Borna Disease Virus Matrix Protein Is an Integral Component of the Viral Ribonucleoprotein Complex That Does Not Interfere with Polymerase Activity[▽]

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We have recently shown that the matrix protein M of Borna disease virus (BDV) copurifies with the affinity-purified nucleoprotein (N) from BDV-infected cells, suggesting that M is an integral component of the viral ribonucleoprotein complex (RNP). However, further studies were hampered by the lack of appropriate tools. Here we generated an M-specific rabbit polyclonal antiserum to investigate the intracellular distribution of M as well as its colocalization with other viral proteins in BDV-infected cells. Immunofluorescence analysis revealed that M is located both in the cytoplasm and in nuclear punctate structures typical for BDV infection. Colocalization studies indicated an association of M with nucleocapsid proteins in these nuclear punctate structures. In situ hybridization analysis revealed that M also colocalizes with the viral genome, implying that M associates directly with viral RNPs. Biochemical studies demonstrated that M binds specifically to the phosphoprotein P but not to N. Binding of M to P involves the N terminus of P and is independent of the ability of P to oligomerize. Surprisingly, despite P-M complex formation, BDV polymerase activity was not inhibited but rather slightly elevated by M, as revealed in a minireplicon assay. Thus, unlike M proteins of other negative-strand RNA viruses, BDV-M seems to be an integral component of the RNPs without interfering with the viral polymerase activity. We propose that this unique feature of BDV-M is a prerequisite for the establishment of BDV persistence.

Matrix proteins of negative-strand RNA viruses (NSV) are critical to viral entry, assembly, and egress from the host cell. In addition to their role in virus budding, M proteins play an active role in viral replication by inhibiting viral transcription, which has been shown for most NSV including influenza virus, vesicular stomatitis virus, rabies virus (RV), respiratory syncytial virus (RSV), measles virus, and other viruses (6, 7, 11, 13, 24, 26, 27, 35, 39). Transcription inhibition by NSV M proteins is presumably an active process and not simply a result of ribonucleoprotein complex (RNP) condensation (10, 11, 21). Independently of the various functions of M, the nature of M-RNP interactions is not well characterized. Direct M-RNA binding has been shown for influenza A virus (36), filoviruses (14), and RSV (27) and has been speculated for RV (11). In addition, there is also strong evidence that protein-protein interactions comprise part of the M-RNP interaction (5, 27, 38). A direct interaction of M with viral nucleoprotein has been postulated for influenza virus, RSV, vesicular stomatitis virus, parainfluenza virus, and measles virus (9, 13, 22, 27).

Borna disease virus (BDV), the prototypic member of the family *Bornaviridae* of the order *Mononegavirales*, is an enveloped, nonsegmented negative-strand RNA virus (2, 28). It causes a persistent infection of the central nervous system of a

wide range of warm-blooded animals, which can result in symptomless viral persistence or severe immune-mediated neurological disease (15, 17, 34). BDV-infected cells typically produce less than 0.05 infectious virus particle per cell (25). BDV is the only known member of the *Mononegavirales* that transcribes and replicates in the nuclei of infected cells (2). This is carried out by an RNP consisting of the viral genome and four nucleocapsid proteins: the nucleoprotein (N), polymerase (L), phosphoprotein (P), and X protein (28). The function of the X protein is unclear, although it can inhibit viral polymerase activity in the BDV minireplicon system (30).

The M protein of BDV is a 16-kDa protein translated from the unspliced M/G/L viral transcript (3, 19). Biochemical studies indicate that BDV-M oligomerizes and stably forms tetramers and octamers (18). Previous work in our laboratory has shown that M can be copurified with tandem affinity purification-tagged N from infected cells, suggesting that M is a stable component of the viral RNP (23). However, due to the lack of M-specific antibodies, studies on the intracellular localization of this protein to support these results have been pending.

We report the generation of an M-specific polyclonal antibody that can be used to detect this protein by an immunofluorescence assay (IFA). Using this antibody, we found that M colocalizes with other nucleocapsid proteins, including BDV-P, in nuclear punctate structures, suggesting an association of M with viral RNPs. In situ hybridization analysis confirmed that M colocalizes with the viral genome in these nuclear structures. Binding studies revealed that, in contrast to other NSV M proteins, BDV-M specifically interacts with P but not with N.

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The interaction of M with P occurs at the N terminus of P and is independent of the ability of P to oligomerize. Surprisingly, expression of M did not reduce but slightly increased the viral polymerase activity in the BDV minireplicon system, while overexpression of M in persistently infected cells did not inhibit viral transcription. M thus appears to be stably bound to RNPs without inhibiting the viral polymerase.

MATERIALS AND METHODS

Cells and viruses. BSR-T7/5, HEK 293 T, and Vero cell lines, and Vero and Oligo (human oligodendroglial) cells persistently infected with BDV strain He/80, were cultured in Dulbecco's modified Eagle's medium-high glucose (4.5%) supplemented with 10% fetal bovine serum, 100 U of penicillin G per ml, 100 µg of streptomycin per ml, and 4 mM glutamine.

M-specific antibody generation. The cDNA carrying the BDV-M open reading frame was amplified from total RNA from C6 cells persistently infected with strain He/80 by reverse transcription-PCR and was cloned into plasmid pGEX. The recombinant protein was expressed in *Escherichia coli* (BL21) as a fusion protein with glutathione *S*-transferase in the presence of isopropyl- β -D-thiogalactopyranoside (IPTG) and was purified with glutathione Sepharose 4B. A portion of the purified protein was cleaved by factor Xa to remove glutathione *S*-transferase. The purification of the recombinant protein was analyzed on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel by staining with Coomassie brilliant blue. The recombinant M protein was used as an immunogen. Rabbits (4 weeks old) were immunized with the recombinant M protein with complete Freund's adjuvant. The polyclonal anti-BDV-M antibody was prepared from the resulting rabbit hyperimmune sera by ammonium sulfate precipitation.

Plasmid construction. PCR was performed with proofreading Turbo *Pfu* DNA polymerase (Stratagene) under standard reaction conditions in a Primus 25 advanced cycler (Peqlab). The integrity of all PCR-derived DNA fragments was verified by sequencing. All primers and sequences are available from the authors upon request.

Plasmid pCA-M was created by amplifying the BDV-M gene from a full-length cDNA copy of the BDV strain He/80 genome by using primers containing restriction sites for the enzymes NotI and BglII. The restricted PCR product was ligated into a NotI- and BglII-linearized pCA vector, which contains a chicken β -actin promoter (30). Plasmid pCA-Flag-M was created by ligating the same insert into the digested pCA-flagP vector (30).

For pull-down assays, the multiple cloning site of plasmid pET15b, which contains an N-terminal hexahistidine tag, was modified. First, the existing BglII site was ablated using site-directed mutagenesis. Next, the plasmid was digested with BamHI and NdeI, and a linker containing restriction sites for NotI and BglII was ligated into the digested vector. Next, genes encoding wild-type (wt) P, wt N, P_{S7-201} (representing the P₁₆ isoform), P₁₋₁₃₅, and P_{LM2G} were isolated from plasmids pCA-P, pCA-N (30), pCA-P_{S7-201}, pCA-P₁₋₁₃₅, and pCA-P_{LM2G} (29), respectively, by digesting these vectors with NotI and BglII and were ligated into NotI- and BglII-linearized pET15b. Deletion mutants pET15b-P₁₁₋₂₀₁, pET15b-P₄₀₋₂₀₁, and pET15b-P₇₂₋₂₀₁ were created by performing PCR with pCA-P as a template, using forward and reverse primers containing a NotI or BglII restriction site, respectively. These amplified fragments were restricted and ligated into the pET15b vector.

Transfections. Cells seeded in 6-well or 12-well plates and grown to 80% confluence were transfected with Metafectene (Biontex) according to the manufacturer's recommendations. Alternatively, 293 T cells seeded in 10-cm-diameter dishes and grown to 80% confluence were transfected using the calcium phosphate method.

Cell extract preparation. For direct Western blot analysis, BDV-infected or uninfected Vero cells in 6-well plates were harvested in phosphate-buffered saline (PBS), pelleted, resuspended in Laemmli buffer (20), and sonicated. For pull-down assays, HEK 293 T cells in 10-cm-diameter plates were suspended in 600 µl buffer A (10 mM HEPES [pH 7.9], 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol [DTT]) and homogenized by three strokes in a Dounce homogenizer. Then 200 µl buffer B (10 mM HEPES [pH 7.5], 1.26 M potassium acetate, 1.5 mM MgCl₂, 0.5 mM DTT) was added, and the extract was homogenized with a further 20 strokes, followed by centrifugation for 20 min at 10,000 × *g* and 4°C. Supernatants were assumed to be whole-cell extracts.

Peptide array. The construction and detection of peptide arrays have been described previously (1, 12, 32). Briefly, peptide arrays composed of overlapping 15-mer fragments with an offset of 3 amino acid (aa) residues per spot, repre-

sented the protein sequences of BDV-M, -X, -P, and -N (all of BDV strain He/80), were blocked, incubated with the M-specific polyclonal serum at a dilution of 1:1,000 in blocking buffer, washed three times, and incubated with an alkaline phosphatase-conjugated rabbit-specific immunoglobulin G antibody. Bound antibodies were visualized by the blue precipitate formed by the substrate 5-bromo-4-chloro-3-indolylphosphate (BCIP)-2,3-bis-(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide (MTT).

Immunofluorescence and Western blot analysis. Immunofluorescence analysis was performed as previously described (32). The M-specific polyclonal antibody was diluted 1:600, the rabbit-derived polyclonal antibodies specific for BDV-N (30) and -P were diluted 1:500 (30), and the mouse-derived monoclonal antibodies specific for BDV-X (32) and BDV-N (Bo18) (16) were diluted 1:200 and 1:50, respectively. Monoclonal anti-Flag M2 and anti-tubulin antibodies (Sigma-Aldrich) were diluted 1:1,000. Western blot analysis was performed using standard techniques and 15% SDS-PAGE gels. The M-specific antibody was diluted 1:800 in 5% low-fat milk powder in PBS and was incubated with membranes overnight at 4°C. After three washes with PBS plus 0.1% Tween 20, antibodies were detected using horseradish peroxidase-coupled goat anti-rabbit antibodies (Jackson ImmunoResearch) diluted 1:1,000 in 5% low-fat milk powder in PBS, and visualization was performed using ECL Plus Western blot detection reagents (GE Healthcare) according to the manufacturer's instructions.

FISH. The strand-specific, digoxigenin (DIG)-labeled RNA probe was synthesized by first linearizing the plasmid template DNA and then performing an in vitro transcription reaction using T7 RNA polymerase and a nucleotide mixture containing DIG-UTP (Roche) according to the manufacturer's instructions. For the BDV genome probe, the previously described plasmid pBS-KS II-p40, which contains the BDV-N gene in a positive orientation downstream from a T7 promoter, was linearized with KpnI and used as a template (33). For the β -actin mRNA probe, plasmid pBSK- β -Actin was linearized with HindIII. After LiCl-ethanol precipitation, the purified probes were cleaved by alkaline hydrolysis to a length of approximately 250 nucleotides and subsequently denatured. Vero or persistently BDV infected Vero cells were seeded onto glass coverslips and grown to near-confluence. Cells were fixed in 4% paraformaldehyde, and proteins were then denatured with 0.1 N HCl for 15 min. Next, the cells were permeabilized with 0.4% Triton X-100, washed, and acetylated using 0.25% acetic anhydride in 0.1 M triethanolamine. After a wash with 2× SSC (1× SSC is 0.15 M NaCl-0.015 M sodium citrate, pH 7.0), cells were incubated 30 min at 55°C in hybridization buffer (50% formamide, 3× SSC, 50 mM HEPES [pH 7.0], 5× Denhardt's solution [1× Denhardt's is 2% Ficoll 400 {wt/vol}, 2% polyvinylpyrrolidone {wt/vol}, and 2% bovine serum albumin {wt/vol}], 200 µg salmon sperm DNA/ml, 100 µg yeast tRNA/ml), followed by a 12-h hybridization in hybridization buffer containing the denatured RNA probe at a concentration of 0.5 µg/ml. The coverslips were then washed in 2× SSC, and detection was carried out using the fluorescent antibody enhancer set for DIG detection (Roche) according to the manufacturer's instructions. For combination fluorescent in situ hybridization (FISH)-IFA, the FISH assay was carried out as described above, and the primary and secondary IFA antibodies were added during the second and third anti-DIG antibody incubations, respectively. The M-specific antibody was diluted 1:600, while the P-specific antibody was used at a dilution of 1:1,000.

Isolation of recombinant proteins. Recombinant proteins were expressed from the pET15b plasmids coding for either wt or mutant P proteins, which contain an N-terminal hexahistidine tag. These plasmids were transformed into *E. coli* strain BL21, grown at 37°C to an optical density at 600 nm of 0.5, induced with 0.05 mM IPTG, and grown another 2 to 4 h at 25°C. After harvest, the cells were resuspended in buffer C (50 mM Tris HCl [pH 8.0], 500 mM NaCl, 5 mM MgCl₂, 5% glycerol, 0.5% NP-40, 2 mM imidazole, 7 mM β -mercaptoethanol), lysed by sonication, and centrifuged 25 min at 10,000 × *g* and 4°C. Supernatants were then incubated 2 h at 4°C with equilibrated Ni²⁺-nitrilotriacetic acid (NTA) agarose beads (QIAGEN). The beads were washed with buffer C containing 30 mM imidazole, and recombinant proteins were eluted in buffer D (20 mM Tris HCl [pH 8.0], 100 mM NaCl, 5 mM MgCl₂, 20% glycerol, 0.1% NP-40, 7 mM β -mercaptoethanol, 250 mM imidazole). Protein concentrations were determined using Rotiquant as recommended by the manufacturer (Roth).

Pull-down assays. Portions (25 µg) of purified hexahistidine-tagged recombinant proteins were incubated with 200 µl Ni²⁺-NTA agarose beads in 1 ml binding buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM MgCl₂, 0.5% NP-40, 1 mM DTT) for 2 h at 4°C. After two washes with 1 ml binding buffer, 20 µl of hybridized beads was incubated with 50 µl of whole-cell extracts containing overexpressed prey protein in 1 ml of hybridization buffer (10 mM Tris-HCl [pH 8.0], 100 mM NaCl, 0.5 mM EDTA, 0.1% NP-40, 1 mM DTT, 10 mM imidazole) for 1 h at 4°C. Beads were then washed four times with hybridization buffer, and bound proteins were eluted in Laemmli buffer and analyzed by Western blot analysis.

FIG. 1. Specificity of the polyclonal anti-BDV-M antibody and intracellular distribution of BDV-M in persistently infected cells. (A) Western blot analysis of total-cell extracts of BDV-infected or uninfected Vero cells using the M-specific polyclonal antibody. The position of BDV-M is indicated. Asterisk indicates nonspecific binding. (B) An array of membrane-bound, overlapping 15-mer peptides (with an offset of 3 amino acid residues per spot) representing the entire amino acid sequence of BDV-M (strain He/80) was incubated with the polyclonal anti-M antibody. Peptide-bound antibodies were identified and visualized as described in Materials and Methods. (C) (Left) Immunofluorescence analysis of BDV-infected Vero cells using the M-specific antibody. (Right) Magnification of the boxed region in the left panel. (D) Immunofluorescence analysis of uninfected Vero cells using the anti-M antibody (left) or 4',6'-diamidino-2-phenylindole (DAPI) (right). (E) Double immunofluorescence analysis using the M-specific antibody together with monoclonal antibodies specific for either BDV-N (upper panels) or BDV-X (lower panels) in BDV-infected Vero cells.

Minireplicon assays. Minireplicon assays were performed using BSR-T7/5 cells, a BHK-21 cell line that stably expresses T7 polymerase (4), in 22-mm-diameter culture dishes as described elsewhere (30). Briefly, semiconfluent cells were transfected with pCA-L, pCA-P, pCA-N, and pT7-gmgC, which contains a T7-driven chloramphenicol acetyltransferase (CAT) gene in the negative orientation between the 3' and 5' noncoding regions of the BDV genome. Reporter gene activity was measured using a CAT enzyme-linked immunosorbent assay kit (Roche) and was normalized for transfection efficiency by measuring light emission from the simultaneous transfection of 100 ng of a plasmid containing a firefly luciferase gene under the control of a T7 promoter (30).

Northern blot analysis. Total RNA was extracted from persistently infected Oligo cells in 35-mm-diameter plates using peqGOLD TriFast reagent (PeqLab) according to the manufacturer's instructions. Equal amounts of RNA (20 μ g) were electrophoresed, transferred to a nylon membrane, and hybridized with radiolabeled DNA probes as previously described (30). Probes used were the previously described BDV-N/P/X probe (31), a BDV-M probe created from a PCR product amplified from vector pCA-M using M-specific primers, and a β -actin mRNA-specific probe synthesized from the restriction-digested insert from pBSK- β -Actin. Hybridized blots were autoradiographed overnight at -80°C on Biomax MR film (Kodak).

FIG. 2. Transient expression of Flag-M results in association with P and N in BDV-infected cells. (A and B) Shown are results of an immunofluorescence analysis of uninfected Vero cells transiently expressing M (A) or Flag-M (B) using the indicated antibodies. (C) Cellular distribution of transiently expressed Flag-M in persistently BDV infected Vero cells. (D) Double immunofluorescence analysis of BDV-infected Vero cells transiently expressing Flag-M, visualized with a monoclonal anti-Flag antibody and polyclonal antibodies specific for either BDV-N (upper panels) or BDV-P (lower panels).

RESULTS

M is located in the nuclei and cytoplasm of infected cells and colocalizes with other viral proteins. In order to study BDV-M in infected cells, we generated a polyclonal antiserum directed against a purified recombinant form of this protein. To confirm the specificity of this antibody, Western blot analysis using total-cell extracts of either BDV-infected or uninfected Vero cells was performed. As shown in Fig. 1A, the antibody recognized a single protein at approximately 16 kDa, which corresponds to a calculated mass for BDV-M of 16,200 Da (19), whereas no such signal was observed in uninfected cells. To identify the linear epitopes of M recognized by the M-specific antibodies and to further confirm the specificity of this serum, peptide arrays representing the entire amino acid sequence of BDV-X, -N, -P, and -M as overlapping membrane-bound 15-mer peptides with an offset of 3 amino acid residues were incubated with the antibody. While five linear epitopes of M were detected by this approach, corresponding to aa 43 to 48, 55 to 63, 70 to 81, 109 to 117, and 121 to 129 (Fig. 1B), no specific signals were observed using the N, P, and X peptide arrays (data not shown). We next analyzed the subcellular localization of M in BDV-infected cells. Staining with anti-M antibodies of Vero cells persistently infected with BDV displayed a cytoplasmic distribution and a punctate nuclear localization of M (Fig. 1C). For uninfected Vero cells, no signals were observed (Fig. 1D). These nuclear "dots" are typical for BDV infection and have been postulated to be the sites of viral transcription and replication (30). Double immunofluorescence analyses were carried out and revealed a colocalization

FIG. 3. BDV-M colocalizes with the viral genome. FISH was performed on infected or uninfected Vero cells using strand-specific RNA probes specific for the BDV genome (A, C, and D) or β -actin mRNA (B) as described in Materials and Methods. DNA was counterstained using 4',6'-diamidino-2-phenylindole (DAPI) (A through D). Combined FISH and immunofluorescence were performed using the strand-specific RNA probes specific for the BDV genome and polyclonal antibodies specific for BDV-P (C) or BDV-M (D). Arrowheads (A) indicate BDV-genome-specific signals.

of M with N (Fig. 1E, upper panels), as well as of M with X (Fig. 1E, lower panels), in BDV-infected Vero cells, confirming that M is indeed localized in these nuclear "dots."

Subcellular distribution of transiently transfected M. To further investigate the association of M with these nuclear punctate structures, the subcellular distribution of transiently expressed M in BDV-infected versus uninfected Vero cells was compared. In uninfected cells, transient expression of wt M or N-terminally Flag-tagged M (Flag-M) resulted in a predominantly diffuse nuclear distribution (Fig. 2A and B). In contrast, expression of Flag-M in BDV-infected cells resulted in a partial relocation of this protein to discrete sites in the nucleus and the cytoplasm (Fig. 2C). To clarify whether nuclear forms of M colocalize with viral nucleocapsid proteins, double immunofluorescence analyses were performed for BDV-infected Vero cells expressing Flag-M by using the anti-Flag monoclonal antibody together with polyclonal antibodies specific for N

FIG. 4. BDV-M binds to the N terminus of P. (A) A cell extract (CE) (lane 1) of 293 T cells containing M or Flag-M was incubated with His-P (lane 3) or His-N (lane 4) that had been purified from *E. coli* and immobilized on Ni^{2+} agarose beads or empty beads (lane 2). After a wash, samples were analyzed by Western blotting for the presence of M or Flag-M (upper panel) using the M-specific polyclonal antibody or a monoclonal antibody directed against the Flag epitope. Equal levels of immobilized His-tagged proteins were confirmed by SDS-PAGE followed by Coomassie staining (lower panel). Arrowheads indicate the positions of purified viral proteins. (B) Schematic of previously mapped interaction domains of BDV-P. (C) BDV-P mutants used in panel D. The two lines in the BDV-P mutant P_{LM2G} indicate the positions of amino acid changes. (D) Binding assay as described for panel A, using the indicated His-tagged BDV-P mutants purified from *E. coli* and a cell extract of 293 T cells expressing Flag-M (upper panels). Equal levels of immobilized His-tagged proteins were confirmed by SDS-PAGE followed by Coomassie staining (lower panels). Arrowheads indicate the positions of purified viral proteins.

or P. As shown in Fig. 2D, Flag-M colocalizes with either N (upper panels) or P (lower panels) in these nuclear dots, implying that exogenous Flag-M can also associate with nucleocapsid proteins. In addition, Flag-M also associates with N and P in punctate structures in the cytoplasm (Fig. 2D).

M colocalizes with the viral genome. Although it can be assumed that the nuclear dots seen in BDV-infected cells, where M and Flag-M associate, represent viral RNPs, colocalization of the viral genome with BDV nucleocapsid proteins has not been directly demonstrated. Therefore, to investigate this, we performed a FISH assay using DIG-labeled RNA probes specific either for the negative orientation of the BDV-N gene, which was presumed to be found only in the viral genome, or for β -actin mRNA as a control. As Fig. 3A shows, in BDV-infected Vero cells, the viral genome could be detected in nuclear dots, while uninfected cells showed no signal. The control probe specific for β -actin mRNA displayed nearly exclusively cytoplasmic staining in BDV-infected cells (Fig. 3B). Colocalization studies using BDV-P-specific antibodies combined with FISH further revealed that BDV-P colocalized

FIG. 5. BDV-M does not block viral polymerase activity. A T7-based BDV minireplicon assay was performed. BSR-T7 cells were transfected with the BDV minigenome-CAT reporter construct, the N, P, and L expression constructs, and the indicated amounts of plasmids expressing M or X. Plasmid amounts used for transfection were kept constant by the addition of a plasmid expressing green fluorescent protein. The transfection mixture also contained a plasmid constitutively expressing luciferase, which served to normalize the variation in transfection efficiency. Complete reaction mixtures in the absence of M- or X-expressing plasmids served as a positive control (+), and the omission of the P-expressing plasmid served as a negative control (-). Minireplicon activity was analyzed by a CAT enzyme-linked immunosorbent assay. The activity observed in the positive-control reaction was arbitrarily set to 100%. Data are means from four independent assays.

in the nuclear dots with the BDV genome (Fig. 3C). Finally, a colocalization of M and the BDV genome in the nuclear punctate structures was observed (Fig. 3D). Taken together, these findings strongly suggest that the nuclear dot structures in BDV-infected cells containing the P, N, and X proteins also contain the viral genome and therefore most likely represent bona fide RNPs. Furthermore, the colocalization of M with the genome suggests that M is a stable component of these structures.

M binds to P but not to N. Like other viral M proteins, BDV-M may associate with the viral RNP by binding to structural proteins of BDV such as N. To test for such interactions, we performed in vitro binding assays, in which N-terminally hexahistidine-tagged, *E. coli*-purified viral N and P proteins were immobilized on agarose beads and incubated with total-cell extracts from 293 T cells expressing recombinant Flag-M. In this assay, Flag-M binds to His-P but not to N (Fig. 4A). Similar results were also obtained using cell extracts from 293 T cells expressing recombinant M (data not shown). P is a scaffold protein that binds to itself as well as to N, X, and L at defined protein domains (Fig. 4B). To investigate whether the M-binding site overlaps with the already defined protein-protein interaction domains of P, we further mapped the region of P necessary for interaction with M. The C-terminal deletion mutant P₁₋₁₃₅, which lacks the L- and N-binding domains, retained the ability to bind to Flag-M (Fig. 4D), as did the mutant P_{LM2G}, which contains 2 substitutions (L141G and M148G), rendering it incapable of homo-oligomerization (29). In contrast, P mutants with N-terminal deletions of 71, 56, 40, or 11 amino acids did not interact with Flag-M, indicating that the N terminus of P is important for the interaction with M. Binding of P to Flag-M and lack of interaction of Flag-M with

FIG. 6. Overexpression of BDV-M in persistently infected cells does not affect viral RNA synthesis. Persistently BDV infected Oligo cells were transfected with the indicated amounts of pCA-M. The total amount of DNA transfected was kept constant by adding pCA-green fluorescent protein (pCA-GFP). After 48 h, total RNA was extracted and subjected to Northern blot analysis (upper panels) using radiolabeled DNA probes specific for either the BDV genome/antigenome (G/AG), BDV transcripts containing the N/P/X genes (1.9, 1.2, and 0.8 kb), the viral M gene (vM), or transcripts containing plasmid-encoded M (pM) or cellular β -actin. The transfection efficiency was approximately 80% as judged by the appearance of green cells. Note that viral transcription is not affected by increasing levels of M. To confirm viral protein expression (lower panels), extracts from transfected cells were subjected to Western blot analysis using antibodies specific for BDV-M and -P and cellular tubulin.

the P mutant lacking the N-terminal 11 aa (P_{Δ11}) or with BDV-N could be further confirmed by immunoprecipitation experiments after coexpression of these proteins in 293 T cells (data not shown). Interestingly, the monomeric P mutant, P_{LM2G}, can still bind to Flag-M, indicating that homo-oligomerization of P is not necessary for M binding (Fig. 4D).

M does not inhibit viral polymerase activity. A typical feature of matrix proteins of negative-strand RNA viruses is the ability to inhibit viral polymerase activity. Based on the ability of BDV-M to interact with P and its association with nucleocapsid proteins in the nuclei of infected cells, we were interested to see whether this protein also exhibits an inhibitory effect on the viral polymerase activity in a manner similar to other viral M proteins. For this purpose, we took advantage of a BDV minireplicon system (30) that allows quantification of the viral polymerase activity. BSR-T7 cells were transfected with L, N, and P expression constructs and with a plasmid expressing a BDV minigenome encoding the CAT protein. The amount of CAT protein measured directly reflects BDV polymerase activity. Increasing amounts of M-expressing plasmids up to 500 ng did not impair polymerase activity. Rather, the CAT protein level increased up to approximately 220% (Fig. 5). In contrast to the effect of M, cotransfection of only 40 ng of X-expressing plasmids resulted in ca. 90% reduction of

the polymerase activity (Fig. 5), as shown previously (30). Minireplicon assays using the P_{Δ11} mutant revealed that this protein is not capable of supporting polymerase activity (data not shown).

To further characterize the effect of M on BDV polymerase activity, we overexpressed M in BDV-infected cells and observed viral transcription and replication. For this purpose, persistently infected Oligo cells were transfected with increasing amounts of M expression plasmids, and total RNA was extracted 48 h later and analyzed by Northern blot analysis using probes specific for the BDV genome/antigenome, viral transcripts, and plasmid-derived M. As shown in Fig. 6, overexpression of endogenous M did not decrease the levels of the viral genome and antigenome or the levels of viral transcripts encoding the N, P, X, and M proteins.

DISCUSSION

In this report, we describe the intracellular localization, interaction partners, and function of the M protein of BDV. We show that M is not only distributed throughout the cytoplasm but can also be found in the nuclear "dot" structures that are characteristic of BDV infection. Furthermore, we show that these dots contain the viral genome in addition to other viral proteins, providing more evidence that these M-containing structures contain viral RNPs. In contrast to other M proteins of NSV, BDV-M binds to the phosphoprotein but not to the nucleoprotein and does not inhibit viral polymerase activity.

Our investigations revealed a colocalization of M with N, P, and X in nuclear punctate structures in BDV-infected cells. These punctate structures, into which transiently transfected L can also integrate (30), have been proposed to be the sites of BDV replication and transcription (30). Our *in situ* hybridization analyses provide the evidence that viral genomic RNA indeed exists at these sites. We therefore postulate that these dots are the major subcellular locations of RNPs in infected cells, since they represent the only sites where all components necessary for RNP formation, as well as M, colocalize.

Apart from virus particle assembly, transcription inhibition is another major function of NSV M proteins, at least for the rhabdoviruses, paramyxoviruses, and orthomyxoviruses (6, 7, 11, 13, 24, 26, 27, 35, 39). However, our data indicate that BDV-M does not inhibit viral polymerase activity either in the BDV minireplicon system or in persistently infected cells (Fig. 5 and 6). In contrast, the presence of M resulted in a stimulation of reporter activity as high as 2.2-fold. In infected cells, overexpression of M did not have a significant effect on the level of viral RNA synthesis, as opposed to RV-M, which inhibited transcription while promoting replication (11). The reasons for the large discrepancy between BDV-M and the matrix proteins of other negative-strand viruses are unclear. In general, transcription inhibition by M proteins is thought to be a stop signal for the polymerase, since enough viral structural proteins are available for particle assembly, and M thus may act as a molecular "switch" (37). However, BDV produces comparatively very low infectious particle titers, a fact that may obviate the need for a strong switch toward assembly. We speculate that the lack of inhibition of viral RNA synthesis that we observed may prevent the BDV viral life cycle from pro-

gressing toward the virion assembly stage, thus promoting the noncytolytic, persistent infection that characterizes this virus.

Although several NSV M proteins have demonstrated RNA-binding capabilities, they can also interact directly with viral nucleocapsid proteins. This has been shown for influenza virus M, for which RNP association is maintained even after deletion of its RNA-binding domain (38). Additionally, an M-N interaction has been demonstrated for several paramyxoviruses (8, 27). Our findings that M specifically binds to the viral nucleocapsid protein P but not to N therefore suggest that the association of M with the viral RNPs may be mediated by P. However, we cannot rule out the possibility that M additionally possesses an RNA-binding activity that influences the binding to the RNP. Binding between M, P, and other viral proteins might occur not only in the nucleus, but also in the cytoplasm, where partial colocalization was observed by IFA. The M-binding site at the N terminus of P does not overlap with the known binding sites for N, X, L, and the homo-oligomerization domain (29, 33). Therefore, binding of M to P might not interfere with the formation of the polymerase complex, thus explaining why the association of M with P does not detract from the cotranscriptional function of P in the BDV minireplicon. Thus, in contrast to M proteins of all other NSV, which are known to interact with the nucleoprotein and to inhibit viral transcription, the unique property of BDV-M to interact with P and not with N might allow unhindered polymerase activity.

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