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Sequence of an influenza virus hemagglutinin determined directly from a clinical sample

(H1 subtype/virus from nasopharynx/polymerase chain reaction/comparison with egg-grown viruses)

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ABSTRACT **The sequence of the HA1 region of the hemagglutinin gene of an influenza virus has been determined without growing the virus in eggs or in cultured cells. The virus used was an H1 strain of influenza A from a clinical specimen taken from a patient in 1987. RNA was extracted directly from virus that had been sedimented out of the transport medium in which the sample had been stored. DNA copies of the hemagglutinin gene, obtained by reverse transcription, were then amplified by the polymerase chain reaction and were sequenced by the dideoxy termination method. The deduced amino acid sequence is highly similar to that of other H1 viruses that had been isolated at about the same time and cultured for a limited number of passages in eggs. Furthermore, the HA1 sequence of progeny virus from this isolate obtained after one passage in chicken embryos is identical to that of the virus obtained directly from the nasopharynx. The results suggest that H1 isolates that have been grown for a limited number of passages in embryonated eggs have HA1 subunits that faithfully represent the virus population in the clinical samples from which they were derived.**

The hemagglutinin (HA) on the surface of the influenza virion is a glycoprotein that has three biological activities that play major roles in the infectious process. The HA attaches the virus to host cells, it is the antigenic target against which neutralizing antibodies are made, and it fuses the viral envelope with the membranes of endocytotic vesicles so that the transcription complex of the virus can enter the cytoplasm of the cell. These three activities are associated with specific regions of the HA (for reviews see refs. 1–3), and amino acid substitutions in these regions can change the ability of the virus to grow in cells of different species. Substitutions on the rim of the receptor binding pocket can change the ability of H3 viruses to bind to specific sialic acid-galactose linkages on host cells (4–7). In addition, the presence of oligosaccharides at specific sites on the HA can determine how well the virus binds to certain cells (8, 9) and how susceptible the HA is to the proteolytic cleavage that is required for the expression of its fusion activity (10–12). Thus, changes in the amino acid sequence of the HA can alter virus host range at the cellular level, and it has been clearly shown that minority forms within a virus population will rapidly predominate if they have a growth advantage during cultivation in the laboratory (2, 8, 13–17).

These observations have led to the concern that minor heterogeneity within clinical samples or mutations that occur during isolation and growth could lead to laboratory-grown influenza virus populations that are significantly different from those in the original clinical samples. Were such selection to occur during growth of the virus in embryonated chicken eggs or in certain cultured cells, virus from those sources would be poorly suited for the study of HA function and for the preparation of vaccines.

The studies presented here were undertaken to determine the sequence of the HA gene directly from the virus within a clinical sample so that nucleotide changes that might be associated with growth in laboratory hosts could be avoided. We have used the polymerase chain reaction (PCR; ref. 18) to amplify DNA made from virus recovered from a patient with influenza and have determined the nucleotide sequence of the HA gene from its 3′ end into the HA2 region. We have used the same procedure to determine the sequence of this region of the HA gene after growth of this isolate in chicken embryos. The deduced amino acid sequences are reported here[§] and are compared to those of viruses of the same subtype isolated since 1977 by growth in embryonated eggs.

MATERIALS AND METHODS

Virus Isolation and Growth. Nasopharyngeal swabs taken for the diagnosis of febrile respiratory illness were used in this study. Samples were taken in January of 1987 at Cardinal Glennon Children's Hospital (Saint Louis, MO). Virus isolation and identification were carried out using tube cultures of primary rhesus monkey kidney cells obtained from Whitaker M.A. Bioproducts. Swabs that contained influenza virus were stored at −70°C in ≈2 ml of transport medium (veal infusion broth containing 0.5% gelatin, penicillin, gentamicin, and amphotericin B).

To obtain egg-grown virus for this study, virus from one of the clinical samples was diluted 1:9 in sterile phosphate-buffered saline and injected into the allantoic cavity of two 10-day-old chicken embryos (0.2 ml per embryo). Allantoic fluid containing ≈1280 hemagglutinating units per ml was harvested after 72 hr of incubation at 34°C and was stored at −70°C.

Plaque-purified influenza A (H1N1), strain WSN, was also used in these studies. This virus has been grown in the laboratory for many generations and for these studies was obtained from Madin–Darby bovine kidney cells.

RNA Extraction. Samples of transport medium or allantoic fluid containing virus were thawed rapidly and diluted with NTE buffer (10 mM Tris·HCl, pH 7.5/100 mM NaCl/1 mM EDTA). Virus from transport medium or allantoic fluid was concentrated by centrifugation at 105,000 × *g* for 2 hr, resuspended in 300 μl of NTE, and quantitated by hemagglutination as described (19). Based on the titer of the

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Abbreviations: HA, hemagglutinin; PCR, polymerase chain reaction.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M33748).

resuspended virus, the clinical samples used in these studies each contained ≈1200 hemagglutinating units of virus.

Twenty microliters of virus suspension containing 50–100 hemagglutinating units was mixed with an equal volume of buffer containing 200 mM Tris·HCl (pH 7.4), 300 mM NaCl, 25 mM EDTA, and 2% SDS and incubated with 100 μg of proteinase K (Boehringer Mannheim) at 37°C for 1 hr. After addition of 10 μg of tRNA, the RNA was extracted with phenol/chloroform and precipitated with ethanol. The RNA was then incubated at 37°C for 30 min with 40 units of RNase-free DNase (Pharmacia), extracted with phenol/chloroform, reprecipitated, and suspended in 10 μl of TE buffer (10 mM Tris·HCl, pH 8.1/1 mM EDTA).

cDNA Synthesis. Reverse transcription of the virion RNA was carried out as described (20) using avian myeloblastosis virus reverse transcriptase obtained from Life Sciences and a primer consisting of a 12-nucleotide sequence that is complementary to the 3′ ends of all of the virion segments. This primer and all others used in this study, unless otherwise noted, were prepared by Paul Wollenzein (Department of Biochemistry, Saint Louis University) using an Applied Biosystems DNA synthesizer and were purified by protocols described by Applied Biosystems. The cDNA·RNA product obtained by reverse transcription was extracted once with phenol/chloroform, precipitated with ethanol, and dissolved in 10 μl of TE buffer.

HA Gene Amplification by the PCR. The entire HA1 region of the HA gene and part of the HA2 region were amplified by using Gene-Amp kits obtained from Cetus/Perkin–Elmer. The reaction mixture (100 μl) contained the cDNA·RNA complex described above, 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 2.5 units of *Thermus aquaticus* (*Taq*) DNA polymerase, 200 μM dNTP, and 20 pmol of each of two primers as indicated in *Results*. The reaction mixture was overlaid with 100 μl of light mineral oil (Sigma) and amplification was carried out for 25 cycles each consisting of 1 min of denaturation at 94°C, 2 min at 35°C for reannealing, and 2 min at 72°C for extension. Following extraction with chloroform to remove the mineral oil, an aliquot of the amplified DNA was analyzed by electrophoresis in 1% agarose gel with a Tris/borate/EDTA buffer system (21). To ensure that there was a sufficient amount of DNA for multiple sequencing reactions, the DNA from the first amplification was purified from low-melting agarose gels and was reamplified as described above.

Nucleotide Sequence Analysis. Double-stranded DNA copies of the HA gene were sequenced directly from the amplified pool by the dideoxy termination method (22). Primers, end-labeled with [γ-^{32}P]ATP (ICN Radiochemicals) by using T4 polynucleotide kinase (Pharmacia), were annealed to 300–400 ng of amplified DNA by boiling for 10 min and allowing the reaction mixture to come to room temperature. The DNA–primer complex was ethanol-precipitated and was sequenced using Sequenase kits (United States Biochemical).

The sequence of both DNA strands was determined from the virus taken directly from the nasopharynx. The six primers used for sequencing the coding strand were based on the published sequences of the WSN and PR8 strains of influenza A (H1N1) (23, 24). They consisted of bases 11–35, 219–233, 384–404, 549–568, 796–813, and 918–932 of plus-strand RNA. The five primers used to sequence the noncoding strand were complementary to nucleotides 1005–986, 692–669, 496–478, 238–219, and 116–99 of the A/SL/2/87 plus strand. The same primers were used to determine the HA sequence of egg-grown virus.

RESULTS

These studies were undertaken to determine the sequence of the HA gene without there having been opportunities for selection during growth of the virus in a laboratory host. The HA1 region and the cleavage region that links HA1 to HA2 have been investigated. The part of the HA gene that was amplified extended from its 3′ end to nucleotide 1095, which is located 31 bases into the HA2 region of this genome segment.

The primers used for preparation of the amplified DNA and their relationship to the HA gene are shown in Fig. 1. Primers I and IV (kindly provided by Andrew Caton, Wistar Institute, Philadelphia) were the first to be used in this study, because they were expected to produce DNA copies that would contain most of the HA1 region of the HA gene (see Fig. 1).

The amplified DNA obtained from two clinical samples by using primers I and IV is shown in Fig. 2*A*. DNA that appeared to be heterogeneous in size but was about 1000 base pairs long was obtained from each. A/SL/2/87 was chosen for use in all subsequent studies.

As shown in Fig. 2*B*, the amplified DNA obtained from this virus could be resolved into three bands by longer periods of electrophoresis. When the individual bands were eluted from a gel like that shown in lane 1 and reapplied to a second gel, each retained its original mobility (lanes 2–4), indicating that they represent discrete populations of molecules. The largest

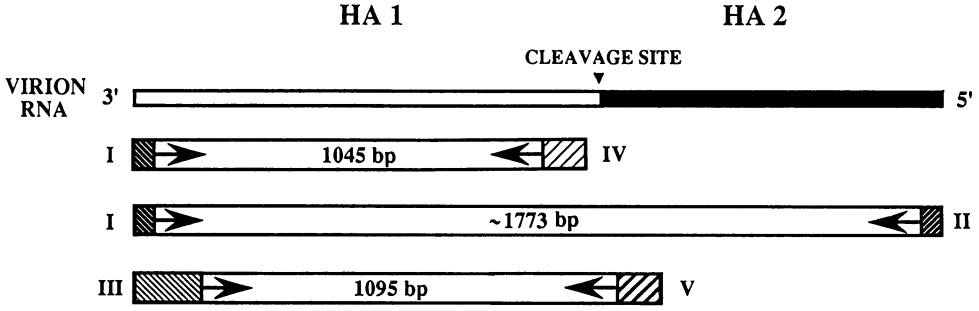

FIG. 1. Primers used in amplification and the DNA products obtained. The primers and their relationship to the HA gene are shown. Primer I, d(AGCAAAAGCAGG), is complementary to the first 12 nucleotides at the 3′ end of all of the virion RNAs. It consists of plus-strand DNA (see text). Primer II, d(AGTAGAAACAAGG), consists of the first 13 nucleotides at the 5′ end of all of the virion RNAs and its minus-strand DNA. Primer III, d(AGCAAAAGCAGGGGAAAATAAAAACAACCAAAATG), is plus-strand DNA and is complementary to the first 35 bases at the 3′ end of A/SL/2/87 HA RNA. Primer IV, d(ATGTTCCTTAGTCCTGTAACCAT), is complementary to nucleotides 1045–1023 of the plus-strand HA DNA. Primer V, d(CAATGAAACCGGCAATGGCTCC), is complementary to nucleotides 1095–1074 of the plus-strand of USSR HA DNA. The regions of the virion RNA represented in amplified DNA synthesized from primers I and IV, I and II, and III and V are also shown. The DNA used for sequencing directly from the nasopharynx was obtained by using primers I and IV in both the first and second amplifications or primers I and II followed by primers III and V (see text). DNA was synthesized from egg-grown A/SL/2/87 by using primers III and V in both amplifications. bp, Base pairs.

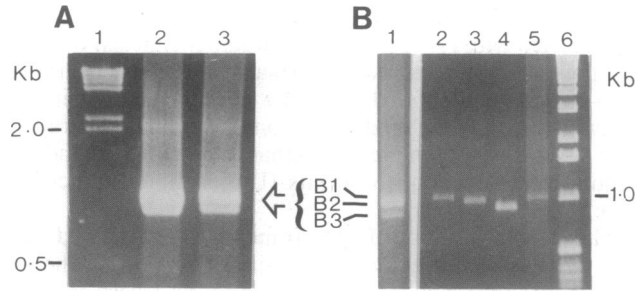

FIG. 2. Agarose gel electrophoresis of the amplified DNA obtained by using primers I and IV. (*A*) Lane 1, *Hin*dIII-digested λ DNA (size markers); lanes 2 and 3, amplified DNA copies of HA RNA from two clinical samples, A/SL/1/87 and A/SL/2/87, respectively. (*B*) Lane 1, amplified A/SL/2/87 HA DNA separated into three bands by electrophoresis for 8 hr at 100 V; lanes 2–4, purified bands B1, B2, and B3, respectively; lane 5, amplified WSN HA DNA; lane 6, marker DNA. kb, Kilobase(s).

of these DNAs, designated B1, had the same mobility as the DNA obtained from the WSN strain of influenza A (H1N1) (lane 5). Amplified DNA obtained from this virus by the same procedure as that used with the clinical samples consistently gave only one band, which was of the size expected from primers I and IV (1045 base pairs). B1 DNA was therefore purified by gel electrophoresis and used for sequence analysis.

To obtain information about the sequence at the cleavage site between HA1 and HA2, we needed DNA copies that extended into the HA2 region. We therefore amplified DNA by using primers I and II (see Fig. 1). Since these primers are complementary to the conserved regions at the two ends of all genome segments (25–27), they were expected to produce complete copies of all eight virion genes, albeit in small amounts. To obtain a sufficient amount of the HA DNA for sequencing, we used these complete DNA copies as templates for reamplification using two new HA-specific primers, III and V. Since we had already determined the sequence of most of the plus-strand HA DNA, one of these new primers (III) was designed to be complementary to the first 35 nucleotides at the 3′ end of the A/SL/2/87 HA gene. This primer is identical to primer I for the first 12 nucleotides but contains 23 additional bases that are specific for the sequence found in A/SL/2/87. Primer III, along with primer V, which is complementary to nucleotides 1095–1074 in the HA2 region of the USSR plus strand (see Fig. 1), produced DNA of one size class. It migrated slightly slower than B1 DNA and had the same *Ava* I and *Ava* II cleavage sites as those in B1 DNA (results not shown). By sequencing both strands of this DNA, we have determined the sequence of the first 31 bases of the HA2 region and have confirmed the sequence that we had previously obtained from B1 DNA. The use of this second method of obtaining amplified DNA has increased the likelihood that our sequence represents that of the majority of the viruses in the clinical sample rather than the sequence of a minority population that was selectively amplified by the primers used.

The nucleic acid sequence of A/SL/2/87 after a single passage in eggs was also determined from DNA obtained by PCR using primers III and V. This DNA, like that obtained directly from the clinical sample when these two primers were used, was homogeneous in size and was slightly larger than B1 DNA. The nucleotide sequence of this DNA was identical to that obtained from virus taken directly from the throat.

The nucleotide sequence of A/SL/2/87 and that of embryonated egg-grown A/TW/1/86 (kindly provided by Nancy Cox, Centers for Disease Control, Atlanta) are highly similar. Differences were observed at seven positions (cytosine in A/TW/1/86 versus uracil in A/SL/2/87 at nucleotides 251, 371, 848, and 1001; guanine versus adenine at nucleotides 748 and 793; and adenine versus guanine at nucleotide 1021). Four of these differences (at residues 251, 371, 848, and 1001) are in the third nucleotide of the codon and do not change the amino acid specified by the sequence. The remaining three (at nucleotides 748, 793, and 1021) change the amino acids at positions 225, 240, and 315 as indicated in Fig. 3. However, two independent reports of the amino acid sequence of A/TW/1/86 have been published (30, 31); they differ at amino acids 138, 240, and 315. As indicated in Fig. 3, the sequence of A/SL/2/87 differs from both of the published sequences of A/TW/1/86 by only one amino acid; aspartic acid (D) is found at position 225 in both nasopharyngeal and egg-grown A/SL/2/87, whereas glycine (G) is found in egg-grown A/TW/1/86. Both of these recent H1 isolates differ significantly from A/USSR/77.

DISCUSSION

Although interest in influenza virus epidemiology and genetics has led to numerous reports concerning the amino acid sequence of the HA gene, all previously reported sequences

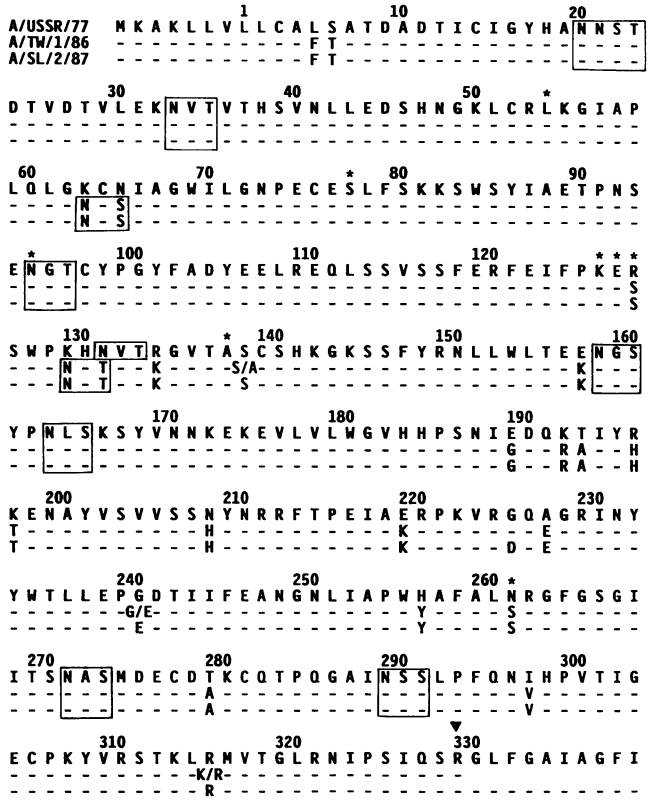

FIG. 3. Amino acid sequence of the HA1 subunit and the HA1/HA2 cleavage region of nasopharyngeal and egg-grown A/SL/2/87 and of two egg-grown H1 strains, A/USSR/77 and A/TW/1/86. The amino acids are numbered to correspond to those in the H3 HA as previously described (28). Amino acids present in the H1 subtype but not in H3 are marked by stars. The glycosylation sites are indicated by boxes. Dashes indicate no difference in sequence from that found in A/USSR/77 (29). For A/TW/1/86, two amino acids are given at three positions. The amino acid above the bar is that found by Cox *et al.* (30) and the one below the bar is that found by Robertson (31). Although both preparations of A/TW/86 that were sequenced contained glycine at residue 225, aspartic acid has been found at that position in numerous egg-grown isolates (see text). Amino acids 330–339 of A/TW/1/86 have not been reported. Arrowhead indicates the proteolytic cleavage site between HA1 and HA2. A single sequence is shown for A/SL/2/87, since nasopharyngeal and egg-grown virus gave identical sequences.

have been derived from virus strains that had been grown either in eggs or in cultured cells. Our approach using the PCR to amplify copies of the HA gene has enabled us to determine the sequence of this viral gene starting with the limited amount of virus that can be recovered from a nasopharyngeal swab taken during the acute stage of illness. In so doing we have been able to determine the sequence of this viral surface glycoprotein without there having been opportunities for selection of variant forms during virus cultivation in eggs or in cell culture.

We have found that the HA gene sequence encompassing the HA1 region and the HA1/HA2 cleavage region of a 1987 isolate is identical when obtained directly from virus in the nasopharynx and from virus grown in chicken embryos. Furthermore, this sequence is strikingly similar to those of other H1 viruses isolated in 1986. In fact, the only difference between A/SL/2/87 and A/TW/1/86 is the presence of aspartic acid instead of glycine at amino acid 225. By analogy to the H3 HA (32), this amino acid is located on the rim of the receptor binding pocket and might be expected to influence the specificity or the affinity of the HA for receptors on host cells from different sources. It has, in fact, been proposed that an aspartic acid to glycine substitution occurs during adaptation of H1 viruses to growth in eggs (33). This is not the case with the isolate described here, since aspartic acid remains at position 225 when A/SL/2/87 is grown for one passage in eggs. In addition, of some 19 H1 viruses isolated since 1977 and sequenced after a limited number of passages in eggs, 8 have aspartic acid, 8 have glycine, and 3 have asparagine at position 225 (30, 31, 33, 34), indicating that all three of these amino acids are compatible with growth of H1 strains in eggs. Thus, the sequence data presented here from DNA synthesized by PCR are consistent with those obtained previously from egg-grown H1 isolates. The accumulated evidence indicates that embryonated eggs do not provide a strong selective pressure for an aspartic acid to glycine substitution at amino acid 225. Whether this change will occur during long-term passage of A/SL/2/87 in eggs or in cultured mammalian cells and whether other amino acid substitutions will accompany it can now be determined.

As indicated in Fig. 3, glutamine has been found at position 226 in A/SL/2/87 and in the two reference strains. This amino acid is also on the rim of the receptor binding pocket and has been implicated in determining the receptor specificity of H3 strains (4). With H3 strains, glutamine at amino acid 226 has been found in virus obtained from avian sources whereas leucine has been found at that position in virus of human and equine origin (35). This is not the case with H1 strains; glutamine has been found at position 226 in all human isolates grown in eggs or cultured cells and, as shown here, in virus obtained directly from the human nasopharynx.

The glycosylation sites on the H1 viruses isolated since 1977 have been highly conserved. As shown in Fig. 3, A/USSR/77 has eight sites whereas A/TW/86 has nine. Except for the additional glycosylation sites at amino acid 63 of the HA1 subunit and the shift of the site from 131 to 129 in the 1986 isolates (30, 31), the H1 strains isolated since 1977 have identical glycosylation sites. Since these same glycosylation sites are found in virus taken directly from the nasopharynx, neither addition nor deletion of glycosylation sites can be attributed to growth of these viruses in the laboratory. It remains to be determined, however, whether all of these sites will be maintained through extensive passage of these viruses in eggs or in cell culture. It is interesting in this regard that the strains of influenza virus that have been grown in the laboratory for many years have functional HA1 subunits with as few as four glycosylation sites, only two of which (those at amino acid 20/21 and 271) are conserved in all strains. In the case of the WSN strain, deletion of a glycosylation site from the tip of the HA actually enhances growth of the virus in some mammalian cells (8, 9), and with an influenza B strain, virus isolated in eggs lacks a glycosylation site that is present on virus isolated from the same source in Madin–Darby canine kidney cells (16). Since deletion of glycosylation sites is clearly tolerated during growth of these viruses in cell cultures, their conservation among new isolates suggests that the oligosaccharides at these sites are critical to the survival of these strains in nature, rather than to the receptor-binding or fusion activity of the HA. These considerations strengthen the previously made proposal that the oligosaccharides may mask antigenic sites (36) that would otherwise contribute to immune surveillance and reduce virus transmission within the human population.

It is important to point out that the information presented here should not be taken to indicate that the virus population within the nasopharynx is homogeneous with respect to its HA sequence. Minor heterogeneity in the HA genes would not be detected by the techniques used here. Since we have sequenced the HA gene directly from amplified DNA pools obtained by two different procedures, we have reduced the chances of seeing random substitutions that could be introduced into the sequence by the *Taq* polymerase. However, this approach also reduces the possibility of detecting minor components within a heterogeneous population. Thus, the deduced amino acid sequence we have obtained is that of the majority component within the clinical samples, and other approaches are needed to determine whether these populations are heterogeneous in HA sequence.

Since only low amounts of virus are needed for sequencing by the technique used here, various questions relating to the virus population within a clinical sample can now be investigated. For example, will virus from other individuals infected within the same epidemic show exactly the same sequence as that reported here, or will the major component found within a clinical sample depend in part on the immune state of the patient? Will there be any changes in HA sequence during the progression of the disease? Will prolonged passage in different laboratory hosts produce different populations from this isolate and, if so, is this due to mutation and selection during cultivation in the laboratory or to selection of different minor components within the clinical sample? With respect to these two alternatives, previous work indicates that both processes occur. On the one hand, mutants with single base substitutions in the HA gene have been found to arise in homogeneous populations derived from single plaques and to replace the original virus strain when the mutant has a selective advantage over the parent in the cell culture used for virus propagation (8, 9). On the other, virus populations obtained directly from patients with influenza have been resolved into subpopulations that differ from one another by as little as a single amino acid (17, 33, 35–37).

The information presented here indicates that growth of new isolates for a limited number of passages in embryonated eggs can be expected to provide H1 stocks with HA1 sequences that faithfully represent those found on virions within the nasopharynx. Whether this is the case with other influenza A subtypes and influenza B viruses can now be determined using the approach employed here. This approach should also be useful in vaccine development and evaluation; it should help to ensure that the virus strains that are incorporated into influenza vaccines have HAs that mirror those found in the human population.

Lastly, the incomplete DNAs in the amplified pools obtained from the clinical samples need to be investigated. As indicated above, these smaller DNAs have been obtained from both of the clinical isolates that we have examined. With A/SL/2/87 they were seen with virus obtained directly from the nasopharynx and from chicken embryo-grown virus when primers I and IV were used during amplification (data not shown), but not when primers III and V were used. They

were not observed when plaque-isolated virus of the WSN strain was amplified using primers I and IV. These DNAs may be generated during amplification as a consequence of some as yet unidentified variation or imperfection in the PCR. However, the conditions under which they are found are consistent with their being authentic DNA copies of defective HA genome segments.

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