Interactions in vivo between the proteins of infectious bursal disease virus: capsid protein VP3 interacts with the RNA-dependent RNA polymerase, VP1

Mirriam G. J. Tacken,1 Peter J. M. Rottier,2 Arno L. J. Gielkens1 and Ben P. H. Peeters1

1 Institute for Animal Science and Health (ID-Lelystad), Department of Avian Virology, PO Box 65, NL-8200 AB Lelystad, The Netherlands
2 University of Utrecht, Department of Infection and Immunity, Utrecht, The Netherlands

Little is known about the intermolecular interactions between the viral proteins of infectious bursal disease virus (IBDV). By using the yeast two-hybrid system, which allows the detection of protein–protein interactions in vivo, all possible interactions were tested by fusing the viral proteins to the LexA DNA-binding domain and the B42 transactivation domain. A heterologous interaction between VP1 and VP3, and homologous interactions of pVP2, VP3, VP5 and possibly VP1, were found by co-expression of the fusion proteins in Saccharomyces cerevisiae. The presence of the VP1–VP3 complex in IBDV-infected cells was confirmed by co-immunoprecipitation studies. Kinetic analyses showed that the complex of VP1 and VP3 is formed in the cytoplasm and eventually is released into the cell-culture medium, indicating that VP1–VP3 complexes are present in mature virions. In IBDV-infected cells, VP1 was present in two forms of 90 and 95 kDa. Whereas VP3 initially interacted with both the 90 and 95 kDa proteins, later it interacted exclusively with the 95 kDa protein both in infected cells and in the culture supernatant. These results suggest that the VP1–VP3 complex is involved in replication and packaging of the IBDV genome.

Introduction

Infectious bursal disease virus (IBDV), a member of the family Birnaviridae (Dobos et al., 1979), is the causative agent of a highly contagious immunosuppressive disease in young chickens. Two distinct serotypes, I and II, have been identified (McFerran et al., 1980; Jackwood & Saif, 1987). All known pathogenic IBDV strains belong to serotype I, whereas serotype II viruses, capable of infecting chickens and turkeys, lack clinical manifestations of the disease (Ismail et al., 1988; Kibenge et al., 1991). IBDV multiplies rapidly in developing B lymphocytes in the bursa of Fabricius, leading to immunosuppression. This increases susceptibility to infections by opportunistic pathogens and reduces the growth rate of surviving animals (for reviews, see Becht & Müller, 1991; Kibenge et al., 1988).

IBDV is an unenveloped, icosahedral virus about 60 nm in diameter (Hirai & Shimakura, 1974). Its genome is composed of two double-stranded (ds) RNA segments designated A and B (Dobos et al., 1979; Müller et al., 1979). The larger segment, A (3·3 kb), encodes a 110 kDa polyprotein (pVP2–VP4–VP3) in a large open reading frame (ORF) (Hudson et al., 1986; Spies et al., 1989), which is cleaved autocatallytically to give pVP2 (48 kDa), VP3 (32 kDa) and VP4 (28 kDa). The viral protease, VP4, is responsible for this self-processing of the polyprotein, but the exact locations of the cleavage sites are unknown (Azad et al., 1987; Jagadish et al., 1988). How further processing of the precursor pVP2 takes place to yield the structural protein VP2 (40 kDa) has not been defined, but cellular proteases are not required for this maturation (Kibenge et al., 1997). Since VP2 does not accumulate intracellularly, as the other viral proteins do, post-translational modification of pVP2 into VP2 probably occurs during or after virus assembly (Müller & Becht, 1982). VP4 has often been described as a minor virion component because it was detected in purified virions prepared by a variety of methods (Kibenge et al., 1988). However, Granzow et al. (1997) showed that VP4 is not a constituent of mature virions but that its presence in virion preparations was due to contaminating VP4-containing type II tubules. The major structural proteins of the virion are VP2 and VP3, both constituents of the proteinaceous capsid of IBDV. VP2 carries major neutralizing epitopes (Azad et al., 1987; Becht et al., 1988), suggesting that it is at least partly exposed...
on the outer surface of the capsid. VP3, the major antigenic component (Fahey et al., 1985), contains a very basic carboxy-terminal region that might interact with the packaged RNA and is therefore expected to be on the inner surface of the capsid (Hudson et al., 1986). In addition to the large ORF, segment A also contains a second ORF, preceding and partially overlapping the polyprotein gene, which encodes VP5 (17 kDa). This non-structural protein has only been detected in IBDV-infected cells (Mundt et al., 1995). VP5 proved to be non-essential for IBDV replication (Mundt et al., 1997) but plays a role in virus pathogenesis (Yao et al., 1998), although its exact function is still unknown. The smaller RNA segment, B (2.9 kb), contains one ORF encoding VP1 (90 kDa), the putative RNA-dependent RNA polymerase (RdRp) (Morgan et al., 1989). The system is based on the juxtaposition, driven by a yeast DNA-binding domain with a transcriptional-activation domain, which results in transcription of a reporter gene (Bartel et al., 1989). The system is based on the juxtaposition, driven by a yeast DNA-binding domain with a transcriptional-activation domain, which results in transcription of a reporter gene (Bartel et al., 1989). The system is based on the juxtaposition, driven by a yeast DNA-binding domain with a transcriptional-activation domain, which results in transcription of a reporter gene (Bartel et al., 1989).

Viral proteins generally function by interactions with viral and/or host-cell proteins. Information about these interactions is thus essential for understanding the infection process. The yeast two-hybrid system is a technique that can be used to identify protein–protein interactions in vivo (Fields & Song, 1989). The system is based on the juxtaposition, driven by a yeast DNA-binding domain with a transcriptional-activation domain, which results in transcription of a reporter gene (Bartel et al., 1989; Chien et al., 1991). Our aim is to use the yeast two-hybrid system to determine specific protein–protein interactions in vivo between the IBDV proteins themselves and between IBDV proteins and cellular proteins. Generating interaction maps in this way may be a valuable first tool for the analysis of protein interactions present within a virus or during infection. Here, we report the evaluation of the interactions between the viral proteins VP1, pVP2, VP3, VP4 and VP5. We found that several complexes can form in yeast cells, some homologous and one heterologous. The heterologous interaction (VP1–VP3) was also detected in vivo in IBDV-infected cells. These results suggest that the different interactions observed may be relevant to the functions of the proteins in the virus replication cycle.

Methods

**Virus, cell line, plasmids and antisera.** The IBDV isolate CEF94 is a derivative of PV1 (Petek et al., 1973). After receiving the PV1 isolate in our laboratory in 1973, we have further adapted this isolate by repeated passage (> 25 times) on either primary chicken embryo fibroblast (CEF) cells or bursa cells.

QT35 cells (Moscovici et al., 1977) were cultured in QT35 medium (Fort-Dodge) supplemented with 5% foetal calf serum. Plasmids pHBl36V (A segment) and pHBl34Z (B segment), which contain full-length genomic cDNA of IBDV strain CEF94, were prepared by using full-length RT–PCR fragments generated from purified dsRNA (H. J. Boot, unpublished results).

A rabbit polyclonal antibody against VP1 was obtained after immunizing rabbits with a gel-purified E. coli expression product consisting of amino acids 580–881 of VP1 of CEF94 (E. Claassen, unpublished results). A monoclonal antibody against VP3 (MAb C3) was kindly provided by H. Müller (University of Leipzig, Germany).

**Construction of two-hybrid expression plasmids.** cDNA coding sequences of VP1, pVP2, VP3, VP4 and VP5 of IBDV strain CEF94 were amplified by PCR by using the Expand high-fidelity PCR system (Boehringer Mannheim). The set of primers used was designed to introduce an EcoRI site at the upstream (5') end and a stop codon plus a SphI, Xhol or Sfil site at the downstream (3') end of each coding sequence (Table 1). Plasmid pHBl36V was used as the DNA template for amplification of the pVP2, VP3, VP4 and VP5 genes and plasmid pHBl34Z was used for amplification of the VP1 gene. The PCR products were precipitated, digested with EcoRI/SphI (pVP2, VP3 and VP5), EcoRI/Xhol (VP1) or EcoRI/Sfil (VP4), gel-purified by the QIAEX-II method (Qiagen) and ligated with T4 ligase (New England Biolabs) into the yeast expression vectors pLexA<sub>β</sub>gal and pB42<sub>Δ</sub> (Clontech). These vectors had previously been digested either with EcoRI/Xhol or with Xhol followed by a treatment with the Klenow fragment of DNA polymerase I and subsequent digestion with EcoRI. The ligation mixture was transformed into E. coli DH5α cells (Life Technologies), which were subsequently grown under ampicillin selection. Plasmid DNA prepared from several independent transformants was screened for the presence of the insert and plasmids from positive clones were sequenced at the fusion junction by cycle sequencing with an ABI 310 sequencer (Perkin Elmer) to ensure correct reading frames.

**Two-hybrid analysis.** All two-hybrid media, buffers and protocols were used as described by Clontech in the manual for the Matchmaker LexA two-hybrid system and in the Clontech yeast protocols handbook. The yeast strain S. cerevisiae EGY48 (Clontech) was first transformed by using the lithium acetate method with the URA3<sup>+</sup> plasmid psop-lacZ (Clontech), which has a lacZ reporter gene preceded by an upstream LexA-binding domain. Transformed cells were amplified and subsequently transformed with pLexA<sub>β</sub>gal (His<sup>+</sup>) and pB42<sub>Δ</sub> (TRP<sup>+</sup>) constructs carrying VP1, pVP2, VP3, VP4 or VP5, in every possible pairwise combination. Control plasmids were pLexA<sub>β</sub>gal-Bicoid (pRFHM1, OriGene), pLexA<sub>β</sub>gal-Lamin C (Clontech), pLexA<sub>β</sub>gal-53 (Clontech), pB42<sub>Δ</sub>-SV40 T (Clontech) and pB42<sub>Δ</sub>-empty vector. This resulted in 43 pairwise transformations (see Tables 2 and 3), which were plated onto SD/Glu/−/His−/Ura−/Trp medium. About 10 His<sup>+</sup> Ura<sup>+</sup> Trp<sup>+</sup> colonies from each transformation were subsequently plated onto SD/Gal/Raf−/His−/Ura−/Trp−/Leu medium. To assess the transcriptional activation of the LEU2 reporter gene and onto SD/Gal/Raf/X-Gal−/His−/Ura−/Trp− medium to assess the transcriptional activation of the lacZ reporter gene. The ability of the LexA fusions used in this study to bind operator DNA was confirmed by a repression assay. For this, yeast strain S. cerevisiae EGY48 was transformed with the URA3<sup>+</sup> plasmid pJK101 (OriGene) and, in parallel, with pJK101 together with one of the LexA<sub>β</sub>gal constructs carrying VP1, pVP2, VP3, VP4 or VP5. Transformed yeast cells were plated onto SD/Glu/−/Ura or SD/Glu/−/His−/Ura medium. Plasmid pJK101 contains a lacZ reporter gene, expression of which is driven by the yeast GAL1 promoter. However, two LexA operator genes have been placed between the GAL1 promoter and the lacZ gene. When a LexA<sub>β</sub>gal fusion protein binds to these operators there will be a decrease in the level of GAL1-driven lacZ expression. A liquid assay to quantify β-galactosidase activities was performed by growing transformants to mid-exponential phase in the appropriate selection medium, SD/Gal/Raf−/−Ura or SD/Gal/Raf−/−His−/−Ura, and using o-nitrophenyl-β-D-galactoside as the chromogenic substrate. Each enzyme activity assay was performed with at least five independent colonies and β-galactosidase specific activities were calculated as described by Clontech.
Table 1. Plasmids and primers used in construction of LexA<sub>BD</sub> and B42<sub>AD</sub> fusion proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>LexA&lt;sub&gt;BD&lt;/sub&gt; plasmid</th>
<th>B42&lt;sub&gt;AD&lt;/sub&gt; plasmid</th>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP1</td>
<td>pLexA&lt;sub&gt;BD&lt;/sub&gt;-VP1</td>
<td>pB42&lt;sub&gt;AD&lt;/sub&gt;-VP1</td>
<td>MT09</td>
<td>cccGAATTCATGAGTCACTTTTCAACAGTCCA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MT10</td>
<td>cggCTCGAGTCATGGCGTTGGCGGCGCTCT</td>
</tr>
<tr>
<td>pVP2</td>
<td>pLexA&lt;sub&gt;BD&lt;/sub&gt;-pVP2</td>
<td>pB42&lt;sub&gt;AD&lt;/sub&gt;-pVP2</td>
<td>MT01</td>
<td>cccGAATTCATGACAAAACCTGCAAGATCAAAACC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MT02</td>
<td>gagCTCGAGTCACCTTATGCGCGCCGATCTGCTTC</td>
</tr>
<tr>
<td>VP3</td>
<td>pLexA&lt;sub&gt;BD&lt;/sub&gt;-VP3</td>
<td>pB42&lt;sub&gt;AD&lt;/sub&gt;-VP3</td>
<td>MT05</td>
<td>cccGAATTCATGTTTTCCCTACAAATCCACCGC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MT06</td>
<td>gagCTCGAGTCACCTGAGTCCCTCATGACAGAC</td>
</tr>
<tr>
<td>VP4</td>
<td>pLexA&lt;sub&gt;BD&lt;/sub&gt;-VP4</td>
<td>pB42&lt;sub&gt;AD&lt;/sub&gt;-VP4</td>
<td>MT03</td>
<td>cccGAATTCAGATTACCTGTCGCGGTGGTCTCC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MT04</td>
<td>gagACCCCTACATTGATAGCTGCGGCGATGTTGGCC</td>
</tr>
<tr>
<td>VP5</td>
<td>pLexA&lt;sub&gt;BD&lt;/sub&gt;-VP5</td>
<td>pB42&lt;sub&gt;AD&lt;/sub&gt;-VP5</td>
<td>MT07</td>
<td>cccGAATTCATGTCAGTAGAGATCAGAAAACCG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MT08</td>
<td>gagCTCGAGTCACCTGGCGTTGGCGGCGCTCT</td>
</tr>
</tbody>
</table>

Results

Interactions between IBDV proteins in a yeast two-hybrid system

In search of protein interactions between the viral proteins of IBDV, we used the LexA-dependent two-hybrid interaction assay (Clontech). cDNA segments encoding the viral proteins of IBDV strain CEF94 were generated by PCR and subcloned into the yeast expression vectors pLexA<sub>BD</sub> and pB42<sub>AD</sub> (Fig. 1).

To detect an interaction between the LexA<sub>BD</sub> and B42<sub>AD</sub> fusion proteins, it is crucial that neither fusion protein has an intrinsic or non-specific ability to activate transcription of the reporter genes. Therefore, all LexA<sub>BD</sub> plasmids were co-transformed with pB42<sub>AD</sub>, lacking an insert to test for intrinsic activation, while all the B42<sub>AD</sub> plasmids were co-transformed with pLexA<sub>BD</sub>-Bicoid or pLexA<sub>AD</sub>-Lamin C to test for specificity of interaction with each of the B42<sub>AD</sub> fusion
Table 2. Interactions between the viral proteins of IBDV strain CEF94 in the yeast two-hybrid system, assayed for leucine autotrophy

Growth was recorded after 2 days when the strain with LexA<sub>BD</sub>-p53 and B42<sub>AD</sub>-SV40 T antigen (positive control) showed clear growth. +, Clear growth (strong interaction); +/−, limited growth (weak interaction); −, no growth (no interaction); ND, not determined. All results shown are representative of at least seven independent transformants.

<table>
<thead>
<tr>
<th>B42&lt;sub&gt;AD&lt;/sub&gt; fusion</th>
<th>LexA&lt;sub&gt;BD&lt;/sub&gt; fusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP1</td>
<td>pVP2</td>
</tr>
<tr>
<td>+/−</td>
<td>−</td>
</tr>
<tr>
<td>pVP2</td>
<td>+</td>
</tr>
<tr>
<td>VP3</td>
<td>+</td>
</tr>
<tr>
<td>VP4</td>
<td>−</td>
</tr>
<tr>
<td>VP5</td>
<td>−</td>
</tr>
<tr>
<td>No insert</td>
<td>−</td>
</tr>
<tr>
<td>SV40 T</td>
<td>ND</td>
</tr>
</tbody>
</table>

Table 3. Interactions between the viral proteins of IBDV strain CEF94 in the yeast two-hybrid system, assayed for β-galactosidase activity

The relative strength of the interaction was judged by the intensity of the blue phenotype after 1 day when the strain with LexA<sub>BD</sub>-p53 and B42<sub>AD</sub>-SV40 T antigen (positive control) had deep-blue colonies. +, Deep-blue colonies (strong interaction); +, light-blue colonies (interaction); −, white colonies (no interaction); ND, not determined. All results shown are representative of at least seven independent transformants.

<table>
<thead>
<tr>
<th>B42&lt;sub&gt;AD&lt;/sub&gt; fusion</th>
<th>LexA&lt;sub&gt;BD&lt;/sub&gt; fusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP1</td>
<td>pVP2</td>
</tr>
<tr>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>pVP2</td>
<td>+</td>
</tr>
<tr>
<td>VP3</td>
<td>+</td>
</tr>
<tr>
<td>VP4</td>
<td>−</td>
</tr>
<tr>
<td>VP5</td>
<td>−</td>
</tr>
<tr>
<td>No insert</td>
<td>−</td>
</tr>
<tr>
<td>SV40 T</td>
<td>ND</td>
</tr>
</tbody>
</table>

proteins. pLexA<sub>BD</sub>-Bicoid and pLexA<sub>BD</sub>-Lamin C are commonly used as control plasmids encoding LexA fused to the Drosophila protein Bicoid (OriGene) and to the human lamin C, respectively, and have been reported not to interact with most other proteins (Bartel et al., 1993; Ye & Worman, 1995; Hughes et al., 1996). The results of the assays for reporter expression of LEU2 (selectable) and lacZ (screenable) under the control of LexA-binding sites are shown in Tables 2 and 3.

None of the fusion proteins activated expression of the LEU2 and lacZ reporter genes intrinsically or non-specifically. Therefore, all interactions between the LexA<sub>BD</sub> and B42<sub>AD</sub> fusion proteins of IBDV may be regarded as specific. Plasmids containing p53 fused to LexA<sub>BD</sub> (Clontech) and SV40 large T antigen (SV40 T) fused to B42<sub>AD</sub> (Clontech) were co-transformed into EGY48 and used as a positive control.

All possible pairwise combinations of plasmids containing the LexA<sub>BD</sub> and B42<sub>AD</sub> fusion proteins were co-transformed into S. cerevisiae strain EGY48. We observed strong homologous interactions of the viral proteins pVP2, VP3 and VP5 (Tables 2 and 3). The strength of these interactions was judged by the intensity of the blue phenotype, which has been suggested to reflect semi-quantitatively the stability of the interaction between the candidate proteins (Estojak et al., 1995; Li & Fields, 1993; Yang et al., 1992).

A possible homologous interaction was found for VP1. The yeast strain with LexA<sub>BD</sub>-VP1 and B42<sub>AD</sub>-VP1 showed limited growth on leucine-selective medium but, however, remained negative for β-galactosidase expression. Measurements of β-galactosidase activity were done after 1 day, when colonies of the positive control (LexA<sub>BD</sub>-p53 with B42<sub>AD</sub>-SV40 T) were deep-blue, because at that time we could distinguish variations in intensity of the blue colour of positive colonies. Beyond 3 days, the true positive colonies all had the same colour intensity. Nevertheless, after 3 days of incubation, we observed some β-galactosidase activity for the homologous interaction of VP1 (data not shown). However, we did not consider this blue phenotype to represent a true positive interaction because at that time-point we also observed weak interactions between some of the B42<sub>AD</sub> fusions and LexA<sub>BD</sub>-Bicoid or LexA<sub>BD</sub>-Lamin C, although not between B42<sub>AD</sub>-VP1 and LexA<sub>BD</sub>-Bicoid or LexA<sub>BD</sub>-Lamin C (data not shown). Moreover, it is known that there is an increased risk of false-positive results after a prolonged incubation due to the sensitivity of the lacZ reporter system. Taken together, the yeast two-hybrid data indicate that VP1 might interact with itself, although this would be a very weak interaction according to our observation that only one of the two reporters was sensitive enough to detect this interaction.

One heterologous interaction was found, between VP1 and VP3. This interaction was found for both reciprocal combinations, although the combination of LexA<sub>BD</sub>-VP1 with B42<sub>AD</sub>-VP3 proved to have a stronger lacZ reporter activity than the combination of LexA<sub>BD</sub>-VP3 with B42<sub>AD</sub>-VP1 (Table 3).

The lack of any significant interaction of the LexA<sub>BD</sub>-VP4 fusion may be a consequence of the protein not entering the yeast nucleus and binding to operators, since it led to almost no decrease in β-galactosidase activity in a repression assay (data not shown). This repression assay exploits the fact that LexA when bound to its operator blocks activation of a constitutively expressed lacZ reporter gene (Brent & Ptashne, 1984).
In summary, the yeast two-hybrid assay demonstrated homologous interactions of pVP2, VP3, VP5 and possibly VP1 and a heterologous interaction between VP1 and VP3.

**VP1 interacts with VP3 in IBDV-infected cells**

Since a complex between VP1 and VP3 may have an important function in the virus replication cycle (see Discussion), we employed a co-immunoprecipitation assay to obtain corroborating evidence for this interaction.

Both IBDV-infected and mock-infected cells were metabolically labelled at 4 h.p.i. with $[^{35}S]$methionine for 4 h. The proteins in the cleared cell lysates were subjected to immunoprecipitation with polyclonal anti-VP1 serum (αVP1) or with a monoclonal antibody against VP3 (αVP3) followed by SDS–PAGE (lanes 1 and 2). Mock-infected cells were used as controls for each immunoprecipitation (lanes 3 and 4). Positions of the viral proteins and molecular size markers (in kDa) are indicated.

In summary, the yeast two-hybrid assay demonstrated homologous interactions of pVP2, VP3, VP5 and possibly VP1 and a heterologous interaction between VP1 and VP3.

**Kinetics of association between the viral proteins VP1 and VP3 in vivo**

A pulse–chase experiment was performed with IBDV-infected cells to assess the rate of complex formation between VP1 and VP3. Both IBDV-infected and mock-infected cells were metabolically labelled at 4 h.p.i. for 1 h and chased for different times. Subsequently, the proteins in the cleared cell lysates as well as in the cell-culture media were immunoprecipitated with antibodies specific for either VP1 or VP3 and analysed by SDS–PAGE. Complexes consisting of VP1 and VP3 were detected in cell lysates directly after the pulse (Fig. 3, lanes 1 and 5). VP1 detected immediately after the pulse again appeared to be present in two forms, of 90 and 95 kDa, in nearly equal amounts (Fig. 3, lanes 1 and 5). However, during the chase, the amount of the 90 kDa protein in the cell lysate decreased more rapidly than the amount of the 95 kDa protein (lanes 1–4). Furthermore, after 5, 9 and 19 h of the

---

**Fig. 2.** Interaction of VP1 and VP3 in IBDV-infected cells. IBDV-infected QT35 cells were pulsed at 4 h.p.i. with $[^{35}S]$methionine for 4 h. At 8 h.p.i., cells were lysed and subjected to immunoprecipitation with polyclonal anti-VP1 serum (αVP1) or with a monoclonal antibody against VP3 (αVP3) followed by SDS–PAGE (lanes 1 and 2). Mock-infected cells were used as controls for each immunoprecipitation (lanes 3 and 4). Positions of the viral proteins and molecular size markers (in kDa) are indicated.
Fig. 3. Kinetics of association between the viral proteins VP1 and VP3 in vivo. IBDV-infected QT35 cells were pulse-labelled at 4 h p.i. for 1 h with [35S]methionine and chased in medium containing an excess of unlabelled methionine. At the times indicated, the cell-culture medium was collected and cells were lysed. Subsequently, the cleared cell lysates (a) and cell-culture media (b) were subjected to immunoprecipitation with polyclonal anti-VP1 serum (αVP1) or with a monoclonal antibody against VP3 (αVP3), followed by SDS-PAGE (lanes 1–8). Mock-infected cells were used as controls for each immunoprecipitation (lanes 9–16). Positions of the viral proteins and molecular size markers (in kDa) are indicated.

In the smaller molecular size region, at least two smaller forms of VP3 were again detected (Fig. 3a, b). Finally, after 5, 9 and 19 h of the chase, two additional bands were detected in the high molecular size region (Fig. 3a, b). These two bands proved to be VP1 specific, as confirmed by Western blot analysis (data not shown).

Discussion

The LexA-dependent yeast two-hybrid system was used to examine all potential interactions existing between the viral proteins VP1, pVP2, VP3, VP4 and VP5 of IBDV. The resulting set of positive pairings are a heterologous interaction between VP1 and VP3 and homologous interactions of pVP2, VP3, VP5 and possibly VP1 (Fig. 4).

The heterologous interaction between VP1 and VP3 was found in different strengths in the reciprocal combinations of the LexA_BD or B42_AD plasmids. Such ‘polarity’ of two-hybrid interactions is frequently observed (Cuconati et al., 1998; Xiang et al., 1995). An interaction can be impaired when a fusion protein is folded improperly or inherently unstable, when its expression is poor or when the fused LexA_BD or B42_AD domain partly occludes the site of interaction.

In order to verify whether VP1 and VP3 can interact physically, their association was analysed further by co-immunoprecipitation studies. We found that VP1 and VP3 interacted in vivo in IBDV-infected cells but not in vitro in a rabbit reticulocyte lysate. In the in vitro experiment, the protein(s) may not be folded in their native conformation, which would hinder the interaction. Likewise, Black et al. (1998) detected interactions in a co-immunoprecipitation assay among the proteins G2R, A18R and H5R of vaccinia virus expressed...
during infection, whereas they failed to detect these interactions when these proteins were synthesized in vitro in a rabbit reticulocyte lysate. The interaction between VP1 and VP3 is specific, since the antibodies used showed no cross-reactivity in a co-immunoprecipitation assay of VP1 and VP3 synthesized separately in vitro. It should also be mentioned that all immunoprecipitations were performed in the presence of a small amount of SDS to disrupt virions. Since this detergent did not disrupt the VP1–VP3 complex, this interaction proved to be relatively strong, which is consistent with the data from the yeast two-hybrid assay.

The interaction between VP1 and VP3 is intriguing, as the known or putative biochemical and biological properties of these proteins do not suggest the likelihood of such an interaction. The complexes consisting of VP1 and VP3 are formed immediately or shortly after translation in the cytoplasm of IBDV-infected cells and are eventually released into the cell-culture medium from 10 h p.i. onwards. Therefore, it is likely that VP1–VP3 complexes are also present in mature virions, since this timing is consistent with the release of extracellular progeny virus particles into the culture medium (Petek et al., 1973).

In our co-immunoprecipitation studies, we detected two proteins in the molecular mass region of VP1, of 90 and 95 kDa. Müller & Becht (1982) and Jackwood et al. (1984) have previously reported the existence of these two polypeptides in different IBDV strains. They indicated that these proteins may have a precursor–product relationship. Since the VP1 specificity of these proteins has been confirmed by Western blot analysis (data not shown), we now have the first evidence that these are indeed two forms of VP1. We found that VP3 interacts with both forms of VP1 immediately or shortly after translation (Fig. 2, lane 2; Fig. 3a, lane 5), but that later during infection VP3 interacts only with the 95 kDa form of VP1 (Fig. 3a and b, lanes 6–8). Further experimentation is required to determine the exact nature of these two forms of VP1 and whether there is a precursor–product relationship between them. The difference in size between these two forms is probably not a consequence of a VP1–RNA complex, since there was no change in size of either the 90 or 95 kDa protein after RNase treatment (data not shown). However, it has been shown for infectious pancreatic necrosis virus that short VP1-linked oligonucleotides can survive RNase treatment, probably due to steric hindrance by the unusually large VP1 (Magyar et al., 1998).

It was noteworthy that, after RNase treatment, the two additional VP1-specific bands detected in the high molecular size region after 5, 9 and 19 h of the chase (Fig. 3a, b) had disappeared, meaning that these bands represented VP1–RNA complexes.

It should also be mentioned that, in addition to the 32 kDa form of VP3, we also detected at least two smaller forms of VP3 (Figs 2 and 3). Such forms are frequently seen in IBDV-infected cells but are usually ignored or confused with VP4. In infectious pancreatic necrosis virus, one such form has been described as VP3a (Dobos, 1995a).

Recently, the interaction between VP1 and VP3 has also been described by Lombardo et al. (1999), who observed the interaction by co-localization and co-immunoprecipitation studies of vaccinia virus-expressed VP1 and VP3. Their and our results raise several interesting possibilities regarding the function of the interaction between VP1 and VP3. The interaction may be involved in the regulation of viral RNA synthesis or may be a part of the replication apparatus, as has been proposed for the interaction between the RdRp and the virus coat protein of tobacco vein mottling virus (Hong et al., 1995). An interaction between the RdRp and the virus coat protein has also been observed for alfalfa mosaic virus (AlMV) (Quadt et al., 1991). In this case, minus-strand synthesis by the AlMV RdRp is inhibited by AlMV coat protein (Quadt et al., 1991). In rotavirus, an interaction between the inner capsid protein, VP6, and the inner core polypeptide, VP3, is necessary for recovery of RNA polymerase activity (Sandino et al., 1994). Alternatively, the interaction between VP1 and VP3 may be involved in virus assembly or encapsidation of the virus. It is known for hepatitis B viruses that an interaction between the viral polymerase and capsid protein is required for encapsidation of the pregenomic RNA (Ziermann & Ganem, 1996). These and other possibilities await further experimental study to elucidate the exact function of the interaction between VP1 and VP3.

Of the homologous two-hybrid interactions found, we did not interpret the VP1–VP1 interaction as a true positive interaction, since this interaction resulted in very weak reporter activity. The signal for this homologous interaction was so weak that lacZ expression was undetectable (Table 3). The weakness of this interaction may be related to an intrinsically weak interaction between VP1 polypeptide chains. However, as mentioned above, weak signals in the two-hybrid assay are not necessarily indicative of the strength of a specific protein–protein interaction; poor or unstable expression, improper folding or steric hindrance of the fused LexA<sub>AD</sub> or B42<sub>BD</sub> domains at the site of interaction may impair the interaction. Using the two-hybrid system, Xiang et al. (1998) reported a very weak interaction between the proteins 3AB and 3CD<sub>p10</sub> of poliovirus, which was observed as a strong interaction when tested by far-Western blotting. It is conceivable that a homologous interaction of VP1 can occur. Xiang et al. (1998) reported an interaction between VPg and the polymerase 3D<sub>p10</sub> of poliovirus. The poliovirus protein VPg is covalently linked to the 5′ ends of both genomic and antigenic viral RNA and 3D<sub>p10</sub> is the RdRp. These authors suggested that a direct interaction between these molecules is involved in the mechanism of initiation of viral RNA synthesis. VP1 of IBDV also exists as a genome-linked protein (VPg) (Müller & Nitschke, 1987). Therefore, an interaction between VPg and VP1 of IBDV may have a similar function. Moreover, it has been suggested that the initiation of viral RNA synthesis
of birnaviruses may involve two VP1 molecules, one serving as a primer and the other for polymerase chain elongation (Dobos, 1995b).

We expected to find an interaction between VP2 and VP3, since these two proteins comprise the proteinaceous capsid of IBDV. Although we used pVP2 instead of mature VP2, this should not influence the results, since Kibenge et al. (1999) showed that processing of pVP2 to VP2 is not necessary for capsid assembly. However, no heterologous interaction between pVP2 and VP3 was detected, only strong homologous interactions of pVP2 and VP3. Of course, false-negative results from the yeast two-hybrid assay are not without precedent, as failure to identify other known protein–protein interactions in the two-hybrid system has been reported (Cuconati et al., 1998; Fields & Sternglanz, 1994; Van Aelst et al., 1993). On the basis of electron micrographs, the subunits of the IBDV capsid are predominantly clustered as trimers (Bottcher et al., 1997). On the outer surface, the trimeric units protrude from a continuous shell of density, and on the inner surface the trimers appear as Y-shaped units. Bottcher et al. (1997) suggested that it is likely that the outer trimers correspond to the protein VP2, carrying the dominant neutralizing epitopes, and that the inner trimers correspond to protein VP3, which has a basic carboxy-terminal tail expected to interact with the packaged RNA. According to this study, it is not surprising to find strong homologous interactions for pVP2 and VP3. Therefore, it is also possible that (p)VP2 only interacts with VP3 when they are both present as a trimer subunit. If so, this cannot be detected in the yeast two-hybrid system. It is worth noting that, in the co-immunoprecipitation studies with anti-VP3 serum, we detected no interaction between VP2 and VP3, consistent with the data obtained with the yeast two-hybrid system. However, as mentioned above, all the immunoprecipitations were performed in the presence of a small amount of SDS, so the presumed VP2–VP3 interaction could have been disrupted.

A homologous interaction was also detected for VP5. Since the exact function of this protein is still unknown, it is difficult to speculate about the functional significance of this interaction. A VP5-deficient virus can replicate in the bursa of inoculated chickens but will not induce bursal lesions (Yao et al., 1998). Whether VP5 must assemble into dimers or multimers to produce its effects is unknown.

One potential drawback of a two-hybrid system arises when the fusion protein fails to localize to the nucleus and to bind operators. This was possibly the case for the LexA<sup>BD</sup>VP4 fusion protein, since this fusion protein showed no significant interaction with other viral proteins. Moreover, we performed a repression assay in which VP4 caused hardly any repression of transcription of the lacZ reporter gene (data not shown). This means that nuclear localization or operator binding of this fusion protein is impaired. However, since the B42<sub>AD</sub>–VP4 fusion protein, which possesses a nuclear localization signal and does not have to bind operators, also showed no interaction with other viral proteins, VP4 is probably not able to form heterologous complexes with the distinct viral proteins. Whether VP4 is able to interact with itself therefore remains uncertain. A homologous interaction of VP4 is not inconceivable, since VP4 aggregates to type II tubules (Graznow et al., 1997).

All of the interactions found between the viral proteins of IBDV were detected in the classical attenuated strain CEF94. To check whether there were significant differences between this attenuated strain and a very virulent strain, we also determined the two-hybrid interactions between the viral proteins of the very virulent isolate D6948, with the exception of VP1. The interactions observed were the same as those found for the classical attenuated strain, indicating that there are probably no great differences between these strains in this respect (data not shown).

Taken together, the interactions observed between the viral proteins of IBDV described in the present study underscore the highly co-ordinated nature of the events in which these proteins must participate during genome expression, replication and assortment. However, extensive studies are still required to confirm the role of these proteins and the functional relevance of the interactions described.

We thank H. Müller (University of Leipzig, Germany) for providing monoclonal antiserum against VP3, H. J. Boot for providing plasmids carrying the full-length genomic cDNA of IBDV and for assistance in the preparation of the manuscript and E. Claassen for the production of polyclonal antiserum against VP1.

References


Received 29 July 1999; Accepted 22 September 1999