INTRODUCTION

Inorganic pyrophosphate (PPi) exists in extracellular fluids (ex: synovial fluid, blood plasma, urine) and plays important physiological roles during bone calcification and mineralization, such as blockage of calcification (Ryan 2001). Deficient PPi promotes pathologic mineralization with basic calcium phosphate (BCP) crystals whereas excess PPi causes calcium pyrophosphate dihydrate (CPPD) crystals accumulation (Altman et al., 1973; Silcox and McCarty, 1974; Ryan 2001). Accumulation of both BCP and CPPD crystals causes serious pathological defects, especially on articular tissue (Ho et al., 2000; Rutsch et al., 2001). Thus, balance of PPi concentration is a crucial factor to keep normal function of bone and joints.

Extracellular/intracellular PPi shuttling is controlled by a membrane protein, ankylosis progressive homolog (Ankh), which is encoded by ankh gene (Ho et al., 2000). The molecular structure of ankh genes has been determined in frog (Nürnberg et al., 2001), chicken (Wang et al., 2005) and mammals (Hakim et al., 1986; Hughes et al., 1995; Ho et al., 2000; Zimin et al., 2009). In zebrafish, two ankh genes, ankha and ankbh, are reported (Ho et al., 2000; Strausberg et al., 2002), but their expression information during early embryogenesis are still limited.

As it might be expected, mutation on ankh gene induces calcification disorder or other pathological defects. For example, craniometaphyseal dysplasia (CMD), a rare inherited disorder of bone in human, is due to ANKH mutation (Nürnberg et al., 2001). The clinical signs and symptoms of CMD include overgrowth and sclerosis of the craniofacial bones and abnormal modeling of the metaphyses of the tubular bones (Nürnberg et al., 2001). In mice, inactivation of ANK leads to generalized, progressive form of arthritis accompanied by mineral deposition, formation of bony outgrowths, and joint destruction (Ho et al., 2000). In zebrafish, two ankh genes, ankha and ankbh, are identified but mutation in zebrafish ankh genes has not discovered thus far.

To elucidate the physiological functions of ank genes during early embryogenesis, it is worthy to analyze comparatively ankh genes across species. Here, we report the spatiotemporal expressions of two zebrafish ankh homolog (ankh) genes by whole mount in situ hybridization and reverse transcriptase polymerase chain reaction (RT-PCR) experiments. This gene expression data will provide more insight into the functional studies of the lower vertebrate ankh genes.
MATERIALS AND METHODS

Fish Embryos Staging

Mature zebrafish (AB strain) were raised at the zebrafish facility of the Life Sciences Development Center, Tamkang University. The fish were maintained at 28°C with a photoperiod of 14 h light and 10 h dark, in an aquarium supplied with freshwater and aeration (Chen et al., 2009; Wang et al., 2009a). Embryos were produced using standard procedures (Westerfield, 1995) and were staged according to standard criteria: hours postfertilization, hpf; or days postfertilization (dpf; Kimmel et al., 1995).

RNA Isolation and Reverse Transcription–Polymerase Chain Reaction (RT–PCR)

We corrected 100 embryos per stage to extract their total RNA. RNA isolation and first-stand cDNA synthesis procedures were according to the previous report with minor modification (Chen et al., 2001; Wang et al., 2009b; Lai et al., 2011). Primer sets (ankha-F: 5'-GGGAGCCTTGTGGATCCACT-3', ankha-R: 5'-TGGGATGACGCACTGCGCAGG-3'; ankhb-F: 5'-GAAGAATGGAGAAGCCGTCAGCA-3', ankhb-R: 5'-ACGGACCATACAGACCGCTG-3'; and β-actin-F: 5'-GTCCCGATCTACAGCTTGGTCG-3', β-actin-R: 5'-GCCGCGCTACCTGTCGCTCG-3') were designed based on the sequences encoding of putative zebrafish ankha, ankhb, and β-actin.

Database Searches and Phylogenetic Analysis

Database searches were carried out using the Blast program at the National Center for Biotechnology Information (Altschul et al., 1997). According to above procedures, cDNA clones encode Ankha and Ankhb were cloned and amplified from embryonic zebrafish mRNA. The presumptive Ankha and Ankhb amino acid sequences were determined with the Wisconsin Sequence Analysis Package v. 10.0 (GCG). The Gap program of that package was used for pair comparisons, and the Pileup and Prettybox programs used for multiple comparisons. ExPASy ProtParam tool (http://expasy.org/tools/protparam.html) was used to predict the pI and MW of Ankha. The Clustalw molecular evolution genetic program was used for our phylogenetic tree analysis (http://www.ebi.ac.uk/clustalw/).

Whole Mount in situ Hybridization, Cryosection and Images

The procedures for whole mount in situ hybridization, and cryosection have been described previously (Pai and Chen, 2010; Peng et al., 2010; Lee et al., 2011), except that ankha and ankhb (this study) were used as probes. They were digoxigenin (DIG)-labeled, after we cloned their partial DNA fragment. For image analysis, all embryos were observed under a microscope (DM 2500, Leica, Germany) equipped with Nomarski differential interference contrast optics (Kramer Scientific) and a digital camera (Cannon, Japan).

RESULTS AND DISCUSSION

Comparison of Deduced Amino Acid Sequences

By searching GenBank, we found two putative zebrafish ankha sequences (ankha: NM_001030259, and ankhb: NM_194370). The deduced amino acid sequence of zebrafish Ankha revealed a 496-amino acid polypeptide, whereas the deduced amino acid sequence of zebrafish Ankhb revealed a 501-amino acid polypeptide (Fig. 1). The zebrafish Ankha/Ankhb polypeptide shares sequence identities of 74–82% of the reported Ank of human, bovine, mouse, rat, chicken, Xenopus, medaka and fugu. In addition, we used the Clustalw program to determine the phylogenetic similarities between zebrafish Ankha/Ankhb and that of other known species. The phylogenetic tree generated by the program showed that zebrafish Ankha/Ankhb (79%) was more closely related to medaka and fugu’s Ank than those from higher vertebrates (data not shown). Moreover, ankha/ankhb gene transcripts from selected vertebrates and their molecular features are summarized in Table. These data reveal that most Ank proteins have alkaline pIs (7.61–8.27), but medaka Ank and zebrafish Ankha/Ankhb proteins share acidic pIs (6.42–6.97).

Developmental Expression of Zebrafish ankha and ankhb

Next, we determined the developmental expressions of zebrafish ankha and ankhb by RT-PCR, and results revealed that endogenous ankha expressed from 6 hpf to 7 dpf, and ankhb expressed from 1-cell (0 hpf) to 7 dpf (Fig. 2). Although RT-PCR products of ankha were undetectable at 1-cell (0 hpf), faint signals were detected by nested RT-PCR analysis (data not shown). These observations indicated that zebrafish ankha and ankhb are maternal inherited genes.

Spatiotemporal Expression of Zebrafish ankha Transcripts during Early Development

To determine the spatiotemporal expression patterns of ankha during early development, we performed whole mount in situ hybridization using an ankha antisense DIG-labeled riboprobe. Zebrafish ankha transcripts were first detected from 1-cell stage to cleavage period (Figs. 3a, 3b), and extended their expression from the gastrula period to the early segmentation stages (Figs. 3c, 3d). At 24-hpf, 36-hpf, 2-dpf, and 3-dpf, the zebrafish ankha transcripts were restricted to trunk and head regions (Figs. 3e–3h). Interestingly, zebrafish ankha transcripts were down-regulated to a
very faint level at 5-dpf (Fig. 3i), but the expressions appeared again at retina at 7-dpf (arrow, Fig. 3j). On the basis of these observations, we conclude that zebrafish ankha is a maternally inherited gene, restricting its expression in head and trunk at early embryonic stages.

Summary of ankha genes from selected vertebrates

<table>
<thead>
<tr>
<th>Species, gene names</th>
<th>Coding region, aa</th>
<th>Mw, kDa</th>
<th>pI</th>
<th>GenBank accession number</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human ANKH</td>
<td>492</td>
<td>54.2</td>
<td>8.00</td>
<td>NM_054027.4</td>
<td>Hughes et al., 1995</td>
</tr>
<tr>
<td>Bos ankha</td>
<td>492</td>
<td>54.2</td>
<td>7.61</td>
<td>NM_01109793</td>
<td>Zimin et al., 2009</td>
</tr>
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<td>Mouse Ank</td>
<td>492</td>
<td>54.3</td>
<td>8.01</td>
<td>NM_020332</td>
<td>Hakim et al., 1986</td>
</tr>
<tr>
<td>Rat Ankh</td>
<td>492</td>
<td>54.3</td>
<td>8.01</td>
<td>NM_053714</td>
<td>Ho et al., 2000</td>
</tr>
<tr>
<td>Chicken ankha</td>
<td>493</td>
<td>54.5</td>
<td>8.27</td>
<td>NM_001012562</td>
<td>Wang et al., 2005</td>
</tr>
<tr>
<td>Xenopus ankha</td>
<td>492</td>
<td>54.0</td>
<td>8.02</td>
<td>NM_001090455</td>
<td>Nürnberg et al., 2001</td>
</tr>
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<td>Medaka ankha</td>
<td>492</td>
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<td>Ensembl</td>
</tr>
<tr>
<td>Fugu ankha</td>
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<td>54.5</td>
<td>7.64</td>
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<td>Ensembl</td>
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<tr>
<td>Zebrafish ankha</td>
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<td>54.6</td>
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<td>NCBI; this study</td>
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<td>Zebrafish ankhh</td>
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<td>55.4</td>
<td>6.97</td>
<td>NM_194370</td>
<td>Ho et al., 2000; this study</td>
</tr>
</tbody>
</table>

Fig. 1. Comparison of the deduced amino acid sequence of zebrafish Ankha/Ankhb with those of other known species. The information was obtained from the GenBank nucleotide sequence database and Ensembl with the following accession numbers: human (NM_054027.4), bovine (NM_001109793), mouse (NM_020332), rat (NM_053714), chicken (NM_001012562), Xenopus (NM_001090455), medaka (ENSORLG00000011729), fugu (ENSTRUG0000008542) and zebrafish Ankh. Amino acid residues similar to those of the zebrafish Ankha/Ankhb are presented in black. TM: transmembrane domain.
Fig. 2. RT-PCR analysis of *ankha* and *ankhb* gene transcripts, using total RNA extracted from the embryos of different developmental stages. Top panel: *ankha*; middle panel: *ankhb*; and bottom panel: loading control (β-actin). Stage of each sample is indicated on the top.

Fig. 3. *ankha* expression during early embryonic stages. (a) One-cell, top view, (b) at 6-hpf stage, lateral view, (c) at 12-hpf and (d) 18-hpf, top view, (e) at 24-hpf and (f) 36-hpf, lateral view, (g–j) at 2-, 3-, 5- and 7-dpf, lateral view.
Again, the spatiotemporal expression patterns of *ankhb* during early development were examined by whole mount in situ hybridization. Results showed that zebrafish *ankhb* transcripts were first observed from 1-cell stage to cleavage period and their expression extended from the gastrula period to the early segmentation stages (Figs. 4a–4c). By 18-hpf, the zebrafish *ankhb* transcripts were detected in somite, and in the head region (Fig. 4d). By 24-hpf, *ankhb* transcripts strongly expressed in the entire head region and in the guts, and that were further confirmed by cryosectioning (Figs. 4e, 4e'). Specially, we found that the expressions of zebrafish *ankhb* were strongly detected in pharyngeal arches at 36-hpf, 2-, 3- and 5-dpf (arrows, Figs. 4f–4i). By 7-dpf, no obvious signals were observed (Fig. 4j). On the basis of these observations, we conclude that zebrafish *ankhb* is a maternally inherited gene, restricting its expression in head, gut as well as pharyngeal arches.

**Comparison of ankh Gene Expression Patterns between Zebrafish and Mouse**

Since *ank* is an evolutionary conserved gene among different vertebrate species, it would be interesting to...
compare their expression patterns between mouse and zebrafish. Though strong ank expression were detected in the developing mouse limbs, the ank mRNAs were also detected in many non-skeletal tissues of adult mouse, including heart, brain, liver, spleen, lung, muscle, and kidney (Ho et al., 2000). Inactivation of ankh not only leads to skeletal defects in mice and human, but also results to increased calcification in kidneys of adult mice (Storm and Kingsley, 1996; Ho et al., 2000). These observations suggest that ank plays an important role in both skeletal and non-skeletal tissues. We have shown that ankha/ankhb transcripts were detected strongly in the presumptive head region (skeletal cell-rich), gut (non-skeletal soft tissue) and pharyngeal arches (skeletal cell-rich) of developing zebrafish embryos. These expression data are consistent with that of mouse ANK. Taken together, we suggest that zebrafish Ankh/Ankhb might play similar roles compare to that of mouse Ank.

In conclusion, this study highlights the distinct expression pattern of two structurally related zebrafish ankha and ankhb genes. They are both maternally inherited genes. Expression of ankha is mainly restricted in head region whereas ankhb is restricted in head region, gut and pharyngeal arches. This information may provide more insight into the molecular structure and expression patterns of the lower vertebrate ank genes.

REFERENCES


