

Arterivirus RNA Synthesis Dissected

Nucleotides, membranes, amino acids, and a bit of zinc...

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1. INTRODUCTION

Over the past ten years, research on by far the largest nidovirus protein, the nonstructural gene 1 or replicase gene, has gone through a number of characteristic stages. First, cDNA copies of various nidovirus replicase genes were cloned and sequenced, with those of the coronaviruses infectious bronchitis virus (IBV) (Bournsnell *et al* 1987) and mouse hepatitis virus (MHV) (Lee *et al* 1991) and the arterivirus equine arteritis virus (EAV) (den Boon *et al* 1991) being the first to be completed. Subsequently, extensive sequence comparisons revealed very interesting similarities/homologies within the nidovirus group (then still classified as corona-, toro-, and arteriviruses) and between nidoviruses and other positive-stranded RNA viruses. About five years later, the taxonomic debate on the position of the various nidovirus subgroups ended at the 1996 International Congress of Virology in Jerusalem. The family *Arteriviridae* and the order *Nidovirales* (containing the *Coronaviridae* and *Arteriviridae*) were formally established to acknowledge both the unique properties of arteriviruses and coronaviruses as well as their intriguing ancestral relationship at the level of replicase genes, genome organization, and replication strategy (Snijder and Spaan 1995, de Vries *et al* 1997).

In the meantime, the experimental characterization of the replicase gene products had mainly been focused on the identification of protease domains

and the elucidation of the pathways used for proteolytic processing of the replicase ORF1a and ORF1ab polyproteins (for a review, see Ziebuhr *et al* 2000). A more recent point of interest is the membrane-association of the nidovirus replication complex. With the advent of reverse genetics systems for arteriviruses in 1996 (Meulenberg *et al* 1998, van Dinten *et al* 1997) and, more recently, for coronaviruses (Almazan *et al* 2000), the stage has now been set for the functional characterization of replicase gene products in the context of their main task in the viral life cycle: RNA synthesis. The size of the nidovirus nonstructural polyprotein varies between 3175 residues for the arterivirus EAV and approximately 7200 amino acids for the coronavirus MHV. This size difference of the replicase again illustrates how both conservation and variation have played a part in nidovirus evolution. In addition to a number of highly conserved domains/functions that can be considered to form the "core" of the nidovirus replicase, each virus (or cluster of viruses) appears to have developed its own set of accessory nonstructural functions.

2. PROTEOLYTIC PROCESSING

Proteolytic processing of nonstructural proteins fulfils a key role in the life cycle of most viruses. In the course of virus evolution, highly specific virus-encoded proteases have evolved and their importance for the regulation of virus replication is becoming more and more evident (for reviews, see Dougherty and Semler 1993, Gorbalenya and Snijder 1996). A comprehensive review on the properties and activities of nidovirus proteases has been published recently in the *Journal of General Virology* (Ziebuhr *et al* 2000). A basic characterization of the various protease domains has been carried out and a cleavage site map has been obtained for prototypic coronavirus and arterivirus replicases. Still, given the size of the nidovirus replicase and the details that have emerged for the replicases of other groups of positive-stranded RNA viruses, it is quite likely that -in processing terms- we have merely revealed the tip of an iceberg. Some of the most challenging topics that remain to be investigated are clearly related to the role of proteolytic processing in the regulation of the nidovirus life cycle. That investigating these aspects is not a simple exercise of "knocking out" cleavage sites, has become clear from our work with the EAV infectious cDNA clone. The systematic mutagenesis of each of the EAV replicase cleavage sites (van Dinten *et al* 1999, M. A. Tijms and E. J. Snijder; unpublished data) has revealed that -basically- they are all essential for normal viral RNA synthesis. This conclusion includes sites that are part of the previously described "minor" pathway for processing of the C-terminal

half of the ORF1a protein (Wassenaar *et al* 1997). This indicates that -as far as the *in vivo* analysis of proteolytic processing by mutagenesis is concerned- a more sophisticated approach is required, for example by using partially cleavable mutant sites and/or partially active protease mutants.

3. MEMBRANE ASSOCIATION OF THE REPLICATION COMPLEX

Having raised antibodies against replicase subunits, using them in immunofluorescence assays to analyze infected cells was a logical next step that was taken by several nidovirus research groups. In Leiden, the initial results obtained for EAV were followed by a comparative analysis for MHV.

Possibly due to the number of labs working on either virus group, the results for arteriviruses seem to be more consistent than those for coronaviruses: all EAV replicase subunits (with the exception of a part of nsp1) and *de novo* RNA synthesis (visualized by BrUTP labeling) colocalize in the perinuclear region of the three cell types tested (Pedersen *et al* 1999, van der Meer *et al* 1998). Also a substantial part of the EAV N protein was found in this region of the cell, but its involvement in genome replication or subgenomic RNA transcription is highly unlikely, since N gene expression can be inactivated without a significant effect on either process (Molenkamp *et al* 2000).

For MHV, the situation is much less clear: different replicase subunits have been reported to localize to different membrane compartments in different cell types (Bost *et al* 2000, Shi *et al* 1999, Sims *et al* 2000, van der Meer *et al* 1999). Co-localization of RNA synthesis and N protein has been reported, but is not as complete as for EAV.

Two technical complications that are sometimes overlooked are (i) the fact that coronavirus-infected cells fuse into syncytia that are unsuitable for a reliable cell biological analysis, and (ii) (true for both virus groups) that replicase processing takes time and antisera do not discriminate between processing intermediates and end products.

There is no doubt that -in the end- we will need the power of the electron microscope to understand the details of nidoviral membrane-associated RNA synthesis. The advantages of a 100-fold higher resolution need hardly be explained, and also allows for the identification of cellular compartments on the basis of their morphology and the presence of well-defined markers. So far, however, only one replicase study for each nidovirus subgroup included electron microscopy (Pedersen *et al* 1999, van der Meer *et al* 1999) and therefore it is good to interpret these data with caution. For the moment, two main differences between arteri- and coronaviruses appear to exist: a

replication complex associated with double ER-derived membranes for arteriviruses (EAV) versus a complex associated with single membranes of another origin, most likely endosomal/lysosomal, for coronaviruses (MHV). The general distribution in the cell of these two replication complexes is somewhat different (Fig. 1): the arterivirus signal is generally closer to the nucleus and a bit less punctate.

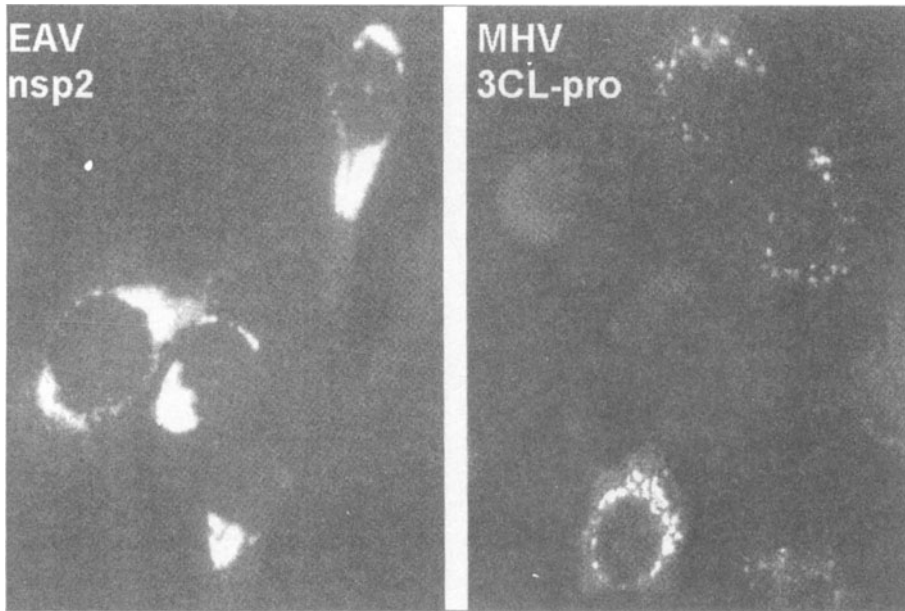


Figure 1. Immunofluorescence staining for specific replicase subunits of the arterivirus EAV (left) in BHK-21 cells and the coronavirus MHV (right) in mouse L cells at a time point around the peak of viral RNA synthesis. For both viruses, the staining is representative for the majority of replicase subunits and *de novo* RNA synthesis (see text).

The formation of paired membranes and double membrane vesicles (DMVs) is a typical feature of arterivirus replication, although the use of double membranes as matrix for RNA synthesis is not restricted to arteriviruses. Double membrane structures in arterivirus-infected cells were seen over 30 years ago and have now been convincingly linked to RNA synthesis. A number of suggestive EM pictures indicate that they originate from ER membranes (Pedersen *et al* 1999), although a thorough biochemical characterization remains to be carried out. We have previously shown that we can induce DMVs that strikingly resemble those in infected cells by expressing EAV nsp2-7, which comprises 1,400 residues of the ORF1a protein, including four major hydrophobic regions. Recently, we have narrowed this down to nsp2 and nsp3 (K. W. Pedersen and E. J. Snijder; unpublished data), which were already known to have a very strong

interaction from our analysis of the proteolytic processing of the ORF1a protein (Snijder *et al* 1994). Co-expression of nsp2 and nsp3, but not the expression of either protein by itself, results in the generation of DMVs.

Clearly we have no well-defined ideas about the way in which the EAV ORF1a proteins associate with membranes. Computer predictions suggest up to 10 trans-membrane domains and furthermore a sort of “processing logic” can be applied (Fig. 2).

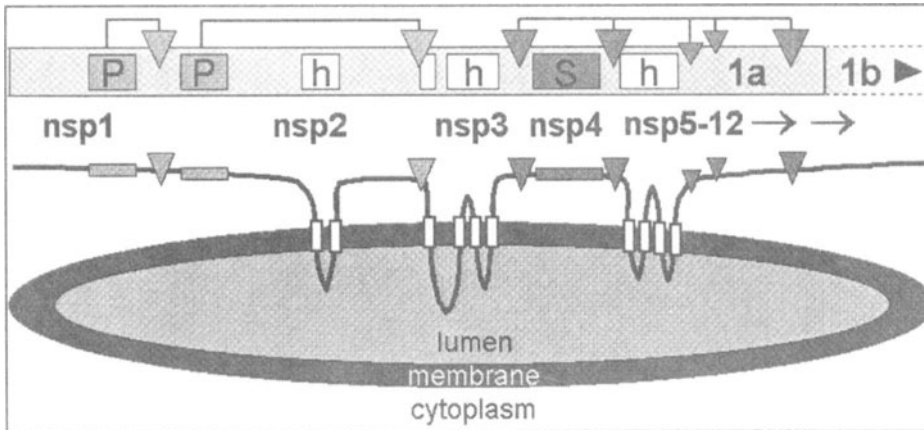


Figure 2. Tentative model for membrane association of the EAV ORF1a protein. The processing scheme of the ORF1a protein is depicted and the hydrophobic domains in nsp2, nsp3 and nsp5 are indicated. The model is based on computer predictions and processing information. Abbreviations: P, papain-like cysteine protease; S, chymotrypsin-like serine protease; h, hydrophobic domain.

For example, the nsp2 protease and the nsp2/3 cleavage site should obviously be on the same side of the membrane, making it logical that the nsp2 hydrophobic domain spans the membrane twice. Likewise, three (and not two or four) is the obvious number of transmembrane segments in the second (largest) hydrophobic domain of nsp3, because otherwise the nsp4 protease would end up in the lumen and not have access to the sites it is known to cleave.

Particularly interesting questions that remain to be addressed relate to (i) the mechanism of translocation, which is probably post-translational since there are no N-terminal signal sequences on the replicase polyprotein, (ii) the coordination of processing and membrane association, (iii) the role of established interactions, like the one between nsp2 and nsp3 (Snijder *et al* 1994) and the cofactor role of nsp2 for the nsp4 protease in cleaving the nsp4/5 site (Wassenaar *et al* 1997), and (iv) the mechanism of the formation of double membranes and DMVs.

4. RNA SYNTHESIS

Following the generation of the EAV infectious cDNA clone in 1996 (van Dinten *et al* 1997), the RNA sequences thought to be involved in discontinuous mRNA transcription were an obvious primary target for site-directed mutagenesis. In our opinion, the discontinuous minus strand extension model (Fig. 3), proposed by Sawicki and Sawicki (Sawicki and Sawicki 1995), is the nidovirus transcription model that is best supported by the experimental data available for corona- and arteriviruses.

According to this model, nidovirus minus strand transcription is either continuous, yielding the genomic minus strand, or discontinuous. The latter process involves a jump, which partially depends on a base pairing interaction between conserved transcription-regulating sequences (TRSs), and yields subgenomic minus strands that serve as templates for the transcription of subgenomic mRNAs. An important aspect of the nidovirus transcription strategy is that the 3' end of the genome contains an array of body TRS sequences, all directing this jump with their own specific activity, and probably also influencing each other's activity in making subgenomic minus strands.

If we try to dissect the discontinuous step in transcription, which may strongly resemble an RNA recombination event (Brian and Spaan, 1997, Nagy and Simon, 1997), we can discriminate at least four steps, each of which may involve specific RNA-RNA and RNA-protein interactions. There is probably attenuation of the transcriptase, translocation of the nascent strand, base-pairing at the leader TRS, and reinitiation of transcription. Although, in our EAV reverse genetics system, we can analyze the impact of mutations only after conversion of the subgenomic minus strand into subgenomic mRNAs, a number of predictions could be made on the basis of the transcription model. First, leader and body TRS mutations were expected to affect base-pairing and identical mutations in leader and body TRSs should complement each other. Second, since not all body TRSs are completely identical, leader TRS mutations might have different effects on the transcription of different subgenomic mRNAs. Third, body TRS mutations may or may not affect attenuation: if they do not, the number of transcriptases reaching upstream body TRSs should remain the same; but if they do for example reduce attenuation, transcription from upstream TRSs may go up.

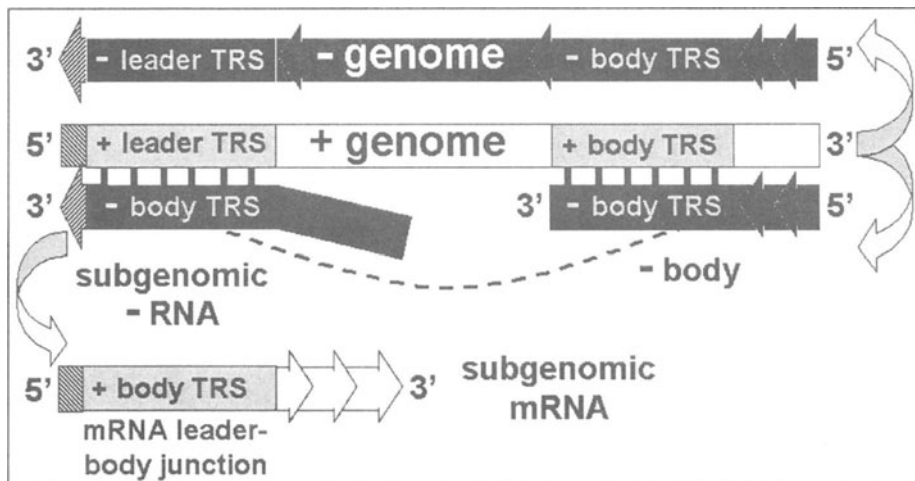


Figure 3. Discontinuous minus strand extension model for nidovirus transcription as proposed by Sawicki and Sawicki (1995). The genomic plus strand is copied into a genomic minus strand that serves as the template for genome replication. In addition, minus strand synthesis can be interrupted at a body TRS, after which the nascent strand is translocated and the TRS complement at the 3' end of the nascent minus strand base-pairs with the genomic leader TRS. Transcription is resumed to add the anti-leader sequence and the resulting subgenomic minus strand (containing the body TRS at its leader-body junction site) is the template for subgenomic mRNA transcription.

Evidence for the importance of the TRS and the existence of a base-pairing interaction between plus leader and minus body TRSs was previously published in a paper by van Marle *et al.* (1999a). However, in this study only the two C residues of the conserved 5' UCAACU 3' sequence that forms the EAV TRS were targeted by mutagenesis. A more recent, comprehensive mutagenesis study of the leader TRS and RNA7 body TRS (A. O. Pasternak, E. van den Born, W. J. M. Spaan, and E. J. Snijder; unpublished data) has revealed that, depending on the nucleotide that is changed, the results can vary quite dramatically. For example, mutants in which identical (leader and RNA7 body) TRS mutations do not restore transcription at all have been obtained. Likewise, body TRS mutations that appear to up-regulate the activity of more upstream body TRSs have been identified. A detailed characterization of these TRS mutants is now in progress.

In addition to RNA primary structure, RNA secondary structure is being studied as a factor that may be involved in the regulation of transcription. A conserved "leader TRS hairpin" has been predicted in the 5' end of all arterivirus genomes and seems a very good candidate to be involved in the base-pairing step of transcription (van Marle *et al* 1999a). At the other end of the genome, a recent analysis of the body TRS regions suggests a link between body TRS activity and its presence in a non-base-paired region of the predicted RNA structure (Pasternak *et al* 2000). Experiments to corroborate this prediction are now in progress.

5. PROTEIN FUNCTIONS IN RNA TRANSCRIPTION

We have previously proposed that the nidovirus replicase may consist of a basic or "core" transcriptase, containing subunits like the RNA-dependent RNA polymerase and helicase found in other systems, that is supplemented with more specialized domains/proteins involved exclusively in subgenomic RNA synthesis (van Marle *et al* 1999a). Thus, one can envision multiple possibilities for discontinuous transcription-specific protein functions, e.g. proteins interacting with body TRSs, leader TRS, or the leader TRS hairpin (Fig. 4).

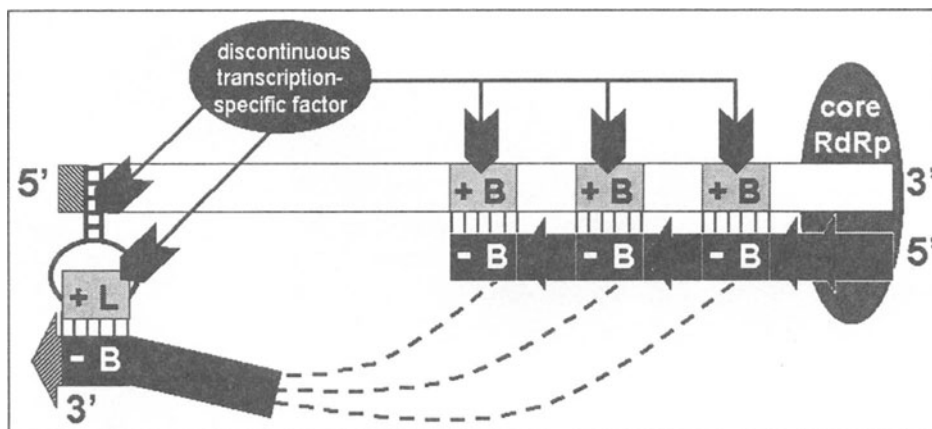


Figure 4. Potential sites for protein-RNA interactions during EAV discontinuous minus strand synthesis. The nidovirus RdRp complex may operate like a core transcriptase that is supplemented with specific protein factors to direct discontinuous minus strand synthesis.

We were confronted with such a transcription-specific function even before the full-length cDNA clone was generated (van Dinten *et al* 1997). Mutant EAV030F had a single replicase mutation in nsp10 (Ser-2429 to Pro)

that selectively knocked out subgenomic RNA transcription, thereby rendering the clone noninfectious. The EAV030F mutation maps to nsp10, which can be considered the most conserved nidovirus replicase subunit, with an N-terminal predicted zinc finger domain (van Dinten *et al* 2000) and a C-terminal helicase, for which unique enzymatic properties were recently revealed that are shared by corona- and arteriviruses (Seybert *et al* 2000). The EAV030F mutation is located in a region that seems to connect zinc finger and helicase (Fig. 5).

We have recently shown that the EAV030F mutant is not completely defective in subgenomic RNA synthesis (van Marle *et al* 1999b). It was estimated that subgenomic plus and minus strand synthesis are about 500-fold reduced, which was supported by a construct in which we inserted the CAT gene at the position of the N protein gene and quantified mRNA7 transcription on the basis of reporter gene expression.

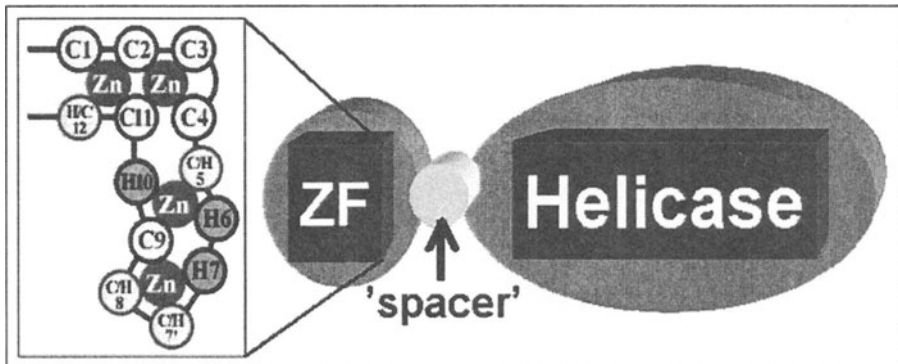


Figure 5. Tentative model for zinc binding by the N-terminal domain of the EAV nsp10 helicase protein (van Dinten *et al* 2000).

More recently, an extensive mutagenesis of the nsp10 zinc finger region and the region containing the original EAV030F mutation was performed, details of which can be found in a paper by van Dinten *et al* (2000). Briefly, all 13 conserved Cys and His residues, which are predicted to be part of an unusual zinc-binding domain (Fig. 5), were found to be important for both genome replication and subgenomic RNA synthesis. However, two additional mutants in the region downstream of the zinc finger displayed EAV030F-like phenotypes and suggested that this region may indeed function as a “spacer” between the zinc finger and helicase domains. The flexibility of this spacer may be essential for a function of nsp10 in subgenomic RNA transcription.

Complementation of the EAV030F defect was attempted using a replicon containing the EAV030F replicase and an IRES/nsp10 cassette in the

genomic 3' end (van Dinten *et al* 2000). Expression of wild-type nsp10 from this locus in the genome was indeed possible, but unfortunately complementation did not occur. Immunofluorescence studies suggested that the IRES-expressed nsp10 did not localize to the replication complex and may have been unable to force the mutant nsp10, which is still active in genome replication, out of the complex.

However, a similar approach for nsp1 was very successful (Fig. 6) and has basically allowed us to establish that nsp1 is a factor that is fully dispensable for genome replication, but essential for discontinuous transcription (M. A. Tijms, L. C. van Dinten, A. E. Gorbalenya, and E. J. Snijder; unpublished data).

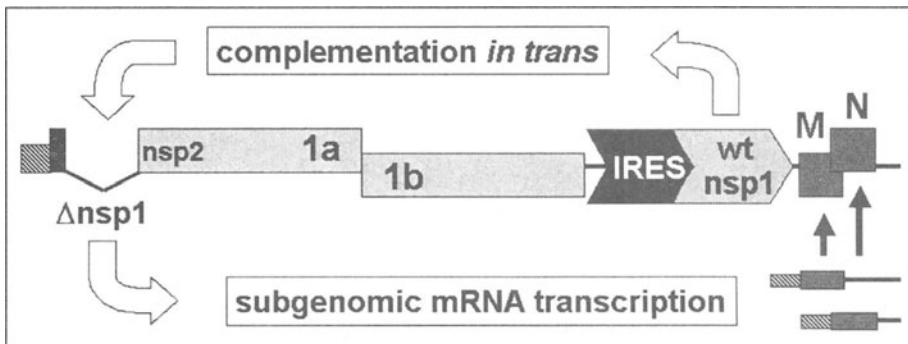


Figure 6. Complementation of the function of EAV nsp1 in subgenomic RNA transcription. First, the nsp1-coding region was deleted from the 5' end of the EAV genome, which resulted in a complete block of subgenomic mRNA transcription. Subsequently, a number of structural genes in the 3' end of the genome were replaced by an IRES element followed by the nsp1 gene. Upon transfection of this construct, nsp1 was expressed from the genomic RNA and subgenomic RNA synthesis (as monitored by the expression of the M and N genes) was found to be restored. These results established the role of nsp1 as a factor specifically involved in subgenomic mRNA synthesis.

Deletion of nsp1 inactivated mRNA synthesis completely, and in this case the IRES-approach yielded a complementation system in which subgenomic RNA synthesis was restored. In contrast to the EAV030F mutant, the block of transcription upon nsp1 deletion seems to be absolute. The identification of a putative zinc finger in the nsp1 N-terminus (M. A. Tijms, L. C. van Dinten, A. E. Gorbalenya, and E. J. Snijder; unpublished data) is an additional intriguing feature of nsp1, since zinc finger domains are widespread in for example transcription factors. Nevertheless, it remains to be established how directly or indirectly nsp1 interacts with the EAV transcription machinery. The fact that the protein does not only colocalize with the viral replication complex, but is also partially found in the nucleus of the infected cell (M. A. Tijms, Y. van der Meer, and E. J. Snijder;

unpublished data), suggests that it may be a multifunctional replicase subunit.

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