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The Avian Influenza Virus Nucleoprotein Gene and a Specific Constellation of Avian and Human Virus Polymerase Genes Each Specify Attenuation of Avian-Human Influenza A/Pintail/79 Reassortant Viruses for Monkeys

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Reassortant viruses which possessed the hemagglutinin and neuraminidase genes of wild-type human influenza A viruses and the remaining six RNA segments (internal genes) of the avian A/Pintail/Alberta/119/79 (H4N6) virus were previously found to be attenuated in humans. To study the genetic basis of this attenuation, we isolated influenza A/Pintail/79 × A/Washington/897/80 reassortant viruses which contained human influenza virus H3N2 surface glycoprotein genes and various combinations of avian or human influenza virus internal genes. Twenty-four reassortant viruses were isolated and first evaluated for infectivity in avian (primary chick kidney [PCK]) and mammalian (Madin-Darby canine kidney [MDCK]) tissue culture lines. Reassortant viruses with two specific constellations of viral polymerase genes exhibited a significant host range restriction of replication in mammalian (MDCK) tissue culture compared with that in avian (PCK) tissue culture. The viral polymerase genotype PB2-avian (A) virus, PB1-A virus, and PA-human (H) virus was associated with a 900-fold restriction, while the viral polymerase genotype PB2-H, PB1-A, and PA-H was associated with an 80,000-fold restriction of replication in MDCK compared with that in PCK. Fifteen reassortant viruses were subsequently evaluated for their level of replication in the respiratory tract of squirrel monkeys, and two genetic determinants of attenuation were identified. First, reassortant viruses which possessed the avian influenza virus nucleoprotein gene were as restricted in replication as a virus which possessed all six internal genes of the avian influenza A virus parent, indicating that the nucleoprotein gene is the major determinant of attenuation of avian-human A/Pintail/79 reassortant viruses for monkeys. Second, reassortant viruses which possessed the viral polymerase gene constellation of PB2-H, PB1-A, and PA-H, which was associated with the greater degree of host range restriction in vitro, were highly restricted in replication in monkeys. Since the avian-human influenza reassortant viruses which expressed either mode of attenuation in monkeys replicated to high titer in eggs and in PCK tissue culture, their failure to replicate efficiently in the respiratory epithelium of primates must be due to the failure of viral factors to interact with primate host cell factors. The implications of these findings for the development of live-virus vaccines and for the evolution of influenza A viruses in nature are discussed.

Epidemic wild-type human influenza A viruses can be attenuated for use as live influenza A virus vaccines for humans by the acquisition, through gene reassortment, of the six genes (internal genes) of avian influenza A viruses which encode proteins other than the surface glycoproteins, i.e., hemagglutinin (HA) and neuraminidase (NA) (8, 16, 18, 25). Previously, it was shown that the acquisition of either the nucleoprotein (NP) gene or the matrix (M) gene of the avian influenza A/Mallard/New York/6750/78 (H2N2) virus was sufficient to attenuate the human influenza A/Udorn/307/72 (H3N2) virus for squirrel monkeys (26). The nucleotide sequences of the NP and M genes of human and avian influenza A viruses were determined, and the predicted amino acid sequences of the encoded proteins were deduced (6, 7). Comparison of the amino acid sequences of the respective avian virus and human virus proteins revealed the existence of host species-specific amino acid substitutions at specific residues (6, 7). We were interested in further evaluating the possibility that these host species-specific amino acid substitutions play important roles in the attenuation of avian-human (AH) influenza A reassortant viruses for primates. We chose to study this possibility by determining whether the NP or M genes of other avian viruses also specified attenuation for primates.

The avian influenza A/Pintail/Alberta/119/79 (H4N6) virus was selected for this study because reassortant viruses containing the six internal genes of the A/Pintail/79 virus were attenuated in squirrel monkeys and humans (8, 25). We sought to characterize the genetic basis of attenuation of these reassortants by isolating reassortant viruses which contain other combinations of genes from the influenza A/Pintail/79 and A/Washington/897/80 (H3N2) viruses and evaluating their level of replication in squirrel monkeys. The failure of either the NP or M gene of the influenza A/Pintail/79 virus to specify attenuation in primates would suggest that the avian influenza virus-specific amino acid substitutions present on that gene were not sufficient to cause attenuation. We observed the following. (i) The NP gene of the avian influenza A/Pintail/79 virus specified attenuation in monkeys, whereas the M gene did not. (ii) A specific constellation of human and avian virus polymerase genes also specified restricted replication in the respiratory

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tract of monkeys. The relevance of these observations to the development of live attenuated influenza A vaccines and to the evolution of influenza viruses in nature is discussed.

MATERIALS AND METHODS

Viruses. The isolation, the biologic cloning in primary chick kidney (PCK) tissue culture, and the characterization of the avian influenza A/Pintail/Alberta/119/79 (H4N6) virus, the human influenza A/Washington/897/80 (H3N2) virus, and the six-gene AH influenza A/Washington/897/80 × A/Pintail/Alberta/119/79 (H3N2) (clone 4) reassortant viruses were described previously (15, 17). (The term six-gene reassortant refers to viruses which acquired the genes which encode the surface glycoproteins HA and NA from the human virus parent and the six internal genes from the avian virus parent).

Production of AH reassortant viruses. Two additional reassortant viruses, clones 2 and 5, were isolated, plaque purified, and grown in eggs in the same manner as clone 4 was (15). All other reassortant viruses were produced by coinfecting PCK or Madin-Darby canine kidney (MDCK) cultures at a multiplicity of infection of 1 with the wild-type influenza A/Washington/80 virus and either the six-gene AH reassortant virus or a subsequently isolated reassortant virus which possessed a mixed constellation of internal genes. The coinfected cultures were incubated at 37°C and harvested 24 h later. Plaque progeny were picked from PCK or MDCK monolayers overlaid with modified Lebowitz-15 medium with HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (M. A. Bioproducts, Walkersville, Md.) plus antibiotics and 0.8% agarose and maintained at 37°C. Plaque progeny were amplified in the allantoic cavity of 9-day-old embryonated eggs, and the genotype of the virus population was determined as described below. Viruses which contained a mixed constellation of internal genes were biologically cloned by plaque-to-plaque passage on PCK or MDCK monolayers and were subsequently amplified in the allantoic cavity of 9-day-old eggs.

Host range phenotype. The infectivity of parental viruses and their reassortant progeny was determined by titration in MDCK and PCK 24-well tissue culture plates maintained at 37°C. Viruses were considered restricted in replication in MDCK if their infectivity for MDCK was 100-fold or more lower than that for PCK. All subsequent studies of viruses which exhibited a host range restriction of replication (*hr* phenotype) were performed with PCK monolayers.

Efficiency of plaque formation. The efficiency of plaque formation of parental viruses and their reassortant progeny was determined at 37, 41, and 42°C on MDCK or PCK as previously described (18).

Genotype of reassortant viruses. The parental origin of the RNA segments of each reassortant virus was determined by comparison of their migration in polyacrylamide gel electrophoresis with those of the corresponding parental genes. Viruses were propagated and purified, and their RNA was extracted as previously described (26), except that virus was pelleted from 10 ml of clarified allantoic fluid through 15 ml of 30% sucrose in STE (0.1 M NaCl, 2.0 mM EDTA, 20.0 mM Tris hydrochloride [pH 7.4]) at 45,000 rpm for 75 min in a Beckman 50.2 Ti rotor at 4°C. The virus pellet was resuspended in 200 μl of STE, the virus was lysed, and its RNA was ethanol precipitated, as previously described (26). If necessary, the viral RNA was extracted twice with phenol and once with chloroform before ethanol precipitation to reduce background on gels.

Purified viral RNA was analyzed in polyacrylamide gels containing 2.6% acrylamide and either 4.5 or 6.0 M urea as previously described (15). Electrophoresis was done for 15 h at 10°C and 84 V. RNA segments were visualized by ammoniacal silver staining of the gels (5).

Determination of coding assignments of viral polymerase genes. Synthetic oligonucleotide primers with sequences specific for each of the three influenza A virus polymerase genes were extended by reverse transcription, with the influenza virus genome RNA as a template. The primers used were: (i) PB2 5'-GAAAGCAGGTCAATT, (ii) PB1 5'-GAAAGCAGGCAAACC, and (iii) PA 5'-GAAAGCAGGTACTGA. (The first nucleotide of each primer was complementary to the fourth nucleotide from the 3' end of the viral RNA.) Dideoxynucleotide chain termination sequencing reactions and gels were as described by Naeve et al. (19).

Studies in squirrel monkeys. The duration and level of replication of the parental viruses and their reassortant progeny were evaluated in squirrel monkeys as previously described (17, 26). Briefly, groups of four monkeys housed in pairs in Horsfal isolation units were inoculated intratracheally with 10^7 50% tissue culture infectious doses ($TCID_{50}$) of a reassortant virus in a 0.5-ml inoculum. Nasopharyngeal swab specimens that were obtained daily for 10 days postinfection and tracheal lavage fluids that were obtained on days 2, 4, and 6 postinfection were inoculated freshly into MDCK or PCK monolayers, and portions were frozen for subsequent titration on appropriate tissue cultures. Four separate studies were performed, and in each study two monkeys received the wild-type human influenza A/Washington/80 virus while two other monkeys received the influenza A/Pintail/79 × A/Washington/80 six-gene reassortant clone 4 virus. In one of these studies, the replication of *hr* viruses was evaluated. PCK tissue culture was used for virus isolation and titration, and the results obtained from control monkeys, which received either wild-type or six-gene reassortant clone 4 virus, were similar to those of other studies in which MDCK tissue was used for virus titration, indicating that the PCK tissue cultures used were competent to quantify virus replication. The mean duration and peak titer of virus shedding were determined for each group of four monkeys which received reassortant virus, and these values were compared with those obtained from each group of eight monkeys which received either wild-type human influenza A virus or six-gene AH reassortant virus.

TABLE 1. Isolation of A/Pintail/79 × A/Washington/80 reassortant viruses

Cross[a]	Virus crossed with A/Washington/80 wild type	Reassortant viruses isolated[b]
A	E-201	39, 82, 202 through 314
B	39	39-20
C	82	82-7, 82-20, 82-31
D	82-20	11, 22
E	22	49
F	208	24, 27
G	24	15

[a] Crosses A to E and G were performed in PCK tissue cultures. Cross F was performed in MDCK tissue culture.

[b] In several instances, more than one isolate with a given genotype was isolated, but only one representative isolate was evaluated.

TABLE 2. Genotype and characterization of AH influenza A/Pintail/79 × A/Washington/80 reassortant viruses in vitro

Clone designation	No. of genes from avian virus	Parental origin of genes in reassortant viruses at indicated locus[a]						Reduction in infectivity in MDCK compared with that in PCK (log_{10} $TCID_{50}$/ml)	Log_{10} reduction in titer (PFU/ml) at indicated temperature compared with 37°C	
		PB2	PB1	PA	NP	M	NS		41°C	42°C
A/Washington/80 wild type	0	H	H	H	H	H	H	−0.7	2.7	≥4.0
278	1	A	H	H	H	H	H	−0.4	1.3	2.8
15	1	H	H	A	H	H	H	−0.6	1.2	≥3.7
264	1	H	H	H	A	H	H	−0.9	1.1	2.2
204	1	H	H	H	H	A	H	−0.8	2.0	≥3.3
82-7	1	H	H	H	H	H	A	−1.1	1.7	≥3.7
251	2	H	H	H	A	A	H	−0.2	0.7	1.4
218	2	A	H	H	H	A	H	0.1	0.7	2.0
228	2	A	H	H	H	H	A	−1.0	0.9	2.4
49	2	H	A	H	H	A	H	4.5[b]	≥3.7	≥3.7
24	2	H	H	A	H	H	A	−1.0	1.9	3.3
39-20	3	A	A	H	H	A	H	2.8[b]	1.7	≥3.6
314	3	A	H	H	A	H	A	−0.1	0.8	1.2
22	3	H	A	H	A	A	H	5.0[b]	2.6	≥3.9
27	3	A	H	H	H	A	A	−0.2	0.4	0.9
11	3	H	H	H	A	A	A	−0.5	0.3	0.6
224	3	H	A	H	A	A	H	4.3[b]	0.9	≥3.0
206	3	A	A	H	A	H	H	2.1[b]	0.1	0.2
226	4	A	H	H	A	A	A	−1.1	0.1	0.3
208G	4	A	H	A	H	A	A	−0.5	1.0	2.3
215	4	H	A	H	A	A	A	5.0[b]	0.1	−0.2
245	4	A	A	H	A	A	H	3.4[b]	0.5	0.1
289	4	A	H	A	A	A	H	−0.8	0.4	1.0
82-31	4	A	A	A	A	H	H	0.4	≥2.3	≥2.3
82-20	5	H	A	A	A	A	A	0.1	≥3.2	≥3.2
4	6	A	A	A	A	A	A	−0.7	−0.1	0.1

[a] A, Gene derived from the avian A/Pintail/79 virus; H, gene derived from the human A/Washington/80 virus. Underlining indicates the gene or combination of genes associated with attenuation.

[b] Viruses which manifest the *hr* phenotype, as defined in Materials and Methods.

RESULTS

Isolation and genotype of reassortant viruses with mixed internal gene constellations. A total of 326 isolates obtained from the mating of the six-gene AH reassortant clone 4 × A/Washington/80 or from an additional six matings of reassortant progeny × A/Washington/80 were characterized (Table 1). One-third of these isolates were reassortant viruses, and viruses with 24 unique genotypes were identified. Although we isolated a reassortant virus (clone 49) which contained the avian influenza virus PB1 and M genes, we were unable to isolate a reassortant which contained only an avian influenza PB1 polymerase gene, despite screening 84 progeny viruses from a mating of clone 49 × A/Washington/80 virus, in which a reassortant frequency of 29% was observed.

To identify unequivocally the origin of the polymerase genes in the reassortants, the partial nucleotide sequences (100 to 200 bases) of each of the polymerase genes in clones 15, 49, and 278 were determined (data not shown) with primers which were specific for each of the three polymerase genes (PB2, PB1, and PA) and which primed both avian and human influenza A virus polymerase genes. Since each of these three reassortant viruses acquired a different influenza virus polymerase gene from the avian virus parent, and since there was 9 to 16% sequence divergence between the respective avian and human influenza A virus genes, it was possible to identify the parental origin of each polymerase gene in each of the three reassortant viruses. The origin of the polymerase genes in each of the other reassortant viruses was then determined by comparing the order of migration of the reassortant virus polymerase genes in polyacrylamide gel electrophoresis with those of clones 15, 49, and 278. The genotypes of the AH influenza A reassortant viruses are shown in Table 2.

The same procedure was applied to influenza A/Mallard/New York/6750/78 × A/Udorn/307/72 reassortant viruses reported earlier (26). The results showed that the avian influenza A/Mallard/New York/6750/78 RNA segments 1, 2, and 3 encoded polymerase proteins PB1, PB2, and PA, respectively.

Host range phenotype. Two reassortant viruses, clones 2 and 5, were isolated on PCK tissue cultures because they were intended for investigational study in humans as candidates for live-virus vaccines, as was clone 4. Both virus suspensions were found to have low infectivity for MDCK tissue cultures, despite high hemagglutination titers and high infectivity titers for PCK tissue culture (data not shown). This finding was unexpected because both the A/Washington/80 and A/Pintail/79 virus parents replicated efficiently on both MDCK and PCK monolayers. Both reassortant viruses were subsequently found to have the same mixed constellation of polymerase genes, and from this we inferred that this constellation of polymerase genes might specify a host-dependent restriction of replication. If this were true, it would be difficult to isolate reassortant viruses with certain

TABLE 3. Association of polymerase genotype and host range restriction in vitro

Polymerase genotype[a]	Virus	Log_{10} reduction in titer ($TCID_{50}$/ml) in MDCK compared with that in PCK
AAH[b]	206	2.1
	39-20	2.8
	245	3.4
	2A1	2.8
		2.8 ± 0.3[c]
HAH	49	4.5
	22	5.0
	224	4.3
	215	5.0
		4.7 ± 0.2[c]

[d]

[a] A, Gene derived from A/Pintail/79 virus; H, gene derived from A/Washington/80 virus.
[b] Genotype is, in order, PB2, PB1, and PA.
[c] Mean ± standard error.
[d] $P < 0.002$ by two-tailed Student's t test.

genotypes in MDCK monolayers since these viruses would be restricted in replication. Because of this possibility, we used PCK tissue for the isolation of the A/Pintail/79 × A/Washington/80 reassortant viruses indicated in Table 1.

The results of this study confirmed the hypothesis that a specific combination of polymerase genes specified the *hr* phenotype. Each reassortant virus which received the polymerase PB1 gene from the A/Pintail/79 virus parent and the polymerase PA gene from the A/Washington/80 virus had an infectivity titer in MDCK monolayers that was significantly lower than that in PCK cultures. The parental origin of the PB2 gene further modified the infectivity of these reassortant viruses for MDCK cultures (Table 3). The presence of an avian influenza virus gene at the PB2 locus (polymerase genotype AAH [PB2, PB1, and PA, respectively] in which A represents a gene derived from avian A/Pintail/79 virus and H represents a gene derived from human influenza A/Washington/80 virus) was associated with host range reduction of infectivity on the order of 100- to 6,000-fold, while the presence of a human virus PB2 gene (genotype HAH) was associated with a 30,000- to 100,000-fold reduction in infectivity ($P < 0.002$).

Replication at temperatures restrictive for wild-type human viruses. Previous studies demonstrated that avian influenza viruses replicate efficiently at 42°C, a temperature restrictive for wild-type human influenza A viruses, and that this phenotype could be transferred to human influenza A viruses via the transfer of one or more avian influenza A virus internal genes (12, 19). (We define the *ts* phenotype in terms of the characteristics of the avian virus parent, i.e., ts^+ viruses form plaques efficiently at 42°C, whereas *ts* viruses manifest 100-fold or greater reduction in plaque formation at elevated temperatures [41 or 42°C] compared with that at permissive temperature [37°C].) Studies with the AH A/Mallard/78 × A/Udorn/72 reassortant viruses indicated that the avian virus polymerase PB1 gene was sufficient, though not essential, for the transfer of the ts^+ phenotype and that other genes such as the avian influenza NP gene could modify the effect of the polymerase gene (26). In the current study, we were unable to associate the transfer of the 42°C growth phenotype with any specific genotype (Table 2). Conversely, there was no specific genotype that was associated with the transfer of the *ts* phenotype of the human virus parent. These negative findings were significant since we were able to associate two other phenotypes with specific genotypes (see above and below). Our results suggested that several different genes of each of the avian and human virus parents play a role in specifying their ts^+ and *ts* phenotypes and that no single gene or combination of genes plays a dominant role.

Level of virus replication in squirrel monkeys. Table 4 shows the relationship between the genotypes of the reassortant viruses, their level of replication in the upper and lower respiratory tract of squirrel monkeys, and their expression of the *hr* phenotype. The NP gene of the A/Pintail/79 virus appeared to play a major role in specifying the attenuation phenotype of these reassortant viruses, since each reassortant virus which inherited the avian NP gene was significantly restricted in mean peak titer of replication in the lower respiratory tract of monkeys compared with that of wild-type human influenza A virus. Viruses which received the A/Pintail/79 virus M gene alone (clone 204) or in combination with the avian influenza PB2 gene (clone 218) were not significantly restricted in replication in monkeys. Thus, the A/Pintail/79 virus M gene did not contribute to attenuation of these reassortant viruses. This was in contrast with our findings in previous studies with A/Mallard/78 × A/Udorn/72 reassortant viruses (26), which showed that both the NP and M genes of the A/Mallard/78 virus contributed to attenuation.

To determine whether the *hr* phenotype observed in vitro correlated with decreased virulence in vivo, we studied the replication of viruses with the polymerase genotype HAH or AAH after inoculation into squirrel monkeys. After intratracheal inoculation, reassortant virus clones 49 and 22 with the polymerase genotype HAH each failed to replicate to detectable levels, although each monkey was infected, as shown by a fourfold rise in serum hemagglutination-inhibiting antibody titer. In contrast, a virus (clone 39-20) with the polymerase genotype AAH was fully competent in the upper respiratory tract of monkeys but was restricted in the lower respiratory tract. To date, of the 36 AH reassortant viruses evaluated for replicative capacity in squirrel monkeys, only the two A/Pintail/79 reassortant viruses which possessed the HAH polymerase genotype uniformly failed to replicate to detectable titer in monkeys (2 of 2 HAH genotype viruses versus 0 of 34 reassortants with other polymerase genotypes, $P < 0.02$, two-tailed Fisher's exact test). Thus, although only two A/Pintail/79 reassortant viruses with the HAH genotype were evaluated in squirrel monkeys and although one of these also possessed the avian virus NP gene whose presence is associated with attenuation, the unique failure of these viruses to replicate to detectable levels provided evidence of the association of the polymerase genotype HAH with host range restriction of replication in vivo.

Although the avian virus NP gene and the polymerase constellation HAH appeared to be the major determinants of attenuation, other genes or gene combinations may play a lesser role. For example, clone 208, which acquired four internal genes from the avian virus parent, was also attenuated in monkeys, despite its lack of the Pintail virus NP gene or the HAH constellation. The results of segregational analysis suggested that this attenuation was due to the combined effects of multiple genes since the single gene substitution reassortant PA polymerase (clone 278) or NS (clone 82-7) viruses were partially attenuated, whereas clones 218, 228, and 24, each of which inherited two of the same four avian influenza virus genes, were not attenuated in monkeys. Additionally, we were unable to isolate a single gene reassortant virus which received only the polymerase

TABLE 4. Comparison of genotype and properties of AH influenza A virus reassortants in vitro and in squirrel monkeys[a]

Clone designation	No. of genes from avian virus	Parental origin of genes in reassortant viruses at indicated locus[b]						Virus replication[c]				Reduction in infectivity in MDCK compared with that in PCK[d]
								Nasopharynx		Trachea		
		PB2	PB1	PA	NP	M	NS	Avg duration of virus shedding ± SE (days)	Mean peak titer ± SE (log_{10} $TCID_{50}$/ml)	Avg duration of virus shedding ± SE (days)	Mean peak titer ± SE (log_{10} $TCID_{50}$/ml)	
A/Washington/80 wild type	0	H	H	H	H	H	H	5.1 ± 0.7	3.4 ± 0.4	4.3 ± 0.7	4.0 ± 0.4	−
Viruses with avian NP gene												
264	1	H	H	H	A	H	H	1.5 ± 0.3	1.5 ± 0.2	1.5 ± 0.5	1.3 ± 0.3	−
251	2	H	H	H	A	A	H	0.5 ± 0.5	0.7 ± 0.2	1.5 ± 1.0	0.9 ± 0.2	−
314	3	A	H	H	A	H	A	1.0 ± 0.0	0.9 ± 0.2	2.5 ± 0.5	2.4 ± 0.1	−
226	4	A	H	H	A	A	A	0.5 ± 0.3	1.0 ± 0.3	1.0 ± 0.6	1.0 ± 0.3	−
Viruses with HAH or AAH polymerase genotype												
22	3	H	A	H	A	A	H	0.0 ± 0.0	≤0.5 ± 0.0	0.0 ± 0.0	≤0.5 ± 0.0	+
49	2	H	A	H	H	A	H	0.0 ± 0.0	≤0.5 ± 0.0	0.0 ± 0.0	≤0.5 ± 0.0	+
39-20	3	A	A	H	H	A	H	5.6 ± 1.9	2.1 ± 0.7	6.0 ± 0.0	2.5 ± 0.4	+
Viruses with avian M gene but lacking other attenuating genes												
204	1	H	H	H	H	A	H	6.0 ± 1.4	3.8 ± 0.5	5.5 ± 0.5	4.6 ± 0.3	−
218	2	A	H	H	H	A	H	6.8 ± 1.4	2.8 ± 0.3	5.0 ± 1.0	3.8 ± 0.5	−
208G	4	A	H	A	H	A	A	2.0 ± 1.4	2.8 ± 1.1	2.5 ± 0.5	1.8 ± 0.3	−
Viruses with other genotypes												
278	1	A	H	H	H	H	H	3.5 ± 0.9	2.1 ± 0.2	4.5 ± 1.0	3.1 ± 0.2	−
82-7	1	H	H	H	H	H	A	2.3 ± 0.3	2.6 ± 0.2	2.0 ± 0.0	3.3 ± 0.3	−
15	1	H	H	A	H	H	H	4.5 ± 1.0	4.3 ± 0.4	2.0 ± 0.8	2.0 ± 0.7	−
24	2	H	H	A	H	H	A	7.5 ± 1.0	5.5 ± 0.3	4.5 ± 1.0	4.8 ± 0.3	−
228	2	A	H	H	H	H	A	7.3 ± 1.2	5.6 ± 0.5	4.0 ± 0.0	4.2 ± 0.7	−
4	6	A	A	A	A	A	A	1.1 ± 0.3	1.9 ± 0.3	2.3 ± 0.5	2.1 ± 0.3	−

[a] Monkeys received 10^7 $TCID_{50}$ of virus. The A/Washington/80 wild-type and the six-gene reassortant clone 4 viruses were each evaluated in eight monkeys, and the other viruses were evaluated in four monkeys each.

[b] A, Gene derived from the A/Pintail/79 virus; H, gene derived from the A/Washington/80 virus. Each virus acquired its HA and NA genes from the A/Washington/80 virus. Underlining indicates the gene or combination of genes associated with attenuation.

[c] Underlined values are significantly different from those for A/Washington/80 ($P < 0.05$ by two-tailed Student t test).

[d] Defined as 100-fold or greater reduction in infectivity for MDCK compared with PCK tissue culture.

PB1 gene from the avian virus parent, but we do not suspect a major independent role for this avian virus gene, since clone 39-20, which acquired the avian virus polymerase PB1 gene in combination with two other avian virus genes, was only partially attenuated in monkeys.

DISCUSSION

In the present study we found that the NP gene of the avian influenza A/Pintail/79 virus plays a major role in the transfer of attenuation to six-gene AH reassortant viruses. Thus, four genotypically different, independently derived AH reassortant viruses, each of which acquired the A/Pintail/79 virus NP gene, were significantly restricted in replication in the lower respiratory tract of squirrel monkeys compared with wild-type human influenza A virus. A similar role of the avian virus NP gene in attenuation of AH reassortant virus for monkeys was observed previously in studies with the avian influenza A/Mallard/78 virus (26). Our findings in these two studies suggest that the NP gene of avian influenza A viruses may be a major determinant of their attenuation for primates, a phenotype which can be transferred to human influenza A viruses by gene reassortment. The specific nucleotide sequences of avian influenza virus NP genes that specify attenuation for primates have not yet been determined.

The M gene of the avian influenza A/Pintail/79 virus, unlike that of the avian influenza A/Mallard/78 virus, does not appear to play a role in the attenuation of AH influenza reassortant viruses for squirrel monkeys. Preliminary nucleotide sequence data (A. J. Buckler-White, manuscript in preparation) indicate that the M gene of the A/Pintail/79 virus contains the avian-specific amino acid substitutions at the host species-specific residues previously described (6); thus, it is reasonable to suggest that these avian virus-specific amino acid substitutions in the M genes of avian influenza A viruses are not sufficient to specify restricted replication in primates, but this requires further study. Because the M gene of the A/Mallard/78 virus was studied with the A/Udorn/72 virus as the donor of human virus genes, whereas the M gene of the A/Pintail/79 virus was studied with the human A/Washington/80 virus parent, we are currently performing additional studies to clarify the role of the M gene of the two avian viruses in the transfer of attenuation.

Characterization of the genetic basis of restriction of replication of influenza A viruses for monkeys or for cells in vitro has revealed two major patterns. The first pattern involves transfer of a phenotype of a parental virus to reassortant progeny through the transfer of one gene or a set of genes (1, 12, 21, 23, 24, 26). The ability of the NP gene of the avian influenza A/Pintail/79 or A/Mallard/78 virus to

transfer the phenotype of restricted replication in nonhuman primates to AH reassortant viruses is an example of this type of inheritance. In the current study, we also observed an example of the second pattern of inheritance, in which a mixed constellation of genes derived from the two parental viruses specifies a phenotype not exhibited by either parent. In this study, both parental viruses replicated efficiently in avian (PCK) and mammalian (MDCK) tissue cultures, and both viruses replicated to detectable levels in squirrel monkeys. In contrast, reassortant viruses with the XAH (X represents a gene derived from either virus parent) polymerase genotype replicated poorly in MDCK cells, and viruses with the HAH polymerase genotype failed to replicate to a detectable level in the respiratory tract of squirrel monkeys. The association of the *hr* phenotype with a specific polymerase genotype indicates that a precise interaction of virus polymerase proteins and cellular factors must occur for efficient viral replication. The efficient replication of both parental viruses in both avian and mammalian tissue cultures demonstrates that each of the polymerase proteins of the two parental viruses can interact efficiently with avian or mammalian cellular factors. Although the polymerase proteins specified by the HAH genotype can interact efficiently with each other and with cellular factors in avian tissue, they fail to interact normally with cellular factors (and possibly with each other) in MDCK cultures or in the respiratory epithelium of squirrel monkeys. This finding of a requirement for a precise interaction of polymerase genes with each other and with host factors is consistent with certain observations from previous studies. It is known that influenza virus gene products interact with cellular factors (13, 27) and that the three polymerase proteins form a complex (9, 14). Our results provide evidence that the interaction of an influenza virus polymerase complex with cellular factors is involved in virus replication in vivo. The biochemical basis of the *hr* phenotype specified by the HAH polymerase constellation of the A/Pintail/79 × A/Washington/80 reassortant viruses remains unknown. Reassortant influenza A/Mallard/78 × A/Udorn/72 viruses which possessed the HAH polymerase constellation were not restricted in MDCK cells or in monkeys, a finding which indicates that this combination of avian and human influenza A virus polymerase genes is not always incompatible with efficient replication in mammalian cells.

An interesting parallel to the association between a specific polymerase genotype and host range restriction of replication is seen in the studies of Beare et al. (3, 4) and Florent et al. (10, 11), who reported the results of studies in volunteers with reassortant viruses derived from human influenza A/PR/8/34 (H1N1) and H3N2 epidemic viruses. Whereas the A/PR/8/34 virus failed to infect seronegative volunteers, reassortant viruses which acquired all six internal genes from the A/PR/8/34 virus and the H3N2 HA and NA genes from epidemic wild-type viruses were virulent in volunteers. This indicated that the avirulence of the A/PR/8/34 virus parent was due to the surface glycoproteins and that the six internal genes did not specify an acceptable level of attenuation. However, each of six vaccine reassortant viruses which acquired two polymerase genes from A/PR/8/34 and the remaining polymerase gene from the respective wild-type virus parent were acceptably attenuated in the volunteers. Thus, the six-gene reassortant A/PR/8/34 viruses replicated efficiently in eggs and in volunteers, whereas the mixed polymerase gene constellation reassortant viruses replicated efficiently in eggs but were restricted in humans, as shown by their attenuation in susceptible volunteers. These results suggest that the A/PR/8/34 reassortant viruses which acquired the mixed constellation of polymerase genes expressed a host range restriction of replication because of the incompatibility of the polymerase gene products and human host cell factors. In the context of the present study, these findings with A/PR/8/34 reassortant viruses define a new class of attenuated viruses that could be used as donors of attenuating genes to new variants of epidemic human influenza A viruses. These new donors differ from previous donors (i.e., *ts*, cold-adapted, or avian influenza A viruses), in that a gene constellation (e.g., the HAH polymerase constellation), rather than a specific gene, specifies the attenuation phenotype. Each of the new donors replicates well in eggs but poorly in primate respiratory tract epithelium. Genotyping techniques currently available are adequate to assure the rapid selection of reassortant viruses derived from these new donors with the desired polymerase genotype. It remains to be determined whether influenza A/Pintail/79 reassortant viruses with the polymerase genotype HAH and the HA and NA genes of new epidemic human influenza A viruses will be acceptably attenuated and immunogenic in humans.

Finally, the results of this study have implications for our understanding of the origin of pandemic influenza A virus strains. Our finding that AH reassortant viruses which acquired avian virus NP genes are restricted in replication in monkeys provides a possible explanation for the conservation of the human virus NP gene during the antigenic shifts of 1957 and 1968 (2, 20, 22) since natural reassortants which acquired an avian virus NP gene might fail to replicate to sufficiently high titer to sustain human-to-human transmission. Similarly, our findings of multiple independent genotypic factors which can restrict replication of AH reassortant viruses in monkeys, together with our inability to isolate viruses with certain reassortant genotypes, suggest that only a limited percentage of the reassortant viruses which might result from coinfection of a host with avian and human influenza A viruses would replicate efficiently in humans. However, our data also show that reassortant viruses with many different constellations of genes can replicate in monkeys to a level similar to that of wild-type human influenza A virus indicating that there are additional factors which make the occurrence of new pandemic influenza A strains a rare event.

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