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Borna Disease Virus (BDV), a Nonsegmented RNA Virus, Replicates in the Nuclei of Infected Cells Where Infectious BDV Ribonucleoproteins Are Present†

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Borna disease virus (BDV) causes neurological disease in a wide range of animal species, providing an important model for studies of persistent viral infection of the central nervous system. In addition, the detection of antibodies that react with BDV antigen in serum from psychiatric patients suggests a role for BDV, or related viruses, in human mental disorders, providing further reason for study of this poorly characterized neurotropic virus. We present evidence that BDV has a nonsegmented negative single-strand RNA genome with the property that viral replication and transcription take place in the nuclei of infected cells where infectious BDV ribonucleoproteins are present. Our results support the view that BDV has unique biological features among animal viruses. Furthermore, the finding that BDV ribonucleoproteins are able to infect susceptible cells raises interesting questions regarding the mechanisms by which some neurotropic viruses may spread through the central nervous system of the infected host without requiring the production of mature infectious virus.

Borna disease (BD) is an infectious neurological disease characterized by profound behavioral abnormalities, inflammatory cell infiltrates in the central nervous system, and the accumulation of BD-specific proteins in limbic system neurons (31; for recent reviews, see references 27, 30, and 38). Although BD has been confirmed to occur as a natural infection only in horses and sheep, the disease can be experimentally transmitted to a wide range of animal species from birds and rodents to nonhuman primates, with variable clinical and pathological manifestations (reviewed in references 30 and 38). Moreover, serological data suggest that the host range of BD may also extend to humans (1, 4, 5, 17, 41, 46).

Despite significant progress in the understanding of BD pathogenesis (21, 34), there is presently little knowledge about its etiological agent, Borna disease virus (BDV) (reviewed in reference 30). Previous studies have led to the proposal that the infectious agent is an enveloped virus (12, 15, 35). In addition, although BDV isolates display remarkable biological differences, all BDVs are characterized by noncytolytic replication and by being highly neurotropic, with cells and tissues of nonneuronal origin exhibiting low susceptibility to BDV infection (22, 30).

The recent isolation of BDV-specific cDNAs (28, 46) has facilitated the initiation of the molecular characterization of this neurotropic virus. Thus, we have previously documented that BDV has an RNA genome (14) which is transcribed in the nucleus of the infected cells. Whereas BDV poly(A)$^+$ transcripts were found to be transported to the cytoplasmic compartment, an additional BDV transcript of approximately 9 kb, apparently nonpolyadenylated and of negative polarity, remained associated with the nuclear fraction (6). However, the size, polarity, polyadenylation, and genomic organization of the BDV genome are subjects of controversy. Thus, although we have previously presented evidence suggesting that the BDV 9-kb RNA is poly(A)$^-$ and of mostly negative polarity (14, 28), others have reported the same RNA to be poly(A)$^+$ with similar amounts of both sense and antisense polarities being present in the infected cells (37–39, 46). Moreover, significant size differences have been reported between the largest BDV RNA detected in BD rat brain and cultured cells persistently infected with BDV, 10 and 8.5 kb, respectively (46), leading to the proposal that the BDV-infected cultured cells mostly contain a defective BDV genome (46). We have also recently shown that BDV infectious particles contain a ~9-kb BDV RNA (6), providing further support for our contention that this RNA constitutes a BDV RNA genomic species. Nevertheless, the very low levels of cell-free virus associated with the BDV infection have hampered the molecular characterization of the BDV genome.

Here we present results from quantitative studies regarding the subcellular distribution, polyadenylation, and polarity of BDV RNAs. We also demonstrate that, in addition to transcription, BDV replication takes place in the nucleus of the infected cells. Moreover, we present evidence that ribonucleoproteins (RNPs) isolated from the nuclear fraction of BDV-infected cells are infectious, whereas the naked RNA extracted from these BDV-RNPs is unable to direct the synthesis of BDV macromolecules on transfection of susceptible cells. Finally, we show that infectious BDV-RNPs contain only one BDV RNA species, the poly(A)$^-$ negative-polarity 9-kb RNA.

Our results provide conclusive evidence that BDV has a nonsegmented negative single-stranded RNA genome whose replication is associated with infectious RNP complexes in the nucleus of the infected cell.

MATERIALS AND METHODS

Cells and virus. The C6 cell line (ATCC CCL 107), derived from a rat glial tumor, and the C6BV cell line, obtained by establishing a persistent BDV infection in C6 cells (8), were grown in Dulbecco's modified Eagle's medium containing

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penicillin, streptomycin, 1% glutamine, and 7% heat-inactivated fetal bovine serum (FBS). BDV stock was prepared from homogenized BDV-infected rat brain as previously described (28). Briefly, brains were removed 3 weeks after infection and a 10% (wt/vol) suspension in Dulbecco's modified Eagle's medium containing 2% FBS was prepared by ultrasonication at 0°C. The homogenate was clarified by centrifugation at 3,000 × *g* for 10 min at 4°C, and the supernatant was recovered, adjusted to 100 μg of bacitracin per ml, and clarified by centrifugation through a 20% sorbital cushion at 100,000 × *g* for 1 h at 4°C. The pellet was resuspended in Dulbecco's modified Eagle's medium containing 2% FBS, and the infectious titer (focus-forming units [FFU] per milliliter) was determined by an immunofocus assay (22, 23). Total C6BV cell extracts were prepared in the same way to determine viral infectivity (FFU per cell). BDV infectious particles were isolated from C6BV cells or BDV persistently infected MDCK cells (MBV cells) (14), passage 15 to 20, as described previously (6) with minor modifications. Briefly, cell monolayers were subjected to a 90-min hypertonic treatment (20 mM Tris HCl [pH 7.4], 250 mM $MgCl_2$) at 37°C to release the cell-bound virus. The supernatant was clarified (2,500 × *g* for 5 min at 4°C) twice, adjusted to 0.002% Zwittergent, incubated for 30 min at 20°C, and adjusted to 100 μg of bacitracin per ml, and virus particles were pelleted through a 20% sorbital cushion at 100,000 × *g* for 1 h at 4°C. The pellet was resuspended in 10 mM Tris HCl (pH 7.5)–100 mM NaCl–50 mM $MgCl_2$ and treated with DNase and pancreatic RNase, both at 50 μg/ml, for 60 min at 30°C. Virus particles were recovered by centrifugation through a 20% sorbital cushion at 100,000 × *g* for 1 h at 4°C.

Animal samples. BD rat brain samples were obtained 3 weeks after inoculating 4- to 6-week-old Lewis rats intracerebrally with 30 μl of BD brain homogenate (3×10^4 FFU). PTI-NB rat brain samples were obtained from BDV-infected newborn rats, which develop a persistent tolerant infection (7).

Radiolabeling conditions and cell fractionation. For in vivo ^{32}P radiolabeling, cells were incubated for 2 h in phosphate-free media supplemented with 2% dialyzed FBS and then labeled with $^{32}P_i$ at specific activity of 2×10^{-6} mCi per cell for 20 h. Nuclei and cytoplasm were partitioned by disruption of the plasma membrane with 0.5% Nonidet P-40 (NP-40) treatment, separation of nuclei from the cytoplasmic fraction by centrifugation, and further washing of the nuclear fraction with deoxycholate and Tween 40 (36).

Northern blot analysis. RNA from BD and PTI-NB rat brain, as well as from total, cytoplasmic, and nuclear fractions of C6 and C6BV cells, was purified by the guanidinium isothiocyanate-acid phenol method (10). Poly(A)$^+$ RNA was purified by two cycles of oligo(dT)-cellulose chromatography as described previously (3). RNA samples were quantified by measurement of A_{260}, size fractionated by 2.2 M formaldehyde–agarose gel electrophoresis, and transferred to nylon membranes by capillarity (42). The RNA samples were cross-linked to the membrane by UV treatment (1.2×10^5 J) and hybridized with the indicated probes. cDNA probes were generated by random primer labeling of the DNA (16), and specific-strand riboprobes were prepared as described previously (33). Hybridizations were routinely conducted with probes of specific activity 5×10^8 to 10×10^8 cpm/μg at a concentration of 2 to 5 ng/ml. Washes were done first at low stringency (2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] at 60°C), followed by high stringency (0.2× SSC at 65°C). The probes were stripped from the blot by boiling three times (15 min each) in 10 mM Tris HCl (pH 8.5)–5 mM EDTA–0.2% sodium dodecyl sulfate (SDS). When indicated, RNA hybridization signals were quantitated by densitometric analysis of the Northern (RNA) blot autoradiograms with an LKB Ultrascan XL Laser Densitometer.

Assay for BDV replication. C6 and C6BV cells (10^7 cells) were radiolabeled with $^{32}P_i$, and total, cytoplasmic, and nuclear RNAs were purified. Procedures were as described above. [^{32}P]RNA was hybridized to a mixture of three oligonucleotides: (i) 5′ GTGGTTTCGCTGGATAGTAGGTAGGGACT GTGGGAATCTC 3′, named AS-rRNA and complementary to nucleotides 3551 to 3590 of the rat 28S rRNA (9); (ii) 5′ CCCTCCGTGAACAAACGCAG 3′, named S-BDV, with BDV genomic polarity complementary to a sequence present in the BDV 40-kDa open reading frame (ORF) (32); and (iii) 5′ GGGCTTCCAACTCCT 3′, named AS-BDV, with BDV antigenomic polarity. AS-rRNA, AS-BDV, and S-BDV were tailed with d(A) and terminal transferase by using a DNA tailing kit (no. 1028 707; Boehringer Mannheim Biochemicals, Indianapolis, Ind.) with an average of 17 d(A) residues added to the 3′ end of each oligonucleotide, and hybridization was conducted at a 3- to 10-fold molar excess of each oligonucleotide in 500 mM NaCl–4 mM $MgCl_2$–10 mM Tris hydrochloride (pH 8.5). After hybridization for 1 h at 37°C, unprotected single-stranded RNA was digested with 25 μg of RNase T_1 per ml and 100 U of RNase T_2 per ml at 30°C for 2 h. Samples were adjusted to 0.5% SDS, digested with proteinase K (100 μg/ml), phenol-chloroform extracted, and ethanol precipitated. RNA-DNA hybrids were purified by oligo(dT) cellulose chromatography, digested with RNase-free DNase I (100 μg/ml) for 45 min at 37°C, proteinase K treated, phenol-chloroform extracted, and ethanol precipitated. Protected RNA was analyzed by denaturing polyacrylamide-urea gel electrophoresis (42).

RNP preparation. Nuclear fractions from C6 and C6BV cells were digested with 100 μg of RNase-free DNase I per ml in buffer I (100 mM KCl, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 10 mM Tris-HCl [pH 7.5]), treated with 0.5% NP-40, layered on a discontinuous glycerol gradient (50 to 25% [vol/vol]) containing 150 mM NaCl, 2 mM dithiothreitol, and 10 mM Tris-HCl (pH 7.5), and centrifuged at 4°C in an SW50.1 rotor at 45,000 rpm for 120 min. A pellet containing RNP and free RNA was resuspended in buffer I and digested with 20 μg of micrococcal nuclease per ml for 15 min at 37°C, and RNPs were recovered by centrifugation through the discontinuous glycerol gradient. RNPs were resuspended in HB buffer (10 mM KCl, 1.5 mM $MgCl_2$, 5 mM dithiothreitol, 10 mM Tris-HCl [pH 7.5]), containing 40% glycerol, and aliquots were made and stored at −70°C. To determine which BDV RNA species were encapsidated in RNP and consequently protected from the nuclease treatment, RNA was extracted from the RNP-containing pellet by the guanidinium isothiocyanate-acid phenol method (10) and analyzed by Northern blot hybridization.

Transfection assays. Procedures for RNA and RNP transfections of C6 cells were as described previously (13, 29). Briefly, cell monolayers were washed with isotonic phosphate-buffered saline (PBS) containing 100 μg of autoclaved gelatin per ml (PBS-G), treated for 30 min at room temperature with 300 μg of DEAE-dextran per ml (molecular mass, 5×10^5 Da)–0.5% dimethyl sulfoxide in PBS-G, and washed once with PBS-G. RNP complexes diluted in 100 μl of PBS-G or RNA diluted in OPTI-MEM (GIBCO-BRL) was allowed to adsorb to the cells for 1 h at room temperature. Finally, the cells were washed twice with PBS-G, and 2 ml of culture medium containing 1% FBS was added per 35-mm dish.

Detection of viral antigens and quantitation of BDV infectivity. The infectivity of viral stocks prepared from BD rat brain or from total C6BV cell extracts was determined by using

the immunofocus assay (22, 23). To detect viral antigens, cells were fixed in acetone-methanol (50:50) and incubated first with antibodies from BD rat (diluted 1:100) and then with fluorescein isothiocyanate-conjugated goat anti-rat immunoglobulin (Boehringer Mannheim Biochemicals) diluted 1:50. To quantify the dose response to RNP complexes on infectivity, we transfected 10^5 C6 cells with the indicated amount of BDV-RNP. At 12 h after transfection, a single-cell suspension was prepared by treatment of the cells with nonenzymatic cell dissociation solution (Sigma), and duplicate aliquots containing 1,000, 200, and 40 C6 transfected cells were mixed with 10^5 C6 cells. Then, 72 h later, the number of FFU was determined by detection of viral antigens by indirect immunofluorescence as described above.

RESULTS

BDV-specific RNAs detected in C6BV cells, BD rat brain, and PTI-NB rat brain. Differences in the size of the largest BDV RNA between BDV-infected cell cultures and BD rat brain have been reported, 8.5 and 10 kb, respectively, suggesting that they may reflect the presence of a defective viral genome in the persistently infected cell cultures (46). Therefore, the use of such BDV-infected cell cultures to biochemically characterize the BDV genome might be questionable. Nonetheless, we have compared the pattern of BDV transcripts in BDV persistently infected C6 (C6BV) cells with that found in BD rat brain, as well as PTI-NB rat brain, and found no differences (Fig. 1). Hybridization with a p24 BDV probe representing the 24-kDa BDV ORF (28, 44, 46) revealed the same BDV-specific transcripts of 0.8, 2.1, 3.5, and approximately 9 kb (which we will refer to as 9 kb) in C6BV cells (Fig. 1A, lane 2), BD rat brain (lane 3), and PTI-NB rat brain (lane 4), whereas hybridization with clone CA BDV probe representing the 5′ part of the BDV genome (11) hybridized to the 9-kb BDV RNA, as well as to two additional BDV-specific RNAs of approximately 6.1 and 7.2 kb, in all three BDV samples (Fig. 1B). In addition, a BDV probe representing the 40-kDa ORF (32, 37) hybridized to the same transcripts of 1.2, 2.1, 3.5, and 9 kb in all three BDV samples (data not shown). RNA obtained from C6 cells or from normal rat brain did not hybridize to the BDV probes (Fig. 1, lanes 1 and 5). Lane 6 corresponds to RNA size markers (0.24- to 9.5-kb RNA ladder [Bethesda Research Laboratories]).

We have previously demonstrated that BDV is not a DNA virus or retrovirus but, rather, has an RNA genome (14). Although preliminary evidence suggested the lack of double-stranded (ds) RNAs associated with the BDV infection, formal proof was not provided. Because of its importance regarding the molecular characterization of BDV, we have further investigated this subject. Hybridization of RNA obtained from C6BV cells to a p24 BDV probe (28, 37, 44, 46) was prevented by previous treatment of the C6BV RNA with 0.1 μg of RNase A per ml at low and high ionic strength (Fig. 2A, lanes 4 and 6). Control experiments with in vitro generated single-stranded (ss) (Fig. 2B, lanes 7 to 10) or ds (Fig. 2B, lanes 1 to 6) BDV RNA indicated that under our experimental conditions, ds RNA was resistant to treatment with 0.1 μg of RNase per ml at high (2× SSC) but not low (0.01× SSC) ionic strength (Fig. 2B, compare lanes 2 and 3). At a higher RNase concentration (1 μg/ml), the in vitro generated BDV ds RNA was not resistant to degradation at high or low ionic strength (Fig. 2B, lanes 4 and 5). BDV ss RNA was sensitive to 0.1 μg of RNase per ml at high ionic strength (Fig. 2B, lane 8). In contrast, BDV-specific RNAs present in C6BV cells (Fig. 2A, lane 7) or in vitro generated ss and ds BDV RNA were resistant to DNase treatment (Fig. 2A, lane 7; Fig. 2B, lanes 6 and 10).

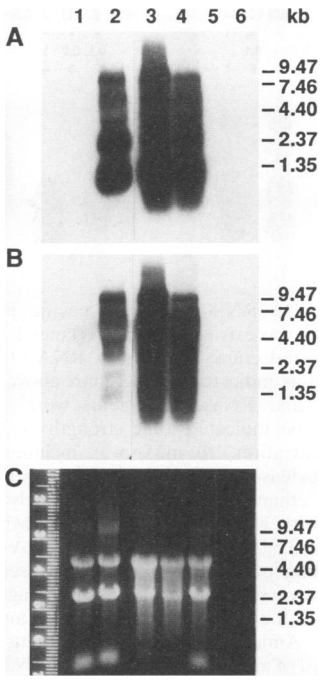

FIG. 1. BDV-specific RNAs detected in C6BV cells, BD rat brain, and PTI-NB rat brain. RNA was extracted from C6 (lanes 1) and C6BV (lanes 2) cells, as well as from BD rat brain (lanes 3), PTI-NB rat brain (lanes 4), and normal rat brain (lanes 5), and analyzed by Northern blot hybridization. (A) Hybridization with a p24 BDV probe (26, 41). (B) Hybridization with clone CA BDV representing the 1.5-kb 5′ end of the BDV genome (11). (C) Ethidium bromide staining showing the 28S (4.7-kb) and 18S (1.8-kb) rRNA. Lane 6, RNA size markers (0.24- to 9.5-kb RNA ladder; no. 5620SA, Bethesda Research Laboratories). Experimental procedures were as described in Materials and Methods.

Polyadenylation, polarity, and cellular distribution of the BDV genome. Total, cytoplasmic, and nuclear RNA, as well as poly(A)$^+$ and poly(A)$^-$ RNA, were prepared from C6 and C6BV cells and analyzed by Northern blot hybridization as described in Materials and Methods. Densitometric analysis of the results presented in Fig. 3A indicated that more than 98% of the 9-kb RNA was recovered in the poly(A)$^-$ fraction, whereas 96, 90, and 92% of the 3.5-, 2.1-, and 0.8-kb BDV transcripts, respectively, detected by a p24 BDV probe, were recovered in the poly(A)$^+$ fraction. Moreover, BDV poly(A)$^+$ transcripts were present in both the cytoplasmic and nuclear fractions but at much higher levels in the former, which contained 80, 91, and 96% of the 3.5-, 2.1-, and 0.8-kb BDV transcripts, respectively. In contrast, the BDV 9-kb poly(A)$^-$ RNA was restricted mostly (93%) to the nuclear fraction. Vimentin, a poly(A)$^+$ cellular mRNA, was found predominantly in the poly(A)$^+$ fraction, whereas histone 2b, a poly(A)$^-$ cellular mRNA, was present only in the poly(A)$^-$ fraction. The recovery efficiency for each fraction was quantitated by using in vitro transcribed radiolabeled RNA with or without a poly(A) tail. Poly(A)$^-$ and poly(A)$^+$ fractions contained less than 1% of the poly(A)$^+$ and poly(A)$^-$ RNA, respectively (data not shown).

Hybridization with specific-strand riboprobes (Fig. 3B), sense and antisense, corresponding to an 875-nucleotide

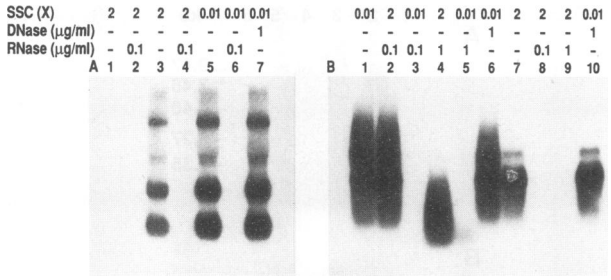

FIG. 2. Sensitivity to RNase of BDV-specific RNAs present in C6BV cells. (A) RNA was extracted from C6 (lanes 1 and 2) and C6BV (lanes 3 to 7) cells, and equal amounts of RNA (10 μg) from each sample were treated as indicated on the chart above the figure. Both RNase-free DNase and RNase treatments were done in a 50-μl reaction volume at the indicated ionic strength at 37°C for 15 min. $MgCl_2$ (final concentration, 10 mM) was included during DNase treatment. After nuclease treatments, samples were extracted with phenol-chloroform, ethanol precipitated, and analyzed by Northern blot hybridization with a p24 BDV probe that hybridizes to BDV transcripts of 0.8, 2.1, 3.5, and 9 kb. (B) BDV ds RNA (lanes 1 to 6) was obtained by annealing of positive- and negative-sense BDV RNAs generated by in vitro transcription of an *Eco*RV fragment (875 nucleotides) obtained from the BDV 40-kDa ORF (32) and subcloned into pBluescript II KS+. Annealing was conducted by using 1 μg of each RNA polarity in 50 μl of annealing buffer (500 mM NaCl, 4 mM $MgCl_2$, 10 mM Tris HCl [pH 8.5]) for 1 h at 60°C. After annealing, the RNA was ethanol precipitated and resuspended in TE (10 mM Tris HCl [pH 8.5], 1 mM EDTA), and aliquots containing 50 ng of RNA were used for the indicated treatments. BDV ss RNA (lanes 7 to 10) was generated by in vitro transcription of one single polarity of the same *Eco*RV fragment. After nuclease treatments, both ds and ss BDV RNAs were processed as described in the legend to panel A.

*Eco*RV fragment within the BDV 40-kDa protein (32, 37) showed that BDV poly(A)$^+$ transcripts are exclusively positive polarity. In contrast, the nuclear poly(A)$^-$ 9-kb BDV RNA is mostly (93%) negative polarity (Fig. 3B and C).

The nuclear contamination of our cytoplasmic fractions was estimated to be 3 to 10% based on the amount of 45S rRNA detected in this fraction by using a specific rRNA probe (data not shown). To evaluate the cytoplasmic contamination of the nuclei, we first demonstrated that lymphocytic choriomeningitis virus (LCMV), an exclusively cytoplasmic virus (25), was able to replicate and transcribe to a similar extent in both C6 and C6BV cells (Fig. 4A). LCMV-infected C6BV cells were fractionated into nuclear and cytoplasmic fractions as described in Materials and Methods, and the RNA was analyzed by Northern blot hybridization with a specific probe for the LCMV nucleoprotein (NP) gene (Fig. 4B). The cytoplasmic contamination of the nuclei was less than 7% based on the levels of LCMV-NP sequences associated with the nuclear fraction, as determined by hybridization to the NP probe.

BDV infectious particles contain the poly(A)$^-$ negative-polarity BDV ~9-kb RNA present in the nucleus of BDV-infected cells. To determine whether the poly(A)$^-$ negative-polarity BDV ~9-kb RNA detected in the nuclear fraction of C6BV cells represents a BDV RNA genomic species, poly(A)$^-$ RNA isolated from BDV infectious particles (Fig. 5, lane 4) was compared with total poly(A)$^-$ RNA isolated from BD rat brain and PTI-NB rat brain (lanes 2 and 3, respectively), as well as nuclear poly(A)$^-$ RNA obtained from C6BV cells (lane 1) and total RNA from BD rat brain (lane 5). Poly(A)$^-$ RNA was selected through one single round of oligo(dT)-cellulose chromatography, which explains the pres-

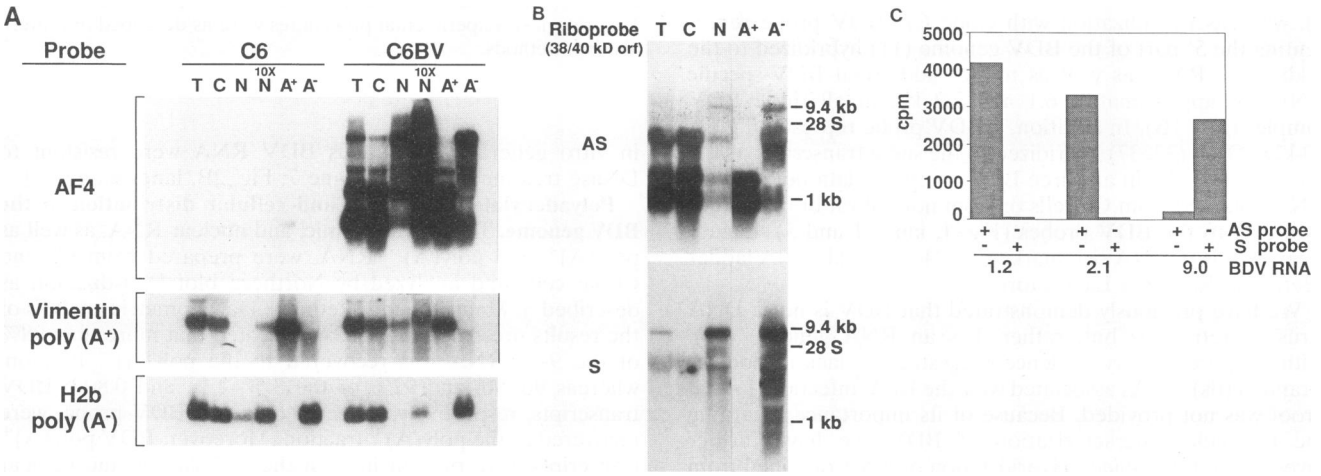

FIG. 3. Subcellular distribution, polyadenylation, and polarity of BDV RNAs. (A) Subcellular distribution and polyadenylation. C6 and C6BV cells were fractionated into nuclear and cytoplasmic fraction as described in Materials and Methods. Total (T), cytoplasmic (C), and nuclear (N) RNA, as well as poly(A)$^+$ (A$^+$) and poly(A)$^-$ (A$^-$) RNA, were analyzed by Northern blot hybridization with the indicated probes. The amount of RNA loaded from each sample corresponded to that obtained from the same number of cells (10^6 cells) in each case, except for the 10×N samples that corresponded to nuclear RNA extracted from 10^7 cells. Probes were AF4, representing the BDV 24-kDa ORF; vimentin, a poly(A)$^+$ cellular mRNA; and histone 2b (H2b), an example of a poly(A)$^-$ cellular mRNA. Experimental procedures were as described in Materials and Methods. (B) Polarity of BDV RNAs. Total (T), cytoplasmic (C), and nuclear (N) RNA, as well as poly(A)$^+$ (A$^+$) and poly(A)$^-$ (A$^-$) RNA, were purified as described in Materials and Methods. RNA samples corresponding to 10^6 cells (T and C), 10^7 cells (N and A$^-$), and 5×10^4 cells (A$^+$) were analyzed by Northern blot hybridization with sense (S) and antisense (AS) strand-specific riboprobes. Both AS and S probes were prepared by in vitro transcription of the *Eco*RV fragment of the BDV 40-kDa ORF described in the legend to Fig. 2. Positions corresponding to 9.4- and 1-kb RNAs, as well as 28S rRNA from HeLa cells (5 kb), are indicated. Experimental procedures were as described in Materials and Methods. (C) To determine the levels of sense and antisense polarities of the 1.2-, 2.1-, and 9-kb BDV RNAs, total RNA was extracted from C6BV cells and fractionated by oligo(dT)-cellulose chromatography, and a duplicate set of poly(A)$^+$ and poly(A)$^-$ RNA samples was size fractionated by 2.2 M formaldehyde–1% agarose gel electrophoresis and transferred to nylon membrane. The membrane was cut into two pieces, each containing one set of samples. One set was hybridized to an S probe and the other was hybridized to an AS probe under the same conditions. The position of each RNA on the membranes was located, and similar membrane surfaces containing the corresponding sample were cut and subjected to Cerenkov counting.

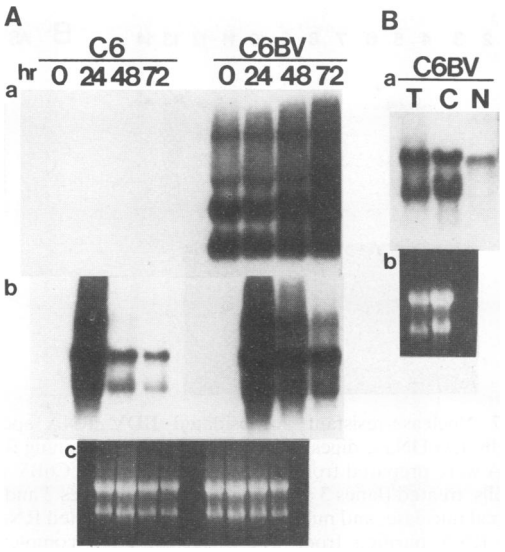

FIG. 4. Replication and transcription of LCMV in C6 and C6BV cells. (A) C6 and C6BV cells were infected with LCMV ARM 53b at a multiplicity of infection of 20 PFU per cell, and at the indicated time points after infection, total RNA was extracted and equal amounts of RNA (10 μg) from each sample were analyzed by Northern blot hybridization. (a) p24 BDV probe. (b) LCMV NP probe, which hybridizes to the S segment of the LCMV genome (S) as well as the NP mRNA (NP). (c) Ethidium bromide staining of the 28S and 18S rRNAs. (B) Levels of LCMV-NP in nuclear fractions of C6BV cells. At 48 h after infection with LCMV ARM 53b at a multiplicity of infection of 20 PFU per cell, C6BV cells were fractionated into nuclear and cytoplasmic fractions as described in Materials and Methods. Total (T), cytoplasmic (C), and nuclear (N) RNAs were extracted, and amounts of RNA of each sample obtained from the same number of LCMV-infected C6BV cells (10^6 cells) were analyzed by Northern blot hybridization with the LCMV-NP probe (a). The amount and quality of the RNA loaded were assessed on the basis of the ethidium bromide staining of 28S (4.7-kb) and 18S (1.8-kb) rRNAs (b). Densitometric analysis indicated that the amounts of LCMV S segment and NP mRNA in the nuclear fraction were 7% and less than 1%, respectively, of the corresponding levels found in the cytoplasmic fraction of the LCMV-infected C6BV cells.

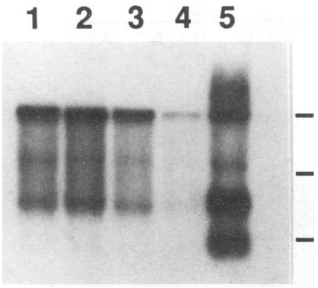

FIG. 5. BDV infectious particles contain the poly(A)$^-$ negative-polarity BDV ~9-kb RNA present in the nucleus of C6BV cells. RNA extracted from the nuclear fraction of C6BV cells, BD rat brain, and BDV infectious particles was fractionated by oligo(dT)-cellulose chromatography (a single round), and the corresponding poly(A)$^-$ fraction together with total RNA from BD rat brain was analyzed by Northern blot hybridization with a p24 BDV probe. Lanes: 1, poly(A)$^-$ nuclear RNA obtained from 5×10^6 C6BV cells; 2, poly(A)$^-$ RNA (6 μg) obtained from BD rat brain; 3, poly(A)$^-$ RNA (10 μg) obtained from PTI-NB rat brain; 4, poly(A)$^-$ RNA isolated from BDV infectious particles prepared from 5×10^8 MBV cells; 5, total RNA (6 μg) obtained from BD rat brain. MBV cells instead of C6BV cells were chosen because of their 10-fold-higher yield of infectious virus particles per cell. Procedures for virus particle preparation, RNA extraction, generation of probes, and hybridization conditions were as described in Materials and Methods. Bars on the right indicate positions corresponding, from top to bottom, to RNA of 9.4, 3, and 0.8 kb.

ence of low levels of poly(A)$^+$ 2.1- and 3.5-kb BDV RNAs in lanes 1 to 3. BDV 9-kb RNA present in the nucleus of C6BV cells was also contained in BDV infectious particles and therefore represents a genomic species.

Subcellular localization of new synthesis of BDV genomic-polarity RNA. The nuclear localization of the BDV 9-kb genomic RNA species (Fig. 3), together with our previous finding that the synthesis of BDV poly(A)$^+$ RNAs takes place in the nucleus (6), suggested that BDV replication might also occur in the nucleus of infected cells. To assay for newly synthesized BDV genomic RNA and the subcellular location of this synthesis, total, cytoplasmic, and nuclear RNA, as well as poly(A)$^+$ and poly(A)$^-$ RNA, was isolated from ^{32}P-radiolabeled C6 and C6BV cells and annealed to a mixture of synthetic oligonucleotides composed of (i) an oligonucleotide, named S-BDV (20 nucleotides), genomic polarity; (ii) an oligonucleotide with BDV antigenomic polarity, named AS-BDV (15 nucleotides); and (iii) an oligonucleotide complementary to a cellular rRNA sequence, named AS-rRNA (40 nucleotides). All three oligonucleotides were previously tailed with dA and terminal transferase, and hybridization was conducted in the presence of a large molar excess of each oligonucleotide with respect to the [^{32}P]RNA. After hybridization, samples were digested with RNases and the protected DNA-RNA hybrids were purified by oligo(dT)-cellulose chromatography. The DNA was eliminated by DNase treatment, and the protected ribo-oligonucleotides were analyzed by acrylamide gel electrophoresis under denaturing conditions (Fig. 6).

Similar levels of the rRNA sequence protected by the oligonucleotide AS-rRNA were found in C6 and C6BV cells (Fig. 6, compare lanes 1 and 2 with lanes 4 and 5), with the cytoplasmic fractions having about 10 times higher levels of the protected rRNA sequence than the corresponding nuclear fractions (compare lanes 2 and 3 with lanes 5 and 6). The BDV-specific oligonucleotides, AS-BDV and S-BDV, did not protect RNA sequences present in C6 cells (lanes 1 to 3). Newly synthesized BDV antigenomic polarity (mRNA polarity) was detected mostly (more than 90%) in the cytoplasm and at lower levels also in the nucleus of C6BV cells, whereas more than 95% of the newly synthesized BDV genomic-polarity sequence was associated with the nuclear fraction. Moreover, only antigenomic polarity was detected in the newly synthesized BDV poly(A)$^+$ RNA (lane 7). In contrast, in addition to genomic polarity, a significant amount of antigenomic polarity was detected in the newly synthesized BDV poly(A)$^-$ RNA (lane 8).

The BDV 9-kb RNA genomic species is associated with infectious BDV-RNPs in the nucleus of infected cells. To investigate whether, similarly to other negative-stranded RNA viruses, BDV RNA genomic species are tightly associated with RNPs, rendering them resistant to nuclease treatment, nuclear fractions from C6BV cells were treated with RNase-free DNase, adjusted to 1% Triton X-100, and then centrifuged through a glycerol step gradient containing 1% Triton X-100. After treatment of the pellet containing free RNA and RNPs with micrococcal nuclease, the RNA was extracted and analyzed by Northern blot hybridization with a p24 BDV probe

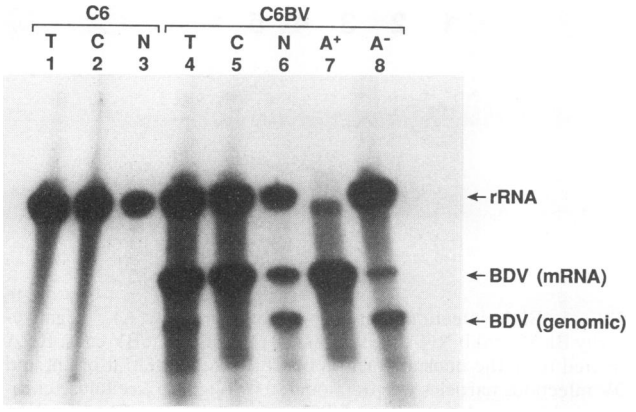

FIG. 6. Subcellular localization of new synthesis of BDV RNA with genomic polarity. Total (T), cytoplasmic (C), and nuclear (N) RNA was isolated from C6 and C6BV cells radiolabeled with $^{32}P_i$ for 20 h. Poly(A)$^+$ and poly(A)$^-$ RNA was obtained by oligo(dT)-cellulose chromatographic fractionation of total RNA. To determine the cellular compartment where the new synthesis of BDV RNA genomic polarity takes place, we used samples in the BDV replication assay described in Materials and Methods. The positions of specific RNA sequences protected from RNase digestion by oligonucleotides AS-rRNA, AS-BDV, and S-BDV are indicated by rRNA, BDV (mRNA), and BDV (genomic), respectively. To quantify the signal corresponding to each protected RNA sequence, in all cases a similar surface of the acrylamide gel containing the pertinent RNase-protected [^{32}P]ribo-oligonucleotide was cut out of the gel and subjected to Cerenkov counting. Background was determined by Cerenkov counting of several pieces of gel from areas not containing samples.

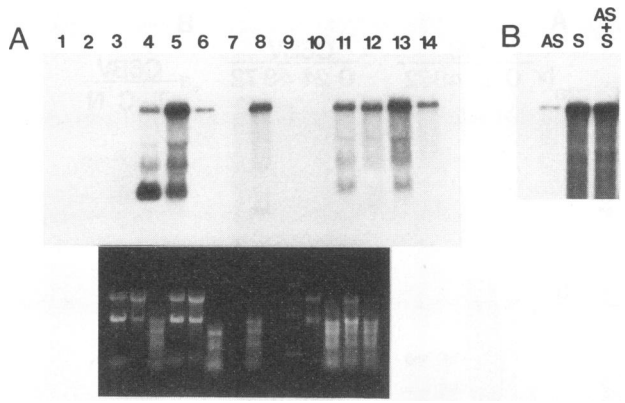

FIG. 7. Nuclease-resistant encapsidated BDV RNA species in C6BV cells. (A) DNase-digested nuclear fractions containing RNP and free RNA were prepared from C6 (lanes 2 and 3) and C6BV (lanes 5 and 6) cells, treated (lanes 3 and 6) or not treated (lanes 2 and 5) with micrococcal nuclease, and nuclease-resistant encapsidated RNAs were purified. RNA purified from C6BV nuclear RNP complexes was fractionated by oligo(dT)-cellulose chromatography to obtain poly(A)$^+$ (lane 7) and poly(A)$^-$ (lane 8) RNA fractions. An equivalent aliquot of the poly(A)$^-$ RNA from lane 8 was treated with RNase (0.1 μg/ml) for 10 min at 37°C (lane 9). Total RNA was purified from C6 (lane 1) and C6BV (lane 4) cells, and RNA size markers (lane 10) were used to estimate the size of BDV RNAs. Samples in lanes 1 and 4 correspond to RNA obtained from 5×10^5 cells. Samples in lanes 2, 3, and 5 correspond to RNA obtained from 2.5×10^6 cells. Samples in lanes 7 and 8 correspond to RNA obtained from 1×10^6 cells. The sample in lane 6 corresponds to RNA obtained from 5×10^5 cells. Lanes 11 through 14 represent mixing experiments. RNA was isolated from C6BV-nuclear RNP (10^6 cells) before (lane 11) and after (lane 12) nuclease treatment. RNA purified from C6BV-nuclear RNP after nuclease treatment (10^6 cells) was mixed with C6BV-nuclear RNP that had not been treated with micrococcal nuclease; the mixture was incubated at 25°C in 25 μl of NTE (150 mM NaCl, 10 mM Tris HCl [pH 8.5], 1 mM EDTA) for 20 min and then treated (lane 14) or not treated (lane 13) with micrococcal nuclease, and the RNA was extracted. Samples were analyzed by Northern blot hybridization with a p24 BDV probe. The lower part of panel A is the ethidium bromide-stained gel before being transferred to the membrane. (B) RNA was purified from micrococcal nuclease treated C6BV-nuclear RNP, and three aliquots, each corresponding to 2×10^6 cells, were analyzed by Northern blot hybridization with strand-specific riboprobes. This panel represents a composite of three independent hybridizations. AS, antisense probe; S, sense probe; AS + S, a mixture of AS and S probes used for the hybridization. Both AS and S probes were adjusted to the same specific activity (5×10^8 cpm/μg), and identical amounts (2 ng/ml) of each probe were used under identical hybridization conditions. Strand-specific riboprobes correspond to the *Eco*RV fragment of the BDV 40-kDa ORF.

(28, 44, 46). Only the BDV 9-kb RNA was protected from nuclease treatment (Fig. 7A, compare lanes 5 and 6). This protected RNA, similar to the BDV 9-kb RNA detected in total nuclear fractions, is also poly(A)$^-$ (Fig. 7A, lanes 7 and 8), and more than 90% is negative polarity as determined by using strand-specific riboprobes (Fig. 7B). Results of reconstruction experiments presented in Fig. 7A, lanes 11 to 14, revealed that the 9-kb BDV RNA present in purified nuclear poly(A)$^-$ RNA from C6BV cells is not protected from nuclease digestion when mixed with nuclear BDV-RNPs. When nuclear RNPs and nuclear poly(A)$^-$ RNA prepared from equal amounts of C6BV cells were mixed, the signal obtained for the BDV 9-kb RNA (Fig. 7A, lane 13) was approximately twofold higher than the one obtained from each independent sample (lanes 11 and 12). Treatment of the mixture with nuclease prior to RNA extraction caused approximately a 50% reduction in the BDV 9-kb signal (lane 14).

Nuclear BDV-RNP were infectious on the basis of their ability to direct synthesis of BDV macromolecules, both protein and nucleic acids, upon transfection of C6 cells. C6 cells transfected with BDV-RNP were recognized by BDV-specific antibodies (Fig. 8a, panel E), displaying the nuclear staining characteristic of the BDV infection (compare panels B and E). Infectivity associated with infectious BDV particles was destroyed by treatment with nonionic detergent (compare panels B and C). In contrast, BDV-RNP remained infectious after the detergent treatment (panel I) but not after protease treatment (panel F). Moreover, naked RNA extracted from BDV-RNP was not infectious as determined by lack of viral antigen and BDV transcript expression after transfection of C6 cells (Fig. 8a, panel H; Fig. 8, lane 3). To estimate the efficiency of RNA transfection, C6 cells were transfected with RNA purified from poliovirus virions and the number of transfected cells was determined by infectious-center assays on HeLa cell monolayers. Results indicated that under our experimental conditions 10^5 infectious-center units per μg of RNA were obtained. Northern blot hybridization experiments in which we compared the hybridization signal obtained with RNA extracted from 50 μl of BDV-RNP with known amounts of BDV RNA sequences generated by in vitro transcription indicated that 50 μl of BDV-RNP complexes contained 5 ng of BDV 9-kb RNA. Somewhat unexpectedly, BDV-RNP were able to direct viral antigen synthesis, although less efficiently, after transfection of C6 cells that were not pretreated with DEAE-dextran (Fig. 8a, panel G; Fig. 8c). C6BV cytoplasmic RNPs displayed 50-fold-lower infectivity than C6BV nuclear RNPs prepared from an

equivalent number of cells. The infectivity of the C6BV cytoplasmic RNPs did not increase when C6BV cytoplasmic RNPs were mixed with C6 nuclear extracts (data not shown). C6 cells transfected with cytoplasmic RNPs from LCMV-infected C6BV cells produced 120 infectious-center units per μl of RNP preparation (1 μl of RNP is equivalent to extract derived from 5×10^5 cells), but no infectious-center units were detected if C6 cells were not treated with DEAE-dextran prior to transfection with LCMV-RNPs (data not shown).

RNA extracted from passage 2 after transfection of C6 cells with C6BV nuclear RNP displayed the same pattern of BDV transcripts as that found in C6BV cells (Fig. 8b, compare lanes 2 and 5), whereas transfection of C6 cells with RNA obtained from BDV-RNP or with nuclear RNP prepared from C6 cells did not lead to the expression of BDV transcripts (Fig. 8b, lanes 1 and 3). Pretreatment of C6 cells with cycloheximide or actinomycin D prior to transfection of C6 cells with BDV-RNP prevented expression of BDV antigens (data not shown). These results support the conclusion that the results presented in Fig. 8a (panels E, G, and I) truly represent new synthesis of viral antigens directed by BDV-RNP rather than merely being the result of input BDV-RNP. Furthermore, total-cell extracts prepared from passage 3 after transfection of C6 cells with BDV-RNP contained infectious BDV particles (0.1 FFU per cell) (data not shown).

To determine whether one RNP particle is sufficient for infection, we conducted dose-response experiments. The results of a dose-response titer determination of the infectivity of RNP-complexes revealed a linear relationship rather than an exponential curve (Fig. 8c), suggesting that interaction with a single particle is sufficient to infect a C6 recipient cell.

Infectious BDV-RNP contain a single RNA species: the poly(A)$^-$, negative-polarity 9-kb RNA. Several attempts to obtain radiolabeled BDV infectious particles failed, probably because of the very low level of cell-free virus associated with the BDV infection. However, the presence of relatively high levels of infectious BDV-RNP in the nucleus of infected cells provided us with a possible alternative approach to determine how many RNA species constitute the BDV genome and thus to establish the segmented or nonsegmented structure of the BDV genome.

Nuclear RNP were prepared from ^{32}P-radiolabeled C6 and C6BV cells (see Materials and Methods) (Fig. 9). [^{32}P]RNA extracted from the nuclear RNP was size fractionated by 2.2 M formaldehyde–0.7% agarose gel electrophoresis and transferred to a nylon membrane. Staining of the membrane with methylene blue allowed us to precisely determine the position of the RNA size markers (Fig. 9A, lane 5). After 24 h of exposure, a single [^{32}P]RNA species of approximately 9 kb was present in RNA obtained from nuclear RNP prepared from C6BV but not from C6 cells (Fig. 9B, compare lanes 1 and 3). The unequivocal RNA nature of the band seen in Fig. 9B, lane 3, was determined by its sensitivity to DNase-free RNase treatment (Fig. 9B, compare lanes 3 and 4). The membrane was allowed to decay for 4 weeks and then hybridized to a p24 BDV probe (25, 44, 46). This probe detected the BDV 9-kb RNA only in RNA obtained from nuclear C6BV cells (Fig. 9C), confirming the viral specificity of the only RNA species found associated with infectious BDV-RNP.

DISCUSSION

We have shown that C6 cells persistently infected with BDV have an identical pattern of BDV transcripts to that found in BD or PTI-NB rat brain (Fig. 1), with the largest detected BDV RNA estimated to be 9 ± 0.4 kb on the basis of its relative electrophoretic migration with respect to the RNA size markers (Fig. 1).

The BDV 9-kb RNA was confined mostly to the nuclear fraction and recovered in the poly(A)$^-$ fraction of the oligo(dT)-cellulose chromatography (Fig. 3A). The detection of vimentin and histone 2b mRNAs exclusively in the poly(A)$^+$ and poly(A)$^-$ fractions, respectively, validated the experimental conditions used for the oligo(dT)-cellulose chromatography, and quantitation of the distribution of in vitro generated radiolabeled RNAs containing or not containing a poly(A)$_n$ tail (average $n = 20$) showed less than 1% of cross-contamination between the poly(A)$^+$ and poly(A)$^-$ fractions under our experimental conditions. Consequently we conclude that the BDV 9-kb RNA present in the nucleus of the C6BV cells should be considered a poly(A)$^-$ RNA, while its presence within BDV infectious particles (Fig. 5) identified this poly(A)$^-$ 9-kb RNA as a BDV RNA genomic species. BDV 9-kb genomic RNA was sensitive to low concentrations of RNase at high ionic strength, indicating the ss nature of this 9-kb BDV RNA (Fig. 2). Under our experimental conditions, we could detect 40-fold-lower levels of BDV 9-kb RNA than that corresponding to the hybridization signal in Fig. 2, lanes 3, 5, and 7. Quantitative studies revealed that approximately 4% of the BDV 9-kb RNA hybridization signal was resistant to digestion with RNase (data not shown), which correlates with the levels of full-length BDV antigenomic RNA, suggesting that it is very unlikely that BDV is a ds RNA virus.

Quantitation of the 9-kb RNA hybridization signals obtained with strand-specific riboprobes showed that 93% of the BDV 9-kb steady-state level present in C6BV cells is negative sense, i.e., complementary to the BDV poly(A)$^+$ mRNAs polarity (Fig. 3B). The inability of the nuclear poly(A)$^-$ RNA obtained from C6BV cells, containing high levels of the BDV 9-kb RNA, to direct the synthesis of BDV proteins in in vitro translation experiments with reticulocyte lysates, in contrast to the ability of the C6BV poly(A)$^+$ RNA to direct the synthesis of the 24- and 39- to 40-kDa BDV polypeptides, as determined by immunoprecipitation of the in vitro-translated proteins with serum from BD rats (data not shown), provided further support for the conclusion that BDV has a negative-stranded RNA genome.

Whether the ratio of antigenomic to genomic polarity for BDV 9-kb RNA found in C6BV cells (approximately 1:13) represents the general situation during BDV replication or, rather, specific regulatory interactions associated with a persistent infection of astrocytes is presently unknown.

Results presented in Fig. 6 revealed that most (>95%) of the newly synthesized BDV genomic-polarity RNA during a 20-h period was poly(A)$^-$ and found largely (>95%) in the nuclear fraction, leading us to conclude that BDV replication takes place in the nucleus of the infected cell. A significant amount, about 8%, of the BDV poly(A)$^-$ RNA newly synthesized during the 20-h radiolabeling period had positive-sense polarity, which probably represents the levels of antigenomic template required for the generation of genomic-polarity RNA. Interestingly, the ratio of antigenomic to genomic poly(A)$^-$ RNA sequences newly synthesized during the 20-h radiolabeling period was very similar to that found for the steady-state level of the BDV 9-kb RNA (compare Fig. 3B and 6). Thus, although our results do not provide direct information about the rate of synthesis of the BDV RNAs, they do suggest that during persistent infection of BDV in astrocytes, the steady-state levels of the negative- and positive-sense BDV 9-kb RNAs probably reflect their corresponding rates of synthesis.

Similar to other negative-strand RNA viruses, it would be

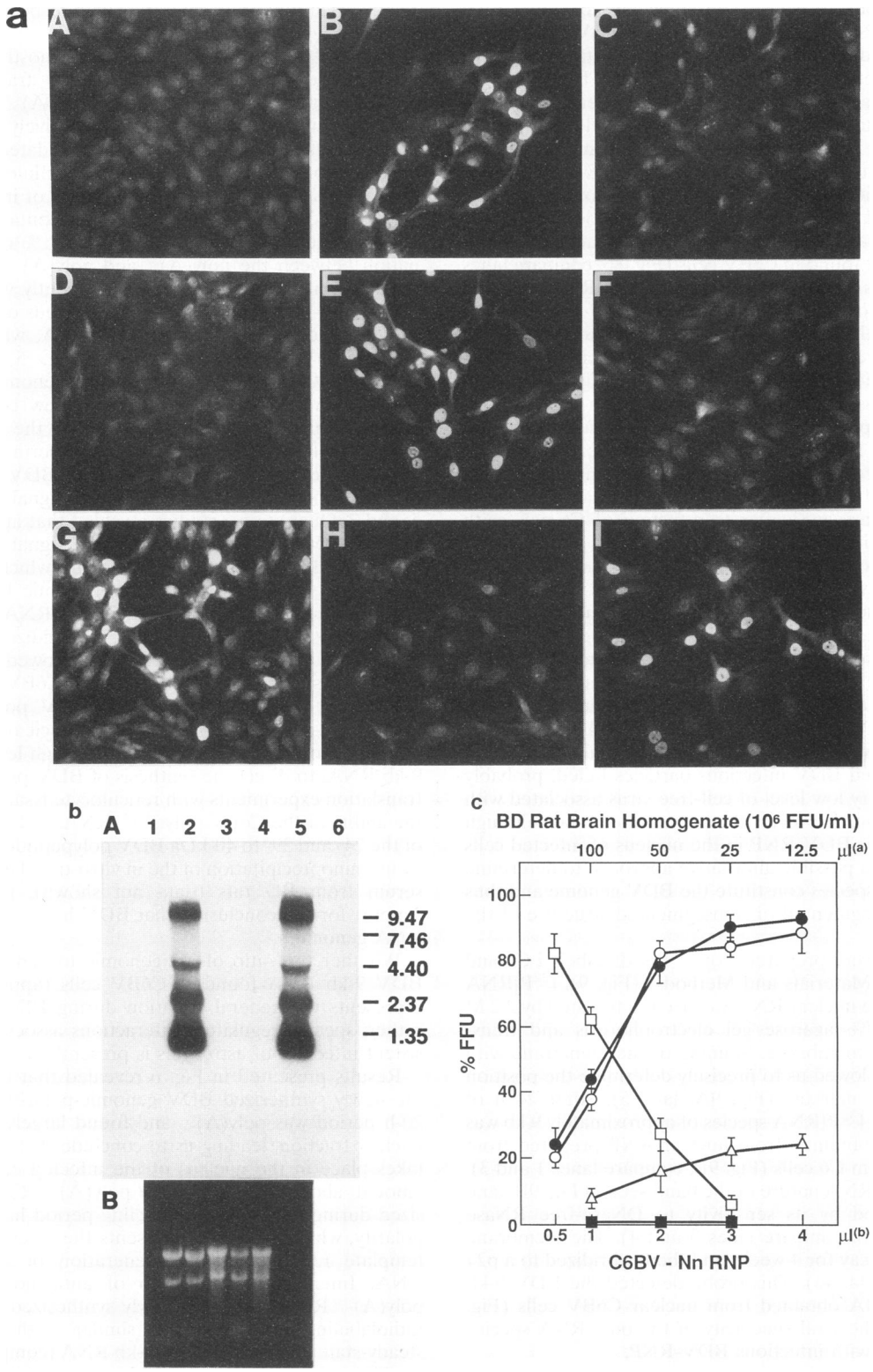

FIG. 8. BDV-RNP complexes present in the nucleus of C6BV cells are infectious. Nuclear RNP complexes were prepared from C6 and C6BV cells as described in Materials and Methods, aliquoted in HB–40% glycerol, and stored at −70°C. The same BDV-RNP preparation was used for all the transfection assays in Fig. 7 and 8. BDV stock (10% [wt/vol] brain homogenate) was prepared and subjected to titer determination as described in Materials and Methods. Transfection of C6 cells with RNP complexes and RNA and detection of BDV viral antigens were conducted as described in Materials and Methods. Infection of C6 cells with BDV stock was conducted as follows. Cells were washed once with medium without serum, and the virus was allowed to adsorb to the cells for 2 h in a final volume of 100 µl of medium containing 2% FBS per 12 cm^2. After adsorption, the inoculum was removed and 1 ml of medium plus 2% FBS per 12 cm^2 was added to the cells. (a) Expression of viral antigens in

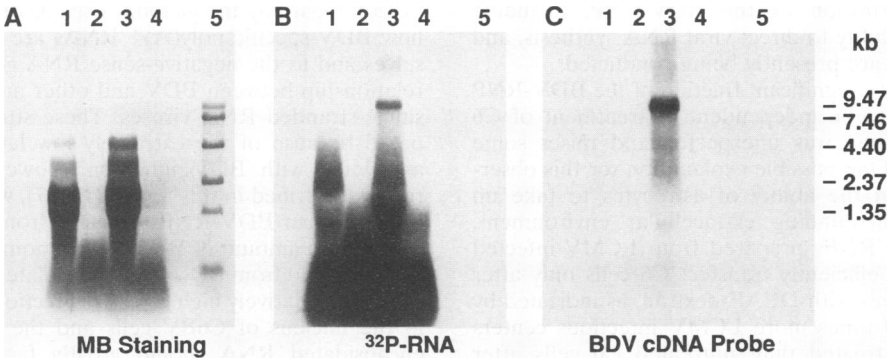

FIG. 9. RNA species contained in infectious BDV-RNPs. C6 and C6BV cells were radiolabeled with $^{32}P_i$, and nuclear RNP were prepared as described in Materials and Methods. Nuclease-resistant encapsidated [^{32}P]RNA species were purified from RNP complexes, and equivalent aliquots of RNA purified from C6-nuclear RNP (lanes 1 and 2) and BDV-RNP (lanes 3 and 4) were treated (lanes 2 and 4) or not treated (lanes 1 and 3) with DNase-free RNase (1 g/ml) for 10 min at 37°C before being size fractionated by 2.2 M formaldehyde–1% agarose gel electrophoresis and transferred to a nylon membrane. (A) RNA samples transferred to the nylon membrane were visualized by methylene blue staining. (B) [^{32}P]RNA species were revealed by exposure of the membrane to an X-ray film (XAR; Kodak) for 20 h. (C) The membrane was allowed to decay for 4 weeks and hybridized to a p24 BDV probe. The exposure time in panel C was 5 h. Lane 5 corresponds to RNA size markers (0.24- to 9.5-kb RNA ladder [Bethesda Research Laboratories]).

expected that the BDV RNA genomic species would be tightly encapsidated into ribonucleocapsid structures (45). Results shown in Fig. 7 indicated that the nuclear poly(A)$^-$ BDV 9-kb RNA, also contained within BDV infectious particles (Fig. 5), was the only BDV RNA associated with RNP complexes on the basis of its resistance to nuclease digestion. However, this result did not exclude the existence of other possible BDV RNA genomic species also associated with RNP complexes, whose sequences are not represented in the BDV cDNA probes currently available. To overcome this problem we isolated nuclear BDV-RNP complexes from ^{32}P-radiolabeled C6BV cells and analyzed the encapsidated nuclease-resistant [^{32}P]RNAs. Only a 9-kb [^{32}P]RNA was resistant to nuclease treatment (Fig. 9B, lane 3). The absence of this RNA in nuclear RNPs prepared from C6 cells (Fig. 9B, lane 1), together with results of hybridization with BDV-specific probe (Fig. 9C, lane 3) proved this RNA to be BDV specific.

It has been reported for other negative-stranded RNA viruses that the RNP complexes, in addition to being the template for transcription and replication, are infectious when transfected into susceptible cells (29, 40, 43). Results shown in Fig. 8 demonstrated that BDV-RNP complexes containing only the 9-kb RNA species have the polymerase activity required for BDV transcription, as well as all the genetic information required to direct the synthesis of BDV macromolecules, both protein and nucleic acids, and the production of infectious BDV particles. As shown in Fig. 8c, after reaching 80 to 85% infection of the cells in the population, no further increase in the percentage of transfected cells was obtained despite the use of increasing amounts of BDV-RNPs. Presently we do not have an explanation for this finding, but it is unlikely that it reflects the presence of a subpopulation of cells refractory to BDV infection, as indicated by the observation that after several passages more than 95% of the transfected cell population expressed viral antigens. One possible explanation is a relationship between the cell cycle and susceptibility to BDV replication, and this is presently under investigation. Furthermore, experiments which aim to obtain a more detailed

C6 cells transfected with BDV-RNPs. Uninfected C6 cells were processed for detection of BDV antigens (panel A). C6 cells (5×10^4 cells) were infected with 100 μl of BDV stock (10^6 FFU/ml) and 72 h later were processed for detection of BDV antigens (panel B). C6 cells were treated as for panel B, but prior to infection the BDV sample was treated with 0.05% NP-40 for 15 min on ice (panel C). C6 cells were transfected with 5 μl of the C6 nuclear RNP preparation (1 μl is equivalent to extract derived from 5×10^5 C6 cells) and 72 h later were processed for detection of BDV antigens (panel D). C6 cells were transfected with 5 μl of C6BV nuclear RNP preparation (1 μl is equivalent to extract derived from 5×10^5 C6BV cells) and analyzed for BDV antigen expression 72 h after transfection (panel E). C6 cells were treated as for panel E, but prior to transfection the BDV-RNP sample was treated with trypsin (250 μ/ml for 10 min at 37°C) (panel F). C6 cells were treated as for panel E but were not treated with DEAE-dextran (panel G). C6 cells were transfected with RNA extracted from 50 μl of C6BV nuclear RNP preparation and 72 h later were processed for detection of BDV antigens (panel H). C6 cells were treated as for panel E, but prior to transfection the BDV-RNP sample was treated with 0.05% NP-40 for 15 min on ice (panel I). (b) C6 cells transfected with BDV-RNPs display the same pattern of BDV transcripts as C6BV cells do. C6 cells (10^5 cells) were transfected with 10 μl of nuclear C6-RNP (lane 1), BDV-RNP (lane 2), or RNA purified from 50 μl BDV-RNP (lane 3) and cultured for two passages (six cell doublings) before total RNA was extracted and compared with RNA extracted from C6 and C6BV cells (lanes 4 and 5, respectively) by Northern blot hybridization with a p24 BDV probe. (A) Hybridization with the BDV probe. Lane 6 corresponds to RNA size markers. (B) Ethidium bromide staining. (c) Dose-response of BDV-RNP-mediated infectivity. C6 cells (5×10^4 cells) were transfected or infected with the indicated amount of nuclear C6BV RNP (BDV-RNP) or BD rat brain homogenate, respectively, and the number of cells expressing BDV antigens (expressed as % FFU) was determined as described in Materials and Methods. (□) cells infected with BD rat brain homogenate; (■) cells infected with BD rat brain homogenate pretreated with 0.05% NP-40 for 15 min on ice; (●) cells transfected with BDV-RNP; (○) cells transfected with BDV-RNP pretreated with 0.05% NP-40 for 15 min on ice; (△) cells not treated with DEAE-dextran prior to transfection with BDV-RNP; (a) amount in microliters of BD rat brain homogenate used in each infection; (b) amount in microliters of BDV-RNP used in each transfection. Results represent the mean and standard deviation of two independent experiments. For each experiment, each amount of both BD rat brain homogenate and BDV-RNP, untreated or NP-40 treated, was assayed in duplicate, as well as each amount of BDV-RNP used to transfect C6 cells that had not been pretreated with DEAE-dextran. The number of FFU obtained in each transfection or infection was determined as described in Materials and Methods.

biochemical characterization of the BDV-RNP, including buoyant density, the ability to direct viral RNA synthesis, and its structural features, are presently being conducted.

The observation that a significant fraction of the BDV-RNP infectivity was found to be independent of treatment of C6 cells with DEAE-dextran was unexpected and raises some interesting questions. One possible explanation for this observation might reside in the ability of astrocytes to take up proteins from the surrounding extracellular environment. However, cytoplasmic RNPs prepared from LCMV-infected C6 cells were able to efficiently transfect C6 cells only after pretreatment of the cells with DEAE-dextran, as indicated by the generation of 100 times more LCMV infectious centers from DEAE-dextran treated than untreated C6 cells after transfection with an equal amount of LCMV-RNP (data not shown). This result suggests that the DEAE-dextran treatment-independent BDV-RNP infectivity is unlikely to be mediated by a nonspecific uptake of RNPs by C6 cells. Moreover, C6 cells transfected with an amount (0.2 μl) of BDV-RNP which initially rendered only 10% of the cells BDV positive, became 100% BDV positive after three passages even though we were unable to detect BDV infectious particles in the supernatant of the culture cells at any time. Whether a similar phenomenon is also found with neuronal cells in vivo remains to be determined. If so, it will be attractive to hypothesize that the dissemination of BDV within the CNS might be mediated at least partially through BDV-RNP complexes with the ability to travel through neuronal networks, without requiring the production of fully mature BDV particles. Such a scenario would offer an explanation of why morphological detection of BDV particles in the CNS of the infected host and infected cultured cells has remained elusive (18, 30, 38), despite the rapid propagation of BDV and relatively high levels of viral transcription and replication. Interestingly, transneuronal transmission of infection mediated by virus-RNP complexes has been proposed to occur during infection with rabies virus, another neurotropic nonsegmented negative-stranded RNA virus (19).

The distribution of BDV antigens in the CNS parallels that of the glutamate and aspartate receptors (18), and astrocytes are known to express receptors for excitatory amino acids (2). Thus, it is conceivable that BDV-RNP complexes might display an affinity for particular neurotransmitter receptors, which would facilitate both their dissemination through specific neuronal networks and the infection of astrocytes and other brain cell types expressing the appropriate receptors for which RNP complexes exhibit affinity.

Among previously characterized animal RNA viruses, only orthomyxoviruses, which have a segmented genome, transcribe and replicate in the nucleus of the infected cell (20, 24). Our experimental results indicate that in addition to transcription (6), replication of BDV, a nonsegmented negative single-stranded RNA virus, takes place in the nucleus of the infected cells. Whether this reflects the fact that, like those of orthomyxoviruses, BDV transcripts are initiated by a cap-snatching mechanism or a need for the host nuclear splicing machinery to generate some of the BDV mRNAs remains to be determined. Nevertheless, our findings point to the unique features of the molecular biology of BDV, the study of which may provide new insights about the biology of animal RNA viruses. It should be noted that the subviral agent hepatitis delta virus, which replicates in nature as a satellite of hepatitis B virus, has also been shown to contain a nonsegmented negative-strand RNA genome that replicates in the nucleus (47).

Further studies aimed at the cloning and complete sequencing of the full-length BDV genome will provide critical information regarding the genomic organization of BDV, including how BDV-specific poly(A)$^+$ RNAs are related among themselves and to the negative-sense RNA genome, as well as the relationship between BDV and other nonsegmented negative single-stranded RNA viruses. These studies have been hampered because of the extremely low levels of cell-free virus associated with BDV infection. However, on the basis of results described in this report (Fig. 7), we estimate that RNA isolated from BDV-RNP prepared from 4×10^5 CBV cells contains an amount of BDV 9-kb genomic RNA equivalent to that obtained from BDV particles isolated from 5×10^8 C6BV cells. The relatively high levels of infectious BDV-RNP present in the nucleus of C6BV cells and the ability to isolate the encapsidated RNA should greatly facilitate the molecular characterization of the BDV genome.

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