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EXPRESSION NOTE

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Tyrosinase gene expression in zebrafish embryos

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Abstract The enzyme tyrosinase is required for the conversion of tyrosine into the pigment melanin. Thus, tyrosinase gene expression is a useful marker for studying the differentiation of melanin-expressing cells during embryogenesis. We describe the spatiotemporal pattern of transcription of the tyrosinase gene and the presence of active enzyme in whole embryos of the zebrafish, *Danio rerio*. At 16.5 h post-fertilisation the tyrosinase gene is transcribed in the dorsal extremity of the developing retinal pigment epithelium, approximately 7 h before visible pigmentation. Shortly thereafter, transcription in neural crest-derived melanocytes is first observed dorsolateral to the mesencephalon and diencephalon and the posterior hindbrain/anterior spinal cord. A wave of gene activation and cell migration is then observed moving towards the posterior of the animal. DOPA staining for tyrosinase activity shows the presence of active enzyme in embryos at least 3 h before visible pigmentation.

Keywords *Danio rerio* · Melanocytes · Melanin · Tyrosinase gene expression · Whole-mount in situ transcript hybridisation

The characteristic yellowish-silver and black striping pattern of the adult zebrafish epidermis is generated by an array of three types of pigmented cell, all derived from the neural crest. The yellowish-silver interstripes of the epidermis are made up of xanthophores and iridophores. The longitudinal dark stripes consist of melanocytes expressing the pigment melanin (reviewed by Kelsh et al. 1996). Melanin is also found in the pigmented epithelial cells of the retina that are derived from the optic vesicle.

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The enzyme tyrosinase is required at three consecutive steps in the synthesis of melanin from the amino acid tyrosine. It converts tyrosine to 3,-4 dihydroxyphenylalanine (DOPA) and then DOPA to DOPAquinone. DOPAquinone reacts spontaneously to form DOPAchrome and then 5,6 dihydroxyindole (DHI). Finally, tyrosinase catalyses the oxidation of DHI to form indole-5,6-quinone which spontaneously forms the melanin biopolymer. (Korner and Pawelek 1982; Hearing 1987). In vitro, melanin can also form spontaneously from DOPAquinone (reviewed in Korner and Pawelek 1982). Loss of tyrosinase activity results in the complete loss of melanin, producing the characteristic albino phenotype of humans and other vertebrates.

In mammals, two enzymes structurally similar to tyrosinase, tyrosinase-related protein 1 (TRP-1) and tyrosinase-related protein 2 (TRP-2), couple to this biosynthetic pathway and modify its components to alter the final constitution of the melanin biopolymer. TRP-2 is a DOPAchrome tautomerase which catalyses the conversion of DOPAchrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) instead of DHI (Tsukamoto et al. 1992). TRP-1 then catalyses the oxidation of DHICA to form an indole-5,6-quinone-2-carboxylic acid, resulting in the production of black eumelanin (Kobayashi et al. 1994).

Transcription of the tyrosinase gene, the TRP genes or the presence of melanin are useful markers for studying neural crest and optic vesicle cell migration and differentiation during embryogenesis. Tyrosinase genes have been described in numerous vertebrates (Inagaki et al. 1994; reviewed in Ferguson and Kidson 1997) including the zebrafish (GenBank Accession No: AJ250302). Interestingly, while pigment rescue studies have been conducted using tyrosinase transgenes (Beermann et al. 1990, 1992; Hyodo-Taguchi et al. 1997) and tyrosinase gene promoter activity has been assayed using various marker genes (Kluppel et al. 1991; Tief et al. 1996, 1997; Schmidt et al. 1998), the spatiotemporal pattern of endogenous tyrosinase gene transcription has not been analysed in whole embryos in a vertebrate. Here we de-

Table 1 Tyrosinase and TRP gene DNA sequences used for phylogenetic analysis. The GenBank accession number of each sequence and the nucleotides used for the analysis are shown

Common name	Species name	Sequence	Sequence accession number	Nucleotides
Frog	*Rana nigromaculate*	Tyrosinase	D12514	130 to 711
Chicken	*Gallus gallus*	Tyrosinase	D88349	118 to 699
Chicken	*Gallus gallus*	TRP-1	AF003631	145 to 735
Chicken	*Gallus gallus*	TYRP2	AF023471	142 to 732
Human	*Homo sapiens*	Tyrosinase	M27160	118 to 699
Human	*Homo sapiens*	TYRP2	S69231	136 to 726
Mouse	*Mus musculus*	Tyrosinase	M20234	118 to 699
Mouse	*Mus musculus*	TRP	X03687	148 to 738
Mouse	*Mus musculus*	TYRP2	X63349	136 to 726
Goldfish	*Carassius auratus*	TRP-1	S71755	130 to 708
Medaka	*Oryzias latipes*	Tyrosinase	AB0322694	121 to 708
Zebrafish	*Danio rerio*	dopachrome tautomerase; TRP2	AF280090	124 to 714
Zebrafish	*Danio rerio*	Tyrosinase	AJ250302	1 to 585

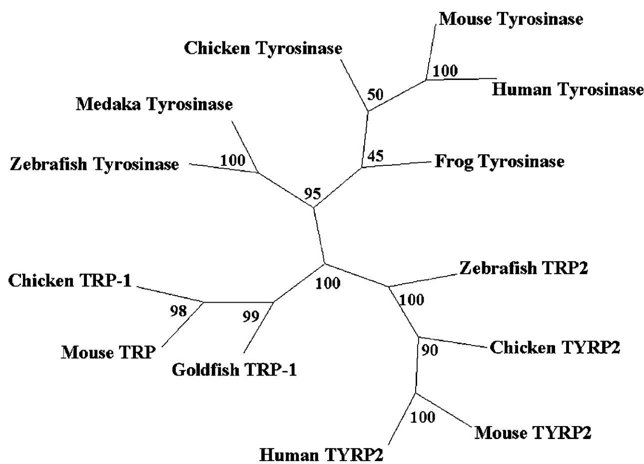


Fig. 1 An unrooted phylogenetic tree of DNA sequences encoding tyrosinase, tyrosinase-related protein (TRP)-1 and TRP-2 from zebrafish and other vertebrates. The tree was constructed using the maximum parsimony method (DNAPARS, Felsenstein 1989). The bootstrap values associated with the likelihood of branch points are indicated as *percentiles*. The strategy used for sequence alignment was as follows: (1) Preliminary alignments were carried out on protein sequences encoded by the regions of DNA sequence shown in Table 1 using the program CLUSTALW (Thompson et al. 1994). (2) The regions of coding DNA corresponding to the edited protein sequences were then aligned according to the information from the protein sequence alignment. Codons were not interrupted. Bootstrapping of the tree constructed using DNAPARS made use of SEQBOOT and a majority rule consensus tree from 1,000 trees was derived with the CONSENSE program. All the programs used were part of the package available on BioNavigator (http://www.bionavigator.com/)

scribe the spatiotemporal pattern of the zebrafish tyrosinase gene by whole-mount in situ transcript hybridisation and we report the detection of active tyrosinase enzyme approximately 3 h before the onset of pigmentation.

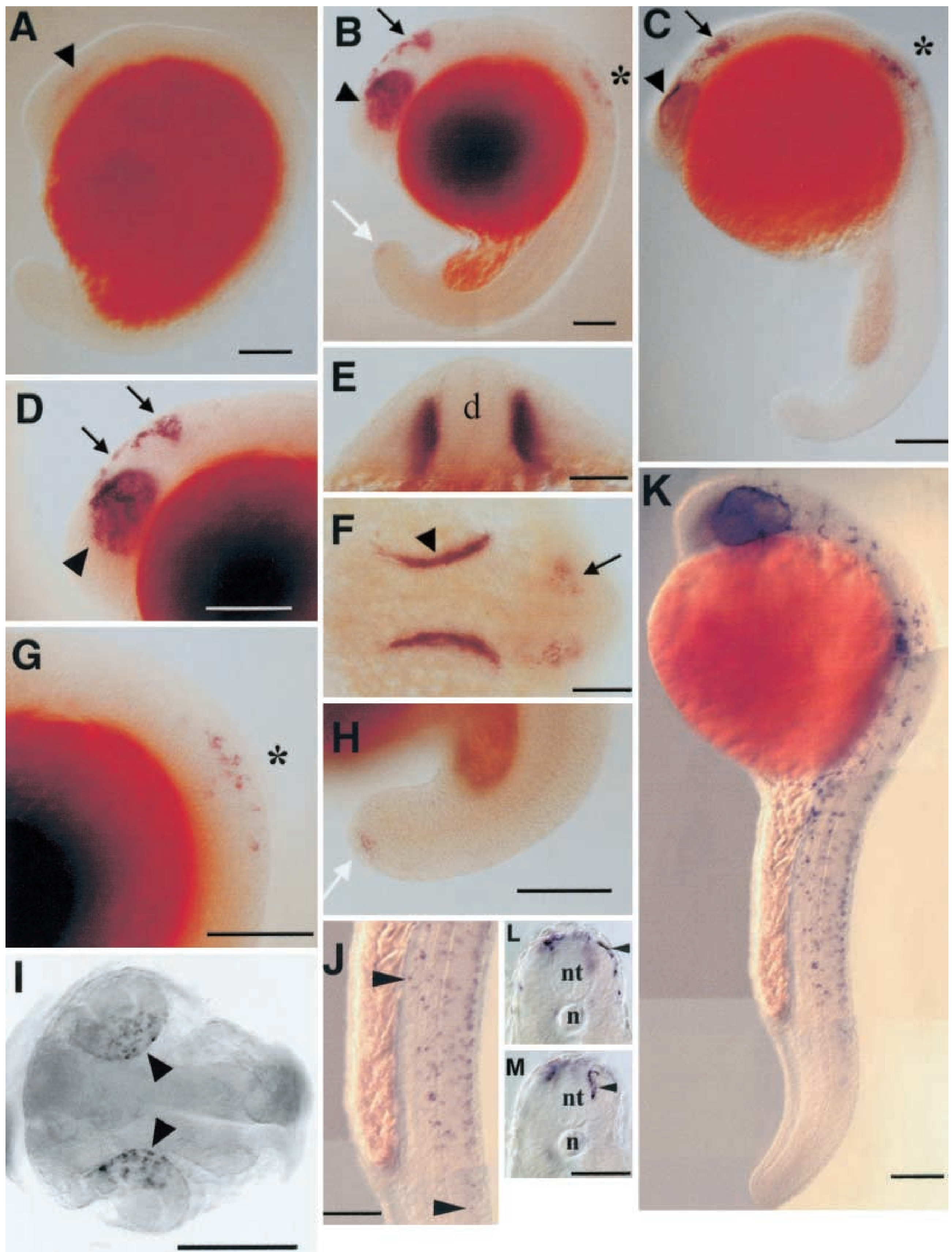
To confirm that the described zebrafish tyrosinase cDNA truly represents a zebrafish tyrosinase gene rather than a TRP-1 or TRP-2 orthologue, we performed a phylogenetic analysis. Table 1 shows the DNA sequences that were compared to construct the phylogenetic tree shown in Fig. 1. As shown in Fig. 1, the zebrafish sequence clearly congregates with other vertebrate tyrosinase genes rather than with the TRP-1 and TRP-2 gene groups.

We then examined the spatiotemporal expression pattern of the zebrafish tyrosinase gene during embryogenesis using whole-mount in situ transcript hybridisation. A digoxigenin-labelled probe was prepared using the zebrafish cDNA as a template and hybridised to whole embryos of various ages according to the method of Jowett (1997). We first observed transcription of the tyrosinase gene at 16.5 h post-fertilisation (hpf) in the dorsal extremity of the developing eye (Fig. 2A). Since melanin is first seen in presumptive retinal pigmented epithelial cells at approximately 24 hpf, tyrosinase gene activity apparently precedes visible melanin accumulation by 7 h.

By 18 hpf tyrosinase gene transcription is observed to span the presumptive pigmented epithelium of the entire eye. Transcription is also apparent in cells (presumably future melanocytes) dorsolateral to the mesencephalon and diencephalon and, at lesser levels, dorsolateral to the developing posterior hindbrain and anterior spinal cord (Fig. 2B,D–H) reflecting the neural crest origin of these cells. A very low level of transcription is observed in cells at the posterior extremity of the extending tail bud in embryos at 18 hpf (Fig. 2B,H). However, dissimilar to other areas of the embryo, we did not observe transcripts in this region at 21 hpf (Fig. 2C).

Due to the presence of melanin in wild-type embryos at 24 hpf, expression of tyrosinase transcripts in 24 hpf and older embryos were observed by inhibiting pigmentation using phenylthiocarbamide (PTC). Embryos were treated with the addition of PTC at 0.2 mM daily from 6 hpf until they were fixed for the in situ hybridisation procedure.

At approximately 25 hpf a wave of anterior to posterior melanocyte differentiation is observed as cells begin transcribing the tyrosinase gene and migrate from the


A
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◀ **Fig. 2A–M** Tyrosinase gene transcription and tyrosinase enzyme activity in zebrafish embryos. Except as indicated, dorsal is *up* and anterior is to the *left*. Developmental stage is given as hours post-fertilisation (hpf) at 28.5°C. **A–D** and **G, H, J, K** Sagittal views at 16.5 hpf (**A**), 18 hpf (**B, D, G, H**), 21 hpf (**C**) and approximately 25 hpf (**J, K**). **E** Transverse optical section through the diencephalon (*d*) at 18 hpf. **F** Dorsal axial view of anterior of embryo at 18 hpf. **I** Dorsal axial view of head of 21.5 hpf embryo. **J** Enlarged view of yolk extension region in **K**. **L, M** Transverse optical sections through yolk extension region of embryo at approximately 25 hpf taken at different focal planes. **A** Transcription in the dorsal extremity of the presumptive retinal pigmented epithelium (*arrowhead*) at 16.5 hpf. By 18 hpf, transcription spans the entire eye (**B, D–F**, *arrowheads*), and is observed in cells dorsolateral to the mesencephalon and diencephalon (*black arrows* in **B, D** and **F**). Lower levels of expression are also observed in cells (indicated by *asterisks*) dorsolateral to the developing posterior hindbrain/anterior spinal cord (**B, G**), and in cells at the posterior extremity of the extending tail bud (*white arrows* in **B** and **H**). **C** Tyrosinase gene transcription at 21 hpf is similar to that at 18 hpf except we could not detect expression in the tail bud. **I** A 21.5 hpf embryo assayed for tyrosinase activity by the conversion of 3,4-dihydroxyphenylalanine (DOPA) into melanin shows indicative staining in cells of the future retinal pigmented epithelium of the eye (*arrow heads*). **J, K** At approximately 25 hpf tyrosinase transcription is seen along most of the anterior-posterior axis. Progressively fewer cells are observed in ventral regions towards the posterior (see region between *arrow heads* in **J**). Transcription is observed in cells in the lateral and medial pathways of melanocyte migration from the neural crest (*arrowheads* in **L** and **M** respectively). The notochord (*n*) and neural tube (*nt*) are indicated. Embryos in **J–M** were treated with phenylthiocarbamide (PTC) to inhibit pigmentation. *Scale bars*: **A–D, G, H, K**, 100 μm; **E, F, I, J, L, M** 50 μm

neural crest to more ventral regions (Fig. 2J,K) via the lateral and medial pathways (Fig. 2L,M) as previously described (Jesuthasan 1996). By approximately 46 hpf this wave of differentiation appears to be complete and the expression pattern resembles that of the wild-type melanin pigmentation pattern (data not shown).

We tested for tyrosinase activity in embryos before the onset of pigmentation by assaying for the conversion of DOPA into melanin as described by Haffter et al (1996; Fig. 2I). We could first detect tyrosinase activity in the presumptive retinal pigmented epithelium of the eye at approximately 21.5 hpf. Thus, in accordance with observations in other vertebrates, tyrosinase activity in the zebrafish can precede visible melanin accumulation by several hours. We could not observe tyrosinase activity in other areas at 21.5 hpf. Since tyrosinase transcription occurs first and at the highest levels in the developing eye, our failure to detect tyrosinase activity in melanocytes at 21.5 hpf or in the eyes of embryos at earlier times is probably due to insufficient sensitivity of the DOPA staining method rather than absence of tyrosinase activity.

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