RNA-Protein Interactions Involved in Persistence of Cytomegalovirus

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Cytomegalovirus

- Enveloped β-Herpes Virus with dsDNA
- Leading cause of congenital birth defects
- Complications for immunocompromised individuals
- Limited number of drugs used for treatment

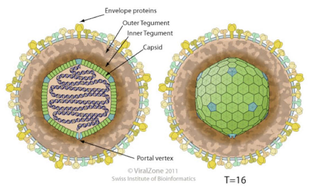

Figure 1: **General structure of HCMV.** dsDNA containing capsid is surrounded by an envelope composed of host cellular membrane.

Noncoding RNA

Noncoding RNA transcripts are not known for being translated into functional proteins and are abundant in genomes across species. Long ncRNA are classified as transcripts greater than 200bp in length and few have been fully characterized. HCMV genome encompasses numerous ncRNA varying from small miRNAs to a long stable intron (1).

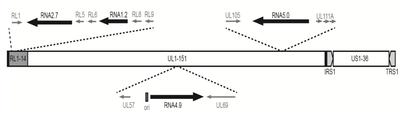

Figure 2: **HCMV ncRNAs.** The HCMV genome encodes multiple ncRNA with a variety of sizes from miRNA to the stable intron RNA5.0 (1).

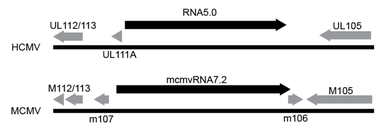

Figure 3: **Stable intro is conserved across all CMV genomes.** Genome sequencing reveals consensus sequences among CMV genomes. HCMV RNA5.0 is the RNA7.2 ortholog of MCMV (2,3).

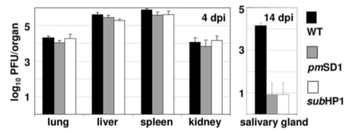

Figure 4: **RNA7.2 is required for persistence.** Mice were infected with 1x10^6 pfu of indicated virus. Levels of PFU/organ supported acute phase of infection 4 dpi. The virus persists in the salivary glands in WT conditions but is not found in mutant strains (3).

Methods

To study the hairpin structure within lnRNA RNA 5.0, we will be utilizing the RNA-Protein Interaction Detection Motif (RaPID). This method allows for biotinylated proteins interacting with our RNA to be analyzed through immunoblotting and Mass Spectrometry. We first have to construct fragments of different lengths for comparison of MS data. These fragments are then cloned into the pGEM vector before enzyme digestions are performed to create compatible overhangs for ligation into the RaPID motif vector. These vectors are then transfected into 293T cells where biotin will be added. Any proteins bound to the RNA will then be biotinylated. These RNA-protein complexes are purified via pull-down assays utilizing streptavidin beads. The samples are then sent for Mass spectrometry analysis while also being analyzed through immunoblot.

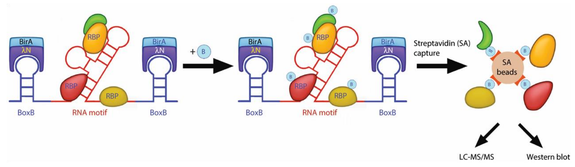

Figure 5 (above): **RaPID Model.** RNA-Protein Interaction Detection (RaPID) motif encodes loops recruiting biotin ligase which biotinylate proteins near the intermediate RNA. Streptavidin beads pull down the biotinylated proteins that are then analyzed by western blot and MS (4) .

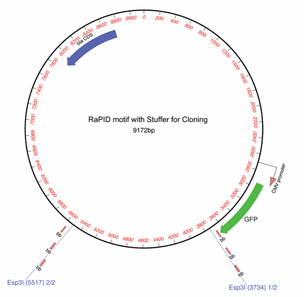

Figure 6: **RaPID Motif Vector.** The RaPID morif vector encodes GFP, ampicillin resistance, two Esp31 enzyme digest sites, and if filled with a stuffer sequence. The motif is 9172bp.

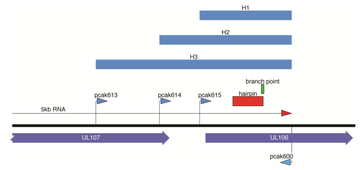

Figure 7: **RNA5.0 fragments from the 3' end.** Different length fragments from the 3' end include the hair-pin structure are inserted into the RaPID motif vector.

To create a control for analyzing MS data, we are building a mutant with a disrupted hairpin structure. Our aim is to reduce the free energy associated with folding to decrease spontaneous folding. Using mFold, we visualized how potential mutations might alter the free energy of folding.

Figure 8: **Predicted structure of RNA 5.0 WT hairpin loop.** The free energy associated with the folding of this loop is -40.00 kJ/mol.

Using Quik-change mutagenesis, we inserted mutation into the different arms of the WT structure. This method allowed for point mutations to be inserted into the sequence of the hairpin. The single mutations decreases ΔG by roughly one third.

Hair Pin Loop Structures

Figure 9: **Predicted structure of hairpin loop with incorporated mutant in the top arm of the loop.** A 6 bp mutation was introduced between the 30-40bp of the loop sequence to disrupt the top of the hair pin structure. The free energy associated with folding is -27.50 kJ/mol.

Figure 10:**Predicted structure of hairpin with site directed mutagenesis mutation in the bottom arm.** A 6 bp mutation was introduced between the 7-14bp of the loop sequence to disrupt the top of the hair pin structure. The free energy associated with folding in -26.90 kJ/mol.

To lower the ΔG further, we combined the mutations made to both arms. This lowered the ΔG to roughly half of what the WT free energy had been. This alteration in the hair pin will allow screening of nonspecific binding proteins to be filtered out of the MS results.

Figure 11: **Predicted structure of Hairpin loop after mutation introduced to both top and bottom sections.** The free energy associated with folding in -14.40 kJ/mol.

Results

We amplified our fragments (H1, H2, and H3). After performing enzyme digests to form compatible overhangs, we ligated the fragments into the pGEM motif vector. Transformation followed by purification produced enough DNA to preform the sequential enzyme digest that would allow for the fragments to be ligated into the RaPID motif vector. We have successfully cloned our fragments into the RaPID motif vector. A primary transfection of H1-RaPID into 293T cells was performed following the Lipofectamine 3000 Reagent protocol. The 293T cells were seeded to be 70-90% confluent at transfection. The lipofectamine reagents are thoughtly mixed and then the DNA is added for the formation of micells. The DNA-lipid complex is then added to the cells. After 24 hours, cells are visualized. The successful transfection will be green as the GFP protein is being translated from the RaPID Motif Vector.

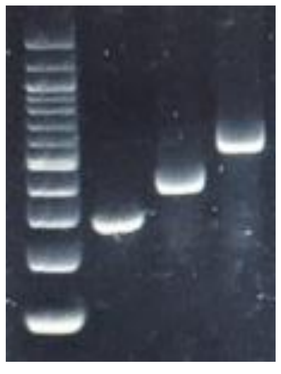

Figure 12: **PCR amplified H1,H2, and H3 fragments.** Fragments were then transformed into P-Gem with amp resistance. Colony PCR screened for correct fragment. Positive samples were sent for sequencing.

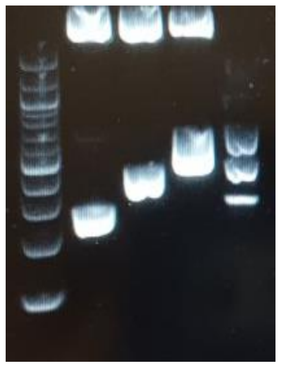

Figure 13: **Enzyme Digests of H1,H2, and H3 compared to original PCR products.** Using Bbs1-HF and HindIII-HF, the fragments now have the correct overhang sequences to be ligated into RaPID motif vector.

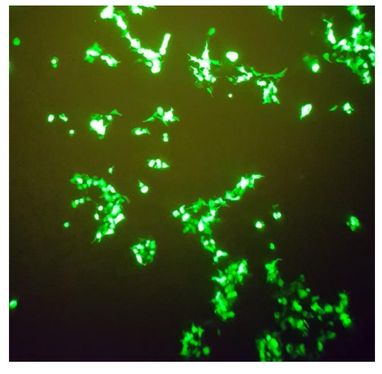

Figure 13: **Primary Transfection of H1-RaPID vector in 293T cells.** Representative photo of transfected cell expressing the GFP protein from the RaPID motif vector.

What's Next?

Now that we have fragments with the correct overhangs, RaPID ligations and then transformations into 293T cells can occur. From there, GFP will be used to identify if transformations were successful. We will biotinylate cells expressing the RaPID vector and preform pull-down assays to determine if proteins are binding to the RNA fragments. Analysis of the proteins will include immunoblotting and MS.

References

1. Schwarz TM, Kulesza CA. 2014. Long ncRNAs expressed during human cytomegalovirus infections. Future Virology 9:587.
2. Caroline AK, Thomas S. 2006. Murine Cytomegalovirus Encodes a Stable Intron That Facilitates Persistent Replication in the Mouse. Proceedings of the National Academy of Sciences of the United States of America 103:18302.
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4. Ramanathan M, Majzoub K, Rao DS, Neela PH, Zarnegar BJ, Mondal S, Roth JG, Gai H, Kovalski JR, Siprashvili Z, Palmer TD, Carette JE, Khavari PA. 2018. RNA-protein interaction detection in living cells. Nature Methods 15:207-212.