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Production of germ-line chimeras in zebrafish by cell transplants from genetically pigmented to albino embryos

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ABSTRACT **To determine whether embryonic cells transplanted from one zebrafish embryo to another can contribute to the germ line of the recipient, and to determine whether pigmentation can be used as a dominant visible marker to monitor cell transplants, we introduced cells from genetically pigmented (donor) embryos to albino recipients at midblastula stage. By 48 hr many of the resulting chimeras expressed dark pigment in their eyes and bodies, characteristics of donor but not albino embryos. By 4–6 weeks of age pigmentation was observed on the body of 23 of 70 chimeras. In contrast to fully pigmented wild-type fish, pigmentation in chimeras appeared within transverse bands running from dorsal to ventral. Pigmentation patterns differed from one fish to another and in almost every case were different on each side of a single fish. At 2–3 months of age chimeras were mated to albino fish to determine whether pigmented donor cells had contributed to the germ line. Of 28 chimeric fish that have yielded at least 50 offspring each, 5 have given rise to pigmented progeny at frequencies of 1–40%. The donor cells for some chimeras were derived from embryos that, in addition to being pigmented, were transgenic for a *lacZ* plasmid. Pigmented offspring of some germ-line chimeras inherited the transgene, confirming that they descended from transplanted donor cells. Our ability to make germ-line chimeras suggests that it is possible to introduce genetically engineered cells into zebrafish embryos and to identify the offspring of these cells by pigmentation at 2 days of age.**

We wish to develop methods for performing insertional mutagenesis in zebrafish. An important tool for such work would be an equivalent of the mouse embryonic stem (ES) cell (1, 2). ES cells are totipotent cells that can be cultured, genetically altered, and then returned to a mouse blastocyst, where they can contribute to all tissues of the resulting chimera, including the germ line. With the use of ES cells that differ genetically in pigmentation from the recipient, chimeras with contributions from the ES cell can be recognized visually by their coat color, as can their offspring that are descendants of the ES cells. The ability to make such germ-line chimeras provides the basis for performing targeted and promoter trap mutagenesis in mice (3–9).

As an important step toward developing an ES-cell equivalent in zebrafish, we wished to determine whether cells from blastula-stage embryos could be transplanted from one embryo to another and participate in forming the germ line of the recipient. We also wished to determine whether pigmentation could be used as a marker to follow descendants of the transplanted cells. Pigmentation would be a particularly useful marker for this purpose in zebrafish, because heavy pigmentation is visible in the eyes and in melanocytes of wild-type fish as early as 1½ to 2 days after fertilization, making pigmented fish readily detectable under a dissecting microscope or even by eye at this stage. Streisinger and colleagues (10, 11) have shown the usefulness of pigmentation as a marker in zebrafish for establishing conditions of γ-ray mutagenesis and for studying cell lineage in the eye.

Kimmel and colleagues (12, 13) have reported success in transplanting undifferentiated cells from one zebrafish embryo to another. Previously, donor cells were marked by injecting fertilized eggs at the one-cell stage or later with fluorescein dextran; several hours later the labeled cells were transplanted from blastula-stage embryos to unlabeled embryos of comparable age. By fluorescence microscopy the donor cells can be seen to participate in normal development over the next few days. We have performed similar experiments using donor cells from genetically pigmented embryos and albino recipients. Albino (*alb-1*) is a spontaneous, recessive mutation of zebrafish whose biochemical basis is not known (14, 15). Whereas wild-type fish acquire heavy pigmentation in their eyes and acquire pigmented melanocytes along the body starting at about 26 hr after fertilization at 28°C, *alb-1/alb-1* fish remain clear until about 2½ to 3 days of age, when they acquire light brown coloration in their eyes and to a limited extent along the body. Adult albinos have light red eyes and pale bodies, while wild-type zebrafish have dark eyes and dark horizontal stripes.

In this report we describe the preparation of chimeras between blastula-stage genetically pigmented and albino zebrafish embryos, the appearance of chimeras at 2–3 days and at 1–3 months of age, and the ability of the genetically pigmented donor cells to contribute to the germ line in chimeras. We discuss the potential of these findings for the development of methods to perform insertional mutagenesis in zebrafish.

MATERIALS AND METHODS

Fish. We obtained an albino (*alb-1/alb-1*) female zebrafish from C. Nüsslein-Volhard (Max-Planck-Institut fur Entwicklungsbiologie, Tubingen, Federal Republic of Germany), mated it to an outbred wild-type fish, also from C. Nüsslein-Volhard, and then bred the F_1 heterozygotes to obtain a good breeding stock of *alb-1/alb-1* fish. Recipient embryos were obtained from matings of *alb-1/alb-1* fish. A number of pigmented donors were used, including our strain of wild type and eggs derived from transgenic lines. The latter were generated following injection of a plasmid containing *lacZ* driven by the Rous sarcoma virus (RSV) long terminal repeat, into fish of mixed genotypes (16). The donor embryo for one of the germ-line chimeras (5-5-3) was derived from a mating between a striped, pigmented, homozygous transgenic male (1/1-1) of transgenic line M9-1 (ref. 16; P. Culp and N.H., unpublished work) that was homozygous for a single integration event (or possibly linked integrations) of the RSV–*lacZ* plasmid, and a nontransgenic female. Thus the donor

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Abbreviations: ES cell, embryonic stem cell; RSV, Rous sarcoma virus.

cells used to make this chimera should be heterozygous for the transgene. The donor embryos for the other germ-line chimeras were derived from a mating of two pigmented, striped, heterozygous transgenics of line M14 (16). None of the fish involved in these experiments were from inbred lines. Fish were raised and maintained as described previously (16) and essentially as described in the Zebrafish Book (17).

Microinjection and Cell Transplantation. Eggs were collected (16) and dechorionated (17) in Holtfreter's solution. In some cases eggs that were to serve as donors were microinjected with a mixture of fluorescein dextran and phenol red (16, 18). Cell transplants were performed on embryos at the midblastula stage (about 1000–2000 cells). Cell transplants were done with a Pasteur pipette whose tip had been pulled in a flame to achieve a bore size of 2–3 cell diameters, and most were performed by hand. A few were performed with the aid of a micromanipulator. Donor cells were loaded by suction from a donor specimen and then injected among the deep cells of a recipient blastoderm at approximately the 1000-cell stage, without damage to the yolk cell. As few as 20 or as many as about 100 cells were injected into a single embryo in different experiments, under a dissecting microscope.

DNA Isolation, PCR, and Southern Blotting. Individual fish were homogenized in 4 M guanidinium isothiocyanate/0.25 mM sodium citrate, pH 7.0/0.5% *N*-lauroylsarcosine/0.1 M 2-mercaptoethanol. This buffer was initially used by Chomczynski and Sacchi (19) for RNA isolation, but we have found that it is also useful in DNA preparation. The sample was extracted once with phenol/chloroform/isoamyl alcohol (25:24:1, vol/vol), and total nucleic acid was precipitated by addition of 3 volumes of ethanol and 0.1 volume of 3 M sodium acetate (pH 5.5). The pellet was dissolved in TE (10 mM Tris/1 mM EDTA, pH 8.0) containing RNase A (100 μg/ml) and incubated at 65°C for 30 min. The sample was again extracted with phenol and precipitated with ethanol as above. The DNA was dissolved in TE.

Approximately 0.5 μg of DNA was used in a PCR as described (20). Primers were homologous to sequences within the RSV long terminal repeat and yielded a PCR product of 386 base pairs. For Southern blot analysis, total DNA (2 μg) was digested, electrophoresed, and blotted onto Hybond-N^+ membrane (Amersham). The blots were prehybridized for 1 hr at 68°C in QuikHyb solution (Stratagene) and hybridized for 2 hr in the same solution with a random-primer-labeled plasmid DNA (13.5 kilobases) containing RSV–*lacZ* sequences (16, 21). This probe detects a major band at about 16 kilobases in *Pst* I digests of DNA from transgenic fish of the M9-1 line. M9-1 was derived from founder M9, which had three integrations of the transgene. M9-1 has the single integration represented by band 1 in the paper of Culp *et al.* (16).

RESULTS

Preparation of Chimeras. The plan of the experiment for generating chimeras is outlined in Fig. 1. Eggs were collected almost simultaneously from genetically pigmented fish and from albino (*alb-1*/*alb-1*) fish and dechorionated with Pronase. In some cases, embryos to be used as donors were injected with fluorescein dextran at the 1-cell stage. Cell transfers were performed at the 1000- to 2000-cell stage. The number of donor cells injected into recipient embryos ranged from about 20 to roughly 100. Survival rates for chimeras and also for dechorionated controls were variable; however, in some batches, survival of chimeras was close to 100%. Altogether we performed 418 transplants on five different days; 135 operated embryos survived and appeared essentially normal at 2 days of age, and 70 survived to sexual maturity.

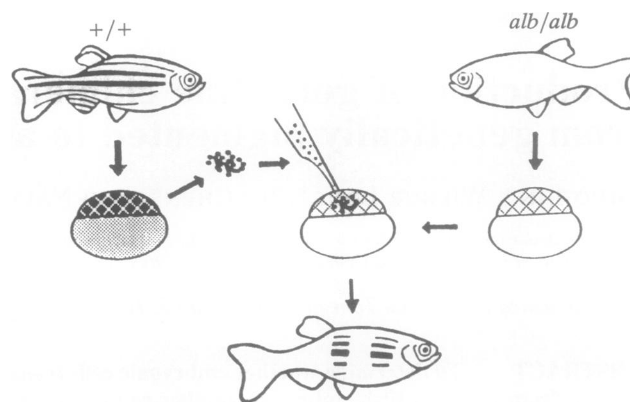

FIG. 1. Diagram of experiment for making zebrafish pigmentation chimeras.

In experiments where donor cells were labeled with fluorescein dextran, we monitored their presence in chimeras by fluorescence microscopy. Fig. 2 shows the appearance of several embryos about 24 hr after receiving a transplant of fluorescein dextran-labeled cells. As expected, transferred cells participated in various lineages.

Appearance of Pigmentation in Chimeras. By 2 days postfertilization, when pigment is clearly visible in the eyes and on the bodies of wild-type fish but not in albino embryos, we observed variegated pigmentation in chimeras. About 85% of the 135 operated embryos possessed pigmentation. It occurred frequently in the eyes, as dark spots on the head, and in some cases in melanophores on the trunk and tail. Fig. 3 shows pigmentation in the eyes and heads of wild-type, albino, and chimeric embryos at 3 days postfertilization.

We raised 70 chimeric fish along with control dechorionated embryos from experiments performed on five different days. Chimeras with one dark and one light or red eye could be seen at all swimming stages. Between about 4 and 8 weeks of age, we began to observe bands of coloration on the trunk and tail of the chimeras. Fig. 4 shows albino and pigmented fish at this stage (*a* and *b*), both sides of two chimeric fish (*c* and *d*), and the pigmented side only of six additional chimeras all of which possessed pigment on just one side of their bodies

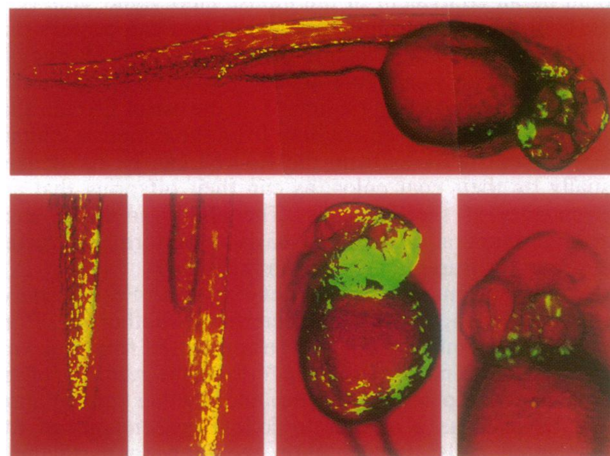

FIG. 2. Participation of transplanted cells in chimeras by 24 hr. Eggs that were to serve as donors were microinjected with fluorescein dextran. After incubation at 27°C overnight the chimeric embryos were examined and photographed separately under the Nomarski channel and fluorescein channel of a confocal microscope (Bio-Rad). The two images were merged and the fluorescent donor cells were shown as green-yellow pseudocolor. Length of the full embryo is about 1.6 mm.

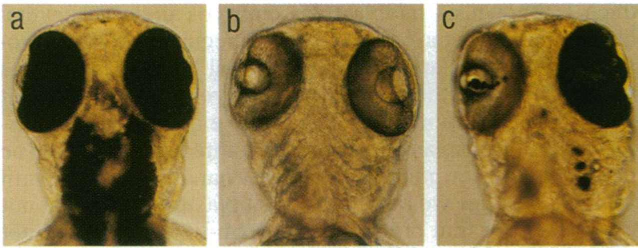

FIG. 3. Pigmentation in a 3-day-old chimera. After incubation at 26–27°C for 3 days, wild-type (*a*), albino (*b*), and chimeric (*c*) embryos were examined and photographed with a Nikon microscope. Size of the fish head is about 500 μm.

(*e–j*). Altogether, 23 of 70 fish had dark pigmentation on their trunk and or tail that could be seen by eye in swimming fish. Some also had dark pigmentation on the head, while yet other fish had pigment only on the head but not on the trunk or tail.

In wild-type fish, dark horizontal stripes alternate with light stripes. Although albino fish do not have dark stripes, they clearly have colorless horizontal stripes. In chimeras, in the regions of the transverse bands, coloration occurs in the normal striped pattern (see Fig. 4). Some of the transverse bands containing dark pigmentation in chimeras are narrow, others wide. If one measures the width of the narrowest bands and the potential length of the midflank stripe, one finds that about 21 narrow bands would be needed to completely fill in the stripe along the length of the fish.

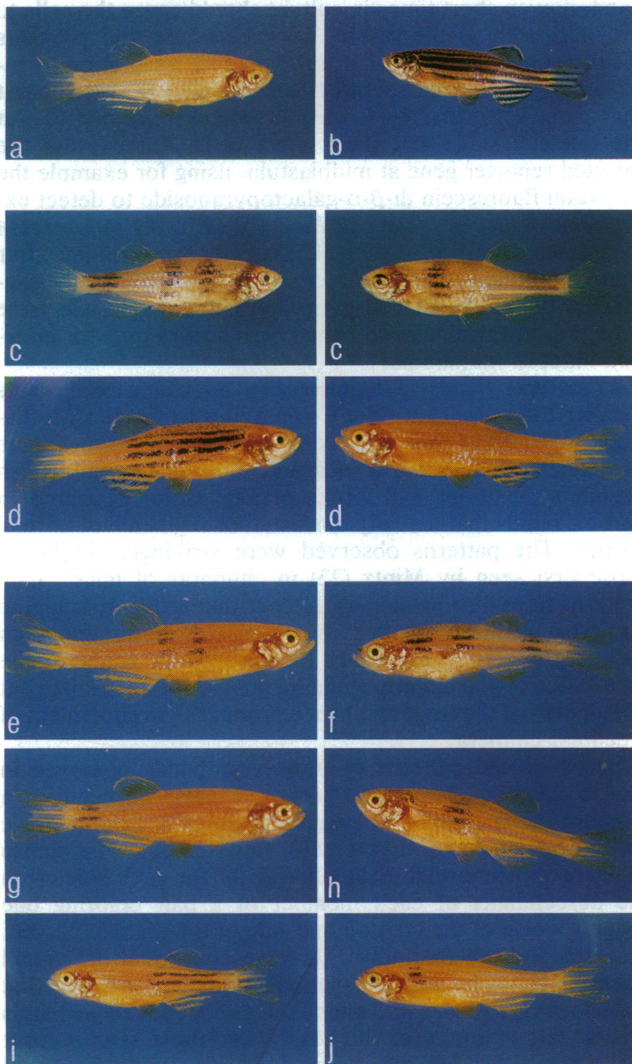

FIG. 4. Pigmentation in adult chimeras. Adult albino (*a*), wild type (*b*), or chimeras (*c–j*) were anesthetized as described (14) and photographed using a Contax MT50 camera mounted with a 1:1 macro lens. Note that for the chimeras in *c* and *d* both sides of each fish are shown, for chimeras in *e–j* only one side. Fish size is about 1 inch.

Germ-Line Chimeras. Chimeras, like control fish, reached sexual maturity beginning at about 2 months of age. We mated the chimeras to albino fish to determine whether they would give rise to any pigmented offspring. So far, 28 chimeras have mated and produced at least 50 offspring each. Of these 28 fish, 23 yielded only albino offspring but 5 yielded pigmented progeny in addition (Fig. 5). As expected, pigmented progeny of chimeras possess the wild-type striped pattern. One of the germ-line chimeras, male 5-5-3, is shown in Fig. 4*i*. Other germ-line chimeras had just one narrow band of pigmentation on the body, or no bands at all. The frequency of pigmented offspring ranged from about 1–2% to 40% for different chimeras (Table 1).

Because little is known about the genes that confer pigmentation in fish, we wished to have additional evidence that pigmented offspring derived from the putative germ-line chimeras were indeed the progeny of the donor cells that had been transplanted into these fish at the blastula stage. The donor cells used to prepare chimera 5-5-3 were heterozygous for a transgene, a RSV–*lacZ* plasmid, and were derived from the transgenic line M9-1 (ref. 16; P. Culp and N.H., unpublished results). Thus, when chimera 5-5-3 is mated to an albino fish, 50% of its pigmented offspring (but none of its albino offspring) should inherit the transgene. We prepared DNA from 5 pigmented and 14 albino offspring of chimera 5-5-3 and analyzed these DNAs by PCR and by Southern blotting. This analysis revealed that three of the five pigmented offspring but none of the albino offspring inherited the transgene. The Southern pattern in the offspring was identical to that seen in other fish of the M9-1 line (Fig. 6). Pigmented offspring from two of the other four germ-line chimeras also inherited the RSV–*lacZ* transgene.

DISCUSSION

Cell transplantation between zebrafish embryos has been used by Ho and Kane (12) and by Hatta *et al.* (13) to study the behavior of cells derived from developmentally important mutants. We are interested in developing the technique as a tool for genetic analysis in zebrafish. The experiments described here demonstrate that cells from midblastula-stage zebrafish embryos can contribute to the germ line after

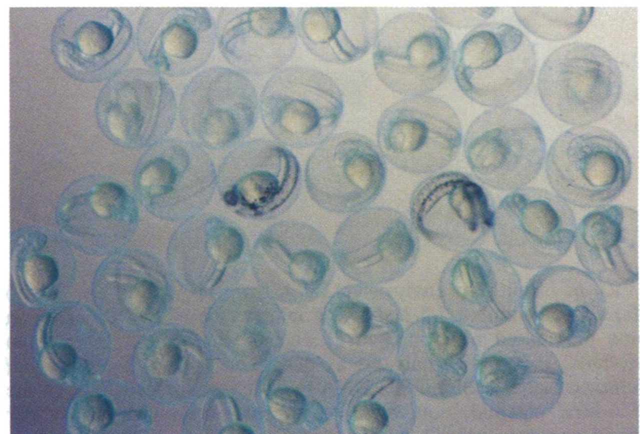

FIG. 5. Progeny of a germ-line chimera. Chimera 5-5-3 (Fig. 4*i*) was pair-mated with an albino female and their offspring were incubated at 27°C for 30 hr and photographed under a dissecting microscope. Diameter of the embryo is about 600 μm.

Table 1. Frequency of pigmented progeny from matings of chimeras with albino fish

	Offspring		
Chimera	No. albino	No. pigmented	% pigmented
5-5-3	450	5	1
4-3/4-6	53	40	43
4-3/4-9	49	8	14
4-3/4-10	141	10	7
4-3/4-16	507	8	2

Offspring were from pair matings of chimeras and albino fish.

transplantation to recipient embryos. The experiments also demonstrate that pigmentation is an excellent marker for detecting germ-line contributions from transplanted cells. An additional observation in these studies was the appearance of transverse bands of pigmentation running from dorsal to ventral on the trunk and tail of chimeras, a result that supports previous evidence that coat color in fish and mice may arise by a similar mechanism (22, 23).

Potential Use of Germ-Line Chimeras for Construction of Genetically Engineered Zebrafish. In the present experiments 20–100 cells were transplanted into recipients consisting of about 1000 cells. A substantial fraction of our chimeras appeared perfectly normal and were fertile. We obtained 5 germ-line chimeras among 28 chimeras that have mated so far and yielded at least 50 offspring each. The frequency of pigmented offspring varied from 1–2% up to 40% for the 5 chimeras. Since zebrafish can yield so many progeny, even a frequency of 1% would be adequate for our purposes. Most important will be obtaining a high frequency of germ-line chimeras. The total number of chimeras examined here is still small, but the preliminary frequency is encouraging. Overall 1 in 5–6 chimeras had germ-line contributions from the donor cells. Interestingly, 4 of the 5 germ-line chimeras were obtained in a single experiment. For this experiment the frequency of germ-line chimeras was 4 out of 14. The fifth chimera was obtained from a second experiment in which the frequency so far is 1 germ-line chimera out of 14. Clearly it will be important to determine accurately the overall frequency of germ-line chimeras and to learn what parameters may influence this frequency.

Donor and recipient embryos in our experiments consisted of about 1000 cells at the time cell transplants were performed. At this stage (10th cleavage plus/minus 1 cleavage),

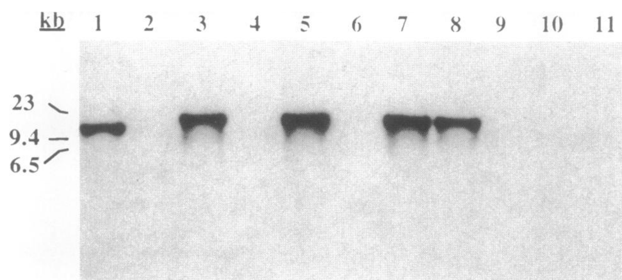

FIG. 6. Southern analysis of DNA from pigmented and albino offspring of chimera 5-5-3 showing inheritance of the transgene in some pigmented offspring. *Pst* I digests of genomic DNA from individual fish were analyzed as described under *Materials and Methods*. Lane 1, albino fish DNA to which was added 50 pg of the RSV-*lacZ* plasmid, equivalent to approximately two copies per diploid genome; lane 2, albino fish DNA; lane 3, DNA from a heterozygous M9-1 donor transgenic fish; lanes 4–8, DNA from five individual pigmented offspring of chimera 5-5-3, three of which inherited the *lacZ* gene; lanes 9–11, DNA from three individual albino offspring of chimera 5-5-3, none of them carried the *lacZ* gene. kb, Kilobase(s).

which is known as midblastula, embryos undergo several changes in cell behavior: cell cycles begin to be asynchronous, a syncytium arising from marginal blastomeres forms around the yolk (the yolk synctial layer), and significant levels of transcription begin (24–26). We chose the midblastula stage for transplants because we wanted donor cells to be undergoing transcription. On the other hand, by waiting until the 1000-cell stage we ran the risk that blastomeres might not be totipotent or that precursors to germ-line cells might not be transplantable. Although to our knowledge germ-line precursors have not been mapped in the developing zebrafish embryo, there has been speculation that cells that give rise to the germ line might arise from the yolk syncytial layer. Since the cells we transplanted were deep cells of the blastoderm, our experiments would seem to show that this is not the case and that future germ cells reside among the free blastomeres. Whether all the blastomeres have this potential remains to be learned.

Our reason for wishing to transplant cells that were undergoing significant levels of transcription is that our goal is to perform insertional mutagenesis on the donor cells before returning them to albino recipients. It may prove possible to grow totipotent blastomeres in culture so that they can be mutagenized by DNA integrations *in vitro*. On the other hand, if even short times in culture should cause the cells to lose totipotency, the results described here and previous results concerning the high frequency of integration of injected DNA into the zebrafish genome (16, 18, 27) suggest that conceivably one could inject promoter trap constructs at the one-cell stage and then select cells that express the injected reporter gene at midblastula, using for example the live stain fluorescein di-β-D-galactopyranoside to detect expression of *Escherichia coli lacZ* (28, 29). As our present experiments suggest, if such cells reenter the germ line after transplantation into albino recipients, then 50% of the pigmented offspring of such germ-line chimeras should have gene trap integrations and hence be heterozygous for insertion-induced mutations.

Transverse Bands of Pigmentation on Chimeras Provide Further Evidence That Coat Color Arises by Similar Mechanisms in Mice and Fish. A striking result was the appearance of pigmentation in the trunks and tails of swimming fish beginning at about 4–8 weeks of age. Color appeared in vertical bands that actually consisted of segments of normal stripes. The patterns observed were strikingly similar to those first seen by Mintz (22) in chimeras of mice. Such patterns have also been seen in trout that arose from albino embryos fertilized by heavily irradiated sperm and then diploidized (23). Presumably this procedure leads to the expression of pigmentation genes from the sperm in some but not all cells of the trout and hence produces the equivalent of a cellular chimera.

Based on the patterns of transverse bands observed in mouse chimeras, Mintz (22) proposed that each band is "a clone of melanoblasts, descended mitotically from a single cell." She concluded that to color fully the coat of a mouse requires 34 primordial melanoblasts, 17 for each side, with the descendants of each member of a pair coloring one stripe's worth of the coat on one side of the animal (22, 30). Jaenisch and collaborators (31) have confirmed the clonal nature of bands by showing that injection of a retrovirus that transduces tyrosinase into albino mice produces single bands on one side of a mouse. Our results and those seen in trout (23) suggest that fish also color their coats with the clonal descendants of a small number of pairs of melanoblast precursors, each member of a pair giving rise independently to a narrow band of color on one side of the animal. The narrowest bands of pigmentation among our chimeras are about 1/21st as long as a normal midflank stripe, measured from the operculum to the caudal peduncle. It is tempting to

say that wider bands are multiples of this width, but the error in the measurements does not permit this conclusion. Nonetheless, the simplest interpretation of our data is that melanophores of the midflank stripe in zebrafish are derived clonally from at least 21 pairs of precursor cells, with the data establishing a minimum number of cells.

By analogy with mouse chimeras, our data argue that *alb-1/alb-1* albino fish possess melanocytes that are unable to produce melanin but that are otherwise normal in their distribution and activities. If these cells were absent in albino fish, we would expect to see pigment distributed in blotches rather than sharp-edged bands, as donor melanocytes would migrate into the surrounding regions (30, 32). The fact that albino fish have red eyes, and hence lack melanin in the pigmented epithelium of the retina, is in agreement with this suggestion, since retinal cells stem from a different cell lineage than do melanocytes.

Conclusion. Current technology in mice, particularly the availability of ES cells and of gene or promoter trap constructs, makes it theoretically feasible to introduce a mutation into virtually any mouse gene that is expressed in an ES cell line (1–9, 33). However, quite apart from the excessive cost of such an approach is the difficulty of studying early developmental processes in the mouse. The zebrafish has the advantage of an easily manipulated embryo that is transparent and accessible. If techniques could be developed for insertional mutagenesis in this vertebrate, it might prove desirable to isolate new mutations in fish, identify those that appear interesting, and then clone their homologues in mice and proceed to targeted mutagenesis in the mouse. Whether it will be possible to achieve the appropriate technology in the fish remains to be seen. The results reported here may prove to be one step toward this goal.

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