VIROLOGY 212, 555–561 (1995)

European Swine Virus as a Possible Source for the Next Influenza Pandemic?

S. LUDWIG,*,[1] L. STITZ,* O. PLANZ,* H. VAN,† W. M. FITCH,† and C. SCHOLTISSEK*,[2]

*Institut für Virologie, Justus-Liebig-Universität Giessen, D-35392 Giessen, Germany; and †Department of Ecology and Evolutionary Biology, University of California at Irvine, Irvine, California 92717

Received April 18, 1995; accepted July 24, 1995

According to phylogenetic data, about 100 years ago an avian influenza virus passed the species barrier (possibly first) to pigs and (possibly from there) to humans. In 1979 an avian influenza A virus (as a whole, without reassortment) again entered the pig population in northern Europe, forming a stable lineage. Here it is shown that the early North European swine viruses exhibit higher than normal evolutionary rates and are highly variable with respect to plaque morphology and neutralizability by monoclonal antibodies. Our results are consistent with the idea that, in order to pass the species barrier, an influenza A virus needs a mutator mutation to provide an additional number of variants, from which the new host might select the best fitting ones. A mutator mutation could be of advantage under such stress conditions and might enable a virus to pass the species barrier as a whole even twice, as it seems to have happened about 100 years ago. This stressful situation should be over for the recent swine lineage, since the viruses seem to be adapted already to the new host in that the most recent isolates — at least in northern Germany — are genetically stable and seem to have lost the putative mutator mutation again.

INTRODUCTION

Pigs seem to exhibit a relatively low species barrier toward infection by avian as well as by human influenza A viruses (for reviews see Webster *et al.*, 1992; Scholtissek *et al.*, 1993) and therefore they might function as "mixing vessels" for the creation of new human pandemic strains by reassortment (for a review see Scholtissek, 1990). However, as a rare event, avian influenza viruses can be introduced into the pig and the human populations without reassortment (Gammelin *et al.*, 1990; Gorman *et al.*, 1990; Webster *et al.*, 1992). It has been discussed that, in order to form a stable lineage in humans, an avian virus has to be passed to pigs first. There it might become adapted to a mammalian species, before it is transferred to humans (Gorman *et al.*, 1991; Scholtissek *et al.*, 1993; Ludwig *et al.*, 1994). About 100 years ago an avian virus was introduced (presumably first) into the pig population and (possibly thereafter) into humans (Webster *et al.*, 1992; Scholtissek *et al.*, 1993). Another introduction of an avian virus as a whole into the pig population occurred in 1979 in northern Europe (Schultz *et al.*, 1991). There are several conceivable mechanisms which might explain how such a rare event can happen. However, a most plausible one is that it might be necessary for the avian virus, before it can form a stable lineage in the new species, to have a mutator mutation in one of the polymerase proteins to provide a larger number of variants, from which the new host can choose the best fitting ones (Scholtissek *et al.*, 1993). Such mutator mutants of an influenza A virus have been found recently by Suárez *et al.* (1992). Because such a mutator mutation is probably advantageous only in a stress situation, like adaptation to the new species, it might easily be lost again after the virus is well adapted.

Here we show that the recent North European, "avian-like" swine viruses exhibit an elevated mutational and evolutionary rate in most genes. Only the rate of the HA1 part of the hemagglutinin (HA) of human strains exceeds that of the swine viruses. The early swine isolates are so variable that efficient plaque purification is not possible and the escape rate in the presence of a specific monoclonal antibody is unexpectedly high. In contrast, the most recent swine isolates are stable again in this respect.

MATERIALS AND METHODS

Virus strains and quantification

The sequences of H1N1 virus strains according to their GenBank Accession Nos. listed in Table 1 were used for the calculations. A/swine/Schleswig-Holstein/1/92 and A/swine/Schleswig-Holstein/1/93 were obtained from Dr. H. Willers (Hannover, Germany). The other H1N1 strains used in this study were obtained from various sources and are part of the collection in Giessen. The plaque assay was performed on primary chicken embryo cells according to Appleyard and Marber (1974).

[1] Present address: Institut für Medizinische Strahlenkunde und Zellforschung, Universität Würzburg, D-97078 Würzburg, Germany.

[2] To whom reprint requests should be addressed. Present address: Dept. of Virology and Molecular Biology, St. Jude Children's Research Hospital, Memphis, TN 38101.

0042-6822/95 $12.00

TABLE 1

Strains and Accession Numbers of the Sequences Used in This Report

HA	
A/USSR/90/77 (3 variants)	K01330, K01331, X00027
A/Brazil/11/78	X00028
A/Kiev/59/79	M38353
A/Lackland/3/78	X00029
A/Finland/20/78	L33757
A/Finland/92/78	L33488
A/England/333/80	X00031
A/India/6263/80	X00030
A/Finland/1/82	*L33489*
A/Ohio/101/83 (5 variants)	M59324–M59328
A/Finland/1/84	L33490
A/Finland/9/84	L33493
A/Yamagata/120/86	*D00841*
A/Singapore/6/86	D00406
A/Taiwan/1/86 (2 variants)	D00407, X17224
A/Yamagata/120/86	D00841
A/SL/2/87	*M33748*
A/NIB/4/88	X59778
A/Finland/75/88	L33755
A/Canada/7/88	L19021
A/Finland/91/89	L33756
A/Czechoslovakia/2/89	L19028
A/Trinidad/2/86	L19012
A/Fiji/2/88	L19011
A/Finland/42/86	L33483
A/Finland/45/87	L33485
A/Finland/53/87	L33486
A/Finland/70/88	L33487
A/Finland/73/88	L33753
A/Victoria/43/88	L19023
A/France/15/88	L19019
A/South Carolina/6/88	*L19025*
A/Czechoslovakia/2/88	L19016
A/Finland/72/88	L33752
A/Sichuan/4/88	L19024
A/Singapore/6/90	*L19026*
A/Singapore/10/90	L20111
A/Arizona/1/90	L19022
A/Stockholm/26/90	L19013
A/Goroka/2/90	L19018
A/Seoul/20/91	L33743
A/Qingdao/28/91	L19017
A/Finland/168/91	L33780
A/Finland/154/91	L33747
A/Finland/164/91	L33749
A/Finland/158/91	L19549
A/Finland/160/91	L33748
A/Finland/188/91	L33750
A/Umea/2/91	L33758
A/Groningen/9938/91	L33745
A/Leningrad/109/91	L33480
A/Netherlands/813/91	L33744
A/Vilnius/48/91	L33482
A/Groningen/9939/91	L33746
A/Finland/198/91	*L33751*
A/Finland/5/84	L33492
A/Fukushima/2/88	L19014
A/Massachusetts/1/90	L19027
A/Suita/1/89 (2 variants)	D13574, D13673
A/sw/Hongkong/1/74	X57491
A/sw/Wisconsin/46/76	NA**
A/sw/New Jersey/11/76	K00992
A/SW/Ehime/1/80	X57494
A/sw/QC/81	U03720
A/sw/QC/91	U03719
A/sw/Wisconsin/3/82	NA**
A/sw/Wisconsin/1915/88	NA*
A/sw/Indiana/1726/88	M81707
A/sw/Nebraska/1/92	L09063
A/sw/Arnsberg/6654/79	Z46437 (This report)
A/sw/Germany/2/81	Z30276 (This report)
A/sw/Germany/8533/91	Z46434 (This report)
A/sw/Schleswig-Holstein/1/92	Z46435 (This report)
M	
A/Fort Monmouth/1/47	U02084
A/Fort Warren/1/50	X08091
A/Singapore/1/57	X08093
A/Leningrad/134/17/57	M81576
A/Leningrad/134/47/57	*M81582*
A/Leningrad/134/57	M81570
A/Ann Arbor/6/60	M23978
A/Korea/426/68	M63531
A/Aichi/2/68	M63515
A/Udorn/307/72	J02167
A/Port Chalmers/1/73	X08092
A/Bangkok/1/79	K01140
A/Memphis/8/88	M63516
A/Guangdong/39/89	L18999, L18995
A/sw/Germany/2/81	M55478
A/sw/Germany/8533/91	Z26861
A/sw/Netherlands/25/80	Z26862
A/sw/Netherlands/12/85	M63525
A/sw/Schleswig-Holstein/1/93	Z46438 (This report)
NP	
A/Hickox/40	M63749
A/FM/47	M63750
A/Fort Warren/50	D00601
A/England/19/55	M63751
A/Singapore/1/57	M63752
A/Loyang/4/57	M76604
A/Ann Arbor/6/60	M23976
A/Victoria/5/68	*M63753*
A/NT60/68	J02137
A/Udorn/307/72	M14922
A/New Jersey/4/76	M76605
A/Texas/1/77	*D00602*
A/California/10/78	D00600
A/Hongkong/5/83	M22577
A/sw/May/54	M63761
A/sw/Wisconsin/1/57	M63762
A/sw/Wisconsin/1/61	M63763
A/sw/Wisconsin/1/67	M76607
A/sw/Tennessee/27/77	M30748
A/sw/Ontario/2/81	M63767
A/SW/Iowa/17672/88	M63768
A/sw/Wisconsin/1915/88	M76608
A/sw/Germany/2/81	M22579
A/sw/Germany/8533/91	Z26856
A/sw/Netherlands/12/85	M30749
A/SW/Italy/839/89	M63772
A/sw/Schleswig-Holstein/1/93	Z46439 (This report)
NS	
A/Bellamy/42	M12596
A/FM/1/47	K00577
A/Fort Warren/1/50	*K00576*
A/Denver/1/57	M12592
A/Ann Arbor/6/60	M12591
A/Berkeley/1/68	M12590
A/Aichi/2/68	*M34829*
A/Udorn/307/72	V01102
A/Alaska/6/77	K01332
A/Houston/24269/85	M17699
A/Equador/4/86	M57641
A/Wyoming/3/87	M57642
A/Stockholm/14/88	M57643
A/sw/Germany/2/81	M55484
A/sw/Germany/8533/91	Z26865
A/sw/Netherlands/25/80	Z26866
A/sw/Schleswig-Holstein/1/93	Z46440 (This report)

Note. NA, no accession No. available; NA*, sequence kindly provided by M. McGregor and V. S. Hinshaw, School of Veterinary Medicine, University of Wisconsin, Madison; NA**, for reference see Luoh *et al.* (1992).

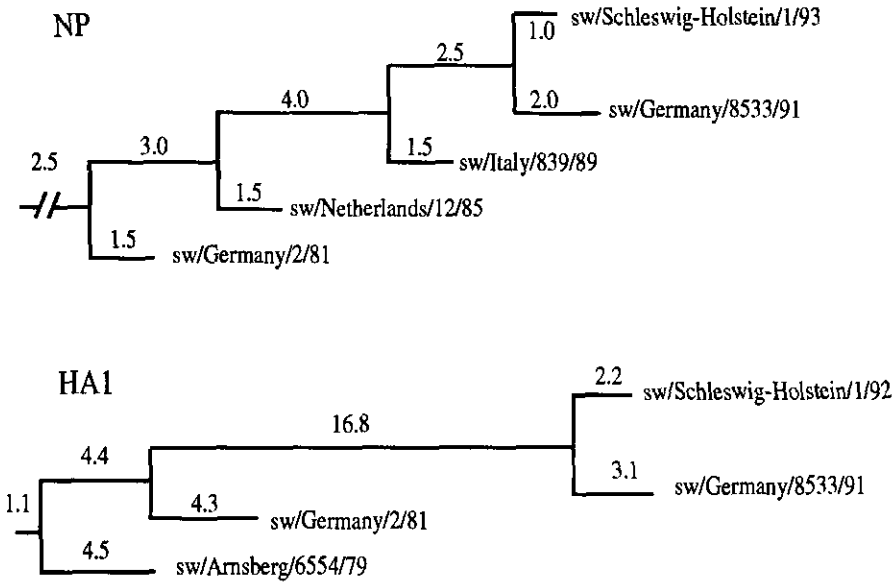

FIG. 1. Phylogenetic trees of the North European swine virus NP and HA1 proteins.

Isolation of viral RNA and sequencing

Viruses were propagated by growing them for 2 to 3 days in 11-day-old embryonated chicken eggs. The viruses were purified from the allantoic fluids and the viral RNA was extracted by the guanidinium–hot phenol method (Maniatis *et al.*, 1982). After reverse transcription DNA was synthesized by PCR according to the protocol of Kawasaki *et al.* (1977) using appropriate primers. Several independent PCR products were either blunt-end ligated into pBluescript II vectors (Alting-Mees and Short, 1989) or were directly sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977) using corresponding oligonucleotide primers.

Monoclonal antibodies (MABs)

MABs against the HA of A/swine/Germany/2/81 were generated as described by Stitz *et al.* (1990), except that octylglycoside-treated HA was used. Tissue culture supernatants from hybridomas were tested for HA by Western blot analysis and in HAI assays. Positive hybridomas were recloned. Mouse MABs were purified by affinity chromatography and were adjusted to a protein concentration of 1 mg/ml. The immunoglobulin isotype for all antibodies was determined to be IgG1 with an agglutination isotyping kit (Serotec, Oxford, England).

Phylogenetic trees

Phylogenetic trees were constructed by means of the parsimony methods of Fitch (1971) and Fitch and Farris (1974). For the construction of the various trees 102 NP, 100 HA, 54 M, and 55 NS sequences of genes or corresponding proteins were used. The branches of the NP and HA1 proteins of North European swine viruses are shown in Fig. 1. The total trees are available on request.

Calculation of the rates of changes of nucleotides and amino acids

The mutational and evolutionary rates were estimated by regression of the years of isolation against the branch distance from the common ancestor node of the nucleotide or the amino acid phylogenetic trees. From the slope of the regression lines the rates of change were calculated as accumulated differences per year and position.

RESULTS

Rates of change of nucleotides and amino acids in North European swine and human influenza virus genes and proteins

In recent phylogenetic studies (Ludwig *et al.*, 1994) it was shown that the NP genes and proteins of the North European swine lineage exhibited a higher evolutionary rate when compared with the human virus lineage. To examine whether this is a general phenomenon or only found for the NP, we have determined the rates of change for the North European swine virus M, NP, NS, and HA genes and proteins and have compared them to corresponding data of the human lineage. The sequences listed in Table 1 were used to count the number of sequence differences. The results are summarized in Table 2. The rates of change of the M, NS, and NP genes of the North European swine viruses were higher than those of human strains; however, the most significant differences between swine and human strains were found in respect to the NP, M, and NS proteins. Note that the differences in rates were more pronounced if the most recent swine strain from 1993 was not used for calculation (see also Fig. 2).

The HA genes and proteins behaved differently. How-

TABLE 2

Evolutionary Rates of the North European Swine Influenza Virus M, NS, NP, and HA1 Part of HA Genes and Proteins Compared to Those of the Human Virus Lineage

M	($\times 10^{-3}$ changes/year/residue)	M gene	M1 protein	M2 protein	No. of strains	Time period
	European swine viruses	1.7	0.09	3.0	5	1980–1993
		(1.9)	(1.0)	(3.0)	(4)	(1980–1991)
	Human virus lineage	1.1	0.09	0.1	13	1947–1989
NS	($\times 10^{-3}$ changes/year/residue)	*NS* gene	*NS1* protein	*NS2* protein	*No. of* strains	Time period
	European swine viruses	2.6	3.7	3.2	4	1980–1993
		(2.6)	*(3.7)*	*(4.0)*	*(3)*	*(1980–1991)*
	Human virus lineage	2.1	3.4	0.5	13	1942–1988
NP	($\times 10^{-3}$ changes/year/residue)		NP gene	NP protein	No. of strains	Time period
	European swine viruses		2.4	1.7	5	1981–1993
			(2.6)	(2.0)	(4)	(1981–1991)
	Classical swine viruses		2.3	1.1	9	1954–1988
	Human virus lineage		1.9	1.4	15	1940–1983
HA1	($\times 10^{-3}$ changes/year/residue)		HA genes	HA proteins	No. of strains	Time period
	European swine viruses		3.7	4.3	4	1979–1993
			(3.9)	(4.3)	(3)	(1979–1992)
	Classical swine viruses		3.2	2.9	10	1974–1992
	Human virus lineage		3.1	5.8	65	1977–1991

Note. Data were calculated from phylogenetic trees as recently described (Ludwig *et al.*, 1991, 1994). Briefly, distances from certain branch points to the tip of each line were plotted versus the year of isolation of each strain. The slope of the regression line, which represents the evolutionary rate (changes/year), was divided into the length of each gene/protein to make the data comparable (changes/year/site).

ever, one has to differentiate between the HA1 and the HA2 regions of the protein in this kind of comparison. While the HA2 protein behaved like the other viral proteins in that the HA2 of the swine viruses exhibited a higher rate of change (data not shown), the HA1 protein behaved the other way around. This reversal of relative rate of accumulating differences between NP and HA1 is clear in Fig. 2. These data imply that the epitopes of HA1 of the human lineage are under a stronger selection pressure than those of the HA1 of the swine lineage.

Analysis of H1 strains in the HAI test using monoclonal antibodies

As shown in Table 3, all North European swine viruses isolated between 1979 and 1993 exhibited similar HAI titers with all four MABs. There was no significant change in antigenicity. The human H1N1 viruses tested did not react. However, there was some variation among the classical swine and avian isolates.

High variation of plaque morphology and of neutralizability of early North European swine viruses

When we tried to plaque-purify the A/Sw/Germany/2/81 strain on primary chicken embryo cells using trypsin in the overlay medium (Appleyard and Marber, 1974), we observed a widely heterogeneous plaque morphology (Table 4). Two MABs were tested for neutralization, MAB 11G4 and MAB 27A5. When these two MABs were used, only MAB 27A5 was able to neutralize all plaque variants. However, MAB 11G4 neutralized only the prevailing small plaque variants, not the larger ones (Table 4). This heterogeneous virus population could be due to mixtures of different viruses forming partial heterozygotes. Such partial heterozygotes segregate on further plaque passages (Scholtissek *et al.*, 1978). However, if small and large plaques were picked and further purified and plaque-passaged, they always split off into small and large plaque variants (with a few intermediates), neutralizable with MAB 11G4 to different degrees (Table 5). Therefore a virus mixture and partial heterozygotes cannot account for the observed variability.

Comparable observations were made with A/Sw/Arnsberg/6554/79) (three consecutive plaque passages, A/Sw/France/Olid/80 (one passage), and A/Sw/Netherlands/12/85 (one passage). However, A/Sw/Germany/8533/91 and A/Sw/Schleswig-Holstein/1/93 exhibited a homogeneous plaque morphology of about 1 mm in di-

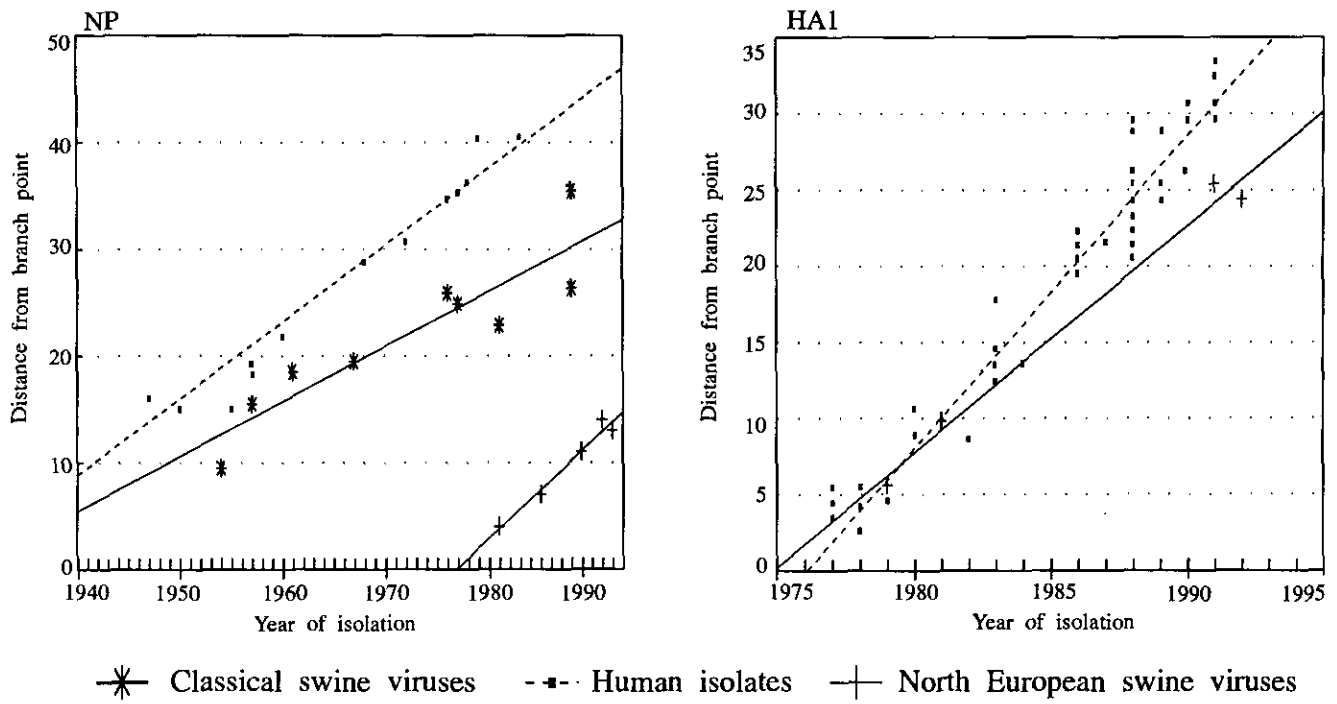

FIG. 2. Graphic comparison of the North European swine virus NP and HA1 rates of changes versus the corresponding rates of the human and the classical swine virus lineages.

TABLE 3

Antigenic Comparison of H1N1 Influenza A Viruses with Four Monoclonal Antibodies Generated to the H1 Hemagglutinin of A/sw/Germany/2/81 in Hemagglutination Inhibition Assays

	Monoclonal antibodies (HI titers)			
Influenza H1N1 strains	22G2	27A5	11G4	22G8
North European swine isolates				
A/sw/Arnsberg/6554/79	32	128	64	64
A/sw/Olida/1/80	32	64	128	32
A/sw/Germany/2/81	64	256	256	128
A/sw/Germany/8533/91	64	128	128	128
A/sw/Schl.-Holstein/1/92	32	256	128	256
A/sw/Schl.-Holstein/1/93	32	256	128	128
Classical swine strains				
A/sw/1976/31	—	64	—	128
A/sw/Wisconsin/1/67	—	256	4	256
A/Wisconsin/3523/88	64	128	128	128
Avian isolates				
A/dk/Alberta/35/76	16	4	32	—
A/dk/Bavaria/2/77	—	—	—	—
A/oystercatcher/Germany/87	—	—	—	—
Human isolates				
A/WSN/33	—	—	—	—
A/FM/1/47	—	—	—	—
A/Loyang/4/57	—	—	—	—

Note. Hemagglutinin inhibition titers are expressed as the reciprocal of the dilution $\times 10^{-1}$ of antibody solution inhibiting 8 hemagglutinating doses of virus.

ameter. Both strains could be neutralized with MAB 27A5 as well as with 11G4 (reduction of plaque yield by at least a factor of 10,000). Various plaque isolates of the two latter strains behaved exactly the same. The same results were obtained when MDCK cells were investigated (data not shown). Thus, this heterogeneity did not depend on the host cells used for the plaque test.

DISCUSSION

According to the genetic data presented in Table 2, the North European swine viruses exhibited an elevated rate of change of all genes tested when compared with human strains. Only the HA1 part of the glycoprotein of North European swine viruses showed a lower evolutionary rate relative to human viruses (Tables 2 and 3, and Fig. 2). There

TABLE 4

Heterogeneity of Plaque Size and Plaque Neutralization by Monoclonal Antibodies of an Early Egg Passage of A/sw/Germany/2/81 (H1N1)

	Plaque forming units		
MAB No.	(1 mm)	(3 mm)	(5 mm)
without MAB	3.4×10^6	4.0×10^5	4.5×10^5
11G4	$<10^3$	4.5×10^5	3.0×10^5
27A5	$<10^2$	$<10^2$	$<10^2$

Note. If other independently obtained allantoic fluids were analyzed, the plaque-size ratio may vary to a certain extent.

TABLE 5

Heterogeneity of Plaque Size and Plaque Neutralization after Several Individual Plaque Passages of A/sw/Germany/2/81 (H1N1)

Plaque isolates	MAB 11G4	Plaque forming units (Plaque size) (1 mm)	(5 mm)
s/s/s/s	−	1.5×10^7	9×10^7
	+	1×10^6	8×10^5
s/s/s/l	−	1×10^7	1.5×10^8
	+	1×10^5	6.5×10^5
s/s/s/s/s	−	2×10^6	$<10^5$
	+	2×10^5	5×10^4
s/s/s/l/l	−	1×10^7	2×10^8
	+	3×10^5	3×10^6
s/s/s/lmab/l	−	5×10^6	1.5×10^8
	+	8×10^6	2×10^8

Note. Small (s, about 1 mm) and large (l, about 5 mm) plaques were picked and propagated in embryonated chicken eggs. The allantoic fluids were taken for the next plaque test. s/s/s/s means that from four consecutive plaque passages each a small plaque was picked; 1 mab means that a large plaque was picked after neutralization with MAB 11G4.

exist at least two possible explanations for our observations: Either the rate of mutation or the rate of evolution has changed. The one does not exclude the other. We are inclined to assume that the elevated rate of change of the genes of the North European swine viruses is mainly due to an increased rate of mutation, possibly evoked by a mutation in one of the polymerase proteins (mutator mutation; Suárez *et al.*, 1992) of the earliest North European swine virus. The unexpected high variability of plaque morphology and escape rate of MAB mutants of the swine viruses isolated between 1979 and 1985 could be explained in the same way (see Tables 4 and 5, note that the late swine isolates of 1991 and 1993 are stable again in this respect). On the other side, the relatively conserved HA1 part of the North European swine isolates (Tables 2 and 3, and Fig. 2) compared with the human viruses might mainly be due to a lower selection pressure by the immune system (lower rate of evolution). Such great differences in rates of evolution have been seen before for the nucleoproteins of the avian viruses on the one side and the human and classical swine viruses on the other side (Gammelin *et al.*, 1990; Gorman *et al.*, 1990). The observation on the lower rate of evolution of the HA1 part of the North European swine viruses is easy to understand taking into account that the globular part of the HA is mainly needed for the virus to escape the immune response of the host. In contrast to humans, most of the pigs do not live long enough to become infected more than once. Thus, the relative antigenic conservation is not surprising and was also shown for the classical swine viruses in North America (Sheerar *et al.*, 1989; Noble *et al.*, 1993). In other words, the *in vitro* selection pressure by the MAB 11G4 is artificial and reveals the mutator mutation. However, such a selection pressure by the immune system is not acting during natural infection of pigs; at least not to the extent that it is during infections of humans.

We might assume that an avian virus might be able to pass the species barrier twice — from birds to pigs and thereafter from pigs to humans — only as long as a mutator mutation is present during the entire period, as might have happened about 100 years ago. If such a mutator mutation is necessary for an avian virus to pass the species barrier to humans via pigs, this threat of passage should be over for the latest "avian-like" swine viruses, because they seem to have already lost their mutator mutation — at least in northern Germany. However, if such "avian-like" swine viruses still containing the putative mutator mutation are still around somewhere else in Europe they might give rise to a pandemic in humans comparable to the Spanish Flu of 1918–1919. The possible transmission of an "avian-like" swine virus as a whole to humans does, of course, not exclude the possibility of transmission of reassortants carrying only one or the other RNA segment of the recent swine strains. Such reassortants were isolated recently from diseased children in the Netherlands (Claas *et al.*, 1994).

ACKNOWLEDGMENTS

This work was supported by the Deutsche Forschungsgemeinschaft, SFB 272, and the Fonds der Chemischen Industrie. We thank Karin Müller and Sabine Klein for expert technical assistance. We thank Dr. H. Willers, Hannover, for providing us with the strains A/sw/Schleswig-Holstein/1/92 and A/sw/Schleswig-Holstein/1/93. We also thank Drs. V. S. Hinshaw and M. W. McGregor for providing sequence data.

REFERENCES

Alting-Mees, M. A., and Short, J. M. (1989). pBluescript II: Gene mapping vectors. *Nucleic Acids Res.* **17,** 9494.

Appleyard, G., and Marber, H. B. (1974). Plaque formation by influenza viruses in the presence of trypsin. *J. Gen. Virol.* **25,** 351–357.

Claas, E. C. J., Kawaoka, Y., De Jong, J. C., Masurel, N., and Webster, R. G. (1994). Infection of children with avian–human reassortant influenza virus from pigs in Europe. *Virology* **204,** 453–457.

Fitch, W. M. (1971). Towards defining the course of evolution: Minimum change for a specific tree topology. *Syst. Zool.* **20,** 406–416.

Fitch, W. M., and Farris, J. S. (1974). Evolutionary trees with minimum nucleotide replacements from amino acid sequences. *J. Mol. Evol.* **3,** 263–273.

Gammelin, M., Altmüller, A., Reinhardt, U., Mandler, J., Harley, V. R., Hudson, P. J., Fitch, W. M., and Scholtissek, C. (1990). Phylogenetic analysis of nucleoproteins suggests that human influenza A viruses emerged from a 19th century avian ancestor. *Mol. Biol. Evol.* **7,** 194–200.

Gorman, O. T., Bean, W. J., Kawaoka, Y., and Webster, R. G. (1990). Evolution of the nucleoprotein gene of influenza A virus. *J. Virol.* **64,** 1487–1497.

Gorman, O. T., Bean, W. J., Kawaoka, Y., Donatelli, I., Guo, Y., and Webster, R. G. (1991). Evolution of influenza A virus nucleoprotein

genes: Implications for the origin of H1N1 human and classical swine viruses. *J. Virol.* **65,** 3704–3714.

Kawasaki, E. S., Clark, S. S., Coyne, M. Y., Smith, S. D., Champlin, R., Witte, O. N., and McCormick, F. P. (1977). Diagnosis of chronic myeloid and acute lymphoic leukemias by detection of leukemia-specific mRNA sequences amplified in vitro. *Proc. Natl. Acad. Sci. USA* **85,** 5698–5702.

Ludwig, S., Haustein, A., Kaleta, E. F., and Scholtissek, C. (1994). Recent influenza A (H1N1) infections of pigs and turkeys in northern Europe. *Virology* **202,** 281–286.

Luoh, S. M., McGregor, M. W., and Hinshaw, V. S. (1992). Hemagglutinin mutations related to antigenic variation in H1 swine influenza viruses. *J. Virol.* **66,** 1066–1073.

Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982). "Molecular Cloning: A Laboratory Manual." Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Noble, S., McGregor, M. S., Wentworth, D. E., and Hinshaw, V. S. (1993). Antigenic and genetic conservation of the hemagglutinin in H1N1 swine influenza viruses. *J. Gen. Virol.* **74,** 1197–1200.

Sanger, F., Nicklen, S., and Coulson, A. R. (1977). Sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.

Scholtissek, C. (1990). Pigs as "mixing vessels" for the creation of new pandemic influenza A viruses. *Med. Principles Pract.* **2,** 65–71.

Scholtissek, C., Rohde, W., Harms, E., Rott, R., Orlich, M., and Boschek, C. B. (1978). A possible partial heterozygote of an influenza A virus. *Virology* **89,** 506–516.

Scholtissek, C., Ludwig, S., and Fitch, W. M. (1993). Analysis of influenza A virus nucleoproteins for the assessment of molecular genetic mechanisms leading to new phylogenetic virus lineages. *Arch. Virol.* **131,** 237–250.

Schultz, U., Fitch, W. M., Ludwig, S., Mandler, J., and Scholtissek, C. (1991). Evolution of pig influenza viruses. *Virology* **183,** 61–73.

Sheerar, M. G., Easterday, B. C., and Hinshaw, V. S. (1989). Antigenic conservation of H1N1 swine influenza virus. *J. Gen. Virol.* **70,** 3297–3303.

Stitz, L., Schmitz, C., Binder, D., Zinkernagel, R., Paoletti, E., and Becht, H. (1990). Characterization and immunological properties of influenza A virus nucleoprotein (NP): Cell-associated NP isolated from infected cells, or viral NP expressed by vaccinia recombinant virus do not confer protection. *J. Gen. Virol.* **71,** 1169–1179.

Suárez, P., Valcácel, and Ortín, J. (1992). Heterogeneity of the mutation rates of influenza A viruses: Isolation of mutator mutants. *J. Virol.* **66,** 2491–2494.

Webster, R. G., Bean, W. J., Gorman, O. T., Chambers, T. M., and Kawaoka, Y. (1992). Evolution and ecology of influenza A viruses. *Microbiol. Rev.* **56,** 152–179.