The Nucleolus Is the Site of Borna Disease Virus RNA Transcription and Replication

J. M. PYPER,* J. E. CLEMENTS, AND M. C. ZINK
Division of Comparative Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Received 13 February 1998/Accepted 3 June 1998

Borna disease virus (BDV) is a neurotropic nonsegmented negative-strand RNA virus with limited homology to rhabdoviruses and paramyxoviruses. A distinguishing feature of BDV is that it replicates in the nucleus of infected cells. Strand-specific probes used for in situ hybridization of infected rat brain showed that there was differential localization of positive- and negative-strand RNAs within the nucleus of neurons. Within nuclei, sense-strand RNAs were preferentially localized within nucleolar regions while genomic-sense RNAs were found in both nucleolar and nonnucleolar regions. These results suggested a role for the nucleolus in BDV replication. Nucleoli isolated from persistently infected neuroblastoma cells contained both genomic and antigenomic BDV RNA species as well as an enrichment of the 39/38-kDa and gp18 BDV proteins. Since the nucleolus is the site of rRNA transcription, we examined BDV transcription in the presence of inhibitors of RNA polymerase I. Inhibition of RNA polymerase I did not affect levels of BDV transcription.

Borna disease virus (BDV) is a neurotropic virus that causes severe neurological disease in its natural hosts (horses, sheep, cattle, and cats) (reviewed in reference 30). The disease is rare, found mostly in central Europe and Scandinavia, and is not readily transmitted between animals. In rats, clinical signs of disease become apparent only when the virus reaches the hippocampus. Hippocampal neurons appear to be most sensitive to the effects of BDV infection and express extremely high levels of BDV antigens and RNA (25, 29).

BDV is a nonsegmented negative-strand (NNS) RNA virus that has recently been classified as a paramyxovirus. However, it is unique among NNS viruses infecting animals in that it replicates in the nucleus (5, 11) and has a number of spliced mRNAs (9, 32). Previous unpublished observations from this laboratory suggested that BDV RNA was concentrated in the nucleoli of infected rat brain neurons. In the present study, we extended these observations by using strand-specific probes for in situ hybridization of sections from infected rat brain. At early time points after infection, these probes revealed a differential pattern of BDV RNA in and around the nucleolus. Sense-strand RNA was detected within the nucleolus, whereas genomic-sense RNA was detected throughout the nucleus. Although BDV RNA and protein species were detected in isolated nucleoli, inhibition of RNA polymerase (Pol) I did not affect synthesis of BDV RNA species.

Localization of BDV RNA in neurons of infected rats. Previous experiments using in situ hybridization suggested that BDV RNA localized in the nucleoli of infected rat brain neurons (unpublished observations). To further examine this possibility, 35S-labeled strand-specific probes were used to detect BDV antigenomic (sense) RNA (Fig. 1a) and BDV genomic RNA (Fig. 1b). Single-stranded probes were made from products of single-sided PCR that specifically amplified one strand of cloned BDV DNA. Clone RT-PCR 5’/3’ was used as a template to make the single-stranded probes; this clone contains ∼300 nucleotides (nt) from each end of the BDV genome and includes coding sequences from the nucleocapsid and Pol genes as well as the terminal noncoding sequences (10). To make a probe that detects sense RNA, clone RT-PCR 5’/3’ was linearized with Smal, and an SP6 primer was used to synthesize sense-strand DNA in a one-sided PCR. This fragment was gel purified and used as the template DNA for making a probe with 35S-dCTP (Amersham) with the oligo Labeling kit (Pharmacia). In a similar fashion, a probe was prepared to detect genomic RNA. Clone RT-PCR 5’/3’ was linearized with SfiI, and a T7 primer was used to synthesize an antisense DNA fragment. The purified fragment was used as a template for making 35S-dCTP-labeled probe. Probes were labeled to a specific activity of 106 cpm/μg and added to the tissue sections at a concentration of 0.2 μg/ml in hybridization buffer. The sections were incubated at 37°C overnight, washed, and dipped in radiographic emulsion (NTB-3; Kodak). Sections were developed after 2 or 3 days of incubation in the dark and counterstained with hematoxylin to identify nuclear structure.

Sagittal sections of brains from pairs of neonatal rats infected for periods of time ranging from 3 days to 7 weeks were examined (total of 18 infected rats). For the earliest two time points (3 and 8 days postinfection), no in situ hybridization signal could be detected. At 12 days postinfection, silver grains could be detected in some of the neurons. Hematoxylin counterstaining was used to reveal the subcellular architecture of these neurons, allowing us to categorize the pattern of grains. Representative cells hybridized with strand-specific probes are shown in Fig. 1. Figure 1a is an example of detection of sense-strand RNA, and Fig. 1b is an example of detection of genomic RNA. At later time points, the density of silver grains was so high that it was impossible to discriminate subnuclear regions in the cells. Therefore, the quantitative analysis was limited to the two animals sacrificed 12 days postinoculation. This time point, when grains were first visible, is expected to represent the earliest stage in replication and thus may more clearly reveal differential subcellular localization of replicating viral RNAs. For each probe and each rat, 100 cells with identifiable nucleoli were examined. Cells were evaluated for the distribu-
tion of grains by examining the relative number of grains over the nucleus and the nucleolus. Although individual cells showed different distributions, overall trends did emerge from an analysis of cells that showed predominantly nucleolar or predominantly nuclear (non-nucleolar) density of grains (Table 1). For both animals, sense RNAs were present at higher levels in the nucleolus than in the nucleus. In contrast, the genomic RNAs tended to be equivalently represented in both nucleolar and nonnucleolar regions, although grains were often concentrated in perinucleolar regions. This examination strongly suggested that the nucleolus was involved in viral RNA synthesis. It further suggested that there was differential retention of antigenomic and genomic RNA species within the nucleolus. The higher levels of antigenomic RNA within the nucleolus suggest its retention as a template for synthesis of the genomic RNA while the higher nuclear (and perinucleolar) levels of genomic RNA suggest that it is being exported from the site of synthesis.

Detection of BDV RNA species in nucleolar fractions. The in situ hybridization data strongly suggested an association of BDV RNA with the nucleolus. To extend this observation, RNA was prepared from subcellular fractions of BDSK cells (28), which are persistently infected human neuroblastoma SKNSH cells (2). The nucleolar isolation protocol of Bolla et al. (3) was used with aliquots saved from intermediate stages of purification. All solutions contained 10 mM vanadyl ribonucleoside complex (Life Sciences) to inhibit RNases during the fractionation procedure. The crude nucleoli were washed with TE (1 mM Tris-HCl [pH 8.0], 1 mM EDTA), digested with RQ1 DNase (Promega), and finally washed with 2 M NaCl to remove any contaminants. RNA was prepared from each fraction (7) and recovered from cytoplasmic, nuclear, and nucleolar fractions as well as from the TE wash of the crude nucleoli but was not recovered from the nonnucleolar nuclear fraction despite the presence of vanadyl ribonucleoside complex in all solutions during the fractionation procedure.

RNA species present in each fraction were identified by Northern blotting analysis with probes specific for genomic and antigenomic BDV RNA as well as a probe specific for 18S rRNA (Fig. 2). Genomic RNA was present at high levels in the

![FIG. 1. In situ hybridization of BDV-infected rat brain 12 days postinfection. Sections were hybridized with single-stranded DNA probes that detect sense-strand RNA (a) and genomic RNA (b). (a) The nucleolus is densely labeled, while there are few grains in the remainder of the nucleus. (b) The nucleolus is spared, but grains are densely distributed at the periphery of the nucleolus and found more diffusely in the nucleoplasm of the neuron. The figure was prepared with Photoshop.](http://jvi.asm.org/)

![FIG. 2. Detection of RNA species in subcellular fractions of BDV-infected cells. RNA isolated from subcellular fractions was subjected to Northern blot analysis with probes specific for BDV genomic RNA (A), BDV sense RNA (B), and 18S rRNA (C). The locations of the full-length genomic and antigenomic RNAs are indicated in panels A and B. Panel B also shows the location of the abundant 0.85-kb mRNA. The location of the mature 18S rRNA species is indicated in panel C; asterisks show the precursor species detected in the nuclear and nucleolar fractions and in the TE wash of nucleoli. The figure was made from scanned fluorographs with Photoshop and Illustrator.](http://jvi.asm.org/)
nucleus, nucleolus, and the TE wash of the crude nuclei, with lower levels detected in the cytoplasmic and whole-cell preparations. Hybridization with a probe that detects mRNAs and antigenomic RNAs showed that full-length antigenomic RNA was present in all fractions, with high levels detected in the cytoplasm, nuclei, and nucleoli. The 0.85- and 2.1-kb mRNA bands are clearly detected in the total and cytoplasmic RNA lanes. However, in the nuclear, nucleolar, and TE wash fractions there is a broad band of ~0.85 to 1 kb in size; the specific mRNA bands are not clearly discernible. Hybridization with an 18S rRNA-specific probe clearly showed the presence of rRNA precursor molecules in the nuclear and nucleolar fractions as well as the TE nucleolar wash. Only mature 18S rRNA was detected in the whole-cell and cytoplasmic RNA preparations.

Detection of BDV proteins in nucleolar fractions. The detection of BDV RNA species in the nucleolar fraction of persistently infected cells led to an examination of the viral protein species present in nucleoli. Many studies have documented punctate nuclear immunofluorescence in BDV-infected cells, but this signal has not been shown to colocalize with nucleoli. Metabolically labeled BDSK cells were subjected to the same fractionation protocol except that a cocktail of protease inhibitors was used instead of the vanadyl ribonucleoside complex. BDSK cells were metabolically labeled overnight with [35S]methionine and [32P]orthophosphate, and the probes were unlabeled RNA species generated in vitro. Four T-25 flasks of BDSK cells were made from scanned fluorographs with Photoshop and Illustrator.

Immunoprecipitation with anti-BDV antisera showed that BDV proteins were differentially represented in the subcellular fractions (Fig. 3A). Detection of the 24-kDa protein was relatively constant among the fractions although slightly lower in the supernatants from the TE and salt washes. In contrast, the level of the 39/38-kDa nucleocapsid protein was slightly elevated in the nuclear fraction compared to that in the cytoplasmic fraction and the whole-cell extract but markedly elevated in the nucleolar fraction and in proteins eluted by the final 2 M NaCl wash of nucleoli. Proteins washed from the crude nucleoli with TE prior to the salt wash contained reduced levels of nucleocapsid protein. Interestingly, gp18 (previously called the 14.5-kDa protein) shows a fractionation pattern similar to that of the nucleocapsid protein.

The efficiency of the fractionation protocol was assessed by immunoprecipitation of the cellular proteins, B23 and SC-35, that served as markers of nucleolar and nonnucleolar nuclear fractions, respectively (Fig. 3B and C). B23 was detected in all fractions but was observed at higher levels in the nuclei, nucleoli, and nucleolar fractions, with the highest concentration in nucleoli (Fig. 3B). Fractions were also immunoprecipitated with anti-SC-35. Little or no SC-35 was detected in immunoprecipitations from the whole-cell lysate, the cytoplasmic fraction, or the salt wash. SC-35 was present in the immunoprecipitations from the nuclear and nonnucleolar fractions as well as the TE supernatant, with highest levels detected in the nonnucleolar fraction (Fig. 3C). Although all immunoprecipitations were subjected to the same stringent washing conditions, numerous background bands were detected only in the nucleolar and nonnucleolar lanes. Because of the background in the nuclear lane, it is impossible to state unequivocally that there is no SC-35 present in the nucleolar preparation, but it is clear that the level is much lower than that in the nonnucleolar fraction. Additionally, the salt wash contains much less SC-35 than does the TE wash, also suggesting that SC-35 has been removed during the nucleolar isolation.

Resistance of BDV RNA synthesis to inhibitors of cellular RNA Pols. The detection of BDV RNA and protein species in the nucleoli suggested a nucleolar functional role in BDV RNA synthesis. One possibility was that RNA Pol I or its cofactors might be involved in BDV RNA synthesis either directly or indirectly. To test this hypothesis, RNA species synthesized in the presence of inhibitors of cellular transcription by RNA Pol I (25 μM camptothecin or 0.04 μg of actinomycin D per ml) or RNA Pol I and Pol II (4 μg of actinomycin D per ml) were analyzed in an RNase protection assay (Fig. 4). For this experiment, the RNA species synthesized during the drug treatments were metabolically labeled with [35S]methionine, and the probes were unlabeled RNA species generated in vitro. Four T-25 flasks of BDSK cells were metabolically labeled in the presence of inhibitors of cellular RNA polymerases. One flask of cells was labeled without drug treatment (control). The other flasks were treated with drugs: 25 μM camptothecin (Pol I inhibition), 0.04 μg (low) of actinomycin D (Pol I inhibition) per ml, or 4 μg (high) of actinomycin D (Pol I and Pol II inhibition) per ml. The flasks were incubated with the drugs in complete medium for 1 h. They were then washed with phosphate-free RPMI medium (Gibco). The labeling medium contained the appropriate drug...
in phosphate-free RPMI supplemented with 10% dialyzed fetal calf serum (Gibco) and 2 mCi of [32P]orthophosphate (New England Nuclear) per ml. Cells were maintained in labeling medium for 5 h, and then RNA was isolated (7). Both the total yield of RNA and the incorporation of radioactivity were determined for each sample.

To detect RNA species transcribed during the drug treatments, unlabeled antisense transcripts were hybridized to the labeled RNA isolated from BDSK cells followed by RNase A digestion and gel analysis. Unlabeled probes specific for BDV RNA, actin mRNA, and 18S rRNA were transcribed in vitro. The BDV 3’ RACE clone (10) contains 314 nt of BDV sequence) and inserting the fragment into pGEM4Z (Promega) with KpnI and BamHI. The cloned DNA was linearized with EcoRI, and antisense RNA was transcribed with T7 RNA Pol.

Hybridizations with the actin and BDV probes contained 10 µg of 32P-labeled total RNA isolated from BDKS cells and 2 µg of unlabeled antisense RNA probe. One microgram of total labeled RNA was hybridized to 2 µg of the 18S rRNA probe to ensure that there was excess probe in the reaction. Multiple probes could not be hybridized in a single tube because of high background levels arising from the digested labeled cellular RNA. Hybridization and digestion conditions have been designated previously (27), except that the digestion buffer contained 100 µg of RNase A per ml and the digestion was allowed to proceed for 1 h.

The level of BDV RNA species synthesized was constant despite the drug treatments (Fig. 4A). In contrast, the drug treatments affected transcription of cellular RNA by RNA Pol I and Pol II (Fig. 4B and C). RNA Pol II activity was assessed with an actin-specific probe (Fig. 4B). The camptothecin treatment caused a slight reduction in expression of actin mRNA, but the low concentration of actinomycin D did not. However, as expected, the high concentration of actinomycin D reduced actin mRNA expression to undetectable levels. An 18S rRNA-specific probe was used to assess the drugs’ effects on RNA Pol I synthetic activity (Fig. 4C). The 18S rRNA probe protects a 200-nt fragment, although an additional band of ~100 nt shows similar responses to the drugs. Both camptothecin and the low concentration of actinomycin D markedly reduced expression of the 18S rRNA species but did not completely inhibit it. However, no 18S rRNA was detected when cells were treated with the high concentration of actinomycin D.

From analysis of 18S rRNA and actin mRNA synthesis, it is clear that 4 µg of actinomycin D per ml inhibited cellular transcription by both RNA Pol I and RNA Pol II. The conditions expected to specifically inhibit cellular transcription by RNA Pol I were not completely effective in blocking 18S rRNA synthesis, although transcription was significantly reduced. However, none of these drug treatments (including the high dose of actinomycin D) affected the level of BDV RNA synthesis, indicating that inhibition of the cellular RNA Pols neither increased nor decreased transcription of BDV RNA.

Conclusions. This study demonstrates that the nucleolus is the site of BDV RNA synthesis. In situ hybridization of newly infected neurons showed a concentration of BDV RNA in and around the nucleolus, with a concentration of antigenic RNA within the nucleolus. The antigenic species are expected to be retained in the nucleolus, while some of the genomic species would be exported to form viral particles. The observation of genomic species at the periphery of the nucleolus suggests export from the site of synthesis. Differential localization of viral RNA species was not detectable at later times of infection in neurons in the rat brains, nor was it detectable by Northern blotting analysis of the persistently infected cells. It is likely that high levels of RNA expression obscure subtle quantitative differences. In the persistently infected cells, both positive- and genomic-sense 9-kb viral RNAs were found in the nucleolus. The presence of both genomic and antigenic BDV RNAs in the nucleolus is consistent with BDV replicative processes occurring in the nucleolus.

A subset of BDV proteins was also concentrated in the nucleolus. If viral RNA synthesis occurs in the nucleolus, the presence of certain BDV proteins would be required in the nucleolus. Viral Pol activity must be present at the site of synthesis, but the protein is presumably present at such low levels as to be undetectable by immunoprecipitation. By analogy with other NNS viruses, the 24-kDa phosphoprotein is expected to play a role in BDV replication. Although nucleoli did not contain enriched levels of the 24-kDa protein, it was present at significant levels within nucleoli. Its presence in multiple cellular compartments may reflect a multifunctional role for this phosphoprotein in addition to its presumed role in replication. The nucleocapsid proteins are expected to be present at high levels at the site of viral RNA replication since genomic and antigenic viral RNAs of NNS viruses are encapsidated at the time of synthesis. The 39/38-kDa nucleocapsid proteins are indeed present at high levels in the nucleolus.

Curiously, we also observed that the level of the gp18 glycoprotein (formerly identified as the 14.5-kDa protein [31]) was elevated in the nucleolus, gp18 is encoded by the third open reading frame of BDV (21). For other NNS viruses, the third open reading frame encodes the matrix protein. The matrix proteins of NNS viruses are believed to form a bridge...
In rhadovirus infections, M binds to the nucleocapsid core. The nucleocapsid core-M protein complexes migrate to regions of the plasma membrane where G protein is concentrated and where the virus buds from the cell (reviewed in reference 35). In contrast, in the classic paramyxoviruses the M protein associates with the viral glycoprotein at the cell surface prior to arrival of the nucleocapsid (reviewed in reference 22).

The nucleolar concentration of gp18 suggests that it associates with newly synthesized nucleocapsids before their export from the nucleolus. Interestingly, antibodies to gp18 have some neutralizing activity against BDV (20). This neutralizing activity may be directed against M-coated nucleocapsids rather than complete virions. BDV-infected cells produce very low levels of infectious particles even though they express high levels of BDV RNA and proteins. Additionally, it has proven difficult to conclusively identify viral particles either in cell culture or in infected animals. This suggests that assembly of complete particles is very inefficient. Immunofluorescent detection of newly infected tissue culture cells usually shows that there are discrete foci of intensely labeled cells surrounded by cells that are labeled less intensely, suggesting cell-to-cell spread of infection. Probably, infection by M-coated nucleocapsids is relatively inefficient, and this infection can be blocked by anti-gp18 antibodies. However, once one cell is infected, adjacent cells can be infected by cell-to-cell transmission of incomplete particles.

The apparent nucleolar involvement suggested by the in situ experiments led us to investigate whether RNA Pol I might be involved in BDV RNA synthesis. BDV RNA synthesis was not affected under conditions that inhibited cellular RNA Pol I, indicating that BDV does not use cellular Pols or cofactors for transcribing and/or replicating its RNA. However, it is possible that other nucleolar proteins are involved, possibly by providing a structural environment for RNA synthesis or a mechanism for export of ribonucleoprotein particles.

The nucleolus is the site of extremely high levels of biosynthetic activity: rRNA species are transcribed and processed, and ribosomal proteins are recruited to the nucleolus for assembly of preribosomes. BDV appears to be one of a small group of unrelated viruses that are known to have coopted certain aspects of nucleolar activity. There are only a few known examples of viral replication in nucleoli. Plant viroids affects the stability and transport of the viral mRNA. Proc. Natl. Acad. Sci. USA 89:11886–11889.

The lentiviruses have made use of nucleoli in a different fashion. The viral proteins Rev and Rex are cellular proteins that subverts nucleolar transport functions. Although no Rev response element-like elements or Rev-like proteins have been described for BDV, it is possible that the virus uses nucleolar transport mechanisms to deliver viral RNA to appropriate cellular compartments.

We thank Michele Neallen, Jeff Spelman, and Kevin Maughan for technical assistance and Krishna Kalicharran, Barry Morse, and other members of the Retrovirus Biology Laboratory for helpful discussions. This work was supported by grant NS31908 from the National Institutes of Health.

REFERENCES


31. Schaedler, R., H. Diringer, and H. Ludwig. 1985. Isolation and character-