Evidence for functional significance of the permuted C motif in Co$^{2+}$-stimulated RNA-dependent RNA polymerase of infectious bursal disease virus

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Segment B of bisegmented infectious bursal disease virus (IBDV) encodes virus protein 1 (VP1), possessing RNA-dependent RNA polymerase (RdRp) activity. This multidomain protein includes an RdRp domain with a non-canonical order of three sequence motifs forming the active site: C–A–B. The A–B–C order of the motifs, as found in RdRps of the majority of viruses, was converted by relocation (permutation) of motif C to a C–A–B order. Due to the unusual location and unproven significance, the motif was named ‘C?’'. This motif includes an Ala–Asp–Asn tripeptide that replaces the C motif Gly–Asp–Asp sequence, widely considered a hallmark of RdRps. In this study, functional significance of the C? motif was investigated by using purified His-tagged VP1 mutants with either a double replacement (ADN to GDD) or two single-site mutants (ADD or GDN). All mutants showed a significant reduction of RdRp activity in vitro, in comparison to that of VP1. Only the least-affected GDN mutant gave rise to viable, albeit partially impaired, progeny using a reverse-genetics system. Experiments performed to investigate whether the C motif was implicated in the control of metal dependence revealed that, compared with Mn$^{2+}$ and Mg$^{2+}$, Co$^{2+}$ stimulated RdRp unconventionally. No activity was observed in the presence of several divalent cations. Of two Co$^{2+}$ salts with Cl$^-$ and SO$_4^{2-}$ anions, the former was a stronger stimulant for RdRp. When cell-culture medium was supplemented with 50 μM Co$^{2+}$, an increase in IBDV progeny yield was observed. The obtained results provide evidence that the unusual Co$^{2+}$ dependence of the IBDV RdRp might be linked to the permuted organization of the motif.

INTRODUCTION

Replication of RNA viruses depends on virus-encoded RNA-dependent RNA polymerases (RdRps) that, in association with other viral and cellular proteins, form replicative complexes (Bartholomeusz & Thompson, 1999; Butcher et al., 2001; Kao et al., 2001; Lai, 1998). The RdRp structures solved to date show that they all have a right-hand-like structure with finger, palm and thumb subdomains (van Dijk et al., 2004). This organization resembles that of other viral and cellular template-dependent single-subunit polymerases, such as DNA-dependent RNA polymerases, reverse transcriptases and DNA-dependent DNA polymerases (Cheetham & Steitz, 2000; Doublie et al., 1999; Ollis et al., 1985). Most RdRp sequence motifs (Koonin, 1991; Poch et al., 1989) are located in the palm subdomain, with motifs A, B and C being the most conserved (Hansen et al., 1997; Ollis et al., 1985; Poch et al., 1989). Typically, these motifs are arranged in the A–B–C sequence order in characterized and putative template-dependent palm-based polymerases. Recently, by using a computer-assisted comparative sequence analysis, a small group of RdRps was identified in which motif C was found upstream of motif A (so-called permuted C–A–B motif arrangement), rather than in the canonical position downstream of motif B (Gorbalenya et al., 2002). These RdRps are encoded by Thosea asigna virus (TaV) and the closely related Euprosterna elaeasa virus (EeV), insect-specific single-stranded RNA viruses with a genome of positive polarity, and double-stranded birnaviruses (Delmas et al., 2004), whose RdRp occupies a domain in the viral protein 1 (VP1). The C–A–B motif organization is compatible with polymerase activity of VP1.

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encoded by infectious bursal disease virus (IBDV) and infectious pancreatic necrosis virus (von Einem et al., 2004; Xu et al., 2004).

Structurally, the C–A–B motif organization is likely to be associated with the unique loop connectivity of the major secondary-structure elements of the palm subdomain at the side opposite to the active site, which is largely formed by the three motifs involved. All of these motifs accepted mutations at otherwise conserved positions of RdRps; these replacements are specific for each lineage, i.e. birnaviruses

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found that IBDV and its polymeerase are highly sensitive to subtle substitutions in the motif C? loop (ADN) that either increased its resemblance to (GDN and ADD) or converted it into (GDD) the canonical sequence. In line with the unique structural properties of the IBDV C? motif, unconventional stimulation of RdRp activity and IBDV replication by Co2++ was observed.

METHODS

Cells. cRNA co-transfection experiments were performed on baby hamster kidney (BHK-21) cells [RIE 194; Collection of Cell Lines in Veterinary Medicine (CCLV), Insel Riems, Germany]. Transfection supernatants were passaged on chicken embryonic cells (CECs) prepared from 11-day-old embryonated eggs of a specific-pathogen-free breed (Lohman). Cells were cultivated in medium 199 (Invitrogen) supplemented with 10% fetal calf serum (FCS; Invitrogen). Growth kinetics were performed in CECs as well as in quail muscle (QM) cells (RIE 466; CCLV) grown in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 10% FCS. High5 cells, a cell clone of insect ovarian cells of Trichoplusia ni (RIE 350; CCLV), were cultivated in serum-free SF900 medium (Invitrogen) and used during transfection experiments for generation of recombinant baculovirus. Cells of a Spodoptera frugiperda (Sf)-derived cell line, Sf9 (RIE 203; CCLV), were maintained in Grace’s insect medium (Invitrogen) supplemented with 10% FCS. Sf9 cells were used for propagation of recombinant baculovirus and preparation of infected cells for protein purification.

Generation of recombinant baculovirus. For expression of VP1, a recombinant baculovirus was generated by using the baculovirus transfer vector pFastBacDual (Invitrogen). The VP1 gene was amplified from plasmid pP2B (Mundt & Vakharia, 1996) by using Deep Vent polymerase (New England Biolabs) and two oligonucleotides (VP1-BacF and VP1-BacHisR; see Supplementary Table S1, available in JGV Online). The PCR fragment was eluted, cleaved with EcoRI/PstI and ligated into appropriately cleaved pFastBacDual to obtain pFastVP1-His. After sequencing, pFastVP1-His was transformed into competent DH10Bac cells (Invitrogen) according to the manufacturer’s instructions. Three white colonies were selected twice on selective plates (as described in the manufacturer’s instructions). Recombinant baculovirus DNA was prepared and subsequently transfected into High5 cells by using Cellfectin reagent (Invitrogen) according to the manufacturer’s protocol. Five days after transfection, the supernatant was passaged by using Sf9 cells to obtain stocks of recombinant baculovirus encoding VP1 of IBDV containing a 6× His sequence at its carboxy terminus (BacVP1-His). In order to exchange certain amino acids in the coding sequence, site-directed mutagenesis was performed (Kunkel et al., 1987). To this end, pFastVP1-His was transformed into Escherichia coli K-12 CJ cells (BioRad) and single-stranded DNA was obtained by following the protocol of Kunkel et al. (1987). For site-directed mutagenesis, four oligonucleotides were used (see Supplementary Table S1, available in JGV Online) to substitute either one (Ala401Gly, Asn403Asp or Asp416Ala) or two (Ala401Gly and Asn403Asp) amino acids. The resulting plasmids, containing mutations in either the C? motif (pGDD-His, pADD-His and pGDN-His) or the A motif (pD461A-His), were sequenced. Appropriate plasmids were selected and recombinant baculoviruses (BacGDN-His, BacADD-His, BacGDD-His and BacD416A-His) were obtained after transfection followed by passaging of the supernatants as described above. Schematic drawings of the obtained plasmids are presented in Fig. 1.

Fig. 1. Schematic representations of plasmids encoding VP1–His and its derivatives. The coding sequence with an artificial 6× His sequence at the C terminus of the RdRp (VP1) of IBDV was cloned into a baculovirus transfer vector. Locations of the RdRp motifs (C?, A, B, E) are shown in the order proposed previously (Gorbalenya et al., 2002). The C? and A motif sequences are specified for the wild type and mutants used in this study. The IBDV A motif conforms to the general formula of RdRp (D-X4–5-E). Amino acids replaced in the mutants are underlined.

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RdRp activity assays. RNA for the RdRp assay was transfected in vitro from plasmid pAP2A (von Einem et al., 2004) by using T7 RNA polymerase. The resulting cRNA was positive-oriented and contained, as well as the complete 5’ and 3’ non-coding regions, a small part of the coding region of segment A. Transcription reactions were treated with DNase I and proteins were extracted with phenol/chloroform; nucleotides were removed by using Micro Bio-Spin 6 columns (BioRad) according to the manufacturer’s instructions. The concentration of cRNA was measured and 1 µg cRNA was used for the RdRp assay. The assay was performed essentially as described previously (von Einem et al., 2004). In reactions where the MgCl2 solution was substituted with CaCl2, CoCl2, CuCl2, FeSO4, FeCl3, MnCl2, NiCl2 or ZnCl2, 2 µl of a 100 mM salt solution in RNase-free water was diluted to a final concentration of 5 mM. All chemicals (Sigma) were of ultrapure quality. After reaction at 37 °C for 1 h, 1 µl proteinase K (20 mg ml−1; Invitrogen) and 1 µl SDS (20%) were added and the resulting mixture was incubated for additional 30 min. After phenol/chloroform extraction, the supernatant was combined with 1 vol. RNA buffer (95% formamide, 0.025% SDS, 0.025% bromophenol blue, 0.025% xylene cyanol, 0.5 mM EDTA). All resulting samples were loaded onto a 5% polyacrylamide gel containing 8 M urea in TBE poured in a Protean XII gel apparatus (Bio-Rad). After electrophoresis overnight, the gel was fixed, incubated with water to remove urea and dried before exposure to an imaging plate (Fujifilm). The exposed imaging plate was read by using a phosphoimager (FLA3000; Fujifilm) with the computer program BAS reader 3.14 (Raytest). Data were analysed by application of the computer program AIDA Image analyse 3.21 (Raytest).

Generation of recombinant IBDV by reverse genetics. For generation of recombinant IBDV, pFastGDD-His, pFastADD-His and pFastGDN-His were cleaved by using EcoRV/DraIII. Fragments encompassing the mutated amino acids (nt 757–1719) were gel-purified and cloned into pP2B cleaved with EcoRV/DraIII (Mundt & Vakharia, 1996). After sequencing, recombinant plasmids containing full-length segment B with the replaced amino acids (pP2B-GDD, pP2B-ADD and pP2B-GDN; Fig. 4) were selected. Plasmids containing segment B and its derivatives (pP2B, pP2B-GDD, pP2B-ADD or pP2B-GDN) and pP2B-D416A (von Einem et al., 2004) were linearized with BsrGI. In vitro-transcribed cRNA of pP2A was co-transfected with cRNA of wild-type or mutated segment B as described previously (Mundt, 1999). Forty-eight hours after transfection, the cells were frozen/thawed and primary CECs were inoculated. When no virus progeny was rescued, three additional blind passages were carried out. In further experiments, VP1–His and its derivatives were purified by metal-ion affinity chromatography (see Supplementary Methods, available in JGV Online). As shown in Fig. 2(a), elution from the Talon matrix yielded a single-band protein of the expected size for each species. Infection of cell cultures with a high m.o.i. (at least 10) and a minimal time span between infection and purification were critical for success of protein purification. By using this protocol, five recombinant proteins (VP1–His, ADD–His, GDD–His, GDN–His and D416A–His) were prepared for subsequent characterization (Fig. 2b). Identities of the different individual proteins were confirmed by combining matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) analysis (according to standard procedures) and Western blotting (using a VP1-specific antiserum) (Birghan et al., 2000; data not shown). The mutant and wild-type protein species were indistinguishable by SDS-PAGE analysis under non-denaturing conditions, indicating the lack of global effects of the mutations on protein folding (data not shown).

Real-time RT-PCR (rRT-PCR) for detection of viral RNA. Supernatants of infected CECs were removed at the appropriate time points and used for the determination of virus titre. Cells were rinsed twice with PBS and RNA was purified by using TRIzol LS (Invitrogen) according to the instructions of the manufacturer. The RNA was dissolved in 50 µl nuclease-free water (Invitrogen). RNA (3 µl) was used for rRT-PCR using a Quantifast Sybr green One-step RT-PCR kit (Invitrogen). For amplification of viral RNA and chicken β-actin mRNA, primer pairs VP1FP/VP1RP and βAEP/βARP (see Supplementary Table S1, available in JGV Online), respectively, were used. The Ct values for both target genes (VP1 and β-actin) of each time point were analysed in parallel, using optimized reaction conditions (50 °C, 10 min; 95 °C, 5 min; 40 cycles of 94 °C, 10 s, with optics off, 60 °C, 30 s with optics on) in a SmartCycler (Cepheid). Presence of the reaction products was verified by melting-point analysis (84.6 and 83.4 °C for VP1 and β-actin RT-PCR products, respectively). The Ct value of the VP1 product was normalized to the Ct value of the β-actin product.

RESULTS

Purification of VP1 species generated by using a baculovirus expression system

The C motif of RdRps is involved in the metal-mediated interaction with incoming NTPs through a conserved Asp residue (Steitz, 1998). In IBDV, its predicted equivalent (motif C?) has unique structural characteristics, including the non-canonical permuted sequence position upstream of motif A and substitutions at two otherwise-conserved positions (Gorbalenya et al., 2002). To gain insight into the functional significance of the C? motif, we sought to characterize mutants of this motif. Five recombinant baculoviruses were generated to express the wild-type VP1 of IBDV strain P2 (BacVP1-His), three C? mutants with either double (BacGDD-His) or single (BacADD-His or BacGDN-His) amino acid replacements, and an A motif mutant (BacD416A-His) that, due to its lack of RdRp activity (von Einem et al., 2004), was provisionally designated a negative control for subsequent experiments. To verify that the different baculoviruses indeed encoded the designed proteins, PCR fragments were amplified from cell-culture supernatants of the infected cells after the second passage by using Deep Vent polymerase (New England Biolabs) and oligonucleotides encompassing the region encoding the C? and A motifs of segment B (see Supplementary Table S1, available in JGV Online). Sequence analysis confirmed the identity of the recombinant baculoviruses (data not shown).

Subtle substitutions in motif C? compromise RdRp activity of VP1

Initially, the VP1 mutants were characterized in a previously developed assay for RdRp activity in vitro (Fig. 2c). It was observed that amino acid replacements in the C? motif inhibited RdRp activity profoundly and, when
combined, cumulatively. The characterized protein species are ranked in the following descending order of observed activity: VP1–His > GDN–His > ADD–His > GDD–His. A 5- and 50-fold decrease in activity was observed for mutants with a single amino acid substitution by a physico-chemically similar amino acid residue at positions 401 (Ala to Gly) and 403 (Asn to Asp), respectively. When combined (Ala401Gly; Asn403Asp), these subtle replacements generated a cumulative effect closely approaching that caused by the dramatic Asp-to-Ala substitution at aa 416 in the A motif that rendered VP1 inactive (von Einem et al., 2004).

In vitro RdRp activity of wild-type and mutant VP1 is stimulated by Co2+, Mn2+ and Mg2+ salts

As it has been shown that the GDD-to-GDN mutation in the C motif of poliomyelitis virus modified the divalent-cation profile of the RdRp (Jablonski & Morrow, 1995), VP1–His and its derivatives (GDN–His, ADD–His and GDD–His) were assayed in the presence of varying cations at different concentrations (1, 5 and 10 mM). In a first series of experiments, the influence of different concentrations of MgCl2 was investigated (Fig. 3). For all protein species, the highest RdRp activity was observed at 5 mM. The relative enzymic activity of the wild-type protein and the mutants was approximately the same as was observed in the initial set of experiments (Fig. 2c). In further experiments, different divalent metal salts (CaCl2, CoCl2, CuCl2, FeSO4, FeCl2, MnSO4, NiCl2 and ZnCl2) were tested for their influence on RdRp activity. The enzymic activity of VP1–His in the presence of 5 mM MgCl2 was taken as 100 %. No RdRp activity was observed for VP1–His or its mutants in the presence of CaCl2, CuCl2, FeSO4, FeCl2, NiCl2 or ZnCl2, whereas MnSO4 and CoCl2 stimulated RdRp activity. These salts stimulated most profoundly at
5 mM, with 4- and 20-fold stimulation of activity by MnSO₄ and CoCl₂, respectively, being observed for VP1–His. In contrast, a stimulation of the GDN–His mutant by these salts was either negligible (MnSO₄) or only 2-fold (CoCl₂). For the ADD mutant, the following residual RdRp activity was observed in the presence of 5 mM of three divalent cation salts: 7 % (Mg²⁺), 3 % (Mn²⁺) and 5 % (Co²⁺) (Fig. 3; data not shown).

**In vivo phenotype of mutants with motif C? substitutions**

The above-described *in vitro* analysis was extended in *vivo* by using reverse genetics. cRNAs transcribed *in vitro* from pP2A and pP2B, pP2B-GDN, pP2B-ADD, pP2B-GDD or pP2B-D416A were co-transfected into BHK-21 cells.
Passaging of supernatants from the transfected BHK-21 cells on CECs showed that only the co-transfection of either pP2A and pP2B or pP2A and pP2B-GDN resulted in infectious virus progeny, named P2r and P2-GDN, respectively (Fig. 5). Transfection experiments were repeated three times followed by three subsequent passages. A similar result was also observed using CECs rather than BHK-21 cells for transfection experiments. Each passage of the supernatant was analysed by Western blotting and immunofluorescence using a polyclonal anti-IBDV serum (Mundt et al., 1995), with only P2r and P2-GDN producing positive results (data not shown). The identity of both viruses was confirmed with RT-PCR and sequence analysis of the obtained fragments (data not shown).

Transfection experiments followed by a passage of the supernatants on CECs were also conducted in the presence of 50 mM FeSO₄, FeCl₂, CuCl₂, MnSO₄ or CoCl₂. In the presence of every divalent ion used, only P2r and P2-GDN were rescued (data not shown).

To characterize the obtained viruses further, growth-kinetics experiments (see Supplementary Methods, available in JGV Online) of the wild-type and GDN mutant viruses rescued after the second passage were performed (Fig. 5). At the five time points characterized and up to 36 h post-infection (p.i.), the relative yield of the P2-GDN mutant was consistently lower. To investigate whether this phenotype was caused by reduced RdRp activity of the GDN mutant, we performed an analysis to estimate the amount of viral RNA by rtRT-PCR. Indeed, the C₅ values obtained for cells infected with wild-type compared with mutant virus (P2r versus P2-GDN) were consistently smaller, indicating a lower RdRp activity of the mutant enzyme.

**IBDV replication is stimulated in the presence of CoCl₂**

To assess a possible effect of divalent cations on replication of wild-type virus in cell culture, cells were infected with P2r (m.o.i. of 1) in the presence of different salts that showed a stimulating effect on RdRp activity (see Supplementary Methods, available in JGV Online). In the first set of experiments, CECs were used. Immediately after virus absorption, CoCl₂ or MnCl₂ was added to the culture medium to a final concentration of 50 μM. Infected CECs with non-supplemented medium containing Mg²⁺, but not Co²⁺ or Mn²⁺, were used as control. This concentration (50 μM) was chosen because CECs tolerated this concentration up to 48 h after exposure, whereas the higher salt concentration (100 μM) proved to be slightly toxic by 24 h after its addition (data not shown). The addition of MnCl₂ inhibited viral production at every time point investigated (12, 24 and 36 h p.i.) (Fig. 6a). In contrast, addition of CoCl₂ to the medium resulted in a small but reproducible increase of viral yield in the cell supernatant, from 3-fold (24 h p.i.) to 5-fold (36 h p.i.). This stimulating effect was confirmed in further experiments using QM cells, where a significant increase in viral titre was observed at 36 h p.i. (Fig. 6b). In contrast, replication of avian reovirus strain 1133 was not affected visibly by the presence of 50 μM CoCl₂ in the CEC medium, indicating that the observed stimulating effect of this salt on IBDV yield is virus-specific (Fig. 6c).
To investigate whether the observed effect on the amount of virus progeny in the presence of divalent ions (here, $\text{Co}^{2+}$ and $\text{Mn}^{2+}$) correlated with accumulation of viral mRNA in CECs (Fig. 6a), rtRT-PCR was performed. For a better discrimination between the $C_t$ values, the obtained RNA was diluted 1:10 before use. Again, accumulation of viral RNA, as measured with $C_t$ values, correlated with the viral titres as expressed by obtained $C_t$ values. It should be mentioned that significant $C_t$ value differences were only observed at two out of three time points characterized (24 and 36 h p.i.).

The higher titres of virus progeny collected in the presence of CoCl$_2$ might be due to selection of mutant viruses. To examine this possibility, we analysed cell supernatants obtained after transfection following one passage on CECs in the absence of CoCl$_2$. The recovered progeny were characterized in both the presence and the absence of 50 $\mu$M CoCl$_2$. No significant difference in the accumulation of progeny over time between viruses rescued in the presence and absence of CoCl$_2$ was found in experiments using different m.o.i.s (0.01, 1 or 10) (data not shown). These data indicate that the presence of CoCl$_2$ during IBDV infection did not favour selection of a high-yield virus in CECs. In order to assay whether the presence of CoCl$_2$ caused a higher mutation rate during replication, the VP2 part of segment A (nt 1–1670) was amplified by RT-PCR and analysed by sequencing. Fifteen plasmids containing the appropriate RT-PCR fragments obtained from each virus progeny (in either the presence or the absence of CoCl$_2$) were sequenced in both directions. Nucleotide-exchange rates were 0.15 and 0.17 nucleotide exchanges per 1000 sequenced nucleotides (with and without CoCl$_2$, respectively).

**DISCUSSION**

In this report, we provide evidence for functional significance of the C$\ddagger$ motif for RdRp activity and viability of a birnavirus that is compatible with its role as a counterpart of the canonical C motif. Further, we show that both RdRp activity and IBDV progeny yield are stimulated by $\text{Co}^{2+}$ unconventionally and relatively strongly, compared with $\text{Mg}^{2+}$ and $\text{Mn}^{2+}$. We speculate that the non-canonical structural organization of the IBDV RdRp could have evolved to utilize $\text{Co}^{2+}$ for its activity to replicate virus.

Among double-stranded RNA (dsRNA) viruses, IBDV was one of the first in which RdRp was provisionally mapped, using a distant sequence similarity to the conserved core of RdRps of positive-sense single-stranded RNA (ssRNA$^+$) viruses (Gorbatenya & Koonin, 1988). When genome sequences of other birnaviruses became available, this initial assignment was confirmed, with a notable exception: unlike three of the four originally identified sequence motifs, A, B and E (called also I, II and IV, respectively), motif C was found not to be conserved (Duncan et al., 1991; Shwed et al., 2002). This finding was surprising given that the C motif, due to its pivotal role in the RdRp structure and function, is uniformly conserved in all other RdRps and related enzymes of viral and cellular origin. A solution to this paradox was proposed by Gorbalenya et al.
(2002). By using a complex profile-based protocol, it was found that RdRps of birnaviruses and ssRNA\(^+\) insect viruses TaV/EeV (currently recognized as members of the family Tetraviridae) adopt a permuted C–A–B motif organization structurally compatible with the palm-fold organization of RdRps. It was also noticed that the permutation correlates with another unusual characteristic: in the permuted RdRps, motifs A, B and C have accepted amino acid substitutions in the positions that remain invariant in other ssRNA\(^+\) and dsRNA viruses (Gorbalenya et al., 2002). For instance, the GDD sequence hallmark of the C motif is replaced by the ADN sequence in the C\? motif of birnaviruses, although it remains unchanged in the C\? motif of the TaV/EeV branch. These RdRp motifs are known to control selective use of NTPs over dNTPs for template-directed synthesis in Mg\(^{2+}\) dependent reactions (Cameron et al., 2003; Crotty et al., 2003). Interestingly, when the conserved positions that are uniquely mutated in the permuted RdRps were probed with similar or other substitutions in the canonical poliovirus RdRp, some of the mutated RdRps (and associated virus replication) acquired an unusual dependence on Mn\(^{2+}\) or Fe\(^{2+}\) (Crotty et al., 2003; Jablonski & Morrow, 1995). Thus, it is conceivable that the permuted RdRps may utilize cation(s) other than Mg\(^{2+}\) for their activity. Indeed, our prior study using non-purified samples of IBDV VP1 showed that Co\(^{2+}\) could stimulate RdRp activity (von Einem et al., 2004).

To study the IBDV VP1 in detail, we have developed a protocol for purification of the recombinant His-tagged VP1. We measured RdRp activity of VP1 by using a previously developed assay that utilizes an internally truncated derivative of the segment A cRNA as a template in a self-primed reaction (von Einem et al., 2004). By using purified VP1, we verified our earlier observation, obtained with cell extracts containing VP1 (von Einem et al., 2004), that this protein possesses RdRp activity. The activity tolerated the artificial 6 × His tag at the VP1 C terminus, but not an Ala replacement of the strictly conserved Asp416 (Asp416Ala) in the A motif (von Einem et al., 2004). Furthermore and in line with results obtained for other dsRNA-containing viruses (bluetongue virus, \(\Phi 6\) phage and human reovirus; Boyce et al., 2004; Makeyev & Bamford, 2000; Tao et al., 2002), our data indicated that cellular and other viral proteins may not be essential for RdRp activity. We cannot exclude the possibility that they could modulate replication and/or transcription of the IBDV genome, because it has been shown that VP3 interacts with VP1 (Lombardo et al., 1999; Tacken et al., 2000), and the formation of VP1–VP3 complexes is probably a key step for the morphogenesis of IBDV particles (Lombardo et al., 1999). Whether amino acid exchanges in the C\? motif influence the VP1–VP3 interaction needs to be investigated in further studies.

Each of two physico-chemically subtle substitutions introduced in the C\? motif inhibited the RdRp activity of VP1. The Ala401Gly replacement converted the ADN motif into a GDN sequence, which is conserved in the C motif of many ssRNA\(^-\) viruses (Poch et al., 1989). This mutant has a decreased RdRp activity in vitro and, accordingly, a debilitating albeit viable phenotype in vivo, when tested by using reverse genetics (Mundt & Vakharia, 1996). The reduced viral titre was indeed caused by a decreased RdRp activity, which was supported by the results of rRT-PCR. In contrast, an Asn-to-Asp replacement at the third position of the C\? motif (Asn403) caused a nearly complete loss of enzymic activity. Accordingly, this mutation resulted in a non-viable virus in vivo. These observations parallel, in one or more aspects, those described for mutants at the first and third positions of the canonical GDD motif in several RNA viruses, including hepatitis C virus (HCV; Benzaghou et al., 2004; Cheney et al., 2002), vesicular stomatitis virus (Sleat & Banerjee, 1993), rabbit hemorrhagic disease virus (Vazquez et al., 2000), bovine viral diarrhea virus (BVDV; Lai et al., 1999) and poliovirus (Jablonski & Morrow, 1995). Collectively, our data strongly support the identification of the C\? motif as a genuine counterpart of the C motif in non-permuted RdRps.

VP1–His is selective in using divalent metals for RdRp activity. Only in the presence of Mn\(^{2+}\), Mg\(^{2+}\) and Co\(^{2+}\) was enzymic activity observed. Other metals (Ca\(^{2+}\), Cu\(^{2+}\), Fe\(^{2+}\), Ni\(^{2+}\) and Zn\(^{2+}\)) were incapable of supporting the VP1–His-catalysed RNA synthesis. A highly selective use of divalent metals (Mg\(^{2+}\), Mn\(^{2+}\), Ca\(^{2+}\) for RdRp activity has also been described for HCV NS5B (Ferrari et al., 1999; Johnson et al., 2000; Ranjit-Kumar et al., 2002). In contrast, a broad range of divalent cations (Cu\(^{2+}\), Ca\(^{2+}\), Mg\(^{2+}\), Fe\(^{2+}\), Ni\(^{2+}\), Co\(^{2+}\) and Mn \(^{2+}\)) was shown to support the RdRp activity of poliovirus 3D\(^{30}\) (Arnold et al., 1999). The observed differences may be either genuine, faithfully reproducing parameters of RdRp activity in vivo, or rather related to differences in assays performed. A molecular mechanism of the interaction between an RdRp and a divalent cation not supporting RdRp activity was elucidated in a structural study of the RdRp of phage \(\Phi 6\) (Salgado et al., 2004). In the presence of Ca\(^{2+}\), the polymerase was shown to be distorted and the processivity of the enzyme was inhibited. Whether similar mechanisms operate in other RdRps remains to be seen.

The Mn\(^{2+}\) stimulation of RdRp activity observed in our experiments parallels that described for RdRps of many viruses, including poliovirus (Arnold et al., 1999), HCV (Ferrari et al., 1999) and BVDV (Lai et al., 1999). In poliovirus, this effect is probably associated with a fast, low-fidelity RNA copying (Arnold et al., 1999) that may be a general phenomenon for RdRps. Mn\(^{2+}\) replaces the commonly used Mg\(^{2+}\) only in specially selected active-site mutants of RdRp of poliovirus (Crotty et al., 2003). In our experiments with wild-type VP1–His, the highest activity was observed in the presence of Co\(^{2+}\) (20- and 4-fold stimulations compared with Mg\(^{2+}\) and Mn\(^{2+}\), respectively); this is highly unusual for an RdRp. For instance, in poliovirus, Co\(^{2+}\) was four times weaker than potent Mn\(^{2+}\) at stimulating RdRp activity (Arnold et al., 1999). In HCV,
Co<sup>2+</sup> blocked RdRp activity altogether (Ranjith-Kumar et al., 2002). According to our data, the Co<sup>2+</sup> dependence of VP1 has characteristics distinguishing it from the Mg<sup>2+</sup> and Mn<sup>2+</sup> dependences. It is modulated selectively by the choice of anion (Cl<sup>-</sup> compared with SO<sub>4</sub><sup>2-</sup>); neither Mg<sup>2+</sup> nor Mn<sup>2+</sup> showed a propensity comparable to that of Co<sup>2+</sup>. In line with these in vitro data, IBVD replication in cell culture was stimulated modestly but reproducibly in a host-independent and virus-specific manner in the presence of 50 μM CoCl<sub>2</sub>. This phenotype was indeed caused by changed RdRp activity, as shown by rtRT-PCR for the presence of viral RNA. It would be interesting to test higher concentrations of CoCl<sub>2</sub>, which, due to cell toxicity, we have not been able to do so far. Collectively, our data strongly support the authenticity of the Co<sup>2+</sup> dependence of the VP1 RdRp.

In summary, our data support a model (Gorbalenya et al., 2002) according to which the C<sub>i</sub> motif of the permuted RdRps is equivalent to the C motif of canonical RdRps. The permuted RdRp organization correlates with the presence of substitutions accepted in several positions of the active site that are strictly conserved in the canonical RdRps. The Co<sup>2+</sup> dependence of the VP1 RdRp presented here indicates that permuted RdRps, also due to these mutations, may have unusual metal requirements.

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