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Title: A novel influenza virus replicon system in yeast identified Tat-SF1 as a stimulatory host factor for viral RNA synthesis

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Abbreviations footnote: cRNA, complementary RNA; RAF-2, viral RNA polymerase activating factor-2; SUB2, suppressor of Brr1-1; Tat-SF1, Tat stimulatory factor1; vRNP, viral ribonucleoprotein complex

Abstract

Influenza viruses infect vertebrates including mammalians and birds. Influenza virus reverse-genetics systems facilitate the study on the structure and function of the viral factors. In contract, less is known about host factors involved in the replication processes. Here, we have developed a replication and transcription system of the negative-strand RNA genome of influenza virus in *Saccharomyces cerevisiae*, depending on viral RNAs, viral RNA polymerases, and nucleoprotein (NP). Disruption of *SUB2* encoding an orthologue of human RAF-2p48/UAP56, a previously identified viral RNA synthesis stimulatory host factor, resulted in reduction of the viral RNA synthesis rate. Using a genome-wide set of yeast single-gene deletion strains, we fished several host factor candidates affecting viral RNA synthesis. We found that among them, Tat-SF1, a mammalian homologue of yeast CUS2, was a stimulatory host factor in the influenza virus RNA synthesis. Tat-SF1 interacted with free NP but not with NP associated with RNA, and facilitates formation of RNA-NP complexes. These results suggest that Tat-SF1 may function as a molecular chaperone for NP as does RAF-2p48/UAP56. This system is proven to be useful for further studies on the mechanism of the influenza virus genome replication and transcription.

Introduction

Viruses are intracellular parasites. Virus replication requires virus-derived factors, and is also totally dependent on host cell functions/machineries. Identification of host factors involved in viral replication is critical to understand the molecular mechanism of virus replication and pathogenicity. In the case of DNA viruses, a number of host factors are identified, and their functional analyses have contributed to not only understanding the molecular mechanism of the viral genome replication and transcription but also leading the study on the eukaryote genome replication and transcription (1). The RNA genome of positive-strand RNA viruses is “infectious”, where “infectious” means that the infectious virus can be recovered when the RNA genome is introduced into cells (2-4). Thus, the reverse-genetic systems using *in vitro* synthesized RNAs of the positive-strand RNA virus was powerful to reveal the role of viral factors and the interaction between viral factors and host factors (5). In contrast, for generation of an infectious negative-strand RNA virus, the negative-strand virus RNA genome should be introduced into cells as complexes with viral RNA polymerases and other viral factors required for RNA-dependent RNA synthesis. Alternatively, the negative-strand RNA genome should be introduced into cells expressing these viral factors.

Influenza virus contains segmented- and negative-strand RNAs as its genome. The influenza virus RNA is associated with viral RNA-dependent RNA polymerases consisting of PB1, PB2 and PA subunits and nucleoprotein (NP) forming viral ribonucleoprotein complexes (vRNP) (6). The vRNP is a basic unit for transcription and replication of the virus genome. It was shown that vRNP complexes isolated from

virions are “infectious” (7). Then, reconstituted vRNP complexes-utilized transfection systems, in which the genome replication and transcription proceed, were reported (8, 9). Recently, the reverse genetics system was established for generation of a recombinant influenza A virus from a set of plasmids (10). With this system, the study on the structure and function of the viral factors has been being carried out extensively (11, 12).

Recent proteomics showed a list of cellular proteins interacting with viral proteins (13). However, there are only a few of host factors identified by functional assays for the viral genome transcription and replication (14-19). Further to identify host factors, a systematic screening system has been needed. Yeast is a good model eukaryotic cell with merits including the well-established genetics and the entire genome information for the genome-wide screening. It has been shown that yeast cells support the replication and transcription of some positive-strand viral RNA genomes such as brome mosaic virus and tomato bushy stunt virus (20, 21).

In this study, to identify host factors systematically, we tried to develop a novel system, in which yeast cells support the replication and transcription of the influenza virus genome depending on transfected vRNP complexes. With this system in hand, we confirmed that the yeast orthologue of a previously identified mammalian host factor is indeed a stimulatory factor for the viral RNA synthesis in yeast cells. In addition, we identified novel host factor candidates for regulation of the virus RNA synthesis using yeast single-gene knockout library. Among then, Tat-SF1 (Tat stimulatory factor 1), a mammalian homologue of a newly identified candidate, CUS2, was a stimulatory host factor in the influenza virus RNA synthesis. Thus, this system could be quite useful to understand the molecular mechanism of the virus replication,

and provide with a novel method for systematic screening of host factors in the influenza virus genome replication processes.

Results

Replication and Transcription of the Influenza Virus Genome in vRNP-transfected Yeast Cells.

First, to examine whether or not the vRNP purified from virions is “infectious” in yeast cells, we introduced vRNP into yeast cells. The synthesis level of viral RNAs, *i.e.*, vRNA, cRNA (complementary RNA; the template for amplification of vRNA), and viral mRNA derived from segment 5 vRNA was analyzed by RT-PCR (Fig. 1*A*). The amount of viral RNAs synthesized in vRNP-transfected yeast cells increased depending on increasing amounts of transfected vRNP. The amount of viral mRNA and vRNA synthesized in yeast cells were more than that of cRNA (Fig. 1*A*, see the PCR result with 22 cycles), as reported previously using infected mammalian cells (12, 22). The similar result was observed for other viral segments (data not shown). These results demonstrate that the viral RNA polymerase and NP are functional in yeast cells. In infected mammalian cells, the primary viral transcription is dependent on infecting vRNP, while the viral genome replication requires newly synthesized viral proteins (12). When vRNP-transfected yeast cells were treated with cycloheximide (CHX), a protein synthesis inhibitor, the synthesis level of viral RNAs was markedly reduced (Fig. 1*B*), indicating that the synthesis of viral RNAs is dependent on newly synthesized proteins. Since the viral mRNA synthesis was also sensitive to CHX treatment, viral mRNA could be synthesized from newly synthesized vRNA as template in this system (12, 22). By western blotting analyses (Fig. 1*C* and Supporting information (SI) Text for detailed discussion), we detected viral proteins synthesized in vRNP-transfected yeast cells. In indirect immunofluorescence assays using anti-NP antibody (Fig. 1 *D*-*L*), NP was

detected in vRNP-transfected yeast cells (Fig. 1*D*), but not in cells treated with CHX (Fig. 1*G*). When vRNP was treated with RNase A prior to transfection, the expression of NP was abolished (Fig. 1*J*), indicating that the viral gene expression was dependent on vRNA in vRNP complexes. Taken altogether, it is concluded that yeast cells support the viral genome replication and viral gene transcription depending on transfected vRNP complexes.

We performed complementation experiments using yeast cells transfected with vRNP complexes devoid of segment 5 vRNA that encodes NP. NP is required for formation of vRNP complexes and efficient elongation of the viral RNA chain (6). We eliminated segment 5 vRNA in vRNP complexes by digestion with RNase H (Fig. 1*M*) (see SI Methods) (23). Then, vRNP complexes lacking segment 5 vRNA (designated depleted vRNP) and mock-digested vRNP complexes were introduced into yeast cells expressing NP or PB2. Figure 1*N* confirmed the expression level of NP and PB2 induced by galactose using pYES2-NP and pYES2-PB2, in which NP and PB2 genes are under the control of the *GAL1* promoter (see SI Methods). In yeast cells transformed with pYES2 or pYES2-PB2, the depleted vRNP did not lead to the synthesis of mRNA and cRNA from segment 3 vRNA (Fig. 1*O*, lanes 3 and 9). In contrast, yeast cells transformed with pYES2-NP could rescue the RNA synthesis (Fig. 1*O*, lane 6) in the galactose induction medium. These results indicate that exogenously added NP complements the system using the depleted vRNP. Further, it is now shown that the replication process in yeast cells is dependent on NP.

Effect of *sub2* Deletion.

We tried to use the system for the functional analysis of RAF-2p48, a

previously identified host factor (14). RAF-2p48 facilitates formation of NP-RNA complexes and stimulates *in vitro* RNA synthesis from a model viral RNA. RAF-2p48 is identical with UAP56, an RNA splicing factor. SUB2, a putative *S. cerevisiae* orthologue of human RAF-2p48/UAP56 (62% amino acid sequence identity), associates with Sm snRNPs and is involved in splicing *in vivo* (24, 25). Prior to genetical analyses of *sub2* in yeast cells, we confirmed that SUB2 stimulates the influenza virus RNA synthesis *in vitro* (SI Fig. 5*A*). The viral RNA synthesis stimulatory activity of SUB2 was as much as 50% of RAF-2p48/UAP56. This result suggests that SUB2 may function as a host factor for the viral genome replication in yeast cells. It is possible that not only conserved but also diverse regions between SUB2 and UAP56 are required for the full activity of RAF-2p48. Then, we examined the effect of the deletion of *sub2* on the viral RNA synthesis in yeast cells. The *sub2* deletion strain was constructed by replacing its ORF with TRP1 maker (see SI Methods). We analyzed the level of viral RNA synthesis in the *sub2* deletion yeast strain by real-time RT-PCR. In the vRNP-transfected *sub2* deletion strain, the rate of cRNA synthesis was reduced to 40% of that in a wild type strain at 9 h post-transfection (SI Fig. 5*B*). This suggests that SUB2 facilitates the viral RNA synthesis as a host factor in yeast cells, and thereby RAF-2p48/UAP56 is a host factor for the viral RNA synthesis in vertebrate cells. The viral RNA synthesis in the *sub2* deletion strain rescued to 60% of that in wild type strain after longer incubation, so that we assume that yeast cells have other host factor(s) that complements the function of SUB2.

Identification of Host Factor Candidates Affecting Viral Genome Replication.

This yeast system could allow us to identify more host factor candidates.

Once we could reach a candidate, we would go back to the vertebrate system to verify and characterize the candidate. To this end, we carried out screening of an yeast single-gene deletion library containing approximately 4,800 strains (80% of all yeast genes in yeast) (26). We first focused on screening of deletion strains lacking 354 genes encoding putative nucleic acid binding/nucleic acid-related functional proteins among the nuclear genes (Categorized by Advanced Search of Saccharomyces Genome Database [http://www/yeastgenome.org/]). Each deletion strain was transfected with vRNP, and the synthesis level of segment 7 cRNA was analyzed by RT-RCR (data not shown). In some deletion strains such as those lacking *lsm12*, *npl3*, etc., viral RNA synthesis was increased compared with the wild type strain. These deleted gene products are candidates for inhibitory factors for the viral RNA synthesis. On the other hand, several strains (*mip6*, *nsr1*, etc.) showed the severe decreased level of viral RNA synthesis. Interestingly, the cRNA synthesis rate was decreased in deletion strains of several factors involved in splicing. The synthesis level of segment 3 cRNA was quantitatively determined by real-time RT-PCR in mutants containing deletions in genes encoding splicing factors (SI Fig. 6). The significant decreased phenotype was found in strains lacking three genes, *isy1*, *msl1* and *cus2*. The human homologue of *ISY1* gene (KIAA1160 cDNA) (27) encodes an unidentified protein. The human MSL1 homologue is U1 snRNP-specific protein A (U1-A) associated with the 164 nucleotide long U1 snRNA (28). CUS2 shares 37% identity with human Tat-SF1 (29), a transcription elongation protein (30-32). Tat-SF1-snRNPs complexes are recruited to the cellular RNA polymerase II elongation complex through the binding of Tat-SF1 and snRNPs to p-TEFb and the nascent splicing substrate, respectively (33).

Tat-SF1 Functions as a Host Factor in Infected Cells.

Next, we attempted to characterize the function of Tat-SF1 in influenza virus genome replication. In order to examine whether Tat-SF1 is involved in the viral RNA synthesis in mammalian cells, we carried out knock-down experiments of Tat-SF1 with the short interference RNA (siRNA) technique. Using the cells in which the amount of Tat-SF1 mRNA was reduced (SI Fig. 7*A*), we determined the synthesis level of segment 7 viral RNAs. The synthesis level of viral cRNA in Tat-SF1 knock-down cells (SI Fig. 7*B*, lane 4) was less than 10% of that in cells transfected with control plasmids (SI Fig. 7*B*, lanes 1 and 3; mock and 6 hour post infection (hpi)). These results suggest that Tat-SF1 plays a role in the viral RNA synthesis, although it is possible that the decreased level of Tat-SF1 may give some effect on splicing of viral and host pre-mRNAs, and thereby have influence in the viral RNA synthesis. In order to rule out the possibility, we compared the ratio of the level of *M1* mRNA to that of *M2* mRNA generated from *M1* mRNA by splicing in both control cells and Tat-SF1 siRNA (siTat-SF1)-transfected cells. The segment 7 vRNA codes for M1 and M2 proteins, which are translated from non-spliced and spliced mRNA, respectively (Fig. 2*B*). In siTat-SF1-transfected cells, the amount of *M1* and *M2* mRNAs were reduced to 75% of that in mock-transfected cells (Fig. 2*A*), while the ratio of the *M1* mRNA level to the *M2* mRNA level in Tat-SF1 knock-down cells was similar to that in control cells (Fig. 2*C*). Next, we carried out complementation experiments for siRNA-introduced cells with a plasmid encoding FLAG-Tat-SF1 containing a silent mutation within the siRNA target sequence (rTat-SF1) (Fig. 2*D*). Transfection of the siTat-SF1 led to 40% reduction in the segment 7 cRNA synthesis in infected cells compared with the level in cells transfected with the control siRNA (Fig. 3*E*), whereas the cRNA synthesis level

was rescued by transfection of FLAG-rTat-SF1 expression plasmid in siTat-SF1-transfected cells. Similar results were obtained in the segment 7 mRNA and vRNA synthesis (Fig. 2 *F* and *G*). These were also the case for the viral RNA synthesis derived from the segment 3 (SI Fig. 8 *A*-*C* and see SI Text for details).

We also examined the viral protein synthesis and the production of infectious influenza virus in siTat-SF1-transfected cells. The expression of NP and PB1 were reduced in siTat-SF1-transfected cells (Fig. 2*H*). The virus titer was examined by the plaque assay (Fig. 2*I*). In the siTat-SF1-transfected cells, the level of infectious progeny viruses was reduced to 25% of that in control cells. Progeny viruses were not recovered from infected control and siTat-SF1-transfected cells in the presence of CHX (data not shown).

Tat-SF1 Stimulates the Viral RNA Synthesis.

Further to examine the mechanism of the Tat-SF1-dependent stimulation of the viral RNA synthesis, we established a HeLa cell line over-expressing FLAG-Tat-SF1. In cells expressing FLAG-Tat-SF1, viral cRNA, mRNA and vRNA synthesis from segment 3 were increased (Fig. 3 *A*-*C*). This was also the case for the viral RNA synthesis derived from the segment 7 (SI Fig. 9 *A*-*C* and see SI Text for further discussion). It was indicated that CHX suppresses viral protein synthesis and blocks vRNA replication (12), while the level of the primary transcription from incoming vRNP is not affected in the present of CHX. Figure 3*D* shows that the primary transcription in the presence of CHX is not affected by expression of FLAG-Tat-SF1. These results suggest that Tat-SF1 stimulates the viral RNA synthesis reaction including replication processes after primary transcription. Thus, it is possible that the

enhancement of the viral mRNA synthesis in the absence of CHX (Fig. 3*B*) is due to the amplified genome by replication.

The effect of the over-expression of FLAG-Tat-SF1 on the viral protein synthesis and the production of infectious progeny viruses were investigated. NP and PB1 were synthesized at the trace level in control cells at 6 h and 8 h post-infection, respectively, whereas the synthesis of NP and PB1 was markedly increased in the cells expressing FLAG-Tat-SF1 (Fig. 3*E*). Further, over-expression of Tat-SF1 led to 5- to 6-fold increase in the progeny virus production compared with control cells (Fig. 3*F*).

Tat-SF1 Facilitates Formation of the vRNA-NP Complex.

To determine a viral factor(s) that interacts with Tat-SF1, we carried out immunoprecipitation assays using recombinant His-myc-Tat-SF1 (designated myc-Tat-SF1 in Fig. 4) with either purified vRNP or micrococcus nuclease-treated vRNP (mnRNP) (Fig. 4*A*). Micrococcus nuclease eliminates vRNA and generates viral proteins free of RNA. His-myc-Tat-SF1 specifically bound to NP among vRNP components when mnRNP was used. This result indicates that Tat-SF1 interacts with NP free of RNA but not NP associated with RNA. Based on this notion, we assumed that Tat-SF1 may have a chaperone-like activity for NP, that is, that Tat-SF1 binds to NP, transfers NP to RNA, and is dissociated from NP during transfer of NP. The addition of increasing amounts of RNA dissociated the Tat-SF1-NP complex (Fig. 4*B*, lanes 6 and 7). This suggests that NP or NP-RNA complex was released from Tat-SF1 by the addition of RNA, and RNA-NP-Tat-SF1 trimeric complex was not formed. Then, we examined the effect of Tat-SF1 on the efficiency of vRNP-NP complex formation (Fig. 4*C*). A ^{32}P-labeled RNA probe was incubated with recombinant NP in the presence or

absence of the recombinant Tat-SF1 and subjected to separation through a 15 to 35% linear glycerol density gradient. After centrifugation, aliquots were fractionated, and RNA-NP complexes were analyzed by a native PAGE. The mobility of RNA-NP complexes was slower than that of the RNA probe only. We found that the formation of vRNA-NP complexes is increased in the presence of Tat-SF1 (Fig. 4*B*, compare lanes 3-5 and lanes 12-14). The shift of RNA probe was not found only in the presence of Tat-SF1 (Fig. 4*C*, lanes 19-27). These results suggest that Tat-SF1 facilitates formation of vRNA-NP complexes possibly by functioning as a chaperone for NP. This observation is exactly the same as that for RAF-2p48 (14), which facilitates the binding of NP to naked viral RNA to form vRNA-NP complexes as active templates for viral RNA synthesis. Tat-SF1 and RAF-2p48 may have the redundant function as NP interacting factors for stimulation of viral RNA synthesis (see discussion).

Discussion

Here, we have described a novel system for replication and transcription of the influenza virus genome of negative polarity in yeast cells. With this yeast system, we could identify Tat-SF1 as a host factor candidate, which is known as a transcription elongation factor of the DNA transcription (30-32). We found that Tat-SF1 stimulates the viral RNA synthesis in a *cell-free* system using vRNP and ApG dinucleotide primer (data not shown). Several splicing proteins may be found to be candidates as host factors for the influenza virus replication process (SI Fig. 6). Some of splicing factors are associated with the Pol II (RNA polymerase II)-dependent transcription complex. Recently, it has been reported that the influenza virus gene transcription is dependent on transcriptionally functional Pol II (34). We assume that vRNP could be associated with Pol II complexes, the factors of which may be utilized as host factors for the efficient viral RNA synthesis.

Tat-SF1 may act as chaperone for NP in formation of RNA-NP complexes. In general, nucleic acid-binding proteins containing a basic domain(s) such as histones and viral basic proteins such as histones and NP tend to aggregate and became inactive in the absence of nucleic acids or appropriate binding proteins such as molecular chaperones including histone chaperone (35-37). Since NP is produced as a form free of RNA in infected cells, the interaction of NP with Tat-SF1 may repress nonspecific aggregation of NP and facilitate the formation of vRNA-NP complex. Previously, RAF-2p48 was identified as a host-derived chaperone for NP that interacts with NP and stimulates the influenza virus RNA synthesis (14). It is possible that Tat-SF1 have a role similar to that of RAF-2p48 for stimulation of the viral RNA synthesis. NP may

utilize redundant host factors in the efficient viral RNA synthesis.

It has been shown that yeast cells support the replication of some positive-strand viral RNA genomes (38). In negative-strand RNA viruses, the expression of viral proteins of vesicular stomatitis virus was observed dependent on primary transcription and translation in yeast cells (39). Here, we show the usefulness of the viral replication system for a negative-strand RNA virus in yeast for identification and characterization of factors involved in the virus replication processes.

Further to use the system for screening of host factors and an inhibitory drug for the influenza virus, vRNA containing a reporter gene could be convenient. To this end, we tried to replace all viral components consisting of vRNP complexes with viral proteins supplied from exogenously added plasmids (data not shown). We constructed plasmids for expression of PB1, PB2, PA, and NP under the control of the *GAL1* promoter, and prepared a model viral RNA (NS-yEGFP RNA) using *in vitro* T7 RNA polymerase-directed transcription system (8, 9), in which the yEGFP coding region is sandwiched with the 5'- and 3'-terminal sequences of segment 8 vRNA encoding NS. The expression of yEGFP was detected from NS-yEGFP RNA in yeast cells expressing three viral RNA polymerase subunits and NP (data not shown). However, the expression level of yEGFP was quite low. This system is under improvement.

Materials and Methods

Yeast Strains and Introduction of DNA and vRNP into Yeast Cells.

Yeast strain YPH499 (*MATa, ura3-52, lys2-801, ade2-101, trp1-63, his3-200, leu2-1*) was used in all experiments. The *sub2* deletion strain was generated by replacement of the entire *sub2* open reading frame with *TRP1* (*sub2*Δ::*TRP1* fragment). The lithium acetate-polyethylene glycol method was used for transformation of yeast cells. Introduction of vRNP was performed according to the procedure for transformation with RNA minor modifications (SI Methods).

Virus Infection.

Preparation of allantoic fluid from influenza A PR/8 virus-infected embryonated chicken eggs and infection process were previously described (19). In this manuscript, HeLa cells were infected at the multiplicity of infection (moi) = 5. Total RNA was prepared using guanidine methods for RT-PCR (SI Methods).

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Figure legends

Fig. 1. Replication and transcription of the influenza virus genome in yeast cells.

(*A*) Yeast spheroplasts were mock-transfected (lane 1) or transfected with vRNP (0.1, 0.3, 1, and 2 μg NP equivalents for lanes 2, 3, 4, and 5, respectively) purified from virions. At 48 h post transfection (hpt), total yeast RNA was extracted and subjected to reverse transcription. PCR were then performed with primer sets specific for negative-sense RNA (vRNA) and positive-sense RNA (mRNA or cRNA) of segment 5 and *ACT1* mRNA. Amplified double-stranded DNAs were subjected to a 7% polyacrylamide gel electrophoresis and visualized by ethidium bromide.

(*B*) Yeast cells transfected with vRNP were incubated in the absence (lanes 1 and 2) or presence of 3 mg/ml (lane 3) and 10 mg/ml (lane 4) of cycloheximide (CHX). RT-PCR was performed with primers specific for segment 3 RNA and *ACT1* mRNA.

(C) The viral protein synthesis in vRNP-transfected yeast and mammalian cells. HeLa cells or yeast cells were transfected with 3 μl of RNP (450 ng NP equivalents). After transfection, cells were incubated in the absence (lanes 1-3 and 5-7) or presence of 100 μg/ml (lane 4) and 3 mg/ml (lane 8) of CHX. We used the previously described method (40) for preparation of total protein from yeast cells. HeLa cell lysates (20 μg) or yeast cell lysates (22.5 μg) were loaded for each lane. Western blotting analyses were carried with anti-NP or anti-β-actin antibodies as a control. Coomassie Briliant Blue (CBB) staining is also shown as a control for preparation of yeast lysates due to lack of an appropriate antibody. The molecular weight marker is shown in lane 9.

(*D-L*) vRNP-transfected yeast cells were immunostained at 24 hpt. NP and DNA were stained with anti-NP antibody (*D*, *G*, and *J*) and DAPI (*E*, *H*, and *K*), respectively.

Overlay; overlay of NP and DAPI staining panels (*F*, *I*, and *L*). Yeast cells were incubated in the absence (*D-F* and *J-L*) or presence (*G-I*) of 5 mg/ml of CHX. Prior to transfection, vRNP were treated with RNase A for 10 min at 37ºC (*J-L*).

(*M-O*) The complementation experiment of segment 5-depleted vRNP. (*M*) Digestion of segment 5 vRNA. The vRNP (3 μg NP equivalents) was mixed with 300 ng of an oligonucleotides corresponding to a part of segment 5 vRNA (Seg. 5 digestion: supplementary experimental procedures), in the presence of 0.4 M NaCl for 5 min at 37°C, and then treated (lane 1) or mock-treated (lane 2) with 30 U of RNase H for 5 min at 37°C. The purified RNA was loaded onto a 3.2% polyacrylamide gel containing 7.7 M urea and visualized by silver staining. Asterisks indicate bands possibly corresponding to digested fragments. (*N*) Western blotting analysis of induced viral proteins. Control (lanes 1 and 2), NP (lanes 3 and 4), and PB2 (lanes 5 and 6). (*O*) RT-PCR analysis of viral RNAs. vRNP and vRNP devoid of segment 5 vRNA (RNase H digestion) were transfected into yeast cells transformed with pYES2 (lanes 1-3), pYES2-NP (lanes 4-6) and pYES2-PB2 (lanes 7-9). Yeast cells were incubated for 24 h in medium containing galactose. RT-PCR analysis was performed with primer sets specific for segment 3 cRNA and mRNA, and *ADH1* mRNA.

Fig. 2. Effect on Tat-SF1 knock-down on virus infection.

(*A*) Total RNA was prepared from HeLa cells which had been transfected with control siRNA or siTat-SF1, and super-infected with influenza virus. Real-time RT-PCR was carried out with primer sets specific for *M1* mRNA, *M2* mRNA, and *β-Actin* mRNA.

(*B*) Representation of *M1* and *M2* mRNA generated from segment 7.

(*C*) The ratio of the amount of *M2* mRNA to that of *M1* mRNA in HeLa cells

transfected with control siRNA or siTat-SF1 and these infected with influenza virus. (*D*-*G*) 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 for 24 h incubation, cells were super-infected with influenza virus. Total RNA was prepared from cells at 3 hour post infection (hpi). Real-time RT-PCR was carried out with primer sets specific for endogenous *Tat-SF1* (*D, Left*), exogenous *FLAG-rTat-SF1* (*D, Right*), segment 7 RNAs (*E*: cRNA, *F*: mRNA, *G*: vRNA), and *β-Actin* mRNA. The results are normalized as the ratio to the level of *β-Actin* mRNA. Error bars show standard deviation.

(*H*) Western blotting analyses of viral proteins. Mock- (lanes 1, 3, 5, and 7) or siTat-SF1 siRNA-transfected (lanes 2, 4, 6, and 8) HeLa cells were infected with influenza virus at the moi of 5. Western blotting analyses were carried with anti-NP, anti-PB1, or anti-β-actin antibodies.

(*I*) Single-step virus growth. Mock- or siTat-SF1 siRNA-transfected HeLa cells were infected with influenza virus at the moi of 0.1. Virus titer was examined by the plaque assay at the indicated times post infection.

Fig. 3. Tat-SF1 as a stimulatory host factor involved in virus RNA synthesis.

(*A*-*D*) Viral RNA synthesis in the presence of a protein synthesis inhibitor. HeLa cells expressing FLAG-Tat-SF1 (Tat-SF1) or control (Neo) HeLa cells were infected with influenza virus in the absence (*A*-*C*) or presence (*D*) of 100 μg/ml of CHX. Real-time RT-PCR was carried out with primer sets for segment 3 cRNA (*A*), segment 3 mRNA (*B* and *D*), segment 3 vRNA (*C*), and *β-Actin* mRNA. Error bars show standard deviation.

(*E*) Western blotting analyses of viral proteins. HeLa cells expressing FLAG-Tat-SF1 (lanes 2, 4, and 6) or control HeLa cells (lanes 1, 3, and 5) were infected with influenza virus at the moi of 1. Western blotting analyses were carried with anti-NP, anti-PB1, or anti-β-actin antibodies.

(*F*) Single-step virus growth. HeLa cells expressing FLAG-Tat-SF1 or control HeLa cells were infected with influenza virus at the moi of 0.1. Virus titer was examined as described.

Fig. 4. Stimulatory activity of Tat-SF1 *in vitro*.

(*A*) The interaction between Tat-SF1 and viral proteins. Immunoprecipitation assays were carried out by anti-myc antibody-conjugated agarose beads using purified His-myc-Tat-SF1 (lanes 6-8) and either vRNP (lanes 4 and 7) or mnRNP (lanes 5 and 8). The affinity beads were washed with IP buffer containing 300 mM KCl. Western blotting analyses were carried with anti-NP, anti-PB1, anti-PB2, anti-PA, or anti-myc antibodies. The input (20%) is shown in lanes 1 and 2.

(*B*) Dissociation of NP-Tat-SF1 complexes by the addition of RNA. An NP-Tat-SF1 complex was reconstituted by mixing purified His-myc-Tat-SF1 (lanes 3, 5, 6, and 7) and mnRNP (lanes 4-7). The mixtures were further incubated in the presence of v53-mer RNA (20 ng (lane 6) and 200 ng (lane 7)). After immunoprecipitation assays using anti-myc antibody-conjugated agarose, western blotting analyses were carried with anti-NP or anti-myc antibodies. The input (20%) is shown in lane 1.

(*C*) Tat-SF1-mediated NP-RNA complex formation. ^{32}P-labeled RNA probe (v53 mer RNA) mixed with recombinant NP was incubated in the presence of BSA (lanes 1-9) or recombinant Tat-SF1 (lanes 10-18), and v53 mer RNA probe mixed with recombinant

Tat-SF1 was incubated in the absence of recombinant NP (lanes 19-27). After sedimentation through a 15 to 35% glycerol density gradient, fractions were collected from the top of the tube. An aliquot of each fraction was analyzed by PAGE on a 6% polyacrylamide gel, and the RNA was visualized by autoradiography. The unbound RNA probe and NP-RNA complexes are indicated by arrowheads.