Structural Basis of the Respiratory Syncytial Virus Polymerase Complexes

Project Objective:

The primary goal of this proposal is to elucidate the structural basis of the RSV synthesis machine, mainly the initiation and elongation stages of RSV transcription carried out by the RSV polymerase (L:P complex).

Project Progress:

1. Preparation and characterization of the RSV polymerase.

Preparing high-quality and large-quantity of RSV L is the critical first step to understanding RSV catalytic core and has been proven challenging[1]. We have successfully co-expressed and co-purified full-length wild-type (wt) RSV RdRP (6x His L and P) and catalytically inactive L(D811A):P using sf21 insect cells, and we also prepared the RSV L alone (**Fig. 1**). We will generate a panel of L fragments with one or two functional domains for simplicity. For large fragments (e.g., RdRP+CAP: 1-1654, 1-1699, 1-1714), we will primarily use insect cells. For smaller fragments (e.g. RdRP: 1-944, 1-961, 50-961; MT+CTD: 1696-2165, 1711-2165, 1725-2165), we will express them in *E. coli* cells.

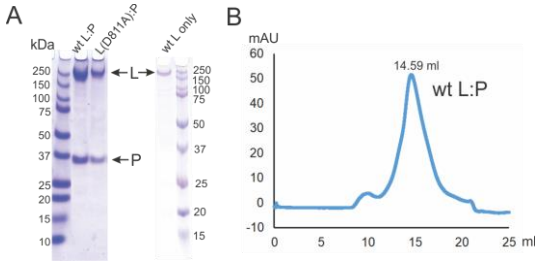

Figure 1: Preparation of the RSV L protein. (A) SDS-PAGE gel shows the expression of full-length wild-type (*wt*) RSV L:P and mutant L_{D811A}:P as well as L only in insect cells. **(B)** The size exclusion chromatography shows the homogeneity. (*Cao et al. Nat Comm 2020*)

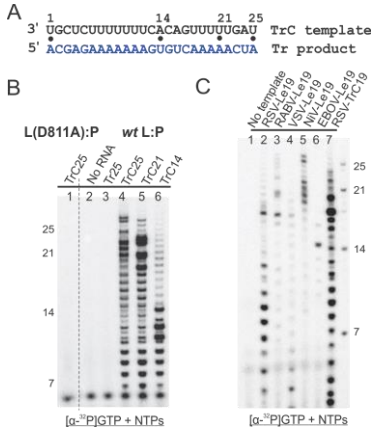

Figure 2: *In vitro* reconstitution of RSV RNA polymerization. (A) the template (TrC) and product (Tr). **(B)** Transcription assay using *wt* and mutant RdRP (L:P) on TrC25, Tr25, TrC21, and TrC14. **(C)** RNA template specificity. The RNA templates from different viruses were used. (*Cao et al. JVI 2020*)

2. In vitro transcription assay. We successfully adopted an *in vitro* transcription assay[1-3] and developed a novel RNA elongation assay to map key promoter features of RSV[4] (**Fig. 2**). Briefly, our assay employs a short RNA template to incorporate [^{32}P]-NTP into the reaction products. Transcription products by wt L:P and mutant L(D811A):P are examined on polyacrylamide gels. We used the first 25 nt of trailer complementary (TrC25) as the template and trailer (Tr25, the product of TrC25) as a negative control[1] (**Fig. 2A**). The results show that wt L:P generates RNA products with TrC25 and different lengths of RNA (e.g., TrC21, TrC14), but not L(D811A):P (**Fig. 2B**). We showed the RNA template specificity by comparing the RSV RdRP activities using leader sequences (Le19) from RSV, rabies (RABV), vesicular stomatitis virus (VSV), Nipah (NiV), and Ebola (EBOV) viruses (**Fig. 2C**). Further, we developed an RNA elongation assay by supplying short primers (3-8 nt) to the RNA template and ruled out the length requirements for proper RNA elongation. We defined the minimum length of the RNA template and fine-mapped the promoter sequence for the RSV RdRP. We will optimize the parameters (i.e., the length and sequence of the template and primers and reaction conditions) to identify suitable homogenous constructs for in-depth structural analysis.

3. Determination of cryo-EM structures of viral polymerases

We have successfully determined multiple high-resolution structures of the polymerases from NNS RNA viruses using single-particle cryo-EM[5,6] (**Fig. 3**). Specifically, I determined the 3.8 Å resolution cryo-EM structure of the vesicular stomatitis virus (VSV) L protein and performed the *de novo* model building of the 2109-residue polypeptide[5] (**Fig. 3A**). Two significant contributions resulted from this work: 1) the determination of the first atomic structure of an asymmetric protein of less than 250 kDa using cryo-EM; 2) the first atomic view of the RNA polymerase of NNS RNA viruses. Recently, we have determined a 3.67 Å cryo-EM structure of the apo RSV polymerase (L:P) using the 200kV Arctica with BioQuantum at the Robert P. Apkarian Integrated Electron Microscopy Core (IEMC) at Emory University (*Cao et al. Nat Comm. 2020*)[6] (**Fig. 3B**).

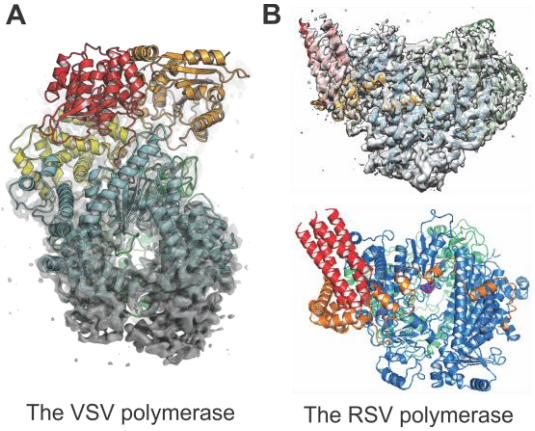

Figure 3: Cryo-EM structures of the polymerases of NNS RNA viruses determined by the PI. (A) 3.8 Å cryo-EM structure of the vesicular stomatitis virus (VSV) polymerase (*Liang et al. Cell, 2015*). **(B)** 3.67 Å cryo-EM structure of the respiratory syncytial virus (RSV) polymerase (*Cao et al. Nat Comm 2020*).

Proposed Project: ***Capture the initiation and elongation stages of RSV transcription***

A major function of the RSV RdRP is the *de novo* RNA synthesis, which requires that the RSV RdRP performs both initiation and elongation functions. Based on known knowledge of RdRPs, we hypothesize that significant rearrangements of functional domains of L occur in response to different stages of RNA synthesis. Therefore, obtaining high-resolution images at key stages of RSV transcription is vital. We will use the *in vitro* polymerization assay to guide the selection of RNA template(s) for the RSV L:P. We will apply a similar strategy to soak an appropriate RNA template and nucleotides or nucleotide analogs into the RSV RdRP to capture the initiation or elongation stages of RSV transcription, as in other RdRPs[7-9]. Preliminary data suggest that after the incubation with the selected RNA template, the RSV L:P:RNA complex showed good homogeneity and similar to that of the *apo* RSV L:P complex, and we will continue to optimize the conditions.

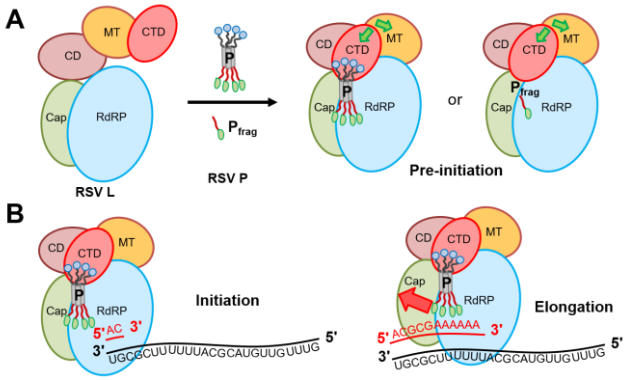

Figure 4: The models of the RSV transcription. (A) The RSV RdRP (L:P), known as the pre-initiation complex. The domains of both monomeric L and tetrameric P are shown. P_{frag} is the minimal P fragment to be identified in this proposal. **(B) Initiation** and **elongation complexes**. The RNA template and RNA transcript are shown as black or red line, respectively. The potential rearrangements of domains are indicated with green or red arrows.

During initiation, the RSV RdRP catalyzes RNA polymerization to *de novo* form a phosphodiester bond between the first and second NTP, without needing a primer. After initiation, the RdRP extends the nascent chain in the elongation stage. Using the template in the transcription assay and selected NTPs, we will trap the initiation and elongation stages of the RSV RdRP. For the initiation complex, we will add only ATP and CTP, which complement the first two residues (3'-UG) in the 3' end of the RNA template. We will add ATP, CTP, and GTP for the elongation complex but not UTP; an 11mer elongation product is expected to form under these conditions. We will also use nucleotide analogs, such as a chain terminator (3'-deoxy GTP), to lock the RdRP at an initiation or elongation stage. For transcription elongation, a pre-formed RNA template and transcript duplex plus additional nucleotides were shown to install the RdRP in a reactive complex successfully We will employ such a strategy for this work (**Fig. 4**).

Preliminary Results:

We used and will continue to use the *in vitro* polymerization assay to guide selecting appropriate RNA template(s) for the RSV L:P. We had negative stain EM images of both RSV L:P and L:P:RNA complex. Preliminary data suggested that after the incubation with the selected RNA template, the RSV L:P:RNA complex showed reasonable homogeneity similar to that of *apo* RSV L:P complex. We have also prepared the cryo-grids for the RSV L:P:RNA complex using similar freezing conditions as the *apo* RSV L:P complex. The initial cryo-screen revealed reasonable ice thickness in the cryo grids (**Fig. 5A, B**), and the particles are readily visible in the grid holes (**Fig. 5C**). We have acquired a small cryo-EM dataset and performed 2D class averages. The class averages show both similar and different views compared to that of *apo* RSV L:P complex (**Fig. 5D**). In summary, we have demonstrated the feasibility of preparing the cryo specimens of the RSV L:P:RNA complex, and ***we successfully determined multiple cryo-EM structures (2.5-3.5 Å) of the RSV polymerase and its complex with RNA templates and RNA products (unpublished) under the current support NCCAT-BAG-BL210628***. Given the exciting preliminary results, we expect to obtain multiple datasets of the complex assemblies to capture different enzymatic stages. Therefore, we wish to request an additional 300KeV Titan Krios time for high-resolution cryo-EM data collection at NCCAT. Once we collect sufficient data, we will obtain the maximum attainable resolution reconstruction of the RNA-bound RSV polymerase using cryo-EM and perform model building and validation.

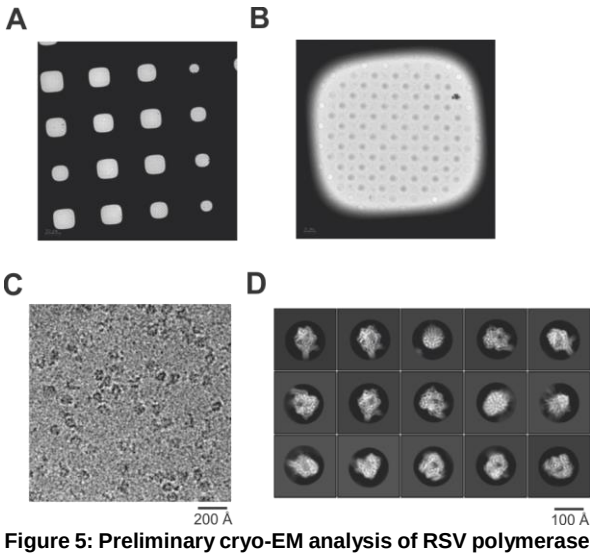

Figure 5: Preliminary cryo-EM analysis of RSV polymerase complex with its RNA (L:P:RNA). (A) The low magnification view of the grid. **(B)** The medium magnification view of a representative grid square. **(C)** The raw image of L:P:RNA complex. **(D)** The class averages show both similar and different views to that of *apo* RSV L:P complex.

REFERENCES:

1 Noton, S. L., Deflube, L. R., Tremaglio, C. Z. & Fearns, R. The respiratory syncytial virus polymerase has multiple RNA synthesis activities at the promoter. *PLoS Pathog* **8**, e1002980, (2012).

2 Morin, B., Rahmeh, A. A. & Whelan, S. P. Mechanism of RNA synthesis initiation by the vesicular stomatitis virus polymerase. *EMBO J* **31**, 1320-1329, (2012).

3 Morin, B., Liang, B., Gardner, E., Ross, R. A. & Whelan, S. P. J. An In Vitro RNA Synthesis Assay for Rabies Virus Defines Ribonucleoprotein Interactions Critical for Polymerase Activity. *J Virol* **91**, (2017).

4 Cao, D., Gao, Y., Roesler, C., Rice, S., D'Cunha, P., Zhuang, L., . . . Liang, B. In Vitro Primer-Based RNA Elongation and Promoter Fine Mapping of the Respiratory Syncytial Virus. *J Virol* **95**, (2020).

5 Liang, B., Li, Z., Jenni, S., Rahmeh, A. A., Morin, B. M., Grant, T., . . . Whelan, S. P. J. Structure of the L Protein of Vesicular Stomatitis Virus from Electron Cryomicroscopy. *Cell* **162**, 314-327, (2015).

6 Cao, D., Gao, Y., Roesler, C., Rice, S., D'Cunha, P., Zhuang, L., . . . Liang, B. Cryo-EM structure of the respiratory syncytial virus RNA polymerase. *Nat Commun* **11**, 368, (2020).

7 Lu, X., McDonald, S. M., Tortorici, M. A., Tao, Y. J., Vasquez-Del Carpio, R., Nibert, M. L., . . . Harrison, S. C. Mechanism for coordinated RNA packaging and genome replication by rotavirus polymerase VP1. *Structure* **16**, 1678-1688, (2008).

8 Tao, Y., Farsetta, D. L., Nibert, M. L. & Harrison, S. C. RNA synthesis in a cage--structural studies of reovirus polymerase lambda3. *Cell* **111**, 733-745, (2002).

9 Gong, P., Kortus, M. G., Nix, J. C., Davis, R. E. & Peersen, O. B. Structures of coxsackievirus, rhinovirus, and poliovirus polymerase elongation complexes solved by engineering RNA mediated crystal contacts. *PLoS One* **8**, e60272, (2013).