A Complex Zinc Finger Controls the Enzymatic Activities of Nidovirus Helicases

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Nidoviruses (Coronaviridae, Arteriviridae, and Roniviridae) encode a nonstructural protein, called nsp10 in arteriviruses and nsp13 in coronaviruses, that is comprised of a C-terminal superfamily 1 helicase domain and an N-terminal, putative zinc-binding domain (ZBD). Previously, mutations in the equine arteritis virus (EAV) nsp10 ZBD were shown to block arterivirus reproduction by disrupting RNA synthesis and possibly virion biogenesis. Here, we characterized the ATPase and helicase activities of bacterially expressed mutant forms of nsp10 and its human coronavirus 229E ortholog, nsp13, and correlated these in vitro activities with specific virus phenotypes. Replacement of conserved Cys or His residues with Ala proved to be more deleterious than Cys-for-His or His-for-Cys replacements. Furthermore, denaturation-renaturation experiments revealed that, during protein refolding, Zn2+ is essential for the rescue of the enzymatic activities of nidovirus helicases. Taken together, the data strongly support the zinc-binding function of the N-terminal domain of nidovirus helicases. nsp10 ATPase/helicase deficiency resulting from single-residue substitutions in the ZBD or deletion of the entire domain could not be complemented in trans by wild-type ZBD, suggesting a critical function of the ZBD in cis. Consistently, no viral RNA synthesis was detected after transfection of EAV full-length RNAs encoding ATPase/helicase-deficient nsp10 into susceptible cells. In contrast, diverse phenotypes were observed for mutants with enzymatically active nsp10, which in a number of cases correlated with the activities measured in vitro. Collectively, our data suggest that the ZBD is critically involved in nidovirus replication and transcription by modulating the enzymatic activities of the helicase domain and other, yet unknown, mechanisms.

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Nidoviruses feature the most complex genetic organization among plus-strand RNA viruses. Their replicase genes encode an exceptionally large number of nonstructural protein domains (7, 27, 40) which mediate the key functions required for genomic RNA synthesis (replication) and subgenomic RNA (sgRNA) synthesis (transcription) (16, 31). The replicase gene is comprised of two open reading frames (ORFs), ORF1a and ORF1b. ORF1a encodes the replicative polyprotein 1a (pp1a), and ORF1b together encode pp1ab (40, 42). Expression of the ORF1b-encoded part of pp1ab requires a −1 ribosomal frameshift during translation, which occurs just upstream of the ORF1a stop codon (3). The backbone of the replicase polyprotein is formed by a series of conserved domains that are arranged in a unique, nidovirus-specific order (5, 7, 8, 27, 29, 40). Some of these domains are rare or absent in other RNA viruses (27, 40, 41).

The nidovirus structural proteins and a number of virus-specific accessory proteins are encoded by up to 12 ORFs located downstream of the replicase gene (11, 30, 32). These ORFs are expressed from a nested set of 3′-coterminal sgRNAs that, in coronaviruses and arteriviruses, possess a common 5′ leader sequence that is identical to the 5′ end of the genome. It is currently believed that these leader-containing sgRNAs are generated from subgenomic minus-strand templates that are produced by discontinuous RNA synthesis (19, 22, 23, 43).

The unique features of nidovirus RNA synthesis are undoubtedly linked with the viral proteome. Previous sequence analyses identified two nidovirus-wide conserved domains that are absent in other RNA viruses (7). One of these is comprised of about 80 to 100 residues, including 12 to 13 conserved Cys/His residues (8, 35). The domain forms the N-terminal part of a protein containing a superfamily 1 helicase domain in its C-terminal half (Fig. 1). Based on its primary structure, the Cys/His-rich segment was proposed to form a complex binuclear Zn-binding domain (ZBD) that may be involved in RNA synthesis (35). Point mutations in the ZBD or a protein segment called the hinge spacer, which is located immediately downstream of the ZBD’s C-terminal Cys residue, disrupted RNA synthesis in equine arteritis virus (EAV), the prototype arterivirus (35). Other mutations in this region selectively impaired sgRNA synthesis (34, 35).

The ZBD-containing protein is known as nonstructural protein 10 (nsp10) in EAV (36) and nsp13 (formerly p66HEL) in human coronavirus 229E (HCoV-229E) (24, 40). Previously, it...
was shown that nsp10 and nsp13 share biochemical properties, including nucleic acid-stimulated ATPase and 5′-to-3′ duplex-unwinding (helicase) activities (24, 25). In this study, we focused on the ZBDs of nsp10 and nsp13 to gain insight into the mechanism(s) underlying the diverse defects of EAV mutants carrying substitutions in the nsp10 ZBD (35). To this end, the ATPase and helicase activities of bacterially expressed nsp10 mutants were characterized. In parallel, we expressed and characterized a similar set of HCoV-229E nsp13 mutants which we hoped would allow us to draw more general conclusions about the structure-function relationships of nidovirus helicases.

We found that point mutations in the ZBD have strong position- and substitution-dependent effects on the ATPase and helicase activities of both the coronavirus and arterivirus enzymes. The fact that replacements predicted to retain the potential Zn$^{2+}$-coordinating activity (Cys for His or His for Cys) proved to be less detrimental than substitution of the same residues with Ala strongly supports the Zn finger function of this domain. In agreement with this interpretation, denaturation-renaturation experiments revealed that, during protein refolding, Zn$^{2+}$ is essential to the restoration of the enzymatic activities of both nsp10 and nsp13. All mutations that inactivated the ATPase and helicase activities of nsp10 blocked EAV genomic RNA and sgRNA synthesis and, consequently, production of virus progeny. In contrast, the in vivo phenotypes of enzymatically active nsp10 mutants varied widely and did not evidently correlate with the levels of the in vitro activities. Collectively, our data imply that the ZBD is involved in nidovirus replication and transcription by controlling the enzymatic activities of the helicase and, possibly, bymodulating other, yet-to-be-defined pathway(s).

**MATERIALS AND METHODS**

**Site-directed mutagenesis.** A recombination-PCR method (39) was used to introduce point mutations into the helicase-encoding sequences of pMal-nsp13 (previously called pMal-HEL) (9) and pMal-nsp10 (25). Mutations in nsp10 that had already been generated in the full-length EAV cDNA clone (35) were
transferred to pMal-nsp10 by exchanging suitable DNA fragments. Table 1 summarizes the proteins characterized in this study.

**Expression and purification of recombinant proteins.** Wild-type and mutant maltose-binding protein (MBP)-HCoV-229E nsp13 and MBP-EAV nsp10 fusion proteins (Table 1) were overexpressed and purified from *Escherichia coli* cells as described previously (9, 25). Aliquots of purified MBP-nsp10 and its mutants were stored at −80°C in buffer A (20 mM Tris-HCl [pH 8.0], 1 M NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol). Aliquots of purified MBP-nsp10 and its mutants were stored at −80°C in buffer B (20 mM Tris-HCl [pH 7.4], 5 mM ATP, 10% glycerol, 5 mM magnesium acetate, 2 mM dithiothreitol, 0.1 mg of bovine serum albumin/ml) with 25 fmol of a twin-tailed (forked) DNA substrate, 5' to 3' (forked) DNA substrate. ATPase activity was determined as described previously (24). In all cases, poly(U) was added to the reaction mixtures at a concentration of 10 mM EDTA. Finally, both aliquots were dialyzed against buffer B.

At higher ionic strength, nsp10 mutants associated more avidly with MBP than did wild-type nsp10. This finding supported the notion that the nsp10 mutants had decreased catalytic activity.

**Helicase assay.** To determine the duplex-unwinding activities, the recombinant proteins were incubated in 40 µM reaction buffer (20 mM HEPES-KOH [pH 7.4], 5 mM ATP, 10% glycerol, 5 mM magnesium acetate, 2 mM dithiothreitol, 0.1 mg of bovine serum albumin/ml) with 25 fmol of a twin-tailed (forked) DNA substrate, 5' to 3' (forked) DNA-T30 (24). The NaCl concentration in the reaction mixtures, resulting from substrate and protein storage buffers, was 25 mM. Following incubation for 30 min at 30°C, the reactions were stopped by the addition of 10 µl of 5% sodium dodecyl sulfate (SDS)-15% Ficoll-100 mM EDTA-0.25% bromophenol blue dye. The reaction products were separated on 10% to 20% gradient polyacrylamide-1× Tris-borate-EDTA gels (acylamide-bisacylamide, 19 to 1) at 4 W until the bromophenol blue dye approached the bottom of the gel. The gels were exposed to X-ray film at −80°C.

**Introduction of nsp10 mutations in EAV full-length cDNA clones.** Previously, the effects of mutations on EAV nsp10 function were analyzed by using a derivative of the EAV infectious cDNA clone pEA5030 that contained several synonymous substitutions (35) which were introduced to engineer or remove restriction sites. One of these mutations, which removed a HindIII restriction site (residues 12303 to 12308) close to the 3' end of the viral cDNA, was later found to affect the fitness of the virus, which became apparent from somewhat delayed
virus replication and progeny titers that were about five times lower than those obtained with the original pEAV030 clone. Consequently, novel nsp10 mutations engineered for this study (Table 1) were tested in a novel full-length clone (pEAN800) that lacked this unfavorable 3'proximal mutation. Virus derived from plasmid pEAN800 was tested extensively and found to be indistinguishable from wild-type virus (data not shown). The previously engineered C2395H, H2399C, and H2414C mutations in nsp10 were also transferred to the pEAN800 backbone to reconfirm the observed phenotypes. Mutations were introduced in an appropriate shuttle vector by standard site-directed PCR mutagenesis as described by Landt et al. (13). After sequence analysis of the complete PCR product, restriction fragments containing the desired mutations were transferred to pEAN800.

**RESULTS**

Selection of ZBD mutations for enzymatic assays. Previously, a large number of EAV mutants with substitutions in the nsp10 ZBD were characterized in tissue culture with an EAV infectious cDNA clone (35). This set of EAV mutants was extended with six novel EAV ZBD mutants (Table 1) which were characterized with respect to genomic RNA and sgRNA synthesis and production of virus progeny. Only two of the six mutants, C2395H and H2414C, proved to be viable, though delayed compared to the wild-type control. In plaque assays, these mutants produced a small-plaque phenotype and strongly reduced virus titers (Fig. 2, bottom panel). The RNA synthesis of the C2395H and H2414C mutants was also studied by Northern blotting (Fig. 3).
characterized by hybridization analysis. RNA derived from wild-type pEAN800 and from both mutant clones was transfected into BHK-21 cells in triplicate, and progeny virus was harvested at complete cytopathic effect. Fresh cells were infected with the three viruses by using a similar multiplicity of infection, and intracellular RNA was isolated at 12 h postinfection, that is, at the end of the first cycle of infection. The presence of the C2395H and H2414C mutations was confirmed by sequence analysis of RT-PCR products. For both mutants, genome replication and sgRNA synthesis could be detected (Fig. 3), but RNA synthesis as a whole was severely reduced compared to the wild-type control, with the reduction being the most severe for the C2395H mutant.

The reverse genetics data obtained in this and a previous study (35) were used to select the most informative ZBD substitutions to be tested in enzymatic assays with bacterially expressed proteins. We decided to characterize mutations that were associated with four different EAV phenotypes (Fig. 2 and 4, bottom panels): (i) no RNA synthesis, (ii) replication of genomic RNA (but no sgRNA synthesis), (iii) genomic RNA and sgRNA synthesis (but no viable virus), and (iv) viable virus but reduced virus titers.

Wild-type and mutant forms of HCoV-229E nsp13 and EAV nsp10 were expressed in E. coli as fusion proteins with the MBP and purified by amylose-affinity chromatography. As negative controls, we used Walker A box (38) mutants of nsp10 (MBP-nsp10_K2534Q) and nsp13 (MBP-nsp13_K5284A) (Table 1), which were previously shown to be ATPase deficient (9, 25, 26). SDS-polyacrylamide gel electrophoresis analysis was used to confirm that each of the mutant proteins could be expressed and purified to the same level as the wild-type proteins, MBP-nsp10 and MBP-nsp13 (compare lanes 2 to 15 with lane 1 in Fig. 5A and compare lanes 2 to 9 with lane 1 in Fig. 5B).

**Fig. 4.** Effects of substitutions and deletions in the EAV nsp10 hinge spacer region. Helicase activity assays were carried out under the same conditions as in the experiment shown in Fig. 2. Lane 1, reaction without protein; lane 2, heat-denatured substrate; lane 3, MBP-nsp10 (wild type [WT]); lane 4, MBP-nsp10_K2534Q; lane 5, MBP-nsp10_S2429P; lane 6, MBP-nsp10_S2429G/P2430G; lane 7, MBP-nsp10_S2429P/P2430S; lane 8, MBP-nsp10_ΔE2427; lane 9, MBP-nsp10_ΔG2428. Below the gel, the relative ATPase activities of the respective proteins are given. The ATPase activity of the wild-type protein, MBP-nsp10, was taken to be 1.0, and all other activities were normalized to this value. In the bottom panel, the tissue culture phenotypes are given for the EAV mutants containing the respective substitutions or deletions in the hinge spacer region (35). +, present; −, absent; ±, strongly reduced; dsDNA, double-stranded DNA; ssDNA, single-stranded DNA.

**Fig. 3.** Hybridization analysis of the RNA synthesis of EAV nsp10 mutants EAV_C2395H and EAV_H2414C. Intracellular RNA was isolated from similar numbers of infected cells at 12 h postinfection. Both genome replication and sgRNA synthesis of the two mutants were reduced compared to a wild-type (WT) control. EAV RNAs 1 to 7 are indicated to the left.
homologs. Collectively, these results establish that the ZBD is indispensable for the ATPase activities of nidovirus helicases. Furthermore, the data support, albeit indirectly, the Zn\textsuperscript{2+} coordinating properties of the conserved Cys and His residues of this domain. The fact that all Cys-to-His and His-to-Cys mutations (including those that gave rise to replication-competent viruses) affected the ATPase activities of nidovirus helicases suggests that the interplay between the zinc-binding and catalytic domains is finely tuned.

**Duplex-unwinding activity of nsp10 is sensitive to substitutions of conserved ZBD Cys and His residues.** As previously reported, nidovirus helicases have 5’-to-3’ duplex-unwinding activities that depend on ATP hydrolysis (24, 25, 32). We therefore sought to investigate whether (and to what extent) reduced ATPase activities would affect the duplex-unwinding activities of the ZBD mutants. As shown for MBP-nsp10 in Fig. 2, there was a clear link between the ATPase and unwinding activities of a given protein. As expected, mutants that lacked ATPase activity also lacked helicase activity (compare lanes 4, 6, 7, and 8 with lane 3). However, the helicase and ATPase activities were not equally affected by a given mutation in all proteins. Such a discrepancy between the observed ATPase and helicase activities was particularly evident in the nsp10_C2395H mutant that, despite considerable ATPase activity (compare lanes 4, 6, 7, and 8 with lane 3). It thus appears that the C2395H mutation inhibits the helicase activity through both ATPase-dependent and -independent pathways. Taken together, the results suggest that ZBD controls the activities of the catalytic domain and indicate that this function may involve ATPase-dependent and -independent determinants.

Zn\textsuperscript{2+} is an essential structural cofactor for coronavirus and arterivirus helicases. The observed strong effects of single ZBD mutations on the enzymatic activities of the catalytic domain strongly suggest that major conformational changes in the ZBD will disrupt the activities of the downstream helicase. Hence, the enzymatic activities of HCoV-229E nsp13 and EAV nsp10 should also be extremely sensitive to Zn\textsuperscript{2+} depletion. To test this hypothesis, purified MBP-nsp13 and MBP-nsp10 were denatured in urea-containing buffer and subsequently renatured in buffer containing either 100 \textmu M zinc acetate or 10 mM EDTA (see Materials and Methods for details). Subsequent ATPase activity assays consistently revealed that proteins refolded in Zn\textsuperscript{2+}-containing buffer had regained ATPase activity, whereas no significant activity was found for proteins refolded in Zn\textsuperscript{2+}-free buffer (Fig. 6). The latter proteins could not be reactivated by supplementing the ATPase reaction buffer with zinc acetate (data not shown). Similar results were obtained in the helicase assay (data not shown). The data lead us to suggest that Zn\textsuperscript{2+} is an essential structural (rather than catalytic) cofactor of nidovirus helicases which, most probably, is required to maintain the enzymatically active conformation. In line with this interpretation, helicase

### Table 2. Relative ATPase activities of MBP–HCoV-229E nsp13 fusion proteins carrying substitutions in the ZBD

<table>
<thead>
<tr>
<th>Protein</th>
<th>Relative ATPase activity</th>
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<tbody>
<tr>
<td>MBP-nsp13</td>
<td>1.0</td>
</tr>
<tr>
<td>MBP-nsp13_K5284A</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>MBP-nsp13_C5003A</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>MBP-nsp13_C5021H</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>MBP-nsp13_C5021A</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>MBP-nsp13_C5024H</td>
<td>1.5</td>
</tr>
<tr>
<td>MBP-nsp13_C5024A</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>MBP-nsp13_C5028R</td>
<td>0.2</td>
</tr>
<tr>
<td>MBP-nsp13_C5050H</td>
<td>0.9</td>
</tr>
<tr>
<td>MBP-nsp13_C5050A</td>
<td>0.6</td>
</tr>
</tbody>
</table>

\*The ATPase activity of the wild-type protein, MBP-nsp13, was taken to be 1.0, and all other activities were normalized to this value. The Walker A box-substituted protein, MBP-nsp13_K5284A, was used as a negative control. Each value represents the average of the results from three independent determinations, which did not vary by more than 15%.

![FIG. 5. Purification of bacterially expressed MBP-EAV nsp10 and MBP-HCoV-229E nsp13 fusion proteins. Fusion proteins (1 \mu g each) were separated by SDS-polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue. Lane M shows the molecular mass markers. (A) MBP-nsp10 fusion proteins. Lane 1, wild-type (WT) MBP-nsp10; lanes 2 to 15, mutant derivatives of MBP-nsp10, with the respective amino acid substitutions indicated above the gel. (B) MBP-nsp13 fusion proteins. Lane 1, wild-type (WT) MBP-nsp13; lanes 2 to 9, mutant derivatives of MBP-nsp13, with the respective amino acid substitutions indicated above the gel.](http://jvi.asm.org/)

![TABLE 2. Relative ATPase activities of MBP–HCoV-229E nsp13 fusion proteins carrying substitutions in the ZBD](http://jvi.asm.org/)
sequence alignments revealed that the ZBD is the only domain in which residues with known zinc-binding potential (Cys, His, and Glu) (35) are conserved among arteriviruses and coronaviruses (data not shown). These results are fully consistent with the genetic and biochemical data presented above and provide further evidence for a control function of the ZBD over the catalytic domain.

**ZBD may provide an essential cis-acting function for the EAV helicase.** To address the mechanism by which the ZBD controls the activities of the catalytic domain, the unwinding activities of two nsp10 derivatives with ZBD deletions were tested. Both proteins proved to be enzymatically inactive and could not be rescued by a separate ZBD provided in trans (data not shown). The helicase activity was also not rescued by intra-allele complementation in an assay with a mixture of two inactive mutants with replacements in either the catalytic domain (the Walker A box mutant protein, MBP-nsp10_K2534Q) or the ZBD (MBP-nsp10_C2377H or MBP-nsp10_H2399C) (Fig. 2, lanes 13 to 15). Although the negative outcome of these experiments may be explained in numerous ways, the results, combined with the helicase activity of the intact nsp10, indicate that the nsp10 enzymatic activity is critically dependent upon ZBD functions provided in cis.

**Enzymatic activities of ZBD hinge spacer mutants.** In addition to mutations in the ZBD itself, we characterized the enzymatic activities of a series of hinge spacer mutations that were proven by reverse genetics to block viral reproduction in tissue culture (35). MBP-nsp10 derivatives with single (MBP-nsp10_S2429P) or double substitutions (MBP-nsp10_S2429G/P2430G and MBP-nsp10_S2429P/P2430S) had almost wild-type ATPase activities, whereas single-amino-acid deletions in this region (MBP-nsp10_DEL2427 and MBP-nsp10_AG2428) led to reduced (but still significant) ATPase activities (Fig. 4). The unwinding and ATPase activities of these mutant proteins were affected to similar extents (compare the data in the top and bottom panels of Fig. 4), indicating that the hinge region may control the nsp10 helicase activity by modulating the associated ATPase activity.

**Complex relationship between effects of EAV ZBD mutations on in vitro enzymatic activities and virus phenotype.** As could be expected from the pivotal role of the helicase for EAV reproduction (35), mutations that inactivated the enzymatic activities of nsp10 also completely blocked sgRNA and genomic RNA synthesis and production of infectious progeny when tested in the EAV infectious clone (Fig. 2, lanes 4, 6 to 8, and 12, and Fig. 4, lane 4). This nonviable phenotype was also seen in 4 other mutants for which low ATPase/helicase activities had been measured in vitro (Fig. 2, lanes 9 and 11, and Fig. 4, lanes 8 and 9). In contrast, all five mutants with ATPase activities of ≥60% of the wild-type level proved to be replication competent, irrespective of the level of their helicase activities and also irrespective of whether the mutation was located in the ZBD or the hinge spacer region. Three of these mutations (with substitutions in the hinge spacer) showed almost wild-type ATPase/helicase activities in vitro but, in the infectious clone, caused severe defects in viral sgRNA synthesis (Fig. 4, lanes 5 to 7). Only two of the ZBD mutations were compatible with virus reproduction. Remarkably, one of these mutations was C2395H, a protein with very weak helicase activity compared to other enzymatically active nsp10 mutants (Fig. 2, lanes 5 and 9 to 11). This comparative analysis shows that, for most mutations, there is no simple correlation between the virus phenotype in tissue culture and the enzymatic activities of the catalytic domain measured in vitro. In our experiments, partial defects in the nsp10 ATPase activity turned out to be more critical to EAV replication than a reduced helicase activity. Furthermore, our data indicate that, besides its involvement in the ATPase/helicase activities, the ZBD-associated hinge spacer may control another yet-to-be identified activity that is required for nidovirus sgRNA synthesis.

**DISCUSSION**

In this study, we provide several lines of evidence to support a zinc-binding activity of the nidovirus-specific ZBD. Furthermore, the ZBD is shown to be of critical importance to the ATPase and duplex-unwinding activities of the C-proximal helicase domain. The observed dependence of the catalytic domain on the N-terminal ZBD seems to be one of the mechanisms by which the ZBD controls key processes of arterivirus (and probably coronavirus) RNA synthesis.

Zn fingers contain conserved Cys and His residues and have been implicated in diverse functions, including protein-protein interactions as well as binding of single- and double-stranded
nucleic acids (12). The fusion of a Zn finger and a helicase domain in a single protein has been identified in a growing number of helicases (1, 17, 37). However, to date, there are only few examples where this structural association was probed experimentally in functional assays. Similar to our data, Poplawski et al. (21) showed recently that a mutation in the ZBD of an archaeal minichromosome maintenance protein, belonging to a protein family that most probably constitutes the eukaryotic replicative helicase, impaired the DNA-binding, ATPase, and unwinding activities of the enzyme, indicating interactions between the Zn finger and enzymatic domains. For RNA viruses, the association of Zn finger and helicase domains in a single protein has only been reported for the order Nidovirales. We have shown here that Zn$^{2+}$ is an indispensable structural component of arterivirus and coronavirus helicases. Our mutagenesis data also indicate that the structures of nidovirus ZBDs are highly constrained and that there is extensive communication between the ZBD and helicase domains. Thus, even substitutions expected to preserve the zinc-binding properties of the domain (Cys→His or His→Cys) significantly reduced and, in some cases, completely abolished the ATPase and unwinding activities of the C-terminal helicase domain.

Using EAV reverse genetics, it has been shown in this and a previous study (35) that most replacements of conserved nsp10 ZBD Cys/His residues result in a complete block of viral RNA synthesis. These in vivo data were now complemented by assessing the enzymatic properties of the ZBD mutants in vitro. We found that, without exception, nondetectable ATPase and helicase activities in vitro correlate with nonviable phenotypes in vivo. However, we also observed that some mutations produced nonviable phenotypes (nsp10_C2412H and nsp10_H2414A), although the proteins possessed significant ATPase and helicase activities. Collectively, the data support the idea that EAV reproduction is most sensitive to the level of nsp10 ATPase activity. Further studies are needed to elucidate the exact mechanism of this dependence.

A potential functional parallel may exist between the nidovirus ZBD/helicase and herpes simplex virus type 1 UL5/UL52, a heteromeric protein complex with zinc-binding and helicase domains. These domains reside in separate proteins that are both necessary for ATPase and unwinding activities (2). Like in our experiments, mutations in the Zn finger of the herpes simplex virus type 1 UL5 protein were shown to affect the enzymatic activities of the associated UL52 helicase (2). We therefore sought to reconstitute a functional nidovirus helicase from separately expressed Zn finger and helicase domains. The inability to reconstitute nidovirus helicase activity by separate expression of the ZBD and the helicase region may be due to purely technical reasons (for example, incorrectly selected domain borders of the zinc-binding and helicase domains). Alternatively, and in our opinion much more likely, nidoviruses may have evolved a helicase whose activities strictly depend on a ZBD to which the catalytic domain remains covalently bound throughout the infection cycle.

The zinc-binding and helicase core domains of nsp10 are thought to be connected by a so-called hinge spacer segment (35). Previously, point mutations in this region were found to selectively disrupt EAV sgRNA synthesis (34, 35). Interestingly, the corresponding proteins with single or double amino acid substi-

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