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# Stage-specific expression of microRNAs during *Xenopus* development

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**Abstract MicroRNAs (miRNAs) repress target genes at the post-transcriptional level and play important roles in development and cell lineage decision. However, in vertebrates, both the targets of miRNAs and their expression profile during development are poorly understood. Here, we report the detailed expression profiles of miRNAs from oocyte stage to tadpole stage in *Xenopus laevis*. As development proceeds, a variety of miRNAs start to be expressed. Most miRNAs emerged at a specific stage and were continuously expressed until the tadpole stage. In addition, we identified a novel miRNA that was expressed only at specific stages of development and that is likely to have roles in midblastula transition.**


*Keywords:* MicroRNA; Small RNA; Midblastula transition; Cell lineage decision; Development; *Xenopus*

## 1. Introduction

MicroRNAs (miRNAs) are non-coding 21–23 nucleotide RNA molecules that regulate the expressions of other genes by inhibiting translation or cleaving complementary target mRNAs [1]. miRNAs originate from genes that are transcribed by RNA pol-II [2]. miRNA primary transcripts (pri-miRNAs) are trimmed into miRNA precursors (pre-miRNAs) by RNaseIII-like enzyme called Drosha [3] and subsequently processed by another RNaseIII-like enzyme (Dicer) into miRNA duplexes [4]. One strand of the duplex (miRNA) is then incorporated into the RNA-induced silencing complex (RISC) and the other strand (miRNA*) is degraded [1].

The total number of miRNAs is estimated to be no more than 255 in the human genome, representing about 1% of the predicted genes in human [5]. Of the estimated 255 human miRNAs, about 200 human miRNAs have been identified and most of these were cloned from tissues and cultured cells [6–8]. The sequences of many miRNAs are conserved among distantly related organisms [9].

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*Abbreviations:* miRNA, microRNA; pri-miRNA, miRNA primary transcript; pre-miRNA, miRNA precursor; MBT, midblastula transition; siRNA, small interfering RNA

miRNAs are not the only functional small RNA in vertebrates. Three other classes of small RNAs have been described in vertebrates: (I) small modulatory RNAs (smRNAs) trigger gene expression through interaction with transcriptional machinery [10], (II) artificial small interfering RNAs (siRNAs) complementary to mRNA or the promoter region and inhibit the gene expression translationally or transcriptionally [11], and (III) endogenous siRNA derived from centromeric repeats are thought to be involved in the formation of the heterochromatin [12].

Previous studies have revealed the importance of miRNAs and other small RNAs in animal and plant development. In *Caenorhabditis elegans*, lin-4 and let-7 miRNAs are expressed at distinct stages of development to regulate the timing of larval developmental transition [13,14]. In vertebrates, post-transcriptional restriction of HOX gene expression is mediated by miRNA [15]. Mutations in the Dicer gene cause germ line defects in *C. elegans* [16], abnormal embryogenesis in *Arabidopsis thaliana* [17], developmental arrest in zebrafish [18] and depletion of ICM in mouse [19].

Despite important roles in development, expression profile of miRNAs in vertebrate development is poorly understood. In the present study, we examined the expression profile of miRNAs in various developmental stages of *Xenopus laevis* and report a new temporally expressed miRNA, maternally derived small RNAs and a small RNA that corresponds to a promoter region.

## 2. Materials and methods

### 2.1. Preparation of oocytes and embryos

Ovaries were surgically removed from three mature *Xenopus laevis* females (Kato S Kagaku) and then suspended in modified Barth's saline (MBS) [88 mM NaCl/1 mM KCl/0.7 mM $CaCl_2$/1 mM $MgSO_4$/2.5 mM $NaHCO_3$/5 mM HEPES, pH 7.4]. The ovaries were cut into pieces and incubated in MBS containing 0.25% collagenase for 2 h at room temperature. A mixture of different size oocytes was filtered through nylon mesh to separate by diameter according to the Dumont stage [20]. To remove the follicular layer completely, oocytes dissected from the ovarian tissues were selected and then treated again with collagenase for 1 h [21]. To obtain ovulated eggs and embryos, females were injected with 500 IU of hCG. Embryos were prepared by artificial fertilization in 0.1× Marc's modified Ringer's (MMR) (100 mM NaCl/2 mM KCl/1 mM $MgSO_4$/2 mM $CaCl_2$/5 mM HEPES, pH 7.4) and maintained at 25 °C, ensuring that they were accurately staged according to the Nieukwoop and Faber stage [22].

### 2.2. Low molecular weight RNA preparation and cloning of small RNA

Total RNA was isolated using the guanidium thiocyanate-phenol-chloroform method [23]. To obtain low molecular weight RNA, total RNA was precipitated by equal amount of PEG solution [1.6 M NaCl,

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13% (w/v, PEG 6000)] and the supernatant was precipitated using isopropanol. For cloning of small RNAs, 50 μg of low molecular weight RNA was used. Small RNA were cloned as described [24].

### 2.3. Bioinformatics analysis

Database searches were performed at the NCBI Blast (http://www.ncbi.nlm.nih.gov/) and the miRNA Registry (http://www.sanger.ac.uk/Software/Rfam/mirna/index.shtml). The secondary structures of sequences covering cloned small RNAs were predicted using MFOLD 3.1 (http://www.bioinfo.rpi.edu/applications/mfold/).

### 2.4. Northern blot analysis

Low molecular weight RNA (5 μg) from each developmental stage was loaded on a 15% denaturing polyacrylamide gel. Probes were prepared and hybridized as described [25]. The oligonucleotide probes were complementary to cloned miRNAs or the spacer promoter sequence. miR-106b sequence was derived from mammal. Imaging plate was exposed for one day or three days according to the signal intensity and signals were detected by using a BAS2500 phosphoimager. Blotted membranes were prepared for each probe.

## 3. Results and discussion

### 3.1. Cloning of small RNAs in various developmental stages

To identify the small RNAs expressed in *Xenopus* development, oocytes and embryos (up to tadpole stage) were collected and then divided into eight classes as described in Table 2. The same number of eggs or embryos from each developmental stage was mixed at each class, and eight independent cDNA libraries were generated from small RNAs in the size range of 15–27 nt by linker ligation method [24]. We obtained a total of 1093 clones with 99–205 clones in each class.

To annotate these clones, vertebrate databases in NCBI Blast and The miRNA registry [26] were used, because the *Xenopus* genome project has not been completed [27]. One or two nucleotides difference was allowed to annotate the clones. We cloned 23 kinds of previously identified miRNAs. Of these 23 kinds, 17 completely matched the mammalian miRNAs, five (miR-20, miR-124, miR-181a, miR-196a, and miR-367) differ from the mammalian sequences by 1 nt, and one (miR-16) differs by 2 nt. In the cloned small RNA, the most abundant classes were miRNA and miRNA* (408 clones), non-coding functional RNA breakdown products such as rRNA (257 clones), tRNA (39 clones) and snRNA (4 clones) (Table 1).

It seems possible that the remaining 386 clones include new miRNAs, because miRNAs have not been identified in *Xenopus* and because mammalian miRNAs have been mainly cloned from adult tissues or cultured cells in previous studies [6–8]. To find miRNAs not registered in the database, we sorted the remaining 386 clones into 203 non-redundant sequences and then searched vertebrate genomes and ESTs for these sequences to obtain the flanking sequences. The obtained sequences were examined to see whether these were fold into a hairpin structure that is characteristic of miRNA precursors. Consequently, three sequences fulfilled this criterion (Fig. 1), suggesting that these three sequences are novel miRNAs.

### 3.2. Novel miRNAs in Xenopus development

Of the 3 novel miRNAs, miR-428 was found in the *Xenopus* EST database, not in other vertebrate databases. However, its 5′ region had some similarity to previously identified mammalian miR-302 and miR-20. Other 2 miRNAs (miR-427, miR-429) or their homologs were found in genomes of other vertebrates. miR-429 was found in the mammalian genome and it is clearly related to miR-200b. miR-427 was found in the zebrafish genome but not in mammalian genomes. However, miR-427 had some similarity to mouse miR-294 (19 nucleotides identity per 23 nucleotides) and human miR-372 (18 nucleotides identity per 23 nucleotides), which were cloned from ES cells [7,28].

### 3.3. Expression pattern of microRNAs in Xenopus development

Twenty-six miRNAs and 2 miRNA*s were isolated by stage specific cloning (Table 2). To examine the expression pattern of cloned miRNA, Northern blot analysis was performed

Table 1
Type and the number of small RNAs cloned from *Xenopus* eggs and embryos

| | Type | Number of clones |
|---|---|---|
| Non-coding RNA | miRNA/miRNA* | 407 |
| | New miRNA[a] | 20 |
| | rRNA | 257 |
| | tRNA | 39 |
| | snRNA | 4 |
| | rRNA spacer promoter | 1 |
| Others | Found in vertebrate EST or genome | 21 |
| | Observed several times[b] | 179 |
| | Observed only once | 165 |
| | Total | 1093 |

[a]miRNAs that were newly identified in this study.
[b]Unknown small RNAs that were not found in databases and were cloned several times at different stages.

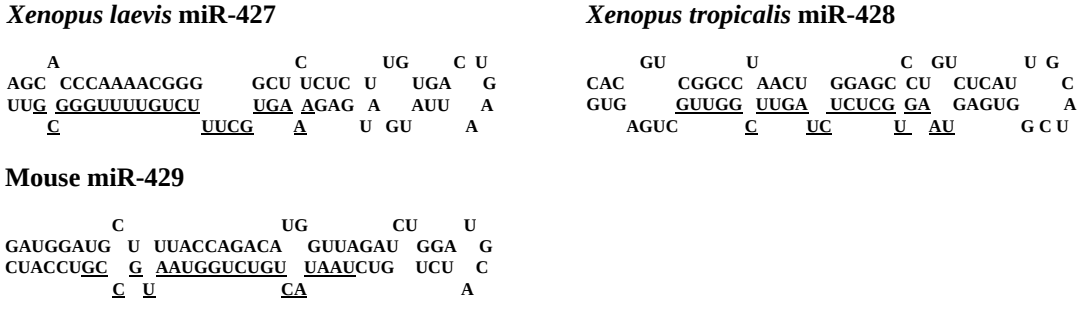


Fig. 1. Predicted secondary structures of miRNA precursors. The putative precursors of miR-427 and miR-428 were derived from *Xenopus* EST, and the putative precursor of miR-429 was derived from mouse genome due to a lack of this miRNA in *Xenopus* database. The miRNA sequences are underlined.

Table 2
miRNAs cloned at defined developmental stages

| miRNA[a] | Sequence[b] | Numbers of clones | | | | | | | | | *Xenopus* EST (stage)[f] | Presence in mammal or zebrafish[g] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Size (nt) | Oocyte, I–VI[c] | Egg, E[d] | Morula, 1–8[e] | Blastula, 9–12 | Gastrula, 13–17 | Neurula, 18–22 | Tailbud, 23–30 | Tadpole, 31–42 | | |
| miR-106b* | CCGCACUGUGGGUACUUGCUGC | 22 | 41 | 35 | 34 | 23 | 32 | 27 | 1 | 2 | | m |
| miR-210 | CUGUGCGUGUGACAGCGGCUGA | 22 | 8 | 8 | 10 | 9 | 9 | 6 | | 1 | | m, z |
| miR-214 | ACAGCAGGCACAGACAGGCAGU | 22 | | 6 | 5 | 3 | 1 | 2 | | | | m, z |
| **miR-427** | **AAAGUGCUUCCUGUUUUGGGCGU** | **23** | | | | **6** | **2** | **7** | | | **BX844791 (10)** | **z** |
| miR-206 | UGGAAUGUAAGGAAGUGUGUGG | 22 | | | | 3 | 4 | 6 | 37 | 45 | | m, z |
| miR-92-1* | AGGUUGGGAUUGGUUGCAAUGCU | 23 | | | | 2 | 3 | 4 | 1 | | BJ042773 (15) | m |
| miR-7 | UGGAAGACUAGUGAUUUUGUUGU | 23 | | | | 1 | | | | 1 | | m, z |
| miR-16 | UAGCAGCACGUAAAUACUGGAG | 22 | | | | 1 | | 1 | | | | m, z |
| miR-19b | UGUGCAAAUCCAUGCAAAACUGA | 23 | | | | 1 | | | | | BJ641506 (10.5) | m |
| miR-200b | UAAUACUGCCUGGUAAUGAUGAU | 23 | | | | 1 | | | | | | m |
| miR-367 | AUUGCACUGUAGCAAUGGUGA | 21 | | | | 1 | | | | | AL662758 (10.5–12) | m |
| **miR-428** | **UAAGUGCUCUCUAGUUCGGUUG** | **22** | | | | **1** | | | | **1** | **AL594612 (10.5–12)** | |
| let-7f | UGAGGUAGUAGAUUGUAUAGU | 21 | | | | 1 | | | | | | m, z |
| miR-20 | CAAAGUGCUCAUAGUGCAGGUAG | 23 | | | | | 1 | 1 | 1 | | BJ641506 (10.5) | m |
| miR-126 | UCGUACCGUGAGUAAUAAUGCG | 22 | | | | | 1 | | | | | m |
| miR-18 | UAAGGUGCAUCUAGUGCAGUUAG | 23 | | | | | | 1 | | | BJ641506 (10.5) | m |
| miR-26a | UUCAAGUAAUCCAGGAUAGGCU | 22 | | | | | | 1 | 5 | 5 | | m, z |
| miR-124 | UAAGGCACGCGGUGAAUACCAA | 22 | | | | | | 1 | | | CN113428 (25–35) | m, z |
| miR-196a | AGGUAGUUUCAUGUUGUUGGGA | 22 | | | | | | 1 | | | | m, z |
| miR-10b | ACCCUGUAGAACCGAAUUUGUG | 23 | | | | | | | 2 | | | m, z |
| miR-1 | UGGAAUGUAAAGAAGUAUGUAU | 22 | | | | | | | 1 | 3 | CD811161 (62) | m, z |
| miR-24 | UGGCUCAGUUCAGCAGGAACAG | 22 | | | | | | | 1 | | | m |
| miR-130b | CAGUGCAAUGAUGAAAGGGCAU | 22 | | | | | | | 1 | | | m |
| miR-133a | UUGGUCCCCUUCAACCAGCUGU | 22 | | | | | | | 1 | | BJ052054 (15) | m, z |
| miR-133b | UUUGGUCCCCUUCAACCAGCUA | 22 | | | | | | | 1 | | | m, z |
| **miR-429** | **UAAUACUGUCUGGUAAUGCCG** | **21** | | | | | | | **1** | | | **m, z** |
| miR-181a | GACAUUCAACGCUGUCGGUGAGG | 23 | | | | | | | | 2 | | m, z |
| miR-301 | AGUGCAAUAGUAUUGUCAAAGCA | 23 | | | | | | | | 1 | | m, z |

[a]Novel miRNAs are showed in bold.
[b]The most frequently obtained sequences are listed.
[c]Roman numerals represent developmental stages at oocyte according to Dumont [20].
[d]E represents ovulated egg.
[e]Arabic numbers represent developmental stages of embryos according to Nieukwoop and Faber [21].
[f]Primary transcripts found in EST are indicated in accession number with cloned stage of the EST.
[g]The presence of homologous miRNAs in mammals and zebrafish is represented as m and z, respectively.

(Fig. 2). Oocytes were divided into three stages and samples were prepared from ten kinds of developmental stages as described in Fig. 2. Since oligonucleotide probes complementary to the miRNA sequences might cross-react to their isoforms (e.g., let7f probe to other let7 isoforms), all of the miRNAs and miRNA*s except miR-133b (which is a homolog of miR-133a) were analyzed. We also analyzed miR-106b, which is another strand of miR-106b* in the miRNA duplex. Distinct

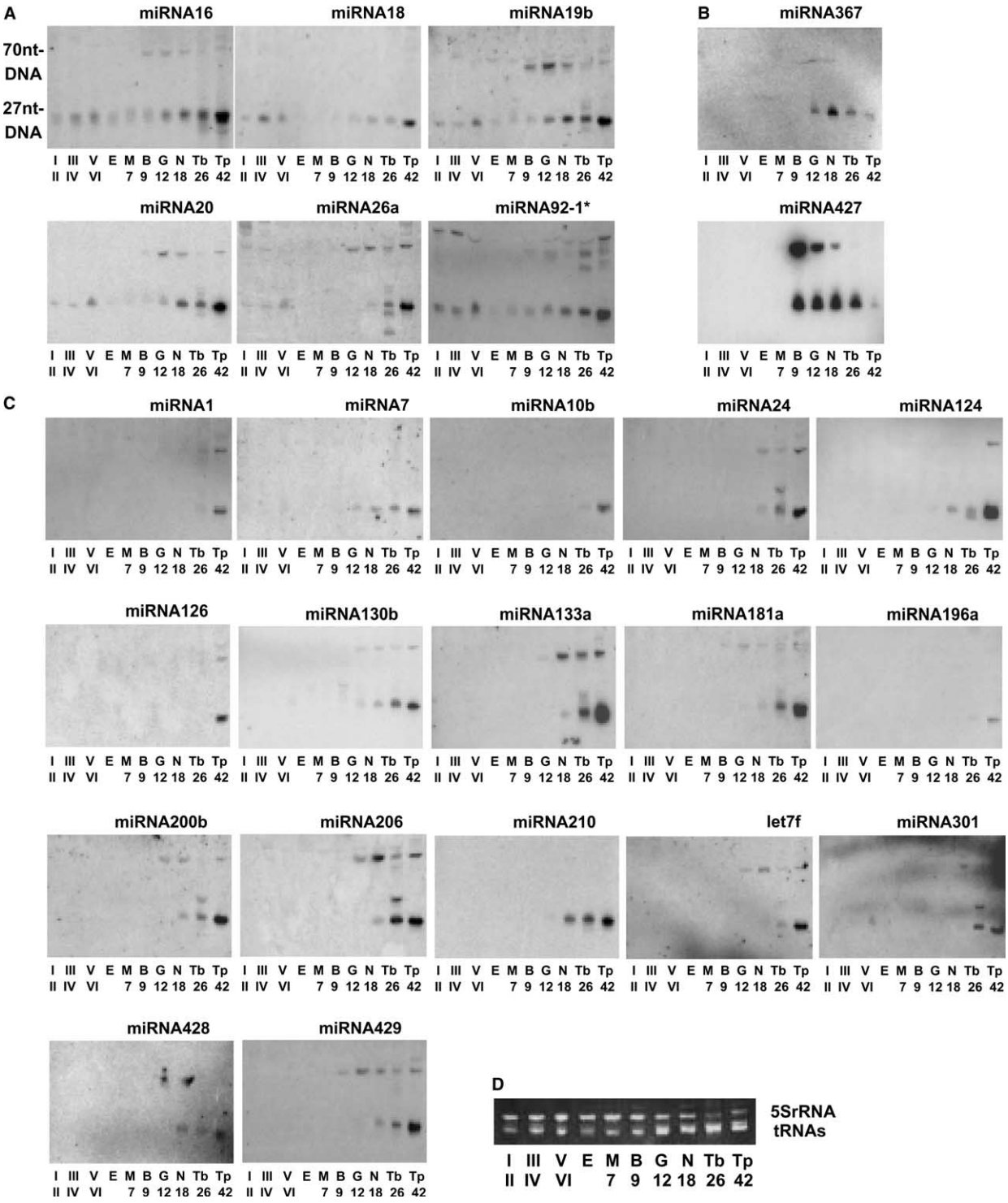


Fig. 2. Expression profile of identified miRNA by Northern blot analysis. Roman numerals represent developmental stages at oocyte according to Dumont. E indicates ovulated egg; M, morula (stage 7); B, blastula (stage 9); G, gastrula (stage 12); N, neurula (stage 18); Tb, tailbud (stage 26); Tp, tadpole (stage 42). (A) miRNAs that are generally expressed. (B) miRNAs that are detectable at defined stages. (C) miRNAs that are detectable from a certain stage after MBT and continue to be detectable until tadpole stage. (D) One-tenth amount of RNA (500 ng) was loaded on each lane and stained with EtBr. 5S rRNA and tRNAs serve as a loading controls.

bands at ~22 nt were observed in all three novel miRNAs (Figs. 2B and C), and other 22 miRNAs except miR-106b, miR-106b* and miR-214 were also detectable. Some discrepancies are observed between cloning and Northern blot results. It is because cloning method includes multiple steps such as gel excision, linker ligation, RT-PCR and concatemerization, so that specific miRNAs would be preferentially amplified at every stage, and because the number that we sequenced would be too small to quantify. miRNAs were categorized into three groups (I–III) according to the Northern blot pattern. Group I miRNAs were detected at the oocyte stage. Their amounts decreased at the ovulated egg and gradually increased thereafter until the tadpole stage (Fig. 2A). This group includes miR-16, miR-18, miR-19b, miR-20, miR-26a and miR-92-1*. Group II miRNAs were temporally expressed at defined stages (Fig. 2B). This group consists of miR-367 and miR-427. Group III miRNAs emerged at a specific stage after stage 9 and were continuously detectable until the tadpole stage (Fig. 2C). This group includes miR-1, miR-7, miR-10b, miR-24, miR-124, miR-126, miR-130b, miR-133a, miR-181a, miR-196a, miR-200b, miR-206, miR-210, miR-301, miR-428, miR-429, and let-7f.

miRNAs of group (I) were detectable almost continuously from the oocyte stage to the tadpole stage. The signals of group I miRNAs diminished from stage V and VI to ovulated egg stage (Fig. 3A). It is possible that the difference in signal between oocytes and eggs is due to the presence of follicle cell around the oocyte, because our method used here might not be sufficient to remove all of the follicle cells completely. However, we could detect the signal in the completely defolliculated oocytes [21] (data not shown). Therefore, miRNAs of group (I) would be expressed generally throughout the development.

miRNAs of group (II) were clearly detectable at definite stages. An intense miR-367 signal was observed at the neurula (stage 18) (Fig. 2B). In miR-427, which was cloned from stage 9 to stage 22 (Table 2), an intense ~22 nt signal was observed definitely from stage 9 to stage 26 (Fig. 2B). A more intense signal of ~70 nt was detected at stage 9. This ~70 nt band is probably a miRNA precursor, because animal miRNA precursors are usually ~70 nt in length [1]. The ~70 nt signal gradually weakened and was difficult to detect after stage 26. These data suggest that miR-427 is transcribed only at the blastula and gastrula stages, and its mature miRNA is transiently expressed only at definite developmental stages. To obtain additional support for this conclusion, we searched the EST database for miR-427 primary transcript. A large number of miR-427 primary transcripts were found in the *Xenopus* EST database. Consistent with the cloning and Northern blot data, all of these ESTs were cloned from an early gastrula library. In *Xenopus* early development, there is a midblastula transition (MBT) that involves initiation of transcription, elongation of the cell cycle, an increase in cell movement, and asynchrony of cell division [29]. MBT occurs at stage 8.5 [29] and this stage corresponds to the time when miR-427 begins to be expressed. Therefore, miR-427 might be involved in developmental transition, as is the case with lin-4 in *C. elegans* [13].

miRNAs of group (III) emerged at specific stage after MBT and were continuously expressed until the tadpole stage. Most of these miRNAs were cloned from adult mammalian organs [6]. Based on their expression patterns, these miRNAs are likely to have some roles in cell lineage decision and maintaining the cell in their differentiated state. It has been reported that non-coding RNA 7H4, which is remnant produced by the cutting of miR-206 precursor from its primary transcript by Drosha, is expressed selectively in the endplate zone of skeletal muscle and is upregulated during early postnatal development in mammals [30]. For these reasons, miR-206, which is the most abundant miRNA that we cloned, might be involved in the formation of the endplate zone of skeletal muscle.

### 3.4. Small RNA that is mapped to rRNA spacer promoter region

We cloned a 22 nt sequence that is mapped to the *Xenopus laevis* spacer promoter region. The typical eukaryotic cell contains hundreds or thousands of rRNA genes organized in head-to-tail tandem arrays located at one or a few chromosomal sites. Each rDNA repeating unit consists of the transcribed pre-rRNA region and repeated intergenic spacer (IGS). Non-coding RNAs of unknown function are transcribed from the IGS spacer promoter, which share ~90% identity with the rRNA gene promoter and terminate upstream of the gene promoter [31] (Fig. 3A).

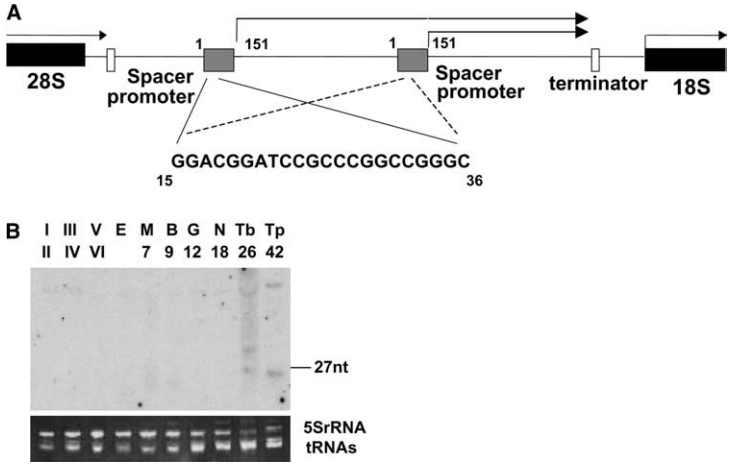


Fig. 3. Small RNA that is mapped to rRNA spacer promoter region. (A) Schematic representation of rRNA spacer and cloned small RNA sequence. Numbers given on the spacer promoter and the small RNA sequence represent the position from the first nucleotide of the spacer promoter. Arrows indicate non-coding RNA transcripts. (B) Northern blot analysis of spacer promoter small RNA. Abbreviations and loading control are described in the legend of Fig. 2.

Table 3
Unknown small RNAs that are cloned several times at different stages

| Small RNA | Sequence[a] | Number of clones | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Size {nt} | Oocyte, I–VI[b] | Egg, E[c] | Morula, 1–8[d] | Blastula, 9–12 | Gastrula, 13–17 | Neurula, 18–22 | Tailbud, 23–30 | Tadpole, 31–42 |
| Small RNA1 | CAUGCGCCACUACUGCCUGGCUCC | 24 | 9 | 8 | 6 | 7 | 9 | 9 | | |
| Small RNA2 | CAGCAGCGGGAACAGUACUGCAG | 23 | 6 | 9 | 6 | 5 | 5 | 8 | | |
| Small RNA3 | GUACCGGGCCCCCCUCGAC | 20 | 4 | | 3 | 3 | 3 | | | |
| Small RNA4 | CCUACUCCCCCGCUGUCGCC | 21 | 3 | 1 | | | 2 | | 1 | |
| Small RNA5 | CGCGACCUCAGAUGGUGGUGCA | 22 | 2 | | 2 | 2 | 1 | 1 | | |
| Small RNA6 | CGAUCCCCGGCAUCUCCACCA | 21 | 1 | 9 | 5 | 2 | 5 | | 1 | |
| Small RNA7 | CUCCUCGCAAACCAACCACGCAUG | 24 | 1 | 8 | 3 | 2 | | | | |
| Small RNA8 | GCGGGAGGGUCGGGCCCGGG | 20 | 1 | 2 | | | | | | |
| Small RNA9 | CCCACAAACCAAACACGCCCG | 21 | 1 | | 1 | | | | | |
| Small RNA10 | UCCCACCGCUGCCACCA | 17 | 1 | | 1 | | | | | |
| Small RNA11 | CCUCCACCGCGGACGUCG | 18 | 1 | | | 2 | | | | |
| Small RNA12 | CCCGCGCCACUACUGCCUGGCUCC | 24 | 1 | | | | 1 | | | |
| Small RNA13 | CGCGCGGGCGGGGCCGGGCCA | 23 | | 2 | 1 | | | | | |
| Small RNA14 | CUGCAUUCGCACGACUCACU | 20 | | 1 | 1 | | | | | |
| Small RNA15 | CCUCCUCCCCCCCGCUGUCGCC | 22 | | | | 5 | 1 | 1 | | |
| Small RNA16 | CUCGCAAACCAACCACGCAUG | 21 | | | | 1 | 1 | | 1 | |

[a]The most frequently obtained sequences are listed.
[b]Roman numerals represent developmental stages at oocyte according to Dumont [20].
[c]E represents ovulated egg.
[d]Arabic numbers represent developmental stages of embryos according to Nieukwoop and Faber [21].

Recent studies have found two types of small RNA that regulate gene expression at the transcriptional level in mammalian cells. One type is smRNAs [10] and the other is siRNAs that induce methylation at the promoter region of DNA [11]. A distinct 22 nt band was detected at the tailbud and tadpole stages by Northern blot using a probe that was antisense to our cloned sequence (Fig. 3B). Therefore, it is possible that our cloned sequence is a functional small RNA such as endogenous smRNA or siRNA rather than a degradation product. However, we cannot exclude the possibility that this signal comes from cross-reaction to a GC-rich 22 nt RNA, as our cloned small RNA showed high GC content (86.4%).

### 3.5. Unknown small RNA that was cloned several times at different stages

Among the unknown sequences that we cloned (Table 1), 16 sequences were cloned several times at different stages (Table 3). Most of these were maternally expressed and were not found at the last stage. These clones had 17–24 nt in length, and had high contents of G and C with an average of 71.6%. These small RNAs do not seem to be miRNAs because miRNAs are usually 21–23 nt in length and do not have such high GC contents [32]. As all of these sequences except small RNA11, which was only 18 nt in length and was found in bacterial genome, were not found in the database of vertebrate and other genomes, we do not know whether any of them are siRNAs or other functional small RNAs. The functions of these unknown small RNAs might become clear after the *Xenopus* genome project is completed.

In summary, in the present study, the expression profiles of miRNAs during *Xenopus* development were revealed. One of the newly identified miRNAs was expressed transiently after MBT, and 17 of 24 examined miRNAs emerged at specific stages of development and were continuously expressed until the tadpole stage. The expression patterns of these 17 miRNAs suggest that they are involved in differentiation. MicroRNAs are differentially expressed during development.

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