Supporting information

Supporting text

The Level of Viral Protein Synthesis in vRNP-transfected Yeast and Mammalian Cells.

To examine the efficiency of viral protein synthesis from vRNP, we examined the synthesis level of NP in vRNP-transfected yeast and mammalian cells (Fig. 1*C*). Yeast spheroplasts were transfected with 3 μl of vRNP (450 ng NP equivalents). At 15 or 20 h post-transfection, yeast lysates were prepared according to the procedure as previously described (1). HeLa cells were transfected with 3 μl of vRNP using DMRIE-C regent (SI Methods). Yeast lysates (22.5 μg/lane) or HeLa cell lysates (20 μg/lane) were separated by 10% SDS-PAGE and analyzed by western blotting using anti-NP antibody. NP was detected in vRNP-transfected yeast and HeLa cells, but not in cells treated with CHX. We also confirmed the efficiency of vRNP transfection by indirect immunofluorescence assay using anti-NP antibody. A few percent of yeast cells showed the detectable level of NP, whereas HeLa cells exhibited a transfection efficiency of ~20% (data not shown).

Effect of Tat-SF1 Knock-down on Virus Infection.

We examined by knock-down experiments whether Tat-SF1 is involved in the viral RNA synthesis. The synthesis level of viral mRNA derived from segment 3 in siTat-SF1-transfected cells was reduced to 70% of that in mock-transfected cells (SI Fig. 8), whereas in siTat-SF1-transfected, that was reduced to 85-90% reduction in the

segment 7 mRNA synthesis (Fig. 2*F*). The overall profile of the synthesis level of vRNA and cRNA in both segments 3 and 7 seemed similar. Segment 7 is one of the late genes. Transcription of the late genes occurs effectively in the late phases of infection. In contrast, the synthesis of viral cRNA and vRNA of all segments initiates from the early phases of infection. Therefore, we assumed that the lower level of the reduction of segment 7 mRNA synthesis in Tat-SF1 knock-down cells could be related to a low transcription level of segment 7 mRNA in the early phases of infection at 3 phi.

Tat-SF1 Stimulates the Viral RNA Synthesis.

We quantitatively examined the amount of viral RNAs derived from segment 3 and segment 7 in virus infected-FLAG-Tat-SF1 over-expression cells. Tat-SF1 efficiently stimulated the synthesis of viral cRNA, mRNA, and vRNA of segment 7 (SI Fig. 9). However, an increase in the level of the segment 3 vRNA synthesis was lower than that in the mRNA synthesis (Fig. 3, compare *B* and *C*). Tat-SF1 has two RNA recognition motifs (RRMs). Tat-SF1 RRM domain is involved in the interaction with the snRNPs through the recognition of a specific RNA molecule. The 3'- and 5'-terminal sequences of the influenza virus genome, consisting of conserved terminal sequences of 10-12 nucleotides followed by segment-specific sequences, are believed to be the regulation site for transcription and replication of the genome. We assumed that the Tat-SF1 sensitivity varies among segments. Together with the discussion in the previous section, the molecular mechanisms for transcription timing in the course of infection (2) and the equality of the replication level among all segment (2) are important open questions to be addressed.

Supporting figure legends

SI Fig. 5. Effect of *sub2* deletion.

(*A*) The stimulatory activity of recombinant RAF-2p48 and SUB2 proteins. *In vitro* viral RNA synthesis was carried out in the absence (lane 1) or presence of increasing amounts (10 ng (lanes 2, 5, and 8), 30 ng (lanes 3, 6, and 9), and 100 ng (lanes 4, 7, and 10)) of recombinant RAF-2p48 (lanes 2-4), SUB2 (lanes 5-7) or bovine serum albumin (BSA, lanes 8-10). The RNA product from the 53 base-long model viral genome is indicated by arrowhead.

(*B*) Effect of *sub2* deletion on viral RNA synthesis. YPH499wt and YPH499Δ*sub2* strains were transfected with vRNP, and incubated for 9, 12, and 15 h, and total RNA was extracted. Real-time RT-PCR was carried out with primer sets specific for segment 7 cRNA and *RDN25-1* rRNA. The total RNA prepared form mock-transfected yeast cells were also analyzed. Error bars, standard deviation.

SI Fig. 6. Comparison of viral RNA synthesis in selected yeast deletion strains.

vRNP was transfected into yeast strains lacking factors related to splicing. Yeast cells were incubated for 12 h. Real-time RT-PCR analysis was performed with primer sets specific for segment 7 cRNA and *RDN25-1* rRNA. Error bars, standard deviation.

SI Fig. 7. Knock-down of Tat-SF1.

(*A*) RT-PCR was carried out using primer sets specific for *Tat-SF1* or *β-Actin* mRNA and total RNA prepared from HeLa cells transfected with pU6-puro plasmid (lanes 1-3) or siRNA (lanes 4-6).

(*B*) HeLa cells were transfected with either pU6-puro-siEGFP (lanes 1 and 3) or pU6-puro-siTat-SF1 (lanes 2 and 4) and then super-infected with influenza virus. Total RNA was prepared from cells at 6 hour post infection (hpi) (lanes 3 and 4). Total RNA prepared from mock-infected HeLa cells was also analyzed (lanes 1 and 2). RT-PCR was carried out with primer sets specific for segment 7 cRNA and *β-Actin* mRNA.

SI Fig. 8. Effect of Tat-SF1 knock-down on virus infection.

(*A*-*C*) HeLa cells were transfected with control siRNA or siTat-SF1. After 72 hpt, cells were transfected with pCAGGS-FLAG-rTat-SF1 and pCAGGS-empty plasmids. After 24 h incubation, cells were super-infected with influenza virus at the moi of 3. Total RNA was prepared from cells at 3 hour post infection (hpi). Real-time RT-PCR was carried out with primer sets specific for segment 3 RNAs (*A*: cRNA, *B*: mRNA, *C*: vRNA), and *β-Actin* mRNA. Error bars show standard deviation.

SI Fig. 9. Tat-SF1 as a stimulatory host factor for influenza virus.

(*A*-*C*) HeLa cells expressing FLAG-Tat-SF1 (Tat-SF1) or control (Neo) HeLa cells were infected with influenza virus at the moi of 3. Real-time RT-PCR was carried out with primer sets for segment 7 RNAs (*A*: cRNA, *B*: mRNA, *C*: vRNA), and *β-Actin* mRNA. The results are normalized as the ratio to the level of *β-Actin* mRNA. Error bars show standard deviation.

Supporting Materials and Methods

Yeast Strains and Culture Conditions.

Yeast strain YPH499 (*MATa, ura3-52, lys2-801, ade2-101, trp1-63, his3-200, leu2-1*) was used in all experiments. Yeast cells were grown at 30ºC in yeast extract-peptone-dextrose medium containing adenine (YPDA). For protein expression induced by galactose, single colonies were picked up and first inoculated into synthetic defined (SD) medium containing 2% glucose. Yeast cells were grown at 30ºC until the optical density at 610 nm (OD_{610}) reached 0.6-0.8. Then, yeast cells were collected by centrifugation at 800 x g and washed twice with phosphate-buffered saline (PBS). The collected yeast cells were cultured in synthetic galactose (SG) medium containing 2% galactose for 6 to 12 h, allowing the induced expression of proteins.

The following plasmids were generated for construction of a *sub2* deletion strain. The sequence of all oligonucleotides and primers used in this study were summarized in SI (Supporting information) Table 1. The *sub2* deletion strain was generated by replacement of the entire *sub2* open reading frame with the *TRP1* (*sub2*Δ::*TRP1* fragment) (3). Promoter and terminator fragments of the *sub2* gene were amplified by RCR with primers SUB2 up-FOR and SUB2 up-REV for the upstream fragment, and SUB2 down-FOR and SUB2 down-REV for the downstream fragment, using the yeast genomic DNA as template. The upstream fragment was digested with *Kpn*I and *Sma*I, and cloned into *Kpn*I- and *Sma*I-digested pBluescript (pBS) plasmid. The resultant plasmid was designed pBS-SUB2-upstream. The downstream fragment was phosphorylated with T4 polynucleotide kinase (TOYOBO), and cloned into pBS-SUB2-upstream plasmid which had been digested with *Bam*HI and

blunted with Klenow fragment (TaKaRa). The generated plasmid, designated pBS-SUB2, contains a unique *Sma*I site between the promoter and the terminator fragments. *TRP1* gene fragment was excised from pRS314 plasmid by digestion with *Eco*O109I and *Ssp*I. To construct a plamid for disruption of *sub2*, designated pBS-SUB2-TRP1, the excised *TRP1* gene fragment was blunted with Klenow fragment, and cloned into *Sma*I-digested pBS-SUB2 plasmid. The *sub2*Δ::*TRP1* fragment was excised from pBS-SUB2-TRP1 by digestion with *Bgl*II and *Ban*III, and then used to transform yeast strain YPH499. *TRP1* transformants were tested for the stability of the *TRP1* phenotype, and *sub2* deletion was confirmed by RT-PCR and genomic PCR.

Introduction of DNA and vRNP Into Yeast Cells.

The lithium acetate-polyethylene glycol method was used to transform yeast cells with plasmid DNAs (4). Introduction of vRNP were performed according to the procedure described for transformation of RNA (5) with minor modifications. Yeast cells were grown at 30ºC in 50 ml of YPDA to a density of 3 x 10^7 cells/ml. The cells were collected by centrifugation, washed with 20 ml of 0.9% NaCl and then with 20 ml of 1 M sorbitol, and resuspended in 20 ml of SCEM (1 M Sorbitol, 0.1 M Sodium citrate (pH 5.8), 10 mM EDTA, and 30 mM 2-Mercaptoethanol). One hundred twenty-five units of Zymolyase-100T (Seikagaku Corporation) were added, and the cells were incubated at 30ºC with occasional mixing. The spheroplasts were collected by low-speed centrifugation, washed with 20 ml of 1 M sorbitol, and then resuspended in 20 ml of STC (1 M Sorbitol, 10 mM Tris-HCl (pH 7.4), and 10 mM $CaCl_2$). The spheroplasts were collected by centrifugation and resuspended in 2 ml of STC. For introduction of vRNP into spheroplasts, spheroplasts (100 μl) were mixed with vRNP.

Spheroplasts mixed with vRNP were incubated at room temperature for 10 min, after which 1 ml of PEG solution (20% polyethylene glycol 6000, 10 mM Tris-HCl (pH 7.4), and 10 mM $CaCl_2$) was added. After further incubation at room temperature for 10 min, the spheroplasts were collected by low-speed centrifugation and resuspended in 1 ml of SOS (1 M Sorbitol, 6.5 mM $CaCl_2$, 1/2 x YPAD or 1/2 x SD).

vRNP Into Mammalian Cells.

Transfection of HeLa cells with vRNP was carried out by using DMRIE-C regent (Invitrogen). HeLa cells were grown on a 3.5-mm-diameter dish at 50% confluence and transfected with DMREI-C regent-vRNP suspension. At 5 h post-transfection, the medium was replaced with fresh growth medium, and cells were further incubated.

Total RNA Extraction from Yeast, RT-PCR, and Genomic PCR.

Extraction of total RNA from yeast cells was performed as previously described (6).

cDNA for detection of segment 5 vRNA was synthesized by reverse transcriptase (TOYOBO) with NP-FOR 1 as primer. This single-stranded cDNA was then PCR-amplified with two specific primers, NP 444-FOR and NP 614-REV. cDNA for detection of segment 5 cRNA was synthesized by reverse transcriptase with NP 3'-END as primer. NP 3'-END primer recognizes the untranslated region of the 3'-terminal sequence of cRNA but not viral mRNA containing poly(A) in stead of the 3' terminal sequence. The single-stranded cDNA was then PCR-amplified with two specific primers, NP 846-FOR and NP 1121-REV. cDNA for detection of segment 5

mRNA was synthesized by reverse transcriptase with oligo-dT as primer. The single-stranded cDNA was then PCR-amplified with two specific primers, NP 846-FOR and NP 1121-REV. cDNA for detection of segment 3 vRNA was synthesized by reverse transcriptase with PA-FOR 1 as primer. The single-stranded cDNA was then PCR-amplified with two specific primers, PA 664-FOR and PA 800-REV. cDNA for detection of segment 3 cRNA was synthesized by reverse transcriptase with PA 3'-END as primer. The single-stranded cDNA was then PCR-amplified with two specific primers, PA 1356-FOR and PA 1683-REV. cDNA for detection of segment 3 mRNA was synthesized by reverse transcriptase with oligo-dT as primer. The single-stranded cDNA was then PCR-amplified with two specific primers, PA 1356-FOR and PA 1683-REV. cDNA for detection of segment 7 vRNA was synthesized by reverse transcriptase with M 67-FOR as primer. The single-stranded cDNA was then PCR-amplified with two specific primers, M 67-FOR and M 200-REV. cDNA for detection of segment 7 cRNA was synthesized by reverse transcriptase with M 3'-END as primer. The single-stranded cDNA was then PCR-amplified with two specific primers, M 897-FOR and M 1007-REV. cDNA for detection of *M1* mRNA was synthesized by reverse transcriptase with oligo-dT as primer. The single-stranded cDNA was then PCR-amplified with two specific primers, M 67-FOR and M 200-REV. cDNA for detection of *M2* mRNA was synthesized by reverse transcriptase with oligo-dT as primer. The single-stranded cDNA was then PCR-amplified with two specific primers, M 897-FOR and M 1007-REV.

For detection of cellular mRNAs, cDNAs were first synthesized with reverse transcriptase and oligo-dT as primer. For the *ACT1* mRNA of yeast, a sense primer, ACT1-FOR and an anti-sense primer, ACT1-REV were used for PCR amplification.

For the *ADH1* mRNA, a sense primer, ADH1-FOR and an anti-sense primer, ADH1-REV were used for PCR. For the *RDN25-1* rRNA, a sense primer, RDN25-1-FOR and an anti-sense primer, RDN25-1-REV were used for PCR. For the *SUB2* mRNA, a sense primer, SUB2 1062-FOR and an anti-sense primer, SUB2-REV were used for PCR. For detection of *TRP1* gene in yeast genome, *TRP1* fragment was amplified by PCR using TRP1-FOR and TRP1-REV as primers and the yeast genomic DNA as template. cDNA for detection of *Tat-SF1* mRNA was synthesized by reverse transcriptase with Tat-SF1 700-REV as primer. This single-stranded cDNA was then PCR-amplified with two specific primers, Tat-SF1 400-FOR and Tat-SF1 700-REV. cDNA for *Tat-SF1* mRNA was synthesized by reverse transcriptase with oligo-dT as primer. This single-stranded cDNA was then PCR-amplified with two specific primers, Tat-SF1 603-FOR1 and Tat-SF1 720-REV (for endogenous *Tat-SF1* mRNA), or Tat-SF1 603-FOR2 and Tat-SF1 720-REV (for exogenous *Tat-SF1* mRNA). For the *β-Actin* mRNA, a sense primer, β-Actin FOR and an anti-sense primer, β-Actin REV were used for PCR. Amplified double-stranded DNAs were subjected to a 7% polyacrylamide gel electrophoresis and were visualized by ethidium bromide. Real-time RT-PCR assays were performed with reverse transcriptase and FullVelocity SYBR Green QPCR Master Mix (STRATAGENE).

Preparation of vRNP Complexes and Thoes Devoid of Segment 5 vRNA.

All viral resources were derived from influenza A/Puerto Rico/8/34 (PR/8) virus. The purification of virions and the isolation of vRNP complexes were as described (7). To prepare vRNP devoid of segment 5 vRNA, vRNP (10 μl containing 1 μg NP equivalents) was incubated at 37ºC for 5 min in presence of 0.6 μl of 5 M NaCl

and 100 ng of oligonucleotides (Seg. 5 digestion) complementary to a part of segment 5 vRNA. Then, the mixture was diluted by adding 40 μl of 12.5 mM Tris-HCl (pH 7.9), 5 mM $MgCl_2$ and 1.25 mM dithiothreitol. Thirty units of RNase H (TaKaRa) were added, and the reaction mixture was incubated at 37ºC for 5 min. The oligonucleotides in the reaction were then completely digested with 4 U of RQ DNase I (Invitrogen) in the presence of 25 U of RNase inhibitor (TOYOBO) at 37ºC for 10 min (8).

***In Vitro* Influenza Virus RNA Synthesis.**

In vitro influenza virus RNA synthesis was performed as previously described (9). Briefly, RNA synthesis was carried out at 30ºC for 60 min in a final volume of 25 μl containing 50 mM HEPES-NaOH (pH 7.9), 3 mM $MgCl_2$, 50 mM KCl, 1.5 mM dithiothreitol, 500 μM each ATP, CTP, and UTP, 25 μM GTP, 5 μCi of [α-^{32}P]GTP (3,000 Ci/mmol), 4 U of RNase inhibitor, 250 μM ApG dinucleotide, 10 ng of a 53-nucletotide-long model RNA template (oligonucleotide: 53-merVwt) of negative polarity, and vRNP (10 ng NP equivalents) as an enzyme source. RNA products were purified, subjected to separation through a 10% PAGE in the presence of 8 M urea, and visualized by autoradiography.

Screening of Host Factors Affecting the Influenza Virus RNA Synthesis Using Yeast Knock-out Strains.

A set of *S. cerevisiae* haploid-deletion strains (BY4741 (*MAT*a, *his3*Δ1, *leu2*Δ0, *met15*Δ0, *ura3*Δ0) or BY4742 (*MAT*α, *his3*Δ1, *leu2*Δ0, *lys2*Δ0, *ura3*Δ0) strain background) were from Open Biosystems. Each yeast strain was transfected with vRNP using the spheroplasts method. At 12 or 16 h after incubation, total RNA was

prepared. The viral RNA synthesis level in each strain was analysized by RT-PCR.

Western Blotting Analysis.

Exponentially growing yeast cells were collected by low-speed centrifugation and washed twice with 0.9% NaCl. Cells were suspended in a buffer (50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 M Sorbitol, and 3 mM Dithiothreitol) containing of 100 μg/ml Zymolyase 100T. Cells were incubated at 30ºC for 60 min with occasional mixing. The spheroplasts were collected by low-speed centrifugation, resuspended in a cell lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% NP-40, and 7 M Urea), and lysis by sonication. The cell lysates were cleared by centrifugation at 15,000 x g for 10 min and subjected to separation through a 7.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blotting with the antibodies where indicated.

Gene Knock-down Experiments.

We used the pU6 plasmid to express 21 nucletotid-long hairpin-type siRNA under the control of the U6 promoter (10) and stealth siRNA (Invitrogen). The following plasmids were generated for selection by puromycin (Invivogen). DNA fragments containing the expression unit of *puromycin N-acetyl-transferase* were digested with *Bam*HI and *Cla*I from pBABE-puro. The fragment encoding *puromycin N-acetyl-transferase* was blunted with Klenow fragment, and cloned into *Ssp*I-digested pU6 plasmid. The resultant plasmid was designated pU6-puro. DNA fragments for targeting *Tat-SF1* and *EGFP* were amplified by PCR using siTat-SF1 FOR and siTat-SF1 REV, and siEGFP FOR and siEGFP REV (11), respectively. PCR products

were digested with *Bsp*MI, and cloned into *Bsp*MI-digested pU6-puro plasmid. The resultant plasmids were designated pU6-puro-siTat-SF1 and pU6-puro-siEGFP. HeLa cells in a 6-mm diameter dish were transfected with pU6-puro-siTat-SF1 or pU6-puro-siEGFP using TransIT LT1 reagent (Mirus). At 24 h after incubation, 2 μg/ml of puromycin was added, and cells were further incubated for 20 h. After the selection in the presence of puromycin, HeLa cells were transferred to the medium in the absence of puromycin. After incubation for 48 h, cells were infected with influenza virus at moi of 3. Total RNA was prepared from infected cells by the guanidine method. Stealth siRNA HTATSF1-HSS120650 (siTat-SF1#1) was 25 base pair-long duplex oligonucleotides with a sense strand of a part of the *Tat-SF1* mRNA sequence. The Stealth RNAi Negative Control Duplexes of Low GC Duplex (Invitrogen) was used as the control for Stealth siRNA of Tat-SF1. HeLa cells were transfected by 50 pmol of Stealth siRNA with 2 μl/well of Lipofectamine2000 transfection reagent (Invitrogen) according to the manufacture's instruction. At 72 h after incubation, cells were infected with influenza virus. Total RNA was prepared from infected cells using the guanidine method.

Construction of Yeast and Mammalian Vectors.

The following plasmids were constructed for expression of influenza virus RNA polymerase subunits and NP in yeast cells. The pYES2 plasmid contains the *GAL1* promoter for inducible expression of exogenous proteins by galactose (12). DNA fragments containing the CYC1 TT (transcription terminator) sequence were amplified by PCR using CYC1 TT-FOR and CYC1 TT-REV as primers and pYES2 as template. PCR products were digested with *Hin*dIII and *Spe*I, and cloned into *Hin*dIII-

and *Xba*I-digested pYES2 plasmid. The resultant plasmid, pYES2-CYC1 TT, contains unique *Hin*dIII, *Bgl*II, *Sma*I and *Bst*EII sites between the *GAL1* promoter and the CYC1 TT. To construct pYES2-PB2-CYC1 TT, DNA fragments corresponding to the PB2 coding sequence were amplified by PCR using PB2-FOR and PB2-REV as primers and pcDNA-PB2 (13) as template. PCR products were phosphorylated with T4 polynucleotide kinase and digested with *Bam*HI, and cloned into *Sma*I- and *Bgl*II-digested pYES2-CYC1 TT. To construct a plasmid for expression of NP, designated pYES2-NP, DNA fragments corresponding to the NP coding sequence were amplified by PCR using NP-FOR 2 and NP-REV as primers and pCAGGS-NP (14) as template. PCR products were digested with *Bam*HI and *Eco*RI, and cloned into *Bam*HI- and *Eco*RI-digested pYES2 plasmid. The following plasmids were constructed for expression of Tat-SF1 in mammalian cells. To construct pCAGGS-FLAG-Tat-SF1, DNA fragments corresponding to the Tat-SF1 coding sequence were amplified by PCR using Tat-SF1-FOR 1 and Tat-SF1-REV 2 as primers and cDNA library from 293T cells as template. PCR product was digested with *Eco*RI, and cloned into *Eco*RI-digested pCAGSS (15). To construct pCAGGS-FLAG-rTat-SF1 (siTat-SF1-resistant), two DNA fragments corresponding to the Tat-SF1 coding sequence were amplified by PCR using Tat-SF1-FOR 1 and Tat-SF1-REV 2 or Tat-SF1-FOR 2 and Tat-SF1-REV 1 as primers and pCAGGS-FLAG-Tat-SF1 as template. PCR products were phosphorylated with T4 polynucleotide kinase and digested with *Eco*RI, and cloned into *Eco*RI-digested pCAGGS.

Antibodies.

Preparations of rabbit anti-PB1, anti-PB2, anti-PA and anti-NP antibodies were described previously (16-18).

FLAG-Tat-SF1 Cell Line.

HeLa cells were co-transfected with pCAGGS-FLAG-Tat-SF1 and pSV2-Neo (encoding neomycin resistance gene). After selection with 1 mg/ml G418 (Nacalai), we confirmed the expression of FLAG-Tat-SF1 by Western blotting analysis using anti-FLAG antibody (Sigma).

Immunoprecipitation Assay.

His-myc-Tat-SF1 (500 ng) was mixed with either vRNP or mnRNP (250 ng of NP equivalents) in IP buffer (50 mM HEPES-NaOH (pH 8.0) and 0.1% NP-40) containing 100 mM KCl and incubated at 25ºC for 60 min. After incubation, anti-myc antibody-conjugated agarose beads (Nacalai) were added, and the mixture was rotated at 25ºC for 60 min. The beads were washed twice with IP buffer containing 300 mM KCl, and immunoprecipitated proteins were separated by 7.5% SDS-PAGE, followed by Western blotting with anti-PB1, anti-PB2, anti-PA, anti-NP, or anti-myc (Nacalai) antibodies.

Single-step Virus Growth.

To determine single-step virus growth, control or siTat-SF1 siRNA-transfected HeLa cells, and HeLa cells expressing FLAG-Tat-SF1 or control HeLa cells were infected with influenza virus at the moi of 0.1. After incubation, the cells were washed

and overlaid with serum-free MEM in absence or presence of 100 μg/ml of cycloheximide. Supernatants were collected at 14 and 18 h (control or siTat-SF1 siRNA-transfected HeLa cells), or 15 and 20 h (HeLa cells expressing FLAG-Tat-SF1 or control HeLa cells) post-infection.

Plaque Assay.

A confluent MDCK cells in a 6-well tissue culture plates were washed with serum-free MEM and then incubated for 1 h at 37ºC with 10-fold serial dilution of virus in 0.5 ml of serum-free MEM. After allowing at 37ºC for 1 h for virus adsorption, the cells were washed with serum-free MEM and then overlaid with MEM containing 0.1% BSA, 0.8% agarose (Sigma), 1 μg/ml trypsin (Nacalai). After incubation at 37ºC for 2 days, plaques were visualized by staining cells with 0.5% amido black.

Preparation of Recombinant Proteins.

The full-length *SUB2* gene was amplified by PCR using SUB2-FOR (His-) and SUB2-REV as primers and the yeast genomic DNA as template. PCR products were phosphorylated with T4 polynucleotide kinase, and cloned into *Eco*RV-digested pBS. The resultant plasmid was designed pBS-His-SUB2. To construct pET-21a-His-SUB2, the DNA fragment corresponding to the *SUB2* coding sequence was excised from pBS-His-SUB2 plasmid by digestion with *Nde*I and *Bam*HI. Then, this DNA fragment was cloned into *Nde*I- and *Bam*HI-digested pET-21a. This plasmid was used for transformation of *Escherichia coli* BL21 (DE3). The His-SUB2 protein was purified using Ni-nitrilotriacetic acid resin. Preparation of His-RAF-2p48/UAP56 and His-NP were described previously (9).

The full-length *Tat-SF1* gene was amplified by PCR using Myc-Tat-SF1 FOR and Tat-SF1 REV3 as primers and the pCAGGS-FLAG-Tat-SF1 as template. PCR products were digested with *Eco*RI and *Hin*dIII, and cloned into *Eco*RI- and *Hin*dIII-digested pFastBacHT. The resultant plasmid was designed pFastBac-myc-Tat-SF1. This plasmid was used for transformation of *Escherichia coli* DH10B. After transformation, we isolated recombinant bacmid DNA encoding His-myc-Tat-SF1 gene. Sf9 cells were cultured in Sf-900 SFM (GIBCO). To generate a recombinant baculovirus for expression of His-myc-Tat-SF1, Sf9 cells were transfected with a recombinant bacmid DNA encoding His-myc-Tat-SF1, and recombinant baculovirus was isolated (Bac-to-Bac Baculovirus Expression System, Invitrogen). For the expression of His-myc-Tat-SF1, Sf9 cells were infected with the recombinant baculovirus at a multiplicity of infection of approximately 10 and then collected at 80 hpi. The recombinant His-myc-Tat-SF1 in infected cell lysates was purified using Ni-nitrilotriacetic acid resin.

Supporting references

1. Yaffe, M. P. & Schatz, G. (1984) *Proc Natl Acad Sci U S A* **81,** 4819-4823.
2. Hatada, E., Hasegawa, M., Mukaigawa, J., Shimizu, K. & Fukuda, R. (1989) *J Biochem (Tokyo)* **105,** 537-546.
3. Lorenz, M. C., Muir, R. S., Lim, E., McElver, J., Weber, S. C. & Heitman, J. (1995) *Gene* **158,** 113-117.
4. Ito, H., Fukuda, Y., Murata, K. & Kimura, A. (1983) *J Bacteriol* **153,** 163-168.
5. Russell, P. J., Hambidge, S. J. & Kirkegaard, K. (1991) *Nucleic Acids Res* **19,** 4949-4953.
6. Leeds, P., Peltz, S. W., Jacobson, A. & Culbertson, M. R. (1991) *Genes Dev* **5,** 2303-2314.
7. Yamanaka, K., Ishihama, A. & Nagata, K. (1990) *J Biol Chem* **265,** 11151-11155.
8. Enami, M. & Enami, K. (2000) *J Virol* **74,** 5556-5561.
9. Momose, F., Basler, C. F., O'Neill, R. E., Iwamatsu, A., Palese, P. & Nagata, K. (2001) *J Virol* **75,** 1899-1908.
10. Miyagishi, M. & Taira, K. (2003) *Oligonucleotides* **13,** 325-333.
11. Ui-Tei, K., Naito, Y., Takahashi, F., Haraguchi, T., Ohki-Hamazaki, H., Juni, A., Ueda, R. & Saigo, K. (2004) *Nucleic Acids Res* **32,** 936-948.
12. Miyaji-Yamaguchi, M., Kato, K., Nakano, R., Akashi, T., Kikuchi, A. & Nagata, K. (2003) *Mol Cell Biol* **23,** 6672-6684.
13. Neumann, G., Watanabe, T., Ito, H., Watanabe, S., Goto, H., Gao, P., Hughes, M., Perez, D. R., Donis, R., Hoffmann, E., Hobom, G. & Kawaoka, Y. (1999) *Proc Natl Acad Sci U S A* **96,** 9345-9350.
14. Turan, K., Mibayashi, M., Sugiyama, K., Saito, S., Numajiri, A. & Nagata, K. (2004) *Nucleic Acids Res* **32,** 643-652.
15. Niwa, H., Yamamura, K. & Miyazaki, J. (1991) *Gene* **108,** 193-199.
16. Kawaguchi, A., Naito, T. & Nagata, K. (2005) *J Virol* **79,** 732-744.
17. Takizawa, N., Watanabe, K., Nouno, K., Kobayashi, N. & Nagata, K. (2006) *Microbes Infect* **8,** 823-833.
18. Naito, T., Momose, F., Kawaguchi, A. & Nagata, K. (2007) *J Virol* **81,** 1339-1349.

SI Table 1. Primers and oligonucleotides used in this study

For yeast genomic DNA and yeast genes

Primer name	Sequence (5' to 3')	Source	Nucleotide positions
SUB2 up-FOR	GTTAGGTACCGAATACTTGGCCAAGATCTTGG	*SUB2* promoter (Chromosome IV)	304495-304516[a]
SUB2 up-REV	ACTTCCCGGGTCCAACAGGCAAACAGTTCCG	*SUB2* promoter (Chromosome IV)	305122-305141[a]
SUB2 down-FOR	CCGTGAATTCAACGTAGTGTAGTCATTTCGTG	*SUB2* terminator (Chromosome IV)	306713-306734[a]
SUB2 dwon-REV	CGTTGAATTCTTCTCCAACATCGATATCAGAG	*SUB2* terminator (Chromosome IV)	307159-307180[a]
ACT1-FOR	ATGGATTCTGAGGTTGCTGCTTTGGTTATT	*ACT1* cDNA	1-30[b]
ACT1-REV	GCGTGAGGTAGAGAGAAACC	*ACT1* cDNA	502-521[b]
ADH1-FOR	ATGTCTATCCCAGAAACTCAAAAAGG	*ADH1* cDNA	1-26[b]

ADH1-REV	AACGTTTTCACCCATGCCGACA	*ADH1* cDNA	219-240[b]
RDN25-1-FOR	CTGGTACCTTCGGTGCCC	*RDN25-1* cDNA	125-142[b]
RDN25-1-REV	CGCTATCGGTCTCTCGCCA	*RDN25-1* cDNA	329-347[b]
SUB2-FOR (His-)	TTTCATATGCATCATCATCATCATCATGGTTCACACGAAGGTGAAGAAGA TTTATT	*SUB2* cDNA	4-29[b]
SUB2 1062-FOR	GCTCGTTACAAGGCTTTCAAAGAT	*SUB2* cDNA	1039-1062[b]
SUB2-REV	CCGGATCCTTAATTATTCAAATAAGTGGACGGATCAATGCCTTC	*SUB2* cDNA	1310-1341[b]
TRP1-FOR	GGACGGCTAGCAACGACATTACTATATATATAATATAGGAAGC	*TRP1* cDNA	1-32[b]
TRP1-REV	GGCCGCGTCTCCCTTGGGCCCGGCAAGTGCACAAACAATACTTAAATA	*TRP1* cDNA	977-1002[b]
CYC1 TT-FOR	CCCCAAGCTTAGATCTGCGCCCGGGTCACCATCATGTAATTAGTTATGTC ACGCTTACATT	*CYC1* terminator	1-28[b]
CYC1 TT-REV	CGGACTAGTGATATCCCGGGGTCTAGAGCAAATTAAAGCCTTCGAGCG	*CYC1* terminator	692-720[b]

[a] Nucleotide positions correspond to those of yeast chromosome IV in the database (*Saccharomyces* Genome Database). *SUB2* is localized between nucleotide positions 305237 and 306577 of chromosome IV. [b] The nucleotide position 1 is set to the 5'-terminus of each mRNA.

For influenza viral genes

Primer name	Sequence (5’ to 3’)	Source	Nucleotide positions
PA-FOR 1	GATCCCGGGCATATGGAAGATTTTGTGCGACAATG	Seg. 3 vRNA	25-47[c]
PA 664-FOR	ATCACAGGAACAATGCGCAAGC	Seg. 3 vRNA	643-664[c]
PA 800-REV	GGTTCAATTCTAGCATTTACTTCTTT	Seg. 3 vRNA	775-800[c]
PA 3’-END	AGTAGAAACAAGGTACTTTTTTGGAC	Seg. 3 cRNA	2208-2233[d]
PA 1356-FOR	CACATTGCAAGCATGAGAAGGAAT	Seg. 3 c/mRNA	1333-1356[d]
PA 1683-REV	GGCACTTCTTAGAAGCATATCTC	Seg. 3 c/mRNA	1661-1683[d]
NP-FOR 1	GGAATTCATATGGCGTCTCAAGGCACCAAACG	Seg. 5 vRNA	46-68[e]
NP 444-FOR	GACGATGCAACGGCTGGTCTG	Seg. 5 vRNA	424-444[e]
NP 614-REV	AGCATTGTTCCAACTCCTTT	Seg. 5 vRNA	595-614[e]
NP 3’-END	AGTAGAAACAAGGGTATTTTTCTTTA	Seg. 5 cRNA	1540-565[f]
NP 846-FOR	CGGTCTGCACTCATATTGAGAGG	Seg. 5 c/mRNA	826-846[f]
NP 1121-REV	GAAAGCTTCCCTCTTGGG	Seg. 5 c/mRNA	1104-1121[f]

M 3'-END	AGTAGAAACAAGGTAGTTTTTTACTC	Seg. 7 cRNA	26-43[g]
M 67-FOR	GTCGAAACGTACGTTCTCTCTATC	*M1* cDNA	44-67[h]
M 200-REV	TCCCCTTAGTCAGAGGTGAC	*M1* cDNA	181-200[h]
M 897-FOR	TAAATACGGACTGAAAGGAG	*M2* cDNA	878-897[h]
M 1007-REV	TTACTCCAGCTCTATGCTGACAAAAT	*M2* cDNA	982-1007[h]
PB2-FOR	CGCGGATCCCGGGCGGCCGCCACCATGGAAAGAATAAAAGAACTAAG AAATCT	*PB2* cDNA	1-29[h]
PB2-REV	GCGCCTCGAGCTACTAGCTTTGATCAACATCATCATT	*PB2* cDNA	754-774[h]
PA-REV	GCGGGGCCCTAACTCAATGCATGTGTAAGGAAGG	*PA* cDNA	2126-2151[h]
NP-FOR 2	TTGGATCCAAAATGGCTACTAAAGGTACTAAAAGATCT	*NP* cDNA	1-27[h]
NP-REV	GGAATTCATCTTAATTGTCGTACTCCTCTGCATTGT	*NP* cDNA	1472-1497[h]

[c, d, e, f and g] Nucleotide positions of influenza viral RNAs, where the nucleotide position 1 is set to the 5'-virus derived terminus of segment 3 vRNA[c], segment 3 cRNA and mRNA[d], segment 5 vRNA[e], segment 5 cRNA and mRNA[f], or segment 7 cRNA and mRNA[g].

[h] The nucleotide position 1 is set to the 5'-virus derived terminus of each mRNA.

For model viral genes and other oligonucleotides

Primer name	Sequence (5’ to 3’)	Source	Nucleotide positions
Seg 5 digestion	GGAATTCATATGGTGATGGAATTGGTCAGAATGATCAAAC	Seg. 5 vRNA	616-640[i]
siTat-SF1 FOR	GGCTCTAGAACCTGCCGGCCACCGATTATTAAAGATTTCATTGT[j]TAGAATT ACATCAAGGG	*Tat-SF1* cDNA	196-206[j]
siTat-SF1 REV	GGCTCTAGAACCTGCTAGCGCATAAAAAGATTACTGAAGATTTCATTGC[j]A TCTCCCTTGATGTAA	*Tat-SF1* cDNA	196-206[j]
siEGFP FOR	GGCTCTAGAACCTGCCGGCCACCGTCATAATGTCTATATTATGG[j]TAGAATT ACATCAAGGG	*EGFP* cDNA	423-443[j]
siEGFP REV	GGCTCTAGAACCTGCTAGCGCATAAAAAGCCACAACGTCTATATCATGG[j]A TCTCCCTTGATGTAA	*EGFP* cDNA	423-443[j]
Tat-SF1 400-FOR	GTTTCATGTTGAAGAAGACAGAAATACAA	*Tat-SF1* cDNA	372-400[j]
Tat-SF1 700-REV	GCGTAATACGACTCACTATAGGGAGATGCACTTCTTCTTCTTCTTTGAGG[j]	*Tat-SF1* cDNA	677-700[j]
Tat-SF1 603-FOR1	TCTGTGGAACTTGCATTAAAACTTTTG	*Tat-SF1* cDNA	577-603[j]

Tat-SF1 603-FOR2	TCCGTGGAGCTGGCCTTG	*Tat-SF1* cDNA	577-594[j]
Tat-SF1 720-REV	CAGCTTCTTCTTATAGTCTTTGCAC	*Tat-SF1* cDNA	696-720[j]
Tat-SF1-FOR 1	CCGAATTCGCCGCCACCATGGACTACAAGGATGACGACGACAAG[l]GGAAT GAGCGGCACCAACTTGGA	*Tat-SF1* cDNA	2-20[j]
Tat-SF1-FOR 2	GACGTGTCTGGTTTGCCTCC	*Tat-SF1* cDNA	407-422[j]
Tat-SF1-REV 1	GGGAATTCTTAAATATCATCGTCATCATCATCGCT	*Tat-SF1* cDNA	2238-2265[j]
Tat-SF1-REV 2	TACATTTGTATTTCTGTCTTCTTCAACATG	*Tat-SF1* cDNA	376-406[j]
Tat-SF1-REV 3	CCCAAGCTTTTAAATATCATCGTCATCATCATCGCT	*Tat-SF1* cDNA	2238-2265[j]
Myc-Tat-SF1 FOR	CCGAATTCGAGCAGAAACTCATCTCTGAAGAGGATCTGGGAATGAGCGG CACCAACTTGGA	*Tat-SF1* cDNA	1-20[j]
β-Actin FOR	ATGGGTCAGAAGGATTCCTATGT	*β-Actin* cDNA	139-161[j]
β-Actin REV	GGTCATCTTCTCGCGGTT	*β-Actin* cDNA	343-360[j]
53-merVwt	AGUAGAAACAAGGGUGUUUUUUCAUAUCAUUUAAACUUCACCCUGCU UUUGCU	Seg. 8 vRNA	1-20[k]

[i and k] Nucleotide positions of influenza viral RNAs, where the nucleotide position 1 is set to the 5'-virus derived terminus of segment 5

vRNA[i] and segment 8 vRNA[k]. [j] The nucleotide position 1 is set to the 5'-terminus of mRNA. [l] Underlines indicate the FLAG-tag sequences.

Figure 1

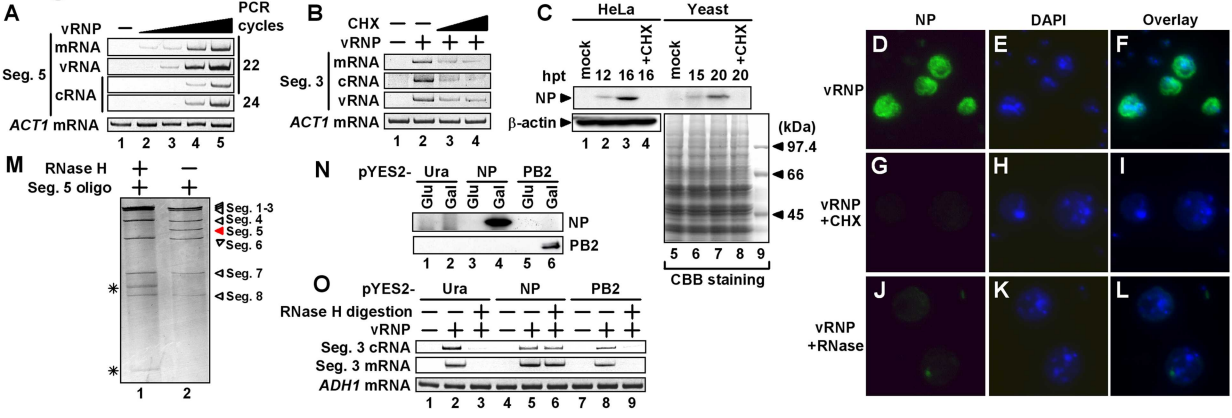

A
vRNP
PCR cycles
Seg. 5
mRNA
vRNA
cRNA
22
24
ACT1 mRNA
1 2 3 4 5
B
CHX
vRNP
Seg. 3
mRNA
cRNA
vRNA
ACT1 mRNA
1 2 3 4
C
HeLa
Yeast
hpt
mock
12 16 16
+CHX
mock
15 20 20
+CHX
NP
β-actin
1 2 3 4
(kDa)
97.4
66
45
5 6 7 8 9
CBB staining
M
RNase H
Seg. 5 oligo
Seg. 1-3
Seg. 4
Seg. 5
Seg. 6
Seg. 7
Seg. 8
1 2
N
pYES2-
Ura
NP
PB2
Glu
Gal
NP
PB2
1 2 3 4 5 6
O
pYES2-
Ura
NP
PB2
RNase H digestion
vRNP
Seg. 3 cRNA
Seg. 3 mRNA
ADH1 mRNA
1 2 3 4 5 6 7 8 9
NP
DAPI
Overlay
vRNP
vRNP +CHX
vRNP +RNase
D
E
F
G
H
I
J
K
L

Figure 2

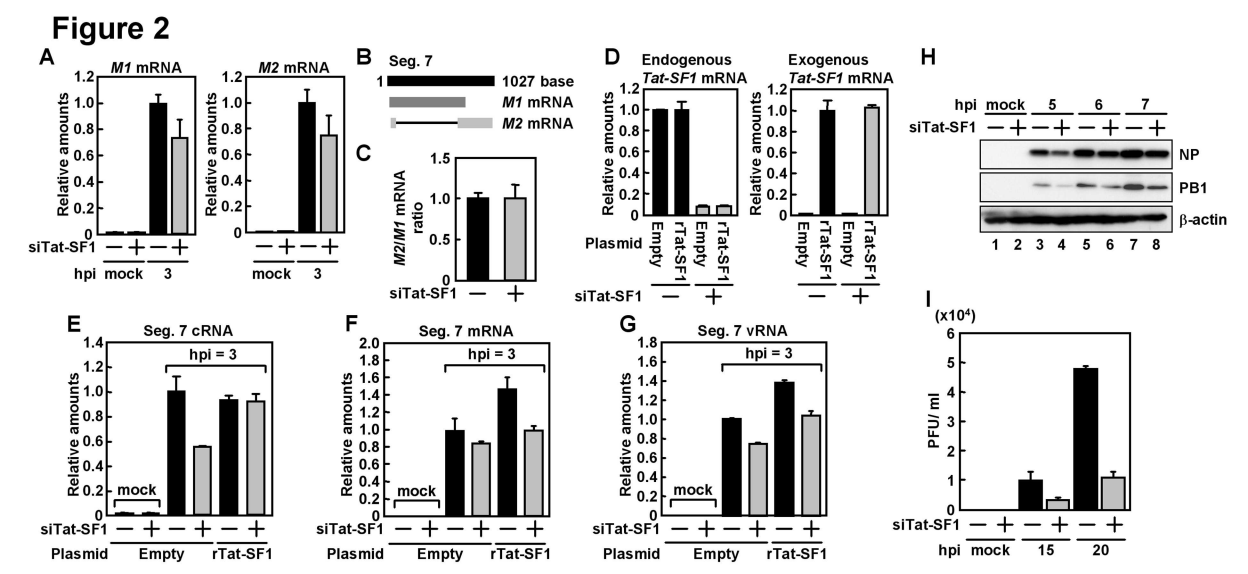

A
M1 mRNA
M2 mRNA
Relative amounts
siTat-SF1
hpi mock 3
B
Seg. 7
1027 base
M1 mRNA
M2 mRNA
C
M2/M1 mRNA ratio
siTat-SF1
D
Endogenous Tat-SF1 mRNA
Exogenous Tat-SF1 mRNA
Plasmid
Empty
rTat-SF1
siTat-SF1
H
hpi mock 5 6 7
siTat-SF1
NP
PB1
β-actin
1 2 3 4 5 6 7 8
E
Seg. 7 cRNA
F
Seg. 7 mRNA
G
Seg. 7 vRNA
hpi = 3
mock
I
(x10⁴)
PFU/ ml
hpi mock 15 20

Figure 3

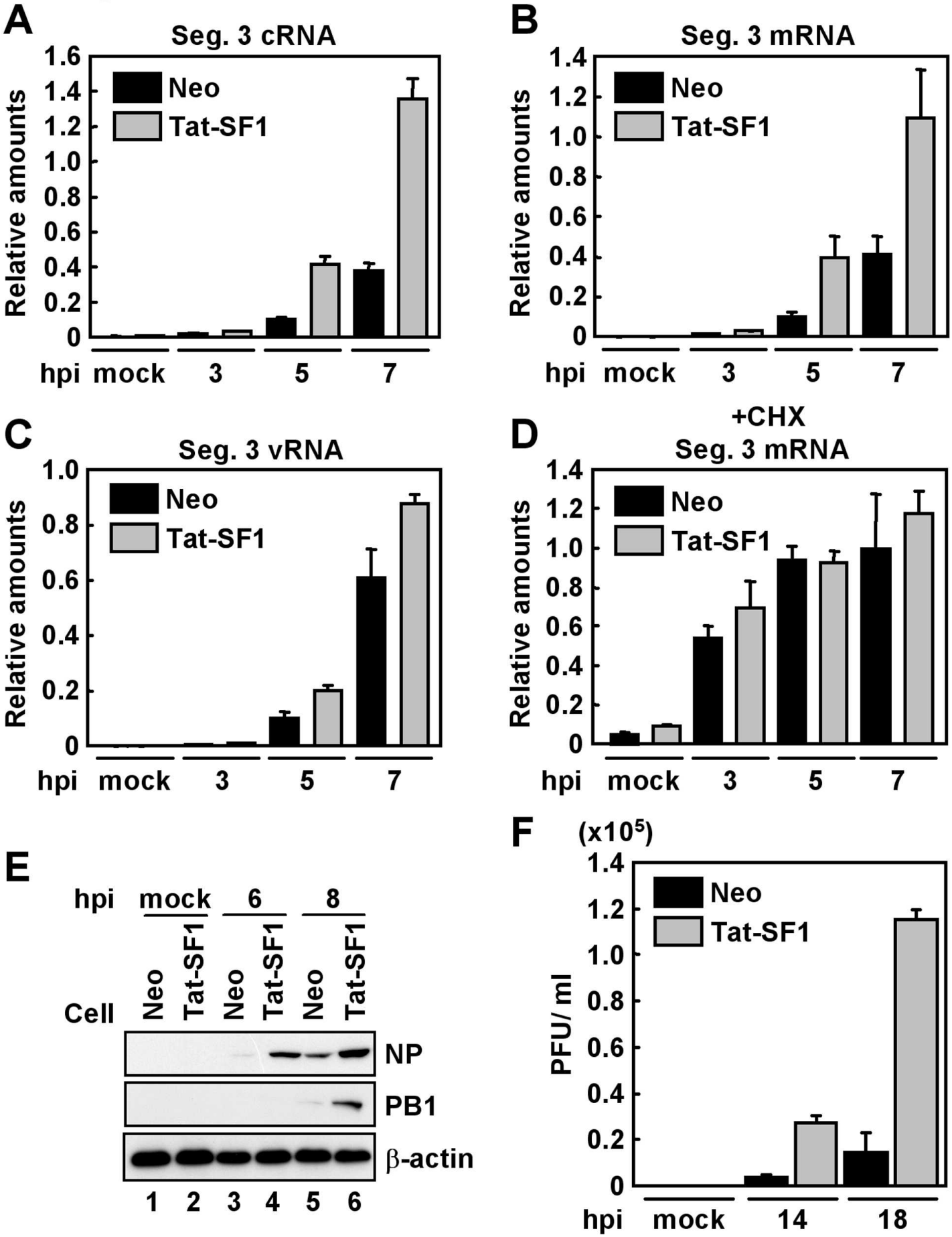
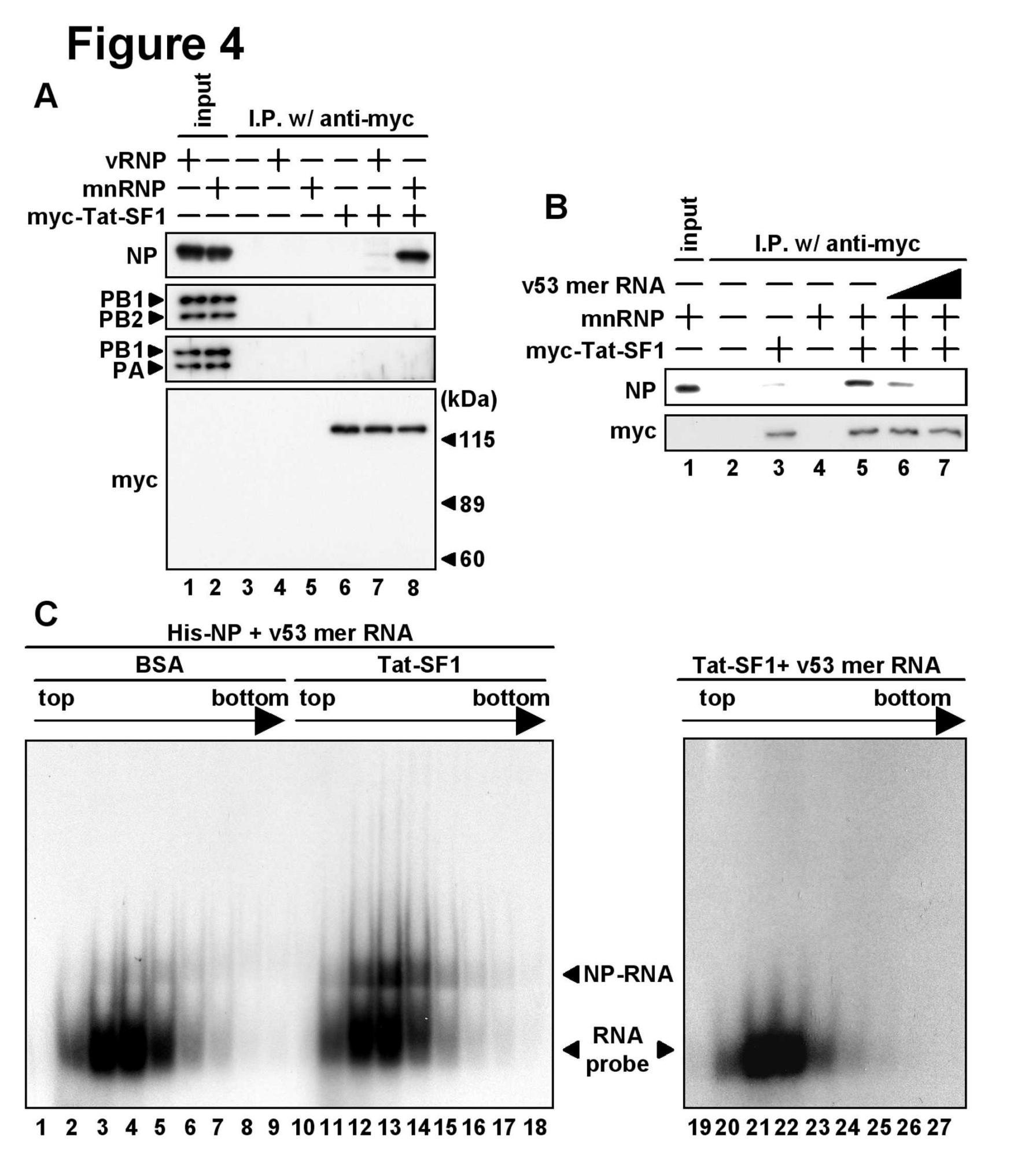
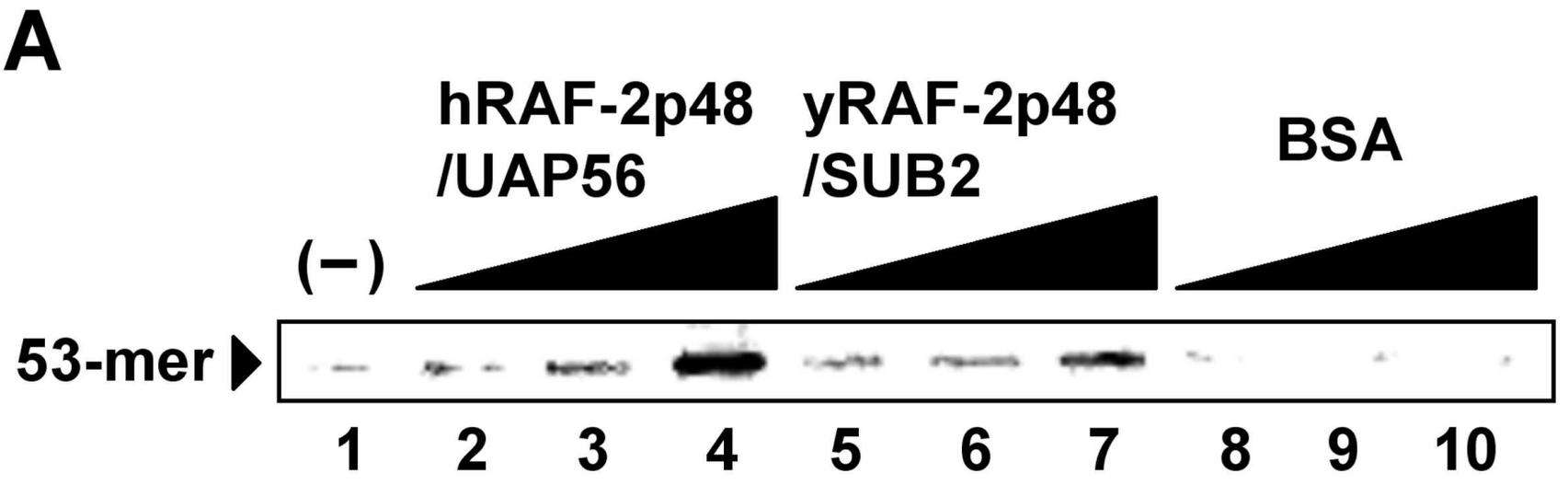
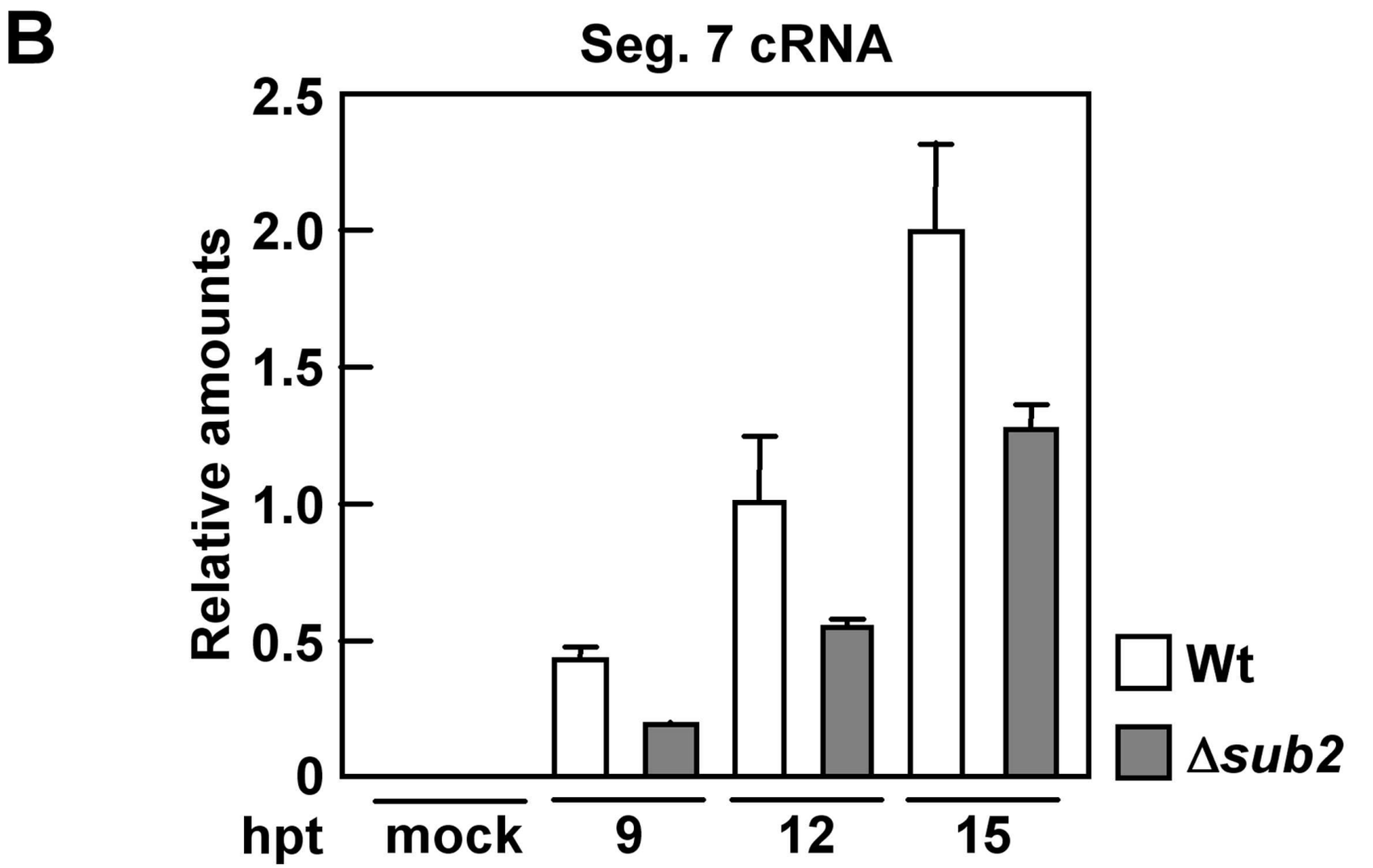

A
Seg. 3 cRNA
Relative amounts
Neo
Tat-SF1
hpi mock 3 5 7
B
Seg. 3 mRNA
Relative amounts
Neo
Tat-SF1
hpi mock 3 5 7
C
Seg. 3 vRNA
Relative amounts
Neo
Tat-SF1
hpi mock 3 5 7
D
+CHX
Seg. 3 mRNA
Relative amounts
Neo
Tat-SF1
hpi mock 3 5 7
E
hpi mock 6 8
Cell Neo Tat-SF1 Neo Tat-SF1 Neo Tat-SF1
NP
PB1
β-actin
1 2 3 4 5 6
F
(x10^5)
PFU/ ml
Neo
Tat-SF1
hpi mock 14 18

Figure 4

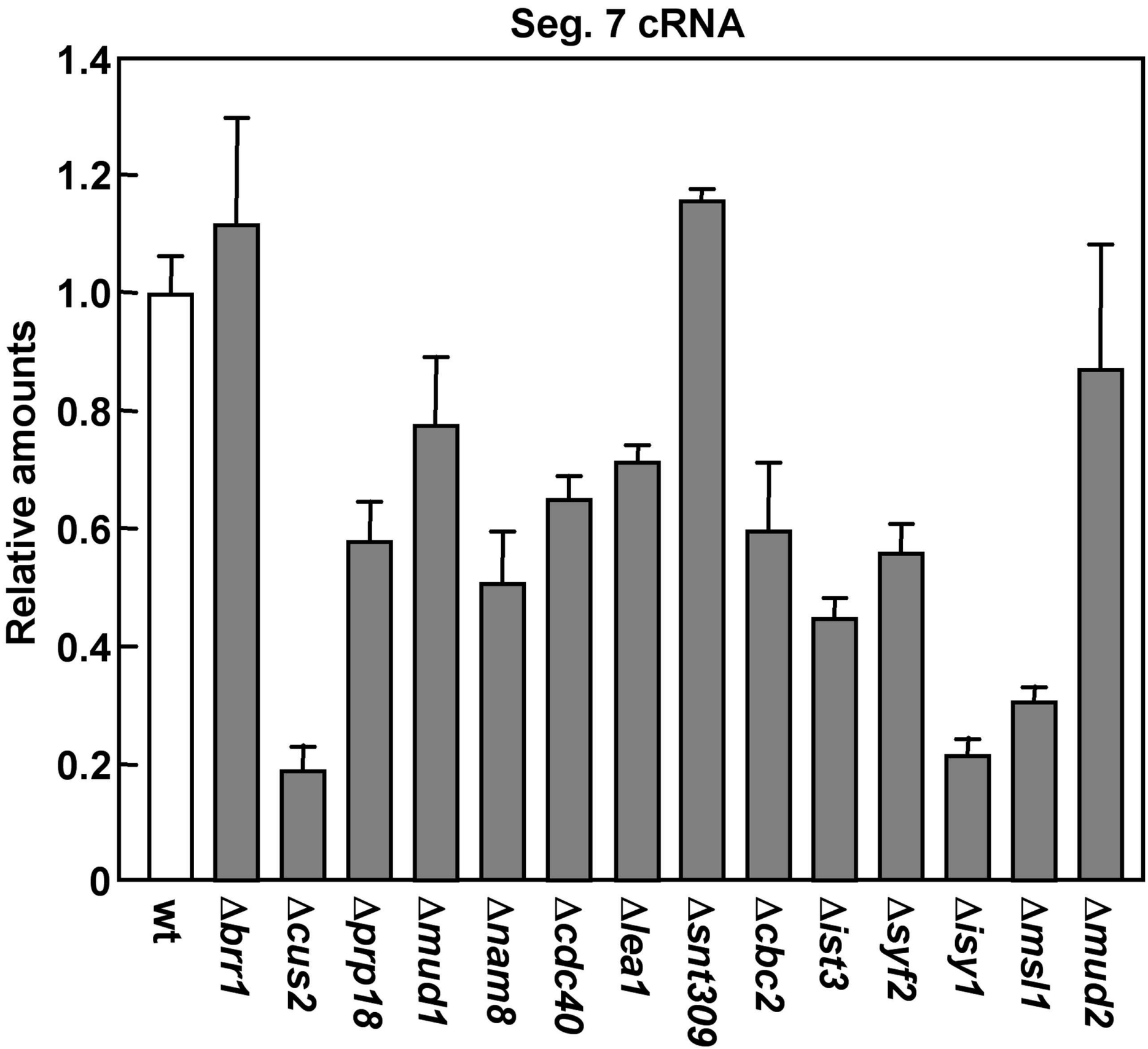

A
input
I.P. w/ anti-myc
vRNP
mnRNP
myc-Tat-SF1
NP
PB1
PB2
PB1
PA
(kDa)
115
89
60
myc
1 2 3 4 5 6 7 8
B
input
I.P. w/ anti-myc
v53 mer RNA
mnRNP
myc-Tat-SF1
NP
myc
1 2 3 4 5 6 7
C
His-NP + v53 mer RNA
BSA
Tat-SF1
top
bottom
top
bottom
Tat-SF1+ v53 mer RNA
top
bottom
NP-RNA
RNA probe
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
19 20 21 22 23 24 25 26 27

SI Figure 5

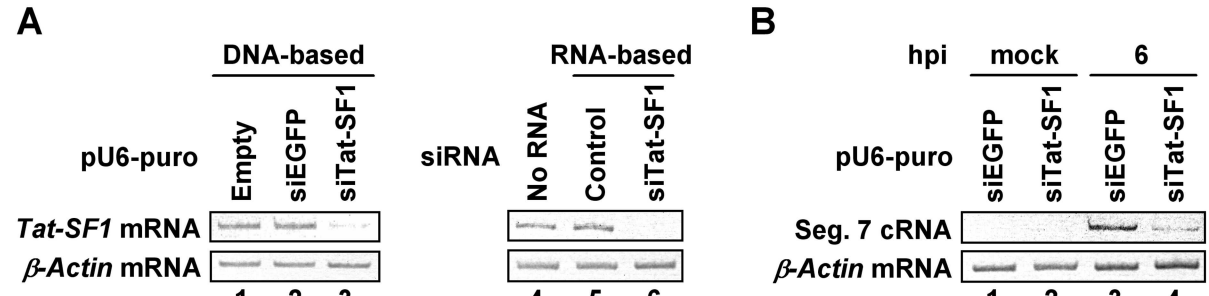

A
hRAF-2p48
/UAP56
yRAF-2p48
/SUB2
BSA
(−)
53-mer
1
2
3
4
5
6
7
8
9
10


B
Seg. 7 cRNA
Relative amounts
2.5
2.0
1.5
1.0
0.5
0
hpt
mock
9
12
15
Wt
Δsub2

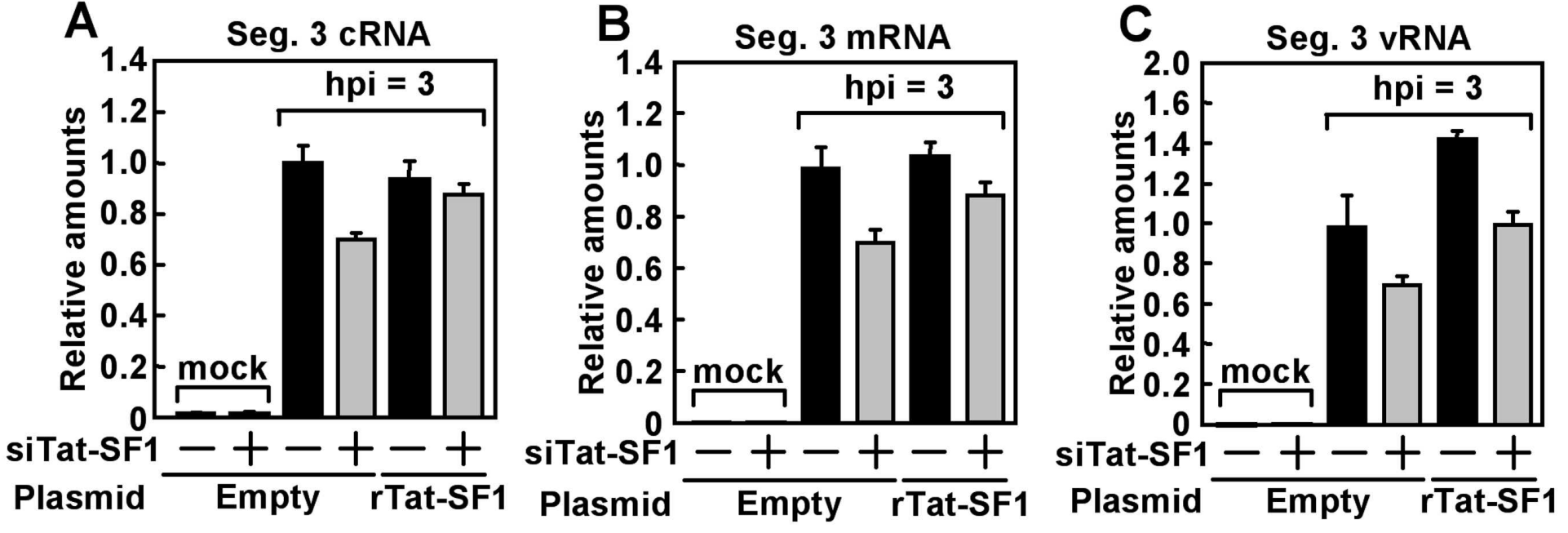
SI Figure 6


Seg. 7 cRNA
Relative amounts
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
wt
Δbrr1
Δcus2
Δprp18
Δmud1
Δnam8
Δcdc40
Δlea1
Δsnt309
Δcbc2
Δist3
Δsyf2
Δisy1
Δmsl1
Δmud2

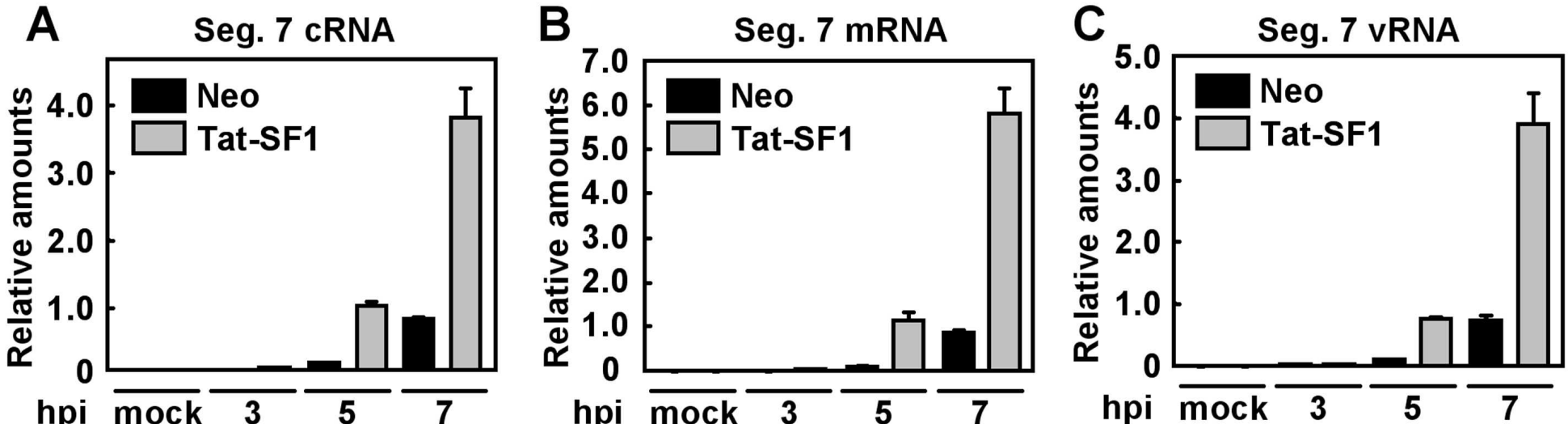
SI Figure 7


A
DNA-based
RNA-based
pU6-puro
Empty
siEGFP
siTat-SF1
siRNA
No RNA
Control
siTat-SF1
Tat-SF1 mRNA
β-Actin mRNA
1 2 3 4 5 6
B
hpi
mock
6
pU6-puro
siEGFP
siTat-SF1
siEGFP
siTat-SF1
Seg. 7 cRNA
β-Actin mRNA
1 2 3 4

SI Figure 8


A
Seg. 3 cRNA
Relative amounts
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
hpi = 3
mock
siTat-SF1 — + — + — +
Plasmid
Empty
rTat-SF1
B
Seg. 3 mRNA
Relative amounts
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
hpi = 3
mock
siTat-SF1 — + — + — +
Plasmid
Empty
rTat-SF1
C
Seg. 3 vRNA
Relative amounts
2.0
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
hpi = 3
mock
siTat-SF1 — + — + — +
Plasmid
Empty
rTat-SF1

SI Figure 9


A
Seg. 7 cRNA
Neo
Tat-SF1
Relative amounts
4.0
3.0
2.0
1.0
0
hpi
mock
3
5
7
B
Seg. 7 mRNA
Neo
Tat-SF1
Relative amounts
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
hpi
mock
3
5
7
C
Seg. 7 vRNA
Neo
Tat-SF1
Relative amounts
5.0
4.0
3.0
2.0
1.0
0
hpi
mock
3
5
7