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Full Length Research Paper

Prokaryotic expression and polyclonal antibody production of transactivator Tas for potential application in detection of human foamy virus infection

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The human foamy virus (HFV) may pose a risk to humans as an infectious agent. The HFV transactivator Tas plays a critical role as a regulatory factor in viral replication, and potently activates gene expression via the 5' LTRs (long terminal repeats) of HFV. However the anti-Tas antibody currently available can rarely be applied in the investigation and diagnosis of HFV because of its high valence. To better understand the possible role of Tas as a potential candidate for the diagnosis and understanding of the infectious mechanism of HFV, the cloning, expression, and purification of Tas are described in this report. Additionally, the preparation of a polyclonal antibody directed against Tas is described. The *Tas* gene was amplified from wild type HFV (pHSRV13) by polymerase chain reation (PCR), subcloned into the pGEX-4T-1 expression plasmid, and then introduced into *Escherichia coli* BL21 (DE3) cells. To make the GST-Tas fusion protein, the recombinant expression plasmid pGEX-4T-1-Tas was induced using isopropyl-β-d-thiogalactoside. Optimal levels of GST-Tas were obtained by optimizing the concentration of IPTG and the induction time. After purification, GST-Tas was used to immunize mice, after which a standard protocol was used to acquire antiserum. Western blot analysis indicated that the prepared antiserum reacted specifically to the recombinant protein when expressed in eukaryotic cells, demonstrating good antigenicity of the antiserum. The HFV Tas protein showed low homology when compared to the foamy virus Tas protein in other species, suggesting that the HFV Tas polyclonal antibody may be specific to HFV Tas. The present study sheds light on the mechanism of the transactivator function and immunogenicity of Tas. Information from this study could be used to develop antibody or antigen detection assays for the clinical detection of the HFV pathogen.

Key words: Human foamy virus (HFV), Tas, polyclonal antibody, expression, western blot.

INTRODUCTION

The foamy virus belongs to the spumavirus genus of retroviruses, and is endemic in most humans, monkeys, and other mammals (such as cats and cattle). Although humans are not a natural host for the foamy virus, they can be infected through zoonosis (Jones-Engel et al., 2005; Khan et al., 2006). Recently, researchers found that foamy virus might also be spread from mother to child and/or through sexual contact with infected women (Betsem et al., 2011). Therefore, it is possible that the foamy virus is similar to human immunodeficiency virus (HIV) and severe acute respiratory syndrome (SARS), because it is a new infectious threat to humans.

Human foamy virus (HFV) was isolated in 1971 from a

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wide tissue tropism commonly found in mammals. The virus can infect cells from many vertebrate species and can also infect diverse tissue types (Achong et al., 1971). Although the infection has not been associated with a specific disease, transgenic mice carrying the *bel* (also called *Tas*) region of HFV displayed a progressive degenerative disease of the central nervous system and the striated muscle (Bothe et al., 1991), which suggested the pathogenic potential of HFV in humans. As a result, concern about an HFV outbreak is increasing and prevention should be stressed.

HFV has the largest known retroviral genome, containing the *gag* (group specific antigen), *pol* (polymerase) and *env* (envelope) structural genes for virion proteins (Cullen et al., 1991; Stange et al., 2005; Liu et al., 2005, 2007 and 2008). In addition to these genes, HFV also has three open reading frames (ORFs) that produce regulatory factors (Mergia et al., 1991; Lochelt et al., 1995). Unlike other retroviruses, the foamy virus uniquely possesses a 5' long terminal repeat (5' LTR) promoter and an internal promoter (IP) (Linial et al., 2000). The first open reading frame, termed *Tas*, is located between the 3' LTR and the *env* gene. The *Tas* gene encodes a DNA-binding, transactivator Tas protein with a molecular mass of 36 kDa. Its transcript relies on the first ORF of the non-structure coding region of the virus genome. Although Tas has the potential ability to transactivate two promoters, it has a higher affinity for the IP compared to the 5' LTR (Bodem et al., 2007), which causes a robust transcriptional transactivation. This event explains why the IP is activated at an early stage to express regulators during the foamy virus life cycle (Mergia, 1994). Currently, Tas has been confirmed as a unique regulator of HFV and is required for the replication and gene expression of HFV (Löchelt et al., 1991, 1993; He et al., 1996; Zhang et al., 2010; Zhu et al., 2011).

This study was performed to determine detection methods for HFV infection. Such HFV detection methods can be used to prevent the potential threat of HFV infection and to provide an essential basis to investigate the novel functions of HFV Tas. In this paper, we cloned the expression plasmid pGEX-4T-1-Tas, purified the HFV Tas protein, and prepared a polyclonal antibody directed against Tas. This polyclonal antibody may be used in HFV serological detection reagents and will serve as a tool for further functional analyses of this gene.

MATERIALS AND METHODS

The *Escherichia coli* strains DH5α and BL21 (DE3) and the pGEX-4T-1 vector were prepared at Wuhan University. T4 DNA ligase, Taq DNA polymerase and all restriction enzymes were purchased from TaKaRa. Protein molecular markers were purchased from Fermentas. PCR product purification kits and plasmid extraction kits were provided by Tian Gen. Five male 6 week old BALB/c mice were purchased from the Animal Holding Unit (AHU) of Tongji Medical College of Huazhong, University of Science and Technology (Wuhan, China).

Construction of the pGEX-4T-1-Tas expression plasmid

The full-length *Tas* gene, which encodes a 300 amino acid protein, was amplified successfully from the HSRV13 plasmid using the following specific primers: forward primer-5'-GTCCCGGGTATGGATTCCTACGAAAAAGA-3' and reverse primer-5'-CCGCTCGAGTTATAAAACTGAATGTTCACCTGACCC -3'. The PCR product was digested with *SmaI* and *XhoI* and subsequently gel purified. The PCR reaction was carried out in a 50 µl total volume using the following reaction conditions: denaturation at 94°C for 5 min followed by 32 cycles of amplification (94°C for 50 s, 55°C for 50 s, 72°C for 60 s). Finally, the recombinant plasmid was verified by double digestion and sequencing.

Expression of GST-Tas by induction of *E. coli* BL21 (DE3)

The transformant containing the pGEX-4T-1-Tas plasmid was cultured overnight at 37°C (shaking at 260 rpm) in 5 ml LB medium. The construct was then subcultured in 500 ml fresh LB containing ampicillin (a concentration of 1:1000) and then grown until the appropriate density (OD600 ≈ 0.6-0.8) was reached. IPTG was added to final concentration of 0.3 mM for 2 h to induce the expression of the fusion protein. As a negative control, bacteria transformed with the empty pGEX-4T-1 vector were treated as described above.

Extraction and purification of the recombinant GST-Tas protein

The 500 ml culture pellet was harvested by centrifugation (4°C, 12000 rpm for 10 min) and resuspended in lysis buffer containing 20 g/ml cell pellet, 1 mg/ml lysozyme and 1 mmol/L PMSF. Then, the cell resuspension liquid was incubated for 30 min on ice and sonicated for 30 min. The cell debris was removed by centrifugation (4°C, 12,000 rpm for 15 min). The supernatant, which is the soluble fraction, was collected and the pellet, which is the insoluble fraction containing inclusion bodies, was re-suspended in lysis buffer. All the fractions were analyzed by 10% Tricine-SDS-PAGE to characterize the presence of recombinant Tas protein. After electrophoresis, the gel was briefly stained with R250 for 4 h and de-stained by methanol. The recombinant protein was affinity-purified with a glutathione-agarose extraction kit for further studies.

Preparation of a polyclonal antibody against Tas

Before injection of the antigen, murine serum was reserved at -80°C. After treatment with Freund's Complete Adjuvant for initial injection (Benjouad, 2009), 150 µg of the Tas fusion protein was used to immunize BALB/c mice. Three booster injections were given (containing 75 µg of fusion protein) at one-week intervals. The antiserum was harvested 7 days after the final injection.

Antiserum titer by ELISA analysis

Antibody titer was measured using an enzyme-linked immunosorbent analysis (ELISA) according to the methods described previously (Li et al., 2011; Lu et al., 2011; He et al., 2011). The purified antigen was diluted to 20 µg/ml in 50 mM carbonate salt buffer (pH 9.6). Then, 100 µl or purified antigen per well was coated on plates. The coated plates were placed at 4°C overnight in carbonate–bicarbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 8.6). After the addition of blocking solution (5% non-fat dry milk and 0.05% Tween-20 in PBS) to each well and incubation at room temperature for 2 h, the plates were washed three times with PBS. Different concentrations of diluted antiserum (ranging

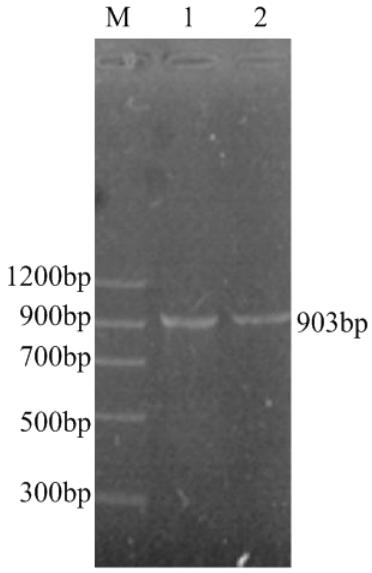

Figure 1. PCR products of the *Tas* gene following 1% agarose gel electrophoresis. Lane M: DNA 100 bp-1200 bp DNA marker; Lane 1 and 2: *Tas* gene.

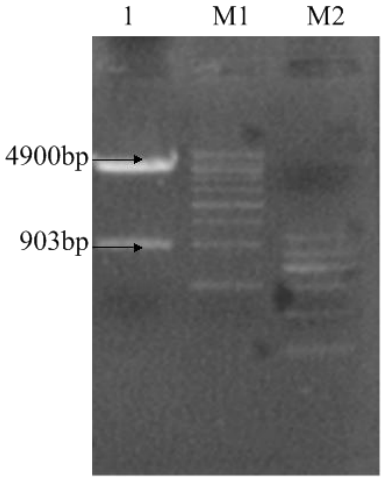

Figure 2. Restriction enzyme digestion analysis of recombinant plasmid. Lane M1: 1 Kb DNA marker; M2: 100 bp-1200 bp DNA marker; Lane 1: *SmaI/XhoI* enzyme-digested product.

from 1:100 to 1:2000) were incubated with the Tas antigen on the plates at 37°C for 2 h. The plates were washed three times with PBS, and goat anti-mouse secondary antibody (1:5000) was applied at 37°C for 1 h. Horseradish peroxidase (HRP) activity on the immunoplate was detected using tetramethyl benzidine (TMB), an enzyme substrate. The reaction was terminated with 2 M H_2SO_4. The antibody titer was detected by measuring absorbance at 450 nm using a microplate reader.

Western blot analysis of the recombinant proteins

HeLa cells were transiently transfected with Tas protein. Cells were washed with D-Hank's buffer twice and then lysed with ice-cold RIPA (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM EDTA, 10 µg/ml leupeptin, 2 mM Na_3VO_4, 15 µg/ml aprotinin and 1 mM PMSF) for 30 min at 4°C with occasional vortexing. The lysates were collected into 1.5 ml tubes and cleared of nuclei by centrifugation at 12,000 g for 10 min. The lysates of transfected HeLa cells were analyzed by 10% SDS-PAGE gels and then transferred electrically to a PVDF membrane at a constant voltage of 100 mV for 2 h. The positive serum sample (1:50 diluted in TBS) was used as the primary antibody (incubated at 37°C for 2 h) and the HRP-conjugated goat anti-mouse IgG (1:5000 dilution) was used as the secondary antibody (incubated at 37°C for 1.5 h). After being washed three times with TBS-T, the membrane was visualized using ECL.

RESULTS

Construction of the pGEX-4T-1-Tas prokaryotic expression plasmid

The *Tas* gene, encoding the transactivator of *spumavirus,* which was amplified by PCR from the pHSRV13 plasmid and then inserted into the expression vector pGEX-4T-1. When resolved on a 1% agarose gel by electrophoresis, the observed length of the target nucleotide sequence was approximately 903 bp, consistent with the expected size (Figure 1). Subsequently, the constructed clone was verified with a double enzymatic digestion (Figure 2). Sequence analysis confirmed that there were no amino acid mutations (data not shown).

Soluble expression and purification of the GST-Tas fusion protein

The confirmed expression vector pGEX-4T-1-Tas was introduced into the *E. coli* strain BL21 (DE3). The fusion protein was induced at 37°C using 0.3 mM IPTG. SDS-PAGE analysis showed a band of approximately 62 kDa, as visualized by Coomassie Brilliant Blue R250 staining. The analysis of the soluble fraction and the cell debris pellet suggested that the majority of the fusion protein is soluble (Figure 3). The target protein was not observed in the control lane containing total cellular protein after IPTG induction. Sonicated cell lysates were purified according to the manufacturer's recommendations (Figure 4).

Specificity analysis by western blot

The purified recombinant Tas protein (GST-Tas) was used to immunize mice, following immunization of the mice, the polyclonal antiserum was successfully obtained. ELISA results showed that various dilutions of the polyclonal

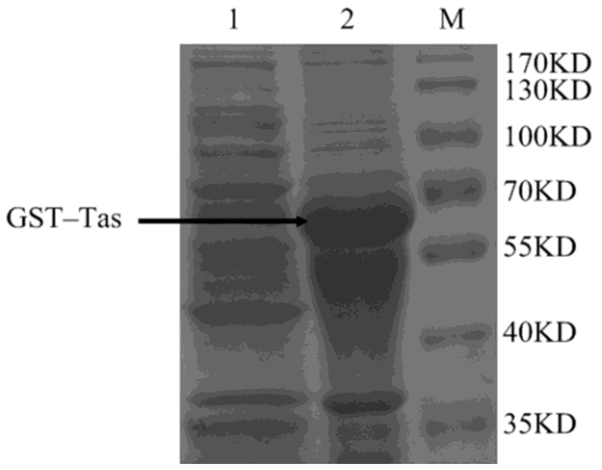

Figure 3. Solubility analysis of the expressed fusion protein by SDS-PAGE. Lane M: protein molecular weight marker; Lane 1: the pellet of GST-Tas induced for 4 h; Lane 2: the supernatant of GST-Tas induced for 4 h.

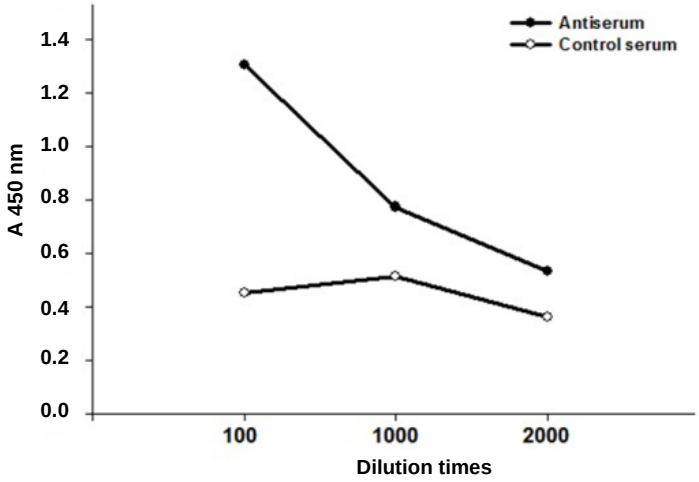

Figure 5. Sensitivity analysis of antiserum by ELISA. The antibody titer of anti-GST-Tas was measured by ELISA. The polyclonal antibody at different dilutions (1:100 to 1:2000) reacted with the recombinant Tas protein.

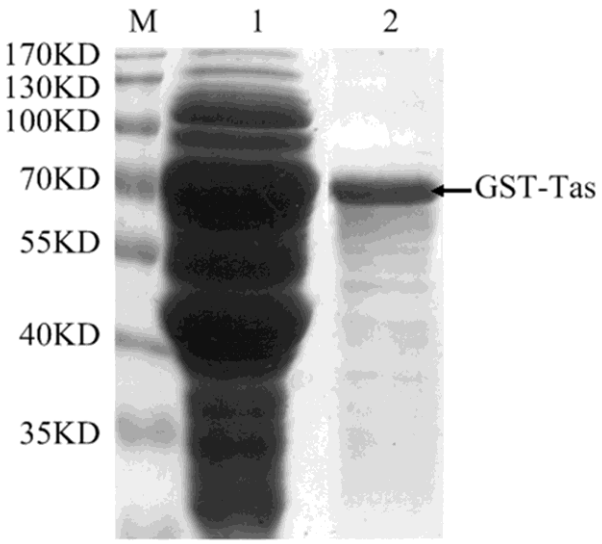

Figure 4. Expression and purification of the GST-Tas protein. Lane 1: unpurified Tas protein obtained at 4 hours post-induction; Lane 2: purified Tas protein obtained at 4 hours post-induction; Lane M: protein molecular weight marker.

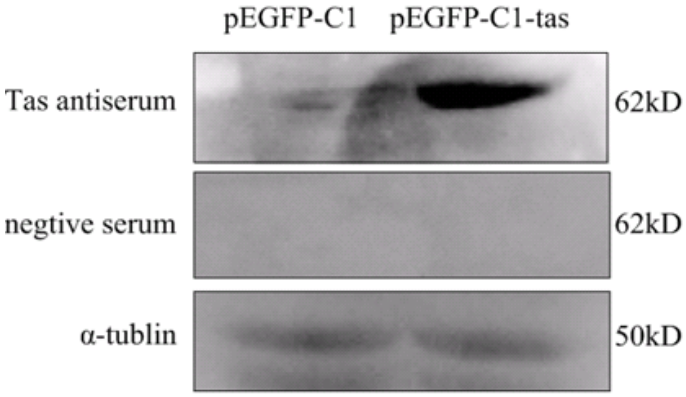

Figure 6. Determination of specificity by western blot analysis. HeLa cells were transfected with the plasmids pEGFP-C1 and pEGFP-C1-Tas in duplicates. 24 h later, cell extracts were analyzed by immunoblotting using an antibody against the Tas protein following SDS-PAGE.

antibody (1:100 to 1:2000) reacted with the recombinant Tas protein (Figure 5). Meanwhile, serum from non-immunized mice did not show detectable precipitation (Figure 5). Western blot analysis was performed to determine the specificity of the antiserum (Figure 6), which revealed that the recombinant Tas protein could be recognized by the anti-Tas polyclonal antibody. However, no obvious band in non-immunized serum control was detected. These results indicate that the antiserum has high specificity for the Tas protein.

DISCUSSION

In this study, the recombinant expression plasmid GEX-4T-1-Tas was successfully constructed. Additionally, a polyclonal antibody against the transactivator Tas protein was produced from mice immunized by the purified GST-Tas protein.

The trans-activator Tas plays an important role in foamy virus replication and latency (Keller et al., 1991; Zhang et al., 2010; Zhu et al., 2011). At the early stage of viral infection, the IP, downstream of *env*, initiates the transcription of non-structural genes, including *Tas*.

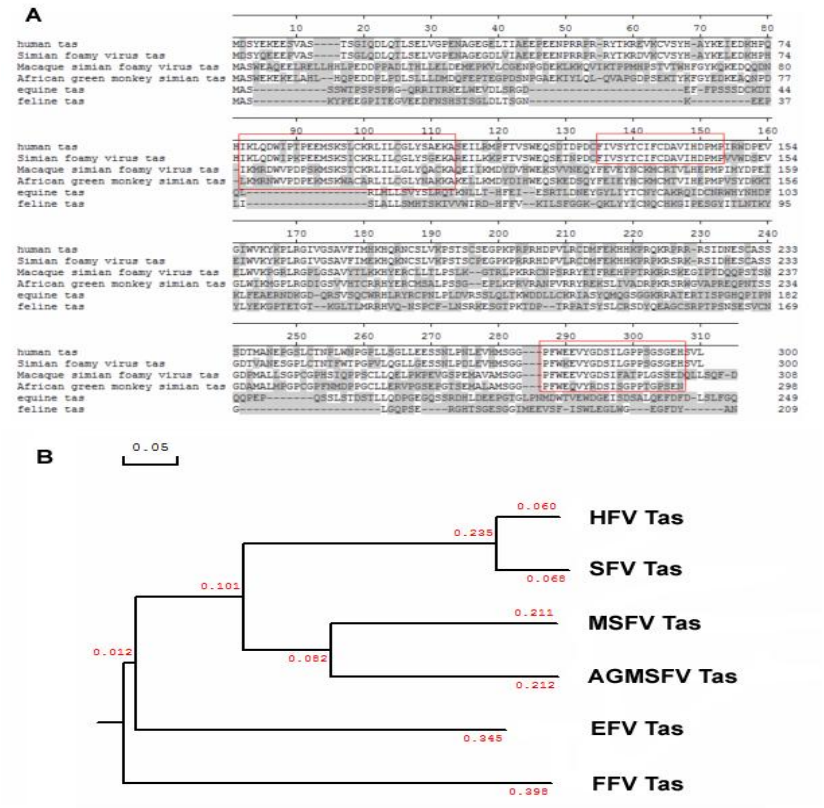

Figure 7. Analysis of homology of foamy virus Tas in different species. The analysis chart originated from amino acid sequences deduced from *Tas* gene sequences after alignment using the DNAMAN program. The accession numbers of the sequences used to analyze Tas proteins are: NP_044282 (human foamy virus Tas), NP_056806 (simian foamy virus Tas), YP_001961124 (macaque simian foamy virus Tas), YP_001956724 (African green monkey simian foamy virus Tas), NP_054718 (equine foamy virus Tas), NP_056917 (feline foamy virus Tas). (A) Comparison of Tas amino acid sequences from various types of foamy viruses. (B) Co-evolutionary relationships of foamy virus Tas phylogenetic trees. The phylogenetic tree shows genetic distances: bars, 0.05 nucleotide substitutions per site. (HFV: human foamy virus; SFV: simian foamy virus; MSFV: Macaque simian foamy virus; AGMSFV: African green monkey simian foamy virus; EFV: equine foamy virus; FFV: feline foamy virus).

Subsequently, the IP is activated by positive feedback associated with Tas regulation. This activation, accompanied by some form of cytokine activity, along with the activation of LTR, results in the expression of structural proteins after IP activation (Mergia, 1994). It is obvious that the processes of gene expression and transcription in foamy virus are complex. Thus, the relationship between Tas and the cytokines involved in its activation of IP requires better understanding.

Thus far, characterization of the Tas protein reported in research papers has relied mainly on the use of fusion proteins with tags such as Myc and Flag. In this study, the valence of the antibody was analyzed by ELISA. Furthermore, the responses and specificities of the antibody were confirmed by the detection of endogenous Tas protein, originating from HFV, as shown by western blot analysis (Figures 5 and 6). Therefore, our studies show that the anti-Tas polyclonal antibody can be used to detect the Tas protein. This anti-Tas antibody could be used to provide assistance to researchers engaging in these mechanical studies in the future.

Foamy virus, specifically simian foamy virus (SFV), was also reported to infect three people (Jones-Engel et al., 2005). Additionally, the human-to-human transmission of SFV by blood transfusion has been investigated in a monkey model (Brooks et al., 2002; Khan et al., 2006). To predict whether the antibody against HFV Tas can be suitable for use with other foamy viruses, we compared the homology of the foamy virus Tas protein in various species (Figure 7 and Table 1, the corresponding

Table 1. Analysis of multiple comparison of the homology of foamy virus Tas.

Homology	HFV (%)	SFV (%)	MSFV (%)	AGMFSV (%)	EFV (%)	FFV (%)
HFV	100					
SFV	87.20	100				
MSFV	41.10	41.40	100			
AGMFSV	40.50	39.90	57.70	100		
EFV	20.80	18.20	20.10	18.50	100	
FFV	25.30	24.90	24.80	27.80	24.6	100

(HFV: human foamy virus; SFV: simian foamy virus; MSFV: Macaque simian foamy virus; AGMSFV: African green monkey simian foamy virus; EFV: equine foamy virus; FFV: feline foamy virus).

sequence in simian (SFV, NP_056806), macaque simian (MSFV, YP_001961124), monkey (AGMFSV, YP_001956724), horse (EFV, NP_054718), and cat (FFV, NP_056917)). The results, indicating low homology, suggest that our polyclonal antibody may have high specificity (Figure 7 and Table 1). In addition, the homology of these Tas proteins in various species share conserved regions. However, there are huge differences between species that should not be ignored (Figure 7). Among the complete sequences, there are two conserved motifs that might be important in the regulation of Tas located between amino acids 83 to 133 and 287 to 307 (Figure 7A). Our future research may utilize these two motifs targets for the production of a vaccine directed against foamy virus infection. Interestingly, HFV and SFV Tas sequences show high homology (87.2%), while comparison between other Tas sequences shows low homology (Table 1). In the phylogenetic trees (Figure 7B), there is evidence that HFV is closer to SFV instead of other foamy viruses. Therefore, the antibody against HFV Tas may be able to detect SFV infection as well as infection. There is a highly conserved region from residues 135 to 153 in both HFV and SFV (Figure 6A). Whether the function of Tas is associated with this region remains to be established.

Still, the pathogenicity of HFV remains controversial. It is known that the low level of viral protein synthesis and the replication of virions make it harmless to the host. Also, it is reported that this virus can be isolated from patients with various neoplastic and degenerative diseases (Aguzzi et al., 1992). In addition, the transactivation mechanism of Tas is unclear, though it is a critical factor in the expression and transcription of HFV. Thus, the importance of detecting the replication and transcription of the foamy virus in infected individuals indicates the need to find a way of measuring the quantity and quality of its transactivator Tas. We hope that the polyclonal antibody against HFV Tas will someday facilitate the research on Tas, and be developed for a clinical assay to detect the foamy virus pathogen.

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