimens can be used. Libraries can be rapidly and efficiently constructed, with yields of 10^6 recombinants from 1 μg of starting mRNA. Moreover, insert-specific probe, equivalent to cDNA probe, can be synthesized from insert sequences isolated from restriction digests of a library in its double-stranded form. Thus, once libraries are constructed, they can be used to generate both subtracted libraries and probes with which to screen them.

Second, once prepared, the libraries can be manipulated in sequential fashion to accomplish different experimental goals. One example of this was the removal of a relatively abundant, highly modulated GFAP sequence from the original scrapie library by specific insert subtraction. Similarly, sequential subtraction with different cDNA libraries could be employed to narrow the scope of sequences represented in a final subtracted library. In many situations it would be desirable to define sequences whose levels are both increased and decreased in a given experimental system. Library subtraction permits the isolation of both classes of transcripts by using the same two starting libraries. Thus, to isolate sequences that are repressed in scrapie-infected brain, one would biotinylate the single-stranded scrapie library and use it to subtract the normal library.

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