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Short Contribution

Expression of the *Xenopus laevis* metallothionein gene during ontogeny

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ABSTRACT Expression of the *Xenopus laevis* metallothionein (MT) gene was studied by *in situ* hybridization throughout development. MT mRNA was detected from the tailbud stage onwards. MT expression was observed in bucco-pharyngeal epithelium, pronephros and liver anlagen, as well as in lens and periventricular areas of the encephalon. MT transcripts, in both larvae and adults, were detected in diverse regions of the central nervous system and in differentiating tissues implicated in detoxification processes: liver hepatocytes, small intestine epithelia and kidney tubules. These data are discussed in the context of MT functions and support a physiological role for MT in growth processes.

KEY WORDS: *metallothionein, development, in situ hybridization, Xenopus laevis*

Metallothioneins are implicated in metal metabolism, cellular repair processes, growth and differentiation (Hamer, 1986). They likely serve as a source of zinc for newly synthesized apoenzymes. Zinc is a cofactor for nearly 300 enzymes (Vallee, 1991) and is also bound to protein domains in many DNA-binding transcription factors (Schmiedeskamp and Klevit, 1994). This metal ion is essential for vertebrate development, since zinc deficiency results in multiple congenital malformations (Webb, 1987). Four classes of MTs have been characterized in mammals. The *MT-I* and *MT-II* genes are expressed in many tissues, and at a particularly high level in liver and kidney. Expression of *MT-III* is restricted to the brain and to male reproductive organs, while that of *MT-IV* is specific to stratified squamous epithelia (Uchida *et al.,* 1991; Palmiter *et al.*, 1992; Quaife *et al.*, 1994; Moffatt and Séguin, 1998). Genetic experiments indicate that MT function is not essential, since mice that cannot synthesize either MT-I or MT-II grow and reproduce normally. Mice lacking MT-III do not reveal any neurological or behavioral deficiencies (Palmiter, 1998). Recent experiments have shown that thiolate ligands in MT confer redox activity on zinc clusters. This strongly suggests that MT would control the cellular zinc distribution as a function of the cellular energy state (Maret and Vallee, 1998).

Involvement of MT in developmental processes has been examined in several species. It was first shown in sea urchins that specific MT isoforms are developmentally regulated (Nemer *et al.*, 1984, 1991). This finding was also subsequently reported in mammals (Andrews *et al.*, 1987, 1991) and in *Drosophila melanogaster* (Silar *et al.*, 1990). One MT isoform (62 amino acids, 20 cysteines) has been characterized in the liver of *Xenopus laevis* (Yamamura and Suzuki, 1983), and the corresponding MT cDNA cloned (Muller *et al.*, 1993; Saint-Jacques and Séguin, 1993). In this work, we studied the localization of MT transcripts by *in situ* hybridization throughout *X. laevis* development to determine if the expression pattern is similar in mammals and in amphibians.

We detected one 0.8 kb MT transcript in RNA extracted from whole ovaries (MT, Fig. 1), but not in RNA from early embryos. Weak expression appears in stage 25/26 embryos and increases in later stages. It was possible to detect earlier signals, i.e. in stage 18 embryos, when analyzing polyA$^+$ mRNAs and using very long exposure times for autoradiograms. Still earlier hybridization signals were observed using RT-PCR amplification. However, MT expression during early *Xenopus* development, in any case, is very low. A noticeable amount of MT transcript is present only throughout larval development (head and trunk samples), and in adult organs (brain, liver, kidney and intestine). The 800bp MT RNA was the only transcript detected in all of our analyses.

MT seems to play a minor role in the control of zinc homeostasis during oogenesis and the first developmental stages in *X. laevis*. For example, treatment with metals does not have a dramatic effect on MT expression during early development: a 10 fold increase in MT mRNA concentration was only observed at stage 26 after

Abbreviations used in this paper: MT, metallothionein.

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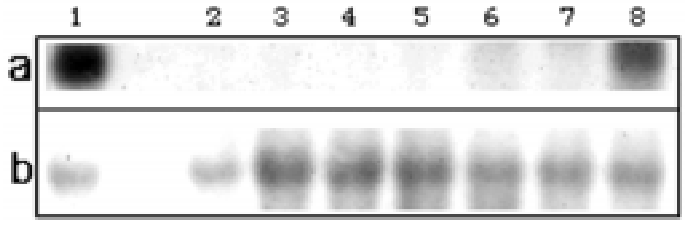

Fig. 1. MT mRNA expression during *Xenopus* development. *RNA was fractionated on 1.2% agarose composite gels (0.6% agarose, 0.6% NuSieve GTG) and hybridized with ^{32}P-labeled X. laevis probes coding for MT (**a**, XL2-MT cDNA, Muller et al., 1993) and 18S rDNA (**b**, control for RNA loading). Total RNA, 20 µg/lane: 1, ovary; 2, early cleavage; 3, blastula; 4, early gastrula; 5, late gastrula; 6, neurula; 7, early tailbud [stage 22]; 8, tailbud [stage 28]. A 0.8 kb MT transcript is detected only in ovary and late tailbud stage embryos.*

adding these metals to the culture medium of stage 8 embryos (Sunderman *et al.*, 1995b). This is in comparison to the 100 fold MT mRNA increase observed when cells in culture are similarly treated (Muller *et al.*, 1993). Amphibian and teleost species which share similar oogenesis processes with *X. laevis* also display identical MT developmental patterns. For example, MT expression during *Xenopus* embryogenesis is similar to that of trout (Olsson *et al.*, 1990). In contrast, axolotl (*Ambystoma mexicanum*, urodele) embryos express MT genes from the blastula stage onwards (Saint-Jacques *et al.*, 1998).

Localization of MT transcripts during embryogenesis was performed by whole-mount *in situ* hybridization. MT RNAs are detected mainly in the anterior part of the gut from stages 33 to 38 (Fig. 2). Strong labeling in endodermal derivatives (bucco-pharyngeal epithelium and outer part of the yolk mass in head or trunk level) is observed (Fig. 3). The pronephric anlage, which is well individualized at these stages, is heavily labeled as well as the developing wolffian duct. A clear hybridization signal is visible in the lens (Fig. 3b) while a weaker signal is observed in the periventricular areas of encephalon and neural tube (Fig. 3b,c). Somites, notochord and lateral plate mesoderm are not labeled. MT gene expression in embryonic cells might be related to a redistribution of the zinc contained in yolk platelets. Vitellus hydrolysis, particularly at the onset of organogenesis, releases large amounts of zinc (Sunderman *et al.*, 1995a) which is then available to induce MT gene expression. The MT protein thus would "buffer" the intracellular zinc concentration.

The same organs express MT throughout *Xenopus* larval development and in the adult. Accumulation of MT transcripts was

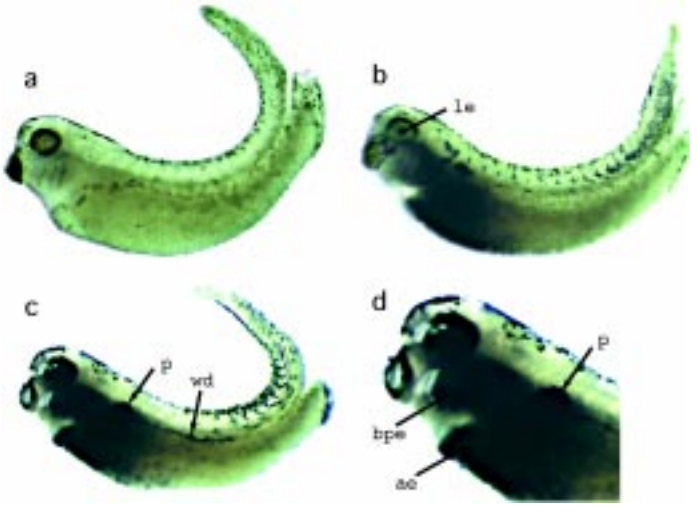

Fig. 2. MT expression in tailbud stage embryos (whole-mount). *Embryos [**(a)** stage 35, **(b)** stage 33, **(c,d)** stage 37] were fixed and processed for whole-mount in situ hybridization using digoxigenin labeled sense **(a)** and antisense **(b-d)** MT riboprobes. The enlargement in **(d)** shows a strong internal labeling including all anterior endoderm. ae, anterior endoderm; bpe, bucco-pharyngeal epithelium; le, lens; p, pronephros; wd, wolffian duct.*

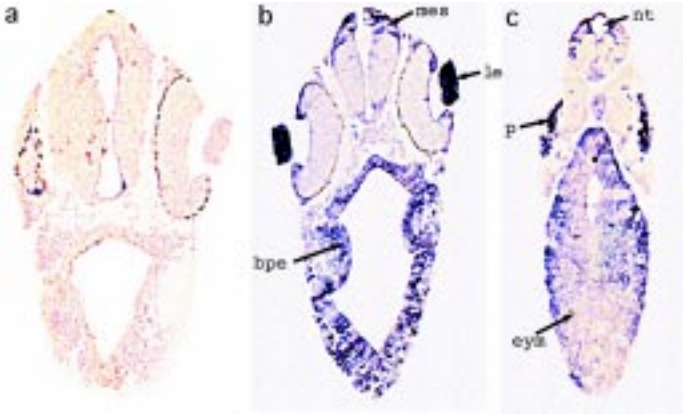

Fig. 3. MT expression in tailbud stage embryos (whole-mount sections). *Transversal sections of whole-mount hybridized embryos: **(a)** sense probe, head level, stage 33, **(b)** antisense probe, head level, stage 33, **(c)** antisense probe, trunk level, stage 37. bpe, bucco-pharyngeal epithelium; eym, endodermal yolk mass; le, lens; mes, mesencephalon; nt, neural tube; p, pronephros.*

observed in liver hepatocyte rows (Fig. 4a,c), larval intestine (Fig. 4c) and proximal kidney tubules (Fig. 4b). The connective and muscular layers of these organs never showed hybridization signals. Strong expression in the anterior part of the intestine, within the predominant columnar absorptive cells, was found during the early climax when feeding begins (stage 45, Fig. 4c). Intestinal primary epithelial cells meanwhile undergo cytolysis, and IFABP (Intestinal Fatty Acid-Binding Protein), a marker of intestinal absorption, is poorly expressed (Shi and Hayes, 1994; Ishizuya-Oka *et al.*, 1997). MT could be required at that time to regulate the level of free radicals or toxic products released by cell cytolysis, and in the subsequent period of rapid differentiation and growth of the adult intestinal tract.

Zinc is a neuromodulator and its level is impaired in some neurological disorders (Ebadi *et al.*, 1995). Expression of the MT gene in the *Xenopus* central nervous system thus deserves special attention. As shown in Figure 5, MT transcripts were found in several parts of the brain, particularly in cellular bodies of periventricular regions. A strong expression was also detected in

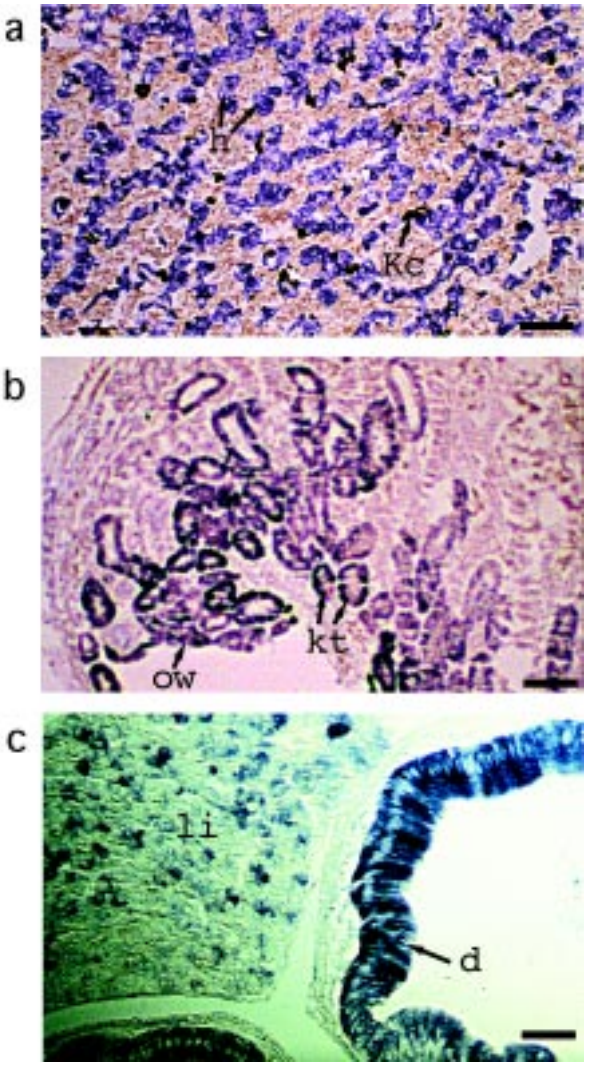

Fig. 4. MT expression in larval and adult organs. *Tissue sections of larval and adult organs embedded in Paraplast were hybridized with antisense MT digoxigenin labeled probes without additional staining.* **(a)** *Adult liver,* **(b)** *adult kidney,* **(c)** *larval liver and intestine, early climax [stage 60]. d, duodenum; h, hepatocytes; Kc, Kuppfer cells; kt, kidney tubule; li, liver; ow, outer wall. Bars,* **(a)** *300 μm,* **(b,c)** *100 μm.*

the infundibulum, and at a lower extent in the hypophysis. This pattern is very similar to that observed in most other studied vertebrates (Hao *et al.*, 1994; Choudhuri *et al.*, 1995). This suggests that MT may play similar functions in all vertebrate brains. However, more complex patterns of MT gene expression have been characterized in species possessing multiple MT isoforms, especially in mammals. For example, the MT-III isoform is particularly abundant in neurons, specifically those sequestering zinc in synaptic vesicles within the cerebral cortex, the hippocampus, the amygdala and the base of the cerebellum (Masters *et al.*, 1994; Erickson *et al.*, 1995).

Taken together, our data demonstrate that the *Xenopus* MT gene is expressed in specific sites at specific times in embryos, larvae and adults. This work focuses on the only MT characterized to date in *Xenopus*. This MT possibly would assume the array of functions of the more specialized MT characterized in mammals (MT-I to MT-IV classes, Palmiter, 1998). Since we were unable to isolate a *Xenopus MT-III* homolog (Muller, unpublished results), and avian species possess a very simple MT gene family (Andrews *et al.*, 1996), it would be interesting to determine if a significant diversification of the MT gene family occurred only in the mammalian lineage during vertebrate evolution.

Experimental Procedures

Embryos

Eggs obtained from gonadotropin-injected *Xenopus* were fertilized *in vitro*, dejellied manually, and cultured in 0.1xMarc's Modified Ringer's solution. Embryos and larvae were staged according to Nieuwkoop and Faber (1967).

Northern blotting

Total RNA was purified with the RNA quick TMII kit (Bioprobe Systems). Polyadenylated RNA was isolated by two runs on oligo (dT) cellulose (Collaborative Research). RNA samples were fractionated on 1.2% agarose (0.6% agarose, 0.6% NuSieve GTG) formaldehyde gels and blotted onto nitrocellulose Hybond N membranes (Amersham). A 752bp cDNA encoding a *Xenopus* metallothionein (MT-XL2, Muller *et al.*, 1993) was labeled by random priming with [α^{32}P]dATP (specific activity: 10^9 dpm/μg).

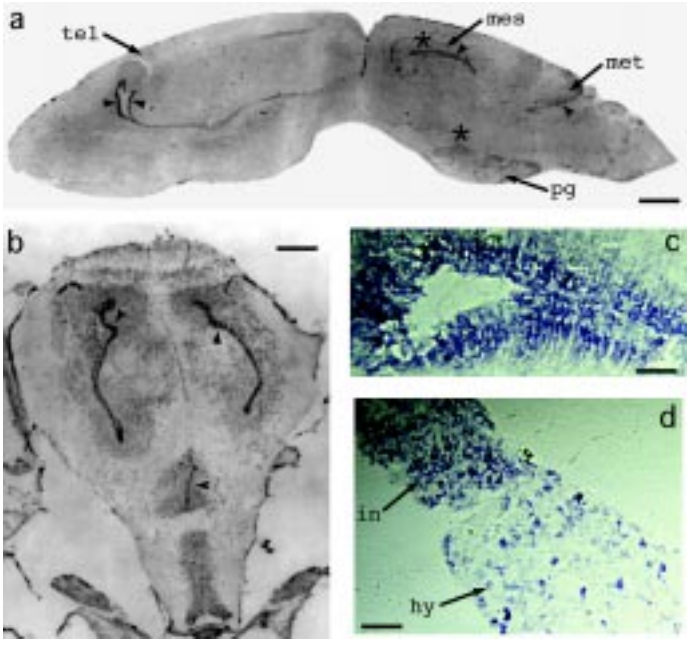

Fig. 5. MT expression in larval and adult brain. (a,b) *Hybridization of brain sections with radioactive antisense probes. Sections were counterstained with 0.1% toluidine blue and examined under brightfield optics.* **(a)** *Parasagittal section of adult brain. Bar, 400 μm* **(b)** *Horizontal section of larval brain [stage 62, climax]. Bar, 300 μm. Arrowheads point to the main labeling sites at the level of periventricular areas.* **(c,d)** *Hybridization with digoxigenin labeled antisense probes. These sections, which are not counterstained, correspond to brain regions indicated by stars in (a).* **(c)** *Enlargement of the periventricular neuroepithelium of the mesencephalon. Bar, 120 μm.* **(d)** *Enlargement of the pituitary gland and infundibulum. Bar, 120 μm. hy, hypophysis; in, infundibulum; mes, mesencephalon; met, metencephalon; pg, pituitary gland (infundibulum and hypophysis); tel, telencephalon.*

Hybridization was carried out overnight at 42°C. Washings were performed in 0.2xSSC+0.5% SDS at 42°C.

In situ hybridization

Whole-mount *in situ* hybridizations were performed on normal embryos with digoxigenin-UTP labeled riboprobes as described by Meyer *et al.* (1997). After examination, tissue sections (10 μm) were performed on these hybridized embryos embedded in paraplast. The DIG-labeled probes were also used for hybridization on tissue sections (7-8 μm) of larvae and adults. Prehybridization was performed 3 h at 65°C and hybridization at 55°C overnight. After treatment with RNAse, washings (2x1 h) were carried out in 0.2xSSC+0.3% CHAPS. Tissue sections without additional staining were examined using bright optics. Radioactive RNA probes were also used for *in situ* hybridization on larval and adult tissue sections (7 μm). Antisense and sense riboprobes were labeled using 5'[α^{35}S]UTP (400 Ci/mmole, Amersham) at a specific activity of 5.10^8 dmp/μg. Slides coated with Amersham LM1 emulsion were exposed for one week at 4°C. These tissue sections were counterstained with 0.1% toluidine blue, mounted in Eukitt, and examined using bright and darkfield optics.

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