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Phenotypic properties resulting from directed gene segment reassortment between wild-type A/Sydney/5/97 influenza virus and the live attenuated vaccine strain

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Abstract

Widespread use of a live-attenuated influenza vaccine (LAIV) in the United States (licensed as FluMist) raises the possibility that vaccine viruses will contribute gene segments to the type A influenza virus gene pool. Progeny viruses possessing new genotypes might arise from genetic reassortment between circulating wild-type (wt) and vaccine strains, but it will be difficult to predict whether they will be viable or exhibit novel properties. To begin addressing these uncertainties, reverse-genetics was used to generate 34 reassortant viruses derived from wt influenza virus A/Sydney/5/97 and the corresponding live vaccine strain. The reassortants contained different combinations of vaccine and wt PB2, PB1, PA, NP, M, and NS gene segments whereas all strains encoded wt HA and NA glycoproteins. The phenotypes of the reassortant strains were compared to wt and vaccine viruses by evaluating temperature-sensitive (*ts*) plaque formation and replication attenuation (*att*) in ferrets following intranasal inoculation. The results demonstrated that the vaccine virus PB1, PB2, and NP gene segments were dominant when introduced into the wt A/Sydney/5/97 genetic background, producing recombinant viruses that expressed the *ts* and *att* phenotypes. A dominant attenuated phenotype also was evident when reassortant strains contained the vaccine M or PA gene segments, even though these polypeptides are not temperature-sensitive. Although the vaccine M and NS gene segments typically are not associated with temperature sensitivity, a number of reassortants containing these vaccine gene segments did exhibit a more restricted *ts* phenotype. Overall, no reassortant strains were more virulent than wt, and in fact, 33 of the 34 recombinant viruses replicated less efficiently in infected ferrets. These results suggest that genetic reassortment between wt and vaccine strains is unlikely to produce viruses having novel properties that differ substantially from either progenitor, and that the likely outcome of reassortment will be attenuated viruses.

Keywords: Influenza virus; Genetic reassortment; Cold-adapted; Temperature-sensitive; Live attenuated vaccine

Introduction

Influenza viruses are members of the *Orthomyxoviridae* family, and as such are enveloped viruses that contain segmented genomes composed of negative-sense, single-stranded RNA (Wright and Webster, 2001). There are three genera of influenza viruses (types A, B, and C), which are categorized based on antigenic differences between the nucleocapsid and matrix proteins. Type A influenza viruses are grouped further into subtypes based on the antigenic properties of the viral-encoded hemagglutinin (HA; subtypes H1 through H16) and

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neuraminidase (NA; subtypes N1 through N9) envelope glycoproteins.

Influenza A and B viruses are important human pathogens responsible for yearly outbreaks of acute respiratory disease (Webster et al., 1992; Wright and Webster, 2001). Individuals can be infected repeatedly with both influenza types, because the antigenic determinants in the HA and NA glycoproteins change periodically providing a means by which the viruses can evade pre-existing humoral immunity. This antigenic variation is driven by two principal mechanisms. Antigenic drift results from the relatively frequent accrual of amino acid substitutions in antigenic determinants caused by nucleotide substitutions introduced by the error-prone viral RNA-dependent RNA replicase (Domingo et al., 1997). Antigenic shift results from the process of genetic reassortment during which entire gene segments are exchanged between related viruses after a permissive host is coinfected with multiple strains. Genetic reassortment plays a key role in emergence of new type A influenza strains, including pandemic viruses, because a diverse gene pool exists in infected birds as well as other animals (Webster et al., 1992).

Live attenuated influenza vaccine (LAIV; FluMist) has been used in the United States since 2003 (Arvin and Greenberg, 2006; Belshe et al., 2004; Harper et al., 2005). Like traditional inactivated influenza vaccines (Gerdil, 2003), LAIV is a trivalent formulation used to vaccinate against two type A strains and one type B strain (Belshe et al., 2004). For vaccines to account for the continuous antigenic evolution that occurs in wt viruses, surveillance data (Stohr, 2003) are studied to identify predominant circulating strains and subsequently select HA and NA antigens expected to provide the best vaccine coverage during the upcoming influenza season. Accordingly, LAIV strains are prepared in the laboratory by genetic reassortment between wt isolates, which supply the appropriate HA and NA genes, and type A or type B master donor viruses (MDV-A or MDV-B) that contribute the attenuated genetic background into which the wt glycoprotein genes are introduced (Maassab and Bryant, 1999; Murphy and Coelingh, 2002).

The attenuated MDV-A genetic background (Maassab, 1967; Maassab and Bryant, 1999) was derived in the 1960s from the A/Ann Arbor/6/60 (H2N2) strain following serial passage of the wt isolate in primary chicken kidney cells cultured at reduced temperature. Adaptation to these highly specific growth conditions resulted in acquisition of numerous mutations (Cox et al., 1988; Herlocher et al., 1993, 1996; Murphy and Coelingh, 2002) and outgrowth of a variant (A/Ann Arbor/6/60*ca*) that expressed a stable cold-adapted (*ca*), temperature-sensitive (*ts*), and attenuated (*att*) phenotype. When type A vaccine strains are produced by reassortment between the MDV-A and a wt isolate, the subsequent genetic selection and screening procedures result in isolation of a new virus with a defined genotype composed of gene segments encoding contemporary wt HA and NA glycoproteins and 6 gene segments (PB2, PB1, PA, NP, M and NS) derived from A/Ann Arbor/6/60*ca*. Consequently, both the vaccine and wt viruses encode identical glycoproteins, but the vaccine strains are genetically and phenotypically distinct because they encode all the A/Ann Arbor/6/60*ca* genetic determinants that restrict replication in the human host including those responsible for the *ca*, *ts*, and *att* phenotypes (Table 1).

During periods of influenza A virus activity in a community, individuals might be exposed to wt virus soon before or soon after vaccination with LAIV leading to coinfection of the nasal epithelium. Under these conditions, virus replication and genetic reassortment might produce progeny that contain novel genotypes, although it remains difficult to predict whether strains containing one or more vaccine virus gene segments will be viable and sufficiently fit to emerge from the general population of replicating virus, and whether the new strains might exhibit novel phenotypic properties. To investigate potential consequences of reassortment between wt and type A vaccine viruses, reverse-genetics (Fodor et al., 1999; Neumann et al., 1999) was used to produce 34 recombinant reassortant strains. These reassortants contained different combinations of gene segments derived from wt A/Sydney/5/97 and the corresponding A/Sydney/5/97 vaccine strain. Consequently, the reassortant strains contained novel gene constellations not previously tested (Jin et al., 2003, 2004; Snyder et al., 1988; Subbarao et al., 1992) that were composed of genes derived from a contemporary wt virus and the much older and divergent A/Ann Arbor/6/60*ca* MDV-A genetic background. Characterization of the recombinant viruses revealed that none replicated more efficiently or were more virulent than wt in infected ferrets, and in fact, many of the reassortant viruses retained properties resembling the attenuated vaccine strain. Taken together, these results indicated that the vaccine virus *ts* and *att* phenotypic markers were dominant even when the relevant gene segments were transferred into a contemporary wt virus genetic background.

Results

Production of recombinant reassortant influenza viruses

Reverse-genetics methodology was used to make a panel of recombinant viruses that contained different combinations of gene segments derived from wt A/Sydney/5/97 influenza virus and the corresponding live vaccine strain. One series of viruses was engineered by introducing one or more vaccine virus gene segments into the wt genetic background, and conversely, a second series was prepared by introducing wt gene segments into the vaccine virus genetic background.

An exhaustive analysis of all possible genotypes that might arise from reassortement between two viruses would require rescue and analysis of 256 recombinant strains (2^8 potential combinations). Since it was impractical to produce over 200 recombinants, the experiments were designed to focus primarily on reassortment of genes encoding components of the viral ribonucleoprotein (RNP) complex (PB2, PB1, PA, and NP). This seemed like a reasonable strategy since it was expected that modification of the viral gene expression and replication apparatus would lead to phenotypic changes, and because it is known that these gene segments (Table 1) are key determinants of vaccine virus phenotype (Cox et al., 1988; Herlocher et al., 1993, 1996; Jin et al., 2003, 2004; Murphy and Coelingh,

Table 1
Amino acid substitutions that distinguish wt A/Sydney/5/97 from vaccine virus

Amino acid substitutions[‡]							
PB2	PB1	PA	NP	M1	M2	NS1	NS2
V67I	P64H	V62I	D18E	V15I	R45H	S3P	S3P
V105M	V114I	N142K	*T23T*	K95R	L54F	I18V	L14M
V114I	M171I	N184S	D34G*	A143V	N82S	V23A	I40L
D120E	I179M	R192H	K65R	F144L	A86S	S28G	L57S
R194Q	V212L	K204R	R77K	A167T		R41K	R86K
I227V	G216S	S208T	R98K	I205V		N48S	T89I
N265S*,†	K327R	C241Y	R103Q	A218V		D53N	
D309E	I336V	K269R	E127D	A219I		K55E	
V382I	E358K	N272D	I186V	T227A		A56T	
S456N	R361S	Y277S	V197I	R231K		H59R	
V461I	K391E*	I311M	S217N	D232N		K66R	
I478V	A401V	K312R	V239M	T239A		T84A	
T491A	E457D	I322V	S286A			I90L	
R526K	K486R	S332P	H334N			L98M	
A559T	M546L	S343A	L343V			N101D	
A569T	D581G*	N383D	V344S			E112A	
V655I	Q584R	I387V	S353I			E125D	
I676T	R621Q	Y437H	N373T			M129I	
S682G	A661T*	I557V	G384R			I144L	
I697L	S741A	*E613E*	E421D			V145I	
D740N		A618T	S423P			E153T	
		L715P	V425I			F166L	
		K716R	T433A			I171N	
			R459Q			N197T	
						N207D	
						G211R	
						Q218stop[#]	
att	***att***	***att***	***att***		***att***		
ts	***ts***		***ts***				
Associated vaccine virus phenotype							

[‡] The data are presented as: Wt residue/amino acid position/vaccine residue.

[†] Substitutions highlighted in gray distinguish wt A/Ann Arbor/6/60 from A/Ann Arbor/6/60*ca* (Cox et al., 1988; Herlocher et al., 1993, 1996; Jin et al., 2003, 2004; Murphy and Coelingh, 2002). Phenotypic properties associated with the vaccine virus substitutions are indicated at the bottom of the table. Two expected codon substitutions (italicized) were not observed in the vaccine strain; the expected change in PA was K613E and T23N in NP.

* Substitutions that have been shown to contribute to the *ts* phenotype.

[#] The MDV-A A/Ann Arbor/6/60*ca* NS1 protein contains a premature stop codon that truncates the polypeptide by 12 amino acids (KRKMARTARSKV).

2002). The analysis was expanded somewhat to include a number of reassortants strains in which the M and NS gene segments were exchanged, because of the importance of NS in immune evasion (Krug et al., 2003) and the association between the *att* phenotype and the vaccine M gene segment (Snyder et al., 1988; Subbarao et al., 1992).

The study also focused on wt virus A/Sydney/5/97 and the vaccine strain encoding the identical glycoproteins. Importantly, wt A/Sydney/5/97 was selected because it consistently spread to the ferret lung after intranasal inoculation (data not shown), which facilitated analysis of the *att* phenotype. It also was important to compare wt, vaccine, and reassortant viruses all encoding identical HA and NA, because this ensured that changes in phenotypic properties would result from reassortment of PB2, PB1, PA, NP, M, or NS, which are the 6 gene segments responsible for the unique properties of the vaccine virus vaccine genetic background (Table 1).

Prior to beginning the molecular cloning necessary to rescue recombinant viruses, the wt and vaccine strains of A/Sydney/5/97 were subjected to complete genomic sequence analysis (Table 1). Comparison of wt and vaccine virus codons revealed numerous amino acid coding changes including those previously associated with the *ts*, *ca*, and *att* phenotypes (shaded in the table). Although sizable, the number of coding changes perhaps was predictable because the vaccine virus (A/Ann Arbor/6/60*ca*) genetic background was adapted to promote growth in chick kidney cells and embryonated eggs at reduced temperature (Maassab and Bryant, 1999), and it was derived over 30 years ago (Maassab, 1967) providing ample time for contemporary wt viruses to diverge considerably from this vaccine progenitor. The divergence in polypeptide coding sequence also implied that exchange of gene segments between these two genetic backgrounds would lead to gene constellation effects [Subbarao et al., 1992 #154], which presumably arise from suboptimal protein-protein interactions.

The 34 reassortant strains selected for the study (Tables 2 and 3) were viable, and they replicated sufficiently in eggs to produce virus stocks that could be used for further characterization. After the genotypes were confirmed by analysis of sequence markers present on each gene segment, studies were

Table 2
Temperature sensitivity of recombinant wt A/Sydney/5/97 influenza virus containing vaccine virus gene segment substitutions

Virus	Recombinant virus genotype								Virus titer[b] (Log_{10} PFU per ml)		EOP[c]	Phenotype[d]
									33 °C	39 °C		
1	PB2	PB1	PA	HA	NP	NA	M	NS	8.5	7.9	0.6	
2	PB2	PB1	PA	HA	NP	NA	M	NS	8.0	7.5	0.5	
3	PB2	PB1	PA	HA	NP	NA	M	NS	8.4	7.1	1.4	
4	PB2	PB1	PA	HA	NP	NA	M	NS	8.3	7.8	0.5	
5	PB2	PB1	PA	HA	NP	NA	M	NS	8.1	<1.7[e]	>6.4[f]	*ts, npl*
6	PB2	PB1	PA	HA	NP	NA	M	NS	8.3	8.0	0.3	
7	PB2	PB1	PA	HA	NP	NA	M	NS	8.3	3.0	5.3	*ts*
8	PB2	PB1	PA	HA	NP	NA	M	NS	8.3	7.8	0.5	
9	PB2	PB1	PA	HA	NP	NA	M	NS	8.3	4.1	4.2	*ts*
10	PB2	PB1	PA	HA	NP	NA	M	NS	8.2	5.4	2.7	*ts*
11	PB2	PB1	PA	HA	NP	NA	M	NS	8.4	<1.7	>6.4	*ts, npl*
12	PB2	PB1	PA	HA	NP	NA	M	NS	8.2	<1.7	>6.5	*ts, npl*
13	PB2	PB1	PA	HA	NP	NA	M	NS	8.1	2.7	5.4	*ts*
14	PB2	PB1	PA	HA	NP	NA	M	NS	8.1	6.8	1.3	
15[g]	PB2	PB1	PA	HA	NP	NA	M	NS	8.4[g]	<1.7	>6.7	*ts, npl*
16	PB2	PB1	PA	HA	NP	NA	M	NS	7.9	7.8	0.1	
17	PB2	PB1	PA	HA	NP	NA	M	NS	7.8	6.7	1.1	
18[g]	PB2	PB1	PA	HA	NP	NA	M	NS	8.2[g]	7.4	0.8	
	Control viruses											
	wt A/Sydney/5/97								8.4	8.4	0.0	*ts, npl*
	Vaccine A/Sydney/5/97								8.3	<1.7	>6.7	

[a] Gene segments are color-coded red (wt) and blue (vaccine). The HA (H3) and NA (N2) gene segments (gray) incorporated in all recombinant strains were identical. The gene segments were cloned from the H3N2 LAIV virus, which contained wt A/Sydney/5/97 glycoprotein coding sequences.
[b] Titers were determined three times and reported as an arithmetic mean value.
[c] Efficiency of plaque formation (EOP): Log_{10} Titer at 33 °C–Log_{10} Titer at 39 °C.
[d] *ts* is defined as having an EOP ≥2.0; *npl*, no plaques at 39 °C.
[e] Limit of detection was 1.7 $logs_{10}$ PFU per ml.
[f] The detection limit (1.7 log_{10} PFU/ml) was used to calculate the EOP for reassortant viruses having no titer at 39 °C.
[g] Two viruses listed in Tables 2 and 3 are identical, but were analyzed in both series of experiments and are included in both tables for clarity. The reassortant virus listed above containing 4 vaccine segments (PB2, PB1, PA, NP) is identical to the virus containing wt M and NS in a vaccine virus background (Table 3). Similarly, the virus listed above containing vaccine virus M and NS gene segments is identical to the vaccine virus in which PB2, PB1, PA, and NP have been replaced with wt gene segments (Table 3).

conducted to evaluate two key phenotypic traits. Expression of the *ts* phenotype was assessed by plaque formation on MDCK cell monolayers at permissive (33 °C) and nonpermissive (39 °C) temperatures, and the *att* phenotype was appraised by quantifying virus replication in the upper and lower respiratory tract of the ferret.

ts phenotype of recombinant reassortant strains

The *ts* phenotype is one of the principal attenuating determinants that limit vaccine virus replication and spread beyond the upper airways (Murphy and Coelingh, 2002). Several earlier studies have shown that multiple gene segments contribute to this property (Table 1) making it a logical phenotypic marker by which to monitor the effect of reassortment. Recombinant viruses were assigned a *ts* phenotype (Tables 2 and 3) if plaque forming efficiency was reduced by 100-fold or more (EOP values ≥2) when cells were incubated at nonpermissive temperature. Replication of the vaccine strain also is sufficiently impaired at nonpermissive temperature to result in a no-plaque (*npl*) phenotype, and those reassortant strains that exhibited this most stringent defect were labeled *ts* and *npl*.

Table 2 summarizes the results obtained when vaccine virus gene segments were transferred into the wt genetic background. Transfer of a single vaccine virus gene was insufficient to convert wt A/Sydney/5/97 into a temperature-sensitive strain (reassortant viruses 1–4, 16, and 17). In contrast, when two vaccine gene segments were transferred (reassortants 5–10 and 18), a number of strains exhibited a *ts* phenotype. Those containing vaccine genotypes PB2-PB1, PB2-NP, PB1-NP, or PA-NP (reassortants 5, 7, 9, and 10) propagated poorly at nonpermissive temperature, and in the case of the PB2–PB1 genotype (reassortant 5), this resulted in the most restrictive *npl* phenotype. The remaining pairs of vaccine gene segments had little effect on plaque forming efficiency (PB2–PA, PB1–PA, or M–NS; reassortants 6, 8, 18).

Most recombinant viruses produced after transfer of 3 (reassortants 11–14) or 4 (reassortant 15) vaccine genes into

Table 3
Temperature sensitivity of recombinant vaccine virus containing wt A/Sydney/5/97 gene segment substitutions

Virus	Recombinant virus genotype								Virus titer[b] (Log_{10} PFU per ml)		EOP[c]	Phenotype[d]
									33 °C	39 °C		
19	PB2	PB1	PA	HA	NP	NA	M	NS	7.4	3.4	4.0	*ts*
20	PB2	PB1	PA	HA	NP	NA	M	NS	7.7	<1.7[e]	>6.0[f]	*ts, npl*
21	PB2	PB1	PA	HA	NP	NA	M	NS	7.5	<1.7	>5.8	*ts, npl*
22	PB2	PB1	PA	HA	NP	NA	M	NS	8.3	<1.7	>6.6	*ts, npl*
23	PB2	PB1	PA	HA	NP	NA	M	NS	7.3	4.9	2.3	*ts*
24	PB2	PB1	PA	HA	NP	NA	M	NS	7.7	<1.7	>6.0	*ts, npl*
25	PB2	PB1	PA	HA	NP	NA	M	NS	7.7	5.1	2.6	*ts*
26	PB2	PB1	PA	HA	NP	NA	M	NS	7.8	3.1	4.7	*ts*
27	PB2	PB1	PA	HA	NP	NA	M	NS	7.6	6.7	0.9	
28	PB2	PB1	PA	HA	NP	NA	M	NS	8.1	<1.7	>6.4	*ts, npl*
29	PB2	PB1	PA	HA	NP	NA	M	NS	7.8	5.5	2.3	*ts*
30	PB2	PB1	PA	HA	NP	NA	M	NS	7.5	6.0	1.5	
31	PB2	PB1	PA	HA	NP	NA	M	NS	7.5	5.9	1.6	
32	PB2	PB1	PA	HA	NP	NA	M	NS	7.4	5.5	1.9	
18[g]	PB2	PB1	PA	HA	NP	NA	M	NS	7.8[g]	7.5	0.3	
33	PB2	PB1	PA	HA	NP	NA	M	NS	8.2	<1.7	>6.5	*ts, npl*
34	PB2	PB1	PA	HA	NP	NA	M	NS	7.8	<1.7	>6.1	*ts, npl*
15[g]	PB2	PB1	PA	HA	NP	NA	M	NS	8.2[g]	<1.7	>6.5	*ts, npl*
	Control viruses											
	wt A/Sydney/5/97								8.4	8.7	–0.0	
	Recombinant vaccine A/Sydney/5/97								8.3	<1.7	>6.7	*ts, npl*

[a–g] See Table 2.

the wt genetic background were temperature-sensitive and several also exhibited the *npl* phenotype (reassortants 11, 12, and 15) like the vaccine control. It was notable that those strains exhibiting the *npl* phenotype all contained both vaccine PB2 and PB1, which was consistent with the results obtained when just two vaccine segments were transferred (see virus 5). In agreement with this trend, reassortant strain 13 lacked the vaccine PB1 gene (vaccine genotype PB2–PA–NP) and retained the ability to produce small plaques at 39 °C. Curiously, a virus containing the vaccine PB1–PA–NP gene segment combination (reassortant 14) exhibited little defect in plaque forming efficiency at nonpermissive temperature despite the fact that some reassortants containing just two of these three gene segments (PB1–NP or PA–NP; reassortants 9 and 10) were temperature-sensitive.

Table 3 summarizes the results produced by transferring genes in the opposite direction; wt gene segments were transferred into the vaccine virus genetic background. The findings revealed that the majority of reassortant viruses were temperature-sensitive and that 8 exhibited the most restrictive *npl* phenotype. All single-gene reassortants remained temperature-sensitive (reassortants 19–22, 33, and 34) although the wt PB2 gene segment did relieve the *npl* phenotype (reassortant 19). Transfer of two wt genes (reassortants 23–28) produced a range of effects. Three reassortants retained the vaccine *ts/npl* phenotype including those that possessed wt genotypes PB2–PA, PA–NP, or M–NS (reassortants 24, 28, and 15), whereas three different wt gene pairs (PB2–PB1, PB2–NP, PB1–PA; reassortants 23, 25, and 26) relieved the *npl* phenotype. A virus containing the wt PB1-NP genotype (reassortant 27) contained just one remaining gene specifying a *ts* determinant and did not exhibit temperature sensitivity. This was consistent with results in Table 2, which indicated that 2 genes containing *ts* determinants were needed to restrict replication. Transfer of 3 or 4 wt genes into the vaccine background (reassortants 29–32 and 18) did relieve the *ts* and *npl* phenotype in most cases with the one exception being the reassortant strain containing the wt PB2–PB1–PA genotype (reassortant 29), which remained temperature sensitive.

Assessment of the att phenotype in infected ferrets

Ferrets are naturally susceptible to infection with human influenza viruses and serve as an important disease model (Maassab et al., 1982). Ferrets also serve as the model to assess the *att* phenotype (Maassab and Bryant, 1999; Maassab et al., 1982). Attenuation is measured by the relative ability to replicate in the lower respiratory tract after intranasal inoculation. Infection with wt viruses generally results in virus propagation in both the nasal epithelium and the lung, whereas replication of vaccine strains is restricted to upper airways. The *ts* phenotype is thought to be one of the important determinants responsible for restricting vaccine virus replication to the cooler upper airways (Murphy and Coelingh, 2002).

When reassortant viruses were tested in ferrets, each recombinant strain was administered to 4–5 animals from a larger study group. All study groups also contained ferrets infected intranasally with control viruses, which included wt

and vaccine strains of A/Sydney/5/97. Studies were considered valid if virus replication in the lung was detected in at least two-thirds of the ferrets infected with wt virus. This helped confirm the absence of pre-existing immunity, which was evaluated in all ferrets by HAI assay prior to the study (see Materials and methods). Nearly all recombinant viruses were tested in at least two independent studies (8–10 animals total), and those few strains that were evaluated in only a single study (5 animals) were negative for replication in the lung. Viruses that failed to propagate in the lung like the vaccine control were assigned the *att* phenotype.

Table 4 summarizes the results obtained after infection with reassortant strains produced in the wt A/Sydney/5/97 genetic background. As expected, all recombinant viruses replicated in the nasal turbinates, though it was evident that some might have achieved considerably lower titers, such as those containing the vaccine PB2–PB1 or PB2–PB1–NP genotypes (viruses 5 and 12). Only one reassortant virus was detected in the lung at wt frequency (virus 17), and all others exhibited reduced capacity to replicate in the lower respiratory tract. This outcome probably reflected the fact that all reassortant strains other then virus 17 carried at least one gene segment that possessed a known attenuating determinant (Table 1). Eight reassortants failed to replicate in the lung and were assigned the corresponding *att* phenotype. These included viruses in which two (PB2–PB1, PB2–NP, PB1–NP, PA–NP; viruses 5, 7, 9, 10), three (PB2–PB1–PA, PB2–PB1–NP, PB2–PA–NP; viruses 11–13), or four (PB2–PB1–PA–NP; virus 15) wt genes were replaced.

Seven reassortant strains listed in Table 4 were designated *att(+/−)*, because these viruses were detected in the lungs of some ferrets, but at a frequency considerably lower than wt, perhaps indicating that these reassortants were partially

Table 4
Effect of vaccine virus gene segment substitutions on replication of recombinant wt A/Sydney/5/97 in the ferret respiratory tract

Virus	Recombinant virus genotype								Nasal turbinates		Lungs		Phenotype[d]
									Number infected[c]	PFU (log_{10}) per g[d]	Number infected[c]	PFU (log_{10}) per g[d]	
1	PB2	PB1	PA	HA	NP	NA	M	NS	14/15	6.0±0.4	5/15 (33%)[e]	2.4±0.4	*att(+/−)*
2	PB2	PB1	PA	HA	NP	NA	M	NS	25/25	6.2±0.3	4/25 (16)	3.0±0.2	*att(+/−)*
3	PB2	PB1	PA	HA	NP	NA	M	NS	20/20	6.7±0.3	5/20 (25)	4.2±1.0	*att(+/−)*
4	PB2	PB1	PA	HA	NP	NA	M	NS	20/20	6.2±0.6	2/20 (10)	3.2±0.1	*att(+/−)*
5	PB2	PB1	PA	HA	NP	NA	M	NS	15/15	4.3±0.8	0/15 (0)	NVD[f]	*att*
6	PB2	PB1	PA	HA	NP	NA	M	NS	15/15	6.6±0.4	3/15 (20)	2.8±0.9	*att(+/−)*
7	PB2	PB1	PA	HA	NP	NA	M	NS	10/10	6.0±0.3	0/10 (0)	NVD	*att*
8	PB2	PB1	PA	HA	NP	NA	M	NS	15/15	6.3±0.3	6/15 (40)	2.7±0.8	*att(+/−)*
9	PB2	PB1	PA	HA	NP	NA	M	NS	13/13	5.3±1.4	0/13 (0)	NVD	*att*
10	PB2	PB1	PA	HA	NP	NA	M	NS	20/20	6.3±0.5	0/20 (0)	NVD	*att*
11	PB2	PB1	PA	HA	NP	NA	M	NS	14/15	5.7±0.8	0/15 (0)	NVD	*att*
12	PB2	PB1	PA	HA	NP	NA	M	NS	10/10	4.2±0.8	0/10 (0)	NVD	*att*
13	PB2	PB1	PA	HA	NP	NA	M	NS	14/14	5.2±1.0	0/14 (0)	NVD	*att*
14	PB2	PB1	PA	HA	NP	NA	M	NS	15/15	6.6±0.8	6/15 (40)	3.2±0.9	*att(+/−)*
15[b]	PB2	PB1	PA	HA	NP	NA	M	NS	13/15	6.2±0.5	0/13 (0)	NVD	*att*
16	PB2	PB1	PA	HA	NP	NA	M	NS	10/10	5.2±0.4	2/10 (20)	2.4±0.5	*att*[h]
17	PB2	PB1	PA	HA	NP	NA	M	NS	10/10	6.9±0.2	8/10 (80)	3.4±0.7	*wt*
18[b]	PB2	PB1	PA	HA	NP	NA	M	NS	10/10	5.8±0.4	1/10 (10)	3.7	*att*[h]
	Control viruses												
	wt A/Sydney/5/97								24/24	6.9±0.3	21/24 (88)	4.0±1.2	*wt*
	Recombinant wt A/Sydney/5/97								19/19	6.7±0.3	13/19 (68)	3.7±1.1	*wt*
	Vaccine A/Sydney/5/97								28/30	5.1±1.0	0/30 (0)	NVD	*att*
	Recombinant vaccine A/Sydney/5/97								14/15	4.4±1.1	0/15 (0)	NVD	*att*

[a] Gene segments are color-coded red (wt) and blue (vaccine). The HA (H3) and NA (N2) gene segments incorporated in all recombinant strains (gray) were identical. The gene segments were cloned from the H3N2 vaccine virus, which contained wt A/Sydney/5/97 glycoprotein coding sequences.

[b] Two viruses listed in Tables 4 and 5 are identical. They were included in both series of experiments described in the tables. These include reassortant strains containing 4 vaccine virus gene segments (PB2, PB1, PA, and NP) as well as virus containing both vaccine M and NS.

[c] Number of animals from which harvested tissue contained virus/total number of animals inoculated.

[d] The average value was calculated (±the standard deviation) from titers obtained from positive tissue samples.

[e] Number in parenthesis is the percentage of total inoculated animals from which harvested lung tissue contained detectable virus.

[f] NVD, No virus detected (limit of detection was 2.0 Log_{10} PFU per g tissue).

[g] Wt control viruses replicated in the lungs of at least 68% of the inoculated animals. Reassortant strains that attained this level of lower respiratory track invasion were labeled wt. Replication of vaccine virus controls was not detected in the lung defining the *att* phenotype. Viruses labeled *att*(+/−) were observed to replicate in lung of some animals, but fewer than wt.

[h] Two viruses were assigned the *att* phenotype despite rare observations of virus replication in the lung. As described in the text, this breakthrough replication was attributed to genetic reversion.

attenuated. For example, virus 4, which contained only the NP gene segment substitution, was found in the lung of just 2 out of 20 animals. Similar findings were obtained with the other single-gene reassortants (viruses 2–4). Several viruses that contained multiple vaccine gene segments also fell into the *att (+/−)* category (vaccine genotypes PB2–PA, PB1–PA, and PB1–PA–NP; viruses 6, 8, 14), which might have been related to the fact that none of these strains exhibited a *ts* phenotype (Table 2). When viruses isolated from lung homogenates were analyzed by nucleotide sequencing, analysis of attenuation markers indicated that these strains did not undergo genetic reversion, but that the breakthrough replication likely was due to a partially attenuated phenotype. Although second-site suppressor mutations cannot be ruled out completely, these *att(+/−)* phenotypes appear to be unique properties resulting from specific gene constellations.

Virus 18, which contained the vaccine M-NS genotype, and the recombinant strain that contained just the vaccine M gene (virus 16), were assigned the *att* phenotype even though 1 or 2 animals, respectively, seemed to contain low levels of virus in the lungs (Table 4). Further evaluation of the lung isolates was not possible because attempts to amplify virus from frozen lung homogenates failed. Consequently, it could not be definitively determined whether the breakthrough replication in one or two animals resulted from an incompletely attenuated phenotype or rare instances of genetic reversion. Nevertheless, the inability to amplify virus from lung tissue supported the conclusion that these reassortants were highly attenuated like the vaccine and likely were present at very low levels in the lung.

When reassortant strains produced in the vaccine virus genetic background were tested in ferrets, all exhibited the *att* phenotype (Table 5). This result demonstrated that the *att* phenotype was quite dominant and that it was difficult to reverse through introduction of wt gene segments. The results also supported conclusions from earlier studies indicating that attenuating mutations resided on multiple gene segments (see Table 1). It was notable as well that there were a number of reassortant viruses (viruses 18, 27, 30–32 and Table 3) that lost their *ts* characteristics yet they remained attenuated.

Comparison of phenotypic traits produced by reassortment

The reassortant viruses were categorized by phenotype in Table 6 to provide a data summary and a different perspective by which to compare all of the strains. Group I contains viruses that exhibited wt traits. There were only two viruses that fit this description including wt A/Sydney/5/97 and the reassortant strain that contained the vaccine NS gene in the wt genetic background (virus 17). The lack of wt-like reassortants

Table 5
Effect of wt A/Sydney/5/97 gene segment substitutions on replication of recombinant vaccine virus in the ferret respiratory tract

Virus	Recombinant virus genotype								Nasal turbinates		Lungs		Phenotype[c]
									Number infected	PFU (log_{10}) per g[c]	Number infected	PFU (log_{10}) per g[c]	
1	PB2	PB1	PA	HA	NP	NA	M	NS	8/10	4.3 ± 1.2	0/10	NVD[d]	*att*
2	PB2	PB1	PA	HA	NP	NA	M	NS	10/10	4.5 ± 1.2	0/10	NVD	*att*
3	PB2	PB1	PA	HA	NP	NA	M	NS	4/5	4.0 ± 0.9	0/5	NVD	*att*
4	PB2	PB1	PA	HA	NP	NA	M	NS	9/10	4.2 ± 1.0	0/10	NVD	*att*
5	PB2	PB1	PA	HA	NP	NA	M	NS	5/5	4.5 ± 1.0	0/5	NVD	*att*
6	PB2	PB1	PA	HA	NP	NA	M	NS	5/5	5.7 ± 0.4	0/5	NVD	*att*
7	PB2	PB1	PA	HA	NP	NA	M	NS	10/10	5.5 ± 0.6	0/10	NVD	*att*
8	PB2	PB1	PA	HA	NP	NA	M	NS	9/10	4.4 ± 0.8	0/10	NVD	*att*
9	PB2	PB1	PA	HA	NP	NA	M	NS	9/10	5.2 ± 1.3	0/10	NVD	*att*
10	PB2	PB1	PA	HA	NP	NA	M	NS	9/10	4.2 ± 1.2	0/10	NVD	*att*
11	PB2	PB1	PA	HA	NP	NA	M	NS	9/10	5.0 ± 1.0	0/10	NVD	*att*
12	PB2	PB1	PA	HA	NP	NA	M	NS	10/10	5.3 ± 1.3	0/10	NVD	*att*
13	PB2	PB1	PA	HA	NP	NA	M	NS	9/10	4.3 ± 1.0	0/10	NVD	*att*
14	PB2	PB1	PA	HA	NP	NA	M	NS	10/10	4.7 ± 1.0	0/10	NVD	*att*
15[g]	PB2	PB1	PA	HA	NP	NA	M	NS	9/10	5.5 ± 1.0	0/10	NVD	*att*
16	PB2	PB1	PA	HA	NP	NA	M	NS	5/5	6.2 ± 03	0/5	NVD	*att*
17	PB2	PB1	PA	HA	NP	NA	M	NS	5/5	5.2 ± 0.4	0/5	NVD	*att*
18[g]	PB2	PB1	PA	HA	NP	NA	M	NS	5/5	5.5 ± 0.3	0/5	NVD	*att*
	Control viruses												
	wt A/Sydney/5/97								20/20	6.9 ± 0.2	16/20 (80%)	3.7 ± 1.0	*wt*
	Recombinant wt A/Sydney/5/97								19/19	6.7 ± 0.3	10/14 (71%)	3.7 ± 1.0	*wt*
	Vaccine A/Sydney/5/97								23/25	4.9 ± 0.9	0/25	NVD	*att*
	Recombinant vaccine A/Sydney/5/97								14/15	4.4 ± 1.1	0/15	NVD	*att*

[a–c] See Table 4.

Table 6
Summary of reassortant virus phenotypes

Virus	Gene segment origin[a]								ts^b phenotype	npl^b phenotype	att^b phenotype
	PB2	PB1	PA	HA	NP	NA	M	NS			
Group I											
wt	PB2	PB1	PA	HA	NP	NA	M	NS	–	–	–
17	PB2	PB1	PA	HA	NP	NA	M	NS	–	–	–
Group II											
1	PB2	PB1	PA	HA	NP	NA	M	NS	–	–	*att*(+/–)
2	PB2	PB1	PA	HA	NP	NA	M	NS	–	–	*att*(+/–)
3	PB2	PB1	PA	HA	NP	NA	M	NS	–	–	*att*(+/–)
4	PB2	PB1	PA	HA	NP	NA	M	NS	–	–	*att*(+/–)
6	PB2	PB1	PA	HA	NP	NA	M	NS	–	–	*att*(+/–)
8	PB2	PB1	PA	HA	NP	NA	M	NS	–	–	*att*(+/–)
14	PB2	PB1	PA	HA	NP	NA	M	NS	–	–	*att*(+/–)
Group III											
16	PB2	PB1	PA	HA	NP	NA	M	NS	–	–	*att*
18	PB2	PB1	PA	HA	NP	NA	M	NS	–	–	*att*
32	PB2	PB1	PA	HA	NP	NA	M	NS	–	–	*att*
31	PB2	PB1	PA	HA	NP	NA	M	NS	–	–	*att*
30	PB2	PB1	PA	HA	NP	NA	M	NS	–	–	*att*
27	PB2	PB1	PA	HA	NP	NA	M	NS	–	–	*att*
Group IV											
7	PB2	PB1	PA	HA	NP	NA	M	NS	*ts*	–	*att*
9	PB2	PB1	PA	HA	NP	NA	M	NS	*ts*	–	*att*
10	PB2	PB1	PA	HA	NP	NA	M	NS	*ts*	–	*att*
13	PB2	PB1	PA	HA	NP	NA	M	NS	*ts*	–	*att*
29	PB2	PB1	PA	HA	NP	NA	M	NS	*ts*	–	*att*
25	PB2	PB1	PA	HA	NP	NA	M	NS	*ts*	–	*att*
26	PB2	PB1	PA	HA	NP	NA	M	NS	*ts*	–	*att*
23	PB2	PB1	PA	HA	NP	NA	M	NS	*ts*	–	*att*
19	PB2	PB1	PA	HA	NP	NA	M	NS	*ts*	–	*att*
Group V											
5	PB2	PB1	PA	HA	NP	NA	M	NS	*ts*	*npl*	*att*
11	PB2	PB1	PA	HA	NP	NA	M	NS	*ts*	*npl*	*att*
12	PB2	PB1	PA	HA	NP	NA	M	NS	*ts*	*npl*	*att*
15	PB2	PB1	PA	HA	NP	NA	M	NS	*ts*	*npl*	*att*
34	PB2	PB1	PA	HA	NP	NA	M	NS	*ts*	*npl*	*att*
33	PB2	PB1	PA	HA	NP	NA	M	NS	*ts*	*npl*	*att*
24	PB2	PB1	PA	HA	NP	NA	M	NS	*ts*	*npl*	*att*
28	PB2	PB1	PA	HA	NP	NA	M	NS	*ts*	*npl*	*att*
20	PB2	PB1	PA	HA	NP	NA	M	NS	*ts*	*npl*	*att*
21	PB2	PB1	PA	HA	NP	NA	M	NS	*ts*	*npl*	*att*
22	PB2	PB1	PA	HA	NP	NA	M	NS	*ts*	*npl*	*att*
vac	PB2	PB1	PA	HA	NP	NA	M	NS	*ts*	*npl*	*att*
	Associated phenotype (Table 1)										
	att	*att*	*att*		*att*		*att*				
	ts	*ts*			*ts*						

[a] Gene segments is color coded to indicate wt (red) and vaccine (blue) as described in Table 2.
[b] The definition of the *ts*, *npl*, and *att* phenotypes are described in Tables 2 and 4.

emphasized the general conclusion that incorporation of vaccine virus genetic material resulted in weakened strains.

Group II is composed of strains that were non-*ts* but exhibited a partially attenuated phenotype. None of these strains contained the vaccine virus M gene consistent with the conclusion that it encodes a strongly attenuating determinant (Table 4). Also, except for virus 14, none of these reassortants contained more than 1 gene associated with temperature sensitivity. This was consistent with the conclusion drawn from Table 2, which indicated that the combined effect of two or more gene segments containing *ts* determinants was necessary to render the wt genetic background temperature-sensitive (also see Table 2). Virus 14, which was the exception, was the unusual recombinant virus that contained three vaccine virus gene segments including two that contained *ts* determinants (vaccine genotype PB1-PA-NP). Further studies will be necessary to understand why this gene constellation did not result in temperature sensitivity.

Group III also contained non-*ts* viruses, but they were fully attenuated (Table 6). All of these strains contained the vaccine M gene segment, consistent with the conclusion (Table 4) that it conferred a dominant *att* phenotype without conferring evident temperature sensitivity. Also, none of these recombinants contained more than one gene associated with temperature sensitivity reinforcing the implication that two or more were

necessary to impart temperature sensitivity on wt/A/Syndey5/97 background.

Group IV contained viruses that exhibited the *ts* and *att* phenotypes, but did not express the most severe no-plaque growth restriction associated with vaccine strains. Viruses 7 and 9, which had vaccine genotypes PB2-NP or PB1-NP, respectively, and contained no other known attenuating gene segments (vaccine PA or M), demonstrated that the *ts* phenotype apparently was sufficient to attenuate replication in the ferret lung and that the more restrictive *npl* phenotype was not absolutely required. It also was notable that none of the Group IV viruses contained the vaccine PB2 and PB1 combination. As described below (see Group V), this supported the conclusion that any strain containing both vaccine PB2 and PB1 expressed the most restrictive *ts/npl* phenotype.

Reassortant strains in Group V resembled the vaccine virus and expressed the *ts/npl/att* phenotype. The PB1 and PB2 genes from the majority of these reassortants were derived from the vaccine, which seemed to be sufficient to produce a vaccine virus-like phenotype (see virus 5). Moreover, only two viruses in Group V did not contain the vaccine PB1-PB2 combination (viruses 20 and 24). Curiously, both of these strains expressed the *ts/npl* phenotype despite their having a composition of *ts* markers (PB1–NP or PB2–NP) that might have been expected to result in expression of temperature-sensitivity but not the most severe no-plaque defect (see viruses 7 and 9 in Group IV). As explained in greater detail below, it appears that the vaccine M gene might enhance expression of the *ts* phenotype, perhaps explaining why viruses 20 and 24 exhibited the *npl* phenotype.

Multiple viruses exhibited properties indicating that the vaccine M gene or the M-NS combination influenced expression of the *ts* phenotype. Table 7 was composed to better illustrate this phenomenon. For example, the phenotype of virus 4 indicated that the vaccine NP gene by itself was insufficient to convert wt A/Sydney into a temperature-sensitive strain, but when vaccine M and NS are added to the composition (virus 29) the resulting reassortant was rendered temperature-sensitive even though neither M nor NS encodes a temperature-sensitive determinant (top of Table 7, viruses 16–18 and Table 1). Further study will be necessary to determine how M and NS might contribute to this gene constellation effect.

Finally, it is worth briefly noting that reassortment did seem to have some effect on replication in the nasal epithelium (Tables 3 and 4) as well. Although there were exceptions to this generalization, and there can be considerable variation in nasal turbinate titers, it appeared that wt A/Syndey/5/97 and reassortants in Group I and II (Table 6) achieved similar titers in the nasal epithelium, which were about 6 $\log_{10}$ PFU or more per gram of tissue. Viruses in Groups III-V, including the vaccine control, generally achieved lower titers that were below 6 $\log_{10}$ PFU per gram. Perhaps this result indicates that viruses expressing the fully attenuated phenotype also exhibit a more generalized replication deficit that reduced titers even in the cooler upper airways.

Discussion

This study was conducted to analyze possible outcomes of reassortment between circulating wt influenza viruses and the attenuated strains contained in the live attenuated vaccine. Recombinant viruses containing different combinations of gene segments derived from the wt and vaccine strains of A/Sydney/5/97 influenza virus were produced and their phenotypic properties were assessed (Tables 2–5, summarized in Table 6). The analysis principally focused on the phenotypic effect produced by different combinations of the 4 gene segments encoding the RNP components (PB2, PB1, PA, and NP), but a limited number of viruses also were constructed to study the

Table 7
Effect of vaccine M and NS gene segments on virus phenotype

Virus	Genotype[a]								*ts*[b] phenotype	*npl*[b] phenotype	*att*[b] phenotype
wt	PB2	PB1	PA	HA	NP	NA	M	NS	–	–	–
17	PB2	PB1	PA	HA	NP	NA	M	NS	–	–	–
16	PB2	PB1	PA	HA	NP	NA	M	NS	–	–	*att*
18	PB2	PB1	PA	HA	NP	NA	M	NS	–	–	*att*
4	PB2	PB1	PA	HA	NP	NA	M	NS	–	–	*att*
29	PB2	PB1	PA	HA	NP	NA	M	NS	*ts*	–	*att*
8	PB2	PB1	PA	HA	NP	NA	M	NS	–	–	*att(+/−)*
25	PB2	PB1	PA	HA	NP	NA	M	NS	*ts*	–	*att*
14	PB2	PB1	PA	HA	NP	NA	M	NS	–	–	*att(+/−)*
19	PB2	PB1	PA	HA	NP	NA	M	NS	*ts*	–	*att*
9	PB2	PB1	PA	HA	NP	NA	M	NS	*ts*	–	*att*
24	PB2	PB1	PA	HA	NP	NA	M	NS	*ts*	*npl*	*att*
13	PB2	PB1	PA	HA	NP	NA	M	NS	*ts*	–	*att*
20	PB2	PB1	PA	HA	NP	NA	M	NS	*ts*	*npl*	*att*

[a,b] See Table 6.

influence of the M and NS gene segments. All recombinant strains encoded wt A/Sydney/5/97 HA and NA ensuring that the phenotypes observed in these studies were not influenced by changes in the surface glycoproteins and were due to reassortment of the unique genetic content that can be supplied by the vaccine virus genetic background. Overall, the results revealed that virtually any of the 6 gene segments donated to a reassortant strain by the vaccine virus genetic background produced detectable growth restriction (Tables 2–6). Perhaps, only exchange of the NS gene segment proved to be neutral (Table 6). Clearly, none of the reassortant viruses appeared to be more virulent than wt A/Sydney/5/97 in infected ferrets, and in fact, 33 of the 34 recombinant strains exhibited some level of attenuation (Tables 4 and 5). These data indicate that natural reassortment between wt type A influenza virus and vaccine strains will lead predominantly to weakened progeny. To generalize this conclusion, additional studies will be necessary that might examine gene segment combinations not tested here or evaluate the effect of using different wt genetic backgrounds.

It also should be noted that this work did not address potential consequences of glycoprotein gene reassortment. The decision to use identical wt HA and NA gene segments in all recombinants seemed reasonable, given that the impetus for conducting this study was to examine how the outcome of reassortment might be affected by the unique genetic content supplied by vaccine viruses. It is also worth emphasizing that the glycoproteins encoded by vaccine viruses are not unique since they originate from circulating field isolates; accordingly, the live vaccines are not expected to donate novel HA or NA gene segments to a reassortant strain.

Conclusions drawn from evaluation of vaccine and wt A/Sydney/5/97 reassortants agreed with earlier results identifying PB2, PB1, and NP as key determinants of the *ts* phenotype. In some of the earlier studies, the data indicated that transfer of vaccine PB2 or PB1 (Jin et al., 2004; Snyder et al., 1988) was sufficient to impart the *ts* phenotype on a non-*ts* genetic background. Results in Tables 2 and 3 also demonstrated that expression of the *ts* and *ts*/*npl* phenotypes correlated with reassortment of the PB1 or PB2 gene segments, but that this effect was only evident when 2 or more vaccine virus gene segments were transferred into the wt A/Sydney/5/97 genetic background. This might be best illustrated in Table 2 where transfer of the vaccine PB1 or PB2 into the wt A/Sydney/5/97 background (viruses 1 and 2) failed to produce a *ts* or *ts/npl* phenotype, whereas transfer of certain pairs of vaccine gene segments (PB2–PB1, PB2–NP, or PB1–NP; viruses 5, 7 and 9) resulted in temperature sensitivity. Similarly, the NP gene contribution to the *ts* phenotype was notable only in combination with other vaccine virus-derived genes (Table 2, PB2–NP, PB1–NP, or PA–NP; viruses 7, 9, and 10) in agreement with results reported previously (Jin et al., 2003). Taken together, these data consistently identify PB2, PB1, and NP as key determinants of the *ts* phenotype, but also indicate that the genetic background used for the analysis significantly influences the expression of this phenotypic trait.

Previous reports have indicated that attenuating mutations reside on 5 different gene segments (PB2, PB1, PA, NP, M) in the A/Ann Arbor/6/60*ca* vaccine virus background (Table 1). The results presented in Tables 4 and 5 provide rather striking support for this conclusion. Transfer of any one of these vaccine gene segments into the wt A/Sydney/5/97 genetic background diminished its ability to replicate in the lungs (Table 4), though single-gene reassortment was not sufficient to completely prevent lower respiratory infection in all cases. In most instances, transfer of two vaccine virus genes (Table 4; PB2–PB1, PB2–NP, PB1–NP, PA–NP, M–NS) was adequate to produce the full *att* phenotype. Consistent with the rather dominant effect of the vaccine genes, it proved to be very difficult to reverse the *att* phenotype of the vaccine virus by replacing gene segments with those from wt A/Sydney/5/97. In fact, of the 18 recombinant viruses prepared in the vaccine virus genetic background (Table 5), none was capable of replicating in the ferret lung.

It was evident that the presence of the vaccine M and NS genes increased the temperature sensitivity of some viruses (Table 7). Additional studies will be necessary to determine whether this effect required the vaccine M, NS, or both genes. It is attractive to predict that the attenuating mutations in the M gene segment, which seem to exert their effect through the M2 polypeptide (Table 1), might play a role in this phenomenon. On the other hand, perhaps the predicted amino acid changes in the vaccine M1 protein (Table 1) contribute to this relatively subtle effect, possibly by altering the interaction between the matrix protein and the nucleocapsid.

There was one particularly intriguing phenotype observed among the reassortant strains. The recombinant virus that contained the vaccine PB1-PA-NP genotype in the wt background (Table 6, virus 14) unexpectedly exhibited wt plaque forming efficiency at 39 °C and was only partially attenuated. This was unexpected because related viruses containing just two of the three vaccine genes (PB1–NP or PA–NP; Table 6, viruses 9 and 10) were temperature-sensitive and attenuated. Additional studies will be required to sort out this curious constellation effect, but perhaps, in this particular reassortant strain, the polymerase complex (Area et al., 2004; Biswas et al., 1998; Biswas and Nayak, 1996; Gastaminza et al., 2003; Gonzalez and Ortin, 1999; Gonzalez et al., 1996; Martin-Benito et al., 2001; Mena et al., 1999; Poole et al., 2004) derived some positive benefit from the fact that PB1 and PA both originated from the same virus. Conceivably, this enhanced the stability or activity of the PB1–PA complex enabling it to function more effectively as a polymerase component, and this partially suppressed the negative consequences of encoding several vaccine proteins. Under these circumstances, this might have allowed the properties of wt PB2 to dominate resulting in a wt-like polymerase complex.

Materials and methods

Cell culture and virology

Madin–Darby canine kidney (MDCK) cells and COS-7 cells were maintained in minimal essential medium (MEM) or Dulbecco's Modification of Eagles Medium (DMEM), respec-

tively, supplemented with 10% fetal bovine serum (FBS). All cells were maintained at 37 °C in 5% CO_2.

The wt isolate of A/Sydney/5/97 originated from the Centers for Disease Control and Prevention, and was obtained from the Vaccine Development Department at Wyeth. Wyeth and Medimmune coproduced the A/Sydney/5/97 LAIV strain (subtype H3N2) used for these studies (Buonagurio et al., 2006). Both viruses were propagated in the allantoic cavity of 9-day old embryonated chicken eggs (Charles River SPAFAS, Inc.), which were inoculated with approximately 10^3 plaque-forming units (PFU) and incubated for 48–52 h at 33 °C. The allantoic fluid from infected eggs was harvested, pooled, and then clarified by centrifugation for 15 min at 500×*g*. Virus stocks were stabilized by addition of 1/10 volume of 10× Sucrose–Phosphate–Glutamate buffer (1× SPG: 0.22 M sucrose, 7.1 mM K_2HPO_4, 3.8 mM KH_2PO_4, 4.9 mM glutamic acid) before storage at −80 °C. Viruses were subjected to genomic consensus sequence analysis as described previously (Buonagurio et al., 2006).

Influenza virus titers were determined by plaque assay. Nearly confluent MDCK cell monolayers cultured in 12-well plates were prepared for infection by aspirating the medium and washing twice with PBS. Virus was serially diluted in PBS supplemented with 0.3% bovine serum albumin (BSA) before the monolayers were infected with 0.1 ml of inoculum for 1 h at 33 °C, at which time 2 ml of agar overlay was added to each well and allowed to solidify at room temperature. The overlay was composed of MEM supplemented with 0.5 μg per ml trypsin-TPCK (Worthington Biochemicals, Inc.), MEM-non-essential amino acids, sodium pyruvate, penicillin, streptomycin, 0.3% BSA, and 0.6% agar (Oxoid, LTD). The infected cells were incubated for 3 days at 33 °C before the overlay was removed and plaques were visualized by staining the monolayer (0.1% crystal violet, 32% ethanol).

Recombinant DNA and influenza virus reverse-genetics

Plasmid DNAs required for recombinant influenza virus rescue (Neumann et al., 1999) were prepared with standard molecular cloning procedures (Ausubel et al., 1987). Influenza virus genomic RNA was purified from wt or vaccine virus stocks by the guanidinium thiocyanate–phenol–chloroform extraction method (Chomczynski and Sacchi, 1987) performed with Trizol reagent (Invitrogen). Viral cDNAs spanning complete gene segments were synthesized by reverse transcription and PCR amplification (RT/PCR) conducted with reagents in the one-step Titan amplification kit (Roche Molecular). The amplified DNAs subsequently were cloned into the pHH21 expression vector to allow synthesis of negative-sense genomic RNA transcripts from a RNA polymerase I transcription unit after transfection of eukaryotic cells (Neumann et al., 1994, 1999) (provided by Y. Kawaoka, University of Wisconsin-Madison). The HA and NA gene segments were cloned only from the A/Sydney/5/97 vaccine virus because they encode polypeptides that are identical to wt (Buonagurio et al., 2006). Both wt and vaccine virus PB2, PB1, PA, and NP coding sequences were cloned into an RNA polymerase II expression vector controlled by the CMV promoter (pCI-Neo, Promega). These plasmids were used to express the viral RNA polymerase subunits and the nucleocapsid protein in transfected cells during virus rescue (Neumann et al., 1999). Plasmid DNA nucleotide sequences were verified by automated cycle-sequencing (Buonagurio et al., 2006) using Big Dye Terminator Mix v3.1 (Applied Biosystems).

Recombinant influenza virus was rescued as described earlier (Neumann et al., 1999) except that COS-7 cells were used rather then 293T cells. Briefly, the cells were grown in six well plates until the monolayers were approximately 70% confluent, at which time cells were transfected with 8 plasmids specifying the desired wt or vaccine virus genome segments, and four plasmids encoding the requisite *trans*-acting polypeptides. DNAs were prepared for transfection by combining 2 μg of each plasmid (24 μg total DNA) in 100 μl of serum-free OptiMEM-I (Invitrogen). Then, 48 μl of transfection reagent (Trans IT LT-1, Panvera) was diluted in 100 μl Opti-MEM I before it was added to the DNA solution and the mixture was incubated at room temperature for 45 min. The total volume of the transfection mixture subsequently was adjusted to 1.0 ml with serum-free OptiMEM-I and transferred to the COS-7 cell monolayers from which the culture medium had been removed. The cells were incubated for 20–24 h at 33 °C (5% CO_2), at which time the transfection medium was replaced by OptiMEM-I containing 0.3% BSA and 0.01% FBS. Seventy-two hours post transfection, the medium containing rescued virus was transferred to a confluent MDCK cell monolayer and allowed to absorb for 2 h. Subsequently, the medium was replaced with OptiMEM-I supplemented with 0.3% BSA and 0.5 μg per ml trypsin. The cells were incubated at 33 °C until cytopathic effect was evident, at which time 100 μl of the supernatant was transferred to a fresh MDCK monolayer to amplify the rescued virus. Virus harvested in the medium supernatant was quantified by plaque assay on MDCK cells and later amplified in 9-day old embryonated eggs, as described above. Reassortant genotypes were verified by partial genomic sequencing of all gene segments, or in some cases by complete genome sequence determination.

Determination of virus phenotype

Parallel plaque assays (see above) conducted with MDCK monolayers incubated at 33 °C and 39 °C were performed to assess the *ts* phenotype. The efficiency of plaquing (EOP) value was determined using the following formula: Log_{10}(virus titer at 33 °C) − Log_{10}(virus titer at 39 °C). Viruses were considered *ts* if EOP values were equal to or greater than 2.0.

The *att* phenotype was evaluated after intranasal inoculation of ferrets with recombinant influenza viruses. All animal care and experimental procedures conformed to guidelines established by the Institutional Animal Care and Use Committee, and the facilities were approved by the American Association for Accreditation and Laboratory Animal Care. Ferrets were obtained from Marshall (North Rose, NY) or Triple-F Farms (Sayre, Pennsylvania) and used for virus challenge at 7 weeks of age. Males were supplied castrated and all animals were vaccinated against canine distemper by the vendor. To confirm

that ferrets used in virus challenge studies were seronegative, animals were housed separately from the general population and serum samples were drawn 1 week prior to shipment. Serum samples were screened routinely for reactivity to A/Sydney/5/97 (H3N2) with a standard hemagglutination inhibition (HAI) assay (WHO, 2002). Periodically, serum samples also were tested for antibodies reactive against both A/Sydney/5/97 and A/New Caledonia/20/99 (H1N1) if an influenza outbreak was suspected in the animal colony. To prepare serum for analysis, nonspecific inhibitors of hemagglutination were minimized by treating the serum with Receptor Destroying Enzyme (Denka Seika Co., LTD) at a 1:4 ratio for 18–20 h at 37 °C. The enzyme and serum complement were inactivated subsequently by incubation at 56 °C for 30 min. The serum was then incubated with a 1/5th volume of a 10% suspension of chicken red blood cells (RBC), which were removed afterward by centrifugation. The treated serum then was used to prepare a two-fold dilution series from which 25 μl was mixed with 4 hemagglutinin units of virus in 96-well, round-bottom plates. Antibody binding was conducted for 30 min at room temperature before 50 μl of a 0.5% RBC suspension was added to each well. The plates were incubated for 45 to 90 min at 4 °C before the HAI titer was reported as the reciprocal value of the last serum dilution that completely inhibited hemagglutination.

Routinely, groups of 4–5 seronegative animals, composed of both males and females, were inoculated to test each recombinant or control virus. Virus was prepared for ferret infection by rapidly thawing frozen stocks in a 33 °C water bath before temporary storage on ice. Each 0.2 ml dose was formulated to contain 7.0 Log_{10} PFU of virus and placed in a tuberculin syringe. Virus was administered to anesthetized animals to prevent sneezing and to ensure uptake of the full virus inoculum. Anesthesia was administered by first placing the ferrets in a chamber receiving 1 l of oxygen containing 5% isoflurane per minute, and was maintained with 1 l of oxygen containing 1–2% isoflurane per minute. The tuberculin syringe was used to administer 0.1 ml of virus formulation per nostril. Animals generally woke from anesthesia within 10 min. Ferrets subsequently were examined daily to identify distressed animals, but no animals died prior to the scheduled end of the experiment on day 3 following virus infection. Ferrets were sacrificed by intraperitoneal administration of sodium pentobarbital (100 mg/kg) before harvesting lung tissue and the nasal turbinates. The lower lobe of the left lung was harvested first to minimize the possibility that nasal turbinate extraction might contaminate the lung with virus from the upper airways. Extracted lung tissue was weighed then immersed in sufficient ice-cold PBS/SPG (PBS adjusted to 1× SPG) to prepare a 10% tissue extract by homogenization (90 s, 8–12,000 rpm, 4 °C) with an Omni Homogenizer (Omni International). Harvested nasal turbinates were placed in 2 ml ice cold PBS/SPG and homogenized 15–30 s with a hand-held mini-homogenizer (Kimble/Kontes Pellet Pestle Mixer). Both lung and nasal turbinate extracts were clarified by centrifugation (500×*g*, 15 min, 4 °C) prior to flash freezing in 0.4 ml aliquots that were stored at −70 °C.

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