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Author(s)	Kojima, Shohei; Honda, Tomoyuki; Matsumoto, Yusuke; Tomonaga, Keizo
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**Heat stress is a potent stimulus for enhancing rescue efficiency of recombinant Borna disease virus**

Shohei Kojima<sup>1</sup>, Tomoyuki Honda<sup>1,†</sup>, Yusuke Matsumoto<sup>1,2</sup>, and Keizo Tomonaga<sup>1,†</sup>

<sup>1</sup>*Department of Viral Oncology, Institute for Virus Research, Kyoto University, 53 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan,* <sup>2</sup>*Department of Microbiology, School of Medicine, Wakayama Medical University, 811-1 Kimiidera, Wakayama 641-8509, Japan*

Running title: Heat stress enhances BDV replication

†To whom correspondence should be addressed.

Department of Viral Oncology, Institute for Virus Research, Kyoto University, 53 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan

Tel: +81-75-751-4034, Fax: +81-75-751-4000

E-mail: [thonda@virus.kyoto-u.ac.jp](mailto:thonda@virus.kyoto-u.ac.jp) and [tomonaga@virus.kyoto-u.ac.jp](mailto:tomonaga@virus.kyoto-u.ac.jp)

## **Abstract**

Recently developed vector systems based on Borna disease virus (BDV) hold promise as platforms for efficient and stable gene delivery to the central nervous system (CNS). However, because it takes several weeks to rescue recombinant BDV (rBDV), an improved rescue procedure would enhance the utility of this system. Heat stress has been reported to enhance the rescue efficiency of other recombinant viruses. Here, we demonstrated that heat stress increased the level of BDV genome in persistently BDV-infected cells without obvious cytotoxicity. Further analyses suggested that the effect of heat stress on BDV infection was not due to the increase in the activity of BDV polymerase. By using heat stress, we succeeded in obtaining more cells in which BDV replication occurred in the initial phase of rBDV rescue. Heat stress is a useful improvement on the published rescue procedure of rBDV. Our findings may accelerate the practical use of BDV vector systems and thus enable broader adoption of this viral vector uniquely suited for gene delivery to the CNS.

**Keywords:** Animal RNA virus, Borna disease virus, Heat stress, Viral vector

## **List of Abbreviations**

BDV, Borna disease virus; BiP, immunoglobulin heavy chain-binding protein; CAT, chloramphenicol acetyltransferase; CDV, canine distemper virus; CNS, central nervous system; DAPI, 4',6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; dpt, days posttransfection; FCS, fetal calf serum; Hsc70, heat shock cognate 70; Hsps, heat shock proteins; L, RNA-dependent RNA polymerase protein; MV, measles

virus; N, nucleoprotein; OL/BDV, persistently BDV-infected OL cells; P, phosphoprotein;  
rBDV, recombinant BDV; RNPs, ribonucleoprotein complexes

## Introduction

BDV belongs to the *Bornaviridae* family within the non-segmented negative-strand RNA viruses. It is characterized by highly neurotropic and persistent infection (1). BDV can infect a wide variety of host species and readily establishes a long-lasting persistent infection in the nucleus without causing apparent cell damage (2, 3). This characteristic makes BDV the only animal RNA virus capable of intranuclear parasitism. Recently, by using a reverse genetics system of BDV, we and others have demonstrated that foreign genes can be stably expressed in brain cells, both *in vivo* and *in vitro*, from an intercistronic region and the 5' end of the BDV genome, respectively (4, 5). These features suggest that BDV could be an ideal vector platform for efficient and stable gene delivery system to the CNS.

A primary roadblock limiting usefulness of the BDV vector system is the low efficiency of rBDV rescue procedure. Successful rescue of rBDV requires several molecular events to occur after transfection of a plasmid expressing the BDV full-length antigenome RNA and helper plasmids expressing N, P and L. At first, viral N, P and L proteins must be synthesized at levels appropriate to initiate the *de novo* encapsidation of antigenome RNA into replication-competent viral RNPs. Second, genome RNA must be transcribed from antigenome RNA. Third, the expression of viral genes from newly formed viral RNPs needs to be at levels sufficient for additional rounds of replication. Exactly which steps may be rate-limiting in successful rescue are unknown, but the efficiency of this relatively rare event may be improved by stimulating one or more of the above processes.

Heat stress is known to enhance the gene expression of many viruses (6, 7). For

example, the RNA-producing activity associated with purified CDV RNPs is increased when they are isolated from cells subjected to heat stress (8). Heat stress also increases MV minireplicon gene expression (6). As a result of the enhancement of viral gene expression, the rescue efficiency of recombinant CDV or MV is improved by heat stress (6, 7). Therefore, we speculated that the efficiency of rBDV rescue could be also improved by heat stress.

Heat stress induces cellular stress responses and the expression of multifunctional proteins called Hsps (9, 10). Some Hsps act as molecular chaperones to assist in proper protein folding (11, 12). In addition to chaperone activity, Hsps play roles in many other biological pathways, such as protein trafficking, regulation of protein function, and DNA replication (11, 13–15). Moreover, Hsps are also involved in the life cycles of various viruses. For example, hsp72 and its constitutively expressed isoform can regulate gene expression of several RNA and DNA viruses through the association with viral nucleocapsid proteins, which may modulate the assembly of viral nucleocapsid (16–18). BDV also takes advantage of host cellular chaperone proteins to control its life cycle (19). For example, the BDV protein X is known to interact with the constitutive isoform of hsp72, Hsc70 (20). Because Hsc70 and a polymerase cofactor, P, share the binding site on X, the interaction of X with Hsc70 results decreased binding of X with P. This leads to an increase in the level of P in the nucleus and stimulates BDV replication. Another example is BiP, also called heat shock 70 kDa protein 5. BiP interacts with the BDV glycoprotein at the cell surface, and several lines of evidence suggest that BiP is involved in the cell surface association of BDV (21). Because of the known interactions between BDV and Hsps, it was reasonable to speculate that heat stress might play a positive role in BDV replication, which could improve the rescue efficiency of rBDV.

In this study, we examined the effect of heat stress on BDV replication. We found that heat stress increases the level of BDV genome RNA in persistently BDV-infected cells. Applying this finding to the rBDV rescue system resulted in an increase in the number of cells in which rBDV replication occurred. All these results suggest that heat stress is a useful improvement on the published rescue procedure of rBDV.

## **Materials and methods**

### **Cells**

Vero cells stably expressing a puromycin resistance gene were cultured in DMEM supplemented with 2% FCS. The OL cell line, derived from a human oligodendroglioma, and the OL/BDV were cultured in DMEM-high glucose (4.5%) supplemented with 5% FCS. 293T cells were cultured in DMEM supplemented with 10% FCS.

### **Plasmid construction**

To generate a minigenome plasmid carrying a *Gaussia* luciferase gene, pCAG-HR, a Pol II-driven minigenome plasmid carrying a CAT gene (22) was modified. Briefly, the *Gaussia* luciferase gene was amplified from a plasmid pGLuc-Basic Vector (New England Biolabs). The CAT gene of pCAG-HR was replaced by the resulting fragment. A catalytically inactive L mutant was created by substituting aspartate with alanine at position 554 of L (23).

### **RNA preparation and real-time RT-PCR**

$2 \times 10^5$  of OL/BDV cells were seeded in 12-well plates. Total RNA was isolated using TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol and then reverse transcription was performed with a Verso cDNA Synthesis Kit (Thermo Fisher Scientific, USA) using oligo-dT primers, a BDV genome-specific primer (5'-TGT TGC GCT AAC AAC AAA CC AAT CAC-3') and a BDV antigenome-specific primer (5'-CAA GCA CTA CAC CAC TGA CA-3'). Real-time PCR was performed by the SYBR-Green PCR Assay and the TaqMan PCR Assay (TOYOBO, Japan) in a 20  $\mu$ l reaction volume, and products were detected with a Rotor-Gene Q System (Qiagen, Germany). PCR reactions were incubated at 95°C for 30 sec, followed by 40 amplification cycles of annealing and extension at 60°C for 30 sec and denaturation at 95°C for 5 sec. GAPDH mRNA was quantified with primers (5'-AGC GAG ATC CCT CCA AAA TC-3' and 5'-AAA TGA GCC CCA GCC TTC TC-3'), and used to standardize the total amount of cDNA. A BDV-forward primer, a BDV-reverse primer and a BDV TaqMan probe were used as described previously (20). Real-time data were analyzed with Rotor-Gene Q software (Qiagen, version 2.1).

### **Western blotting**

$1 \times 10^4$  of OL/BDV cells were seeded in 24-well plates and incubated at 37°C for 24 hr, followed by 4- or 12-hr incubation at either 37°C or 40°C. The cells were lysed with SDS sample buffer. The total cell lysate was subjected to SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, USA). Antibodies used in this study were as follows; a mouse anti-BDV N (HN321), a rabbit anti-BDV P (HB03), a mouse anti-BDV P (HP062), a rabbit anti-BDV X and a mouse anti-Tubulin (Sigma-Aldrich, USA) antibody.



### **Immunofluorescence analysis**

$1.5 \times 10^5$  of OL/BDV cells were plated on coverslips and incubated at 37°C for 24 hr, followed by either 4- or 12-hr incubation at either 37°C or 40°C. Cells were next fixed for 10 min in 4% paraformaldehyde, permeabilized by incubation in PBS containing 0.25% Triton X-100 for 10 min, then treated with PBS containing 1% bovine serum albumin for 1 hr. The cells were then incubated with the anti-BDV N, P and X antibodies for 1 hr. This was followed by incubation with appropriate Alexa Fluor-conjugated secondary antibodies (Invitrogen). The cells were counterstained with DAPI for 30 min. A confocal laser-scanning microscope ECLIPSE Ti (Nikon Inc., Japan) was used for cell immunofluorescence imaging and data collection.

### **BDV reconstitution assay**

rBDV were reconstituted by reverse genetics technology established by our group (5).  $5 \times 10^5$  293T cells were seeded in 6-well plates and incubated at 37°C for 24 hr. The cells were transfected with 2 µg of pFct-BDV P/M-GFP (5), 0.25 µg of pCA-N (22), 0.025 µg of pCXN2-P (22), and 0.25 µg of pCA-L (22) using Lipofectamine 2000 (Invitrogen). At 1 day and 4 dpt, the cells were cultured at either 37°C or 40°C for either 4 or 12 hr, and at 2 dpt, the cells were passaged to 10-cm dishes. GFP positive cells were counted by fluorescent microscopy.

### **Minigenome assay**

A minigenome assay was carried according to the protocol described previously (20) with several modifications. Briefly,  $2 \times 10^5$  293T cells were seeded in 12-well plates and

incubated at 37°C for 24 hr. The cells were transfected with 0.25 µg of a Pol II-driven minigenome plasmid as described except encoding the *Gaussia* luciferase gene instead of the CAT gene, 0.25 µg of pCA-N, 0.025 µg of pCXN2-P, and 0.25 µg of pCA-L using Lipofectamine 2000 (Invitrogen). Thirty-six-hr after transfection, the cells were further cultured at either 37°C or 40°C. At 4, 6, 8 and 10 hr after the change in temperature, *Gaussia* luciferase activity was measured by using the *Gaussia* Luciferase Assay kit (New England Biolabs) according to the manufacturer's instructions in a single-well luminometer (Berthold Lumat LB 9507, Germany). The activity at the indicated incubation time after the change in temperature was normalized with that using the catalytically inactive L mutant-expressing plasmid instead of pCA-L (23).

#### **WST-1 assay**

$1 \times 10^5$  of OL/BDV cells were seeded in 24-well plates. The plates were incubated at 37°C for 24 hr, followed by 4- or 12-hr incubation at either 37°C or 40°C. Then, the cells were incubated at 37°C for another 1 hr with Premix WST-1 (Takara Bio Inc., Japan). Medium was measured at 440 nm in a microplate reader (SH-9000 Lab, Corona Electric, Japan).

## **Results**

### **Increase in the level of BDV genome RNA in persistently BDV-infected cells by heat stress**

To determine if heat shock could potentially increase the rescue efficiency of rBDV, we first examined its effects on BDV in the setting of established infection. To this end, we analyzed the effect of heat stress on BDV replication and transcription in OL/BDV cells.

The cells were incubated either at 37°C or at 40°C for 12 or 24 hr. The amount of BDV genome RNA in OL/BDV cells cultivated at 40°C was increased compared to that at 37°C (Fig. 1a). On the other hand, the level of BDV mRNA was not altered by heat stress for 12 or 24 hr (Fig. 1b). These results indicate that heat stress increases the level of BDV genome RNA, but not BDV mRNA, in OL/BDV cells. We then determined the minimum duration of heat stress required for the effect on BDV infection described above. The level of BDV genome RNA was increased after cultivation at 40°C for 4 hr, but not for 2 hr (Fig. 1c). In contrast, the amount of BDV mRNA was slightly increased after heat stress for 2 hr, but not 4 hr (Fig. 1d). These results suggest that cultivation at 40°C for at least 4 hr increases the level of BDV genome RNA in OL/BDV cells. Because BDV genome RNA is transcribed from antigenome RNA, we next examined whether the level of BDV antigenome RNA was also increased in the heat-stressed OL/BDV cells. After cultivation of OL/BDV cells at 40°C, the level of BDV antigenome RNA was also upregulated (Fig. 1e). Cultivation of OL/BDV cells at 40°C for 4 or 12 hr did not induce obvious cytotoxicity (Fig. 2). These results suggest that heat stress increased the levels of both BDV genome and antigenome RNAs, but not BDV mRNAs, without obvious cytotoxicity.

#### **Effect of heat stress on BDV proteins or BDV polymerase activity**

To determine how heat stress increases the level of BDV genome RNA, we next evaluated whether heat stress influenced the levels of viral proteins. Because it has been reported that the N-to-P stoichiometry affects BDV polymerase activity (24), we first quantified these proteins. As shown in Figure 3a, the levels of the N and P proteins were unaffected by heat stress. Previous evidence indicates that nucleocytoplasmic trafficking of BDV proteins plays a critical role in BDV replication (19). Therefore, we examined the changes

of subcellular distribution of viral proteins by heat stress. As shown in Figures 3 b and c, heat stress did not grossly alter the distribution of viral proteins. It has been reported that the X protein is translocated into the nucleus by heat stress. The discrepancy between the previous and this studies in the effect of heat stress on the intracellular localization of the X protein may be explained by the different protocols of heat stress, at 44°C for 60 min in the previous study and at 40°C for 4 or 12 hr in this study. Finally, we evaluated the activity of BDV polymerase in heat stressed cells using a BDV minigenome assay. In the assay, mRNA of a reporter gene is transcribed by BDV polymerase from the plasmid-driven BDV minigenome RNA. After the assembly of BDV minigenome RNPs in 293T cells at 37°C, the cells were further incubated either at 37°C or 40°C. After the change in temperature, reporter gene expression from minigenome RNA at 40°C was similar to that at 37°C (Fig. 4), consistent with Figures 1 b and d. These results suggest that the effect of heat stress on BDV is not due to the increase of BDV polymerase activity. Rather, the number of BDV RNPs active in replication might be increased, resulting in the increased level of BDV genome RNA.

#### **Increase of GFP-positive cells in 293T cells expressing components of BDV RNPs carrying a GFP gene by heat stress**

Since heat stress increased the level of BDV genome RNA, we reasoned that it could potentially improve the rescue efficiency of rBDV. We summarize the published rescue procedure of rBDV in Figure 5a (5). In the first week, replication-competent BDV RNPs are reconstituted in 293T cells expressing the BDV full-length antigenome RNA and helper proteins, N, P, and L. From 3 to 8 dpt, rBDV propagates into co-cultured Vero cells. Then, a few weeks after cocultivation with Vero cells, rBDV propagates throughout the

Vero cells. We hypothesized that the increased level of BDV genome RNA by heat stress might improve the rescue efficiency of rBDV (Figs. 5 b and c). We chose two conditions, 40°C for 4 or 12 hr, as heat-stressed conditions. We transfected a plasmid expressing the BDV full-length antigenome RNA carrying a GFP reporter gene and helper plasmids expressing N, P, and L to 293T cells. The transfected 293T cells were subjected to heat stress on 1 and 4 dpt. Because a reporter GFP gene is transcribed from rBDV genome RNA, the expression of GFP ensures that rBDV replication has occurred in the cells. We observed an increase in the number of GFP-positive cells in 4 hr heat-stressed compared to the unstressed condition at 6 dpt, but not 2 dpt (Fig. 5b). At 4 dpt, the number of GFP-positive cells tended to be increased, although the change was not significant (Fig. 5b). Then, we evaluated whether heat stress for 12 hr increases the number of GFP-positive 293T cells at 4 dpt. As shown in Figure 5c, the number of GFP-positive cells in the condition of heat stress for 12 hr was increased at 4 dpt. The number of GFP-positive cells was comparable to that in unstressed condition at 6 dpt (Figs. 5 b and c). All of these results suggest that heat stress increases the number of BDV replication-positive cells, which is likely to improve the rescue efficiency of rBDV.

## **Discussion**

In this study, we demonstrated that heat stress increases the level of BDV genome RNA, but not BDV mRNA, in OL/BDV cells (Fig.1). Heat stress did not affect the expression levels or the subcellular distributions of viral proteins in OL/BDV cells (Fig. 3). Furthermore, heat stress did not induce obvious cytotoxicity (Fig. 2). All of these observations indicate that heat stress is a potent stimulus to enhance rescue efficiency of

rBDV. Therefore, by using heat stress, we modified the reconstitution step of the published BDV rescue procedure and found that heat stress indeed improved the number of BDV replication-positive cells (Fig. 5).

The mechanism responsible for the increase in BDV genome RNA in OL/BDV cells after heat stress may be related to enhanced BDV polymerase activity or the increased number of BDV RNPs active in replication. Using a BDV minigenome assay, we revealed that heat stress did not increase the expression level of a minigenome reporter gene (Fig. 4), suggesting that BDV polymerase activity itself can not explain the increased level of BDV genome RNA after heat stress. Therefore, we speculate that the increase of the number of replication-competent BDV RNPs may be a cause of the increase of BDV genome RNA. Although we could not exclude the possibility that only the replicase activity of BDV polymerase, but not the transcriptase activity, is activated by heat stress, it also results in the increase of BDV RNPs. Collectively, it is reasonable that heat stress upregulates BDV genome RNA at least by increasing the number of BDV RNPs.

Increase in the level of BDV genome RNA resulting from heat stress of cells strongly indicate host cellular proteins as mediators of these effects. Many Hsps were upregulated by our heat stress conditions (data not shown). Our results are consistent with the proposed role of the Hsps already known to be involved in the life cycle of BDV. For example, Hsc70 mainly exerts its effect on BDV replication, rather than transcription (20), in keeping with the effect seen in the study. Therefore, some of Hsps induced by heat stress could mediate the effect of heat stress. Attempts to identify host cellular proteins responsible for the effects of heat stress are on-going. Preliminary data suggests that the over-expression of any Hsps individually is not sufficient for increasing the level of BDV genome RNA in the absence of heat stress. We consider it likely that the effect of heat

stress requires Hsps in combination with other cellular proteins induced by heat stress. Further investigation is required to identify cellular proteins responsible for the effect of heat stress.

In sum, we identified heat stress as a stimulus that increases the number of 293T cells in which BDV replication occurs in the initial reconstitution step of the rBDV rescue procedure. We propose two strategies to improve the rescue procedure of rBDV. First, by subjecting the cells to heat stress in the initial reconstitution step, the increased BDV replication-positive 293T cells will propagate more efficiently into co-cultured Vero cells and increase the rescue efficiency of rBDV. Second, we might shorten the time of the initial reconstitution step, because the increased number of BDV replication-positive 293T cells in the heat-stressed condition might be enough to seed on-going infection in Vero cells at an earlier time point than in the published protocol. Overall, further optimization of the rescue procedure will be helpful to facilitate use of this novel BDV vector system. Improvement of rescue efficiency of rBDV by our modification will emphasize the potency of a novel BDV vector system as a good system not only for efficient and safe gene delivery into the CNS but also for various applications to basic research fields such as stem cell biology.

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## Disclosure

The authors declare no conflicts of interest.

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## Figure legends

Fig. 1 Increase in the level of BDV genome RNA in OB cells by heat stress. (a, b) Increase of the levels of BDV genome RNA (a) and mRNA (b) after heat stress for 12 or 24 hr. OL/BDV cells were grown at either 37°C or 40°C for 12 or 24 hr. The levels of BDV genome RNA and mRNA were determined by qRT-PCR and the results are given in percent related to unstressed control. (c, d) Increase of the levels of BDV genome RNA (c) and mRNA (d) after heat stress for 2 or 4 hr. After 24 hr at 37°C, OL/BDV cells were grown at either 37°C or 40°C for 12 or 24 hr. (e) Increase of the levels of BDV antigenome RNAs after heat stress for 2, 4, 12 or 24 hr. After 24 hr at 37°C, OL/BDV cells were grown at either 37°C or 40°C for 2, 4, 12 or 24 hr. The levels of BDV antigenome RNAs were determined by qRT-PCR and the results are given in percent related to unstressed control. Values are expressed as the mean + S.D. \*  $P < 0.05$ , \*\*  $P < 0.01$ , n.s. no significance (Student's  $t$  test). At least three independent experiments were performed.

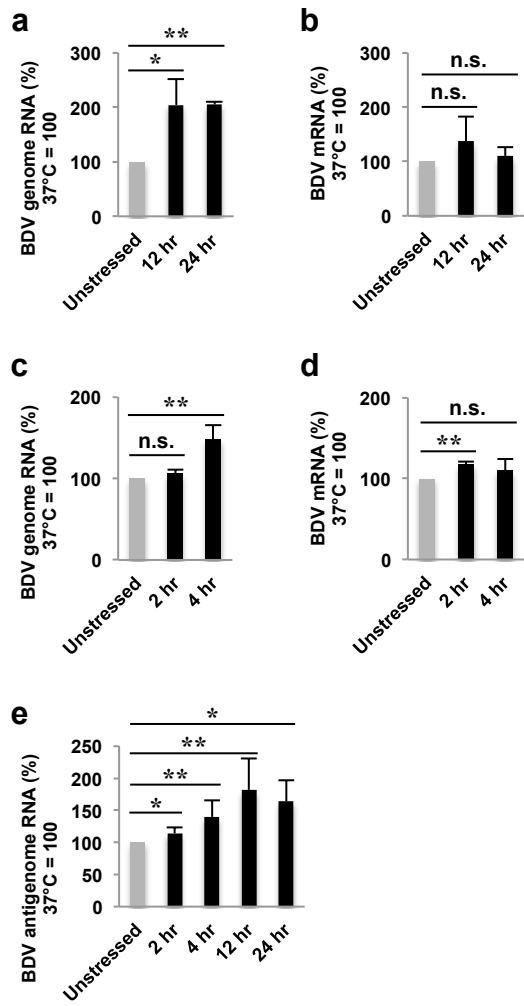
Fig. 2 Cell viability after heat stress. Cell viability after heat stress for 4 or 12 hr was analyzed by the cell number (a) and WST-1 assay (b). Values are expressed as the mean + S.D. n.s. no significance (Student's  $t$  test). At least three independent experiments were performed.

Fig. 3 Effect of heat stress on BDV proteins. (a) The level of BDV proteins after heat stress. After 24 hr at 37°C, OL/BDV cells were incubated at either 37°C or 40°C for the indicated time. The levels of N, P, and tubulin were assessed by Western blotting. (b)

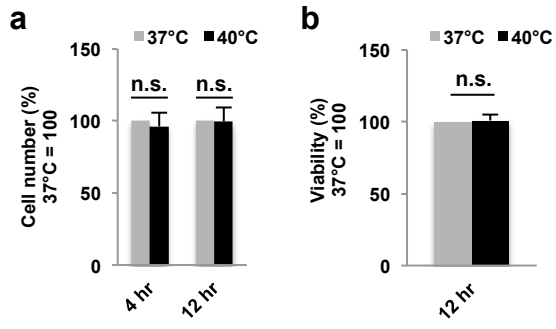
Subcellular localization of BDV proteins, N and P, after heat stress. Heat-stressed or unstressed OL/BDV cells were fixed and stained using anti-N and anti-P antibodies. (c) Subcellular localization of BDV proteins, X and P, after heat stress. Heat-stressed or unstressed OL/BDV cells were fixed and stained using anti-X and anti-P antibodies. The cells were counterstained with DAPI. Bars, 5  $\mu\text{m}$ .

Fig. 4 Minigenome assay at either 37°C or 40°C. At 36 hr after transfection of BDV minigenome carrying a *Guassia* luciferase gene, the transfected cells were further incubated at either 37°C or 40°C for the indicated time. The results are given in percent related to unstressed control at the indicated time. Values are expressed as the mean + S.D. n.s. no significance (Student's *t* test). At least three independent experiments were performed.

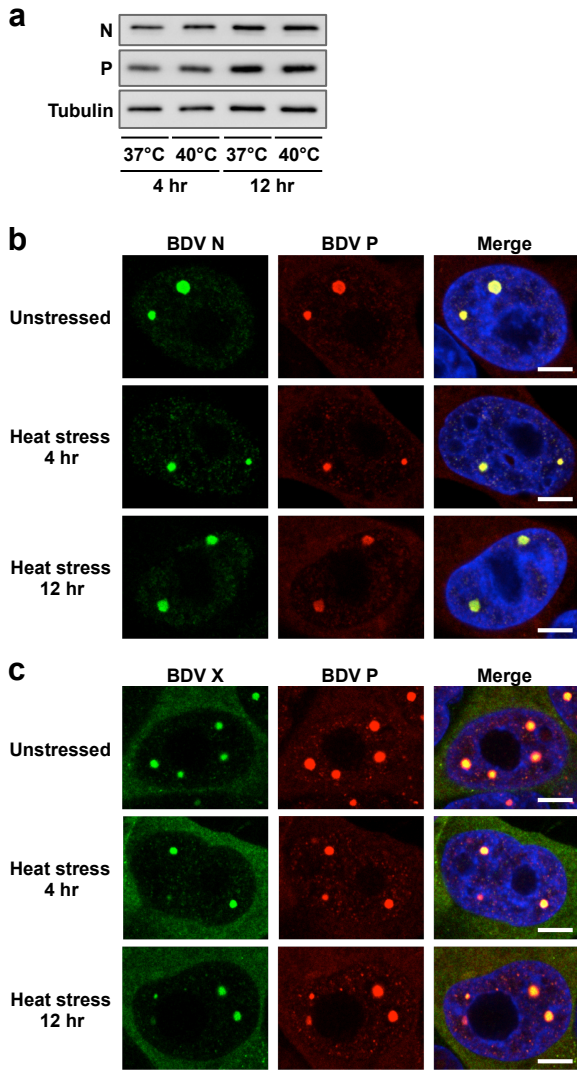
Fig. 5 Increase of GFP-positive cells in 293T cells expressing components of BDV RNPs carrying a GFP gene by heat stress. (a) Outline of rBDV rescue procedure. (b, c) Increase of GFP-positive cells by heat stress. 293T cells were transfected a plasmid expressing the BDV full-length antigenome RNA carrying a reporter GFP gene and helper plasmids expressing N, P, and L. The transfected 293T cells were subjected to heat stress for 4 (b) or 12 hr (c) at 1 and 4 dpt. The number of GFP-positive cells at the indicated time point was measured by fluorescent microscopy. Values are expressed as the mean + S.D. \*  $P < 0.05$ , \*\*  $P < 0.01$ , n.s. no significance (Student's *t* test). At least three independent experiments were performed.



**Figure 1**

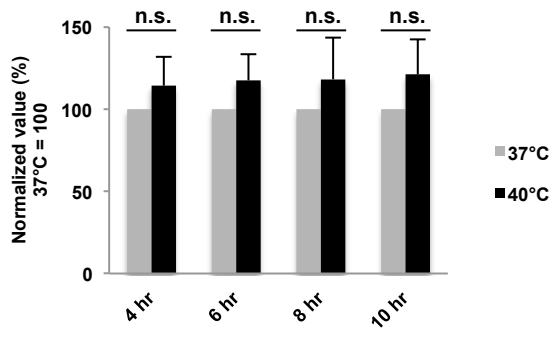


**Figure 2**

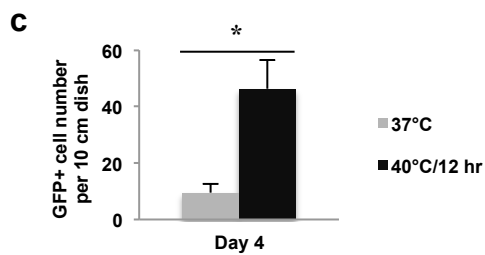
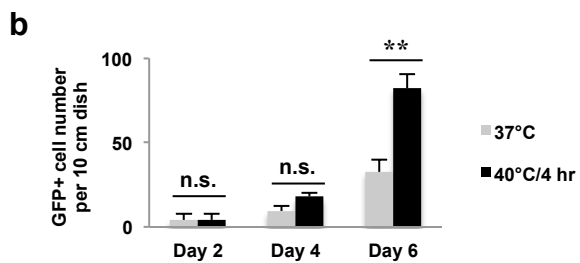
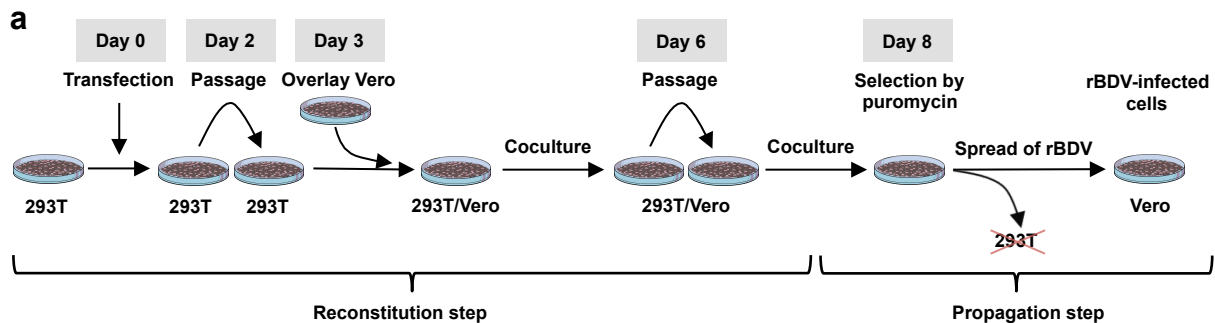


**Figure 3**





**Figure 4**



**Figure 5**