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The Induction of a Specific Pigment Cell Type by Total Genomic DNA Injected into the Neural Crest Region of Fish Embryos of the Genus *Xiphophorus**

Jürgen Vielkind**, Hertraut Haas-Andela***, Ursula Vielkind**, and Fritz Anders
Institute of Genetics, Justus Liebig University of Giessen, D-6300 Giessen, Federal Republic of Germany

Summary. We report genetic transformation in an intact higher organism, i.e., in xiphophorine fish. The gene to be transferred (*Tu*) is responsible for the formation of T-melanophores in the platyfish and is involved in the formation of melanomas in platyfish-swordtail hybrids. After injection of *Tu*-donor DNA into the neural crest region of embryos from *Tu*-free fish, some of the recipients developed T-melanophores. In a few cases, one or two single T-melanophores were formed during late embryogenesis. In most cases, many T-melanophores developed in young fish and were arranged in several colonies or in a pattern. DNase-degraded *Tu*-donor DNA, *Tu*-free fish DNA, as well as DNA from *E. coli* and adenovirus-2, did not induce T-melanophores. When using DNA from different strains of *Tu*-donor fish which differed in a regulating gene linked to *Tu*, the percentages of fish showing T-melanophores paralleled the degree of phenotypic expression of the *Tu* gene in the DNA donor. The results suggest that the *Tu* gene has been successfully transferred together with the linked regulating gene.

Introduction

Genetic transformation, i.e., the expression of foreign genes in the recipient cells, provides a powerful tool to study the structure and function of genes. Despite many efforts to achieve genetic transformation in eukaryotes (see Ottolenghi-Nightingale, 1974; Klingmüller 1976), it was only in the last few years that it could be demonstrated without any doubt, both for cells in culture (see Pellicer et al. 1980; Willecke 1980) and, only recently, for a multicellular organism, the mouse (Brinster et al. 1981). The reasons why so many trials have failed are mainly due to the complexity of eukaryotic cells and whole organisms, in both morphological and genetic terms. Although, for technical reasons, it is easier to use cells in culture rather than the whole organism, the study of genes in this case is restricted to those which are expressed in cultured cells. For studying the structure and function of genes which are involved in developmental processes, the developing multicellular organism is required. Several approaches have been followed to achieve this goal, e.g., to introduce foreign genes via cells (Brinster 1974; Mintz and Illmensee 1975), nuclei (Illmensee and Hoppe 1981), or naked DNA (Jaenisch and Mintz 1974; Gordon et al. 1980; Brinster et al. 1981) into mouse blastocysts or eggs.

The present paper reports on the introduction of a gene responsible for the determination of a specific pigment cell, the *macromelanophore*, into early fish embryos via total genomic DNA. The experiment takes advantage of the platyfish-swordtail melanoma system which has been recently reviewed and discussed elsewhere (Vielkind and Vielkind 1982). The main features of this system can be summarized as follows.

Various platyfish strains show different patterns of black spots on their skin. These spots represent clusters of macromelanophores, a cell type which is formed in addition to the regular melanophores. Each spot pattern is due to a sex chromosome-linked gene complex consisting of the *macromelanophore gene* and an allele of a regulating gene, termed *linked R gene*. Further regulating genes, termed *non-linked R genes*, are located on chromosomes other than the gene complex. Some swordtail strains do not develop macromelanophores. They are considered not to possess the macromelanophore gene and its regulating genes. Platyfish-swordtail hybrids carrying the macromelanophore gene develop benign to malignant melanomas (see Gordon 1959; Anders 1967; Anders et al. 1973a, b).

Transplantation experiments have shown that the macromelanophores originate from the same neural crest precursor cells as the regular melanophores (Humm and Young 1956). Cytological studies on the differentiation of regular melanophores and macromelanophores (Diehl 1975) and on spots and melanomas (Vielkind et al. 1971a; Vielkind 1976) have shown that the determination of macromelanophores takes place at a very early stage of pigment cell differentiation. Thus, the macromelanophore-determining factor is supposed to act in melanoblasts before they enter differentiation into the regular melanophores.

The determination of melanoblasts to enter the macromelanophore pathway is evaluated by Anders et al. (1974, 1980) as neoplastic transformation, since they claim to have evidence that the macromelanophore is a terminally differentiated tumor cell. The macromelanophore was therefore named *transformed melanophore* or *T-melanophore*, and its determining factor was named "*tumor gene*" (*Tu*). In the present report, we will use the terms *Tu* gene and T-melanophore.

The following working hypothesis is put forward in order to explain the formation of spot patterns and melanomas in xiphophorine fish: the *Tu* gene acts in melanoblasts and induces a cellular alteration leading to the formation of T-melanophores.

* Part of this work has been done by H.H. for her doctoral thesis

** *Present Address:* Environmental Carcinogenesis Unit, British Columbia Cancer Research Centre, 601 West 10th Avenue, Vancouver, B.C., Canada V5Z 1L3

*** *Present Address:* Institute of Human Genetics, Justus Liebig University of Giessen, D-6300 Giessen, Federal Republic of Germany

Offprint requests to: J. Vielkind

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The linked *R* gene is thought to limit *Tu* expression to a certain number of melanoblasts and to a defined body region, while the various non-linked *R* genes are thought to suppress cell division and/or to promote differentiation in the cells altered by the *Tu* gene. The result is a characteristic T-melanophore spot pattern. When *R* genes are eliminated by genetic crosses or are impaired by mutation, the *Tu* gene acts in more melanoblasts, and the altered cells proliferate at an uncontrolled rate. Depending on how many *R* genes are missing, the altered cells form extended spot patterns or local melanomas. In the absence of intact *R* genes, melanomas are formed over the whole body of the fish, and the macromelanophores appear already during embryogenesis.

The Strategy of the Experiment

In planning a transformation experiment in xiphophorine fish, we wished to attain the following objectives: (i) The application of the donor DNA to the cells to be transformed, either directly or in such a way that the DNA will reach these cells before it is degraded. To do this we have chosen recipient cells whose location has been identified and which are known to express the gene to be transferred. (ii) The detection of a single transformed cell within the entire organism. As is known from experiments in cell culture systems, DNA-mediated transformation of a cell is a rare event. To circumvent this limitation, we applied the transforming DNA at a developmental stage when a large number of recipient cells were present. To allow for the detection for even a single transformed cell per individual, we applied donor DNA to cells which are still dividing and chose a recipient genotype which allows an unlimited expression of the transferred gene.

Based on the knowledge of the genetic and developmental conditions leading to melanoma formation in xiphophorine fish, we have designed the transformation experiment outlined in Fig. 1.

As a phenotypic marker, we chose the formation of T-melanophores because these cells can be easily detected and diagnosed. While the regular melanophores are about 100 μm in diameter and have a stellate shape with long narrow dendrites, the T-melanophores reach up to 500 μm in diameter and have a more compact shape. In addition, the T-melanophores tend to overlap each other and to form clusters in contrast to the regular melanophores which remain single and are evenly distributed.

The gene to be transferred is the *Tu* gene responsible for T-melanophore formation. The donor DNA is isolated from fish of a *Tu*-genotype and is transferred to fish which do not possess this gene and therefore do not develop T-melanophores. The recipient fish also lack all *Tu*-controlling *R* genes in order to favour *Tu* expression.

The cells in which the transferred gene can be expressed are the melanoblasts. These cells develop from neural crest cells at an early stage of embryogenesis, divide rapidly, and migrate into the skin where they will later differentiate into melanophores (Tavolga 1949; Weston 1963; Borack 1972; Mayer 1973; Chen et al. 1974; Turner et al. 1975). It therefore seems reasonable to supply the *Tu*-donor DNA to the neural crest cells immediately before they start to migrate, so that about 1,000 prospective melanoblasts can be treated simultaneously. In a recipient lacking all *R* genes, a *Tu*-gene taken up into one of these cells should be expressed in all daughter cells, and the transformed cells should proliferate at an uncontrolled rate. Thus, the transformation event should be amplified twice, first, by normal divisions of the prospective melanoblasts during embryogenesis, and second, by proliferation of the altered melanoblasts. Due to the migration of melanoblasts into all areas of the fish body and the tendency of the T-melanophores to cluster, we would expect several colonies of T-melanophores scattered over the

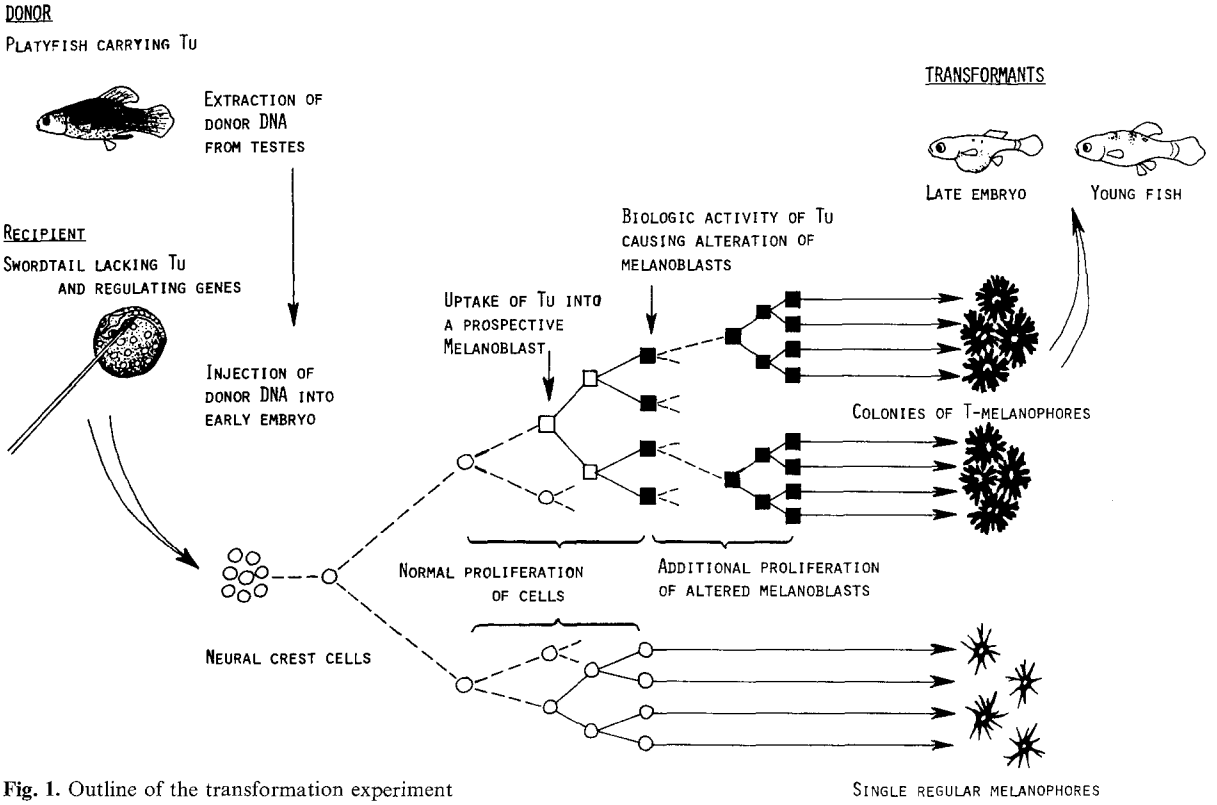

Fig. 1. Outline of the transformation experiment

fish body. These colonies would represent cell clones which are all derived from a single transformed melanoblast. Due to the absence of *R* genes in the recipient fish, the T-melanophores should occur early during development, thereby reducing the observation period required for the detection of a transformation event.

About ten years ago, we started to perfect conditions for the planned transformation experiment (Vielkind 1971; Vielkind et al. 1971b, 1973, 1976). We developed a method for culturing in vitro the embryos of the viviparous fish (Haas-Andela 1976), improved our method for isolating a very clean, high molecular weight DNA (Schwab and Vielkind 1973), and followed the fate of heterologous DNA in the recipient embryos after various modes of application (Vielkind et al. 1971b, 1973; Schwab et al. 1976; Vielkind and Giergsdies 1976).

Materials and Methods

a) Experimental Animals

Inbred strains of the viviparous cyprinodont fish *Xiphophorus helleri, X. (Platypoecilus) maculatus* and *X. (P.) variatus* were used. These fish strains originated from wild fish collected from various river systems in Central America by Gordon and coworkers (see Kallman 1975). Most of them have been maintained in the aquarium of the Institute of Genetics at Giessen since 1964. The mutant strains have been maintained and inbred since 1973.

Recipient Fish. As recipients for DNA, embryos from a *Tu*-free strain of the swordtail *X. helleri guentheri* as well as embryos from the *Tu*-free segregants of *X. mac./X. hell./X. hell.* backcross hybrids (*Tu-free hybrids*) were used. While the swordtails lack the *Tu*-controlling *R* genes, the hybrids carry several nonlinked *R* genes according to the segregation of chromosomes derived from the platyfish.

Donor Fish. Tu-free DNA was obtained from both types of the recipient fish (Fig. 2a) as well as from a *Tu*-free strain of the platyfish *X. maculatus* (Fig. 2b). Different types of *Tu-donor DNA* were obtained from *Tu*-carrying strains of the platyfish *X. maculatus* and the platyfish *X. variatus* (Fig. 2c to 2h).

All the *Tu*-donors carried two of several macromelanophore gene complexes (macromelanophore *genes*, according to Gordon 1927, 1948) which contain the *Tu* gene as a common constituent but which are assumed to differ from each other in a regulating gene linked to *Tu*. These gene complexes therefore induce different macromelanophore (or T-melanophore) spot patterns. The gene complexes selected for the gene transfer experiment were the following: the wild-type gene complexes *Sd* (Spotted dorsal), *Sp* (Spotted), *Sr* (Striped) and the X-ray-induced mutant gene complexes *Sd'* (mutation of *Sd*), *Sr'* (mutation of *Sr*) and *Li'* (translocation of *Li*, Lineatus, from the *Li*-chromosome of *X. variatus* to the *Dr*-chromosome of *X. maculatus; Li'* is identical to *Dr Li* in earlier publications (for details, see Anders et al. 1973a, b).

The genetic constitution of the male donor fish carrying macromelanophore gene complexes is listed in Table 1. Since the gene complexes are located on the sex chromosomes (either X or Y), the male donor fish were either heterozygous or homozygous for the specific complex, depending on the sex determination modus of the strain (XX females and XY males, or WY females and YY males; see Gordon and Gordon 1957; Kallman 1975). The *Tu*-donor fish can be grouped into three classes of genotypes according to assumptions about the state of the *Tu*-linked regulating gene and the manifestation of their T-melanophore spot patterns:

(i) In the strains *X. mac. Sd/Sr* and *X. mac. Sp/Sr*, the *Tu* gene of each gene complex is well controlled by an *intact* linked regulating gene (*R*). This situation has been termed *Tu R*. Fish of these genotypes show normal spot patterns built up by a limited number of T-melanophores (Fig. 2c and d).

(ii) In the strains *X. mac. Sd'/Sr'* and *X. mac Sr'/Sr'*, the *Tu* gene of each gene complex is controlled by a *slightly impaired* linked regulating gene (*R'*). This situation has been termed *Tu R'*. Fish of these genotypes show enhanced spot patterns with an increased number of T-melanophores (Fig. 2e and f).

(iii) In the strains *X. mac. Li'/Sr'* and *X. var. Li'/Sr'*, the *Tu* gene of the mutant gene complex *Li'* is controlled only by a *strongly impaired* linked regulating gene (*R''*). This situation has been termed *Tu R''*. Fish of the *Li'* genotype show a further enhancement of the spot pattern due to a drastic increase in the number of T-melanophores (Fig. 2g and h).

b) Isolation and Purification of Fish DNA

Fish DNA was extracted from the testes of 6 to 8-month-old males. For each preparation, the testes of about 20 animals were used. The fish were killed in ice water in order to minimize the action of DNases. The testes were removed under sterile conditions and gently homogenized in 8 ml of TNE buffer (0.01 M Tris-HCl, 0.1 M NaCl, 0.001 M EDTA, pH 7.4) at 4° C in a Potter glass homogenizer. After adding 25% sodium dodecylsulfate to a final concentration of 1%, the homogenate was lysed by incubation at 60° C for 20 min. After cooling to room temperature, the lysate was treated with pronase (200 µg/ml, 1 h at 37° C). The DNA was precipitated by adding 2 volumes of cold 95% ethanol and dissolved in 8 ml of TNE buffer. The DNA solution was treated with RNase (100 µg/ml, 30 min at 37° C) and treated again with pronase. The DNA was again precipitated with cold ethanol.

For further purification in a CsCl density gradient, the DNA was dissolved in 0.1 M borate buffer, pH 9.2; 6.5 g of CsCl (suprapur, Merck) were added to 5 ml of the DNA solution (maximum DNA content, 100 µg/ml) to give a final density of 1.710 g/cm^3. This solution (about 6.7 ml) was transferred into 12 ml polyallomer tubes and overlaid with silicon oil. The gradients were run in a W60 rotor of a Christ Omega II ultracentrifuge at 33,000 rpm and 22° C for 60 h. After centrifugation, the tubes were pierced at the bottom, and fractions of three drops each were collected under constant pressure of silicon oil. The DNA-containing fractions were pooled and diluted with one volume of 1/10 × SSC (sodium saline citrate; 0.15 M NaCl, 0.015 M Na-citrate). The DNA was precipitated with cold ethanol, washed for 30 min in ethanol/NaCl-EDTA (2/1; v/v) and resolved in 0.5 ml of sterile SSC. The DNA content of the solution was determined by measuring the UV absorption at 260 nm. The DNA solution was diluted with SSC to a final concentration of 600 µg DNA/ml and stored at 4° C.

The fish DNA isolated by this procedure was highly purified (absorbance ratios: 250/260 nm, 0.90; 280/260 nm, 0.54; 290/260 nm, 0.25) and of high molecular weight (5–6 × 10^7 daltons). Its buoyant density in CsCl was 1.6985 g/cm^3 and its density in Cs_2SO_4 was 1.4232, corresponding to a GC-content of 38.5%. A detailed characterization of the donor DNA has been given elsewhere (Vielkind 1979).

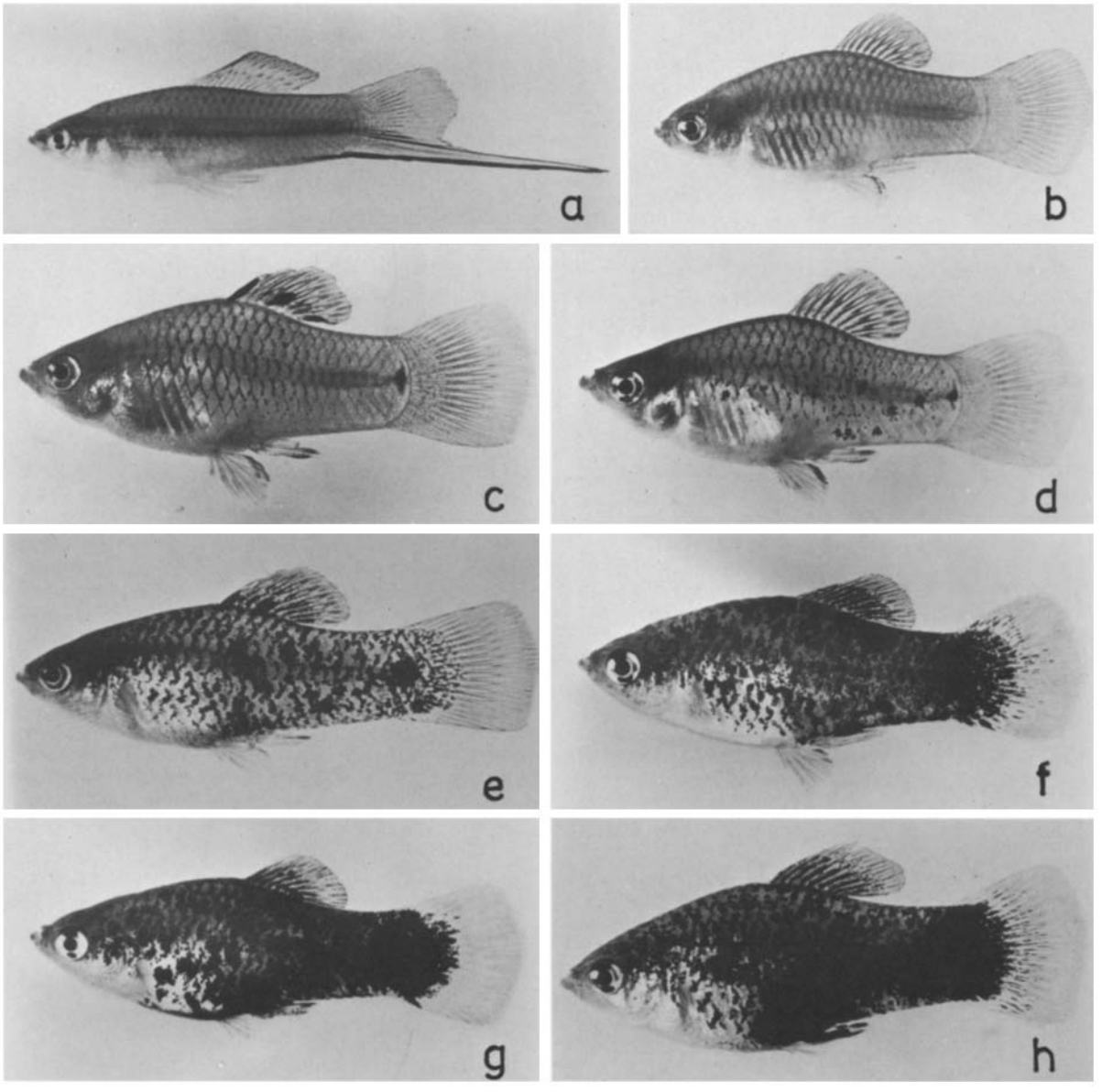

Fig. 2a–h. Male donor fish used in the transformation experiment. **a** *X. helleri guentheri*; the *Tu*-free *X. mac./X. hell./X. hell.* backcross hybrids show an identical phenotype (embryos of both these genotypes were used as recipients); **b** *X. maculatus* lacking the *Tu* gene; **c** *X. maculatus Sd/Sr*; **d** *X. maculatus Sp/Sr*; **e** *X. maculatus Sd′/Sr′*; **f** *X. maculatus Sr′/Sr′*; **g** *X. maculatus Li′/Sr′*; **h** *X. variatus Li′/Sr′*

c) Isolation and Purification of Bacterial DNA

Bacterial DNA for control experiments was isolated from *Escherichia coli* 15 T^-, as described earlier (Schwab and Vielkind 1973), except that deproteinization was done with pronase (see above) instead of chloroform. After pronase digestion, the bacterial DNA was further purified in the same manner as the fish DNA.

d) Adenovirus-2 DNA

Adenovirus-2 DNA was kindly provided by Dr. E.L. Winnacker, Institute of Biochemistry, Munich (F.R.G.).

e) Digestion of DNA with DNase

DNA at a concentration of 600 µg/ml was digested with 60 µg DNase I (Calbiochem, Giessen, F.R.G.) in 1 ml of PBS containing 0.003 M $MgCl_2$ for 2 h at 37° C. The reaction was stopped by adding 1 volume of chloroform/isoamylalcohol (24:1; v/v). The solution was then centrifuged for 30 min at 30,000 × *g* and 4° C. The aqueous phase was recovered, centrifuged again, and mixed with 1 volume of SSC.

f) Isolation and Culture of Recipient Embryos

Embryos of early developmental stages were isolated from pregnant females of the recipient genotypes 11 days after the last brood had hatched. The embryos were washed twice in sterile, modified PBS (Dulbecco's phosphate buffered saline; diluted 8:3 with conditioned tank water). Their developmental stages were checked according to the description given by Tavolga (1949). Only embryos of the stages 10 to 12 were selected (Fig. 3). After injection of DNA (see below), each embryo was washed twice in sterile PBS and transferred into a test tube containing

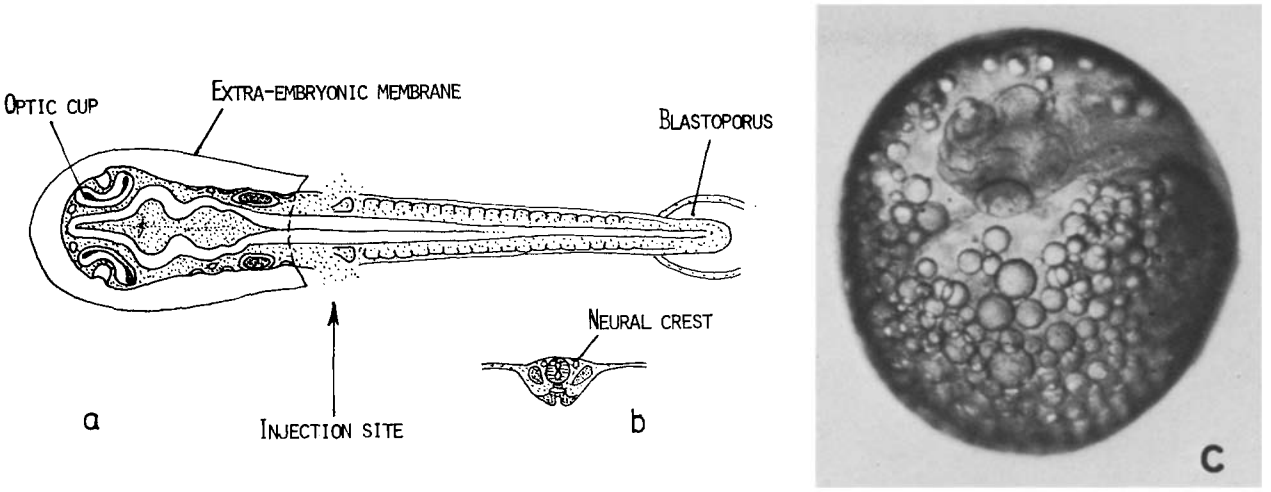

Fig. 3a–c. Early embryonic stages of *Xiphophorus helleri* used as recipient for donor DNA: **a** Top view of stage 11, according to Tavolga (1949), 3.9 days old, total length 1.7 mm. **b** Cross section of stage 11 at the level of the injection site. **c** Top view of stage 12, 4.2 days old, total length 1.8 mm

2 ml of sterile culture medium. This medium consisted of PBS supplemented with 250 mg glucose, 250 mg casein hydrolysate (Roth), and 2 ml of chick embryo extract (Gibco Bio-Cult) per liter (see Haas-Andela 1976). The medium contained 100 IU/ml of penicillin and 100 µg/ml of streptomycin. According to the experience of Humm and Young (1956), the medium was prepared with conditioned tank water (filtered and sterilized) instead of distilled water. The embryos were maintained in the dark at temperatures of 28–29° C. When they reached the last stage of embryonic development (stage 26, reduction of the extraembryonic membranes and reduction of the yolk), 5 to 10 embryos were placed together with their culture medium into a Visking dialysis bag (Serva, pore size 15 Å). The dialysis bags were placed horizontally into conditioned tank water in which adult fish had been kept for at least four weeks. After 15 to 24 h, the young fish were released into the conditioned tank water. Initially, the water level was very low (max. of 3 cm). After one week, the water level was gradually increased and the water temperature was lowered to about 26° C. The young fish were fed with powdered dry food (Tetramin) and nauplii of *Artemia salina* and were raised to adulthood.

g) Injection of DNA into Embryos

The injection of donor DNA into the fish embryos was carried out under sterile conditions by using a micromanipulator (Leitz). The latter was equipped with a glass needle having an inner diameter of 10 to 20 µm and with a glass stick having a small hole into which the embryo could be placed into a proper orientation. The DNA solution (600 µg DNA/ml SSC) was diluted with PBS containing phenol red (100 µg/ml) to give a final concentration of 300 µg DNA/ml). This solution was injected into the neural crest region of the embryo (see Fig. 3). The injection was carried out by passing the injection needle through the yolk sac, since earlier studies had shown that DNA injected directly into the neural crest region was rapidly released into the culture medium (Schwab et al. 1976). The injection procedure was controlled under a stereo microscope by watching the flow of the stained DNA solution into the embryo. About 0.05 to 0.09 µg of DNA were injected per embryo. Due to the injection procedure, the molecular weight of the DNA was reduced from $5–6 \times 10^7$ daltons to $3–4 \times 10^7$ daltons (Vielkind 1979).

h) Observation of Recipients and Registration of Abnormalities in Development and Pigment Cell Formation

The DNA-treated embryos, as well as untreated controls, were checked under a stereo microscope every 2–3 days. After being transferred into tank water, the young fish were checked macroscopically about twice a week. Developmental disorders and any alteration in the normal melanophore pattern of the recipients were documented photographically. Embryos and very young fish were placed directly on cotton wool in cold water, while fish of three months or more were first narcotized in MS 222 (Sandoz). Photographs were made by using a Zeiss microscope equipped with a Contarex camera, bellows, and Luminar 16 or Luminar 25 (for magnifications between 7× and 18×) or a Zeiss Tessovar magnifier pickup unit (for magnifications between 1× and 7×).

Results

a) Absence of Unspecific Alterations which Might Interfere with T-Melanophore Formation

It has been reported that DNA can induce developmental disorders (Martinovitch et al. 1962) and mutations (Fahmy and Fahmy 1965) which give rise to unspecific phenotypic changes. We therefore investigated the extent to which alterations in the formation of pigment cells occur in fish embryos treated with an unspecific DNA.

Recipient embryos were treated with *E. coli* DNA (about 1,500 embryos), DNase-degraded *E. coli* DNA (about 1,000 embryos), adenovirus-2 DNA (450 embryos) and were compared to those treated with DNA-free injection buffer only (520 embryos). In all groups, malformations occurred at a frequency of 4–5%, which is twice as high as in untreated in vitro-cultured embryos (see Haas-Andela 1976). Most of these fish showed a curvature of the spine, while some showed a shortening of the body and a distinct indentation behind the head. Since the malformations occurred with the same frequency in DNA-treated individuals as in those treated with injection buffer only, they are most probably due to a mechanical disrupture of tissues during injection. As in untreated in vitro-cultured embryos, about 70% of the individuals of each group survived the stage of birth and developed into fertile adults. This indicates that

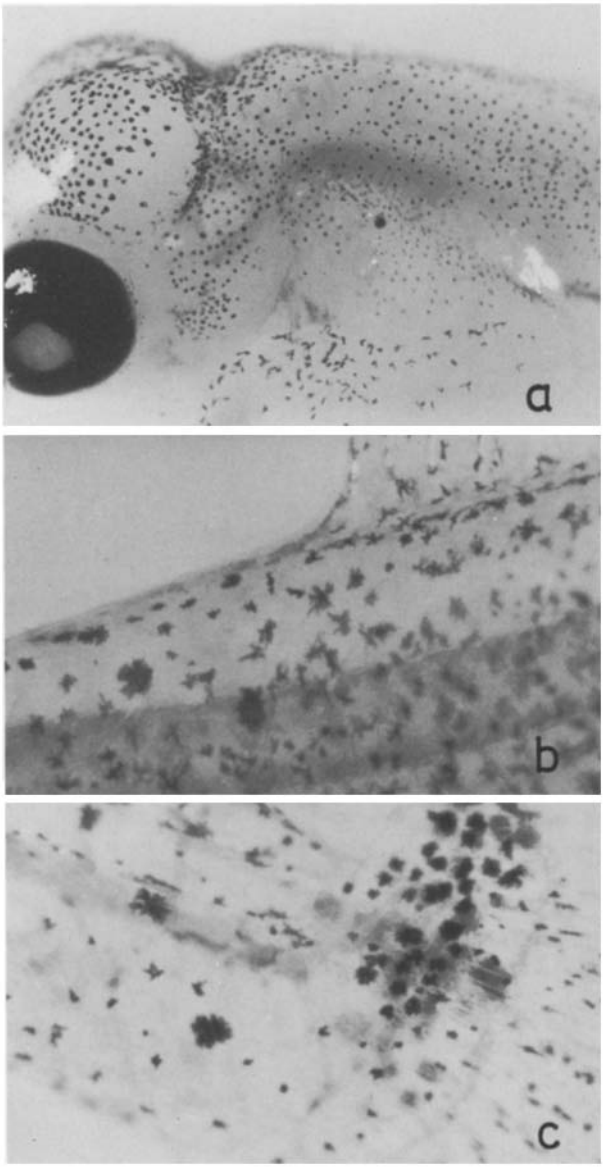

Fig. 4a–c. The appearance of large pigment cells during embryonic development in recipients treated with *Tu*-donor DNA: **a** A single large pigment cell in an *X. helleri* embryo (stage 20) after treatment with DNA from *X. maculatus Sd'/Sr'* (*Tu R'/Tu R'*-DNA). **b** Two large pigment cells in a *Tu*-free hybrid embryo (stage 24) after treatment with DNA from *X. maculatus Sr'/Sr'* (*Tu R'/Tu R'*-DNA). **c** For comparison, T-melanophores in an embryo (stage 20) of the donor strain *X. maculatus Sr'/Sr'*

neither the injection procedure itself nor the various DNA preparations affect the viability of the recipients. None of these individuals showed any alteration in the formation of pigment cells, and not a single cell was observed which resembled a T-melanophore.

b) Induction of T-Melanophores by Tu-Donor DNA

To induce T-melanophore formation in fish by transferring the *Tu* gene, total genomic DNA from *Tu*-carrying fish (*Tu*-DNA) was injected into embryos of fish which ordinarily do not develop T-melanophores.

The genotypes of the various donor and recipient fish used in these experiments are given in Table 1. In control experiments, DNase-degraded *Tu*-DNA, as well as DNA from fish lacking the *Tu* gene (*Tu*-free DNA) were used.

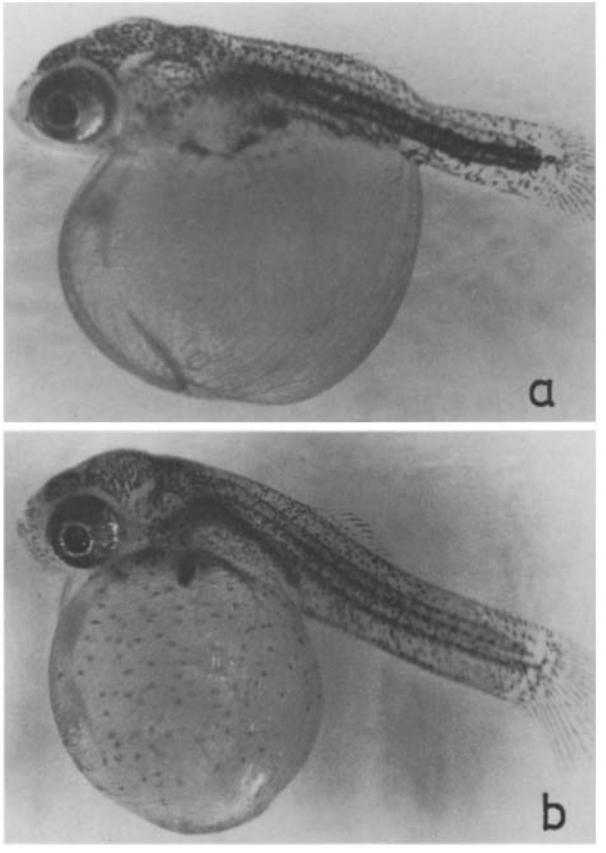

Fig. 5. a Aggregation of large pigment cells along the spinal cord of a recipient embryo (*X. helleri*) after treatment with DNA from *X. maculatus Sr'/Sr'* (*Tu R'/Tu R'*-DNA). **b** For comparison, untreated embryo of *X. helleri*

In all groups of recipients treated with DNA from a *Tu*-donor, a certain percentage of individuals showed pigment cells which were extremely large and which had broad dendrites. These cells differed significantly from the regular melanophores of the recipient fish but greatly resembled the T-melanophores of the donor fish. As can be seen from the data presented in Table 1, they occurred in both types of recipients and they appeared in embryos as well as in young fish. Such large pigment cells have never been observed in individuals treated with DNase-degraded *Tu*-DNA or with any of the *Tu*-free DNAs.

In embryos, one to three large pigment cells appeared 11–13 days after injection of the *Tu*-DNA, at about the stages 20 to 22. They were located either in the region near the yolk sac (Fig. 4a) or in the region between the head and the dorsal fin (Fig. 4b). The large pigment cells induced by the *Tu*-DNA looked identical in appearance to the T-melanophores of *Tu*-donor fish (Fig. 4c). In two *X. helleri* recipient embryos, an accumulation of pigment cells along the spinal cord was observed after treatment with *Tu*-DNA from *X. maculatus Sr'/Sr'* or *X. maculatus Li'/Sr'*, respectively (Fig. 5a; for comparison, see control embryo in Fig. 5b). These cells occurred 11 days after injection of the DNA, and they increased in number during further development of the embryos. Both embryos died at the stage of birth. Light microscopic examination demonstrated that the accumulated cells along the spinal cord were much larger and showed broader dendrites than the regular melanophores which, in a low number, are always present in this area.

In 2 to 7-month old fish which had been treated with *Tu*-DNA during embryogenesis, small dark spots were observed

Table 1. Development of T-melanophores in recipient fish treated with *Tu*-donor DNA during early development

Type of donor DNA[a]	Donor fish[b]	Recipient fish	Number of embryos treated	Number (%) of fish that survived the stage of birth	Number (%) of embryos showing T-melano-phores	Number (%) of young fish showing T-melano-phores
Tu R/*Tu R*-DNA	*X.mac. Sd/Sr*	*X. helleri*				
	Sp/Sr		421	287 (68.2)	2 (0.5)	0
	X.mac. Sd/Sr	*Tu*-free hybrids				
	Sp/Sr		114	74 (65.0)	0	0
Total:			535	361 (67.5)	2 (0.4)	0
Tu R'/*Tu R'*-DNA	*X.mac. Sd'/Sr'*	*X. helleri*	242	170 (70.2)	3 (1.2)	3 (1.8)
	X.mac. Sr'/Sr'		280	198 (70.6)	5 (1.8)	5 (2.5)
	X.mac. Sd'/Sr'	*Tu*-free hybrids	290	213 (73.4)	1 (0.3)	4 (1.9)
	X.mac. Sr'/Sr'		240	176 (73.3)	3 (1.2)	3 (1.7)
Total:			1,052	757 (72.0)	12 (1.1)	15 (2.0)
Tu R'/*Tu R'*-DNA	*X.mac. Li'/Sr'*	*X. helleri*	312	204 (65.4)	3 (1.0)	16 (7.8)
	X.var. Li'/Sr'		384	268 (69.8)	6 (1.6)	20 (7.5)
	X.mac. Li'/Sr'	*Tu*-free hybrids	204	135 (66.2)	2 (1.0)	9 (6.7)
	X.var. Li'/Sr'		132	89 (67.5)	2 (1.5)	6 (6.7)
Total:			1,032	696 (67.5)	13 (1.3)	51 (7.5)
DNase-degraded *Tu R'*/*Tu R'*-DNA	*X.mac. Li'/Sr'*	*X. helleri*	710	482 (68.0)	0	0
Tu-free DNA	*X. helleri*	*X. helleri*	762	528 (69.3)	0	0
	Tu-free hybrids		216	143 (66.2)	0	0
	Tu-free *X.mac.*		184	121 (65.8)	0	0
	X. helleri	*Tu*-free hybrids	436	287 (65.8)	0	0
	Tu-free hybrids		234	192 (82.0)	0	0
	Tu-free *X.mac.*		178	119 (66.8)	0	0
Total:			2,010	1,390 (69.2)	0	0

[a] *Tu* designates the *Tu* gene; *R*, *R'*, and *R''* are different alleles of a linked regulating gene which results in differences in the expression of *Tu*

[b] The gene complexes *Sr*, *Sd*, etc. are described in Materials and Methods

which were due to colonies of 10–40 large pigment cells. The number of colonies per fish usually ranged between 3 and 5, and all colonies of any one fish appeared at about the same time, as did all the cells in any one colony. The colonies were located near the dorsal fin or between the head and the dorsal fin (Fig. 6a). In two fish, more than 10 colonies were scattered over the whole body (Fig. 6b). During further development, cells increased in size and tended to overlap with their dendrites (Fig. 7a). When compared to the T-melanophores of *Tu*-donor fish of the same age (Fig. 7c), the large pigment cells looked identical to the T-melanophores. In some near-adult fish, the large pigment cells were arranged in patterns (Fig. 7b), including a crescent in the caudal peduncle, a net-like accumulation of large pigment cells along the margins of the scales in a limited body area, a black stripe along one side of the midline of the body.

Individual large pigment cells were frequently surrounded by a halo in which the regular melanophores were lacking. Such a halo was more prominent in embryos than in young fish. In all embryos and in most of the young fish, the large pigment cells degenerated after a certain period and were removed by macrophages. They completely disappeared 7–9 days after appearance in embryos, and 3–4 weeks after appearance in young fish, and were never observed to reappear in these individuals. In all fish showing a pattern-like arrangement of the large pigment cells, the cells did not degenerate but were stable for several months until the fish died.

Thus, the large pigment cells observed in recipients treated with *Tu*-donor DNA showed all the characteristics of T-melanophores: they were of the same size and morphology, they appeared in colonies, and they tended to overlap each other with their dendrites. The induction of halo formation and the stimulation of macrophage activity, both of which occur regularly in *Tu*-donor fish in response to T-melanophore formation (Gordon and Lansing 1943; Schmidt 1978), strongly support the view that the large pigment cells induced by *Tu*-donor DNA represented true T-melanophores.

c) Frequencies of Fish Showing T-Melanophores

The data given in Table 1 show that *Tu*-DNAs of different types resulted in different frequencies of fish showing T-melanophores.

Fig. 6 a, b. The appearance of large pigment cells during development of young fish in recipients treated with *Tu*-donor DNA: **a** Several colonies of large pigment cells between the head and the dorsal fin of a 3-month-old *Tu*-free hybrid after treatment with DNA from *X. variatus Li'/Sr'* (*Tu R''/Tu R'*-DNA). **b** More than 10 small colonies of large pigment cells scattered over the whole body of a 3-month-old *X. helleri* after treatment with DNA from *X. maculatus Sd'/Sr'* (*Tu R'/Tu R'*-DNA)

Different *Tu*-DNAs of the same type, however, gave almost identical results. There were no significant differences between the results obtained from the two types of recipient fish, *X. helleri* and *Tu*-free hybrids.

When the donor DNA was obtained from platyfish strains in which the *Tu*-linked regulating gene was intact (*Tu R/Tu R*-DNA), only 0.4% of the embryos and none of the young fish developed T-melanophores. Donor DNA from strains in which the regulating gene was slightly impaired (*Tu R'/Tu R'*-DNA) yielded about 1% in embryos and about 2% in young fish. When the donor DNA was derived from strains in which the linked regulating gene was strongly impaired on one chromosome and slightly impaired on the homologous chromosome (*Tu R''/Tu R'*-DNA), about 1% of embryos developed T-melanophores, while in young fish this portion was markedly increased to about 7%. It should be noted that the two DNAs of the latter type derived from different species (*X. maculatus* and *X. variatus*) which had the *Tu*-carrying chromosomes in common.

The data show that the efficiency of the *Tu*-DNA depended on the *Tu R*-type of the DNA and was directly correlated with the degree of defect in the *R* gene linked to *Tu*. This correlation was only true for the frequencies of fish showing T-melanophores, while the number of T-melanophores per fish was not correlated with the type of donor DNA. The data further show that, except for the *Tu R* complex, neither the genetic background of the donor fish nor that of the recipient fish influenced the outcome of the experiment.

Discussion

The injection of DNA from *Tu*-donor fish into the neural crest region of the recipient embryos resulted in the formation of large pigment cells in a certain percentage of the individuals. These cells showed all the characteristic features associated with T-melanophores. We concluded that the presence of these typical T-melanophores was due to transformation and not to other causes: Fish of the recipient genotypes have been kept in the aquarium for many generations without developing any T-melanophores, neither spontaneously nor after treatment with X-rays or chemical carcinogens. Thus the objection that the DNA treatment raised only the background level of spontaneously occurring phenotypic changes (see Fox et al. 1971; Siniscalco 1972; Ottolenghi-Nightingale 1974) cannot be made. There is some evidence from other systems that heterologous DNA is more rapidly degraded in the recipient than homologous DNA (Gradmann-Rebel and Hemleben 1976) and that DNA degraded to smaller molecules can cause mutations in the recipient (Fahmy and Fahmy 1965). It might be argued that the degradation products of the heterologous platyfish-derived donor DNA had induced a mutation in a silent *Tu* gene of the recipient swordtail or hybrid fish, resulting in the formation of T-melanophores. However, neither DNA from a *Tu*-free platyfish strain nor the even more heterologous DNA from *E. coli* or adenovirus-2 were able to induce T-melanophores.

Thus the induction of T-melanophores definitely depended on the presence of the *Tu* gene in the donor DNA. The specificity of the *Tu*-donor DNA is further supported by the fact that the percentages of recipient fish showing T-melanophores were positively correlated with the degree of *Tu*-expression in the donor which, in turn, depended on the degree of impairment of the *Tu*-linked regulating gene. The most plausible explanation for this correlation is that the *Tu* gene and the *R* gene had been cotransferred. Some implications of this result on the function of the *Tu* gene and the *R* gene have been published elsewhere in a preliminary form (Vielkind et al. 1978, 1979).

Taken together, our results strongly suggest that the *Tu R* gene complex had been taken up by a melanoblast, had been integrated, and had become biologically active. This interpretation is supported by the results of experiments which demonstrated the presence of ColE1 plasmid DNA in the nuclear DNA fraction of recipient embryos (Dippel and Vielkind, unpublished data). The question as to whether part of the plasmid DNA had been integrated into the chromosomal DNA could not be clearly answered, but the experiments show that the injected DNA reaches the cell nuclei of the embryos. Since the DNA of the *Tu*-donor fish certainly has a higher degree of sequence homology to the recipient DNA than the plasmid DNA, it may have a greater chance of becoming integrated (see Wigler et al. 1979). Although integration of the donor DNA is not essential for its expression (Fox and Yoon 1970; Brown and Gurdon 1977), it would stabilize the newly acquired information, thus favouring its transmission during subsequent cell divisions.

The percentages of recipients showing T-melanophores ranged between 0.4% and almost 8%, depending on the type of *Tu*-donor DNA. These frequencies are high when compared to the transformation frequencies in other systems. We believe that the discrepancy in frequencies is only apparent: The number of prospective melanoblasts at the time of DNA injection has been estimated to be about 1,000. Thus the frequency of a transformation event on a cellular basis is in the range of 10^{-5}, the same order of transformation frequencies reported for cell culture systems (Pellicer et al. 1978; Wigler et al. 1978; Willecke

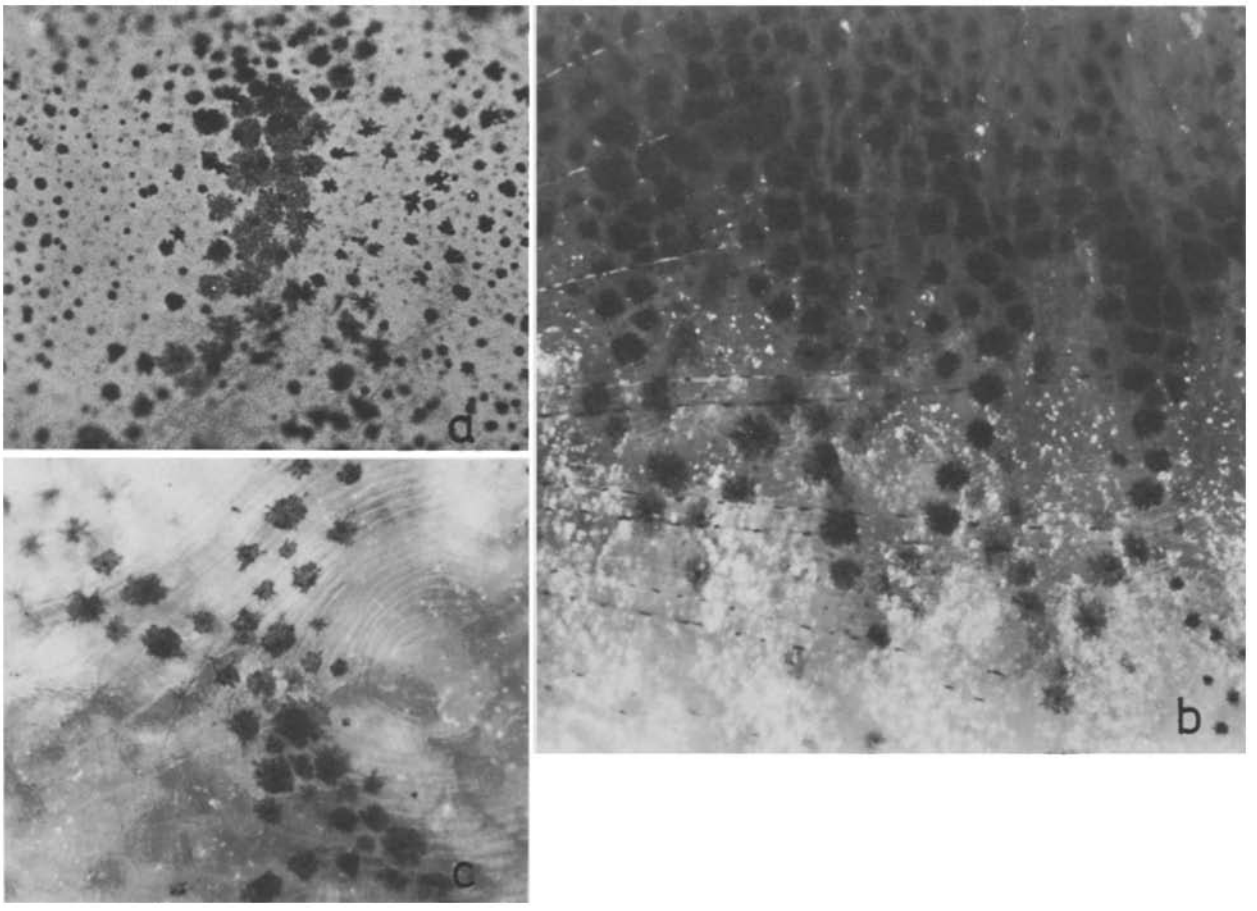

Fig. 7a–c. Large pigment cells arranged into colonies or patterns in recipients treated with *Tu*-donor DNA: **a** Pigment cell colony in a 3-month-old *X. helleri* after treatment with DNA from *X. variatus Li'/Sr'* (*Tu R''/Tu R'*-DNA). **b** Pigment cells forming a net-like pattern in a 4.5-month-old *Tu*-free hybrid after treatment with DNA from *X. maculatus Sr'/Sr'* (*Tu R'/Tu R'*-DNA). **c** For comparison, T-melanophores in a 4-month-old *X. maculatus Sd/Sr*

et al. 1979) as well as for *Ephestia* (Caspari et al. 1975) and *Drosophila* (Fox et al. 1971; Germeraad 1976; Fox 1977).

In most transformants, the transformation is due to a single event. In the group of recipients with the highest number of young fish showing T-melanophores (51 out of 696 individuals treated with the *Tu R''/Tu R'*-type of donor DNA), it was calculated by using Poisson's formula that two events may have occurred in about 2 of 51 individuals.

In contrast to the frequency of fish showing T-melanophores, the number of T-melanophores formed per individual was not correlated with the type of *Tu*-donor DNA but rather with the developmental age of the recipient. While in embryos one or two single T-melanophores were formed, the number of T-melanophores in young fish was fairly high, and the cells tended to be clustered in colonies. The differences between embryos and young fish can be explained by assuming that the donor DNA injected into the neural crest region of the embryos had been taken up into melanoblasts of different developmental stages (see Chen et al. 1974; Turner et al. 1975). In most of the recipients showing T-melanophores, these cells occurred in *randomly distributed colonies*. This suggests that in these individuals the *Tu* gene had been taken up into a prospective melanoblast which was just developing from the neural crest. The descendants of this cell migrated into the skin in different directions, resulting in more or less random distributions over the fish body. Due to the lack of *R* genes in the recipient fish, those cells which expressed the *Tu* gene were allowed to undergo further cell divisions so that each of these cells gave rise to a colony of T-melanophores. In some recipients which had almost reached adulthood, the T-melanophores were not clustered in colonies but were arranged in a *pattern-like manner*, i.e., along the margins of scales, along the midline of one body side, or in the caudal peduncle. Similar arrangements can also be observed with regular melanophores, especially in adult fish. This suggests that in these individuals the *Tu* gene had been taken up into a melanoblast which was already determined to migrate into a specific direction. Like the regular melanoblasts, the descendants of this cell followed certain pathways so that they became arranged in a pattern-like manner. In the case of embryos, the formation of *only one or two* T-melanophores near the injection site suggests that the *Tu* gene had been taken up into a melanoblast which had reached its final location and had already entered differentiation. In such a cell, the lack of *R* genes could hardly stimulate further division so that the transformed cell developed immediately into one or two single T-melanophores.

In summary, all our results strongly suggest that we have succeeded in transferring the *Tu* gene which is responsible for T-melanophore formation in *Xiphophorus*.

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