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Mutations in the Hemagglutinin Receptor-Binding Site Can Change the Biological Properties of an Influenza Virus

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Avian influenza virus reassortants containing human influenza virus hemagglutinins do not replicate in ducks. Two mutations in the receptor-binding site of a human hemagglutinin at residues 226 and 228 allowed replication in ducks. The mutations resulted in a receptor-binding-site sequence identical to the known avian influenza virus sequences.

Influenza viruses interact with host cell sialyloligosaccharide receptors via a surface glycoprotein, the hemagglutinin (HA) (3). The tertiary structure of the HA has been solved, and a putative sialic acid-binding site has been proposed (13). Nucleotide sequence analysis of HA genes coding for proteins with different oligosaccharide-binding specificities revealed residue 226 of HA1 to be a part of the receptor-binding site (RBS) located in a pocket on the distal tip of the molecule (7). Although these studies suggested that variations in receptor specificity may influence biological properties of the virus, they did not provide information on the biological consequences of such changes. We report here evidence that mutations in the HA RBS at residues 226 and 228 in HA1 may change the tissue tropism of the virus.

To examine the role of the HA in tissue tropism, we produced reassortants between an avian influenza virus strain, A/Mal/NY/6750/78 (H2N2) (Mal/NY), which replicates in the intestinal tracts of ducks, and a human strain, A/Udorn/307/72 (H3N2) (Udorn), which does not. Thirty-four reassortants were genotyped by hybridization, tested for their ability to replicate in ducks, and analyzed for antigenic variation with monoclonal antibodies (5). Eight reassortants had the Udorn HA and all the other genes from Mal/NY; only one of these reassortants, R2, replicated in ducks after oral inoculation (5). Reassortant R2 was selected for more detailed study.

Preliminary antigenic analysis of the R2 HA with monoclonal antibodies specific for antigenic sites A, B, and C on H3 molecules (Mem/1/71) revealed that the R2 HA no longer reacted with antibodies specific for antigenic site B (5). These results suggested that mutations in the HA had indeed occurred, changing the antigenic character of the HA and perhaps altering the tissue tropism of the virus.

To rule out the possibility of mutations in gene segments other than the HA bearing responsibility for the change in tropism of this reassortant, we independently segregated the HA of R2 onto a human virus, A/Bel/42 (H1N1) (Bel), and subsequently transferred it back to its avian parent, Mal/NY. If R2 had acquired mutations in other gene segments critical for the altered tissue tropism and if the mutations in the HA gene were insufficient to alter the phenotype, then one would not expect the backcross reassortant to replicate in the intestinal tracts of ducks. The R2-Bel reassortant, which contained all genes but the HA gene from Bel, failed to replicate in ducks, indicating that the enterotropism phenotype is polygenic. The backcross reassortant R2-Mal/NY, which contained only the R2 HA gene and the other seven genes from Mal/NY, had the same phenotype as the original R2 virus. Although not constituting formal proof of the absence of other mutations, the results suggest that the R2 phenotype was determined by mutations in the Udorn HA and probably not by mutations in other gene segments in the R2 virus.

To identify the location of these mutations, the RNA segments coding for the HA of both the parental Udorn strain and the R2 mutant were sequenced. The following oligodeoxynucleotides were synthesized by phosphoramidite chemistry on an Applied Biosystems DNA synthesizer and were used to prime dideoxynucleotide chain termination sequencing reactions (8): 1AGCAAAAGCAGG, 201CTGGTTCAGAGT, 412TGCCTCGTCAGG, 618GGGGTTCACCAC, 801ATACTGGTAATT, 1002GTTAAGCAAAAC, 1203CAAATCAATGGG, 1401TCGGAAATGAAC, and 1601GTCAGGATACAAA, numbered in accordance with the sequence of A/Mem/102/72 cDNA (10). The 6-μl reaction mixture contained Tris-hydrochloride (pH 8.0), 0.045 M; magnesium chloride, 0.0045 M; potassium chloride, 0.045 M; dithiothreitol, 0.009M; dATP, dGTP, dCTP, and dTTP, 0.000045 M each; human placental RNase inhibitor (Biotec Inc.), 3 U; reverse transcriptase (Life Sciences), 5 U; 5 μCi of [α-^{32}P]dATP; and ddATP, ddGTP, ddCTP, and ddTTP, 0.000045 M each. The mixture was incubated at 37°C for 30 min followed by a 30-min chase with a 0.000166 M concentration of each of the four deoxynucleotides (1). The products were analyzed on 12 and 8% polyacrylamide gels. The only nucleotide sequence differences revealed in the R2 HA were in codons for residues 226 and 228 of HA1 in the RBS. The leucine at residue 226 in the Udorn strain was changed to glutamine in R2 as a result of a codon change from CTG to CAG, and the serine at 228 in the Udorn strain was changed to glycine in R2 as a result of a codon change from AGT to GGT (Fig. 1).

Studies by Rogers et al. (7) have shown that an amino acid change at residue 226 correlates with receptor specificity in vitro and with sensitivity of the virus to neutralization by a glycoprotein inhibitor present in horse serum. Molecules with leucine at 226 recognize *N*-acetylneuraminic acid α2, 6 galactose (SAα2, 6 Gal) linkages in glycoproteins and are sensitive to horse serum inhibitor, whereas those with glutamine at 226 recognize SAα2, 3 Gal linkages and are insensitive to horse serum inhibitor.

Since R2 had acquired a mutation at residue 226, the HAs of two additional reassortants with the same genotype, R3 and R4, were sequenced through the RBS region and tested

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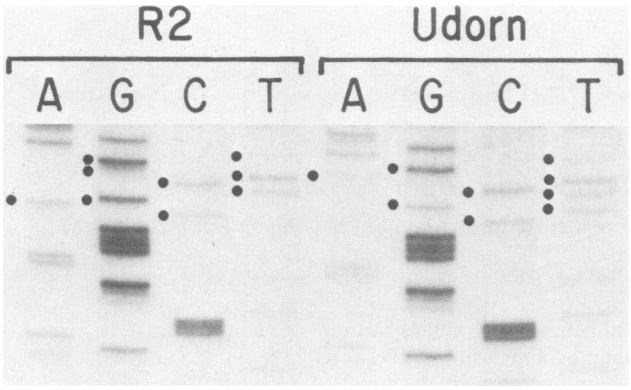

FIG. 1. Nucleotide sequence changes in the HA genes of an influenza virus tissue tropism mutant (R2) and its parental type (Udorn). The sequences shown include nucleotides 732 to 765 primed with the 618GGGGTTCACCAC primer. The only base changes in the R2 sequence relative to Udorn are included in the sequences highlighted by closed circles at amino acid residues 226 and 228 in HA1.

for sensitivity to horse serum inhibitor and for replication in ducks (Table 1). Virus HAs with leucine at 226 were sensitive to the inhibitor, whereas those with glutamine at 226 were insensitive, in accordance with the results of Rogers et al. (7). The parental Udorn virus and reassortant R3, both of which have the wild-type RBS sequence, did not replicate in ducks. In contrast, R4, with a single mutation at 226 (Leu to Gln), did replicate in avian bursal tissue when inoculated rectally, and R2, with the same mutation at 226 and an additional mutation at 228 (Ser to Gly), replicated in bursal tissue and in the cells lining the intestinal tracts of ducks. These point mutations in the RBS are likely to have altered the tissue tropism of the reassortants, suggesting that such mutations may have a pronounced effect on the spread of the virus to susceptible tissues and may influence host range as well.

We presume that these mutations alter the receptor specificity of the HA, but at this time we do not have specific information on the type of receptors on avian cells. If mutations in the RBS do change receptor specificity in vivo as demonstrated in vitro (7), then one might expect to find conservation in RBS sequences among viruses infecting a particular tissue or perhaps a specific host. Examination of 27 HA1 sequences now reported in the literature reveals that 4 can be considered to be avian strains (A/duck/Ukraine/63, fowl plague virus, A/Seal/Mass/1/80, and reassortant R2), and all have the same RBS sequence, Gly-Gln-Ser-Gly-Arg-Ile (4, 6, 11). The remaining 23 HAs have different RBS sequences. We suggest that the RBS sequence on avian influenza virus HAs is conserved as a result of selection by the host cell. Indeed, host cell selection of antigenic variants has recently been reported (9).

It should be noted that R4, although able to replicate in avian bursal tissue, can only do so if admininstered by rectal inoculation and not if administered orally. It is possible that the R4 HA is acid labile and will not withstand passage through the gizzard, where the pH is as low as 2.9 (12). Two possible explanations for this are that (i) the R4 RBS sequence has serine at 228, possibly leading to lability at low pH, or (ii) although the R2 and Udorn HAs were completely sequenced, R4 was sequenced only through the RBS, and thus, additional mutations which affect the phenotype could exist in the R4 HA.

The fact that two amino acid changes in the RBS can alter the antigenicity of the molecule is not surprising, since antigenic site B is immediately adjacent to, or part of, the RBS. Furthermore, these mutations could easily alter the conformation of the molecule to prevent the binding of certain monoclonal antibodies. Studies are under way to determine the binding sites of antibodies which no longer bind to the HA because of a mutation in the RBS.

A previous report by Both et al. (2) suggested that a single point mutation in the HA of influenza virus A/NJ/76 alters the virulence of the virus for pigs. Unfortunately, the results presented are difficult to interpret; the high-growing H strain HA gene was completely sequenced, but the low-growing L strain was not. The L strain HA1 sequence was missing 68 amino acids, and the HA2 sequence was not determined.

From our studies, it is clear that sequence changes in the RBS of the HA may have a profound effect on the biology of influenza viruses in nature. Changes at 226 (Leu to Gln) and at 228 (Ser to Gly) permit a virus to transit the low pH of the gizzard, replicate in cells lining the intestinal tract, and be spread by fecal contamination of the water supply. Viruses possessing all of the avian genes required for replication in ducks but lacking the changes at 226 and 228 in the RBS cannot replicate in the intestinal tracts of ducks. Viruses with a mutation at 226 in HA1 have the ability to replicate in bursal tissue but not in the cells of the intestinal tract. This would suggest that changes at 226 and 228 in a human HA can make that molecule avian and influence both the ability to transit the digestive tracts of ducks and the tissues in which it replicates. We emphasize the fact that additional avian genes are required for replication in these tissues;

TABLE 1. RBS sequences, sensitivity to neutralization by horse serum inhibitor, and replication in ducks after rectal inoculation of reassortant influenza viruses[a]

Virus	Amino acid at HA1 residue:						HSI sensitivity[b]	Replication in ducks[c]	
	225	226	227	228	229	230		Bursa	Intestine
Udorn	Gly	Leu	Ser	Ser	Arg	Ile	+	−	−
R2	Gly	Gln	Ser	Gly	Arg	Ile	−	+	+
R3	Gly	Leu	Ser	Ser	Arg	Ile	+	−	−
R4	Gly	Gln	Ser	Ser	Arg	Ile	−	+	−

[a] The complete HA1 sequences of Udorn and reassortant R2 were determind with synthetic oligodeoxynucleotides to prime dideoxynucleotide sequencing reactions containing total virion RNA, reverse transcriptase, and ^{32}P-labeled deoxynucleotides as described previously (1,8). Reassortant R3 and R4 RBS sequences were obtained with primer 618GGGGTTCACCAC. Reassortant influenza viruses were prepared as previously described (5).

[b] Sensitivity to horse serum inhibitor (HSI) was determined in hemagglutination inhibition assays (4) with commercially available horse serum; +, hemagglutination inhibition titer of 10,000; −, hemagglutination inhibition titer of <10.

[c] Duck enterotropism was assayed in groups of five 4-week-old Pekin ducks after rectal inoculation of virus (12); + indicates that high titers (10^5 to 10^6 50% egg infective doses per g) of virus were isolated from bursal or intestinal tissues collected from the birds and that the virus was antigenically and genetically identical to the input virus; − indicates that no virus was isolated.

however, the first step in the replication cycle, virus attachment to appropriate host cell receptors, is clearly influenced by the amino acid sequences in the RBS.

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