rat brain protein but did bind to glutamate cross-linked by glutaraldehyde to rat brain proteins. Solid-phase absorption of the antibody to glutamate conjugated to bovine serum albumin eliminated positive staining.

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6. The identity of 18 neurons from the arcuate and paraventricular nuclei as neuroendocrine was confirmed in another experiment in which cells projecting to the vascular system were labeled by retrograde axonal transport of intravenous injections of horseradish peroxidase [R. D. Broadwell and M. W. Brightman *J. Comp. Neurol.* **166**, 257 (1976)]. Presynaptic glutamate-immunoreactive boutons were found on each cell (C. Decavel and A. N. van den Pol, in preparation).
7. The number of gold particles per bouton varied with the concentrations of antisera. The ratio of labeling of highly immunoreactive to less reactive structures was similar over several antisera concentrations. The presence of high levels of glutamate in presynaptic boutons supports, but does not prove, the possibility that it is released. All neurons probably contain some glutamate. Those cells that use glutamate as a neurotransmitter appear to maintain higher concentrations of glutamate in their axon terminals than other cells do (*8, 9, 19*), as shown ultrastructurally by ratios of immunogold particles over different cells.
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12. A Zeiss IM35 inverted microscope with a ×20 Olympus ultraviolet objective (numerical aperture = 0.70) was fitted with a computer-controlled shutter to block fluorescent light when a video frame was not being recorded. A Hamamatsu SIT video camera was interfaced with an ITI 151 video processor driven by an IBM AT computer. Processed images were stored on a 2023F Panasonic laser disk recorder. Additional details are found in A. H. Cornell-Bell, S. M. Finkbeiner, M. S. Cooper, S. J. Smith, *Science* **247**, 470 (1990) A Bio-Rad confocal scanning laser microscope was also used to confirm our results..
13. Although our results indicate an important role of kainate and quisqualate receptors in excitatory transmission of hypothalamic neurons, these experiments do not rule out a contribution from the NMDA receptor. Under the appropriate conditions, such as depolarization to about −30 mV, glutamate could induce inward current flow through NMDA receptor–activated channels, and an NMDA component to the decay phase of the EPSP in some paraventricular nucleus neurons could be detected. Nonetheless, our data suggest that NMDA receptors contribute to synaptic transmission less in the hypothalamus than in other brain areas such as the cerebral cortex or hippocampus.
14. Several studies in the supraoptic and paraventricular nuclei have combined intracellular recording and staining with vasopressin and oxytocin-neurophysin immunocytochemistry, antidromic stimulation, Nissl counterstaining, or visual determination of location in slices or sections; the purpose of these multidisciplinary studies has been to identify the electrophysiological properties of the major groups of neurons and neuroendocrine cells in these nuclei [R. D. Andrew, B. A. MacVicar, F. E. Dudek, G. I. Hatton, *Science* **211**, 1187 (1981); P. Cobbet, K. G. Smithson, G. I. Hatton, *Brain Res.* **362**, 7 (1986); J. G. Tasker and F. E. Dudek, *J. Physiol. (London)*, in press; N. W. Hoffman, J. G. Tasker, F. E. Dudek, *Soc. Neurosci. Abstr.* **15**, 1088 (1989) (*2*). The 13 paraventricular cells had high input resistance (200 to 500 megohms), a regular or slowly adapting discharge of action potentials with no low-threshold potential to a depolarizing current pulse, and linear current-voltage relation to about −100 mV. Identical neurons in the paraventricular nucleus have been injected with Lucifer Yellow or biocytin and shown to be magnocellular (Tasker and Dudek) and neurophysin-positive (Hoffman *et al.*); therefore, this cell type has been identified as a neurosecretory cell. These electrophysiological properties are identical to those recorded in the supraoptic nucleus (*2*), which contains primarily neurosecretory cells. The other seven cells displayed low-threshold calcium spikes and strong time-dependent inward rectification [P. Poulain and B. Carette *Brain Res. Bull.* **19**, 453 (1987); Tasker and Dudek; Hoffman *et al.*] This latter cell type is neurophysin-negative and is probably not neurosecretory. EPSPs in all of these cells were blocked in a dose-dependent manner by CNQX.
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A Borna Virus cDNA Encoding a Protein Recognized by Antibodies in Humans with Behavioral Diseases

SUSAN VANDEWOUDE, JUERGEN A. RICHT, MARY CHRISTINE ZINK, RUDOLF ROTT, OPENDRA NARAYAN, JANICE E. CLEMENTS

Borna disease virus (BDV) causes a rare neurological disease in horses and sheep. The virus has not been classified because neither an infectious particle nor a specific nucleic acid had been identified. To identify the genome of BDV, a subtractive complementary DNA expression library was constructed with polyadenylate-selected RNA from a BDV-infected MDCK cell line. A clone (B8) was isolated that specifically hybridized to RNA isolated from BDV-infected brain tissue and BDV-infected cell lines. This clone hybridized to four BDV-specific positive strand RNAs (10.5, 3.6, 2.1, and 0.85 kilobases) and one negative strand RNA (10.5 kilobases) in BDV-infected rat brain. Nucleotide sequence analysis of the clone suggested that it represented a full-length messenger RNA which contained several open reading frames. In vitro transcription and translation of the clone resulted in the synthesis of the 14- and 24-kilodalton BDV-specific proteins. The 24-kilodalton protein, when translated in vitro from the clone, was recognized by antibodies in the sera of patients (three of seven) with behavioral disorders. This BDV-specific clone will provide the means to isolate the other BDV-specific nucleic acids and to identify the virus responsible for Borna disease. In addition, the significance of BDV or a BDV-related virus as a human pathogen can now be more directly examined.

BORNA DISEASE IS AN INFECTIOUS neurological disease that occurs sporadically in horses and sheep in Central Europe (*1*). Brain homogenates from infected animals can be used to infect a large number of animal species, from rodents to nonhuman primates (*2–4*). Studies in rats have shown that the agent is highly neurovirulent and invades the brain from peripheral sites by axonal transport (*2*). It replicates in specific groups of neurons in the cerebral cortex and causes biphasic behavioral disease; the short-term effects of Borna disease include aggression and hyperactivity, and the long-term effects include apathy and eating disorders (*3*). In tree shrews (*Tupaia*

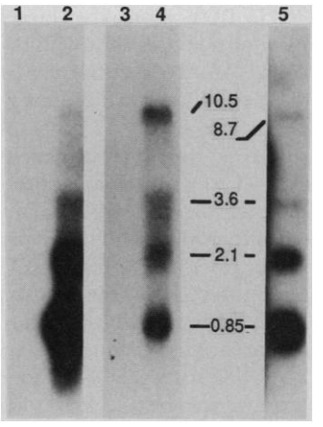

Fig. 1. Specific hybridization of a cDNA clone to RNA isolated from BDV-infected cells and rat brain. Northern blot analysis of RNA (10 μg per lane) from: uninfected MDCK cells (lane 1); BDV-infected MDCK cells (lane 2); uninfected rat brain (lane 3); BDV-infected rat brain (lane 4); and BDV-infected MDCK cells (lane 5). The Northern blot was hybridized with a nick-translated probe made from DNA from the B8 clone (*11*). The rat brain RNA was obtained 6 weeks after inoculation of 1-day-old Lewis rats. Standard molecular techniques were used (*18*). We constructed the cDNA libraries using poly(A)-selected RNA from uninfected and infected MDCK cells using reagents and methods in the Invitrogen Librarian kit. The modified Gubler-Hoffman technique (*19–22*) was used for the synthesis of the cDNA; cDNAs were not size-selected. Colonies of the subtracted library were grown in the presence of IPTG and lifted onto nylon membranes. The microwave method was used to fix proteins to the filters (Invitrogen). A mix of monoclonal antibodies to the 38/39-kD Borna-specific antigen was used to detect clones expressing Borna-specific proteins. The DNA from nine positive clones was nick-translated and used as a probe on Northern blots of RNA from BDV-infected and uninfected rat brain. Standard techniques were used to prepare RNA (*23, 24*). Samples were run on a formaldehyde denaturing gel and transferred to nylon membranes (*25*). Sizes were determined by comparison with a radiolabeled RNA ladder (Bethesda Research Laboratories). Specific hydridization to the RNA from infected brain was detected in one of the nine clones (clone B8).

glis), infection is associated with disruption in social interactions (*4*). Recent studies demonstrate the presence of antibodies against BDV in humans with psychiatric illnesses that include personality disorders and schizophrenia (*5, 6*). The BDV virus replicates in cell cultures and rat brain with the synthesis of 38/39-, 24-, and 14-kD proteins (*7*). However, the virus has not been classified because neither a particle nor a specific nucleic acid has been identified in infectious material.

To identify and clone a BDV-specific nucleic acid, we constructed a subtractive cDNA library from the mRNA isolated from uninfected and BDV-infected MDCK cell lines. We performed two rounds of selection to enrich the cDNA library for BDV-specific cDNAs (Fig. 1). The vector used for cloning contained a polylinker in the *lac z* gene into which inserts were cloned; this allowed bacterial expression of a fusion protein. Colonies were grown in the presence of isopropylthiogalactoside (IPTG) to induce expression from the *lac z* promoter and were screened with monoclonal antibodies specific for the BDV 38/39-kD protein (*7*). Nine antibody-positive clones were identified. The DNA from these clones was nick-translated and hybridized to Northern blots containing RNA from uninfected and BDV-infected rat brain. One clone specifically hybridized to RNA from infected rat brain and showed no hybridization to RNA from uninfected rat brain (Fig. 1). This clone, called B8, contained a 700-bp BDV-specific insert that is an apparently full-length mRNA. The B8 clone hybridized to four RNAs of 10.5 or 8.7, 3.6, 2.1, and 0.85 kb in BDV-infected rat brain and cell cultures (Fig. 1). These four RNAs were enriched by polyadenylate [poly(A)] selection and are apparently (+)strand mRNAs. Further, it was reproducibly found that the largest RNA species in rat brain was 10.5 kb, whereas in BDV-infected cell cultures the largest species was 8.7 kb (Fig. 1, lane 5). This difference may reflect the presence of a defective genome in the persistently infected cell cultures.

To determine the polarity of the RNA species in the BDV-infected cell cultures and tissues, we made strand-specific RNA probes from the B8 clone. The polarity of the B8 clone was determined by sequence analysis; at one end of the clone a stretch of A's was found and at the other end several ATGs were found preceding open reading frames (ORFs). The (−)strand probe showed a similar hybridization pattern to the nick-translated B8 clone and identified four BDV-specific transcripts (10.5, 3.6, 2.1, and 0.85 kb) in BDV-infected rat brain RNA (Fig. 2A). This (−)strand probe also hybridized to RNAs of the same size from poly(A)-selected RNA (Fig. 2A). The presence of apparently subgenomic mRNAs (3.6, 2.1, and 0.85 kb), all of which hybridize with the (−)strand of the B8 clone, suggest that these mRNAs contain common sequences. In contrast, the (+)strand probe hybridized only to a 10.5-kb RNA in BDV-infected rat brain (Fig. 2B).

To eliminate the possibility that the BDV agent is a DNA virus or that the B8 clone represents a cellular gene whose expression was increased in BDV-infected cells, we did Southern hybridization with DNA isolated from uninfected and BDV-infected rat brain and MDCK cells. A single-copy gene equivalent of the B8 clone was run in parallel and was detected under the hybridization conditions used (*8, 9*). When the nick-translated B8 clone was used as a probe (*10*), no hybridization was detected on Southern blots containing 10 μg of DNA from either the BDV-infected or uninfected tissues and cells (*11*).

Nucleotide sequence analysis of the B8 clone revealed two overlapping ORFs (Fig. 3). The larger ORF contained 218 amino acids coding for a protein of 24 kD. Amino acid sequence analysis of peptides from the 24-kD protein of BDV confirms that the amino acid sequence of this ORF corresponds to the 24-kD protein (*12*). The small

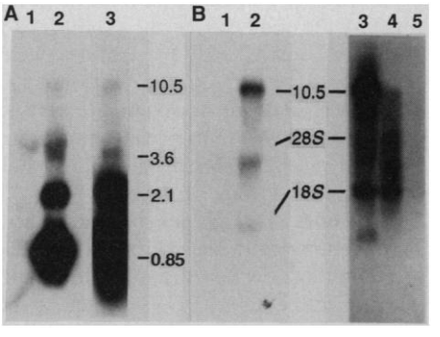

Fig. 2. Determination of polarity of BDV-specific RNAs. (**A**) Negative strand [^{32}P]UTP (uridine 5′ triphosphate)-labeled RNA transcripts of clone B8 hybridized to Northern blots containing: 7.5 μg of RNA from uninfected rat brain (lane 1); 7.5 μg of RNA from BDV-infected rat brain (lane 2); and 2.0 μg of poly(A)-selected RNA from BDV-infected rat brain (lane 3). (**B**) Positive strand [^{32}P]UTP-labeled RNA transcripts of clone P4 hybridized to Northern blots containing: 7.5 μg of RNA from uninfected rat brain (lane 1) and 7.5 μg of RNA from BDV-infected rat brain (lane 2). RNA from BDV-infected rat brain was heated at 90°C for 2 min and chromatographed on oligo(dT) cellulose and RNA was used in Northern blots: 5 μg of total RNA from BDV-infected rat brain (lane 3); 3 μg of RNA from oligo(dT) column wash (lane 4); and 3 μg of poly(A)-containing RNA (lane 5). Clone P4 was constructed by subcloning the entire B8 cDNA insert into the PGEM3 vector to enable transcription of the B8 clone in both directions using T7 polymerase (Promega). Sequence analysis revealed that T7 polymerase would direct transcription of the (+)strand of the P4 cDNA clone and the (−)strand of the B8 cDNA clone. After linearizing the plasmids with restriction enzyme digestion, we used T7 polymerase to direct RNA synthesis in vitro for both constructs. [^{32}P]UTP was used in the reaction to label the transcripts. RNA was isolated from tissues, and cells and Northern blots were prepared as described in Fig. 1. Poly(A) RNA was selected by chromotography on oligo(dT) cellulose. Standard conditions were used for hybridization to Northern blots. Stringent washes were performed using 0.1× standard saline citrate at 65°C for 2 hours.

S. VandeWoude, Colorado State University, Lab Animal Resources, Painter Center, Fort Collins, CO 80532.
J. A. Richt, M. C. Zink, O. Narayan, J. E. Clements, Division of Comparative Medicine, Johns Hopkins University, School of Medicine, Baltimore, MD 21205.
R. Rott, Institute of Virology, Justus-Liebig-University-Giessen, Federal Republic of Germany.

Fig. 3. Amino acid sequence of two ORFs in the B8 cDNA clone. (**A**) The large ORF contains 218 amino acids and the methionine residue is at base pair 39 in the B8 clone. (**B**) The small ORF contains 78 amino acids and overlaps the first ORF. The methionine residue is at base pair 88 in the B8 clone (*26, 27*).

ORF contained 78 amino acids coding for a protein of 9 kD. The nucleotide sequence and the amino acid sequence of both ORFs were searched against the GenBank and European Molecular Biology Laboratory (EMBL) databases, and no significant similarities were detected between the B8 clone and any viral or cellular gene.

To determine whether the B8 clone encoded BDV-specific proteins, we transcribed the clone in vitro in both orientations. The RNAs were translated in a rabbit reticulocyte lysate with ^{35}S-labeled methionine, and the proteins were analyzed by immunoprecipitation with polyclonal and monoclonal antibodies to BDV (Fig. 4). In parallel, poly(A)-selected RNA from BDV-infected rat brain was translated and analyzed by immunoprecipitation with the same BDV-specific antibodies (Fig. 4). RNA corresponding to the (+)strand of the B8 clone directed the synthesis of four proteins (24, 15.5, 14.5, and 13 kD) that were recognized by BDV-specific polyclonal antibodies. In addition, some monoclonal antibodies to both the 38/39-kD and the 24-kD BDV proteins immunoprecipitated the same bands. The recognition of the 24-kD protein by monoclonal antibodies against the 38/39-kD protein has been observed previously (*12*). The mRNA from rat brain directed the synthesis of the 38/39-, 24-, 15.5-, 14.5-, and 13-kD proteins that were immunoprecipitated by the BDV-specific polyclonal and monoclonal antibodies. These data provide further evidence that the B8 clone is a BDV-specific nucleic acid and encodes BDV-specific proteins.

Antibodies to BDV have been identified in the serum of psychiatric patients by indirect immunofluorescence (*5, 6*). To examine whether antibodies to BDV in human sera would recognize the virus-specific proteins encoded by the B8 clone, we tested sera from normal controls (*5*) and from psychiatric patients (*5*) with behavioral disorders from areas in Germany endemic for BDV. The serum from two of the patients had been shown by indirect immunofluorescence to have antibodies to BDV antigens in infected MDCK cells. One serum recognized the 24-kD BDV protein and one the 38-kD BDV protein on protein immunoblots of homogenates of BDV-infected rat brain (*13*). To determine if antibodies in these human sera would recognize the 24-kD protein encoded by the B8 clone, we analyzed ^{35}S-labeled protein translated in vitro (Fig. 4) by immunoprecipitation. In three of the patients' sera, including the serum previously shown by protein immunoblot to recognize the 24-kD protein, antibodies recognized the 24-kD protein (Fig. 5). It has been observed by protein immunoblot analysis of human sera that either the 24-kD or the 38/39-kD protein, but not both, is recognized. Recognition of both proteins is characteristic of immune serum from animals infected with BDV. There was little or no antigenic response to the BDV protein in seven normal controls. These results suggest the presence of BDV or a BDV-related virus in humans; a role for such an agent in human disease remains to be determined. However, this cross-reactivity may allow the use of cloned BDV proteins and nucleic acid to identify and isolate a BDV-related human virus.

The B8 cDNA clone characterized in this report represents a highly specific nucleic acid probe for the BDV agent. In situ hybridization using the B8 clone on sections of uninfected and BDV-infected rat brain showed hybridization only to RNA in infected brain. Hybridization was localized to neurons in the cerebral cortex, thalamus, the dentate gyrus, and the CA4 and CA3 regions of the pyramidal gyrus of the hippocampus. This pattern of hybridization is remarkably similar to the localization of BDV antigen established by classical histopathological and immunocytochemical techniques (*14*). The B8 clone most likely represents a cDNA copy of the 0.85-kb mRNA species. Nucleotide sequence analysis showed that the cDNA contained two ORFs, one of which most likely corresponds to the 24-kD protein observed in BDV-infected cells. The fact that this clone strongly hybridizes to the two other small mRNAs (3.6 and 2.1 kb) suggests that these mRNAs share common sequences. They could represent a nested set of partially overlapping mRNAs or mRNAs with common 5′ leader or 3′ specific sequences. The antigenic cross-reaction between the 38/39- and 24-kD proteins suggests that the coding regions for

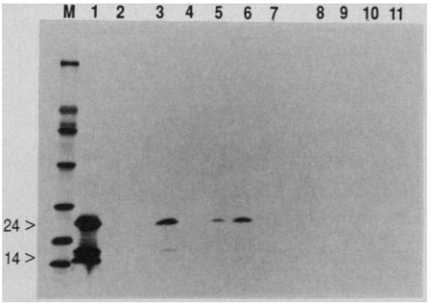

Fig. 5. Analyses of antibodies in human serum by immunoprecipitation of the BDV-specific proteins translated from the B8 clone. RNA transcribed in vitro from the P4 clone [(+)strand RNA] was translated in vitro as described in Fig. 4. The lanes contain 1×10^5 cpm of [^{35}S]methionine-labeled proteins immunoprecipitated with the following sera: BDV-infected rat (lane 1); normal rat (lane 2); human anti-BDV-p24 (lane 3); human anti-BDV-p38/39 (lane 4); patient 112 (lane 5); patient 114 (lane 6); patient 115 (lane 7); and normal human sera (lanes 8 to 11). Lane M contains molecular weight markers (^{14}C-labeled), from top to bottom: 200-, 92.5-, 69-, 46-, 30-, 21.5-, and 14.3-kD proteins.

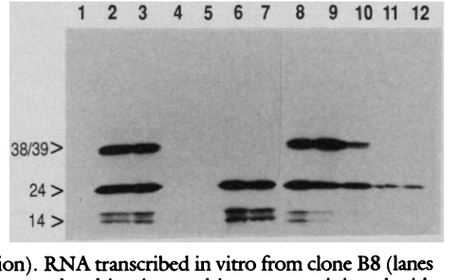

Fig. 4. Identification of BDV-specific proteins encoded by the B8 clone. Poly(A)-selected RNA from uninfected rat brain (lane 1) and BDV-infected rat brain (lanes 2, 3, 8, 9, and 10) were translated in vitro and immunoprecipitated with polyclonal anti-BDV sera from rat (lanes 2, 3, and 8), rabbit (lanes 1 and 5), or monoclonal antibodies to the 38/39-kD protein (lane 9) and the 24-kD protein (lane 10). In vitro transcription with T7 polymerase synthesized the (−)strand RNA from the B8 clone and (+)strand RNA from the P4 clone (B8 subcloned in reverse orientation). RNA transcribed in vitro from clone B8 (lanes 4 and 5) and clone P4 (lanes 6, 7, 11, and 12) was translated in vitro and immunoprecipitated with polyclonal anti-BDV sera from rat (lanes 4 and 8), rabbit (lanes 5 and 7), or monoclonal antibodies to the 38/39-kD protein (lane 11) and 24-kD protein (lane 12). Controls were in vitro translation reactions without RNA, which were immunoprecipitated with polyclonal anti-BDV sera from rats, rabbit, 38/39-kD monoclonal antibodies, and 24-kD monoclonal antibodies. There was no protein detected in any of these controls. Rat brain RNA was isolated and poly(A)-selected (*15, 20, 21*). Poly(A)-selected RNA (0.5 μg) was translated in vitro using staphylococcal nuclease–treated rabbit reticulocyte lysate (Promega) and [^{35}S]methionine as a label. In vitro transcription of clones B8 and P4 was carried out with 1 μg of linearized DNA template in a standard reaction with T4 polymerase and m′G (5′) pppG (cap analog) (Stratagene). This RNA (0.1 μg) was used in the in vitro translation system described above. Immunoprecipitation (using 1×10^5 cpm per sample) was carried out at 37°C for 1 hour (polyclonal sera) or 4 hours (monoclonal sera), protein G–Sepharose beads were added, and the mixture was incubated overnight at 4°C (*14*). Samples were run on 5 to 20% gradient SDS-polyacrylamide gels, and fluorography was performed (Entensify, New England Nuclear). Gels were dried and exposed at −70°C.

the proteins overlap. The (+)strand and (−)strand probes hybridize with equal intensity to 10.5-kb RNA species in BDV-infected rat brain RNA, strongly suggesting that the 10.5-kb RNA species represents the viral genome. Isolation of the BDV agent will be necessary to determine whether the virus contains a (+)strand, (−)-strand, or double-stranded RNA genome. The biology of the BDV agent resembles rabies virus in that both viruses are extremely neurotropic (*2*). Lymphocytic choriomeningitis virus (LCM) and BDV have similar immunopathological mechanisms that contribute to disease (*15*). Both rabies virus and LCM viruses have (−)strand genomes. However, the production of small cross-hybridizing mRNAs by the BDV agent is similar to the organization and gene expression observed for coronaviruses (*16*). Thus, the isolation of cDNAs for the other BDV mRNAs and the genomic BDV RNA will be required to resolve the question of the genetic organization of the BDV.

While this paper was in preparation, another cDNA clone for BDV was reported that has a similar but not identical hybridization pattern to RNA in BDV-infected rat brain (*17*). The conclusion of Lipkin and co-workers was that BDV is a (−)strand virus. However, our data do not support that conclusion because equal quantities of positive- and negative-polarity 10.5-kD RNA were detected in BDV-infected rat brain. Further, the nucleotide sequence of the B8 clone contains a normal amount of the dinucleotide CG, which is more characteristic of (+)strand RNA than (−)strand RNA viruses. The molecular clone described in this report will allow further studies on the pathogenesis of Borna disease and the extension of such studies to human disease.

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27. Single-letter abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

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