A Large Imprinted microRNA Gene Cluster at the Mouse Dlk1-Gtl2 Domain

Hervé Seitz, Hélène Royo, Marie-Line Bortolin, et al.

*Genome Res.* 2004 14: 1741-1748
Access the most recent version at doi:10.1101/gr.2743304

**Supplemental Material**  
http://genome.cshlp.org/content/suppl/2004/08/13/gr.2743304.DC1.html

**References**  
This article cites 66 articles, 31 of which can be accessed free at:  
http://genome.cshlp.org/content/14/9/1741.full.html#ref-list-1

Article cited in:  
http://genome.cshlp.org/content/14/9/1741.full.html#related-urls

**Email alerting service**  
Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or click here

---

To subscribe to *Genome Research* go to:  
http://genome.cshlp.org/subscriptions

---

Cold Spring Harbor Laboratory Press
A Large Imprinted microRNA Gene Cluster at the Mouse Dlk1-Gtl2 Domain

Hervé Seitz, Hélène Royo, Marie-Line Bortolin, Shau-Ping Lin, Anne C. Ferguson-Smith, and Jérôme Cavaille

Over 200 distinct genes encoding 21- to 25-nucleotide (nt)-long noncoding RNAs (miRNAs) have been identified through either computer-assisted approaches or cdNA cloning strategies in many organisms, including worm, plants, flies, mouse, and human (Lai 2003; Bartel 2004). They are encoded within irregular hairpins and generated by a two-step mechanism involving Drosha and Dicer, two distinct dsRNA-specific ribonucleases belonging to the RNAse III family. First, Drosha cleaves a larger RNA precursor (the pri-miRNA) in the nucleus to release RNAs encoding 70-nt-long hairpins. Second, Dicer excises these precursors to generate 21- to 25-nt-long miRNAs that are incorporated into the RISC/miRNP complexes containing proteins belonging to the Argonaute family (Bernstein et al. 2001; Hutvagner et al. 2001; Ketting et al. 2001). Then, the mature 21- to 23-nt-long miRNAs are transported from the cytoplasm to the nucleus, where they guide the RISC/miRNP complexes to their targets, leading to either RNA degradation (RNA interference or RNAi) or translation repression (Hutvagner and Zamore 2002; Reinhart et al. 2002; Llave et al. 2003). Indeed, miRNAs perfectly complementarily to the RNA target guide sequence-specific RNA cleavages (they function like small interfering RNAs or siRNAs), whereas miRNAs imperfectly complementarily to the target repress its translation by an as-yet-unknown mechanism.

In plants, miRNAs exhibit perfect or near-perfect complementarities with their targets, and they can either guide miRNA degradation (Llave et al. 2002; Palatnik et al. 2003; Tang et al. 2003; Xie et al. 2003) or inhibit their translation (Aukerman and Sakai 2003; Chen 2004). Remarkably, most of predicted target miRNAs encode for transcription factors known to regulate leaf and flower development, embryonic patterning, and timing transition to flowering (Rhoades et al. 2002). By contrast, animal miRNAs seem to act predominantly as translational repressors, and they have been shown to play a key role in the establishment of temporal and spatial gene expression patterns (Lee et al. 1993; Reinhart et al. 2000; Johnston and Hobert 2003), in the regulation of tissue growth, apoptosis, and fat metabolism (Brennecke et al. 2003; Xu et al. 2003) and in the differentiation of hematopoietic lineages (Chen et al. 2004). However, although many computationally predicted miRNA targets have been recently reported in flies and in mammals (Enright et al. 2003; Lewis et al. 2003; Stark et al. 2003), only a very few miRNA targets have been experimentally tested in vivo, and other RNA-mediated gene silencing mechanisms can also be envisaged. In this regard, RNAi-related mechanisms are also required for the silencing of heterochromatin in Schizosaccharomyces pombe (Hall et al. 2002; Reinhart and Bartel 2002; Volpe et al. 2002; Schramke and Allshire 2003; Verdelet et al. 2004), for the programmed chromosomal rearrangements in Tetrahymena (Yao et al. 2003) and for the transcriptional gene silencing in plants and in Drosophila (Mette et al. 2000; Hamilton et al. 2002; Pal-Bhadra et al. 2002, 2004; Zilberman et al. 2003). Thus, 21- to 25-nt-long RNAs are involved in a range of eukaryotic gene regulation phenomena, and their potential as modulators of gene expression is just beginning to be appreciated.

Genomic imprinting refers to an epigenetic phenomenon by which a subset of mammalian autosomal genes, the so-called imprinted genes, are expressed in a parent-of-origin specific manner (only one of the two parental alleles is expressed). Most of these imprinted genes are clustered within large chromosomal domains harboring both paternally and maternally expressed imprinted genes. Molecular mechanisms underlying genomic imprinting are not fully understood but include allele-specific DNA methylation and core histone modifications, cis-acting elements...
Multiple sequence alignments revealed that 15 predicted miRNA genes have sequences related to the previously described mir-154 gene precursor (Lagos-Quintana et al. 2002; Seitz et al. 2003), and a subsequent BLAST search with lower stringency conditions allowed us to identify eight additional human mir-154-related sequences (Figs. 2B, 3A), although six do not have clear mouse counterparts. These mir-154-related sequences have been grouped into the A-type family (A1 to A24 gene copies). Interestingly, within the same 40-kb interval other hairpins could also be ranked together into families of closely related sequences: the B-type family (six and three gene copies in human and mouse, respectively) and the C-type family (four gene copies both in human and in mouse; Figs. 2B, 3B,C). Thus, overall a total of 40 conserved in silico–predicted human miRNA genes, all of them mapping in the same relative orientation, have been isolated downstream of the C/D snoRNA gene cluster (Fig. 2B), and to our knowledge, they represent the largest cluster of miRNA genes described to date. A-, B-, and C-type pre-miRNA sequences appear to be related to each other (particularly at their terminal 5’ and 3’ ends; data not shown), and this might suggest that the three miRNA families have evolved through duplications from a common miRNA gene ancestor.

**Experimental Detection of the In Silico-Predicted miRNAs**

The expression of all the predicted miRNA species was first monitored in mouse by Northern blot analysis with oligonucleotide probes complementary to either the 5’ or the 3’ arm of the predicted fold-back structures. We failed to detect convincing signals for most of them, including mir-154 and mir-134, which were originally cloned from cDNA libraries (Lagos-Quintana et al. 2002; data not shown). Indeed, only miR-C3, miR-A24, and B-type miRNAs gave positive signals in total RNA isolated from whole embryo or adult brain (data not shown). Nevertheless, based on our current knowledge of the relative position of the mature miRNA within the pre-miRNA (Lim et al. 2003), we designed a novel specific set of primers for several miRNAs distributed along the cluster (Supplemental data S1) and performed a more sensitive primer extension analysis. By using this approach, we mapped the 5’ end of small RNA species encoded within pre-miR-22 (3’ arm), pre-miR-A24 (3’ arm), pre-miR-B4 (3’ arm), pre-miR-B6 (3’ arm), pre-miR-C2 (3’ arm), pre-miR-C3 (3’ arm), pre-miR-154 gene (5’ arm), pre-miR-134 (5’ arm), pre-miR-K (3’ arm), pre-miR-M (3’ probe), and pre-miR-N (3’ probe; Table 1; data not shown). Because of potential cross-hybridization between related miRNA sequences, we have been unable to test unambiguously each miRNA gene candidate, hence the possibility that some of the in silico–predicted miRNA variants might not be expressed cannot be formally ruled out. However, three re-
Based on conservation criteria between human and fish that these miRNA sequences are specific to mammalian species. Caenorhabditis elegans shown). BLAST searches also failed to detect significant hits in baviy et al. (2003).

Recently cloned miRNAs—miR-323, miR-329, and miR-300—are encoded within the 3’ strand of the A1, A2, and A9 pre-miRNA gene precursors (Houbaviy et al. 2003; Kim et al. 2004), respectively, and miRNAs encoded by a single-copy gene (miR-D, G, H, I, K) are indicated as black arrows. Note that four miRNA genes—miR-A22, miR-K, miR-A23, and miR-A24—are embedded within a conserved CpG island (indicated by a gray rectangle) from which the most conserved sequences lie within the pre-miRNA genes (data not shown). Dotted lines denote splicing events identified by ESTs analysis. Numbering indicates the relative position of the miRNA gene cluster (in nucleotides) within the BAC AL132709 sequence. The picture is drawn to scale.

**Figure 2** Identification of computationally predicted miRNA genes at the human imprinted 14q32 domain. (A) Distribution of the conserved hairpins between human and mouse along the 1-Mb-long imprinted 14q32 domain. Vertical bars indicate the number of in silico–predicted hairpins found within each 50-kb-long region. Blue bars indicate the number of human hairpins at the 14q32 domain; red bars, the number of conserved hairpins between mouse and human; and yellow bars, the number of conserved hairpins giving rise to a miRscan score >10 (Lim et al. 2003). The relative positions of the overlapping human BACs covering the analyzed domain are indicated below the histogram. (B) A large cluster of miRNA genes mapping downstream from the C/D snoRNA gene cluster. miRNA genes belonging to the A-, B-, and C-types are represented by green, red, and blue arrows, respectively, and miRNAs encoded by a single-copy gene (miR-D, G, H, I, K) are indicated as black arrows. Note that four miRNA genes—miR-A22, miR-K, miR-A23, and miR-A24—are embedded within a conserved CpG island (indicated by a gray rectangle) from which the most conserved sequences lie within the pre-miRNA genes (data not shown). Dotted lines denote splicing events identified by ESTs analysis. Numbering indicates the relative position of the miRNA gene cluster (in nucleotides) within the BAC AL132709 sequence. The picture is drawn to scale.


Because of their location within a previously characterized imprinted domain, we next examined whether the novel miRNA genes are subjected to genomic imprinting. As shown in Figure 4C, E15.5 embryos with a maternal uniparental disomy of chromosome 12 (matUPD12) express all the miRNAs that we have tested, whereas embryos having two paternal chromosome 12 copies (patUPD12) do not, showing that these miRNA genes are expressed at roughly the same levels not only in the head but also in the trunk and the placenta, indicating that other tissues distinct from the brain express them as well (Fig. 4A; data not shown). In neonates (P0, P2, P7), miRNAs are still detected in the trunk, even though we noticed a reproducible decrease in the expression level for most of them after birth (Fig. 4B).

Curiously, although each primer extension product generally appears as a band doublet for a given miRNA, for samples extracted from the head or the trunk in neonates, several miRNAs exhibit a different intensity ratio between the two vicinal cDNA bands. The biological relevance of this difference, if any, remains unclear (see also Table 1). Further studies are needed to identify cell type–specific expression patterns as well as mechanisms of miRNA gene regulation.
though we can not exclude that these observations have a biological significance, we believe that our primer extension assay is not quantitative enough to reproducibly observe the expected twofold increase in the matUPD embryos. An IG-DMR located between the \( \text{Dlk1} \) and \( \text{Gtl2} \) genes has been recently shown to play a key role in controlling imprinted expression within the whole locus (Fig. 1). Deletion of the unmethylated IG-DMR from the maternally inherited chromosome results in the silencing of all the maternally expressed imprinted genes together with loss of imprinting (biallelic expression) of the maternally repressed genes. When inherited from the paternal allele, the deletion of the methylated IG-DMR does not affect imprinted gene expression (Lin et al. 2003). All the miRNAs that we have checked were absent from total RNA extracted from embryos having a maternal deletion of the IG-DMR, whereas those with the paternally inherited deletion had miRNA expression similar to that of the control unmanipulated littermates (Fig. 4D). We therefore conclude that, similar to the other maternally expressed noncoding genes (anti-\( \text{Rtl1} \), \( \text{Gtl2} \), and the C/D snoRNA genes; Lin et al. 2003), the expression of the miRNA gene cluster is controlled by this distal cis-acting element.

**The miRNAs Are Processed From Large Noncoding RNAs**

All the miRNA genes described in this study share the same tissue-specific expression pattern with strongest expression in the adult brain. A similar gene expression pattern has been previously reported for the other neighboring maternally expressed noncoding RNA genes (\( \text{Gtl2} \) and C/D RNA genes; Schmidt et al. 2000; Cavaille et al. 2002). Very little is known about miRNA transcription and its regulation in mammals (Bartel 2004). Although a few of these clustered miRNA genes are embedded within introns or exons of \( \text{Mirg} \), a maternally expressed gene lacking a conserved protein-coding potential (Seitz et al. 2003), the genomic organization of most of them is still unclear (Figs. 2B, 5). Each pre-miRNA gene might be transcribed from its own tissue-specific promoter or alternatively it might be processed from (some) polycistronic-like RNA(s) as already reported for other clustered miRNAs (Lee et al. 2002). To address this second possibility, we have used RT-PCR to detect primary precursors encompassing several miRNAs relatively close to each other. As shown in Figure 5, transcripts encompassing several neighboring miRNAs are readily detected. Although we cannot rule out that some miRNA genes are transcribed from their own promoter, it seems more likely that most, if not all, pre-miRNA genes are generated through RNA processing either from polycistronic RNA and/or from intronic sequences rather than being individually expressed as \(~70\)-nt-long primary transcripts. Thus, the novel clustered miRNA genes share a number of common properties with the upstream C/D snoRNA genes (Cavaille et al. 2002). Both classes of small noncoding RNA genes are (1) relatively weakly conserved during evolution, (2) organized as repeated arrays and processed from larger noncoding RNA genes, (3) transcribed in the same direction from the maternal chromosome only, (4) regulated by the IG-DMR, and (5) expressed in a tissue-specific manner with strongest expression in the adult brain.
brain. Thus, it is tempting to speculate that C/D RNAs and miRNAs are processed from a single, large primary transcript that might initiate at the Gtl2 promoter. Future work is now needed to fully characterize this atypical and complex transcription unit.

**DISCUSSION**

Most imprinted genes play important roles in embryonic and/or placental growth (Tycko and Morison 2002) and developmental abnormalities in mouse and human have been previously associated with the lack of expression of the maternally inherited genes at distal 12 and 14q32, respectively (Georgiades et al. 2000; Kurosawa et al. 2002). Given the known roles of miRNAs in development (Carrington and Ambros 2003), it is likely that these novel imprinted miRNAs mediate a function during mouse development. In contrast to the mir-127 and mir-136 genes encoded in the antisense strand of the 

---

**Table 1. List of Experimentally Detected or Cloned miRNAs at the Mouse Distal 12 Domain**

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Sequence (5’ to 3’)</th>
<th>Maternally expressed miRNAs</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-342</td>
<td>UCCUCAACAGAAAUCUCCACCUUC</td>
<td>n.d.</td>
<td>Kim et al. 2004; this study</td>
</tr>
<tr>
<td>miR-345</td>
<td>UCCUCAACAGAAAUCUCCACCUUC</td>
<td>n.d.</td>
<td>Kim et al. 2004; this study</td>
</tr>
<tr>
<td>miR-127</td>
<td>UCCUCAACAGAAAUCUCCACCUUC</td>
<td>+</td>
<td>Lagos-Quintana et al. 2002; Seitz et al. 2003</td>
</tr>
<tr>
<td>miR-136</td>
<td>ACICCAACAUUUGUUGUUGUGAAGAA</td>
<td>+</td>
<td>Lagos-Quintana et al. 2002; Seitz et al. 2003</td>
</tr>
<tr>
<td>miR-134</td>
<td>ACICCAACAUUUGUUGUUGUGAAGAA</td>
<td>+</td>
<td>Lagos-Quintana et al. 2002; this study</td>
</tr>
<tr>
<td>miR-154/mir-A19-5’</td>
<td>UAUGUUGUAUGGUUCACAUCUU</td>
<td>+</td>
<td>Lagos-Quintana et al. 2002; this study</td>
</tr>
<tr>
<td>miR-323/mir-A1-3’</td>
<td>GCACAUUUGUUGUUGUUGUGAAGAA</td>
<td>n.d.</td>
<td>Kim et al. 2004; this study</td>
</tr>
<tr>
<td>miR-329/mir-A2-3’</td>
<td>AACACACCCACACUUCACUUCUU</td>
<td>n.d.</td>
<td>Kim et al. 2004; this study</td>
</tr>
<tr>
<td>miR-300/mir-A9-3’</td>
<td>UAICCAACAGAAAUCUCCACCUUC</td>
<td>n.d.</td>
<td>Houbaviy et al. 2003; this study</td>
</tr>
<tr>
<td>miR-409/mir-A22-3’</td>
<td>GAACAUUUGUUGUUGUUGUGAAGAA</td>
<td>n.d.</td>
<td>this study</td>
</tr>
<tr>
<td>miR-410/mir-A24-3’</td>
<td>AAUAUAACACAGAUGGCCUGUU</td>
<td>+</td>
<td>this study</td>
</tr>
<tr>
<td>miR-376/mir-B4</td>
<td>UACUGUUGUUGUUGUUGUGAAGAA</td>
<td>+</td>
<td>this study</td>
</tr>
<tr>
<td>miR-376/mir-B6</td>
<td>UACUGUUGUUGUUGUUGUGAAGAA</td>
<td>+</td>
<td>this study</td>
</tr>
<tr>
<td>miR-411/mir-C2</td>
<td>AACACACCCACACUUCACUUCUU</td>
<td>+</td>
<td>this study</td>
</tr>
<tr>
<td>miR-380-3p/mir-C3</td>
<td>AAUAUAACACAGAUGGCCUGUU</td>
<td>+</td>
<td>this study</td>
</tr>
<tr>
<td>miR-299/mir-D</td>
<td>UGACUAUCACACCCACACUUCACUUCUU</td>
<td>n.d.</td>
<td>Houbaviy et al. 2003; this study</td>
</tr>
<tr>
<td>miR-412/mir-K</td>
<td>ACICCAACAUUUGUUGUUGUGAAGAA</td>
<td>n.d.</td>
<td>this study</td>
</tr>
<tr>
<td>miR-337/mir-M</td>
<td>UACACCCACACACUUCACUUCUU</td>
<td>+</td>
<td>Kim et al. 2004; this study</td>
</tr>
<tr>
<td>miR-370/mir-N4</td>
<td>GCCGCCCGCGCGCGCGACCGGUGGUG</td>
<td>+</td>
<td>this study</td>
</tr>
<tr>
<td>miR-341*</td>
<td>UCCUCAACAGAAAUCUCCACCUUC</td>
<td>n.d.</td>
<td>Kim et al. 2004</td>
</tr>
</tbody>
</table>

---

**Notes:**

1. Novel miRNA sequences have been submitted to the miRNA registry (http://www.sanger.ac.uk/cgi-bin/Rfam/mirna/browse.pl) and they have been given new names in accordance to the miRNA registry numbering (e.g. mir-A22-3’ is also called miR-409 in the miRNA registry).

2. The exact length of the miRNAs which have been detected solely by primer extension is not known. Thus, the two last nucleotides at the 3’-termini are in italics as they are only predicted based on a 23 nt theoretical long RNA species. Primer extension assay generates two cDNA products with a size differing by one nucleotide. Based on the miR-134 and miR-154 sequences obtained independently by cloning strategies (Lagos-Quintana et al. 2002), we have considered the shorter cDNA product as the correct 5’-termini while the longer one might correspond to in vivo processing. 

3. miR-323/A1, miR-329/A2, miR-A9, miR-A22 and miR-A24 are processed from the 3’ strand of the A1, A2, A9, A22 and A24 gene copies, respectively suggesting that both strands of A-type can potentially be converted to miRNAs.

4. Depending upon RNA samples, we could also detect a ladder-like pattern superimposed to the mature 5’ end of miR-N. Thus, this miRNA might not fulfill the stringent criteria described in (Ambros et al. 2003).

5. miR-341 was not found in our in silico search as it is not conserved at the human inprinted 14q32 interval. n.d. indicates not determined.
silencing of target sequences in plants (Aufsatz et al. 2002). Thus, an emerging theme is the idea that noncoding RNAs have key functions in genome modification. Whether the clustered miRNAs can control gene expression at the transcriptional level is still an open and challenging question. We noticed that four miRNA genes—miR-A22, miR-A23, miR-A24, and miR-K—are embedded within a conserved CpG island located at the 3′ end of the miRNA gene cluster. The methylation status of this CpG-rich domain is under investigation.

Did this large cluster of miRNAs emerge within this imprinted domain before or after the onset of imprinting at this region? If the former, did it confer some aspect of imprinting control, or if the latter, is the imprinted expression of these small hairpin RNAs a mere consequence of more widespread expression of noncoding RNAs from the maternal chromosome? One thought-provoking idea pertaining to the evolution of the imprinting mechanism suggests that intragenomic parasitic sequences, including repeated sequences, retrotransposons, and other related elements, are heritably and variably silenced in order that they are rendered transcriptionally inactive, “hidden” by heterochromatinization, and/or immobilized (Barlow 1993; Bird 1997; Yoder et al. 1997; Martienssen 1998; Whitelaw and Martin 2001). Although this hypothesis was based on a function for DNA methylation, it could equally be applied to a small RNA-mediated mechanism. Thus, one can speculate that these repeated small RNA genes might have integrated into this chromosomal domain prior to the emergence of its imprinting, and their subsequent gene amplification may have been perceived by the

Figure 4  Expression of in silico–predicted miRNA genes. Tissue-specific (A) and developmental gene expression pattern (B) of the miRNAs analyzed by primer extension assay. H indicates head; T, trunk. 5.8S and let-7 probes have been used as gel loading controls (Northern blot analysis). (C) The miRNA genes are only expressed from the maternally inherited chromosome. Ten micrograms of total RNA extracted from wild-type embryos, embryos with maternal uniparental disomy for chromosome 12 (matUPD12), or embryos with paternal uniparental disomy for chromosome 12 (patUPD12) have been subjected to primer extension assay. (D) The miRNA imprinted gene expression is controlled by the intergenic germline-derived differentially methylated region (IG-DMR). Ten micrograms of total RNA extracted from embryo with a deletion of the IG-DMR from either the maternally or the paternally inherited chromosomes (as specified at the top of the picture) have been subjected to primer extension analysis.

Figure 5  Detection of large transcripts overlapping several miRNA genes. Ten micrograms of DNAse I–, proteinase K–treated total RNAs extracted from the head of mouse embryo (E17.5) was subjected to RT-PCR with appropriate primers (numbered arrows) mapping outside from the ~70-nt pre-miRNA genes. The location of the primers and expected sizes of the amplification products are shown on the top part of the figure (not drawn to scale). Primer sequences are available on request. RT indicates reverse transcriptase (Superscript II, Invitrogen).
host as “foreign DNA,” resulting in imposition of epigenetic regulation to the surrounding protein-coding genes. Conversely, the miRNA gene cluster might have been generated after the establishment of the imprinting, perhaps due to a specific chromatin architecture prone to gene duplication and/or to the different behavior of the two parental chromosomes, which is permissive for nondisruptive insertion. It remains to be determined whether the parental origin specific nature of the miRNA expression contributes to, or is a consequence of, genomic imprinting.

An exhaustive characterization of all the epigenetic marks along this large imprinted domain together with genetically engineered mouse models is now required to fully appreciate the potential involvement of these imprinted miRNA genes in mouse development and/or epigenetic control.

METHODS

Unless otherwise noted, all techniques for manipulating nucleic acids were performed according to standard protocols.

Oligodeoxynucleotides

They were all synthesised by Y. de Préval (LBME) on a PerSeptive Biosystems Expedite apparatus. Primer sequences are available on request.

Searching for miRNA Genes at Human Imprinted 14q32 Domain

BAC sequences around DLK1/GTL2 positions were retrieved from the Human Genome Project Working Draft (http://genome.ucsc.edu/) and 100-nt-long stem-loop structures with a ΔG < -30 kcal/mole were systematically identified within the -1-Mb-long imprinted 14q32 region by using RNAfold software from http://www.ncbi.nlm.nih.gov/Structure/pubmed/PubMed/11503. We then retained the conserved hairpin structures between the murine and human orthologous imprinted loci (according to BLAT search at http://genome.ucsc.edu/) that give rise to a miRscan score >10 by using the software developed by D.P. Bartel and his colleagues (http://genes.mit.edu/mirsscan/). Sequence alignment of the miRNA genes were obtained by MultiAlin (http://prodes.toulouse.inra.fr/multalin/multalin.html), and conserved nucleotides were shaded by GeneDoc (http://www.Cris.com/~ketchup/genedoc.shtml).

Experimental Detection of the miRNAs

In silico–predicted miRNA genes were validated either by Northern analysis or by primer extension. Total RNA was isolated by the guanidinium thiocyanate method from mouse tissues or from whole embryos freshly prepared according to French institutional and United Kingdom Home Office guidelines. For Northern blot analyses, 15 to 20 µg of RNA were electrophoresed on a 15% acrylamide /7 M urea gel. Northern blot analyses, 15 to 20 µg of DNAse I–, protease K–treated total RNA was separated on a denaturing 15% acrylamide/7 M urea gel. Electrophoresis in 0.5 × TBE buffer was performed onto nylon membranes (Hybond N+) and the resulting cDNA products were annealed with a (32P)–labeled oligodeoxynucleotide probes, through an overnight incubation in 5 × SSC, 1% SDS, 150 µg/ml yeast tRNA, and 5 × Denhardt’s. Membranes were washed twice with 2 × SSPE and 0.1 % SDS at room temperature before autoradiography. For primer extension assay, after a heat denaturation step (5 min at 75°C), 10 µg of total RNA was annealed with a (32P)–labeled and gel-purified specific primer (see Supplemental data) and reverse transcribed during 2 h at 42°C by AMV reverse transcriptase (Promega), and the resulting cDNA products were analyzed on a 15% acrylamide /7 M urea gel.

ACKNOWLEDGMENTS

This work has been initiated in the Dr. Jean-Pierre Bachelier’s laboratory, and we thank him very much for his permanent support and for the critical reading of the manuscript. We are also grateful to D. Morello for her help with mouse manipulation. Work in the laboratory of J.C. is supported by the Ministère de l’Éducation Nationale, de la Recherche et de la technologie (Agence de la Recherche et de la technologie) and work in the laboratory of A.F.S. is funded by a CR-UK grant. S.–P.I. is funded by a graduate research scholarship from the Taiwanese Government; H.S., by a PhD Fellowship Allocation de Moniteur Normalien (Ministère de l’Éducation Nationale, de la Recherche et de la technologie). The authors declare that they have no competing financial interests.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 USC section 1734 solely to indicate this fact.

NOTE ADDED IN PROOF

Human miRNAs derived from our computationally predicted pre-miR-B3 and pre-miR-A23 have been experimentally cloned by Suh et al. (2004). They have been named miR-368 and miR-369, respectively.

REFERENCES


Imprinted microRNA Genes
enzyme Dicer in the maturation of the let-7 small temporal RNA. Science 293: 834–838.


