Molecular Interactions of the Infectious Bursal Disease Virus Proteins

MIRRIAM TACKEN

Cover photo: IBDV particle, enlarged 4 million times;
Three dimensional map of Infectious Bursal Disease Virus, computed from cryoelectron micrographs. © B. Böttcher and R.A. Crowther

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Molecular Interactions of the Infectious Bursal Disease Virus Proteins

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General Introduction

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THE DISEASE

DEFINITION

Infectious bursal disease (IBD) is a highly contagious, immunosuppressive infection of immature chickens caused by the infectious bursal disease virus (IBDV) (Fig. 1). The disease is characterised by the destruction of the lymphoid organs, in particular the bursa of Fabricius where the virus infects actively dividing and differentiating lymphocytes of the B-cell lineage. The infection, when not fatal, causes an immunosuppression, in most cases temporary, depending on the dose and virulence of the strain, the age and breed of the animals, and the presence or absence of passive immunity.

No evidence exists of transmission of IBDV to non-avian species, including humans; the disease thus has no direct impact on public health.

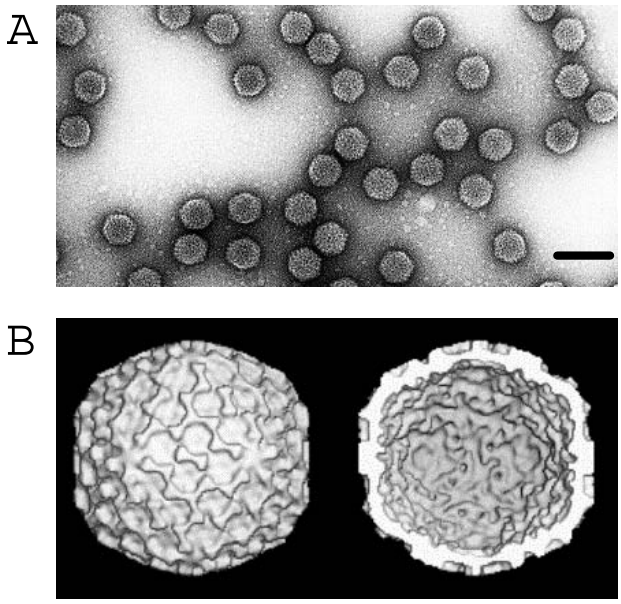


Figure 1. Structure of the IBDV virion (Bottcher *et al.*, 1997; with kind permission of R.A. Crowther). (A) Electron micrograph of purified IBDV virions; bar, 100 nm. (B) Three-dimensional map of the IBDV capsid; the outer (left) and inner (right) surfaces of the particle are viewed along a threefold axis of icosahedral symmetry.

SHORT HISTORY AND EPIDEMIOLOGY

The syndrome, which emerged in 1957 (Cover, 1960), was formally documented in 1962 by Cosgrove (Cosgrove, 1962) in broiler flocks near the town of Gumboro, Delaware (USA). Later outbreaks were subsequently referred to as 'Gumboro disease' after the geographic location. Originally the condition was named 'nephritis-nephrosis syndrome of chickens' because of prominent kidney lesions. After it became evident that an enlarged bursa of Fabricius with endematous swelling and haemorrhagic lesions is the prominent feature of this disease, the term 'infectious bursal disease' has become commonly accepted. Despite confusion regarding the identity of the causal agent, which was originally isolated by Winterfield *et al.* (1962), Koch's postulates were satisfied by Snedeker *et al.* (1967), who isolated a virus from the bursa of Fabricius of affected birds and subsequently produced an attenuated vaccine. Based on its morphology, IBDV was tentatively classified sequentially as a picornavirus (Cho & Edgar, 1969), an adenovirus (Almeida & Morris, 1973), a reovirus (Lukert & Davis, 1974; Kosters *et al*., 1972) and an orbivirus (Hirai & Shimakura, 1974; Harkness *et al*., 1975), until the essential structural features of purified virus were determined. The IBD agent became then designated a birnavirus (Dobos *et al*., 1979) based on the presence of two double-stranded RNAs (dsRNA) (Muller *et al*., 1979*a*) and unique biophysical characteristics (Dobos *et al*., 1979).

Between 1960 and 1964, the disease spread to most regions of the USA (Lasher & Davis, 1997), reaching Europe in the years 1962 to 1971 (Faragher, 1972). From 1966 to 1974, the disease was detected in the Middle East, southern and western Africa, India, the Far East and Australia (Faragher, 1972; Firth, 1974; Jones, 1986; Lasher & Shane, 1994; van den Berg, 2000; van der Sluis, 1999). Infectious bursal disease is currently an international problem: 95% of the 65 countries that responded to a survey conducted by the Office International des Epizooties (OIE) in 1995 declared cases of infection (Eteradossi, 1995), including New Zealand which had been free of disease until 1993 (Jones, 1986).

ANTIGENETIC AND VIRULENCE VARIATIONS

Until 1985, the IBDV field strains (classical strains) isolated were of relative low virulence, causing only 1-2% of specific mortality, and outbreaks were satisfactorily controlled by vaccination. However, since 1985 antigenic and pathogenic variant strains of IBDV, distinct from these classical strains, with increased specific mortality have been described in different parts of the world (reviewed in van den Berg, 2000). In the USA, new strains responsible for up to 5% of specific mortality were described (Rosenberger & Cloud, 1986). These virus isolates were designated

antigenic-variant viruses since they were antigenically different from the classical strains isolated before 1985. They infected broiler chickens possessing relatively high levels of maternal antibodies and were highly immunosuppressive (Box, 1989).

Later on, after 1988, in Europe (Chettle *et al*., 1989), and subsequently in Japan (Nunoya *et al*., 1992; Lin *et al*., 1993), a new class of pathotypic variants with high mortality rates (50-60% in laying hens and 25-30% in broilers) were observed. These isolates caused up to 100% mortality in specific-pathogen-free (SPF) chickens (Nunoya *et al*., 1992; van den Berg *et al*., 1991) and were therefore designated very virulent IBDV (vvIBDV) strains. They rapidly spread all over Asia and to other major parts of the world (reviewed in Eteradossi, 1995; van den Berg, 2000). Australia, New Zealand, Canada and the north of USA, however, have so far remained unaffected (Snyder, 1990; Proffitt *et al*., 1999; Sapats & Ignjatovic, 2000; Nevalainen *et al*., 1999; Czifra & Janson, 1999) by these acute forms of vvIBDV (Fig. 2).

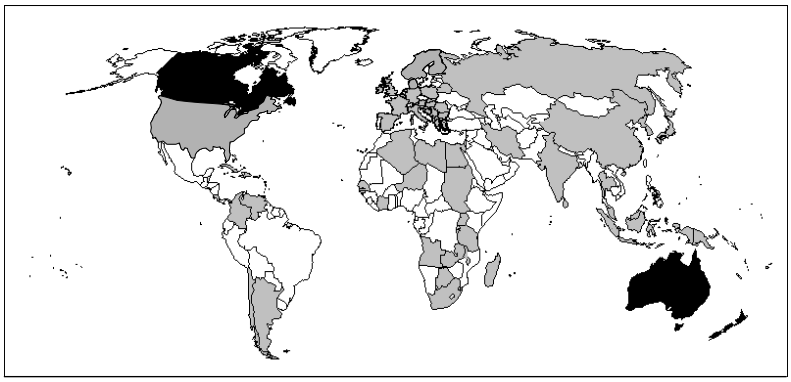


Figure 2. Worldwide geographical distribution of the two forms of IBDV (updated from van den Berg, 2000). In gray, countries where both acute and immunosuppressive forms have been reported. In black, countries where no acute but only immunosuppressive forms have been reported. In white, countries with no reported cases of IBDV (van den Berg, 2000, with kind permission of T.P. van den Berg, N. Eterradossi and Avian Pathology).

These vvIBDV strains caused disease even in the presence of protective maternal antibody against the classical vaccine strains, similar to the antigenic-variant strains (Chettle *et al*., 1989). However, in contrast to the latter, the vvIBDV strains exhibit an enhanced virulence while having the same antigenic structure as classical IBDV isolates (Brown *et al*., 1994). The underlying molecular mechanism for the enhanced virulence of vvIBDV has not been elucidated.

TAXONOMY AND CLASSIFICATION

Infectious bursal disease virus is a member of the birnavirus genus, family *Birnaviridae* (Leong *et al*., 2000). This family was established in 1986 to describe and classify a group of animal viruses that carry a bisegmented dsRNA genome as their distinguishing characteristic (Dobos *et al*., 1979). The two main representatives of this virus family are the infectious pancreatic necrosis virus (IPNV) of young salmonid fish and the causative agent of infectious bursal disease of chicken (IBDV). Other virus members belonging to this family are the tellina virus (TV), oyster virus (OV) and crab virus of bivalve molluscs, and the Drosophila X virus (DXV) of the fruit fly (*Drosophila melanogaster*) (Leong *et al*., 2000). A virus with birnavirus characteristics has been isolated also from European eels (EV) affected by stomatopapillomas. Tumors could, however, not be induced by EV, although the virus causes 50% mortality in young eels. In mammals the only birnavirus-like agents have been observed in faecal samples of several species of vertebrates (Chandra, 1997). Differences from true birnaviruses have been noted in size, length of genome segments and buoyant density; the designation 'picobirnaviruses' has therefore been proposed (Pereira *et al*., 1988; Chandra, 1997). The role of these agents in outbreaks of diarrhoea is uncertain.

The name *birna* highlights the most important feature of these viruses: *bi* signifies the bisegmented nature of the viral genome as well as its double-strandedness and *rna* indicates the nature of the viral nucleic acid. The family contains three genera: Genus Aquabirnavirus (type species: IPNV), Genus Avibirnavirus (type species: IBDV) and Genus Entomobirnavirus (type species: DXV) (Table 1).

Chickens are the only avian species known to exhibit clinical symptoms upon an IBDV infection. Turkeys, ducks, and ostriches are susceptible to infection with IBDV but are resistant to clinical disease (Lukert & Saif, 1997; McNulty *et al*., 1979). Two serotypes of IBDV have been described, distinguished by cross-neutralisation and cross-protection tests. All viruses capable of causing disease in chickens belong to serotype I. Serotype II virus, on the other hand, has been isolated from turkeys having coryza and diarrhoea, but it remains unclear whether this serotype has any true pathogenic significance (Cummings *et al*., 1986). Infections of chickens with serotype II virus do not cause clinical manifestations or noticeable lesions.

In addition to serological classification, the viral strains of IBDV may be classified according to virulence. The serotype II strains may be considered apathogenic and the pathogenic serotype I strains can be subdivided into classical virulent, antigenic-variant or very virulent isolates.

Family	Genus	Type Species	Acronym
Birnaviridae	*Aquabirnavirus*	infectious pancreatic necrosis virus	IPNV
	Avibirnavirus	infectious bursal disease virus	IBDV
	Entomobirnavirus	Drosophila X virus	DXV

Genome: dsRNA

Host: Invertebrates; Vertebrates

Derivation of Name: *Aqua*: from Latin *aqua*, "water"
Avi: from Latin *avis*, "bird"
Bi: from Latin prefix *bi*, "two", signifies the bisegmented nature of the viral genome as well as the presence of dsRNA
Entomo: from Greek *entomon*, "insect"
Rna: from *ribo nucleic acid*, indicating the nature of the viral genome

Table 1. Taxonomic Structure of the Family *Birnaviridae*

PATHOGENESIS AND CLINICAL SIGNS

The preferred host of the virus is young chickens in which a clinical disease develops, while the infection is essentially subclinical in older birds. The incubation period is very short: two to three days. Under natural conditions, the most common mode of infection appears to be via the oral route. From the gut, the virus is transported to other tissues by phagocytic cells, most likely resident macrophages. The target organ of IBDV is the bursa of Fabricius at its maximum development, which is the specific source for B-lymphocytes in avian species. *In vivo* and *in vitro* studies have shown that the virus has a predilection for actively dividing immunoglobulin M (IgM)-bearing B-lymphocytes (Ivanyi & Morris, 1976; Kaufer & Weiss, 1980). Bursectomy can prevent illness in chicks infected with virulent virus (Hiraga *et al*., 1994). The severity of the disease is directly related to the number of susceptible cells present in the bursa of Fabricius; therefore, the highest age at which the animals are susceptible is between 3 and 6 weeks, when the bursa of Fabricius is fully developed. This age dependent susceptibility is broader in the case of vvIBDV strains (van den Berg *et al*., 1991; Nunoya *et al*., 1992). By 13 h post-infection, virus-containing cells appear in the bursa and the virus spreads rapidly through the bursal follicles. Virus replication leads

to extensive lymphoid cell destruction in the medullary and the cortical region of the follicles (Tanimura & Sharma, 1997). The cellular destructive process may be accentuated by apoptosis of virus-free bystander cells (Tanimura & Sharma, 1998). At 16 h post-infection, a second and pronounced viraemia occurs with secondary replication in other organs leading to disease and death (Muller *et al*., 1979*b*). Similar kinetics are observed for vvIBDV strains but replication at each step is amplified. T lymphocytes are resistant to infection with IBDV (Hirai & Calnek, 1979). Although the thymus undergoes marked atrophy and extensive apoptosis of thymocytes during the acute phase of virus infection, there is no evidence that the virus actually replicates in thymic cells (Tanimura & Sharma, 1998; Sharma *et al*., 1989). Gross and microscopic lesions in the thymus are quickly overcome and the thymus returns to its normal state within a few days of virus infection.

Clinical signs associated with acute disease include anorexia, depression, ruffled feathers, diarrhoea, prostration and death. The incidence of mortality is highly variable ranging from 100% to negligible. The most conspicuous lesions concern the bursa of Fabricius. The organ is enlarged, endematous and in some cases haemorrhagic. Petechial or diffuse haemorrhages may be found in muscular tissue. Lymphoid follicles of the bursa of Fabricius are totally necrotic, and in surviving birds the follicles are devoid of lymphoid cells (Fig. 3). Mortality commences on the third day of infection, reaches a peak by day four, then drops rapidly, and the surviving chickens recover a state of apparent health after five to seven days. Disease severity depends on the age and breed sensitivity of the infected birds, the virulence of the strain and the degree of passive immunity. Schematically, the global situation can be divided into three principal clinical forms, as follows:

a) the classical form, as described since the early 1960s and caused by the classical strains of IBDV, where specific mortality is relatively low, and the disease is most often subclinical, occurring after a decline in the level of passive antibodies (Faragher, 1972).

b) the immunosuppressive form, caused by the pathogenic antigenic-variant strains, with an increased mortality and which partially resists neutralisation by antibodies against the classical IBDV strains (Jackwood & Saif, 1987; Snyder, 1990).

c) the acute form, caused by vvIBDV strains, which is characterised by an acute rogressive clinical disease, leading to high mortality rates; mortality occurs even in the presence of a moderate level of maternal antibodies (Chettle *et al*., 1989; Stuart, 1989; van den Berg *et al*., 1991).

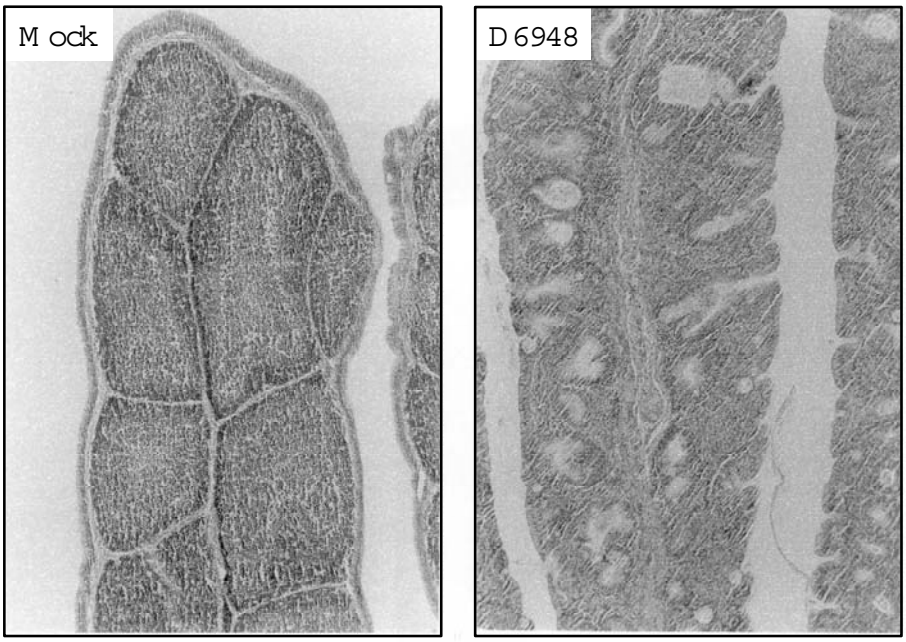


Figure 3. Hematoxylin-eosin-stained sections of the bursa of Fabricius at day 13 post infection of SPF chickens either mock infected (Mock) or infected with wild-type vvIBDV D6948 (D6948). The mock-infected chickens show no damage of the follicular structure of the bursa, while the vvIBDV infected chickens show that the virus completely destroyed the bursa follicles and induced cystic formation.

Recovery from disease or from subclinical infection is followed by immunosuppression with more serious consequences if infection occurs early in life. The most severe and long-lasting immunosuppression occurs when day-old chickens are infected by IBDV (Faragher *et al*., 1974; Sharma *et al*., 1989; Allan *et al*., 1972; Sharma *et al*., 1994). In field conditions, this rarely occurs since chickens tend to become infected later at approximately two to three weeks of age, when maternal antibodies decline. It has been reported that the virus has an immunosuppressive effect at least until the age of six weeks (Giambrone, 1979; Lucio & Hitchner, 1980; Wyeth, 1975).

ECONOMIC IMPACT

Economic losses in the poultry industry result from high mortality and morbidity rates due to the acute form of the disease and from a subclinical infection in chickens below 3 weeks old characterised by B-cell dependent immunodeficiency (Kibenge *et al*., 1988*a*; van der Sluis, 1999). The latter condition enhances the susceptibility of chickens to other infections and depresses the response of infected chickens to

vaccines against other diseases such as Newcastle disease, Marek's disease and infectious bronchitis. Because vaccination is the principal method of viral disease control in commercial poultry worldwide (Lasher & Shane, 1994), IBDV should be considered as one of the most important viral pathogens of the commercial poultry industry.

PREVENTION AND CONTROL

IBDV is a highly stable virus under different environmental conditions. It can persist in poultry houses for several weeks even after thorough cleaning and disinfection (Lukert & Hitchner, 1984). It is more resistant to heat and ultraviolet light than reovirus (Petek *et al*., 1973) and is resistant to ether and chloroform. It is inactivated at pH 12.0, but remains infectious at pH 2.0 (Benton *et al*., 1967). This complicates attempts to control infections by hygienic means and makes vaccination inevitable.

In the past, a combination of live and inactivated vaccines used in the parent breeder flocks was sufficient to induce the production of high levels of maternal antibody in the broiler progeny, which prevented early infections and therefore immunosupression. However, most intermediate vaccines are presently inadequate in providing protection against vvIBDV. Some of the less attenuated ('hot') vaccine strains with acceptable reduction of virulence are given after determining the optimum age of vaccination using a formula which predicts the decline in maternal antibody (Kowenhovan & van den Bos, 1993). With the increase in knowledge about the molecular structure and immunology of IBDV, better attenuated and genetically engineered vaccines are being developed.

THE VIRUS

VIRUS STRUCTURE AND GENOMIC ORGANISATION

IBDV is a small, non-enveloped virus with a bisegmented dsRNA genome. The virion has a single capsid shell of icosahedral symmetry with a diameter of 60-70 nm (Fig. 1). Electron cryomicroscopy and image processing analysis showed that the structure of the virion is based on a T=13 lattice formed by trimer-clustered subunits (Bottcher *et al*., 1997; Caston *et al*., 2001). The external surface of the virion is composed of 260 protruding trimeric subunits formed by VP2 (Bottcher *et al*., 1997). The inner capsid is built of 200 Y-shaped trimeric subunits formed by VP3 (Bottcher *et al*., 1997). Five different classes of trimers can be distinguished according to their different local environment (Bottcher *et al*., 1997; Caston *et al*., 2001).

The viral genome structure and organisation are presented in figure 4. The larger segment A (approximately 3,260 base pairs [bp]) contains two open reading

frames (ORFs). The larger ORF encodes a 110 kDa polyprotein (pVP2-VP4-VP3) that is autocatalytically cleaved by the viral protease VP4 to yield the viral proteins pVP2 (also known as VPX; 48 kDa), VP3 (32 kDa) and VP4 (28 kDa) (Sanchez & Rodriguez, 1999; Birghan *et al.*, 2000; Lejal *et al.*, 2000). During *in vivo* virus maturation pVP2 is further processed, by site-specific cleavages at its carboxy end, most likely by VP4 protease activity, generating mature VP2 (40 kDa) and four small peptides (Da Costa *et al.*, 2002). A small ORF preceding and partially overlapping the larger ORF of Segment A encodes VP5 (17 kDa) (Mundt *et al.*, 1995). The smaller genome segment B (approximately 2,827 bp) contains one large ORF encoding VP1 (90 kDa), the viral RNA polymerase which exists both as a free protein and as a genome-linked protein (VPg), covalently attached to the 5' end of the RNA (Muller & Nitschke, 1987; Spies *et al.*, 1987).

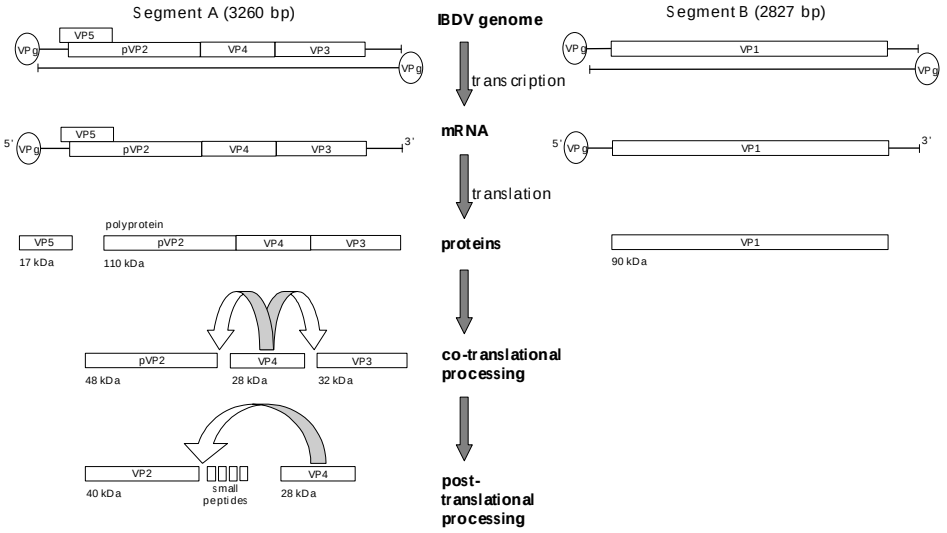


Figure 4. Schematic representation of the genomic organization of IBDV. The gene arrangements of both genome segments A and B are shown. VPg denotes the genome-linked form of VP1 that is covalently attached to the 5' end of the RNA. The molecular masses of the proteins (in kDa) are indicated.

The VP1 ORFs and proteins of pathogenic serotype I and nonpathogenic serotype II strains share an 89% nucleotide and 93-98% amino acid sequence homology, respectively (Brown & Skinner, 1996). The RNA-dependent RNA polymerase (RdRp) consensus sequence motifs (Bruenn, 1991; Shwed *et al.*, 2002) are conserved in the VP1 proteins of both IBDV serotypes. In segment A, lower nucleotide (83-84%) and amino acid (90%) sequence identities occur between coding regions of serotype I and II strains (Kibenge *et al.*, 1991). This is mainly attributed to a hypervariable region

corresponding to the serotype-specific epitope(s) in the structural protein VP2 (Bayliss *et al*., 1990).

The 5' and 3' noncoding termini of the IBDV genome segments resemble those of other segmented RNA viruses such as reovirus (Antczak *et al*., 1982) and influenza virus (Stoeckle *et al*., 1987), where both 5' and 3' termini are homologous between the genome segments. The 5' noncoding terminal sequence in both genome segments of IBDV consists of a 32-nucleotide consensus sequence, the 3' noncoding terminal sequence of a conserved pentamer (Kibenge *et al*., 1996; Mundt & Muller, 1995). These segment-specific 5' and 3' end conserved regions also contain perfect inverted terminal repeats (ITRs) of 6 and 12 nucleotides in segment A and B, respectively (Mundt & Muller, 1995). Furthermore, the 5' and 3' noncoding termini of the IBDV genome segments have the potential to form stem and loop secondary structures (Boot *et al*., 1999; Kibenge *et al*., 1996; Mundt & Muller, 1995).

VIRAL PROTEINS

VP1, the RNA-dependent RNA polymerase of the virus, is present in small amounts in the virion (≈ 3% of the virion protein mass; Dobos *et al*., 1979), both as a free polypeptide and as a genome-linked protein (VPg) (Muller & Nitschke, 1987; Kibenge & Dhama, 1997). In IPNV, VP1 is linked to the 5' ends of both genome segments by a serine-5'-GMP phosphodiester bond (Calvert *et al*., 1991). Since IBDV and IPNV behaved similarly during *in vitro* guanylylation reactions (Dobos, 1993), VP1 of IBDV is also considered to be attached to a guanine residue at the 5' terminus of the genome segments. Whether the 5' termini of the noncoding strands also have this covalently linked VPg is not known. Furthermore, as has been shown to occur *in vitro,* VPg may act as a primase in the generation of viral mRNAs, and as a result becomes part of these mRNAs through covalent linkage at their 5' ends (Dobos, 1995; Magyar *et al*., 1998). Notably, the VPg protein of IBDV is unusually large compared to other VPg's (e.g. adeno- and picornavirus) and its functioning both as a primer and as a polymerase is without precedent. Thus, by their genome-linked protein and their protein-primed RNA synthesis birnaviruses are unique among the double-stranded RNA viruses. Recently, it has been demonstrated that VP1 forms complexes with the capsid protein VP3, when expressed in a heterologous mammalian expression system, suggesting an additional role of VP1 in virus morphogenesis (Lombardo *et al*., 1999).

VP2 and VP3 are the major viral structural proteins, forming the outer and inner layers of the proteinaceous capsid of the virion, respectively. VP2 contains the antigenic region responsible for the induction of neutralising antibodies and for serotype specificity (Fahey *et al*., 1989). This protein is highly hydrophobic and

conformation dependent, as demonstrated by the observation that all neutralising monoclonal antibodies (Mabs) react in immunoprecipitation but not in Western blot (Oppling *et al*., 1991; Schnitzler *et al*., 1993; van den Berg *et al*., 1996). Furthermore, it has been reported that transient expression of VP2 *in vitro* leads to the induction of apoptosis (Yao & Vakharia, 2001; Fernandez-Arias *et al*., 1997). VP3 has group-specific antigens that are recognised by non-neutralising antibodies (Oppling *et al*., 1991; Becht *et al*., 1988). The protein contains charged residues at its carboxy-terminal domain, a domain suggested to be involved in either packaging or stabilising the RNA genome within the interior of the viral capsid (Hudson *et al*., 1986; Bottcher *et al*., 1997). Expression of the IBDV structural genes in various expression systems revealed the production of virus-like particles (VLPs) of IBDV. Extensive studies of these particles has given more insight into capsid morphogenesis. Single expression of VP3 does not yield any type of particle (Martinez-Torrecuadrada *et al*., 2000*b*). The expression of pVP2 alone, leads to the formation of twisted tubular structures and isometric particles (Caston *et al*., 2001; Chevalier *et al*., 2002). Single expression of VP2 results in the spontaneous formation of dodecahedral particles which may assemble into larger, fragile icosahedral capsids built up by 12 dodecahedral capsids (Caston *et al*., 2001). Only expression of the polyprotein gives rise to the formation of virus-like particles (VLPs) with a size and shape very similar to those of authentic IBDV particles. It has also been demonstrated that co-expression of the polyprotein with VP1 results in the formation VLPs in which VP1 becomes incorporated (Lombardo *et al*., 1999). The final processing of pVP2 to VP2, however, does not occur in VLPs. Recently, it was shown that the environment of the C-terminal domain of VP3 functions as an important switch controlling virus morphogenesis. The fusion of an exogenous protein to the C-terminus of the inner VP3 capsid protein appeared to trigger efficient VLP formation and processing of pVP2 to VP2 in a recombinant expression system (Chevalier *et al*., 2002).

VP4 is a non-structural protein associated with the formation of specific microtubules present in infected cells (Granzow *et al*., 1997). This is in contrast with previous studies describing this protein as a minor structural component present in mature virions purified by various methods (reviewed in Kibenge *et al*., 1988*a*). However, Granzow *et al*. (1997) demonstrated that VP4 is not a constituent of mature virions but that its presence in virion preparations was due to a contamination with the VP4-containing type II tubules. VP4 is the virus-encoded protease that shares some characteristics with bacterial Lon proteases (Sanchez & Rodriguez, 1999; Birghan *et al*., 2000; Lejal *et al*., 2000). It is involved in the auto-processing of the polyprotein producing pVP2, VP3 and itself (Azad *et al*., 1987). The amino acids responsible for this proteolytic activity have recently been

characterised as a serine-lysine catalytic dyad (Birghan *et al*., 2000). In addition, the primary cleavage sites at the pVP2-VP4 and VP4-VP3 junctions of the polyprotein have been identified (Sanchez & Rodriguez, 1999; Lejal *et al*., 2000). Previously, the polyprotein cleavage sites were assumed to be located at the dibasic residues $R_{(452)}$-$R_{(453)}$ and $K_{(722)}$-$R_{(723)}$, as predicted by Hudson *et al.* (1986). The corrected processing sites were, however, redefined as being located at amino acid residues $A_{(512)}$-$A_{(513)}$ and $A_{(755)}$-$A_{(756)}$ and these sites are characterised by the (Thr/Ala)-X-Ala↓Ala motif (Lejal *et al*., 2000). As determined by N-terminal sequencing, three (possibly four) additional cleavage sites for VP4, involved in the pVP2 maturation process, are present in the C-terminal domain of pVP2 (Da Costa *et al*., 2002).

VP5 was first described in IPNV (Havarstein *et al*., 1990) and has been identified more recently in IBDV infected cells (Mundt *et al*., 1995). This non-structural protein proved to be non-essential for viral replication and infection but important for virus-induced pathogenicity (Mundt *et al*., 1997; Yao *et al*., 1998), although its exact function is still unknown. The protein is highly basic, cysteine-rich, and its sequence is conserved among all pathogenic strains of IBDV (>95% identity). Sequence-based topology predictions indicated that VP5 is a class II membrane protein, having an intracellular N-terminal tail, a transmembrane helix and an extracellular C-terminal region (Lombardo *et al*., 2000). Additionally, it was shown that VP5 accumulates within the host cell plasma membrane (Lombardo *et al*., 2000). Expression of VP5 resulted in the alteration of cell morphology, the disruption of the plasma membrane, and a drastic reduction of cell viability (Lombardo *et al*., 2000). Furthermore, the protein is capable of inducing a programmed cell death response in cell culture (Yao & Vakharia, 2001). VP5 is therefore suggested to play an important role in the release of the IBDV progeny.

IBDV REPLICATION

A characteristic of the pathogenic IBDV strains, especially vvIBDV strains, is their inability to grow in cell culture. Usually, these strains can be adapted to replicate and produce cytopathic effect (CPE) in primary cell cultures of chicken origin by extensive passaging either in chicken embryos or in cell culture using primary chicken embryo cells, primary chicken embryo fibroblast cells (CEF), or cell lines of avian or mammalian origin such as quail-derived cells (QT35, QM5 or QM7) and Vero cells (Lukert & Davis, 1974; Kibenge *et al*., 1988*b*). Adaptation of wild-type IBDV, however, always seems to correlate with attenuation (Yamaguchi *et al*., 1996*b*; Yehuda *et al*., 1999).

The first step in virus infection is the attachment of the virus to a specific receptor on the surface of susceptible host cells. According to the results of a flow

cytometric virus binding assay by Ogawa *et al*. (1998) the IBDV host range is mainly controlled by the presence of a virus receptor composed of an N-glycosylated protein associated with the B-lymphocytes represented mostly by IgM-bearing cells. Although some progress has been made in the identification of membrane proteins possibly involved in the entry of IBDV into chicken embryo fibroblasts or into chicken lymphocytes (Nieper & Muller, 1996; Setiyono *et al*., 2001), the actual nature of the receptor is still unknown.

Recently, it was shown that wild-type vvIBDV, with its selective tropism for chicken B-lymphocytes, is able to replicate in non-B-lymphoid cells once it is artificially (transfection of cDNA) introduced in these cells (Boot *et al*., 2000*a*). The inability of vvIBDVs to infect non-B-lymphoid cells is therefore likely due to their inability to recognise a certain receptor and/or their inability to enter the cell. In contrast, cell culture adapted vvIBDV is able to enter and replicate in both B-lymphoid and non-B-lymphoid cells. It has therefore been speculated that the typical B-lymphoid cell tropism of vvIBDV strains might be related to the recognition of an IBDV specific B-lymphoid cell receptor whereas cell culture adapted IBDV isolates recognise a general IBDV receptor present on a wide range of cells (Boot *et al*., 2000*a*). Previously, it was shown that the viral factor responsible for propagation in non-B-lymphoid cells is located on VP2 (Lim *et al*., 1999; Mundt, 1999; Yamaguchi *et al*., 1996*a*; van Loon *et al*., 2002). In addition, there are several studies in which amino acid changes of vvIBDV isolates, resulting from adaptation to non-B-lymphoid cell cultures, have been mapped (Lim *et al*., 1999; Mundt, 1999; Yamaguchi *et al*., 1996*a*; van Loon *et al*., 2002). Important amino acids for propagation in non-B-lymphoid cells were found within the hypervariable region of VP2 (i.e. amino acids at position 253, 279, and 284) (Lim *et al*., 1999; Mundt, 1999; van Loon *et al*., 2002).

The consecutive steps involved in the replication of IBDV or of other birnaviruses have not been traced individually. The virus replicates in the cytoplasm and a single cycle of replication takes 10-12 hr at 37 °C (Petek *et al*., 1973; Nick *et al*., 1976). *In vitro* ssRNA synthesis studies showed that the RNA polymerase synthesises viral ssRNA by an asymmetric, semiconservative, strand-displacement mechanism, whereby the nascent strand displaces one of the parental strands (Spies *et al*., 1987; Mertens *et al*., 1982). Two genome-length 24S mRNAs hybridising to the 2 segments were detected both *in vivo* (Somogyi & Dobos, 1980) and *in vitro* (Mertens *et al*., 1982). In both cases, birnaviruses were transcriptionally active without the need for uncoating or for degradation of the capsid (Spies *et al*., 1987). The 24S ssRNA as well as 14S dsRNA are synthesised *in vitro* (Spies *et al*., 1987; Mertens *et al*., 1982; Somogyi & Dobos, 1980), where the 24S ssRNA component is believed to be the viral RNA that serves as the template for the synthesis of

complementary strands to form dsRNA (Somogyi & Dobos, 1980). More recent experiments indicate that virion-associated VP1 catalyses a guanylylation reaction which serves to prime viral RNA synthesis; apparently only the plus strands of the two genome segments are synthesised *in vitro* which remain base-paired to their templates (Dobos, 1995) (Fig. 5). The initiation of viral RNA synthesis has been suggested to involve either 2 VP1 molecules, one serving as a primer and the other as the polymerase for chain elongation, or just a single VP1 molecule serving both functions (Dobos, 1995).

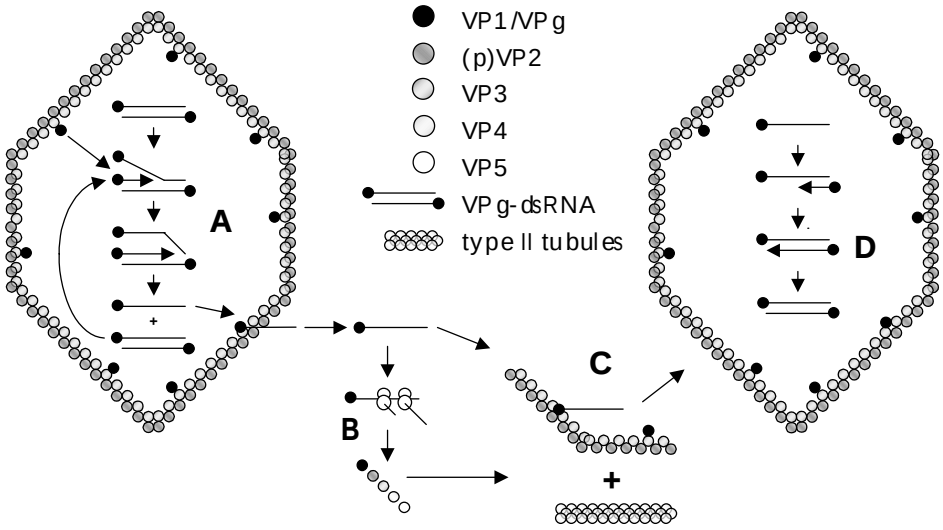


Figure 5. Schematic representation of a possible IBDV replication mechanism. (A) RNA polymerase activity of VP1 has no need for uncoating or degradation of the capsid; VP1 primed, semiconservative strand-displacement transcription, by which the plus strands synthezised remain base-paired to the template. (B) Translation of viral mRNAs and proteolytic processing of the polyprotein. (C) Capsid protein assembly and virus maturation; formation of VP4-containing type II tubules. (D) VPg-ssRNA serving as template for the generation of VPg-dsRNA.

Regulated expression of the viral genes may be essential for the multiplication of IBDV. IBDV specific polypeptides are identified in chicken bursal lymphoid cells as early as 90 min after infection and in the culture medium of such cells from 6 h after infection (Muller & Becht, 1982). The first step governing the IBDV capsid assembly is the autoproteolytic cleavage of the polyprotein, generating pVP2, VP4 and VP3. Moreover, recent data indicate that IBDV assembly is coupled with the polyprotein cleavage in a cleavage-restricted manner (Birghan *et al.*, 2000). The VP4 protease would therefore be a key regulator in the assembly process. Furthermore, it is

expected that VP3, together with its role in providing structural integrity to the IBDV capsid, acts as an internal scaffold to control the particular type of capsid protein assembly (Martinez-Torrecuadrada *et al*., 2000*a*; Chevalier *et al*., 2002). Since VP2 does not accumulate intracellularly, as do the other viral proteins, the pVP2-to-VP2 conversion might be associated with the last steps of the viral cycle (Muller & Becht, 1982). Recently, it was shown that three (most probably four) small peptides resulting from the pVP2 maturation process are associated with the virus particle (Da Costa *et al*., 2002). Two of these peptides proved to be essential for virus viability and are thus likely to play an important role in capsid assembly, genome encapsidation, and/or genome entry into the target cell. As is the case with other naked viruses, it is expected that the release of IBDV requires the alteration of the cell membrane. Both VP2 and VP5 have been implicated to play an important role in the release of the viral progeny by their capability to induce apoptosis in cell culture (Yao & Vakharia, 2001; Fernandez-Arias *et al*., 1997).

RESEARCH TOOLS

IBDV REVERSE GENETICS

The development of a reverse genetics system for IBDV, which enables one to generate virus entirely from cloned cDNA, and hence allows the genetic manipulation of the virus, has progressed rapidly over the past decade. These techniques provide the tools for studying the biological role of the different coding and noncoding regions of the viral genome.

The basis for the development of such a system for IBDV has been the construction of a full-length cDNA clone. This starts with the reverse transcription of the viral RNA into a single-stranded DNA copy, which is subsequently converted into a stable double-stranded form. The first IBDV reverse genetics system reported was based on the full-length cDNA cloning of the IBDV segments in a vector, producing full-length RNAs and the subsequent transfection thereof into eukaryotic cells, allowing the generation of completely new synthetic viruses (Mundt & Vakharia, 1996).

Changes or additions to the original system have included the development of improved methods for reverse transcription, polymerase chain reaction (PCR) and for cloning of full-length segments of both strands of IBDV (Akin *et al*., 1999; Boot *et al*., 2000*b*). More recently, simplifications of the IBDV reverse genetics systems have been proposed for the generation of synthetic particles by direct transfection of cDNA vector as opposed to transfection of RNA. In the present study, we made use of the reverse genetics system reported by Boot *et al.* (1999),

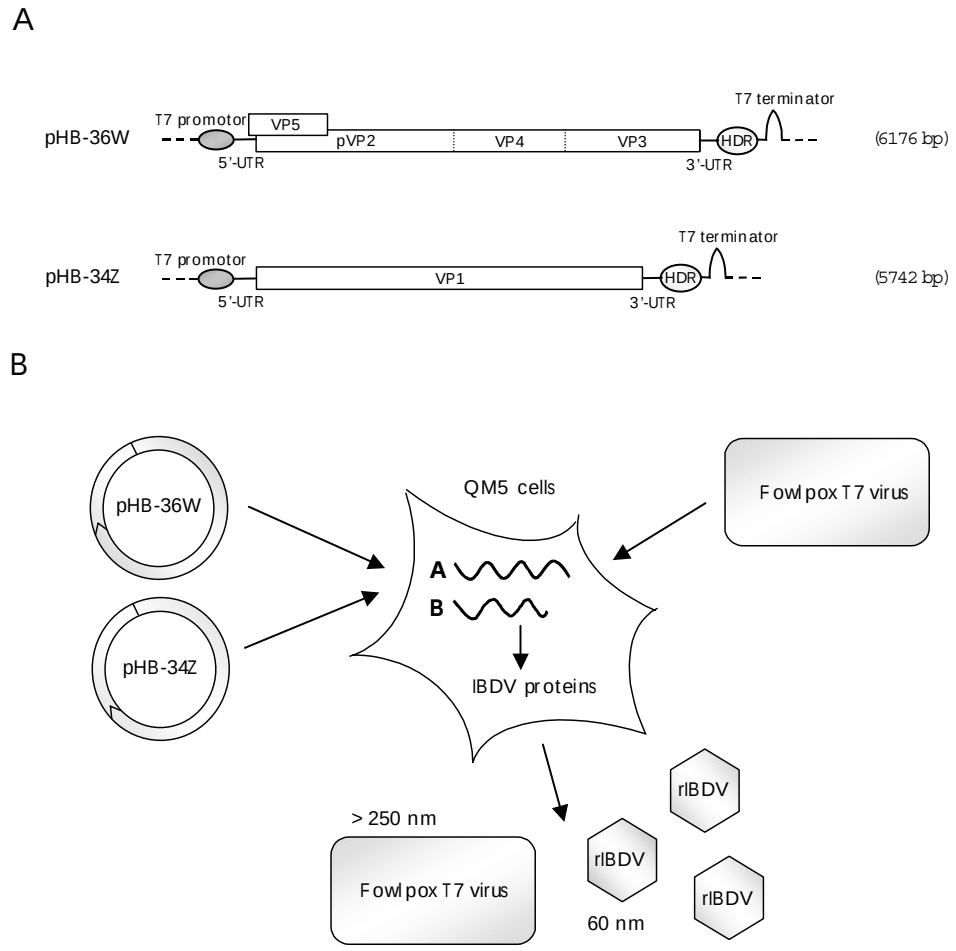


Figure 6. Schematic representation of the IBDV reverse genetics system reported by Boot *et al.*, 1999. (A) Plasmids containing a full-length IBDV A-segment cDNA (pHB-36W) or B-segment cDNA (pHB-34Z); boxes indicate open reading frames (ORFs), and several functional genetic elements such as a hepatitis delta ribozyme (HDR) and a T7 RNA polymerase promoter and terminator sequences are indicated. (B) In vivo Fowlpox T7 expression system. Eukaryotic cells (i.e. QM5 cells) are infected with a recombinant Fowlpox virus that expresses functional T7 RNA polymerase. The subsequent co-transfection of the plasmids pHB-36W and pHB-34Z allows the generation of both Fowlpox T7 virus and new synthetic IBDV. By filtration through a 100 nm filter the Fowlpox T7 virus can be completely removed from the rescued IBDV (rIBDV).

where infectious IBDV was rescued from plasmids that contained full-length IBDV cDNAs behind a T7 promoter, by transfecting these plasmids into cells that had

been infected with a recombinant Fowlpox virus that expressed T7 RNA polymerase (Fig. 6). Another reverse genetics system which was reported only once relies on the transfection of vector plasmids containing the cDNAs under a cytomegalovirus (CMV) promoter (Lim *et al.*, 1999).

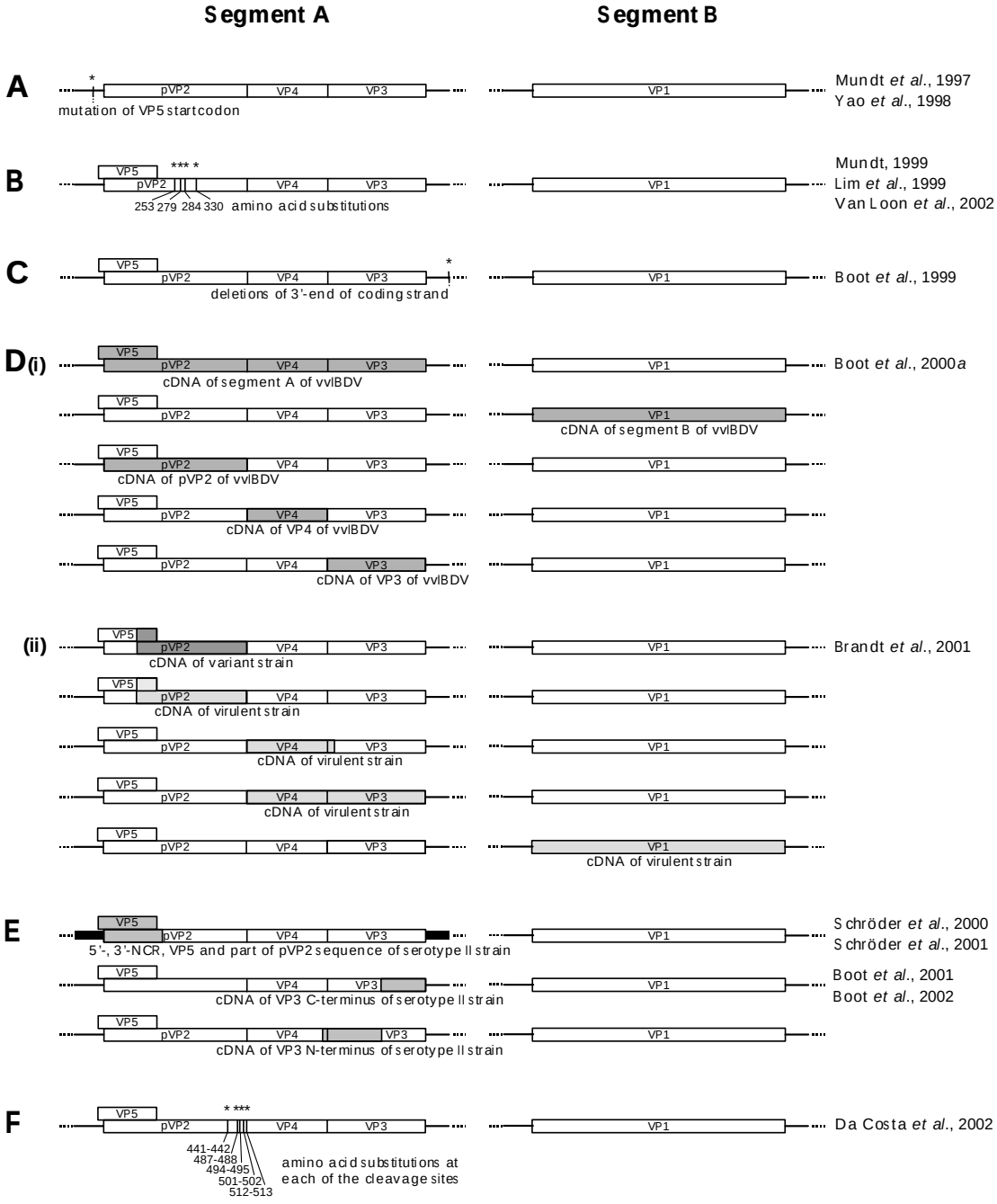
The genetic engineering of IBDV reassortants, recombinants and mutants has been (see figure 7), and will be, of considerable help to elucidate the role of segments, genes, regions or even single nucleotides and amino acids in the IBDV life cycle.

THE YEAST TWO-HYBRID SYSTEM

The yeast two-hybrid system provides a yeast-based genetic assay for detecting protein-protein interactions. The system has rapidly become an attractive method because it allows the selection of genes encoding potentially interacting proteins without the need for protein purification. Prior to the development of the yeast two-hybrid system, both the identification of physical protein-protein interactions and their subsequent functional characterisation traditionally relied upon time and labour intensive biochemical approaches. The yeast two-hybrid system presents three major advantages over alternative assays for gene identification. First, since it is based on a powerful selection scheme performed with a convenient microorganism, it allows very high numbers of potential coding sequences to be assayed in a relatively simple experiment. Second, it relies on an assay performed *in vivo* and is thus not limited by the artificial conditions of *in vitro* assays. Finally, since it is based on a physical binding assay, a wide variety of protein-protein interactions can be detected and characterised following one single commonly used protocol.

The original yeast two-hybrid GaL4 system, developed in 1989 by Fields and Song (Fields & Song, 1989), exploits the fact that the yeast transcriptional activator GaL4 has a separable DNA binding domain ($GaL4_{BD}$) and activation domain

Figure 7. Schematic representation of different applications of the reverse genetics systems. (A) VP5 gene knockout mutant of IBDV to study the function of VP5. (B) Site-directed mutagenesis of different amino acid residues in (p)VP2 to examine their role in the adaptation of IBDV to cell culture. (C) Evidence for involvement of the 3'-terminal sequence in genome replication. (D) Chimeric viruses to study the molecular determinants of virulence, cell tropism and pathogenic phenotype of IBDV; (i) chimera's of a classical IBDV and a vvIBDV, and (ii) of an attenuated vaccine virus and a variant or virulent virus. (E) Interserotypic chimeric viruses to study the different pathotypes of IBDV serotype I and II. (F) Site-directed mutagenesis of the pVP2 maturation cleavage sites to study the function of the four small pVP2 derived peptides.


Segment A
Segment B
A
mutation of VP5 start codon
Mundt *et al*., 1997
Yao *et al*., 1998
B
253 279 284 330 amino acid substitutions
Mundt, 1999
Lim *et al*., 1999
Van Loon *et al*., 2002
C
deletions of 3'-end of coding strand
Boot *et al*., 1999
D(i)
cDNA of segment A of vvIBDV
cDNA of segment B of vvIBDV
cDNA of pVP2 of vvIBDV
cDNA of VP4 of vvIBDV
cDNA of VP3 of vvIBDV
Boot *et al*., 2000*a*
(ii)
cDNA of variant strain
cDNA of virulent strain
cDNA of virulent strain
cDNA of virulent strain
cDNA of virulent strain
Brandt *et al*., 2001
E
5'-, 3'-NCR, VP5 and part of pVP2 sequence of serotype II strain
cDNA of VP3 C-terminus of serotype II strain
cDNA of VP3 N-terminus of serotype II strain
Schröder *et al*., 2000
Schröder *et al*., 2001
Boot *et al*., 2001
Boot *et al*., 2002
F
441-442/
487-488
494-495
501-502
512-513
amino acid substitutions at each of the cleavage sites
Da Costa *et al*., 2002

($GaL4_{AD}$); neither can activate transcription on its own (Chien *et al.*, 1991). Transcriptional activation is detected only when the binding domain is linked to its DNA recognition sequence and is also tethered, though not necessarily covalently, to the activation domain. The yeast two-hybrid system involves fusing the $GaL4_{BD}$ to a protein 'X' (bait) and the $GaL4_{AD}$ to a protein 'Y' (prey). If the bait and prey protein interact, then a functional GaL4 is restored and transcriptional activation can be detected. If binding sites for GaL4 are placed upstream of a reporter gene, transcriptional activation can be monitored easily.

Over the last decade, a variety of versions of the two-hybrid method has been developed (Fashena *et al.*, 2000; Legrain *et al.*, 2001; Vidal & Legrain, 1999). The LexA-based version which we have used in this study, utilises the same basic idea as the GaL4 system except that the DNA binding protein is the *Escherichia coli* (*E. coli*) LexA protein while the activation domain is the 88-residue acidic *E. coli* peptide B42 (Gyuris *et al.*, 1993). Two different vectors, expressing bait and prey fusions of these domains, are used to transform a yeast strain possessing a dual reporter system responsive to transcriptional activation through the LexA operator (see figure 8). Furthermore, the LexA-based system has been designed with a galactose-inducible promoter that has utility in studies of heterologous fusion proteins that are toxic or lethal in yeast. Yeast cells carrying recombinant plasmids are grown and maintained on glucose medium, which represses the expression of fusion proteins. Plating the yeast strain on galactose containing medium induces the expression of the fusion proteins for experimental purposes. Inducible expression means there is less opportunity for the foreign fusion protein to have a toxic effect on the yeast host and thus be eliminated from the pool of potentially interacting proteins. The LexA-based system in this two-hybrid format is therefore often referred to as an 'interaction trap' (Golemis & Khazak, 1997; Mendelsohn & Brent, 1994).

The yeast two-hybrid system also has some potential drawbacks. For example, the system is limited to proteins that can be transported to the nucleus, which may prevent its use with certain extracellular and membrane proteins. Proteins must be able to fold and exist stably in yeast cells and to retain activity as fusion proteins. The use of protein fusions also means that the site of interaction may be occluded by one of the transcription factor domains. Interactions dependent on a posttranslational modification that does not occur in yeast cells will not be detected. Many proteins, including those normally not involved in transcription, may activate transcription when only fused to a DNA-binding domain (Ma & Ptashne, 1987); this activation prevents a library screen from being performed. However, it is often possible to delete a small region of a protein that activates transcription and hence to remove the activation function while retaining other properties of the

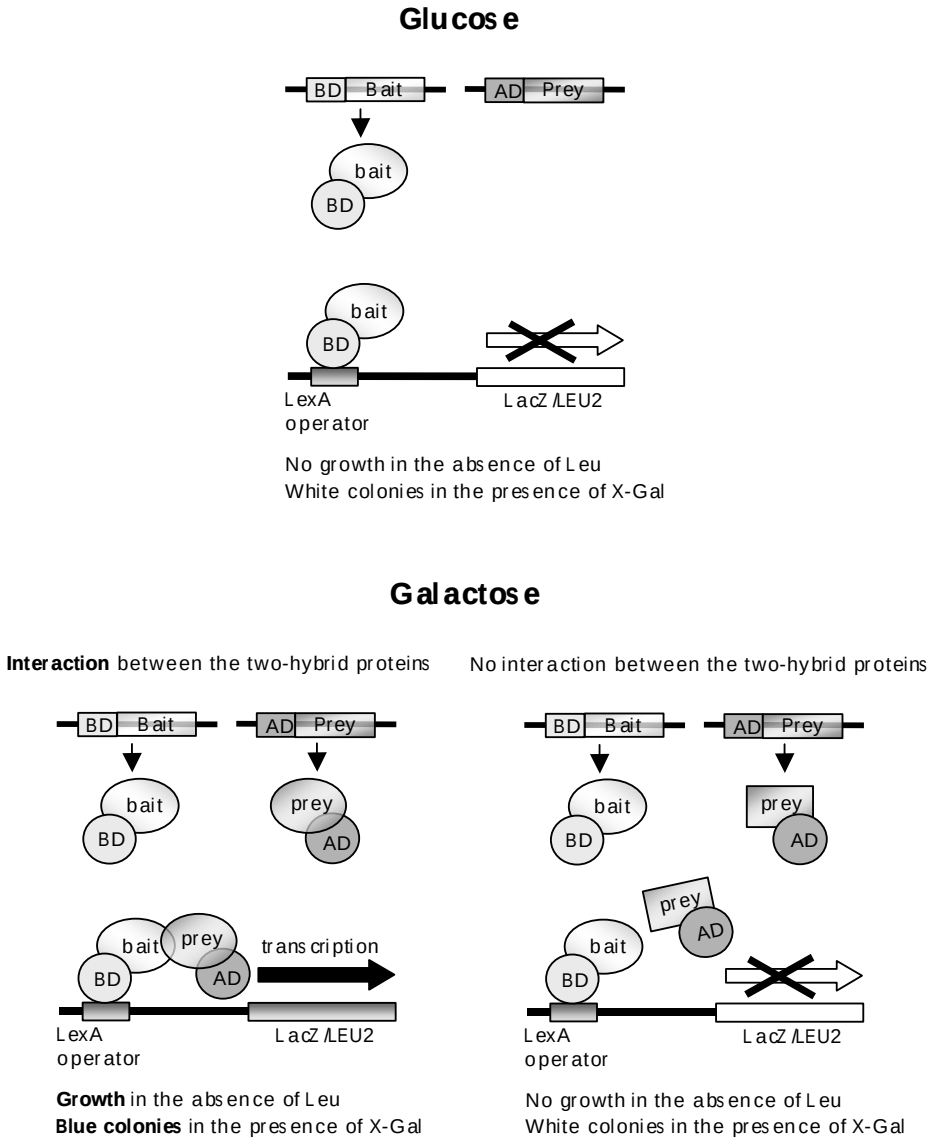


Figure 8. Schematic representation of the LexA-based yeast two-hybrid system. Yeast cells are co-transformed with a bait and prey vector, coding for the LexA DNA-binding domain (DB) fused to a bait protein and the B42 activation domain (AD) fused to a prey protein (i.e. a library member), respectively. The bait is expressed under the control of the constitutive ADH1 promoter, whereas the expression of the prey is directed by the conditional GAL1 promoter, which is induced in galactose-containing medium but repressed in glucose-containing medium. The yeast strain contains two LexA operator-responsive reporters, one a chromosomally integrated copy of the LEU2 gene (required for growth on medium lacking leucine), the second a plasmid bearing a LacZ gene (causing yeast to turn blue on medium containing X-gal). If the prey is induced and the encoded protein does not interact specifically with the bait protein the two reporters are not

activated. If the prey encoded protein interacts with the bait it will result in the activation of the two reporters.

protein. The most common problem associated with a library search is the identification of false positives: fusion proteins that activate transcription when expressed alone, or show a high level of non-specific interactions. A very useful table of commonly identified two-hybrid false positives has been compiled (http://www.fccc.edu:80/research/labs/golemis/InteractionTrapInWork; a web page which gives details of false positives identified in two-hybrid screens). The elimination of false positives, however, requires extensive control experiments: clones must be tested alone and in combination with a number of unrelated bait fusion proteins. Finally, some proteins may be able to interact in yeast cells whereas they do not in their natural environment, simply because they are never expressed at the same time or in the same tissue or subcellular compartment. Thus it is important to confirm a potential interaction by a different strategy or method. A good first step to show biological significance is to verify the interaction by a biochemical technique, preferably co-immunoprecipitation from a cell in which both proteins are expressed. Ideally, the next step would involve a functional assay to show that both proteins are involved in the same biological process.

PROTEIN-PROTEIN INTERACTION MAPS: A LEAD TOWARDS CELLULAR FUNCTIONS

Viruses are obligatory intracellular parasites. For their multiplication they are essentially dependent on components and machineries provided by the host cell. Interactions between viral and host cell proteins occur at all stages of the infection process. Viruses recognize their target cell through interaction with specific receptors and/or other components on the cell membrane resulting in virion internalization. Gene expression of most DNA viruses is mediated by cellular polymerases and regulated largely by cellular transcription factors. Most RNA viruses replicate and transcribe their genomes by RNA-dependent RNA synthesis, a process foreign to eukaryotic cells. Therefore, many of the factors that are normal components of cellular RNA translation are subverted to play an integral or regulatory role in the replication and transcription of viral RNA. These replication complexes often include transient or long-lived interactions with host proteins for structural purposes or to recruit regulatory and catalytic functions. It is now well established that coupling of the different, sequential steps of virus replication is central to the overall infectious cycle of many RNA viruses. Identification of cellular interaction partners of viral proteins is therefore likely to provide a more complete

understanding of the dynamics of RNA replication, virus-mediated cellular modulation and host-range restriction.

AIM OF RESEARCH AND SCOPE OF THESIS

As we argued above, a detailed understanding of the IBDV infection process will require a full description of all interactions that the viral proteins have with themselves, with each other, and with host cell components. At the start of this project very little was known about this. What was known was a partly incorrect composition of the viral particle and a first cryoelectron microscopy structure of the virion (Bottcher *et al.*, 1997), on the basis of which some interaction could be predicted. No host components interacting with viral proteins had been reported yet. The aim of this thesis therefore was to initiate making the inventory of interactions essential to the IBDV life cycle. To this end, we employed the then recently developed yeast two-hybrid system.

First, we focussed on the homo- and heteromeric interactions of the known viral proteins. Proteins usually do not function as individual molecules. Very often they assemble into larger complexes, either by homo-oligomerization or by interaction with other proteins. Virus assembly is an obvious (and extreme) example of this as it is driven by the unique interactions between the viral structural components. Our evaluation revealed that several complexes of the viral proteins could form in yeast cells, some homologous and one heterologous (**Chapter 2**). The heterologous interaction, between VP1 and VP3, was also detected *in vivo* in IBDV-infected cells. Then, we identified the domains responsible for the interaction between VP1 and VP3. By using the yeast two-hybrid system as well as by mutagenesis of an infectious cDNA clone of IBDV we mapped the domain in VP3 interacting with VP1 to the extreme carboxy-terminal domain of the polypeptide. This interaction appeared to be crucial for the production of infectious progeny. These investigations also revealed that VP3 additionally binds to viral dsRNA, both of segment A and B (**Chapter 3**).

The results from a yeast two-hybrid search for candidate cellular proteins of the bursa of Fabricius interacting with VP1, pVP2, VP3 and VP5 are presented in **Chapter 4**. Putative biological implications for some of the interactions found are discussed. The yeast two-hybrid screening using VP1 as bait allowed the isolation of eight candidate cDNA clones with full-sequence identity to the carboxy-terminal domain of eukaryotic initiation factor 4AII (eIF4AII), a key component in the initiation of eukaryotic translation. In **Chapter 5** we have followed up on this potential interaction. The specific interaction of VP1 with this particular eIF4AII carboxy-

terminal domain was confirmed by co-immunoprecipitation. Interestingly, no interaction was observed between VP1 and full-length eIF4AII.

The study presented in **Chapter 6** describes a further investigation of the potentially self-interacting viral proteins of IBDV. We initially reevaluated the interactions between the viral proteins VP1, pVP2, VP3, VP4 and VP5, using the coding sequences of pVP2, VP3 and VP4 based on the recently corrected polyprotein processing sites. The approach of using yeast two-hybrid interaction screens with protein segments, combined with radio-immunoprecipitation assays, to map individual contact sites on the viral proteins, yielded valuable information, in particular on the importance of the amino-terminal region in the homologous interaction of VP3.

Finally, a comprehensive overview of the results described in chapters 2-6 is given in **Chapter 7**.

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CHAPTER 2

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

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ABSTRACT

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 of 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 kDa and 95 kDa. Whereas VP3 initially interacted with both the 90 kDa 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, 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 with 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 RNA (dsRNA) 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 autocatalytically 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 which 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 nonstructural 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.*, 1988; Spies *et al.*, 1987). This protein is present as a free polypeptide and as a genome-linked protein, called VPg, within virions (Müller & Nitschke, 1987).

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 protein-protein interaction, of a yeast DNA-binding domain with a transcriptional activation domain, which results in transcription of a reporter gene (Bartel *et al.*, 1993; 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 virion 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.

MATERIALS AND METHODS

VIRUS, CELL LINE, PLASMIDS AND ANTISERA

The IBDV isolate CEF94 is a derivate 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 pHB36W (A-segment) and pHB34Z (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 *Eco*RI site at the upstream (5') end and a stop codon plus a *Sal*I, *Xho*I or *Stu*I site at the downstream (3') end of each coding sequence (Table 1). Plasmid pHB36W was used as the DNA template for amplification of the pVP2, VP3, VP4, and VP5 genes and plasmid pHB34Z was used for amplification of the VP1 gene. The PCR products were precipitated, digested with *Eco*RI/*Sal*I (pVP2, VP3, and VP5), *Eco*RI/*Xho*I (VP1) or *Eco*RI/*Stu*I (VP4), gel-purified by the QIAEX-II method (QIAGEN), and ligated with T4 ligase (New England BioLabs) into the yeast expression vectors pLexA$_{BD}$ and pB42$_{AD}$ (Clontech). These vectors had previously been digested either with *Eco*RI/*Xho*I, or with *Xho*I followed by a treatment with the Klenow fragment of DNA polymerase I and subsequent digestion with *Eco*RI. The ligation mixture was transformed into

Escherichia coli DH5-α 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.

Protein	$LexA_{BD}$ plasmid	$B42_{AD}$ plasmid	Primer	
			Name	Sequence
VP1	$pLexA_{BD}$-VP1	$pB42_{AD}$-VP1	MT09	ccgGAATTC*ATGAGTGACATTTTCAACAGTCCA*
			MT10	cgtCTCGAG**TCA***TGGCTGTTGGCGGCTCTCC*
pVP2	$pLexA_{BD}$-pVP2	$pB42_{AD}$-pVP2	MT01	ccgGAATTC*ATGACAAACCTGCAAGATCAAACCC*
			MT02	gatcGTCGAC**TCA***CCTTATGGCCCGGATCATGTCTTTG*
VP3	$pLexA_{BD}$-VP3	$pB42_{AD}$-VP3	MT05	ccgGAATTC*CGTTTCCCTCACAATCCACGCGAC*
			MT06	gatcGTCGAC**TCA***CTCAAGGTCCTCATCAGAGAC*
VP4	$pLexA_{BD}$-VP4	$pB42_{AD}$-VP4	MT03	ccgGAATTC*AGGATAGCTGTGCCGGTGGTCTCC*
			MT04	gtAGGCCT**TCA***TTTGATGAACGTTGCCCAGTTGGG*
VP5	$pLexA_{BD}$-VP5	$pB42_{AD}$-VP5	MT07	ccgGAATTC*ATGGTCAGTAGAGATCAGACAAACG*
			MT08	gatcGTCGAC**TCA***CTCAGGCTTCCTTGGAAGGTC*

Table 1. Plasmids and primers used in construction of $LexA_{BD}$ and $B42_{AD}$ fusion proteins. Gene fusions were made by PCR amplification of cDNA flanked by engineered restriction sites and ligation into corresponding restriction sites of vectors $pLexA_{BD}$ and $pB42_{AD}$. The primer sequences are listed 5' to 3'. Capital letters in italics denote homology with IBDV sequences. The restriction site in each primer is underlined. Additional nucleotides 5' of the restriction site are in lower case letters. Bold letters indicate an in-frame stop codon.

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*$^+$ plasmid p8op-lacZ (Clontech), which has a *Lac*Z reporter gene preceded by an upstream LexA binding domain. Transformed cells were amplified and subsequently transformed with $pLexA_{BD}$ (*HIS3*$^+$) and $pB42_{AD}$ (*TRP1*$^+$) constructs carrying VP1, pVP2, VP3, VP4 and VP5, in every possible pairwise combination. Control plasmids were $pLexA_{BD}$-Bicoid (pRHFM1, OriGene), $pLexA_{BD}$-Lamin C (Clontech), $pLexA_{BD}$-53 (Clontech), $pB42_{AD}$-

SV40 T (Clontech), and pB42$_{AD}$-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$^+$ Ura$^+$ Trp$^+$ 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.

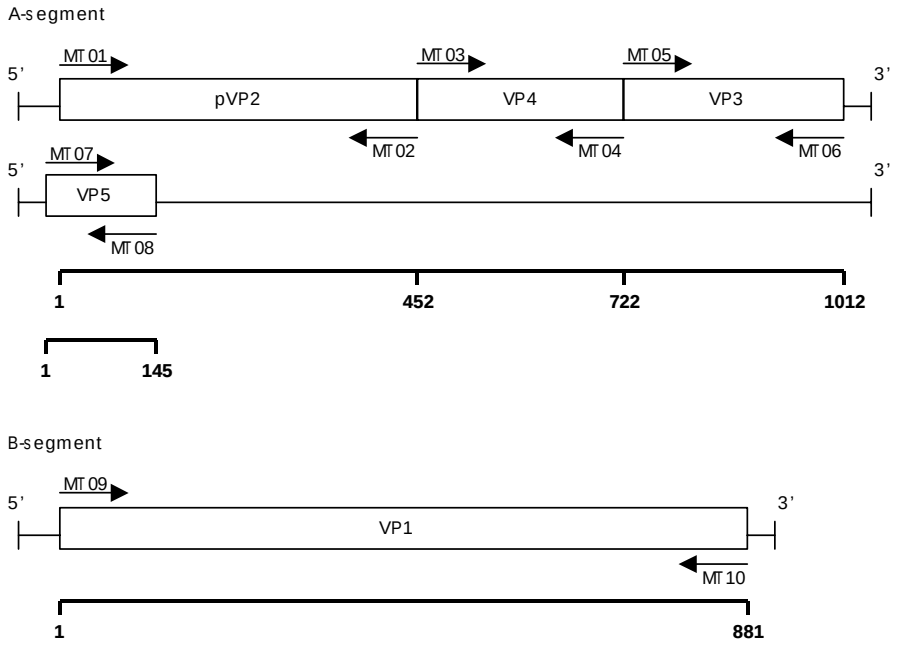

Figure 1. Construction of two-hybrid fusion proteins. Schematic representation of genome segments A and B of IBDV strain CEF94. The numbers under the lines below represent amino acid positions of expressed regions. The positions and names of the oligonucleotide primers used in PCR amplification of various regions are indicated. The boundaries between the viral proteins pVP2, VP4 and VP3 are deduced from the putative cleavage sites reported by Hudson *et al.* (1986).

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*$^+$ plasmid pJK101 (OriGene) and, in parallel, with pJK101 together with one of the pLexA$_{BD}$ 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 operators have been placed between the GAL1

promoter and the *lacZ* gene. When a $LexA_{BD}$ fusion protein binds to these operators there will be a decrease in the level of GAL1-driven *lacZ* expression. A liquid assay to quantitate ß-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-ß-D-galactoside as the chromogenic substrate. Each enzyme activity assay was performed with at least five independent colonies and ß-Galactosidase specific activities were calculated as described by Clontech.

RADIOLABELING OF INFECTED CELLS, IMMUNOPRECIPITATION AND GEL ELECTROPHORESIS

Confluent monolayers of QT35 cells (25 cm^2) were infected with IBDV strain CEF94 at a m.o.i. of 10 or mock-infected. At 2.5 h postinfection (p.i.), cells were starved of methionine for 1.5 h in methionine-free EMEM medium (Gibco/BRL). At 4 h p.i., cells were either pulsed for 4 h or, in case of the pulse-chase radiolabeling, for 1 h, with 20 µCi/ml [^{35}S]methionine (Amersham) in methionine-free EMEM medium. The latter were subsequently chased for different times in QT35 medium. After the 4 h-pulse or the pulse-chase period, the cell-culture medium was discarded (in case of the 4 h-pulse experiment) or collected (in case of the pulse-chase experiment) and brought to a concentration of 1x PBS-TDS lysis buffer with a 5x PBS-TDS lysis buffer stock solution (5 % Triton X-100, 2.5 % sodium deoxycholate, 0.5 % SDS, 0.7 M NaCl, 14 mM KCl, 50 mM Na_2HPO_4, 7.5 mM KH_2PO_4). The cell monolayers were washed three times with ice-cold phosphate-buffered-saline (PBS) and solubilized in 1x PBS-TDS lysis buffer for 10 min at room temperature. Both the medium and cell lysates were then frozen and thawed twice, and clarified by centrifugation for 20 min at 13,000 rpm in a microfuge. All lysates were precleared with Protein A Sepharose (Amersham) before being immunoprecipitated with anti-VP1 or anti-VP3 antibodies. Protein A Sepharose-bound immune complexes were washed three times in 1x PBS-TDS lysis buffer and eluted in 20 µl sodium dodecyl sulfate (SDS) sample buffer (60 mM Tris-HCl [pH 6.8], 2.5% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.1% bromophenol blue). Proteins were resolved on 12% separating gels by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by phosphor imaging (STORM-840, Molecular Dynamics) and ImageQuaNT software (Molecular Dynamics).

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$_{BD}$ and pB42$_{AD}$ (Fig. 1).

To detect an interaction between the LexA$_{BD}$ and B42$_{AD}$ fusion proteins, it is crucial that neither fusion protein has an intrinsic or nonspecific ability to activate transcription of the reporter genes. Therefore, all LexA$_{BD}$ plasmids were cotransformed with pB42$_{AD}$ lacking an insert to test for intrinsic activation, while all the B42$_{AD}$ plasmids were cotransformed with pLexA$_{BD}$-Bicoid or pLexA$_{BD}$-Lamin C to test for specificity of interaction with each of the B42$_{AD}$ fusion proteins. pLexA$_{BD}$-Bicoid and pLexA$_{BD}$-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$_{BD}$ and B42$_{AD}$ fusion proteins of IBDV may be regarded as specific. Plasmids containing p53 fused to LexA$_{BD}$ (Clontech) and SV40 large T antigen (SV40 T) fused to B42$_{AD}$ (Clontech) were cotransformed into EGY48 and used as a positive control.

All possible pairwise combinations of plasmids containing the LexA$_{BD}$ and B42$_{AD}$ fusion proteins were cotransformed into the *S. cerevisiae* EGY48 strain. 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 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$_{BD}$-VP1 and B42$_{AD}$-VP1 showed limited growth on Leucine-selective medium but, however, remained negative for ß-galactosidase expression. Measurements of ß-galactosidase activity were done after one day, when colonies of the positive control (LexA$_{BD}$-p53 with B42$_{AD}$-SV40 T) were deep blue, because at that time we could distinguish variations in intensity of the blue color of positive colonies. Beyond three days, the true positive colonies all had the same color intensity. Nevertheless,

	$LexA_{BD}$ fusion							
$B42_{AD}$ fusion	**VP1**	**pVP2**	**VP3**	**VP4**	**VP5**	**Bicoid**	**Lamin C**	**p53**
VP1	+/–	–	+	–	–	–	–	ND
pVP2	–	+	–	–	–	–	–	ND
VP3	+	–	+	–	–	–	–	ND
VP4	–	–	–	–	–	–	–	ND
VP5	–	–	–	–	+	–	–	ND
No insert	–	–	–	–	–	–	–	ND
SV40 T	ND	ND	ND	ND	ND	ND	ND	+

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 $LexA_{BD}$-p53 and $B42_{AD}$-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.

	$LexA_{BD}$ fusion							
$B42_{AD}$ fusion	**VP1**	**pVP2**	**VP3**	**VP4**	**VP5**	**Bicoid**	**Lamin C**	**p53**
VP1	–	–	+	–	–	–	–	ND
pVP2	–	++	–	–	–	–	–	ND
VP3	++	–	++	–	–	–	–	ND
VP4	–	–	–	–	–	–	–	ND
VP5	–	–	–	–	++	–	–	ND
No insert	–	–	–	–	–	–	–	ND
SV40 T	ND	ND	ND	ND	ND	ND	ND	++

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 intensity of the blue phenotype after 1 day when the

strain with $LexA_{BD}$-p53 and $B42_{AD}$-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.

after three days of incubation, we observed some ß-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 $B42_{AD}$ fusions with $LexA_{BD}$-Bicoid or $LexA_{BD}$-Lamin C as well, although not between $B42_{AD}$-VP1 and $LexA_{BD}$-Bicoidor $LexA_{BD}$-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 $LexA_{BD}$-VP1 with $B42_{AD}$-VP3 proved to have a stronger *LacZ* reporter activity than the combination $LexA_{BD}$-VP3 with $B42_{AD}$-VP1 (Table 3).

The lack of any significant interaction of the $LexA_{BD}$-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 ß-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 labeled at 4 h postinfection, with [^{35}S]methionine, for 4 h. The proteins in the cleared cell lysates were subjected to immunoprecipitation with a rabbit polyclonal antibody against VP1 or a monoclonal antibody against VP3 and analysed by SDS-PAGE. In addition

to the protein against which the antibodies were directed, each antibody co-immunoprecipitated the other protein, indicating that an interaction had taken place between them (Fig. 2). The observation that only a small amount of VP1 was co-precipitated by the antibody against VP3 (lane 2) may be a consequence of the use of a limiting amount of anti-VP3 antibodies that was unable to precipitate all of the VP3. VP3 is probably synthesized in much larger quantities than VP1, since analysis of the protein composition of IBDV has shown that VP3 constitutes 40%, whereas VP1 constitutes only 3% of the protein in the infectious virus (Dobos *et al.*, 1979; Kibenge *et al.*, 1988). Therefore, precipitation of a fraction of VP3 will precipitate only that proportion of VP1 that is interacting with it, whereas precipitation of all the VP3 will precipitate all of the interacting VP1.

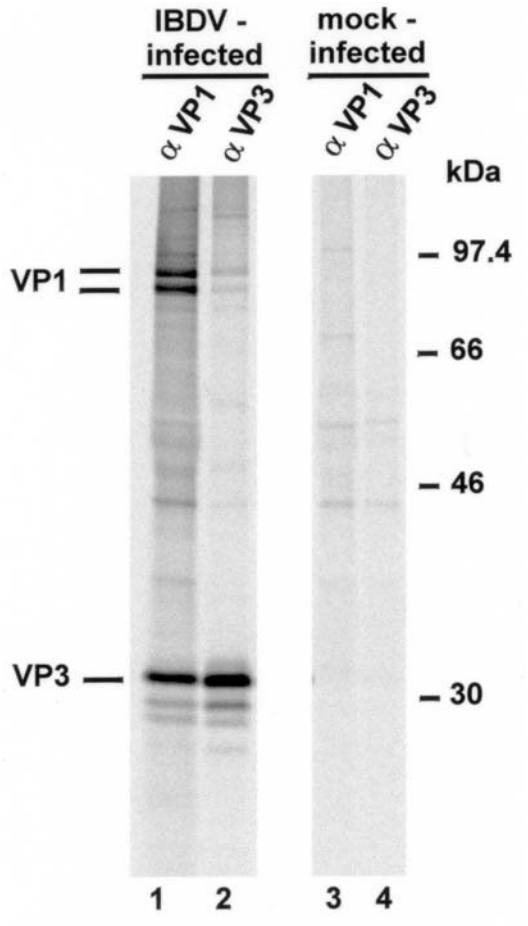

Figure 2. Interaction of VP1 and VP3 in IBDV-infected cells. IBDV-infected QT35 cells were pulsed at 4 h p.i. with [^{35}S]methionine for 4 h. At 8 h p.i., cells were lysed and subjected to immunoprecipitation with polyclonal anti-VP1 serum (αVP1) or with a monoclonal antibody against VP3 (αVP3) followed 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.

A noteworthy observation was the appearance of two proteins at the position of VP1 (90 kDa and 95 kDa), although the existence of these two polypeptides has

previously been reported for different IBDV strains (Müller & Becht, 1982; Jackwood *et al.*, 1984). The same protein bands were obtained when the immunoprecipitate was treated with RNAse prior to SDS-PAGE (data not shown), indicating that the difference in size between the two proteins was not due to the presence of a VP1-RNA complex.

The other bands obtained in the precipitates with lower apparent molecular sizes were VP3 specific, as they were precipitated by anti-VP3 and also co-precipitated by anti-VP1 antibodies, whereas they were absent in the mock-infected control. Moreover, the VP3 specificity of these bands, as well as the VP1 specificity of the 90 and 95 kDa band, was confirmed by Western blot analysis (data not shown).

To test for nonspecific co-immunoprecipitation of VP1 and VP3, we synthesized and radiolabeled VP1 and VP3 seperately *in vitro* in a rabbit reticulocyte lysate and tested both antibodies for cross-reactivity. We found that the anti-VP1 and anti-VP3 antibodies each precipitated only their cognate protein (data not shown). In addition, we analysed whether VP1 and VP3 could also interact *in vitro* by co-expression in a rabbit reticulocyte lysate. However, the results showed that neither VP1 nor VP3 co-precipitated *in vitro* (data not shown).

In summary, the heterologous interaction between VP1 and VP3 that was detected in the two-hybrid system could be confirmed by co-immunoprecipitation using IBDV-infected cells.

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 labeled at 4 h postinfection 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. 3A, lanes 1 and 5). VP1 detected immediately after the pulse, again appeared to be present in two forms, 90 kDa and 95 kDa, in nearly equal amounts (Fig. 3A, 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 to 4). Furthermore, at 5, 9 and 19 h of the chase, VP3 interacted only with the 95 kDa form of VP1 and not with the 90 kDa form (lanes 6-8). The same phenomenon was detected in the cell-culture media. Here, the complexes consisting of VP1 (95 kDa) and VP3 were detected from 5 h of chase onwards, that is 10 h p.i. (Fig. 3B). This timing is consistent with the release of

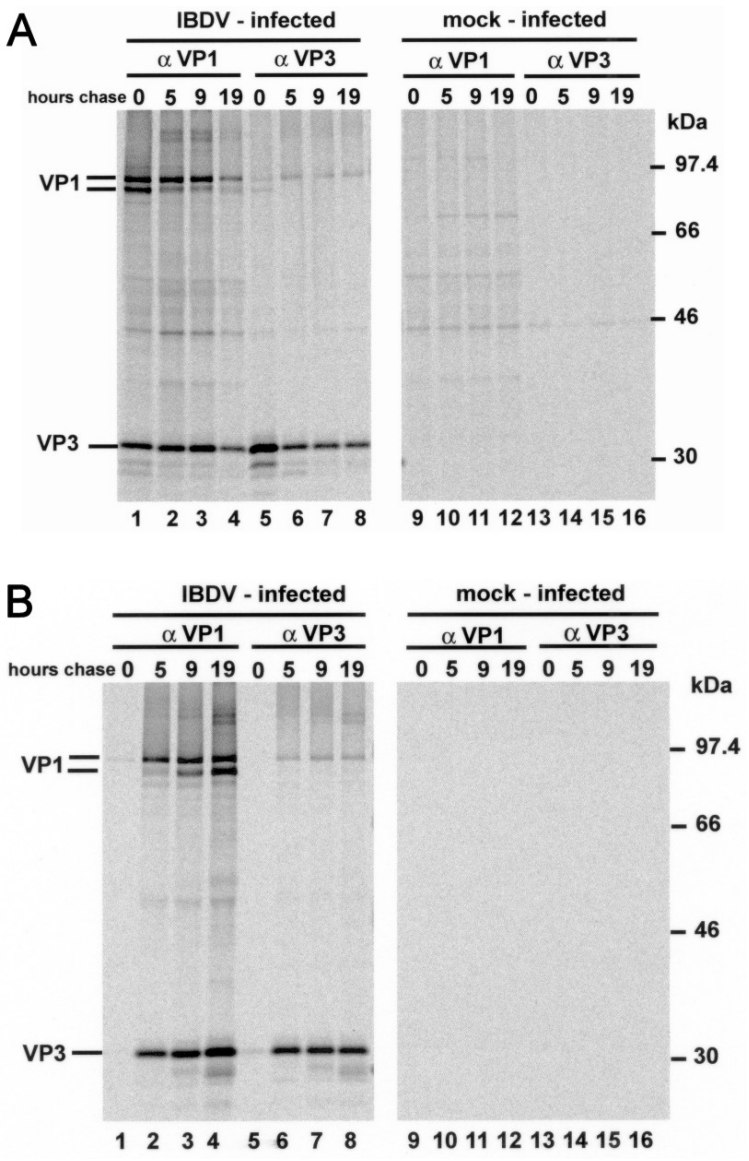

Figure 3. Kinetics of association between the viral proteins VP1 and VP3 *in vivo*. IBDV-infected QT35 cells were pulse-labeled at 4 h p.i. for 1 h with [^{35}S]methionine and chased in medium containing an excess of unlabelled methionine. At the times indicated, the cell-culture medium was collected 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.

progeny virus particles into the culture medium (Petek *et al*., 1973).

In the smaller molecular size region, at least two smaller forms of VP3 were again detected (Fig. 3A and B).

Finally, after 5, 9 and 19 h of the chase, two additional bands were detected in the high molecular size region (Fig. 3A and 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 of 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 seperately *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 postinfection 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 kDa 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, and 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 to 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 kDa 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 and 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, 1995*a*).

Recently, the interaction between VP1 and VP3 has also been described by Lombardo *et al*. (1999) who observed the interaction by co-localisation 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 their 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.

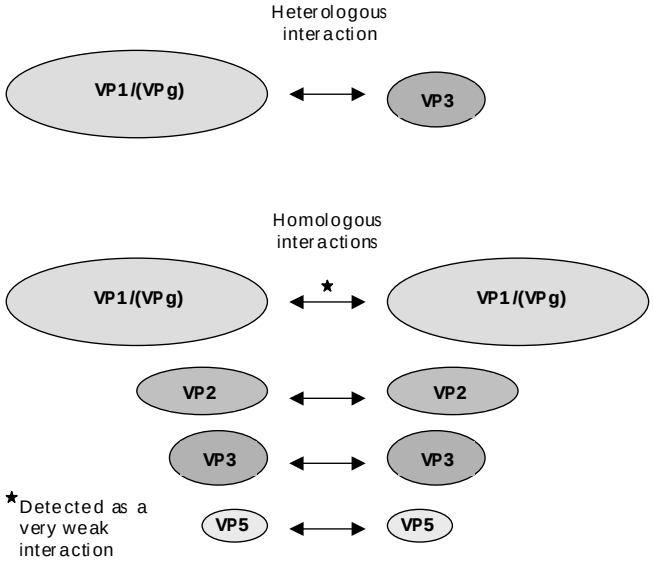

Figure 4. Protein linkage map of the viral proteins of IBDV suggested by data obtained by the yeast two-hybrid system.

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 undectable (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 specific protein-protein interaction; poor or unstable expression, improper folding, or steric hindrance of the fused $LexA_{BD}$- or

$B42_{AD}$-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 $3CD^{pro}$ 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 $3D^{pol}$ of poliovirus. The poliovirus protein VPg is covalently linked to the 5' ends of both genomic and antigenomic viral RNA and $3D^{pol}$ is the RNA-dependent RNA polymerase. These authors suggested that a direct interaction between these molecules is involved in the mechanism of initiation of viral RNA synthesis. VP1 of IBDV exists also 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, 1995*b*).

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 trimer units protrude from a continuous shell of density, and on the inner surface these 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 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 interacts only 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-immunoprecipiation 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 unkown.

One potential drawback of a two-hybrid system arises when the fusion protein fails to localize to the nucleus and bind operators. This was possibly the case for the $LexA_{BD}$-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 $B42_{AD}$-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 (Granzow *et al.*, 1997).

All the interactions found between the viral proteins of IBDV were detected in the classical attenuated strain CEF94. To check whether there are 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 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 coordinated 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.

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CHAPTER 3

Infectious bursal disease virus capsid protein VP3 interacts both with VP1, the RNA-dependent RNA polymerase, and with viral double-stranded RNA

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ABSTRACT

Infectious bursal disease virus (IBDV) is a double-stranded RNA (dsRNA) virus of the *Birnaviridae* family. Its two genome segments are encapsidated together with multiple copies of the viral RNA-dependent RNA polymerase, VP1, in a single-shelled capsid that is composed of VP2 and VP3. In this study we identified the domains responsible for the interaction between VP3 and VP1. Using the yeast two-hybrid system we found that VP1 binds to VP3 through an internal domain, while VP3 interacts with VP1 solely by its carboxy-terminal 10 amino acids. These results were confirmed by using a reverse genetics system that allowed us to analyze the interaction of carboxy-terminally truncated VP3 molecules with VP1 in infected cells. Co-immunoprecipitations with VP1- and VP3-specific antibodies revealed that the interaction is extremely sensitive to truncation of VP3. The mere deletion of the C-terminal residue reduced co-precipitation almost completely and also fully abolished production of infectious virions. Surprisingly, these experiments additionally revealed that VP3 also binds to RNA. RNase treatments and RT-PCR analyses of the immunoprecipitates demonstrated that VP3 interacts with dsRNA of both viral genome segments. This interaction is not mediated by the carboxy-terminal domain of VP3 since C-terminal truncations of 1, 5 or 10 residues did not prevent formation of the VP3-dsRNA complexes. VP3 seems to be the key organizer of birnavirus structure as it maintains critical interactions with all components of the viral particle: itself, VP2, VP1 and the two genomic dsRNAs.

INTRODUCTION

Infectious bursal disease virus (IBDV) is the causative agent of a highly contagious disease of young chickens. IBDV multiplies rapidly in developing B lymphocytes in the bursa of Fabricius, leading to immunosuppression and increased susceptibility to other diseases. Of the two IBDV serotypes, only serotype 1 is pathogenic to chickens (Jackwood *et al.*, 1982). IBDV belongs to the family *Birnaviridae* (Dobos *et al.*, 1979). Members of this family are characterized by a double-stranded RNA (dsRNA) genome consisting of 2 segments (A and B) that are packaged within a single shelled icosahedral capsid of 60 nm.

The smaller genome segment B of IBDV (approximately 2.9 kb) encodes a single protein, viral protein 1 (VP1; 91 kDa). This protein is the putative RNA-dependent RNA polymerase (RdRp) (Bruenn, 1991). It has the intriguing feature of occurring in virions in two forms, both covalently bound to the 5' ends of the genomic dsRNA segments (viral protein genome-linked; VPg) (Dobos, 1993), and as

a free polypeptide. The larger dsRNA segment A (approximately 3.3 kb) contains two partly overlapping open reading frames (ORFs). The first ORF encodes a non-structural protein, VP5 (17 kDa). This protein proved to be non-essential for viral replication and infection (Granzow *et al.*, 1997; Mundt *et al.*, 1997; Yao *et al.*, 1998). The second ORF encodes a 110 kDa polyprotein, which is autocatalytically cleaved to give pVP2 (48 kDa), VP4 (28 kDa) and VP3 (32 kDa). The viral protease VP4, through a catalytic site belonging to the Lon-protease family, is responsible for this self-processing of the polyprotein (Birghan *et al.*, 2000; Lejal *et al.*, 2000; Sanchez & Rodriguez, 1999). During virus maturation, the precursor pVP2 is further processed into mature VP2 (40 kDa) probably as a result of a site-specific cleavage of pVP2 by VP4 protease activity (Kibenge *et al.*, 1997; Lejal *et al.*, 2000).

VP2 and VP3 are the major viral structural proteins. They form the proteinaceous capsid, the structure of which was shown by cryoelectron microscopy and image reconstruction to exhibit a T=13 lattice (Bottcher *et al.*, 1997; Caston *et al.*, 2001). The capsid shell is composed of homotrimeric subunits of VP2 and VP3; 260 VP2 trimers constitute the outer surface while the 200 Y-shaped VP3 trimers line the inner surface. Consistently, VP2 carries the major neutralizing epitopes (Azad *et al.*, 1987; Becht *et al.*, 1988) while VP3, by virtue of its basic carboxy-terminal domain, has been suggested to interact with the packaged dsRNA (Bottcher *et al.*, 1997; Hudson *et al.*, 1986).

While these ultrastructural studies already provided important insight into the general architecture of the virus, essentially nothing is yet known about the specific interactions between the different viral components and how these give rise to the formation of viral particles. We and others recently reported that VP3 is able to associate with VP1 (Lombardo *et al.*, 1999; Tacken *et al.*, 2000). We showed that VP1-VP3 complexes are formed in the cytoplasm of IBDV-infected cells and eventually released into the cell culture medium, suggesting that the viral polymerase is incorporated into virions through interactions with the inner capsid protein (Tacken *et al.*, 2000). Likewise, Lombardo *et al.* (1999) showed that VP1 is efficiently incorporated into IBDV virus-like particles (VLPs) produced in mammalian cells co-expressing the IBDV polyprotein and VP1.

In the present study we have followed up on these supposedly essential interactions. By using the yeast two-hybrid system as well as by mutagenesis of an infectious cDNA clone of the virus we mapped the domain in VP3 interacting with VP1 to the extreme carboxy-terminal tail of the polypeptide. This interaction appeared not to be required for negative-strand RNA synthesis but to be crucial for the production of infectious progeny virus as deletion of just 1 carboxy-terminal residue already abolished virus replication. These investigations also revealed that

VP3 additionally binds to viral dsRNA, both of the A segment and of the B segment, and that it does so through a domain distinct from that binding to VP1.

MATERIALS AND METHODS

CELLS, VIRUSES, PLASMIDS AND ANTISERA

QM5 cells (Antin & Ordahl, 1991) were cultured in QT35 medium (Gibco-BRL) supplemented with 5 % fetal calf serum (FCS) and 2% antibiotic solution ABII (1,000 U of penicillin [Yamanouchi], 1 mg of streptomycin [Radiumfarma], 20 μg of amphotericin B [Fungizone], 500 μg of polymixin B, and 10 mg of kanamycin/ml) in a CO_2 (5%) incubator at 37°C. The classical IBDV isolate CEF94 is a derivate of PV1 which has been adapted for growth in cell cultures (Boot *et al.*, 1999; Petek *et al.*, 1973). Recombinant fowlpox virus expressing the T7 polymerase gene (FPV-T7) (Britton *et al.*, 1996) was received from the laboratory of M. Skinner (Compton Laboratory, Berks, United Kingdom). The preparation of the plasmids pHB36W (A-segment) and pHB34Z (B-segment) which contain full-length genomic cDNA of IBDV strain CEF94, has been described (Boot *et al.*, 1999). Polyclonal rabbit antisera against VP1 and VP3 were produced by injecting rabbits with purified recombinant VP1 or VP3 (Boot *et al.*, 2000*a*; Tacken *et al.*, 2000). Monoclonal antibody directed against VP3 (MAb C3) was kindly provided by H. Müller (University of Leipzig, Germany).

CONSTRUCTION OF TWO-HYBRID EXPRESSION PLASMIDS

Copy DNA encoding the full-length sequences of VP1 and VP3 of IBDV strain CEF94, or defined parts thereof (see figure 1), were amplified by PCR by using the Expand high fidelity PCR system (Boehringer Mannheim). Plasmid pHB36W was used as the DNA template for preparing the VP3 constructs and plasmid pHB34Z for the VP1 constructs. The sets of primers used were designed to introduce an *Eco*RI site at the upstream (5') end and a stop codon plus either a *Sal*I site (VP3 constructs) or a *Xho*I site (VP1 constructs) at the downstream (3') end of each coding sequence (Table 1). PCR products were precipitated, digested with *Eco*RI/*Sal*I (VP3 PCR fragments) or *Eco*RI/*Xho*I (VP1 PCR fragments), gel purified by the QIAEX-II method (QIAGEN), and ligated with T4 DNA ligase (New England BioLabs) into the yeast expression vectors $pLexA_{BD}$ and $pB42_{AD}$ (Clontech). These vectors had previously been digested with *Eco*RI/*Xho*I. The ligation mix was transformed into *Escherichia coli* DH5-α 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 using an ABI 310 sequencer (Perkin Elmer) to ensure correct reading frames.

TWO-HYBRID ANALYSIS

All two-hybrid media, buffers, and protocols were prepared and 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 transformed by using the lithium acetate method with the *URA3*$^+$ plasmid p8op-lacZ (Clontech), which has a *Lac*Z reporter gene preceded by an upstream LexA binding domain. Transformed cells were amplified and subsequently co-transformed with pLexA$_{BD}$ (*HIS3*$^+$) and pB42$_{AD}$ (*TRP1*$^+$) constructs carrying the VP1 cDNA (or fragments thereof) and the VP3 cDNA (or segments thereof). Control plasmids were pLexA$_{BD}$-Bicoid (pRHFM1, OriGene) and pB42$_{AD}$-empty vector. Transformants were plated onto SD/Glu/-His/-Ura/-Trp medium. His$^+$ Ura$^+$ Trp$^+$ 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/5-bromo-4-chloro-3-indolyl ß-D-galactopyranoside (X-gal)/-His/-Ura/-Trp medium to assess the transcriptional activation of the *LacZ* reporter gene. Transformants containing plasmids pLexA$_{BD}$-53 (Clontech) and pB42$_{AD}$-SV40 T (Clontech), in which 53 codes for the murine p53 protein known to physically associate strongly with SV40 large T antigen (Chien *et al*., 1991; Li & Fields, 1993), served as a positive control to assess assay conditions.

MODIFICATION OF THE FULL-LENGTH cDNA CLONE OF IBDV SEGMENT A

Plasmid pHB36W was used for oligonucleotide-directed mutagenesis to introduce stop codons in the 3' terminal region of the coding strand of VP3 by PCR. Primers p139, p140, p141, and p142 (Table 1) containing nucleotide substitution(s) to create these stop codons were used in combination with primer p138 (Table 1).

The resulting PCR fragments were digested with *Bgl*II and *Kpn*I, agarose gel purified (Qiaex gel extraction kit), and ligated (Rapid ligation kit, Boehringer-Mannheim) into the full-length segment A clone (pHB36W), which had been digested with the same restriction enzymes. The ligation mixture was subsequently used to transform *Escherichia coli* DH5-α cells (Life Technologies). To confirm the correct nucleotide sequence of the pHB36W derivates, designated pHB36W-VP3Δ1(tag), pHB36W-VP3Δ1(tga), pHB36W-VP3Δ5(tga), and pHB36W-VP3Δ10(tga), respectively, we determined the nucleotide sequence of the exchanged part (*Bgl*II/*Kpn*I fragment). Two independently obtained plasmids of each pHB36W

derivate were used for *in vitro* transcription/translation and for transfection of QM5 cells.

IN VITRO TRANSCRIPTION AND TRANSLATION

In vitro T7 polymerase-driven expression was carried out by using the TNT Quick coupled transcription/translation system (Promega) as described by the manufacturer, except that reactions were performed in a final volume of 2.5 μl. Plasmid pHB36W or its derivatives (0.4 μg) were used as template. The resulting viral proteins were resolved in 12% separating gels by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by autoradiography.

TRANSFECTION OF QM5 CELLS

QM5 cells were grown to 80% confluency in 60-mm dishes and infected with FPV-T7 (multiplicity of infection [MOI] = 3). After 1 h, the cells were washed once with 5 ml QT35 medium and covered with 5 ml of Optimem 1 (Gibco-BRL). In the meantime, 2.0 μg of DNA was mixed with 25 μl Lipofectamine (Gibco-BRL) in 0.5 ml Optimem 1 and kept at room temperature for at least 30 min. The QM5 cells were subsequently covered with 4 ml of fresh Optimem 1, and the DNA-Lipofectamine mixture was added. The transfection was performed overnight (18 h) in a 37°C incubator (5.0% CO_2). The transfected monolayer was rinsed once with QT35 medium and fresh QT35 medium supplemented with 5% FCS and 2% ABII was added. The plates were further incubated for another 24 h.

RADIOLABELING OF TRANSFECTED CELLS, IMMUNOPRECIPITATION AND GEL ELECTROPHORESIS

At 48 h post-transfection, cells were starved for 1 h in methionine-free Eagle's minimal essential medium (EMEM) (Gibco-BRL). Cells were then labeled for 3 h with

Table 1. Nucleotide sequences and positions of the primers used for VP1 and VP3 deletion mutagenesis, for the generation of mutant segment-A cDNA constructs, and for RT-PCR analysis

[a] The primer sequences are listed 5' to 3'. Restriction sites are in italics. Additional nucleotides 5' of a restriction site are in lowercase. Boldface letters indicate an in-frame stop codon. Mutated nucleotides are underlined.

[b] A+ and B+, A and B segment plus strands, respectively; A– and B–, A and B segment minus strands, respectively.

[c] (+) RNA, plus strand RNA; (–) RNA, minus strand RNA.

Primer	Nucleotide sequence[a]	Position[b]	Purpose[c]
p009	ccg*GAATTC*ATGAGTGACATTTTCAACAGTCCAC	B+ (nt 112)	VP1 and VP1ΔC derivates
p110	cgt*CTCGAG***TCA**TGGCTGTTGGCGGCTCTCC	B– (nt 2754)	VP1 and VP1ΔN derivates
p075	ccg*GAATTC*CTCTTGATCCCTAAAGTTTGGGTG	B+ (nt 202)	VP1ΔN30
p076	ccg*GAATTC*GTTTTGCAGCCACGGTCTCTGC	B+ (nt 292)	VP1ΔN60
p078	ccg*GAATTC*CCCCAATGCGTACCCGCCAGAC	B+ (nt 472)	VP1ΔN120
p080	ccg*GAATTC*GAGGTCGCCACTGGAAGAAACC	B+ (nt 652)	VP1ΔN180
p082	ccg*GAATTC*GGCGACTTTGAGGTTGAAGATTAC	B+ (nt 832)	VP1ΔN240
p084	ccg*GAATTC*AAGAAGCTACTCAGCATGTTAAGTG	B+ (nt 1012)	VP1ΔN300
p086	ccg*GAATTC*AATAACGTGTTGAACATTGAAGGGTG	B+ (nt 1192)	VP1ΔN360
p088	ccg*GAATTC*GAGGCAAACTGCACTCGCCAAC	B+ (nt 1372)	VP1ΔN420
p089	ccg*GAATTC*CAAACATGGGCCACCTTTGCCATG	B+ (nt 1462)	VP1ΔN450
p104	cgt*CTCGAG***TCA**TCTGCTCGTTCCTGCTCCGAG	B– (nt 2664)	VP1ΔC30
p103	cgt*CTCGAG***TCA**CTGTGGGTTCTTGACTTCTGGG	B– (nt 2574)	VP1ΔC60
p101	cgt*CTCGAG***TCA**CTTGGACTTGTGGAGTTTCTCGG	B– (nt 2394)	VP1ΔC120
p099	cgt*CTCGAG***TCA**GGGCTTGGGGGGTACTGGCTTG	B– (nt 2214)	VP1ΔC180
p097	cgt*CTCGAG***TCA**GCAGGCTTTGTTCAGGAGTGGG	B– (nt 2034)	VP1ΔC240
p095	cgt*CTCGAG***TCA**GAGATCTTTGCTGTATGTAGCTGAC	B– (nt 1854)	VP1ΔC300
p093	cgt*CTCGAG***TCA**CTCAATTGATTTGAACTCCTCGCTG	B– (nt 1674)	VP1ΔC360
p091	cgt*CTCGAG***TCA**GGCAATGTTCATGGCAAAGGTGG	B– (nt 1494)	VP1ΔC420
p090	cgt*CTCGAG***TCA**GGCTTGCATGTGTTGGCGAGTG	B– (nt 1404)	VP1ΔC450
p057	tc*GAATTC*GCTTCAGAGTTCAAAGAGACCCCC	A+ (nt 2396)	VP3 and VP3ΔC derivates
p006	gatc*GTCGAC***TCA**CTCAAGGTCCTCATCAGAGAC	A– (nt 3166)	VP3 and VP3ΔN derivates
p061	aagt*GTCGAC***TCA**GCTTGGCCCTCGGTGCCCATTG	A– (nt 2782)	VP3ΔC129
p060	ccg*GAATTC*GGCCAGCTAAAGTACTGGCAGAAC	A+ (nt 2786)	VP3ΔN129
p063	aagt*GTCGAC***TCA**ATAGACTTTGGCAACTTCGTCTATG	A– (nt 2977)	VP3ΔC64 and VP3ΔN64,C64
p062	ccg*GAATTC*AACGCACCACAAGCAGGCAGCAAG	A+ (nt 2588)	VP3ΔN64
p064	gatc*GTCGAC***TCA**CCAGCGGCCCAGCCGACCAG	A– (nt 3136)	VP3ΔC10
p065	gatc*GTCGAC***TCA**TGTTGGAGCATTGGGTTTTGGCTTG	A– (nt 3106)	VP3ΔC20
p066	gatc*GTCGAC***TCA**TGGTAGAGCCCGCCTGGGATTGCG	A– (nt 3076)	VP3ΔC30
p067	gatc*GTCGAC***TCA**CAGCTCCATCGCAGTCAAGAGCAGATC	A– (nt 3046)	VP3ΔC40
p068	gatc*GTCGAC***TCA**CATCTGTTCTTGGTTTGGGCCACGTC	A– (nt 3016)	VP3ΔC50
p069	gatc*GTCGAC***TCA**GTTGATTTCATAGACTTTGGCAACTTCG	A– (nt 2986)	VP3ΔC60
p106	gatc*GTCGAC***TCA**AAGGTCCTCATCAGAGACGGTCC	A– (nt 3163)	VP3ΔC1
p107	gatc*GTCGAC***TCA**GTCCTCATCAGAGACGGTCCTG	A– (nt 3160)	VP3ΔC2
p108	gatc*GTCGAC***TCA**ATCAGAGACGGTCCTGATCCAGC	A– (nt 3154)	VP3ΔC4
p109	gatc*GTCGAC***TCA**GACGGTCCTGATCCAGCGGCC	A– (nt 3148)	VP3ΔC6
p110	gatc*GTCGAC***TCA**CCTGATCCAGCGGCCCAGCC	A– (nt 3142)	VP3ΔC8
p111	tc*GAATTC*GAAGAACAAATCCTAAGGGCAGCTAC	A+ (nt 2882)	VP3ΔN162
p112	tc*GAATTC*GAAATCAACCATGGACGTGGCCC	A+ (nt 2978)	VP3ΔN194
p113	tc*GAATTC*CCAAAGCCCAAGCCAAAACCCAATG	A+ (nt 3074)	VP3ΔN226
p114	tc*GAATTC*ATCAGGACCGTCTCTGATGAGGAC	A+ (nt 3137)	VP3ΔN248
p138	CTCAAAGAAGATGGAGACC	A+ (nt 2704)	pHB36W derivates
p139	CA*GGTACC*TCA**CTA**AAGGTC	A– (nt 3177)	pHB36W-VP3Δ1(tag)
p140	CA*GGTACC*TCA**TCA**AAGGTCC	A– (nt 3177)	pHB36W-VP3Δ1(tga)
p141	CA*GGTACC*TCACTCAAGGTCCTC**TCA**AGAGAC	A– (nt 3177)	pHB36W-VP3Δ5(tga)
p142	CA*GGTACC*TCACTCAAGGTCCTCATCAGAGACGGTCCT**TCA**CCAGC	A– (nt 3177)	pHB36W-VP3Δ10(tga)
pA(+)	CCCTGACCCTGTGTCCCCCACAGTC	A– (nt 295)	RT, (+)RNA segment A
pAC1	GATCGGTCTGACCCCGGGGGAG	A+ (nt 3)	PCR, segment A specific
pANC1	TAGGTCGAGGTCTCTGACCTGAGAG	A– (nt 264)	PCR, segment A specific
pA(–)	GTGCATGCAGAGAAGAGCCGGTTGGC	A+ (nt 2852)	RT, (–)RNA segment A
pANC2	ACCCGCGAACGGATCCAATTTGGG	A– (nt 3256)	PCR, segment A specific
pAC2	ATCTACGGGGCTCCAGGACAGGC	A+ (nt 2912)	PCR, segment A specific
pB(+)	GGGCGATGTGTTGGGTAGTACTTTGGG	B– (nt 458)	RT, (+)RNA segment B
pBC1	ATACGATGGGTCTGACCCTCTGGG	B+ (nt 3)	PCR, segment B specific
pBNC1	GGAAGTACTCCTGATCTCCAATAGGG	B– (nt 433)	PCR, segment B specific
pB(–)	GGGTTCCCACTCGACGAGTTCCTAGC	B+ (nt 2077)	RT, (–)RNA segment B
pBNC2	CCCCCGCAGGCGAAGGCCGGG	B– (nt 2822)	PCR, segment B specific
pBC2	TGAGCTGTCAGAGTTCGGTGAGGC	B+ (nt 2112)	PCR, segment B specific

20 μCi of [^{35}S]methionine (Amersham)/ml in methionine-free EMEM. At the end of the labeling, the cell cultures were lysed on ice in 1x phosphate-buffered saline (PBS)-TDS lysis buffer, using a 5x PBS-Triton-sodium deoxycholate-SDS (TDS) lysis buffer stock solution (5 % Triton X-100, 2.5 % sodium deoxycholate, 0.5 % SDS, 0.7 M NaCl, 14 mM KCl, 50 mM Na_2HPO_4, 7.5 mM KH_2PO_4). Cell debris was removed by centrifugation at 4°C for 20 min at 13,000 × *g*. All lysates were pretreated with Protein A Sepharose (Amersham) prior to being immunoprecipitated with polyclonal anti-VP1 serum or monoclonal anti-VP3 (MAb C3) serum. Protein A Sepharose-bound immune complexes were washed three times in 1x PBS-TDS lysis buffer and eluted in 30 μl sodium dodecyl sulfate (SDS) sample buffer (60 mM Tris-HCl [pH 6.8], 2.5% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.1% bromophenol blue). Proteins were resolved in 12% separating gels by SDS-PAGE and visualized by autoradiography.

RNase TREATMENT OF IMMUNOPRECIPITATES

Before SDS-PAGE the Protein A Sepharose-bound immune complexes were resuspended in 10 μl of 10 mM Tris-HCl (pH 7.5) and treated as follows. For RNaseONE treatment, the immunoprecipitates were incubated for 45 min at 37°C with 1,000 U/ml RNaseONE (Promega) in 10 mM Tris-HCl (pH 7.5), 5 mM EDTA, and 200 mM sodium acetate. RNaseONE, a RNase able to cleave a phosophodiester bond between any two ribonucleotides, catalyzes the degradation of ssRNA to cyclic nucleotide monophosphate intermediates (Promega). For RNaseA treatment, the immunoprecipitates were incubated for 45 min at 37°C with RNaseA at a concentration of 100 μg/ml in 10 mM $MgCl_2$. RNaseA is often considered as an ssRNA-specific enzyme, though at a low salt concentration (i.e. 10 mM $MgCl_2$) it degrades both ssRNA and dsRNA (Libonati & Sorrentino, 2001). At the end of the RNase treatments the sepharose beads were collected and washed three times with 1x PBS-TDS lysis buffer, after which the immune complexes were eluted in 30 μl SDS-sample buffer and subjected to SDS-PAGE.

RT-PCR ANALYSIS OF IMMUNOPRECIPITATES

For the negative- and positive-strand specific reverse transcription (RT-)PCR of segment A and B, 1 μl of the respective immunoprecipitates was used. To prime cDNA synthesis on the genomic positive and negative strand of segment A we used oligonucleotide pA(+) and pA(–), that hybridized to nt 271-295 and 2852-2877 of the plus and minus strand, respectively (Table 1). To prime cDNA synthesis on the genomic positive and negative strand of segment B we used oligonucleotide pB(+) and pB(–), that hybridized to nt 432-458 and 2077-2102 of the plus and minus strand,

respectively (Table 1). For reverse transcription, samples containing 1 µl of the immunoprecipitate and 2 pmol primer in a total volume of 10 µl were incubated at 98°C for 2 min and immediately chilled on ice. Subsequently, 10 µl of an RT-mix containing 2 x Superscript II first strand synthesis buffer (Gibco-BRL), 20 mM DTT, 1 mM of each dNTP and 200 U of Superscript II (Gibco-BRL) were added. For the negative control reaction, the addition of Superscript II enzyme was omitted. The RT reaction was carried out at 50°C for 60 min, after which the incubation was continued at 70°C for 15 min to inactivate the RT. The reaction products were amplified by PCR using primer pairs pAC1/pANC1 for the plus strand of segment A (yielding a PCR product of 262 bp corresponding to nt 3-264), pAC2/ pANC2 for the minus strand of segment A (yielding a 345 bp PCR product corresponding to nt 2912-3256), pBC1/pBNC1 (yielding a 431 bp PCR product corresponding to nt 3-433) for the plus strand of segment B and pBC2/pBNC2 for the minus strand of segment B (yielding a 711 bp product corresponding to 2112-2822) (Table 1). The PCR consisted of 25 cycles, each comprising 15 sec of denaturation at 94°C, 30 sec of annealing at 68°C, and 45 sec of elongation at 72°C. The 25 cycles were followed by a 90 sec incubation at 72°C.

DETECTION OF INFECTIOUS RESCUED IBDV

To rescue infectious virus, transfected cells and culture fluid were freeze-thawed three times and the supernatant was filtered through a 200 nm pore size filter (Acrodisc; Gelman Sciences) to remove the FPV-T7 and cellular debris. The cleared supernatant was either stored at –20°C or used directly for further analysis. Infectious rescued IBDV (rIBDV) was detected by inoculating a subconfluent monolayer of QM5 cells with part of the cleared supernatant. After incubation for 48 h at 37°C the cells were washed with PBS, dried, and stored at –20°C until an immunoperoxidase monolayer assay (IPMA) was performed using a polyclonal antiserum against VP3 (Wensvoort *et al*., 1986).

RESULTS

A CARBOXY-TERMINAL BINDING SITE IN VP3 IS RESPONSIBLE FOR VP1-VP3 INTERACTION

The yeast two-hybrid system (Fields & Song, 1989; Fields & Sternglanz, 1994; Warbrick, 1997) is an increasingly popular tool for the detection of protein-protein interactions. Here we used the system to map the domains responsible for the VP1-VP3 interaction. Various truncated versions of VP1 and VP3 were inserted into the plexA_{BD}

('bait') and $pB42_{AD}$ ('prey') yeast expression plasmids. An interaction between the bait and prey fusion proteins allows yeast cells containing both plasmids to grow on medium lacking leucine and to produce β-galactosidase (β-gal), giving rise to blue colonies on X-Gal-containing medium. The strength of the protein-protein interaction can be judged by the intensity of the blue phenotype, allowing a semi-quantitative evaluation of the interaction between the candidate proteins (Estojak *et al.*, 1995; Li & Fields, 1993; Yang *et al.*, 1992). As positive controls, a combination of plasmids $pLexA_{BD}$-VP1 and $pB42_{AD}$-VP3, as well as the reciprocal combination $pLexA_{BD}$-VP3 and $pB42_{AD}$-VP1, each one containing a complete gene, were tested and these produced the expected phenotypes. Individually, or in combination with the respective control plasmids $pLexA_{BD}$-bicoid and $pB42_{AD}$-empty, these constructs were negative in both assays, confirming the specificity of the test system.

To map the interacting domain of VP3, five different deletion mutants were initially generated: VP3ΔC129 and VP3ΔN129, in which the carboxy-terminal and amino-terminal halves of VP3 were deleted; VP3ΔC64 and VP3ΔN64, lacking 64 amino acids at either end; and VP3ΔN64,C64, missing 64 amino acids from both termini of the protein (Fig. 1B-I). Similarly, a large series of amino- and carboxy-terminal deletion mutants were generated for VP1 (Fig. 1A). The coding region of each of the deletion mutants was expressed by both $pLexA_{BD}$ and $pB42_{AD}$ expression vectors, and the products were tested for their capability to interact with wild-type VP1 or VP3. To rule out the possibility of non-specific transactivation of the reporter genes, all constructs were additionally assayed for reporter gene activation when expressed either alone or together with the respective control plasmids $pLexA_{BD}$-bicoid and $pB42_{AD}$-empty. Since all constructs proved to be negative in these tests (data not shown), all interactions between the $LexA_{BD}$ and $B42_{AD}$ fusion proteins were regarded as specific. The results of the assays for the VP1-VP3 interaction are presented in figure 1. Of the VP1 deletion mutants both carboxy- and amino-terminally truncated fusion proteins scored positive in the test assessing production of β-gal and growth on medium lacking Leu, except for the mutants with an amino- or carboxy-terminal truncation of 240 amino acids or larger (Fig. 1A). These results suggest that the internal core domain of VP1 is essential for VP3 interaction. For the domain mapping of VP3 the results showed that a deletion of 64 amino acids or more at the carboxy terminus completely abolished binding to VP1, whereas the deletions at the amino terminus had no effect on this interaction (Fig. 1B-I). This indicated that the carboxyl terminus of VP3 is critical for the VP1-VP3 interaction.

To determine the interacting domain in VP3 more precisely, we constructed and tested six progressive carboxy-terminal deletion mutants, lacking 10, 20, 30, 40, 50, and 60 amino acids. None of these mutants showed reporter gene activation,

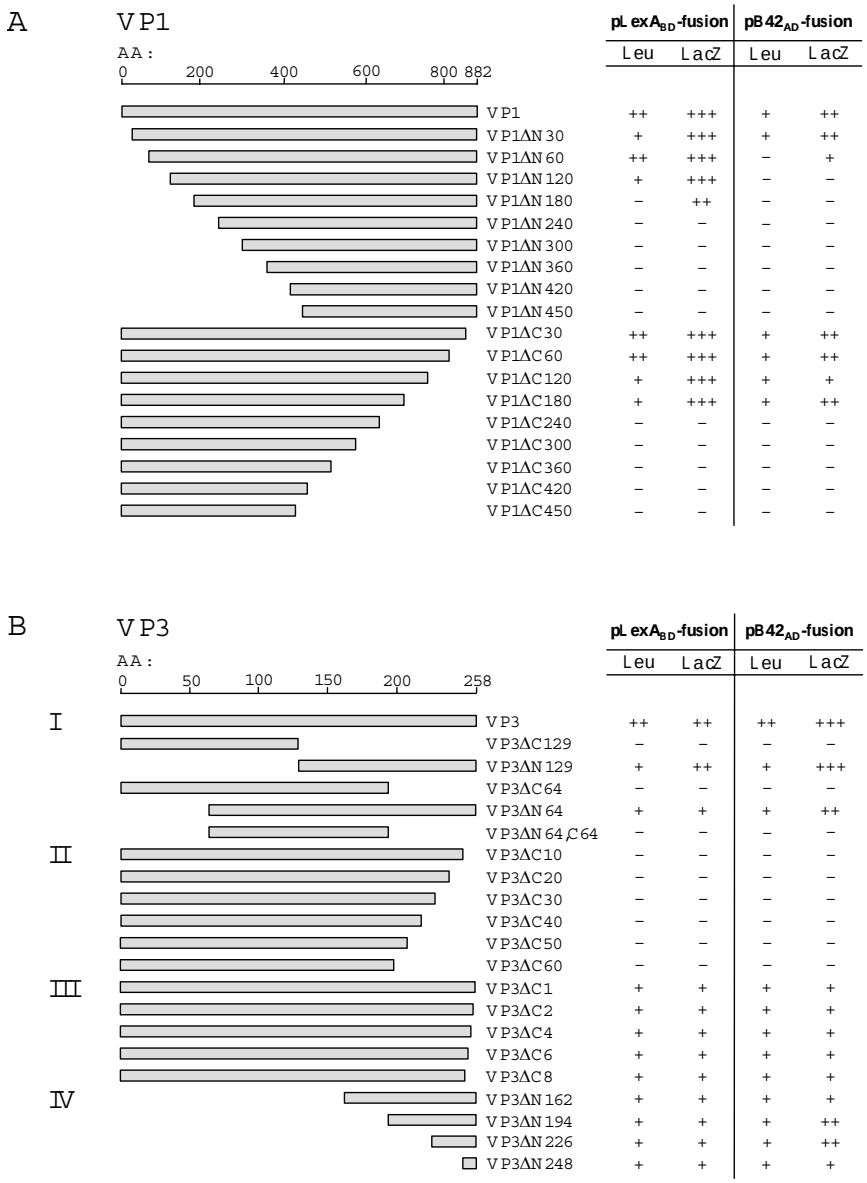


Figure 1. Mapping of the VP1-VP3 binding domain(s) by using deletion mutagenesis and the yeast two-hybrid system. Schematic representations of the deletion mutations in VP1 and VP3 are presented. VP1 and VP3 deletion mutants were cloned into pLexA$_{BD}$ and pB42$_{AD}$ yeast expression vectors and tested for their ability to interact with full-length VP3 or full-length VP1, respectively. Interactions were assayed for leucine autotrophy (LEU) and for β-galactosidase activity (LacZ). LEU:++, clear growth (strong interaction); +, growth (interaction); –, no growth (no interaction). LacZ: +++, deep-blue colonies (very strong interaction); ++, blue colonies (strong interaction); +, light-blue colonies (interaction); –, white colonies (no interaction). All results shown are representative of at least seven independent transformants.

suggesting that the binding domain of VP3 is located within the very last 10 amino acids of the carboxyl terminus (Fig. 1B-II). We therefore constructed and tested five additional carboxy-terminal deletion mutants lacking 1, 2, 4, 6, and 8 amino acids. Each of these deletion mutants had VP1 binding activity, albeit activity weaker than that of wild-type VP3 (Fig. 1B-III). To confirm these findings, we also tested a series of VP3 proteins with amino-terminal deletions by removing up to 248 of the 258 amino acids of the protein. All these truncated proteins, even the mutant VP3ΔN248, consisting of just the 10-residue carboxy-terminal domain of VP3, conferred growth on Leu$^-$ medium and gave blue colonies on X-gal-containing medium (Fig. 1B-IV).

Collectively, these yeast two-hybrid experiments consistently identified the 10-amino-acid carboxy-terminal peptide of VP3 as the VP1 interaction domain. To independently confirm this conclusion and to study the function of the VP1-VP3 interaction, we made use of our reverse genetics system (Boot *et al.*, 2000*b*) to generate viral VP3 carboxy-terminal deletion mutants.

IN VIVO INTERACTION BETWEEN VP1 AND CARBOXY-TERMINALLY TRUNCATED VP3

To test for a functional role of VP1-VP3 interaction during IBDV replication, mutant IBDV cDNA clones of the full-length segment A were constructed by introducing stop codons in the 3' terminus of the coding strand to generate carboxy-terminally truncated VP3 mutants. cDNA clones pHB36W-VP3Δ10(tga), pHB36W-VP3Δ5(tga), pHB36W-VP3Δ1(tga), and pHB36W-VP3Δ1(tag), containing a stop at the codon positions 10, 5, or 1 preceding the natural stop codon of the VP3 gene, were prepared (Fig. 2). In all mutants the original codon was replaced by a TGA stop codon except in clone pHB36W-VP3Δ1(tag) where a TAG stop codon, which required only a single point mutation (GAG to TAG), was used,

To analyze the expression and processing of the viral proteins of the mutated A segments, *in vitro* transcriptions and translations were performed by using a rabbit reticulocyte system. The radiolabeled translation products were subsequently analyzed by SDS-PAGE and autoradiography (Fig. 3). In all cases the viral polyprotein was processed normally into pVP2, VP3, and VP4. As predicted, the apparent molecular mass of VP3 decreased with increasing deletions: the removal of 5 or 10 carboxy-terminal amino acids gave rise to an apparent reduction in molecular mass of some 1 to 2 kDa.

To study more directly the role of the VP3 carboxy-terminal domain in interaction with VP1, we co-expressed the proteins in cells using the co-transfection system described previously (Boot *et al.*, 2000*b*). The wild-type and mutated full-length segment A cDNA clones, pHB36W, pHB36W-VP3Δ1(tag), pHB36W-VP3Δ1(tga), pHB36W-VP3Δ5(tga), and pHB36W-VP3Δ10(tga), were each transfected into QM5

cells together with either the wild-type full-length segment B cDNA clone (pHB34Z) or a mutant full-length segment B cDNA clone, named pMB13 (Fig. 4A). The last clone was used as a negative control for virus replication. In this clone, the stem-loop structure in the 3' terminus of the coding strand predicted by Boot *et al.* (1999), was changed into a more stable stem-loop structure by the replacement of three adjacent cytosine residues (nt 2802-2804) by three guanines, which resulted in a construct failing to yield virus in combination with wild-type segment A cDNA (Boot *et al.*, 2001).

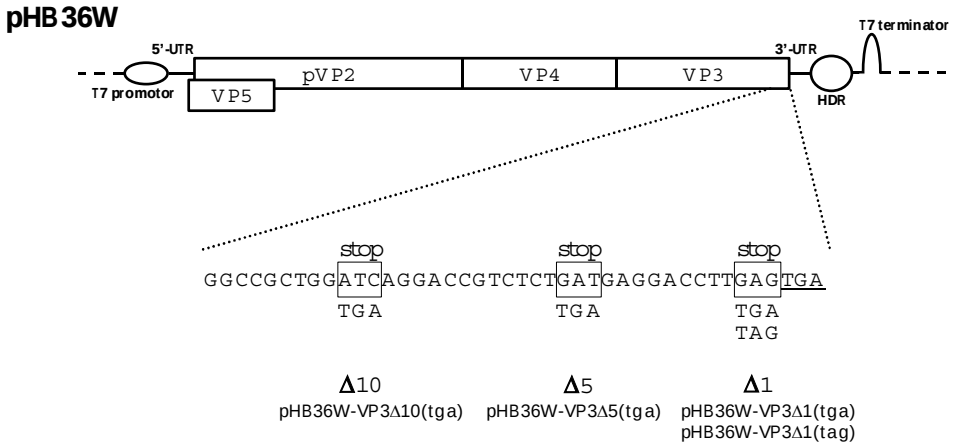


Figure 2. Schematic representation of mutagenesis of the segment A cDNA clone. Plasmid pHB36W, containing the full-length cDNA sequence of segment A, preceded by a T7 promoter sequence and followed by the autocatalytic hepatitis delta virus ribozyme (HDR) and a T7 terminator, is shown at the top of the figure. The nucleotide sequence of the 3' terminus of the ORF for the polyprotein is depicted below. The TGA stopcodon of VP3 is underlined. Mutated nucleotides are boxed and introduced stopcodons are indicated below in bold letters. The generated plasmids are named pHB36W-VP3Δ10(tga), pHB36W-VP3Δ5(tga), pHB36W-VP3Δ1(tga) and pHB36W-VP3Δ10(tag), according to the position and kind of stopcodon in the 3' terminus of the coding sequence of VP3.

Forty-eight hours post-transfection, cells were metabolically labeled for 3 hours with [^{35}S]methionine and subsequently subjected to immunoprecipitation with either anti-VP1 or anti-VP3 serum. The immunoprecipitates obtained were analyzed by SDS-PAGE and the labeled proteins visualized by autoradiography. As shown in the upper panel of Fig. 4B, when using the anti-VP3 serum, comparable amounts of the full-length and the truncated VP3 proteins were detected in the transfected QM5 cells. VP1 was clearly co-precipitated with full-length VP3 but only in minor amounts with the carboxy-terminally truncated VP3Δ1 (lanes 1-3). The immunoprecipitations

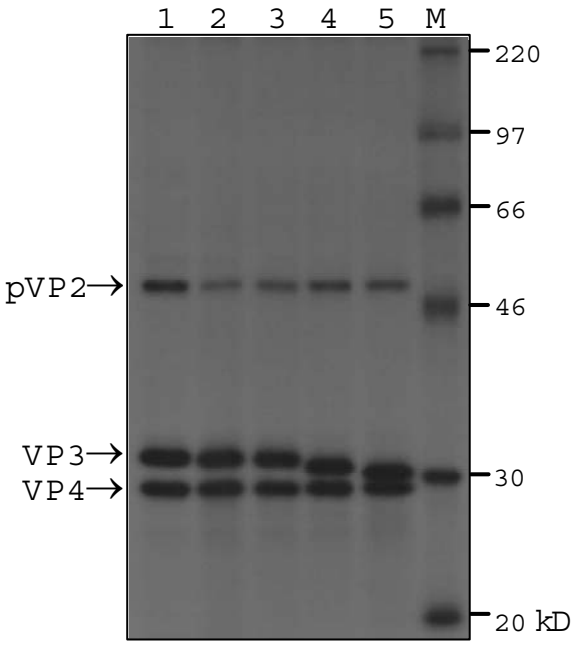


Figure 3. Autoradiogram of an SDS-PAGE analysis of a coupled *in vitro* transcription/translation reaction. Plasmids containing the full-length A-segment, pHB36W (lane 1), pHB36W-VP3Δ1(tag) (lane 2), pHB36W-VP3Δ1(tga) (lane 3), pHB36W-VP3Δ5(tga) (lane 4), pHB36W-VP3Δ10(tga) (lane 5) were used as DNA template to produce the viral proteins. The positions of the viral proteins and the sizes of the marker proteins (lane M) are indicated.

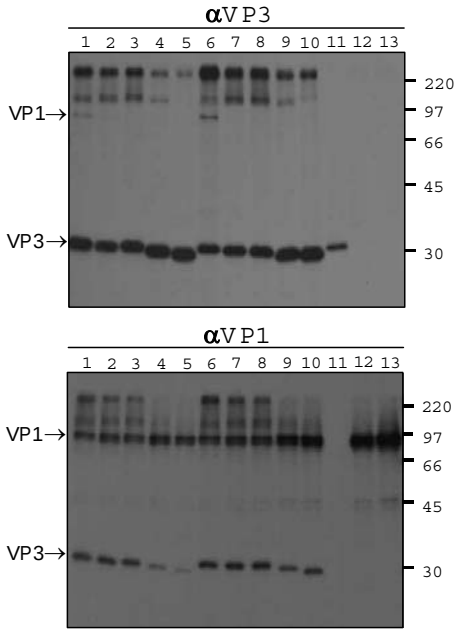
of VP3Δ5 and VP3Δ10 (lanes 4-5) completely failed to co-precipitate detectable amounts of VP1. This is in contrast with the anti-VP1 immunoprecipitations where VP1 and VP3 were both present in all immunoprecipitates (lanes 1-5). The same results were found with the pMB13 co-transfected QM5 cells (lanes 6-10) indicating that productive viral replication is not required for VP1-VP3 binding. For IBDV strain CEF94 we recently showed free VP1 protein to occur in two forms with molecular masses of 90 and 95 kDa (Tacken *et al*., 2000). In IBDV-infected cells both these forms appeared to interact with VP3 initially after their translation, though the interaction

Figure 4. Radio-immunoprecipitation analysis of VP1-VP3 interaction in transfected QM5 cells. (A) QM5 cells were (co-)transfected with the indicated plasmids containing either the wild-type or a mutated cDNA of the A-segment and/or B-segment. At 48 h post transfection cells were metabolically labeled for 3 h with [^{35}S]methionine. Subsequently, cells were lysed and immunoprecipitated with anti-VP3 serum (αVP3) or with anti-VP1 serum (αVP1) followed by SDS-PAGE, either directly (B) or after an RNaseONE (C) or RNaseA treatment (D). Positions of the viral proteins and sizes of marker proteins (in kDa) are indicated.

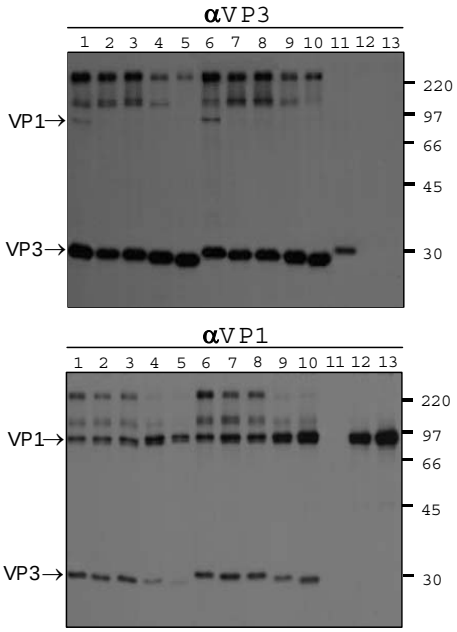
A Transfection

	A segment	B segment
1	pHB36W	pHB34Z
2	pHB36W-VP3Δ1(tag)	,,
3	pHB36W-VP3Δ1(tga)	,,
4	pHB36W-VP3Δ5(tga)	,,
5	pHB36W-VP3Δ10(tga)	,,
6	pHB36W	pMB13
7	pHB36W-VP3Δ1(tag)	,,
8	pHB36W-VP3Δ1(tga)	,,
9	pHB36W-VP3Δ5(tga)	,,
10	pHB36W-VP3Δ10(tga)	,,
11	pHB36W	-
12	-	pHB34Z
13	-	pMB13

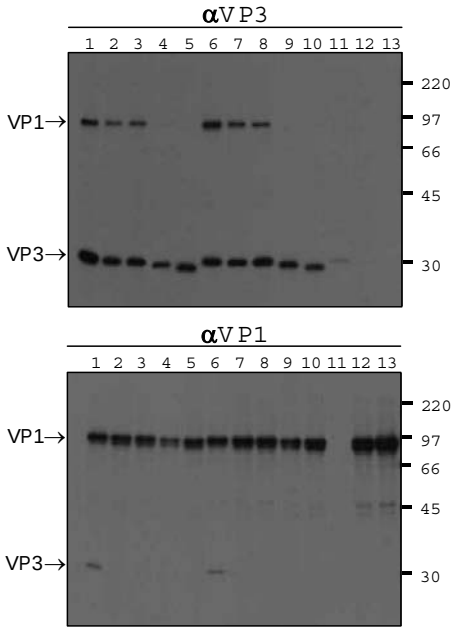
B Immunoprecipitation



C RNaseONE /Immunoprecipitation



D RNaseA /Immunoprecipitation



with the 90 kDa form gradually became undetectable thereafter (Tacken *et al.*, 2000). These observations explain the presence of the two discrete bands of free VP1 in the 90-95 kDa size range seen in the anti-VP1 immunoprecipitates (Fig. 4B-D), while only the 95 kDa band appears in the anti-VP3 immunoprecipitates: apparently only the 95 kDa form of VP1 was interacting with VP3 and therefore co-precipitated when using the anti-VP3 serum.

Furthermore, with the exception of the controls of transfected A-segment or B-segment alone (lanes 11-13), all immunoprecipitations revealed the presence of several bands in the high molecular mass region of > 97 kDa (lanes 1-10). Our previous studies have shown that these bands are VP1 specific and represent VP1-RNA complexes, as ascertained by Western blot analysis using VP1-specific sera and RNase treatment (Tacken *et al.*, 2000). It was obvious that these VP1-RNA complexes were present in both the VP1-specific and the VP3-specific immunoprecipitations. Since, in the latter case, neither VP3Δ5 nor VP3Δ10 were able to co-precipitate VP1, we speculated that the presence of these VP1-RNA complexes in the VP3-specific immunoprecipitates might be a consequence of a direct VP3-RNA interaction, rather than of a VP1-VP3 interaction. This assumption would also explain the unexpected co-precipitation of VP3Δ5 and VP3Δ10 proteins using the VP1 antibodies. The observation that the amount of co-precipitated VP3 correlated with the amount of precipitated VP1-RNA complex rather than with the amount of precipitated VP1 (lanes 1-5 and 6-10) also supports this interpretation. We therefore analyzed the sensitivity of the immune complexes to RNase.

VP3 INTERACTS BOTH WITH VP1 AND WITH IBDV SPECIFIC dsRNA

To study the VP1-VP3 interaction per se, i.e. without the interference of RNA-mediated co-immunoprecipitation, we performed an RNase treatment on all the VP1-specific and VP3-specific immunoprecipitates. To distinguish between ssRNA and dsRNA we performed an RNaseONE as well as an RNaseA treatment, thereby degrading specifically ssRNA or both ssRNA and dsRNA, respectively. The RNaseONE treatment failed to degrade the VP1-RNA complexes (Fig. 4C). The electrophoretic patterns of both the VP1- and VP3-specific co-immunoprecipitates were indistinguishable from those presented in Fig. 4B, indicating that the integrity of the VP1-RNA complexes is not dependent on ssRNA. In contrast, in the RNaseA treated samples, no high molecular mass (>97 kDa) complexes were present (Fig. 4D; lanes 1-13). Hence, the VP1-RNA complexes were maintained by dsRNA. Furthermore, in the VP3-specific immunoprecipitations, VP1 was co-precipitated with full-length VP3 and, to a lesser extent, with VP3Δ1, but not with VP3Δ5 and VP3Δ10. The reciprocal immunoprecipitations fully confirmed these results. The VP1 antiserum co-

precipitated full-length VP3 and, again in much lower amounts, VP3Δ1 (clearly seen after a prolonged exposure of the autoradiograph; data not shown). Obviously, VP3Δ5 and VP3Δ10 were no longer co-precipitated in the absence of dsRNA. Apparently, VP3 is able to associate with the VP1-dsRNA complexes through direct VP3-dsRNA interaction.

It was therefore of interest to determine whether the dsRNA in these complexes represented IBDV genome segment A- and/or B- specific sequences. To this end we performed an RT-PCR analysis. The untreated, RNaseONE and RNaseA treated VP3-specific immunoprecipitates were analyzed for the presence of positive and negative stranded genomic RNA. As expected, RT-PCR products specific for the 5' end of both strands of both segments were obtained from the untreated and RNaseONE treated samples but not from the RNaseA treated samples (Table 2; data of the RNaseONE treated samples not shown). The finding that no product was obtained when reverse transcriptase was omitted from the reaction before PCR, indicates that the PCR products were derived from RNA, not from contaminating DNA. In cells transfected with either pHB36W or pHB34Z alone, no RT-PCR products were obtained, confirming the absence of IBDV specific VP1-dsRNA complexes in these immunoprecipitates. Plasmids pHB36W and pMB13, a combination known to be incapable of generating infectious progeny virus when transfected into cells, appeared to be RNA-replication competent as shown by the RT-PCR products obtained (Table 2). Hence, the RNaseONE-resistant but RNaseA-sensitive high molecular mass material in the radio-immunoprecipitation analysis of this combination (Fig. 4B-D, lanes 6-10) was of IBDV-specific dsRNA origin. Infection induced in cells by using pMB13 and pHB36W is probably affected at the level of particle assembly or the particles produced are somehow not infectious.

As the RT-PCR analysis was performed with primers designed to amplify 5' end sequences from plus as well as minus strand RNA, the results indicate that full-length dsRNA was present in the immunoprecipitates. Consequently, the higher molecular mass material of ≥ 220 kDa that had barely entered the gel in the immunoprecipitation assays, most likely represents the full-length dsRNA-VPg complexes. The smear below it suggests the presence of incomplete dsRNA molecules such as VPg linked to partial dsRNA molecules, consisting of one full-length strand and one partial, complementary strand; this material runs faster than the full-length dsRNA-VPg complexes and appears as a smear due to its heterogeneity (Cho *et al.*, 1993; Kordyban *et al.*, 1997). When full-length dsRNA-VPg complexes from another birnavirus, infectious pancreatic necrosis virus (IPNV), were treated with RNase by Magyar *et al.* (1998), VPg molecules were obtained carrying oligonucleotides that had survived the enzymatic treatment, supposedly due to

Transfected plasmids		Strand-specific RT-PCR[a]							
		Untreated				RNaseA treated			
A segment	B segment	A+	A–	B+	B–	A+	A–	B+	B–
pHB36W	pHB34Z	+	+	+	+	–	–	–	–
pHB36W -VP3Δ1(tag)	pHB34Z	+	+	+	+	–	–	–	–
pHB36W -VP3Δ1(tga)	pHB34Z	+	+	+	+	–	–	–	–
pHB36W -VP3Δ5(tga)	pHB34Z	+	+	+	+	–	–	–	–
pHB36W -VP3Δ10(tga)	pHB34Z	+	+	+	+	–	–	–	–
pHB36W	–	–	–	–	–	–	–	–	–
–	pHB34Z	–	–	–	–	–	–	–	–
pHB36W	pMB13	+	+	+	+	–	–	–	–

Table 2. RT-PCR analysis of the anti-VP3 immunoprecipitation reactions of Fig.4B and 4D.

[a] Specific positive sense and negative sense primers of segments A and B (A(+), A(–), B(+) and B(–), respectively) were used for RT. Following RT, the reaction products were amplified by PCR using specific primer pairs based on 5' terminal sequences of both the positive and negative strands of segments A and B (for details, see Materials and Methods). The plus and minus signs indicate the presence or absence of a specific PCR product. Controls, in which reverse transcriptase was omitted from the reaction, were negative.

steric hindrance by the unusually large VPg. The two discrete bands seen migrating at ≈ 110-150 kDa might therefore represent two forms of VPg linked to short oligonucleotides that were inacessible for RNase. Interestingly, these short VPg-linked oligonucleotides were not resistant to RNaseA treatment.

Altogether, these observations supported the results of the yeast two-hybrid screen with the truncated VP3 deletion mutants. In addition, they revealed that the (co-)precipitated VP1-RNA complexes contained genomic dsRNA and that VP3 is able to associate with these complexes by interacting directly with the dsRNA.

VP1-VP3 INTERACTION IS ESSENTIAL FOR THE GENERATION OF INFECTIOUS PROGENY

To analyze the VP3 truncated viral mutants, VP3Δ1(tag), VP3Δ1(tga), VP3Δ5(tga) and VP3Δ10(tga), for their ability to produce infectious virus we examined the transfection supernatants of these mutants for the presence of recombinant IBDV. Aliquots of the supernatants were inoculated onto fresh monolayers of QM5 cells which were subsequently incubated for 48 h and fixed. An IBDV-specific antibody assay was used to analyze whether infection had occurred. In none of the assays IBDV proteins could be detected in the QM5 monolayer except for the wild-type

situation where unmodified segments A and B had originally been transfected (data not shown). These results indicate that all mutant viruses were unable to generate infectious progeny.

DISCUSSION

Little is yet known about the nature and significance of the interactions between the viral components during the IBDV life cycle. Here we focussed on the viral capsid protein VP3. We demonstrated that its interaction with VP1 is critically mediated by its carboxy terminal domain. The removal of just the terminal residue already affected its association with VP1 and inhibited the production of infectious progeny completely. The VP3 protein also appeared to interact with both segments of the viral dsRNA. Our results thus point to an essential role for VP3 in virus morphogenesis, it being involved not only in the formation of the capsid shell but also in the incorporation of the genome and the viral RNA polymerase.

The VP3-domain interacting with VP1 was unambiguously mapped to the carboxy-terminal tail by two independent approaches. In yeast two-hybrid analyses the ability to associate with VP1 was lost when 10 or more terminal residues of VP3 were removed while, conversely, the corresponding decapeptide by itself was able to establish interaction. These assays additionally demonstrated the VP1 protein to interact with VP3 through its more internal domain, but further mapping thereof was not pursued. Site-directed mutagenesis of our infectious cDNA clone revealed that in IBDV infection the interaction between the two proteins is even more sensitive to changes in the VP3 carboxy-terminal tail. The mere deletion of the ultimate residue dramatically affected the strength of their association, as judged by the almost complete loss of their interaction in the co-immunoprecipitation assay and the complete loss of infectious virus production.

These studies unexpectedly led us to the detection of the VP3-dsRNA interactions. The key observation here was that higher molecular mass material, which we mentioned earlier to contain VPg-linked RNA (Tacken *et al.*, 2000), was immunoprecipitated with anti-VP3 serum from lysates of cells undergoing infection with VP3Δ5 and VP3Δ10 mutant virus (Fig. 4B). Because this material was unlikely to have been precipitated through interaction of the mutant VP3 molecules with VPg (free VP1 was clearly not co-precipitated from these lysates) a direct VP3-RNA interaction seemed most plausible. RNase treatments subsequently confirmed this interpretation demonstrating in addition that the interaction was double-strand specific: RNaseA but not the single-strand specific RNaseONE degraded the immune complexes. Furthermore, RT-PCR analysis substantiated that the RNA was

indeed of IBDV origin and that it contained both positive and negative strands of each of the two genome segments. As the RT-primers were designed to amplify the 5' end sequence from the plus or the minus RNA strand, the results indicate that full-length dsRNA had been produced. Less dsRNA-linked VP1 was detected in cells expressing the C-terminally truncated proteins VP3Δ5 and VP3Δ10 than when wild-type VP3 or VP3Δ1 were expressed, suggesting that the VP3 truncations are either affecting the capacity of VP3 to associate with dsRNA or impairing dsRNA synthesis by affecting the capacity of VP1 to function as a replicase, transcriptase or primase. Wild-type VP3 was found predominantly in association with the dsRNA-linked form of VP1, relatively little being associated with free VP1. This may indicate that not all free VP1 is bound to VP3 during infection. Alternatively, the observation might have been brought about by the experimental conditions used: differences in efficiencies with which the different complexes (VP3/VP1 vs. VP3/VPg-dsRNA) had been immunoprecipitated or the amount of anti-VP3 antibodies having been limiting. VP3 is probably synthesized in excess of VP1 (VP3 constitutes 40% of the virion protein, VP1 only 3%; Dobos *et al*., 1979). Limiting VP3 antibodies would be expected to favour the precipitation of dsRNA-VP1/VP3 complexes over free VP1/VP3 complexes as the former probably carry relatively more VP3. Finally it is of note that the same autoradiographic patterns were obtained in different RIP-assays, both with transfected and with infected cells, using different labeling periods and different labeling times (data not shown), indicating that the results obtained in the present co-immunoprecipitation analysis were not skewed by unlabeled VP1 and VP3 formed prior to metabolic labeling.

Our results finally provide experimental evidence for the long anticipated role of VP3 in binding to viral nucleic acids. Already in 1986 Hudson *et al.* (1986) proposed such a role on the basis of the polypeptide's primary sequence. These investigators noticed that the predicted carboxy-terminal domain, with 12 positively charged residues (and 8 prolines) among its last 40 residues, is highly basic in nature. Interestingly, the very terminal stretch of this domain is on the contrary very acidic, with 4 negatively charged amino acids being present within the last 5 residues. This suggests that VP3 might possess two functional domains within its carboxy- terminus: the extreme terminal part that binds to VP1 and the positively charged domain immediately upstream thereof that binds the viral dsRNA. Future mutation analyses will be required to experimentally confirm the role of this latter domain.

Knowledge about the morphogenesis of birnaviruses is still very limited. Most of what we know came from studies in which IBDV cDNAs were expressed in cells using vaccinia- or baculovirus systems (Caston *et al*., 2001; Fernandez-Arias *et al*., 1998; Lombardo *et al*., 1999; Martinez-Torrecuadrada *et al*., 2000; Vakharia, 1997). These

studies revealed that virus-like particles indistinguishable from authentic virions can be assembled just from the segment A derived polyprotein precursor (Fernandez-Arias *et al*., 1998; Lombardo *et al*., 1999). It appeared that correct particle assembly is not dependent on the presence of viral RNA or VP1, nor of the small protein VP5 also specified by segment A, but that it merely requires the proteins pVP2 and VP3, the latter acting as an internal scaffold (Martinez-Torrecuadrada *et al*., 2000). VP1 was co-incorporated into such particles, and to an extent similar to that in virions, when its cDNA was co-expressed with segment A cDNA (Lombardo *et al*., 1999). These investigations in addition pointed to an important role of the carboxy-terminal tail of pVP2 in the assembly of the T=13 capsid, this terminal region being crucially involved in the control of the interactions between VP2 trimers and between VP2 trimers and VP3 trimers (Caston *et al*., 2001).

The results demonstrate that the VP3 protein is a key organizer in birnavirus morphogenesis. By its interactions with all the structural components in the virion it appears to create the interior architecture that is required for the proper execution of the replication and transcription activities that are the hallmark of double-stranded RNA viruses. It is now clear that VP3 entertains interactions with itself, with VP2, with free VP1 molecules, most likely also with genome-bound VP1, and with the dsRNA segments A and B. Different domains in the protein are responsible for these different interactions. The carboxy-terminal domains binding to the dsRNAs and to VP1 are, for instance, distinct from the region involved in the homotypic interactions giving rise to the VP3 trimers that have been observed in viral particles by the ultrastructural analyses (Bottcher *et al*., 1997; Caston *et al*., 2001; M. G. J. Tacken *et al*., unpublished data). We have not yet explored the domain(s) in the homotrimeric VP3 complex interacting with the homotrimeric VP2 complexes that constitute the outer surface of the viral capsid.

Of particular interest are the interactions of VP3 with VP1 and VPg. These interactions are obviously of critical importance as the various activities embodied in the polymerase protein are essential for the virus life cycle. Though we did not provide direct evidence for it, we assume that VP3 does interact with VPg and that the interacting domain in VP1 and VPg is the same. Free VP1 molecules present in virions have been shown to carry covalently bound, short stretches of viral RNA, presumably 5'-terminal sequences (Kibenge & Dhama, 1997). Their association with VP3 suggests that VP1 does not bind through the domain that contains the serine residue by which these oligonucleotides are attached, and by which also VPg is linked to genomic RNA. It is therefore more likely that VP1 and VPg both bind through another, but identical domain. It will be interesting to find out if and how the virus regulates the number of free and genome-bound VP1 molecules assembled in

each viral particle. Higher resolution structural analyses may shed light on this question and establish whether free VP1 and VPg assume distinct and specific positions within the internal virion cavity. Precise spatial arrangements are likely to be required within the virion for the expeditious functioning of this replication and transcription machine.

Double-stranded RNA viruses of higher eukaryotes invariably contain multiple genome segments. It is still an unresolved issue how these segments are correctly assorted during particle assembly. Among these viruses the birnaviruses are exceptional because of their genome-linked polymerase protein VPg, the other viruses all having a cap structure associated with the 5'end of the positive RNA strand of each segment. A role of the VPg moiety in the selection of the genome segments is therefore conceivable. Alternatively, this selection might be achieved by VP3, through its capacity of binding dsRNA. This capacity might of course also serve to assist the proper positioning of the two dsRNA segments within the virion. We have not investigated whether dsRNA binding activity of VP3 is sequence-specific and, if so, whether the sequences recognized are segment-specific or occur in the common terminal genomic regions. If this dsRNA binding activity would indeed operate at the level of segment selection during particle assembly we should assume that VP3 binds to specific double-stranded regions within the viral mRNAs, as all dsRNA viruses are assumed to synthesize their negative RNA strand only after the packaging of their mRNAs. The specific encapsidation of IBDV mRNAs might thus resemble the mechanism by which the hepatitis B virus assembles its pregenomic RNA. Here the viral polymerase binds to a specific stem-loop structure present in viral mRNAs which subsequently leads to the polymerase-dependent encapsidation of the viral nucleic acid (Bartenschlager & Schaller, 1992; Ziermann & Ganem, 1996). Much work will be required to resolve these issues, not only for IBDV but as well for the other dsRNA viruses. It is expected that the continued molecular virological studies in combination with the advancing ultrastructural analyses will allow us to answer the many outstanding questions concerning the biology of these viruses.

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CHAPTER 4

A yeast two-hybrid search for candidate cellular proteins interacting with the infectious bursal disease virus proteins VP1, pVP2, VP3 and VP5

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ABSTRACT

The yeast LexA interaction trap was used to screen a cDNA library from bursae of 3-weeks-old chickens in order to identify proteins that interact with the viral proteins VP1, VP3, or VP5 of the classical infectious bursal disease virus (IBDV) strain CEF94 or, with pVP2 of the very virulent IBDV strain D6948. In each case a large number of cDNA clones was obtained, which were partially sequenced. Searches in databases with these cDNAs identified some known proteins. We report here the identification of several candidate interactors for the viral proteins VP1, pVP2, VP3, and VP5 of IBDV. Putative biological implications for some of the interactions found are discussed. Whether these cellular proteins are true interactors remains to be established.

INTRODUCTION

Infectious bursal disease virus (IBDV) is the causative agent of infectious bursal disease, a highly contagious immunosuppressive disease in young chickens. IBDV, a member of the genus Avibirnavirus in the family Birnaviridae (Dobos *et al.*, 1979), replicates specifically in developing B lymphocytes in the bursa of Fabricius. During replication, viral proteins induce apoptosis, resulting in a rapid depletion of B lymphocytes (Vasconcelos & Lam, 1995; Jungmann *et al.*, 2001). Infection of 3- to 6-week-old chickens with IBDV causes an acute disease characterized by high morbidity and mortality. Surviving chickens as well as younger chickens that are infected with IBDV generally show no clinical signs, but may be immunosuppressed during their remaining lifetimes. The pathogenic serotype I IBDV isolates are subdivided into classical, antigenic-variant, and very virulent isolates. Very virulent IBDV (vvIBDV) strains are unable to infect non-B-lymphoid cells.

IBDV is a non-enveloped, icosahedral virus with a diameter of 60 nm (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). Segment A encodes a polyprotein (pVP2-VP4-VP3) that is processed into the major structural proteins VP2 and VP3 (Hudson *et al.*, 1986) by VP4, a virus-encoded protease that shares some characteristics with bacterial Lon proteases (Sanchez & Rodriguez, 1999; Birghan *et al.*, 2000; Lejal *et al.*, 2000). The non-structural VP4 protein is mainly associated with type II tubules of 24 nm in diameter (Granzow *et al.*, 1997). VP2 and VP3 form the outer and inner layers, respectively, of the icosahedral virions (Bottcher *et al.*, 1997). Processing of the precursor pVP2 into mature VP2 occurs during or after virus assembly (Müller & Becht, 1982), presumably by VP4 protease activity (Kibenge

et al., 1997; Lejal *et al.*, 2000). A second open reading frame in segment A encodes a small, non-structural protein VP5 (Mundt *et al.*, 1995). This protein proved to be non-essential for IBDV replication (Mundt *et al.*, 1997). Although the exact function of VP5 is still unknown, Yao *et al.* (1998) showed that it plays a role in virus pathogenesis. Segment B encodes the putative RNA-dependent RNA polymerase, VP1. This protein is present within virions both as a free polypeptide and as a genome-linked protein, called VPg, attached to the 5' end of the positive strands of the two genomic segments (Müller & Nitschke, 1987). Whether the 5' termini of the noncoding strands also have this covalently linked VPg is not known. Also unclear is the nature of the 5' termini of birnaviral mRNAs. Whereas Spies & Müller (1990) reported that these mRNAs may contain a cap structure, Dobos found that VP1 lacks the enzymatic activities for generating such a cap structure (Dobos, 1993). Although in vitro synthesized mRNA containing a cap structure has been used for the rescue of infectious IBDV after transfection into eukaryotic cells (Mundt & Vakharia, 1996) this does not prove that viral mRNA is indeed capped and not VPg-linked. Moreover, it has been shown for other viruses that the VPg can be replaced by a cap structure to rescue infectious virus from cloned cDNA (Boyer & Haenni, 1994).

In general, viral replication is the result of a complex interplay between functions encoded by the viral and host genomes. While purification of replication complexes has allowed some insight as to the participation of host proteins in this process (Hayes & Buck, 1993; Quadt *et al.*, 1993), genetic approaches are required for the identification and analysis of these host proteins. The yeast two-hybrid system is a powerful tool to screen DNA libraries for proteins that interact with a specific protein of interest (Fields & Song, 1989). The system is based on the functional reconstitution of a transcriptional activator. The target protein (bait) is fused to a DNA-binding domain (BD) while library encoded proteins (prey) are fused to the activation domain (AD) (Bartel *et al.*, 1993; Chien *et al.*, 1991). Interaction between bait and prey hybrids reconstitutes the transcriptional activation function and stimulates reporter gene expression. This procedure has permitted the characterization of protein associations *in vivo* where biochemical experiments, such as copurification, failed to show interaction (Huet *et al.*, 1994; Brondyk *et al.*, 1995; Rossi *et al.*, 1996), likely due to kinetic parameter constraints (Brent, 1996).

Recently, we have used the yeast two-hybrid system to analyse the intermolecular interactions between the viral proteins of IBDV themselves (Tacken *et al.*, 2000). In the present study, we aimed to identify cellular partners for the viral proteins and have applied the inducible LexA-dependent yeast two-hybrid system to screen a cDNA library from bursae of 3-weeks-old chickens using VP1, VP3, and

VP5 of IBDV strain CEF94, as well as pVP2 of vvIBDV strain D6948, as a bait. Very virulent IBDV was taken as the source of pVP2 rather than the cell culture adapted IBDV strain CEF94 as this is the protein responsible for the particular B-lymphoid cell tropism (Lim *et al.*, 1990; Mundt, 1999; Boot *et al.*, 2000). The LexA-based interaction trap offers inducible expression of the library fusion proteins, which allows less opportunity for the foreign fusion proteins to have a toxic effect on the yeast host and thus to be eliminated from the pool of potentially interacting proteins. We found that several host cell proteins are able to form complexes with the viral proteins of IBDV in yeast cells.

MATERIALS AND METHODS

BACTERIAL AND YEAST STRAINS

Escherichia coli strain DH5-α (Life Technologies) was used for subcloning. *Saccharomyces cerevisae* strain EGY48 (MATα, his3, trp1, ura3, $LexA_{op(x6)}$-*LEU2*) was used for the interaction trap. Yeast strain EGY[p8oplacZ] is the host strain EGY48 transformed with the autonomously replicating reporter plasmid p8op-LacZ (Clontech).

PLASMIDS AND CDNA LIBRARY

The plasmids $pLexA_{BD}$-VP1, $pLexA_{BD}$ -VP3 and $pLexA_{BD}$ -VP5 containing cDNA-coding sequences of VP1, VP3, and VP5, respectively, of IBDV strain CEF94, have been described previously (Tacken *et al.*, 2000). Plasmids $pLexA_{BD}$-pVP2 and $pLexA_{BD}$-VP4 were constructed as follows. Plasmid pHB-22R, containing the consensus cDNA of the A-segment of the vvIBDV strain D6948 (Boot *et al.*, 2000) was used as a template to generate the cDNA encoding pVP2 by PCR using the Expand high fidelity PCR system (Boehringer Mannheim). Similarly, plasmid pHB-36W, containing the consensus cDNA of the A-segment of the IBDV strain CEF94 (Boot *et al.*, 1999) was used as the template to generate the cDNA encoding VP4. The sets of primers used for pVP2 (upstream: 5'-CCGGAATTCATGACAAACCTGCA AGATCAAACCC-3'; downstream: 5'-GATCGTCGACTCACCTTATGGCCCGGATCA TGTCTTTG-3') and for VP4 (upstream: AGGAATTCGCCGACAAGGGGTACGAGGT AGTC-3'; downstream: 5'-TAACTCGAGTCAGGCCATGGCCAGGTCGTACTGGC-3') were designed to introduce an *Eco*RI site at the upstream (5') end and a stop codon plus a *Sal*I or *Xho*I site, respectively, at the downstream (3') end of the coding sequence. The PCR products were precipitated, digested with *Eco*RI/*Sal*I or *Eco*RI/*Xho*I, gel purified by the QIAEX-II method (QIAGEN), and ligated with T4 ligase (New England BioLabs) into the yeast expression vector $pLexA_{BD}$ (Clontech). This vector had previously been

digested with *Eco*RI/*Xho*I. The ligation mix was transformed into *Escherichia coli* DH5-α cells, 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 (containing $pLexA_{BD}$-pVP2 or $pLexA_{BD}$-VP4) were sequenced at the fusion junction by cycle sequencing using an ABI 310 sequencer (PE Applied Biosystems) to ensure correct reading frames.

Control plasmids $pLexA_{BD}$-53 (human p53 gene in $pLexA_{BD}$) and $pB42_{AD}$-SV40 T (SV40 large T antigen in $pB42_{AD}$) were from Clontech.

For the generation of a $pB42_{AD}$ cDNA plasmid library total RNA was isolated from bursae of 3-weeks-old chickens in a one-step protocol with acidic guanidinium thiocyanate, phenol, and chloroform (Chomczynski & Sacchi, 1987) and used for poly(A)$^+$ RNA purification with Mini-Oligo(dT) Cellulose Spin Columns (5 Prime → 3 Prime, Inc.) according to the manufacturer's instructions. Five micrograms of poly(A)$^+$ RNA was used for cDNA synthesis by a custom cDNA library service (OriGene Technologies, Inc.). Details of construction: cDNA was made using an *Xho*I-oligo d(T) primer. An *Eco*RI adapter was ligated onto the 5' end of the cDNA. Subsequently, the cDNA was cut with *Xho*I (internal sites were protected) and size-fractionated before cloning into *Eco*RI-*Xho*I sites of $pB42_{AD}$. Transformation into bacterial cells yielded 4.8×10^6 independent clones. Some 85% of the clones contained an insert, with an average insert size of about 1 kb and an insert size range of 0.4-2.5 kb, which was determined by purifying plasmid DNA from 48 random clones.

TWO-HYBRID LIBRARY SCREENING

All two-hybrid media, buffers, and protocols were as described in the Clontech Manual for the Matchmaker LexA Two-Hybrid System and in the Clontech Yeast Protocols Handbook. The yeast strain *S. cerevisiae* EGY48[p8op-lacZ] (Clontech), containing two reporter genes, *LEU2* and *Lac*Z, under the control of two independent promotors, was first transformed by using the lithium acetate method with the $pLexA_{BD}$ (*HIS3*$^+$) constructs carrying VP1, pVP2, VP3, VP4, and VP5, respectively. The resulting strains were selected and grown in SD/Glu/-His/-Ura medium. Stable expression of the LexA hybrid proteins was verified by Western blot analysis according the manufacturer's instruction (Clontech). The ability of the LexA fusions to bind operator DNA, as well as the inability of these LexA fusions for autonomous reporter gene activation, was confirmed by a repression and an activation assay, respectively, as described previously (Tacken *et al*., 2000). The obtained yeast strains EGY48[p8op-LacZ] harboring $plexA_{BD}$-VP1, $plexA_{BD}$-pVP2, $plexA_{BD}$-VP3, $plexA_{BD}$-VP4, or $plexA_{BD}$-VP5 were transformed according to the lithium

acetate protocol with plasmid DNA from the $pB42_{AD}$-bursae-cDNA library. Double transformants were grown on SD/Glu/-His/-Ura/-Trp medium to select for the presence of both types of hybrid plasmids and the *LacZ* reporter plasmid. Only when $>10^6$ independent clones were obtained the primary library cotransformants were harvested, pooled, and plated on SD/Gal/Raf/-His/-Ura/-Trp/-Leu medium to screen for transcriptional activation of the *LEU2* reporter gene. Yeast strain EG48[p8op-LacZ] transformed with an empty vector construct, $pB42_{AD}$-empty, was used as negative control, whereas yeast strain EG48[p8op-LacZ] transformed with $pLexA_{BD}$-53 and $pB42_{AD}$-SV 40 T was used as positive control. After 4 days of incubation at 30 °C, Leu+ colonies were patched onto SD/Glu/X-gal/-His/-Ura/-Trp medium as well as SD/Gal/Raf/X-gal/-His/-Ura/-Trp medium to screen for transcriptional activation of the *LacZ* reporter gene. Colonies and the library plasmids they contained were tentatively considered positive if they were blue on the Gal/Raf containing plates but not blue or only faintly blue on the Glu containing plates. Colonies of the LacZ+ clones were restreaked, at least once, onto SD/Glu/-His/-Ura/-Trp plates to isolate single colonies and were retested for both Leucine autotrophy and β-galactosidase activity. Positive candidate clones were analyzed by sequencing. For this purpose, yeast cells of a single colony were resuspended in 40 µl H_2O and broken up by a heat-treatment of 5 minutes at 100 °C followed by a vortex-treatment for 1 minute in the presence of glass beads. Cell debris was removed by centrifugation and 4 µl aliquots of the supernatants were used as template for PCR amplification of the bursa derived cDNA inserts in $pB42_{AD}$ using the $pB42_{AD}$ specific primers 5' CCAGCC TCTTGCTGAGTGGAGATG 3' (upstream) and 5' GTAGACAAGCCGACAACCTTGATTGG 3' (downstream). PCR products were purified using a PCR purification kit (Qiagen) and analyzed by sequencing.

DNA SEQUENCING AND SEQUENCE ANALYSIS

Partial sequence analysis of cDNAs was performed by cycle sequencing (BigDye terminator kit, PE Applied Biosystems) with an ABI 310 sequencer (PE Applied Biosystems). Sequence similarity searches of the databases were conducted by using the default settings of the BLAST program (Altschul *et al.*, 1990) on the National Center for Biotechnology Information World Wide Web server.

RESULTS

In search of cellular proteins interacting with IBDV proteins, we performed a LexA-dependent two-hybrid screen. Bursae of 3-weeks-old chickens were chosen as a source for poly(A)$^+$ RNA, from which a directionally cloned cDNA library in the $pB42_{AD}$

vector was generated. cDNA segments encoding VP1, VP3, VP4, and VP5 of IBDV strain CEF94, and pVP2 of vvIBDV strain D6948 were generated by PCR and subcloned into the yeast expression vector pLexA$_{BD}$. The generated pLexA$_{BD}$ fusion plasmids were introduced into yeast cells carrying two reporter genes, *LEU2* integrated in the genome, and *LacZ* located on the p8op-LacZ reporter plasmid. Protein expression of the LexA fusion plasmids was verified by Western analysis (data not shown). Activation and repression assays (Golemis *et al*., 1996) confirmed that the fusion protein by itself did not activate the reporter genes and that it was localized in the nucleus (data not shown). The yeast strains obtained, harboring plexA$_{BD}$-VP1, pLexA$_{BD}$-pVP2, plexA$_{BD}$-VP3, plexA$_{BD}$-VP4 or pLexA$_{BD}$-VP5, were transformed with the pB42$_{AD}$-bursae-cDNA library and plated on glucose SD medium lacking his, ura, and trp. All transformations yielded >10^6 independent clones except for the yeast strain harboring pLexA$_{BD}$-VP4 (Table 1).

Yeast strain EGY48[p8op-LacZ] harboring the following plasmids		**Number of independent cDNA clones**				
		Primary transformants[a] (cfu)	**1st screening**		**rescreening**	
pLexA$_{BD}$ -fusion:	pB42$_{AD}$ -fusion:		**Leu+[b]**	**LacZ+[c]**	**Leu+**	**LacZ+**
VP1	bursae-cDNA	2.0 x 10^6	3825	329	329	192
pVP2	bursae-cDNA	2.1 x 10^6	130	54	54	48
VP3	bursae-cDNA	3.0 x 10^6	226	45	45	44
VP4	bursae-cDNA	5.0 x 10^3	nd	nd	nd	nd
VP5	bursae-cDNA	1.7 x 10^6	326	194	165	77

Table 1. Compilation of Yeast two-hybrid screening results. Yeast strain EGY48[p8oplacZ] was sequentially transformed, first with a pLexA$_{BD}$ plasmid encoding one of the IBDV proteins VP1, pVP2, VP3, VP4, or VP5, and then with the pB42$_{AD}$-bursae-cDNA library. In a first screening, yeast clones were initially tested for leucine autotrophy (Leu+) and subsequently Leu+ clones were assayed for β-galactosidase activity (LacZ+). In a rescreening all isolated Leu+/LacZ+ clones were assayed for a second time for expression of both reporter genes analogous to the first screening. cfu; colony-forming units. nd; not determined.

[a] Total approximate number of transformants obtained on SD/glu/-His/-Ura/-Trp plates.

[b] Yeast clones were scored as Leu+ if they grew on SD/Gal/Raf/-His/-Ura/-Trp plates but not on SD/Glu/-His/-Ura/-Trp plates.

[c] Yeast clones were scored as LacZ+ if they turned blue on SD/Gal/Raf/X-gal/-His/-Ura/-Trp but not or only faintly blue on SD/Gal/Raf/X-gal/-His/-Ura/-Trp plates.

Since a saturating screen of the library requires at least 10^6 independent transformants, the library screen with VP4 was not further analyzed. For the screenings with VP1, pVP2, VP3, and VP5, all primary transformants were pooled and subsequently plated for Leu+ selection on galactose/raffinose SD medium lacking his, ura, trp, and leu. Yeast transformed with an empty vector construct, $pB42_{AD}$-empty, was used as a negative control, whereas yeast transformed with $pLexA_{BD}$-53 and $pB42_{AD}$-SV 40 T was used as a positive control. All Leu+ colonies were subsequently replica-plated to glucose-, as well as galactose/raffinose- SD medium

Number of clones with same sequence information	**Proteins of database matching the cDNA sequence of the respective cDNA clones**
42	*Cytochrome C oxidase*
21	Unknown
16	Paladin [accession no: NM_013753]
8	Eukaryotic translation initiation factor 4A, isoform 2 (eIF4AII)
7	*NAD(+)-isocitrate dehydrogenase*
5	Unknown
4	Unknown
4	RAN binding protein 1 (RanBP1)
4	Putative cyclin G1 interacting protein [accession no: NP_006340]
3	Unknown
3	*Ribosomal protein*
2	Unknown
2	Unknown
2	Unknown
2	Unknown
2	Unknown
1	Enhancer of rudimentary homolog [accession no: NP_004441]
1	*Zinc finger transcription factor* [accession no: AAC05500]
	(27 x)[a)] Unknown
	(36 x)[b)] ND
Total number of positive cDNA clones: 129	

Table 2. Putative cellular interactors of VP1. The yeast LexA interaction trap was used to screen a cDNA library from bursae of 3-weeks-old chickens in order to identify proteins that interact with VP1 of IBDV strain CEF94. Positive cDNA clones were selected on the basis of their ability to activate transcription of both *LEU2* and *LacZ* reporter genes under the control of LexA binding sites. Sequence comparisons of the respective cDNAs were performed using the default settings of the BLAST program the National Center for Biotechnology Information World Wide Web server. Proteins that are notoriously found as non-specific interactor in a two-hybrid assay are in italics. Unknown; no similarities were found. ND; not done (cDNA has to be sequenced).

[a)] Number of clones, each with a different sequence information.

[b)] Number of clones that were not sequenced.

Number of clones with same sequence information	Proteins of database matching the cDNA sequence of the respective cDNA clones
6	Unknown
5	Unknown
3	Unknown
3	Unknown
2	Calmodulin
2	*Ubiquitin*
2	*Ribosomal protein*
2	Unknown
1	Sin3-associated polypeptide 18 (Sap18) [accession no: NP_033145]
1	Ornithine decarboxylase antizyme (ODC-AZ)
1	Hypothetical protein KIAA0168 [accession no: P50749]
	(11 x)[a)] Unknown
	(9 x)[b)] ND
Total number of positive cDNA clones: 48	

Table 3. Putative cellular interactors of pVP2. The yeast LexA interaction trap was used to screen a cDNA library from bursae of 3-weeks-old chickens in order to identify proteins that interact with pVP2 of vvIBDV strain D6948. For further details see legend of Table 2.

[a)] Number of clones, each with a different sequence information.

[b)] Number of clones that were not sequenced.

Number of clones with same sequence information	Proteins of database matching the cDNA sequence of the respective cDNA clones
6	RAN binding protein 1 (RanBP1)
5	*Ferritin, heavy chain*
4	Unknown
4	Thymocyte protein cThy28kD [accession no: AAA75591]
2	Unknown
2	Unknown
2	Unknown
1	Embryonic ectoderm development protein [accession no: AAC53302]
1	Pyruvate kinase
1	*Cytochrome C oxidase*
1	*Ribosomal protein*
	(5 x)[a)] Unknown
	(10 x)[b)] ND
Total number of positive cDNA clones: 44	

Table 4. Putative cellular interactors of VP3. The yeast LexA interaction trap was used to screen a cDNA library from bursae of 3-weeks-old chickens in order to identify proteins that interact with VP3 of IBDV strain CEF94. For further details see legend of Table 2.

[a)] Number of clones, each with a different sequence information.

[b)] Number of clones that were not sequenced.

Number of clones with same sequence information	Proteins of database matching the cDNA sequence of the respective cDNA clones
8	Unknown
7	*Elongation factor 1-alpha*
4	Guanine nucleotide binding protein (G protein) [accession no: NP_006089]
4	Ovoinhibitor [accession no: AAA48994]
3	Phosphoglycerate kinase (PGK)
3	Unknown
2	Unknown
1	Gamma-immunoglobulin heavy chain [accession no: CAA30161]
1	Elonase [accession no: BAA07132]
1	Ornithine decarboxylase antizyme (ODC-AZ)
1	Death associated protein 5 (DAP5) [accession no: CAA61857]
1	DNA polymerase epsilon subunit B
1	Beta-tubulin
1	Clusterin
1	Inorganic pyrophosphatase
1	Phosphoglucanate dehydrogenase
1	Hypothetical protein P15-2 [accession no: NP_061168]
1	Peptidylprolyl isomerase E (cyclophilin E) [accession no: NP_006103]
1	*HSPC322* [accession no: AAF29010]
1	RAN binding protein 16 (RanBP16)
1	RNA-binding protein siahBP [accession no: AAD44358]
	(19 x)[a)] Unknown
	(13 x)[b)] ND
Total number of positive cDNA clones: 77	

Table 5. Putative cellular interactors of VP5. The yeast LexA interaction trap was used to screen a cDNA library from bursae of 3-weeks-old chickens in order to identify proteins that interact with VP5 of IBDV strain CEF94. For further details see legend of Table 2.

[a)] Number of clones, each with a different sequence information.

[b)] Number of clones that were not sequenced.

containing X-gal and lacking his, ura, and trp, to assess the transcriptional activation of the LacZ reporter. About 10-50% of the screened Leu+ colonies turned blue on the galactose/raffinose plates and not or only faintly blue on the glucose plates (Table 1). All positive colonies were restreaked at least once on glucose SD medium lacking his, ura, and trp, to allow segregation of some multiple $pB42_{AD}$-bursae-cDNA library plasmids within single colonies while maintaining selective pressure for the plasmids. Consequently, these restreaked colonies were rescreened for expression of *LacZ* and *LEU2* reporter genes. The rescreenings led to the isolation of a number of distinct clones as putative interactors for VP1, pVP2, VP3, and VP5 as compiled in Table 1. Partial sequences of the cDNA inserts of these clones were determined, and aligned to protein sequences in data banks. The results of these analyses are documented in Table 2 – 5. As is clear from these tables, for each viral protein a

number of different sequences were obtained with different frequencies. While many of these sequences had significant homology to known proteins, for several no such match was found. We also obtained several protein scores that are notoriously found as non-specific interactors in two-hybrid screens. Finally, for some clones the sequence analysis failed for unknown reasons. Putative biological implications for several interactions found are discussed below.

DISCUSSION

Little is known about the intermolecular interactions of the viral proteins of IBDV and host cell proteins. In general, viral proteins often function by contacting other proteins, particularly proteins that regulate the different cellular processes such as transcription, translation, replication, signal transduction, cell cycling, and programmed cell death. In this way the virus interferes with these processes to adjust the conditions for its optimal replication. Finding interacting partners of viral proteins can thus reveal much about the function of these proteins. Here we used the inducible LexA-dependent two-hybrid system in yeast to search for cellular partners of the viral proteins VP1, VP3, and VP5 of IBDV strain CEF94, and pVP2 of vvIBDV strain D6948. The screening of a cDNA library from bursae of 3-weeks-old chickens allowed the isolation of a number of putative cellular interactors. Because of the large amounts of information flowing from these interaction experiments, it is difficult to make a qualitative interpretation. Which of the individual interactions found are meaningful? Many of the proteins identified in interactor hunts are non-specific interactors: they appear to interact with a number of different unrelated LexA fusions (Chien *et al.*, 1994). Interestingly, the commonly isolated non-specific interactors, which include ribosomal proteins, cytochrome oxidase, proteasome subunits, mitochondrial proteins, ferritin, NADH dehydrogenase, zinc finger proteins, elongation factors, and other proteins (http://www.fccc.edu/research/labs/golemis/main_false.html), are not isolated in every interactor hunt, and in fact do not appear to interact with every bait. For example, frequently a non-specific interactor will interact with just 30% of different bait proteins (Finley & Brent, 1997). Conversely, these commonly found non-specific interactors are occasionally found to be true specific interactors. Furthermore, we tend to give less weight to interactions between proteins that are so ubiquitous in the life of the cell (e.g., members of the ubiquitin system or heat shock proteins) that the interactions might be meaningful but relatively uninformative.

From our two-hybrid screen with VP1 we obtained 8 independent yeast clones harboring cDNAs encoding the eIF4AII C-terminal domain. Eukaryotic

initiation factor 4A, an essential component of the translation initiation system, normally binds to the initiation complex in a cap-mediated way (Gingras *et al.*, 1999). A direct interaction between VP1 and eIF4A might therefore be feasible since the viral mRNAs of IBDV are likely to lack a cap-structure, carrying a covalently linked VP1 (VPg) at their 5' end instead. Furthermore, we found a total of 21 independent cDNA clones each coding for the same unknown protein, and a total of 16 cDNA clones encoding paladin, as putative cellular partners for VP1. Paladin, obtained in a subtractive screen of mouse gastrulation (Pearce *et al.*, unpublished data, 1996), however, is also a novel protein with unknown function. It is therefore difficult to speculate about a possible function of these two putative interactors. An approach to search for more information would be to assume that these new proteins function in a network of protein interactors and to use them as bait in an additional two-hybrid screening to identify other members of the network. Repeating this process would further increase the chances of isolating a previously characterized protein, or one the sequence of which might provide clues to its function. Another putative interactor found for VP1 was RanBP1. It is worth noting that RanBP1 was found as putative interactor for both VP1 and VP3. RanBP1, a RanGTP-binding protein, is located in the cytoplasm and has been implicated in the release of nuclear export complexes from the cytoplasmic side of the nuclear pore complex (Görlich & Kutay, 1999; Kehlenbach *et al.*, 2001). Since VP1 and VP3 also interact with each other we could speculate that these proteins are involved in a network of proteins functioning in nucleocytoplasmic transport. However, considering that the known interactor for RanBP1, Ran, requires only a strech of five acidic amino acids for its interaction with RanBP1 (Kehlenbach *et al.*, 2001), our observed putative interactions of VP1 and VP3 with RanBP1 might simply be non-specific. Other putative interactors found for VP1 were either unknown proteins, notorious non-specific interactors, or interactors unlikely to be meaningful.

For the interaction hunt with pVP2 we used the gene from a vvIBDV strain rather than from the cell culture adapted IBDV strain CEF94 as bait, since VP2 of the vvIBDV strain is responsible for the particular B-lymphoid cell tropism (Lim *et al.*, 1990; Mundt, 1999; Boot *et al.*, 2000). Recently, it was found that wild-type vvIBDV is able to replicate in non-B-lymphoid cells once it is artificially (transfection of cDNA) introduced into these cells (Boot *et al.*, 2000). Therefore, the inability of vvIBDV strains to infect non-lymphoid cells is likely due to the inability to recognize a certain receptor and/or the inability to enter the cell. It was speculated that the typical B-lymphoid cell tropism of vvIBDV strains might be related to the recognition of an IBDV specific B-lymphoid cell receptor whereas classical IBDV strains recognize a general IBDV receptor present on a wide range of cells (Boot *et al.*, 2000). Thus, by

using the vvIBDV pVP2 protein as bait in our screening we thought we might have a chance to find a candidate B-lymphoid specific IBDV receptor, though this thought is also somewhat impetuous for various reasons. For instance, to accomplish a receptor-interaction it is usually necessary that the protein in question is in its natural conformation, so there might be only a receptor-interaction with mature VP2, and not with the premature form pVP2. Furthermore the pVP2 and, in addition, the VP3 coding sequences that we have used as bait, were based on the putative polyprotein cleavage sites reported by Hudson *et al.* (1986). Recently, the exact proteolytic processing sites at the pVP2-VP4 and VP4-VP3 junctions of the polyprotein of IBDV were identified (Sanchez & Rodriguez, 1999). Hence, the pVP2 and VP3 coding sequences we have used were therefore incorrect. Also, the virus specific receptor might be composed of glycosylated proteins (Ogawa *et al.*, 1998). Interactions dependent on a posttranslational modification, such as glycosylation, do not occur in yeast cells and therefore will not be detected. Finally, there are some drawbacks of the yeast two-hybrid system: (i) the two-hybrid system is limited to proteins that can be localized to the nucleus, which may prevent its use with certain extracellular proteins (ii) the bait and prey protein must be able to fold and exist stably in yeast cells and retain activity as fusion protein (iii) the use of protein fusions means that the site of interaction may be occluded by one of the fusion domains. Taken all together, it thus might be unlikely to find a possible IBDV-specific receptor in a yeast two-hybrid hunt. Nevertheless, as nothing is known about the existence of possible cellular interactors for pVP2, we also aimed to search for such putative cellular partners. Our interaction hunt yielded 48 putative interactors (Table 3). Most of these did not have any obvious homology to known proteins and their significance thus remains unclear. Of those that had a match in the database a Sap18 homologue and ornithine decarboxylase antizyme (ODC-AZ) are worth mentioning. Sap18 is a polypeptide that is associated with the mammalian transcriptional repressor Sin3 (Zhang *et al.*, 1997). pVP2 might somehow contribute to the regulation of gene expression through the recruitment of the Sap18 homologue and/or the Sap18-Sin3 complex. ODC-AZ is the central element in a feedback loop that controls the cellular level of polyamines. Polyamines play key roles in processes ranging from the functioning of certain ion channels (Williams, 1997), nucleic acid packaging, DNA replication, apoptosis, transcription and translation. The expression of ODC-AZ genes in vertebrates requires a specific +1 translational frameshift. This frameshifting occurs in the decoding of the antizyme (AZ) gene, which consists of two partially overlapping open reading frames. The AZ protein binds to ornithine decarboxylase (ODC) (Murakami *et al.*, 1992*a*; Li & Coffino, 1993, 1994), inhibits it (Heller *et al.*, 1976) and targets it for degradation (Murakami *et al.*, 1992*b*; Murakami

et al., 1999). ODC catalyzes the first and rate-controlling step in the synthesis of polyamines, conversion of ornithine to putrescine. Increasing polyamine levels induce frameshifting in AZ mRNA and so increase the level of AZ (Gesteland *et al*., 1992; Rom & Kahana, 1994). Since AZ negatively regulates the synthesis and uptake of polyamines, the frameshifting is the sensor for the autoregulatory circuit. Our observation that a viral protein might bind to AZ is potentially interesting. Often virus particles contain polyamines packaged with the viral components suggesting that polyamines are in some way important for viral replication. Like the AZ gene the IBDV A segment has two overlapping open reading frames. It is therefore tempting to speculate that the cellular polyamine levels somehow regulate the differential expression of the VP5 and pVP2-VP4-VP3 open reading frames through programmed frame-shifting. Remarkable enough, ODC-AZ was found as a putative interactor both for pVP2 and for VP5. Nevertheless, we should bear in mind that ODC-AZ was only found once in both screenings whereas a library that has been screened to exhaustion usually represents each interactor cDNA more than once in the putative positives. Whether ODC-AZ is a true interactor is therefore at this point very speculative.

By the two-hybrid screen with VP3, we isolated only a few cDNA clones with sequence homology to known proteins. Among them: RanBP1 (discussed above), Thymocyte protein cThy28kD, and embryonic ectoderm developmental protein (EED). cThy28kD is a nuclear phosphoprotein that is highly expressed in the bursa, thymus and spleen. It is thought to have a functional role in bursal lymphocyte apoptosis (M.M. Compton; personal communication). EED belongs to the Polycomb-Group (PcG) proteins, which form multimeric protein complexes involved in maintaining the transcriptional repressive state of genes over successive cell generations (van Lohuizen, 1998). At this point in time it is difficult to speculate about a putative relationship between VP3 and cThy28kD or EED. Besides, these putative interactors should first be confirmed with a bait protein of VP3 that is based on the recently identified (Sanchez & Rodriguez, 1999) VP4-VP3 proteolytic processing site of the IBDV polyprotein.

The interaction hunt with VP5 yielded quite a number of different putative interactors. As discussed, most two-hybrid approaches inevitably produce false positives. Possibly VP5 is a “sticky” protein and therefore associates with multiple non-specific interactors. Therefore, it is difficult to speculate which of the putative interactors found for VP5 are potential candidates to pursue as biologically relevant. Despite this, possibly worth mentioning are guanine nucleotide binding protein (G protein), ovoinhibitor, and death associated protein 5 (DAP5) as putative interactors. G-protein [accession no: NP_006089], homologous to chicken B complex

protein, is a membrane-associated protein (Guillemot *et al*., 1989). Lombardo *et al*. (2000) hypothesised that accumulation of VP5, a class II membrane protein, in the plasma membrane leads to a progressive alteration of the plasma membrane that facilitates the release of the virus progeny. Although speculative, an interaction between VP5 and G-protein might be involved in the process of release. Ovoinhibitor is a multidomain Kazal-type proteinase inhibitor with each domain containing an actual or putative reactive site for a serine proteinase (Saxena & Tayyab, 1997). Whether there may be a biological relevant relation between IBDV VP4, which is delineated as a new type of viral serine protease (Lejal *et al.*, 2000), and VP5 might be too speculative. Finally, although it was only found once in the screening, death associated protein 5 (DAP5) might also be worth mentioning. If DAP-5 proves to be a true interactor this interaction is interesting. DAP-5 is a putative modulator of programmed cell-death (Levy-Strumpf *et al.*, 1997). Since the mechanism of IBDV-induced apoptosis is still unknown this putative interaction might therefore be a first clue to unravelling this mechanism.

Finally, a two-hybrid screen for putative cellular interactors of VP4 failed. For some reason the transformation efficiency of the bursae-cDNA library into the yeast host strain harboring VP4 as bait was too low. At least 10^6 independent transformants are required before a meaningful screen can be done, while we obtained only $5x10^3$ transformants with the VP4 expressing yeast cells.

Taken altogether, the yeast two-hybrid screenings described here allowed the isolation of several putative cellular interactors for the viral proteins VP1, pVP2, VP3 and VP5 of IBDV. Although the candidates found in a two-hybrid assay do not address the biological significance of the interactions, several interactions that we observed may have a real *in vivo* function. A first step to demonstrate biological relevance is to verify these interactions by a different, biochemical assay, preferably by a co-precipitation from a cell in which both proteins are expressed. Ideally, the next step would involve a functional assay for the interactor, to show, for example, that the protein is involved in the same biological process as the viral protein. We hope that our ongoing studies will indeed elucidate the biological significance of some of the putative interactors in the IBDV life cycle.

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CHAPTER 5

VP1, the RNA-dependent RNA polymerase and genome-linked protein of infectious bursal disease virus, interacts with the carboxy-terminal domain of translational eukaryotic initiation factor 4AII

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ABSTRACT

Infectious bursal disease virus (IBDV), a member of the *Birnaviridae* family, is a non-enveloped, double-stranded RNA (dsRNA) virus. Viral protein 1 (VP1), the putative RNA-dependent RNA polymerase, occurs in virions both as a free polypeptide and as a genome-linked protein, called VPg. To gain more insight in its function, we initiated a yeast two-hybrid screen of a cDNA library from the bursa of Fabricius of 3-weeks-old chickens, using VP1 as bait. With this approach, we isolated eight cDNA clones with complete sequence homology to the carboxy-terminal domain of eukaryotic translation initiation factor 4AII (eIF4AII). These clones behaved as *bona fide* VP1 partners in yeast two-hybrid control crosses. The association was confirmed by co-immunoprecipitation analyses of the co-expressed VP1 protein and carboxy-terminal domain of eIF4AII. eIF4A plays an essential role in the initiation of translation of both capped and uncapped mRNAs. Its association with IBDV VP1 or, rather, with viral mRNA-linked VPg points to an important function of this viral protein in IBDV mRNA translation. An interaction between VP1 and full-length eIF4AII was, however, not observed. In view of the known two-domain structure of the eIF4AII protein it is conceivable that the interaction of VP1 with the full-length molecule requires collaborating proteins that open up its structure and expose the VP1-binding site in the carboxy-terminal domain. The biological relevance of the potential VPg-eIF4AII interaction is discussed.

INTRODUCTION

Infectious bursal disease virus (IBDV) is the causative agent of a highly contagious disease of young chickens that causes significant losses to the poultry industry. In the infected animal, IBDV targets the developing B lymphocytes in the bursa of Fabricius, in which it multiplies rapidly, leading to immune suppression. This condition increases the animal's susceptibility to opportunistic infections with other pathogens and reduces the growth rate of surviving animals (Kibenge *et al.*, 1988).

IBDV belongs to the genus *Avibirnavirus* of the family *Birnaviridae* (Dobos *et al.*, 1979). The virus consists of a non-enveloped capsid, 60 nm in diameter, that contains two segments of double-stranded RNA (dsRNA) of 3.3 kb (segment A) and 2.9 kb (segment B). Segment A contains two partially overlapping open reading frames (ORFs). The smaller ORF, located at the 5' end, encodes VP5. This 17 kDa protein, detected only in IBDV-infected cells (Mundt *et al.*, 1995), is not required for virus replication but plays a role in viral pathogenesis (Mundt *et al.*, 1995; Mundt *et al.*, 1997). The large ORF encodes a 110 kDa polyprotein precursor that is

autocatalytically cleaved to produce pVP2 (48 kDa), VP3 (32 kDa), and VP4 (28 kDa). VP4 is the protease responsible for the processing of the polyprotein. In mature virions, pVP2 is further processed into VP2 (40 kDa) (Da Costa *et al*., 2002). VP2 and VP3 are the structural proteins that constitute the viral capsid.

Segment B contains one ORF which encodes VP1, the putative RNA-dependent RNA polymerase (RdRp). This 90 kDa protein is responsible for transcription (plus-strand or mRNA synthesis) and replication (minus-strand synthesis) (Spies *et al*., 1987). Moreover, it interacts with VP3, an association likely to be essential for IBDV particle morphogenesis (Lombardo *et al*., 1999; Tacken *et al*., 2000; Tacken *et al*., 2002). VP1 is present within virions both as a free polypeptide and as a genome-linked protein, called VPg, attached to the 5' end of the positive strands of both genomic segments (Muller & Nitschke, 1987). RNA polymerase activity can be demonstrated in purified virions without any pretreatment (Spies *et al*., 1987) indicating that, like with reovirus (Skehel & Joklik, 1969), transcription can be initiated without the need for uncoating. *In vitro*, transcription by the virion RdRp is primed by VP1 and proceeds via an asymmetric, semiconservative strand-displacement mechanism (Spies, *et al*., 1987). The initiation of viral mRNA synthesis may therefore involve either two VP1 molecules, one serving as a primer and the other as a polymerase for chain elongation, or just a single VP1 molecule performing both these functions (Dobos, 1995). The newly synthesized viral mRNAs contain a covalently linked VP1 at their 5' end and, similar again to reovirus mRNAs, lack a poly(A) tail at their 3' end. The IBDV VP1 protein is unusually large compared to other VPg's (e.g. adeno- and picornavirus) and its function as primer as well as polymerase is without precedent.

Viruses are obligatory intracellular parasites. For their multiplication they are essentially dependent on components and machineries provided by the host cell. Interactions between viral and host cell proteins occur at all stages of the infection process. Viruses recognize their target cell through interaction with specific receptors and/or other components on the cell membrane resulting in virion internalization. Gene expression of most DNA viruses is mediated by cellular polymerases and regulated largely by cellular transcription factors. Most RNA viruses replicate and transcribe their genomes by RNA-dependent RNA synthesis, a process foreign to eukaryotic cells. Therefore, many of the factors that are normal components of cellular RNA translation are subverted to play an integral or regulatory role in the replication and transcription of viral RNA. These replication complexes often include transient or long-lived interactions with host proteins for structural purposes or to recruit regulatory and catalytic functions. It is now well established that coupling of the different, sequential steps of virus replication is

central to the overall infectious cycle of many RNA viruses. Identification of cellular interaction partners of viral proteins is therefore likely to provide a mre complete understanding of the dynamics of RNA replication, virus-mediated cellular modulation and host-range restriction.

Recently, we analysed the intermolecular interactions between the IBDV proteins using the yeast two-hybrid system (Tacken *et al.*, 2000; Tacken *et al.*, 2003). In the present study, we aimed at identifying potential cellular partners for the viral RdRp and genome-linked protein, VP1. To this end we applied the inducible LexA-dependent yeast two-hybrid system to screen a cDNA library from bursae of 3-weeks-old chickens using VP1 as a bait. The LexA-based interaction trap offers inducible expression of the library fusion proteins, which allows less opportunity for the foreign fusion proteins to exhibit a toxic effect on the yeast host and thus to be eliminated from the pool of potentially interacting proteins. The screening allowed the isolation of eight candidate cDNA clones coding for the carboxy-terminal domain of RNA-helicase translation initiation factor eIF4AII, a key component in the initiation of eukaryotic translation. Co-immunoprecipitation analyses confirmed the interaction between VP1 and the carboxy-terminal domain of eIF4AII *in vivo*.

MATERIAL AND METHODS

CELLS, VIRUSES, BACTERIAL AND YEAST STRAINS

QM5 cells (Antin & Ordahl, 1991) were cultured in QT35 medium (Gibco-BRL) supplemented with 5% fetal calf serum (FCS) and 2% antibiotic solution ABII (1,000 U of penicillin [Yamanouchi], 1 mg of streptomycin [Radiumfarma], 20 μg of amphotericin B [Fungizone], 500 μg of polymixin B, and 10 mg of kanamycin/ml) in a CO_2 (5%) incubator at 37°C. The classical IBDV isolate CEF94 is a derivate of PV1 which has been adapted for growth in cell cultures (Boot *et al.*, 1999). Recombinant fowlpox virus expressing the T7 polymerase gene (FPV-T7) (Britton *et al.*, 1996) was received from the laboratory of M. Skinner (Compton Laboratory, Berks, United Kingdom). *Escherichia coli* strain DH5-α (Life Technologies) was used for general DNA manipulation, and KC8 (Clontech) for rescuing prey plasmids from yeast cotransformants. Yeast (*Saccharomyces cerevisiae*) strain EGY48 (MATα, his3, trp1, ura3, $LexA_{op(x6)}$-*LEU2*) was used for the interaction trap. Yeast strain EGY[p8oplacZ] is the host strain EGY48 transformed with the autonomously replicating reporter plasmid p8op-LacZ (Clontech).

ANTIBODIES

The rabbit anti-VP1 serum directed against amino acids 580-881 of VP1 of IBDV strain CEF94 has been described previously (Tacken *et al*., 2000). The polyclonal rabbit antiserum against eIF4A was a gift from of Dr. C. Kuhlemeier (University of Berne, Switserland) and monoclonal mouse anti-eIF4A antibody was a gift from Dr. H. Trachsel (University of Berne, Switserland). The c-myc monoclonal antibody was purchased from Clontech.

PLASMIDS AND cDNA LIBRARY

Plasmids pLexA$_{BD}$-VP1 and pB42$_{AD}$-VP1, both containing the cDNA-coding sequence of VP1 of IBDV strain CEF94, have been described previously (Tacken *et al*., 2000). Plasmid pB42$_{AD}$-c-eIF4AII(full-length) encoding the full-length cDNA sequence of chicken bursa-specific eIF4AII (Gen-Bank accession number AF515726) was used as the DNA template for preparing c-eIF4AII constructs and plasmids pTZ18R-4A and pTZ19-4AII (Scheper *et al*., 1992) encoding the full-length cDNA sequences of mouse eIF4AI and eIF4AII, respectively, for preparing m-eIF4AI and m-eIF4AII constructs. The full-length coding sequences of eIF4A were subcloned in-frame with the LexA DNA-binding domain in pLexA$_{BD}$ (Clontech), the B42-activation domain in pB42$_{AD}$ (Clontech), and the c-myc epitope tag in pGBKT7 (Clontech). The carboxy-terminal (C) domain coding sequences of eIF4A, c-eIF4AII(C-domain), m-eIF4AI(C-domain) and m-eIF4AII(C-domain), starting at Leu244 (Leu243 for m-eIF4AI), and the amino-terminal (N) domain coding sequences of eIF4A, c-eIF4AII(N-domain), m-eIF4AI(N-domain) and m-eIF4AII(N-domain), from Met1 to Thr243 (Thr242 for m-eIF4AI), as well as the carboxy-terminal 107aa (C-107aa) coding sequences of mouse eIF4A, m-eIF4AI(C-107aa) and m-eIF4AII(C-107aa), starting at Ser301 (Ser300 for m-eIF4AI), were obtained by PCR using appropriate primers on pB42$_{AD}$-c-eIF4AII(full-length), pTZ18R-4A and pTZ19-4AII, and then subcloned into pLexA$_{AD}$, pB42$_{AD}$ and pGBKT7. The subcloning of the pB42$_{AD}$-cDNA library fragments c-eIF4AII(C-107aa), c-eIF4AII(C-109aa) and c-eIF4AII(C-122aa) in the *Eco*RI site of pLexA$_{BD}$ and pGBKT7 gave plasmids pLexA$_{BD}$-c-eIF4AII(C-107aa), pLexA$_{BD}$-c-eIF4AII(C-109aa), pLexA$_{BD}$-c-eIF4AII(C-122aa), and pGBKT7-c-eIF4AII(C-107aa) and pGBKT7-c-eIF4AII(C-122aa). Each construct was sequenced to verify correctness of the sequences and fusions. The preparation of the plasmid pHB34Z which contains the full-length genomic cDNA of segment B of IBDV strain CEF94, has been described previously (Boot *et al*., 1999). Control plasmids pLexA$_{BD}$-53 (human p53 gene in pLexA$_{BD}$), pB42$_{AD}$-SV40 T (SV40 large T antigen in pB42$_{AD}$) and pLexA$_{BD}$–Lamin C (human Lamin C gene in pLexA$_{BD}$) were obtained from Clontech. Plasmids pLexA$_{BD}$-pVP2, pB42$_{AD}$-pVP2,

pLexA$_{BD}$-VP3 and pB42$_{AD}$-VP3 contain cDNA-coding sequences of pVP2 and VP3 of IBDV strain CEF94 (Tacken *et al*., 2003).

For the generation of the pB42$_{AD}$ cDNA plasmid library, total RNA was isolated from bursae of 3-weeks-old chickens in a one-step protocol with acidic guanidinium thiocyanate, phenol, and chloroform (Chomczynski & Sacchi, 1987) and used for poly(A)$^+$ RNA purification with Mini-Oligo(dT) Cellulose Spin Columns (5 Prime $\rightarrow$ 3 Prime, Inc.) according to the instructions. Five micrograms of poly(A)$^+$ RNA was used for cDNA synthesis by a custom cDNA library service (OriGene Technologies, Inc.). This library contained 4.8 x 10^6 independent clones.

TWO-HYBRID LIBRARY SCREENING

All yeast two-hybrid media, buffers, and protocols were as described in the Clontech Manual for the Matchmaker LexA two-hybrid system and in the Clontech Yeast Protocols Handbook. The yeast strain EGY48[p8op-lacZ] was first transformed by using the lithium acetate method with the pLexA$_{BD}$ (*HIS3*$^+$) construct carrying VP1. Stable expression of the LexA hybrid protein was verified by Western blot analysis according to the manufacturer's instruction. The ability of the LexA fusion to bind operator DNA, as well as the inability of the LexA fusion for autonomous reporter gene activation, was confirmed by a repression and an activation assay, respectively, as described previously (Tacken *et al*., 2000). The obtained yeast strain EGY48[p8op-LacZ] harbouring plexA$_{BD}$-VP1 was transformed according to the lithium acetate protocol with plasmid DNA from the pB42$_{AD}$-bursae-cDNA library. Double transformants were grown on SD/Glu/-His/-Ura/-Trp medium to select for the presence of both types of hybrid plasmids and the *LacZ* reporter plasmid. The primary transformant cells (2x10^6 CFU) were pooled and plated on SD/Gal/Raf/-His/-Ura/-Trp/-Leu medium to screen for transcriptional activation of the *LEU2* reporter gene. Yeast strain EG48[p8op-LacZ] transformed with the empty vector construct, pB42$_{AD}$-empty, was used as negative control, whereas yeast strain EG48[p8op-LacZ] transformed with pLexA$_{BD}$-53 and pB42$_{AD}$-SV 40 T was used as positive control. After 4 days of incubation at 30°C, Leu+ colonies were patched onto SD/Glu/X-gal/-His/-Ura/-Trp medium as well as SD/Gal/Raf/X-gal/-His/-Ura/-Trp medium to screen for transcriptional activation of the *LacZ* reporter gene. Colonies were tentatively considered positive if they were blue on the Gal/Raf containing plates but not blue or only faintly blue on the Glu containing plates. Colonies of the LacZ+ clones were restreaked, at least once, onto SD/Glu/-His/-Ura/-Trp plates to allow possible segregation of multiple cDNA library plasmids within single colonies, and were retested for both Leucine autotrophy and β-galactosidase activity. Positive clones were analysed by sequencing. For this purpose, yeast cells of a single colony were

resuspended in 40 µl H_2O and broken by a heat-treatment of 5 min at 100°C followed by a vortex-treatment for 1 min in the presence of glass beads. Cell debris was removed by centrifugation and 4 µl aliquots of the supernatants were used as template for PCR amplification of the bursa derived cDNA inserts in $pB42_{AD}$ using the $pB42_{AD}$ specific primers 5' CCAGCCTCTTGCTGAGTGGAGATG 3' (upstream) and 5' GTAGACAAGCCGACAACCTTGATTGG 3' (downstream). PCR products were purified using a PCR purification kit (Qiagen) and analysed by sequencing.

Yeast clones harbouring plasmids encoding candidate interacting proteins were grown in SD/Glu/-Trp medium and library plasmids were isolated using Method 1 (described by Clontech in the Yeast Protocols Handbook) and transformed into electrocompetent *Escherichia coli* KC8. Bacterial transformants were selected on M9 minimal medium (Clontech Yeast Protocols Handbook). Library plasmids were repurified from bacterial transformants and used to retransform EGY[p8op-lacZ] yeast cells together with control plasmids ($pLexA_{BD}$-Lamin C, $pLexA_{BD}$-pVP2 and $pLexA_{BD}$-VP3) or for subcloning the cDNA library insert in $pLexA_{BD}$ and pGKT7.

DNA SEQUENCING AND SEQUENCE ANALYSIS

Sequence analysis of cDNAs was performed by cycle sequencing (BigDye terminator kit, PE Applied Biosystems) with an ABI 310 sequencer (PE Applied Biosystems). Sequence similarity searches of the databases were conducted by using the default settings of the BLAST program (Altschul *et al.*, 1990) on the National Center for Biotechnology Information World Wide Web server (http://www.ncbi.nlm.nih.gov/BLAST/).

TRANSFECTION OF QM5 CELLS

QM5 cells were grown to 80% confluency in 60-mm dishes and infected with FPV-T7 (multiplicity of infection [MOI] = 3). After 1 h, the cells were washed once with 5 ml QT35 medium and covered with 5 ml of Optimem 1 (Gibco-BRL). In the meantime, 2.0 µg of DNA was mixed with 25 µl Lipofectamine (Gibco-BRL) in 0.5 ml Optimem 1 and kept at room temperature for at least 30 min. The QM5 cells were subsequently covered with 4 ml of fresh Optimem 1, and the DNA-Lipofectamine mixture was added. The transfection was performed overnight (18 h) in a 37°C incubator (5.0% CO_2). The transfected monolayer was rinsed once with QT35 medium and fresh QT35 medium supplemented with 5% FCS and 2% ABII was added. The plates were incubated for another 24 h.

RADIOLABELING OF TRANSFECTED CELLS AND IMMUNOPRECIPITATION

At 48 h post-transfection, cells were starved for 1 h in methionine-free EMEM medium (Gibco-BRL). Cells were then labeled for 3 h with 20 μCi/ml of [^{35}S]methionine (Amersham) in methionine-free EMEM medium. At the end of the labeling, the cell cultures were lysed on ice in 1x PBS-TDS lysis buffer, using a 5x PBS-TDS lysis buffer stock solution (5% Triton X-100, 2.5% sodium deoxycholate, 0.5% sodium dodecyl sulfate (SDS), 0.7 M NaCl, 14 mM KCl, 50 mM Na_2HPO_4, 7.5 mM KH_2PO_4). Cell debris was removed by centrifugation at 4°C for 20 min at 13,000 × *g*. All lysates were pretreated with Protein A Sepharose (Amersham) before they were used for immunoprecipitation with polyclonal anti-VP1 serum or monoclonal anti-c-myc serum. Protein A Sepharose-bound immune complexes were washed three times in 1x PBS-TDS lysis buffer and eluted in 30 μl SDS sample buffer (60 mM Tris-HCl [pH 6.8], 2.5% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.1% bromophenol blue). Proteins were resolved in 18% separating gels by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by autoradiography.

IBDV INFECTION AND IMMUNOBLOTTING

Monolayer cultures of 80% confluency of QM5 cells were infected with IBDV strain CEF94 (MOI = 5), or mock-infected. After 1 h of virus absorption, QT35-medium (1% serum) was added to the cells, and incubation was continued until the indicated times (time zero was the time when virus was added to the cells). Where appropriate, 15 min prior to harvesting, cells were washed 3 times with methionine-free EMEM medium (Gibco-BRL) and subsequently labeled for 15 min with 20 μCi/ml of [^{35}S]methionine (Amersham) in methionine-free EMEM medium (Gibco-BRL). Cell extracts were prepared on ice with 1x PBS-TDS lysis buffer. Labeled proteins were subjected to SDS-PAGE (12%) and visualized by autoradiography. Alternatively, unlabeled samples were transferred onto Immobilon-P (Millipore) and probed with rabbit antibodies specific for eIF4A (1:3,000). Thirty ng of purified rabbit eIF4AI (Scheper *et al.*, 1992) was used as a positive control for the immunoblotting. Detection on X-ray film was achieved by using peroxidase-linked goat anti-rabbit immunoglobulins (Ig) (1:3,000; DAKO) and chemiluminescent reagents (Pierce).

RESULTS

VP1 INTERACTS WITH THE C-TERMINAL DOMAIN OF eIF4AII

To investigate the possible interaction of VP1 with cellular proteins, the LexA-dependent yeast two-hybrid interaction assay was used. To this end, the VP1 gene

was subcloned into the yeast expression vector pLexA$_{BD}$. The generated pLexA$_{BD}$–VP1 fusion plasmid was introduced into yeast cells carrying two reporter genes, i.e. *LEU2* integrated in the genome, and *LacZ* located on the p8op-LacZ reporter plasmid. Expression of the VP1-fusion protein was verified by Western blot analysis (data not shown). Activation and repression assays (Golemis *et al.*, 1996) confirmed that the fusion protein by itself did not activate the reporter genes and that it was localized to the nucleus (data not shown).

A yeast two-hybrid cDNA library in pB42$_{AD}$, constructed from poly(A)+ RNA from bursae of 3-weeks-old chickens, was screened with pLexA$_{BD}$–VP1 used as a bait. Plasmids from positive clones were isolated and the inserts were sequenced in both directions. The identity of the respective genes was determined by BLAST analysis. Of the eleven putative VP1 binding proteins that were found more than twice, three were listed by the "Interaction Trap Table of False Positives" (http://www.fccc.edu/research/labs/golemis/main_false.html) as notoriously non-specific interactors, and five were classified as proteins of unknown function, i.e. having no known functional or structural homologues. Other interacting proteins were RAN binding protein 1 (RanBP1) and the putative cyclin G1 interacting protein [accession no: NP_006340]. However, the clone that was frequently recovered (eight isolates) and, in addition, exhibited a more robust β-galactosidase activity, corresponded to part of the translational eukaryotic initiation factor 4AII (eIF4AII) and would thus be the chicken representative of this initiation factor. For this reason we focused our attention on the study of the interaction between VP1 and eIF4AII. An additional consideration was the recent demonstration of another interaction of a viral RdRp, the NS5B protein of hepatitis C virus, with (human) eIF4AII (Kyono *et al.*, 2002).

The eight recovered eIF4AII-homologous cDNAs ranged in size from 983 to 1028 nt. They all mapped to the 3' end of the eIF4AII gene sequence, five clones encoding 107 aa, one clone encoding 109 aa and two clones encoding 122 aa of the eIF4AII carboxy-terminus (Fig. 1; indicated by asterisks). All cDNAs had the same 662 nt-3' UTR in which two potential polyadenylation signals occur. The eight cDNAs were derived from at least two different mRNA populations since a comparison of their sequences showed the occurrence of two nucleotide differences in the coding region neither of which, however, affected the predicted amino acid sequence of eIF4AII. As the actual N-terminus of the chicken eIF4AII was unknown we isolated and sequenced the full-length cDNA clone of this protein from our bursae library. A 1845 nt cDNA sequence was obtained for the chicken eIF4AII gene (c-eIF4AII; Gen-Bank accession number AF515726). Its coding sequence of 1221 nt

predicts a 407 aa protein. The 3'terminal part of the gene corresponding to the aa sequence that was common to all eight VP1-interacting eIF4AII-cDNAs showed

```
c-eIF4AII  1    MSGGSADYSRDHGGPEGMEPDGVIESNWNEIVDNFDDMNLKESLLRGIYA
m-eIF4AII  1    --------N-E-------D------------------------------
m-eIF4AI   1    --ASQDSR---N•--D----E------------S------S---------

c-eIF4AII  51   YGFEKPSAIQQRAIIPCIKGYDVIAQAQSGTGKTATFAISILQQLEIDLK
m-eIF4AII  51   ------------------------------------------------EF-
m-eIF4AI   50   --------------L-----------------------------I-----

c-eIF4AII  101  ETQALVLAPTRELAQQIQKVILALGDYMGATCHACIGGTNVRNEMQKLQA
m-eIF4AII  101  --------------------------------------------------
m-eIF4AI   100  A-------------------VM--------S-----------A-V----M

c-eIF4AII  151  EAPHIVVGTPGRVFDMLNRRYLSPKWIKMFVLDEADEMLSRGFKDQIYEI
m-eIF4AII  151  --------------------------------------------------
m-eIF4AI   150  -----I-------------------Y----------------------D-

c-eIF4AII  201  FQKLSTNIQVVLLSATMPMDVLEVTKKFMREPIRILVKKEELTLEGIKQF
m-eIF4AII  201  ------S----------T----------D------------------
m-eIF4AI   200  -----S-T----------S----------D------------------
                                                122          109
c-eIF4AII  251  YINVEREEWKLDTLCDLYETLTITQAVIFLNTRRKVDWLTEKMHARDFTV
m-eIF4AII  251  --------------------------------------------------
m-eIF4AI   250  --------------------------------------------------
                107
c-eIF4AII  301  SALHGDMDQKERDVIMREFRSGSSRVLITTDLLARGIDVQQVSLVINYDL
m-eIF4AII  301  --------------------------------------------------
m-eIF4AI   300  --M-----------------------------------------------

c-eIF4AII  351  PTNRENYIHRIGRGGRFGRKGVAINFVTEEDKRILRDIETFYNTTVEEMP
m-eIF4AII  351  --------------------------------------------------
m-eIF4AI   350  ------------------------M-------T----------SI----

c-eIF4AII  401  MNVALDI
m-eIF4AII  401  -------
m-eIF4AI   400  L------
```

Figure 1. Comparison of the amino acid sequence for chicken eIF4AII (c-eIF4AII) predicted from full-length cDNA, mouse eIF4AII (m-eIF4AII) and mouse eIF4AI (m-eIF4AI). Amino acid sequences were aligned using MSA Version 2.1 provided on the World Wide Web server. Dashes indicate amino acids identical to that of the chicken eIF4AII. The dot indicates a gap inserted by the alignment program. The asterisks mark the start of the the amino acid sequences of the VP1-interacting bursae library clones pB42AD-eIF4AII(C-122aa), pB42AD-eIF4AII(C-109aa) and pB42AD-eIF4AII(C-107aa). Bold letters indicate the start of the carboxyl-terminal domain of the "dumbbell" structure of eIF4A according to the published crystal structure of yeast eIF4A (Caruthers *et al.*, 2000). Conserved motifs of eIF4A are boxed. The accession numbers are as follows: c-eIF4AII (AF515726), m-eIF4AII (S00985) and m-eIF4AI (S00986).

complete sequence identity. Some differences were, however, observed in its 612 nt-3' UTR, which lacked a stretch of 53 nucleotides, showed one transition (T to C), and contained an additional three nucleotides (CTT) at the extreme 3' end (Fig. 2). The deduced amino acid sequence of chicken eIF4AII was compared to that of eIF4AII and eIF4AI from mouse (Fig. 1). The comparison indicates that the chicken protein is more similar to mouse eIF4AII (98% identity) than to mouse eIF4AI (91% identity). Both isoforms, eIF4AI and eIF4AII, are involved in translation initiation and are functionally interchangeable (Yoder-Hill *et al*., 1993). More recently, a third member of the eIF4A family, eIF4AIII, has been identified but this protein is more distantly related and its involvement in translation initiation has been questioned in view of its predominant nuclear occurrence (Holzmann *et al*., 2000; Li *et al*., 1999).

3'-UTR

```
eIF4AII(full-length)    1       TAATCCCTGGAGAGGAGATGGTTTGAATGCAGTGCTCGCTGTTGCTGAATAGGCGATTCA
eIF4AII(C-122aa)        1       ------------------------------------------------------------

eIF4AII(full-length)    61      CTTGCATTGTGCTTCTTTCTTTGGGGATATATTGAATCTTGTCTCAATGCTCATAACGGA
eIF4AII(C-122aa)        61      ------------------------------------------------------------

eIF4AII(full-length)    121     TCAGAAATACAGATTTTTGATAAGCGAAGCGACTTTTTGTCGTGAGCTCTTGTGGGGAAA
eIF4AII(C-122aa)        121     ------------------------------------------------------------

eIF4AII(full-length)    181     GCCATTGGCTTTATCCACTTTAGGGTTAGA****************************
eIF4AII(C-122aa)        181     ----------------------------CTGTTGGGGTTGGGTGGAAAGTCATGGGGT

eIF4AII(full-length)    211     **********************AATTTATTTCCTAGTTCCATAGAAGTGGTTGTATTAG
eIF4AII(C-122aa)        241     CTGTAAATTTTTTCTTTATTAGA-----------------------------------

eIF4AII(full-length)    248     ATGTTCTTTATCATTTAATAATTTACTTATGGACTAAAAGATATAAGTGCTGTATAAAAT
eIF4AII(C-122aa)        301     ------------------------------------------------------------

eIF4AII(full-length)    308     CAGCCAATTATGTTAAACTAGCCTACCTTCCTTTATTGTATGTACTTACCCTTCAGATTG
eIF4AII(C-122aa)        361     ------------------------------------------------------------

eIF4AII(full-length)    368     AATTGGAAAGGCCTTTCAAAATCTCAAAACTTTTATAAGAGCATTAAAATGCATTTTCGT
eIF4AII(C-122aa)        421     ------------------------------------------------------------T--

eIF4AII(full-length)    428     TTGATATGTATTTATTCAATAAAGTATTTAATTAGTGGTAAGTGTGATCTGGACCCTGTT
eIF4AII(C-122aa)        481     ------------------------------------------------------------

eIF4AII(full-length)    488     GCTAAGCCCCAGCAAGCAAGCAATCATACTATTGTCTTAGTTAGGGTTAAACCCAGTAAA
eIF4AII(C-122aa)        541     ------------------------------------------------------------

eIF4AII(full-length)    548     ATTTGCCATATTGCACATGTCTTAATGAAGTTTGAATGTTCAATAAAATACTTCTATATT
eIF4AII(C-122aa)        601     ------------------------------------------------------------

eIF4AII(full-length)    608     CACTTAAA
eIF4AII(C-122aa)        661     --***AAA
                                     Δ
```

Figure 2. Sequence alignment of the 3' UTRs of chicken bursa eIF4AII full-length cDNA and the VP1-interacting chicken bursa eIF4AII(C-122aa) cDNA. The stop codon is given in bold letters. Dashes indicate identical residues and asterisks mark missing nucleotides. The polyadenylation signals are underlined and the position of poly(A) addition for both cDNAs is indicated by an open triangle beneath the sequence.

VP1 INTERACTS ONLY WITH THE C-TERMINUS OF eIF4AII IN THE YEAST TWO-HYBRID SYSTEM

In order to check the specificity of our observed VP1-eIF4AII interaction, the VP1-interacting eIF4AII library clones were retransformed back to yeast either alone or in combination with VP1, with the binding domain vector alone, or with different proteins unrelated to VP1: pVP2 and VP3 of IBDV, and Lamin C.

In addition, full-length eIF4AII, termed c-eIF4AII(full-length), was assayed in the yeast two-hybrid system in the same way as the eIF4AII library clones, termed c-eIF4AII(C-107aa), c-eIF4AII(C-109aa) and c-eIF4AII(C-122aa). All transformants failed to induce the reporter genes except for the combination of the three eIF4AII library clones with VP1 (Table 1). A similar result, although with a weaker interaction strength, was obtained for the reciprocal combinations of bait and prey fusion-proteins (Table 1).

These results demonstrate that eIF4AII(C-107aa), eIF4AII(C-109aa) and eIF4AII(C-122aa) are true positive VP1 interaction partners in the two-hybrid system. They also show that full-length eIF4AII did not interact with VP1 in this assay.

CHARACTERIZATION OF THE VP1-INTERACTING DOMAINS OF eIF4AI AND eIF4AII

The crystal structure of eIF4A has been reported as a "dumbbell" structure consisting of two compact domains connected by an extended linker (Caruthers *et al*., 2000). Since the VP1-interacting eIF4AII library clones encode almost the complete carboxyl-terminal domain of this "dumbbell" structure (Fig.1) it was of interest to know whether the interaction is indeed limited to this particular domain. To this end, the carboxyl-terminal domain (Fig. 1, c-eIF4AII residues 244-407) and the amino-terminal domain (Fig. 1, c-eIF4AII residues 1-243) of the eIF4AII–"dumbbell" structure were independently assayed for interaction with VP1 in the two-hybrid system.

The assay confirmed that the carboxyl-terminal domain of eIF4AII, termed eIF4AII(C-domain), but not its amino-terminal domain, termed eIF4AII(N-domain), was able to interact with VP1. It also appeared that the interaction of eIF4AII(C-domain) with VP1 was weaker than that of the shorter C-terminal domains of the eIF4AII library clones (Table 1). The reciprocal combination of the respective bait and prey fusion-proteins gave the same result, although again, as was observed with the eIF4AII library clones, the interaction strength of this combination was weaker.

We also investigated whether the interaction of eIF4AII with VP1 is isoform-II specific. To this end the two isoforms eIF4AI and eIF4AII of mouse (m-eIF4AI and m-eIF4AII) had to be used because the existence and hence the sequence of a putative chicken eIF4AI is unknown. Along with these constructs we additionally

$LexA_{BD}$ fusion	$B42_{AD}$ fusion	LEU[a]	LacZ[b]
VP1	c-eIF4AII(full-length)	–	–
VP1	c-eIF4AII(C-107aa)	+++	++
VP1	c-eIF4AII(C-109aa)	+++	++
VP1	c-eIF4AII(C-122aa)	+++	++
VP1	c-eIF4AII(C-domain[c])	+	+
VP1	c-eIF4AII(N-domain[d])	–	–
c-eIF4AII(full-length)	VP1	–	–
c-eIF4AII(C-107aa)	VP1	+	+/–
c-eIF4AII(C-109aa)	VP1	+	+/–
c-eIF4AII(C-122aa)	VP1	+	+/–
c-eIF4AII(C-domain)	VP1	+/–	+/–
c-eIF4AII(N-domain)	VP1	–	–
VP1	m-eIF4AII(full-length)	–	–
VP1	m-eIF4AII(C-107aa)	+++	++
VP1	m-eIF4AII(C-domain)	+	+
VP1	m-eIF4AII(N-domain)	–	–
m-eIF4AII(full-length)	VP1	–	–
m-eIF4AII(C-107aa)	VP1	+	+/–
m-eIF4AII(C-domain)	VP1	+/–	+/–
m-eIF4AII(N-domain)	VP1	–	–
VP1	m-eIF4AI(full-length)	–	–
VP1	m-eIF4AI(C-107aa)	+	+
VP1	m-eIF4AI(C-domain)	+/–	–
VP1	m-eIF4AI(N-domain)	–	–
m-eIF4AI (full-length)	VP1	–	–
m-eIF4AI(C-107aa)	VP1	–	–
m-eIF4AI(C-domain)	VP1	–	–
m-eIF4AI(N-domain)	VP1	–	–

Table 1. Interactions between IBDV VP1 and different lengths of chicken bursa-specific eIF4AII (ceIF4AII), mouse eIF4AII (meIF4AII) and mouse eIF4AI (meIF4AI) in the Yeast Two-Hybrid System

[a] Interactions were assayed for leucine autotrophy (LEU): +++; clear growth (strong interaction); +, growth (interaction); +/–, limited growth (weak interaction); –, no growth (no interaction). All controls, including the fusion-proteins by themselves or in combination with Lamin C, or the viral proteins pVP2 and VP3, remained negative.

[b] Interactions were assayed for β-galactosidase activity (LacZ): ++; deep-blue colonies (strong interaction); +, blue colonies (interaction); +/–, light-blue colonies (weak interaction); –, white colonies (no interaction). All controls, including the fusion-proteins

by themselves or in combination with Lamin C, or the viral proteins pVP2 and VP3, remained negative.

[c,d] C-domain and N-domain encodes the carboxyl- and amino-terminal domain of the "dumbbell" structure of eIF4AII (Caruthers *et al.*, 2000).

tested the N- and C-terminal domains of both these mouse-specific eIF4A isoforms, as well as a truncated form encoding 107 aa of the C-terminus resembling the shortest bursa-specific eIF4AII library clone. It is of interest to note that the amino acid sequence of m-eIF4AII(C-107aa) is 100% identical to that of c-eIF4AII(C-107aa) (Fig. 1). The results showed that for both mouse-specific eIF4A isoforms I and II the 107 aa polypeptide bound to VP1, although the strength of the interaction with the isoform I polypeptide was weaker. In the reciprocal combination this truncated protein, m-eIF4AI(C-107aa), even failed to activate *LEU* or *LacZ* reporter activity. Consistently, also the C-terminal domains of both m-eIF4AII and m-eIF4AI were able to interact with VP1, although the isoform I interaction appeared to be very weak as only one of the two reporters was sensitive enough to allow detection of this interaction. Moreover, the C-domain of m-eIF4AI failed to interact with VP1 in its reciprocal combination of bait and prey. Finally, the N-terminal domain as well as the full-length form both of m-eIF4AI and of m-eIF4AII, were unable to interact with VP1. All these results are similar to those found for the chicken-specific eIF4AII(N-domain) and eIF4AII(full-length).

Altogether, these results indicate that the carboxy-terminal domain of both eIF4A isoform I and II is able to interact with VP1, but that isoform II has the strongest interaction.

VP1-eIF4AII(C-TERMINUS) INTERACTION IS NOT DEPENDENT ON YEAST-SPECIFIC FACTORS

Next we wanted to confirm the specificity of the interaction of eIF4AII(C-terminus) with VP1 in an independent manner. With this aim we co-expressed the VP1 protein with various forms of the eIF4A proteins in avian QM5 cells using a transient fowlpox virus expression system, and analysed association of the proteins by radio-immunoprecipitation (RIP) assay. For this, cDNA of chicken eIF4AII and of mouse eIF4AI and eIF4AII, as well as derivates thereof (i.e. fragments encoding the polypeptides C-107aa, C-domain and N-domain) were cloned as a fusion protein with a c-myc epitope tag in a transcription plasmid behind a T7 RNA polymerase promoter. We fused the c-myc tag (N-terminally) to eIF4A because no antibody against eIF4A(C-terminus) was available. For the expression of VP1 we used plasmid

pHB34Z, in which the full-length genomic cDNA of segment B of IBDV is cloned between a T7 RNA polymerase promoter and the autocatalytic hepatitis delta virus ribozyme sequence (Boot *et al*., 1999). The eIF4A encoding plasmids were co-transfected with plasmid pHB34Z into QM5 cells that were previously infected with a recombinant fowlpoxvirus expressing T7 RNA polymerase (Britton *et al*., 1996). Forty-eight hours post-transfection, cells were metabolically labeled for 3 h with [^{35}S]methionine, cell lysates were prepared and subjected to immunoprecipitation with either anti-VP1 or anti-c-myc serum. The immunoprecipitates obtained were analysed by SDS-PAGE and the labeled proteins visualized by autoradiography. Mock-transfected cells and cells transfected with either pHB34Z or the eIF4A-encoding plasmids alone were used as controls for each immunoprecipitation.

Both antibodies were found to precipitate only their cognate protein, indicating that they did not exhibit cross-reactivity (Fig. 3A, lanes 13-15 and Fig. 3B, lanes 6-8; data of eIF4A-derivatives not shown except for c-eIF4AII(C-107aa)). Using the anti VP1-serum, comparable amounts of VP1 were detected in the different transfected QM5 cells (Fig. 3A, lanes 1-12). Full-length c-eIF4AII, m-eIF4AI and m-eIF4AII each failed to be co-precipitated with VP1 (Fig. 3A, lanes 1, 5 and 9). In contrast, all C-107aa constructs were co-precipitated with VP1, indicating that these polypeptides were interacting with VP1 (Fig. 3A, lanes 2, 6 and 11). Notably, the binding of m-eIF4AI(C-107aa) to VP1 (Fig. 3A, lane 6) appeared to be weaker than that of m-eIF4AII(C-107aa) (Fig. 3A, lane 11). Of the C-domain derivatives, m-eIF4AI failed to be co-precipitated (Fig. 3A, lane 7), whereas both c-eIF4AII and m-eIF4AII were co-precipitated though in much lower quantities than their C-107aa derivative (Fig. 3A, lane 3 and 10). No co-precipitation with VP1 was observed of any of the N-domain derivatives (Fig. 3A, lanes 4, 8 and 12). Using the anti-c-myc antiserum for the reciprocal immunoprecipitations, all tagged eIF4A-derivatives were clearly precipitated. However, in neither case could any co-precipitating VP1 be detected (Fig. 3B, lanes 1-4; data of the m-eIF4A(-derivatives) not shown). This was equally the case when we tested the derivative c-eIF4AII(C-122aa) for interaction with VP1 (Fig. 3B, lane 5).

eIF4A IS NOT CLEAVED IN IBDV-INFECTED CELLS

The observation that only the carboxy-terminal domain of eIF4A, not the full-size protein was able to interact with VP1 prompted us to test the idea that in IBDV-infected cells the eIF4A protein might be proteolytically cleaved to generate a carboxy-terminal polypeptide. Such a process would not be without precedent as a similar cleavage has been demonstrated for eIF4AI in foot-and-mouth disease virus (FMDV)-infected cells (Li *et al*., 2001). Thus, we assessed the effect of IBDV infection

on host eIF4A by immunoblotting of cell extracts with a polyclonal antibody specific for eIF4A.

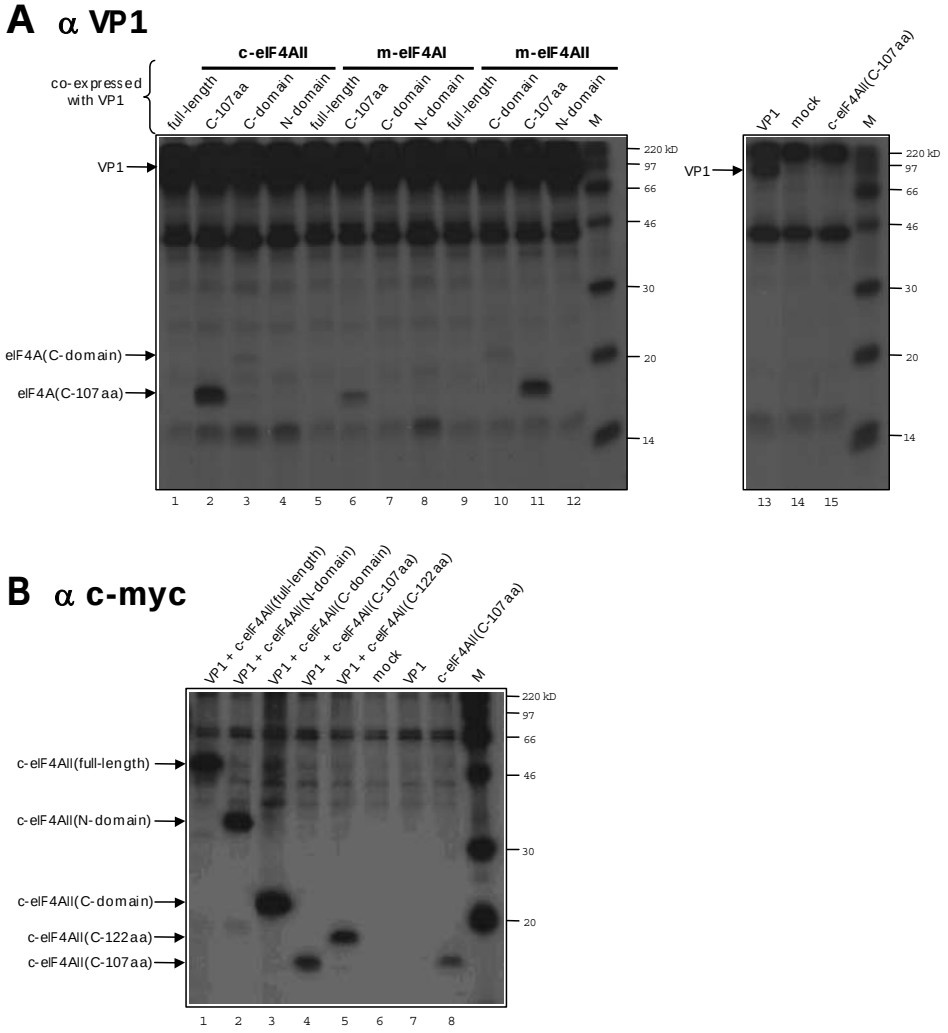

Figure 3. Radio-immunoprecipitation analysis of VP1-eIF4A interaction in transfected QM5 cells. QM5 cells were (co-)transfected with plasmids pHB34Z and/or pGBKT7-eIF4A(derivatives) expressing the indicated polypeptides. At 48 h post transfection cells were metabolically labeled for 3 h with [^{35}S]methionine. Subsequently, cells were lysed and immunoprecipitated with anti-VP1 serum (A) or with anti-c-myc serum (B) followed by SDS-PAGE (18% polyacrylamide) and autoradiography. Mock-transfected cells were used as a control for each immunoprecipitation. Positions of the precipitated polypeptides and sizes of marker proteins (M) are indicated.

First, host cell protein synthesis and production of virus-encoded proteins were monitored as a function of time during infection (Fig. 4A). QM5 cells were infected with IBDV and labelled with [^{35}S]-methionine for 15 min at various time points until 24 h post-infection (p.i.). As controls, mock-infected cells were similarly labelled. Cell extracts were prepared, proteins were electrophoresed in a polyacrylamide gel and the labelling pattern visualized by autoradiography. No shut-off of host cell polypeptide synthesis was observed in infected cells indicating that the IBDV infection generally did not interfere with host protein translation. Virus-specific protein synthesis was first detected at 4 h and was maximal between 6 and 12 h p.i. The cells started to show cytopathic effect (CPE) by about 12 h p.i. while at 24 h p.i., when the experiment was terminated, nearly all cells appeared to have succumbed. At this stage almost no protein synthesis was detected.

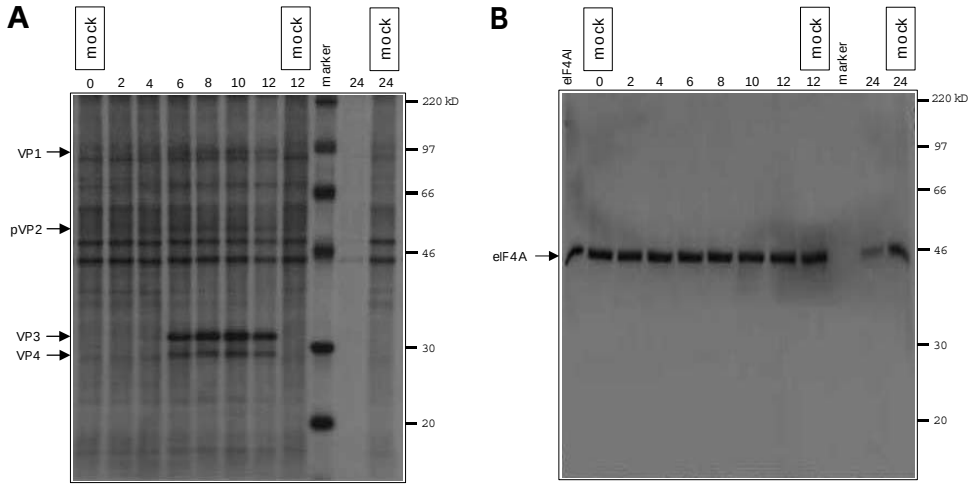

Figure 4. Analysis of host cell protein synthesis within IBDV-infected cells. QM5 cells were infected with IBDV strain CEF94. (A) Fifteen min prior to the indicated times (hours post-infection) cells were labeled for 15 min with [^{35}S]methionine and subsequently lysed. The cell extracts were analysed by SDS-PAGE (12% polyacrylamide) and autoradiography (A). Alternatively, cells infected in parallel with those analysed in panel A were harvested at the same times without metabolic labelling. Cell extracts were prepared and analysed by SDS-PAGE (12% polyacrylamide) and immunoblotting with polyclonal anti-eIF4A serum (B). Mock-infected QM5 cell extracts (lanes mock) were analysed in parallel at indicated times. Purified eIF4AI was used as a positive control for the immunoblotting (lane eIF4AI). Positions of viral proteins, eIF4A and molecular size markers are indicated.

The effect of IBDV infection on eIF4A was assessed by immunoblotting of the gel followed by probing of the blotted proteins with a specific anti-eIF4A serum (Fig. 4B).

Full-length eIF4A migrated at about 46 kD as a doublet, most likely representing the eIF4AI and eIF4AII isoforms, which were present in both infected and mock-infected cells. It was apparent that the infection with IBDV did not result in a specific cleavage of eIF4A nor in a general proteolytic degradation of the protein as no cleavage products nor a significant loss of full-length eIF4A was detected during this time course under these conditions. The decrease of the overall quantity of full-length eIF4A at 24 h p.i. was attributed to the severe CPE at this stage of infection.

DISCUSSION

It is becoming remarkably clear that the birnavirus VP1 protein is an impressively multifunctional viral component, exhibiting a range of activities throughout the viral life cycle. Firstly, as the RNA-dependent RNA polymerase, VP1 carries out all those diverse functions required for the replication and transcription of the binary dsRNA genome that occur within viral particles and in the cell's cytoplasm (Spies *et al*., 1987). Secondly, it acts as a primase in the generation of viral mRNAs, as a result becoming part of these mRNAs through covalent linkage at their 5' end (Magyar *et al*., 1998). Furthermore, by its interaction with the inner capsid component VP3 (Lombardo *et al*., 1999; Tacken *et al*., 2000; Tacken *et al*., 2002) the protein is also considered to be instrumental in the encapsidation of viral RNAs during particle morphogenesis. VP1 also associates with itself, but the significance of this interaction has not yet been established (Tacken *et al*., 2000). In the present study we focussed, for the first time, on interacting host cell partners of VP1 by using the yeast two-hybrid system. Our observation that VP1 specifically binds to the C-terminus of eukaryotic translation initiation factor eIF4AII suggests an involvement of this protein in translation initiation. This interaction therefore further extends the list of potential functions of this viral protein.

eIF4A is the prototypic member of the DEAD-box family of RNA helicases (Linder *et al*., 1989). Together with eIF4B it participates in the initiation of polypeptide synthesis by facilitating the melting of RNA secondary structures present in the 5' UTR of mRNAs required for the efficient recruitment of the 40S ribosomal subunit (reviewed in Gingras *et al*., 1999). eIF4A is a loosely bound subunit of the eIF4F complex, of which eIF4E (cap-binding protein) and eIF4G (scaffold component) are the other subunits. Eukaryotic cytoplasmic mRNAs contain a cap structure at their 5'-terminus. The cap structure is required for efficient attachment of the mRNA to the 40S ribosomal subunit. This attachment is mediated by eIF4F (through binding of eIF4E to the cap) and by ribosome-bound eIF3. eIF4A is an essential factor, required for both cap-dependent and cap-independent translation (Blum *et al*., 1992).

An interaction of VP1/VPg with eIF4AII is intriguing particularly because IBDV mRNAs are not capped but possess a covalently linked VPg at their 5' ends. A direct interaction of eIF4A with VPg may be a key event in the establishment of IBDV infection. It may reveal a novel cap-independent mechanism for the initiation of translation in which the binding of eIF4A to VPg mediates the recruitment of the 40S ribosomal subunit to the 5' end of the viral mRNA. Thus, while eIF4E is essential for initiation of translation of capped mRNAs, and proteolytically cleaved eIF4G for that of picornaviral uncapped mRNA (Lamphear *et al.*, 1995), eIF4A may have a similar function for IBDV mRNA.

For a subset of viral and cellular mRNAs, cap-independent initiation of translation is accomplished by internal binding of the ribosome at or upstream of the initiation codon mediated by an internal ribosomal entry site (IRES) in the 5' UTR (reviewed in Martinez-Salas *et al.*, 2001). The possibility of IRES-mediated cap-independent translation has not been investigated for IBDV, nor for other birnaviruses, but is not unlikely to occur. Although the nucleotide sequence of the 5' UTR of IBDV RNA is relatively short (segment A: 96 nt, segment B: 111 nt) compared to that of many viral IRES-containing mRNAs (about 200-500 nt), both segments have a theoretical potential to form Y-shaped or hairpin stem and loop secondary structures (Kibenge *et al.*, 1996; Mundt & Muller, 1995). These structures resemble those proposed to occur in some cellular IRESes (Le & Maizel, 1997). However, there is no convincing evidence supporting the idea that these cellular IRES structures are of significance for translation initiation. More interestingly, it was found that some mRNAs may recruit ribosomes by base pairing with ribosomal RNA (Chappell *et al.*, 2000; Owens *et al.*, 2001; Zhou *et al.*, 2001). Sequences as short as 9 nt have been shown to possess such IRES activity (Chappell *et al.*, 2000). These short IRES-modules have complementarity to the 18S rRNA occurring in 40S ribosomal subunits. A similar feature might be the case for IBDV mRNA. A highly conserved sequence of 13 nt in the 5' UTR of both segments, located exactly 19 nt upstream of the respective start codons for the nearest ORF (VP5 gene in segment A, VP1 gene in segment B), has complementarity to the chicken reticulocyte 18S rRNA and could thus function as a binding site for the 40S ribosomal subunit (Mundt & Muller, 1995). A tenable hypothesis is that an interaction of the C-terminus of eIF4A with VPg might be required to open mRNA secondary structure to allow ribosome binding.

The eIF4AII encoding cDNA clones that were identified in the yeast two-hybrid screen all corresponded to the carboxy-terminal domain of the protein. The specific interaction of VP1 with this particular eIF4AII domain was confirmed in various experiments, not only in the two-hybrid system but also in the co-immunoprecipitation assays. No association of VP1 was observed with full-length

eIF4AII, also not in IBDV-infected cells (data not shown) by different co-immunoprecipitation assays. We then investigated whether in infected cells an eIF4AII carboxy-terminal fragment is generated by some kind of cleavage, similar to what has been found for eIF4AI in FMDV-infected cells (Li *et al*., 2001). No breakdown of eIF4AII was, however, observed, the protein appearing as stable as in non-infected cells. Our interpretation of these results is that the formation of VPg-eIF4AII complexes might be conformation dependent and transient. In its free form the initiation factor does not expose its VP1 binding site. Only when engaging in the formation of a translation initiation complex this site becomes available as a result of conformational changes induced in the eIF4AII structure by its interaction with other partners involved in the assembly of the complex such as eIF4G or 3' end mRNA elements. In fact, various studies suggest that eIF4A does indeed adopt different conformations. (i) Mammalian eIF4A undergoes a cycle of ligand-dependent conformational changes as it binds its substrates, hydrolyzes ATP, and releases products (Lorsch & Herschlag, 1998*a*; Lorsch & Herschlag, 1998*b*). (ii) In its crystallized form, eIF4A has a 'dumbbell' structure in which the amino- and carboxyl-terminal domains occur as separately folded entities connected by an extended, 11-residue linker (Caruthers *et al*., 2000). In solution, the linker between the domains is relatively flexible, allowing eIF4A to adopt many different conformations (Caruthers *et al*., 2000). (iii) eIF4A is expected to interact only transiently with eIF4G, cycling in and out of the eIF4F complex, thereby changing its conformation (Pause *et al*., 1994). Altogether, these considerations support our interpretation, although we can of course not exclude that full-length eIF4A has an overall weak affinity for VP1.

The immunoprecipitation analyses demonstrated that co-expressed VP1 and c-myc-tagged carboxy-terminal eIF4AII polypeptides form stable complexes, particularly VP1/C-107aa complexes, that are efficiently immunoprecipitated with VP1 antibodies (Fig. 3A). Somewhat surprisingly, these complexes were not precipitable in the reciprocal assay using c-myc antibodies (Fig. 3B). The c-myc antibodies clearly recognized the tagged c-eIF4AII proteins but no VP1 was co-precipitated. It seems feasible that the binding of antibody to the tag induces a conformational change in the eIF4AII polypeptide that destabilizes its complex with VP1. The tag was fused to the amino-terminus of the c-eIF4AII polypeptides. We have not tested whether attachment to the carboxy-terminus of the polypeptides would have made a difference.

It is quite remarkable that our two-hybrid screening only yielded eIF4A-derivatives of isoform type-II as VP1-interacting proteins and that no clones of the functionally equivalent isoform type-I were isolated. eIF4AII strongly resembles eIF4AI (in mice the two isoforms have 91% identity) though its tissue-specific expression

(Nielsen & Trachsel, 1988) and its developmental regulation (Morgan & Sargent, 1997) differ from those of eIF4AI. While the carboxy-terminal polypeptides (C-107aa) of both the mouse-specific eIF4AI and eIF4AII isoforms were able to interact with VP1, the strength of the interaction with isoform type-I was consistently weaker. This suggests that VP1 preferentially associates with eIF4AII. Data indicate that this isoform is also held more strongly in the eIF4F complex than is eIF4AI (Conroy *et al.*, 1990).

The interaction of VP1 with the carboxy-terminal domain of eIF4AII reported here suggests yet another viral strategy on the protein translation battlefield. Future studies will be required to confirm and establish the functional significance of this interaction for viral multiplication, particularly whether VP1, when linked to viral RNA (i.e. as VPg) can indeed bind the initiation factor and support translation initiation of viral RNA, without compromising host mRNA translation. Another interesting question relates to the possible occurrence of 5'-3' interactions in the IBDV mRNAs and to the binding partners that mediate these interactions. These studies will undoubtedly reveal additional striking features of the biology of these fascinating viruses.

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CHAPTER 6

Homotypic interactions of the infectious bursal disease virus proteins VP3, pVP2, VP4 and VP5: mapping of the interacting domains

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CHAPTER 6 **HOMOLOGOUS INTERACTIONS OF IBDV PROTEINS**

ABSTRACT

Infectious bursal disease virus (IBDV), a non-enveloped double-stranded RNA virus of chicken, encodes five proteins. Of these, the RNA-dependent RNA polymerase (VP1) is specified by the smaller genome segment, while the large segment directs synthesis of a non-structural protein (VP5) and a structural protein precursor from which the capsid proteins pVP2 and VP3 as well as the viral protease VP4 are derived. Using the recently redefined processing sites of the precursor we have re-evaluated the homotypic interactions of the viral proteins using the yeast two-hybrid system. Except for VP1, which interacted weakly, all proteins appeared to self-associate strongly. Using a deletion mutagenesis approach we subsequently mapped the interacting domains in these polypeptides, where possible confirming the observations made in the two-hybrid system by performing co-immunoprecipitation analyses of tagged protein constructs co-expressed in avian culture cells. The results revealed that pVP2 possesses multiple interaction domains, consistent with available structural information about this external capsid protein. VP3-VP3 interactions were mapped to the amino-terminal part of the polypeptide. Interestingly, this domain is distinct from two other interaction domains occurring in this internal capsid protein: while binding to VP1 has been mapped to the carboxy-terminal end of the protein, interaction with the genomic dsRNA segments has been suggested to occur just upstream thereof. No interaction sites could be assigned to the VP4 protein; any deletion applied abolished its self-association. Finally, one interaction domain was detected in the central, most hydrophobic region of VP5, supporting the idea that this virulence determinant may function as a membrane pore-forming protein in infected cells.

INTRODUCTION

Infectious bursal disease (IBD), also known as Gumboro disease, is an acute contagious viral disease of young chickens that is of major importance for the poultry industry (Cheville, 1967). The IBD virus (IBDV) causes a severe immunosuppression by destroying B-lymphoid cells present in the bursa of Fabricius followed by bursal atrophy. The immunosuppression leads to an increased susceptibility to other pathogens and reduces the growth rate of surviving animals (Kibenge *et al.*, 1988). To date, two distinct serotypes (I and II) of IBDV have been identified (Jackwood *et al.*, 1982). The pathogenic serotype I viruses are isolated from chickens and vary in virulence. Serotype II viruses are nonpathogenic and occur in turkeys and chickens (Jackwood *et al.*, 1982; McFerran *et al.*, 1980).

IBDV belongs to the family *Birnaviridae* (Leong *et al.*, 2000). Members of the family contain a double-stranded RNA (dsRNA) genome consisting of two segments, designated A and B, within an unenveloped single-shelled icosahedral capsid of 60 nm in diameter (Dobos *et al.*, 1979; Muller *et al.*, 1979). The smaller segment B (approximately 2.9 kb) encodes viral protein 1 (VP1; 95 kDa), the putative RNA-dependent RNA polymerase (RdRp) (Bruenn, 1991; Macreadie & Azad, 1993; Spies *et al.*, 1987). This polypeptide is present in the virion both as a free protein and as a genome-linked protein, called VPg, attached to the 5' end of the positive strands of the two genomic segments (Dobos, 1993; Spies & Muller, 1990). Segment A (approximately 3.3 kb) contains two partially overlapping open reading frames (ORFs). The first, smaller ORF encodes nonstructural protein VP5, which is not essential for viral replication *in vitro* but important for virus-induced pathogenicity (Mundt *et al.*, 1997; Yao *et al.*, 1998). The larger ORF encodes a 110 kDa polyprotein that is autocatalytically cleaved, rendering three polypeptides: pVP2 (48 kDa), VP3 (32 kDa) and VP4 (28 kDa). VP4, a serine-lysine protease (Birghan *et al.*, 2000), is responsible for this self-processing (Lejal *et al.*, 2000; Sanchez & Rodriguez, 1999). It is a nonstructural protein mainly associated with type II tubules of 24 nm in diameter (Granzow *et al.*, 1997). pVP2 is further processed at its carboxy terminus to become VP2 (40 kDa) (Da Costa *et al.*, 2002; Lejal *et al.*, 2000). VP2 and VP3 are the structural proteins forming the outer and inner layers of the virion, respectively (Bottcher *et al.*, 1997; Caston *et al.*, 2001). Recently we showed that VP3 interacts not only with VP1 but also with the genomic dsRNA segments A and B, the former interaction being of crucial importance for IBDV replication (Tacken *et al.*, 2002).

Interactions among viral proteins and between viral and host proteins play a central role in the infection process. These interactions include transient as well as long-lived associations. Often, these interactions establish essential functional complexes responsible for processes such as viral genome replication, RNA transcription and translation, virus assembly and virus release. Identification of such protein-protein interactions is therefore of vital importance for a better understanding of the dynamics of viral multiplication.

We have recently been using the yeast two-hybrid system in various interaction studies of the IBDV proteins. Thus, we detected a number of candidate cellular interaction partners for the IBDV proteins VP1, pVP2, VP3 and VP5 (Tacken *et al.*, 2001). One of these was further explored revealing an interaction between VP1 and translational eukaryotic initiation factor 4AII (eIF4AII) (M.G.J. Tacken, A.A.M. Thomas, B.P.H. Peeters, P.J.M. Rottier and H.J. Boot, submitted). We also used this system to analyse the intermolecular interactions between the IBDV proteins themselves (Tacken *et al.*, 2000). Though the yeast assays did not allow us to detect

an interaction between the two capsid proteins pVP2 and VP3, an interaction between the latter and VP1, presumed to draw the polymerase into assembling viral particles, was clearly demonstrated. Whereas these studies also revealed homotypic interactions of the IBDV structural proteins, the recent redefinition o processing scheme of the structural protein precursor (Lejal *et al.*, 2000; Sanchez & Rodriguez, 1999) now casts some doubt on these observations. In the present study we have first re-evaluated our earlier findings based on the newly established processing sites. This confirmed the homotypic interactions found for pVP2, VP3, VP4 and VP5, which we now also demonstrated by co-immunoprecipitation of co-expressed tagged and untagged forms of the proteins. We subsequently carried out deletion studies to map the self-interacting domains in each of these proteins. It appears that the different proteins interact through different domains which for the VP3 protein identified yet another domain in addition to the ones shown recently to be involved in associating with VP1 and with genomic dsRNA of both segment A and B.

MATERIALS AND METHODS

CELL LINE, VIRUS, PLASMID, AND BACTERIAL AND YEAST STRAINS

QM5 cells (Antin & Ordahl, 1991) were cultured in QT35 medium (Gibco-BRL) supplemented with 5 % fetal calf serum (FCS) and 2% antibiotic solution ABII (1,000 U of penicillin [Yamanouchi], 1 mg of streptomycin [Radiumfarma], 20 μg of amphotericin B [Fungizone], 500 μg of polymixin B, and 10 mg of kanamycin/ml) in a CO_2 (5%) incubator at 37°C. Recombinant fowlpox virus expressing the T7 polymerase gene (FPV-T7) (Britton *et al.*, 1996) was received from the laboratory of M. Skinner (Compton Laboratory, Berks, United Kingdom). The preparation of the plasmid pHB36W, which contains the full-length genomic cDNA of segment A of IBDV strain CEF94, has been described (Boot *et al.*, 1999). *Escherichia coli* (*E. coli*) strain DH5-α (Life Technologies) was used during DNA manipulations. Yeast strain *Saccharomyces cerevisiae* (*S. cerevisiae*) EGY48 (MATα, his3, trp1, ura3, $LexA_{op(x6)}$-*LEU2*) was used for the yeast two-hybrid analyses. Yeast strain *S. cerevisiae* EGY48[p8oplacZ] is the host strain EGY48 transformed with the autonomously replicating reporter plasmid p8op-LacZ (Clontech).

CONSTRUCTION OF TWO-HYBRID EXPRESSION PLASMIDS

The plasmids $pLexA_{BD}$-VP1, $pLexA_{BD}$-VP5, $pB42_{AD}$-VP1, $pB42_{AD}$-VP5, containing the cDNA sequence encoding VP1 or VP5 of IBDV strain CEF94, have been described previously (Tacken *et al.*, 2000). Copy DNA encoding the full-length sequences of

pVP2, VP3 and VP4 of IBDV strain CEF94, as well as defined parts of VP5, pVP2, VP3, and VP4 (see Fig. 2), were amplified by PCR by using the Expand high fidelity PCR system (Boehringer Mannheim). Plasmid pHB36W was used as a template. The sets of primers used were designed to introduce an *Eco*RI site at the upstream (5') end and a stop codon plus either a *Sal*I site or a *Xho*I site at the downstream (3') end of each coding sequence (Table 1). The PCR products were precipitated, digested with *Eco*RI/*Sal*I or *Eco*RI/*Xho*I, gel purified by the QIAEX-II method (QIAGEN), and ligated with T4 ligase (New England BioLabs) into the yeast expression vectors pLexA$_{BD}$ and

Primer	Nucleotide sequence[a]	Orientation	Polypeptide
p001	ccg*GAATTC*ATGACAAACCTGCAAGATCAAACCC	+	pVP2, pVP2ΔC128 and pVP2ΔC256
p122	ccg*GAATTC*TTCCAAGGAAGCCTGAGTGAACTG	+	pVP2ΔN128 and pVP2ΔN128/C128
p120	ccg*GAATTC*CTGGGCGCCACCATCTACCTC	+	pVP2ΔN256
p054	aagtG*TCGAC***TCA**GGCGAGAGTTAGCTGCCTTATGC	–	pVP2, pVP2ΔN128 and pVP2ΔN256
p157	taa*CTCGAG***TCA**AACCAGGTTCTTTGCTAGTTCAGG	–	pVP2ΔC128 and pVP2ΔN128/C128
p119	taa*CTCGAG***TCA**TACAAGGCCGTGGACGCTTGTTTG	–	pVP2ΔC256
p057	tc*GAATTC*GCTTCAGAGTTCAAAGAGACCCCC	+	VP3, VP3ΔC64, VP3ΔC129 and VP3ΔC161
p131	ccg*GAATTC*GTGTTCATGTGGCTGGAAGAGAATG	+	VP3ΔN32/C64, VP3ΔN32/C129, and VP3ΔN32/C161
p062	ccg*GAATTC*AACGCACCACAAGCAGGCAGCAAG	+	VP3ΔN64 and VP3ΔN64/C64
p060	ccg*GAATTC*GGCCAGCTAAAGTACTGGCAGAAC	+	VP3ΔN129
p006	gatc*GTCGAC***TCA**CTCAAGGTCCTCATCAGAGAC	–	VP3, VP3ΔN64 and VP3ΔN129
p063	aagt*GTCGAC***TCA**ATAGACTTTGGCAACTTCGTCTATG	–	VP3ΔC64, VP3ΔN64/C64 and VP3ΔN32/C64
p061	aagt*GTCGAC***TCA**GCTTGGCCCTCGGTGCCCATTG	–	VP3ΔC129 and VP3ΔN32/C129
p132	aagt*GTCGAC***TCA**CCTCTGTGCTTCCTCTGGTGTGG	–	VP3ΔN32/C161 and VP3ΔC161
p055	ag*GAATTC*GCCGACAAGGGGTACGAGGTAGTC	+	VP4, VP4ΔC61 and VP4ΔC122
p144	ccg*GAATTC*AAAGCATTGAACAGCAAAATG	+	VP4ΔN60 and VP4ΔN60/C61
p124	ccg*GAATTC*TGGGACGACAGCATTATGCTGTCC	+	VP4ΔN121
p143	taa*CTCGAG***TCA**GGCCATGGCCAGGTCGTAC	–	VP4, VP4ΔN60 and VP4ΔN121
p125	taa*CTCGAG***TCA**GGCGAGCTTGGTGCTTCTAAAGC	–	VP4ΔC61 and VP4ΔN60/C61
p123	taa*CTCGAG***TCA**GACATCATCTATTGGGACAACGGTG	–	VP1ΔC122
p007	ccg*GAATTC*ATGGTCAGTAGAGATCAGACAAACG	+	VP5ΔC37, VP5ΔC73 and VP5ΔC55
p130	ccg*GAATTC*GGCGTCCATTCCGGACGACACC	+	VP5ΔN36, VP5ΔN36/C37 and VP5ΔN36/C55
p128	ccg*GAATTC*TGGATTCCCTGGCTCAATTGTGGG	+	VP5ΔN72 and VP5ΔN72/C37
p145	ccg*GAATTC*CTACAAGTTCGATCAGATGCTCCTG	+	VP5ΔN90
p008	gatc*GTCGAC***TCA**CTCAGGCTTCCTTGGAAGGTC	–	VP5ΔN36, VP5ΔN72 and VP5ΔN90
p129	taa*CTCGAG***TCA**TTGTAACTGGCCGGTAGGTTCTG	–	VP5ΔC37, VP5ΔN36/C37 and VP5ΔN72/C37
p146	ttg*CTCGAG***TCA**TTCCCATTGCTCTGCAGTGTGTAG	–	VP5ΔC55 and VP5ΔN36/C55
p127	taa*CTCGAG***TCA**GGGAAAAAGACAATTAGCCCTGAC	–	VP5ΔC73

Table 1. Sequences of primers used for the construction of the yeast two-hybrid expression plasmids

[a] The primer sequences are listed 5' to 3'. Restriction sites are in italics. Nucleotides 5' of a restriction site which are unable to hybridize with the IBDV cDNA are in lower case. Bold letters indicate an in-frame stop codon.

$pB42_{AD}$ (Clontech). These vectors had previously been digested with *Eco*RI/*Xho*I. The ligation mix was transformed into *E. coli* DH5-α cells, 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 using an ABI 310 sequencer (PE Applied Biosystems) to ensure correct reading frames. Control plasmids $pLexA_{BD}$-53 (human p53 gene in $pLexA_{BD}$), $pB42_{AD}$-SV40 T (SV40 large T antigen in $pB42_{AD}$) and $pLexA_{BD}$-Lamin C (human Lamin C gene in $pLexA_{BD}$) were from Clontech.

TWO-HYBRID ANALYSIS

All two-hybrid media, buffers, and protocols were as described in the Clontech Manual for the Matchmaker LexA Two-Hybrid System and in the Clontech Yeast Protocols Handbook (Clontech). The yeast strain *S. cerevisiae* EGY48[p8op-lacZ], which contains two reporter genes, *LEU2* and *Lac*Z, under the control of two independent promoters, was transformed by using the lithium acetate method with $pLexA_{BD}$ (*HIS3*$^+$) and $pB42_{AD}$ (*TRP1*$^+$) constructs carrying VP1, pVP2, VP3, VP4 and VP5, in every possible pairwise combination. Control plasmids were $pLexA_{BD}$-Lamin C (Clontech), $pLexA_{BD}$-53 (Clontech), $pB42_{AD}$-SV40 T (Clontech), and $pB42_{AD}$-empty vector. This resulted in 37 pairwise transformations (see Table 2 and 3) that were plated onto SD/Glu/-His/-Ura/-Trp medium. About 10 His$^+$ Ura$^+$ Trp$^+$ 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/5-bromo-4-chloro-3-indolyl ß-D-galactopyranoside (X-gal)/-His/-Ura/-Trp medium to assess the transcriptional activation of the *LacZ* reporter gene.

Stable expression of the hybrid proteins was verified by Western blot analysis according the manufacturer's instructions (Clontech). 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*$^+$ plasmid pJK101 (OriGene) and, in parallel, with pJK101 together with one of the $pLexA_{BD}$ constructs carrying VP1, pVP2, VP3, VP4, or VP5. Transformed yeast cells were plated onto SD/Glu/-Ura or SD/Glu/-His/-Ura medium, respectively. Plasmid pJK101 contains a *LacZ* reporter gene whose expression is driven by the yeast GAL1 promoter. However, two LexA operators have been placed between the GAL1 promoter and the *lacZ* gene. When a $LexA_{BD}$ fusion protein binds to these operators there will be a decrease in the level of GAL1-driven *lacZ* expression. A liquid assay to quantify ß-galactosidase activities was performed by growing transformants to mid log phase in the appropriate selection medium, SD/Gal/Raf/-Ura or SD/Gal/Raf/-His/-Ura, and

using *O*-nitrophenyl-ß-D-galactoside as the chromogenic substrate. Each enzyme activity assay was performed with at least five independent colonies and ß-Galactosidase specific activities were calculated as described by Clontech.

CONSTRUCTION OF THE EXPRESSION PLASMIDS FOR CO-IMMUNOPRECIPITATION ANALYSIS

The c-Myc-tag and hemagglutinin (HA)-tag fusions were cloned as follows. The pVP2, VP3, VP4, VP5 (or defined parts thereof) containing inserts were excised from their respective pLexA$_{BD}$-vectors described above, through restiction enzyme digestion with either *Eco*RI/*Xho*I or *Eco*RI/*Sal*I. These fragments were gel purified by the QIAEX-II method (QIAGEN) and ligated with T4 ligase (New England BioLabs) into the vectors pGBKT7 and pGADT7 (Clontech). The vector pGBKT7 had previously been digested with *Eco*RI/*Sal*I and the vector pGADT7 with *Eco*RI/*Xho*I. The ligation mix was transformed into *E. coli* DH5-α cells, which were subsequently grown either under kanamycin (pGBKT7) or ampicillin (pGADT7) 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 using an ABI 310 sequencer (PE Applied Biosystems) to ensure correct reading frames.

TRANSFECTION OF QM5 CELLS

QM5 cells were grown to 80% confluency in 60-mm dishes and infected with FPV-T7 (multiplicity of infection [MOI] = 3). After 1 h, the cells were washed once with 5 ml QT35 medium and covered with 5 ml of Optimem 1 (Gibco-BRL). In the meantime, 2.0 µg of DNA was mixed with 25 µl Lipofectamine (Gibco-BRL) in 0.5 ml Optimem 1 and kept at room temperature for at least 30 min. The QM5 cells were subsequently covered with 4 ml of fresh Optimem 1, and the DNA-Lipofectamine mixture was added. The transfection was performed overnight (18 h) in a 37°C incubator (5.0% CO_2). The transfected monolayer was rinsed once with QT35 medium and fresh QT35 medium supplemented with 5% FCS and 2% ABII was added, and the plates were incubated for another 24 h.

RADIOLABELING OF TRANSFECTED CELLS AND IMMUNOPRECIPITATION

At 48 h post-transfection, cells were starved for 1 h in methionine-free EMEM medium (Gibco-BRL). Cells were then labeled for 3 h with 20 µCi/ml of [^{35}S]methionine (Amersham) in methionine-free EMEM medium. At the end of the labeling, the cell cultures were lysed on ice in 1x PBS-TDS lysis buffer, using a 5x PBS-TDS lysis buffer

stock solution (5 % Triton X-100, 2.5 % sodium deoxycholate, 0.5 % SDS, 0.7 M NaCl, 14 mM KCl, 50 mM Na_2HPO_4, 7.5 mM KH_2PO_4). Cell debris was removed by centrifugation at 4°C for 20 min at 13,000 × *g*. All lysates were pretreated with Protein A Sepharose (Amersham) before they were used for immunoprecipitation with either monoclonal anti-c-Myc serum (Clontech) or polyclonal anti-HA serum (Clontech). Protein A Sepharose-bound immune complexes were washed three times in 1x PBS-TDS lysis buffer and eluted in 30 µl sodium dodecyl sulfate (SDS) sample buffer (60 mM Tris-HCl [pH 6.8], 2.5% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.1% bromophenol blue). Proteins were resolved in 18% separating gels by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by autoradiography.

RESULTS

INTERACTIONS OF pVP2, VP3, pVP4, VP1 AND VP5 WITH THEMSELVES AND EACH OTHER ASSAYED IN THE YEAST TWO-HYBRID SYSTEM

The ability of the full-length viral proteins, VP1, pVP2, VP3, VP4 and VP5 to interact with themselves or with each other was re-evaluated using the polypeptides pVP2, VP3 and VP4 based on the corrected polyprotein cleavage sites (compare Fig. 1C with 1D). cDNA fragments of IBDV strain CEF94 encoding these polypeptides were generated by PCR and subcloned into the plasmids $pLexA_{BD}$ and $pB42_{AD}$ for two-hybrid analysis. The plasmid $pLexA_{BD}$ constitutively expresses proteins as carboxy-terminal fusions to a 202 amino acids sequence of the bacterial lexA DNA binding domain (BD). The plasmid $pB42_{AD}$ was used as the expression vector for all transcriptional activation domain (AD) fusion proteins. This plasmid expresses proteins under the control of the galactose-inducible GAL1 promoter as carboxy-terminal fusions to a simian virus SV40 (SV40) nuclear localization sequence, an influenza virus hemagglutinin epitope tag, and the B42 AD. Protein expression of the fusion plasmids was verified by Western blot analysis (data not shown).

Plasmids $pLexA_{BD}$ and $pB42_{AD}$ expressing VP1, pVP2, VP3, VP4 and VP5 fusion proteins were co-transformed in all possible pairwise combinations into the yeast strain *S. cerevisae* EGY48[p8opLacZ]. This strain has the upstream activating sequences of the chromosomal LEU2 gene replaced with six LexA operators and contains the p8op-LacZ reporter plasmid encoding the *LacZ* gene under the control of eight LexA operators. Potential interactions were then scored by testing for growth in synthetic complete medium lacking leucine and for production of β-

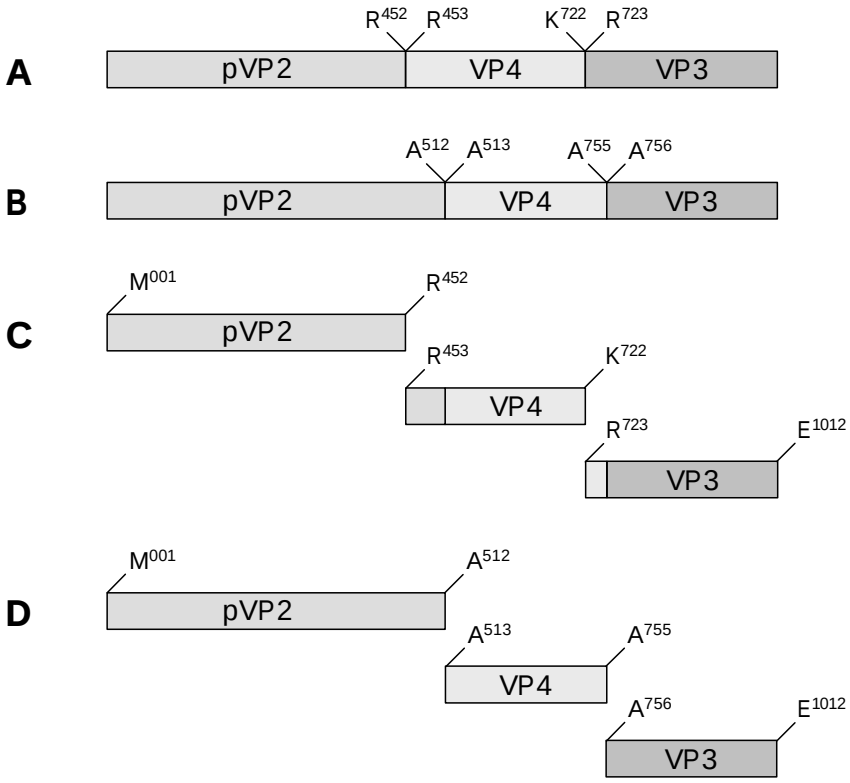


Figure 1. Comparison of the previously and currently used two-hybrid fusion proteins encoding the viral proteins pVP2, VP4 and VP3 of IBDV. (A and B) Schematic representation of the IBDV polyprotein; the pVP2-VP4 and VP4-VP3 junctions of the polyprotein are indicated based (A) on the putative cleavage sites reported by Hudson *et al.* (Hudson *et al.*, 1986) and (B) based on the recently corrected cleavage sites (Lejal *et al.*, 2000; Sanchez and Rodriguez, 1999). (C and D) Schematic representation of the polypeptides used for the two-hybrid analysis; (C) previously used (Tacken *et al.*, 2000) and (D) currently used. The first and last amino acid as well as the position of expressed regions are indicated.

galactosidase (β-gal) as observed by the appearance of blue colonies on X-Gal-containing medium. The strength of the protein-protein interaction was judged by the intensity of the bleu phenotype or by the time required for growth on mediumlacking leucine. At least seven independent transformants of each strain were screened. Additionally, all fusions were tested for intrinsic or nonspecific activation. The $pB42_{AD}$ fusion plasmids were co-transformed with LexA fused to the human Lamin C protein to test for specificity of interaction with each of the BD fusions while all the $pLexA_{BD}$ fusion plasmids were co-transformed with an empty AD to test for intrinsic activation. None of the fusions did intrinsically or nonspecifically activate expression of the LEU2 and LacZ reporters, confirming the specificity of the

test system for IBDV proteins. Plasmids containing sequences encoding p53 fused to LexA and plasmids with sequences of SV40 large T antigen fused to B42, were co-

$LexA_{BD}$ fusion: / $B42_{AD}$ fusion:	VP1	pVP2	VP3	VP4	VP5	Lamin C	53
VP1	+/-	-	+++	-	-	-	nd
pVP2	-	+++	-	-	-	-	nd
VP3	+++	-	+++	-	-	-	nd
VP4	-	-	-	+++	-	-	nd
VP5	-	-	-	-	+++	-	nd
no insert	-	-	-	-	-	-	nd
SV40 T	nd	nd	nd	nd	nd	nd	+++

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 1 day when the strain with $LexA_{BD}$-p53 and $B42_{AD}$-SV40 T antigen (positive control) showed clear growth. +++, clear growth after one day (very strong interaction); +/-, limited growth (weak interaction); -, no growth (no interaction); nd, not determined. All results shown are representative of at least 7 independent transformants.

$LexA_{BD}$ fusion: / $B42_{AD}$ fusion:	VP1	pVP2	VP3	VP4	VP5	Lamin C	53
VP1	-	-	+	-	-	-	nd
pVP2	-	+++	-	-	-	-	nd
VP3	+++	-	+++	-	-	-	nd
VP4	-	-	-	+++	-	-	nd
VP5	-	-	-	-	+++	-	nd
no insert	-	-	-	-	-	-	nd
SV40 T	nd	nd	nd	nd	nd	nd	+++

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 intensity of the blue phenotype after 1 day when the strain with $LexA_{BD}$-p53 and $B42_{AD}$-SV40 T antigen (positive control) had deep blue colonies. +++, deep blue colonies (very strong interaction); +, light blue colonies (interaction); -, white colonies (no interaction); nd, not determined. All results shown are representative of at least 7 independent transformants.

transformed into EGY48[p8oplacZ] and used as a positive control. Strains containing these fusion proteins showed visible growth in Leu⁻ medium after 2 days, and deep blue colonies on X-Gal containing medium after 1 day. We observed strong homologous interactions for the viral proteins pVP2, VP3, VP4 and VP5, a possible though weak homologous interaction for VP1 and one heterologous interaction between VP1 and VP3 (Tables 2 and 3).

When we compare these results with those obtained previously (Tacken *et al.*, 2000), there is one apparent difference. The homologous interaction of VP4, which we clearly observe in our present study, was not detected in our earlier work. Apparently, the amino-terminal extension abolished the protein's ability to interact with itself.

DELETION MAPPING OF THE SELF-INTERACTING DOMAINS OF THE IBDV PROTEINS USING THE YEAST TWO-HYBRID SYSTEM

To study the domains involved in the homotypic interactions of the pVP2, VP3, VP4 and VP5 proteins, five different deletion mutants were initially generated for each protein (Fig. 2A-D, no. 2-6; dark grey depicted polypeptides). To simplify interpretation of the results, we divided each of the viral proteins into four regions, designated REGION I to IV, as shown in Fig. 2. All the deletion mutants were expressed by both the pLexA$_{BD}$ and the pB42$_{AD}$ expression vectors and the fusion products were tested for their capability to interact with their respective full-length protein. To rule out the possibility of non-specific transactivation of the reporter genes, all constructs were additionally assayed for reporter gene activation when expressed either alone or together with the control plasmids pLexA$_{BD}$-Lamin C or pB42$_{AD}$-empty. All constructs proved to be negative in these tests (data not shown).

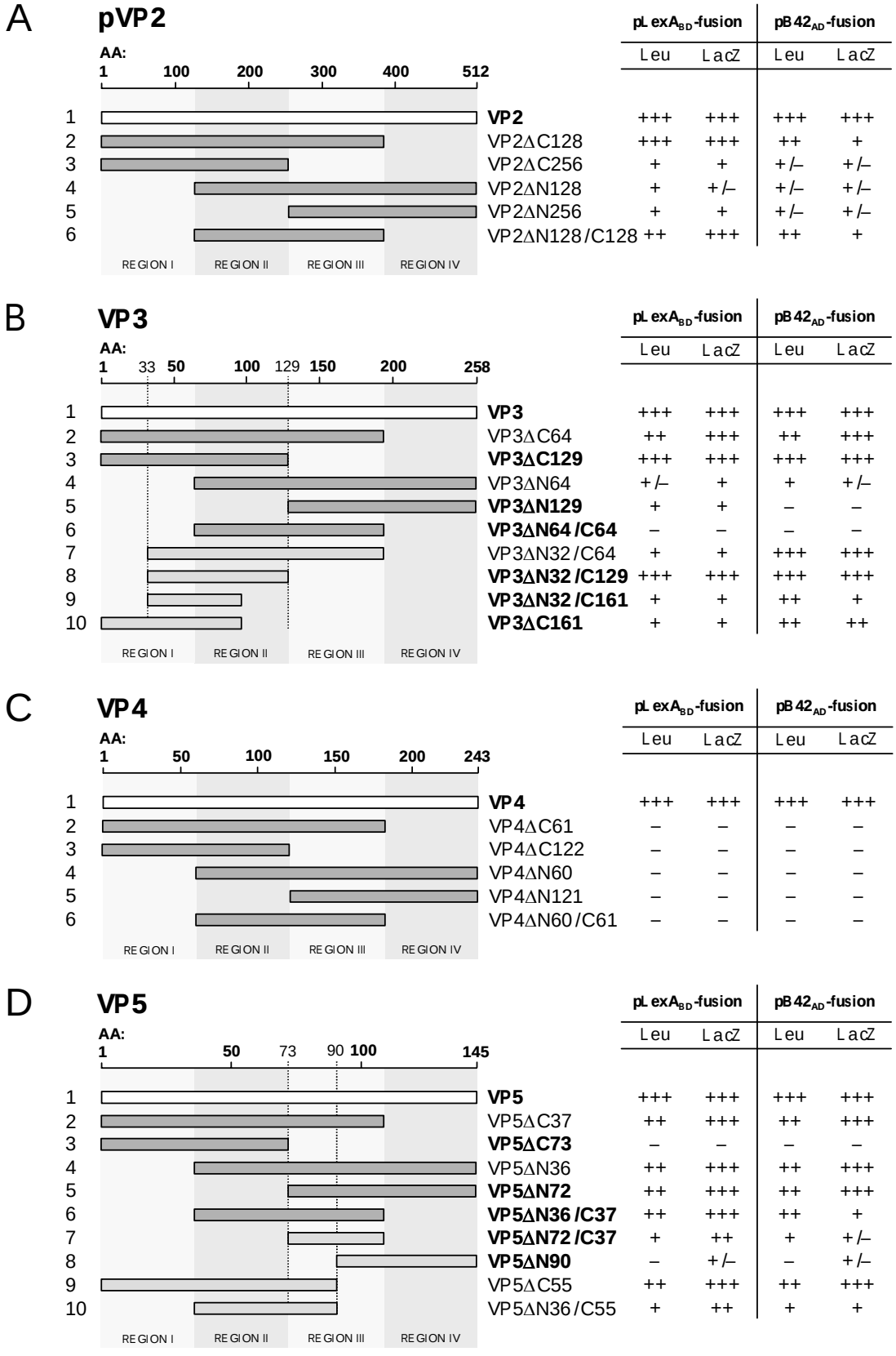
The results of the assays are presented in Fig. 2. Of the pVP2 deletion mutants all truncated fusion proteins scored positive in the β-gal and Leu⁻ growth tests, albeit with some variation in interaction strength (Fig. 2A, no. 2-6). The observation that both the amino-terminal and the carboxy-terminal half of the pVP2 protein were able by themselves to interact with the full-size polypeptide suggests that the homotypic interactions between pVP2 molecules are based on more than one contact site. For VP3 the critical domains for interaction are located in the amino-terminal part. Carboxy-terminal deletion of one-fourth or half of the protein as in VP3ΔC64 and VP3ΔC129 was without measurable effect on the strength of the interaction with full-length VP3 (Fig. 2B, no. 2-3). Truncations, however, by 64 and 129 amino acids at the amino terminus were detrimental for their interaction with full-length VP3: the scores with these polypeptides were (very) weak or even negative (Fig. 2B, no. 4-6). VP4-VP4 interaction appeared to be extremely sensitive to

modifications. Any deletion that we applied appeared to be invariably lethal (Fig. 2C, no. 2-6). VP5-VP5 association on the other hand was quite tolerant to deletions. All truncations except for the construct VP5ΔC73 (Fig. 2D, no. 3), which lacks the sequence for the carboxy-terminal half of the protein, scored positive in both the β-gal and Leu⁻ growth test (Fig. 2D, no. 2-6). These results suggest that REGION III is responsible for VP5 homomeric interaction.

FINE MAPPING THE SELF-INTERACTION SITES OF VP3 AND VP5 USING THE YEAST TWO-HYBRID SYSTEM

To determine the putative self-interacting domain of VP3 and VP5 more precisely, we constructed and tested four additional progressive deletion mutants for each protein. For VP3 a series of constructs was generated encoding internal core protein fragments (Fig. 2B, no. 7-9) as well as an amino-terminal protein fragment (Fig. 2B, no. 10), each comprising a part of REGION I and/or II. When these constructs were assayed in the yeast two-hybrid system homotypic interactions were observed for all these polypeptides, though for three of them the interaction strength measured in the pairing of pLexA$_{BD}$-VP3(deletion mutant) with pB42$_{AD}$-VP3(full-length) was significantly lower than that in the reciprocal pairing of pB42$_{AD}$-VP3(deletion mutant) with pLexA$_{BD}$-VP3(full-length) (Fig. 2B, no. 7, 9 and 10). Such polarity of two-hybrid

Figure 2. Deletion mapping of the regions in pVP2, VP3, VP4 and VP5 responsible for homologous interaction by using the yeast two-hybrid system. All of the deletion mutants were cloned into pLexA$_{BD}$ and pB42$_{AD}$ yeast expression vectors and tested for their ability to interact with full-length pVP2 (A), VP3 (B), VP4 (C) or VP5 (D). Left: Overview of the deletion mutants that were assayed. The graded line above each panel denotes the amino acid (AA) positions of the primary full-length sequence of each viral protein. A breakdown of the polypeptides into REGIONS I-IV is shown by shaded areas. Polypeptides depicted dark grey were used for an initial mapping; polypeptides depicted light grey were assayed subsequently for fine mapping. The polypeptides denoted in bold-face type were additionally assayed for interaction with their full-length cognate protein *in vivo* by co-immunoprecipitation analysis (see text). Right: Interaction data obtained with double transformants. Interactions were assayed for leucine autotrophy (Leu) and for β-galactosidase activity (LacZ). Leu: +++, clear growth after one day (very strong interaction); ++, clear growth after two days (strong interaction); +, growth (interaction); +/–, limited growth (weak interaction); –, no growth (no interaction). LacZ: +++, deep blue colonies (very strong interaction); ++, blue colonies (strong interaction); +, light blue colonies (interaction); +/–, very light blue (weak interaction); –, white colonies (no interaction). All results shown are representative of at least 7 independent transformants.



		pLexA$_{BD}$-fusion		pB42$_{AD}$-fusion	
		Leu	LacZ	Leu	LacZ
A					
1	**VP2**	+++	+++	+++	+++
2	VP2ΔC128	+++	+++	++	+
3	VP2ΔC256	+	+	+/–	+/–
4	VP2ΔN128	+	+/–	+/–	+/–
5	VP2ΔN256	+	+	+/–	+/–
6	VP2ΔN128/C128	++	+++	++	+
B					
1	**VP3**	+++	+++	+++	+++
2	VP3ΔC64	++	+++	++	+++
3	**VP3ΔC129**	+++	+++	+++	+++
4	VP3ΔN64	+/–	+	+	+/–
5	**VP3ΔN129**	+	+	–	–
6	**VP3ΔN64/C64**	–	–	–	–
7	VP3ΔN32/C64	+	+	+++	+++
8	**VP3ΔN32/C129**	+++	+++	+++	+++
9	**VP3ΔN32/C161**	+	+	++	+
10	**VP3ΔC161**	+	+	++	++
C					
1	**VP4**	+++	+++	+++	+++
2	VP4ΔC61	–	–	–	–
3	VP4ΔC122	–	–	–	–
4	VP4ΔN60	–	–	–	–
5	VP4ΔN121	–	–	–	–
6	VP4ΔN60/C61	–	–	–	–
D					
1	**VP5**	+++	+++	+++	+++
2	VP5ΔC37	++	+++	++	+++
3	**VP5ΔC73**	–	–	–	–
4	VP5ΔN36	++	+++	++	+++
5	**VP5ΔN72**	++	+++	++	+++
6	**VP5ΔN36/C37**	++	+++	++	+
7	**VP5ΔN72/C37**	+	++	+	+/–
8	**VP5ΔN90**	–	+/–	–	+/–
9	VP5ΔC55	++	+++	++	+++
10	VP5ΔN36/C55	+	++	+	+

interactions has frequently been observed (Cuconati *et al.*, 1998; Xiang *et al.*, 1995) and is most likely an inherent consequence of the two-hybrid assay. The fused $LexA_{BD}$- or $B42_{AD}$-domain might partly occlude the site of interaction, or the fusion proteins may be folded improperly, be unstable or poorly expressed. The VP3 mutant termed VP3ΔN32/C129, however, interacted in both its polarities with the full-length VP3 (Fig. 2B, no. 8) as strongly as full-length VP3 interacted with itself (Fig. 2B, no. 1). Based on these results we conclude the internal core protein fragment encompassing residues 33 to 129 (i.e. REGION II and half of REGION I) to be essential for VP3 homomeric interaction (Fig. 2B, region between dotted lines).

The constructs that were generated for the further mapping of the interaction site in VP5 consisted either of the complete REGION III (Fig. 2D, no. 7) or of half of it, extended on one side with REGION IV, REGION II, or REGION I + II (Fig. 2D, no. 8-10). When the combined results of the assays are considered it is clear that the REGION III amino-terminal half (residues 73-90; Fig. 2D, region between dotted lines) is essential for homotypic VP5 association, with flanking domains additionally contributing to the interaction. This is seen most convincingly by looking at the effect of extending the non-functional amino-terminal half of VP5 (REGION I + II; VP5ΔC73; Fig. 2D, no. 3) with the residue 73-90 fragment (VP5ΔC55; no. 9), which renders the polypeptide strongly association-competent. Extending this construct further with the remaining part of REGION III (no. 2) does not increase the interaction strength any further. Conversely, the mere addition of the residue 73-90 fragment to the virtually inactive carboxy-terminal 55-residue polypeptide VP5ΔN90 (no. 8) generates a highly interactive polypeptide VP5ΔN72 (no. 5) to which further amino-terminal extension (VP5ΔN36; no. 4) does not seem to add much. Though we did not test the residue 73-90 fragment independently it is clear that the strength of its interaction with full-size VP5 would not be very high and that it requires the flanking regions as illustrated by the extended polypeptides VP5ΔN72/C37 (no. 7) and VP5ΔN36/C55 (no. 10) each of which binds with moderate strength.

HOMOLOGOUS INTERACTIONS OF THE FULL-LENGTH IBDV PROTEINS pVP2, VP3 AND VP4 ASSAYED BY CO-IMMUNOPRECIPITATION ANALYSIS

To obtain corroborating evidence for the interactions detected by the two-hybrid analyses, we employed a radio-immunoprecipitation (RIP) assay using a co-transfection system in which the respective proteins are transiently co-expressed in QM5 cells. To this end, we used plasmid pHB36W, in which the full-length genomic cDNA of segment A of IBDV is cloned in a transcription plasmid between a T7 RNA polymerase promoter and the autocatalytic hepatitis delta virus ribozyme sequence

(Boot *et al.*, 1999). Furthermore, for the co-expression of the viral proteins we used the transcription plasmid pGBKT7, in which the cDNA of each of the viral proteins, pVP2, VP3, VP4 and VP5, was cloned behind a T7 RNA polymerase promoter sequence, in fusion with a sequence encoding the c-Myc epitope tag. By using these tagged fusion proteins and anti-c-Myc antibodies, it was possible to specifically precipitate only one of the two homologous interacting proteins and to distinguish these proteins by a difference in their electrophoretic mobility (i.e. comparing the tagged and the untagged protein) when analysing the (co-) precipitates by SDS-PAGE. The molecular size of the fused c-Myc tag, including a small linker-peptide, is approximately 12 kDa.

The plasmids encoding the c-Myc-tagged proteins were co-transfected with plasmid pHB36W into QM5 cells that had previously been infected with a

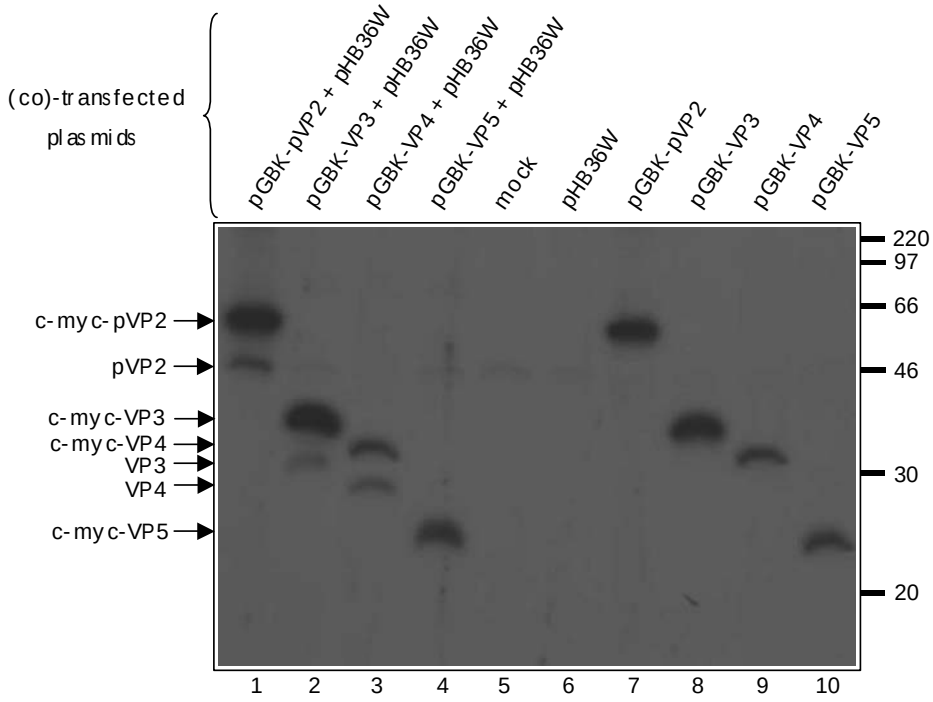


Figure 3. Radio-immunoprecipitation analysis of homologous interactions of the full-length viral proteins pVP2, VP3, VP4 and VP5 of IBDV in transfected QM5 cells. QM5 cells were (co-)transfected with the indicated plasmids expressing either the full-length untagged viral proteins pVP2, VP3, VP4 and VP5 (pHB36W) or a full-length viral protein, pVP2, VP3, VP4 or VP5, fused to a c-Myc-tag (pGBKT7). At 48 h post transfection cells were metabolically labeled for 3 h with [^{35}S]methionine. Subsequently, cells were lysed and immunoprecipitated with anti c-Myc serum, followed by SDS-PAGE. Positions of the viral (fusion-) proteins and sizes of marker proteins (in kDa) are indicated.

recombinant fowlpox virus that expresses T7 RNA polymerase (Britton *et al.*, 1996). Forty-eight hours post-transfection, cells were metabolically labeled for 3 hours with [^{35}S]methionine and subsequently subjected to immunoprecipitation with anti-c-Myc serum. The immunoprecipitates obtained were analyzed by SDS-PAGE and the labeled proteins visualized by autoradiography. Mock-transfected cells and cells transfected with either pHB36W or the c-Myc encoding plasmids alone, were used as controls for each immunoprecipitation.

As is clear from the results shown in Fig. 3, the c-Myc antibody precipitated only the c-Myc-tagged fusion proteins, indicating that it had no cross-reactivity (lanes 5-10). Furthermore, in the immunoprecipitations of the cell lysates of the co-transfected cells, the full-length untagged viral proteins pVP2, VP3 and VP4, were each clearly co-precipitated with their cognate c-Myc-tagged fusion protein (lanes 1-3). However, untagged full-length VP5 failed to be co-precipitated with c-Myc-tagged VP5 (lane 4). The observation that the relative amounts of tagged pVP2 and VP3 versus untagged pVP2 and VP3 are substantially different, whereas tagged and untagged VP4 showed nearly equivalent amounts, may be a consequence of different transfection efficiencies. It is also possible that the tagged pVP2 and VP3 proteins additionally interacted with themselves, whereas tagged VP4 did not, or to a lesser extent.

FINE MAPPING OF HOMOTYPIC INTERACTIONS OF VP3 AND VP5 BY CO-IMMUNOPRECIPITATION ANALYSIS

Using this *in vivo* co-immunoprecipitation assay, we additionally analyzed several VP3 and VP5 truncation mutants assayed earlier in the two-hybrid system (Fig. 2B, no. 3, 5, 6 and 8-10; Fig. 2D, no. 3 and 5-8; polypeptides denoted in bold-faced type) for their ability to be co-precipitated with their full-length cognate protein. For this purpose, the pGBKT7 plasmids coding for the c-Myc-tagged VP3 or VP5 full-length protein were co-expressed with a plasmid, pGADT7, encoding a VP3- or VP5-deletion mutant protein in fusion with an influenza virus hemagglutinin (HA) epitope tag. By using two different tags we were able to assay for protein-protein interaction with two different antibodies, specifically precipitating either the c-Myc-tagged full-length protein or the HA-tagged deletion mutant. Furthermore, by swapping the c-Myc- and HA-fusions, the assay was additionally performed reciprocally, namely by co-expressing HA-tagged full-length VP3 or VP5 together with c-Myc-tagged VP3 or VP5 deletion mutant proteins.

Control experiments performed for each immunoprecipitation using radiolabeled lysates of mock-transfected cells and of cells transfected with either the pGBKT7 or the pGADT7 plasmid alone, demonstrated that both the c-Myc-

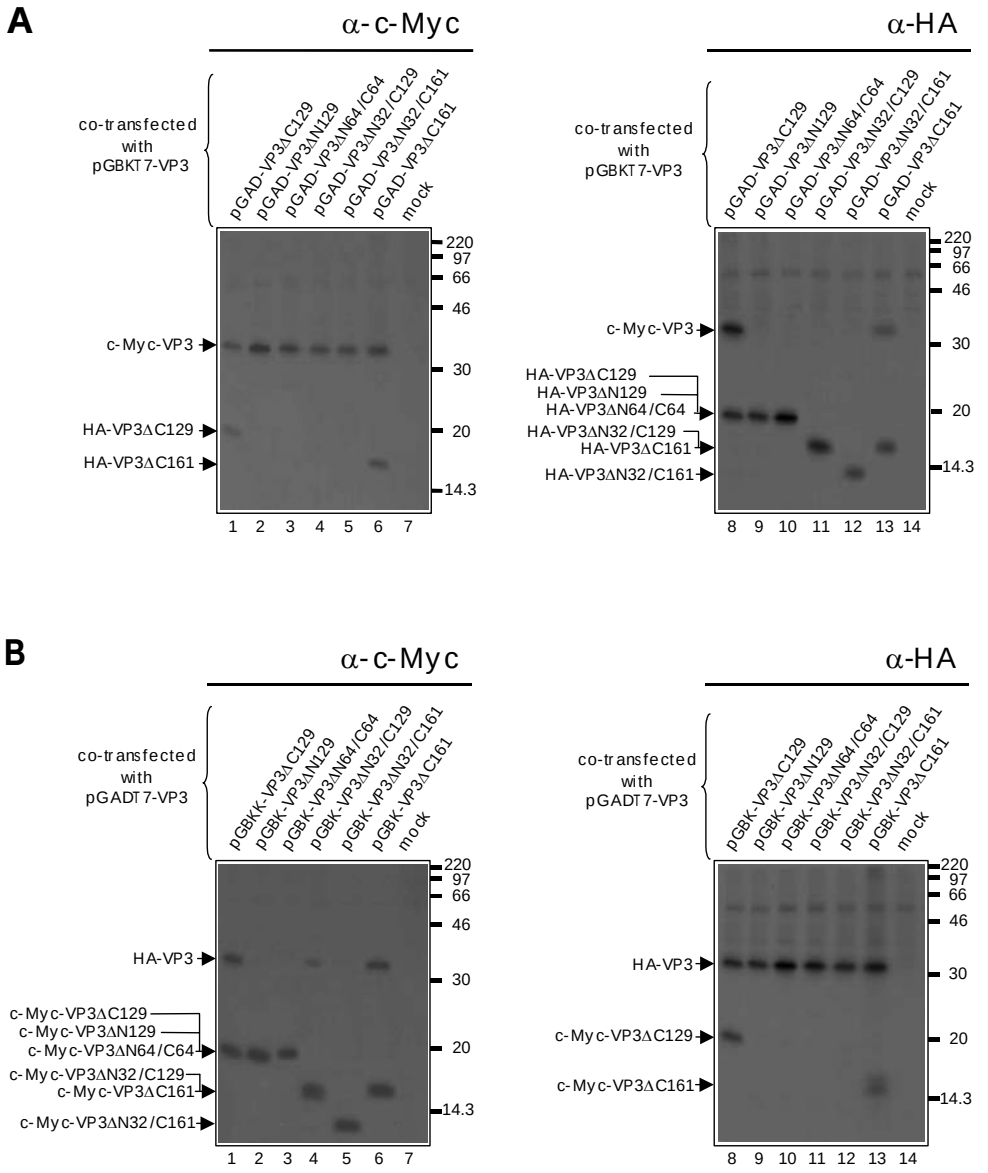


Figure 4. Radio-immunoprecipitation analysis of truncated VP3 proteins in transfected QM5 cells. The VP3 mutant constructs (typed in bold-face in Fig. 2B) were cloned into pGBKT7 and pGADT7 expression vectors and the expressed polypeptides were tested for their ability to interact with full-length VP3. QM5 cells were co-transfected with the indicated plasmids expressing either the full-length, c-Myc-tagged VP3 protein (pGBKT7) together with a HA-tagged VP3 mutant polypeptide (pGADT7) (A), or a c-Myc-tagged VP3 mutant polypeptide together with the full-length HA-tagged VP3 protein (B). At 48 h post transfection cells were metabolically labeled for 3 h with [^{35}S]methionine. Subsequently, cells were lysed and proteins were immunoprecipitated with anti c-Myc serum or anti HA serum, followed by SDS-PAGE. Positions of the viral (fusion-) proteins and sizes of marker proteins (in kDa) are indicated.

and the HA-antibodies lacked cross-reactivity against the respective fusion-proteins (data not shown). When using the c-Myc antibodies for the precipitation of c-Myc-tagged VP3, only the HA-tagged mutant proteins VP3ΔC129 and VP3ΔC161 were found to be co-precipitated (Fig. 4A, lanes 1 and 6). The same result was observed when using the HA-antibodies, where c-Myc-tagged full-length VP3 was only co-precipitated with the HA-tagged VP3ΔC129 and VP3ΔC161 proteins (Fig. 4A, lanes 8 and 13). Furthermore, reversing the c-Myc- and HA-tags did not affect the observed interactions, thus confirming the results (Fig. 4B, lanes 1, 6, 8 and 13). In addition, an interaction was observed for mutant protein VP3ΔN32/C129, though weak and only in one orientation. When using the c-Myc antibodies, HA-tagged full-length VP3 was observed to be co-precipitated with the HA-tagged VP3ΔN32/C129 (Fig. 4B, lane 4). For VP5, none of the deletion mutants tested was found to be co-precipitated with full-length VP5, either way and irrespective of the orientation of the tags (data not shown).

DISCUSSION

In the present study we confirmed and extended our earlier analysis of the homotypic interactions of the IBDV proteins. The use of the recently refined cleavage map of the structural protein precursor allowed the detection of a novel homologous interaction for VP4 by the yeast two-hybrid system, in addition to the self-association observed earlier for pVP2, VP3 and VP5 as well as – though weakly – for VP1.

A further characterization of the strongly interacting pVP2, VP3, VP4 and VP5 proteins was undertaken both by using the yeast two-hybrid system and by performing co-immunoprecipitation analyses of co-expressed cDNA constructs. Through deletion mutagenesis we were able to map the self-interacting regions in all of the proteins except for VP4 which appeared to be very sensitive to modifications. The data on VP3 allow us now to design a domain map in which the different regions of the protein involved in homomeric and heteromeric interactions can be assigned.

The IBDV capsid is an icosahedron with a T=13 lattice composed of trimeric subunits (Bottcher *et al.*, 1997; Caston *et al.*, 2001). The outer face of the particle is composed of 260 trimeric VP2 clusters. Closely apposed to the inside of this protein layer are 200 Y-shaped trimeric VP3 structures. This arrangement not only predicts the existence of homomeric interactions of the two capsid proteins, their intimate association dictates the occurrence of heteromeric contacts as well. However,

while the self-association of both proteins came out convincingly, we failed to detect pVP2/VP3 interactions in our assays. The reasons for this most likely relate to the assay systems that we used. The two-hybrid system, for instance, would be inadequate if we assume that the interactions only occur between the trimeric units. Also, if such heteromeric complexes would have been formed in the co-expression system, they may not have been sufficiently stable to survive the immunoprecipitation conditions, e.g. due to the low concentration of SDS present. It is of note that VP2/VP3 complexes were also not detected in infected cell lysates (Tacken *et al.*, 2000) and that the assembly of virus-like particles from these proteins somehow requires the VP3 protein to be 'activated', as their formation could only be achieved after modification of its carboxy terminus (Chevalier *et al.*, 2002).

We observed strong interactions between full-length VP3 molecules in our two-hybrid assays. These interactions were subsequently narrowed down by protein truncation to the amino-terminal region of the molecule. Specifically, a polypeptide comprising residues 1-97 was identified as the domain responsible for homomeric interaction, since this region proved to interact with its full-length cognate protein in both the yeast two-hybrid and the co-immunoprecipitation assay. Interestingly, this domain is clearly distinct from the regions in the VP3 molecule known to interact with other components in the virion. VP3 has been shown by us (Tacken *et al.*, 2000) as well as by others (Lombardo *et al.*, 1999) to bind to the viral RNA-dependent RNA polymerase, VP1. We were able to map the domain in VP3 responsible for this interaction to the very carboxy-terminal 10 residues (Tacken *et al.*, 2002). In the same study we additionally demonstrated, that VP3 also interacts with both dsRNA segments of the viral genome. The domain responsible for this association has not been determined yet, but has been implicated to occur in the carboxy-terminal region as well, in a domain spanning residues 221-247, a highly basic region with interspersed proline residues (Bottcher *et al.*, 1997; Hudson *et al.*, 1986; Tacken *et al.*, 2002). Importantly, deletion of the VP1-binding domain in VP3 did not abolish VP3-dsRNA interactions (Tacken *et al.*, 2002) nor did it affect VP3-VP3 interaction (unpublished results). Conversely, deletion of the self-interacting domain of VP3 did not affect the VP1 binding activity (unpublished results). Altogether, these data are consistent with the independent functioning of three domains in the VP3 protein as we depicted in the domain model of the protein presented in figure 5. The drawing shows the homomeric association function localized in the amino-terminal one-third of the molecule, the VP1-binding segment at the opposite end of the molecule, and the dsRNA-binding domain just preceding the latter.

Unlike VP3 (Martinez-Torrecuadrada *et al.*, 2000*b*), (p)VP2 has been shown to have a strong tendency to self-assemble giving rise to different types of particles

depending on the conditions, varying from rigid, flexible and twisted tubules to smaller or larger virus-like particles (Caston *et al.*, 2001; Chevalier *et al.*, 2002; Fernandez-Arias *et al.*, 1998; Lombardo *et al.*, 1999; Martinez-Torrecuadrada *et al.*, 2000*a*; Martinez-Torrecuadrada *et al.*, 2000*b*). Detailed electron cryomicroscopy and image processing analyses of different types of particles clearly revealed the trimeric VP2 structures in which each molecule can be subdivided into three subdomains, each able of interacting in a non-equivalent fashion with another VP2 molecule of the same or of an adjoining trimer (Caston *et al.*, 2001). This constellation would predict that removal of one such subdomain does not necessarily disable the interactions by the others. Our observations support this view, as the deletions of different domains of the pVP2 molecule appeared not to abolish the association capacity of the remaining polypeptides. This was also not the case after truncation of a carboxy-terminal 128-residue segment, which contains the 50-60 residues tail supposed to play a crucial role in the control of the interactions between trimers of VP2 as well as between trimers of VP2 and VP3 (Caston *et al.*, 2001). Without this tail domain independently expressed VP2 assembles into T=1

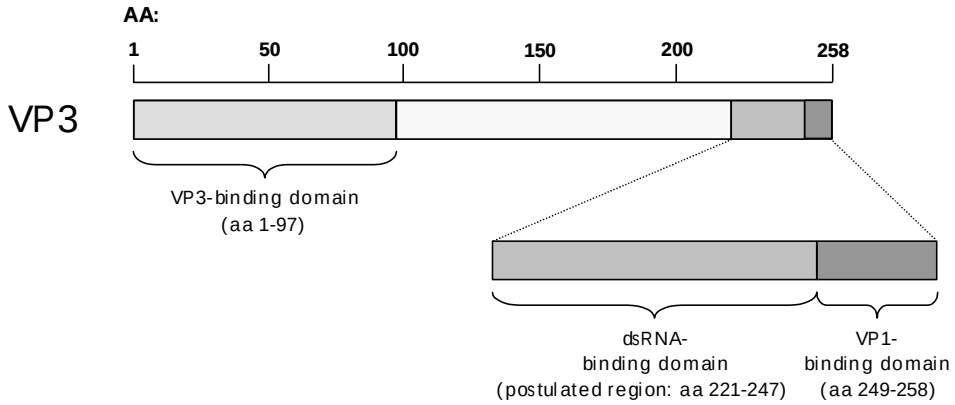


Figure 5. A domain model for VP3. Functional binding domains are indicated as shaded boxes. The binding site for VP1 has been mapped previously to the extreme carboxy terminus (Tacken *et al.*, 2002), while the approximate one-third amino-terminal region was presently shown to be responsible for homomeric interaction. The site for dsRNA interaction has not been precisely defined yet though has been postulated to comprise the indicated stretch of amino acids (Caston *et al.*, 2001; Hudson *et al.*, 1986; Tacken *et al.*, 2002). The graded line above the upper bar representing the VP3 polypeptide indicates the amino acid (AA) residue numbers.

capsids (Caston *et al.*, 2001), while this domain is essential for normal virion morphogenesis in IBDV infected cells (Da Costa *et al.*, 2002).

VP4, the viral protease, was anticipated to exhibit homologous interaction. In IBDV infected cells this protein assembles into distinct tubular aggregates, 24-26 nm in diameter, known as type II tubules (Granzow *et al.*, 1997). Though the function of these structures in viral infection is still unclear, they might serve to inactivate excess protease activity, thereby preventing lethal damage to the virus or to the cells. Attempts to identify interacting domain(s) in the polypeptide failed; none of the deletions tested preserved the ability of the protein to associate with its full-length counterpart in the yeast two-hybrid assay. Negative results in the two-hybrid assay often result from protein instability, but that appeared not to be the case here. All described fusion proteins were analyzed by immunoblotting and appeared to accumulate in high amounts and with the expected size (data not shown). Our observations are therefore most compatible with the terminal domains interacting with each other to stabilize a conformation that is required for homotypic association. Deletion of either terminus disrupts this conformation and abolishes the interaction. This is also consistent with our initial inability to detect the homotypic interaction of VP4 in the yeast two-hybrid system (Tacken *et al.*, 2000). The VP4 protein that we tested in those studies carried a 61 residue amino-terminal extension while lacking 34 residues at its carboxy-terminal, both due to the use of incorrect precursor cleavage sites (Fig. 1C). Either or both these defects apparently interfered with self-association.

The exact function of VP5 is still unknown. The protein is highly basic, cysteine-rich, and its sequence is conserved among all serotype I IBDV strains (>95% identity). A VP5 deficient virus can replicate in the bursa of inoculated chickens but does not induce bursal lesions (Yao *et al.*, 1998). It has also been demonstrated that this VP5 deficient mutant IBDV remains closely associated with and is not efficiently released from infected cells (Yao & Vakharia, 2001). The protein has therefore been speculated to somehow facilitate progeny virus release (Yao & Vakharia, 2001). Recently, it was shown that VP5 accumulates within the host cell plasma membrane (Lombardo *et al.*, 2000). Expression of VP5 resulted in alteration of cell morphology, disruption of the plasma membrane, and in a drastic reduction of cell viability (Lombardo *et al.*, 2000). Furthermore, the protein is capable of inducing a programmed cell death response in culture cells (Yao & Vakharia, 2001). Sequence-based topology predictions indicated that VP5 is a class II membrane protein, having an intracellular amino-terminal tail, a transmembrane helix and an extracellular carboxy-terminal region (Lombardo *et al.*, 2000). Interestingly, the putative self-interacting domain of VP5 that we mapped in our two-hybrid analysis

to the domain comprising residues 73-90 corresponds almost exactly to the predicted transmembrane region (residues 69-88) of the protein. Due to the common α-helical structure of transmembrane domains, many are considered to form channels or pores in the membrane by a so-called 'barrel-stave' mechanism (reviewed in (Ojcius & Young, 1991)). The observation that the plasma membrane of VP5 expressing cells was permeable not only to small molecules, but also to macromolecules such as lectin and IgG molecules (Lombardo *et al.*, 2000) strongly supports the hypothesis that VP5 molecules indeed form pore structures within the plasma membrane. In addition, these observed changes in membrane permeability are similar to those produced in *E. coli* by the viral proteins E1 of hepatitis C virus, M2 of influenza virus, gp41of human immunodeficiency virus and 3AB of poliovirus, whose transmembrane domains are also thought to cause pore formation (Arroyo *et al.*, 1995; Ciccaglione *et al.*, 2001; Guinea & Carrasco, 1994; Lama & Carrasco, 1992; Lama & Carrasco, 1995). The 'barrel-stave' mechanism involves three major steps: (i) binding of monomers to the membrane; (ii) insertion into the membrane to form a pore; and (iii) progressive recruitment of additional monomers to increase the pore size (Shai, 1995). Initial assembly of monomers on the surface of the membrane must occur before the peptide is inserted, since it is energetically unfavourable for an amphipatic α-helix to traverse the membrane as a monomer. A homotypic interaction of VP5 through a self-interacting domain located within its predicted membrane-spanning region is therefore consistent with the hypothesis that VP5 molecules co-assemble at the host plasma membrane to form a pore structure. It would hence be interesting to evaluate whether mutations of conserved amino acids in the putative transmembrane region of VP5 would affect its membrane-permeabilizing activity.

Shortcomings of the individual methods used in this study necessitate a cautious interpretation of data observed just by one type of assay. This is for instance obvious from those cases where we were unable to confirm protein interactions by the co-immunoprecipitation assay that had been convincingly demonstrated in the yeast system, as exemplified by some of the VP3 and all VP5 truncation constructs that were tested by both approaches. Such inconsistencies between the two techniques are frequently observed (Vidal & Legrain, 1999; Warbrick, 1997). Our yeast two-hybrid deletion analysis of amino- and carboxy-terminally truncated VP3 molecules showed that neither the first 32 nor the last 129 amino acids of VP3 were necessary for VP3-VP3 interaction. However, the strongly interacting deletion mutant VP3ΔN32/C129, which lacks both these terminal domains, was found not to be efficiently co-immunoprecipitated when co-expressed with full-length VP3. On the other hand, the VP3 deletion mutant

VP3ΔC161, which differs from mutant VP3ΔN32/C129 only by having its amino terminus intact, clearly interacted with full-length VP3 in both the two-hybrid and the co-immunoprecipitation assays. Because the two-hybrid $LexA_{BD}$ and $B42_{AD}$ fusion proteins were stably expressed and negative for self-activation of the LEU2 and LacZ reporter genes, we attach much value to the positive results obtained with the yeast two-hybrid system. Thus, the deletion mutant VP3ΔN32/C129 most likely behaved as a bona fide VP3 partner in the yeast two-hybrid system. In contrast, there are many possible causes for negative results in the immunoprecipitation assay. One is the effect of the c-Myc or HA tags used in this assay. These extensions might sterically hinder interaction of the fusion proteins or reduce their interaction strength. Alternatively, their interaction might become destabilized by the binding of antibodies to the tags. While these considerations leave us with uncertainties about some of our – unconfirmed – observations and at the same time ask for additional, independent interaction assays, the combined results of our study provide a good basis for the further analysis of these important interactions in the future.

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CHAPTER 7

Summary and General Discussion

SUMMARY AND GENERAL DISCUSSION

The research described in this thesis was carried out in order to contribute to a better understanding of all interactions that the viral proteins of IBDV have with themselves, with each other, and with host cell proteins. At the time this project was started, very little was known about the interactions among the viral proteins of IBDV and between the viral and host cell proteins. What was known was a partly incorrect composition of the viral particle and a first cryoelectron microscopic study of the virion (Bottcher *et al*., 1997), on the basis of which some interactions could be predicted. No host components interacting with viral proteins had been reported yet.

To obtain a better insight into the different interactions essential in the IBDV life cycle, we employed the, at that time, recently developed yeast two-hybrid system. The generation of interaction maps in this way showed to be a valuable first tool for the analysis of protein interactions present within the virion or during infection.

The next paragraphs summarise and discuss the studies as described in the various chapters (2-6). At the end a brief description of future perspectives is given.

HOMOMERIC INTERACTIONS BETWEEN THE VIRAL PROTEINS OF IBDV

The first experiments were set up to identify interactions between the known proteins of IBDV themselves. The IBDV capsid is an icosahedron with a T=13 lattice composed of trimeric subunits (Bottcher *et al*., 1997; Caston *et al*., 2001). The outer face of the particle is composed of 260 trimeric VP2 clusters. Closely apposed to the inside of this protein layer are 200 Y-shaped trimeric VP3 structures. This arrangement not only predicts the existence of homomeric interactions of the two capsid proteins, their intimate association dictates the occurrence of heteromeric contacts as well. In this thesis, we showed that both capsid proteins appeared to self-associate strongly, though a heteromeric interaction of these two proteins was not found (Chapter 2 and 6). However, if this heteromeric interaction only occurs between the trimeric units of VP2 and VP3, the yeast two-hybrid system might simply have been inadequate to detect this interaction. Furthermore, it was shown recently that the assembly of virus-like particles (VLPs) from VP2 and VP3 somehow requires the VP3 protein to be 'activated' as the formation of VLPs could only be achieved after modification of the carboxy terminus of VP3 (Chevalier *et al*., 2002).

Deletion mapping of the regions in VP2 responsible for homologous interaction revealed that VP2 possesses multiple interaction domains (Chapter 6). This is consistent with available structural information about this external capsid

protein. Detailed electron cryomicroscopy and image processing analyses of different types of particles clearly revealed the trimeric VP2 structures in which each molecule can be subdivided into three subdomains, each capable of interacting in a nonequivalent fashion with another VP2 molecule of the same or an adjoining trimer (Caston *et al*., 2001).

The amino-terminal 97 amino acids of VP3, was identified as the domain responsible for VP3 homomeric interaction (Chapter 6). The functional relevance of this binding domain is discussed below (see **The interacting domains of VP3**).

VP4, the viral protease, was anticipated to exhibit homologous interaction. In IBDV-infected cells this protein assembles into distinct tubular aggregates, 24-26 nm in diameter, known as type II tubules (Granzow *et al*., 1997). In our initial yeast two-hybrid screening we made use of a VP4 two-hybrid fusion protein that was based on the presumed cleavage sites of the structural protein precursor reported by Hudson *et al*. in 1986. Here, we failed to detect the homomeric interaction of these VP4 molecules (Chapter 2). However, later these processing sites appeared to be incorrect and the exact cleavage sites were newly established in 1999 and 2000 by Sanchez & Rodriguez and Lejal *et al*., respectively. We therefore re-evaluated our findings and this allowed the detection of the homologous interaction of VP4 (Chapter 6). Attempts to identify interacting domain(s) in this polypeptide failed; VP4-VP4 interaction appeared to be very sensitive to modifications in the polypeptide (Chapter 6).

The exact function of VP5 is still unknown. The protein is dispensable for virus replication in vitro but important for pathogenicity (Mundt *et al*., 1997; Yao *et al*., 1998). It has recently been shown that VP5 is a cytolytic protein that accumulates within the plasma membrane of infected cells and promotes the egress of progeny virions (Lombardo *et al*., 2000; Yao & Vakharia, 2001). It was hypothesized by Lombardo *et al*. (2000) that VP5 molecules might form pore structures within the host plasma membrane. Hence, the homotypic interaction of VP5 that we have described in this thesis (Chapters 2 and 6) would fit in this hypothesis. Interestingly, the putative self-interacting domain of VP5 that we mapped in our two-hybrid analysis to the domain comprising residues 73-90, corresponds almost exactly to the predicted transmembrane region (residues 69-88) of the protein (Lombardo *et al*., 2000). Due to the common α-helical structure of transmembrane domains, many are considered to form channels or pores in the membrane by a so-called "barrel-stave" mechanism (reviewed in Ojcius & Young, 1991). The barrel-stave mechanism involves three major steps: (i) binding of monomers to the membrane; (ii) insertion into the membrane to form a pore; and (iii) progressive recruitment of additional monomers to increase the pore size (Shai, 1995). Initial assembly of monomers on the

surface of the membrane must occur before the peptide is inserted, since it is energetically unfavorable for an amphipatic α-helix to traverse the membrane as a monomer. A homotypic interaction of VP5 through a self-interacting domain located within its predicted membrane-spanning region is therefore consistent with the hypothesis that VP5 molecules co-assemble at the host plasma membrane to form a pore structure. It would hence be interesting to evaluate whether mutations of conserved amino acids in the putative transmembrane region of VP5 would affect its membrane-permeabilizing activity.

VP1 also associates with itself but the significance of this interaction has not yet been established (Chapters 2 and 6). It is conceivable that a homologous interaction of VP1 can occur. Xiang *et al*. (1998) reported an interaction between VPg and the polymerase $3D^{pol}$ of poliovirus. The poliovirus protein VPg is covalently linked to the 5' end of both genomic and antigenomic viral RNA and $3D^{pol}$ 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) (Muller & 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 as polymerase for chain elongation (Dobos, 1995*b*).

HETEROMERIC INTERACTIONS BETWEEN THE VIRAL PROTEINS OF IBDV

We found that a heterologous complex between VP1 and VP3 can be formed in yeast cells (Chapter 2). This heteromeric interaction was also detected *in vivo* in IBDV infected cells (Chapter 2). Our results are consistent with the results described by Lombardo *et al*. (1999), who observed the interaction of VP1 with VP3 by co-localization and co-immunoprecipitation studies of vaccinia virus-expressed VP1 and VP3. Their and our results raised several interesting possibilities regarding the function of the VP1-VP3 interaction. We therefore have followed up on this supposed essential interaction. By using the yeast two-hybrid system as well as by mutagenesis of an infectious cDNA clone of IBDV we mapped the domain in VP3 interacting with VP1 to the extreme carboxy-terminal tail of the polypeptide (Chapter 3).

Recently, Maraver *et al*. (2003*a*) reported similar results. They established the VP1 binding motif of VP3 to be present within the highly charged 16-amino-acid stretch on the C terminus of VP3. These results are comparable with ours; the VP3 domain interacting with VP1 in our yeast two-hybrid analyses was mapped to its carboxy-terminal 10 amino acids (Chapter 3). Unfortunately, Maraver *et al*. (2003*a*)

did not test a VP3 deletion mutant lacking its 10 carboxy-terminal residues in their assay. In spite of this, there is one other obvious difference between these two studies. The domain mapping of VP3 that Maraver *et al.* used to identify the VP1 binding motif was based on co-immunoprecipitation analysis of vaccinia virus-expressed VP1 and VP3(-mutants), whereas the co-immunoprecipitation analysis that we used to confirm our yeast-two hybrid analysis was based on an IBDV reverse genetics system. This allowed us to analyze the interaction of carboxy-terminally truncated VP3 molecules with VP1 in infected cells. By using this strategy we found that *in vivo* the removal of just the terminal residue was already sufficient to affect its association with VP1 and also to fully abolish the production of infectious progeny (Chapter 3). Maraver *et al.*, on the other hand, studied the role of the VP1 binding motif *in vivo* using only the complete 16-amino-acid stretch of the extreme C terminus of VP3 (Maraver *et al.*, 2003*a*). They analyzed the effect of Trojan peptides (synthetic peptides) containing this 16-amino-acid stretch of the C terminus of VP3, on virus replication. They showed that the presence of these Trojan peptides in IBDV-infected cells specifically reduced infective virus production.

Altogether, their and our results demonstrate that the formation of VP1-VP3 complexes plays a crucial role during virus morphogenesis.

INTERACTIONS BETWEEN THE VIRAL COMPONENTS OF IBDV: PROTEIN-dsRNA INTERACTIONS

In 1986 Hudson *et al.* (1986) already suggested that VP3, by virtue of its basic carboxy-terminal domain, might interact with the packaged viral dsRNA genome. Later, in 1999, studies performed with the aquabirnavirus infectious pancreatic necrosis virus (IPNV) showed the presence of VP3 in ribonucleoprotein complexes isolated from virus particles (Hjalmarsson *et al.*, 1999). These complexes were shown to contain VP3 and RNA. However, it was not determined whether VP3 directly bound to RNA molecules or to VP1 complexed to dsRNA (Hjalmarsson *et al.*, 1999). Additionally, VP3-RNA complexes could also be purified from supernatants of IPNV-infected cell cultures, although the nature of the bound RNA was not determined (Hjalmarsson & Everitt, 1999). These observations along with the fact that VP3 coats the inner surface of the virus particle (Bottcher *et al.*, 1997; Caston *et al.*, 2001) emphasized the possibility that VP3 indeed establishes direct interactions with the virus genome.

In this thesis, we provided the first experimental evidence for this long-anticipated role of VP3 in binding to viral nucleic acids (Chapter 3). RNase treatments and reverse transcription-PCR analyses of VP1- and VP3-immunoprecipitates (i.e. prepared from lysates of cells undergoing infection)

demonstrated that VP3 interacts with dsRNA of both viral genome segments (Chapter 3). Additionally, we showed that this interaction is not mediated by the extreme carboxy-terminal end of VP3 since C-terminal truncations of 1, 5, or 10 residues did not prevent formation of the VP3-dsRNA complexes (Chapter 3).

Recently, Kochan *et al.* (2003) further characterized the RNA-binding activity of VP3 and showed that VP3 interacts with RNA in a sequence-independent fashion. The lack of any binding specificity for ssRNA probes that they used in their study might indicate that, as is the case with other virus-encoded nucleoproteins (Gott *et al.*, 1993; Klein *et al.*, 2000; Muriaux *et al.*, 2001; Skuzeski & Morris, 1995), other factor(s) could be necessary to confer specificity. VP1 is the most likely candidate for this role. Interestingly, VP3 also bound dsRNA and ssDNA, but not dsDNA. These binding characteristics are usually found in RNA-binding proteins, especially in dsRNA-binding proteins (Draper, 1999). Furthermore, according to the results of Kochan *et al.* (2003) the VP3 ssRNA-binding domain is located within a highly conserved 69-amino-acid stretch close to the N-terminus of the protein. Whether this domain also comprises the dsRNA-binding motif is currently under investigation by this group. It is, however, obvious that this domain is different from the highly basic carboxy-terminal domain that was predicted by Hudson *et al.* (1986) to be involved in binding to viral nucleic acids. As VP3 does not comprise any of the known RNA or DNA binding motifs that have previously been described in the literature such as zinc fingers, arginine-rich regions or RGG-box sequences (Burd & Dreyfuss, 1994; Kochan *et al.*, 2003), the exact domain of VP3 that comprises the dsRNA-binding motif therefore still remains a subject for further investigation.

THE INTERACTING DOMAINS OF VP3

It is now clear that VP3 entertains interactions with itself (Chapters 2 and 6), with VP2 (Bottcher *et al.*, 1997; Caston *et al.*, 2001), with free VP1 molecules (Chapters 2 and 3; and Lombardo *et al.*, 1999; Maraver *et al.*, 2003*a*), most likely also with genome-bound VP1 (Chapter 3), and with dsRNA segments A and B (Chapter 3; and Kochan *et al.*, 2003). Different domains in the protein are responsible for these different interactions. The carboxy-terminal domain binding to VP1, for instance, proved to be distinct from the region binding to the dsRNAs (Chapter 3; and Kochan *et al.*, 2003) and from the region responsible for homomeric interaction (Chapter 6). These data indicate that these three domains of VP3 can function independently from each other.

In this thesis, the amino-terminal part of VP3 comprising residues 1-97, was identified as the domain responsible for homomeric interaction (Chapter 6). Maraver *et al.* (2003*b*), on the contrary, recently mapped an oligomerization

domain of VP3 within a 24-amino-acid stretch near the carboxy-terminal end of the polypeptide, which partially overlaps the VP1 binding domain. Their results are (solely) based on the existence of a ladder of high-molecular mass VP3-specific protein bands, ranging from 65 to 170 kDa, in samples prepared from insect cells expressing His-tagged VP3 fusion proteins that proved to be resistant to denaturation. Comparing this study to the data presented in this thesis indicates that there are obviously some discrepancies. For instance, we have never seen such a ladder of high-molecular mass, denaturation resistant, VP3-specific protein bands in similar experiments, when expressing influenza virus hemagglutinin (HA)- and/or c-*Myc*-tagged VP3 fusion proteins in avian cells (Chapter 6). In contrast, we showed that, under denaturating conditions, all of the existing viral component interactions, with the exception of the VPg-dsRNA interactions, were completely disrupted (Chapters 2, 3 and 6). Furthermore, we showed that deletion of the VP1-binding domain in VP3, comprising the 10-amino-acids stretch at its carboxy-terminal end, or even a deletion of the complete carboxy-terminal half of the protein (hence, in addition comprising the VP3 oligomerization domain mapped by Maraver *et al.*, 2003*b*), did not affect the VP3-VP3 interaction in two of our different and independent assays (Chapter 6 and unpublished results). It is difficult to speculate about the reasons underlying these conflicting results. Understanding the discrepancies between these two different studies on the mapping of the homomeric interaction domain of VP3 will thus need further research.

It has frequently been seen, by us as well as by others, that VP3 occurs in (at least) two electrophoretic forms in IBDV-infected cells (Chapter 2). One of these bands has the expected size of VP3 (32 kDa) while the other seems to be smaller (28 kDa). The existence of two VP3 species has also been reported for IPNV (Dobos & Rowe, 1977; Hjalmarsson & Everitt, 1999; Dobos, 1995*a*). The nature of the smaller VP3 species, however, remained unclear. Pulse-chase analyses indicated that only full-length VP3 is incorporated in IBDV virions (Chapter 2). Additionally, mass spectrometry of purified IBDV particles revealed the presence of only the full-length VP3 (M.G.J. Tacken, H.J. Boot, B.P.H. Peeters, P.J.M. Rottier, B. Delmas and E. Mundt, unpublished results). This suggests that the shorter form might have additional, non-structural, functions. We speculated that it could be generated by internal initiation of translation at an AUG codon located 17 residues downstream from the bona fide initiator codon. Alternatively, the full-length protein might undergo a proteolytic trimming. The importance of the AUG codon was assessed by reverse genetics (M.G.J. Tacken, H.J. Boot, B.P.H. Peeters, P.J.M. Rottier, B. Delmas and E. Mundt, unpublished results). Our results showed that the IBDV mutants having the AUG codon (Methionine) exchanged for a CTC codon (Leucine) or an ATT codon

(Isoleucine) were reduced in their efficiency to generate recombinant IBDV (Table 1), though still able to accumulate both VP3 species in infected cells (Fig. 1).

	$TCID_{50}$ [a]
rIBDV[b]	6.6
IBDV-segmentA[c]	-
mIBDV[d]-M772A	-
mIBDV-M772T	-
mIBDV-M772P	-
mIBDV-M772N	-
mIBDV-M772Q	-
mIBDV-M772E	-
mIBDV-M772V	-
mIBDV-M772L	3.3
mIBDV-M772I	2.3
mIBDV-M772G	-
mIBDV-M772S	-

Table 1. Effect of substituting methionine at position 772 of the polyprotein on replication. The effect was assessed by reverse genetics; derivates of either plasmids pHB-36W (A-segment of serotype I strain CEF94; Boot *et al*., 1999) or p2A (A-segment of serotype I of strain P2; Mundt *et al*., 1996) were transfected in combination with plasmids pHB-34Z (B-segment of strain CEF94; Boot *et al*., 1999) or p2B (B-segment of strain P2; Mundt *et al*., 1996) respectively, into IBDV susceptible cells and the resulting IBDV titer was determined 24 h after transfection.
[a] 50% tissue culture infective doses ($TCID_{50}$) is as Log 10 values
[b] rIBDV; unmodified rescued IBDV (positive control)
[c] only the IBDV-segment A was transfected (negative control)
[d] mIBDV; mutagenized IBDV having the methionine at position 772 of the polyprotein exchanged for the amino acid as indicated

Unfortunately, attempts to N-terminally sequence both VP3 species to determine whether the smaller VP3 species had been proteolytically trimmed at its N-terminus have failed as it turned out that these proteins were blocked (M.G.J. Tacken, H.J. Boot, B.P.H. Peeters, P.J.M. Rottier, B. Delmas and E. Mundt, unpublished results). Interestingly, Maraver *et al*. (2003*b*) recently showed that the VP3 protein synthesized by recombinant baculoviruses (rBVs) in insect cells, is proteolytically

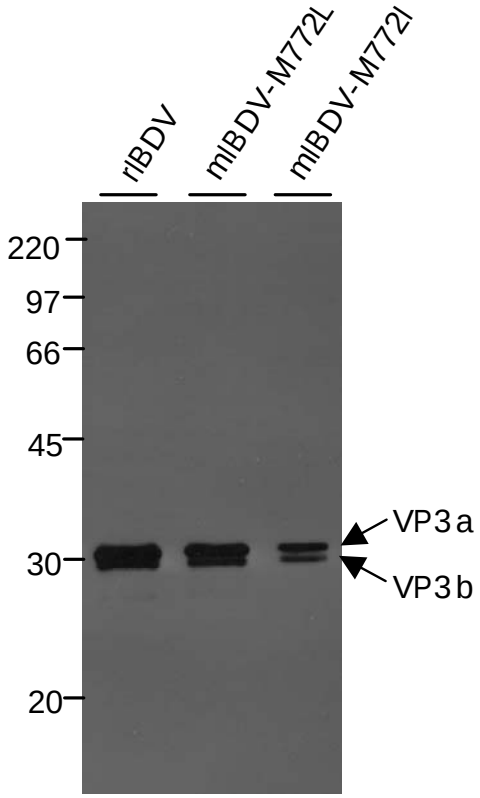

Figure 1. Western blot analysis of VP3 in rIBDV, mIBDV-M772L and mIBDV-M772I (see Table 1) infected avian cells. VP3 proteins (indicated by arrows) were visualized using a VP3 specific monoclonal antibody. The position of marker proteins and their sizes (kDa) are indicated on the left.

cleaved at the C-terminal end of the protein, leading to the accumulation of a product lacking the 13 C-terminal residues. It would be interesting to determine whether this VP3-derived product is identical to the smaller VP3 species observed in IBDV-infected cells. If so, it would be additionally interesting to analyze its possible role during virus replication. This VP3-derivative lacking its 13 C-terminal residues undoubtedly has lost its VP1-binding activity, though, does it still possess its homomeric and dsRNA-binding activities? Maraver *et al.* (2003*b*) additionally showed that coexpression of VP1 prevents VP3 trimming and restores virus like particle (VLP) formation in insect cells. They speculated that the formation of VP3-VP1 complexes might hinder the VP3 cleavage site, and thus protect the VP3 C-terminal tail against proteolysis.

INTERACTIONS BETWEEN VIRAL AND HOST CELL PROTEINS

Identification of cellular interaction partners of the viral proteins of IBDV could provide a more complete understanding of the dynamics of RNA replication, virus-mediated cellular modulation and host-range restriction. In this thesis, we presented a yeast two-hybrid search for candidate cellular proteins interacting with the IBDV proteins VP1, pVP2, VP3, and VP5 (Chapter 4). We used the inducible LexA-dependent yeast two-hybrid system to screen a cDNA library from bursae of Fabricius of 3-weeks-old chickens. We found that several host cell proteins were able to form complexes with the IBDV proteins in yeast cells (Chapter 4). The putative biological implications for some of the interactions found were discussed in Chapter 4. A first step to demonstrate biological relevance was to verify these interactions by a different biochemical assay. For this purpose, we performed a series of co-immunoprecipitations using different antibodies that were kindly provided by several groups (Table 2). However, as yet, only one of these virus-host interactions found in yeast could be convincingly confirmed by co-immunoprecipitation analysis, i.e. the interaction between VP1 and the carboxy-terminal domain of translation eukaryotic initiation factor 4AII (eIF4AII). Therefore, we have followed up on this supposedly essential interaction in Chapter 5.

CHARACTERIZATION OF THE PUTATIVE INTERACTION BETWEEN VP1 AND eIF4AII

Eukaryotic initiation factor 4A is an essential component of the translation initiation system. Normally it binds to the initiation complex in a cap-mediated way (Fig. 2A) (Gingras *et al.*, 1999). An interaction of VP1 (or VPg) with eIF4AII is intriguing particularly because IBDV mRNAs are not capped but possess a covalently linked VPg at their 5' end. A direct interaction of eIF4A with VPg may therefore be a key event in the establishment of IBDV infection. Possibly, it represents a novel cap-independent mechanism for the initiation of translation in which the binding of eIF4A to VPg mediates the recruitment of the 40S ribosomal subunit to the 5' end of the viral mRNA. Whereas an interaction between the cap-structure and eIF4E is required for normal capped translation, an interaction between proteolytically cleaved eIF4G and an internal ribosome entry site for uncapped picornaviral mRNA

Table 2. Antibodies used for co-immunoprecipitation analysis. The following antibodies were used for co-immunoprecipitation analysis, in order to verify the biological relevance of some of the interactions found in yeast between IBDV and host cell proteins described in chapter 3. Other antibodies used in these co-immunoprecipitation analyses which are not listed as they were achieved from our own laboratory, were anti-VP1, anti-VP2, anti-VP3 and anti-VP5.

Potential cellular interactors of VP1		
Paladin		
anti-paladin	polyclonal (rabbit)	Dr. J. Pearce, Wellcome CRC Institute, University of Cambridge, UK.
NAD(+) Isocitrate dehydrogenase		
anti-Idh	polyclonal (rabbit)	Dr. H. van der Spek, Swammerdam Institute for Life Sciences, University of Amsterdam, The Netherlands.
RAN binding protein 1 (RanBP1)		
anti-RanBP1	polyclonal (goat)	Santa Cruz Biotechnology, Inc.
Eukaryotic translation initiation factor 4A, isoform 2 (eIF4AII)		
anti-eIF4AII	polyclonal (rabbit)	Dr. C. Kuhlemeier, University of Berne, Switserland
anti-eIF4AII	monoclonal (mouse)	Dr. H. Trachsel, University of Berne, Switserland
Potential cellular interactors of VP2		
Ornithine decarboxylase antizyme (ODC-AZ)		
anti-ODC-AZ	polyclonal (rabbit)	Dr. J.L.A. Mitchell, Center for Biochemical and Biophysical Studies, Northern Illinois University, USA
anti-ODC-AZ	polyclonal (rabbit)	Dr. O.A. Jänne, Biomedicum Helsinki, University of Helsinki, Finland
anti-ODC-AZ	polyclonal (rabbit)	Dr. S. Matsufuji, Jikei University School of Medicine, Minato-ku, Tokyo, Japan
anti-ODC-AZ	monoclonal (mouse)	Dr. S. Matsufuji, Jikei University School of Medicine, Minato-ku, Tokyo, Japan
Sin3-associated polypeptide 18 (Sap18)		
anti-SAP18	polyclonal (rabbit)	Dr. A. Kuzmichev and Dr. D. Reinberg, Howard Hughes Medical Institute, University of Medicine and Dentistry of New Jersey, Piscataway, New Jersey, USA
anti-SAP18	polyclonal (rabbit)	Dr. S. Hanes, New York State Department of Health & State University of New York-Albany, USA
Potential cellular interactors of VP3		
RAN binding protein 1 (RanBP1)		
anti-ranbp1	polyclonal (goat)	Santa Cruz Biotechnology, Inc.
Thymocyte protein cThy28kD		
anti-cThy28	polyclonal (rabbit)	Dr. M.M. Compton, Department of Poultry Science, University of Georgia, Athens, Greece
Embryonic ectoderm development protein (EED)		
anti-EED	polyclonal (rabbit)	Dr. A.P. Otte, BioCentrum Amsterdam, University of Amsterdam, The Netherlands
Potential cellular interactor of VP5		
Ornithine decarboxylase antizyme (ODC-AZ)		
anti-ODC-AZ	polyclonal (rabbit)	Dr. J.L.A. Mitchell, Center for Biochemical and Biophysical Studies, Northern Illinois University, USA
anti-ODC-AZ	polyclonal (rabbit)	Dr. O.A. Jänne, Biomedicum Helsinki, University of Helsinki, Finland
anti-ODC-AZ	polyclonal (rabbit)	Dr. S. Matsufuji, Jikei University School of Medicine, Minato-ku, Tokyo, Japan
anti-ODC-AZ	monoclonal (mouse)	Dr. S. Matsufuji, Jikei University School of Medicine, Minato-ku, Tokyo, Japan Tokyo, Japan

translation (Lamphear *et al*., 1995), an interaction between VPg and eIF4A may have a similar function in translating uncapped IBDV mRNA. Two possible models describing how an eIF4A-VPg interaction might induce the recruitment of the 40S ribosomal subunit for IBDV initiation of translation are dicussed below (see also figure 2).

The association of VP1 with the carboxy-terminal domain of eIF4AII was found in yeast (Chapters 4 and 5) and confirmed by co-immunoprecipitation analyses (Chapter 5). Interestingly, no interaction was observed between VP1 and full-length eIF4AII, nor with its functionally equivalent isotype eIF4AI. We also investigated whether in infected cells an eIF4AII carboxy-terminal fragment is generated by some kind of cleavage, similar to what has been found for eIF4AI in foot-and-mouth disease virus (FMDV)-infected cells (Li *et al*., 2001). No breakdown of eIF4AII was, however, observed, the protein being as stable as in non-infected cells (Chapter 5).

In view of the known two-domain structure of the eIF4AII protein (Caruthers *et al*., 2000) it is conceivable that the interaction of VP1 with the full-length molecule requires collaborating proteins that open up its structure and expose the VP1-binding site in the carboxy-terminal domain. Based on our observations described in Chapter 5 and on published information we propose two possible models for the initiation of translation of IBDV mRNAs, which may hold as well for other birnaviridae (Fig. 2). We postulate that binding of VP1 to eIF4A is required for the recruitment of the translational machinery, which in turn promotes 43S pre-initiation complex binding to the mRNA. The molecular interactions that enable the incoming 43S pre-initiation complex to bind mRNA are thought to involve interaction of the eIF3 component of 43S complexes with eIF4G. However, in yeast a direct eIF3-eIF4G interaction has not been reported. The two models we propose differ primarily in the role played by eIF4G. Model 1 (Fig. 2B) states that eIF4A bound to VPg attracts eIF4G. In this model, binding of the 43S pre-initiation complex is mediated by an interaction of eIF3 with eIF4G. It is assumed that eIF4G is not preassembled in the eIF4F complex but, rather, interacts with the 43S pre-initiation complex prior to mRNA recruitment. This has been postulated before (Gingras *et al*., 1999; Joshi *et al*., 1994; Lamphear *et al*., 1995) and is based on the documented interaction of *in vitro* synthesized eIF4G with the 43S pre-initiation complex in the absence of mRNA (Joshi *et al*., 1994). The second model, which we favor, proposes that VPg executes the pivotal functions of both eIF4E and eIF4G by delivering eIF4A to the 5' end of the viral mRNA, where it is appropriately positioned to unwind secondary structure and provide a single-stranded RNA region as a landing pad for the 43S pre-initiation complex (Fig. 2B; Model 2). This model suggests that due to the unwound secondary

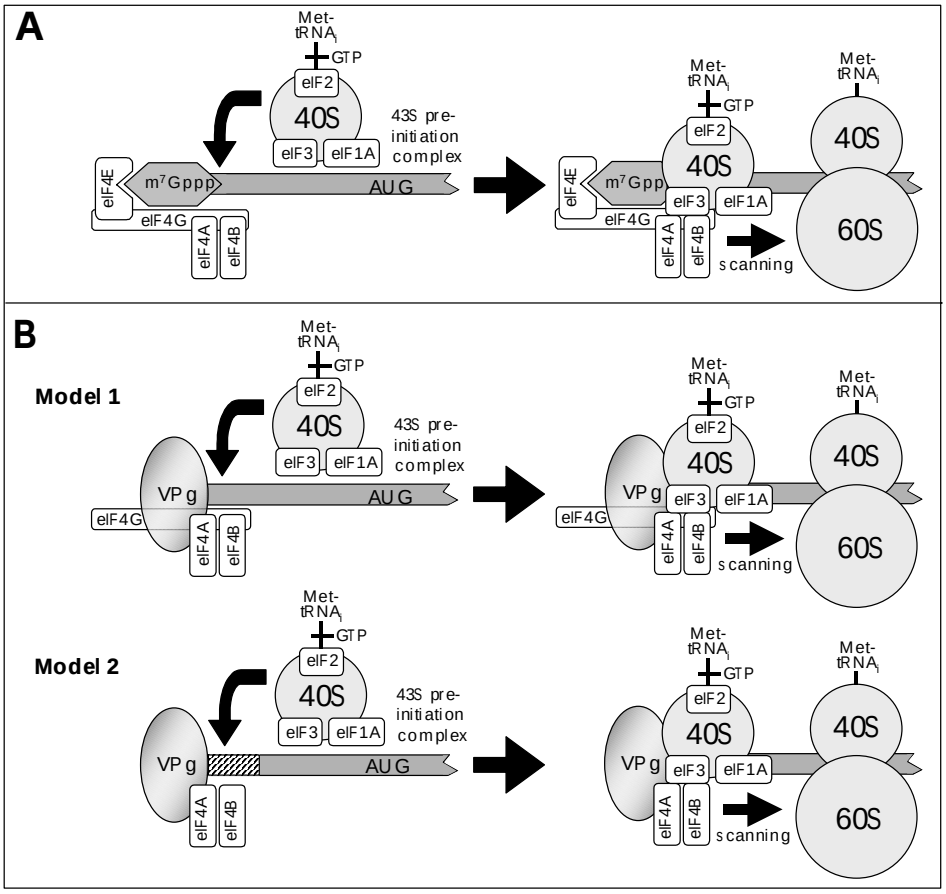

Figure 2. Hypothetical models for cap-independent initiation of translation of IBDV mRNA, in comparison with cap-dependent initiation of translation of its host cell mRNAs. (A) A typical capped mRNA of the eukaryotic host cell. eIF4F (i.e. eIF4E, eIF4G and eIF4A) binds to the 5' cap and eIF4B joins eIF4A to unwind secondary structure of the mRNA. eIF3, eIF1A and the ternary complex (i.e. eIF2, GTP and initiator methionyl-tRNA (Met-tRNAi)), all bound to the 40S ribosomal subunit, form the 43S pre-initiation complex. This complex binds downstream of the cap and then scans the 5' leader to locate the first AUG initiation codon. Subsequently the 60S subunit joins to form the translationally competent 80S ribosome. (B) Uncapped IBDV mRNA with VPg covalently linked at the 5' end. Model 1: eIF4A bound to VPg attracts eIF4G that serves as landing platform for the 43S pre-initiation complex. The binding of the 43S pre-initiation complex to mRNA presumably occurs via eIF3-eIF4G interaction. Model 2: VPg interacts with eIF4A and executes the function of both eIF4E and eIF4G in cap-dependent translation, by bridging eIF4A to the mRNA. After sufficient mRNA secondary structure is unwound, the 43S pre-initiation complex binds the mRNA, possibly by base pairing between the putative 18S rRNA binding site (a 32 nt consensus sequence present in both IBDV segments; depicted as a dashed box) and its complement within the 40S ribosomal subunit.

structure in the 5' region of the mRNA, the 43S pre-initiation complex is able to bind to the mRNA, presumably by base pairing between the putative binding site for 18S rRNA, present in both IBDV segments (Mundt & Muller, 1995), and its complement within the ribosomal RNA in the 40S ribosomal subunit. Alternatively, the binding of the 43S pre-initiation complex to the mRNA could be mediated by an eIF3-RNA interaction. Functional eIF3-mRNA interactions for translation initiation have been described for foot-and-mouth disease virus (FMDV), hepatitis C virus (HCV) and classical swine fever virus (CSFV) (reviewed in Martinez-Salas *et al*., 2001). However, these interactions involve extended tertiary RNA structures which do not seem to occur in the 5' UTR of IBDV mRNAs. This model is based on the fact that the activity of eIF4G for IBDV translation may be redundant. Various activities of eIF4G are not required such as its binding to eIF4E, its docking to MAP kinase-interacting protein kinase-1 (Mnk1) to allow eIF4E phosphorylation, and its interaction with poly(A)-binding protein (PABP) to bridge the 5' cap structure to the 3' poly(A) sequence (Gingras *et al*., 1999). Interestingly, during FMDV-infection eIF4G is proteolytically cleaved, thereby separating the eIF4E- and PABP-binding domain from the eIF4A- and eIF3-binding domain (Lamphear *et al*., 1995). As we assessed by Western blot analysis, a similar cleavage of eIF4G did not take place in IBDV infected cells (data not shown).

It has been argued that communication between the 5' and 3' ends of mRNAs is important for regulation and efficiency of mRNA translation (Sachs, 2000). This view gained support by the demonstrated circularisation of a large number of viral and nonviral mRNAs that lack a cap or a poly(A) tail (Guo *et al*., 2001 and references therein). It is therefore expected that also the uncapped and non-polyadenylated IBDV mRNAs require a functional interaction between terminal RNA elements similar to that observed between the canonical 5' cap and 3' poly(A) tail. It is exciting to speculate that the predicted stem-loop structure of the 3' terminus of the IBDV mRNAs (Boot *et al*., 1999), might be involved in such a closed loop complex, operating as a surrogate for the poly(A) tail in enhancing the translation of viral mRNAs. Our finding that VP3 interacts both with VP1 and with the genomic dsRNA segments of IBDV (Chapter 3), and hence possibly also with the dsRNA region of the stem-loop structure, adds to the interest of this hypothesis.

In contrast to many viruses that induce host translational shut-off, IBDV infection is not associated with a decrease in host protein synthesis (Chapter 5). How, then, do IBDV mRNAs adequately compete for available translation factors? The supposed binding of eIF4A to VPg most likely occurs transiently, where bound eIF4A is expected to be readily exchanged with free eIF4A, similar to when it recycles through the eIF4F complex (Pause *et al*., 1994). As eIF4A is the most

abundant of the translation initiation factors present in cells (three- to sixfold in relative excess over other initiation factors; Duncan & Hershey, 1983), a possible sequestering of eIF4A by VPg would not be detrimental for cellular translation. Moreover, a possible partial depletion of available eIF4A, sequestered by VPg, mainly affects the eIF4AII isoform, leaving the pool of ubiquitous and functionally equivalent eIF4AI accessible for host translation. Furthermore, in both our proposed models the cap-binding protein eIF4E, the least abundant of the initiation factors (Duncan *et al.*, 1987), is supposed not to be required for the translation of the uncapped IBDV mRNAs, leaving host translation unaffected. Finally, phosphorylated eIF4E-binding protein 1 (4E-BP1), which liberates and activates eIF4E (Gingras *et al.*, 1999), remained unaffected upon IBDV infection (data not shown), consistent with our observed lack of inhibition of host cell protein synthesis by IBDV infection.

CLOSING REMARKS AND FUTURE PROSPECTIVES

In conclusion, the experiments described in this thesis showed that yeast two-hybrid interaction mapping provided a valuable (first) tool for the identification and molecular characterization of different interactions occurring in the IBDV life cycle.

An association of eIF4AII with IBDV VP1 or, rather, with viral mRNA-linked VPg points to an important function of this viral protein in IBDV mRNA translation. However, future studies will be required to confirm and establish the functional significance of this interaction for viral multiplication. It would be interesting to know whether VP1, when linked to viral RNA (i.e. as VPg) can indeed bind the initiation factor and support translation initiation of viral RNA, without compromising host mRNA translation. Another interesting question relates to the occurrence of 5'-3' interactions in the IBDV mRNAs and to the binding partners that mediate these interactions.

The VP3 protein seems to be the key organizer in birnavirus morphogenesis. By its interactions with all the structural components in the virion it appears to create the interior architecture that is required for the proper execution of replication, transcription and assembly activities of these viruses. Nevertheless, knowledge about the morphogenesis of birnaviruses is still very limited and, while finishing the manuscript of this thesis, many questions remain. For instance, as VP1 resides inside the viral particle and VP3 forms trimeric subunits located at the inner surface of the virion shell, it is an intruiging question of how the VP1 binding activity of VP3 is regulated, given that only a few VP1 molecules are encapsidated into a virion. It will also be interesting to find out if and how the virus regulates the numbers of free and genome-bound VP1 molecules assembled in each viral particle. Furthermore, it is still an unresolved issue how the two genomic dsRNA segments are correctly assorted

during particle assembly. A role for the VPg moiety in the selection of the genome segments is conceivable. Alternatively, this selection might be achieved by VP3 through its capacity to bind dsRNA. However, this capacity might of course also only assist in the proper positioning of the two dsRNA segments within the virion. Nevertheless, if this dsRNA binding activity would indeed operate at the level of segment selection during particle assembly we should assume that VP3 binds to specific double-stranded regions within the viral mRNAs, as all dsRNA viruses are assumed to synthesize their negative RNA strand only after the packaging of their mRNAs. The specific encapsidation of IBDV mRNAs might thus resemble the mechanism by which the hepatitis B virus assembles its pregenomic RNA. Here the viral polymerase binds to a specific stem-loop structure present in viral mRNAs which subsequently leads to the polymerase-dependent encapsidation of the viral nucleic acid (Bartenschlager & Schaller, 1992; Ziermann & Ganem). Much work will be needed to resolve these issues, not only for IBDV but as well for other dsRNA viruses.

Continued molecular virological studies in combination with ultrastructural analyses are likely to be required to answer the many outstanding questions concerning the biology of these viruses.

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CHAPTER 7

Nederlandse Samenvatting

Moleculaire Interacties van de Infectious Bursal Disease Virus Eiwitten

NEDERLANDSE SAMENVATTING

Het infectious bursal disease virus (IBDV), ook wel het Gumboro virus genoemd, veroorzaakt de zeer besmettelijke ziekte infectieuze bursitis (IBD) bij kippen. De ziekte werd voor het eerst in 1957 waargenomen in de Amerikaanse plaats Gumboro (Delaware), vandaar het eponiem “De ziekte van Gumboro”. Het virus heeft zich inmiddels wereldwijd verspreid. De ziekte komt uitsluitend voor bij gevogelte (kippen en kalkoenen) en wordt gekenmerkt door de vernietiging van de lymphoide organen, met name de bursa van Fabricius, waar het virus de actief delende en differentiërende B-lymphocyten infecteert. De ziekte kan fataal zijn of een immunosuppressie veroorzaken die dan vaak van tijdelijke aard is, afhankelijk van de dosis en virulentie van de IBDV stam, de leeftijd van de dieren en het dierenras, en van de aan- of afwezigheid van passieve immuniteit. Het IBDV is een dubbelstengs RNA (dsRNA)-virus dat twee segmenten (A en B) bevat en daardoor behoort tot de familie *Birnaviridae* en het geslacht avibirnavirus. Tot deze familie behoren ook het infectious pancreatic necrosis virus (IPNV), voorkomend bij vissen, het tellina virus (TV) en het oyster virus (OV), voorkomend bij tweekleppige schelpdieren, en het Drosophila X virus (DXV), voorkomend bij fruitvliegjes.

Het virus is zeer stabiel en persistent, dat wil zeggen moeilijk te verwijderen met desinfecterende middelen. De ziekte vormt een grote bedreiging voor de kippenhouderij en is alleen beheersbaar door middel van een strict en intensief vaccinatieprogramma. IBD is al enkele decennia endemisch in Nederland en Europa, waarbij een evenwicht bleek te bestaan tussen de weerstand van gevaccineerde dieren en het veldvirus. Vanaf 1988 veroorzaakte het voorkomen van “very virulent” (vv) IBD virussen uitbraken met een zeer hoge uitval, ondanks vaccineren. Elders in de wereld (U.S.A.) zijn variantstammen geisoleerd die in antigene eigenschappen verschillen van de eerder circulerende (klassieke) virusstammen en waartegen de klassieke vaccins minder bescherming bieden.

Er is weinig bekend over de biochemische gebeurtenissen tijdens de replicatie van birnavirussen in het algemeen en van IBDV in het bijzonder. Om meer te weten te komen over dit proces is het onder andere van belang om meer inzicht te krijgen in de verschillende interacties die plaatsvinden tijdens de virale levenscyclus van IBDV. Bij aanvang van dit onderzoek was hierover nog weinig bekend. Wel werden er enkele interacties voorspeld op basis van een cryo-electronenmicroscopische structuur van het viron, maar over eiwit-eiwit interacties tussen virus en gastheer was nog helemaal niets bekend. Het doel van dit onderzoek was daarom een inventarisatie te maken van de interacties die plaatsvinden tussen de virale eiwitten van IBDV onderling en tussen virus en gastheer

eiwitten. Hiertoe werd gebruik gemaakt van het destijds net ontwikkelde yeast two-hybrid systeem. Dit is een techniek waarmee op een relatief eenvoudige manier *in vivo* specifieke eiwit-eiwit interacties in gist aangetoond kunnen worden. De techniek kan gebruikt worden om na te gaan of er interacties bestaan tussen twee bekende eiwitten of om een bibliotheek van eiwitten te screenen op interactie met een bekend targeteiwit. Vervolgens kan er met behulp van plaatsgerichte mutagenese achterhaald worden waar zich de precieze bindingsdomeinen van één of beide eiwitten bevinden.

Allereerst werden de interacties tussen de virale eiwitten van IBDV onderling geanalyseerd. Het genoom van IBDV codeert voor een vijftal virale eiwitten, VP1-VP5; drie hiervan zijn structureel, dat wil zeggen komen voor in rijpe virus deeltjes (VP1, VP2 en VP3), en twee zijn niet-structureel (VP4 en VP5). VP2, VP3 (beide capside eiwitten) en VP4 (een protease) worden gevormd door autoproteolytische klieving van een precursor (het polyproteine) die gecodeerd wordt door segment A. VP5 (exacte functie onbekend) wordt ook gecodeerd door segment A, maar vanaf een apart open leesraam. VP1 tenslotte, RNA polymerase, wordt gecodeerd door segment B. Het yeast two-hybrid systeem werd gebruikt om alle mogelijke combinates tussen deze vijf eiwitten te testen op interactie. Uit deze experimenten bleek dat verschillende complexen in gist gevormd kunnen worden, enkele homologe interacties, van VP2, VP3, VP5 en mogelijk van VP1, en één heterologe interactie tussen VP1 en VP3. De homologe interactie van VP1 was zeer zwak en werd om die reden niet als overtuigend positief gerekend. Aanvankelijk werd er geen homologe interactie voor VP4 waargenomen. Echter, in deze eerste two-hybrid screening werd gebruik gemaakt van hybride constructen welke gebaseerd waren op de tot dan toe veronderstelde klievingsplaatsen in het IBDV polyproteine. Later bleek dat deze processing sites afweken van de onlangs opnieuw bepaalde klievingsplaatsen. Om die reden is daarna nog een tweede two-hybrid screening uitgevoerd met nieuwe hybride constructen gebaseerd op deze nieuw bepaalde klievingsplaatsen. Hieruit bleek dat, naast de al eerder aangetoonde interacties, ook VP4 in staat was tot het vormen van een homologe interactie.

Vervolgens werd het bestaan van de heterologe interactie VP1-VP3 verder geanalyseerd in IBDV-geinfecteerde cellen door middel van co-immunoprecipitatie studies. Hieruit bleek dat de VP1-VP3 complexen worden gevormd in het cytoplasma van de gastheercel en later tijdens de infectie in het celculture medium terecht komen, hetgeen erop duidt dat deze interactie ook plaatsvindt in mature virions. Om de functie van deze interactie nader op te kunnen helderen werd daarna met behulp van plaatsgerichte mutagenese achterhaald waar zich de precieze bindingsdomeinen van deze twee eiwitten bevinden. Deze mapping-

studie werd gedaan door verschillende VP1 en VP3 deletie-constructen te testen op interactie met respectievelijk full-length VP3 en VP1 in het yeast two-hybrid systeem. Hieruit bleek dat het VP3-bindingsdomein van VP1 zich in een intern gelegen domein van het eiwit bevindt, en dat VP3 uitsluitend via zijn 10 meest carboxy-terminaal gelegen aminozuren met VP1 interacteert.

Om de functionaliteit van de VP1-VP3 interactie te bepalen werd gebruik gemaakt van IBDV reverse genetics. Bij reverse genetics wordt gebruik gemaakt van een infectieuze cDNA kloon van het virus. Met behulp van recombinant DNA technieken kunnen mutaties in deze cDNA kloon worden aangebracht. Door middel van een reverse genetic systeem wordt vervolgens dit cDNA omgezet in mRNA zodat het virus kan repliceren. Het recombinante IBDV kan uit een celkweek gezuiverd en geanalyseerd worden. Van dit systeem werd gebruik gemaakt voor het testen van een drietal verschillende VP3 mutanten. Het bleek dat bij een verwijdering van 1 aminozuur (het meest carboxy-terminale aminozuur van VP3; VP3ΔC1) de VP1-VP3 interactie *in vivo* nog wel, hetzij verzwakt, mogelijk was, maar dat bij een deletie van 5 of 10 aminozuren van de carboxy-terminus van VP3 (VP3ΔC5 en VP3ΔC10) deze interactie niet meer mogelijk was. Verder bleek dat geen enkele van deze drie mutanten in staat was om (infectieuze) virusdeeltjes te vormen in het reverse genetics systeem. Geheel onverwacht leidde dit onderzoek tevens tot het aantonen van de in de literatuur al lang gesuggereerde interactie van VP3 met het virale dsRNA genoom van IBDV. Door middel van RNase- en reverse transcriptase PCR analyse kon aangetoond worden dat VP3 in staat was om zowel segment A als B van het IBDV genoom te binden. Omdat ook VP3ΔC1, VP3ΔC5 en VP3ΔC10 in staat waren het virale dsRNA te binden bleek dat het dsRNA bindind domein van VP3 onafhankelijk functioneert van het VP1-bindingsdomein. Uit deze resultaten blijkt dat VP3 een essentiële rol speelt in de virus morfogenese, waarbij het niet alleen betrokken is bij de vorming van de capside struktuur maar ook bij de incorporatie van het genoom en het virale RNA polymerase, VP1.

Ook de homologe interacties, gevonden voor VP2, VP3, VP4 en VP5, werden verder gekarakteriseerd door middel van domein mapping, volgens eenzelfde strategie als voor de mapping van de VP1-VP3 interactie. De resultaten van deze mappings studie werden daarna, althans daar waar dat mogelijk was, bevestigd in een niet yeast two-hybrid gerelateerd systeem, door middel van co-immunoprecipitatie studies. Uit dit onderzoek is gebleken dat VP2 meerdere, van elkaar onafhankelijke, bindingsdomeinen heeft voor homologe interactie, hetgeen overeenkomt met de structurele informatie die over dit capside eiwit bekend is (gebaseerd op een cryo-electronenmicroscopische studie van virus-like particles). De VP3-VP3 interactie werd gemapt op het amino-terminale deel van het eiwit.

Tevens is gebleken dat dit domein voor homologe interactie onafhankelijk kan functioneren van het VP1-bindingsdomein dat op het carboxy-terminale uiteinde van het eiwit gelegen is. Hiermee is duidelijk dat de drie bindingsdomeinen van VP3, het VP1-bindingsdomein, het dsRNA bindingsdomein en het homologe interactie domein, verschillend zijn gesitueerd in het eiwit en waarschijnlijk ook allemaal onafhankelijk van elkaar kunnen functioneren. Voor VP4 bleek het niet mogelijk om volgens deze strategie een domein voor homologe interactie te mappen. Dit eiwit bleek namelijk zeer gevoelig voor deleties. Tenslotte werd er voor VP5 een centraal gelegen domein gemapt als bindingssite voor homologe interactie. Dit domein omvat het meest hydrofobe gebied van VP5, een verondersteld transmembraan gebied. Omdat onlangs aangetoond is dat VP5 accumuleert in het plasmamembraan van de gastheercel en ook betrokken is bij het vrijkomen van nieuw gevormde virusdeeltjes, is het mogelijk dat dit domein voor homologe interactie betrokken is bij de vorming van een plasma membraan-porie in de gastheercel.

Voor het identificeren van interacties tussen virus- en gastheer-eiwitten werd een bibliotheek van eiwitten die tot expressie komen in de bursa van Fabricius van jonge kippen (3 weken oud), gescreend op interactie met de virale eiwitten VP1, VP2, VP3 en VP5 van IBDV. Hierbij werd voor ieder van deze virale eiwitten een aantal mogelijke, zowel bekende als nog onbekende, cellulaire interactoren gevonden. Hoewel voor de meeste van deze interacties nog aangetoond dient te worden of ze daadwerkelijk plaatsvinden tijdens een IBDV infectie, werd de biologische betekenis van sommige van deze veronderstelde interacties uitgebreid bediscussieerd in dit proefschrift.

Voor een van de gevonden virus-gastheer interacties werd de associatie bevestigd. Dit betrof de interactie tussen VP1 en het carboxy-terminale 1/3 deel van de eukaryotische translatie initiatie factor 4A-isovorm 2 (eIF4AII). Dit cellulaire eiwit is een essentieel component bij de initiatie van translatie, waarbij mRNA vertaald wordt in eiwit. Vrijwel alle eukaryotisch cytoplasmatische mRNAs hebben een cap struktuur aan hun 5' uiteinde en normaal gesproken bindt eIF4AII voor eiwitsynthese aan het initiatie complex op een cap-afhankelijke wijze. Een interactie tussen VP1 en eIF4AII is daarom heel interessant omdat de virale mRNAs van IBDV geen cap-structuur hebben aan het 5' uiteinde maar een covalent gebonden VP1 molecuul (VPg genaamd). Een directe interactie tussen VPg en eIF4AII zou daarom een essentiele stap kunnen zijn voor de initiatie van virale eiwitsynthese. Opvallend was echter dat alleen het carboxy-terminale 1/3 deel van eIF4AII, en dus niet het volledige eiwit, interactie vertoonde met VP1. Omdat het eIF4AII eiwit een kenmerkende struktuur heeft, bestaande uit twee domeinen, lijkt het aannemelijk

dat een interactie tussen VP1 en volledig eIF4AII mogelijk alleen kan plaatsvinden wanneer andere eiwitten helpen om deze struktuur zodanig te openen dat de VP1-bindingssite gelegen in het carboxy-terminale domein van dit eiwit vrij komt te liggen. In dit proefschrift zijn twee mogelijke modellen beschreven over hoe een VPg-eIF4AII interactie zou kunnen plaatsvinden en daarmee betrokken zou zijn bij de initiatie van de virale mRNA translatie.

Samenvattend heeft het yeast two-hybrid systeem bewezen een waardevolle techniek te zijn voor het in kaart brengen van de interacties die plaatsvinden in de virale levenscyclus van IBDV. Met name de specifieke interacties die plaatsvinden tussen de virale componenten onderling zijn goed in kaart gebracht. Tevens zijn er verscheidene cellulaire kandidaat eiwitten gevonden die mogelijk een essentiele interactie aangaan met de virale eiwitten. Het is te verwachten dat deze kennis verder onderzoek naar IBDV zal stimuleren. De verwachting is dat zal deze kennis in de toekomst zal kunnen bijdragen aan preventie en controle van IBD.

Appendices

ABBREVIATIONS

aa	amino acid
Ab	Antibody
AD	Activation Domain
AlMV	Alfalfa Mosaic Virus
amp	ampicillin
ATP	Adenosine Triphosphate
AZ	Antizyme
β-Gal	β-Galactosidase
BD	Binding Domain
BSA	Bovine Serum Albumin
bp	base pair
C	Carboxy
cDNA	copy DNA
CEF	chicken embryo fibroblast
CFU	Colony-Forming unit
CMV	Cytomegalovirus
co-IP	co-Immunoprecipitation
CPE	Cytopathic effect
CSFV	Classical Swine Fever Virus
CThy28kD	Thymocyte protein
DAP5	Deatch Associated Protein 5
DNA	Deoxyribonucleic Acid
DNAse	Deoxyribonuclease
dsDNA	double-stranded DNA
dsRNA	double-stranded RNA
DXV	Drosophila X Virus
4E-BP1	eIF4E-Binding Protein 1
E. coli	*Escherichia coli*
EED	Embryonic Ectoderm Developmental protein
eIF	eukaryotic Initiation Factor
EM	Electron Microscopy
EV	European Eels Virus
FCS	Foetal Calf Serum
FMDV	Foot and Mouth Disease Virus
FPV-T7	Fowlpox Virus expressing the T7 polymerase gene
Gal	Galactose

Glu	Glucose
GTP	Guanosine Triphosphate
HA	Haemagglutinin
HCV	Hepatitis C Virus
HDR	Hepatitis Delta Ribozyme
IBD	Infectious Bursal Disease
IBDV	Infectious Bursal Disease Virus
Ig	Immunoglobulin
IP	Immunoprecipitation
IPMA	Immunoperoxidase Monolayer Assay
IPNV	Infectious Pancreatic Necrosis Virus
IRES	Internal Ribosomal Entry Site
ITR	Inverted Terminal Repeat
kb	kilobase
kDa	kilo Dalton
Mab	Monoclonal antibody
Met-tRNA$_i$	initiator methionyl-tRNA
mIBDV	mutagenized IBDV
Mnk1	MAP kinase-interacting protein kinase-1
m.o.i.	multiplicity of infection
mRNA	messenger RNA
N	Amino
NADH	reduced form of the Nicotinamide Adenine Dinucleotide
ND	Not Determined
nt	nucleotide
ODC	Ornithine Decarboxylase
ODC-AZ	Ornithine Decarboxylase Antizyme
OIE	Office International des Epizooties
ORF	Open Reading Frame
OV	Oyster Virus
PABP	Poly(A)-Binding Protein
PBS	Phosphate-Buffered Saline
PBS-TDS	Phosphate-Buffered Saline - Triton-sodium Deoxycholate-SDS
PcG protein	Polycomb-Group protein
PCR	Polymerase Chain Reaction
PGK	Phosphoglycerate Kinase
p.i.	post-infection
Raf	Raffinose

RanBP1	RanGTP-binding protein 1
RdRp	RNA-dependent RNA polymerase
RGG box	Arginine-Glycine-Glycine (Arg-Gly-Gly) box
rIBDV	rescued IBDV
RIP	Radio-Immunoprecipitation
RNA	Riboucleic acid
RNAse	Ribonuclease
RRL	Rabbit Reticulocyte Lysate
rRNA	ribosomal RNA
RT	Reverse Transcriptase
RT-PCR	Reverse Transcriptase - Polymerase Chain Reaction
Sap18	Sin3-associated polypeptide 18
S. cerevisiae	*Saccharomyces cerevisae*
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis
SPF	Specific Pathogen Free
ssDNA	single-stranded DNA
ssRNA	single-stranded RNA
SV40	Simian Virus 40
$TCID_{50}$	50% Tissue Culture Infective Dose
tRNA	transfer RNA
TV	Tellina Virus
UTR	Untranslated Region
VLP	Virus-Like Particle
VP	Viral Protein
VPg	genome-linked Viral Protein
vvIBDV	very virulent IBDV
wt	wild-type

DANKWOORD

De Finish! Eindelijk, de eindstreep is in zicht! Het was een ware marathon, of zeg maar gerust een ultraloop. Tijdens mijn onderzoeksperiode zijn er momenten geweest dat ik dacht nooit aan het schrijven/afronden van mijn proefschrift toe te komen. Het was een leerzame maar vooral drukke tijd welke vaak gepaard ging met vallen en opstaan. Maar eindelijk is het nu dan toch zover gekomen en is het moment aangebroken om de laatste regels te vullen en er daadwerkelijk een punt achter te zetten.

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CURRICULUM VITAE

Mirriam Tacken zag haar eerste daglicht op 18 april 1970 in Horst (Noord-Limburg) en bracht haar jeugd door in dit prachtige dorpje. Zij volgde de MAVO opleiding aan de 'Jacob Merlo Horstius' te Horst, waarna de Middelbare Laboratorium Opleiding (MLO) werd voltooid aan het MBO-college Noord-Limburg te Venlo.

In 1989 is zij begonnen met het Hoger Laboratorium Onderwijs (HLO) aan de Hogeschool Eindhoven. Tijdens deze opleiding werd gekozen voor de studierichting algemene microbiologie met moleculaire biologie en biochemie als specialisatie. De stage en afstudeeropdracht werden uitgevoerd bij, respectievelijk, de research groepen moleculaire biologie en biochemie, van de afdeling Animal Health Research van het concern Ciba-Geigy (huidige naam: Novartis) te Bazel, Zwitserland, onder leiding van Dr. B. Suri. In juli 1993 werd de studie afgerond met het behalen van de Ingenieurs titel (Ing.).

Datzelfde jaar is zij begonnen met de studie Biologie, fysiologisch-biochemische richting, aan de Faculteit der Natuurwetenschappen van de Katholieke Universiteit van Nijmegen. Tijdens de doctoraalfase verrichtte zij haar bijvakstage binnen de afdeling Endocrinologie van de firma Organon Akzo-Nobel te Oss, onder begeleiding van Dr. R.G.J.M. Hanssen en Dr. A. Wiersma, en Prof. Dr. E.W. Roubos van de Katholieke Universiteit van Nijmegen. De hoofdvakstage werd verricht bij de afdeling Experimentele Plantkunde, onderzoeksgroep Celbiologie van de Plant, onder leiding van Prof. Dr. C. Mariani en onder begeleiding van Dr. B.H.J. de Graaf. Het doctoraal examen in de Biologie werd in maart 1997 *met genoegen* afgelegd waarbij de daarbij behorende Doctorandus titel (Drs.) werd verkregen.

Van juni 1997 tot maart 2002 was zij aangesteld als Assistant In Opleiding (AIO) bij de toenmalige afdeling Aviaire Virologie van het DLO-Instituut voor Dierhouderij en Diergezondheid, het ID-DLO (huidige naam: Animal Sciences Group WUR), te Lelystad, onder leiding van Dr. A.L.J. Gielkens. Hier werd, onder begeleiding van Dr. B.P.H. Peeters en Dr. H.J. Boot, en Prof. Dr. P.J.M. Rottier van de faculteit Diergeneeskunde van Universiteit Utrecht, het in dit proefschrift beschreven onderzoek uitgevoerd. Naast het begeleiden van studenten en het bezoeken van verschillende congressen, volgde zij een AIO opleidingsprogramma aan de onderzoeksschool 'Eijkman Graduate School for Immunology and Infectious Diseases' (voorheen 'Graduate School Infection and Immunity') van de faculteit Diergeneeskunde van Universiteit Utrecht.

Sinds september 2002 is zij werkzaam als postdoc bij de afdeling Kindergeneeskunde van het VU Medisch Centrum te Amsterdam onder leiding van

Prof. Dr. M.S. van der Knaap. Hier wordt onderzoek gedaan aan ziekten van de witte stof van de hersenen bij kinderen.

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