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Two primate-specific small non-protein-coding RNAs in transgenic mice: neuronal expression, subcellular localization and binding partners

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ABSTRACT

In a rare occasion a single chromosomal locus was targeted twice by independent Alu-related retroposon insertions, and in both cases supported neuronal expression of the respective inserted genes encoding small non-protein coding RNAs (npcRNAs): BC200 RNA in anthropoid primates and G22 RNA in the *Lorisoidea* branch of prosimians. To avoid primate experimentation, we generated transgenic mice to study neuronal expression and protein binding partners for BC200 and G22 npcRNAs. The BC200 gene, with sufficient upstream flanking sequences, is expressed in transgenic mouse brain areas comparable to those in human brain, and G22 gene, with upstream flanks, has a similar expression pattern. However, when all upstream regions of the G22 gene were removed, expression was completely abolished, despite the presence of intact internal RNA polymerase III promoter elements. Transgenic BC200 RNA is transported into neuronal dendrites as it is in human brain. G22 RNA, almost twice as large as BC200 RNA, has a similar subcellular localization. Both transgenically expressed npcRNAs formed RNP complexes with poly(A) binding protein and the heterodimer SRP9/14, as does BC200 RNA in human. These observations strongly support the possibility that the independently exapted npcRNAs have similar functions, perhaps in translational regulation of dendritic protein biosynthesis in neurons of the respective primates.

INTRODUCTION

Even after discovering that RNA itself has catalytic activity (1,2) the doctrine remained: the few surviving functional RNAs, including ribosomal RNAs, tRNAs and others are fossilized remnants of the long gone RNA/RNP worlds. Recent developments forced us to rethink. Experimental and computational RNomics uncovered a plethora of non-protein coding RNAs (npcRNAs) [reviewed in (3,4)]. The discovery of non-protein coding RNAs that arose relatively recently in diverse metazoan lineages, such as BC1 RNA in rodents (5), sphinx RNA in *Drosophila melanogaster* (6), the bsr transcript (7) that harbors RBII-36 snoRNA in the *Rattus* genus (8), BC200 RNA in anthropoid primates (9), and G22 RNA in the *Lorisoidea* branch of prosimians (10) indicates that functional non-protein coding RNAs are not merely fossils from the RNA and RNP worlds. Retroposition is much more pervasive in the evolution of genes (protein coding and other) and genomes. Continued recruitment (or exaptation) of novel RNA molecules including snoRNAs and miRNAs into cellular processes underscores the significance of studying biogenesis of npcRNAs and their cellular functions, such studies will aid in establishing molecular causes of physiological differences of species (11–14). The timing of biogenesis of BC1 and BC200 npcRNAs is well documented. BC1 RNA arose by retroposition of a $tRNA^{Ala}$ after the mammalian radiation in rodents. BC1 RNA is prevalently expressed in neurons and transported to dendritic processes (15). Targeted deletion of its gene reduces exploratory behavior and increases anxiety of mice (16,17). BC200 RNA (200 nt in length) arose independently by retroposition of a monomeric Alu element into a locus that permitted expression in primate neurons. It has been shown to be dendritic in human brain (9). Interestingly, both RNAs despite their different evolutionary origins might have analogous functions (18). BC1 and BC200

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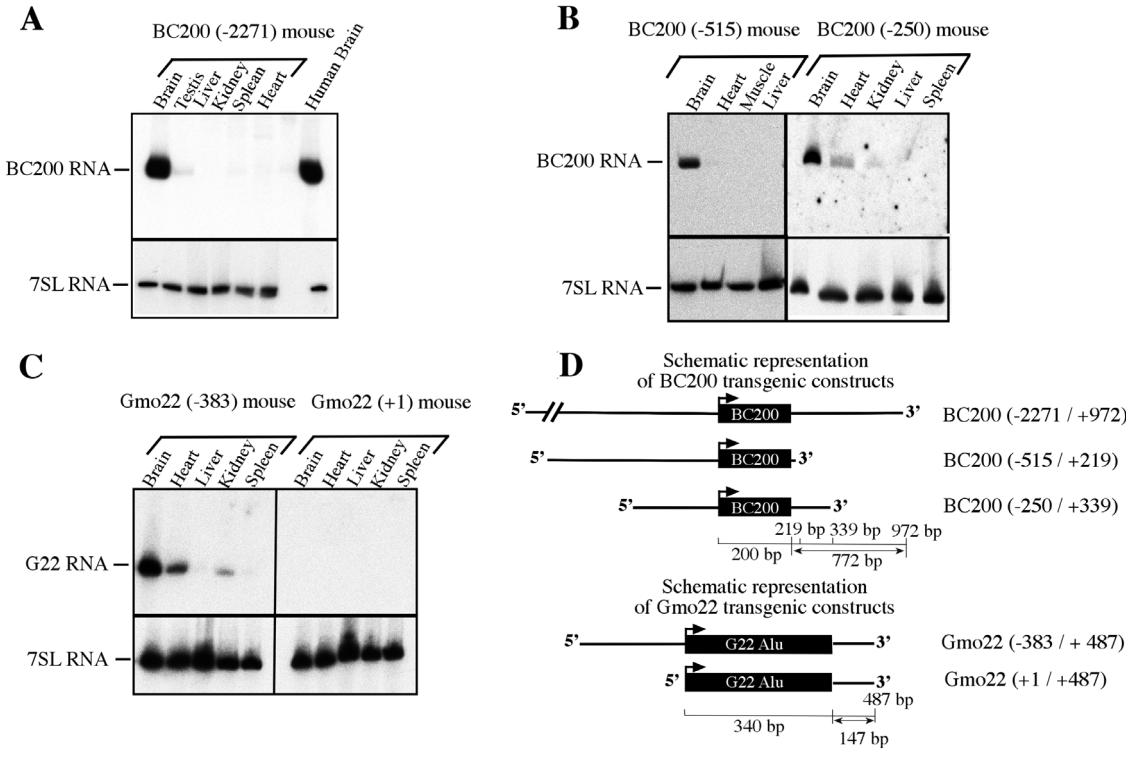

Figure 1. Northern blot analysis of RNA from BC200 and Gmo22 transgenic mice. Northern blot hybridization of total RNA isolated from various tissues of BC200 (−2271)—(**A**); BC200 (−515), BC200 (−250)—(**B**); Gmo22 (−383) and Gmo22 (+1)—(**C**) transgenic mice as indicated in the figures was performed. Hybridization was carried with ^{32}P-labeled oligonucleotide probes: BC207–BC200 RNA-specific (A and B) and G22-3′-specific probe for G22 RNA (C). As a loading control, all blots were hybridized with ^{32}P-labeled 7SL_3′ oligonucleotide—specific probe for 7SL RNA. Respective RNAs are indicated. (**D**) Schematic representation of various constructs having deletions at 5′ and 3′ flanking regions for BC200 and Gmo22 transgenic mice.

RNAs inhibit translation *in vitro* as well as in transfected cells. At lower concentrations, however, Alu RNAs appear to stimulate translation (19). Consequently, the BC RNAs have been suggested to regulate translation in dendritic microdomains (19–22). Such a role is supported by identification of protein partners that play roles in translation including SRP9/14 for BC200 RNA (23,24) and PABP for both BC1 and BC200 RNAs as well as for transcribed SINEs with poly(A) tails (18,20,25). When the BC RNAs are preincubated with PABP prior to the *in vitro* translation assay, the inhibitory effect is less pronounced (20). Hence other possible functions for BC1 and BC200 RNAs have to be entertained (20).

Previously, we demonstrated that the ancestrally unoccupied G22/BC200 RNA locus was independently targeted by two different but related SINE elements, once in strepsirrhines and once in anthropoid primates (10). Thus, G22 RNA, derived from a dimeric Alu element, inserted into the same locus as BC200 RNA at a different time in a different primate lineage (10). Moreover, it has been shown for Lorisiformes and Anthropoidea that both insertions are transcriptionally active and mainly brain-specific. Based on HeLa cell transfection experiments, it was proposed that the BC200/G22 locus harbors promoter elements required for maintaining the tissue-specific transcription of both genes (10). As studying functional aspects of these molecules in primates is hampered by a number of ethical or conservational restrictions, we generated transgenic mice that simulate expression of BC200 and G22 npcRNAs in the original primate species. Here we report studies on their transcriptional regulation, cellular and subcellular location as well as their interaction with protein partners.

MATERIALS AND METHODS

BC200 and Gmo22 transgenic mice

The 3.2 kb EcoRI–HindIII DNA fragment for BC200 (−2271) transgenic mice was released from the λ bacteriophage BC200-9a, with the respective restriction enzymes (26) (Figure 1D). DNA fragments for the production of BC200 (−515) and BC200 (−250) transgenic mice were PCR amplified from λ bacteriophage BC200-9a (26) (Figure 1D). The following pairs of oligonucleotides were used for PCR amplification: BC200 (−515): BC2TR2dir 5′-TTGAGACC-TTGAGGGGCTTTT-3′ and BC2TR4rev 5′-CTTCCAGGC-AAGAATATGAAAAG-3′; BC200 (−250): BC2TR4dir 5′-AT-TTAAATTCAGAGCCAAAAG-3′ and BC2TR2rev 5′-TACTG ATTAAAAAAGTAAAAAG-3′. PCR fragments were cloned into the pBluescript vector and sequenced. DNA fragments for Gmo (−383) and Gmo (+1) transgenic mice were obtained from the plasmids pGmo—(−383) and pGmo—(+1), respectively (10) (Figure 1D). All DNA fragments were released by respective restriction endonucleases and purified through agarose gel electrophoresis.

For transgenic mice production, DNAs were diluted up to the concentration of 2 μg/ml and injected into the pronuclei

of B6D2F1 mice (Harlan Winkelman GmbH, Germany) according to protocol (27), which were subsequently transferred into the oviduct of 0.5-day pseudopregnant, CD-1 foster mice.

DNA tail biopsy and Southern blot analysis

DNA from 3-week-old mouse-tails was extracted as described (28). Approximately 5 μg of genomic DNA was digested with PstI or EcoRI, fractionated on 0.8% agarose gels, and transferred to GeneScreen nylon membranes (NEN DuPont) by positive pressure blotting. Membranes were hybridized with a ^{32}P-labeled probe for respective genes, and washed twice with 0.5× SSPE, 0.5% SDS at 65°C and exposed to MS film (Kodak) at −80°C overnight.

RNA blot analysis

Total RNA was extracted from different mouse tissues using TRIzol reagent (Gibco BRL) according to the manufacturer's instructions. RNA was separated on 6% polyacrylamide/7M urea gels and electro-transferred to positively charged nylon membranes (Qiagen or Ambion). After ultraviolet (UV) cross-linking, membranes were hybridized at 58°C overnight in 1 M sodium phosphate pH 6.2, 7% SDS with ^{32}P-labeled oligonucleotide probes: for BC200 RNA—BC207: 5′-cTTG-TTGCTTTGAGGGAAGTTACGCTTATTTggtac-3′; for G22 RNA—G22-3′: 5′-TGTGCCCTGAGCAGAGGGCAG-TGGCACCGGAGCCCAC-3′; and as a control, probe for 7SL RNA was used, 7SL_3′: 5′-AAGAGACGGGGTCTC-GCTATG-3′. Nucleotides in lower case are unrelated to the BC200 sequence. After hybridization, membranes were washed twice with 1× SSC, 0.1% SDS for 20 min at 58°C and exposed to MS film (Kodak) at −80°C overnight.

DIG-labeled *in situ* hybridization

Digoxigenin (DIG)-labeled riboprobes for hybridization with BC200 RNA were generated from plasmid pKK536-6, which contained 71 nt (position 119–189) from the 3′-unique domain of the human BC200 gene cloned into pBluescript KS (+) between the KpnI and SacI sites of the MCS. Following linearization with either KpnI [antisense (AS)] or SacI [sense (S)], DIG-labeled RNA probes were *in vitro* transcribed using T7 or T3 polymerases, respectively, and the DIG RNA Labeling Kit (Roche Molecular Biochemicals) according to the manufacturer's instructions.

DNA templates featuring a T7 promoter followed by the S and AS sequence for part of G22 RNA (position 205–278) were generated by PCR amplification from pGmo- (−383) plasmid using pairs of oligonucleotides: G22antiRev 5′-GG-AGTCTGAGGTTGCAGTGGGC-3′ and G22T7antiF 5′-TA-ATACGACTCACTATAGGTCGAGACAGAGTTCCACC-CTG-3′, for AS probe G22sensRev 5′-GTCGAGACAGAG-TTCCACCCTG-3′ and G22T7sensF 5′-TAATACGACT-CACTATAGGGAGTCTGAGGTTGCAGTGGGC-3′ for the S probe. G22 RNA probes were *in vitro* transcribed from the respective PCR products in the presence of DIG-modified UTP according to the manufacturer's instructions (Roche Molecular Biochemicals).

BC200 and Gmo22 RNA transgenic mice, 6–10 weeks old, and their wild-type littermates were transcardially perfused with 0.1 M phosphate-buffered saline (PBS), pH 7.2 followed by freshly prepared 4% paraformaldehyde in PBS (PFA). The brains were removed and postfixed overnight at 4°C. The next morning, 30 μm coronal sections were cut on a vibrating microtome (Leica) and, for ease of handling, further fixed in PFA for one week at 4°C. Prior to hybridization, free-floating sections were washed 3 × 5 min with PBS, incubated in Proteinase K (1 μg/ml) for 30 min at 37°C, incubated for 2 × 5 min in freshly prepared glycine (0.1 M) and finally rinsed 2 × 15 min in 2× SSC. Prehybridization [50% Formamide, 1.2 M NaCl, 0.02 M Tris (pH 7.5), 2× Denhardts, 2 mM EDTA, 1 mg/ml denatured salmon sperm DNA, 1 mg/ml total yeast RNA and 0.1 mg/ml yeast tRNA] was at 50°C for 1 h in a humid chamber. Hybridization using 200 ng/ml DIG-labeled RNA probes in prehybridization buffer plus 10% dextran sulfate was overnight at 50°C in a humid chamber. The sections were washed 2 × 5 min with 2× SSC, digested with RNase A (2 μg/ml) for 30 min at 37°C, washed 3 × 15 min with 2× SSC at 50°C and finally washed under high stringency 3 × 30 min with 0.1× SSC, 0.1% sodium pyrophosphate. To detect the DIG label, the sections were washed 3 × 5 min with PBS, blocked (1% Blocking Reagent, Roche Molecular Biochemicals) for 60 min at RT and incubated overnight at 4°C with sheep anti-DIG-AP (1:500 in blocking buffer, Roche Molecular Biochemicals). After washing 4 × 5 min with PBS and equilibrating 2 × 5 min in 0.1 M NaCl, 0.1 M Tris (pH 9.5), 0.05 M $MgCl_2$, the antibody was detected enzymatically using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as a substrate. The sections were finally washed, mounted, and coverslipped with VectaMount (Vector Laboratories, Inc., Burlingame, USA). Hybridization signals were photographed using a Nikon E600 microscope and Open Lab Software (Improvision, Coventry UK).

In situ hybridization of mouse embryos

In situ hybridization on embryonic sections of BC200 transgenic mice was performed as described previously (9). On day E15 or E18 embryos were removed from the uteri of pregnant mice, washed twice with PBS pH 7.2, fixed in 4% paraformaldehyde in PBS overnight at 4°C, and equilibrated in ascending concentrations of sucrose (up to a 20%). Embryos were embedded with Tissue Tek OCT medium (SAKURA) and sagittal cryo-sections of 15 μm thickness were generated. Probes (AS or S) specific for BC200 RNA were *in vitro* transcribed in the presence of [α-^{35}S]UTP, from the template pKK536-6 (see above), and linearized with KpnI or SacI, respectively. Following hybridization (see above) using 5 × 10^6 c.p.m./ml of ^{35}S-labeled RNA probe, slides were washed for 30 min at room temperature in 2× SSC and then digested with 30 μg/ml RNase A at 37°C for 30 min. Subsequently, slides were washed for 60 min with 2× SSC at 50°C and finally under high stringency for 3 h at 50°C in 0.1× SSC, 0.05% Na-pyrophosphate and 14 mM 2-mercaptoethanol with gentle stirring. Wash solution was cooled down to room temperature, and slides were dehydrated through 50, 70 and 90% ethanol containing 0.3 M NH_4OAc for 2 min each. Vacuum dried sections were exposed to X-Omat film (Kodak).

Immunoprecipitation

Immunoprecipitation was performed as described previously (18). Anti-PABP antibody (18) and anti SRP9 antibody (24) were immobilized on protein G-sepharose beads (Pharmacia) and washed thrice with IP buffer [12.5 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM $MgCl_2$, 0.25% NP-40 and 5% glycerol]. The beads were incubated with the antibody overnight at 4°C. The unbound antibody was washed (4 times) with the IP buffer. The bound beads were subsequently incubated for 2–3 hours with crude brain extracts (S2) from BC200 (−2271) and Gmo22 (−383) transgenic mice, at 4°C. Following incubation, the beads were washed with IP buffer (4–5 times) to remove the unbound sample. RNA was extracted from the bound complexes using TRIzol reagent (Gibco BRL). The RNA was analyzed by northern blot analysis (see above).

T7 *in vitro* transcription of BC200 RNA and G22 RNA

BC200 and G22 npcRNAs were *in vitro* transcribed from DNA templates containing a T7 promoter. DNA template for *in vitro* transcription of BC200 RNA was obtained from pPBC200 plasmid (23) digested with DraI restriction endonuclease. G22 RNA was transcribed directly from PCR product amplified from pGmo- (−383) plasmid using the following oligonucleotides: Gmo22T7_F 5′-TAATACGACTCACTATAGGCCGGGCGCGGTGGCTCA-3′ and GmoTn-Rev 5′- AGGGAAAAATATGAAAAGGGGATTTGGG-3′. Transcription with T7 RNA polymerase was performed in a reaction volume of 200 µl (29). RNAs were separated on denaturing 8% polyacrylamide, 7 M urea gels and passively eluted in 0.3 M NaOAc buffer (pH 5.2), 1 mM EDTA, 0.2% phenol at 4°C overnight. Subsequently, RNAs were EtOH precipitated and dissolved in 30 µl H_2O.

Gel shift assay

For binding assays to human PABP, BC200 RNA and G22 RNA were *in vitro* transcribed from plasmid and PCR templates (see above) containing a T7 promoter in the presence of [α-^{32}P]UTP using 250 U of T7 RNA polymerase followed by gel purification (see above). Before incubation with recombinant PABP protein, RNAs were heat denatured (2 min at 85°C) and subsequently cooled in ice. Complex formation of recombinant PABP with BC200 RNA and G22 RNA was performed in a reaction volume of 20 µl, containing 10 mM Tris–HCl (pH 7.4), 100 mM KCl, 2.5 mM $MgCl_2$, 2 mM DTT, 5% glycerol, 0.5% Triton X-100, 2 µg heparin, 1 µg BSA, 2.5 µg tRNA and 5 U of ribonuclease inhibitor (RNasin, MBI). Aliquots (0.05 pmol) of RNAs were incubated with increasing concentrations of PABP as indicated in Figure legends (Figure 7). Competition assays were performed under above binding conditions with constant concentrations of PABP around 25 nM and with increasing concentrations of competitor RNA (Figure 7). All reactions were incubated for 30 min at 4°C, followed by 10 min at room temperature. RNA and RNA–protein complexes were separated on native 6% (w/v) polyacrylamide gels, containing 1× TBE [90 mM Tris, 64.6 mM boric acid, 2.5 mM EDTA (pH 8.3)]. Electrophoresis was performed at 20°C in 0.5× TBE running buffer for 10–12 h. Gels were fixed in 10% acetic acid, 25% 2-propanol for 30 min at room temperature, dried by vacuum blotting and exposed to MS film (Kodak) at −80°C overnight.

The apparent K50 values of the RNA–protein complexes were defined as the protein concentration at which 50% of the input RNA was shifted to an RNP complex (30).

Native gel electrophoresis

The native complexes from the S2 brain extracts were separated on a composite gel containing 4% acrylamide/bisacrylamide (80:1, w/w) and 0.4% (w/v) agarose as described (31). Following electrophoresis, the RNPs and RNAs were electroblotted on Nylon membrane (Ambion). The RNA was immobilized by UV cross-linking, the membrane was then hybridized with ^{32}P-labeled BC207 oligonucleotide probe (see above) complimentary to the unique region of BC200 RNA. Hybridization and washings were performed as described (31).

Mouse housing conditions

All lines were established by breeding male and female founders with C57/Bl6J mice. Non-transgenic siblings were used as control animals. Mice were bred at the central animal facility of the University Clinic, Münster in a temperature (21°C) controlled room with 12:12 h light/dark cycle and housed under non-enriched standard conditions. Pups were weaned 19–23 days after birth and females were kept separately from males. Mice were housed in standard [27 (l) × 21 (w) × 15 (h) (cm)] or [42 (l) × 27 (w) × 15 (h) (cm)] cages, for up to three or seven littermates, respectively.

RESULTS

Flanking promoter elements in the BC200 RNA locus are responsible for tissue-specific transcription

In order to investigate the transcriptional activity of the BC200/G22 locus in transgenic mice, we designed various constructs in which the 5′ flanking regions were sequentially deleted: BC200 (−2271), BC200 (−515), Gmo22 (−383), BC200 (−250), Gmo22 (+1) (Figure 1D). In two of the BC200 constructs, the 3′ flanks were also shortened. For example in BC200 (−250), 139 nt instead of 772 nt 3′ flanks [as in BC200 (−2271)] were retained. Finally, in BC200 (−515), the 3′ flank was completely removed except for 19 nt that follow the 200 nt corresponding to the BC200 RNA coding region (see Figure 1D). This should address the possibility whether elements that influence transcription are also located downstream of BC200 RNA gene.

When total RNA from different tissues of three independent BC200 (−2271) and BC200 (−515) transgenic mouse lines was analyzed we observed that the brain-specific expression pattern of BC200 RNA (with low expression level in testis) (Figure 1B and C) is largely reproduced and comparable to native BC200/G22 activity in Anthropoidea and Lorisiformes (9,10,32,33). However, one of the transgenic mouse lines, BC200 (−515), showed a low transcriptional activity of BC200 gene in the heart (Figure 1B). The differences in transcription patterns of the BC200 RNA gene within BC200 (−515) transgenic mouse lines most likely reflect the effect of the position of integration in the

mouse genome with the concomitant absence of insulation caused by the shorter 5′ flanks, which would fortuitously provide additional enhancer(s) to the 5′-flanking region, rather than being an effect of regulator element(s) deletion located in the 3′-flanking region of the BC200 locus.

It has been shown that upstream sequence elements in the BC200/G22 locus of Lorisiformes and Anthropoidea contain conserved external promoter elements similar to RNA polymerase II and other pol III genes (10). For example, a conserved TATA-like motif maps to around position −20 and −30; a second conserved sequence block, positioned ∼70 bp from the insertion point (coinciding with the +1 position of the transcript) is similar to the proximal sequence element (PSE-motif). Finally, a distal sequence element (DSE-like motif) is located ∼220 bp upstream from the +1 transcriptional start of both npcRNAs (10). Additional elements are expected to be responsible for tissue-specific expression. We decided to test whether shortened flanks are still sufficient to maintain tissue-specific transcription of BC200 and G22 transgenes in mice. Two similar constructs were generated, BC200 (−250) and Gmo22 (−383). The construct BC200 (−250) has 250 bp of the upstream sequence from human and Gmo22 (−383) has 383 bp from the *Galago moholi* locus, presumably involved in regulation of transcription of BC200 RNA and G22 RNA, respectively (Figure 1D) (10). From these constructs we generated three independent lines for BC200 (−250) and Gmo22 (−383) transgenic mice. Northern blot analysis using total RNA extracted from different tissues showed that both RNAs were predominantly expressed in the brain (Figure 1B and C). Once more, we observed low levels of transcription of the BC200 and G22 transgenes in heart and additionally in kidney. Thus, we confirmed that the predicted regulatory elements located in the 5′-flanking region in the BC200 locus are sufficient to maintain transcriptional activity of the retroposed elements with high-expression in the brain, although repression in other tissues was somewhat diminished.

Next, we generated three independent transgenic mouse lines with construct Gmo22 (+1), in which the complete 5′ flanking G22 RNA sequence was removed (Figure 1D). In order to analyze these transgenic mice, total RNA from different tissues was extracted and northern blot hybridization was performed with a G22 RNA-specific probe. We failed to detect even a weak signal on our blots indicating that the G22 dimeric Alu element is transcriptionally inactive in the transgenic mice. Based on these results, we confirmed our prediction that regulatory elements upstream of BC200 and G22 RNA sequences in the BC200/G22 locus are important for their transcription. Importantly, our data support the notion that in intact organisms external promoter elements are required for transcriptional activity of SINE retronuons (10).

BC200 RNA and G22 RNA are localized to dendrites

Anthropoidea BC200 RNA is selectively expressed in the nervous system (9,33,34). The distribution of human BC200 RNA shows a clear dendritic localization in the brain (9,34). Using BC200 (−2271) transgenic mice, we examined whether the BC200 RNA specifically expressed in the mouse brain had a similar subcellular localization pattern as native BC200 RNA in human brain (9). *In situ* hybridization on coronal sections through the hippocampus of BC200 (−2271) transgenic mice using an AS probe designed against the unique region of BC200 RNA clearly showed that BC200 RNA is expressed in the CA1, CA2 and CA3 pyramidal cells of the hippocampus (Figure 2A), where RNA is found in the somatic cytoplasm and in both apical and basal dendrites (Figure 2B). Dendritic localization is fairly uniform, except in *stratum lucidum* of area CA3, the terminal fields of the dentate mossy fibers (Figure 2A), as is the case for phylogenetically unrelated rodent BC1 RNA. There is little or no expression in the granular cells of the dentate gyrus (Figure 2A). In the cortex, BC200 RNA is expressed in all layers (I–VI), but most prominently in the large pyramidal cells of layer V (Figure 2C). As in the hippocampus, labeling in the cortex was evident in both somatic cytoplasm and dendrites. In amygdala, where the basal lateral area is more intensely labeled than the central area (Figure 2D), higher magnification reveals clear dendritic staining (Figure 2E).

As G22 RNA also is predominantly and specifically expressed in the brain (Figure 1C), we examined whether G22 RNA exhibits a dendritic localization pattern in the corresponding transgenic mice similar to BC200 RNA. Indeed, G22 RNA was somatodendritically localized (see Figure 3A and C). While in the hippocampus of BC200 (−2271) transgenic mice there is intense dendritic staining in stratum radiatum of the CA1 and CA2 regions, in Gmo22 (−383) transgenes, not all cells are stained in the CA1 and CA2 region, however, the dendrites in the stratum radiatum of CA1 and CA2 region show substantial labeling, albeit, weaker in comparison to BC200 RNA. This might simply be due to differences in expression levels of G22 RNA and BC200 RNA in the investigated transgenic mice. Unlike BC200 transgenes, where there are variable levels of BC200 RNA in the cells of the different cortical layers, in Gmo22 transgenes, the RNA shows a uniform distribution in all layers. Presumably, due to the weaker expression levels of G22 RNA there is no clear dendritic signal observed in the cortical neurons. Apart from exhibiting common dendritic localization patterns, BC200 RNA and G22 RNA have different expression patterns in other areas of the brain. For example, while BC200 RNA is expressed at very low levels, if at all, in the dentate gyrus, G22 RNA is expressed at high levels in dentate gyrus, evident by the intense staining in the somata as well as the corresponding dendritic field of dentate gyrus (Figure 3A and C, higher magnification not shown). Control S probe in each case did not show any specific labeling (Figure 3A–D). Furthermore, sections of wild-type mouse brains were used as an additional control in hybridizations with both AS and S probes for both BC200 and G22 RNA. As expected, there is no specific labeling with either S or AS probe (Figure 3D). In order to compare the signal in dendrites, the non-dendritic tubulin mRNA was included as a negative control (Figure 3E). We observed an intense signal in the cell bodies but clearly no signal in dendritic fields.

Prenatal expression of BC200 RNA in transgenic mice

As there are indications that BC1 RNA is expressed prenatally in the developing nervous system (35), and switched

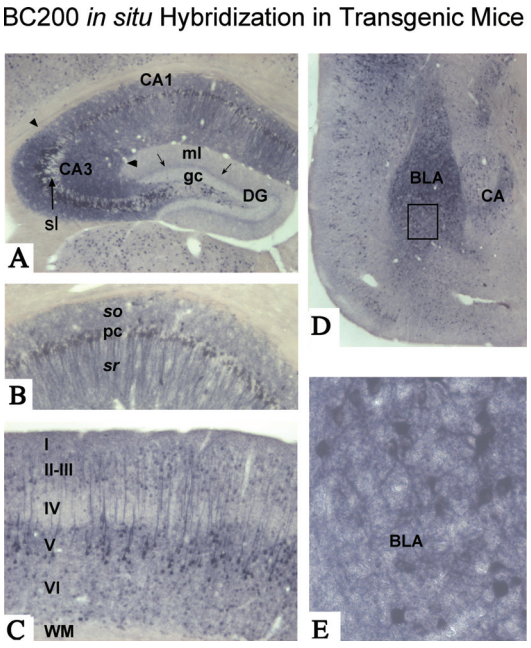

Figure 2. *In situ* hybridization of transgenic BC200 RNA. (**A**) In the hippocampus there is robust labeling in the cell bodies and dendrites of the hippocampus proper. (**B**) An enlargement of hippocampal area CA1 shows clear labeling in *stratum radiatum* containing the apical dendrites of the pyramidal cells. (**C**) In the cortex overlying the hippocampus, corresponding to primary somatosensory cortex, BC200 RNA is present in most cells in all the layers; however, dendritic localization is particularly evident in the large pyramidal cells of layer V. (**D**) Amygdala, BC200 RNA is unevenly distributed in the various nuclei of the amygdala. The basal lateral amygdala is more strongly labeled than is the central amygdala. (**E**) Dendrites in the basal lateral amygdala are clearly stained. Abbr. BLA, basal lateral amygdala; CA, central amygdala; DG, dentate gyrus; gc, granule cells; ml, molecular layer; pc, pyramidal cells; *sl, stratum lucidum*; *so, stratum oriens; sr, stratum radiatum*; WM, white matter; I–VI, cortical layers.

on when nerve cells start to communicate with each other (36), we examined expression of BC200 RNA in embryos of transgenic mice. On embryonic day 15, labeling was weak and possibly, like BC1 RNA (37), also detectable in non-neuronal cells. A later embryonic stage (E18) showed prominent expression in the brain, spinal cord and in a different plane in the retina (Figure 4).

The transgenic BC200 RNP has similar mobility as native BC200 RNP

In order to determine whether BC200 RNA in transgenic mice forms an RNP similar to the native BC200 RNP, we performed native gel electrophoresis with crude cytoplasmic extracts (S2) prepared from human brain and transgenic mouse brain as described (18); *in vitro* transcribed RNA was also loaded as a control. The RNPs and the RNA were transferred onto nylon membrane and hybridized with ^{32}P-labeled oligonucleotide BC207, complementary to the unique region of BC200 RNA. RNP from BC200 transgenic mice, BC200 (−2271), and the native BC200 RNP from human brain migrate with similar mobilities, indicating that they have comparable sizes and in all likelihood similar protein components (Figure 5). In either case, free RNA could not be detected in the crude extracts (Figure 5). Next, we checked whether the protein partners of BC200 RNP in the transgenic mice are identical to those of the native BC200 RNP in order to validate the mouse model for further functional studies.

BC200 RNA and G22 RNA bind common protein partners, SRP9/14 and PABP

Recently, it was shown that PABP and SRP9/14 proteins were part of the BC200 RNP complex in the human brain (18,24). Moreover, it has been reported that Alu RNAs have a potential to interact with the heterodimer SRP9/14 protein (23,38). In order to investigate whether the BC200 RNA and G22 RNA had corresponding protein partners in the respective transgenic mice, immunoprecipitations were carried out against PABP and heterodimer SRP9/14 proteins using anti-PABP (18) and anti-SRP9 (24) antibodies, respectively. Crude S2 brain extracts were prepared from BC200 (−2271) and Gmo22 (−383) transgenic mice as reported previously (18). Both npcRNAs were efficiently immunoprecipitated with the antibodies mentioned above but not with pre-immune sera (Figure 6A and B). This confirms that the BC200 RNP complex formed in BC200 (−2271) transgenic mice brain is similar to that of native BC200 RNP in the human brain containing at least PABP and the protein heterodimer SRP9/14, and that the mouse model can be used for further studies. Moreover, the G22 RNP formed in Gmo22 (−383) transgenic mice has at least three common protein partners with human BC200 RNA, indicating that the two RNPs are similar and therefore could be involved in related functions in the brain.

In vitro binding studies of BC200 RNA and G22 RNA with PABP

To further study similarities or differences of the primate BC200 RNA and G22 RNA with respect to their interaction with PABP, we investigated in detail the binding properties of both npcRNAs.

First, we tested the ability of PABP to interact selectively and specifically with BC200 RNA. We examined whether selective binding between recombinant PABP and *in vitro* transcribed BC200 RNA could be detected by gel retardation assays in the presence of vast excess of non-specific yeast tRNA (molar ratio 1:2000). Consistent with previous reports (18), we were able to detect two distinct complexes in our assay that might correlate with two potential PABP binding sites in the 41 nt long A-rich region of BC200 RNA (Figure 7B). The BC200 RNA–PABP complex was formed with an apparent K50 value of 16 nM. We also performed competition assays with unlabeled BC200 RNA as a competitor (see Materials and Methods) (Figure 7D). The intensity of the upper shifted band was completely abolished with a 16-fold excess of unlabeled BC200 RNA, and with 80-fold excess, both bands were mostly competed (Figure 7D).

We next tested whether *in vitro* transcribed G22 RNA would interact with human recombinant PABP in the presence of a vast excess of non-specific yeast tRNA (see above). When increasing amounts of PABP were added to

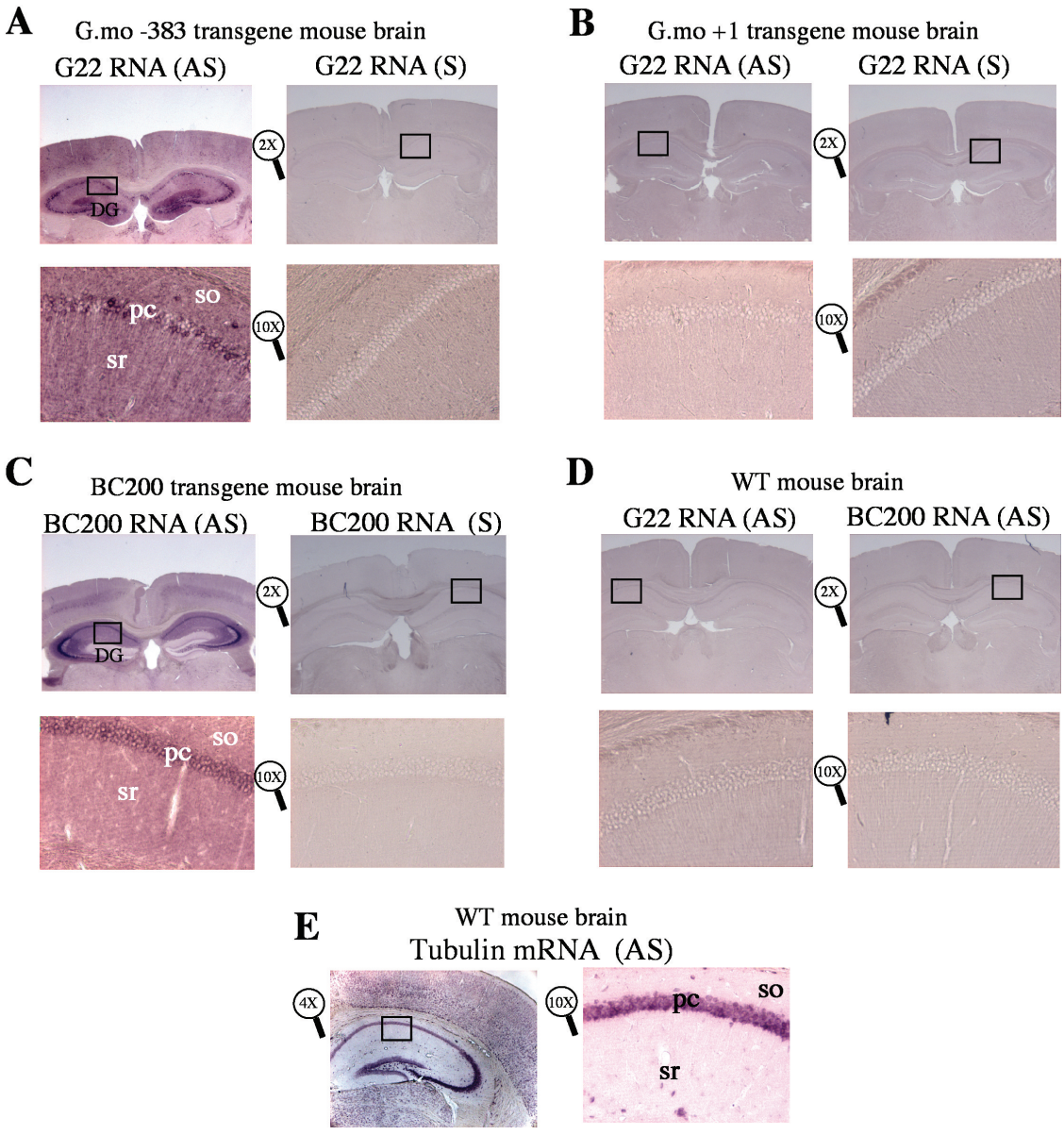

Figure 3. *In situ* hybridization of transgenic BC200 and G22 RNA. (**A**) Dendritic localization of Gmo22 RNA, substantial labeling was observed in the CA1, CA2 and CA3 regions of the hippocampus. At higher magnification of cells in the CA1 region, labeling is evident up to the distal ends of the dendrites. In the dentate gyrus (DG), labeling in the dendritic field was more intense than in the CA1-CA3 dendritic fields. The control, with S probe did not show any labeling. (**B**) There is no significant labeling observed for G22 RNA in Gmo +1 transgenic animals with either the AS probe or the contol (S) probe. The higher magnification further confirms that there is no specific labeling. (**C**) Dendritic localization of BC200 RNA, intense signal was observed in the CA1, CA2 and CA3 areas of the hippocampus and in some of the cortical layers (Figure 2). The *stratum lucidum* does not show any labeling. The higher magnification reveals strong labeling in the cell bodies as well as the dendritic field of CA1. The dentate gyrus shows very low signal if at all in comparison to other areas of the brain. The control (S) probe does not show any labeling. (**D**) *In situ* hybridization experiments with (AS) probes for both BC200 RNA and G22 RNA on the sections of wild-type (WT) mice, as a control, did not show any significant labeling as evident at both lower and higher magnifications. (**E**) *In situ* hybridization showing the localization of tubulin mRNA. There is intense labeling in all the regions of hippocampus, CA1, CA2 and CA3, and also the cells in the dentate gyrus. In the cortex, the cells in almost all the layers are stained intensely. The signal is distinctly confined to the somatic region in all the cells that are labeled. The higher magnification further shows that the dendritic field in the hippocampus is devoid of any labeling.

G22 RNA, we detected two distinct mobilities of the RNP complex as we did with the BC200/PABP RNP. The G22–PABP RNP complex was formed with an apparent K50 value of 12 nM (Figure 7A). In experiments where ^{32}P-labeled G22 RNA was competed from the G22/PABP RNP with unlabeled G22 RNA, we observed similar results as with the BC200 RNA complex (Figure 7C and D). Thus, we demonstrated specificity of the G22 RNA–PABP interaction. To gain further insight into the interaction between the investigated RNAs and PABP, we performed competition experiments, whereby ^{32}P-labeled G22 RNA and BC200 RNA were competed from their RNPs with unlabeled BC200 RNA and G22 RNA, respectively (Figure 7E and F).

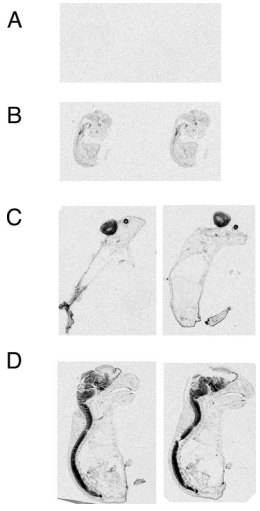

Figure 4. BC200 RNA expression in mouse embryos. (**A**) E15 embryos, sagittal middle sections, S control. (**B**) E15 embryos, sagittal central sections through the spinal cord; AS probe. (**C**) E18 embryos, sagittal lateral sections through the retina; AS probe. The BC200 signal is seen in brain and retina. (**D**) E18 embryos, sagittal central sections through the spinal cord; AS probe. The BC200 signal is seen in brain, spinal cord and retina.

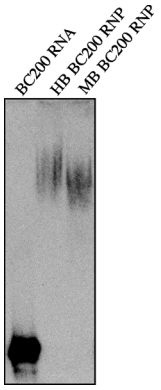

Figure 5. Comparison of human and transgenic mouse BC200 RNPs. S2 extracts from both human and transgenic mouse brains were separated on a native gel and the resulting blot was hybridized with ^{32}P-labeled oligonucleotide (BC207) complimentary to the unique region of the human BC200 RNA gene. The first lane contains *in vitro* transcribed BC200 RNA.

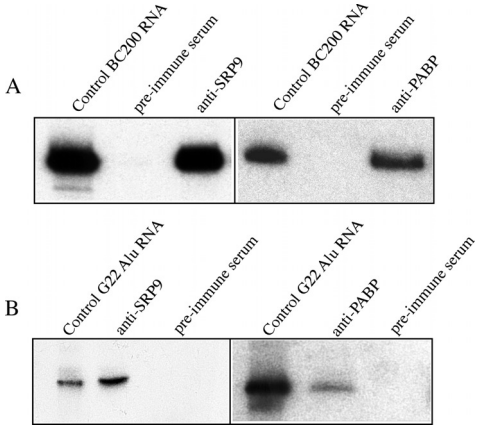

Figure 6. Immunoprecipitation of BC200 and G22 RNPs from transgenic mouse brain. (**A** and **B**) Samples of the S2 fractions from BC200 (−2271) and Gmo22 (−383) transgenic mouse brains (same amount in each case) were immunoprecipitated with antibodies against PABP and SRP9 proteins. RNA was extracted from the precipitated complexes and separated on 7 M urea/8% polyacrylamide gels, and the resulting transfer blots were hybridized as described (see Materials and Methods). As a control in each case, RNA from complexes immunoprecipitated from the crude extract using pre-immune serum (PABP and SRP9) was loaded. *In vitro* transcribed BC200 RNA and G22 RNA were loaded as an additional control (first lane in each case).

Unlike BC200 RNA, which has a 41 nt long A-rich region in its central part, G22 RNA contains two A-rich regions of 15 nt each, in the center and near the 3′ end. When ^{32}P-labeled G22 RNA was competed with unlabeled BC200 RNA for binding with PABP, we observed reduced intensity of the upper shifted band with 5- to 80-fold excess of competitor (Figure 7E). At similar conditions ^{32}P-labeled BC200 RNA was competed with unlabeled G22 RNA. The reduction in the intensities of the shifted bands was observed with 3- to 160-fold excess of unlabeled G22 RNA (Figure 7F).

The slight differences observed in competition might be due to co-operative binding of two PABP molecules to the longer A-rich region of BC200 RNA in comparison to individual binding of monomers to the two separated binding sites in G22 RNA. Our results clearly demonstrate that both BC200 and G22 npcRNAs form specific RNP complexes with PABP. Thus, it is tempting to speculate that the G22 RNP in Lorisiformes brain might be involved in functions similar to its analog, the anthropoid BC200 RNP, e.g. possibly in the regulation of dendritic translation (20–22).

DISCUSSION

Usually, novel genes arise by duplication of existing ones. There are two major modes of duplication: (i) a segment containing an entire gene (usually several, and up to hundreds or thousands of kilobases, occasionally even entire chromosomes or genomes) duplicate or (ii) an RNA transcript of a gene is converted into cDNA by the action of the enzyme reverse transcriptase and inserted at random positions of the genome. The latter requires fortuitous juxtaposition to promoter elements and, as a result, leads with lower frequency to functional gene products that can be exapted into variant and, over time, novel functions (11). The unsuccessful events are prevalent in genomes of multicellular species and are known as retropseudogenes, SINEs and the like. Multiple retroposon targeting of the same locus is rare as there are potentially more than a hundred thousand potential integration sites. If it occurs, the precise integration at a given location is less surprising due to the consensus sequence 5′-TT/AAAA-3′ of preferred integration sites. In the case presented here, it is quite unusual that both transcripts have been conserved in their respective lineages for at least 35 million years. This is a strong indication for recruitment (exaptation) into a function resulting in purifying selection. Nevertheless, the respective genes are relatively young and establishing their functions is not trivial, especially when they occur in species that are not amenable to experimentation due to conservational or ethical reasons. One way to circumvent these limitations is the generation of transgenic animals that express the genes to be studied. Their expression patterns may provide the first leads concerning their function.

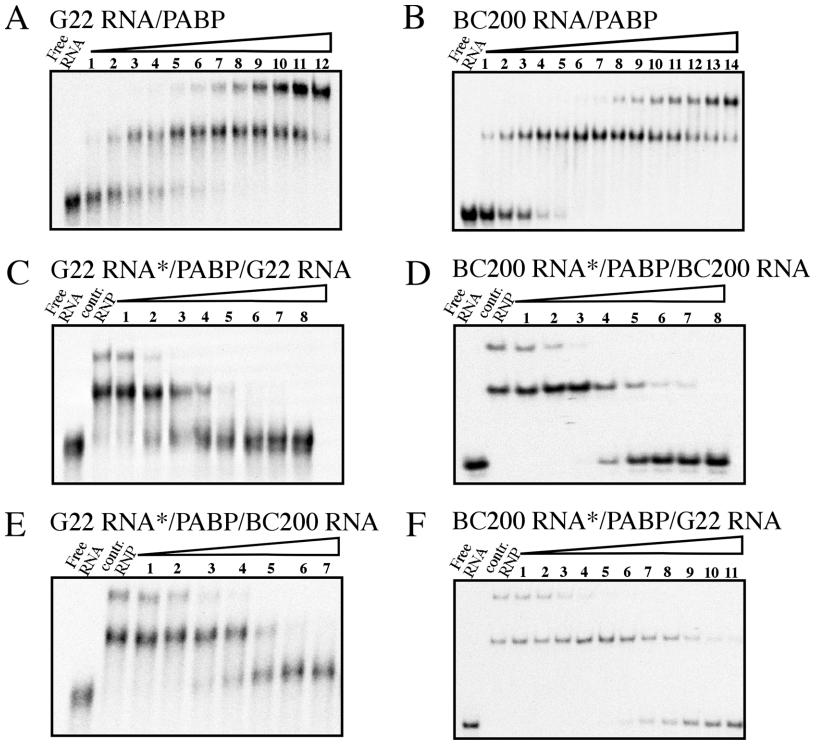

Figure 7. Interaction of human PABP with BC200 RNA and G22 RNA detected by gel retardation analysis. (**A** and **B**) Interaction of human PABP with G22 RNA and BC200 RNA, respectively. ^{32}P-labeled RNAs were incubated with human recombinant PABP protein as follows: free RNA, no protein was added; lanes 1–12: 1, 2, 3, 4, 6, 8, 12, 16, 25, 32, 50 and 62 nM PABP was added; in (B) lanes 13 and 14 represent increasing concentrations of PABP up to 100 and 125 nM, respectively. RNP complex formation between (**C**) ^{32}P-labeled G22 RNA and PABP; (**D**) ^{32}P-labeled BC200 RNA and PABP, in the presence of unlabeled G22 RNA and BC200 RNA, respectively for (C and D). (**E**) Competition assay between ^{32}P-labeled G22 RNA and PABP, in the presence of unlabeled BC200 RNA. (**F**) Competition assay between ^{32}P-labeled BC200 RNA and PABP, in the presence of unlabeled G22 RNA. Lanes indicated as follows: free RNA, no protein or competitor RNA was added; contr. RNP, initial RNP complex formed in the presence of 25 nM of PABP, no competitor RNA was added; (C–E) lanes 1–7: 2, 10, 20, 40, 80, 150 and 200 nM competitor RNA was added to the initial RNP complex; lanes 8 for (C and D), represent the same conditions as lanes 7, except that no PABP protein was added in reactions. (F) Lanes 1–11: 2, 4, 8, 17, 35, 70, 140, 200, 280, 400 and 560 nM G22 RNA was added to the initial RNP complex.

Furthermore, in case of RNA molecules, delineation of their complexed protein partners provides clues to their functions.

We generated at least three founder lines for each construct in order to exclude atypical expression patterns due to contributions of the genomic integration loci. While tissue-specific expression patterns were identical in founder lines that were generated from the same transgene, we could observe differences in their distribution regarding cell-types, (such as cortical neurons; data not shown). This could be ascribed to slight differences in expression of the respective constructs when integrated in different chromosomal loci. This is more pronounced when less flanking regions are present. These shorter flanks (mainly 5′) might have lost regulatory sequences or simply provide less buffering against influences from the loci of integration. For example, BC200 RNA with 2271 nt of 5′ flanking sequences revealed signals on northern blot only in brain and at a reduced level in the testis (Figure 1A). Even in the presence of only 515 nt upstream, expression was mostly restricted to these organs (Figure 1B, left panel). In contrast, when only 250 nt were upstream of BC200 RNA or 383 nt upstream of G22 RNA, we observed, in addition to expression in the brain, noticeable signals in heart and kidney, but not in liver and spleen (Figure 1B, right panel, Figure 1C, left panel). Expression levels of the −515 BC200 RNA construct indicate that, if any, at most 19 nt at the 3′ flank are necessary for tissue-specific expression. Apart from the internal box-A and box-B RNA polymerase III promoter elements, which are probably necessary for basal transcription levels (10), the regulatory elements for efficient, cell-type-specific and developmentally regulated expression of BC200 and G22 RNAs must reside within 515 nt upstream from the transcriptional start and, if at all, 19 nt downstream of the transcriptional termination.

Our results indicate that the region upstream of the BC200 RNA gene (−515−−250) might be involved in repressing expression in tissues other than brain and testes. More likely, flanking regions simply protect from position effects.

In line with earlier cell transfection experiments (10), wherein removal of the entire 5′ flanking sequences resulted in a sequence with intact internal box-A and box-B RNA polymerase III promoter elements and 147 nt 3′ flanking sequences (Figure 1D), expression of the dimeric Alu element-derived G22 RNA was completely abolished (Figure 1C, right panel). This is convincing proof that these elements are not sufficient for transcription *in vivo*, at least under normal conditions.

Moreover, our data indicate that the Alu-derived retroposons harboring intact internal promoter elements are ‘dead on arrival’ (39), unless they are fortuitously furnished by

the locus with its own regulatory elements, an event exceedingly rare.

Expression of BC200 RNA in transgenic mice revealed cell-type-specific expression that generally is analogous to the pattern observed in human brain sections (Figure 2) and to the rodent analogue BC1 RNA. Similarly, the transgenic BC200 RNA is upregulated during embryonic development of the nervous system (Figure 4). Early embryonic expression appears to be reduced but more promiscuous with respect to tissue or cell-types. Importantly, as in primates, BC200 RNA was transported to dendrites (Figure 3). Congruence of BC200 RNA and G22 RNA transgene expression is not too surprising due to the high-level of sequence similarity. A surprising divergence between all BC200 RNA and G22 RNA transgenes is the extreme difference (absence and high-level expression) of the respective RNAs in dentate gyrus.

Several hundred mRNAs are estimated to be dendritic. Consistently, non-protein coding RNAs that play a role in synthesis or regulatory aspects of translation can be found in dendrites. Examples for the former are rRNA (40,41) and tRNAs (42), for the latter moderate levels of SRP RNA (36), high-levels of BC1 and BC200 RNA and most recently even a micro RNA [miR-134; (43)]. Because of the great similarity of G22 RNA, especially to BC200 RNA (basically, G22 is a dimer of BC200 RNA), it is not surprising that G22 RNA is transported into dendrites. More work in transgenic models is needed to delineate the structures and/or motifs that are responsible for dendritic localization (44).

Both BC RNAs and miR-134 are possibly involved in the regulation of translation (20–22,43,45). Deletion of BC1 RNA leads to a behavioral phenotype (see above) and miR-134 is thought to control translation of the synaptic Limk1 protein, thereby regulating the size of dendritic spines. It would not be surprising if G22 RNA had a function in a prosimian branch that closely corresponds to that of BC200 RNA in anthropoid primates. Observations reported here support the assumption that G22 RNA shares PABP as a major protein partner with BC1 RNA and BC200 RNA and the SRP protein dimer SRP9/14 with BC200 RNA. The observation that the K50 values of interaction of PABP with BC200 and G22 RNAs are slightly different (Figure 7), might be due to the distribution of the A-rich sequences on both RNAs. A rather long stretch (41 nt) is located in the center of BC200 RNA, while G22 RNA features two shorter A-rich regions (15 nt each) between the monomers and 3′ to the right monomer.

Of the dendritic small npcRNAs, the best-studied is BC1 RNA in rodents. Although it is not ancestrally related to the primate dendritic npcRNAs characterized in this paper, it might be a functional analogue due to a similar tripartite domain structure (a 5′ domain with similarity to a SINE, a central A-rich domain, and a 3′ unique non-repetitive domain) and, as mentioned above, shared protein binding partners. It will be interesting to test whether mice transgenic for BC200 RNA or G22 RNA, when crossed into BC1 RNA-deleted mice, will exhibit compensation of the reduced exploratory behavior and increased anxiety of KO mice.

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