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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

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A B S T R A C T

Coxsackieviruses B (CV-B) (*Picornaviridae*) are a common infectious cause of acute myocarditis in children and young adults, a disease, which is a precursor to 10–20% of chronic myocarditis and dilated cardiomyopathy (DCM) cases. The mechanisms involved in the disease progression from acute to chronic myocarditis phase and toward the DCM clinical stage are not fully understood but are influenced by both viral and host factors. Subgenomic replicons of CV-B can be used to assess viral replication mechanisms in human cardiac cells and evaluate the effects of potential antiviral drugs on viral replication activities. Our objectives were to generate a reporter replicon from a cardiotropic prototype CV-B3/28 strain and to characterize its replication properties into human cardiac primary cells. To obtain this replicon, a cDNA plasmid containing the full CV-B3/28 genome flanked by a hammerhead ribozyme sequence and an MluI restriction site was generated and used as a platform for the insertion of sequences encoding emerald green fluorescent protein (EmGFP) in place of those encoding VP3. *In vitro* transcribed RNA from this plasmid was transfected into HeLa cells and human primary cardiac cells and was able to produce EmGFP and VP1-containing polypeptides. Moreover, non-structural protein biological activity was assessed by the specific cleavage of eIF4G1 by viral $2A^{pro}$. Viral RNA replication was indirectly demonstrated by inhibition assays, fluoxetine was added to cell culture and prevented the EmGFP synthesis. Our results indicated that the EmGFP CV-B3 replicon was able to replicate and translate as well as the CV-B3/28 prototype strain. Our EmGFP CV-B3 replicon will be a valuable tool to readily investigate CV-B3 replication activities in human target cell models.

1. Introduction

Enteroviruses (*Picornaviridae* family) are ubiquitous and common human pathogens responsible for a wide range of clinical manifestations ranging from subclinical to severe or fatal acute infections in immunocompetent adults or children. Among group B Enteroviruses, coxsackieviruses type B (CV-B), and mainly CV-B3, are considered to be a common cause of acute myocarditis in children and young adults, a disease, which is a precursor to 10–20% of chronic myocarditis cases as well as dilated cardiomyopathy (prevalence = 7 cases/100,000 s leading cause of heart transplantation worldwide) (Nguyen et al., 2013; Rakar et al., 1997). The mechanisms involved in the disease progression from acute to chronic myocarditis phase and toward the DCM clinical stage are not fully understood but are influenced by both viral and host factors (Chase and Semler, 2012; Sin et al., 2015; Ventéo et al., 2010).

CV-Bs are small, naked, single-stranded positive RNA viruses of about 7.5 kb. Their genome is flanked on the 5′ end by a noncoding region (5′ NCR), which is crucial for the initiation of replication and translation of the viral genome (Sharma et al., 2009). The genome is covalently linked to the virus-encoded peptide VPg (3B) at its 5′ terminus and is polyadenylated at its 3′ terminus. Naked viral RNA is sufficient to initiate replication when introduced into the cytoplasm since it can act directly as a messenger RNA (mRNA)

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(Racaniello, 2007). The genome encodes a single open reading frame (ORF) of approx. 2200 amino-acids. The polyprotein synthesized subsequently to virus entry and uncoating is processed during and following translation by viral proteinases ($2A^{pro}$, $3C^{pro}$ and $3CD^{pro}$) into precursor polypeptides: P1 that will constitute capsid proteins (VP1-4); P2 that will be cleaved to produce $2A^{pro}$ as well as 2BC and 2C, proteins involved in cytoplasmic membrane reorganization and genomic replication; and P3, which is cleaved into proteins involved in protein processing and viral RNA replication (*e.g.*, $3C^{pro}$, $3CD^{pro}$, VPg, and $3D^{pol}$). In enterovirus-infected cells, translation of 5′ capped cellular mRNAs is shut-off due to $2A^{pro}$ cleavage of the eIF4G component of the eIF4F cap-binding protein complex (Hsu et al., 2009). CV-B translation is, however, initiated using an internal ribosome entry site (IRES) in the 5′ NCR in a cap-independent manner (Fernández-Miragall et al., 2009). For picornaviruses, a *cis*-acting replication element (cre) is present in genomic RNAs and is necessary for genome replication. In CV-B3 RNA, the cre has been mapped to a region within the 2C coding region (van Ooij et al., 2006).

Replicons are genomic replication competent elements that cannot produce infectious particles (Kaplan and Racaniello, 1988). The co-transfection of 'helper' viruses or plasmids providing capsid proteins *in trans*- can be used to restore this ability. CV-B replicon systems provide a unique opportunity of studying wild-type or genetically modified CV-B3 replication in the absence of infectious virus. In addition, they can facilitate the analysis of translation and RNA replication of circulating strains of CV-B under BSL2 laboratory conditions.

A sub-genomic replicon of the cardiotropic CV-B3/28 strain could be used to study viral replication mechanisms and evaluate the effects of antiviral drugs on viral replication activities in cardiac cells. To obtain this replicon, a cDNA plasmid containing the full CV-B3/28 genome flanked by a T7 promoter followed by a hammerhead ribozyme sequence and an MluI restriction site was used for the insertion of EmGFP in place of the VP3 gene. This paper describes the construction and the characterization of this new EmGFP CV-B3 replicon encoding the full repertoire of non-structural proteins to provide an easy method of studying replication, based upon live-cell imaging together with quantitation of fluorescence in human target cell models.

2. Material and methods

2.1. Cell culture

HeLa cells were grown in Eagle's minimal essential medium (MEM, Life Technologies) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% penicillin/streptomycin. A549 cells were grown in DMEM medium and supplemented with same amounts of complements. Cells were passaged using trypsin/EDTA twice a week. Human primary cardiomyocytes (HCM, Promocell GmbH) were grown in myocytes growth medium containing 5% Fetal Calf Serum (FCS), 0.5 ng/ml Epidermal Growth Factor, 2 ng/ml Basic Fibroblast Growth Factor and 5 μg/ml of Insulin. Cells were passaged using trypsin/EDTA once a week, and medium was changed every 2 days. Both cell types were grown as monolayers at 37 °C in humidified 5% CO_2 atmosphere.

2.2. Plasmid constructs

2.2.1. EmGFP purification

Sequences encoding the EmGFP gene were excised from a bacterial expression plasmid pRSET-EmGFP Bacterial Expression Vector (Life Technologies). The plasmid was linearized with HindIII restriction enzyme (New England Biolabs Inc.). The amplification of EmGFP gene was carried out by PCR with KAPA2G Robust DNA Polymerase (KapaBiosystems). Briefly 100 ng of linearized plasmid was added to a reaction mix composed of 5 μl of 5X KAPA2G Buffer A, 0.5 μl of 10 mM dNTP Mix, 1.25 μl of 10 μM EmGFP5FW primer, 1.25 μl of 10 μM EmGFP3RV primer, 0.2 μl of 5 U/μl KAPA2G Robust DNA Polymerase and 16 μl of DEPC water. Primers were phosphorylated on their 5′ termini; the sequences are described in Table 1. The PCR cycle was composed of an initial denaturation stage of 3 min at 95 °C, followed by 35 amplification stages with a denaturation step of 20 s at 95 °C, a hybridization step of 20 s at 65 °C, an elongation step of 30 s at 72 °C and a final elongation stage of 1 min at 72 °C. The amplicon length was verified by agarose gel electrophoresis, and the EmGFP band was excised and purified with PureLink Quick Gel Extraction Kit (Life Technologies). The sequence obtained does not contain a final stop codon.

Table 1
Primers sequences used to insert EmGFP into CV-B3/28 genome.

Primers	Sequence
EmGFP3RV	5′P-CTT-GTA-CAG-CTC-GTC-CAT-GCC-GA-3′
EmGFP5FW	5′P-ATG-GTG-AGC-AAG-GGC-GAG-GAG-3′
CVB3-28 2432-2452	5′-TCG-CAG-GAA-AAC-TTT-TTC-CAG-3′
CVB3-28 1759-1736	5′-AGT-ATT-CAT-GGT-TGG-TAA-GCC-CTG-3′
CVB3-28 1736-1759	5′CAG-GGC-TTA-CCA-ACC-ATG-AAT-ACT-3′

2.2.2. CV-B3/28 genome amplification

A TOPO XL plasmid containing the full CV-B3/28 Genome flanked by a T7 promoter followed by a hammerhead ribozyme sequence and an MluI restriction site, was used for the insertion of EmGFP in place of the VP3 gene. The plasmid sequence was amplified by PCR primers excluding VP3, generating an amplicon of about 10.3 kb. Briefly, TOPO XL CV-B3/28 plasmid was linearized by BglII (New England Biolabs Inc.), and 50 ng was added to a reaction mix composed of 10 μl of 5X KAPA HiFi Fidelity Buffer, 1.5 μl of 10 mM dNTP Mix, 1.5 μl of 10 μM CVB3-28 2432–2452 primer, 1.5 μl of 10 μM CVB3-28 1759–1736 primer, 1 μl of 1 U/μl KAPA HiFi DNA Polymerase (KapaBiosystems) and 35 μl of DEPC water (Table 1). The PCR cycle was composed of an initial denaturation stage of 3 min at 95 °C, followed by 35 amplification stages with a denaturation step of 20 s at 98 °C, a hybridization step of 15 s at 65 °C, an elongation step of 10 min 30 s at 72 °C and a final elongation stage of 10 min 30 s at 72 °C. The PCR product was recovered following agarose gel electrophoresis and purified with PureLink Quick Gel Extraction Kit (Life Technologies). The amplicons contained the complete plasmid sequence with a nearly complete deletion of VP3 sequences. Only 21 nucleotides encoding the 5′ end N-terminal amino acids and 21 nucleotides encoding the C-terminal amino acids of VP3 were maintained, allowing the insertion of EmGFP coding sequences in frame.

2.2.3. Blunt ligation of purified EmGFP and CV-B3/28 fragments

Purified amplicons were quantified by spectrophotometry method (Picodrop Ltd) before ligation using T4 DNA Ligase (Life Technologies). EmGFP amplicons carried phosphate on their 5′ extremity thanks to the primers, allowing a direct ligation without prior phosphate addition steps. Briefly, 30 fmol of CV-B3/28 amplicon and 105 fmol of EmGFP amplicon were added to 4 μl of 5X DNA Ligase Reaction Buffer, 1 μl of T4 DNA Ligase (Life Technologies) and DEPC water to a final volume of 20 μl. The reaction was incubated 24 h at 14 °C.

2.2.4. E.coli *transformation*

The ligation products were transformed into competent Top10 *Escherichia coli* (Life Technologies). The ligation reaction (2.5 μl) was incubated with 50 μl of competent cells for 5 min on ice,

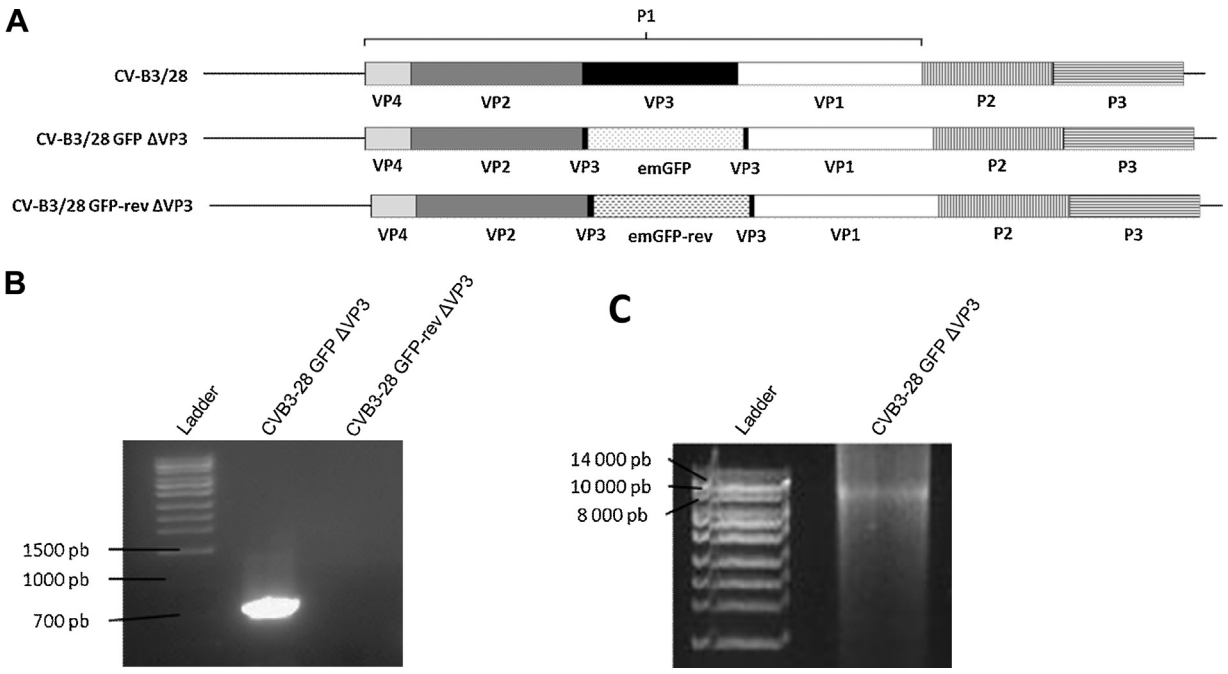

Fig. 1. Construction of CV-B3/28 expressing EmGFP genome. (A) Genome of CV-B3/28 clones. The cardiotropic strain CV-B3/28 was used to insert EmGFP in VP3 region. Two clones were generated, with a productive EmGFP (CV-B3/28 GFP ΔVP3) and a reverse sequence of EmGFP (CV-B3/28 GFP-rev ΔVP3). (B) PCR screening of the correct orientation of EmGFP into CV-B3/28 genome. The forward primer binds in the remaining 5′ VP3 region and the reverse primer binds the EmGFP 3′ extremity. 1: CV-B3/28 GFP ΔVP3. 2: CV-B3/28 GFP-rev ΔVP3. (C) The transcripted viral RNA length (7500 nt) was assessed by gel agarose electrophoresis.

followed by 40 s at 42 °C and 2 min on ice before incubating with 250 μl of SOC medium (Life Technologies) at 37 °C. Transformed cultures were grown on LB agar plates containing 50 μg/ml kanamycin (Life Technologies) overnight at 37 °C. Colonies were screened for the presence of EmGFP gene by PCR. The positive clones were cultured in 5 ml LB Broth (Sigma) containing 50 μg/ml kanamycin, followed by purification with the High Purity Plasmid Miniprep Kit (Neo Biotech). Purified plasmids were quantified by spectrophotometry method (Picodrop Ltd.) and linearized by digestion with restriction enzyme MluI (New England Biolabs Inc.). PCR amplification of a region overlapping the conserved 5′ end of the VP3 and EmGFP genes verified the correct EmGFP orientation. Briefly 20 ng (1 μl) of linearized plasmid were added to a reaction mix composed of 5 μl of 5X KAPA2G Buffer A, 1 μl of 10 mM dNTP Mix, 2 μl of 10 μM CVB3-28 1736–1759 primer, 2 μl of 10 μM EmGFP3RV primer, 0.4 μl of 5 U/μl KAPA2 G Robust DNA Polymerase (KapaBiosystems) and 38.6 μl of DEPC water. Primer sequences used are described in Table 1. The PCR cycle was composed of an initial denaturation stage of 3 min at 95 °C, followed by 45 amplification stages with a denaturation step of 30 s at 95 °C, a hybridization step of 30 s at 57 °C, an elongation step of 1 min at 72 °C and a final elongation stage of 1 min at 72 °C. A positive signal of amplification verified the correct EmGFP sequence insertion.

2.3. Transcription

Selected plasmids were linearized with restriction enzyme MluI and transcribed into RNA using a MEGAscript T7 Transcription Kit (Ambion). Synthesized RNAs were purified with EZ-10 Total RNA MiniPrep Kit (Biological Industries) and quantified by spectrophotometry (Picodrop Ltd.).

2.4. Transfection

After trypsinization, 1×10^5 cells/well were seeded in 24-well plates (Nunclon delta surface, Thermo Scientific). The plates were incubated overnight at 37 °C. Cells were washed with DPBS and 400 μl of opti-MEM GlutaMAX was added. The transfection mixtures consisted in 400 ng of synthesized RNA or 400 ng of pmaxGFP (Lonza) and 1 μl of Lipofectamine 2000 in Opti-MEM Glutamax. The mixtures were incubated 25 min at room temperature and 100 μl of mixture were added to the cells, and the plates were transferred to a cell incubator for 3 h at 37 °C. Following incubation, the medium containing the mixture was removed, and the cells were washed once with PBS followed by addition of 500 μl of MEM 2% FBS. Finally, the plates were incubated at 37 °C for the indicated times.

2.5. Immunofluorescence staining

Cells were cultivated in 24-well plate containing a round coverslip (Bellco). The cells were fixed with a cold methanol-acetone mixture (3:1) and incubated for 10 min at −20 °C. The wells were then washed 3 times with PBS (Biomérieux). The saturation step was performed by adding 200 μl of PBS supplemented with 1% BSA (Sigma–Aldrich) and 2% FBS (Fetal Bovine Seurm (Life Technologies)). VP1 specific antibody (Monoclonal Mouse Anti-Enterovirus Clone 5-D8/1, Dako) was used at a dilution of 1:500 in PBS supplemented with 1% BSA and 2% SVF, and incubated for 1 h at 37 °C. After 3 washes in PBS, the primary antibody was replaced with 200 μl of secondary antibody [goat anti-mouse IgG (H + L), Alexa Fluor 647 Conjugate, Life Technologies] at a dilution of 1:400 in PBS supplemented with 1% BSA, 2% SVF and incubated at 37 °C for 1 h. The wells were washed, and 200 μl of DAPI (Invitrogen) at a dilution of 1:10,000 in PBS was added for 2 min at room temperature followed by a wash with PBS. Finally the coverslips were removed from the wells and mounted on a slide with Ultramount Permanent Mounting Medium (Dako). Slides were read with an Axiovert 200 (Zeiss).

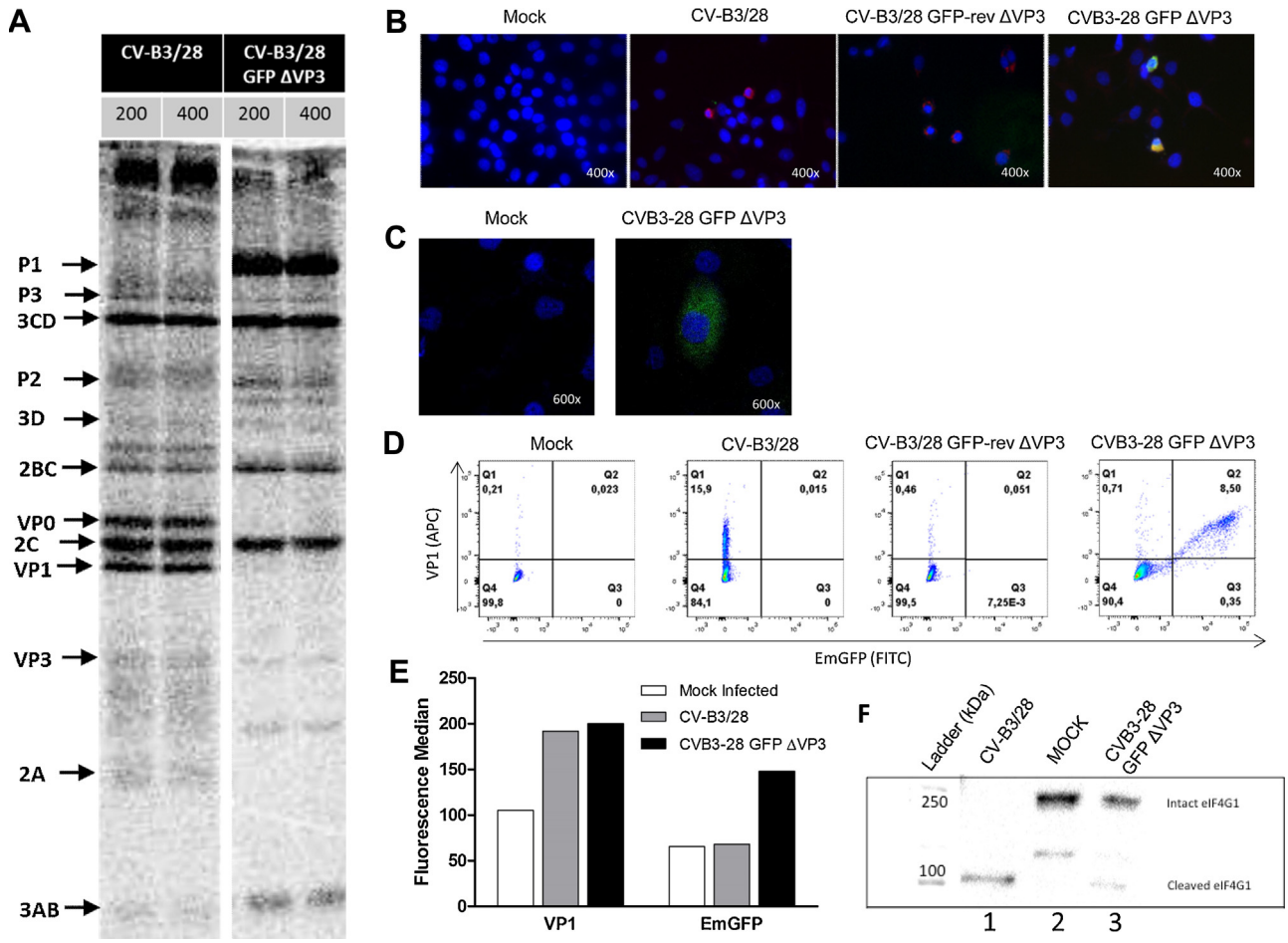

Fig. 2. Viral protein synthesis activity of CV-B3/28 GFP-rev ΔVP3 and CV-B3/28 GFP ΔVP3. (A) *In vitro* translation assays. *In vitro* transcribed RNAs (200 or 400 ng) were incubated with HeLa S10 extract in presence of ^{35}S Methionine. Compared to the original gel, the image shown in the panel has been cropped. (B) Viral protein production was assessed in HeLa cells by immunofluorescent staining of VP1 (red) and EmGFP (green). Colocalization of VP1 and GFP is indicated in yellow. Cellular nuclei were stained by DAPI (blue). (C) EmGFP signal (green) was observed in transfected primary human cardiomyocytes. Cellular nuclei were stained by DAPI (blue). (D) Similar analysis were conducted by flow cytometry at 4 h post transfection, assessing the expression of both VP1 signal (*Y* axis) and EmGFP (*X* axis) in transfected HeLa cells. (E) Fluorescence median of cells in flow cytometry allowed evaluating the expression of VP1 and EmGFP in HeLa cells. (F) Western-blot analysis of VP1 and GFP from HeLa cells 24 h post transfection infected by CV-B3/28 (Lane 1) or CV-B3/28 GFP ΔVP3 (Lane 3) or mock infected (Lane 2).

2.6. Flow cytometry

Transfected cells were analyzed by flow cytometry for the presence of VP1 or GFP. After trypsinization and harvesting in 2 ml of DPBS-20% FBS, the cells were fixed with 1% PFA (paraformaldehyde) for 10 min at 4 °C followed by 10 min of permeabilization using triton 0.1%. Cell pellets were washed, and incubated with a 1:100 dilution of anti-VP1 antibody (Dako) for 30 min on ice. The primary antibody was removed by washing in DPBS-2% FBS, and cell pellets were incubated for an additional 30 min with a 1:500 dilution of anti-mouse Alexa Fluor 647 antibody (Life Technologies). After a final wash, the cells were resuspended in 500 μl of 1% PFA before analyzing with a BD FACSARIA III flow cytometer. The data were analyzed with FlowJo v10.7 (Tree Star Inc.).

2.7. Western blot to detect eIF4G1

Total proteins were extracted using cold RIPA buffer (Sigma–Aldrich) with Protease Inhibitor Cocktail® (Roche) and fractionated by SDS-PAGE on 4–15% acrylamide gradient gel (Mini-PROTEAN® TGX, Biorad). Proteins were then electroblotted onto 0.45 μm nitrocellulose membrane, which was blocked with Tris-buffered saline-Tween 20 (TBST: 20 mM Tris, 0.5% Tween 20) containing 5% bovine serum albumin. The membrane was incubated overnight at 4 °C with a 1:500 dilution of polyclonal eIF4G1 Rabbit IgG (15704-1-AP, Proteintech). The membrane was washed 3 times with TBST before incubation with peroxidase-linked anti-rabbit secondary antibody (Thermo-Fisher GE healthcare Biosciences). After 3 washes with TBST, immunoreactive proteins were detected using ChemiDoc™ MP System (Biorad) with enhanced chemiluminescence substrate (GE Healthcare).

2.8. Infectious viral particle

The detection of infectious viral particles in culture supernatants was determined using a plaque assay. Six-well plates were seeded at a density of 0.8×10^6 A549 cells per well, transferred into a cell incubator and incubated overnight to produce a confluent cell monolayer. The next day, the cells were washed with DMEM without FBS and serial tenfold dilutions of the transfected cell supernatant prepared in serum free DMEM were added to the corresponding wells. The plates were incubated for 1 h at 37 °C with 5% CO_2, with gentle rocking every 10 min allowing uniform viral adsorption. After incubation, medium was removed and replaced with an overlay of 1% agarose (Life Technologies) and a 2X solution containing MEM 10X, 4 mM L-glutamine, 1% penicillin/streptomycin, 2 mM sodium bicarbonate 7.5% and 4% FBS (Life Technologies). After 10 min allowing overlay solidifica-

tion, the plates were incubated for 72 h at 37 °C. Agarose overlays were carefully removed, and the cells were stained with a crystal violet solution for 5 min, followed by washing with sterile water. Plaques were counted to determine the yields titer was expressed as PFU (plaque-forming units per ml).

2.9. In vitro *translation analysis*

To assess viral protein synthesis, an *in vitro* translation assay was carried out by incorporating ^{35}S-methionine into newly-synthesized proteins. Briefly, 400 or 200 ng (1 μl) of synthetic RNA was added to a reaction containing 1 μl all-4-mix (1 mM ATP, 250 μM CTP, 250 μM GTP, and 250 μM UTP, 16 mM HEPES pH 7.4, 60 mM KOAc, 30 mM creatine-phosphate, and 400 μg creatine kinase), 1 μl of ^{35}S-methionine and 7 μl of HeLa S10 cytoplasmic extract as previously described (Cathcart et al., 2013). The mixture was incubated for 6 h at 30 °C. Laemmli sample buffer (10 μl) was added to the mixture and incubated for 3 min at 100 °C. The samples were subjected to electrophoresis on a 12.5% polyacrylamide gel containing SDS at 110 V overnight. The gel was washed 3 times in DMSO for 30 min, once in DMSO-PPO for 30 min, once in deionized water for 45 min and dried. The gel was an exposed to X-ray film overnight prior to developing using the Molecular Imager FX (Biorad).

2.10. *Replication inhibition assay*

Fluoxetine is a selective serotonin reuptake inhibitor, which has been shown to inhibit enterovirus replication (Zuo et al., 2012). Fluoxetine chlorhydrate 20 mg/5 ml oral solution (ARROW) was used in this assay. Confluent HeLa cell monolayers were trypsinized and 1×10^5 cells were cultured in a 24-well plate for flow cytometry analysis or on glass coverslips for immunofluorescence assays, then incubated at 37 °C, 5% CO_2 with a complete medium (MEM, Life Technologies). After 24 h, the 80%-confluent cells were washed twice with DPBS, and 500 μl of OptiMEM Glutamax with no serum was added. Thirty minutes prior to transfection, fluoxetine was diluted in the same medium to achieve a final concentration of 5 μM or 10 μM. The transfection was carried out as described above. Transfected cells were incubated for 12 h at 37 °C in the presence of the indicated concentrations of fluoxetine. After incubation, cells were washed with DPBS and trypsinized. Trypsin was inactivated using 2 ml of DPBS with 20% FBS and the cells were pelleted by centrifugation. After washing, the pellets were resuspended in 500 μl of DPBS before analysis by flow cytometry.

The cells used for immunofluorescence assays were washed once with DPBS, and then fixed and permeabilized using a cold methanol-acetone mix (3:1) for 10 min at −20 °C. A dilution of 1:10,000 of DAPI was used to stain cell nuclei before observing under the microscope. Post-acquisition, images were processed using Fiji (ImageJ) software (Schindelin et al., 2012). To quantify the number of EmGFP positive cells, images were taken from representative fields for a total of approx. 5000 cells per incubation condition.

3. Results

3.1. *Generation of a CV-B3 replicon containing the EmGFP sequence inserted in place of the viral VP3 gene*

The cloning strategy allowed the generation of full-length CV-B3 cDNA replicons containing the EmGFP sequences in place of the viral VP3 sequences (Fig. 1A). This construct was generated using a plasmid containing the full length CV-B3/28 sequence (Chapman et al., 1994). The viral genome was flanked by a hammerhead ribozyme; this autocatalytic sequence eliminated terminal non-viral sequences that are inserted at the 5′ terminus of the viral genomic RNA during the production of synthetic viral RNAs, thereby allowing us to obtain the same properties as authentic viral RNA with the exception of the absence of VPg covalently linked to the 5′ terminus. The 3′ extremity terminus of the viral cDNA contained an MluI restriction site preceded by a nucleotide poly(A) sequence, allowing an authentic 3′ end without any non-viral plasmid sequences. The orientation of the EmGFP coding sequence in cDNA replicons was assessed by PCR assays and clones with inserts in both the sense and reverse orientations were selected (Fig. 1B). The replicons with a sense and reverse direction were respectively named CV-B3/28 GFP ΔVP3 and CV-B3/28 GFP-rev ΔVP3 (Fig. 1A). Transcription of the different replicons leads to an expected 7500 nucleotide long synthetic RNA (Fig. 1C). These transcripts were used *in vitro* to assess protein synthesis and genomic replication activities in HeLa-229 cells and HCM.

3.2. *Viral protein synthesis of EmGFP replicons in HeLa cells and human cardiac cells*

Using our different cDNA replicons, synthetic viral RNAs were transcribed and the viral protein synthesis ability of the replicons was assessed by an *in vitro* translation assay. Viral RNAs were incubated with HeLa-S10 cytoplasmic extracts in the presence of ^{35}S-radiolabeled methionine. The results showed a protein translation pattern for the replicons, with or without EmGFP, similar to that of wild-type CV-B3/28 RNA (Fig. 2A). The only differences observed were the lack of processing of the P1 precursor containing EmGFP sequences in place of VP3 sequences. This is due to the previously described effects of altering the folding of the P1 precursor by insertion of non-authentic amino acid sequences (Ypma-Wong et al., 1988). However, the processing of the non-structural precursors was not affected by the EmGFP sequences, validating our replicons for further *in vitro* characterization.

Synthetic viral RNAs were then transfected into HeLa cells to assess *in vivo* translation and replication of the viral genome using EmGFP cytoplasmic fluorescence. VP1 synthesis was detected in CV-B3/28 prototype strains as well as in both CV-B3/28 GFP ΔVP3 and CV-B3/28 GFP-rev ΔVP3 replicons (Fig. 2B). As expected EmGFP cytoplasmic fluorescence was only detected in CV-B3/28 GFP ΔVP3 transfected HeLa cells where it co-localized with VP1 staining (Fig. 2B). EmGFP signal was not detected in CV-B3/28 and CV-B3/28 GFP-rev ΔVP3 transfected HeLa cells (Fig. 2B). The EmGFP signal was observed in human primary cardiomyocytes with a same pattern (Fig. 2C). Moreover, flow cytometry allowed us to quantitatively compare fluorescence levels produced between the replicons and the prototype strain after VP1 staining. At 4 h post-transfection, the CV-B3/28 RNA was expressed in more cells than the CV-B3/28 GFP ΔVP3 replicon (15.91% *vs.* 8.50%) or the CV-B3/28 GFP-rev ΔVP3 (15.91% *vs.* 0.5%) (Fig. 2D). The wild-type viral RNA showed similar intra-cytoplasmic VP1 fluorescence median values as the CV-B3/28 GFP ΔVP3 replicon (183 *vs.* 190; Fig. 2E), indicating that these two viruses produced similar amounts of viral proteins. As expected, EmGFP signal was detectable in CV-B3/28 GFP ΔVP3 but not in CV-B3/28 transfected cells. Biological activities of non-structural viral proteins were assessed through the specific proteinase activity of $2A^{pro}$ on eIF4GI transcription factor, leading to the shut off of cellular translation during CV-B infection. Non-cleaved eIF4GI translation factor has a molecular weight of about 250 kDa and the $2A^{pro}$ cleavage yield a 100 kDa specific product (Castelló et al., 2011). EIF4GI cleavage was detected by Western-Blot in both CV-B3/28 and CV-B3/28 GFP ΔVP3 infected HeLa cells (Fig. 2F), demonstrating sufficient maturation and conserved biological activities of non-structural viral proteins in CV-B3/28 GFP ΔVP3 replicon. The difference between CV-B3/28 and CV-B3/28 GFP ΔVP3 cleavage levels is explained by the variation of infected

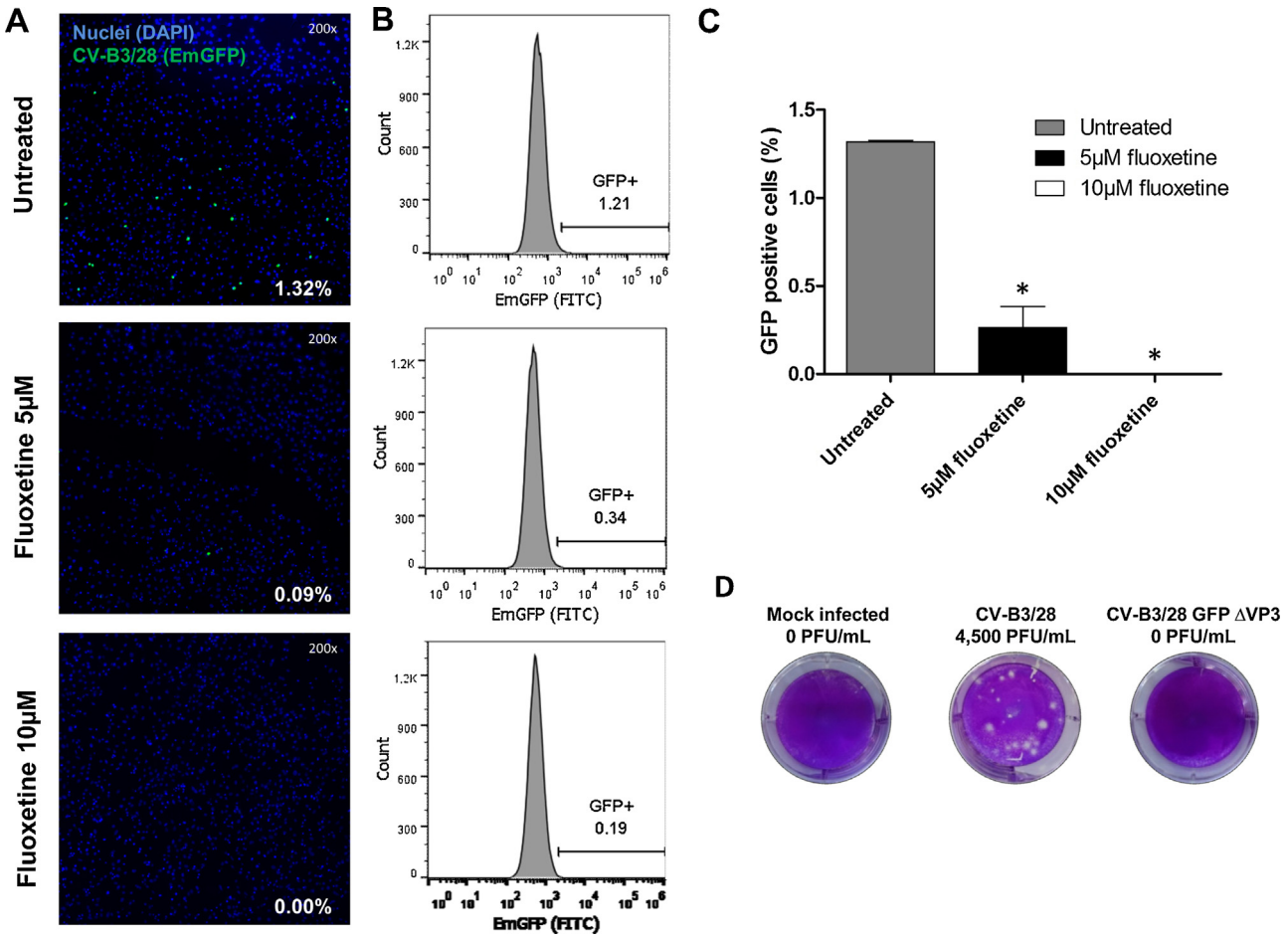

Fig. 3. CV-B3/28 EmGFP replication inhibition using fluoxetine-HeLa cells were transfected with 400 ng of CV-B3/28 EmGFP ΔVP3 RNA, and treated with 0, 5 and 10 μM of fluoxetine for 12 h. (A) Images were captured using a fluorescence microscope. EmGFP signal was normalized to DAPI signal using ImageJ software and the percentage of infected cells is displayed in the bottom right corner of each panel. (B) Flow cytometry: percentage of EmGFP expressing cells. (C) Quantification of the signal acquired in microscopy in 12 representative fields for each of the tested conditions. The untreated cells showed approximately 1.32% of GFP-expressing cells, significantly different ($p < 0.001$, two-way ANOVA, SAS 9.4) from the positive signal in fluoxetine 5 μM and fluoxetine 10 μM with a percentage respectively 0.09% and 0.00%. $^{*}p < 0.001$. (D) Infectious particle production. Transfected cell culture supernatants were harvested and added to A549 cell monolayer. After 72H PFU were counted.

cells number (15.91% *vs.* 8.50% at 4 h). CV-B3/28 virus is able to spread from cell to cell, leading to an increasing number of cells with cleaved eIF4G1 whereas CV-B3/28 GFP ΔVP3 $2A^{pro}$ can only be active in primary transfected cells.

3.3. RNA replication and production of infectious viral particles in human target cells

Viral RNAs were transfected in the presence of two different concentrations (5 μM and 10 μM) of fluoxetine, an inhibitor of enterovirus replication (Zuo et al., 2012). No notable cytotoxicity was observed in mock-transfected cells. The compound did not interfere with the EmGFP signal, hence no difference between the untreated or 5 μM and 10 μM fluoxetine treated wells in the pmaxGFP transfected HeLa cells (61.9%, 63.7% and 61.3% respectively) was observed (data not shown). Both fluoxetine doses demonstrated efficient reduction of GFP positive cells following CV-B3/28 GFP ΔVP3 transfection. Quantification of positive cells by microscopy indicated 1.32% of GFP positive cells in the untreated wells, and 0.09 and 0% respectively in 5 μM and 10 μM fluoxetine-treated wells ($P < 0.001$ two-way ANOVA; Fig. 3A–B panel). The flow cytometry analysis showed similar results (untreated: 1.21%, 5 μM fluoxetine: 0.34%, 10 μM fluoxetine: 0.19%; Fig. 3C). The different levels of viral proteins produced by the CV-B3/28 and the CV-B3/28 GFP ΔVP3 replicons in the transfected HeLa cells could be explained by the inability of CV-B3/28 GFP ΔVP3 replicon to produce infectious particles (Fig. 3D). Infectious particle production was then investigated and no PFU were detected in CV-B3/28 GFP ΔVP3 transfected cells (Fig. 3D). This property allows the study of replication and translation activities of the input RNA in human transfected cells without the production of infectious virus.

4. Discussion

In the present study, we designed a method allowing the generation of an novel CV-B3 replicon carrying an EmGFP in place of VP3 gene, without any major genomic length or sequence modifications. In HeLA 229 cells as well in primary human cardiac cells, the genomic RNA replication and translation of our subgenomic replicon appeared similar to those demonstrated by the cardiotropic CV-B3/28 strain, except for the lack of infectious viral particle production. Therefore, our CV-B3 replicon could be used as a template to study replication and translation activities in different continuous and primary human cell lines. Moreover, it could be used as a platform to easily generate genetically-modified viral forms detected and characterized in acute and chronic CV-B3 induced human pathologies. Previously, other researchers have made several constructs of CV-B3. In 2004, a replicon depleted of the entire P1 region and containing eGFP and luciferase as reporters

was constructed (Meyer et al., 2004). Other investigators described constructs with GFP inserted upstream of the polyprotein (Lim et al., 2005) or following VP1 (Zeng et al., 2013), resulting in infectious viruses with slightly decreased infectivity. The choice of EmGFP as reporter was motived by its improved maturation, folding and brightness at cell culture conditions (37 °C) (Shaner et al., 2005). Our CV-B3/28 EmGFP ΔVP3 construct shares 94.15% identity with CV-B3/28. This accounts for our observation that genomic replication and translation activities of our subgenomic replicon appeared very similar to the cardiotropic CV-B3/28 strain, except for the lack of infectious viral particle production. Moreover, this replicon can be easily and safely used in a viral BSL2 laboratory because no infectious viral particles can be produced. In addition, the EmGFP CV-B3 replicon provides an easy method of studying CV-B3 replication using live-cell imaging and quantitation of fluorescence measured by fluorescence or confocal microscopy and flow cytometry devices.

Our main objectives were to generate a novel replicon from a cardiotropic prototype CV-B3/28 strain and to characterize its replication properties into human cardiac primary cells. To this aim, we constructed a CV-B3 replicon that could be used to quantify protein synthesis and genomic RNA replication activity of a previously described cardiotropic strain (Chapman et al., 1994). The EmGFP gene was used as a reporter and inserted into the VP3 region of the CV-B3/28 genome. The correct insertion of the reporter and the quality of the transcripts were assessed by RT-PCR and electrophoresis respectively (Fig. 1). The protein synthesis was evaluated by *in vitro* translation assays to validate the constructions, and viral RNAs were then transfected into HeLa cells and primary human cardiomyocytes (Fig. 2). As expected, our replicons could not produce any infectious particles due to the lack of VP3 (Fig. 3). Taken together these results indicated that the GFP insertion prevented the viral protein P1 maturation and, therefore, infectious viral particle production and further rounds of infection of surrounding cells. Interestingly, our VP3 deleted replicons appeared to be able to generate all of the viral non-structural proteins. Proteinase 2A activity was assessed by Western blot analysis of eIF4G1 cleavage that revealed its specific cleavage products highlighting the cellular translation shut-off and the conserved activities of the non-structural viral protein.

In the present report, the viral replication was indirectly demonstrated using fluoxetine, a chemical which inhibits the synthesis of negative- strand RNAs by either interfering with 2C protein activity or the rearrangement of cellular membranes by the viral 2BC or 3A proteins (Zuo et al., 2012). Our results showed detectable EmGFP in untreated cells and demonstrated a significant EmGFP signal reduction in the presence of fluoxetine. These data indicated that genomic RNA replication was necessary for EmGFP detection in transfected cells (Fig. 3A–C). Finally, this EmGFP CV-B3 replicon appeared to be a valuable tool to assess the existence of CV-B3 replication and translation activities in various human target cells and, more specifically, in human cardiomyocytes infected during acute and chronic human EV infections. Indeed, EmGFP production by replicons could reflect both genomic replication and translation levels in transfected human cells, and these viral replication activities could be easily quantified by fluorescence microscopy or flow cytometry assays. Moreover, our construct could be easily altered in sequences encoding one or more viral non-structural proteins to investigate the potential impact of specific mutations on picornavirus replication activity in various human target cells such as cardiomyocytes.

In conclusion, our novel EmGFP CV-B3 replicon provides an easy method for studying viral replication, based upon live-cell imaging together with quantitation of fluorescence in transfected cells. Moreover, our EmGFP CV-B3 replicon can be used as a template to characterize the potential impact of various natural mutations occurring throughout the CV-B genome on the virus biology in different *in vitro* models. Finally, the EmGFP CV-B3 replicon can be used to assess the effects of potential antiviral drugs on viral replication activities in human cardiac myocytes.

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