2. Materials and Methods

2.1. Materials

2.1.1. Chemicals:

Acrylamide (40%, 20:1)	Roth	Karlsruhe, Germany
Agar	Difco	Kansas City, USA
Agarose	Gibco	Betheseda, USA
Ammonium acetate	Sigma	München, Germany
Ampicillin	Serva	Heidelberg, Germany
Bacto-Tryptone	Difco	Kansas City, USA
Bacto-Agar	Difco	Kansas City, USA
Bacto-Yeast-Extract	Difco	Kansas City, USA
Bis-Acrylamide	Serva	Heidelberg, Germany
Blocking reagent	Roche	Basel, Switzerland
Bromophenol blue	Sigma	München, Gramany
BSA	Biomol	Hamburg, Germany
Chloroform	Merck	Darmstadt, Germany
Diethyl pyrocarbonat (DEPC)	Sigma	München, Germany
Dimethyl sulfoxid (DMSO)	Merck	Darmstadt, Germany
Dithiothreitol	Sigma	München, Germany
dNTP (100 mM)	Amersham	Braunschweig, Germany
λ-DNA/HindIII/EcoRI marker	Fermentas	Vilnius, Lithuania
Ethanol	Merck	Darmstadt, Germany
Ethidium bromide	Serva	Heidelberg, Germany
Ficoll 400	Promega	Madison, USA
Formaldehyde	Merck	Darmstadt, Germany
Formamide	Fluka	New Ulm, Germany
Glycerol	Sigma	München, Germany
Hexamers	Gibco	Betheseda, USA
IPTG	Biomol	Hamburg, Germany
Ketavert (ketamine 100 mg/ml)	Pharmacia	Erlangen, Germany
Mineral oil	Sigma	München, Germany
Phenol	Sigma	München, Germany
Penicillin-Streptomycin (10u/ml)	Gibco	Betheseda, USA
Rompun (xylazine 2%)	Bayer	Leverkusen, Germany
SDS	Serva	Heidelberg, Germany
TEMED	Gibco	Betheseda, USA
Triton X-100	Serva	Heidelberg, Germany
Tris	Sigma	München, Germany
TRIzol reagent	Gibco	Betheseda, USA
Urea	Sigma	München, Germany
X-Gal	Biomol	Hamburg;Germany
ΦX174-DNA/*Hae*III-Marker	Fermentas	Vilnius, Lithuania
Xylencyanol	Bio-Rad	München, Germany

2.1.2. Cell culture media:

DMEM	Gibco	Betheseda, USA
Trypsine-EDTA (x1)	Gibco	Betheseda, USA
FCS	Gibco	Betheseda, USA

2.1.3. Enzymes:

Alkaline phosphatase	Promega	Madison, USA
DNase A	Roche	Basel, Switzerland
DNase I (RNase-free)	Roche	Basel, Switzerland
MMLV	Gibco	Betheseda, USA
DNA ligase	Gibco	Betheseda, USA
Proteinase K	Sigma	München, Germany
Restriction enzymes	Amersham	Freiburg, Germany
RNase A	Roche	Basel, Switzerland
RNasin	Promega	Madison, USA
T4 -DNA-Ligase	Promega	Madison, USA
Taq-DNA-Polymerase	Gibco/BRL	Betheseda, USA

2.1.4. Kits:

Ambion RPA II kit	AMS Biotechnology	Whitney, UK
QIAquick Gel Extract Kit	Qiagen	Hilden, Germany
Plasmid Maxi Kit	Qiagen	Hilden, Germany
5´RACE-Kit	Gibco	Betheseda, USA
3´RACE-Kit	Gibco	Betheseda, USA
Prime-It-Labeling Kit	Stratagene	La Jolla, USA
Transcription Kit	Stratagene	La Jolla, USA
Ambion mMessage mMachaine Kit	AMS Biotechnology	Whitney, UK

2.1.5. Equipment for molecular biology:

Agarose gel electrophoresis apparatus	Biometra	Niedersachsen, Germany
Analytic balance	Sartorius Analytic	Göttingen, Germany
Bio-imaging Analyzer BAS 2000	FUJIX	Tokyo, Japan
Centrifuge 5415C	Eppendorf	Hamburg, Germany
Centrifuge Sorvall RC 5C	Heraeus	Hanau, Germany
Incubator (bacteria culture) B6120	Heraeus	
Liquid scintillation system Beckman LS6000SC	Beckman	Hanau, Germany Minnesota, USA
Membrane filter (0.22 µm, 0.45 µm)	Millipore	
Osmometer	Fiske	Morlsheim, Germany
Peltier thermal Cycler PTC-200	Biozym	Norwood, USA
Power supply for the gel chamber	Appligene	Oldendorf, Germany
Quartz-cuvettes	Hellma	Illkirch , France
Quickspin™ columns, Sephadex G-50	Roche	Mühlheim, Germany

Saran film	Roth	Basel, Switzerland
Slab gel dryer: SGD4050	Appligene	Karlsruhe, Germany
Strips for the PCR	Biozym	Illkirch , France
Superfrost plus slides	Menzel Glaeser	Oldendorf, Germany
Thermomixer 5437	Eppendorf	Braunschweig, Germany
UV/ visible spectrophotometer	Appligene	Hamburg, Germany
UV Stratalinker 1800	Stratagene	Illkirch , France
Vortex: VibroFix	Janke & Kunkel-IKA	La Jolla, USA
Vertical polyacrylamide gel electrophoresis apparatus	Sigma	Heitersheim, Germany München, Germany
Whatman 3MM paper	Whatman	Madison, USA

2.1.6. Solutions and buffers:

Ampicillin	50 µg/ml H2O
Antifade solution	90% glycerol 0.02 M Tris-HCl pH-8.0 2.3% DABCO 0.5 µg/ml propidium iodide
Blocking reagent	1% in 4× SSC
Denaturation solution	1.5 M NaCl 0.5 M NaOH
Denhart Solution (x100)	2% BSA 2% Ficoll 400 2% Polyvinylpyrrolidone Store at -20°C in aliquots
DEPC H_2O	0.1% Diethyl-Pyrocarbonat in H_2O bidest 37ºC over night, autoclave
Depurinization solution	0.25 M HCl
dNTP-Mix (5 mM)	5 mM dATP 5 mM dGTP 5 mM dCTP 5 mM dTTP in TE buffer
Ear buffer	100 mM Tris pH 8.5 5 mM EDTA 200 mM NaCl 0.2% SDS
Ethidium bromide	1 mg/ml H_2O bidest

Formamide	50% in 2x SSC
Fish Water	60 mg "Instant Ocean" per liter dH2O.

Fixing solution

Fix buffer: 4% sucrose, 0.15 mM CaCl2, 0.1 M PO4 pH 7.3.
For general fixation: 1.5% glutaraldehyde, 0.5% paraformaldehyde in fix buffer.
For antibody staining: 4% paraformaldehyde in fix buffer.

GTE-buffer	50 mM Glucose 10 mM EDTA 25 mM Tris-HCl (pH 8.0)
Hybridization Buffer	0.4% SDS 8x Denhardt solution 2% Sarcosyl 8x SET
Hybridization mixture	50% formamide 10% dextran sulphate 50 mM sodium phosphate 0.1% SDS 1x Denhardt solution
in situ Staining Buffer	100 mM Tris pH 9.5 50 mM MgCl2 100 mM NaCl 0.1% Tween-20 1 mM Levamisol (add fresh
Loading buffer	40% sucrose 0.05% bromphenolblue 0.05% xylencyanol in TE buffer
Lysis buffer	50 mM tris-HCl (pH 8.0) 100 mM NaCl 100 mM EDTA 1% SDS
Neutralization solution	1.5 M NaCl 1 M tris-HCl (pH 7.0)
PBS buffer	130 mM NaCl 7 mM Na_2 HPO4 4 mM NaH_2 HPO4

PMSF	100 mM phenylmethylsulfonylfluoride in isopropanol
Pepsin solution	0.1 mg/ml in 0.01M HCl
Pronase:	5 mg/ml pronase diluted to 1 mg/ml in embryo medium
Protein Extraction Buffer	10 mM tris, pH 7.4 2% Triton-X 100 1 mM PMSF 1 mM aprotinin 1 mM leupeptin 1 mM trypsin inhibitor
PTU	0.003% 1-phenyl-2-thiourea in 10% Hank's saline
Proteinase K	10 mg/ml in H_2O bidest
Paraformaldehyde	4% paraformaldehyde in 1x PBS
RNase A, stock	4 mg/ml in H_2O bidest
SDS/Running Buffer	25 mM Tris 192 mM Glycine 1% SDS
SET (20x) pH 7.2	3 M NaCl 0.4 M tris 20 mM EDTA
Solution E1	50 mM tris pH 8.0 10 mM EDTA 100 µg/ml RNase
Solution E2	200 mM NaOH 1% SDS
Solution E3	3.1 M potassium acetate pH 5.5
SSC (20x)	3 M NaCl 0.3 M Na citrate (pH 7.0)
TAE-Buffer (50x)	200 mM tris 200 mM acetic acid 5 mM EDTA pH 8.0

TBE-buffer (5x)	445 mM tris-HCl (pH 8.0) 445 mM boric acid 10 mM EDTA
TE-buffer (1x)	10 mM tris-HCl (pH 8.0) 1 mM EDTA
TE/RNase-buffer	20 µg/ml RNase A in TE buffer
Transfer Buffer	
	20 mM Tris pH 8.0
	150 mM Glycine
	20% methanol
Washing solution	0.2x SET 0.2% SDS
X-Gal solution	50 mg / 800 µl DMSO

2.1.7. Sterilization of solutions and equipments:

All solutions, which are not heat sensitive, were sterilized at 121°C, 105 Pa for 60 min in an autoclave. Heat sensitive solutions were filtered through a disposable sterile filter (0.22 to 0.45 µm pore size). Plastic ware was autoclaved as above. Glassware was sterilized overnight in an oven at 220°C.

2.1.8. Media, antibiotics, and agar-plates:

Media for bacteria:

LB Medium (pH 7.5)	1% bacto-trypton 0.5% yeast extract 0.5% NaCl
LB-Agar	1% bacto-trypton 0.5% yeast extract 0.5% NaCl 1.5% agar

The LB medium was prepared with distilled water, autoclaved, and stored at 4°C.

Antibiotics:

Master solution for ampicillin was prepared as 50 mg/ml, sterile filtered and stored at -20°C. The antibiotic was added after the autoclaved medium has cooled down to a temperature lower than 55°C with a final concentration 50 µg/ml.

Amp / IPTG / X-Gal plates

LB-agar with 100 µg/ml ampicillin, 4 µg/ml IPTG and 50 µg /ml X-Gal was poured into Petri dishes. The dishes were stored at 4°C.

Media for cell culture

Feeder-cells medium	DULBECCO´s MEM (DMEM) 0.1 mM non essential amino acids 1 mM sodium pyruvate 10^{-6} M ß-Mercaptoethanol 2 mM L-Glutamine 10% fetal calf serum (FCS) 1 u/ml penicillin-streptomycin

Gels

1% agarose gel	1 g agarose 100 ml 1xTAE 50 µl EtBr (1 mg/ml)
2% agarose gel	2 g agarose 100 ml 1xTAE 50 µl EtBr (1 mg/ml)
5% PAA-gel (for RPA):	28 g urea 12 ml 5x TBE 480 µl APS 10 ml AA/BAA (20:1) 64 µl Temed

2.1.9. Vectors used for the cloning and sequence analysis

pGEM-T	Promega	Wisconsin, USA
pcDNA3.1(-)	Invitrogen	USA
pCS2+	Columbia university	USA

2.2. Methods

2.2.1. Animals

Different strains of zebrafish were kept in local tap water at 28 °C, and fed well under a 14:10-h light-dark photoperiod, controlled by automatic timer according to standard conditions (Westerfield 2000; The zebrafish book, ZFIN). Zebrafish strains selected to breed were put in breeding tanks for collecting fertilized eggs. The eggs were collected within 5 min after fertilization in Petri dishes with egg water.

2.2.2. Removing the chorions from the zebrafish embryos

The embryo chorions can be removed mechanically or by an enzymatic method.
First, by using only forceps (Dumont No. 5) a small hole is made in the chorion, and then it is turned upside down, so that the embryos become free from the chorion. In the second method, the embryos are treated with the enzyme pronase (2 mg/ml in egg water, 1 min, 28.5 °C). This way makes the chorion easier to remove, and then the embryos are rinsed with egg water 3 times to remove the pronase. Usually the embryos become free from their chorions during the rinse. The free embryos can be transferred using a Pasteur pipette to a Petri dishes with egg water and grow till the desired embryo stages.

2.2.3. Extraction and purification of RNA from zebrafish embryos

Zebrafish embryos and adults were anesthetized with buffered MS-222 and then appropriate amounts of embryos and adult tissues were collected and homogenized in trizol solution. Tissue homogenates were then mixed with 20% of chloroform for trizol (0.2 ml chloroform for each 1 ml trizol), shaken and vortexed vigorously for at least 15 seconds, let sit at room temperature for 10 min, then centrifuged for at least 15 min at 12000 rpm at 4°C the upper, aqueous phase (ca. 60% of total volume) was transferred to a fresh tube, by avoiding transfer of any interphase, mixed it immediately by inverting tubes 5-8 times with an equal volume of isopropanol, incubated at room temperature for 5 min and then centrifuged for 10 min at 10000 rpm at 4°C. The supernatant was discarded and the pellets were precipitated by another centrifugation, washed with 70% ethanol, dried, resuspended in nuclease-free water, and stored at -20°C. The amount and quality of the total RNA were determined by measuring the absorbance at 260 and 280 nm with a spectrophotometer.

$$[\text{RNA}] \text{ in } \mu g/\mu l = \frac{40X \text{ dilution factor} \times OD}{1000}$$

2.2.4. RT-PCR analysis

Total RNAs extracted from zebrafish tissues and embryos were treated with DNase I (Promega, Madison, WI) to remove DNA contamination. For cDNA synthesis, ≈3–5 µg of total RNA were reverse-transcribed in a final volume of 20 µl containing: 0.5 mM dNTPs, 2.5 µM oligo(dT)20, 250 ng of random primers, 5 mM dithiothreitol, 40 units of RNase inhibitor, and 200 units of SuperScript III RT (Invitrogen, Carlsbad, CA) for 45 min at 42°C, followed by a 95°C incubation for 10 min. For PCR amplification, 4 µg of cDNA were used as template in a 50-µl final reaction volume containing 0.25 mM dNTPs, 2.5 units of ExTaq polymerase, and 0.2 µM of each primer. Thirty cycles were performed for each reaction.

2.2.5. DNA transformation in bacteria

2.2.5.1. Preparation of competent E.coli bacteria

LB medium (100 ml) was inoculated with a single colony of E.coli (strain DH5α) and the culture was grown at 37° C to OD 0.6. Bacteria were centrifuged (10 min, 4°C, 3000 rpm) and the pellet was resuspended in 50 ml of sterile 50 mM CaCl2 solution (4°C) and incubated on ice for 30 min. The suspension of bacteria was centrifuged (10 min, 4°C, 3000 rpm) and the pellet was resuspended in 10 ml of sterile 50 mM CaCl2 (4°C) with 15% glycerol. The mixture was dispensed into aliquots of 80 µl and stored at -80° C.

2.2.5.2. Ligation of DNA fragments

The ligation of an insert into a vector was carried out in the following reaction mix:

30 ng vector DNA
50-100 ng insert DNA
1 µl ligation buffer (10x)
1 µl T4 DNA ligase (5 U/µl)
in a total volume of 10 µl, filled up with H2O bidest.

The ligations were carried out at 16°C overnight.

2.2.5.3. Transformation of competent bacteria

Transformation of the bacteria was done by gently mixing one aliquot of competent bacteria (80 µl) with 10 µl of ligation reaction or with 50 ng of pure plasmid DNA. The reaction was placed on ice for 30 min. In the meantime, LB plates were placed at 37°C to warm up. The cells were then heat-shocked at 42°C for 90 sec and placed on ice for 2 more min. In order to accelerate bacteria growth, 850 µl LB medium was added to the reaction which was incubated

at 37°C for 60 min with shaking. After incubation, an aliquot of 150 µl was spread on Amp/X-Gal/IPTG plate. The remaining cells were shortly centrifuged, redissolved in 100 µl LB medium and spread on another Amp/X-Gal/IPTG plate. The plates were incubated overnight at 37°C. The selection for the presence of the lacZ gene was carried out by the usual blue-white screening method (Sambrook et al., 1989).

2.2.5.4. Isolation of DNA

2.2.5.4.1. Isolation of genomic DNA from mouse tails

One cm of the tail from a mouse was incubated in 700 µl of Lysis buffer containing 35 µl proteinase K (10 mg/ml) at 55°C overnight with shaking. In the morning the samples were incubated in ice for 10 min, then mixed with 300 µl of 6 M NaCl, and kept on ice for 5 more min. After centrifugation (14 000 rpm, 4ºC, 10 min) the aqueous phase was transferred into a new tube and incubated for 15 min at 37°C with 5 µl of RNase A (4 mg/ml). DNA was precipitated by adding 1 ml of isopropanol and centrifuged (14 000 rpm, 4ºC, 15 min). The pellet was washed with 75% ethanol, dried and resolved in 100-200 µl of TE-buffer. The concentration of extracted DNA was measured. DNA was kept at -20ºC.

2.2.5.4.2. Isolation of genomic DNA from cultured cells

Genomic DNA was isolated from 10 cm culture dish with confluent cells. One ml of Ear buffer containing 1 mg/ml proteinase K were added to each dish and incubated overnight at 37°C in the CO_2 incubator. In the morning, lysates were transferred into a 2 ml eppendorf tube, DNA was precipitated with 1 ml of isopropanol, centrifuged, washed with 75% ethanol and dried. After adding of 100-500 µl of TE/RNase buffer, DNA was incubated for 1 h at 55°C to dissolve and then frozen.

2.2.5.4.3. DNA extraction from agarose gel

PCR fragments, which were used for cloning, labeling, and microinjections were gel extracted using QIAquick Gel Extract Kit (Qiagen). The extraction procedures recommended by the supplier were followed.

2.2.5.4.4. Isolation of plasmid DNA from Escherichia coli

2.2.5.4.4.1. Mini preparation

Three ml of LB medium with 50 µg/ml ampicillin was inoculated with a single E.coli colony and incubated overnight at 37°C with shaking. Two ml of this culture was centrifuged at 14000 rpm for 1 min. The pellet was resuspended in 300 µl of solution E1. Cells were lysed by adding 300 µl of solutions E2. Equal amount of solution E3 was added to the tube, and mixed immediately by inverting. Cell debris and chromosomal DNA were pelleted by centrifugation at 14 000 rpm, RT for 5 minutes. The supernatant was transferred into a new tube and 0.6 ml of isopropanol was added to precipitate the DNA. After centrifugation (14 000 rpm, RT, 10 min) and washing with 70% ethanol, air-dried pellet was resuspended in 50µl TE buffer. 2-5 µl of DNA was taken for the further digestion. DNA was kept at -20°C.

2.2.5.4.4.2. Maxi preparation

Plasmid DNA was isolated from 300 ml of night cell culture using Qiagen Plasmid Maxi Kit according to the manufacturer's instruction. The DNA was usually dissolved in 200-600 µl of TE buffer and kept at -20°C.

2.2.6. DNA sequencing

The DNA samples were submitted to automatic sequencing using the thermo sequenase fluorescent-labeled primer reaction. The sequencing was performed by Invitek (Berlin-Buch, Germany).

2.2.7. TPH2 mRNA synthesis and microinjection

The full length TPH2 cDNA was cloned in pSC2+ expression vector and fused with Myc His. The plasmid DNAs were linearized with ApaI, and were transcribed in vitro with SP6 RNA polymerase using mMESSAGE mMACHINE kit (Ambion).

Transcription reaction:

1 ug linearized DNA template
10 ul 2x NTP/CAP mix
2 ul 10x reaction buffer (Sp6)
2 ul SP6 polymerase

filled with H_2O to 20 ul total volume, incubated at 37 °C for 2h. Then, 1 ul DNase was added and incubated additional 20 min to remove the DNA template.

To purify the RNA, we used a kit (Clean up RNA, QIAGEN), and the pellet was dissolved in RNase free DEPC water, the concentration was measured and the RNA was stored at –80 °C. For mRNA injections, 50 pg mRNA was injected into 1-4-cell stage zebrafish embryos. To confirm the Myc-His-TPH2 protein synthesis in the fish embryos, we extracted the embryo proteins for Western blot using Myc-His antibody.

2.2.8. Riboprobes synthesis for in situ hybridization

The DNA fragments for zebrafish RNA probes (nucleotides 300–600) were obtained by PCR and inserted into the pGEM-T or pGEM-T Easy vector (Promega). Purified plasmids were then linearized by restriction enzyme digestion, and in vitro transcription was performed with T7 or SP6 RNA polymerase, in the presence of digoxigenin (dig)-UTP (Roche). To purify the ribo-probe, we used a kit (Clean up RNA, QIAGEN).

2.2.9. Whole mount in situ hybridization

Zebrafish embryos, ~ 5–10 individuals for each stage, were fixed with 4% paraformaldehyde overnight at 4 °C, and then washed several times with phosphate-buffered saline (PBS). Fixed samples were rinsed with PBST (0.2% Tween 20, 1.4 mM NaCl, 0.2 mM KCl, 0.1 mM Na2HPO4, and 0.002 mM KH2PO4; pH 7.4), treated with 10 µg/ml proteinase K for 5–20 min, washed with PBST several times, and then postfixed in 4% paraformaldehyde in PBS for 20 min. After a brief washing with PBST, the embryos were incubated with hybridization buffer (HyB) containing 50% formamide, 5x SSC, and 0.1% Tween 20 for 5 min at 65°C. Prehybridization was performed in HyB+, which is the hybridization buffer supplemented with 500 µg/ml yeast tRNA and 50 µg/ml heparin (Sigma) for 2-5 h at 65°C. After prehybridization, samples were incubated in 100 ng of the RNA probe in 200 µl of HyB+ at 65°C overnight for hybridization. Embryos were then washed at 65°C as follow:

1: Hyb buffer, at 67°C, 20 min (1x)

2: 50% SSCT/ 50% Formamide, at 67°C, 3x 20 min

3: 75% SSCT/ 25% Formamide, at 67°C, 1x 20 min

4: SSCT at 67°C, 2x 20 min

5: SSCT (0.2 x) at 67°C 4x 30 min

6: PBT, 67°C, 5min

After serial washings, embryos were incubated in blocking solution containing 5% sheep serum and 10 mg/ml BSA in PBST for 2 h and then incubated in 1:2,000 alkaline phosphatase-conjugated anti-dig antibody in blocking solution over night at 4 °C. After the reaction, samples were washed with PBST several times and transferred to the staining buffer. The staining reaction was held with NBT and BCIP in staining buffer until the signal was sufficiently strong. Samples were observed and documented with a binocular microscope (Leica). Images were processed with Adobe Photoshop software.

2.2.10. Immunohistochemistry

Zebrafish embryos were dechorionated and fixed in 4% paraformaldehyde for 4 h at room temperature or overnight at 4°C. After being washed several times in PBS. Embryos between 1d-5d, permeate with collagenase solution in PBS for 30 mins, and then washed several times with PBS, then the embryos were blocked for 1 h at room temperature in blocking solution (5% sheep serum, 2% BSA, 1% DMSO and 0.1% tween 20 in PBS). Embryos were then incubated with 1:100 blocking solution-diluted primary antibodies overnight at 4°C, then washed several times in washing solution (2% BSA in 0.1% tween 20 in PBS), and then incubated with 1:200 blocking solution-diluted secondary antibodies at 4°C overnight. Then embryos were washed again several times and observed and documented with a binocular microscope (Leica).

2.2.11. Alcian blue staining

For skeletal analysis, we used alcian blue dye that stains the extracellular matrix associated with chondrocytes. Anaesthetized larvae were fixed in 4% paraformaldehyde at room temperature for several hours or overnight at 4°C, and then transferred into a 0.1% solution of alcian blue dissolved in 80% ethanol 20% acetic acid. After staining in this solution overnight, embryos were rinsed in ethanol and rehydrated gradually into PBS. Pigmentation was then removed by bleaching in 3% hydrogen peroxide 1% potassium hydroxide for several hours. Stained embryos in 80% glycerol were photographed by a binocular microscope (Leica).

2.2.12. DASPEI live staining

Zebrafish embryos 2, 3, 4, and 5 days old, were immersed in 1mM DASPEI (2-(4-dimetyl-aminostyryl)-N-ethyl pyridinium iodide; Molecular Probe, Oregon) in E3 (5mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$) for 20 minutes according to Balak et al. (1990).

They were rinsed thoroughly in E3, anaesthetised with MESAB (0.5 mM 3-aminobenzoic acid ethyl ester, 2 mM Na_2HPO_4), and mounted in methylcellulose in a depression slide.

2.2.13. Tunel assay (apoptosis test)

After the fish embryos were dechorionated and fixed in 4% paraformaldehyde, they were washed several times in PBS and permeated with methanol for 30 min at –20 °C, and then rehydrated in methanol/PBT (3:1, 1:1, 1:3), 5 min each step, and then washed 5 min in PBT, and then the embryos were digested in Proteinase K (10 ug/ml) for 5 min at room temprature (for embryos older than 16 somites 10 min), and again washed several times in PBT, and refixed for 20 min at room temprature, and again washed in PBT. Then the embryos were treated with pre-chilled solution (ethanol/acetic acid 2:1), for 10 min at –20 °C, washed several times in PBT, at the end incubated in the fluorescent labelling solution for 1h at 37 °C, according to the instruction in Cell Death kit (Roche), and then rinsed 3x in PBT and observed and documented by a binocular microscope (Leica).

2.2.14. Enzyme activity assay

2.2.14.1. Phenylalanine hydroxylase (PAH)

PAH catalyzes the conversion of phenylalanine to tyrosine. The assay for the hydroxylase, we used, is based on the colorimetric determination of tyrosine by the nitrosonaphthol procedure.

Reagents

1. Phenylalanine, 20 mM
2. Potassium phosphate, 1.0 M, pH 6.80
3. Triphospho pyridine nucleotide (TPN), 2.5 mM
4. Glucose-6-phosphate, 250 mM
5. Glucose-6-phosphate dehydrogenase
6. Catalase, 2 mg/ml, 78,000 units/ml
7. 6,7-Dimethyltetrahydropterin, 1 mM, dissolved in 5 mM HCl
8. Dihydropteridine reductase
9. Enzyme fraction to be assayed.

Procedure

The reaction mixture was prepared by the addition of 0.1 ml of each solution to test tubes cooled in ice. Water was added so that the final volume will be 1.0 ml. The last two additions of 0.1 ml were the hydroxylase and the tetrahydropterin. The mixture was incubated at 25°C in open tubes for 30 min with shaking, then stopped by the addition of 2 ml of 12 % trichloroacetic acid, (TCA). A zero-time control, where the TCA was added prior to any enzymes, serves as blank. The precipitated protein was removed by centrifugation, and

tyrosine was determined on a 2.0 ml aliquot of the supernatant fluid. A tyrosine standard containing the same amount TCA as the experimental tubes was carried through each assay.

Tyrosine determination by the Nitrosonaphthol procedure

Reagents

1. 1-Nitroso-2-naphthol, 0.1%, in 95% ethanol
2. Nitric acid, 1:5, containing 0.5 mg/ml $NaNO_2$ and 10 ml of concentrated nitric acid and diluted up to 50 ml with water; this solution must be prepared every few days.

Procedure

2 ml of TCA supernatant solution was added into the tube of the stoppered reaction. Then it was mixed with 1 ml of the nitrosonaphthol solution. The tubes were incubated at 55 °C for 30 min and then cooled to room temperature. Ethylene dichloride (10 ml) was added, and mixed. The tubes were centrifuged at 200 g for 5 min, and approximately 3 ml of the clear supernatant solution were pippeted into a cuvette and the optical density was determined at 450 nm.

Definition of unit and specific activity

One unit of phenylalanine hydroxylase is defined as that amount which catalyzes the formation of 1 µmol of tyrosine per min under conditions of the assay. Specific activity is units per mg of protein.

2.2.14.2. Tryptophan hydroxylase (TPH)

TPH enzyme activity of transfected COS7 cells was determined using an adapted method as described (Gal and Patterrson; 1973), monitoring 5 hydroxy tryptophan (5-OHTrp) accumulation by HPLC in the presence of NSD1015, an inhibitor of aromatic amino acid decarboxylase in the reaction mixture. Transfected COS7 were harvested with a scraper and washed twice with phosphate buffered saline, resuspended in 75 mM Tris acetate buffer (pH 7.5), and lysed by sonication. After withdrawal of an aliquot for protein determination, the homogenates were immediately preincubated in 100 µl buffer containing 2 mg/ml catalase, 25 mM DTT, 100 µM Fe(NH4)2(SO4)2 for 10 min at 30°C in the dark. The preincubated samples were incubated at 37°C for 30 min after addition of 400 ul 15 mM Tris-acetate buffer (pH6.4) containing at final concentration of 300 µM 6-methyl-tetrahydropterin and 2 mM NSD1015. The reaction was stopped by addition of perchloric acid to a final concentration of 300 mM. 5-OH Trp concentration and TPH activity was determined using HPLC.

2.2.15. Western Blot

Preparation of the fish embryos in sample buffer:

The yolk sac of the fish embryos should be removed, because the yolk contains a high concentration of protein, by incubation of the embryos in 0.3 M sucrose for 30 min, under shaking. Then the embryos have to be dissolved in sample buffer (6 embryos/10 µl sample buffer).

Sample buffer:

- 12.5 ml 2 M Tris pH 6.8
- 4 g SDS
- 0.1 g Bromphenol blue
- 20 ml Glycerol
- 2 ml β-mercaptoethanol
- Water up to 50 ml

The sample buffer can be stored at – 20 °C for a long time.

The dissolved embryos are loaded in a SDS gel, run at 20 mA per gel until the dye front is close to the bottom, after that the proteins are be transferred to a nitrocellose membrane at 250 mA in transfer buffer for 1-4 h. The blotting membrane is incubated first with blocking buffer (5% dry milk in TBS) overnight at 4 °C or 2 h at room temperature (RT) and, next incubated with the primary antibody for 1 h in blocking buffer at RT. Then the blot is washed 3x10 min in washing buffer (TBS containing 0.1% Tween 20) with shaking. After that, the blot is again incubated with the secondary antibody conjugated with peroxidase for 1 h in blocking buffer at RT, and then washed 3x10 min in washing buffer with shaking. Then the blot is treated with ECL solution for 1 min, and exposed to X-ray film for 1-30 min to develop the picture of the protein bands.

2.2.16. Cell culture

2.2.16.1. Embryonic stem cells (ES cells)

ES cells were grown on primary mouse embryonic fibroblast feeder layer in Dulbecco modified Eagle medium (DMEM) containing 10% fetal calf serum, 100 IU/ml penicillin, 100 µg/ml streptomycin, and were supplemented with Leukaemia inhibitory factor (LIF) ESGRO® (1000 units/ml) to prevent its differentiation. The best colonies are rounded or oval in shape with a phase bright edge. ES cells were electroporated with the gene targeting construct to generate transgenic mice as described in 3.7.

2.2.16.2. COS7 cells

Cells were obtained from the American Type Culture Collection (Rockville, MD), and grown in Dulbecco modified Eagle medium (DMEM) containing 1 mg/ml glucose, and was supplemented with 10% fetal calf serum, 100 IU/ml penicillin, and 100 g/ml streptomycin.

Cells were transiently transfected with full length cDNA for the Drosophila melanogaster genes Henna or DmTPH cloned in the expression vector pcDNA3.1(+), in which gene expression is controlled by the cytomegalovirus (CMV) promoter. $Ca_3(PO_4)_2$ was used for cell transfection, and neomycin for cell selection as described by Walther et al. (2002).

2.2.17. Synthetic oligonucleotides

2.2.17.1. Primers (Biotez ;Berlin-Buch, Germany)

Symbol	Sequence (5´→ 3´)	Orientation	Ann. Temp.
1. Zebrafish:			
Tryptophan hydroxylase isoforms (TPH)			
DRTPH5	TTCAAGGACAATGTCTATCG	sense	54
DRTPH13	GGGAGTCGCAGTGTTTGATG	antisense	60
DRTPH23	TGTACCCACAATGCTTTGTC	antisense	56
TPHD25	TACCTGCAGAACCTGCCTCT	sense	60
TPHD23	AGAGAAGACCAGCCCCGTAT	antisense	60
TPH2r15	GTGTGAACTCCAAAGCAGCA	sense	58
TPH2r13	TGGTATTCCTTCCCCATCTG	antisense	58
TPH2r25	TGGTGTGAACTCCAAAGCAG	sense	58
TPH2r23	CAATCCACCCAAAACACACA	antisense	56
Aromatic amino acid decarboxylase (AAAD)			
DRAAAD15	ACCATCCTGACCGATCTGAG	sense	60
DRAAAD13	CGTACATGCGGAACACAAAC	antisense	58
DRAAAD25	GGCGATGTGGGTAAAGAAAA	sense	56
DRAAAD23	AGCAGTTCCTGAAGCAGCTC	antisense	60
Serotonin Transporter (5HTT)			
DR5HTTb5	CTTCAGCCTGTGGAAAGGAG	sense	60
DR5HTTb3	AACCCACCAAAAGTGCTGTC	antisense	58
DR5HTTa5	AACTCAATCTCTCCCGCTGA	sense	58
DR5HTTa3	CCTTCCAGACCTGCAAATGT	antisense	58
Serotonin Receptors			
DR5HT1A5	GACCTTATGGTGTCGGTGCT	sense	60
DR5HT1A3	AGGACCAGCATGAGGATGAG	antisense	60
DR5HT1B5	TTTGAAGCAGGACCTTTGCT	sense	56
DR5HT1B3	GCGCTGATGGAGTCACTGTA	antisense	60
DR5HT1E5	ACTGGCGGTGACAGATTTTC	sense	58
DR5HT1E3	GACACAGAAGGCATGCTTGA	antisense	58
DR5HT5A25	TCAGCCAGAACAGCTCCTTT	sense	58
DR5HT5A23	TCCTCCGAATACGTCTCACC	antisense	60
5HT2B5	TGGGCAGCGCTGCTCATTCT	sense	63
5HT2B3	CTTCATTGTCTGTCAACAGC	antisense	56
Danio 2B5	CGCTGCTCATTCTTCTGGTC	sense	60
Danio 2B3	GTGTTTCATAAACCGCTTTGAGT	antisense	59
DR 5HT2B5	AGAATGGCTAACGTCAGGC	sense	57
DR 5HT2B3	TAGACCAGCGGGTTGATGCC	antisense	63
DR5HT2C5	GCGCTCTCTGTCCTATTTGG	sense	60
DR5HT2C3	ACCCAACACTTTGGATGCTC	antisense	58

DR5HT35	TACAAACCCATCCAGGTGGT	sense	58
DR5HT33	GCAACAGCTTCACCACAAAA	antisense	56
rec35	ATGATGGAGAATGGGAGCTG	sense	58
rec33	CAGCGTCTTCATGGAAGTGA	antisense	58
DR5HT4b5	CCGCACTTGGTAATTTGCTT	sense	56
DR5HT4b3	GAAAGCCACAGCTGAACACA	antisense	58
DR5HT5A15	TGCTGTACGGCTAGCATTTG	sense	58
DR5HT5A13	GTTGGAGTAACCCAGCCAGA	antisense	60
DR5HT5A25	TCAGCCAGAACAGCTCCTTT	sense	58
DR5HT5A23	TCCTCCGAATACGTCTCACC	antisense	60
DR5HT65	TCCTCATTATCTCCCCGTTG	sense	58
DR5HT63	GTGGTACACACTCGCACACC	antisense	63
DR5HT75	CGTCGGAAGTTCTCAATGGT	sense	58
DR5HT73	TGCCGATGAAGATATTGCAG	antisense	56

Neural Crest Cells Markers

AP2α 5	GCTCCTCCGCTGTCTCATAC	sense	63
AP2α 3	GGATATGGAAACGGGACCTT	antisense	58
Crestin 5	CCCACACCATGAACACTCAG	sense	60
Crestin 3	CACTTGATTCCCACGAGCTT	antisense	58
Sox9b5	AGCAAAACACTCGGCAAACT	sense	56
Sox9b3	CGAAGGCCTCTATGTTGGAG	antisense	60
Foxd35	CCAAGAGCAGCCTGGTAAAG	sense	60
Foxd33	TCTGGTCCCTGAGAATGTCC	antisense	60
dlx2b5	TGGCTCGGATATGCATACAA	sense	56
dlx2b3	CTGGGTCTTCTGGAATCTGC	antisense	60
dlx6a5	TTCATGGAGTTTGGACAGCA	sense	56
dlx6a3	TTACCTGCGTCTGTGTGAGC	antisense	60
HNK15	CAAGGACATAGTAGAGGTGG	sense	58
HNK13	CTCCTCAAACAGCTCCAAGC	antisense	60
edn15	CTTTGGTGCTCCAGCATCTT	sense	58
edn13	CAGCTTGTGTTTGCATTGCT	antisense	56
endra5	AACCAGGTGTCCAGTTCAGG	sense	60
endra3	TTTCTCATCGGACAGTGACG	antisense	58
ednrb15	TTGGTGGCTTTTCAGCTTCT	sense	56
ednrb13	TTATGGCTGATCCTCGCTCT	antisense	58
Sox9a5	AAAGCGGATCTGAAACGAGA	sense	56
Sox9a3	CTGGTGGCTGTCGGAATAGT	antisense	60
Prep1.15	CGGCAAAGCCTTCTTTAGTG	sense	58
Prep1.13	TGTCTCGCTGTTCATCTTGG	antisense	58

Col2a15	TGGTAGAGATGGTGCTGCTG	sense	60
Col2a13	TCCTGCCTCACCCTTATCAC	antisense	60
Goosecoid5	CCGGTTGTGTTCTCCAACTT	sense	58
Goosecoid3	CGACGTCAGGGTATTTCGTT	antisense	58
Hoxa25	CCCAGAGTACCCTTGGATGA	sense	60
Hoxa23	CATTGTTTGCCACTTGTTCG	antisense	56
Hoxb1b5	GGACACAGCAATTTCTGCAA	sense	56
Hoxb1b3	CCTTCTCAAGTTCCGTGAGC	antisense	60

Neural Marker

Fez5	CAGGGGAGCATTATGCACTT	sense	58
Fez3	GCGCTTTCACTCCATTCTTC	antisense	58
Pax6.15	TGCTGATGCAAAAGTCCAAG	sense	56
Pax6.13	ACCATCTTGATTGGGCTGTC	antisense	58
Foxb1.25	CAGAAACCGCCGTACTCCTA	sense	60
Foxb1.23	CCCAGGTTGTAGCTGGACAT	antisense	60
Zash1a5	CGTAAACCAGCAGCAGTTCA	sense	58
Zash1a3	AGCGTCTCCACTTTGCTCAT	antisense	58
Zash1b5	GAGAAGCAGGACAGGGAGTG	sense	63
Zash1b3	CATGTGGTGAAGTCCAGCAG	antisense	60

2.Drosophila melanogaster:

Phenylalanine hydroxylase

Henna3	TTGTGAAAATGTACCAGCGGC	sense	60
Henna5	ATGAATTCTACTCAGACGCGC	antisense	60

Tryptophan hydroxylase

TPHDM5	AGTAGCCTAGCAAGTGCAGTGCAG	sense	67
TPHDM3	CATTGCTGTCCATTTCCATTCC	antisense	60

3. Mouse:

mTPH15	GCGAAGGAAGACGTTATGGA	sense	58
mTPH13	GTTCAGCCAAGAGAGGAACG	antisense	60
mTPH25	GAGCAGCAAGACAGCAGTTG	sense	60
mTPH23	GCCACAGTACTTGGTCAGCA	antisense	60
Neo1L	GTTGTGCCCAGTCATAGCCGAATAGCC	antisense	71
NTPHIn23c	GCCTAATGATGAAAGTGGGAT	sense	57

2.2.17.2. Morpholio antisense oligonucleotides (Gene Tools ; Philomath, OR 97370, US)

Foxd3 5UTR : TGCTGCTGGAGCAACCCAAGGTAAG
Sox10 5UTR : ATGCTGTGCTCCTCCGCCGACATCG
5HT2B ATG : CCTGACGTTAGCCATTCTGCCTTGG
TPH15UTR : TTGCTGATGTATAAACACGCCCTGG
TPH2 ATG :GAACATCATCATGGCAGGTTGCATC