Int. J. Dev. Biol. 47: 299-302 (2003)

Short Communication

Expression of Muscle LIM protein during early development in *Xenopus laevis*

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ABSTRACT We have isolated the *Xenopus* homologue of Muscle LIM protein (MLP, CRP3) and examined its expression during early embryonic development. MLP is only expressed in the differentiated heart during early development and is expressed in a subset of other striated muscles during later stages. There is no MLP expression during primary myogenesis in the somites, although it is found in adult skeletal muscle.

KEY WORDS: *Xenopus, MLP, CRP3, cardiogenesis, heart*

Muscle LIM protein (MLP or CRP3) is a member of the LIM domain-containing cysteine-rich protein (Csrp) family that usually have restricted expression patterns and play important roles development (Dawid *et al.*, 1998). MLP contains two LIM domains that are each followed by a glycine-rich domain, however MLP lacks other known protein motifs classifying it as a member of the LIM-only subset of this protein family. MLP has been shown to potentiate skeletal muscle differentiation in cell culture and its expression is limited to skeletal and cardiac muscle in adult mammals (Arber *et al.*, 1994). Mice lacking MLP usually die of dilated cardiomyopathy shortly after birth (Arber *et al.*, 1997). The dilated cardiomyopathy exhibited by these mice share many of the same physiological features as dilated cardiomyopathy in humans (Ecarnot-Laubriet *et al.*, 2000; Esposito *et al.*, 2000; Su *et al.*, 2001) and patients with chronic heart failure show decreased expression of MLP (Zolk *et al.*, 2000).

Several observations suggest that MLP could play a role in the differentiation of striated muscle in vertebrates. Although the majority of the protein is found in the cytoplasm, associated with actin-based filaments, there is a portion that is found in the nucleus (Arber and Caroni, 1996). Expression of MLP enhances the ability of myogenic regulatory factors to cause muscle differentiation in C2 myoblasts (Arber *et al.,* 1994) indicating that it may play a role in the early differentiation process. The possibility of a direct role in skeletal muscle differentiation is supported by the observation that MLP can directly bind the myogenic, basic helix-loop-helix (bHLH) proteins but not other bHLH proteins (Kong *et al.,* 1997). In addition, the closely related CRP1 and CRP2 promote the differentiation of smooth muscle development through interactions with GATA proteins and serum response factor (Chang *et al.*, 2003).

Despite MLP's potential role in the differentiation of striated muscle, a detailed analysis of its expression pattern in early *Xenopus* embryos has not been done. MLP expression has been demonstrated in the murine heart at e8.0 and in other striated muscles from e13.5 (Harrod *et al.*, 1996; Jain *et al.*, 1998). In order to examine MLP expression, we have cloned a *Xenopus* gene product with high sequence similarity to MLP (Fig. 1A). The nucleotide sequence is more closely related to mammalian *CRP3* sequences than to *CRP1* or *CRP2* (Fig. 1B). In addition, partial *Xenopus* sequences have been identified that correspond to *CRP1* (GenBank Accession number: BU904058) and *CRP2* (GenBank Accession number: BJ098908) and these are distinct from our sequence. The sequence data coupled with the expression pattern below strongly suggests that our *MLP* sequence represents the *Xenopus* homologue of *MLP/CRP3*.

MLP first appears in the developing myocardium at stage 26/27 (Fig. 2 A,F). This is the same time that most genes involved in cardiac contraction begin to be expressed (Mohun *et al.*, 2000). Surprisingly, we do not detect *MLP* expression in the developing somites indicating that, in *Xenopus*, the initial differentiation of skeletal muscle precursors does not involve MLP. At stage 36, the expression of *MLP* is still restricted to the heart (Fig. 2B). It was not until later stages (stage 41) that additional

Abbreviations used in this paper: MLP, Muscle LIM protein.

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0214-6282/2003/$25.00

Printed in Spain
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sites of expression were identified. Expression in the intermandibularis muscle and in the paired lymph hearts, a small contractile structure, was observed (Fig. 2 C,D,E). *MLP* is eventually expressed in both the adult heart and skeletal muscle, as detected by northern blot analysis (Fig. 2G), suggesting that *MLP* expression is limited to post-metamorphic skeletal muscle.

The expression pattern of *Xenopus MLP* corresponds to other *MLP* genes in that the expression is limited to striated muscle (Fig. 2) whereas CRP1 and CRP2 are expressed in a wider array of tissues (Henderson *et al.*, 1999; Henderson *et al.*, 2002). We saw no expression below the somites, a region fated to give rise to smooth muscle cells in *Xenopus* (Oka *et al.*, 2000). To confirm that *MLP* is expressed in the myocardium and not endocardium, as suggested in sections, we exposed embryos to retinoic acid at stage 15, a treatment known to inhibit myocardial differentiation (Drysdale *et al.*, 1997). Treatment of retinoic acid was able to block expression of MLP indicating that the expression is myocardial (Fig. 3). This result also indicates that the MLP expression is a result of myocardial differentiation as retinoic acid blocks the differentiation of the myocardium but not the expression of all genes expressed in the heart prior to differentiation (Jiang *et al.*, 1999).

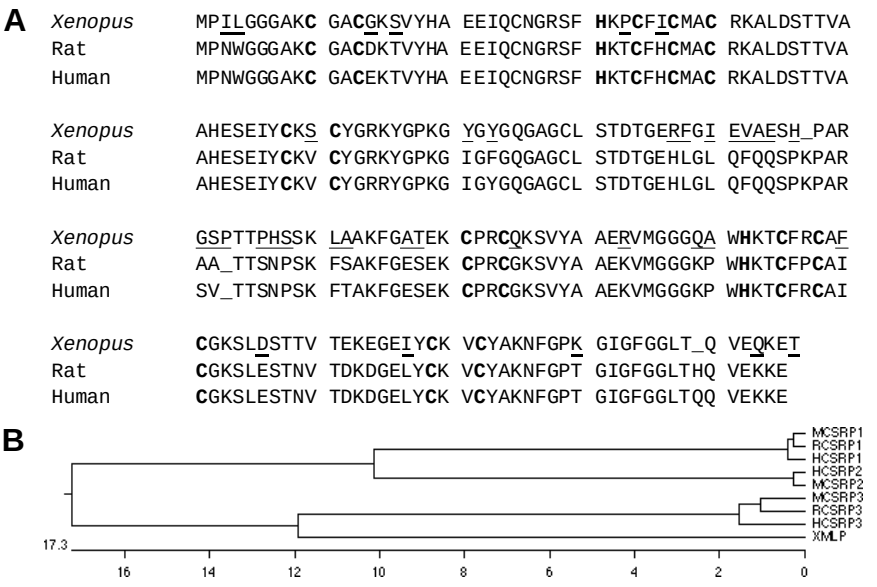

Fig. 1.Sequence analysis of *Xenopus* MLP. **(A)** *A comparison of the* Xenopus, *rat, and human MLP amino acid sequences (GenBank accession numbers AAF78241, X81193 and AAA92571 respectively). Differences in the* Xenopus *MLP sequence, when compared to the mammalian sequences, are underlined. The cysteine/histidine residues that form the two zinc fingers are in bold and are conserved across all three sequences.* **(B)** *A dendrogram showing the relationship of XMLP to other members of the CRP family showing that the* Xenopus *sequence clusters with other vertebrate CRP3 sequences rather than CRP1 or CRP2.*

MLP can interact with the myogenic regulatory factors and can promote differentiation of skeletal muscle in cultured cells (Kong *et al.*, 1997). In *Xenopus*, *MyoD* is first expressed prior to gastrulation (Harvey, 1990; Hopwood *et al.*, 1992). *Myf5* and *Mrf4* are also expressed well before *MLP* (Hopwood *et al.*,

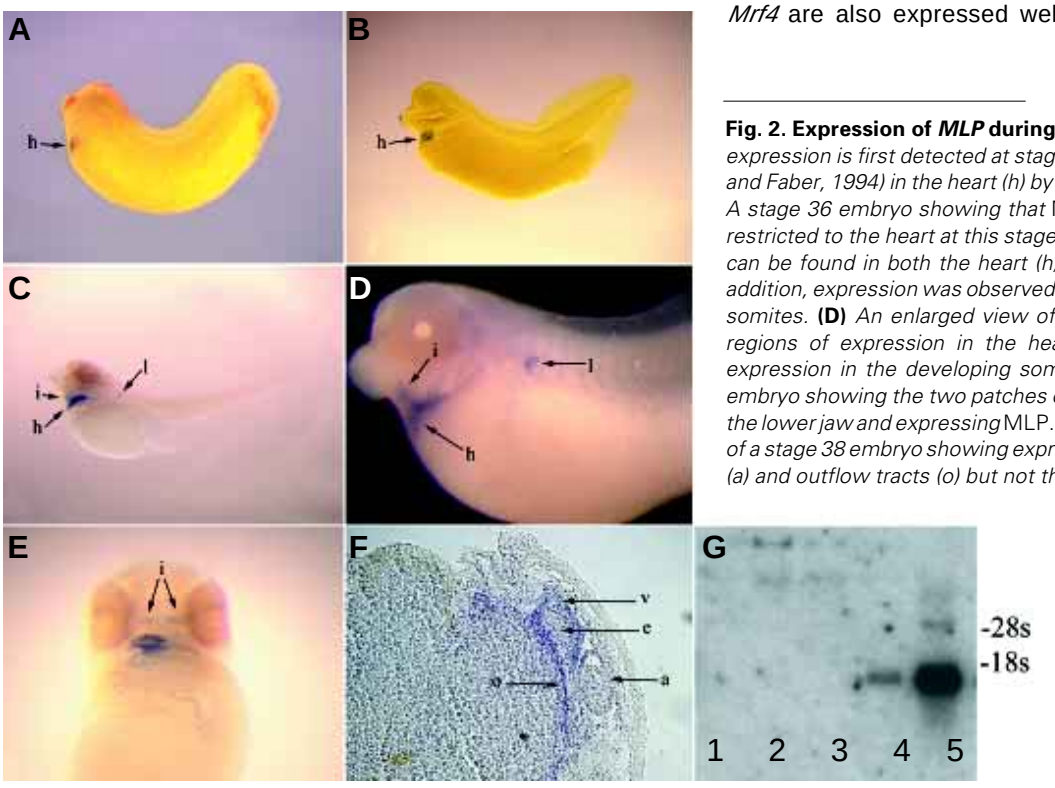

Fig. 2. Expression of *MLP* during *Xenopus* embryogenesis. (A) MLP *expression is first detected at stage 27 (staged according to Nieuwkoop and Faber, 1994) in the heart (h) by whole mount* in situ *hybridization.* **(B)** *A stage 36 embryo showing that* MLP *expression (seen in blue) is still restricted to the heart at this stage.* **(C)** *At stage 40/41,* MLP *expression can be found in both the heart (h) and intermandibularis (i) muscle. In addition, expression was observed in the lymph heart (l) near the anterior somites.* **(D)** *An enlarged view of a stage 40/41 embryo showing the regions of expression in the head and clearly showing the lack of expression in the developing somites.* **(E)** *Ventral view of a* Xenopus *embryo showing the two patches of intermandibularis muscle spanning the lower jaw and expressing* MLP. **(F)** *A frontal section through the heart of a stage 38 embryo showing expression of* MLP *in the ventricle (v), atria (a) and outflow tracts (o) but not the endocardium (e).* **(G)** *Northern blot of adult intestine (1), kidney (2), liver (3), skeletal muscle (4), and heart (5) RNA probed with* MLP. *There was approximately equal loading in each lane based on rRNA levels.* MLP *expression is restricted to the heart and skeletal muscle. As in mammals, a higher level of expression was observed in the heart, in which a second, larger transcript was also observed (Arber* et al., *1994).*

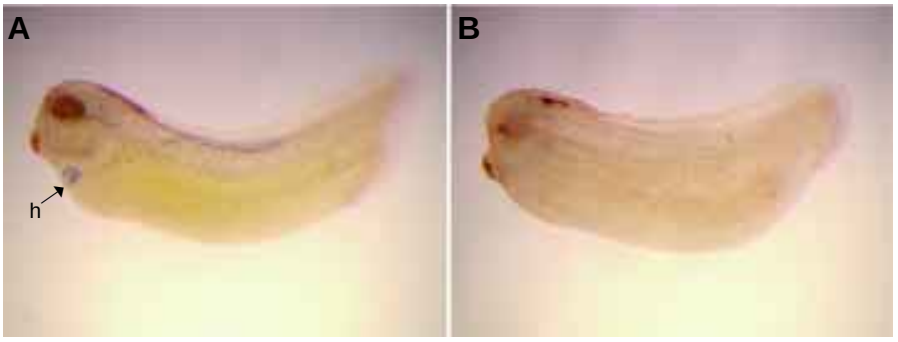

Fig. 3. *MLP* expression is disrupted by retinoic acid. *Expression of* MLP *in* **(A)** *a normal heart (h, arrow) and* **(B)** *an embryo treated with 1 μM retinoic acid. Treatment with retinoic acid results in a complete loss of* MLP *expression in the heart region.*

1991; Jennings, 1992). This would indicate that, at least in *Xenopus*, interactions with the myogenic regulatory proteins do not occur until after primary myogenesis. Myogenin is not required for primary myogenesis in *Xenopus* and is not expressed until the transition from embryonic to adult muscle during metamorphosis (Nicolas *et al.*, 1998), and thus specific interactions between MLP and myogenin remain a possibility.

Although we know that MLP is required for normal cytoarchitecture and function in the heart (Arber *et al.*, 1997), its function remains elusive. We have demonstrated that in *Xenopus*, *MLP* expression is associated with an unusual subset of striated musculature. However, the timing of expression of *MLP* in striated muscle in *Xenopus* indicates that it cannot play a role in the initial differentiation of these tissues. It is hoped that by understanding this pattern, a greater insight into the function of MLP will result.

Experimental Procedures

To obtain the clone, a rat *MLP* probe (kindly provided by S. Arber) was used to probe an adult *Xenopus* heart cDNA library (kindly provided by J. Catt, NIH). Whole mount *in situ* hybridization performed according to Harland (1991). Northern blot analysis was performed using standard procedures with random-primed *Xenopus* MLP cDNA as a probe. The phylogenetic relationship of the *CRP* sequences was done with MegAlign from the DNASTAR sequence analysis package (DNASTAR Inc.)

Embryos were treated with 1 μM retinoic acid at stage 15 according to Drysdale *et al.,* 1997. Embedding an embryo in paraplast, after whole mount *in situ* hybridization, and sectioning at 15 μm was done to generate the histological section. All embryos were staged according to Nieuwkoop and Faber (1994).

Acknowledgements

This work was supported by a grant from the Canadian Institutes of Health Research, the National Sciences and Engineering Research Council of Canada and a Research Scholarship from the Heart and Stroke Foundation of Canada (TAD).

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Received: June 2002
Reviewed by Referees: July 2002
Modified by Authors and Accepted for Publication: February 2003
Edited by: Thomas D. Sargent