Development of recombinant rabies viruses vectors with Gaussia luciferase reporter based on Chinese vaccine strain CTN181

Jialiang Du [a], Qing Tang [a,*], Ying Huang [a], Willoughby E. Rodney [b], Lihua Wang [a], Guodong Liang [a]

[a] State Key Laboratory for Infectious Diseases Prevention and Control, State Key Laboratory for Molecular Virology and Genetic Engineering, Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, 155 Changbai St. Changping Dist., Beijing 102206, China
[b] Children's Research Institute, Children's Hospital of Wisconsin, 8701 Watertown Plank Road, Wauwatosa, WI 53226, USA

ABSTRACT

The recombinant *rabies virus* (RV) vectors encoding the secreted gene marker Gaussia luciferase (Gluc) were generated based on Chinese vaccine strain CTN181. Vectors included replication competent CTN-Gluc, CTN/G_{Q333R}-Gluc, in which the amino acid in position 333 of glycoprotein was mutated from glutamine (Q) to arginine (R), and replication constrained CTNΔG-Gluc, in which the glycoprotein encoding gene (G) was deleted. The growth of recombinant RVs in transfected cells was confirmed through biochemical assays of Gluc activities. Gluc expression in recombinant CTNΔG-Gluc virus was highest while that in CTN/G_{Q333R}-Gluc virus was lowest. The optimal time to harvest recombinant RVs was determined and the function of pathogenic and nonpathogenic rabies glycoprotein in virus recovery was examined. The addition of glycoprotein was slightly beneficial for virus recovery and the titer of rescued virus was lowered even when the amino acid in G333 position of glycoprotein was mutated from nonpathogenic Gln to pathogenic Arg. *Conclusions*: Viral vectors based on a human rabies vaccine strain CTN181 were successful. Gluc was useful as an *in vitro* gene marker for monitoring the growth of recombinant RVs iteratively in cell culture.

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1. Introduction

Methods to rescue infectious *rabies virus* (RV) entirely from cloned cDNA became possible in 1994 (Schnell et al., 1994). Reverse genetics technology has since improved (Inoue et al., 2003; Ito et al., 2003) and enabled the construction of replication competent (Foley et al., 2002; Hosokawa-Muto et al., 2006; Liu et al., 2010; McGettigan et al., 2003; McKenna et al., 2003, 2007; Mebatsion et al., 1996; Wanjalla et al., 2010; We et al., 2010; Zhao et al., 2010) and replication constrained (Cenna et al., 2008, 2009; Etessami et al., 2000; Gomme et al., 2010; Ito et al., 2005; Morimoto et al., 2005; Wickersham et al., 2007, 2010) RV vectors carrying foreign gene(s). One of the main merits of RV-based vectors is the high and stably targeted presentation of foreign genes *integrated* into the permissive region of RV genome, in the form of an extra transcription unit and in permissive cell lines complemented for replication constrained vectors. Other advantages of RV-based vectors include expressing transgenes at extremely high levels (Mebatsion et al., 1996), eliminating the major source of cytotoxicity (Dietzschold et al., 2008), genetically targeting infection to susceptible cells (Wickersham et al., 2010), and inducing strong humoral and cellu-

lar immune responses against infectious diseases especially which has no effective vaccines (McKenna et al., 2003). Previously, SAD B19 strain of RV was mainly and successfully used as a seed for the construction of viral vectors for generating live vaccines for HIV/SIV, HCV or SARS-CoV (Faber et al., 2005b; Faul et al., 2009; McKenna et al., 2003; Siler et al., 2002) and delivering cytokines as chemokines, cytochrome C, TNF-α and IFN-β (Pulmanausahakul et al., 2001; Faber et al., 2005a; Faul et al., 2008; Zhao et al., 2009; Kuang et al., 2009). However, since even SAD B19 and its derivate SAD I16 were pathogenic when inoculated directly into mouse brains, further efforts to attenuate the virus are necessary (Vos et al., 1999; Mebatsion, 2001; Rasalingam et al., 2005). Therefore, in this report, the highly attenuated Chinese human RV vaccine strain CTN181 (Du et al., 2008) is recommended as a more suitable candidate seed strain for development of new viral vectors.

Another requirement is a novel gene marker for rapid and convenient evaluation of the growth and proliferation of recombinant RV vectors because cytopathic effect (CPE) cannot be induced even with highly pathogenic RVs. In previous studies, the beta-galactosidase (β-gal), bacterial chloramphenicol acetyltransferase (CAT), green fluoresence protein (GFP), and firefly luciferase (luc) were used as reporter molecules to monitor the replication of recombinant RVs *in vitro* and/or *in vivo* (Conzelmann and Schnell, 1994; Mebatsion et al., 1996; Huang et al., 2010; Liang et al., 2010). However, there is need for a gene marker which can more easily
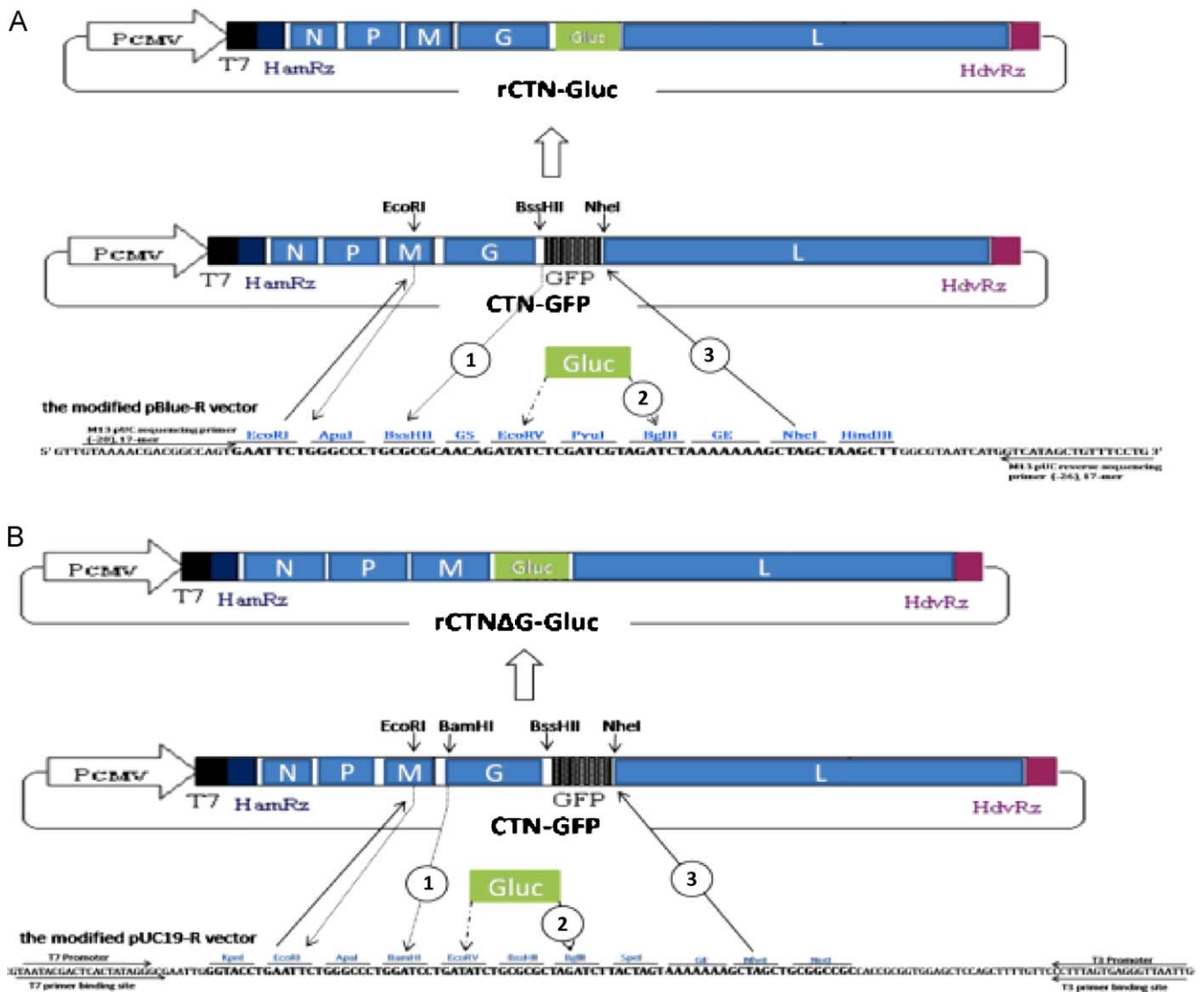
Fig. 1. Schematic diagram for the construction of rCTN-Gluc (A) and rCTNΔG-Gluc (B).

and sensitively monitor the growth and proliferation of recombinant RV vectors. The Gluc possesses a natural secretory signal and upon expression is secreted from cultured mammalian cells into the cell medium in an active form, which allows for multiple samples to be assayed from the same transfected cells at different times. In addition, Gluc assay generates over 1000-fold higher bioluminescent signal intensity in cultured mammalian cells, compared to Firefly and Renilla Luciferases (Tannous et al., 2005). In this study, we investigated the feasibility of Gluc reporter assay for convenient, sensitive and temporal monitoring of the growth and proliferation of recombinant RV vectors.

The Gluc gene was cloned into three genome plasmids including the full-length genome of CTN (rCTN), rCTN with the G_{Q333R} mutation (rCTN/G_{Q333R}, in which the Gln in 333 position of glycoprotein was mutated to Arg), and rCTN with G-deficient (rCTNΔG, in which the G gene was deleted) to form rCTN-Gluc, rCTN/G_{Q333R}-Gluc, rCTNΔG-Gluc, on the basis of previously established reverse genetics system based on CTN181 strain (Huang et al., 2010). The three recombinant viral vectors were produced in transfected cells and confirmed infectious. Gluc activities were used to indicate and compare the growth of different recombinant RV vectors in cell culture. Moreover, the best harvest time for the recombinant RV vectors were determined by the quantitative detection of Gluc expression.

These results suggest that human rabies vaccine strain CTN181 could be a potential candidate for establishment of viral vectors including replication competent and replication constrained vectors carrying foreign genes.

2. Materials and methods

2.1. Cells culture

Baby hamster kidney (BHK-21) cells (ATCC, Rockville, MD) and mouse neuroblastoma (MNA) cells (kindly provided by Wuhan Institute of Biologic Product, Wuhan, China) were maintained in Dulbecco's modified Eagle's medium (D-MEM) containing 10% heat-inactivated fetal bovine serum (FBS) (Gibco-BRL, Gaithersburg, MD) at 37 °C in a humidified 5% CO_2 atmosphere.

2.2. Construction of recombinant RV clones with encoding segment of Gaussia luciferase

Using conventional cloning techniques, the recombinant RV vectors were synthesized (as shown in Fig. 1) based on the previously established reverse genetics system CTN-GFP (Huang et al., 2010).

Table 1
The single strand nucleotide acid and primer sequences used in this study for cloning and RT-PCR.

Names	Sequence (5′–3′)
MCS-F	GAATTCTGGGCCCTGCGCGC*AACA*GATATCTCGATCGTAGATCT*AAAAAAA*GCTAGCTAAGCTT
MCS-R	CTTAAGACCCGGGACGCGCG*TTGT*CTATAGAGCTAGCATCTAGA*TTTTTTT*CGATCGATTCGAA
MCS-F′	CTGAATTCTGGGCCCTGGATCCTGATATCTGCGCGCTAGATCTTACTAGT*AAAAAAA*GCTAGCTGC
MCS-R′	CATGGACTTAAGACCCGGGACCTAGGACTATAGACGCGCGATCTAGAATGATCA*TTTTTTT*CGATCGACGCCGG
M-EcoRI	GGGTATGGTGTATCAACATGAATTC
G-AACA	TAGGATCCTTGAGGGA*TGTT*AAAAG
Gluc-EcoRV	ATCGATATCGGATCCAGCCACCAT
Gluc-BglII	CTAGATCTAGATGCATGCTCGAGC
G$_{Q333R}$-F	TACAAATCGGTC**CGA**ACTTGGGATGAGATCATCCCC
PB-F	TACCTTTACATTTTGAGCCTCTTGG
PB-R	GGATCAATCATCATGGTGAGAGTTT
PU-F	AGGCAGGTCATCTCCTTCATATTTC
PU-R	CTGGATCAATCATCATGGTGAGACA

First, rCTN-Gluc was constructed as follows: the multi-cloning sites (MCS) of pUC19 vector (NEB, U.S.A.) was replaced to EcoRI–ApaI–BssHII–*AACA*–EcoRV–PvuI–BglII–*polyA*–NheI–HindIII, formed by annealing of two artificially synthesized, complemented single DNA chains named MCS-F and MCS-R (Table 1) to designate pUC19-R. Then, a 1930 nt-length EcoRI–BssHII DNA fragment including the latter half of the M gene, the M–G intergenic region (IGR), the complete G gene was cloned into the EcoRI–BssHII site of pUC19-R to designate subclone pUC19-R′. The amplified Gluc gene with the primer pairs Gluc-F and Gluc-R (Table 1) was cloned into the EcoRV–BglII site of pUC19-R′ to generate plasmid pUC19-R′-Gluc. Subsequently the EcoRI–NheI fragment in CTN-GFP was replaced by EcoRI–NheI fragment from pUC19-R′-Gluc to form full-length rCTN-Gluc construct.

Next, the plasmid rCTN/G$_{Q333R}$-Gluc was constructed using a specific single-site mutation primer G$_{Q333R}$-F (the codon mutated from CAA to CGA as shown in bold and underlined in Table 1) and the QuikChange Multi-Mutagenesis kit (Stratagene, U.S.A.) according to the manufacturer's instruction using rCTN-Gluc plasmid as the template. Briefly, 10× QuikChange Multi reaction buffer 2.5 μl, QuikSolution 0.5 μl, rCTN-Gluc plasmid 1 μl, mutation primer G$_{Q333R}$-F 2 μl, dNTP mix 1 μl, QuikChange Multi enzyme blend 1 μl, double-distilled H$_2$O 17 μl were added together to final volume of 25 μl, and the reaction was performed in a PCR cycler with condition of pre-denaturation at 95 °C for 1 min, and 30 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, extension at 65 °C for 30 min. Following temperature cycling, the reaction was placed on ice for 2 min to cool the reaction to ≤37 °C. Dpn I restriction enzyme (1 μl) digested the parental ds-DNA (non-mutated rCTN-Gluc) at 37 °C for 1 h. After transformation, several clones were picked out for confirmation by DNA sequencing.

Finally, the plasmid rCTN△G-Gluc was constructed as follows. The MCS of pBluescript II SK (+) vector (Stratagene, U.S.A.) was replaced by KpnI–EcoRI–ApaI–BamHI–EcoRV–BssHII–BglII–SpeI–*polyA*–NheI–NotI, formed by annealing the single DNA chains named MCS-F′ and MCS-R′ in Table 1, to designate pBlue-R. Then, a 305 nt-length amplified EcoRI–BamHI DNA fragment including the latter half of the M gene, the M–G intergenic region and the transcriptional start signal (AACA) of G gene with the primer pair M-EcoRI and G-AACA was cloned into the EcoRI–BamHI site of pBlue-R to designate subclone pBlue-R′. The amplified Gluc above was cloned into the pBlue-R′ to generate pBlue-R′-Gluc plasmid, finally the EcoRI–NheI fragment in CTN-GFP were replaced by EcoRI–NheI fragments from pBlue-R′-Gluc to form the rCTN△G-Gluc construct.

Both of the original and artificially introduced transcriptional start and stop signals (as shown in italic and underlined in Table 1) could be used for transcription of the Gluc gene and each of the recombinant plasmids was confirmed by restriction enzyme digestion (Fig. 2) and sequencing.

2.3. Recovery of recombinant rabies viruses from cloned cDNAs

The recombinant RV clones including rCTN-Gluc, rCTN/G$_{Q333R}$-Gluc and rCTN△G-Gluc were recovered as before (Huang et al., 2010). Also we subcloned a PCR-amplified cDNA fragment of the full-length G gene into pVAX1 (Invitrogen, U.S.A.) and named the resulting plasmid pVAX-G as an additional helper plasmid. Briefly, 80% confluent BHK-21 cells cultured in six-well plate were transfected using Lipofectamine 2000 reagent (Invitrogen, U.S.A.) with 2 μg of rCTN-Gluc, rCTN/G$_{Q333R}$-Gluc or rCTN△G-Gluc, 1 μg of pVAX-N, 0.5 μg of pVAX-P, 0.5 μg of pVAX-L, and/or 0.4 μg of pVAX-G. The transfected cells were maintained for 3–5 days at 37 °C in a humidified 5% CO$_2$ incubater. The supernatants and transfected cells of replication competent viruses were subjected to three freeze–thaw cycles, and were then added onto MNA cells and incubated for 3 days at 34 °C to allow virus propagation while that of G-deficient virus was harvested and stored at −80 °C. The rescued recombinant RVs were designated as CTN-Gluc, CTN/G$_{Q333R}$-Gluc and CTN△G-Gluc.

2.4. Confirmation of recovered recombinant RVs by IFA and RT-PCR

At four days post-transfection, the transfected cells were examined by the immunofluorescence assay (IFA) to validate the formation of RNPs inside the cells. To further confirm whether the recovered RVs were derived from rCTN-Gluc, rCTN/G$_{Q333R}$-Gluc and rCTN△G-Gluc, the reverse transcription polymerase chain reaction (RT-PCR) was performed with specific primers. Total RNA from
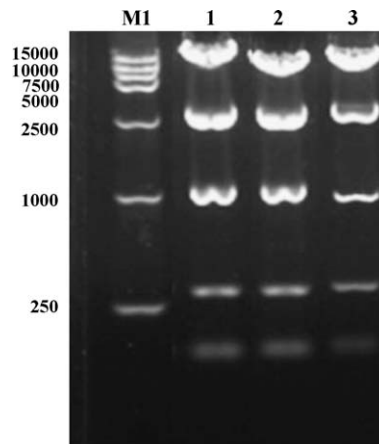

Fig. 2. Confirmation of rCTN-Gluc, rCTN△G-Gluc, rCTN/G$_{Q333R}$-Gluc plasmids by EcoRV digestion. Lanes 1 (rCTN-Gluc) and 3 (rCTN/G$_{Q333R}$-Gluc): 10407, 3160, 1148, 322 and 102 bp); Lane 2 (rCTN△G-Gluc): 8791, 3160, 1148, 322 and 102 bp.

the supernatant from the infected MNA cells was extracted using TRIZOL reagent (Invitrogen, U.S.A.), the single-stranded cDNA was synthesized using Ready-To-Go You-Prime First-Strand Beads (GE, UK) with random primer pdN(6) and the PCR reaction was performed using the single-stranded cDNA as a template. Primer pair PB-F and PB-R was used to confirm the recovered RV from rCTNΔG-Gluc by producing a 727 bp fragment, while PU-F and PU-R to confirm the recovered RVs from rCTN-Gluc and rCTN/G_{Q333R}-Gluc by producing a 709 bp fragment each. DNA sequencing was carried out to reconfirm the mutation of the recombinant RVs from rCTN/G_{Q333R}-Gluc. In order to identify false positive results due to the presence of recombinant cDNA clones in the supernatant of infected MNA cells, PCR was also performed using DNA isolated from supernatant of cells infected with rescued viruses as template.

2.5. Gluc activity assay

For time points 24, 48, 72 and 96 h post-transfection, as little as 15 μl supernatant was collected for Gluc assay using the commercial kit (NEB, U.S.A.) according to the manufacturer's instructions. Briefly, 50 μl of the 1X GLuc assay working solution was added to the collected 15 μl supernatant and the luminescence was measured promptly with a luminometer.

2.6. Titration of the recombinant RVs

BHK-21 cells were infected with 5-fold serial dilutions of transfected cell supernatants and incubated in 96-well plates at 37 °C for 24 h. The cells were then fixed in 80% (v/v) acetone and stained with Rabies DFA Reagent as mentioned above. Foci were counted under a fluorescence microscope and calculated as focus forming units/ml (FFU). All titrations were performed in duplicate wells, and the average value was calculated as the titer.

3. Results

3.1. Construction and confirmation of the recombinant cDNA clones

Through the artificially modified subclones pUC19-R′ and pBlue-R′, the replication competent (rCTN-Gluc and rCTN/G_{Q333R}-Gluc) and G-deficient (rCTNΔG-Gluc) cDNA clones based on CTN181 strain were established. The novel gene marker Gluc was inserted into the G–L and M–L intergenic region (IGR) respectively and the original pseudogene region was deleted. All the recombinant cDNA clones were subjected to restriction endonuclease digestions (Fig. 2), and then further confirmed by DNA sequencing. The results showed that the nucleotide sequences of the cDNA clones were obtained successfully with the expected nucleotide deletion and mutation after recombination and cloning. In addition, among the three cDNA clones, the IGRs between G and Gluc genes, Gluc and L genes were substituted and shortened to six nucleotides (GCGCGC and GCTAGC), respectively (data not shown) compared with 24 nucleotides between G–L IGR in the genome of parental CTN181 virus.

Moreover, the fourth helper plasmid pVAX-G was constructed, which transient expresses and provides glycoproteins for virus assembly in transfenction procedure in trans. The results of enzyme digestion and sequencing were not shown.

3.2. Rescue and confirmation of recombinant RVs from cloned cDNAs

The optimal transfection efficiency in BHK-21 cells was obtained by co-transfection with 2 μg rCTN-Gluc, rCTN/G_{Q333R}-Gluc or rCTNΔG-Gluc, and 1 μg pVAX-N, 0.5 μg pVAX-P, 0.5 μg pVAX-L and/or 0.4 μg pVAX-G in which the ratio between the genome plasmids and helper plasmids was similar as the previous report (Wickersham et al., 2010; Huang et al., 2010). Four days after transfection, the recombinant RV granules could be stained in the cytoplasm and membrane of the transfected BHK-21 cells (Fig. 3). The recovered viruses were named CTN-Gluc, CTNΔG-Gluc and CTN/G_{Q333R}-Gluc, respectively. This indicates that the recombinant RVs carrying Gluc gene were successfully rescued.

The RT-PCR results (Fig. 4) showed that the expected fragments were produced from the recombinant RVs (Fig. 4 lanes 1–3) but no product was produced from DNA extracts of supernatant of infected MNA cells (Fig. 4 lanes 4–6), which indicates that the DNA fragments did not originate from the cDNA clones rCTN-Gluc, rCTN/G_{Q333R}-Gluc and rCTNΔG-Gluc used for transfection and the G gene was indeed absent from the rCTNΔG-Gluc genome and the sequencing result also confirmed that the 333 position of G gene was successfully mutated from nonpathogenic Gln (Q) to pathogenic Asp (R) in the rCTN/G_{Q333R}-Gluc clones.

3.3. Gluc activity assay

The quantitative detection of Gluc activity in culture supernatant was performed to analyze and validate the efficiency of the rescued recombinant RV vectors. Briefly as little as 15 μl of culture supernatant was collected at different time points post-transfection (24 h, 48 h, 72 h, and 96 h) and used for Gluc activity detection with a luminometer. The results of Gluc activity assay in Fig. 5 showed that the expression of Gluc began from 24 h after transfection, increased gradually and there was no dramatic difference among the three expression systems from the three recombinant vectors before 48 h after transfection. Then the Gluc activity reached a peak at 72 h in the two of them (8.1–8.8×10^4 RLU in CTNΔG-Gluc and 3.5–5.3×10^4 RLU in CTN-Gluc) and at 96 h in CTN/G_{Q333R}-Gluc (0.6–2.5×10^4 RLU). After 72 h post-transfection, the Gluc activity decreased in CTNΔG-Gluc and CTN-Gluc systems rapidly or moderately while activity kept increasing in CTN/G_{Q333R}-Gluc system to 96 h or beyond.

3.4. Titers of recovered viruses in BHK-21 cells without amplification

Supernatants of the transfected cells with CTN-Gluc, CTNΔG-Gluc, and CTN/G_{Q333R}-Gluc recombinant RVs were titrated respectively for virus production. The result showed that the titers of the recombinant RVs in the condition of co-transfecting with 3 or 4 helper plasmids were as follows: the titers of CTNΔG-Gluc and CTN-Gluc were 7.8×10^4 (cotransfecting with the addition of pVAX-G) and 3.2–4.2×10^4 FFU/ml respectively at 72 h after transfection, and the titer of CTN/G_{Q333R}-Gluc was 1.1–1.7×10^4 FFU/ml at 96 h after transfection.

4. Discussion

This is the first report of a rabies virus vector based on a human vaccine strain compared with the previous rescued RVs including SAD-B19 (Schnell et al., 1994), RC-HL (Ito et al., 2001), HEP-Flury (Inoue et al., 2003) and ERA (Wu and Rupprecht, 2008). We recommend a more safe and effective seed virus for new rabies virus vector development. The genome of CTN181 strain has been characterized (Du et al., 2008). CTN-based human and animal rabies vaccine produced in Vero and primary hamster kidney cells were studied and used in China (Yu, 2006; Zhang et al., 2002; Cheng et al., 2008). Furthermore, our previous studies in the field of biological properties of CTN181 strain using the recombinant CTN-GFP virus demonstrated that it can stably expressing GFP in vitro and in vivo
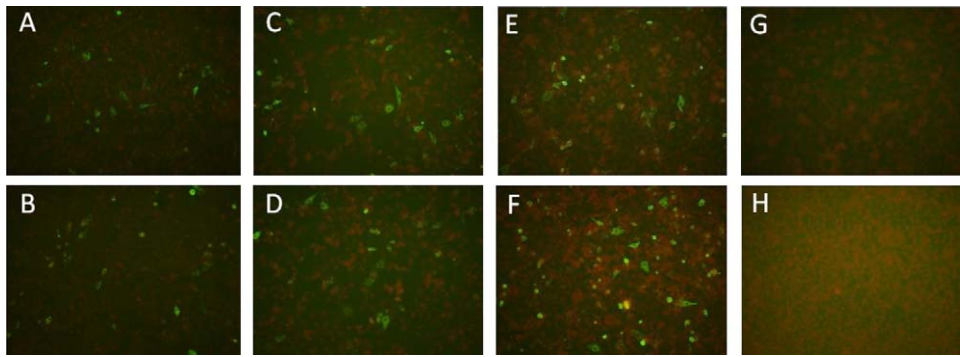
Fig. 3. Staining of rabies virus granules in BHK-21 cells 4 days after transfection. (A, C, E) Cells transfected with rCTN-Gluc, rCTN/G_{Q333R}-Gluc and rCTNΔG-Gluc plus pVAX1-N, P, L. (B, D, F) Cells transfected with rCTN-Gluc, rCTN/G_{Q333R}-Gluc and rCTNΔG-Gluc plus pVAX1-N, P, G, L. (G–H) Negative control.
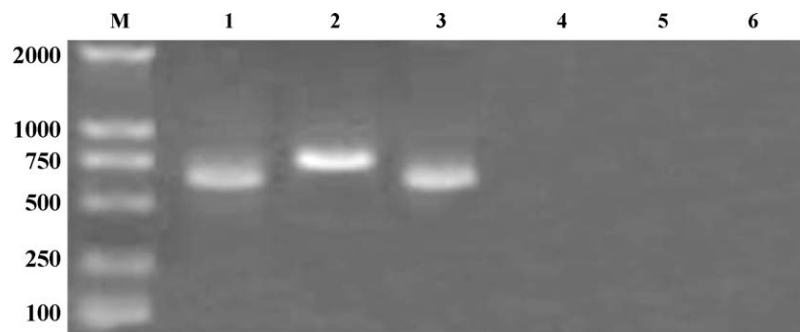

Fig. 4. RT-PCR and PCR products of CTN-Gluc, CTNΔG-Gluc and CTN/G_{Q333R}-Gluc virus were electrophoresed through 1% agarose gels, lanes 1–3, RT-PCR product of the recovered CTN-Gluc, CTNΔG-Gluc and CTN/G_{Q333R}-Gluc virus (length 709, 727, 709 bp). Lanes 4–6, PCR product with DNA extracts.
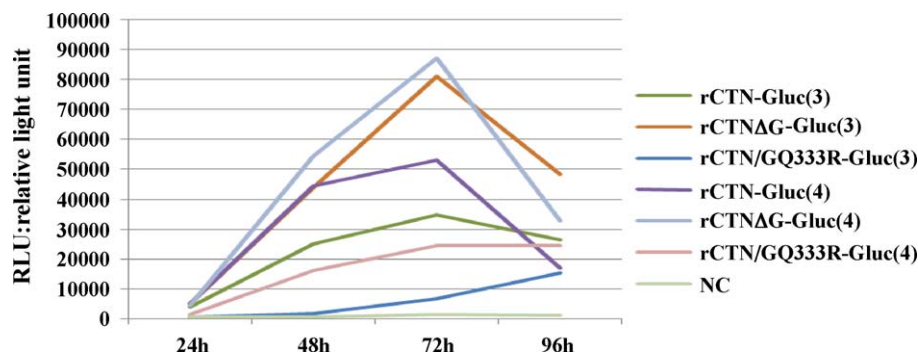

Fig. 5. Time-course of Gluc activity in cell culture medium co-transfected with rCTN-Gluc, CTNΔG-Gluc, CTN/G_{Q333R}-Gluc and the corresponding helper plasmids. The number in the parentheses (3 or 4) represents the genome plasmids transfected with three essential helper plasmids or extra addition of G-helper plasmid.

and it was nonlethal even inoculated intracerebrally in 4-week-old mice (Huang et al., 2010). Moreover, a biomolecular contrast agent, ferritin, was also successfully expressed, which suggests the possible application of this rabies vector for diagnostic or therapeutic gene delivery into the central nervous system (Liu et al., 2010).

In this study, we used the traditional molecular cloning techniques to generate the replication competent (CTN-Gluc and CTN/G_{Q333R}-Gluc) and G-deficient (CTNΔG-Gluc) RV vectors with the Gluc reporter gene from cloned cDNAs of the highly attenuated human rabies vaccine strain CTN181 used in China. Two subclones pBlue-R′ and pUC19-R′, which were designed based on the commercial vectors pBluescript II SK (+) and pUC19, facilitated the construction of recombinant RV vectors in our study because difficulties were faced in directly cloning the short Gluc gene (0.6 kb) into large RV vectors (replication competent vector was 15 kb and G-deficient vector was 13 kb). The BssHII and NheI sites in the G–L IGR of CTN-GFP are not always suitable for cloning of

transgenes. After transfection, a significant amount of RV granules was detected, which demonstrated that our established recombinant CTN181-based viral vectors could function well as predicted. In addition to the existence of virus granules, the RT-PCR results provided further evidence of marker gene expression in the transfected cells. Moreover, we found that the recombinant RV from rCTNΔG-Gluc without pVAX-G as an additional helper plasmid cannot infect fresh BSR cells while that from rCTNΔG-Gluc with pVAX-G can, when the recombinant RVs were propagated in MNA cells, which Is in accordance with the characterization of replication deficient RVs (Gomme et al., 2010; Etessami et al., 2000; Cenna et al., 2009).

Recently, the naturally secreted Gluc from the marine copepod *Gaussia princeps* has been demonstrated to be a sensitive and quantitative biomarker for monitoring biological processes *in vitro* and *in vivo* (Tannous, 2009; Chen et al., 2010; Zhu et al., 2011; Griesenbach et al., 2011; Feng et al., 2010; d'Enfert et al., 2010). It has several advantages over other commonly used reporters as

described in Section 1 part. Here, we seek to evaluate Gluc as a novel gene marker for real-time monitoring the growth of recombinant RV vectors in transfected cells and the results of Gluc activity assay facilitated the confirmation and comparison of different vectors.

First, the optimal time to harvest CTN-Gluc and CTNΔG-Gluc was determined to be 72 h after transfection, while that for harvest of CTN/G_{Q333R}-Gluc could be delayed to 96 h or even longer. Before this study, the harvest time for recombinant RVs depends on many factors, such as the concentration of different plasmids, condition of transfected cells, transfection techniques and so on. In present, the introduction of Gluc provides an indication for the harvest of different recombinant RVs through quantitative detection which is more convenient to perform, instead of empirical judgment which depends on the experience accumulation in a certain lab or group.

Second, compared with CTN-Gluc and CTN/G_{Q333R}-Gluc, CTNΔG-Gluc has potential to express gene marker Gluc at a higher level, which was the same as the result of a previous study that G-deficient vector expresses transgenes at extremely high levels (Wickersham et al., 2007). The gene marker Gluc was inserted in the form of an intact transcriptional unit including transcriptional start signal AACA, open reading frame of Gluc gene and transcriptional stop signal poly(A)$_7$. Therefore, Gluc can be transcribed and translated the same as virus proteins. Because there are five genes (totally 13510 nt) and Gluc gene is in the fourth position of CTNΔG-Gluc genome while there are six genes (totally 15133 nt) and Gluc gene is in the fifth position of CTN-Gluc and CTN/G_{Q333R}-Gluc genome, virus transcription and replication of recombinant CTNΔG-Gluc is greater than that of recombinant CTN-Gluc and CTN/G_{Q333R}-Gluc illustrating transcriptional attenuation.

Third, the G protein supplied in the recovery procedure can be slightly helpful for the formation of virus assembly. In this study, all the recombinant plasmids were transfected with/without the helper plasmid expressing RV glycoprotein. Gluc activity in systems with an G-helper plasmid was slightly higher than that in systems just with the three essential N, P and L-helper plasmids, although not dramatically (Fig. 5), which was consistent with previously study (Morimoto et al., 2000).

Fourth, one promising application of RV-based vectors is for gene transfer and genetic engineering vaccines for other infectious diseases. High-level expression of therapeutic genes or antigens of other pathogens may be necessary for optimal use. Virus yield in MNA cells was quite low when compared to the parental virus and other RV-based vectors reported previously (Schnell et al., 1994; Ito et al., 2001; Inoue et al., 2003; Wu and Rupprecht, 2008), and the same as the previously reported recombinant CTN-GFP and CTN-ferritin viruses (Huang et al., 2010; Liu et al., 2010). The reason for this is not clear. The efficiency of rescued virus is affected by multiple factors (Inoue et al., 2003). Before the study, we suspected whether the nonpathogenic glycoprotein plays a role in the low rescued virus titer. Previous studies had shown that the attenuation of RV is associated with its ability to induce apoptosis in neuronal cells, and that the G protein is responsible for triggering the apoptosis cascade (Jackson and Rossiter, 1997). Unfortunately, the results from this study showed that the level of rescued virus titer was lowered even when the amino acid in G333 position of glycoprotein was mutated from nonpathogenic Gln to pathogenic Asp, which rejects our hypothesis.

In conclusion, although the low virus titer needs to be improved and further examinations including virus stability and safety in cell culture and animal inoculation are also required, this study has proved that human rabies vaccine strain CTN181 is a promising viral vector including replication competent and replication constrained vectors. These vectors might be further used in the research of recombinant vaccines for some viral diseases when the pathogen cannot be easily cultivated.

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