

1 **Epistatic interactions can moderate the antigenic effect of substitutions**  
2 **in hemagglutinin of influenza H3N2 virus**

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12 Running Title: Context-dependent effect of mutations in H3N2 virus

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19 **Abstract**

20 We previously showed that single amino acid substitutions at seven positions  
21 in hemagglutinin determined major antigenic change of influenza H3N2 virus.  
22 Here, the impact of two such substitutions was tested in eleven representative  
23 H3 hemagglutinins to investigate context-dependence effects. The antigenic  
24 effect of substitutions introduced at hemagglutinin position 145 was fully  
25 independent of the amino acid context of the representative hemagglutinins.  
26 Antigenic change caused by substitutions introduced at hemagglutinin position  
27 155 was variable and context-dependent. Our results suggest that epistatic  
28 interactions with contextual amino acids in the hemagglutinin can moderate the  
29 magnitude of antigenic change.

30 Influenza viruses of the H3N2 subtype have been circulating in humans since  
31 1968 and are a major cause of annual epidemics. Antibodies against the  
32 hemagglutinin (HA) surface glycoprotein can neutralize the virus and are a  
33 critical component of our immune defense against influenza viruses. However,  
34 the HA changes over time to escape from recognition by neutralizing antibodies  
35 present in the human population. The antigenic evolution of H3N2 viruses was  
36 previously mapped using hemagglutination inhibition (HI) assay data spanning  
37 a 35-year period (1). During this period, 11 genetically and antigenically distinct  
38 clusters emerged that comprise viruses of high antigenic similarity, each of  
39 which was consecutively replaced by a new cluster of antigenically distinct  
40 viruses (Fig 1A). Antigenic cluster transitions, the major antigenic changes  
41 between clusters, were subsequently shown to be predominantly caused by  
42 single amino acid substitutions on seven key positions adjacent to the HA  
43 receptor binding site (RBS) (2). Most positions were involved in cluster  
44 transitions multiple times suggesting that possibilities for antigenic change of  
45 influenza viruses are restricted (Fig. 1B and 1C).

46

47 Epistatic interactions can shape the evolution of influenza viruses (3–6). For  
48 example, intragenic epistasis in HA has been suggested to limit the rate of  
49 antigenic evolution and to inhibit the reversal of RBS substitutions to ancestral  
50 genotypes (5–8). An important question that remains unanswered is whether  
51 the HA amino acid context in which a substitution occurs determines its ability  
52 to escape from antibody recognition.

53

54 To answer this question, we selected two substitutions that were responsible  
55 for major antigenic change during H3N2 virus antigenic evolution (Fig. 1B).  
56 Substitution 155TY was responsible for the first antigenic cluster transition of  
57 the H3N2 virus in 1972, and 155YH together with 159SY and 189KR caused  
58 an antigenic cluster transition in 1987 (2). Substitution 145NK first caused an  
59 antigenic cluster transition in 1989 after 21 years of H3N2 virus evolution in  
60 humans (1, 2). The same substitution was responsible for another cluster  
61 transition six years later. We introduced the substitutions as single mutations  
62 into the HA genes of viruses representing the 11 antigenic clusters (Fig. 1D).  
63 Depending on the amino acid at position 155 or 145, we introduced either 155Y  
64 or T, or 145K or N in the HA genes (Fig. 1D). Viruses with naturally occurring  
65 145SN substitutions were detected from 1973 onwards (1). Between 1975 and  
66 1989 nearly all isolated strains had 145N. However, the 145SN substitution did  
67 not contribute to major antigenic change during this period (2). When  
68 representative viruses had 145S we therefore introduced 145K, but not 145N.  
69 Substitution 155H was involved in the cluster transition that occurred in 1987  
70 and 155H remained dominant between 1987 and 2002. For representative  
71 viruses with 155H, two modified HA genes containing either 155T or 155Y were  
72 constructed (Fig 1D). All introduced substitutions resulted in substantial  
73 changes in the biophysical properties of the amino acids. Plasmids containing  
74 wildtype or modified HA genes were used to generate recombinant viruses  
75 consisting of the (modified) HA gene and remaining genes of A/Puerto  
76 Rico/8/34 by reverse genetics (9). The presence of introduced mutations and  
77 absence of unwanted changes in HA was confirmed by Sanger sequencing.  
78 Subsequently, the antigenic properties of recombinant viruses were analyzed

79 in HI assays using the previously defined panel of ferret antisera listed in Table  
80 S1 (2, 10). To test the antigenic difference between 155T and 155Y in  
81 representative viruses with 155H we compared the HI results of the 155T and  
82 155Y mutants.

83

84 Mutants with substitutions at HA position 155 in HK68, EN72, VI75, TX77,  
85 SY97, and FU02 representative viruses were substantially antigenically  
86 different from their respective wildtype viruses, with up to 64-fold differences in  
87 HI titers (Fig. 2A). The 155TY amino acid difference at position 155 had a small  
88 antigenic effect in the HA context of all but one of the remaining representative  
89 viruses (SI87, BE89, BE92, WU95). Additionally, substitutions 155HT and HY  
90 had no or only modest antigenic effects in these four representative viruses—  
91 none had a more than 2-fold HI titer reduction against sera raised to viruses  
92 with homologous wildtype HAs. In the SY97 HA the 155T mutant was  
93 substantially antigenically different from the wildtype virus, but the 155Y mutant  
94 was not. Of the five representative viruses with a naturally present 155H, the  
95 155TY amino acid difference thus had a substantial antigenic effect only in the  
96 context of a single HA. These data strongly suggest that the modest effect of  
97 the 155TY difference in multiple HAs was due to the amino acid context in which  
98 it was introduced. Thus, although the TY substitution at position 155  
99 substantially changed the antigenic properties in more than half of the HAs  
100 tested here, the HA amino acid context in which this substitution occurs may  
101 dampen its ability to escape from antibody recognition.

102

103 In contrast, mutants with substitutions on position 145 of the same set of  
104 representative HAs were each antigenically distinct from their respective  
105 wildtype viruses (Fig. 2B). Thus, the magnitude of antigenic change caused by  
106 145 NK or KN substitutions appears not to be affected by the HA amino acid  
107 context.

108

109 Substitution 145NK was first observed when it caused the antigenic cluster  
110 transition from the SI87 to the BE89 cluster (Fig. 1A and 1B). When 145K was  
111 introduced in the HA of viruses representing the antigenic clusters that  
112 circulated prior to the SI87 cluster (HK68, EN72, VI75, TX77, and BK79), it  
113 caused similar escape from inhibition by antisera to contemporary or previously  
114 circulating strains as did 145K in the SI87 representative virus (Fig. 2B). We  
115 therefore next compared the magnitude of antigenic escape by 145K to that of  
116 the cluster-transition substitutions that occurred naturally before 1989 (Fig. 1).  
117 In this analysis, only antisera to strains from the same or previous antigenic  
118 clusters as the representative virus were included, thus testing escape from  
119 antibodies induced to previously circulating strains. The magnitude of the  
120 antigenic differences caused by 145K were similar to those caused by the  
121 naturally occurring substitutions that were responsible for antigenic cluster  
122 transitions before 1989 (Fig. 2C). Thus, if viruses with 145K had appeared  
123 before the BE89 antigenic cluster they may have been sufficiently antigenically  
124 different from earlier H3N2 viruses to provide escape from population immunity.  
125  
126 The central question addressed in this study was if the antigenic effect of  
127 substitutions in HA is dependent on the amino acid context in which they occur.

128 We answered this question using two substitutions known to be responsible for  
129 escape from population immunity in the past and the same analysis methods  
130 that were used to determine the contribution of these substitutions to antigenic  
131 evolution (2). The data generated for this study reflect the ability of the test  
132 viruses to escape from binding by antibodies in polyclonal ferret antisera at a  
133 fixed HA-activity. The magnitude of antigenic change caused by the introduced  
134 substitutions in the representative hemagglutinins far exceeds the antigenic  
135 change observed in studies using hemagglutinins with different binding avidities  
136 (11-13). Additionally, the large titer differences observed between sera tested  
137 to the same virus, up to 6.8 log<sub>2</sub> HI titer differences between sera, suggest that  
138 our results are not simply a reflection of the small differences that are the  
139 resultant of variations in receptor avidity.

140

141 HA amino acid context did not affect the magnitude of antigenic change caused  
142 by substitutions introduced on position 145, nor of the majority of substitutions  
143 introduced at position 155. Thus, the ability to cause antibody escape in the  
144 HAs tested here was largely independent of the amino acid context. While these  
145 results are in agreement with the recurrent use of seven key positions for major  
146 antigenic change during influenza H3N2 virus evolution and emphasize the  
147 potential importance of these key positions for future antigenic change (2), the  
148 data also suggest that epistatic interactions govern the antigenic effect caused  
149 by the substitutions introduced HA position 155.

150

151 The differences in magnitude of the antigenic effects of 155T and 155Y  
152 substitutions versus the context-independent antigenic changes caused by the

153 145N and 145K substitutions in different amino acid backgrounds may be due  
154 to differences in local HA structure (Fig. 3). Position 155 is located in the  
155 depression between the 190-helix that contains conserved position 195Y and  
156 a loop that contains conserved position 153W, which are fundamental  
157 components of the RBS (14, 15). In contrast, position 145 is located on a  
158 protruding loop that may have fewer structural constraints. Therefore, the  
159 substitutions introduced at position 145 may possibly have a larger impact on  
160 the local HA structure than the substitutions introduced at position 155, resulting  
161 in the more pronounced antigenic changes in the mutants with a substitution at  
162 position 145 observed here.

163

164 The ability of a new antigenic variant to escape from population immunity  
165 depends on the distance between the antigenic variant and the contemporary  
166 epidemic virus, which should be sufficiently large to escape from neutralization  
167 by antibodies to currently circulating viruses. Additionally, the direction of  
168 antigenic evolution should be away from all previously circulating viruses to  
169 maximize escape from recognition by antibodies to viruses responsible for  
170 earlier epidemics. We have here focused on testing the magnitude of antigenic  
171 change caused by substitutions in different HA amino acid contexts because  
172 limitations inherent to our experimental setup preclude meaningful analysis of  
173 directionality. Although our data indicate that the magnitude of antigenic change  
174 by 145K in HAs representative of early evolution strains equals that of the  
175 naturally selected escape mutants, we can make no claims about the  
176 directionality of antigenic change. Earlier work showed that epistatic  
177 interactions can affect the directionality of antigenic evolution because



178 introduction of co-occurring mutations with cluster-transition substitutions  
179 changed the directionality of the mutant viruses without adding to the antigenic  
180 distance (2). Although many evolutionary variables may determine which  
181 viruses eventually cause an epidemic, viruses with naturally selected escape  
182 mutations perhaps had a more favorable direction of antigenic evolution  
183 compared to viruses with 145K in HAs prior to 1989, which could explain why  
184 145K escape mutants did not emerge during the first decades of H3N2 virus  
185 evolution.

186

187 In summary, the requirement that substitutions occur in an HA context that is  
188 permissive for the protein changes that induce antibody escape suggests that  
189 the magnitude of antigenic change depends on epistatic interactions.  
190 Understanding the role of epistasis in antigenic evolution will help to evaluate  
191 the epidemic potential of newly emerging viruses.

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FIG 1 Experimental background and viruses used in this study. (A) Antigenic map of H3N2 virus antigenic evolution between 1968 and 2003. Open squares and colored circles indicate antisera and epidemic viruses, respectively. The viruses are color coded according to the antigenic cluster to which the virus belongs. Both horizontal and vertical axes indicate antigenic distance, the spacing between gridlines is one antigenic unit which equals a two-fold difference in the HI assay. Letters and <sup>[L]</sup><sub>[SEP]</sub>digits in antigenic cluster names refer to the location and year of <sup>[L]</sup><sub>[SEP]</sub>isolation of the first vaccine strain in that cluster (HK, Hong Kong; EN, England; VI, Victoria; TX, Texas; BK, Bangkok; SI, Sichuan; BE, Beijing; WU, Wuhan; SY, <sup>[L]</sup><sub>[SEP]</sub>Sydney; FU, Fujian). The large circles indicate the viruses used in this study to represent the antigenic cluster clusters. (B) Substitutions responsible for antigenic cluster transitions as defined in (2). (C) Amino acid positions responsible for major antigenic change during H3N2 virus antigenic evolution plotted on an A/Aichi/2/68 HA trimer (PDB accession code 5HMG). Monomers are shown in black, grey, and white, while the RBS is in yellow. Amino acid positions 145 and 155 are indicated in red and blue, respectively, while the remaining key positions are indicated in orange. (D) Mutants constructed for this study. Cluster representative viruses had the HA amino acid consensus sequence of all viruses in that cluster (described in (2)). BI, Bilthoven; NL, The Netherlands.

FIG 2 (A) HI titer differences between viruses with wild type and 155 mutant HAs. Each symbol represents the log<sub>2</sub> HI titer difference for an individual antiserum between a representative virus and a mutant with 155TY or 155YT, or between mutants with 155HT and 155HY (indicated as 155TY for SI87, BE89, BE92, WU95, and SY97). Color coding indicates the corresponding antigenic clusters for the strains used to raise the antisera (Fig 1A). The 2-fold difference in HI titer considered to be the error of the HI assay is indicated by the dashed horizontal line. (B) HI titer differences between viruses with wildtype HA and 145K or 145N mutants. Symbols as in panel A. (C) HI titer differences between cluster representatives, 145K mutants, and cluster-transition mutants. Each symbol represents the log<sub>2</sub> HI titer difference for an individual antiserum between viruses with wildtype and 145K mutant HA or between the wildtype and cluster-transition mutant virus. For the analysis in panel C only antisera to strains from the same or preceding antigenic clusters as the representative virus were included. Color coding as in panel A. Shapes indicate the individual antisera used for this analysis. HI data are available from Table S2.

FIG 3 Cartoon representation of the A/Aichi/2/68 RBS area. Positions 155 and 145 are indicated in blue and red, respectively. Positions 195Y and 153W, which are conserved among influenza A virus subtypes (14, 15), are indicated in pink.

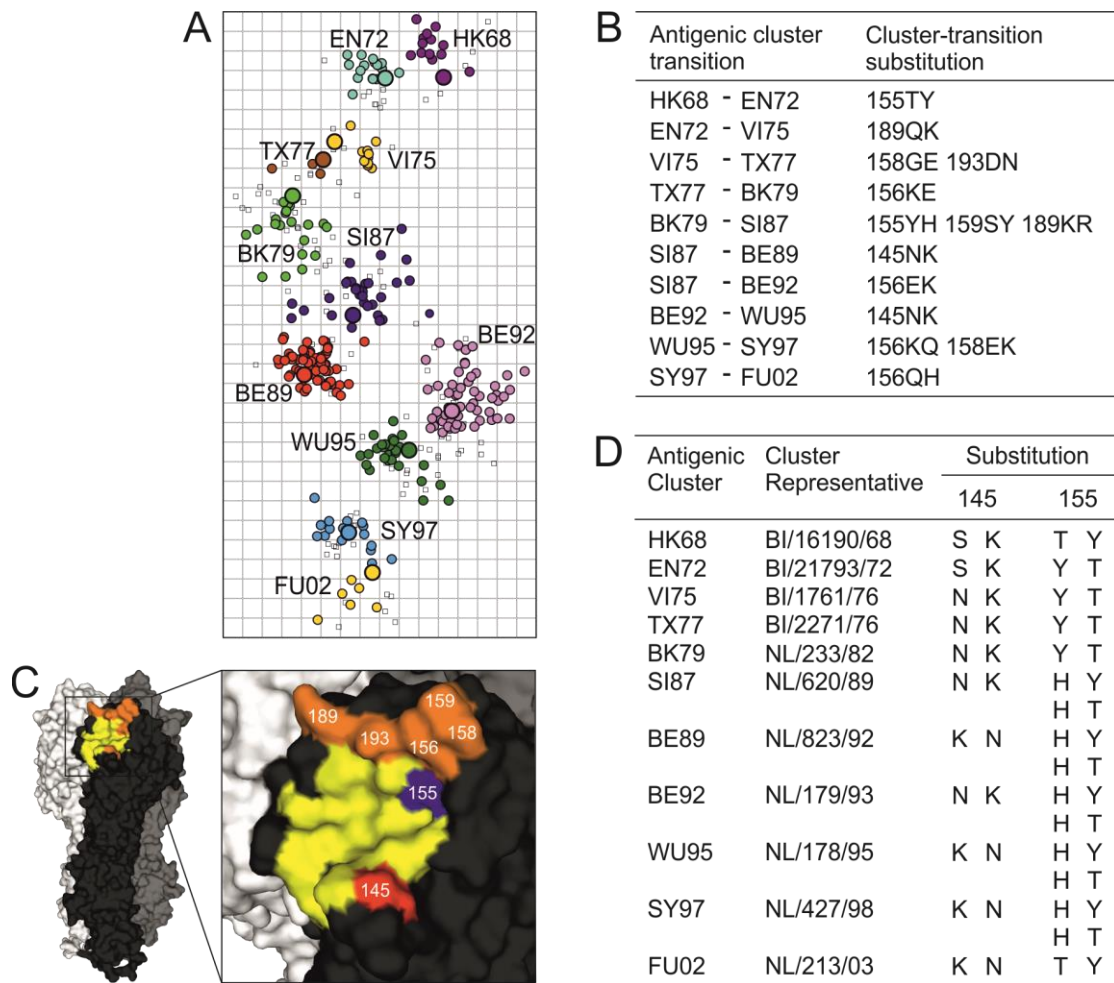


Fig 1

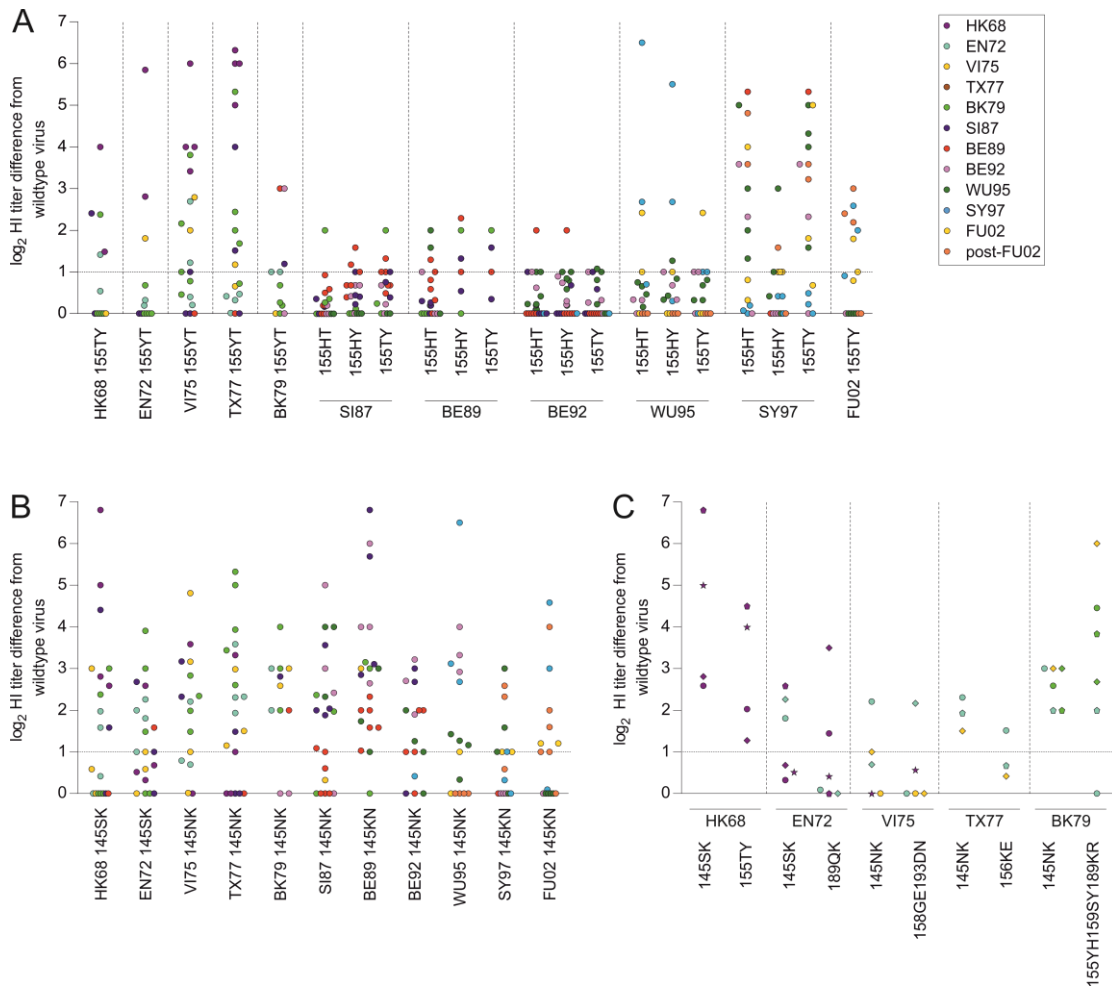


Fig 2

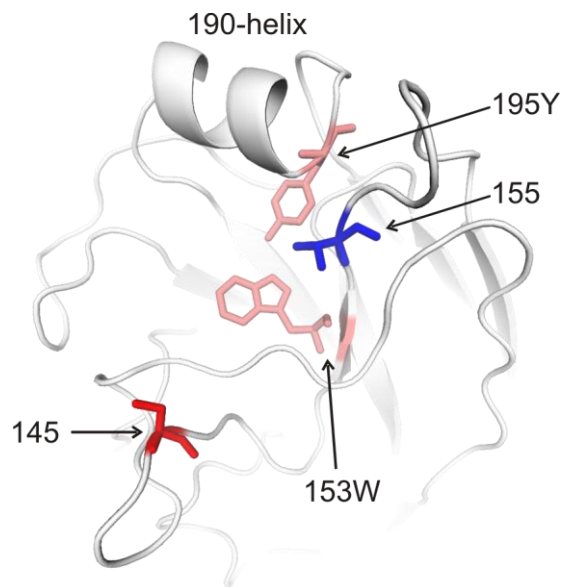


Fig 3