MATERIALS AND METHODS

Comparative RNA structure analysis
Initial computer-aided predictions of complete segment 8 sequences from representative strains of influenza A and B were produced using the algorithms simulating folding during RNA synthesis (Gultyaev, 1991; Gultyaev et al., 1995). For these initial predictions, one influenza A strain was chosen from each group according to the clustering of the sequences into 9 main lineages (Kawaoka et al., 1998; Basler et al., 2001) – human, swine, four avian and two equine lineages belonging to the clade (“allele”) A and one avian lineage of clade B. The details of local structures predicted for different viruses were also verified by the folding algorithms capturing equilibrium (Zuker, 2003) and kinetic (Xayaphoummine et al., 2005) properties of RNA folding. Finally, the conservation of the structures was checked by BLAST (Altschul et al., 1990) sequence comparison of all sequences present in GenBank (3017 strains at the moment of writing). The BLAST option “search for short, nearly exact matches” was used with sequence queries comprising local structures.

Synthesis of RNA transcripts
Transcripts corresponding to nts 498-526 of segment 8 mRNA were synthesized by T7-directed transcription from DNA templates generated by PCR on plasmids harboring the complete segment 8 cDNA of various strains. Primers used for PCR, were FLU7 (5'-TAATACGACTCATAAGGAGAAATCTCACCATTACCTTCTTCC-3', T7 promoter sequence in bold) and FLU8 (5'-CATTCCATTCAAGTCCGATG-3'). Derivatives C1, D2, E2, F2, and H2, were obtained by cloning of the appropriate oligonucleotide pairs into PfoI and Psp51I sites of pRF599 (gift of Dr. R. Fouchier). pRF599 harbors a cDNA copy of the A/NL/219/03 [H7N7] NS segment. PCR with oligo's FLU7 and FLU8 generated the DNA templates for transcription. Transcripts were purified by double isopropanol precipitation as described before (Olsthoorn et al., 1999).

Structure probing of RNA transcripts
RNA transcripts were dephosphorylated and subsequently 5'-radiolabeled as described by Nagel et al. 2002. Labeled RNA was purified by double isopropanol precipitation. RNA was incubated at room temperature in probing buffer (10 mM sodium cacodylate pH7.2, 10mM MgCl$_2$, 50 mM NaCl, 10 µg yeast tRNA) with water or enzyme. After 30 min at room temperature an equal volume of formamide loading buffer was added and samples were heated for 3 min. at 95ºC and subsequently electrophoresed in a 10% polyacrylamide/8M urea gel. Gel was dried and exposed to a phosphorimaging screen.

NMR
Synthetic oligonucleotides - HPLC grade - of 49 nt corresponding to positions 523-571 of DKSH01 or VT04 RNA were purchased from IBA (Goettingen, Germany), and dissolved in 10 mM sodium phosphate buffer pH 6.0, 90%.10% H$_2$O/D$_2$O. The 2D NOESY spectrum was recorded at 288 K on a Varian Inova 600 MHz spectrometer using a 200 ms mixing time.

REFERENCES


Supplementary figure 1S. **Structure probing of A/PR/8/34, DKSH01, and VT04 transcripts (95 nt).** 5′-radiolabeled RNA was incubated at room temperature in probing buffer (10 mM sodium cacodylate pH7.2, 10mM MgCl₂, 50 mM NaCl, 10 μg yeast tRNA) with water or 0.01 units of RNase T1. After 30 min at room temperature an equal volume of formamide loading buffer was added and samples were heated for 3 min. at 95°C and subsequently electrophoresed in a 10% polyacrylamide/8M urea gel. Gel was dried and exposed to a phosphorimaging screen. RNase T1-susceptible G-residues are indicated at the right side of the gel.
Supplementary figure 2S. **Shifting the equilibrium between pseudoknot and hairpin conformation.** Via site-directed mutagenesis changes were introduced into 95-nt transcripts to deliberately affect this equilibrium. In C1 the equilibrium is shifted toward the pseudoknot conformation. In F2 and H2 the equilibrium is shifted toward the hairpin conformation. In D2 and E2 the equilibrium is comparable to that of natural DKSH01 RNA (see Fig. 1C). Nucleotide changes with respect to the sequence of E2 are indicated in red boxes. Note that C1 and F2 have changes at both sides of the pseudoknot and hairpin. For clarity the surrounding and loop nucleotides are not shown. Image in the middle shows an agarose gel loaded with the indicated transcripts, and stained with ethidium bromide (see also legend to Fig. 1C).
Supplementary figure 3S. Structure probing of C1, E2, and F2 transcripts (95 nt).
A. 5’-radiolabeled RNA was incubated at room temperature in probing buffer (10 mM sodium cacodylate pH 7.2, 10 mM MgCl₂, 50 mM NaCl, 10 µg yeast tRNA) with the indicated enzyme(s). After 30 min at room temperature an equal volume of formamide loading buffer was added and samples were heated for 3 min. at 95°C and subsequently electrophoresed in a 10% polyacrylamide/8M urea gel. Gel was dried and exposed to a phosphorimaging screen. Lanes 1, 6, and 11 are control samples (no enzyme added). Lanes 2, 7, and 12 were incubated with 0.01 units; lanes 3, 8, and 13 with 0.001 units RNase T1. Lanes 4, 9, and 14 were incubated with 4 units; lanes 5, 10, and 15 with 0.1 units of nuclease S1. RNase T1-susceptible G-residues are indicated at the right side of the gel. B. Migration of the 5’-radiolabeled RNAs in a native 10% acrylamide gel. "hp" and "pk" mark the position of the hairpin and pseudoknot conformers, respectively. In this gel, E2 consists of approx. 75% hairpin and 25% pseudoknot conformers as determined by phosphorimaging.
Supplementary figure 4S. **2D-NOESY NMR experiment of the VT04 hairpin.** A synthetic oligonucleotide of 49 nt corresponding to positions 523-571 of A/Vietnam/1194/04 (except for position 538 which was A) was measured as described under Materials and Methods. The sequential imino to imino proton NOE walk is depicted by the black lines.
Supplementary figure 5S. 2D-NOESY NMR experiment of the ‘minimal’ pseudoknot. A synthetic oligonucleotide of 49 nt corresponding to positions 523-571 of DKSH01 was measured as described under Materials and Methods. The NOE walk is depicted by the red lines. Black lines with question marks indicate unassigned crosspeaks.