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Arterivirus RNA-dependent RNA polymerase: Vital enzymatic activity remains elusive

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ABSTRACT

All RNA viruses encode an RNA-dependent RNA polymerase (RdRp), which in arteriviruses is expressed as the C-terminal domain of nonstructural protein 9 (nsp9). Previously, potent primer-dependent RdRp activity has been demonstrated for the homologous polymerase subunit (nsp12) of the distantly related coronaviruses. The only previous study focusing on the *in vitro* activity of nsp9 of an arterivirus (equine arteritis virus; EAV) reported weak *de novo* polymerase activity on homopolymeric RNA templates. However, this activity was not retained when Mn^{2+} ions were omitted from the assay or when biologically relevant templates were supplied, which prompted us to revisit the biochemical properties of this polymerase. Based on the properties of active-site mutants, we conclude that the RNA-synthesizing activities observed in *de novo* and primer-dependent polymerase and terminal transferase assays cannot be attributed to recombinant EAV nsp9-RdRp. Our results illustrate the potential pitfalls of characterizing polymerases using highly sensitive biochemical assays.

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Introduction

Polymerases, enzymes that catalyze the templated synthesis of polynucleotides in the 5′–3′ direction, are encoded by all organisms and RNA viruses, as well as some DNA viruses. Reflecting the principal differentiation into DNA- and RNA-based processes and functions, they can be grouped into four classes, each possessing a distinct combination of specificities for their substrate (NTPs or dNTPs) and template (RNA or DNA) under physiological conditions. Despite these fundamental differences in terms of substrate requirements, many polymerases in these four classes, including all characterized RNA-dependent RNA polymerases (RdRps), employ the same catalytic mechanism and a similar three-dimensional fold, which resembles the shape of a right hand with finger, thumb, and palm domains (Iyer et al., 2003; Ng et al., 2008). At the sequence level, these polymerases share motifs A and C, which reside in the most conserved palm domain (Delarue et al., 1990). A few conserved residues, primarily aspartates, located in these motifs are involved in (d)NTP binding and/or catalysis (Arnold et al., 2005; Arnold and Cameron, 2004) and consequently their replacement should abolish or at least severely decrease nucleic acid synthesis (Ng et al., 2008).

Based on their requirements for initiation of nucleic acid synthesis, two types of polymerases are recognized: primer-dependent and *de novo*-initiating enzymes (Ng et al., 2008; van Dijk et al., 2004). The latter, to our knowledge exclusively (DNA- or RNA-dependent) RNA polymerases, are capable of positioning the first two NTPs, typically two purines, in a manner that allows the formation of a starting dinucleotide. In contrast, primer-dependent polymerases are unable to accommodate the stable association between the first (d)NTP and the template that is required for *de novo* initiation. Consequently, the formation of a first dinucleotide is an energetically extremely unfavorable event and to overcome this problem short RNA primers must be recruited, placed on the template, and extended. For this purpose, organisms and viruses have evolved different priming mechanisms that are all supported by additional proteins or domains. They may involve the synthesis of short RNA fragments (by e.g. eukaryotic DNA primase (Pellegrini, 2012)), the formation of covalent RNA-protein complexes (e.g. picornavirus VPg-RNA complexes (Steil and Barton, 2009)), or the utilization of tRNAs (by lentivirus tRNA-binding domains (Kleiman, 2002)) or cap-bearing 5′ fragments cleaved from cellular mRNAs (generated by influenza virus, bunyavirus, and arenavirus endoribonuclease and cap-binding domains (Boivin et al., 2010; Morin et al., 2010; Reguera et al., 2010)).

In viruses of the order *Nidovirales* (comprising the families *Arteriviridae*, *Coronaviridae*, *Mesoniviridae*, and *Roniviridae*), which are characterized by their large to exceptionally large single-stranded RNA genomes (de Groot et al., 2012; Lauber et al.,

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2012), a canonical RdRp is expressed from ORF1b as part of the pp1ab replicase polyprotein. After proteolytic cleavage, a protein subunit (nonstructural protein 9 (nsp9) in *Arteriviridae*, nsp12 in *Coronaviridae*) harboring conserved RdRp motifs in its C-terminal two-thirds is released (Gorbalenya et al., 1989; Lauber et al., 2012; Lehmann et al., 2015; van Dinten et al., 1996; Ziebuhr and Siddell, 1999). This cleavage product is a key subunit of the membrane-associated multi-subunit enzyme complex that mediates the synthesis of diverse viral RNAs (Sawicki et al., 2007; van Hemert et al., 2008a; van Hemert et al., 2008b). This complex has been characterized *in situ* and through reconstitution of its activities *in vitro*. In one of these studies severe acute respiratory syndrome coronavirus (SARS-CoV) was proposed to express a second, non-canonical RNA polymerase subunit: the ORF1a-encoded nsp8 (Imbert et al., 2006), which was described as an obligatory *de novo*-initiating polymerase capable of synthesizing products of less than six nucleotides (Imbert et al., 2006). Since nsp12 was found to be primer-dependent (te Velthuis et al., 2010a), it was speculated that the two proteins may work sequentially on the same template, with nsp8 providing the primers required by the nsp12 “main RdRp”. Although supported by some biochemical studies on nsp8 of other coronaviruses (Xiao et al., 2012), other results called into question this clear division of labor between nsp8 and nsp12 (te Velthuis et al., 2012; Ahn et al., 2012). Most recently, the addition of nsp7 and nsp8 was shown to activate the primer extension activity of SARS-CoV nsp12 in an *in vitro* assay (Subissi et al., 2014). The same protein combination was required for *de novo* initiation of RNA synthesis. The nsp7-nsp8 complex by itself, on the other hand, did not show any polymerase activity and was therefore proposed to serve as an activator and processivity factor for the nsp12 RdRp.

Besides SARS-CoV nsp12, RdRp activity was studied previously for only one other nidovirus “main RdRp”, nsp9 of the arterivirus equine arteritis virus (EAV; Beerens et al., 2007). In that study, weak *de novo* RdRp activity was reported on poly-uridine (pU) and poly-cytidine (pC) single-stranded RNAs, while no primer extension or terminal transferase activity, i.e. the non-templated elongation of RNA strands, was detected. Thus, it was concluded that EAV nsp9 activity is restricted to *de novo* initiation. However, the relevance of the observed activity for virus replication remained uncertain since activity on templates containing appropriate virus-specific sequences could not be detected and the *in vitro* activity required the presence of Mn^{2+}, which is known to relieve template requirements for other polymerases (Arnold et al., 1999). One possible explanation for the lack of initiation on virus-specific templates could be that additional co-factors, e.g. higher-order RNA structures or proteins, are needed for genuine *de novo* initiation *in vivo*. Therefore the aim of this study was to characterize the RNA polymerase activity of EAV nsp9 in more detail. We report the results of a carefully controlled study involving several preparations of purified recombinant EAV nsp9, including the wild-type protein and a set of active-site mutants, which were tested for *de novo* and primer-dependent polymerase and terminal transferase activities. However, the RdRp domain of recombinant EAV nsp9 did not specifically display any of these activities and we were thus unable to reproduce the published polymerase activity of the protein. Furthermore, we noticed a striking resemblance between the product profile of one of the tested nsp9 preparations and that of T7 phage RNA polymerase. Our results emphasize the need to employ diverse controls when utilizing highly sensitive biochemical assays.

Results and discussion

Expression and purification of EAV nsp9 using two vectors

Previously, the purification and *de novo* polymerase activity of recombinant EAV nsp9 were described (Beerens et al., 2007). In that study the viral protein (which will be referred to as nsp9/pDEST) was expressed from a pDEST vector with a C-terminal hexahistidine tag in *E. coli* BL21 (DE3). Typically, an unknown fraction of such bacterially expressed proteins may contain an N-terminal formylmethionine, due to saturation of the endogenous protein processing pathway by nsp9 overexpression. Such an N-terminal extension would not be consistent with the authentic N-terminus of nsp9, which is expected to be a glycine residue derived from the proteolytic release of nsp9 from the pp1ab polyprotein by nsp4-mediated cleavage of the Glu1677↓Gly1678 site (Snijder et al., 1996). As it was previously reported for SARS-CoV nsp8 and nsp12 that such foreign N-terminal sequences may influence RdRp activity and stability, respectively (te Velthuis et al., 2010a, 2012), we decided to also express EAV nsp9 as part of a ubiquitin fusion protein by using a pASK vector (Gohara et al., 1999; te Velthuis et al., 2010a) yielding a product that will hereafter be referred to as nsp9/pASK. Co-expression of the ubiquitin-specific protease UBP1, which can remove the N-terminal ubiquitin fusion partner *in bacterio*, enabled us to reproduce the natural glycine N-terminus of nsp9. An additional advantage of the pASK vector is that it allows expression to be driven by the endogenous pool of *E. coli* RNA polymerase after induction with anhydrotetracycline. In contrast, nsp9/pDEST was expressed from a T7 promoter. The potential presence of the phage T7 RNA polymerase in the ultimate nsp9 preparations, with previously demonstrated weak RdRp activity (Beerens et al., 2007), could be of concern.

Both recombinant nsp9 variants were expressed under identical culture conditions and batch purified using metal ion chromatography, with Co^{2+} targeting the C-terminal hexahistidine tag of both polypeptides. As Fig. 1A shows, both proteins could be obtained with similar purity, but nsp9/pASK was expressed in higher quantities than nsp9/pDEST. Attempts to further purify the proteins by gel filtration did not result in a significant improvement, as judged by silver staining of SDS-PAGE gels (data not shown).

T7 RNA polymerase contamination may account for de novo activity observed with EAV nsp9/pDEST preparations

The preparations of nsp9/pDEST and nsp9/pASK were tested side-by-side in a *de novo* polymerase assay as described before (Beerens et al., 2007). The only noteworthy difference was the length of the pU template, which was 30 nucleotides in our experiments compared to an undefined mixture containing RNAs of up to 300 nucleotides in the study of Beerens *et al.* Unfortunately, neither of the nsp9 variants showed any activity on this template. Next, we tested for RdRp activity using a template whose 3′-terminal dinucleotide matched the CC dinucleotide that is present immediately upstream of the poly(A) tail at the 3′ end of the EAV genome. Indeed, as previously shown for homopolymeric pC templates, nsp9/pDEST exhibited some activity with this RNA template, while nsp9/pASK remained essentially inactive (Fig. 1B, middle and left panel, respectively, lanes R_1).

The nsp9/pDEST protein differed from nsp9/pASK in having an artificial N-terminal residue and in being produced by induction of T7 RNA polymerase expression. We reasoned that the latter feature, expression of an additional polymerase, may be linked to the (gain of) activity observed for the nsp9/pDEST preparation. To test this hypothesis, we included a highly diluted sample (0.01 U/μl final concentration) of a commercially available T7 RNA polymerase in our assays. Since this enzyme is DNA-dependent, we included two single-stranded DNA templates: a DNA variant (D_1) of the RNA template used and a DNA template containing the complement of the T7 promoter sequence (D_4). Surprisingly, T7 RNA polymerase was active on all of these templates under the employed conditions (Fig. 1B, right panel) with the expected preference for DNA templates.

Strikingly, the nsp9/pDEST preparation showed the same overall pattern, including the preference for DNA templates, as the commercial T7 RNA polymerase. In line with this notion, an nsp9/pASK preparation gained *de novo* activity once it was expressed in BL21 (DE3) after addition of IPTG (data not shown). Hence, this circumstantial evidence suggested that contaminating T7 RNA polymerase, rather than EAV nsp9 itself, was responsible for the *de novo* polymerase activity observed. We investigated whether a gel filtration step with a low flow rate (0.3 ml/min) would remove the suspected trace contamination of T7 RNA polymerase (molecular weight 99 kDa) from a preparation of nsp9/pDEST (molecular weight 78 kDa), but this was not found to be the case (data not shown).

In conclusion, our results revealed that the polymerase assay employing ^{32}P-labeled NTPs, which was used in this and previous studies, is sensitive enough to detect the activity of trace amounts of contaminating T7 RNA polymerase. This polymerase was also able to act on templates lacking the established T7 promoter requirements. Whether or not this contamination was also present in the nsp9 preparations described in Beerens et al. (2007), and later on also by te Velthuis et al. (2010b), cannot be established with certainty as the experiments presented here and those published previously differed in some respects. Particularly the previously used purification protocol could not be reproduced in our experiments due to technical difficulties with the described purification buffer, which in our hands induced protein precipitation during purification. Also, the fact that we did not detect any RdRp activity for nsp9/pDEST on a pU template, and therefore also not for any potential contaminant, may argue in favor of the detection of genuine nsp9 activity in those previous studies. In this context, it is noteworthy that thus far the two reported coronavirus RdRp activities (associated with nsp8 and nsp12) were addressed in six independent biochemical studies (Ahn et al., 2012; Imbert et al., 2006; Subissi et al., 2014; te Velthuis et al., 2010a, 2012; Xiao et al., 2012), none of which succeeded in exactly reproducing the results of any of the others. This may indicate that nidovirus RdRps are highly delicate proteins that may respond to minute changes during purification or in their reaction environment.

EAV nsp9/pASK preparations possess primer-dependent polymerase and terminal transferase activity on RNA substrates

Besides testing for *de novo* activity, we analyzed whether EAV nsp9 may possess primer-dependent polymerase activity, like its coronavirus homolog nsp12 (Subissi et al., 2014; te Velthuis et al., 2010a). We used a similar assay as the one described above, but this time provided partially double-stranded templates. We found that both nsp9 preparations were enzymatically active on these templates and showed the highest extension activity if both the template and primer were RNAs (Fig. 1C, left and middle panel). The impact of the type of substrate showed that the observed activity correlated directly with the addition of nucleic acid substrates, hence was not based on a co-purified *E. coli*-derived RNA or DNA substrate. Furthermore, as the presence of a DNA template significantly decreased processivity, it also demonstrated that the responsible polymerase was RNA dependent. Interestingly, while the use of a DNA primer in combination with an RNA template completely precluded extension of the primer (no products in the 20–39 nucleotide size range), a radiolabeled 40-nucleotide product was detected. This suggested that the polymerase possessed terminal transferase activity, but only on RNA substrates. To investigate this further, we also compared the elongation of single-stranded RNA and DNA substrates in an assay otherwise identical to the one used for measuring primer-dependent polymerase activity (Fig. 1D). As expected, both nsp9 preparations showed a clear selectivity in favor of RNA. In this context, it is also noteworthy that neither the primer extension nor the terminal transferase assay buffer included Mn^{2+} ions, which can favor activity on sub-optimal templates (Arnold et al., 1999). Together with the demonstrated DNA specificity of T7 RNA polymerase (Fig. 1C and D, right panels) this supports the reliability of these assays with respect to reproducing physiologically relevant substrate preferences.

To conclude the characterization of the polymerase, its nucleotide preference was examined. To this end, a primed RNA template (Fig. 2A) was first elongated in the presence of a low concentration of radiolabeled ATP, resulting in frequent abortion of transcription after incorporation of the first nucleotide. Subsequently, either dATP or ATP was supplied in a concentration that should allow restarting and completion of the reaction (Fig. 2B). As expected, addition of ATP enabled the synthesis of almost fully extended products, while dATP did not support any extension beyond one or two nucleotides (Fig. 2C). In agreement with the lack of DNA primer extension and the known inability of the prototypic poliovirus RdRp to further extend deoxynucleotide chains (Arnold and Cameron, 2004; Gohara et al., 2000), we thus conclude that the observed activity originated from an RNA-dependent RNA polymerase.

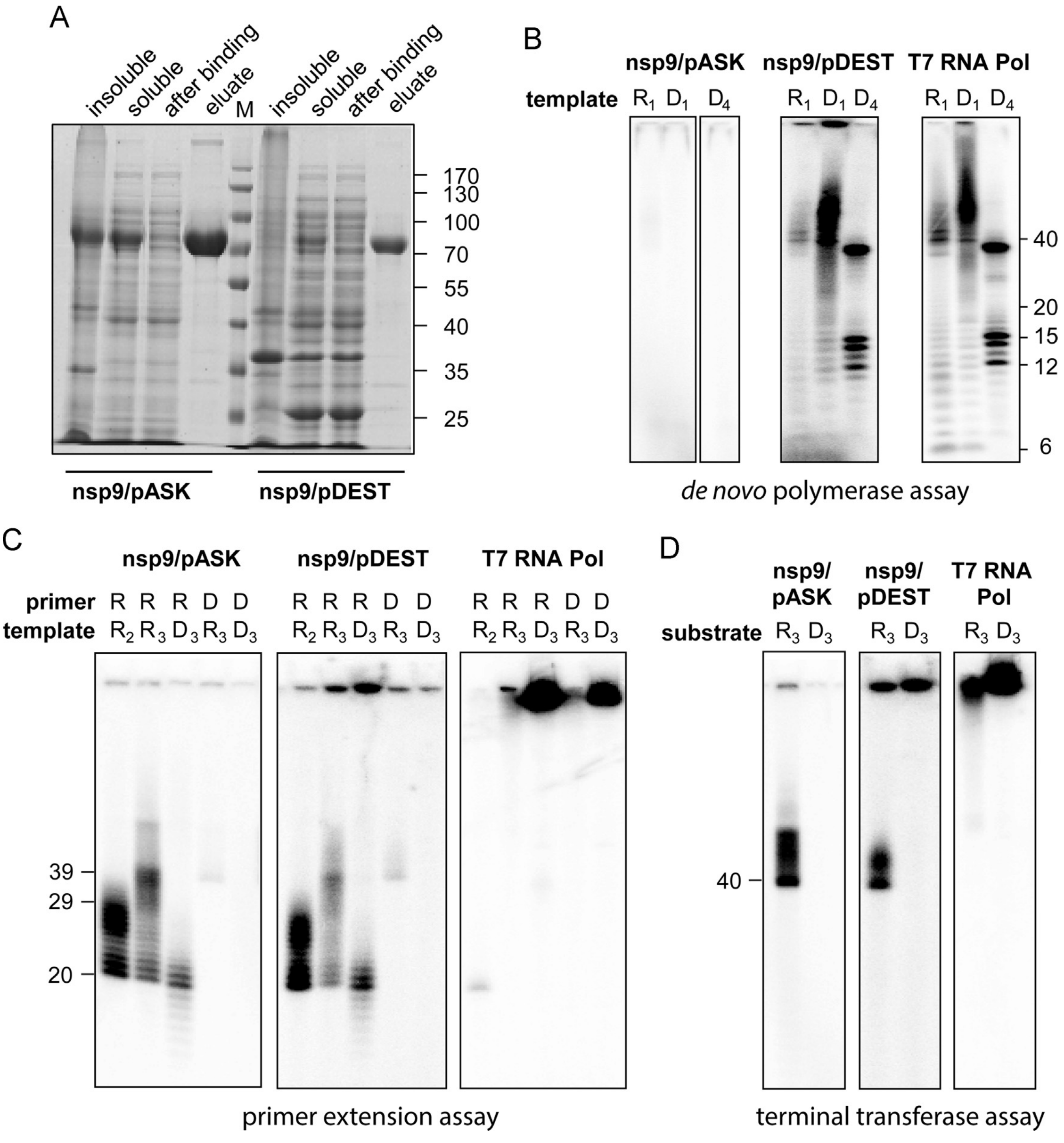
EAV mutants carrying replacements of active-site aspartates of the nsp9 RdRp

Polymerase activity is primarily based on a two-metal-ion mechanism involving several residues. In contrast to other catalytic mechanisms, which may feature a single or a few absolutely required residues, these individual amino acids work in concert during metal catalysis to provide a framework for metal ions and substrates to bind. Consequently, instead of abolishing all activity, the substitution of single residues in the active site of an RdRp may merely reduce these binding affinities. Thus, depending on the individual contribution of a residue, such replacements may be more or less detrimental to the enzyme's function, and consequently to virus viability.

To establish whether the observed activity was associated with the RdRp domain of EAV nsp9, we substituted several key residues of its (predicted) active site (Beerens et al., 2007; Den Boon et al., 1991) with alanine. Using a full-length cDNA clone, we engineered EAV nsp9 mutants with alanine substitutions of each of the four conserved aspartates of motifs A (mutants D445A, D450A and double mutant D445A/D450A) and C (mutant D560A and double mutant D559A/D560A), which in better characterized polymerases are known to coordinate the essential metal ions or interact with the NTP's 2′ and 3′ hydroxyl groups. In agreement with their expected essential role, and with our preliminary observations for equivalent SARS-CoV nsp12 mutants (unpublished data), each of the aspartate-to-alanine substitutions tested had a severe impact on viral replication. Whereas all double mutations tested were lethal, viruses carrying single mutations apparently retained a low level of RNA synthesis, ultimately leading to reversion to the wild-type genome sequence later in the experiment (by 48 h p.t.; see also Supplementary Table S2). In all cases, a single point mutation was sufficient to restore the codon for the wild-type residue. Nevertheless, the results were somewhat unexpected given the universal conservation of all four aspartates in positive-stranded RNA virus RdRps. To our knowledge replication of mutants with a single replacement of these active site residues, even though severely decreased and undetectable until reversion had occurred, has not been reported for any other RNA virus thus far.

Observed primer extension and terminal transferase activities cannot be attributed to EAV nsp9 RdRp

Following the reverse genetics studies on the RdRp active site of nsp9, we transferred the same mutations to the nsp9/pASK expression construct to obtain negative controls for the biochemical RdRp assays described in the previous paragraphs. None of the double, triple, or quadruple aspartate-to-alanine substitutions tested showed a decreased primer extension activity compared to two independently purified batches of wild-type recombinant nsp9/pASK (Fig. 3). Likewise, D445A and D560A mutant proteins retained terminal transferase activity (data not shown). Thus, the observed activities either derived from a second active site within nsp9, which was not targeted by mutagenesis, or originated from a different (contaminating) protein altogether. To discriminate between these possibilities, we tested whether it was


A
insoluble
soluble
after binding
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M
insoluble
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170
130
100
70
55
40
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25
nsp9/pASK
nsp9/pDEST
B
nsp9/pASK
nsp9/pDEST
T7 RNA Pol
template
R1 D1 D4
R1 D1 D4
R1 D1 D4
40
20
15
12
6
de novo polymerase assay
C
nsp9/pASK
nsp9/pDEST
T7 RNA Pol
primer
R R R D D
R R R D D
R R R D D
template
R2 R3 D3 R3 D3
R2 R3 D3 R3 D3
R2 R3 D3 R3 D3
39
29
20
primer extension assay
D
nsp9/ pASK
nsp9/ pDEST
T7 RNA Pol
substrate
R3 D3
R3 D3
R3 D3
40
terminal transferase assay

Fig. 1. Expression, purification, and RNA polymerase activities of two recombinant EAV nsp9-His preparations. R and D indicate the use of RNA and DNA templates, respectively. Identical numbers indicate templates with equivalent sequences. Template sequences are listed in Supplementary Table S1. Product lengths (nt) are indicated on the right or left side of the figures. (A) Coomassie brilliant blue-stained SDS-PAGE gel of samples taken during metal ion chromatography using Co^{2+}. Insoluble and soluble: respective fractions after cell lysis; after binding: unbound protein after removal of Co^{2+} resin; eluate: elution fraction after purification. The molecular weight of nsp9-His is 78 kDa. Size markers are depicted on the right in kDa. (B) *de novo* polymerase assay using nsp9 expressed from pASK (nsp9/pASK; final protein concentration 2 μM) or pDest (nsp9/pDEST; final protein concentration 0.6 μM) vectors, or using commercial T7 RNA polymerase (0.05 U per sample). Assays were performed in the presence of 1.5 mM ATP, 0.7 mM GTP, 0.7 mM UTP and 0.17 μM [α-^{32}P]CTP. Products longer than template length, 30 nt for R_1 and D_1, result from terminal transferase activity acting on either the template or the newly synthesized strand. D_4 template length 45 nt (C) Results of a primer extension assay (primer length 19 nt) using recombinant nsp9/pASK (final protein concentration 1 μM), nsp9/pDEST (final protein concentration 0.3 μM), or commercial T7 RNA polymerase (0.025 U per sample), in the presence of 50 μM ATP and 0.17 μM [α-^{32}P]ATP. Products longer than template length (29 nt for R_2, 39 nt for R_3 and D_3) must have resulted from terminal transferase activity acting on either the template or the newly synthesized strand. (D) Results of a terminal transferase assay. The signal at the very top of the gel likely represents products of >200 nt that cannot be resolved in the high-percentage acrylamide gel used here. Note that products resulting from end-labeling with ATP may be further extended by a back-priming mechanism.

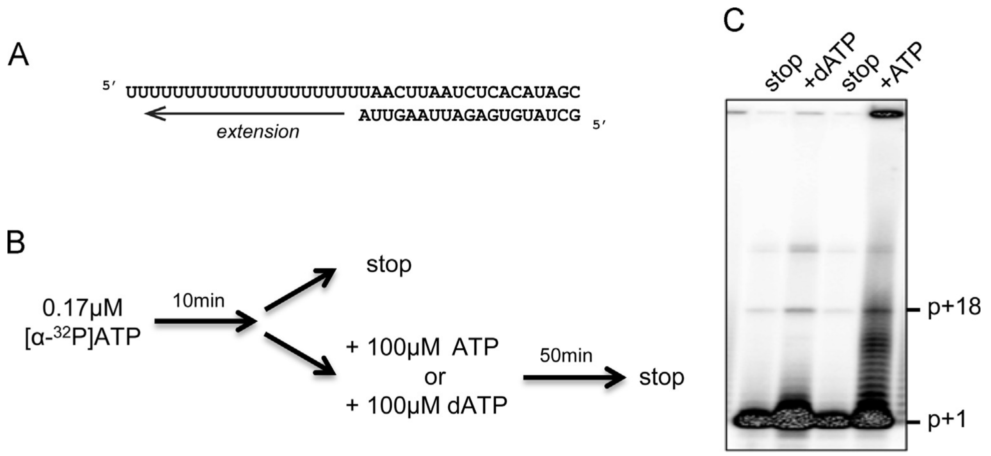

Fig. 2. Stop-and-go primer extension assay using EAV nsp9-His expressed from a pASK vector and primer/template R/R_3 in the presence of ATP or dATP. (A) Sequence of primer/template. (B) Schematic representation of the experimental design. (C) Polymerase products after interrupted and resumed synthesis. The sizes of primer extension products are indicated on the right.

possible to separate nsp9-containing fractions from biochemically active ones during purification of the quadruple nsp9/pASK aspartate-to-alanine mutant. To this end, the wash steps of the previously established purification protocol were modified in either of two ways; first, a decreasing salt gradient was introduced to weaken (disrupt) hydrophobic interactions between a contaminant and nsp9, and second, an increasing imidazole concentration was employed in order to eliminate any contaminant from the Co^{2+}-resin. As shown in Fig. 4, the NaCl elution fraction and wash steps 2 and 3 of the imidazole gradient contained almost identical amounts of nsp9-His, as judged by SDS-PAGE, while two of these three fractions were inactive in the polymerase assay. This partial correlation between the presence of recombinant nsp9-His and primer extension activity could be due to either the presence of two forms of nsp9, enzymatically active and defective, or the presence of a second enzyme responsible for the activity. Although the nature and origin of the possible heterogeneity of nsp9 remains elusive, a candidate protein from *E. coli* that may account at least for the contaminating terminal transferase activity is polyA polymerase I (PAP I), which has been reported to add a polyA tail to RNA substrates in a template-independent manner *in vitro* (Yehudai-Resheff and Schuster, 2000). Whether or not this enzyme or other *E. coli* terminal transferases would also be capable to extend primers in a template-dependent manner is, to our knowledge, currently unknown, but seems unlikely given PAP I's physiological role.

Conclusion

In this study we could neither confirm the previously reported *de novo* polymerase activity nor detect any other RNA polymerase activity specifically originating from purified recombinant EAV nsp9-His, indicating that the *in vitro* characterization of the arterivirus RdRp presents a formidable challenge. One possibility is that the activity depends on the presence of one or more (viral) protein co-factor(s) or specific RNA sequences that we have not been able to identify thus far, despite attempts to supplement RdRp assays with several small products from the nsp6-8 region (not shown). While the reasons underlying the differences with earlier studies on nsp9 remain to be elucidated, the outcome of the present study emphasizes the need for designing proper controls, especially when utilizing highly sensitive biochemical assays for the characterization of enzymes with low activity. Furthermore, it illustrates that *in vitro* assays may reveal activities that are not biologically relevant under physiological conditions or in the presence of interaction partners that may alter substrate preferences by modifying an enzyme's conformation. Being aware of these pitfalls probably is one of the most fundamental prerequisites for the deduction of biological roles from biochemical assays.

Material and methods

Protein expression and purification

C-terminally His-tagged fusion proteins of wild-type and mutant EAV nsp9 were expressed under the control of a tetracycline promoter from a pASK vector in the *E. coli* BL21 derivative C2523/pCG1 as described (Gohara et al., 1999; te Velthuis et al., 2012). As a reference, nsp9-His_6 was expressed from a previously used pDEST construct in *E. coli* BL21 (DE3) cells after IPTG induction under otherwise identical conditions. Proteins were purified by metal affinity chromatography using Co^{2+} (Talon beads) as described (te Velthuis et al., 2012) using a buffer containing 20 mM HEPES, pH 7.5, 500 mM NaCl, 10% glycerol (v/v), 10 mM imidazole, and 5 mM β-mercaptoethanol unless stated otherwise. Where indicated, a second purification step using a Superdex 200 10/300 GL gel filtration column and a buffer containing 20 mM HEPES, pH 7.5, 300 mM NaCl, and 1 mM DTT was performed at 4 °C using a flow rate of 0.3 ml/min.

RNA polymerase assays

Three different types of RNA polymerase assays were performed: *de novo* initiation, primer extension, and terminal transferase assays. For *de novo* assays samples contained 10 mM Tris, pH 8.0, 5 mM KCl, 25 mM NaCl (including 20 mM from the protein storage buffer), 6 mM $MgCl_2$, 1.5 mM $MnCl_2$, 1.5 mM DTT, 12.5% glycerol (including 10% from the protein storage buffer), 0.005% Triton X-100, 1.5 U RiboLock RNase inhibitor (Thermo Scientific), 0.5 μM single-stranded nucleic acid template, 1.5 mM ATP, if required 0.7 mM GTP and 0.7 mM UTP, 0.17 μM [α-^{32}P]CTP (Perkin Elmer, 3000 Ci/mmol), and 2 μM nsp9/pASK or 0.6 μM nsp9/pDEST or 0.05 U T7 RNA polymerase from a commercial source (Life Technologies). Primer extension and terminal transferase assays were performed in 20 mM Tris, pH 8.0, 10 mM KCl, 20 mM NaCl (including 10 mM from the protein storage buffer), 6 mM $MgCl_2$, 1 mM DTT, 10% glycerol (including 5% from the protein storage buffer), 0.01% Triton X-100, 0.5 U RiboLock RNase inhibitor, 1 μM

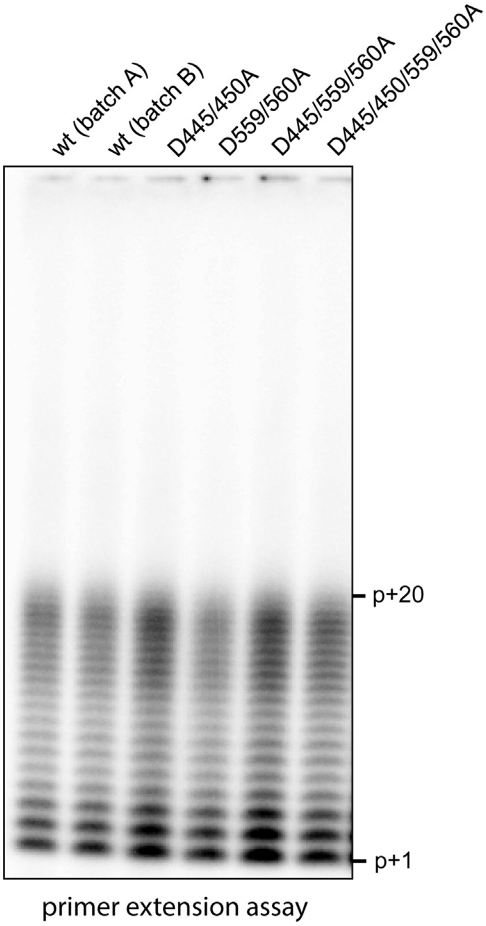

Fig. 3. Primer extension assay on primer/template R/R_3 using wild-type EAV nsp9-His expressed from a pASK vector and mutants in which essential aspartate residues of the RdRp domain were replaced with alanine (D445 and D450 of motif A, D559 and D560 of motif C). The sizes of primer extension products are indicated on the right; reactions were performed in the presence of 50 μM ATP and 0.17 μM [α-^{32}P]ATP.

partially double-stranded (primer extension) or single-stranded (terminal transferase) nucleic acid, 50 μM ATP, 0.17 μM [α-^{32}P]ATP (Perkin Elmer, 3000 Ci/mmol), and 1 μM nsp9/pASK or 0.3 μM nsp9/pDEST or 0.025 U T7 RNA polymerase (Life Technologies). Sequences of used nucleic acids are listed in Supplementary Table S1.

Nucleic acids were annealed with complementary primers by heating to 95 °C for 2 min, then keeping them at 52 °C for 30 min, and finally letting them cool to room temperature in 30 min.

In all three assays, samples were incubated for 1 h at 30 °C before the reaction was stopped by addition of an equal volume of formamide-based gel loading buffer (95% formamide, 18 mM EDTA, 0.025% SDS, xylene cyanol, bromophenol blue) and 2 min denaturation at 95 °C. Products were separated by gel electrophoresis in 20% polyacrylamide gels (19:1) containing 7 M urea. Gels were run in 0.5x Tris–borate–EDTA (TBE) buffer and phosphorimaging was performed using a Typhoon variable mode scanner (GE Healthcare). Band intensities were analyzed with ImageQuant TL software (GE Healthcare).

EAV reverse genetics

Codons specifying conserved nsp9 residues were replaced with alanine codons using the QuikChange protocol and were introduced into full-length cDNA clone pEAV211 (van den Born et al., 2005; van Dinten et al., 1997) using appropriate shuttle vectors and restriction enzymes. Synthesis of full-length RNA and transfection of BHK-21 cells was performed as described previously (Nedialkova et al., 2010). Transfected cells were monitored until 72 h post transfection (p.t.) by immunofluorescence microscopy using antibodies directed against the nsp3 and N proteins as described (van der Meer et al., 1998). To monitor the production of viral progeny, supernatants were harvested at 48 h p.t. and plaque assays were performed as described (Nedialkova et al., 2010). To verify the presence of the introduced mutations or reversions in viable mutants, fresh BHK-21 cells were infected with supernatants harvested at 72 h p.t., RNA was isolated with TriPure after 18 h, and the nsp9-coding region was amplified by RT-PCR and sequenced.

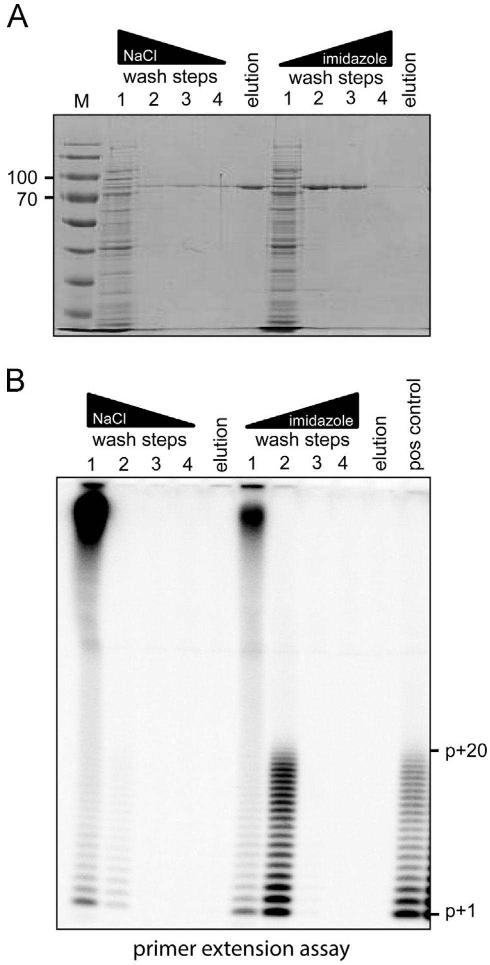

Fig. 4. Correlation between EAV nsp9-containing fractions and primer extension activity. (A) Coomassie brilliant blue-stained SDS-PAGE gel of samples taken during the purification of nsp9/pASK by Co^{2+} affinity chromatography using wash buffers with either a decreasing NaCl concentration or an increasing imidazole concentration. Size markers are depicted on the left in kDa. (B) The samples shown in A were examined for primer extension activity on primer/template R/R_3. The sizes of primer extension products are indicated on the right.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2015.10.002.

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