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Intragenic Suppression of a Deletion Mutation of the Nonstructural Gene of an Influenza A Virus

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The influenza A/Alaska/77 (H3N2) virus mutant 143-1 is temperature sensitive (*ts*) due to a spontaneous in-frame 36-nucleotide deletion in the nonstructural (NS) gene segment, which leads to a 12-amino-acid deletion in the NS1 protein. In addition, it has a small-plaque phenotype on MDCK cell monolayers. However, phenotypically revertant (i.e., ts^+) viruses were isolated readily following replication of the 143-1 virus both in vitro and in vivo. In order to determine the genetic mechanism by which escape from the *ts* phenotype occurred, we performed segregational analysis and found that an intrasegmental suppressor mutation caused the loss of the *ts* phenotype. Nucleotide sequence analysis revealed the presence of an intragenic mutation in each of the ts^+ phenotypic revertant viruses, involving a substitution of valine for alanine at amino acid 23 of the NS1 protein. This mutation resulted in acquisition of the ts^+ phenotype and also in the large-plaque phenotype on MDCK cells, characteristic of the wild-type A/Alaska/77 parent virus. This amino acid substitution is predicted to generate an area of alpha helix in the secondary structure of the amino-terminal portion of the NS1 protein of the revertant viruses which may compensate for loss of an alpha-helical region due to the deletion of amino acids 66 to 77 in the NS1 protein of the 143-1 virus.

The genome of influenza A virus consists of eight segments of single-stranded negative-sense RNA encoding at least 10 viral polypeptides (11). The NS gene segment is the smallest of these gene segments and encodes two polypeptides found in infected cells but not in purified virions, referred to as NS1 and NS2. Few temperature-sensitive (*ts*) or conditionally lethal mutations of the NS gene segment are available, and relatively little is known regarding the roles of the NS1 and NS2 proteins in influenza virus replication.

Recently, an influenza A virus with an unusual NS gene segment was isolated during reassortment between the cold-adapted (*ca*) influenza A/Ann Arbor/6/60 virus and the wild-type (*wt*) influenza A/Alaska/77 (H3N2) virus (13). The reassortant virus, denoted CR43-3, derived the hemagglutinin (HA) and neuraminidase (NA) gene segments from the influenza A/Alaska/77 virus and the PB1, PB2, PA, nucleoprotein (NP), and matrix protein (M) gene segments from the *ca* influenza A/Ann Arbor/60 virus, but the NS gene segment did not appear to be derived from either parent virus. Nucleotide sequence analysis subsequently demonstrated that the NS gene segment of the CR43-3 virus was derived from that of the A/Alaska/77 virus by an in-frame 36-nucleotide deletion, which led to a predicted deletion of amino acids 66 to 77 of the NS1 protein (2). The CR43-3 virus was *ts*, with a shutoff temperature for plaque formation of 37°C, and manifested a host range phenotype not seen with either parent virus, characterized by restricted replication in Madin-Darby canine kidney (MDCK) cells compared with replication in primary chick kidney (PCK) cells (13).

In order to evaluate specifically the contribution of the NS deletion to the phenotypes of the CR43-3 virus, a backcross mating of this virus with the A/Alaska/77 *wt* virus was performed (23). Reassortant virus 143-1 was isolated, which derived the NS gene segment bearing the deletion mutation from the CR43-3 virus and all other gene segments from the A/Alaska/77 *wt* virus. The 143-1 virus was *ts*, with a shutoff temperature for plaque formation of 37°C, but had only a partial-host-range phenotype, manifested by a reduction in the size of the plaques formed on MDCK cells. These phenotypes could also be transferred with the NS gene segment from the 143-1 virus to the *wt* influenza A/Bethesda/85 virus (23).

The significant degree of temperature sensitivity associated with the deletion in the NS gene segment of the 143-1 virus suggested that this gene segment could be used as a genetically stable component of a live attenuated influenza virus vaccine. Therefore, further studies to evaluate the 143-1 virus in animals with a 37°C body temperature were performed. However, the 143-1 virus replicated efficiently in hamsters and chimpanzees, suggesting that the *ts* phenotype of this virus was host dependent (23). In addition, viruses which had recovered the ability to grow in MDCK cells at 40°C (i.e., ts^+ viruses) were isolated from these animals, suggesting that the defect in replication imposed by the NS deletion could be compensated for, most likely by one or more second-site mutations (23). Analysis of such mutations may provide insight into the structural requirements for NS gene function or into possible interactions with other gene products. Therefore, in the current study, we determined the nucleotide sequence of the NS gene segment of the 143-1 virus and analyzed the mechanisms by which the *ts* phenotype of the 143-1 virus was lost during in vivo and in vitro passage. The results of these studies suggest that the *ts* and plaque-size phenotypes of the 143-1 virus can be directly attributed to the deletion in the NS gene and that loss of these phenotypes can occur by intragenic suppression by a point mutation.

MATERIALS AND METHODS

Viruses and cells. The isolation, biological cloning, and characterization of the *wt* influenza A/Alaska/77 (H3N2) and A/Bethesda/85 (H3N2) viruses and of the 143-1 virus con-

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taining the NS gene segment bearing the deletion mutation have been described previously (21, 23). The *ts* virus C1332-8 was isolated from the nasopharynx of seronegative chimpanzee 1332 on the eighth day following intratracheal inoculation with the 143-1 virus. The ts^+ virus C1331-5 was isolated from the nasopharynx of seronegative chimpanzee 1331 on the fifth day following intratracheal inoculation with the 143-1 virus (23). The ts^+ virus H2-7 was isolated from nasal turbinates of a cyclophosphamide-treated hamster on the seventh day following intranasal inoculation with the 143-1 virus (23). All viruses were isolated on MDCK cells (23). Each of these viruses was biologically cloned by plaque-to-plaque passage in MDCK cells at 33°C and expanded by a single passage in 9-day-old embryonated hen eggs.

The 143-1 virus was also subjected to serial passage in MDCK cells at gradually increasing temperatures. The 143-1 virus was first biologically cloned by plaque-to-plaque passage and grown in MDCK cells at 33°C. This seed pool was then serially passaged in a MDCK monolayer culture with a fluid overlay at 38, 39, and 40°C. Cell culture supernatants from wells inoculated with the highest dilution of virus that induced cytopathic effect at the highest temperature were used for the next passage until a virus which grew equally well at 40°C as at 33°C was isolated after four passages. This virus, denoted P4-40 (passage 4 at 40°C), was then biologically cloned by plaque-to-plaque passage in MDCK cells at 40°C and studied further. As a control, a parallel series of passages of the 143-1 virus at 33°C were also performed.

Efficiency of plaque formation. The level of temperature sensitivity of the wild-type viruses, the 143-1 virus, and the revertant viruses was determined by plaque assay on MDCK cells as described previously (19). The shutoff temperature for plaque formation was defined as the lowest temperature at which a greater than 100-fold reduction in plaque titer relative to that at 33°C occurred. Viruses with a shutoff temperature of 39°C or less were considered *ts*.

Genetic reassortment. A backcross of each of the ts^+ isolates of the 143-1 virus with the A/Bethesda/85 *wt* virus was performed by previously described methods (19, 23). Briefly, confluent MDCK monolayers were coinfected with the A/Bethesda/85 virus and either the 143-1 virus or one of the ts^+ isolates at a multiplicity of infection of approximately 5 for each virus. After a 24-h incubation at 33°C, the cell culture supernatant was plaqued on MDCK cells at 33°C. Individual reassortant plaques were picked, and the parental origin of the viral gene segments was determined as described below. Reassortant viruses deriving the NS gene segment from the 143-1 parent virus or the ts^+ derivative were further plaque passaged and then tested for the temperature-sensitive phenotype as described above. Reassortant viruses exhibiting a shutoff temperature of 39°C or less were considered temperature sensitive.

Genotype analysis. Individual virus plaques were inoculated directly into 9-day-old embryonated hen eggs. Viral RNA was purified from allantoic fluid, and the genotype of each reassortant virus was determined by comparison of the migration of the viral RNA gene segments with those of the parent virus RNA gene segments on 2.6% polyacrylamide gels containing 4.5 or 6 M urea after silver staining of the gels, as described previously (26).

Nucleotide sequence analysis. The nucleotide sequences of the NS gene segments of the A/Alaska/77 *wt* virus, the *ts* 143-1 virus, and the ts^+ isolates were determined by direct dideoxynucleotide sequencing of cDNA copies of the NS gene segment generated by polymerase chain reaction (PCR) (10). Briefly, approximately 1 μg of purified virus RNA and the synthetic DNA primer 5′-AGCAAAAGCAGG were heated to 95°C for 1 min, annealed on ice, and added to a first-strand cDNA synthesis reaction mix containing avian myeloblastosis virus reverse transcriptase (Life Sciences, St. Petersburg, Fla.) in PCR buffer (50 mM KCl, 50 mM Tris [pH 8.4], 25 mM $MgCl_2$, 100 μg of bovine serum albumin per ml) with 1.25 mM each deoxynucleoside triphosphate. Following a 1-h incubation at 42°C, the products were added directly to a PCR reaction mix also containing the reverse primer 5′-AGTAGAAACAAGG and 2 U of *Taq* polymerase (Cetus Corp., Norwalk, Ohio). The double-stranded DNA product corresponding to the NS gene segment was purified by polyacrylamide gel electrophoresis and sequenced directly with Sequenase version 2.0 (US Biochemical, Cleveland, Ohio) following the manufacturer's instructions except for the addition of dimethyl sulfoxide at the annealing step (29). Oligonucleotide primers for PCR and nucleotide sequencing reactions were obtained from Genetic Designs, Houston, Tex. The nucleotide sequence of both strands of the cDNAs was determined. Nucleotide sequence analysis and peptide secondary-structure predictions were done with the programs of the University of Wisconsin genetics group (9) on a VAX computer.

RESULTS

Characterization of revertant viruses. In a previous study, phenotypically revertant viruses were isolated from animals experimentally infected with the 143-1 virus (23). In order to determine the mechanism by which these viruses escaped from the temperature-sensitive phenotype specified by the NS1 deletion mutation, three of the viruses isolated from animals were selected for further study and their shutoff temperature was determined after biological cloning. These results are shown in Table 1. Virus C1332-8, isolated after 8 days of replication in a chimpanzee, manifested significantly restricted replication at 39°C. In contrast, virus C1331-5, isolated after 5 days of replication in a chimpanzee, and virus H2-7, isolated after 7 days of replication in an immunosuppressed hamster, were able to form plaques as efficiently at 40°C as at 33°C, a phenotype clearly different from that of the 143-1 virus administered to these animals. We also subjected the 143-1 virus to selective pressure by serial passage of this virus in MDCK cells at progressively increasing temperatures, as described in Materials and Methods. The ability of the viruses isolated at each passage level to form plaques at elevated temperatures is also shown in Table 1. A marked change in shutoff temperature was observed between passage levels 2 and 3, at which point the virus recovered the ability to replicate efficiently at 40°C. As a control, the 143-1 virus was also passaged in a parallel series of titrations in MDCK cells at 33°C. The virus recovered after the fourth such passage showed no change in its level of temperature sensitivity.

The 143-1 virus also manifested a partial-host-range phenotype, characterized by a small-plaque phenotype on MDCK cells at the permissive temperature of 33°C. The plaque morphology on MDCK cells at 33°C of the viruses analyzed in this study is shown in Fig. 1. The 143-1 virus exhibited a small-plaque morphology on MDCK cells, while the A/Alaska/77 *wt* virus formed large, clear plaques on these cells. The H2-7, C1331-5, and P4-40 viruses also formed large, clear plaques on MDCK cells, similar to those of the A/Alaska/77 virus, while the *ts* virus C1332-8 mani-

TABLE 1. Efficiency of plaque formation at permissive and nonpermissive temperatures of the A/Alaska/77 and 143-1 viruses and of viruses isolated after in vivo and in vitro replication of the 143-1 virus

Virus	Passage history of 143-1	Reduction in plaque titer (log_{10}) at indicated temp vs titer at 33°C			Shutoff temp (°C) for plaque formation[a]
		38°C	39°C	40°C	
A/Alaska/77	NA[b]	0.00	0.00	0.63	≥41
143-1	NA	>3.00	>4.00	>4.00	≤38
C1332-8	8 days in chimpanzee 1332	0.93	>3.00	>4.00	≤39
C1331-5	5 days in chimpanzee 1331	0.00	0.00	0.78	≥41
H2-7	7 days in hamster 2	0.09	0.20	1.00	≥41
P1-38	MDCK cells at 38°C (P1)[c]	>2.00	>3.00	>3.00	≤38
P2-39	39°C passage of P1	1.16	>3.00	>3.00	39
P3-39	39°C passage of P2	0.35	0.61	1.70	≥41
P4-40	40°C passage of P3	0.50	0.20	0.27	≥41
P4-33	4 passages at 33°C	2.30	>3.00	>3.00	≤38

[a] Shutoff temperature is defined as the lowest temperature at which a greater than 100-fold reduction in plaque titer occurs relative to the plaque titer at 33°C.
[b] NA, not applicable.
[c] P1, passage 1.

fested a small-plaque phenotype on these cells, similar to that of the 143-1 virus.

Genetic analysis. In order to determine the genetic mechanism by which the phenotypic revertants escaped from the *ts* phenotype, we generated reassortants between the 143-1 virus or the ts^+ phenotypic revertant viruses and the A/Bethesda/85 (H3N2) *wt* virus. The A/Bethesda/85 virus was chosen as the *wt* virus parent because it replicated efficiently at 40°C and each of its gene segments could be readily distinguished from those of the revertant viruses by polyacrylamide gel electrophoresis (data not shown). The genotype of the reassortant progeny viruses was then determined, and reassortant viruses which derived the NS gene segment from the 143-1 virus or one of the revertant viruses were plaque purified and tested for the *ts* phenotype. Reassortant viruses exhibiting a shutoff temperature of 39°C or less were considered *ts*. Under these circumstances, reassortant viruses which derive the NS gene segment from the

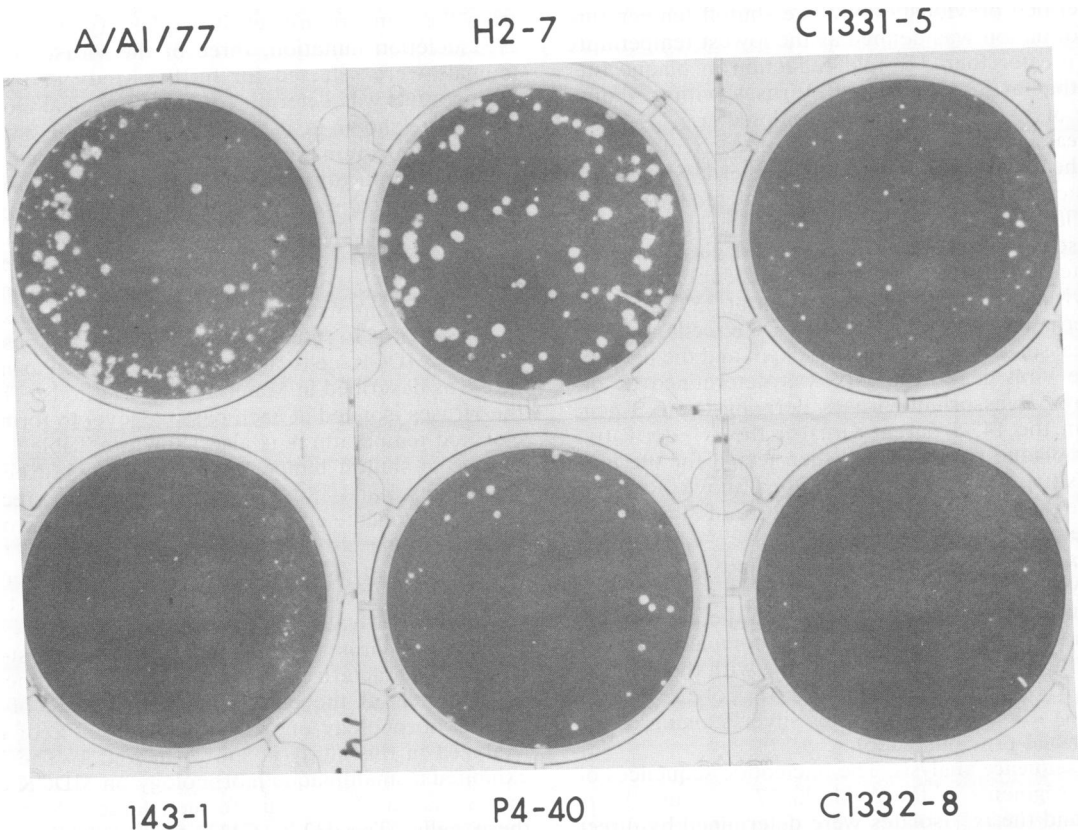

FIG. 1. Plaque morphology of the A/Alaska/77, 143-1, H2-7, P4-40, C1331-5, and C1332-8 viruses. Biologically cloned viruses were grown on MDCK cells under an agarose overlay for 72 h at 33°C. Cells were fixed with Formalin and stained with crystal violet.

TABLE 2. Results of reassortment between the 143-1 virus and the A/Bethesda/85 virus

Reassortant	Origin[a] of gene segment: 1	2	3	4	5	6	7	8	Phenotype
8	B	A	A	B	B	A	A	A	*ts*
9	B	B	A	B	B	B	B	A	*ts*
14	A	A	A	A	B	A	B	A	*ts*
18	A	A	B	B	B	A	A	A	*ts*
23	B	A	B	B	A	B	A	A	*ts*
27	A	A	A	A	A	A	B	A	*ts*
33	A	A	A	B	B	A	A	A	*ts*

[a] A, gene segment derived from the 143-1 virus; B, gene segment derived from the A/Bethesda/85 virus.

143-1 virus should be *ts*. If an extrasegmental suppressor mutation were present in the ts^+ revertant viruses, then reassortant viruses which derive the NS gene segment from a revertant virus but in which the gene segment bearing the suppressor mutation is replaced by the corresponding gene segment of the A/Bethesda/85 virus should also be *ts*. Alternatively, if the mechanism of reversion were intrasegmental suppression, or true reversion (unlikely in the case of a large deletion mutation), then each reassortant virus deriving the NS gene segment from a ts^+ revertant virus should be ts^+ irrespective of the A/Bethesda/85 genes present in the reassortant.

The results of this analysis are shown in Tables 2 through 5. As expected, viruses which received the NS gene segment from the 143-1 virus and a variety of other gene segments from the A/Bethesda/85 virus were *ts* (Table 2). Each reassortant virus which received the NS gene segment from the A/Bethesda/85 virus was ts^+ (data not shown). This confirms the ability of the NS gene segment of the 143-1 virus to transfer the *ts* phenotype to the A/Bethesda/85 virus (23).

In contrast, the NS gene segments of the C1331-5, H2-7, and p4-40 viruses were unable to transfer the *ts* phenotype to the A/Bethesda/85 virus (Tables 3 through 5). In each case, reassortant viruses which derived the NS gene segment from one of the revertant viruses together with one or more gene segments of the A/Bethesda/85 virus retained the ability to replicate efficiently at 39°C. Each of the A/Bethesda/85 virus genes (except the NS gene) was present in one or more of these reassortants. In addition, the NS gene segment present in the ts^+ revertant viruses was identical to that of the 143-1 virus by polyacrylamide gel electrophoresis, indicating that true reversion had not occurred. These results suggest that an intrasegmental suppressor mutation was responsible for the loss of the *ts* phenotype by the ts^+ phenotypic revertant viruses C1331-5, H2-7, and P4-40.

Nucleotide sequence analysis. To determine the sequence of the intrasegmental suppressor mutation, we next determined the nucleotide sequences of the NS gene segments of the A/Alaska/77, 143-1, C1331-5, H2-7, and P4-40 viruses. Because multiple attempts to sequence the RNA of the NS gene segment of the 143-1 virus directly were unsuccessful (due to the relatively poor growth of this virus and consequent difficulties in template preparation), we directly sequenced PCR-generated cDNA copies of these gene segments. The nucleotide sequence information generated by this approach should accurately represent the sequence of the virion RNA, since products with random PCR-induced errors at any specific site would be expected to constitute minor subpopulations of the bulk product. Each mutation was confirmed by sequencing of templates generated by at least two different PCR amplifications of virion RNA.

TABLE 3. Results of reassortment between the C1331-5 virus and the A/Bethesda/85 virus

Reassortant	Origin[a] of gene segment: 1	2	3	4	5	6	7	8	Phenotype
3	B	B	B	B	B	*	B	C	ts^+
5	B	B	C	B	C	B	C	C	ts^+
8	C	C	C	B	B	C	B	C	ts^+
22	C	C	C	C	B	C	C	C	ts^+
30	B	C	B	C	C	C	C	C	ts^+
51	C	C	C	B	B	B	B	C	ts^+
54	C	C	B	B	B	B	C	C	ts^+
55	B	C	C	C	B	B	C	C	ts^+
57	C	C	B	C	C	B	B	C	ts^+
63	C	C	C	C	C	B	*	C	ts^+
65	B	B	B	C	B	C	C	C	ts^+
67	C	B	B	B	C	B	B	C	ts^+
70	C	C	C	B	B	*	C	C	ts^+
74	B	C	B	B	B	C	B	C	ts^+
79	C	B	B	B	C	B	C	C	ts^+
81	C	C	B	B	B	B	B	C	ts^+
106	C	B	C	C	C	C	B	C	ts^+

[a] C, gene segment derived from the C1331-5 virus; B, gene segment derived from the A/Bethesda/85 virus; *, parental origin of gene segment could not be determined unequivocally.

The nucleotide sequence of the NS gene segment of the 143-1 virus was identical to that of the A/Alaska/77 virus except for the 36-nucleotide deletion, confirming that additional mutations in the NS gene had not been selected in the generation of reassortant 143-1. The nucleotide sequence of the NS gene segment of the *ts* C1332-8 virus was identical to that of the 143-1 virus. However, the nucleotide sequences of the NS gene segments of the ts^+ C1331-5, H2-7, and P4-40

TABLE 4. Results of reassortment between the H2-7 virus and the A/Bethesda/85 virus

Reassortant	Origin[a] of gene segment: 1	2	3	4	5	6	7	8	Phenotype
1	B	B	B	B	B	B	B	H	ts^+
5	H	B	H	H	H	B	H	H	ts^+
12	H	H	B	H	B	H	B	H	ts^+
16	B	B	B	B	H	H	B	H	ts^+
17	B	*	H	B	B	H	B	H	ts^+
19	B	H	H	B	H	B	*	H	ts^+
20	B	B	B	B	B	H	H	H	ts^+
41	B	B	B	B	B	B	B	H	ts^+
48	H	B	H	B	B	H	B	H	ts^+
49	H	H	H	B	B	H	H	H	ts^+

[a] H, gene segment derived from the H2-7 virus; B, gene segment derived from the A/Bethesda/85 virus; *, parental origin of gene segment could not be determined unequivocally.

TABLE 5. Results of reassortment between the P4-40 virus and the A/Bethesda/85 virus

Reassortant	Origin[a] of gene segment: 1	2	3	4	5	6	7	8	Phenotype
1	B	B	B	P	*	*	B	P	ts^+
3	B	B	B	P	*	*	P	P	ts^+
18	P	B	P	B	B	B	B	P	ts^+
28	P	P	P	P	B	P	P	P	ts^+
34	P	P	P	B	B	P	B	P	ts^+
37	P	P	P	P	B	*	B	P	ts^+
40	B	B	P	P	P	P	P	P	ts^+
49	P	P	B	B	B	P	P	P	ts^+
51	P	P	P	B	B	B	B	P	ts^+
54	P	P	B	P	B	B	P	P	ts^+
57	B	B	P	P	B	B	P	P	ts^+
59	B	B	P	P	P	P	B	P	ts^+

[a] P, gene segment derived from the P4-40 virus; B, gene segment derived from the A/Bethesda/85 virus; *, parental origin of gene segment could not be determined unequivocally.

viruses each contained a U to C transition at nucleotide 94 (mRNA sense), leading to a predicted change from valine to alanine at amino acid 23 of NS1. Because this change occurs within the NS2 intron, no alteration in NS2 would be predicted to occur. The mechanism of escape of these viruses from the *ts* phenotype was therefore attributable to intragenic suppression. The C1331-5 virus also had a noncoding nucleotide change from U to C at nucleotide 110. The sequences of the NS genes of the C1331-5, H2-7, and P4-40 viruses were otherwise identical to that of the 143-1 virus.

In order to determine when in the course of serial in vitro passage of the P4-40 virus the U to C change at nucleotide 94 occurred, we determined the nucleotide sequence of the NS gene segment from nucleotides 50 to 150 of viruses from each passage level. As shown in Fig. 2, the change from valine to alanine occurred between passage 2 and passage 3 of this virus, coincident with a significant change in shutoff temperature for plaque formation from 39 to more than 40°C. This change was seen more clearly after further passage and biological cloning of this virus.

The potential structural consequences of the amino acid change present in the revertant viruses, as predicted by computer analysis of secondary structure (6), are shown in Fig. 3. The 12-amino-acid deletion in the NS1 protein of the 143-1 virus disrupts a region of alpha helix predicted to occur approximately between amino acids 51 and 84 of the wild-type NS1 protein. The change from valine to alanine at amino acid 23 in the revertant viruses is predicted to result in generation of a new region of alpha helix between approximately amino acids 18 and 27 of the NS1 protein. The effects of these changes on the three-dimensional structure of the NS1 protein are unknown.

DISCUSSION

In a previous study, the 143-1 virus was shown to have two phenotypes which could be transferred with the NS gene segment to a *wt* influenza A virus, a shutoff temperature for plaque formation on MDCK cells of 37°C (*ts* phenotype) and a small-plaque phenotype on MDCK cells (23). In the current study, we confirmed the ability of the NS gene

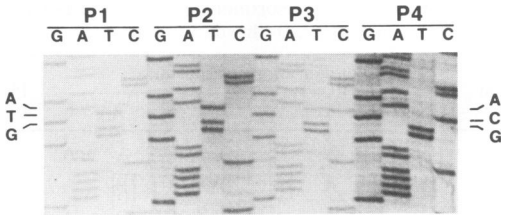

FIG. 2. Dideoxy sequencing of the plus strand of a PCR copy of the NS gene segment of the 143-1 virus after serial passage at 38°C (P1), 39°C (P2 and P3), and 40°C (P4). A substitution of C for T at nucleotide 94 can be seen to occur between the second and third passage.

segment of the 143-1 virus to transfer these phenotypes to the *wt* influenza A/Bethesda/85 virus and showed that the nucleotide sequence of the NS gene segment of the 143-1 virus was identical to that of the A/Alaska/77 virus except for the 36-nucleotide deletion. Thus, the two phenotypes of the 143-1 virus can be directly attributed to the 12-amino-acid deletion in the NS1 protein of this virus.

When the 143-1 virus was administered to hamsters and chimpanzees in the previous study (23) or passaged in vitro at elevated temperatures in this study, viruses which concurrently lost the two phenotypes of the 143-1 virus were isolated. Loss of the *ts* and small-plaque phenotypes occurred concurrently in each of the ts^+ phenotypic revertant viruses. These observations suggest that the products of the NS gene segment, either alone or in combination with those of other viral genes, play a role in the host range of influenza A viruses (4, 24, 27).

The genetic mechanism by which the phenotypically revertant viruses lost the *ts* phenotype was shown to be the development of an intragenic suppressor mutation. Nucleotide sequencing revealed that there was a single-amino-acid difference between the NS gene segment of the 143-1 virus and the corresponding genes of the revertant viruses, a change from valine to alanine at amino acid 23 of the NS1 protein. Intragenic suppression of point mutations in *ts* mutants of influenza A viruses has been reported previously (18, 26), as has intragenic suppression of *ts* and other mutations of animal viruses, including vesicular stomatitis virus, poliovirus, foot-and-mouth disease virus, vaccinia virus, and Sindbis virus (12, 14, 17). Intragenic suppression of a deletion mutation of an influenza virus has not been reported previously.

The mechanism by which this mutation affects the stability of the NS1 protein is unclear. The deletion of the 12 amino acids of NS1 in virus 143-1 disrupts a region of alpha helix in the NS1 protein that extends approximately from amino acids 51 to 84 of the wild-type sequence. Recently, an additional *ts* mutant of the influenza A/Udorn/72 virus was described in which this alpha-helical region was disrupted by a change from lysine to asparagine at amino acid 62 of the NS1 protein (8). These observations suggest that this alpha-helical region of NS1 plays an important role in the function or structural stability of the NS1 protein. In the ts^+ revertant viruses analyzed in this study, a single-amino-acid change to alanine at position 23 was able to restore this lost function. This mutation occurs within the amino-terminal half of the NS1 protein, which has been suggested to be the active portion of the molecule (20). However, the presence of a valine at amino acid 23 of the NS1 protein is not conserved within human influenza A viruses (3), suggesting that the

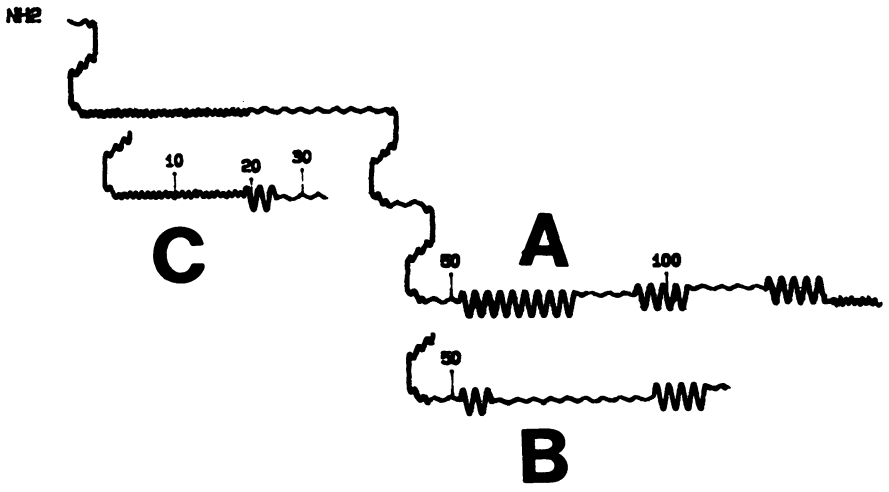

FIG. 3. Chou-Fasman peptide structure predictions of the amino-terminal 150 amino acids of the NS1 protein. (A) Structure of the NS1 protein of the A/Alaska/77 virus; (B) structure of the 143-1 virus around the deletion (the structure of the NS1 protein of this virus would otherwise be identical to that of the A/Alaska/77 virus); (C) 1331-5 virus sequence at the site of the intragenic suppressor mutation (the structure of the NS1 protein of this virus would otherwise be identical to that of the 143-1 virus). Helices are shown with a sine wave, beta sheets are shown with a sharp sawtooth wave, turns are shown with 180° turns, and coils are shown with a dull sawtooth wave.

structure of this particular region may tolerate some variability. While the change to alanine is a conservative one, computer analysis predicts that this substitution would result in conversion of a region of beta-sheet structure to one of alpha helix, approximately from amino acids 18 to 27 of NS1. It is therefore possible that this new region of alpha helix is able to substitute for the loss of the original alpha-helical structure. However, more definitive analysis of the functional significance of these mutations awaits the development of a direct in vitro assay of NS protein function.

The observation that a single point mutation may result in the loss of a phenotype specified by a deletion mutation is relevant to the development of live virus vaccines. Animal viruses bearing a number of types of missense mutations, including *ts*, *ca*, monoclonal antibody-resistant (*mar*), and protease activation (*pa*) mutations have been evaluated as potential live attenuated vaccines. In the case of the *ts*, *mar*, and *pa* mutants, reversion to the wild-type phenotype following replication in vitro or in vivo has been observed (5, 16, 28), and this possibility represents a major concern in the use of missense mutations in the construction of such vaccines. This has been particularly true in the development of *ts* influenza A viruses as live virus vaccines, where phenotypic reversion of a *ts* influenza A virus containing two independent *ts* lesions was observed following prolonged replication in a seronegative child (25, 26). To date, reversion of influenza virus vaccines containing the attenuating genes of the *ca* influenza A/Ann Arbor/6/60 virus has not been observed, possibly because this virus possesses four independent attenuating mutations (22).

Viable animal viruses bearing large deletions represent an attractive alternative to missense mutations in the construction of live attenuated vaccines, since the attenuation phenotype imposed by such mutations should be more stable (15). In addition, the recent development of techniques for in vitro mutagenesis of influenza A viruses provides a means for the ready construction of such mutations (7). The results of the current study, however, suggest that even this approach will not eliminate the need for concern about possible reversion, since both intragenic and potentially extragenic suppressor mutations may develop and alter the phenotypes specified by deletion mutations. A similar need for concern has been suggested by the observation that second-site mutations in the simian virus 40 VP1 gene can suppress a large deletion in the agnogene of this virus following replication in vitro (1). Therefore, it may be necessary to construct influenza viruses bearing deletion mutations in several gene segments to ensure adequate genetic stability. Studies to address the feasibility of this approach are in progress.

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