VIROLOGY 179, 853–856 (1990)

SHORT COMMUNICATIONS

Molecular Characterization of the Borna Disease Agent

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Received May 11, 1990; accepted August 13, 1990

Borna disease (BD) is a neurologic syndrome characterized by profound disturbances in behavior and the accumulation of specific antigens in limbic system neurons. The potency of brain homogenates from animals with BD to cause disease in normal animals is reduced by exposure to detergents. We have recently described isolation and characterization of clones derived from the BD agent. Here we present evidence that suggests that the BD agent is a negative-sense, single-strand RNA virus. The 8.5-kb genome of this virus appears to be associated with nuclei and encodes two major RNA transcripts of 2.1 and 0.8 kb. © 1990 Academic Press, Inc.

The Borna disease (BD) agent is an unclassified pathogen that causes immune-mediated neurologic disease characterized by marked abnormalities in behavior in a wide variety of host species (*1–5*). Recent reports indicate that the BD agent may have a role in human neuropsychiatric diseases (*6–8*). It has been suggested that the BD agent is likely to be an enveloped virus because the potential for brain homogenates from infected animals to cause disease is reduced by exposure to detergents (*1, 9*).

We have reported isolation of BD agent cDNA clones using a library prepared from the brain of a rat with acute BD (*10*). These clones detected RNA transcripts with MW 8.5, 2.1, and 0.8 kb in BD rat brain. BD cDNAs did not detect BD nucleic acid sequences in Southern hybridizations using DNA extracted from BD rat brain. This result led us to conclude that the transcripts were not host-encoded. *In situ* hybridization studies showed that BD transcripts were not distributed in all cells in BD rat brain. Thus, inability to detect BD DNA sequences in BD rat brain did not exclude the possibility that the BD agent could be either a DNA virus or a retrovirus.

In this paper we present hybridization experiments using BD cDNA clones and a cell line persistently infected with the BD agent (MBV) (*11*). Our results indicate that the BD agent is not a retrovirus or a DNA virus. In addition, we show that the two smaller RNA transcripts (2.1 and 0.8 kb) are poly(A)$^+$ and that the 8.5-kb transcript appears to be associated with nuclei.

Figure 1 demonstrates that all of the cells in the MBV cell line contained BD-specific antigens and BD nucleic acids (Figs. 1B and D). In contrast, none of the cells in the normal control cell line contained either BD-specific antigens or BD nucleic acids (Figs. 1A and C). Thus, the MBV cell line was suitable for addressing the possibility that the BD agent might have a DNA genome present at the level of a single copy per cell.

As shown in Fig. 2, a BD cDNA probe did not detect BD sequences in *Eco*RV-digested genomic or episomal DNA from MBV cells or MDCK cells. Clone pAB5 was used for these experiments because its cDNA insert has an 0.8-kb *Eco*RV restriction fragment. Therefore, failure of pAB5 to detect BD nucleic acids was not due to random integration of BD sequences into the host genome. The sensitivity of our hybridization procedure for detection of BD sequences was less than 1 copy per cell genome. Probe pAB5 detected 1 pg of the 0.8-kb *Eco*RV fragment corresponding to 0.5 copies per genome (Fig. 2, lane 6). Furthermore, when filters were hybridized with a somatostatin cDNA probe (*12*), we detected this single-copy gene in genomic DNA from both MDCK cells and MBV cells (Fig. 2B). These results indicate that the BD agent is unlikely to be either a retrovirus or a DNA virus.

To determine whether MBV cells could serve as a system for studying the molecular biology of the BD agent we analyzed BD transcripts in these cells by Northern hybridization. MBV cells contained the same three major transcripts (8.5, 2.1, and 0.8 kb) previously identified in BD rat brain (Fig. 3A, lanes 2 and 7). In some experiments we detected an additional band with MW of 3.5 kb (Fig. 3A, lane 2, and Fig. 3B, lane 3). Although this additional transcript was not always present, we believe it to be BD-specific. One explanation for ambiguity could relate to the nature of the probe used. For example, it is possible that pAF4 probe contains a small amount of sequence representing the 3.5-kb

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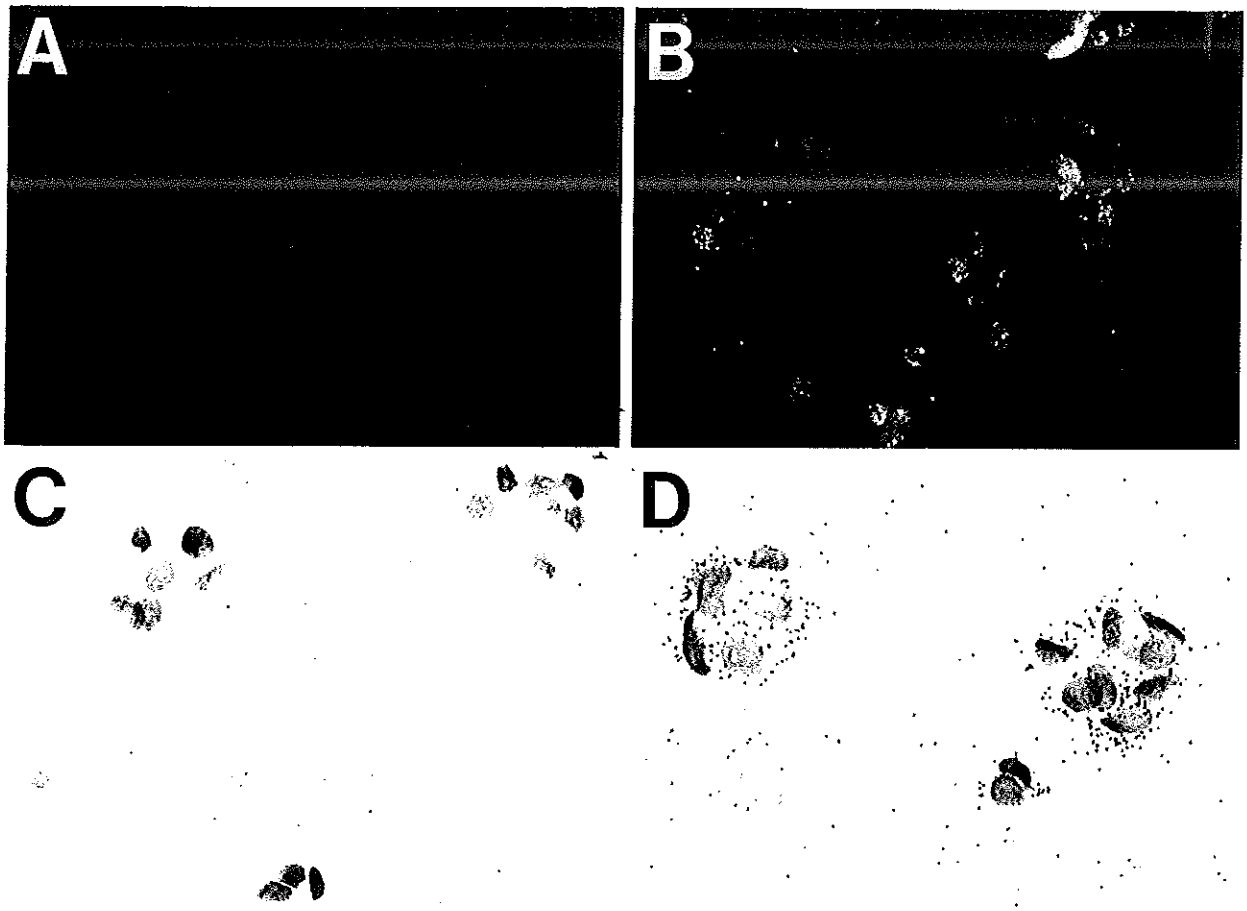

FIG. 1. BD antigens and BD nucleic acids in persistently infected MDCK cells (MBV). Normal MDCK cells (A, C) or MBV cells (B, D) were grown to equivalent density on chamber slides. To detect BD antigens (A, B), cells were fixed in acetone, incubated first with antibodies from a rabbit with BD, and then incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin (Caltag, South San Francisco, CA). To detect BD nucleic acids (C, D), cells were fixed by immersion in 4% buffered paraformaldehyde and then hybridized to BD agent riboprobes pAF4-SP6 and pAF4-T7 as described (*10*). Identical patterns of hybridization were obtained with both probes. Only results with pAF4-T7 are shown.

transcript. Alternatively, the level of the 3.5-kb transcript might be variable.

Oligo(dT)-cellulose chromatographic fractionation showed that the 2.1- and 0.8-kb transcripts are poly(A)$^+$ (Fig. 3A, lanes 8 and 9, and Fig. 3B). Interestingly, when we compared total RNA (nuclear and cytoplasmic) with cytoplasmic RNA, we found that the 8.5-kb transcript was absent from the latter (compare lanes

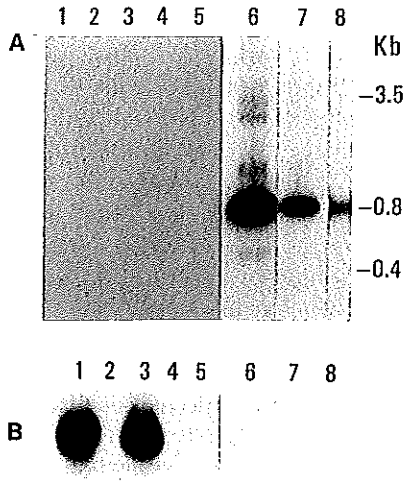

FIG. 2. Southern hybridization experiments indicate that the BD agent is not a DNA virus or a retrovirus. Genomic and episomal DNA from MDCK and MBV cells was extracted as described (*15, 16*). All DNA samples were digested with *Eco*RV, proteinase K treated (50 μg/ml) in the presence of 0.5% SDS, phenol–chloroform extracted, and ethanol precipitated. The samples were quantified by 260 nm absorbance, size-fractionated by 0.75% agarose gel electrophoresis, and transferred to nylon membranes as described (*17*). Hybridization and washing conditions were as described (*10*). Probes [^{32}P]cDNA pAB5 and [^{32}P]cDNA somatostatin were prepared by nick-translation (*18*). Samples were (lane 1) MDCK genomic DNA (15 μg); (lane 2) MDCK episomal DNA (7 μg); (lane 3) MBV genomic DNA (15 μg); (lanes 4 and 5) MBV episomal DNA (8 and 20 μg, respectively); (lanes 6, 7 and 8) pAB5 cDNA, *Eco*RV-digested (1, 10, and 100 pg, respectively). Probes used were (A) ^{32}P-pAB5 *Eco*RV fragment and (B) ^{32}P-cDNA somatostatin.

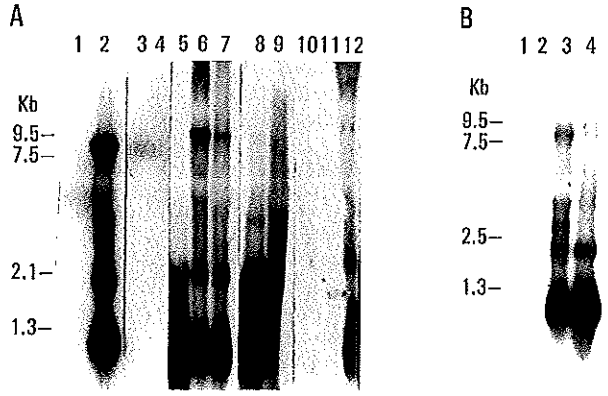

FIG. 3. Characterization of BD transcripts in MBV cells and BD rat brain. (A) Transcripts with MW 8.5, 2.1, and 0.8 kb were detected in BD rat brain (lane 2) and MBV cells (lane 7), but not in normal rat brain (lane 1) or in MDCK cells (lane 4). Only the 2.1- and 0.8-kb BD transcripts were detected in cytoplasmic extracts from MBV cells (lane 5). The 8.5-kb BD transcript was enriched in the nuclear fraction from MBV cells (lane 6). Only the 2.1- and 0.8-kb transcripts were detected in the poly(A)$^+$ fractions of RNA from MBV cells (lane 8). RNase treatment prevented hybridization of BD cDNA probes to RNA from MBV cells (lane 10) or BD rat brain (lane 11). DNase did not affect hybridization of BD probes to RNA from MBV cells (lane 12) or BD rat brain (not shown). Size-markers apply only to lanes 1 and 2. (B) Only the 2.1- and 0.8-kb transcripts were detected in poly(A)$^+$ fractions of RNA from BD rat brain (lane 4). Samples in A were (lanes 1 and 2) total RNA (10 μg) from normal and BD rat brain, respectively, (lane 3) 10 μg of MDCK cytoplasmic RNA, (lane 4) 10 μg MDCK total RNA, (lane 5) 10 μg MBV cytoplasmic RNA, (lane 6) MBV nuclear RNA (amount corresponding to 20 times more cells than used for cytoplasmic fraction), (lane 7) 10 μg MBV total RNA, (lane 8) 2 μg MBV poly(A)$^+$ RNA, (lane 9) 10 μg MBV poly(A)$^-$ RNA, (lanes 10 and 11) 10 μg of total RNA extracted from MBV cells or BD rat brain, respectively, treated with RNase A, (lane 12) 10 μg of MBV total RNA treated with DNase I. Samples in B were (lane 1) 10 μg normal rat brain total RNA, (lane 2) 1.5 μg normal rat brain poly(A)$^+$, (lane 3) 10 μg BD rat brain total RNA, (lane 4) 1.5 μg BD rat brain poly(A)$^+$. Total RNA was isolated as described (*19*). To isolate cytoplasmic or nuclear RNA, cells were harvested and resuspended in 150 m*M* NaCl/10 m*M* Tris–HCl, pH 8.5/1.5 m*M* $MgCl_2$/0.5% NP-40. After 10 min at 0–4°, to allow cell lysis, the cell extracts were centrifuged at 1000 *g* for 5 min. The nuclear pellet was resuspended in 100 m*M* Tris–HCl, pH 7.5/12.5 m*M* EDTA/150 m*M* NaCl/1% SDS and passaged five times through a 21 gauge needle. Both cytoplasmic supernatants (adjusted to 1% SDS) and resuspended nuclei were incubated for 30 min at 55 with 200 μg/ml proteinase K. Following phenol–chloroform extraction and ethanol precipitation, total nucleic acid from nuclei was digested using 1 unit/μg of RNase-free DNase in 50 m*M* Tris–HCl, pH 7.5/10 m*M* $MgCl_2$ for 30 min at 37°, phenol–chloroform extracted, and ethanol precipitated. RNase treatment: 10 μg of total RNA extracted from MBV cells or BD rat brain was digested for 1 hr at 37° in 1× SSC having 1 μg/ml of RNase A. DNase treatment: 10 μg of total RNA from MBV cells or BD rat brain was digested for 1 hr at 37° in 10 m*M* Tris–HCl, pH 7.5/100 m*M* NaCl/10 m*M* $MgCl_2$ using 20 units of RNase-free DNase I. Poly(A)$^+$ fractions were purified through oiligo(dT)-cellulose chromatography as described (*20*). Samples were boiled for 3 min before salt addition and chromatography purification were performed. These precautions were taken to prevent artifacts due to annealing between poly(A)$^+$ and poly(A)$^-$ species if they had complementary sequences. Samples were quantified by 260 nm absorbance, size-fractionated by 2.2 *M* formaldehyde/1% agarose gel electrophoresis, and transferred to nylon membranes as described (*21*). Hybridization and washing conditions were as described (*10*). pAF4 [^{32}P]cDNA labeled by nick-translation (*18*) was used as probe.

5, 6, and 7, Fig. 3A). The nuclear fraction of MBV cells was highly enriched for the 8.5-kb transcript (Fig. 3A, lanes 5, 6, and 7). The 2.1- and 0.8-kb transcripts were also detected in the nuclear fraction, though at much lower levels, (Fig. 3A, lanes 5, 6, and 7). It remains to be determined whether the presence of the 2.1- and 0.8-kb transcripts in RNA extracted from nuclei represent incomplete purification of the nuclear fraction. An alternate explanation may be that BD mRNAs are transcribed in nuclei and then transported to the cytoplasm.

To address the possibility that the BD agent might be a double-strand RNA virus, BD rat brain RNA and MBV RNA were exposed to pancreatic RNase prior to use in Northern hybridization experiments. Treatment with RNase, but not with DNase, prevented hybridization to BD cDNA probes (Fig. 3A, lanes 10, 11, and 12). These results suggested that BD agent is not a double-strand RNA virus.

The Southern hybridization experiments presented argue strongly against the possibility of the BD agent being either a DNA virus or a retrovirus. Our previous results using single-stranded RNA probes to determine the polarity of BD transcripts (*10*), together with sensitivity to pancreatic RNase and oligo(dT) fractionation data here described, suggest that the BD agent is likely to be a negative-sense single-strand RNA virus.

To our knowledge, influenza virus is the only animal RNA virus with nuclear localization for transcription and replication (*13, 14*). Our data indicated that the 8.5-kb transcript is associated with the nucleus. Whether BD agent replication or transcription occurs in the nuclear compartment remains to be addressed.

We recognize that the BD cDNAs isolated may not represent all BD transcripts. It is possible then, that the BD agent may encode other poly(A)$^+$ transcripts in addition to the 2.1- and 0.8-kb RNAs that we have detected. Furthermore, though unlikely, it is possible that the BD agent is a segmented RNA virus and that all the cDNA clones we have isolated correspond to one unique segment. Despite these concerns, we propose that the BD agent is likely to be a negative-sense, single-strand RNA virus with an 8.5-kb genome associated with nuclei, encoding at least two major mRNAs of 2.1 and 0.8 kb.

ACKNOWLEDGMENTS

We thank M. Oldstone and M. Salvato for helpful suggestions, J. Kil for excellent technical assistance and S. Herzog and R. Rott for

use of the MBV cell line. J.C.T. is supported by a postdoctoral fellowship from the Fullbright Organization. K.M.C. has a Merck Clinician Scientist Award. W.I.L. is supported by a CIDA (NS-01026) from the National Institutes of Health.

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