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Hemagglutinin of swine influenza virus: A single amino acid change pleiotropically affects viral antigenicity and replication

(antigenic variation/point mutation/nucleotide sequence/viral genetics/virulence)

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ABSTRACT **The complete nucleotide sequence has been obtained of the H1 hemagglutinin (HA) gene of a high-yielding (H) mutant of the A/NJ/11/76(H1N1) strain of swine influenza virus in studies of a viral reassortant (X-53a) bearing this gene. This determination has permitted comparison with human influenza H1N1 prototype viruses A/WSN/33 and A/PR/8/34, with which 80% and 94% amino acid homology was found between HA1 and HA2, respectively. Partial sequences have been determined for other viral reassortants containing either H or L (low-yielding phenotype) genes derived from A/NJ/11/76. Sequence of the HA1 region of an L mutant prototype was virtually completed and differed from that of the H mutant by only four amino acid changes. Sequence analysis of four other viruses was restricted to regions of the HA with which monoclonal antibodies capable of distinguishing L and H mutants are presumed to react. Therefore, changes in these sequences are relevant to changes in viral phenotype. Change at residue 155 from Gly to Glu is associated with change from L to H HA phenotype. This site, structurally equivalent to amino acid 158 on the Wiley *et al.* HA model [Wiley, D. C., Wilson, I. A. & Skehel, J. J. (1981) *Nature (London)* 289, 373–378] is near the tip of the HA monomer adjacent to the proposed receptor binding site and therefore credibly could influence both viral antigenicity and replication. Because both L and H variants exist in nature and because revertants may be selected in the laboratory as replication variants in the absence of immunoselection, these studies provide evidence for fortuitous antigenic change in association with change in biological function, which is determined by a single base change.**

Hemagglutinin (HA) L and H mutants of contemporary strains of swine influenza virus differ pleiotropically in their capacity to replicate in chicken embryos, Madin–Darby canine kidney cells (MDCK cells), and the respiratory tract of swine (1–3). Although antigenically identical in tests with conventional polyclonal antibodies (1), these mutants can be distinguished with certain cross-absorbed heterotypic antisera or with monoclonal antibodies (4). The L and H mutants have been intensively studied because (*i*) they are commonly found in field isolates from swine as dimorphic variants, in which one may be detected in primary passage in the absence of the other (2), or in early passage isolates from humans (1, 2) or turkeys (unpublished data); (*ii*) genetic evidence suggests that the variants reflect point mutations in the HA gene (1); and (*iii*) it has seemed important to identify a mutational site that concomitantly influences both the antigenicity of virus and replication in its natural host.

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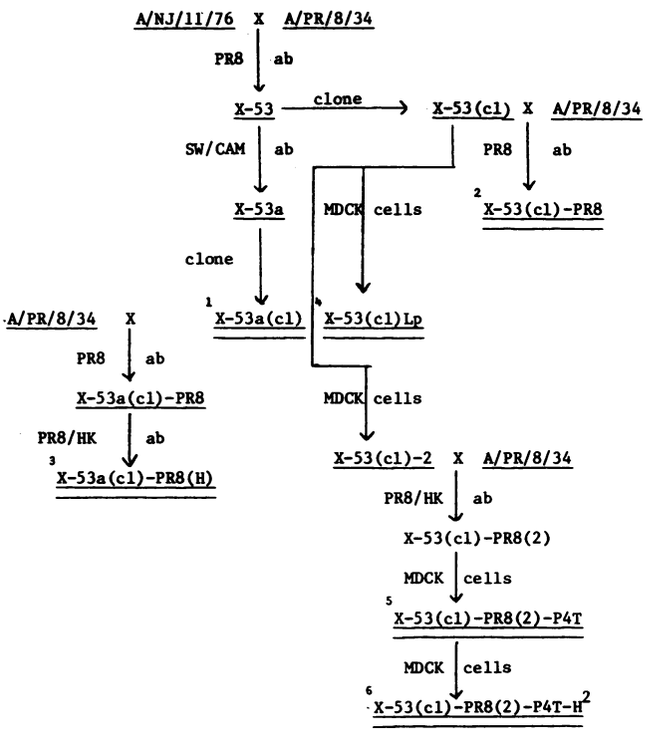

FIG. 1. Genealogy of reassortant viruses on which nucleotide sequence analysis of the HA gene has been performed (numbered, double-underlined viruses). The details of passage history are shown in Table 1. All reassortants are definitively derived from uncloned A/NJ/11/76(H1N1) swine influenza virus and A/PR/8/34(H1N1). PR8 ab, antibody to PR8 virus; SW/CAM ab, antibody to A/SW/CAM/39(H1N1) virus; PR8/HK ab, antibody to reassortant of PR8 and Hong Kong (H3N2) viruses containing PR8 HA and Hong Kong neuraminidase. X-53 viruses (L phenotype) are on the right and X-53a (H phenotype) are on the left. Repeated reassortment with PR8 virus has yielded viruses (designated by PR8 suffix) containing all PR8 genes, except for that coding for the (swine) HA. When not specified (i.e., MDCK cells) passage has been in chicken embryos.

MATERIALS AND METHODS

Viruses. The derivation and properties of the basic reassortant viruses X-53 and X-53a used in the present experiments have been presented in detail (1). These viruses were derived from genetic reassortment of the A/PR/8/34 (H1N1) (hereafter referred to as PR8) and A/NJ/11/76 (H1N1) (swine) influenza viruses. X-53 (L phenotype) and X-53a (H phenotype) viruses

Abbreviations: HA, hemagglutinin; HI, hemagglutination-inhibition.

Table 1. Detailed passage history of viruses on which nucleotide sequence analysis of portions of the HA gene was performed

Virus	Immediate antecedent*	Passage history†‡§
1	X-53a	E E E X-53a(cl) E E E E E → RNA (1)
2	X-53	E E E E E E X-53(Cl) × PR8 E E E → X-53(cl)-PR8 E E → RNA (P) (P) (P) (2) (P) (P)
3	X-53a(cl) × PR8 (1)	E E E E → X-53a(cl)-PR8 E E E E E E E E E X-53a(cl)-PR8(H) E → RNA (P) (P) (P) (P) (3)
4	X-53(cl)	E E E M M M M E X-53(cl)Lp → RNA (4)
5	X-53(cl)	M M E M E E X-53(cl)(2) × PR8 E E → X-53(cl)(2)PR8(2) M M M M M E X-53(cl)-PR8(2) p4t ↓ RNA (P) (P) (5)
6	X-53(cl)-PR8(2)-p4t (5)	M M E E M M E X-53(cl)-PR8(2)-p4t-H^2 E → RNA (6)

Note: × PR8 indicates reassortment with A/PR/8/34 virus during dual infection of chicken embryo allantoic sac.
* See Fig. 1.
† The letter E designates a single passage in the allantoic sac of the chicken embryo.
‡ P designates passage with antibody to the PR8 (A/PR/8/34) virus HA.
§ The letter M designates a single passage in MDCK cells.

have only HA and neuraminidase genes from the swine influenza virus parent (1, 5). In other reassortants (X-53-PR8 and X-53a-PR8) the swine influenza virus HA RNA has been segregated into viruses deriving all other RNAs from A/PR/8/34 virus. The genealogy or derivation from the above viruses of viruses with different biologic characteristics or passage history is shown in Fig. 1. The details of passage of the six viruses of which the HA RNAs were subjected to nucleotide sequence analysis are presented in Table 1. These viruses will be identified below by the numerical designations shown in Tables 1 and 2 and in Fig. 1.

Antibody Preparations and Antigenic Analysis. The nature of antibody preparations employed and the methods for viral antigenic analyses have been described (4).

Plaquing and Plaque Inhibition. Plaquing and plaque inhibition of viruses was carried out in MDCK cells with trypsin-containing minimal essential medium by methods previously described (6, 7).

Definition of Viral Phenotype. Criteria for the definition of the antigenic and biologic phenotypes of the mutant viruses have been presented in earlier papers (1, 4). In brief, reassortant viruses of L phenotype are relatively low yielding in chicken embryos (HA titers < 1:2,048) and produce small plaques (1–3 mm in diameter) in MDCK cells. The H phenotype is the reciprocal; though the plaque sizes overlap, the mean plaque size is greater with H reassortants. Very few L and H mutant viruses have been studied in swine, but L viruses are more infectious for swine than H mutants by a factor of at least 1,000-fold (2). Antigenic phenotype is defined with monoclonal antibodies, as indicated in Table 2 and specifically described in ref. 4.

Cloning and Nucleotide Sequence Analysis of Swine Influenza HA Genes. A DNA copy of the HA gene for virus 1 was synthesized and cloned as described (8). This was subjected to sequence analysis either directly (9) or by the "dideoxy" method (10) after subcloning fragments into the bacteriophage M13 mp7.1 (11). The cloned HA gene was also digested with suitable restriction endonucleases to generate DNA fragments that were used as primers for cDNA synthesis by reverse transcriptase by using genomic RNA from viruses 1–6 as template (12). By the

Table 2. Summary of representative reassortant viruses on which nucleotide sequence analysis of HA RNA has been carried out

Virus		log 2 HI titers with antibody				Antigenic phenotype	Biologic phenotype	HA residue in H1 antigenic regions				
Number	Name	SW/CAM*	9C8†	PR8/HK*	Sa-13‡			153§	155¶	159	228	84
1	X-53a(cl)	<1	1	2	7	H	H	Lys	Glu	Pro	Ser	
2	X-53(cl)-PR8	2	6	<1	4	L	L	Glu	Gly	Pro	Asn	(Lys → Asn)
3	X-53a(cl)-PR8(H)	<1	1	<1	<1	Non-L Non-H	H	Lys	Glu	Thr	Asn	
4	X-53(cl)-Lp	6	5	<1	1	L	L**	Lys	Gly	Pro	Asn	
5	X-53(cl)-PR8(2)-p4t (recloned)	5	7	<1	<1	L	L	Lys	Gly	Pro	Asn	
6	X-53(cl)-PR8(2)-p4t-H^2 (L to H revertant)	<1	1	—	7	H	H	Lys	Glu	Pro	Asn	

HI, hemagglutination-inhibition.
* Polyclonal antibody (rabbit serum).
† X-53-PR8 monoclonal antibody (mouse ascites fluid).
‡ Monoclonal antibody (mouse ascites fluid).
§ H1 antigenic region Sb.
¶ H1 antigenic region Sa.
‖ Structurally equivalent to residue 158 in H3 model.
** Large plaque L mutant not yet tested in swine for definitive biophenotype.

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   1 --UAGAAACA AGGGUGUUUU UUCUCAUGUC UCUGAAAUCC UAGUUUUAAA UACAUAUUCU
  61 GCACUGUAAA GACCCAUUAG AGCACAUCCA AAAACUGAUU GCCCCCAGGG AGACCAACAG
 121 UACCAAUGAA CUGGCGACAG UUGAAUAGAU CGCCAAAAUC UGGUAAAUUC UUGUUGACUC
 181 CAACUUUACU CCAUCUAUCU CCUCUCUGUU UAGCUUUGAU UCUUCUGAGU ACUUUGGGUA
 241 AUCAUAAGUC CCAUUUUUGA CGCUCUCCAU GCAUGUAUCA UCACAUUUGU GAUAGAAUUC
 301 AAAGCAGCCA UUUCCAAUUU CUUUGGCAUU GUUCCUUAGC UGGCUUCUUA CUUUCUCAUA
 361 CAGGUUCUUU ACAUUCGAGU CGUGGAAAUC CAGAGUUCUU UCAUUUUCCA AUAGAACCAA
 421 CAGUUCUGCA UUGUAUGUCC AAAUAUCCAG AAAACCAUCA UCAACCUUUU UGUUUAAAUU
 481 CUCUAUUCUU UUUUCCAAGU GGUUGAAUUC UUUACCCACU GCCGUGAAUU GCGUGUUCAU
 541 CUUUUCAAUA ACAGAAUUUA CUUUGUUAGU GAUCCCGUCA AUGGCAUUUU GUGUGCUCCU
 601 UUGGUCCGCU GCAUAUCCUG AUCCCUGUUC AUUCUGAUGG UGAUAACCAU ACCAUCCAUC
 661 UAUCAUUCCU GUCCAUCCCC CCUCAAUAAA ACCAGCAAUG GCUCCAAACA GACCUCUAGA
 721 UUGAAUAGAC GGGACAUUCC UUAGUCCUGU AGCCAUUCUC AAUUUUGUGC UUUUGACAUA
 781 UUUUGGACAU UCUCCAAUUG UGACUGGAUG UAUAUUCUGA AAUGGGAGGC UGGUGUUUAU
 841 GGCACCCUUG GGUGUUUGAC ACUUCGUGUU ACAAUCAUGA ACUGGUGCAU CCCAAAUUAU
 901 AAUACCAGAU CCAGAACCUC UAUUCAUUGC GAAAGCAUAU CUUGGUACCA CUAGAUUUCC
 961 AGUUGCUUCA AAUGUUAUUG UGUCUCCAGG UUCUAUUAGU GUCCAGUAAU AGCUCAUCCU
1021 CCCUGCUUGA CCUCUCACCU UGGGUCUUGC UGCUAUUUCU GGCUUGAACU UUCUGUUAUA
1081 UUUUGAUGAC CCUACAAAAA CAUAGGCAUC UGCAUUUUGG UAGAGACUUU GUUGAUCAGU
1141 ACUGGUAGGU GGAUGAUGAA UGCCCCAUAG CACAAGGACU UCCUUCCCUU UAUUGUUAAC
1201 AUAGGAUUUG CUGAGCUUUG GGUAUGAAUU UUCUUUUUUC ACCAGCCAUA UUAAAUUUCU
1261 GUAGAAGCUG UUUGCUCCAG CAUAAGGGCA UGCUGCCGUC ACACCUCUGU UUGUUUCAUG
1321 AUUGGGCCAC GAACUUGUCU UGGGGAAUAU CUCGAAUCUU UCAAAUGAUG ACACUGAGCU
1381 CAACUGCUCU CUCAGCUCUU CAUAAUUGAU GAAAUCUCCU GGGUAACAUG UCCCAUUGUC
1441 UGAUUUCGAU GUUUCCACAA UGUAAGACCA UGAGCUUACU GUGAGUAGUA AUUCACAUUC
1501 UGGGUUUCCC AAAAGCCUAC CGGCAAUGUU ACAUUUACCC AAGUGCAAUG GGGCUAUCCC
1561 CCCCAGUUUA CAUAGUUUCC CGUUAUGUCU GUCUUCAAGA AGAUUAACAG AGUGUGUUAC
1621 UGUUACAUUC UUUUCUAGUA CUGUAUCAAC AGUGUCAGUU GAAUUAUUUG CAUGAUAACC
1681 UAUACAUAGU GUGUCUGCAU UUGUGGCUGC AAAUGUACAU AACAAGACUA AUAGUAUUGC
1741 CUUCAUUUCG GUGGCUUUUA UUUUCCCCUG CUUUUGCU
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FIG. 2. Nucleotide sequence of the HA gene of swine influenza virus strain A/NJ/11/76 (H mutant) from reassortant virus X-53a.

use of dideoxynucleoside triphosphates (10), sequence information from the same region of the HA gene was obtained and compared for the various mutants.

RESULTS

Sequence of the H-Phenotype Swine Influenza Virus HA Gene. The complete nucleotide sequence was determined for the HA gene of H-phenotype virus 1 (Fig. 2) by the strategy shown in Fig. 3. Swine influenza HA has been grouped with human isolates such as A/PR/8/34 and A/WSN/33 in the H1 subtype (13). There is approximately 80% and 94% amino acid homology in HA1 and HA2, respectively, between virus 1 and these strains. The differences among them are summarized in Fig. 4. The nucleotide and inferred amino acid sequences of the H-phenotype virus 1 provide the basis for comparison with other viruses with different antigenic and biological phenotypes.

Sequence Analysis of HA RNAs from Other Related Viral Reassortants. Considering the available information on the structure, function, and antigenic regions of the influenza HA and the conserved nature and primarily structural role of HA2 (16), we concentrated our sequence analysis efforts on the HA1 region of the L phenotype HA RNAs. To compare HAs of the L and H phenotypes, partial nucleotide sequences for the HA genes of viruses 2–6 were determined indirectly by using primer DNA fragments from the cloned virus 1 (12). The sequence of the HA1 region of the gene of L-prototype virus 2 was virtually completed, except for nucleotides 1–65, 235–305, 800–880, and 1,048–1,061, which encode amino acids −1 to −11 (in the signal peptide), 51–74, 239–266, and 322–326, respectively. Comparison of the nucleotide sequences encoding HA1 for viruses 1 and 2 revealed point mutations at bases 335 (A → C), 540 (A → G), 547 (A → G), and 766 (G → A), resulting in changes at amino acid residues 84, 153, 155, and 228, respectively (Table 2). Determination of the nucleotide sequences encoding these residues in other closely related L-phenotype viruses 4 and 5 showed no differences among them with respect to residues 153 (Lys) or 228 (Asn). Therefore, interest focused on amino acid 155 as the probable determinant of L or H viral phenotype. All viruses of L phenotype had Gly at this site and the

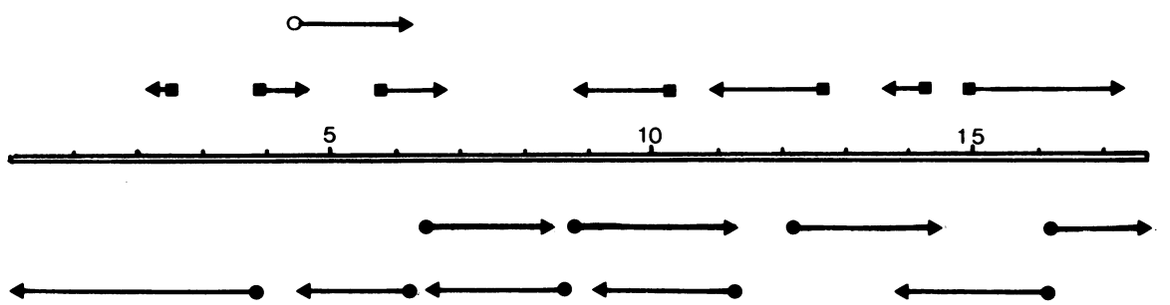

FIG. 3. Strategy for determining the nucleotide sequence of the cloned DNA copy of the swine influenza HA gene. The numbers refer to the nucleotides × 10^{-2}. The sequence was determined either by copying cDNA from swine influenza genome RNA (○) (12), by the method of Maxam and Gilbert (■) (9), or by the dideoxy method (10) after subcloning fragments into bacteriophage M13 mp7.1 (●) (11).

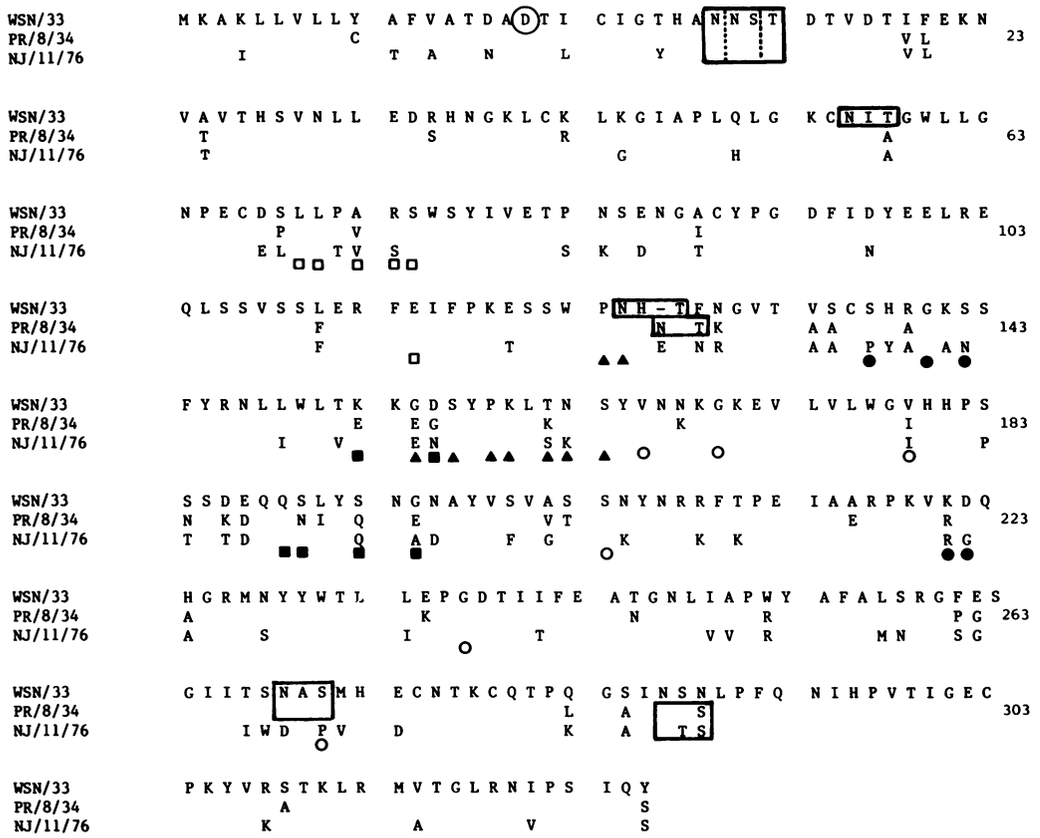

FIG. 4. Summary of amino acid differences in HA1 between A/WSN/33 (14), A/PR/8/34 (Cambridge) (15), and A/NJ/11/76 (X53a) by using the single-letter amino acid code (12). Boxed residues indicate potential sites for carbohydrate attachment. The NH_2-terminal residue of mature HA is circled. Antigenic regions defined for A/PR/8/34 (Mt. Sinai) (17) are indicated: ▲, Sa; ■, Sb; □, Cb; ●, Ca_2; and ○, CA_1. A/NJ/11/76 (X53a) also differs from A/WSN/33 in HA2 at residues 39 (WSN X53a; K → R), 46 (N → D), 72 (N → H), 77 (M → I), 113 (L → S), 123 (K → R), 127 (N → R), 146 (N → D), 147 (E → T), 153 (R → K), 172 (K → E), 181 (M → T), 182 (G → R), and 183 (V → I).

two H-phenotype viruses had Glu.

Our attention was further directed to this site by parallel studies with monoclonal antibodies to the Sa antigenic site of A/PR/8/34 virus, in which it was found that L and H phenotypes could be antigenically distinguished (4). [Due to the conservation of key residues among influenza virus subtypes (16), amino acid 155 of swine influenza virus HA is structurally equivalent to residue 158 in H3 subtype viruses and lies within the H1 Sa antigenic site (17).] The conclusion that amino acid 155 is indeed the relevant residue is reinforced by phenotypic and biological comparison of viruses 5 and 6. Virus 5 was cloned by five successive plaque-to-plaque passages in MDCK cells (Table 1). After additional MDCK and chicken embryo passages, virus 6 emerged as a large plaque-forming (biologic) mutant of H antigenic phenotype. Corresponding to this change in antigenic and biologic phenotype, amino acid 155 changed from Gly to Glu (Table 2), as a result of a point mutation (G → A) at nucleotide 547.

Virus 3 (non-L, non-H antigenically) was selected with PR8/HK antiserum from H reassortant X-53a(cl)-PR8 (Fig. 1). It is nonreactive with antibodies specific for either L or H mutants. Its biologic phenotype must be provisionally defined as H on the basis of its yield in chicken embryos and large plaque size, but it has not yet been administered to swine. Like viruses of H phenotype, it has Glu at position 155, but, unlike H viruses 1 and 6 (and indeed all of the other viruses studied), as a result of a C → A mutation at nucleotide 558, it has Thr rather than Pro at nearby position 159. The change here at this "anchor residue" (16) might well influence the binding of antibody to the region containing residue 155.

DISCUSSION

The Swine Influenza Virus (X53a) HA Sequence. The complete nucleotide sequence for the H-phenotype swine influenza virus (X-53a) has been determined (Fig. 2). The inferred amino acid sequence revealed, as expected, a high degree of homology with the HA proteins of other H1 subtype viruses. The swine HA gene encodes a signal sequence of 17 amino acids and HA1 and HA2 regions of 326 and 222 amino acids, respectively; a single Arg residue links HA1 and HA2. In comparing the HA amino acid sequences for A/WSN/33, A/PR/8/34, and X-53a, all classified as members of the H1 subtype, it is evident that in HA1 amino acid changes are widely scattered, making the significance of many differences difficult to assess (Fig. 4). However, two features are worth comment: (*i*) there are notable clusters of amino acid differences, particularly between X-53a and the other viruses, in and adjacent to the regions corresponding to the known antigenic sites of the H1 HA molecule (17)—in particular, the Ca2, Cb, and Sb sites; (*ii*) compared with A/WSN/33, the swine HA has lost three carbohydrate attachment sites (at Asn residues 56, 127, and 269) and gained one (at Asn residue 287). Moreover, the Hsw HA retains no attachment sites where the presence of carbohydrate could potentially mask proposed antigenic regions (16, 17). This may explain the drift in the amino acid sequence of the Hsw HA, compared with those of A/WSN/33 and A/PR/8/34 in the region of residues 127–142 and 267–272 (Fig. 4). The latter region, which retains a carbohydrate site in A/PR/8/34 and did not change in laboratory-selected variants (17), is structurally equivalent to H3 residues 269–274—i.e., the C antigenic site (16).

Comparison of L and H Phenotype HAs. Our comparison of nucleotide sequences for the HA1 regions of L and H phenotype reassortants 1 and 2 has demonstrated few differences. It is notable that two of the four differences found are within an antigenic region defined for the closely related PR8 virus and that monoclonal antibodies to this (Sa) region can distinguish between the L and H mutants. When virus 6, the H revertant of L phenotype virus 5, was studied by sequence analysis of the viral RNA through the Sa region, only a single deduced amino acid change was found at position 155, although the revertant virus 6 had changed both in biologic and antigenic phenotype. It is significant that this revertant emerged as a high-yielding (biologic phenotype) variant in MDCK cells and was not selected with antibody.

These data, together with those obtained from study of the other reassortants, indicate that Gly at position 155 predicts L phenotype and Glu predicts H phenotype. An exception is virus 3, which has lost reactivity with H-reactive antibody during anti-H immunoselection, but which retains Glu at position 155. However, the Pro to Thr change at neighboring residue 159 might alter antibody binding to the Sa site without affecting whatever function of Glu determines biologic phenotype.

The large plaque L variant, virus 4, was selected for sequence analysis with the expectation that its changed biologic phenotype might reflect mutation of other viral genes as has been observed with other large plaque mutants of other viruses. In this case, the retention of Gly at position 155 can be correlated with its retained L antigenic phenotype.

Biological Implications of the Mutation. Mutation at residue 155 [corresponding to position 158 on the Wiley *et al.* H3 HA model (16)] is at a credible site to influence viral biologic activity. This residue is near the tip of the HA monomer immediately adjacent to the proposed receptor binding site. Two functions have been unequivocally ascribed to the HA molecule that could be expected to significantly influence viral replication: (*i*) attachment to cellular receptors and (*ii*) mediation of infection of the cell after posttranslational modification of the HA by cellular proteases (18). In view of the evidence that the key mutation is at a site at or near the receptor pocket at the top of the HA spike, it seems unlikely that the mutants differ with respect to the second function and probable that the mutation influences cellular attachment in some way. (We have found no difference in the cleavage of HA or protease susceptibility of L and H prototypes.) Accordingly, we have studied the absorption kinetics of L and H reassortants in MDCK cell monolayers (in which replication differences are manifest) but have not found significant differences (data not shown). In studies of attachment of the virus to receptors of erythrocytes of a variety of species we have found differences in hemagglutination pattern only with sheep erythrocytes. L mutants bind with slightly greater avidity to sheep erythrocytes, as reflected by slower elution of virus and disagglutination of cells under conditions in which neuraminidase activity is absent or abolished.

The present experiments provide biochemical confirmation of previously presented genetic evidence (1) that change in viral replicative capacity or virulence (plaque size) may coincidentally be associated with antigenic change related to a single mutation. By inference, minor antigenic variation as defined by highly specific (monoclonal) antibody is not necessarily selected for as a reciprocal of modulation by host antibody but can be driven by primary selection for changes in the HA that affect its biological function. It should be stressed that H mutants arise in the absence of immunoselection in host systems favoring their replication, in which case the coincident minor antigenic change appears to be essentially fortuitous (19). Minor antigenic variation occurs also in A/NWS/33 (H1N1) HA mutants selected for different trypsin sensitivity (20). Additional evidence for nonimmunologic selection of antigenic variants comes from recent studies of influenza B-virus subpopulations in which antigenicity varies with different host cell tropisms (21). Indeed, the phenomenon may be a general one. Pathogenicity of fixed rabies virus strains for adult mice depends on the presence of an antigenic determinant on the viral glycoprotein (22), whereas HA antigenic variants of reovirus type 3 are changed in neurovirulence (23).

It is not surprising that changes affecting viral replication and virulence might occur at antigenic sites, because such sites must be at the exterior of the molecule in accessible locations for interaction with host cells.

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