Supplementary Methods

Viral stocks, viral proteins and nucleic acids

The purified viral stock of influenza virus, A/Panama/2007/1999(H3N2) was a kind gift from Dr H. Kumihashi, Research Foundation for Microbial Diseases, Osaka University. A/Aichi/2/1968(H3N2) was grown in embryonated chicken eggs and purified as described previously (Kanaseki *et al.*, 1997). To evaluate the aptamer-binding region in the viruses, we purified the HA protein (consisting of the HA1–HA2 complex) from the viruses. In brief, the virus stock (1·2–1·4 mg viral protein in 1·5–2·0 ml PBS), containing about 30 % sucrose, was diluted 1·5-fold with Tris buffer [50 mM Tris, 25 mM NaCl (pH 7·5)], and was centrifuged (for 30 min at 45 000 ***g*** and 4 °C) to remove the sucrose. The pellet was suspended in 0·25 ml Tris buffer, mixed with 0·06 ml 20 % (w/w) Triton X-100 and incubated for 2 h at 37 °C. After centrifugation for 30 min at 45 000 ***g*** and 4 °C, the supernatant was applied to an ion-exchange column (Vivapure spin column, type S mini H; Sartorius AG). The protein eluted from the column was layered on a stepwise sucrose-density gradient [20–60 % (w/w) sucrose in PBS] and was centrifuged for 20 h at 160 000 ***g*** and 4 °C. The sample was fractionated and examined by SDS-PAGE. The fractions containing the purified HA protein were collected and stored at –80 °C until use. Similarly, the HA protein was purified from strains A/Wyoming/3/2003, A/Sydney/05/97, and A/Wuhan/359/95 of H3N2.

The RNA pool (30N) used in the selections was described previously (Fukuda *et al.*, 2000). The RNA pool contained a core of 30 random bases flanked by primer 5′-GGGAGAATTCCGACCAGAAG-N30-CCTTTCCTCTCTCCTTCCTCTTCT-3′. The primers used for the amplification of the pool were 5′-AGTAATACGACTCACTATAGGGAGAATTCCGA CCAGAAG-3′ (designated 39.N30) and 5′-AGAAGAGGAAGGAGAGAGGAAAGG-3′ (designated 24.N30). In the selection cycles, yeast tRNA (Boehringer Mannheim) was used as a non-specific competitor. To prepare the shorter variant of the influenza aptamer, we synthesized the DNA template 5′-AGTAATACGACTCACTATAGGGTTAGCAGTCGGCATGCGGTACAGAC AGACCC-3′ corresponding to the P30-10-16 aptamer. Using the T7 promoter forward primer and the corresponding reverse primer (5′-GGGTCTGTCTGTACCGCATGCCG-3′), the above DNA template was converted into double-stranded DNA and used for *in vitro* transcription to prepare the RNA for the binding analysis.

Analysis of aptamers and binding assay

To obtain the individual aptamers, the amplified PCR product from cycle 10 was ligated directly into the pCRII vector (Invitrogen) according to the manufacturer's protocol. DNA was isolated from individual clones by the alkaline-lysis method and was sequenced with a Dye

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Terminator Sequencing kit (Applied Biosystems) on a DNA sequencer (model 373A; Applied Biosystems). To evaluate the binding activities of the RNA pools from different selection cycles, as well as those of the individual aptamers, internally labelled RNA was prepared by using 0·5 mCi (18·5 MBq) [α-^{32}P]CTP ml^{-1}. The binding and *in vitro* transcription conditions were similar to those used for the selection except for the molar ratio of RNA to virus or HA protein (20 nM RNA and 1·25 mg virus ml<MIN>1 or 200 nM HA). The filters were washed with 1 mlof binding buffer and air-dried and the radioactivity was quantified with an image analyser (BAS2000; Fuji Film). To ensure that the binding was specific, we added a 10-fold molar excess of tRNA as a non-specific competitor in the binding reaction. To determine the aptamer-binding region on the virus, we isolated the HA proteins from the A/Aichi and A/Panama strains of influenza virus and directly tested the binding of aptamers to these proteins, using conditions similar to those used for the whole virus (filter-binding assay).

Binding assay using SPR

Kinetic measurements of aptamer binding were conducted by using the SPR method on a BIAcore 2000, with a streptravidin-coated sensor-chip, from BIAcore. The association and dissociation kinetics of the P30-10-16-HA complex and the P30-10-16C-HA complex were evaluated. To determine the affinity constants of the selected aptamers, we prepared the aptamers with 24-mer poly(A) nucleotides at the 3′ end, which could anneal to the complementary biotinylated oligo(dT) [5′-Biotin-(T)$_{24}$-3′]. A spacer sequence (C-nucleotide)$_4$ between the poly(A) sequence and the 3′-end sequence of the aptamer sequence was also used, in order to avoid overlapping between the hybridizing region and the aptamer-binding site near the chip. To prepare this extended RNA at the 3′ end, we used two primers: a forward primer similar to that used in the selection and a 3′-end primer [5′-(T)$_{24}$-(G)$_4$-AGAAGAGGAA GGAGAGAGGAAAGG-3′]. The double-stranded DNA template was generated by PCR and transcribed *in vitro*, as mentioned above. Initially, the biotinylated oligo(dT)$_{24}$ was attached to the streptavidin (SA chip; BIAcore) by dissolving the oligo(dT) in binding buffer (5 μM final concentration) and injecting it for 12–24 s to obtain a response of 1000 resonance units (RU), at a flow rate of 5 μl min^{-1}. The excess or unbound biotinylated oligo(dT) was washed with binding buffer, at a flow rate of 20 μl min^{-1} for 10 min. To analyse the binding kinetics of the aptamer, 20 μl (50 nM final concentration) aptamer was injected at a flow rate of 2 μl min^{-1} for 10 min, which resulted in an increase of about 1200 RU upon aptamer binding to the complementary biotinylated primer. Various concentrations of HA (6·6–20 nM) were injected into the flow cell at a flow rate of 20 μl min^{-1} for 3 min, to obtain the binding kinetics. After each measurement, the sensor chip was washed with a buffer solution, followed by 10 mM NaOH, before the next injection. For a comparative study, the commercially available antibody against the HA of

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A/Panama (catalogue no. MAB8254; Chemicon International) was injected at various concentrations between 6 and 100 nM. The data were fitted to a local fit of the kinetic simultaneous K_a/K_d model, on the assumption of Langmuir (1 : 1) binding. For analysing the binding ability of aptamer to three other HAs derived from closely related isolates (A/Wyoming/3/2003, A/Sydney/05/97 and A/Wuhan/359/95), we have used conditions similar to those mentioned above. The HA concentrations tested for the binding analysis ranged from 50 to 500 nM.

Phosphate modification and interference analysis

In order to map the binding sites of the HA within the selected aptamer, a phosphate modification assay was performed. *In vitro*-transcribed RNA was treated with calf intestine phosphatase for 1 h at 37 °C, extracted with phenol and precipitated with ethanol. The RNA was labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase, electrophoresed on a 10 % polyacrylamide/7 M urea gel and eluted from the gel. The labelled RNA was allowed to fold into a tertiary structure by heating at 95 °C for 2 min and cooling slowly to room temperature. The 5′ end-labelled RNA (1100×10^3 c.p.m.) was dissolved in buffer [20 mM HEPES (pH 8·0), 1 mM EDTA and 2·5 μg tRNA] and was mixed with 5 μl saturated *N*-nitroso-*N*-ethyl urea in ethanol. After modification at 90 °C for 2 min, the reaction was stopped by the addition of 15 μg carrier tRNA and the sample was recovered by ethanol precipitation. To allow the formation of the aptamer–HA complex, 20 pmol treated sample was denatured in 25 μl selection-binding buffer at 92 °C for 2 min and left to cool at room temperature for 10 min. The aptamer–HA complex was separated by passage through a nitrocellulose filter and was partially hydrolysed in a solution (200 μl) of 100 mM Tris/HCl (pH 9·0) at 50 °C for 5 min. The RNA-cleavage products were recovered by ethanol precipitation and loaded on a 10 % polyacrylamide/7 M urea gel. An aliquot of the alkaline hydrolysates of the aptamer and samples digested with RNase T1 were co-electrophoresed for band identification. The gel was dried and exposed on an X-ray film for autoradiography.

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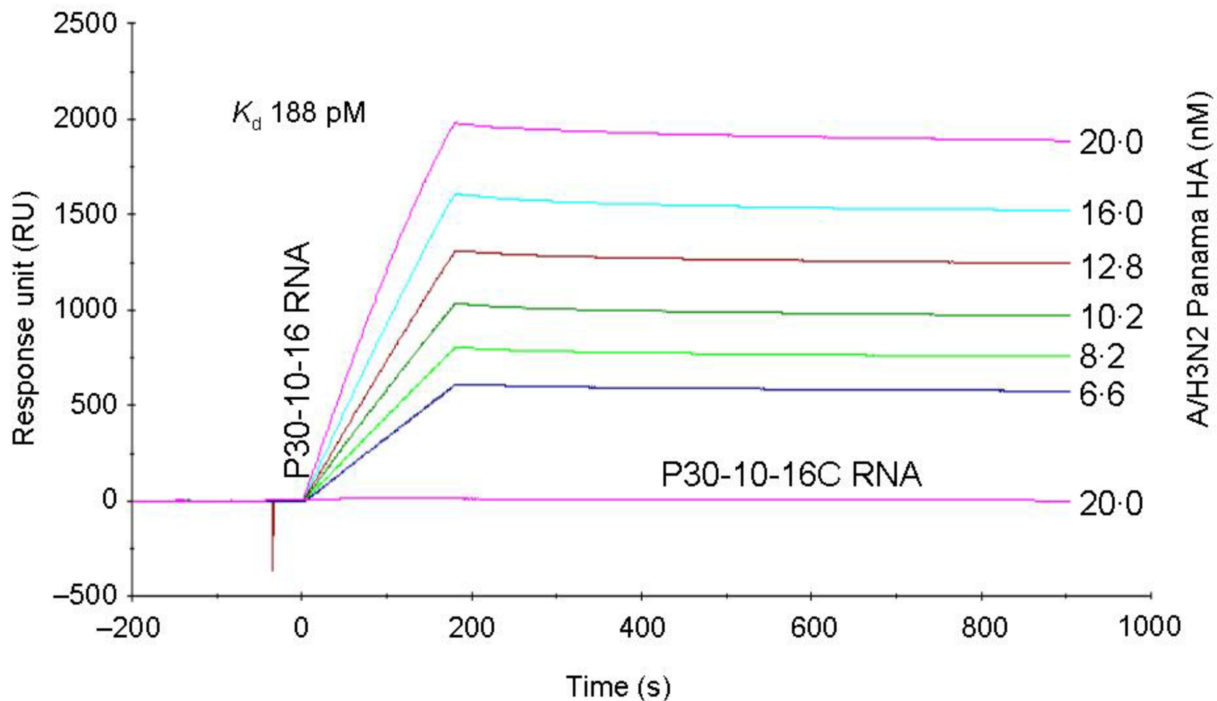
Supplementary Fig. S1. SPR analysis of the P30-10-16-HA complex. Twenty microlitres (50 nM final concentration) aptamer was injected at a flow rate of 2 μl min^{-1} for 10 min. Sensogram runs with HA of A/Panama/2007/1999(H3N2) was injected into the flow cell at a flow rate of 20 μl min^{-1} for 3 min.



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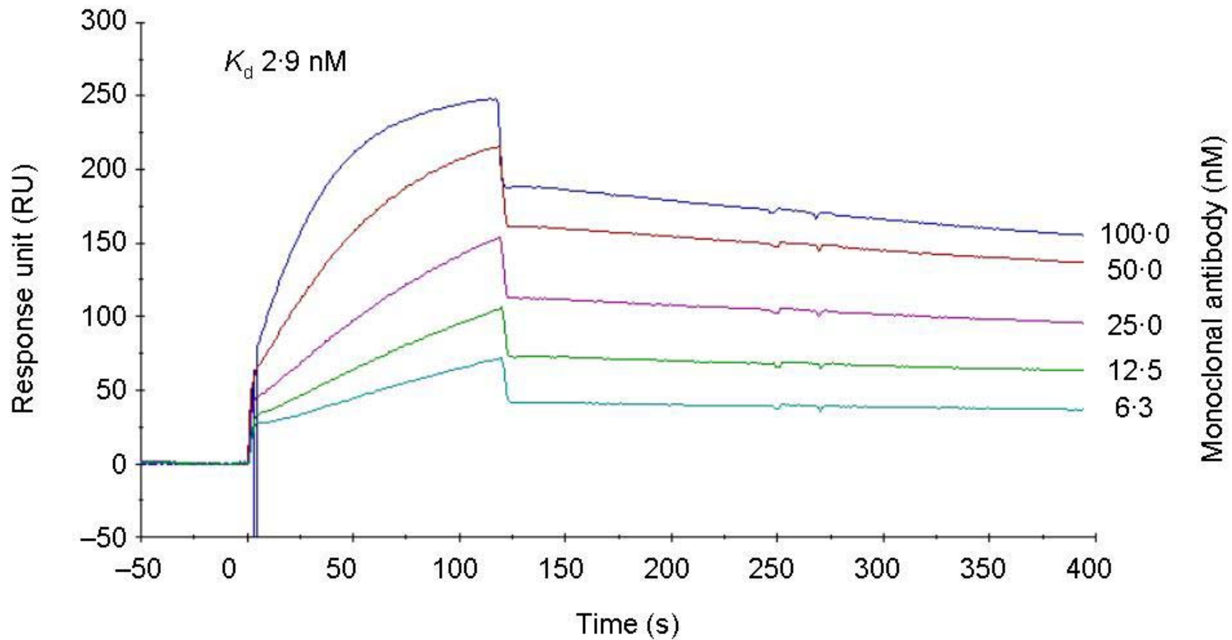
Supplementary Fig. S2. SPR analysis of the mAb–HA complex. The commercially available antibody against the HA of A/Panama was injected at various concentrations between 6 and 100 nM. The data were fitted to a local fit of the kinetic simultaneous K_a/K_d model, on the assumption of Langmuir (1 : 1) binding.



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