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Antigenic and Genetic Variation in Influenza A (H1N1) Virus Isolates Recovered from a Persistently Infected Immunodeficient Child

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Antigenic and genetic variations have been analyzed in eight consecutive isolates recovered from a child with severe combined immunodeficiency syndrome persistently infected with naturally acquired type A (H1N1) influenza virus over a 10-month period. Hemagglutination inhibition reactions and T_1 oligonucleotide fingerprinting demonstrated that these viruses were related to strains causing outbreaks in the United States at that time (1983 to 1984) but that antigenic and genetic differences between consecutive isolates could be detected. This variation between isolates was examined further by sequencing the RNAs encoding the HA1 region of the hemagglutinin (HA) and the nucleoprotein (NP) in five of the consecutive isolates. Multiple point mutations were detected in both genes, and a deletion of one amino acid was detected in the HA. Depending on the isolates compared, 5.8×10^{-3} to 17×10^{-3} substitutions per nucleotide site per year were detected in the RNAs encoding the HA1, and 3.5×10^{-3} to 24×10^{-3} substitutions per nucleotide site per year were detected in the NP gene. Fifty-four percent of the base changes in the HA1 and 73% in the NP led to amino acid substitutions. A progressive accumulation of mutations over time was not observed, suggesting that the genetic diversity of these viruses may best be interpreted as the result of shifts in the population equilibrium (quasispecies) of replicating variant genomes.

The epidemiologic success of influenza virus is due in large part to variation in the two surface glycoproteins of the virus, hemagglutinin (HA) and neuraminidase (1, 53). Sequencing studies of the HA genes of field strains (reviewed in reference 58) have shown that for the HA (the major viral antigen against which neutralizing antibodies are primarily directed), variation occurs predominantly in the HA1 domain and appears to cluster in five main antigenic regions (56, 57, 59). Evolution of the HA of influenza A viruses is characterized by an accumulation of amino acid changes in the antigenic sites following a generally linear evolutionary pathway with a fairly constant and rapid rate of change (4, 27, 44, 54). However, new epidemic strains of influenza A virus do not always evolve from the previous one (4, 10). Fewer sequencing studies have been undertaken on influenza virus genes encoding nonsurface proteins of the virus; however, available information suggests that internal proteins, like the nonstructural proteins, also evolve along a linear pathway, albeit at a slower rate (5). The higher rate of coding nucleotide substitution in the HA gene compared with other genes has been taken as evidence that immune selection is an important factor in the evolution of the HA (4, 34, 42, 54, 56).

Other studies have documented genomic and antigenic diversity between influenza viruses isolated during an epidemic period (4, 9, 10), during an outbreak in a defined location (9, 35, 38, 41), during a single epidemic in semiclosed communities (39, 40), or as single isolates (31, 33). Genetic heterogeneity is thought to be due to the rate of mutation of the influenza virus genome (28, 43) and the selection of variants in a partially immune population. Additional diversity is generated by reassortment of the segmented influenza virus genome during mixed infections with cocirculating variant influenza viruses (35, 61).

Because consecutive isolates over an extended period are rarely available from the same person, little is known about the extent and patterns of influenza virus variation during prolonged replication in a single individual. Previously, we reported the case of a 6-month-old patient with severe combined immunodeficiency syndrome (SCID) who developed a 10-month-long chronic infection with naturally acquired influenza A virus (26). SCID is a congenital immunodeficiency characterized by an absence of both T-cell and B-cell functions and requires the isolation of the patient in a sterile environment. The influenza virus isolates recovered from this patient provided an opportunity to examine the evolution of influenza virus in a single individual under circumstances of (i) a highly diminished selective pressure from the immune system and (ii) an unlikely reinfection of the individual with other cocirculating influenza virus variants.

In the present study, we describe the antigenic and molecular properties of these sequential influenza virus isolates, and through sequencing, we examine the extent of variation of two influenza virus genes, one encoding the highly variable HA1 domain of the external HA glycoprotein and the other encoding the type-specific nucleoprotein (NP) that is, in field isolates, less variable than the HA.

MATERIALS AND METHODS

Viruses. Influenza A (H1N1) virus specimens were isolated from nasal secretions taken from a 6-month-old SCID patient at about monthly intervals over a 10-month period

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(from May 1983 to February 1984). Viruses were isolated on 15 separate occasions. For virus isolation, undiluted nasal washes were used to inoculate primary monkey kidney cells, and thereafter isolates were passaged twice undiluted in MDCK cells (49). Titers of the virus ranged from 10^4 to 10^5 PFU/ml. Eggs were inoculated with 0.2 ml of undiluted MDCK material. Hemagglutination titers of the viruses in the first egg passage ranged from 1:64 to 1:518. Influenza A H1N1 virus A/Ohio/4/83 (A/OH/83), collected on 6 January 1983 in the same state in which the patient lived, was provided by the Ohio Department of Health, Columbus. Other viruses used in this study were A/Georgia/79/83 (A/GA/83) and the vaccine strain A/Chile/1/83 (A/CH/83), collected during April 1983 and July 1983, respectively. Both viruses were representative of strains causing major influenza outbreaks at the time the child underwent treatment. The antigenic properties, T_1 fingerprints, and HA1 sequences of these two viruses have been reported previously (10, 13, 44). All viruses were grown in the allantoic cavity of 11-day-old embryonated eggs at a low multiplicity of infection and were passaged three to four times.

HI tests. Hemagglutination inhibition (HI) tests were carried out according to standard procedures using trypsin and periodate-treated postinfection ferret serum (32). For monoclonal antibody (MAb) analysis, receptor-destroying enzyme was used to treat mouse ascitic fluids (gift from R. G. Webster) containing MAbs directed specifically against the HA of A/USSR/90/77 (H1N1) as described previously (20, 52).

RNA extraction and T_1 oligonucleotide mapping. Viruses were purified by polyethylene glycol precipitation and sucrose density gradient centrifugation, and RNA was extracted from purified virions as previously described (11). For T_1 oligonucleotide fingerprinting, purified RNA was digested with T_1 RNase, and the resultant oligonucleotides were labeled at the 5′ ends with [γ-^{32}P]dATP and T4 polynucleotide kinase. In the subsequent two-dimensional mapping, electrophoresis was performed at pH 3.5 in a 10% polyacrylamide–6 M urea gel for the first dimension and at pH 8.3 in a 21.8% polyacrylamide gel buffered with Tris-borate for the second dimension (11).

RNA sequencing. RNA sequence analysis was performed by the dideoxy chain termination method, using synthetic oligodeoxynucleotide primers and reverse transcriptase essentially as described elsewhere (12) except that 10 μCi of [α-^{35}S]dATP (Amersham Corp.; specific activity, >1,000 Ci/mmol) for each 5.5 μl of reaction mixture was used and reverse transcriptase incubations and chase were done at 42°C for 20 min. Primers 5′d (AAGCAGGGGAAAATA AAA), 5′d (AATCATGGTCCTACATTG), 5′d (TTTTACA GAAATTTGCTA), 5′d (GAAAATGCTTATGTCTCT), and 5′ (GGAAATCTAATAGCGCCA), complementary to viral RNA sequences beginning at nucleotides 6, 304, 513, 666, and 819, respectively, were used to obtain sequences of the HA1 domain of the HA gene. Primers 5′d (AGCAGGGTA GATAAT), 5′d (GCACCGAGCTTAAACTCA), 5′d (CGAA TCTGGCGC), 5′d (GGGATCAATGAT), 5′d (TGGACCT GCCGT), 5′d (GAGGAGTACAAATTGCTT), and 5′d (GT GCAAAGAAACCTTC), complementary to viral RNA sequences beginning at nucleotides 7, 176, 397, 643, 888, 1127, and 1285, respectively, were used to obtain sequences of the NP gene. The reaction products were separated by electrophoresis in 0.35-mm-thick 5, 6, or 8% polyacrylamide–8.3 M urea gels containing 100 mM Tris-borate (pH 8.3) and 5 mM EDTA. After electrophoresis, gels were soaked in 5% methanol–5% acetic acid, dried, and autoradiographed overnight at room temperature. Nucleotide sequences were stored and analyzed in a Digital Corporation VAX computer using version 5.3 of the sequence analysis software package of the University of Wisconsin Genetics Computer Group (14).

Nucleotide sequence accession numbers. GenBank accession numbers for sequences determined in this work are as follows: HA1 isolate A, M59324; HA1 isolate C, M59325; HA1 isolate D, M59326; HA1 isolate F, M59327; HA1 isolate H, M59328; NP isolate A, M59329; NP isolate C, M59330; NP isolate D, M59331; NP isolate F, M59332; NP isolate H, M59333; A/OH/83, M59334.

RESULTS

Case history and antigenic analysis. The diagnosis of SCID was made when this male child was 6 months old, a few days after he presented to Cincinnati Children's Hospital Medical Center on 17 May 1983. The patient was then placed in laminar-airflow isolation. Influenza A H1N1 virus was first isolated from nasal secretions of the patient on 24 May 1983. At that time the patient exhibited fever, profuse rhinorrhea, cough, rhonchi and rales, and patchy infiltrate in lingular areas. Thereafter he experienced eight similar episodes which were self-limited and not associated with isolation of bacterial pathogens. Influenza virus was isolated during these episodes and at each attempt over a 10-month period (from May 1983 to February 1984). Eight of 15 isolates were selected for this study; they were designated A to H in sequential order according to date of collection. All of them except isolate G were collected during febrile episodes. The course of immunological treatment of the patient and dates of isolation of the selected viruses are summarized in Fig. 1. Regular administration of intramuscular gamma globulin (HI titer to both H1N1 and H3N2 subtypes of influenza A virus of <1:10) was initiated in June 1983 as standard maintenance prophylaxis and continued biweekly until February 1984. The child underwent bone marrow transplants on 17 August 1983 and 17 November 1983; the second of these was successful. Titers of the virus in the nasal wash specimens ranged from 10^4 to 10^5 PFU/ml until 26 January 1984 (11 weeks after the second transplant). Thereafter, titers dropped to 10^2 PFU/ml, and on 14 February 1984, influenza A virus was isolated for the last time. In March 1984, 15 weeks after the second transplant, influenza A H1N1 virus subtype-specific cytotoxic T-lymphocyte activity as well as specific antibody (HI, ≥1:320) to H1N1 strains but not to H3N2 or influenza B virus (HI, <1:10) was first detected. More details of the case history have been published elsewhere (26).

Table 1 summarizes the antigenic properties of these viruses. The standard HI tests with postinfection ferret serum derived against several representative H1N1 reference strains showed that these isolates were closely related to the reference strain A/CH/83, which was causing major influenza outbreaks at that time. However, two of the isolates, C and D, exhibited a higher level of reactivity with all the reference ferret antisera. Analysis with MAbs revealed that viruses E, F, G, and H, isolated after the successful bone marrow transplant but before detectable HI antibody developed, no longer reacted with MAbs W18 and 22/1, indicating that antigenic changes had occurred in the HAs of these viruses. The MAbs and the sites they recognize on the HA polypeptide have been described previously (36, 52).

Genetic analysis of virus isolates. T_1 oligonucleotide fingerprinting was used to characterize total genomic RNA of the

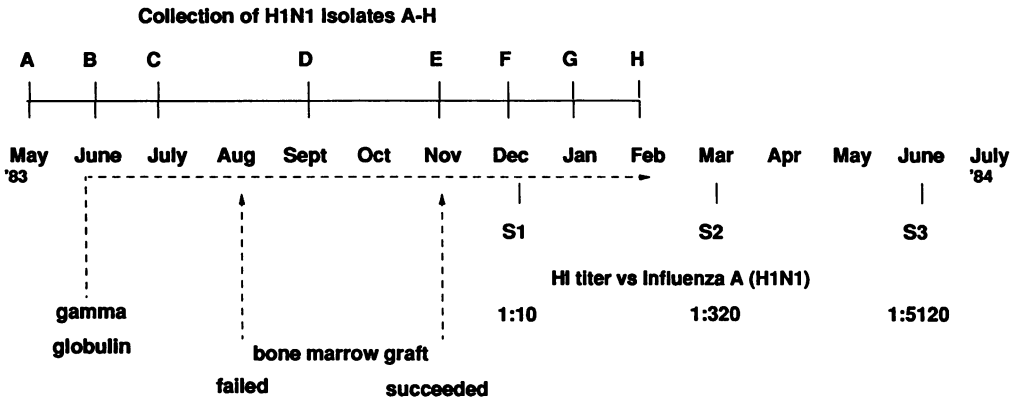

FIG. 1. Course of immunological treatment and influenza virus isolations (designated A to H) in a 6-month-old child with SCID who was persistently infected with naturally acquired type A (H1N1) influenza virus. Human gamma globulin (HI titer to influenza A H1N1 and H3N2 viruses of less than 1:10) was administered intramuscularly from June 1983 to February 1984. Analysis of serum samples from the patient (S1, S2, and S3) indicate that influenza A virus-specific antibody to H1N1 strains (HI, >1:320) but not to H3N2 or influenza B (HI, <1:10) was first detected in March 1984 (4 months after the successful bone marrow graft).

isolates from the patient. Diagrams in Fig. 2 compare the fingerprints of isolate A with typical 1983 epidemic strains (Fig. 2A) and of isolates A to H with each other (Fig. 2B). The isolates from the patient had oligonucleotide fingerprints closely related to the 1983 viruses A/GA/83, A/CH/83, and A/OH/83, which were widely circulating in the general population. However, pairwise comparison of the fingerprints of the eight sequential virus isolates (Fig. 2B, A to H) revealed differences between all but isolates G and H. A sequential accumulation of oligonucleotide changes in the virus isolates over time was not observed. Instead, new oligonucleotides often were not present in subsequent isolates (Fig. 2).

To analyze these genetic differences in greater detail, we sequenced the RNAs encoding the HA1 domain of the HA and the NP of isolates A, C, D, F, and H. Nucleotide and deduced amino acid sequences of the HA1 domain are shown in Fig. 3 and 4, respectively. These sequences were compared with each other and with those of A/CH/83 and A/GA/83 (10). The sequence of the HA1 of the first isolate (isolate A) from the child was identical to that of A/GA/83 or A/CH/83 except for a silent mutation at position 852 which is conserved in all the consecutive isolates. This position is not changed in any of the H1N1 influenza virus field strains sequenced to date. All sequences, including that of A/GA/83, differed from the A/CH/83 sequence at position 486, resulting in one substitution at amino acid 138 which appears to be unique to this reference strain (8a, 44).

The nucleotide and amino acid changes in the HA1 domain between the initial isolate (isolate A) and consecutive isolates are summarized in Table 2. Thirteen point mutations and one amino acid deletion were detected; 54% of the point mutations led to amino acid substitutions, all of them located in or very close to antigenic sites (44). The nucleotide sequence of the HA1 domain of each isolate differed from that of the initial virus (isolate A) in 0.09 to 0.9% of positions. Although variation in the HA1 domain was extensive, the mutations were not always sequentially acquired.

TABLE 1. Antigenic analysis of influenza virus isolates from patient

Reference and test virus[a]	HI titer with								
	Postinfection ferret antiserum to:					USSR/77 MAb[b]			
	USSR/77	BR/78	ENG/80	IND/80	CH/83	W18	22/1	70/1	264
USSR/77	320	640	640	160	160	6,400	3,200	25,600	12,800
BR/78	80	640	640	160	320	1,600	3,200	25,600	800
ENG/80	80	640	640	320	640	1,600	800	12,800	800
IND/80	160	320	160	640	640	400	800	12,800	<
CH/83	20	160	80	160	160	200	200	1,600	<
A (5/83)[c]	40	80	160	80	80	800	200	800	<
B (6/83)	40	160	160	160	160	1,600	200	400	<
C (7/83)	640	640	640	640	640	1,600	200	6,400	<
D (9/83)	320	320	320	320	320	800	200	3,200	<
E (11/83)	160	80	80	160	160	<	<	200	<
F (12/83)	160	160	160	160	160	<	<	200	<
G (1/84)	160	160	160	160	160	<	<	400	<
H (2/84)	160	320	160	320	160	<	<	400	<

[a] USSR/77, A/USSR/90/77; BR/78, A/Brazil/11/70; ENG/80, A/England/333/80; IND/80, A/India/6263/80; CH/83, A/Chile/1/83.

[b] The sites in the HA polypeptide recognized by each monoclonal antibody have been described elsewhere (36) and involve the following amino acids: W18, amino acid 125c; 22/1, amino acids 129, 133, and 157; 70/1, amino acid 157; and 264, amino acids 189 and 190. <, Less than 100.

[c] Date of isolation (month/year) of viruses from patient is given in parentheses for isolates A to H.

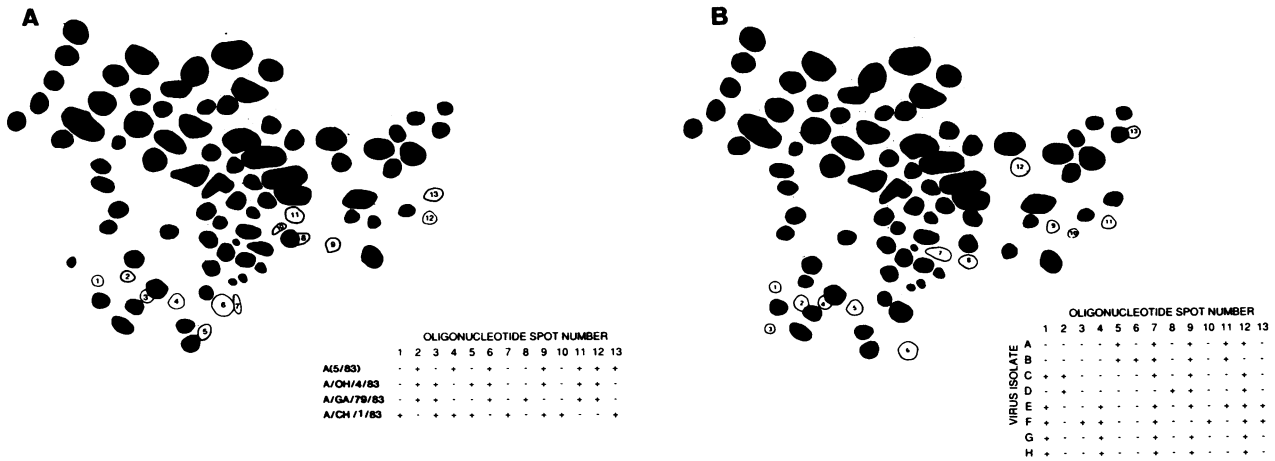

FIG. 2. T_1 oligonucleotide fingerprint analysis of the influenza virus isolates recovered from the patient. (A) Diagram comparing the fingerprints of three 1983 reference strains: A/OH/83, A/GA/83, and A/CH/83 (published elsewhere [10]) and the initial isolate, A, from the patient. (B) Diagram comparing the fingerprints of consecutive isolates A to H. Solid and empty spots denote oligonucleotides respectively common to and variable among fingerprints of the viruses compared. The presence (+) or absence (−) of the variable oligonucleotides in each virus isolate is indicated in the bottom half of each diagram.

Between viruses A and C or D, collected 2 and 4 months apart, respectively, there was one amino acid change in site Sa (amino acid 158) and two additional silent mutations in virus D. The nucleotide change at position 294 in virus C was not counted because both nucleotides C and T appeared at this position in the first virus isolate. The nucleotide substitutions described above were not observed in isolates F and H, which were collected after the successful bone marrow graft. Instead, different nucleotide changes appeared, giving rise to amino acid substitutions in or near antigenic sites Cb, Sa, and Sb in addition to the deletion of amino acid 134 in site Ca. Five of these changes, including the deletion, were detected in the last isolate, and no additional changes were observed.

Rate of fixation of mutations has been defined as the number of mutations per unit time which become dominant among replicating genomes (17). When the first isolate is compared with each consecutive isolate, this rate ranged from 5.8×10^{-3} to 17×10^{-3} substitutions per nucleotide site per year in contrast to the rate of 5.4×10^{-3} substitutions per nucleotide site per year recorded for H1N1 influenza virus field strains (58) or the rate of 3.8×10^{-3} substitutions per nucleotide site per year determined for the two 1983 epidemic variants analyzed here.

Figures 5 and 6 show the nucleotide and deduced amino acid sequences, respectively, of the NP genes of isolates A, C, D, F, and H. The NP gene of A/OH/83 was also sequenced and compared with NP sequences of A/USSR/90/77 and A/Brazil/11/78 (2). Sequences from these three field strains are closely related to those of the isolates from the child. For example, the NP sequences of A/OH/83 and isolate A differed at nucleotide positions 146, 298, 1175, and 1455. These substitutions result in three amino acid replacements at positions 34, 85, and 377, two of which are not observed in isolates C and D from the patient. The amino acid change at position 34 and the silent mutation at position 1455 are conserved in all consecutive isolates.

The nucleotide and amino acid changes in the NP between isolate A and consecutive isolates are summarized in Table 3. Eleven nucleotide changes in the NP sequences of these viruses were detected; 73% of these changes led to amino acid substitutions. Isolate C differed from isolate A in six nucleotide and six amino acid positions; five of these changes were conserved in the next consecutive isolate, D, which also exhibited an additional silent mutation. None of the changes were maintained in the subsequent isolates, F and H. These two viruses had identical NP sequences that differed from that of the first isolate in four nucleotide and two amino acid positions and from those of isolates C and D in nine nucleotide and nine amino acid positions.

Thus, the NP gene of each isolate differed from that of the initial virus in 0.27 to 0.40% of nucleotide positions, representing rates of fixation of mutations of 3.5×10^{-3} to 24×10^{-3} substitutions per nucleotide site per year. These rates are significantly higher than the rate of 2.5×10^{-3} substitutions per nucleotide site per year determined for the human lineage of NP genes (2, 24) or the rate of 4.5×10^{-3} substitutions per nucleotide site per year calculated for the NP genes of the three H1N1 field strains compared in this study. Therefore, the NP gene exhibited extensive nucleotide and amino acid sequence variation, following a pattern of mutation in consecutive isolates similar to that observed for the HA gene.

DISCUSSION

Influenza virus infections in humans are normally self-limited and of short duration. The persistent excretion of influenza virus over a 10-month period by the SCID patient reported here is a rare event. In this study, we examined antigenic and genetic variations in consecutive influenza virus isolates recovered from the immunodeficient patient.

Antigenic analysis (Table 1) and T_1 oligonucleotide fingerprinting (Fig. 2) demonstrated that these isolates were similar to influenza A (H1N1) viruses that were circulating widely in the United States when the child became ill. Differences in the pattern of antigenicity (Table 1) and oligonucleotide changes between fingerprints (Fig. 2) of consecutive isolates demonstrated that the virus underwent variation during this chronic infection. Sequencing two of

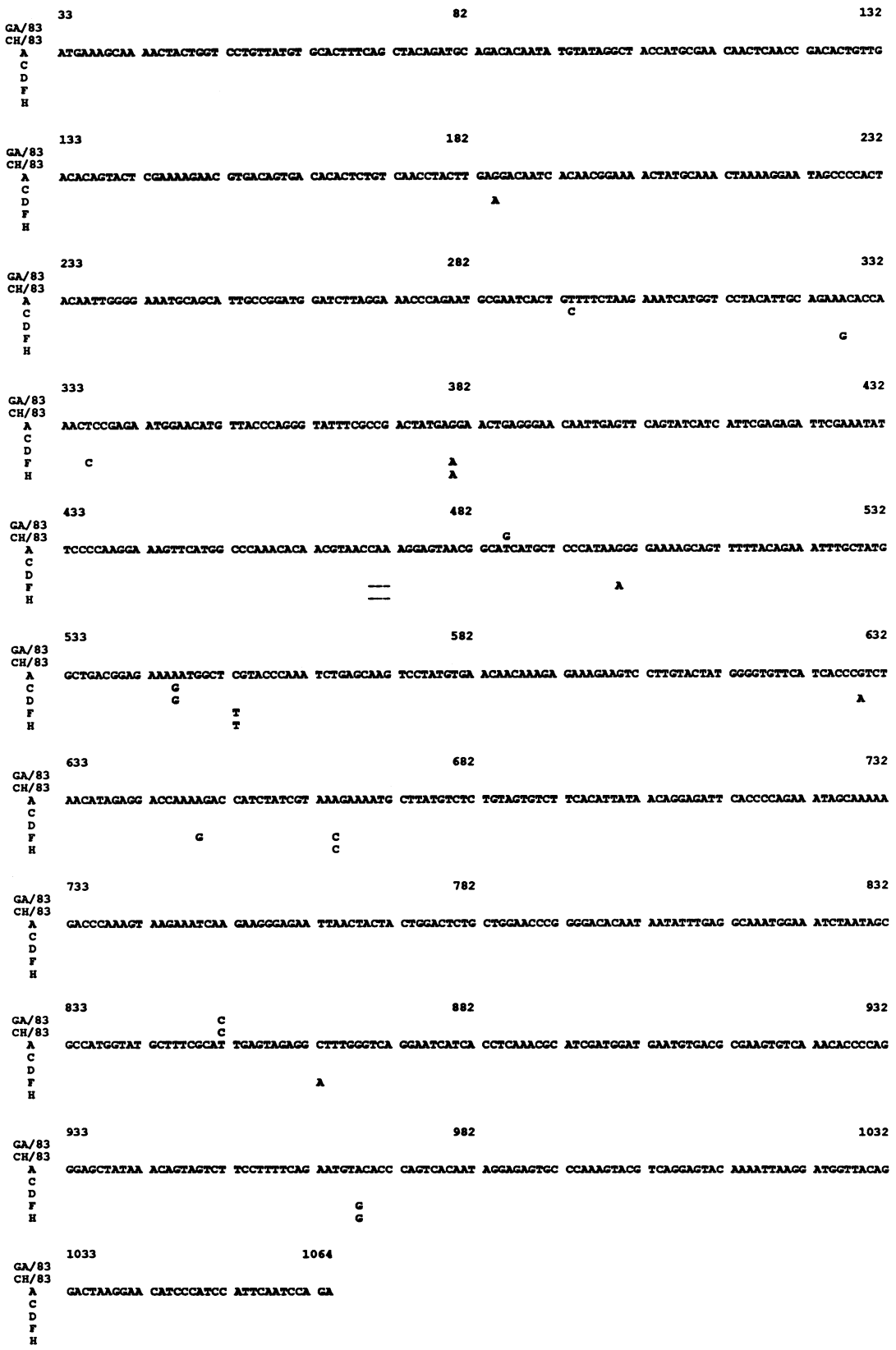

FIG. 3. Nucleotide sequences encoding the HA1 domain of the HA gene (starting at the initiation codon at base 33) of isolates A, C, D, F, and H and two 1983 reference strains, A/GA/83 and A/CH/83. Differences between the sequence of the initial isolate, A, printed in full, and other isolates and reference strains are shown. Dashes (–) under bases 470 to 472 in viruses F and H indicate deleted nucleotides.

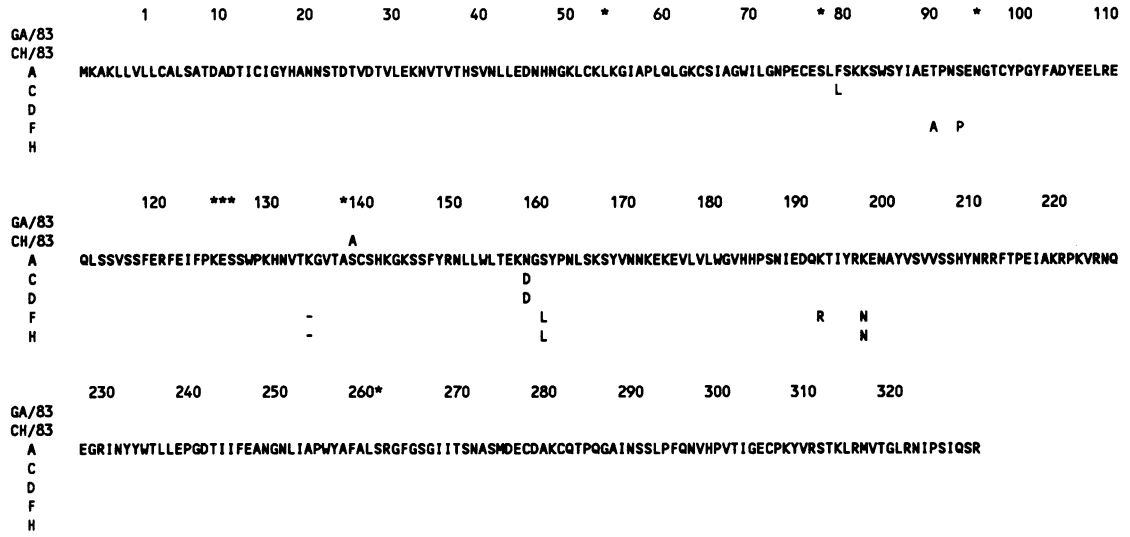

FIG. 4. Amino acid sequences of the HA1 domain of the HA deduced from the nucleotide sequences of isolates A, C, D, F, and H and reference strains A/GA/83 and A/CH/83 shown in Fig. 3. The amino acids are numbered to correspond to the numbering for the H3 subtype, according to the alignment of Winter et al. (60), with additional amino acid residues present in the H1 subtype sequence marked by an asterisk. These additional residues are numbered by a reference to the previous residue followed by a letter designation (e.g., 53a, 77a, 125a, 125b, 125c). The dash (–) under amino acid 134 in viruses F and H indicates a deleted amino acid.

the influenza virus genes (HA and NP) in five isolates showed multiple nucleotide and amino acid substitutions in each gene (Tables 2 and 3). However, in agreement with the T_1 fingerprinting analysis, there was a pattern of nonprogressive fixation of mutations in consecutive isolates, in contrast with the evolutionary pattern observed for influenza virus field strains (4, 10, 44). In some isolates, collected only 4 to 8 weeks apart, the number of substitutions observed both for the HA1 and NP corresponded to the number fixed after several years of evolution of influenza virus field strains. These results suggest that related but novel virus populations predominated in each isolation.

The deletion of residue 134 in isolates E and F is adjacent to amino acid 133, thought to be involved in binding MAb 22 (36). It is likely that this deletion caused the lack of reactivity of both isolates with this MAb (Table 1). Loss of reactivity of isolates E and F with MAb W18 could not be attributed to a particular amino acid substitution. Viruses C and D had a common amino acid substitution from Asn to Asp at position 158 (Table 2), a change that encodes the loss of a potential glycosylation site. This change could cause the different reactivity of the two isolates with polyclonal sera (Table 1). Viruses F and H share the loss of this potential glycosylation site through a substitution of Ser to Leu at amino acid 160 but did not exhibit a similar change in antigenicity. These differences may arise from additional substitutions in isolates F and H that affect binding.

All amino acid changes in the HA1 domain of the isolates occurred in or near antigenic sites (Table 2). Since antigenic domains are located in loops exposed on the surface of the

TABLE 2. Sequence variation in HA1 domains of influenza virus isolates from patient

Nucleotide no.	Nucleotide changes[a] in isolate:					Amino acid		Antigenic site[b]
	A (5/83[c])	C (7/83)	D (9/83)	F (12/83)	H (2/84)	Substitution	No.	
186	GAG	GAG	GAa	GAG	GAG	No change		
294	T/cTT	cTT	TTT	TTT	TTT	Leu/Phe	79	Cb
327	ACA	ACA	ACA	gCA	ACA	Thr/Ala	90	Near Cb
336	TCC	TCC	TCC	cCC	TCC	Ser/Pro	93	Near Cb
380	GAG	GAG	GAG	GAa	GAa	No change		
470–472	ACC–AAA	ACC–AAA	ACC–AAA	AC---A	AC---A	Thr-Lys/Thr	134	Ca
500	AAG	AAG	AAG	AAa	AAG	No change		
546	AAT	gAT	gAT	AAT	AAT	Asn/Asp	158	Sa
553	TCG	TCG	TCG	TtG	TtG	Ser/Leu	160	Sa
629	CCG	CCG	CCa	CCG	CCG	No change		
649	AAG	AAG	AAG	AgG	AAG	Lys/Arg	192	Sb
665	AAA	AAA	AAA	AAc	AAc	Lys/Asn	197	Sb
863	GGC	GGC	GGC	GGa	GGC	No change		
968	GTA	GTA	GTA	GTg	GTg	No change		

[a] Indicated by small letters in the codons.
[b] As defined by Caton et al. (6).
[c] Collection date (month/year) of isolate.

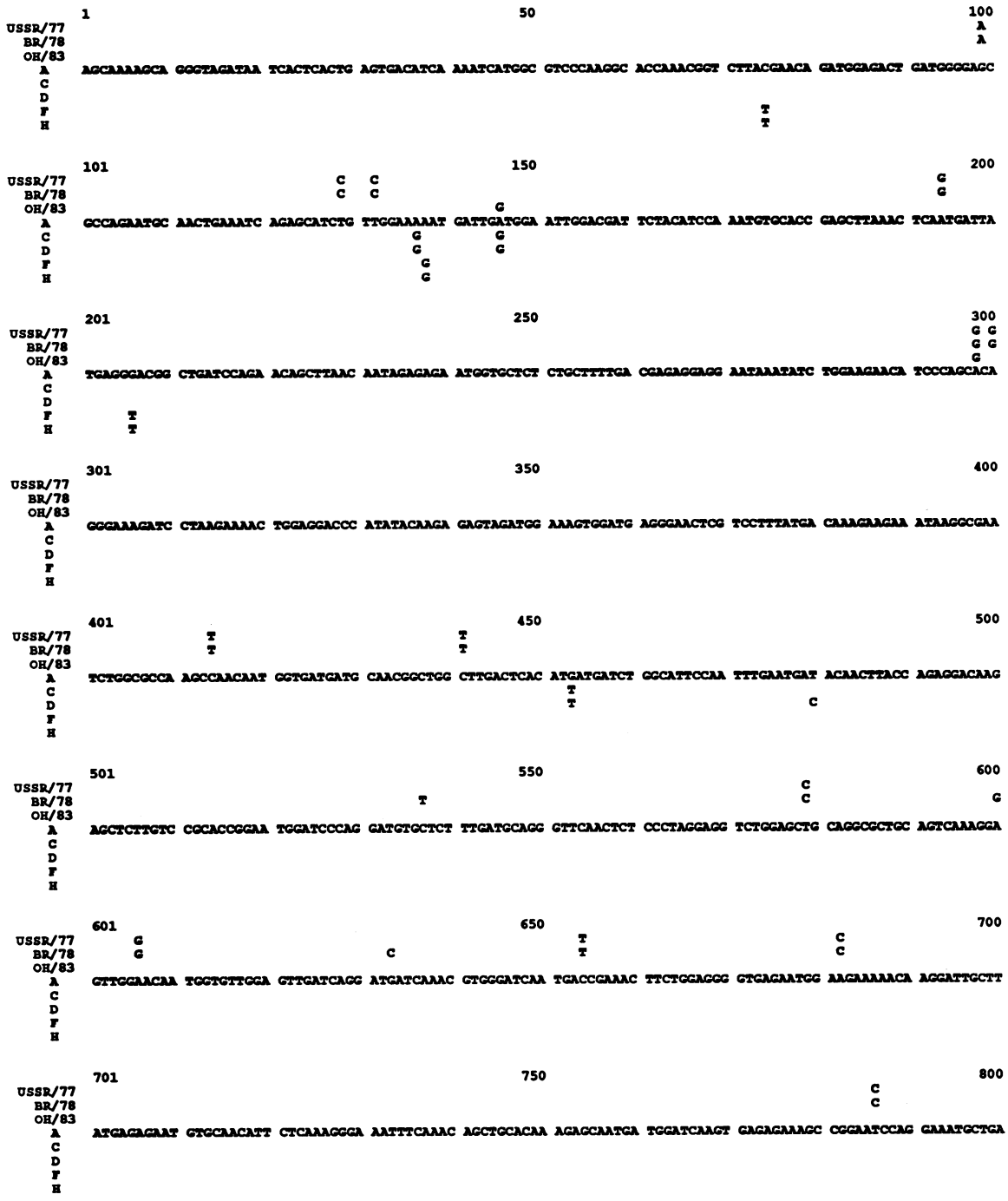

FIG. 5. Nucleotide sequences of the NP gene of isolates A, C, D, F, and H and three H1N1 reference strains, A/USSR/77, A/Brazil/78 (BR/78), and A/OH/83. Differences between the sequence of the initial isolate, printed in full, and other isolates and reference strains are shown.

protein (57, 58), where alterations occur without changing structural or functional features of the HA, variation in antigenic regions need not be the result of immune selection. In support of this idea are the recent observations of Diez et al., who reported the selection of antigenic variants of foot-and-mouth disease virus (FMDV) upon serial passage in cell culture (16) or in virus rescued from BHK-21 cell lines persistently infected with FMDV (15) despite the absence of antibodies. In addition, the virus rescued from the persistent infection showed an elevated number of coding mutations (15). Similarly, antigenic variants have been detected upon adaptation of FMDV to Spinner suspension cultures (3) and in herpes simplex virus during persistence in cell culture (25). Extensive variation has also been observed in certain

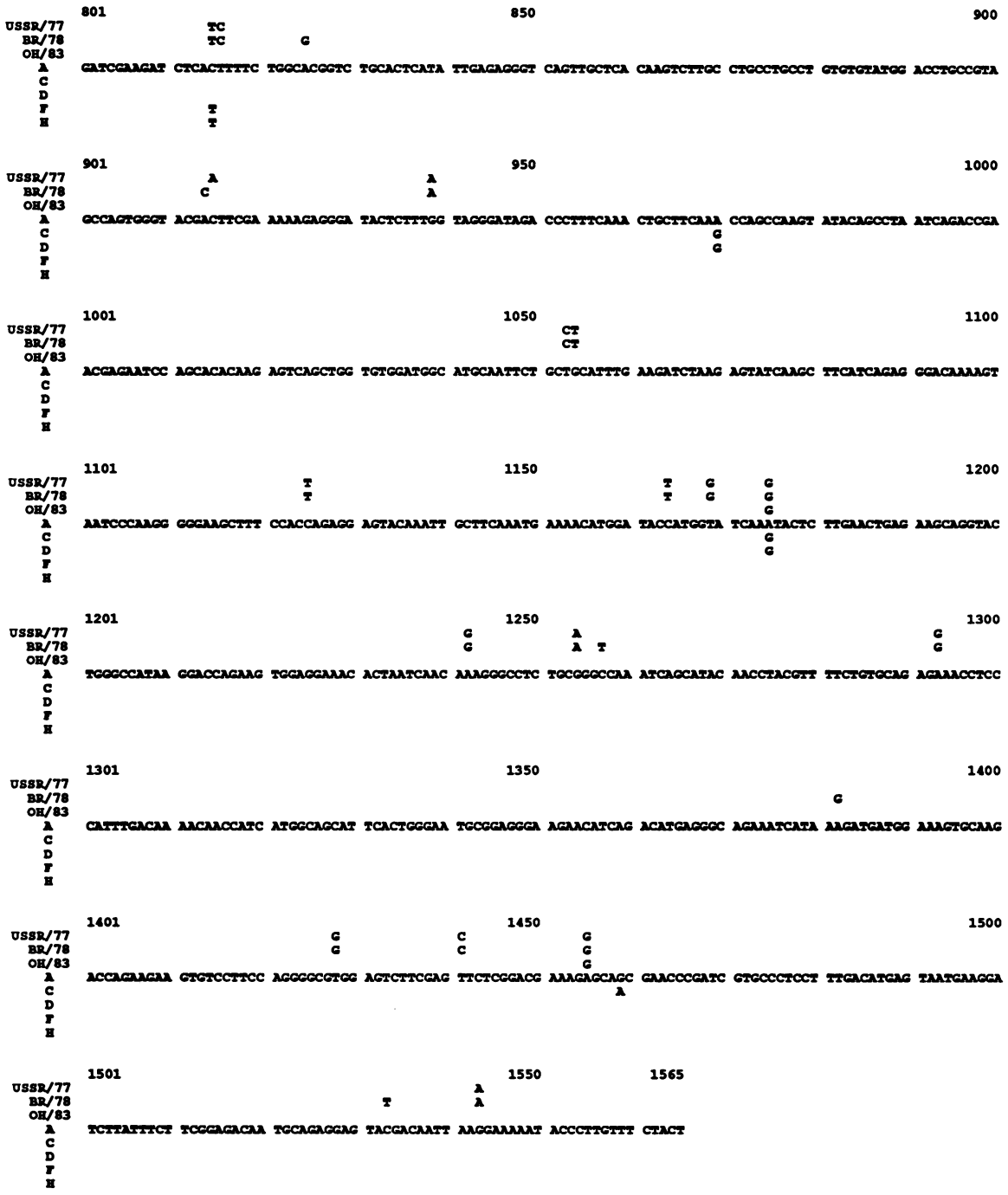

FIG. 5—*Continued.*

regions of the envelope glycoprotein (gp120) of the human immunodeficiency virus in the absence of antibody responses from infected persons (8).

The only demonstrable immune capability of this patient was natural killer activity (see reference 26 for the immunological aspects of the case). However, it cannot be completely ruled out that natural killer activity (51) and/or the repeated administration of intramuscular human immunoglobulin (titer of less than 1:10 to both influenza A H1N1 and H3N2 viruses) provided enough immune pressure to select for the variants. Likewise, it is possible that some level of immune activity acquired from the bone marrow donor contributed to selection of the changes observed in isolates collected after the successful bone marrow graft.

The relatively high number of coding changes observed in the NP genes of the isolates (Table 3) cannot be explained on the basis of antibody selection, since it has been suggested that selection pressure in the NP genes of human influenza virus is exerted by host-specific adaptation (24) and/or the host cytotoxic T-cell response (22, 50).

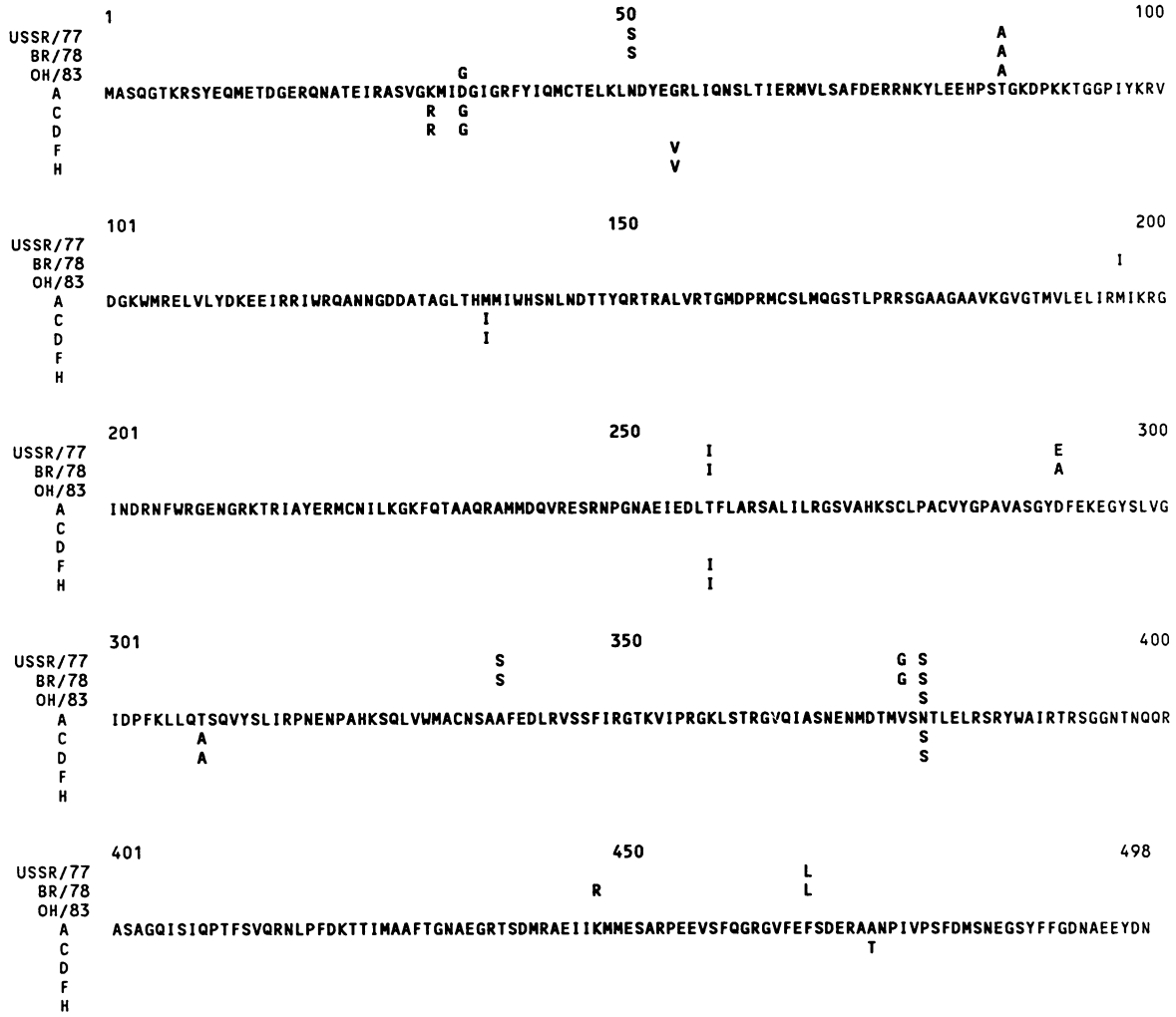

FIG. 6. Amino acid sequences of the NP deduced from the nucleotide sequences of isolates A, C, D, F, and H and reference strains A/USSR/77, A/Brazil/78 (BR/78), and A/OH/83 shown in Fig. 5.

Factors that may have contributed to the observed variation in the influenza virus isolates during the course of this prolonged infection in an immunodeficient host are (i) a change in the number of total virus replications in this special host; (ii) an unusual replication event, such as a mutation in a polymerase gene increasing the gene's rate of copy error; and (iii) laboratory selection of variants. It is unlikely that our observations are due to laboratory selection of variants, because isolates were initially obtained in MDCK cells, high titers of virus were present at each isolation (including egg passages), and the isolates were not cloned or passaged at terminal dilution. No amino acid changes were observed in those positions identified to change during propagation of H1N1 viruses in eggs (31, 45) or in other residues involved in the receptor binding site (55, 56). Furthermore, the same level of variation is not observed in the child's initial isolate (isolate A), which had the same passage history as the other consecutive isolates, or for field isolates received at our laboratory that are routinely cultivated in a similar way (4, 10, 44).

Results presented here for influenza A (H1N1) virus agree with reports of increased genetic and antigenic variations observed in immunocompromised hosts during chronic or persistent infections established by other RNA viruses (rotavirus [30], coxsackievirus [37], FMDV [23], measles virus [7], vesicular stomatitis virus [29], visna virus [47], equine infectious anemia virus [46]; reviewed in reference 17). The factors responsible for the increased variation are thought to be based on conditions that promote disequilibrium of viral populations (reviewed in reference 48). In recent years, detailed antigenic and molecular analyses of RNA viruses have shown that RNA viral populations are extremely heterogeneous (17). Taken together, these studies suggest that each RNA population consists of a distribution of related nonidentical genomes rather than a single defined species (17). Such distributions have been called quasispecies (reviewed in reference 21), a term that reflects both the heterogeneity of viral RNA populations and the competitive replication among continuously arising variant genomes (18, 19). These rapidly mutating populations can remain stable under certain conditions of replication, but any change in environmental conditions that offers a previously disadvantaged variant in a virus population the opportunity to compete favorably with the predominant virus (for example, immune response or selection of MAb-resistant mutants) can shift the equilibrium and drive virus evolution. In this context, the

TABLE 3. Sequence variation in NP of influenza virus isolates from patient

Nucleotide no.	Nucleotide changes[a] in isolate:					Amino acid	
	A (5/83[b])	C (7/83)	D (9/83)	F (12/83)	H (2/84)	Substitution	No.
75	TAC	TAC	TAC	TAt	TAt	No change	
137	AAA	AgA	AgA	AAg	AAg	Lys/Arg	31
138	AAA	AgA	AgA	AAg	AAg	No change	
146	GAT	GgT	GgT	GAT	GAT	Asp/Gly	34
206	GGA	GGA	GGA	GtA	GtA	Gly/Val	54
453	ATG	ATt	ATt	ATG	ATG	Met/Ile	136
480	GAT	GAT	GAc	GAT	GAT	No change	
815	ACT	ACT	ACT	AtT	AtT	Thr/Ile	257
970	ACC	gCC	gCC	ACC	ACC	Thr/Ala	309
1175	AAT	AgT	AgT	AAT	AAT	Asn/Ser	377
1459	GCG	aCG	GCG	GCG	GCG	Ala/Thr	472

[a] Indicated by the small letters in the codons.
[b] Collection date (month/year) of isolate.

genetic diversity of these influenza virus isolates obtained from a chronically infected immunodeficient child likely reflects fluctuations in the population equilibrium of variants rather than an increased mutation rate.

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