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SHORT REPORT

R7A mutation in N protein renders temperature sensitive phenotype of VSV by affecting its replication and transcription in vitro

Longyun Chen[1] · Yanyan Zhou[1] · Min Zhao[1] · Huimin Chen[1]

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Abstract

Viral genomic RNA encapsidated by nucleoprotein (N) forms functional template for the transcription and replication of vesicular stomatitis virus (VSV). The crystal structure of the N-RNA complex shows that RNA is tightly sequestered between the two lobes of the N protein. The residue (R7) in N-terminal arm of N is of great importance to the formation of functional N-RNA template. In our study, we found that single amino acid substitution (R7A) resulted in the loss of CAT expression in vitro minigenome system at 37 °C. But the R7A had little effect on CAT expression at 31 °C. Further analysis showed that R7A had great effects on the RNA synthesis and the formation of cytoplasmic inclusions of VSV only at 37 °C not at 31 °C. For the further investigation of the effect of R7A on virus replication, we checked the dominant-negative effect of N_{R7A} in minigenome system and the single step curve of recombinant virus with R7A mutation in N protein ($rVSV_{R7A}$) under 37 °C and 31 °C separately. Our results showed that the mutation of R7A within the N-terminal arm of N affected both replication and transcription and induced VSV to become temperature sensitive.

Keywords VSV · Replication and transcription · R7A · Temperature sensitive

Introduction

Vesicular stomatitis virus is considered as the prototypic rhabdovirus within the family *Rhabdoviridae*. It contains a genomic RNA of 11,161 nucleotides. The viral genome carries five genes according to the gene order N, P, M, G, and L [1–3]. Nucleocapsid protein-associated genome is utilized as the template for transcription and replication of vesicular stomatitis virus and other non-segmented negative-strand (NNS) RNA viruses [4, 5]. VSV strain used in our study comes from San Juan strain and belongs to Indiana serotype. Previous study has demonstrated that naked genome RNA cannot be the efficient template for RNA synthesis of VSV [6, 7]. The genomic RNA of VSV encapsidated by N protein forms the N-RNA template. The N-RNA template associated with the viral P and L proteins comprises the viral RNA-dependent RNA polymerase (RdRp). RdRp is responsible for the replication and transcription of the virus [7–9].

During the replication cycle of VSV, the nascent RNA encapsidated by the N protein is necessary to form biologically active N-RNA template. It is well known that the interaction between N protein and genomic RNA is not sequence specific. The N protein can make N-RNA complex with cellular RNAs without P protein. With the presence of the P protein, N protein could encapsidate the viral RNA specifically. Furthermore, the interaction of N–P is important for maintaining the N protein in a soluble and encapsidation-competent form [10]. Earlier studies revealed that five amino acids of the carboxy-terminal (C-terminal) are important for encapsidation and genome replication [11]. Some key amino acids in the amino-terminal (N-terminal) arm have been demonstrated to be critical for the transcription and replication. Previous studies also showed that the 350 residues of N-terminal may contribute to RNA binding. The deletion of the N-terminal arm (amino acids 1–22) of N prevents the formation of N-RNA complex [12, 13].

The structure of the N protein in complex of N-RNA was determined by X-ray crystallography (PDB: 2GIC). Each N protein begins with a flexible N-terminal arm which descends into the N-terminal lobe and ascends into the

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✉ Huimin Chen
chm1215@hbtcm.edu.cn

[1] Hubei University of Chinese Medicine, Basic Medical College, Wuhan 430065, China

C-terminal lobe [14]. In the N-RNA complex, one N molecule interacts with three neighboring N molecules through three types of contact (contact I, II, and III). The N-terminal arm of N protein is involved in contact I and III [13, 15]. Our previous study revealed that three triple-amino-acid substitutions (TVK4-6A3, RII7-9A3, and VIV13-15A3) and one single-amino-acid substitution (R7A) resulted in the loss of RNA synthesis at 37 °C [16].

At the same time, we found that the activity of N_{R7A} was different at 37 °C and 31 °C. The effect and molecular mechanism of R7A on the replication and transcription of VSV at 37 °C and 31 °C has not been clearly elucidated. In this study, we study the effect of R7A on the function of N protein at 37 °C and 31 °C separately. The VSV minigenome system was used to demonstrate that mutation of R7A within the N-terminal arm of N protein inhibited viral replication and transcription at 37 °C. We also found that the mutation of R7A had effect on the formation of cytoplasmic inclusions and N_{R7A} had dominant-negative effect in VSV minigenome system at 37 °C. Consistent with previous study, the titer of passage 1 $rVSV_{R7A}$ decreased greatly at 37 °C. However, all these effect was weakened at 31 °C.

Materials and methods

Plasmid constructs

To identify the effect of R7A on the function of N protein at different temperature, we constructed plasmids with mutation (R7A) in the N gene of VSV. pBS-N cDNA was used as template for genetic manipulations to generate constructs encoding c-Myc epitope tagged wild-type (wt) and mutant N in plasmids pGBKT7 and pCAGGS. Plasmids of pCAGGS encoding HA-tagged wt P protein and L protein were generated via genetic manipulations. The VSV genome containing a point mutation (R7A) in the N gene was constructed as follows: The fragments with R7A mutation of the N gene were obtained by overlapping PCR. Then, the PCR products were inserted into pVSVFL(+) via ligase-independent cloning method to substitute wt N gene. All constructs were verified by sequencing.

VSV CAT minigenome assay

BHK-21 cells, infected with vTF7-3 in six-well plates, were transfected with either BD-N_{wt} or BD-N_{R7A} plus pGADT7-P (AD-P), pBS-L and pVSV-CAT2 encoding VSV minigenome [7]. At 40 h post-transfection, cell lysates were collected and subjected to CAT enzyme-linked immunosorbent assay (ELISA) for the detection of CAT expression level as described in previous study [17]. The expression levels of N_{wt} and N_{R7A} proteins were detected via Western blotting with monoclonal anti-Myc antibody (sc-40). The relative CAT expression level supported by only P and L was used as a mock control. All assays were repeated three times.

RNA extraction and relative quantitative real-time RT-PCR

Following the manufacturer's instructions, total RNA was extracted using a RNeasy mini kit (Qiagen). 1 μg total RNA of each sample was treated by DNaseI (Invitrogen) and used as reverse transcription (RT) templates. As described in our previous study, specific primer for cDNA/antigenome detection, and the oligo-dT was used for cDNA/mRNA detection [7]. Real-time PCR was performed on real-time system (Bio-rad) using SsoFast EvaGreen Supermix (Bio-rad) with gene-specific primer for antigenomic cDNA, CAT mRNA, or β-actin mRNA. We used β-actin as the reference gene. RNA synthesis level supported by only P and L was used as a mock control.

Confocal microscope

Cytoplasmic inclusions formed in RNA synthesis of VSV have been seen via confocal microscopy in previous studies [18]. BSR-T7 cells (stably expressing T7 RNA polymerase) in six-well plates were transfected with plasmid combinations encoding the following: (i) pCAGGS-N_{wt} and pVSVFL(+), which carry the full length of the genome of VSV; (ii) pCAGGS-$N_{wt,}$ pCAGGS-P, pCAGGS-L, and pVSVFL(+), which carry the full length of the genome of VSV; and (iii) pCAGGS-N_{R7A}, pCAGGS-P, pCAGGS-L, and pVSVFL(+). After 40 h of transfection, the medium was discarded. Cells were washed three times with ice-cold phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde for 20 min at room temperature. After being washed three times with PBS, the cells were incubated in 0.2% Triton-X100 for 20 min and washed with ice-cold PBS. Then, the cells were blocked with 3% bovine serum albumin (BSA) in PBS for 30 min at room temperature and incubated with rabbit polyclonal anti-Myc antibody (Santa Cruz 1:200) or mouse monoclonal anti-HA antibody (Sigma 1:2000) for 2 h at room temperature. Afterward, the cells were washed three times with 1% BSA and incubated with the goat anti-rabbit immunoglobulin antibody Rhodamine (Thermo 1:100) or goat anti-mouse immunoglobulin Fluorescein (Thermo 1:200) secondary antibody for 1 h at room temperature. After being washed three times with 1% BSA, the coverslips were turned over and put on a glass slide with a drop of Fluoroshield (Sigma) containing DAPI (4, 6-diamidino-2-phenylindole) for nuclear staining. Confocal images were collected via an Olympus confocal FV1000 microscope.

In vivo coimmunoprecipitation

BHK-21 cells were infected with vTF7-3 for 1 h at a multiplicity of infection (MOI) of 10 in six-well plates. Then the cells were transfected with the plasmids in the presence of Lipo-fectamine 2000 (Invitrogen). After 24 h of transfection, cell lysates were prepared as described by Chen et al. [17]. Myc-tagged N_{wt} or N_{R7A} were co-expressed with HA-tagged P to assess the interaction of N–P. Precleared supernatants of lysates were incubated with a polyclonal anti-Myc Antibody for 4 h at 4 °C with rotation. After centrifugation, supernatants were mixed with protein G Sepharose 4 Fast Flow medium and rotated overnight at 4 °C. Beads were washed five times with washing buffer. The beads were boiled in 2X sodium dodecyl sulfate (SDS) loading buffer, and proteins were detected by Western blotting using an anti-Myc or anti-HA antibodies.

Recovery of recombinant virus

80% confluence BHK-21 cells in six-well plates were infected with vTF7-3 at an MOI of 1. Then, the cells were transfected with pBS-N (5 μg), pBS-P (3 μg), pBS-L (1 μg), and pVSVFL(+) (5 μg) or pVSVFL(+) with R7A mutation in the N gene (5 μg). The transfection medium was discarded at 6 h post-transfection, and the cells were incubated with 2 ml of DMEM-10% FBS for 2 days at 37 °C. After that, the cells were frozen and thawed three times, and the clarified supernatants were collected via centrifugation. The supernatants passed through 0.22 μm filters to remove vTF7-3 and were layered onto a fresh BHK-21 cell monolayer for amplification at 37 °C. Then, the supernatants were harvested and titrated. Agar plugs were picked up during titration for the isolation of single recovered recombinant VSV. The agar plugs were incubated in 500 μl Opti-MEM overnight at 4 °C, and 250 μl of it was added to the fresh BHK-21 cell monolayer for the amplification of the recombinant virus.

Results

R7A within the N-terminal arm of N has different effect on the expression of CAT at 37 °C and 31 °C

It has been proved that the N-terminal arm of the N is critical for the function of N protein [13]. Our previous study showed that N_{R7A} can't support CAT expression of the VSV minigenome at 37 °C [16]. In this study, we checked the function of N_{R7A} in VSV minigenome system at 37 °C and 31 °C separately. As shown in Fig. 1b, the relative CAT expression supported by N_{R7A} was only about 5% of that supported by Nwt, although the expression levels of N_{R7A} and N_{wt} were similar, thereby indicating that N_{R7A} was barely active in the minigenome assay at 37 °C. Next, we found the relative CAT expression supported by N_{R7A} was about 60% of that supported by N_{wt} at 31 °C (Fig. 1c). Clearly, the mutation of R7A in N-terminal of N protein had little effect on the CAT expression at 31 °C.

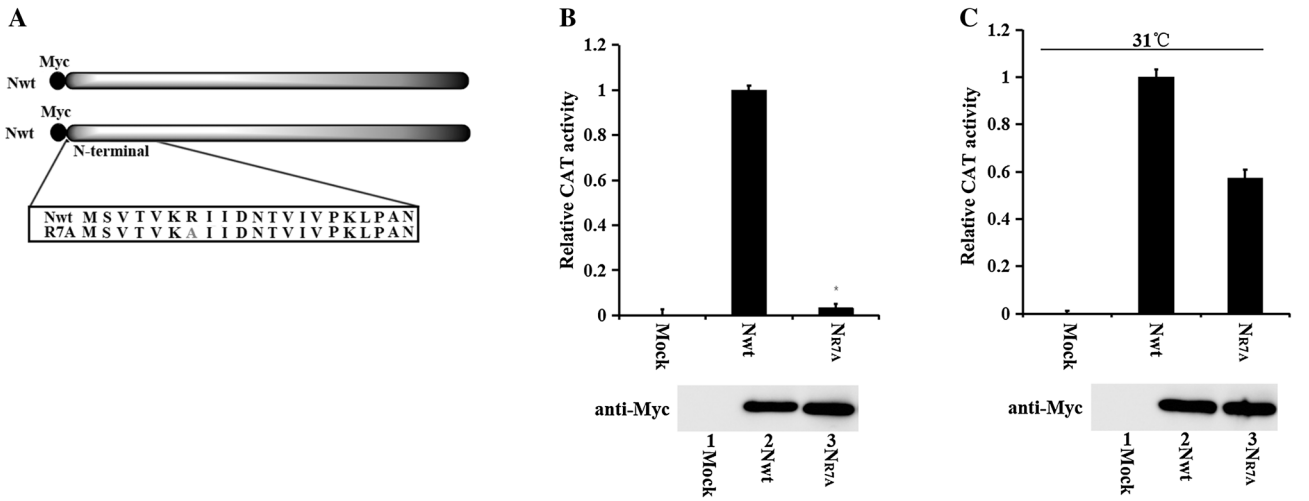

Fig. 1 The effect of R7A in the N-terminal of N_{wt} on the expression of CAT at 37 °C and 31 °C. **a** Schematic representation of full-length VSV N_{wt} and N with R7A mutation in the N-terminal. **b**, **c** BHK-21 cells were infected with vTF7-3 and transfected with plasmids as described in Materials and Methods. In the minigenome assay, relative CAT expression levels supported by N_{wt} and N_{R7A} were analyzed via CAT ELISA. The relative CAT expression level supported by only P and L was used as a mock control. The CAT expression level supported by N_{wt} was used as a positive control and defined as 100%. The expression of N_{wt} and N_{R7A} was detected via Western blotting with anti-Myc antibody. *$P < 0.05$ versus N_{wt} group

The effect of R7A on the replication and transcription of the VSV minigenome was weakened at 31 °C

In our previous study, we demonstrated that the defects in either transcription or replication could result in the loss of CAT expression [7]. We have found that N_{R7A} did not support CAT expression at 37 °C, but it can support CAT expression at 31 °C in this study. So we next sought to determine whether N_{R7A} has effect on the transcription or replication of CAT minigenome at 31 °C by real-time PCR. Compared to the replication and transcription levels supported by N_{wt}, the results showed that the replication level supported by N_{R7A} reduced about 70% (Fig. 2a), and the transcription level supported by N_{R7A} reduced about 80% (Fig. 2c) at 37 °C. However, the replication level supported by N_{R7A} increased to 60% of that supported by N_{wt} at 31 °C (Fig. 2b). We also found the transcription level supported by N_{R7A} increased at 31 °C (Fig. 2d). Thus, we assumed that R7A had an effect on both transcription and replication at 37 °C, but the effect was weakened at 31 °C.

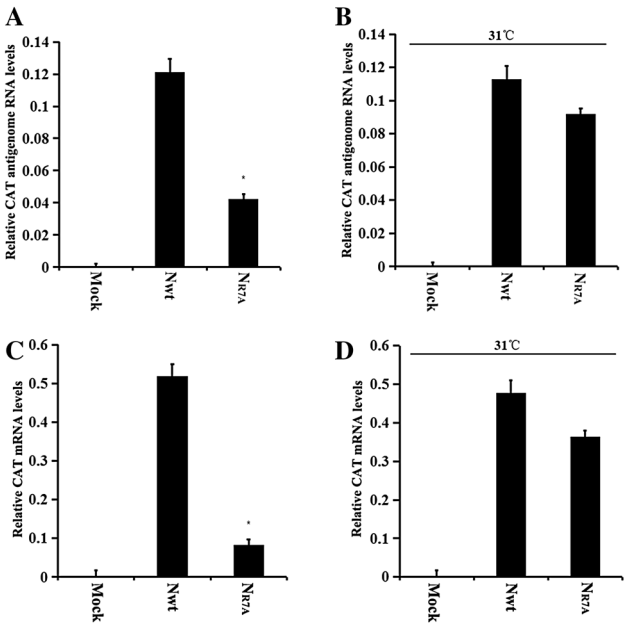

Fig. 2 The effect of N_{R7A} on the replication and transcription of the VSV minigenome. **a**, **b** RNA synthesis analysis of N_{wt} and N_{R7A} at 37 °C and 31 °C. Transfection was performed as described in Materials and Methods. CAT antigenomic RNA level supported by N_{R7A} relative to that supported by N_{wt} was analyzed via real-time RT-PCR. β-actin was used as a reference gene. **c**, **d** CAT mRNA level supported by N_{R7A} relative to that supported by N_{wt} was analyzed via real-time RT-PCR. β-actin was used as a reference gene. *$P < 0.05$ versus N_{wt} group

N_{R7A} couldn't support the formation of cytoplasmic inclusions efficiently at 37 °C

Previous study showed that VSV-infected cells can form cytoplasmic inclusions during protein synthesis as the predominant sites of mRNA synthesis in the cell [18]. Thus, we sought to determine whether N_{R7A} could form cytoplasmic inclusions at 37 °C or 31 °C. The plasmids encoding N_{wt} or N_{R7A}, P, L, and genomic RNA of VSV were co-transfected into BSR-T7 cells. After 30 h of transfection, we observed the formation of cytoplasmic inclusions in cells expressing N_{wt}, P, L, and genomic RNA. When the N_{wt} was replaced by N_{R7A}, the formation of inclusion bodies couldn't be observed in cells obviously at 37 °C although the co-localization of N_{R7A} and P was observed. However, we could observe the formation of inclusion body supported by N_{R7A} with P, L, and genomic RNA at 31 °C (Fig. 3). These results suggest that N_{R7A} was defective on the formation of cytoplasmic inclusions at 37 °C, and it could support the formation of cytoplasmic inclusions at 31 °C.

N_{R7A} had dominant-negative effect on N_{wt} function at 37 °C

In our previous study, N_{R7A} exhibited much lower activity of RNA synthesis than N_{wt} at 37 °C, but it maintained the ability to interact with P at 37 °C [16]. We also found that N_{R7A} protein could interact with P protein at 31 °C (Fig. 4a). Therefore, we sought to determine whether N_{R7A} protein have dominant-negative effect on the function of N_{wt} in CAT minigenome assay at 37 °C or 31 °C. Our results showed that with the increase of N_{R7A} protein expression, the expression of CAT supported by N_{wt} gradually decreased, even below 12% compared to the positive control at 37 °C. On the contrary, the expression of CAT supported by N_{wt} didn't decrease greatly with the expression of N_{R7A} protein at 31 °C, even though the expression of N_{R7A} increased gradually (Fig. 4b). These suggested that expression of N_{R7A} didn't interrupt the normal function of N_{wt} significantly in the minigenome assay at 31 °C.

The phenotype of passage 1 (P1) $rVSV_{R7A}$ was temperature sensitive

Our results showed that N_{R7A} was transcription and replication defective in minigenome assay. So we try to determine whether R7A mutation within N gene had effect on the phenotype of recombinant virus. The mutation of R7A was engineered into the N gene of VSV genome. Recombinant virus with a mutation of R7A was recovered successfully at 37 °C and 31 °C separately. We observed that virus expressing N_{R7A} only can replicate to titers 1000-fold lower than that of wt VSV at 37 °C at 24 hpi, but can replicate to titers

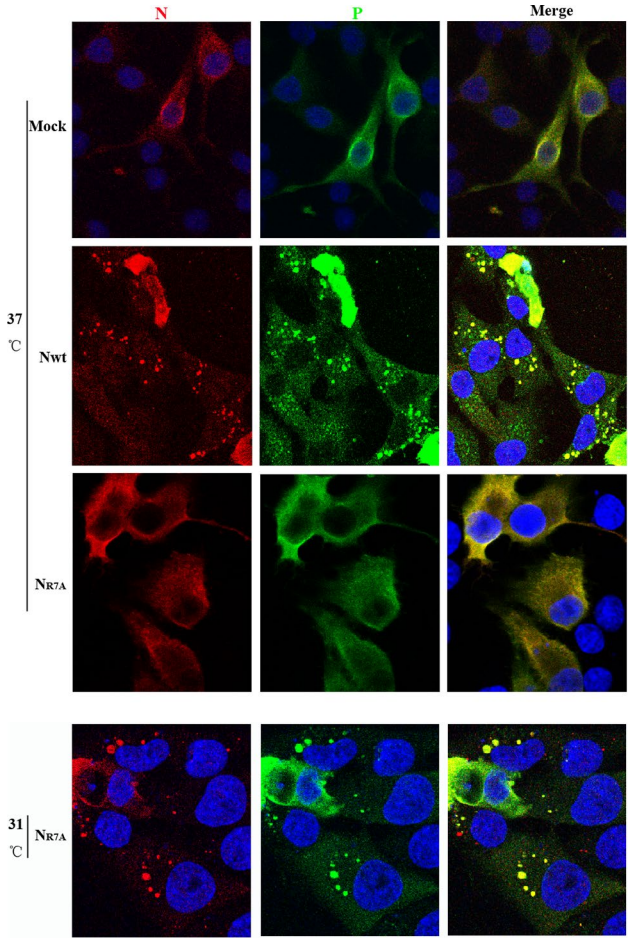

Fig. 3 Cytoplasmic inclusions cannot be observed in the presence of N_{R7A} at 37 °C. BSR-T7 cells were transfected with plasmids pCAGGS-N_{wt} or pCAGGS-N_{R7A} in addition to plasmids encoding HA-P, L, and and the VSV genome. At 30 h post-transfection, cells were fixed, stained, and visualized via confocal microscopy as described in Materials and Methods. N_{wt} but not N_{R7A} co-expressed with P, L, and antigenome formed cytoplasmic inclusions at 37 °C. The formation of cytoplasmic inclusions supported by N_{R7A} was observed at 31 °C. BSR-T7 cells transfected with the plasmid pCAGGS-N_{wt} plus HA-P and antigenome were used as a negative control. Nucleus was stained by DAPI

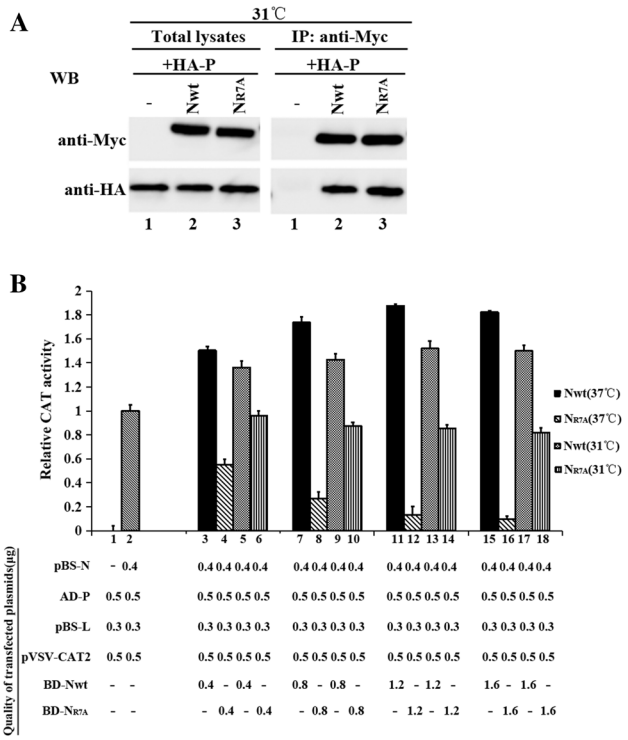

Fig. 4 The dominant-negative effect of N_{R7A} in the minigenome system at 37 °C and 31 °C. **a** Myc-tagged N_{wt} or N_{R7A} and HA-tagged P were co-expressed in BHK cells at 31 °C. (Left) The expression of N_{wt}, N_{R7A} and P was detected via Western blot analysis using anti-Myc and anti-HA monoclonal antibodies. (Right) Immunoprecipitation was performed using anti-Myc polyclonal antibody, and immune complexes were detected with anti-Myc and anti-HA monoclonal antibodies. **b** BHK-21 cells were infected with vTF7-3 and transfected with plasmids pBS-N (0.4 μg), AD-P (0.5 μg), pBS-L (0.3 μg) and pVSV-CAT2 (0.5 μg) plus gradually increased concentration of the plasmids which encoding Myc-tagged N_{wt} and N_{R7A} individually. The relative CAT expression level of cells transfected with only plasmids pBS-N (0.4 μg), AD-P (0.5 μg), pBS-L (0.3 μg), and pVSV-CAT2 (0.5 μg) was used as a positive control and defined as 100%

20-fold lower than that of wt VSV at 31 °C at 24 hpi (Fig. 5). In our experiment, we sequenced the N gene and confirmed the R7A mutation in the viral genome. We also confirmed that R7A mutation in the mutant VSV was stable after eight serial passages at 37 °C and 31 °C. The results demonstrated that the P1 rVSVR7A was temperature sensitive.

Discussion

It is well known that RNP complex consisting of the virus genomic RNA encapsidated by the N protein is the template for transcription and replication for VSV and other NNS RNA viruses. Several studies have shown that the levels of transcription and replication directed by the RNP template can be affected by single amino acid substitution in the N protein [19–21]. In this study, we have examined the role of residue (R7) located in the N-terminal arm of the N protein in viral RNA synthesis at 37 °C and 31 °C separately.

The N-terminal arm residue (R7) was chosen based on several conclusions from previous studies. It is located in the portion of the N-terminal arm that interacts with the C-lobe and the C-loop of neighboring N protein monomer to form a three-component interaction network. In addition, the single mutation of 21 amino acids in the N-terminal arm showed that only the residue (R7) plays a key role in the function of N [16, 22]. In this study, the target residue (R7) was replaced with alanine. The results suggested that the N_{R7A} had different activity at 37 °C and 31 °C (Fig. 1). In our study, the effects of R7A on N protein were analyzed

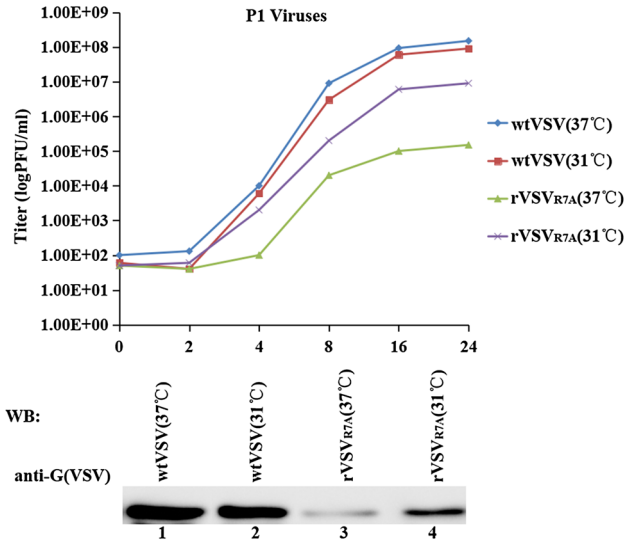

Fig. 5 The characterization of recombinant viruses. The growth curve of the rVSVwt and $rVSV_{R7A}$ passaged at 37 °C and 31 °C. BHK-21 cells were infected with the recombinant viruses at an MOI of 3, and supernatants were harvested at 0, 2, 4, 8, 16, and 24 h postinfection. Plaque assay was used to determine the titers of virus at each time point. Replication levels of the P1 viruses were compared at 37 °C and 31 °C separately

from the following aspects: (i) the levels of transcription and replication of CAT minigenome; (ii) the formation of cytoplasmic inclusions; (iii) the dominant-negative effect on Nwt; and (iv) the phenotype of P1 $rVSV_{R7A}$.

Here, we identified that R7A within the N-terminal arm of N had little effect on the transcription and replication of CAT minigenome at 31 °C, in spite of the fact that N_{R7A} was severely debilitated in its ability to support the transcription and replication at 37 °C (Fig. 2). Previous study has demonstrated that viral derived inclusions are sites of RNA synthesis in cells infected with VSV. It pointed out that the cytoplasmic inclusions formed with viral protein expression and contained the viral machinery necessary for RNA synthesis [18]. So we decided to check the effect of R7A on the formation of cytoplasmic inclusions. Plasmids encoding N_{wt} or N_{R7A}, P, L and antigenomic RNA were co-transfected into BSR-T7 cells. 30 h after transfection, Nwt, N_{R7A} and P proteins were stained and observed under laser scanning confocal microscope. Inclusion bodies were observed in about 30% of the cells transfected with Nwt. When N_{wt} was replaced by N_{R7A}, no obvious inclusion bodies were observed in the cells at 37 °C. However, inclusion bodies supported by N_{R7A} could be observed obviously in the cells at 31 °C (Fig. 3).

In our previous study, we have demonstrated that N_{R7A} maintained the N–N as well as N-P interaction compared to N_{wt} [16]. Because N_{R7A} is defective in transcription and replication, we speculate that N_{R7A} may have the dominant-negative effect on Nwt. Our results confirm this speculation at 37 °C. However, we found the dominant-negative effect of N_{R7A} was weakened at 31 °C (Fig. 4). Then we sought to determine whether N_{R7A} have different effect on the virus life cycle at 37 °C and 31 °C. To that end, R7A was engineered into the N gene in the context of the VSV genome. Recombinant virus with mutation of R7A was recovered successfully at 37 °C and 31 °C separately. The growth curve of the virus showed that P1 $rVSV_{R7A}$ was temperature sensitive (Fig. 5). In our study, we had confirmed that R7A mutation in the mutant VSV was stable after eight serial passages at 37 °C and 31 °C. We also had checked if any compensatory mutations and found two second-site mutations could compensate the effect of R7A mutation after eight serial passages. We stated these in our previous article [23]. As a strategy to protect nonhuman primates, VSV-based vaccines have been extensively explored against a wide range of viruses. For HIV and Ebola, rVSV-based vaccines have proven to be promising according to the clinical trials [24–30]. Furthermore, rVSV-ΔM51 with the deletion of methionine at position 51 in the M protein has been demonstrated to be a highly attenuated VSV vector in vivo and considered to be a potent viral vaccine vector and anti-cancer agents [31–34]. So we consider that $rVSV_{R7A}$ could be used for the development of viral vaccines and cancer therapy.

In summary, our work presented here indicates that the mutation of R7A within N protein has great effect on the transcription and replication at 37 °C and this kind of effect could be weakened at 31 °C. Our study demonstrated that the mechanism of this effect was related to the formation of inclusion bodies and N_{R7A} can't support the formation of inclusion bodies efficiently at 37 °C. In our further study, we will try to optimize the temperature sensitive $rVSV_{R7A}$ for the development of vaccines and oncolytic viruses.

Author contributions LYC, YYZ and HMC conceived the study. LYC, YYZ, MZ and HMC participated in the conduct of the study. LYC and HMC drafted and reviewed the manuscript. All authors critically revised and approved the manuscript.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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