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A biochemical portrait of the nidovirus RNA polymerases and helicase

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Chapter 10

A discussion of
nidovirus RNA synthesis

Introduction

Daily, numerous RNA molecules are created inside the eukaryotic cell. In the nucleus for example, RNAs of several hundred to thousand bases long are synthesised by RNA polymerases operating at local concentrations of $\sim 1 \mu\text{M}$ [130,131,438]. However, most of these RNAs need to be shortened through internal splicing events or extended at their 3' end by polyA polymerases before they can become functional [415,439]. RNA modifications are also made in the cytoplasm, where various protein complexes regulate, for instance, i) the stability of miRNAs through the 3' addition of adenosines [440], ii) the degradation of miRNAs by synthesising polyU tails on their 3' ends [441], iii) the efficiency of mRNA translation by restoring or extending their polyA tails [442], or iv) the turnover of ribosomal and mitochondrial RNAs through their ability to add polyadenylyl or heteromeric tails to these molecules [443]. In addition, many enzymes are also involved in the cleavage of RNA molecules, such as exo- and endonucleases, which may be involved in RNA metabolism in general, the maturation of Okazaki fragments or defences against pathogens with RNA genomes [444,445]. Furthermore, motor proteins such as helicases play a crucial role in regulating the dynamics of nucleic acid substrates and the molecules that bind them, including some RNA polymerases that need to switch from initiation to extension, *i.e.*, 'escape' their promoter.

Strikingly and unique for RNA pathogens, many of these functions were also predicted by comparative sequence analysis or found via screening methods in the nidovirus genomes [58,84,273]. This virus group consists of enveloped positive stranded RNA (+RNA) viruses and unites the *Corona-*, *Roni-* and *Arteriviridae* based on comparative sequence analyses, phylogenetic analyses and a similar genome organisation [58,84]. So far, the existence of a large number of these enzymatic functions has been verified using *in vitro* methods, while others were added by accidental discoveries. Together they demonstrate that nidoviruses encode two different RNA polymerase activities [154,156,196] (chapter 3-5), an RNA helicase [109,110], two separate exonuclease activities [105] (chapter 9), an endonuclease [269,446], single strand RNA (ssRNA) binding proteins [270,447], and two methyltransferases [272,273]. Although the identification and characterisation of these RNA proteins is of medical interest - partly since nidoviruses were the protagonists in a number of pandemics, including the 2003 outbreak of the lethal severe acute respiratory syndrome coronavirus (SARS-CoV) [58,65,437,448] and because the development of anti-nidoviral strategies greatly depends on our understanding of the function of these enzymes in the nidovirus replication cycle - they may one day also give us an answer to the question "What constitutes a nidovirus replicase and why do nidoviruses need this enzymatic diversity, while other RNA viruses do not?"

Nidovirus RNA synthesis, a little background...

All nidovirus replicase enzymes, putatively with the exception of at least one actively or passively contributing host factor [290,313], are encoded by the nidovirus genome [58,390]. To accommodate the large number of replicase enzymes, approximately two-thirds of the nidovirus genome is dedicated to encoding two large polyproteins that contain all the mature viral replicative enzymes or non-structural proteins (nsps). Nidovirus genomes are further marked by their polycistronic nature, meaning that they encompass multiple open reading frames (ORFs). To express these ORFs, which are all situated downstream of the replicase gene and encode the structural and accessory proteins required for, *e.g.*, virion formation, nidoviruses also employ a unique discontinuous RNA synthesis process. Quintessentially, this process involves the synthesis and subsequent transcription of a nested set of subgenome (sg) -RNA molecules [266,416,449]. These types of -RNA molecules are both 3'- and 5'-co-terminal with each other and the genome-length -RNA - also referred to as the replicative intermediate when associated with a complementary genomic +RNA and replicase enzymes -, but by definition shorter in length as they progressively lack internal ORFs of the genome. Interestingly, various lines of research have indicated that the replicative intermediate must be derived from a continuous mode of RNA synthesis, whereas a discontinuous mode, including a strand transfer step, is required to produce sg -RNAs [265,416,449]. To produce capped and polyadenylated subgenome and genomic +RNAs, both types of -RNA molecules need to be transcribed [51,265,449].

In spite of having similar genomic structures, the replicase gene is significantly larger in CoVs than in arteriviruses (*i.e.*, encoding ~4000 compared to ~6500 amino acids). Correspondingly, the CoV, torovirus and ronivirus genomes are dramatically larger than those of arteriviruses and have been found to range up to 31 kb [35]. Regardless of these size differences, the replication and transcription of the nidoviral genomes has long been believed to be catalysed by the same conserved viral RNA-dependent RNA polymerase (RdRp) activity [58,84,196,274]. More recent evidence and the observations from chapter 4 and 5 suggest, however, that the CoV and arterivirus RdRps are different and that a second RdRp is involved in CoV RNA synthesis as well [154,155,156]. Whether this second RdRp is crucial for the replication of these large genomes remains to be investigated in detail, but initial experiments suggest that the lysine to alanine mutation at position 58 in nsp8 (*i.e.*, K58A) is lethal for SARS-CoV (Posthuma, Zevenhoven-Dobbe, te Velhuis and Snijder, unpublished results).

The initial expression of the viral replicase enzymes and the RdRp(s) as large polyproteins and their subsequent release from these polyproteins by viral proteases is vital for nidovirus replication. Furthermore, the maturation of nsps appears to follow a conserved pattern across the nidovirus order [44,323]. Although various explanations can be offered to explain this expression strategy, it is presently believed that the above

process chiefly evolved to establish control over replicase assembly and the activation of the enzymes that are part of it. Additional fine-tuning of the molecular ratios between the nidoviral replicase proteins - and thus the regulation of activities in the replication and transcription complex (RTC) - is likely provided by a ribosomal frameshift signal encoded inside the RdRp-coding region. During translation, this structure can interact with and regulate the ribosome and thereby establish a down-regulated expression of the nsps downstream of the frameshift (including the conserved viral polymerase and helicase functions) relative to those encoded upstream [45,323]. Whether this structure also influences (*e.g.*, stalls) the viral polymerase or helicase is presently unknown.

A much overlooked element that is present on all nidoviral mRNAs and required for efficient translation is the 3' polyA tail [418]. It is presently unknown at which stage polyadenylation - which as we described in chapter 8 may be performed by SARS-CoV nsp(7+8) - is initiated on the 3' untranslated region (UTR) present in each sg mRNA and the genome. In fact, it first needs to be established whether the same RdRp that catalyses +RNA synthesis can also perform this activity - in which case polyadenylation would be continuous with extension - or whether two different RdRps, in line with polyadenylation in eukaryotic cells [415,450], are required to complete +RNA synthesis and termination. In this light, it is interesting to note that no clear CoV nsp8 homologue is present in the arterivirus genome, suggesting that both functions may be united in the arterivirus RdRp nsp9, but physically separated in CoVs. Interestingly, if we observe the RNA products in Fig. S1 of chapter 7, we may indeed reach the conclusion that EAV nsp9 is able to perform terminal transferase activity as well as copy an RNA template.

The experiments in chapter 8 suggest SARS-CoV nsp8 may initiate -RNA synthesis on the 3' terminal cytosine of the genome in addition to polyadenylation. The former activity would rule out the attachment of a polyU tail at the 5' ends of the -RNAs. However, the presence of such a tail was reported for bovine CoV -RNAs in 1991 [451] and not necessarily excluded in poly(dT) binding experiments of isolated viral dsRNAs [418], suggesting that -RNAs may become polyU-tailed after the initiation of -RNA synthesis or that -RNA synthesis is significantly different *in vivo* (due to additional protein factors or regulating signals) than could currently be assessed *in vitro*.

Proofreading

Ranking first in genome size among all other RNA viruses, the large genomes of the corona, bafini-, toro- and roniviruses are regarded as unique products of +RNA virus evolution [58,323,437]. As mentioned in chapter 9 and discussed by Gorbalenya *et al.* [35], the faithful replication of such gigantic RNA sequences is a precarious undertaking, particularly if one assumes that their viral polymerases have the same incorporation fidelity as other viral RdRps (~1 error in every 10^4 bases). Interestingly, it was argued that the 3'-to-5' exonuclease function encoded in these large genomes may have facilitated

nidovirus genome expansion by 'adding' a new level of error control to the nidoviral RTC [35,437], an observation that appears to be corroborated by the difference in mutation frequency between wild-type and nsp14 exonuclease knockout CoVs [106,107].

If we consider just their basic function, replicases are already truly remarkable enzymes. Whereas most enzymes either have a relaxed substrate specificity in order to utilise a wide range of relatively similar substrates or a very strict specificity to select a single substrate from a pool of (near) homologues, polymerases essentially do both and are able to adapt their substrate specificity as they translocate along the nucleic acid template [436]. To appreciate this, one merely has to observe the following: the free energy difference in solution between the formation of Watson-Crick base pairs and non-Watson-Crick base pairs is 1-5 kcal/mol (2-8 K_bT) [452,453]. That isn't much, since the incorporation of an NTP already gives us ~11 kcal/mol (~18 K_bT). Further, if we use the above values to calculate the bare error frequency using $\Delta\Delta G = RT\ln(k_c/k_i)$ - where k is the binding constant for correct (k_c) and incorrect bases (k_i), T the temperature, and R molar gas constant - we get an estimate of the basic polymerase error that is around one misincorporation in every 5-150 bases [436]. If we compare this to the observed error rate in RNA viruses (~ 10^{-3} - 10^{-4}) or eukaryotes (10^{-5} - 10^{-9}), it is clear that base pairing in itself cannot account for the selectivity in the polymerase reaction.

The polymerase is thus able to enhance the free energy differences between correct and incorrect base incorporations, a value that can be obtained from the relative rate of incorporating correct and incorrect base pairs, weighted by the concentration of each base pair [436,454]:

$$\text{relative rate} = \frac{(k_{cat} \cdot K_m)_{correct} \cdot [\text{correct base}]}{(k_{cat} \cdot K_m)_{incorrect} \cdot [\text{incorrect base}]}$$

Although these values have been obtained for the well-studied poliovirus RdRp [56,180], they are currently unknown for the nidovirus polymerases and we are thus unable to put a quantitative measure on the fidelity of the nidovirus RNA polymerase. In fact, we only have the qualitative evidence from chapter 9 and the deep-sequencing of passaged virus genomes by Eckerle *et al.* [106,107] for arguing that the CoVs encode a mechanism that has the ability to recognise and correct mismatches in the nascent RNA strand.

Given that two exonucleases have been discovered in the CoV genome and only one has so far been shown to be capable of recognising mismatches, it is of course tempting to hypothesise that the nidovirus proofreading mechanism combines both enzymes to improve the fidelity. However, one might just as well argue that they should work in separate complexes, if only to achieve a higher overall replication rate and efficiency. Here, the latter simply follows from the fact that an interplay between nsp12 and nsp14

would not only require the canonical transfer of the nascent strand from the polymerase active site (E_{pol}) to the exonuclease active site of the RdRp (E_{exo}) [52,436], but it would also entail a subsequent transfer from E_{exo} to the active site of the dedicated exonuclease nsp14 (E_{ExoN}). Clearly, such a multi-step correction process would significantly slow down RNA synthesis and likely be far too elaborate and 'costly' to support just a three-fold larger genome. Interestingly, if we analyse the recently published pair-wise interaction studies between the SARS-CoV nsp's, we find that nsp14 only binds to SARS-CoV's second polymerase, nsp8, and not to nsp12 [85,289,423]. In turn, this suggests that each polymerase may have its own exonuclease to improve the overall fidelity of RNA synthesis that may itself consist of two or more separated processes. However, until we observe a difference in the relative incorporation rate between a CoV nsp12 E_{exo} mutant or a wild-type CoV nsp12 in the presence of a wild-type nsp14 relative to wild-type CoV nsp12 alone, multiple explanations for the observed phenomena are still possible.

Nidovirus RNA products

Regardless of the nidovirus replicase composition and whether it can exist in two or more molecularly and functionally different entities, the polymerases need to direct the catalysis of a 3'- and 5'-coterminally nested set of sg mRNAs. Most of these molecules serve as templates for the translation of only their 5'-proximal ORF, although some are functionally polycistronic and can thus be translated into more than one protein [58,390]. As outlined above, it is now understood that the production of these molecules is orchestrated during -RNA synthesis [265,266,416]. In addition, it is clear that it must involve both a discontinuous step, which produces sg -RNAs, and at least two continuous processes that yield i) the full-length anti-genome template for replication and ii) each type of viral mRNAs [51,265,449].

Crucial parameters that influence and ultimately characterise discontinuous RNA synthesis are the pausing/dissociation frequency of the nidovirus RdRps, the base-pairing interactions between sense and antisense transcription-regulating RNA sequences (TRSs) [455,456], and various protein factors that may switch the replicase from a continuous to a discontinuous mode or just generally stimulate template switching [457,458]. Furthermore, research into the TRSs in arteri- and coronavirus genomes revealed that the genomic sequence surrounding the TRS and the proximity of the TRS to the genomic 3' end might play a role in discontinuous RNA synthesis as well [265,267,422,459,460]. Presently, however, we do not have an estimate of the RdRp processivity, as we lack knowledge of basic parameters like the nucleotide incorporation rate under single cycle conditions (in nt/s) and the RdRp dissociation rate (in s^{-1}). The ratio of these two parameters would easily provide us with an estimate of how far a typical nidovirus polymerase can extend a given RNA molecule before it becomes prone to dissociation, and thus how processive it will be on average. Furthermore, we could use one or both of these

relatively simple parameters to screen the influence of other nsps on the polymerase activity and thereby build up a much wider knowledgebase that could help explain the interplay and composition of the replicase. Of course, the apparent disparity between the continuous and discontinuous processes does not necessarily depend on the regulation of an inherently highly processive RdRp. In fact, the replicase may be able to synthesise long RNAs just as well by using a non-processive RdRp if the polymerase is frequently replaced with 'fresh' polymerase subunits, putatively in a fashion that resembles the polymerase turn-over of the DNA replication machinery [461].

A more direct study estimate of the replicase activity may be obtained with single-molecule force-spectroscopy studies, such as those shown in chapters 6 and 7. These experiments can provide insight into the sequence and force dependency of the enzyme under study, and the enzyme's processivity under various conditions. The data presented in chapter 7 already provide such information for the EAV helicase nsp10 - a component of the EAV replicase and involved in discontinuous RNA synthesis [403] - and demonstrate the effect of co-factors and the local sequence on nsp10's tendency to pause. It is tempting to speculate that this information gives us a glimpse of the processivity of the replicase as well if we assume that the RdRp nsp9 follows in the wake of nsp10. Indeed, given nsp9's inability to displace strands downstream of its polymerase direction itself (chapter 7, Fig. S1) it would be highly dependent on a helicase function and putatively forced to pause at regular intervals if nsp10 fails to unwind the dsRNA. On the other hand, the RdRp-helicase tandem may also be envisioned to be more processive and efficient than each enzyme is on its own: 1) the helicase can unwind the dsRNA, thereby allowing the polymerase to use a locally single-stranded template and reach its optimal incorporation rate, while 2) the helicase can achieve a higher unwinding velocity since the polymerase may function as a moving roadblock behind the helicase and prevent it from translocating backwards and away from the unwinding fork [386,399].

Initiation of RNA synthesis and complex formation

As mentioned above, all nidovirus replicase enzymes are initially part of large polyproteins. Interestingly, these polyproteins contain trans-membrane proteins that can associate the replicase with cellular membranes [58,72,294,390,462,463,464]. Although this process is often considered to be a strategy that establishes a vital (micro)-environment for viral RNA synthesis and a protection of viral replication intermediates and triphosphate-containing, uncapped RNAs from host defence mechanisms [185,228,235], it is in theory also a mechanism to control viral anti-host defence enzymes as they i) include the vital viral proteases required for polyproteins processing and the putative activation of enzymatic functions [44,88] and ii) may inadvertently disrupt cellular regulatory processes that depend on ubiquitination such as organelle biogenesis, ribosome biosynthesis and cellular transcription [465,466,467]. Furthermore, it allows the polyproteins

to be a vehicle that not only contributes to the regulation of the RdRp activity and the assembly of the replicase, it theoretically also enables them to control the initiation of RNA synthesis and thus assist in regulating the RdRp. I will discuss this in the next two sections below.

If we assume that a limited multiplicity of infection (MOI) facilitates, on average, only one infection event per cell, only a single viral genome will be released per cell. Under such conditions, the initial level of viral protein synthesis will thus be limited and the starting concentration of viral proteins correspondingly low. Consequently, without any build-in strategy to ensure that translation is immediately followed by the association of the newly synthesised viral RdRp with this singular viral genome, the likelihood of a chance encounter - even if we account for microdomain formation in the crowded cytoplasm [468,469] - is likely minute. Moreover, given the extensive protein network that surrounds the ER membrane at cellular homeostasis, it seems improbable that the effects of a single translational event and the concomitant insertion of the viral transmembrane domains would suffice to induce sufficient membrane-pairing and curvature to, *e.g.*, produce an invagination that could confine the viral genome. Furthermore, the activities of the viral replicase likely depend on several more protein factors than just the RdRp [111,457] (chapter 7), so in order to support a rapid initiation of viral RNA synthesis, the viral genome must encode signals that establish a long enough association with the nascent polyprotein to allow the formation of the most optimal platform for -RNA synthesis.

Taking the above, the polyprotein-based expression of the viral replicase proteins, and the conserved membrane-association strategies into consideration, the association of the viral genome with the RdRp or other replicase proteins may thus be achieved as follows: i) a direct integration of the transmembrane proteins into the ER membrane by and near the ER translocon (a protein complex that resides in the ER membrane and uses the surrounding phospholipids as medium for protein insertion [470,471]), ii) the production of the viral RdRp and its immediate association with the scaffold of transmembrane proteins (likely via the polyprotein), iii) and the continued association of the genome with the site of transmembrane insertion, possibly due to the presence of multiple translating ribosomes on the genome (*i.e.*, through polysome formation [472,473]). Interesting though inconclusive in light of point iii) is the observation by Sawicki *et al.* that -RNA synthesis is four times more sensitive to translation inhibition than +RNA synthesis [474].

Although association of the RdRp with the membrane scaffold is likely achieved through the covalent linkage of the RdRp to the membrane-spanning subunits in the polyproteins, activation of the RdRp may require its release from the polyproteins, particularly given the importance of the SARS-CoV N-terminal RdRp domains for activity (chapters 3 and 5). It was therefore intriguing to note that nsp8, which is expressed

at equal ratio's with the trans-membrane subunits of the replicase, is able to recognise the 3' UTR of the SARS-CoV genome and use it as template for *de novo* RNA synthesis (chapter 8).

Initiation, continued...

The low viral protein levels that characterise the early stages of infection may also have selected for an RdRp initiation mechanism that requires as few cofactors as possible. This may, for instance, explain why the RdRp subunit of most +RNA viruses studied to date is sufficient to catalyse the condensation of ribonucleoside triphosphates *in vitro* [134,277]. As demonstrated in chapters 3 and 5, both SARS-CoV polymerases nsp12 and nsp(7+8) are active under such conditions, although it is presently not clear how well this activity reflects the incorporation rate *in vivo*. Interestingly, the hypothesised selection for a rapid initiation mechanism may also clarify why SARS-CoV nsp8 initiates dinucleotide formation in absence of a template, even as part of polyproteins nsp7-8 and nsp7-10 (chapter 5, te Velhuis and Snijder, unpublished observations). Such a feature may stabilise its binding to the genomic 3' end and thus facilitate a more rapid transition from translation to the initiation of -RNA synthesis. In addition, such a system predicts that nsp8 would function primarily *in cis*, a hypothesis that is attractively well in line with *in vivo* observations for MHV nsp8 [475].

However, among +RNA viruses like the picornaviruses and the flaviviruses, there is a tendency to use and initiate on RNA structures encoded in the 5' UTR, and not to utilise a multimeric, additional polymerase like nsp8. Poliovirus 3D^{pol}, for instance, initiates RNA synthesis by adding uridylyl moieties to the viral protein VPg using an internal genomic region as template [197], but requires structures in the 5' UTR to coordinate its association with the polyA tail and the initiation of -RNA synthesis [197,206,207]. Flavivirus initiation by NS5 on the other hand immediately starts -RNA synthesis on a 5' UTR promoter, but then similarly relocates to the 3' end of the genome to produce a replicative intermediate [205,223,226].

A factor contributing to the differences between nidoviruses and other +RNA viruses may be the formation of ribonucleoprotein (RNP) complex that associates with the ends of the viral genome, a process that is required for proper flavivirus and picornavirus replication [197,199,223,406]. However, (RNP-based) circularisation of nidovirus genomes has been postulated as well [476] and, together with a sequence-induced folding of the genome [459], provides an attractive mechanism that brings together the body and leader TRSs in order to facilitate strand transfer during discontinuous RNA synthesis.

Presently, there is insufficient evidence to conclude that circularisation of the genome and 3' UTR-based initiation of RNA synthesis by nsp8 are mutually exclusive. A further analysis of the various protein-protein and RNA-protein interactions will, however, be a daunting, but likely also a highly rewarding task. One can think of *in vitro* studies in

which nsp8 RNA synthesis is studied in the presence of both genomic ends and one or more of the various protein co-factors that were found to interact with nsp8 in pair-wise protein-protein interaction studies [289,477]. In addition, recent advances in single-molecule FRET technologies now allow investigators to use multiple FRET pairs, which can facilitate studies of the interactions of multiple biological molecules at the same time [439]. Consequently, such an approach would provide a quantitative and real-time measure of how well RNA synthesis is performed by nsp8 [or nsp(7+8), 7-8 and 7-10] and whether the dissociation constants and incorporation rates are altered in the presence of both genomic ends or co-factors like ssRNA binding proteins, the helicase, or the TRS-unwinding nucleocapsid protein [447].

Finding the replicase in the membrane stacks...

After initiation of -RNA synthesis, nidovirus RNA synthesis is generally assumed to proceed rapidly, since virus-specific radio-active signals in metabolic labelling experiments can already be detected within minutes [418]. Interestingly, as nidovirus infection progresses, the membrane-bound complexes also start to induce various membrane structures, including double membranes, CMs and DMVs [67,68,381,478]. Due to their characteristic morphology and association with nidovirus infections, they have often been used as a signature readout for the efficiency of nidovirus infections and putative sites of replication, and have therefore drawn substantial attention and research investments [70,479]. Interestingly, the involvement of DMVs in nidovirus replication has also been subject to much debate ever since electron tomography failed to visualise clear connections between the inside of SARS-CoV-induced DMVs and the cytosol [68], an observation that is in contrast with the replication vesicles of most other +RNA viruses [75,228]. Furthermore, immunolabelling of CoV-infected cells, showed that the viral nsps, including the second RdRp nsp8, preferentially co-localise with CMs instead of DMVs, suggesting that CMs may be the actual sites of RNA synthesis [68].

Conclusive evidence that establishes that CMs are the sites of RdRp activity in the infected cell is presently unavailable, however. For instance, viral dsRNA, putatively representing the replicative intermediate, has so far been mainly found inside DMVs [68]. In addition, the RNA-synthesising activity of membrane-associated RTCs isolated from infected cells has been shown to be insensitive to both nuclease and protease digestions in absence of detergents, suggesting that active RTCs are protected by their membranes and, serving as a reality check, that membranes, active RdRps and viral nucleic acid are associated with one another [290,313].

Several explanations have been offered to justify the above, ostensibly paradoxical observations for the non-overlapping locations of the viral RNA and the replicase enzymes, including analyses made for flaviviruses in which only a fraction of the replicase proteins was found to be actively contributing to RNA synthesis [68,480]. Still, if we then

assume that DMVs are indeed sealed and that they give a correct indication of the location of active nidovirus RTCs, how do we ratify the impossible diffusion of nucleoside triphosphates into these vesicles and the possibly even more challenging export of viral RNAs out of them and into the cytoplasm for translation or packaging? Furthermore, the increased stability of dsRNA molecules inside these vesicles seems at odds with the reported high turnover of negative strands in MHV infections [481].

Concluding remarks

The outbreak of SARS caused significant economical damages and many human casualties [65,448]. However, in the wake of the 2003 pandemic, the efforts to understand CoV infection and replication increased, and the expansion of our knowledge of nidoviruses has certainly followed suit. Unfortunately, still many nidovirus secrets remain well hidden under wraps and our present understanding of the viral replicase continues to be significantly fragmented.

It is likely that future studies will strongly depend on the outcomes of the sustained development of nidovirus *in vitro* and surrogate systems. The latter may of course utilise the fact that membrane modifications can be induced by the membrane nsps alone and may offer valuable insight into the (micro)-environment that nurtures viral RNA synthesis *in vivo*. Indeed, it is presently largely ambiguous what this environment is and what the RdRp requires to perform all its documented functions *in vitro*. The studies presented in chapters 3-5 and 7-9, should therefore be interpreted with caution and only be regarded as the first steps towards the reconstitution of an active nidoviral replicase *in vitro* given that they still require significantly more quantitative analyses and extensive comparisons with *in vivo* data.

Of course, the RdRp itself should be part of further future scrutiny as well, if only to explain the integration of the exonuclease and polymerase activities in SARS-CoV nsp12 and how this affects the fidelity of the virus in cell culture. Lastly, a better understanding and identification of the nidovirus RNA structures in the 3' and 5' UTRs will likely become important as well, because they appear to play a crucial role in various activities of the viral replicase. Together, the insights that ensue from all these future endeavours will likely allow us to better comprehend what components the nidovirus replicase is made of and how they integrate to give the wide array of phenomena that we can see to date.

