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Evolution

Neural BC1 RNA as an evolutionary marker: Guinea pig remains a rodent

(non-messenger-RNA/recruited retroposon/exaptation/rodent specific/rodent monophyly)

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ABSTRACT **The traditional morphologically grounded placement of South American guinea pig-like rodents (Caviomorpha) within one of the two rodent suborders, Hystricognathi, has been disputed by recent analysis of protein and nucleic acid sequence data. The Caviomorpha and possibly all Hystricognathi would be considered a separate order, distinct from the other rodent suborder, Sciurognathi, and thus of the order Rodentia, and would be placed closer phylogenetically to other mammals [Graur, D., Hide, W. A. & Li, W.-H. (1991) *Nature (London)* 351, 649–652]. To address the discrepancy between morphological comparisons and sequence analyses, we have applied an alternative form of molecular analysis. We demonstrate that BC1 RNA, a neural-specific small cytoplasmic RNA that is the product of a retropositionally generated gene (a gene derived by reverse transcription of RNA followed by insertion of the DNA copy into the genome), is present in Sciurognathi and guinea pig but not in other mammalian orders including Lagomorpha, Artiodactyla, and Primates. The species-confined, tissue-specific expression of a retroposed sequence therefore supports the morphological evidence for monophyly of Rodentia inclusive of guinea pig and demonstrates the usefulness of such molecular genetic markers. Furthermore, the conservation and tissue-specific expression of the BC1 RNA gene in the two divergent rodent suborders suggests that this macromolecule has been exapted into a functional role (i.e., coopted into a variant or novel function) in the rodent nervous system.**

Based on a large body of morphological evidence, the order Rodentia, containing approximately half of all mammalian species (1), has been subdivided into two suborders (2). The Sciurognathi include true rats, mice, hamsters, and squirrel-like rodents, while the Hystricognathi, also representing a group of Old and New World rodent families, are now primarily restricted to Africa and South America. Examples of this diverse suborder include guinea pig, chinchilla, and porcupine. However, challenging this morphologically established phylogenetic scheme of Rodentia is an apparent "molecular paradox"—namely, most guinea pig protein and nucleic acid sequences exhibit an unusually high level of dissimilarity when compared with their rodent orthologues (refs. 3–5, but also see ref. 6).

A recently proposed solution (7, 8) would define Caviomorpha and possibly the entire suborder Hystricognathi as a separate mammalian order distinct from Rodentia. Unfortunately, the proposal of a separate order addresses the molecular data but leads to incongruence with paleontological information and anatomical data (9). Thus, well-established morphological synapomorphies between Sciurognathi and Hystricognathi would be reinterpreted as homoplasies (10). Furthermore, the molecular evidence underlying this extreme proposal must also be reevaluated. For example, Allard *et al.* (10) have shown that using the same method of comparative sequence analysis as Graur *et al.* (7)—maximum parsimony—but dependent upon the use of an outgroup, either monophyly or polyphyly can be supported. Reexamination of the data by Hasegawa *et al.* (11) using the maximum likelihood method also does not support rodent polyphyly. These contrasting studies highlight, as has been previously noted (12), that caution is required in inferring phylogenies strictly from the comparison of amino acid and protein coding nucleic acid sequences.

Alternative methods of molecular analyses may aid in reaching a consensus on phylogenetic placement where morphology and standard DNA and amino acid sequence analysis cannot. The presence of repetitive sequences [such as *Alu* elements and tRNA-derived retroposons (13–18), pseudogenes (19, 20), and internal gene duplications (21, 22)], newly arisen genes (23), the differential expression patterns of preexisting (24–26) or newly arisen genes, and gene loss or inactivation (27, 28) would provide "time-landmarks of evolution" (29) and would constitute molecular genetic markers. Such characters may prove particularly useful in cases where radiations have occurred relatively fast with lineages connected by rather short internodes.

BC1 RNA (30) is encoded by a tRNA-derived retroposon such that the 5′ domain of the encoded RNA shares 80% sequence similarity with $tRNA^{Ala}$.† This neural-specific small cytoplasmic RNA (33) is complexed with proteins to form a ribonucleoprotein particle (34–36) and is located in the somata and dendrites of a specific subset of neurons (37). BC1 RNA has been detected in several sciurognathid rodents other than rats, and its brain-specific expression pattern has been shown to be conserved (38). We examined whether BC1 RNA was present and maintained its neural-specific expression pattern in different mammalian orders, including guinea pig, as well as determined its cDNA sequences in several rodents‡ to provide molecular evidence for rodent evolutionary relationships.

METHODS

Animals. Tissue from the following animals for RNA isolation was used: guinea pig (*Cavia porcellus*; Hartley

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†The BC1 RNA gene was generated by duplication of $tRNA^{Ala}$ (31). Most likely, at least one of the steps involved retroposition (an event where RNA is reverse-transcribed into a DNA copy followed by insertion into the genome), a notion supported by the hallmark A-rich region (32) located at the 3′ end of the putative founder $tRNA^{Ala}$ domain. In the new genomic environment, transcription of the modified tRNA ends at an RNA polymerase III transcription termination site further downstream, accounting for the greater length of BC1 RNA (i.e., the internalization of the A-rich region and the acquired 3′ unique domain; see Fig. 2).

‡The cDNA sequences reported in this paper have been deposited in the GenBank data base (accession numbers U01304, U01309, and U01310 for guinea pig, Syrian Golden hamster, and mouse, respectively).

strain, male), Syrian Golden hamster (*Microcricetus aureus*; female), mouse (*Mus musculus*; C57BL/6 strain, female), rat (*Rattus norvegicus*; Sprague–Dawley strain, male), and rabbit (*Oryctolagus cuniculus*; New Zealand White Strain, female). Cow (*Bos taurus*) brain RNA was a gift from T. Kirchausen (Harvard University).

RNA Blots. Isolation of RNA, electrophoresis (20 μg of total RNA per lane) on 1.2% agarose gels containing formaldehyde, blotting to GeneScreen nylon membrane (NEN), immobilization by UV-irradiation (39), and hybridization (without formamide) have been carried out by standard techniques (40). The amount and integrity of the loaded RNA were determined by staining the gel with ethidium bromide (data not shown). An oligodeoxynucleotide, HT005 (5′-A_3GGTTGTGTGTGCCAGTTACCTTGT$_9$GGTCT$_5$GTTA-T_4GTCT$_5$-3′), complementary to the 60 3′-most nucleotides in rat BC1 RNA (33) was 5′-end-labeled with ^{32}P and used as a probe (40). The filter was washed at 42°C in 5× SSC (40) and exposed for 6 hr on Fuji RX x-ray film at −80°C with intensifier screen.

RNA samples (30 μg each) from guinea pig brain, liver, and kidney were separated on a 6% acrylamide gel (19:1 acrylamide/bismethyleneacrylamide) containing 7 M urea and were transferred to GeneScreen. An oligodeoxynucleotide, GU033, complementary to the 3′ region of guinea pig BC1 RNA (5′-AAAGGTTGTTTGTGTGCGCAGTTACCTTGT-TTG-3′) was 5′-end-labeled with ^{32}P and used as a probe for hybridization at 50°C in 20% formamide (40). The final wash was in 0.5× SSC at 55°C, and the filter was exposed overnight on Fuji RX x-ray film at −80°C with intensifier screen.

Construction, Isolation, and Characterization of cDNA to Rodent BC1 RNA. An oligo(dC) tail was added to the 3′ ends of total RNA from mouse, Syrian (Golden) hamster, and guinea pig brains by the method of Devos *et al.* (33, 41). cDNA synthesis and cloning into Lambda ZAP phage (Stratagene) was performed as described (33). The respective libraries were screened with oligodeoxynucleotide HT005 (see above) and/or probes corresponding to the 5′ domain of rat BC1 RNA (33). Inserts from 15 guinea pig, 20 mouse, and 20 Syrian hamster clones were sequenced by the dideoxy chain-termination method (42, 43).

5′-End Determination of Rat BC1 RNA. The exact 5′ end of rat BC1 RNA has been determined by RACE (rapid amplification of cDNA ends) PCR as described (44). Briefly, 1 μg of total rat brain RNA was converted into cDNA by using the cDNA cycle kit (Invitrogen), the manufacturer's instructions, and oligonucleotide WC002 (5′-GGTTGTGTGTGC-CAGTTACC-3′) as a reverse-transcription primer. The 3′ end of the first-strand cDNA was then tailed by using dTTP and terminal transferase (Boehringer Mannheim). The tailed cDNA was PCR-amplified for 30 cycles (denaturation for 30 sec at 94°C, annealing for 1 min at 55°C, and extension for 2 min at 72°C; initial denaturation was for 4 min at 94°C, and final extension was for 10 min at 72°C) with primers HT021 (5′-GCCTTCGAATTCAGCACCAAAAAAAAAAAA-AAAAA-3′) and WC006 (5′-GCCTTCGAATTCGGTTGT-GTGTGCCAGTTACCTTG-3′). The products were amplified an additional 30 cycles (conditions as above) with the adapter primer HT023 (5′-GCCTTCGAATTCAGCACC-3′). After digestion with *Eco*RI, the PCR products were cloned into Lambda ZAP II (Stratagene). About 1000 plaques were screened with an oligonucleotide complementary to the internal 5′ domain of BC1 RNA, JB-E (5′-GGCAAGCGCTC-TACCACTGAGCTAAATCCCCAG-3′). Inserts from several positive clones were sequenced.

Sequence Data Analysis. Sequences were initially compared and aligned by using the program BESTFIT and PILEUP, respectively, in the Genetics Computer Group suite of programs (version 7.0; ref. 45) made available by the Department of Biomathematics, Mount Sinai School of Medicine; in some instances, the sequences were further aligned manually.

RESULTS AND DISCUSSION

BC1 RNA Expression Is Rodent Specific. To establish whether BC1 RNA is present in mammalian species other than rodents, an oligonucleotide probe was designed complementary to the 3′-terminal 60 nucleotides of rat BC1 RNA and then was used to screen RNA blots under very-low-stringency conditions (Fig. 1*A*). No RNA of a similar size to rat BC1 RNA was detectable in rabbit or bovine neural tissue. A band of slightly slower electrophoretic mobility was detected, however, in human brain-derived RNA.

To identify this RNA, a human brain cDNA library was screened by using the 3′ oligonucleotide probe under similar conditions (46). All 16 clones characterized contained BC200 RNA, a primate neural-specific small cytoplasmic RNA (46, 47). However, BC200 RNA is phylogenetically distinct from BC1 RNA and consequently does not constitute a primate BC1 RNA orthologue. Isolation and sequence analysis of the BC200 RNA gene reveals that BC200 RNA represents a transcriptionally active monomeric *Alu* element (61), which was detected on the BC1 RNA blots in Fig. 1*A* because of the low degree of sequence similarity between the 3′ ends of these RNAs (see Fig. 2 and ref. 46). Therefore, BC1 RNA is not conserved in lagomorphs, artiodactyls, or primates.

BC1 RNA Is Conserved and Expressed in a Neural-Specific Manner in Guinea Pig. We next probed, under high-stringency conditions, RNA extracted from guinea pig (Fig. 1*B*). An RNA species of the appropriate size that is present only in brain but not liver or kidney was identified, suggesting that BC1 RNA is present in guinea pig and maintains its

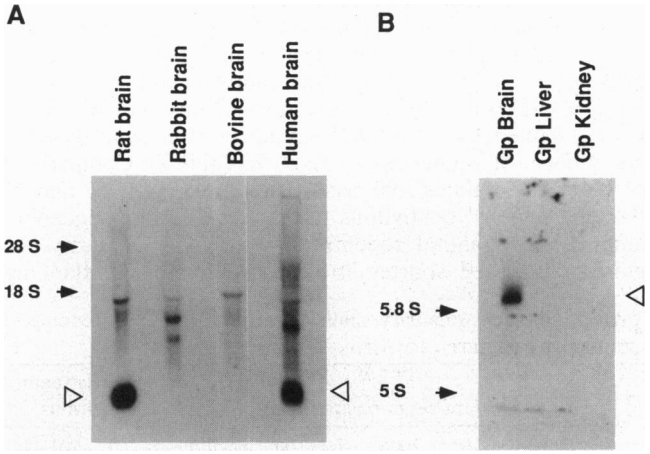

FIG. 1. Detection of small RNAs in mammalian brain tissues. (*A*) RNA blot hybridization on a 1.2% agarose gel with 20 μg of rat, rabbit, bovine, and human brain RNA using the HT005 probe complementary to the 60 3′-most nucleotides of rat BC1 RNA (33). The blot was performed under low-stringency conditions (final wash at 42°C in 5× SSC). Open arrowheads depict the position of small RNAs in the size range of BC1 and BC200, while arrows indicate the positions of 18S and 28S ribosomal RNA. Several bands located below 18S RNA are detected in the lanes corresponding to rabbit and human brain. They are also present in RNA extracted from rabbit liver (data not shown) and represent nonspecific signals due to the low washing stringencies employed. (*B*) RNA blot hybridization on a denaturing polyacrylamide gel with 30 μg of guinea pig (Gp) brain, liver, and kidney RNA using a probe (GU033) complementary to the nonrepetitive region of guinea pig BC1 RNA. Arrows indicate the positions of 5S (120 nucleotides) and 5.8S (160 nucleotides) ribosomal RNA, and the open arrowhead indicates the position of guinea pig BC1 RNA.

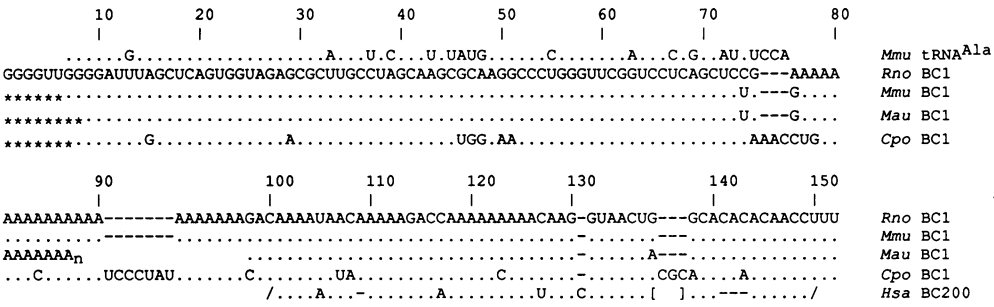

FIG. 2. cDNA sequence alignment of BC1 RNA from rat (Rno), mouse (Mmu), Syrian golden hamster (Mau), and guinea pig (Cpo). The sequence of rat BC1 RNA is from ref. 33. Virtually all cDNA sequences isolated from the same species were identical among clones except for the length of the longer oligo(A) stretches in the center of the molecule (an artefact most likely introduced by reverse transcription) and the length of the 5′ end. The actual length of the oligo(A) stretches has been derived from genomic sequences (48) except in the Syrian hamster, where the uncertainty is indicated by A_n. The exact 5′ end of rat BC1 RNA has been determined by RACE (rapid amplification of cDNA ends) PCR. All eight clones analyzed as described under *Methods* exhibited the 5′ sequence shown. The cDNAs of mouse, hamster, and guinea pig do not extend to the terminal 5′ end of BC1 RNA. This region (from six to eight nucleotides) is indicated by asterisks. Genomic sequences (48) of the respective genes indicate that they are all identical with the rat sequence (for Syrian hamster, a pseudogene exhibits the same 5′ sequence). A minor variation of cDNA clones was observed at the 3′ ends, which usually ended in stretches corresponding to two to four uridine residues. This may be due to 3′ degradation of the RNA (e.g., during experimental manipulation) or a naturally occurring polymorphism of BC1 RNA. Numbering is with reference to the rat BC1 RNA sequence (33). Positions 1–65 of $tRNA^{Ala}$ and positions 6–71 of rat BC1 RNA exhibit 80% sequence similarity (determined as described in ref. 45 with default settings). The nucleotides that differ in mouse mature $tRNA^{Ala}$ (31) from the 5′ domain of BC1 RNAs are shown. The region of human (Hsa) BC200 RNA (positions 134–187; ref. 46) that has 91% sequence similarity (ref. 45; gap weight 2.0, length weight 0.3) to rat BC1 RNA (positions 101–149) is shown between slashes in the last line. In all cases dots correspond to identical positions and hyphens mark gaps introduced for optimal alignment. Brackets in the BC200 sequence indicate eight additional nucleotides and one substitution (instead of G-137 in rat BC1).

neural-specific expression pattern. The presence of BC1 RNA in guinea pig was conclusively established by isolating and sequencing its cDNA. Comparison of the guinea pig and several Sciurognathi BC1 cDNA sequences is shown in Fig. 2.

A further level of analysis was performed by comparing the coding and flanking regions of the BC1 RNA gene between rat, mouse, Chinese hamster, and guinea pig. As would be expected for sequences encoding a functional RNA, a marked contrast between the level of sequence conservation found between the coding regions and the lower levels present in flanking regions was found (Table 1). Among Sciurognathi (rat, mouse, and Chinese hamster), BC1 RNA coding sequences exhibit 99% similarity while sciurognathid and guinea pig sequences share 89% (Table 1). Comparison of blocks containing 100 base pairs (bp) of the 5′ and 3′ flanking regions, contiguous to the RNA coding sequence, among sciurognathid rodents shows >88% similarity. In guinea pig, even shorter stretches of immediate flanking regions diverge further, requiring the introduction of numerous gaps for alignment (48). These divergence differences between coding and flanking regions, especially apparent in the guinea pig, strongly support that BC1 RNA has been conserved.

Table 1. Percent sequence similarities of the BC1 RNA coding and flanking regions

Rodent sequence comparisons	Upstream positions		RNA coding region	Downstream positions	
	−101/−200	−1/−100		1–100	101–200
m/r	90	96	99	96	90
m/h	89	95	99	88	80
r/h	88	95	99	88	79
gp/r		77*	89	75*	

The sequences aligned in Fig. 2 correspond to the central column (RNA coding region) in this table. Upstream flanking sequences are preceded by a "−" sign; downstream flanking sequences are numbered 1–200. Flanking sequences (from ref. 48) were artificially grouped into blocks of 100 nucleotides. Alignment was done as described (45) with the default settings (gap weight 5.0, length weight 0.3) except for the alignment of guinea pig flanking sequences (marked with an asterisk), where the gap weight was reduced to 2.5 and the length weight to 0.2. m, Mouse; r, rat; h, Chinese hamster; gp, guinea pig.

*Only 52 nucleotides 5′ and 70 nucleotides 3′ to the guinea pig BC1 RNA coding sequence were aligned to avoid the introduction of numerous gaps.

Rodent Monophyly Is Supported by the Presence and Expression Pattern of BC1 RNA. Using BC1 RNA as a molecular genetic marker to account for the expression of the RNA in Sciurognathi and Hystricognathi and its absence from other mammals permits three different evolutionary scenarios to be defined. To discriminate between rodent monophyly and polyphyly, these scenarios encompass two possible phylogenetic trees (Fig. 3, as adapted from ref. 7). The data are most consistent with rodent monophyly wherein guinea pig (and possibly all Hystricognathi) represents a suborder of Rodentia.

In the first scenario, a monophyletic scheme wherein Sciurognathi and Hystricognathi jointly diverge from other mammals is depicted. The retroposition event generating the BC1 RNA gene and transcriptional activation would occur after the joint divergence but prior to the suborders themselves branching (Fig. 3*A*). In the second scenario, the BC1 RNA gene would be generated prior to the branching of the Sciurognathi and Hystricognathi (Fig. 3*B*). The most parsimonious tree favors rodent monophyly: transcriptional activation need only occur once prior to suborder branching (tree I). For polyphyly, two independent neural-specific transcriptional activation events would be required (tree II). In the third scenario, both retroposition and transcriptional activation would occur prior to the branching of the Sciurognathi and Hystricognathi (Fig. 3*C*). This scenario requires the BC1 RNA gene to be deleted or transcriptionally inactivated in artiodactyls and primates. While we cannot exclude this final scenario, compatible with either monophyly (tree I) or polyphyly (tree II), it is less likely than the others because it requires an additional gene inactivation event in some lineages. Nonetheless, if tree II is correct, the Sciurognathi and Hystricognathi would have to be grouped close together without intervening orders (not expressing BC1 RNA), otherwise additional independent deletion or transcription inactivation events would be required. Graur, Li, and colleagues (49) have recently published a phylogenetic tree for mammals

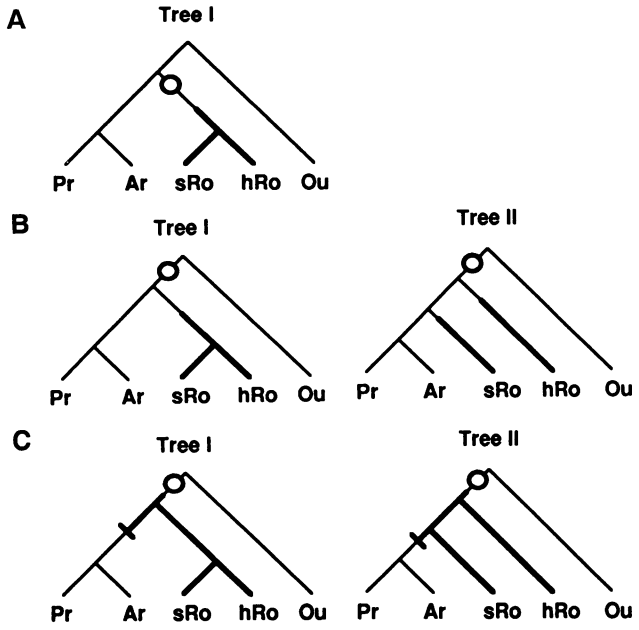

FIG. 3. Two phylogenetic trees (adapted from ref. 7) for hystricognathid (hRo) and sciurognathid rodents (sRo), artiodactyls (Ar), primates (Pr), and an outgroup (Ou). Tree I represents rodent monophyly and tree II represents polyphyly. In *A* the BC1 RNA gene arose (represented by open circle) after the lineage leading to both rodent suborders branched off. Transcriptional activity (represented by enhanced lines) persists after branching of the suborders. In *B* the gene arose prior to the branching of the mammals depicted in the two trees, but transcriptional activation occurred after rodent branching. In *C* transcriptional activation occurred also prior to the branching of the mammals depicted. However, the gene or its transcription was inactivated (short perpendicular bar) prior to the branching of primates and artiodactyls.

inferred from cytochrome *b* sequences by the neighbor-joining method. With respect to the sciurognathid rodents, the human–elephant cluster branches off prior to the branching of the guinea pig–porcupine cluster. This tree would thus necessitate at least two separate events of BC1 gene loss or inactivation, again favoring tree I in Fig. 3*C*, consistent with rodent monophyly.

Recently, the method of maximum-parsimony sequence analysis has been used in an attempt to discern the evolutionary relationships of rodents (7, 10). A key assumption in the analysis proposing rodent polyphyly (7) assumes rate homogeneity between Sciurognathi and Hystricognathi. This hypothesis is not supported by our data. The contrasting results obtained can therefore be explained by the fact that unequal evolutionary rates between species can lead to incorrect interpretations with this form of molecular analysis (7–12).

Evolutionary Implications of BC1 RNA. The presence of BC1 RNA in nervous system tissue of rats, mice, hamsters (Sciurognathi) and guinea pigs (a member of the Hystricognathi) and its absence from lagomorphs, artiodactyls, and primates provides molecular biological evidence consistent with a large body of morphological evidence (1, 2) supporting rodent monophyly. Therefore, the expression of a macromolecule can constitute a molecular genetic marker or molecular synapomorph to establish phylogenetic relationships among mammalian orders. [The absence of BC1 RNA in rabbits does not necessarily rule out the proposed relationship between Rodentia and Lagomorpha in the cohort Glires (discussed in refs. 9 and 50).]

Furthermore, our data illustrates that BC1 RNA has most likely been coopted into a functional role (i.e., exapted).§ This is suggested (*i*) by its unusual anatomical and subcellular location (37), wherein only ribosomal RNA as polysomes and a small number of messenger RNAs are known to share this dendritic subcellular localization (52–54); (*ii*) by its complexation with proteins to form a ribonucleoprotein particle (34–36); and (*iii*) by its conserved sequence in rodents for ≥55 million years.¶ If true, the evolutionary data on the retropositionally derived BC1 RNA further suggest that functional RNAs are not merely remnants of the RNA world. RNAs can, as is the case with proteins, yield new variants by gene duplication via recombination (56) or retroposition (57, 58). These molecules are then potentially available for recruitment (exaptation, defined in refs. 58–60) into different tasks (their encoding genes termed xaptonuons or xaptogenes; ref. 58). The contributions of tRNAs and tRNA-derived molecules to various functions of the genome and the cell may therefore be broader and more varied than previously anticipated.

§Recruitment of new RNAs such as BC1 RNA and BC200 RNA may enhance the efficiencies of preexisting proteins or ribonucleoprotein (RNP) complexes. The RNA-encoding genes would then be under functional constraints. In the case of BC1 RNA and BC200 RNA, both may have been independently recruited, as shown by their distinct phylogenies, into a variant (possibly regulatory) role that may be unique to specific neurons of rodents and primates, respectively. However, our data cannot rule out the existence of functional orthologues in other mammals whose coding sequences during the last 60–100 million years have diverged so highly as to preclude their detection by cross-hybridization. However, when one considers the relatively short evolutionary time scale and the level of sequence conservation among rodent BC1 RNAs, this would be highly unlikely. RNA analogues, generated from different ancestor genes and thus nonhomologous to either BC1 RNA or BC200 RNA, may have been recruited into the hypothetical preexisting protein or RNP complexes to similarly enhance function. The necessary numerous and separate events to account for parallel establishment of analogous RNAs in many other mammalian or even vertebrate lineages would also be highly unlikely. Therefore, the simplest explanation, yet not without precedent in evolution, is generation and recruitment of a variant gene into a different functional role in one or a few lineages (for example, see ref. 51).

¶Among all rodents examined, the BC1 RNA coding region is much more conserved than its flanking regions (see Table 1). Yet, a sequence similarity of 89% between the rat and guinea pig BC1 RNA coding region is significantly lower than similarity levels observed between most small nuclear RNA and small cytoplasmic RNA mammalian orthologues. Possibly the BC1 locus was for a time evolving more rapidly after the branching of the two rodent suborders. This rapid rate slowed, at least among sciurognathids during the last 20–30 million years, as evidenced by the fact that rat, mouse, and hamster BC1 RNAs exhibit 99% similarity. An analogous observation of relatively high levels of replacement polymorphism after the separation of two species was made for parts of the chimeric jingwei gene in *Drosophila* (55).

Note Added in Proof. While this paper was in press, a reevaluation of anatomical data and a survey of molecular data in view of the hypothesis of rodent polyphyly (7, 8) was published by Luckett and Hartenberger (ref. 62). Their analyses strongly corroborate rodent monophyly while lending little or no support to rodent polyphyly.

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