nature portfolio

Peer Review File

RNA synthesis and substrate analog inhibition in the CCHFV polymerase

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Any redactions in this file are there to maintain patient confidentiality, the confidentiality of unpublished data, or to remove third-party material.

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)
In this manuscript Gao et al present the CryoEM structure of the L protein of CCHFV at different conformational states, its apo form, bound to the 5' promoter and in a functional state corresponding to replication elongation. The structures reveal the structural evolution of the L proteins of bunyavirus into macropolymerases including additional insertions and structural motifs which appear involved in many steps of the RNA synthesis and transcription by cap snatching. This structure represents the cenit of the structural characterization of L proteins after an extensive work on virus representatives of all families during a decade. It is thus a very attended structure by the community and is relevant to have a complete view of Bunyavirus replication. The ms includes in cellula minireplicon studies with structure-based mutagenesis proving the importance for replication and transcription of the new findings and an in vitro RNA synthesis assay with nucleotides analogs showing specific inhibition by nucleotide analog XTP. Methods and data statistics are properly described and provided (with the exception of EC14b, see below). In conclusion is a beautiful work extremely informative on the replication of a terrible emerging human pathogen which is increasingly circulating in wild stock at a global scale and eventually infecting humans with terrible consequences.

There are however issues to be addressed:

1- The most relevant structure is the EC14 (RNA synthesis elongation state). From this data two other structures were obtained: EC14a and EC14b, at slightly lower resolution and the later showing important details as the FID domain and template RNA extra density. EC14b structure should be incorporated to the table and the model shown in Fig 2 A and B should be made available and also appear in the table. Consider some mutants were made based on EC14b structure and that figure 2 A shows the structure and electrostatics.

2- RNA density interpretation: The method for the assembly of the EC14 structure consists on the synthesis of a dsRNA inside the active site chamber resulting in the synthesis of 14 nts from a 3nt primer. In the chamber we only see 10nt of the 14 expected.
2a- There is a problem with the basepairing regarding U4002 and G4005 conformation of the B chain that should be fixed.
2b- Here the RNA template used for the EC14 assembly seems to appear in the active site and bound to the UPD (T38b), this is interpreted as two molecules bound (T38a and b). However there is a discontinuous density connecting the T38b to the 3’ of the nascent strand in the active site suggesting that they could be the same RNA molecule (see attachment). Since the density of the dsRNA does not fully allow a proper base identification (therefore the RNA identity) and since T38 is quite a long molecule; could not be that T38a and b are actually the same molecule? Authors should consider this option.
2c- There is density to build additional 5 base pairs in T38b. Does this make sense with the sequence of T38 (which is not fully provided for that region in fig C)?.

Please provide the full sequence and check the possibility of an extension of T38 molecule by a T38 template simulating transcription initiation-elongation.

These issues do not affect the robustness, validity and reliability of the conclusions made of the paper based on a very

accurate structural interpretation of the protein by comparing with homologs and a clear biochemical and in cellula characterisation. However,the figure 1c (RNA sequences) seems inconsistent with the densities found in EC14 as if the EC14 was obtained differently as described…. There should be an explanation.

Minor comments:
ln120, instead of "traditional" use "conserved".
Ln128 citation 18 seems not the good one
Ln 36 RdRP protein
ln190 generic downstream template binding? Please here and all along the text specify if is template or product RNA binding

It seems in fig s9 the 50° turn is for the panel below and not for the panel on the right.

Ln336 When you speak about T38b/P3b interactions, since P3b is 3nts long and not 8nts long as density shows,are you sure P3b is the one hybridizing?

Referee #2

(Remarks to the Author)
In this manuscript the authors present three high-resolution cryo-electron microscopy single-particle analysis (cryoEM SPA) structures of the Crimean-Congo hemorrhagic fever virus (CCHFV) L protein. These structures provide the first detailed structural insights into this viral polymerase, highlighting key features of this protein and allowing comparison with related RNA-dependent RNA polymerases from other negative-strand RNA viruses. The authors describe the structures in much detail and used a minireplicon system with interface mutants to confirm the importance of specific domain interactions for polymerase activity and furthermore tested polymerase activity of CCHFV, LASV and RVFV L proteins in the presence of six different nucleotide analogues. While these structures are a great accomplishment with strong impact for the field of bunyaviruses, the overall scientific strategy and the analysis is somewhat inconsistent or at least not clearly explained.
For example, the inhibitor studies were extended to LASV and RVFV (belonging to the viral families phylogenetically closest to nairoviruses) while the structural comparisons of the L protein were made generally to LACV (belonging to a different order within bunyaviruses) and influenza viruses (generally not further defined but summarized as Orthomyxoviridae although also significant differences are present between these species). It appears that there is also quite a lot to do to put this work in relation to other published studies and support the claim made in the discussion: “Along with available structural and functional data in the literature, this work allows us to propose an integrated model for CCHFV RdRP replication/transcription with testable hypotheses”. Especially the similarities to others have been brushed over somewhat, although these will be key for the development of antiviral strategies potentially with broad activity against bunyaviruses.
The mutagenesis appears scientifically flawed as there were no transfection controls or expression tests of the L protein performed, therefore the respective conclusions are unfortunately not reliable. Mutagenesis was not done for specific features pointed out such as the PR-loop or the PE, etc. The inhibitor studies are a nice addition but also seem like a superficial add-on rather than a thorough analysis with different templates and comprehensive controls (or structural evidence).
Therefore, this manuscript “reveals unique features related to additional structural elements and their interactions with the conserved parts” but unfortunately it doesn’t go much further than presenting new structures without a clear and critical interpretation.

Specific comments:

Abstract
Line 21: “extending RNA binding paths on both sides of RdRP active site and creating interaction networks to restrict core structure dynamics likely for ultra-capability in processive RNA synthesis.” I don’t understand what this sentence should mean, it is very speculative and does as such not belong in an abstract.

Line 23: “inhibits” grammar

Line 24: “rarely observed in negative-strand RNA viruses” What does this mean? Structurally visualized? Detected?

Introduction:
Line 34: “Leviviridae” does not exist anymore, as far as I know. Please check the latest virus taxonomy (ICTV taxonomy browser)
In general, the authors often use taxonomic terms instead of specific virus names which is problematic when generalizing things for a certain group of viruses (e.g. Orthomyxoviridae) without delineating it clearly from other viruses in the higher ranking order and when it is unclear why a family taxon has been used when this family is the only member in an order which is the only order in a class (example Cystoviridae within Vidaviricetes). If a feature has been found in one virus species, this does not by itself mean it is representative for an entire family, order or class of viruses.

Line 44: “L denotes the large segment of the virus genome that also encodes the L protein” This is not quite correct. L stands for “large” and is also used in L RNA describing the larger RNA segment. However, it also describes the L protein as “large” viral protein independent of the encoding RNA segment naming.

There is a BioRxiv preprint available on CCHFV L protein structure (https://doi.org/10.1101/2025.10.21.683639). The authors should include a comparison of what is similar and additional between these two studies.

Line 72: “extra-large L protein” non-scientific

Line 73: “previously unidentified cross-region interactions” How can this be previously unidentified if these are proposedly the first structures of CCHFV?

Line 78: Reference 13 needs to be included again.

Results:
Line 99: “In Endo-resolved homologous structures, lid interacts extensively with Endo” Please be specific.

Line 111: “. With this EC structure, we are able to define the entire architecture of CCHFV L” 78% does not represent the entire architecture of the CCHFV L.

It is interesting that the authors compare the architecture of CCHFV L with LACV and orthomyxoviruses, while phenuiviruses and arenaviruses are phylogenetically much closer to the nairoviruses and there are many structures available as well.

Line 134: “Although not by design, the T38b-UPD and P3b-Endo interactions greatly help understand both RNA synthesis and cap-snatching.” How? RNa bound to an RNA endonuclease is barely a surprise, what’s new about this? Be specific!

There is no data demonstrating the exact metal ions observed. The authors need to explain why they modeled which ion at which site. It is not obvious to everyone, especially in this journal with this broad readership. What’s the evidence here?

Line 144: “imperfectly complementary region near the 3’-end” Please be more specific, this is not clear, references point towards 2 review articles, this doesn’t provide any clarity.

What is the proposed function of the iGate? It is entirely the same in all recorded states, Rna present or not. What does it “gate”? The “equivalent substructure” supposed to be present in other bunyaviruses is not clear for me.

Line 157: “Even no distal duplex is present” Do you mean “even though”?

Line 158: “where lysine and arginine residues cluster at the downstream direction of the hook binding pocket (Fig. S5B).” This does not become apparent from this figure, which is about conservation.

Line 176 & 178: the 5’ RNA is referred to as EC14 but in line 172 & 179 the overall EM map is referred to as EC14 structure. The nomenclature is confusing and should be cleaned up.

Line 185: “Lysing” typo

The authors should compare their structural data, e.g. for the endonuclease domain to the other published data on nairovirus L protein from crystallography: https://doi.org/10.1101/2025.10.28.684947 as well as cryo-EM https://doi.org/10.1101/2025.10.21.683639 and integrative modelling/predictions https://doi.org/10.1074/jbc.RA118.004373

Line 194: Sentence requires a literature reference since a very broad statement is made about -ssRNA endonucleases.

Line 245: instead of saying ‘the two parts of the block’ the authors could just say between the endo and the CBD/mid-link.

Line 256: the phrase ‘is still allowed but with restrictions by these interactions’ is unclear.

Line 263: ‘It turned out that’ non-scientific language

Line 264: “It turned out that all ten mutations reduced the minigenome replication level, albeit to various extent (Fig. 3, B-C, 1.4-57% of WT-level), supporting the functional importance of these interaction networks.” This is a very superficial analysis of these mutants and the conclusion is not supported. The mutant proteins might be not expressed, structurally unstable or functionally impaired. Does a reduction to 57% of the WT indeed mean the function is impaired? The WT values are also fluctuating quite a bit (75-130%). Also, the results of independent repeats seem to vary quite a lot in comparison. Did the authors compensate for transfection efficiency in any way?
I request also an explanation on the statistical analysis. With an n=2 and a relation of all values of mutants as % of the WT, a statistical test for significance is difficult as the WT does not have a distribution (as it is set to 100%) while the mutants do have a distribution around a average value. Please specify the statistical test used to label the columns with asterisks.

Line 270: “immediate chain terminating NAs are rarely reported for this large group of RNA viruses” What is meant here? Like approved drugs on the market? Or published manuscripts with NAs acting as a chain terminators?

Line 276: optional: please add the names of the two viruses referred to and referenced.

Line 280: The EC14 assembly assay is not mentioned in the methods section. Is it just the addition of the EC14 RNA to the L

protein and then incubation at 22°C? Is it what you refer to as CCHFV RdRP elongation complex assembly assay? Please clarify/simplify in the text.

Line 286: “In the presence of ATP/UTP, the main products produced by CCHFV RdRP were 9-mer and 10-mer, with the latter arising from a template-G:product-U misincorporation” .This conclusion is neither well explained nor logical. If there was a misincorporation of a canonical NTP, why would this product of misincorporation then get enriched in the reaction if the nucleotides needed to continue the synthesis were still all there. A misincorporation does usually not per se terminate RNA synthesis and the following nucleotides in the template were again U, which was present in the reaction. Why would production selectively stop there? Is this a specific feature of nairovirus polymerase compared to LASV and RVFV? How would this look with a different template?

Line 290: Comparison across CCHFV, LASV and RVFV are not really appropriate given the fact that the LASV and RVFV samples did not have RNA that was adapted to the respective viruses and had higher protein and RNA concentration in the assay and were subsequently 10-fold diluted (which is still equivalent to a 3-fold higher protein concentration than for the CCHFV samples).

The authors test several NA inhibitors but the analysis they provide is very superfivial. An outcompetition of a misincorporation by an NA with the correct nucleobase is not a great achievement in absence of the correct NTP, it is expected.
Do the authors suggest that MTP is being incorporated as well without any immediate inhibitory effect?

Discussion:
Line 306: “Besides seven RdRP catalytic motifs, replication-related modules or elements common for segmented -ssRNA virus include promoter binding platform formed by PA-C-like and fingers, initiation-related priming element (PE, also known as priming 308 loop) and prime-and-realign loop (PR-loop), and elongation-related lid, while the most notable Nairoviridae-specific modules
310 include FID and UPD.” This suggests that the features mentioned in the first half of the sentence are all also present in nairoviruses.

Line 313: “ds Polymycoviridae” misleading. ds what? Are there other (ss) Polymycoviridae?

Line 319: “have the aforementioned dual-function feature still awaits structural evidence” Please be specific about the aforementioned dual function feature. It actually needs also functional evidence, which could have been addressed by the authors. The authors define the priming element/priming loop in CCHFV L (“PE (residues 2839-2857 in CCHFV L) is largely disorderedl). n the following sentences the authors define the PR-loop (“The PR-loop (residues 2304-2315 in CCHFV L)”). I am missing the explanation why CCHFV L would need both a priming loop AND a prime-and-realign loop, this is usually not the case in other RdRps. More importantly, the functional evidence for this definition of priming elements is entirely missing. This part of the structure is solely described in the discussion but the speculative character is not made clear enough.

Line 334: ‘when switching’ instead of ‘if switching’. If would indicate you don’t know whether this switch happens, while you have data in which you observe this.

Line 335: ‘is’ instead of ‘are’

Line 335: “Although the EC14 structure are not obtained using a capped primer, its polymerase elongation state and the T38b/P3b interactions with the CBD-/Endo-containing major regions allow us to reasonably judge what state it likely mimics in replication/transcription and what types of conformational changes are required to achieve other important states during cap-snatching and RNA synthesis.” I couldn’t disagree more. Observing short RNAs bound to single domains does not mimic the domain movements or provide any information on the orchestration of these movements. This space could be used much better to discuss the value and limitations of the presented data and compare to published work. While the authors acknowledge that “In transcription-related LACV structures, the relative CBD/Endo orientations relative to RdRP are diverse, while the interactions between CBD and Endo are also highly variable” and “In corresponding IAV structures, while the placement of the CBD and Endo relative to RdRP are consistent, the orientations of them, in particular CBD, can be quite different.” the authors “propose that CCHFV L could fulfill most transcription/replication tasks without large-scale relative movement of its three major regions”. All this is based on the first 3 structures of this protein of which only one is actually visualizing a functional complex.

Line 363: ‘length of about’

Line 364: “to separate the two RNA strands, marking the entering of early elongation stage” Why is this still early elongation when the template-product duplex get separated? What do the authors denote the phase after prime and realignment is finished and before the strand separation happens?

Line 373: which accompanying work?

Line 374: “For both replication and transcription, the ss upstream template eventually reaches the secondary binding site,” It is interesting that this binding site has not been formally identified, experimentally proven or the proposed binding pocket analysed in the structure. Yet, the authors describe what is supposed to happen.

Line 379: “Both FID and UPD could contribute to EC processivity through interactions with downstream template and upstream product, respectively, likely ensures enough processivity to synthesize the L transcript that is about twice the size of those in other Bunyaviricetes systems.” Why haven’t the authors tested this hypothesis with a mutational study to provide functional evidence for some of the special structural features?

Line 393: Sentence is not clear. What assessment exactly?

Line 398: ‘drastic difference’ statement is not appropriate given the concerns stated for line 290.

Methods:
It would be helpful for the reader to add a table with all the RNA sequences used in this work. That way readers do not have to jump back and forth to look for sequences that are mentioned by acronyms somewhere in the text or method section. For the cryoEM work the sequences only appear in Figure 1 but are not mentioned in the related methods section and for the NA intervention assays only the LASV and RVFV sequences are mentioned in the related methods section.

Line 448: Information on the what happened with these samples is missing. Presumably this assay led to the gel shown in Figure 1? If so, how was this gel run and stained?

Line 467: Reference for Topaz is missing. Even if you used it through CryoSPARC, Topaz is an outside software that needs to be reference separately. CryoSPARC just provides a wrapper. The correct reference can be found in the CryoSPARC Guide.

Line 511: A minimum of three independent experiments is required for such an experiment to perform a robust statistical analysis.

Line 514: Information about the imaging is required. Especially whether the entire dish with cells i.e. all cells were imaged or whether only a selected region was imaged and quantified.

Line 515: The minireplicon evaluation appears to be biased. There does not seem to be a way to normalise for fluctuations in transfection efficiencies. Fractions of eGFP positive cells vary strongly even within the WT data points (lowest and highest point are more than 50% apart). Therefore, comparing to the WT rate of eGFP positive cells is not significant nor does it allow conclusions about the protein stability or ability to be enzymatically active. Strong fluctuations between experimental sets can be seen in at least four different mutants, sometimes with discrepancies of up to 50%.

Figures
When showing cryoEM maps, especially detailed views of specific regions, it is helpful to mention the contour level at which the map is being shown in the figure (e.g. Fig. S3, S5, S6). It is mentioned in the methods but this information belongs to the figure caption.
Labels should not be on top of structures. This makes it hard to read everything.

Fig 1: the gel in panel C is not described or explained in the figure caption. EC14 is the name of one the structures presented, yet this panel shows RNA sequences and a gel. Is the CCHFV model shown in panel D the EC14 structure or another? Maybe add this information in the caption? Panel A representation is a bit unclear with the dashed lines between CCHFV L and the other two viruses. The labels for EC14, apo and 5’-cRNA bound are also not obvious to find initially and could be overlooked given the panel labels are the same font and size.

Fig 2: Dashed magenta line in panel A EC14b and panel B is not explained. What is it meant to show?

Fig 3: For the minireplicon data, three independent experiments are expected. Also, for four sets of mutations there is a strong difference in the values from the first and second experiment. Could this be explained?
Panel C caption is missing a description of what the white and black circles represent. Presumably data points from the two independent experiments?
Panel B: Images for M1-10 are all missing scale bars and image M10 looks like it may be at a different magnification?

Fig 4: RdRP intervention? Inhibition of RdRP activity is the more appropriate description.
It is not convincing that the red triangle points at a 10 nt product including an incorporated inhibitor. It could also be a 9 nt product with the inhibitor incorporated. Controls like L + template/no NTPs, L + template and primer/no NTPs are also missing. There is an endonuclease activity present in L, how did the authors verify that none of the products observed are derived from RNA cleavage?

Fig 5: A sub-panel structure of a-h is very confusing when having an A-C panel structure at the same time, maybe use I, II, III etc. Also it should become clearer which proposed arrangements of domains and RNA during the processes is based on the real structural evidence presented. Here I would argue, that the authors have not at all addresses transcription in their experiments and therefore it is entirely speculative how domains would be arranged.

Fig S1: Scale bar is 50 nm for micrograph but you have a second scale bar for 2D classes, either mention both or none, since the scale bars shown have their length written next to them.

Fig S2: “the root-mean-square deviation (RMSD) values of superimposed L protein a-carbon atoms of apo and cRNA-bound structures are 1.3 Å (40.6% residue coverage) and 1.2 Å (46.5% residue coverage), respectively, and are 0.8 Å (40.9% residue coverage) and 0.9 Å (47.3% residue coverage), respectively, if excluding the lid residues.” It is not clear to me if the authors mean X% of the total residues built in the models or X% of the L protein sequence.

S3: When EM maps are presented, information about the map contour levels are required.
Legend for panel B: please rephrase from more clarity. For example, remove “and absent in apo/5’-cRNA-bound” in line 43. The following sentence suggests that everything that is shown in panel B is not visible/resolved in the apo/5’-cRNA-bound data.

S5: The 5’ hook labelling for LASv L is incorrect. The sequence of the RNA hook starts with G at position 0, which is a non-templated extra G.

S7: The topology of nairovirus endonucleases was proposed in a paper by Holm et al. 2018 (10.1074/jbc.RA118.004373), the authors did neither cite this work nor compare their data to the published model.

Citations:
The authors cite many review articles instead of the original works. This is not best scientific practice. I would recommend to acknowledge the original work.

Structures:
The deposited structural models seem to be of high quality. Some domains seem to not be well resolved in some maps (e.g. the PA-C like region). However, no major problems have been detected upon inspection.
Final note: How about Arg3813 stacking onto the RNA nucleotide to separate product and template? The authors haven’t even mentioned this residue nor validated the role using mutagenesis.

Referee #3

(Remarks to the Author)
In this manuscript, Jia and colleagues report three high-resolution (2.7–3.0 Å) cryo-EM structures of the CCHFV L protein in apo and RNA-bound conformations, resolving up to 78% of the full-length polymerase. Despite its extraordinary size of over 4,000 residues, the authors show that CCHFV-L adopts a conserved overall architecture comparable to that of related viral polymerases, such as those from influenza, Lassa, and La Crosse viruses. In contrast to these polymerases, CCHFV-L contains additional domains, including the upstream product-binding domain (UPD) and the finger-insertion domain (FID). The structures of these domains, proposed to act as extended RNA-binding motifs, are described in detail, although their functions remain uncharacterised in this study. Beyond the structural analyses, the authors provide data on the importance of a nairovirus-specific interaction network between the Endo, RdRP, and CBD domains using minireplicon assays. They also demonstrate inhibition of RNA synthesis with a ribose-2′-modified nucleotide analogue.
Overall, this manuscript presents novel and compelling findings that significantly advance our understanding of the architecture and potential regulatory mechanisms of nairovirus polymerases. While this reviewer cannot comment on the structural data, the functional assays would benefit from additional biological replicates, more rigorous statistical treatment, and improved data presentation. A more comprehensive characterisation of the role of the proposed ion-binding sites and the Endo-RdRP-CBD interaction network would further strengthen the manuscript.

Specific points:

1. Figure 1c: Explain what is shown in this panel and how the image was obtained; this information is missing from both the figure legend and the Materials and Methods section. Additionally, clarify why bands at approximately 14 nt are considered degradation products in lane 5 but genuine products in lanes 8 and 9. All relevant bands should be clearly identified and labelled.
2. Figure 2b: The legend states that the R/K residues in regions I and II are shown as sticks; however, these are barely visible in the figure. A clearer depiction would facilitate interpretation of the minigenome data presented in Figure 3.
3. Figure 3c: The minireplicon assay panel shows activity data from 10 different mutants located in regions potentially involved in RNA binding or interdomain interactions. Except from the FID:RNA mutants, theses residues are not introduced or discussed earlier in the text. To aid interpretation, please elaborate on the relevance of these sites, the rationale for the amino acid substitutions, and indicate which structural figures illustrate the corresponding interactions.
4. Figure 3c. Please include information on the expression levels of each mutant protein; reduced activity could result from lower expression rather than disruption of specific interactions.
5. Figure 3c. This panel contains data from two independent experiments, each performed in triplicate. The results appear to differ substantially between experiments for some mutants; therefore, an additional biological replicate is recommended. Please also clarify whether the 2x3 values were treated as independent data points in the ANOVA analysis. It is advisable to first average technical triplicates before performing the analysis. However, two independent replicates are insufficient to robustly estimate variance for ANOVA.
6. Figure 4: Please include a schematic representation of the templates, primers, and products used, similar to that shown in Figure 1, to improve clarity. Indicate whether the same templates were used for all three polymerases, and provide the corresponding sequences both in the figure and in the Methods section. Also state whether the experiments were reproducible across different protein preparations, and how many times each assay was performed.

7. Language and clarity. The manuscript would benefit from moderate language editing, including correction of grammatical errors and typos (e.g., lysine at line 185), consistent terminology, and reduced use of abbreviations (e.g., “+”-denotion, iGate, NA). Some statements could also be clarified (e.g., “major functional regions”, “ultra-capability” in the abstract, “PA-like UPD” at line 201). Additionally, the use of black outlines around letters and numbers in the figures makes the text difficult to read.

Referee #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Referee #5

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)
The authors have considered my critiques and addressed them. Some uncertainties regarding what is observed in the maps remain, but I acknowledge that the signals I refer to are far from unambiguous. I am therefore satisfied with the additional clarifications provided.

I understand that the paper presents functional data on the newly identified residues involved in elongation, rather than other processes. This aligns with the new findings and, in my view, is relevant. The study does not address initiation or cap snatching (which involve the PR-loop or PE) in the structural or functional analyses provided. However, elongation is a process that occurs after cap snatching or replication initiation, so it is natural to discuss the implications of the findings in this context. Of course, this needs to be properly explained in the manuscript, and I believe it is.

Referee #3

(Remarks to the Author)
This is a revised version of a manuscript on the CCHFV L protein previously considered by Nature. The authors have made a substantial effort to address the numerous comments raised by the reviewers. Importantly, they now include expression levels of mutant L proteins (Fig. 3b), provide a clearer justification for the selection of amino acid residues for mutagenesis, and have repeated key experiments to enable statistical analysis.
One remaining issue concerns Fig. 1c, which I still find difficult to interpret despite the revised description and figure legend. It is unclear why bands that appear similar are interpreted as background in some lanes but as products in others. The authors should label the relevant products directly in the figure, as they already do for the presumed degradation products in lane 5 using black arrowheads.

Version 2:

Reviewer comments:

Referee #3

(Remarks to the Author)
The authors have labelled the relevant bands and I have no further comments.

Point-by-point responses to reviewers' comments for manuscript tracking #: 2025-10-27418

Insights into RNA synthesis and substrate analog inhibition in the CCHFV RNA-dependent RNA polymerase

by Hengxia Jia, Bo Tang, Shunli Liu, Xin Wen, Fan Wu, Xiao Hu, Hu Zhou, Tingting Chong, Lincan Lv, Qiaojie Liu, Guibo Rao, Mingyu Wei, Xuping Jing, Sheng Cao, Fei Deng, Zhihong Hu, Bo Shu, Rui Gong, Jiqin Wu, Manli Wang, Peng Gong

(Our responses are shown in boldface text with grey background)

Referees' comments:

Referee #1 (Remarks to the Author):

In this manuscript Gao et al present the CryoEM structure of the L protein of CCHFV at different conformational states, its apo form, bound to the 5' promoter and in a functional state corresponding to replication elongation. The structures reveal the structural evolution of the L proteins of bunyavirus into macropolymerases including additional insertions and structural motifs which appear involved in many steps of the RNA synthesis and transcription by cap snatching. This structure represents the cenit of the structural characterization of L proteins after an extensive work on virus representatives of all families during a decade. It is thus a very attended structure by the community and is relevant to have a complete view of Bunyavirus replication. The ms includes in cellula minireplicon studies with structure-based mutagenesis proving the importance for replication and transcription of the new findings and an in vitro RNA synthesis assay with nucleotides analogs showing specific inhibition by nucleotide analog XTP. Methods and data statistics are properly described and provided (with the exception of EC14b, see below). In conclusion is a beautiful work extremely informative on the replication of a terrible emerging human pathogen which is increasingly circulating in wild stock at a global scale and eventually infecting humans with terrible consequences.

We thank this reviewer for all the comments and concerns. Our responses are listed below, and we deposited the EC14b structure (PDB entry 25XO) according to this reviewer's suggestion.

There are however issues to be addressed:

1- The most relevant structure is the EC14 (RNA synthesis elongation state). From this data two other structures were obtained: EC14a and EC14b, at slightly lower resolution and the later showing important details as the FID domain and template RNA extra density. EC14b structure should be incorporated to the table and the model shown in Fig 2 A and B should be made available and also appear in the table. Consider some mutants were made based on EC14b structure and that figure 2 A shows the structure and electrostatics.

We have deposited the EC14b structure and related data are included in Extended Data Table 1 and Fig. 3a-c, and Fig. 2a-b.

2- RNA density interpretation: The method for the assembly of the EC14 structure consists on the synthesis of a dsRNA inside the active site chamber resulting in the synthesis of 14 nts from a 3nt primer. In the chamber we only see 10nt of the 14 expected.

Within the 14-nt product, the four nucleotides at the very upstream (5'-direction) are not modelled due to weak EM density. We added a few sentences to explicitly describe the density in the active site chamber and the related model building, and presented a stereo-pair image of densities overlaid with structural model in Extended Data Fig. 3b to show the consistence between density and model.

2a- There is a problem with the basepairing regarding U4002 and G4005 conformation of the B chain that should be fixed.

We fixed the base conformation issue accordingly to this comment and the correction is reflected in the updated EC14 and the new EC14b deposition (PDB entries: 25XN and 25XO).

2b- Here the RNA template used for the EC14 assembly seems to appear in the active site and bound to the UPD (T38b), this is interpreted as two molecules bound (T38a and b). However there is a discontinuous density connecting the T38b to the 3' of the nascent strand in the active site suggesting that they could be the same RNA molecule (see attachment). Since the density of the dsRNA does not fully allow a proper base identification (therefore the RNA identity) and since T38 is quite a long molecule; could not be that T38a and b are actually the same molecule? Authors should consider this option.

Based on this reviewer's suggestion, we performed an experiment to test such a possibility. The data in Extended Data Fig. 3e showed that T38 was not extended by the same NTP combination used in EC14 assembly, ruling out this possibility.

2c- There is density to build additional 5 base pairs in T38b. Does this make sense with the sequence of T38 (which is not fully provided for that region in fig C)?.

Please provide the full sequence and check the possibility of an extension of T38 molecule by a T38 template simulating transcription initiation-elongation.

The density for T38b is not as explicit as that of the aforementioned template-product duplex. However, the junction of double-stranded and single-stranded regions and the global folding of the stem-loop are quite clear, allowing confident building of a total of 6 nucleotides including 2 base pairs around the junction. We presented the rational for model building of this region by showing the densities and RNA secondary structure side-by-side in Extended Data Fig. 3a.

All RNA sequences used in this study have been shown in different figures and as a complete collection in Extended Data Table 2.

These issues do not affect the robustness, validity and reliability of the conclusions made of the paper based on a very accurate structural interpretation of the protein by comparing with homologs and a clear biochemical and in cellula characterisation. However,the figure 1c (RNA sequences) seems inconsistent with the densities found in EC14 as if the EC14 was obtained differently as described…. There should be an explanation.

Please refer to our responses to comments 2b and 2c.

Minor comments:

ln120, instead of "traditional" use "conserved".

Modified as suggested.

Ln128 citation 18 seems not the good one

The original citation (PMID 18769709) of the 627-domain has been used.

Ln 36 RdRP protein

Modified as suggested.

ln190 generic downstream template binding? Please here and all along the text specify if is template or product RNA binding

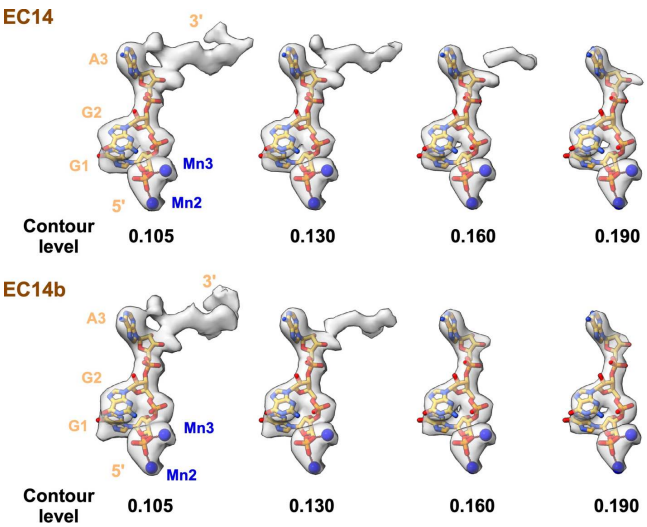
Modified wherever appropriate.

It seems in fig s9 the 50° turn is for the panel below and not for the panel on the right.

The turning symbol is correct. In the revised manuscript, the panels a-b of Extended Data Fig. 9 were removed according to revision of the discussion section.

Ln336 When you speak about T38b/P3b interactions, since P3b is 3nts long and not 8nts long as density shows,are you sure P3b is the one hybridizing?

The densities at the 3'-direction of the P3b model are much weaker than those of P3b (see a comparison at different contour levels below), and they are not explicit enough for confident model building. Hence, either P3b (provided in excess for EC assembly) or dissociated P3-derived products can occupy the Endo active site, and we choose to stay with only building P3b in the EC14/EC14b structures. We added related descriptions in the figure legends of Extended Data Fig. 3a, to discuss these possibilities.



Referee #2 (Remarks to the Author):

In this manuscript the authors present three high-resolution cryo-electron microscopy single-particle analysis (cryoEM SPA) structures of the Crimean-Congo hemorrhagic fever virus (CCHFV) L protein. These structures provide the first detailed structural insights into this viral polymerase, highlighting key features of this protein and allowing

comparison with related RNA-dependent RNA polymerases from other negative-strand RNA viruses. The authors describe the structures in much detail and used a minireplicon system with interface mutants to confirm the importance of specific domain interactions for polymerase activity and furthermore tested polymerase activity of CCHFV, LASV and RVFV L proteins in the presence of six different nucleotide analogues. While these structures are a great accomplishment with strong impact for the field of bunyaviruses, the overall scientific strategy and the analysis is somewhat inconsistent or at least not clearly explained.

For example, the inhibitor studies were extended to LASV and RVFV (belonging to the viral families phylogenetically closest to nairoviruses) while the structural comparisons of the L protein were made generally to LACV (belonging to a different order within bunyaviruses) and influenza viruses (generally not further defined but summarized as Orthomyxoviridae although also significant differences are present between these species). It appears that there is also quite a lot to do to put this work in relation to other published studies and support the claim made in the discussion: “Along with available structural and functional data in the literature, this work allows us to propose an integrated model for CCHFV RdRP replication/transcription with testable hypotheses”. Especially the similarities to others have been brushed over somewhat, although these will be key for the development of antiviral strategies potentially with broad activity against bunyaviruses. The mutagenesis appears scientifically flawed as there were no transfection controls or expression tests of the L protein performed, therefore the respective conclusions are unfortunately not reliable. Mutagenesis was not done for specific features pointed out such as the PR-loop or the PE, etc. The inhibitor studies are a nice addition but also seem like a superficial add-on rather than a thorough analysis with different templates and comprehensive controls (or structural evidence).

Therefore, this manuscript “reveals unique features related to additional structural elements and their interactions with the conserved parts” but unfortunately it doesn’t go much further than presenting new structures without a clear and critical interpretation.

We thank this reviewer for all the comments and concerns and the efforts to make a collection of comments from other two reviewers. In the revised version, we presented more enzymology and virology data to better present our findings

and removed or reduced the contents that are not functionally addressed in the manuscript. More specifically, the functional data address the interactions between the CCHFV-specific structural elements (such as FID and UPD) and the conserved parts of L by the minigenome study and the NA lead discovery by the enzymology study. More rigorous experimental settings have been applied to both studies as suggested by the reviewers. Our responses to specific concerns and comments are listed below.

Specific comments:

Abstract

Line 21: “extending RNA binding paths on both sides of RdRP active site and creating interaction networks to restrict core structure dynamics likely for ultra-capability in processive RNA synthesis.” I don’t understand what this sentence should mean, it is very speculative and does as such not belong in an abstract.

We removed this part of the sentence.

Line 23: “inhibits” grammar

Corrected.

Line 24: “rarely observed in negative-strand RNA viruses” What does this mean? Structurally visualized? Detected?

We removed this part of the sentence and specifically describe the meaning of it in the results section as rarely observed in enzymology and structural studies.

Introduction:

Line 34: “Leviviridae” does not exist anymore, as far as I know. Please check the latest virus taxonomy (ICTV taxonomy browser)

Updated.

In general, the authors often use taxonomic terms instead of specific virus names which is problematic when generalizing things for a certain group of viruses (e.g. Orthomyxoviridae) without delineating it clearly from other viruses in the higher ranking order and when it is unclear why a family taxon has been used when this family is the only member in an order which is the only order in a class (example Cystoviridae within Vidaviricetes). If a feature has been found in one virus species, this does not by itself mean it is representative for an entire family, order or class of viruses.

We agree with this reviewer for being more specific in taxonomy related descriptions and modified our text wherever appropriate.

Line 44: “L denotes the large segment of the virus genome that also encodes the L protein” This is not quite correct. L stands for “large” and is also used in L RNA describing the larger RNA segment. However, it also describes the L protein as “large” viral protein independent of the encoding RNA segment naming.

Annotation of “L” modified based on this comment.

There is a BioRxiv preprint available on CCHFV L protein structure (https://doi.org/10.1101/2025.10.21.683639). The authors should include a comparison of what is similar and additional between these two studies.

A comparison with this and another recently published study (PMID 41991608) is included in the discussion. Basically, these studies report structures similar to the apo and cRNA-bound structures in our manuscript.

Line 72: “extra-large L protein” non-scientific

We removed “extra-large”.

Line 73: “previously unidentified cross-region interactions” How can this be previously unidentified if these are proposedly the first structures of CCHFV?

We removed “previously unidentified”.

Line 78: Reference 13 needs to be included again.

Reference added as suggested.

Results:

Line 99: “In Endo-resolved homologous structures, lid interacts extensively with Endo” Please be specific.

Examples are included in the revised description.

Line 111: “. With this EC structure, we are able to define the entire architecture of CCHFV L” 78% does not represent the entire architecture of the CCHFV L.

We have replaced entire with “relatively complete”.

It is interesting that the authors compare the architecture of CCHFV L with LACV and orthomyxoviruses, while phenuiviruses and arenaviruses are phylogenetically much closer to the nairoviruses and there are many structures available as well.

We included the structural comparison of SFTSV (*Phenuviridae*) and LASV (*Arenaviridae*) in Extended Data Fig. 5.

Line 134: “Although not by design, the T38b-UPD and P3b-Endo interactions greatly

help understand both RNA synthesis and cap-snatching." How? RNa bound to an RNA endonuclease is barely a surprise, what's new about this? Be specific!

Specific descriptions are included.

There is no data demonstrating the exact metal ions observed. The authors need to explain why they modeled which ion at which site. It is not obvious to everyone, especially in this journal with this broad readership. What's the evidence here?

We modeled these metal ions with coordination geometry (tetrahedral for Zn^{2+} in particular) and distance (all shorter than 2.5 Å) considered. We added description related to this and labeled the coordination interactions and distances in Fig. Extended Data Fig. 3a.

Line 144: "imperfectly complementary region near the 3'-end" Please be more specific, this is not clear, references point towards 2 review articles, this doesn't provide any clarity.

Research articles of IAV and LACV and the more relevant review have been provided as references.

What is the proposed function of the iGate? It is entirely the same in all recorded states, Rna present or not. What does it "gate"? The "equivalent substructure" supposed to be present in other bunyaviruses is not clear for me.

We only kept the description of the substructure for its association with the two zinc ions and no longer kept the nomenclature.

Line 157: "Even no distal duplex is present" Do you mean "even though"?

The distal duplex is not included in our experimental design for EC assembly, but its binding site can be identified. We modified the related descriptions for better clarity.

Line 158: "where lysine and arginine residues cluster at the downstream direction of the hook binding pocket (Fig. S5B)." This does not become apparent from this figure, which is about conservation.

In the initial submission, we referred to the wrong panel. In the revised version, these lysine/arginine residues are shown in Extended Data Fig. 6c.

Line 176 & 178: the 5' RNA is referred to as EC14 but in line 172 & 179 the overall EM map is referred to as EC14 structure. The nomenclature is confusing and should be cleaned up.

In the revision, we specifically refer to the global map or the density of the FID

region.

Line 185: “Lysing” typo

Corrected.

The authors should compare their structural data, e.g. for the endonuclease domain to the other published data on nairovirus L protein from crystallography: https://doi.org/10.1101/2025.10.28.684947 as well as cryo-EM https://doi.org/10.1101/2025.10.21.683639 and integrative modelling/predictions https://doi.org/10.1074/jbc.RA118.004373

While the second work does not include structure of endonuclease, the first work (published in Nucleic Acids Res) and the modeling work define about the same segment of L (residues 585-898). Therefore, we compared the boundaries of our Endo definition (residues 464-928) with the NAR work. Basically, the $Endo_{NE}$ (residues 464-583) and a 30-residue C-terminal is part of the RNA binding groove are not included in the published work likely due to exclusion in protein construct design. Descriptions related to this comparison have been included in the revision (main text and Extended Data Fig. 7b).

Line 194: Sentence requires a literature reference since a very broad statement is made about -ssRNA endonucleases.

Reference added as suggested.

Line 245: instead of saying ‘the two parts of the block’ the authors could just say between the endo and the CBD/mid-link.

Modified as suggested.

Line 256: the phrase ‘is still allowed but with restrictions by these interactions’ is unclear.

We replace the description with “could be moderately restricted”.

Line 263: ‘It turned out that’ non-scientific language

Modified as suggested.

Line 264: “It turned out that all ten mutations reduced the minigenome replication level, albeit to various extent (Fig. 3, B-C, 1.4-57% of WT-level), supporting the functional importance of these interaction networks.” This is a very superficial analysis of these mutants and the conclusion is not supported. The mutant proteins might be not expressed, structurally unstable or functionally impaired. Does a reduction to 57% of the WT indeed mean the function is impaired? The WT values are also fluctuating quite

a bit (75-130%). Also, the results of independent repeats seem to vary quite a lot in comparison. Did the authors compensate for transfection efficiency in any way?
I request also an explanation on the statistical analysis. With an n=2 and a relation of all values of mutants as % of the WT, a statistical test for significance is difficult as the WT does not have a distribution (as it is set to 100%) while the mutants do have a distribution around a average value. Please specify the statistical test used to label the columns with asterisks.

Based on this reviewer and reviewer 3's comments, we performed the minigenome experiments with transfection control in an n=3 setting, while Western blot analysis was carried out to confirm the expression of L protein. Statistical analyses were done using the average of each set and the statistical test used are include in the figure legends. Four additional mutations were included in the revision and the details are included in the revision of results and Fig. 3, and more specific interpretation of the data are provided.

Line 270: "immediate chain terminating NAs are rarely reported for this large group of RNA viruses" What is meant here? Like approved drugs on the market? Or published manuscripts with NAs acting as a chain terminators?

We meant in the literature. We specified this in the revision.

Line 276: optional: please add the names of the two viruses referred to and referenced.

Virus names added as suggested.

Line 280: The EC14 assembly assay is not mentioned in the methods section. Is it just the addition of the EC14 RNA to the L protein and then incubation at 22°C? Is it what you refer to as CCHFV RdRP elongation complex assembly assay? Please clarify/simplify in the text.

The reaction scheme used for CCHFV RdRP EC assembly was taken to assess the effect brought by CTP analogs. We rewrote this sentence and clarify this fact.

Line 286: "In the presence of ATP/UTP, the main products produced by CCHFV RdRP were 9-mer and 10-mer, with the latter arising from a template-G:product-U misincorporation" .This conclusion is neither well explained nor logical. If there was a misincorporation of a canonical NTP, why would this product of misincorporation then get enriched in the reaction if the nucleotides needed to continue the synthesis were still all there. A misincorporation does usually not per se terminate RNA synthesis and the following nucleotides in the template were again U, which was present in the

reaction. Why would production selectively stop there? Is this a specific feature of nairovirus polymerase compared to LASV and RVFV? How would this look with a different template?

In the revised manuscript, we tested CTP analogs on different templates, designed additional templates to assess all 4 RNA base variants of XTP (renamed as XCTP, and the other three being XGTP, XATP, and XUTP). It turned out that similar chain terminating effect after a misincorporation event was observed on two different templates (Extended Data Fig. 9d-e, T38 and T43-1 templates).

Line 290: Comparison across CCHFV, LASV and RVFV are not really appropriate given the fact that the LASV and RVFV samples did not have RNA that was adapted to the respective viruses and had higher protein and RNA concentration in the assay and were subsequently 10-fold diluted (which is still equivalent to a 3-fold higher protein concentration than for the CCHFV samples).

Virus-specific hook RNA was used for each virus (sequences have been provided in Extended Data Table 2), and the micromolar (vs. sub-micromolar for CCHFV) concentrations of RdRP and RNA were used for LASV and RVFV following the reaction settings in our recently published LASV work (PMID 39737930) to ensure enough amount of complex assembly because we found that sub-micromolar concentrations of RdRP and template resulted in much lower activities than the CCHFV system, possibly due to lower affinity of LASV/RVFV RdRP with the template. We have added descriptions to explain why higher concentrations of RdRP and template were used for LASV and RVFV.

The authors test several NA inhibitors but the analysis they provide is very superfivial. An outcompetition of a misincorporation by an NA with the correct nucleobase is not a great achievement in absence of the correct NTP, it is expected.

In the revised version, we addressed the chain terminating feature and virus-specificity (vs. LASV and RVFV) for all four RNA base variants of XNTP, and assessed the competition potential of them, using XUTP-HCV RdRP system for comparison. The details are included in the results section and Fig. 4 and Extended Data Fig. 9.

Do the authors suggest that MTP is being incorporated as well without any immediate inhibitory effect?

Yes. We have added description to description of the data related to MTP.

Discussion:

Line 306: “Besides seven RdRP catalytic motifs, replication-related modules or elements common for segmented -ssRNA virus include promoter binding platform formed by PA-C-like and fingers, initiation-related priming element (PE, also known as priming 308 loop) and prime-and-realign loop (PR-loop), and elongation-related lid, while the most notable Nairoviridae-specific modules

310 include FID and UPD.” This suggests that the features mentioned in the first half of the sentence are all also present in nairoviruses.

Due to lack of experimental evidence in *Nairoviridae*, we removed PR-loop in our discussion.

Line 313: “ds Polymycoviridae” misleading. ds what? Are there other (ss) Polymycoviridae?

We edited this sentence to clarify the meaning of +ss and ds as +ss and ds RNA virus groups.

Line 319: “have the aforementioned dual-function feature still awaits structural evidence” Please be specific about the aforementioned dual function feature. It actually needs also functional evidence, which could have been addressed by the authors. The authors define the priming element/priming loop in CCHFV L (“PE (residues 2839-2857 in CCHFV L) is largely disorderedI). n the following sentences the authors define the PR-loop (“The PR-loop (residues 2304-2315 in CCHFV L)”). I am missing the explanation why CCHFV L would need both a priming loop AND a prime-and-realign loop, this is usually not the case in other RdRps. More importantly, the functional evidence for this definition of priming elements is entirely missing. This part of the structure is solely described in the discussion but the speculative character is not made clear enough.

Dual function has been specified as participation in both polymerase initiation and elongation. As mentioned in the response to previous comment, PR-loop is not discussed in content directed describing CCHFV RdRP.

Line 334: ‘when switching’ instead of ‘if switching’. If would indicate you don’t know whether this switch happens, while you have data in which you observe this.

Modified as suggested.

Line 335: ‘is’ instead of ‘are’

Corrected.

Line 335: “Although the EC14 structure are not obtained using a capped primer, its polymerase elongation state and the T38b/P3b interactions with the CBD-/Endo-containing major regions allow us to reasonably judge what state it likely mimics in replication/transcription and what types of conformational changes are required to achieve other important states during cap-snatching and RNA synthesis.” I couldn’t disagree more. Observing short RNAs bound to single domains does not mimic the domain movements or provide any information on the orchestration of these movements. This space could be used much better to discuss the value and limitations of the presented data and compare to published work. While the authors acknowledge that “In transcription-related LACV structures, the relative CBD/Endo orientations relative to RdRP are diverse, while the interactions between CBD and Endo are also highly variable” and “In corresponding IAV structures, while the placement of the CBD and Endo relative to RdRP are consistent, the orientations of them, in particular CBD, can be quite different.” the authors “propose that CCHFV L could fulfill most transcription/replication tasks without large-scale relative movement of its three major regions”. All this is based on the first 3 structures of this protein of which only one is actually visualizing a functional complex.

We have taken the suggestions of this reviewer, and removed speculative content of the discussion and focus more on the limitation of the current study.

Line 363: ‘length of about’

Corrected.

Line 364: “to separate the two RNA strands, marking the entering of early elongation stage” Why is this still early elongation when the template-product duplex get separated? What do the authors denote the phase after prime and realignment is finished and before the strand separation happens?

We typically define the phases as initiation and elongation. The stages between initiation and elongation are defined as “transition between initiation and elongation”. Here we tend to define the late elongation stage at which a relative complete interaction network has been established between the polymerase and RNA that may include interactions at the ss upstream product and template, the secondary binding site (if present), etc. We understand that in some of the literature early and late elongation are referred to the above-mentioned

transition and elongation, respectively. In the revised version, we have tried to be clear of the definition with the nomenclature in the literature mentioned.

Line 373: which accompanying work?

This is a mistake and the related description is removed.

Line 374: “For both replication and transcription, the ss upstream template eventually reaches the secondary binding site,” It is interesting that this binding site has not been formally identified, experimentally proven or the proposed binding pocket analysed in the structure. Yet, the authors describe what is supposed to happen.

We agree that the “secondary binding site” awaits more evidence, and state this only as a possibility in the revised version.

Line 379: “Both FID and UPD could contribute to EC processivity through interactions with downstream template and upstream product, respectively, likely ensures enough processivity to synthesize the L transcript that is about twice the size of those in other Bunyaviricetes systems.” Why haven’t the authors tested this hypothesis with a mutational study to provide functional evidence for some of the special structural features?

In the revised version, both FID-RNA and UPD-RNA interactions are probed in the minigenome assay. However, systems capable of characterizing an EC with established FID/UPD interactions or evaluate the processivity of genome-segment-length RNA synthesis have not been established. These limitations have been included in the discussion.

Line 393: Sentence is not clear. What assessment exactly?

Clarified as enzymology and structural assessment.

Line 398: ‘drastic difference’ statement is not appropriate given the concerns stated for line 290.

This part of the discussion has been modified based on additional data characterizing NAs and the differences in assay settings are carefully discussed where necessary.

Methods:

It would be helpful for the reader to add a table with all the RNA sequences used in this work. That way readers do not have to jump back and forth to look for sequences that are mentioned by acronyms somewhere in the text or method section. For the cryoEM work the sequences only appear in Figure 1 but are not mentioned in the

related methods section and for the NA intervention assays only the LASV and RVFV sequences are mentioned in the related methods section.

Full sequences have been provided in Extended Data Table 2, and most of them have been also included in related figures including Fig. 1.

Line 448: Information on the what happened with these samples is missing. Presumably this assay led to the gel shown in Figure 1? If so, how was this gel run and stained?

Information related to gel in Fig. 1 has been provided in the Methods and figure legends.

Line 467: Reference for Topaz is missing. Even if you used it through CryoSPARC, Topaz is an outside software that needs to be reference separately. CryoSPARC just provides a wrapper. The correct reference can be found in the CryoSPARC Guide.

Reference of Topaz software has been included.

Line 511: A minimum of three independent experiments is required for such an experiment to perform a robust statistical analysis.

As mentioned above, three independent experiments were performed and the data are presented in Fig. 3.

Line 514: Information about the imaging is required. Especially whether the entire dish with cells i.e. all cells were imaged or whether only a selected region was imaged and quantified.

The entire dish was imaged and has been described in the revised methods.

Line 515: The minireplicon evaluation appears to be biased. There does not seem to be a way to normalise for fluctuations in transfection efficiencies. Fractions of eGFP positive cells vary strongly even within the WT data points (lowest and highest point are more than 50% apart). Therefore, comparing to the WT rate of eGFP positive cells is not significant nor does it allow conclusions about the protein stability or ability to be enzymatically active. Strong fluctuations between experimental sets can be seen in at least four different mutants, sometimes with discrepancies of up to 50%.

The minigenome data in the revised version included transfection control and three independent experiments. The protein expression was confirmed by Western blot data. Please refer to data presented in Fig. 3 and Extended Data Fig. 8 for details.

Figures

When showing cryoEM maps, especially detailed views of specific regions, it is helpful to mention the contour level at which the map is being shown in the figure (e.g. Fig. S3, S5, S6). It is mentioned in the methods but this information belongs to the figure caption.

Labels should not be on top of structures. This makes it hard to read everything.

We took the suggestion to mention map contour levels within the figure or in the figure legends. We tried to minimize labeling on top of structure throughout the manuscript.

Fig 1: the gel in panel C is not described or explained in the figure caption. EC14 is the name of one the structures presented, yet this panel shows RNA sequences and a gel. Is the CCHFV model shown in panel D the EC14 structure or another? Maybe add this information in the caption? Panel A representation is a bit unclear with the dashed lines between CCHFV L and the other two viruses. The labels for EC14, apo and 5'-cRNA bound are also not obvious to find initially and could be overlooked given the panel labels are the same font and size.

Descriptions related to the gel have been included in the figure legends. Panel a has been optimized based on the suggestions.

Fig 2: Dashed magenta line in panel A EC14b and panel B is not explained. What is it meant to show?

This line is used to classify the labeled R/K residues. It has been explained in the revised figure legends.

Fig 3: For the minireplicon data, three independent experiments are expected. Also, for four sets of mutations there is a strong difference in the values from the first and second experiment. Could this be explained?

Please refer to our responses above.

Panel C caption is missing a description of what the white and black circles represent. Presumably data points from the two independent experiments?

In part based on the suggestion of reviewer 3, the average value of each independent set has been used for statistical analyses and plotting in the revised Fig. 3.

Panel B: Images for M1-10 are all missing scale bars and image M10 looks like it may

be at a different magnification?

Scale bar for all images is the same and is labeled in the first image in revised Extended Data Fig. 8.

Fig 4: RdRP intervention? Inhibition of RdRP activity is the more appropriate description.

Modified as suggested.

It is not convincing that the red triangle points at a 10 nt product including an incorporated inhibitor. It could also be a 9 nt product with the inhibitor incorporated. Controls like L + template/no NTPs, L + template and primer/no NTPs are also missing. There is an endonuclease activity present in L, how did the authors verify that none of the products observed are derived from RNA cleavage?

In the revised manuscript, the test of all four XNTPs consistently showed faster migration of the XNMP-containing RNA. Possibilities arisen from endonuclease activities have been ruled out based on data obtained using Stains-All staining (for possible degradation of template) and ^{32}P-labeling (for XNMP-containing products) gels.

Fig 5: A sub-panel structure of a-h isvery confusing when having an A-C panel structure at the same time, maybe use I, II, III etc. Also it should become clearer which proposed arrangements of domains and RNA during the processes is based on the real structural evidence presented. Here I would argue, that the authors have not at all addresses transcription in their experiments and therefore it is entirely speculative how domains would be arranged.

Romain letters have been used as suggested. We removed the transcription part and use dashed lines, question marks, and the figure legends to indicate uncertainties.

Fig S1: Scale bar is 50 nm for micrograph but you have a second scale bar for 2D classes, either mention both or none, since the scale bars shown have their length written next to them.

Scale bar description removed in the legends as suggested.

Fig S2: “the root-mean-square deviation (RMSD) values of superimposed L protein a-carbon atoms of apo and cRNA-bound structures are 1.3 Å (40.6% residue coverage) and 1.2 Å (46.5% residue coverage), respectively, and are 0.8 Å (40.9% residue coverage) and 0.9 Å (47.3% residue coverage), respectively, if excluding the lid

residues.” It is not clear to me if the authors mean X% of the total residues built in the models or X% of the L protein sequence.

The percentage is referred to the total residues built. We have clarified this in the revised figure legends.

S3: When EM maps are presented, information about the map contour levels are required.

Contour levels have provided.

Legend for panel B: please rephrase from more clarity. For example, remove “and absent in apo/5’-cRNA-bound” in line 43. The following sentence suggests that everything that is shown in panel B is not visible/resolved in the apo/5’-cRNA-bound data.

We rewrote Extended Data Fig. 3 legends with this comment considered. It is true that everything shown in the original panel b is not visible/resolved in apo/5’-cRNA-bound data.

S5: The 5’ hook labelling for LASv L is incorrect. The sequence of the RNA hook starts with G at position 0, which is a non-templated extra G.

We corrected the position labeling for LASV hook RNA in revised Extended Data Fig. 6a.

S7: The topology of nairovirus endonucleases was proposed in a paper by Holm et al. 2018 (10.1074/jbc.RA118.004373), the authors did neither cite this work nor compare their data to the published model.

We have added this citation wherever appropriate.

Citations:

The authors cite many review articles instead of the original works. This is not best scientific practice. I would recommend to acknowledge the original work.

We have replaced some of the review articles with original research article as suggested.

Structures:

The deposited structural models seem to be of high quality. Some domains seem to not be well resolved in some maps (e.g. the PA-C like region). However, no major problems have been detected upon inspection.

Also with reviewer 1’s comments considered, we went through the three original structures and the newly added EC14b structure. Necessary model modification

and refinement have been done for EC14 and the data have been re-deposited in PDB/EMDB.

Final note: How about Arg3813 stacking onto the RNA nucleotide to separate product and template? The authors haven't even mentioned this residue nor validated the role using mutagenesis.

This residue was mentioned in original Fig. 5, its legends, and in the discussion section. We have added related reference of the first proposal of a structurally equivalent residue in LACV. Since this manuscript focus on the CCHFV-specific interactions and NA inhibition mechanism in functional studies, mutagenesis at this site has not been explored.

Referee #3 (Remarks to the Author):

In this manuscript, Jia and colleagues report three high-resolution (2.7–3.0 Å) cryo-EM structures of the CCHFV L protein in apo and RNA-bound conformations, resolving up to 78% of the full-length polymerase. Despite its extraordinary size of over 4,000 residues, the authors show that CCHFV-L adopts a conserved overall architecture comparable to that of related viral polymerases, such as those from influenza, Lassa, and La Crosse viruses. In contrast to these polymerases, CCHFV-L contains additional domains, including the upstream product-binding domain (UPD) and the finger-insertion domain (FID). The structures of these domains, proposed to act as extended RNA-binding motifs, are described in detail, although their functions remain uncharacterised in this study. Beyond the structural analyses, the authors provide data on the importance of a nairovirus-specific interaction network between the Endo, RdRP, and CBD domains using minireplicon assays. They also demonstrate inhibition of RNA synthesis with a ribose-2′-modified nucleotide analogue.

Overall, this manuscript presents novel and compelling findings that significantly advance our understanding of the architecture and potential regulatory mechanisms of nairovirus polymerases. While this reviewer cannot comment on the structural data, the functional assays would benefit from additional biological replicates, more rigorous statistical treatment, and improved data presentation. A more comprehensive characterisation of the role of the proposed ion-binding sites and the Endo-RdRP-CBD interaction network would further strengthen the manuscript.

We thank this reviewer for all the comments and concerns. In the revised manuscript, the minigenome data have been obtained based on this reviewer and review 2's suggestions. In the revised manuscript, we only made conservative comments about the zinc ion binding sites and the interaction network related the Endo-RdRP-CBD, and rather focus on the CCHFV-specific regions such as UPD and FID in the virology study and the NA inhibition mechanism in the enzymology study. The details of our responses are listed below.

Specific points:

1. Figure 1c: Explain what is shown in this panel and how the image was obtained; this information is missing from both the figure legend and the Materials and Methods section. Additionally, clarify why bands at approximately 14 nt are considered degradation products in lane 5 but genuine products in lanes 8 and 9. All relevant bands should be clearly identified and labelled.

Information related to Fig. 1c have been provided in the figure legends and methods section. An Endonuclease defective L mutant (D693A-E825A) was used in comparison with the WT L to show that the 14 nt product is from synthesis but not from degradation of endonuclease cleavage (Extended Data Fig. 3e).

2. Figure 2b: The legend states that the R/K residues in regions I and II are shown as sticks; however, these are barely visible in the figure. A clearer depiction would facilitate interpretation of the minigenome data presented in Figure 3.

In revised Fig. 2b, we used spheres to show the β-carbon of these residues and made a better contrast of them.

3. Figure 3c: The minireplicon assay panel shows activity data from 10 different mutants located in regions potentially involved in RNA binding or interdomain interactions. Except from the FID:RNA mutants, theses residues are not introduced or discussed earlier in the text. To aid interpretation, please elaborate on the relevance of these sites, the rationale for the amino acid substitutions, and indicate which structural figures illustrate the corresponding interactions.

We added introduction of all 14 mutants (additional 4 mutants were tested in the revision) in the revised version where appropriate.

4. Figure 3c. Please include information on the expression levels of each mutant

protein; reduced activity could result from lower expression rather than disruption of specific interactions.

Western blot data for WT and mutant L proteins have been provided in Fig. 3b.

5. Figure 3c. This panel contains data from two independent experiments, each performed in triplicate. The results appear to differ substantially between experiments for some mutants; therefore, an additional biological replicate is recommended. Please also clarify whether the 2x3 values were treated as independent data points in the ANOVA analysis. It is advisable to first average technical triplicates before performing the analysis. However, two independent replicates are insufficient to robustly estimate variance for ANOVA.

We took this suggestion and obtained the minigenome data in a 3*3 setting, and use the average value of three technical triplicates for statistical analyses.

6. Figure 4: Please include a schematic representation of the templates, primers, and products used, similar to that shown in Figure 1, to improve clarity. Indicate whether the same templates were used for all three polymerases, and provide the corresponding sequences both in the figure and in the Methods section. Also state whether the experiments were reproducible across different protein preparations, and how many times each assay was performed.

Based on this suggestion, we have provided the information of the RNA in Fig. 4, and Extended Data Fig. 9 and Table 2. Descriptions related to reproducibility has been provided in the methods.

7. Language and clarity. The manuscript would benefit from moderate language editing, including correction of grammatical errors and typos (e.g., lysine at line 185), consistent terminology, and reduced use of abbreviations (e.g., “+”-denotion, iGate, NA). Some statements could also be clarified (e.g., “major functional regions”, “ultra-capability” in the abstract, “PA-like UPD” at line 201). Additionally, the use of black outlines around letters and numbers in the figures makes the text difficult to read.

We have tried to improve the language of this manuscript. Some abbreviations including the iGate have been removed also based on the comments from other reviewers.

Referee #4 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Please refer to our responses to reviewer 2.

Referee #5 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Please refer to our responses to reviewer 2.

Point-by-point responses to reviewers' comments for manuscript tracking #: 2025-10-27418

RNA synthesis and substrate analog inhibition in the CCHFV polymerase

by Hengxia Jia, Bo Tang, Shunli Liu, Xin Wen, Fan Wu, Xiao Hu, Hu Zhou, Tingting Chong, Lincan Lv, Qiaojie Liu, Guibo Rao, Mingyu Wei, Xuping Jing, Sheng Cao, Fei Deng, Zhihong Hu, Bo Shu, Rui Gong, Jiqin Wu, Manli Wang, Peng Gong

(Our responses are shown in boldface text with grey background)

Referees' comments:

Referee #1 (Remarks to the Author):

The authors have considered my critiques and addressed them. Some uncertainties regarding what is observed in the maps remain, but I acknowledge that the signals I refer to are far from unambiguous. I am therefore satisfied with the additional clarifications provided.

We thank this reviewer for all the comments and concerns during the entire evaluation process.

I understand that the paper presents functional data on the newly identified residues involved in elongation, rather than other processes. This aligns with the new findings and, in my view, is relevant. The study does not address initiation or cap snatching (which involve the PR-loop or PE) in the structural or functional analyses provided. However, elongation is a process that occurs after cap snatching or replication initiation, so it is natural to discuss the implications of the findings in this context. Of course, this needs to be properly explained in the manuscript, and I believe it is.

Limitations of the current work and perspectives are included in the discussion.

Referee #3 (Remarks to the Author):

This is a revised version of a manuscript on the CCHFV L protein previously considered by Nature. The authors have made a substantial effort to address the numerous comments raised by the reviewers. Importantly, they now include expression levels of mutant L proteins (Fig. 3b), provide a clearer justification for the selection of amino acid residues for mutagenesis, and have repeated key experiments

to enable statistical analysis.

One remaining issue concerns Fig. 1c, which I still find difficult to interpret despite the revised description and figure legend. It is unclear why bands that appear similar are interpreted as background in some lanes but as products in others. The authors should label the relevant products directly in the figure, as they already do for the presumed degradation products in lane 5 using black arrowheads.

We thank this reviewer for all the comments and concerns during the entire evaluation process. We have added labels to better distinguish degradation and synthesized products both in Fig. 1c and Extended Data Fig. 3e.