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Evolution of H5 subtype avian influenza A viruses in North America

M. García [a], D.L. Suarez [a], J.M. Crawford [a], J.W. Latimer [a], R.D. Slemons [b], D.E. Swayne [a], M.L. Perdue [a,*]

[a] *USDA, Agriculture Research Service, Southeast Poultry Research Laboratory, 934 College Station Road, Athens, GA 30605, USA*

[b] *Department of Veterinary Preventive Medicine, College of Veterinary Medicine, The Ohio State University, 1900 Coffey Road, Columbus, OH 43210, USA*

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Abstract

The phylogenetic relationships of the hemagglutinin (HA) and non-structural (NS) genes from avian influenza (AI) H5 subtype viruses of North American origin are presented. Analysis of the HA genes of several previously uncharacterized isolates from waterfowl and turkeys provided clear evidence of significant sequence variation and existence of multiple virus clades or sub-lineages, maintained in migratory waterfowl. Phylogenetic analysis of NS gene sequences further demonstrated multiple sub-lineages and also demonstrated re-assortment of two NS alleles in wild duck populations. Based on currently available HA_1 gene sequences, at least four clades exist with waterfowl isolates included in three of the four groups. The most genetically unstable of these sub-lineages is composed of recent poultry isolates from the outbreak of AI in Central Mexico. This group of viruses, which replicated unabated in chickens for at least 16 months, exhibited an increased rate of mutation in both the HA and NS gene. Comparison of the HA_1 sequence data for all available North American H5 subtype viruses demonstrated minimal variation both in and around the amino acids predicted to be involved in the HA receptor binding site. The sequences also revealed that migratory waterfowl, live poultry market chicken, and turkey isolates uniformly lack a glycosylation site at amino acid 236 in the HA protein which is present in commercial chicken isolates. © 1997 Elsevier Science B.V.

Keywords: Avian influenza virus; Hemagglutinin protein; Non-structural protein; Viral evolution

1. Introduction

Avian influenza viruses of 13 different hemagglutinin (H1-H13) and nine different neu-

* Corresponding author.

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raminidase (N1-N9) subtypes have been isolated from migratory waterfowl in North America as indicated by surveillance studies from 1974 to 1988 (Slemons et al., 1974; Slemons and Easterday, 1977; Hinshaw et al., 1980; Halvorson et al., 1982; Deibel et al., 1985; Nettles et al., 1985; Stallknecht et al., 1990; Slemons et al., 1991; Graves, 1992). Initially, migratory waterfowl were proposed to play a major role in the transport and dissemination of influenza viruses (Slemons et al., 1974). The widely distributed influenza virus pool observed in migratory waterfowl later suggested these as the host reservoir for influenza viruses that cause outbreaks in domestic poultry (Hinshaw et al., 1980). These proposals are consistent with the common occurrence of low pathogenicity (LP) outbreaks in poultry. For example, LP outbreaks caused by influenza viruses of several subtypes are common among range-reared turkey flocks in Minnesota, where the outbreaks coincide with an increase in waterfowl populations during their southward migration through the state (Halvorson et al., 1982). In contrast to the wide variety of AI subtypes involved in outbreaks of low or moderate pathogenicity, highly pathogenic (HP) AI outbreaks have been restricted to a few viruses of the H5 and H7 subtypes (Wood et al., 1993; Senne et al., 1996a).

Although the expression of the HP phenotype among AI viruses has been linked to multiple genes (Rott et al., 1979), structural features in the hemagglutinin glycoprotein are a primary determinant of an isolate's pathogenicity (Webster and Rott, 1987; Kawaoka and Webster, 1988). Analyses of H5 viruses isolated during outbreaks in poultry in North America have demonstrated that HP viruses most likely emerge from LP forms of the virus after acquiring specific mutations in the HA gene (Kawaoka et al., 1984; Horimoto et al., 1995a; García et al., 1996). Previous phylogenetic analysis of the HA gene, based upon partial HA_1 sequences from H5 viruses, provided additional evidence indicating that HP H5 viruses do not constitute unique lineages but rather arise from LP precursors introduced into poultry probably from aquatic birds (Rohm et al., 1995).

Three outbreaks of highly pathogenic AI of the H5 subtype have occurred in North America. The prototype H5 viruses were isolated from turkeys in Ontario during 1966 where the virus caused a limited HP outbreak (Lang et al., 1968). The second HP outbreak was in Pennsylvania during 1983 and 1984 (Eckroade and Bachin, 1987), while the third occurred more recently in central Mexico during 1994 to 1995 (Senne et al., 1996b). Precise identification of sources of virus causing HP outbreaks in poultry has remained elusive. The sporadic isolation of H5N2 viruses from chickens in live poultry markets since 1986 suggests that in addition to turkeys, these birds may be a potential source of H5 LP viruses for poultry (Senne et al., 1992). In order to identify all the potential reservoirs of H5 influenza viruses infecting poultry it is important to study the genetic relationship among viruses from all available hosts including aquatic birds, live market birds and commercial poultry. This will allow a clearer understanding of the epidemiological significance of each host in the generation of HP AI outbreaks. This study presents analysis of the HA_1 and the NS gene sequences of H5 viruses isolated in North America during the past 29 years. It demonstrates that avian influenza viruses of the H5 subtype continue to undergo dynamic evolutionary variation including apparent variation in mutation rates among different host species and gene re-assortment among mixed virus populations in the same host species.

2. Materials and methods

2.1. Viruses

A total of 26 H5 viruses were included in these phylogenetic analysis: 22 from North America and four isolates from Europe. Among the North America viruses, six were from migratory waterfowl, one isolate from shore birds, one from emu, two from live poultry market chickens, four from turkeys and eight from commercial chickens. In this study sequences of the HA_1 plus the N-terminal six amino acids of the fusion peptide were obtained for six waterfowl, three turkey, and one chicken isolate. The complete sequence for the NS gene RNA was obtained for 20 isolates. The

Table 1
H5 avian influenza virus isolates utilized in the phylogenetic analysis

Viruses	Highly pathogenic	Abbreviation	HA sequence accession number	NS sequence accession number
A/turkey/Ontario/7732/66(H5N9)	Yes	TOn66[a]	M30122[h]	U85376[g]
A/turkey/Wisconsin/68(H5N9)	No	TWi68[a]	U79456[g]	U85378[g]
A/mallard/Wisconsin/34/75(H5N6)	No	MWi75A[b]	U79451[g]	U85379[g]
A/mallard/Wisconsin/169/75(H5N3)	No	MWi75B[b]	U79452[g]	U85375[g]
A/mallard/Wisconsin/428/75(H5N3)	No	MWi75C[b]	U79453[g]	U85380[g]
A/duck/Michigan/80(H5N2)	No	DMi80[d]	U79449[g]	U853811[g]
A/turkey/Minnesota/3689-1551/81(H5N2)	No	TMn81[a]	U79454[g]	U85382[g]
A/chicken/Pennsylvania/1/83(H5N2)	No	CPe83E[a]	M18001[h]	NA[i]
A/chicken/Pennsylvania/1370/83(H5N2)	Yes	CPe83L[a]	M1O243[h]	NA[i]
A/mallard/Ohio/556/87(H5N9)	No	MOh87[c]	U67783[g]	U85377[g]
A/mallard/Ohio/345/88(H5N2)	No	MOh88[c]	U79450[g]	NA[i]
A/ruddy turnstone/Delaware/244/91(H5N2)	No	RDe91	UO5330[h]	NA[i]
A/chicken/Pennsylvania/13609/93(H5N2)	No	CPe93[a]	U05331[h]	U85383[g]
A/chicken/Florida/25717/93(H5N2)	No	CFl93	U05332[h]	NA[i]
A/emu/Texas/399243/93(H5N2)	No	ETx93[a]	U28919[h]	U85384[g]
A/chicken/Mexico/31381-7/94(H5N2)	No	CMx93[e]	U37165[h]	U85385[g]
A/chicken/Hidalgo/26654-1368/94(H5N2)	No	CHi94[e]	U37172[h]	U85386[g]
A/chicken/Mexico/31381-1/94(H5N2)	No	CJa94A[e]	U37166[h]	U85387[g]
A/chicken/Mexico/31381-2/94(H5N2)	No	CJa94B[e]	U37167[h]	U85388[g]
A/chicken/Queretaro/14588-19/95(H5N2)	Yes	CQ95A[e]	U37182[h]	U85389[g]
A/chicken/Queretaro/7653-20/95(H5N2)	Yes	CQ95B[e]	U79448[g]	U85390[g]
A/turkey/Minnesota/10734/95(H5N2)	No	TMn95[e]	U79455[g]	U85391[g]
A/chicken/Scotland/59(H5N1)	Yes	CS59	X07826[h]	AF009898[g]
A/duck/Ireland/113/83(H5N8)	Yes	DI83	M18450[h]	NA[i]
A/turkey/Ireland/1378/83(H5N8)	Yes	TI83[f]	M18451[h]	U85392[g]
A/turkey/England/50-92/91(H5N1)	Yes	TE91[f]	Wood et al., 1994	U85447[g]

[a] Viruses obtained from the repository of the Southeast Poultry Research Laboratory, USDA, ARS (Athens, GA).
[b] Viruses isolated by Slemons and Easterday; obtained from the repository of Dr. B.C. Easterday at University of Wisconsin (Madison, WI).
[c] Viruses obtained from the repository of Dr. R. Slemons at Ohio State University (Columbus, OH).
[d] Viruses obtained from the repository of Dr. R. Webster at St. Jude Children's Research Hospital (Memphis, TN).
[e] Viruses obtained from the repository of the National Veterinary Service Laboratory (NVSL) (Ames, IA).
[f] Viruses obtained from the repository of Dr. D. Alexander at Central Veterinary Laboratory (Surrey, UK).
[g] Sequences obtained during this study.
[h] Sequences obtained from GeneBank.
[i] Not available.

remaining sequences were obtained from GenBank. A complete list of the viruses and sequences utilized in this study are presented in Table 1. Viruses were propagated in the allantoic cavity of 10-day-old chicken embryos as previously described by Perdue et al. (1990).

2.2. Sequence analysis

Viral RNA extraction, amplification by reverse transcriptase-polymerase chain reaction (RT-PCR) and sequencing of PCR products of the HA gene were performed as previously described by García et al., 1996. The NS gene of selected isolates was amplified by RT-PCR using primers consisting of the conserved 5′ terminal 12 bases and the 3′ terminal 13 bases coupled to a 12 base extension containing four uracils. The amplified PCR product containing the full length NS gene cDNA was extracted from an agarose gel and inserted into a plasmid vector, (pAMP1; Life Technologies, Gaithersburg, MD). The plasmid was used to transform DH5α cells (Life Technologies) and positive colonies were screened with cDNAs specific for the NS gene. Positive colonies were grown overnight and plasmid extracted with the High Pure Plasmid Isolation Kit (Boehringer Mannheim, Indianapolis, IN). All sequences were obtained in both directions on an ABI automated sequencer (Smith et al., 1986) using the manufacturer's suggested protocols. Primer sequences are available upon request.

2.3. Sequence data analysis

Nucleotide sequences, prediction of amino acid sequences and alignments were completed using the GeneWorks 2.45 software (Intelligenetics, Mountain View, CA), or DNASTAR (Madison, WS). Phylogenetic analysis and pair-wise distances between gene sequences were calculated with PAUP software, Version 3.1.1 (Swofford, 1989). Phylogenetic trees were generated utilizing heuristic searches with the neighbor-joining method. Error associated with tree structures was evaluated by bootstrap analyses.

3. Results and discussion

Analysis of phylogenetic relationships among the HA genes based on nucleotide and amino acid sequences exhibited a divergence of H5 subtypes into the two geographically distinct lineages previously reported (Rohm et al., 1995), a North American and a European lineage. Although the topology and distribution of sequences in phylogenetic trees (phylograms) based on nucleotides and amino acids varied slightly, four sub-lineages or clades were easily defined for the North American viruses. The groups are designated as I-IV (Fig. 1). Clade I includes the prototype highly pathogenic H5 isolate from Ontario in 1966, a turkey isolate, a waterfowl origin virus MWi75A and the two 1983 Pennsylvania outbreak viruses (CPe83E, CPe83L) which share a common ancestor. Clade II includes primarily duck isolates with sequences (MWi75B and MWi75C) that clearly share a common ancestor with the MOh88 sequence. The DMi80 and TMn81 isolates included in this clade share a common ancestor as illustrated by the clustering in the nucleotide constructed phylogram, but in the amino acid phylogram TMn81 was less distinctly classified. As such, the synonymous changes indicate a single clade where the non-synonymous changes indicate separate clades. Clade III has sequences from shore bird (RDe91), live poultry market chickens (CFl93, CPe93), ratite (ETx93) and turkey (TMn95) viruses sharing a common ancestor. Clade IV includes sequences from viruses isolated during the recent outbreak of HP AI in Mexico. Within this sub-lineage viruses CQ95A and CQ95B were characterized as highly pathogenic. In the interest of streamlining data presentation, only a few Mexican isolates were selected to represent the diversity of the 18 viruses previously characterized in this clade (García et al., 1996). In both phylograms, clade IV sequences from HP and LP viruses share an apparent common ancestor. Waterfowl and shore bird virus sequences are found among three of the four sub-lineages indicating that multiple H5 virus sub-lineages are maintained in aquatic birds. Furthermore, sequences from waterfowl viruses MWi75A, MWi75B and MWi75C, isolated during the same year by the same laboratory, are found in two clearly separated clades of the phylogenetic tree.

The results illustrated in Fig. 1 clearly show that multiple populations or lineages of H5 viruses are present at the same time in the same geographic location in a waterfowl population. Pair-wise distances between H5 subtype influenza viruses from migratory waterfowl and poultry

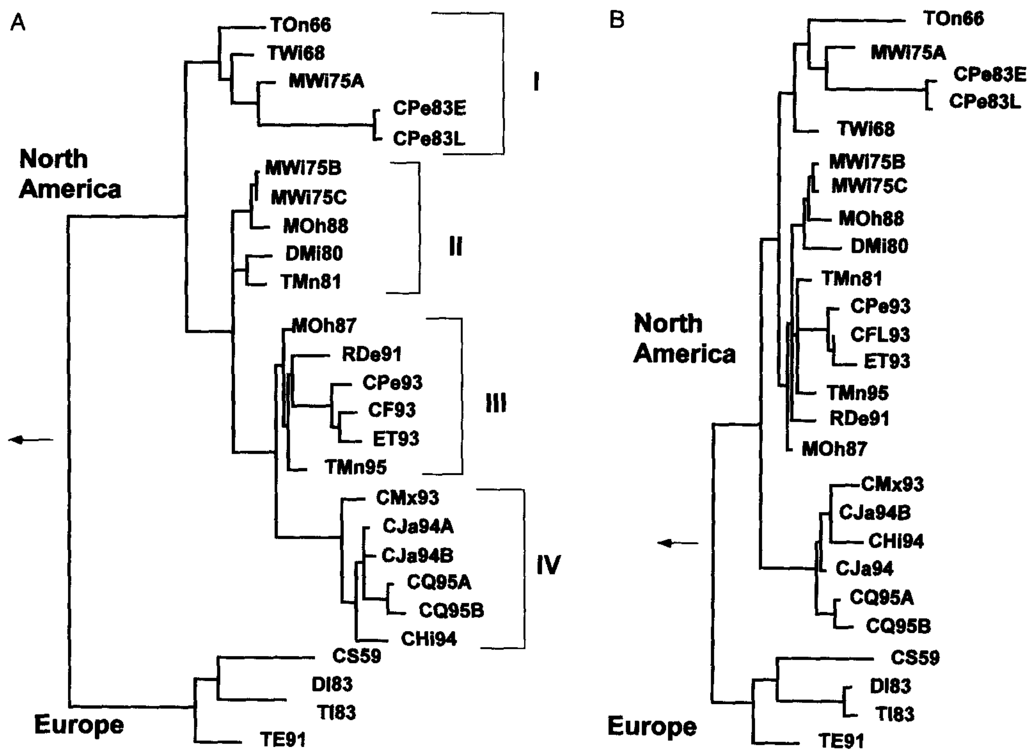

Fig. 1. Phylogenetic trees of the HA gene from H5 influenza viruses. (A) The phylogram constructed for 26 H5 viruses utilizing complete nucleotide coding sequences of the HA_1 subunit and the N-terminal fusion peptide sequences. The tree was rooted to the H2 HA_1 sequence of A/mallard/Montana/61 (H2N2) (GeneBank accession number L11136). A single nucleotide tree was obtained after 2000 bootstrap re-samplings. (B) The amino acid consensus tree from 15 trees obtained after 1000 bootstrap re-samplings. The lengths of the horizontal lines are proportional to the nucleotide and amino acid changes between sequences. Vertical lines separate progeny virus lineages at the point where they branch from a theoretical common ancestor. The arrow at the left indicates the direction of the A/mallard/Montana/61 (H2N2) from the root node. Abbreviations of viruses are listed in Table 1.

origin viruses indicate that a close phylogenetic relationship exists, particularly between sequences from turkey and waterfowl origin viruses. Nucleotide distance comparisons between HA_1 sequences of waterfowl and turkey origin viruses showed that viruses MOh87 and TMn95 have the highest HA_1 nucleotide sequence similarity, with a nucleotide pair-wise distance of 1.7%. However, MOh87 and TMn81 have the highest HA_1 amino acid similarity, followed by MOh87 and TMn95. The amino acid pair-wise distance shared by MOh87 and TMn81 is 1.8%, and 2.1% for MOh87 and TMn95. Temporal and spatial epidemiological evidence has linked influenza infections of turkeys in Minnesota with viruses isolated from waterfowl in the area (Halvorson et al., 1982). The high sequence similarity observed between the waterfowl origin virus MOh87 and the Minnesota turkey viruses (TMn81 and TMn95) HA_1 sequences is the first molecular epidemiological evidence that links influenza H5 infection in Minnesota turkeys with a waterfowl origin virus. Furthermore, the high level of sequence similarity indicates that a stable H5 gene lineage has been maintained in the waterfowl, and that viruses carrying that particular HA gene lineage have been sporadically introduced into Minnesota turkey flocks.

Influenza surveillance studies of live poultry markets have demonstrated that, recently, the most common influenza virus isolated from this environment is the H5N2 subtype, isolated from

chickens, ducks, guinea fowl, turkey, quail, pigeon and chuckar partridge (Senne et al., 1992). This virus subtype reappeared in chickens from live poultry markets in 1993 (Saito et al., 1994). Comparison of nucleotide and amino acid distances between HA_1 sequences of waterfowl and all chicken origin viruses demonstrates that MOh87 and CFl93 share the highest HA similarity followed by MOh87 and CPe93. The nucleotide and amino acid pair-wise distances for MOh87 and CFl93 were 2.8% and 3.3%, while for MOh87 and CFl93 were 2.9% and 3.6%, respectively. The close phylogenetic relationship between the HA sequences of waterfowl virus MOh87 and live poultry market chicken viruses, CFl93 and CPe93, indicates that a waterfowl virus may have been the source of the 1993 live poultry market viruses.

The apparent differences in the genetic stability, over time, between clade IV and the other clades, led us to consider the mutation rate among these H5 strains. Measuring the mutation rate of avian influenza virus genes has classically involved direct comparison of isolates, oftentimes from various orders of birds, and expressing the variation over time (see Gorman et al., 1992). This may not be a valid approach, however, for measuring an accurate rate as multiple species are involved and multiple introductions of virus have occurred. We previously published a mutation rate for the isolates from Mexico based upon the assumption that there was a single introduction and the same virus was allowed to replicate unabated in the poultry populations in Mexico (García et al., 1996). When the clades in Fig. 1A were compared, the sub-lineage with the lowest rate of nucleotide and coding changes per year was sub-lineage II, composed of mostly waterfowl viruses isolated during a 13 year period. Clades I and III included sequences from viruses originating from a wider variety of hosts showed slightly higher mutation rate. Though perhaps not accurate, the estimated average mutation rates for H5 virus sub-lineages I, II, and III fall within the estimated evolutionary rates values obtained for human influenza H1 and H3 genes which range from 0.61 to 7.0×10^{-3} nucleotide substitutions per site per year (Gorman et al., 1992). In contrast, clade IV, composed of sequences from chicken-origin H5N2 viruses from Mexico, acquired 28.1×10^{-3} nucleotide and 8.8×10^{-3} coding changes per site per year.

These results suggest that nucleotide substitution rates in the HA gene of H5 subtype influenza viruses increase significantly once the virus is introduced into commercial poultry. There were only two isolates sequenced from the 1983 poultry outbreak in the northeast US, so it is not known how rapidly these viruses were evolving in the field. The relationships in clade I, however, suggest that the CPe83E and CPe83L isolates from this period had undergone significant sequence variation from their progenitor population. At this time, there are no other studies where HA sequences from multiple viruses isolated during a single AI poultry outbreak have been compared. Therefore we cannot presently determine if the higher apparent mutation rate observed for the viruses of Mexican origin is a unique characteristic of this particular outbreak or common to any situation in which unrestricted replication in commercial poultry is allowed.

The phylogenetic relationship of several subtype H5 North American isolates based upon the NS gene sequences is shown in Fig. 2. The phylogram readily identifies the two previously described alleles of the NS gene (Treanor et al., 1989; Ludwig et al., 1991). There also appear to be multiple sub-lineages of viruses circulating in migratory waterfowl based upon the NS sequence data. As in the case of the HA gene data, this is most obvious from the finding that viruses isolated at the same time in the same laboratory from a single survey of mallards, yields two distinct alleles of the NS gene. They are found in the isolates which were members of the two separate clades defined by the HA gene phylogram, namely the MWi75A and MWi75C. The fact that the MWi75B isolate contains a hemagglutinin virtually identical to the MWi75C and an NS coding sequence most closely related to the MWi75A A allele is most easily explained by concluding that re-assortment of genes between two distinct sub-lineages of H5 viruses occurred in these wild duck populations. The isolates from Mexico shown in the Fig. 2, as was the case for the HA gene, also demonstrated a higher substitution rate in the NS

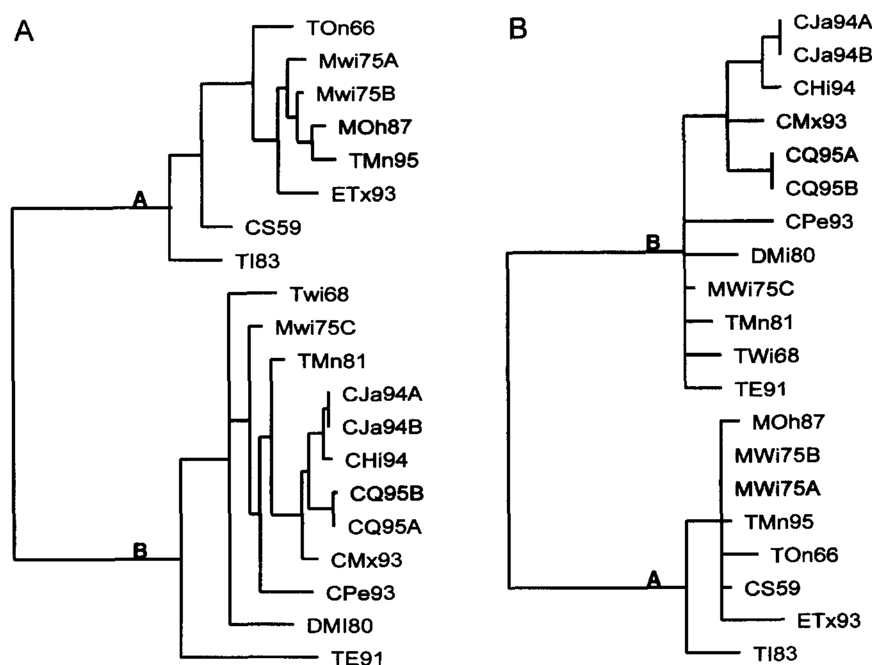

Fig. 2. Phylogenetic comparison of NS segment cDNA sequences. Several of the isolates used in the HA gene analysis were subjected to cloning and sequencing of the NS RNA. The sequences were analyzed as in Fig. 1. (a) Relationship based on nucleotide sequences. (b) Relationship based on NS1 amino acid sequences. A and B on the cladogram denote the two different alleles previously identified (Treanor et al., 1989).

sequences (19.5×10^{-3} per site per year) when compared to the published rates of change for the NS gene (1.94×10^{-3} per site per year and 1.78×10^{-3} per site per year; see Gorman et al., 1992).

Examination of the HA sequences in this study led to two additional observations regarding the structure of the HA_1 segment in H5 subtype viruses. With the exception of the sequences for the TOn66 and the RDe91 isolates, all sequences examined contained completely conserved sequences in the areas surrounding the proposed receptor binding site (Fig. 3a). Although it is well known that receptor site sequences are highly conserved among the type A influenza viruses, this is the first report of enough sequences to establish this exclusively in the H5 subtype. The single amino acid differences in the TOn66 and RDe91 may be due to observed variation in tissue tropism of these two strains (Van Campen et al., 1989; Saito et al., 1994). The second observation was that all commercial chicken isolates possessed a potential glycosylation site specified at position 236 which was absent from the other isolates (Fig. 3b).

The number and distribution of glycosylation sites along the HA_1 subunit has been implicated in establishing tissue tropism (Inkster et al., 1993), and virulence has been associated with the loss of a glycosylation site at position 11 in field isolates of H5 subtypes (Kawaoka et al., 1984) and in laboratory derivatives passed in 14-day-old chicken embryos (Horimoto et al., 1995b). In this survey, both virulent and avirulent strains were shown to lack the site at asparagine 11, and four potential glysosylation patterns were identified for natural isolates. An alpha-carbon tracing model of the consensus H5 hemagglutinin sequence predicted the position of the potential glycosylation at site 236 was on the surface of the HA molecule globular head near the receptor binding pocket at 220-224 (data not shown). Thus the acquisition of glycosylation at site 236 may be an adaptive fea-

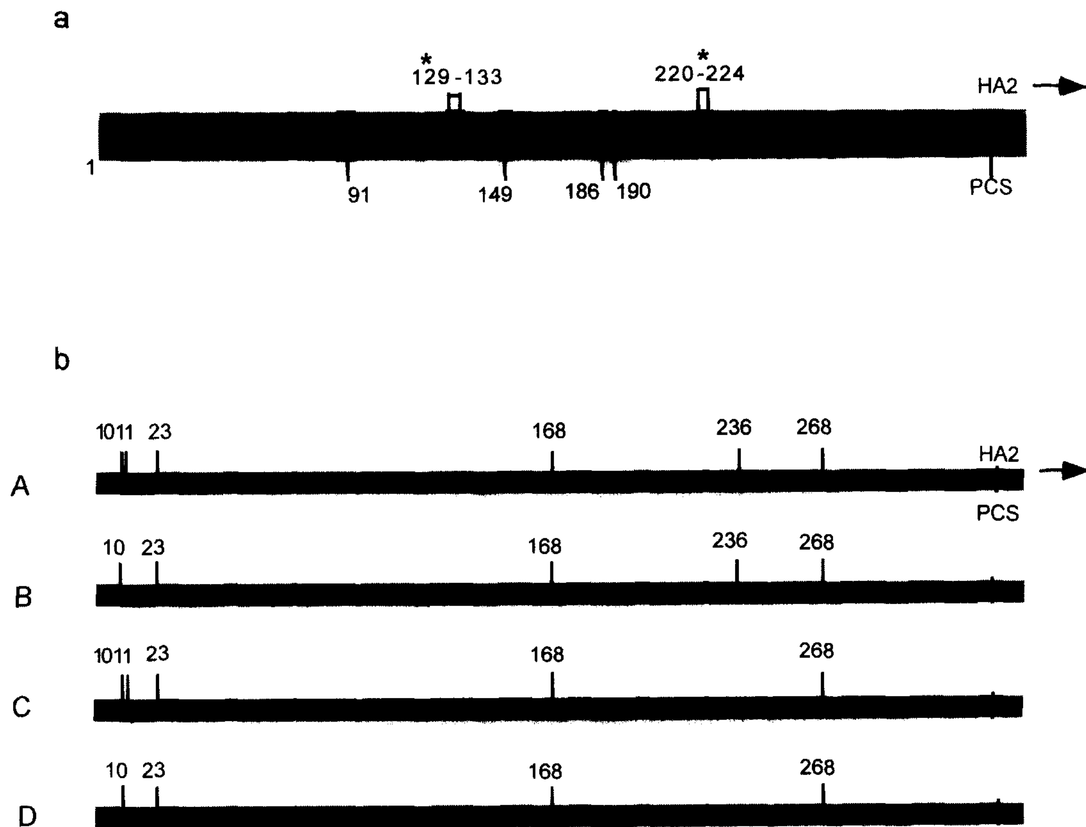

Fig. 3. Consensus structural features of H5 hemagglutinin genes. (A) Schematic diagram of the HA_1 subunit and fusion peptide indicating positions of amino acids predicted to form the receptor binding pocket of the HA molecule for avian influenza H5 virus. Amino acid positions are indicated by the numbers below and above the diagram. Position 1 was given to the first residue of the coding sequence of the HA_1 subunit. The proteolytic cleavage site (PCS) is indicated at amino acid position 326. The receptor binding site is formed by amino acids 129–133 (Ser-Gly-Val-Ser-Ser), 220–224 (Asn-Gly-Gln-Ser-Gly), 91 (Tyr), 149 (Trp), 186 (Glu) and 190 (Leu). The dark boxes indicate the conserved amino acids which surround the receptor binding amino acids. Amino acid substitutions were observed at position 129 (Ser → Thr) for the HA_1 sequence of RDe91 and at position 223 (Ser → Asn) for the HA1 sequence of TOn66. The position of the amino acid substitutions are marked with an asterisk. (B) Potential glycosylation site distribution patterns among North American H5 viruses. PCS, proteolytic cleavage site. The amino acid positions for the asparagine (Asn) residue in the potential glycosylation sequences are shown as follows: (A) sequences of CPe83E, CMx93, CJa94A, CJa94B, CQ95A, and CQ95B; (B) sequences of CPe83L and CHi94; (C) sequences of TOn66, TWi68, MWi75A, MWi75B, MWi75C, DMi80, TMn81, MOh87, MOh88, RDe91, CPe93, CFl93 and TMn95; (D) ETx93 sequence.

ture of H5 viruses associated with efficient replication and/or transmissibility in commercial chickens. Studies are in progress to determine whether this is the case, as such a marker site might be useful in predicting the length of time an AI virus has been in a particular population of hosts.

These data clearly indicate both a substantial level of sequence variation in the hemagglutinin gene, and re-assortment of gene segments resulting in stable sub-lineages in wild waterfowl populations. There is an apparent significant increase in rates of change and possible adaptive changes occurring when the viruses enter commercial poultry. It is not known whether this is due to increased immune pressure, transfer between avian orders, a real increase in mutation rate, or simply due to the increased concentration of available hosts and increased number of isolates.

An important meaning of the findings, however, is that recently proposed vaccination protocols for controlling the HP H5 subtype strains may ultimately face the same inherent antigenic drift problems faced when vaccinating against human type A influenza viruses.

It has been previously suggested (Gorman et al., 1992; Webster et al., 1992) that avian influenza viruses are in 'evolutionary stasis', having adapted to a primordial reservoir of feral waterfowl and thus undergoing negative genetic selection to remain in such an adaptation. These conclusions were reached based upon phylogenetic analysis of primarily the nucleoprotein (NP) gene (Webster et al., 1992), indicating a lower level of positive selection in the NP gene sequences when avian strains are compared to human or swine strains. However, the data in this report based on the HA and NS genes do not appear consistent with the idea of 'evolutionary stasis'. Conversely, they suggest that avian influenza viruses continue to evolve at a significant rate in various orders, families, genus and species of the class Aves.

References

Deibel, R., Emord, D.E., Dukelow, W., Hinshaw, V.S., Wood, M.W., 1985. Influenza viruses and paramyxoviruses in ducks in the Atlantic flyway, 1977-1983, including H5N2 isolate related to the virulent chicken virus. Avian Dis. 29, 970–985.

Eckroade, R.J., Bachin, L.A., 1987. Avian influenza in Pennsylvania-the beginning. In: Proceedings 2nd International Symposium on Avian Influenza. US Animal Health Association, Athens, GA, pp. 22-32.

García, M., Crawford, J., Latimer, J.W., Rivera-Cruz, E., Perdue, M.L., 1996. Heterogeneity in the haemaglutinin gene and emergence of the highly pathogenic phenotype among recent H5N2 avian influenza viruses in Mexico. J. Gen. Virol. 77, 1493–1504.

Gorman, O.T., Bean, W.J., Webster, R.G., 1992. Evolutionary processes in influenza viruses: divergence, rapid evolution, and stasis. Curr. Top. Microbiol. Immunol. 176, 75–97.

Graves, I.V., 1992. Influenza viruses in birds of the Atlantic flyway. Avian Dis. 36, 1–10.

Halvorson, D., Karunakaran, D., Senne, D., Kelleher, C., Bailey, C., Abraham, A., Hinshaw, V.S., Newman, J., 1982. Epizootiology of avian influenza-simultaneous monitoring of sentinel ducks and turkeys in Minnesota. Avian Dis. 27, 77–85.

Hinshaw, V.S., Webster, R.G., Turner, B., 1980. The perpetuation of orthomyxoviruses and paramyxoviruses in Canadian waterfowl. Can. J. Microbiol. 26, 622–629.

Horimoto, T., Rivera, E., Pearson, J., Senne, D., Krauss, S., Kawaoka, Y., Wester, R.G., 1995a. Origin and molecular changes associated with emergence of a highly pathogenic H5N2 influenza virus in Mexico. Virology 213, 223–230.

Horimoto, T., Ito, T., Alexander, D.J., Kawaoka, Y., 1995b. Cleavability of hemagglutinin from an extremely virulent strain of avian influenza virus containing a unique cleavage site sequence. J. Vet. Med. Sci. 57, 927–930.

Inkster, M.D., Hinshaw, V.S., Schulze, I.T., 1993. The hemagglutinins of duck and human H1 influenza viruses differ in sequence conservation and glycosylation. J. Virol. 67, 7436–7443.

Kawaoka, Y., Webster, R.G., 1988. Molecular mechanism of acquisition of virulence in influenza virus in nature. Microb. Pathog. 5, 311–318.

Kawaoka, Y., Naeve, C.W., Webster, R.G., 1984. Is virulence of H5N2 influenza viruses in chickens associated with loss of carbohydrate from the hemagglutinin?. Virology 139, 303–316.

Lang, G., Narayan, O., Rouse, B.T., Ferguson, A.E., Connell, M.C., 1968. A new influenza virus A infection in turkeys. II. A highly pathogenic variant, A/turkey/Ontario/7732/66. Can. Vet. J. 9, 151–160.

Ludwig, S., Schultz, U., Mandler, J., Fitch, W.M., Scholtissek, C., 1991. Phylogenetic relationship of the nonstructural (NS) genes of influenza A viruses. Virology 183, 566–577.

Nettles, V.F., Wood, J.M., Webster, R.G., 1985. Wildlife surveillance associated with an outbreak of lethal H5N2 avian influenza in domestic poultry. Avian Dis. 29, 733–741.

Perdue, M.L., Wainright, P., Brugh, M., 1990. Effects of chicken embryo age on time death following infection with avian influenza viruses: implications for distinguishing highly pathogenic isolates. Virus Res. 16, 137–152.

Rohm, C., Horimoto, T., Kawaoka, Y., Suss, J., Webster, R.G., 1995. Do hemagglutinin genes of highly pathogenic avian influenza viruses constitute unique phylogenetic lineages?. Virology 209, 664–670.

Rott, R., Orlich, M., Scholtissek, C., 1979. Correlation of pathogenicity and gene constellation of Influenza A viruses. III. Non-pathogenic recombinants derived from highly pathogenic parent strains. J. Gen. Virol. 44, 471–477.

Saito, T., Horimoto, T., Kawaoka, Y., Senne, D.A., Webster, R.G., 1994. Emergence of a potentially pathogenic H5N2 influenza virus in chickens. Virology 201, 277–284.

Senne, D.A., Pearson, J.E., Panigrahy, B., 1992. Live poultry markets: a missing link in the epidemiology of avian influenza. In: Proceedings 3rd International Symposium on Avian Influenza, Madison, WI, pp. 50-58.

Senne, D.A., Panigrahy, B., Kawaoka, Y., Pearson, J.E., Suss, J., Lipkind, M., Kida, H., Webster, R.G., 1996a. Survey of the hemagglutinin (HA) cleavage site: sequence of H5 and H7 avian influenza viruses: amino acid sequence at the HA

cleavage site as a marker of pathogenicity potential. Avian Dis. 40, 425–437.

Senne, D.A., Rivera, E., Panigrahy, B., Fraire, M., Kawaoka, Y., Webster, R.G., 1996b. Characterization of avian influenza H5N2 isolates recovered from chickens in Mexico. In: Jensen, M.M., Ceniceros, M.A. (Eds.), Proceedings of the 45th Western Poultry Disease Conference, Cancun, Mexico. University of California, Davis, Conferences Services, pp. 5-8.

Slemons, R.D., Easterday, B.C., 1977. Type-A influenza viruses in the feces of migratory waterfowl. J. Am. Vet. Med. Assoc. 171, 947–948.

Slemons, R.D., Johnson, D.C., Osborn, J.S., Hayes, F., 1974. Type-A influenza viruses isolated from wild free-flying ducks in California. Avian Dis. 18, 119–124.

Slemons, R.D., Shieldcastle, M.C., Heyman, L.D., Bednarik, K.E., Senne, D.A., 1991. Type A influenza viruses in waterfowl in Ohio and implications for domestic turkeys. Avian Dis. 35, 165–173.

Smith, L.M., Sanders, J.Z., Kaiser, R.J., Hughs, P., Dodd, C., Connell, C.R., Heines, C., Kent, S.B.H., Hood, L.E., 1986. Fluorescence detection in automated DNA sequence analysis. Nature 321, 673–681.

Stallknecht, D.E., Shane, S.M., Zwank, P.J., Senne, D.A., Kearny, M.T., 1990. Avian influenza viruses from migratory and resident ducks in coastal Louisiana. Avian Dis. 34, 398–405.

Swofford, D. (1989). PAUP: Phylogenetic Analysis Using Parsimony, Version 3. Illinois Natural History Survey, Champaign, IL.

Treanor, J.J., Snyder, M.H., London, W.T., Murphy, B.R., 1989. The B allele of the NS gene but not the A allele, attenuates a human influenza A virus for squirrel monkeys. Virology 171, 1–9.

Van Campen, H., Easterday, B.C., Hinshaw, V.S., 1989. Virulent avian influenza A viruses: their effect on avian lymphocytes and macrophages in vivo and in vitro. J. Gen. Virol. 70, 2887–2895.

Webster, R.G., Rott, R., 1987. Influenza virus A pathogenicity: the pivotal role of the hemagglutinin. Cell 50, 665–666.

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

Wood, G.W., McCauley, J.W., Bashiruddin, J.B., Alexander, D.J., 1993. Deduced amino acid sequences at the hemagglutinin cleavage site of avian influenza A viruses of H5 and H7 subtypes. Arch. Virol. 130, 209–217.

Wood, G.W., Banks, J., McCauley, J.W., Alexander, D.J., 1994. Deduced amino acid sequences of the haemagglutinin of H5N1 avian influenza virus isolates from an outbreak in turkeys in Norfolk, England. Arch. Virol. 134, 185–194.