erb-B*[a]: An "Ignition Spark" for the *Xiphophorus* Melanoma Machinery?

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Introduction

Neoplasia is not limited to human beings, or to mammals, but can develop in all taxonomic groups of the recent Eumetazoa and even in multicellular plants. It therefore appears to be inherent to the multicellular organization of life [1]. The oncogenes that are associated with human cancer are also distributed throughout the animal kingdom [2–9]. Moreover, tumor-suppressor genes [10] that may control the expression of oncogenes and the manifestation of a tumor phenotype have been identified in humans and were also detected in the invertebrate *Drosophila melanogaster* and lower vertebrates of the genus *Xiphophorus* [11–17]. According to one current concept, carcinogenesis is a multistep process that includes activation of one or more "dominant acting oncogenes" and the inactivation of tumor-suppressor genes [18, 19]. The lower vertebrate genus *Xiphophorus* offers the possibility to study both the activation of oncogenes and the inactivation of tumor-suppressor genes.

Members of the genus *Xiphophorus*, teleost fish, inhabit freshwaters of the Atlantic drainage systems of Central America [20]. Eighteen species have evolved [21–24], comprising innumerable races and populations which, besides their meristic characters, display a population-specific homogeneous coloration [25–27] composed of melanophores, pterinophores, and purinophores [28–30]. In addition to the homogeneous pigmentation, certain populations exhibit black spot patterns which are composed of giant melanophores [31]. Comparative histological, ultrastructural, and biochemical studies have shown that the giant pigment cells are actually neoplastically transformed pigment cells (Tr melanophores) which in the purebred fish are restrained from proliferation by terminal differentiation [30].

Xiphophorus collected from wild populations in their natural habitat and bred in closed stocks in the laboratory are almost completely insusceptible to neoplasia, i.e., are insensitive to mutagenic carcinogens and tumor promoters, whereas certain hybrids between different populations develop neoplasms spontaneously or after treatment with carcinogens [32–35].

The ability of certain purebreds to form spots in distinct compartments of the body and the capability of certain hybrids to develop melanoma spontaneously or after treatment with carcinogens is coded by a sex chromosomal gene complex, which is accessory in the genome; this complex harbors a Mendelian factor which appears as an oncogene and which was arbitrarily symbolized "tumor gene" *Tu* [13, 32–34, 36]. This complex consists of (a) the pterinophore locus (*Ptr*) which is responsible for pterinophore differentiation, (b) the compartment-specific loci (R_{Co}) which restrict both pterinophore and Tr melanophore differentiation to a distinct part of the body, and (c) the melanophore

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locus (*Mel-Tu*) which, if impaired to *Mel-Tu'*, neoplastically transforms the melanin-containing pigment cells [13, 32, 37]. Both R_{Co} and *Mel-Tu*, which together form the so-called *Tu* complex, are impaired in the accessory *Tu* complex and are therefore oncogenic. The activation of the oncogenic potential of the *Tu* complex may occur in the soma (induced melanoma) or in the germline (spontaneous melanoma in the progeny) [13] by mutation. The activity of the *Tu* complex is regulated by the nonlinked tumor-suppressor gene *Diff*, which if present in the homozygous state, restrains the transformed pigment cells from proliferation by terminal differentiation [13–15, 37].

Comparative studies on the inheritance of restriction fragments with polymorphic lengths in fish with and without the accessory *Tu* complex revealed that *Mel-Tu'* contains a v-*erb*-B-related gene [7, 40–44] that is well known in humans as c-*erb*-B [38, 39]. This gene was named x-*erb*-B* (x-*egfr*-B) by our group [40–42] and X*mrk* by others [43]; in the following pages it will be referred to as x-*erb*-B*. Cloning and subsequent sequencing of the fragments with polymorphic length and a complete cDNA showed that x-*erb*-B* is closely related to the human c-*erb*-B-1 (h-*egf-r*) gene and that it encodes a receptor tyrosine kinase which apparently is a member of the epidermal growth factor receptor (EGF-R) family [9, 40–43, 45]. Southern blot analyses showed that x-*erb*-B* is not only part of the accessory *Mel-Tu'* locus, but also of a locus that is indispensable in the fish; this locus probably harbors the indispensable *Tu* complex, which appears to be inherent in the genome of *Xiphophorus* irrespective of whether these animals have inherited the susceptibility to melanoma [7, 9, 40]. The indispensable, x-*erb*-B* was named x-*erb*-B*^{i}; the x-*erb*-B* which is part of the oncogenic *Mel-Tu'* is accessory in the genome of the fish and was designated x-*erb*-B*^{a} [9, 41]. The sequences of the x-*erb*-B* fragments of polymorphic length showed that there are some differences between x-*erb*-B*^{i}, the X-chromosomal x-*erb*-B*^{a}, and the Y-chromosomal x-*erb*-B*^{a}, respectively (Zechel, Schleenbecker unpublished data and [6–9, 45]). It appears that – at least in some cases – x-*erb*-B*^{i} is also located on the *Mel-Tu'*-carrying sex chromosomes and that x-*erb*-B*^{a} evolved from it by gene duplication [46]. Some species of *Xiphophorus* (e.g., *X. helleri*) have not evolved sex chromosomes, and in these cases x-*erb*-B*^{i} may be located on the corresponding chromosome [7].

It appears that x-*erb*-B* is not the EGF-R gene of the fish but is a slightly different gene [9, 40]. The gene encoding the xiphophorine EGF-R has been partly cloned and sequenced; it was named c-*erb*-B (= x-*egfr*-A, x-*egf-r*) [7, 40–42]. The xiphophorine c-*erb*-B is apparently not part of the *Mel-Tu'* locus and is not inherited in parallel with spots and melanoma [9]; therefore, it appears to be of minor importance for the formation of melanoma.

In this paper we show that c-*erb*-B may be of some relevance for the manifestation of the tumor phenotype. However, since x-*erb*-B**a* is located within the accessory *Mel-Tu'* it appears to be the most likely candidate to act as an "ignition spark" for the *Xiphophorus* melanoma machinery. To verify the importance of x-*erb*-B* in melanoma formation, we studied the inheritance of x-*erb*-B*^{i} and x-*erb*-B*^{a} in fish mutants insusceptible to melanoma or susceptible to inducible and spontaneous melanoma. We show that the indispensable x-*erb*-B*^{i} is expressed in nontumorous tissues of fish harboring and lacking the accessory *Mel-Tu'*. Furthermore, we describe the expression of x-*erb*-B*^{i} and the overexpression of x-*erb*-B*^{a} in different melanomas. The complex relation between the inheritance of x-*erb*-B*, pp60^{x-src} activity, and inositol-lipid turnover are discussed, as well as the possibility of cooperation between x-*erb*-B* and other genes.

Results and Discussion

The x-erb-B^{*i}, *x-erb-B*^{*a}, *and c-erb-B Genes*

Inheritance

To verify the importance of x-*erb*-B^{*a} for melanoma formation we studied the inheritance of all three v-*erb*-B-related genes, namely x-*erb*-B^{*i}, x-*erb*-B^{*a}, and c-*erb*-B (= x-*egf-r*) in fish harboring and lacking the accessory *Tu* complex. We shall recapitulate here that some of the *Tu* complex-mediated spot (Tr melanophore) patterns of the purebreds [13, 21–29] give rise to a melanoma following appropriate interpopulation or interspecific hybridization. Depending on the specificity of the inherited *Tu* complex, melanoma formation may not be inducible [47], or melanoma formation may occur spontaneously or after induction with promoters and initiators [33]. Studies on polymorphic lengths of restriction fragments (RFLP analyses) showed that the x-*erb*-B^{*a} genes map to the *Tu* complexes which are terminally located on the X- and Y(Z)-chromosomes of *X. maculatus* and *X. variatus* [48]. More specifically, this was shown by the detection of *Eco*R1 fragments specific for the x-*erb*-B^{*a} of the respective *Tu* complex: fragments of 4.9 kilobases (kb) and 11.0 kb were assigned to the *Tu* complex of a certain X-chromosome of *X. maculatus* and *X. variatus*, respectively; the *Tu* complexes of a certain *X. maculatus* Y- and Z-chromosome harbor a 6.7-kb fragment. The x-*erb*-B^{*i} and the c-*erb*-B (x-*egf-r*) are not located within the respective loci.

We studied the inheritance of x-*erb*-B*-(x-*erb*-B^{*i}- and x-*erb*-B^{*a}-) and c-*erb*-B-(x-*egf-r*-)specific restriction fragments in purebred *X. xiphidium* and *X. andersi*, as well as in different purebreds and hybrids harboring and lacking aberration in the *Tu*-complexes.

Figure 1 (A, B, left) shows that Southern analyses with a v-*erb*-B-specific probe

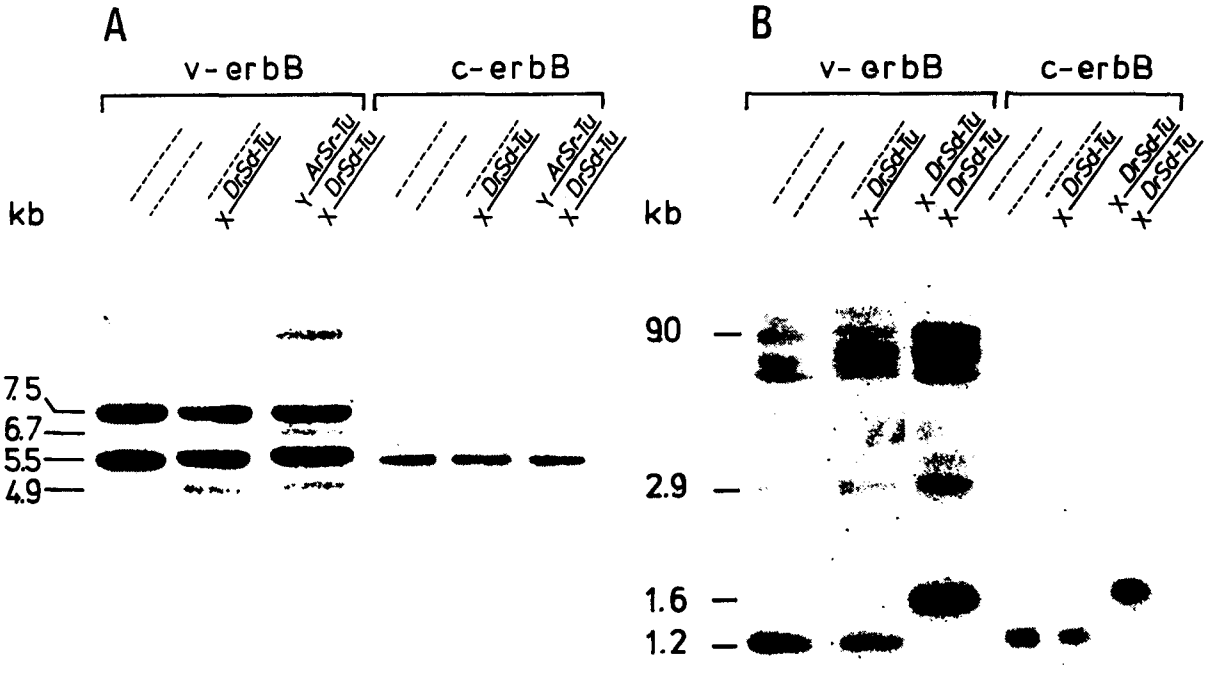

Fig. 1 A, B. Restriction fragments homologous to both v-*erb*-B and the xiphophorine EGF receptor gene c-*erb*-B (= x-*egf-r*). Hybridization against **A** *Eco*R1 and **B** *Hin*dIII-digested genomic DNA from *X. helleri* from Rio Lancetilla (---/---), *X. maculatus* from Rio Jamapa (male, X *Dr Sd-Tu*/Y *Ar Sr-Tu;* female X *Dr Sd-Tu*/X *Dr Sd-Tu*), and a BC hybrid *mac/hell* (X *Dr Sd-Tu*/---). Filters were probed with v-*erb*-B under stringent conditions (40% formamide; 1 × SCC/1% SDS, 65 °C) and with c-*erb*-B under high-stringency conditions (50% formamide; 0.1 × SSC/1% SDS, 68 °C)

Table 1. Presence of x-*erb* B*-specific *Eco* R1 fragments in purebreds and hybrids with and without aberrant sex chromosomes

Species (river provenance)/ interspecific hybrid	m, f / F_1/BC	Phenotypically detected chromosome aberration (sex, *Pter*, *Tu* complex)		x-*erb* B* fragments (kb)	No. of fish analyzed[a]
X. maculatus (Rio Jamapa)	f	X *F Dr Sd-Tu* / X *F Dr Sd-Tu*	–	7.5, 4.9	16
X. maculatus (Rio Jamapa)	m	Y *M Ar Sr-Tu* / X *F Dr Sd-Tu*	–	7.5, 6.7, 4.9	22
X. helleri (Rio Lancetilla)	f	———— / ————[b]	–	7.5	82
X. helleri (Rio Lancetilla)	m	———— / ————[b]	–	7.5	147
X. helleri (Rio Lancetilla)	f	*Db-Tu* / *Db-Tu*	–	7.5	2
X. helleri (Rio Lancetilla)	m	*Db-Tu* / *Db-Tu*	–	7.5	2
mac/hell	BC_n	X *F Dr Sd-Tu* / ————	–	7.5, 4.9	22
	BC_n	X *F Dr Sd-Tu'* / ————	*Sd-Tu* mutation	7.5, 4.9	5
	BC_n	X *F Dr* ——— / ————	*Sd-Tu* deletion	7.5, 4.9	32
	BC_n	X *F Dr* ——— / ————	*Sd-Tu* deletion	7.5	1
	BC_n	X *F Dr* ——— / ————	*Sd-Tu* deletion	12.5, 7.5	4
	BC_n	X *F Dr* ——— / ————	*Sd-Tu* deletion	13.0, 7.5	10
	BC_6	X *F Dr* ——— / ————	*Li-Tu* deletion	7.5, 4.9	15
mac/hell	BC_n	*Sd-Tu* / ————	*Sd-Tu* translocation	7.5, 4.9	10
	BC_6	*Sd-Tu* / ————	*Sd-Tu* translocation	7.5, 4.9	6
mac/hell	BC_n	Y *M Ar Sr-Tu* / ————	–	7.5, 6.7	8
	BC_n	X *F Dr Ar Sr-Tu* / ————	*Ar Sr-Tu* translocation	7.5, 6.7	9
	BC_n	X *F Dr ar* ——— / ————	*Sr-Tu* deletion	7.5	11
	BC_1	Y *M* ——— / ————	*Ar Sr-Tu* deletion	7.5	4
	BC_1	*Sr-Tu* / ————	*Sr-Tu* translocation	7.5, 6.7	2
X. variatus (Rio Panuco)	f	X *F Ye Li-Tu* / X *F Ye Li-Tu*	–	11.0, 7.5	8
X. variatus (Rio Panuco)	m	Y *M Or Pu-Tu* / X *F Ye Li-Tu*	–	11.0, 7.5	16
var/hell	BC_n	X *F Ye Li-Tu* / ————	–	11.0, 7.5	4
var/mac	F_1	X *F Ye Li-Tu* / X *F Dr Sd-Tu'*	*Sd-Tu* mutation	11.0, 7.5, 4.9	5
var/mac/hell	BC_5	X *F Dr Li-Tu* / ————	*Li-Tu* translocation	7.5, 4.9	15
X. maculatus (belize River)	f	W *F* ——— / Z *M Br Ni-Tu*	–	7.5, 6.7	9
X. maculatus (Belize River)	m	Z *M Br Ni-Tu* / Z *M Br Ni-Tu*	–	7.5, 6.7	13
X. maculatus (domestic stock)	f	W *F C''-Tu* / Z *M Br N'-Tu*	*Ni-Tu* mutation	14.0, 7.5, 4.9	1
X. maculatus (domestic stock)	m	Z *M Br N'-Tu* / Z *M Br N'-Tu*	*Ni-Tu* mutation	7.5, 4.9	1
mac/hell	F_1	W *F C''-Tu* / ————	*Ni-Tu* mutation	14.0, 7.5	3
	F_1	Z *M Br N'-Tu* / ————	*Ni-Tu* mutation	7.5, 4.9	2

Table 1 (continued)

Species (river provenance)/ interspecific hybrid	m, f / F_1/BC	Phenotypically detected chromosome aberration (sex, *Pter*, *Tu* complex)		x-*erb* B* fragments (kb)	No. of fish analyzed[a]
X. maculatus (Belize River)	f	W *F* / Z *M Br Ne-Tu*	–	7.5, 6.7	4
X. maculatus (Belize River)	m	Z *M Br Ne-Tu* / Z *M Br Ne-Tu*	–	7.5, 6.7	9
mac/hell	BC_3	Z *M Br Ne-Tu* / ----------	–	7.5, 6.7	5
	BC_3	Z *M Br Ne-Tu* / ----------	–	8.5, 7.5, 6.7	1

[a] All fish were analyzed individually;

[b] *X. helleri* has not evolved sex chromosomes; dashes indicate the corresponding chromosome; m, male; f, female; F_1, hybrid; BC_n, highly backcrossed individuals (higher than BC_7); sex, sex locus; *M*, *F*, sex-determining region; *Ptr* = pterinophore pattern: *Dr*, dorsal red; *Ar*, anal red; *Ye*, yellow; *Or*, orange; *Br*, brown. *Tu* complex, Tr melanophore pattern: *Sd*, spotted dorsal; *Sr*, stripe sided; *Db*, dabbed; *Sd'*, spotted dorsal, modified; *Li*, lineatus; *Pu*, punctatus; *Ni*, nigra; *N'*, patched nigra; *Ne*, nigra extended; *C''*, phenotypically similar to crescent' (*C'*).

revealed a differing distribution of several v-*erb*-B homologous (A) *Eco*R1 and (B) *Hin*dIII fragments in the spotted *X. maculatus* from Rio Jamapa (male, Y *Ar Sr-Tu*/X *Dr Sd-Tu;* female, X *Dr Sd-Tu*/X *Dr Sd-Tu*), the nonspotted *X. helleri* from Rio Lancetilla (---/---; the accessory *Tu* complex and x-*erb*-B*[a] are lacking), and the melanoma-bearing interspecific hybrid (X *Dr Sd-Tu*/---) (symbols, see Table 1); *X. helleri* was used as a suitable recurrent parent to generate these BC hybrids. Besides the *Eco*R1 fragments of 4.9 kb and 6.7 kb that map to the sex chromosomal *Tu* complexes *Sd-Tu* and *Sr-Tu*, respectively, Fig. 1 A (section v-*erb*-B) shows two v-*erb*-B-homologous *Eco*R1 fragments of 5.5 kb and 7.5 kb that are present in all fish of the genus *Xiphophorus* analyzed to date, irrespective of whether these animals harbor accessory *Tu* complexes. Hybridization of the v-*erb*-B-specific probe against DNA preparations of *X. xiphidium* from Rio Soto la Marina reveals accessory *Eco*R1 fragments of 2.6 and/or 2.8 kb, one of which is always present in F_1 hybrids of *X. xiphidium*/*X. andersi;* these fragments, therefore, may also represent parts of a x-*erb*-B*[a] (not shown). As mentioned above, *X. helleri*, in general, neither harbors an accessory *Tu* complex nor any of the x-*erb*-B*[a]-specific *Eco*R1 fragments. As an exception, a certain race of *X. helleri* may develop a spot pattern called "dabbed" (*Db-Tu*) which, however, does not give rise to melanoma formation following interspecific hybridization [47], thus far we could not assign any accessory x-*erb*-B*[a]-specific *Eco*R1 fragment to the *Db-Tu* (Table 1). Provided the presence of an accessory *Eco*R1 fragment, that is to say the presence of a certain x-*erb*-B*[a], is a prerequisite for the susceptibility to melanoma, then the interspecific hybrids carrying the *Db-Tu* are insusceptible to neoplasia, possibly because they do not harbor any of the accessory x-*erb*-B* gene fragments that we mentioned in the last paragraph. It remains unclear whether a

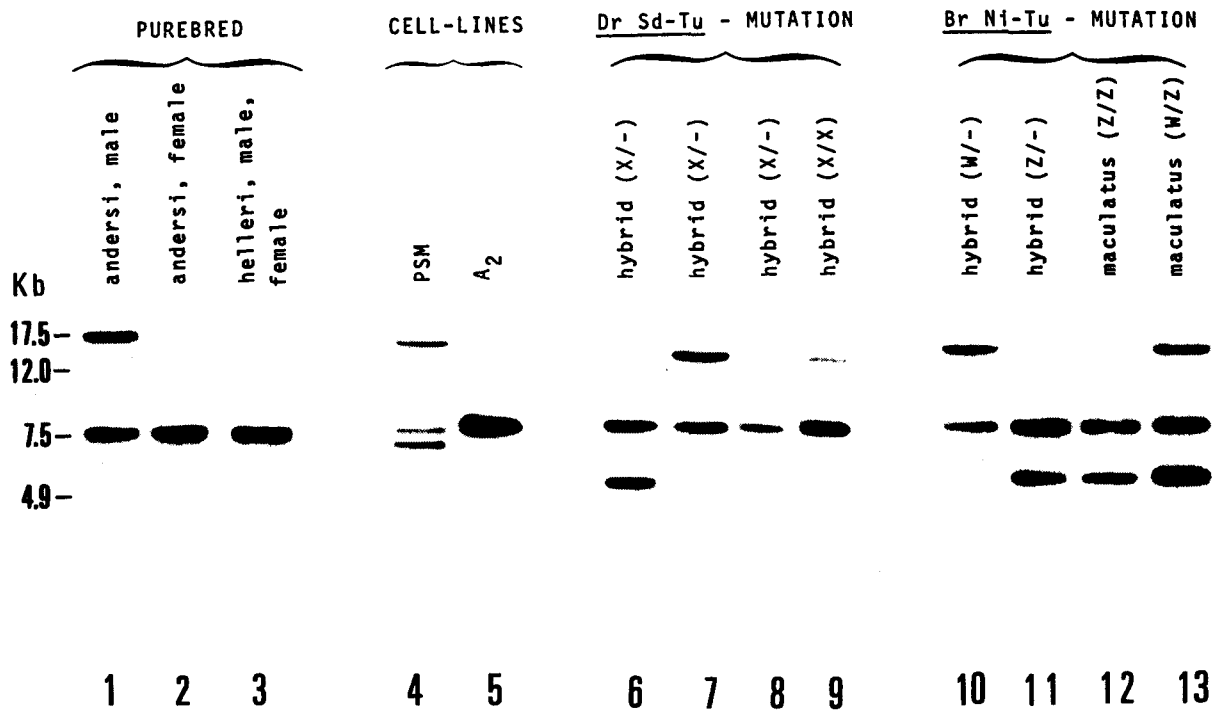

Fig. 2. Inheritance of x-*erb*-B*^{i} and x-*erb*-B*^{a} in the purebreds *X. andersi* (Rio Atoyac) and *X. helleri* (Rio Lancetilla), in the cell lines PSM and A_2, and in purebreds and/or hybrids that display mutations of the X-chromosomal *Dr Sd-Tu* and the Z-chromosomal *Br Ni-Tu*, respectively (Southern blot analysis). The 7.5-kb *Eco*R1 fragment is indispensable (x-*erb*-B*^{i}), while the *Eco*R1 fragments of 17.5, 16, 14, 13, 12.5, 6.7 and 4.9 kb are accessory (x-*erb*-B*^{a}). Filters were probed with x-*erb*-B*-specific probes under high stringency conditions (50% formamide; 0.1 × SSC/1% SDS, 68 °C)

mutation in x-*erb*-B*^{i} may cause the formation of the dabbed spot pattern.

Figure 1 (A, B, right) shows that hybridization of the c-*erb*-B- (x-*egf-r*-) specific fragment against *Eco*R1-digested DNA led to the detection of a single 5.5-kb band; this is true for all genotypes analyzed [7, 9]. In contrast, this fragment detects *Hin*dIII fragments of polymorphic length (RFLP). This RFLP is not inherited in parallel with the X-chromosome and the tumor phenotype: The 1.6-kb band of *X. maculatus* undergoes a successive substitution for the 1.2-kb band of *X. helleri* in the hybrids during the process of introgressive hybridization.

When hybridized against *Eco*R1-digested genomic DNA, the cloned 4.9-kb and 6.7-kb *x-erb*-B*^{a}-specific fragments detect themselves and generally a 7.5-kb band [9, 40]. The 7.5-kb band shows the presence of the indispensable x-*erb*-B*^{i} (Fig. 2, lane 3). Hybridization of these probes against genomic DNA from *X. andersi* from Rio Atoyac [23] revealed the presence of the indispensable 7.5-kb *Eco*R1 fragment in females (Fig. 2, lane 2); besides this fragment, the probe detected an additional *Eco*R1 fragment of 17.5 kb in the male (Fig. 2, land 1).

In Southern hybridizations of the *x-erb*-B*-specific probes against *Hin*dIII-digested DNA (data shown in [7]), the presence of the X-chromosomal x-*erb*-B*^{a} is detectable by the polymorphic length of the *Hin*dIII restriction fragments. The length of the *Sd-Tu*-specific *Hin*dIII fragment is 7.2 kb. The presence of the indispensable x-*erb*-B* as well as the presence of the Y-chromosomal x-*erb*-B*^{a} is marked by a 9.2-kb band.

To answer the question whether inactivation or loss of the above-mentioned x-*erb*-B*^{a} fragments and x-*erb*-B*^{a} genes will result in loss of the capability to form melanoma spontaneously or after induction with carcino-

gens, we bred mutants showing phenotypically detectable mutations of the sex chromosomal gene complex including *Ptr* and the *Tu* complex. Several of the mutants used were genetically and phenogenetically analyzed in 1973 [32, 40]. Since then, more new mutants have been isolated and studied (for photographs, see [7, 32, 34]; A. Anders, unpublished data). The mutations studied in this work concern the X-chromosome and the Y-chromosome of *X. maculatus* from Rio Jamapa, and the Z-chromosome of *X. maculatus* from Belize River. We would like to point out here that the gene complexes of interest are arranged in an uniform order on the respective chromosomes with (a) the sex-determining region proximal to the centromer, followed by (b) the *Ptr* loci (*Dr*, *Ar*, *Br*) and (c) the accessory *Tu* complexes (*Sd-Tu*, *Sr-Tu*, *Ni-Tu*, *Ne-Tu*) (symbols see Table 1). The pterinophore loci serve as phenotypically detectable markers in purebreds and hybrids with and without aberrant sex chromosomes; the activity of the accessory *Tu* complex in question is monitored by the formation of spots in the purebred and the susceptibility to melanoma in the hybrids.

The X-chromosome of *X. maculatus* from Rio Jamapa shows the following gross constitution: X *F Dr Sd-Tu* (*F*, female; *Dr*, dorsal red; *Sd-Tu*, Tr melanophores in the dorsal fin). Hybrids (*mac/hell*) carrying this chromosome show a dorsal reddish coloration and develop melanoma spontaneously in the dorsal fin; the presence of x-*erb*-B*^{a} in the *Sd-Tu* region is disclosed by the 4.9-kb *Eco*R1 fragment (Table 1). The 4.9-kb fragment is also present in the mutant *Sd'-Tu*, which shows an enlarged spot pattern in the purebred and a melanoma which may cover large parts of the dorsal part of the body as well as parts of the fins and the mouth.

We isolated five types of hybrids with aberrant X-chromosomes; these hybrids appeared to be "loss of function" mutants, since they lost the ability to form dorsal melanomas (may be referred to as *Sd-Tu* deletion); the *Dr* locus mediates a dorsal reddish coloration of the body and thereby serves as a marker for the presence of the X-chromosome. One type of mutant lost both the ability to form a melanoma and the x-*erb*-B*^{a}-specific 4.9-kb band (Fig. 2, lane 8). Two types of mutant retained the 4.9-kb fragment (Fig. 2, lane 6). Two other types of mutant lost the 4.9-kb band but gained a 12.5-kb and 13-kb fragment, respectively (Fig. 2, lanes 9 and 7; Table 1). These data show that loss of function of the *Sd-Tu* locus in the different reddish mutants is caused by different events. In three cases the loss of function appears to involve gross structural changes in the x-*erb*-B*^{a} locus. Molecular analysis of a comparable mutant with a 12-kb *Eco*R1 fragment showed that the loss of function mutation may be due to a insertion [43]. Since the 4.9-kb and the 6.7-kb x-*erb*-B*-specific probes used for the present analysis cannot detect the complete x-*erb*-B*^{a} gene [7], and since the Southern blot hybridization cannot monitor minor changes in a gene (e.g., point mutations), it remains unclear whether the mutants with the "intact" 4.9-kb fragment show a mutation of the x-*erb*-B*^{a} locus. Since these nonspotted animals develop melanoma with low frequency following treatment with carcinogens [40], it appears that the oncogenic potential of the *Sd-Tu* locus, and possibly of the respective x-*erb*-B*^{a} locus, may have been inactivated in this mutant by a point-mutation or other small structural changes.

Besides the *Sd-Tu* "deletion," we studied two mutants which phenotypically exhibit a translocation of the *Sd-Tu* locus onto an autosome of *X. helleri*. The mutations occurred independently and had different influences on the sex determination processes in the hybrids; both mutants, however, lost their reddish coloration but retained the ability to develop a dorsal melanoma and the x-*erb*-B*^{a}-specific 4.9-kb band (Table 1).

The Y-chromosome of *X. maculatus* from Rio Jamapa shows the following

gross constitution: Y *M Ar Sr-Tu* (*M*, male; *Ar*, anal red; *Sr-Tu*, stripe sided, Tr melanophores on the body side). Hybrids (*mac/hell*) carrying this chromosome show a reddish coloration of the whole body and may develop a clonal melanoma after treatment with tumor-initiating agents [13, 33, 35]; the presence of x-*erb*-B*^{a} in the *Sr-Tu* region is disclosed by a 6.7-kb *Eco*R1 fragment (Fig. 1).

We studied four types of mutational changes at the *Ar Sr-Tu* locus: (a) two types of change which appear to be due to a translocation of the terminal region of the Y-chromosome onto the X-chromosome in *X. maculatus* and onto an autosome in *X. helleri*, and (b) two types of change that apparently involve a "loss of function" or a deletion of the *Sr-Tu* locus. The results are summarized in Table 1. The type of mutant which gained the X/Y-chimeric chromosome (X *F Dr Ar Sr-Tu*) lost both the ability to develop melanoma spontaneously and the *Sd-Tu*-specific 4.9-kb *Eco*R1 fragment, but gained the sensitivity to initiators and the 6.7-kb *Eco*R1 fragment. Mutation of X *F Dr Ar Sr-Tu* (may be referred to as *Sr-Tu* deletion) in a BC hybrid resulted in progeny that showed an intense reddish coloration but lacked the ability to develop a melanoma spontaneously or after treatment with carcinogens; these animals neither harbor the 4.9-kb nor the 6.7-kb *Eco*R1 fragment (Table 1). Another type of *Sr-Tu* deletion was observed in an *X. maculatus* purebred: The animals lost the reddish coloration of the anal fin and the Tr melanophore pattern "stripe sided"; interspecific hybridization of these mutants (*mac/hell*) resulted in an F_1 generation that was free of Tr melanophores when it inherited the Y-chromosome; both the purebreds and the hybrids that carry the aberrant Y-chromosome only harbor the indispensable 7.5-kb fragment. The second translocation event was also observed in the purebred *X. maculatus:* the animals lost the reddish coloration of the anal fin but retained the spot pattern "stripe sided" and the 6.7-kb *Eco*R1 fragment. The respective BC hybrids (*mac/hell*) lack the reddish coloration, but show, besides intense green and blue stripes, an enlarged *Sr-Tu* spot pattern; these animals possess the 6.7-kb *Eco*R1 fragment. The hybrids are now being tested for their sensitivity to carcinogens. The investigation of the accessory *Tu* complex of the *X. maculatus* Y-chromosome strongly suggests that the presence of a functional x-*erb*-B*^{a} copy is a prerequisite for the capability to develop melanoma after treatment with tumor-initiating agents.

The Z-chromosome of *X. maculatus* from Belize River (male, ZZ; female, WZ) shows the following gross constitution: Z *M Br Ni-Tu* or Z *M Br Ne-Tu* (*M*, male; *Br*, brown; *Ni-Tu*, large black spots on the body side, nigra [47]; *Ne-Tu*, body side is almost completely black, nigra extended). The accessory *Tu* complexes *Ni-Tu* and *Ne-Tu*, respectively, harbor an x-*erb*-B*^{a} that was diclosed by a 6.7-kb *Eco*R1 fragment [40]. The W-chromosome does not harbor an x-*erb*-B*^{a} that can be detected by the polymorphic length of *Eco*R1 fragments (Table 1). Hybrids (*mac/hell*) carrying one of the mentioned Z-chromosomes show a brownish coloration of the body and may spontaneously develop melanoma.

We shall firstly describe a new type of *Ni-Tu* mutant and secondly discuss an aberrant *Eco*R1 fragment pattern in one female with *Ne-Tu;* the results are summarized in Table 1. The mutation of the *Ni-Tu* locus resulted in a changed Tr melanophore pattern in the purebred *X. maculatus:* the animals show a modified spot pattern on the body side and a new pattern in the caudal fin. The aberrant *Ni-Tu* was named *N'-Tu* (patched nigra), while the new spot-pattern was designated *C''-Tu*, because of its similarity to the pattern *C'-Tu* [47]. Hybridization of the x-*erb*-B*-specific probe against *Eco*R1-digested DNA of the female (WZ) reveals bands of 14 kb, 7.5 kb (x-*erb*-B*^{i}), and 4.9 kb (Fig. 2, lane 13). The male (ZZ) shows bands of 7.5 kb and 4.9 kb (Fig. 2, lane 12). Hybrids (*mac/*

hell) with the pattern *C″-Tu* inherited the 14-kb and the 7.5-kb bands, while hybrids with *N′-Tu* inherited the 7.5-kb and the 4.9-kb bands (Fig. 2, lanes 10 and 11). These data support the idea that the 4.9-kb fragment can be assigned to the Z-chromosomal *N′-Tu*, while the 14-kb fragment is probably part of the W-chromosomal *C″-Tu*. We assume that a crossover between the W- and Z-chromosomes changed the Z-chromosomal *Ni-Tu*-region, including the x-*erb*-B*^{a}, and, furthermore, generated an accessory W-chromosmal x-*erb*-B*^{a} that is detectable by the polymorphism of restriction fragment lengths. We wonder whether the changes of the W- and Z-chromosomal x-*erb*-B*^{a} imply changes in the susceptibility of the mutant to neoplasms. Investigation of the susceptibility of (highly backcrossed) hybrids to neoplasms on the one hand, and the molecular structure of the x-*erb*-B*^{a}-fragments on the other hand, will give some information of both the structural changes of the W- and Z-chromosomes and their relevance for melanoma formation.

The "*Ne-Tu* mutant" was detected when we analyzed the x-*erb*-B*-specific *Eco*R1 fragments of BC hybrids harboring the Z *M Br Ne-Tu* chromosome of *X. maculatus* (Belize River); the hybrid did not exhibit changes of the phenotype. Hybridization of the x-*erb*-B*-specific probe against digested DNA disclosed the expected 6.7-kb and 7.5-kb fragments and an additional one of 8.5 kb (Table 1). This additional band may possibly indicate a point mutation in one allele (generation and/or deletion of an *Eco*R1 site) and/or duplication of an x-*erb*-B* gene.

Expression in a Melanoma Cell Line, the Melanoma, and Non-melanomatous Cell Lines

Preparations of total RNA and polyA^{+}-selected RNA were subjected to northern blot analyses using rat β-actin [49], *Drosophila* β_1-tubulin [50], and *Xiphophorus* rRNA [51, 52] as a control for the amount of RNA applied to the nylon membrane; moreover, the filters were stained with methylene blue and the stain was scanned with a densitometer. To calculate the size of a detected mRNA, we coseparated a commercially available size marker (RNA ladder, BRL) and/or human 28 *S* and 18 *S* rRNA on the formaldehyde agarose gels, and utilized *Xiphophorus* 28 *S* rRNA (3.3 kb [51]) and 18 *S* rRNA (1.8 kb [51]) as internal size markers.

Melanoma Cell Line PSM. The PSM cell line was derived from a hereditary melanoma of a hybrid exhibiting a Tr melanophore pattern on the body side [54]. This cell line, which was immortalized during subculturing, exhibits tyrosinase activity [54] and is able to form foci in the culture dish and colonies in soft agar ([55], H. Schäfer-Pfeiffer, K. Krüger, C. Zechel, unpublished data). Hybridization of the x-*erb*-B*-specific probes against digested DNA revealed *Eco*R1 fragments of 16 kb, 7.5 kb, and 6.7 kb (Fig. 2, lane 4), and *Hin*dIII fragments of 9.2 kb and 4.8 kb [7]. The fragments of 7.5 kb and 6.7 kb probably represent x-*erb*-B*^{i} and the Y-chromosomal x-*erb*-B*^{a}, respectively, while the 16-kb fragment may be the result of a rearrangement of a x-*erb*-B* gene.

Both types of gene, the x-*erb*-B* (x-*erb*-B*^{i} and x-*erb*-B*^{a}) and the c-*erb*-B (x-*egf-r*), are expressed in PSM cells [7, 40, 56]. Two x-*erb*-B*-specific transcripts (4.6 kb and 4.0 kb) were detected; the 4.6-kb transcript is a mRNA of low abundance while the 4.0-kb mRNA is highly abundant (Table 2; Fig. 3). Northern hybridization against varying amounts of polyA^{+}-selected RNA proved the mRNA character of the 4.6-kb and 4.0-kb transcripts (smaller RNA species, see p. 223). The 4.6-kb mRNA probably represents the transcript of the x-*erb*-B*^{i} gene, while the 4.0-kb mRNA is probably transcribed from the x-*erb*-B*^{a} locus disclosed by the 6.7-kb Southern band; thus far, we have not been able to assign any detectable transcript to the locus disclosed by the 16-kb Southern fragment. Our studies on the expression of x-*erb*-B* genes in

Table 2. Expression of c-*erb* B (x-*egf-r*) and x-*erb* B* in xiphophorine cell lines, embryos, juvenile fish, tissues of the adult fish, and melanomas

Origin of RNA	c-*erb* B (=x-*egf-r*) 4.5-kb mRNA	Indispensable x-*erb* B* 4.6-kb mRNA	Accessory x-*erb* B*	
			4.0-kb mRNA	8.0-kb mRNA
Cell lines				
A2	+(+)	++(+)	0	0
PSM	++ [a]	+	++++	0
Sd' Sr'	n.t.	0	0	0
Golden	n.t.	0	0	0
Embryo, stage				
0	+++	+++	0	0
1–4	++	++	0	0
5–9	++	+(+)	0	0
10–12	++	+(+)	0	0
13–14	++	+(+)	0	0
15–16	++	+(+)	0	0
17–20	++	++	0	0
21–24	++	+(+)	0	0
25–26	++	+(+)	0	0
Juvenile fish, day after birth				
1–10	++	++	0	0
Adult fish, tissue				
Brain	+	+	0	0
Eye	+	+	0	0
Fin	n.t.	+	0	0
Gill	+++	++(+)	0	0
Heart	+	0	0	0
Kidney	+++		0	0
Liver	0	0	0	0
Muscle	0	0	0	0
Skin	n.t.	+	0	0
Spleen	+(+)	0	0	0
Testis	+	+	0	0
Melanoma				
Spontaneous				
benign	0–+	+	+–++	0
malignant	+–++	+	++–++++	0
Promoted				
benign	+–++	+	+–++	0
malignant	++	+	++–++++	0
malignant [b]	+	(+)	0	+–+++
Initiated				
benign	0–+	+	+–++	0
malignant	+	+	++–+++	0

o, not detectable; +, detectable (low expression); ++, expression; +++, high expression; ++++, very high expression; ( ), intermediate; n.t., not tested.

[a] mRNA of 4.3 kb.

[b] Fish with the X-chromosomal *Li-Tu* express a x-*erb* B*-specific RNA of 8.0 kb.

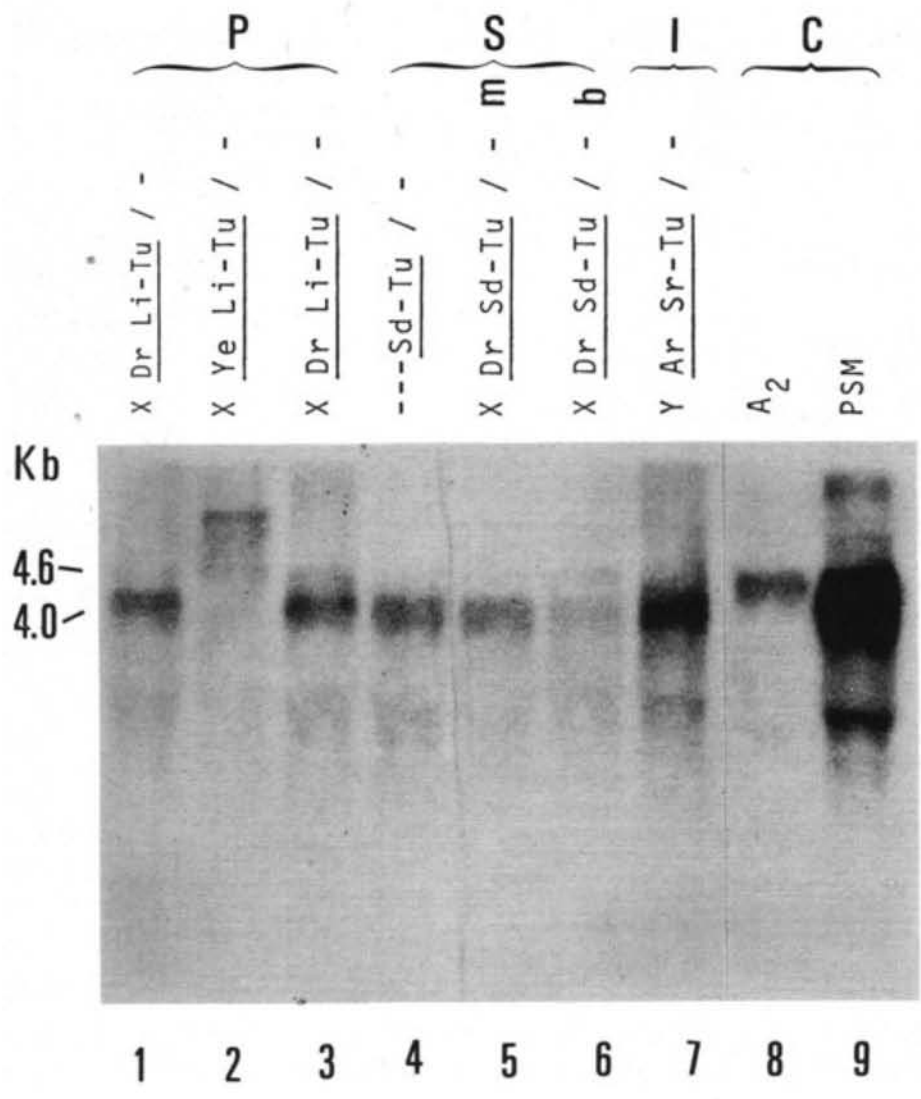

Fig. 3. Expression of x-*erb*-B* genes in: *P*, promoted melanomas; *S*, spontaneous melanomas; *I*, initiated melanomas; and *C*, cell lines (*m*, malignant melanoma; *b*, benign melanoma). RNA filters were probed with x-*erb*-B*-specific probes under high stringency conditions (50% formamaide; 1 × SSC/1% SDS, 68 °C). For symbols, see Table 1

transformed and nontransformed tissues and studies on the transcriptional activation [45] support the idea that x-*erb*-B* mRNAs of different size are transcribed from distinct genes, namely x-*erb*-B*^{i} and x-*erb*-B*^{a}. The mRNA transcribed from the c-*erb*-B (x-*egf-r*) locus is 4.3 kb in size (Fig. 4).

Cell Line A_2. The A_2 cell line [57] was derived from embryos of *X. xiphidium*, and harbors the oncogenic Y-chromosomal *Tu* complex *F1-Tu* [45]. A_2 cells are immortalized, but do not form colonies in soft agar [55]. Hybridization of the x-*erb*-B* specific probes against digested DNA revealed an *Eco*R1 fragment of 7.5 kb (Fig. 2, lane 5) and *Hin*dIII fragments of 9.2 kb and 4.8 kb [7]. Hybridization against polyA^+-selected RNA and total RNA revealed a x-*erb*-B*-specific transcript of 4.6 kb (Fig. 3). Expression of a single c-*erb*-B-specific transcript of 4.5 kb was clearly detected in polyA^+-selected RNA [7].

Cell Lines Sd′Sr′ and Golden. Both cell lines were established from epithelial tissues of adult fish [58], and show an epithelial cell-like shape. They were subcultured approx. 30 times and cannot therefore yet be referred to as immortalized.

Sd′Sr′ cells were derived from a *X. maculatus* male (Y *Ar Sr′-Tu*/X *Dr Sd′-Tu*) which is homozygous for the tumor suppressor *Diff* and therefore terminally differentiates the Tr melanophores in the

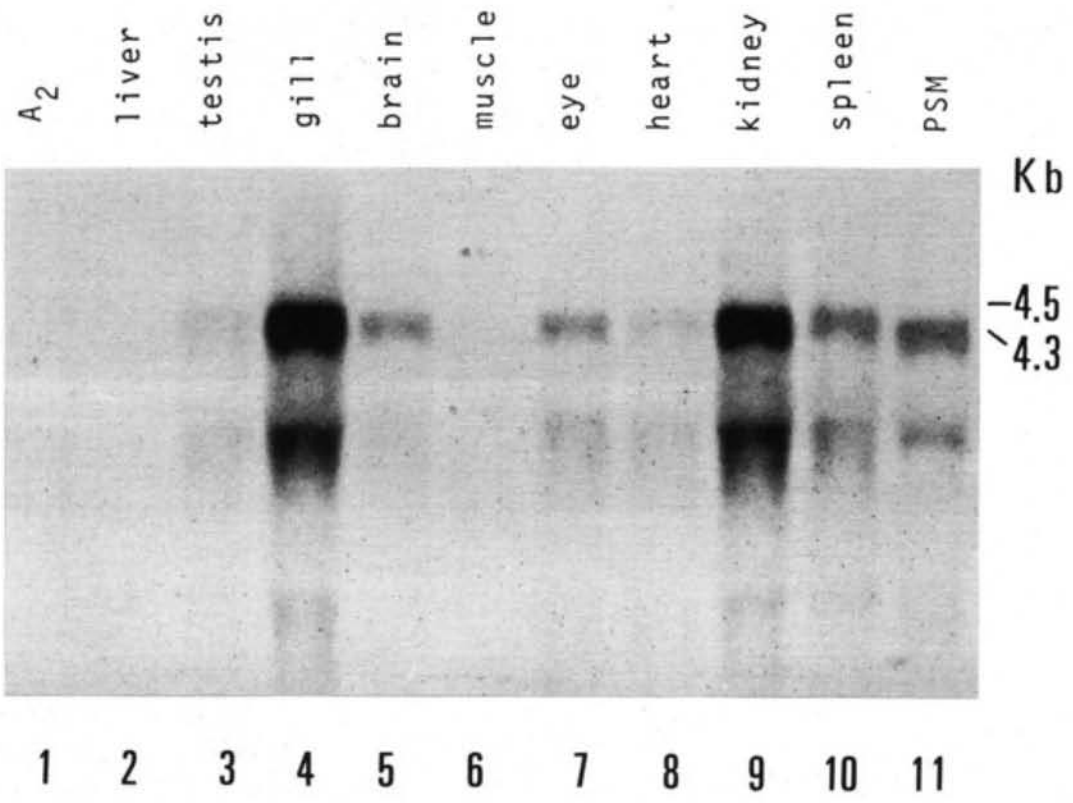

Fig. 4. Expression of the xiphophorine EGF receptor gene c-*erb*-B (= x-*egf-r*) in the cell lines A_2 and PSM, and in tissues of the adult nontumorous fish. Conditions, see Fig. 3

dorsal fin and on the body side. Sd'Sr' cells show the expected x-*erb*-B*-specific Southern fragments of 7.5 kb, 6.7 kb, and 4.9 kb (not shown) but do not express any detectable x-*erb*-B^{*i} or x-*erb*-B^{*a} transcripts. It must be shown by further studies whether the lack of x-*erb*-B* expression (x-*erb*-B^{*i} and x-*erb*-B^{*a}) in the Sd'Sr' cell is due to the activity of *Diff*, or whether the x-*erb*-B* loci are kept silent in these epithelial cell-like cells by any other factor.

Golden cells [58] were isolated from a BC hybrid that carries the "golden" gene (see [8, 59]) but lacks an oncogenic *Tu* complex. The Golden cell does not possess any of the "polymorphic" x-*erb*-B^{*a} fragments and does not show detectable amounts of x-*erb*-B* transcripts (not shown).

Spontaneous Melanoma. Following appropriate interpopulational or interspecific hybridization, melanoma develops spontaneously in hybrids harboring the chromosome X *Dr Sd-Tu* or the chimeric chromosome ---*Sd-Tu* (phenotypically detected translocation of *Sd-Tu* onto an *X. helleri* autosome). Presence of the *Diff* gene results in development of a benign melanoma, while absence of *Diff* allows development of a malignant melanoma [13, 32]. The hybrids possess the x-*erb*-B^{*i} and the X-chromosomal x-*erb*-B^{*a} (Table 1). The x-*erb*-B*-specific probes detect mRNAs of 4.6 kb and 4.0 kb in both the benign and the malignant melanoma of the hybrid X *Dr Sd-Tu*/– (Fig. 3, lanes 5 and 6) and ---*Sd-Tu*/– (Fig. 3, lane 4), respectively. The amount of 4.0 kb mRNA is high in melanoma, the highest level of mRNA being detected in the malignant melanoma (Fig. 3; Table 2). We suppose that the 4.0-kb mRNA is transcribed from the x-*erb*-B^{*a}-locus, while the 4.6-kb mRNA, which is detectable in a low amount in the melanoma, may be transcribed from the x-*erb*-B^{*i} locus (see expression in PSM cells). The c-*erb*-B- (x-*egf-r*-)specific probe generally detects a mRNA of 4.5 kb in the spontaneous melanoma (not shown); the amount of mRNA detected in the malignant melanoma exceeds that detected in the benign melanoma (Table 2).

Promoted Melanoma. Amongst others things [8, 35], hybrids carrying the chromosome X *Ye Li-Tu* or the chimeric chromosome X *Dr Li-Tu* (*Li-Tu* translocation onto a *X. maculatus* X-chromosome; Table 1) are sensitive to tumor-promoting agents such as steroids [35, 60] or ultraviolet radiation [61]. Treatment of the hybrid X *Dr Li-Tu* (4.9-kb x-*erb*-B* fragment) with promoters resulted in development of a fast-growing malignant melanoma, which expresses the 4.6-kb and overexpresses the 4.0-kb x-*erb*-B*-specific mRNA (Fig. 3, lanes 1 and 3; Table 2). The malignant melanoma of the X *Ye Li-Tu*-hybrid (11-kb x-*erb*-B*^{a} fragment) expresses mRNAs of 4.6 kb and 8 kb (Fig. 3, lane 2). Our data indicate that the 8-kb mRNA is specifically transcribed from the *Li-Tu* locus of the *X. variatus* X-chromosome (Table 2; [52]). The x-*erb*-B- (x-*egf-r*-) specific probe detects a mRNA of 4.5 kb in hybrids with a promoted melanoma, the expression detected in a benign melanoma being lower than that in a malignant melanoma (Table 2).

Initiated Melanoma. Up to 20% [37] of the hybrids carrying the *X. maculatus* chromosome Y *Ar Sr-Tu* (6.7-kb x-*erb*-B*^{a} fragment) or the chimeric chromosome X *Dr Ar Sr-Tu* (*Ar Sr-Tu* translocation onto the *X. maculatus* X-chromosome; 6.7-kb fragment; Table 1) develop a clonal melanoma after treatment with tumor-initiating agents such as X-rays [63], *N*-methyl-*N*-nitrosourea (MNU), *N*-ethyl-*N*-nitrosourea (ENU), and others [33, 35–37]. The clonal melanoma expresses two mRNAs of 4.6 kb and 4.0 kb (Fig. 3, lane 7), the 4.0-kb mRNA probably being transcribed from the *Sr-Tu* locus. The highest amount of 4.0 kb mRNA was detected in fast-growing malignant melanomas. In any initiated melanoma, however, the amount of 4.0-kb mRNA greatly exceeds that of the 4.6-kb mRNA. As we have shown for the spontaneous melanoma and the promoted melanoma, in the initiated melanoma the amount of the 4.0-kb transcript is also positively related to the degree of malignancy (Table 2). Expression of the c-*erb*-B- (x-*egf-r*-)specific 4.5-kb mRNA was detectable in almost all of the initiated melanomas (Table 2).

In summary, the northern blot analyses revealed x-*erb*-B* specific transcripts of 8 kb, 4.6 kb, and 4.0 kb, and c-*erb*-B- (x-*egf-r*-)specific transcripts of 4.5 kb and 4.3 kb in preparations of total RNA and poly A^{+}-selected RNA. The results reveal that x-*erb*-B*^{a} is expressed or overexpressed in the melanoma and the melanoma cell line PSM. It appears that the mRNAs of 4.0 kb and 8 kb are probably specifically transcribed from the x-*erb*-B*^{a} genes in the melanoma; presumably, the 4.0-kb mRNA is specific for certain *X. maculatus* x-*erb*-B*^{a}, while the 8.0-kb mRNA is transcribed from the *X. variatus Li-Tu* locus. The 4.6-kb mRNA is probably transcribed from the x-*erb*-B*^{i} locus. The xiphophorine c-*erb*-B gene is also expressed in almost all of the melanomas analyzed and may be important for the manifestation of the tumor phenotype. To learn about the function of x-*erb*-B* (x-*erb*-B*^{i} and x-*erb*-B*^{a}) and c-*erb*-B in nontumorous tissues and to verify that the transcripts of 4.0 kb and 8 kb are tumor-specific, we studied the expression of both types of v-*erb*-B-related gene in nontumorous tissues of fish with and without accessory *Tu* complexes.

Expression in Nontumorous Tissues of Fish With and Without Accessory Tu Complex

Preparations of total RNA were subjected to northern blot analyses using the controls described previously (p. 221). Poly A^{+}-selected RNA of stage 21 embryos (stage according to [59]) and kidneys were analyzed to verify the mRNA character of the transcripts detected in embryonic or adult tissues.

Nontumorous Tissues of Fish Without Accessory *Tu* Complex. The embryos and juvenile and adult fish subjected to northern analyses contained the genetic in-

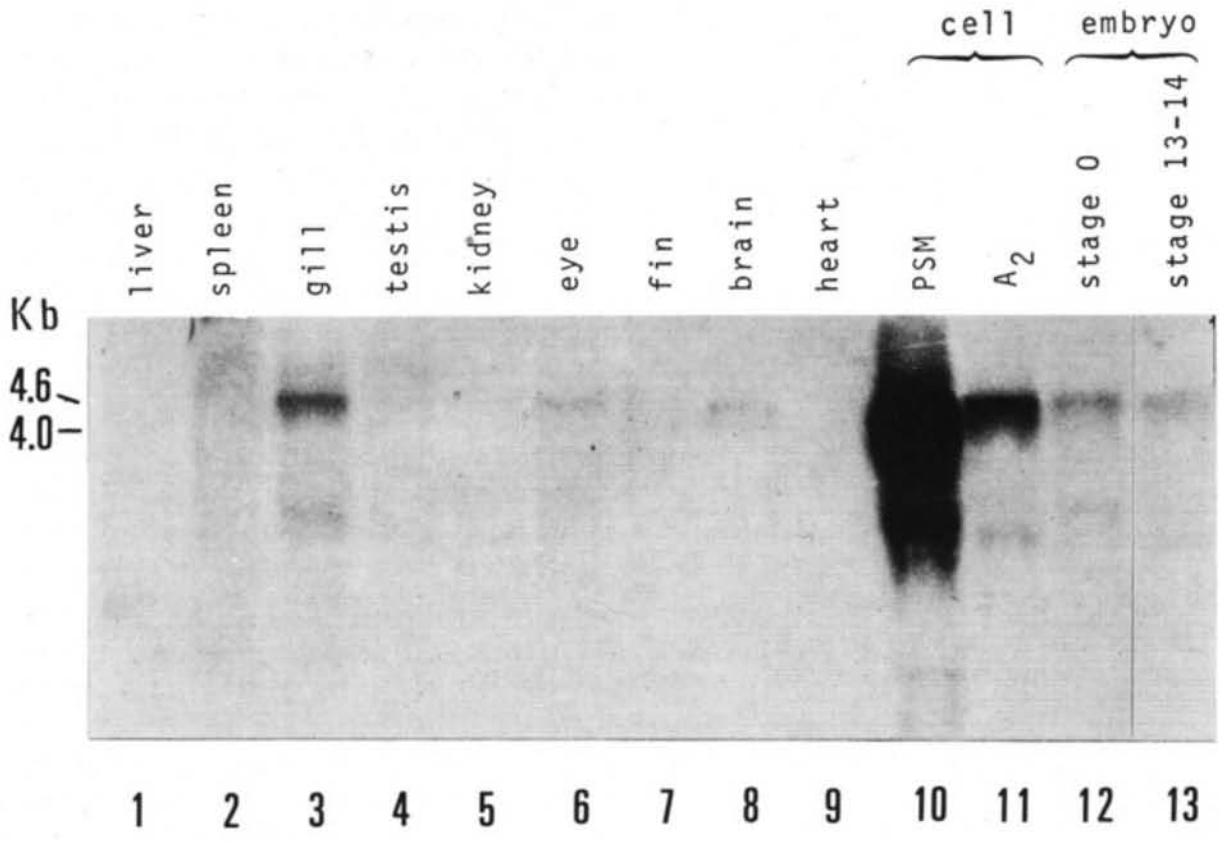

Fig. 5. Expression of x-*erb*-B* genes in tissues of the adult nontumorous fish, in PSM and the A_2 cells, and in embryos (stage 0, maternal RNA; stage 13–14, early organogenesis) of nontumorous fish. Conditions, see Fig. 3

formation of *X. helleri* from Rio Lancetilla which possesses x-*erb*-B*^{i} but lacks x-*erb*-B*^{a} (Table 1). In the embryo (Table 2; stages according to [59]), the x-*erb*-B*-specific probes detect only one type of mRNA, namely the 4.6-kb mRNA (Fig. 5, lanes 12 and 13), which – in the case of the melanoma – we suspected to be transcribed from the x-*erb*-B*^{i} locus (Table 2). The 4.6-kb transcript is stored in the unfertilized egg (stage 0). The amount of x-*erb*-B*^{i} mRNA decreases during early blastula formation (stages 1–4), slightly increases when invagination of the neural keel takes place (stages 5 and 6), and stays almost constant up to organogenesis. The amount of x-*erb*-B*^{i} transcript is enhanced during late organogenesis (stages 17–20), slightly decreases during late embryogenesis (stages 20–26), and stays almost constant during the first 10 days after birth (Table 2). In the adult fish (Table 2, Fig. 5), a considerable amount of the x-*erb*-B*^{i}-transcript is detectable only in the gills; expression of x-*erb*-B*^{i} is low in the eye and brain, and very low, but detectable, in testes, fins, and skin. Hybridization against polyA^{+}-selected RNA revealed that the 4.6-kb mRNA is the only mRNA expressed in nontumorous tissues of *Xiphophorus*. RNA species of larger or smaller size (e.g., RNA of approx. 3 kb) which were observed in preparations of total RNA of, for example, the gills (Figs. 5, 6) are not detectable in preparations of polyA^{+}-selected RNA and therefore may be considered as a x-*erb*-B*^{i}-precursor RNA, specific degradation products, or something else.

Our results clearly reveal that the expression of x-*erb*-B*^{i} is important for embryogenesis and for some tissues in the adult fish. The expression of x-*erb*-B*^{i} in the embryo and in the gills, both of which contain proliferating cells, indicate that x-*erb*-B*^{i} may be important for the regulation of proliferation processes. Since x-*erb*-B* codes for a protein of the EGF-R family and is expressed in proliferating nontumorous or tumorous cells, x-*erb*-B*^{i} and x-*erb*-B*^{a}, respectively, may be

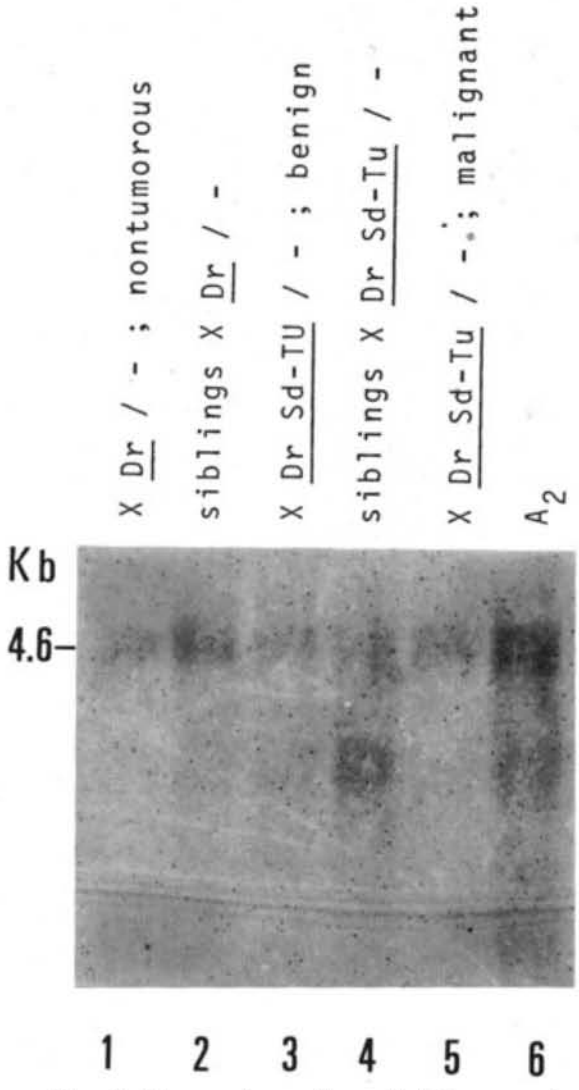

Fig. 6. Expression of x-*erb*-B* genes in the gills of hybrids that have inherited the X-chromosomal 4.9-kb x-*erb*-B*[a] fragment and in the gills of siblings from the same crossing that have not inherited the X chromosomal fragment. *1*, X *Dr*/–, reddish tumorfree hybrid with an aberrant X chromosome; *2*, nonreddish siblings of X *Dr*/– (the aberrant X chromosome is lacking); *3*, X *Dr-Sd-Tu*/–, reddish hybrid with a benign dorsal melanoma; *4*, tumorfree siblings of X *Dr Sd-Tu*/–, benign/malignant (X chromosome is lacking); *5*, X *Dr-Sd-Tu*/–, reddish hybrid with a malignant dorsal melanoma. Conditions, see Fig. 3; symbols, see Table 1

involved in a mitosis-stimulating pathway comparable to that described for the human EGF-R [64].

The EGF-R gene (c-*erb*-B) of *Xiphophorus* is differentially expressed during embryogenesis (Table 2), with the lowest amount of 4.5-kb transcript during early organogenesis (stages 13 and 14) and the highest expression detected in the unfertilized egg (stage 0). The expression of c-*erb*-B stays at an almost constant level during late embryogenesis (stages 20–25), birth (stage 26), and for the first 10 days after birth (Table 2). In the adult fish, c-*erb*-B is highly expressed in the gills and in the kidney (Fig. 4; Table 2). Expression was also detected in spleen, brain, and the eyes, while low expression was observed in testis and heart. The size of the c-*erb*-B transcript detected in embryonic and adult tissues, as well as the size of those transcripts observed in the melanomas and the A_2 cells, was 4.5 kb. The 4.3-kb c-*erb*-B transcript was only detected in the PSM cells and may disclose a modified EGF-R gene that is specific for PSM cells. Smaller RNA species (approx. 3 kb; Fig. 4) were observed in some preparations of total RNA; since these RNA species were not detected in polyA^+-selected RNA, they probably represent precursors of the mRNA, or specific degradation products (small RNA species in Fig. 4), or something else.

The EGF-R gene of *Xiphophorus* appears to be important for the development of the embryo and for some tissues in the adult fish, especially the gills and the kidneys. Possible, c-*erb*-B is functionally involved in stimulating mitosis in the course of the regeneration of the epithelial cells of both the gills and the kidneys. Expression of the human EGF-R gene was observed in nontumorous renal tissues, human renal cancer, and a number of other human epithelial malignancies [65].

Nontumorous Tissues of Fish With Accessory *Tu* Complex. To verify that the 4.0-kb mRNA is a tumor-specific transcript of x-*erb*-B*[a], we analyzed nontumorus tissues of hybrids that possess a normal or aberrant *X. maculatus* X-chromosome, or lack such a chromosome. Since our experiments showed that the x-*erb*-B*[i]-specific mRNA is well detectable in the gills, we studied the expression of x-*erb*-B* in the gills of hybrids with X *Dr Sd-Tu*, X *Dr* (phenotypically

detected *Sd-Tu* deletion) and the *Dr Sd-Tu* and *Dr*-free siblings of these hybrids. Hybrids with X *Dr Sd-Tu* possess the indispensable 7.5-kb x-*erb*-B*^{i} fragment and the 4.9-kb x-*erb*-B*^{a} fragment. We detected only one type of RNA, namely the 4.6-kb x-*erb*-B*^{i}-specific transcript, in total RNA from hybrids with benign melanoma (presence of *Diff*), hybrids with malignant melanoma (absence of *Diff*), and the Tr melanophore-free siblings of these hybrids (Fig. 6, lanes 3–5). A transcript of the same size was detected in the gills of the hybrids with X *Dr*, which possess the 7.5-kb and the 4.9-kb x-*erb*-B* fragments, as well as in the gills of their X *Dr*-free siblings (Fig. 6, lanes 1 and 2). The amount of 4.6-kb mRNA was almost the same in all five cases.

The data described in the preceding paragraphs indicate that the x-*erb-B*^{*a} locus is transcribed tumor specifically, while the indispensable x-*erb*-B*^{i} locus is transcribed in nontumorous tissues of fish harboring and lacking accessory *Tu* complexes. The regulation, that is to say the activation or inactivation, of both the "proto-oncogenic" x-*erb*-B*^{i} and the onocogenic x-*erb*-B*^{a} needs further investigation.

X-erb-B^{*a} *and Other Genes: Possible Cooperation?*

Nothing is known about the key genes critical in starting the molecular and biochemical machinery that initiates a tumor. Fortunately, from studies of the polymorphic length of restriction fragments of xiphophorine oncogenes, we can discriminate the parental origin of a certain oncogene in a particular hybrid [8]. We shall concentrate on hybrids that have inherited the *X. maculatus* X-chromosome or Y-chromosome, which in the hybrid (*mac/hell*) mediates the formation of a melanoma spontaneously or after induction with tumor-initiating agents, respectively. In our highly backcrossed hybrids bearing a melanoma or sensitive to initiators, the sex chromosome with the accessory *Tu* complex is the only chromosome that originates from *X. maculatus;* 47 chromosomes originate from *X. helleri.* The molecular and the biochemical machinery which causes the development of the melanoma in a BC hybrid is, therefore, mainly run by *X. helleri*-derived genes, while the signal for starting the machinery must be created and transmitted by the *X. maculatus* sex chromosome. Since this gene must be located in the region of the accessory *Tu* complex, it appears that the signal for the initiation of the processes preceding the morphological realization of the melanomas probably comes from the x-*erb*-B*^{a}. Besides x-*erb*-B*^{a} [40] and x-*erb*-B*^{i} [46], none of the 14 oncogenes that we detected in the genome of *Xiphophorus* could be assigned to the sex chromosomes and the accessory *Tu* complexes [8].

A possible way in which x-*erb*-B*^{a} could initiate the processes leading to melanoma development has not yet been investigated. So far, we have found that x-*erb*-B*^{a} is expressed and overexpressed in melanomas that developed spontaneously or after induction with carcinogens; the highest amount of x-*erb*-B*^{a} transcripts was observed in spontaneous and promoted malignant melanomas of hybrids carrying a certain *X. maculatus* X-chromosome [62].

The machinery necessary for melanoma formation remains poorly understood, but probably involves activity of several *X. helleri*-derived oncogenes and their products. We showed that the c-*erb*-B (x-*egf-r*) is expressed in almost all melanomas and, therefore, may be of some relevance for the melanoma machinery. Another gene, namely the xiphophorine *src* gene, x-*src*, is also somehow involved in melanoma formation. It was shown earlier that x-*src* is probably of importance for the melanoma machinery and may be related to the initial events: Biochemical studies, especially immunoprecipitation assays, showed that the xiphophorine counterpart to the Rous sarcoma virus oncogene product, namely

the pp60^{x-src} kinase, is active in several healthy tissues of *Xiphophorus*, particularly in brain tissues [2, 3, 34]. In hybrids that inherited the *Tu* complex *Sd-Tu* and, therefore, develop melanoma spontaneously, the pp60^{x-src} activity in the brain was three times higher than that in the brain of their tumorfree siblings (*Sd-Tu* is lacking). Moreover, the activity in the melanoma was elevated up to three times above that of the already elevated activity in the brain of the melanoma-bearing fish [8, 66]. Depending on the genetically determined degree of malignancy, the pp60^{x-src} activity of a particular fish may be increased in parallel in both the melanoma and the brain [2, 66].

The turnover of inositol lipids [67, 68] was recently found to correlate with the inheritance of accessory *Tu* complexes containing x-*erb*-B*^{a} on the one hand and with the pp60^{x-src} activity on the other hand [8, 41, 69, 70]. Phosphoinositide turnover and pp60^{x-src} kinase activity were found consistently elevated in the spontaneous melanoma and the healthy brain of hybrids carrying the *Tu* complex *Sd-Tu* before and after the onset of the malignant melanoma [70]. Predictions can be made about whether a certain individual that harbors an accessory x-*erb*-B*^{a}-containing *Tu* complex is prone to develop a spontaneous malignant melanoma. Hybrids that have inherited the *Tu* complex *Sr-Tu*, and therefore require carcinogen-induced somatic mutations of particular regulatory genes of this *Tu* complex for initiation of a melanoma, show standard levels of phosphoinositide turnover and pp60^{x-src} activity in the brain; only malignant melanomas induced in these genotypes show elevated inositol incorporation [69] and elevated pp60^{x-src} activity [66]. The malignant melanoma of these animals cannot be predicted by the inositol lipid turnover and pp60^{x-src} kinase activity. The complex correlation between the inositol lipid turnover and pp60^{x-src} activity and the genetically determined melanoma formation, respectively, indicate that the inositol lipids and the x-*src* are probably involved in the processes preceding the morphological realization of the melanoma. This correlation is intimately coupled with the inheritance of x-*erb*-B*^{a}. Further studies are required to show whether the activity of x-*erb*-B*^{a} or other genes could regulate inositol lipid turnover and influence pp60^{x-src} activity in the melanoma.

Northern blot analyses showed that, besides x-*erb*-B*, c-*erb*-B, and x-*src*, the xiphophorine *ras* gene (x-*ras*) is expressed in the melanoma [8, 56]; the x-*ras* protein could possibly be related to a putative signal transduction pathway that causes phophatidyl inositol-1,4-biphosphate degradation. Such a signal transduction pathway could also be influenced by the x-*erb*-B*, the c-*erb*-B (x-*egf-r*), or other gene products. Transcripts of the putative x-*sis* gene (x-*pdgf*) that codes for the platelet-derived growth factor (PDGF) of the fish [6] were detected in several melanomas [62], while the putative x-*pdgf-r* gene that encodes the xiphophorine PDGF receptor [6, 71] is expressed in all tumors analyzed [62]. We assume that x-*sis* and x-*pdgf-r* may mediate tumor growth by an autocrine or paracrine mechanism; furthermore, via its receptor, PDGF could have the opportunity to regulate a signal transduction pathway with some relevance for the tumor machinery. mRNAs of genes related to the human *erb*-A [7, 9] (see also [72]) are poorly detectable in melanomas [62].

x-*erb*-B*^{a} is the most likely candidate to initiate the complex of processes preceding the morphological realization of the melanoma. However, generation of transgenic fish [73, 74] which specifically develop melanomas following induction of a transfected x-*erb*-B*^{a} gene are needed to prove this hypothesis. The process of melanoma initiation may require collaboration of x-*erb*-B*^{a}, inositol lipid turnover, and pp60^{x-src} activity, while the melanoma machinery may involve the genes x-*erb*-B*^{i}, c-*erb*-B (x-*egf-r*), x-*ras*, x-*pdgf*, and x-*pdgf-r*.

Summary and Conclusions

The polymorphic length of certain restriction fragments so far have revealed only a certain v-*erb*-B-related gene that is inherited along with the accessory oncogenic *Tu* complexes which mediate the development of melanomas in *Xiphophorus* after appropriate interpopulational or interspecific hybridization. This gene, named x-*erb*-B*, codes for a tyrosine receptor kinase that belongs to the EGF-receptor family. Two types of x-*erb*-B* gene, namely x-*erb*-B*^{i} and x-*erb*-B*^{a}, exist in the genome of a melanoma-bearing hybrid. One of them, namely x-*erb*-B*^{i}, is indispensable in the genome of the fish and the other, namely x-*erb*-B*^{a}, is accessory in the genome of the fish. The latter is part of the melanoma-determining oncogenic gene complex and exists at least in three allelic forms. These forms can be assigned to the *Tu* complexes that are responsible for the development of the (a) spontaneous, (b) initiated, and (c) promoted melanoma, respectively. Studies of the inheritance and expression of x-*erb*-B*^{a} in mutants susceptible or insusceptible to melanoma indicated that x-*erb*-B*^{a} seems to act as the "ignition spark" of the *Xiphophorus* melanoma machinery. In particular, the expression data support the hypothesis that x-*erb*-B*^{a} is specifically activated in the tumor: x-*erb*-B*^{a} is highly expressed or overexpressed in the melanoma, the amount of x-*erb*-B*^{a}-specific mRNA (4.0 kb) being positively related to the malignancy of the melanoma. Expression of the indispensable x-*erb*-B*^{i} (4.6-kb mRNA) is also detectable, but comparatively low in the melanoma. x-*erb*-B*^{i} is probably involved in the regulation of the proliferation of embryonic tissues and epithelial tissues in the adult fish. In nontumorous tissues, e.g., the gills, of both fish harboring and lacking accessory *Tu* complexes, only the x-*erb*-B*^{i}-specific mRNA is detectable, the amount of 4.6-kb transcript detected in the tumor fish and in the tumor-free siblings being almost the same. The activation of the "proto-oncogenic" x-*erb*-B*^{i} probably occurred by gene duplication and mutation and resulted in the generation of the oncogenic x-*erb*-B*^{a}. This gene's oncogenicity is presumably suppressed by the *Diff* gene in the purebred; in the hybrid however, where crossing-related impairment of the *Diff* gene took place, the inheritance and expression of x-*erb*-B*^{a} is associated with the susceptibility to melanoma.

The c-*erb*-B (x-*egf-r*) gene of *Xiphophorus* which codes for the xiphophorine EGF receptor is probably involved in signal transduction processes in embryonic tissues and in epithelial tissues in the adult. Besides being expressed in healthy tissues, c-*erb*-B is expressed in almost all spontaneous or induced melanomas analyzed. The expression detected in the malignant melanoma exceeds that in the benign melanoma. A transcript of aberrant size, 4.3 kb instead of 4.5 kb, was only observed in the melanoma cell line PSM. Since, thus far, amplification or rearrangement of the c-*erb*-B gene has not been observed in fish susceptible to melanoma, we conclude that the xiphophorine EGF receptor gene is not involved in the initiation of the key processes preceding melanoma. The c-*erb*-B gene, however, may be important for the manifestation of the tumor phenotype by taking part in the melanoma machinery.

Besides x-*erb*-B*^{i}, x-*erb*-B*^{a}, and c-*erb*-B, several oncogenes are expressed in the melanoma. Expression of the x-*src* gene, that is to say pp60^{x-src} activity and phosphatidylinositol turnover, are elevated in healthy tissues of hybrids susceptible to spontaneous melanoma and in the melanoma itself; the elevation in the melanoma exceeds that in the nontumorous tissues of the fish. The elevation of both pp60^{x-src} activity and inositol lipid turnover are in parallel with the degree of malignancy of the tumor and the inheritance of a certain x-*erb*-B*^{a}.

We conclude that the signal for initiation of the key processes preceding melanoma, including the elevation of inositol

lipid turnover and pp60^{x-src} activity, is associated with the inheritance and expression of the accessory x-*erb*-B*[a].

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