

Temporal and spatial expression patterns of *FoxN* genes in *Xenopus laevis* embryos

MAXIMILIAN SCHUFF, ANTJE RÖSSNER, CORNELIA DONOW and WALTER KNÖCHEL*

Abteilung Biochemie, Universität Ulm, Ulm, Germany

ABSTRACT Using RT-PCR and *in situ* hybridisation, we have analysed the temporal and spatial expression patterns of *Xenopus Fox* genes of subclass N. By screening cDNA libraries and by RT-PCR using embryonic RNA and primers derived from EST analyses, we could isolate *FoxN2*, *FoxN4*, *FoxN5* and different isoforms of *FoxN3*. *FoxN2* and *FoxN3* transcripts were found during all developmental stages including early cleavage and tailbud stages. *FoxN5* transcripts were only present at early cleavage stages, while *FoxN4* expression began after midblastula transition. Spatial expression of *FoxN2* was first detected in the early eye field and later, in the branchial arches, the vagal ganglion and in the developing retina. *FoxN3* transcripts were found within the animal cap. In post-gastrula embryos, neural crest cells and the early eye field showed strong expression of *FoxN3*. At late tadpole stages, the branchial arches were stained. *FoxN4* was expressed in the early eye field and later in the developing retina cells, the nephrostomes of the pronephric kidney and in the midbrain. A ubiquitous expression of *FoxN5* was found in early cleavage stage embryos.

KEY WORDS: *X. laevis*, *FoxN* transcription factor, eye anlage, nephrostome, branchial arch

Forkhead box (Fox) transcription factors are involved in a variety of biological processes, such as cell proliferation, maintenance of pluripotency and cellular differentiation. According to conserved amino acid positions within the DNA binding winged-helix domain, they are divided into subclasses (FoxA - FoxS) (Kaestner *et al.*, 2000). Subclass N gained special interest, because *Foxn1* (*whn*) was found to be mutated in nude mice lacking immune defence (Nehls *et al.*, 1994). *Foxn1* knockout in mice results in downregulation of hair keratins, athymia and abnormal morphogenesis of the epidermis and hair follicles. Furthermore, *Foxn1* is a downstream target of the Wnt pathway (Balciunaite *et al.*, 2002). *FOXN2/HTLF* (Human T-cell Leukemia Factor) was identified in search of transcription factors binding the LTR of the human T-cell leukemia virus (Li *et al.*, 1992). *FOXN3/CHES1* (checkpoint suppressor 1) suppresses the lethality, UV sensitivity and a G2 checkpoint defect of a *mec1* null mutation in yeast and plays an essential role in cell cycle regulation (Pati *et al.*, 1997; Scott *et al.*, 2003). It was found to be downregulated in oral squamous cell carcinoma (OSCC) (Chang *et al.*, 2005). *Foxn4* is expressed in the retina (Gouge *et al.*, 2001). A knockout analysis in mice revealed that *Foxn4* is involved in the development of amacrine and horizontal cells during retinogenesis. The retinogenic factors Math3, NeuroD1 and Prox1 have been identified as putative downstream targets (Li *et al.*, 2004). *FOXN5/R1*, *FOXN6/R2* and

their homologues in the rat and the mouse were identified in silico. It is known that human *FOXN6* RNA is expressed in breast cancer cell lines and primary breast cancer (Kato and Kato, 2004a; 2004b).

While the information on mammalian FoxN factors is steadily increasing, so far little is known about members of this subclass in *Xenopus*. We therefore analysed the sequences of the *Xenopus laevis* FoxN subclass by blasting the human, mouse and zebrafish sequences against several *Xenopus laevis* EST libraries and, simultaneously, suggest their genomic organisation by blasting the genome of *Xenopus tropicalis*.

Results and Discussion

FoxN1 is predicted in the *Xenopus tropicalis* genome on scaffold 50 by *Ensembl GeneView*. Blasting this sequence we found two corresponding non-overlapping EST clones from *Xenopus tropicalis* (DN049598 and DT425724) derived from adult kidney and skin. However we failed to amplify this sequence from cDNA of *Xenopus tropicalis* stage 25 embryos. EST clones from *Xenopus laevis* embryos are not known. We suppose that *FoxN1*

Abbreviations used in this paper: EST, expressed sequence tag; Fox, forkhead box transcription factor, HTLF, Human T-cell leukemia factor.

*Address correspondence to: Dr. W. Knöchel, Abteilung Biochemie, Universität Ulm, Albert-Einstein-Allee 11, D-89081 Ulm, Germany.
Fax: +49-(0)-731-502-3277. e-mail: walter.knoechel@medizin.uni-ulm.de

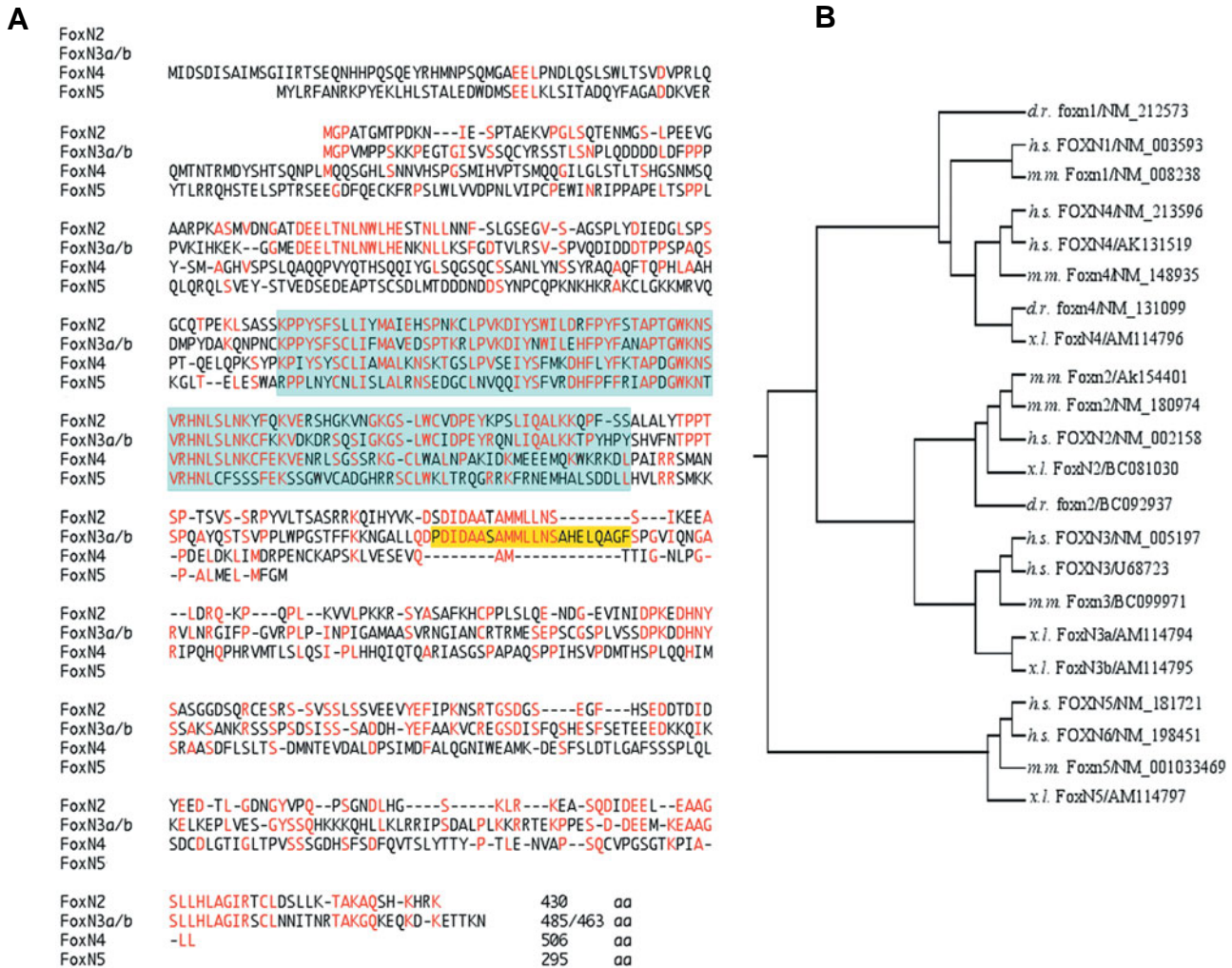


Fig. 1. The FoxN subclass in *Xenopus laevis*. (A) Comparison of amino acids of FoxN proteins. Identical amino acids are shown in red, deletions are indicated by dashes. The fork head domains and the amino acids encoded by an additional exon VI (present in FoxN3a but not in FoxN3b) are coloured. (B) Phylogenetic tree of FoxN proteins of different species (d.r., *Danio rerio*; h.s., *homo sapiens*; m.m., *mus musculus*; x.l., *Xenopus laevis*) created by ClustalW. The FoxN proteins can be classified into three branches (FoxN1/4, FoxN2/3 and FoxN5/6 paralogues (Kato and Kato, 2004b)).

exists in *Xenopus laevis*, but is only expressed at later stages.

FoxN2 was first identified by overlapping EST clones (BU909915, CA988030, CF27159) of *Xenopus laevis* which encode the C-terminal part and two overlapping EST clones from *Xenopus tropicalis* (CX795231, CX800360) encoding the putative N-terminal part. Blast analysis of these EST clones led to the full-length cDNA clone BC081030 (Klein *et al.*, 2002). The open reading frame of *FoxN2* encodes 430 amino acids (Fig. 1A) sharing 67% identity with the human sequence (NM_002158).

FoxN3 was identified from the 5'-EST clone (BJ046265) and the 3'-EST clone of *Xenopus tropicalis* (CX855631) and *Xenopus laevis* (BJ074333). Using primers derived from these EST clones, we succeeded in PCR amplification of the complete *Xenopus* sequence. Interestingly, *FoxN3* shows alternative splicing, because cloning of the PCR fragments yielded two ORF clones of different length, which differ in an additional 66 bp encoding exon VI (see Fig. 2). It seems, the longer isoform is also present in mice

as we identified this exon in the genomic sequence of mouse *Ches1* and we found a putative *Foxn3* EST clone (BG082349) containing this exon. Moreover, 5'-RACE of *Xenopus laevis* *FoxN3* revealed two different leader exons which were also identified in the genome of *Xenopus tropicalis* (see Fig. 2). Sequences of the long variant containing exons I and VI (*FoxN3a*) and of the short variant containing exon II and lacking exon VI (*FoxN3b*) were deposited at EMBL (EMBL accession numbers AM114794 and AM114795, respectively).

FoxN4 was discovered in the *X. tropicalis* EST clone CX964167. Additional database analyses led to two other non-overlapping *Xenopus laevis* EST clones (BJ061286, DR724233), coding for the N-terminal part of *FoxN4*. A PCR fragment derived from these EST clones was used for library screening and an incomplete cDNA containing the 3'-end was isolated. The entire coding sequence was amplified from stage 32 *Xenopus laevis* cDNA (EMBL accession number AM114796).

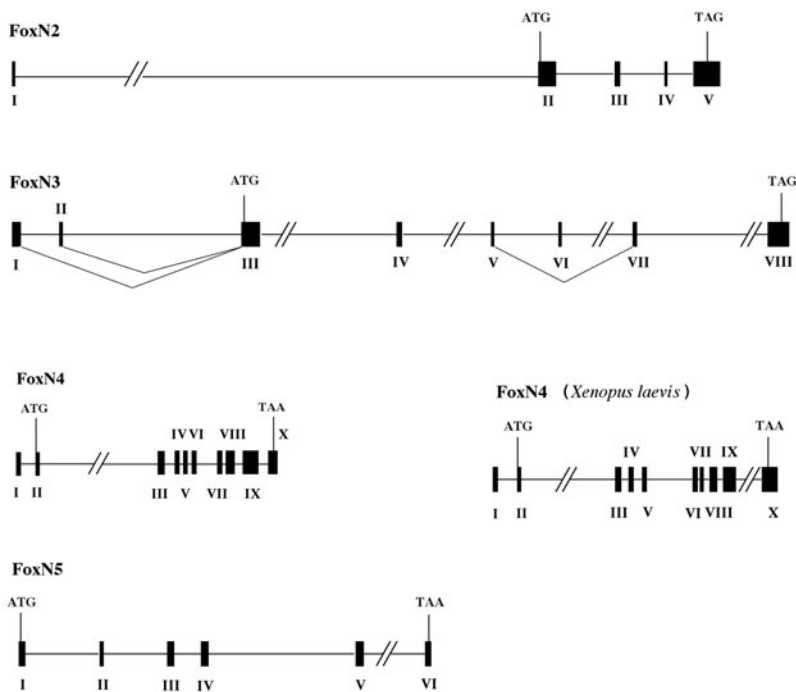


Fig. 2. Genomic organisation of FoxN genes in *Xenopus tropicalis*. Mapping of gene structure was analysed by Ensembl. Exons are numbered with Roman numerals. Genes have been identified at scaffold 111 (FoxN2), scaffold 22 (FoxN3), scaffold 9 (FoxN4) and scaffold 75 (FoxN5). The FoxN4 gene has also been isolated and sequenced in *Xenopus laevis*.

FoxN5 was identified from the *Xenopus laevis* EST clone CA791267. A PCR fragment derived from this EST clone was used for screening a gastrula stage cDNA library. Four identical cDNA clones were isolated that all contain an open reading frame of 885 bp (deposited under EMBL accession number AM114797). A comparison with corresponding sequences of mouse, human and the *X. tropicalis* EST clone (CR761025), which contains a stop codon in the 5'-UTR, suggests that the open reading frame is complete. The resulting protein shares only an identity of 23% with human FOXN5 (NM_181721). The X-chromosomal-located human FOXN6 (NM_198451) encodes a protein that shares 31% identity with the *Xenopus laevis* protein, but FOXN6 is regarded as a retrotransposon of an ancestral FOXN5 during evolution (Katoh and Katoh, 2004b). Since no other FoxN homologues were found in *Xenopus laevis*, we report this sequence as *FoxN5*. Additional evidence for this nomenclature comes from the genomic localisation and its surroundings. In the human chromosome 11 we found *Archain* flanking FOXN5. The *Xenopus laevis* cDNA clone matched on scaffold 75 of the *X. tropicalis* genome, where we identified the predicted *Archain* gene of *Xenopus*.

Fig. 1A shows an alignment of the amino acid sequences of *Xenopus laevis* FoxN proteins. Aside from their rather conserved winged helix domains, FoxN2 and FoxN3 share additional conserved motifs within their N-terminal and C-terminal parts. In contrast, FoxN4 and FoxN5 are less conserved outside their fork head domains and the sequence of FoxN5 deviates by various substitutions even within the fork head domain. Fig. 1B shows a phylogenetic tree of all known FoxN proteins including their

different isoforms from human, mouse, *Xenopus laevis* and zebrafish. While FoxN2, FoxN3 and FoxN4 proteins of *Xenopus laevis* are rather close to their orthologues from other species, *Xenopus* FoxN5 shows more distance to the human and mouse orthologues. Splicing of exon VI in FoxN3 leading to different protein variants was also observed for human FOXN3 (see NM_005197 and U68723 in Fig. 1B).

Fig. 2 compares the genomic organisation of the FoxN subfamily in *Xenopus tropicalis*. Most FoxN genes in *Xenopus tropicalis* contain a leader exon with the exception of FoxN5. FoxN3 has two alternatively used leader exons separated by 1.2 kb. Moreover, exon VI is found to be alternatively spliced in embryonic RNA. The FoxN4 gene was additionally analysed from *Xenopus laevis*. Screening of a genomic library led to the isolation of two recombinant phages containing this gene. The structures of the orthologous genes are very similar, except that insertion or deletion of DNA sequences led to increased or decreased sizes of introns, respectively. A comparison of the genomic structures of *Xenopus tropicalis* FoxN5 and human FOXN5 reveals identical numbers of five exons. In contrast human FOXN6 is a mono-exonal gene (data not shown).

The temporal expression patterns of *Xenopus laevis* FoxN genes (Fig. 3) were analysed by RT-PCR using RNA of different developmental stages (Nieuwkoop and Faber, 1967). FoxN2 expression was observed at all analysed stages including the early cleavage stages. The amount of transcripts is slightly decreased during gastrulation and neurulation (stages 10-20), but at stage 30, FoxN2 is upregulated again and continues to be present until stage 45. A similar pattern was observed for the two variants of FoxN3, which differ by alternative splicing of exon VI. The variant containing exon VI migrates more slowly and seems to be less abundant. Both transcripts are maternally synthesized, are present during early cleavage stages and decrease during gastrulation. An upregulation of both splice variants occurs at stage 25 and persists until stage 45. We also looked for the utilization of the two different leader exons and found that both of

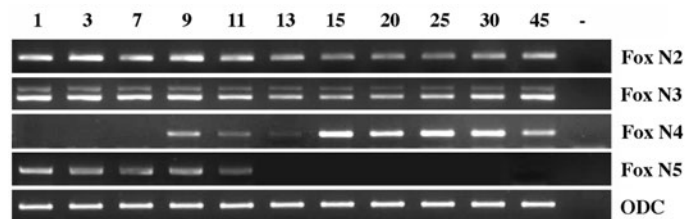


Fig. 3. Temporal expression of FoxN genes during *Xenopus* development. Expression was analysed by RT-PCR using RNA isolated from embryos at the indicated developmental stages (Nieuwkoop and Faber, 1967). Primers for FoxN3 were selected to discriminate between FoxN3a (upper band) and FoxN3b (lower band). Ornithine decarboxylase (ODC) was used as an internal control. Lane (-) shows a negative control in absence of RNA.

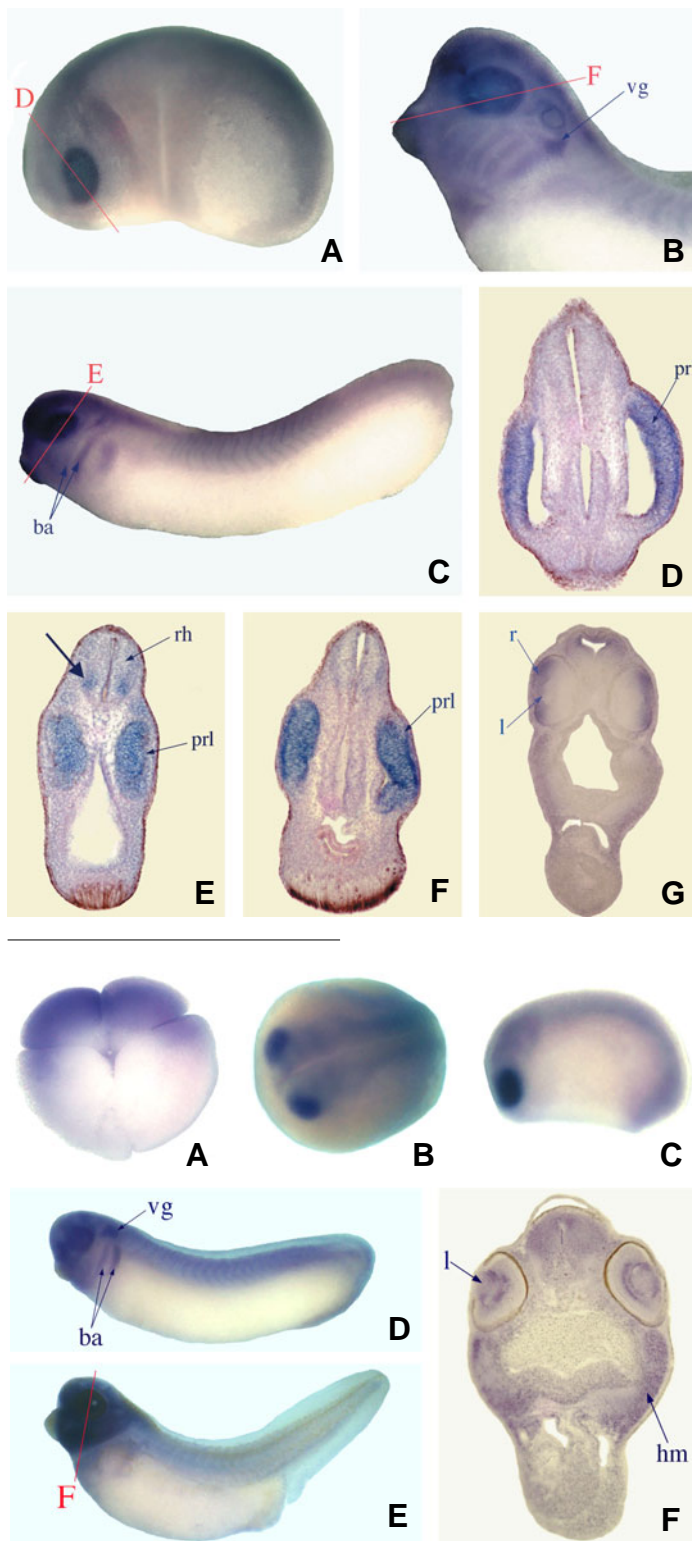


Fig. 5 (Above). Whole mount *in situ* hybridisation of *FoxN3*. (A) Transversal razor blade sectioned 8-cell stage embryo, animal cells are on top. (B) Dorsal view of a stage 23 embryo. Lateral views of (C) stage 26, (D) stage 29 and (E) stage 38 embryos. (F) Transversal section of an embryo at stage 38. The red line in (E) indicates plane of section shown in (F). *ba*, branchial arches; *hm*, head mesenchyme; *l*, lens; *vg*, vagal ganglion.

Fig. 4 (Left). Whole mount *in situ* hybridisation of *FoxN2*. (A) Stage 26, lateral view; (B) magnification of the head of a stage 33/34 embryo, lateral view; (C) lateral view of a stage 29 embryo. The red lines in (A-C) denote transversal sections shown in (D-F). (D) Stage 26; (E) stage 29; (F) stage 33/34. (G) A transversal section through the head of an embryo at stage 36. *ba*, branchial arches; *l*, lens; *prl*, presumptive retinal layer; *rh*, rhombencephalon; *r*, retina; *vg*, vagal ganglion. The fat arrow in (E) denotes the *FoxN2* stained cell layer, lateral to the ventricular zone.

them are used for the variants including and lacking exon VI (data not shown). Thus, at least four different sequences of *FoxN3* are present during *Xenopus* embryogenesis. However, the temporal expression profiles are very similar. *FoxN4* expression was not observed during early cleavage stages but showed an initial increase after midblastula transition. Transcripts diminish during gastrulation, but are strongly enhanced again at stage 15 and remain until stage 45. *FoxN5* is expressed maternally and transcripts are rapidly degraded during gastrulation. At the beginning of neurulation, *FoxN5* expression is already very weak and no transcripts are detected at stage 15.

The spatial expression patterns were analysed by whole mount *in situ* hybridisation (Harland, 1991). The earliest localized expression of *FoxN2* is visible at stage 23 in the eye anlagen (data not shown). At stage 26, an intense staining has been observed in the developing eye (Fig. 4A). At stage 29, *FoxN2* transcripts are also localised in the branchial arches and the brain (Fig. 4C). Fig. 4B shows a magnification of the head of a stage 33/34 embryo with *FoxN2* expression in the vagal ganglion. Transversal sections of stained embryos (Fig. 4D-G) reveal that *FoxN2* expression is localised in the prospective retinal layer and a layer of cells lateral to the ventricular zone (Fig. 4E). At stage 36, when the retinal precursor cells begin to differentiate, retinal expression of *FoxN2* is downregulated. Note, that *FoxN2* expression is absent from the lens (Fig. 4G). Interestingly, expression in the branchial arches and the cells located lateral to the ventricular zone was also observed in the mouse embryo (Tribioli *et al.*, 2002), suggesting that both genes act as functional orthologues.

Fig. 5 shows the spatial expression of the *FoxN3* gene. At early cleavage stages, *FoxN3* transcripts are localized within the animal half (Fig. 5A). At gastrulation, the expression expands over the whole embryo, but does not include the future endodermal cells of the blastopore (data not shown). During neurulation, an intense staining in the prospective eye field is observed. Another expression domain was found in two stripes lateral to the neural tube, i.e., in the neural crest cells (Fig. 5B, stage 23 embryo). Fig. 5C shows that *FoxN3* transcripts are strongly enriched in the eye vesicles (embryo stage 26, lateral view). From stage 29 onwards, *FoxN3* transcripts are observed predominantly in the eye, the branchial arches and in the vagal ganglion (Fig. 5D). At stage 38, we observe diffuse staining of the whole head (Fig. 5E). However, a section reveals relatively stronger staining in the head mesenchyme and in the eye lens (Fig. 5F).

Fig. 6 shows the expression of the *Xenopus FoxN4* gene. Similar to the mouse orthologue (Gouge *et al.*, 2001), *FoxN4* transcripts are predominantly localised in the developing eye. A weak staining is observed in the eye anlage at stage 18 (Fig. 6A). Between stages 21 and 26, *FoxN4* expression becomes stronger in the eye field (see Figs. 6B-D). At stage 29/30, an intense staining is observed within the eye vesicle, in the midbrain and in the nephrostomes of the pronephric kidney (Fig. 6E). *FoxN4*

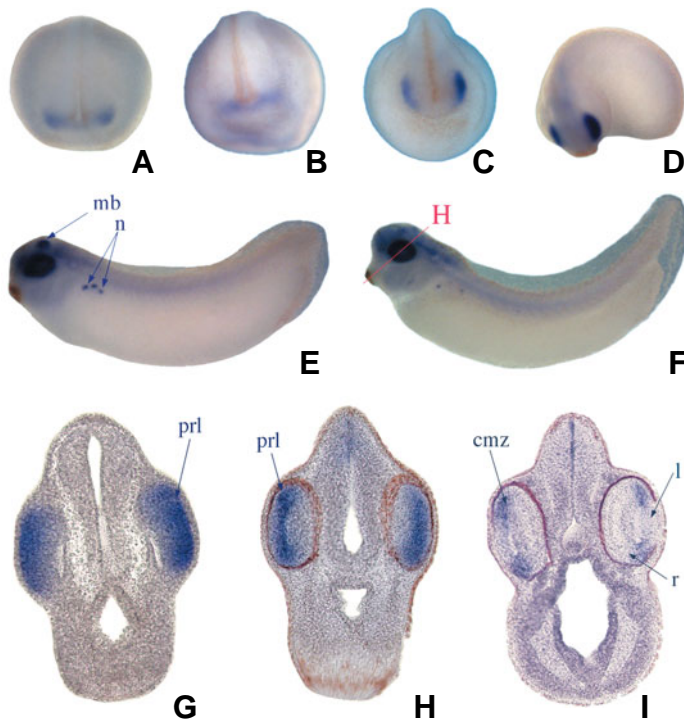


Fig. 6. Whole mount *in situ* hybridisation of *FoxN4*. Anterior view of a (A) stage 18, (B) stage 21 and (C) stage 23 embryo. (D) Half-lateral view of a stage 26 embryo. Lateral view of a (E) stage 29/30 and (F) stage 33/34 embryo. The red line in (F) denotes the plane of the transversal sections shown in (H-I). (G) Transversal section of the head of a stage 26 embryo. (H) Section of a stage 33/34 embryo. (I) Section of a stage 38 embryo. cmz, ciliary marginal zone; l, lens; mb, midbrain; n, nephrostomes; prl, presumptive retinal layer; r, retina.

expression is found in the prospective retinal layer, but is absent from the lens placode (Fig. 6G-I). After stage 30, expression of *FoxN4* in the nephrostomes is downregulated. At stage 33/34, it is largely absent, while the staining in the eye is still visible (Fig. 6F). Transversal section of a stage 26 embryo shows an intense and clearly restricted staining of *FoxN4* in the prospective retinal layer (Fig. 6G). After stage 33/34, expression of *FoxN4* in the eye was downregulated (Fig. 6H). At stage 38, only a few cells are stained in the peripheral region of the retina (Fig. 6I). This staining co-localises with the ciliary marginal zone (CMZ), which is assumed to be a retinal stem cell pool in amphibians, reptiles and birds, but not in mammals.

The expression of *FoxN5* was found to be ubiquitous during early cleavage and gastrula stages. In post-gastrula stage embryos, no staining was visible (data not shown). We suggest that maternal transcripts are evenly distributed in dividing cells, but are degraded until the onset of neurulation.

Experimental Procedures

Total RNA was extracted from *Xenopus laevis* embryos using TRIzol (Gibco) reagent and RNeasy Cleanup (Qiagen). cDNA was synthesised with Transcriptor First Strand cDNA Synthesis Kit (Roche). Primers used for RT-PCR were:

FoxN2 forw: 5'-TCT CTG ACA TCT TCT TGC CCG TT-3'
rev: 5'-GTG CAG CCA GTT CAA GTT GGT CA-3'
FoxN3 forw: 5'-GGC AAA GGT TCT CTG TGG TGC A-3'
rev: 5'-CTG AGG CAG CCA TTG CTC CAA TA-3'
FoxN4 forw: 5'-AGG GCA CTG TGT TCT CTC CTT TG-3'
rev: 5'-CCA GGA GAG TGT ACA TTA TTT GA-3'
FoxN5 forw: 5'-TCT CCT ACA GCA CTT GAA GAC TG-3'
rev: 5'-GAT CCA CTC AGG GCA TGG AAT CA-3'
ODC forw: 5'-GGG CAA AGG AGC TTA ATG TG-3'
rev: 5'-CAT TGG CAG CAT CTT CTT CA-3'

Sequences were amplified from cDNA obtained by RT-PCR or from Lambda ZAP gastrula and Lambda ZAP tadpole cDNA *Xenopus laevis* libraries (Stratagene) with Taq Polymerase (ABgene) and cloned in pDrive cloning vector (Qiagen). For the genomic *FoxN4* sequence, we screened a genomic Lambda Fix library (Stratagene) using *FoxN4* cDNA as labelled probe. EST database analyses and comparative genomics analyses on FoxN orthologues and multiple sequence alignments were performed by using programs: <http://www.ncbi.nlm.nih.gov/blast>, <http://www.ensembl.org/index.html> and <http://www.ebi.ac.uk/clustalw/>.

Primers used for cloning of complete ORF sequences were:

FoxN2 forw: 5'-cgg gat ccc gat GGG TCC AGC AAC TGG AAT GAC T-3'
rev: 5'-cgg ctc gag cgg TTG TGA GAT TGT GCC TTT GCA GT-3'
FoxN3 forw: 5'-cgg ctc gag cgg ATG GGT CCA GTC ATG CCT CCT AG-3'
rev: 5'-gcc tat gta CAT TAG AAT TCA CCT GAC GTG CTG-3'
FoxN4 forw: 5'-cgg ctc gag cgg AAT GAT AGA CAG TGA CAT CTC TGC-3'
rev: 5'-gcc tat gta ATA CAT AGA GAA GTC ATC ACA GT-3'

5'-RACE was performed using GeneRacer Kit, Invitrogen. PCR was done with T3 or T7 primers and a gene-specific primer using a Lambda ZAP cDNA library. A second semi-nested PCR was carried out with a second gene-specific primer. The PCR products were sequenced and cloned. Whole mount *in situ* hybridisations were done according to standard procedures (Harland, 1991). For vibratome sectioning the embryos were embedded in agarose (2%) or in gelatine/albumine. Serial vibratome sections (10-50 μ m) were processed.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 497/A3).

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Received: November 2005

Reviewed by Referees: January 2006

Modified by Authors and Accepted for Publication: February 2006