JOURNAL OF VIROLOGY, Mar. 1985, p. 771–775
0022-538X/85/030771-05$02.00/0

Vol. 53, No. 3

Nucleoprotein and Membrane Protein Genes Are Associated with Restriction of Replication of Influenza A/Mallard/NY/78 Virus and Its Reassortants in Squirrel Monkey Respiratory Tract

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Received 13 September 1984/Accepted 9 November 1984

An avian influenza A virus, A/Mallard/NY/6750/78(H2N2), was restricted in replication in the respiratory tract of squirrel monkeys. Avian-human influenza A reassortant viruses possessing the six RNA segments coding for nonsurface proteins (i.e., internal genes) of this avian virus were as restricted in replication in squirrel monkeys as their avian influenza parent. These findings indicated that restriction of replication of the avian influenza virus is a function of one or more of its internal genes. For an investigation of which of the avian influenza genes was responsible for restricted replication in the respiratory tract of primates, reassortant viruses were produced that contained human influenza virus surface antigens from the A/Udorn/72(H3N2) virus and one or more of the internal genes derived from the avian influenza virus parent. Avian-human reassortant influenza A viruses containing only the nucleoprotein or matrix protein RNA segment from the avian influenza virus parent were as restricted in their growth as an avian-human influenza reassortant virus containing each of the six avian influenza internal genes. In addition, an avian-human influenza reassortant virus possessing only the avian RNA 1 and nonstructural genes (which by themselves do not specify restricted replication) manifested a significant reduction of virus replication in squirrel monkey tracheas. Thus, the avian nucleoprotein and matrix genes appear to play a major role in the host range restriction exhibited by the A/Mallard/78 virus and its reassortants, but the combination of RNA 1 and nonstructural genes also contributes to restriction of replication.

Live attenuated influenza A viruses have been produced by transfer of genes from an attenuated donor virus to new epidemic influenza A viruses (5). Since resistance to influenza A virus is mediated by the development of an immune response to the hemagglutinin (HA) and neuraminidase (NA) glycoproteins, live attenuated reassortant vaccine strains were selected in which genes for these surface antigens were derived from the epidemic virus; the nonsurface protein ("internal") genes were derived from the attenuated parent (1). Evaluation of avian influenza A viruses as donors of attenuating genes to human influenza virus was initiated because there is evidence that several of the genes of avian and human influenza viruses have undergone significant divergence in nucleotide sequence as these viruses have evolved in their separate hosts (2, 3, 9, 15, 16, 18). Because of these differences, it was considered likely that some avian influenza A viruses would grow poorly in cells of primate respiratory epithelium and thereby be attenuated. Because of the significant divergence of nucleotide sequence of avian and human influenza virus genes, it was also considered likely that attenuated avian-human reassortant influenza A viruses would retain their attenuated characteristics after limited replication in humans.

During a survey of avian influenza A viruses, it was observed that the A/Mallard/NY/6750/78(H2N2) strain was significantly restricted in squirrel monkey respiratory tracts compared with a wild-type human influenza A virus (11). Avian-human influenza A reassortant viruses possessing the six internal RNA segments of this avian virus were as restricted in their replication in squirrel monkeys as their avian influenza A virus parent (13). These findings indicated that restriction of replication of the avian influenza virus is a function of one or more of its internal genes. For an investigation of which avian influenza gene or combination of genes was responsible for restricted replication in primates, reassortant viruses were produced that contained the human influenza virus surface antigens from the A/Udorn/72(H3N2) virus and one or more of the internal genes derived from the avian influenza virus parent. Comparison of the level of replication of these avian-human influenza A reassortant viruses in squirrel monkeys suggested that the avian influenza virus nucleoprotein (NP) and matrix (M) genes play a major role in the host range restriction exhibited by the A/Mallard/78 virus and its reassortants.

MATERIALS AND METHODS

Viruses. A plaque-purified suspension (three plaque-to-plaque passages) of the A/Udorn/307/72(H3N2) wild-type virus was previously shown to be virulent for humans and squirrel monkeys (12, 14). This human influenza A virus was mated with the avian A/Mallard/NY/6750/78(H2N2) virus, and an avian-human influenza reassortant virus was produced that contained the HA and NA genes of the human influenza A virus; the other six RNA segments were derived from the avian influenza A virus parent (13). This reassortant virus was designated Mal × Ud-1A.

Production of avian-human influenza A virus reassortants. For production of the desired reassortant viruses, Madin-Darby canine kidney (MDCK) cultures were coinfected with the A/Udorn/307/72 human virus and the Mal × Ud-1A reassortant at a multiplicity of infection of 5 as previously

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TABLE 1. Generation of reassortants containing a mixed constellation of avian and human influenza internal genes

Mating	Human A/Udorn/72 influenza virus mated with the following virus:	No. of plaque progeny genotyped	No. of reassortants identified	Clone designation of new unique reassortants that were identified
A	Mal × UD-1A[a]	57	10	1-8
B	1[b]	20	11	9-10
C	4[b]	31	24	11-18
D	8[b]	20	15	19

[a] Avian influenza A/Mallard/78 × human A/Udorn/72 reassortant with the six internal genes of the former virus and the HA and NA genes of the latter virus.

[b] Clones 1, 4, and 8 were progeny from mating A; the genotypes of these reassortants are indicated in Table 2.

described (13). The coinfected cultures were harvested 24 h later, and plaque progeny were picked from MDCK monolayer cultures overlaid with Eagle no. 2 medium and 0.8% agarose containing antibiotics and trypsin and maintained at 37°C as previously described (13). The plaque progeny were then amplified in the allantoic cavities of 10-day-old eggs, and the genotype of each plaque population was determined as outlined below. Each of the plaque progeny contained the HA and NA genes of the A/Udorn/72 virus because these genes were present in each parent. Those progeny containing a mixed constellation of internal genes were further cloned by a plaque-to-plaque passage on MDCK cells and subsequently amplified in the allantoic cavities of 10-day-old embryonated eggs.

Efficiency of plaque formation. The efficiency of plaque formation of parental viruses and their reassortant progeny was determined at 37 and 42°C on MDCK monolayer cultures as previously described (13).

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 that of the corresponding parental genes. Viruses were propagated and purified before extraction of RNA as previously described (8) or by a modification of the procedure allowing small-scale purification of 12 viruses simultaneously. When the modified technique was used, virus was pelleted from the clarified allantoic fluid of 1 or 2 eggs at 36,000 rpm for 60 min in a Beckman 50 Ti rotor at 4°C. The virus pellet was suspended in 0.5 ml of STE (0.1 M NaCl, 2.0 mM EDTA, 20.0 mM Tris-hydrochloride [pH 7.4]) and pelleted through 7 ml of 30% sucrose in STE at 36,000 rpm for 90 min at 4°C in a 50 Ti rotor. The second virus pellet was suspended in 200 μl of STE and incubated with 250 μg of proteinase K (EM Labs) and 0.4% sodium dodecyl sulfate for 20 min at 56°C. After the addition of 30 μl of 10× LiCl buffer (1.4 M LiCl, 0.1 M sodium acetate, 5% sodium dodecyl sulfate [pH 4.9]), the RNA was precipitated by the addition of 0.8 ml of ethanol. Purified virion RNA was analyzed by polyacrylamide gel electrophoresis as described previously (10). RNA segments were visualized by ammoniacal silver staining of the gels (4).

Monkey studies. Reassortant progeny were evaluated for their level of replication in squirrel monkeys (*Saimiri sciureus*) as previously described (11). Briefly, each reassortant virus was used to inoculate a group of four monkeys; 0.5 ml of virus ($10^{7.0}$ 50% tissue culture infective doses [$TCID_{50}$]) was instilled into the trachea of each monkey. A nasopharyngeal swab was collected daily for 10 days, and a tracheal lavage was obtained on days 2, 4, and 6. The nasopharyngeal swabs and tracheal lavages were inoculated freshly onto MDCK monolayers in 24-well plates, and portions were frozen for subsequent titration. Each frozen specimen was titrated individually. A mean $\log_{10}$ titer was determined for each group of monkeys on each day of sampling. The A/Udorn/72 and Mal × Ud-1A viruses used in the monkey studies were passaged in MDCK cell cultures to approximate the number of passages of the reassortant viruses in this cell line. Four separate studies were performed in monkeys, and in each study two monkeys were inoculated with the A/Udorn/72 human virus and another group of two monkeys was inoculated with the Mal × Ud-1A reassortant. Mean values ($TCID_{50}$/ml) on the eight animals inoculated with A/Udorn/72 or the Mal × Ud-1A reassortant were determined and served as the basis for evaluating the degree of restriction of each reassortant tested.

RESULTS

Production and genotype of avian-human influenza A reassortants. From the mating of A/Udorn/72 and the Mal × Ud-1A reassortant, 57 progeny viruses were characterized and 10 were found to be reassortants (Table 1). Among these 10 reassortants, eight distinct genotypes were identified; these were designated clones 1 through 8. Three of these 8 new reassortant viruses were then backcrossed with the

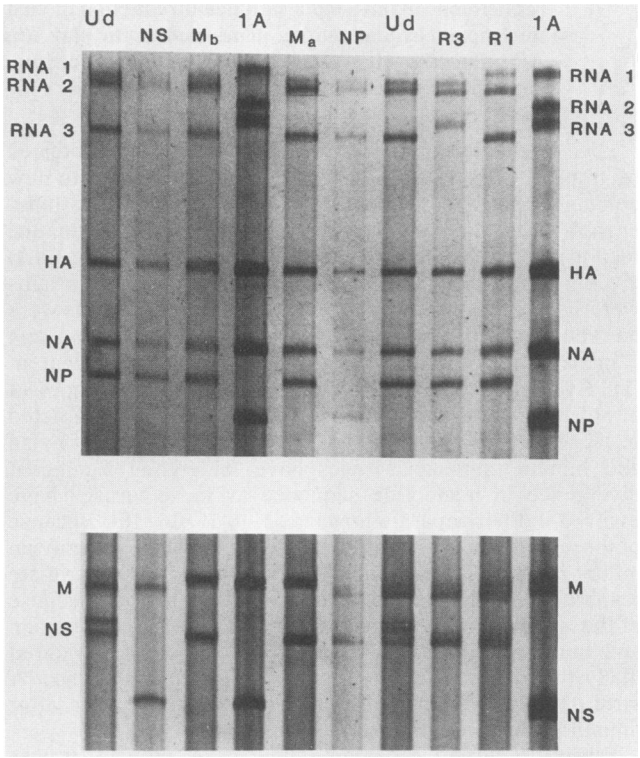

FIG. 1. Polyacrylamide gel electrophoresis of virion RNAs from human A/Udorn/72 (Ud), avian Mal × Ud-1A (1A) reassortant, and single gene substitution reassortants possessing only an RNA 1 (R1), RNA 3 (R3), NP, M, or nonstructural protein (NS) gene of avian influenza origin. Each virus possessed the HA and NA genes of the human A/Udorn/72 parent. M_a and M_b indicate two independent isolates of a reassortant possessing only the M gene derived from the avian influenza virus. The 16-cm gels contained 2.6% acrylamide and either 4.5 M (top) or 7.0 M (bottom) urea. Electrophoresis was for 20 h at 5°C and 100 V.

TABLE 2. Genotype, level of replication in monkeys, and efficiency of plaque formation of avian-human influenza reassortant viruses

Virus[a]	No. of genes derived from A/Mallard/78 avian virus	Parental origin of genes in avian-human influenza A reassortant virus[b]						Virus replication[c]				Log_{10} reduction[d] of plaque formation in MDCK cells at 42°C compared with 37°C
								Trachea		Nasopharynx		
		1	2	3	NP	M	NS	Mean peak titer (log_{10} $TCID_{50}$/ml) ± SE	Avg duration of virus shedding (days) ± SE	Mean peak titer (log_{10} $TCID_{50}$/ml) ± SE	Avg duration of virus shedding (days) ± SE	
A/Udorn/72	0	U	U	U	U	U	U	4.9 ± 0.3	5.5 ± 0.3	4.4 ± 0.4	7.8 ± 0.7	≥5.0
19	1	M	U	U	U	U	U	5.2 ± 0.2	6.0 ± 0.0	4.8 ± 0.1	7.8 ± 0.8	1.9*
10	1	U	U	M	U	U	U	5.4 ± 0.7	5.0 ± 0.6	**2.3 ± 0.2**	**4.0 ± 0.9**	3.6
17	1	U	U	U	M	U	U	**≤0.5 ± 0.0**	**0.0 ± 0.0**	**1.7 ± 0.2**	**1.0 ± 0.0**	5.7
6	1	U	U	U	U	M	U	**2.6 ± 1.1**	**3.0 ± 0.6**	**1.0 ± 0.2**	**1.8 ± 0.5**	≥5.5
13	1	U	U	U	U	M	U	**1.9 ± 0.6**	**2.0 ± 0.8**	3.1 ± 0.6	**2.8 ± 1.4**	4.4
2	1	U	U	U	U	U	M	4.4 ± 1.1	5.0 ± 0.6	3.4 ± 0.5	5.0 ± 1.2	≥5.4
11	2	U	U	U	U	M	M	**1.5 ± 0.7**	**1.5 ± 1.0**	**2.3 ± 0.9**	**2.5 ± 0.9**	≥4.9
12	2	M	U	U	U	U	M	**3.3 ± 0.8**	**3.5 ± 1.0**	2.9 ± 0.9	**2.5 ± 1.2**	1.2*
15	2	M	U	U	M	U	U	**2.4 ± 0.7**	**2.5 ± 1.0**	2.6 ± 1.1	**2.5 ± 1.0**	4.8
18	2	U	U	U	M	U	M	**1.4 ± 0.4**	**1.5 ± 0.5**	**1.8 ± 0.8**	**0.5 ± 0.3**	≥5.6
3	2	U	U	M	U	U	M	5.1 ± 0.7	6.0 ± 0.0	2.4 ± 1.0	4.5 ± 2.1	3.9
14	3	M	U	U	U	M	M	NT[e]	NT	NT	NT	0.6*
16	3	M	U	U	M	U	M	NT	NT	NT	NT	0.2*
19	3	U	U	M	M	U	M	**0.9 ± 0.3**	**1.0 ± 0.6**	**1.6 ± 0.2**	**1.0 ± 0.0**	6.0
1	4	M	U	M	M	M	U	NT	NT	NT	NT	3.2
8	4	U	M	M	M	U	M	NT	NT	NT	NT	2.5
4	5	M	M	U	M	M	M	**1.7 ± 0.2**	**2.0 ± 0.0**	**2.2 ± 0.3**	**1.5 ± 0.5**	1.6*
5	5	M	U	M	M	M	M	**0.8 ± 0.3**	**0.5 ± 0.5**	**2.2 ± 0.3**	**1.0 ± 0.0**	0.5*
7	5	U	M	M	M	M	M	**1.7 ± 0.4**	**2.0 ± 0.0**	**0.9 ± 0.2**	**0.8 ± 0.3**	1.8*
Mal × Ud-1A	6	M	M	M	M	M	M	**1.0 ± 0.4**	**0.8 ± 0.5**	**1.8 ± 0.3**	**1.9 ± 0.4**	0.7*

[a] Numerals indicate clone designations of avian-human influenza A reassortants. See Table 1.
[b] 1, 2, and 3 indicate RNA 1, RNA 2, and RNA 3, respectively. U, Gene derived from A/Udorn/72 virus; M, Gene derived from A/Mallard/78 virus. Each reassortant virus has the A/Udorn/72 HA and NA genes.
[c] Monkeys received $10^{7.0}$ $TCID_{50}$ of virus and were infected in each instance. The A/Udorn/72 and Mal × Ud-1A viruses were each evaluated in eight monkeys, and the reassortants were tested in four monkeys. Values are means from four animals. Boldface values are significantly less than those for A/Udorn/72 virus.
[d] Average of two or more tests. Asterisks indicate values similar to those of the Mal × Ud-1A parent.
[e] NT, Not tested.

A/Udorn/72 virus, and 11 additional reassortant genotypes were generated (Table 1). From these four matings (i.e., A to D), 48 reassortant viruses representing 18 distinct genotypes were obtained (Table 1). The genotypes of the six reassortants that contained only one avian influenza virus gene are shown in Fig. 1. In each instance, the parental origin of the single internal gene that was derived from the A/Mallard/78 avian virus was clearly established. A reassortant virus containing only the avian influenza RNA 2 segment was not recovered.

Level of viral replication in monkeys. The relationship between the level of replication of each reassortant virus in vivo and its genotype is shown in Table 2. Avian-human influenza A reassortant viruses that contained only an RNA 1, RNA 3, or nonstructural (NS) protein RNA segment of avian influenza origin grew to the same level in the trachea as their human influenza A/Udorn/72 parent. Reassortant clone 10 (containing only avian influenza RNA 3) exhibited some reduction in replication in the upper respiratory tract. Importantly, reassortants containing only the avian influenza NP or M gene were significantly restricted in their replication in the upper and lower respiratory tracts. Each reassortant virus that contained an avian influenza M gene or NP gene in combination with one or more other avian influenza genes also exhibited significant restriction in the upper and lower respiratory tracts. These observations suggest that the M and NP genes constitute the major determinants of host range restriction exhibited by the avian A/Mallard/78(H2N2) virus and its reassortants. The avian-human reassortant clone 12 that possessed only RNA 1 and NS avian influenza genes (which by themselves did not specify restriction of replication) exhibited a reduced level of virus replication in the upper and lower respiratory tracts. This suggests that a combination of avian influenza internal genes other than M and NP also contribute to the observed host range restriction.

Genes responsible for replication of the avian influenza virus at elevated temperature. Avian influenza viruses replicate efficiently at 42°C, a temperature restrictive for replication of human influenza A viruses (11). We previously demonstrated that one or more of the avian influenza A virus internal genes were responsible for this phenotype (13). The availability of the avian-human influenza A reassortant viruses permitted us to examine which avian influenza gene or combination of genes was needed for replication at 42°C (Table 2). The avian-human influenza reassortant virus clone 19 that possessed only RNA 1 of avian influenza origin resembled the Mal × Ud-1A virus in its lack of significant temperature sensitivity, i.e., it was less than 100 times reduced in titer at 42°C. We have arbitrarily defined a 10^2

reduction of plaque formation at 42°C as significant because none of the avian influenza A viruses or their six internal avian influenza gene reassortants tested have exhibited a 10^2 reduction in efficiency of plaque formation at 42°C (9, 11, 13). Thus, the avian influenza RNA 1 gene provides a function essential for replication at 42°C. However, the genetic control of this phenotype is more complex and cannot be explained solely by the action of a single avian influenza gene. Each of the avian-human influenza reassortants that contain an A/Mallard/78 RNA 1 gene replicates efficiently at 42°C, except for clone 15 and clone 1, both of which also contain an avian influenza NP gene. This suggests that the avian influenza NP gene can modify the effect of the avian influenza RNA 1 gene on growth at 42°C. Clearly, avian genes other than RNA 1 also contribute to replication at 42°C. For instance, clone 7 contains all avian influenza internal genes except for RNA 1, yet it replicates well at 42°C. Considered together, these observations suggest that more than one gene contributes to the ability of the A/Mallard/78 virus to replicate at 42°C, but the RNA 1 gene appears to play a major role.

DISCUSSION

The avian A/Mallard/NY/6750/78(H2N2) influenza virus replicates efficiently in ducks but poorly in monkeys. When the internal genes of this avian virus were reassorted with those of a virulent human influenza A virus, reassortants with 18 distinct genotypes were recovered. All of these reassortants derived their surface antigen genes from the human influenza virus. Evaluation of these avian-human influenza reassortant viruses for level of replication in the squirrel monkey respiratory tract revealed that the NP and M genes of the avian influenza virus were major determinants of the host range restriction exhibited by this avian influenza virus and its reassortants. In addition, a reassortant possessing the RNA 1 and NS genes from the avian influenza parent exhibited significant restriction of replication in the upper and lower respiratory tract. This suggests that other internal genes, which by themselves do not restrict replication, can act together to effect a reduction in replication, but the precise mechanism of this interaction remains to be determined. The role of the RNA 2 segment in host range restriction could not be assessed because an avian-human influenza reassortant containing only the avian influenza RNA 2 segment was not isolated.

The mechanism by which the avian influenza NP and M genes restrict replication in monkey respiratory tracts is not known. The NP gene is monocistronic and codes for a virion-associated protein (20). The genetic relatedness of NP genes of avian and mammalian influenza A viruses was recently studied by nucleic acid hybridization (3). The avian strains fell into one of two groups; the equine virus strains comprised two other groups, and the human and swine influenza viruses together constituted a fifth distinct group (3). These findings suggest that the NP protein may play an important role in host specificity. Thus, sequence divergence of human and avian influenza NP genes may explain the NP-mediated host range restriction observed in the present study. However, specific sequence divergences are probably required for restriction because some avian influenza viruses replicate efficiently in primates (11).

The RNA segment coding for the M proteins is known to be bicistronic; it codes for M1 and M2 proteins (6, 7). Another smaller protein may also be coded by this RNA segment (7). The M1 protein is virion associated, whereas the M2 protein is not. Thus, either, or both, of the M1 and M2 proteins could be major determinants of the restriction observed in the present study. Interestingly, recent sequence analysis indicates that the divergence in amino acid sequence between the avian and human influenza M2 proteins is greater than between the corresponding M1 proteins (9). This suggests that the M2 protein may be an important determinant of host range of influenza A viruses. Currently, the nucleotide sequences of the M and NP genes of the avian A/Mallard/78 virus are being determined; this should allow us to define the extent and location of divergence of these influenza genes from the corresponding genes of various human influenza A viruses that have been sequenced to date.

An attempt was made to identify the avian influenza virus genes that specify replication at 42°C. This property was found to be relatively complex. Initially, an avian-human influenza reassortant that contained only the avian influenza RNA 1 gene was found to grow well at 42°C, suggesting that this gene is a determinant of growth at high temperature. However, the effect of RNA 1 could be suppressed by an avian influenza NP gene as indicated by the *ts* phenotype of reassortants that derived only their RNA 1 and NP genes from the A/Mallard/78 virus. RNA 1 was not the only determinant of efficient replication at 42°C since a reassortant lacking avian influenza RNA 1 but possessing the other five avian influenza internal genes replicated efficiently at 42°C. Considered together, these findings indicate that more than one of the avian influenza genes specifies replication at 42°C and that interaction of the avian influenza genes to produce this phenotype is complex. The genetic basis for restriction of growth in monkeys is different from that responsible for growth at 42°C in cell culture because these two properties segregate independently during gene reassortment.

The findings from the present study have implications for the use of avian influenza A viruses as donors of genes to attenuate human influenza A viruses. Since the avian NP and M genes are both able to attenuate human influenza virus for monkeys, restoration of virulence of an avian-human influenza reassortant for humans would require appropriate genetic changes in both of these genes or suppression of both genes. Previously it was observed that an influenza A virus with *ts* point mutations affecting two separate genes was able to escape its attenuation phenotype after replication in experimental animals or susceptible volunteers (19). It is likely that the divergence of amino acid sequence of the avian influenza M and NP proteins from that of their human influenza virus counterparts is considerably more extensive than that of influenza virus *ts* mutants from their wild-type virus parent. If this prediction is correct, it would be unlikely that avian influenza genes could easily develop the number of mutations required for restoration of virulence, especially during a restricted infection of susceptible individuals. Also, the avian influenza RNA 1 and NS genes contribute to attenuation, and this provides an additional degree of stability to avian-human influenza reassortant viruses bearing the six internal avian influenza genes. Studies are being carried out in humans and monkeys to assess the level of phenotypic stability conferred on avian-human influenza reassortant viruses by the six avian A/Mallard/NY/6750/78 internal genes.

Two questions have been raised regarding the use of live influenza A reassortant virus vaccines in humans. First, a major concern is that reassortant progeny might have pathogenic properties that differ significantly from either parent, e.g., neurovirulent reassortants might arise from non-neurovirulent parents (17). Second, if a live avian-human influenza

reassortant were used in an open population, genetic reassortment with naturally circulating strains might lead to the generation of potentially harmful avian-human influenza reassortants. Observations made during the current study are relevant to these concerns. Fifteen genetically distinct avian-human influenza reassortants were tested in monkeys, and none of these viruses grew better than the virulent human influenza virus that served as the donor of human influenza genes. Although non-respiratory tissues were not sampled for virus or studied histopathologically, there was no evidence that any of the reassortants exhibited unusual pathogenic properties, i.e., none of the monkeys developed unusual symptoms indicative of involvement of other organ systems such as the central nervous system. Also, none of the monkeys became more ill or shed more virus than monkeys infected with the more virulent of the two parental viruses, i.e., the human influenza A/Udorn/72 virus. Indeed, 12 of the 18 reassortants exhibited evidence of restriction of virus replication and attenuation. Thus, in this limited study, viruses representing 15 of 64 (23%) of the possible avian-human influenza reassortants did not exhibit unexpected biological properties, indicating that it is reasonable to continue the evaluation of these potential live virus vaccines in humans.

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