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Genetic Variants of Influenza A/Taiwan/1/86 Cocirculating in Canada during the Winter of 1986 to 1987

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The first isolate of influenza virus in Canada during the winter of 1986 to 1987 was a genetic variant of A/Taiwan/1/86. This genetic variant type was the predominant strain obtained from several of the western provinces. The variant strains were antigenically indistinguishable from A/Taiwan/1/86 but were remarkably distinct by T1 oligonucleotide mapping. T1 mapping of individual genome segments indicated that the variants evolved from an A/Taiwan/1/86-like virus through the accumulation of point mutation or deletion or insertion events and probably do not contain foreign genes. The relative distribution of genetic variation was approximately equal among the individual genes, with the possible exception of segments 1 or 2 that were analyzed in combination and thus could not be individually associated with the observed variation.

Given that the molecular genetic basis for influenza virus pathogenicity (ability to cause disease) is largely unknown (19, 21), it is presently not possible to predict disease or epidemic potential from viral structure or properties (11), although some advances have been made in this area. Surveillance for the identification of antigenic variants is the basis for monitoring influenza epidemic potential, since antigenic variation is a prerequisite for the epidemic emergence of antigenically novel strains.

Sequence analysis of the HA1 portion of the hemagglutinins of unusual variants of H1 (17) and H3 (2) subtypes has demonstrated a consistent alteration in amino acids in positions 188 to 193, providing some predictive value in the detection of amino acid changes in this region (11). This region has been identified as one of the four major antigenic sites of hemagglutinin (3, 24). Advancements have been achieved with respect to the rapid identification of antigenic variants; however, the a priori interpretation of such data with respect to epidemic potential cannot be done without relying on further analysis of isolates to identify those that are emerging as the most successful strains.

Instances of large genomic change have been associated with major epidemic activity and are thus relevant to predicting epidemic potential. Recently, extensive genomic variation detected in viruses of the A/Brazil/11/78 strain was shown to be due to reassortment with H3N2 viruses (1, 25). These A/California/10/78-like viruses became epidemically prevalent in the absence of antigenic variation (14). More recently, the extensively genetically altered A/Chile/1/83 strain has emerged to become epidemically active (11).

This paper describes the occurrence of a novel genetic type of A/Taiwan/1/86 that was prevalent in Canada in the winter of 1986 to 1987. This type of variant was remarkable, not only because of its extensive genetic variation but also because of its antigenic conservation, being indistinguishable from the prototype by hemagglutination inhibition assay. The genetic structure of these viruses was analyzed and compared to ascertain whether reassortment was the basis of its variation. T1 mapping of individual genome segments indicated that these viruses had likely evolved from an A/Taiwan/1/86-like predecessor without the introduction of foreign genes from H3N2 strains.

MATERIALS AND METHODS

Viruses. Human isolates of A/Taiwan/1/86-like strains (28 isolates) were provided by Canadian public health laboratories in the winter of 1986 to 1987. Isolates of A/Taiwan/1/86 were sent from all the provinces west of, and including, Quebec. The early isolates that were subjected to molecular analysis are shown in Table 1. All the isolates were from geographically distinct locations and thus were more likely to represent distinct outbreaks.

The following H1N1 prototype strains were obtained from the reference collection of the Influenza Section, Laboratory Centre for Disease Control, Ottawa, Ontario, Canada: A/Brazil/11/78, A/England/333/80, A/Victoria/7/83, A/Dunedin/27/83, A/Chile/1/83, and A/Taiwan/1/86, as well as the H3N2 strains A/Caen/1/84 and A/Mississippi/1/85. Viruses were grown in the allantoic cavities of chicken embryos; incubation was for 48 h at 34°C.

[^{35}S]Methionine labeling of protein. Confluent monolayers of Madin Darby canine kidney cells in 35-mm-diameter dishes were washed twice with phosphate-buffered saline before inoculation with 0.2 ml of stock virus to produce a multiplicity of infection of 10 to 100. Adsorption was for 0.5 h at 34°C in a 5% CO_2 atmosphere. Cultures were overlaid with Eagle minimum essential medium containing Earle salts supplemented with penicillin (final concentration, 100 U/ml) and streptomycin (final concentration, 100 μg/ml) and incubated at 34°C for 12 h before labeling. Phosphate-buffered saline-washed (twice) infected cells were pulse-labeled for 0.5 h with 0.5 ml of L-methionine-free minimum essential medium supplemented with 20 μCi of [^{35}S]methionine (1,500 Ci/mmol).

SDS-PAGE and Cleveland peptide mapping. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method of Laemmli (12) was used; gels were made with an acrylamide-*N*,*N*'-methylene bisacrylamide ratio of 75:1. Partial proteolysis for the purpose of peptide mapping was carried out by the method of Cleveland et al. (4). The NS1 protein (nonstructural protein 1) and M1 protein (matrix protein 1) comigrated on SDS-PAGE for specific strains. To obtain these proteins in purified form, preparative gels were made with an acrylamide-*N*,*N*'-methylene bisacrylamide ratio of 18.75:1 to separate the NS1 and M1 proteins (8). Papain was used for mapping M1 and NS1 proteins; three

TABLE 1. Histories of A/Taiwan/1/86-like isolates that were molecularly characterized

Isolate	Patient age (yr)	Collection date	Place of origin
RV150-86	31	25 November 1986	Silvan Lake, Alberta, Canada
RV4-87	36	17 December 1986	Toronto, Ontario, Canada
RV6-87	23	21 December 1986	Wiarton, Ontario, Canada
RV8-87	1	29 December 1986	Ottawa, Ontario, Canada
RV9-87	13	10 December 1986	Regina, Saskatchewan, Canada
RV10-87	7	10 December 1986	Prince Albert, Saskatchewan, Canada

quantities of enzyme were used: 250, 25, and 2.5 ng. *Staphylococcus aureus* V8 protease was used to map NP (nucleocapsid) proteins; three quantities of enzyme were used: 400, 40, and 4 ng. NP proteins were mapped on 15% acrylamide gels, whereas the M1 and NS1 proteins were mapped on 17.5% acrylamide gels.

^{32}P labeling and electrophoresis of RNA. Madin Darby canine kidney cells were infected as described above for methionine labeling, except that monolayers in 60-mm-diameter dishes were washed with normal saline after infection and 1 mCi of carrier-free ^{32}P orthophosphoric acid was applied in 1 ml of phosphate-free minimum essential medium. At 4 h postinfection, a further 4 ml of phosphate-free medium was added, and infection was allowed to proceed overnight. The virus was purified by absorption to guinea pig erythrocytes as described by Cowley et al. (5). The viral RNA was phenol extracted twice, ethanol precipitated, and then suspended in sample buffer containing 4 M urea, 0.1% SDS, 0.1% bromphenol blue, and 15% glycerol. Samples were heated in a boiling water bath for 20 s, followed by quenching on ice. Electrophoresis was done at 20°C for 16 h at 120 V in gels composed of 4.5% acrylamide, 0.06% *N,N'*-methylene bisacrylamide, 125 mM Tris hydrochloride (pH 8.0), 6 M urea, 0.4% SDS, 0.005% (vol/vol) TEMED (*N,N,N',N'*-tetramethylethylenediamine), and 0.005% ammonium persulfate. The pH 8.9 tank buffer consisted of 26 mM Tris base, 26 mM glycine, and 0.05% SDS.

RNase T1 oligonucleotide mapping of genomic RNA. The method of Pederson and Haseltine (16) was used with slight modification. Calf alkaline phosphatase treatment was omitted, since it was unnecessary, given the 5' sequence of influenza virus RNA (20). Virus was purified by two cycles of centrifugation through sucrose step gradients composed of three zones, 30, 50, and 60%. The viral RNA was obtained from virus by protease K and SDS treatment, followed by two phenol-chloroform extractions, one chloroform extraction, and precipitation with ethanol (2.5 volumes) and 3 M sodium acetate (pH 5.4) (0.1 volume) as described by Maniatis et al. (13). For each T1 map, 2 μg of RNA was used.

The analysis of genome segments involved the isolation of segments in a polyacrylamide gel, followed by elution as described by Jeppessen et al. (10) Genomic RNA was subjected to electrophoresis as described above, except that the buffer was 42 mM Tris hydrochloride (pH 6.8). RNA bands were detected by staining in 2 μg of ethidium bromide per ml, excised, extruded through a 22-gauge needle into 3 ml of buffer; elution occurred by diffusion during incubation at 37°C overnight. RNA was phenol extracted before T1 oligonucleotide mapping was done.

Hemagglutination inhibition test. The hemagglutination inhibition test was done in micro-assay using 96-well microdilution plates. The assay was done as previously described (23). Nonspecific inhibitors were inactivated by neuraminidase (*Vibrio cholerae*) and heat (56°C for 0.5 h) treatment.

RESULTS

All of the 28 influenza A viruses submitted to the Influenza Section, Laboratory Centre for Disease Control, for reference analysis in 1986 to 1987 were serologically indistinguishable from A/Taiwan/1/86 by hemagglutination inhibition assay with fowl antisera; this was confirmed for several of these isolates by the WHO Collaborating Center for Reference and Research on Influenza, Centers for Disease Control, Atlanta, Ga., with ferret antisera that were more discriminatory than fowl antisera (data not shown).

Oligonucleotide mapping was performed on the genomic RNA of several of the early A/Taiwan/1/86 isolates obtained from separate locations (see Materials and Methods). All isolates possessed distinct oligonucleotide maps; however, the isolates were not homogeneous in their similarity to the reference strain, making up two groups on the basis of pattern (Fig. 1). Isolates were either similar to the prototype, possessing the expected number of oligonucleotide changes (approximately 6/year), such as RV4-87, RV6-87, and RV8-87 (that had 5 to 8 changes), or were very distinct from the

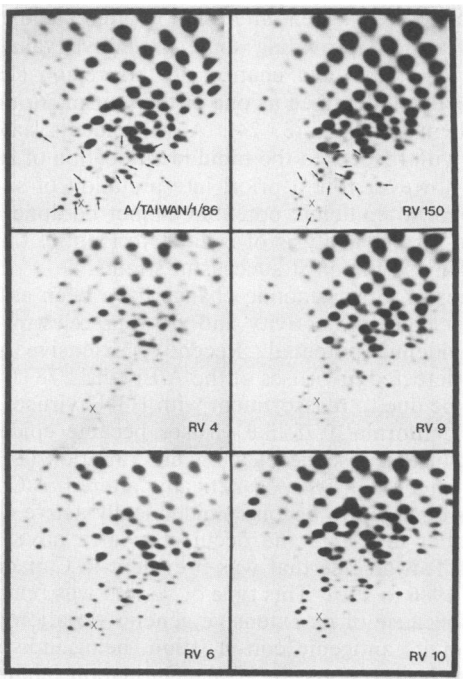

FIG. 1. Oligonucleotide maps of A/Taiwan/1/86 and five Canadian isolates from the winter of 1986 to 1987. The position of the xylene cyanol marker dye is indicated (×). For the RV150-86 and A/Taiwan/1/86 maps, the oligonucleotides that differ for each of these two maps, relative to each other, are indicated by arrows. Abbreviations: RV 150, RV150-86; RV 4, RV4-87; RV 9, RV9-87; RV 6, RV6-87; RV 10, RV10-87.

TABLE 2. Quantitative comparison of differences in RNase T1-generated oligonucleotides of A/Taiwan/1/86-like isolates versus H1N1 and H3N2 prototype strains

Virus strain	No. of oligonucleotide differences	
	A/Taiwan/1/86	RV150-86
A/Taiwan/1/86	0	31
RV4-87	8	34
RV6-87	5	36
RV8-87	6	37
RV150-86	31	0
RV9-87	34	5
RV10-87	32	3
A/Chile/1/83	>50	>50
A/Mississippi/1/85	>50	>50

prototype strain possessing 31 to 34 oligonucleotide differences, such as RV150-86, RV9-87, and RV10-87 (Table 2). The first genetic variant isolate was RV150-86, collected on 25 November 1986. The variant strains were homogeneous, possessing similar oligonucleotide maps with few changes. Oligonucleotide map patterns of other reference strains of the H1N1 subtype (A/Brazil/11/78, A/England/333/80, A/Victoria/7/83, A/Dunedin/27/83, and A/Chile/1/83) were much more dissimilar to RV150-86 (>50 changes) than was A/Taiwan/1/86 (data not shown). Similarly, the H3N2 strains A/Caen/1/84, and A/Mississippi/1/85 were very dissimilar from RV150-86 (data not shown).

RNA and protein electrophoresis. The preceding oligonucleotide mapping analysis indicated that specific A/Taiwan/1/86 strains were genetically altered, relative to other cocirculating strains that were more prototypelike. Electrophoresis of genomic RNA and protein was done to assess whether specific portions of the genome or specific viral proteins were detectably changed. The following viruses were analyzed: A/Taiwan/1/86; A/Mississippi/1/85; prototype-like strains RV4-87 and RV6-87; variant-like strains RV150-86, RV9-87, and RV10-87.

The RNA segments of prototypelike strains were indistinguishable from A/Taiwan/1/86 by electrophoresis (Fig. 2). The genetic variant types were all distinguishable from the prototype by RNA electrophoresis, possessing segments 6, 7, and 8 of lesser mobility. The variants RV9-87 and RV10-87 were distinguishable from RV150-86 on the basis of electrophoretic mobility of segments 1 and 2; segments 1 and 2 comigrated for RV150-86 but migrated as a doublet for RV9-87 and RV10-87.

The variant-type viruses all produced the same protein profiles on electrophoresis. The profiles of variant-type virsues were distinct from the prototype as a result of altered mobility of the NS1 protein (Fig. 3). The M1 and NS1 proteins of A/Taiwan/1/86 comigrate. The prototypelike viruses possessed polypeptides that were indistinguishable from A/Taiwan/1/86, except for RV4-87 that possessed a novel protein subjacent to NS2 (nonstructural protein 2) (data not shown).

Oligonucleotide map comparison of individual genome segments. The genetic variants of A/Taiwan/1/86 were the result of either point mutation, reassortment, or a combination of these processes. If the A/Taiwan/1/86 variants are reassortants, they will possess novel genes derived from other influenza A viruses. The only other subtype of influenza A virus that was cocirculating in humans between 1985 and 1986 was H3N2, of which the then current strain was A/Mississippi/1/85. To test the possibility that RV150-86 was a genetic reassortant of A/Taiwan/1/86 and an H3N2 strain, such as A/Mississippi/1/85, the individual genome segments of these three strains were compared by oligonucleotide mapping. Genomic RNA was fractionated by PAGE. All segments were resolved, except for segments 1 and 2. The purified segments, as well as a mixture of segments 1 and 2, were subjected to oligonucleotide mapping and compared to determine the proportion of common oligonucleotides (Fig. 4; Table 3). Only the largest oligonucleotides in each pattern

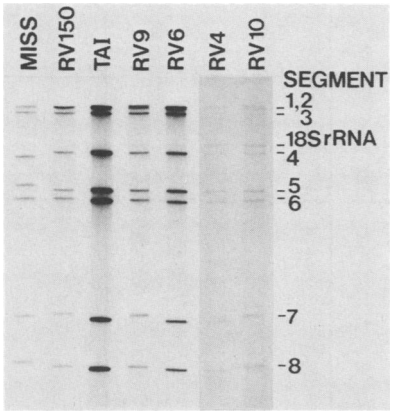

FIG. 2. PAGE of genomic RNA from A/Taiwan/1/86, A/Mississippi/1/85, and clinical isolates. Abbreviations: MISS, A/Mississippi/1/85; RV150, RV150-86; TAI, A/Taiwan/1/86; RV9, RV9-87; RV6, RV6-87; RV4, RV4-87; RV10, RV10-87. Viral RNA was labeled with ^{32}P and analyzed on 4.5% acrylamide gels. The segment numbers and the 18S rRNA are indicated to the right of the gel.

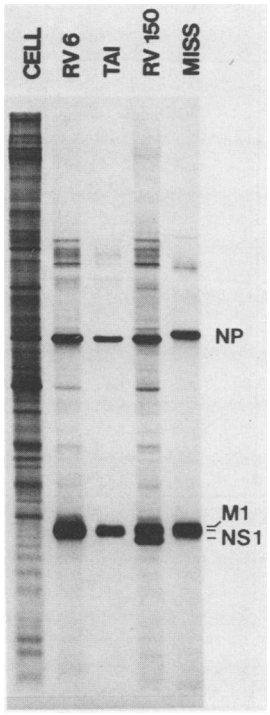

FIG. 3. SDS-PAGE of [^{35}S]methionine-labeled infected-cell proteins. Abbreviations: CELL, uninfected cell control; RV6, RV6-87; TAI, A/Taiwan/1/86; RV 150, RV150-86; MISS, A/Mississippi/1/85. The positions of the NP and M1 proteins and the alternate positions of NS1 protein are indicated.

TABLE 3. Occurrence of RV150-86 oligonucleotides in individual genome segments of A/Taiwan/1/86 and A/Mississippi/1/85

Genome segment (protein)[a]	No. of oligonucleotides[b]	No. (%) of nucleotides in common with:	
		A/Taiwan/1/86	A/Mississippi/1/85
1+2 (PB2+PB1)	30	17 (57)	8 (27)
3 (PA)	15	12 (80)	3 (20)
4 (HA)	28	17 (61)	2 (7)
5 (NP)	19	14 (74)	8 (42)
6 (NA)	17	11 (65)	3 (18)
8 (M1, M2)	9	7 (78)	2 (22)
8 (NS1, NS2)	17	13 (76)	4 (24)

[a] The proteins coded by the individual segments are indicated. Abbreviations: PB2, basic transcriptase associated with protein 2; PB1, basic transcriptase associated with protein 1; PA, acidic transcriptase-associated protein; HA, hemagglutinin; NP, nucleocapsid protein; NA, neuraminidase; M1, matrix protein 1; M2, matrix protein 2; NS1, nonstructural protein 1; NS2, nonstructural protein 2.

[b] Represents the size of the subset, the group of largest oligonucleotides, of each RV150-86 pattern that was compared.

were compared. On analysis of the resulting oligonucleotide maps, it was observed that the segment 3 preparations contained significant quantities of oligonucleotides from the adjacent segments 1 and 2. It was therefore necessary to subtract this background before making comparisons; the spots specific for segment 3 of RV150 are indicated by arrows in Fig. 4.

All of the RV150 genome segments shared the majority of their large oligonucleotides with the corresponding A/Taiwan/-1/86 segments and possessed fewer common oligonucleotides with A/Mississippi/1/85. The altered oligonucleotides were distributed among all of the genome segments, although this cannot be said unequivocally with respect to segments 1 and 2, since they were analyzed as a mixture. The individual genome segments possessed a relatively uniform proportion of mutation, as would be expected from silent mutation rates (9). The segment 1 and 2 combination was more variant relative to the prototype than any other individual segment. Since the distribution of variation cannot be discerned between segments 1 and 2, it is not clear whether one or both of these segments account for the observed variation. Either both segments are equally variant or one or other of these segments is extensively variant, as a result of reassortment or random mutation. Comparison of segments 1 and 2 from RV150 with those of A/Mississippi/1/85 indicates that if one of these segments is a foreign gene, it did not originate from an A/Mississippi/1/85-like virus, since this segment combination was less similar than the A/Taiwan/1/86 combination. Hayashida et al. (9) showed that segment 2 was the most conserved amino acid sequence but the most varied nucleotide sequence; the latter finding was thought to be anomalous by the authors and possibly due to genetic exchange. It is possible that segment 2 is responsible for most of the variation detected for the combination of segment 1 and 2 detected by T1 mapping.

Peptide mapping. Peptide mapping by limited proteolysis and SDS-PAGE fractionation of NP, M1, and NS1 proteins from A/Taiwan/1/86, A/Mississippi/1/85, RV150-86, and RV6-87 was done to assess the extent of missense mutation.

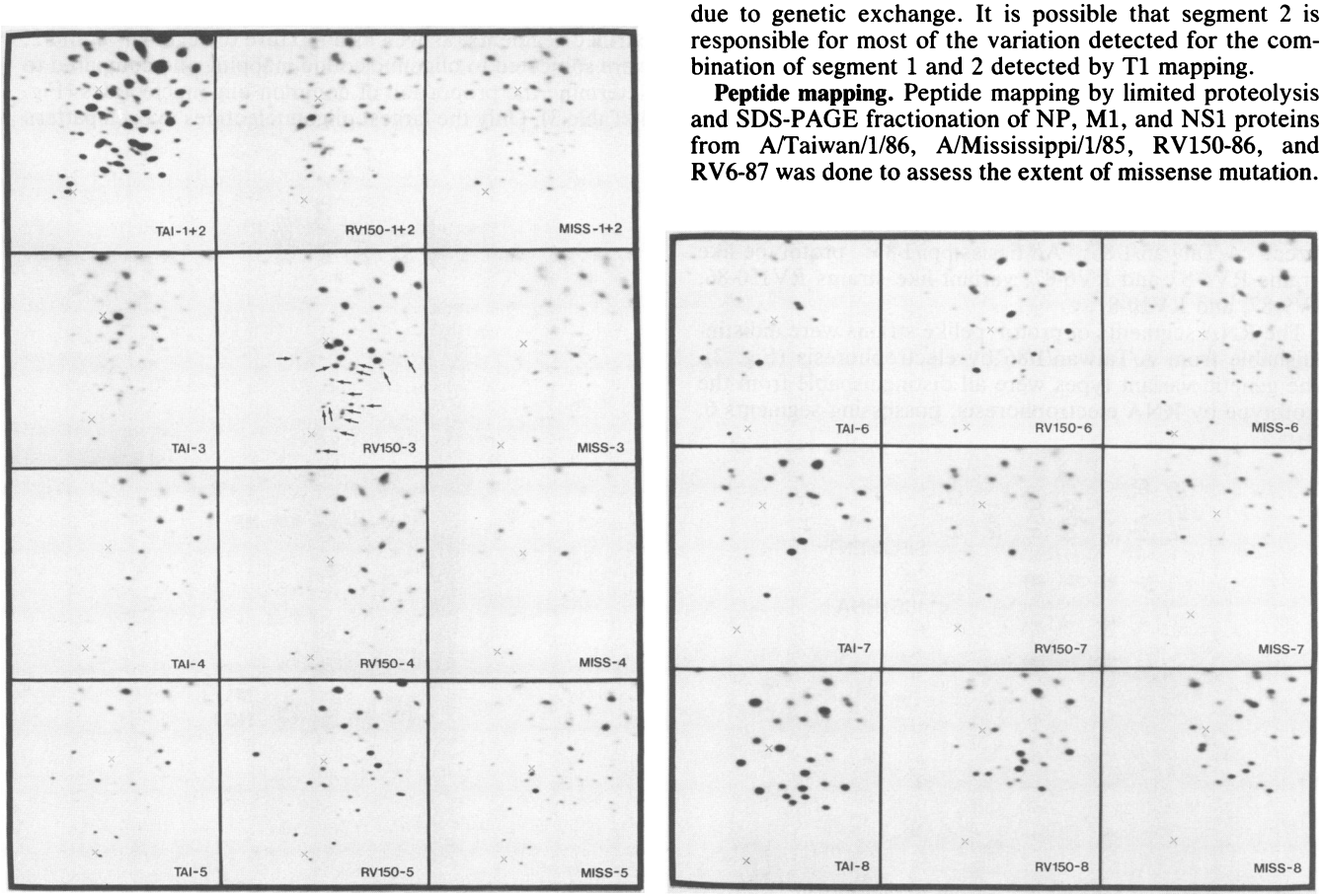

FIG. 4. Oligonucleotide maps of individual genome segments from A/Taiwan/1/86 (TAI), A/Mississippi/1/85 (MISS), and RV150-86 (RV150). The oligonucleotide maps are labeled with respect to the virus (before the hyphen) and genome segment number (after the hyphen). The positions of reference dyes are indicated (×); the xylene cyanol is the lower reference, and bromphenol blue is the upper reference. Since the segment 3 maps contain some background from segments 1 and 2 (1 + 2), the spots specific for segment 3 of the RV150-86 map are indicated by arrows.

These proteins were selected because of their abundance in infected cells. Papain was used to map NS1 and M1 proteins, whereas *S. aureus* V8 protease was used for mapping NP proteins; three concentrations of enzyme were used for each analysis (only the NS1 maps are shown). The peptide maps of the A/Mississippi/1/85 proteins were all distinct from A/Taiwan/1/86 and RV150-86 proteins, indicating further that the RV150-86 segments coding for these proteins did not come from A/Mississippi/1/85. The RV6-87 peptide maps were indistinguishable from the corresponding peptide maps of A/Taiwan/1/86. No detectable change was observed between RV150-86 and the prototype strain with respect to their M1 protein peptide maps, whereas very subtle changes were observed in the NP peptide maps and extensive pattern changes were observed for the NS1 proteins (Fig. 5).

The structural mapping of individual segments and proteins showed that RV150-86 did not possess any H3N2 genome segments from contemporary strains and indicates that RV150-86 probably acquired its extensive genetic changes relative to A/Taiwan/1/86 through random mutation.

DISCUSSION

This paper demonstrates the occurrence and characterization of extensively mutated genetic variants of A/Taiwan/1/86, typified by RV150-86, that were prevalent in Canada in the winter of 1986 to 1987. These genetic variants were antigenically indistinguishable from prototype A/Taiwan/1/86 by hemagglutination inhibition assay. Oligonucleotide map analysis of the hemagglutinin-coding genome segment clearly showed that mutations had altered at least eight oligonucleotides in this gene; however, it was not known whether the amino acid coding had been affected. Missense mutations in regions of the hemagglutinin that do not affect hemagglutination, either directly or indirectly, would be undetectable by hemagglutination assay. It is also possible that single epitope changes in the receptor portion of the hemagglutinin would be undetectable with immune sera (22).

The genetic variants were as distinct from the prototype as had been observed for reassortants of H1N1 that emerged in 1978 (A/California/10/78-like strains). A/California/10/78 was a reassortant between A/Brazil/11/78 H1N1 virus and an H3N2 virus (25). These reassortants became prevalent in the United States and Asia in 1979 (14). A/California/10/78 possessed four genome segments encoding internal genes from the H3N2 strain and was antigenically like A/Brazil strains. The T1 oligonucleotide maps of A/California/10/78 and A/Brazil/11/78 differed by 30 oligonucleotide spots (11). A distinct reassortant differing from A/California/10/78 by possessing a H3N2-derived segment 7 and an A/USSR/1/77-like hemagglutinin was also detected at this time (15). Oligonucleotide mapping of individual genome segments of RV150-86 indicated that the A/Taiwan/1/86 genetic variant (RV150-86) did not contain foreign genes derived from contemporary H3N2 strains and thus likely arose as a consequence of random mutation. It was also possible that genes from another H1N1 virus had been introduced into RV150-86 by reassortment. This however was not evident from T1 map comparison with several H1N1 prototype strains (A/Brazil/11/78, A/England/333/80, A/Victoria/7/83, A/Dunedin/27/83, and A/Chile/1/83), since the RV150-86 specific group of oligonucleotides (Fig. 1) was not present in the oligonucleotide maps of these H1N1 prototypes (data not shown). Questions remain as to the origin of such extensive mutation in these variants.

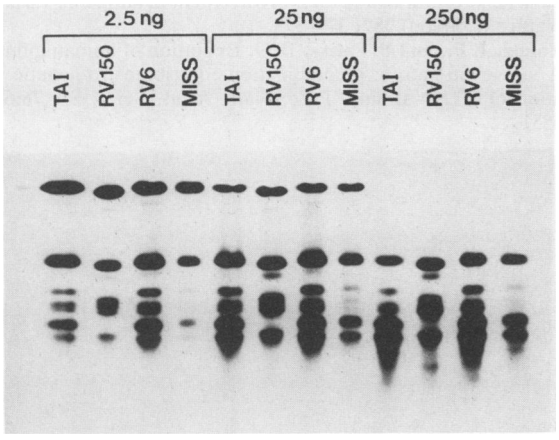

FIG. 5. V8 peptide maps of NS1 proteins from A/Taiwan/1/86, A/Mississippi/1/85, RV6, and RV150. Peptides were resolved in 17.5% acrylamide gels. The quantities of protease used are shown. Abbreviations: TAI, A/Taiwan/1/86; RV150, RV150-86; RV6, RV6-87; MISS, A/Mississippi/1/85.

Cocirculation of antigenic variants is not common but has been described in several instances (11). The cocirculation of genetic variants has been observed for reassortants of A/Brazil/11/78, but the means of detecting such variants is a relatively recent development and thus cocirculation of genetic variants may have gone undetected in the past.

RV150-86 possessed approximately five times the mutations as would be expected for an influenza virus that has evolved for 1 year. A possible explanation is that RV150-86-like strains diverged from the A/Taiwan/1/86 lineage 5 years ago after their common ancestor acquired the A/Taiwan/1/86 antigenic specificity and were undetected before 1986. The fact that A/Taiwan/1/86 is most similar to A/Hong Kong/2/82-like strains (17) indicates that A/Taiwan/1/86-like strains could have coevolved with RV150-86-like strains for the last several years. Alternatively, RV150-86 may have evolved at five times the normal rate as a result of a higher inherent capability to mutate such as is seen for mutator phenotypes in bacteria (6) or as occurs during persistent viral infection (7).

Genetic variants appeared to be common in Canada, particularly in the western provinces; each of the three isolates from Alberta and Saskatchewan that were subjected to oligonucleotide mapping were genetically variant strains. Fourteen of 28 A/Taiwan/1/86-like isolates sent to the Laboratory Centre for Disease Control for reference analysis possessed variant-like NS1 proteins when analyzed by SDS-PAGE. The numbers of total isolates possessing the variant-type protein profile obtained from the individual provinces, listed from east to west, were as follows: Quebec, 0 of 3; Ontario, 2 of 9; Manitoba, 1 of 1; Saskatchewan, 2 of 2; Alberta, 2 of 2; and British Columbia, 7 of 10 (data not shown). Since the variant is antigenically identical to the prototype, the consequence of its prevalence may not be greater than that of A/Taiwan/1/86; however, it is significant and somewhat compelling that these variants may possess a selective advantage since the variants have emerged from the A/Taiwan/1/86 virus population. The alternative possibility is that RV150-86 viruses do not have a selective advantage relative to A/Taiwan/1/86-like strains and that they have cocirculated at a constant relative frequency for the past year without detection; this possibility cannot presently be excluded but seems unlikely, given the extent of

molecular characterization currently used for influenza surveillance.

Mutants that are conserved must be considered to be of epidemiological significance. Since RV150-86 is antigenically indistinguishable from prototype A/Taiwan/1/86, there should be little consequence to its spread, since there is an A/Taiwan/1/86 vaccine program in place and the incidence of A/Taiwan/1/86-like virus is being monitored. It is possible that antigenic variants of RV150-86 would have increased epidemic potential relative to A/Taiwan/1/86 if such variants had an increased ability to spread, as well as antigenic novelty. Antigenic variation in RV150-86-like strains may be of greater relevance than in the prototype strains and should be monitored. Monitoring will require oligonucleotide mapping or electrophoresis of RNA or protein of A/Taiwan/1/86-like strains, in addition to the conventional serological analysis.

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