

A serine-to-asparagine mutation at position 314 of H5N1 avian influenza virus NP is a temperature-sensitive mutation that interferes with nuclear localization of NP

Nattamon Siboonnan · Wittawat Wiriyarat · Chompunuch Boonarkart ·
Warunya Chakritbudsabong · Anan Jongkaewwattana · Pilaipan Puthavathana ·
Prasert Auewarakul · Ornpreeya Suptawiwat

Received: 28 August 2012 / Accepted: 21 November 2012 / Published online: 11 January 2013
© Springer-Verlag Wien 2013

Abstract We have generated a temperature-sensitive (ts) mutant from a human isolate of the H5N1 avian influenza virus by classical adaptation in cell culture. After 20 passages at low temperature, the virus showed a ts phenotype. The ts mutant also showed an attenuated phenotype after nasal inoculation in mice. Using reverse genetics, we generated reassortants carrying individual genomic segments of the wild-type and mutant viruses in an A/Puerto Rico/8/34 background, 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 cells.

Introduction

A cold-adapted (ca) phenotype is defined as the ability to replicate efficiently at a low temperature, whereas a 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 lose their replicative capacity at a temperature above 39 °C, have been successfully used as live attenuated nasal vaccines. Two ca/ts influenza vaccine strains, A/Ann Arbor 6/60 (H2N2) and A/Leningrad/134/47/57 (H2N2), are currently available [1, 2]. Genetic determinants of ca and ts phenotypes of these viruses have been well characterized [2, 3].

H5N1 avian influenza virus is a highly virulent virus that is capable of infecting a wide range of avian and mammalian species, including humans. The virus caused an explosive outbreak in Southeast Asia in 2003–2004 and spread rapidly to other geographic regions. Despite its 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 [4]. 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 the 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 the human population [5]. 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 [6]. Lowering the

N. Siboonnan · C. Boonarkart · P. Puthavathana ·
P. Auewarakul · O. Suptawiwat (✉)
Department of Microbiology, Faculty of Medicine Siriraj
Hospital, Mahidol University, 2 Prannok Road, Bangkoknoi,
Bangkok 10700, Thailand
e-mail: ornpreeya@yahoo.com

W. Wiriyarat · W. Chakritbudsabong
Department of Preclinic and Applied Animal Science,
Faculty of Veterinary Science, Mahidol University,
Nakhonpathom 73170, Thailand

A. Jongkaewwattana
National Center of Genetic Engineering and Biotechnology,
Pathumthani 12120, Thailand

temperature setting of H5N1 avian influenza virus by in vitro adaptation may provide a clue on how the virus would evolve in a growth environment with a lower temperature in the 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

The viral isolate and in vitro adaptation

The viral isolate A/Thailand/SP83/2004 (H5N1) was adapted to growth at low temperature by serial passages with gradually decreasing temperature [6]. We used low-passage virus, which had been propagated in MDCK (Madin-Darby canine kidney) cells at 37 °C for eight passages. We initially grew the virus in MDCK cells in minimum essential medium (MEM) containing 1 µg L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin per ml at 30 °C for five passages until the titers at this temperature approached the initial titers at 37 °C. The virus was subsequently propagated at 27 °C, and then at 25 °C for nine and six passages, respectively.

Growth temperature characterization

MDCK (Madin Darby canine kidney) cells were seeded at a density of 5.5×10^5 /well in six-well plates overnight at 37 °C. Cells were then washed two times with serum-free MEM containing 1 µg TPCK-treated trypsin per ml. Viruses were inoculated to the cells at 55 TCID₅₀ (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 a 5% CO₂ incubator, and medium was then added to adjust the volume to 3 ml. The cells were incubated at 33 °C, 37 °C or 40 °C in a 5% CO₂ incubator. At 6, 24, 30, and 48 or 53 hours post-infection, supernatants were collected, and hemagglutination (HA) and TCID₅₀ titers were determined. The growth kinetics assay was performed in triplicate.

For TCID₅₀ titration, 3×10^6 MDCK cells in 96-well plates were inoculated with twofold serially diluted viral supernatant. The inoculated cells were maintained in 1X MEM supplemented with 1 µg TPCK-treated trypsin per ml at 37 °C in a CO₂ incubator. At 24 hours post-infection, the medium was removed, and the cells were washed and fixed in 80% acetone for 60 minutes at 4 °C. Endogenous peroxidase activity was quenched by incubating in 3% H₂O₂ for 30 minutes at room temperature. Viral antigen in the fixed cells was then detected using an NP-specific

monoclonal antibody (Millipore, USA) and a horseradish-peroxidase-conjugated anti-mouse IgG antibody (Southern Biotech Associates Inc., Birmingham, USA). Eight wells of uninfected cell controls were included in each plate, and an OD (optical density) value greater than the mean + 2SD of the cell controls was considered positive. The TCID₅₀ titer was then calculated from the number of positive wells, using Reed and Muench method.

Animal inoculation

Female 6- to 8-week-old BALB/c mice were inoculated intranasally with A/Thailand/SP83/2004 (H5N1) wild-type or the ts virus at 10¹ to 10³ PFU/ml. Six mice were used in each group. All mice were monitored for weight loss and survival for 2 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 described previously [7]. Amplified fragments were cloned into pHw2000 reverse genetics plasmid, sequenced, and used for reconstruction of reassorted reverse genetic viruses as described previously [8, 9]. Briefly, HEK-293 cells cocultured with MDCK cells on a 6-well plate were transfected with 1 µg each of the eight plasmids using Lipofectamine™ 2000 (Invitrogen, USA). After 30 hours, viruses were rescued from the 293T cells by adding 1 ml of Opti-MEM® I Reduced Serum Medium containing 2 µg of TPCK-treated trypsin per ml. The plate was then incubated at 37 °C for 24–48 hours. After the incubation, the presence of rescued virus was determined by observing cytopathic effect (CPE) and testing for HA titer.

Transfection and subcellular localization of viral NP

In order to study subcellular localization of wild-type and mutant NP, HEK-293 cells were transfected with pHw2000 carrying wild-type and mutant NP genes using DMRIE-C Reagent (Invitrogen, USA). Transfected cells were maintained at 33 °C, 37 °C and 40 °C in CO₂ incubators. At 48 hours post-transfection, cells were washed with PBS, fixed with 4% paraformaldehyde at room temperature for 20 minutes, and permeabilized with 0.5% Triton X-100 for 15 minutes. NP protein was then detected using a specific monoclonal antibody (MILLIPORE, USA) and Alexa Fluor® 568-conjugated anti-mouse IgG antibody (Invitrogen, USA). 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

Genes encoding components of the viral polymerase complex, comprising PB2, PB1, PA, and NP of PR8 or three of the four PR8 genes plus individual gene from SP83 or SP83/20 were introduced into HEK-293 cells together with pPolI-GFP by transfection using DMRIE C Reagent (Invitrogen, USA). The transfected cells were incubated at 33 °C, 37 °C or 40 °C for 72 hours. Finally, transfected cells were harvested for GFP (green fluorescence protein) expression analysis by flow cytometry. The plasmid pPolI-GFP is similar to the previously described pPolI-sNA-GFP [10], except that it does not encode the secreted NA. It was designed to produce only negative-stranded GFP RNA from the polI promoter. Because the negative-stranded RNA contains a viral UTR at each end, it can be transcribed by the viral polymerase complex to express the GFP.

Results

The ts mutant

The in vitro-adapted virus, designated A/Thailand/SP83/20/2004 (SP83/20), showed a ts phenotypes (Fig. 1). At 40 °C, it grew to a titer 4-6 log lower than that of the wild-type SP83 virus, whereas at 37 °C, both the 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. 1b). 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 replication efficiency at various temperatures were also reflected in their plaque sizes (Fig. 1c). In intranasal

Fig. 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 determined by plaque assay. Three independent experiments were performed. The viruses showed different plaque sizes at different temperatures in accordance with the growth kinetics (c)

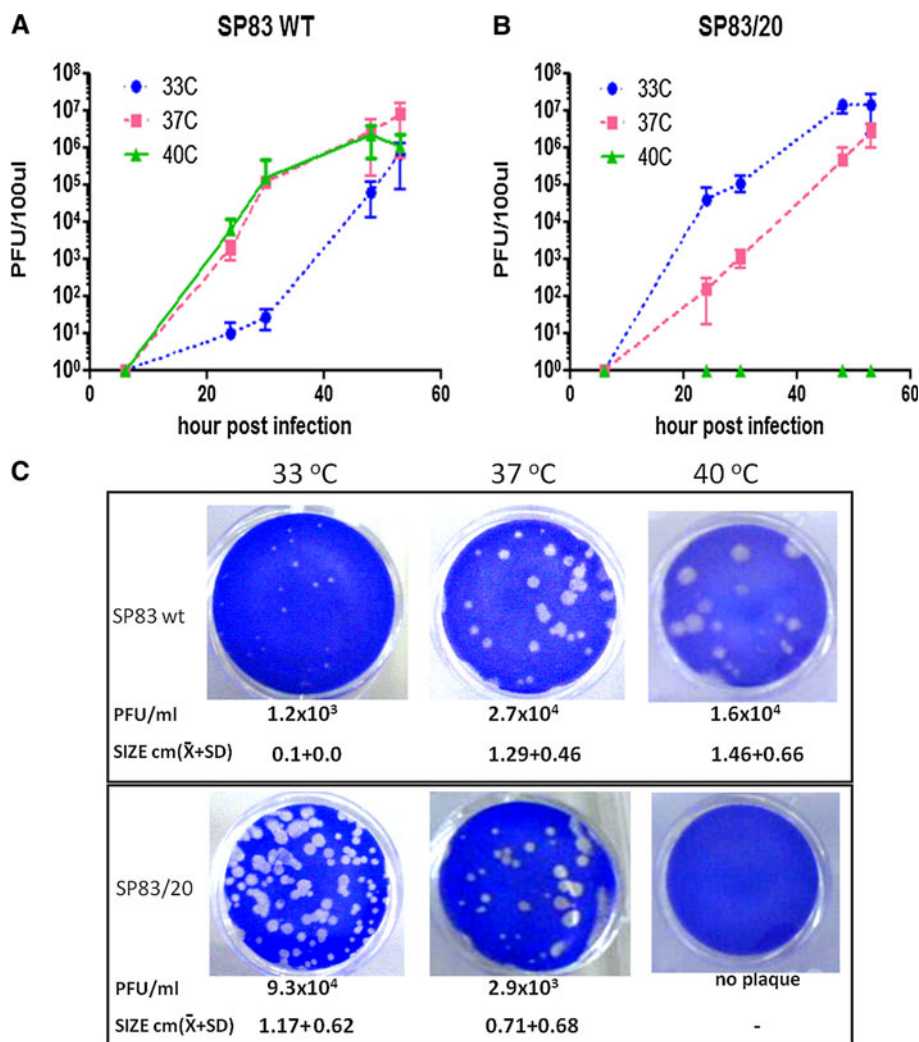
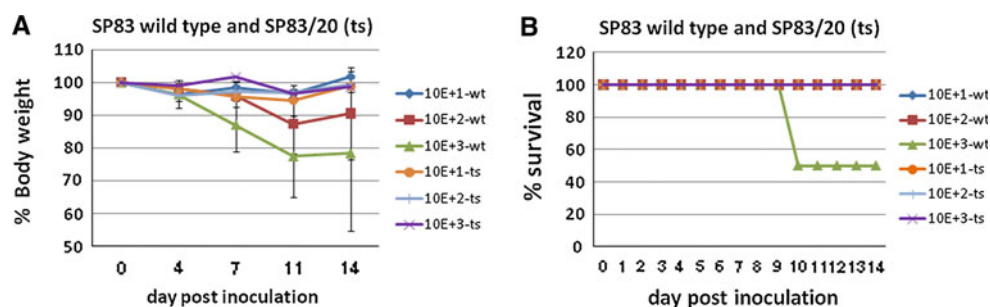


Fig. 2 Weight loss (a) and survival rates (b) of BALB/C mice infected intranasally with wild-type SP83 and SP83/20 at dosages of 10^1 (◆, ●), 10^2 (■, +), and 10^3 (▲, ×) TCID₅₀, suggesting a lower virulence of SP83/20



inoculation experiments in mice, infection with SP83/20 resulted in less weight loss and a higher survival rate than with the wild-type virus (Fig. 2a and b). All of the mice infected with SP83/20 survived, and none of them showed any weight loss, whereas the mice infected with 10^2 and 10^3 TCID₅₀ of the wild type SP83 showed significant weight loss, and 50% of the 10^3 TCID₅₀ group died. This suggested that SP83/20 was less virulent than the wild-type virus.

Genetic determinant of the ts phenotype

Sequences of all genomic segments of SP83 and SP83/20 were compared. Three mutations were found in PB2, and one each in PB1, PA and NP (Table 1). In order to identify genetic determinants of the ts phenotype, we generated reassortants with individual genomic segments of SP83 and SP83/20 in the background of the vaccine strain A/Puerto Rico/8/34 (PR8). We could generate viable viruses for all the genomic segments except for the PB2 of SP83/20. This indicated that the mutations in PB2 of SP83/20 made it 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.

The growth phenotype of these reassortants was characterized by comparing the growth kinetics of reassortants carrying PB1, PA, and NP from SP83/20 and PB2 of SP83 with individual mutations of SP83/20 to that of reassortants carrying wild-type SP83 genes in MDCK cells. 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 shown). None of these viruses exhibited the ts phenotype. In contrast, the reassortant carrying SP83/20 NP (rPR8-NP-SP83/20) replicated poorly only at 40 °C in MDCK cells (Fig. 3a). Moreover, the polymerase complex carrying NP-SP83/20 showed much lower fluorescent intensity and lower numbers of fluorescent cells at 40 °C in the polymerase activity assay, indicating a functional defect at high temperature (Fig. 3b). 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, the subcellular localization of the wild-type and mutant NP protein at 33 °C, 37 °C and 40 °C was determined. After transfection of HEK-293 cells, both the wild-type and mutant NP protein showed predominant nuclear localization at 33 °C and 37 °C. However, at 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

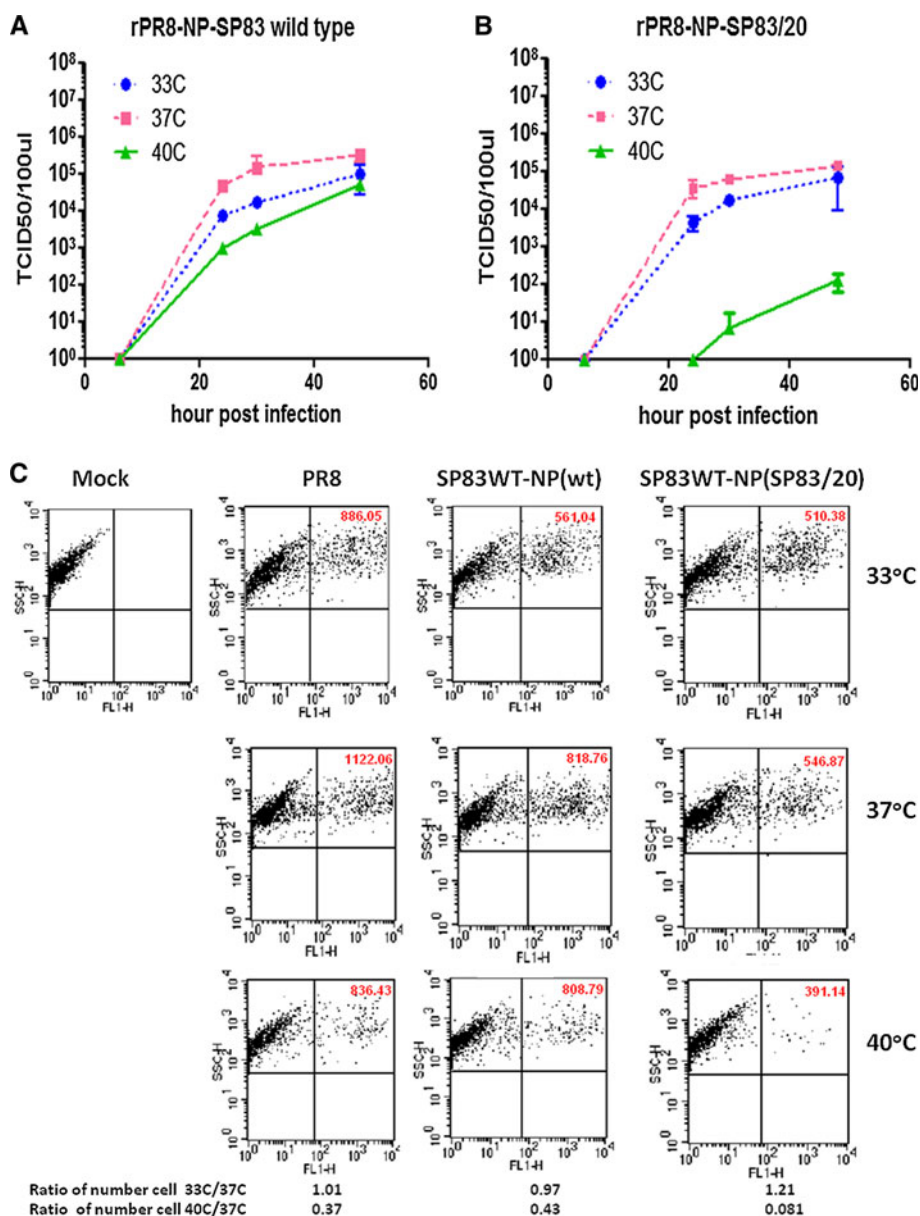
Temperature-sensitive mutants are common tools for studying essential functions of viruses. The ability to replicate at the permissive temperature allows the mutant to be selected, propagated and studied, while the non-permissive temperature allows the defect to be mapped and studied.

Table 1 Mutations in the SP83/20 sequence compared to the wild-type SP83 sequence

Gene (GenBank accession number)	Amino acid position	Strains	
		SP83wt	SP83/20
PB2 (ABO10164)	194	Q (Gln)	H (His)
	195	D (Asp)	Y (Tyr)
	591	Q (Gln)	R (Arg)
PB1 (ABO21686)	554	D (Asp)	N (Asn)
PA (ABP35616)	448	A (Ala)	E (Glu)
NP (ABO21700)	314	S (Ser)	N (Asn)

The GenBank accession numbers of the wild-type sequences are shown

Fig. 3 Kinetic growth curve of rPR8-NP-SP83 virus (left) and rPR8-NP-SP83/20 virus (right) in MDCK cells at 33 °C, 37 °C and 40 °C (a). Viral titers from each time point were determined by TCID₅₀ assay. The data were derived from three independent experiments. The defect at 40 °C of the NP-SP83/20 mutant in viral polymerase complex activity was shown by co-transfecting HEK-293 cells with plasmids expressing the polymerase complexes and pPolII-GFP reporter plasmid and incubating at 33 °C, 37 °C or 40 °C. The level of GFP expression was analyzed by flow cytometry (b). The numbers represent geometric means of fluorescent intensity of cells in the upper right quadrants. The ratios of the numbers of cells in the upper right quadrants at different temperatures are shown at the bottom of the panel

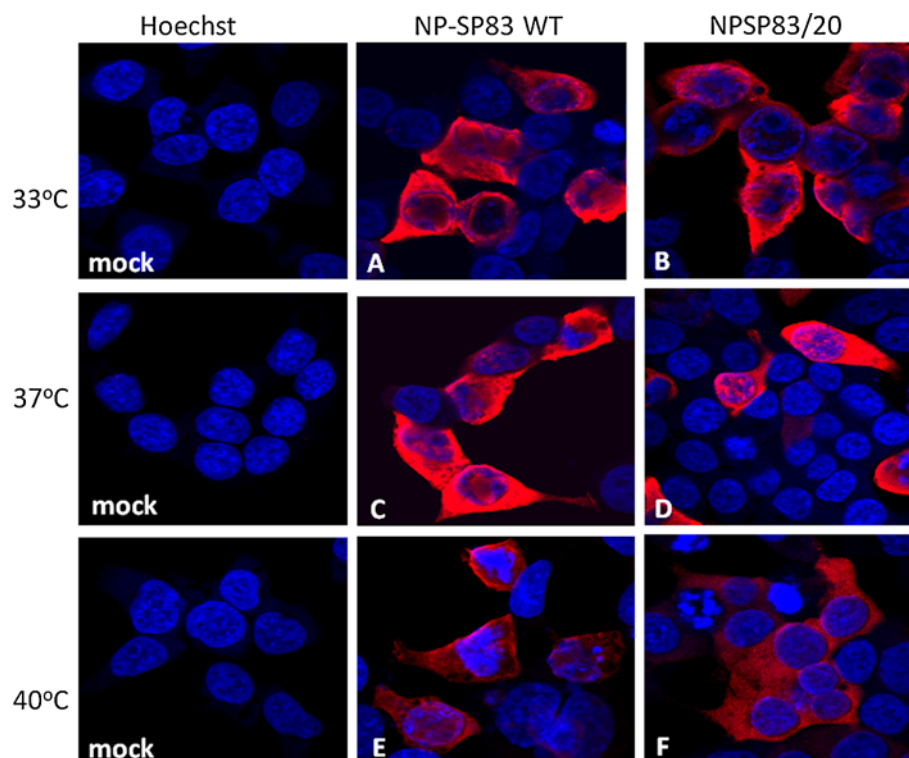


Previously, the S314N mutation was reported to be a ts mutation in A/WSN/33/ts56 virus [11]. In contrast to our data showing the defect in nuclear localization, the S314N mutation in A/WSN/33/ts56 was reported to cause a defect in its interaction with viral ribonucleoprotein (RNP) but not in its nuclear localization. It should be noted that the data showing normal nuclear localization of the NP S314N mutant of A/WSN/33/ts56 in that report was not directly compared to those obtained with the wild-type virus. Furthermore, it was shown previously that an N319K mutation in the NP gene of an H7N7 avian influenza virus contributed to adaptation of the virus to mammalian hosts by enhancing binding to importin alpha 1 and the nuclear accumulation of the NP protein [12]. The fact that the same S314N mutation was found in the two ts strains suggests

that this mutation is a common mechanism for influenza viruses to adapt to low temperature. On the other hand, the adjacent localization of the S314N and N319K mutations and the similar effect on nuclear localization of the NP protein suggest that this region is functionally important for the nuclear transport of the NP protein.

It is not clear whether the other observed mutations in PB2, PB1, and PA affected viral replication in any other way. Interestingly, the PB2 Q591R mutation was previously reported to play a crucial role in adaptation of the virus to mammalian hosts in the 2009 H1N1 pandemic influenza virus and some strains of H5N1 avian influenza virus [13, 14]. The PB2 Q591R in our ts strain might be therefore be a result of viral adaptation to mammalian cell culture but not the lower growth temperature.

Fig. 4 Localization of NP-SP83 wt (A, C, E) and NP-SP83/20 (B, D, F) in 293T cells at 33 °C, 37 °C and 40 °C. 293T cells were transfected with 1 µg of plasmid and analyzed by indirect immunofluorescence assay using an NP-specific monoclonal antibody and Alexa Fluor® 568-conjugated secondary antibody. Cell nuclei were stained with Hoechst dye



The transport of vRNPs across the nuclear membrane by NP is a key event for the influenza virus life cycle. NP has been shown to be sufficient to mediate the nuclear import of vRNAs [15–17]. The NP can interact with karyopherin (importin) α because of its three nuclear localization signals (NLSs). An unconventional NLS (M₁ASQGTKRSYEQM₁₃) is at the very N-terminus. The second NLS (K₁₉₈RGINDRNFWRGFNGRRTR₂₁₆) in the central part of NP appears to be weaker than the upstream NLS [18, 19]. The third NLS has been proposed to be located between amino acids 320 and 400 [20, 21]. The S314N mutation is located near this third NLS.

In summary, we describe an 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 at non-permissive temperature and contributed to the ts phenotype.

Acknowledgements This work was supported by Thailand Research Fund (TRG5180011). S.N. and B.C. were supported by the postgraduate scholarship and research assistant programs, respectively, of the Faculty of Medicine Siriraj Hospital, pHw2000 plasmid was kindly provided by R.G. Webster.

References

- Glezen WP (2004) Cold-adapted, live attenuated influenza vaccine. *Expert Rev Vaccines* 3:131–139
- Isakova-Sivak I, Chen LM, Matsuoka Y, Voeten JT, Kiseleva I, Heldens JG, den Bosch H, Klimov A, Rudenko L, Cox NJ, Donis RO (2011) Genetic bases of the temperature-sensitive phenotype of a master donor virus used in live attenuated influenza vaccines: A/Leningrad/134/17/57 (H2N2). *Virology* 412:297–305
- Jin H, Lu B, Zhou H, Ma C, Zhao J, Yang CF, Kemble G, Greenberg H (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* 306:18–24
- Hatta M, Hatta Y, Kim JH, Watanabe S, Shinya K, Nguyen T, Lien PS, Le QM, Kawaoka Y (2007) Growth of H5N1 influenza A viruses in the upper respiratory tracts of mice. *Plos Pathogens* 3:1374–1379
- Scully MA, Gillim-Ross L, Santos C, Roberts KL, Bordonali E, Subbarao K, Barclay WS, Pickles RJ (2009) Avian Influenza virus glycoproteins restrict virus replication and spread through human airway epithelium at temperatures of the proximal airways. *Plos Pathogens* 5:e1000424
- Maassab HF (1967) Adaptation and growth characteristics of influenza virus at 25 degrees c. *Nature* 213:612–614
- Hoffmann E, Stech J, Guan Y, Webster RG, Perez DR (2001) Universal primer set for the full-length amplification of all influenza A viruses. *Archives of Virology* 146:2275–2289
- Hoffmann E, Krauss S, Perez D, Webby R, Webster RG (2002) Eight-plasmid system for rapid generation of influenza virus vaccines. *Vaccine* 20:3165–3170
- Hoffmann E, Neumann G, Kawaoka Y, Hobom G, Webster RG (2000) A DNA transfection system for generation of influenza A virus from eight plasmids. *Proc Natl Acad Sci USA* 97:6108–6113
- Wanitchang A, Patarasirin P, Jengam J, Jongkaewwattana A (2011) Atypical characteristics of nucleoprotein of pandemic influenza virus H1N1 and their roles in reassortment restriction. *Arch Virol* 156:1031–1040
- Medcalf L, Poole E, Elton D, Digard P (1999) Temperature-sensitive lesions in two influenza A viruses defective for replicative transcription disrupt RNA binding by the nucleoprotein. *J Virol* 73:7349–7356

12. Gabriel G, Herwig A, Klenk HD (2008) Interaction of polymerase subunit PB2 and NP with importin alpha1 is a determinant of host range of influenza A virus. *Plos Pathogens* 4:e11
13. Mehle A, Doudna JA (2009) Adaptive strategies of the influenza virus polymerase for replication in humans. *Proc Natl Acad Sci USA* 106:21312–21316
14. Yamada S, Hatta M, Staker BL, Watanabe S, Imai M, Shinya K, Sakai-Tagawa Y, Ito M, Ozawa M, Watanabe T, Sakabe S, Li C, Kim JH, Myler PJ, Phan I, Raymond A, Smith E, Stacy R, Nidom CA, Lank SM, Wiseman RW, Bimber BN, O'Connor DH, Neumann G, Stewart LJ, Kawaoka Y (2010) Biological and structural characterization of a host-adapting amino acid in influenza virus. *Plos Pathogens* 6:e1001034
15. O'Neill RE, Jaskunas R, Blobel G, Palese P, Moroianu J (1995) Nuclear import of influenza virus RNA can be mediated by viral nucleoprotein and transport factors required for protein import. *J Biol Chem* 270:22701–22704
16. Wu WW, Pante N (2009) The directionality of the nuclear transport of the influenza A genome is driven by selective exposure of nuclear localization sequences on nucleoprotein. *Virology* 393:103–111
17. Wu WW, Sun YH, Pante N (2007) Nuclear import of influenza A viral ribonucleoprotein complexes is mediated by two nuclear localization sequences on viral nucleoprotein. *Virology* 357:103–111
18. Weber F, Kochs G, Gruber S, Haller O (1998) A classical bipartite nuclear localization signal on Thogoto and influenza A virus nucleoproteins. *Virology* 250:9–18
19. Ozawa M, Fujii K, Muramoto Y, Yamada S, Yamayoshi S, Takada A, Goto H, Horimoto T, Kawaoka Y (2007) Contributions of two nuclear localization signals of influenza A virus nucleoprotein to viral replication. *J Virol* 81:30–41
20. Bullido R, Gomez-Puertas P, Albo C, Portela A (2000) Several protein regions contribute to determine the nuclear and cytoplasmic localization of the influenza A virus nucleoprotein. *J Gen Virol* 81:135–142
21. Wang P, Palese P, O'Neill RE (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* 71:1850–1856