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Isolation and Expression Study of a Maternally Expressed Novel *Xenopus* Gene *Xem1* Encoding a Putative Evolutionarily Conserved Membrane Protein

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A novel *Xenopus* maternally expressed gene, *Xem1*, was isolated by differential display PCR and 5′-RACE. *Xem1* coded for a putative transmembrane protein of 172 amino acids. Sequence analysis, including the clustering and reconstruction of ESTs (Expressed Sequence Tags), revealed that homologs of Xem1 are widely distributed in eukaryotic phyla, suggesting that Xem1 is a member of evolutionarily conserved proteins. Expression of *Xem1* mRNA occurred from the previtellogenic stage and its level increased during oogenesis, maintained throughout oocyte maturation to blastula stage and then decreased in post gastrula stages. In cleavage stage, *Xem1* RNA was distributed uniformly, and in adult, occurred predominantly in ovary and testis. We assume that *Xenopus Xem1* may have its function in gametogenesis and in early phase of embryogenesis, whose function may be related to transport mechanism of small molecular weight substances like metal ions, from analogy to the function of its homologs in other organisms.

We previously applied differential display methods (1, 2) to analyze gene expression profiles in early embryogenesis of *Xenopus laevis* (3). During the work, we cloned a differentially displayed cDNA band, Jn6-3, which had no similarity to the sequences deposited in database. The expression pattern in embryos of this gene showed a down-regulated one (high level occurrence from cleavage to blastula stage and low level occurrence in later stages) (3).

In the present study, we constructed its full-length cDNA by 5′-RACE and named the gene as *Xem1* (*Xenopus m*aternally expressed mRNA *1*). Based on the sequence obtained, we carried out homology search, including clustering and reconstruction of homologous ESTs, with the results that *Xem1* is a member of a novel gene family of transmembrane proteins, widely conserved in eukaryotic species ranging from yeast to human. We report here the results of the sequence analysis and RT-PCR comparing the expression of *Xem1* in oocytes, embryos and adult tissues.

MATERIALS AND METHODS

Preparation of full-length cDNA for Jn6-3. The upstream region of Jn6-3 was obtained by 5′ RACE (Rapid Amplification of cDNA Ends) method (4) using a kit obtained from BRL (MA). Briefly, the cDNA was synthesized from blastula stage RNA using the JpS1 primer (5′-CATCTGCTTTCAAACAG-3′) and SuperScript II reverse transcriptase, tailed with dA by terminal deoxynucleotidyl transferase, and used as a template to amplify 5′ end of cDNA using oligo-dT primer and gene-specific primers, JpS2 (5′-CACACCATACTTTCCAGATG-3′) and JpS3 (5′-GGTAGTTTTGGGTAGCAAAC-3′). Amplified products were subcloned into pT7Blue vector (Novagen, WI) and sequenced by dideoxy chain termination method using Labstation Thermo Sequenase labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham, UK). The cDNA was amplified by RT-PCR and subjected to direct cycle sequencing to eliminate the effect of misincorporated nucleotides at the step of PCR as described in Results section.

Sequence analysis. Sequence data obtained were subjected to homology search using BLAST and FASTA programs on the cs6400 computer at Human Genome Center, Institute of Medical Science, University of Tokyo.

Assembly and reconstitution of ESTs were performed using DNASIS (HITACHI Software Engineering, Japan), GENETYX-MAC/ATSQ (Software Development, Japan) and GENETYX-MAC (Software Development) softwares on a personal computer. The discrepancy among EST sequences were manually edited to deduce consensus sequence for each species.

Nucleotide sequence data of *Xem1* reported in this paper was deposited in DDBJ, EMBL and GenBank nucleotide sequence databases (accession number, AB000458).

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Preparations of oocytes, embryos and adult tissues. Ovary was excised from a chilled *Xenopus laevis* female, and oocytes were obtained by digestion of clumps of the ovary by 1 mg/ml collagenage (SIGMA, MO) in modified Barth's solution (88 mM NaCl, 1 mM KCl, 0.7 mM $CaCl_2$, 1 mM $MgSO_4$, 2.5 mM $NaHCO_3$, 10 mM HEPES, pH 7.6). Oocytes collected were staged according to Dumont (5). Maturation was induced in stage VI oocytes by treatment with 2 mM progesterone (SIGMA) in OR2 solution (82.5 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM Na_2HPO_4, 5 mM HEPES, pH 7.8) (6). Completion of maturation was confirmed by the appearance of a white spot at the animal side.

Unfertilized eggs were squeezed out manually from females which had been injected with 250 units of a gonadotropic hormone, Gonatropin (Teikokuzoki, Japan). Eggs were artificially fertilized, dejelled in 2 % cysteine-HCl (pH 7.9), and cultured in 0.1 × Steinberg's solution (6 mM NaCl, 0.067 mM KCl, 0.034 mM $Ca(NO_3)_2$, 0.083 mM $MgSO_4$, 1 mM HEPES, pH 7.4) at 20-21°C until desired stages (7).

All the adult tissues except testis were obtained from mature females chilled in ice water using forceps and scissors. Tissues were rinsed with OR2 solution and kept frozen until analyzed.

RNA extraction and RT-PCR assay. RNAs were isolated from oocytes, embryos and adult tissues by a modified single-step acid-guanidine thiocyanate-phenol-chloroform method (8) using TRIZOL reagent or TRIZOL-LS reagent (BRL, MA). RNAs were purified by salt and ethanol precipitation. For RT-PCR analysis, RNAs were converted into the first strand cDNAs using oligo-$(dT)_{12\text{-}18}$ primer. The RT-PCR mixture containing cDNA and specific primer sets (10 pmol each) was subjected to 21 thermal cycles (94°C for 30 sec, 60°C for 1 min and 72°C for 1 min). Primers used for *Xem1* had the sequences: 5′-AGTCACACTCCAAGCGGAAG-3′ and 5′-AAGAAACATGGAGAAGTGTG-3′. Primers used to detect cytoskeletal actin transcript in adult tissues were 5′-CTGAGTTCATGAAGGATCAC-3′ and 5′-AAATTTACAGGTGTACCTGC-3′. Products were resolved on a polyacrylamide gel and stained with SYBR Green I (Molecular Probe, OR).

RESULTS

Previous studies showed that Jn6-3, a DNA fragment of 282 bp isolated by FDD (Fluorescent Differential Display) method, representing only partial 3′-side sequence of a maternally-inherited mRNA, had no homology to sequences deposited in database (3). Therefore, we first determined the full-length sequence for the mRNA by 5′ RACE technique using the RNA prepared from blastulae (4). The final product obtained from 5′-RACE was 1.2 kb in length and sequence data confirmed its continuity with Jn6-3. To eliminate the artificial base substitution during the PCR, we amplified the cDNA by RT-PCR and subjected it to direct cycle sequencing, and confirmed the nucleotide sequence of 1393 bp in length shown in Fig. 1. The size of the cDNA obtained (ca 1.4 kb) roughly coincided with the size previously estimated (1.6 kb) from the Northern blot signal for Jn6-3 RNA (3), suggesting that the *Xem1* cDNA obtained is the full or nearly full-length cDNA.

As shown in Fig. 1, the cDNA contained a single open reading frame (ORF) of 516 bp with an upstream in-frame stop codon. The ORF encoding a polypeptide of 172 amino acid residues was highly hydrophobic in nature, with two potential transmembrane helices as shown in its hydropathy profile (Fig. 2). These results suggest that the cDNA encodes an integral membrane protein. Sequence analysis by PSORT program (9) predicted that the protein coded by the cDNA may be localized in mitochondrial inner membrane (certainty = 0.825) or in plasma membrane (certainty = 0.600), although the validity of the prediction awaits further study.

When we compared the amino acid sequence of Xem1 with those deposited in database using BLASTP and FASTA programs, two putative transmembrane portions (a.a. residues 23-39, and 133-149) (Fig. 1, underlines) were found to have significantly high homology to those of previously reported proteins, such as YHX5/CTR2/YHR175W (an yeast protein predicted from systematic genomic sequencing of chromosome VIII) (10), L9931.6/YLR411W (also an yeast hypothetical protein), F58G6.2 and F58G6.3 (two *C. elegans* hypothetical proteins predicted from genome sequencing) (11), PIM (Polymorphic Immunodominant Molecule protein found in protozoan parasite *Theileria parva*) (12), QP protein (glutamine- and proline-rich protein from *Theileria parva*) (13) and COPT1 (Copper Transporter 1 of *Arabidopsis*) (14).

We performed TBLASTN or TFASTA search against dbEST (15) in order to obtain more information about the related proteins from other sources. Xem1 showed significantly high homology to putative protein products expected to occur from the analysis of ESTs from human, mouse, *C. elegans*, *Arabidopsis* and rice. We retrieved these ESTs, and used them as "probes" for another homology search to identify overlapping ESTs that had not been hit in the initial search. By repeating such homology search cycles (we named this "cDNA walking *in silico*", because it is like cDNA walking on computer) and retrieving the nucleotide sequence data from the opposite end of each Washington U-Merck cDNA clone (16), we collected a group of ESTs presumably derived from a single message, joined them and assembled the data into a single unity.

Thus, complete sequences of homologous proteins were deduced from ESTs in human, mouse and *Arabidopsis*, whereas only partial structures were revealed in rice and *C. elegans*. In *C. elegans*, however, complete structure of two putative Xem1 homologs were deduced by integrating the assembled EST data with the genomic sequence data (11). Alignment of proteins identified by these homology searches revealed a family of Xem1 proteins as summarized in Fig. 3. They seem to be classified into two subfamilies; one having homology to Xem1 only in the hydrophobic domains (F52G6.2, F52G6.3, PIM, QP protein, YHX5/CTR2/YHR175W, L9931.6/YLR411W and COPT1) and the other having homology not only in the two hydrophobic domains but also in the regions between them (Xem1 homologs of *Xenopus*, human, mouse, *C. elegans*-1 (F31E8.4), *C. elegans*-2 (F27C1.2) and *Arabidopsis*). Figure 4 shows a group of Xem1 homologs which had high homology.

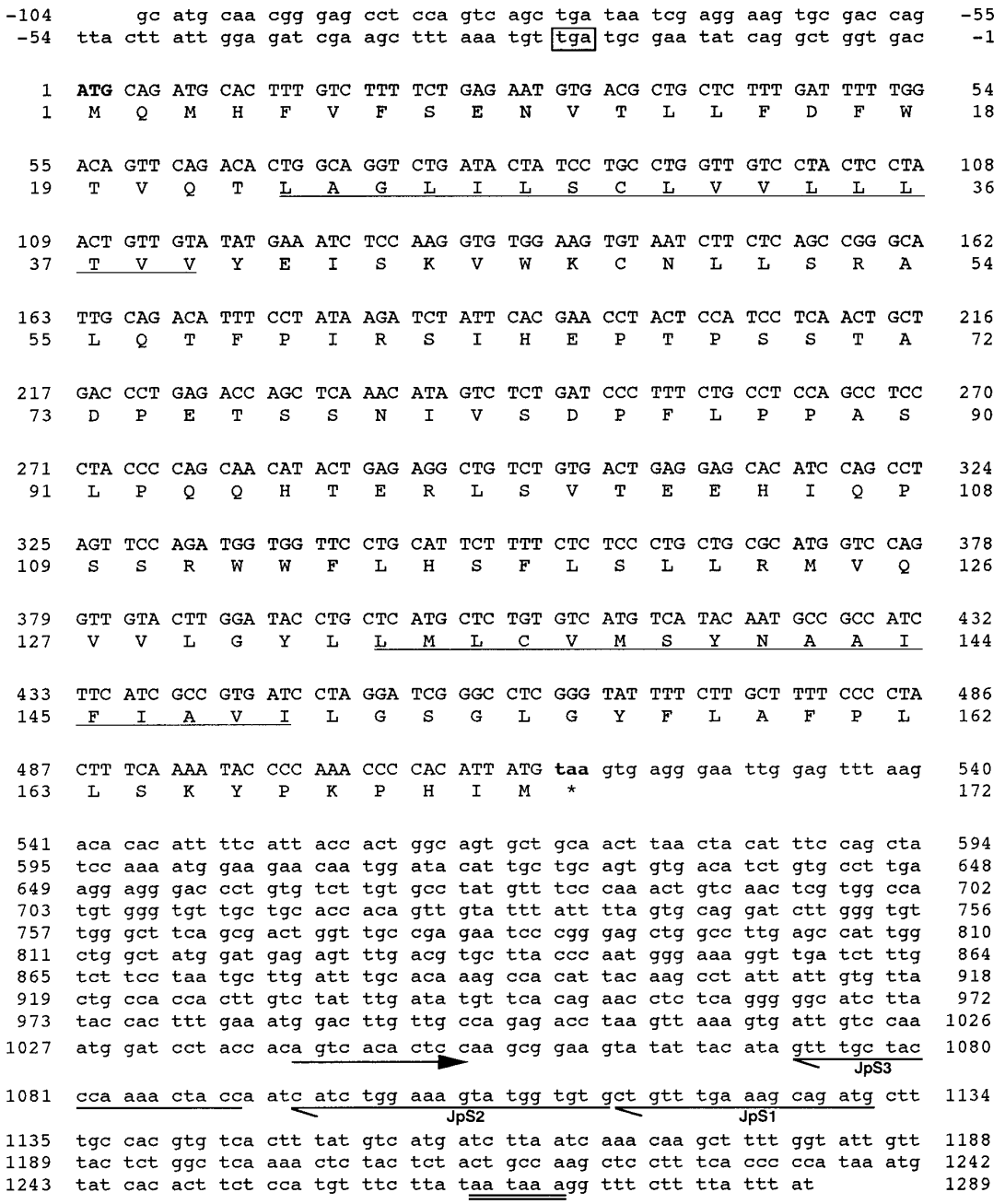

FIG. 1. Structure of Xem1. Nucleotide and deduced amino acid sequences of cDNA for *Xem1*, the gene from which Jn6-3 had been derived. Coding sequence is in capital letters and UTR's in small letters. Translational start codon and stop codon are in bold. Two predicted transmembrane domains are underlined. The putative polyadenylation signal was doubly underlined. The upstream in-frame stop codon is boxed. Arrow indicates the sequence matched with the arbitrary primer used for the initial differential display PCR which identified the Jn6-3. Half-headed arrows indicate the sequences used for primers in 5′ RACE.

From these results we predict that Xem1 has a considerably large number of homologous proteins in a wide range of eukaryotic phyla.

We followed the changes in the level of *Xem1* signal during oogenesis, oocyte maturation and embryogenesis of *Xenopus laevis* by RT-PCR (Fig. 5A). The fluorescent intensity analysis of the amplified products revealed that during oogenesis the level of *Xem1* transcript detected at stage I oocyte increased and peaked at stage III, but thereafter maintained with only a slight decrease until stage VI (Fig. 5B). During oocyte maturation, the level of Xem1 signal remained essentially unchanged, though it decreased slightly after 5 hr, as is known for most of the maternal mRNAs (17).

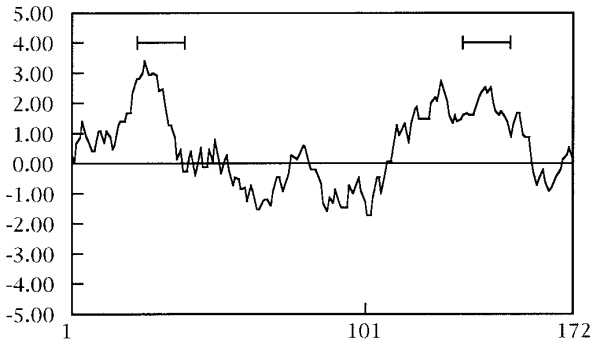

FIG. 2. Hydropathy profile of the amino acid sequence of Xem1. Hydropathy profile of the amino acid sequence of Xem1. The method of Kyte and Doolittle (23) was employed using a window size of 11 successive residues. Predicted transmembrane domains are indicated by cross bars.

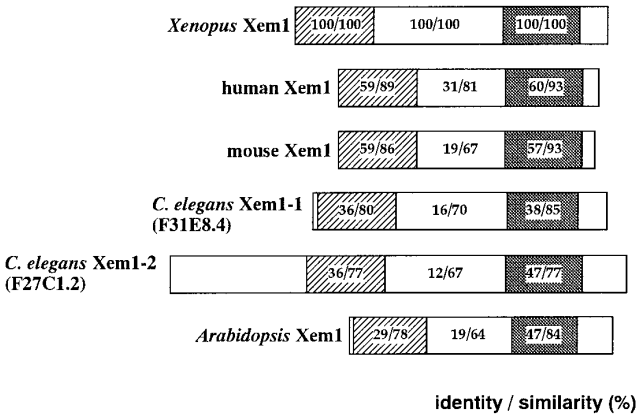

FIG. 4. Structure of Xem1 homologs. Hatched and dotted boxes indicate the conserved hydrophobic domains shown in Fig. 3. Numbers in boxes represent identity and similarity expressed in percentile. The boxes are drawn proportionally to their sizes.

The level of *Xem1* transcript present in the egg did not change through cleavage to blastula stage, but at and after gastrula stage became quite low, and the level did not increase even at tadpole stage, a finding which further confirms the "down-regulated" nature of this gene transcript (3). Essentially the same results were obtained under different thermal cycling, and therefore, we concluded that the temporal changes in the

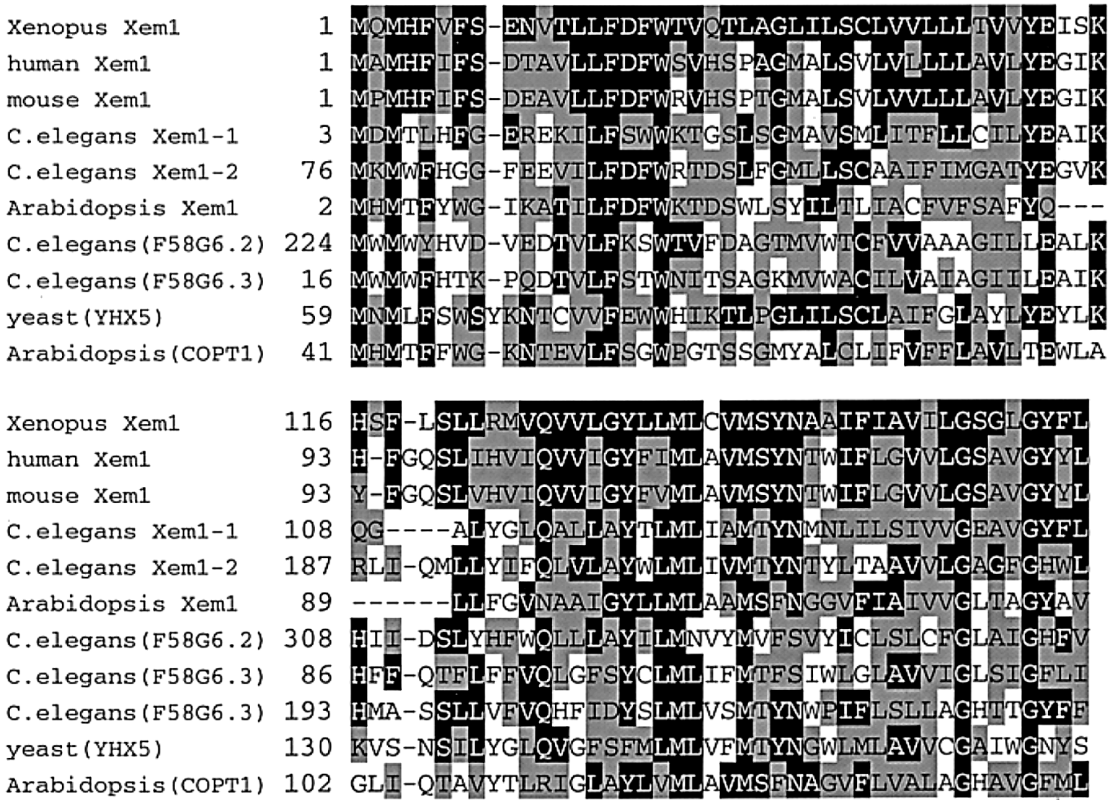

FIG. 3. Conserved regions in *Xenopus* Xem1 and other proteins. Identical and similar positions are shaded in black and gray, respectively. Xem1 homologs (human, mouse *C. elegans*-1, -2, *Arabidopsis*) were reconstructed by ESTs in each species. *C. elegans* Xem1 homologs were reconstructed with the aid of genomic sequence data of F27C1.2 and F31E8.4. F58G6.2 and F58G6.3 are hypothetical proteins in *C. elegans* genome. YHX5/CTR2/YHR175W is yeast hypothetical protein. COPT1 is a *Arabidopsis* protein. The number at the left of each indicates the amino acid residue in each protein. See text for references for these sequences.

level of *Xem1* transcript shown in Fig. 5B was reliable. We examined by RT-PCR if there is regional difference in the distribution of *Xem1* transcript in blastomeres of 8-cell stage embryos. For this purpose, we dissected embryos into four different parts (animal-dorsal, animal-ventral, vegetal-dorsal and vegetal-ventral), but results obtained showed the uniform distribution of *Xem1* transcript within the embryo (data not shown).

Tissue distribution pattern of *Xem1* transcript was examined also by RT-PCR. In this experiment, the signal (157 bp) for cytoskeletal actin mRNA as a ubiquitous mRNA was examined for a reference mRNA. As shown in Fig. 6 strong signal (225 bp) for *Xem1* was observed in testis and ovary, and only weak signal occurred in a wide variety of tissues, including muscle, liver, lung, stomach, kidney, heart, intestine and brain under the conditions in which the signal for cytoskeletal actin mRNA was detected at more or less similar levels in all the tissues. Although the high level occurrence of *Xem1* in ovary was consistent with the maternal nature of this transcript, its high level occurrence in testis was an unexpected result, which suggest that *Xem1* gene has some essential role in gametogenesis not only in female but also in male.

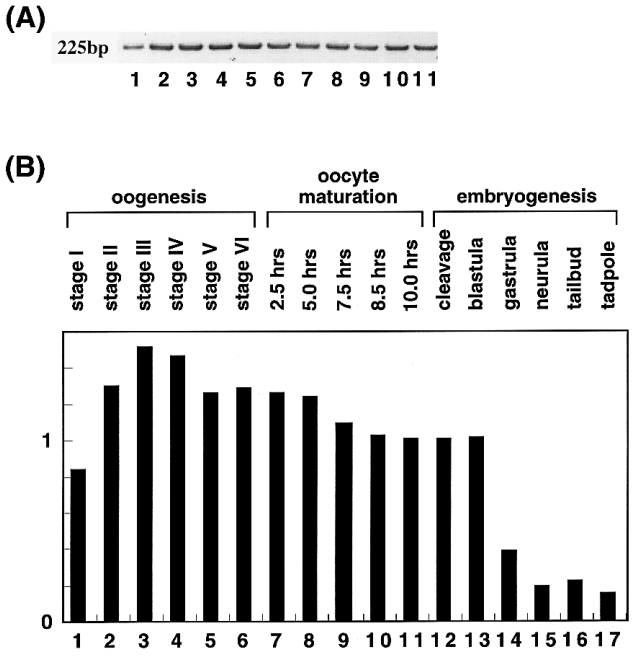

FIG. 5. Expression of *Xem1* transcript. Expression of *Xem1* transcripts during oogenesis, oocyte maturation, and embryogenesis as studied by RT-PCR. Total RNA corresponding to 0.025 oocyte was used for the analysis of oocytes at different stage (one full-grown oocyte contains 4 μg of total RNA and younger oocytes contain smaller amounts of RNA), whereas 25 ng of RNA were used for assays with embryos. Lanes 1 to 6, stages I to VI oocyte, respectively; lanes 7 to 11, stage VI oocyte treated with progesterone for 2.5, 5.0, 7.5, 8.5 and 10.0 hrs, respectively; lane 12 (cleavage); lane 13 (blastula); lane 14 (gastrula); lane 15 (neurula); lane 16 (tailbud); lane 17 (early-tadpole). (A) Expression of *Xem1* transcripts during oogenesis and oocyte maturation by RT-PCR. (B) A graphical expression of the result in (A). The intensity of each band in (A) was measured by a fluorescent image analyzer (FluorImager SI, Molecular Dynamics). Note that the values for lane 11 and lane 12 were taken as 1.0 for lanes 1 to 11 and lanes 12 to 17, respectively, since fertilization does not alter the mRNA content.

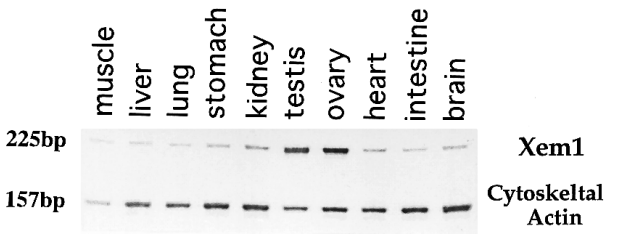

FIG. 6. Distribution of *Xem1* transcript in adult tissues. For RT-PCR, 25 ng of total RNAs from each tissue was used. The cytoskeletal actin as a ubiquitous mRNA was used as a reference.

DISCUSSION

In the present study, we determined the full-length or nearly full-length structure of cDNA for maternally provided and developmentally down-regulated gene, *Xem1*. From its structure, this gene seems to encode a transmembrane protein in a variety of eukaryotic phyla. Despite its conserved structure which was revealed by sequence analysis, no clues to functions are currently available for *Xenopus* Xem1 protein. To obtain insights for its possible function in embryos, we inserted *Xem1* ORF into *Xenopus* globin 5′ and 3′ UTRs in a derivative of pSP64T (18), pSP36T (a gift from Drs. E. Amaya and M. Kirschner), and microinjected the *in vitro* transcribed mRNA into *Xenopus* fertilized eggs. In this experiment, however, no modification of development was observed, although injection of mRNAs for other proteins such as S-adenosylmethionine decarboxylase (19) and activin receptor (20) interfered with normal development.

By sequence analysis, however, COPT1 from *Arabidopsis* and yeast YHX5/CTR2/YHR175W, both have been shown to be transporters of copper (14), were found to be very close homologs of *Xenopus* Xem1. From sequence and distribution data in adult tissues and embryos, we tentatively postulate that *Xenopus* Xem1 may play a role in membrane transport mechanism in various cells especially in those in germ line and possibly in embryos at very early stage. In this relation, it is intriguing to note that some metals such as zinc and iron have been reported to be accumulated during oogenesis (21), and peroxovanadium compounds induce maturation in *Xenopus* oocytes (22).

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bryos and in *in vitro* transcription and microinjection experiment of *Xem1* mRNA, respectively. This work was partly supported from grants from Ministry of Education, Science, Sports and Culture, Japan and Japan Society for the Promotion of Science.

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