

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 log₂ 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 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 ^[L]_[SEP]digits in antigenic cluster names refer to the location and year of ^[L]_[SEP]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, ^[L]_[SEP]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 log₂ 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 log₂ 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.

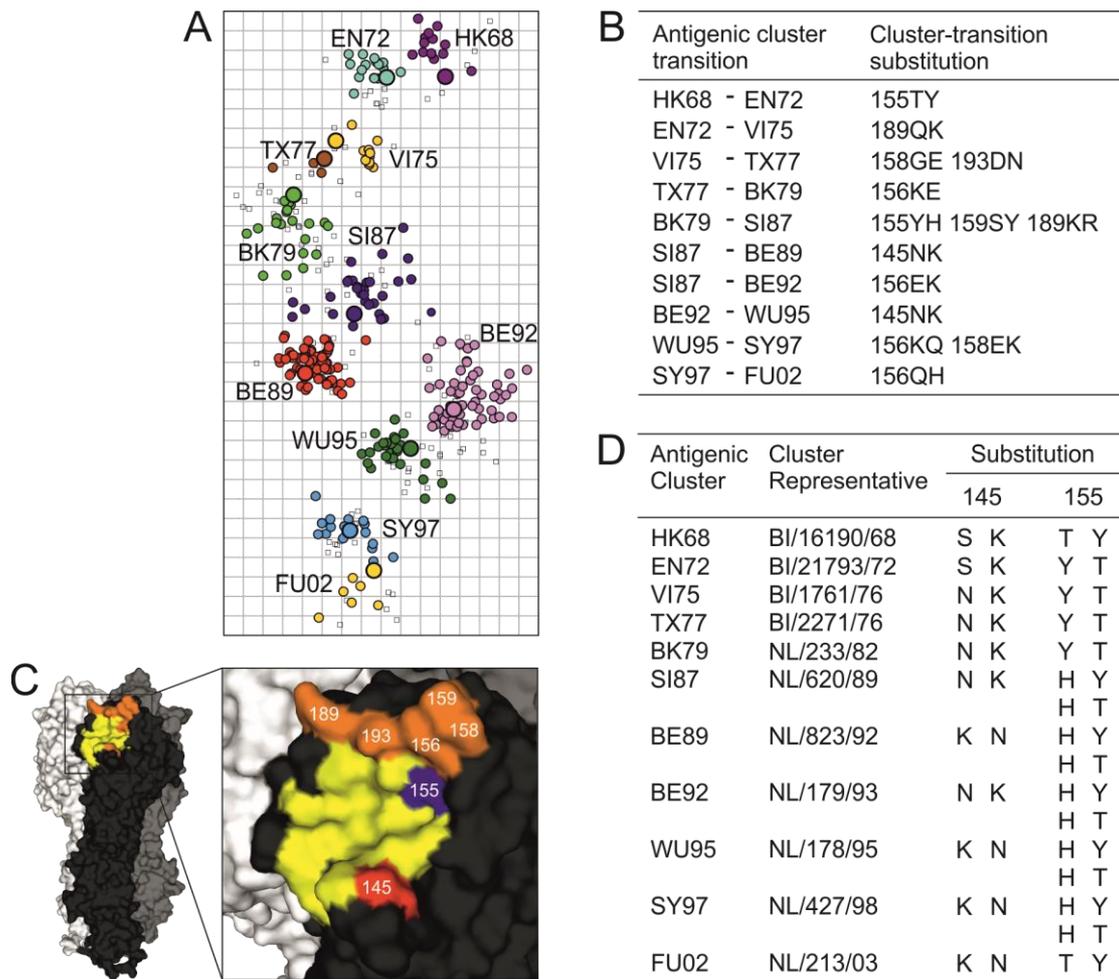


Fig 1

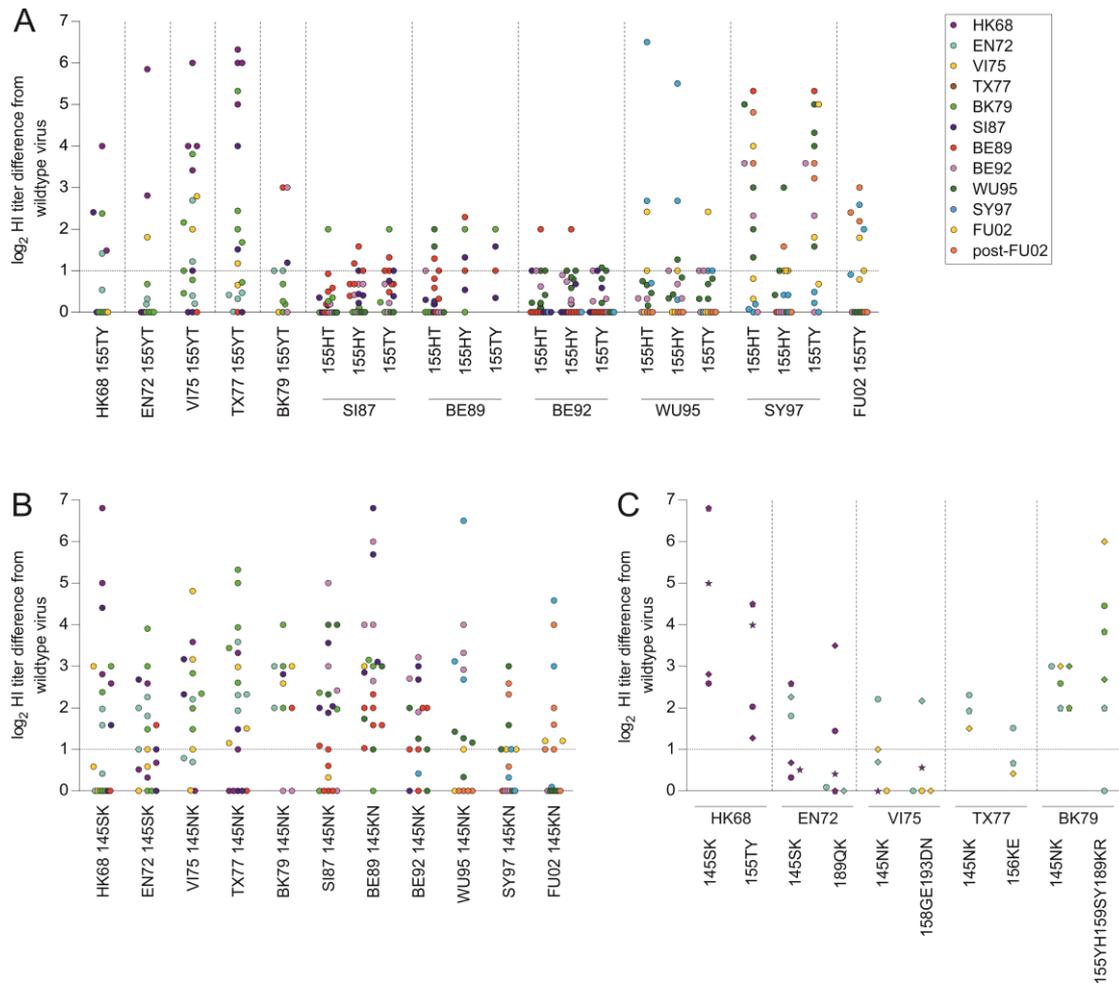


Fig 2

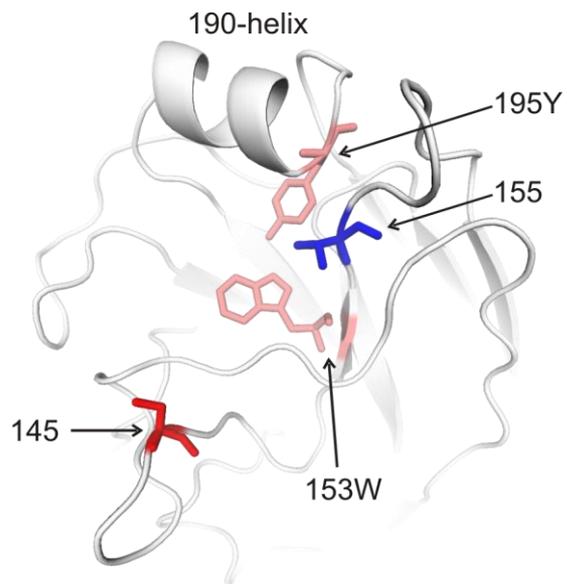


Fig 3