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Investigating properties of wildtype and mutant Barmah Forest virus replicases

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Investigating properties of wildtype and mutant Barmah Forest virus replicases

Abstract:

Barmah Forest virus (BFV) is an alphavirus that is mostly spread in Australia and it causes disease in humans upon infection that may manifest as arthritis, myalgia, fever and rash. For this reason, BFV is a cause for concern in puclic health and often a subject in alphavirus related research. In the current work, we observed that BFV replicase activates the type I IFN response in the absence of a viral template, and that mutations in viral replicase proteins may boost IFN production. Furthermore, we looked into the effect that these mutations may possess on the replication and transcription of the BFV replicase using the trans-complementation system. These experiments aid in understanding how alphaviruses influence host cells and vice versa, which could provide useful information in the research for antiviral agents.

Keywords: alphavirus, Barmah Forest virus, BFV replicase, alphavirus non-structural proteins

CERCS code: B230

Barmah Forest viiruse metsiktüüpi ja mutantsete replikaaside omaduste uurimine

Lühikokkuvõte:

Alfaviirus BFV on levinud peamiselt Austraalias. BFV põhjustab inimesi nakatades haiguslikke sümptomeid, mida iseloomustavad liigesvalud, lihasvalud, palavik ja lööve ning oma levikupiirkondades kujutab BFV poolt põhjustatud haigus tervishoiuprobleemi. Seetõttu on BFV alfaviiruste teemalistes uurimustes tihti esindatud. Käesolevas bakalaureusetöös kirjeldasime BFV replikaasi omadust aktiveerida tüüp I IFN vastust sõltumata viiruslike matriits-RNA-de olemasolust rakus ja uurisime mõne mutatsiooni mõju sellele omadusele. Nende samade mutatsioonide mõju analüüsisime ka replikaasi võimes sünteesida viiruslikke RNA-sid. Uurimuse tulemused aitavad mõista kuidas alfaviirused mõjutavad peremeesrakke ning ka vastupidi ja siinne informatsioon võib olla kasulik viirusvastaste ravimite leidmisel.

Märksõnad: alfaviirus, Barmah Forest viirus, BFV replikaas, alfaviiruste mittestruktuursed valgud

CERCS kood: B230

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TERMS, ABBREVIATIONS AND NOTATIONS

aa – Amino acid

AUD – Alphavirus unique domain

BFV – Barmah Forest virus

CHIKV – Chikungunya virus

CMV – Cytomegalovirus

CSE – Conserve structural element

CPV – Cytopathic vesicles

C – Capsid protein

dsRNA – double stranded RNA

EEEV – Eastern Equine Encephalitis Virus

EILV – Eilat virus

FBS – Fetal bovine serum

FLuc – Firefly luciferase

gRNA – Genome/genomic RNA

GLuc – Gaussia luciferase

HVD – Hypervariable domain

IFN-β – Interferon Beta

icDNA – Infectious Clone

IMDM – Iscove's Modified Dulbecco's Medium

Km – Kanamycin

LB – lysogeny broth

MAYV – Mayaro virus

nsP – Non-structural protein

NRAMP – natural resistance-associated macrophage proteins

NLS – nuclear localization signal

nt – nucleotide

ONNV – O'nyong'nyong virus

PAMP – Pathogen associated molecular pattern

PRR – Pattern recognition receptor

RRV – Ross River virus

RdRp – RNA-dependent RNA polymerase

SAV – Salmonid Alphavirus

SFV – Semliki Forest virus

SINV – Sindbis virus

sgRNA – Sugnomic RN

SINV – Sindbis virus

TF – TransFrame

IFN – Interferon

UTR – Untranslated region

VEEV – Venezuelan Equine Encephalitis virus

WEEV – Western Equine Encephalitis virus

ZBD – Zinc-binding domain

INTRODUCTION

Viruses are non-cellular obligatory endoparasites which means that they rely on the host cell to complete their life cycle. Viral infections are often harmful to hosts and can subsequently lead to illness and sometimes death, and as (recent) history shows, viruses can cause outbreaks of epidemics and pandemics. For this reason, viruses have long been and remain important subjects in scientific research. Virology looks into viral infection caused pathologies, and extends to the field of immunology, which studies the relationships between the virus and host organisms, describes the molecular biology of viruses, and much more, in order to further extend our understanding of these infectious agents and contribute to our efforts in controlling viral infections. Thus, in the case of many viruses, many antiviral strategies have been developed to battle these pathogens.

Alphaviruses are positive-sense RNA genomic viruses from the *Togaviridae* family. Currently, over 30 species of alphaviruses have been discovered. Alphaviruses are arboviruses as they are mostly distributed by suitable mosquito vectors, and their transmission to human hosts can result in significant pathologies. Namely, alphavirus infection can lead to the development of arthralgia, myalgia, fever, rash, and in some cases, chronic arthritis. However, no specific antiviral drugs or vaccines are available to manage these infections clinically. An example of an alphavirus pathogenic in human hosts is Barmah Forest virus (BFV) which is mostly spread in Australia and is a cause of concern for public health in areas of mosquito vector occurrence. For this reason, BFV has been the subject in many alphavirus related research.

In the current work, we described, for the first time, an interesting property of the BFV replicase and its relationship to the host's type I interferon response. Furthermore, we investigated how several mutations in the non-structural proteins affect the aforementioned property. The same mutations were investigated in the aspect of viral RNA replication to describe the effects that these mutations may hold on the replicase activities. The experimental work conducted here further extends on the knowledge we hold on alphaviruses and may contribute to future research on antiviral strategies.

1. LITERATURE REVIEW

1.1. General overview on alphaviruses

The genus *Alphavirus* consists of positive-sense single-stranded RNA genomic viruses which belong to the *Togaviridae* family and the realm *Riboviria*. Currently, over 30 species of alphaviruses have been described (Chen et al., 2018). Alphaviruses are zoonotic pathogens transmitted majorly by mosquitoes to a wide range of vertebrate hosts, mostly mammals and birds but there are also alphaviruses that infect fish (Zaid et al., 2020). There are exceptions, such as Eilat virus (EILV), which can solely infect insect cells (Nasar et al., 2012).

Alphaviruses can be classified as New World and Old World alphaviruses based on their global localization, evolution, pathogenicity and vector to host interaction (Cappuccio & Maisse, 2020). The New World alphaviruses include Venezuelan Equine Encephalitis virus (VEEV), Eastern Equine Encephalitis virus (EEEV) and Western Equine Encephalitis virus (WEEV) are which are found both in North and South America, while the Old World alphaviruses such as Sindbis virus (SINV), Semliki Forest virus (SFV), Barmah Forest virus (BFV), Ross River virus (RRV), Chikungunya virus (CHIKV) and O'nyong-nyong virus (ONNV) are mainly found in Asia, Africa and Australia. Mayaro Virus (MAYV) is an exception in this classification, as it was once classified as an Old World alphavirus belonging to the SFV serocomplex, yet it is found in North and South America (Cappuccio & Maisse, 2020; Garmashova et al., 2007). Thus, due to the fast global spreading of these viruses and their arthropod vectors, this Old and New World classification has become outdated (Cappuccio & Maisse, 2020). A more informative categorization is the serocomplex classification based on alphavirus genetic similarity and their serological reactivity. There are eight serocomplexes with different alphavirus species associated with each complex (Forrester et al., 2012). The SFV complex is presently the largest, and its members (in addition to SFV) are mostly arthritogenic alphaviruses such as CHIKV, ONNV and RRV but also hosts New World alphaviruses like MAYV and Una virus. Another larger complex would be the VEEV serocomplex. In addition to the eponymous species, the WEEV complex also includes a number of representatives and actually one of the most studied Old World alphavirus – SINV (Chen et al., 2018). The EEEV serocomplex is associated with two virus species, these being EEEV and Madariaga virus, mainly found in North and South America (Chen et al., 2018; Silva et al., 2017). The BFV, Middelburg and Ndumu virus complexes presently include

solely their eponymous representatives (Chen et al., 2018). Lastly, there is the insect host specific EILV complex, which in addition to EILV itself, is proposed to include the more recently discovered Taï Forest alphavirus (Hermanns et al., 2017; Nasar et al., 2012). There is still the question of the aquatic alphaviruses Southern Elephant Seal virus (SESV) and Salmon Pancreas Disease virus (SPDV), which yet remain unclassified in any of the established alphavirus serocomplexes (Chen et al., 2018).

Although alphavirus infection in humans rarely results in fatalities, there are clinical manifestations associated with its infection. Alphaviruses can also be divided into two categories based on their pathogenic profiles: arthritogenic alphaviruses (namely Old World alphaviruses) and encephalitic alphaviruses (namely New World alphaviruses) (Cappuccio & Maisse, 2020). Arthritogenic alphavirus infections caused by such agents as CHIKV, ONNV, BFV and RRV can manifest in humans as florid rash, headache, fever, fatigue, polyarthritis, myalgia, and can sometimes lead to chronic illness (Suhrbier et al., 2012). Encephalitic alphaviruses belong mostly to the WEEV, VEEV and EEEV serocomplexes. Upon human infection, the clinical manifestations are debilitating febrile disease and encephalomyelitis. In difficult situations, it can consequently lead to the host's death (Cappuccio & Maisse, 2020). Mosquito vectors as earlier stated, aid in the propagation of alphavirus infection and the fast distribution of these infectious agents could be based on seasonal activity such as temperature and rainfall (Jacups et al., 2008). Different mosquito vectors have been noted to distribute several species of alphavirus across various geographical region (Fig.1) namely; CHIKV is one of the widest spread alphavirus, it is transmitted by *Aedes aegypti and Aedes albopictus* both in the Caribbean , America and other tropical regions (Fig.1) (Burt et al., 2017). ONNV mostly distributed in East Africa, is transmitted by *Anopheles funestus* and *Anopheles gambiae* (Fig.1) (Rezza et al., 2017). The BFV and RRV are transmitted by *Culex annulirostris*, *Aedes vigilax, Aedes notoscriptus* and *Aedes aegypti,* mostly in Australia and surrounding regions (Jacups et al., 2008). For more examples of mosquito vectors and associated alphaviruses, see figure below (Fig.1).

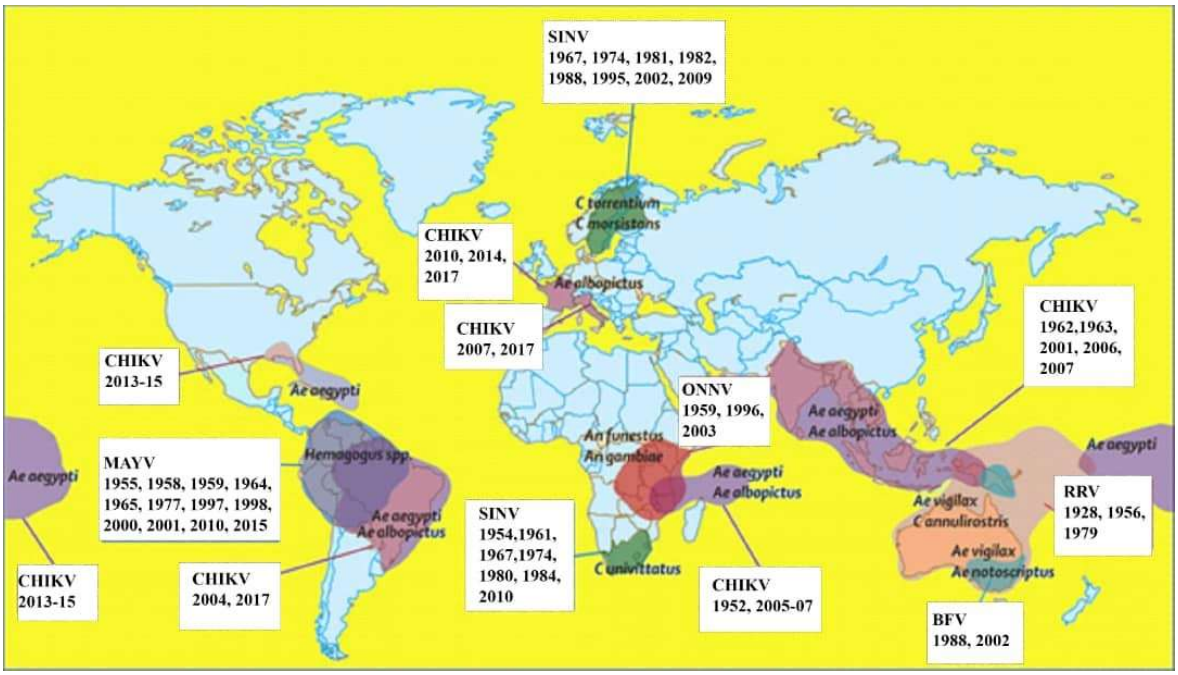

Figure 1.**Notable outbreaks of arthritogenic alphaviruses.** Arthritogenic alphavirus regions of infection, the years of prominent infection and their associated mosquito vectors involved in the transmissions in those regions are indicated. Modified from (Zaid et al., 2020).

1.2. Virion structure

The alphavirus virion has a diameter of approximately 70 nm. It consists of a single copy of the positive-sense RNA genome enclosed in 240 copies of capsid proteins (CP) that form the nucleocapsid,a host plasma membrane derived lipid bilayer, which acts as a bridge between the nucleocapsid and the glycoproteins, and the glycosylated proteins E1 and E2 which are embedded into the bilipid layer to form the viral envelope (Fig. 2b) (Jose et al., 2009; Strauss & Strauss, 1994). There are a total of 240 copies of CP in the mature virion, which form an icosahedron of T=4 symmetry. E1 and E2 form stable heterodimers also of 240 copies which are organized into trimers that form 80 spike-like structures that protrude on the outer surface of the virion (Fig 2.a) (Kuhn, 2013; Voss et al., 2010). E1 and E2 are anchored to the bilipid membrane via their C-terminal domains which include transmembrane helices, and by reaching the other side of the membrane, they also interact in a 1:1 ratio with the CP in the nucleocapsid (Tang et al., 2011)

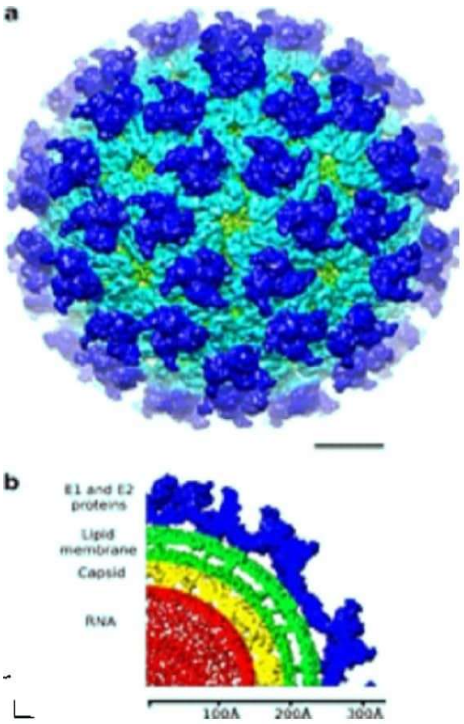

Figure 2. **The structure of the alphavirus virion as exposed by Cryo-EM imaging of the BFV virion**. **(a)** The intact ~70 nm diameter virion is shown with the glycoprotein outer layer still intact, exhibiting the characteristic spikes formed by the E2-E1 heterodimer trimers. **(b)** The cutout section of the virion. The protruding glycoproteins E1 and E2 are shown in in blue. Below that, the host cell derived lipid membrane is shown in green. The capsid proteins are shown in yellow which cover the viral genomic RNA (shown in red) to compose the viral nucleocapsid. Modified from (Kostyuchenko et al. 2011).

1.3. Alphavirus genome

The alphavirus positive-sense RNA genome is approximately 11.7 kb in length. The genomic RNA molecule contains a 5' 7-methylguanosine cap and a 3' polyadenylated (polyA) tail, by which it mimics cellular mRNA molecules as the first synthesis event following its release into the cytoplasm is translation of viral proteins, and secondly, to evade the host's immune response early on (Strauss & Strauss, 1994). The alphavirus genome contains two open reading frames (ORFs) (Fig.3) which divide the genome into two major regions. The 5'-terminal two thirds of the RNA genome encodes for the nonstructural proteins (nsP) via the direct translation of the precursor non-structural polyprotein P1234 or P123, which are subsequently processed into individual nsP-s (nsP1, nsP2, nsP3 and nsP4). The 3' one-third of the genome encodes for the structural precursor polyprotein, which occurs not through direct translation from the genome but is rather translated from the subgenomic RNA (sgRNA). The structural polyprotein also goes through processing to yield the individual

structural proteins C, E2, E3, 6K/TF, E1 and their intermediate cleavage products (Fig.3) (Kuhn, 2013) .

The ORFs are flanked with UTRs: the 5’ UTR found at the 5’ end of the genome, 3’UTR immediately precedes the poly(A) tail, and another UTR lies in between the two ORFs. These UTRs contains 4 cis-elements, also known as conserved sequence elements (CSE), which are essential for viral replication and transcription (Hyde et al., 2015).

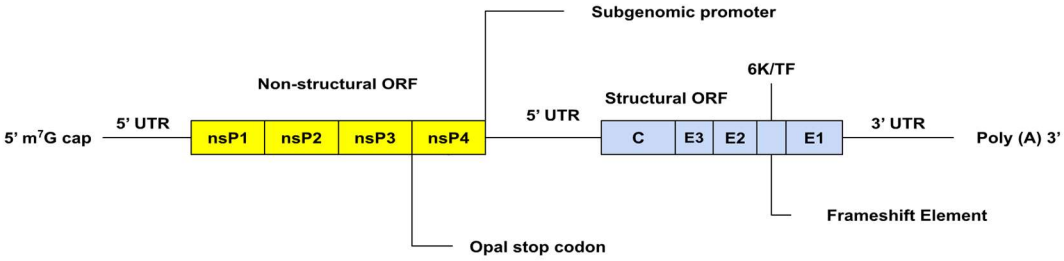

Figure 3. A general overview of the organization of the Alphavirus genome. The Alphavirus genome is a positive-sense RNA molecule that possesses a 5'-m^{7}G Cap-(7-methylguanosine cap) and 3'-Poly(A) (polyadenylated) tail. The two ORFs are flanked by the 5’ UTR, 3’ UTR and a 5’ UTR for the subgenomic region that lies between the two ORFs. The first ORF (indicated in yellow) encodes for the nonstructural polyproteins. The opal stop codon that lies between the junction of nsP3 and nsP4 has been indicated within this ORF. The second ORF encodes for the structural polyproteins. Modified from (Götte et al., 2018).

1.4. Nonstructural Proteins

Alphavirus nsPs are translated first as polyprotein precursors, either as the shorter and more abundant P123 form or as P1234. The reason for such different translation products lies between nsP3 and nsP4, for there is a UGA codon, otherwise known as an opal stop codon, separating the nucleotide sequences for these proteins. This stop codon is described as being “leaky”, as from time to time, readthrough of this opal codon occurs and instead, the longer P1234 non-structural polyprotein is produced (Strauss & Strauss, 1994). Not all isolates of SFV and ONNV possess the UGA codon and instead encode for arginine in that position, resulting in the non-structural polyprotein to be synthesized only as the P1234 form (Strauss et al., 1988; Tuittila et al., 2000). It is proposed that the termination sequence influences the viruses biological properties, as the SFV strain A7(74), which possesses the opal codon, causes more severe clinical symptoms in mice than does the SFV6 strain, which encodes for arginine instead (Saul et al., 2015; Tuittila et al., 2000). The non-structural polyproteins are

subsequently processed to individual nsP-s and their cleavage intermediates (Strauss & Strauss, 1994). The functions and characteristics of individual nsPs are reviewed in this section.

nsP1 is approximately 60 kDa in molecular weight (Kuhn, 2013). It has several functions in alphavirus replication and is an indispensable component of the replication complex or replicase, as are all four of the nsPs (Kuhn, 2013). nsP1 is known to have both methyltransferase and guanylyltransferase activities involved in the capping of the nascent viral genome and sgRNA (Ahola & Kääriäinen, 1995; Laakkonen et al., 1994). It is an important property of alphaviruses since the RNA synthetic events take place in the cytoplasmic compartment, thus alphaviruses need to supply their own mechanism for the addition of the 5' cap (Kuhn, 2013). For alphaviruses, the viral RNA syntheses are membrane-associated processes, where the membrane binding is mediated by nsP1 (Rupp et al., 2015). nsP1 is the only membrane-bound protein of the alphavirus replicase, and post-translational palmitoylation further increases its affinity to bind to the plasma membrane and cytopathic vesicles (CPV) (Spuul et al., 2007)**.** CPVs are modified endosomes and lysosomes, which are major sites of RNA synthesis in the alphavirus infection cycle. The involvement and interaction of nsP3 with nsP1 is important to bind replicases to CPV membranes rather than the plasma membrane (Salonen et al., 2003).

nsP2 is the largest alphavirus nsP with a molecular weight of ~90 kDa and it has various enzymatic and non-enzymatic activities (Kuhn, 2013)**.** The N-terminal domain of nsP2 contains several enzymatic activities that are essential for viral RNA synthesis; namely, it acts as an RNA triphosphatase, a nucleoside triphosphatase, and as an RNA helicase (Rupp et al., 2015). Through its RNA triphosphatase activity, nsP2 is also involved in the capping process of viral genomic and sgRNAs, as it processes newly synthesized molecules and prepares them for capping by nsP1. The RNA helicase activity aids the RNA synthetic processes by unwinding duplex structures during replication and transcription of viral RNAs, and its nucleoside triphosphatase activity is needed to supply energy to the helicase. The C-terminal domain contains the viral papain-like cysteine protease, which cuts nonstructural polyprotein into its intermediate forms and individual non-structural proteins, which can then be utilized in their various functions during the infection cycle. In its C-terminal domain, nsP2 also

contains a nuclear localization signal (NLS) which significance is described in the subsection 1.7. (Jose et al., 2009; Merits et al., 2001).

nsP3 (~60 kDa) is divided into three domains: the Macro domain found in the N-terminal portion of the polyprotein, the central part contains the alphavirus unique domain (AUD), and the C-terminal contains the hypervariable domain (Götte et al., 2018; Jose et al., 2009). The macro domains in CHIKV and VEEV have been reported to possess ADP-ribose and RNA-binding actions (Malet et al., 2009). Furthermore, as an enzyme, nsP3 was later reported to possess ADP-ribosylhydrolase activity, removing ADP-ribose groups from ADP-ribosylated proteins. Inactivating mutations in this active site hindered the replication of CHIKV in mammalian and insect cells (McPherson et al., 2017). There are four conserved cysteine residues in AUD which coordinate the binding of a zinc ion and each of the cysteines were found to be required for virus replication. As a result, this region is also known as the zinc-binding domain (ZBD) (Shin et al., 2012). Mutational studies have further shown that this domain is vital for subgenomic RNA synthesis and binds viral RNA (Gao et al., 2019). The HVD, as the name implies, varies quite significantly in different alphaviruses and for this reason, alphavirus nsP3 sizes can be quite different too: ONNV nsP3 is 570 aa residues and BFV nsP3 469 aa residues in length (Götte et al., 2018). The aforementioned difference in some alphavirus strains in either encoding the leaky opal stop codon or the arginine codon also lies in HVD (Strauss & Strauss, 1994). Mutational studies have shown that this domain also permits some level of insertions and deletions. This feature serves useful, for example, in immunofluorescence experiments as it allows for insertion of fluorescent marker proteins (Götte et al., 2018). HVD also hosts several serine and threonine residues which allow for post-translational phosphorylation of nsP3. In the case of SFV, it has been shown that nsP3 phosphorylation is not essential for viral fitness, yet as for CHIKV, lack of nsP3 phosphorylation resulted in a non-infectious genome (Teppor et al., 2021; Vihinen et al., 2001). nsP3 cellular localization has mostly been characterized as being cytoplasmic, where they either co-localize with other nsPs in the replicase complexes or separately from the replicase as pronounced foci (Panas et al., 2012; Pietilä et al., 2018; Vihinen et al., 2001). The focal nsP3 localization pattern has been linked to interactions with Ras-GAP SH3-domain-binding protein (G3BP) to inhibit the host cells antiviral efforts to produce stress granules (Panas et al., 2012). Recently, it has been shown in our workgroup that nsP3 of BFV, in addition to previously described localization patterns, is also transported into the nuclei

of infected mammalian cells. This work also included the description of an NLS which mapped to the junction of AUD and HVD, and the disruption of nuclear localization resulted in an attenuated phenotype when growth kinetics were observed in mouse embryonic fibroblasts (Omler et al., unpublished data). In the present study, the effects of this mutation are also investigated for using a mutant BFV replicase.

nsP4 (~70 kDa) (Kuhn, 2013) is the alphavirus RNA-dependent RNA polymerase (RdRp) which includes the signature GDD (Gly-Asp-Asp) motif seen in different viral RdRps in its catalytic site (Kamer & Argos, 1984). nsP4 is active as a polymerase only as a member of the replication complex, as nsP4 separation from other nsPs inhibits the expression of RdRp functions (Lemm & Rice, 1993). The C-terminal domain, which holds the RdRp activity, is very conserved and exhibits high homology to other viral RdRps (Kamer & Argos, 1984). The shorter N-terminal domain is specific to alphaviruses (Rupp et al., 2011). The most N-terminal residue is a tyrosine, which renders free nsP4 highly unstable and when the protein lacks interaction with other replicase components, it leads to degradation by the ubiquitin-dependent pathway (de Groot et al., 1991; Jose et al., 2009). nsP4 interacts with the rest of the replicase via the N-terminal domain, and such interactions stabilize the protein (Lemm & Rice, 1993). nsP4 has also been shown to possess adenylyltransferase activity which is essential for viral genome and sgRNA poly(A) tail maintenance (Jose et al., 2009).

1.5. Alphavirus infection cycle

The natural *in vivo* infection by alphavirus begins with a bite of an infected arthropod vector during feeding, and this results in the virus being transmitted from the mosquito's salivary gland into the epidermis and then into the subcutaneous cells resulting in local epidermal inflammation (Lim et al., 2018). The actual infection can initiate when suitable host receptors are expressed that alphaviruses can specifically bind to via their E2 proteins. Alphaviruses utilize different receptors for entry, as no universal receptors have been described for all members of the genus (Holmes et al., 2020). For SINV, divalent metallic ion plasma membrane transporters NRAMP (natural resistance-associated macrophage proteins) have been identified as receptors to gain entry to both mammalian and insect cells (Stiles & Kielian, 2011). Laminin receptors have also been identified as high-affinity receptors for SINV in mammalian cells and for VEEV in insect cells (Ludwig et al., 1996; Wang et al.,

1992). Alphaviruses may supplement their attachment by using attachment factors like heparan sulfate, which acts as a binding receptor for SINV (Smit et al., 2002). C-type lectins also act as attachment factors for SFV, CHIKV and SINV (Holmes et al., 2020). It has also been suggested that CHIKV may use prohibitin as receptors to enter mammalian cells (Wintachai et al., 2012). Several alphaviruses, such as CHIKV, RRV, MAYV, BFV and ONNV, enter host cells using matrix remodeling associated protein 8 (Mxra8) (Fig.4). Yet several other alphaviruses, such as SFV, Una virus and Middelburg virus, did not utilize Mxra8 for entry (Zhang et al., 2018).

Following successful receptor binding, the virion is taken into the cell by clathrin-mediated endocytosis, placing the virion into an endosomal compartment (DeTulleo & Kirchhausen, 1998). The pH level decreases in the endosome, which triggers the restructuring of the E1–E2 dimer to expose the fusion peptide in E1 (Glomb-Reinmund & Kielian, 1998). This process causes fusion of the viral membrane with the endosomal membrane resulting in the release of the nucleocapsid into the cytoplasm and with the aid of ribosomes, the nucleocapsid content is unpacked almost immediately, and the virus genome is released into the cytoplasm (Wengler & Wengler, 2002).

After the release of the genomic RNA, the precursor non-structural polyproteins are translated as either P123 or P1234, the occurrence of the opal codon has been described above (Strauss & Strauss, 1994). Non-structural polyproteins are then proteolytically processed by nsP2, and the first in *cis* cleavage results in the release of nsP4, which is then recruited to form the complex P123+nsP4. That is known as the alphavirus early replication complex, as it uses the genomic RNA as a template to synthesize complementary negative-strand RNA (Merits et al., 2001). nsP1 in *cis* cleavage from P123 follows to yield the nsP1+P23+nsP4 replicative complex, which is known as the intermediate replicase. This replicase complex synthesizes both the positive and negative strand viral RNAs but with more affinity toward genomic RNA production (Lemm et al., 1994). nsP2 and nsP3 are finally separated by the in *trans* cleavage activity of P23 to result in the formation of late replication complexes nsP1+nsP2+nsP3+nsP4 (Strauss & Strauss, 1994). Furthermore, with the accumulation of free nsP2, the processing becomes more rapid to only yield the late replicase and with this, the shift towards positive strand RNA synthesis, where sgRNA synthesis occurs at a higher rate (Shirako & Strauss, 1994).

As previously noted, alphavirus RNA synthesis occurs in association with cellular membranes (Strauss & Strauss, 1994). More precisely, at the plasma membrane and on the membranes of CPVs, alphavirus replication induces the formation of numerous small spherules of roughly 50 nm in diameter, that serve as enclosed sites for RNA production (Peränen et al., 1995). The replication complex is situated at the neck of these structures, while the RNA products remain inside the invaginations. The spherules help hide the replicative dsRNA intermediate and uncapped RNA species from being recognized by the host cell in order to avoid antiviral responses (Spuul et al., 2010).

The sgRNA is translated to produce the structural polyprotein, in which the components lie in the following order: C-PE2(E3+E2)-6K/TF-E1 (Strauss & Strauss, 1994). The capsid protein C is thus the first to be translated, and it cleaves the polyprotein precursor autocatalytically and co-translationally in *cis* to release itself (Nicola et al., 1999). Once C protein is cleaved, it remains in the cytoplasm where it recognizes specific packaging signals from the newly synthesized 5' part of the full-length genomic RNA. Thus, ensuring a full-length genome is packaged into the nucleocapsid (Frolova et al., 1997; Leung et al., 2011). The cleavage of C also exposes the N-terminus of PE2, which includes a signal peptide for the transport of the remaining polyprotein into the endoplasmic reticulum (ER) (Lobigs et al., 1990; Strauss & Strauss, 1994). In the ER, the polyprotein is processed by cellular proteases to first yield PE2 and 6K-E1 and further cleavage results in the formation of PE2-E1 heterodimers (Strauss & Strauss, 1994). The latter is subsequently transported through the Golgi complex, where cellular furin cuts E3 from the dimer and the resulting E2-E1 heterodimers are then transported to the plasma membrane, where they form trimers. The E2-E1 trimers then interact with the nucleocapsid, initiating final virion formation and budding from the plasma membrane to acquire the bilipid membrane (Strauss et al., 1995; Zhao & Garoff, 1992). A graphic overview of the alphavirus infection cycle is given in figure 4.

The small E3 protein is somewhat necessary for the coordinated transport of PE2-E1 dimers to the Golgi complex and is not a vital component of mature virions, as not all alphavirus virions possess these proteins (Strauss & Strauss, 1994). The short proteins 6K and TF (TransFrame) are similar products of structural protein synthesis, as their N-terminal portions remain the same, but a -1 ribosomal frameshift can occur to produce TF with a different

C-terminus instead. This results in the absence of E1 production in the respective polyprotein. Both 6K and TF are included in virions at small quantities, but their biological significance is not clear (Ramsey & Mukhopadhyay, 2017).

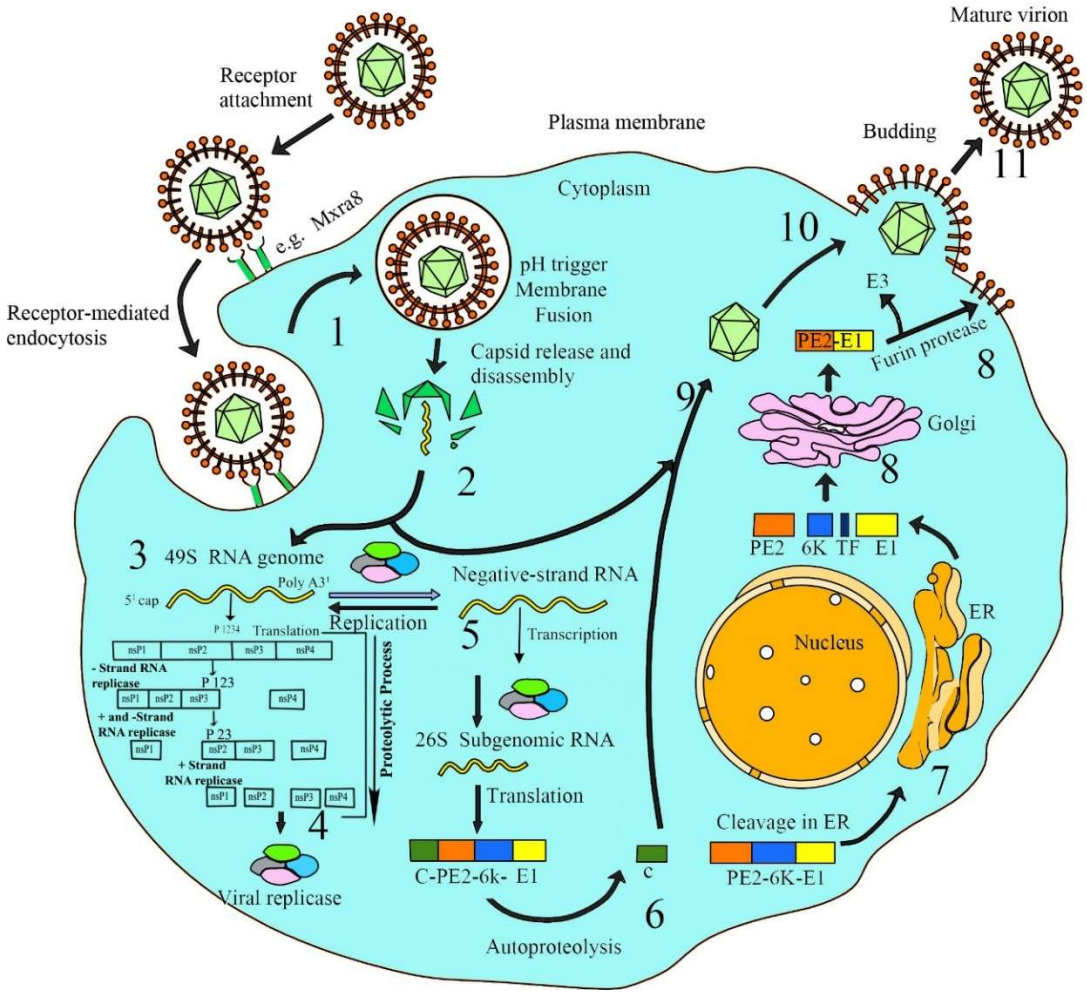

Figure 4. **Overview of the alphavirus infection cycle.** (1) Alphavirus entry through receptor binding and endocytosis. (2) The fusion of the viral and endosomal membranes occurs as the endosomal compartment is acidified. The nucleocapsid disassembly follows and viral genome is allowed to be released into the cytoplasm. (3 and 4) The viral genome is directly translated to yield the nonstructural polyprotein, which is then cleaved into the individual nonstructural proteins and processing intermediates that make up the viral replicase in a sequential manner. (5) The replicase complex is formed, which generates complementary full-length negative-strand RNA intermediates to the viral genome. These serve as precursor for the synthesis of genomic and subgenomic RNAs. The subgenomic RNA translates for the structural proteins. (6 and 9) The C protein is the first structural protein cleaved by autoproteolysis to form the nucleocapsid. (7) The remaining structural proteins are transported to and cleaved at the ER. (8) These structural proteins are moved through the Golgi complex

to the plasma membrane and simultaneously processed to prepare for virion assembly. (10) The mature E2-E1 trimers bound to the plasma membrane interact with the nucleocapsid and initiate the virion assembly and subsequent budding from the plasma membrane. Modified from (Abdelnabi & Delang, 2020).

1.6. Alphavirus Trans-Replicase System

The alphavirus replicase can be expressed as a separate element from the rest of the viral proteins while still retaining its RNA replicative functions. This property has been utilized to design the alphavirus trans-replicase system, where functions of the replicase can be investigated outside of the context of *bona fide* viral infection. In this case, the viral replicase and its template are expressed from different plasmids, and there are various advantages associated with the use of a trans-replicase system. For example, the independence of this system ensures genetic integrity, allowing for a detailed analysis of recombinant replicases, which include mutations such as substitutions, deletions, and insertions that possess a debilitating effect on the viral replicase which may be otherwise lethal to the virus and may revert in the case of infectious virus studies (Utt et al., 2016). example is also seen in the study of the biogenesis of spherules, where it was determined that the length of the replicated RNA determined the size of the formed spherules (Spuul et al., 2010).

Furthermore, such a system can be used to study properties of otherwise high containment level viruses, such as CHIKV, in a regular cell culture setup (Utt et al., 2016). This system can also be used in comparative studies to analyze the effects that certain mutations may exhibit on the efficiencies of RNA replication and transcription. It is important to note that the terms “replication and transcription” refer, in such studies, to producing genomic RNA and sgRNA respectively. In such studies, the recombinant templates are altered to encode for reporter proteins, such as luciferases, allowing for highly sensitive measurement of RNA replication and transcription. It has been established that major portions of the alphavirus genome can be removed and replaced with such marker proteins. Some regulatory elements need to be retained for the effective recognition by the replication complex and these are: the 5’ UTR and some parts that encode for nsP1, the 3’ UTR and the polyA tail, and the subgenomic promoter (Fig. 5). Thus, most of the sequences encoding for nsPs and all of the structural proteins can be replaced by sequences encoding for the expression of appropriate markers, for example, Firefly luciferase (FLuc) and Gaussia luciferase (GLuc), respectively (Fig. 5) (Spuul et al., 2011; Utt et al., 2016). Such templates have been previously prepared

in our research group as DNA constructs, and similarly, plasmid constructs have been made to express viral replicases of several alphaviruses, and such a system has been utilized in the current work as well. The replicase expression constructs include, first of all, the complete sequence for the non-structural polyprotein, the expression of which has been subjected to the control of the cytomegalovirus (CMV) early promoter to allow for RNA replication upon transfection. To conclude the transcription, Simian virus 40 (SV40) termination sequence follows the sequence of the non-structural protein (Fig. 5) (Götte et al., 2020; Lello et al., 2020). The same wildtype (wt) BFV replicase, its RdRp catalytic site mutant (negative control) and a recombinant BFV template construct were used in the current work (Götte et al., 2020).

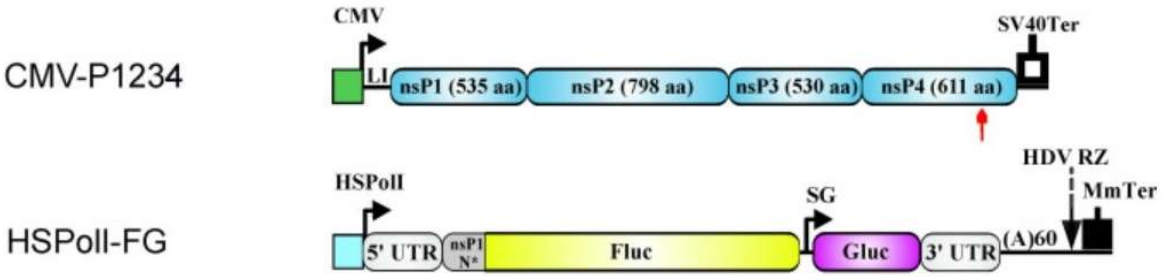

Figure 5. A schematic representation of the components of an alphavirus trans-replication system. CMV-P1234 represents the viral replicase construct. The non-structural proteins are represented in blue. The CMV early promoter, allowing for replicase RNA transcription in transfected mammalian cells is shown in green. The replicase sequence is concluded with the termination signal from SV40 (SV40Ter). HSPol1-FG represents the recombinant viral template construct. The human RNA polymerase I, which allows for the template RNA transcription in transfected cells, is indicated in light blue. This is followed by the 5' UTR of the respective alphavirus studied and a short sequence from nsP1. The remainder of the non-structural polyprotein encoding sequence has been replaced with the sequence encoding for FLuc. The viral subgenomic promoter drives the expression of GLuc, which replaces the viral structural protein ORF. This if followed by the viral 3' UTR and a polyA tail and a further termination element. Taken and shown here with permission from the author (Lello et al., 2020).

1.7. Type I IFN response in alphavirus infection

In the case of many viral infections, including alphavirus infection, the type I IFN response serves as the first line of protection facilitated by the host. The secretory effector molecules of the type I IFN pathway are IFN-α and IFN-β, which upon expression, bind to transmembrane IFN-α and IFN-β receptors (IFNAR) on outer cell surfaces to activate interferon stimulated genes via the Janus kinase signal transducer and activator transcription (JAK/STAT)

pathway to stimulate an antiviral state. Such activation results in the inhibition of viral entry, replication, virion packaging and budding (Fros & Pijlman, 2016).

The pathway is first induced by the recognition of pathogen-associated molecular patterns or PAMPS, which symbolize the molecular signature characteristics of different groups of pathogens. An example of alphavirus PAMPs are dsRNA replicative intermediates, and RNA that lacks the 5' cap (Carpentier & Morrison, 2018). During alphavirus infection, these elements are recognized by such cytoplasmic receptors as RIG-I (retinoic acid-inducible gene1), MDA5 (melanoma differentiation-associated protein 5), TLR3 (toll-like receptor 3) and TLR7 (Nikonov et al., 2013). It has been shown that in the case of alphavirus infection, uncapped RNA is mainly detected by RIG-I and dsRNA mainly by MDA5 (Akhrymuk et al., 2016).

Alphaviruses possess their own means to circumvent the host innate immune response. For the Old World alphavirus, nsP2 has been reported to possess an NLS and free nsP2 is localized also in the nucleus. Once there, nsP2 triggers the degradation of RNA polymerase II catalytic subunit (RPB1), which causes transcriptional shutdown in the host cells, leading to the cytopathic effect in mammalian cells (Akhrymuk et al., 2012; Garmashova et al., 2006). However, in the case of the New World alphaviruses, the C protein form a complex with the nuclear transporters to inhibit cellular transcription by blocking the nuclear pore. Consequently, nuclear trafficking is hindered, causing a reduced mRNA transcription of the host, that also results in translational shutdown (Garmashova et al., 2007). Recent studies done wt SINV 6K and TF proteins, showed that TF protein can also inhibit type 1 IFN responses by palmitoylation mechanism (Rogers et al., 2020).

1.8. On Barmah Forest virus

BFV was first isolated in 1974 from a pool of *Culex annulirostris* mosquitoes captured in the Australian states Victoria and Queensland (Michie et al., 2020). It was described to be an alphavirus sometime later in the 1980's and further genome sequencing and virion structure analyses have cemented this classification (Dalgarno et al., 1984; Kostyuchenko et al., 2011; Lee et al., 1997). For a long time, it has been considered an Australian alphavirus, but a more recent discovery has indicated an isolated case in Papua New Guinea as well (Caly et al., 2019; Jacups et al., 2008).

In 1988, BFV infection was linked to human illness for the first time (Michie et al., 2020). The clinical manifestations describing BFV caused disease in humans include febrile illness, myalgia, arthralgia (sometimes chronic arthritis) and severe rash. These symptoms are similar to disease caused by RRV, another alphavirus widely spread in Australia and these similarities are thought to cause some misdiagnosis of causative agents (Flexman et al., 1998). Over the past 10 years, over 900 cases of BFV infection have been reported in Australia, and thus remains a cause of concern for public health (http://www9.health.gov.au/cda/source/cdaindex.cfm, Australian Department of Health, Notifiable Diseases Surveillance System, Australia, 2021).

Several mosquito species have been described as vectors for BFV, including *Culex annulirostris*, *Aedes vigilax, Aedes normanensis, Aedes notoscriptus* and *Verrallina funereal* (Caly et al., 2019; Jacups et al., 2008).

2. EXPERIMENTAL WORK

2.1. Aims of the Thesis

The aim of this thesis was to assess the ability of the BFV replicase in inducing the type I IFN response in a human cell line in the absence of a viral template. We also investigated whether several mutations in BFV nsP-s influence this property. Furthermore, we looked into the effect that these mutations may possess on the replication and transcription efficiencies of the BFV replicase using the trans-complementation system. The mutations in question are in nsP2 (T1325P), nsP3 (K1651D) and nsP4 (V1911D), where in brackets the amino acid positions and relevant substitutions are indicated with respect to the full-length nonstructural protein sequence. The respective BFV mutant replicases were designated as BFV^{T1325P}-Rep, BFV^{K1651D}-Rep and $BFV^{T1325P+V1911D}$-Rep, where the latter possesses mutations both in nsP2 and nsP4.

The objectives of the thesis were as follows:

1. the cloning of mutant BFV replicases' DNA constructs harbouring the mutations in nsP2, nsP3 and nsP2+nsP4 as described above;

2. to assess whether BFV replicase activates the type I IFN response in HEK293T cell line in the absence of a viral template;

3. to investigate if the aforementioned mutations influence type I IFN induction in HEK 293T cells in the absence of a viral template;

4. to assess how the aforementioned mutations influence BFV replication and transcription efficiencies in HEK 293T cells.

2.2. Materials and methods

2.2.1 Cloning of the mutant BFV replicase DNA constructs

The materials taken as basis for mutant BFV replicases were the previously constructed BFV wt replicase DNA clone based on the pMC-GTU vector backbone containing a selective marker for Kanamycin (Km) resistance (BFV-Rep) (Lello et al. 2020), which was used for vector acquisition, and mutant BFV infectious clone DNA constructs (based on a pUC57 vector), which were used for insert fragments acquisition (Omler et al. unpublished data). These DNA clones were all previously constructed by my colleagues in our research group. Three different BFV infectious clones were used, which included mutations either in the nsP2 coding region (T1325P), nsP3 (K1651D) or the nsP2 and nsP4 coding regions (T1325P+V1911D).

For the vector DNA preparation, the BFV-Rep construct was cleaved using FastDigest restriction enzymes AdeI and NheI (Thermo Scientific™) according to the manufacturer's protocol. The reaction mixture was incubated at 37 °C for 30 min after which the alkaline phosphatase FastAP (Thermo Scientific™) was added into the reaction mixture, which was then incubated for a further 30 min. The restriction mixture was then column purified (Zymo Research DNA Clean & Concentrator™ Kit) and checked for correct restriction fragment sizes by agarose gel electrophoresis using 0.8 % TAE agarose gel. For the insert DNA preparation, the above mentioned mutations containing BFV infectious clone DNA-s were cleaved using the same restriction enzymes and also incubated for 1 h at 37 °C. Following the incubation, the restriction fragments were separated by agarose gel electrophoresis on a 0.8% TAE agarose gel and the correct bands were excised. The gel fragments were column purified using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research). The DNA ligation with the acquired vector and insert DNA-s was performed with the T4 DNA Ligase and the supplied 5x T4 DNA Ligase Buffer (Invitrogen™) in a final volume of 20 ul and the ligation mixtures were incubated overnight at 12 °C.

The ligation mixtures were used to transform competent E. coli XL-10 strain cells by the heat shock method. For this, the ligation mixtures were added to 200 µl of competent cell aliquots and incubated for 30 min on ice. This was followed by a 1.5 min long heat shock at 42 °C after which the samples were again incubated on ice for 3 min. After the incubation, 800 µl of LB (Lysogen Broth) medium was added to each sample which were then incubated at 37 °C for 1 h. The transformation samples were then centrifuged at 4000 x g for 5 min to pellet the cells. After discarding most of the supernatant, the pellet was resuspended in the leftover ~50 ul media and plated onto LB agar and Km containing Petri dishes and incubated at 37 °C for 16 h. The formed bacterial colonies were subsequently selected for propagation in 3 ml of LB medium, which was supplemented with Km in a final concentration of 25 µg/ml, and the samples were incubated in a shaking incubator at 37 °C at 220 rpm for 16 h. The plasmid DNA was then purified using a commercial miniprep kit (FavorPrep™ Plasmid Extraction Mini Kit, Favorgen Biotech Corp.). The plasmids were then confirmed for integrity and quality by restriction fragment analysis by gel electrophoresis on a 0.8% TAE agarose gel, the concentrations were measured using the Nanodrop 2000c spectrophotometer and sequences confirmed by Sanger sequencing. From each pool of mutation harbouring samples, single samples were selected for amplification in larger quantities of 50 ml in SOY medium which was supplemented with Km in a final concentration of 25 ul/mg. These samples were then purified using the NucleoBond® Xtra Midi EF kit (Macherey-Nagel) for high quality and concentration endotoxin free DNA sample acquisition. The purified DNA samples were measured for concentrations using the Nanodrop 2000c spectrophotometer (Thermo Scientific™) and confirmed by Sanger sequencing.

2.2.2. Cell lines and cell culturing

The used cell lines and their culturing conditions are listed below:

1. **HEK293T cells** (human embryonic kidney cell line) were cultured in DMEM (Dulbecco's Modified Eagle's Medium, Gibco®) supplemented with 10% fetal bovine serum (FBS, Pan Biotech™). For transfection experiments on 24-well cell culture plates, on the previous day, $4x10^5$ cells were seeded into each well.
2. **Cop5 cells** (murine fibroblast cell line) were cultured in IMDM (Iscove's Modification of DMEM, Corning®) with supplemented with 10% FBS (Pan Biotech™). For

transfection experiments on 24-well cell culture plates, on the previous day, $2x10^5$ cells were seeded into each well.

Both cell lines were incubated for either propagation or experimental procedures at 37 °C with an atmospheric CO_2 concentration of 5% and at high humidity conditions.

2.2.3. DNA transfection into HEK293T cells

DNA transfections were performed with ~90% confluent HEK293T cell monolayers in 24-well plates. DNA transfections into HEK293T cells were done using Lipofectamine LTX® with PLUS™ reagent (Invitrogen™). For the type I IFN induction experiments, 1 µg of DNA plasmids encoding for BFV-Rep (wild type replicase), BFV-Rep-GAA (inactive replicase negative control), BFV^{T1325P}-Rep, BFV^{K1651D}-Rep, $BFV^{T1325P+V1911D}$-Rep and SFV-Rep were used. For the trans-complementation experiments, 0.5 µg of DNA plasmids encoding for the aforementioned BFV replicases and 0.5 µg of DNA plasmids encoding for the recombinant BFV template expressing FLuc and GLuc (HSPol1-FG-BFV) were used, bringing the total DNA amounts to 1 µg per transfection. Reagent volumes for transfection reaction mixtures were based on previous optimisation experiments by my colleagues and used in same amounts for both sets of experiments: 3.5 µl of LTX reagent and 1.8 ul of PLUS reagent was used per 1 µg of DNA plasmids. In one tube, DNA and PLUS reagent mixtures were prepared in Opti-MEM (Opti-MEM™ Reduced Serum Medium 1x, Gibco™) and in another tube, Opti-MEM and LTX reagent mixtures were prepared in Opti-MEM. The two mixtures were then combined, bringing the total volume per transfection mixtures to 100 µl and these were incubated at room temperature for 10 min. During the incubation period, culturing growth medium was aspirated from cell monolayers and substituted with 400 ul of fresh DMEM growth medium. Following the 10 min incubation, 100 ul of reaction mixtures were added to each well on the 24-well plates.

In the case of the type I IFN induction experiments, the transfected cells were incubated for 48 h post transfection before lysis and further total RNA purification. For the trans-complementation experiments, the transfected cells were incubated for 18 h post transfection before lysis and subsequent luciferase assays.

2.2.4. RNA purification, RNA transfection into Cop5 cells and measuring IFN-β levels by ELISA assay

Total RNA samples were column purified from HEK293T cells transfected with BFV-Rep, BFV-Rep-GAA, BFV^{T1325P}-Rep, BFV^{K1651D}-Rep, BFV$^{T1325P+V1911D}$-Rep and SFV-Rep using a commercial RNA purification kit (Quick-RNA^{TM} MiniPrep Kit, Zymo Research) according to the manufacturer's protocol. The RNA samples were checked for integrity by agarose gel-electrophoresis on a 1% TAE agarose gel and the RNA concentrations were determined with the Nanodrop 2000c spectrophotometer (Thermo Scientific).

The Lipofectamine^{TM} 2000 transfection reagent (Invitrogen^{TM}) was used to transfect RNA into ~90% confluent Cop5 cells on 24-well cell culture plates, where 5 µl of Lipofectamine^{TM} 2000 reagent was used to transfect 5µg of RNA. For a single transfection the mixtures were prepared as follows: in one tube, 5µg of RNA sample was diluted in Opti-MEM to bring the mixture volume to 50 ul, and in another tube, 5 ul of Lipofectamine^{TM} 2000 reagent was diluted in 45 µl Opti-MEM. The dilutions were then mixed together (bringing the total volume to 100 µl) and incubated at room temperature for 10 min. Growth media was then aspirated from the cells and replaced with 400 µl of fresh IMDM growth medium into each well. Following the incubation, 100 ul of transfection mixture was added to each well and the cells were placed into the incubator for 4-5 h, after which the media was again replaced.

The total incubation time was 48 h, after which the media was collected to be assayed for IFN-β concentrations. The sample IFN-β levels were measured using VeriKine Mouse Interferon Beta ELISA Kit (PBL Assay Science) according to the manufacturer's protocol.

2.2.5. Trans-complementation assay

The DNA transfections performed for this assay are described in the subsection 2.2.3.. The luciferase assay was done using the Dual Luciferase® Reporter Assay System Kit (Promega) and a description on the used kit reagent volumes follow. The transfected HEK293T cells in 24-well plates were lysed in 100 ul of Passive Lysis Buffer and incubated for 15 min at room temperature on a tabletop shaker set to 500 rpm. Tubes for each sample were prepared with 20 µl Luciferase Assay Substrate dilutions and a reaction to detect FLuc signals was initiated by the addition of 4 µl of sample lysate. Very briefly, the tube was mixed by vortexing and luciferase signal was immediately measured using the GloMax® 20/20 luminometer

(Promega). This was followed by the addition of 20 µl of Stop & Glo® reagent dilution to the same sample which was then briefly vortexed, and measured for GLuc signals by luminometry.

2.2.6. Data analysis and visualisation

Statistical analysis and data visualisation was performed using the GraphPad Prism 9 software. The type I IFN induction and trans-complementation assay data were analysed for statistical significance by unpaired Student's T-test.

2.3. Results and discussion

2.3.1. Cloning of mutant BFV replicases

The mutant BFV replicases were based on a previously constructed wt BFV replicase expression plasmid made in our work group, pMC_GTU_BFV _Repl, which will be referred to in the current work as BFV-Rep. This construct was used to produce the vector DNA in the cloning process. To acquire the insertion fragments with the mutation harbouring sequences, previously constructed BFV infectious DNA clones were used. Namely, they were three BFV infectious clones with mutations in nsP2, nsP3 and nsP2+nsP4 encoding sequences. The mutation in nsP2 (T1325P) lies near the end of the protein's sequence and includes a point mutation that changes the threonine in position 1325 to be encoded for proline. The mutation in nsP3 (K1651D) results in the expression of aspartic acid instead of lysine in position 1651. This mutation lies in the NLS of nsP3 and disrupts the nuclear localization of the protein (Omler et al., unpublished data). The nsP2+nsP4 (T1325P+V1911D) infectious clone includes firstly the aforementioned mutation in nsP2 (T1325P) and a second mutation in nsP4, which allows for the expression of aspartic acid instead of valine in position 1911. The amino acid positions for the mutation sites are described with respect to the full-length BFV non-structural polyprotein sequence.

The cloning was conducted according to a single ligation step protocol. The unique restriction sites for the enzymes NheI and AdeI in the BFV genome DNA complement allowed for the cleavage of a 5931 bp DNA fragment (that spans for most of the sequence encoding for the non-structural polyprotein) from the BFV-Rep to be used directly as a vector, and the infectious clone DNAs, to be directly used as inserts. The cloning products were confirmed

by Sanger sequencing for the complete non-structural polyprotein sequence. The resulting mutant BFV replicase DNA constructs including the aforementioned mutations were designated as follows: BFV^{T1325P}-Rep, BFV^{K1651D}-Rep and BFV$^{T1325P+V1911D}$-Rep. A schematic overview of the replicase constructs is given in figure 6.

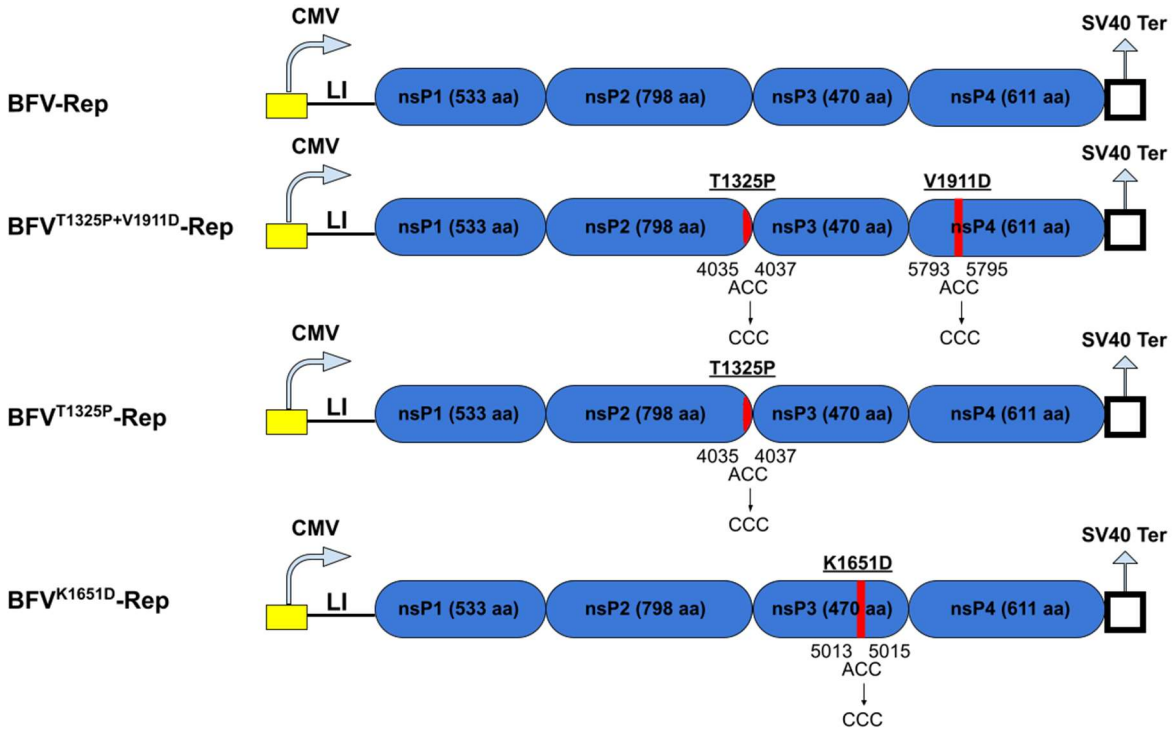

Figure 6. Schematic representation of wt BFV replicase and the constructed mutant replicases. The BFV nsPs are represented in blue with their sizes in amino acid residues indicated within each protein. The positions of the mutations have been indicated in red with their respective positions in the BFV non-structural polyprotein indicated underlined above the schematics. Below the mutation positions are indicators of the respective changes in the DNA codons allowing for the amino acid changes where the numbers are marked with respect to the full length BFV genome. CMV (with arrow) – cytomegalovirus early promoter; LI – herpes simplex virus 1 leader sequence; SV40 Ter – Simian virus 40 RNA transcription termination signal.

2.3.2. Replication and transcription of recombinant viral RNAs by BFV^{T1325P}-Rep, BFV^{K1651D}-Rep and BFV$^{T1325P+V1911D}$-Rep

Using the trans-complementation system, we looked into the effect that the aforementioned mutation may exhibit on the properties of BFV in viral RNA replication and transcription. We co-transfected HEK293T cells with BFV-Rep, its RdRp inactive catalytic site mutant BFV-Rep-GAA (serving as the negative replicase control), BFV^{T1325P}-Rep, BFV^{K1651D}-Rep

and BFV$^{T1325P+V1911D}$-Rep with a recombinant BFV viral template expressing for FLuc and GLuc (HSPol1-BFV-FG). In previous studies, the wt BFV replicase (BFV-Rep) and HSPol1-BFV-FG template have been effectively used to describe these properties in various scenarios (Götte et al., 2020; Lello et al., 2020). Thus, BFV-Rep was also used as an experimental positive control. The co-transfected cells were lysed 18 h post transfection and measured for the levels of FLuc and GLuc expression which, as described above, indicate efficiencies of viral RNA replication and transcription, respectively. The measured values for luciferase activity are returned by the luminometer as relative light units or RLUs. The data for FLuc and GLuc signal intensities are presented here as fold changes or ratios, where the signal intensities for BFV-Rep-GAA are taken as the baseline points (mean values of BFV-Rep-GAA transfected cells' FLuc and GLuc measurements are taken as 1), and the values for the assayed replicases are compared to the baseline values and shown as how many times the signal is higher than it is for the negative control (BFV-Rep-GAA).

It was observed that viral replication was very much boosted in the case of BFV^{T1325P}-Rep when compared to BFV-Rep (Fig. 7). To a lesser yet significant extent, the mutations in nsP2 and nsP4 together also boosted the replication efficiency when compared to the wt replicase (Fig. 7). Interestingly, the disruption of the NLS causes much lower levels of viral RNA replication, as BFV^{K1651D}-Rep FLuc signals were significantly lower than those of BFV-rep (Fig. 7).

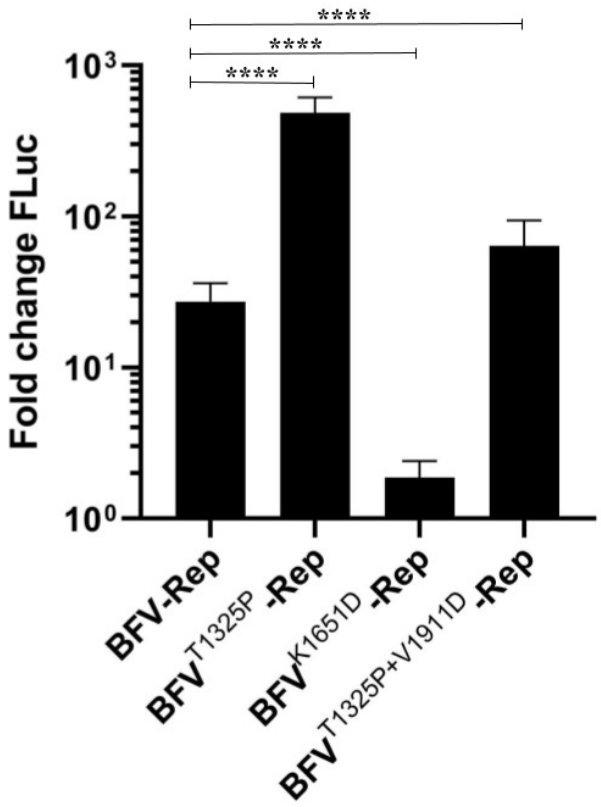

Figure 7. Viral RNA replication by BFV-Rep and mutant replicases. HEK293T cells were transfected with BFV-Rep, BFV-Rep-GAA, BFV^{T1325P}-Rep, BFV^{K1651D}-Rep and BFV$^{T1325P+V1911D}$-Rep, and analyzed for FLuc levels at 18 h post transfection. Each column represents 9 data points from 3 independent experiments. p-values are indicated as follows: p<0.05*, p<0.01**, p<0.001***, p<0.0001****.

Similarly, for viral RNA transcription, the lone mutation in nsP2 resulted in higher levels of GLuc when compared to the wt replicase (Fig. 8). Another similarity was observed for BFV^{K1651D}-Rep, as the transcription efficiency was also significantly lower than it was for BFV-Rep (Fig. 8). The joint effect of the mutations in BFV$^{T1325P+V1911D}$-Rep apparently does not affect viral RNA transcription, at least in the case of BFV (Fig 8).

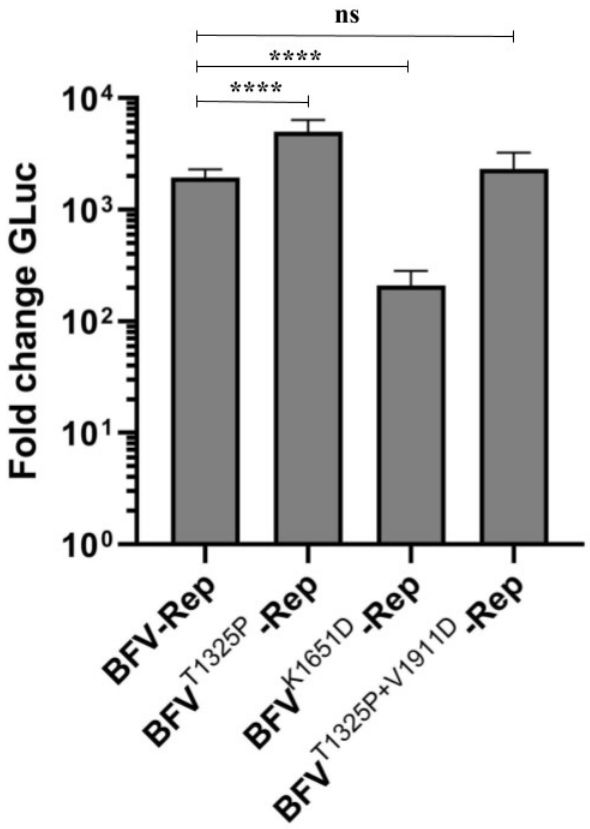

Figure 8. Viral RNA transcription by BFV-Rep and mutant replicases. HEK293T cells were transfected with BFV-Rep, BFV-Rep-GAA, BFV^{T1325P}-Rep, BFV^{K1651D}-Rep and BFV$^{T1325P+V1911D}$-Rep, and analyzed for GLuc levels at 18 h post transfection. Each column represents 9 data points from 3 independent experiments. p-values are indicated as follows: p<0.05*, p<0.01**, p<0.001***, p<0.0001****.

Previous results from our research group show that the mutation in nsP3 (K1651D), which disrupts the NLS, results in lower propagation rates in the case of a viral infection in earlier timepoints in murine cells but not in Vero cells (African green monkey kidney epithelium cells) (Omler et al., unpublished data). The data here somewhat supports the observation, as by the 18 h timepoint, both the replication and transcription rates of viral RNAs were much lower for BFV^{K1651D}-Rep when compared to BFV-Rep. This effect may be host cell type specific, as the current experiment was conducted in a human cell line, but differences were previously observed in murine and non-human primate cells. This property should be investigated further in future studies.

The observed mutation in nsP2 (T1325P) lies in the vicinity of the cleavage site that separates nsP2 and nsP3 during the polyprotein processing. There is no current evidence on it, but the mutation under scrutiny here could affect the polyprotein processing and this may result in the higher synthesis rates of the viral RNAs, as has been shown before for mutations that slow down the processing of this cleavage site (Bartholomeeusen et al., 2018).

2.3.2. Induction of the type I IFN response facilitated by BFV-Rep, BFV^{T1325P}-Rep, BFV^{K1651D}-Rep and BFV$^{T1325P+V1911D}$-Rep

It is very common that alphavirus infection results in the activation type I IFN response in many cell types competent to express such a system. However, it has also been shown by utilizing only the replicase element of the trans-replication system, that SFV replicase can induce high levels of IFB-β in the absence of any viral templates when expressed in Cop5 cells. The same study showed that the replicase was using cellular RNAs in the cytoplasm as templates to produce RNA species that serve as PAMPs to activate interferon production (Nikonov et al., 2013). As a part of this thesis, we sought to determine whether BFV replicase also exhibits such an activity in the absence of viral RNA templates. Firstly, we transfected HEK293T cells with BFV-Rep, BFV-Rep-GAA (serving as the replicase negative control) and SFV-Rep, with the latter serving as an experimental positive control. Then, we purified the total RNA fraction from the transfected cells. We used these RNA samples for another round of transfections into Cop5 cells to analyze for expressed IFN-β levels in these murine cells. We did not measure for IFN levels from transfected HEK293T cells directly, as they lack an intact type I IFN system and it has been shown that purified RNA from SFV replicase transfected cells is also sufficient to activate type I IFN response (Hornung et al., 2002; Nikonov et al., 2013). Furthermore, the BFV replicase exhibits very similar RNA replicative activity in HEK293T cells to SFV replicase, but yet unpublished data from our workgroup showed that such high similarities were not observed for Cop5 cells (Lello et al., 2020).

We measured IFN-β levels from the transfected Cop5 cells and observed elevated levels of IFN-β from BFV-Rep transfected cells when compared to the negative control. The signals for IFN-β were nowhere near the levels detected for SFV-Rep (Fig. 9b), yet they were roughly twice that were measured for BFV-Rep-GAA, showing statistically significant difference (Fig. 9a).

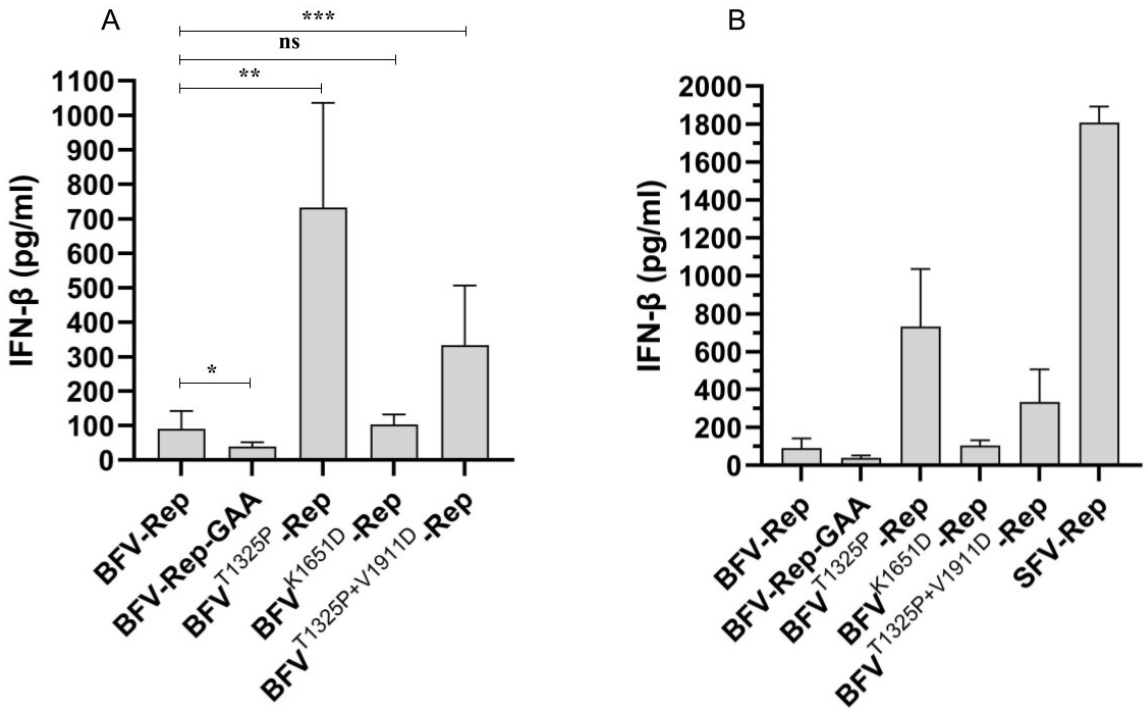

Figure 9. IFN-β induction in wt BFV replicase and mutant BFV replicases' transfected Cop5 cells. (A) Cop5 cells were transfected with RNA extracts from transfected HEK293T cells and after 48h, the supernatant was analyzed by ELISA assay for IFN-β levels which are presented by concentration (pg/ml). Statistical significance is presented in p-values as follows: ns (not significant); $p<0.05$*; $p<0.01$**; $p<0.001$***. **(B)** This figure acts as a supplement to (A) to show the levels detected in SFV-Rep (experimental positive control) transfected cells, otherwise, the data remains the same. Each column represents the means of 7 datapoints from three independent experiments with the exception of BFV-Rep-GAA, where a single data point was omitted as it was contaminated by a BFV-Rep sample.

We then analyzed BFV^{T1325P}-Rep, BFV^{K1651D}-Rep and BFV$^{T1325P+V1911D}$-Rep for such a property as well. Firstly, we saw that the lack of nuclear localization for nsP3 did not influence this property, as the IFN-β levels were very comparable between BFV^{K1651D} and BFV-Rep (Fig. 9a). On the other hand, the T1325P mutation in nsP2 boosted the IFN-β levels quite significantly and higher levels were also observed for BFV$^{T1325P+V1911D}$-Rep, when compared to BFV-Rep (Fig. 9a).

The ability of the wt BFV replicase to induce the type I IFN response in the absence of viral RNA templates has not been reported before. According to unpublished studies conducted

in our research group, this is also not a universal attribute of alphavirus replicases, as preliminary data shows that only some alphaviruses possess such a property. Thus, the exact significance of this occurrence is still to be determined. As discussed above, the T1325P mutation may influence the processing of the cleavage site between nsP2 and nsP3, and the slowdown of which has previously been shown to induce higher levels of type I IFN induction upon viral infection. It was proposed to be the effect of lower amounts of free nsP2 in the infected cell, which acts antagonistically towards host transcriptional events (Gorchakov et al., 2008). This may also be the case here, but further research is needed to conclude how this mutation influences the expression of viral proteins. It may also be that BFV^{T1325P}-Rep shows a higher affinity towards utilizing any suitable RNA templates, including those serving as cellular RNAs to produce PAMPs, as BFV^{T1325P}-Rep replicated recombinant viral RNA templates at a higher rate. BFV$^{T1325P+V1911D}$-Rep is more modest in these activities when compared to BFV^{T1325P}-Rep, yet retaining higher viral RNA replication rates than BFV-Rep. The V1911D mutation alone can not be introduced into the BFV genome, as such a virus is not rescuable. Understandably, it would be interesting to know how this mutation influences viral replication and transcription, which could be studied using the trans-replication system, but such an expression construct was not cloned for the thesis at hand due to time constraints. Let it remain as a subject for future research.

SUMMARY

BFV is an alphavirus of clinical importance found mostly in Australia. Humans serve as hosts for BFV and can experience symptoms such as arthralgia, myalgia, excessive rash and fever. These infections may be asymptomatic, but in some cases, result in chronic illness that can last up to months. For the past 10 years, there have been nearly 1000 confirmed cases annually for BFV related illness.

BFV, like other alphaviruses, activates the type I IFN response component of the innate immune system upon infection in several cell types. And as a result, the virus too has to cope with this activated antiviral response, and several strategies to circumvent the effects of such an effective immune response are utilized by the virus. It has been previously shown that the SFV replicase activates the type I IFN response in the absence of viral RNA templates and it has been proposed that this is a strategy of the host cells in amplifying PAMP production in infected cells for enhanced detection of viral infections. This has also been established not to be a universal property of alphavirus replicases, and in the current thesis, we sought to investigate if this was the case for BFV replicase as well.

To investigate this, we transfected mammalian cells with BFV-Rep and observed that, when compared to the replicase negative control, the wt replicase indeed induced the expression of higher levels of IFN-β in the absence of viral RNA templates. This is the first time such an attribute has been described for BFV. As part of this thesis, we also constructed mutant BFV replicases harbouring mutations in nsP2, nsP3 and a double mutant with mutations in nsP2 and nsP4 to study the effects of these mutations on the aforementioned property and the effects these mutations hold over viral RNA replication and transcription. BFV^{K1651D}-Rep retained similar IFN-β levels in transfected cells, indicating that the disruption of the previously described NLS does not influence this property of the replicase. T1325P mutation on the other hand (present both in BFV^{T1235P}-Rep and $BFV^{T13225P+V1911D}$-Rep) boosted the induction of IFN-β significantly, when compared to BFV-Rep. Viral RNA replication efficiency was also boosted for BFV^{T1235P}-Rep and $BFV^{T1325P+V1911D}$-Rep and viral transcription rates for BFV^{T1325P}-Rep when compared to BFV-Rep. We concluded that the T1325P mutation could affect the replicase's affinity to utilize RNA templates of any origin, as it also induced much higher levels of IFN-β in the absence of viral templates. Yet the exact analysis of this shall remain as topics for further research.

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