หนังสืออ้างอิง

Gabriel, G., A. Herwig, et al. (2008). "Interaction of polymerase subunit PB2 and NP with importin alpha1 is a determinant of host range of influenza A virus." PLoS Pathog 4(2): e11.

Krug, R. M., M. Ueda, et al. (1975). "Temperature-sensitive mutants of influenza WSN virus defective in virus-specific RNA synthesis." J Virol 16(4): 790-6.

Li, Z., Y. Jiang, et al. (2006). "The NS1 gene contributes to the virulence of H5N1 avian influenza viruses." J Virol 80(22): 11115-23.

Mandler, J. and C. Scholtissek (1989). "Localisation of the temperature-sensitive defect in the nucleoprotein of an influenza A/FPV/Rostock/34 virus." Virus Res 12(2): 113-21.

Medcalf, L., E. Poole, et al. (1999). "Temperature-sensitive lesions in two influenza A viruses defective for replicative transcription disrupt RNA binding by the nucleoprotein." J Virol 73(9): 7349-56.

Noton, S. L., M. Simpson-Holley, et al. (2009). "Studies of an influenza A virus temperature-sensitive mutant identify a late role for NP in the formation of infectious virions." J Virol 83(2): 562-71.

Peiris, M. (2006). "Pathogenesis of avian flu H5N1 and SARS." Novartis Found Symp 279: 56-60; discussion 60-5, 216-9.

Portela, A. and P. Digard (2002). "The influenza virus nucleoprotein: a multifunctional RNA-binding protein pivotal to virus replication." J Gen Virol 83(Pt 4): 723-34.

Seo, S. H., E. Hoffmann, et al. (2002). "Lethal H5N1 influenza viruses escape host anti-viral cytokine responses." Nat Med 8(9): 950-4.

Shapiro, G. I. and R. M. Krug (1988). "Influenza virus RNA replication in vitro: synthesis of viral template RNAs and virion RNAs in the absence of an added primer." J Virol 62(7): 2285-90.

Szretter, K. J., S. Gangappa, et al. (2007). "Role of host cytokine responses in the pathogenesis of avian H5N1 influenza viruses in mice." J Virol 81(6): 2736-44.

5. output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

จากการวิจัยในโครงการนี้สามารถศึกษาพบตำแหน่งนิวคลีโอไทด์และกรดอะมิโนที่สำคัญต่อการเป็น temperature sensitive phenotype (Ts) ของไวรัส H5N1 ได้ อีกทั้งยังเข้าใจกลไกการทำงานของกรดอะมิโนดังกล่าวในการแสดงออกซึ่งลักษณะ Ts phenotype

โครงการวิจัยดังกล่าวได้กำลังทำการส่ง manuscript เพื่อลงตีพิมพ์ในวารสารต่างประเทศในหัวข้อเรื่อง " A serine to asparagines mutation at position 314 of H5N1 avian influenza virus NP gene is a temperature-sensitive mutation that interfere with nuclear localization of NP protein"

ภาคผนวก

Manuscript

A serine to asparagines mutation at position 314 of H5N1 avian influenza virus NP gene is a temperature-sensitive mutation that interfere with nuclear localization of NP protein

Siboonnan N, Wiriyarat W[1], Boonarkart C, Jongkaewwattana A[2], Puthavathana P, Auewarakul P, Suptawiwat O

Department of Microbiology, Faculty of Medicine Siriraj Hospital, and [1] Faculty of Veterinary Science, Mahidol University, Bangkok, Thailand, [2] National Center of Genetic Engineering and Biotechnology, Bangkok

We have generated a temperate sensitive (ts) mutant from a human isolate of H5N1 avian influenza virus by classical adaptation in cell culture. After 20 passages at low temperature, the virus showed ts phenotype. The ts mutant also showed attenuated phenotype after nasal inoculation in mice. Using reverse genetics, we generated reassortants carrying individual genomic segment of the mutant, and found that the nucleoprotein (NP) gene could confer the ts phenotype. This mutant NP contains a serine to asparagine mutation at position 314 (S314N). The mutant NP protein showed a defect in nuclear localization at high temperature in mammalian but not in avian cells.

Introduction

Cold-adapted (ca) phenotype is defined as an ability to replicate efficiently at a low temperature, whereas ts phenotype is defined as an absence or marked reduction of replication at a non-permissive high temperature. Some ca/ts influenza strains, which replicate well at 25°C but lost replicative capacity at a temperature above 39°C, have been successfully used as live-attenuated nasal vaccines. These viruses replicate in nasal cavity, where the temperature is low, and do not replicate well in deeper parts of respiratory tract. Two ca/ts influenza vaccine strains, A/Ann Arbor 6/60 (H2N2) and A/Leningrad/134/47/57 (H2N2), are currently available (Garmashova, Polezhaev et al. 1984; 2003; Mossad 2003; Glezen 2004). Genetic determinants of ca and ts phenotypes of these viruses have been well characterized (Donabedian, DeBorde et al. 1987; Klimov, Egorov et al. 1995; Jin, Lu et al. 2003; Hoffmann, Mahmood et al. 2005; Chen, Aspelund et al. 2006; Chen, Aspelund et al. 2008)

H5N1 avian influenza virus is a highly virulent virus capable of infecting a wide range of avian and mammalian species including human. The virus caused explosive outbreak in Southeast Asia in 2003-2004 and spread rapidly to other geographic regions. Despite the ability to infect mammals, the virus still maintains many of its avian virus characteristics, including receptor usage preference and the ability to grow at 40°C, which is the body temperature of birds. This ability to replicate well at high temperature may contribute to its high virulence in mammals, in which increased body temperature or fever is an innate defense capable of suppressing replication of other viruses. Differences in temperature tropism between avian and human influenza viruses also contribute to the avian-human interspecies barrier. Avian influenza viruses are optimized to replicate at higher temperature and replicate poorly at a temperature lower than human body temperature. Because the temperature in human upper airway is considerably lower than the core body temperature, seasonal influenza viruses have to be able to replicate at this temperature in order to infect and transmit efficiently in human

population. The optimal temperature for most seasonal influenza viruses is 33°C. For H5N1 avian influenza virus to become successful in transmitting among humans, it would likely have to adapt its temperature tropism profile.

In vitro adaptation is a simple way to yield viruses with different growth characteristics. Lower the temperature setting of H5N1 avian influenza virus by in vitro adaptation may provide a clue on how the virus would evolve to growth environment with lower temperature in human upper airway. We therefore generated an H5N1 strain with a lower optimal growth temperature by in vitro adaptation, and studied its growth characteristics and genetic determinants.

Materials and Methods

Viral isolate and the in vitro adaptation

The viral isolates A/Thailand/SP83/2004 (H5N1) was adapted to growth at low temperature. We used low-passage virus, which had been propagated in MDCK (Madin-Darby Canine Kidney) cells at 37°C for 8 passages. We initially grew the virus in MDCK cells in Minimum Essential Medium (MEM) supplemented with 10% heat-inactivated fetal calf serum (FBS) at 30°C for 5 passages until the titers in this temperature approached the initial titers at 37°C. The virus was subsequently passaged at 27°C and then at 25°C for 9 and 6 passages, respectively.

Growth temperature characterization

MDCK (Madin Darby canine kidney) and CEF (Chiken embryo fibroblast) cells were seeded at a density of 5.5 x 10^5/ well in six-well plates overnight at 37°C. Cells were then washed two times with serum free media MEM containing 1μg/ml of L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) -treated Trypsin. Viruses was inoculated to the cells at 55 $TCID_{50}$ (50% tissue culture infectious dose) in a total volume of 200 μl, and allowed to

adsorb for one hour at 33 °C, 37 °C or 40°C in 5% CO_2 incubator, then media was added to adjust the volume to 3 ml. The cells were incubated at 33 °C, 37 °C or 40°C in 5% CO_2 incubator. At 6, 24, 30, and 48 or 53 hours post-infection, supernatants were collected and hemagglutination (HA) and $TCID_{50}$ titers were determined. The growth kinetic assay was performed in triplicate.

For HA titration, 50 µl of 0.5% goose red blood cells in 1X PBS pH 7.2 was mixed with 50 µl of two-fold serially diluted viral supernatant in U-shape microtiter plates and incubated at 4°C for 30 minutes. The last dilutions with complete HA were read as HA titers. For $TCID_{50}$ titration, $3x10^6$ MDCK cells in 96-well plates were inoculated with two-fold serially diluted viral supernatant. The inoculated cells were maintained in 1XMEM media supplemented with 1 ug/ml TPCK at 37 °C in a CO_2 incubator. At 24 hours post-infection, the media was removed, the cells were washed and fixed in 80% for 60 minutes at 4 °C. Endogenous peroxidase activity was quenched by incubating in 3% H_2O_2 for 30 minutes at room temperature. Viral antigen in the fixed cells was then detected by a NP-specific monoclonal antibody (MILLIPORE, USA) and a horse-radish peroxidase-conjugated anti-mouse IgG antibody (Southern Biotech Associates Inc., Birmingham,USA). 8 wells of uninfected cell controls were included in each plate, and OD (optical density) of over mean+2SD of the cell controls was considered positive. TCID50 titer was then calculated from the number of positive wells using Reed and Muench method.

Animal inoculation

Female 6-8 week-old BALC/c mice were intranasally inoculated with A/Thailand/SP83/2004(H5N1) wild type or the ts virus at 10^1 to 10^4 PFU/ml. Six mice were used in each group. All mice were monitored for weigh loss and survival rate for 3 weeks. The experiment was performed in animal isolators in a BSL-3 facility.

Viral gene cloning and construction of reassortants

Viral genomic segments were amplified by RT-PCR using universal primers as previously described. Amplified fragments were cloned into pHw2000 reverse genetics plasmid, sequenced, and used for reconstruction of reassorted reverse genetic viruses as previously described. Briefly, 1 μg each of the 8 plasmids were transfected into HEK-293 cells cocultured with MDCK cells on a 6-well plate using Lipofectamine™ 2000 (Invitrogen, USA). After 24 hours, viruses were rescued from 293T cell by adding 1 ml of Opti-MEM® I Reduced Serum Medium containing 2μg/ml of TPCK-treated trypsin. The plate was then incubated at 37°C for 24-48 hours. After 24-48 hours of incubation, the presence of rescued virus was determined by observing cytopathic effect (CPE) and testing for HA titer.

The construction of the reporter plasmid, designated pPolI-GFP, was carried out by replacing ORF of segment 7 of A/PR/8/34 expressed in the backbone of a bi-directional plasmid, pHW2000, with EGFP protein, resulting in pHW-GFP. Consequently, the GFP transcript was flanked by 3'UTR and 5'UTR of the segment 7 of either influenza A virus. The CMV promoter of pHW-GFP plasmid was further removed to allow only expression of negative-sense RNA from human RNA polymerase I promoter, giving rise to a final construction of pPolI-GFP. The removal of CMV promoter was carried out by hi-fidelity PCR amplification of pHW-GFP using the primer pair containing BamHI restriction site (underlined): Forward:5-'TATAGGATCCCTGGCTTATCGAAATTAATAC-3' and Reverse : 5'TATAGGATCCCGACGTCAGGTGGCACTTTTCG. The PCR product was purified and double digested with BamHI and DpnI (Fermentas) to generate cohesive ends and eliminate the residual template, respectively. Subsequently, the plasmid was allowed to self-ligate before transformation into DH10B competent cells. The absence of the CMV promoter in PolI-sNA-GFP plasmid was verified by direct nucleotide sequencing.

Transfection and subcellular localization of viral NP

In order to study cellular localization of wild type and mutant NP, pHw2000 carrying wild type mutant NP genes was transfected into HEK-293 cells using DMRIE-C reagent (Invitrogen, USA). Transfected cells were maintained at 33 or 40°C in CO_2 incubators. At 48 hours post-infection, cells were fixed in 80% cold acetone at 4°C for 30 minutes. NP protein was then detected using a specific monoclonal antibody (MILLIPORE, USA) and a FITC-conjugated anti-mouse IgG antibody. Cells were counterstained with Hoechst dye nuclear stain (Invitrogen, USA) and examined under a laser scanning confocal microscope (LSM 510 Meta, Zeiss, Jena, Germany).

Polymerase activity assay

A PB2 expression plasmid from SP83 wild type (PB2 627E) showed only low level of GFP expression when was cotransfected with pPolI-GFP reporter plasmid and other 3 polymerase complex expression plasmids of SP83 wild type; therefore PB2 expression plasmid from another viral strain (PB2 627K) was used instead in the polymerase assay.

Viral polymerase complex comprising PB2 of A/open billed stork/Nakhonsawan/BBD1821J/05 (H5N1) and PB1, PA and NP form SP83 wild type or SP83/20 were transfected with pPolI-GFP reporter plasmid into HEK-293 cells by DMRIE C reagent. All transfected cells were incubated at 33°C, 37°C and 40°C for 72 hours. Finally, transfected cells were harvested for GFP expression analysis by flow cytometry.

Results

The ts mutant

The in vitro-adapted virus, designated A/Thailand/SP83/20/2004 (SP83/20) showed ts phenotypes (Fig 1). At 40°C it grew to a titer 4- 6 log lower than that of wild type SP83 virus, whereas at 37°C both wild type and mutant viruses gave comparable infectious titers. The wild type virus replicated well at 37 °C and 40°C yielding a titer that was slightly higher than at

33°C (Fig. 1a). This reflected the avian phenotype of the virus. In contrast, SP83/20 replicated poorly at 40°C with a titer 5 - 7 log lower than at 33°C (Fig. 1 b). The SP83/20 virus also replicated better at 33°C than at 37°C, which indicates a shift in its optimal growth temperature. The differences in the replication efficiency at various temperatures were also confirmed by plaques sizes (Fig.1 c). In mice intranasal inoculation experiments, SP83/20 virus showed lower weight loss and higher survival rate than wild type virus (Figure 2). These suggested that SP83/20 has lower virulent than the wild type virus.

Genetic determinant of the ts phenotype

Sequences of all genomic segments of SP83 and SP83/20 were compared. GenBank accession numbers of these sequences are shown in Table 1. We found three mutations in PB2 and one each in PB1, PA and NP (Table 2). In order to identify genetic determinants of the ts phenotype, we initially attempted to reconstruct reverse genetic viruses using all genomic segments of SP83 and reassortants carrying each individual gene from SP83/20 in the background of SP83. Unfortunately, after multiple attempts we could not get any viable virus. In order to test whether the cloned viral genomic segments were functional, we generated reassortants with individual genomic segments of SP83 and SP83/20 in the background of the vaccine strain A/Puerto Rico/8/64 (PR8). We could generate viable viruses for all the genomic segments except for the PB2 of SP83/20. This indicated that these genomic segments were functional and that the mutations in PB2 of SP83/20 made it defective or incompatible with the PR8 genomic background. In order to individually characterize the mutations, we introduced each of these three mutations into PB2 of SP83, and we were able to generate three reverse genetic viruses each carrying SP83-PB2 with single mutation.

We next characterized the growth phenotype of these reassortants. We compared growth kinetics of reassortants carrying PB1, PA, NP from SP83/20, and PB2 of SP83 with individual mutations of SP83/20 to that of reassortants carrying wild type SP83 genes in both

MDCK and CEF cell lines. Reassortants carrying mutant PB2, PB1, and PA grew to comparable or slightly lower titers at 33°C, 37°C, and 40°C as compared to reassortant viruses with wild type genes (data not showed). None of these viruses exhibited ts phenotype. In contrast, the reassortant carrying SP83/20 NP (rPR8-NP-SP83/20) replicated poorly at 40°C only in MDCK cells (Fig. 3a) but not in CEF cells (Fig. 3b). Moreover, activity of polymerase complex carrying NP-SP83/20 showed a functional defect at 40°C comfirming the replication defect of the reassortant (Fig. 3c). . Because the NP of SP83/20 contains only one mutation (S314N), we can conclude that the S314N mutation contributed to the ts phenotype of this strain.

The S314N mutation caused a defect in nuclear accumulation of the NP

In order to provide a mechanistic explanation for the effect of the NP S314N mutation, we studied subcellular localization of wild type and mutant NP protein at 33 and 40°C. After transfection into HEK-293 cells, both wild type and mutant NP protein showed predominant nuclear localization at 33°C. However, at 37°C and 40°C the NP S314N was detected mainly in the cytoplasm, whereas the wild type NP showed normal nuclear localization (Fig. 4). This indicated that the S314N mutation in the NP of H5N1 virus caused a defect in nuclear localization at high temperature.

Discussion

A/Ann Arbor/6/60 (H2N2) and A/Leningrad/134/47/57 (H2N2) are ca, ts viruses, which are used as master donor strains for live-attenuated influenza vaccines. Genetic determinants of ts phenotype of the A/Ann Arbor/6/60 (H2N2) were mapped to PB2, PB1, and NP. Likewise, the genetic determinants of ts phenotype of the A/Leningrad/134/47/57 were mapped to PB2, PB1 and PA. Although the NP of A/Ann Arbor/6/60 carries ts genetic

determinant, the ts mutation in this NP is D34G, which is different from the S314N mutation found to be the ts determinant in our mutant. Some other ts mutations in NP were also reported.

Temperature sensitive mutant is a common tool to study essential functions of viruses. The ability to replicate at permissive temperature allows the mutant to be selected, propagated and studied, while the non-permissive temperature allows the defect to be mapped and studied. Previously, the S314N was reported to be ts mutations in A/WSN/33/ts56 virus. In contrast to our data showing the defect in nuclear localization, the S314N mutation in A/WSN/33/ts56 was reported to cause a defect in its interaction to viral RNA but not nuclear localization. It should be noted that the subcellular localization of the mutant NP of A/WSN/33/ts56 in that report was not directly compared to that of wild type virus. Nevertheless, the fact that the same mutation was found in the two strains suggests that this mutation is a common mechanism for influenza viruses to adapt to low temperature.

The vRNPs transport across nuclear membrane by NP is a key event for influenza virus life cycle. NP is shown to be sufficient to mediate the nuclear import of vRNAs(O'Neill, Jaskunas et al. 1995; Wu, Sun et al. 2007; Wu and Pante 2009). The NP can interact with karyopherin (importin) α because of its three nuclear localization signal (NLS). An unconventional NLS (M_1ASQGTKRSYEQM_{13}) is at the very N-terminus. The second NLS residues (K_{198} RGINDRNFWRGFNGRRTR$_{216}$) in the central part of NP appeared to be weaker than the upstream NLS (Weber, Kochs et al. 1998). The third NLS was proposed to be located between amino acid 320 and 400 (Wang, Palese et al. 1997; Bullido, Gomez-Puertas et al. 2000). The S314N mutation is located near this third NLS. Whether the mutation influents the NLS function directly requires further study. It is interesting that the effect of S314N mutation was specific to mammalian cells. This suggests that the mutation caused a defect in interaction of the NP to a cellular factor in mammalian cells. In summary, we describe a S314N mutation in the NP gene of H5N1 avian influenza virus selected by an adaptation to

lower growth temperature, and show that it caused a defect in nuclear accumulation of NP in non-permissive temperature and contributed to the ts phenotype.

Legends

Figure 1 Kinetic growth curve of A/Thailand/SP83/2004(H5N1) virus (a) and SP83/20 virus (b) in MDCK cells at 33 °C, 37 °C and 40 °C. Viral titers from each time point were titrated by plaque assay. Three independent experiments were performed. The viruses showed different plaque sizes at different temperature in accordance with the growth kinetics (c)

Figure 2 Weight loss (left panel)and survival rates (right panel) of BALC/C mice infected with various dosages ofwild type SP83 (upper panel) and SP83/20 (lower panel).

Figure 3 Kinetic growth curve of rPR8-NP-SP83 virus (left) and rPR8-NP-SP83/20 virus (right) in MDCK cells (a) and CEF cells (b) at 33 °C, 37 °C and 40 °C. Viral titers from each time point were titrated by TCID50 assay. The data were derived from three independent experiments. Activity of viral polymerase complexes carrying SP83 WT-NP(wt) or SP83 WT-NP(SP83/20). Polymerase expression plasmids and pPolI-GFP reporter plasmid were transfected into HEK-293 cells and were incubated at 33 °C, 37 °C and 40 °C for 3 days. The level of GFP expression was analyzed by flow cytometry. The numbers represent geometric means of fluorescent intensity of cells in right upper quadrants. .

Figure 4 Localization of NP-SP83/20 (A,C) and NP-SP83 wt (B,D,) in 293T cells at 33°C and 40 °C. 293T cells were transfected with 1 μg of plasmid and analyzed by indirect immunofluorescence assay using a NP-specific monoclonal antibody and FITC-conjugated secondary antibody. Cells nuclei were stained with Hoechst dye.

Table 1 GenBank accession numbers of Influenza A virus (A/Thailand/SP83/2004(H5N1)) amino acid sequences were shown.

gene	**GenBank Accession number** **Influenza A virus (A/Thailand/SP83/2004(H5N1))**
PB2	ABO10164
PB1	ABO21686
PA	ABP35616
NP	ABO21700
HA	ABP51984
NA	ABP52013
M	ABP35619-ABP35620
NS	ABO21701-ABO21702

Table 2 Summary the sequence comparison of H5N1 strain A/Thailand/SP83/2004 from database in the GenBank, SP83wt, and SP83/20.

Gene	Amino acid position	Strain		
		SP83-GenBank	SP83wt	SP83/20
PB2	194	Q (Glu)	Q (Glu)	H (His)
	195	D (Asp)	D (Asp)	Y (Tyr)
	591	Q (Glu)	Q (Glu)	R (Arg)
PB1	554	D (Asp)	D (Asp)	N (Asn)
PA	448	A (Ala)	A (Ala)	E (Glu)
NP	314	S (Ser)	S (Ser)	N (Asn)

References

(2003). "Influenza virus vaccine live intranasal--MedImmune vaccines: CAIV-T, influenza vaccine live intranasal." Drugs R D **4**(5): 312-9.

Bullido, R., P. Gomez-Puertas, et al. (2000). "Several protein regions contribute to determine the nuclear and cytoplasmic localization of the influenza A virus nucleoprotein." J Gen Virol **81**(Pt 1): 135-42.

Chen, Z., A. Aspelund, et al. (2006). "Genetic mapping of the cold-adapted phenotype of B/Ann Arbor/1/66, the master donor virus for live attenuated influenza vaccines (FluMist)." Virology **345**(2): 416-23.

Chen, Z., A. Aspelund, et al. (2008). "Molecular studies of temperature-sensitive replication of the cold-adapted B/Ann Arbor/1/66, the master donor virus for live attenuated influenza FluMist vaccines." Virology **380**(2): 354-62.

Donabedian, A. M., D. C. DeBorde, et al. (1987). "Genetics of cold-adapted B/Ann Arbor/1/66 influenza virus reassortants: the acidic polymerase (PA) protein gene confers temperature sensitivity and attenuated virulence." Microb Pathog **3**(2): 97-108.

Gabriel, G., A. Herwig, et al. (2008). "Interaction of polymerase subunit PB2 and NP with importin alpha1 is a determinant of host range of influenza A virus." PLoS Pathog **4**(2): e11.

Garmashova, L. M., F. I. Polezhaev, et al. (1984). "[Cold-adapted strain of A/Leningrad/134/47/57 (H2N2)--a special attenuation donor of live influenza vaccine for children and the isolation of its recombinants]." Vopr Virusol **29**(1): 28-31.

Glezen, W. P. (2004). "Cold-adapted, live attenuated influenza vaccine." Expert Rev Vaccines **3**(2): 131-9.

Hoffmann, E., K. Mahmood, et al. (2005). "Multiple gene segments control the temperature sensitivity and attenuation phenotypes of ca B/Ann Arbor/1/66." J Virol **79**(17): 11014-21.

Jin, H., B. Lu, et al. (2003). "Multiple amino acid residues confer temperature sensitivity to human influenza virus vaccine strains (FluMist) derived from cold-adapted A/Ann Arbor/6/60." Virology **306**(1): 18-24.

Klimov, A. I., A. Y. Egorov, et al. (1995). "Genetic stability of cold-adapted A/Leningrad/134/47/57 (H2N2) influenza virus: sequence analysis of live cold-adapted reassortant vaccine strains before and after replication in children." J Gen Virol **76 (Pt 6)**: 1521-5.

Krug, R. M., M. Ueda, et al. (1975). "Temperature-sensitive mutants of influenza WSN virus defective in virus-specific RNA synthesis." J Virol **16**(4): 790-6.

Li, Z., Y. Jiang, et al. (2006). "The NS1 gene contributes to the virulence of H5N1 avian influenza viruses." J Virol **80**(22): 11115-23.

Mandler, J. and C. Scholtissek (1989). "Localisation of the temperature-sensitive defect in the nucleoprotein of an influenza A/FPV/Rostock/34 virus." Virus Res **12**(2): 113-21.

Medcalf, L., E. Poole, et al. (1999). "Temperature-sensitive lesions in two influenza A viruses defective for replicative transcription disrupt RNA binding by the nucleoprotein." J Virol **73**(9): 7349-56.

Mossad, S. B. (2003). "Demystifying FluMist, a new intranasal, live influenza vaccine." Cleve Clin J Med **70**(9): 801-6.

Noton, S. L., M. Simpson-Holley, et al. (2009). "Studies of an influenza A virus temperature-sensitive mutant identify a late role for NP in the formation of infectious virions." J Virol **83**(2): 562-71.

O'Neill, R. E., R. Jaskunas, et al. (1995). "Nuclear import of influenza virus RNA can be mediated by viral nucleoprotein and transport factors required for protein import." J Biol Chem **270**(39): 22701-4.

Peiris, M. (2006). "Pathogenesis of avian flu H5N1 and SARS." Novartis Found Symp **279**: 56-60; discussion 60-5, 216-9.

Seo, S. H., E. Hoffmann, et al. (2002). "Lethal H5N1 influenza viruses escape host anti-viral cytokine responses." Nat Med **8**(9): 950-4.

Shapiro, G. I. and R. M. Krug (1988). "Influenza virus RNA replication in vitro: synthesis of viral template RNAs and virion RNAs in the absence of an added primer." J Virol **62**(7): 2285-90.

Szretter, K. J., S. Gangappa, et al. (2007). "Role of host cytokine responses in the pathogenesis of avian H5N1 influenza viruses in mice." J Virol **81**(6): 2736-44.

Wang, P., P. Palese, et al. (1997). "The NPI-1/NPI-3 (karyopherin alpha) binding site on the influenza a virus nucleoprotein NP is a nonconventional nuclear localization signal." J Virol **71**(3): 1850-6.

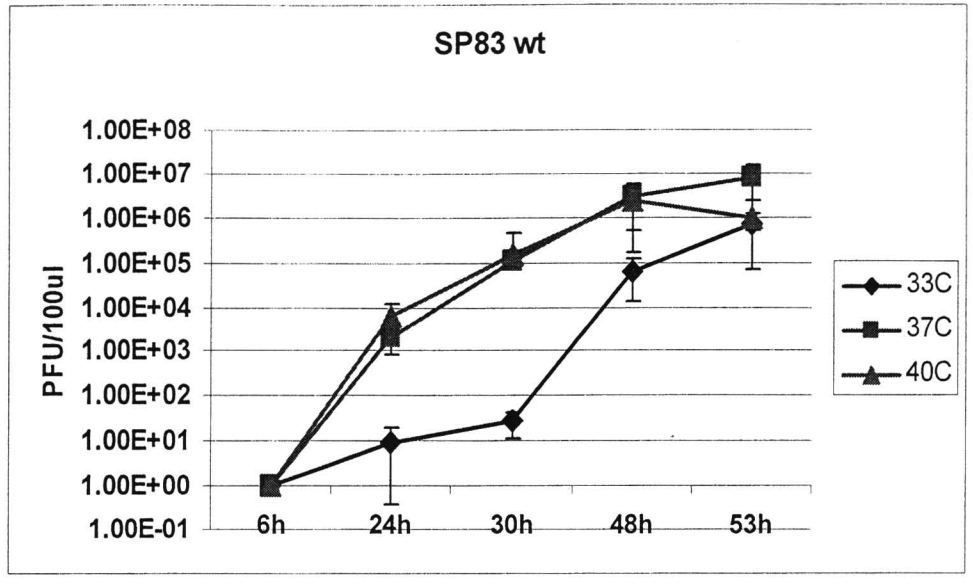
Weber, F., G. Kochs, et al. (1998). "A classical bipartite nuclear localization signal on Thogoto and influenza A virus nucleoproteins." Virology **250**(1): 9-18.

Wu, W. W. and N. Pante (2009). "The directionality of the nuclear transport of the influenza A genome is driven by selective exposure of nuclear localization sequences on nucleoprotein." Virol J **6**: 68.

Wu, W. W., Y. H. Sun, et al. (2007). "Nuclear import of influenza A viral ribonucleoprotein complexes is mediated by two nuclear localization sequences on viral nucleoprotein." Virol J **4**: 49.

Figure 1

a


SP83 wt
PFU/100ul
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
1.00E-01
6h
24h
30h
48h
53h
33C
37C
40C

b

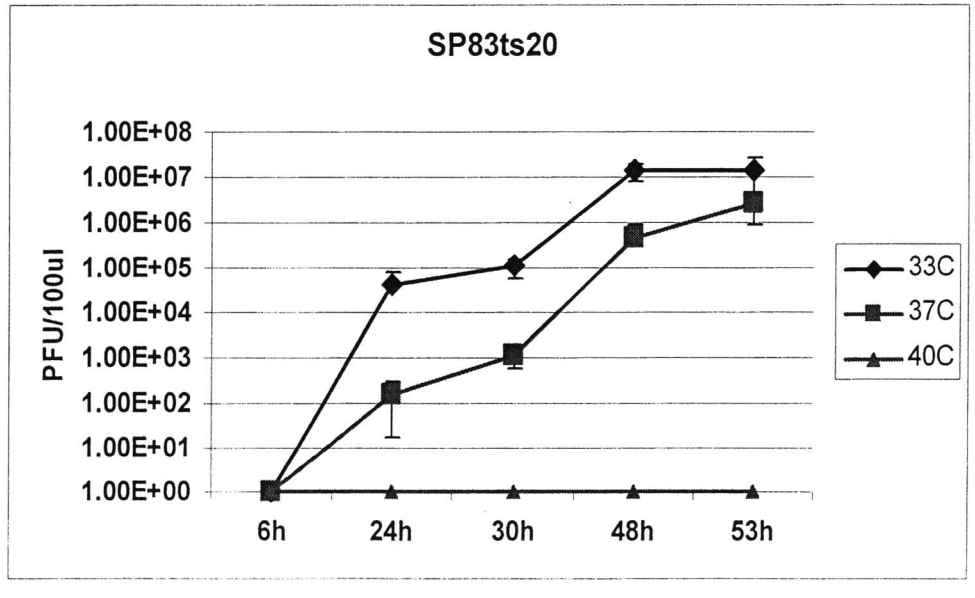

SP83ts20
PFU/100ul
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
6h
24h
30h
48h
53h
33C
37C
40C

Fig. 1 c

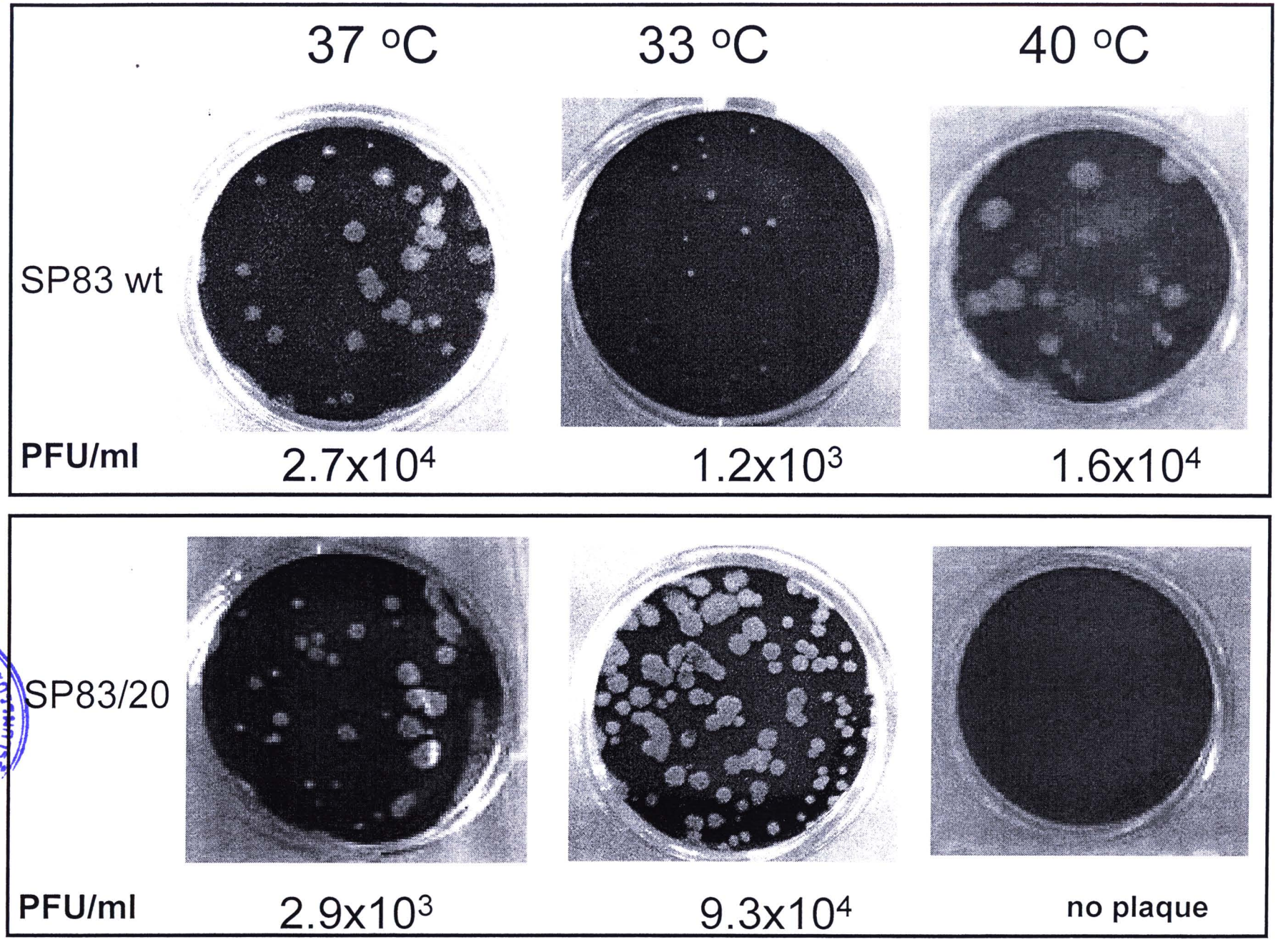
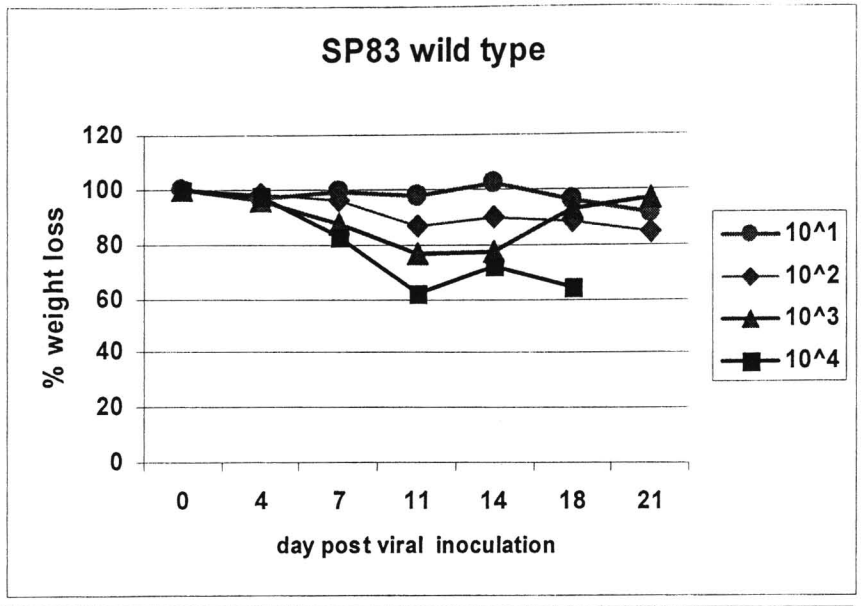
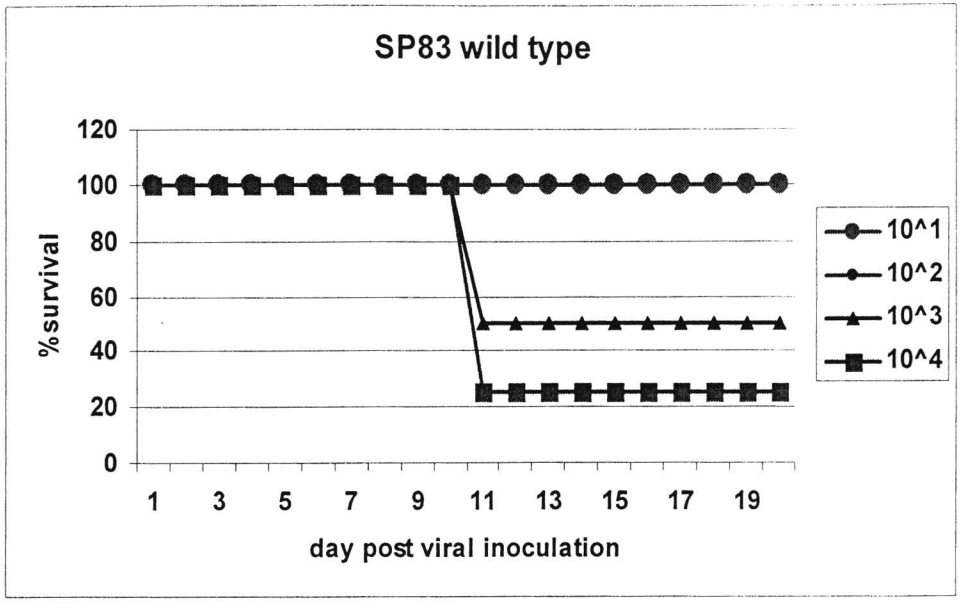
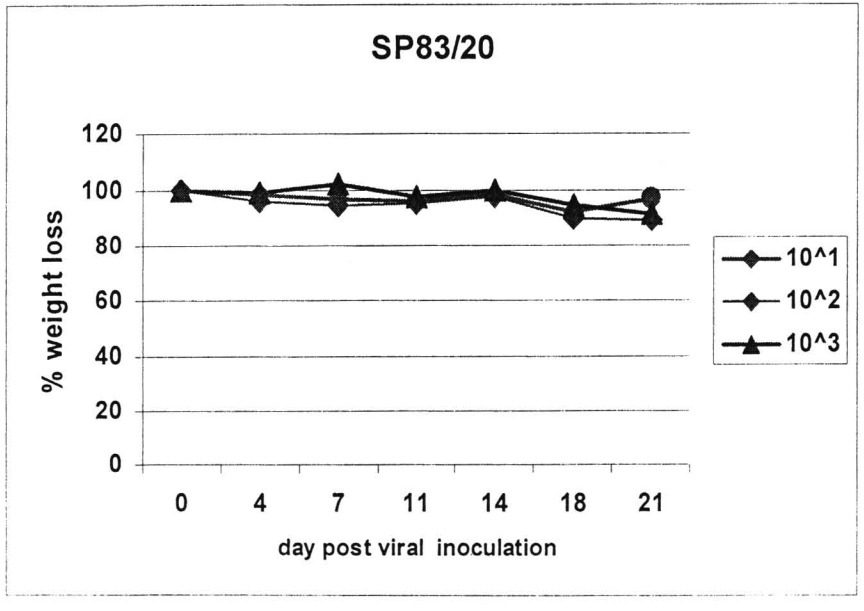

37 °C
33 °C
40 °C
SP83 wt
PFU/ml
2.7x10^4
1.2x10^3
1.6x10^4
SP83/20
PFU/ml
2.9x10^3
9.3x10^4
no plaque

Figure 2


SP83 wild type
% weight loss
120
100
80
60
40
20
0
0
4
7
11
14
18
21
day post viral inoculation
10^1
10^2
10^3
10^4


SP83 wild type
%survival
120
100
80
60
40
20
0
1
3
5
7
9
11
13
15
17
19
day post viral inoculation
10^1
10^2
10^3
10^4


SP83/20
% weight loss
120
100
80
60
40
20
0
0
4
7
11
14
18
21
day post viral inoculation
10^1
10^2
10^3

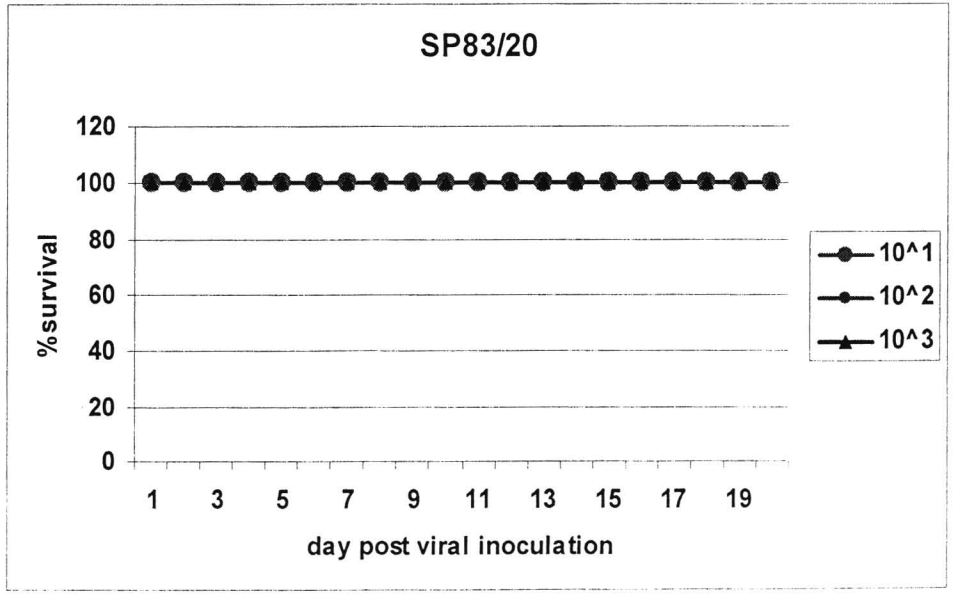
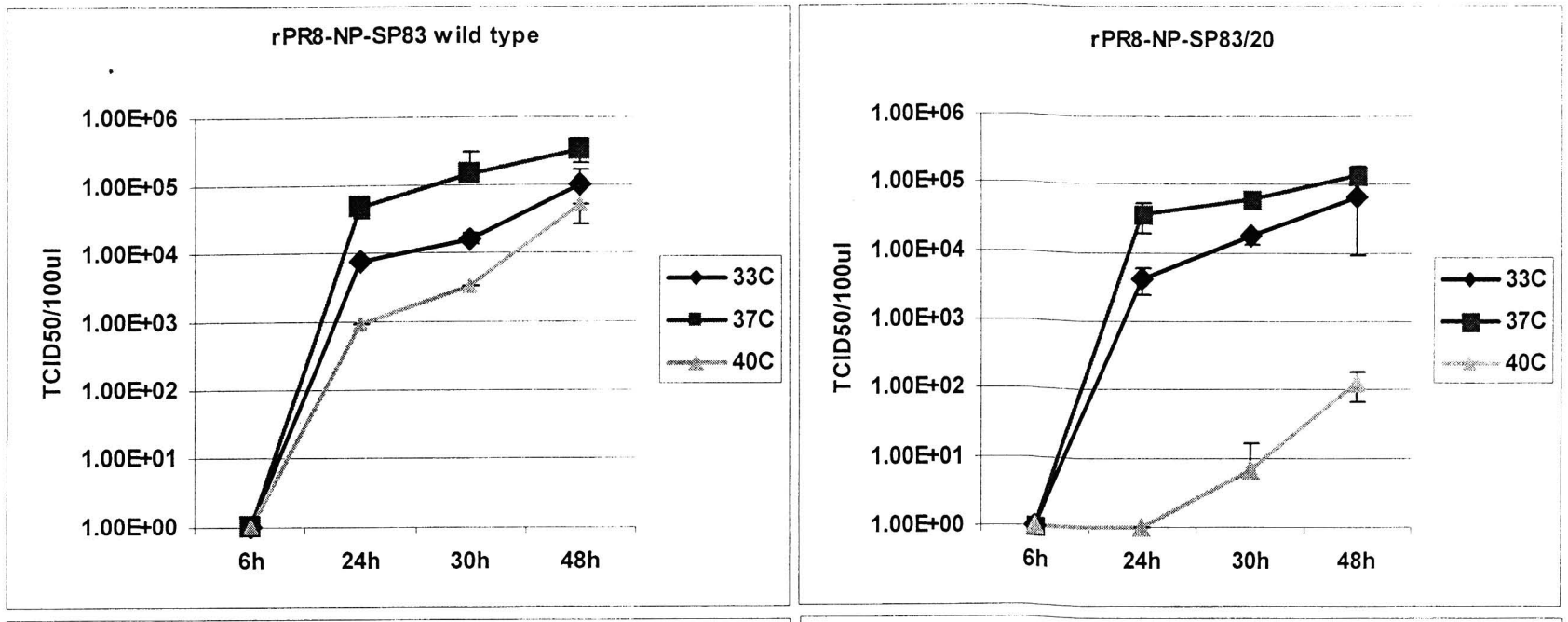
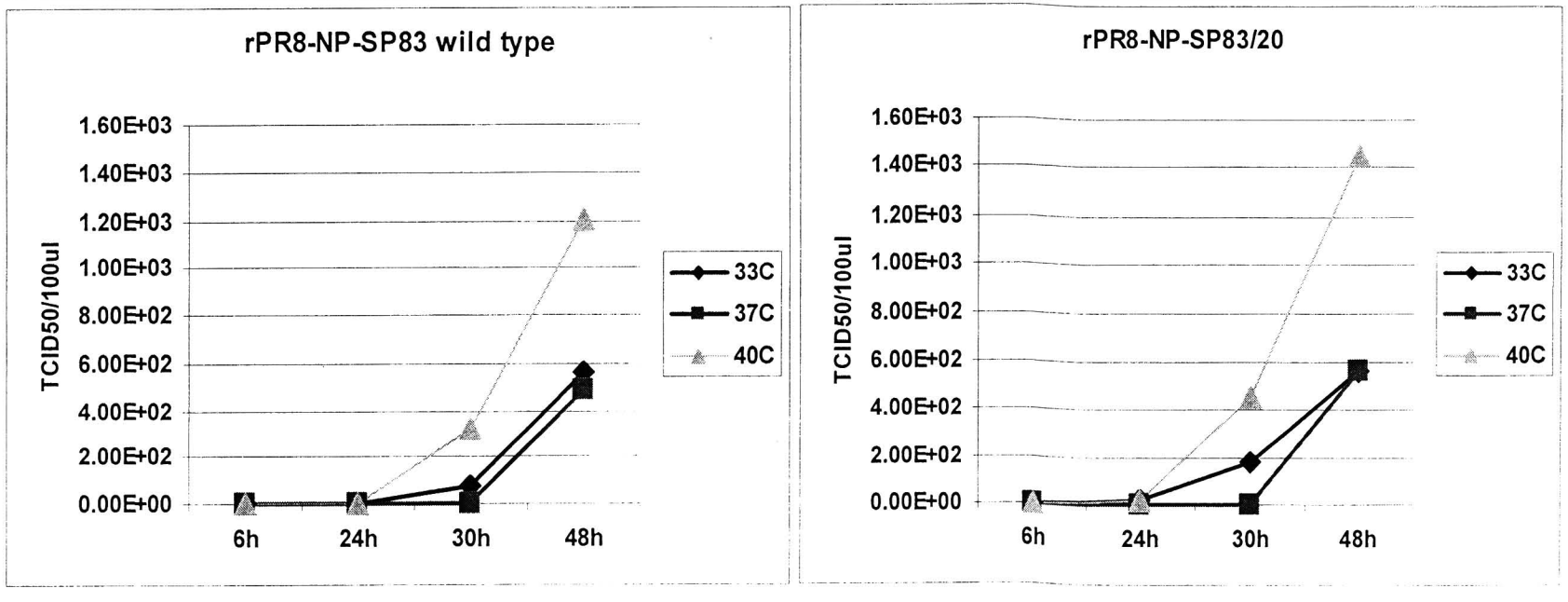

SP83/20
%survival
120
100
80
60
40
20
0
1
3
5
7
9
11
13
15
17
19
day post viral inoculation
10^1
10^2
10^3

Figure 3

a


rPR8-NP-SP83 wild type
TCID50/100ul
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
6h
24h
30h
48h
33C
37C
40C
rPR8-NP-SP83/20
TCID50/100ul
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
6h
24h
30h
48h
33C
37C
40C

b


rPR8-NP-SP83 wild type
TCID50/100ul
1.60E+03
1.40E+03
1.20E+03
1.00E+03
8.00E+02
6.00E+02
4.00E+02
2.00E+02
0.00E+00
6h
24h
30h
48h
33C
37C
40C
rPR8-NP-SP83/20
TCID50/100ul
1.60E+03
1.40E+03
1.20E+03
1.00E+03
8.00E+02
6.00E+02
4.00E+02
2.00E+02
0.00E+00
6h
24h
30h
48h
33C
37C
40C

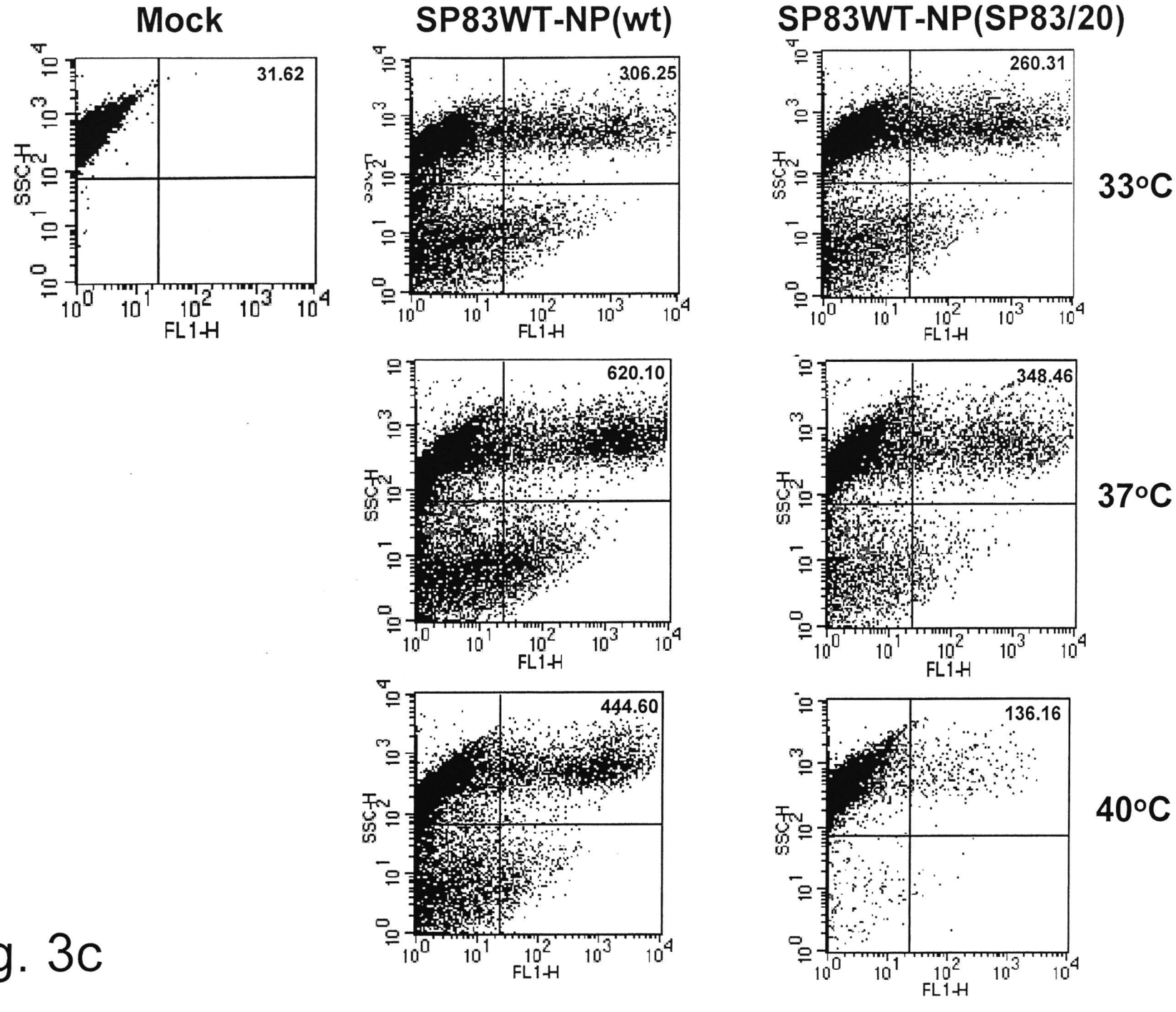

Mock
SP83WT-NP(wt)
SP83WT-NP(SP83/20)
31.62
306.25
260.31
620.10
348.46
444.60
136.16
33°C
37°C
40°C
SSC-H
FL1-H

Fig. 3c

Figure 4