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A Mutation in the PA Protein Gene of Cold-Adapted B/Ann Arbor/1/66 Influenza Virus Associated with Reversion of Temperature Sensitivity and Attenuated Virulence

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Reassortant SG3 inherits only the acidic polymerase (PA) protein gene from the cold-adapted B/AA/1/66 influenza virus (ca B/AA/1/66) and all remaining genes from a virulent, wild-type virus. This reassortant demonstrates attenuated virulence in ferrets and expresses a ts phenotype characteristic of the ca parent. During virulence evaluation of SG3, a virulent, non-ts revertant virus (designated SG3rFL) was isolated from the lungs of one ferret. In order to determine whether the reversion of SG3 resulted from mutation of the PA gene and/or as the result of extragenic supressor mutations, the revertant PA gene of SG3rFL was transferred to a reassortant (SG3r) inheriting only the revertant PA gene from SG3rFL and all remaining genes from SG3. Reassortant SG3r was non-ts and virulent, indicating that mutation of the PA gene was sufficient for the reversion of the ts and attenuation phenotypes expressed by SG3rFL. The nucleotide and predicted amino acid sequences of the SG3rFL PA gene were determined and compared to those of wt and ca B/AA/1/66. The predicted PA proteins of wt and ca B/AA/1/66 are known to differ by six amino acid substitutions including a valine to methionine substitution at residue 431. The PA proteins of ca B/AA/1/66 and SG3rFL were distinguished by only the single amino acid substitution of methionine to isoluecine also occurring at residue 431. Thus, the methionine residue was implicated in the attenuation of ca B/AA/1/66 and its reassortants. The hydropathic properties of valine, isoleucine, and methionine suggested that reversion involved the restoration of hydrophobic character at this site. © 1988 Academic Press, Inc.

INTRODUCTION

The cold-adapted B/Ann Arbor/1/66 influenza virus (ca B/AA/1/66) was developed as a source of attenuating genes for the production of live, type B influenza virus vaccines (Maassab and DeBorde, 1985). 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. Preliminary investigations indicate that vaccine candidate strains derived from ca B/AA/1/66 express stable attenuation in ferrets and humans (Davenport *et al.*, 1977; Monto *et al.*, 1982; Reeve *et al.*, 1982). In addition to attenuation, ca B/AA/1/66 expresses temperature-sensitive (ts) and cold-adapted (ca) phenotypes which are also transferred to reassortant progeny through the inheritance of ca B/AA/1/66 genes. A recent analysis of reassortant viruses has demonstrated that inheritance of the PA gene from ca B/AA/1/66 is necessary for the acquisition of the ts phenotype and is primarily but, not entirely, responsible for the expression of attenuated virulence in ferrets (Donabedian *et al.*, 1987).

Sequence analysis of ca B/AA/1/66 and its progenitor wild-type virus has revealed 105 nucleotide substitutions encoding 26 predicted amino acid substitutions to be present in the six "internal" protein genes of the ca virus. Of these, 17 nucleotide substitutions encoding six predicted amino acid substitutions reside in the ca B/AA/1/66 PA gene (DeBorde *et al.*, 1988). We are interested in identifying those substitutions in ca B/AA/1/66 which are responsible for the acquisition of temperature sensitivity and attenuated virulence in order to (1) investigate the potential stability of attenuation; (2) describe the underlying molecular basis for the expression of attenuated virulence; and (3) identify unambiguous markers of attenuation to be used in screening vaccine candidate strains.

The isolation of SG3rFL,[2] a virulent, non-ts revertant virus, was reported previously (Donabedian *et al.*, 1987). This virus was recovered from a ferret infected with SG3, a temperature-sensitive and attenuated

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[2] For convenience, reassortants are identified by an identification (ID) code which indicates those RNA segments (genes) inherited from ca B/AA/1/66 and/or the revertant virus. The two-letter prefixes SG and MG stand for single gene and multiple gene reassortant. For example, SG3 refers to the reassortant inheriting only RNA 3 (PA gene) from ca B/AA/1/66 and all remaining genes from wt B/HN/1732/76. The two-letter suffix FL modifies this scheme by indicating the inheritance of all other genes from the revertant virus rather than wt B/HN/1732/76. Finally, the revertant PA gene has been designated as '3r' to distinguish it from the ca B/AA/1/66 PA gene.

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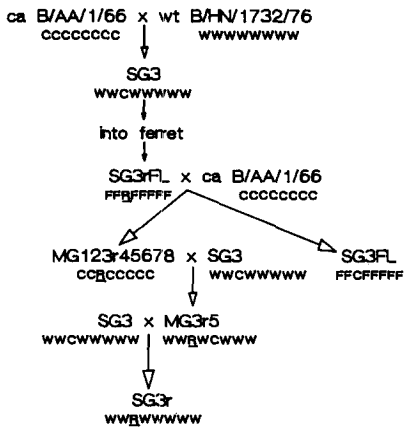

FIG. 1. Summary of reassortment. This flow diagram summarizes the derivation of SG3, the isolation of the revertant virus SG3rFL, and the strategy used to segregate and transfer the revertant PA gene (*R*) to SG3. The gene composition of each reassortant is shown. The letter F indicates the wild-type genes derived from the ferret isolate which potentially bear suppressor mutations.

reassortant which inherits only the PA gene from ca B/AA/1/66 and all remaining genes from the virulent wild-type influenza virus, B/Houston/1732/76 (wt B/HN/1732/76). Polyacrylamide gel electrophoresis of both SG3 and SG3rFL vRNAs at different temperatures revealed variant migration of only the PA and HA genes of SG3rFL (Donabedian *et al.*, 1987). Since the ca B/AA/1/66 PA gene is instrumental in the expression of the ts and attenuation phenotypes, this observation suggested that reversion of SG3 resulted from mutation of its PA gene.

The present report describes reassortment and sequence analysis of the SG3rFL revertant PA gene. In conjunction with the sequence determination of the wt and ca B/AA/1/66 PA genes (DeBorde *et al.*, 1988), these investigations have discounted the significance of extragenic suppression and allowed the identification of an attenuating lesion in the ca B/AA/1/66 PA protein.

MATERIALS AND METHODS

Viruses and cells

Influenza B virus reassortant SG3 was produced by reassortment of ca B/AA/1/66 and wt B/HN/1732/76 as described previously (Donabedian *et al.*, 1987). The virulent, non-ts revertant virus SG3rFL was recovered from the lungs of one ferret infected with SG3 (Donabedian *et al.*, 1987).

The preparation, maintenance, and infection of Madin–Darby canine kidney (MDCK) cells (Flow laboratories, Rockville, MD) and primary chick kidney (PCK) cells were described previously. (Maassab, 1969; Odagiri *et al.*, 1982; Maassab and DeBorde, 1983). Except where otherwise stated, all media used to propagate virus in MDCK cells contained 1 μg/ml L-(tosylamido 2-phenyl)ethyl chloroethyl ketone (TPCK) trypsin (Worthington Biochemicals, Bedford, MA).

Reassortment and the determination of reassortant gene composition

Ten culture tubes of PCK cells were infected with 3 PFU/ml of both SG3rFL and ca B/AA/1/66 in 0.3 ml. After adsorption for 1 hr at room temperature, the inoculum was replaced with 1 ml of 2× Eagle's medium and incubation was continued for 2 days at 33°. Viral progeny were passed undiluted once in the presence of ferret antiserum (hemagglutination/inhibition titer = 1:80) raised against wt B/HN/1732/76. The virus in each tube from the second passage was amplified in eggs and their gene compositions were determined by polyacrylamide gel electrophoresis of vRNA. This pro-

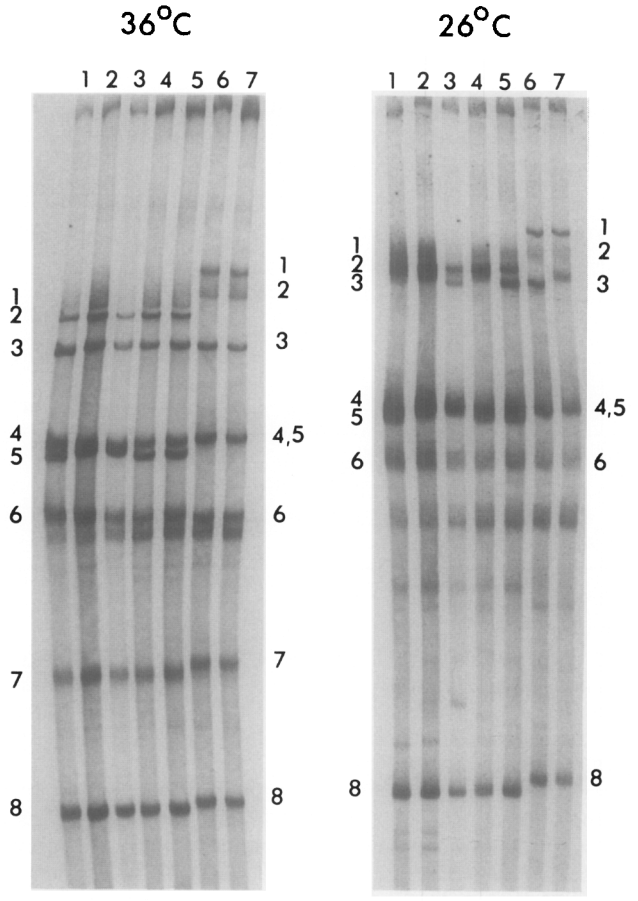

FIG. 2. Reassortant gene compositions. [^{3}H]uridine-labeled vRNA was subjected to electrophoresis in mixed polyacrylamide gels without stacks at 36 and 26°. Lane 1, wt B/HN/1732/76. Lane 2, SG3FL. Lane 3, SG3rFL. Lane 4, SG3. Lane 5, SG3r. Lane 6, MG123r45678. Lane 7, ca B/AA/1/66.

cedure yielded a reassortant (MG123r45678) which inherited only the revertant PA gene from SG3rFL and all remaining genes from ca B/AA/1/66.

The transfer of the revertant PA gene to reassortant SG3 required two successive matings in MDCK cells using antiserum against ca B/AA/1/66 as described above. Initially, reassortants MG123r45678 and SG3 were mated to yield a reassortant (MG3r5) inheriting only the revertant PA and HA genes from MG123r45678 and all remaining genes from SG3 and subsequently, reassortants MG3r5 and SG3 were mated to yield a single gene reassortant (SG3r) inheriting only the revertant PA gene and all remaining genes from SG3. At each stage, the appropriate reassortants were purified by one plaque to plaque purification in MDCK cells at 33° and amplified in eggs. The gene compositions of reassortants were verified after plaque purification.

Preparation and polyacrylamide gel electrophoresis of [^{3}H]uridine-labeled vRNA

[^{3}H]uridine-labeled vRNA was prepared from viruses grown in PCK cells as described previously (Donabedian *et al.*, 1987). Virus pelleted from infected tissue culture media was resuspended in 300 μl of 0.3 *M* sodium acetate, pH 6.0, and incubated at 37° for 10 min in the presence of 500 μg/ml proteinase K. SDS was added to 0.5% and incubation was continued for 10 min. Viral RNA was precipitated overnight at −20° in 2.5 vol of 95% ethanol.

The parental origin of reassortant vRNA segments was determined by observing differential electrophoretic migration of labeled vRNA. Polyacrylamide gel electrophoresis was performed at 36–38° and 26° using mixed agarose–polyacrylamide gels as described previously (Donabedian *et al.*, 1987) except that, in some instances, the stacking portion of the gel was omitted. The differences in migration between ca B/AA/1/66 and wt B/HN/1732/76 RNA segments were small, but consistently reproducible using this method. In addition, the origin of the PA gene was verified by sequence analysis after each reassortment experiment.

Virus infectivity titration and virulence assays

The titration of virus infectivity by plaque assay in MDCK cells and the evaluation of virulence in ferrets were performed exactly as described previously (Donabedian *et al.*, 1987).

Nucleotide sequence determination

The nucleotide sequences of the SG3, SG3rFl, MG123r45678, and SG3r PA genes were determined by a combination of three sequencing techniques. The first 40 nucleotides of the vRNA 3′ termini of the SG3rFL PA gene was sequenced by the direct chemical method described by Peattie (1979). RNA ligase end-labeling with [^{32}P]cytidine bis-phosphate was performed as described by DeBorde *et al.* (1988). The 3′

TABLE 1

RELATIVE INFECTIOUS TITERS (PFU/ml) OF REASSORTANTS AT 33 AND 39° [a]

Virus	Gene segment origin [b] 1	2	PA 3	NP 4	HA 5	NA 6	M 7	NS 8	ID code	Mean infectious titer (log_{10} PFU/ml ± SE) at 33°	Mean log_{10} reduction of infectious titers (PFU/ml ± SE[c]) at 39° vs 33°	Phenotype[d]
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
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	Cv	W	Wv	W	W	W	SG3rFL	7.4 ± 0.2	0.95 ± 0.3	Non-ts
VIII-2-2	C	C	Cv	C	C	C	C	C	MG123r45678	8.4 ± 0.1	>4.0	ts
XVI-17-2	W	W	Cv	W	W	W	W	W	SG3r	8.6 ± 0.2	1.4 ± 0.2	Non-ts
XI-13-I[e]	W	W	C	W	Wv	W	W	W	SG3FL	8.2 ± 0.02	>4.0	ts

[a] The infectious titers of SG3r, MG123r45678, and SG3FL are compared with those previously reported for their parental strains (Donabedian *et al.* 1987). The values shown were determined from three or more experiments. SE, sample error.

[b] Gene segment origin is indicated as follows: C, RNA segment is originally derived from ca B/AA/1/66; W, RNA segment is originally derived from wt B/HN/1732/76; Cv and Wv, variant genes of the revertant virus isolated from ferret lungs. Gene coding assignments were determined as described by Donabedian *et al.* (1987).

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

[d] As with the cold-adapted parent, reassortants that demonstrate no infectious titer at 39° are temperature-sensitive (ts).

[e] All genes except the PA gene are inherited from SG3rFL.

termini of the SG3, MG123r45678, and SG3r PA genes were determined by dideoxynucleotide chain termination sequencing of poly(A)$^+$ PA RNA using a [γ-32P]ATP-labeled oligonucleotide primer specific for nucleotides 85–100. This technique, designed for the sequencing of low abundance poly(A)$^+$ RNAs, was described by Geliebter *et al.* (1986). The remaining nucleotides were determined by dideoxynucleotide chain termination sequencing as described by DeBorde *et al.* (1986) and incorporating terminal deoxynucleotidyl transferase enzyme to resolve all ambiguities.

RESULTS

Derivation of reassortants inheriting the revertant PA gene

The reversion of SG3 resulted from either mutation of the PA gene and/or extragenic suppressor mutations. Reassortment was employed, essentially, to replace the PA gene of SG3 with the revertant PA gene of SG3rFL in order to distinguish among these alternatives. In so doing, the revertant PA gene was segregated from potential extragenic suppressor mutations (i.e., the remainder of the SG3rFL genome). Since only the PA and HA genes of SG3 and SG3rFL could be distinguished by electrophoresis, it was first necessary to transfer the revertant PA gene to a carrier virus, all of whose genes could be distinguished from those of SG3. For this purpose, a mating of SG3rFL and ca B/AA/1/66 was performed to derive MG123r45678. The subsequent transfer of the revertant PA gene required two successive matings with SG3. This reassortant strategy is summarized in Fig. 1. On the basis of polyacrylamide gel electrophoresis, the resulting reassortant (SG3r) was genetically equivalent to SG3 except for the difference detected in their PA genes. The gene compositions of these reassortants at 36° and 26° are shown in Fig. 2.

Phenotypic characterization of reassortants

The ts properties in MDCK cells of SG3r and its parental strains are shown in Table 1. As with ca B/AA/1/66, reassortants that demonstrate no infectious titer at 39° express the ts phenotype. In contrast to SG3, SG3r failed to express the ts phenotype. Rather, SG3r demonstrated a $\log_{10}$ reduction of infectious titers at 39° as compared to 33° similar to that of SG3rFL and wt B/HN/1732/76. This result established that mutation of the PA gene was sufficient for the reversion of the ts phenotype expressed by SG3rFL. Mutation of the PA gene was also necessary for such reversion since expression of the ts phenotype by SG3FL was not suppressed. SG3FL inherits only the PA gene from ca B/AA/1/66 and all remaining genes from SG3rFL. Thus, although the presence of extragenic suppressor mutations was not strictly excluded, these data demonstrated that such mutations would not be sufficient for reversion of the ts phenotype.

To determine whether mutation of the PA gene was associated with reversion to virulence, SG3r was adminstered to ferrets (Table 2). Virulence was evaluated on the basis of fever and viral replication in the nasal turbinates and lungs. As with SG3rFL and wt B/HN/1732/76, SG3r grew well in the turbinates, induced a significant febrile response, and was isolated from the lungs at a similar frequency. Thus, mutation of the PA gene was sufficient for the reversion to virulence expressed by SG3rFL.

The ts and attenuation properties of the carrier virus (MG123r45678) were determined to investigate the effect of the revertant PA gene when inherited in combination with other ca B/AA/1/66 genes. This reassortant inherits the revertant PA gene from SG3rFL and all remaining genes from ca B/AA/1/66. Like ca B/AA/1/66, MG123r45678 expressed the ts and attenuation phenotypes (Tables 1 and 2). Reassortant analysis indicates that inheritance of the ca B/AA/1/66 PA gene is required for the expression of the ts phenotype and attenuation equivalent to ca B/AA/1/66 (Donabedian *et al.,* 1987). Since the revertant PA gene lost the ability to confer these properties to a single gene reassortant (i.e., SG3r), this result suggested that the revertant PA gene retained one or more ts and attenuating mutations which were phenotypically expressed only in combination with other ca B/AA/1/66 genes.

Sequence analysis of the revertant PA gene

To identify the mutation(s) in the PA gene associated with phenotypic reversion, the complete nucleotide sequence of the SG3rFL PA gene was determined and compared with that of ca B/AA/1/66. Nucleotides 2–2306 of the SG3, MG123r45678, and SG3r PA genes were also sequenced to verify the identity of these genes.

The revertant PA genes of SG3rFL, MG123r45678, and SG3r were distinguished from the PA genes of ca B/AA/1/66 and SG3 by only two nucleotide substitutions (Fig. 3). As indicated in Table 3, the substitution of uracil with cytosine at nucleotide 1886 did not alter the predicted amino acid sequence of the revertant PA protein. However, the substitution of guanine with adenine at nucleotide 1322 resulted in the predicted amino acid substitution of methionine in the PA protein of ca B/AA/1/66 with isoleucine in the revertant PA protein at residue 431. There was no obvious reason

to conclude that a nucleotide substitution at a silent position in the body of the PA gene would be phenotypically expressed. Thus, sequence analysis suggested that the single predicted amino acid substitution of methionine with isoleucine occurring at residue 431 in the PA gene of SG3 was responsible for its loss of the ts phenotype and the corresponding reversion to virulence.

DISCUSSION

Inheritance of the PA gene from ca B/AA/1/66 confers the ts phenotype and a significant degree of attenuated virulence to reassortant progeny using wt B/HN/1732/76 as the other parent (Donabedian *et al.*, 1987). The reversion of these phenotypes in a reassortant (SG3) inheriting only the PA gene from ca B/AA/1/66 was shown to result from mutation of this gene rather than as the result of extragenic suppression. The possibility that extragenic suppression was responsible for reversion was discounted by the inability of the revertant PA gene to confer the ts and attenuation phenoytpes in a genetic background equivalent to that of SG3. It is unlikely that SG3r possesses a "new" extragenic suppressor mutation since reassortment and biological cloning were performed at 33°, a permissive temperature providing no selective advantage to non-ts progeny. Furthermore, polyacrylamide gel electrophoresis revealed no differences in migration of SG3 and SG3r gene segments apart from the PA gene.

The predicted amino acid sequence of the ca B/AA/1/66 PA protein is distinguished from that of the wt B/AA/1/66 PA protein by six amino acid substitutions, including a valine to methionine substitution at residue 431 (DeBorde *et al.*, 1988). At the protein level, reversion of the ts and attenuation phenotypes corresponded with the substitution of this methionine residue with isoleucine (Table 3). The loss of attenuation which resulted from this substitution established the involvement of the methionine residue at position 431 in the attenuation of ca B/AA/1/66 and its reassortants. In addition, this result demonstrated the absolute genetic correlation of attenuation and expression of the ts phenotype conferred by the PA protein gene of ca B/AA/1/66.

We interpret the expression of the ts and attenuation phenotypes by MG123r45678 as evidence for the presence of at least one additional attenuating mutation in the revertant PA gene (see Results). Such mutations would be expressed only in combination with one or more other ca B/AA/1/66 genes. Alternatively, it is possible that, in combination with other ca B/AA/1/66 genes, the isoleucine residue at position 431 in the

TABLE 2

VIRULENCE EVALUATION IN FERRETS[a]

Virus	Gene segment origin[b] 1 PA	2 NP	3 HA	4 NA	5 M	6 NS	7	8	ID code	Mean peak temp. ±SE (no. with fever ≥103.4°F/no. infected)	*Lung* No. positive/No. infected Day 2	Day 4	*Turbinate* mean $\log_{10}$ PFU/ml ± SE Day 2	Day 4
ca B/AA/1/66	C	C	C	C	C	C	C	C		103.1 ± 0.6* (1/6)	0/3	0/3	3.7 ± 0.4	2.9 ± 0.2
VIII-2-2[a]	C	C	Cv	C	C	C	C	C	MG123r45678	103.0 ± 0.8* (1/6)	0/3	0/3	4.8 ± 0.3	4.7 ± 0.04
I-1-6	W	W	C	W	W	W	W	W	SG3	102.6 ± 0.9* (1/6)	1/3	0/3	5.4 ± 0.4	4.6 ± 0.6
I-1-6 FL	W	W	Cv	W	Wv	W	W	W	SG3rFL	103.9 ± 0.2 (6/6)	1/3	1/3	6.9 ± 0.3	6.0 ± 0.4
XVI-17-2	W	W	Cv	W	W	W	W	W	SG3r	104.1 ± 0.2 (6/6)	2/3	1/3	5.9 ± 0.07	5.2 ± 0.08
wt B/HN/1732/76	W	W	W	W	W	W	W	W		103.8 ± 0.2 (12/12)	2/6	2/6	6.1 ± 0.2	5.4 ± 0.1

[a] The virulence of SG3r and MG123r45678 is compared with that previously reported for their parental strains (Donabedian *et al.*, 1987). Ferrets were inoculated intranasally with 6.5 $\log_{10}$ PFU except for those infected with MG123r45678 which received 7.1 $\log_{10}$ PFU. Values were determined from 10% lung and turbinate suspensions titrated in MDCK cells. SE, sample error.

[b] Gene segment origin is indicated as follows: C, RNA segment is originally derived from ca B/AA/1/66; W, RNA segment is originally derived from wt B/HN/1732/76; Cv and Wv, variant genes of ts-revertant virus isolated from ferret lung. Gene coding assignments were determined as described by Donabedian *et al.*, 1987).

* Mean differs significantly ($P < 0.01$) from wt B/HN/1732/76 by Student's *t* test.

TABLE 3

NUCLEOTIDE AND AMINO ACID SUBSTITUTIONS IN THE REVERTANT PA GENE COMPARED WITH THE wt AND ca B/AA/1/66 PA GENES

wt B/AA/1/66	Nucleotide	^{1317}CCC	GUG	GAG	. . . . .	GGG	ACU	CAA^{1889}
	Amino acid	$_{430}$Pro	Val	Gln	. . . . .	Gly	Thr	Gln$_{620}$
ca B/AA/1/66		CCC	AUG	GAG	. . . . .	GGG	ACU	CAA
		Pro	Met	GLN	. . . . .	Gly	Thr	Gln
SG3rFL		CCC	AUA	CAG	. . . . .	GGG	ACC	CAA
		Pro	Ile	Gln	. . . . .	Gly	Thr	Gln

Note. Sequence data for wt and ca B/AA/1/66 are from DeBorde *et al.* (1988).

revertant PA gene is responsible for expression of the ts and attenuation phenotypes by MG123r45678.

Ramig and Fields (1979) described three categories of mutation resulting in the phenotypic reversion of reovirus ts mutants. These are (1) true reversion, in which mutation restores the wild-type sequence and hence, phenotype; (2) pseudoreversion by intragenic suppression, in which a second mutation, occurring in the same gene as the original mutation, restores the wild phenotype; and (3) pseudoreversion by extragenic suppression, in which a second mutation in a different gene restores the wild phenotype. By this scheme, pseudorevertants retain the original mutation (in a suppressed state) and consequently, are genetically distinguishable from true revertants. The present investigation provides a specific example of reversion requiring a more detailed explanation.

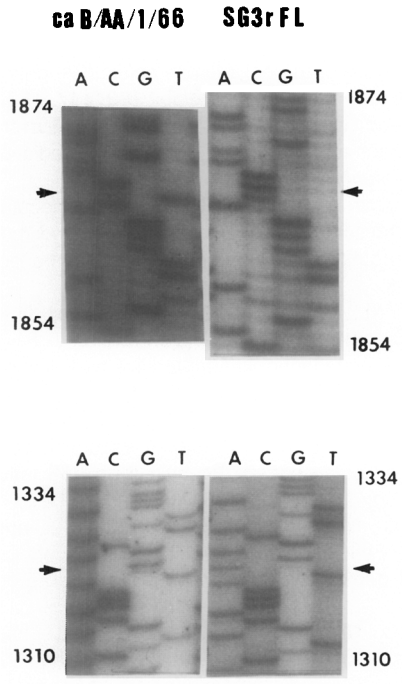

FIG. 3. Nucleotide sequence comparison of the ca B/AA/1/66 and SG3rFL PA genes. Arrows indicate the two nucleotide substitutions, adenosine for guanine at position 1322 and cytosine for uracil at position 1886 found in the SG3rFL PA gene.

As discussed, genetic analysis demonstrated that reversion of SG3 did not result from extragenic suppression. Since the revertant PA protein retains five of the six amino acid substitutions which distinguish the wt and ca B/AA/1/66 PA proteins (DeBorde *et al.*, 1988), the substitution of methionine with isoleucine may have resulted in the intragenic suppression of an attenuating lesion constituted by one or more of these other substitutions. Alternatively, the methionine residue at position 431 of the ca B/AA/1/66 PA protein may constitute the actual attenuating lesion. At the nucleotide level, reversion was associated with a guanine to adenine nucleotide substitution at position 1322 which modified the coding significance of the original adenine to guanine nucleotide substitution at position 1320 of the ca B/AA/1/66 PA gene (Table 3). Thus, although the revertant PA gene retained the original mutation, reversion corresponded with the substitution of an amino acid at the same position as the original mutation. This substitution did not restore the original amino acid sequence, but may have led to a condition analogous to true reversion.

Sequence analysis of the PA genes of wt B/HN/1732/76 and wt B/Singapore/222/79, as well as wt B/AA/1/66, has revealed valine at amino acid residue 431 (DeBorde *et al.*, 1988; Akoto-Amianfu *et al.*, 1987). Since wt B/AA/1/66, wt B/HN/1732/76, and SG3rFL all fail to express the ts phenotype and are virulent, we reason that the isoleucine residue of SG3rFL and the valine residue of wild-type isolates share some property which allows the PA proteins of these viruses to function efficiently at supraoptimal temperatures. As described by Kyte and Doolittle (1982) the water-vapor free energies and interior–exterior distribution of amino acid side chains of valine and isoleucine are sufficiently similar to warrant assignment of the two highest hydropathy index values (4.2 and 4.5) to these residues. By these criteria, methio-

nine is somewhat less hydrophobic (index value = 1.9). The effects of these differences are illustrated in Fig. 4. Hydropathy plots representing the region of the wt B/AA/1/66 and revertant PA proteins around amino acid 431 are almost superimposable and both deviate from that of ca B/AA/1/66. Thus, we speculate that reversion may involve the restoration of hydrophobic character at this site.

Extragenic suppression is thought to occur through compensating alterations of a protein that is in physical contact with the product of the gene bearing the original mutation (Jarvik and Botstein, 1975). By analogy, intragenic suppression may be thought to occur through compensating alterations at one location of a given protein that is in physical contact with a different location, bearing the original mutation, of the same protein. In this case, whether or not the revertant PA gene was intragenically suppressed, the mutation involving residue 431 identified a region of functional significance in the PA protein of influenza B viruses.

The three polymerase proteins of influenza B viruses are presumed to be the functional analogs of the influenza A polymerase proteins. The PA protein of influenza A viruses may be directly involved only in synthesis of the viral RNA genome (Krug *et al.*, 1975; Braam *et al.*, 1983); however, no specific function has yet been attributed to the PA protein. Interestingly, position 431 falls near the COOH-terminal end of a short homologous region in the PA proteins of influenza A and B viruses. Residues 421–432 of the wt B/AA/1/66 PA protein and residues 425–436 of the A/NT/60/68 PA protein are 75% homologous whereas, homology between these two proteins is 38% overall (DeBorde *et al.*, 1987).

Stable attenuation is a necessary component of a suitable live vaccine candidate. Since mutation is essentially a random event, it is impossible to ensure completely stable attenuation. However, the probability that mutation will result in a reversion to virulence is greatly diminished by the accumulation of attenuating mutations. It follows that the degree of stability can be estimated by determining the number of attenuating mutations present in a given virus (Scholtissek and Rott, 1984). Thus, in the absence of procedures to incorporate site-directed mutations into viable negative-strand viruses, the isolation and analysis of revertants provides a useful method to investigate virulence and its attenuation at the level of protein structure.

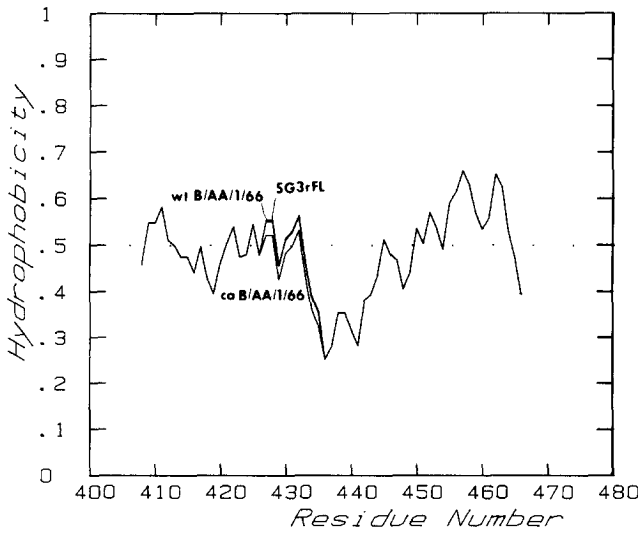

FIG. 4. Effect of amino acid substitution on the hydropathy of the PA protein around residue 431. The wt B/AA/1/66, ca B/AA/1/66, and SG3rFL nucleotide sequences, respectively, predict valine, methionine, and isoleucine at residue 431. Hydropathy plots were generated using the MSEQ computer program (Black and Glorioso, 1986). Points above the centerline represent hydrophobic residues. Plots for wt and ca B/AA/1/66 were from the sequence data of DeBorde *et al.* (1988).

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REFERENCES

AKOTO-AMANFU, E., SIVASUBRAMANIAN, N., and NAYAK, D. P. (1987). Primary structure of the polymerase acidic (PA) gene of an influenza B virus (B/Sing/222/79). *Virology* **159,** 147–153.

BLACK, S. D., and GLORIOSO, J. C. (1986). MSEQ: A microcomputer-based approach to the analysis, display, and prediction of protein structure. *Biotechniques* **4,** 448–460.

BRAAM, J., ULMANEN, I., and KRUG, R. M. (1983). Molecular model of a eukaryotic transcription complex: Functions and movements of influenza P proteins during capped RNA-primed transcription. *Cell* **34,** 609–618.

DAVENPORT, F. M., HENNESSEY, A. V., MAASSAB, H. F., MINUSE, E., CLARK, L. C., ABRAMS, G. D., and MITCHELL, J. R. (1977). Pilot studies on recombinant cold-adapted live type A and B influenza virus vaccines. *J. Infect. Dis.* **136,** 17–25.

DEBORDE, D. C., DONABEDIAN, A. M., HERLOCHER, M. L., NAEVE, C. W., and MAASSAB, H. F. (1988). A sequence comparison of wild-type and cold-adapted B/Ann Arbor/1/66 influenza virus genes. *Virology* **163,** 429–443.

DEBORDE, D. C., NAEVE, C. W., HERLOCHER, M. L., and MAASSAB, H. F. (1986). Resolution of a common RNA sequencing ambiguity by terminal deoxynucleotidyl transferase *Anal. Biochem.* **157,** 275–282.

DEBORDE, D. C., NAEVE, C. W., HERLOCHER, M. L., and MAASSAB, H. F. (1987). Nucleotide sequences of the PA and PB1 genes of B/Ann/Arbor/1/66 virus: Comparison with genes of B/Lee/40 and type A influenza viruses. *Virus Res.* **8,** 33–41.

DONABEDIAN, A. M., DEBORDE, D. C., and MAASSAB, H. F. (1987). Genetics of cold-adapted B/Ann Arbor/1/66 influenza virus reassortants: The acidic polymerase (PA) protein gene confers tem-

perature sensitivity and attenuated virulence. *Microbiol. Pathol.* **3,** 97–108.

GELIEBTER, J., ZEFF, R. A., MELVOLD, R. W., and NATHENSON, S. G. (1986). Mitotic recombination in germ cells generated two major histocompatability complex mutant genes shown to be identical by RNA sequence analysis: K^{bm9} and K^{bm6}. *Proc. Natl. Acad. Sci. USA* **83,** 3371–3375.

JARVIK, J., and BOTSTEIN, D. (1975). Conditional-lethal mutations that suppress genetic defects in morphogenesis by altering structural proteins. *Proc. Natl. Acad. Sci. USA* **72,** 2738–2742.

KRUG, R. M., UEDA, M., and PALESE, P. (1975). Temperature-sensitive mutants of influenza WSN virus defective in virus-specific RNA synthesis. *J. Virol.* **16,** 790–796.

KYTE, J., and DOOLITTLE, R. F. (1982). A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **157,** 105–132.

MAASSAB, H. F (1969). Immunologic characteristics of cold-adapted influenza virus. *J. Immunol.* **102,** 728–732.

MAASSAB, H. F., and DEBORDE, D. C. (1983). Characterization of an influenza host range mutant. *Virology* **119,** 82–95.

MAASSAB, H. F., and DEBORDE, D. C. (1985). Development and characterization of cold-adapted viruses for use as live virus vaccines. *Vaccine* **3,** 355–369.

MONTO, A. S., MILLER, F. D., and MAASSAB, H. F. (1982). Evaluation of an attenuated, cold-recombinant influenza B virus vaccine. *J. Infect. Dis.* **145,** 57–64.

ODAGIRI, T., DEBORDE, D. C., and MAASSAB, H. F. (1982). Cold-adapted recombinants of influenza A viruses in MDCK cells. I. Development and characterization of A/Ann Arbor/6/60 × A/Alaska/6/77 recombinant viruses. *Virology* **119,** 82–95.

PEATTIE, D. A. (1979). Direct chemical method for sequencing RNA. *Proc. Natl. Acad. Sci. USA* **76,** 1760–1764.

RAMIG, R. F., and FIELDS, B. N. (1979). Revertants of temperature-sensitive mutants of reovirus: Evidence for frequent extragenic suppression. *Virology* **92,** 155–167.

REEVE, P., PIBERMANN, M., BACHMAYER, H., LIEHL, E., MORITZ, A., GANZINGER, U., HOFMANN, H., and KUNZ, C. (1982). Studies in man with a cold-recombinant live influenza B virus vaccine. *J. Med. Virol.* **92,** 1–9.

SCHOLTISSEK, C., and ROTT, R. (1984). Correlation between the loss of the temperature-sensitive phenotype and pathogenicity of fowl plague virus mutants in the chicken. *Virus Res.* **1,** 117–131.