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RNA Aptamers Specifically Interact with the Prion Protein PrP

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We have isolated RNA aptamers which are directed against the recombinant Syrian golden hamster prion protein rPrP23-231 (rPrP^c) fused to glutathione *S*-transferase (GST). The aptamers did not recognize the fusion partner GST or the fusion protein GST::rPrP90-231 (rPrP27-30), which lacks 67 amino acids from the PrP N terminus. The aptamer-interacting region of PrP^c was mapped to the N-terminal amino acids 23 to 52. Sequence analyses suggest that the RNA aptamers may fold into G-quartet-containing structural elements. Replacement of the G residues in the G quartet scaffold with uridine residues destroyed binding to PrP completely, strongly suggesting that the G quartet motif is essential for PrP recognition. Individual RNA aptamers interact specifically with prion protein in brain homogenates from wild-type mice (C57BL/6), hamsters (Syrian golden), and cattle as shown by supershifts obtained in the presence of anti-PrP antibodies. No interaction was observed with brain homogenates from PrP knockout mice (*prn-p$^{0/0}$*). Specificity of the aptamer-PrP interaction was further confirmed by binding assays with antisense aptamer RNA or a mutant aptamer in which the guanosine residues in the G tetrad scaffold were replaced by uridine residues. The aptamers did not recognize PrP27-30 in brain homogenates from scrapie-infected mice. RNA aptamers may provide a first milestone in the development of a diagnostic assay for the detection of transmissible spongiform encephalopathies.

Prions are thought to be involved in the pathogenesis of transmissible spongiform encephalopathies such as scrapie, bovine spongiform encephalopathy (BSE), and Creutzfeldt-Jakob disease (26) (for a review, see reference 7). According to the protein-only hypothesis (1, 12, 26), the crucial step in prion propagation is the conversion of PrP^c into its structural and infectious isoform PrP^{Sc}. Knockout mice lacking the *prn-p* gene were found to be resistant to infection with PrP^{Sc}-containing material, demonstrating that PrP^c is necessary for the development of transmissible spongiform encephalopathies (3, 4). Recent transmission studies of the BSE agent for mice suggest that PrP^{Sc} might be involved in species adaptation but that an unidentified agent may actually transmit BSE (21).

Full-length PrP^{Sc} identified in scrapie-associated fibrils (2, 16, 17) can be processed by proteinase K to proteinase K-resistant PrP27-30 lacking 67 amino acids from the amino terminus (5, 27–29). PrP27-30 is present in an aggregated form in amyloid-like rods (27–29) and scrapie-associated fibrils (16). A proteinase K-sensitive, soluble, and presumably noninfectious version of PrP27-30, designated sPrP27-30 (25), which was recently identified in human platelets, might also act as a precursor of proteinase K-resistant PrP27-30.

The biochemical processes involved in prion propagation, prion processing, and prion protein aggregation are largely unknown. Their study requires a diagnostic reagent able to distinguish between PrP^c and its various isoforms. Although PrP-specific antibodies have been described, none of these achieved the required discrimination (13, 14). We thus turned to the method of in vitro selection of large combinatorial libraries of randomized RNA or single-stranded DNA (ssDNA) sequences (reviewed in references 11 and 24). In vitro selection, or SELEX, has proven extremely successful in the identification of nucleic acid ligands, designated aptamers, which bind target molecules with high affinity and specificity, in some cases exceeding that of antibodies (19).

For SELEX, protein targets must be fully accessible in a stable, soluble, and native conformation. We thus used a previously described GST::rPrP^c fusion protein (35), the glutathione *S*-transferase (GST) domain of which serves as a useful ligand for the immobilization of the fusion protein. Using a complex library of randomized RNA sequences, we identified and characterized a series of PrP-specific RNA aptamers and studied their binding properties under different conditions by gel retardation assays. The binding site of the selected RNA aptamers to PrP was mapped with a series of PrP peptides fused to GST (6, 35). In addition, the aptamers were analyzed for specific binding to PrP^c or PrP^{Sc} in crude brain homogenates from uninfected mice, hamsters, and cattle and scrapie-infected mice and *Prn-p$^{0/0}$* mice. Binding specificity was assayed in supershift experiments with PrP^c-specific antibodies and with antisense aptamers and mutant aptamers in which the G residues in the G scaffold were replaced with U residues.

MATERIALS AND METHODS

In vitro selection. The RNA pool, designated M111.1, was prepared as described previously (8). M111.1 carries a randomized sequence of 74 nucleotides flanked by two 20-mer primer binding sites and displays a pool complexity of 1.03×10^{15} (10). Radiolabelled RNA M111.1 (6.8 nmol in the first cycle, 1.82 nmol in the second cycle, 914 pmol in the third cycle, 665 pmol in the fourth cycle, 2.07 nmol in the fifth cycle, 831 pmol in the sixth cycle, 2.7 nmol in the seventh cycle, and 1.94 nmol in the eighth and ninth cycles) was incubated with GST immobilized on glutathione Sepharose (Pharmacia) corresponding to 185 pmol of immobilized GST in binding buffer (8 mM Na_2HPO_4–0.87 mM KH_2PO_4–136 mM NaCl–112.6 mM KCl–2 mM dithiothreitol–2 mM $MgCl_2$) at

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37°C. In cycles 1 to 7, beads were collected by centrifugation at 700 × *g* for 10 min. In cycles 8 and 9, the negative selection was performed twice for 30 min as described above. The first eight cycles of the cognate selection were carried out with the supernatant from the negative selections with immobilized recombinant GST::PrP^{c}23-231 (53 pmol) (35). After 60 min of incubation, nonbinding RNAs were removed by washing with binding buffer, and the binding RNAs were eluted with elution buffer (8 M urea–100 mM sodium citrate [pH 8.0]–3 mM EDTA). The RNA was extracted with phenol (pH 5.0)-chloroform and ethanol precipitated. In cycles 9 to 11, selected RNA (40 pmol in the 9th cycle and 4 pmol in the 10th and 11th cycles) was incubated with GST::PrP^{c}23-231 in solution (160 pmol in the 9th cycle and 40 pmol in the 10th and 11th cycles) for 60 min at 37°C in binding buffer. RNA-protein complexes were separated from unbound RNA by electrophoresis on a 0.7% native agarose gel as described previously (37). The shifted RNA/GST::PrP^{c}23-231 complex was excised and purified by Quiagen extraction according to the manufacturer's protocol. The amplification protocol for binding RNAs was identical in each cycle; 50% of the extracted RNA was subjected to a reverse transcription reaction, 50% of the resulting cDNA was amplified by PCR, and 50% of the PCR-DNA was in vitro transcribed. RNA from cycle 11 was reverse transcribed and PCR amplified (9). The cDNA from 20 clones was restricted with *Eco*RI and *Bam*HI, subcloned into pGEM-3-Zf(−), and sequenced.

RNA preparation. RNA aptamer motifs I, II, and III were synthesized from individual aptamer clones by in vitro transcription with T7 RNA polymerase, or we amplified the relevant cDNA coding for Ap1, Ap2, and Ap3 from plasmids pGEM-3-Zf(−)::Ap1, pGEM-3-Zf(−)::Ap2, and pGEM-3-Zf(−)::Ap3, respectively, by PCR with PCR primer I (5′) CCGAATTCTAATACGACTCACTA TAGGAGTCAGCCTTCACTGC and PCR primer II (3′) GTGGATCCGAC CGTGGTGCC. RNA aptamers Ap1, -2, and -3 were synthesized by in vitro transcription with T7 RNA polymerase. Ap1 antisense RNA was synthesized by in vitro transcription with SP6 RNA polymerase. Ap1 (29 mer) was synthesized by PCR with an oligodeoxyribonucleotide (5′) GGCTAATACGACTCACT ATAGGGAATTTGAGGGACGATGGGGAAGTGGG, PCR primer III (5′) GGCTAATACGACTCACTATA, and PCR primer IV (5′) CCCACTTCC CCACCGTC, followed by in vitro transcription with T7 RNA polymerase. The mutant aptamer Ap1 (G→U) was synthesized by PCR with oligodeoxyribonucleotide (5′) GGCTAATACGACTCACTATAGCAATGCGTTGTGTTT AATT TGATTTACGATTTTGAAGTTTTGACGAATGACTCATTGC, PCR primer III, and PCR primer V (5′) GCAATGAGTCATTCGTCAAA, followed by in vitro transcription with T7 RNA polymerase. The RNA was phenol-chloroform extracted, ethyl alcohol precipitated, and purified by polyacrylamide gel electrophoresis and Sephadex G-50 spin columns.

Gel retardation assay. Radiolabelled RNA generated by in vitro transcription in the presence of [α-^{32}P]GTP or [α-^{32}P]UTP (30) and protein or brain homogenates were incubated in binding buffer (described above) for 60 min at 37°C in the absence (for brain homogenates) or presence (for recombinant GST::rPrP^{c}23-231) of 1 μg of bovine serum albumin per μl and loaded onto a 0.7% native agarose gel. With brain homogenates, 20 U of RNasin (Promega) was added. After electrophoresis, gels were fixed and dried or blotted onto nitrocellulose and analyzed by autoradiography.

Supershift assay. Antibodies Ra18 or Ra37-15 were preincubated with brain homogenate from hamster (kindly provided by C. I. Lasmézas) at 4°C in binding buffer (see above). After 60 min, the binding reaction was supplemented with 2 pmol of aptamer Ap1 and incubated for 45 min at 37°C before a mixture of yeast tRNAs (5 μg total; Boehringer Mannheim, catalog no. 109 495) was added. The reaction mixture was incubated for 15 more min, followed by the addition of 6 μl of sample buffer comprised of 40% glycerol in 0.6× phosphate-buffered saline (PBS). The mixture was analyzed on a 0.7% agarose gel. After electrophoresis at 6 V/cm, the gel was dried or blotted onto nitrocellulose and analyzed by autoradiography.

Preparation of brain homogenates. Ten percent (wt/vol) brain homogenates from wild-type mice (C57BL), PrP knockout mice (PrP$^{0/0}$), hamsters (Syrian golden), cattle, and scrapie-infected mice (strain 79A; 120 or 150 days postinfection) were prepared as follows. Brains were homogenized in 9 volumes of PBS supplemented with 0.5% Nonidet P-40, 0.5% deoxycholic acid, and 0.6 mM phenylmethylsulfonyl fluoride. After centrifugation (8,000 × *g* for 10 min), the supernatants were aliquoted and stored at −80°C. The protein concentration was measured by a bicinchoninic acid assay.

For supershift experiments, brain homogenates from Syrian golden hamster were obtained from C. I. Lasmézas, Commissariat à l'Energie Atomique, Fontenay-aux-Roses, France. Whole brain hemispheres were homogenized at 20% (wt/vol) in a 5% glucose solution. The homogenates were further diluted to 5% in PBS containing 1 mM phenylmethylsulfonyl fluoride (final concentration) and centrifuged at 12,000 rpm (in an Eppendorf centrifuge) for 15 min at 4°C. The supernatant was assayed in supershift experiments.

Proteins and antibodies. GST, GST::rPrP23-231 (GST::rPrP^{c}), GST::rPrP90-231 (GST::rPrP27-30), GST::P23-52, GST::P53-93, GST::90-109, GST::P129-175, GST::P180-210, and GST::P218-231 from the Syrian golden hamster were prepared as described previously (6, 35, 38). Polyclonal antibody Ra18 directed against a peptide corresponding to amino acids (aa) 37 to 53 from hamster PrP, polyclonal antibody Ra37-15 directed against a peptide corresponding to aa 206 to 225 from hamster PrP, and polyclonal antibody Ra5 directed against a peptide corresponding to aa 95 to 110 of the hamster or mouse prion protein were prepared as described previously (13).

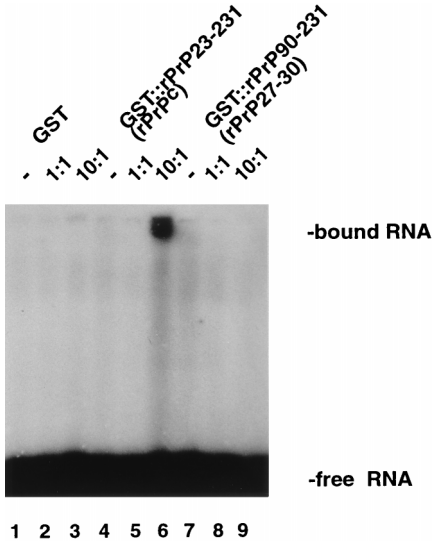

FIG. 1. RNA aptamers directed against rPrP23-231 (rPrP^{c}) from hamster after 11 cycles of in vitro selection interact specifically with GST::rPrP23-231. After 11 cycles of in vitro selection, 4 pmol of radiolabelled RNA was incubated in the absence of protein (lanes 1, 4, and 7) and in the presence of 4 (lane 2) and 40 (lane 3) pmol of GST, 4 (lane 5) and 40 (lane 6) pmol of GST::rPrP23-231, and 4 (lane 8) and 40 (lane 9) pmol of GST::rPrP90-231 and analyzed on a 0.7% agarose gel.

Proteinase K assays. Ten microliters of each individual brain homogenate was incubated in the absence or presence of 10 or 20 μg of proteinase K per ml (Sigma) at 37°C. Reactions were terminated after 30 min by the addition of sodium dodecyl sulfate (SDS) sample buffer, incubated for 10 min at 94°C, and analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting. When they were used in gel retardation assays, brain homogenates were preincubated with 20 μg of proteinase K per μl for 30 min at 37°C.

SDS-polyacrylamide gel electrophoresis and Western blotting. Protein samples were analyzed on SDS–12.5% polyacrylamide gels (20), blotted onto nitrocellulose (32), and developed in the presence of the Ra5 antibody.

RESULTS

In vitro selection of RNA aptamers that bind to rPrP23-231 (PrP^{c}) fused to GST. Recombinant rPrP23-231 (rPrP^{c}) from the Syrian golden hamster fused to GST (35) and a complex pool of about 10^{15} different RNA molecules randomized at 74 nucleotide positions (8) were subjected to an in vitro selection procedure. The selection protocol included a negative selection on immobilized GST-glutathione Sepharose in cycles 1 to 8 to remove non-PrP-specific sequences. After eight selection cycles, about 2% of the total input RNA bound to the glutathione-GST::rPrP23-231 column but not to a glutathione-GST or glutathione column. To increase the stringency of the selection, three additional cycles with free GST::rPrP23-231 in a gel retardation assay were performed. After a total of 11 cycles of iterative selection and amplification, RNA aptamers which bind specifically to rPrP23-231 (rPrP^{c}) fused to GST could be isolated (Fig. 1, lane 6). The aptamers do not bind to GST or to rPrP90-231 (rPrP27-30) (38) fused to GST (Fig. 1, lanes 1 to 3 and 7 to 9, respectively), suggesting that the selected aptamers bind to the N-terminal region of rPrP23-231.

RNA from cycle 11 was reverse transcribed, and the resulting pool of cDNAs was converted into double-stranded DNAs by PCR, cloned into pGEM-3-Zf(−), and sequenced. Fourteen of the twenty clones sequenced (70%) contained four sets of three highly conserved consecutive guanosine residues separated by single-stranded regions between four and seven nu-

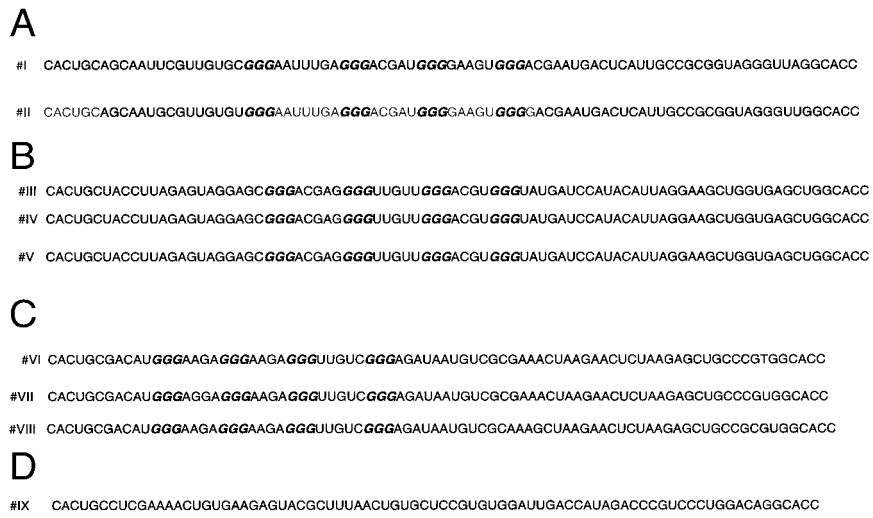

FIG. 2. Sequences of selected RNA aptamers directed against rPrP^{c}23-231 from hamster fused to GST by in vitro selection. The aptamers belong to several groups of molecules. RNA aptamers of motif I (A), motif II (B), and motif III (C) can have G quartet motifs and recognize rPrP23-231 (rPrP^c) but not rPrP90-231 (rPrP27-30). (D) Aptamers which have no G quartet motif do not bind to GST::rPrP^c, GST::rPrP27-30, or GST.

cleotides long. These guanosine-rich consensus motifs are flanked by two variable regions of predominantly Watson-Crick covariation (Fig. 2). The distributions of selected RNA aptamers in the clones sequenced were as follows: for motif I, 10%; for motif II, 15%; for motif III, 15%. Thirty percent of the sequenced DNA molecules code for RNAs which may contain G quartets but have unique loop regions. The remaining 30% of the selected RNA aptamers did not contain any G quartet motifs. The presence of repetitive guanosine triplets suggests that these aptamers can form a G quartet, a secondary-structure motif well known to stabilize RNA molecules (Fig. 3). Among the selected RNA aptamers, eight (40%) could be grouped into three classes based on their homology within the three single-stranded loop regions (Fig. 2). While individual members of each class had identical putative G tetrad and loop regions, they showed significant covariation in the Watson-Crick helix.

Monoclonal RNA aptamers Ap1, Ap2, and Ap3 bind specifically to rPrP23-231 (rPrP^c) fused to GST. Representative monoclonal RNA aptamers from classes I (Ap1; Fig. 3A), II (Ap2; Fig. 3C), and III (Ap3; Fig. 3D) interact specifically with rPrP23-231 (rPrP^c) from Syrian golden hamster fused to GST in solution, as determined by a gel retardation assay. None of the three aptamers binds to the recombinant GST-fused prion protein rPrP90-231 (rPrP27-30) (Fig. 3A, C, and D, lanes 3), demonstrating that the molecules distinguish between recombinant rPrP^c and recombinant rPrP27-30. Members of the aptamer class that lacks the G quartet scaffold (Fig. 2D) did not bind to GST, GST::rPrP23-231, or GST::rPrP90-231 (data not shown). We also constructed a 29-mer version of Ap1 which corresponds exactly to the motif that lacks the complete stem region, depicted in Fig. 3B. This truncated mutant had the same binding characteristics the full-length Ap1 did, demonstrating that the stem is not necessary for PrP recognition (Fig. 3B, lane 2).

G quartets of the aptamer are essential for PrP^c recognition. To further investigate whether the G residues are essential for building up the G quartet scaffold, we replaced the guanosine residues with uridine residues. The interaction of Ap1 with PrP^c (Fig. 4B, lane 2) was completely abolished with G→U Ap1 (lane 4), demonstrating that the G residues are essential for PrP recognition. Application of an antisense Ap1 aptamer complementary to Ap1 also failed to interact with PrP^c (Fig. 4A, lane 4), confirming that the G quartet scaffold is required for PrP recognition. In addition, unlabelled Ap1 is able to compete in a dose-dependent manner for the binding of radiolabelled Ap1 to rPrP23-231 fused to GST (Fig. 4C, lanes 3 to 6), while unselected RNA failed to compete for the Ap1-PrP^c binding (data not shown), confirming the specificity of the Ap1-PrP^c interaction.

Mapping of the PrP-aptamer binding site. To map the interaction site of Syrian golden hamster PrP^c to RNA aptamers containing motifs I (Ap1) and II (Ap2), we employed a series of recombinant prion peptides (6, 35) in the gel retardation assay. Only peptide $P_{23\text{-}52}$ was found to interact with RNA aptamers Ap1 and Ap2 (Fig. 5, lanes 2), demonstrating that these amino terminal amino acid residues of PrP are essential for interaction with the aptamer. Ap3 (Fig. 3D) also interacts with the amino terminal end of PrP (data not shown).

Interaction of the RNA aptamer with a protein in brain homogenates from mice, hamsters, and cattle. To investigate whether RNA aptamers are able to recognize authentic PrP^c in crude protein extracts, brain homogenates from mice were assayed for PrP-RNA interactions in gel retardation assays. Brain homogenates from wild-type mice incubated with radiolabelled RNA aptamer Ap1 yielded a band which showed significantly reduced electrophoretic mobility compared to that of free RNA (Fig. 6A, lanes 3 and 1, respectively). No interaction was observed with brain homogenates from isogenic PrP knockout mice (*Prn-p*$^{0/0}$) (Fig. 6A, lane 2), suggesting that the aptamers interact specifically with authentic mouse PrP^c. To further prove the specificity of the PrP-aptamer recognition, we employed the antisense Ap1 construct in binding assays with brain homogenates from transgenic mice overexpressing PrP^c (Tg35). Used as controls, both antisense aptamer Ap1 and the G→U mutant Ap1 failed to interact with authentic PrP (Fig. 6B, lanes 3 and 5).

To test for the species specificity of the aptamer-PrP interaction, we also analyzed brain homogenates from hamsters and cattle. Figure 6C demonstrates that Ap1 and Ap2 also interact with PrP^c from hamsters (lanes 2 and 5), mice (lanes 4, 7, and 10), and cattle (lane 11).

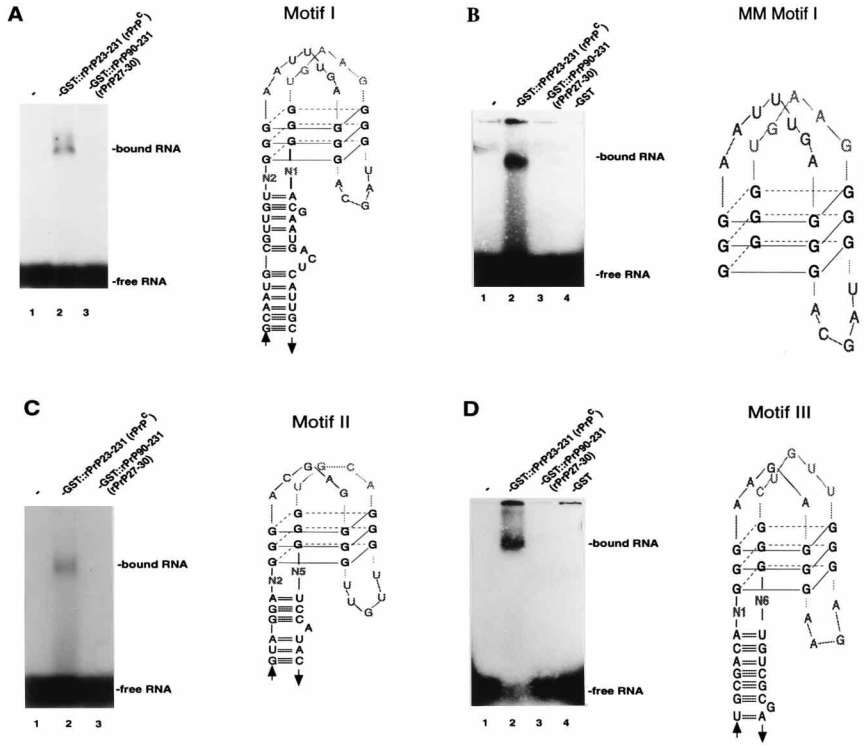

FIG. 3. RNA aptamer motifs I, II, and III recognize the recombinant prion protein rPrP23-231 (rPrP^c) but not rPrP90-231 (rPrP27-30) from hamster. (A) Four picomoles of labelled RNA Ap1 (motif I; lanes 1 to 3) was incubated in the presence of 40 pmol each of recombinant GST::rPrP23-231 (rPrP^c) (lane 2) and GST::rPrP90-231 (rPrP27-30) from Syrian golden hamster (lane 3). (B) Four picomoles of labelled RNA Ap MM (29 mer; MM motif I; lanes 1 to 4) was incubated in the presence of 40 pmol (each) of recombinant GST::rPrP23-231 (rPrP^c) (lane 2), GST::rPrP90-231 (rPrP27-30) from Syrian golden hamster (lane 3), and GST (lane 4). (C) Four picomoles (each) of labelled RNA Ap2 (motif II; lanes 1 to 3) was incubated in the presence of GST::rPrP23-231 (rPrP^c) (lane 2) and GST::rPrP90-231 (rPrP27-30) (lane 3) from hamster. Assays were analyzed on 0.7% agarose gels. (D) Four picomoles (each) of labelled RNA Ap3 (motif III; lanes 1 to 4) was incubated in the presence of 40 pmol (each) of recombinant GST::rPrP23-231 (rPrP^c) (lane 2) and GST::rPrP90-231 (rPrP27-30) from Syrian golden hamster (lane 3) and GST (lane 4).

Aptamer/protein recognition in brain homogenates from uninfected and scrapie-infected mice. Brain homogenates from wild-type mice and Tg35 mice which overexpress PrP^c interacted with the RNA aptamer (Fig. 6D, lanes 3 and 4; PrP$^{0/0}$ negative control, lane 5). A considerably weaker interaction was observed with brain homogenates from scrapie-infected mice (Fig. 6D, lane 2). To exclude the possibility that the weaker signal in these homogenates is due to the absence of PrP^{Sc} in the supernatant because of possible precipitation of this isoform, we preincubated the brain homogenates in the presence or absence of proteinase K and performed a Western blot analysis of the supernatant fractions. Full-length PrP^c with a molecular mass of ~33 kDa present in the brain homogenates of uninfected animals was sensitive to proteinase K (Fig. 6E, lanes 1 to 3). In contrast, full-length PrP^{Sc} from infected animals can be processed to proteinase K-resistant material represented in three bands between 27 and 30 kDa which are characteristic of PrP27-30 (Fig. 6E, lanes 4 to 9). These data demonstrate that PrP^c and PrP^{Sc} are present in the supernatant under our aptamer binding assay conditions. As expected, PrP^c reveals proteinase K sensitivity, whereas full-length PrP^{Sc} present in scrapie-infected brain homogenates (2) is processed to PrP27-30. The proteinase K treatment of the brain homogenates resulted in a complete loss of the interaction with the aptamers (Fig. 6D, lanes 6 to 9), demonstrating that the aptamer does not bind to PrP27-30 due to the lack of 67 aa at the amino terminus.

Aptamer Ap1 generates a supershift in the presence of anti-PrP antibodies and brain homogenates from hamster. To verify the specificity of the PrP^c-aptamer complex, we included anti-PrP antibodies in the gel retardation assay with brain homogenates from hamster. PrP-specific antibody Ra37-15 directed against aa 206 to 225 from hamster PrP (Fig. 7, lane 7) resulted in a supershift of the Ap1-PrP complex (Fig. 7, lane 7). This result confirms the specific recognition of PrP by Ap1 in the brain homogenates and demonstrates that the carboxy terminus of PrP is not recognized by the aptamer. A second antibody, Ra18, also supershifted with Ap1, again confirming the specific PrP^c recognition by the aptamer (Fig. 7, lane 3). The fact that Ra18, which is directed against aa 37 to 53 of the prion protein, interacts with PrP^c and Ap1 together with the finding that a peptide encompassing aa 23 to 52 of the prion protein (Fig. 5, lanes 2) interacts with the aptamer suggests that it is the ultimate amino terminus (aa 23 to 36) of the PrP which is recognized by the aptamer. We applied preimmune serum as a negative control in the gel retardation assay, which neither interacted with the aptamer (Fig. 7, lane 5) nor resulted in a supershift in the presence of the brain homogenates (Fig. 7, lane 6). Antibodies Ra18 (Fig. 7, lane 4) and Ra37-15 (Fig. 7, lane 8) alone did not interact with the aptamer. Ap1 also failed to interact with antibodies directed against foreign proteins such as the laminin receptor (data not shown).

DISCUSSION

The following three questions were addressed in this study. (i) Can RNA aptamers which recognize the prion protein be isolated? (ii) How do these aptamers bind to prion proteins?

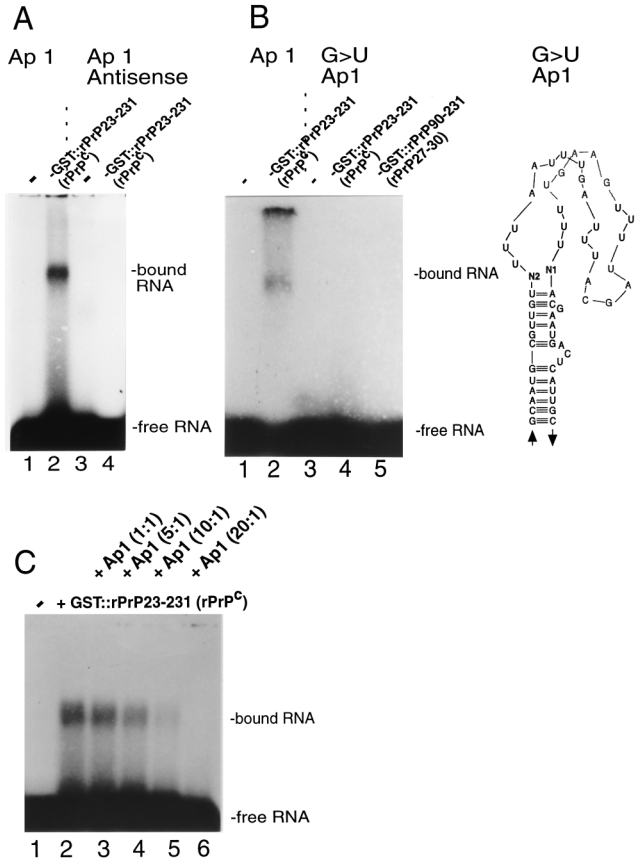

FIG. 4. Antisense Ap1 and G→U Ap1 do not interact with rPrP^c. Unlabelled Ap1 competes for the binding of labelled Ap1 to rPrP^c. (A) Four picomoles (each) of labelled Ap1 (motif I; lanes 1 and 2) and Ap1 antisense (lanes 3 and 4) was incubated in the presence of 40 pmol of recombinant GST::rPrP23-231 (rPrP^c) (lanes 2 and 4). (B) Four picomoles of labelled Ap1 (motif I; lanes 1 and 2) was incubated with GST::rPrP^c (lane 2), and 4 pmol of labelled G→U Ap1 (lanes 3 to 5) was incubated in the presence of GST::rPrP23-231 (rPrP^c) (lane 4) and GST::rPrP90-231 (lane 5). (C) Two picomoles of labelled Ap1 (lanes 1 to 6) was incubated with 40 pmol of GST::rPrP23-231 (lanes 2 to 6) and in the presence of 2 pmol (lane 3), 10 pmol (lane 4), 20 pmol (lane 5), and 40 pmol (lane 6) of unlabelled Ap1. Samples were analyzed on a 0.7% agarose gel.

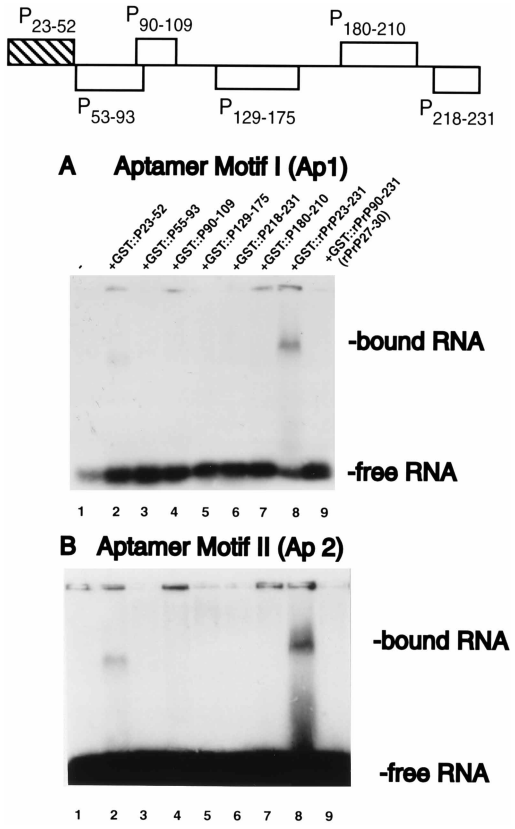

FIG. 5. Mapping of the RNA aptamer motif I (Ap1) and the motif II (Ap2)-PrP interaction site. Four picomoles of labelled RNA aptamer motif I (Ap1; lanes 1 to 9) (A) and 4 pmol of labelled RNA aptamer motif II (Ap2; lanes 1 to 9) (B) were incubated in the presence of 40 pmol (each) of GST::rPrP23-231 (rPrP^c) (lanes 8), GST::rPrP90-231 (rPrP27-30) (lanes 9) from hamster and 20 pmol each of GST::P_{23-52} (lanes 2), GST::P_{53-93} (lanes 3), GST::P_{90-109} (lanes 4), GST::$P_{129-175}$ (lanes 5), GST::$P_{218-231}$ (lanes 6), and GST::$P_{180-210}$ (lanes 7). Reaction assays were analyzed on 0.7% agarose gels. At the top is a schematic representation of the hamster PrP region. Hatched box, PrP region interacting with the aptamers; empty boxes, PrP region not interacting with the aptamers.

(iii) Do the aptamers exhibit enough affinity and specificity to unambiguously detect prion proteins in crude brain homogenates from infected and noninfected animals?

RNA aptamers specifically recognize recombinant prion protein rPrP^c. The first question was addressed by employing an in vitro selection approach to identify RNA aptamers which bind to PrP^c. Seventy percent of the selected RNA aptamers revealed primary sequence homologies, suggesting that their secondary structure contains a three-layered G tetrad motif. RNAs which contain putative G quartet motifs were grouped into three families with different stem-and-loop regions. All these aptamers interacted with recombinant PrP^c but not with rPrP27-30. The remaining 30% of the sequenced RNAs did not contain putative G quartet motif and did not bind to rPrP^c, rPrP27-30, or GST.

To further specify the interaction of the aptamers with the recombinant prion protein, we constructed an RNA molecule containing the G quartet scaffold of motif I without the stem and additional sequences. This aptamer also interacted with rPrP^c but not with rPrP27-30, demonstrating that these 29 nucleotides are sufficient for PrP recognition.

In addition, we competed the interaction of radiolabelled Ap1 to rPrP^c with increasing amounts of unlabelled Ap1 in a dose-dependent manner. In contrast, unselected RNA failed to compete for the Ap1-rPrP^c binding, confirming the specific binding of Ap1 to rPrP^c.

The G quartet scaffold is essential for PrP recognition. To determine whether the G tetrads are essential for PrP binding, we replaced the guanosine residues with uridine residues in Ap1. The G→U aptamer failed to bind to rPrP^c, demonstrating that the G residues are essential for interaction with PrP. Application of an antisense Ap1 in PrP binding assays also failed to interact with rPrP^c, providing more evidence that G quartets are essential for aptamer binding. In addition, competition experiments in the presence of unlabelled aptamers and unselected RNAs confirmed the specificity of the aptamer-PrP interaction.

Several in vitro-selected nucleic acids which contain G tetrad motifs have been described. A guanosine-rich 15-mer ssDNA which binds and inhibits the human blood-clotting factor thrombin has been identified (24). Nuclear magnetic resonance structural analysis revealed that the motif consists of a highly compact tertiary structure and includes two layers of G tetrads, two T-T loops, and one TGT loop (23, 33, 34). Lauhon and Szostak (22) isolated the first RNA structure which contains a proposed two-layered G quartet structure and binds

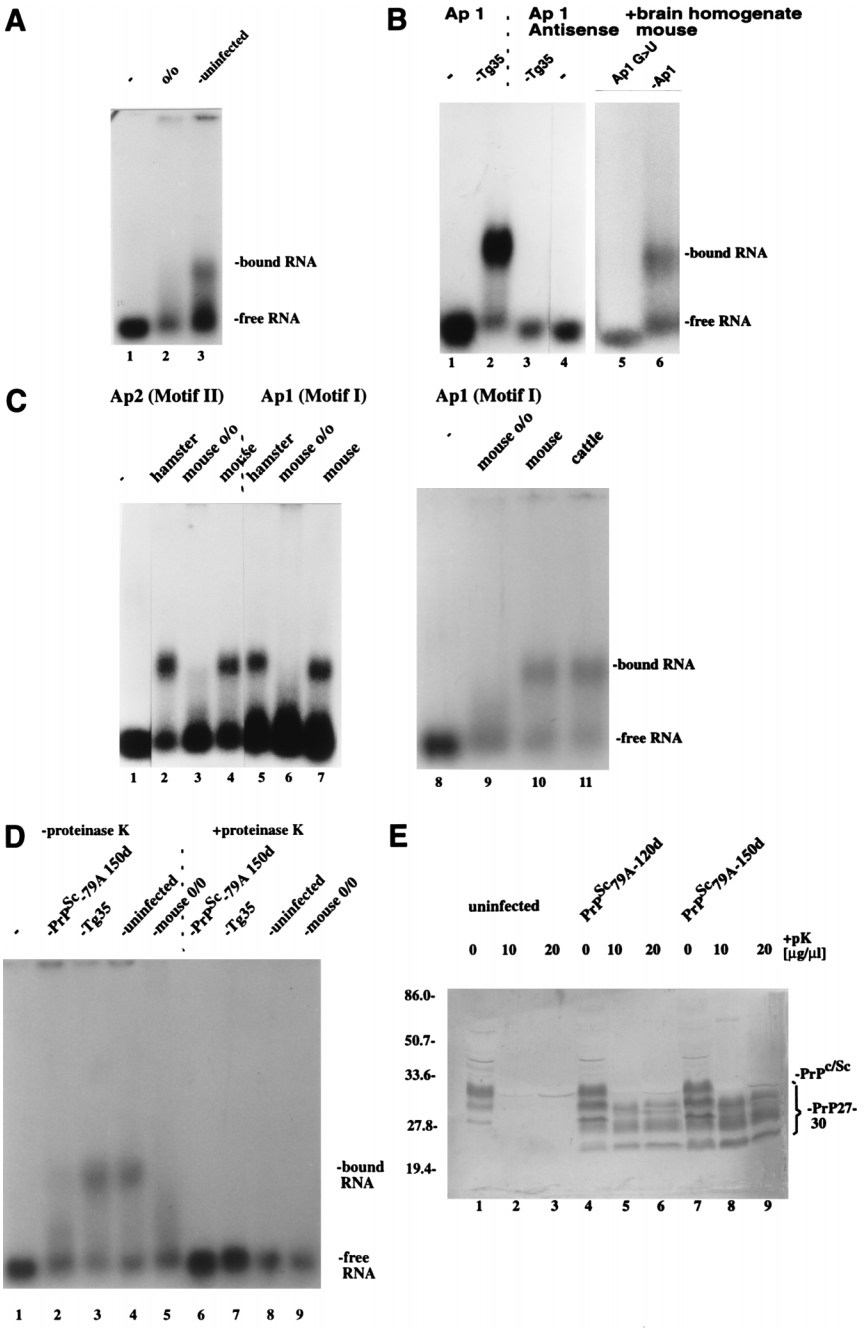

FIG. 6. Interaction of brain homogenates from different species with RNA aptamers directed against prion protein PrP^c. (A) One picomole (each) of radiolabelled RNA aptamer motif I (Ap1) (lanes 1 to 3) was incubated in the presence of 10 μl of brain homogenate (each) of *PrP*$^{0/0}$ mice (lane 2) and wild-type mice (lane 3) and analyzed on a 0.7% agarose gel. (B) Two picomoles (each) of radiolabelled RNA aptamer motif I (Ap1) (lanes 1 and 2) and 2 pmol of radiolabelled Ap1 antisense aptamer (lanes 3 and 4) were incubated in the presence each of 10 μl of brain homogenate of Tg35 mice (lanes 2 and 3); 2 pmol each of Ap1 G→U (lane 5) and Ap1 (lane 6) was incubated with each of 10 μl of brain homogenate from wild-type mice and analyzed on a 0.7% agarose gel. (C) One picomole (each) of radiolabelled RNA Ap2 (lanes 1 to 4) and RNA Ap1 (lanes 5 to 11) was incubated in the presence of 10 μl (each) of hamster (lanes 2 and 5), cow (lane 11), *PrP*$^{0/0}$ mouse (lanes 3, 6, and 9), and wild-type mouse (lanes 4, 7, and 10) brain homogenates and analyzed on a 0.7% agarose gel. (D) One picomole (each) of radiolabelled RNA Ap1 (lanes 1 to 9) was incubated in the presence of 10 μl (each) of brain homogenates pretreated (lanes 6 to 9) or not pretreated (lanes 2 to 5) with proteinase K originating from uninfected mice (lanes 4 and 8), scrapie-infected mice (PrP^{Sc}-79A-150d; lanes 2 and 6), PrP^c-overexpressing mice (Tg35; lanes 3 and 7), and PrP knockout mice (*PrP*$^{0/0}$; lanes 5 and 9) and analyzed on a 0.7% agarose gel. (E) Ten microliters (each) of brain homogenates (10% [wt/vol]) from uninfected mice (lanes 1 to 3) and scrapie-infected mice 120 (lanes 4 to 6) and 150 (lanes 7 to 9) days postinfection were incubated with 10 (lanes 2, 5, and 8) and 20 (lanes 3, 6, and 9) μg of proteinase K for 30 min at 37°C, analyzed on a 12.5% polyacrylamide gel, and blotted onto nitrocellulose. The blot was developed with the Ra5 antibody directed against a peptide corresponding to aa 95 to 110 of mouse PrP. Molecular size markers (in kilodaltons) are indicated on the left.

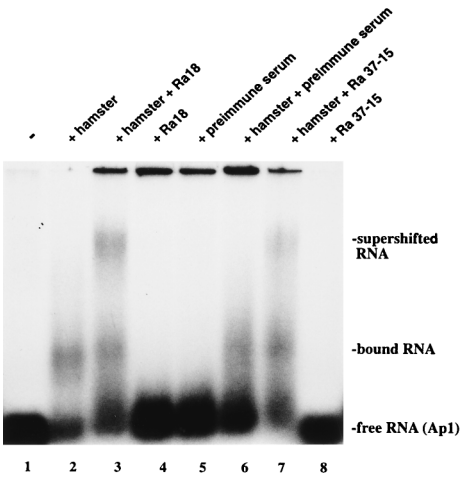

FIG. 7. Interaction of brain homogenates from hamster with RNA aptamer Ap1 and PrP-specific antibodies directed against prion protein PrP^c. Two picomoles (each) of radiolabelled RNA aptamer motif I (Ap1) (lanes 1 to 8) was incubated in the presence of 10 μl of brain homogenate (20 μg/μl) from hamster (lane 2), 10 μl of brain homogenate from hamster plus 12 μl of the Ra18 antibody (lane 3), 12 μl of the Ra18 antibody in the absence of brain homogenate (lane 4), 12 μl of preimmune serum (lane 5), 10 μl of brain homogenate from hamster plus 12 μl of preimmune serum (lane 6), 10 μl of brain homogenate from hamster plus 12 μl of the Ra37-15 antibody (lane 7), and 12 μl of Ra37-15 antibody in the absence of brain homogenate (lane 8). Samples were analyzed on a 0.7% agarose gel.

specifically to the cofactor riboflavin. The G quartets were proposed on the basis of covariations among different selected sequences. An ATP-binding ssDNA aptamer was also found to contain G-rich covariations. The model proposed for the ATP-binding DNA structure was based on a stable framework composed of two stacked G quartets (18). An ssDNA motif which is rich in G residues and bound to L-arginine was suggested to contain mixed guanine and adenine quartets (15). Finally, RNA aptamers containing 2′-NH_2-modified sugars were isolated for binding to human immunoglobulin E (39). Secondary-structure analysis suggested that the 2′-NH_2 RNA aptamers contain G quartets. The G tetrad motif thus seems to represent a recurring structural element in the selection of various functional RNAs. In all cases, including the one reported here, the aptamers were found to be highly specific for the ligand they were selected for, despite the fact that they all contain a guanosine quartet secondary-structure framework. G quartets also represent an important structural element in a number of natural nucleic acid sequences, including telomeric structures (40), and have been discussed for human immunodeficiency virus type 1 RNA dimerization (36).

RNA aptamers interact with the N terminus of rPrP. Aptamers Ap1 and Ap2 bind to the N terminus of PrP^c as shown in a binding study with selected peptides which span almost the entire region of the prion protein. The only peptide for which an interaction was observed was $P_{23\text{-}52}$, demonstrating that the amino terminus of PrP is specifically recognized by the aptamer. The failure of the aptamers to bind to rPrP27-30 or to native PrP27-30 can readily be explained by the lack of the 67 amino acids in the N terminus of this protein.

RNA aptamers interact specifically with native PrP^c from hamster, mouse, and cow. Do the RNA aptamers recognize native PrP^c? They do interact with a protein in brain homogenates from different species. The most conclusive evidence indicating that this interaction occurs with authentic prion protein was from the negative-control experiment with brain homogenates from *prn-p*$^{0/0}$ mice, in which this interaction was not observed. As the only difference between the two brain homogenates is that the latter lacks PrP^c, the interaction obtained with the homogenates from wild-type and PrP-overexpressing Tg35 mice can reasonably be attributed to a specific aptamer-PrP recognition.

To further confirm the specific recognition of PrP^c by the RNA aptamer, we performed the gel retardation assay in the presence of anti-PrP antibodies. The polyclonal antibodies Ra18 and Ra37-15 directed against aa 37 to 53 and 206 to 225 of the hamster prion protein, respectively, resulted in a supershift of the aptamer-PrP^c complex. No supershift was observed with preimmune serum or a non-PrP binding antibody. These data confirmed that the protein which is recognized by the aptamer in brain homogenates is indeed PrP^c. Moreover, the fact that the Ra18 antibody, which is directed against aa 37 to 53 of the hamster or mouse prion protein, interacts with the PrP^c-Ap1 complex together with the finding that the peptide encompassing aa 23 to 52 of the prion protein interacts with the aptamer suggests that the ultimate amino terminus (aa 23 to 36) of the prion protein interacts with the aptamer.

The specificity of the PrP-aptamer interaction was further confirmed by the finding that an antisense version of Ap1 did not result in a band shift when it was incubated with brain homogenates from Tg35 mice. In addition, G→U Ap1 failed to interact with these brain homogenates, confirming that the G quartet scaffold is required for PrP recognition.

Binding of the aptamers to PrP^c contained in brain homogenates from hamsters, mice, and cattle implies that the interaction is likely to occur within a region in which the prion proteins of these different species are highly homologous. The N terminus of PrP^c is indeed highly conserved among these species (31).

RNA aptamers do not interact with native PrP27-30. When brain homogenates from scrapie-infected mice were used in the gel retardation assay, a very weak band shift with radiolabelled aptamer was observed. This signal may have been due to some residual full-length PrP^c in the brains of infected animals. The fact that the shifted band disappeared after treatment with proteinase K is not related to the failure of the aptamers to detect the infectious isoform PrP^{Sc} but can be explained by the fact that treatment with proteinase K is known to remove the N terminus of PrP^{Sc}. However, this signal was abolished after pretreatment of the homogenate with proteinase K, demonstrating that the binding is not due to proteinase K-resistant PrP27-30. It is important that the failure of the aptamers to recognize PrP27-30 cannot be attributed to the possibility that it is insoluble and therefore not present in the homogenates, because Western blot analysis showed that proteinase K-resistant PrP27-30 was present in the supernatant in our assay. The aptamer-protein interaction signal with brain homogenates from uninfected and PrP^c-overexpressing mice (Tg35) was abolished after the homogenates were treated with proteinase K. Western blot analysis of the uninfected brain homogenate demonstrated that PrP^c was soluble and sensitive towards proteinase K under our assay conditions.

Our data show that in vitro selection can be applied to isolate highly PrP^c-specific RNA aptamers. On the basis of the present study, it can reasonably be anticipated that the screening of large combinatorial libraries of nucleic acids against insoluble amyloid rods, fibrillous plaques of PrP^{Sc}, or purified PrP^{Sc} from ScN_2a cells might lead to nucleic acid aptamers which specifically recognize PrP^{Sc}. Such nucleic acid ligands may be directly applicable to diagnosis of transmissible spongiform encephalopathies.

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