Direct evidence for a RNA packaging signal-mediated virus assembly mechanism

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Introduction

RNAs have a wide variety of biological functions, possessing the ability to alter their secondary and tertiary structures in order to present different functional states. The genomes of single-stranded, positive-sense RNA viruses have historically been seen as a passenger in the process of genome compaction and packaging when forming virions. Previous studies focussed on the viral coat proteins (CP) driving assembly via charge neutralisation alone. This mechanism cannot account for the high level of viral genome packaging specificity seen in almost all cases. We demonstrated previously, using single molecule fluorescence correlation spectroscopy (smFCS), that the model system, MS2 bacteriophage, preferentially packages its own RNA genome via binding of its cognate CP to multiple regions of degenerate sequence/structure within the RNA that we term packaging signals (PS). This sequence-specific binding is accompanied by a collapse in the hydrodynamic radius (Rh) of the genome, which is essential for it to fit inside its capsid. These cognate CP-PS interactions also serve to increase the specificity, fidelity and efficiency of the virion assembly process.
Recent work with the model virus Satellite Tobacco Necrosis Virus (STNV), demonstrates that not only does the RNA genome present PSs to cognate CPs to assist with assembly, but these PSs act cooperatively to increase the fidelity and yield of assembled capsids. One consequence is that the relative PS spacing within the STNV genome radically effects assembly fidelity. Indeed, we can recreate *in vitro* the assembly properties of STNV RNA with a completely artificial sequence encompassing appropriately spaced minimal CP recognition features. We have also obtained unambiguous evidence confirming PS-mediated assembly of infectious MS2 using CLIP-SEQ.

Results

SELEX was performed on the STNV CP, identifying RNA aptamers that bind with high affinity, all of which possessed an -AXXA- loop motif. These were compared to the STNV-1 genome, identifying several aptamers with high sequence homology to the genome, the best of which is termed B3. B3 has been shown to promote efficient STNV capsid assembly *in vitro* in reassembly assays at micromolar concentrations. In order to unpick the mechanistic detail, however, required analysis at a more likely *in vivo* concentration (nanomolar). We used recently developed smFCS assays to monitor STNV assembly at these concentrations by monitoring the Rh of dye-labelled RNA. We showed using B3, and a variant replacing the replacing the -AXXA- motif with –UUUU- (in order to keep the overall electrostatics the same,) that full capsid assembly was possible using a single PS, and that assembly was sequence dependant, with the 4U variant only forming unstable species.

In order to test whether a similar assembly would occur with a genomic fragment, this assay was performed on the first 127 nt of the STNV-1 genome, named PS1-5. This was the 5′ end of the genome, encompassing the first 5 putative PSs. The middle PS, PS3, was also homologous to the B3 aptamer. PS1-5, and several variants replacing the -AXXA- motifs of each loop with 4 U's were fluorescently labelled, and tested with the aforementioned smFCS assays as before. This genomic fragment highlighted the cooperative nature of the PS mediated assembly mechanism, as an initial collapse in the Rh of the RNA occurs, (dependant on the presence of the wild type PS3 sequence,) with a more rapid assembly step to *T*=1 VLPs, (dependant on the presence of the wild type sequence of the flanking loop

motifs,) in comparison to assembly with B3 alone (Figure 1A). Additional experiments varying the relative spacing between these 5 PSs showed that precise placement of CPs along this RNA fragment was an absolute necessity for faithful VLP assembly (figure 1B).

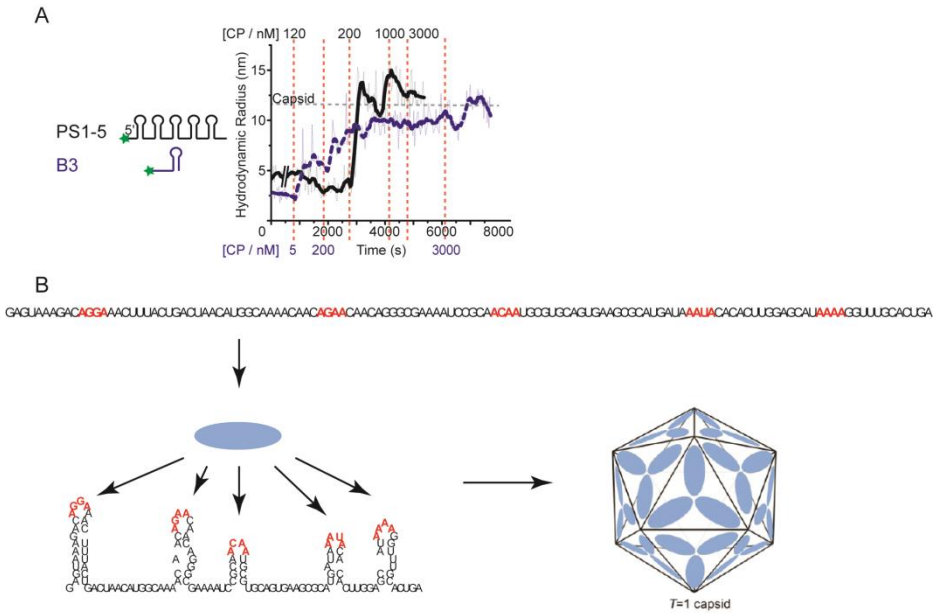

Figure 1: PS-mediated assembly of STNV. **(a)** Comparison of STNV VLP assembly using one PS (B3, dashed blue) vs 5 (PS1-5, black). CP titrations are shown by red dashed lines. B3 slowly forms a *T*=1 capsid in titrations up to 3 µM CP, while PS1-5 shows an initial Rh drop, followed by a highly co-operative assembly at 200 nM CP. **(b)** Multiple, short, degenerate CP-recognition motifs within the STNV genome (highlighted in red) when presented appropriately by RNA folding into stem-loops, are bound co-operatively at high affinity (low nM) by cognate CPs, leading to efficient capsid formation.

We showed previously using smFCS that MS2 bacteriophage preferentially packages its own genome, and exhibits the cooperative nature one expects from a PS-mediated assembly. We have also predicted the 60 packaging signals we expect to find within the MS2 genome. Working with Prof Cheng Kao's group at the University of Indiana in Bloomington we have verified these predictions experimentally. The MS2 virion was subjected to CLIP-SEQ (Figure 2).which uses UV irradiation to crosslink the RNA genome to the protein shell of MS2, these points of contact corresponding to PSs. The bound RNA was then sequenced by Next Generation sequencing and the reads aligned to the genome. Peaks of positive sequence matches correspond almost perfectly with previous PS predictions.

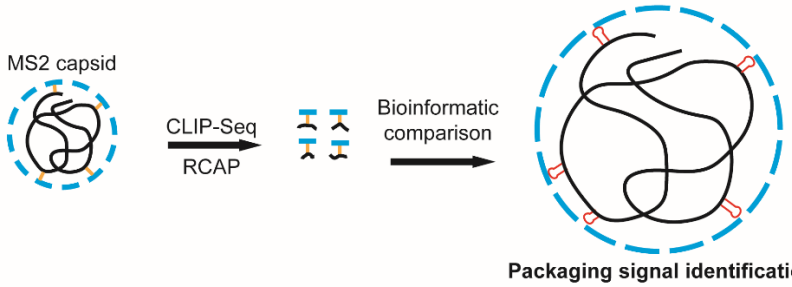

Figure 2: CLIP-Seq and RCAP techniques identify the RNA and CP interfaces in infectious virions, confirming the predicted packaging signals within this phage.

In summary, we have confirmed the presence of predicted packaging signals in the RNA phage MS2, using a combination of independent *in silico* and *in vitro* experimental techniques. SmFCS assays have been used to pick apart the mechanistic details that are involved in PS-mediated assembly. This work has led to a new, non-electrostatic paradigm

for ssRNA virus assembly (Prevelige, P. (2015) Follow the Yellow Brick Road: a paradigm shift in virus assembly. *J. Mol. Biol.* **428**:416-8).

Publications

Patel N., Dykeman E.C., Coutts R.H.A., Lomonossoff G.P., Rowlands D.J., Phillips S.E.V., Ranson N., Twarock R., Tuma R. & Stockley P.G. (2015) Revealing the density of encoded functions in a viral RNA. *Proc. Natl. Acad. Sci. USA* **112**:2227-2232.

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Collaborators

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