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## An in situ hybridization screen for the rapid isolation of differentially expressed genes

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**Abstract** To rapidly isolate genes specifically expressed during medaka development we generated a cDNA library enriched for genes expressed in the head region of the developing embryo. Clones were spotted on filters automatically and preselected for abundantly expressed genes by hybridizing them with a probe derived from RNA of undifferentiated totipotent cells. Of the nonhybridizing clones 153 were chosen randomly and further analyzed by whole-mount in situ hybridization. There were 67 selected clones differentially expressed in the developing embryos, and 48 of these were expressed in the developing head. Differentially expressed genes were either of novel type or showed homology to known genes containing DNA binding motifs or to putative housekeeping genes.

**Key words** Random picking · In situ hybridization · Medaka fish · Development · ATPase · Malate dehydrogenase

### Introduction

Mutagenesis screens followed by cloning of the genes causing the mutant phenotype have been a very successful approach in the fruitfly *Drosophila melanogaster* to identify players involved in developmental processes (Nüsslein-Volhard and Wieschaus 1980). Many of the

factors responsible for establishing the invertebrate body plan have been identified. Homologues of the genes initially isolated on the basis of the mutant phenotype in *Drosophila* have subsequently been isolated in many vertebrate systems such as mouse, *Xenopus*, and zebrafish. To isolate novel genes essential for vertebrate embryonic development large-scale mutagenesis screens in zebrafish have been carried out (Driever et al. 1996; Haffter et al. 1996).

In addition, random picking of cDNA libraries and subsequent in situ hybridization analysis have been performed (Kopczynski et al. 1998; Gawantka et al. 1998; B. Thiesse, personal communication). The random picking approach described here was designed to obtain genes expressed during early development.

We generated a cDNA library enriched for genes expressed in the developing head region during the outbudding of the optic vesicle (4-somite stage; Iwamatsu 1994). This library was spotted onto filters by a robot and preselected for abundantly expressed clones by hybridizing them with a probe generated from undifferentiated embryonic stem cells (MES1; Hong et al. 1996). Negative clones were analyzed by in situ hybridization. Of all the cDNAs 44% were expressed in a distinct pattern in the developing embryo, and 31% showed expression in the head and the eye. Clones with an interesting pattern were sequenced. Among the sequenced cDNAs we found clones with homology to known genes containing both DNA binding domains and novel genes. Interestingly, putative “housekeeping genes” were found to be specifically expressed.

### Material and methods

#### cDNA library

The library was generated as described elsewhere (Henrich et al. 1997). In brief, total RNA was isolated from the head region of about 450 stage 20 (4-somite stage) embryos. Poly(A)<sup>+</sup> RNA was subsequently isolated using the Oligotex-RNA-Kit (Qiagen). We used 300 ng poly(A)<sup>+</sup> RNA for generation of the library following

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**Table 1** Selection of sequences of differentially expressed clones

Clone no.	Homology	Expression (st. 21)	Accession no.
<b>Ribosomal genes</b>			
554-02-a18	Ribosomal protein L11	Ubiqu. strong	AJ238271
554-35-a15	40S ribosomal Protein S24	Lens, somites	AJ238272
554-36-b16	Ribosomal protein L7	Tailbud	AJ238273
554-37-d08	40S Ribosomal protein	Somites	AJ238274
554-37-d20	Ribosomal protein L18	Lens, somites	AJ238235
<b>Putative housekeeping genes</b>			
554-37-b02	<i>ATPase subunit 6</i>	Lens, nr, tectum (st. 24) (Fig. 1)	AJ012191
554-35-c23	<i>Malate dehydrogenase</i>	Endoderm, gut (Fig. 3)	AJ012193
554-35-c24	<i>Pyruvate dehydrogenase</i>	Lens placode	AJ238011
554-36-a14	Ferritin H3	Head ectoderm	AJ238012
554-36-a24	Cytoplasmic actin	Axonal tracts (st. 24)	AJ238106
554-02-a03	<i>NADH dehydrogenase</i>	Nose, lens (st. 24)	AJ238025
554-37-b18	<i>Cytochrome oxidase</i>	mb, eye	AJ242591
554-37-d24	<i>Elongation factor-1<math>\beta</math></i>	Early head, mb, hb placodes	AJ237772
555-01-a06	<i>Phospholipid hydroperoxide glutathione peroxidase</i>	Epiblast	AJ242590
<b>Regulatory genes</b>			
554-37-b10	Nucleic acid binding protein	Lens, dors retina, mb (st. 24) (Fig. 2)	AJ012192
554-01-b09	Heat shock protein 27; $\alpha$ -crystallin B-chain	Lens pit	AJ238100
554-02-a05	Human melanoma antigen	Lenscore (st. 24)	AJ238101
554-35-a07	Macrophage migration inhibitor factor	nr, lens	AJ238102
554-35-b02	Tumor metastasis inhibitor factor	hb placodes, eyes, retina	AJ238104
554-35-c15	Chromobox protein	mb	AJ238107
554-35-c21	Heat shock protein 10	Lens placode, mb	AJ238010
554-35-c10	Eukaryotic initiation factor; methionine amino peptidase	Lenscore, retina (st. 24)	AJ238105
554-37-b24	GTP binding protein	Retina	AJ238023
<b>Novel genes</b>			
554-37-d14		mb, eye (st. 24)	AJ238099
554-35-b01		Tailbud	AJ238103
554-36-b12		Lateral mesoderm	AJ238024
554-34-F05		Tailbud, eye	AJ237771
555-01-b10		Lateral mesoderm	AJ012190

the Superscript Plasmid System (Life Technology). The longest fractions of the cDNA were directionally ligated to the plasmid vector pSport1 (*NotI-SalI* precut). Plasmids were electroporated into highly competent electroMAX DH10B *Escherichia coli* cells (Life Technologies) yielding a library with an initial complexity of 400,000 independent clones. The primary library was plated onto 22×22 cm agarose plates at a density of 2500 colonies per plate. Well separated colonies were identified and picked into 384-well microtiter plates by an automatic device. These master plates were replicated, and clones were spotted onto 22×22 cm hybond N<sup>+</sup> nylon filters by a robot. The library is represented on one filter containing 27,648 clones.

The library is made available through the resource center in Berlin (RZPD, <http://web.rzpd.de/cgi-bin/newlib#MEDAKA-cDNA>). The clone number consists of the number of the library (554), the number of the microtiter plate, and the coordinates of the clone within this plate.

#### Medaka stocks

Wild-type *Oryzias latipes* from a closed stock at the Max Planck Institute for Biophysical Chemistry were kept as described elsewhere (Köster et al. 1997).

#### Filter hybridization

Total RNA was isolated (Chomczynski and Sacchi 1987) from medaka MES1 cells (Hong et al. 1996). The subtraction probe was

prepared from 1  $\mu$ g reverse-transcribed total RNA using d(T)<sub>12-18</sub> primers. Filters were prehybridized for 30 min and hybridized for 16 h at 65°C in 7 ml Church buffer (Church and Gilbert 1984) using 1×10<sup>6</sup> cpm/ml. The filters were washed for 1 h at 65°C and exposed for 5 h.

#### Whole-mount in situ hybridization

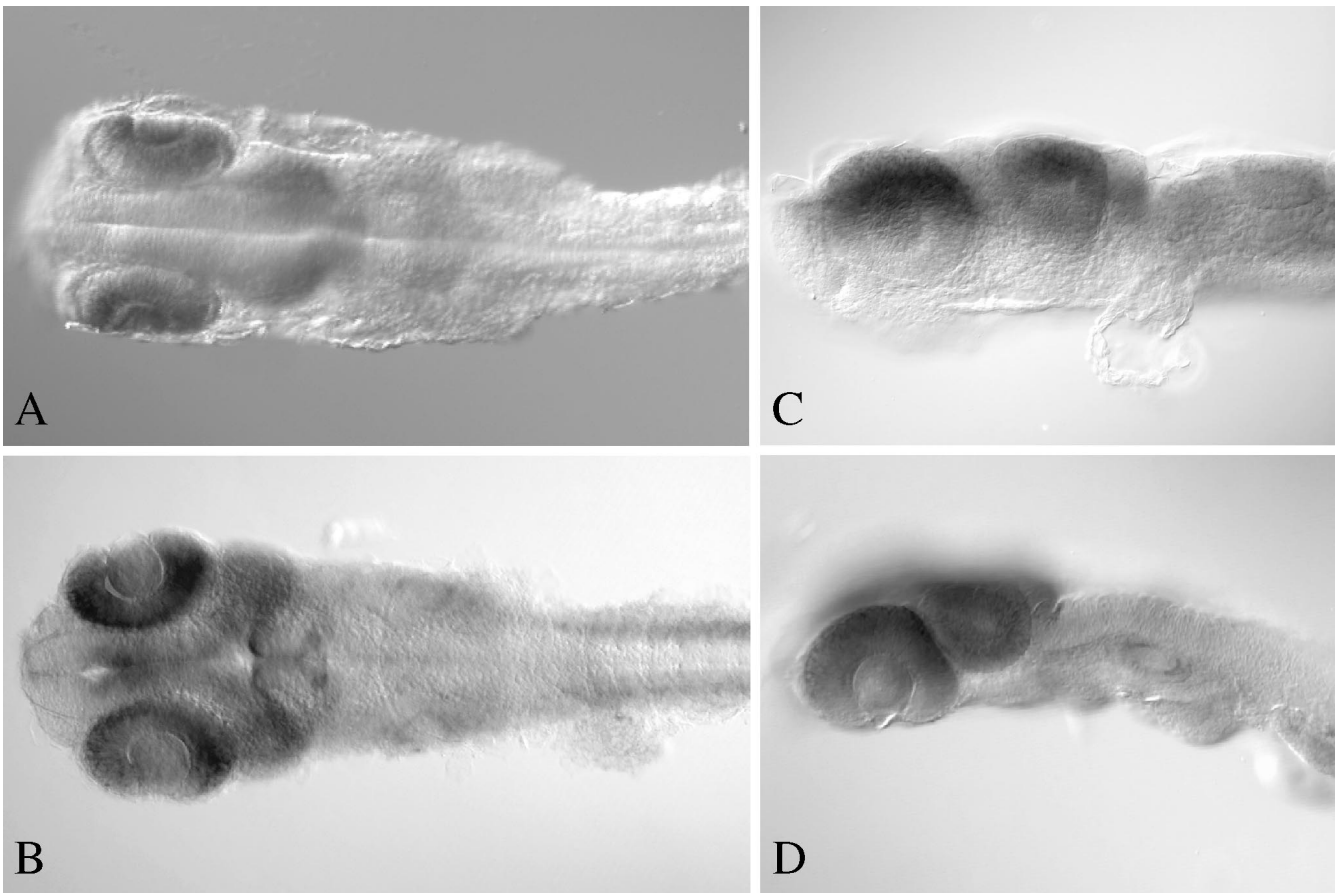
Whole-mount in situ hybridization was performed using digoxigenin-labeled RNA probes as described elsewhere (Loosli et al. 1998). In situ hybridizations for the screening procedure were carried out by a robot (in situ pro, Abimed, Germany). A description of all expression patterns obtained with the picked cDNAs is available upon request.

#### Vibratome sections

Vibratome sections were prepared following standard procedures (Bober et al. 1994).

## Results and discussion

To isolate genes expressed during the early steps of head development we dissected the head regions of four-somite stage embryos. This material was used to isolate



**Fig. 1A–D** Expression pattern of clone 554-37-b02. **A,B** Dorsal views; *left* anterior. **C,D** Lateral views; *left* anterior. **A,C** 12-somite stage, st. 23. **B,D** 18-/19-somite stage, st. 25

poly(A)<sup>+</sup> RNA and subsequently generate a primary cDNA library (Henrich et al. 1997). The initial complexity of the library was 400,000 independent clones, 27,648 of which were spotted onto a filter by a robot.

To exclude genes that are expressed in undifferentiated MES1 cells the spotted library was hybridized with a probe generated by reverse transcription of mRNA isolated from MES1 cells (Hong et al. 1996). This preselection resulted in a strong hybridization signal in 15% of the clones. Nonhybridizing clones represent genes expressed during early head formation that are not abundantly transcribed in MES1 cells. Of these clones 153 were randomly chosen for in situ hybridization. Of these, 19% ( $n=29$ ) showed no hybridization signal, 37% ( $n=57$ ) were ubiquitously expressed, and 44% ( $n=67$ ) showed differential expression in the developing embryo. Many of these were expressed in all tissues, but were clearly upregulated in certain regions of the embryo. Of these, 72% ( $n=48$ ) were expressed in developing head and eye structures such as mid- and hindbrain, lens, optic vesicles, and neuroretina.

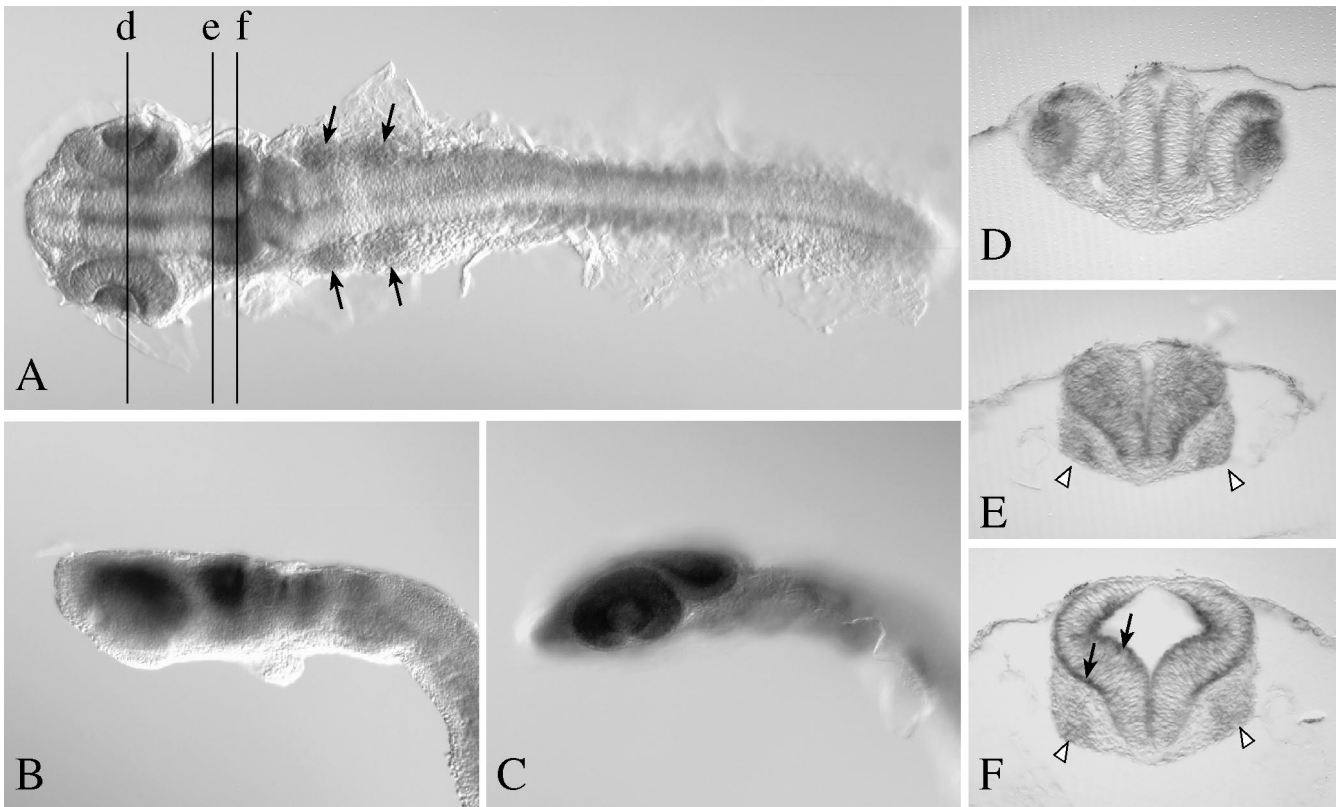
Sequence analysis revealed that about 50% of the differentially expressed genes were homologous to genes regarded as housekeeping genes or ribosomal proteins

(Table 1). About one-third of the differentially expressed clones showed homology to putative regulatory proteins. Five showed no homology to any known gene. Following section discusses examples of clones expressed in the developing head as well as examples of differentially expressed “housekeeping genes.”

The sequence of clone 554-37-b02 (352 bp) contains an uninterrupted open reading frame, which reveals high homology to the 3' region of ATPase subunit 6 (81% amino acid identity). Weak expression starts at the 2-somite stage (st. 19; data not shown) in the head region of the embryo. At the 9-somite stage signals in the lens, the inner portion of the neuroretina, and the optic tectum appear (Fig. 1A, C). At 18-/19-somite stage (st. 25; Fig. 1B, D) expression is down-regulated in the lens and expression in the ear and the somites starts.

The finding of an ATPase expressed in the developing lens is accord with the proposed role of ATPase in lens differentiation. It is thought to be involved in the regulation of the osmotic pressure of the lens (Graw et al. 1990). Consistently enhanced ATPase activity has been detected in several cataract mutants.

Over a range of 148 bp clone 554-37-b10 (324 bp) shows 85% nucleotide identity to exon 5 of human cellular nucleic acid binding protein, a known zinc finger containing protein with the capacity to bind both ssDNA and RNA (Flink et al. 1998). Initially this clone is ubiquitously expressed at low levels in the entire embryo (da-



**Fig. 2A–F** Expression pattern of clone 554-37-b10. **A** Dorsal view; *left* anterior. **B,C** Lateral views; *left* anterior. **D–F** Transverse sections at the levels indicated by the *lines* in **A**. **A,B,D–F** 12-somite stage, st. 23. **B** 18-/19-somite stage, st. 25

ta not shown). At the 12-somite stage strong expression is detected in the lens and dorsal retina (Fig. 2A, B, D), midbrain (Fig. 2A, E, F), and rhombencephalic placodes (Fig. 2A, arrows). Transverse sections show a strong uniform expression in the anterior midbrain (Fig. 2E), whereas at more posterior levels expression is restricted to the inner and outermost layers of the lateral tectum (Fig. 2F, arrows). An additional expression domain can be detected in the lateral head mesenchyma at the level of the midbrain (Fig. 2E, F, arrowheads).

The insert of clone 554-35-c23 (1206 bp) contains an open reading frame that shows highest homology to the  $\alpha$ -chain of cytoplasmic pig malate dehydrogenase (90% amino acid similarity). Expression starts at the 6-somite stage (st. 21) in two domains ventrolateral to the neural tube at the level of the anterior hindbrain (Fig. 3A, B). During subsequent development these domains fuse (Fig. 3C, E), forming the gut tube (Fig. 3F). This fused domain buds out at the anterior part and forms a T-like structure seen from the dorsal side (Fig. 3G). An additional endodermal expression domain emerges at the 9-somite stage ventral to the neural tube starting at the level of the sixth somite and extending posterior (Fig. 3D). During subsequent development the expression level in this domain increases (Fig. 3H). In the optic vesicles and the presumptive midbrain weak expression is

detectable starting at the 6-somite stage (Fig. 3C, D, E). At the 18-somite stage these expression domains become restricted to the neuroretina and the two lobes of the tectum (Fig. 3G, H).

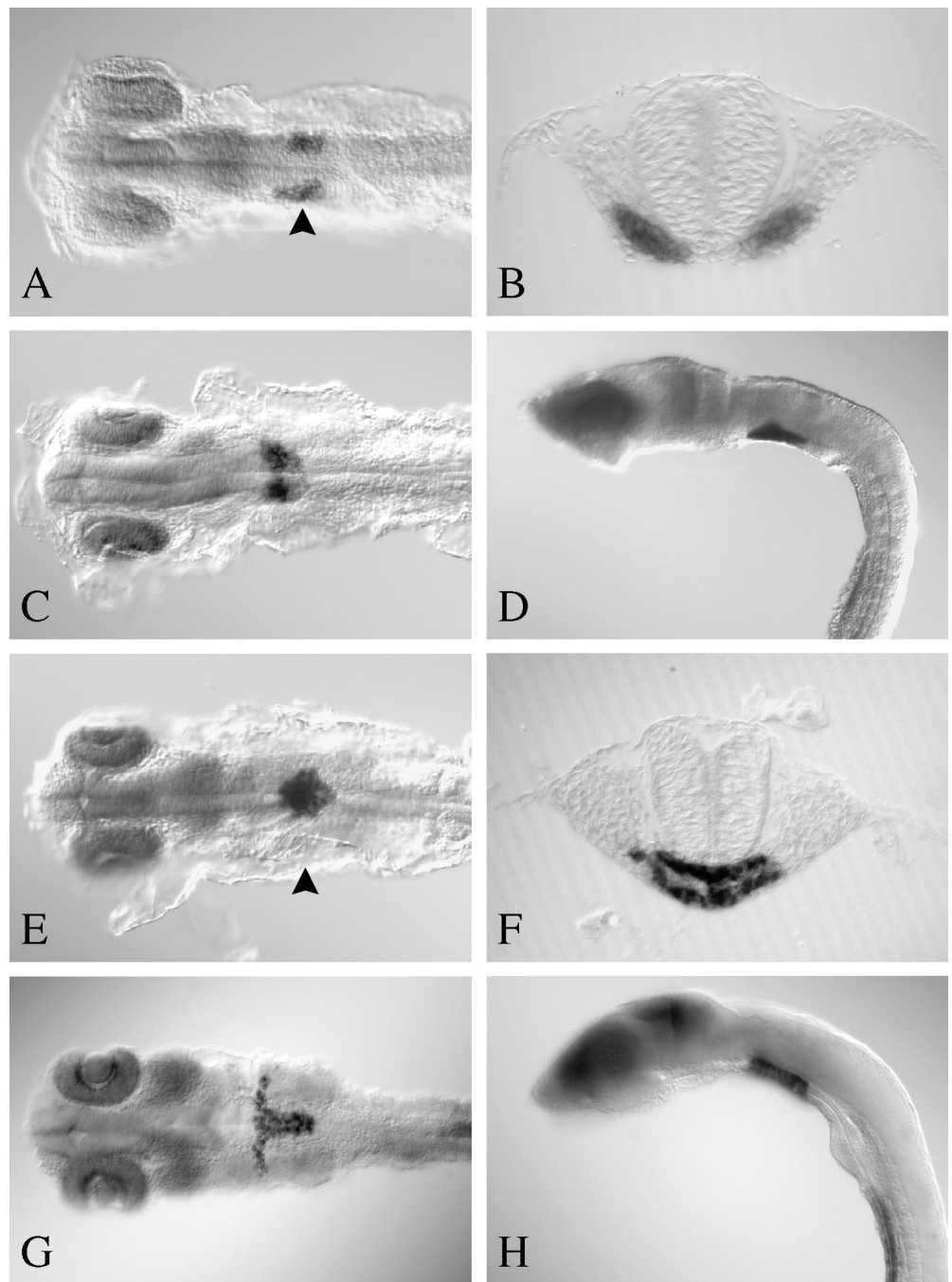
Biochemical analysis shows cytoplasmic malate dehydrogenase to be expressed in all tissues but predominantly in the liver of adult fish (Basaglia 1989). It is surprising that the medaka homologue of malate dehydrogenase is expressed in very distinct domains in early development. Interestingly, the malate dehydrogenase we isolated is expressed at highest levels in a region of the endoderm that later gives rise to the liver prior to the onset of organogenesis. This early expression makes this clone an interesting candidate direct target gene for liver specific regulators such as HNF-4 (Duncan et al. 1994).

Despite a subtractive hybridization step still many abundantly expressed genes were present in the library. Normalization of the library would help to avoid this problem. The differential expression of so-called housekeeping genes can be explained by their control by developmental regulators. Developmental pathways finally must result in the regulation of housekeeping genes. Proliferative tissues need more energy and cell shape changes need the control of cytoskeletal components.

Still, random picking of a preselected cDNA library and subsequent *in situ* analysis is a simple technique that allows the rapid identification of novel genes for any given aspect of early development.



**Fig. 3A–H** Expression pattern of clone 554-35-c23. **A,C,E,G** Dorsal views; *left anterior*. **D,H** Lateral views; *left anterior*. **B,F** Transversal sections at the level indicated in **A** and **E**, respectively. **A,B** 6-somite stage, st. 21. **C** 9-somite stage, st. 22. **D–F** 12-somite stage, st. 23. **G,H** 18-/19-somite stage, st. 25



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## References

- Basaglia F (1989) Some aspects of isozymes of lactate dehydrogenase, malate dehydrogenase and glucosephosphate isomerase in fish. *Comp Biochem Biophys* 92:213–226
- Bober E, Franz T, Arnold HH, Gruss P, Tremblay P (1994) Pax-3 is required for the development of limb muscles: a possible role for the migration of dermomyotomal muscle progenitor cells. *Development* 120:603–612
- Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156–159
- Church GM, Gilbert W (1984) Genomic sequencing. *Proc Natl Acad Sci USA* 81:1991–1995

- Driever W, Solnica-Krezel L, Shier AF, Neuhaus SCF, Malicki J, Stemple DL, Stanier DYR, Zwartkruis F, Abdelilah S, Rangini Z, Belag J, Boggs C (1996) A genetic screen for mutations affecting embryogenesis in zebrafish. *Development* 123:37–46
- Duncan SA, Manova K, Chen WS, Hoodless P, Weinstein DC, Bachvarova RF, Darnell JE, Jr (1994) Expression of transcription factor HNF-4 in the extraembryonic endoderm, gut, and nephrogenic tissue of the developing mouse embryo: HNF-4 is a marker for primary endoderm in the implanting blastocyst. *Proc Natl Acad Sci USA* 91:7598–7602
- Flink IL, Blitz I, Morkin E (1998) Characterization of cellular nucleic acid binding protein from *Xenopus laevis*: expression in all three germ layers during early development. *Dev Dyn* 211:123–130
- Gawantka V, Pollet N, Delius H, Vingron M, Pfister R, Nitsch R, Blumenstock C, Niehrs C (1998) Gene expression screening in *Xenopus* identifies molecular pathways, predicts gene function and provides a global view of embryonic patterning. *Mech Dev* 77:95–141
- Graw J, Reitmeir P, Wulff A (1990) Osmotic state of lenses in three dominant murine cataract mutants. *Graefes Arch Clin Exp Ophthalmol* 228:252–254
- Haffter P, Granato M, Brand M, Mullins MC, Hammerschmidt M, Kane DA, Odenthal J, van Eeden FJ, Jiang YJ, Heisenberg CP, Kelsh RN, Furutani-Seiki M, Vogelsang E, Beuchle D, Schach U, Fabian C, Nüsslein-Volhard C (1996) The identification of genes with unique and essential functions in the development of the zebrafish, *Danio rerio*. *Development* 123:1–36
- Henrich T, Burgdorf C, Lehrach H, Wittbrodt J (1997) Generation of two spotted plasmid cDNA-libraries of the head region of stage 20 and whole stage 17 medaka embryos. *Fish Biol J Medaka* 9:41–45
- Hong Y, Winkler C, Scharl M (1996) Pluripotency and differentiation of embryonic stem cell lines from the medakafish (*Oryzias latipes*). *Mech Dev* 60:33–44
- Iwamatsu T (1994) Stages of normal development in the medaka *Oryzias latipes*. *Zool Sci* 11:825–839
- Kopczynski CC, Noordermeer JN, Serano TL, Chen WY, Pendleton JD, Lewis S, Goodman CS, Rubin GM (1998) A high throughput screen to identify secreted and transmembrane proteins involved in *Drosophila* embryogenesis. *Proc Natl Acad Sci USA* 95:9973–9978
- Köster R, Stick R, Loosli F, Wittbrodt J (1997) Medaka spalt acts as a target gene of hedgehog signaling. *Development* 124:3147–3156
- Loosli F, Köster RW, Carl M, Krone A, Wittbrodt J (1998) Six3, a medaka homologue of the *Drosophila* homeobox gene sine oculis is expressed in the anterior embryonic shield and the developing eye. *Mech Dev* 74:159–164
- Nüsslein-Volhard C, Wieschaus E (1980) Mutations affecting segment number and polarity in *Drosophila*. *Nature* 287:795–801
- Ye Q, Worman HJ (1996) Interaction between an integral protein of the nuclear envelope inner membrane and human chromodomain proteins homologous to *Drosophila* HP1. *J Biol Chem* 271:14653–14656