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Developing an *in vitro* enzymatic assay for the hepatitis E virus RNA-dependent RNA polymerase

James S. Porterfield Prize for International Virology 2015 - Report

Research visit to:

Research group Prof. Dr. Bruno Coutard – Prof. Dr. Bruno Canard

Architecture et Function des Macromolecules Biologiques (AFMB) Laboratory

Aix-Marseille Université

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1. Introduction

Hepatitis E virus (HEV) is a positive-sense single-stranded RNA virus classified in the *Hepeviridae* family. The virus is transmitted feco-orally and although most infections remain asymptomatic, HEV is one of the most common causes of acute viral hepatitis and is known to cause large water-borne epidemics in developing countries.[1,2] Such outbreaks are mainly due to infections with genotype 1 and are typically associated with high mortality rates in pregnant women.[3,4] Genotypes 3 and 4 are the main causes of hepatitis E in industrialized countries where infections occur mostly through consumption of undercooked pig meat.[5] Genotype 3 infections may evolve to chronicity in immunocompromised patients (e.g. transplant recipients, HIV-infected individuals,…) and often require either dose reductions of immunosuppressant drugs and/or treatment with ribavirin or pegylated alpha interferon.[6] Ribavirin is the drug of choice in most patients,[7] but has several side effects and treatment failure occurs occasionally.[8,9] We analyzed the viral genomes in two cases of ribavirin failure and identified the G1634R mutation associated with the onset of treatment failure.[8] This mutation does not result in an altered ribavirin susceptibility in vitro, but increases viral replication which may explain the apparent clinical resistance to ribavirin.[8] Recently, an additional patient was identified with the G1634R mutation and two additional mutations in the viral RNA-dependent RNA polymerase (RdRp) which altered viral fitness and ribavirin susceptibility (manuscript submitted for publication).

To understand the basic functioning of the HEV RdRp and elucidate the underlying molecular mechanisms of HEV polymerase mutations (such as G1634R), an enzymatic assay for the HEV RdRp would be crucial. To this end, we initiated a collaboration with the “Architecture et Function des Macromolecules Biologiques” (AFMB) laboratory in Marseille, France. The group of Bruno Canard and Bruno Coutard is specialized in elucidating the structure and function of viral proteins and developing corresponding enzymatic assays. Their attempts to express a functional HEV RdRp using bacterial and mammalian expression systems were not successful however. As an alternative, we attempted to recapitulate the activity of the HEV RdRp *in vitro* using crude replication complexes isolated from hepatoma cells stably transfected with a subgenomic HEV replicon.

2. Work performed during the research stay

In preparation of the research stay in Marseille, I constructed a selectable subgenomic HEV replicon. To this end, the *Gaussia* luciferase gene in the Kernow-C1 p6/luc reporter replicon[10] was replaced with a neomycine resistance gene. Capped RNA transcripts from the resulting p6/Neo construct were transfected into Huh7 hepatoma cells. Cells supporting stable replication of the replicon were selected by addition of geneticin (G418) to the culture medium. After expansion, crude replication complexes were isolated from Huh7 p6/Neo cells by differential ultracentrifugation of cell lysates.

The isolated crude replication complexes were used in Marseille to perform a viral RNA replication assay according to procedures established for other positive-sense single-stranded RNA viruses.[11–15] Reactions were performed using following components:

	Final concentration
Replication complexes (in TN buffer* + 15% glycerol)	
HEPES pH 7.5	50 mM
DTT	10 mM
KCl	10 mM
Actinomycin D	10 µg/mL
Creatine phosphokinase	20 U/mL
Creatine phosphate	10 mM
RNasin	0.8 U/µL
$MgCl_2$	3 mM
GTP	0.5 mM
ATP	1 mM
CTP	0.5 mM
UTP	0.04 mM
α-^{32}P-UTP	10 µCi / 50 µL
H_2O ad 50 µL	

* TN buffer = 10mM Tris pH7.8 - 10mM NaCl

Negative control reactions were included without Mg^{2+} and an alternative reaction with Mn^{2+} instead of Mg^{2+} was performed as well. Reactions were incubated at 30°C and stopped after 0, 1 or 2 hours by addition of EDTA. RNA was ethanol-precipitated from reactions and analyzed by

formaldehyde-agarose gel electrophoresis. No newly synthesized (i.e. radioactive bands) could be detected.

Experiments were repeated under slightly altered conditions, but again no RNA replication could be observed. We hypothesize that an additional host factor is required for efficient RNA replication or that the current protocol does not allow isolation of viable HEV replication complexes. Other protocols for replication complex isolation and *in vitro* RdRp assays are currently being explored.

3. Gains of the research visit

Despite the fact that we were unable to develop a HEV polymerase assay, the research visit to the AFMB lab in Marseille was very valuable and has allowed me to gain important experience in biochemical techniques, enzymatic assays and working with radioactivity. I also experienced a different research environment and significantly expanded my network. In fact, the research visit also contributed to another collaboration between our lab in Leuven and AFMB regarding the HEV macrodomain. The paper resulting from this collaboration has recently been submitted for publication.

4. Acknowledgements

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5. References

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