

## Genetics of cold-adapted B/Ann Arbor/1/66 influenza virus reassortants: the acidic polymerase (PA) protein gene confers temperature sensitivity and attenuated virulence

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The cold-adapted B/Ann Arbor/1/66 influenza virus (ca B/AA/1/66) expresses temperature-sensitive (ts), cold-adapted (ca) and attenuation phenotypes. Reassortants which inherit one or more genes from ca B/AA/1/66 and all other genes from a virulent, wild-type influenza virus, B/Houston/1732/76, were produced and evaluated in order to identify the gene(s) responsible for the ts, ca and attenuation phenotypes. Only reassortants which inherited the PA gene from ca B/AA/1/66 expressed the ts phenotype in MDCK cells at 39°C. None of the reassortants tested expressed the ca phenotype in embryonated eggs at 25°C. The virulence of several reassortants was evaluated in ferrets. Inheritance of the PA gene from ca B/AA/1/66 was correlated with significant febrile attenuation and the apparent restriction of viral replication in the lower respiratory tract. Isolation of a virulent, non-ts revertant virus inheriting only the PA gene from ca B/AA/1/66 established a direct relationship between expression of the ts phenotype and attenuated virulence. Evidence for the contribution of at least one other gene from ca B/AA/1/66 to attenuation was observed. Thus, based on the methods used to determine reassortant gene compositions, these results indicate that the PA gene is primarily responsible for attenuation of ca B/AA/1/66 and its reassortants.

*Key words:* Cold-adapted influenza B virus; vaccine; temperature-sensitive; acidic polymerase.

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### Introduction

The cold-adapted B/Ann Arbor/1/66 influenza virus (ca B/AA/1/66) has been developed for the production of live vaccines in a manner analogous to that of cold-adapted A/Ann Arbor/6/60 influenza virus (ca A/AA/1/66).<sup>1-3</sup> Preliminary studies indicate that vaccine candidate strains derived from ca B/AA/1/66 are attenuated, genetically stable and antigenic in humans.<sup>4-6</sup> Like its type A counterpart, ca B/AA/1/66 also expresses two temperature-dependent replication phenotypes *in ovo* and *in vitro*. These phenotypes are temperature sensitivity (ts), the restriction of viral replication at 39°C; and cold adaptation (ca), the efficient replication of virus at 25°C. The ts and ca

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phenotypes of the ca A/AA/6/60 virus have been associated with the attenuation of virulence in animals and man.<sup>7-11</sup>

The properties of temperature sensitivity, cold adaptation and attenuation can be transferred to reassortant progeny by the inheritance of RNA segments (genes) from ca B/AA/1/66. The vaccine candidates produced in our laboratory are reassortants which inherit the six genes encoding the internal proteins from ca B/AA/1/66 and the hemagglutinin (HA) and neuraminidase (NA) genes from a relevant wild-type virus. Although such candidates are attenuated, the particular segments of ca B/AA/1/66 which confer attenuation and the mechanisms by which attenuation is expressed are unknown. We have been interested in identifying the genes of ca B/AA/1/66 that are responsible for attenuation in order to: (1) assess the value of the ts and ca phenotypes as laboratory markers of attenuation; (2) investigate the potential stability of attenuation; and (3) ultimately characterize attenuating lesions at the sequence level. For these purposes, a set of reassortants inheriting one or more genes from ca B/AA/1/66 and their remaining genes from a virulent, wild-type virus, B/Houston/1732/76 (wt B/HN/1732/76), were produced and evaluated for virulence in ferrets and for the expression of the ts and ca phenotypes.

## Results

### *The PA gene of ca B/AA/1/66 determines temperature sensitivity*

The derivation of reassortants inheriting only one gene from ca B/AA/1/66 (single gene reassortants) was accomplished for all genes except the nucleoprotein (NP) gene. The ts properties of these reassortants in Madin–Darby canine kidney (MDCK) cells are shown in Table 1. As with the cold-adapted parent, reassortants that demonstrate no infectious titer at 39°C are temperature-sensitive (ts). Of the single ca gene reassortants tested, only the reassortant inheriting the PA gene (SG3\*) from ca B/AA/1/66 was temperature-sensitive at 39°C as demonstrated by the absence of a detectable infectious titer. The log<sub>10</sub> reduction of infectious titers at 39°C as compared to 33°C of all other single gene reassortants were similar to that of wt B/HN/1732/76.

The ts properties of reassortants inheriting more than one gene from ca B/AA/1/66 (multiple gene reassortants) were also determined to investigate the expression of the ts phenotype by the combination of ca B/AA/1/66 genes. As before, the expression of the ts phenotype was consistently correlated with the inheritance of only the PA gene from ca B/AA/1/66. Reassortants that failed to express the ts phenotype included a reassortant (MG12478) that inherited all internal protein genes from ca B/AA/1/66 except the PA gene. Thus, the PA gene of ca B/AA/1/66 was responsible for the expression of temperature sensitivity.

Reassortants MG124678, MG12478 and MG1247 were somewhat more temperature-sensitive at 39°C than the wt parent was. These reassortants probably possess one or more 'leaky' ts mutations. In experiments using MG12478, plaque titration at 40°C or 41°C did not result in further restriction and plaques isolated at 39°C remained partially temperature-sensitive (data not shown). Thus, partial temperature sensitivity was neither indicative of a ts mutation requiring higher temperatures for full expression nor was it attributable to genetic instability of the ts phenotype. A single gene reassortant inheriting the NP gene from ca B/AA/1/66 was not isolated. However, comparison of MG1247 with MG127 indicated that while the inheritance of only the

\* For convenience, reassortants are identified by an identification (ID) code which indicates the RNA segments inherited from ca B/AA/1/66. The two-letter prefixes SG and MG stand for single gene and multiple gene reassortant. For example, MG12478 refers to the reassortant inheriting multiple genes from ca B/AA/1/66, namely, RNAs 1, 2, 4, 7 and 8.

**Table 1** Relative infectious titers (pfu/ml) of reassortants at 33°C and 39°C<sup>a</sup>

Clone	Gene segment origin <sup>b</sup>								ID code	Mean infectious titer (log <sub>10</sub> pfu/ml ± SE) at 33°C	Mean log <sub>10</sub> reduction of infectious titers (pfu/ml ± SE <sup>c</sup> ) at 39°C vs 33°C	Phenotype <sup>d</sup>
	1	2	PA 3	NP 4	HA 5	NA 6	M 7	NS 8				
ca B/AA	C	C	C	C	C	C	C	C		7.4 ± 0.2	>4.0	ts
wt B/HN	W	W	W	W	W	W	W	W		8.6 ± 0.1	1.1 ± 0.4	non-ts
II-47-1	C	W	W	W	W	W	W	W	SG1	8.4 ± 0.05	1.0 ± 0.4	non-ts
II-29-4	C	W	W	W	W	W	W	W	SG1	7.1 ± 0.09	0.76 ± 0.07	non-ts
II-41-4	C	W	W	W	W	W	W	W	SG1	7.6 ± 0.2	0.82 ± 0.2	non-ts
II-47-4	W	C	W	W	W	W	W	W		8.8 ± 0.6	0.96 ± 0.2	non-ts
I 1-6	W	W	C	W	W	W	W	W	SG3	8.8 ± 0.2	>4.0	ts
I 1-6 FL	W	W	Cr	W	Wr	W	W	W		7.4 ± 0.2	0.95 ± 0.3	non-ts
II-38-3	W	W	W	W	C	W	W	W	SG5	8.9 ± 0.2	0.89 ± 0.6	non-ts
II-28-1	W	W	W	W	W	C	W	W	SG6	8.0 ± 0.2	1.1 ± 0.08	non-ts
V 76-1	W	W	W	W	W	W	C	W	SG7	9.2 ± 0.2	0.96 ± 0.2	non-ts
II-53-6	W	W	W	W	W	W	W	C	SG8	8.7 ± 0.3	1.2 ± 0.3	non-ts
II-43-2	C	W	C	W	C	C	C	C	MG135678	7.1 ± 0.5	>4.0	ts
II-43-4	C	W	W	W	C	C	C	C	MG15678	7.2 ± 0.5	0.98 ± 0.4	non-ts
III-58-3	C	C	C	C	W	C	C	C	MG1234678	7.8 ± 0.2	>4.0	ts
IV-27-1	C	C	W	C	W	C	C	C	MG124678	8.3 ± 0.3	1.8 ± 0.5	non-ts
IV-27-4	C	C	W	C	W	W	C	C	MG12478	8.3 ± 0.2	2.1 ± 0.8	non-ts
IV-27-5	C	C	W	C	W	W	C	W	MG1247	8.0 ± 0.5	2.2 ± 0.7	non-ts
IV-27-3	C	C	W	W	W	W	C	W	MG127	8.7 ± 0.05	0.53 ± 0.06	non-ts
IV-28-6	W	C	C	C	C	W	W	W	MG2345	8.7 ± 0.3	>4.0	ts
II-41-2	W	C	C	W	W	W	W	W	MG23	8.0 ± 0.1	>4.0	ts
II-19-3	W	W	C	W	C	W	W	W	MG35	8.5	>4.0	ts
II-51-8	C	W	W	W	C	C	C	W	MG1567	7.7 ± 0.06	0.99 ± 0.2	non-ts
II-14-1	W	C	W	W	W	C	C	C	MG2678	8.8 ± 0.1	1.4 ± 0.3	non-ts

<sup>a</sup> Except for reassortant II-19-3, the values shown were determined from three or more experiments. SE = Standard error.

<sup>b</sup> Gene segment origin is indicated as follows—C: RNA segment is derived from ca B/AA/1/66; W: RNA segment is derived from B/HN/1732/76; W<sub>r</sub>: RNA segment matches the indicated parent but, the encoded protein varies from both parental strains; Cr and Wr: variant genes of ts-revertant virus isolated from ferret lungs. Gene coding assignments were determined as described in Materials and methods.

<sup>c</sup> A log reduction of >4.0 indicates the absence of infectious titer at 39°C. The titration of low dilution inocula results in the destruction of the cell monolayer without detectable plaque formation.

<sup>d</sup> As with the cold-adapted parent, reassortants that demonstrate no infectious titer at 39°C are temperature-sensitive (ts).

NP gene from ca B/AA/1/66 would not be sufficient for the expression of the ts phenotype, it could be associated with the expression of partial temperature sensitivity, either by itself, or in combination with the PB1, PB2 and/or M genes.

### Evaluation of the ca phenotype in eggs

Replication of ca B/AA/1/66 at 25°C in embryonated chicken eggs was clearly distinguished from that of wt B/HN/1732/76 (Table 2) on the basis of the log<sub>10</sub> reduction of infectious titers (EID<sub>50</sub>/ml) at 25°C as compared with 33°C. Viruses that express the ca phenotype were defined as those demonstrating less than a 2 log<sub>10</sub> reduction of infectious titers at 25°C versus 33°C, that is, they should replicate with roughly the same efficiency as the ca parent does. By this criteria, none of the reassortants tested expressed the ca phenotype. However, with respect to the wt parent, several reassortants demonstrated substantially improved replication at 25°C versus 33°C as indicated in Table 2. Among the single gene reassortants, only the reassortant inheriting the HA gene from ca B/AA/1/66 (SG5) demonstrated improved replication at 25°C. Although a single gene reassortant inheriting the NP gene from ca B/AA/1/66 was not isolated, such a reassortant would not be expected to express cold adaptation since MG124678, MG12478 and MG1247 failed to demonstrate even intermediate replication at 25°C.

All multiple ca gene reassortants inheriting the HA gene from ca B/AA/1/66 also demonstrated improved replication at 25°C. However, evidence for contributions from other genes was observed since MG1234678 (inheriting only the HA gene from the wt parent) demonstrated intermediate replication and two multiple gene reassortants (MG135678 and MG2345), inheriting genes from the ca parent in addition to the HA

**Table 2** Evaluation of the ca phenotype in embryonated eggs

Clone	Gene segment origin <sup>a</sup>								ID code	Infectious titer (log <sub>10</sub> EID <sub>50</sub> /ml ± SE <sup>b</sup> ) at 33°C	Log <sub>10</sub> reduction of infectious titers (EID <sub>50</sub> /ml ± SE <sup>c</sup> ) at 25°C vs 33°C	Phenotype <sup>d</sup>
	1	2	PA 3	NP 4	HA 5	NA 6	M 7	NS 8				
ca B/AA	C	C	C	C	C	C	C	C		7.7 ± 0.3	0.9 ± 0.4	ca
wt B/HN	W	W	W	W	W	W	W	W		8.5 ± 0.1	4.9 ± 0.2	non-ca
II-41-4	C	W	W	W	W	W	W	W	SG1	9.5 ± 0.0	5.2 ± 0.4	non-ca
II-47-4	W	C	W	W	W	W	W	W		8.8 ± 0.2	> 5.2	non-ca
I-1-6	W	W	C	W	W	W	W	W	SG3	9.5 ± 0.0	5.5 ± 0.3	non-ca
I-1-6 FL	W	W	Cr	W	Wr	W	W	W		8.5 ± 0.2	4.5 ± 0.4	non-ca
II-38-3	W	W	W	W	C	W	W	W	SG5	9.0 ± 0.2	3.6 ± 0.3	int.
II-28-1	W	W	W	W	W	C	W	W	SG6	9.2 ± 0.2	5.2 ± 0.4	non-ca
V-76-1	W	W	W	W	W	W	C	W	SG7	8.5 ± 0.0	4.8 ± 0.2	non-ca
II-53-6	W	W	W	W	W	W	W	C	SG8	9.0 ± 0.3	4.8 ± 0.4	non-ca
II-43-2	C	W	C	W	C	C	C	C	MG135678	6.9 ± 0.2	2.2 ± 0.3	int.
II-43-4	C	W	W	W	C	C	C	C	MG15678	9.5 ± 0.0	3.5 ± 0.2	int.
III-58-3	C	C	C	C	W	C	C	C	MG1234678	9.4 ± 0.08	3.9 ± 0.2	int.
IV-27-1	C	C	W	C	W	C	C	C	MG124678	8.5 ± 0.0	4.8 ± 0.2	non-ca
IV-27-4	C	C	W	C	W	W	C	C	MG12478	9.2 ± 0.2	4.8 ± 0.4	non-ca
IV-27-5	C	C	W	C	W	W	C	W	MG1247	8.5 ± 0.0	4.5 ± 0.3	non-ca
IV-27-3	C	C	W	W	W	W	C	W	MG127	9.0 ± 0.3	5.0 ± 0.4	non-ca
IV-28-6	W	C	C	C	C	W	W	W	MG2345	9.2 ± 0.2	2.3 ± 0.4	int.
II-41-2	W	C	C	W	W	W	W	W	MG23	8.5 ± 0.4	4.8 ± 0.4	non-ca
II-19-3	W	W	C	W	C	W	W	W	MG35	8.9 ± 0.2	3.3 ± 0.3	int.
II-51-8	C	W	W	W	C	C	C	W	MG1567	9.4 ± 0.1	3.5 ± 0.4	int.
II-14-1	W	C	W	W	W	C	C	C	MG2678	8.2 ± 0.2	4.5 ± 0.4	non-ca

<sup>a</sup> Gene segment origin is indicated as follows—C: RNA segment is derived from ca B/AA/1/66; W: RNA segment is derived from wt B/HN/1732/76; W<sub>v</sub>: RNA segment matches the indicated parent but, the encoded protein varies from both parental strains; Cr and Wr: variant genes of the ts-revertant virus isolated from ferret lungs. Gene coding assignments were determined as described in Materials and methods.

<sup>b</sup> SE = standard error.

<sup>c</sup> As with the cold-adapted parent, reassortants that demonstrate less than a 2 log<sub>10</sub> reduction of infectious titers at 25°C vs 33°C are cold-adapted (ca). Reassortants demonstrating improved replication at 25°C vs 33°C in comparison to the wild-type parent are intermediate (int.).

gene, demonstrated further improvement with respect to SG5. Comparison of MG15678 with MG135678, as well as MG124678 with MG1234678, indicated that the PA gene of ca B/AA/1/66 increased the degree of cold adaptation in certain multiple gene reassortants. Interestingly, the inheritance of only the HA and PA genes from the wt parent was sufficient to abrogate cold adaptation in MG124678 but, inheritance of only these genes from the ca parent was not sufficient to confer the ca phenotype on MG35.

### Evaluation of virulence

In order to determine the relative contributions of ca B/AA/1/66 genes to attenuation, a limited number of reassortants were assayed for virulence in ferrets (Table 3). Virulence was evaluated on the basis of fever and viral replication in the upper respiratory (nasal turbinates) and lower respiratory (lungs) tracts. The virulence of ca B/AA/1/66 was clearly distinguished from that of wt B/HN/1732/76 using the ferret model system. The wt parent produced fever in all ferrets, replicated well in the turbinates, and was isolated from the lungs of 4 of 12 infected ferrets. In contrast, the ca parent produced fever in only 1 of 6 ferrets, replicated poorly in the turbinates and was not isolated from the lungs.

Significant febrile attenuation was expressed only by the two reassortants (SG3 and MG23) inheriting the PA gene from ca B/AA/1/66. Like the ca parent, the mean peak temperature of ferrets infected with either reassortant was significantly less than that of the wt virus. Although the precise level of febrile attenuation attributable to the inheritance of the PA gene was variable, these results indicated that the PA gene of ca B/AA/1/66 was a major contributor to the attenuation of virulence. This observation was corroborated by the febrile response to MG12478, which induced a

**Table 3** Febrile response and viral replication in ferrets

Clone	ID code	Mean peak Temp $\pm$ SE <sup>a</sup>	No. of ferrets with fever $\geq$ 103.4/ No. infected	Lung <sup>b</sup>		Turbinate <sup>b</sup> mean	
				No. positive/ No. infected day 2	No. infected day 4	log <sub>10</sub> pfu/ml $\pm$ SE day 2	log <sub>10</sub> pfu/ml $\pm$ SE day 4
ca B/AA/1/66		103.1 $\pm$ 0.6*	1/6	0/3	0/3	3.7 $\pm$ 0.5	2.9 $\pm$ 0.2
I-1-6	SG3	102.6 $\pm$ 0.9*	1/6	1/3	0/3	5.4 $\pm$ 0.3	4.6 $\pm$ 0.6
II-41-2	MG23	103.3 $\pm$ 0.6*	3/6	0/3	0/3	6.0 $\pm$ 0.2	5.5 $\pm$ 0.2
IV-27-4	MG 12478	103.6 $\pm$ 0.6	9/12	0/6	0/6	6.1 $\pm$ 0.4	5.0 $\pm$ 0.1
V-76-1	SG7	103.7 $\pm$ 0.4	10/12	1/6	2/6	6.5 $\pm$ 0.2	6.1 $\pm$ 0.4
I-1-6 FL		103.9 $\pm$ 0.2	6/6	1/3	1/3	6.9 $\pm$ 0.3	6.0 $\pm$ 0.4
II-41-4	SG1	103.8 $\pm$ 0.2	6/6	1/3	2/3	7.0 $\pm$ 0.3	6.2 $\pm$ 0.2
wt B/HN/1732/76		103.8 $\pm$ 0.2	12/12	2/6	2/6	6.1 $\pm$ 0.3	5.4 $\pm$ 0.1

<sup>a</sup>\* = mean differs significantly ( $P < 0.05$ ) from wt B/HN/1732/76 by Student's  $t$ -test. SE = standard error.

<sup>b</sup> Values determined from 10% lung and turbinate suspensions.

mean peak temperature significantly higher than those induced by the ca parent and SG3. This result also suggested that the PA gene from ca B/AA/1/66 may be essential for the maximum attenuation of a vaccine candidate strain produced with ca B/AA/1/66.

With the exception of one ferret (see below), SG3 and MG23 were not recovered from the lungs of infected animals, tentatively indicating a role for the PA gene in the restriction of viral replication in the lower respiratory tract. The apparent restriction of replication observed for MG12478 in the lungs of infected ferrets suggested that at least one, as yet unidentified, gene other than the PA gene from ca B/AA/1/66 was also involved in attenuation. By themselves, RNA 1 and the matrix (M) gene were excluded as potential contributors, since SG1 and SG7 demonstrated virulence comparable to that of wt B/HN/1732/76.

The level of viral replication in ferret turbinates was determined at 2 and 4 days after infection (Table 3). All viruses grew well in the turbinates except ca B/AA/1/66. It could not be determined which genes of the ca parent were responsible for this character. Fever was frequently observed in groups of ferrets demonstrating mean virus titers in turbinates at day 2 of 6.0 log<sub>10</sub> pfu/ml or greater. The mean turbinate titer at day 2 of ferrets demonstrating fever was 6.5  $\pm$  0.5 log<sub>10</sub> pfu/ml. The mean turbinate titer at day 2 of ferrets not demonstrating fever was 5.2  $\pm$  1.1 log<sub>10</sub> pfu/ml. These two samples yield a  $t$ -statistic of 4.5 ( $P = 1.0 \times 10^{-4}$  at 31 d.f.), suggesting a relationship between viral replication in ferret turbinates and the presence or absence of fever.

A weaker, but still significant, relationship was also observed between the occurrence of fever at any time and mean virus titer in the turbinates at day 4. However, this relationship was not correlated with late occurring or enduring fevers and probably reflects the stronger relationship found to occur earlier in infection. The apparent rate at which virus titers in the turbinates fell between days 2 and 4 differed among the viruses assayed. No relationship between the reduction of turbinate titer with segment composition or fever could be inferred from the data.

#### *Isolation and characterization of the ts-revertant I-1-6 FL*

As mentioned, virus was recovered from the lungs of one ferret infected with SG3. Since this reassortant was temperature-sensitive in MDCK cells at 39°C, it was not

expected to replicate at what should be the restrictive temperature of the ferret lower respiratory tract.<sup>12</sup> As a result, it was of interest to determine if this virus had undergone modification of its ts phenotype. Plaque titration at 39°C revealed a virus sub-population, designated I-1-6 FL, which had lost temperature sensitivity (Table 1). When administered to ferrets, I-1-6 FL produced fevers comparable to and was recovered from the lungs at the same frequency as the wt parent (Table 3). Thus, loss of the ts phenotype by SG3 was accompanied by a corresponding reversion to virulence. Since the PA gene of ca B/AA/1/66 was previously shown to be responsible for its ts phenotype, this result established a direct relationship between attenuation and the expression of the ts phenotype by ca B/AA/1/66.

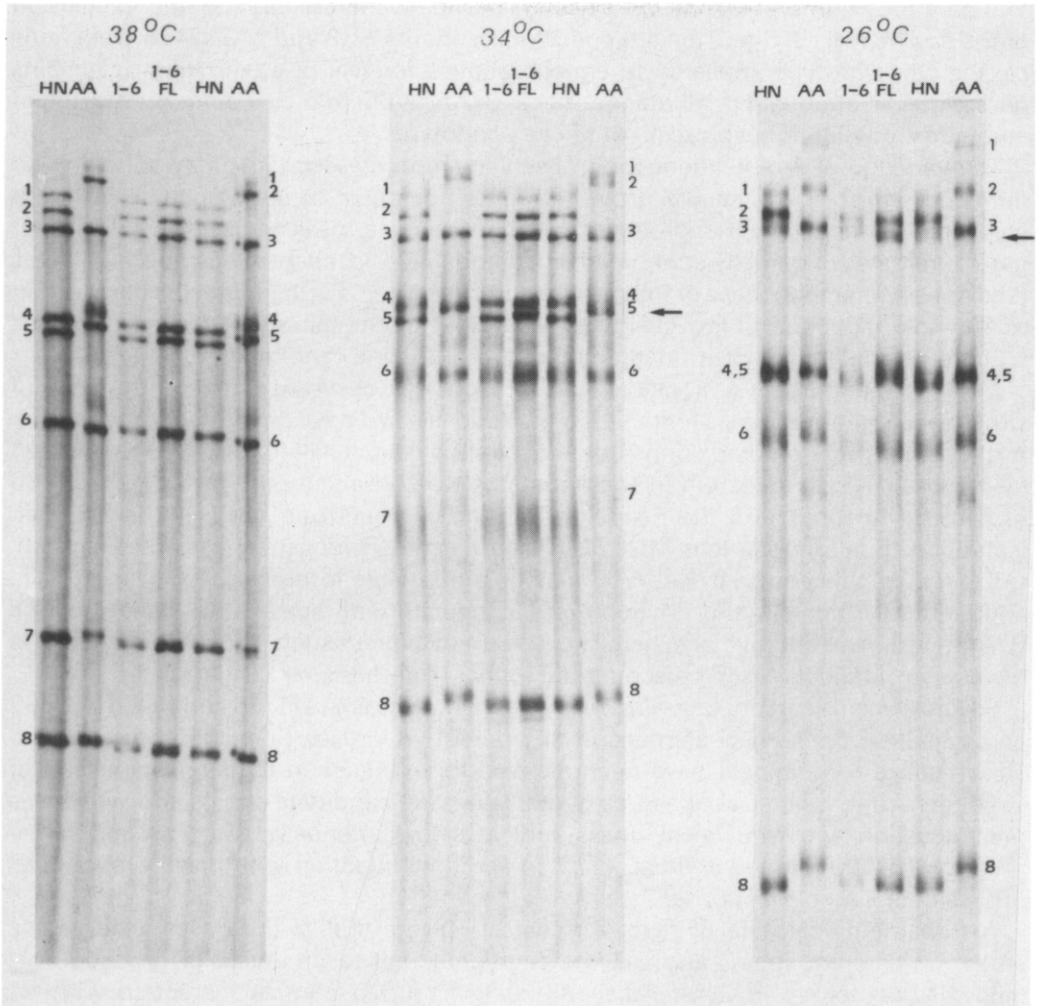
In order to investigate the genetic changes that may have occurred in the ts-revertant I-1-6 FL, the migration of I-1-6 FL and SG3 RNA segments were compared at different temperatures on mixed polyacrylamide-agarose gels (Fig. 1). Only the PA and HA genes of I-1-6 FL demonstrated altered migration. Altered migration was clearly observed at 26°C for the PA gene and at 34°C for the HA gene. Comparison of I-1-6 FL and SG3 proteins on 5–13% SDS-polyacrylamide gels did not reveal any differences in migration (not shown). In addition, the HA proteins of wt B/HN/1732/76, I-1-6 FL, and SG3 were antigenically indistinguishable by hemagglutination inhibition. These results suggested that small changes in the PA and possibly HA genes of SG3 have resulted in its loss of the ts phenotype and attenuation. However, a more rigorous determination of the presence or absence of extragenic suppressor mutations is required.

## Discussion

Cold-adapted B/AA/1/66 was developed as a donor of attenuating genes for the production of live influenza virus vaccines. In addition to attenuation, ca B/AA/1/66 expresses the ts and ca phenotypes. Allied with electrophoretic characterization of the segmented influenza virus genome, the ts and ca phenotypes are routinely used as *in vitro* markers to identify vaccine candidate strains without specific knowledge of their association with attenuation.<sup>4,5,13</sup> Through genetic reassortment, one or more genes of ca B/AA/1/66 can be segregated among reassortant progeny that inherit their remaining genes from a virulent wild-type virus. Based on our determination of reassortant gene compositions, the contributions of several genes to temperature sensitivity, cold adaptation and the attenuation of virulence were described.

The data showed that inheritance of the PA gene from ca B/AA/1/66 was responsible for the transfer of the ts phenotype to wt B/HN/1732/76. The possibility that spontaneous mutations may influence or be responsible for the expression of the ts phenotype, rather than the inheritance of genes from ca B/AA/1/66, is discounted by the consistent expression of the ts phenotype only by multiple gene reassortants inheriting the PA gene from the ca parent. For example, it is unlikely that the variant HA protein of reassortant II-47-4 (inheriting only RNA 2 from ca B/AA/1/66) suppresses temperature sensitivity in this reassortant since inheritance of RNA 2 from the ca parent is unassociated with the ts phenotype in multiple gene reassortants. Similar reasoning discounts the influence of potentially undetected mutations in reassortant II-47-4 and, by comparing the appropriate reassortants, in the other single gene reassortants as well.

Inheritance of the PA gene from ca B/AA/1/66 was also found to correlate with significant attenuation of virulence. Furthermore, evaluation of a virulent, non-ts revertant reassortant (I-1-6 FL) demonstrated that attenuation conferred by the inheritance of the PA gene from ca B/AA/1/66 was mediated by the expression of its



**Fig. 1.** Genetic variation in the ts-revertant I-1-6 FL.  $^3\text{H}$ uridine-labeled vRNA was subjected to electrophoresis in mixed agarose-polyacrylamide gels at 38°C, 34°C, and 26°C. Arrows indicate variant segments in I-1-6 FL. HN: wt B/HN/1732/76. AA: ca B/AA/1/66; reassortant I-1-6 (SG3). 1-6 FL: ts-revertant I-1-6 FL.

ts phenotype. As a result, the ts phenotype should be considered an important laboratory marker of attenuated virulence for the ferret animal model.

In contrast to the ts phenotype, the ca phenotype could not be associated with a single gene. Efficient growth at 25°C apparently involved several mutations, some of which required the interaction of genes for expression. We hypothesize that the transfer of wt genes to an otherwise ca virus may disable replication at 25°C in the same sense that the inheritance of genes bearing ts lesions disable replication at elevated temperatures. Alternatively, the apparent contributions of the PA and HA genes of ca B/AA/1/66 may suggest that these genes act cooperatively with other genes to effect the expression of the ca phenotype. The complexity of such an interaction is indicated by comparison of reassortants MG135678 and MG2345 (i.e. several genes would be involved). In either case, among reassortants produced with wt B/HN/1732/76, the minimum number of genes from the ca parent sufficient for the expression of the ca phenotype equivalent to ca B/AA/1/66 *in ovo* would include the HA and PA genes.

The data provide no evidence for a relationship between attenuation and expression of the *ca* phenotype. Since the attenuated reassortants SG3 and MG12478 were non-*ca*, the *ca* phenotype appeared to emerge above the level of attenuation. It remains possible that attenuating mutations in *ca* B/AA/1/66 form a necessary subset of mutations required for expression of the *ca* phenotype.

The variation of febrile attenuation observed among reassortants may be related to differences in their replication in the turbinates compared to the *ca* parent. For both cold-adapted and conditional-lethal *ts* mutant viruses, peak virus titers in hamster nasal turbinates of partially attenuated or reactogenic cold-adapted vaccine candidates tend to be higher than those of fully attenuated viruses.<sup>14,15</sup> Furthermore, the observation of 100-fold reduction in the level of replication in the human nasopharynx correlates with the attenuation of cold-adapted influenza A vaccine candidates.<sup>11</sup>

In the present study, a significant association was observed between the level of virus titers in the turbinates and the febrile response two days after infection. A similar relationship is encountered during influenza A virus infection of ferrets.<sup>16-18</sup> Pronounced restriction of viral replication in the turbinates was observed only for *ca* B/AA/1/66 (Table 3). Inheritance of the PA gene from the *ca* parent did not result in a similar restriction. Since reassortant MG12478 also did not demonstrate restricted growth, the low virus titers of *ca* B/AA/1/66 may be attributable to one or both of its surface antigens. Alternatively, the PA gene may cooperate with some other gene(s) of *ca* B/AA/1/66 in an additive or synergistic fashion to express such restriction. The results of vaccine candidate testing discount the former hypothesis.

Several vaccine candidate strains derived from both *ca* B/AA/1/66 and *ca* A/AA/6/60 and inheriting the surface antigens from different wt viruses (including both H1 and H3 influenza A subtypes) have been assayed for virulence in ferrets. Comparison of virus titers in turbinates between *ca* parents, vaccine candidate strains and wt viruses after infection with equivalent doses, indicates that attenuated viruses consistently produce 10–100 fold lower titers.<sup>3-5,13,19</sup> In addition, infection with these viruses does not result in a febrile response.

Histopathological data of ferret turbinates indicate that at the doses used in this study, *ca* parental strains and vaccine candidates fail to produce or produce only mild histopathology in ferret nasal turbinates.<sup>4,5,13,19</sup> In contrast, infection with wt viruses always produces severe histopathology. Thus, cold-adapted viruses grow to lower titers in nasal turbinates and fail to induce an inflammatory response comparable to wt viruses. Since fever coincides with the inflammatory response and inflammation is essentially a response to tissue injury,<sup>20</sup> the restricted replication of *ca* B/AA/1/66 in the URT appeared to be an important determinant of attenuated virulence.

Genetically stable attenuation is a necessary component of a suitable vaccine donor strain. Since mutation is essentially a random event, it is impossible to ensure genetic stability. However, the probability that mutation will result in a reversion to virulence is greatly diminished by the accumulation of attenuating lesions. Evidence for the involvement of at least two genes (PA and one or more genes of MG12478) in the attenuation of *ca* B/AA/1/66 was observed. Thus, the stability of attenuation observed during limited trials of vaccine candidates produced with *ca* B/AA/1/66 would be expected.<sup>4-6</sup> Unfortunately, conditional-lethal *ts* donor strains possessing two attenuating lesions have been found to revert in children.<sup>21</sup> As a result, to be reasonably confident that attenuation conferred by *ca* B/AA/1/66 is acceptably stable for use as a live vaccine, it will be necessary to demonstrate additional attenuating lesions.

Finally, attenuation equivalent to *ca* B/AA/1/66 could not be attributed to any of the reassortants tested. A recent analysis of single gene reassortants derived from *ca* A/AA/6/60 (performed in collaboration with M. H. Synder, NIAID, Bethesda, MD)



has also failed to attribute complete attenuation to the inheritance of single genes (H. F. Maassab, personal communication). The full understanding of the attenuated virulence expressed by cold-adapted influenza viruses will require further investigation of cumulative or synergistic effects that may result from the interaction of ca B/AA/1/66 genes.

## Materials and methods

*Viruses and cells.* The derivation of ca B/AA/1/66 from an epidemic strain of type B influenza was described by Maassab and DeBorde.<sup>3</sup> Wild-type B/HN/1732/76 was obtained from Dr Robert Couch, Baylor University, Houston, TX. Prior to reassortment, the wt virus was cloned by two successive plaque isolations in MDCK cells.

The preparation, maintenance and infection of MDCK cells (Flow Laboratories, Rockville, MD) and primary chick kidney (PCK) cells were described previously.<sup>7,22,23</sup> Except where otherwise stated, all media used to propagate virus in MDCK cells contained 1  $\mu\text{g/ml}$  L-(tosylamido-2-phenyl)ethyl chloroethyl ketone (TPCK) trypsin (Worthington Biochemicals, Bedford, MA).

*Production of reassortants.* Reassortants were produced by mating ca B/AA/1/66 and wt B/HN/1732/76 using a modification of the infectious center technique of Nakajima and Sugiura.<sup>24</sup> PCK or MDCK cells were dually infected with 1–10 pfu/ml of each parental strain. After 1 hour of adsorption at room temperature, the cells were washed once with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  deficient HBSS and dispersed by treatment with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  deficient HBSS containing 0.5% trypsin and 2% EDTA for 1 minute at room temperature. This solution was removed and incubation was continued for 3–5 minutes at 35°C. The dispersed cells were suspended in 10 ml of HBSS and pelleted at 1000 *g* for 10 minutes. The cell pellet was resuspended and diluted so that the appropriate cell monolayer, grown in a 25  $\text{cm}^2$  tissue culture vessel, was seeded with approximately 10, 100, or 500 infected cells. The cells were allowed to settle for 5 minutes and 5 ml of overlay media was added. The overlay media used for PCK cells was composed of Medium 1,<sup>25</sup> 0.8% agar (BBL Microbiology Systems, Cockeysville, MD), and 0.01% DEAE-dextran (Pharmacia, Uppsala, Sweden). The overlay media used for MDCK cells was composed of Medium 199, 0.8% agarose (FMC, Rockville, ME) and 5  $\mu\text{g}/\mu\text{l}$  TPCK trypsin. After 2 days of incubation at 33°C, the cell monolayers were stained by the addition of 4 ml of the appropriate overlay media containing 0.01% neutral red. Individual plaques were picked on the third or fourth day after plating, suspended in 1 ml of E-MEM, diluted 10-fold and replated as described above. The virus from individual plaques was replated to isolate pure reassortant clones. The virus isolated from individual plaques in the second plating was amplified at 33°C in eggs and gene compositions were determined by polyacrylamide gel electrophoresis of viral RNA (vRNA). Isolates identified as reassortants were purified by one plaque to plaque purification in MDCK cells at 33°C and amplified in eggs. The gene compositions of reassortants were verified after plaque purification.

*Preparation of [<sup>3</sup>H]uridine-labeled vRNA.* [<sup>3</sup>H]uridine-labeled vRNA was prepared from viruses grown in PCK cells. Confluent PCK cells grown in two 25  $\text{cm}^2$  tissue culture vessels were infected and each incubated in the presence of 250  $\mu\text{Ci}$  of [5-,6-<sup>3</sup>H]uridine (35–50 Ci/mole, Amersham, Arlington Heights, IL) in 1.5 ml of 2 $\times$  Eagle's medium at 33°C. After 4 hours 3 ml of fresh 2 $\times$  Eagle's medium was added and incubation was continued for 2 days.

Infected tissue culture media was clarified and the virus pelleted through a 30% sucrose cushion in STE buffer (0.1 M Tris-HCl, 0.001 M EDTA, pH 7.4) as described previously.<sup>23</sup> Viral RNA was phenol-chloroform extracted and resuspended in RNA loading buffer.<sup>23</sup>

*Polyacrylamide gel electrophoresis of vRNA.* The parental origins of reassortant RNA segments were routinely determined by polyacrylamide gel electrophoresis of vRNA at 38°C and 30°C. The differences in migration between ca B/AA/1/66 and wt B/HN/1732/76 RNA segments (particularly for RNA segment 3) were small, but consistently reproducible using this method. In addition, the inheritance of the SG3 and I-1-6 FL PA genes from ca B/AA/1/66 was verified by sequence analysis (data not shown).

Polyacrylamide gel electrophoresis was performed using mixed agarose-polyacrylamide gels as described by Odagiri *et al.*<sup>22</sup> Temperature control was accomplished by submerging the gels

in running buffer (1×TBE and 0.1% SDS) in an apparatus which was equilibrated in a heated 25 gallon fish tank. RNA samples were electrophoresed for 20 hours at a constant voltage of 240 V. The gels were dried and fluorographed as described previously.<sup>22</sup>

*Coding assignments of RNA segments 3–8.* The coding assignments of ca B/AA/1/66 and wt B/HN/1732/76 RNA segments 3–8 were determined by (1) observing corresponding inheritance of RNA segments and proteins; and (2) by electroblot transfer of electrophoresed vRNA followed by hybridization with cDNA synthesized using primers specific for RNA segments 4–8 of the B/Lee/40 influenza virus.

The mapping strategy of corresponding inheritance was described by Racaniello and Palese<sup>26</sup> and performed using standard techniques for protein electrophoresis.<sup>27,28</sup> During this analysis, reassortant II-47-4 was observed to possess a variant HA protein as demonstrated by the altered migration of its HA2 subunit (not shown). This variant arose during reassortment or during initial amplification of the plaque isolate, presumably as the result of random mutation(s). We have indicated this gene to be inherited from wt B/HN/1732/76 since reassortant II-47-4 was antigenically indistinguishable by hemagglutination inhibition.

The hybridization strategy was described by Dahlberg and Stellwag<sup>29</sup> and exploited previously established coding assignments<sup>26</sup> and sequence data<sup>30–34</sup> for B/Lee/40 influenza virus RNA segments. Primers were obtained from Dr Clayton Naeve, St Jude Children's Research Hospital, Memphis, TN. Complementary DNA was synthesized and labeled with [<sup>32</sup>P]-dATP (1000–1500 Ci/mole, NEN) in a double-volume 'A' sequencing reaction as described by DeBorde *et al.*<sup>35</sup> with the dideoxy-ATP being excluded. RNA segments 3–8 were found to respectively encode the PA, NP, HA, M and non-structural (NS) proteins<sup>36</sup> (and unpublished data) as indicated in Tables 1 and 2. The basic polymerase proteins, PB1 and PB2, were not assigned.

*Virus infectivity titration by plaque assay.* Plaque assays were performed essentially as described previously.<sup>37,38</sup> Monolayers of MDCK cells grown in 6- or 24-well plates were infected with 10-fold serial dilutions of virus. Each dilution was infected into four replicate wells. The inoculum was removed and the cells were overlaid with plaquing media composed of 1×L-15 media, 0.002% phenol red, 0.8% agarose and 5 µg/ml TPCK trypsin. Infected cultures were incubated in parallel at 33°C and 39°C for 4 days. On the second day after infection, a second overlay of plaquing medium containing 0.01% neutral red was added.

*Virus infectivity titration in embryonated eggs.* Ten- or eleven-day-old embryonated eggs were inoculated allantoically through a side-puncture with 0.1 ml of 10-fold serially diluted virus. Each dilution was inoculated into 4 or more eggs. The puncture was sealed with paraffin and the eggs were incubated in parallel at 25°C for 5 days and at 33°C for 3 days. Approximately 1 ml of allantoic fluid was harvested from each egg and the presence of virus was determined by hemagglutination of 0.5% chicken red blood cells. A 50% egg infectious dose (EID<sub>50</sub>) was calculated for each virus at 25°C and 33°C using the Spearman–Karber method.<sup>39</sup>

*Virulence assays in ferrets.* Neutered, male ferrets vaccinated against canine distemper (Marshall Research Farms, North Rose, NY) were used when 8–12 weeks old. Before infection, one third of the ferrets were screened for serum antibody against ca B/AA/1/66 and wt B/HN/1732/76 by hemagglutinin inhibition.<sup>40</sup> All ferrets tested demonstrated titers of less than 1:2.

Virus was administered intranasally to six ferrets at a dose of 6.5 log<sub>10</sub> pfu (MDCK, 33°C) in 1 ml. Three ferrets were sacrificed at 2 and 4 days after infection. The nasal turbinates and a portion of the lower right lobe of the lung were excised and prepared as 10% tissue suspensions in E-MEM medium as described previously.<sup>4</sup> The infectious titers of lung and turbinate suspensions were determined by plaque assay in MDCK cells at 33°C. Rectal temperatures were taken twice daily.

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