Supporting Information

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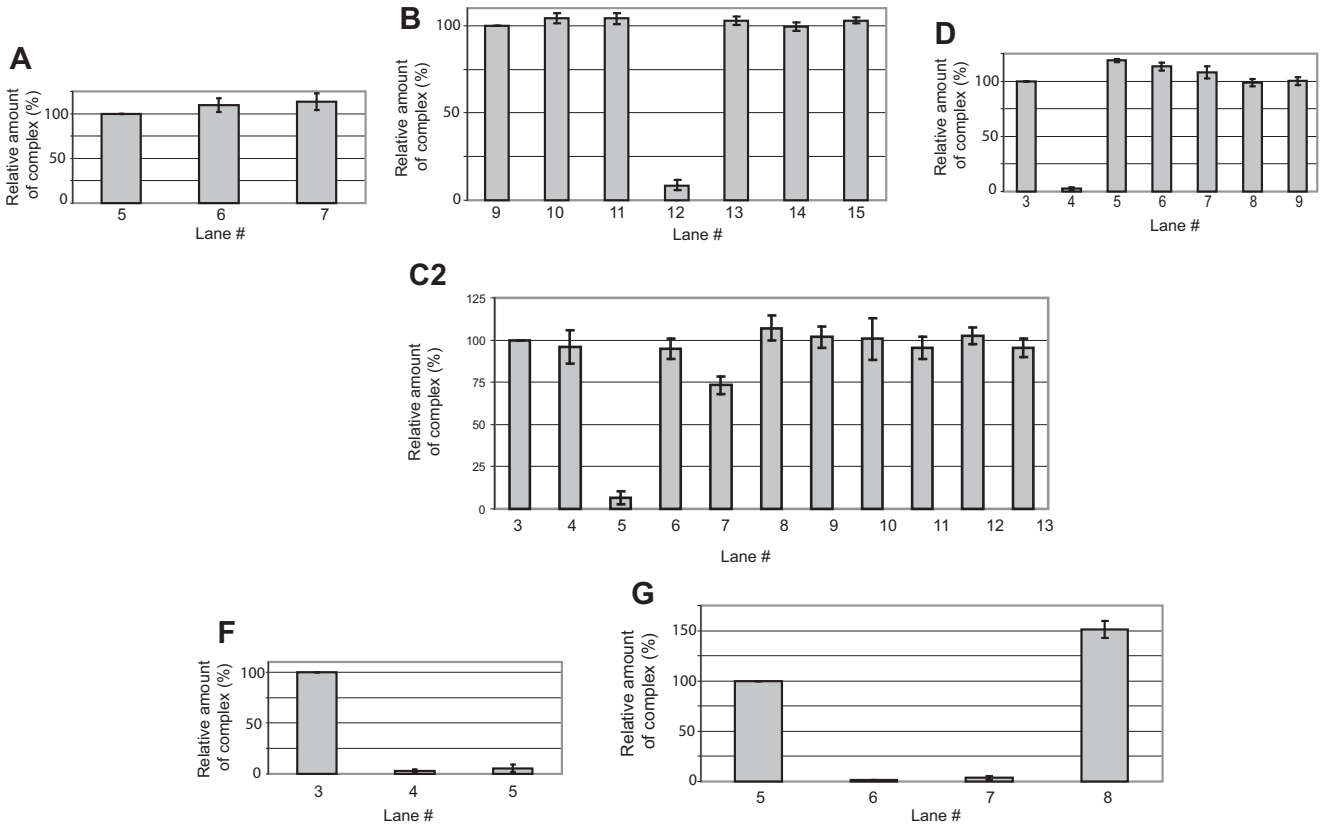

Fig. S1. Quantification of the experiments shown in Fig. 1. *A*, *B*, *C2*, *D*, *F*, and *G* correspond to the same panels in Fig. 1; for coherence there is no *C1* and no *E* in this figure. Only the lanes in which both single-stranded negative-sense RNA segments (vRNAs) have been coincubated were quantified. The lane numbers below each histogram match with the lanes numbers in Fig. 1. The amount of intermolecular complex present in each lane was normalized relative to the amount of complex obtained when wild-type (WT) vRNAs 2 and 8 were coincubated in the absence of oligo. Results are expressed as the mean ± SEM [$n = 4$ (*A*), 3 (*B*), 3 (*C2*), 5 (*D*), 13 (*F*), and 8 (*G*)].

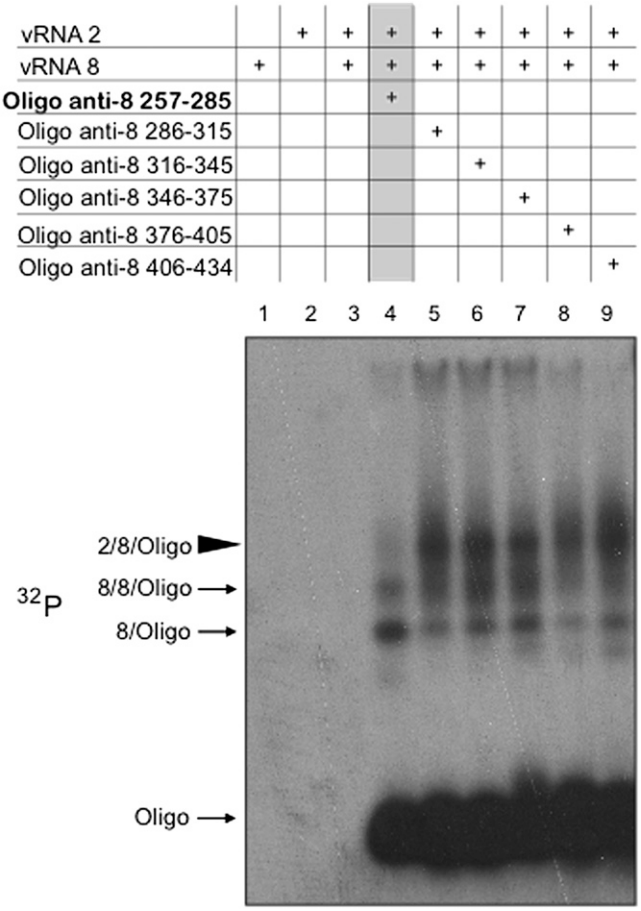

Fig. S2. Precise mapping of the region of vRNA 8 interacting with vRNA 2 using oligos. Autoradiography of the experiment shown in Fig. 1 *C1* and *C2*, in which trace amounts of ^{32}P-labeled oligos were included. Positions of the unbound oligos and of the oligos annealed to the monomeric and dimeric forms of vRNA 8 and to the heterodimeric vRNA 2/vRNA 8 complex are indicated.

A

vRNA 2 3′-AACCUUCGUUACCGAAAGGAA-5′
mRNA 2 5′-UUGGAAGCAAUGGCUUUCCUU-3′

vRNA 2^{Mut} 3′-AAUCUCCGAUACCGCAAGGAA-5′
mRNA 2^{Mut} 5′-UUAGAGGCUAUGGCGUUCCUU-3′

codon	UUG --> UUA		GAA--> GAG		GCA --> GCU		GCU--> GCG	
amino acid	L	L	E	E	A	A	A	A

B

vRNA 8 3′-GUUUUAUCGGUAACGAAGGUC-5′
mRNA 8 5′-CAAAAUAGCCAUUGCUUCCAG-3′

vRNA 8^{Mut} 3′-GUUUUAGCGGUAUCGGAGAUC-5′
mRNA 8^{Mut} 5′-CAAAAUCGCCAUAGCCUCUAG-3′

codon	AUA --> AUC		AUU --> AUA		GCU --> GCC		UCC --> UCU	
amino acid	I	I	I	I	A	A	S	S

Fig. S3. Mutations introduced in vRNAs 2 (*A*) and 8 (*B*) do not change the protein sequences. Mutations are indicated in the (–) strand vRNAs and in the corresponding (+) strand mRNAs. The codons that are affected by point substitutions are indicated, together with the corresponding amino acids.

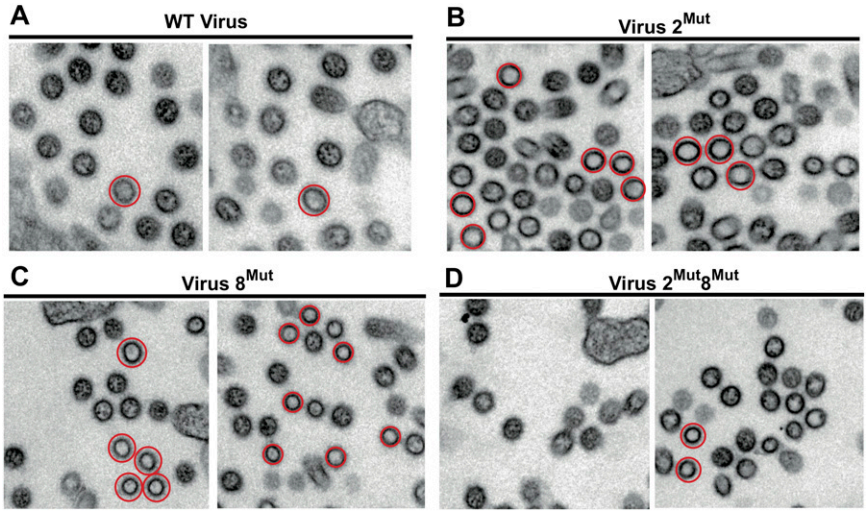

Fig. S4. Additional cross-sections of WT and mutant viruses. WT viruses (*A*) and viruses containing mutations in (*B*) segment 2 (2^{Mut}), (*C*) segment 8 (8^{Mut}), and (*D*) the virus containing mutations in both segments ($2^{Mut}8^{Mut}$) were observed by electron microscopy. Cross-sections revealing a complete matrix layer without any dots corresponding to vRNPs inside are circled in red.

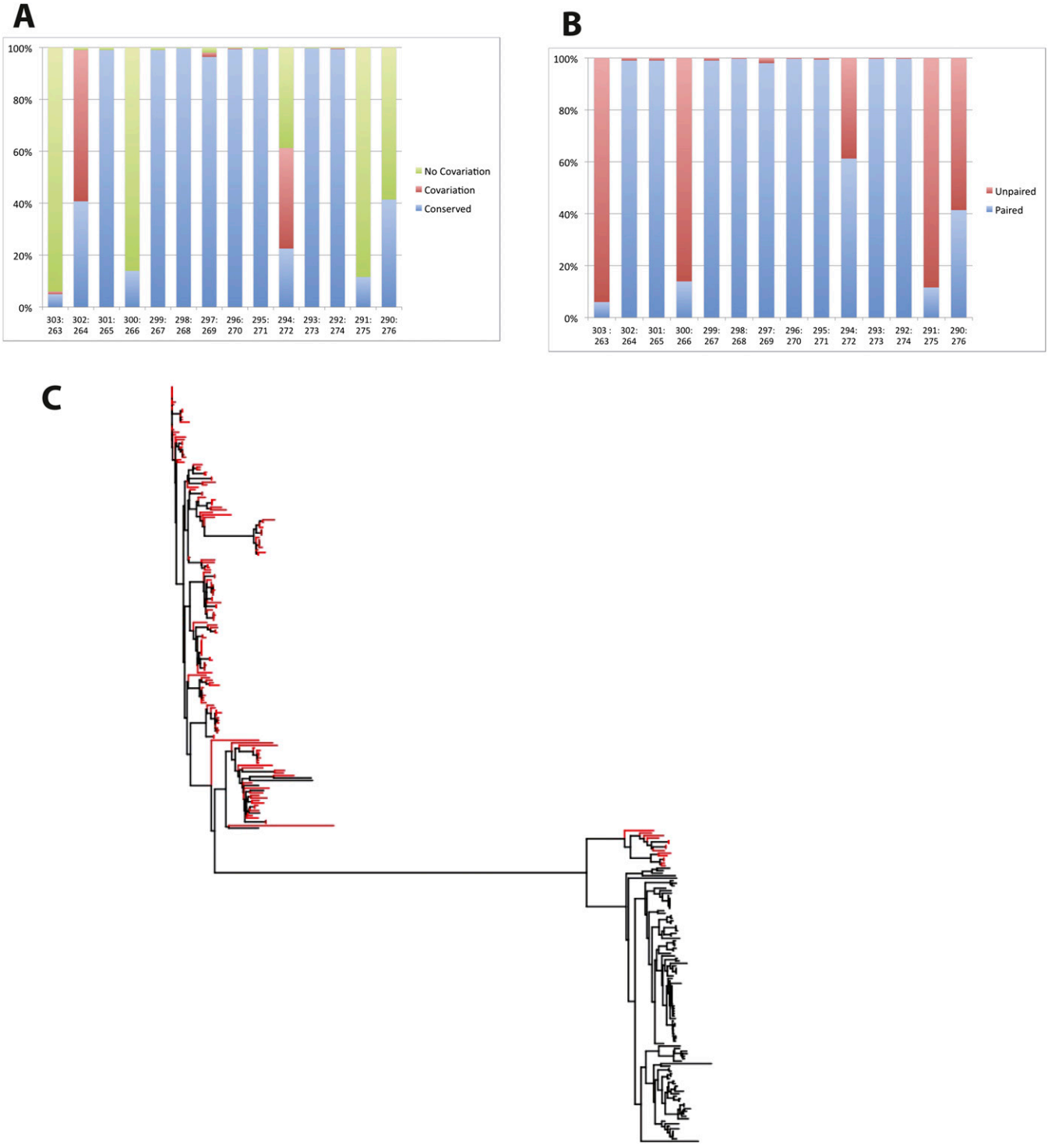

Fig. S5. Conservation of the interactions between vRNAs 2 and 8 among avian H5N2 influenza A viruses (IAVs). Three hundred eight complete sequences from the National Center for Biotechnology Information database were analyzed. (*A*) Conservation and covariation (or absence of) nucleotides (nts) 290–303 of vRNA 2 and nts 263–276 of vRNA 8. Significant covariation to maintain base-pairing is found at base pairs 302:264 (vRNA 2:vRNA 8) and 294:272. (*B*) Fraction of matched and mismatched base pairs at each position. Sixty-one percent of the avian H5N2 IAVs can form at least eight consecutive base pairs thanks to covariations at positions 294:272. (*C*) Phylogenetic tree of segment 8. The strains in which nt 272 of vRNA 8 can form a stable base pair with nt 294 of vRNA 2 are indicated in red.

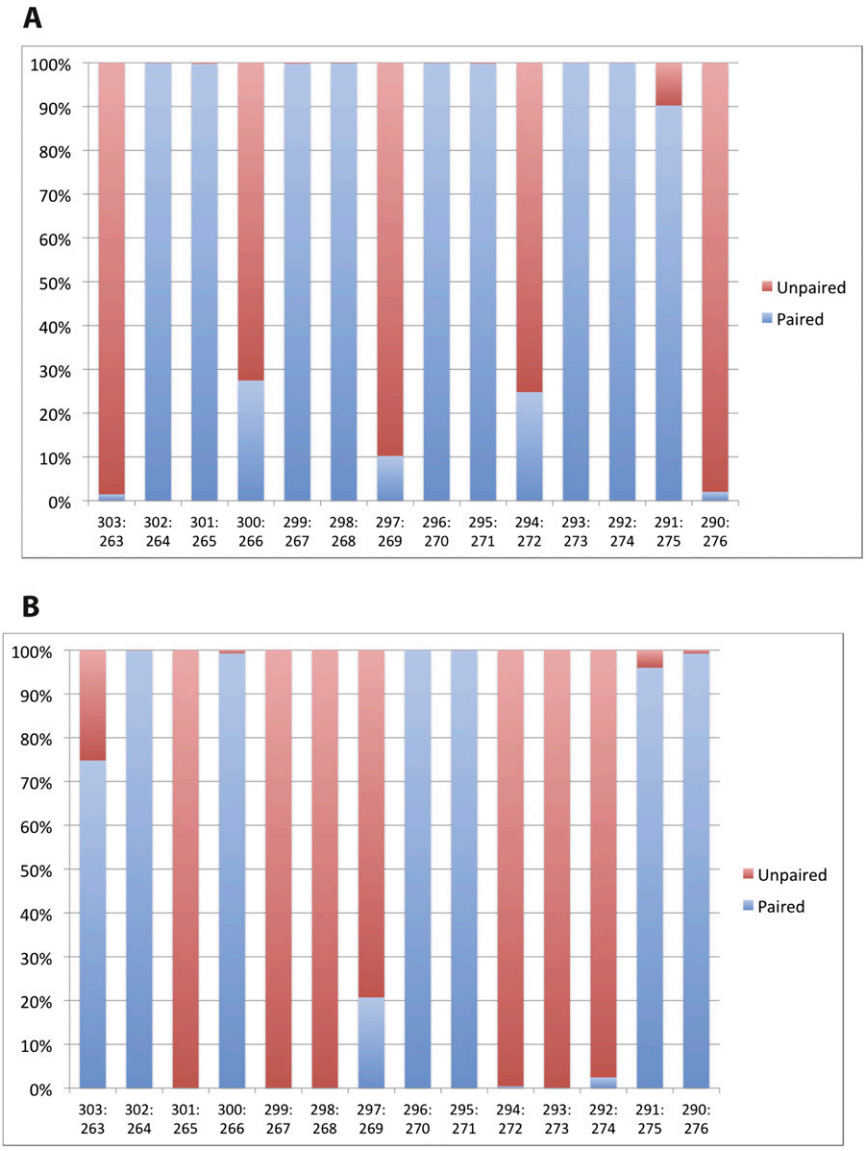

Fig. S6. The vRNA 2/ vRNA 8 interaction is not conserved among human IAVs. Base-pairing possibilities between the loci involved in the interaction between vRNAs 2 and 8 in A/Finch/England/2051/91 (H5N2) have been analyzed in 5,191 human H1N1 IAVs (*A*) and 3,123 human H3N2 IAVs (*B*). Because of limited base-pairing possibilities at positions 297:269, 294:272, and 300:266, only 275 of 5,191 human H1N1 IAVs can form at least eight consecutive base pairs. Among the 3,123 human H3N2 IAVs, none can form more than three consecutive base pairs.

Table S1. Relative amounts of vRNAs 2, 6, 7, and 8 in Madin–Darby canine kidney cells 3 h postinfection with WT, 2^{Mut}, 8^{Mut}, or $2^{Mut}8^{Mut}$ virus

	vRNA			
Virus	7	6	2	8
WT	100 (70–136)	100 (87–108)	100 (77–126)	100 (86–115)
2^{Mut}	100 (68–140)	72 (54–94)	87 (65–111)	106 (92–122)
8^{Mut}	100 (78–128)	130 (120–140)	144 (116–178)	137 (115–161)
$2^{Mut}8^{Mut}$	100 (74–135)	77 (71–82)	111 (91–135)	105 (93–117)

Infected cells 3 h postinfection, with relative amounts normalized to vRNA 7. vRNA 7 was used as a reference to normalize cells infected with different viruses. The amount of vRNAs 2, 8, and 6 in cells infected with mutant viruses was then compared with those infected with WT virus. RT-quantitative PCR was performed, as described in *Materials and Methods*. Data were obtained from two independent experiments performed in triplicate. The 95% confidence interval is indicated between parentheses.

Table S2. Competition between WT and mutant vRNAs 2 and 8 for packaging into progeny virions

Experimental conditions and results	Transfection/infection no. 1	2	3	4	5	6	7	8
Transfected plasmid	2^{WT}	2^{WT}	8^{WT}	8^{WT}	2^{Mut}	2^{Mut}	8^{Mut}	8^{Mut}
Infecting virus	$2^{Mut}8^{Mut}$	2^{Mut}	$2^{Mut}8^{Mut}$	8^{Mut}	WT	8^{Mut}	WT	2^{Mut}
Competing vRNAs	$2^{WT} + 2^{Mut}$		$8^{WT} + 8^{Mut}$		$2^{WT} + 2^{Mut}$		$8^{WT} + 8^{Mut}$	
Most frequent progeny virus	$2^{Mut}8^{Mut}$	WT	$2^{Mut}8^{Mut}$	WT	WT	$2^{Mut}8^{Mut}$	WT	$2^{Mut}8^{Mut}$
Fraction of most frequent virus	34/40 (85%)	22/25 (88%)	14/16 (88%)	26/40 (65%)	31/35 (89%)	26/38 (68%)	32/36 (89%)	21/38 (55%)
*P**	<0.0001		0.0002		<0.0001		<0.0001	

Two hundred ninety-three T cells were first transfected with the plasmid indicated in the first row and then infected with the virus indicated in the second row, resulting in the competition between the vRNAs indicated in the third row.

*Probability that the (WT or mutant) vRNA partner does not influence packaging of the competing WT and mutant vRNAs.