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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)
<i>xtr-let-7a</i>	UGAGGUAGUAGGUUGUAUAGUU	22	<i>xtr-mir-128a</i>	UCACAGUGAACCCGGUCUCUUUU	22
<i>xtr-let-7b</i>	UGAGGUAGUAGUUUGUGUAGU ^b	21	<i>xtr-mir-129</i>	CUUUUUGCCGGUCUGGGCUUGC	21
<i>xtr-let-7c</i>	UGAGGUAGUAGGUUGUAUAGUU	22	<i>xtr-mir-130a</i>	CAGUGCAAUGUUAAAAGGGCAU	22
<i>xtr-let-7e</i>	UGAGGUAGUAGGUUGUUUAGUU ^b	22	<i>xtr-mir-130b</i>	CAGUGCAAUGAUGAAAAGGGCAU	22
<i>xtr-let-7f</i>	UGAGGUAGUAGAUUGUAUAGUU	22	<i>xtr-mir-130c</i>	CAGUGCAAUUAAAAGGGCAU	22
<i>xtr-let-7g</i>	UGAGGUAGUUUGUACAGU	21	<i>xtr-mir-132</i>	UAACAGUCUACAGCCAUGGUCG	22
<i>xtr-let-7i</i>	UGAGGUAGUUUGUGUCUGU	21	<i>xtr-mir-133a</i>	UUGGUCCCCUUAACCAGCUGU	22
<i>xtr-mir-1a</i>	UGGAAUGUAAAAGUAUGUA	21	<i>xtr-mir-133b</i>	UUGGUCCCCUUAACCAGCUGA	22
<i>xtr-mir-1b</i>	UGGAAUGUAAAAGUAUGUA	21	<i>xtr-mir-133c</i>	UUGGUCCCCUUAACCAGCUGC	22
<i>xtr-mir-7</i>	UGGAAAGUAGUGAUUUUGUUG	22	<i>xtr-mir-133d</i>	UUGGUCCCCUUAACCAGCUGC	22
<i>xtr-mir-9a</i>	UCUUUGGUUAUCUAGCUGUAUGA	23	<i>xtr-mir-135</i>	UAUGGCUUUUUAUCCUUAUGUGA	23
<i>xtr-mir-9a*</i>	UAAAGCUGAUAACCGAAAGU	21	<i>xtr-mir-137</i>	UAUUGCUUAAGAAUACCGCUAG	22
<i>xtr-mir-10a</i>	UACCCUGUAGAUCCGAAUUGUG	23	<i>xtr-mir-138</i>	AGCUGGUGUUGUGAAUC	17
<i>xtr-mir-10b</i>	UACCCUGUAGAAACCGAAUUGU	22	<i>xtr-mir-139</i>	UCCACAGUGCAUGUGUCU	18
<i>xtr-mir-15a</i>	UAGCAGCACAUAAUGGUUUUGUG	22	<i>xtr-mir-140</i>	AGUGGUUUUACCCUAUGGUAG	21
<i>xtr-mir-15b</i>	UAGCAGCACAUCAUGAUUUUGCA	22	<i>xtr-mir-142-5p</i>	CAUAAAAGUAGAAAGCACUAC	20
<i>xtr-mir-16a</i>	UAGCAGCACGUAUUUUGGUG	22	<i>xtr-mir-142-3p</i>	UGUAGUGUUUCCUUAUUUUGGA	23
<i>xtr-mir-16b</i>	UAGCAGCACGUAUUUUGGUG	22	<i>xtr-mir-143</i>	UGAGAUGAAGCACUGUAGCUCG	22
<i>xtr-mir-17-5p</i>	CAAAGUGCUUACAGUGCAGGUAGU	24	<i>xtr-mir-144</i>	UACAGUAUAGAUGAUGUACUAC	22
<i>xtr-mir-17-3p</i>	ACUGCAGUGAAGGCACUUGU	20	<i>xtr-mir-145</i>	GUCCAGUUUUCCAGGAAUCCCUU	24
<i>xtr-mir-18a</i>	UAAGGUGCAUCUAGUGCAGAUAG	23	<i>xtr-mir-146</i>	UGAGAACUGAAUUCCAUAGGUU	22
<i>xtr-mir-18a*</i>	ACUGCCCUAAGUGUCUUCUUCU	21	<i>xtr-mir-146b</i>	UGAGAACUGAAUUCCAUAGGACU ^a	22
<i>xtr-mir-18b</i>	UAAGGUGCAUCUAGUGCAGGUAG	23	<i>xtr-mir-147</i>	UUGUGUGGAAAUGCUUCUUAU ^{a, c}	20
<i>xtr-mir-18b*</i>	UAGCCCUAUAUGUCUUCUUUU	20	<i>xtr-mir-148a</i>	UCAGUGCAUUAACAGAAUUGU	22
<i>xtr-mir-19b</i>	UGUGCAAUCCAUUGCAAACUGA	23	<i>xtr-mir-148b</i>	UCAGUGCAUACACAGAACUUUGU	22
<i>xtr-mir-20a</i>	UAAAGUGCUUUAUAGUGCAGGUAG	23	<i>xtr-mir-150</i>	UCUCCCAACCCUUGUACCCAGAG	22
<i>xtr-mir-20b</i>	CAAAGUGCUCAUAGUGCAGGUAGU	24	<i>xtr-mir-153</i>	UGCAUAGUCACAAAGGAGA	20
<i>xtr-mir-20b*</i>	ACUGUAAUUAUGGGCACUUAAC	20	<i>xtr-mir-155</i>	UUAUAGCUAAUUCGUAUAGGGG	22
<i>xtr-mir-22</i>	AAGCUGCCAGUUUAAGAACUCGU	22	<i>xtr-mir-181a</i>	AACAUCUACCGUCUGCGGUGAGU	23
<i>xtr-mir-23a</i>	AUCACAUUGCCAGGGAUUUCC	21	<i>xtr-mir-181a-1*</i>	ACCAUCGAUCGUGUACUGUACA	22
<i>xtr-mir-23b</i>	AUCACAUUGCCAGGGAUUUACCA	22	<i>xtr-mir-181a-2*</i>	ACCAUCGCGCCUUGACUGUACC ^{a, d}	22
<i>xtr-mir-24a</i>	UGGCUCAGUUCAGCAGGAACAG	22	<i>xtr-mir-181b</i>	AACAUCUACUUGCUGUCGGUGGG	22
<i>xtr-mir-25</i>	CAUUGCAGUUGUCUCGGUCUGA	22	<i>xtr-mir-182</i>	UUUGGCAAUUGGUAAGAACUCACA	22
<i>xtr-mir-26</i>	UUCAAAGUAAUCCAGGAUAGGC	21	<i>xtr-mir-182*</i>	UGGUUUCUAGACUUGCCCAUCA	21
<i>xtr-mir-27a</i>	UUCACAGUGGCUAAGUUCGCG	21	<i>xtr-mir-183</i>	UAUGGCACUGGUAAGAUUCACUG	22
<i>xtr-mir-27c</i>	UUCACAGUGGCUAAGUUCGCG	21	<i>xtr-mir-189</i>	GUGCCUACUGAACUGAUUACAGU	23
<i>xtr-mir-27b</i>	UUCACAGUGGCUAAGUUCGCG	21	<i>xtr-mir-192</i>	AUGACCUAUGAAUUGACAGCC	21
<i>xtr-mir-29a</i>	UUUGGCACUUUGAAAUCGGUU	21	<i>xtr-mir-194-1</i>	UGUAACAGCAACUCCAUUGGGA	22
<i>xtr-mir-29b</i>	UAGCACCAUUUGAAAUCAGUGUU	23	<i>xtr-mir-196a</i>	UAGGUAGUUUUAUGUUGUUG	22
<i>xtr-mir-29c</i>	UAGCACCAUUUGAAAUCGGUUU	23	<i>xtr-mir-199a</i>	CCCAGUGUUUCAGACUACCCUUC	23
<i>xtr-mir-29d</i>	UAGCACCAUUUGAAAUCAGUGUU	23	<i>xtr-mir-199a*</i>	UACAGUAUGUCUGACAUUUGUU	22
<i>xtr-mir-30a-5p</i>	UGUAAACAUCUCCGACUGGAAG	22	<i>xtr-mir-200a</i>	UAACACUGUCUGGUAACGAUGU	22
<i>xtr-mir-30a-3p</i>	CUUUUCAGUCAGAUUUUGCAGC	22	<i>xtr-mir-200b</i>	UAAUACUGCCUGGUAUUGAGAC	23
<i>xtr-mir-30b</i>	UGUAAACAUCUCCGACUCAGCU	22	<i>xtr-mir-202</i>	AGGGGCAUAGGGCAUUGGGAAA	22
<i>xtr-mir-30c</i>	UGUAAACAUCUCCGACUCAGC	23	<i>xtr-mir-202*</i>	UUUCCUAUGCAUUAUCCUUUU	22
<i>xtr-mir-30d</i>	UGUAAACAUCUCCGACUGGAAG	22	<i>xtr-mir-204</i>	UUCCUUUGUCAUCCUUAUGCCU	22
<i>xtr-mir-30d*</i>	CUUUUCAGUCUGGUGUUUGCUCG	22	<i>xtr-mir-206</i>	UGGAAUGUAAGGAAGUGUGUGG	22
<i>xtr-mir-30e</i>	UGUAAACAUCUCCGACUGGAAG	22	<i>xtr-mir-208</i>	AUAAGACGAGCAUUAAGCUGUGU	22
<i>xtr-mir-30e*</i>	CUUUUCAGUCUGGUAUGUUACAGC	22	<i>xtr-mir-210</i>	CUGUGCGUGUGACAGCGGCUAA	22
<i>xtr-mir-31</i>	GGCAAGAUUGUGGCAUAGCUG	21	<i>xtr-mir-214</i>	ACAGCAGGCACAGACAGGCAG	21
<i>xtr-mir-31b</i>	GGCAAGAUUGUGGCAUAGCUG ^a	20	<i>xtr-mir-215</i>	AGUACCUAUGAAAUGACAGCC	21
<i>xtr-mir-33a</i>	GUGCAUUGUAGUUGCAUUG	19	<i>xtr-mir-217</i>	UACUGCAUCAGGAACUGAUUGGAU	24
<i>xtr-mir-34a</i>	UGGCAGUGUCUUAGCUGGUUGUU	23	<i>xtr-mir-218</i>	UUGUGCUUGAUUAACCAUGU	21
<i>xtr-mir-34b</i>	AGGCAGUGUAGUAGCUGAUUUGU	23	<i>xtr-mir-221</i>	AGCUACAUUGUCUGCUGGGUUUC	23
<i>xtr-mir-92a</i>	UAUUGCAGUUUGCCGCGCUG	21	<i>xtr-mir-222</i>	AGCUACAUCUGGCUACUGGGUUC	24
<i>xtr-mir-93a</i>	AAAGUGCUGUUCGUGCAGGUAG	22	<i>xtr-mir-301</i>	CAGUGCAAUAGUAUUGUCAAAAGC	23
<i>xtr-mir-93b</i>	AAGUGCUGUUCGUGCAGGUAG	22	<i>xtr-mir-320</i>	AAAAGCUGGGUUGAGAGGUGA ^a	21
<i>xtr-mir-96</i>	UUUGGCACUAGCACAUUUUUGC	22	<i>xtr-mir-338</i>	UCCAGCAUCAGUGAUUUUUGUGG	23
<i>xtr-mir-98</i>	UGAGGUAGUAAGUUGUAUUGUU	22	<i>xtr-mir-363-5p</i>	CGGGUGGAUCACGAUGCAAUUU	22
<i>xtr-mir-99</i>	AACCCGUAAGAUCCGAUCUUGUG	22	<i>xtr-mir-363-3p</i>	AAUUGCAGCGUAUCCAUUGUA	22
<i>xtr-mir-100</i>	AACCCGUAAGAUCCGAUCUUGUG	22	<i>xtr-mir-365</i>	UAAUGCCCCUAAAAUCCUUAU	22
<i>xtr-mir-101a</i>	UACAGUAUCUGUAUAAACUGAAG	22	<i>xtr-mir-375</i>	UUUGUUCGUUCGCGCUGCUUA ^a	22
<i>xtr-mir-103</i>	AGCAGCAUUGUACAGGGCUAUGA	23	<i>xtr-mir-383</i>	AGAUCAGAAAGGUAUUGUGGCU	22
<i>xtr-mir-106</i>	CAAAAGUGCUUUAUAGUGCAGGUAG	23	<i>xtr-mir-427-1</i>	GAAAGUGCUUUUCUGUUUUGGGCG ^a	23
<i>xtr-mir-107</i>	AGCAGCAUUGUACAGGGCUAUGA	23	<i>xtr-mir-427-2</i>	GAAAGUGCUUUUCUGUUUUGGACG ^a	23
<i>xtr-mir-122</i>	UGGAGUGUGACAAUGGUGUUUGU	23	<i>xtr-mir-427-3</i>	GAAAGUGCUUUUCUGUUUUGGGCG ^a	23
<i>xtr-mir-124</i>	UAAGGCAACGCGGUGAAUGCCA	21	<i>xtr-mir-428</i>	UAAUGUCUCUUAUGUUCGGUUU	22
<i>xtr-mir-125a</i>	UCCUGAGACCUCUAAACCGUG	23	<i>xtr-mir-429</i>	UAAUACUGUCUGGUAUUGCCG	21
<i>xtr-mir-125b</i>	UCCUGAGACCUCUAAACCGUGA	22	<i>xtr-mir-451</i>	AAACCUGUACCAUUAUCUGAGUUU	23
<i>xtr-mir-126</i>	UCGUACCGUGAGUAUAUAGC	21	<i>xtr-mir-455</i>	UAUGUGCCUUGGACUACUACG	22
<i>xtr-mir-126*</i>	CAUUAUUACUUUUGGUACCGG	21	<i>xtr-mir-499</i>	UUAAGACUUGCAGUGAUGUUAA	23

^aNew miRNAs species identified in this study.^bPotentially different annotation if seed sequence conservation is considered. *xtr-let-7b* [UGAGGUAGUAGUUUGUGUAGU] could be *xtr-let-7g*; *xtr-let-7e* [UGAGGUAGUAGGUUUUAGUU] could be *xtr-let-7a*.^cSequence [UUUGUGGAAUUGCUUCUUAU] is predicted to be *xtr-mir-147*; accession number is pending experimental validation.^dSequence [ACCAUCGCGCCUUGACUGUACC] 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)				
<i>xtr-let-7b</i>	<i>xtr-mir-20a</i>	<i>xtr-mir-125a</i>	<i>xtr-mir-148b</i>	<i>xtr-mir-208</i>
<i>xtr-let-7f</i>	<i>xtr-mir-26</i>	<i>xtr-mir-125b</i>	<i>xtr-mir-150</i>	<i>xtr-mir-214</i>
<i>xtr-let-7g</i>	<i>xtr-mir-27a</i>	<i>xtr-mir-126</i>	<i>xtr-mir-153</i>	<i>xtr-mir-215</i>
<i>xtr-let-7i</i>	<i>xtr-mir-29b</i>	<i>xtr-mir-126*</i>	<i>xtr-mir-155</i>	<i>xtr-mir-218</i>
<i>xtr-mir-1a</i>	<i>xtr-mir-30b</i>	<i>xtr-mir-130a</i>	<i>xtr-mir-181a</i>	<i>xtr-mir-301</i>
<i>xtr-mir-1b</i>	<i>xtr-mir-30e-5p</i>	<i>xtr-mir-130b</i>	<i>xtr-mir-181a-1*</i>	<i>xtr-mir-320</i>
<i>xtr-mir-7</i>	<i>xtr-mir-30e-3p</i>	<i>xtr-mir-130c</i>	<i>xtr-mir-181a-2*</i>	<i>xtr-mir-338</i>
<i>xtr-mir-9a</i>	<i>xtr-mir-31</i>	<i>xtr-mir-133a</i>	<i>xtr-mir-181b</i>	<i>xtr-mir-363</i>
<i>xtr-mir-9a*</i>	<i>xtr-mir-31b</i>	<i>xtr-mir-133c</i>	<i>xtr-mir-182</i>	<i>xtr-mir-363*</i>
<i>xtr-mir-15a</i>	<i>xtr-mir-33a</i>	<i>xtr-mir-133d</i>	<i>xtr-mir-182*</i>	<i>xtr-mir-383</i>
<i>xtr-mir-15b</i>	<i>xtr-mir-34a</i>	<i>xtr-mir-135</i>	<i>xtr-mir-194</i>	<i>xtr-mir-428</i>
<i>xtr-mir-16a</i>	<i>xtr-mir-34b</i>	<i>xtr-mir-139</i>	<i>xtr-mir-196a</i>	<i>xtr-mir-429</i>
<i>xtr-mir-16b</i>	<i>xtr-mir-92a</i>	<i>xtr-mir-140</i>	<i>xtr-mir-199a</i>	<i>xtr-mir-451</i>
<i>xtr-mir-17-5p</i>	<i>xtr-mir-93a</i>	<i>xtr-mir-142-5p</i>	<i>xtr-mir-199a*</i>	<i>xtr-mir-455</i>
<i>xtr-mir-17-3p</i>	<i>xtr-mir-93b</i>	<i>xtr-mir-142-3p</i>	<i>xtr-mir-200a</i>	<i>xtr-mir-499</i>
<i>xtr-mir-18a</i>	<i>xtr-mir-96</i>	<i>xtr-mir-143</i>	<i>xtr-mir-200b</i>	
<i>xtr-mir-18a*</i>	<i>xtr-mir-98</i>	<i>xtr-mir-145</i>	<i>xtr-mir-202</i>	
<i>xtr-mir-18b</i>	<i>xtr-mir-103</i>	<i>xtr-mir-146</i>	<i>xtr-mir-202*</i>	
<i>xtr-mir-18b*</i>	<i>xtr-mir-106</i>	<i>xtr-mir-146b</i>	<i>xtr-mir-204</i>	
<i>xtr-mir-19b</i>	<i>xtr-mir-107</i>	<i>xtr-mir-148a</i>	<i>xtr-mir-206</i>	
INTERGENIC MiRNAs (22)				
<i>xtr-let-7c</i>	<i>xtr-mir-27b</i>	<i>xtr-mir-30d*</i>	<i>xtr-mir-147</i>	<i>xtr-mir-427-2</i>
<i>xtr-mir-10a</i>	<i>xtr-mir-29a</i>	<i>xtr-mir-122</i>	<i>xtr-mir-189</i>	<i>xtr-mir-427-3</i>
<i>xtr-mir-10b</i>	<i>xtr-mir-29c</i>	<i>xtr-mir-124</i>	<i>xtr-mir-221</i>	
<i>xtr-mir-23b</i>	<i>xtr-mir-29d</i>	<i>xtr-mir-132</i>	<i>xtr-mir-375</i>	
<i>xtr-mir-24a</i>	<i>xtr-mir-30d</i>	<i>xtr-mir-138</i>	<i>xtr-mir-427-1</i>	
EXONIC MiRNAs (10)				
<i>xtr-mir-20b</i>	<i>xtr-mir-22</i>	<i>xtr-mir-133b</i>	<i>xtr-mir-192</i>	<i>xtr-mir-222</i>
<i>xtr-mir-20b*</i>	<i>xtr-mir-99</i>	<i>xtr-mir-183</i>	<i>xtr-mir-210</i>	<i>xtr-mir-365</i>
INTRONIC AND INTERGENIC MiRNAs (6)				
<i>xtr-let-7e</i>	<i>xtr-mir-30c</i>	<i>xtr-mir-128a</i>		
<i>xtr-mir-27c</i>	<i>xtr-mir-101a</i>	<i>xtr-mir-137</i>		
INTRONIC AND EXONIC MiRNAs (8)				
<i>xtr-let-7a</i>	<i>xtr-mir-25</i>	<i>xtr-mir-30a-3p</i>	<i>xtr-mir-129</i>	
<i>xtr-mir-23a</i>	<i>xtr-mir-30a-5p</i>	<i>xtr-mir-100</i>	<i>xtr-mir-217</i>	
MiRNAs ACROSS-INTRON/EXON JUNCTION (1)				
<i>xtr-mir-144</i>				

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

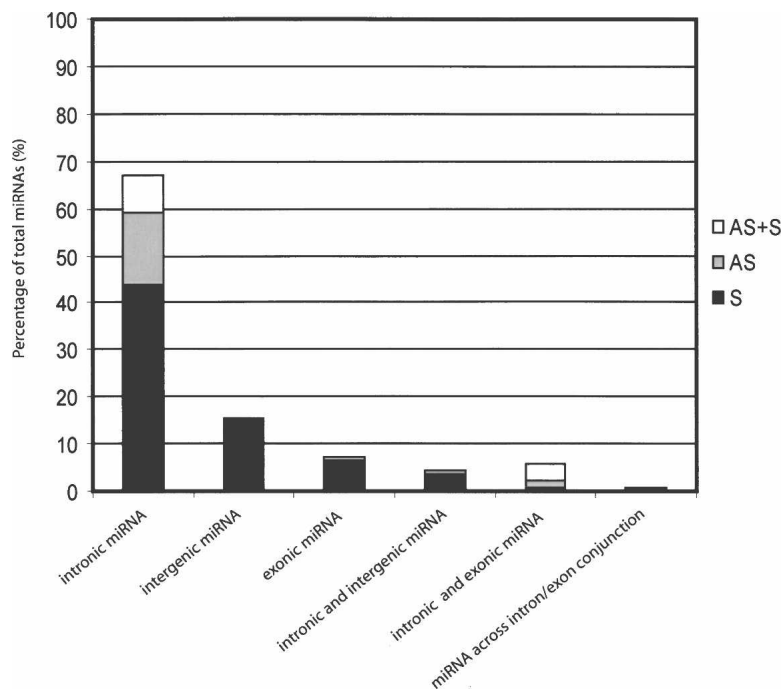


Figure 1. Genomic distribution of identified *Xenopus tropicalis* microRNAs. Graphical representation of the *X. tropicalis* miRNA's genomic organization. For intronic miRNAs, the solid sector of the bar indicates the proportion of miRNA genes positioned on the coding strand (S), the gray sector indicates 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)
<i>xtr-let-7a/xtr-mir-125a</i>	Xentr3/scaffold_285: 948731–961757	+	13,027
<i>xtr-mir-1a/xtr-mir-133c</i>	Xentr3/scaffold_220: 919435–941946	+	22,512
<i>xtr-mir-1a/xtr-mir-133a</i>	Xentr3/scaffold_549: 334888–337393	+	2505
<i>xtr-mir-1b/xtr-mir-133d</i>	Xentr3/scaffold_977: 368847–369356	–	509
<i>xtr-mir-15a/xtr-mir-16a</i>	Xentr3/scaffold_643: 310251–310484	–	233
<i>xtr-mir-15b/xtr-mir-16b</i>	Xentr3/scaffold_150: 310275–310469	–	193
<i>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</i>	Xentr3/scaffold_212: 656631–657356	+	725
<i>xtr-mir-17-5p/xtr-mir-17-3p/xtr-mir-18a/xtr-mir-18a* /xtr-mir-20a/xtr-mir-92/</i>	Xentr3/scaffold_894: 85148–85887	–	633
<i>xtr-mir-23b/xtr-mir-24a/xtr-mir-27b</i>	Xentr3/scaffold_528: 833673–834573	+	935
<i>xtr-mir-27a/xtr-mir-23a</i>	Xentr3/scaffold_829: 483579–484270	–	691
<i>xtr-mir-27c/xtr-mir-27c</i>	Xentr3/scaffold_1:7020632–7028355	–	7723
<i>xtr-mir-25/xtr-mir-93b</i>	Xentr3/scaffold_814: 522886–523081	–	194
<i>xtr-mir-29a/xtr-mir-29/xtr-mir-129</i>	Xentr3/scaffold_20: 657670–660400	+	70,450
<i>xtr-mir-29b/xtr-mir-29c</i>	Xentr3/scaffold_393: 249789–253020	+	3232
<i>xtr-mir-30a/xtr-mir-30c</i>	Xentr3/scaffold_67: 1391637–1398956	–	7319
<i>xtr-mir-30b/xtr-mir-30d</i>	Xentr3/scaffold_90: 1522361–1526686	–	4326
<i>xtr-mir-30c/xtr-mir-30e</i>	Xentr3/scaffold_399: 513153–514163	–	1011
<i>xtr-mir-34b/xtr-mir-34b</i>	Xentr3/scaffold_1083: 172336–180020	+	7684
<i>xtr-mir-34b/xtr-mir-34b</i>	Xentr3/scaffold_18: 3288131–3294119	+	5987
<i>xtr-mir-98/xtr-let-7f</i>	Xentr3/scaffold_750: 538453–538808	+	427
<i>xtr-mir-99/xtr-let-7c</i>	Xentr3/scaffold_116: 668725–669496	+	770
<i>xtr-mir-128a/xtr-mir-153</i>	Xentr3/scaffold_31: 3158967–2956054	–	20,2914
<i>xtr-mir-130b/xtr-mir-130c</i>	Xentr3/scaffold_118: 53604–61323	–	7718
<i>xtr-mir-137/xtr-mir-137</i>	Xentr3/scaffold_36: 1251053–1267094	–	16,032
<i>xtr-mir-142/xtr-mir-142</i>	Xentr3/scaffold_100: 1012673–1013940	–	1267
<i>xtr-mir-143/xtr-mir-145</i>	Xentr3/scaffold_493: 665585–666622	+	1037
<i>xtr-mir-181a/xtr-mir-181b</i>	Xentr3/scaffold_858: 464202–464748	+	545
<i>xtr-mir-181a/xtr-mir-181b</i>	Xentr3/scaffold_1164: 125236–129501	+	4266
<i>xtr-mir-183/xtr-mir-96 / xtr-mir-182</i>	Xentr3/scaffold_20: 2863123–2867042	+	3834
<i>xtr-mir-199a/xtr-mir-214</i>	Xentr3/scaffold_74: 833054–838267	+	5213
<i>xtr-mir-202/xtr-mir-202</i>	Xentr3/scaffold_305: 963500–971292	–	7791
<i>xtr-mir-215/xtr-mir-194</i>	Xentr3/scaffold_39: 2646993–2647935	–	943
<i>xtr-mir-221/xtr-mir-222</i>	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-mir-142-3p* 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.

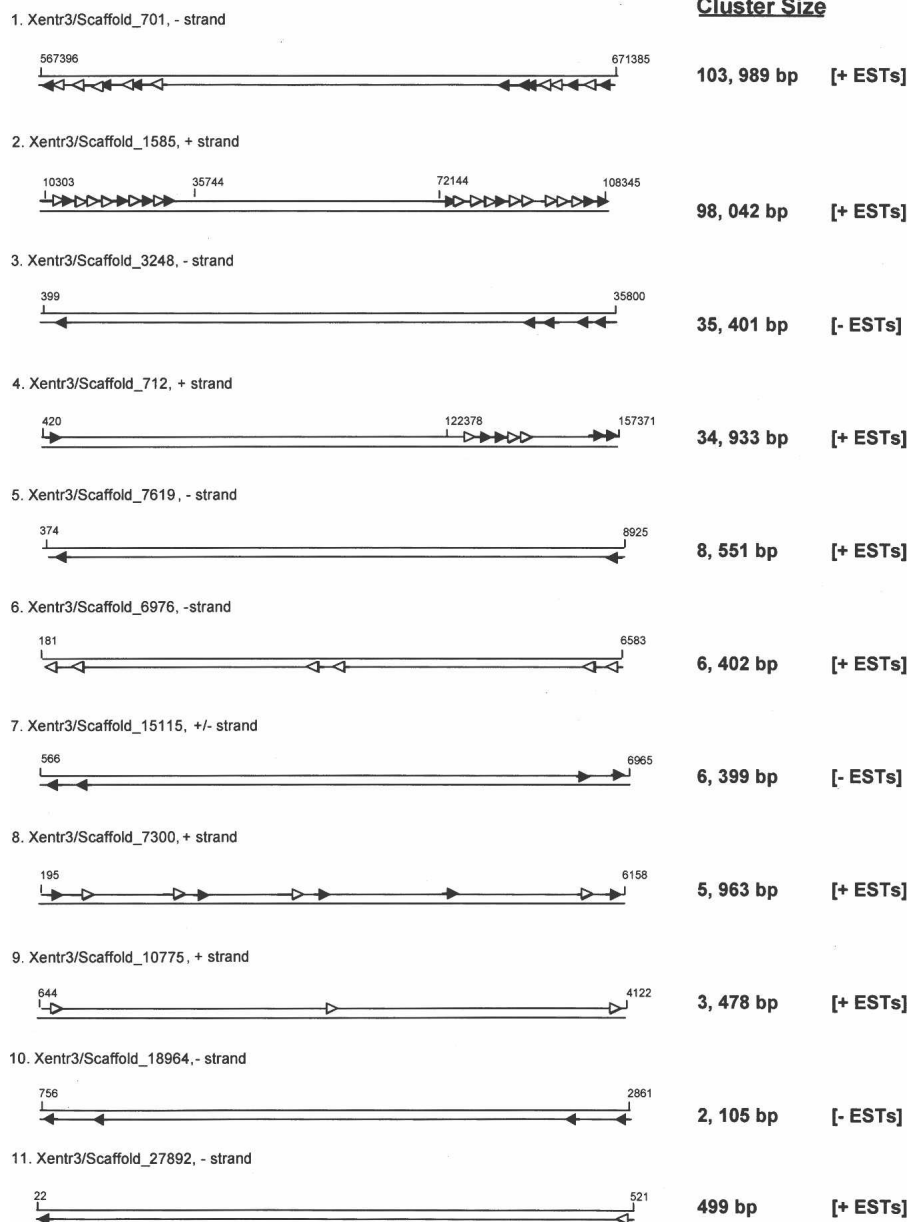
***Xenopus tropicalis* xtr-miR-427 clusters**

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-

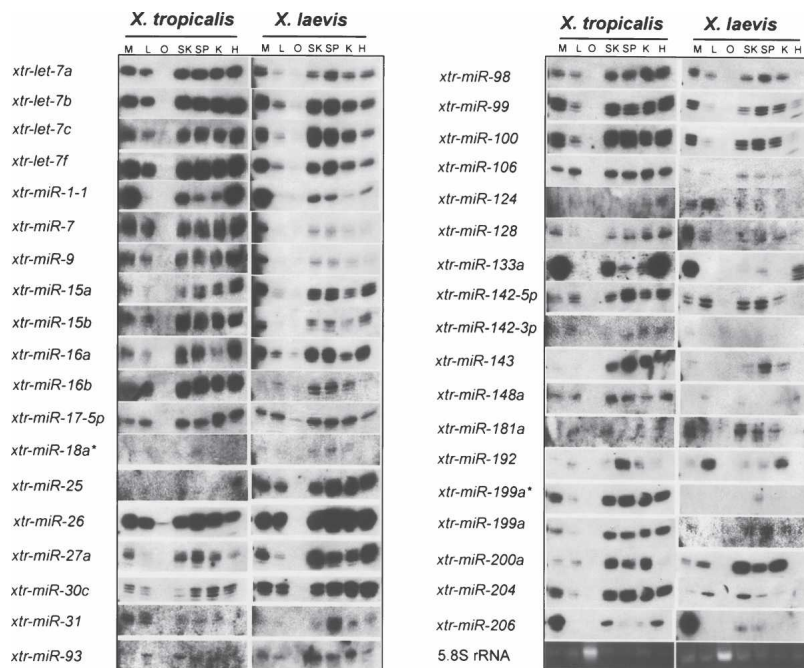


Figure 3. Northern blot validation of computationally identified *Xenopus tropicalis* and *Xenopus laevis* miRNAs. Total RNA was isolated from *X. tropicalis* and *X. laevis* oocytes and adult tissues, resolved on denaturing polyacrylamide gels, electroblotted to nylon membranes, and hybridized with 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 pre-dominance 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,

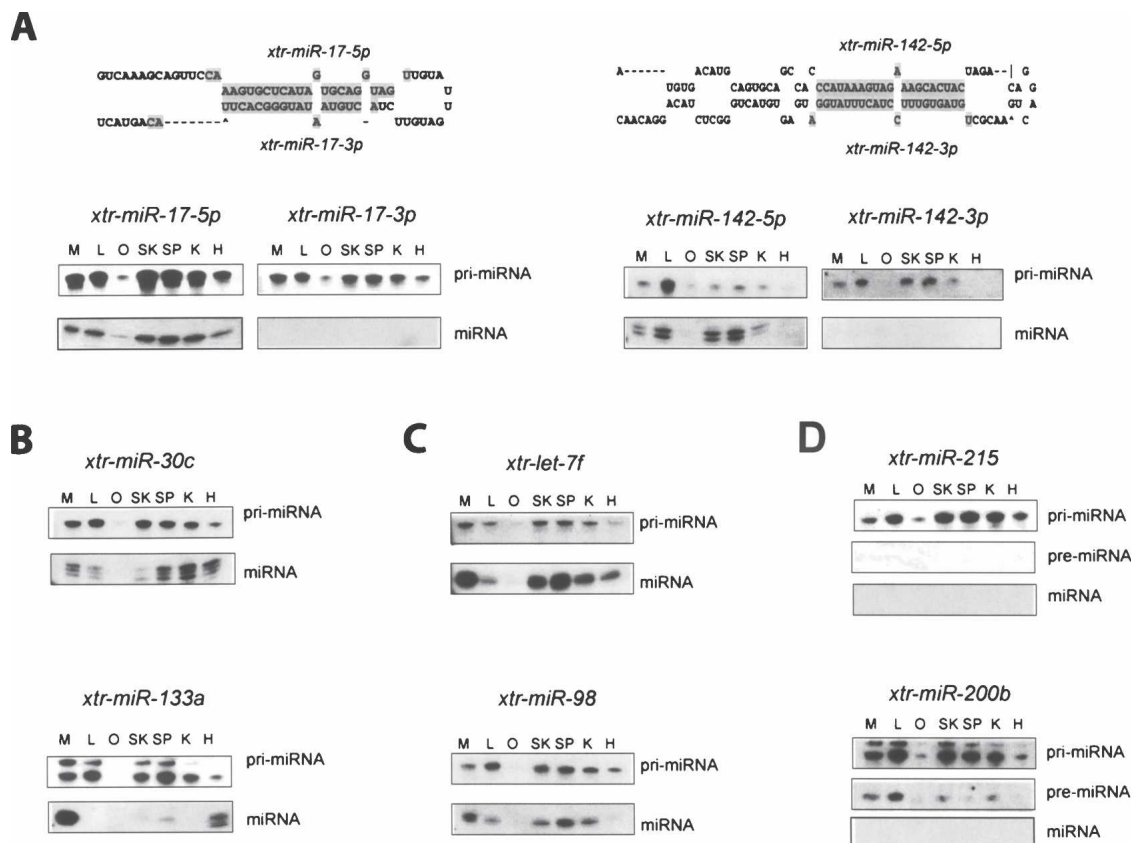


Figure 4. Differential expression of *Xenopus laevis* miRNAs and pri-miRNAs. Total RNA was isolated from *X. laevis* oocytes and adult tissues were resolved on denaturing polyacrylamide gels, electroblotted to nylon membranes, and hybridized with DNA oligonucleotides complementary to computationally 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 μ g 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.

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