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Persistence of the influenza A/WSN/33 virus RNA at midbrain levels of immunodefective mice

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Strains of influenza A virus are known to infect specific subpopulations of neurons in the mouse brain. Here we report that all segments of the genome of the neurotropic influenza A virus, strain WSN/33, can persist in the brains of immunodefective transporter associated with Antigen Processing 1 (TAP1) mutant mice. Ten to 17 months after injection of virus into the olfactory bulbs, viral RNA encoding the nonstructural NS1 protein was detected in sections from the brain at midbrain levels by RT-PCR in almost all animals. Both negative-strand genomic RNA (vRNA) and positive-strand RNA, including mRNA, were found. RNA encoding nucleoprotein and polymerases, which form the replicative complex of the virus, were detected in fewer brains. RNA encoding envelope proteins were found only in occasional brains. No viral cDNA could be identified. This observation shows that certain regions of the brain in immunodefective mice may harbor the genome of influenza A virus including the NS1 gene, the products of which may play a regulatory role in host-cell metabolism. *Journal of NeuroVirology* (2001) **7,** 117–124.

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Introduction

Influenza A virus infections in humans have been associated with disturbances in nervous system functions, but the pathogenetic mechanisms behind such complications are not clear. Some strains of influenza A virus, adapted to undergo replication in the mouse brain, can infect subpopulations of neurons. For example, the mouse-neuroadapted strain, WSN/33, which is derived from the first influenza A virus isolated (for review, see Ward, 1996), can cause a lethal infection and targets neurons in the hippocampus and substantia nigra following intracerebral or systemic injections (Takahashi *et al*, 1995; Yamada *et al*, 1996). To identify target areas for this virus in the brain, we previously injected virus into the olfactory bulb, which resides within a potential natural route of infection for this air-borne virus, and traced its spread in the mouse brain. Such injections produced a nonlethal infection with viral antigens localized to the anterior olfactory nuclei and serotonergic raphe nuclei in the brainstem, which project directly to the olfactory bulbs, as well as to dopaminergic neurons in the ventral tegmentum area (VTA). The virus infected ependymal cells in ventricles and attacked the underlying medial habenular and midline thalamic nuclei as well (Mori *et al*, 1999). Although infectious virus was cleared from the brains within 12 days postinfection (p.i.), viral antigens were still detected in the VTA and dorsal and medial raphe nuclei 35 days p.i. in immunodefective TAP1 −/− mice.

By using TAP1−/− mice, unable to efficiently clear a virus infection through the $CD8^+$ T cell-mediated immune response, we addressed the question whether central nervous system tissue can harbor components of influenza A virus for longer periods of time. This model was also used to seek persisting viral genetic material at levels of the upper brainstem, sites that are targeted by the primary infection. In particular, interest was focused on persistence of the nonstructural NS1 gene, the gene product of which regulates the host-cell macromolecular synthesis and, thus, has a potential to affect neuronal function. We also analyzed in what form viral RNA persists in the brain, i.e., the polarity of the RNA strands, and whether reverse transcripts (i.e., cDNA) of the viral genes are present, as was earlier described

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during persistent lymphocytic choriomeningitis virus infection (Klenerman *et al*, 1997). Our results show that RNA corresponding to all eight viral segments could be detected at levels of the upper brainstem in some, and NS1 RNA in almost all, mice even 17 months after injection of the WSN/33 influenza A virus into the olfactory bulb.

Results

NS1 RNA from influenza A/WSN/33 persists for 17 months in the mouse brain

During an acute infection we have previously identified viral antigens in consecutive sections through the brain at different levels (Mori *et al.* 1999). In order to examine whether the gene for NS1 can persist in the same regions of the brain, we sampled brain material and analyzed these by RT-PCR. NS1 RNA was detected 10–17 months after the infection and found in 10 out of 12 brains examined (Table 1). NS1 RNA was detected in sections containing those neurons that are targeted during an acute infection, and was particularly conspicuous in anterior olfactory nuclei and midbrain levels (Figure 1A, B). Message for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected by RT-PCR and was determined to be within the linear range of amplification after 26 cycles (Figure 1C). NS1 RNA could not be detected in brains from non-infected TAP1 mice (data not shown).

Both negative- and positive-strand RNA are present

When analyzing the polarity of the NS1 RNA (Figure 2A) isolated from brains sampled at 12 and 17 months p.i., both negative- (vRNA) and positive-strand (mRNA and/or cRNA) RNA was detected. Out of the four samples tested, negative-strand RNA was detected in two and positive-strand RNA detected in three samples, respectively (Figure 2B). In order to determine if mRNA was present, oligo (dT) priming of the RT reaction was performed. NS1 sequences could subsequently be amplified by the use of specific primers in two out of five brain samples tested (Figure 2C).

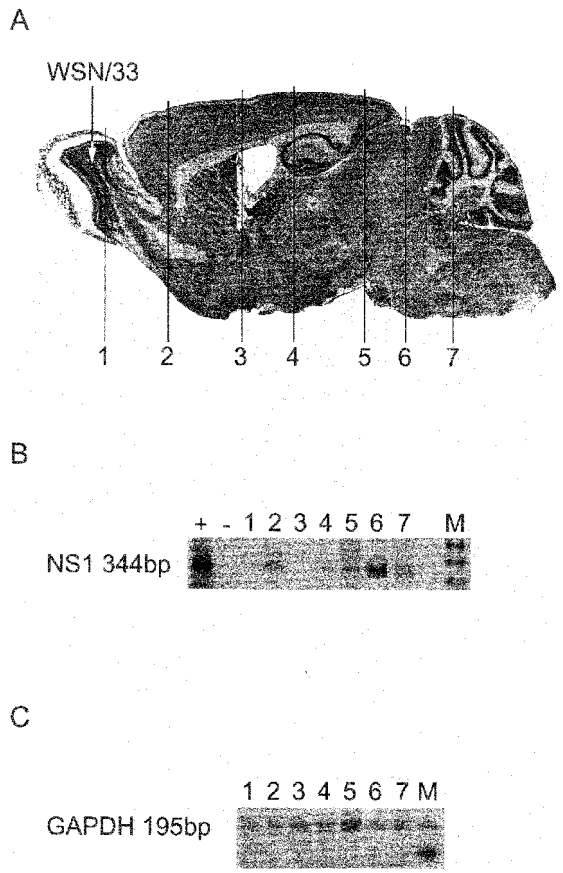

Figure 1 (**A**) A sagittal section through a mouse brain. Numbers (1–7) indicate levels from which coronal sections where sampled for analyses. (**B**) RNA encoding NS1 detected by PCR in the sections, 10 months p.i. Positive (+) and negative (−) control, size marker (M). (**C**) RNA encoding GAPDH detected by PCR in the same sections as in **B**. Size marker (M).

Table 1 Influenza A virus RNA detected in mouse brains at different time points postinfection (p.i.)[a]

Gene	*10 months p.i.*	*12 months p.i.*	*17 months p.i.*	*Total*
NS1[b]	2/3	3/3	5/6	10/12
NP[b]	2/3	2/3	3/6	7/12
M1/2[b]	1/3	1/3	1/6	3/12
NA[c]	2/3	1/3	0/6	3/12
HA[c]	0/3	1/3	1/6	2/12
PA[d]	1/1	1/2	0/4	2/7
PB1[d]	nd	1/3	0/4	1/7
PB2[d]	1/1	0/2	1/4	2/7

NS: Nonstructural protein; NP: Nucleoprotein; M: Matrix protein; NA: Neuraminidase; HA: Hemagglutinin; PA, PB1, PB2 subunits of polymerase. Not determined (nd).
[a]Numbers detected/Numbers tested.
[b]35 cycles of amplification.
[c]Nested/seminested PCR.
[d]45 cycles of amplification.

RNA corresponding to all viral segments is detected 17 months after infection in the mouse brain

When extending the analysis of the same brains 10, 12, and 17 months p.i. to the other viral segments, RNA encoding nucleoprotein (NP) and matrix (M) protein was detected together with RNA encoding the three polymerases (PA, PB1, and PB2) and the two surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) (Table 1). These other viral segments, and in particular those encoding the envelope and polymerase proteins, were detected in fewer brains as compared to NS1 even after using nested/seminested PCR (HA and NA), or increasing the number of amplifications cycles to 45 (PA, PB1, and PB2). This might reflect differences in the number of template copies or a difference in sensitivity between the different primers used. None of these

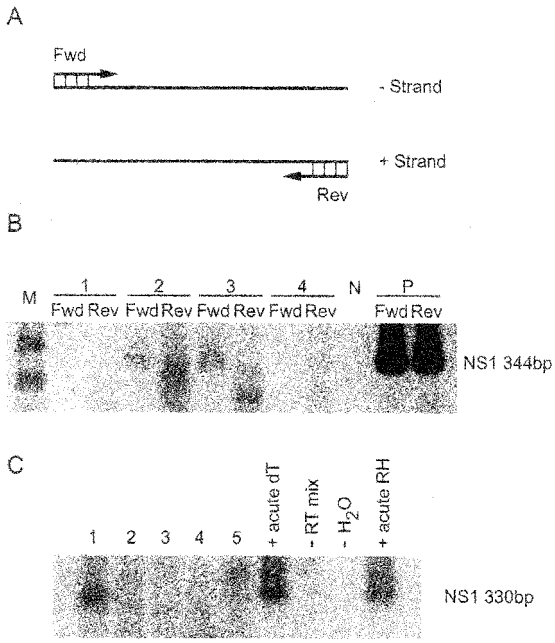

Figure 2 (**A**) Strand specific reverse transcription. Forward primer NS-141 (Fwd) anneals to negative-strand RNA and reverse primer NS-484 (Rev) anneals to positive-strand RNA. (**B**) PCR reaction using primers NS-141 and NS-484 on strand specific reverse transcribed RNA from sections of four mouse brains (1–4). Size marker (M), RNA extracted from a mouse brain 7 days p.i. (P) and negative control (N). (**C**) Amplification of a fragment of the NS1 gene, using primers NS505 and NS835, from oligo (dT), primed RNA extracted from five mouse brains 17 months p.i. (lanes 1–5), oligo (dT) primed RNA from a mouse brain 7 days p.i (+ acute dT), RT mix with no RNA added (− RT mix), water as template in the PCR reaction (−H_2O) and amplification of random primed cDNA from mouse brain 7 days p.i. (+ acute RH).

Table 2 Sequencing of PCR products

Gene	*Mouse brain*[a]	*Clone number*	*Sequence 5′ to 3′*[b]	*Base substitution*
NS1	A-12	FA23	49–685	279C→T
				522C→A
				582A→G
				657A→T
		FA31	49–460	222G→A
				279C→T
				385A→G
	B-17	FA24	49–685	279C→T
				357G→A
		FA32	49–460	279C→T
	C-17	FA25	49–460	279C→T
		FA33	380–685	None
NP	B-17	F10	86–626	146G→A
				359T→G
M1/2	D-10	F11	619–878	846G→C
HA	E-12	F12	1098–1459	None
NA	F-12	F36b	258–489	None
		F36c	258–489	None
PA	G-10	F27	1355–1595	None
PB1	F-12	FA35a	1598–1877	1648G→A
				1694A→G
		FA35c	1598–1877	1605T→C
				1738A→G
PB2	G-10	FA29	1143–1283	None
	C-17	FA30	1143–1283	None
	Virus inoculum			
NS1		FA26	49–460	119A→G
				260A→G
				279C→T
				303A→G
				379G→A
		FA34	380–685	None

[a]Letters indicate individual animals. Numbers refers to months p.i.
[b]Position according to the numbering in GenBank reference sequence. For the respective GenBank accession numbers see Table 3.

viral segments could be detected in brains from non-infected TAP1 mice.

Influenza A is not reverse transcribed into DNA
In sections adjacent to those containing NS1 RNA we tried to amplify NS1-, NP-, M-, and HA-specific sequences by PCR on isolated DNA. We could not detect reverse transcripts in any of these sections, indicating that transcription of viral cDNA does not explain the persistence of the viral RNA.

Sequencing of PCR products
To verify the identity of the different PCR products, cloning and sequencing was performed. The NS1 gene was sequenced from the virus inoculum and brain material sampled 12 and 17 months p.i. (Table 2). Sequence variations exist between the different NS1 clones, obtained within one animal as well as between animals. Sequence variations were also detected in the obtained sequences encoding NP, M and PB1 as compared to the respective reference sequences deposited in GenBank. As no NP sequence from the influenza A/WSN/33 strain was available in GenBank, the obtained NP sequence was deposited and given the accession number AF306656. Sequences of HA, NA, and PB2 were identical to those in GenBank.

Viral proteins could not be detected
No NS1 immunoreactivity could be detected in any of the NS1 positive brains (10, 12, or 17 months p.i.) by either Western blot or immunohistochemistry. Immunostaining of the brains using polyclonal anti-WSN/33 antiserum was also negative in brain sections parallel to sections positive for viral RNA. As positive control for the NS1 and polyclonal anti-WSN/33 antiserum immunoreactivity brains sampled at 7 days p.i. were used.

Neuronal destruction
In order to determine the extent of neuronal destruction, sections were examined histologically. In the olfactory bulbs, an almost total loss of the cells in the central part of the granular layer, with remnants of calcified necrosis was consistently observed in all mice. All mice also showed loss of ependymal

cells particularly in the anterior part of the lateral ventricles on the injected side. The medial habenular and midline thalamus displayed, in some cases, an almost total loss of neurons. Calcified necrotic areas occurred in the midbrain of an occasional mouse. These alterations were similar to what we have observed in brains examined 35 days p.i. (Mori *et al*, 1999). In this set of brain sections, a few $CD4^+$ T cells were present, mainly scattered in the brainstem of the infected mouse.

Discussion

The present study shows that RNA encoding all influenza A viral proteins can persist at midbrain levels following injection of the virus into the olfactory bulbs of immunodefective TAP1−/− mice. Since viral cDNA could not be detected, the ability of this RNA virus to persist in the brain probably involves some form of continuous replication and restricted expression of its components (for review, see Kristensson and Norrby, 1986). The limited tissue destruction in the brains did not overtly increase over time, which indicates that the cytolytic effects of the virus infection have been restricted. As for other cytolytic RNA virus infections that may persist in the brain, reduced cytolytic activity may occur by (a) evolution of nonlytic viral mutants, (b) infection of cells not vulnerable to the lytic viral components, or (c) restricted replication of virus and/or expression of its components in the brain (for review, see Ahmed *et al*, 1996).

The mechanisms, by which influenza A virus exerts its cytolytic effects, still remain to be clarified in detail. However, the viral neuraminidase and NS1 proteins appear to be involved (Ludwig *et al*, 1999). The NS1 protein, for which the RNA was detected in 10 out of 12 brains in the present study, is not part of the viral particles. It is encoded by the colinear mRNA transcribed from the 8th segment of the viral genomic RNA and regulates the host cell metabolism to favor virus replication by mediating retention of cellular mRNA in the nucleus and inhibition of pre-mRNA splicing (Chen *et al*, 1999; Nemeroff *et al*, 1998). By switching off the host cell protein synthesis, it can cause the death of an infected cell (Ludwig *et al*, 1999). Weakly cytolytic mutant influenza A/WSN can, however, persist without producing any detectable progeny virus in MDCK cells and with relatively constant levels of expression of the NS gene (Urabe *et al*, 1994). Furthermore, mutations of the NS1 gene have been observed during persistence of both influenza virus A and C in cell cultures (Lucas *et al*, 1988; Marschall *et al*, 1999). When comparing the obtained sequences of the NS1 gene isolated from mouse brains sampled 12 and 17 months p.i. with the GenBank sequence (M12597), we found minor nucleotide substitutions. These variations occurred not only between the different animals but also within individual animals. Since the virus was not plaque purified, these variations may reflect heterogeneity of the virus stock used for injection, although RT-PCR artifacts can not be excluded. It is, at this point, not known whether these variations are associated with altered cytopathogenicity of the virus.

Although the infiltration of $CD4^+$ T cells presently observed may indicate a continuing low grade expression of specific MHC-viral peptide complexes, activated T lymphocytes may also persist long after viral proteins and nucleic acids have become undetectable in the brain (Hawke *et al*, 1998). However, since in these immunodefective mice we found not only genomic RNA but also mRNA encoding the NS1 protein, it is possible that translation of NS1 message occurs, but below the level of detection and in nontoxic concentrations. Consistent with observations on other persistent RNA virus infections in the brain, e.g., measles virus in which envelope components (matrix, fusion, and hemagglutinin) are downregulated while the nucleoproteins are expressed at higher levels (for review, see Schneider-Schaulies *et al*, 1999), a number of NS1 RNA containing sections also contained RNA encoding both NP and the polymerases. The products of these genes constitute the minimal subset of viral proteins needed for replication of all viral genes (Huang *et al*, 1990), which would provide a mechanism for maintaining the observed components of the virus in the brains.

In humans, influenza A virus may occasionally invade the host from the primary site of replication in the epithelium of the respiratory tract. Symptoms of acute encephalitis or encephalopathy have been described (for review, see Murphy and Webster, 1996), and influenza A virus antigens have been detected in ependymal cells at autopsy (Frankova *et al*, 1977). Influenza A mRNA was found recently in the CSF from children with influenza menigoencephalitis or encephalopathy by RT-PCR (Fujimoto *et al*, 1998). A persistent infection with influenza A virus has been observed in an immunodeficient child. Genetic diversity of the virus was found in nasal secretion sampled over a 10-month period. This diversity probably reflected heterogeneity of the viral RNA population, since no progressive accumulation of mutations was observed (Rocha *et al*, 1991). Although much disputed, certain epidemiological data have associated the 1918/19 influenza epidemic with *Encephalitis lethargica* that was followed by postencephalitic parkinsonism characterized by neurofibrillary degeneration of neurons in the substantia nigra (e.g., Ravenholt and Foege, 1982). Our present finding of persistent influenza A virus RNA in the central nervous system at levels of the midbrain should prompt future studies. Thus, it will be important to analyze whether the corresponding gene products, in particular those encoded by the most frequently detected NP and NS1 genes, affect neuronal function and development.

Materials and methods

Virus and virus titration
Influenza virus A/WSN/33 was obtained from Dr. S Nakajima (The Institute of Public Health, Tokyo, Japan), and propagated on Madin-Darby canine kidney (MDCK) cell monolayers in MEM (Life Technologies, Paisley, Scotland) supplemented with 2 mM L-glutamine, 0.01 M HEPES buffer and 0.2% BSA (all obtained from Sigma, St. Louis, MO, USA) and penicillin G (50 IU/ml)/streptomycin (50 μg/ml) (Life Technologies). The virus titer was 5×10^7 PFU/ml, as determined by plaque assay on MDCK cells in the absence of trypsin (Tobita *et al*, 1975).

Mice, virus inoculation, and brain sampling
Mice with targeted disruption of the TAP1 gene were derived from a breeding pair obtained from Dr L Van Kaer (Vanderbilt University, Nashville, TN, USA). Four-week-old TAP1−/− mice, an equal number of both sexes, were used for infection. Following anesthetization by the intraperitoneal administration of 7.2% chloral hydrate, they were stereotactically injected into the right main olfactory bulb with 1 μl (5×10^4 PFU) of the virus suspension as described previously in detail (Mori *et al*, 1999). Groups of mice, 3–6 in each, were sampled 7 days and 10, 12, and 17 months p.i. A noninfected animal was included as a negative control. Under deep anesthesia the mice were sacrificed, the brains dissected out and snap frozen in isopentane on dry ice. Cryostat sections, 14-μm thick, were cut at 7 levels through the brain: olfactory bulbs (4.5–4.0 mm anterior to bregma), anterior olfactory nuclei (3.0–2.5 mm anterior to bregma), striatum (0.5–0 mm anterior to bregma), thalamus (1–1.5 mm posterior to bregma), upper brainstem (3–3.5 mm and 4–4.5 mm posterior to bregma), and middle brainstem (5.0–5.5 mm posterior to bregma) (Franklin and Paxinos, 1997). Sections were taken for PCR analyses and adjacent sections for histological and immunohistochemical examinations.

RNA extraction
Total RNA was extracted from cryostat sections of influenza infected mouse brains by the acid phenol method (Chomczynski and Sacchi, 1987) or by the use of the RNeasy Kit (Qiagen, GmbH, Germany). Viral RNA from MDCK culture supernatants was isolated by the QIAmp viral RNA Mini Kit (Qiagen). Total RNA was quantified by spectrophotometry (Ultrospec Plus, Pharmacia LKB Biotechnology). RNA was treated with 1 U of amplification grade DNase I (Life Technologies, Paisley, UK) for 15 min at room temperature, inactivated by the addition of 2.5 mM EDTA, and incubation for 10 min at 65°C according to manufacturer's instructions.

Reverse Transcription
DNase-treated RNA (200–500 ng) was reverse transcribed in a 20 μl reaction solution containing the following reagents from Life Technologies: 500 ng of oligo (dT) primer, 250 ng of random primers or 2 pmoles of gene specific primers, 1× RT buffer, 10 mM DTT, and 0.5 mM each of dNTPs. The reaction was heated to 72°C for 2 min and chilled on ice before the addition of 200 U of MoMLV reverse transcriptase (Superscript II). cDNA synthesis was allowed to proceed for 1 h at 37°C. The reaction was then inactivated by heating to 70°C for 15 min.

PCR
One μl of random primed cDNA was amplified in a 25 μl PCR reaction with the following reagents, 100 ng of each primer, 0.5 U Platinum *Taq* (Life Technologies), 1 × PCR buffer, 4 mM $MgCl_2$, and 0.4 mM each of dGTP, dATP, dTTP and dCTP (Life Technologies). A GeneAmp PCR system 2400 or 9700 (P-E Biosystems, Foster City, CA, USA) with the following cycling conditions was used: denaturation at 94°C for 20 s, annealing at 58–60°C for 30 s, and extension at 72°C for 50 s for 35 cycles with NS, NP, M, HA, and NA primers. For amplification of the polymeras genes 45 cycles were used. Detection of GAPDH within the linear range of amplification was done using 18, 22, 26, and 30 cycles. For detecting HA or NA a second round of amplification was performed using nested/semi-nested primers. One μl of the first round amplification reaction was added to a 25 μl PCR reaction, with conditions identical to those described previously. The primer sequences are given in Table 3. PCR products were electrophoresed in a 2% agarose gel and visualized using SYBR Gold Stain (Molecular Probes, Eugene, OR, USA) on a UV-transilluminator (GelDoc 2000, Bio-Rad, Hercules, CA, USA).

Detection of NS1 mRNA
One μl of oligo (dT) primed cDNA was amplified in a 25-μl PCR reaction, with the following reagents: 1 μM of each primer, 0.2 mM dNTP's, 1 × Advantage 2 PCR buffer, and 1 × Advantage 2 polymerase mix (Clontech, Palo Alto, CA, USA), using 1 μM each of the NS505 and NS835 primers with the following cycling conditions: 2 min at 94°C, 94°C for 15 s, annealing and extension at 68°C for 90 s for 45 cycles with a final extension at 68°C for 10 min. PCR products were electrophoresed and visualized as described before.

DNA extraction
DNA was extracted from brain regions adjacent to those containing RNA encoding NS1 from one animal 12 months p.i., and five animals 17 months p.i., giving a total number of 30 samples. A noninfected animal was included as a negative control. The EasyDNA Kit (Invitrogen, Carlsbad, CA, USA), including RNase treatment (40 μg/ml for 30 min at 37°C) was used according to the manufacturer's instructions. Ethanol precipitated DNA was reconstituted in 10 mM Tris-Cl, 1 mM EDTA buffer and stored at 4°C.

Table 3 PCR primer sequences

Gene	*5′-position*	*Polarity*	*Sequence 5′ to 3′*	*GenBank*[a]
NS1/2	28	Sense	TGGATCCAAACACTGTGTCAA	M12597
	141[b]	Sense	GATCAGAAGTCCCTAAGAGGAAGAG	
	360	Sense	GCAGGCCCTCTTTGTATCAG	
	505	Sense	TTTCACCACTGCCCTCTCTT	
	484[b]	Anti-sense	TCTTCGGTGAAGGCCCTTAGTAAT	
	705	Anti-sense	TAATTGTTCCCGCCATTTTC	
	835	Anti-sense	TCTCTTGCTCCACTTCAAGC	
	862	Anti-sense	AAATAAGCTGAAACGAGAAAGTTCTTA	
NP	54	Sense	TCAAGGCACCAAACGATCTTAGGAACAGAT	M30746
	656	Anti-sense	CGATCATTGATCCCACGTTTGATCATTCTG	
M1/2	599	Sense	CAGAGGCCATGGATATTGCT	X08088
	898	Anti-sense	CTCTGGCACTCCTTCCGTAG	
HA	964	Sense	TACACCCAGTCACAATAGGAGAGTG	J02176
	1078[c]	Sense	TTGCTGGTTTTATTGAGGGG	
	1479[c]	Anti-sense	ACTCAAAACACCCATTTCCG	
	1509	Anti-sense	CCATGCATTCATTGTCACACTTGTGG	
NA	238	Sense	ATCTCTTTGTCCCATCCGTG	L25817
	366[c]	Anti-sense	AAGGTCCTGCATTCCAAGTG	
	509	Anti-sense	ACCAAGCAACCGATTCAAAC	
PA	1335	Sense	CATTGCAAGCATGAGAAGGA	X17336
	1615	Anti-sense	TTGGGTCAGTGAGGGAAAAC	
PB1	1578	Sense	CGAGTCTGCGGACATGAGTA	J02178
	1897	Anti-sense	ATAAACGCCCCTGGTAATCC	
PB2	1123	Sense	GTTGGGAGAAGAGCAACAGC	J02179
	1303	Anti-sense	GATTCGCCCTATTGACGAAA	
GAPDH	351	Sense	CCATGGAGAAGGCCGGGG	M32599
	545	Anti-sense	CAAAGTTGTCATGGATGACC	

[a]GenBank accession numbers of sequences used for primer design and alignments. (NP sequence from influenza A/WS/33 strain.) GAPDH primer sequences from reference Der *et al* (1997).
[b]Primers used separately for strand specific reverse transcrition.
[c]Primers used for nested/seminested PCR.

DNA PCR

10–50 ng of DNA was amplified by using the following primer pairs; NS141 and NS484, NP54 and NP656, M624 and M923, or HA964 and HA1456 in a 25 μl reaction mixture, as described before. The PCR was performed in the GeneAmp PCR system 2400 using the following cycling conditions: heat activation of the polymerase for 2 min at 94°C, followed by 40 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s with a final extension at 72°C for 10 min. PCR products were electrophoresed and visualized as described previously.

Cloning and sequencing

PCR products were directly ligated into a plasmid vector (pCR 2.1-TOPO, Invitrogen). Recombinant plasmids found to contain an insert by PCR using specific primers were sequenced using T7 and M13 reverse sequencing primers by the fluorescent dideoxy terminator method of cycle sequencing on a Perkin-Elmer, Applied Biosystems Division (Perkin Elmer) 377 automated DNA sequencer (Core facilities at the Karolinska Institutet). The resulting sequences were compared to each other using the PILEUP program (Wisconsin Package Version 9.0, Genetics Computer Group, Madison, WI, USA) and to previously reported sequences using BLASTN and BLASTX algorithms.

Western blotting

Five 14-μm sections from influenza A/WSN/33 infected brain were treated with 300 μl RIPA buffer (1 × PBS, 1% igepal CA-630 [Sigma], 0.5% sodium deoxycholate [Sigma], 0.1% lauryl sulfate [Sigma]) containing 30 μg PMSF at 4°C for 30 min. Whole cell lysate was immunoprecipitated with Protein A/G PLUS-Agarose (Santa Cruz Biotechnology, Santa Cruz, CA, USA) according to manufacturer's instructions together with primary antibody, anti-NS1 rabbit antiserum (generous gift from Dr A Nieto, Centro Nacional de Biotecnologia, Madrid, Spain), 1:400, at 4°C overnight. The immunopreciptate was size fractionated on a 12% Tris-Glycine gel (Novex, San Diego, CA, USA) and the electrophoresed proteins were transferred to an Immobilon-P^{SQ} PVDF membrane (Millipore Co, Bedford, MA, USA) according to manufacturer's instructions. After electrotransfer, the PVDF membrane was blocked with 5% fat-free milk (Biorad) in PBS containing 0.05% Tween-20 (Sigma Chemicals) for 2 h at room temperature. The primary antibody, anti-NS1 rabbit antiserum, was diluted 1:400 in blocking solution and the membrane was incubated overnight at 4°C. The membrane was washed with PBS-Tween-20 three times at 37°C. Following incubation with swine anti-rabbit coupled to HRP antibody (DAKO, Glostrup, Denmark), diluted 1:2000 in blocking solution, at room temperature for 2 h and washing with

PBS-Tween-20, immunopositive bands were visualized using a chemiluminescent ECL kit (Amersham Pharmacia Biotech, Buckinghamshire, England) and Hyperfilm ECL (Amersham Pharmacia Biotech).

Immunohistochemistry
For immunostaining of influenza A antigens the following antisera were used: the polyclonal rabbit anti-NS1 antiserum (1:400) and a rabbit polyclonal anti-WSN/33 antiserum (1:1,000; a generous gift from Dr S Nakajima). $CD4^+$ T cells were immunostained with a rat monoclonal anti-CD4 antibody (L3T4; PharMigen, San Diego, CA, USA), 1:200. The antisera were diluted in 2% normal swine serum and the monoclonal antibodies in 2% normal rat serum (both from DAKO) in PBS with 0.3% Triton X-100. The sections were incubated for 12 h at 4°C. As secondary antibodies, biotinylated swine anti-rabbit Ig's and rabbit anti-rat Ig's (DAKO) were used, respectively. They were diluted 1:400 in 2% normal swine or rat serum in PBS, 0.3% Triton X-100 and the sections were incubated for 1 h at room temperature. Immunopositivity was visualized after incubation with avidin-biotinylated horseradish peroxidase (DAKO) for 30 min followed by 0.02% hydrogen peroxide and 0.02% 3-amino-9- ethylcarbazole (Sigma) in sodium-acetate buffer, pH 5.3, for 4 min. After thorough washing, the sections were mounted in glycerol-gelatin. An adjacent series of sections was stained with cresyl violet-acetic acid or hematoxylin-eosin for histological examination and cytoarchitectural verification.

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References

Ahmed R, Morrison LA, Knipe DM (1996). Persistence of Viruses. In: *Fields virology*. Fields BN, Knipe DM, Chanock RM, Melnick JL, Roizman B, Shope RE (eds). Philadelphia: Lippincott-Raven Publishers, pp 219–249.

Chen Z, Li Y, Krug RM (1999). Influenza A virus NS1 protein targets poly(A)-binding protein II of the cellular 3′-end processing machinery. *EMBO J* **18:** 2273–2283.

Chomczynski P, Sacchi N (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162:** 156–159.

Franklin K, Paxinos G (1997). *The mouse atlas in stereotaxic coordinates*. San Diego: Academic Press.

Frankova V, Jirasek A, Tumova B (1977). Type A influenza: postmortem virus isolations from different organs in human lethal cases. *Arch Virol* **53:** 265–268.

Fujimoto S, Kobayashi M, Uemura O, Iwasa M, Ando T, Katoh T, Nakamura C, Maki N, Togari H, Wada Y (1998). PCR on cerebrospinal fluid to show influenza-associated acute encephalopathy or encephalitis. *Lancet* **352:** 873–875.

Hawke S, Stevenson PG, Freeman S, Bangham CR (1998). Long-term persistence of activated cytotoxic T lymphocytes after viral infection of the central nervous system. *J Exp Med* **187:** 1575–1582.

Huang TS, Palese P, Krystal M (1990). Determination of influenza virus proteins required for genome replication. *J Virol* **64:** 5669–5673.

Klenerman P, Hengartner H, Zinkernagel RM (1997). A non-retroviral RNA virus persists in DNA form. *Nature* **390:** 298–301.

Kristensson K, Norrby E (1986). Persistence of RNA viruses in the central nervous system. *Annu Rev Microbiol* **40:** 159–184.

Lucas WT, Whitaker-Dowling P, Kaifer CR, Youngner JS (1988). Characterization of a unique protein produced by influenza A virus recovered from a long-term persistent infection. *Virology* **166:** 620–623.

Ludwig S, Pleschka S, Wolff T (1999). A fatal relationship—influenza virus interactions with the host cell. *Viral Immunol* **12:** 175–196.

Marschall M, Helten A, Hechtfischer A, Zach A, Banaschewski C, Hell W, Meier-Ewert H (1999). The ORF, regulated synthesis, and persistence-specific variation of influenza C viral NS1 protein. *Virology* **253:** 208–218.

Mori I, Diehl AD, Chauhan A, Ljunggren HG, Kristensson K (1999). Selective targeting of habenular, thalamic midline and monoaminergic brainstem neurons by neurotropic influenza A virus in mice. *J Neurovirol* **5:** 355–362.

Murphy B, Webster R (1996). Orthomyxoviruses. In: *Fields virology*. Fields BN, Knipe DM, Chanock RM, Melnick JL, Roizman B, Shope RE (eds). Philadelphia: Lippincott-Raven Publishers, pp 1397–1445.

Nemeroff ME, Barabino SM, Li Y, Keller W, Krug RM (1998). Influenza virus NS1 protein interacts with the cellular 30 kDa subunit of CPSF and inhibits 3' end formation of cellular pre-mRNAs. *Mol Cell* **1:** 991–1000.

Ravenholt RT, Foege WH (1982). 1918 influenza, encephalitis lethargica, parkinsonism. *Lancet* **2:** 860–864.

Rocha E, Cox NJ, Black RA, Harmon MW, Harrison CJ, Kendal AP (1991). Antigenic and genetic variation in influenza A (H1N1) virus isolates recovered from a persistently infected immunodeficient child. *J Virol* **65:** 2340–2350.

Schneider-Schaulies J, Niewiesk S, Schneider-Schaulies S, ter Meulen V (1999). Measles virus in the CNS: the role of viral and host factors for the establishment and maintenance of a persistent infection. *J NeuroVirol* **5:** 613–622.

Takahashi M, Yamada T, Nakajima S, Nakajima K, Yamamoto T, Okada H (1995). The substantia nigra is a major target for neurovirulent influenza A virus. *J Exp Med* **181:** 2161–2169.

Tobita K, Sugiura A, Enomote C, Furuyama M (1975). Plaque assay and primary isolation of influenza A viruses in an established line of canine kidney cells (MDCK) in the presence of trypsin. *Med Microbiol Immunol (Berl)* **162:** 9–14.

Urabe M, Tanaka T, Tobita K (1994). Use of competitive PCR to estimate the level of NS gene persisting in MDCK cells which survived productive replication of a mutant of influenza virus A/WSN. *J Virol Methods* **49:** 361–366.

Ward AC (1996). Neurovirulence of influenza A virus. *J Neurovirol* **2:** 139–151.

Yamada T, Yamanaka I, Takahashi M, Nakajima S (1996). Invasion of brain by neurovirulent influenza A virus after intranasal inoculation. *Parkinsonism and Related Disorders* **2:** 187–193.