Int. J. Dev. Biol. 49: 981-984 (2005)
doi: 10.1387/ijdb.052070jl

Developmental Expression Pattern

Developmental expression of *Xenopus Fragile X mental retardation-1* gene

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ABSTRACT Dysregulation of *Fragile X mental retardation-1 (Fmr1)* gene expression results in an inherited form of mental retardation known as the Fragile X syndrome (FXS). *Fmr1* is a highly conserved gene with a broad yet distinctive expression pattern during vertebrate development. Here, we examined the expression pattern of *Fmr1* during *Xenopus* embryonic development. Zygotic expression of *Fmr1* began just prior to gastrulation and gradually increased during subsequent embryonic stages. By *in situ* hybridization, *Fmr1* transcripts were detected by early tailbud stage and showed robust expression in the central nervous system (CNS), eye and pharyngeal arches. By late tailbud stage, *Fmr1* expression became stronger in the CNS and craniofacial regions including the ear vesicle and eye. In addition, the notochord expressed high levels of *Fmr1* transcripts in the late tailbud stage embryos. In the tadpole brain, the olfactory bulb and cerebellum exhibited strong *Fmr1* expression. The developmental expression pattern of *Fmr1* is consistent with the wide range of abnormalities observed in FXS. Further, our findings indicate that *Xenopus* will serve as an excellent model to study the developmental basis of this disease.

KEY WORDS: *Fmr1, Fragile X syndrome, neural crest, Xenopus*

Fragile X syndrome (FXS) is the most common form of inherited mental retardation, with an incidence of about 1:4000 in males and 1:8000 in females. Individuals with FXS display moderate to severe mental retardation as well as craniofacial abnormalities and connective tissue dysplasia (Hagerman and Hagerman, 2002). Fragile X mental retardation-1 (Fmr1) gene encodes an RNA-binding protein, FMRP, which is highly conserved during evolution. FMRP consists of two K-homology (KH1-2) domains and an RGG-box domain (Fig. 1A). It also has a nuclear localization (NLS) and an export (NES) signal (Fig. 1A). Recently, the RGG-box has been demonstrated to mediate binding to a subset of RNAs with G-quartet structure (Darnell *et al.*, 2001). *Xenopus* FMRP had 90%, 95%, 90%, 100% and 61% amino acid sequence identities to NLS, KH1, KH2, NES and RGG box domains of human FMRP, respectively (Fig. 1A). Phylogenetic analysis further revealed that the amino acid sequences of *Xenopus* FMRP is closer to human FMRP than either zebrafish or *Drosophila* (Fig. 1B), suggesting that it will serve as a useful model to study FMRP function(s).

Northern blot analysis of developing embryos indicated that *Xenopus Fmr1* has a large maternal component, most of which disappeared prior to gastrulation (Fig. 2). Zygotic expression of *Fmr1* began at about gastrula (st. 10) and gradually increased from neurula (st. 12) to early tadpole stage (st. 47). During early to late tailbud stage, *Fmr1* expression intensified in the craniofacial region with prominent staining in the pharyngeal arches (Fig. 3A). This distribution resembled the expression pattern of neural crest marker *AP-2*α (compare Fig. 3A and 3B). However, unlike the *AP-2*α expression at this stage (Luo *et al.*, 2002), *Fmr1* transcript levels were higher in the CNS and eye (Fig. 3A, E and G).

Horizontal sections of an early tailbud stage embryo further revealed that endodermal, mesodermal and mesenchymal (neural crest) regions of the pharyngeal arches expressed *Fmr1* (Fig. 3A and D). By early tadpole stage (st. 35), forebrain, midbrain, hindbrain regions and the notochord showed high *Fmr1* expression (Fig. 3C, F and G). Similar findings in the CNS (Agulhon *et al.*, 1999) and notochord (Hergersberg *et al.*, 1995) have been observed in mice. Again, craniofacial regions including the ear vesicles (Fig. 3F) and eye (Fig. 3G) displayed high *Fmr1* transcript levels at this stage. Furthermore, in the late tadpole (st. 45) CNS, the olfactory bulbs and the cerebellum displayed strong *Fmr1* expression (Fig. 4).

Abbreviations used in this paper: CNS, central nervous system; Fmr, Fragile X mental retardation; FXS, Fragile X syndrome

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0214-6282/2005/$25.00

Printed in Spain
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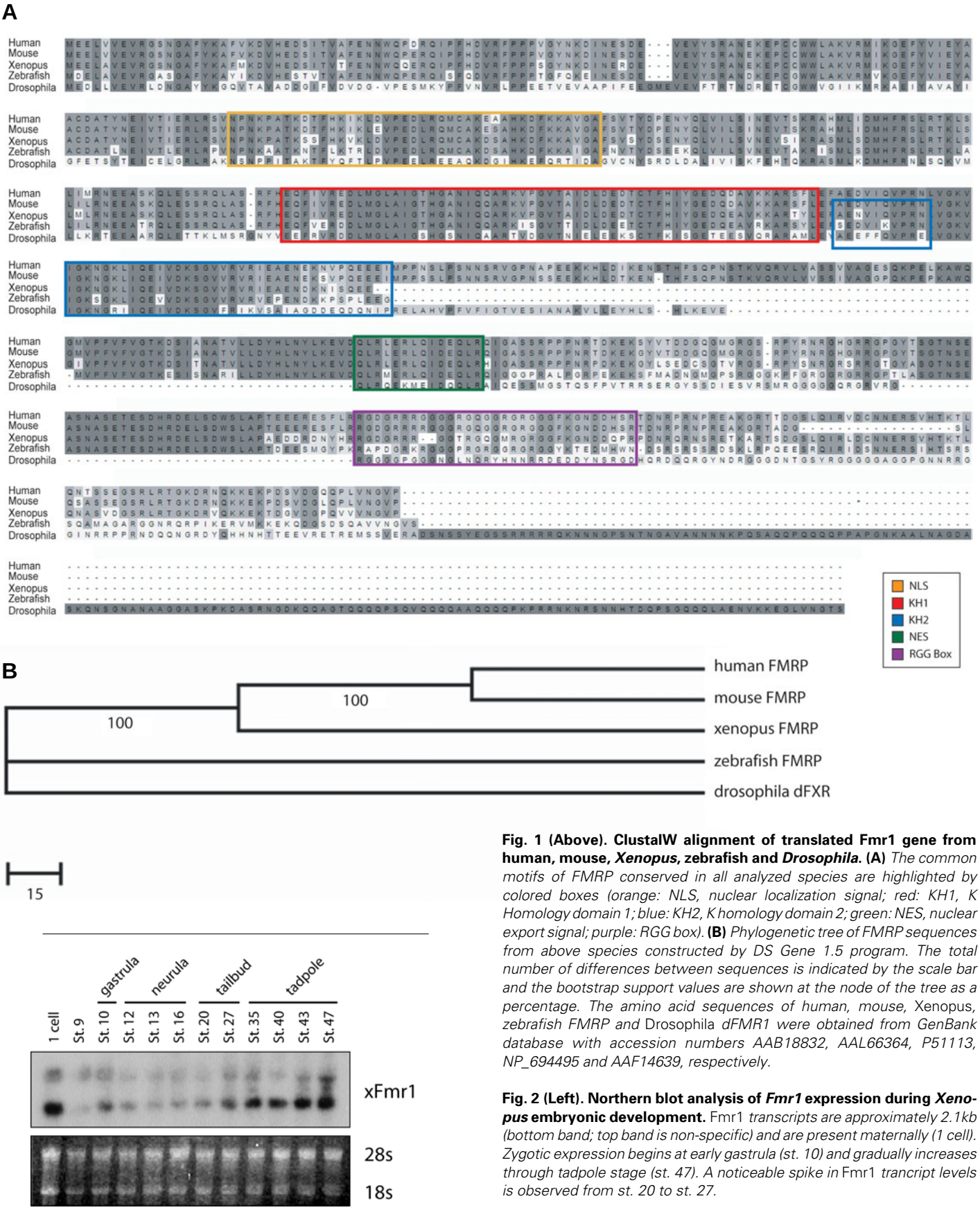

Fig. 1 (Above). ClustalW alignment of translated Fmr1 gene from human, mouse, *Xenopus*, zebrafish and *Drosophila*. (A) *The common motifs of FMRP conserved in all analyzed species are highlighted by colored boxes (orange: NLS, nuclear localization signal; red: KH1, K Homology domain 1; blue: KH2, K homology domain 2; green: NES, nuclear export signal; purple: RGG box).* **(B)** *Phylogenetic tree of FMRP sequences from above species constructed by DS Gene 1.5 program. The total number of differences between sequences is indicated by the scale bar and the bootstrap support values are shown at the node of the tree as a percentage. The amino acid sequences of human, mouse,* Xenopus, *zebrafish FMRP and* Drosophila *dFMR1 were obtained from GenBank database with accession numbers AAB18832, AAL66364, P51113, NP_694495 and AAF14639, respectively.*

Fig. 2 (Left). Northern blot analysis of *Fmr1* expression during *Xenopus* embryonic development. Fmr1 *transcripts are approximately 2.1kb (bottom band; top band is non-specific) and are present maternally (1 cell). Zygotic expression begins at early gastrula (st. 10) and gradually increases through tadpole stage (st. 47). A noticeable spike in* Fmr1 *trancript levels is observed from st. 20 to st. 27.*

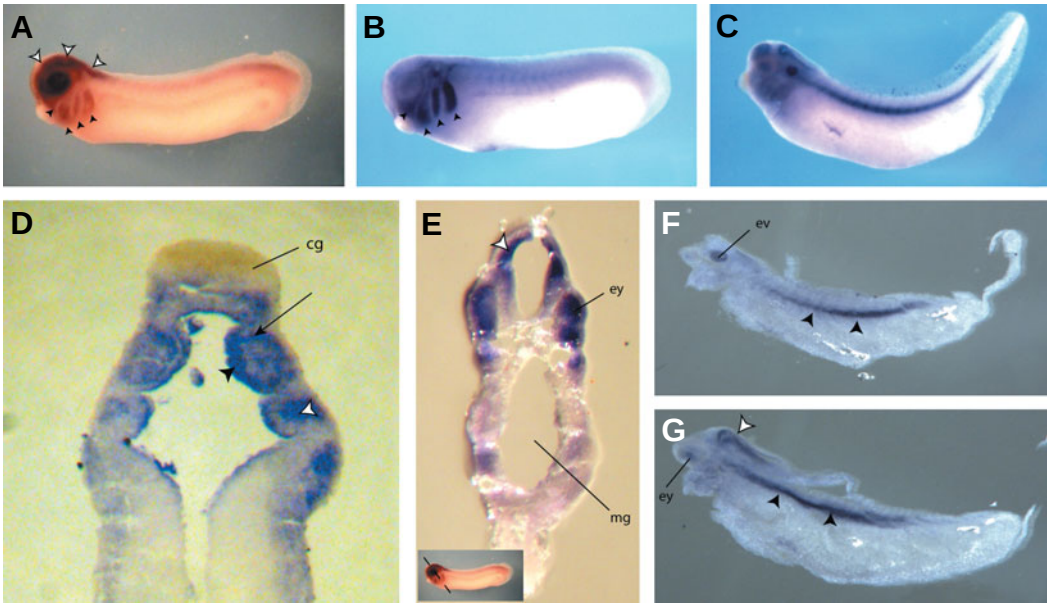

Fig. 3. Whole mount *in situ* hybridization of developing *Xenopus* embryos for *Fmr1*. (A) *Early tailbud stage embryo (st. 26) showing* Fmr1 *expression in the CNS (white arrowheads) and pharyngeal arches (black arrowheads).* **(B)** *Expression of* AP-2α*, a neural crest marker at tailbud stage (st. 26). In comparison to* Fmr1 *expression pattern,* AP-2α *expression is mostly limited to the pharyngeal arches at this stage (black arrowheads).* **(C)** Fmr1 *expression at early tadpole stage embryo (st. 26; see F and G for details).* **(D)** *Horizontal section of st. 26 embryo at the level of cement gland showing* Fmr1 *expression in endodermal (black arrowhead), mesodermal (black arrow) and mesenchymal (white arrowhead) regions of the pharyngeal arches, with slight staining in the ectodermal region. The difference in staining intensity between the left and right side is due to the oblique nature of the sectioned tissue. Cement gland (cg) is negative for* Fmr1 *expression.* **(E)** *Oblique transverse section of tailbud stage embryo (st. 26). Strong expression is observed in the eye evagination (ey) and in CNS (white arrowhead). Insets show approximate region that has been sectioned (mg: midgut).* **(F,G)** *Sagittal section of late tailbud stage embryo depicted in Fig. 3C showing strong expression in the CNS (G: white arrowhead), craniofacial regions, notochord (F,G: black arrowheads), ear vesicle (F: ev) and eye (G: ey).*

In this paper, we demonstrated the developmental expression pattern of *Fmr1* in *Xenopus* embryos. Although previous studies have hinted at *Fmr1* expression in the neural crest cells and branchial arches in mice (Agulhon *et al.*, 1999, de Diego Otero, 2000), our present data provide a clear evidence for *Fmr1* expression in these regions. Because various tissue structures affected in FXS are derivatives of cranial neural crest, our findings carry important implications for understanding the FXS pathogenesis. Therefore, further examination of the role of *Fmr1* expression in the pharyngeal arches as well as various craniofacial regions would undoubtedly enhance our understanding of the Fragile X pathology. Finally, since many tools are available to study various developmental processes using *Xenopus* development, this system should serve as an excellent animal model to investigate the role of *Fmr1* expression during embryonic development.

Experimental Procedures

Protein alignment and phylogenetic analysis

The protein multiple sequence alignment was constructed with DS Gene 1.5 (Accelrys). Phylogenetic analysis was performed with DS Gene 1.5 using the Neighbor Joining Method and bootstrap analysis was performed with 1,000 resampled datasets.

Northern blot hybridization

RNAs were analyzed on Glyoxal gels (Ambion, Inc). Northern blots were washed in 0.2X SSPE at 65ºC. Fmr1 probe was labeled with 32P-dCTP by primer extension (Amersham Pharmacia).

In situ *hybridization*

Whole-mount *in situ* hybridization was performed as described previously (Harland, 1991) using digoxygenin antisense probes for *Xenopus* Fmr1 and AP-2α (Luo *et al.*, 2003). *Xenopus* Fmr1 antisense probe was synthesized from pGEM72-xFmr1 plasmid (Siomi *et al.*, 1995) containing the full-length *Xenopus* Fmr1 using primers: forward 5'-cgg cga tct aga CTC CAA TGG AGC TTT CTA C-3'; reverse 5'-gca taa gga gct cTC AAT TGC AGT CAC CCC AG-3' (Xba1 and Sac1 sites are underlined in the primer sequence, respectively). The PCR product was digested with Xba1 and Sac1, gel purified and subcloned into Xba1 and Sac1 sites in pBluescript SK- vector (Stratagene). It was then linearized with Xba1 and *in vitro* transcribed in the presence of digoxygenin and T3 polymerase

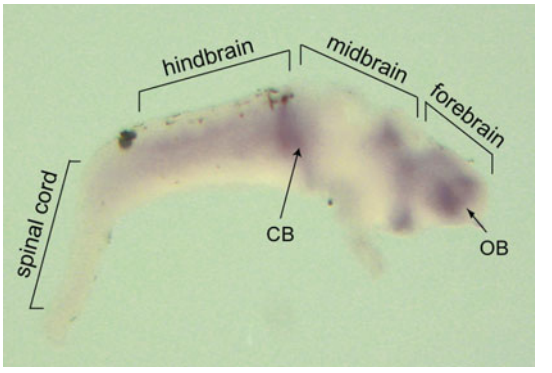

Fig. 4. Whole-mount *in situ* hybridization of *Fmr1* in tadpole brain. *The forebrain, midbrain and hindbrain (lateral view) show moderate to heavy* Fmr1 *expression. Abbreviations: ob, olfactory bulb; cb, cerebellum.*

(Roche Molecular).

Acknowledgements

*We thank Dr. Gideon Dreyfuss for the pGEM72-*Fmr1 *plasmid and Dr. Kim Mowry and Dr. Tracy Kress for advice and access to their Xenopus facility. We also thank Dr. Yanhui Zhang for technical assistance. This work was supported by NIMH Predoctoral NRSA fellowship MH065094-02 (J.H.L) and by grants from National Institutes of Health (NS39321; RR15578). We also thank NICHD for their support.*

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Received: May 2005
Reviewed by Referees: June 2005
Modified by Authors and Accepted for Publication: July 2005
Edited by: George Malacinski