Xenopus microRNA genes are predominantly located within introns and are differentially expressed in adult frog tissues via post-transcriptional regulation

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The amphibian *Xenopus* provides a model organism for investigating microRNA expression during vertebrate embryogenesis and development. Searching available *Xenopus* genome databases using known human pre-miRNAs as query sequences, more than 300 genes encoding 142 *Xenopus tropicalis* miRNAs were identified. Analysis of *Xenopus tropicalis* miRNA genes revealed a predominate positioning within introns of protein-coding and nonprotein-coding RNA Pol II-transcribed genes. MiRNA genes were also located in pre-mRNA exons and positioned intergenically between known protein-coding genes. Many miRNA species were found in multiple locations and in more than one genomic context. MiRNA genes were also clustered throughout the genome, indicating the potential for the cotranscription and coordinate expression of miRNAs located in a given cluster. Northern blot analysis confirmed the expression of many identified miRNAs in both *X. tropicalis* and *X. laevis*. Comparison of *X. tropicalis* and *X. laevis* blots revealed comparable expression profiles, although several miRNAs exhibited species-specific expression in different tissues. More detailed analysis revealed that for some miRNAs, the tissue-specific expression profile of the pri-miRNA precursor was distinctly different from that of the mature miRNA profile. Differential miRNA precursor processing in both the nucleus and cytoplasm was implicated in the observed tissue-specific differences. These observations indicated that post-transcriptional processing plays an important role in regulating miRNA expression in the amphibian *Xenopus*.

[Supplemental material is available online at www.genome.org.]

MicroRNAs are small noncoding RNAs of between 19 and 23 nucleotides in length that play fundamental roles in regulating gene expression in both plants and animals (Carrington and Ambros 2003; Bartel 2004; Valencia-Sanchez et al. 2006). MiRNAs base pair with messenger RNA and the established RNA:RNA duplex promotes mRNA cleavage and/or inhibition of translation, thus down-regulating the synthesis of the encoded protein. Estimates of miRNAs in a given organism number in the hundreds with each miRNA targeting its own set of 1–50 messenger RNAs (John et al. 2004; Bentwich 2005). It is now estimated that ~30% of human protein-coding genes are post-transcriptionally regulated by miRNAs (Lewis et al. 2005; Rajewsky 2006).

MiRNAs are well conserved in evolution, particularly in animals, suggesting that they play conserved functions in different organisms (Pasquinelli et al. 2000; Chen and Rajewsky 2007). They are often expressed in a cell-specific manner or during different stages of development, thus suggesting regulatory roles in cellular differentiation and organism development (Ason et al. 2006; Sood et al. 2006). More recently, some miRNAs have also been implicated in human cancer, heart failure, and neurodegenerative diseases (Bilen et al. 2006; Kloosterman and Plasterk 2006; van Rooij et al. 2006).

Analysis of miRNA genes has revealed a variety of genomic organizations (Lai et al. 2003; Rodriguez et al. 2004; Griffiths-Jones et al. 2006). A large fraction of animal miRNAs are encoded within introns of protein-coding genes, most often on the coding strand of the pre-mRNA. A few are located with pre-mRNA exons, while others are positioned within intergenic regions. Most pri-miRNAs are transcribed by RNA Pol II and subsequently polyadenylated (Cai et al. 2004; Lee et al. 2004) before cleavage in the nucleus by the RNase III endonuclease Drosha, which is found in the microprocessor complex (Seitz and Zamore 2006). The pre-miRNA is then exported to the cytoplasm using the exportin 5 pathway (Lund et al. 2004). It is further cleaved by the endonuclease Dicer and the resulting ~22-nt miRNA/miRNA* duplex is incorporated into the RNA-induced silencing or RISC complex (Tang 2005).

A number of approaches have been used to define miRNAs in various organisms. The cloning and sequencing of total small RNAs of appropriate size from isolated tissues or whole organisms has led to the identification of many miRNA species (for review, see Berezikov et al. 2006). Alternatively, computational approaches such as miRscan and miRseeker (Lai et al. 2003; Lim et al. 2003a,b) have identified miRNAs using either homology searches or finding potential RNA hairpins that exhibit those features characteristic of the double-stranded stem–loop structures of the folded pre-miRNA (Rodriguez et al. 2004; Weber 2005). A distinct advantage of computational searching is the ability to identify miRNAs that are often only expressed in specific tissues or stages of development or at low-copy number that may be missed by cloning and sequencing approaches.

The objective of this study was to computationally identify *Xenopus* miRNA genes, characterize their genomic organization, and then examine their expression in adult frog tissues. We present here 142 *Xenopus* miRNAs homologs. Like other vertebrates, ~75% of these miRNAs are located within pre-mRNA introns. Analysis of miRNA expression in both *Xenopus tropicalis* and *Xenopus laevis* revealed the differential expression of some

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Table 1. *Xenopus tropicalis* microRNAs

MiRNA	Sequence	Length (nt)	MiRNA	Sequence	Length (nt)
xtr-let-7a	UGAGGUAGUAGGUUGUAUAGUU	22	*xtr-mir-128a*	UCACAGUGAACCGGUCUCUUUU	22
xtr-let-7b	UGAGGUAGUAGUUUGUGUAGU[b]	21	*xtr-mir-129*	CUUUUUGCGGUCUGGGCUUGC	21
xtr-let-7c	UGAGGUAGUAGGUUGUAUGGUU	22	*xtr-mir-130a*	CAGUGCAAUGUUAAAAGGGCAU	22
xtr-let-7e	UGAGGUAGUAGGUUGUUUAGUU[b]	22	*xtr-mir-130b*	CAGUGCAAUGAUGAAAGGGCAU	22
xtr-let-7f	UGAGGUAGUAGAUUGUAUAGUU	22	*xtr-mir-130c*	CAGUGCAAUAUUAAAAGGGCAU	22
xtr-let-7g	UGAGGUAGUUGUUUGUACAGU	21	*xtr-mir-132*	UAACAGUCUACAGCCAUGGUCG	22
xtr-let-7i	UGAGGUAGUAGUUUGUGCUGU	21	*xtr-mir-133a*	UUGGUCCCCUUCAACCAGCUGU	22
xtr-mir-1a	UGGAAUGUAAAGAAGUAUGUA	21	*xtr-mir-133b*	UUGGUCCCCUUCAACCAGCUA	22
xtr-mir-1b	UGGAAUGUUAAGAAGUAUGUA	21	*xtr-mir-133c*	UUGGUCCCCUUCAACCAGCUGC	22
xtr-mir-7	UGGAAGACUAGUGAUUUUGUUG	22	*xtr-mir-133d*	UUGGUCCCCUUCAACCAGCCGC	22
xtr-mir-9a	UCUUUGGUUAUCUAGCUGUAUGA	23	*xtr-mir-135*	UAUGGCUUUUUAUUCCUAUGUGA	23
xtr-mir-9a*	UAAAGCUAGAUAACCGAAAGU	21	*xtr-mir-137*	UAUUGCUUAAGAAUACGCGUAG	22
xtr-mir-10a	UACCCUGUAGAUCCGAAUUUGUG	23	*xtr-mir-138*	AGCUGGUGUUGUGAAUC	17
xtr-mir-10b	UACCCUGUAGAACCGAAUUUGU	22	*xtr-mir-139*	UCUACAGUGCAUGUGUCU	18
xtr-mir-15a	UAGCAGCACAUAAUGGUUUGUG	22	*xtr-mir-140*	AGUGGUUUUACCCUAUGGUAG	21
xtr-mir-15b	UAGCAGCACAUCAUGAUUUGCA	22	*xtr-mir-142-5p*	CAUAAAGUAGAAAGCACUAC	20
xtr-mir-16a	UAGCAGCACGUAAAUAUUGGUG	22	*xtr-mir-142-3p*	UGUAGUGUUUCCUACUUUAUGGA	23
xtr-mir-16b	UAGCAGCACGUAAAUAUUGGGU	22	*xtr-mir-143*	UGAGAUGAAGCACUGUAGCUCG	22
xtr-mir-17-5p	CAAAGUGCUUACAGUGCAGGUAGU	24	*xtr-mir-144*	UACAGUAUAGAUGAUGUACUAC	22
xtr-mir-17-3p	ACUGCAGUGAAGGCACUUGU	20	*xtr-mir-145*	GUCCAGUUUUCCCAGGAAUCCCUU	24
xtr-mir-18a	UAAGGUGCAUCUAGUGCAGAUAG	23	*xtr-mir-146*	UGAGAACUGAAUUCCAUAGGUU	22
xtr-mir-18a*	ACUGCCCUAAGUGCUCCUUCU	21	*xtr-mir-146b*	UGAGAACUGAAUUCCAUGGACU[a]	22
xtr-mir-18b	UAAGGUGCAUCUAGUGCAGUUAG	23	*xtr-mir-147*	UUGUGUGGAAAUGCUUCUAU[a, c]	20
xtr-mir-18b*	CUGCCCUAAAUGCUCCUUUU	20	*xtr-mir-148a*	UCAGUGCAUUACAGAACUUUGU	22
xtr-mir-19b	UGUGCAAAUCCAUGCAAAACUGA	23	*xtr-mir-148b*	UCAGUGCAUCACAGAACUUUGU	22
xtr-mir-20a	UAAAGUGCUUAUAGUGCAGGUAG	23	*xtr-mir-150*	UCUCCCAACCCUUGUACCAGAG	22
xtr-mir-20b	CAAAGUGCUCAUAGUGCAGGUAGU	24	*xtr-mir-153*	UUGCAUAGUCACAAAAGUGA	20
xtr-mir-20b*	ACUGUAAUAUGGGCACUUAC	20	*xtr-mir-155*	UUAAUGCUAAUCGUGAUAGGGG	22
xtr-mir-22	AAGCUGCCAGUUGAAGAACUGU	22	*xtr-mir-181a*	AACAUUCAACGCUGUCGGUGAGU	23
xtr-mir-23a	AUCACAUUGCCAGGGAUUUCC	21	*xtr-mir-181a-1**	ACCAUCGAUCGUUGACUGUACA	22
xtr-mir-23b	AUCACAUUGCCAGGGAUUACCA	22	*xtr-mir-181a-2**	ACCAUCGGCCGUUGACUGUACC[a, d]	22
xtr-mir-24a	UGGCUCAGUUCAGCAGGAACAG	22	*xtr-mir-181b*	AACAUUCAUUGCUGUCGGUGGG	22
xtr-mir-25	CAUUGCACUUGUCUCGGUCUGA	22	*xtr-mir-182*	UUUGGCAAGGUAGAACUCACA	22
xtr-mir-26	UUCAAGUAAUCCAGGAUAGGC	21	*xtr-mir-182**	UGGUUCUAGACUUGCCAACUA	21
xtr-mir-27a	UUCACAGUGGCUAAGUUCCGC	21	*xtr-mir-183*	UAUGGCACGGUAGAAUUCACUG	22
xtr-mir-27c	UUCACAGUGGCUAAGUUCUGC	21	*xtr-mir-189*	GUGCCUACUGAACUGAUAUCAGU	23
xtr-mir-27b	UUCACAGUGGCUAAGUUCUGC	21	*xtr-mir-192*	AUGACCUAUGAAUUGACAGCC	21
xtr-mir-29a	UAGCACCAUUUGAAAUCGGUU	21	*xtr-mir-194-1*	UGUAACAGCAACUCCAUGUGGA	22
xtr-mir-29b	UAGCACCAUUUGAAAUCAGUGUU	23	*xtr-mir-196a*	UAGGUAGUUUCAUGUUGUUGG	22
xtr-mir-29c	UAGCACCAUUUGAAAUCGGUUAU	23	*xtr-mir-199a*	CCCAGUGUUCAGACUACCUGUUC	23
xtr-mir-29d	UAGCACCAUAUGAAAUCAGUGUU	23	*xtr-mir-199a**	UACAGUAGUCUGCACAUUGGUU	22
xtr-mir-30a-5p	UGUAAACAUCCUCGACUGGAAG	22	*xtr-mir-200a*	UAACACUGUCUGGUAACGAUGU	22
xtr-mir-30a-3p	CUUUCAGUCAGAUGUUUGCAGC	22	*xtr-mir-200b*	UAAUACUGCCUGGUAAUGAUGAC	23
xtr-mir-30b	UGUAAACAUCCUACACUCAGC	22	*xtr-mir-202*	AGGGGCAAAGUGCAUGGGAAAA	22
xtr-mir-30c	UGUAAACAUCCUACACUCUCAGC	23	*xtr-mir-202**	UUUCCUAUGCAUAUACCUCUUU	22
xtr-mir-30d	UGUAAACAUCCCCGACUGGAAG	22	*xtr-mir-204*	UUCCCUUUGUCAUCCUAUGCCU	22
xtr-mir-30d*	CUUUCAGUCUGGUGUUUGCUGC	22	*xtr-mir-206*	UGGAAUGUAAGGAAGUGUGUGG	22
xtr-mir-30e	UGUAAACAUCCUUGACUGGAAG	22	*xtr-mir-208*	AUAAGACGAGCAUAAAGCUUGU	22
xtr-mir-30e*	CUUUCAGUCGGAUGUUUACAGC	22	*xtr-mir-210*	CUGUGCGUGUGACAGCGGCUAA	22
xtr-mir-31	GGCAAGAUGUUGGCAUAGCUG	21	*xtr-mir-214*	ACAGCAGGCACAGACAGGCAG	21
xtr-mir-31b	GGCAAGAUGCUGGCAAGCU[a]	20	*xtr-mir-215*	AUGACCUAUGAAAUGACAGCC	21
xtr-mir-33a	GUGCAUUGUAGUUGCAUUG	19	*xtr-mir-217*	UACUGCAUCAGGAACUGAUUGGAU	24
xtr-mir-34a	UGGCAGUGUCUUAGCUGGUUGUU	23	*xtr-mir-218*	UUGUGCUUGAUCUAACCAUGU	21
xtr-mir-34b	AGGCAGUGUAGUUAGCUGAUUGU[b]	23	*xtr-mir-221*	AGCUACAUUGUCUGCUGGGUUUC	23
xtr-mir-92a	UAUUGCACUUGUCCCGGCCUG	21	*xtr-mir-222*	AGCUACAUCUGGCUACUGGGUCUC	24
xtr-mir-93a	AAAGUGCUGUUCGUGCAGGUAG	22	*xtr-mir-301*	CAGUGCAAUAGUAUUGUCAAAGC	23
xtr-mir-93b	AAGUGCUGUUCGUGCAGGUAG	22	*xtr-mir-320*	AAAAGCUGGGUUGAGAGGUGA[a]	21
xtr-mir-96	UUUGGCACUAGCACAUUUUUGC	22	*xtr-mir-338*	UCCAGCAUCAGUGAUUUUGUUGG	23
xtr-mir-98	UGAGGUAGUAAGUUGUGUGGU	22	*xtr-mir-363-5p*	CGGGUGGAUCACGAUGCAAUUU	22
xtr-mir-99	AACCCGUAGAUCCGAUCUUGUG	22	*xtr-mir-363-3p*	AAUUGCACGGUAUCCAUCUGUA	22
xtr-mir-100	AACCCGUAGAUCCGAACUUGUG	22	*xtr-mir-365*	UAAUGCCCUAAAAAUCCUUAU	22
xtr-mir-101a	UACAGUACUGUGAUAACUGAAG	22	*xtr-mir-375*	UUUGUUCGUUCGGCUCGCGUUA[a]	22
xtr-mir-103	AGCAGCAUUGUACAGGGCUAUGA	23	*xtr-mir-383*	AGAUCAGAAGGUGAUUGUGGCU	22
xtr-mir-106	CAAAGUGCUUAUAGUGCAGGUAG	23	*xtr-mir-427-1*	GAAAGUGCUUUCUGUUUUGGGCG[a]	23
xtr-mir-107	AGCAGCAUUGUACAGGGCUAUCA	23	*xtr-mir-427-2*	GAAAGUGCUUUCUGUUUUGGACG[a]	23
xtr-mir-122	UGGAGUGUGACAAUGGUGUUUGU	23	*xtr-mir-427-3*	GAAAGUGCUUUCUGCUUUUGGGCG[a]	23
xtr-mir-124	UAAGGCACGCGGUGAAUGCCA	21	*xtr-mir-428*	UAAGUGCUCUCUAGUUCGGUUG	22
xtr-mir-125a	UCCCUGAGACCCUUAACCUGUG	23	*xtr-mir-429*	UAAUACUGUCUGGUAAUGCCG	21
xtr-mir-125b	UCCCUGAGACCCUAACUUGUGA	22	*xtr-mir-451*	AAACCGUUACCAUUACUGAGUUU	23
xtr-mir-126	UCGUACCGUGAGUAAUAAUGC	21	*xtr-mir-455*	UAUGUGCCUUUGGACUACAUCG	22
xtr-mir-126*	CAUUAUUACUUUUGGUACGCG	21	*xtr-mir-499*	UUAAGACUUGCAGUGAUGUUUAA	23

[a]New miRNAs species identified in this study.
[b]Potentially different annotation if seed sequence conservation is considered. *xtr-let-7b* [UGAGGUAGUAGUUUGUGUAGU] could be *xtr-let-7g*; *xtr-let-7e* [UGAGGUAGUAGGUUGUUUAGUU] could be xtr-let-7a.
[c]Sequence [UUGUGUGGGAAAUGCUUCUAU] is predicted to be *xtr-mir-147*; accession number is pending experimental validation.
[d]Sequence [ACCAUCGGCCGUUGACUGUACC] is temporally designated as pre-miRNA *xtr-mir-181a-2**; accession number not assigned.

Table 2. Genomic organization of identified *Xenopus tropicalis* microRNAs

INTRONIC MiRNAs (95)

xtr-let-7b	xtr-mir-20a	xtr-mir-125a	xtr-mir-148b	xtr-mir-208
xtr-let-7f	xtr-mir-26	xtr-mir-125b	xtr-mir-150	xtr-mir-214
xtr-let-7g	xtr-mir-27a	xtr-mir-126	xtr-mir-153	xtr-mir-215
xtr-let-7i	xtr-mir-29b	xtr-mir-126*	xtr-mir-155	xtr-mir-218
xtr-mir-1a	xtr-mir-30b	xtr-mir-130a	xtr-mir-181a	xtr-mir-301
xtr-mir-1b	xtr-mir-30e-5p	xtr-mir-130b	xtr-mir-181a-1*	xtr-mir-320
xtr-mir-7	xtr-mir-30e-3p	xtr-mir-130c	xtr-mir-181a-2*	xtr-mir-338
xtr-mir-9a	xtr-mir-31	xtr-mir-133a	xtr-mir-181b	xtr-mir-363
xtr-mir-9a*	xtr-mir-31b	xtr-mir-133c	xtr-mir-182	xtr-mir-363*
xtr-mir-15a	xtr-mir-33a	xtr-mir-133d	xtr-mir-182*	xtr-mir-383
xtr-mir-15b	xtr-mir-34a	xtr-mir-135	xtr-mir-194	xtr-mir-428
xtr-mir-16a	xtr-mir-34b	xtr-mir-139	xtr-mir-196a	xtr-mir-429
xtr-mir-16b	xtr-mir-92a	xtr-mir-140	xtr-mir-199a	xtr-mir-451
xtr-mir-17-5p	xtr-mir-93a	xtr-mir-142-5p	xtr-mir-199a*	xtr-mir-455
xtr-mir-17-3p	xtr-mir-93b	xtr-mir-142-3p	xtr-mir-200a	xtr-mir-499
xtr-mir-18a	xtr-mir-96	xtr-mir-143	xtr-mir-200b	
xtr-mir-18a*	xtr-mir-98	xtr-mir-145	xtr-mir-202	
xtr-mir-18b	xtr-mir-103	xtr-mir-146	xtr-mir-202*	
xtr-mir-18b*	xtr-mir-106	xtr-mir-146b	xtr-mir-204	
xtr-mir-19b	xtr-mir-107	xtr-mir-148a	xtr-mir-206	

INTERGENIC MiRNAs (22)

xtr-let-7c	xtr-mir-27b	xtr-mir-30d*	xtr-mir-147	xtr-mir-427-2
xtr-mir-10a	xtr-mir-29a	xtr-mir-122	xtr-mir-189	xtr-mir-427-3
xtr-mir-10b	xtr-mir-29c	xtr-mir-124	xtr-mir-221	
xtr-mir-23b	xtr-mir-29d	xtr-mir-132	xtr-mir-375	
xtr-mir-24a	xtr-mir-30d	xtr-mir-138	xtr-mir-427-1	

EXONIC MiRNAs (10)

xtr-mir-20b	xtr-mir-22	xtr-mir-133b	xtr-mir-192	xtr-mir-222
xtr-mir-20b*	xtr-mir-99	xtr-mir-183	xtr-mir-210	xtr-mir-365

INTRONIC AND INTERGENIC MiRNAs (6)

xtr-let-7e	xtr-mir-30c	xtr-mir-128a	
xtr-mir-27c	xtr-mir-101a	xtr-mir-137	

INTRONIC AND EXONIC MiRNAs (8)

xtr-let-7a	xtr-mir-25	xtr-mir-30a-3p	xtr-mir-129
xtr-mir-23a	xtr-mir-30a-5p	xtr-mir-100	xtr-mir-217

MiRNAs ACROSS-INTRON/EXON JUNCTION (1)

xtr-mir-144

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miRNAs in different adult tissues, suggesting significant post-transcriptional regulation of miRNA expression. These initial investigations also help to establish *Xenopus* as a model amphibian for further investigations of miRNA biogenesis and function during development.

Results

Computational identification of *Xenopus* microRNAs

Human pre-miRNA sequences were obtained from the microRNA database (miRBase: Giffiths-Jones et al. 2006) of the Sanger Institute and used as queries to search for *Xenopus* miRNA homologs using available *X. tropicalis* databases. Although still under construction, the *X. tropicalis* genome is most complete, consisting of DNA scaffolds assembled from overlapping contig sequences (Klein et al. 2002). Three different *X. tropicalis* databases were used (JGI, Ensembl, and the UCSC *X. tropicalis* genome server) as well as available EST databases (TIGR, Sanger, and the NCBI databases) (Smalheiser 2003; Li et al. 2006). The homology search was carried out simultaneously using all of the genomic and EST databases (see Supplemental Fig. 1 for the search strategy).

A total of 142 putative *X. tropicalis* miRNAs were identified in our computational search (Table 1; Supplemental Table 1).

Included in these 142 species are 28 of the 29 homologs previously shown to be differentially expressed in *X. laevis* during embryogenesis (Watanabe et al. 2005). During the course of this investigation, many of our identified miRNAs also appeared on the Sanger microRNA registry (http://microrna.sanger.ac.uk/cgi-bin/sequences/mirna_summary.pl?org=xtr).

Each of the identified *Xenopus* miRNAs is able to fold into the hairpin–loop structure characteristic of a folded pre-miRNA. Folding analysis was first accomplished using Mfold (Zuker 2003) and the resulting secondary structures manually adjusted using more recently established criteria for pre-miRNA identification as detailed in the Methods and Supplemental Figure 1. (The folding of those *X. tropicalis* miRNAs not exhibiting 100% miRNA similarity with its human homolog are shown folded as pre-miRNAs in Supplemental Table 1.) These computational approaches were most productive in identifying miRNAs for *X. tropicalis*. However, some *X. laevis* miRNAs were also identified in our search and they are designated in Supplemental Tables 2 and 3.

Xenopus miRNA-coding sequences are predominantly located within introns of RNA Pol II-transcribed host genes

The available *X. tropicalis* genome databases enabled a determination of miRNA genomic context for all 142 *X. tropicalis* species
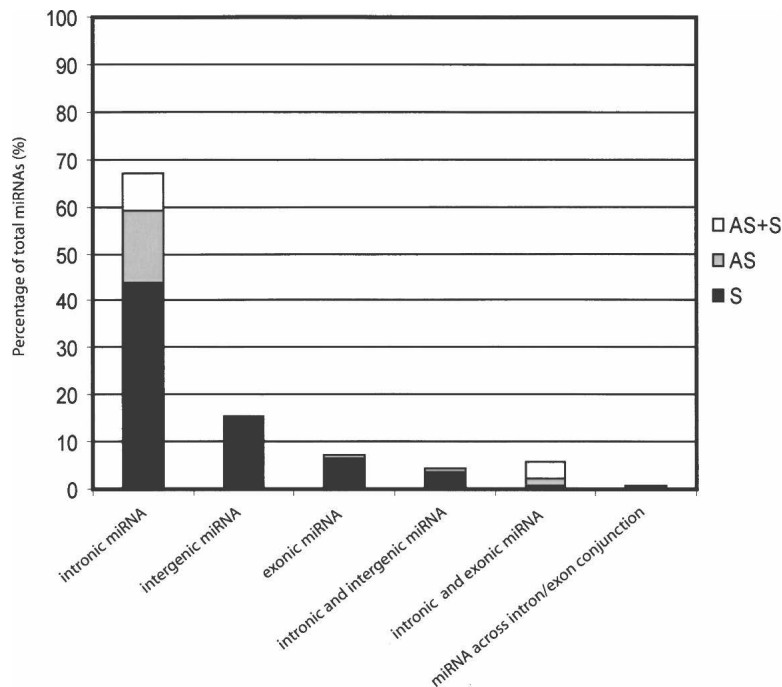
Figure 1. Genomic distribution of identified *Xenopus tropicalis* microRNAs. Graphical representation of the *X. tropicalis* miRNA's genomic organization. For intronic miRNAs, the solid sector of the bar indicates the proportion of miRNA genes positioned on the coding strand (S), the gray sector indicates the proportion of miRNA genes located on the noncoding strand (AS), and the white sector indicates miRNA genes found on both coding and noncoding strands (AS + S).

(Supplemental Table 2). Approximately 90% of the miRNAs are found exclusively in a single genomic context even if a given miRNA is present in the genome in multiple copies. The predominant context is miRNA positioning within introns (77%) of RNA Pol II-transcribed genes (Table 2; Fig. 1). A few are located in pre-mRNA exons (~7%) with slightly more located within intergenic regions (~15%).

The *X. tropicalis* intronic miRNAs can be grouped into three types. Some miRNAs are located within introns of known protein-coding genes. Others are encoded in putative introns, predicted to be within hypothetical protein-coding sequences (designated in Supplemental Table 2 by scaffold or protein ID numbers). MicroRNAs were also identified within designated introns of "non-spliced EST" sequences (Mott 1997) as listed in the UCSC and Ensembl genome server data bases (see Supplemental Table 3). The transcription of miRNA precursors for those encoded in known protein-coding sequences and those identified in the EST database is clear. Transcription of miRNAs encoded in hypothetical putative protein-coding sequences may be less certain. However, a majority of these are also found in the EST database, indicating that they are also transcribed (Supplemental Table 3). Comparison of the *Xenopus* intronic miRNAs with their human homologs revealed strong conservation in genomic location (see Supplemental Table 4).

Xenopus miRNA genes are clustered in the genome

Analysis of miRNA gene location within the *X. tropicalis* genome frequently revealed multiple miRNAs clustered on a given DNA scaffold. Almost 50% of the 142 identified *X. tropicalis* miRNA species were observed to occur at least once in a miRNA cluster (Table 3). For many of the miRNAs appearing in a cluster, addi-

tional copies of the same miRNA are often found elsewhere as a single, nonclustered miRNA-coding sequence. A given cluster could be a simple repeat of a single miRNA species (e.g., *xtr-mir-137/ xtr-mir-137 cluster* [Xentr3/scaffold 36] and *xtr-mir-202/xtr-mir-202* [Xentr3/ scaffold 305]). However, the vast majority of clusters possessed different miRNAs. Interestingly, several clusters were found to be repeated in the *Xenopus* genome. Repeated clusters could be identical in miRNA composition, such as the *xtr-mir-34b/xtr-mir-34b* cluster found on two different DNA scaffolds (Xentr3/ scaffolds 194, 350, and 366). Also observed was a *X. tropicalis* miRNA *xtr-mir-17/xtr-mir-92* cluster that is designated the "oncogene cluster" in human (He et al. 2005). This cluster possesses six miRNAs (*xtr-mir-17-5p/xtr-mir-17-3p/xtr-mir-18a/xtr-mir-18a*/xtr-mir-20a/xtr-mir-92*; Xentr3/scaffold 894).

A particularly striking example of miRNA clustering was the *Xenopus xtr-mir-427* coding sequences. We have identified ~80 *xtr-mir-427* genes in the *X. tropicalis* genome (Supplemental Table 2), many of them clustered on specific DNA scaffolds as shown in Figure 2. The length of DNA scaffold containing *xtr-mir-427* miRNAs varied greatly from only several hundred to more than 100,000 bp. The close proximity of some clustered miRNAs suggests cotranscription in a single pri-miRNA transcript, whereas distantly positioned miRNAs may suggest otherwise. Strikingly, analysis of available *X. tropicalis* EST data indicates that all but two indicated clusters could well be transcribed as a single, common precursor transcript (designated as +ESTs). Even more striking was the observation that some of the miRNAs within a cluster were absent from the EST (open triangles), indicating differential processing of the pri-miRNA transcript.

Expression of *X. tropicalis* and *X. laevis* miRNAs

Total RNA was prepared from *X. tropicalis* and *X. laevis* adult tissues and miRNA expression assessed using Northern blot analysis. Of the 63 miRNAs assessed, 47 species or 77% (designated in Supplemental Table 1) were observed in one or more of the tissue-specific RNA preparations (38 of these are presented in Fig. 3). Those miRNAs tested but not detected may be expressed in other tissues not examined, expressed during specific stages of embryogenesis, or expressed at such low levels as to make detection by Northern blot analysis difficult. Most of the observed miRNAs were expressed in almost all of the adult tissues assayed. The lack of miRNA expression in oocytes is consistent with miRNA roles in regulating gene expression during embryogenesis and organism development.

The expression patterns were comparable for most of the miRNAs in both *X. tropicalis* and *X. laevis* tissues. However, apparent quantitative differences in the expression patterns of some miRNAs were observed between the two different *Xenopus* species. These miRNAs included *xtr-mir-98, xtr-mir-133a, xtr-mir-*

Table 3. *Xenopus tropicalis* microRNA clusters

miRNA gene cluster	Cluster location	Strand (− /+)	Length (nt)
xtr-let-7a/xtr-mir-125a	Xentr3/scaffold_285: 948731–961757	+	13,027
xtr-mir-1a/xtr-mir-133c	Xentr3/scaffold_220: 919435–941946	+	22,512
xtr-mir-1a/xtr-mir-133a	Xentr3/scaffold_549: 334888–337393	+	2505
xtr-mir-1b/xtr-mir-133d	Xentr3/scaffold_977: 368847–369356	−	509
xtr-mir-15a/xtr-mir-16a	Xentr3/scaffold_643: 310251–310484	−	233
xtr-mir-15b/xtr-mir-16b	Xentr3/scaffold_150: 310275–310469	−	193
xtr-mir-106/xtr-mir-18b/xtr-mir-18b*/xtr-mir-19b /xtr-mir-20b/ xtr-mir-20b*/xtr-mir-92a/xtr-mir-363-5p /xtr-mir-363-3p	Xentr3/scaffold_212: 656631–657356	+	725
xtr-mir-17-5p/xtr-mir-17-3p/xtr-mir-18a/xtr-mir-18a* /xtr-mir-20a/xtr-mir-92/	Xentr3/scaffold_894: 85148–85887	−	633
xtr-mir-23b/xtr-mir-24a/xtr-mir-27b	Xentr3/scaffold_528: 833673–834573	+	935
xtr-mir-27a/xtr-mir-23a	Xentr3/scaffold_829: 483579–484270	−	691
xtr-mir-27c/xtr-mir-27c	Xentr3/scaffold_1:7020632–7028355	−	7723
xtr-mir-25/xtr-mir-93b	Xentr3/scaffold_814: 522886–523081	−	194
xtr-mir-29a/xtr-mir-29/xtr-mir-129	Xentr3/scaffold_20: 657670–660400	+	70,450
xtr-mir-29b/xtr-mir-29c	Xentr3/scaffold_393: 249789–253020	+	3232
xtr-mir-30a/xtr-mir-30c	Xentr3/scaffold_67: 1391637–1398956	−	7319
xtr-mir-30b/xtr-mir-30d	Xentr3/scaffold_90: 1522361–1526686	−	4326
xtr-mir-30c/xtr-mir-30e	Xentr3/scaffold_399: 513153–514163	−	1011
xtr-mir-34b/xtr-mir-34b	Xentr3/scaffold_1083: 172336–180020	+	7684
xtr-mir-34b/xtr-mir-34b	Xentr3/scaffold_18: 3288131–3294119	+	5987
xtr-mir-98/xtr-let-7f	Xentr3/scaffold_750: 538453–538808	+	427
xtr-mir-99/xtr-let-7c	Xentr3/scaffold_116: 668725–669496	+	770
xtr-mir-128a/xtr-mir-153	Xentr3/scaffold_31: 3158967–2956054	−	20,2914
xtr-mir-130b/xtr-mir-130c	Xentr3/scaffold_118: 53604–61323	−	7718
xtr-mir-137/xtr-mir-137	Xentr3/scaffold_36: 1251053–1267094	−	16,032
xtr-mir-142-5p/xtr-mir-142	Xentr3/scaffold_100: 1012673–1013940	−	1267
xtr-mir-143/xtr-mir-145	Xentr3/scaffold_493: 665585–666622	+	1037
xtr-mir-181a/xtr-mir-181b	Xentr3/scaffold_858: 464202–464748	+	545
xtr-mir-181a/xtr-mir-181b	Xentr3/scaffold_1164: 125236–129501	+	4266
xtr-mir-183/xtr-mir-96 / xtr-mir-182	Xentr3/scaffold_20: 2863123–2867042	+	3834
xtr-mir-199a/xtr-mir-214	Xentr3/scaffold_74: 833054–838267	+	5213
xtr-mir-202/xtr-mir-202	Xentr3/scaffold_305: 963500–971292	−	7791
xtr-mir-215/xtr-mir-194	Xentr3/scaffold_39: 2646993–2647935	−	943
xtr-mir-221/xtr-mir-222	Xentr3/scaffold_143: 2026361–2027010	+	649

142-5p, and xtr-mir-143 (Fig. 3). Differences in expression patterns could reflect species-specific differences in transcription or differential pri-mRNA processing. Some clustered miRNAs exhibited comparable expression patterns (e.g., *xtr-mir-99/xtr-let-7c* cluster), consistent with cotranscription in a common pri-miRNA as previously suggested (Lagos-Quintana et al. 2001; Baskerville and Bartel 2005). However, this was not true for all miRNA clusters (see below).

Evidence for the regulated, tissue-specific expression of *X. laevis* miRNAs

More detailed examination of selected *X. laevis* miRNAs indicated their post-transcriptional regulation. Northern blot analysis of the *xtr-mir-17-5p/xtr-mir-17-3p* and *the xtr-mir-142-5p/xtr-miR142-3p* miRNAs transcribed in common pre-miRNAs revealed that while both pri-miRNA precursors were transcribed in all adult frog tissues, only *xtr-mir-17-5p* and *xtr-mir-142-5p* were observed as mature miRNAs (Fig. 4A). This suggested differential processing of the pre-miRNA in the cytoplasm to produce the miRNA found on the 5′ strand, but not on the 3′ "passenger strand". Northern blot analysis of both *xtr-mir-30c* and *xtr-mir-133a* revealed different expression patterns of the pri-miRNA precursor and the mature miRNA (Fig. 4B). The distinct tissue-specific expression patterns of precursor and mature miRNAs suggested differential pri- or pre-miRNA processing and/or differential stabilization of the mature miRNA. The observation of two pri-miRNA precursors may also suggest

the transcription of *xtr-mir-133a* pri-miRNA from different promoters or the differential processing of the pri-miRNA transcript.

Both *xtr-mir-98* and *xtr-let-7f* are clustered on a 427-nt DNA fragment (Xentr3_750) and can reasonably be assumed to be cotranscribed as a common pri-miRNA precursor transcript. The size of the pri-miRNA transcript observed is consistent with the cotranscription of these three miRNAs. Notably, while the patterns of *xtr-let-7f* and *xtr-mir-98* pri-miRNA expression are identical, the patterns of tissue-specific expression for the two mature miRNAs are clearly different (Fig. 4C). Particularly prominent is the presence of xtr-let-7f miRNA in heart tissue, but the absence of *xtr-mir-98* in the same tissue. This suggested the differential processing of the pri-miRNA precursor or the pre-miRNA intermediate to produce *xtr-let-7f* but not *xtr-mir-98*.

Finally, both mature *xtr-mir-215* and *xtr-mir-220b* were not observed in initial Northern blot analysis. However, additional examination revealed the presence of pri-miRNA primary transcripts for both miRNAs in all adult tissues and the presence of *xtr-mir-200b* pre-miRNA in some adult tissues (Fig. 4D). These observations suggested the lack of *xtr-mir-215* pri-miRNA primary transcript processing to produce either pre-miRNAs or mature miRNAs in all tissues and the differential processing of *xtr-pre-mir-200b* in some tissues, along with the possible disruption of pre-miRNA transport and/or cytoplasmic Dicer processing in all tissues.
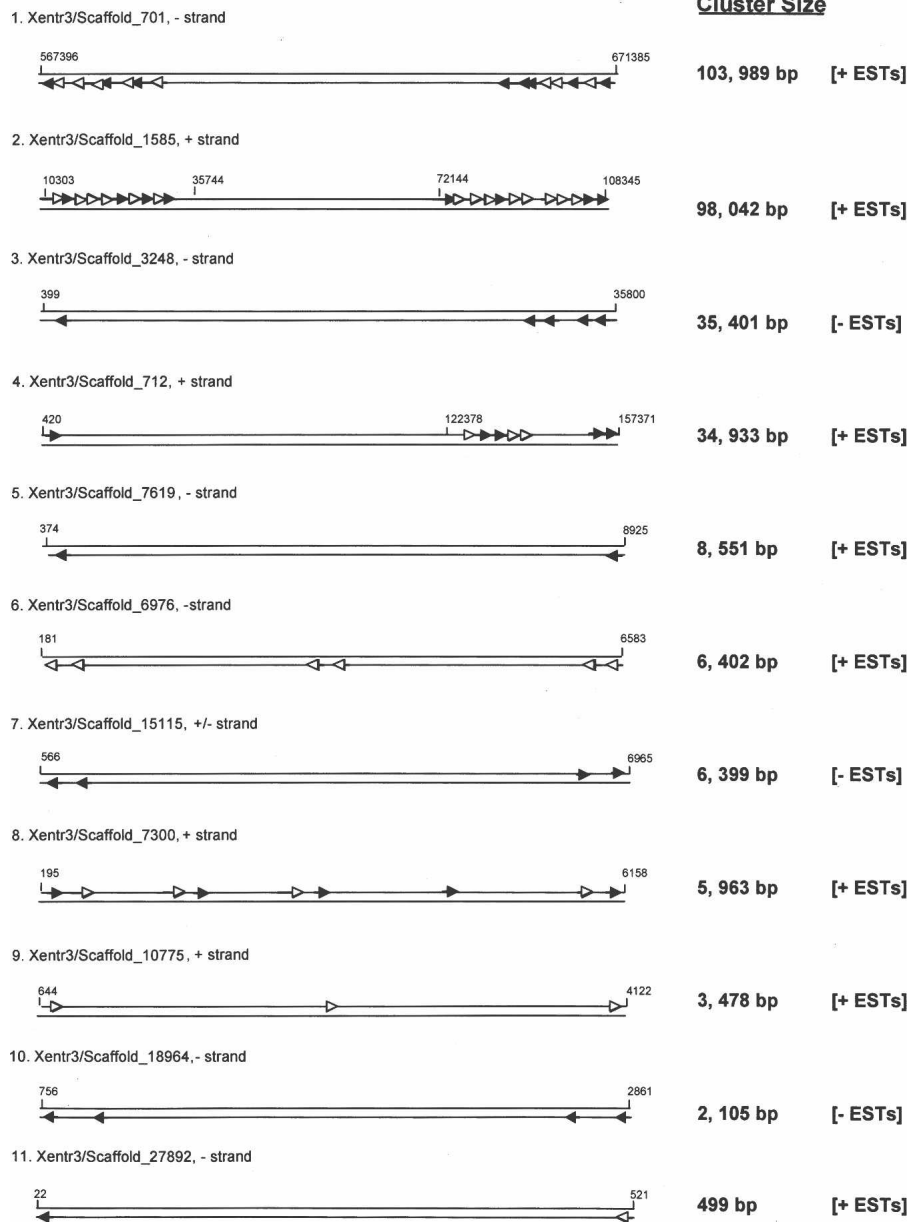
Xenopus tropicalis xtr-miR-427 clusters

Figure 2. *Xenopus tropicalis mir-427* clusters. Schematic presentation of identified *X. tropicalis mir-427* clusters. Specific scaffolds are designated with scaffold boundary nucleotides indicated at each terminus. The largest to smallest DNA scaffolds are presented from *top* to *bottom* with the scaffold total length in nucleotides indicated at the *side*. Arrows indicate individual *xtr-mir-427* genes. For some clusters, the open triangles indicate absent miRNAs in transcribed ESTs. The *top* strand of each scaffold is the +DNA strand and the *bottom* strand is the −DNA strand. +ESTs indicate evidence for cotranscription of each cluster (Supplemental Table 3).

Discussion

A computational approach using human pre-miRNA query sequences has identified more than 300 miRNA genes encoding some 142 *Xenopus* miRNA species. *Xenopus* miRNA genes are predominantly located within introns and highly clustered in the genome. Using Northern blot analysis as well as the EST data-

bases, a substantial proportion of the identified *Xenopus* miRNAs have been verified as expressed RNA transcripts. The shared expression patterns of almost all *X. tropicalis* and *X. laevis* miRNAs in the various adult tissues are consistent with highly conserved miRNA function in vertebrates. However, a few probed miRNAs exhibited tissue-specific differences in these two *Xenopus* species, suggesting perhaps species-specific miRNA expression. Most striking however, was the observation of post-transcriptional regulation of miRNA expression at both nuclear and cytoplasmic processing steps.

Xenopus miRNAs exhibit several genomic contexts, but their predominant positioning within pre-mRNA introns is similar to other vertebrates, including human, where it has been estimated that 70% of the miRNAs are intronic (Rodriguez et al. 2004). The intronic miRNAs are located within many known protein-coding host genes, but also found within putative, unidentified Pol II transcripts and within EST sequences. Notably, many of the *Xenopus* intronic miRNAs are encoded within the same host gene as their human homolog, extending their conservation beyond simply the pre-miRNA-coding sequence (Supplemental Table 4). In addition, this conservation also extends to clusters of intronic miRNAs conserved in both human and *Xenopus*. Conserved miRNA clusters within the same host gene suggest the possible coordinated expression of both host pre-mRNA and included miRNAs as previously suggested (Ying and Lin 2006).

Like other vertebrates, miRNA clustering is a prominent feature in the *Xenopus* genome. Approximately half of the identified *Xenopus* miRNAs are found in clusters in varying combinations. The clustering of *Xenopus* miRNA genes could also have important implications for the coordinated, tissue-specific expression of clustered miRNAs during development. Certainly, pri-miRNAs containing clustered miRNAs have been reported (Lau et al. 2001; Cai et al. 2004) and some *Xenopus* RNA transcripts found in the EST databases support clustered miRNA cotranscription in a common pri-miRNA. Some clustered *Xenopus* miRNAs are positioned on lengthy DNA fragments, raising the question as to the maximum size of pri-miRNA transcripts. Bartel and coworkers (Baskerville and Bartel 2005) have suggested pri-miRNAs as large as 50 kb, and some of the transcripts found in the *Xenopus* EST database support this proposition. Comparison of human miRNA clusters (Al-
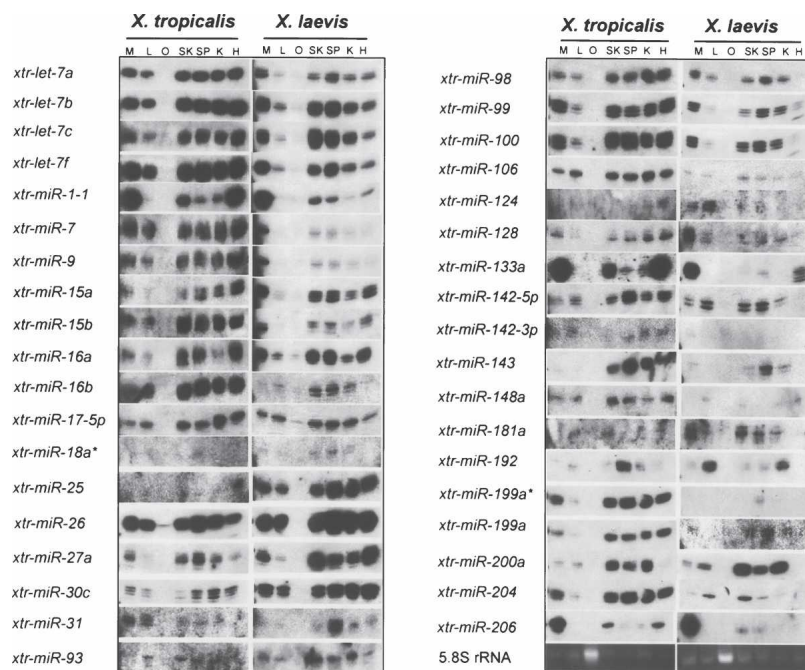
Figure 3. Northern blot validation of computationally identified *Xenopus tropicalis* and *Xenopus laevis* miRNAs. Total RNA was isolated from *X. tropicalis* and *X. laevis* oocytes and adult tissues, resolved on denaturing polyacrylamide gels, electroblotted to nylon membranes, and hybridized with DNA oligonucleotides complementary to computationally identified *Xenopus* miRNAs. Specific *Xenopus* miRNAs sequences probed with complementary DNA oligonucleotides are indicated at *left*. *Xenopus* tissues extracted for RNA analysis are indicated at the *top*. (M) Muscle; (L) liver; (O) oocyte; (SK) skin; (SP) spleen; (K) kidney; (H) heart.

tuvia et al. 2005) with the *Xenopus* miRNA clusters has revealed many of the same clustered miRNAs, yet miRNA clustering in human and *Xenopus* is not entirely conserved, possibly suggesting differences in coordinated miRNA transcription in these two vertebrates.

Of particular importance will be defining promoters for pri-miRNA transcription of intergenic, intronic, and clustered miRNAs. Analysis of miRNA synthesis has revealed that the majority of pri-miRNAs are transcribed by Pol II polymerase and possess 5′ caps and 3′ poly (A) tails (Cai et al. 2004; Lee et al. 2004), although recent work has shown that some pri-miRNAs are transcribed by RNA Pol III (Borchert et al. 2006). The predominance of intronic miRNAs has implied cotranscription with the host pre-mRNAs and pre-miRNA processing concurrent with host pre-mRNA splicing (Cai et al. 2004; for review, see Kim and Nam 2006). However, differing pre-mRNA/pri-miRNA expression profiles with respect to their mature, fully processed miRNA may suggest miRNA-specific promoters within the host gene (Thomson et al. 2007). Alternatively, regulated alternative pre-mRNA splicing coupled with intronic miRNA processing could explain the different expression profiles observed. Clearly, for those miRNAs positioned with a host gene intron but on the noncoding strand, pri-miRNA-specific promoters will be required.

Further examination of transcribed *Xenopus* miRNA sequences in the EST databases may suggest an even more complex picture of miRNA transcription and processing. Those miRNAs occurring in the EST databases are indicated in Supplemental Tables 2 and 3. These previously designated "spliced" miRNAs are flanked by upstream and downstream transcript corresponding to the genomic sequences flanking the pre-miRNA sequence.

However, far more intriguing is the occurrence of so-called "non-spliced" RNA transcripts (Mott 1997). These RNA transcripts consist of transcribed upstream and downstream genomic sequence, but lack the pre-miRNA sequence. These RNAs suggest that during transcription, a processing event has excised the pre-miRNA sequence. Fully, more than 70% of the *Xenopus* miRNAs identified in this study are found in these nonspliced EST transcripts, consistent with cotranscriptional processing of pre-miRNA sequences. For the intronic miRNAs, this may suggest a complex biogenesis pathway distinct from pre-mRNA transcriptional events.

Finally, analysis of *Xenopus* miRNA expression has revealed unanticipated expression profiles for some *Xenopus* pri-miRNAs and their processed, mature miRNAs. Differences in tissue-specific expression between the two *Xenopus* species were surprising, yet other laboratories have reported similar observations (Ason et al. 2006; Sood et al. 2006). Unfortunately, Northern blot analysis is not sufficiently quantitative to make definitive statements about miRNA amounts, and more detailed analysis awaits quantitative PCR investigation. Evidence has recently been presented indicating differential pri-miRNA processing of some clustered miRNAs (Samols et al. 2005; Obernosterer et al. 2006) and our observations also indicated the differential processing of the miR-98/*let-7f/let-7a-1* cluster. However, most striking were the distinctly different, tissue-specific expression patterns of some *Xenopus* pri-miRNAs with respect to their processed mature miRNAs. Similar observations have been made by Hammond and coworkers (Thomson et al. 2007) as well as Martinez and coworkers (Obernosterer et al. 2006). Collectively, these observations suggest significant post-transcriptional regulation in the miRNA biogenesis pathway at various processing steps in both the nucleus and cytoplasm. The consequences of such regulation could have a large impact on the ultimate expression of a specific miRNA species. Future experiments concerning miRNA transcription and post-transcriptional processing are likely to reveal a complex and highly regulated pathway for miRNA expression.

Methods

Computational identification of *Xenopus* microRNAs

Human pre-miRNA sequences were retrieved from miRbase::sequences (http://microrna.sanger.ac.uk/sequences/) and used as query sequences against available *X. tropicalis* genomic databases (Xentr3-Oct. 2004 assembly and the Xentr4-Aug. 2005 assembly with most of our reported data obtained from the 2004 assembly and designated as Xentr3). This computational approach is summarized in Supplemental Figure 1 and consisted of homology searching, alignment and annotation,
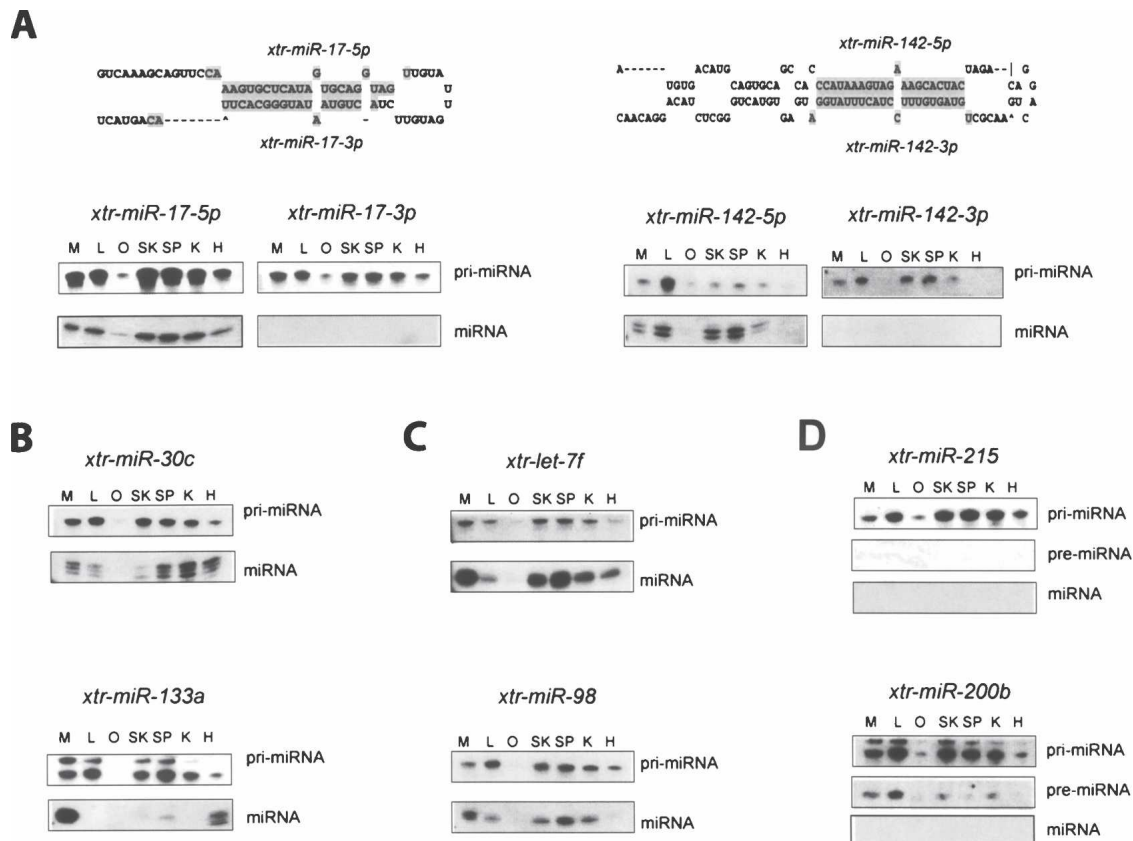
Figure 4. Differential expression of *Xenopus laevis* miRNAs and pri-miRNAs. Total RNA was isolated from *X. laevis* oocytes and adult tissues were resolved on denaturing polyacrylamide gels, electroblotted to nylon membranes, and hybridized with DNA oligonucleotides complementary to computationally identified *Xenopus* miRNAs. Specific *Xenopus* miRNAs sequences probed are indicated at the *top* and detected miRNA transcripts indicated at *right*. *Xenopus* tissues extracted for RNA analysis are indicated at the *top*. (M) muscle; (L) liver; (O) oocyte; (SK) skin; (SP) spleen; (K) kidney; (H) heart. (*A*) Differential expression of *Xenopus laevis* miRNAs located on the 5′ and 3′ arms of the same pre-miRNA. The folded structure of these pre-miRNAs is illustrated. (*B*) Tissue-specific expression of *Xenopus laevis* miRNAs. (*C*) Tissue-specific expression of *Xenopus laevis* miRNAs clustered on the same pre-miRNA transcript. (*D*) Differential expression of transcribed *Xenopus laevis* pri-miRNA precursors at the level of pre-miRNA and/or miRNA processing.

and pre-miRNA secondary structural analysis. The *X. tropicalis* genome database is presently under construction (Klein et al. 2002) and consists of sequenced DNA fragments or "scaffolds" and overlapping "contig" sequences. The three genomic databases utilized were the JGI *X. tropicalis* genome server (two servers on the JGI Web site: http://genome.jgi-psf.org/Xentr3/Xentr3.home.html and http://genome.jgi-psf.org/Xentr4/Xentr4.home.html), Ensembl *X.tropicalis* (http://www.ensembl.org/Xenopus_tropicalis/blastview), and the UCSC *X. tropicalis* genomic server (http://www.genome.ucsc.edu/cgi-bin/hgBlat). The human pre-miRNA query sequences were used in either BLAT or BLASTN algorithms for searching. An alternative search path utilized the available EST databases: TIGR's *Xenopus* EST database (http://tigrblast.tigr.org/tgi/ with options for *X. laevis* and *X. tropicalis*) and the Sanger *Xenopus* EST project (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/x_tropicalis). Homology searching was carried out in all genomic and EST databases with initial analysis revealing that pre-miRNA sequences were more useful than the shorter miRNA sequences. Preliminary results provided sequence alignments as well as the candidate *Xenopus* miRNA genomic locations with possible host genes.

Candidate *Xenopus* miRNAs were further analyzed for their folded structures with specific pre-miRNA secondary structural features being used as filters in the verification process. Identified

Xenopus sequences (60–120 nt long) were folded using the Mfold program (http://www.bioinfo.rpi.edu/applications/mfold/old/rna/) and manually adjusted according to specific criteria based upon recent analysis and definition of conserved or preferred pre-miRNA structures. These features included (1) the length of the pre-miRNA core region between 60 and 80 nt (Ambros et al. 2003), (2) a pre-miRNA hairpin stem is ≥22 nt (deduced from the listed microRNAs in the microRNA registry database), (3) a loop size of ≥10 nt (Zeng et al. 2005), (4) hairpin bulges within the base-paired stem of not more than 6 nt (Ambros et al. 2003; Weber 2005), (5) an initial preferred nucleotide of U (Lau et al. 2001), (6) well-conserved miRNA seed sequences or nucleotides 2–7 (Lewis et al. 2004), (7) total G:U base pairs within the hairpin stem of <5 nt (Bonnet et al. 2004), and (8) similar locations of the mature human and *Xenopus* microRNAs on the same hairpin arm.

Northern blot analysis

Total RNA of adult female *X. tropicalis* and *X. laevis* frogs was prepared from various harvested tissues by extraction using TRIzol reagent according to the manufacturer's protocols (Invitrogen). A total of 10 μg of total tissue RNA was resolved on 12% denaturing polyacrylamide gels containing 7 M urea and then electroblotted onto Genescreen Nylon membrane (NEN) in TBE buffer. Northern blots were dried and then incubated over-

night in 10 mL of ULTRAhyb-Oligo (Ambion) containing 5′-³²P radio-labeled oligonucleotides (21–23 nt) complementary to the predicted microRNA sequences. Prehybridization and hybridization procedures were performed at 37°C as previously detailed (Liu et al. 2005). Nylon membranes were washed three times for 10 min at room temperature in 2X SSC containing 0.5% SDS and then once for 15 min at 42°C in 2X SSC containing 0.5% SDS (Bentwich 2005). Both phosphorimaging and autoradiography were used to reveal hybridized microRNAs, pri-miRNAs, and pre-miRNAs.

Acknowledgments

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