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HMG-X, a *Xenopus* gene encoding an HMG1 homolog, is abundantly expressed in the developing nervous system

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Abstract We used a PCR-based subtraction cloning procedure with Concanavalin A-treated and untreated animal caps from stage 9 *Xenopus* embryos to search for genes the expression of which is induced during neurogenesis. One of these genes was found to encode a homolog of mammalian HMG 1 and 2, hence named *HMG-X*. *HMG-X* mRNA was maternally transmitted, up-regulated in neuroectoderm-derived tissues throughout early development, and eventually down-regulated in all adult tissues examined except ovary. Our data suggest that we have identified a gene for a member of the HMG1/2 family that could have an important role in neurogenesis.

Key words: PCR; cDNA subtraction; Animal cap; Neural development; In situ hybridization

1. Introduction

Recent studies have led to the identification of some of the endogenous molecules, such as activin [1] and noggin [2], involved in the process of neural induction in *Xenopus* embryo. The signals elicited by these inducers must eventually be transmitted into the nucleus of ectodermal cells and alter their gene expression; however, the molecular events in this later step of neural induction remain largely obscure.

The mammalian high mobility group proteins, HMG1 and 2, are known as non-histone nuclear proteins which are ubiquitously expressed and display a high degree of conservation across species, implying their role in some fundamental biological process. They have been postulated to be a structural component of chromatin or to operate as DNA chaperones [3–5]. Furthermore, roles in replication, repair and recombination have been suggested since the HMG proteins bind to DNA in a sequence non-specific manner, preferentially to single-stranded DNA [6]. The HMG1/2 may also play a role in gene regulation by inducing conformational changes in DNA [7,8] and facilitating cooperative interactions between *cis*-acting elements and sequence-specific DNA-binding proteins [9–11].

Here we have attempted to identify genes up-regulated after neural induction of *Xenopus* ectoderm in vitro, and isolated a cDNA encoding a protein (designated HMG-X) exhibiting a high degree of homology to mammalian HMG1 and 2.

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Abbreviations: HMG, high mobility group protein; PCR, polymerase chain reaction; Con A, Concanavalin A; EF-1α, elongation factor-1α; bp, base pairs; kb, kilobase.

2. Materials and methods

2.1. Neural induction in animal caps

Eggs of *Xenopus laevis* were obtained as described [12]. Embryos were staged according to Nieuwkoop and Faber [13]. Eighty animal caps from stage 9 embryos were incubated for the initial 3 h with or without 500 μg/ml of Con A using a modification of the method of neural induction reported by Takata et al. [14] and Grunz [15], which will be described elsewhere in detail (Hatada et al., in preparation). Neural induction in sampled animal caps was confirmed after 3 days by direct microscopic observation of brain-like structures (Fig. 1) and immunohistochemistry with monoclonal antibody NEU-1 directed against neuronal cells [6] (data not shown).

2.2. Construction of PCR-based directional library

Approximately 30 ng of poly(A)$^+$ RNA obtained from induced (+) and uninduced (−) animal caps was reverse transcribed using the primer 5′-GAGAGAGACCCGGGACGATGAGCTGTGAATCTC-GAG(T)$_{16}$-3′. After second strand synthesis [17] and blunt-ending, *Mlu*I-linkers, prepared by annealing the oligos 5′-GGACTATGTGA-CGCGTATGG-3′ and 5′-pCCATACGCGTCACATAG-3′, were ligated to the ends of the cDNAs. After *Sma*I digestion, the excess linkers and DNA fragments of less than 400 bp were removed by gel-filtration. The larger DNA was amplified by PCR for library construction and for probes for use in differential screening (see below) using the primers 5′-GGACTATGTGACGCGTATGG-3′ and 5′-GACGATGAGCT-CTGATCTCG-3′. The reaction mixture was first incubated at 96°C for 5 min and subjected to 30 cycles of PCR (1 min at 96°C, 1 min at 55°C and 2 min at 67°C). A second round of PCR (additional 30 cycles in fresh reaction mixture) was performed using one-tenth of the initial amplification product. The cDNA of more than 400 bp selected by agarose gel-electrophoresis was digested with *Mlu*I and *Xho*I, and then directionally cloned between the corresponding sites of the pGEM7Zf(−) vector (Promega).

2.3. Subtractive hybridization and differential screening

Using a modification of the method reported by Rubenstein [18], 1 μg of single-stranded antisense DNA (ssDNA) from the (+) library was subtracted twice with 20 μg of sense RNA in vitro transcribed from the (−) library with 5% UTP-biotin (Clontech) in unmodified UTP. The remaining DNA was subjected to 30 cycles of PCR using above set of primers, and cloned into pGEM7Zf(−). This sub-library consists of approximately 30,000 clones. We have screened about 2,000 clones using ^{32}P-labeled probes generated by PCR from the (+) and (−) templates described above. Candidate cDNAs for differentially expressed genes were further examined by Northern blot hybridization using total RNA extracted from Con A-treated and untreated animal caps.

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Cloning and sequence analysis of HMG-X *cDNA*

Ve screened a unidirectional embryonic cDNA library which we had structed earlier using standard techniques [12] with a cDNA probe ne #12) obtained from our PCR-based subtraction procedure. The leotide sequence of clone #12.9 (approximately 1.2 kb) was deter- ied and has been submitted to the DDBJ, EMBL and GenBank abases under accession number D30765. Structural homology was lyzed using TFASTA and PILEUP [19] obtained from Wisconsin netics Computing Group.

Northern blot hybridization

1.2 kb *Eco*RI–*Xho*I fragment excised from clone #12.9 was used template for making *HMG-X* probes. For controls, PCR-generated NA fragments of *Xenopus* c-*src* [20] and EF-1α [21] were used. bridization and densitometry were performed as described [12].

Whole-mount in situ hybridization

Digoxigenin (DIG)-labeled RNA probes in sense and antisense ori- ations were prepared from clone #12.9 using the DIG RNA Label- Kit (Boehringer). The probe size was reduced to approximately 200 by limited alkaline hydrolysis. Whole-mount in situ hybridization performed essentially as described [22].

Results

Isolation of cDNA clones up-regulated in animal caps during neural development

Neural tissue-like structures such as neural tubes, with or hout eyes, were induced in about 90% of the animal caps lays after Con A treatment, while only ciliated epithelial ues were formed in untreated samples (Fig. 1).

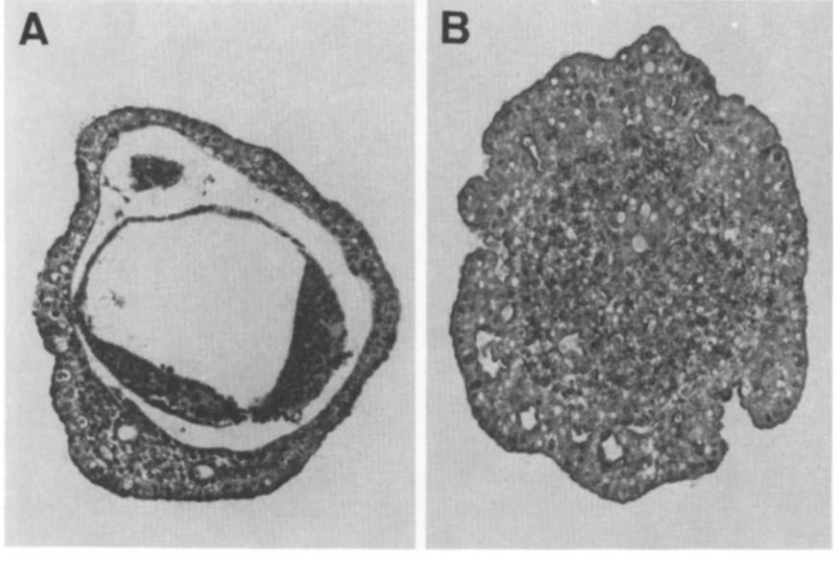
Poly(A)$^+$ RNA extracted from the Con A-treated and un- ated animal caps was amplified by RNA-PCR and used for nstructing unidirectional cDNA libraries (designated (+) and library, respectively). The average size of the inserts esti- ted by random sampling was about 420 bp (range, 400–600). Single-stranded DNA from the (+) library was subtracted h biotinylated RNA transcribed from the (−) library. A rtion of the resulting sub-library was subjected to differential eening using radiolabeled, PCR-amplified cDNAs from Con treated and untreated animal caps. Among 2,000 clones ran- mly picked from the sub-library, 43 clones gave significantly onger signals with the probe from Con A-treated animal caps. Sequencing and database searches for these 'up-regulated' clones revealed that two of them, #12 (ca. 200 bp) and #14 (ca. 180 bp), contained novel sequences. Most of the other clones were derived from RNA species especially abundant in embryo, such as tRNAs, 12 S rRNA, and mRNA for EF-1α.

Clone #12 encodes a protein similar to mammalian HMG1 and 2

We screened a previously constructed unidirectional total embryonic cDNA library [12] with a cDNA probe based on clone #12 and obtained 13 positive clones out of 50,000 pla- ques. We determined its total sequence of the longest cDNA, clone #12.9 (Fig. 2A). The cDNA consists of 1080 bp and contains a single, long open reading frame of 636 bp preceded by three in-frame termination codons. The first methionine in the open reading frame is in a context appropriate for transla- tion initiation [23], and the predicted protein product consists of 212 amino acids with a calculated molecular mass of about 24,200. There are two mRNA-destabilizing signals, ATTTA [24], in the 3′ non-coding region. A database search with the predicted amino acid sequence revealed that it shares a high degree of homology with mammalian HMG1 and 2, as well as trout HMG-T; hence we named it HMG-X (Fig. 2B). HMG-X has the canonical primary structure of an HMG1/2 family pro- tein, consisting of two repeats of the HMG box [25] and a carboxy-terminal acidic tail.

Expression of HMG-X *mRNA*

A 1.2 kb transcript of *HMG-X* was detected in embryos at stages 0–35 by Northern blot hybridization (Fig. 3A). Since zygotic transcription is not active until the midblastula transi- tion [26], the signals detected both in the ovary and stages 0–6 embryo strongly suggests maternal transmission of *HMG-X* mRNA, although the transcript size of the latter is slightly larger, possibly because of elongation of the poly(A) tail as has been observed for the non-histone protein B4 [27]. As com- pared to the relatively invariable expression of c-*src* [28], *HMG-X* was up-regulated after stage 8 and peaked around stage 12–14, followed by down-regulation at stage 20–25 and up-



. 1. Neural induction of animal caps with Con A. (A) Animal caps treated with Con A formed a neural tube-like structure after 3 days. (B) Untreated mal caps formed a ciliated epithelial cell mass.

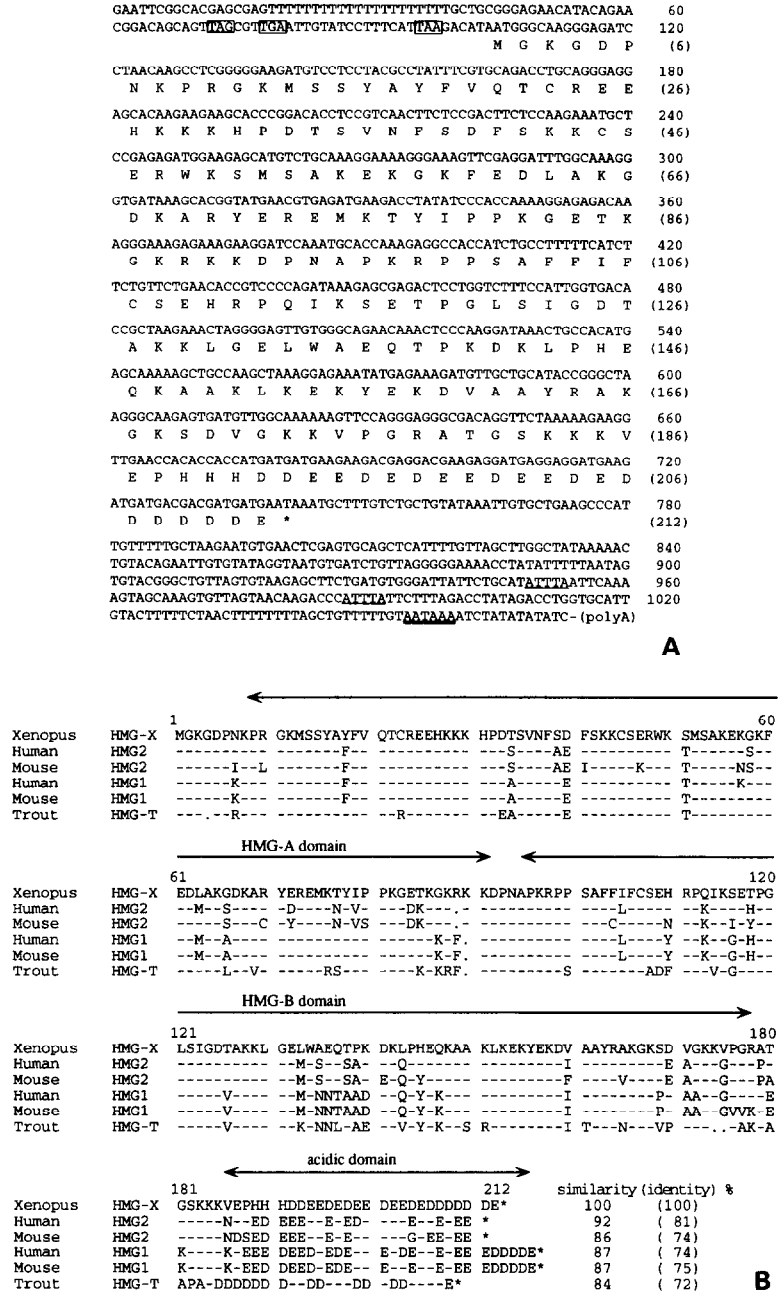


Fig. 2. Structure of *HMG-X* cDNA and its encoded protein. (A) Nucleotide sequence of the #12.9 cDNA and predicted amino acid sequence. Three in-frame termination codons preceding the first methionine are boxed. The two mRNA-destabilizing signals are underlined, and the polyadenylation signal is double-underlined. (B) Alignment of HMG1/2 family protein sequences. The amino acid residues identical between HMG-X and each homolog are indicated by a dash (–).

regulation again at stage 35 (Fig. 3B). In all the adult tissues examined, except for the ovary, two faint bands of about 2 kb and 1.2 kb were identified (Fig. 3C). The 2 kb transcript was not seen in adult ovary or embryonic samples.

HMG-X mRNA expression was further analyzed by whole-mount in situ hybridization with embryos at various stages of development (Fig. 4A–J). In the blastula (stage 10), weak signals of *HMG-X* expression were detected in the animal hemisphere (Fig. 4A). In the late gastrula (stage 12), the transcript was detected throughout the ectoderm (Fig. 4B). At this stage the ectoderm covers the entire surface of the embryo except for the yolk plug. In the early neurula (stage 15), the signal was detected throughout the neural plate, except for areas along the midline (Fig. 4C and D). In the tailbud (stage 22), expression was seen along the neural tube and its derivatives, including optic and otic vesicles, as well as some non-neural tissues such as the branchial arch anlagen (Fig. 4E and F). In stage 25 larva, signals along the neural tube were diminished especially in the middle part of the body, while expression remained intense in brain, optic and otic vesicles, branchial arches and tail bud (Fig. 4G and H). In stage 32 tadpoles, signals were found in the differentiating nervous system, including brain, eyes, ears, spi-

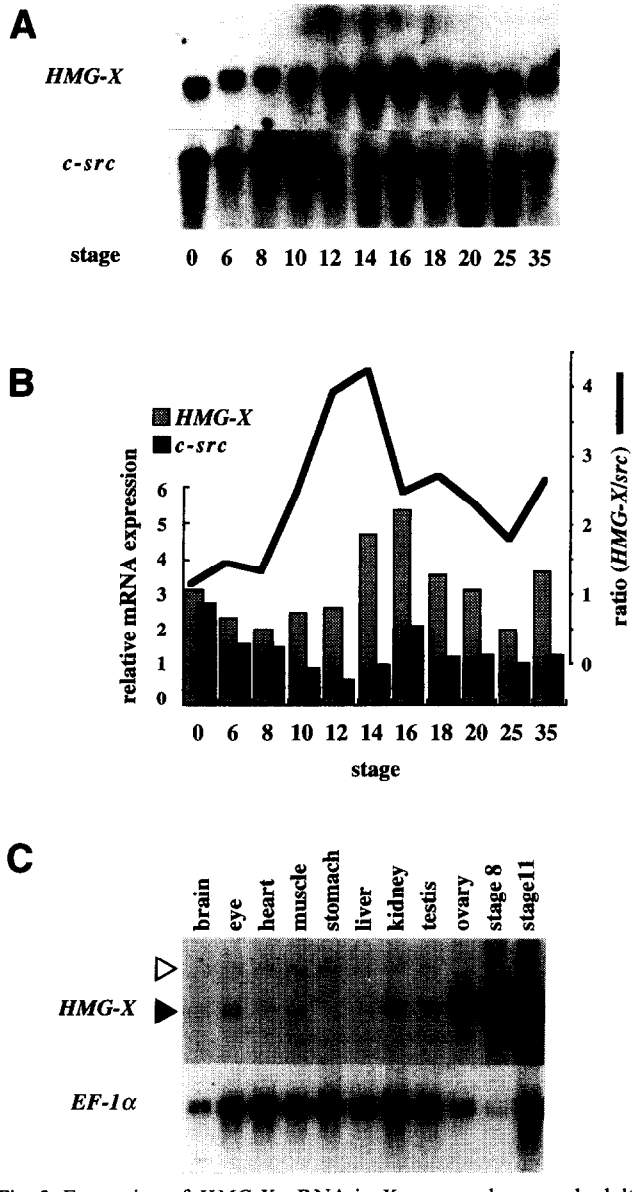


Fig. 3. Expression of *HMG-X* mRNA in *Xenopus* embryos and adult tissues. (A) Northern blot analysis. Each lane contained 2.5 μg of poly $(A)^+$ RNA isolated from whole embryos. (B) Densitometric analysis of the data presented in (A). The shaded and black bars represent intensities of the *HMG-X* and c-*src* bands, respectively. The bent line indicates their ratio. (C) Northern blot analysis. Each lane contained 2.5 μg of poly$(A)^+$ RNA from adult *Xenopus* tissues and whole embryos for comparison. The open and filled arrowheads indicate the 2.0- and 1.2-kb transcript, respectively.

nal cord and intercostal nerves, and also in the branchial arches and tail (Fig. 4I and J). Apparently, the total signal level at this stage was more than that at stage 25, consistent with the up-regulation from stage 25 to 32 on the Northern blot analysis. No signals were seen in the notochord or cement gland throughout development up to stage 32, which was confirmed by observing axial sections of the hybridized samples (data not shown). Control samples hybridized with the *HMG-X* sense probe showed no detectable signals in any of the stages (data not shown).

4. Discussion

We have shown that *HMG-X* mRNA is likely to be maternally transmitted and is highly expressed in neuroectoderm-derived tissues during embryogenesis and in the adult ovary, that the expression of *HMG-X* gene is regulated in a tissue- and stage-specific manner, and that its expression pattern cannot be explained solely on the basis of the growth activity or the origin of the cells. Interestingly, down-regulation of HMG1 protein in rat myoblast cell lines during in vitro myogenesis has been reported [29], supporting, albeit indirectly, the idea of stage-specific expression of the *HMG1/2* family of genes. These findings are not compatible with the idea that sequence-specific HMG proteins are cell-type specific, while sequence non-specific HMG proteins are ubiquitous [3]. Since more than one HMG1/2 family of genes are found in the genomes of *Drosophila* and *Saccharomyces cerevisiae*, it seems reasonable to predict that additional HMG1/2-related gene(s) in *Xenopus* may also show a distinct tissue- and stage-specific expression pattern. The bulk of earlier evidence suggests that HMG1 and 2 do not recognize specific DNA sequences but rather exert their function by somehow regulating the activities of other, sequence-specific DNA-binding proteins such as progesterone receptor [30]. If this is the case, HMG-X protein could participate in neural fate determination by regulating, through modulation of chromatin configuration, the ability of transcription factors to induce the expression of a specific set of genes. Our preliminary microinjection experiments indicated that over-expression of *HMG-X* mRNA in the 2-cell-stage embryo was not sufficient to induce neural tissue ectopically (unpublished). However, our negative results with wild-type *HMG-X* mRNA do not rule out a regulatory role of its product in neural development.

The molecular mechanism of Con A action on neural induction in *Xenopus* animal caps is far from clear. The possibility cannot be excluded that an unidentified impurity in the Con A preparations might actually be the active principle (unpublished). In light of the recent findings that inhibition of the activin signal results in neural induction [31], possibly the active

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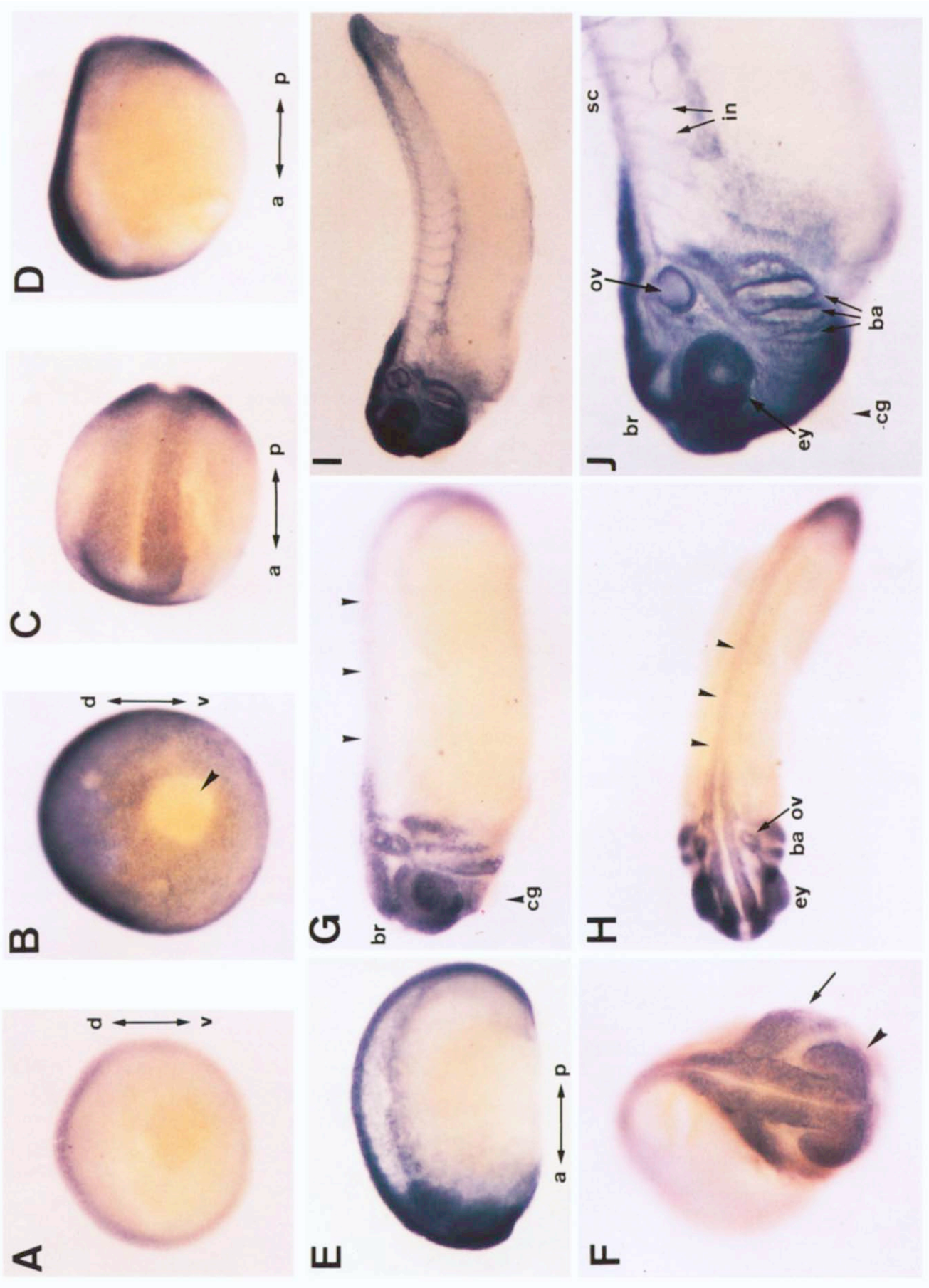
Fig. 4. Whole-mount in situ hybridization analysis of *HMG-X* mRNA in *Xenopus* embryos. (A) Vegetal view of a stage 10 embryo (blastula) showing minimal expression in vegetal hemisphere. (B) Vegetal view of a stage 12 embryo (late gastrula). *HMG-X* is expressed throughout the ectoderm but not in the yolk plug (arrowhead). Note that the signal is more intense in the dorsal region (presumptive neuroectoderm) than in the ventral region (presumptive epidermal ectoderm). (C) Dorsal and (D) lateral views of a stage 15 embryo (neurula), showing expression along the neural plate and in head and tail regions. Note the minimal expression along the midline and down-regulated expression in non-neural ectoderm. (E) Lateral and (F) anterior views of a stage 22 embryo (tailbud), showing intense expression along the neural tube, optic vesicles (arrowhead), branchial (arrow) and tail regions. (G) Lateral and (H) dorsal views of a stage 25 embryo. Expression in the middle part of the neural tube was reduced (arrowheads). (I) Lateral view of a stage 32 embryo, showing intense expression in the head organs and tail. (J) Higher magnification of (I). Note the expression along the intercostal nerves (arrows). a, anterior; p, posterior; d, dorsal; v, ventral; br, brain; ey, optic vesicle and eye; ov, otic vesicle; ba, branchial arches; cg, cement gland; sc, spinal cord; in, intercostal nerves.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ɔle in the Con A preparation interferes with the activin
ng pathway. Another possibility is that it might activate
ı kinase C, resulting in neural induction [32]. Thus the
event in neural induction using this system is unknown;
heless, we consider this in vitro neural induction system
l source of presumptive neuroectoderm undergoing de-
ation and development toward neural tissues in a syn-
zed manner. Another merit of using the *Xenopus* system
we can follow the expression patterns of isolated genes
early embryo by whole-mount in situ hybridization and
eir activity by injecting the mRNAs. It has been found
MG-2 protein is down-regulated during brain develop-
n the rat [33], however, no information about its expres-
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aining large quantities of animal caps undergoing syn-
ɔus neural development did not prove practical. In order
rcome this obstacle we employed a PCR-based subtrac-
oning strategy. However, one of the drawbacks in this
ıch was that we had to use PCR-amplified cDNA and
ɛ that all the mRNA species were amplified to the same
, which may not always have been the case. Out of 2,000
screened, we found two clones (#12 described here and
he expression of which is prominently up-regulated dur-
ɔ neural induction in vitro. Clone #14 was recently found
ɔde a glutamine synthase (unpublished data), a homolog
ch is highly induced in glial cells during development of
ɛtina [34]. The majority of the clones obtained, however,
ıe relatively abundant genes with fundamental functions.
stance, EF-1α mRNA is markedly up-regulated during
us embryogenesis [21], while the amount of 12 S rRNA
riable [35]. Therefore some of the abundant cDNA spe-
ɛm to have been preferentially amplified by PCR. Never-
ɨ, this approach worked well and we expect to obtain
up-regulated' clones from the libraries we generated dur-
s study (to date we have screened less than 7% of the total
b-library). Characterization of such genes should yield
information about the changes in gene activities during
ɛnesis.

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