Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

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Guest Editor's Introduction

Editorial for "Methods to characterize virus small RNAs and RNA structures"

RNA viruses represent serious threats to the welfare of the global community as evidenced by the most recent viral pandemic caused by SARS-CoV-2, the etiological agent of COVID-19. A common feature of this large family of viruses is that they store their genetic material in the form of RNA, a highly adaptable and engaging biomolecule that orchestrates essential steps of viral replication cycles. Upon infection, a single genome can reprogram the cellular environment to sustain efficient viral protein production and RNA synthesis, from which progeny viral particles assemble. The cellular stage of viral RNA replication involves numerous RNA-RNA and RNA-protein interactions that regulate both viral and cellular biology. Interactions that involve viral-derived small RNAs are just one example by which viruses adapt to sustain efficient cellular replication; however, their mechanisms of action and host pathways usurped are gradually coming into focus. The purpose of this collection is to highlight the emerging roles of virus-derived small RNAs and some of the experimental techniques that are available to better understand their cellular mechanisms of action.

The articles in this issue focus on three areas: biogenesis and functions/regulation mechanisms of mammalian virus-derived small RNAs; biogenesis and functions/regulation mechanisms of plant virus-derived small RNAs; and techniques to probe viral RNA structures and interactions. The first three articles examine various aspects of biosynthesis, functions, and regulatory mechanisms of mammalian virus-derived small RNAs (vsRNAs). The article by Lee et al. [1] examines picornavirus vsRNAs derived from highly structured genomic regions, e.g., the internal ribosome entry site (IRES), by host Dicer enzyme. The Shi laboratory discovered vsRNAs in human cells infected by enterovirus A71 (EV-A71) [2]. While their functions are largely unknown, the IRES stem-loop II (SL-II)-derived vsRNA1 inhibits EV-A71 IRES-dependent translation in cells and in vitro. As such, vsRNA1 inhibits virus replication, which suggests vsRNAs may contribute to antiviral immunity. Lee et al. [1] describe key methods for characterizing vsRNAs including annotation by next-generation sequencing; assay of their abundance by Northern blotting; their Dicer-dependent synthesis by in vitro cleavage assays and Dicer:RNA gel-shift assays; and their inhibitory effect on IRES-dependent translation by employing in vitro translation assays. The second paper by Li and Brewer [3] builds upon methods described by Lee et al. [1] to examine mechanisms by which vsRNAs inhibit IRES-dependent translation. In addition to vsRNA1, inhibition requires IRES-associated trans-acting factors (ITAFs), i.e., host proteins. Earlier work showed that stem-loop II (SL-II) in the EV-A71 IRES is a hot spot for association with host RNA-binding proteins, including hnRNP A1, mRNA decay factor AUF1, mRNA stability factor HuR, and RISC subunit Ago2 [4]. Li and Brewer [3] describe methods for functional analyses of vsRNA1-mediated repression of IRES activity. These include construction of bicistronic IRES-luciferase reporters, protein-RNA interaction assays, knockdown of RNA-binding proteins using RNA interference, and assessing effects of vsRNAs and host RNA-binding proteins on viral protein synthesis and replication. Importantly, these methods should be applicable to other virus-derived, small RNAs as well. The third article by Jiang et al. [5] in this section describes an in vitro transcription system to produce virus-specific ssRNAs and dsRNAs. ssRNAs are produced by traditional DNA-templated transcription using T7 DNA-dependent RNA polymerase. Of course, these templates can encode RNA corresponding to any viral genomic or subgenomic RNA sequences. For synthesis of dsRNA, they employ the RNA-dependent RNA polymerase phi6 and a ssRNA template to produce dsRNA. One of the advantages of this enzymatic approach is that it minimizes problems that can arise upon annealing two complementary RNA strands, e.g., secondary structure within a ssRNA due to self-annealing; and low yields of long dsRNAs. These RNAs can be subsequently modified (e.g., phosphorylated, capped) depending on the application. Jiang et al. [5] demonstrate two biological applications for their virus-specific RNAs: (i) *Giardia intestinalis* or human Dicer enzymes for cleavage of dsRNAs to generate swarms of Dicer-substrate siRNA (DsiRNA). These swarms target multiple genomic sequences. The authors demonstrate their efficacy towards inhibiting replication of several influenza A strains in human primary cells. (ii) Their second application is delivering viral dsRNA mimics into mammalian cells to study activation of innate immune responses. By example, the authors demonstrate differences in RNA length-dependent recognition by RIG-I-like receptor (RLR) proteins RIG-I and MDA5 and subsequent production of antiviral cytokines.

The next three articles comprise the second section, which examines various aspects of biosynthesis, functions, and regulatory mechanisms of plant virus-derived small RNAs. For antiviral surveillance, infected plants generate virus-derived, small interfering RNAs (vsiRNAs) via Dicer-like enzymes (DCL) for RNA interference. High throughput sequencing technologies, coupled with the requisite computational tools for management of small RNA sequence (sRNA-Seq) data, provide extraordinary opportunities to address fundamental questions in virology. The article by Vivek et al. [6] reviews the state of the art regarding deep sequencing of sRNA pools and bioinformatic tools for diagnosis of known, and discovery of novel, plant viruses/viroids. Specifically, the authors delve into experimental issues, such as upstream sample preparation, library preparation and processing, and sequencing. They then discuss bioinformatic strategies, addressing facets such as, pre-processing of sRNA reads and viral contig assembly, databases and genomic subtraction, and benchmarking. Thus, the authors nicely

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describe optimization strategies, caveats, and best practices. The next article in the plant virus section by Zhao et al. [7] describes experimental approaches to determine which functional pathway(s) novel vsRNAs may trigger within an infected host for RNA silencing-mediated antiviral activity. Upon infection, virus RNA can yield two types of vsRNAs – siRNA or miRNA. The Argonaute protein Ago 1 is essential for the miRNA pathway, and Ago2 is essential for the siRNA pathway. The authors employ a virus-insect vector model – rice stripe tenuivirus (RSV) infection of small brown planthoppers (*Laodelphax striatellus*, Fallen). Their previous work identified four vsiRNAs derived from RSV in infected planthoppers, rice, and tobacco. They utilize Ago1- and Ago2-specific antibodies for RNP-immunoprecipitation (RIP) and qPCR to identify which vsRNA associates with Ago1 or Ago2 to obtain clues as to the function of each vsRNA. Interestingly, the authors found that two of the vsRNAs were not present in either Ago1- or Ago2-immunoprecipitated complexes. Their conclusion: regulatory pathways other than miRNA- or siRNA-mediated might be utilized by infected cells. The authors note their methods may be suitable for studying other sRNAs as well. The third and final article in the section on plant viruses describes methods to map transcription start sites (TSSs) in bogomoviruses. These ssDNA viruses cause disease in important crops including pepper, cotton, and tomatoes. Mapping TSSs provides important information about virus gene regulation and identification of novel RNAs. Mapping all bogomovirus 5′-ends remains uncompleted and transcriptomic analyses for many species are very limited. As such, the article by Arif et al. [8] describes an interesting method to map TSSs using mixed infections of the tobacco-related plant *Nicotiana benthamiana* with rice stripe tenuivirus (RSV) and three separate bogomoviruses – Corchorus yellow vein virus (CoYVV), Ramie mosaic virus (RamV), and Cotton leaf curl Multan virus (CLCuMuV). RSV 5′-end caps its RNAs via a "cap-snatching" mechanism by which the virus cleaves capped mRNAs of co-infecting viruses (and host mRNAs) at a site 10–15 nucleotides downstream of the cap. The capped RNA fragment serves as a primer to initiate RSV genome transcription. High throughput sequencing of snatched capped-RNA leaders are mapped onto each bogomovirus genome to identify mRNA 5' ends, i.e. TSSs. Their results show that begomoviruses use many different TSSs to transcribe the same gene, producing many different mRNA isoforms containing the corresponding open reading frame.

The final six articles comprise the third section of this special issue and address techniques that can inform on the structure–function properties of viral RNAs across multiple scales of biological resolution. Viral RNA structures represent genetically encoded elements that participate in numerous types of interactions to coordinate efficient replication within the cellular environment. Knowledge of the mechanisms by which these virus-host complexes contribute to pathogenesis requires a comprehensive assessment of viral RNA interactions. First, the article by Haddad et al. [9] offers a perspective on how integrating techniques that probe different properties of RNA interactions provide a framework to better understand the complexities of RNA viruses within the cellular environment. By combining various methods, the authors suggest that the emerging molecular descriptions of virus-host complexes will facilitate the discovery of novel antiviral targets and assist in interpreting their biological functions. Summary descriptions of the different technologies can be found in the perspective by Haddad et al. [9]; therefore, brief overviews of the invited author contributions are provided here. The article by Andrews et al. [10] describes an *in silico* approach (ScanFold) to discover thermodynamically stable RNA structures across a virus genome. Since RNA structure is linked to function, ScanFold provides a facile method to take a single viral RNA sequence and identify predictive RNA structural elements. The underlying principle is that functional RNA structures can be identified by tiling across a viral RNA genome and computing folding free energies for all base pair possibilities to calculate a z-score. Local viral RNA sequences are then ranked by z-score to identify genome regions with the propensity to form thermodynamically stable (functional) structures. Since some viral-derived small RNAs function through feedback loops by interacting with viral genome, ScanFold should assist in identifying their putative receptor sites. The next two articles by Tomezsko and Rouskin [11] and Lukasiewicz and Contreras [12] describe orthogonal tools to experimentally probe viral RNA structure. First, Tomezsko and Rouskin [11] steps through the process of globally determining viral RNA structures *in vitro* and *in vivo* using DMS-MaPseq. Here, the small molecule dimethyl sulfate (DMS) preferentially methylates unpaired or dynamic adenosine and cytosine residues within a viral RNA genome. Those modified residues are encoded as mutations during reverse transcription by the thermostable group II intron reverse transcriptase (TGIR-II) and subsequently detected by deep sequencing. The modification indices can be incorporated into RNA folding algorithms to determine experimental structures of viral genomes. By comparison, the method described by Lukasiewicz and Contreras [12] directly probes the accessibility of unpaired regions within RNA structure by using an antisense platform that has been engineered to express GFP only when hybridized to the appropriate RNA target. Using this system, regions that are unpaired in viral RNA genomes can be assessed independent of a predicted or experimental RNA structure. Both technologies should prove beneficial to understanding the mechanisms of action of viral-derived small RNAs.

The last two papers in this section provide very different perspectives of viral RNA genomes. The paper by Mugisha et al. [13] describes how binding sites for proteins can be mapped to viral RNA genomes. Cross-linking immunoprecipitation coupled with sequencing (CLIP-seq) identifies protein binding sites along viral RNAs with nucleotide resolution. Since some viral-derived small RNAs modulate RNA binding protein interactions with their RNA targets [2,4], CLIP-seq can help resolve the exact mechanism of action under conditions where concentrations of the vsRNAs can be adjusted. The final paper in this edition by Kotar et al. [14] covers the application of NMR spectroscopy to probe, with high-resolution, the structural dynamics of viral RNA elements. As described by Kotar et al. [14], advances *in vitro* transcription procedures, isotopic labeling strategies, and data acquisition have pushed the size limit of RNA structures that can be studied by NMR spectroscopy. Unlike the other techniques described in this edition, NMR spectroscopy provides atomic-resolution detail about viral RNA structures. Thus, NMR can be used to characterize the mechanisms of action of vsRNAs by determining their stereochemical properties either free or when bound to their cognate genomic receptors.

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