Numerous microRNPs in neuronal cells containing novel microRNAs

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ABSTRACT

Spinal muscular atrophy (SMA) is a common neurodegenerative disease that is caused by deletions or loss-of-function mutations in the Survival of Motor Neuron (SMN) protein. SMN is part of a large complex that functions in the assembly/restructuring of ribonucleoprotein (RNP) complexes. We recently showed in HeLa cells that two components of the SMN complex, Gemin3 and Gemin4, together with the argonaute protein eIF2C2, also associate with microRNAs (miRNAs) as part of a novel class of RNPs termed miRNPs. Here we report on miRNPs isolated from neuronal cell lines of mouse and human, and describe 53 novel miRNAs. Several of these miRNAs are conserved in divergent organisms, including rat, zebrafish, pufferfish, and the nematode *Caenorhabditis elegans*. The chromosomal locations of most of the novel miRNAs were identified and indicate some phylogenetic conservation of the likely precursor structures. Interestingly the gene locus of one miRNA, miR-175, is a candidate region for two neurologic diseases: early-onset parkinsonism (Waisman syndrome) and X-linked mental retardation (MRX3). Also, several miRNAs identified as part of miRNPs in these cells appear to constitute two distinct subfamilies. These subfamilies comprise multiple copies of miRNAs on different chromosomes, suggesting an important function in the regulation of gene expression.

Keywords: Gemin3; miRNAs; SMA; motor neurons

INTRODUCTION

MicroRNAs (miRNAs) are a large family of short (20–24-nt) single-stranded noncoding regulatory RNAs that includes the small temporal RNAs (stRNAs) lin-4 and let-7 (Ambros and Horvitz 1984; Reinhart et al. 2000). Based on knowledge gained from studies on stRNAs, miRNAs are thought to modulate gene expression by partially base-pairing with target mRNA sequences. Members of this new class of RNAs have been found in several metazoans including *Caenorhabditis elegans* (Lau et al. 2001; Lee and Ambros 2001), mouse (Lagos-Quintana et al. 2002), and humans (Lagos-Quintana et al. 2001; Mourelatos et al. 2002). miRNAs appear to be derived from the processing of larger precursors of approximately 70 nt or longer. The precursors are predicted to form imperfect stem-loop structures from which the mature miRNAs are excised from one half of the stem by a process that involves the RNAse III enzyme Dicer (Hutvagner et al. 2001).

miRNAs were recently shown to be components of a novel class of ribonucleoprotein (RNP) complexes termed miRNPs (Mourelatos et al. 2002). These complexes sediment as 15S particles in sucrose gradients and were found to contain the proteins Gemin3 (a DEAD-box putative RNA helicase; Charroux et al. 1999), Gemin4 (Charroux et al. 2000), and eIF2C2, a member of the argonaute family of proteins (Mourelatos et al. 2002). Gemin3 and Gemin4 are also components of the SMN complex, a large multiprotein complex containing the Survival of Motor Neuron (SMN) protein, Gemin2 (formerly SIP 1; Liu et al. 1997), Gemin5 (Meister et al. 2001; Gubitz et al. 2002), Gemin6 (Paushkin et al. 2002; Pellizzoni et al. 2002), and Gemin7 (Baccon et al. 2002). The SMN complex functions in the assembly/restructuring of RNPs, including spliceosomal small nuclear RNPs (snRNPs; Fischer et al. 1997; Pellizzoni et al. 1998; Meister et al. 2001), and possibly small nucleolar RNPs (snoRNPs; Jones et al. 2001; Pellizzoni et al. 2001a), heterogeneous nuclear RNPs (hnRNPs; Mourelatos et al. 2001), and transcriptosomes (Pellizzoni et al. 2001b).

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Deletion or loss-of-function mutations in SMN cause spinal muscular atrophy (SMA), a common genetic disease characterized by progressive degeneration of motor neurons (Melki 1997). Because Gemin3 and Gemin4 are shared components of the SMN and miRNP complexes, deletion or loss-of-function mutations of SMN in SMA may also affect the activity of miRNPs due to possible redistribution or changes in the levels of Gemin3 and Gemin4. Thus, it is possible that specific or general changes in the activity of the miRNPs play a role in the development of SMA. However, it was not known whether Gemin3 also associates with miRNAs in motor neuron cells, which are specifically affected in SMA. Furthermore, miRNAs associated with miRNPs in this cell type have not been previously identified. Here we report on the characterization of miRNAs associated with miRNPs isolated from neuronal cells. We identified 53 novel miRNAs from mouse and human neuronal cell lines, several of which are phylogenetically conserved in divergent organisms. The predicted precursor structure of most of these miRNAs was determined from known genomic sequences. Several miRNAs were found to constitute distinct subfamilies comprising multiple copies on different chromosomes, suggesting that these miRNAs play an important role in the regulation of gene expression.

RESULTS AND DISCUSSION

Gemin3 associates with miRNAs in neuronal cell lines

Because SMA specifically affects motor neurons, we wished to study miRNPs in well characterized neuronal cell lines. To do so, total extracts from mouse motor neuron MN-1 cells (Salazar-Grueso et al. 1991; Brooks et al. 1997) and human retinoblastoma Weri cells (McFall et al. 1977) were incubated with either the anti-Gemin3 antibody (11G9) or with the nonimmune mouse serum (NMS) as control. The immunoprecipitates were digested with proteinase K, and the associated RNAs were isolated. The extracted RNAs were 3′-end-labeled with [5′-^{32}P]-pCp, resolved by gel electrophoresis, and visualized by autoradiography. As shown in Figure 1, short (~24-nt) RNAs were specifically coimmunoprecipitated with the anti-Gemin3 antibody but not with the control antibody in both MN-1 and Weri cells. This indicates that Gemin3 is associated with small RNAs not only in HeLa cells (Mourelatos et al. 2002), and suggests that Gemin3 interacts with miRNAs in various cell types including motor neuron cells, as part of miRNPs.

Identification and characterization of miRNAs

To identify the miRNAs in these cells, the RNAs were cloned as previously described (Elbashir et al. 2001; Mourelatos et al. 2002). A total of 107 clones from Weri and MN-1 cells were sequenced. We found 41 different miRNAs associated with miRNPs in Weri cells, six of which were also found with miRNPs in HeLa cells (Table 1, miRNAs in bold letters; Mourelatos et al. 2002). Several miRNAs previously identified from total RNA, including let-7, miR-15, miR-20, miR-21, miR-29a, and the more recently described miR-15b, miR-27b, miR-29c, and neuronal-specific miR-124a (Lagos-Quintana et al. 2002) were also found, indicating that miRNPs also play a role in the metabolism of these miRNAs. Interestingly, 25 novel miRNAs were found in the miRNP from human neuronal cells, and three additional miRNAs were identified by sequence homology to the new miRNAs identified in the present study. Most miRNAs can be localized in the human genome, and are highly conserved in other organisms with little sequence variation. The precursor sequence and secondary structure of miRNAs that are found on several chromosomes in one species and/

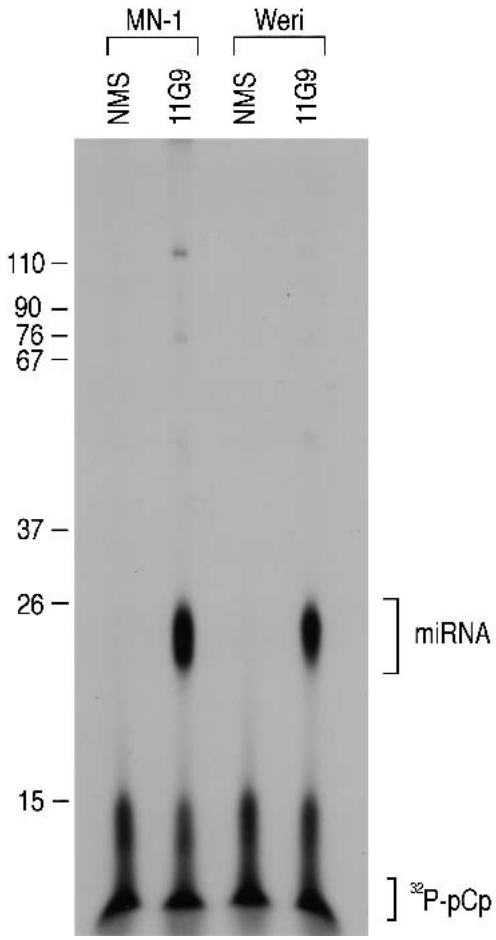

FIGURE 1. miRNPs from mouse and human neuronal cells. Total extracts from MN-1 (mouse) and Weri (human) cells were incubated with either nonimmune mouse serum (NMS) as a control or the anti-Gemin3 antibody (11G9). Immunoprecipitates were treated with proteinase K, and associated RNAs were purified by phenol-chloroform extraction and precipitated. Isolated RNAs were 3′-end-labeled with [5′-^{32}P]-pCp and resolved by electrophoresis on 15% denaturing polyacrylamide gels. *Left:* molecular weight marker.

TABLE 1. miRNA sequences associated with miRNPs in the Weri human neuronal cell line

miRNA	Number of clones	miRNA sequence	Size (nt)	Source	Predicted Precursors						
					Hs (Chr.)	Dr	Fr	Dm	Ce	Mm	Rn
let-7a	1	UGAGGUAGUAGGUUGUAUAGUU	22	Hs	+ (9, 11, 22)	-	+	+	+	+	+
miR-15a	1	UAGCAGCACAUAAUGGUUUGUGA	23	Hs	+ (13)	+	+	-	-	-	-
miR-15b	1	UAGCAGCACAUCAUGGUUUAC	21	Hs, Mm	+ (3)	-	-	-	-	+	-
miR-19a	4	UGUGCAAAUCUAUGCAAAACUGA	23	Hs	+ (13)	-	+	-	+	-	+
miR-19b	4	UGUGCAAAUCCAUGCAAAACUGA	23	Hs	+ (13, X)	-	+	-	-	-	+
miR-20	8	UAAAGUGCUUAUAGUGCAGGUAG	23	Hs	+ (13)	-	+	-	-	-	+
miR-21	1	AGUUAGCUUAUCAGACUGAUGUUGA	25	Hs	+ (13)	-	+	-	-	-	+
miR-27b	1	GUUCACAGUGGCUAAGUUCUG	21	Hs, Mm	+ (9)	-	+	-	-	+	+
miR-29a	1	UAGCACCAUCUGAAAUCGGUUA	22	Hs	+ (7.1, 7.2)	-	-	-	-	+	+
miR-29c	1	UAGCACCAUUUGAAAUCGGUUA	22	Hs	+ (1)	-	+	-	-	-	+
miR-91	5	CAAAGUGCUUACAGUGCAGGUAG	23	Hs	+ (13)	-	+	-	-	-	+
miR-92	3	UAUUGCACUUGUCCCGGCCUGU	22	Hs	+ (13, X)	-	+	+	-	-	+
miR-93	1	AAAGUGCUGUUCGUGCAGGUAG	22	Hs	+ (7.1, 7.2)	-	+	-	-	+	-
miR-94	1	UAAAGUGCUGACAGUGCAGAUA	22	Hs	+ (7.1, 7.2)	-	-	-	-	+	-
miR-106	1	AAAAGUGCUUACAGUGCAGGUAGC	24	Hs	+ (X)	-	+	-	-	-	-
miR-124a	3	UAAGGCACGCGGUGAAUGCCAA	22	Hs	+(8.1, 8.2, 20)	-	+	+	+	-	-
miR-172	2	UGGCAGUGUCUUAGCUGGUUGUU	23	Hs	+ (1)	-	+	-	-	+	-
miR-173	1	CACGGGGAGGUAGUGACGAAA	21	Hs	+ (2.1, 2.2)	-	-	+	-	-	+
miR-174	1	UAUGGCACUGGUAGAAUUCACU	22	Hs	+ (7)	+	+	+	-	-	-
miR-175	1	CAAGUCACUAGUGGUUCCGUUUA	23	Hs	+ (X)	-	-	+	-	+	-
miR-176	1	GGACUGCCUCAGCUGUGC	18	Hs	+ (2)	-	-	-	-	-	-
miR-177	1	GUGUCCUAAGGUGAGCUCAG	20	Hs	+ (8)	-	-	-	+	+	+
miR-178	1	AACAUUCAACGCUGUCGGUGAGUU	25	Hs, Mm	+ (1, 9)	-	+	-	-	+	+
miR-179	1	GGUUGAUAGGUCGGGGGUGUAA	22	Hs, Mm	-	-	-	-	-	-	-
miR-180	1	AUUUCAGGUGAAGUUUCAAGAGUC	24	Hs, Mm	-	-	-	-	-	-	-
miR-181	1	GUGCUAGGGAUUGGGGCUUG	20	Hs, Mm	-	-	-	-	-	+	+
miR-182a	1	CAAAGUGCUGUUAGUGCAGGUAG	23	Hs	-	-	+	-	-	-	+
miR-182b	-	CAAAGUGCUGUUAUUGCAGG	20	-	+ (11)	-	-	-	-	-	+
miR-183	2	ACUACGAAUGAUAACAUCCGUGG	23	Hs	-	-	-	-	-	-	-
miR-184	1	AUGCAAGUCGAGCUUGAAGUUUC	23	Hs	-	-	-	-	-	-	-
miR-185	1	AUCGUUAUUAUCGAUGGCGUGA	22	Hs	-	-	-	-	-	-	-
miR-186	1	CGGCCUUAGUCGUCGGGGUGAUU	23	Hs	-	-	-	-	-	-	-
miR-187	1	AGGAGCACAGCUGGGUAUCUAAGU	24	Hs	-	-	-	-	-	-	-
miR-188	1	CAACCUUGGGAUACCACCCUGUA	23	Hs	-	-	-	-	-	-	-
miR-189	1	CGUAGACCCGAAACCGGGUGAC	22	Hs	-	-	-	-	-	-	-
miR-190	1	UACCGCAUAAUGUUGAAAGAUGG	23	Hs	-	-	-	-	-	-	-
miR-191	1	CGGUUCAUACCCGAAGGGUCGCAA	24	Hs	-	-	-	-	-	-	-
miR-192	1	AUAACGUUGAAAGAUGGCAUC	21	Hs	-	-	-	-	-	-	-
miR-193	1	CGAGCCGUCGUAGACCACGACGUU	24	Hs	-	-	-	-	-	-	-
miR-194	1	GCCGUCGUCGACGAGUGCACUU	22	Hs	-	-	-	-	-	-	-
miR-195	1	CAAAGUGCUUACAGUUCAGGUAG	23	Hs	-	-	-	-	-	-	-
miR-196a	1	UAAGGGCUGGGUCCGUCGGGCU	22	Hs, Mm	-	-	-	-	-	-	-
miR-196b	-	UAAGGGCUGGGUCCGUCGGGCU	22	-	+ (13)	-	-	-	-	-	+
miR-196c	-	GGGCUGGGCCGGUCGGGC	18	-	+ (10)	-	-	-	-	-	-

The short (22–25 nt) RNAs associated with miRNPs in Weri cells were cloned as previously described (Elbashir et al. 2001), and a total of 64 clones were sequenced. **miRNAs** in bold letters were previously shown to associate with miRNPs in HeLa cells. The miRNA sequences are shown 5′ to 3′. The source (Hs; *Homo sapiens,* Mm; *Mus musculus*) indicates the species of the cell line from which the miRNAs were identified. In this column, - indicates miRNAs identified by sequence homology in the human genome. (Chr.); chromosomal localization of the miRNA in the human genome. Dr; *Danio rerio,* Fr; *Fugu rubripes,* Dm; *Drosophila melanogaster,* Ce; *Caenorhabditis elegans,* Mm; *Mus musculus,* Rn; *Rattus norvegicus.* + indicates fully conserved miRNAs, and + in red color represents conserved miRNAs containing one or two mismatches.

or in several species are often moderately conserved (data not shown). Seven miRNAs were identified in both Weri and MN-1 miRNPs (Table 1). Of these RNAs, three could not be mapped in either the human or mouse genome, and one, miRNA-181, is found in the mouse and rat genomes but not in the available sequences of the human genome. The miRNAs that have no database entry do not correspond to any known structural or messenger RNAs, and thus are most likely present in repetitive genomic regions or in genomic domains that are not yet fully sequenced.

Including the miRNAs found in both cell lines (Table 1), 40 different miRNAs were found associated with miRNPs in MN-1 cells (Table 2; Mourelatos et al. 2002). These include 10 miRNAs previously shown to be in miRNPs in HeLa cells (miRNAs in bold letters), and miR-23b and miR-26b that were recently described from total mouse RNA (Lagos-Quintana et al. 2002). We found 23 new miRNAs in the miRNPs from mouse neuronal cells, and identified two additional miRNAs by sequence homology in the human genome. Similarly to human miRNAs, most mouse sequences are conserved and can be localized by database search in the genome of at least one organism. The miRNAs that have not been localized in the human genome are either not present, not sufficiently conserved in human to be identified, or present in genomic regions that are not yet fully sequenced. Although several of the miRNAs were analyzed

TABLE 2. miRNA sequences associated with miRNPs in the MN-1 mouse neuronal cell line

miRNA	Number of clones	miRNA sequence	Size (nt)	Source	Predicted Precursors						
					Hs (Chr.)	Dr	Fr	Dm	Ce	Mm	Rn
miR-16	1	UAGCAGCACGUAAAUAUUGGCG	22	Mm	+ (3, 13)	-	+	-	-	+	-
miR-19a	1	UGUGCAAAUCUAUGCAAAACUGA	23	Mm	+ (13)	-	+	-	+	-	+
miR-19b	2	UGUGCAAAUCCAUGCAAAACUGA	23	Mm	+ (13, X)	-	+	-	-	-	+
miR-21	1	UAGCUUAUCAGACUGAUGUUGAC	22	Mm	+ (13)	-	+	-	-	-	+
miR-22	1	AAGCUGCCAGUUGAAGAACUG	21	Mm	+ (17)	-	-	-	-	+	+
miR-23b	2	AUCACAUUGCCAGGGAUUACCAC	23	Mm	+ (9)	-	+	-	-	-	-
miR-24	2	UGGCUCAGUUCAGCAGGAACAG	23	Mm	+ (9, 19)	-	+	-	-	+	+
miR-26b	1	UUCAAGUAAUUCAGGAUAGGUUU	23	Mm	+ (2)	-	+	-	-	+	+
miR-27	2	UUCACAGUGGCUAAGUUCCGC	21	Mm	+ (19)	-	+	-	-	+	+
miR-103	1	AGCAGCAUUGUACAGGGCUAUGA	23	Mm	+ (5, 10, 20)	-	+	-	-	+	+
miR-197	1	CAAAGAAUUCUCCUUUUGGGCUU	23	Mm	+ (1)	-	-	-	-	-	-
miR-198	1	ACUGGACUUGGAGUCAAAAGG	21	Mm	+ (4)	-	-	-	-	-	-
miR-199a	1	UGAUUCGGUGGGUGGUGGUGC	21	Mm	-	-	-	-	-	-	-
miR-199b	-	UGAUUCCGUGGGUGGUGGUGC	21	-	+ (19)	-	-	-	-	-	+
miR-199c	-	UGAUUCUGUGGGUGGUGGUGC	21	-	+ (11, 17)	-	-	-	-	+	+
miR-200	1	UGUAAACAUCCCCGACUGGAA	22	Mm	+ (8)	-	+	-	-	+	-
miR-201	1	CCUGGGUGCCAAGUUUUGAC	20	Mm	+ (4)	-	-	-	-	-	-
miR-202	1	CAGUGCAAUGUUAAAAGGGCAU	22	Mm	+ (11)	-	+	-	-	-	+
miR-203	1	GUGCUAGGAUUGGGGCU	17	Mm	+ (4)	-	-	-	-	-	+
miR-204	1	ACAGUAGUCUGCACAUUGGUUA	22	Mm	+ (1, 9.19)	-	-	-	-	+	+
miR-205	1	AGCCAAUGGUGCGAAGCUA	19	Mm	+ (8, 11, 17)	-	-	-	-	+	-
miR-206	1	UGUUGAAAAAGCAUGGGAUG	20	Mm	+ (13)	-	-	-	-	+	+
miR-207	1	UAAAGUGCUGACAGCUCAGAUA	22	Mm	-	-	-	-	-	-	-
miR-208	1	CCGUAGGCCGUUGAAGCGAUC	21	Mm	-	-	-	-	-	-	-
miR-209	1	UUAGUAUGGUUGCCUUCCAA	20	Mm	-	-	-	-	-	-	+
miR-210	1	UCGUCCCGAGACCGAUUAUUU	21	Mm	-	-	-	-	-	-	-
miR-211	1	GACCUGAGAGGGUGAUCGGCCAC	23	Mm	-	-	-	-	-	-	-
miR-212	1	AGGCAUGGCCAGGUUGAAGCGAGG	24	Mm	-	-	-	-	-	-	-
miR-213	1	GUACUUAGUAGAGCAGCCAC	20	Mm	-	-	-	-	-	-	-
miR-214	1	GGCGUAAAGGGAGCGUACGCGGAU	24	Mm	-	-	-	-	-	-	-
miR-215	1	AGACCCACCAGGCGUUCGGCC	21	Mm	-	-	-	-	-	-	-
miR-216	1	UUAGAUGAGAUAACAGGUUUCU	22	Mm	-	-	-	-	-	-	-
miR-217	1	CAAAGCAGCAGUAUCGCCU	19	Mm	-	-	-	-	-	-	-
miR-218	1	AUGCCUGUCGGUUACUGCCUGCU	23	Mm	-	-	-	-	-	-	+
miR-219	2	CAGACAGGGUGUACAUGACCUU	22	Mm	-	-	-	-	-	+	-

The short (22–25 nt) RNAs associated with miRNPs in MN-1 cells were cloned as previously described (Elbashir et al. 2001), and a total of 43 clones were sequenced. **miRNAs** in bold letters were previously shown to associate with miRNPs in HeLa cells. The miRNA sequences are shown 5′ to 3′. The source (Hs; *Homo sapiens,* Mm; *Mus musculus*) indicates the species of the cell line from which the miRNAs were identified. In this column, - indicates miRNAs identified by sequence homology in the human genome. (Chr.); chromosomal localization of the miRNA in the human genome. Dr; *Danio rerio,* Fr; *Fugu rubripes,* Dm; *Drosophila melanogaster,* Ce; *Caenorhabditis elegans,* Mm; *Mus musculus,* Rn; *Rattus norvegicus.* + indicates fully conserved miRNAs, and + in red color represents conserved miRNAs containing one or two mismatches.

by northern blotting, it is not certain whether these are novel neuronal-specific or ubiquitously miRNAs. The miRNAs with an entry in the human genome database are predicted to fold into stem-loop structures with surrounding genomic sequences (Fig. 2). The predicted secondary structure of the miRNA precursors localized on more than one chromosome or that can be mapped in the genome of other species is often partially conserved (data not shown). Two of the miRNAs, miR-23b and miR-27b, were found as part of a novel cluster in the human genome on chromosome 9 (Fig. 3A; solid boxes). In addition, two computer-predicted putative miRNA precursors were found surrounding miR-27b (dashed boxes in Fig. 3).

Interestingly, miR-175 from Weri cells, which is located on the X chromosome in humans and is conserved in *D. melanogaster* and *M. musculus*, was also found as part of a longer human Expressed Sequence Tag (EST). This EST contains the miR-175 miRNA precursor and possibly another miRNA precursor that was computer-predicted, and encodes a putative isoform of the epsilon subunit of the gamma-aminobutyric acid (GABA) A receptor (Fig. 3A; Wilke et al. 1997). This receptor is a multisubunit chloride channel that inhibits synaptic transmission in the central nervous system. The gene locus of the epsilon subunit is a candidate region for two neurologic diseases: early-onset parkinsonism (Waisman syndrome; Gregg et al. 1991), and X-linked mental retardation (MRX3; Gedeon et al. 1991). Thus, it will be of interest to determine whether miR-175 synthesis or activity are affected and whether deregulation of this miRNA plays a role in the development of these diseases.

Two distinct subfamilies of miRNAs associate with miRNPs in neuronal cells

Analysis of the miRNPs indicates that they contain a large family of highly conserved miRNAs in this cell type (Fig. 3B). This miRNA family encompasses at least nine mem-

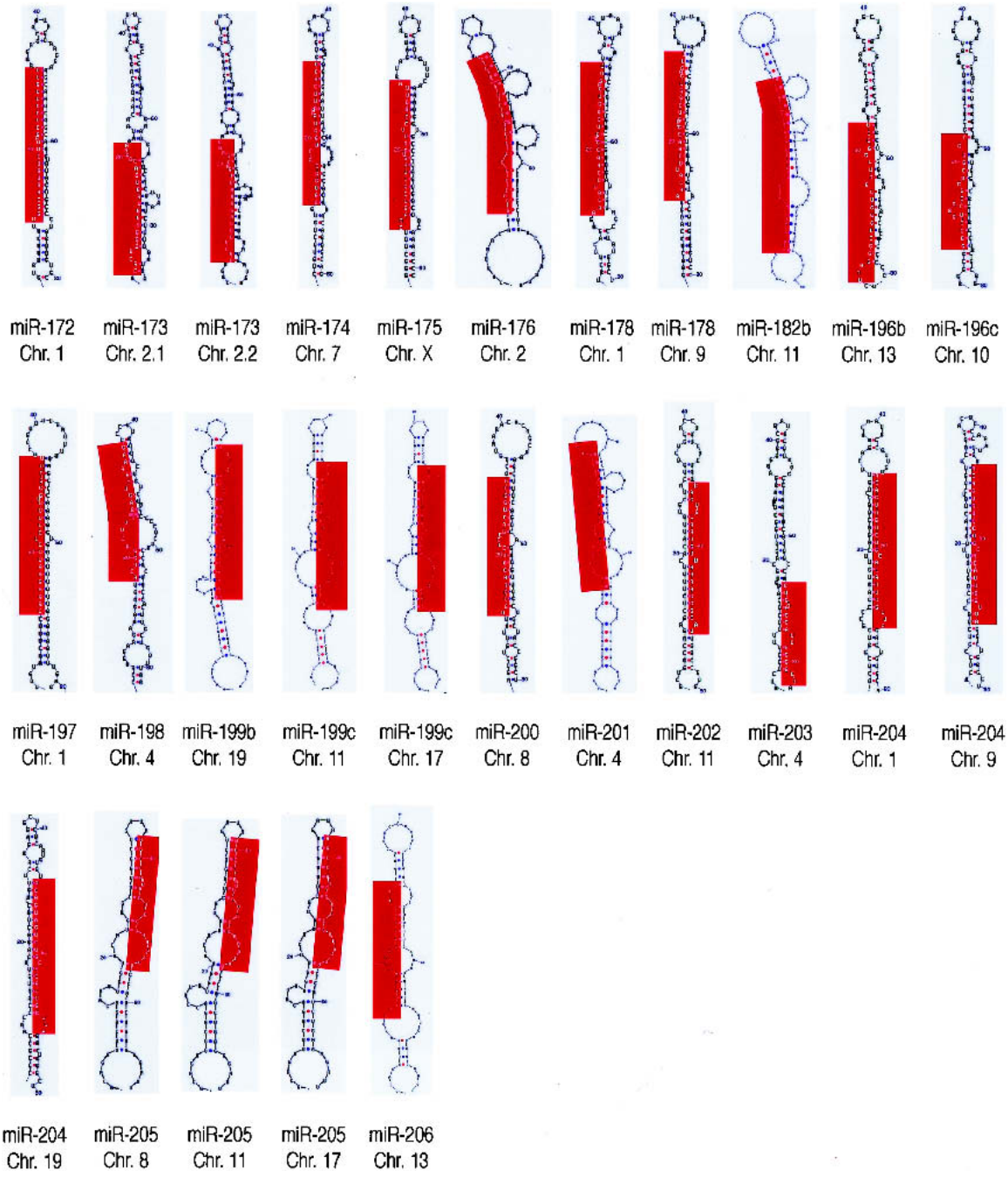

FIGURE 2. Predicted secondary structures of the novel human miRNA putative precursors. Human genomic sequences upstream and downstream of the novel miRNAs were folded with the computer program mfold. Predicted secondary structures are shown 5′ to 3′. Red areas represent the mature miRNA. Chromosome number represents the localization of the miRNA in the human genome.

bers, including miR-182a, miR-182b, miR-188, and miR-207, which are described herein. This result suggests that these miRNPs may play an important role in the maturation and/or delivery of their miRNAs to a common class of putative RNA targets in neuronal cells. In addition, several miRNAs with homologous 5′ ends were found in miRNPs (Fig. 3C). Most members of this miRNA class were associated with miRNPs identified from the neuronal cells, including the novel miR-29c, miR-187, and miR-217. The 5′ end of several miRNAs was recently found to be complementary to the K box, Brd box, and GY box motifs found in the 3′ UTR of basic helix-loop-helix (bHLH) repressors and the Bearded (Brd) family members (Lai 2002). The K and Brd box sequences mediate negative posttranscriptional

mRNA regulation by affecting the polyadenylation status of transcripts, resulting in decreased stability and translational efficiency of the mRNA (Lai and Posakony 1997; Lai et al. 1998). Thus, the identification of these miRNA families should help in the identification of putative mRNA targets and in the elucidation of their functions.

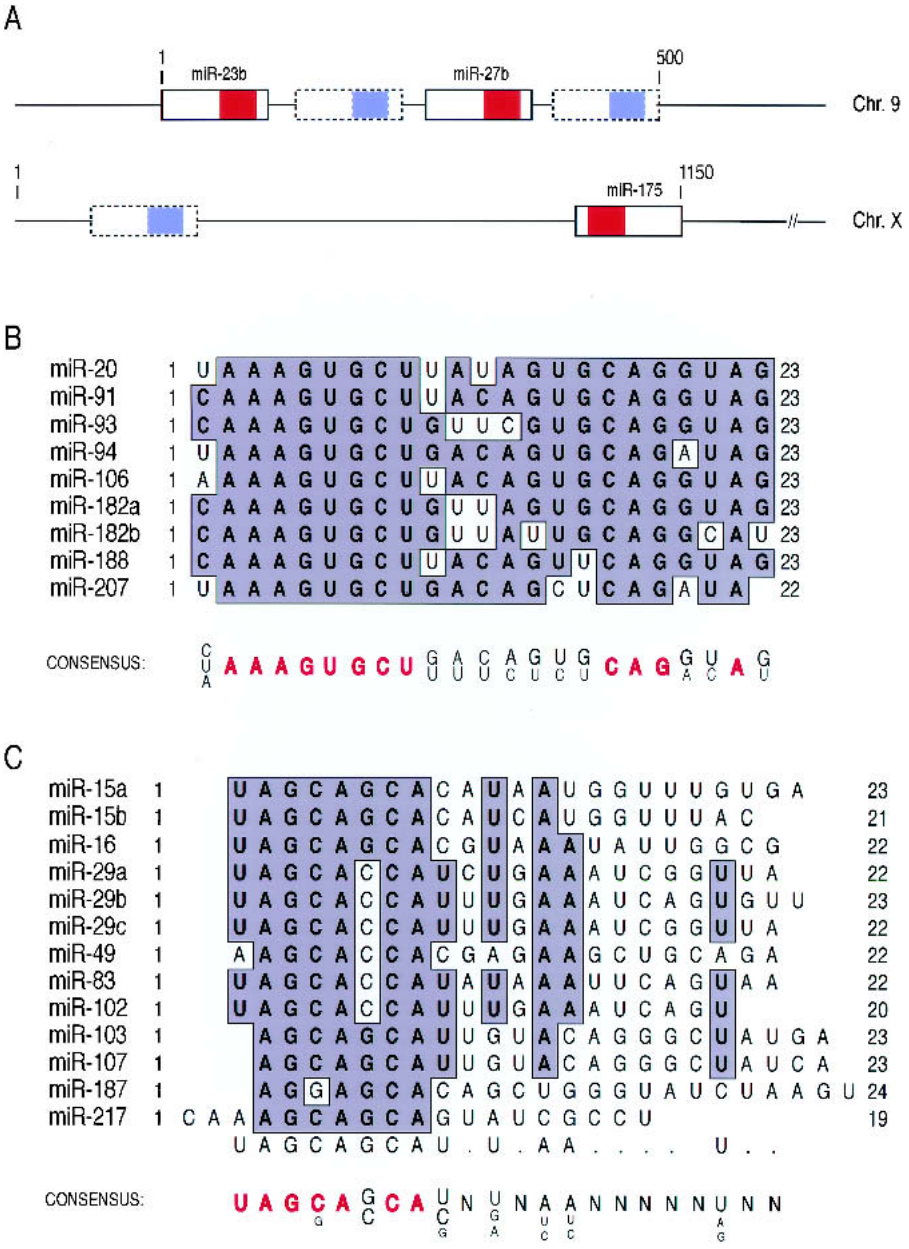

FIGURE 3. Novel miRNA clusters and families of miRNA paralogs associated with miRNPs in neuronal cells. (*A*) Novel miRNA clusters. The putative precursors of identified miRNAs are presented as solid boxes, and the mature miRNAs identified in the study are shown in red. Computer-predicted putative miRNA precursors are presented as dashed boxes, and the predicted mature miRNAs are shown in light blue. The chromosomal localization in the human genome is indicated on the *right*. The size of the region containing the miRNAs is indicated at the *top* of each cluster. (*B*) miRNPs contain a conserved miRNA family in neuronal cells. miRNA family members associated with miRNPs in neuronal cells are aligned. The names of miRNAs are indicated on the *left*. miRNA length is shown at the end of each miRNA. Conserved residues are in bold letters and gray shaded areas. Consensus is shown at the *bottom*: Nucleotides shown in red were invariable. (*C*) miRNPs contain a class of miRNAs with homologous 5′ ends in neuronal cells. miRNA class members with homologous 5′ ends are aligned. The names of miRNAs are indicated on the *left*. miRNA length is shown at the *end* of each miRNA. Conserved residues are in bold letters and gray shaded areas. Consensus is shown at the *bottom*: Nucleotides shown in red were invariable.

MATERIALS AND METHODS

Cell culture and cell extract preparation

MN-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; complete DMEM). Weri cells were grown in DMEM supplemented with 10% fetal bovine serum. At 80% confluence, cells (1×10^7) were collected by centrifugation at 1000 *g* for 15 min at 4°C. The cell pellet was washed with cold phosphate-buffered saline (PBS), and with RSB 200 (20 mM Tris-HCl pH 7.4, 2.5 mM magnesium chloride, 200 mM sodium chloride). Cells were resuspended in one volume of lysis buffer (RSB 200 containing 0.05% NP-40, RNAsin (1 U/μL) and protease inhibitors (Complete™; Roche), and disrupted by brief sonication. Extract was clarified by centrifugation at 11,000 *g* for 15 min at 4°C.

RNA immunoprecipitation, 3′-end labeling, and miRNA cloning

For immunoprecipitation, cell extract was incubated for 1 h at 4°C with either normal mouse serum (NMS) as control, or with anti-Gemin3 (11G9) antibodies immobilized on GammaBind™ G Sepharose™ (Amersham Pharmacia Biotech). Immunoprecipitates were washed five times with lysis buffer, treated with DNAse I (0.5 U/μL; Roche) for 15 min at 30°C, followed by proteinase K digestion (0.2 μg/μL; Roche) for 30 min at 37°C. RNA was extracted with phenol followed by two phenol/chloroform extractions and ethanol precipitation. The RNA was 3′-end labeled with [5′-^{32}P]-pCp and analyzed on a denaturing 15% polyacrylamide gel. The ~22-nt miRNAs were cloned by using the protocol developed by Elbashir and coworkers (2001).

RNA analysis

miRNA sequences were determined by automated DNA-cycle sequencing. Chromosomal locations and genomic se-

quences of the novel miRNAs were identified by searching various genomes with the Basic Local Alignment Sequence Tool (BLAST®) algorithm, available at the National Center for Biotechnology Information (NCBI). Secondary structures of the likely precursors of the novel miRNAs were predicted by folding upstream and downstream genomic miRNA sequences with the computer program mfold (Zuker et al. 1999). miRNA alignments were established with the macvector program.

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REFERENCES

Ambros, V. and Horvitz, H.R. 1984. Heterochronic mutants of the nematode *Caenorhabditis elegans*. *Science* **226:** 409–416.

Baccon, J., Pellizzoni, L., Rappsilber, J., Mann, M., and Dreyfuss, G. 2002. Identification and characterization of Gemin7, a novel component of the SMN complex. *J. Biol. Chem.* **277:** 31957–31962.

Brooks, B.P., Paulson, H.L., Merry, D.E., Salazar-Grueso, E.F., Brinkmann, A.O., Wilson, E.M., and Fischbeck, K.H. 1997. Characterization of an expanded glutamine repeat androgen receptor in a neuronal cell culture system. *Neurobiol. Dis.* **3:** 313–323.

Charroux, B., Pellizzoni, L., Perkinson, R.A., Shevchenko, A., Mann, M., and Dreyfuss, G. 1999. Gemin3: A novel DEAD box protein that interacts with SMN, the spinal muscular atrophy gene product, and is a component of gems. *J. Cell Biol.* **147:** 1181–1194.

Charroux, B., Pellizzoni, L., Perkinson, R.A., Yong, J., Shevchenko, A., Mann, M., and Dreyfuss, G. 2000. Gemin4. A novel component of the SMN complex that is found in both gems and nucleoli. *J. Cell Biol.* **148:** 1177–1186.

Elbashir, S.M., Lendeckel, W., and Tuschl, T. 2001. RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes & Dev.* **15:** 188–200.

Fischer, U., Liu, Q., and Dreyfuss, G. 1997. The SMN-SIP1 complex has an essential role in spliceosomal snRNP biogenesis. *Cell* **90:** 1023–1029.

Gedeon, A., Kerr, B., Mulley, J., and Turner, G. 1991. Localisation of the MRX3 gene for non-specific X linked mental retardation. *J. Med. Genet.* **28:** 372–377.

Gregg, R.G., Metzenberg, A.B., Hogan, K., Sekhon, G., and Laxova, R. 1991. Waisman syndrome, a human X-linked recessive basal ganglia disorder with mental retardation: Localization to Xq27.3-qter. *Genomics* **9:** 701–706.

Gubitz, A.K., Mourelatos, Z., Abel, L., Rappsilber, J., Mann, M., and Dreyfuss, G. 2002. Gemin5, a novel WD repeat protein component of the SMN complex that binds Sm proteins. *J. Biol. Chem.* **277:** 5631–5636.

Hutvagner, G., McLachlan, J., Pasquinelli, A.E., Balint, E., Tuschl, T., and Zamore, P.D. 2001. A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. *Science* **293:** 834–838.

Jones, K.W., Gorzynski, K., Hales, C.M., Fischer, U., Badbanchi, F., Terns, R.M., and Terns, M.P. 2001. Direct interaction of the spinal muscular atrophy disease protein SMN with the small nucleolar RNA-associated protein fibrillarin. *J. Biol. Chem.* **276:** 38645–38651.

Lagos-Quintana, M., Rauhut, R., Lendeckel, W., and Tuschl, T. 2001. Identification of novel genes coding for small expressed RNAs. *Science* **294:** 853–858.

Lagos-Quintana, M., Rauhut, R., Yalcin, A., Meyer, J., Lendeckel, W., and Tuschl, T. 2002. Identification of tissue-specific microRNAs from mouse. *Curr. Biol.* **12:** 735–739.

Lai, E.C. 2002. Micro RNAs are complementary to 3′ UTR sequence motifs that mediate negative post-transcriptional regulation. *Nat. Genet.* **30:** 363–364.

Lai, E.C. and Posakony, J.W. 1997. The Bearded box, a novel 3′ UTR sequence motif, mediates negative post-transcriptional regulation of Bearded and Enhancer of split Complex gene expression. *Development* **124:** 4847–4856.

Lai, E.C., Burks, C., and Posakony, J.W. 1998. The K box, a conserved 3′ UTR sequence motif, negatively regulates accumulation of enhancer of split complex transcripts. *Development* **125:** 4077–4088.

Lau, N.C., Lim, L.P., Weinstein, E.G., and Bartel, D.P. 2001. An abundant class of tiny RNAs with probable regulatory roles in *Caenorhabditis elegans*. *Science* **294:** 858–862.

Lee, R.C. and Ambros, V. 2001. An extensive class of small RNAs in *Caenorhabditis elegans*. *Science* **294:** 862–864.

Liu, Q., Fischer, U., Wang, F., and Dreyfuss, G. 1997. The spinal muscular atrophy disease gene product, SMN, and its associated protein SIP1 are in a complex with spliceosomal snRNP proteins. *Cell* **90:** 1013–1021.

McFall, R.C., Sery, T.W., and Makadon, M. 1977. Characterization of a new continuous cell line derived from a human retinoblastoma. *Cancer Res.* **37:** 1003–1010.

Meister, G., Eggert, C., Buhler, D., Brahms, H., Kambach, C., and Fischer, U. 2001. Methylation of Sm proteins by a complex containing PRMT5 and the putative U snRNP assembly factor pICln. *Curr. Biol.* **11:** 1990–1994.

Melki, J. 1997. Spinal muscular atrophy. *Curr. Opin. Neurol.* **10:** 381–385.

Mourelatos, Z., Abel, L., Yong, J., Kataoka, N., and Dreyfuss, G. 2001. SMN interacts with a novel family of hnRNP and spliceosomal proteins. *EMBO J.* **20:** 5443–5452.

Mourelatos, Z., Dostie, J., Paushkin, S., Sharma, A., Charroux, B., Abel, L., Rappsilber, J., Mann, M., and Dreyfuss, G. 2002. miRNPs: A novel class of ribonucleoproteins containing numerous microRNAs. *Genes & Dev.* **16:** 720–728.

Paushkin, S., Gubitz, A.K., Massenet, S., and Dreyfuss, G. 2002. The SMN complex, an assemblyosome of ribonucleoproteins. *Curr. Opin. Cell Biol.* **14:** 305–312.

Pellizzoni, L., Kataoka, N., Charroux, B., and Dreyfuss, G. 1998. A novel function for SMN, the spinal muscular atrophy disease gene product, in pre-mRNA splicing. *Cell* **95:** 615–624.

Pellizzoni, L., Baccon, J., Charroux, B., and Dreyfuss, G. 2001a. The survival of motor neurons (SMN) protein interacts with the snoRNP proteins fibrillarin and GAR1. *Curr. Biol.* **11:** 1079–1088.

Pellizzoni, L., Baccon, J., Rappsilber, J., Mann, M., and Dreyfuss, G. 2002. Purification of native survival of motor neurons complexes and identification of Gemin6 as a novel component. *J. Biol. Chem.* **277:** 7540–7545.

Pellizzoni, L., Charroux, B., Rappsilber, J., Mann, M., and Dreyfuss, G. 2001b. A functional interaction between the survival motor neuron complex and RNA polymerase II. *J. Cell Biol.* **152:** 75–85.

Reinhart, B.J., Slack, F.J., Basson, M., Pasquinelli, A.E., Bettinger, J.C., Rougvie, A.E., Horvitz, H.R., and Ruvkun, G. 2000. The 21-nucleotide let-7 RNA regulates developmental timing in *Caenorhabditis elegans*. *Nature* **403:** 901–906.

Salazar-Grueso, E.F., Kim, S., Kim, H. 1991. Embryonic mouse spinal cord motor neuron hybrid cells. *Neuroreport* **2:** 505–508.

Wilke, K., Gaul, R., Klauck, S.M., and Poustka, A. 1997. A gene in human chromosome band Xq28 (GABRE) defines a putative new subunit class of the GABAA neurotransmitter receptor. *Genomics* **45:** 1–10.

Zuker, M., Mathews, D.H., and Turner, D.H. 1999. *Algorithms and thermodynamics for RNA secondary structure prediction: A practical guide.* Kluwer Academic, Dordrecht, The Netherlands.