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Four Viral Genes Independently Contribute to Attenuation of Live Influenza A/Ann Arbor/6/60 (H2N2) Cold-Adapted Reassortant Virus Vaccines

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Clinical studies previously demonstrated that live influenza A virus vaccines derived by genetic reassortment from the mating of influenza A/Ann Arbor/6/60 (H2N2) cold-adapted (*ca*) donor virus with epidemic wild-type influenza A viruses are reproducibly safe, infectious, immunogenic, and efficacious in the prevention of illness caused by challenge with virulent wild-type virus. These influenza A reassortant virus vaccines also express the *ca* and temperature sensitivity (*ts*) phenotypes in vitro, but the genes of the *ca* virus parent which specify the *ca*, *ts*, and attenuation (*att*) phenotypes have not adequately been defined. To identify the genes associated with each of these phenotypes, we isolated six single-gene substitution reassortant viruses, each of which inherited only one RNA segment from the *ca* parent virus and the remaining seven RNA segments from the A/Korea/1/82 (H3N2) wild-type virus parent. These were evaluated in vitro for their *ca* and *ts* phenotypes and in ferrets, hamsters, and seronegative adult volunteers for the *att* phenotype. We found that the polymerase PA gene of the *ca* parent specifies the *ca* phenotype and that the PB2 and PB1 genes independently specify the *ts* phenotype. The PA, M, PB2, and PB1 genes of the *ca* donor virus each contribute to the *att* phenotype. The finding that four genes of the *ca* donor virus contribute to the *att* phenotype provides a partial explanation for the observed phenotypic stability of *ca* reassortant viruses following replication in humans.

The influenza A/Ann Arbor/6/60 (H2N2) cold-adapted (*ca*) virus is being considered as a donor of genes to virulent wild-type viruses for the production of attenuated reassortant viruses for use in humans as live influenza A virus vaccines. The *ca* donor virus expresses three phenotypes: (i) the *ca* phenotype, i.e., the ability to replicate efficiently on primary chicken kidney (PCK) tissue culture at 25°C, a temperature restrictive for wild-type virus; (ii) the temperature sensitivity (*ts*) phenotype, i.e., restriction of replication on Madin-Darby canine kidney (MDCK) tissue culture at 39°C in contrast to wild-type human influenza A viruses, which replicate efficiently at temperatures up to 40 to 41°C; and (iii) the attenuation (*att*) phenotype, i.e., restricted replication in the respiratory tracts of ferrets, hamsters, or humans. Six-gene *ca* reassortant viruses, which acquire the RNA segments that encode the hemagglutinin and neuraminidase from virulent wild-type virus and the remaining six RNA segments (internal genes) from the *ca* donor virus, also express each of these phenotypes. The genetic basis of each of these three phenotypes is undefined. Because reassortants derived from the influenza A/Ann Arbor/60 *ca* donor virus exhibit the characteristics desired for a live attenuated influenza A virus vaccine for use in humans, there is interest in the mechanisms of attenuation of these reassortant viruses. Since a virus which is attenuated as a result of a large number of mutations on several RNA segments presumably would be more stable than one which contains only one or two attenuating mutations, there is a need to determine the number of genes bearing *att* mutations.

To identify the genes associated with the *ca*, *ts*, and *att* phenotypes, we isolated six single-gene substitution reassortant viruses, each of which inherited only one RNA segment from the *ca* donor virus and the remaining RNA segments from the A/Korea/1/82 (H3N2) wild-type virus parent. We also isolated additional reassortant viruses which acquired more than one gene from the *ca* donor virus and the remaining genes from the wild-type virus parent. These reassortant viruses and the wild-type virus parent were evaluated for their expression of the *ca* and *ts* phenotypes in vitro and for evidence of attenuation in ferrets and hamsters and in seronegative adult volunteers. The results demonstrated that the *ca* phenotype is specified by the polymerase PA gene and the *ts* phenotype is specified by each of the polymerase PB2 and PB1 genes of the *ca* donor virus. We also found that four genes of the *ca* donor virus independently contribute to attenuation of influenza A *ca* reassortant vaccine viruses.

MATERIALS AND METHODS

Viruses. The isolation, biological cloning, and production of the influenza A/Korea/1/82 (H3N2) wild-type virus (lot E-186) in the allantoic cavities of eggs were described previously (23). The A/Korea/82 × A/Ann Arbor/6/60 six-gene *ca* reassortant virus (CR 59-19) was isolated and biologically cloned, as previously described (6). Reassortant viruses T_2 (36-3-1), T_3 (25-1-7), and T_6 (6-1-1) were previously described by Odigari et al. (19). These viruses were

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restudied here because they represented single-gene or mixed-gene reassortant viruses that are useful for segregational analysis and because there is confusion in the literature concerning their genotypes (19, 20).

Additional reassortant viruses were produced by coinfecting specific-pathogen-free primary chicken kidney (PCK) tissue cultures at a multiplicity of infection of 1 with the A/Korea/1/82 wild-type virus and either the A/Korea/82 six-gene reassortant *ca* 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 monolayers overlaid with Lebowitz-15 medium modified with *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (M. A. Bioproducts, Walkersville, Md.) plus antibiotics and 0.8% agarose and maintained at 34 to 37°C. Plaque progeny were amplified in the allantoic cavities of 9-day-old embryonated eggs, and the genotype of the virus population was determined as described below. Viruses which contained a mixed population of internal genes were biologically cloned by plaque-to-plaque passage on PCK monolayers and amplified by passage in PCK monolayers with fluid overlay.

The final virus suspensions administered to volunteers were grown in 9-day-old specific-pathogen-free eggs (SPAFAS, Inc., Norwich, Conn.) and tested for the presence of adventitious agents by Louis Potash (Flow Laboratories, Inc., McLean, Va.). The virus suspensions administered to volunteers in this study and their infectivity titers in Madin-Darby canine kidney (MDCK) monolayers were as follows: six-gene *ca* (E-204), $10^{7.5}$ 50% tissue culture infectious doses ($TCID_{50}$)/ml; PB2 reassortant (E-260), $10^{7.6}$ $TCID_{50}$/ml; PB1 reassortant (E-251), $10^{7.1}$ $TCID_{50}$/ml; PA reassortant (E-242), $10^{7.0}$ $TCID_{50}$/ml; NP reassortant (E-234), $10^{8.4}$ $TCID_{50}$/ml; M reassortant (E-239), $10^{8.1}$ $TCID_{50}$/ml; and NS reassortant (E-233) $10^{8.0}$ $TCID_{50}$/ml (in each case the single-gene reassortant viruses are designated by the gene derived from the *ca* donor virus and E-204, E-260, etc., represent lot designations).

Efficiency of plaque formation at permissive and restrictive temperatures. The efficiency of plaque formation of parental and reassortant viruses in PCK tissue cultures at 25°C was determined and compared with that at temperatures (33 to 36°C) permissive for both wild-type and A/Ann Arbor/60 *ca* virus. The efficiency of plaque formation of each virus in MDCK monolayers at 38, 39, and 40°C was also compared with that at permissive temperatures.

Genotype of reassortant virus. 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 and RNA was extracted as previously described (26) with the modification described by Snyder et al. (22a). Purified viral RNA was analyzed by using polyacrylamide gels containing 2.6% acrylamide and 7.0 M urea or 3.0% acrylamide and 4.5 M urea as described previously (13). Electrophoresis conditions are described in the legend to Fig. 1. RNA segments were visualized by ammoniacal silver staining (2).

The polymerase proteins encoded by each of the RNA segments 1, 2, and 3 of our reassortant viruses were identified by comparing the migration of viral RNAs with those of the A/Ann Arbor/60 *ca* virus in a gel system in which the order of migration of viral polymerase genes had been defined previously (24).

Studies with ferrets. The duration and level of replication of parental viruses and the single-gene reassortant viruses were evaluated by using ferrets as previously described (11). Briefly, in each experiment, a group of nine ferrets were anesthetized and inoculated intranasally with 10^7 $TCID_{50}$ of a single-gene-substitution reassortant virus. Also, in each experiment, groups of three ferrets each were inoculated with 10^7 $TCID_{50}$ of either the wild-type or the six-gene *ca* reassortant A/Korea/82 virus. On days 2, 4, and 8 postinfection, one-third of the ferrets in each group were sacrificed, a 10% (wt/vol) suspension of the nasal turbinates and lungs was prepared, and the virus titers were determined in MDCK tissue culture and expressed in terms of PFU per gram of tissue.

Studies with hamsters. Studies of the replication of the wild-type, six-gene *ca*, and single-gene reassortant viruses in 5-week-old female Golden Syrian hamsters were performed as described previously (25). Groups of 24 hamsters were anesthetized and inoculated intranasally with 10^5 $TCID_{50}$ of a single-gene reassortant or parental virus in a 0.1-ml suspension. Four to six hamsters in each group were sacrificed on each of days 1 to 4 postinfection, a 10% (wt/vol) suspension of the nasal turbinates and lungs of each hamster was prepared, and the virus titers were determined in MDCK tissue culture and expressed in terms of $TCID_{50}$ per gram of tissue.

Studies with seronegative adult volunteers. Study protocols were approved by the respective clinical research committees of the National Institute of Allergy and Infectious Diseases, the University of Maryland School of Medicine, the University of Rochester School of Medicine, and the Johns Hopkins Medical Institutions. Healthy adults between the ages of 18 and 40 years who were not taking medication, did not have a history of influenza vaccination, and had a hemagglutination-inhibiting antibody titer in serum of 1:8 or less were recruited from among community members in Maryland or Rochester, N.Y. Descriptions of the clinical study protocols were published previously (16). Volunteers who received wild-type or single-gene reassortant virus were inoculated intranasally with 10^6 $TCID_{50}$ of virus in a 0.5-ml inoculum and were housed on an isolation ward at one of the participating institutions, where they were observed daily for at least seven consecutive days after inoculation. Volunteers who received the A/Korea/82 six-gene *ca* reassortant virus were inoculated intranasally with $10^{6.5}$ $TCID_{50}$ in a 0.5-ml inoculum as outpatients. Outpatient volunteers recorded their vital signs four times per day and were observed daily for 4 days. For the criteria for illness, see Table 5, footnote *e*.

The titer of hemagglutination-inhibiting antibody in serum was determined as previously described with the influenza A/Korea/82 (H3N2) virus or a reassortant virus which contained the hemagglutinin (H3) of the homologous A/Philippines/2/82 and the N7 neuraminidase of A/equine-1 virus. The amount of serum immunoglobulin G (IgG) or nasal wash IgA antibody to purified hemagglutinin-neuraminidase prepared from A/Korea/82 virus was determined by an enzyme-linked immunosorbent assay (ELISA) as previously described (10, 16, 17). The concentration of IgA in nasal wash was determined by using an ELISA capture assay with goat anti-human IgA (Sigma Chemical Co., St. Louis, Mo.) as capture antibody followed by a ladder of nasal wash, rabbit anti-human IgA, goat anti-rabbit IgG conjugated with alkaline phosphatase, and *p*-nitrophenyl phosphate as substrate. The diluents and incubation periods for this assay were the same as for our ELISA assay described above. ELISA values obtained by using nasal wash specimens from volun-

teers were compared with a standard curve obtained from analysis of human colostral IgA (Sigma), and the IgA antibody concentration in nasal wash specimens from volunteers was calculated. We determined the IgA concentrations of approximately 50 specimens by using both this new ELISA capture technique and our previous method, single radial immunodiffusion (16), and found that the levels were 10-fold higher with the new technique. Thus, to maintain consistency in our determination of influenza virus-specific ELISA antibody titers, we found it necessary to transform the ELISA hemagglutinin-neuraminidase titers, using as our standard an IgA concentration of 100 mg/dl rather than 10 mg/dl as used in our previous studies (15, 16, 23, 24).

Nasal wash specimens for virologic study were collected before virus inoculation and daily for the duration of observation. The method used for virus isolation was published previously (16). Isolation of virus from nasal wash specimens or a fourfold rise in influenza virus-specific antibody titer was considered evidence of infection. Data from all infected volunteers were used in the calculations of mean duration and mean peak titer of virus shedding.

Studies with volunteers were performed between November 1984 and July 1986. A total of six inpatient studies were performed, and in four of these studies, two or more viruses were administered. The control groups which received wild-type or six-gene *ca* reassortant viruses were studied concurrently with the groups which received the single-gene reassortant viruses. The six-gene *ca* reassortant virus was administered to volunteers as part of another study performed by M.L.C. which will be published elsewhere (S. A. J. Goings et al., manuscript in preparation).

Statistical methods. Comparisons of means were performed by using Dunnett's test for multiple comparisons (7). Fisher's exact test with Bonferoni's correction for multiple comparisons (9) was used for comparison of ratios. All tests were two tailed.

RESULTS

Isolation and genotype of reassortant viruses. Reassortant viruses, each of which acquired one or more RNA segments derived from the A/Ann Arbor/60 *ca* donor virus, were isolated from the mating of the A/Korea/82 six-gene *ca* reassortant × A/Korea/82 wild-type viruses or from an additional mating of reassortant progeny × A/Korea/82 wild-type virus (Table 1). To determine the order of migration of the RNA segments encoding polymerase proteins PB2, PB1, and PA, the migration in gel electrophoresis of viral RNA of the reassortant viruses was compared with that of the A/Ann Arbor/60 *ca* parental virus under conditions in which the order of migration of the polymerase genes is known (24) (data not shown). The coding assignments of the RNA segments 1, 2, and 3 of the A/Ann Arbor/60 *ca* virus in this gel system were previously determined by hybridization of the RNA with radiolabeled DNA probes specific for each of the polymerase PB2, PB1, and PA genes (24). To determine unequivocally the coding assignments of the polymerase genes of three *ca* reassortant viruses previously isolated by Odigari et al. (19), we determined the genotypes of those viruses by using the same comparative gel electrophoresis methodology described above (data not shown). The genotypes of these reassortants and of the reassortant viruses isolated in the current study are shown in Table 2. Polyacrylamide gels showing the genotype of each of the single-gene reassortant viruses isolated in the current study are shown in Fig. 1.

TABLE 1. Isolation of A/Ann Arbor/60 *ca* × A/Korea/82 wild-type reassortant viruses

Mating	Virus mated with A/Korea/82 wild type	Reassortant viruses isolated[a]
A	A/Ann Arbor/60 *ca*	CR59-19
B	A/Korea/82 *ca* CR59-19	Clones 69 (NS), 18 (NP), 50 (PB2, PB1, PA, M, and NS), 96 (M and NS)
C	Clone 96	Clone 7 (M)
D	Clone 50	Clones 6 (PA), 46 (PB1), 24 (PB2 and NS), 9 (PB1 and NS), 29 (PB2, M and NS)
E	Clone 24	Clone 118 (PB2)

[a] Designations in parentheses indicate the gene or genes that derived from the *ca* donor virus.

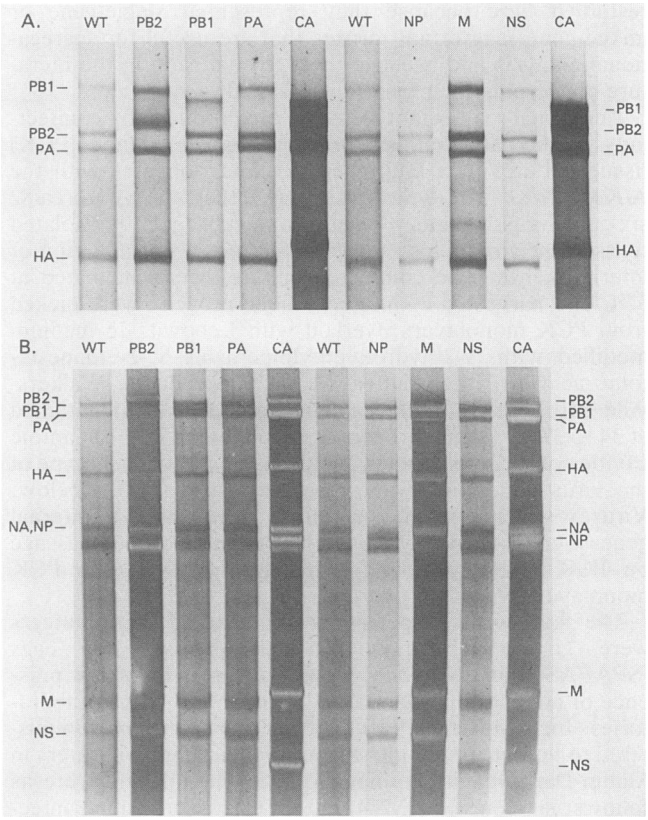

FIG. 1. Genotype of A/Korea/1/82 × A/Ann Arbor/6/60 *ca* reassortant viruses. (A) A 3.0% acrylamide–4.5 M urea gel was used. Samples were electrophoresed for 24 h at 5°C and 90 V constant voltage. (B) A 2.6% acrylamide–7.0 M urea gel was used. Samples were electrophoresed for 15 h at 5°C and 100 V constant voltage. Lanes for both gels: WT, A/Korea/82 wild type; CA, A/Ann Arbor/60 *ca* virus; PB2, clone 118; PB1, clone 46; PA, clone 6; NP, clone 18; M, clone 7; NS, clone 69. Abbreviations: HA, hemagglutinin; NA, neuraminidase.

Efficiency of plaque formation at permissive and restrictive temperatures. To determine which RNA segments of the influenza A/Ann Arbor/60 *ca* virus encode the *ca* and *ts* phenotypes, we evaluated the ability of the reassortant viruses to form plaques on PCK tissue culture at 25°C and at 33 to 35°C and on MDCK tissue at 33 to 36°C and at 38, 39, and 40°C (Table 2).

Results with the A/Korea/82 reassortant viruses showed

TABLE 2. Comparison of the genotype and the *ca* and *ts* phenotypes of parental and reassortant viruses

Clone designation	Parental origin of genes in viruses at indicated locus[a]								Log_{10} reduction in PFU/ml at indicated temp compared with permissive temp[b]					
									MDCK			*ts* phenotype[c]	PCK 25°C	*ca* phenotype[d]
	PB2	PB1	PA	HA	NA	NP	M	NS	38°C	39°C	40°C			
Viruses derived from A/Korea/82														
Single-gene reassortant														
118	A	W	W	W	W	W	W	W	1.4	4.5	≥4.9	+	≥2.6	−
46	W	A	W	W	W	W	W	W	0.7	2.0	≥4.5	+	≥2.8	−
6	W	W	A	W	W	W	W	W	0.0	1.1	3.5	−	1.5	+
18	W	W	W	W	W	A	W	W	0.3	0.8	2.0	−	≥3.6	−
7	W	W	W	W	W	W	A	W	0.3	0.6	1.3	−	≥3.6	−
69	W	W	W	W	W	W	W	A	0.1	0.4	1.4	−	3.0	−
Multiple-gene reassortant														
29	A	W	A	W	W	W	A	A	NT[e]	3.2	≥5.0	+	0.5	+
9	W	A	W	W	W	W	W	A	NT	3.2	≥5.1	+	≥2.6	−
24	A	W	W	W	W	W	W	A	NT	4.8	≥5.0	+	≥3.4	−
A/Korea *ca* CR59-19	A	A	A	W	W	A	A	A	1.5	3.6	≥4.3	+	0.2	+
A/Korea wild type	W	W	W	W	W	W	W	W	0.0	0.5	1.5	−	≥3.3	−
Viruses derived from A/Alaska/77														
T2	W	W	A	W	W	W	W	W	NT	−0.1	0.3	−	NT	+[f]
T3	W	W	A	W	A	W	W	W	NT	0.7	0.6	−	NT	+[f]
T6	W	A	A	W	A	W	W	W	NT	4.2	≥6.1	+	NT	+[f]
ca donor virus														
A/Ann Arbor/60 *ca*	A	A	A	A	A	A	A	A	1.5	3.7	≥4.0	+	0.1	+

[a] A, Gene derived from the A/Ann Arbor/60 (H2N2) *ca* donor virus; W, gene derived from the wild-type parent virus.
[b] Permissive temperature is 33 to 36°C.
[c] Viruses whose ability to form plaques at 39°C is reduced 100-fold compared with that at the permissive temperature were considered *ts*.
[d] The *ca* phenotype is defined as a less than 100-fold reduction in plaque formation at 25°C compared with that at the permissive temperature.
[e] NT, Not tested.
[f] As reported previously by Odigari et al. (19).

that the acquisition of the PA polymerase gene of the *ca* donor virus was sufficient to transfer the *ca* phenotype to a reassortant virus. No other RNA segment of the *ca* virus parent was associated with the transfer of the *ca* phenotype. Both the A/Alaska/77 and the A/Korea/82 reassortant viruses which received only the PA gene of the *ca* parent expressed the *ca* but not the *ts* phenotype. Considered together, these results showed that the *ts* phenotype was associated with different RNA segments of the *ca* donor virus than was the *ca* phenotype. The primary role of the PB2 polymerase gene of the A/Ann Arbor/60 *ca* virus in the transfer of the *ts* phenotype was shown by the near identity of the degree of restriction of replication at each elevated temperature of the clone 118 (PB2) reassortant virus and those of the six-gene *ca* reassortant and the *ca* parent virus (the designation in parentheses represents the single gene derived from the *ca* donor virus). Data for clones 29 and 24 confirmed that the *ts* phenotype cosegregates with the PB2 gene of the *ca* donor virus. The results also indicated that polymerase PB1 of the *ca* donor virus played a role in specifying the *ts* phenotype, although this role was quantitatively less important than that of polymerase PB2, as shown by the lower temperature sensitivity of clone 46 (PB1) at 39°C compared with that of clone 118 (PB2). Consistent with the interpretation that the PB1 gene specifies the *ts* phenotype, we found that the A/Alaska/77 reassortant virus T6, which was *ca* and *ts*, inherited only the PA and PB1 genes of the *ca* donor.

Level of replication and virulence in ferrets. The ferret is a well-characterized model for comparing the virulence of *ca* reassortant and wild-type influenza A viruses (11). Ferrets infected with wild-type virus sustain a high level of virus replication in the upper respiratory tracts and a moderate level in the lower respiratory tracts and develop fever and coryza. By contrast, during infection, six-gene *ca* reassortant viruses are not recoverable from the lower respiratory tract, nor do signs of illness develop. The single-gene reassortant virus clones 46 (PB1), 118 (PB2), 6 (PA), and 7 (M) were attenuated in ferrets, as shown by their greatly reduced replication in the lower respiratory tracts of ferrets after intranasal inoculation (Table 3). The incidence of coryza or fever in ferrets infected with one of the single-gene reassortant clone 46 (PB1), 118 (PB2), 6 (PA), or 7 (M) viruses was significantly lower than that in ferrets infected with wild-type virus. It is interesting that the reduction in the incidence of coryza was not accompanied by a reduction of virus replication in the nasal turbinates. The reason for this is not understood.

Level of replication and virulence in hamsters. It is likely that the evidence of attenuation of some of the single-gene reassortant viruses we observed in the ferret model was due to temperature sensitivity of these viruses. If this were true, the relevance of that attenuation for humans would remain unclear, because the normal body temperature of ferrets (approximately 39.4°C) is higher than that of humans (37°C). In the study of *ts* viruses, good correlation between the level of temperature sensitivity of an influenza A virus, the level of replication in the lower respiratory tracts of hamsters, and the level of attenuation in humans was observed (3, 22). Thus, it seemed that hamsters, whose normal body temper-

TABLE 3. Infection of ferrets with influenza A/Korea/82 *ca* reassortant or wild-type viruses

Clone designation	Gene(s) derived from A/Ann Arbor/60 *ca*	No. tested/ day[a]	Mean titer ± SE (log_{10} PFU/g of tissue) in nasal turbinates on day[b]:			Mean titer ± SE (log_{10} PFU/g of tissue) in lungs on day[b]:			% with coryza	% with fever
			2	4	8	2	4	8		
118	PB2	3	6.1 ± 0.5	5.3 ± 0.2	1.9 ± 0.5	≤1.4 ± 0.0[c]	≤1.4 ± 0.0[c]	≤1.4 ± 0.0	55	0[c]
46	PB1	3	7.8 ± 0.3	6.8 ± 0.6	≤1.4 ± 0.0	2.2 ± 0.8[c]	≤1.4 ± 0.0[c]	≤1.4 ± 0.0	33[c]	22[c]
6	PA	3	7.6 ± 0.4	7.6 ± 0.0	3.7 ± 1.2	≤1.4 ± 0.0[c]	≤1.4 ± 0.0[c]	≤1.4 ± 0.0	33[c]	0[c]
18	NP	3	6.1 ± 0.5	6.3 ± 0.3	2.0 ± 0.6	4.5 ± 0.8	3.9 ± 0.7	≤1.4 ± 0.0	100	55
7	M	3	7.7 ± 0.1	7.4 ± 0.3	≤1.4 ± 0.0	≤1.4 ± 0.0[c]	≤1.4 ± 0.0[c]	≤1.4 ± 0.0	22[c]	33
69	NS	3	6.0 ± 0.2	5.5 ± 0.1	≤1.4 ± 0.0	3.3 ± 0.2	3.0 ± 0.2	≤1.4 ± 0.0	66	66
A/Korea *ca*	Six internal genes	6	6.1 ± 0.4	5.7 ± 0.6	≤1.4 ± 0.0	≤1.4 ± 0.0[c]	≤1.4 ± 0.0[c]	≤1.4 ± 0.0	17[c]	0[c]
A/Korea wild type	None	6	7.5 ± 0.3	6.9 ± 0.3	1.7 ± 0.3	4.6 ± 0.5	4.1 ± 0.5	1.7 ± 0.3	100	72

[a] Anesthetized ferrets received 10^7 $TCID_{50}$ of virus intranasally.
[b] Ferrets were sacrificed on the indicated day; 10% (wt/vol) suspensions of nasal turbinate and lung tissues were made and cultured in MDCK tissue cultures (two wells per dilution).
[c] $P < 0.05$ compared with the group which received wild-type virus.

ature is the same as that of humans, would be better than ferrets as a model in which to evaluate the contribution of the *ts* phenotype to the restriction of virus replication in the lower respiratory tract. As a corollary, it was possible that a difference in the level of virus replication in the lower respiratory tract between the PB1 and PB2 single-gene reassortant viruses would be revealed in hamsters but not in ferrets, whose pulmonary temperature would be highly restrictive for both viruses. The results are given in Table 4. We found that the single-gene reassortant clone 118 (PB2) virus failed to replicate to detectable levels in the lower respiratory tracts of hamsters after intranasal inoculation: a finding similar to that for the six-gene *ca* reassortant virus. Consistent with its level of expression of the *ts* phenotype in vitro, clone 46 (PB1) was less restricted than clone 118 (PB2) in the lower respiratory tracts of hamsters. Surprisingly, clone 7 (M) was restricted in replication in the lungs of hamsters, although it was not *ts* in vitro. We observed no evidence of restriction of replication of the non-*ts* single-gene reassortant clone 6 (PA) virus in hamsters. Although the six-gene *ca* reassortant virus was restricted in replication in the upper respiratory tracts of hamsters, none of the single-gene reassortant viruses showed restriction of replication at that site. Despite this unrestricted replication for up to 4 days, each of 41 viruses isolated from the nasal turbinates or lungs of 22 hamsters infected with the clone 6 (PA) reassortant virus retained the *ca* phenotype, as indicated by their ability to replicate efficiently at 25°C. Similarly, each of 23 isolates of clone 118 (PB2) obtained from the upper respiratory tracts of hamsters retained the *ts* phenotype.

Studies with adult volunteers. In previous studies of attenuated reassortant influenza A viruses, it was observed that the level of virus replication in the nasopharynges of volunteers correlated with the virulence of the virus (22, 23). Therefore, in the present study, the mean duration and peak titer of virus shedding in volunteers infected with a reassortant virus were compared with those in volunteers infected with wild-type virus as a quantitative measure of the level of attenuation of each of the A/Korea/82 × A/Ann Arbor/6/60 *ca* reassortant viruses. Using these criteria, we found that reassortant clones 6 (PA) and 7 (M) were significantly restricted in replication in the upper respiratory tracts of volunteers compared with the wild-type virus (Table 5). Reassortant clone 18 (NP) was also restricted in replication, although the reduction in the mean peak titer of virus replication failed to achieve statistical significance at the $P = 0.05$ level. However, even viruses which showed restriction of replication in the upper respiratory tracts of volunteers replicated to higher titer than did the six-gene *ca* reassortant, indicating that acquisition of the PA or M genes transferred only partial attenuation. The frequency and degree of illness produced by the wild-type virus infection were too low to allow a meaningful comparison of the level of clinical viru-

TABLE 4. Replication of influenza A/Korea/82 *ca* reassortant or wild-type virus in the respiratory tracts of hamsters[a]

Clone designation	Gene(s) derived from A/Ann Arbor/60 *ca*	Nasal turbinates		Lungs	
		No. of hamsters	Mean titer ± SE (log_{10} $TCID_{50}$/g of tissue)	No. of hamsters	Mean titer ± SE (Log_{10} $TCID_{50}$/g of tissue)
118	PB2	6	5.8 ± 0.2	6	<1.5 ± 0.0[b]
46	PB1	6	5.1 ± 0.3	6	3.0 ± 0.4[b]
6	PA	6	5.6 ± 0.2	6	5.6 ± 0.2
18	NP	6	4.7 ± 0.3	5	4.6 ± 0.6
7	M	6	5.0 ± 0.3	5	2.7 ± 0.4[b]
69	NS	6	5.7 ± 0.2	4	5.1 ± 0.9
A/Korea *ca*	Six internal genes	6	3.4 ± 0.5[b]	6	<1.5 ± 0.0[b]
A/Korea wild type	None	6	5.4 ± 0.2	6	5.6 ± 0.5

[a] Anesthetized 4-week-old female Golden Syrian hamsters received $10^{5.0}$ $TCID_{50}$ of virus in 0.1 ml intranasally. For each virus, groups of four to six hamsters were sacrificed daily, a 10% (wt/vol) suspension of nasal turbinate and lung tissue was made, and the virus titer was determined in MDCK culture. The number of infected animals and the titer achieved on the day of maximum virus shedding are reported.
[b] $P < 0.05$ compared with the group which received wild-type virus.

TABLE 5. Infection of seronegative adult volunteers with A/Korea/82 *ca* reassortant or wild-type viruses

Clone designation	Gene(s) derived from A/Ann Arbor/60 *ca*	Dose[a] (log_{10} $TCID_{50}$)	No. of volunteers	% Infected[b]	Virus shedding			% with immune response[d]	% of volunteers with illness	
					% of volunteers	Duration[c] (days)	Mean peak titer[c] (log_{10} $TCID_{50}$/ml)		Fever	Any illness[e]
118	PB2	6.0	8	75	50	3.0 ± 1.1	2.4 ± 0.7	63	0	13
46	PB1	6.0	10	90	80	5.2 ± 0.7	2.2 ± 0.4	70	0	10
6	PA	6.0	15	87	67	3.5 ± 0.6	1.8 ± 0.3[f]	87	0	20
18	NP	6.0	15	67	60	4.0 ± 0.7	1.9 ± 0.4	67	0	7
7	M	6.0	15	100	93	5.0 ± 0.4	1.5 ± 0.1[f]	64	0	0[f]
69	NS	6.0	9	78	78	5.9 ± 1.1	3.5 ± 0.7	78	0	11
A/Korea *ca*	Six internal genes	6.5	55	73	11	0.3 ± 0.1[f]	0.7 ± 0.1[f]	73	0	0[f]
A/Korea wild type	None	6.0	14	86	86	5.1 ± 0.3	3.4 ± 0.5	86	7	36

[a] Seronegative (hemagglutination inhibition titer, ≤1:8) volunteers received 0.5 ml of virus intranasally. Viral genotypes are described in the text and shown in Fig. 1. After inoculation, volunteers were examined and nasal wash specimens were collected daily for 4 (six-gene *ca* virus) or 7 to 10 (other groups) days.

[b] Virus recovery or an antibody titer increase signified infection.

[c] Data from each infected volunteer were used for calculations. Data are expressed as mean ± standard error of the mean.

[d] A significant increase in antibody titer was documented by a fourfold or greater rise in serum hemagglutination inhibition, serum IgG ELISA or nasal wash IgA ELISA antibody titer.

[e] Volunteers were considered ill if they developed any of the following syndromes: fever (oral temperature, >37.8°C), systemic illness (myalgias or chills and sweats), upper respiratory tract illness (rhinitis, pharyngitis, or both observed on two consecutive days), or lower respiratory tract illness (persistent cough on two or more consecutive days).

[f] $P < 0.05$ compared with the group which received wild-type virus.

lence of the single-gene reassortant viruses. Significant restriction of replication in the upper respiratory tract was not observed for the PB2 or PB1 single-gene reassortant viruses. Each of 21 isolates of clone 6 (PA) obtained from 10 volunteers retained the ability to replicate at 25°C, and each of 13 isolates of clone 118 (PB2) obtained from 4 volunteers retained the *ts* phenotype.

DISCUSSION

We isolated and evaluated single- and multiple-gene substitution reassortant viruses derived from the mating of influenza A/Ann Arbor/6/60 *ca* (H2N2) virus with a current epidemic wild-type virus in an effort to determine the genetic basis of the *ca*, *ts*, and *att* phenotypes of *ca* reassortant viruses. To minimize the possibility of selecting revertant viruses, we performed all of our mating, cloning, and amplification procedures at temperatures permissive for both the *ca* and wild-type virus parents. Our analysis revealed that the PA gene of the *ca* donor virus plays a major role in the transfer of the *ca* phenotype. Odagiri et al. (19) previously demonstrated that acquisition of a single gene, which in this study we have identified unequivocally as the polymerase PA gene of the A/Ann Arbor/60 *ca* virus, was sufficient for the transfer of the *ca* phenotype. The current results confirm their earlier findings and extend them by demonstrating that no other internal gene of the A/Ann Arbor/60 *ca* virus is capable by itself of transferring the *ca* phenotype to reassortant viruses.

In the current study, segregational analysis with our A/Korea/82 reassortant viruses and the previously described A/Alaska/77 (19) reassortant viruses demonstrated independent contributions by the A/Ann Arbor/6/60 *ca* virus polymerase PB2 and PB1 genes to the *ts* phenotype. This is consistent with the results of previous studies in which complementation analysis demonstrated a role for PB1 and PB2 in the *ts* phenotype (21, 25). In one study (5), each of the genes of the *ca* parent virus was represented in ts^+ viruses. However, these ts^+ viruses were isolated at 39°C, a temperature which favors ts^+ revertants. Thus, phenotypic reversion cannot be excluded as an explanation of that result. In contrast, when Cox et al. isolated A/Ann Arbor/60 *ca* × wild-type reassortant viruses at permissive temperatures, the polymerase PB2 and M genes of the *ca* parent virus were present in each of three *ts* reassortants (5). However, the M gene, but not the PB2 gene, was also present in ts^+ reassortant viruses. Those results suggested a major role for the PB2 gene in specifying the *ts* phenotype, but they were insufficient to exclude the possibility that the M gene of the *ca* parent was also involved in the transfer of this phenotype. The current study provided evidence that the M gene of the *ca* donor virus is not required for expression of the *ts* phenotype by reassortants which derived their polymerase PB2 gene from the *ca* donor virus.

After defining the genes of the A/Ann Arbor/6/60 *ca* virus which specify the *ca* and *ts* phenotypes, we evaluated virus replication of our single-gene substitution reassortants, six-gene *ca* reassortant, and wild-type viruses in animals and volunteers to define the internal genes of the *ca* parent which specify the *att* phenotype. We found that in ferrets the PB1, PB2, PA, or M gene was able to greatly restrict replication in the lower respiratory tracts. In hamsters the PB2 gene played a major role, whereas the PB1 gene made a lesser contribution. The M gene of the *ca* donor virus also played a role in attenuation, whereas the PA gene did not specify *att* for hamsters. Since influenza A viruses which are attenuated for humans owing to temperature sensitivity are restricted in replication in the lungs of hamsters (3, 22), this finding suggests that the PA gene produced *att* through a mechanism other than temperature sensitivity. It remains to be demonstrated whether that mechanism is related to cold adaptation. The failure to demonstrate a contribution by the PA gene to *att* in hamsters is not surprising, since virulence of influenza A viruses in humans and rodents was previously shown to segregate independently (12). Interestingly, we observed evidence of restriction of replication of the single-gene reassortant viruses in the lower respiratory tracts, but not in the upper respiratory tracts, of ferrets and hamsters.

This was true even for clone 7 (M), which closely resembled the wild-type virus in terms of lack of temperature sensitivity in vitro. In contrast, in humans, restriction of virus replication in the upper respiratory tract was noted for two single-gene reassortant viruses. This indicates that one mechanism of expression of the *att* phenotype involves host tissue-specific restriction of replication.

In humans, the PA and M genes of the *ca* donor virus contribute to the *att* phenotype. However, none of our single-gene reassortant viruses was as attenuated in humans as the six-gene *ca* reassortant virus was. Previously we demonstrated that a reassortant virus which contained both the PA and M genes of the *ca* virus parent was as attenuated in volunteers as the homologous six-gene *ca* reassortant virus was (23). This suggests that the attenuation specified by each of the A/Ann Arbor/60 *ca* virus PA and M genes is additive and both genes must be transferred to a reassortant virus to achieve the level of attenuation observed in six-gene *ca* reassortant virus vaccines. Additional support for this suggestion of additive effect on attenuation by the PA and M genes is revealed by a reanalysis of a previous study of A/Alaska/6/77 (H3N2) *ca* reassortant viruses in volunteers (14). The A/Alaska/77 CR 31 clone 3 *ca* reassortant virus, which received the M gene from the wild-type virus parent and the five other internal genes from the *ca* virus parent, was attenuated in seronegative adult volunteers, but two other reassortant viruses, which received both the PA and M genes from the *ca* donor virus, manifested an additional 10-fold restriction of replication. A similar 10-fold difference in the level of replication was observed in the present study between either the clone 6 (PA) or 7 (M) virus and the six-gene *ca* reassortant virus. Although PA and M represent one combination of *ca* virus genes which together specify the full *att* phenotype, the currently available data are not sufficient to exclude the possibility that other combinations of A/Ann Arbor/60 *ca* virus genes also specify the full *att* phenotype.

Studies with ferrets and hamsters further indicated that the A/Ann Arbor/60 *ca* virus polymerase PB2 and PB1 genes, each of which specifies the *ts* phenotype, also contribute to the attenuation phenotype. The degree of temperature sensitivity specified by these genes was sufficient to significantly restrict virus replication in the lower respiratory tract of an animal whose normal body temperature is the same as that of humans. Since we sampled virus replication only in the upper respiratory tracts of volunteers, we lack data to confirm the restriction of replication of the PB1 and PB2 single-gene-substitution reassortant viruses in the lower respiratory tracts of humans. However, on the basis of the hamster data, we infer that both the PB1 and PB2 genes contribute to the *att* phenotype in humans, but that this is manifested only in their lower respiratory tracts. Thus, we would suggest that four genes, PB1, PB2, PA, and M, of the A/Ann Arbor/6/60 *ca* donor virus contribute to the *att* phenotype in humans.

Previous studies demonstrated the safety of immunization of seronegative adults and fully susceptible children with *ca* reassortant viruses (1, 4, 8, 14, 15, 18, 28). However, experience with *ts* viruses indicates that a virus which contains two single-step *ts* mutations on separate RNA segments can revert to virulence (27). In contrast, the current study provides evidence that mutations on at least four RNA segments of the *ca* donor virus specify attenuation for humans. This indicates that the likelihood for reversion of *ca* reassortant virus vaccines is lower than that for the previously studied *ts* viruses. We provide for the first time a biological basis for the observed stability of these vaccines in humans.

Further studies are needed to identify the nucleotide sequences that specify the *ca*, *ts*, and *att* phenotypes. Comparison of the nucleotide sequences of the genes of the *ca* donor virus which contribute to attenuation with those of the wild-type parental virus should provide information concerning the number and nature of mutations on each gene that specifies each of the characteristic phenotypes. Studies of revertant viruses isolated in vitro or after replication in ferrets would help characterize the importance of individual mutations. Clinical isolates could then be studied to determine the frequency with which genetic reversion occurs for each of the lesions which specify attenuation of *ca* reassortant virus vaccines.

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