1. PUBLISHABLE SUMMARY

Coxsackie B viruses (CVB), which belong to the *Enterovirus* genus of the *Picornaviridae* family, have non-enveloped icosahedral capsids composed of 60 copies each of the four structural proteins (VP1 to VP4), enclosing a single-stranded, positive-sense RNA genome of ~7,400 nucleotides. The genome consists of a single open reading frame encoding 11 mature proteins, flanked on both the 5' and 3' ends by two non coding regions (NCRs). The enterovirus 5’ NCR is ~750 nucleotides in length and has several complex RNA secondary structures including an internal ribosome entry site (IRES) involved in cap-independent translation and a 5' terminal cloverleaf or stem-loop I (S-L I) required for replication of the viral genome. Stem-loop I includes approximately the first 110 nucleotides of the 5’ NCR and is organized into one stem (a) and three stem-loop structures (b, c, d), which bind cellular and viral proteins involved in virus RNA synthesis. In the cytoplasm of infected cells, cellular poly(rC)-binding protein 2 (PCBP2) and viral protein 3CDpro bind, respectively, stem-loops b and d of stem-loop I at the 5’ end of the enterovirus genome to form a ribonucleoprotein complex required for the initiation of anti-genomic, negative-strand RNA synthesis as well as genomic positive-strand RNA synthesis (Figure 1). PCBP2 also binds to a C-rich stretch of nucleotides located 3’ of the stem ‘a’ in S-L I.In turn, heterogeneous nuclear ribonucleoprotein C (hnRNPC) is thought to bind to the cloverleaf at the 3' end of anti-genomic (negative-strand) viral RNA to facilitate the initiation of positive-strand genomic RNA synthesis. These newly-synthesized positive-strand RNAs are either used as messenger RNAs for further rounds of translation and RNA replication or as genomic RNAs packaged into virions for subsequent rounds of infection.

The CVBs are common human pathogens, transmitted through fecal-oral and respiratory routes, and are considered as the main cause of acute myocarditis in children and young adults in developed countries. Following the acute phase, myocarditis can become chronic and evolve toward the clinical end-stage of dilated cardiomyopathy (DCM), in which the heart becomes enlarged and its systolic function is impaired, leading to heart transplantation. During the progression of disease from acute myocarditis to DCM, the virus can be detected in the myocardium. A correlation between enterovirus replication and poor clinical outcomes has been demonstrated,suggesting that continued virus replication is involved in the progression of the disease. Moreover, expression of viral 2A proteinase alone in a murine model of CVB3 myocarditis has been shown to generate dilated cardiomyopathy. Expression of 2A can cause significant impairment of cardiomyocyte function through proteolytic cleavage of dystrophin, resulting in a decrease in cell contractility, an increase in membrane permeability, and a focal spread of the virus to adjacent cells. However, the molecular mechanisms by which the virus can persist in the heart from acute myocarditis to DCM are poorly understood, thereby limiting the development of new specific therapeutic strategies.

Recently, Bouin and colleagues reported CVB3 strains harboring 5’ terminal genomic RNA deletions, ranging from 15 to 48 nucleotides in length, in explanted heart tissue collected from a 47-year-old immunocompetent woman suffering from idiopathic DCM. These terminal deletions, which partially removed the 5’S-L I, mirrored results previously obtained by Chapman and colleagues, who demonstrated that terminally deleted (TD) mutations can occur in a patient naturally infected with CVB2 and suffering from fulminant myocarditis, in the heart and the pancreas of mice inoculated with wild-type CVB3, and during CVB3 passages in primary cell cultures. However, these recent findings showed for the first time the existence of terminally deleted (TD) CVB genomic RNA populations in a patient suffering from chronic cardiomyopathy.

Deletions involving sequences within the 5’S-L Istructure of genomic RNAs are known to cause profound changes in viral biology by reducing viral replication,when compared to wild-type viruses. Indeed, the discovery of TD mutations in heart biopsies was associated with low viral load and abnormal positive- to negative-strand viral RNA ratios, close to one rather than the high ratios (30 to 70) normally seen in enterovirus-infected cellswith wild-type viruses. Moreover, TD variants of CVB3 replicate in both cell culture and tissues from experimentally-inoculated mice and from naturally infected humans without apparent cytopathic effect. Overall, 5’ terminal deletions within genomic RNAs could be a mechanism by which CVB can persist covertly in the heart long after the acute infection cycle with limited, but progressive, damage to the myocardium. A prolonged, persistent viral infection could then explain how chronic cardiomyopathies develop *via* the continuous synthesis of viral proteins with pro-apoptotic, immuno-modulating, or other harmful activities for cardiac cell structures and functions.

To date, translation and RNA replication of 5’ terminally deleted CVB3 in a cardiac cell model have not been studied. Moreover, the mechanism by which deletions within the 5’ S-L I affect the replication of the viral RNA is still unknown. The aim of this study was to assess in vitro the consequences of the 5’ S-L I deletions observed *in vivo* on CVB3 replication. Using cell-free extracts of uninfected HeLa cells and cell culture transfection of luciferase replicons in two types of cardiac myocytes, we evaluated translation and RNA replication of full-length and 5’ terminally deleted CVB3 RNAs. These experiments demonstrated that CVB3 RNAs harboring 5’ terminal deletions from 8 to 49 nucleotides can be translated as efficiently as those of wild-type strains (Figure 2). However, transfection of reporters RNAs harboring these deletions into cultured cardiomyocytes demonstrated a loss of detectable RNA amplification signal beyond the translation of input RNAs (Figure 3-5). Replication experiments *in vitro* in cell free HeLa cell extracts confirmed these findings, showing greatly reduced levels of negative- and positive-strand RNA synthesis in each of the terminally-deleted RNAs. Among the terminally deleted CVB3 RNAs tested, RNA synthesis was observed only for the ones harboring deletions of either 8 or 21 nucleotides. The only RNAs produced from these deleted templates were double-stranded (ds) RNAs [without detectable single-stranded (ss) RNAs], suggesting an abnormal positive- to negative-strand RNA ratio close to 1,similar to the ratios found *in vivo* for TD viruses (Figure 6).

Partial deletion of the 5’S-L I sequences would be predicted to alter the binding sites of proteins involved in CVB RNA synthesis, possibly providing an explanation for the low levels of TD RNA replication. To address this possibility, we used RNA mobility shift assays to investigate the binding of host protein PCBP2 and viral protein 3CDpro to the 5’ end of deleted forms of positive-strand RNA. We found that binding of these cellular and viral replication factors was qualitatively conserved for the 5’ terminally deleted viruses, independent of the size of the deletion; however, the stability and/or protein binding affinity of the ribonucleoprotein complexes decreased with the increase in size of the deletion, since they required higher protein concentrations to form (figure 7). Overall, this study provides an *in vitro* analysis of RNP complexes that may be involved in persistent forms of CVB3 observed *in vivo* from heart tissues. Our data provide a possible explanation for the low levels of RNA replication of deleted strains as a result of the consequences of the deletions on the formation of the RNP complex. In addition, these data raise the question of the capacity of some of the deleted strains to replicate autonomously, since no significant levels of RNA synthesis were detected for 5’ terminal deletions larger than 21 nucleotides.

In conclusion, dilated cardiomyopathy is the leading cause of heart transplantation in developed countries, and coxsackie B viruses are detected in about one third of idiopathic, dilated cardiomyopathies. 5’ terminal deletions of the viral genome involving an RNA secondary structure required for RNA replication have been recently reported as a possible mechanism of virus persistence in the human heart. These mutations are likely to disrupt the correct folding of an RNA secondary structure required for RNA replication. In this report, we demonstrate that viruses with these 5’ terminally deleted genomic RNAs can synthesize viral proteins and genomic RNAs in human cardiac myocytes. Our results provide a possible connection of the activities directed by viral RNAs harboring 5’ terminal deletions to previous studies linking persistent viruses to dilated cardiomyopathy pathophysiology, viral 2A proteinase-mediated dystrophin cleavage, and local, continuous inflammatory responses responsible for immune-pathological damage. Moreover, we show that the binding of cellular and viral replication factors to viral RNA is conserved despite genomic deletions, but that the impaired RNA synthesis associated with terminally deleted viruses could be due to loss of stability of the ribonucleoprotein complexes formed.

2. USE AND DISSEMINATION OF FOREGROUND

Section A (public) – DISSEMINATION MEASURES

**Dissemination activities**

The original results related to the research conducted under the cardiovir project were presented (i) during a seminar given February 5th, 2015 in the University of California Irvine, (ii) during a seminar given September 22nd, 2015 in the University of Poitiers and (iii) at the meeting of the French Society for Microbiology (22-23 Marsh 2016) at the Pasteur Institute.

**Publications**

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| **LIST OF SCIENTIFIC PUBLICATIONS, STARTING WITH THE MOST IMPORTANT ONES** | | | | | | | | | | |
| No. | Title / DOI | Main author | Title of the periodical or the series | Number,  date or  frequency | Publisher | Place of publication | Date of  publication | Relevant pages | Open access ? | Type |
| 1 | Functional consequences of RNA 5’ terminal deletions on coxsackievirus B3 RNA replication  and ribonucleoprotein complex formation. | Lévêque N, Garcia M, Bouin A, Nguyen JHC, MyPhuong T, Andréoletti L, Semler BL. | Journal of Virology | In press | American Society for Microbiology (ASM) | United States | In press | In press | Yes | Peer-reviewed |
| 2 | A 21st century perspective of poliovirus replication.  doi: 10.1371/journal.ppat.1004825 | Lévêque N, Semler BL. | PLoS Pathogen | 11(6) | Plos | United States | 2015 Jun 4 | e1004825 | Yes | Peer-reviewed |
| 3 | Construction of a subgenomic CV-B3 replicon expressing emerald green fluorescent protein to assess viral replication of a cardiotropic enterovirus strain in cultured human cells.  doi: 10.1016/j.jviromet.2016.01.005. | Wehbe M, Huguenin A, Lévêque N, Semler BL, Hamze M, Andreoletti L, Bouin A. | Journal of Virol ogical Methods | 230 | Elsevier | Netherlands | 2016 Apr | 1-8 | Yes | Peer-reviewed |
| 4 | Viral Determinants of miR-122-Independent Hepatitis C Virus Replication.  doi: 10.1128/mSphere.00009-15 | Hopcraft SE, Azarm KD, Israelow B, Lévêque N, Schwarz MC, Hsu TH, Chambers MT, Sourisseau M, Semler BL, Evans MJ. | mSphere | pii | ASM | United States | 2015 Nov 25 | e00009-15. | Yes | Peer-reviewed |