Mouse microRNA profiles determined with a new and sensitive cloning method

Shuji Takada[1], Eugene Berezikov[2], Yoshihiro Yamashita[1], Mariana Lagos-Quintana[3], Wigard P. Kloosterman[2], Munehiro Enomoto[1], Hisashi Hatanaka[1], Shin-ichiro Fujiwara[1], Hideki Watanabe[1], Manabu Soda[1], Young Lim Choi[1], Ronald H. A. Plasterk[2], Edwin Cuppen[2] and Hiroyuki Mano[1,4,*]

[1]Division of Functional Genomics, Jichi Medical University, 3311-1 Yakushiji, Shimotsukeshi, Tochigi 329-0498, Japan, [2]Hubrecht Laboratory, Uppsalalaan 8, Utrecht, The Netherlands, [3]Laboratory of RNA Molecular Biology, Rockefeller University, 1230 York Avenue, New York, NY 10021, USA and [4]CREST, Japan Science and Technology Agency, Saitama 332-0012, Japan

ABSTRACT

MicroRNAs (miRNAs) are noncoding RNA molecules of 21 to 24 nt that regulate the expression of target genes in a post-transcriptional manner. Although evidence indicates that miRNAs play essential roles in embryogenesis, cell differentiation and pathogenesis of human diseases, extensive miRNA profiling in cells or tissues has been hampered by the lack of sensitive cloning methods. Here we describe a highly efficient profiling method, termed miRNA amplification profiling (mRAP), as well as its application both to mouse embryos at various developmental stages and to adult mouse organs. A total of 77 436 Small-RNA species was sequenced, with 11 776 of these sequences found to match previously described miRNAs. With the use of a newly developed computational prediction algorithm, we further identified 229 independent candidates for previously unknown miRNAs. The expression of some of these candidate miRNAs was confirmed by northern blot analysis and whole-mount *in situ* hybridization. Our data thus indicate that the total number of miRNAs in vertebrates is larger than previously appreciated and that the expression of these molecules is tightly controlled in a tissue- and developmental stage-specific manner.

INTRODUCTION

MicroRNAs (miRNAs) are short noncoding RNA molecules that inhibit gene expression through incomplete base pairing with the 3′-untranslated region (3′-UTR) of target mRNAs (1,2). The miRNA system is conserved from worms to mammals and contributes to the regulation of a wide variety of cellular functions. In *Caenorhabditis elegans*, for instance, larval development is regulated by a set of miRNAs that include members of the *lin-4* and *let-7* families (3,4), and the function of *Dicer1*, which encodes an enzyme essential for miRNA biogenesis, is indispensable for mouse embryonic development (5). Furthermore, the miRNA miR-181 has been implicated in the differentiation of mouse B lymphocytes (6).

Evidence indicates that miRNAs also play a role in the pathogenesis of human disorders including cancer. The expression profiles of miRNAs are thus effective for classification of human cancers (7,8). Human *let-7* miRNAs target transcripts of the proto-oncogene RAS and are down-regulated in a large proportion of lung cancer specimens (9). Localization of miRNA genes to the fragile sites of human chromosomes indicates that many more miRNAs may be linked to carcinogenesis (10).

Although the recent public miRNA registry (miRBase release 7.1 at http://microRNA.sanger.ac.uk) contains 326 entries for human miRNAs, a large number of additional human miRNAs are thought to exist (11,12). Given the relation of miRNAs to cell growth and differentiation and to human disease, it is important to compare the expression profiles of miRNAs (both known and unidentified previously) among normal tissues and clinical specimens. Such studies have been hampered, however, by the lack of sensitive cloning methods for miRNAs. Current standard procedures for miRNA isolation require several 100 μg of total RNA as a starting material (13), an amount that is difficult to obtain from small tissues or clinical specimens. To overcome such limitations, we have developed a highly sensitive cloning method for miRNAs, which we have termed miRNA amplification profiling (mRAP).

*To whom correspondence should be addressed. Tel: +81 285 58 7449; Fax: +81 285 44 7322; Email: hmano@jichi.ac.jp

MATERIALS AND METHODS

mRAP

A Small-RNA fraction was directly isolated from cells with the use of a mirVana miRNA Isolation Kit (Ambion). In our experience, the yield of Small-RNA with this kit was about 40–50% of that for total RNA obtained by conventional methods from the same number of cells. A portion of this Small-RNA fraction together with size markers (19, 24 and 33 nt) was subjected to electrophoresis on a 15% polyacrylamide gel under denaturing conditions. The region of the gel containing RNA of 19–24 nt was excised, and the RNA molecules were recovered, dephosphorylated by incubation for 30 min at 50°C with calf intestinal alkaline phosphatase (New England Biolabs) and ligated to the 3′ adaptor [5′-(Pu)uuAACCGCGAATTCCAG(idT)-3′], where lowercase letters indicate RNA, uppercase letters indicate DNA, Pu denotes 5′-phosphorylated uridine, and idT represents 3′-inverted deoxythymidine (Dharmacon). The ligated RNA was subjected to reverse transcription with PowerScript reverse transcriptase (Clontech) and the RT primer (5′-GACTAG-CTGGAATTCGCGGTTAAA-3′) in the presence of the 5′ adaptor (5′-GACCACGCGTATCGGGCACCACGTATG-CTATCGATCGTGAGATGGG-3′). The products were amplified by PCR for 32 cycles of incubation at 95°C for 30 s and 65°C for 30 s with AmpliTaq Gold DNA polymerase (Applied Biosystems), the 5′ PCR primer (5′-GCGTATCGG-GCACCACGTATGC-3′), and the 3′ PCR primer (5′-GAC-TAGCTTGGTGCCGAATTCGCGGTTAAA-3′). The resulting amplicons were fractionated by electrophoresis, and those from 90 to 95 bp were eluted, digested with BanI endonuclease (New England Biolabs), and subjected to concatamerization with the use of a Ligation High Kit (Toyobo, Osaka, Japan). Products from 500 to 2000 bp were isolated by electrophoresis and cloned into the pGEM-Teasy vector (Promega). A more detailed description of the mRAP protocol is provided as Supplementary Data on the NAR web site.

Prediction of novel miRNAs

Base calling and quality trimming of sequence chromatograms were performed with phred software (14). After masking of vector and adaptor sequences and removal of redundancy, inserts of ≥18 bp were mapped to genomes (ncbi35 assembly for human, ncbim34 assembly for mouse) with the use of the megablast program in the NCBI software suite (ftp://ftp.ncbi.nlm.nih.gov/blast). For every genomic locus that matched an insert, repeat annotations were retrieved from the Ensembl database (http://www.ensembl.org) and repetitive regions were discarded. Genomic regions containing inserts with 100 nt flanking sequences were retrieved from Ensembl, and a sliding window of 100 nt was used to calculate RNA secondary structures with RNA-fold software from the Vienna RNA Secondary Structure Package (15).

To detect homologous hairpins in other genomes, we performed a BLAST search with mature regions of each RNA sequence against human, mouse, rat, dog, cow, opossum, chicken, zebrafish and fugu genomes. Hits of ≥20 nt with an identity of ≥70% were extracted from the genomes together with flanking sequences of a size similar to that observed for the original hairpins. Extracted sequences were checked for hairpin structures with the use of RNAfold, and positive hairpins were aligned with the original hairpin with CLUSTAL W (16). For remaining hairpins, randfold (17) values were calculated for every sequence in an alignment by mononucleotide shuffling and 1000 iterations. A cutoff of 0.01 was used for randfold, and only regions that contained a hairpin below this cutoff for at least one species in an alignment were considered as candidates of miRNA genes. Berezikov *et al.* (18) describe the computational method for prediction of miRNAs in more detail.

Northern blot analysis

Small-RNA fractions (0.1 to 0.5 µg) were subjected to electrophoresis on a 15% polyacrylamide gel under denaturing conditions, and the separated molecules were transferred electrophoretically to a Hybond-N nylon membrane (Amersham Biosciences). The membrane was incubated with ^{32}P-labeled locked nucleic acid (LNA) corresponding to mature miRNA sequences in ULTRAhyb-Oligo solution (Ambion), and signals were detected with a BAS-1500 image analyzer (Fuji Photo Film).

Whole-mount *in situ* hybridization

Whole-mount *in situ* hybridization was performed as described (19). LNA-modified oligonucleotides were synthesized by Thermo Electron (Ulm, Germany), and digoxigenin labeling was performed with a DIG Oligonucleotide 3′ End Labeling Kit (Roche Diagnostics, Penzberg, Germany).

RESULTS

Development of mRAP

To isolate miRNAs from small quantities of RNA, we first tried to amplify the miRNA fraction by incorporating simple PCR steps into the conventional miRNA cloning procedures (13). However, all such trials resulted in an amplification of non-specific products from degraded RNAs and adaptor-primer concatamers without miRNA-derived cDNAs (as shown in Figure 1A). To circumvent this limitation, we invented the mRAP procedure by utilizing (1) the SMART method (Clontech) for an efficient cDNA amplification and (2) a long, sophisticated 5′ adaptor. All nucleotide sequences of the 5′ adaptor originally invented by Lagos-Quintana (13), SMART IIA oligonucleotide (Clontech), and a BanI site (for a uni-directional concatamerization of PCR products) were incorporated into our initial 5′ adaptor sequence, which was subsequently optimized by addition/removal of nucleotides to reduce non-specific PCR products. In addition, the length of the 5′ adaptor (46 bases) was determined so that the miRNA-derived products can be easily separated from the two major byproducts (see Figure 1A).

In the mRAP procedure, isolated Small-RNA molecules are first ligated at their 3′ end to a 3′ adaptor and then reverse-transcribed with the use of a primer (RT primer) complementary to the 3′ adaptor (Figure 1A). Because of the fact that certain reverse transcriptases possess terminal deoxynucleotidyl transferase activity a few nucleotides
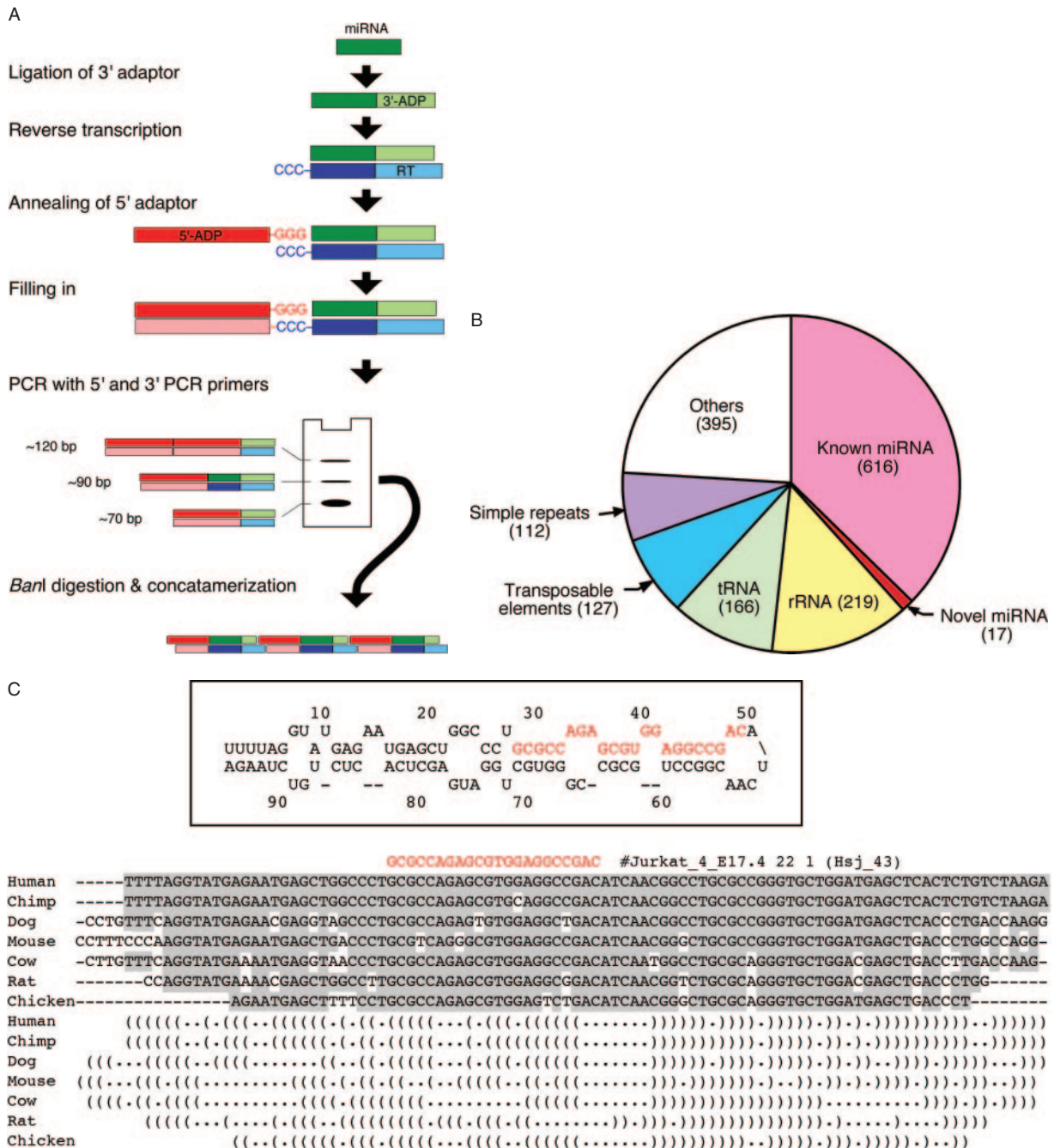
Figure 1. The mRAP protocol and its application to Jurkat cells. (**A**) Isolated Small-RNA molecules are ligated to the 3′ adaptor (3′-ADP) and subjected to reverse transcription with the RT primer. After annealing of the 5′ adaptor (5′-ADP) to the poly(C) overhang at the 3′ end of the synthesized cDNAs, the latter are subjected to PCR with the 5′ and 3′ PCR primers. After an extensive cloning/sequencing of the PCR products, we noticed that, of the three major sizes of amplicon generated, only the middle one includes cDNAs derived from miRNAs. The large product of ∼120 bp is composed of two 5′ adaptors and one 3′ adaptor without miRNA sequences. The small product of ∼70 bp is, on the other hand, composed of only one 5′ adaptor and one 3′ adaptor. The product of ∼90 bp are thus isolated, digested with BanI, and self-ligated to yield concatamers. (**B**) Among 1652 mRAP clones of Jurkat cells that matched the human genome sequence, 616 clones corresponded to known miRNAs, 17 are candidates for novel miRNAs and 219 corresponded to rRNAs, 166 to tRNAs, 127 to transposable elements, 112 to simple repeats and 395 to other genomic sequences that do not fold into a hairpin or otherwise fail the miRNA prediction pipeline. (**C**) Alignment of the nucleotide sequence (red) of one predicted novel miRNA (Hsj_43) with genomic sequences of human, chimpanzee, dog, mouse, cow, rat and chicken. Nucleotides conserved between human and other species are shaded in gray. Possible base pairing schemes for the respective Hsj_43 precursors are shown below the aligned sequences and, for the human sequence, in the upper inset.

(mostly deoxycytidine) are added to the 3′ end of each cDNA strand (20). After the annealing of a 5′ adaptor to the poly(C) overhang of the cDNAs, PCR is performed with 5′ and 3′ PCR primers to exponentially amplify the cDNAs. The isolated cDNAs are digested with the BanI restriction endonuclease and self-ligated to generate concatamers, which are then inserted into a plasmid vector for nucleotide sequencing.

As a test case, we first applied mRAP to 5 μg of a Small-RNA fraction isolated from the human T cell line Jurkat. The procedure readily generated >1 × 10⁴ colony-forming units of the concatamer library. A total of 958 clones was randomly chosen from the library and subjected to nucleotide sequencing. Each plasmid insert consisted of multiple short cDNAs (average of 2.59 cDNAs per insert), and the dataset contained a total of 2392 such cDNAs of ≥18 bp. The 1652 cDNA sequences that passed quality assessment were subjected to computational screening for previously unidentified miRNAs with an algorithm developed in-house. In brief, after filtering of repeat, rRNA, tRNA and small nucleolar (snoRNA) sequences, the remaining sequences predicted to fold into stable stem–loop structures were selected and checked for overlap with known miRNA genes (18).

As shown in Figure 1B, the Jurkat dataset contained 616 clones of known miRNAs (corresponding to 60 independent miRNAs) and 17 clones of newly predicted miRNAs (corresponding to 15 independent miRNAs) (see Supplementary Tables S1 and S2). The proportion of miRNA clones among our Jurkat cDNA sequences (38.3%) was slightly smaller than that (46.9%) obtained by the conventional method by Lagos-Quintana *et al.* (13).

One such candidate for the novel miRNA sequences ('Hsj_43' according to our tentative nomenclature system) is shown aligned with vertebrate genomes in Figure 1C. The precursor of this miRNA is presumed to comprise 95 nt in human, and its nucleotide sequence is conserved among various vertebrates and can fold into an incompletely complementary hairpin structure (Figure 1C).

To determine whether mRAP is able to efficiently isolate miRNAs from a small number of cells, we prepared a Small-RNA fraction (7 μg, 700 and 70 ng, respectively) from 1 × 10⁶, 1 × 10⁵ and 1 × 10⁴ Jurkat cells. We found that mRAP readily generated >1 × 10⁴ colony-forming units of concatamer libraries from all three samples (data not shown). Nucleotide sequencing of randomly chosen clones revealed that the most abundant hsa-miR-142-3p occupies 36.6% (26 reads out of 71 total miRNA reads), 26.1% (24 out of 92) and 20.0% (17 out of 85) of total miRNA clones isolated from the 1 × 10⁶, 1 × 10⁵ and 1 × 10⁴ cells, respectively (data not shown). Similarly, another abundant miRNA, hsa-miR-143, could be found in 11.3% (8 reads), 9.8% (9 reads) and 15.3% (13 reads) of miRNAs from the 1 × 10⁶, 1 × 10⁵ and 1 × 10⁴ cells, respectively. The proportion of isolated rRNAs was also constant among the samples, indicating the high fidelity of mRAP even when performed with a small number of cells. These data confirmed that mRAP is highly sensitive for characterization of miRNA profiles, needing <0.1% of the initial RNA quantity required for current methods (13).

miRNA profiling of mouse embryos

We next applied mRAP to obtain miRNA profiles of mouse. We first isolated mouse embryos at 6.5, 7.5, 8.5, 9.5, 10.5, 11.5, 12.5, 13.5, 14.5, 15.5 and 17.5 days postcoitum (dpc) and subjected them to miRNA profiling. A total of 25 944 small cDNAs was sequenced for all embryos (average of 2359 clones per embryo); 3362 of these clones corresponded

to 150 known miRNAs (miRBase release 7.1), and 198 of them corresponded to 75 novel miRNAs (see Supplementary Tables S3 and S4). These data indicated that many miRNAs are expressed from an early stage of embryogenesis (at 6.5 dpc, for instance, 9.63% of Small-RNA species corresponded to miRNAs). Furthermore, novel candidate miRNAs were detected throughout embryogenesis; the proportion of novel miRNAs among all known and unknown miRNAs was 6.54 ± 3.67% (mean ± SD) for the developmental stages examined.

The expression profiles of miRNAs at each developmental stage of the mouse embryo are summarized in Figure 2A and Supplementary Table S3. Whereas some miRNAs, (such as mmu-mir-124a) are expressed throughout embryonic development, many others are expressed only at specific stages. Expression of mmu-mir-206, e.g. was almost undetectable up to 13.5 dpc but was increased markedly at 14.5 dpc and thereafter. Expression of mmu-mir-148a was largely restricted to 10.5 dpc, at which time it constituted 16.07% of all miRNAs. Similarly, 24 cDNA clones (7.55% of all miRNA species) derived from the mouse embryo at 7.5 dpc corresponded to mmu-mir-23b, whereas only 0 to 3 such clones were identified at other stages of development.

We performed Northern blot analysis to confirm the miRNA profiles identified by mRAP screening. As shown in Figure 2B, northern analysis revealed that the expression of mmu-mir-206 increased progressively with time of embryonic development, whereas that of mmu-mir-124a remained relatively stable (with a slight increase apparent at 13.5 to 15.5 dpc). Direct comparison revealed that the temporal profiles of mmu-mir-206 expression determined by northern blot analysis and by mRAP were similar, with a slight difference in detection sensitivity (Figure 2C).

Northern analysis also detected the putative novel miRNA Mmj_157 at an appropriate size and with preferential expression in mid to late stages of embryogenesis (Figure 2B). We examined the localization of putative miRNAs in whole-mount preparations of mouse embryos at 10.5 dpc by *in situ* hybridization with LNA-modified DNA as a probe. Some of the novel miRNAs were found to be expressed in a tissue-specific manner. Both Mmj_163 and Mmj_157 putative miRNAs were detected specifically in the central nervous system, with the former being preferentially expressed in the telencephalon and the latter in the myelencephalon (Figure 2D). Despite its abundance in the central nervous system of embryos, we were not able to detect Mmj_157 in adult brain (Figure 3A and Supplementary Table S5), indicating that expression of this putative miRNA is both spatially and temporally restricted. In adult mice, a substantial amount of Mmj_157 was apparent only in the placenta, in which it constituted 12.85% of all miRNA clones.

miRNA profiling of adult mouse organs

We next determined the miRNA profiles for 21 organs of the adult mouse with the mRAP procedure. A total of 51 492 clones derived from Small-RNAs (average of 2452 clones per organ) was sequenced and found to include 8141 clones of known miRNAs and 287 clones of novel candidate miRNAs. The distribution of abundant miRNAs in each organ is shown schematically in Figure 3A, with the complete
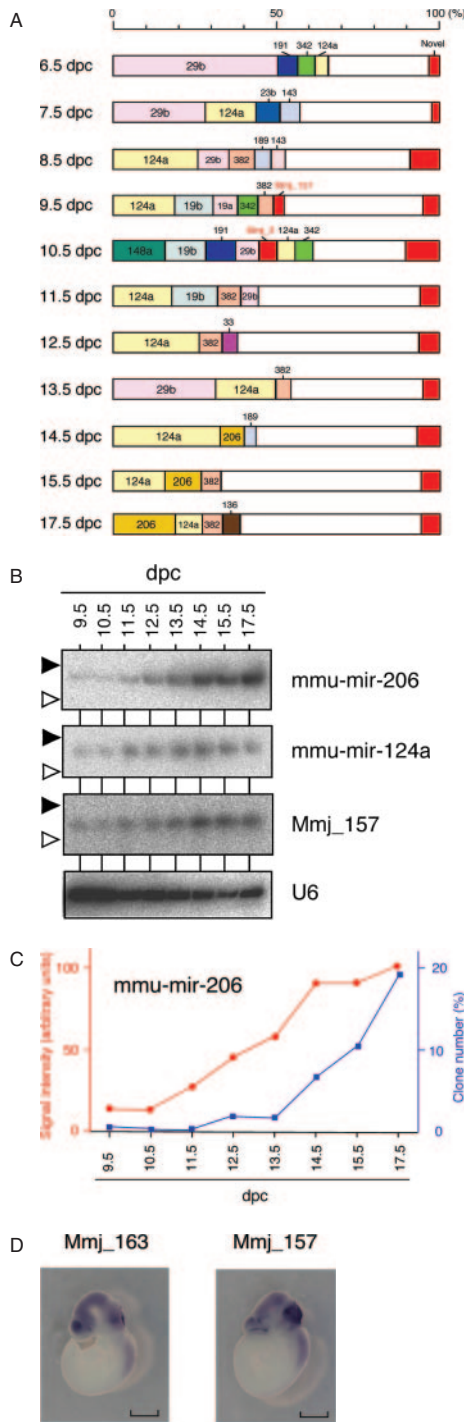
Figure 2. Expression profiles of miRNAs in the mouse embryo. (**A**) The percentage of each miRNA among the total miRNA population was calculated for mouse embryos at the indicated stages of development (6.5 to 17.5 dpc). Abundant miRNAs are shown color-coded, with candidates for novel miRNAs in red. (**B**) Northern blot analysis of the Small-RNA fraction isolated from mouse embryos at the indicated developmental stages (9.5 to 17.5 dpc). The blot was probed with oligonucleotides specific for mmu-mir-206, mmu-mir-124a or Mmj_157; a probe for U6 small nuclear RNA was used as an internal control. Closed and open arrowheads indicate the positions of 24 and 19 nt, respectively. (**C**) Expression level of mmu-mir-206 during mouse embryogenesis as determined from the northern blot in (B) (red line) and from the mRAP dataset (blue line). (**D**) Whole-mount *in situ* hybridization of mouse embryos at 10.5 dpc with LNA-modified probes specific for Mmj_163 or Mmj_157. Scale bar, 1 mm.

dataset being presented in Supplementary Table S5. Some miRNAs, including mmu-mir-124a and mmu-mir-143, were found to be expressed ubiquitously among organs, whereas many others were abundant in only a subset of organs, with their relative expression (clone number) varying markedly among such organs. Marked expression of mmu-let-7b, for example, was apparent only in kidney, lung and ovary, and the proportion of mmu-mir-382 among all miRNA clones was >1% only in brain and placenta.

Candidates for novel miRNAs were found in the proportion of 4.20 ± 4.75% (mean ± SD) of all miRNA species for each organ. Similar to known miRNAs, expression of these candidate miRNAs was found to be regulated in a tissue-dependent manner (Supplementary Table S5). We did not detect a correlation between the miRNA profiles and germ-layer origins of organs.

Northern blot analysis confirmed the organ-specific expression of known and novel miRNAs in the adult mouse (Figure 3B). Expression of Mmj_157 was found to be restricted to the placenta and ovary, consistent with the mRAP data (Figure 3C). Northern analysis revealed expression of mmu-mir-122a to be largely liver-specific (with a low level of expression also apparent in stomach), again consistent with the expression profile obtained by mRAP (Figure 3B and C).

DISCUSSION

We have thus developed a sensitive method for miRNA profiling and have applied this method to obtain the first extensive miRNA profiles of the mouse. Our screening identified 229 putative novel miRNAs (corresponding to 260 loci on mouse chromosomes). Sequence conservation of our novel miRNA candidates among different species is summarized in Supplementary Table S6. In compliance with criteria for miRNA annotation, we require several independent lines of experimental evidence (e.g. cloning and northern blot analysis, or cloning from several libraries) to define a novel miRNA as a bona fide miRNA (21,22). If experimental evidence is limited (e.g. cloned only from one library), novel miRNAs are considered as candidates and are annotated correspondingly (Supplementary Table S7).

It should be noted that, since three Gs are added to the 5′-termini of miRNAs in mRAP (Figure 1A), it might be difficult to precisely determine the 5′ ends of miRNAs especially when the genomic sequence adjacent to mature miRNAs contains Gs. Thus, it is possible that the nucleotide sequences of our novel miRNA candidates in Supplementary Tables S2 and S4 will contain inappropriate Gs at the 5′-termini.

Although, we sequenced 77 436 mouse Small-RNA species, many miRNAs were isolated only once in each tissue or embryo (Supplementary Tables 3 and 5), suggesting that the overall mouse miRNA catalog may not have been fully revealed. Furthermore, given the stringent parameters in our computational screening, it is possible that some bona fide novel miRNAs in our dataset were inappropriately dropped at this *in silico* step. Screening for novel miRNAs by a microarray approach with the same computational algorithm identified a different, but partially overlapping, set of

candidate miRNAs in mouse (18). Similarly, our screening for miRNAs in human clinical specimens by mRAP resulted in the isolation of a set of candidate novel miRNAs that include many with no mouse orthologs either in our dataset or in the miRBase depository (S. Takada, Y. Yamashita, E. Berezikov, Y.L. Choi, S. Fujiwara, M. Enomoto, H. Hatanaka, H. Watanabe, M. Soda, R.H.A. Plasterk, E. Cuppen and H. Mano, manuscript submitted). It is thus likely that the mouse genome encodes additional miRNAs yet to be discovered.

Isolation of novel miRNAs has been attempted to date through a variety of approaches. Lagos-Quintana *et al.* (13) compared miRNA profiles among mouse organs by a conventional miRNA cloning procedure. They identified that three miRNAs are expressed in a tissue-specific manner; mmu-mir-1 in heart, mmu-mir-124a in brain and mmu-mir-122a in liver, all of which is in a very good agreement with our observation (see Figure 3A and Supplementary Table S5). On the other hand, Barad *et al.* (23) chose oligonucleotide microarrays to compare miRNA profiles among five human tissues. Again, they revealed a tissue-specific expression of hsa-miR-122a and hsa-miR-124a, which matches our results.

Mineno *et al.* (24) recently analyzed miRNA expression with the massively parallel signature sequencing (MPSS) technology among three developmental stages (9.5, 10.5 and 11.5 dpc) of mouse embryo. Many of their 'top 20 miRNA signatures' can be observed in our dataset. For instance, their result reveals that the expression of mmu-mir-199a was increased from 9.5 to 11.5 dpc of mouse embryo. Our data demonstrates that the augmentation of mmu-mir-199a expression further continues to 15.5–17.5 dpc (Supplementary Table S3) of embryo. Similarly, both of our and Mineno's data indicate that mmu-mir-19b is abundantly expressed at 9.5–11.5 dpc of mouse embryo (Supplementary Table S3). Additionally, one of the abundant novel miRNAs in our embryo dataset, Mmj_157, was also counted for many times as miRNA426 in the data of Mineno *et al.* There may be, however, some difference between these two datasets. One of the highly expressed miRNAs in mouse embryo, mmu-mir-124a, in our data are missed from that of Mineno *et al.* Our northern blot analysis in Figure 2B supports the expression of mmu-mir-124a in embryo.

To directly compare our mRAP data with those by other high-throughput methods, we then hybridized RNA from
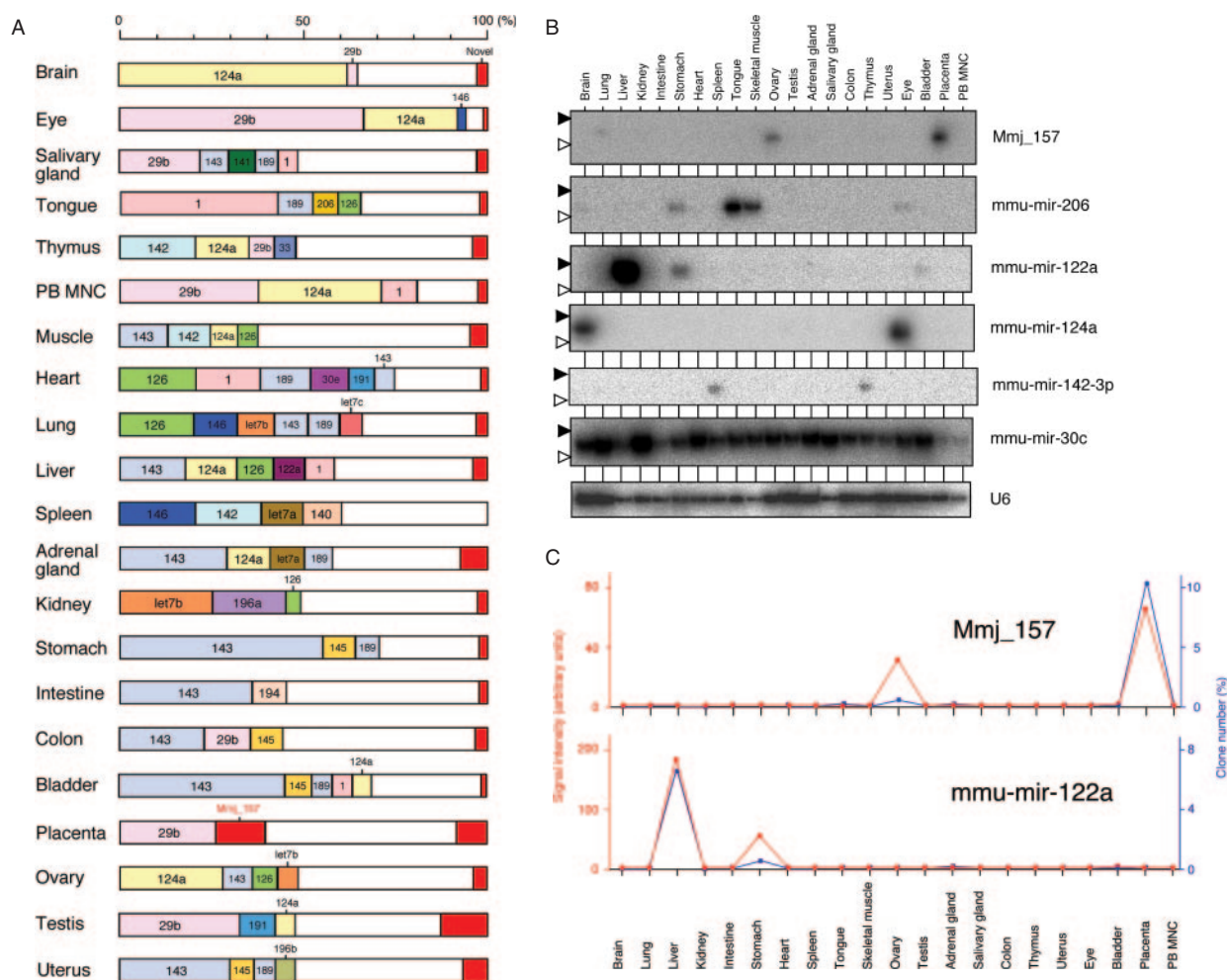

Figure 3. Expression profiles of miRNAs in adult mouse organs. (**A**) The percentage of each miRNA among the total miRNA population was calculated for the indicated organs of the adult mouse and is shown schematically as in Figure 2A. PB MNC, peripheral blood mononuclear cells. (**B**) Northern blot analysis of the Small-RNA fraction from the indicated adult mouse organs with probes specific for the indicated RNA species. (**C**) Expression levels of Mmj_157 or mmu-mir-122a in adult mouse organs as determined from the northern blot in (B) and from mRAP data.

Jurkat cell line to miRCURY LNA microarrays (Exiqon, Vedbaek, Denmark) to quantitate miRNA amounts. Hsa-miR-142, the most abundant miRNA in our Jurkat dataset (Supplementary Table S1), was indeed identified as one of the strongest signals in the array data (data not shown). However, with regard to another abundant miRNA hsa-miR-143 in our dataset, the microarray could give a hybridization signal only at the intensity of backgrounds (data not shown). Northern blot analysis clearly confirmed the expression of hsa-miR-143 in Jurkat cells (Supplementary Figure S1), supporting our mRAP data. Caution should thus be taken to estimate the miRNA profiles based on some type of microarrays.

We also quantitated the expression level of mmu-mir-122a, mmu-mir-185 and let-7-a with the TaqMan MicroRNA assay (Applied Biosystems) in mouse brain, liver and heart. Relative expression intensity of mmu-mir-122a to that of let-7-a was 1.056×10^{-4} for brain, 7.227 for liver and 1.230×10^{-4} for heart, indicating the liver-specific expression of mmu-mir-122a. On the other hand, the TaqMan assay revealed a weak but ubiquitous expression of mmu-mir-185; its relative expression level to that of let-7-a was 5.759×10^{-3} for brain, 3.816×10^{-3} for liver and 6.769×10^{-3} for heart. Both of these data are highly compatible with our dataset (Supplementary Table S5).

Given that mRAP is able to provide an miRNA profile with as few as 1×10^4 cells, it opens up the possibility of direct characterization of miRNAs in small amounts of tissue, such as those available for mouse embryos (as demonstrated in the present study) and fresh human specimens. Indeed, with mRAP, we have characterized miRNA profiles even for small papillary muscles of the human heart ventricle (S. Takada, R. Kaneda, E. Berezikov, Y. Yamashita, Y.L. Choi, S. Fujiwara, M. Enomoto, H. Hatanaka, H. Watanabe, M. Soda, R.H.A. Plasterk, E. Cuppen and H. Mano, manuscript submitted). Our present miRNA profiling in mouse has shown that such profiles vary markedly among tissues and developmental stages. An important application of mRAP will be determination of whether expression of miRNAs is associated with human disease by analysis of fresh human tissue specimens.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR online.

ACKNOWLEDGEMENTS

Conflict of interest statement. None declared.

REFERENCES

1. Meister,G. and Tuschl,T. (2004) Mechanisms of gene silencing by double-stranded RNA. *Nature*, **431**, 343–349.
2. Bartel,D.P. (2004) MicroRNAs: genomics, biogenesis, mechanism and function. *Cell*, **116**, 281–297.
3. Abbott,A.L., Alvarez-Saavedra,E., Miska,E.A., Lau,N.C., Bartel,D.P., Horvitz,H.R. and Ambros,V. (2005) The let-7 MicroRNA family members mir-48, mir-84, and mir-241 function together to regulate developmental timing in *Caenorhabditis elegans*. *Dev. Cell*, **9**, 403–414.
4. Lee,R.C., Feinbaum,R.L. and Ambros,V. (1993) The *C. elegans* heterochronic gene lin-4 encodes Small-RNAs with antisense complementarity to lin-14. *Cell*, **75**, 843–854.
5. Bernstein,E., Kim,S.Y., Carmell,M.A., Murchison,E.P., Alcorn,H., Li,M.Z., Mills,A.A., Elledge,S.J., Anderson,K.V. and Hannon,G.J. (2003) Dicer is essential for mouse development. *Nature Genet.*, **35**, 215–217.
6. Chen,C.Z., Li,L., Lodish,H.F. and Bartel,D.P. (2004) MicroRNAs modulate hematopoietic lineage differentiation. *Science*, **303**, 83–86.
7. Lu,J., Getz,G., Miska,E.A., Alvarez-Saavedra,E., Lamb,J., Peck,D., Sweet-Cordero,A., Ebert,B.L., Mak,R.H., Ferrando,A.A. *et al.* (2005) MicroRNA expression profiles classify human cancers. *Nature*, **435**, 834–838.
8. Calin,G.A., Ferracin,M., Cimmino,A., Di Leva,G., Shimizu,M., Wojcik,S.E., Iorio,M.V., Visone,R., Sever,N.I., Fabbri,M. *et al.* (2005) A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. *N. Engl. J. Med.*, **353**, 1793–1801.
9. Johnson,S.M., Grosshans,H., Shingara,J., Byrom,M., Jarvis,R., Cheng,A., Labourier,E., Reinert,K.L., Brown,D. and Slack,F.J. (2005) RAS is regulated by the let-7 microRNA family. *Cell*, **120**, 635–647.
10. Calin,G.A., Sevignani,C., Dumitru,C.D., Hyslop,T., Noch,E., Yendamuri,S., Shimizu,M., Rattan,S., Bullrich,F., Negrini,M. *et al.* (2004) Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. *Proc. Natl Acad. Sci. USA*, **101**, 2999–3004.
11. Berezikov,E., Guryev,V., van de Belt,J., Wienholds,E., Plasterk,R.H. and Cuppen,E. (2005) Phylogenetic shadowing and computational identification of human microRNA genes. *Cell*, **120**, 21–24.
12. Bentwich,I., Avniel,A., Karov,Y., Aharonov,R., Gilad,S., Barad,O., Barzilai,A., Einat,P., Einav,U., Meiri,E. *et al.* (2005) Identification of hundreds of conserved and nonconserved human microRNAs. *Nature Genet.*, **37**, 766–770.
13. 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.
14. Ewing,B. and Green,P. (1998) Base-calling of automated sequencer traces using phred. II. Error probabilities. *Genome Res.*, **8**, 186–194.
15. Hofacker,I.L. (2003) Vienna RNA secondary structure server. *Nucleic Acids Res.*, **31**, 3429–3431.
16. Thompson,J.D., Higgins,D.G. and Gibson,T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.*, **22**, 4673–4680.
17. Bonnet,E., Wuyts,J., Rouze,P. and Van de Peer,Y. (2004) Evidence that microRNA precursors, unlike other non-coding RNAs, have lower folding free energies than random sequences. *Bioinformatics*, **20**, 2911–2917.
18. Berezikov,E., van Tetering,G., Verheul,M., van de Belt,J., van Laake,L., Vos,J., Verloop,R., van de Wetering,M., Guryev,G., Takada,S. *et al.* (2006) Many novel mammalian microRNA candidates identified by extensive cloning and RAKE analysis. *Genome Res.*, in press.
19. Kloosterman,W.P., Wienholds,E., de Bruijn,E., Kauppinen,S. and Plasterk,R.H. (2006) *In situ* detection of miRNAs in animal embryos using LNA-modified oligonucleotide probes. *Nature Meth.*, **3**, 27–29.
20. Chenchik,A., Zhu,Y.Y., Diatchenko,L., Li,R., Hill,J. and Siebert,P.D. (1998) In Siebert,P.D. and Larrick,J. (eds.), *Gene Cloning and Analysis by RT–PCR*. BioTechniques Books, MA, pp. 305–319.
21. Ambros,V., Bartel,B., Bartel,D.P., Burge,C.B., Carrington,J.C., Chen,X., Dreyfuss,G., Eddy,S.R., Griffiths-Jones,S., Marshall,M. *et al.* (2003) A uniform system for microRNA annotation. *RNA*, **9**, 277–279.

22. Berezikov,E., Cuppen,E. and Plasterk,R.H. (2006)
    Approaches to microRNA discovery. *Nature Genet.*, **38**,
    S2–S7.
23. Barad,O., Meiri,E., Avniel,A., Aharonov,R., Barzilai,A., Bentwich,I.,
    Einav,U., Gilad,S., Hurban,P., Karov,Y. *et al.* (2006) MicroRNA
    expression detected by oligonucleotide microarrays: system
    establishment and expression profiling in human tissues. *Genome Res.*,
    14, 2486–2494.
24. Mineno,J., Okamoto,S., Ando,T., Sato,M., Chono,H., Izu,H.,
    Takayama,M., Asada,K., Mirochnitchenko,O., Inouye,M. *et al.* (2006)
    The expression profile of microRNAs in mouse embryos. *Nucleic Acids
    Res.*, **34**, 1765–1771.