Overlap of Interaction Domains Indicates a Central Role of the P Protein in Assembly and Regulation of the Borna Disease Virus Polymerase Complex*

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The active polymerase complex of Borna disease virus is composed of the viral proteins N, P, and L. The viral X (negative regulatory factor) protein acts as a regulator of polymerase activity. Interactions of P with N and X were previously studied, but interactions with L were poorly defined. Using a mammalian two-hybrid system, we observed that L specifically interacts with P but not with N, X, or itself. Mapping of the L-binding domain in the P molecule revealed that it overlaps with two adjacent domains required for multimerization and interaction with N. Competition experiments showed that the interaction between L and P was inefficient when N was present, indicating that L may preferentially interact with free P in infected cells. Interestingly, a multimerization-defective P mutant maintained the ability to interact with L, N, and X but failed to support reporter gene expression from an artificial Borna disease virus minigenome. Furthermore, dominant negative effects on minigenome activity were only observed when P mutants with an intact multimerization domain were used, suggesting that P multimers, rather than monomers, exhibit biological activity. P mutants lacking functional interaction domains for L or N still formed complexes with these viral proteins when wild-type P was available as a bridging molecule, indicating that P multimers have the potential to act as scaffolds on which the RNA polymerase complex is assembled.

The active transcription and replication complex of non-segmented negative strand RNA viruses, termed ribonucleoprotein complex (1), consists of the viral polymerase (L),1 the phosphoprotein (P), the nucleoprotein (N), and the viral RNA (2). P is a co-transcriptional factor of L, whereas N encapsidates the viral genome to form N-RNA complexes (3). For Sendai virus, a prototype paramyxovirus, it was shown that P acts as a scaffold protein, which brings L into close proximity to the N-RNA complex (4), thus allowing RNA synthesis. In the absence of P, the N-RNA complexes are not recognized by L (5). The viral P protein exerts this scaffolding function by employing independent binding sites for L and the N-RNA complex (6). P of Sendai virus contains an additional but distinct binding domain for free N (not bound to viral RNA) (7) and a domain required for multimer formation (8, 9). Complex formation between P and free N prevents aggregation of N (7). Oligomerization is an essential prerequisite for Sendai virus P function in viral transcription and replication (4). The precise function of oligomeric P is unclear. It is assumed that oligomer formation facilitates movement of P (and most likely of the complete polymerase complex) on the RNA template, thereby maintaining polymerase processivity (4). A requirement of P oligomers for viral RNA synthesis was also described for P proteins of other viruses of the order Mononegavirales, including vesicular stomatitis virus (10, 11) and human parainfluenza virus type 3 (12). For the P protein of Sendai virus and human parainfluenza virus type 3, a defined coiled coil region is sufficient for oligomer formation (8, 13). By contrast, vesicular stomatitis virus P oligomerization only occurs prior to phosphorylation of two amino acid residues in the N-terminal acidic region (11). Interestingly, phosphorylation-negative vesicular stomatitis virus P mutants are fully active in replication but defective in transcription (14), suggesting that the viral transcriptase and replicase are two distinct entities with different subunit composition.

BDV is the prototype member of the family Bornaviridae in the order Mononegavirales. It replicates in the nucleus of infected cells (15, 16) and uses alternative splicing for maturation of viral transcripts (17, 18). BDV persistently infects the central nervous systems of a broad range of warm-blooded animals (19). Depending on host and age, BDV infection can result in symptomless viral persistence or severe immune-mediated neurological disease (20). Seroepidemiological surveys suggest that BDV or a related virus can infect humans (21), although the association with specific diseases awaits further clarification. The viral genome includes six open reading frames (ORFs) encoding N, X, P, matrix protein (M), glycoprotein (G), and L (22, 23). Similar to other members of the family Mononegavirales, BDV-P can form oligomers and is able to interact with N and L (24, 25). BDV-P also interacts with the viral X (negative regulatory factor) protein (24). The domains mediating the interactions with N and X were mapped, whereas the domain of P required for the interaction with L was unknown. Functional analysis of the BDV polymerase complex based on artificial minigenomes demonstrated that N, P, and L are sufficient for viral replication and transcription and that X has a negative regulatory function on polymerase activity (26, 27). It was further observed that viral RNA synthesis was only efficient
when the N to P ratio was ~10:1 (26, 27), indicating that the P protein is a key regulator of polymerase activity.

In analogy to P proteins of other Mononegavirales, we hypothesized that the ability of BDV-P to function as a transcriptional cofactor is dependent on oligomer formation. Using a mammalian two-hybrid system to detect protein-protein interactions and a viral minireplisome assay to study functional aspects of the BDV polymerase, we mapped the L-binding site on P and found that it overlaps with the domains for interaction with N and itself. We further showed that the viral negative regulatory factor X can interact with P and P–N complexes, and we provide evidence that P multimers are of critical importance for polymerase activity. Based on these various results, we suggest that P multimers act as central regulatory elements of assembly and activity of the polymerase complex.

**Experimental Procedures**

**Plasmid Constructions**—Primer sequences used in PCR to clone wild-type and mutant BDV sequences into the various vectors are available on request. PCR was performed with proofreading Fwo DNA polymerase (Princeton, New Jersey). DNA fragments were purified using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Digested plasmids were visualized on a 0.8% agarose gel.

To assemble cDNAs encoding VP16/L and Gal4/L fusion proteins in the pCA expression vector, the VP16 transactivation domain and the Gal4 DNA-binding domain were amplified from pVP16/P and pGal4/P (24) using primer pairs VP16(+, EcoRI) and VP16(–, NolI) or Gal4(+, EcoRI) and Gal4(–, NolI). The PCR fragments were EcoRI/NolI-digested and ligated together with the NotI/DraIII fragment (~750 bp) excised from pCA-FLAGL (27) into EcoRI/DraIII-opened pCA-FLAGL plasmid, resulting in expression constructs pCA-VP16/L and pCA-Gal4/L. To generate vectors pCA-VP16/X and pCA-VP16/X, the L ORF was excised from pCA-VP16/L by NotI/HindIII digestion and then inserted into pCA-expression vector plasmid, resulting in expression constructs pCA-VP16/L and pCA-VP16/X, respectively. Because NotI and XbaI create identical nucleotide overhangs, the plasmid was digested and ligated into NotI/XbaI-opened pCA-FLAGL plasmid, resulting in expression constructs pCA-VP16/L and pCA-VP16/X, respectively. The expression constructs were subjected to sequence analysis.

**Cells and Transfections**—Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum for 293T cells (human embryonic kidney) and with 10% fetal calf serum plus 1.0 mg/ml G418 for BSR-T7 cells (baby hamster kidney) stably expressing the T7 RNA polymerase (28). The cells were kept at 37°C in a 5% CO₂ humidified atmosphere.

Seminconfluent layers of 293T and BSR-T7 cells were grown in 15-mm (24-well) or 25-mm (12-well) dishes before transfection with the various plasmids using Metafectene (Biontex, Munich, Germany). DNA dilutions were prepared in 25 µl of DMEM for the transfection of the 15-mm dishes and in 50 µl of DMEM for the transfection of the 25-mm dishes. The DNA preparations were subsequently mixed with 25 µl of DMEM containing 2.5 µl of Metaffectene and 50 µl of DMEM containing 5 µl of Metafectene, respectively. The transfection solutions were incubated for 30 min at room temperature and then applied directly to the cell supernatants.

**Mammalian Two-Hybrid Assay**—Seminconfluent 293T cells in 24-well dishes were transfected with the indicated plasmids as described above. Twenty-four h post-transfection, firefly and Renilla luciferase expression were analyzed using the dual luciferase kit from Promega, basically as described by the manufacturer’s protocol. Briefly, the cells were washed once with PBS (10 mM lysis buffer) and buffered with 10% methanol on a shaking table. Ten µl of the cellular extract were mixed with 50 µl of ice-cold firefly luciferase substrate (Promega), and the emitted photons were measured as relative light units for 10 s in a Lumat LB9501 luminometer (Berthold). Then 50 µl of ice-cold stop and glow Renilla luciferase substrate (Promega) was added to the mixture, and the Renilla luciferase-mediated light emission was measured again for 10 s in the LB9501 luminometer.

**Immunoprecipitation and Western Blot Analysis**—Seminconfluent 293T cells in 25-mm dishes were transfected with the indicated amount of plasmids as described above. Twenty-four h post-transfection, the cells were washed in 500 µl of ice-cold phosphate-buffered saline and lysed in 200 µl of lysis buffer (50 mM Tris, pH 7.5, 1.5 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 0.1% deoxycholate) supplemented with 8 µl of a 1:100 dilution of pCA-FLAGN (27) expressing the T7 RNA polymerase (28). The cells were kept at 37°C in a 5% CO₂ humidified atmosphere. Based on these various results, we suggest that P multimers act as central regulatory elements of assembly and activity of the polymerase complex.

**RESULTS**

**Regulation of BDV Polymerase**

In analogy to P proteins of other Mononegavirales, we hypothesized that the ability of BDV-P to function as a transcriptional cofactor is dependent on oligomer formation. Using a mammalian two-hybrid system to detect protein-protein interactions and a viral minireplisome assay to study functional aspects of the BDV polymerase, we mapped the L-binding site on P and found that it overlaps with the domains for interaction with N and itself. We further showed that the viral negative regulatory factor X can interact with P and P–N complexes, and we provide evidence that P multimers are of critical importance for polymerase activity. Based on these various results, we suggest that P multimers act as central regulatory elements of assembly and activity of the polymerase complex.
Regulation of BDV Polymerase

RESULTS

Identification of the L-binding Domain in BDV-P by Mammalian Two-hybrid Analysis—To study the interaction of BDV-L with itself and with other viral proteins in the mammalian two-hybrid assay (29), we generated expression constructs pCA-VP16/L and pCA-Gal4/L that code for the complete L protein of BDV fused at the N terminus to either the transactivation domain of VP16 or the DNA-binding domain of Gal4. Semiconfluent 293T cells were co-transfected with pCA-VP16/L and either pGal4/N, pGal4/P, pGal4/X (24), or pCA-Gal4/L, respectively. The transfection mixtures further contained two different reporter constructs, one containing a Gal4 promoter-controlled firefly luciferase gene and one containing a constitutively expressed Renilla luciferase gene. Firefly luciferase activity (which indicates protein-protein interaction) was normalized for transfection efficacy by taking the Renilla luciferase activity into account. Expression of VP16/L together with Gal4/P resulted in >200-fold increased luciferase activity over the Gal4/MxA negative control (Fig. 1A). No enhanced luciferase expression was detected when VP16/L was expressed with Gal4/N, Gal4/X, Gal4/L, or the Gal4/MxA negative control (Fig. 1A), indicating specific interaction between the viral L and P proteins. BDV-L still interacted exclusively with BDV-P when the VP16 and Gal4 fusion partners were swapped (data not shown).

Unmodified N and X proteins were expressed together with VP16/L and Gal4/L to determine whether their presence would interfere with the L-P interaction. The X interaction domain is located between P residues 72 and 87 (30), and the N interaction domain includes P residues 197–201 (24). Co-transfection of pCA-N (encoding BDV-N) reduced luciferase activity some 5-fold (Fig. 1B). By contrast, co-transfection of pCA-X (encoding BDV-X) had no detectable impact on the L-P interaction (Fig. 1B). These data indicated that the domain of P required for interaction with L overlapped at least partially with the N but not with the X interaction domain.

Because X acts as regulator of BDV transcription and replication (27), we asked whether P would mediate the association of X with L and N. To address this question, we determined whether unmodified wild-type P could serve as a bridging molecule in the mammalian two-hybrid assay that brings Gal4/X into physical contact with either VP16/L or VP16/N (see schematics included in Fig. 1, C and D). In the presence of unmodified wild-type P, strongly enhanced luciferase activity was observed (Fig. 1, C and D) indicating that the formation of XPL and XPN complexes was induced.

To map the domain in P responsible for binding to BDV-L, we generated a series of constructs in which various N- or C-terminally truncated P proteins were fused to Gal4 (Fig. 2A). Truncations were designed to selectively remove one or more of the previously defined domains on BDV-P known to mediate interaction with the viral proteins N, X, and P (24). Selective elimination of parts of the N-binding domain (Gal4/P1–135) or of the complete X-binding domain (Gal4/P194–201) did not significantly alter the ability of the resulting P fusion proteins to interact with VP16/L (Fig. 2B). In contrast, elimination of the P oligomerization domain (Gal4/P135–197 and Gal4/P172–201) abolished the interaction with VP16/L almost completely, suggesting that this region contains essential sequences for interaction with L. The Gal4/P1–172 fusion protein did not interact with VP16/L, whereas Gal4/P1–183 did (Fig. 2B), indicating that P residues 172–183 (which are not required for P oligomerization) are important for the interaction with L. Because Gal4/P1–135–201 (but not Gal4/P1–201) interacted with VP16/L (Fig. 2B), amino acid positions 135 and 183 determine the N- and C-terminal boundaries of the L interaction domain on BDV-P, as indicated in Fig. 2A.

A Monomeric Variant of BDV-P Retains the Ability to Interact with L, N, and X—Mammalian two-hybrid analyses suggested that BDV-P can oligomerize (24). To determine whether P monomers can interact with other viral proteins, we mutated a leucine zipper motif in the oligomerization domain of P (Fig. 1D).
by inserting two point mutations that replaced leucine 141 and methionine 148 by glycine residues. The resulting construct encoding PLM2G was further engineered to encode a variant with an N-terminal FLAG tag (FLAGPLM2G). An additional construct was generated encoding a FLAGPLM2G variant lacking four amino acids at the C terminus (FLAGPLM2G/197).

The effect of these mutations on oligomerization was first analyzed in a co-immunoprecipitation experiment in which 293T cells were co-transfected with these constructs and a vector (pCA-haP) encoding N-terminally HA-tagged wild-type P. Western blot analysis showed that the extent of expression of all FLAG-tagged P variants was comparable (data not shown). Protein complexes were isolated using a mouse anti-FLAG monoclonal antibody M2 and protein A-coated Sepharose beads. After intense washing, protein A-bound complexes were eluted and analyzed using a goat anti-HA polyclonal antibody. All constructs containing an intact P oligomerization domain (FLAGPwt, FLAGP56–201, FLAGP1–172, FLAGP1–183) were able to co-precipitate HA-labeled P, whereas FLAGGFP and FLAGP1–135 were not (Fig. 3B). FLAGP56–201 co-precipitated HA-labeled P with greatly reduced efficacy. FLAGPLM2G had lost this residual activity (Fig. 3B). These results strongly suggested

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**FIG. 2.** Identification of the domain in BDV-P responsible for interaction with L. A, schematic linear representation of P showing the previously defined domains responsible for direct interaction with BDV-N, -P, -X (gray boxes). The translation initiation site of a naturally occurring N-terminally truncated BDV-P isoform (P56–201) is indicated. The horizontal black bar indicates the location of the newly identified L interaction domain. Note that the N-terminal boundary of the N interaction domain is not mapped. B, mammalian two-hybrid analysis of the VP16/L interaction with N- and C-terminally truncated P mutants fused to the Gal4-binding domain. Semiconfluent 293T cells in 24-well plates were transfected with 0.4 μg of pCA-VP16/L, 0.2 μg of the indicated pGal4 constructs, 0.2 μg of pluc and 0.05 μg of pRL-SV40. All transfection mixtures were prepared as duplicates and used to transfect two wells in parallel. One day post-transfection the cells were lysed and analyzed for firefly and Renilla luciferase using the dual luciferase kit. Values were normalized to the MxA Renilla value as described in the legend to Fig. 1. Shown are the normalized firefly luciferase values. The value of wild-type BDV-P was set to 100%. One typical result of three independent experiments is shown. RLU, relative light units.

**FIG. 3.** Altered interaction of BDV-P mutants with defective oligomerization domain. A, schematic linear representation of P showing the domains responsible for direct interaction with BDV-N, -P, -X, and -L. The relevant amino acids (single letter code) of a potential leucine zipper motif and two point mutations introduced in construct PLM2G are shown. B, co-immunoprecipitation of HA-tagged BDV-P by various P mutants. Semiconfluent 293T cells in 12-well plates were transfected with 1 μg of pCA-haP and 1 μg of the indicated pCA-FLAGP constructs. Twenty-four h post-transfection, the cells were lysed, and P complexes were immunoprecipitated. The precipitated material was analyzed by Western blotting using a goat anti-HA polyclonal antibody. The gel position of HA-tagged P is indicated. The strong bands at ~50 kDa contain the heavy chain of the immunoprecipitating antibody. It served as control to verify loading of comparable amounts of sample into each lane. C, interaction of PLM2G and PLM2G/197 with BDV-L, -N, -P, and -X in the mammalian two-hybrid assay. Semiconfluent 293T cells in 24-well plates were transfected with 0.2 μg of pluc, 0.05 μg of pRL-SV40, and 0.2 μg of the indicated pGal4/P constructs. The transfection mixtures further contained either 0.4 μg of pCA-VP16/L or 0.2 μg of pCA-VP16/N, pCA-VP16/P, and pCA-VP16X, respectively. All transfection mixtures were prepared as duplicates and used to transfect two wells in parallel. One day post-transfection, the cells were lysed and analyzed for firefly and Renilla luciferase expression. Normalization to the Pwt/Renilla value was done as described in the legend to Fig. 1. The value for Gal4/Pwt was set to 100%. The panels show average values of at least three independent experiments. The standard deviations are indicated. RLU, relative light units.
that the leucine-zipper motif within the oligomerization domain is essential for the formation of the P oligomers.

To assess the ability of $P_{\text{LMEG}}$ and $P_{\text{LMEG/197}}$ to interact with L, N, P, and X in the mammalian two-hybrid assay, we inserted their coding regions into vector pGal4/P, resulting in constructs pGal4/$P_{\text{LMEG}}$ and pGal4/$P_{\text{LMEG/197}}$, respectively. Gal4/$P_{\text{LMEG}}$ and Gal4/$P_{\text{LMEG/197}}$ both failed to induce luciferase activity above background level when co-expressed with VP16/P (Fig. 3C), confirming that the oligomerization potential of these molecules is strongly reduced. Lack of activity of Gal4/$P_{\text{LMEG}}$ in this assay presumably reflects lower sensitivity of the mammalian two-hybrid assay compared with the immunoprecipitation assay. As expected based on the known position of the N and P interaction domains (24), VP16/N interacted with Gal4/$P_{\text{LMEG}}$ but not with Gal4/$P_{\text{LMEG/197}}$. Interestingly, abolition of the oligomerization capacity enhanced the interaction of P with X almost 2-fold, suggesting that extensive P multimerization might interfere with efficient X-P interaction. Most importantly, Gal4/$P_{\text{LMEG}}$ and Gal4/$P_{\text{LMEG/197}}$ both strongly interacted with VP16/L (Fig. 3C), demonstrating that oligomerization of P is not a prerequisite for interaction with L.

P oligomers had previously not been demonstrated with biochemical techniques. To find out which oligomeric forms might exist, we expressed wild-type P (and $P_{\text{LMEG}}$ as negative control) in 293T cells and analyzed cell lysates by gel filtration. The bulk of wild-type P eluted between the positions of the marker proteins bovine serum albumin (66 kDa) and cytochrome c (12.4 kDa), indicating that this mutant is between the ovalbumin marker (44.5 kDa) and cytochrome (76–81 kDa), suggesting the predominant interaction of P is not a prerequisite for interaction with L.

![Image](77x604 to 287x738)

**Fig. 4.** P has a high tendency to form oligomers. S100 extract of 293T cells expressing wild-type P (top panel) or $P_{\text{LMEG}}$ (lower panel) was subjected to gel filtration on a Superose 12 column. Column fractions of 500 μl were collected and analyzed by 12% SDS-PAGE followed by Western blotting using a monoclonal anti-P rabbit antiseraum. The gel filtration properties of blue dextran 2000 (void volume), apo-transferrin (76–81 kDa), bovine serum albumin (BSA, 66 kDa), ovalbumin (OVA, 44.5 kDa), and cytochrome c (Cyto. c, 12.4 kDa) are indicated.

![Image](335x382 to 545x737)

**Fig. 5.** Functional analysis of BDV-P variants in a viral minireplicon assay. A, BSR-T7 cells in 12-well plates were transfected with 400 ng of pTT-gmgA, 100 ng of pBST-luc, 500 ng of pCA-N, 300 ng of pCA-L, and 25 ng of the indicated pCA-P constructs. As a negative control, a plasmid encoding the L protein of measles virus (MVL(--) was used instead of pCA-L. B, BSR-T7 cells in 12-well plates were transfected with 400 ng of pTT-gmgA, 100 ng of pBST-luc, 500 ng of pCA-N, 25 ng of pCA-P, and 300 ng of pCA-L. In addition, 25 ng of pCA-FLAGP or the indicated pCA-FLAGP constructs were added to each sample. Seventy-two h post-transfection, the cells were lysed and analyzed for CAT and luciferase expression by enzyme-linked immunosorbent assay and luciferase-mediated light emission, respectively. Shown are CAT values that were normalized for transfection efficacy. The values represent the average of at least three independent experiments. The standard deviations are indicated. Single asterisk indicates a statistically significant ($p < 0.05$) inhibition of CAT expression compared with the pCA-FLAGFP control as determined by the Student’s t test. Double asterisks indicate a statistically highly significant inhibition ($p < 0.01$). Abs., absorbance.
VP16/N and Gal4/P1–197, respectively, which do not interact.

RLU

293T cells in 24-well plates were transfected with 0.2 plasmids as indicated. pGal4/P1–172, pGal4/P1–197, and pCA-VP16/N might induce interaction between VP16/L and Gal4/P1–172 or determined whether co-expression of unmodified wild-type P directly (see Fig. 2).

We found that oligomerization-competent P mutants continued to act as dominant negative factors in this assay even when they lacked the ability to interact with N and L. Interestingly, mutant FLAG-P1-M2G that oligomerized only poorly retained residual interference activity, which disappeared after the oligomerization potential was further diminished by introducing an additional mutation. These data collectively demonstrated that oligomeric rather than monomeric P exhibits high biological activity. Gel filtration studies further revealed that BDV-P could form stable dimers, trimers, and possible tetramers, suggesting that one of these oligomeric states represents the biological active form of this protein.

The conclusion that P is only active in its oligomeric state helps to draw a picture of the active BDV polymerase complex. As suggested by our results, L is unable to interact directly with N. We therefore assume that P oligomers represent the critical link that connects L with encapsidated viral RNA. Oligomeric P may simultaneously interact with several partners, thereby acting as a scaffold, which brings L in close proximity to the N-RNA complex. We assume that interaction with L and N-RNA is accomplished by different P subunits of the multimeric complex. A similar scenario of P-mediated polymerase assembly was suggested for Sendai virus, where interaction of P and L is believed to induce a subtle conformational change in the P multimer, which allows neighboring P molecules to interact with the N-RNA complex (4). Using a modified mammalian two-hybrid system in which interaction is mediated by a bridging molecule, we were able to demonstrate that oligomeric P can indeed interact with L. With the same system, we also showed that oligomeric P can interact with N. These results support the view that the various subunits of the P multimer are capable of engaging in different interactions, bringing L and encapsidated RNA into close contact. Restricted interaction of L may prevent inactivation of polymerase molecules through non-productive binding to free N molecules that are abundantly present in infected cells. Association of N with viral RNA might induce a conformational change in N, which in turn might expose a binding site for the P-L complex. In this context, it is of interest to note that two independent binding sites for P have been described (31).

The main conclusion of the present work is that P oligomerization is essential for the formation of active BDV polymerase.

**DISCUSSION**

Available information from Sendai virus and some other non-segmented negative strand RNA viruses indicates that P proteins may serve as a scaffold for the assembly of the viral polymerase complex (4, 11, 12). It was previously shown that P of BDV can form oligomers and that it can interact with N, X, and L (24, 25). In this work, we found that the L polymerase subunit of the BDV polymerase exclusively binds to P but fails to interact with N, X, and itself, adding weight to the assumption that P plays a central role in polymerase complex formation. Mapping studies showed that the L-binding domain of P is partially overlapping with the domains required for oligomerization and N binding. The tight packaging of the P-, L-, and N-binding domains in the C-terminal 70 residues of the P molecule suggested a strong interdependence of the various interactions. In agreement with this view, we found that the L-P interaction was strongly inhibited when N was present, indicating that L preferentially interacts with free P.

From interaction studies with mutant P1-M2G that carries an inactive leucine zipper motif, we arrived at the conclusion that oligomerization does not strongly influence the ability of P to interact with the other viral proteins. However, although monomeric P interacted with viral proteins similar to its wild-type counterpart, it was inactive when used in place of wild-type P in a BDV polymerase reconstitution assay, indicating that oligomerization is critical for its function. Because P mutants with other minor modifications were also inactive in this system, the possibility remained that inactivity in the minireplicon assay was not caused by the inability of P1-M2G to form multimers. However, additional work with various P mutants clearly demonstrated that the oligomerization domain of P is indispensable for activity. We initially observed that wild-type P with a short N-terminal tag (FLAG-P) lost the ability to reconstitute active BDV polymerase complexes and simultaneously gained the ability to act as a dominant negative factor when used in combination with wild-type P. Using this system, we could show that dominant negative effects of FLAG-P were only observed with P mutants that retained the ability of form oligomers. We found that oligomerization-competent P mutants continued to act as dominant negative factors in this assay even when they lacked the ability to interact with N and L. Interestingly, mutant FLAG-P1-M2G that oligomerized only poorly retained residual interference activity, which disappeared after the oligomerization potential was further diminished by introducing an additional mutation. These data collectively demonstrated that oligomeric rather than monomeric P exhibits high biological activity. Gel filtration studies further revealed that BDV-P could form stable dimers, trimers, and possible tetramers, suggesting that one of these oligomeric states represents the biological active form of this protein.

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complexes. We suggest a model for the assembly and the regulation of the BDV polymerase complex, which shows that by interactions with the different subunits of the P multimer, L and the viral N-RNA complex are brought into close contact. At an optimal N to P ratio of ~10:1 (27), N-RNA template may carry a moderate number of P molecules that probably show a regular distribution on the molecule as suggested for Sendai virus. In analogy with the “cartwheeling” model of Sendai virus (4), the LP complex of BDV could glide over the P-N-RNA complex by consecutive interaction of one P subunit of the P-L complex with one P subunit of the P-N-RNA complex. This model would readily explain why excess of P exhibits a strong inhibitory effect on polymerase activity (26, 27). Unfavorably high amounts of P might increase the frequency of P molecules on the N-RNA template. If the polymerase uses P-N complexes to “walk” on the N-RNA template, the polymerase may not process quickly when P is abundantly present on the template.

We recently showed that X acts as a regulator of the polymerase complex through interaction with P (32). Other negative strand RNA viruses also code for proteins that act as negative regulatory factors. The M1 protein of the influenza A virus and the Z protein of the lymphocytic choriomeningitis virus exhibit an inhibitory effect on viral polymerase activity (33, 34). The NS1 and M2–2 proteins of respiratory syncytial virus are potent inhibitors of viral transcription and replication (35, 36). Similarly, the V and C proteins of Sendai virus, the V protein of measles virus, and the NSs protein of bunyamwera virus were shown to interfere with viral genome replication (37–39). In these cases, the interactions of negative regulatory factors with components of the viral polymerase complexes are poorly characterized, and direct association with the polymerase complex has not been demonstrated. From previous work, it was clear that the X protein of BDV can physically interact with P (24, 34, 40). We therefore tested whether X could directly associate with P-L and P-N complexes in our modified mammalian two-hybrid-based system, in which interaction is mediated by bridging proteins. Results from these binding studies indicate that X-P-L and also X-P-N complexes are formed. Based on immunofluorescence studies of either BDV-infected cells or transfected cells overexpressing X, it was previously suggested that X might act by sequestration of P to the cytoplasm, thereby limiting the availability of P for viral RNA synthesis in the nucleus (30, 32). Although translocation of P into the cytoplasm might partially explain the effect of X, our new data suggest that X could also act more directly in the nucleus, namely by association with the viral polymerase complex through binding to P. This interaction would inhibit the formation of functional polymerase complexes by blocking the association of P-L with P-N-RNA complexes or, alternatively, by blocking the gliding process. A picture thus emerges from these studies that shows that the BDV polymerase complex is highly regulated. It supports previous speculation that a high complexity of polymerase regulation is a prerequisite for the persistent lifestyle of BDV.

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