Orange: Comments on influenza genetic stability

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Evolution and Ecology of Influenza A Viruses

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There is nothing permanent except change.
Heraclitus

INTRODUCTION

Influenza is the paradigm of a viral disease in which continued evolution of the virus is of paramount importance for annual epidemics and occasional pandemics of disease in humans. The gene pool of influenza A viruses in aquatic birds provides all the genetic diversity required for the emergence of pandemic influenza viruses for humans, lower animals, and birds. In humans, pigs, and horses, influenza A viruses show both antigenic drift and genetic shift. In contrast, there is emerging evidence that avian influenza viruses are in evolutionary stasis. The purpose of this review is to establish that rapid evolution in influenza A viruses in humans and other mammals has continued since the beginning of recorded medical history and is dependent on periodic introductions of gene segments or entire influenza viruses from the avian influenza virus gene pool. In aquatic wild birds, influenza virus appears to be fully adapted to its host and causes no disease signs. The understanding of the ecology of influenza viruses in this benign reservoir of aquatic birds is imperative if we wish to find ways to intervene and reduce or prevent the occasional catastrophic pandemics such as the one that decimated the human population of the world in 1918 after the appearance of "Spanish" influenza.

Continuing evolution is most prominent in the surface glycoproteins of influenza viruses but also occurs in each of the eight gene segments of both type A and type B influenza viruses. The variability results from accumulation of molecular changes in the eight RNA segments that can occur by a number of different mechanisms including (i) point mutations (antigenic drift), (ii) gene reassortment (genetic shift), (iii) defective-interfering particles, and (iv) RNA recombination. Each of these mechanisms may contribute to the evolution of influenza viruses.

Mutations, including substitutions, deletions, and insertions, are one of the most important mechanisms for producing variation in influenza viruses. The lack of proofreading among RNA polymerases contributes to replication errors on the order of 1 in 10^4 bases (64, 168). This contrasts with the much higher replication fidelity found among DNA polymerases, i.e., errors on the order of 1 in 10^9 bases per replication cycle. Each round of RNA virus replication results in a mixed population with many variants, most of which are not viable, but some of which have potentially advantageous mutations that can become dominant under the right selective conditions. The surprise from recent studies on influenza viruses is not the extent of genetic variation, but rather the apparent genetic stability of influenza viruses in aquatic avian reservoirs.

Since influenza viruses have segmented genomes, reassortment is an important mechanism for producing diversity very rapidly; it occurs among influenza A viruses in nature and is important in the appearance of pandemics in human populations. Reassortment has been demonstrated between influenza B virus strains in the laboratory but is probably not important for producing novel gene combinations, for there is no known influenza B virus gene pool except in humans. Other mechanisms for producing genetic variation include defective-interfering particle-mediated interference and intramolecular recombination. Although defective-interfering particles can influence evolution by reducing the yields of nondefective particles and modifying pathogenicity (168), their role in influenza virus evolution has not received much attention. Although intramolecular recombination in negative-stranded viruses is rare, recent studies have shown one instance of insertion of cellular mRNA sequences into the hemagglutinin (HA) gene with acquisition of virulence (85). This provides another mechanism for providing rapid evolutionary changes.

Ecology concerns the interrelationships of organisms and their environment. In this report, we will deal mainly with the interaction of influenza viruses with aquatic birds and how this permits the perpetuation of all known influenza virus subtypes in nature. This includes virus adaptation in different hosts, transmission, and avoidance of the host immune system. We will also deal with the geographical and seasonal distribution of influenza viruses in avian species from a global perspective.

Since influenza viruses have been collected for more than 90 years from many hosts in different geographical regions, they provide a unique resource for examining the ecology and evolution of an RNA virus. Influenza viruses were isolated at the beginning of the 20th century from chickens (A/Brescia/1902 [H7N7]), from pigs in the late 1920s, from humans in the early 1930s, from horses and domestic ducks in the 1950s, from terns in 1961, and from many waterfowl and shorebirds since 1974 (59, 76). The early influenza viruses were initially maintained by animal-to-animal passage and later by freeze-drying; reliable low-temperature storage (−70°C) has been available since the 1950s. The advent of rapid sequencing technology has provided a plethora of sequence information which has permitted phylogenetic analysis for most of the influenza virus genes. Comparison between the phylogenetic trees of the different gene segments of influenza A viruses has not previously been done and is one of the topics of this review.

After the appearance of the Asian/57 and Hong Kong/68 human pandemic influenza viruses, Martin Kaplan in the World Health Organization encouraged investigators to study influenza viruses in lower animals and birds in order to elucidate the origin of pandemic influenza viruses. Studies on the ecology of influenza A viruses established that all known influenza A viruses are perpetuated in aquatic birds, and a hypothesis was proposed (59) that aquatic birds are the primordial source of all influenza viruses in other species. This review uses the currently available sequence information about each influenza virus gene segment to compare their phylogenetic relationships and show how this information relates to our knowledge of the ecology of influenza viruses. Such information is used to test the hypothesis that aquatic birds are the source of all influenza viruses in other species. Finally we examine this information for the purpose of developing strategies that could prevent the appearance of future epidemics or pandemics of influenza viruses.

STRUCTURE AND FUNCTION OF THE INFLUENZA VIRUS VIRION

Current knowledge of the molecular biology of influenza viruses has been recently reviewed in extensive detail by Lamb (95), and individual references are not given. This section presents a summary of that knowledge as background information.

Components of the Virion

Influenza A viruses are members of the *Orthomyxoviridae* family. They are differentiated from type B and C influenza

viruses on the basis of the identity of the major internal protein antigens, the nucleoprotein (NP) and matrix (M1) proteins. On initial isolation, influenza A viruses are small (80 to 120 nm in diameter), pleomorphic particles that later become generally spherical. These particles consist of a host-derived lipid bilayer envelope in which the virus-encoded glycoproteins HA and neuraminidase (NA) and M2 are embedded; an inner shell of matrix protein; and, at the center, the nucleocapsids of the viral genome (Fig. 1). The genome of influenza A viruses consists of eight unique segments of single-stranded RNA, which are of negative polarity (i.e., complementary to the mRNA sense). The RNA is loosely encapsidated by multiple NP molecules. Complexes containing the three viral polymerase proteins (PB1, PB2, and PA) are situated at the ends of the nucleocapsids.

To be infectious, a single virus particle must contain each of the eight unique RNA segments. Available evidence suggests that incorporation of RNAs into virions is at least partly random. The random incorporation of RNA segments allows the generation of progeny viruses containing novel combinations of genes (i.e., genetic reassortment) when cells are doubly infected with two different parent viruses.

The eight influenza A viral RNA segments encode 10 recognized gene products. These are PB1, PB2, and PA polymerases, HA, NP, NA, M1 and M2 proteins, and NS1 and NS2 proteins.

PB2 polymerase. PB2 polymerase is encoded by RNA segment 1, the slowest-migrating RNA species by gel electrophoresis. It is a member of the protein complex providing viral RNA-dependent RNA polymerase activity. It is known to function during initiation of viral mRNA transcription as the protein which recognizes and binds the 5′ capI structures of host cell mRNAs for use as viral mRNA transcription primers. Endonucleolytic cleavage of these cap structures from host mRNAs is also at least in part a function of PB2. The role of PB2 in the other virus-directed RNA synthetic processes, i.e., synthesis of full-length template cRNA and new negative-sense viral RNA (vRNA), is not known since these processes do not require host cap priming. Newly synthesized PB2 proteins migrate to the nucleus of infected cells.

PB1 polymerase. PB1 polymerase is encoded by RNA segment 2; it functions in the RNA polymerase complex as the protein responsible for elongation of the primed nascent viral mRNA and also as elongation protein for template RNA and vRNA synthesis. PB1 proteins localize in the nucleus of infected cells.

PA polymerase. PA polymerase is encoded by RNA segment 3. It also localizes in the infected cell nucleus and is a member of the RNA-dependent RNA polymerase complex along with PB1 and PB2, but its role in viral RNA synthesis is unknown. There is evidence for possible roles as a protein kinase or as a helix-unwinding protein.

Hemagglutinin. The HA protein is an integral membrane protein and the major surface antigen of the influenza virus virion. It is responsible for binding of virions to host cell receptors and for fusion between the virion envelope and the host cell. HA is encoded by RNA segment 4. It undergoes three kinds of posttranslational processing: proteolytic cleavage, glycosylation, and fatty acid acylation. Newly synthesized HA is cleaved to remove the amino-terminal hydrophobic sequence of 14 to 18 amino acids, which are the signal sequence for transport to the cell membrane. Carbohydrate side chains are added, whose number and position vary with the virus strain. Palmitic acid is added to cysteine

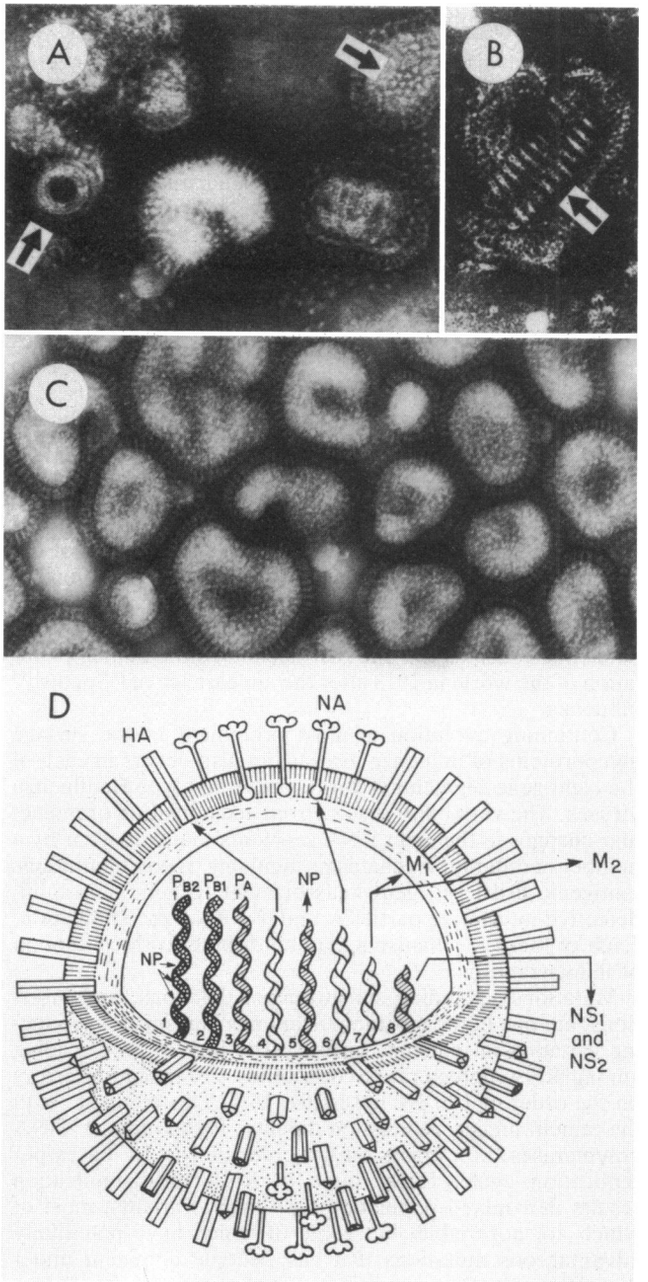

FIG. 1. Structure of the influenza virus virion. (A) Micrograph showing a partially lysed influenza virus virion with a helical internal component (left-hand arrow). An extended helix obtained by Titron X-100 treatment of the virus is also visible (right-hand arrow). (B) A ribonucleoprotein particle segment of influenza virus examined by immunogold labeling with antibodies to polymerase protein is shown (arrow). The dense gold particle locating the polymerase proteins is at one end. (C) Electron micrograph of negatively stained A/WSN/34 (H1N1) influenza virus. The virions are roughly spherical and contain glycoprotein spikes. (D) Diagram of the virion. The glycoprotein spikes are approximately 16 nm long and are of two kinds: the HA predominates, and the NA occurs in patches. Inside the lipid bilayer are eight segments of single-stranded RNA, each of which codes for one protein (sometimes two). The NP-RNA-polymerase complex occurs in a helix (see panel A), not as individual segments indicated by the diagram. Electron micrographs were taken by Gopal Murti.

residues near the HA carboxy terminus. The final processing step is cleavage of the HA into two subunits, HA1 and HA2 (uncleaved HA is called HA0), connected by disulfide linkages. This cleavage is accomplished by host-produced trypsinlike proteases and is required for infectivity because virus-cell fusion is mediated by the free amino terminus of HA2. The fully processed HA thus consists of HA1 of (typically) about 324 amino acids plus variable carbohydrate, and HA2 of (typically) about 222 amino acids plus variable carbohydrate plus 3 palmitate residues.

HA molecules form homotrimers during maturation. The three-dimensional structure of a complete HA trimer has been determined. In essence, each HA molecule consists of a globular head on a stalk. The head is made up exclusively of HA1 and contains the receptor-binding cavity as well as most of the antigenic sites of the molecule. The stalk consists of all of HA2 and part of HA1. The carboxy-terminal region of HA2 contains the hydrophobic transmembrane sequence and a terminal cytoplasmic anchor sequence where palmitate is attached.

Owing to error-prone viral RNA polymerase activity, influenza virus HA is subject to a very high rate of mutation, estimated at about 2×10^{-3} base substitutions per position per virus generation, or about one base substitution in the HA gene per virus generation. Selection for amino acid substitutions is driven at least in part by immune pressure, as the HA is the major target of the host immune response. Although the amino acids making up the receptor-binding site, as well as cysteine and most proline residues, are highly conserved, the remainder of the HA molecule is highly mutable. In nature there are presently 14 recognized subtypes of HA (called H1, H2, etc.), which differ by at least 30% in the amino acid sequence of HA1 and which are serologically not cross-reactive (Table 1). Subtypes may include several variant strains which are partially serologically cross-reactive.

Nucleoprotein. NP is encoded by RNA segment 5. It is transported into the infected cell nucleus, where it binds to and encapsidates viral RNA. In addition to its structural role, NP is believed to play a role in the switching of viral RNA polymerase activity from mRNA synthesis to cRNA and vRNA synthesis. NP is abundantly synthesized in infected cells and is the second most abundant protein in the influenza virus virion. It is phosphorylated; the pattern of phosphorylation is host cell dependent and may be related to viral host range restriction. NP is also a major target of the host cytotoxic T-cell immune response.

Neuraminidase. NA, encoded by RNA segment 6, is also an integral membrane glycoprotein and a second major surface antigen of the virion. NA cleaves terminal sialic acid from glycoproteins or glycolipids. Thus, it functions to free virus particles from host cell receptors, to permit progeny virions to escape from the cell in which they arose, and so facilitate virus spread.

NA is glycosylated and possesses an amino-terminal hydrophobic sequence which functions both as signal for transport to the cell membrane and as transmembrane domain; it is not cleaved away. The distribution of NA has not been conclusively resolved; immunogold-labeling experiments suggest that the NA tetramers are not evenly distributed over the virion envelope, as is HA, but aggregate into patches or caps. The complete three-dimensional structure of an NA tetramer, bound to antibody, has been determined.

Like HA, NA is highly mutable with variant selection partly in response to host immune pressure. Nine subtypes of NA (called N1, N2, etc.) have been identified in nature; they are not serologically cross-reactive (Table 1). Different variants of several subtypes are known.

TABLE 1. HA and NA subtypes of influenza A viruses isolated from humans, lower mammals, and birds

Subtype	Virus found in species of origin[a]			
	Humans	Swine	Horses	Birds
HA				
H1	PR/8/34	Sw/Ia/15/30	–[b]	Dk/Alb/35/76
H2	Sing/1/57	–	–	Dk/Ger/1215/73
H3	HK/1/68	Sw/Taiwan/70	Eq/Miami/1/63	Dk/Ukr/1/63
H4	–	–	–	Dk/Cz/56
H5	–	–	–	Tern/S.A./61
H6	–	–	–	Ty/Mass/3740/65
H7	–	–	Eq/Prague/1/56	FPV/Dutch/27
H8	–	–	–	Ty/Ont/6118/68
H9	–	–	–	Ty/Wis/1/66
H10	–	–	–	Ck/Ger/N/49
H11	–	–	–	Dk/Eng/56
H12	–	–	–	Dk/Alb/60/76
H13	–	–	–	Gull/Md/704/77
H14	–	–	–	Dk/Gurjev/263/82
NA				
N1	PR/8/34	Sw/Ia/15/30	–	Ck/Scot/59
N2	Sing/1/57	Sw/Taiwan/70	–	Ty/Mass/3740/65
N3	–	–	–	Tern/S.A./61
N4	–	–	–	Ty/Ont/6118/68
N5	–	–	–	Sh/Austral/1/72
N6	–	–	–	Dk/Cz/56
N7	–	–	Eq/Prague/1/56	FPV/Dutch/27
N8	–	–	Eq/Miami/1/63	Dk/Ukr/1/63
N9	–	–	–	Dk/Mem/546/74

[a] The reference strains of influenza viruses, or the first isolates from that species, are presented.
[b] –, not found in this species.

M1 protein. Influenza virus RNA segment 7 is bicistronic, encoding both M1 and M2 proteins. Colinear transcription of segment 7 yields mRNA for the matrix protein. This is the most abundant protein in the influenza virus virion. Matrix protein forms a shell surrounding the virion nucleocapsids, underneath the virion envelope. In the infected cell it is present in both cytoplasm and nucleus. It has no known enzymatic activity, although it has been speculated to play an important role in initiating progeny virus assembly.

M2 protein. The mRNA for M2 is also transcribed from RNA segment 7. It is derived from the colinear (M1) transcript by splicing. M2 is an integral membrane protein, whose membrane-spanning domain also serves as a signal for transport to the cell surface. It is present as a tetramer in large amounts on the infected cell surface, and a small amount is found in the virion. It is believed to act as a proton channel to control the pH of the Golgi during HA synthesis and to allow acidification of the interior of the virion during virus uncoating.

Nonstructural NS1 and NS2 proteins. RNA segment 8 encodes the two nonstructural proteins NS1 and NS2. NS1 mRNA is colinear with the vRNA, whereas NS2 mRNA is derived by splicing. These proteins, particularly NS1, are abundant in the infected cell (NS1 primarily in the nucleus, NS2 primarily in the cytoplasm) but are not incorporated into progeny virions. Both proteins play roles in virus replication, but those roles have not been fully defined. NS2 appears to modulate the synthesis of NS.

Influenza Virus Replication Cycle

The expression and replication of influenza viruses have been extensively reviewed by Krug et al. (91), and this section serves as background information. An influenza virus particle with cleavage-activated HA binds to cells via interaction between the receptor-binding site of HA and the terminal sialic acid of the cell surface receptor glycoprotein or glycolipid. Following binding, the attached virion is endocytosed by the cell. The low pH of the endocytotic vesicle triggers a conformational change in cleaved HA which is believed to facilitate insertion of the hydrophobic free amino terminus of HA2 into the vesicular membrane, initiating fusion of the viral and vesicular membranes. Fusion releases the contents of the virion into the cytoplasm of the cell.

The nucleocapsids of the parent virus migrate into the host cell nucleus, and their associated polymerase complexes begin primary transcription of mRNA. The primary transcripts are used for translation of viral proteins, which in the early stage of infection are predominantly NP and NS1. Translation of host mRNAs is blocked. Newly synthesized NP and NS1 migrate to the nucleus. It is believed that the increased concentration of free NP triggers the shift from mRNA synthesis to cRNA and vRNA synthesis by the infecting viral genome. Newly synthesized vRNAs are encapsidated in NP within the nucleus and function as templates for secondary transcription of viral mRNAs. Later in infection, the principal translation products are M1, HA, and NA proteins. HA and NA proteins are posttranslationally processed and transported to the cell surface, where they integrate into the cell membrane.

Recent evidence suggests that buildup of M1 protein in the nucleus is associated with the migration of nucleocapsids out of the nucleus for assembly into progeny viral particles in the cytoplasm. Few details of the assembly process are known. Generally, a viral core of nucleocapsids becomes encased in a shell of M1 protein and buds outward through the cell membrane, enclosing itself within a bubble of membrane as its own envelope, complete with the viral surface glycoproteins. Interactions between M1 and the cytoplasmic domains of HA, NA, or M2 have been proposed as signals for budding. NA activity of progeny virions releases them from the host cell.

With most influenza viruses, the final step in virus maturation is extracellular; this is the cleavage of HA0 into HA1 and HA2 by host proteases. Cleaved HA is relatively unstable at low pH; therefore, with avian influenza viruses, which are transmitted primarily by the fecal-oral route, cleavage probably occurs after excreted virions have entered into their new host and passed through the stomach. HAs of the highly virulent avian influenza viruses are probably cleaved intracellularly. HAs of mammalian influenza viruses are probably cleaved by extracellular proteases of the respiratory tract in either the original or the new host.

RESERVOIRS OF INFLUENZA A VIRUSES IN NATURE

Influenza A viruses infect a variety of animals, including humans, pigs, horses, sea mammals, and birds. Recent phylogenetic studies of influenza A viruses have revealed species-specific lineages of viral genes and have demonstrated that the prevalence of interspecies transmission depends on the animal species. They have also revealed that aquatic birds are the source of all influenza viruses in other species, as illustrated in Fig. 2.

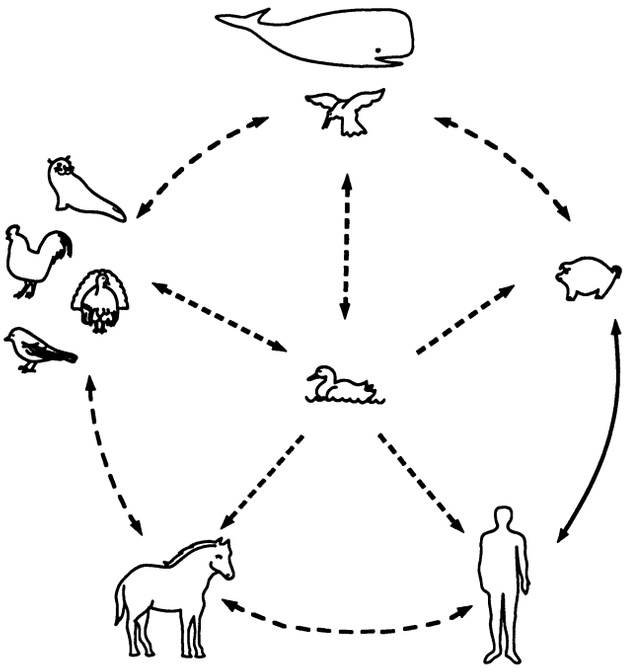

FIG. 2. Reservoir of influenza A viruses. The working hypothesis is that wild aquatic birds are the primordial reservoir of all influenza viruses for avian and mammalian species. Transmission of influenza virus has been demonstrated between pigs and humans (solid lines). There is extensive evidence for transmission between wild ducks and other species, and the five different host groups are based on phylogenetic analysis of the NPs of a large number of different influenza viruses (46).

Influenza Viruses in Birds: Wild Ducks, Shorebirds, Gulls, Poultry, and Passerine Birds

Extensive surveillance of birds during an outbreak of Newcastle disease in poultry in California showed that many nonpathogenic influenza A viruses could be isolated from birds (160). Subsequently, influenza viruses were isolated from wild birds, captive caged birds, and domestic ducks, chickens, and turkeys, leading to the realization that nonpathogenic avian influenza viruses are ubiquitous, particularly in aquatic birds (5), and that all of the different subtypes of influenza A viruses (H1 to H14 and N1 to N9) are perpetuated in aquatic birds, particularly in migrating waterfowl (62).

The disease signs associated with influenza A virus infections in avian species vary considerably with the strain of virus. Infections caused by most strains of influenza virus are completely asymptomatic; however, a few strains produce systemic infection accompanied by central nervous system involvement, with death occurring within 1 week. Viruses in the latter category include a few members of the H5 and H7 subtypes, such as A/FPV/Dutch/27 (H7N7) and A/Chicken/Pennsylvania/1370/83 (H5N2).

In wild ducks, influenza viruses replicate preferentially in the cells lining the intestinal tract, cause no disease signs, and are excreted in high concentrations in the feces (up to $10^{8.7}$ 50% egg infectious doses per g) (188). Avian influenza viruses have been isolated from freshly deposited fecal material and from unconcentrated lake water. This information indicates that waterfowl have a very efficient way to transmit viruses; i.e., via fecal material in the water supply.

If one considers that a large number of susceptible young ducks gather each year on Canadian lakes, it is understandable that many birds are infected by virus shed into the lake water. This would explain the high incidence of virus infection in Canadian ducks, particularly juveniles. Transmission by feces also provides a way for ducks, as they migrate through an area, to spread their viruses to other domestic and feral birds.

The avirulent nature of avian influenza infection in ducks may be the result of virus adaptation to this host over many centuries, creating a reservoir that ensures perpetuation of the virus. This speculation strongly suggests that ducks occupy a unique and very important position in the natural history of influenza viruses. Influenza viruses of avian origin have been implicated in outbreaks of influenza in mammals, such as seals (181, 182), whales (53), and pigs (147) in Europe, as well as in domestic poultry, especially turkeys (53) (see below).

Longitudinal studies of wild ducks in Canada from 1976 to 1989 revealed the following: (i) a high percentage (up to 20%) of juvenile birds have influenza virus infection when the birds congregate before migration; (ii) none of the birds show any symptoms of infection; and (iii) multiple subtypes of influenza virus are enzootic (62). These and other studies performed elsewhere established that 13 of the 14 HA subtypes and all the 9 known NA subtypes of influenza viruses are maintained in wild ducks; so far, the H13 subtype has been isolated only from shorebirds and gulls.

In wild ducks in the northern hemisphere, influenza viruses predominate in August and September. Juvenile birds are infected as they congregate in marshaling areas in Canada prior to migration, when up to 30% of birds hatched that year are shedding influenza viruses. This annual epidemic in wild ducks of influenza that causes no disease signs must affect the majority of juvenile birds. During southern migration from Canada the birds continue to shed virus, and by the time they reach the lower Mississippi in November the frequency falls to only 1.6 to 2% (167, 186); in December and January in Louisiana it falls to 0.4% (167). Samples collected from wild ducks as they arrive back in Canada after spring migration show a 0.25% isolation rate (unpublished data), which is sufficient to indicate that influenza viruses are brought back by the ducks. Certain subtypes of influenza viruses predominate in wild ducks in a particular flyway, but the predominant virus differs from one flyway to another and from year to year (63).

Systematic studies on ducks and whistling swans from Siberia that overwinter in Japan reveal an influenza virus isolation rate of between 0.5 and 9% during the winter months (127–129, 175). The virus isolation rate varied from year to year but was higher than that found during the winter months among ducks in the United States, reinforcing the idea that these viruses are perpetuated year round in a particular avian species. The predominant subtypes differ from year to year and during a season, as has been found in systematic studies in wild ducks in North America.

Influenza viruses with a variety of HA and NA subtypes have also been isolated from wild waterfowl in other parts of the world including Russia (99, 100), southern China (154), western Europe (4, 158, 169), Israel (97), and Australia (38, 103), demonstrating the worldwide extent of avian influenza virus gene pools in nature. Recent phylogenetic studies indicate that influenza viruses in Eurasia and Australia are genetically distinct from those in North America (37, 47), presumably owing to confinement of birds to the distinct flyways of each hemisphere. These studies demonstrate that

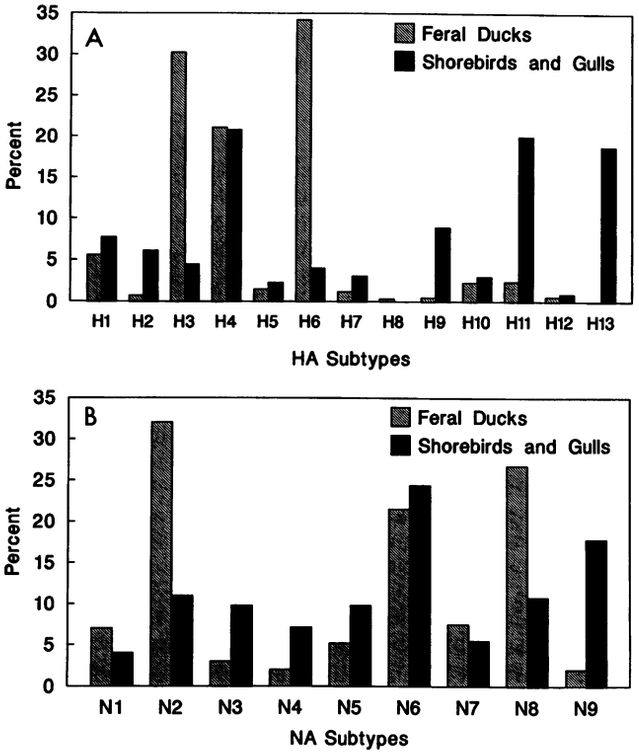

FIG. 3. Distribution of HA and NA subtypes of influenza A viruses in wild ducks and shorebirds plus gulls. (A) HA distribution. The 14 HA subtypes are unevenly distributed in ducks, shorebirds, and gulls; H3, H4, and H6 are most prevalent in ducks, whereas H4, H9, H11, and H13 are most prevalent in shorebirds and gulls. H8 is rare in ducks and has not been isolated from gulls. H13 has so far been found only in gulls. H14, a new subtype, is not shown and has been isolated from ducks. (B) NA distribution. All NA subtypes are found in ducks, gulls, and shorebirds. The N2, N6, and N8 subtypes are most prevalent in wild ducks, whereas N6 and N9 are most prevalent in shorebirds and gulls. The numbers for wild ducks are from surveillance for 13 years (1978 to 1990) in Alberta, Canada, and the numbers for shorebirds and gulls are from surveillance for 5 years (1986 to 1990) in Delaware Bay and adjacent coastlines of the United States.

evolution of influenza viruses can be influenced by the physical barriers that prevent intermixing of their hosts.

Influenza viruses have also been isolated sporadically from shorebirds, including gulls, terns, shearwaters, guillemots, and sandpipers (14, 59). A systematic study of shorebirds and gulls at Delaware Bay on the east coast of the United States revealed that influenza A viruses are prevalent in spring (May and June [up to 30%]) and fall (September and October [up to 8%]) (76). Most of the different HA and NA subtypes have been isolated, including H13. The predominant subtypes of virus in these birds differ from those in ducks (Fig. 3A). H3 and H6 viruses are prevalent in ducks; however, only a few H3 and H6 viruses have been isolated from shorebirds and gulls. By contrast, the predominant subtypes of viruses from shorebirds and gulls (H4, H9, H11, and H13) represent only a minor fraction of duck viruses. Although not as evident as the HA subtypes, the predominant NA subtypes in ducks (N2, N6, and N8) differ from those in shorebirds and gulls, in which N6 and N9 are the prevalent subtypes (Fig. 3B). The vast majority of these viruses are nonpathogenic in chickens and ducks, but the

original isolate from terns in South Africa (A/Tern/S.A./61 [H5N3]) (14) is highly pathogenic for domestic poultry. Experimental infections of Peking ducks with shorebird and gull isolates showed that only half of the shorebird and gull isolates have the potential to replicate in ducks. These studies established that the gene pool of influenza viruses in shorebirds and gulls is different from that in ducks.

Two groups of influenza viruses have been isolated from domestic poultry. The first group of viruses include the H5 and H7 subtypes and are highly virulent, causing 100% mortality experimentally. These viruses were known to be the causative agents of fowl plague, and later, in 1955, they were identified by Schafer as influenza viruses (144). The others are less virulent, have a variety of HA subtypes, and cause no death in experimental infection. The major determinant of the difference in virulence between the two groups of viruses is susceptibility of the HA to cellular proteases, which cleave it into HA1 and HA2 (187). Disease signs vary, depending on the species and age of poultry, strain of virus, and accompanying bacterial infection. Typical signs of highly pathogenic viruses in chickens or turkeys include decreased egg production; respiratory signs; rales; excessive lacrimation; sinusitis; cyanosis of unfeathered skin, especially of the combs and wattles; edema of the head and face; ruffled feathers; diarrhea; and nervous disorders.

These virulent avian viruses appear to originate from aquatic birds, because they have no other known reservoirs. However, compelling evidence that influenza viruses have been transmitted from pigs to turkeys is also available (60). Influenza viruses in live poultry markets in large cities such as New York and Miami may be the immediate origin of these viruses. Influenza viruses genetically closely related to those which caused the outbreak in Pennsylvania in 1983 to 1984 (e.g., A/Chicken/Pennsylvania/1370/83 [H5N2]) were isolated from birds in these markets for several years after the virus was eradicated from domestic poultry farms in Pennsylvania (183).

Influenza viruses have been isolated less frequently from passerine birds; however, studies during the highly pathogenic H7N7 outbreak in Australia in 1986 established that starlings and sparrows are susceptible to infection and are potential spreaders of avian influenza viruses (31, 122). Influenza viruses have also been isolated from other species of birds, including pet birds, which were either inapparently infected (123) or dead (6, 71, 107, 121). Since the prevalence of influenza viruses in these birds is limited, their role in evolution and maintenance of the viruses is unknown.

These studies have established the existence of a vast reservoir of influenza A viruses in aquatic birds. The evidence that all of the current mammalian influenza A viruses are probably derived from aquatic birds indicates that a vast influenza gene pool exists for future mammalian influenza viruses in nature.

Influenza Viruses in Pigs

Two subtypes of influenza A viruses, H1N1 and H3N2, have been isolated from pigs. These include classic swine H1N1, avianlike H1N1 and human- and avianlike H3N2 viruses. Influenza in swine was first observed in the United States during the catastrophic 1918 to 1919 human influenza pandemic. Signs of the disease in pigs, as in humans, consist of nasal discharge, coughing, fever, labored breathing, and conjunctivitis (152, 153). Pneumonia develops after intratracheal administration of the virus (192). As indicated by the isolation of A/Swine/Iowa/15/30 (H1N1) by Shope (152) and retrospective serological studies in humans, the classic swine virus is antigenically similar to the type A influenza virus responsible for the human pandemic (111). Swine influenza virus has remained in the swine population and has been responsible for one of the most prevalent respiratory diseases in pigs in North America.

Studies of pigs in the United States from 1976 to 1978 showed that swine influenza viruses were prevalent throughout the pig population, with approximately 25% of the animals having evidence of infection (57). The swine viruses were isolated throughout the year, contrary to earlier suggestions that the virus appeared on a seasonal basis and persisted through interepizootic periods by a complicated mechanism involving earthworms and lungworms (153).

Recent serological studies of pigs in the United States demonstrated that classic H1 influenza viruses continue to circulate at high frequency among pigs in the north-central United States (average incidence, 51%). Subtype H3 viruses antigenically similar to current human H3 viruses were circulating at a low frequency in 1988 to 1989 (average, 1.1%), particularly in the southeastern United States, although no virus isolation has been made since 1980 (25). At this time, swine influenza is not a major economic burden to farmers, and there have been no recent attempts to control this virus by vaccination.

Outbreaks of influenza in swine in Europe since 1980 have been associated with influenza A viruses that are antigenically and genetically distinguishable from classic swine (H1N1) viruses isolated from pigs in North America (132). The viruses are similar to H1N1 viruses isolated from ducks. The signs of disease have been typical of classic swine influenza, with high fever, 100% morbidity, and low mortality. The available evidence suggests that avian (H1N1) influenza viruses were transmitted to pigs and are causing significant disease (147). The epizootiology of influenza in swine in Europe is further complicated by the reintroduction of classic swine (H1N1) influenza virus into Italy from North America in 1976 (120), although this type of virus seems to have been replaced by avianlike H1N1 viruses in the mid-1980s (36a)

H3N2 viruses, antigenically similar to human strains, were also identified in swine in North America, Europe, and Asia and do not cause clinical signs of disease (93). These viruses, maintained in European pigs, now produce typical swine influenza symptoms. Preliminary data suggest that their NP and NS genes are replaced with those of avianlike H1N1 viruses cocirculating in Europe (23a). Some of the H3N2 swine viruses isolated in Asia, however, are entirely avianlike (86).

In pigs, in which swine influenza viruses (H1N1) are enzootic and H3N2 viruses are either enzootic or periodically introduced from humans, reassortants possessing H1N2 (Hsw1N2) have been detected in Japan (170). The isolation of this virus demonstrates that genetic reassortment can occur in nature between influenza A viruses in pigs, although this reassortant virus did not result in an epidemic strain.

During 1976, swine influenza virus (H1N1) was isolated from military recruits at Fort Dix, one of whom died of the disease. Antigenically and genetically indistinguishable isolates were subsequently obtained from a man and a pig on the same farm in Wisconsin (57). These studies confirmed the earlier serological and virus isolation studies that had implicated swine viruses in human disease (145). Serological studies of slaughterhouse workers indicated that swine influenza viruses are transmitted to humans relatively fre-

quently (up to 20% of workers in 1977 had antibodies to swine influenza virus), but in the recent past none of these incidents has resulted in an epidemic of disease in humans. However, the swine virus is still occasionally isolated from humans with respiratory illness (32) and occasionally is lethal (141).

The above information indicates that pigs serve as major reservoirs of H1N1 and H3N2 influenza viruses and are frequently involved in interspecies transmission of influenza viruses. Although these strains have shown a limited capacity to spread from pigs to humans, their maintenance in pigs and the frequent introduction of new viruses (46, 47, 86, 147) from other species may be important in the generation of pandemic strains of human influenza.

Influenza Viruses in Horses

Influenza viruses have probably been maintained in horses for centuries, based on the description of equine diseases, but the first isolation of virus was made in 1956 (164). Two different subtypes of influenza A virus (H3N8 and H7N7) have been identified in horses. The latter is commonly known as equine 1 virus, and the former is known as equine 2 virus. Both viruses produce similar disease signs in horses (e.g., dry hacking cough, fever, loss of appetite, muscular soreness, and tracheobronchitis), but infections produced by equine 2 virus are usually more severe (15, 20, 104). Secondary bronchial pneumonia almost always accompanies equine influenza. A high incidence of inflammation of the heart muscle (interstitial myocarditis) has been recorded in horses during and after A/Equine/Miami/1/63 (H3N8) (equine 2 virus) infections (44). These two types of viruses (equine 1 and 2 viruses) have cocirculated in equine hosts (176), providing the opportunity for genetic reassortment. Antigenic analysis failed to detect the predicted reassortants, but competitive RNA-RNA hybridization (10) and nucleotide sequence analyses (47) indicate that genetic exchange of viral genes encoding internal proteins has occurred in nature.

The H7N7 virus may have disappeared from the horse population, as the virus has not been isolated from horses since 1977. However, recent worldwide serological surveillance demonstrated that the H7N7 viruses may still be circulating at marginal levels in Central Asia (179a.) Recently, equine influenza outbreaks were observed in South Africa, India, and China, where equine influenza viruses were not known to be circulating. The causative agent of the South African outbreak was identified as an H3N8 virus, which was probably introduced by horses from the United States (79). The virus responsible for the Chinese outbreak is especially intriguing. Although it contains the same surface antigens, H3N8, as the other common equine 2 influenza viruses, its genetic features are avianlike, indicating that another H3N8 virus from birds was recently introduced into horses (50). How, from the vast number of avian influenza viruses with different HA and NA subtypes that can be found in nature, this H3N8 virus came to be introduced into horses remains unknown. However, avian influenza viruses with this combination of surface proteins (H3N8) may have a selective advantage over other subtypes for replication in horses. Seroarcheological studies indicate that an H3N8 virus appeared in 1890 in the human population, coincident with historical records of an influenza pandemic in that year. This development has a precedent in Europe, where an H1N1 virus was introduced from birds into pigs in the early 1980s.

Phylogenetic studies indicate that the common equine H3 HA gene was introduced into horses from birds long ago and has been maintained in this species for a long time (77). Similar analysis of the other genes from equine influenza viruses also showed that exchange of the influenza virus genes between horses and other species is limited, in contrast to frequent interspecies transmission between pigs and other species (47, 86). This suggests that horses may be an isolated or dead-end reservoir for influenza A viruses.

Influenza Viruses in Other Species: Seals, Mink, and Whales

In 1979 to 1980, approximately 20% of the harbor seal (*Phoca vitulina*) population of the northeastern coast of the United States died of a severe respiratory infection with consolidation of the lungs, typical of primary viral pneumonia (43). Influenza virus particles were found in high concentrations in the lungs and brains of the dead seals and were subtyped as H7N7 (e.g., A/Seal/Massachusetts/1/80). Competitive RNA-RNA hybridization showed that all the genes were closely related to those from different avian influenza virus strains. Biologically, however, the virus behaved more like a mammalian strain, replicating to high titer in ferrets, cats, and pigs. By contrast, it replicated poorly in avian species, produced no disease signs, and was not shed in feces. This is probably due to its rapid adaptation to mammalian hosts. It also showed the potential to cause conjunctivitis in humans, but did not spread further. Infected persons recovered without complications, and antibodies to the virus did not develop in the serum of infected individuals (181). In squirrel monkeys, the seal influenza virus replicated in the lungs and nasopharynx after intratracheal administration (112) and in the conjunctiva after administration into the eye. In one monkey that died of pneumonia, the virus was recovered from the spleen, liver, muscles, and lungs, indicating that the virus has the capability for systemic spread in primates.

It is not known whether the A/Seal/Mass/1/80 virus originated by transmission from birds or whether influenza in seals had previously escaped detection. Serological and biological information favors the first explanation because surveillance studies provided no serological evidence for influenza infection in surviving animals on the New England coast. Additionally, there has been no further evidence of this virus in seals since 1980.

The A/Seal/Mass/1/80 (H7N7) influenza virus provided the first evidence that a strain deriving all of its genes from an avian influenza virus can be associated with severe disease in a mammalian population in nature. This raises the possibility that some human or animal influenza viruses are derived directly from avian strains, as we postulate for the 1918 pandemic strain (see below).

Another strain of influenza virus was isolated from the lungs and brains of harbor seals (*P. vitulina*) found dead on the New England coast of the United States in 1983 (58). It was associated with low mortality (approximately 2 to 4%) and was subtyped as H4N5. Each of the eight RNA segments was related to those from avian influenza viruses but was distinguishable from those in the A/Seal/Mass/1/80 (H7N7) virus. The isolation of a second influenza A virus from seals suggests that this species may be important in the ecology of influenza viruses.

Influenza A viruses (H13N2 and H13N9) have also been isolated from the lungs and hilar nodes of one stranded pilot whale, although the relationship between influenza virus infection and stranding remains unknown. Genetic analysis

of the H13N9 whale virus has shown that the virus was recently introduced from birds (27, 56). Influenza A viruses (H1N3) have also been isolated from the lungs and a liver of whales (Balaenopteridae) in the South Pacific (101).

Influenza viruses have been isolated from mink raised on farms. These viruses, which were of avian origin (H10N4), caused systemic infection and disease in the mink and spread to contacts (90). The potential susceptibility of mink to influenza A viruses from a variety of animal species had been demonstrated experimentally before this outbreak (108, 126).

These findings demonstrate that interspecies transmission of influenza A viruses occurs relatively frequently, mainly from birds to mammalian species. The epidemics tend to be self-limiting, and the newly introduced viruses do not seem to be maintained in animal species such as seals, whales, and mink.

Molecular Determinants of Host Range Restriction

The preceding sections demonstrate that influenza viruses in nature exhibit partial restriction of their host ranges: it is possible to distinguish groups of viruses which are rarely detected in animals other than their normal host. Restriction is said to be partial because of the evidence for occurrences of interspecies virus transmission, e.g., the finding of avian H1N1 viruses in swine in Europe, human H3N2 viruses in swine, swine H1N1 viruses in humans, and other avian viruses in seals, whales, and mink. The determinants of host range restriction have not been completely elucidated, but clearly a variety of different factors are involved, and probably any of the influenza virus gene products may be the determining factor for specific virus-host combinations.

Attention has focused upon the HA as a primary determinant of host range, because of its role in host cell recognition and attachment. Although all HA subtypes have been detected in avian species, particular HA subtypes are characteristic of the viruses commonly found in mammalian species: H1, H2, and H3 in humans; H1 and H3 in swine; and H3 and H7 in horses. The basis for these limitations is unknown. It is possible that some unrecognized feature of these subtypes makes them peculiarly well adapted for growth in those mammals. Alternatively, their current status may be an accident of recent history. We have no idea what influenza virus subtypes might have infected humans prior to about 120 years ago.

Cleavability of the HA is an important determinant of the virulence of avian influenza viruses, but probably not of host range. It has been observed that in tissue culture, viruses with highly cleavable HA can adsorb to, enter, and synthesize HA which is cleaved in cells that are still nonpermissive for virus multiplication (26). Trypsinlike proteases capable of cleaving HA are likely to be found in all mammals and birds, supplied by the resident bacterial flora or by host secretions such as digestive enzymes. Therefore, it is unlikely that host-specific differences in the ability to cleave HA are important for host range restriction. However, cleaved HA is less stable at acidic pH than uncleaved HA is (146). Thus, cleavability may affect host range if the route of virus transmission in a host requires the virus to pass through the stomach, as in ducks.

The receptor specificity of influenza virus HA depends on the host species from which they are isolated. Human H3 viruses bind almost exclusively to sialyloligosaccharides terminated by SAα2,6Gal, whereas avian and equine H3 viruses preferentially bind SAα2,3Gal (138–140). This difference in specificity is tightly associated with the presence, in the structure of the HA receptor-binding site, of Leu-226 in the human H3 viruses (as well as the humanlike swine H3 viruses), instead of Gln-226 in the avian or equine viruses. H1 viruses isolated from humans after 1977, swine H1 viruses, and human H2 viruses are also preferentially SAα2,3Gal specific (137, 195). However, these viruses, as well as all other influenza A viruses, possess Gln-226. Also, passage of human or swine H1 and H3 viruses in embryonated chicken eggs can alter the receptor-binding specificity, antigenicity, or growth properties of the progeny virus, making it more avianlike, although with a mutating residue 226 (75, 135, 137, 172). Thus, there are other amino acid positions in HA which are accessory receptor-binding determinants.

The amino acids identified as constituting the heart of the HA receptor-binding site (Tyr-98, Trp-153, His-183, Glu-190, Leu-194, and Gln [Leu]-226) (189, 191) are essentially invariant features of HA of influenza A viruses. Immediately adjacent, Ser-227 is highly characteristic of duck viruses of all subtypes as well as human/swine H3 viruses, whereas Ala/Gly-227 is present in HA of some other mammalian viruses. Gly-228 covaries with Gln-226; it is conserved in all duck and mammalian viruses except in the human/swine H3 HA, which have Leu-226–Ser-227–Ser-228. Substitution of Gln-226–Ser-227–Gly-228 into a human H3 virus permitted virus replication in ducks, whereas substitution of Gln-226–Ser-227–Ser-228 did not (114). The importance of these distinctions in host range discrimination is not understood.

Along with the human/swine H3 HA, the most unusual HA receptor-binding site belongs to the H13 viruses, which are also distinguished by occurring in nature commonly in shorebirds but not at all in ducks. H13 HA possesses most of the conserved amino acids noted above, but has Arg/Lys-227 instead of Ser-227 characteristic of duck HAs. Also, H13 HA uniquely possesses Trp-229, whereas HAs of all other sequences subtypes, regardless of the host, have Arg-229 (27). Again, the significance of this is not understood; however, the H13 HA is not the sole factor restricting H13 virus replication in ducks (see below).

Competitive inhibitors of HA receptor binding, present in the serum of many species, could also play a role in host range restriction. The α_2-macroglobulin of horse and guinea pig serum contains a receptor analog which is an inhibitor of human H3 HA, whereas equine and avian H3 HA, which recognize different sialic linkages, are resistant (138). Other inhibitors have been identified in the serum of other species including humans (143).

There is evidence for a role of influenza virus NA in host range discrimination. Changes in NA can sometimes alter virulence properties of the virus (12, 171) or the ability of a virus to form plaques in tissue culture (119, 150). The common influenza viruses of mammals appear restricted in their NA as well as their HA subtypes (N1 and N2 in human and swine viruses, N7 and N8 in equine viruses), but the features of NA responsible for this restriction are unknown. There is no simple linkage of particular NA subtypes with particular HA subtypes. Some combinations are much more common than others, and indeed 45 of 126 possible HA-NA subtype combinations have not yet been found in nature.

The internal genes of influenza A viruses (PB1, PB2, PA, NP, M1, M2, NS1, and NS2) may also play roles in host discrimination. Phylogenetic analysis has identified more or less host-specific lineages of each of these genes (see below), but the significance of the mutations that specifically characterize these lineages awaits analysis. It has been proposed

that host-specific phosphorylation patterns of NP can affect the replication of influenza viruses (88, 89, 148). Also, avian NP specifically causes restricted replication of otherwise human viruses in squirrel monkeys (162). Reassorted constellations of the three polymerase genes, sometimes also including NP, have been associated with attenuation of virulence or replicative ability in specific hosts (142); for example, substitution of avian for human PB1 in a human virus attenuates its replication in MDCK cells and squirrel monkeys but not in chicken kidney cells (162), whereas the PB2 gene of fowl plague virus apparently determines its ability to replicate in mammalian cell cultures (7, 72). Similarly, substitution of some avian NS genes (the B allele) for human NS in a human virus results in attenuation in squirrel monkeys (174).

Host range discrimination is thus a polygenic trait. The specific contribution of each gene will be much better understood once the functions of each gene product and its interactions with host cell factors are known in detail. Until then it will be difficult to predict with certainty the ability of a novel reassortant virus to infect an alternate host.

Mechanism for Perpetuating Influenza Viruses in Avian Species

There is convincing evidence that all 14 subtypes of influenza A viruses are perpetuated in the aquatic bird populations of the world, especially in ducks, shorebirds, and gulls; there is no evidence that influenza viruses persist for extended periods in individual animals. This indicates that some mechanism has evolved for maintaining influenza viruses in aquatic avian species; in this section we will consider the possibilities.

The maintenance of influenza virus in the aquatic bird population differs from the maintenance of influenza virus in humans in that large numbers of susceptible juveniles are introduced yearly after spring hatching. After the annual autumn epidemic of influenza virus in juvenile ducks, it is unlikely that many remain uninfected. The majority of infected birds are presumably immune to reinfection with the predominant influenza subtype. This probably influences the changes in the subtype predominating in a particular flyway from year to year.

Several possibilities have been suggested for the perpetuation of influenza viruses in the aquatic bird populations of the world. These are discussed below.

Continuous circulation in aquatic bird species. The information discussed above suggests that each of the influenza virus subtypes could be maintained in the wild-duck population in North America. The detection of low levels of influenza viruses throughout the winter months in North America and in Japan and detection of virus in ducks as they arrive back in Canada at the beginning of the breeding season support this notion.

Circulation between different avian species. Since influenza viruses are prevalent in shorebirds in the spring and in wild ducks in the fall, there may be interspecies transmission. About half of the influenza viruses isolated from gulls and shorebirds will experimentally infect ducks (76); however, sampling of shorebirds in Alberta, Canada, during August and September failed to reveal any influenza viruses in shorebirds and gulls when they were prevalent in ducks.

Persistence in water or ice. When wild ducks are present in August and September, influenza viruses can be isolated from lake water without concentration. It is possible that influenza viruses are preserved frozen in ice or in lake water and reinfect ducks in the spring. Tests of lake water in the winter and spring have so far failed to detect influenza viruses. The infectivity of influenza viruses in water is dependent on the virus strain tested and the salinity, pH, and temperature of the water; at 17°C some strains remain infectious for up to 207 days, and at 4°C they remain infectious for longer times (165, 166), raising the possibility of persistence of influenza virus in water when the ducks are absent.

Persistence in individual animals. Although virus shedding from the intestinal tract in some ducks can continue for 2 to 4 weeks (62), there is no evidence that continued shedding occurs. The possibility has been raised that influenza virus persists in an integrated or episomal form in the genetic material of humans or lower animals. To determine whether influenza virus can persist in the tissues of ducks, by some as yet unexplained mechanism, the polymerase chain reaction method of gene amplification was used to detect influenza virus in experimentally infected ducks (179). Infectious virus and RNA cleared concurrently after oral infection of ducks with influenza virus. There was no evidence from polymerase chain reaction analysis of the HA gene for persistence of viral genetic information.

There is ample evidence from several laboratories (34, 41, 136) that influenza viruses can produce persistent infections in cell culture. The conditions that lead to this state are still not understood. There is much less evidence for persistent infections of animals, although there are reports of extending shedding of influenza viruses from immunocompromised animals including nude mice (194).

There is evidence that H3N2 influenza viruses can persist in pigs after disappearing from the human population (52, 157), but some of these H3N2 strains were most probably derived from recent infection with H3N2 avian influenza A viruses (86).

Continuous circulation in subtropical and tropical regions. There is increasing evidence that in the tropical and subtropical regions of the world, influenza viruses of humans are isolated year round (134), whereas in temperate climates, influenza is a winter disease and the virus is infrequently isolated in the summer months. Influenza viruses have been isolated year round from domestic ducks in Hong Kong (154). Although surveillance studies for influenza viruses in wild ducks and shorebirds have not been done in tropical regions, the possibility has to be considered that the epicenter of influenza virus perpetuation is in tropical regions of the world and that ducks, shorebirds, and gulls transport viruses from the tropical regions to the temperate regions during spring migration. The argument against a tropical epicenter is that high-density congregations of wild birds are not found in these regions of the world.

At this time the most convincing data support the first alternative, i.e., that there is continuous circulation of influenza viruses in wild ducks with very low levels of detectable virus while the birds are in their overwintering sites in the subtropics.

EVOLUTIONARY PATHWAYS

Evolutionary Patterns among Influenza A Viruses

Interspecies transmissions, combined with isolation of host species, contribute to the evolutionary divergence of viruses because of the separation of host-specific virus gene pools. Barriers to frequent interspecies transmissions maintain the separation of progeny and parent virus gene pools

and allow independent evolution of host-specific strains. These barriers may be in the form of infrequent likelihood of transmission because of different ecologies of host species, a lack of infectivity of the virus in new hosts, or interference from established viruses mediated by host immunity. Partitioning of avian influenza virus gene pools can result from geographic separation of waterfowl populations by separation of flyways and breeding and overwintering grounds. This mechanism has been suggested for the divergence of H4 HA lineages in avian viruses (37). The subdivision of host populations provides a great deal of heterogeneity to virus populations and enhances the maintenance of a large number of virus subtypes.

Interspecies transmission of viruses does not necessarily result in a net gene flow between host-specific virus gene pools; reassorted progeny virions with new genes may have lower fitness (reduced replication and virus shedding) relative to virions with host-adapted genes and thus may not persist. In mixed infections of different virus strains in the same host cells, gene segments of the different strains behave like alleles in populations of eukaryotic organisms; all possible combinations of gene subtypes are theoretically generated, but only certain combinations may be viable. Independent reassortment of some genes may not be observed; such genes constitute gene constellations. Reasons for a lack of independent evolution among virus genes vary. One possibility is that protein-protein interactions preclude independent evolution of the associated genes located on different gene segments; i.e., reassorted viruses involving these genes may have relatively low fitness. In this situation, the evolution of these genes and their proteins is interdependent and is referred to as coevolution; reciprocal evolution is required to maintain the fitness of the progeny virions. Another factor contributing to the formation of distinct gene constellations is parallel host-specific evolution of virus proteins. The loss of host-adapted genes through reassortment may decrease the fitness of progeny virions. The third factor contributing to the formation of distinct gene constellations is chance. Reassortment may not decrease fitness but does not occur because isolation of host specific virus gene pools provides few opportunities for mixed infections with other host-specific virus strains. Thus, although interspecies transmission and the segmented genome of influenza viruses facilitates the generation of new virus genotypes in a virus gene pool, the successful introduction of new genes may be subject to conditions that preclude some genotypes or make them unlikely to appear.

Selection Pressures and Evolutionary Constraints on Influenza Viruses

Each virus gene may evolve differently because of different selective pressures and evolutionary constraints. Genes that code for surface proteins (HA and NA) may be subject to strong selection pressure by neutralizing antibodies of host immune systems. Genes coding for internal proteins (e.g., NP) may not be subjected to strong host immune selection pressure but are thought to undergo significant host-specific adaptive evolution (42, 46). Internal protein genes such as those coding for polymerases (e.g., PB2) may have virus-specific functional constraints on evolution (48). Because of host immune selection pressure, surface proteins are expected to evolve more rapidly and to be replaced by reassortment more frequently. Reassortant viruses with new genes for surface proteins have a selective advantage over the parent virus to which the host has had considerable antigenic exposure; the new viruses are able to escape (at least temporarily) the host immune response. If these new viruses are sufficiently infectious, they can cause pandemics and replace previous strains, resulting in antigenic shifts (184). Thus, surface protein genes may not be expected to have a long evolutionary history within hosts that subject the virus to considerable immune selection pressure, e.g., humans. Internal protein genes that show a high degree of host-specific evolution and cause virus attenuation when experimentally reassorted with different host-specific viruses (e.g., the NP gene) (148, 162, 173) are not expected to reassort frequently. However, internal protein genes that evolve slowly and do not show a high degree of host-specific evolution may be replaced more frequently because new reassorted viruses are not likely to show any attenuation (e.g., PB1 genes in human viruses) (77). In the case of PB1 genes, virus-specific constraints on the viral polymerases may prevent any significant host-specific divergent evolution. Thus, the conserved nature of these proteins does not present a barrier to reassortment; i.e., reassorted viruses do not suffer a loss of relative fitness.

Host-Specific Evolution of Influenza Virus Genes

The putative role of NP as a determinant of host range (148, 162, 173) has led to its use as a model for long-term host-specific evolution of influenza viruses (42, 46, 47). Using RNA hybridization techniques, Bean (10) showed that NP genes fall into five host-specific groups. Subsequently, Gorman et al. (46, 47) showed in phylogenetic analyses that NP genes have evolved into five major host-specific lineages that correspond to five NP gene RNA hybridization groups (10) (Fig. 4). These lineages are (i) EQPR56 (Equine/Prague/56) (equine 1 viruses); (ii) recent equine viruses (equine 2 viruses), i.e., those related to Equine/Miami/63; (iii) human viruses joined with classic swine viruses, i.e., those related to Swine/Iowa/15/30; (iv) H13 gull viruses; and (v) all other avian viruses. Geographic patterns of evolution are evident in avian virus NP genes; North American, Australian, and Old World isolates form separate sublineages.

At present, phylogenies for the six internal influenza genes that include H1N1 and H3N2 human and swine viruses have been determined (PB1 [77], PA [125], PB2 [48], NP [42, 46, 47], M [73], and NS [80]). Phylogenies for genes of HA surface protein subtypes H3 and H4 are available (9, 37). Generalized phylogenetic trees (cladograms) showing the major branching topologies for the phylogenies of these genes are shown in Fig. 4. Of the eight gene phylogenies shown in Fig. 4, none show identical topologies. This indicates that past reassortment events and extinction have affected patterns of evolution for each gene.

For gene phylogenies that include EQPR56 (equine 1 virus), this virus always shows an outgroup relationship to other virus lineages. This pattern indicates that the EQPR56 is the most divergent and possibly the oldest of the nonavian influenza A virus lineages. As such, its relationship to other gene lineages permits us to estimate the relative order or ages of divergence (vicariance) among the gene lineages. Cladograms for the NP and PA genes are very similar and would be identical if the EQPR56 PA gene (sequence not done) shows the same relative position (Fig. 4). The close match in the phylogenies of NP and PA genes indicates that they share a common evolutionary history in each host; they form a gene constellation and apparently have not reassorted independently.

The M gene shows significant differences from the NP and

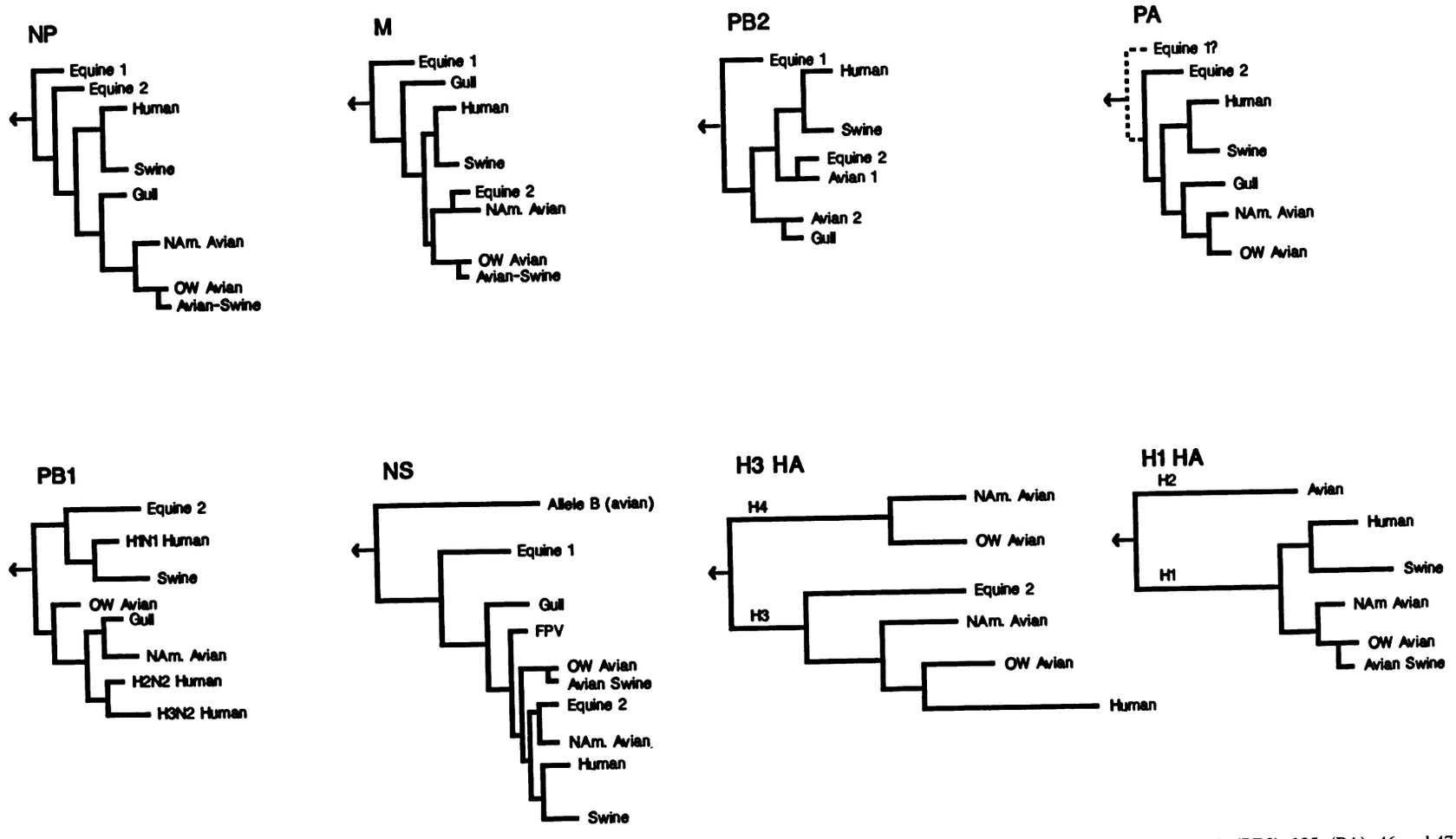

FIG. 4. Generalized phylogenies (cladograms) of influenza virus genes. Nucleotide phylogenies represented are taken from references 77 (PB1), 48, (PB2), 125, (PA), 46 and 47 (NP), 78 (H1HA), 9 (H3HA), 73 (M), and 80 (NS). Phylogenies were determined with PAUP software version 2.4 (David Swofford, Illinois Natural History Survey), which uses a maximum-parsimony algorithm to find the shortest trees. The horizontal distance is proportional to the minimum number of nucleotide differences needed to join the gene sequences (no scale is given). Vertical lines are used for spacing branches and labels. The arrow at the left of each tree represents the node connecting the influenza B virus homolog. Equine 1 is represented by the Eq/Prague/56 (H7N7) virus isolate. Equine 2 is represented by recent H3N8 equine viruses. Unless noted otherwise, human represents human H1N1, H2N2, and H3N2 viruses. Swine represents classic swine viruses (i.e., those related to Sw/Iowa/15/30), Gull refers to H13 gull viruses, FPV refers to fowl plague viruses, NAm. Avian refers to North American avian viruses, OW Avian refers to Old World or Eurasian avian viruses, and Avian Swine refers to avianlike H1N1 swine viruses. There are two distinct avian lineages in the PB2 tree, Avian 1 and Avian 2, which contain Eurasian and North American avian viruses, respectively.

PA cladograms. First, the equine 2 virus M genes appear to have been recently derived from North American avian viruses, in contrast to the much older origin of equine 2 virus NP and PA genes. Second, the H13 gull virus M gene is closer to EQPR56 and more distant from other virus M genes, suggesting that the gull virus M gene may have an older origin than the H13 gull NP and PA genes. Like the recent equine virus M genes, the PB2 gene of H13 gull viruses shows a more recent origin than the NP, PA, and M gull virus genes.

In the NS gene phylogeny there is a very old "B allele" (174) avian lineage that predates the divergence of the EQPR56 (equine 1 virus) lineage. The H13 gull virus NS gene shows the same relative evolutionary position as the H13 gull virus M gene; i.e., the origins of these genes are older than the origin of the NP, PA, PB1, and PB2 gull virus H13 genes. The fowl plague virus NS genes, which are from the earliest avian virus isolates, appear to be derived from a lineage that diverged prior to the split between Old World avian, North American avian, and human and classical swine groups. The recent equine virus NS genes, like the M and PB2 genes, is derived from the North American avian virus group and shows a more recent origin than NP, PA, and PB1 genes.

The PB1 lineage shows less congruence with other gene lineages. Human virus PB1s show three different origins: H1N1 human viruses form a sister group to classical swine viruses, and H2N2 and H3N2 human viruses each form separate sublineages derived from avian virus PB1s. The H13 gull virus PB1 gene, like the PB2 gene, appears to have a more recent origin than the other genes, but the recent equine virus PB1 appears to have an older origin like the NP and PA genes.

A comparison of nucleotide and amino acid sequences of H3 HA genes shows that the progenitor of the 1968 human pandemic (Hong Kong) strain was derived from an avian virus very similar to those currently circulating in ducks in Asia, and the transfer of the avian virus H3 gene to human viruses probably occurred in 1965 (9). Since then, the H3 human viruses have diverged rapidly from this progenitor. This rapid accumulation of nucleotide and amino acid changes is in contrast to the much lower rates observed in avian H3 viruses, and, like the avian virus NP, the avian virus H3 protein appears to be in evolutionary stasis. Unlike human H3 viruses, the equine 2 (H3N8) viruses apparently diverged from an avian ancestor much earlier as indicated from their greater distance from avian H3 virus relatives. Each of four H3 swine virus isolates analyzed appears to represent independent introductions into pigs, two having been derived from human viruses and two from avian viruses (9). These last examples support the concept that swine may serve as intermediates in the transmission of avian influenza viruses or their genes to the human virus gene pool.

Common Ancestry for Human and Classic Swine Virus Genes

In all of the phylogenies of the internal protein genes and the surface protein H1 HA gene, the human and classic swine virus lineages show a sister group relationship, indicating that they share a common ancestor (Fig. 4). Analyses of amino acid trees of NP, PB2, and M genes show that the common ancestors are all avianlike (46–48, 73). Antigenic studies of human and classic swine virus N1 NA proteins (see e.g., reference 83) predict the same sister group relationship. The common sister group relationship for all genes of human and classic swine viruses and the closeness of the common ancestors to avian virus proteins suggest that the human and classic swine virus ancestor was not a reassortant virus but an entirely new avian-derived virus (46, 48). It is important to note that the H1N1 human virus as originally constituted prior to 1918 has since been reassorted twice: in 1957 the H1 HA, N1 NA, and PB1 genes were replaced with new avianlike genes, and in 1968 the H2 HA and PB1 genes were again replaced with avianlike genes (77) (Fig. 4).

Evolutionary Rates of Influenza Virus Genes

Current paradigms of influenza A virus evolution are based on studies of human viruses, for which host immune selection pressure is potentially high and virus surface proteins are expected to evolve rapidly. For human influenza viruses, the H3 HA surface protein genes are evolving much more rapidly than the internal protein genes PB1, PB2, PA, NP, and M1 (Table 2). The proportion of silent to total nucleotide changes in surface versus internal protein genes reveals differences in evolution. Among the internal protein genes PB2, NP, and M1, the proportion of silent changes is much higher than for the surface protein H3 HA (81 to 96% versus 57% [Table 2]). Furthermore, a comparison of coding and noncoding evolutionary rates for human virus genes shows that they are evolving at different rates. For example, the M1 and M2 genes of the M gene segment are evolving very differently from each other and fit the model of internal versus external protein gene evolution just discussed. M2, a minor influenza virus surface protein gene, is evolving much more rapidly than the internal M1 matrix protein gene, and a higher proportion of the nucleotide changes in the M2 gene are nonsilent. M1 is evolving very slowly and shows almost no accumulation of coding changes over a 55-year period (Table 2) (73).

Homologous genes from host-specific virus strains also evolve at different rates. Recent evolutionary studies of influenza virus NP genes have shown that avian virus genes are evolving more slowly than those in human viruses and that avian virus proteins are highly conserved, showing no net evolution over the past 60 years. Within the Old World avian virus NP gene lineage there is no accumulation of amino acid or coding changes; all accumulating nucleotide changes are silent. Classic swine virus NP genes are evolving similarly to human virus NP genes, but at the protein (amino acid) level the swine virus NPs are more conserved. Genes of H3N8 equine 2 viruses are evolving more slowly than human or swine viruses.

Dates of Emergence of Influenza Virus Strains

Evolutionary rates estimated from phylogenetic analyses can be used to estimate dates of emergence or origin of new virus lineages (Table 3). Analyses of NP, PB2, and M gene segments estimate 1905 to 1914 for the appearance of the common ancestor of human and classic swine H1N1 viruses (46–48, 73). Estimation of this common-ancestor date assumes that the evolutionary rate has remained constant from the origin to the present, which may not be realistic. If the evolutionary rates were somewhat higher during the first few years after the appearance of this avianlike virus in human and swine hosts, the date of origin may be later than 1905 to 1914. H1N1 avianlike viruses appeared in swine in Europe in 1979 (147) and have continued to circulate and evolve. The NP genes of these new viruses evolved at a higher rate than NP genes in human and classic swine viruses over the period

TABLE 2. Nucleotide evolutionary rates for influenza A virus genes based on phylogenetic analyses

Gene segment subtype (host)	Nucleotide evolutionary rate (10^{-3} bases/yr) for[a]:			% Silent changes	N[b]	Period	Reference
	Total segment	Coding changes	Silent changes				
PB1 (human)	0.87				3	1957–1968	77
PB2 (human)	1.82	0.15	1.67	92	7	1933–1988	48
PA (human)	1.32				4	1934–1968	125
HA							
H3[c] (human)	7.0				16	1968–1979	16
H3 (human)	4.44	1.91	2.53	57	14	1968–1986	9
H3[c] (equine)	2.8				2	1963–1979	33
H3 (equine)	1.74	0.45	1.29	74	13	1963–1986	9
H1[c] (human)	4.3				14	1977–1983	133
H1 (human)	0.61				5	1933–1980	78
H1 (swine)	1.26				9	1930–1988	78
NP (human)	2.20				9	1934–1983	8
NP (human)	2.18	0.42	1.76	81	16	1933–1983	46
NP (swine)	2.12	0.22	1.90	90	11	1930–1988	46
NP (avian-swine)	2.88	0.47	2.40	83	3	1981–1989	46
NP (equine)	0.78	0.21	0.57	73	4	1963–1986	47
NP (avian)	1.21	0	1.21	100	19	1927–1982	46
M (human)	1.08				13	1933–1988	73
M1	0.83	0.03	0.80	96	13	1933–1988	73
M2	1.36	0.46	0.90	66	13	1933–1988	73
M (swine)	1.30				8	1930–1988	73
M1	1.43	0.05	1.38	97	8	1930–1988	73
M2	0.91	0.48	0.43	47	8	1930–1988	73
NS (human)	1.94				9	1942–1985	23
NS (human	1.78				14	1933–1977	80

[a] Evolutionary rates shown are for the entire gene segment, coding changes, and silent or noncoding changes. Coding changes reflect amino acid or protein evolution.
[b] N, number of virus isolates included in the estimation of evolutionary rates.
[c] Based on HA1 subunit sequence.

1930 to 1988. The evolution of these H1N1 avianlike swine viruses may serve as a model for early evolution of H1N1 human and classic swine viruses. By applying the evolutionary rate of the NP genes of H1N1 avianlike swine viruses to pre-1930 human and classic swine virus NP genes, the estimated date becomes 1918, which is in closer agreement with historical records and retrospective serological evidence (see, e.g., references 30 and 111).

TABLE 3. Estimated dates of origin of influenza A viruses[a]

Gene	Host/virus	Date of origin	Reference
NP	Human	1914	47
NP	Human	1912	46
NP	Classic swine	1913	46
NP	Old World avian	1912	47
NP	Old World avian	1904	46
NP	Avianlike swine	1979	46
PB2	Human	1910	48
NS	Human	1915–1920	118
NS	Human	1921	80
NS	Classic swine	1901	80
M	Human	1915	118
M	Human	1905	73
M	Classic swine	1912	73

[a] Estimated dates of origin were estimated from regressions of phylogenetic distances of virus genes against dates of isolation.

Unlike mammalian viruses, estimation of dates of origin for avian virus gene lineages has not been generally successful. In most cases, there is a lack of correspondence between the date of virus isolation and relative position in phylogenetic trees, e.g., North American avian virus NPs (46), PB2 (48), M (73), and H3 (9). This situation is probably the result of a combination of an insufficient sample of avian virus isolates, the suspected high diversity of avian virus gene lineages, and the impact of evolutionary stasis on avian virus proteins. Fortunately, NP genes of Old World avian strains show some consistency in dates of isolation and tree position, thus permitting estimation of an evolutionary rate and date of a common ancestor. The rate of avian virus NP gene evolution is lower than for human and swine viruses, and the protein shows no evolution over more than 60 years of avian virus isolates (Table 2). A minimum estimated date for the common ancestor of Old World avian virus NP genes may be 1904; severe constraints on avian virus protein evolution result in some silent back mutations not being reflected in phylogenetic analyses, and therefore the lengths of the deep internal branches are underestimated (47). By using 1904 as reference point, the relative ages of other avian NP gene lineages can be estimated. For example, Australian avian, North American avian, and H13 gull virus NP gene lineages must have diverged from the Old World avian lineage before

TABLE 4. Similarities of influenza A and B virus gene sequences

Gene	% Sequence similarity[a]		Reference
	Nucleotide	Amino acid	
PB1	59.5–60.7	58.3–61.0	35, 77, 82, 159
PB2	45.4–46.5	37–38	48
PA		38	3, 35
HA[b]	36–48	24–39	92
NA[c]			
NP	33.6–35.5	37	19, 47, 98, 115
M1	33	25	18, 73
M2		14	18
NS	30	9.7–16.2	17, 80

[a] Similarities represent percent identity of aligned sequences. No data are available for NA genes of influenza A viruses, but the sequence of influenza B is known (151).

[b] The lower value represents percent identity for HA1, and the higher value represents identity for HA2.

[c] Homology for the region between amino acid residues 116 and 363 of the influenza B virus NA protein sequence.

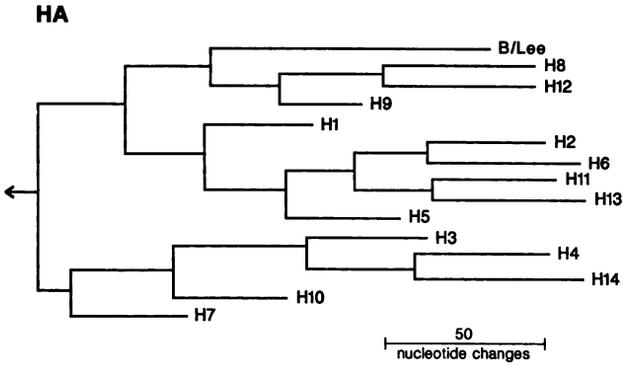

FIG. 5. Phylogeny of influenza virus HA gene subtypes. This phylogenetic tree is similar to that in reference 81, except that this tree is unrooted (does not set the B virus HA as an outgroup) and has a different topology. The analysis was based on aligned residues 63 to 343 (H3 numbering) of HA1 genes, and the phylogenetic tree was determined with PAUP software version 2.4. The number of variable nucleotides represented is 198, the total tree length is 1,021 steps (nucleotide changes), and the consistency index (proportion of changes due to forward mutations) is 0.453. Horizontal distance is proportional to the minimum number of nucleotide changes to join HA gene sequences. Vertical lines are of arbitrary length for spacing branches and labels. The arrow indicates the midpoint of the tree.

1904. Considering the relatively great distance of the isolates from the Old World lineage and the relatively slow evolution in avian virus genes, the H13 gull and North American avian viruses may have diverged many centuries ago from common ancestors shared with Old World avian viruses.

Origin of Influenza A, B, and C Viruses

The general structural features and genome organization of influenza A, B, and C viruses suggest that they share a common ancestry distinct from other negative-strand RNA viruses (36, 161). Of the three virus types, A and B viruses are much more similar to each other in genome organization and protein homology than to C viruses (94, 161), which suggests that influenza C viruses diverged well before the split between A and B viruses (161). The more distant relationship of influenza C virus to the A and B viruses is demonstrated in phylogenetic analyses of NP genes (42). Among the homologous genes that have been compared for influenza A and B viruses, the polymerase genes show the highest level of homology, and the organization and size of these genes have remained more similar than for other genes (Table 4) (95). The 60% level of homology between the nucleotide sequences of PB1 genes of A and B viruses is greater than the lowest homologies seen among nucleotide sequences of HA1 subunits of the 14 HA subtypes of influenza A viruses (31.3 to 60.6%) (81). By comparison, the nucleotide sequences of the HA1 subunits of influenza A and B viruses show 36% homology (92). The greater range in variation among influenza A virus HA1 nucleotide sequences than between influenza B virus HA1 and their influenza A virus counterparts suggests that influenza B viruses diverged from the A viruses after divergence of the early ancestors of present-day HA subtypes. This suggestion is further supported by a phylogenetic analysis of partial HA1 gene sequences (Fig. 5), which shows the influenza virus B/Lee/40 HA as being more closely related to the HA lineage containing H9, H8, and H12 subtypes than to two other major HA lineages. This analysis suggests that prior to the divergence of influenza B virus, the ancestral avian virus HA subtypes had already diverged and that the B virus HA and the present 14 avian virus HA subtypes were derived from these ancestral subtypes.

Influenza B and C viruses are human viruses and are not found in avian hosts, although the C viruses have been isolated from pigs and dogs; this has led to the suggestion that pigs serve as an alternate host reservoir for B and C viruses (51). Influenza B and C viruses exhibit slower evolution (especially the C viruses) than human influenza A viruses (2, 22, 23, 130, 196). Influenza C viruses cause only infrequent outbreaks of mild respiratory disease (74) and occur primarily in young children (124). Similarly, influenza B viruses typically cause milder respiratory illness than influenza A viruses and infect primarily children. Although influenza A viruses infected children and the elderly, they differ from the B and C viruses by their continually evolving antigenic character and their ability to reinfect adults. These differences among the A, B, and C viruses have profound effects on their patterns of evolution. Influenza A viruses in humans evolve along a single branch lineage, which indicates that there are no cocirculating strains of the same subtype and that the virus evolves by clonal reconstitution following widespread extinction (21). In contrast, the B and C viruses exhibit multiple lineage evolution, which indicates the presence of cocirculating strains within the human population (21, 196). The pattern of epidemiology and evolution of the B and C viruses suggests that they are at or approaching an evolutionary equilibrium with their human hosts, whereas the A viruses are not and are prevented from it by perturbations arising from reassortment of human virus genomes with those from avian viruses (see, e.g., reference 131).

Assuming that the B and C viruses originated from avian viruses, they may be viewed as evolutionary relicts of previously widespread avian virus strains. To account for their high degree of divergence from the A viruses and their relative evolutionary equilibrium, they probably diverged many centuries to thousands of years ago from avian influenza virus ancestors. Modern influenza B and C viruses do not reassort with influenza A viruses and leave viable progeny. To explain this genetic incompatibility, there had to be little or no gene flow between human and avian virus

gene pools after the introduction of avian-derived influenza viruses into human hosts. During a long period of isolation, human influenza B and C viruses underwent host-specific adaptive evolution and accumulated enough mutations that reassortant viruses were no longer viable. This proposal suggests that there have been fundamental changes in the ecology of human influenza viruses during human history; i.e., during recent history there has been less isolation of human and avian virus gene pools and, as a result, human influenza viruses have been prevented from reaching an evolutionary equilibrium with their hosts by an irregular infusion of avian virus genes into the human virus gene pool.

Shared characters between influenza A and B viruses are primitive; i.e., they were derived from a common ancestor. Thus, we expect that older influenza A virus lineages should share more characters with the B viruses than would those in more recent lineages. For example, phylogenetic analyses show that the NP, PB2, M, and NS genes of influenza A virus EQPR56 (equine 1 virus) are consistently closer to influenza B virus gene homologs; this pattern suggests that the EQPR56 viruses diverged from avian influenza viruses earlier than other nonavian lineages did.

Evidence for an Avian Origin of the Influenza Viruses

The common features of influenza A and B viruses leave little doubt that they share a common ancestor. Although the historical record is incomplete, enough evidence is available to provide some insight into the origin of these viruses. The origin of the influenza viruses actually addresses two related, but distinct questions: (i) in which host did the first influenza virus evolve, and (ii) in which host(s) are the nearest common ancestors of each of the influenza virus gene segments? In the sections that follow, we present summaries of phylogenetic analyses that provide strong evidence that all of the current gene segment lineages circulating in both mammals and birds originated from avian influenza viruses.

In any long-continued host-parasite relationship there will be selection in the host to eliminate or lessen the deleterious effects of the parasite. In the parasite, there will be reciprocal selection for mutations that reduce host mortality and for mutations that reduce the deleterious effects of the host on the parasite. At equilibrium, the virus would be expected to replicate efficiently, cause minimal disease to the host, infect a high proportion of the host population, be perpetuated in host populations, and show increased genetic diversity with time. Stabilizing selection acts to maintain an optimally adapted phenotype but does not mean that genetic diversity is decreased; over time the accumulation of silent genetic mutations in different virus populations can lead to significant lineage divergence in nucleotide sequences of virus genes, but amino acid sequences of the proteins (virus phenotypes) are highly conserved, e.g., avian influenza virus genes and proteins (46–48). In relationships in which the generation time of the parasite is much shorter than that of the host, we can expect the parasite to evolve rapidly toward an adaptive equilibrium or optimum. Failure of this reciprocal host-parasite adaptation process is likely to lead to the extinction of one or the other.

A number of features of avian influenza viruses suggest that waterfowl may be the original hosts. Influenza viruses in wild waterfowl populations are ubiquitous, infection is nearly always asymptomatic, and large amounts of virus are shed by infected birds (181). In addition there is considerable genetic diversity in avian viruses; 14 HA and 9 NA subtypes persist and circulate in the avian host reservoir. Each HA and NA subtype appears to be antigenically and phenotypically homogeneous and relatively genetically conserved when compared with the antigenic, phenotypic, and genetic distinctness of each subtype. The selective pressure that would have caused diversification and the reasons for the continued coexistence of this array of avian virus subtypes remain unknown. The very high level of conservation observed in proteins of avian viruses suggests that an adaptive optimum has been nearly achieved. The apparent evolutionary stasis of these proteins suggests, further, that within the normal avian host population, any modification of the protein sequence is likely to prove detrimental in the long run. Therefore, avian influenza viruses and their waterfowl hosts appear to provide a classic example of an optimally adapted system. The very low levels of evolution observed for avian virus proteins suggest that many centuries have been required to generate the current genetic diversity and distinct separation of avian virus HA and NA subtypes.

There is only one known report of a virulent virus causing disease in a wild bird population (Tern/South Africa/61) (14). In domestic fowl and mammals, outbreaks and epidemics of influenza are relatively frequent but unpredictable and are usually accompanied by disease symptoms and mortality. Only a few of the numerous avian virus subtypes have been observed in nonavian hosts. Evolution of virus proteins in nonavian hosts typically shows a rapid accumulation of mutations away from avianlike forms, which indicates an avian origin for these viruses. This is exemplified by evolution of human and classic swine viruses. The high degree of adaptation of avian viruses to their natural hosts, the considerable genetic diversity of avian virus subtypes, and the evolutionary stasis of avian virus proteins suggest that influenza viruses are a long-established pathogen in wild birds and more transient in other hosts. The evolutionary stasis exhibited by avian virus proteins is all the more remarkable considering the geographic separation of avian virus populations and ongoing reassortment of HA, NA, and other genes of avian influenza viruses.

Reconstruction of Influenza Virus Phylogenies

Phylogenetic trees represent hypotheses about evolutionary relationships among taxa. The phylogenetic analysis used in these studies (maximum parsimony) makes the assumption that the shortest mutational path connecting the taxa under study will provide a useful hypothetical evolutionary pathway. Thus, our phylogenetic analysis makes no assumptions about relationships based on historical evidence, dates of isolation, or measures of antigenic and genetic similarity. These other sources of evidence provide independent tests of hypotheses about relationships.

Interpretation of relatively recent events in the evolution of influenza virus RNA segments is straightforward, and the avian origin of some of the genes of current mammalian viruses is easily seen (Fig. 4). For example, genetic and phylogenetic analyses have now demonstrated that all genes that have appeared in mammalian virus gene pools over the past century have an avian origin (see, e.g., references 9, 47, 48, 73, and 77). Each influenza virus gene phylogeny represents only a partial history of virus evolution, but consideration of all gene phylogenies together reveals a more complete picture of virus evolution. A comparison of branching patterns among influenza virus gene phylogenies (Fig. 4) demonstrates that reassortment of nonavian with avian viruses has occurred repeatedly over the evolutionary history of influenza viruses. The different relative positions of

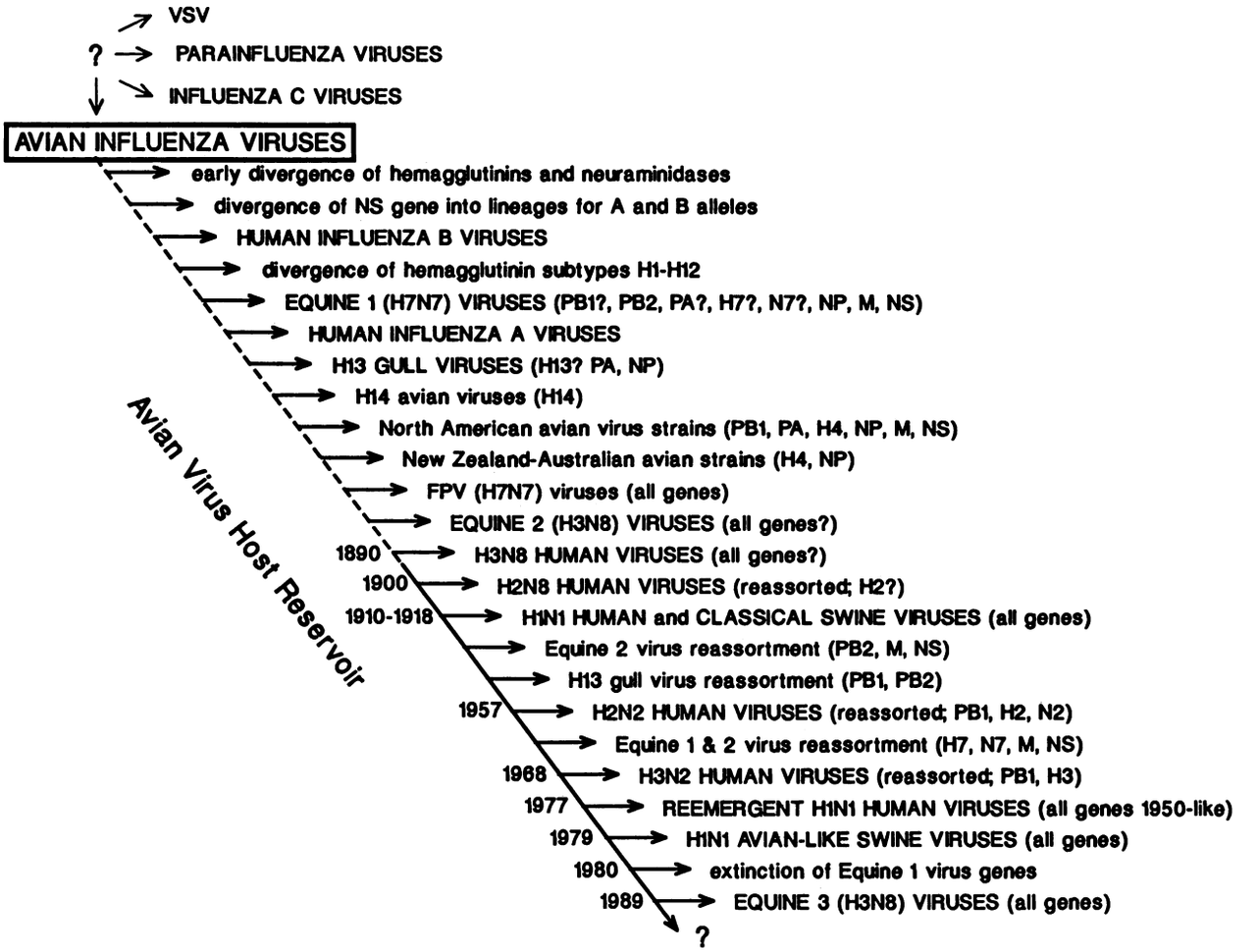

FIG. 6. Summary of the evolution of influenza viruses. The vicariant events (those that lead to lineage divergence) depicted in this figure are in rough chronological order and have been deduced from collective analysis of influenza virus gene phylogenies (e.g., Fig. 5), antigenic and genetic data, and historical records. The dashed line indicates an indeterminant time frame. The genes involved in evolutionary events are included in parentheses. The EQUINE 3 viruses at the bottom of the figure refer to new avianlike H3N8 viruses that have recently appeared in northeastern China (50). VSV, vesicular stomatitis virus.

gene lineages among nonavian viruses indicate that the genes of host-specific viruses are of different relative ages. This suggests that a consequence of the appearance of new reassorted viruses has been the extinction of previous strains or their genes. Finally, these phylogenetic analyses demonstrate that whole avian viruses can be transmitted to new hosts, displace previous strains, and evolve independently from their avian ancestors. A recent example of transmission of a whole avian influenza virus into a new host was the introduction of avian H1N1 viruses into swine in Europe (46–48).

Interpretation of long branches proximal to the root or midpoint of phylogenetic trees and speculation on the ultimate origins of a lineage must proceed with caution. Phylogenetic trees generated in this manner have no inherently favored point of origin or direction. The direction of evolution can be inferred by reference to dates of isolation, and hypothetical points of origin can be estimated from the genetic distances on the tree if the rates of mutation have remained constant for all branches within a lineage. Since it is clear that the rates of mutation are greater in mammalian than in avian hosts (Table 2), it is necessary to understand how this may distort phylogenies of virus evolution. All of the phylogenies represented in Fig. 4 show mammalian virus genes branching from the base of the tree. This does not imply that they represent the most primitive forms of the gene, but simply that they have diverged relatively farther and possibly earlier than other strains analyzed. In all cases the phylogenies are consistent with the hypothesis that influenza viruses have been continuously evolving in waterfowl and that at various times these viruses have been introduced into other avian and mammalian hosts. These introductions are manifested by the formation of new host-specific lineages due to new introductions of influenza viruses or virus genes from avian hosts. This is our current working hypothesis for the origin of influenza viruses (Fig. 6).

Historical Summary of Influenza Virus Evolution

A historical summary of the major vicariant events (events that result in a separation or generation of new gene pools and lead to lineage divergence from a common ancestor) in the evolution of influenza viruses is presented in Fig. 6. Divergence of the major lineages of HA and NA subtypes

and the split between NS A and B alleles represent possibly the oldest vicariant event in the evolution of influenza A viruses. The diversity of HA and NA subtypes among the older virus isolates (1902 to 1956) and the distinct pattern of evolutionary divergence among all known HA subtypes (81) indicate that their divergence predates the 20th century. Moreover, the large differences among some avian virus gene lineages and the apparent slow evolution of avian virus genes (9, 46–48) suggest that some HA and NA groups and NS A and B alleles diverged many centuries ago. As discussed above, the divergence of human influenza B viruses from avian influenza viruses must also be quite old, probably occurring after the divergence of ancestors of the major avian HA subtypes but before the appearance of the most recent avian HA subtypes (e.g., H13, H4, and H14) (Fig. 5 and 6).

The oldest vicariant event among mammalian influenza A viruses is the divergence of EQPR56 (equine 1) viruses. Gorman et al. (47) provide 1800 as a latest estimate of the date of the divergence of the EQPR56 virus. The divergence of other host-specific virus gene lineages occurred after this (during the 19th century). For example, the origin of the HA, M, NS, NP, and PA genes of present-day H13 gull viruses probably occurred in two separate reassortment events during the 19th century. Other major 19th century events include the origin of North American avian virus strains, recent equine strains, and fowl plague viruses (Fig. 6).

Present-day human viruses originated in the early 20th century, just prior to the 1918 pandemic, and classic swine viruses were probably derived from human viruses during the 1918 pandemic (46). Reassortment in human, recent equine, and possibly H13 gull viruses has occurred since the 1920s. The most recent events have been the appearance of a new avian-derived H1N1 virus in European swine populations in 1979 and the H3N8 virus in horses in Northeastern China in 1989. The new swine influenza virus has continued to evolve and circulate in European swine populations for more than 10 years. The new H3N8 influenza virus in China is also continuing to evolve (50).

PROPOSALS FOR THE ORIGIN OF HUMAN PANDEMIC STRAINS

Since the first human influenza virus was isolated in 1933, new subtypes of human type A influenza viruses have occurred in 1957, when the H2N2 subtype (Asian influenza) replaced the H1N1 subtype, in 1968, when the Hong Kong (H3N2) virus appeared, and in 1977, when the H1N1 virus reappeared (Fig. 7). Each of these new subtypes first appeared in China, and anecdotal records suggest that previous epidemics also had their origin in China. Serological and virological evidence suggests that since 1889 there have been six instances of the introduction of a virus bearing an HA subtype that had been absent from the human population for some time. For the HA there has been a cyclical appearance of the three human subtypes with the sequential emergence of H2 viruses in 1889, H3 in 1900, H1 in 1918, H2 again in 1957, H3 again in 1968, and H1 again in 1977 (Fig. 7).

One of the central questions to be answered is that of where the novel strains of human pandemic viruses come from. If this can be answered, there may be ways to prevent them.

Antigenic and Sequence Comparisons of Influenza Viruses from Different Hosts

Seroarcheology, the detection of antibodies to earlier influenza virus infection in the sera of elderly people, has provided indirect evidence for the prior circulation of contemporary strains of human influenza viruses. Thus, tests of serum of elderly persons collected before the Asian pandemic of 1957 showed that 26% of persons had antibodies to an influenza virus related to A/Japan/305/57 (H2N2) (106) and 90% of persons born prior to 1877 had antibodies that inhibited A/Hong Kong/1/68 (H3N2) (Fig. 7). Similar serological studies indicated the circulation of H1N1 influenza viruses in humans between 1908 and 1919 (105). Seroarcheological analysis indicates that the N8 NA was associated with H3 in 1900 (84). Seroarcheology, although imprecise, does provide evidence that (i) only influenza viruses of the H1, H2, and H3 subtypes have infected humans since the mid-19th century and (ii) there is cyclical alternation of the H1, H2, and H3 viruses as the predominant strains in humans. This raises the question of whether the remaining HA subtypes (H4 through H14) have the potential to infect humans.

Antigenic comparisons of human H1, H2, and H3 influenza viruses with strains in lower animals and birds established that each subtype has a counterpart in aquatic avian species. The H1 variants circulating in humans in the early 1930s were similar to swine influenza viruses and were later shown to be antigenically similar to viruses from wild ducks (61). The Asian/57 H2 influenza viruses of humans were shown to be antigenically similar to viruses from ducks (185), and the Hong Kong/68 H3 influenza viruses were similar to equine 2 (178) and duck (28) influenza viruses. The antigenic similarities were later confirmed by peptide-mapping analysis of the HA protein (96) and sequence analysis of the HA gene (33, 39), strongly supporting the suggestion for reassortment in the emergence of new human pandemic strains. Mainly on the basis of serological evidence, a revised system of nomenclature for influenza viruses was developed in 1971 (193); it reflects the serological interrelationships among influenza viruses from humans, lower animals, and birds (Table 1).

Genetic Evidence

Genetic evidence for the origin of human pandemic strains obtained by RNA-RNA hybridization (149) and by oligonucleotide mapping (117) has shown that the HA and NA of the pandemic strains were most closely related to those of the avian viruses. The HA of the prototype human H3 virus, A/Aichi/2/68, is more closely related to that of avian H3 viruses than to that of equine H3 viruses (33, 39, 86). Recently we have shown that not only the genes encoding the surface proteins, but also the PB1 gene, were derived from avian influenza viruses in both the 1957 and 1968 strains, although we do not know why the gene encoding the polymerase protein was introduced from avian species on each occasion (77). Therefore, both the pandemic strains in 1957 and 1968 are derived by genetic reassortments between human and avian viruses. We do not know from which part of the world those avian genes originated. More extensive phylogenetic analysis may allow the geographical identification of the origin of the responsible avian viruses.

Genetic analysis suggests that the 1918 pandemic strain had a different origin from the 1958 and 1968 strains. As discussed in the preceding section, phylogenetic studies

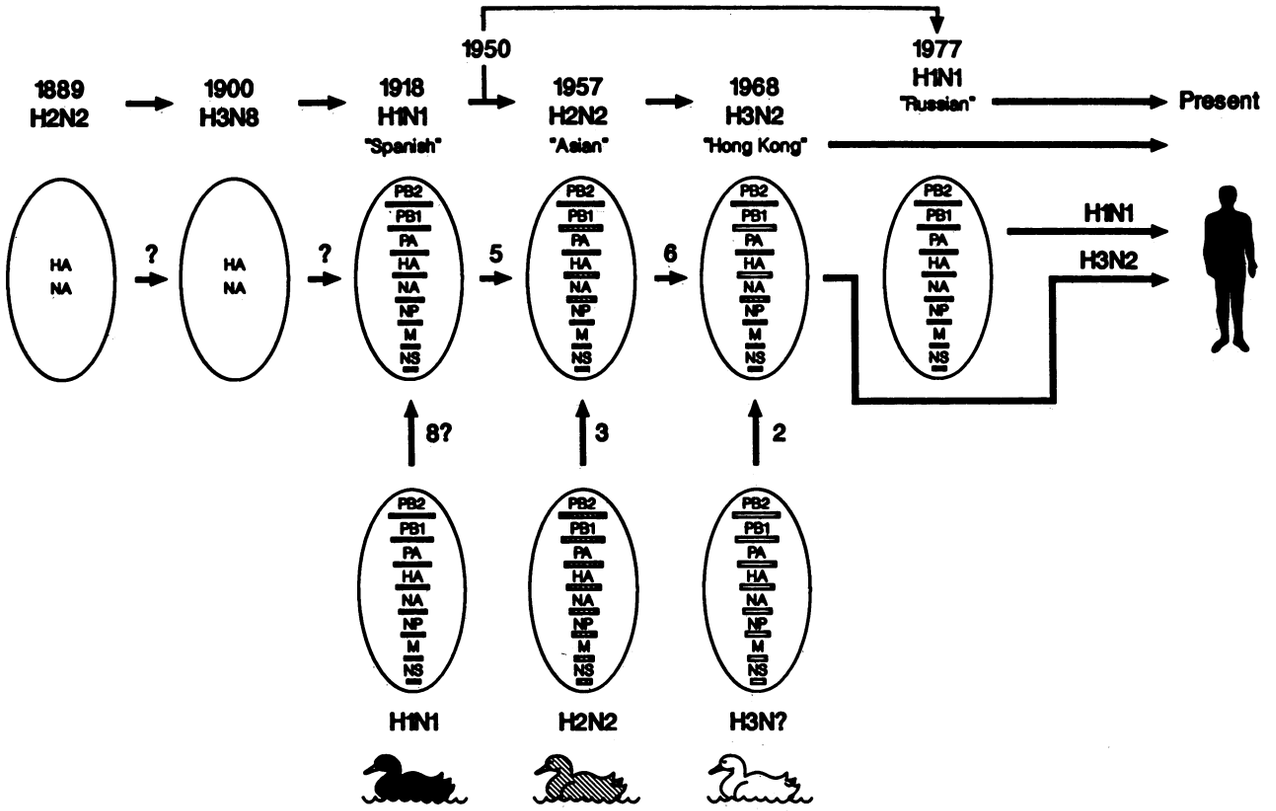

FIG. 7. Postulated evolution of the influenza A viruses currently circulating in humans. Seroarcheology suggests that H2N2 and H3N8 influenza viruses circulated in humans in 1989 and 1990, respectively. Phylogenetic evidence suggests that an influenza virus possessing eight gene segments from avian influenza reservoirs was transmitted to humans and pigs before 1918 and replaced the 1900 strain. This virus mutated, was carried from North America to Europe by American troops, and caused the catastrophic Spanish influenza pandemic of 1918. In 1957 the Asian pandemic virus acquired three genes (PB1, HA, and NA) from the avian influenza gene pool in wild ducks by genetic reassortment and kept five other genes from the circulating human strain. After the Asian strain appeared, the H1N1 strains disappeared from humans. In 1968 the Hong Kong pandemic virus acquired two genes (PB1 and HA) from the duck reservoir by reassortment and kept six genes from the virus circulating in humans. After the appearance of the Hong Kong strain, the H2N2 Asian strains were no longer detectable in humans. In 1977 the Russian H1N1 influenza virus that had circulated in humans in 1950 reappeared and spread in children and young adults. This virus probably escaped from a laboratory and has continued to cocirculate with the H3N2 influenza viruses in the human population.

indicate a close relationship among all of the genes of classic swine H1N1 and human influenza A viruses (46). Estimates provided by these analyses suggest a date between 1905 and 1914 for the origin of the common ancestor of human and classic swine viruses, whose genes were avianlike. Hence, a totally new H1N1 virus of avian origin (not a reassortant) could have appeared in human or swine populations before the 1918 influenza pandemic and replaced the previously existing human virus strains. Whether the virus was first introduced into humans and then transmitted to pigs, or vice versa, remains unknown.

Mechanism(s) for the Appearance of New Human Pandemic Strains of Influenza Virus

The new subtypes of influenza viruses that have appeared in humans, as occurred in 1957 (Asian influenza), in 1968 (Hong Kong influenza), and in 1977 (Russian influenza), have several characteristics in common: (i) their appearance was sudden, (ii) they first occurred in China, (iii) they were antigenically distinct from the influenza viruses then circulating in humans, and (iv) they are confined to the H1, H2, and H3 subtypes.

On the basis of phylogenetic evidence presented above, the most likely explanation for the appearance of new pandemic strains in humans is that they were derived from avian influenza viruses either after reassortment with the currently circulating human strain or by direct transfer (Fig. 7). There is ample evidence for genetic reassortment between human and animal influenza A viruses in vivo (180), and genetic reassortment has also been detected in humans (29). Genetic and biochemical studies conclude that the 1957 and 1968 strains arose by genetic reassortment. As described above, the 1957 Asian H2N2 strain obtained its HA, NA and PB1 genes from an avian virus and the remaining five genes from the preceding human H1N1 strain (45, 77, 149).

The new pandemic strain that occurred in Hong Kong in 1968 contained the H3 HA and PB1 genes from an avian donor (39, 77) and the N2 NA and five other genes from the N2N2 strain circulating in early 1968. The HA of the human Hong Kong/68 strain differed by only seven amino acids from Asian avian H3 strains (9a), providing the strongest evidence as yet that the Hong Kong virus originated by reassortment between an avian influenza virus and the circulating human strain.

The second way that new pandemic viruses could occur in the human population would be if an avian strain or a strain

from another mammal became infectious for humans. The phylogenetic evidence discussed above favors this mechanism for the appearance of the Spanish influenza virus in 1918. The periodic transmission of swine influenza viruses to humans that presumably occurred at Fort Dix and the isolation of virtually identical viruses from pigs and humans (57, 141) leave no doubt that such transmissions occur in nature and may be more frequent than originally thought. Most of these transmissions are dead-end transmissions in that the viruses have little or no capacity to secondarily transmit to human contacts and initiate an epidemic. However, the disastrous pandemic virus of 1918 to 1919 did possess this property.

A third explanation for the origin of pandemic viruses is that the new virus which may have caused an epidemic many years previously remained hidden and unchanged in some place since that time. The appearance of Russian influenza (H1N1) provided support for this concept. This virus appeared in Anshan, northern China, in May of 1977 and subsequently spread to the rest of the world; it is identical in all genes to the virus that caused a human influenza epidemic in 1950 (116). Where was this virus for 27 years? The possible explanations include preservation in a frozen state, preservation in an animal reservoir, or retention in an integrated, as yet undetected form in the genetic material of a human or lower animal. The animal reservoir option is unlikely, for the accumulation of mutations would have continued. There is no evidence for integration of influenza genetic material into the host genome, leaving the most likely explanation that in 1977 the H1N1 virus was reintroduced to humans from a frozen source.

From the above discussion it is apparent that there are multiple ways in which pandemic influenza viruses can arise, and each has probably played a role in the evolution of the influenza viruses currently circulating in humans. The primordial source of all influenza virus genes is from the aquatic bird reservoir, and many mutations and reassortments occur during adaptation to the human host.

Is the Pig a "Reassortment Vessel?"

Current human influenza viruses are believed to have arisen by genetic reassortment between previous human influenza viruses and nonhuman viruses. Where did the reassortment between genes of human and avian influenza viruses occur?

Reassortment requires simultaneous infection of a host animal with both avian and human influenza viruses. The pig has been the leading contender for the role of intermediate host for reassortment: swine are the only mammalian species which are domesticated, are reared in abundance, and are common hosts for human influenza viruses. The evidence supporting the role of the pig is as follows. (i) Pigs are susceptible to infection by subtype H1N1 and H3N2 influenza viruses of both human and avian origin, although the mechanism by which pigs contract avian viruses is undetermined. Pigs probably acquire human viruses by inhalation of aerosols during periods of close contact. (ii) Humans occasionally contract influenza viruses from pigs, such as in 1976 or 1988, when isolated cases of swine influenza in humans caused deaths. About 10% of persons with occupational exposure to swine develop antibody to swine influenza virus (145). (iii) Genetic analyses indicate that genes for most internal proteins of human influenza viruses share a common ancestor with the equivalent genes of most swine influenza viruses, but not with the equivalent genes of other mammalian influenza viruses, after diverging from the avian virus lineage. (iv) There is no evidence that humans are susceptible to natural infection with true avian influenza viruses.

There has not been any "smoking-gun" influenza virus that appeared in swine before starting a human pandemic. Subtype H2 viruses such as caused human disease from 1957 to 1968 have never been detected in swine. Subtype H3 viruses of both avian and human origin have been detected in pigs but not before 1968. The mass-vaccination strategy in response to the Fort Dix swine influenza incident in 1976 was predicted on the belief that H1 virus would be the next pandemic strain; instead, it quickly died out in humans. The avianlike H1N1 virus which emerged and became established in pigs in Europe (147) might have been another candidate as the next human pandemic strain, but if so it was probably forestalled by the accidental reemergence of true human H1N1 viruses in 1977. Swine are not routinely screened for influenza virus infection, and such surveys that have been done (see, e.g., references 25 and 57) concentrated their attention on the H1 and H3 subtypes already known to be harbored by swine. Under these conditions a smoking gun may not be spotted. Greater attention must be given to the possibility of swine infection with the subtypes H4 to H14.

The molecular determinants of viral host range restriction in any species are not thoroughly understood (see the section on molecular determinants of host range restriction). Because avian influenza viruses do not normally infect humans, the mechanism by which avian influenza viruses may adapt to growth in humans has not been examined. Therefore the necessity for preadaptation of avian influenza viruses or their genes to a mammalian host such as swine, before they can become infectious for humans, has not been established. Ferrets and squirrel monkeys can be experimentally infected with different avian influenza viruses, with virus shedding and seroconversion. Over 25% of volunteers inoculated with high doses of avian influenza viruses (subtypes H4, H6, and H10) shed virus and displayed mild disease symptoms, but produced no detectable hemagglutinating inhibiting antibody (13). The failure of a swine virus such as the Fort Dix virus to displace the established human influenza virus (A/Victoria/3/75 [H3N2]) as the dominant strain in a population, even when introduced into the closed, relatively crowded, and immunologically naive Fort Dix community, suggests that swine-adapted viruses are not, after all, very well adapted to humans.

The emergence of new human pandemic viruses is rare, occurring at unpredictable intervals on the order of decades. It is possible that these rare events are caused by rare circumstances, which may never be specifically identified. One could speculate, for instance, about humans with mild influenza infections bathing in virus-contaminated duck ponds. Currently available evidence suggests that the common circumstance of close contact between humans and pigs facilitates the spread of particular viruses, the swinelike H1 and humanlike H3 viruses, among these two species. Pigs might be the reassortment vessel for generation of some novel pandemic viruses, although not necessarily for all.

Is there an Epicenter for Influenza Viruses?

Historical records and the appearance of the Asian, Hong Kong, and Russian pandemic strains of influenza virus in China suggest that the majority of pandemics of human influenza since about 1850 have originated in China. The exception seems to be Spanish influenza, which may have

originated in military camps in Kansas and was taken to Europe by U.S. troops in 1918 (30).

The possibility has been raised that southern China is an influenza epicenter (155). Unlike the temperate or subarctic regions of the world, where influenza in humans is a winter disease, in the tropical and subtropical regions of China influenza occurs year round (134). In China, influenza A viruses of all subtypes are prevalent in ducks and in water frequented by ducks (49) and the different subtypes are present year round with a peak incidence in summer months (154). In China and other areas of Southeast Asia, influenza viruses of the H1N1 and H3N2 subtypes are prevalent in pigs year round (49, 156).

These studies establish that influenza viruses occur in humans, pigs, and aquatic birds in China, but how is this different from other tropical and subtropical regions of the world? Ecological studies show that influenza viruses occur in each of these species in all countries where tests have been done; these countries include temperate as well as tropical climates (59, 100). In temperate climates influenza in people and pigs is a winter disease and usually occurs when free-flying aquatic birds are absent. The tropical and subtropical regions of the world include Southeast Asia (including Southern China), India (including Pakistan and Bangladesh), Central Africa, and Central America. If we examine the distribution of people, pigs, and ducks in these countries, we find that the human population is largest in India and China, smaller in Central Africa, and smallest in Central America. The pig population is largest in China, small in India and Central America, and moderate in Central Africa. The distribution of wild and domestic ducks in the world is influenced largely by the availability of surface water, and they are prevalent in all regions except the Antarctic. The domestic duck population is largest in China and smaller in the other regions; however, aquatic birds migrate through or overwinter in subtropical and tropical regions.

Religious customs may influence the regions where influenza may originate. Pigs are a common domestic animal throughout the world, but their incidence is influenced by religion, social customs, and climate. Pigs are not an approved source of protein in the Muslim and Jewish religions and are considered dirty animals in India, where up to 80% of the population are vegetarians. Since pigs may play an important role in interspecies transmission of influenza, they may be the limiting factor in some countries and may explain why influenza viruses do not occur in all tropical regions of the world. These considerations leave southern China as a possible region where influenza viruses cocirculate in people, pigs, and ducks, thus providing the opportunity for interspecies transmission and genetic exchange among influenza viruses.

Although the above considerations about the regions of the world where influenza pandemics originate are interesting, they are still merely speculations. Only circumstantial evidence exists for the appearance of pandemic influenza viruses in southern China. Epidemiological studies of the frequency of influenza virus transfer between species have not been done, and they merit attention. The molecular tools are at hand for answering these questions. There may be additional unknown ecological features, such as air pollution, genetic susceptibility, host range, and local customs, that influence the appearance of influenza viruses.

Over the past few years, an increasing number of strains from the People's Republic of China have been included in the annual vaccine recommendations by the World Health Organization. For the 1990 to 1991 influenza season, the World Health Organization recommended vaccination with three strains of Asian origin including A/Shanghai/16/89 (H3N2), A/Taiwan/1/86 (H1N1), and B/Yamagata/16/89. The possibility exists that southern China is the epicenter for epidemic as well as pandemic influenza viruses.

Further studies on influenza in Southeast Asia are necessary to determine whether an epicenter does exist in this region and whether it is an important source of strains for inclusion in vaccines.

Why Do Some Virus Strains Die Out?

One noteworthy characteristic of the history of influenza virus in humans is that when a new virus subtype appears and causes a pandemic, the previously circulating subtype usually disappears. This probably occurred in 1918 when emerging H1N1 viruses replaced H3-like viruses, in 1957 when the H2N2 subtype replaced H1N1, and 1968 when H3N2 replaced H2N2. It did not occur in 1977, when H1N1 viruses reemerged after a 20-year absence from humans. Since 1977, H1N1 and H3N2 viruses have cocirculated.

The reasons for the sudden disappearance of previously circulating human strains are unknown. Presumably the earlier strain was somehow disadvantaged in comparison with the novel one, despite the benefit of years of adaptation to humans. The obvious disadvantage is that it has already elicited widespread immunity in the human population. Another hypothetical disadvantage may be that after a period of evolution in humans, the older strain has reached a biological limit: it can no longer produce a viable variant which differs antigenically from previously occurring variants, or alternatively new variants lead in the direction of decreased fitness of the virus for growth in human hosts (190). There is insufficient evidence to support this from current knowledge of the evolution of the H1 and H2 human viruses that died out, and it will be interesting to compare in fitness the evolutionary course of the human H1 viruses from 1950 to 1957 and again from 1977 to the present.

Virus disappearance may be explained by a mechanism for systematic interference between competing strains; i.e., infection with one subtype of virus elicits cross-protection against a different subtype. Sonoguchi et al. (163) observed significantly fewer second infections than expected if there were no cross-protection. Frank et al. (40) did not find this quantitative difference in another study, but both studies found that when second infections did occur, the severity and duration of symptoms were reduced. This suggests that in an individual, the first influenza A infection in an influenza season suppresses a second influenza A infection, even of different subtype, but not an influenza B infection, nor an influenza A infection in a following year. This suppression is likely to result in decreased virus shedding even if symptoms are produced. It is interesting that, according to a recent simulation of influenza virus epidemic spread by Ackerman et al. (1), viral interference resulting in a 50% decrease in second virus shedding or a 25% decrease in host susceptibility to second virus attack would be very difficult to actually detect by standard epidemiological survey.

Suppression cannot be mediated by anti-HA antibody. The phenomena of intrinsic viral interference and interferon-mediated interference may provide cross-protection, but only during or within a few days of the initial viral infection. We propose that over a longer term of 1 to 2 months, suppression is due to the $CD8^+$ T-lymphocyte response to the shared internal proteins of human influenza A virus. The $CD8^+$ T-lymphocyte response is considered important for

recovery from influenza infection but is not generally protective by itself against subsequent infection. The frequency of virus-specific $CD8^+$ T-lymphocyte precursors declines over the months following virus infection (109), and the time needed for recall of a cell-mediated immune response becomes protracted, allowing a subsequent infection to become established and progress to disease (in the absence of specific antibody). However, it is conceivable that over a relatively brief period following acute viral infection, a heightened $CD8^+$ T-lymphocyte response against influenza A virus internal proteins could effectively curtail a subsequent influenza A infection. In 1968, antibody to N2 NA could also have played a suppressive role.

Even the latest variant of a previously circulating virus subtype is relatively limited in its ability to spread rapidly, owing to widespread herd immunity induced by earlier variants that are cross-reactive to some degree. A virus of novel subtype can spread without restraint; e.g., newly arising pandemic strains typically infect a higher than normal fraction of the human population, and in 1918 and 1957, pandemic outbreaks preceded the start of the normal influenza season (reviewed in references 30 and 87). Thus in a pandemic year the novel strain will probably be the first to infect most people. If, as proposed above, the first influenza A infection in an individual indeed suppresses subsequent influenza A infections in the same year, maintenance of the older subtype will be handicapped by the decreased susceptibility of hosts who would otherwise be susceptible to it (owing to insufficient antibody) and the consequent reduction of total shedding of the older virus.

Would this be sufficient to cause the extinction of a subtype? The events of 1977 to 1979 suggest that it is likely. In 1977 subtype H1N1 viruses had been absent from humans for only 20 years; therefore, probably half of the human population already possessed some degree of immunity to them when they reemerged. Within this half, H3N2 virus circulation could proceed almost normally. Additionally, in 1977 an important variant virus, A/Texas/77, was replacing the previous A/Victoria/75-like viruses as the predominant H3N2 strain. Yet despite these favorable circumstances, H3 viruses almost completely disappeared in the following year (24). Without them, H3 viruses might have disappeared from humans completely.

Among animal influenza viruses there is no parallel to this phenomenon of sudden virus replacement and extinction. Human H3 viruses have periodically entered the swine populations since 1968, without replacing H1 viruses as the major influenza virus of swine. In horses H7N7 and H3N8 viruses cocirculated for over a decade, although H7N7 viruses eventually became very rare or extinct (179a). In waterfowl many virus subtypes cocirculate continually. Although not every subtype is detected in a given year, it is believed that each subtype is maintained at low frequency. There is no evidence that any subtype has become globally extinct throughout the waterfowl reservoir.

Do human influenza viruses of "extinct" subtypes survive in hidden reservoirs? It is unlikely that isolated small groups of humans are refuges for epidemic viruses, because such viruses would rapidly induce group immunity and be extinguished before significantly new antigenic variants could be generated. Instead, Monto and Maassab (110) proposed that "extinct" human influenza viruses continue to circulate at very low frequency, like the more rare influenza viruses of waterfowl or like interepidemic (summertime) influenza in humans. There is no evidence that this occurs in humans over decades without an outbreak, and we await the emergence of the next pandemic strain to clarify the matter. The 1977 reemergence of the H1N1 subtype is an exceptional case: this virus from 1950 almost certainly escaped back into nature from frozen storage.

Alternative Theories for the Appearance of Human Influenza Viruses

There are two other controversial theories for the appearance of human influenza virus. These are controversial because although they seem to explain the occurrence of simultaneous disease outbreaks without discernable connection, there is no biological evidence to support them. One explanation, by Hope-Simpson and Golubev (65–67), is that influenza viruses are spread by humans who are themselves asymptomatic carriers, rather than directly from one acutely infected host to the next. These carriers supposedly once suffered an acute influenza infection. In these latently infected carriers, seasonal changes correlated with solar irradiation serve to reactivate the virus infection, but in rare cases it may presumably remain dormant until many decades have passed. However, despite some amazing claims, there has been no confirmed instance of latent influenza infection in humans or other animals. It is now known that influenza and other respiratory viruses do not completely cease to circulate during interepidemic periods, although the incidence and severity of disease are greatly reduced. This can account for the separate development of epidemic foci during the "flu season" without necessity for viral latency. The second controversial model is from Hoyle and Wickramasinghe (68, 69), who claim that influenza viruses are brought to Earth by comets. Within the upper atmosphere there may be several related virus variants drifting about, and every 10 years or so the Earth picks up a new group of virus strains from a different comet. Seasonal epidemics are the result of seasonal changes in atmospheric circulation which temporarily bring down large numbers of virus particles to the surface, where they may be inhaled by humans or animals. These authors have recently attempted to link the incidence of influenza pandemics with the sunspot cycle (70). However, in reality the occurrence of pandemics is fairly well distributed over all phases of the sunspot cycle (102, 177). There is absolutely no direct evidence in support of this view, which ignores the most fundamental features of virus biology, and influenza virologists consider it to be without merit. Yet it reappears from time to time in the popular press and the correspondence pages of *Nature*. Henderson et al. (54, 55) statistically analyzed and rejected three versions of cometary models for influenza virus evolution in favor of continuous biological evolution in humans.

The comet theory and the latency theory both seem to be attempts to design models of virus ecology which fit epidemiological information that is viewed as not in accord with standard models of virus transmission. In our view, defects of standard influenza epidemiology are more simply explained by a background of subclinical infections during interepidemic periods.

Can the Emergence of Pandemic Strains be Prevented?

With the realization that there is a reservoir of all known influenza A virus subtypes in aquatic wild birds in nature, recommendations have been made by agricultural authorities to prevent direct or indirect contact between domestic poultry and wild birds. One of the classic mistakes made by chicken and turkey farmers is to raise a few domestic ducks

on a pond near their poultry barns, for these birds attract wild ducks. Each of the highly pathogenic outbreaks of avian influenza that have occurred in recent years—H5N2 in chickens and turkeys in Pennsylvania and surrounding states in 1983 to 1984 (11), H5N8 in turkeys in Ireland in 1983 (113), and H7N7 in chickens in Victoria, Australia, in 1985 (31)—could probably have been prevented if the domestic poultry had been raised in ecologically controlled houses that maintained a high standard of security and had limited access.

Turkeys raised on open range in Minnesota are frequently infected with influenza viruses. In a 2-year period, 97 flocks had virological or serological evidence of influenza virus infection (53). Eight different serotypes were isolated from the turkeys, and antigenically similar viruses were isolated from sentinel ducks. Straightforward preventative measures have been recommended to minimize contact between the reservoir of influenza viruses in nature and domestic poultry. These are all aimed at minimizing contact between domestic poultry and wild birds, feces, and contaminated water and include the following. (i) Do not walk directly from outside environments into poultry houses without washing boots. (ii) Do not use untreated pond water for watering poultry. The frequency of influenza virus infection in turkeys raised in "closed" houses is much lower than among turkeys raised on the range.

If we assume that people, pigs, and aquatic birds are the principal variables associated with the interspecies transfer of influenza virus and the emergence of new human pandemic strains, it may be possible to influence the occurrence of human pandemics of influenza. The principles applied to preventing outbreaks of influenza in domestic animals should be equally applicable here. We know that pandemic strains of human influenza emerge only rarely; however, the available information indicates that interspecies transmission of influenza viruses may not be so rare, for up to 10% of persons with occupational exposure to pigs develop antibodies to swine influenza virus (145). We know that the majority of transfers of influenza viruses from pigs to humans are dead-end transfers in that they do not spread efficiently from human to human. As indicated above, we do not know the frequency of virus transfer between the suspect species in southern China. If there is an epicenter for pandemic influenza, and if there is a detectable frequency of transfer between people, pigs, and ducks, and if we understand the ecological and agricultural features involved in the transfer, pandemics may be preventable. If pigs are the major mixing vessel for influenza viruses, changes in the agricultural practices that separate pigs from people and ducks could conceivably prevent future pandemics.

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