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Comparison of Complete Amino Acid Sequences and Receptor-Binding Properties among 13 Serotypes of Hemagglutinins of Influenza A Viruses

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We determined the sequences of 7 serotypes (H4, H6, H8, H9, H11, H12, and H13) of hemagglutinin (HA) genes, which have not been reported so far. The coding regions consisted of 1692 nucleotides in H4, 1698 in H6, 1695 in H8, 1680 in H9, 1695 in H11, 1692 in H12, and 1698 in H13, and specified 564, 566, 565, 560, 565, 564, and 566 amino acids, respectively. By comparison of amino acid sequences, 13 HA serotypes could be divided into two families, i.e., an H1 group (H1, H2, H5, H6, H8, H9, H11, H12, and H13) and an H3 group (H3, H4, H7, and H10). The relationship was essentially similar to that reported by Air from the comparison of 80 amino-terminal amino acid sequence of 12 HA serotypes (G.M. Air, 1981, *Proc. Natl. Acad. Sci. USA* 78, 7639–7643). Though a considerable amino acid sequence difference exists between certain HA serotypes, several amino acid residues in fusion peptides ($HA_2$1–11) and receptor-binding sites ($HA_1$98, −134, −138, −153, −183, and −195) were shown to be conserved among the 13 HA serotypes. Human H1 and avian H3, H4, H8, and H10 viruses preferentially bound NeuAcα2,3Gal sequences, whereas human H2 and H3 and avian H6 and H9 viruses bound NeuAcα2,6Gal sequences, although the amino acid residues at position 226 of human H2 and avian H6 and H9 serotype HAs are glutamine. These results show that the amino acid residue at position 226 is not necessarily a determinant of receptor specificity for all serotypes. © 1991 Academic Press, Inc.

INTRODUCTION

The hemagglutinin (HA) glycoprotein of influenza A virus performs two crucial functions in the early stage of virus infection. First, HA is responsible for binding of the virus to cell surface receptors (Hirst, 1941) and, second, it mediates liberation of the viral genome into the cytoplasm through membrane fusion (Maeda and Ohnishi, 1980; Huang *et al.*, 1981; Lenard and Miller, 1981). Since the three-dimensional structure of H3 HA was determined by X-ray analysis (Wiley *et al.*, 1981; Wilson *et al.*, 1981), functional regions of H3 HA protein, i.e., the receptor-binding site and fusion peptide, have been located on the three-dimensional model. HA is also known to be the major variable antigen (Webster *et al.*, 1983), and influenza A viruses isolated from birds and mammals are subdivided into 13 serotypes by antigenic differences. The primary structures of 6 (H1, H2, H3, H5, H7, and H10) of the 13 serotypes had been determined by 1988 (for references, see Klenk and Rott 1988), and in 1989 those of H4 and H13 were reported (Chambers *et al.*, 1989; Donis *et al.*, 1989). The sequences of the other 5 serotypes (H6, H8, H9, H11, and H12) remained to be determined, even though the sequences of the 3′ end of the viral genomic RNA had been determined (Air, 1981).

The study by Rogers *et al.* (1983) showed that the amino acid residue at position 226 of H3 HA is involved in receptor specificity and sensitivity to neutralization by a glycoprotein inhibitor present in horse serum. Recently, Daniels *et al.* (1987) showed that a deletion mutant ($HA_1$224–230 deleted) retained receptor-binding activity, but lost the ability of discrimination between NeuAcα2,6Gal and NeuAcα2,3Gal linkages. This finding suggested that residue 226 of H3 HA is included in the region which determines linkage specificity but is less directly involved in the receptor binding itself. We showed, however, a significant role of residue 226 in receptor binding for H1 HA (Nobusawa and Nakajima, 1988). A difference in the role of residue 226 between H1 and H3 HA in receptor binding shows a difference in responsibility of amino acid residues located at the same position.

In the present study we determined the nucleotide sequences of genes of 7 serotypes of HA and deduced the amino acid sequences. Amino acid sequences of the receptor-binding sites and its vicinity were compared among 13 serotypes. Receptor specificity was studied for 9 serotypes.

Sequence data from this article have been deposited with the DDBJ, EMBL, and GenBank Data Libraries under Accession Nos. D90302–D90308.

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MATERIALS AND METHODS

Viruses and virus RNA

The influenza viruses A/Turkey/Ontario/6118/68 (H8N4), A/Duck/Alberta/60/76 (H12N5), and A/Gull/Maryland/704/77 (H13N6) were generously provided by Dr. H. Kida (Hokkaido University). Other viruses used for cDNA cloning included A/Duck/Czechoslovakia/56 (H4N6), A/Shearwater/Australia/1/72 (H6N5), A/Turkey/Wisconsin/66 (H9N2), A/Duck/England/56 (H11N6). A/Duck/Ukraine/1/63 (H3N8) and A/Tern/South Africa/61 (H5N3) were also used for the study of receptor specificity. These viruses were grown in 11-day-old embryonated chicken eggs and were purified by centrifugation through 30–60% (w/v) discontinuous sucrose gradients in a Beckman SW28 rotor. Virion RNA for cloning was extracted by the hot phenol method (Palese and Schulman, 1976).

Cloning of the HA gene and determination of nucleotide sequences

HA-cDNA was prepared by reverse transcription of virion RNA according to the method of Gubler and Hoffman (Gubler and Hoffman, 1983). Oligonucleotide primer (5′ AGCAAAAGCAGG 3′) complementary to the 3′ terminus of the negative strand RNA was used for cDNA cloning. Double-stranded cDNAs were separated by electrophoresis through agarose gels (0.8% in Tris–EDTA–acetate buffer, pH 8.0) and a cDNA band corresponding to a length of about 1700 bp was eluted. Poly(dC)-tailed cDNA was inserted into poly(dG)-tailed plasmid pBR322 at PstI site. After transfection of *Escherichia coli* C600 with the recombinant DNA, colony hybridization was performed. Several oligonucleotides were synthesized, according to partial sequences of the 3′ end of the negative-strand RNA of HA genes (Air, 1981), and utilized as hybridization probes for screening for a clone of each HA serotype. The oligodeoxyribonucleotides used as probes were 5′ TGGTCCTACATAGTGGA 3′ for H6, H8, and H9; 5′ ATGGTCCTACATAGT 3′ for H11 and H12; and 5′ ATATGCCTGGGACATCA 3′ for H4 and H10. Since not all the virus strains used in this study are the same as those in the study of Air (1981), the synthetic oligonucleotides may not have been completely complementary to cDNA of the respective HA serotype. The conditions for colony hybridization were therefore relaxed initially and filters were washed at increasing temperatures until the probes were dissociated from nonspecific DNA. The colonies showing positive hybridization were purified, and DNAs were isolated from these colonies for subcloning into phage M13 mp18 or mp19. For DNA sequence analysis dideoxy chain termination method was used (Sanger *et al.*, 1977).

Transfection and generation of recombinant virus stocks

SVHA recombinant DNAs were introduced into subconfluent monolayers of CV-1 cells together with helper virus DNA by DEAE-dextran method (McCutchan and Pagano, 1968) as described previously (Nobusawa *et al.*, 1987). For characterization of the HA expressed from SVHA, subconfluent monolayers of CV-1 cells were infected with virus stock containing SVHA recombinant virus and helper virus dl-1055.

Mutagenesis

The procedure used for site-specific mutagenesis was described previously (Nobusawa *et al.*, 1985). M13 recombinant phages containing a mutant HA DNA were screened by plaque hybridization. After the presence of desired base substitutions was confirmed by sequencing using dideoxy chain termination method (Sanger *et al.*, 1977), the DNA fragments containing substitutions in the HA cDNA of M13 recombinant phages were exchanged with the corresponding region in the SVHA-2 (Nobusawa *et al.*, 1987).

Indirect immunofluorescence

Monolayers of CV-1 cells were infected with each recombinant virus stock. At 48 to 60 hr postinfection, infected cells were examined either for cell surface expressed HA protein without fixation or for intracellular HA protein after fixation with ethanol/acetone (1:1). Indirect immunofluorescence staining with rabbit anti-A/USSR/77 HA serum generously provided by Dr. K. Nerome (National Institute of Health, Tokyo, Japan) was performed as described previously (Nobusawa *et al.*, 1985).

Receptor specificity of influenza virus hemagglutinin for sialyloligosaccharides of gangliosides

The assay system was designed to determine the ability of ganglioside-coated erythrocytes to undergo influenza virus-mediated hemolysis at a low pH at 37° as well as agglutination at pH 7.2 at 4°, as described in previous papers (Suzuki *et al.*, 1986, 1989).

RESULTS

Comparison of nucleotides and amino acid sequences among 13 serotype HAs

The nucleotide sequences of the HA genes of H4 (A/Duck/Czechoslovakia/56), H6 (A/Shearwater/Australia/1/72), H8 (A/Turkey/Ontario/6118/68), H9 (A/Turkey/Wisconsin/66), H11 (A/Duck/England/56), H12 (A/Duck/Alberta/60/76), and H13 (A/Gull/Maryland/

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TABLE 1

Serotype	Amino acid number[a] −15	−10	−1	+1
(a) Comparison of amino acid sequences of signal peptides among the 13 serotype HAs				
H1	M K A N L L V L L C A L A A A D A			D
H2	M A I I Y L I L L F T A V R G			D
H5	M E E I V L L F A I V S L A R S			D
H6	M I A I I V V A I L A T A G R S			D
H11	M E K T L L F A A I F L C V K A			D
H13	M A L N V I A T L T L I S V C V H A			D
H9	M E T K A I I A A L L M V T A A N A			D
H8	M E K F I A I A T L A S T N A			Y
H12	M E K F I I L S T V L A A S F A			Y
H7	M N T Q I L V F A L V A V I P T N A			D
H10	M Y K V V V I I A L L G A V K G			L
H4	M L S I V I L F L L I A E N S S			Q
H3	M K T I I A L S Y I F C L A L G			Q

Serotype	Amino acid number 185	190	200	210	
(b) Comparison of amino acid sequences of membrane anchor region among the 13 serotype HAs					
H1	Q I L A I Y S T V A S S L V L L V S L G A I S F W M C S				(−24.8)
H2	Q I L A I Y A T V A G S L S L A I M M A G I S F W M C S				(−28.7)
H5	Q I L S I Y S T V A S S L A L A I M V A G L S F W M C S				(−27.8)
H6	Q I L A I Y S T V S S S L V L V G L I I A V G L W M C S				(−29.9)
H11	K I L S I Y S C I A S S L V L A A L I M G F M F W A C S				(−28.7)
H13	K A L S I Y S C I A S S V V L V G L I L S F I M W A C S				(−27.1)
H9	K I L T I Y S T V A S S L V L A M G F A A F L F W A M S				(−28.7)
H8	K I L S I Y S T V A A S L C L A I L I A G G L I L G M Q				(−24.2)
H12	K I L S I Y S S V A S S L V L L L M I I G G F I F G C Q				(−26.4)
H7	V I L W F S F G A S C F L L L A I A V − G L V F − I C V				(−36.7)
H10	I I L W F S F G E S C F V L L A V V M − G L V F F − C L				(−34.7)
H4	I I L W I S F S I S C F L L V A L L L A − F I L W A C Q				(−39.8)
H3	W I L W I S F A I S C F L L C − V V L L G F I M W A C Q				(−41.4)

Note. Hydrophobic values are given in parentheses. Dashes are inserted for maximum sequence homology.

[a] The amino acid numbering used for the H3 HA (Wilson *et al.*, 1981) was used. The signal peptidase cleavage site is between the amino acid residues at positions −1 and +1.

704/77) serotype viruses were determined by sequencing cloned DNAs. The coding regions of H4, H6, H8, H9, H11, H12, and H13 serotype HA genes consisted of 1692, 1698, 1695, 1680, 1695, 1692, and 1698 nucleotides and specified 564, 566, 565, 560, 565, 564, and 566 amino acids, respectively. Therefore the complete amino acid sequences are now available for HAs of all 13 serotypes. The nucleotide sequences of H4 and H13 HA genes have recently been determined for the same strains used in this study (Donis *et al.*, 1989; Chambers *et al.*, 1989). Our sequences are different from the above-reported sequences in 14 nucleotides and 4 amino acids for H4, and in 8 nucleotides and 2 amino acids for H13 HA. The discrepancy might be due to different passage histories of the virus strains used.

The amino-terminal signal peptide cleavage site has not been identified directly by amino acid sequencing. By analogy with H1, H2, H5, and H7 (Air, 1981; Kawaoka *et al.*, 1984) we assumed that the amino terminus of HA1 is aspartic acid for H6, H9, H11, and H13 serotype HAs and glutamine for H4 as shown by Donis *et al.* (1989). According to the (−3, −1)-rule of Von Heijne (1986), it was unlikely that the HA1s of H8 and H12 have aspartic acid at its amino terminus as had been suggested (Air, 1981). Using an algorithm which predicts cleavage sites for signal peptidase (Von Heijne, 1986), we presumed that the amino terminus of

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FIG. 1. Comparison of amino acid sequences of 13 HA serotypes. The sequences are aligned according to Kawaoka *et al.* (1984) and Feldmann *et al.* (1988). Dashes are inserted to give maximam sequence homology. The conserved amino acid residues are in bold face. The amino acid numbering for H3 serotype is shown on the right side. Numbering is restarted for HA2 in (b). H1; A/PR/8/34 (Winter *et al.*, 1981)); H2; A/Japan/305/57 (Gething *et al.*, 1980); H3; A/Aichi/2/68 (Verhoeyen *et al.*, 1980); H4, A/Duck/Czechoslovakia/56; H5; A/Chick/Pennsylvania/1370/83 (Kawaoka *et al.*, 1984); H6; A/Shearwater/Australia/1/72; H7; A/FPV/Rostock/34 (Porter *et al.*, 1979); H8; A/Turkey/Ontario/6118/68; H9; A/Turkey/Wisconsin/66; H10; A/Chicken/Germany/N/49 (Feldmann *et al.*, 1988); H11; A/Duck/England/56; H12; A/Duck/Alberta/60/76; H13; A/Gull/Maryland/704/77.

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b

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H1  GLFGAIAGFIEGGWTGMIDGWYGYHHQNEQGSGYAADQKSTQNAINGITNKVNSVIEKMNIQFTAVGKEFNKLEKRMENL
H2  GLFGAIAGFIEGGWQGMVDGWYGYHHSNDQGSGYAADKESTQKAFDGITNKVNSVIEKMNTQFEAVGKEFGNLERRLENL
H3  GLFGAIAGFIENGWEGMIDGWYGFRHQNSEGTGQAADLKSTQAAIDQINGKLNRVIEKTNEKFHQIEKEFSEVEGRIQDL  80
H4  GLFGAIAGFIENGWQGLIDGWYGFRHQNAEGTGTAADLKSTQAAIDQINGKLNRLIEKTNDKYHQIEKEFEQVEGRIQDL
H5  GLFGAIAGFIEGGWQGMVDGWYGYHHINGQGSGYAADKKSTQKAIDGITNKVNSIIDKMNTQFEAVGREFNNLERRIENL
H6  GLFGAIAGFIEGGWTGMIDGWYGYHHENSQGSGYAADRESTQKAVDGITNKVNSIIDKMNTQFEAVDHEFSNLERRIDNL
H7  GLFGAIAGFIENGWEGLVDGWYGFRHQNAQGEGTAADYKSTQSAIDQITGKLNRLIEKTNQQFELIDNEFTEVEKQIGNL
H8  GLFGAIAGFIEGGWSGMIDGWYGFHHSNSEGTGMAADQKSTQEAIDKITNKVNNIVDKMNREFEVVNHEFSEVEKRINMI
H9  GLFGAIAGFIEGGWPGLVAGWYGFQHSNDQGVGMAADKGSTQKAIDKITSKVNNIIDKMNKQYEVIDHEFNELEARLNMI
H10 GLFGAIAGFIENGWEGMVDGWYGFRHQNAQGTGQAADYKSTQAAIDQITGKLNRLIEKTNTEFESIESEFSETEHQIGNV
H11 GLFGAIAGFIEGGWPGLINGWYGFQHRDEEGTGIAADKESTQKAIDQITSKVNNIVDRMNTNFESVQHEFSEIEERINQL
H12 GLFGAIAGFIEGGWPGLVAGWYGFQHQNAEGTGIAADRDSTQRAIDNMQNKLNNVIDKMNKQFEVVNHEFSEVESRINMI
H13 GLFGAIAGFIEGGWPGLINGWYGFQHQNEQGTGIAADKESTQKAIDQITTKINNIIDKMNGNYDSIRGEFNQVEKRINML

H1  NKKVDDGFLDIWTYNAELLVLLENERTLDFHDSNVKNLYEKVKSQLKNNAKEIGNGCFEFYHKCDNECMESVRNGTYDYP
H2  NKRMEDGFLDVWTYNAELLVLMENERTLDFHDSNVKNLYDKVRMQLRDNVKELGNGCFEFYHKCDDECMNSVKNGTYDYP
H3  EKYVEDTKIDLWSYNAELLVALENQHTIDLTDSEMNKLFEKTRRQLRENAEEMGNGCFKIYHKCDNACIESIRNGTYDHD 160
H4  ENYVEDTKIDLWSYNAELLVALENQHTIDVTDSEMNKLFERVRRQLRENAEDKGNGCFEIFHKCDNNCIESIRNGTYDHD
H5  NKNLEDGFIDVWTYNAELLVLMENERTLDLHDSNVKNLYDKVRLQLRDNAKEWGNGCFEFYHKCDNECMESVRNGTYNYP
H6  NKRMEDGFLDVWTYNAELLVLLENERTLDLHDANVKNLYERVKSQLRDNAMILGNGCFEFWHKCDDECMESVKNGTYDYP
H7  INWTKDFITEVWSYNAELLVAMENQHTIDLADSEMNKLYERVRKQLRENAEEDGTGCFEIFHKCDDDCMASIRNNTYDHS
H8  NDKIDDQIEDLWAYNAELLVLLENQKTLDEHDSNVKNLFDEVKRRLSANAIDAGNGCFDILHKCDNECMETIKNGTYDHK
H9  NNKIDDQIQDIWAYNAELLVLLENQKTLDEHDANVNNLYNKVKRALGSNAVEDGNGCFELYHKCDDQCMETIRNGTYDRQ
H10 INWTKDSITDIWTYNAELLVAMENQHTIDMADSEMLNLYERVRKQLRQNAEEDGKGCFEIYHTCDDSCMESIRNNTYDHS
H11 SKHVDDSVVDIWSYNAQLLVLLENEKTLDLHDSNVRNLHEKVRRMLKDNAKDEGNGCFTFYHKCDNKCIERVRNGTYDHK
H12 NSKIDDQITDIWAYNAELLVLLENQKTLDEHDANVRNLHDRVRRVLRENAIDTGDGCFEILHKCDNNCMDTIRNGTYNHK
H13 ADRIDDAVTDIWSYNAKLLVLLENDKTLDMHDANVKNLHEQVRRELKDNAIDEGNGCFELLHKCNDSCMETIRNGTYDHT

H1  KYSEESKLNREKVDGVKLESMGI-YQILAIYSTVASSLVLLVSLGAISFWMCS-NGSLQCRICI
H2  KYEEESKLNRNEIKGVKLSSMGV-YQILAIYATVAGSLSLAIMMAGISFWMCS-NGSLQCRICI
H3  VYRDEALNNRFQIKGVELKS-GYKDWILWISFAISCFLLC-VVLLGFIMWACQR-GNIRCNICI 221
H4  IYRDEAINNRFQIQGVKLTQ-GYKDIILWISFSISCFLLVALLLA-FILWACQ-NGNIRCQICI
H5  KYSEESKLKRKEIDGIKWESMG-TYQILSIYSTVASSLALAIMVAGLSFWMCS-NGSLQCRICI
H6  KYQDESKLNRQEIESVKLESLG-VYQILAIYSTVSSSLVLVGLIIAVGLWMCS-NGSMQCRICI
H7  KYREEAMQNRIQIDPVKLSS-GYKDVILWFSFGASCFLLLAIAV-GLVF-ICVKNGNMRCTICI
H8  EYEEEAKLERSKINGVKLEEN-TTYKILSIYSTVAASLCLAILIAGGLILGMQ-NGSCRCMFCI
H9  KYQEESRLERQKIEGVKLESEG-TYKILTIYSTVASSLVLAMGFAAFLFWAMS-NGSCRCNICI
H10 QYREEALLNRLNINPVKLSS-GYKDIILWFSFGESCFVLLAVVM-GLVFF-CLKNGNMRCTICI
H11 EFEEESKINRQEIEGVKLDSSGNVYKILSIYSCIASSLVLAALIMGFMFWACS-NGSCRCTICI
H12 EYEEESKIERQKVNGVKLEENS-TYKILSIYSSVASSLVLLLMIIGGFIFGCQ-NGNVRCTFCI
H13 EYAEESKLKRQEIDGIKLKSEDNVYKALSIYSCIASSVVLVGLILSFIMWACS--GNCRFNVCI
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FIG. 1—*Continued*

HA1 is at Tyr-16 for H8 and Tyr-17 for H12 HA (Table 1a).

With the above seven HA serotypes, proteolytic cleavage of HA0 to HA1 and HA2 was thought to take place at the carboxyl side of arginine at position 329 (H3 numbering) as with other HA serotypes. There is a single arginine at the cleavage site between HA1 and HA2 (Fig. 1). The HA2 amino terminus starts at glycine residue following arginine. Mature HAs, therefore, consist of the HA1 subunit containing 327 (H6), 326 (H4 and H8), 325 (H11), 324 (H12 and H13), and 319 (H9) amino acids and the HA2 subunit containing 223 (H11 and H13), 222 (H6, H8, H9, and H12), and 221 (H4) amino acids.

The putative membrane anchor regions at the carboxy terminus of HA2 lie between HA_2185 and HA_2211 (H3 numbering; Table 1b) and the nearly entire stretch is hydrophobic, as are the signal peptides. The hydrophobicity value of each membrane anchor peptides in the 13 serotype HAs is shown in parentheses in Table 1b. These values show that the membrane anchor regions of the H3 group is more hydrophobic than that of the H1 group. The significance of higher hydrophobic value of the H3 group remains unknown. HA_2187 leucine, (H3 numbering) is conserved all through the 13 serotypes, while several amino acid residues are conserved within each of the H1 or H3 groups [Ile-186, Ile-189, Tyr/Ser-190, Phe-191, Ser-194, Cys-195, Ser/Phe-196, Leu-199, Cys-210 (H3 numbering)] and among some serotypes within each group [Gln/Lys-185, Phe/Ile-189, Leu/Phe-205 (H3 numbering), etc.] as shown in Table 1b.

There are 4, 8, 10, 7, 7, 9, and 7 potential glycosylation sites (Asn–X–Ser/Thr) on H4, H6, H8, H9, H11, H12, and H13 HAs, respectively. The sites conserved among two or more members of the 13 HA serotypes are the residues Asn-20,21 (H1, H2, H6, H8, and H11), Asn-21 (H9 and H13), or Asn-22 (H3, H4, H7, and H10) of HA1 and the residue 154 of HA2 (H3 numbering).

Homology of amino acid sequences among the 13 serotype HAs

The deduced amino acid sequences of the coding regions for the 13 serotype HAs are aligned with each

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other (Fig. 1). Thirteen cysteine residues, 12 of which may compose six disulfide bonds and the 18 glycine and 6 proline residues are conserved among the 13 serotypes. Moreover, fusion peptides (14 amino acids in length) at the amino terminus of HA2 are strictly conserved except for the residue at position 12 (Fig 1b).

For the purpose of phylogenetic analysis of all HA serotypes, the degree of amino acid sequence homology among the 13 serotypes was calculated using the Biosequence Analysis System of the Institute of Medical Science, University of Tokyo, run on a VAX-11/750 computer system. After transformation of the sequence homology into amino acid differences and application of Kimura's (1983) correction method to the differences, phylogenetic analysis was carried out by the modified Farris method (Tateno *et al.*, 1982) and neighbor joining method (Saitou and Nei, 1987). Both methods gave essentially the same phylogenetic tree topology. The result obtained by the modified Farris method is shown in Fig. 2. The phylogenetic tree is similar to those suggested by Air (1981) and Hinshaw (1982), except that the relationships among the H8, H9, and H12 are different between ours and theirs. The 13 serotypes are arranged in Table 2 in the same order as in the phylogenetic tree (Fig. 2). Comparison of the entire amino acid sequence homology of the 13 serotypes confirmed the validity of the previous division of 12 serotypes into two groups from the comparison of 80 amino-terminal amino acid sequence (Air, 1981). In this study, the 13 serotypes are divided into an H1 group comprising H1, H2, H5, H6, H8, H9, H11, H12, and H13 serotypes and an H3 group comprising H3, H4, H7, and H10 serotypes. H13 HA is included in the H1 group and has 59.3% homology with H11 HA and 52.2% homology with H9 HA, but has less than 50% homology with the other serotype HAs in the H1 group. Amino acid sequence homology within each group ranges from 47.5 to 74.1% (group H1) and 49.6 to 66.5% (group H3) (Table 2). In contrast, similarity ranges from 39.8 to 44.4% between serotypes belonging to different groups. However, the similarity of the HA2 subunit is about 50% even between groups and this shows that the HA2 subunit is more conserved than the HA1 subunit. In group H1, the closest pair is H2 and H5 (74.1%) followed by the pairs of H1–H2 (67.2%), H8–H12 (66.7%), H1–H5 (65.5%), and H9–H12 (65.1%). In group H3, the closest pairs are H3 and H4 (66.5%) and H7 and H10 (65.3%). Other pairs in group H3 have about 50% homology. Comparison of entire amino acid sequences shows that the regions conserved among several closely related serotypes are not localized in a particular region, but the extent of homology is roughly uniform throughout the HA protein.

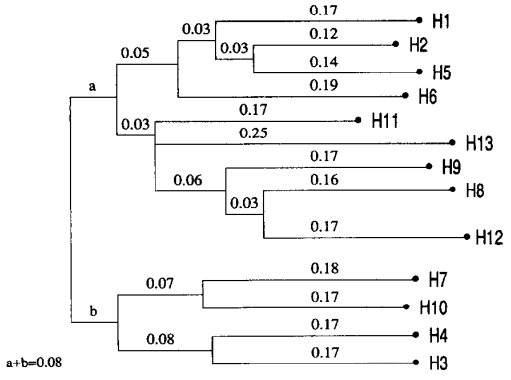


FIG. 2. Phylogenetic tree for the 13 serotype HAs. The distance matrix was calculated from amino acid identity values shown in Table 2 by the method of Dayhoff (1972) and the dendrogram was constructed from using the modified Farris method (Tateno *et al.*, 1982). The root of the dendrogram could not be estimated by this method. Branch length represents genetic distance. Viruses in this figure are the same as those in Fig. 1.

Comparison of the receptor-binding site among the 13 serotypes

Examination of amino acid sequences involved in receptor binding in the 13 serotype HAs revealed the following. Several amino acids known to form the receptor-binding cavity and the "second shell" in H3 HA (H3 numbering: Tyr-98, Gly-134, Ala-138, Trp-153, and His-183, Tyr-195) (Weis *et al.*, 1988) are conserved all through the 13 serotypes (Table 3). Other amino acids relatively well conserved are Ser-136 (Thr in H1, H6, H7, H10, H11, and H13), Leu-194 (Ile in H1), Gly-225 (Asp in H1), Gly-228 (Ser in H3 and H13), and Arg-229 (Trp in H13). Additionally, the 7 serotype HAs sequenced in this study possessed Gln-226, which is known to be involved in differential receptor specificity in the H3 serotype.

Receptor-binding specificity for several HA serotype influenza isolates

Receptor-binding specificity was assayed with gangliosides containing sugar chains with either *N*-acetylneuraminic acid (NeuAc) or *N*-glycolylneuraminic acid (NeuGc) and with either NeuAcα2,3Gal or NeuAcα2,6Gal linkage for the HA of human and avian origin, and of various serotypes (H1 through H10 with the exception of H7) (Table 4). All viruses bound more strongly to the *N*-acetylneuraminic acid species of sialic acid than to those of the *N*-glycolyl type. It is worth noting that A/PR/8/34 (H1N1), A/Duck/Ukraine/1/63 (H3N8), A/Duck/Czechoslovakia/56 (H4N6), A/Tern/South Africa/61 (H5N3), A/Turkey/Ontario/6118/68(H8N4), and A/Chicken/Germany "N"/49 (H10N7)

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preferentially bound to NeuAcα2,3Gal-containing oligosaccharides, whereas, A/Japan/305/57(H2N2), A/Aichi/2/68 (H3N2), A/Shearwater/Australia/1/72(H6N5), and A/Turkey/Wisconsin/66(H9N2) bound to NeuAcα2,6Gal-containing oligosaccharides.

Comparison of the structure of the receptor-binding site and its vicinity

Comparison of amino acid sequences of the receptor-binding site among the 13 serotypes showed that there are several amino acid residues which compose the receptor-binding site but are not conserved. A variant of H3 HA from which residues $HA_1$224–230 consisting the left edge of the receptor-binding site are deleted retained receptor-binding activity (Daniels *et al.*, 1987). To compare the structural requirement of H1 and H3 HAs for receptor-binding activity, we prepared a similar mutant H1 HA protein expressed from the SVHA recombinant virus. A stretch of wild-type H1 (A/USSR/90/77) HA gene in the SVHA-2 (Nobusawa *et al.*, 1988) was replaced by a mutant sequence. In the

TABLE 2

AMINO ACID SEQUENCE HOMOLOGY[a] AMONG HA MOLECULE OF VIRUSES REPRESENTING EACH OF THE 13 SEROTYPES

	H1	H2	H5	H6	H11	H13	H9	H8	H12	H7	H10	H4	H3
H1		67.2 (58.7) [79.6]	65.5 (55.7) [79.8]	60.8 (50.1) [76.4]	55.5 (51.1) [61.9]	48.6 (42.7) [57.2]	53.0 (45.1) [64.5]	52.6 (46.5) [61.5]	49.8 (43.9) [58.4]	41.6 (33.8) [53.0]	43.5 (36.4) [53.9]	41.8 (35.4) [51.1]	42.6 (35.2) [53.4]
H2			74.1 (66.7) [84.9]	62.2 (50.3) [79.5]	55.0 (50.6) [61.4]	47.5 (42.9) [54.2]	53.2 (46.5) [63.0]	49.7 (43.0) [59.5]	48.2 (43.6) [54.9]	43.1 (36.1) [53.3]	43.9 (37.7) [53.0]	40.4 (35.2) [48.0]	41.7 (36.0) [50.0]
H5				61.2 (50.0) [77.6]	56.2 (51.2) [63.5]	49.2 (43.4) [57.7]	51.5 (42.6) [64.5]	50.3 (41.9) [62.2]	48.3 (41.3) [58.5]	44.4 (36.8) [55.5]	43.2 (35.8) [54.0]	41.1 (35.0) [50.0]	43.2 (37.3) [51.8]
H6					55.5 (50.2) [63.2]	49.6 (43.0) [59.2]	54.7 (48.3) [64.0]	52.8 (46.8) [61.6]	51.0 (45.1) [59.8]	42.4 (35.6) [52.3]	41.9 (34.5) [52.7]	40.8 (33.6) [51.3]	41.5 (35.8) [49.8]
H11						59.3 (50.9) [71.6]	53.9 (45.8) [65.7]	50.9 (41.8) [64.2]	51.2 (41.4) [65.5]	41.0 (34.5) [50.5]	43.3 (36.7) [52.9]	42.3 (34.0) [54.4]	43.8 (36.3) [54.8]
H13							52.2 (42.6) [66.2]	49.3 (40.8) [61.7]	50.5 (41.1) [64.2]	39.8 (33.0) [49.7]	40.2 (32.8) [51.0]	40.3 (30.9) [54.0]	39.9 (31.5) [52.2]
H9								64.7 (61.0) [70.1]	65.1 (62.3) [69.2]	42.5 (34.3) [54.5]	43.3 (36.3) [53.5]	43.1 (37.9) [50.7]	41.7 (35.7) [50.5]
H8									66.7 (61.0) [75.0]	40.9 (33.6) [51.6]	42.6 (36.3) [51.8]	43.5 (37.2) [52.7]	42.6 (35.2) [53.4]
H12										43.9 (36.3) [55.0]	43.2 (35.2) [54.9]	43.6 (36.6) [53.8]	42.7 (35.3) [53.5]
H7											65.3 (53.8) [82.1]	49.6 (36.5) [68.7]	49.6 (38.0) [66.5]
H10												51.7 (42.1) [65.7]	52.2 (42.0) [67.1]
H4													66.5 (53.8) [85.0]
H3													

Note. H1:A/PR/8/34; H2:A/Jap/305/57; H3:A/Aichi/2/68;H4:A/Duck/Czechoslovakia./56; H5:A/Chick/Penn/1370/83; H6:A/Shearwater/Australia/1/72; H7:A/FPV/34; H8:A/Turkey/Ontario/6118/68; H9:A/Turkey/Wisconsin/66; H10:A/Chick/Germany "N"/49; H11:A/Duck/Eng/56; H12:A/Duck/Alberta/60/76; H13:A/Gull/Maryland/704/77.

[a] Figures not parenthesized are percent homology between whole HA molecule including the connecting peptide or arginine residue between the HA_1 and HA_2 subunits, but without a signal peptide. Percentages of amino acid identity among HA1 and HA2 subunits are shown in the upper or lower parentheses, respectively. Percent identity was calculated, deletions or insertions being counted as mismatches.

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TABLE 3

COMPARISON OF AMINO ACID SEQUENCES AROUND THE RECEPTOR-BINDING SITE AMONG THE 13 SEROTYPE HAS

	Amino acid number[a]						
Serotype	98	153	155	183	190	194	195
	(a) Receptor-binding site						
H1	Y	W	T	H	D	I	Y
H2	Y	W	T	H	E	L	Y
H5	Y	W	I	H	E	L	Y
H6	Y	W	I	H	E	L	Y
H11	Y	W	I	H	E	L	Y
H13	Y	W	I	H	E	L	Y
H9	Y	W	T	H	E	L	Y
H8	Y	W	T	H	E	L	Y
H12	Y	W	T	H	E	L	Y
H7	Y	W	L	H	E	L	Y
H10	Y	W	V	H	E	L	Y
H4	Y	W	V	H	E	L	Y
H3	Y	W	T	H	E	L	Y

	Amino acid number[a]					
Serotype	224	225	226	227	228	229
	(b) Left edge of receptor-binding pocket					
H1	R	D	Q	A	G	R
H2	N	G	Q	G	G	R
H5	N	G	Q	S	G	R
H6	N	G	Q	R	G	R
H11	N	G	Q	A	G	R
H13	N	G	Q	R	S	W
H9	N	G	Q	Q	G	R
H8	R	G	Q	Q	G	R
H12	R	G	Q	Q	G	R
H7	N	G	Q	S	G	R
H10	N	G	Q	S	G	R
H4	R	G	Q	S	G	R
H3	R	G	L	S	S	R

	Amino acid number[a]				
Serotype	134	135	136	137	138
	(c) Right edge of receptor-binding site				
H1	G	V	T	A	A
H2	G	G	S	R	A
H5	G	V	S	S	A
H6	G	V	T	R	A
H11	G	V	T	A	A
H13	G	T	T	S	A
H9	G	T	S	R	A
H8	G	T	S	K	A
H12	G	T	S	K	A
H7	G	T	T	S	A
H10	G	T	T	K	A
H4	G	K	S	G	A
H3	G	G	S	N	A

[a] The amino acid numbering used for the H3 HA (Wilson *et al.*, 1981) was used.

mutant seven residues are deleted: 224–230(SVHA-del-2). This mutant HA protein was expressed in CV-1 cells from the recombinant virus vector SVHA-del-2. Expression of the HA protein on the infected cell surface was determined by indirect immunofluorescence staining using rabbit anti-A/USSR/77 serum. While the mutant HA of SVHA-del-2 was demonstrated on the infected cell surface by immunofluorescence staining, hemadsorption and fusion activity could not be detected (data not shown). These findings suggest that the role of the left edge of the receptor-binding site in H1 HA is different from that in the H3 HA.

DISCUSSION

We determined the nucleotide sequences of H4, H6, H8, H9, H11, H12, and H13 serotype HAs of influenza A viruses in the present study. Amino acid sequences are now available for all 13 serotypes. Alignment of amino acid sequences was carried out with conserved cysteine residues. It showed that the proportion of conserved amino acid residues all through the 13 serotype HAs is 12% (42/346) for HA1 and 30% (67/225) for HA2. All conserved amino acid residues including 13 cysteines, 18 glycines, and 6 prolines seem to be important in maintaining the basic structure of the HA protein.

Several amino acid residues forming the receptor-binding cavity and the second shell in H3 HA are conserved all through the 13 serotype HAs. Basic structure of the receptor-binding site is, therefore, similar among the 13 serotype HAs. While crucial amino acids for receptor binding are conserved, only Gly-134 is conserved on the right edge (134–138) of the receptor-binding site all through the 13 serotype HAs. Ala-138 is conserved in the strains studied here, but not in other strains (A/USSR/77, A/WSN/34). Several amino acid substitutions were found in the right edge of the pocket, but the atom which is presumed as interacting with sialic acid in the H3 HA (Weis *et al.*, 1988) is conserved in either the main chain or the side chains of a substituting amino acid residue at the same position in the other serotype HA as in the H3 serotype. For instance, the hydroxyl group in the side chain of the residue Ser-136 presumed as interacting with sialic acid in H3 is conserved on the side chain of the substitute residue threonine in other serotypes (H6, H7, H10, H11, and H13). On the other hand, the significance of the left edge for the receptor-binding activity might be different from that of the right edge, since not all the amino acid residues on the left edge in H3 HA seem to interact with sialic acid (Weis *et al.*, 1988). Moreover, the left edge appears to be one of the determinants of receptor specificity (Rogers *et al.*, 1983), but does not appear to be pivotal for receptor binding in H3 serotype

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HA, because the residue 224–230 constituting the left edge is deleted in a variant of H3N2 virus (Daniels *et al.*, 1987). By contrast, H1 serotype HA expressed from SVHA-del-2 with the same amino acid deletion lost its receptor-binding activity. The left edge, therefore, seems to be pivotal for receptor binding for H1 HA. The significance of the left edge of the pocket for receptor-binding activity might be different between the two HA serotypes.

The receptor specificity of the HA has been considered to be one of the determinants of the tissue tropism and host range in influenza virus. It was reported that all human isolates of the H3 serotype agglutinated the erythrocytes containing the NeuAcα2,6Gal linkage but not those bearing the NeuAcα2,3Gal linkage, whereas antigenically similar isolates from avian and equine species preferentially bound the erythrocytes containing the NeuAcα2,3Gal linkage (Rogers and Paulson, 1983). The amino acid residue at position 226 in H3 serotype HA is known to be a determinant of receptor specificity; i.e., H3 HA that recognizes NeuAcα2,6Gal linkage has leucine, while that which is NeuAcα2,3Gal-specific has glutamine at position 226 (Rogers *et al.*, 1983). We investigated then the relationship between the amino acid residues at position 226 and receptor specificity with H1, H2, H3, H4, H6, H8, H9, and H10 HAs. In contrast to H3 HA, H2, H6, and H9, HAs having glutamine at position 226 preferentially recognized NeuAcα2,6Gal linkage (Table 4). These findings suggested that the amino acid residue at position 226 is not necessarily the determinant of receptor specificity. Our previous paper showed that H1 serotype HA with leucine at position 226 expressed from SVHA-330 has no hemadsorption activity (Nobusawa *et al.*, 1988). Suzuki *et al.* (1989) showed recently that a single amino acid substitution of (Ser-205 to Thr) altered the receptor specificity of H3 HA. In this study, however, the amino acid residues at position 205 were valine (H1), glycine (H2, H5, H6, and H7), serine (H3, H4), threonine (H8, H9), and glutamic acid (H10), and no correlation could be found between the amino acid residue at position 205 and receptor specificity.

In this study, the receptor specificity with H1, H2, H3, H4, H5, H6, H8, H9, and H10 HAs was examined by hemolysis assay with gangliosides containing either NeuAcα2,3Gal or NeuAcα2,6Gal. In the previous paper, receptor specificity was examined by adsorption to derivatized human erythrocytes containing either NeuAcα2,6Gal or NeuAcα2,3Gal linkage. The amount of virus adsorbed was quantitated by the neuraminidase activity (Paulson and Rogers, 1986). In this study, on the other hand, ganglioside–erythrocytes were prepared by incorporating gangliosides into desialylated chicken erythrocytes and the adsorbed virus was measured by virus-mediated hemolysis. By these two methods, the degree of receptor specificity examined for certain strains was not always the same, though similar preference in the linkage specificity was observed. In the case of A/Aichi/2/68, Rogers and Paulson (1983) observed no virus adsorbed to derivatized erythrocytes containing NeuAcα2,3Gal, but we detected a small amount of virus bound to NeuAcα2,3Gal-containing erythrocyte. This discrepancy may due to the different amount of virus used for the assay; i.e., Suzuki *et al.* (1986) used 2^7–2^{10} HA units, in contrast to 2^2 HA units used by Rogers and Paulson (1983). The differences in species of erythrocyte and in the method of reconstructing erythrocytes may also cause a difference in the amount of virus bound to reconstructed erythrocytes.

Subdivision of serotypes of influenza A viruses was based on the antigenic relationships in double immuno-

TABLE 4

RECEPTOR SPECIFICITY OF INFLUENZA VIRUSES (HUMAN AND AVIAN ISOLATES)

Chicken erythrocytes desialylated and then coated with	Receptor specificity[a]								
	Human isolates			Avian isolates					
	H1N1	H2N2	H3N2	H3N8	H4N6	H5N3	H6N5	H8N4	H9N2	H10N7
Sialylparagloboside										
NeuAcα2,3Gal	63 ± 5	51 ± 4	49 ± 6	78 ± 7	78 ± 10	77 ± 8	52 ± 7	61 ± 5	41 ± 3	82 ± 10
NeuAcα2,6Gal	29 ± 4	83 ± 9	85 ± 7	53 ± 7	65 ± 6	62 ± 5	79 ± 4	28 ± 3	61 ± 5	51 ± 6
NeuGcα2,3Gal	0	0	3 ± 1	4 ± 1	5 ± 2	5 ± 1	6 ± 2	2 ± 1	5 ± 1	4 ± 1

Note. H1, A/PR/8/34; H2, A/Jap/305/57; H3, A/Aichi/2/68, A/Duck/Ukraine/63; H4, A/Duck/Czechoslovakia/56; H5, A/Tern/South Africa/61; H6, A/Shearwater/Australia/1/72; H8, A/Turkey/Ontario/6118/68; H9, A/Turkey/Wisconsin/66; H10, A/Chick/Germany "N"/49.

[a] The receptor specificity was expressed by virus-mediated hemolysis at low-pH of desialylated and ganglioside-coated chicken erythrocytes. The recovery of hemolysis by ganglioside coated erythrocytes were calculated by (absorbance at 540 nm of the supernatant of virus-mediated hemolysis of ganglioside coated erythrocytes—absorbance at 540 nm of the supernatant of virus-mediated hemolysis of desialylated erythrocytes)/pmol ganglioside attached to 10^6 erythrocytes.

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diffusion tests (WHO Memorandum, 1980). In contrast to the absense of antigenic relationship among 13 HA serotypes by double immunodiffusion tests, certain degrees of amino acid homology were found to exist. A homology as high as 74% (66.7% in HA1) was found between H2 and H5 serotypes. After submission of this manuscript Kawaoka *et al.* (1990) reported the nucleotide sequence of a new serotype, H14. They showed that H14 is closely related to H4 in amino acid sequence homology (68.5% for HA1, 92.3% for HA2). This is even higher than the homology found between the thus far most closely related H2 and H5 serotypes. They discussed that near identity in HA2 between H4 and H14 argues for very recent divergence of these two serotypes. On the other hand, the amino acid sequence homology within a serotype is 80% or more even between virus strains readily distinguishable by polyclonal antibodies (Nestorowicz *et al.,* 1987; Kawaoka *et al.,* 1989; Donis *et al.,* 1989). As substitution of a single amino acid out of five to six residues which usually make up an antigenic determinant may profoundly alter the antigenicity, antigenic change is not predicted from amino acid sequence change. Amino acid sequence homology of 68.5% or less for the HA1 subunit may serve, however, as a rough indication for different serotypes.

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