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The Biology of an Oncogene, Based Upon Studies on Neoplasia in *Xiphophorus*

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Xiphophorus, including swordtails and platyfish, is a genus of small viviparous freshwater fish from Central America that serves increasingly as a laboratory animal [1]. For 25 years we have used *Xiphophorus* for studies on neoplasia, which can easily be induced in hybrids between descendants of different provenance [2]. Although neoplasia of these animals is rather well understood in terms of formal genetics, the molecular basis of this phenomenon was extremely resistant to any elucidation. Recently a promising approach to the study of neoplasia in *Xiphophorus* at the molecular level has been undertaken in a cooperative work of the laboratories of H. Bauer (Institut für Virologie, Giessen), W. and H. Kersten (Institut für Physiologische Chemie, Erlangen), S. Nishimura (National Cancer Center Research Institute, Tokyo), and our laboratories. The present review will trace some steps of our studies that led to the detection of a cellular oncogene and a prominent regulatory gene.

A. The Taxonomic Groups of *Xiphophorus*

The genus *Xiphophorus* lives in genetically isolated populations in brooks, rivers, lakes, ponds, and pools, and has evolved into innumerable genotypically and phenotypically distinguishable groups [1]. Based on certain morphological and ecological characters, 17 of these groups have been listed as species [3].

All individuals of this genus, however, can be hybridized in the laboratory without difficulty, and all hybrids are fertile. This, together with the findings on the conformity of genome organization [4], the low degree of enzyme polymorphism [5, 6], and the normal chromosome pairing during meiosis in the hybrids [7], led to the conclusion that the taxonomic differences between these groups of *Xiphophorus* are not at the species level, but at the level of elementary local populations as well as ecological and geographical races.

B. Insusceptibility to Neoplasia in Wild Populations and Their Purebred Laboratory Descendants

Tens of thousands of individuals from different wild populations of *Xiphophorus* have been collected by several investigators (see [2]), but no tumors were detected. In the progeny of the wild populations, which in the case of *X. helleri* from Rio Lancetilla and *X. maculatus* from Rio Jamapa have been bred in the laboratory since 1939 (about 80 and 120 generations, respectively), no tumors occurred. About 10,000 specimens of purebred descendants of the wild populations have been treated with powerful carcinogens such as benzo(a)pyrene, N-methyl-N-nitrosourea (MNU), and X-rays, but none developed neoplasia. These animals are highly insusceptible to neoplasia (Table 1, first part).

* This contribution appears also in the 33rd Mosbach Colloquium: "The Biochemistry and Differentiation of Morphogenesis", also published by Springer

	No. of survivors		No. of neoplasms	
	MNU	X-rays	MNU	X-rays
Purebred				
X. maculatus	410	3405	0	0
X. variatus	ca 100	ca 500	0	0
X. xiphidium	ca 100	ca 100	0	0
X. helleri	415	ca 2000	0	0
X. cortezi	ca 100	ca 100	0	0
	ca 7200		0	
Hybrids				
F_1	470	ca 1000	18 (4%)	0
$F_2 - F_{24}$; BC_n	8258	3587	826 (10%)	163 (5%)
	ca 13,500		1007 (7.5%)	

Table 1. Neoplasia in *Xiphophorus* 1 year after treatment with MNU (10^{-3} M; four times for 1 h at 2-week intervals) and X-rays (1000 R; three times for 45 min at 6-week intervals)

C. Susceptibility to Neoplasia in Laboratory Hybrid Populations

In contrast to the animals from purebred wild populations, animals from laboratory hybrid populations derived from crosses between the purebred descendants of wild populations may be susceptible to neoplasia. Following treatment with carcinogens, depending on the wild populations used for hybridization, about 1%–4% of the F_1-hybrids develop neoplasia. Tumor incidence increases in the second hybrid generation (F_2) up to about 10% and remains stable in the succeeding generations, which have been tested up to F_{24} (Table 1, second part).

D. Classification of Neoplasms

As compiled from the results of several investigators in our laboratories [8] 805 of 10,195 (8%) hybrids which survived treatment with MNU and X-rays developed a large variety of different neoplasms. Most of the neoplasms were classified as neurogenic and mesenchymal, with melanoma, neuroblastoma, and fibrosarcoma being the predominant types. Epithelial neoplasms were less frequent but comprised the largest diversity (Table 2).

E. Assignment of Cancer Susceptibility to Chromosomes

To study what may be the crucial differences between the fish that were insusceptible and those that were susceptible to cancer, we attempted to assign the carcinogen-triggered neoplasms to chromosomes. For this study 65 defined genotypes of *X. maculatus, X. xiphidium, X. variatus, X. cortezi, X. helleri,* and their hybrids were employed [9, 10]. The genotypes exhibit, or lack, specific color patterns or enzyme markers which are due to the expression of specific genes, of which each is located on a different chromosome. We used mainly backcrosses, which were selectively bred for a specific phenotypic marker, and thereby for a specific chromosome. Such backcrosses segregate into 50% animals carrying the marker chromosome, and 50% lacking this chromosome.

Neoplasia could be assigned specifically to many different chromosomes. In the example shown in the scheme of Fig. 1 almost exclusively the backcross (BC) segregants exhibiting the stripes inherited from *X. maculatus* were susceptible. They developed melanoma, neuroblastoma, ephithelioma, and fibrosarcoma. Some of these hybrids developed several tumors of different types. This is not to say that all fish exhibiting the stripes develop neoplasia; but almost all neoplasms develop in those animals that belong to the group of

Table 2. Neoplasms induced in $F_2 - F_{24}$ and BC_n generations (MNU: 6608 survivors; X-rays: 3587 survivors; total: 10,195)

Type of neoplasm	No. of neoplasms			Incidence (%) based on total No. of survivors	
	MNU	X-rays		MNU	X-rays
Neurogenic					
Melanoma (benign)	135	93		2.12	2.6
Melanoma (malignant)	138	34	491	2.09	0.95
Neuroblastoma	84	7		1.27	0.2
Epithelial					
Squamous cell carcinoma	6	0		0.09	0
Epithelioma	19	6		0.28	0.17
Carcinoma (low-differentiated)	3	4		0.05	0.11
Carcinoma (high-differentiated)	2	5		0.03	0.14
Adenocarcinoma (kidney)	8	2	78	0.12	0.05
Adenocarcinoma (thyroid)	2	3		0.03	0.08
Papilloma	9	0		0.14	0
Hepatoma	5	1		0.07	0.03
Acanthoma	3	0		0.04	0
Mesenchymal					
Fibrosarcoma	190	6		2.87	0.17
Rhabdomyosarcoma	33	2	236	0.5	0.05
Lymphosarcoma	1	0		0.01	0
Reticulosarcoma	4	0		0.06	0
Total	642	163	805		

805 of 10,195 (7.9%) hybrids developed neoplasia; 92% of the hybrids were sufficiently protected

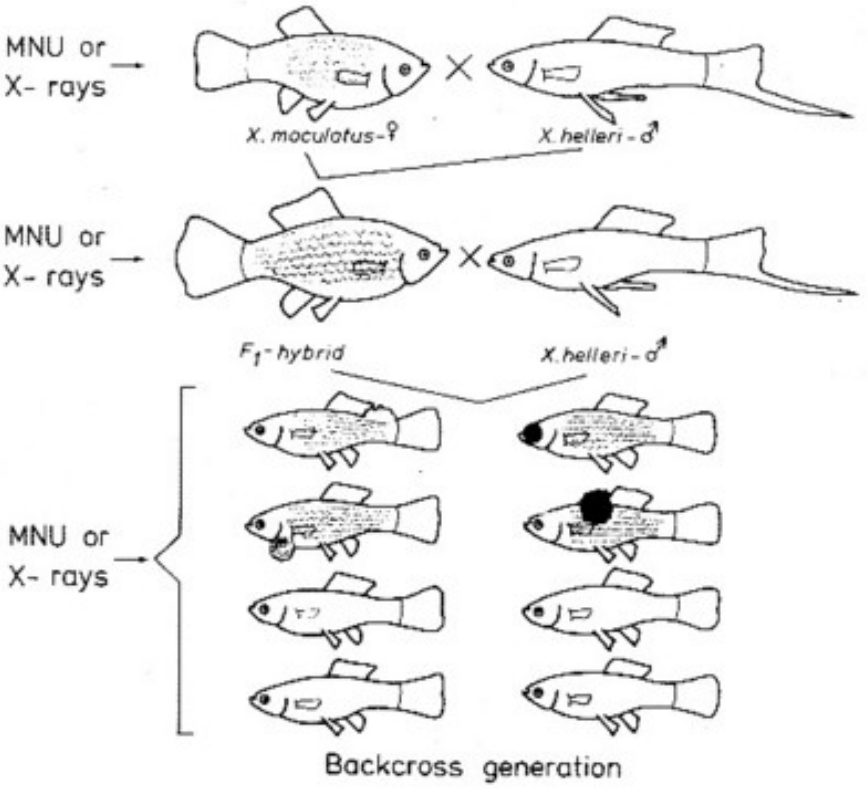

Fig. 1. Crossing scheme showing the assignment of carcinogen-triggered neoplasms to backcross segregants exhibiting stripes that are inherited from *Xiphophorus maculatus*. For details see text

BC segregants exhibiting stripes. Thus, the susceptibility to develop different kinds of neoplasms apparently depends on the marker chromosome which is responsible for the stripes. There are other examples in which susceptibility to neurogenic and epithelial neoplasia could be assigned to a certain chromosome while susceptibility to neoplasms of mesenchymal origin depends on a different chromosome [10]. Other examples have shown that susceptibility to develop melanoma may depend on a Y-chromosome, an X-chromosome, or an autosome. For all backcross hybrids tested so far, susceptibility to develop neoplasia is apparently chromosome specific.

F. Assignmentof Cancer Susceptibility to Oncogenes and Regulatory Genes

Information about the genes underlying susceptibility to carcinogen-triggered neoplasia comes from the analysis of the hereditary trait of certain "spontaneously" developing melanomas, pterinophoromas, neuroblastomas, thyroid carcinomas, kidney adenocarcinomas, and reticulosarcomas in hybrids. These neoplasms are very rare compared with the carcinogen-triggered tumors and therefore have been considered as a curiosity by many cancer researchers. They can, however, be produced at the will of the experimenter and have therefore been studied by many investigators [1]. They have contributed many important facts for developing ideas about how other animals, including humans, might inherit cancer [11, 12].

In the following chapters we shall mainly deal with the genes involved in melanoma development because their phenotypic effect, due to the enlarged shape and heavy pigmentation of the transformed cells, can easily be observed without killing the fish. Even a single transformed cell can be distinguished from a regular pigment cell.

Crosses of a spotted *X. maculatus* (platyfish) with a nonspotted *X. helleri* (swordtail) result in F_1-hybrids that develop benign melanoma instead of spots (Figs. 2, 3). Backcrosses of the F_1-hybrids using the swordtail as the recurrent parent result in offspring (BC_1), 50% of which exhibit neither spots nor melanoma while 25% develop benign melanoma (like the F_1), and 25% develop malginant melanoma. Further backcrosses of the fish (not shown in Fig. 3) carrying benign melanoma with the swordtail result in a BC_2 that exhibits the same segregation as the BC_1. The same applies for further backcrosses. Backcrossing of the fish carrying malignant melanoma with the swordtail results in a BC_2 in which 50% of the animals do not develop melanoma, while the remaining 50% develop malignant melanoma. In contrast, backcrossing of the melanoma-bearing hybrids using the platyfish as the recurrent parent results in a gradual suppression and finally disappearance of neoplasia in the succeeding generations.

These results, with the inclusion of cytogenetic findings, were interpreted as follows [2]: The spots and their corresponding genes are a specific accessory of the platyfish. The melanomas of the hybrids are closely related to the spots which actually are extreme benign melanomas. On the other hand the swordtail lacks both the corresponding spots and genes (Fig. 3).

The genetic information for neoplastic transformation of pigment cells in encoded in a "tumor gene" (*Tu*) which is inherited by the platyfish. About 70 crossovers, deletions, duplications, and translocations show that *Tu* is located at the end of the X-chromosome and is under control of linked and nonlinked regulatory genes [13]. In the platyfish used in this experiment a "major" pigment cell-specific regulatory gene (*R*) linked to *Tu* as well as two "minor" regulatory genes, which compartment-specifically suppress melanoma formation in the dorsal fin (R_{Df}) and the posterior part of the body (R_{Pp}), are mutated to R', R_{Df}', and R_{Pp}', respectively, and can no longer suppress *Tu*. Evidence for this comes from the appearance of some transformed pigment cells in the dorsal fin and in the skin of the posterior part of the body. The regulatory gene that actually suppresses tumor formation in the platyfish used in this experiment is the homozygous nonlinked "differentiation gene" (*Diff*) which can easily be detected by the esterase marker (*Est-1*) closely linked to *Diff* [6, 14, 15]. Further regulatory genes also present in the system are not taken into consideration.

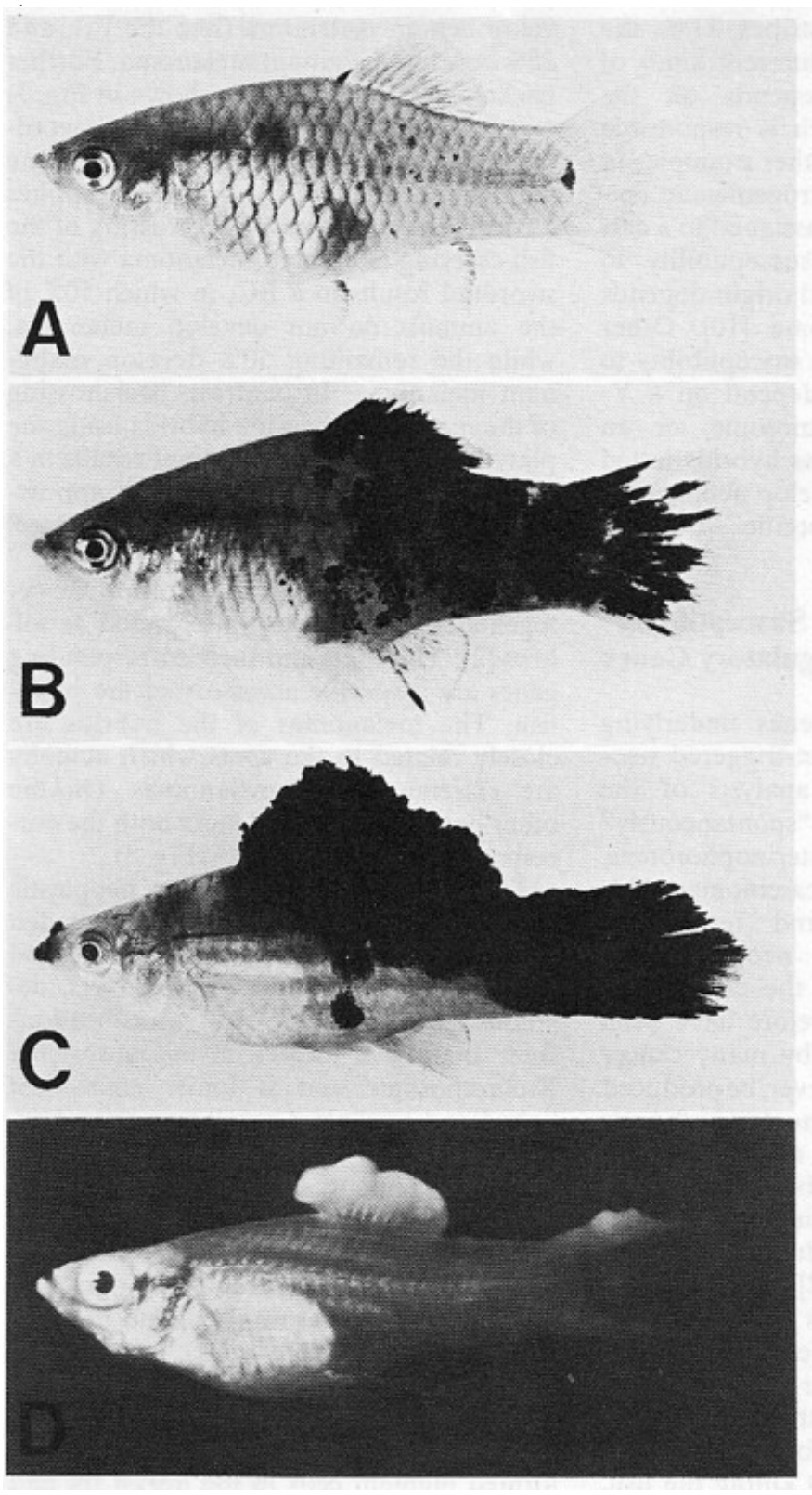

Fig. 2 A–D. Spots and melanomas in *Xiphophorus.* **A** *X. maculatus* from Rio Jamapa (Mexico); "spotted dorsal fin" (mutation R_{Df}) and "spotted posterior part" of the body (mutation $R_{Pp'}$). **B** F_1-hybrid between *X. maculatus* **A** and *X. helleri* from Rio Lacetilla (Honduras) exhibiting benign melanoma instead of spots. **C** BC-hybrid **B** with *X. helleri* as the recurrent parent exhibiting malignant melanoma instead of spots. **D** Albino BC-hybrid exhibing malignant amelanotic melanoma. **A–C** correspond to the respective schematic drawings shown in Fig. 3

Following crossings and backcrossings using the swordtail as the recurrent parent, the chromosomes of the platyfish are replaced by the homologous chromosomes of the swordtail, resulting in the gradual disintegration of the regulating gene system for *Tu*.

In contrast, following backcrossings of the melanoma-bearing hybrids with the platyfish as the recurrent parent, the chromosomes carrying regulatory genes are reintroduced into the descendants. This results in a reconstruction of the original regulating gene system that suppresses the activity of *Tu*.

"Spontaneous" development of melanoma as well as its suppression following the appropriate crossing procedures was found in several experimental hybrid populations derived from different purebred populations of different geographical or ecological origin.

In order to disclose the genetic basis for the bulk of neoplasms that develop following treatment with a carcinogen, we have modified the experiment shown in Fig. 3 to the experiment shown in Fig. 4: The *R' R_{Df}' R_{Pp}' Tu* chromosome was replaced by the *R R_{Df} R_{Bs}' Tu* chromosome, the "major" *R* of which is nonmutated and active. Since this *R* is inherited along with *Tu*, neoplasia does not develop spontaneously in the hybrids. Following treatment with carcinogens, those hybrids carrying the *R-Tu* chromosome but lacking *Diff* (determined by the esterase) are highly susceptible to neoplasia. In this case development of neoplasia requires only impairment, or

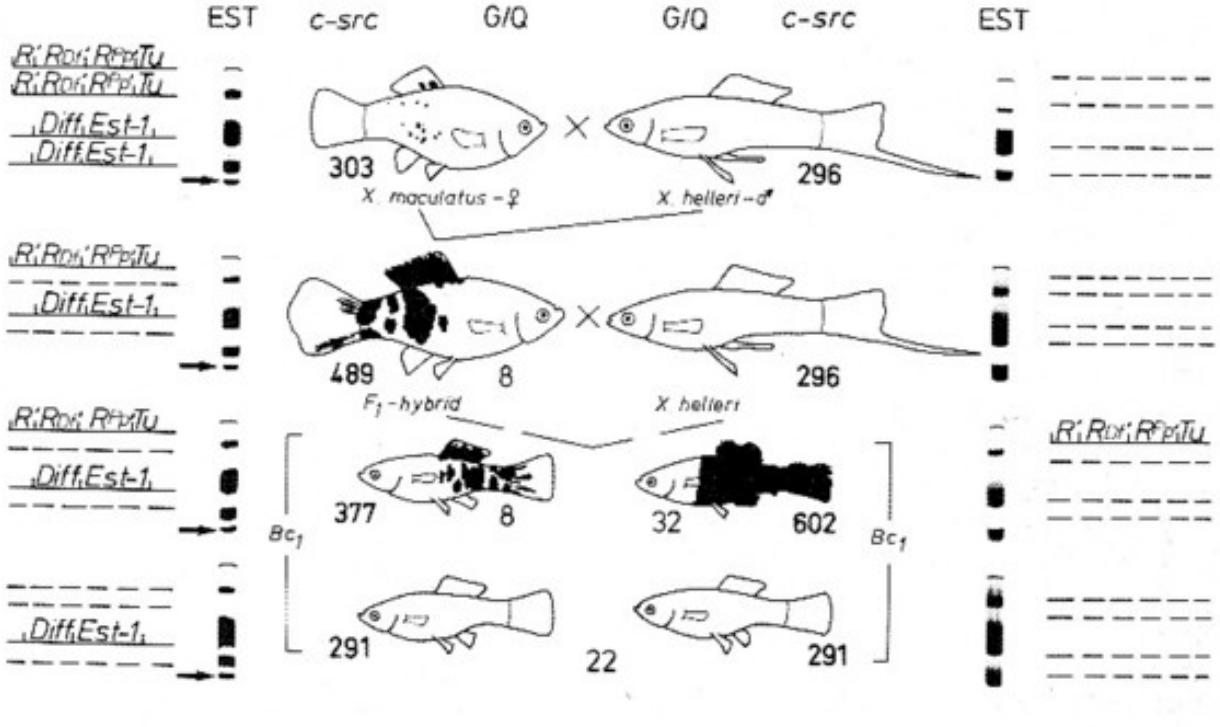

Fig. 3. Crossing scheme which displays the genetic conditions for the "spontaneous" development of spots (Fig. 2A), benign melanoma (Fig. 2B), and malignant melanoma (Fig. 2C). —— chromosomes of *X. maculatus;* –– chromosomes of *X. helleri; Tu,* tumor gene; R_{Pp}' and R_{Df}', impaired regulatory genes controlling *Tu* in the compartments of the posterior part of the body (Pp) and the dorsal fin (Df); R', impaired regulatory gene specific to pigment cells but nonspecific to the compartments; *Diff*, regulatory gene controlling differentiation of neoplastically transformed cells; *Est-1*, locus for esterase – 1 of *X. maculatus. EST,* esterases (polyacrylamide gel electrophoresis from homogenates of the eye); note linkage of *Diff* and *Est-1* (see *arrows*). *c-src,* $pp60^{c\text{-}src}$ kinase activity (cpm/mg protein; 53K; see Fig. 11); note basic and excessive activity, and correlation between *c-src* expression and *Tu* expression. G/Q, ^{3}H-guanine incorporation in position 34 (anticodon) of tRNAs for Tyr, Asn, Asp, and His (pmol/A_{260}; see Fig. 15); note low incorporation in tumors of *Diff* animals, indicating high Q content, and high incorporation in the melanoma of *Diff*-lacking animals, indicating low Q content (combined from data of [2, 6, 13, 14, 30, 31, 34, 40, 46]). For details see text

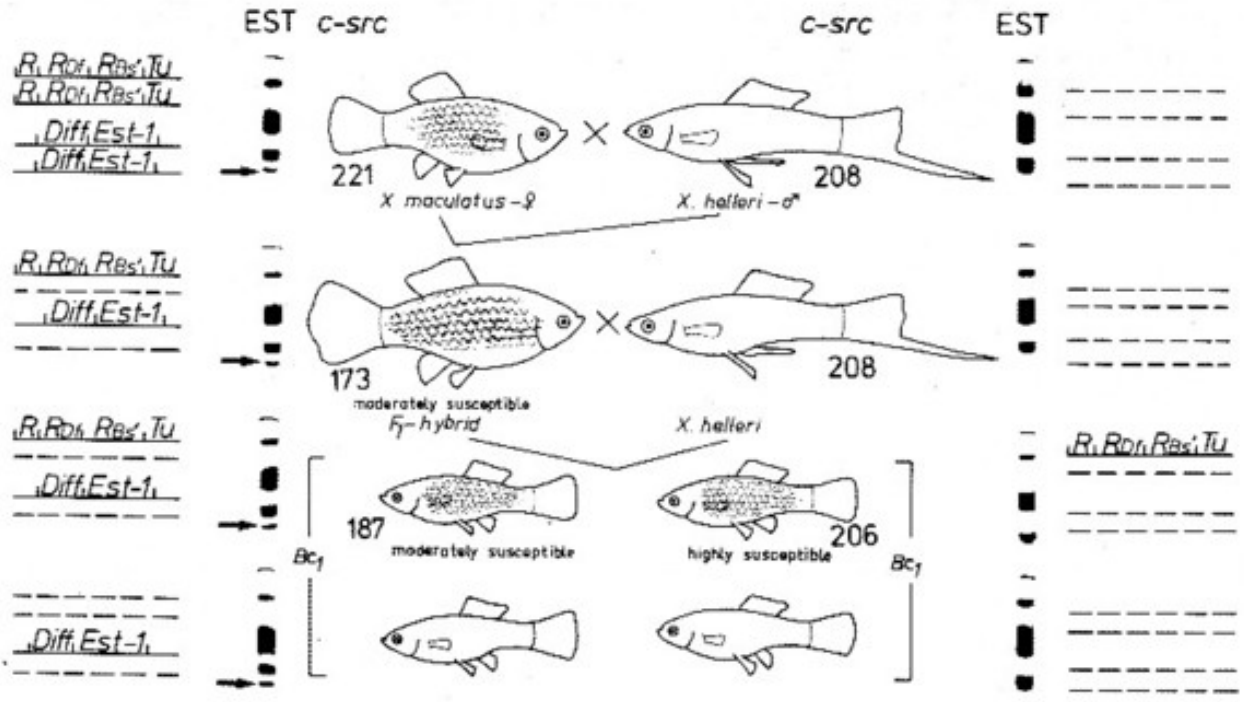

Fig. 4. Crossing scheme displaying the genetic conditions for susceptibility to carcinogen-dependent neoplasia. The highly susceptible genotype is highly sensitive to the carcinogenic (mutagenic) trigger. Abbreviations according to Fig. 3 $R_{Bs'}$, impaired the regulatory gene controlling *Tu* in the compartment of the entire side of the body (Bs) (combined from data of [6, 16, 31, 34]). For details see text

deletion, of a single *R* gene by the carcinogen in a somatic cell [16].

In conclusion, the regulating gene systems confidently suppressing *Tu* in the nonhybrids become disorganized if chromosomes derived from different populations are combined in the hybrids by the experimenter. This implies that, in purebred fish, the *Tu* and its sets of regulatory genes are population-specifically coadapted by natural selection. In any case the genetic information coding for neoplastic transformation can be traced to a *Tu* which is present in the different cell types and is normally under control of population-specific and cell-type-specific polygenic systems of linked and nonlinked regulatory genes, which suppress the development of the various types of potential neoplasms (Fig. 5). According to the formal assignment of different neoplasms to a particular chromosome, the particular *Tu* that codes for neoplastic transformation of pigment cells may also code for transformation of cells of mesenchymal and epithelial origin (see Figs. 1, 5).

G. Enhancement of Melanin Pigmentation in Melanoma as an Epiphenomenon of *Tu* Expression

To study the relationship between melanoma formation and melanin synthesis [17, 18] we separated both processes by introgression of an albino gene into melanoma-bearing hybrids. The result was albinos which developed melanomas that completely lacked the melanin (Fig. 2D). This indicates that enhanced melanin pigmentation of melanomas is an epiphenomenon of neoplastic transformation of pigment cells exerted by *Tu*.

H. The Competent Cells for the Activity of *Tu* in the Pigment Cell System

The precursors of the melanin-producing pigment cells of *Xiphophorus,* like those of other vertebrates, originate from the neural crest, and migrate to their final destination (see Fig. 5). They divide and undergo dif-

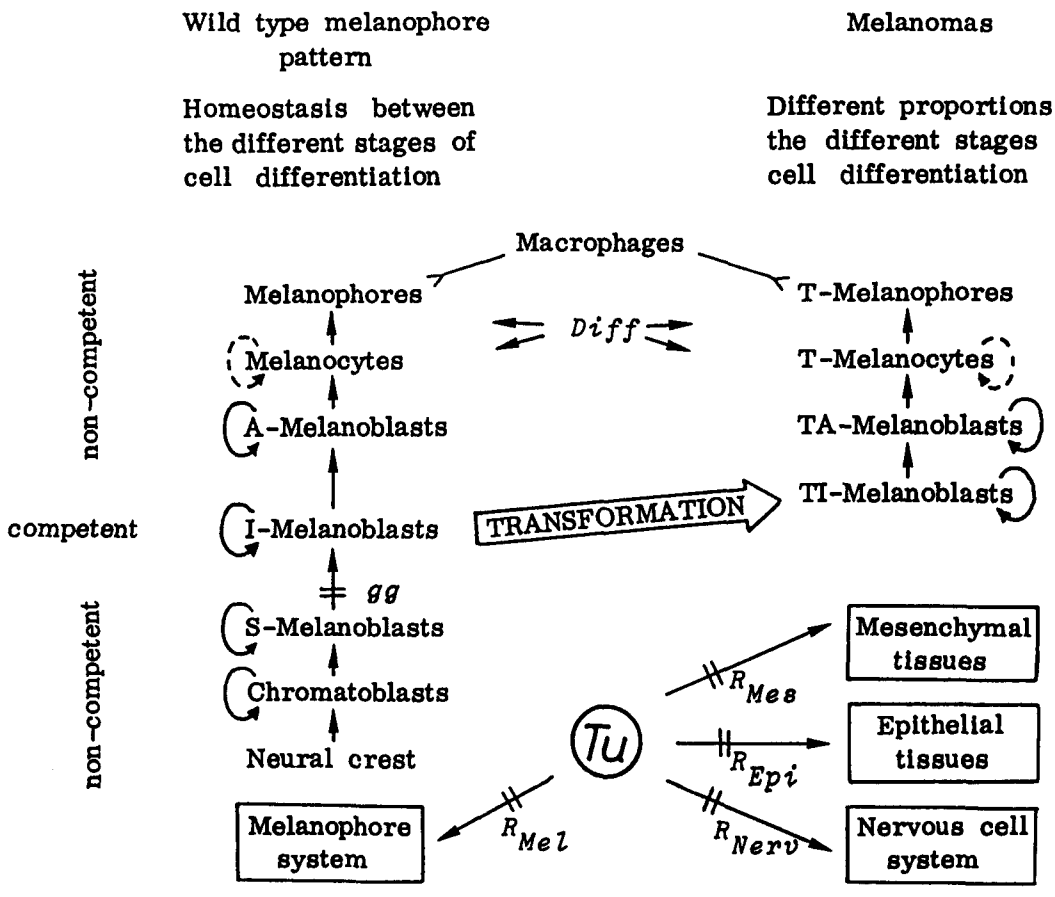

Fig. 5. Schematic presentation of the differentiation of normal and neoplastically transformed pigment cells. Only the intermediate melanoblasts (I-melanoblasts) are competent for transformation. *Tu,* tumor gene; R_{Mel}, pigment cell-specific regulatory genes for control of *Tu; gg,* homozygous "golden" mutation that blocks pigment cell differentiation; R_{Nerv}, R_{Epi}, R_{Mes}, regulatory genes that control *Tu* in the nervus cell system, the epithelial tissues and the mesenchymal tissues, respectively; *Diff,* regulatory gene which supports cell differentiation. Macrophages attack only the terminally differentiated pigment cells (both normal and transformed). (According to a scheme in [2], modified.) For details see text

ferentiation through the stages of chromatoblasts, stem(S)-melanoblasts, intermediate(I)-melanoblasts, advanced(A)-melanoblasts, melanocytes, and, finally, differentiate to melanophores, which are incapable of dividing. At a certain age the melanophores are removed by macrophages. Supply comes from S-melanoblasts [2, 19, 20].

A-melanoblasts, melanocytes, and melanophores have never been observed to undergo neoplastic transformation. Thus, these cells appear to be noncompetent for the *Tu* activity. On the other hand, in genotypes carrying a mutation that arrests differentiation at the stage of S-melanoblasts, melanomas cannot develop until exogenous promoters push differentiation of S-melanoblasts to the stage of I-melanoblasts [21]. These studies show that also the neural crest cells, chromatoblasts, and S-melanoblasts are noncompetent. We therefore conclude that the only stage of differentiation in which the pigment cells are competent for the transforming activity of *Tu* is the stage of I-melanoblasts. These cells become transformed to TI-melanoblasts. We assume that the principle of competence of a cell to the transforming activity of *Tu* applies also for other kinds of neoplasms.

It has not been possible to show so far whether *Tu* is still active in the transformed cells (T cells) for the maintenance of the neoplastic state. In any case the TI-melanoblasts continue to differentiate to TA-melanoblasts, T-melanocytes and, finally, to T-melanophores that are incapable of dividing. This process corresponds to differentiation of the nontransformed pigment cells. It is not under the control of *Tu* but under the control of the

differentiation gene *Diff*. This, furthermore, implies that *Tu* does not specify the degree of malignancy of the melanoma (see later).

I. The Genuine Effect of *Tu* in the Pigment Cell System

Information about the genuine effect of *Tu* comes from a balanced laboratory stock carrying a lethal *Tu* translocation that originated according to Fig. 6. *Tu* from an X-chromosome of *X. maculatus* becomes translocated to an autosome of *X. helleri* and, in its new position, is not under control of its former linked regulatory genes (*R*, R_{Df}, etc.). The progeny of this stock segregates into 50% carrying the nonlinked *Diff* which survive, while the corresponding 50% lacking *Diff* is lethal. As a consequence of the Mendelian inheritance of the *Tu* translocation through the germ line, and the lack of *Diff*, *Tu* becomes active in the developing progeny as soon as the pigment cell precursors differentiate to the competent I-melanoblasts.

This process starts in the 5-day-old embryos. Some time later some single dividing T-melanocytes appear at the peduncle of the tail fin of the embryo (Fig. 7). Neoplastic transformation continues in all areas of the developing embryo, where a pigment cell precursor becomes competent, thus building the lethal "whole body melanoma". This reflects the genuine effect of the completely derepressed *Tu* on the pigment cell system [13].

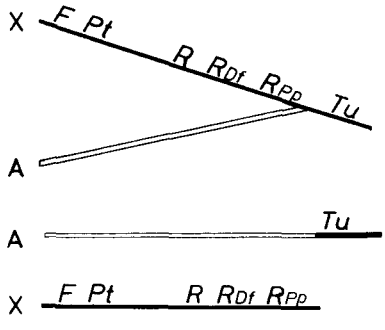

Fig. 6. Translocation of the tumor gene (*Tu*) from the X-chromosome (X) of *X. maculatus* to an autosome (A) of *X. helleri*. Note separaion of *Tu* from its linked regulatory genes (*R*, R_{Df}, R_{Pp}; see Fig. 3). *F*, sex-determining region of the X-chromosome; *Pt*, pterinophore locus. For the phenotypic effect see Fig. 7

It is suggestive to assume that *Tu* might exert an essential function in the early embryo which is related to the neural crest and its derivatives. In normal embryogenesis this function becomes switched off or choked by the regulatory genes prior to the 5th day of embryonic life. If, however, the regulatory genes (i.e., the entire switch) are lacking, *Tu* continues to exert its early embryo-specific function, which as a process of misguided cellular development transforms the competent cells to the neoplastic state.

J. Indispensable and Accessory *Tu* Copies

In the sex chromosomes of the platyfish 30 deletions involving *Tu* have been characterized genetically, and some of the major deletions involving both the *Tu* and its linked regulatory genes, additionally to the genetic results, were cytologically observed [13, 22]. All deletions are nonlethal. Even the loss of a *Tu*-containing segment of the X-chromosome (one Giemsa band), in the homozygous condition in the female or in the hemizygous state in the male, apparently has no detectable effect on viability. This, together with the fact that the swordtail used in our crossing experiments populationspecifically lacks the *Tu* (see Figs. 3, 4), led us to the conclusion that the *Tu* so far considered is not essential but is an accessory to the fish [2]. This is not to say that the *Tu* has no normal function. One could, for instance, assume that additional copies of *Tu* that are indispensable to the fish are present in the autosomes and may compensate for the loss of the sex chromosome-linked *Tu* loci according to a gene dosage compensation mechanism which warrants normal functions. Support for the assumption of multiple copies of *Tu* per haploid genome comes from the following experiment. Platyfish, carrying the deletion of the Giemsa band that involves the accessory *Tu*, were crossed according to the procedure outlined in Figs. 3, 4, with the swordtail populationspecifically lacking the accessory *Tu*. No tumors developed in the hybrids. Following treatment of the backcross hybrids with MNU, however, melanomas developed which were specifically localized at the upper part of the tail

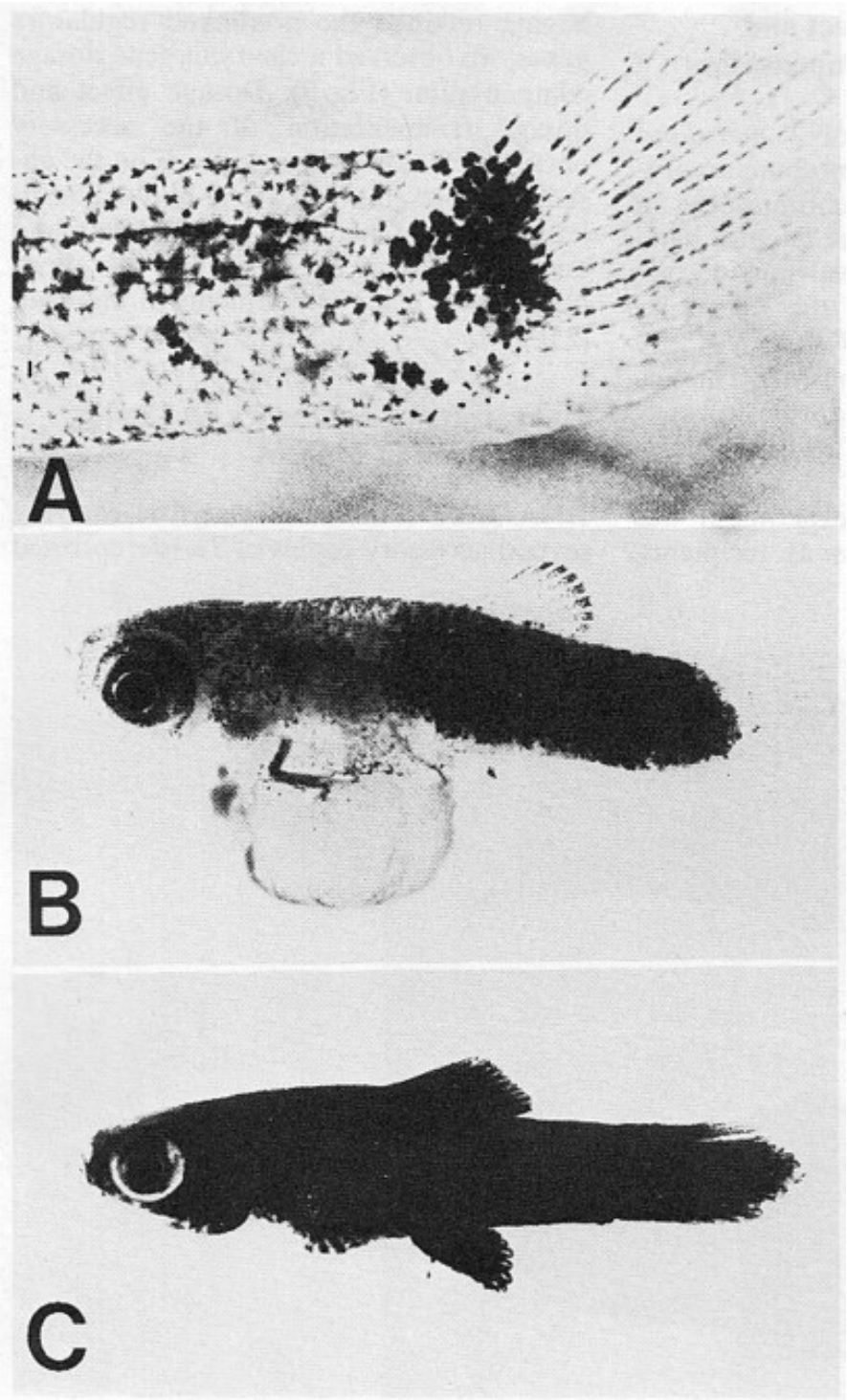

Fig. 7A–C. The genuine effect of the tumor gene *Tu* (corresponding to the scheme of translocation shown in Fig. 6). **A** Tail of a 10-day-old embryo (3 mm in length) exhibiting some T-melanocytes at the peduncle of the tail fin. **B** The same fish, 5 days later (4 mm in length). **C** Neonate of the same genotype (6 mm in length)

fin. These neoplasms could be assigned to an autosome. Thus it appears that the platyfish, besides the easily detectable accessory *Tu* copies contains additional ones that require more intricate experiments for their detection.

Our experience that all individuals of all groups of *Xiphophorus* can contribute to susceptibility to neoplasia in the hybrids suggests that all individuals contain *Tu* copies that are indispensable for the fish, and may contain accessory *Tu* copies. Up to ten accessory copies of the repressed *Tu* could be introduced into a laboratory stock by crossings. No effect on viability could be observed.

K. Oncogene Dosage Effect and Oncogene Dosage Compensation

Both the X-chromosome of *X. maculatus* containing *Tu* (the X-chromosome according to Fig. 3) and the X-chromosome of *X. maculatus,* having lost the Giemsa band carrying *Tu,* were introduced into the genome of *X. helleri* lacking the regulatory genes for the accessory *Tu.* X^{Tu} X^{Del} × X^{Tu} Y matings were accomplished. The segegating offspring had none, one, or two, respectively, accessory *Tu* copies and showed a clear-cut gene dosage effect (Fig. 8). If, however, the experiment was modified by using animals as recipients, having retained the nonlinked regulatory genes, we observed a clear-cut gene dosage compensation (Fig. 9). Dosage effect and dosage compensation of the accessory oncogene *Tu,* therefore, depends on the absence or presence, of the nonlinked regulatory genes. Oncogene dosage effect and oncogene dosage compensation has been observed in many experiments of this kind [23].

L. Transfer of Accessory *Tu* Copies by Injection of DNA

DNA from laboratory platyfish carrying several accessory copies of *Tu* (derepressed

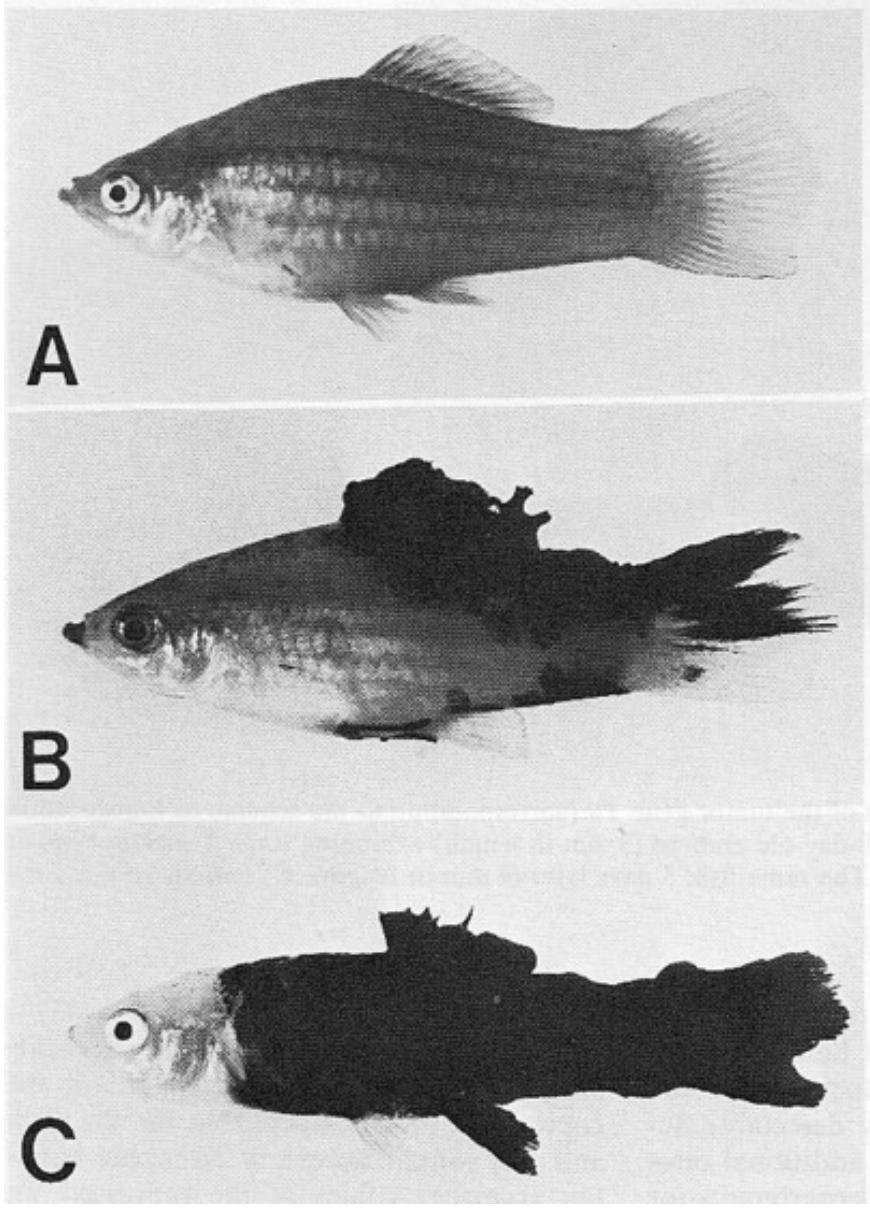

Fig. 8A–C. *Gene-dosage effect* of the (incompletely) *derepressed* tumor gene (*Tu*). **A** No *Tu* (not a single transformed pigment cell). **B** One dosage of *Tu* (melanoma formation). **C** Double dosage of *Tu* (double effect in melanoma formation). Compare with Fig. 9

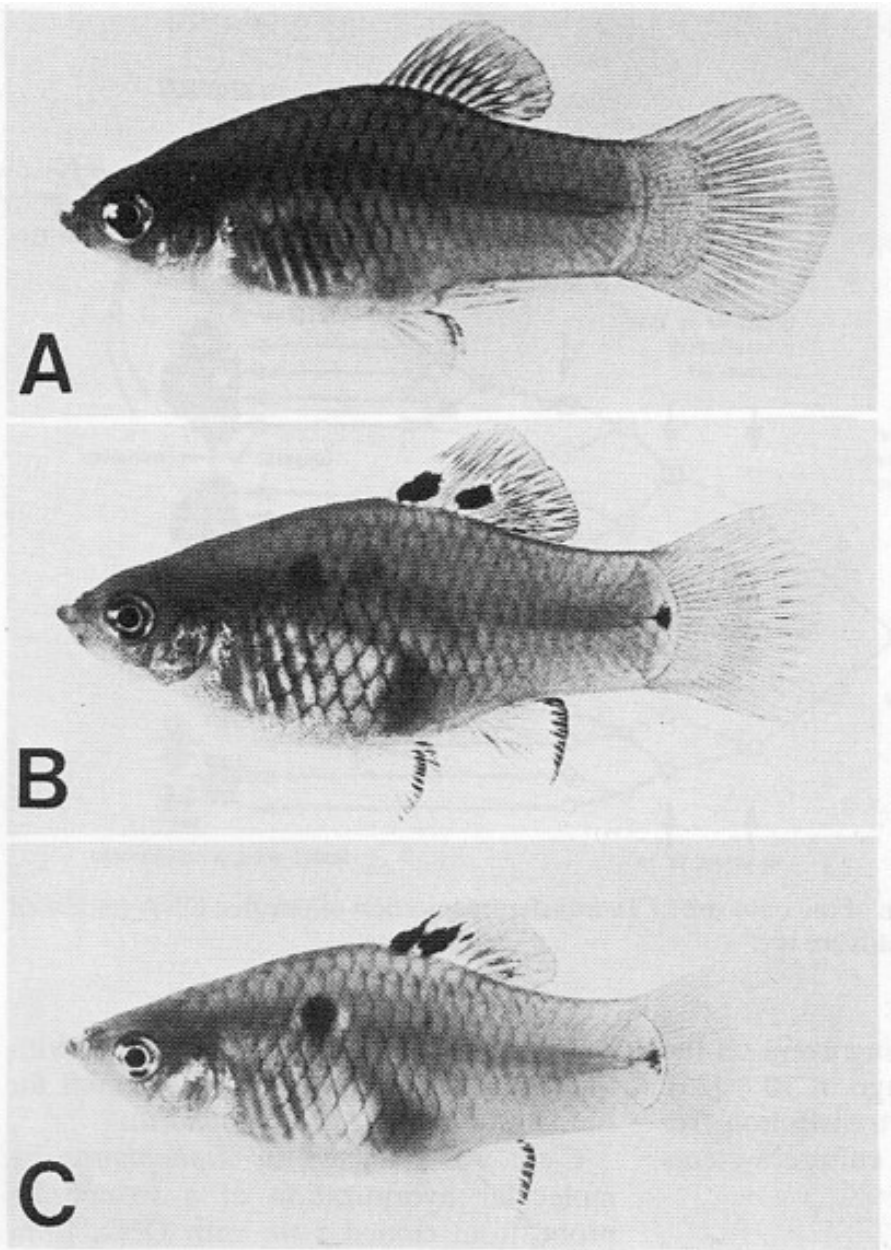

Fig. 9 A–C. *Gene dosage compensation* of the *repressed* tumor gene (*Tu*). **A** No *Tu* (not a single transformed pigment cell). **B** One dosage of *Tu* (spots consisting of transformed pigment cells; see dorsal fin). **C** Double dosage of *Tu* (effect shows no difference to that of one dosage). Compare with Fig. 8

as well as repressed) was injected into the neural crest region of early embryos of the swordtail which lacked both the accessory *Tu* copies and the regulatory genes (Fig. 10). The injected DNA may maintain its high molecular weight for about 2 h and thereafter becomes degraded to pieces which are too small to contain genetic information [24]. Following the successful uptake of *Tu* by an S-melanoblast of the embryo, this cell may later become competent to the *Tu* activity by differentiation to an I-melanoblast, which eventually may become neoplastically transformed to a TI-melanoblast. Additional proliferation of T cells amplifies the original transforming effect of *Tu*, and the result becomes visible as the transformed cells differentiate to the easily detectable colonies of T-melanocytes and T-melanophores [25].

Depending on the type of the *Tu* donor DNA (cotransfer of intact or damaged regulatory genes) the percentage of recipients showing T-melanocytes and T-melanophores ranged from 0.4% to almost 8% (total number of survivors tested in these experiments: 1390). Since the number of the target cells (pigment cell precursors) at the time of DNA injection has been estimated to be about 1000, the

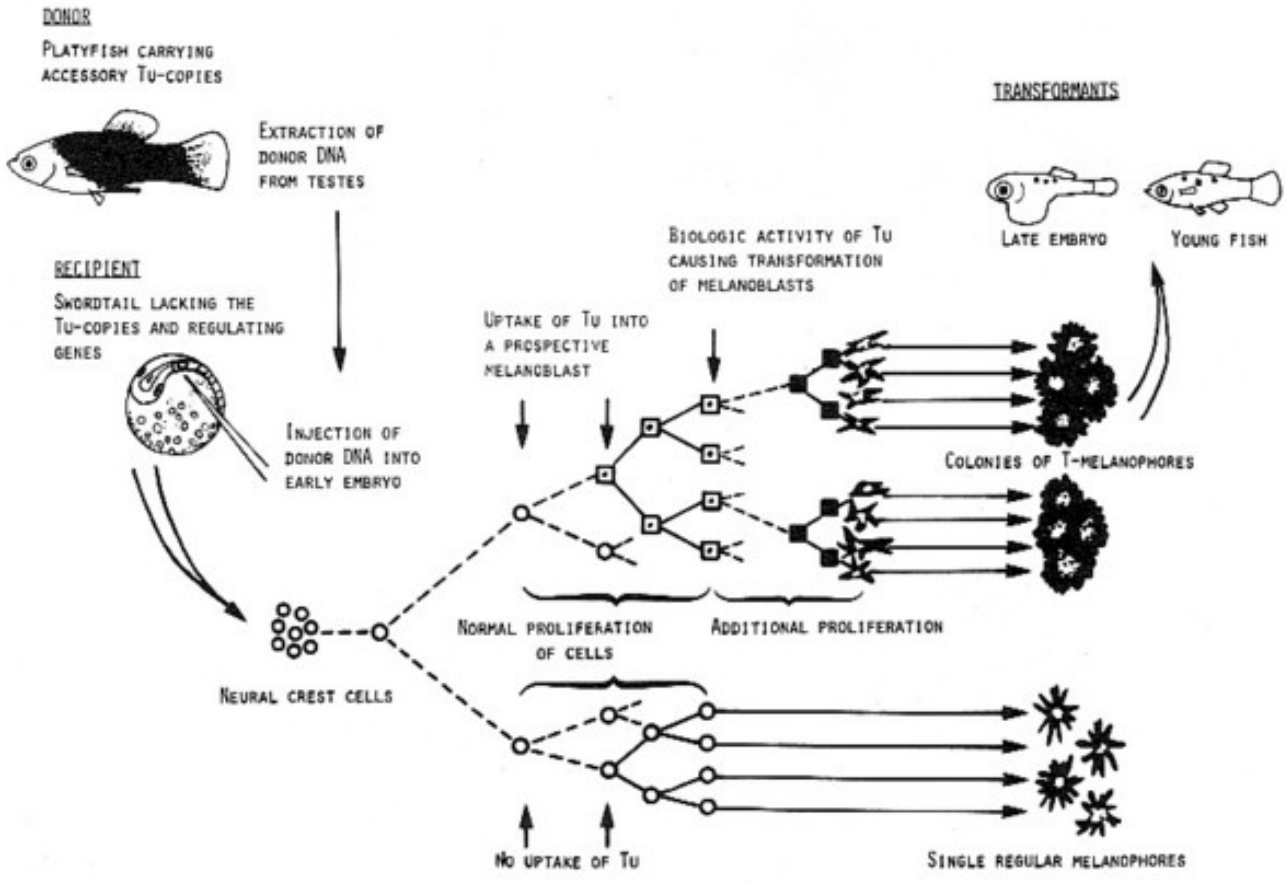

Fig. 10. Schematic presentation of the outcome of *Tu* transfer by injection of purified DNA (modified from a scheme in [25]). For details see text

frequency of the transforming event on the cellular basis is in the range of 10^{-5} [25]. This is the same order of transfection frequencies reported for cell culture systems [25–27].

M. The *c-src* Oncogene in the *Tu* Melanoma System

Several virological observations came to our knowledge which could be of interest for a molecular interpretation of the *Tu* gene: In chicken it was found that the oncogene *v-src* from Rous sarcoma virus (RSV) has a counterpart, *c-src*, in the noninfected cells [28]. *C-src* or at least a similar gene was also found in mammals including mouse, calf, and humans [29]; and commercial DNA derived from salmon [29] obviously contains the same gene. There is, however, no convincing evidence to relate the cellular *src* or its gene product, a 60,000-dalton phosphoprotein with kinase activity ($pp60^{c\text{-}src}$), to neoplasia that depends on conditions other than virus infections (see discussions in [30, 31]). With this background we started the search for *c-src* in the genome of *Xiphophorus*.

C-src was detected in *Xiphophorus* by molecular hybridization of a *src*-specific probe from cloned *v-src* with DNA from fish [32]. To identify $pp60^{c\text{-}src}$, brains of the fish were labeled with ^{32}P-orthophosphate, and brain extracts were immunoprecipitated with antisera from RSV tumor bearing rabbis (TBR-serum) followed by polyacrylamide gel electrophoresis. The 60K protein detected in the gel has a tyrosine-specific kinase activity, and represents the $pp60^{c\text{-}src}$ [31].

The kinase activity was measured according to Fig. 11 [33] and then determined (see legends of Figs. 3, 11) in several tissues including skin, liver, spleen, testes, brain, and melanoma. Brain and melanoma always had the highest kinase activity. Genotype-specific differences in kinase activity showed an identical trend in both brain and melanoma [31]. To compare *c-src* expression in nontumorous and tumorous fish, kinase activity was mainly determined in the brains of these fish.

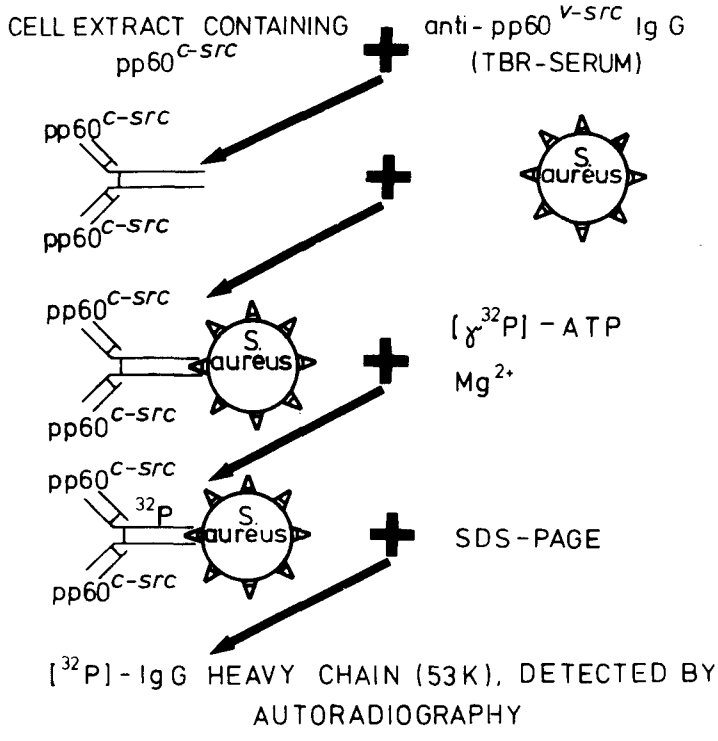

Fig. 11. Assay for pp60$^{c\text{-}src}$ kinase activity according to Collet and Erikson ([33], modified; see also [30, 31, 34])

To study the possible relation between neoplasia and *c-src* expression we manipulated neoplasia in *Xiphophorus* according to the three genetic experiments outlined in Figs. 3, 4 and 12 [34].

1. In the experiment recorded in Fig. 3 the purebred *X. maculatus* carrying two repressed copies of the accessory *Tu*, as well as the purebred *X. helleri* and the BC-hybrids lacking the *Tu*, display the same activity of *c-src* kinase. This activity appears to be the basic expression of *c-src*. In contrast, the melanoma-bearing hybrids which contain the derepressed *Tu* show an increase of *c-src* activity, with the malignant melanoma bearing BC-hybrids displaying the highest activities.

2. In the experiment recorded in Fig. 4 all purebred and hybrid animals, irrespective of the lack and the dosage of *Tu* but dependent upon the nontumorous state exerted either by several regulatory genes or by a linked *R* alone (see the highly susceptible genotype), display a uniform *c-src* activity which seems to represent the basic *c-src* expression, as in the purebred animals and *Tu* lacking hybrids in Fig. 3.

3. In littermates (Fig. 12) which are genetically identical except for the lack of the accessory *Tu* and the presence of one or two partially derepressed accessory *Tu* copies, *c-src* displays a kinase activity that increases stepwise in parallel to the lack and the dosage of *Tu*, which, in their turn, determine whether the animals will develop no tumors, slowly growing tumors, or fast-growing tumors. Table 3 shows additional experiments of the same kind that yielded similar results.

The main results of these experiments are that the nontumorous fish display a basic expression of *c-src* which in the tumorous fish may increase stepwise under two different conditions, namely (a) the stepwise derepression of an accessory *Tu*, and (b) the stepwise introduction of additional copies of a derepressed accessory *Tu*. Since the measurements were accomplished in the brains of the fish the increase of the activity of *c-src* is related di-

Table 3. pp60$^{c\text{-}src}$ associated kinase ativity in brain extracts specified by cpm per milligram soluble protein[a] in F_2-segregants (Data from [34])

Tu gene Complex[c]	Dosage of *Tu*[b]		
	–/–	*–/Tu*	*Tu/Tu*
Striped	90	200	390
Dabbed^{BR}	170	190	390
Dabbed^{RI}	200	260	1240

[a] Three to 8 brains per measurement
[b] One gel each
[c] Different gels each

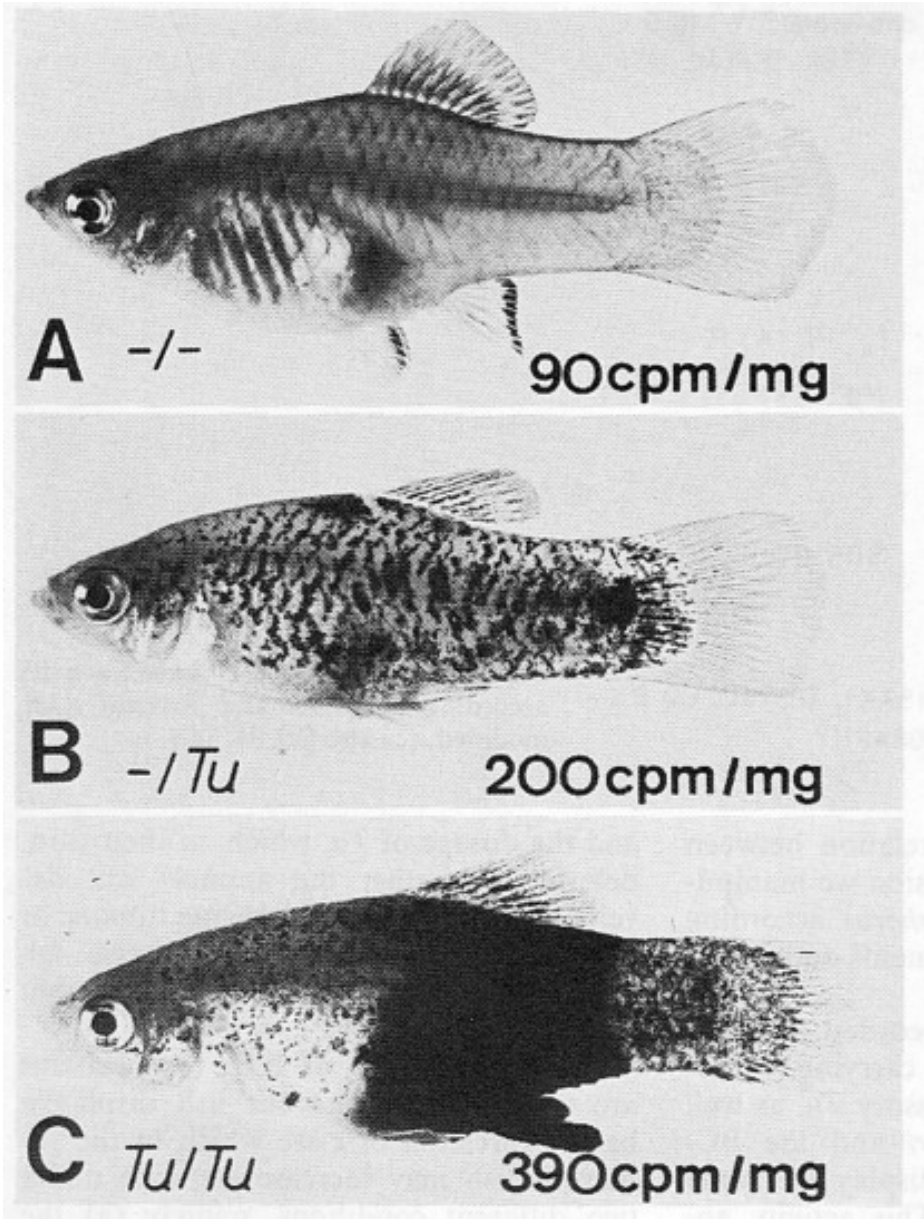

Fig. 12 A–C. Correlation between gene dosage effect of *Tu* (phenotype of the tumor) and gene dosage effect of *c-src* (pp60$^{c\text{-}src}$ kinase activity) in littermates containing **A** no accessory *Tu,* **B** one dosage of *Tu,* **C** double dosage of *Tu.* The genetic backgrounds of the fish are identical. **A** *Tu* is deleted in the germ line. **B, C** The pigment cell-specific *R* linked to *Tu* is impaired by germ line mutation [13, 23]. Kinase data from [34]

rectly to the activity of *Tu*, and does not represent an epiphenomenon of melanoma formation, such as the elevated activity of many enzymes, [5, 6, 14, 15] enhanced melanin synthesis [18], certain chromosome aberrations [35], etc.

These findings suggest several possibilities for an interpretation of how *Tu* might be related to *c-src*: (a) *Tu* might be independent from *c-src*, and the correspondence between both *Tu* and *c-src* is due to linkage relationship. (b) *c-src* might represent a regulatory gene for *Tu* or *vice versa*. (c) *Tu* might consist of different oncogenes that are responsible for different kinds of neoplasia and *c-src* is one of these genes. (d) *Tu* might be identical to *c-src*, and this oncogene is capable of coding for a large variety of neoplasia. At present we cannot decide on a special interpretation. Additional data are required.

N. Distribution of *c-src* in the Animal Kingdom

The presence of *c-src* in the genome of different animals such as chicken, salmon [29], and *Xiphophorus* led to the more systematic search for this oncogene in additional taxonomic groups of animals. Firstly, different groups of *Xiphophorus* and different fish

Table 4. Expression of pp60$^{c\text{-}src}$ kinase in brain extracts of different fish species [31]

Xiphophorus helleri	from Belize River
X. helleri	from Rio Lancetilla
X. maculatus	from Belize River
X. maculatus	from Rio Jamapa
X. maculatus	from Rio Usumacinta
X. cortezi	
X. variatus	from Rio Coy
X. variatus	from Rio Panuco
Girardinus falcatus	
Girardinus metallicus	
Poecilia sphenops	
Belonesox belizanus	
Heterandria bimaculata	
Xenotoca eiseni	

genera more or less related to *Xiphophorus* were investigated. All fish tested (Table 4) show a pp60$^{c\text{-}src}$ kinase activity indicating that *c-src* must be present [31]. In addition, *c-src* was evidenced by its kinase activity in a large variety of metazoans other than fish, ranging from mammals to sponges, which, together with the results from other laboratories are listed in Table 5. *C-src* was not found in protozoa, algae, or higher plants [36].

The distribution of the cellular counterpart of the virl *v-src* brings about the idea that *c-src* might have evolved together with the multicellular organization of the animals, and that neoplasia might be a character that is closely related to this evolution.

O. The Regulatory Gene *Diff* in the *Tu* Melanoma System

The gene *Diff* is one of the most prominent regulatory genes known in the melanoma system of *Xiphophorus*. As shown in Fig. 3, benignancy and malignancy in the hybridization-conditioned melanomas depend upon the presence or absence of the chromosome carrying *Diff*. Biochemical markers for this chromosome, i.e., the esterase *Est-1* and the isozyme A of the glyceraldehyde-3-phosphate dehydrogenase [6] have confirmed that the *Diff*-carrying chromosome is derived from the platyfish, the source of accessory *Tu*.

P. The Major Characters of *Diff* Expressions

The clear-cut Mendelian segregation of benignancy and malignancy has provided the opportunity to study the basic differences between the benign and the malignant state of the melanomas (Fig. 13, Table 6). The cytological, fine structural, biochemical, and biological data suggest that *Diff* promotes the differentiation of T cells: If *Diff* is lacking, the majority of the melanoma cells persist in the stage of the poorly differentiated, continuously dividing TA-melanoblasts and T-melanocytes, and only few cells differentiate to the final stage of the T-melanophores. If, however, the *Diff* is present, the majority of the melanoma cells become terminally differentiated to T-melanophores, whereas only a few cells remain in the stage of TA-melanoblasts and T-melanocytes. The T-melanophores at a certain age are removed by macrophages ([14, 18, 20, 38], see also Fig. 5). This process is antagonistic to the permanent supply of melanoma cells from S-melanoblasts, and thus the melanoma is rendered benign [20, 21].

Table 5. *c-src* in eukaryotes (from [36])

Mammals	Bony fish	Cartilaginous fish	Insects
Humans [29]	Flat fish	Shark	Cockroach
Calf [29]	Sea robin		*Drosophila* [37]
Rat	Mackerel	Jawless fish	
Mouse	Roach	Lamprey	Sponges
	Gudgeon		Marine sponge
Birds	*Xiphophorus*	Acrania	Freshwater sponge
Chicken	Salmon [29]	*Amphioxus*	
Quail	Codfish		
	Cichlid		

Q. Diffusiveness of a *Diff* Dependent Product Involved in Differentiation

Transplantation experiments, including the composition of chimeras by fusion of parts of early embryos, have shown that pigment cell precursors present in the transplants taken from fish carrying *Tu* but lacking *Diff* (see Fig. 3) become incompletely differentiated and give rise to malignant melanoma if transplanted into embryos lacking *Tu* and *Diff*. If, however, the pigment cell precursors of the same genotype were transplanted into *Tu*-lacking embryos that contain the *Diff*, the cells of the developing melanoma become terminally differentiated and regain their distance regulation (Fig. 14). Thus the effect of *Diff* on the differentiation of the neoplastically transformed pigment cells can be traced to a diffusible substance [39]. The nature of this substance is unknown.

R. Modified tRNAs Involved in *Diff* Dependent Differentiation

There is considerable evidence for the involvement of tRNA containing modifications of the nucleotides in the process of cell differentiation in normal and neoplastic tissues [40]. Many studies were focused on a family of tRNAs including $tRNA^{Asn}$, $tRNA^{Asp}$, $tRNA^{His}$, and $tRNA^{Tyr}$ which may contain queuosine (Q) instead of guanosine (G) in the first position of the anticodon (position 34). Q is a hypermodified G. The more the differentiation progresses, the more G is replaced by Q in position 34 [40–46]. The method to estimate the G : Q ratio in a given popu-

Table 6. The Gene *Diff* in *Tu/-Xiphophorus* [2, 6, 14, 18, 20, 21, 38, 39, 40, 46]

Diff/–	–/–
Benign melanoma	Malignant melanoma
Differentiated	Poorly differentiated
Slow-growing	Fast-growing
Noninvasive	Invasive
Nonlethal	Lethal
Difficult to transplant	Easily transplantable
Difficult to promote	Promotion by testosterone, cAMP, corticotropin, BrdUrd, nutrient factors, etc.
Regression following testosterone treatment, etc.	No regression
No vascularization	Vascularization
Weak effect of external factors on growth rate	Drastic effect of external factors on growth rate (temperatue, salinity, cyclic Bt_2AMP, corticotropin, BrdUrd, etc.)
No effect of nutrient factors	Drastic effect of nutrient factors (amino acids)
Many macrophages	Few macrophages
T-melanophores prevail	TA-melanoblasts and T-melanocytes prevail
Endopolyploid and multinucleated	Diploid and uninucleated
Mature melanosomes	Immature melanosomes
Lack of ER and Golgi complexes	Well-developed ER and Golgi complexes
Low enzyme activities	High enzyme activities (tyrosinase, $LDH^{b}B4$, MDH^{c}, etc.)
Low rate of thymidine incorporation	High rate of thymidine incorporation and DNA synthesis
Low pteridine contents	High pteridine contents
First position of the anticodon of $tRNA^{Asp}$, $tRNA^{Asn}$, $tRNA^{Tyr}$, $tRNA^{His}$ contains predominantly queuosine	First position of the anticodon of $tRNA^{Asp}$, $tRNA^{Asn}$, $tRNA^{Tyr}$, $tRNA^{His}$ contains predominantly guanosine
Diff product is diffusible	No product

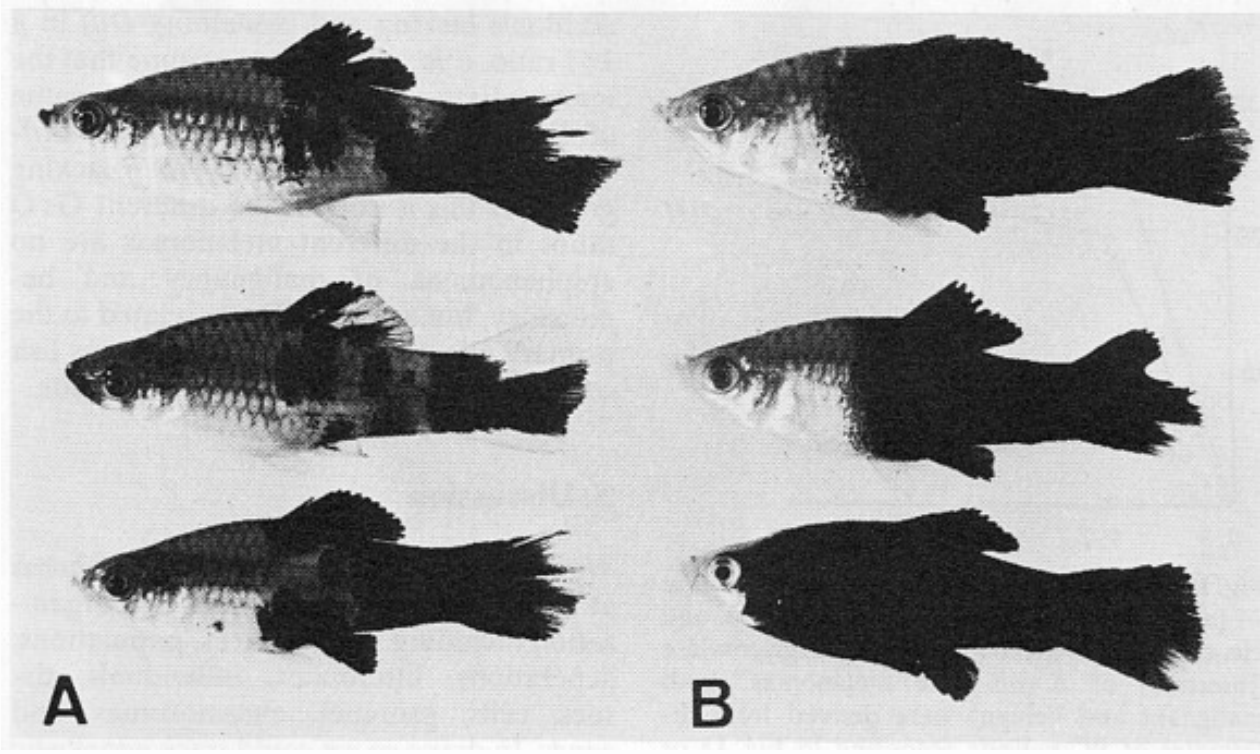

Fig. 13A, B. Segregants of littermates **A** containing one dosage of the differentiation gene (*Diff*) and **B** lacking *Diff*. Segregation of animals carrying benign and malignant melanoma is according to that of the backcross generation shown in the schematic drawings of Fig. 3

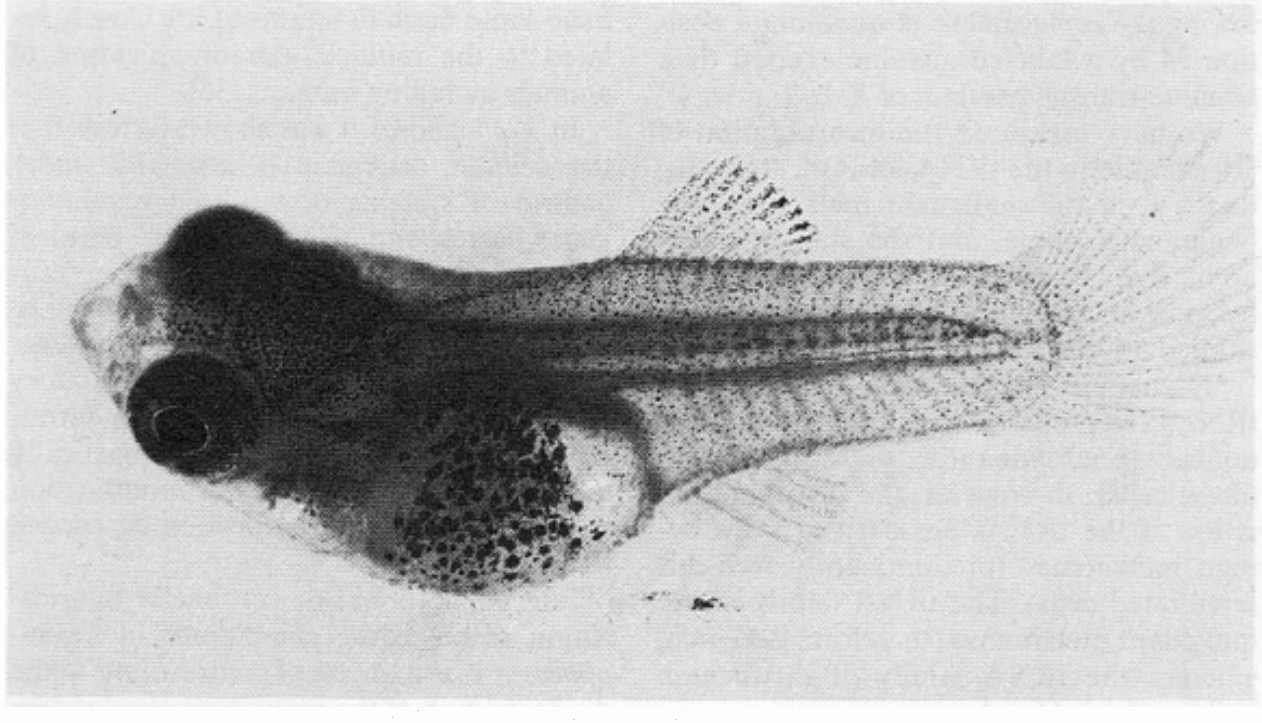

Fig. 14. Secondary chimera composed by transplantation of tissues containing precursor cells of malignant melanoma that originated from a young BC-hybrid containing *Tu* but lacking *Diff* (see malignant melanoma developing BC-segregant in Fig. 3), to a littermate lacking *Tu* but containing *Diff* (see the nontumorous BC-segregant at bottom left in Fig. 3). Note terminal differentiation and distance (density) regulation of the transformed cells of the transplant according to the *Diff* genotype of the host (from [39]). For details see text

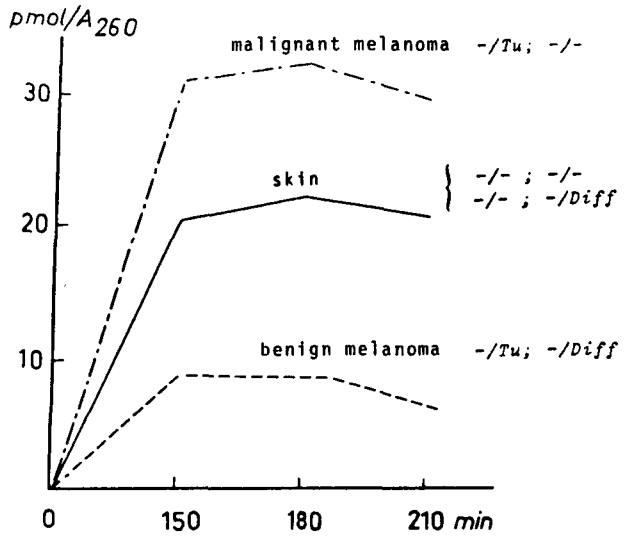

Fig. 15. Incorporation of ^{3}H-guanine in position 34 (anticodon) of tRNAs for Tyr, Asn, Asp, and His catalyzed by tRNA-guanine-transglycosylase (insertase) of *E. coli*. The melanomas (both malignant and benign) were derived from littermates of BC-hybrids according to Fig. 13 or Fig. 3, respectively. The skin was derived from both *Diff*-containing and *Diff*-lacking nontumorous BC-segregants according to Fig. 3. Compare with G/Q data shown in Fig. 3 (from [40, 46]). For details see text

lation of the tRNA family consists in following the replacement of quanine in position 34 by a labeled guanine exerted by a guanine-transglycosylase of *E. coli*.

We have measured the incorporation of ^{3}H-guanine in the tRNA for Asn, Asp, His, und Tyr in the malignant melanoma, the benign melanoma, and the skin of melanoma-free littermates. In addition, F_1-hybrids carrying benign melanomas were studied ([40, 46], Figs. 3, 15):

^{3}H-guanine incorporation is high if the tRNAs are prepared from malignant melanomas (predominantly poorly differentiated cells). In contrast, the incorporation is low if the tRNAs are derived from benign melanomas (predominantly well-differentiated cells). The tRNA family of the malignant melanomas, therefore, is G-rich, whereas the tRNA family of the benign melanomas is Q-rich.

^{3}H-guanine incorporation in the skin of nontumorous littermates is intermediate between those of the malignant and the benign melanomas. Since the nontumorous fish (like the tumorous ones) consist of individuals lacking and containing *Diff* in a 1 : 1 ratio, it is suggestive to assume that the intermediate data represent a mean value of ^{3}H-guanine incorporation in the *Diff*-containing group and in the *Diff*-lacking group. If this is correct the different G : Q ratios in the different melanomas are no epiphenomena of malignancy and benignancy, but are very closely related to the primary effect of *Diff* that in tumorous fish converts the malignant to the benign state.

S. Discussion

We have studied neoplasia of *Xiphophorus* at different levels of the biological organization including species, races, populations, generations, littermates, individuals, tissues, cells, genomes, chromosomes, and genes. In doing so we could trace neoplastic transformation to the activity of one or several copies of the oncogene *Tu* which shows a relation to a cellular counterpart of the transforming *src* oncogene of avian sarcoma virus, the *c-src* [30]. The normal function of *c-src* remained unknown. Since *c-src*, however, was also found in all individuals of all metazoans tested, and was not found in protozoans and plants, it might have some basic functions of life closely related to the multicellular organization of animals including humans [36].

In *Xiphophorus* it was shown further that the cellular oncogene is normally under control of systems of multiple regulatory genes corresponding to regulator genes of bacteria and phages. Some of the regulatory genes are located on other chromosomes than those bearing an oncogene. One of the most prominent regulatory genes appears to be responsible for terminal differentiation of the neoplastically transformed cells exerted via modification of nucleosides in the anticodon of certain tRNAs [40].

Interpopulational or interracial hybridization in preceding generations in *Xiphophorus* is the main event contributing to the disintegration of the regulatory gene system for the oncogene. Germ line mutations that may also disturb the regulatory gene systems are probably less important than hybridization because they are always rare, or may become repaired. Somatic mu-

tations and tumor promotion, which are the majority of carcinogenic triggers, may complete this disintegration. The majority of the neoplasms of *Xiphophorus* belongs to the types that are triggered by carcinogens or promoters on a competent genetic background like their counterparts in humans, which represent about 90% of all human neoplasms (see [8]).

The phenomenon of introducing susceptibility to neoplasia by means of hybridization is not limited to *Xiphophorus*. Many examples have been cited from the animal kingdom [8]. It appears that in animals from wild populations neoplasia is difficult to induce and "spontaneously" developing neoplasms are rare, while in animals of hybrid origin (domesticated and laboratory animals; naturally occurring and experimentally produced hybrids) neoplasia is easily inducible and the incidence of "spontaneously" developing neoplasms is high.

While we do not have hybridization in human beings comparable to hybridization of domesticated or laboratory animals such as fish and mice, it is suggestive to speculate how much effect hybridization may have had on the high tumor incidence observable in some of our highly developed nations. Such speculations are probably of little value in the fight against cancer, but in our search for the cause of human neoplasia they could help to realize the factors that make an individual susceptible to neoplasia and, therefore, sensitive to the carcinogens of our environment.

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