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A fragile X mental retardation-like gene in a cnidarian

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Abstract

The fragile X mental retardation syndrome in humans is caused by a mutational loss of function of the fragile X mental retardation gene 1 (*FMR*1). FMR1 is an RNA-binding protein, involved in the development and function of the nervous system. Despite of its medical significance, the evolutionary origin of *FMR*1 has been unclear. Here, we report the molecular characterization of *HyFMR*1, an *FMR*1 orthologue, from the cnidarian hydroid *Hydractinia echinata*. Cnidarians are the most basal metazoans possessing neurons. *HyFMR*1 is expressed throughout the life cycle of *Hydractinia*. Its expression pattern correlates to the position of neurons and their precursor stem cells in the animal. Our data indicate that the origin of the fraxile X related (FXR) protein family dates back at least to the common ancestor of cnidarians and bilaterians. The lack of FXR proteins in other invertebrates may have been due to gene loss in particular lineages.

Keywords: FMR1; FMRP; FXR; *Hydractinia*; Evolution; Hydrozoa

1. Introduction

The fragile X syndrome is the most common form of inherited mental retardation in humans. The disease is caused by a loss of function mutation in the fragile X mental retardation gene, *FMR*1, mostly due to the expansion of a CGG repeat in the 5′ untranslated region of the gene. Repeat expansion is followed by hypermethylation resulting in the transcriptional silencing of the *FMR*1 gene (Pieretti et al., 1991; Verkerk et al., 1991).

FMR1 (also termed fragile X mental retardation protein [FMRP]) is an RNA binding protein that contains at least three types of RNA binding motifs: a ribonucleoprotein K homology domain (KH domain; FMR1 has two such domains), an arginine and glycine rich domain (RGG box) and an N-terminal NDF domain shown to have RNA and protein binding properties (Siomi et al., 1994; Adinolfi et al., 2003; Ramos et al., 2003a). The so-called FMR1/FXR interaction domain located near the amino terminus of the protein is responsible for the dimerization and interaction with the two other members of the same protein family (Zhang et al., 1995; Siomi et al., 1996). A ribosomal interaction domain mediates interactions with the 60 S ribosomal subunit (Khandjian et al., 1996; Siomi et al., 1996; Tamanini et al., 1996; Feng et al., 1997). Due to the presence of a nuclear export signal sequence (NES), it has been proposed that FMR1 may shuttle between the nucleus and cytoplasm while carrying its target RNAs (Eberhart et al., 1996; Bardoni et al., 1997). Functional studies have revealed a role of FMR1 in dendritic and synapse development (Laggerbauer et al., 2001; Zhang et al., 2001; Morales et al., 2002; Sung et al., 2003). Recently, FMR1 has also been proposed to function in the RNAi machinery as it was shown to be present in the RISC, the

Abbreviations: cDNA, complementary to RNA; FMR1, fragile X mental retardation gene 1; FMRP, fragile X mental retardation protein; FXR, fraxile X related; i-cells, interstitial cells; NDF, N-terminal domain of FMR1; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends.

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RNA-induced silencing complex (Caudy et al., 2002; Jin et al., 2004).

Vertebrate genomes contain one copy of the *FMR*1 gene and two autosomal paralogues termed fraxile X related 1 (*FXR*1) and *FXR*2 (Zhang et al., 1995). The evolutionary history of the three genes is unclear. The first invertebrate orthologue of *FMR*1 was isolated from *Drosophila* (Wan et al., 2000) and was termed *dFMR*1. Its gene product exhibits a very high sequence similarity with the human and other vertebrate FXR proteins and is the only invertebrate FXR characterized so far. Database searches of available complete genome sequences of *Caenorhabditis elegans* and *Saccharomyces cerevisiae* have not revealed any *FMR*1/*FXR* homologue (Wan et al., 2000).

We have isolated and analyzed the cDNA of a member of the *FXR* gene family from the marine hydroid *Hydractinia echinata* (for a description of the animal, see Frank et al., 2001). This animal belongs to the most ancient extant eumetazoan phylum, the Cnidaria. The fact that cnidarians are the most basal, living metazoans possessing a nervous system is intriguing in this regard. It raises the possibility that the function of FMR1 has been conserved during the evolution of the Eumetazoa, and that it has been primarily related to the development of the nervous system.

2. Materials and methods

2.1. Isolation of HyFMR1 full-length cDNA

A cDNA fragment of 600 bp with a predicted amino acid sequence showing high similarity to FXR proteins was isolated during a differential gene expression analysis from an arrayed, nonamplified cDNA library. The full-length cDNA was obtained by rapid amplification of cDNA ends (RACE) polymerase chain reaction (PCR) according to the SMART RACE protocol (Clontech) from polyp and larvae cDNA. The oligonucleotides 5′-ACAAGGGGCCAATATC-CAAG-3′ and 5′-TCTGGCTCAGGGTCAATCTTTA-3′ were used for the 3′ and 5′ RACE, respectively. PCR products were cloned into a pGEM-T vector (Promega) according to the manufacturer's instructions and sequenced from both ends using T7 and SP6 primers. We have then designed primers including the start and stop codons and amplified the entire coding region from polyp and larvae cDNA (*Hydractinia* larvae do not feed, excluding contamination from foreign nucleic acids), as well as from a genomic template. The identity of the gene was verified by sequencing. We have termed the gene *HyFMR*1 for *Hydroid FMR*1.

2.2. Database search and phylogenetic analysis

To identify all available FMR1/FXR sequences from public databases, we used the human and insect FXR proteins or their conserved domains as queries for BLAST searches of EMBL/Genebank and the uncompleted genomic sequence data and expressed sequence tags (EST) listed by KEGG (http://www.genome.ad.jp/kegg/catalog/org_list.html) or GenomeWeb (http://www.hgmp.mrc.ac.uk/GenomeWeb/). FMR1/FXR sequences obtained by TBLASTN searches only were further transcribed and translated into amino acid sequences using the HUSAR package (http://genome.dkfz-heidelberg.de). For phylogenetic studies, the protein sequences were aligned using CLUSTAL W. The alignment is available upon request. Phylogenetic inference was carried out using the neighbor joining and the Bayesian phylogenetic method. For neighbor-joining analysis, the PHYLIP 3.5 software package (http://evolution.genetics.washington.edu/phylip.html) based on a PAM001 distance matrix implemented in the program was used. The robustness of the tree was tested by bootstrap analysis with 1000 replications. Bayesian phylogenetic analysis was performed by the MrBayes 3.0 beta4 program (Huelsenbeck and Ronquist, 2001). The WAG model with gamma distribution of substitution rates was applied. Prior probabilities for all trees were equal, the starting trees were random, and tree sampling was performed every 10th generation. Metropolis-coupled Markov chain Monte Carlo sampling was performed with one cold and three heated chains that were run for 40000 generations. Posterior probabilities were estimated on 2000 trees (burnin=2.000). The tree presented here was visualized using TreeView (http://taxonomy.zoology.gla.ac.uk/rod/rod.html).

2.3. In situ hybridization

A digoxigenin-labeled RNA probe of 268 bases was generated and used in all in situ hybridization experiments. Two linearized pGEM-T plasmids (for sense and antisense probes) containing an insert of the cDNA from position 797 to 1065 were used as templates for in vitro transcription according to the manufacturer's protocol (MBI). In situ hybridization was performed as described (Gajewsky et al., 1996) with the following modifications. (1) Hybridization was carried out at 54 °C for 16 h. (2) Incubation with the Fab fragments of the anti-digoxigenin–alkaline–phosphatase conjugate antibody (Roche) was overnight at 4 °C. Antibody dilution was 1:10000.

2.4. Northern blot

Total RNA was isolated from different developmental stages using the acid guanidinium thiocyanate method, separated on a 1% agarose–formaldehyde gel (7 μg per lane) and blotted onto a Hybond-N membrane. Polyps were starved for 48 h before processing to exclude any nucleic acid contamination from preyed *Artemia*. The RNA was bound to the membrane by baking for 30′ at 120 °C. *HyFMR1* RNA was detected by hybridization with two

digoxigenin-labeled probes. One was the same as used for in situ hybridization. The other represented position 1046–1223 of the coding region. Both probes yielded the same results. Hybridization and washing were carried out at high stringency. Antibody detection was as described above.

2.5. Immunohistochemistry

Anti-RFamide antibodies were a kind gift from Prof. Günter Plickert (University of Cologne). Immunohistochemistry was carried out as previously described (Plickert and Kroiher, 1988) and observed under a fluorescence microscope.

3. Results

3.1. Full-length cDNA and predicted amino acid sequence

Using RACE PCR, we have isolated a cDNA clone of 1992 bp. The cDNA contained an open reading frame of 1863 bp, encoding a putative protein of 621 amino acid residues with a predicted molecular mass of 70.6 kDa. The nucleotide sequence is deposited at the EMBL database under accession number AJ829441. The predicted amino acid sequence showed the highest similarity with the human FMR1 in the first 2/3 of the protein. Similarity decreased towards the C-terminus known to be less conserved among

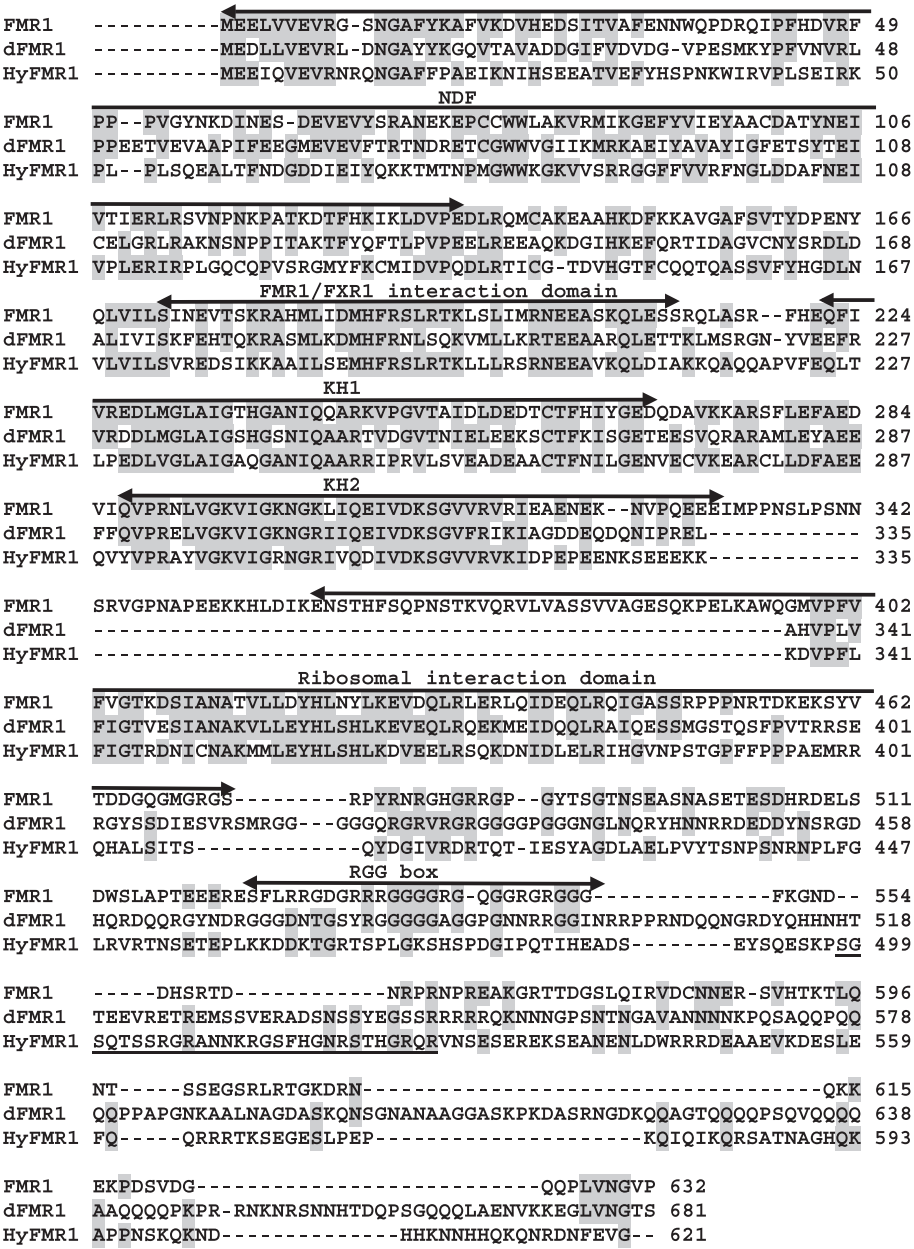

Fig. 1. Alignment of HyFMR1 with its human and *Drosophila* orthologues. Conserved amino acids are shaded. Domain structure is indicated by arrows. The area containing HyFMR1's putative RGG box is underlined.

all FXR family members (Wan et al., 2000). Typical FMR1/FXR structural elements were found in HyFMR1 (Fig. 1), including an N-terminal NDF domain, a FMR1/FXR interaction domain, 2 KH domains and a ribosomal interaction domain (Fig. 1). The first KH domain is nearly 60% identical to the human FMR1 and more than 50% to the *Drosophila* homologue, dFMR1. The second KH domain displays 50% identity to the human FMR1 and 70% to the dFMR1. Highly conserved isoleucine residues in the first KH domain are also found in HyFMR1 as well as the first isoleucine in the KH2 domain. Instead of the second conserved isoleucine in KH2, however, HyFMR1 contains valine (Fig. 1). HyFMR1 also shares more than 40% sequence identity with the human and *Drosophila* homologues in the regions previously delineated as FMR1/FXR binding domain and ribosomal interaction domain (Siomi et al., 1996). A putative RGG box is found at the C-terminus of HyFMR1 (Fig. 1).

3.2. Phylogenetic analysis

Using both the neighbor-joining and the Bayesian phylogenetic methods, we have constructed a phylogenetic tree for the FXR protein family (Fig. 2). Members of the vertebrate FXR2 clustered together. The vertebrate FXR1 proteins formed a sister group to FXR2, and the two groups clustered together to form the sister group to the vertebrate FMR1 cluster. The two characterized invertebrate proteins (dFMR1 and HyFMR1) together with the predicted tunicate protein formed the sister group to all vertebrate proteins. The predicted tunicate *FMR*1 homologue, *cFMR*1, was the only representative of the FXR family in the genome of the tunicate *Ciona intestinalis* according to our database search.

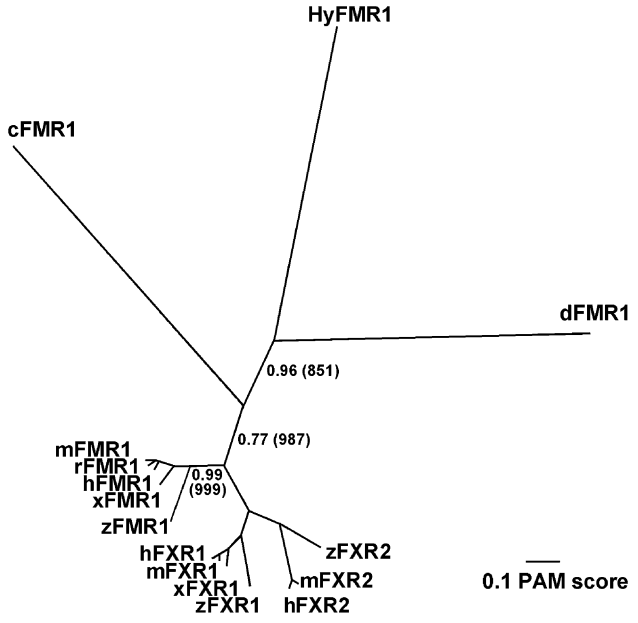

Fig. 2. Phylogenetic analysis of FXR protein family members. Bayesian probabilities and bootstrap values (given in parentheses) are shown only when lower than 1.0 or 1000, respectively. c—*Ciona*, d—*Drosophila*, h—human, m—mouse, r—rat, x—*Xenopus*, z—zebrafish.

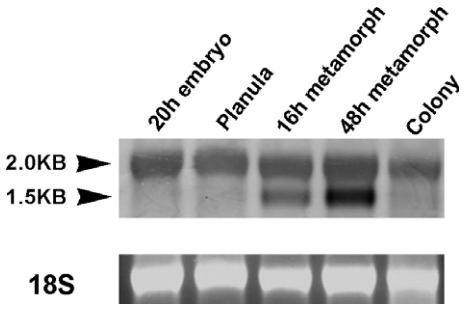

Fig. 3. Comparative Northern blot analysis of several *Hydractinia* life stages.

3.3. Northern blot analysis

To reveal the temporal pattern of expression and transcript complexity of *HyFMR*1, we examined a series of developmental stages by Northern blot analysis. A transcript of about 2 kb, corresponding to the full length cDNA, was detected in all life stages (Fig. 3), including the unfertilized egg (not shown). An additional, smaller transcript of 1.5 kb appeared during late metamorphosis (Fig. 3) and may represent alternative polyadenylation or promoter usage. The level of expression of the shorter transcript in this stage was markedly higher than that of the full-length transcript. It decreased during the end of metamorphosis, reaching undetectable levels in fully developed polyps (Fig. 3).

3.4. In situ hybridization

We have also studied the spatial and temporal expression of *HyFMR*1 during the entire life cycle of *Hydractinia* by whole mount in situ hybridization (Fig. 4). *HyFMR*1 mRNA was already expressed in released eggs (not shown). Very early embryos showed ubiquitous expression (Fig. 4A, compare sense probe Fig. 4B). Following gastrulation, at about 18 h post fertilization, expression was endodermal (Fig. 4C). However, in later embryonic stages (about 22 h), high mRNA levels were detected also in the ectoderm (Fig. 4D). In the larval stage, *HyFMR*1 expression was ectodermal, most prominent at the anterior first third of the body column (Fig. 4E). After the onset of metamorphosis, *HyFMR*1 was strongly expressed in the ectoderm (Fig. 4F). At early metamorphosis (<10 h postinduction), the most prominent signal was visible in the region of attachment to the substratum (not shown). About 18 h postinduction, the ectodermal staining was mostly localized at the base and the middle of the body column and less in the head region of the developing polyp. In the young primary polyp (48 h postinduction), expression was ectodermal, and the same expression pattern was also

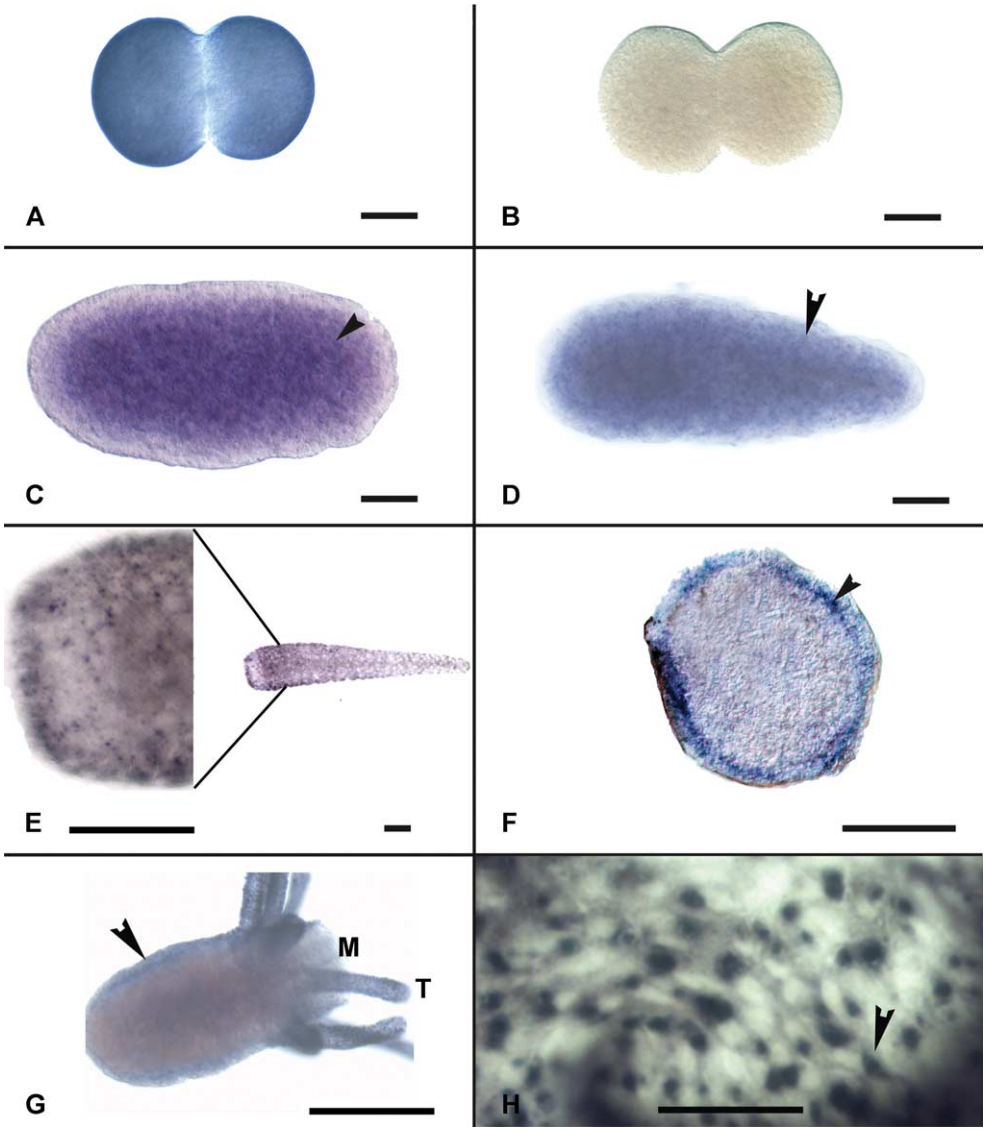

Fig. 4. Whole mount in situ hybridization of *Hydractinia* with a *HyFMR*1 probe. (A) Two cell stages. (B) Two cell stages, sense probe. (C) 18- embryo. Arrowhead points to endodermal expression. (D) 22-h embryo. Arrowhead points to ectodermal expression. (E) A 3-day-old planula larva. The anterior end is showed in higher magnification. (F) A 16-h metamorphosing polyp viewed from beneath. Arrowhead points to ectodermal expression. (G) A mature feeding polyp. Arrowhead points to ectodermal expression. Mouth and tentacle are indicated by M and T, respectively. (H) A higher magnification of the ectoderm from the head region of a mature, feeding polyp, showing cells resembling neurons (arrowhead). Scale bars are approximately 50 μm in panels (A–F), 75 μm in panel (G) and 20 μm in panel (H).

found in feeding and sexual polyps (Fig. 4G) isolated from mature colonies.

3.5. Localization of HyFMR1 expression

High magnification images of in situ hybridization preparations revealed cells with neuronal morphology and position (Fig. 4H). To colocalize neurons with *HyFMR*1 positive cells, we performed immunohistochemistry with antibodies directed against the neuropeptide RFamide, a neuronal marker in hydroids (Grimmelikhuijzen, 1985). Anti-RFamide positive neurons constitute only a small fraction of all neurons. Nevertheless, comparison between RFamide immunohistochemical preparations and in situ hybridization with *HyFMR*1 probes suggested that *HyFMR*1 is indeed expressed in neurons. Large neurosensory cells around the mouth were visible in both in situ hybridization and RFamide immunohistochemistry (Fig. 5).

4. Discussion

The FXR protein family was originally described in vertebrates, where its members fulfill a role in neuronal development and synapse plasticity, mostly through translational repression of specific neuronal mRNAs (Laggerbauer et al., 2001; Zhang et al., 2001; Sung et al., 2003). The first report of an FMR1 homologue outside the Vertebrata

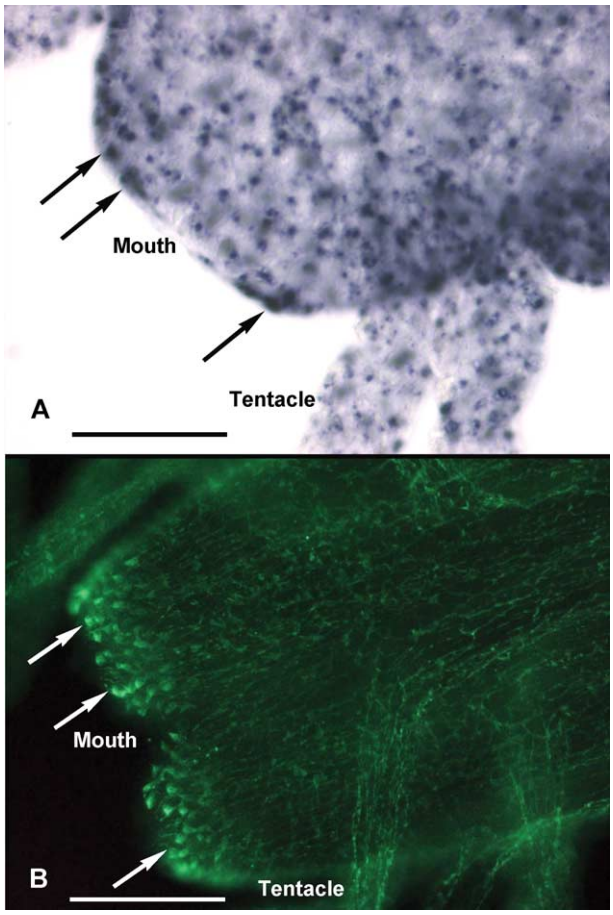

Fig. 5. A comparison of in situ hybridization (A) and anti-RFamide immunohistochemistry (B) of the head region of a mature feeding polyp. Arrows point to neurosensory cells around the mouth. M—mouth, T—tentacle. Scale bars are approximately 40 μm.

came from a study on *Drosophila*. Functional studies in this animal have revealed that dFMR1 is involved in neurite extension, guidance and branching (Zhang et al., 2001; Morales et al., 2002). Loss of function mutations in dFMR1 also causes behavioral defects in flies (Dockendorff et al., 2002; Inoue et al., 2002). It was therefore surprising that no *FMR*1 homologue was found in *C. elegans*, which also possesses a nervous system (Wan et al., 2000). Our discovery of an FMR1 homologue in a cnidarian readdresses the issue of the origin and ancestral function of FXR proteins. Our database searches failed to find an FMR1-related protein in any organism lower than cnidarians. We conclude that the FXR family was already present in the common ancestor to cnidarians and bilaterians. The gene may have been lost in the lineage leading to *C. elegans*. In support of this hypothesis, our database searches revealed some expressed sequence tags (EST) with an FMR1-like sequence in other nematodes. The flatworm *Schisostoma japonicum* genome also contains a gene with high similarity to FMR1 (Hu et al., 2003).

Our phylogenetic analysis revealed three main clusters within the FXR family in vertebrates: FMR1, FXR1 and FXR2. The *Drosophila* genome contains only one copy of the gene, which has a similar function like the vertebrate FMR1 and shows highest sequence similarity to it. HyFMR1 appears to be the only FXR family representative in *Hydractinia*. Our search in >4000 *Hydractinia* ESTs and >70000 ESTs from the hydroid, *Hydra*, did not reveal any additional FXR-like sequence, although the existence of such a gene can at present not be ruled out. Therefore, our data suggest that two successive gene duplication events have resulted in the FXR1 and FXR2 subfamilies, respectively (see also Kirkpatrick et al., 2001). Our database search revealed that the genome of the tunicate, *C. intestinalis*, contains only one copy of an *FMR*1-like gene. Based on the phylogenetic position of tunicates within the Chordata, we conclude that the gene duplication events have occurred in the vertebrate ancestor after the divergence of the Urochordata.

The predicted gene product of *HyFMR*1 contains typical FMR1/FXR domains, indicating that the domain architecture directing specific molecular interactions has been established very early in evolution. Highest sequence similarity was found in the first 2/3 of the protein, the region containing the NDF, two KH domains, the dimerization domain and the ribosomal interaction domain. The highly conserved isoleucine residue in the second KH domain is substituted by valine in HyFMR1. A point mutation at this position (I304N) in the human *FMR*1, which replaces isoleucine with asparagine, leads to a nonfunctional protein and also causes the disease (Siomi et al., 1994; Ramos et al., 2003b). We argue that the folding of HyFMR1 is not affected since the isoleucine in the *Hydractinia* protein is substituted by valine, another hydrophobic amino acid.

*HyFMR*1 is also a maternal transcript as its mRNA was detected by Northern blot and in situ hybridization in unfertilized eggs (not shown). Expression was ubiquitous in early embryos and became endodermal following gastrulation. Shortly thereafter, in the preplanula stage, endodermal expression was complemented by ectodermal expression. Endodermal expression reached undetectable levels in all later life stages. In planula larvae and metamorphosed polyps, expression was restricted to the base of the ectoderm, corresponding to the position of nerve cells. Hydroid's nerve cells are arranged as an irregular network, mainly located at the base of the ectoderm (Grimmelikhuijzen, 1985). The nervous system is dynamic. Throughout the life cycle of *Hydractinia*, neurons are lost, and new ones differentiate from a population of totipotent stem cells called interstitial cells or i-cells that also give rise to nematocytes, gland cells and gametes (Müller et al., 2004). The nervous system is already established at the planula stage, and sensory neurons at the anterior part of the larva are involved in the release and the transmission of internal metamorphosis-triggering signals (Schmich et al., 1998). Larval neurons undergo apoptosis shortly after the induction of metamorphosis, and new adult neurons differentiate in the forming and growing polyp. I-cells first appear in embryonic

endoderm following gastrulation. Some of them migrate to the ectoderm in the preplanula stage to become larval nerve and stinging cells. During metamorphosis, most i-cells migrate to the ectoderm and remain ectodermal during the rest of the animal's life. Ganglion cells differentiate during metamorphosis first in the basal region of the future polyp. In later metamorphic stages, they are found in the ectoderm at a higher position along the body column. Finally, as the new polyp forms, they appear mostly in the ectoderm of the head and the tentacles. *HyFMR*1's expression pattern was concordant with the position of nerve cells and/or their precursors throughout the hydroid's life cycle, resembling that of its *Drosophila* and mammalian homologues (Devys et al., 1993; Tamanini et al., 1997; Agulhon et al., 1999; Wan et al., 2000). This and the highly conserved sequence suggest that HyFMR1 plays a role in the development and maintenance of the nervous system also in hydroids.

In addition to the full-length mRNA of *HyFMR*1, which was expressed in all life stages of the animal, we have also detected a smaller band by Northern blot that may represent a splice variant, alternative polyadenylation or start codon usage. The *HyFMR*1 isoform is highly expressed only during late metamorphosis while down-regulated in other stages. The isoform transcript lacks approximately 500 bases, probably at one of its ends since RT–PCR with primers spanning the entire coding sequence failed to show a second band (not shown). Its expression pattern during polyp development might point to a function in this process.

The discovery of a *FMR*1 homologue in a cnidarian, the oldest, living animal phylum possessing nerve cells, suggests that, in contrast to previous opinions (Wan et al., 2000), this gene family arose earlier in metazoan evolution and was already present in the common ancestor to cnidarians and bilaterians. If indeed no FXR protein exists in metazoans lower than Cnidaria, it would support an ancient function related to nerve development and function. It is not clear which protein undertook this function in metazoans that are lacking a copy of this gene in their genome, and whether *C. elegans* is indeed a unique case in this regard, as other nematodes do possess *FMR*1-like genes (see above). Further research on *HyFMR*1 and the protein it encodes may uncover the original function this protein fulfilled in the development of the oldest nervous system. Interestingly, hydroids' nervous systems differ from that of higher animals in their dynamic character, plasticity and regenerative ability. Studies on this topic may be of assistance in the attempts to understand the mechanistic basis of the disease and render *Hydractinia* a new model system to study the fragile X mental retardation syndrome.

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