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Phenotypic rescue of the albino mutation in the medakafish (*Oryzias latipes*) by a mouse tyrosinase transgene

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Abstract

Mutations of the tyrosinase gene are one common cause of a similar phenotype in all vertebrates, known as albinism. In an attempt to contribute to an understanding of the genetic hierarchy governing the development of pigmentation, we have used a mouse tyrosinase minigene under the control of its 5.2 kb upstream promoter region to rescue two different albino mutations in the medakafish, *Oryzias latipes*. Around hatching stages an almost perfect phenocopy of the wildtype pigmentation was obtained. Subsequent ectopic melanin overproduction indicated a possible incompatibility of the heterologous mouse promoter for stable expression during the entire ontogenesis. Like in some tyrosinase transgenic mouse lines a strong variegation effect was observed. The transgene-mediated pigmentation phenotype was obtained up to the eighth offspring generation. The phenotypic effects of the tyrosinase transgene in different albino mutant strains places the i^3-locus upstream and the *b*-locus downstream of the tyrosinase locus i^1 in the genetic hierarchy leading to wildtype pigmentation.

Keywords: Albino; Phenotypic rescue; Medaka; Tyrosinase; Transgenic fish; Pigmentation development

1. Introduction

Albinism is a pigmentation disorder which becomes apparent as a partial or total lack of the wildtype melanin coloration. Many different genetic defects can lead to albinism. They may affect the multiple steps of development and differentiation of the melanocytic lineage (Boissy and Nordlund, 1997) or the physiology of the terminally differentiated pigment cell, the classical example being a mutation in the gene encoding tyrosinase, the enzyme that produces melanin. The introduction of the mouse tyrosinase gene in albino strains of the *c*-series of alleles of mice which are tyrosinase deficient was found to overcome their deficiency and to produce wildtype pigmentation (Beermann et al., 1990; Klüppel et al., 1991; Schedl et al., 1993; Montoliu et al., 1996). This represents a classical example for the phenotypic rescue of a mutation. Such experiments are required for the ultimate functional verification of a mutant gene which in general is predicted from the lack of this function and/or the primary structural defect associated with the mutant allele. We have chosen albino medakafish for a similar experiment because the application of the transgenic technology to this model organism in combination with a successful rescue of the albino mutation should be of specific interest for developmental biological questions for many reasons. Small laboratory fish species, including the well-known zebrafish, are currently used for genetic approaches to unravel fundamental mechanisms in developmental biology and many related fields. At present a plethora of mutants affecting the diverse processes of interest are collected (Driever et al., 1994; Mullins et al., 1994; Ozato and Wakamatsu, 1994; for detailed information see special issue Development 123, 1–481, 1996). Future work will concentrate on the identification, isolation and structural characterization of the mutant genes. The further success of this genetic approach will then depend on the availability of efficient transgenic technologies, which at

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present are not sufficiently developed in fish (Iyengar et al., 1996). Stable transgenic lines rather than transient transgenics (which are always posing the problem of mosaic expression) are a prerequisite for the analysis of developmental genes especially during organogenesis and late embryonic development. Developmental genes which have been isolated via positional cloning have to be regarded as candidate genes for a given phenotype as long as their identity has not been proven in a mutant rescue experiment. In order to avoid the many complications emerging from mosaic expression of the re-introduced wildtype gene in the mutant embryo stable transgenic lines have to be established. The medaka so far is unique in the possibility of delivering foreign DNA directly to the nucleus of immature oocytes by microinjection (Ozato et al., 1986; Ozato et al., 1989), while all other techniques for producing transgenic fish work through injection into the cytoplasm of early embryos.

Using a wildtype tyrosinase gene for a mutant rescue has several applications. If the wildtype pigmentation can be restored, tyrosinase-based constructs can be used for efficient detection of gene expression in albino recipients in 'enhancer trap' strategies for the identification of developmental genes or as a marker in transposon-constructs in mutagenesis approaches. Finally, rescue of albino mutants will be useful to analyze the different developmental steps during differentiation of the pigment cell lineage.

In medaka, so far seven loci affecting melanin pigmentation have been described (Egami et al., 1990). In this study, three of those, *b*, i^1 and i^3, were used for rescuing the albino phenotype. In fish homozygous for *b*-alleles, black pigment cells are absent from the skin, however, retinal pigmentation and melanophores of the choreoidea are phenotypically wildtype. In the amelanotic skin of *b*/*b* fish tyrosinase gene expression and enzymatic activity are like in wildtype fish (Hishida et al., 1961; Inagaki et al., 1994). Nothing is known about the *b*-gene and its biological function. The two other mutants, i^1 and i^3, cause ocularcutaneous albinism. They belong to separate non-linked complementation groups, thus constituting two independent albino loci (Tomita, 1992). Some interaction between the mutant *b* and i^1 alleles has been observed, as ibR strain fish (i^1/i^1; *b*/*b*) show pale darkish pigmentation of the eye starting around the hatching stage. The dermal melanophores, however, are not pigmented at any stage. On the other hand, i^1 mutant fish of the iBR strain, which are wildtype at the *b*-locus, have no melanin at any time in any organ or tissue. No tyrosinase activity is detected in i^1/i^1 albino fish. The recessive i^1-allele phenotype was found to be due to an insertional disruption of the tyrosinase gene by the transposable element *Tol1* leading to premature termination of the transcript (Koga et al., 1995). The homozygous i^3-locus mutant phenotype is independent of the *b*-locus and does not have melanin in the retina or in melanophores at any stage of development. i^3/i^3 albinos show a weak tyrosinase activity in embryos and fry, but no activity in adult fish (Yamamoto, 1975; Egami et al., 1990; Y.H.-T. and M.S., data not shown). The genetic defect causing the i^3 mutation is unknown.

Here we report on the phenotypic rescue of the i^1 and i^3 mutation in stable transgenic medaka, produced by germinal vesicle injection using a tyrosinase minigene from the mouse under the transcriptional control of its own upstream region. The data show that the i^3 gene acts upstream and the *b*-gene downstream of i^1 in the genetic hierarchy governing pigment cell development.

2. Results

In the transient expression assay (Winkler et al., 1991; Winkler et al., 1992) cytoplasmic injection of pmTyr4 into two-cell stage embryos of i-3bR and iBR (n = 178) did not lead to any phenotypic change in pigmentation of the 102 embryos that developed to the hatching stage. This method has reliably yielded informative phenotypes with respect to the function of ectopically expressed transgenes (Winkler et al., 1994; Wittbrodt and Rosa, 1994; Oliver et al., 1996). However, in these cases strong overexpression was achieved and the expression is highly mosaic. The tyrosinase promoter is weak compared to the viral promoters used in the above studies and pigmented cells constitute only a minor fraction (much less than 1%) of the embryo. In a second approach, we therefore used injection into the germinal vesicle of premature oocytes (Ozato et al., 1986). More than 1000 oocytes were injected, of which approximately 70% ovulated, 50% were successfully fertilized and 10% developed to the hatching stage (Table 1 and data not shown). In injected embryos of H04C in this and other experiments (data not shown), no phenotypic changes were observed. However, in 18 (approximately 20%) of the surviving embryos of the albino strains (i-3bR, ibR and iBR) at around stage 28, several black pigment cells of exactly the same phenotype as wildtype melanophores appeared in the eye leading to a mosaic distribution of pigmentation (Fig. 1a) and on the head. In approximately 2000 non-injected control albino embryos analyzed in our laboratories, not a single one showed this eye pigmentation phenotype, nor was melanin pigmentation observed elsewhere during development. All pigmented fish from the injection experiment showed abnormalities like growth retardation and deformed head structures to a different degree and none was able to hatch. Injected embryos that survived after hatching, however, had no detectable melanin. Thirty-six individuals were raised to adulthood. Seven fish of the i-3bR strain and three fish of the ibR genotype that had been injected with pmTyr4 were mated to uninjected fish of the same genotype. Offspring was obtained from seven fish (Table 2). From six of the seven founder fish again some embryos at late embryogenesis (around stage 28) showed mosaic eye pigmentation. The phenotype of these matched the pigmented F_0 animals. In addition, some embryos developed very pronounced pigmentation

Table 1
Survival of embryos and number of pigmented F_0 fish after pmTyr4 injection

Strain	Number of oocytes			Number of embryos			Number of embryos with pigmented eyes[a]	Number of fish (adults)[b]
	Injected	Ovulated	Fertilized	At stage 19	At stage 26	At hatching		
i-3bR	189	143	112	48	36	16	6	7
ibR	191	154	88	29	19	12	2	6
iBR	279	181	140	77	40	20	4	7
H04C	30	23	18	18	10	9	–[c]	6
Total	689	501 (73%)	358 (52%)	172 (25%)	105 (15%)	57 (8%)	12 (2%)	26 (4%)

[a]Mosaic pigmentation of the eye, abnormal development, lethal.
[b]No phenotypic effect on pigmentation observed.
[c]Because in this genotype the retina is pigmented, a phenotypic effect of pmTyr4 would be undetectable.

of the whole body (Fig. 1b). In i-3bR embryos at hatching stage, black pigment cells appeared on the dorsal head region and on the dorsal midline of the trunk. These melanophores were of slightly less dentritic shape and sometimes fewer in number if compared to that of wildtype embryos. Shortly thereafter, a diffuse black pigmentation was seen dispersed over the body and the blood became completely black. In ibR embryos, the eyes were not mosaic but uniformly black like in wildtype embryos. The number of skin melanophores appeared to be slightly smaller compared to i-3bR embryos. Some ibR embryos also progressed through wildtype body pigmentation, hyperpigmentation and black blood. Some embryos were able to hatch, but all pigmented embryos eventually died within 1 or 2 days after hatching (Fig. 2).

Histological analysis confirmed that at the onset of melanin pigmentation in the transgenic embryos pigmented cells appeared only at those locations where pigment cells are found in wildtype embryos and hatchlings, namely the retina, chorioidea and the integument. At later stages of development, however, large pigment loaded cells appeared at ectopic sites, e.g. the somites, the ventral mesenchyme, or in the developing fin (Fig. 3).

As in F_0, all surviving F_1 fish were unpigmented. When those fish were bred further (F_2–F_8, or outcrossed to parental albino strains), the same phenomena were observed (Table 3, Fig. 2). A representative crossing experiment is given in Table 4.

Southern blot analysis of F_0 founders (approximately 10 months old) revealed large but varying amounts of the transgene DNA in several tissues. The expected restriction fragments of the injected DNA were detected (Fig. 4a). The fate of the transgene construct was followed by PCR analyses (Fig. 4b). All F_0-fish tested as adults (at least older than 3 months of age) were positive for the presence of the foreign DNA (fish A, muscle; fish C, muscle and liver; fish D, muscle and ovary; fish I, muscle, testes and liver) although no melanin pigmentation was observed. Similarly in non-expressing F_1 (14 of 20 tested) and F_2 (three of four tested) the transgene was present in adults and in pigmented as well as non-pigmented progeny.

In summary, of the seven fertile F_0 albino fish (out of 36 grown to adulthood) used for genetic analysis six had pigmented offspring. The frequency ranged from 1.2 to 57%. Approximately half of the $F_1 \times F_1$ crossings (range 40–65%) yielded F_2 sibships with pigmented embryos with frequencies from 0.7 to 14.6%. Expression of the transgene has so far been observed up to the eighth generation.

3. Discussion

Injection of a mouse tyrosinase minigene under the control of its own regulatory sequences led to a phenotypic rescue of the albino mutation in ibR and i-3bR medakafish during late embryogenesis. Pigmentation of the eyes and the appearance of melanocytes/melanophores in the embryonic integument was evident like in wildtype medaka. Slight phenotypic differences in the transgenics such as a fewer number of pigmented cells and a later onset of pigmentation may be either due to the genetic background (e.g. the *R*-locus) or the heterologous origin of the promoter.

The phenotypic effects obtained after expression of the mouse tyrosinase in the different pigmentation mutants of the medaka give some information on the genetic network determining pigmentation. Reintroduction of a functional tyrosinase gene, although of heterologous (mouse) origin, restored the normal melanin production and led, at least for the period around hatching, to a wildtype pigmentation phenotype in i^1/i^1 embryos which have a disrupted tyrosinase gene (Koga et al., 1995). This is comparable to the phenotypic effect of the same construct in transgenic albino mice (Beermann et al., 1990; Klüppel et al., 1991). No effect was obtained in the *b*-mutant (H04C strain). Thus, the *b*-locus must act downstream of the tyrosinase-encoding albino gene, i^1, consistent with normal levels of tyrosinase enzyme activity in this mutant. The i^3 mutant was rescued by tyrosinase transgene expression in an almost similar fashion as the i^1 mutant. As the i^3-locus is non-allelic and non-linked to i^1, it obviously acts upstream of i^1. The hypothesis that i^3 might encode a regulator for i^1 is supported by the fact that some basal tyrosinase activity is still found in i^3/i^3 embryos, probably as a result of leakiness of the i^3 mutation.

The overproduction and ectopic expression of melanin

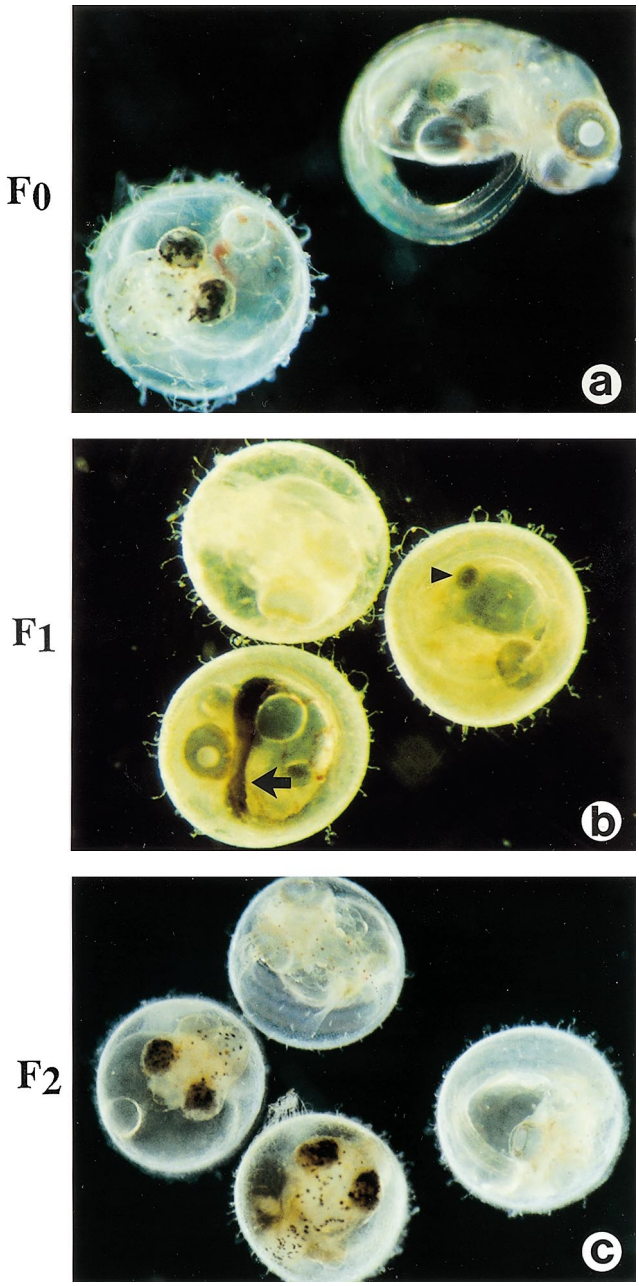

Fig. 1. (a) Control albino embryo (right) and injected (F_0) embryo (left), at the hatching stage (8 days of development). The injected embryo exhibits scattered melanophores on the dorsocranium and mosaic pigmentation of the eye. (b) Non-pigmented (upper left) and pigmented F_1 embryos, with ectopic melanin in the gall bladder (arrowhead) and the pericard (arrow). In the pigmented embryos the eyes are fully pigmented. (c) Non-pigmented and pigmented F_2 offspring embryos.

observed in later stages of development is most likely due to the non-homologous regulatory sequences and the adjacent mouse genomic DNA contained within plasmid pmTyr4. It has been shown that as little as 270 bp of 5′-flanking sequence of the mouse tyrosinase gene is sufficient to give a correct temporal and spatial expression pattern in transgenic albino mice (Klüppel et al., 1991), but so far unidentified distant DNA-elements interact with the proximal regulatory sequence (Porter and Meyer, 1994) thereby modulating the intensity of the pigmentation phenotype. From other studies on transgenic fish there is accumulating evidence that the correct expression of genes that normally are under a fine-tuned differential regulation regime may only be obtained using the homologous control regions (see Iyengar et al., 1996). After the recent cloning of the medaka tyrosinase cDNA (Inagaki et al., 1994) and parts of the transcribed region of the genomic locus (Koga et al., 1995), such sequences may be available in the near future. It can be expected that a full and stable rescue of the albino mutation in medaka will be reached if the homologous sequences are used for gene transfer. Thereby all problems arising from phenomena which are connected to activities of the mouse promoter not faithfully reproducing the activity of the endogenous promoter will be circumvented. This would add fish to the list of vertebrates where the tyrosinase gene is a useful marker for the identification of transgenic animals.

A totally unexpected finding was the phenotypic variation in all transgenic lines and the observation that non-expressing albino fish gave rise to expressing offspring over many generations. This phenomenon, however, made it possible that the transgene could be transmitted despite the lethality of ectopic melanin overproduction in later stages of development and that it was not totally ‘diluted out’ after a few generations. Variegated expression of transgenes and interindividual differences in transgene expression are known phenomena in mice (Martin and Whitelaw, 1996). Our findings are reminiscent of those in stable transgenic lines of black nonagouti mice carrying a tyrosinase promoter/SV40E transforming gene and of genetically albino mice with a metallothionein promoter/mouse tyrosinase gene construct (Bradl et al., 1991; Mintz and Bradl, 1991) or the human tyrosinase gene (Porter and Meyer, 1994). In some stable lines, in the retina, choroidea and the skin, mosaic coloration and interindividual differences in the degree of transgene expression were seen, while other lines were phenotypically uniform for many generations. This clonal variation in gene expression within the same animal and between genotypically extremely uniform animals was explained as the result of unstable transgene expression as a consequence of transgene integration into particular chromosomal regions. Such regions may be inheritedly prone to destabilize the function of a linked gene possibly affecting events in establishing chromatin configuration, methylation, splicing of transcripts, or other processes within the same tissue or even between different individuals of the same genotype. This view is supported by mosaic expression of specific autosomal genes, e.g. in the intestine of the rat (Rubin et al., 1989) or in transgenic mice (Sweetser et al., 1988). As an alternative explanation for the phenomenon that we observed, we cannot exclude that at least some of the injected DNA was not integrated into the host DNA and transmitted extrachromosomally. Unequal somatic distribution could then also result in mosaic expression. However, the reappearance of this phenomenon in

Table 2
Number of offspring and pigmentation of embryos of medaka injected with pmTyr4

Individual	Genotype	Sex	Number of		Number of embryos with	
			Fertilized eggs	Normally hatching fry	Pigmented eyes	Pigmented body
A	i-3bR	M	0	–	–	–
B	i-3bR	F	465	193	102	8
C[a]	i-3bR	F	191	119	19	1
D[a]	i-3bR	F	543	467	10	1
E	ibR	M	403	400	–[b]	0
F	ibR	F	172	83	–[b]	1
G	ibR	F	40	24	–[b]	1
H	i-3bR	M	0	–	–	–
I	i-3bR	M	1134	792	10	1
J	i-3bR	M	0	–	–	–

[a]Malformation of the eyes (iris closed).
[b]No observation.

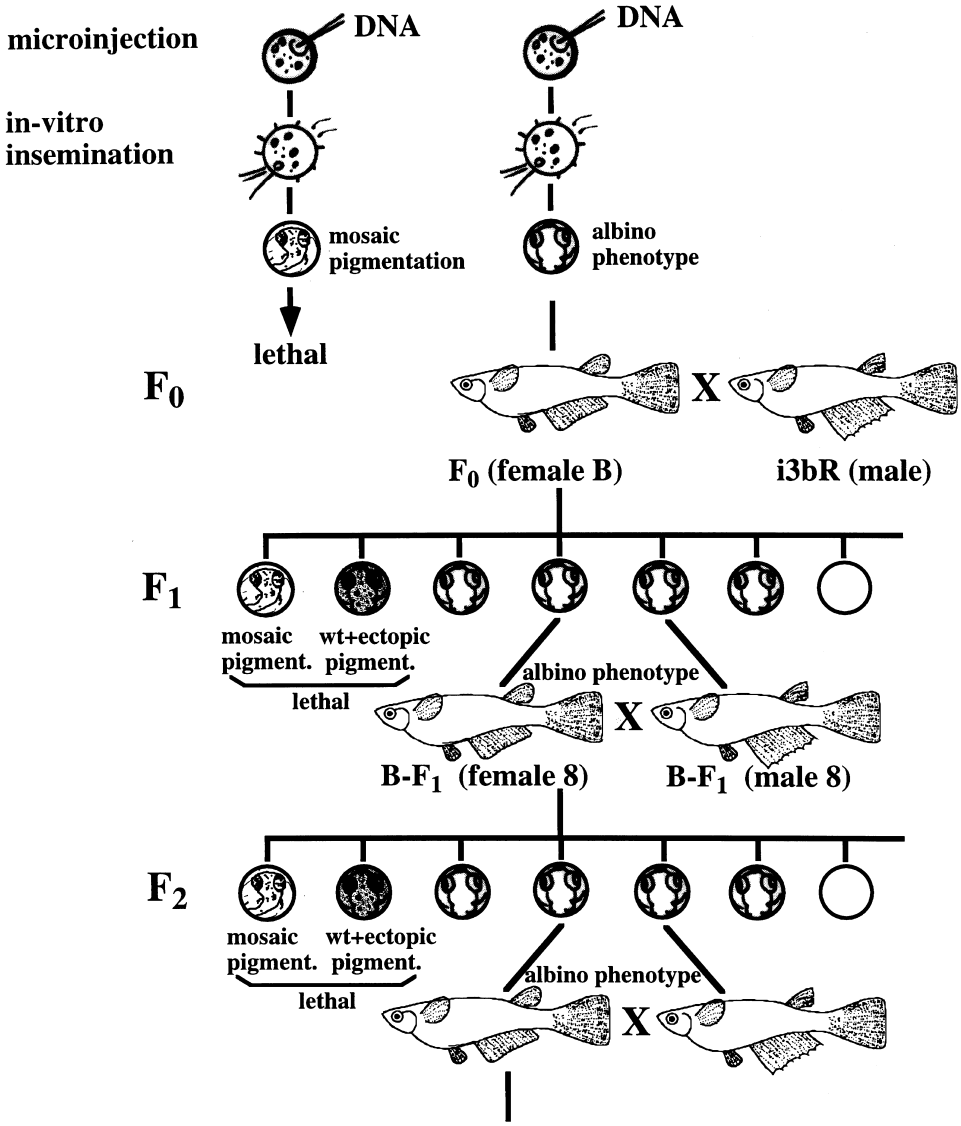

Fig. 2. Schematic representation of the production of transgenic albino medakafish and reappearance of pigmentation in succeeding generations. For details see text.

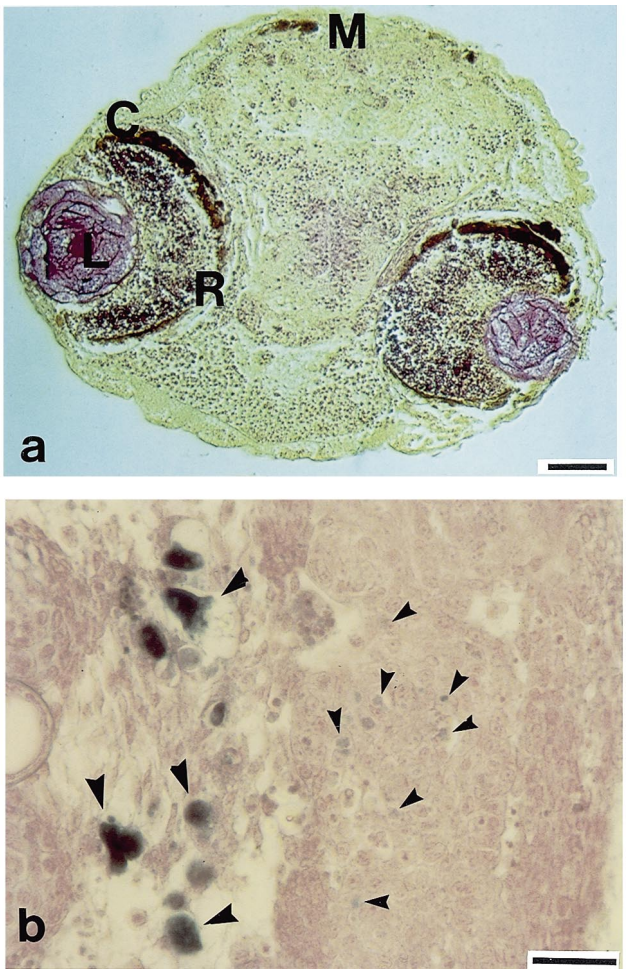

Fig. 3. Histological analysis of transgenic albino medaka embryos exhibiting different degrees of melanin pigmentation. (a) Transversal section of the head of a transgenic embryo showing melanophores (arrowheads) in the retina (R), chorioidea (C) and the meninx (M). L, lens; B, brain. HPT stain, bar represents 50 μm. (b) Transversal section of the trunk region showing ectopic expression of melanin in the ventral mesenchyme. Blue stained cells contain either melanin (large arrowheads) or melanin precursors (small arrowheads). Schmorl stain, bar represents 20 μm.

succeeding generations up to F_8 makes this explanation unlikely.

In a comparable attempt to rescue a mutant phenotype in medaka, Matsumoto et al. (1992) used a similar mouse tyrosinase cDNA under the control of its own regulatory region. As recipient the orange-red variety of medaka corresponding to the H04C strain used in our study was employed that lacks melanin-containing cells in the skin. Fish were obtained that exhibited the full wildtype pigmentation and were viable. Surprisingly, the transgenic founder female was not mosaic in soma or germline as seen in all other stable transgenic experiments with medaka performed so far (Ozato et al., 1989; Kinoshita et al., 1996; Toyohara et al., 1996). The phenotypic result was also unexpected as the orange-red variety of medaka is due to a mutation that does not affect tyrosinase on the mRNA (Inagaki et al., 1994) or the protein/enzyme level (Hishida et al., 1961; Hirose and Matsumoto, 1993). In our experiments we did not observe any phenotypic effects after injection of the tyrosinase construct into embryos of the orange-red variety. In the tyrosinase-negative albino medaka, however, a transient phenotypic rescue of the wildtype pigmentation was obtained that was accompanied by mosaic expression, F_0 germline mosaicism and an absolute lethality at the latest at larval stages of the expressed transgene. This was obviously due to the high levels of toxic melanin metabolites.

Two other studies so far have been concerned with experiments to rescue mutant phenotypes in small laboratory fish. In the zebrafish, transient DNA expression from injected DNA of bone morphogenetic protein 4 (BMP 4) only partially rescued a mutation that affects dorsoventral patterning, while injection of RNA was sufficient to fully restore the wildtype phenotype in a related mutant affecting the same process (Hammerschmidt et al., 1996). In medaka a transgenic line was established where the gulonolactone oxidase was introduced to rescue the metabolic deficiency caused by the absence of this enzyme activity in wildtype fish (Toyohara et al., 1996). Although enzyme activity could be detected in transgenic fish, the biochemical phenotype, namely production of ascorbic acid, could not be rescued.

Table 3

Summary of matings of F_1 fish and number of pairs that produced pigmented embryos in F_2 or backcross generations

Mating group	Number of pairs tested	Number of pairs that produced embryos with	
		Mosaic eye pigmentation	Pigmented body
$BF_1 \times BF_1$	12	5	2
$CF_1 \times CF_1$	20	13	7
$DF_1 \times DF_1$	13	–[b]	3
$BF_1 \times P$[a]	4	1	1
$DF_1 \times P$[a]	10	4	2

[a]Backcross to non-injected fish of the same genotype.
[b]No observation.

Table 4

Number of embryos with pigmented eyes and body in F_2 sibling from transgenic fish B

$BF_1 \times BF_1$ crossbreed no.	Number of fertilized embryos	Number of embryos with	
		Pigmented eyes	Pigmented body
7	61	0	0
8	295	13	6
9	145	1	0
10	132	1	0
11	154	0	0
12	226	21	12
13	152	0	0
14	190	2	0
15	60	0	0
16	27	0	0
17	113	0	0
18	34	0	0

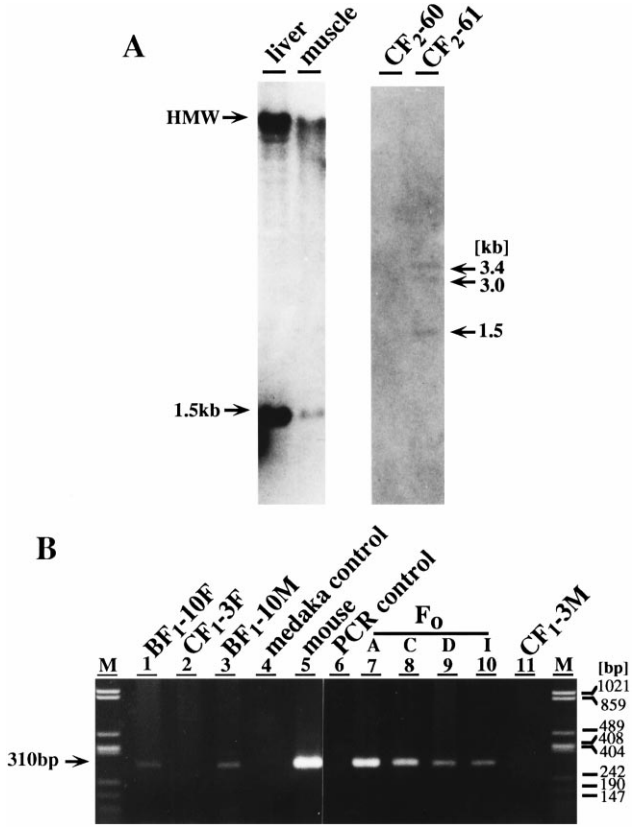

Fig. 4. (a) Southern blot analysis of F_0 fish A and two F_2 fish. *Xba*I digested DNA was hybridized with the pTyrCAT (–3.7) probe. The 1.5 kb band represents the internal *Xba*I fragment from pmTyr4 and the HMW band from F_0 contains the flanking vector sequences. The additional bands in the F_2 embryo 61 might represent junction fragments or rearranged sequences. (b) PCR analysis of adult F_0 fish and F_1 embryos with the mouse tyrosinase gene specific primers Tyr3/Tyr4. Lanes 1–3 and 11, F_1; lanes 7–10, F_0; lane 4, non-injected medaka (Carbio strain); lane 5, mouse (BalbC strain); lane 6, PCR reaction control without template DNA.

The possibility of obtaining a phenotypic rescue of a mutant adds to some other recent developments in medaka like the so far unique possibility of producing stable transgenic lines via DNA transfer to the oocyte nucleus (Ozato et al., 1989; Kinoshita et al., 1996) and the early embryo (Toyohara et al., 1996), the establishment and long-term propagation in vitro of embryonic stem cell-like tissue cultures (Wakamatsu et al., 1994; Hong and Schartl, 1996; Hong et al., 1996) and the isolation of an actively transposing mobile element (Koga et al., 1996). Thus, the medaka-fish may be a useful system for the in vivo manipulation of gene function and the experimental modulation of a phenotype by ectopic gene expression.

4. Experimental procedures

4.1. Experimental animals

All medaka fish were maintained in the aquarium of the NIRS and the Biocenter Würzburg under standard conditions (Hyodo-Taguchi and Egami, 1985). The strains that were used in this study are given in Table 5.

Founder fish of the i-3bR, ibR and iBR (Egami et al., 1990) strains were obtained from Dr Tomita of Nagoya University and given to the NIRS. They were maintained by mass mating as a closed colony.

4.2. Plasmids

For microinjection the pmTyr4 construct (Beermann et al., 1990) was used. pmTyr4 contains a mouse tyrosinase minigene consisting of 5.2 kb 5′ upstream sequences, exon 1, intron 1 and exons 2–5 attached to SV40 splice and polyadenylation sites with the 66 bp small T intron cloned into a modified Bluescript vector (Stratagene). pTyrCAT (–3.7) (Klüppel et al., 1991) was used to generate a hybridization probe from the mouse tyrosinase promoter to avoid cross-hybridization with the medaka tyrosinase coding sequence. The 2.05 kb *Hind*III fragment was isolated which contains a 1.5 kb *Xba*I fragment.

4.3. Microinjection

Microinjection into the germinal vesicle was performed essentially as described (Ozato et al., 1989). Plasmid DNA for injection was extracted and purified by caesium chloride density gradient centrifugation (see Winkler et al., 1991). Undigested plasmids were diluted to a final concentration of 10 μg/ml in 0.1 mM Tris, 0.01 mM EDTA and 20–30 pl were injected into each germinal vesicle at a constant flow rate. In vitro maturation, in vitro insemination and embryo culture were done according to Ozato et al. (1986, 1989). Embryo stages are according to Iwamatsu (1994).

4.4. DNA analysis

DNA from adult fish was obtained as described (Schartl, 1988) and DNA from pooled or single embryos was extracted with the addition of carrier DNA from adult non-transgenic medaka (Carbio strain) according to Winkler et al. (1991). For Southern analysis 5 μg of DNA was

Table 5
Strains used in this study

Strain	Phenotype	Genotype[a]	Tyrosinase activity
H04C[b]	Orange-red	(*b*/*b*; *R*/*R*)	Wildtype
ibR	Albino	(i^1/i^1; *b*/*b*; *R*/*R*)	No activity
iBR	Albino	(i^1/i^1; *B*/*B*; *R*/*R*)	No activity
i-3bR	Albino	(i^3/i^3; *b*/*b*; *R*/*R*)	Very low in embryo, no activity in adults

[a]Nomenclature of *i*-alleles according to Hori; see medakafish homepage on the World Wide Web (http://biol1.bio.nagoya-u.ac.jp:8000/).
[b]H04C (Hyodo-Taguchi and Egami, 1985), an inbred strain of the orange-red variety, is homozygous recessive at the *b*-locus. The *R* alleles govern the deposition of orange-red pigmentation in xanthophores (Yamamoto, 1975) giving these fish a light yellow pigmentation.

digested with *Xba*I and hybridized with the 2.05 kb *Hind*III fragment of pTyrCAT (–3.7) under conditions of high stringency (50% formamide, 42°C). Washing conditions were 0.1 SSC, 1% SDS at 68°C. For amplification of a transgene-specific fragment, the following oligonucleotides were used according to Beermann et al. (1990): TYR1 (5′-GAG CCT TAC TTG GAA CAA GCC-3′) located at position +1475 of the tyrosinase sequence (Müller et al., 1988); and TYR2 (5′-CTG CTC CCA TTC ATC AGT TCC-3′) derived from the SV40 small T antigen and located at position +4522 of the published sequence (Buchman et al., 1980). The fragment size generated by PCR using these primers was 515 bp. A second pair of primers, TYR3 (5′-CAG GCA GAG GTT CCT GCC AG-3′) and TYR4 (5′-GTG GGG ATG ACA TAG ACT GA-3′) gave rise to a 310 bp fragment from the mouse tyrosinase gene. PCR was done with 50 pM of each primer for 35 cycles consisting of 1 min denaturation at 92°C, 1 min annealing at 59°C for TYR1/2 and 60°C for TYR3/4 and 1 min extension at 72°C. In cycle 1, time 1 was 2 min and in cycle 35, time 3 was 4 min. For negative control, either no DNA was added to the reaction tube or DNA from non-injected medaka (strain Carbio) and *Xiphophorus maculatus* (strain Rio Jamapa, *Mdl*^{Sp}-X*mrk* = *Tu-Sp*) was used. In positive control reactions genomic DNA from BalbC mice (for primers TYR3/4) and plasmid pmTyr4 (for primers TYR1/2) was used.

4.5. Histological analysis

Embryos were fixed in Bouin's fluid, dehydrated and embedded in paraffin. Thin sections were stained with haematoxylin/phloxine/tartracine (HPT). For detection of melanin precursors the ferri-ferrocyanide staining procedure according to Schmorl was used and the sections were counterstained with nuclear fast red (see Romeis, 1989).

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