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Continuous Passaging of a Recombinant C-Strain Virus in PK-15 Cells Selects Culture-Adapted Variants that Showed Enhanced Replication but Failed to Induce Fever in Rabbits

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Classical swine fever virus (CSFV) is the etiologic agent of classical swine fever, a highly contagious disease that causes significant economic losses to the swine industry. The lapinized C-strain, a widely used vaccine strain against CSFV, has low growth efficiency in cell culture, which limits the productivity in the vaccine industry. In this study, a recombinant virus derived from C-strain was constructed and subjected to continuous passaging in PK-15 cells with the goal of acquiring a high progeny virus yield. A cell-adapted virus variant, RecCpp80, had nearly 1,000-fold higher titer than its parent C-strain but lost the ability to induce fever in rabbits. Sequence analysis of cell-adapted RecC variants indicated that at least six nucleotide changes were fixed in RecCpp80. Further adaption of RecCpp80 variant in swine testicle cells led to a higher virus yield without additional mutations. Introduction of each of these residues into the wild-type RecC backbone showed that one mutation, M979R (T3310G), located in the C-terminal region of E2 might be closely related to the cell-adapted phenotype. Rabbit inoculation revealed that RecCpp80$_{+10}$ failed to induce fever in rabbits, whereas RecCpp40$_{+10}$ caused a fever response similar to the commercial C-strain vaccine. In conclusion, the C-strain can be adapted to cell culture by introducing specific mutations in its E2 protein. The mutations in RecCpp80 that led to the loss of fever response in rabbits require further investigation. Continuous passaging of the C-strain-based recombinant viruses in PK-15 cells could enhance its in vitro adaption. The non-synonymous mutations at 3310 and 3531 might play major roles in the enhanced capacity of general virus reproduction. Such findings may help design a modified C-strain for improved productivity of commercial vaccines at reduced production cost.

Keywords: Classical swine fever, C-strain, cell culture adaptation, fever in rabbits

Introduction

Classical swine fever virus (CSFV) is the etiologic agent of classical swine fever (CSF), a highly contagious disease that causes significant economic losses to the swine industry [1]. It is a single-strand, positive-sense RNA virus belonging to the genus *Pestivirus* within the family *Flaviviridae* [2]. CSFV has a genome size of about 12.3 kb that comprises a 5′-untranslated region (UTR), a 3′-UTR, and a single large open reading frame encoding a polyprotein of 3,898 amino acids, which is processed by both cellular and viral proteases into four structural proteins (Core, Erns, E1, andE2) and eight non-structural proteins (Npro, p7, NS2, NS3, NS4A, NSS4B, NS5A, and NS5B) [3].

Systemic prophylactic immunization with live attenuated vaccines is effective for controlling CSF [4, 5]. The commercially available CSFV vaccine used in China is based on the Chinese strain (C-strain) that was attenuated after serial passages of the CSFV strain in rabbits [6, 7]. The vaccine could be produced from spleen and lymph nodes of rabbits inoculated with the attenuated strain or by in vitro cultivation of the virus in primary bovine testicle cells

or the swine testicle (ST) cell line [6]. Cell culture systems, such as the porcine kidney epithelial cell line (PK-15), could be optimized for large-scale production of vaccines with low cost and have become the common sources for C-strain vaccines [6, 8]. However, the rabbit-adapted C-strain is known to grow suboptimally in cell lines. In addition, genetic recombinant C-strain viruses also yielded low titers of progeny viruses and exhibited slow growth in some cell lines [9, 10]. The relative low titer of the C-strain virus is a limiting factor of productivity for the vaccine industry.

Viruses could become cell-adapted with increased yield upon repeated subculturing [11–15]. In this study, a recombinant virus was constructed from C-strain vaccine and subjected to continuous passaging in PK-15 cells with the goal of acquiring a high progeny virus yield. A cell-adapted virus variant emerged after 80 passages and had 1,000-fold higher virus titers than its parent strain. This adapted strain could be used to study strategies for the improved production efficiency of C-strain-based recombinant vaccines and to examine the mechanisms of CSFV replication.

Materials and Methods

Cells, Virus, and Antibody

The ST cells (swine testicle cells) and PK-15 cells (porcine kidney epithelial cells) were cultured in Dulbecco's minimal essential medium (DMEM) (Hyclone, Thermo Scientific, USA) with 10% fetal calf serum (Hyclone) and antibiotics. The vaccine C-strain was obtained from China Animal Husbandry Industry Co. Ltd. (Beijing, China) and virus stocks were prepared by passages in PK-15 cells [16]. The murine monoclonal antibody (mAb) 6B8 against E2 protein was described in our previous study [17]. The goat anti-mouse IgG conjugated with Alexa Fluor 488 was purchased from Molecular Probe (Life Technologies, USA).

Construction of the C-Strain-Based Infectious Clone

Six fragments that cover the full-length C-strain genome (GenBank: HM175885) were amplified by RT-PCR with the corresponding primer pairs (Table S1). The BamHI site in the middle of the genome was utilized in subcloning to assemble the two fragments (CF123 and CF456, Fig. 1). Briefly, the T7-CF1 fragment was assembled into pA plasmid (modified pACYC low copy plasmid) by XbaII and SpeI digestion to generate pA-T7-CF1. The second fragment CF2 was cloned into intermediate plasmid pGEM by SpeI /PstI and then digested by PstI/SalI for ligation with the third fragment CF3 digested with the same enzyme pair, resulting in an intermediate pGEM-CF23. The CF23 fragment was transfered from pGEM to pA-T7-CF1 after digestion with SpeI/SalI. This resulted in recombinant plasmid pA-CF123 covering the 5′-half genome. The recombinant plasmid pB-CF456 containing the 3′-half genome was obtained by restriction enzyme digestion as follows: The CF4 fragment was assembled into pB plasmid (modified pBR322 plasmid) by BamHI/NcoI digestion to generate pA-CF4. The CF5 fragment was digested by NcoI/NotI and cloned into pB-CF4 to generate pB-CF45. The CF6 fragment was further cloned into pB-CF45 by BstBI/SalI digestion, resulting in pB-CF456. The full-length cDNA clone pA-RecC (T7-CF123+CF456) was generated from pB-CF456 using BamHI/SalI and ligated into the pA-CF123 digested with the same enzyme pair.

Generation of the Recombinant C-Strain Virus and Cell Adaptation Process

In vitro rescue of the C-strain-based infectious clone was described in our previous study [16]. Briefly, the pA-RecC plasmid was

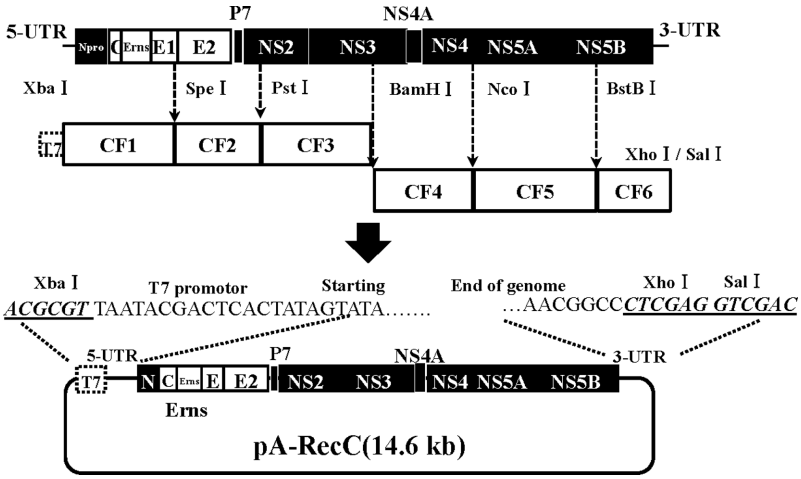

Fig. 1. Schematic representation of the strategy applied for assembly of the full-length C-strain infectious cDNA clone.
Six fragments (CF1 to CF6) covering the whole viral genome were amplified by PCR using primer pairs indicated in Table S1. The XbaI site along with the T7 promoter was fused anterior to 5′-UTR in CF1 by PCR using primer F1-upper, while XhoI and SalI enzyme sites were introduced to the end of 3′-UTR in CF6. The C-strain infectious clone pA-RecC was constructed as described in Materials and Methods.

linearized by XhoI and purified using the phenol-chloroform method. Full-length genomic RNA was transcribed from linearized pA-RecC using the in vitro T7 Megascript system (Ambion, USA) and precipitated with LiCl. The RNA transcript was then transfected into PK-15 cells by electroporation at 150 V with the Gene Pulser Xcell electroporator (Bio-Rad, USA). The cells were then plated in 25-cm^2 flasks and incubated for 4 days at 37°C and 5% CO_2 to rescue the recombinant virus RecC. Continuous passaging of the RecC virus was conducted by subculturing the electroporated cells into new T25 flasks (in 1:4 ratio) with trypsin-EDTA treatment every 4 days. The cells of each passage were collected and stored at −80°C as virus stocks for further detection. In parallel, a small portion of each passage was seeded in a 24-well plate to examine expression of the viral E2 protein by immunostaining with anti-E2 mAb 6B8 as described below. For further adaptation of RecCpp40 and RecCpp80 in ST cells, infected cultures in PK-15 cells at indicated passages were inoculated onto a fresh ST cell monolayer in T25 flasks, and the virus samples were collected after 4 days of incubation by repeated freezing and thawing. For the next rounds of passages (up to passage 10), the cell lysates after each passage were clarified by centrifugation and the supernatant samples containing virus particles were used to infect new ST cell monolayers.

Virus Titration and Indirect Immunofluorescence

The recombinant viruses from different passages were titrated using the end-point dilution method. PK-15 cells were inoculated with 10-fold serial dilutions of viral suspensions and seeded onto cells in 96-well plates (10^4 cells in 100 µl per well). After incubation for 96 h, viral particles were detected by indirect immunofluorescence using anti-E2 mAb 6B8 as described elsewhere [16]. Titers were calculated according to the method of Reed and Münch and presented as median tissue culture infective dose ($TCID_{50}$)/ml.

Growth Kinetics of the Recombinant Viruses

Growth of the recombinant viruses at different passages was evaluated relative to the wild-type C-strain virus. The PK-15 or ST cell monolayers were seeded in 24-well plates and infected with 150 $TCID_{50}$ viruses (multiplicity of infection, MOI=0.001). After 1 h adsorption at 37°C (set as time 0), the supernatant samples were removed and a volume of 0.7 ml of fresh medium was added to the wells. Samples of whole cultures (both cells and culture supernatants) were collected at 12, 24, 48, 72, and 96 h post-infection (hpi) for virus titration. The attached cells in separate plates at the same time points were lysed for analysis of intracellular viral RNA copies.

Viral RNA Extraction and Quantification

Viral RNA was extracted from the lysates of infected cells using the RNAprep Pure Tissue Kit (Tiangen, China) and subjected to cDNA synthesis using the GoScript Reverse Transcriptase Kit (Promega, USA) with the downstream primer 5-UTR-L (5′-CTCCCAGCACGTGGTGTGATTTC-3′) or β-actin-L (5′-GTGATC TCCTTCTGCATCCTGTC-3′). The cDNAs were then amplified with the primer pair 5′-TGGGTGGTCTAAGTCCTGAGTA-3′ (sense) and 5′-CTCCCAGCACGTGGTG TGATTTC-3′ (antisense, targeting the 5′-UTR). RNA copy numbers were determined from the standard curve derived from serial dilutions of the plasmid containing 5′-UTR of the C-strain cDNA. The β-actin gene transcripts of cell lysates were also determined with the primer pair 5′-CTCGATCATGAAGTGCGAC-3′ (sense) and 5′-GTGATC TCCTTCTGCATCCTGTC-3′ (antisense). Viral RNA copies of each test sample were normalized to β-actin transcripts of corresponding time points and expressed as Lg (Virus RNA).

Sequence Analysis of High- and Low-Passage Recombinant Viruses

To examine any mutations important for in vitro growth of culture-adapted viruses, RNA extracts (the same method mentioned above) of RecCpp40, RecCpp80, and vaccine C-strain were reverse transcribed using reverse primers (lownew primers in Table S2). Eight fragments covering the genome were amplified as detailed: fragments CF2 to CF5 were amplified by nest-PCR using corresponding primer pairs in Table S1. Fragments CF1 and CF6 contain untranslated regions difficult for amplification and sequencing, and thus each fragment was split into two short fragments for nest-PCR (Table S2), named as CF1a, CF1b, CF6a, and CF6b. Finally, a total of eight fragments were cloned into pUC19 and six colonies of each fragment were sent to Lifetechnologies (Shanghai, China) for conventional sequencing. In case of any random mutations caused by the RT-PCR and sequencing process, the whole sequencing process was performed six times.

Site-Directed Mutagenesis

For generation of C-strain-based mutant viruses based upon the above comparative sequence analysis of the genomes of both the low- and high-passage viruses RecCpp40 and RecCpp80, the infectious clone pA-RecC was used as the template in which specific nucleotides were mutated one by one using the Quick Change XL Site-Directed Mutagenesis Kit (Stratagene, USA). The primers were designed by the Quick-Change Primer Design Program (http://www.stratagene.com) (Table S3). The extracted mutant pA-RecC plasmids were sent to Lifetechnologies for conventional sequencing, and the expected nucleotide changes in each mutant infectious clone were verified by sequencing. The resulting infectious clones were used to rescue the mutant viruses in ST cells using the procedure described above.

Rabbit Inoculation

Twenty-four New Zealand white rabbits (about 2 kg) were randomly assigned to four groups (six per group). Each rabbit in groups I and II was inoculated intravenously with 1 ml of $RecCpp40_{+10}$ or $RecCpp80_{+10}$ (40- or 80-passage viruses with additional 10 passages before inoculation, 10^4 $TCID_{50}$/ml) via the ear vein. Rabbits in group III were inoculated with one dose (1 dose/ml) of C-strain vaccine (positive control) and those in

group IV, with 1 ml of DMEM (containing 10% FBS) per rabbit as the negative control. Rectal temperature was examined at 8-h intervals. At 28 days post-inoculation, blood samples were collected from all rabbits for serum separation. Antibody responses were measured by indirect ELISA on the E2-coated wells, as previously reported [17]. The animal experiments were approved by the Laboratory Animal Management Committee of Zhejiang University (Approval No. 20141102).

Statistical Analysis

One-way ANOVA was used and followed by pair-wise comparison between viral strains using the Student-Newman-Keuls method or Student's *t*-test, where appropriate, to extract statistical differences for viral titers and genomic RNA [18]

Results

Generation of Culture-Adapted RecC Viruses

PK-15 cells containing viral RNA delivered by electroporation were passaged every 3 days. Anti-E2 monoclonal antibody was used to determine expression of E2 by indirect immunofluorescence. In the initial passages, only E2 protein expression was observed in PK-15 cells. Virus particles were detected in the cell lysates or in the culture supernatants from the 10^{th} passage. To obtain higher titers, we completed a total of 110 passages (Fig. 2). Viral particles began to appear in the culture supernatant after 10 passages with low level of titers. By titrating the virus stocks collected at every 10 passage, we found that the titer increased with continuous passaging to $10^{4.0}$ $TCID_{50}$/ml at passage 40 and rose to a peak of $10^{6.0}$ $TCID_{50}$ $TCID_{50}$/ml at passage 80 (Fig. 2, with the virus stocks named by their passage times, such as RecCpp40 or RecCpp80). There was significant increase of virus titers in passages between 10 and 15/20, 15/20 and 40/60, as well as 40/60 and 80 ($p < 0.05$). No further increase was seen after passage 80. Continuous culture stopped at passage 110. Increased growth was also apparent in additional 10-passage subcultures in ST cells with RecCpp80, as compared with RecCpp40 ($p < 0.05$). These results indicate that the recombinant virus RecC was successfully rescued and continuous passaging led to increased virus yield.

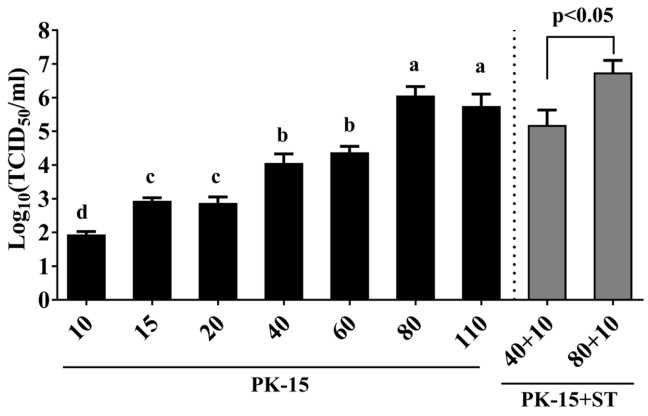

Fig. 2. Titration of cell culture-adapted RecC virus variants after continuous passaging.

The rescued virus in PK-15 cells was passaged every 4 days. Infected PK-15 cells were collected at every passage for titration. A total of 110 passages were conducted. The titers of RecC virus variants were determined by the end-point dilution method in PK-15 cells ($TCID_{50}$/ml). The titers between passages labeled with different lower-case letters differed at $p < 0.05$ by the Student-Newman-Keuls method. RecCpp40 and RecCpp80 were then subjected to further passaging in swine testicle cells for another 10 rounds. The titers of $RecCpp40_{+10}$ and $RecCpp80_{+10}$ were analyzed by Student's *t*-test.

In Vitro Growth Kinetics of PK-15 Cell Culture-Adapted RecC Viruses Derived from Different Passages

To determine if the recombinant viruses enhanced their replication potential upon continuous passages in PK-15 cells, the virus stocks derived from different passages were used in the growth kinetics assay using PK-15 cells or ST cells at MOI of 0.001. Figs. 3A and 3B show that high-passage viruses RecCpp80 and RecCpp110 produced about $10^{5.0\text{-}5.5}$ $TCID_{50}$/ml at 96 hpi in both cell lines, whereas the titers of RecCpp20, RecCpp40, and their parent C-strain virus were around $10^{3.0}$ $TCID_{50}$/ml (statistically significant compared with RecCpp80 or RecCpp110 at 96 hpi; $p < 0.05$). RT-qPCR revealed that viral RNA accumulation within infected cells at 96 hpi did not show significant differences ($p = 0.21$ by one-way ANOVA) among the recombinant viruses of different passages on either cell line (Figs. 3C and 3D, shown as normalized genomic RNA copies). These data suggest that RecC at higher passages in PK-15 cells might have adaptive mutations that favor their replication in both cell lines. Because RecCpp80 and RecCpp110 had similar in vitro growth potential, we chose RecCpp80 as the culture-adapted virus for further studies.

In Vitro Growth Properties of ST Cell Culture-Adapted RecC Viruses

Continuous passage of RecC in PK-15 cells led to adaptation in both PK-15 and ST cells. The virus stock of RecCpp40 or RecCpp80 from PK-15 cells was individually passaged for 10 rounds in ST cells, resulting in two virus pools with titers of $10^{5.0}$ $TCID_{50}$/ml ($RecCpp40_{+10}$) or $10^{6.5}$

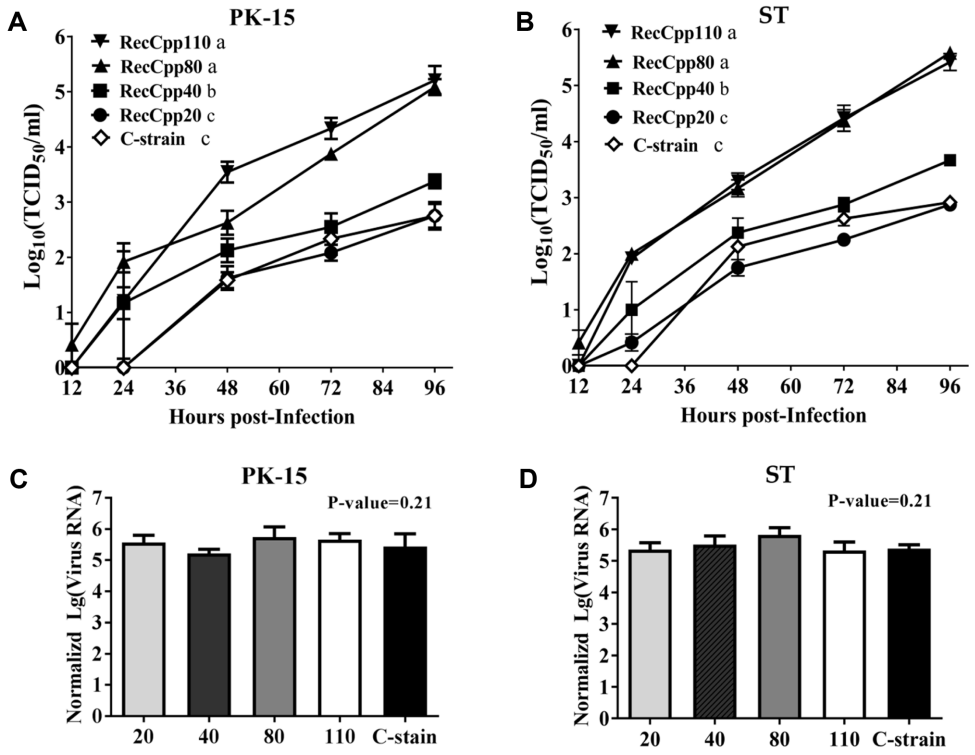

Fig. 3. In vitro growth kinetics of the parent C-strain and PK-15 cultured virus variants RecCpp20, RecCpp40, RecCpp80, and RecCpp110 in PK-15 or swine testicle (ST) cell lines.

The cells were infected (MOI = 0.001) either with C-strain or with any of the RecC virus variants. Virus yields obtained at the indicated hours post infection (hpi) were titrated in PK-15 cells (**A**) or ST cells (**B**). Infected cell lysates were also collected at 72 hpi for quantification of the intracellular viral RNA by RT-qPCR (**C** and **D**). Data are the mean ± SD from three independent experiments. In panels A and B, the virus variants labeled with different lower-case letters (after the figure labels) differed signigicantly ($p < 0.05$) at 96 hpi by the Student-Newman-Keuls method. One-way ANOVA was used for viral genomic RNA levels (panels C and D, Lg equals to log10, similarly hereinafter).

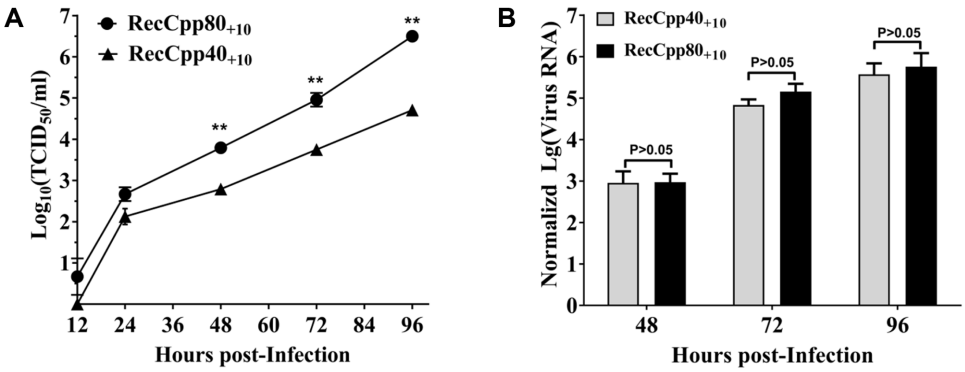

Fig. 4. In vitro growth characteristics of the RecCpp40$_{+10}$ and RecCpp80$_{+10}$ virus variants.

Swine testicle cell monolayers were infected (MOI = 0.001) with RecCpp40$_{+10}$ and RecCpp80$_{+10}$. Virus samples were collected at indicated times post infection for titration (**A**) and intracellular viral RNA quantitation (**B**). Data represent the mean ± SD from three independent experiments. The difference of $TCID_{50}$ between RecCpp40$_{+10}$ and RecCpp80$_{+10}$ was significant ($p < 0.01$) at 48, 72, and 96 hpi, whereas the RNA copies between these two virus variants did not differ significantly ($p > 0.05$).

$TCID_{50}$/ml (RecCpp80$_{+10}$), respectively, showing significant differences ($p < 0.05$, Fig. 2). To further characterize the growth potential of such adapted recombinant viruses, ST cells were infected in parallel with RecCpp40$_{+10}$ or

RecCpp80$_{+10}$ at MOI of 0.001, and the total viruses produced were titrated in ST cells. Fig. 4A shows that RecCpp80$_{+10}$ produced a higher titer that reached $10^{6.5}$ TCID$_{50}$/ml by 96 hpi, whereas the corresponding titer for RecCpp40$_{+10}$ was $10^{4.75}$ TCID$_{50}$/ml, significantly lower than RecCpp80$_{+10}$ ($p < 0.01$ by two-tailed Student's *t*-test). Moreover, there were no significant differences of viral RNA between RecCpp80$_{+10}$ and RecCpp40$_{+10}$ during infection in ST cells (Fig. 4B).

Cell-Adapted Virus Variant RecCpp80$_{+10}$ Failed to Induce Fever in Rabbits

C-strain is able to induce typical fever responses in rabbits. To assess whether continuous passaging of the C-strain-based recombinant viruses in cell lines would affect its adaptation to rabbits, RecCpp40$_{+10}$ and RecCpp80$_{+10}$ were tested for fever responses in parallel with their parent C-strain (as positive control). Rabbits inoculated with

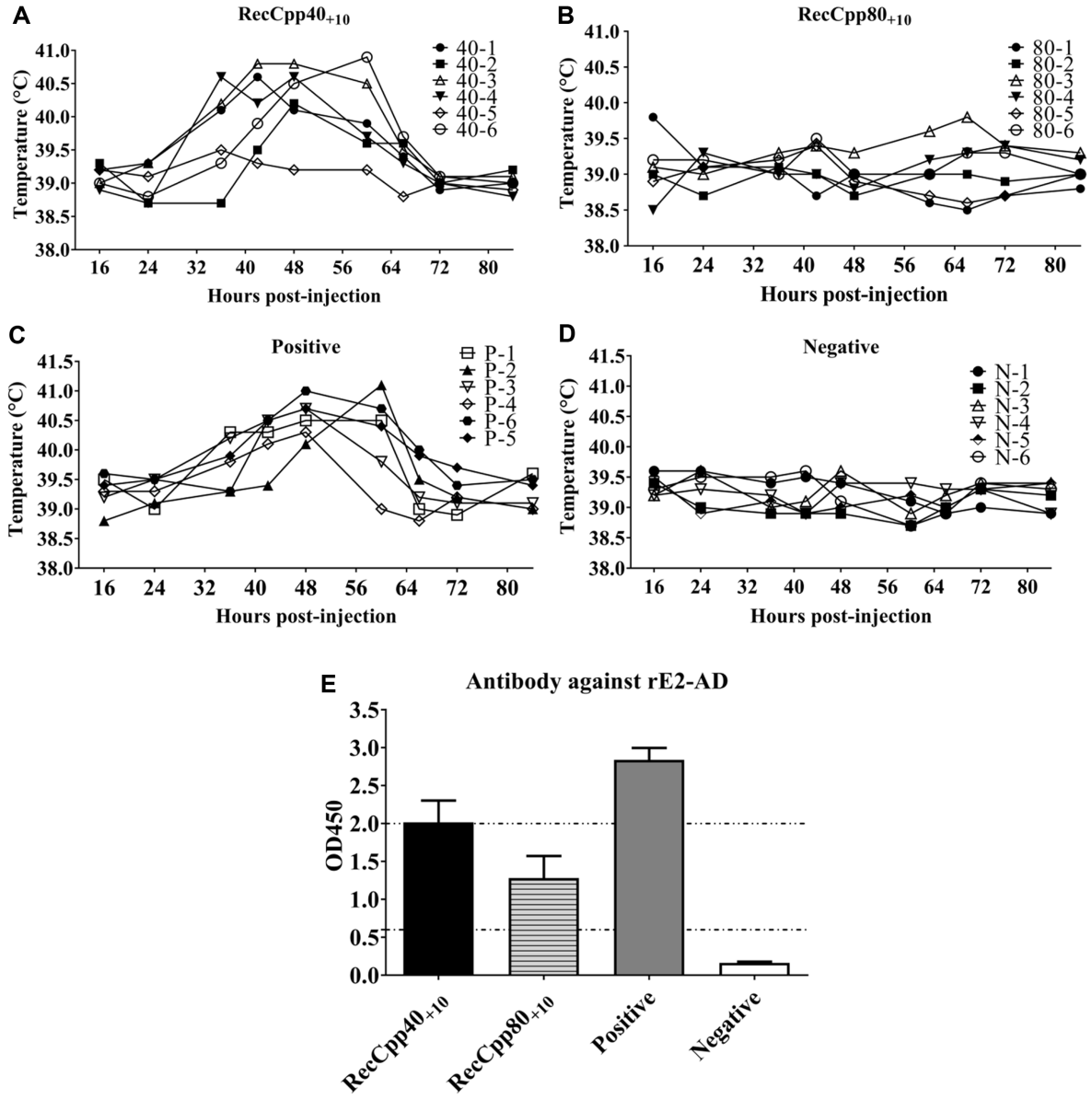

Fig. 5. Fever responses and antibody levels in rabbits inoculated with RecCpp40$_{+10}$ and RecCpp80$_{+10}$.

Twenty-four 2-kg weight New Zealand white rabbits were randomly assigned to four groups (6 per group) and were inoculated intravenously with indicated viruses or DMEM control via the ear vein. Rectal temperature was measured every 8 h post-inoculation to record the fever response. At 28 days post-inoculation, sera of all rabbits were harvested to monitor the anti-E2 antibodies by indirect ELISA. An OD$_{450}$ value higher than 2.0 was considered as a strong positive sample after vaccination, whereas an OD$_{450}$ value lower than 0.6 was treated as a negative sample.

RecCpp40$_{+10}$ and C-strain exhibited increased rectal temperature from 36 hpi that lasted for 16–24 h (Figs. 5A and 5C). The mock-infected rabbits did not show any temperature changes (Fig. 5D). However, RecCpp80$_{+10}$ did not induce a fever response as well (Fig. 5B). Indirect ELISA demonstrated that rabbits inoculated with all RecC viruses were positive for E2 specific antibodies, although there were differences in the antibody levels between or among the viruses (Fig. 5E). These results indicate that both recombinant viruses induced antibody responses in rabbits, but only the low-passage RecC40$_{+10}$ induced fever.

Sequence Analysis of the Adapted RecC Virus Variants

Sequencing analysis revealed that RecCpp40 contained three nucleotide mutations relative to the C-strain (Table 1). Mutations from A to G or from A to T were detected at position 26 of 5′-UTR in some of its clones. The mutation from C to A at 1802 resulted in S476R substitution in Erns, a protein located on the surface of CSF virions and involved in virus-cell attachment [19, 20]. The T to A mutation of 3310 caused a M979K substitution in the E2 protein. RecCpp80 harbored six different mutations relative to parental C-stain (Table 1). The two mutations A to T in 26 and C to A in 1802 were identical to RecCpp40. However, RecCpp80 also had mutation at 3310 but was different from RecCpp40 by having T to G with M979R substitution. Three additional amino acid substitutions were found in RecCpp80 only: G to A at 83 in 5′-UTR, G to T at 3531 with V1053L in E2, and C to T at 8268 with L2645F in NS4B. Sequencing was also conducted with both RecCpp40$_{+10}$ and RecCpp80$_{+10}$, and no additional mutations were seen during additional passages in ST cells.

Effects of Individual Mutations on Growth of the C-Strain-Based Mutant Viruses

An early study showed that S476R mutation could enhance CSFV replication in PK-15 cells [21]. Site-directed mutagenesis was performed only with the five mutations based on the RecCpp80 virus, with the C-strain infectious clone as the backbone, to assess the effect of each mutation on virus replication. Considering that more passages in cells might cause additional mutations in the viral genome, the mutant viruses were obtained from electroporated ST cells at passage 10 and named by their mutation positions. The titers for RecC-26, RecC-83, RecC-3310, and RecC-3531 viruses were about 10^4 to 10^5 $TCID_{50}$/ml. RecC-8268 was not included for growth kinetics because of its low titer (only 100 $TCID_{50}$/ml). In vitro growth kinetics in the ST cells showed that mutation at nt3310 (RecC-3310) significantly improved growth, with $10^{5.0}$ $TCID_{50}$/ml at 96 hpi, relative to the other two mutant viruses RecC-26 and RecC-83, and also the C-strain virus ($p < 0.05$). However, this single mutation did not seem to be sufficient to confer cell culture adaptation since the virus titer of RecCpp80$_{+10}$ was nearly 25-fold higher than RecC-3310 ($p < 0.05$). Resequencing of RecC-26, RecC-83, and RecC-3531 at passage 10 showed

Table 1. Mutations in recombinant viruses RecCpp40 and RecCpp80 as compared with C-strain (GenBank: HM175885).

Gene segments	Position	RecCpp40			RecCpp80		
		Nucleotide change	Amino acid change (position)	Clones	Nucleotide change	Amino acid change (position)	Clones
5-UTR	26	A → G	N/A	2/6	A → G	N/A	3/6
	26	A → T	N/A	4/6	A → T	N/A	3/6
	83				G → A	N/A	5/6
Erns	1802	C → A	Ser → Arg (476)	3/6	C → A	Ser → Arg (476)	6/6
E2	3310	T → A	Met → Lys (979)	6/6	T → G	Met → Arg (979)	6/6
	3509				C → T	N/A	3/6
	3531				G → T	Val → Leu (1043)	4/6
NS3	5903	T → C	N/A	6/6	T → C	N/A	6/6
	6309	T → C	N/A	6/6	T → C	N/A	6/6
NS4B	7586				G → T	N/A	2/6
	8036	T → C	N/A	6/6	T → C	N/A	6/6
	8268				C → T	Leu → Phe (2645)	4/6
NS5A	8519	C → T	N/A	5/6	C → T	N/A	6/6

All nonsynonymous and silent mutations (N/A, not applicable or no amino acid changes) are listed. Eight clones of each RecC virus were sequenced and the numbers of clones containing the indicated mutations are shown.

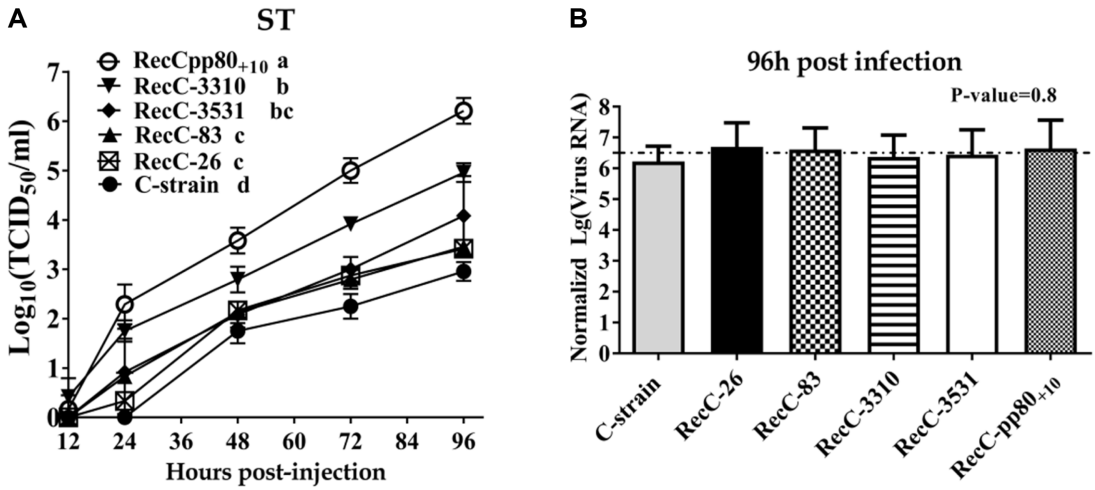

Fig. 6. Effects of four individual mutations at 26, 83, 3310, and 3531 on in vitro replication of the recombinant CSFVs.
Cell monolayers were infected (MOI = 0.001) with individual mutant viruses RecC-26, RecC-83, RecC-3310, and RecC-3531. Virus titration was conducted at the indicated times. Intracellular virus RNA levels at 96 hpi were analyzed by RT-qPCR. In panel A, the virus variants labeled with different lower-case letters (after the figure labels) differed significantly ($p < 0.05$) at 96 hpi by the Student-Newman-Keuls method.

that some of the mutant viruses already acquired mutations with S476R (T1802A) and M979L (T3310A) during the 10-round passages in ST cells. Besides this, RecC-3310 also showed S476R (T1802A) mutation at passage 10.

Discussion

The C-strain has been considered as the most effective and safe live attenuated vaccine that could induce rapid and long-lasting immunity against most CSFV isolates [6, 22–24]. Different from other two cell-adapted vaccine strains, GPE$^-$ and Thiverval, C-strain was attenuated by serial passages in rabbits and exhibited weak growth in cell cultures [9, 10, 25]. This is an apparent drawback to the vaccine industry. Therefore, selection of cell-adapted C-strain viruses and identification of its encoding amino acids critical for adaptation would be important for C-strain vaccine production. In HCV, successive passaging of different isolates in cell cultures resulted in several cell-adapted variants that contain specific amino acid changes in different genes [14, 26–28]. Here, we report a cell-adapted CSFV variant, RecCpp80, that exhibited good growth in ST cells with over 1,000-fold higher titer than its parent C-strain.

We found that the recombinant viruses with notably enhanced growth in both PK-15 and ST cells emerged at around 80 passages. Because there were virtually no significant differences of RNA replication among the recombinant viruses obtained at passages 20, 40, 80, and 110, we suppose that the cell-adapted phenotype of the C-strain in PK-15 or ST cells may not be due to increased replication of the genomic RNA, but to enhanced capacity of general virus reproduction (shown as total virus yield). This was confirmed by the growth curves and RNA replication levels of recombinant viruses RecCpp40$_{+10}$ and RecCpp80$_{+10}$ that were subjected to 10 more passages in ST cells.

RecCpp40 was then selected together with RecCpp80 for genomic sequencing for comparison with their parent C-stain. Since CSFV does not cause any cytopathic effect in the PK-15 or ST cell line, we could not purify RecCpp40 and RecCpp80 by the plaque assay. Thus, the existence of more than one virus sequence variant at each cell passage could be possible with different degrees of dominance of one variant over several others within the population. Unless explicitly indicated, mutations described in Table 1 were given out of a total of six sequenced. There are four mutations, in a total of six, that are unique for RecCpp80: G to A at 83 in 5′-UTR, 3310 from T to G (M979R in E2), G to T at 3531 (V1053L in E2), and C to T at 8268 (L2645F in NS4B). The S476R mutation in protein Erns is consistent with a previous report that this mutation would enhance the interaction between CSFV and cell surface heparin sulfate to facilitate virus attachment and invasion [21]. However, the S476R mutation is also found in RecCpp40, which exhibited moderate growth in both ST and PK-15 cells. Thus, we only focused on the other five mutations that have not been reported.

By sited-directed mutagenesis, we attempted to rescue mutant viruses containing each one of the above five single

mutations. Sequencing analysis revealed that mutant viruses RecC-26, RecC-83, and RecC-3531 also acquired S476R (C1802A) and M979R (T3310G) mutations during the rescuing process, thus bearing triple mutations. RecC-3310 also had additional mutation of S476R. Thus, it is possible that S476R is a common mutation of cell-cultured CSFV as was seen with the *Brassica* strain subjected to continuous passage [21]. Because both RecC-3310 and RecC-3531 ended with higher titers than the others, the nonsynonymous mutations at 3310 and 3531 could be important for cell culture adaptation. Further research is required to see if the mutation at 3310 from T to A (M979L) or from T to G (M979R) is important for virus replication, or if combined mutations at 1802, 3310, and/or 3531 are essential for replication of the C-strain virus. Notably, the two mutations M979R and V1043L located in the C-terminus of E2 are known to be outside of the antigenic domains of E2 [29]. Therefore, such cell culture adapted viruses would not affect the antigenicity of E2. Similar results were also shown in previous reports that CSFV could reach a high titer and their antigenicity did not undergo any change after cell culture adaptation [30-32]. Since additional 10 passages, in ST cells, of the PK-15-cultured RecCpp80 did not lead to any further mutations, we suggest that the mutant virus at passage 80 could be genetically stable.

The quality and efficacy of the commercial C-strain vaccine has been routinely tested by rabbit inoculation [6] based on its adaption to rabbits with a typical fever response [22]. RecCpp80 did not induce a fever response in rabbits, whereas RecCpp40 induced a similar fever response as its parent C-strain. The antibody responses of each group of rabbits were confirmed by ELISA, indicating that all viruses were successfully inoculated into animals. This finding indicates that the high passage process in cell culture might have changed the adaption phenotype of the C-strain in rabbits. In fact, the molecular basis of C-strain adaption in rabbits has not yet been elucidated. A recent report suggests that both UTRs of the C-strain were essential for fever response, but not necessary for virus replication in rabbits [22]. Another molecular maker for rabbit adaption of lapinized CSFVs is the 12-nt insertion (CUUUUUUCUUUU) in the 3′-UTR [33]. In the present study, there were two mutations in the 5′-UTR of RecCpp80, and the 12-nt insertion sequence was stable. Thus, the effects of these two mutations in the 5′-UTR, either alone or in combination, on fever induction require further investigation. Besides this, both RecC26 and Rec83 at passage 10 also contained S476R (T1802A) and M979L (T3310A) and might have contributed to the successful rescue.

Taken together, this study clearly indicates that continuous passaging of the C-strain-based recombinant viruses in PK-15 cells could enhance its in vitro adaptation that resulted in about 1,000-fold increase of the virus yield. The nonsynonymous mutations at 3310 and 3531 might play major roles in enhanced in vitro replication of CSFV. Further studies are required to investigate if particular substitution at aa979 or combined substitutions of the three residues found in Erns and E2 would enhance viral replication to a level close to RecCpp80. The mutations in RecCpp80 that led to the loss of fever response in rabbits also await further investigation. Such findings may help design a modified C-strain for improved productivity of commercial vaccines at reduced production cost.

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