

VP1 of infectious bursal disease virus is an RNA-dependent RNA polymerase

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Segment B of the bisegmented, double-stranded RNA genome of infectious bursal disease virus (IBDV) encodes the viral protein VP1. This has been presumed to represent the RNA-dependent RNA polymerase (RdRp) as it contains motifs that are typical for the RdRp of plus-strand RNA viruses. Here it is demonstrated that baculovirus-expressed wild-type but not motif A mutated VP1 acts as an RdRp on IBDV-specific RNA templates. Thus, on a plus-strand IBDV segment A cRNA template, minus-strand synthesis occurred in such a way that a covalently linked double-stranded RNA product was generated (by a 'copy-back' mechanism). Importantly, enzyme activity was observed only with templates that comprised the 3' non-coding region of plus-strand RNAs transcribed from IBDV segments A and B, indicating template specificity. RdRp activity was shown to have a temperature optimum of 37 °C and required magnesium ions for enzyme activity. Thus, it has been demonstrated unequivocally that VP1 represents the RdRp of IBDV.

Received 30 October 2003

Accepted 21 April 2004

INTRODUCTION

Infectious bursal disease virus (IBDV) is the causal agent of a highly contagious, immunosuppressive disease in chickens, first described by Cosgrove (1962). IBDV belongs to the genus *Avibirnavirus* of the family *Birnaviridae* (Leong *et al.*, 2000). Its genome consists of two segments, A and B, of double-stranded RNA encapsidated by a single-shelled icosahedral capsid of 60 nm diameter. The larger genome segment A encodes a polyprotein of approximately 110 kDa which is autoproteolytically cleaved by a *cis*-acting viral protease (Birghan *et al.*, 2000; Lejal *et al.*, 2000) to give rise to the viral proteins (VP) VP2, VP3 and VP4. A second open reading frame that precedes and partially overlaps the polyprotein coding sequence (Spies *et al.*, 1989) encodes VP5. Although VP5 has been detected in IBDV-infected chicken embryo cells (CECs) as well as in bursal cells of IBDV-infected chickens (Mundt *et al.*, 1995), it is not required for virus replication (Mundt *et al.*, 1997). Genome segment B encodes a 97 kDa protein designated VP1. There is evidence (Kibenge & Dhama, 1997) that VP1 is associated with the 5'-terminal IBDV RNA sequences, and that it interacts with VP3 (Lombardo *et al.*, 1999; Tacken *et al.*, 2000).

The coding region of IBDV VP1 (Morgan *et al.*, 1988) was shown to contain motifs I (now termed motif A), II (motif

B), III (motif C) and IV (motif E) that are common to RNA-dependent RNA polymerases (RdRp) of positive-strand RNA (ssRNA+) viruses (Gorbalenya & Koonin, 1988). Subsequent analyses revealed that the originally described motif C is not conserved in other birnaviruses (Shwed *et al.*, 2002) and may be fortuitous. Its genuine counterpart, which includes the Ala–Asp–Asn rather than the common Gly–Asp–Asp tripeptide, was identified upstream of motif A using a sophisticated computer-assisted analysis that involved newly determined sequences of ssRNA+ *Thoesa asigna* and *Euprosterina eleasa* viruses (Gorbalenya *et al.*, 2002). This unique structural organization of VP1 may be compatible with RdRp (Gorbalenya *et al.*, 2002) as well as other enzymic activities suggested in early studies (Spies *et al.*, 1987; Spies & Müller, 1990), although neither of them has been demonstrated yet.

To verify RdRp activity of the IBDV VP1, we expressed this protein in the baculovirus system and analysed its properties *in vitro*.

METHODS

Generation of recombinant baculovirus expressing VP1. For expression of VP1, a recombinant baculovirus was generated using the baculovirus transfer vector pAcSG2 and the Baculo Gold system (PharMingen; Becton Dickinson). The VP1 gene was amplified from

Table 1. Oligonucleotides used for cloning procedures

Composition and location of the oligonucleotide primers used for amplification and site-directed mutagenesis. Nucleotides changed by mutagenesis are shown in capital letters; the changed coding nucleotide triplet is in bold type; nucleotides specific for IBDV are in italics. The restriction enzyme cleavage sites used are marked and underlined. Positions where primers bind (nucleotide number) and numbering of amino acids are in accordance with the published sequence of strain P2 (Mundt & Müller, 1995).

Name	Sequence	Orientation	Position
VP1-BacF	<i>EcoRI</i> CTCGAGGAATTCattctgcccacatgagtgacattttcaac	Sense	101–129
VP1-BacR	<i>PstI</i> GGTACCCTGCAGtttagcggctctccttttggcgttggccgccg	Antisense	2719–2748
D416A	gtactcaattg CT ctagagaag	Sense	1347–1368

plasmid pP2B (Mundt & Vakharia, 1996) by Deep Vent polymerase (New England Biolabs) using appropriate oligonucleotides (VP1-BacF, VP1-BacR; Table 1). The PCR fragment was eluted and cloned blunt-ended into the *SmaI* restriction enzyme cleavage site of pUC18 to obtain pUC18-VP1. After verification by sequencing, pUC18-VP1 was cleaved with *EcoRI/PstI* and the eluted fragment encompassing the VP1 coding region was ligated into *EcoRI/PstI*-cleaved pAcSG2 to obtain pAcSG2-VP1. To exchange Asp at amino acid position 416 of VP1 with Ala, site-directed mutagenesis was performed (Kunkel *et al.*, 1987). To this end, pP2B was cleaved with *EcoRI/PstI* and the complete cDNA sequence of segment B was ligated into the plasmid pBluescript II SK+ (pBSSK-P2B). After transformation into *Escherichia coli* strain CJ (Bio-Rad), single-stranded DNA was generated and site-directed mutagenesis performed using oligonucleotide D416A (Table 1). After sequencing, the generated plasmid pBSSK-P2BD416A was cleaved with *DraIII/EcoRV* and ligated into appropriately cleaved pP2B to obtain pP2BD416A. This plasmid was then cleaved with *DraIII/PstI* and the 1879 bp fragment obtained was ligated into appropriately cleaved pAcSG2-VP1 to obtain pAcSG2-VP1D416A. Plasmid pAcSG2-VP1 is shown schematically in Fig. 1.

Sf9 cells were transfected using either pAcSG2-VP1 or pAcSG2-VP1D416A and Baculo Gold DNA, as recommended by the manufacturer. The obtained baculovirus expressing VP1 (VP1-Bac) or the mutant protein (VP1D416A-Bac), a baculovirus expressing NS5B of hepatitis C virus (HCV; NS5B-Bac, Behrens *et al.*, 1996), and wild-type *Autographa californica* nuclear polyhedrosis baculovirus (AcPNV) (PharMingen) were used for infection of *Sf9* cells at m.o.i. 1. At 72 h after infection, mock-infected and infected *Sf9* cells were harvested by centrifugation at 250 g for 10 min at 4 °C. Cells were washed once in ice-cold PBS. Resuspension of the pellet and preparation of cytoplasmic extracts were performed essentially as described by Behrens *et al.* (1996). The lysates obtained were stored in aliquots at –70 °C.

Lysates were tested for the presence of VP1 and NS5B by discontinuous SDS-10% PAGE (Sambrook *et al.*, 1989) followed by blotting onto a nitrocellulose membrane (Schleicher and Schüll, Germany). VP1 was detected with rabbit anti-VP1 serum (Birghan *et al.*, 2000) and NS5B with rabbit anti-NS5B serum (Behrens *et al.*, 1996). Antigen-antibody complex was visualized by incubation with diluted horseradish peroxidase-conjugated goat anti-rabbit antibodies (Sigma) followed by an enhanced chemoluminescence system (Pierce) as described by the manufacturer.

Substrate for RNA polymerase assay. To assay for polymerase activity, cRNA transcripts were originated from plasmid pP2A (Birghan *et al.*, 2000) and pP2B (Mundt & Vakharia, 1996). To this end, pP2A was cleaved with *RsrII/BglII* to remove nearly the complete coding region of segment A (nucleotides 201–3020). After fill-in using Klenow enzyme and ligation, a plasmid was obtained

containing the 5' and 3' non-coding regions (NCRs) of segment A and a small part of the coding region of the polyprotein gene, nt 131–201 (pΔP2A, Fig. 1). pP2B was cleaved with *Bsu36I/BsrGI* to delete a large part of the coding region of VP1 (nt 231–2538). The remaining plasmid was treated as described above to obtain a

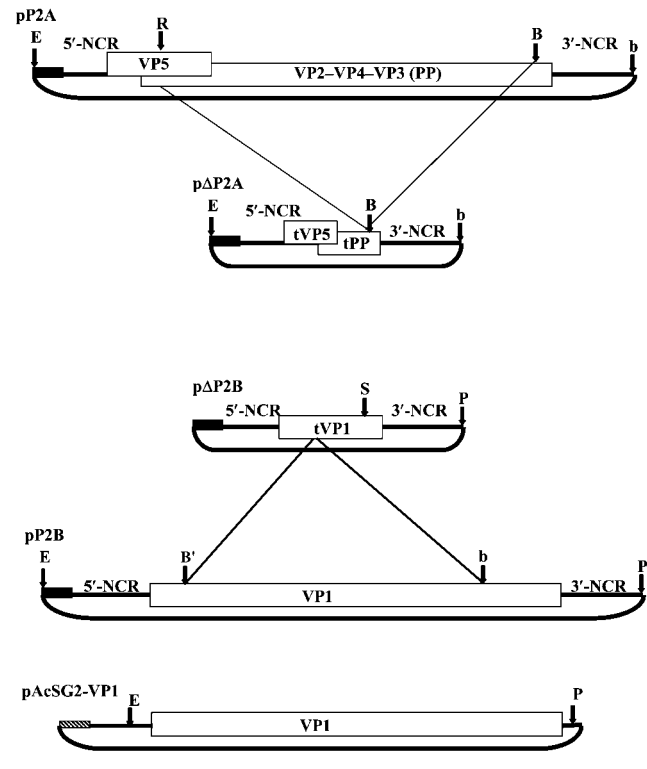


Fig. 1. Construction of cDNA clones of segments A and B of IBDV. Map of the genomic organization of IBDV segments A (pP2A) and B (pP2B) cloned as full-length cDNA in the plasmid pUC18. Coding sequences for viral proteins (VP) encoded by segment A (VP5, VP2–VP4–VP3 as polyprotein PP) and segment B (VP1) are depicted by an open box and appropriate NCRs are marked. T7 promoter is depicted by a solid rectangle. Plasmid pΔP2A encoded a truncated VP5 (tVP5) and a truncated polyprotein (tPP); pΔP2B encodes a truncated VP1 (tVP1). Plasmids for expression of VP1 in pAcSG2-VP1 are also shown. Enzymes used for cloning procedures and linearization, respectively, of plasmids (see Methods): E, *EcoRI*; B, *BglII*; b, *BsrGI*; B', *Bsu36I*; P, *PstI*; R, *RsrII*; S, *SgrAI*.

plasmid which contained the 5' and 3' NCRs and a small part of the coding region of segment B (p Δ P2B, Fig. 1). p Δ P2A was linearized with *Bsr*GI or *Bgl*II and p Δ P2B was cleaved with *Sgr*AI or *Pst*I. The samples were digested with proteinase K (0.5 mg ml⁻¹)/SDS (0.5%) and purified by phenol/chloroform extraction. T7 polymerase reaction was carried out using the following conditions in a 25 μ l sample: 2 μ g linearized DNA, 1 \times transcription buffer (Promega), 10 mM DTT, 0.5 mM of each nucleotide, 1 μ l RNasin (Promega), 1 μ l T7 RNA polymerase. After incubation at 37 °C for 60 min, 1 μ l DNase I (Roche) was added for degradation of plasmid DNA. The plus-stranded cRNAs obtained were separated from nucleotides using Probe Quant G50-Micro Columns (Amersham Pharmacia) following the manufacturer's instructions, ethanol precipitated, and subsequently used for further experiments.

cRNA transcribed from p Δ P2A was ³²P-labelled during the transcription reaction and eluted from a 1% agarose gel. To this end, approximately 1 μ g linearized p Δ P2A (5 μ l) was added to a mixture consisting of 5 μ l 5 \times transcription buffer (Promega), 2.5 μ l 100 mM DTT (Promega), 3 μ l of an rNTP mixture (ATP, CTP, GTP, 10 mM each; Roche), 1 μ l of 1 mM UTP (Roche), 5 μ l [α -³²P]UTP [10 μ Ci, 3000 Ci mmol⁻¹ (111 TBq mmol⁻¹); ICN], 1.5 μ l nuclease-free water, 1 μ l RNasin (Promega) and 1 μ l T7 RNA polymerase (Promega). After 1 h incubation at 37 °C, reaction products were separated in a 1.5% low-melting-point agarose gel (Seakem Agarose; Biozym) containing 0.5 μ g ethidium bromide. The appropriate RNA was excised and 400 μ l of a buffer containing 5% lithium dodecyl sulphate, 100 mM Tris/HCl pH 8.0, 0.5 M LiCl, 10 mM EDTA and 5 mM DTT (Sawicki & Sawicki, 1990) was added. The mixture was then incubated at 70 °C for 10 min, followed by phenol/chloroform extraction and ethanol precipitation.

In vitro RdRp assays using Sf9 cell extracts. The assay was performed using cytoplasmic extracts and *in vitro* transcribed cRNA derived from p Δ P2A or p Δ P2B. For the assay, to 10 μ l of the cytoplasmic extract were added 8 μ l 100 mM Tris/HCl pH 7.5, 2 μ l 100 mM MgCl₂, 4 μ l 10 mM DTT, 1 μ l 1 M KCl, 0.8 μ l 50 mM EDTA, 0.5 μ l RNasin (20 units, Promega), 2 μ l actinomycin D (1 mg ml⁻¹), 2 μ l of ATP, CTP and GTP (each 10 mM), 5 μ l cRNA dissolved in RNase-free water (approx. 1 μ g) and 1 μ l [α -³²P]UTP (10 μ Ci). After incubation of the reaction mixture at 22 °C for 2 h, reaction products were subjected to proteinase K (0.5 mg ml⁻¹) digestion in 1 vol. 2 \times proteinase K buffer (300 mM NaCl, 10 mM Tris/HCl pH 7.5, 1% SDS) at 37 °C for 30 min, and then isolated. After phenol/chloroform extraction, glycogen (Roche) was added and RNA was ethanol-precipitated.

In a modified polymerase assay [α -³²P]UTP-labelled cRNA (see above) was used. To this end, radioactively labelled [α -³²P]cRNA was ethanol-precipitated and sedimented by centrifugation. After resuspension in RNase-free water, 5 μ l was added to the reaction mixture instead of non-labelled cRNA, and 2 μ l 10 mM UTP was added. Purification of reaction products was performed as described above.

RNase digestion. RNase digestion was performed using RNase If (New England Biolabs) for specific digestion of single-stranded RNA, or using RNase III (New England Biolabs) which specifically cleaves double-stranded RNA into fragments of 12–15 bp with two-base 3' overhangs. For RNase If assay, reaction products from the polymerase assay were ethanol-precipitated, sedimented by centrifugation, resuspended in 20 μ l buffer (50 mM Tris/HCl pH 8.0, 10 mM MgCl₂, 1 mM DTT, 100 mM NaCl) and 20 U enzyme was added. To assay for double-stranded RNA, reaction products were resuspended in an appropriate buffer (10 mM Tris/HCl pH 8.0, 10 mM MgCl₂, 1 mM DTT, 100 mM NaCl) and 0.2 U RNase III was added. For incubation with RNase T1 (Roche) enzyme was added directly to the RdRp reaction to a final concentration of

1 U μ l⁻¹. Reaction mixtures were incubated at 37 °C (RNase III, RNase If, RNase T1) for 30 min, digested by incubation with proteinase K/SDS, and purified as described above.

Analysis of RNA. Reaction products were sedimented, washed with 70% ethanol and resuspended in loading buffer (95% formamide, 20 mM EDTA pH 8.0, 0.05% bromophenol blue, 0.05% xylene cyanol). Resuspended RNA was separated in a 5% acrylamide gel containing 8 M urea in TBE using a 60 cm sequencing apparatus (Gibco-BRL) at 70 W for 2 h. Gels were fixed, incubated with water to remove urea, and dried before exposure at -70 °C for at least 1 week to an X-OMAT AR film (Eastman Kodak) using an intensifying screen.

Preparation of RNA markers. To obtain [α -³²P]UTP-labelled RNA markers, pP2A was cleaved with *Rsr*II, *Nde*I and *Sac*I at positions 201, 647 and 868, respectively, of the full-length cDNA sequence of segment A of IBDV strain P2 (Mundt & Müller, 1995). The labelling reaction for cRNA using T7 polymerase, and purification of the three radioactively labelled cRNAs of lengths 201, 647 and 848 nt, were performed as described above.

RESULTS

Expression of wild-type and mutated VP1 in insect cells

To verify RdRp activity of VP1, we established recombinant baculoviruses expressing the entire VP1 gene (VP1-Bac). As a control we generated a recombinant baculovirus that expressed a VP1 with an amino acid exchange (Asp-416 to Ala) in the presumed A motif (VP1D416A-Bac). This mutation was aimed at abolishing the putative polymerase activity, as Asp-416 in addition to other Asp residues has been suggested to be important for binding divalent cations crucial for the catalytic mechanism (Gorbalenya *et al.*, 2002).

Analysis of lysates of *Sf9* cells infected with the two recombinant baculoviruses (VP1-Bac and VP1D416A-Bac) showed after probing with the anti-VP1 antiserum that IBDV VP1 protein was expressed (Fig. 2a). The specificity of the detected signal was proved using lysates of mock-infected and IBDV (strain P2)-infected CECs, prepared as described previously by Mundt *et al.* (1995). In the P2-infected CECs we detected the full-sized protein, while in the lysates of VP1-Bac- and VP1D416A-infected *Sf9* cells we detected VP1 as well as some degradation products which were not detectable in the control lysates. In a parallel experiment we expressed the HCV NS5B RdRp using an analogous baculovirus approach. The expression of the protein was also confirmed by Western blots applying an anti-HCV NS5B antiserum.

VP1 catalyses RNA polymerization *in vitro*

To assess RdRp activity in lysates containing baculovirus-directed VP1, we established a conventional RdRp assay which contained cytoplasmic extracts of *Sf9* cells, including the heterologously expressed protein, actinomycin D to inhibit DNA-dependent RNA polymerases and unlabelled rNTPs and α -³²P-labelled UTP. As a template, we employed

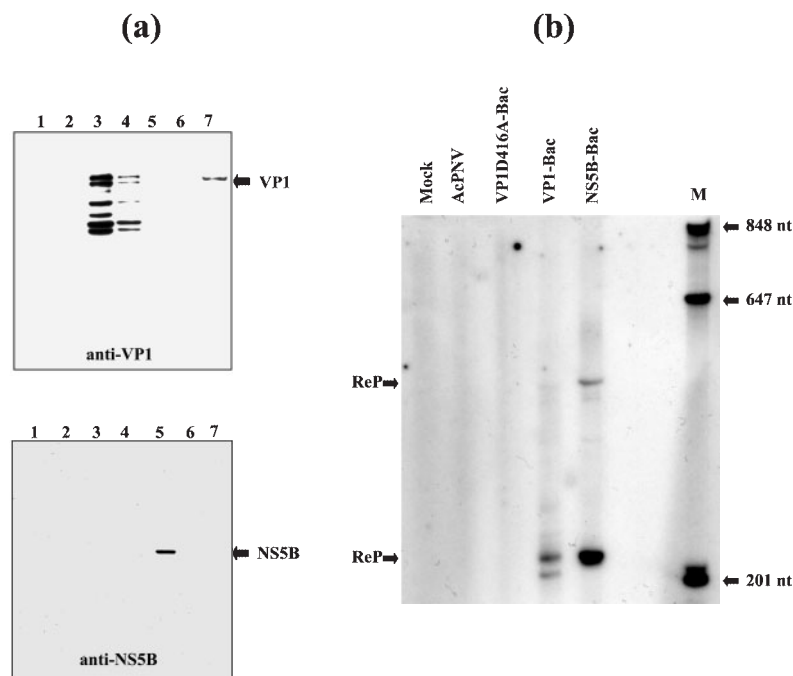


Fig. 2. VP1 expressed with the baculovirus system shows RdRp activity. (a) Expression of VP1, VP1D461A and NS5B of HCV was performed in *Sf9* cells. These were either mock-infected (lane 1) or infected with wild-type baculovirus (lane 2), or with recombinant baculovirus encoding the mutated VP1D416A (lane 3), authentic VP1 (lane 4), or NS5B of HCV (lane 5). As controls, lysates of uninfected CECs (lane 6) or CECs infected with IBDV strain P2 (lane 7) were used. The lysates obtained were separated in 10% SDS-PAGE gel and membranes incubated after blotting with rabbit antisera raised against VP1 and NS5B of HCV. Locations of proteins are marked by arrows. (b) RdRp activity was detected in cytoplasmic extracts of VP1- and NS5B-expressing *Sf9* cells. A 444 nt *in vitro* synthesized transcript of Δ P2A was incubated with lysates of non-infected (Mock), wild-type baculovirus-infected (AcNPV), VP1D416A-Bac-infected (VP1D416A-Bac), VP1-Bac-infected (VP1-Bac), and NS5B-Bac-infected *Sf9* cells (NS5B-Bac). Labelling was performed using [32 P]UTP, and reaction products (ReP) were separated on a denaturing 5% polyacrylamide/8 M urea gel. 32 P-labelled marker RNAs (M) are marked by arrows. Fixed and dried gel was exposed to X-ray film.

a plus-stranded cRNA which was transcribed from p Δ P2A after its linearization with *Bsr*GI (length 444 nt, termed RNA-A444) (see Methods); this RNA contained both NCRs of segment A of IBDV. Analogous experiments with the HCV NS5B were performed to confirm that our assay was functional. In all reactions analysed we observed a faint band migrating at high retardation in the analytical gel (Fig. 2b). A probable origin of this product was resolved in a separate experiment (see below). Our analysis of the reaction products also revealed a labelled and faster-migrating band that was detected in the presence of IBDV VP1 or HCV NS5B but not in control lysates (mock-infected and AcNPV-infected *Sf9* cells) or VP1 Δ 416A-Bac-infected *Sf9* cells. Hence, these data indicated that the lysates of VP1-Bac-infected cells contained an RdRp activity similar to that present in the lysates of NS5B-Bac-infected cells (Behrens *et al.*, 1996).

In the next experiment, we tested cytoplasmic extracts of VP1-Bac-infected and AcNPV-infected *Sf9* cells in a parallel approach, using either the above-described assay with unlabelled RNA-A444 substrate and the labelled nucleotide or, alternatively, with a mixture that contained unlabelled nucleotides and a 32 P-labelled RNA-A444 substrate. As shown in Fig. 3(a), we detected the aforementioned product with a faster electrophoretic mobility

independently of the nature of the reaction mixture in the assays that contained VP1. Thus, in addition to confirming that VP1 has polymerase activity, these experiments demonstrated that the enzyme used the input RNA-A444 transcript as a substrate. The labelling of the previously unlabelled input RNA in the absence of VP1 is likely to be explained by the activity of terminal nucleotidyl transferases (TNTs) that are present in the cytoplasmic extracts of *Sf9* cells (Behrens *et al.*, 1996).

Analysis of reaction products

To test whether the products detected were single-stranded or double-stranded RNAs, they were digested with RNase If, which is specific for ssRNA; or with RNase III, which specifically hydrolyses dsRNA (Fig. 3b). Thus, the upper product detectable in each of the reactions was identified as ssRNA because it disappeared on incubation with RNase If (Fig. 3b, lane 1). This confirmed our view that it represented the substrate RNA-A444, which was probably radioactively labelled by the TNT activity present in the cytoplasmic extracts of *Sf9* cells. Conversely, the lower reaction product, which appeared only in the VP1-containing reactions, was hydrolysed by RNase III, indicating that it was dsRNA (Fig. 3b, lane 1). These results showed that the specifically VP1-catalysed reaction product was dsRNA.

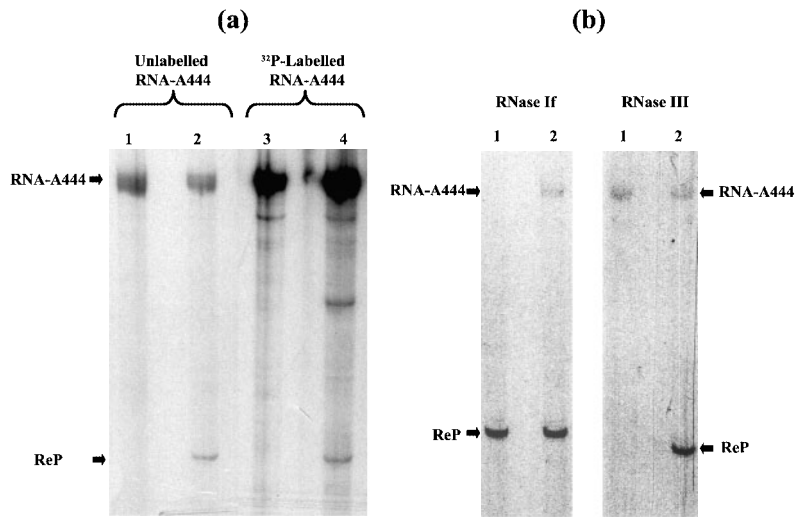


Fig. 3. Characterization of reaction products. (a) A 444 nt *in vitro* synthesized transcript of Δ P2A (RNA-A444) was used as a substrate for enzyme activity. Non-labelled (lanes 1 and 2) and ³²P-labelled RNA-A444 (lanes 3 and 4) of Δ P2A was incubated with lysates of either wild-type baculovirus-infected (lanes 1 and 3) or VP1-Bac-infected *Sf9* cells (lanes 2 and 4). Reaction products were separated using a denaturing 5% polyacrylamide/8 M urea gel. Substrate RNA (RNA-A444) and reaction products synthesized by a 'copy-back' mechanism are marked with arrows. (b) Characterization of reaction products by nuclease treatment. *In vitro* transcribed RNA-A444 was incubated with lysates of *Sf9* cells infected with VP1-Bac, purified by phenol/chloroform extraction and ethanol precipitation. Precipitated products were incubated with either RNase If or RNase III (lane 1), or mock-incubated in the appropriate buffer (lane 2). Reaction products (ReP) were analysed on a 5% polyacrylamide/8 M urea gel.

The IBDV RdRp operates by substrate priming

In order to characterize the product of VP1-mediated reaction further, we analysed it under highly denaturing conditions and tested its accessibility to the ss-specific RNase T1 (Fig. 4). We performed the RdRp assay with cytoplasmic extracts of VP1-Bac-infected and AcNPV-infected *Sf9* cells using either ³²P-labelled RNA-A444 (lanes 1–4) or unlabelled RNA-A444 (lanes 6 and 7) templates. Half of each of the assay mixtures was exposed to RNase T1 at 37 °C; the reaction products in each case were resuspended in loading buffer, heated to 95 °C and separated on a pre-heated (approx. 80 °C) denaturing (8 M urea) polyacrylamide gel. In the non-T1-treated samples containing VP1 and a hot or cold template, we observed the dsRNA product (see above) that migrated at a high electrophoretic mobility in the gel (lanes 3 and 6). In addition, a band that migrated significantly slower than the input ³²P-labelled RNA-A444 was detected in lane 3 (dHP band). We interpreted the slower-migrating product as corresponding to the denatured ss form of the reaction product. We believe that weak labelling and only partial denaturation of the reaction product were the reasons for the lack of a slower-migrating product in lane 6. This partial denaturation was also evident in lane 3. As the ds (lane 3) and denatured products (lanes 4 and 7) after T1 digestion were found to be labelled in reactions that involved either a labelled or an unlabelled template, we concluded that the original template RNA was part of the reaction product (see above). Thus, in line with previous observations obtained with poliovirus and HCV RdRp, it became apparent that the fast-migrating reaction product probably corresponded to ds hairpin that was generated by the

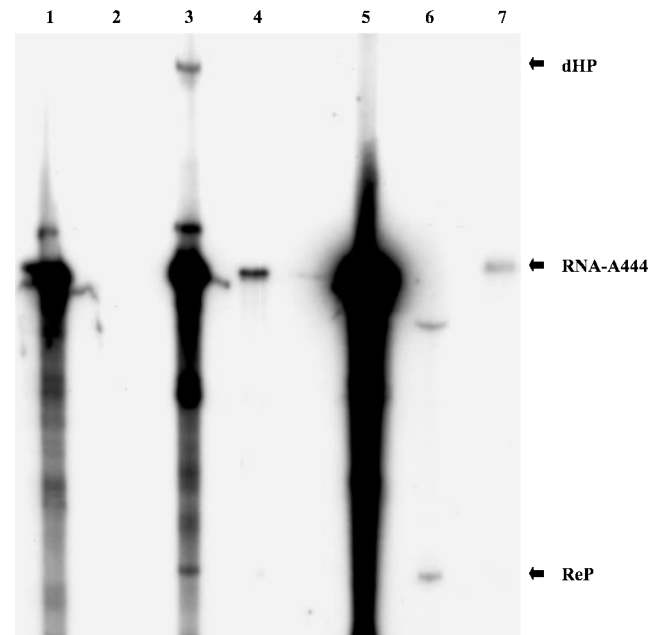


Fig. 4. RdRp products after RNase T1 treatment. ³²P-Labelled RNA-A444 (lanes 1–4) or non-labelled RNA-A444 (lane 6 and 7) was incubated with lysates of AcNPV-infected (lanes 1 and 2) or VP1-Bac-infected (lanes 3, 4, 6, 7) *Sf9* cells. Lane 5 shows ³²P-labelled RNA-A444 without incubation with cellular lysates. Reaction products were either RNase T1-treated (lanes 2, 4, 7) or left untreated (lanes 1, 3, 6), heated in loading buffer for 5 min at 95 °C and separated in a pre-heated denaturing 5% polyacrylamide/8 M urea gel. ReP, Reaction product; dHP, denatured hairpin.

'copy-back' RNA synthesis mechanism involving template priming. This notion was clearly confirmed in the course of the experiments that applied RNase T1: in the reactions utilizing VP1-Bac lysate, unlabelled RNA template and a labelled nucleotide, we detected a product that was sensitive to the T1 digestion and had the same electrophoretic mobility as the input RNA-A444 in the denaturing gel. These properties indicate that the primary product of VP1-mediated reaction corresponded to a hairpin made up from the original template and a complementary negative strand synthesized *de novo*. T1 converted this hairpin into the dsRNA molecule that was denatured under the conditions of the urea gel into two ss molecules, one of which – labelled negative single-strand – remained detectable.

We thus concluded that RNA synthesis by the VP1 polymerase (as determined under the conditions of our assay system) followed a template priming and 'copy-back' mechanism.

Template specificity of VP1

The next experiment was aimed at testing the substrate specificity of VP1 RNA polymerase (Fig. 5). For this purpose, two different IBDV segment A-specific ssRNAs were tested in the polymerase assay described above, utilizing either IBDV VP1 or HCV NS5B. The first substrate corresponded to RNA-A444, which was transcribed *in vitro* from p Δ P2A after linearization with *Bsr*GI, and which contained the 5' as well as the 3' NCR of segment A (Fig. 5a, lanes 5–7). The second substrate tested was a 201 nt RNA (RNA-A201) transcribed from p Δ P2A after linearization with *Bgl*III; it contained only the 5' NCR of segment A (Fig. 5a, lanes 2–4). After incubation of the RNA-A444 template

with lysates containing VP1 or NS5B, in each case we observed the known fast-migrating hairpin product (lanes 6 and 7). However, while the HCV NS5B polymerase was found to be active on both RNA substrates (lanes 4 and 7), which was explained by its unspecific polymerase activity described earlier (Behrens *et al.*, 1996), this was not the case with VP1. VP1 was evidently inactive on the RNA-A201 substrate that lacked the 3' NCR of segment A (lane 3). To support this finding, we used two additional substrates in addition to RNA-A444 and RNA-A201 for RdRp polymerase assay using lysates containing VP1 (Fig. 5b). To this end, p Δ P2B was linearized with *Sgr*AI and *Pst*I to obtain cRNA transcripts lacking the 3' NCR of segment B (RNA-B317) and containing both 5' and 3' NCRs of segment B (RNA-B520), respectively. Here, we observed that VP1 was inactive with RNA-B317 (lane 3). In contrast, a reaction product was observed when RNA-B520 was used in the assay. Hence, we concluded that the 3' NCR of segments A and B was critical for VP1-directed RNA polymerization *in vitro*.

Factors affecting VP1 polymerase activity

Finally, we analysed the effects of temperature and divalent cations on the replicase activity of VP1. In a first series of experiments, we observed that the amount of the RdRp-specific reaction product accumulated with increasing incubation temperature up to 37 °C, and decreased at 40 °C (Fig. 6). Interestingly, the signal of the slower-migrating ssRNA product disappeared with increasing temperature, indicating that the TNT-mediated labelling of the input cRNA was temperature-sensitive. Furthermore, beginning at 30 °C an additional reaction product was observed which migrated faster than the reaction product accumulated at 15 and 22 °C. As the additional product

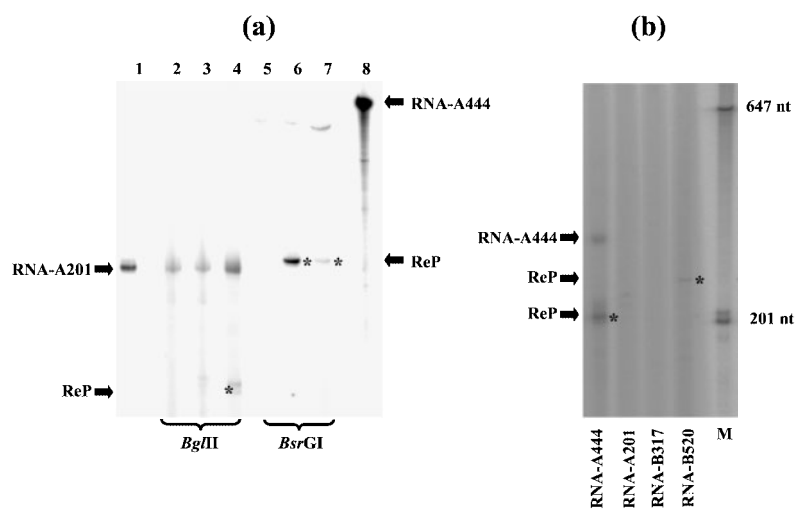


Fig. 5. RdRp activity of VP1 depends on the 3' NCR. (a) Substrate RNA transcripts of different lengths (201 nt without 3' NCR of segment A designated RNA-A201, lanes 2–4; 444 nt with 3' NCR of segment A, RNA-A444, lanes 6–8) were incubated with lysates of AcPNV-infected (lanes 2 and 5), VP1-Bac-infected (lanes 3 and 6) and NS5B-Bac-infected (lanes 4 and 7) Sf9 cells. Lane 1 shows ³²P-labelled RNA-A201; ³²P-labelled RNA-444 is visible in lane 8. (b) RNA-A444, RNA-A201 and two transcripts obtained from p Δ P2B (317 nt without 3' NCR of segment B designated RNA-B317; 520 nt with 3' NCR of segment B designated RNA-B520) were incubated with VP1-containing lysates. Reaction products were analysed using a denaturing 5% polyacrylamide/8 M urea gel. Locations of substrate RNA transcripts, reaction products (ReP) and ³²P-labelled marker RNAs are shown by arrows.

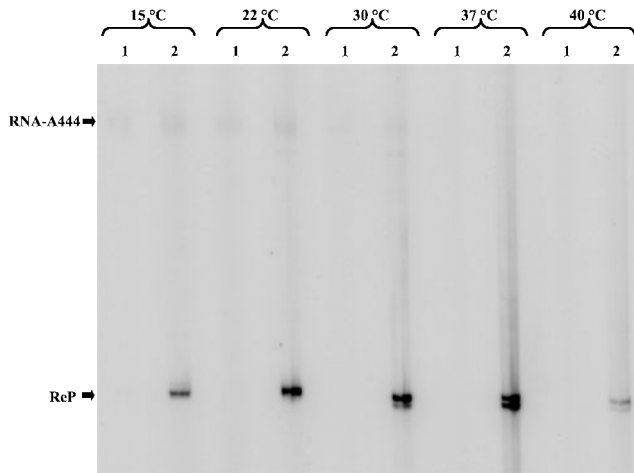


Fig. 6. Optimal temperature for RNA synthesis. Assays were performed with lysates of either AcPNV-infected (lane 1) or VP1-Bac-infected (lane 2) *Sf9* cells. Incubation temperatures are indicated. Reaction products (RNA-A444, ReP) were separated in a denaturing 5% polyacrylamide/8 M urea gel.

was not detectable after incubation with control lysates, it was concluded that it was also the result of VP1 activity.

The final experiments demonstrated that VP1 is a metal-activated polymerase that uses divalent metals for nucleotide polymerization (Fig. 7). Omission of Mg^{2+} resulted in a complete block of VP1 RdRp activity. However, experiments using $CoCl_2$ or $MnCl_2$ instead of $MgCl_2$ yielded replication products, indicating that cations other than magnesium also support the polymerase activity of VP1. In contrast, other divalent cations ($CaCl_2$, $CuCl_2$, $FeSO_4$, $NiCl_2$) did not support VP1 polymerase activity.

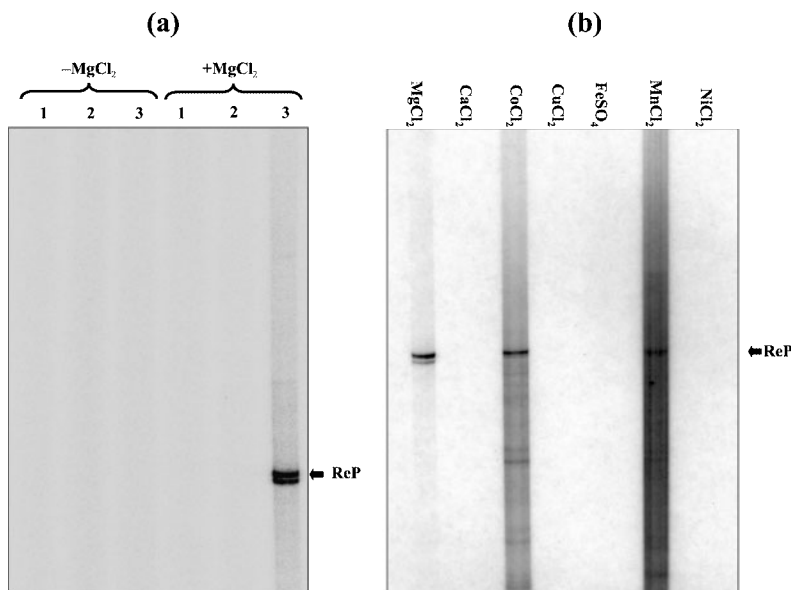


Fig. 7. Polymerase activity is dependent on divalent cations. (a) RdRp assays using lysates of non-infected (lane 1), AcPNV-infected (lane 2) and VP1-Bac-infected (lane 3) *Sf9* cells were performed at 37 °C without or with $MgCl_2$. Reaction products were analysed on a 5% polyacrylamide/8 M urea gel. (b) Mg^{2+} ions can be replaced by different divalent cations. The following compounds were added to lysates of VP1-Bac-infected *Sf9* cells: $MgCl_2$, $CaCl_2$, $CoCl_2$, $CuCl_2$, $FeSO_4$, $MnCl_2$, $NiCl_2$. Solutions were added to the reaction mixture at the same molarity as $MgCl_2$. After incubation at 37 °C for 1 h, purified reaction products were analysed using a 5% polyacrylamide/8 M urea gel. Location of reaction products (ReP) is shown by arrows.

DISCUSSION

For members of the family *Birnaviridae*, little is known about the replication mechanism of the viral RNA genome. As with positive-strand RNA viruses, they encode their own RdRp for autonomous genome replication. A first indication that VP1 of IBDV may represent the RdRp came from computer-assisted analysis that revealed the conservation of the RdRp motifs of ssRNA + viruses in this protein (Gorbalenya & Koonin, 1988). A recent extension of this analysis showed that the birnavirus VP1 contains an unconventional permuted organization of the RdRp motifs (Gorbalenya *et al.*, 2002; see Introduction). Although Spies & Müller (1990) showed that polymerase activity was associated with highly purified IBDV particles, the protein responsible was not identified. The aim of this study was to identify and characterize the RdRp activity in IBDV VP1.

Although *E. coli*-expressed VP1 failed to show polymerase activity (data not shown), we were able to demonstrate RdRp activity in lysates of insect cells infected with a recombinant baculovirus expressing VP1. This report provides the first direct evidence that VP1 of IBDV is indeed the RdRp of the virus. Related to this finding, we ruled out the possibility that RdRp activity requires an interaction between the two IBDV proteins VP1 and VP3 (Lombardo *et al.*, 1999; Tacken *et al.*, 2000) as well as binding of double-stranded IBDV RNA by VP3 (Tacken *et al.*, 2002). This is the second, after phage $\phi 6$ (Makeyev & Bamford, 2000), dsRNA virus for which *in vitro* RdRp activity was shown to be associated with a single protein species. In contrast for the dsRNA rotavirus, two virus proteins (VP1 and VP2) were found to be necessary for polymerase activity (Patton *et al.*, 1997).

In our VP1-mediated RdRp assay we detected two types of product. Using the ss-specific RNase If, we proved that

the slower-migrating reaction product was ssRNA template likely modified by cellular TNT. The faster-migrating RNA product, which was exclusively synthesized in the presence of VP1, was shown to be a hairpin RNA. The detected RdRp activity was due to VP1 rather than a contaminating cellular protein that was proved using the VP1-D416A mutant which carries an Ala replacement of the highly conserved Asp residue in the motif A responsible for divalent cation binding (Gorbalenya *et al.*, 2002); with this mutant no polymerase activity was detected. The latter result provides a first evidence for the vital character of the RdRp motif A in the IBDV VP1 and it was recently extended by showing that the VP1-D416A mutant does not produce a viable progeny *in vivo* in cotransfection experiments using the reverse genetics system for IBDV (Mundt & Vakharia, 1996) (data not shown).

Optimization of the reaction conditions for RdRp activity of VP1 showed that larger amounts of reaction product could be obtained at 37 °C. Spies & Müller (1990) stated that the polymerase activity detected within purified IBDV particles reached a peak at 40 °C. In our experiments VP1 activity decreased significantly at 40 °C, which may be due to the different reaction conditions. Using VP1-Bac at incubation temperatures above 22 °C, an additional faster-migrating reaction product was observed. The nature of this is unknown, but it is probably a reaction product of VP1 activity, as it was also dependent on the presence of Mg²⁺ ions. Mg²⁺ ions were essential for RdRp activity of VP1, but could be partially replaced by Mn²⁺ or Co²⁺ ions. This indicated that for IBDV VP1, like other polymerases, metal ion co-ordination is important in the polymerization reaction. Similar results were obtained for synthesis of dsRNA using purified IBDV particles (Spies & Müller, 1990). In this system, use of Mn²⁺ instead of Mg²⁺ decreased polymerase activity by 80% compared with the main reaction product consisting of hairpin RNA, in line with the results described here. For the related infectious pancreatic necrosis virus, it has been shown that absence of Mg²⁺ prevented synthesis of dsRNA using purified viral particles (Dobos, 1995).

The finding that the template cRNA was labelled in the cell lysates even in the absence of VP1 (upper product) may be explained by the TNT activities that are present in Sf9 extracts. TNT activity has also been described for RdRps of RNA viruses including NS5B of HCV (Behrens *et al.*, 1996; Ranjith-Kumar *et al.*, 2001), poliovirus 3D^{pol} RdRp (Neufeld *et al.*, 1994) and Q β phage replicase (Bausch *et al.*, 1983). Thus, the VP1 of IBDV itself may contain TNT activity whose analysis requires further experimental studies.

The data presented suggest that the mechanism of RNA synthesis by VP1 *in vitro* probably follows a 'copy-back' mechanism, in which the template's 3' end primes the synthesis of an antisense strand that falls back to form an RNA hairpin. It is interesting that, in contrast to HCV and many other RNA viruses, the polymerase activity of VP1 in IBDV appeared to be strictly dependent on the 3' terminal

sequences of genomic segments A and B. The molecular basis for this dependence remains to be elucidated, although it might be related to the permuted structural organization of VP1 polymerase. Thus, the IBDV polymerase in combination with the 3' end of the genomic RNA may define a certain specificity in the first replication step. A similar specificity was observed with the rotavirus system (Patton *et al.*, 1997) where the very 3' end of the 3' NCR of gene 8 was shown to be necessary for RdRp activity of VP1 + VP2, and thus it may be a special feature of segmented dsRNA viruses.

The data presented represent an important starting point for better understanding of the IBDV life cycle.

ACKNOWLEDGEMENTS

We are grateful to Thomas Mettenleiter for stimulating discussions and critical reading of the manuscript. We also thank Angela Hillner and Dietlind Kretzschmar for excellent technical assistance. This study was supported by DFG grant MU 1244/1-3 and Intervet International, Boxmeer, The Netherlands.

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