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Isolation and characterization of Borna disease agent cDNA clones

(limbic system/behavioral disorders/central nervous system infection)

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ABSTRACT **Borna disease (BD) is a neurologic syndrome characterized by behavioral disturbances and the accumulation of specific proteins in limbic system neurons. A viral etiology has been proposed because BD can be induced in birds, rodents, and primates by inoculation with filtered brain homogenates from animals with BD. We report here the isolation and preliminary characterization of cDNA clones from a rat with BD. These clones hybridized to specific transcripts in BD rat brain and arrested *in vitro* translation of BD proteins. *In situ* hybridization experiments using RNA probes prepared from these clones showed an abundance of these transcripts in limbic system neurons. Northern (RNA) hybridizations using these RNA probes indicated that the BD agent is probably a virus with major transcripts of 8.5, 2.1, and 0.8 kilobases.**

Borna disease (BD) is an immune-mediated neurologic syndrome characterized by profound abnormalities in behavior. Although BD was initially described as an epidemic disease in horses and other livestock, the disease has been experimentally induced in a wide variety of vertebrates, including birds, rodents, and primates, through inoculation of filtered brain extracts from infected animals (1–6). Because the potency of such extracts to induce disease is reduced by exposure to UV or detergents, the BD agent has been suggested to be an enveloped virus (1, 7).

Animals with BD produce antibodies that recognize 60 kDa, 38/40-kDa, 25-kDa, and 14.5-kDa proteins in brains of infected animals and in infected cell cultures (1, 3, 8, 9). These proteins are thought to be encoded by the BD agent because they are present in all animals with BD and because antibodies from one host species recognize the same panel of proteins in other species with BD (1, 3, 8, 9).

The behavioral manifestations of BD vary with the host species. Birds and livestock show motor disturbances (1, 3). Rats have a biphasic course. The acute phase is characterized by displays of aggression, hyperactivity, and ataxia; this phase is followed over a period of several weeks by listlessness, blindness, and, in some animals, by paralysis or obesity (4, 5). These biphasic abnormalities in rat behavior have been correlated with specific alterations in neurotransmitter mRNA levels (6). Tree shrews show abnormalities in social and sexual behaviors (2). In each of these host species, BD proteins are present primarily in limbic system neurons (1, 3, 5). Recent studies suggest that the host range for BD may extend to man. Antibodies to BD proteins have been described in patients with bipolar depressive disorders, in intravenous drug abusers, and in individuals seropositive for human immunodeficiency virus (10–12).

Interest in BD as a model for virus-induced behavioral disorders and recognition of the potential role of BD in human neuropsychiatric disease led us to attempt characterization of the BD agent. Because classical methods for purification of viral particles had not been successful, we adopted a recombinant DNA approach to study this unusual infectious agent. We report here the isolation and preliminary characterization of cDNA clones that appear specific for the BD agent.

MATERIALS AND METHODS

BD Brain Homogenates. Stocks of BD brain homogenate were prepared by inoculating neonatal Lewis rats intracerebrally with brain homogenate from an adult Lewis rat with acute BD (5). Three weeks after inoculation brains were removed and Dounce-homogenized into a 20% suspension (wt/vol) in Eagle's minimal essential medium/2% fetal calf serum (GIBCO), clarified by centrifugation at 2000 × *g* for 10 min at 4°C, and stored at −70°C. Brain homogenates were inoculated into cultures of fetal rabbit brain to determine the titer of infectivity by immunofluorescent assay for BD-specific antigens (5). The titer of brain homogenates (tissue culture 50% infective dose) was ≈4 × 10^6 units/ml.

Animals. To induce acute BD, 4- to 6-week-old inbred male Lewis rats (Charles River Breeding Laboratories, Wilmington, MA, or Research Institute of Scripps Clinic, La Jolla, CA) were inoculated intracerebrally with 10^5 tissue culture 50% infective dose units of BD brain homogenate (5). Three weeks after inoculation rats were sacrificed to extract nucleic acids or to obtain brain sections for immunohistochemistry and *in situ* hybridization. One rat from each cohort of ten inoculated with BD brain homogenate was examined histologically for the presence of inflammatory infiltrates and BD antigens in brain. Normal rats and rats inoculated intracerebrally with brain homogenates from normal adult rats were used as controls.

Nucleic Acid Extraction and Purification. RNA was extracted from brains or lymphoid tissues (lymph nodes, spleen, and thymus) of normal or BD rats by homogenization in guanidinium isothiocyanate and centrifugation through cesium chloride (13). Poly(A)$^+$ and poly(A)$^-$ RNA fractions were isolated by oligo(dT)-cellulose chromatography (14). DNA was extracted from brains of normal or BD rats using SDS and proteinase K (15). The quantity and integrity of DNA and RNA were measured by spectrophotometry and ethidium bromide staining intensity in agarose gels.

BD cDNA Library Preparation and Screening. A cDNA library of 200,000 recombinants in pGEM3 (Promega Biotec) was prepared from 10 μg of BD rat brain poly(A)$^+$ RNA by using the method of Gubler and Hoffman (16). The library was screened first with a ^{32}P-labeled cDNA probe prepared from BD rat brain poly(A)$^+$ RNA enriched for BD-specific sequences by subtraction with normal rat brain cDNA (17). The clones that hybridized with this subtracted probe were

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Abbreviation: BD, Borna disease.

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replicated and screened three times by differential colony hybridization (18) with ^{32}P-labeled probes prepared from BD or normal rat brain cDNA. Clones were then hybridized with a ^{32}P-labeled cDNA probe prepared by using poly(A)$^+$ RNA from rat lymphoid tissues to distinguish host sequences representing infiltrating inflammatory cells. Clones that did not hybridize with the lymphoid cDNA probe were considered candidates for BD-specific cDNAs. These clones were selected for further analysis by restriction mapping, Northern (RNA) and Southern hybridization, and hybrid arrest experiments.

Probes. ^{32}P-labeled cDNA probes for Northern and Southern hybridizations were prepared by nick-translation (19) or random hexanucleotide-primed labeling (20) of plasmid DNA. ^{32}P-labeled RNA probes for Northern hybridizations were prepared from plasmid cDNA templates according to protocols from Promega Biotec. ^{35}S-labeled RNA probes for *in situ* hybridization experiments were prepared as described (21). RNA probes were analyzed by size fractionation on denaturing agarose gels to ensure that they represented the entire cDNA template.

Northern Hybridization. Ten micrograms of RNA from normal or BD rat brain was size-fractionated on 2.2 M formaldehyde/1.2% agarose gels and transferred to nylon membranes as described (22). Membranes were hybridized with ^{32}P-labeled cDNA probes or ^{32}P-labeled RNA probes at 68°C in 6× SSC (1× SSC is 0.15 M sodium chloride/0.015 M sodium citrate)/5× Denhardt's solution (1× Denhardt's solution is 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin)/10 mM EDTA/4 mM sodium pyrophosphate/0.5% SDS/salmon sperm DNA at 250 μg/ml/yeast tRNA at 250 μg/ml for 18 hr and then washed in 0.2× SSC/0.1% SDS at 68°C for 30 min before autoradiography. To reduce background hybridization to ribosomal RNA, membranes hybridized with RNA probes were exposed to RNase A at 100 μg/ml/2× SSC at 37°C for 30 min. RNA size markers including vesicular stomatitis virus [11 kilobases (kb)], poliovirus (7.2 kb), and a commercial RNA ladder (Pharmacia LKB) were used to determine the approximate molecular sizes of RNA transcripts.

Southern Hybridization. Fifteen micrograms of *Eco*RI-digested genomic DNA from normal or BD rat brain was size-fractionated on 1% agarose gels. After depurination, denaturation in alkali, and neutralization, DNA was transferred to nylon membranes, as described (23). Conditions for hybridization, washing, and autoradiography were the same as for Northern hybridizations. *Eco*RI-digested BD plasmid DNA was included in these experiments as a control for sensitivity.

***In Vitro* Translation, Hybrid Arrest, and Immunoprecipitation.** Two-microgram samples of poly(A)$^+$ RNA from normal or BD rat brain were used for *in vitro* translation with rabbit reticulocyte lysates (Promega Biotec). Translated proteins were immunoprecipitated with serum from rabbits with BD and staphylococcal protein A (Pansorbin, Calbiochem) (24). In hybrid arrest experiments (25), 1-μg samples of individual linearized BD cDNA clones or plasmid DNA without insert were hybridized to poly(A)$^+$ RNA before *in vitro* translation. Immunoprecipitated proteins were analyzed by SDS/PAGE and autoradiography.

***In Situ* Hybridization.** Protocols for *in situ* hybridization have been described (21). Normal or BD rats were perfused with 4% buffered paraformaldehyde; brains were fixed overnight and embedded in paraffin. Five-micrometer sagittal sections were collected onto chrom/alum-coated slides. Paraffin was removed with xylene, and sections were treated with proteinase K and 0.05 N HCl to facilitate probe penetration. Hybridization occurred overnight at 52°C in 50% (vol/vol) formamide/0.75 M NaCl/0.02 M Pipes/0.01 M EDTA/10% dextran sulfate/5× Denhardt's solution/50 mM dithiothreitol/0.2% SDS/salmon sperm DNA at 100 μg/ml/yeast tRNA at 100 μg/ml with 20 ng of probe per slide. Slides were rinsed in 4× SSC/300 mM β-mercaptoethanol, incubated with RNase A, and then washed in 2× SSC at 56°C. Slides were dipped in NTB-2 emulsion (Eastman Kodak) for autoradiography at 4°C for 48 hr and then developed in D-19 solution (Eastman Kodak) and fixed in 30% (wt/vol) sodium thiosulfate.

RESULTS

BD Proteins Are Encoded by Poly(A)$^+$ RNAs. Standard recombinant DNA methods depend upon oligo(dT)-primed synthesis of cDNA from poly(A)$^+$ termini of mRNAs. To determine whether BD resulted in transcription of specific poly(A)$^+$ RNAs, poly(A)$^+$ RNA fractions from normal or BD rat brain were translated *in vitro*, and products were immunoprecipitated with serum from normal rabbits or rabbits with BD. Proteins of 38/40 kDa, consistent with major BD antigens, were immunoprecipitated only from translation reactions containing BD rat brain poly(A)$^+$ RNA (Fig. 1). These results indicated that mRNAs specific to BD were contained in the poly(A)$^+$ RNA fraction and supported the use of this fraction and oligo(dT)-priming reactions for synthesis of BD-specific cDNA clones.

Isolation of BD cDNAs. A plasmid cDNA library containing 200,000 individual recombinants was constructed from BD rat brain poly(A)$^+$ RNA; 40,000 recombinants in this library were screened with a cDNA probe prepared from BD rat brain enriched for BD-specific sequences by subtraction with normal rat brain cDNA (see *Materials and Methods*). Approximately 1000 clones were identified as potential BD-

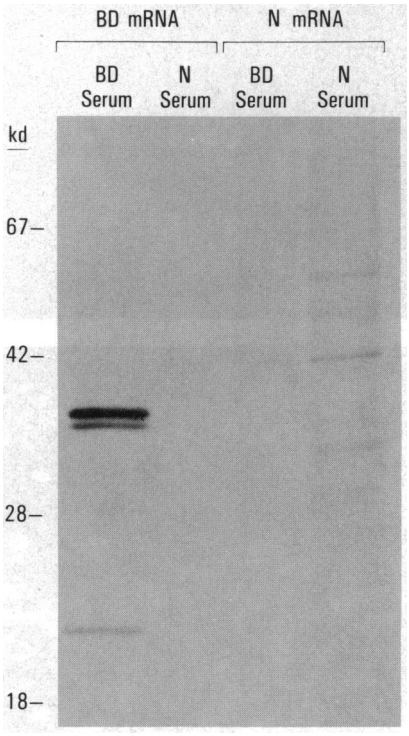

FIG. 1. Immunoprecipitation of BD-specific *in vitro* translation products with serum from a rat with BD. Poly(A)$^+$ RNA extracted from the brain of a normal rat (N) or a rat with BD was translated *in vitro* by using rabbit reticulocyte lysates. [^{35}S]Methionine-labeled translation reactions were incubated first with serum from a normal rabbit (N serum) or a rabbit with BD (BD serum) and then with staphylococcal protein A. Immunoprecipitation products were analyzed by autoradiography after SDS/PAGE. BD serum immunoprecipitated 38/40-kDa and 20-kDa proteins from *in vitro* translation reactions with BD poly(A)$^+$ RNA.

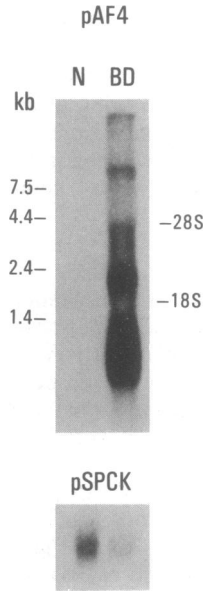

FIG. 2. BD-specific RNA transcripts in BD rat brain. Total cell RNA extracted from the brains of normal (N) or BD rats was size-fractionated by electrophoresis under denaturing conditions, transferred to nylon membranes, hybridized with either pAF4 (BD cDNA clone) or pSPCK (cholecystokinin cDNA), and analyzed by autoradiography. Clone pAF4 hybridized with three RNA transcripts in BD rat brain (8.5, 2.1, and 0.8 kb). Hybridization signal intensity with pSPCK was higher in N brain than in BD brain.

specific clones in this initial screening. Three subsequent rounds of screening by differential hybridization with cDNA probes prepared from BD versus normal rat brain reduced the number of potential BD-specific recombinants to 67. These 67 recombinants were screened with a cDNA probe prepared from lymphoid tissues to identify clones derived from host inflammatory cells. Eleven clones did not hybridize with the lymphoid tissue cDNA probe. Restriction analysis of these 11 clones showed that 7 were unique (4 clones were identical).

Characterization of BD Clones. To confirm that the 7 unique cDNA clones represented BD-specific sequences, plasmid DNAs from individual clones were nick-translated and used as probes in Northern hybridization experiments with total RNA extracted from normal or BD rat brain. A cDNA clone encoding cholecystokinin (26) was also used as a probe to control for the quantity and integrity of brain mRNA.

All BD clones hybridized to abundant 8.5- and 0.8-kb transcripts present only in BD rat brain. In addition, 4 of 7 BD clones hybridized to a 2.1-kb transcript restricted to BD rat brain.

Detection of the three BD-specific transcripts by clone pAF4 is shown in Fig. 2. Identical results were obtained with clone pAB5 (see below). Cholecystokinin mRNA was abundant in normal rat brain; hence, the lack of pAF4 hybridization to normal rat brain RNA was not due to nonspecific RNA degradation (Fig. 2). The observation that cholecystokinin

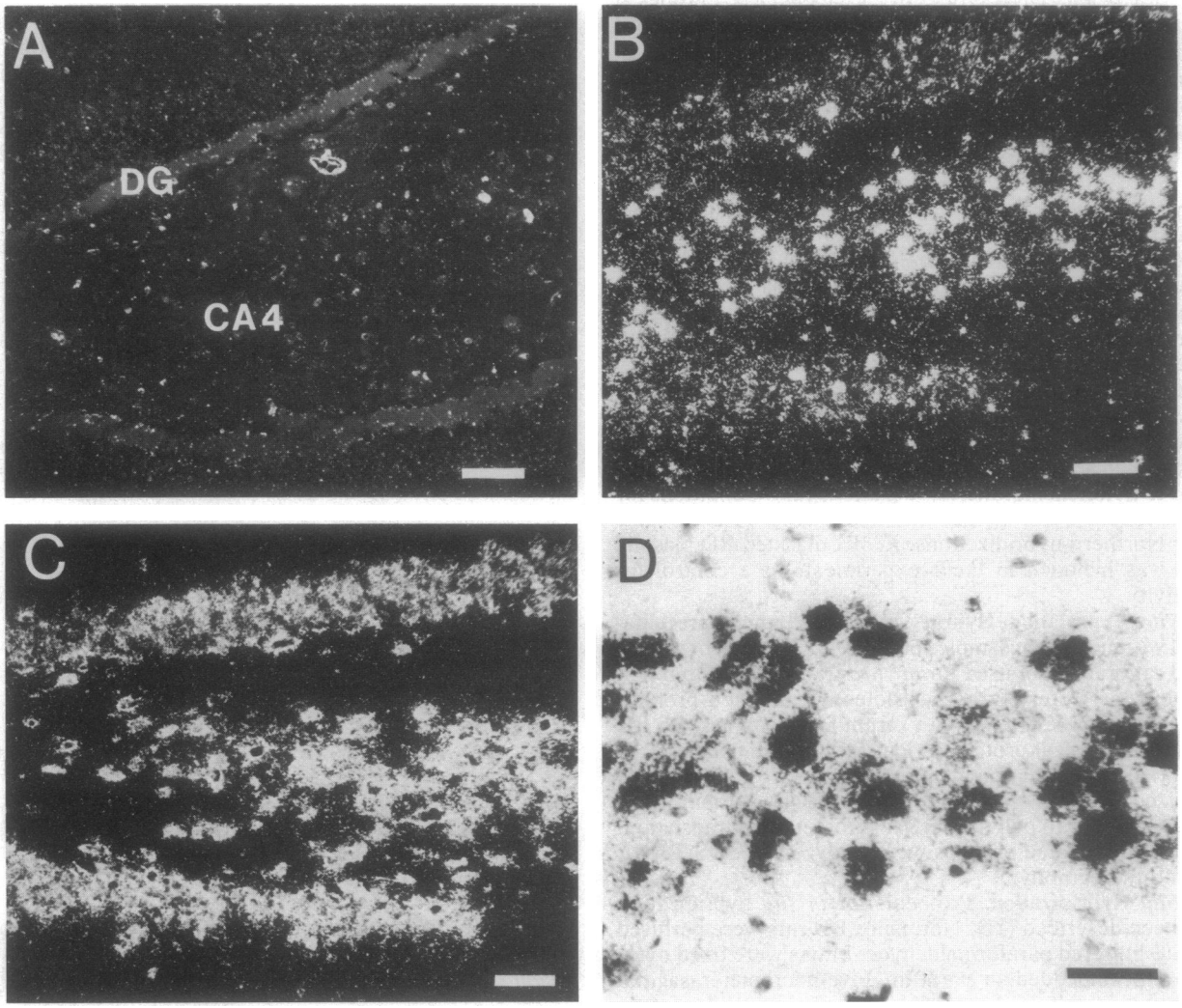

FIG. 3. *In situ* hybridization of BD RNA probes to RNA transcripts in BD or normal (N) rat brain. Five-micrometer sections through hippocampus of N rat (*A*) or BD rat (*B*, *C*, and *D*) hybridized with either pAF4-SP6 (*A* and *B*), or pAF4-T7 (*C* and *D*) photographed in dark-field (*A*, *B*, and *C*) or bright-field (*D*) condition. (*D*) Detail of sector CA4 from *C*. Identical patterns of hybridization were seen with pAF4-SP6 and pAF4-T7, although pAF4-T7 hybridized with higher intensity (see *Results*). DG, dentate gyrus; CA4, sector CA4 of hippocampus. (Bars = 50 μm in *A*, *B*, and *C*; bar = 25 μm in *D*.)

mRNA levels were reduced in brains of rats with BD was consistent with earlier reports (6).

To determine whether these cDNA clones represented host transcripts induced by BD or mRNAs encoded by the putative BD agent genome, Southern hybridizations were performed using nick-translated BD clones pAF4 or pAB5 and genomic DNA extracted from brains of normal rats or rats with BD. Defined quantities of pAF4 or pAB5 plasmid DNA were added to samples of genomic DNA to ensure sufficient sensitivity to detect single-copy genes. Filters were also hybridized with a cDNA probe encoding the single-copy cholecystokinin gene. No hybridization to genomic DNA was seen in either normal or BD rat brain samples in spite of sensitivity to 0.2 copies per genome (data not shown). These results were confirmed with slot blots of DNA from normal and BD rat brain with the same controls as for Southern hybridizations.

Distribution of BD Nucleic Acids in BD Rat Brain. In rats, the onset of clinical disease has been shown to coincide with the appearance of BD proteins in pyramidal neurons in hippocampus (5). To confirm the specificity of isolated cDNA clones for BD, *in situ* hybridization studies were performed with brain sections from normal and BD rats. Complementary RNA probes were prepared from BD clone pAF4 by using the opposing SP6 or T7 promoters of the pGEM vector.

pAF4-SP6 and pAF4-T7 RNA probes hybridized specifically to nucleic acids in BD brains. Normal rat brain did not hybridize with either BD probe. In BD rat brain, BD-probe hybridization signal was distributed in neurons in layers 4 and 5 of cortex and in brainstem but was most dense over limbic structures including thalamus and sectors CA3 and CA4 in hippocampus. The intensity of hybridization signal was higher with pAF4-T7 probes than with pAF4-SP6 probes. A typical pattern of *in situ* hybridization in hippocampus is shown in Fig. 3. Normal hippocampus did not hybridize with probes prepared from either pAF4-SP6 (Fig. 3*A*) or pAF4-T7 (data not shown). In contrast, both BD probes hybridized in BD hippocampus (Fig. 3 *B*, *C*, and *D*). In Fig. 3, the higher intensity of signal seen with the pAF4-T7 probe is readily apparent in the dentate gyrus. Identical patterns of hybridization were seen with both BD probes when duration of autoradiography with pAF4-T7 experiments was decreased to 50% of that allotted for pAF4-SP6 experiments. Pretreatment of tissue sections with RNase A prevented hybridization; pretreatment with DNase had no effect (data not shown).

Relative Polarity of BD-Specific Transcripts. Because the complementary RNA probes, pAF4-SP6 and pAF4-T7, both hybridized to RNA in BD rat brain sections, it was important to determine the polarity of each of the three BD-specific transcripts detected by pAF4 cDNA. To address this question, pAF4-SP6 and pAF4-T7 RNA probes were used in Northern hybridization experiments. Both RNA probes hybridized specifically to BD rat brain RNA. RNA probe pAF4-SP6 hybridized only to an 8.5-kb transcript in BD rat brain RNA. RNA probe pAF4-T7 hybridized primarily to a 2.1- and an 0.8-kb transcript in BD rat brain RNA. RNA probe pAF4-T7 also hybridized at low intensity to an 8.5-kb transcript in BD rat brain (Fig. 4). This confirmed the *in situ* hybridization results and demonstrated that transcripts with complementary sequence are present in BD rat brain.

Correlation of BD cDNA Clones with BD-Specific Proteins. Hybrid-arrest experiments were performed to determine which of the BD-specific proteins were encoded by the isolated BD cDNAs. Poly(A)$^+$ RNA from BD rat brain was hybridized to plasmid DNA from individual BD clones and translated *in vitro* by using rabbit reticulocyte lysates. The protein products were immunoprecipitated with serum from rabbits with BD and analyzed by SDS/PAGE and autoradiography. Controls included mock hybrid-arrest experiments that used plasmid DNA without cDNA insert or plasmid DNA containing a normal rat gene (somatostatin), *in vitro*

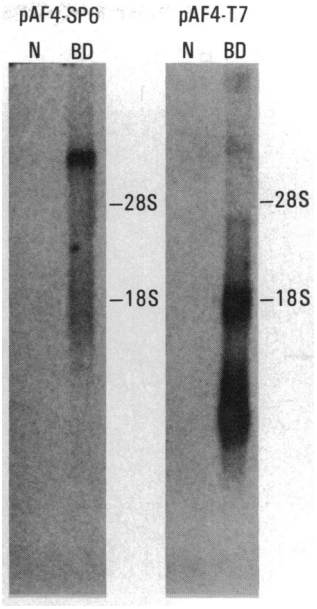

FIG. 4. Polarity of BD-specific RNA transcripts in BD rat brain. Total-cell RNA extracted from brains of normal (N) or BD rats was size-fractionated by electrophoresis under denaturing conditions, transferred to nylon membranes, hybridized with RNA probes transcribed from promoters flanking the cDNA insert in BD clone pAF4, and analyzed by autoradiography. RNA probe pAF4-SP6 hybridized to an 8.5-kb transcript in BD rat brain. RNA probe pAF4-T7 hybridized strongly to 2.1- and 0.8-kb transcripts and minimally to an 8.5-kb transcript in BD rat brain.

translation of poly(A)$^+$ RNA from normal rat brain, and immunoprecipitation of translation products with serum from normal rabbits.

Several proteins were immunoprecipitated by BD serum after *in vitro* translation of BD poly(A)$^+$ RNAs (Fig. 5 *A* and *B*, lanes 3). Hybridization of BD poly(A)$^+$ RNA with BD clone pAB5 blocked translation of 38/40-kDa proteins (Fig. 5*A*). Identical results were obtained in hybrid-arrest experiments with four other BD cDNA clones. In contrast, hybridization of BD poly(A)$^+$ RNA with BD clone pAF4 prevented translation of a 20-kDa protein (Fig. 5*B*). Hybridization of BD poly(A)$^+$ RNA to vector DNA (Fig. 5 *A* and *B*) or a rat somatostatin cDNA clone (data not shown) did not affect translation of BD-specific proteins.

DISCUSSION

Several lines of evidence support the identification of cDNA clones derived from BD rat brain as representing transcripts from the BD agent. (*i*) These cDNAs represent RNAs specific for BD. In Northern hybridizations these cDNAs detected RNA transcripts in BD rat brain that were not present in normal rat brain. Further, as shown by *in situ* hybridization studies, the distribution of these transcripts in BD rat brain correlated precisely with described distribution of BD antigens in infected rat brain (5). (*ii*) These cDNAs were not transcribed from host genomic sequences. Both Southern and slot-blot hybridizations with sensitivity to 0.2 copy per genome provided no evidence of complementary sequence in either normal or BD rat brain DNA. (*iii*) Hybrid-arrest experiments with BD cDNA clones prevented translation of BD-specific proteins. One of these proteins, the 38/40-kDa antigen, has been previously reported in BD. In contrast, the 20-kDa protein has not been described. It remains to be determined whether the 20-kDa protein is another specific BD antigen or undergoes posttranslational modification in infected cells to form the 25-kDa protein not found in *in vitro* translation products.

The BD agent cannot be assigned to a specific virus class from these studies. Southern and slot-blot hybridization experiments with BD probes have not detected DNA target sequences; however, the BD agent could still be encoded by a DNA genome. Although controls were adequate to detect single-copy genes, BD proteins and transcripts are not distributed equally in all brain cells. Hence, these experiments would not have detected BD-agent DNA if it were present in only a small percentage of brain cells.

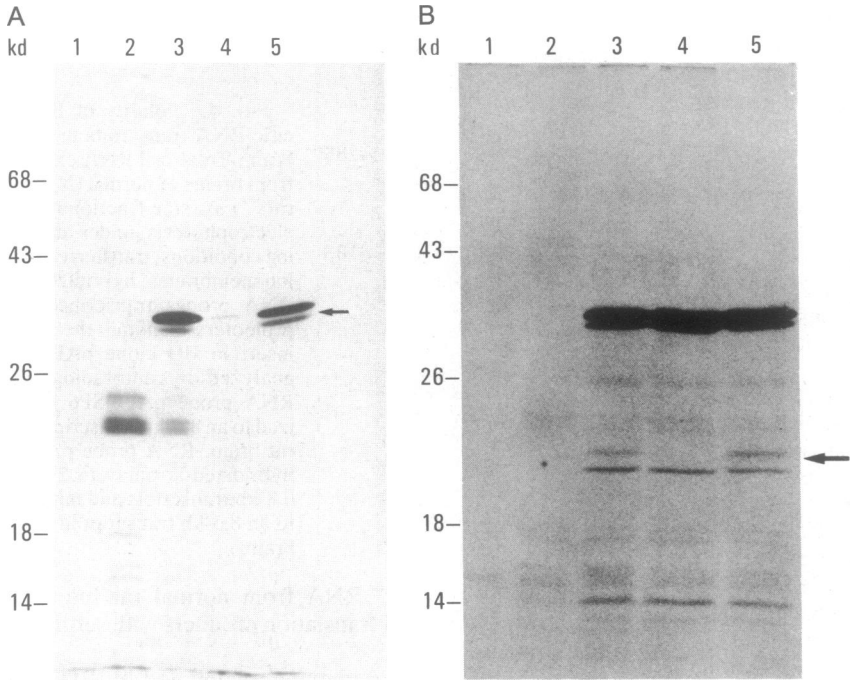

FIG. 5. BD cDNA clones hybrid-arrest *in vitro* translation of BD RNAs. Poly(A)$^+$ RNA extracted from the brain of a normal (N) or BD rat was translated *in vitro* by using rabbit reticulocyte lysates. [^{35}S]Methionine-labeled translation reactions were immunoprecipitated with serum from a N rabbit or a rabbit with BD and staphylococcal protein A. Immunoprecipitation products were analyzed by autoradiography after SDS/PAGE. Poly(A)$^+$ RNA was hybridized to BD cDNA (lanes 4) or plasmid DNA (lanes 5) before *in vitro* translation. Lanes: 1, N RNA, BD serum; 2, BD RNA, N serum; 3, BD RNA, BD serum; 4, BD RNA hybridized with BD cDNA, BD serum; 5, BD RNA hybridized with plasmid DNA, BD serum. (*A*) Hybrid-arrested translation of 38/40-kDa (kd) protein by BD cDNA pAB5 (arrow). (*B*) Hybrid-arrested translation of 20-kDa protein by BD cDNA pAF4 (arrow). Hybridization of BD RNA with plasmid DNA (lanes 5) or rat somatostatin cDNA (data not shown) did not prevent translation of BD RNAs. Nonspecific background bands migrating at 22–24 kDa were seen after immunoprecipitation of BD *in vitro* translation products with serum from N or BD rabbits. The intensity of these bands was higher in *A* than in *B*. Duration of autoradiographic exposure for *B* was longer than for *A* to facilitate visualization of the 20-kDa-BD translation product.

Three BD-specific RNA transcripts were detected in BD brains: 8.5 kb, 2.1 kb, and 0.8 kb. Northern hybridization experiments using strand-specific RNA probes (pAF4-SP6 and pAF4-T7) showed that the most abundant 8.5-kb transcript was complementary to the 2.1- and 0.8-kb transcripts. These data could be explained by postulating the BD agent to be a negative-strand RNA virus. Were this the case, the abundant 8.5-kb RNA might represent the viral genome. The 2.1-kb and 0.8-kb transcripts could be overlapping mRNAs complementary to the major 8.5-kb transcript. The minor 8.5-kb transcript, representing the same strand as the two smaller transcripts, might be a replication intermediate. Recently, Northern hybridization experiments with oligo(dT)-fractionated RNA from BD rat brain indicated that the 2.1-kb and 0.8-kb transcripts are poly(A)$^+$ (J. C. de la Torre and W.I.L., unpublished work). These results lend additional support to the hypothesis that the BD agent is a negative-strand RNA virus.

In summary, these findings support the hypothesis that BD has an infectious etiology and indicate that the BD agent is likely to be an RNA virus with a genome of ≈8.5 kb. Definitive characterization of the BD agent will require sequence analysis and isolation of BD virions. Future work must be directed toward understanding the molecular biology of this infectious agent, its catholic host range, and tropism for selected CNS cell populations. The BD cDNA clones described here will be powerful tools for exploring the potential role of BD as a pathogen in human neurologic and psychiatric diseases.

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