Search for Genes Critical for the Early and/or Late Events in Carcinogenesis: Studies in *Xiphophorus* (Pisces, Teleostei)*

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A. Introduction

I. Historical Background

The concept of genes that code for neoplastic transformation, called "oncogenes," originates from two sources, virology and animal genetics. The virological source can be traced back to the year 1910 when Peyton Rous discovered the virus that causes sarcoma in chickens. It took, however, about 60 years until evidence was produced that the cancer determinants located in the genome of this and related viruses (retroviruses) are truly genes [1, 2]. The source in animal genetics dates from 1929, when Myron Gordon, Georg Häussler, and Curt Kosswig independently discovered that the F_1 hybrids between certain domesticated ornamental breeds of the Central American fish species *Xiphophorus maculatus* (platyfish) and *X. helleri* (swordtail) spontaneously develop melanoma that is inherited in the hybrid generations like the phenotype of any normal Mendelian gene located in the genome of the fish. The basic idea in both the retrovirus and the *Xiphophorus* model is that oncogenes present in the genome of animals are activated by changes in structure (point mutation, translocation, truncation) and/or changes in expression (ectopic expression, unscheduled expression), and that products of the activated genes mediate the neoplastic transformation of a target cell [3–6].

In addition, a recent extension of the oncogene hypothesis is that "tumor-suppressor genes" or "antioncogenes" control the expression of oncogenes and the manifestation of a tumor phenotype [7, 8]. Such "oncostatic genes" have been identified in several systems: firstly, the retinoblastoma gene in humans [9, 10], secondly, the lethal giant larvae gene in *Drosophila* [11, 12], and thirdly, the differentiation gene *Diff* in *Xiphophorus* [3, 13].

While many investigators have focussed their attention on the role of retroviral oncogenes (symbolized by v-*onc*s) in neoplastic transformation, we concentrated on cellular oncogenes (symbolized by x-*onc*s) and the oncostatic genes that might be involved in the early and late events in carcinogenesis in *Xiphophorus*.

Our research on oncogenes in *Xiphophorus* began in 1957 with systematic crossings between populations, races, and species and with mutagenesis studies in purebred and hybrid fish. *Xiphophorus* from wild populations of the natural habitat and *Xiphophorus* bred from wild populations in the laboratory are almost completely insusceptible to neoplasia, i.e., insensitive to mutagenic carcinogens and tumor promotors, whereas hybrids derived from crossings between different wild populations develop neoplasms spontaneously or after treatment with carcinogens [14–16]. Subsequently we found that melanoma and a large variety of other neoplasms developing either spontaneously or after treatment with carcinogens can be as-

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signed to a particular Mendelian gene located on particular sex chromosomes [3, 14, 16, 17]. This gene is an "oncogene" by definition, and was designated as "tumor gene," *Tu*. The phenotypic expression of *Tu* is regulated by systems of modifying genes that may stimulate (*S* genes) or repress (*R* genes) *Tu* activity [18]. The genetic make-up of the modifying gene system is highly different among the species of the genus *Xiphophorus*, less different among the races of a certain species, but still different among populations of a certain race. Evidence for this assumption comes from the observation that the degree of malignancy of melanomas in interspecific hybrids depends on the parental genomes [18, 19].

Studies on transformed pigment cells in purebreds and interspecific hybrids led to the characterization of the *Tu* and the *R* genes by means of classical pheno- and cytogenetic methods, however little is known about the *S* genes. The *Tu* gene, although detected in fish with transformed melanocytes (Tr melanocytes) is also present in all specimens of wild populations of *Xiphophorus*, irrespective of the phenotypic expression of Tr melanocytes. It is postulated that *Tu* fulfills an essential, so far unknown function (indispensable *Tu*), and that its function in fish developing tumors is an accessory one (accessory *Tu*). In the wild populations malignant expression of *Tu* is under stringent control exerted by *R* genes that are organized mainly as members of three interrelated *R* gene systems: (a) *Tu*-linked tissue-specific *R* genes (*R-mel, R-neu, R-epi, R-mes*) which, if impaired, lost, or translocated, permit the *Tu*-encoded tumor formation in the pigment cell system and neurogenic, epithelial, and mesenchymal tissues [3]; (b) *Tu*-linked compartment-specific *R* genes, which restrict spots and melanomas to distinct compartments of the body; 14 compartment-specific *R* genes (*R-co*) have been identified which, if impaired, correspond to sites of the body where the spots in the purebreds and the melanomas in the hybrids occur [3]; (c) the *Tu*-non-linked modifying genes (*R* and *S* genes), which control proliferation and differentiation of the transformed pigment cells, e.g., the prominent *R* gene *Diff* which was disclosed by the clear-cut 1:1 segregation between the BC hybrids bearing malignant melanomas and those bearing benign melanomas [13, 20, 21]. *Tu, R-mel,* and *R-co* are closely linked to each other and form a Mendelian entity designated as "tumor gene complex" (*Tu* complex). *Tu* complexes that are accessory in the fish and determine Tr melanophore patterns are the subject of the first part of the present article and modifying genes that of the second part.

II. Approach

Table 1 shows the gross constitution of the *Tu* complex-containing region of the sex chromosomes of *Xiphophorus* in wild-type order and after X-ray induced structural changes. Most of the mutants used were genetically and phenogenetically analyzed in 1973 (for photographs see [14]). Since then, more and new mutants have been isolated and studied (for photographs see [22]). The respective Mendelian genes on the chromosomes are arranged in a uniform order with (a) the sex-determining region proximal to the centromer, followed by (b) the pterinophore loci (*Dr, Ar, Ye, Or, Br*) and (c) the melanophore loci (*Sd-Tu, Sr-Tu,* etc.). Purebred animals containing a *Tu* complex exhibit spots. If, however, the *Tu* complex is present in *X. maculatus*/*X. helleri* hybrids, melanoma develops either spontaneously or following treatment with carcinogens. Animals lacking the *Tu* complex are almost completely incapable of developing spots or melanoma [15, 16]. Although the *Tu* complexes are rather well understood in terms of Mendelian genetics, studies undertaken at the molecular level have failed so far to characterize them and to distinguish between those that are indispensable and those that are accessory. Not only have no gene products of the *Tu* complexes been identified, but also no

Table 1. Wild-type and structural mutants in *Xiphophorus*

				1973	1988
A	FM.....	*X. helleri,*	wild type, Rio Lancetilla		
X	F *Dr, Sd-Tu*	*X. maculatus,*	wild type, Rio Jamapa		
X	M *Ar, Sr-Tu*	*X. maculatus,*	wild type, Rio Jamapa		
X	F *Ye, Li-Tu*	*X. variatus,*	wild type, Rio Panuco		
Y	M *Or, Pu-Tu*	*X. variatus,*	wild type, Rio Panuco		
Z	M *Br, Ni-Tu*	*X. maculatus,*	wild type, Belize River		
Z	M	*X. maculatus,*	Rio Usomacinta		
X	F,	Deletions,	no *Tu* complex left	1	14
Y	M,	Deletions,	no *Tu* complex left		3
X	F *Dr,*	Deletions,	pterinophore locus left	2	48
Y	M *Ar,*	Deletions,	pterinophore locus left		4
Z	M *Br,*	Deletions,	pterinophore locus left		3
X/Y	M *Dr, Sr-Tu*	Crossovers		1	2
A/X	FM..., *Sd-Tu*	Interspecific translocations		1	11
A/Y	FM..., *Sr-Tu*	Interspecific translocations		1	3
X/X	F *Dr, Li-Tu*	Interspecific crossovers		1	2
X+Y	F *Ye, Li-Tu, Or, Pu-Tu*	Unequal crossovers, duplications		4	5
A/X	FM..., *Dr,*	Interspecific translocation and deletion		1	
Y+X	M *Ar, Sr, Sd-Tu*	Unequal crossover		1	4
X+Y	F *Dr, Ar, Sr-Tu*	Deletions and crossover		1	1
X/Y	F *Dr, Ar,*	Deletions/deletions/crossovers			3
Total				14	103

A, Autosome of *X. helleri* homologous to the sex chromosomes of *X. maculatus*; X, Y, Z, sex chromosomes; F, M, female- and male-determining regions. Pterinophore loci: *Dr,* dorsal red; *Ar,* anal red; *Ye,* yellow; *Or,* orange; *Br,* brown; *Ni,* nigra. Melanophore loci: *Sd,* spotted dorsal; *Sr,* striped; *Li,* lineatus; *Pu,* punctatus.

alterations have been observed which would be expected to appear when the *Tu* complexes switch over to tumorigenic potency. Moreover, the *Tu*-nonlinked *R* genes and the *S* genes are poorly defined in terms of Mendelian and molecular genetics. The approach was to identify and to map sequences strictly correlated with the inheritance of the tumor phenotype, that is to say, of the *Tu* complexes and the modifying genes, and to study their expression.

We had probes specific for 15 molecularly defined viral oncogenes at our disposal when we started our search for genes structurally and functionally related to the genetic factors determining neoplasia in *Xiphophorus*. Southern blot analyses and some sequence data revealed that almost all oncogenes corresponding to the probes are present in the genome of all individuals of *Xiphophorus* tested so far [16, 23–28]; only *ros* and *mos* could not be identified in the fish. Some of the xiphophorine cellular oncogenes (x-*onc* genes) show restriction fragment length polymorphisms (RFLP), the patterns of which have evolved differently in the various taxonomic groups of fish [22, 27, 28]. For instance, the pattern of the lengths of the restriction fragments of x-*sis* is specific to each of the different species but there is no RFLP within each of the species; actually these species show a monomorphism of the restriction fragment length of x-*sis* [27]. In contrast, the pattern of lengths of the restriction fragments of x-*erb*A and x-*erb*B is species nonspecific but is specific to the different races and populations of the species. The lengths of certain fragments of x-*erb*B are even different in females and males of

the same population [22, 28]. We used the RFLP phenomenon as an indicator for the Mendelian inheritance of the x-*oncs* through the purebred and hybrid generations. If a certain oncogene fragment is inherited independently from the inheritance of spot or melanoma formation, one can conclude that the respective oncogene is not "critical" for the first step of melanoma formation. This is not to say that such an oncogene is not involved in melanoma formation at all; for instance, x-*src*, x-*sis*, x-*ras*, x-*myc* are expressed in the melanoma [27, 29, 30, 31] and are certainly implicated in tumor growth or tumor progression, but they are contributed by *X. helleri* to the hybrid while the appearance of spots or melanoma is contributed by *X. maculatus*. These genes, therefore, are not candidates for the primary event leading to melanoma.

B. Results and Discussion

I. Oncogenes that Might Be Considered "Critical" for the Early Events in Carcinogenesis

1. x-*erb*B Restriction Fragment Length Polymorphism

In the following paragraphs we concentrate on certain viral *erb*B (v-*erb*B) homologous DNA fragments because they are so far the only fragments that show the same inheritance as the susceptibility to melanoma. These fragments correspond to the x-*erb*B gene that represents a xiphophorine epidermal growth factor (EGF) receptor gene ([22]; for an overview on v-*erb*B and c-*erb*B see [32]).

Figures 1 and 2 show that Southern analyses revealed a different distribution of several *Eco*RI fragments of x-*erb*B in the different purebred and hybrid genotypes. Fragments of 3.5 kb and 4.3 kb, and some bands larger than 12 kb, are distributed in all populations and species of the fish genus without any as yet detectable pattern. Two fragments comprising 5.5 and 7.5 kb are constantly present in all individuals of all populations of *Xiphophorus* tested and, therefore, appear to be located on an autosome and to be structurally unrelated to the *Tu* complexes which determine the spot pattern and the melanoma formation. Three fragments, however, comprising 4.9, 6.7, and 11.5 kb are restricted to individuals exhibiting the sex-chromosome-linked spot patterns or melanomas. The latter fragments claimed our special interest.

The 4.9-kb *Eco*RI fragment is restricted to all individuals of *X. maculatus* from Rio Jamapa (female XX, male XY; Fig. 1, lanes a and b) exhibiting the X-chromosomal *Dr Sd-Tu* complex (dorsal red, spotted dorsal). The 6.7-kb *Eco*RI fragment is linked to the Y-chromosomal *Ar Sr-Tu* (anal red, stripe sided) from the same population and to the Z-chromosomal *Br Ni-Tu* (brown, nigra) from *X. maculatus* from Belize River (female WZ, male ZZ; lane d). The 11.5-kb fragment is specific to the X-chromosomal *Ye Li-Tu* (yellow, lineatus) of *X. variatus* from the Rio Panuco lane c. *X. maculatus* from Rio Usomacinta (lane e) and *X. helleri* from Rio Lancetilla (lane f) that lack both the sex-chromosomal pterinophore locus and the *Tu* complex (no spot patterns occur) lack also the sex-chromosomal restriction fragments. The latter results confirm that the sex-chromosomal spot patterns and the sex-chromosomal v-*erb*B related *Eco*RI fragments are linked to each other.

To assign the X-, Y- and Z-specific fragments more specifically to melanoma we introduced normal and structurally mutated sex chromosomes of *X. maculatus* and a normal X chromosome of *X. variatus* into the genome of *X. helleri* by introgression comprising mostly more than five backcrosses. The BC hybrids, which as expected segregated into equal portions of siblings exhibiting or lacking melanomas, were examined in parallel to the purebreds (Fig. 2; compare with Fig. 1). The 4.9-kb fragment was found in the normal and tumorous tissues of the melanoma developing BC segregants that carry the X-chromosomal *Dr Sd-Tu*

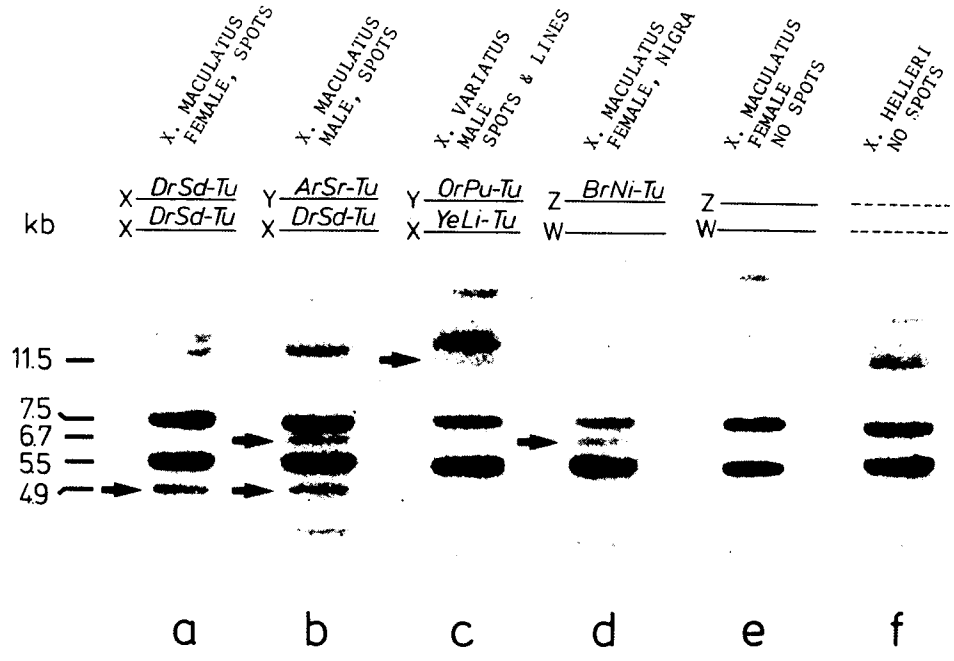

Fig. 1. Assignment of three v-*erb*B homologous fragments (*arrows*) to the spot determining sex chromosomes (Southern blot) of purebred xiphophorine fish. *X,Y,W,Z,* sex chromosomes. *Dashes* indicate autosomes of *X. helleri* that are homologous to the sex chromosomes of *X. maculatus* and *X. variatus*. For gene symbols and phenotypes of the animals see Table 1

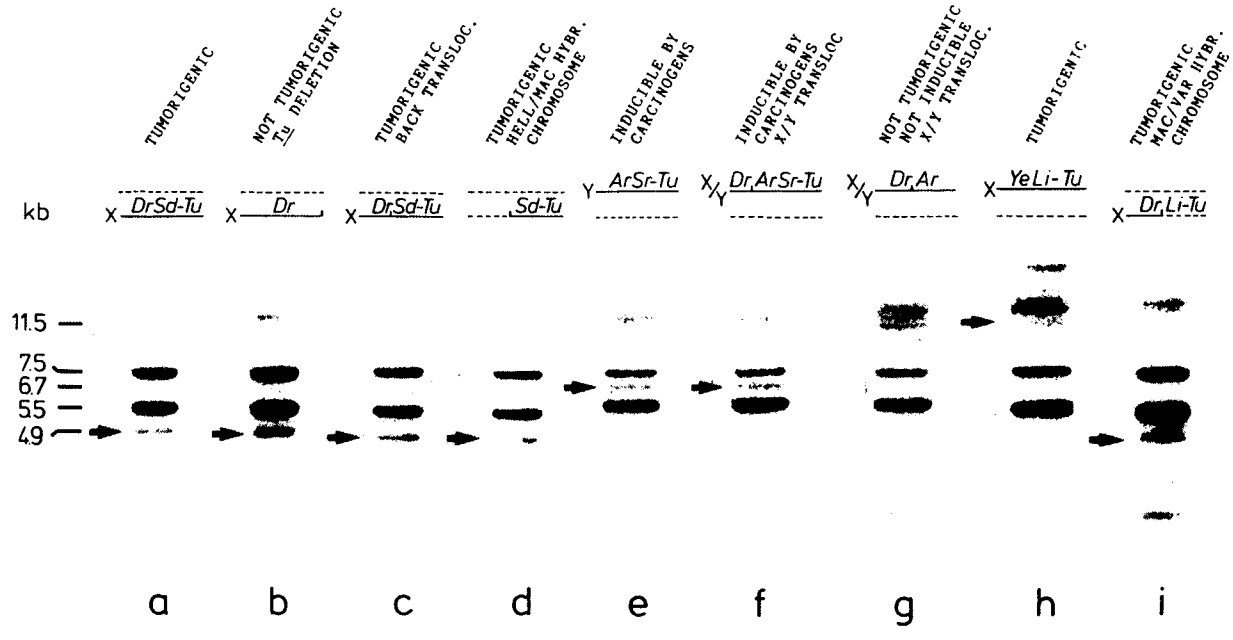

Fig. 2. Assignment of the three v-*erb*B homologous EcoRI fragments detected in the purebreds (see Fig. 1), to the melanoma-determining region of the tumor gene complexes in *X. maculatus*/*X. helleri* and *X. variatus*/*X. helleri* hybrids carring normal and structurally changed chromosomes. (For symbols and phentoypes see Table 1)

complex but was not found in the melanoma-free segregants (siblings) lacking this X chromosome (not shown in Fig. 2; the restriction fragment pattern was identical to that of the purebred *X. helleri*). The 6.7-kb fragment was identified in all BC hybrids exhibiting the Y-chromosomal *Ar Sr-Tu* complex but was not detected in the siblings not having inherited this Y chromosome. The Z-chromosomal 6.7-kb fragments were not investigated in the hybrids. The 11.5-

kb fragment was identified in the normal tissues of all melanomatous BC hybrids displaying the X-chromosomal *Ye Li-Tu*. These results confirm the linkage of the three sex-chromosomal fragments to the respective sex chromosomes of *X. maculatus* and *X. variatus* that transmit the capability of the spontaneous or induced development of melanoma through the hybrid generations.

2. Location of the Xiphophorine *erb*B Restriction Fragments

Cytogenetic observations on 88 X-ray-induced and spontaneous structural chromosome changes have shown that the *Tu* complexes and the adjacent pterinophore loci are terminally located on the sex chromosomes [3, 14]. Our present study on the three sex-chromosome-specific *Eco*RI Southern fragments disclosed new details of the genetic make-up of the region where the information for pigment cell transformation is located.

a) The 4.9-kb Fragment. A melanoma-free red individual of *Dr Sd-Tu* BC hybrid segregants was propagated to a red substrain which turned out to be completely incapable of developing melanoma spontaneously and almost completely incapable of developing melanoma following treatment with mutagenic carcinogens and tumor promoters. The breeding and treatment experiments suggest that the red substrain had lost the *Sd-Tu* complex but had retained its *Dr* locus on its X chromosome (Fig. 2, lane b). Southern analysis showed that the X-chromosomal 4.9-kb band is still present in the mutant. Provided that the terminal chromosome deletion was created by an unequal crossing over, the finding of the *Sd-Tu* deletion suggests the existence of the corresponding translocation of the platyfish *Sd-Tu* to a chromosome of *X. helleri* thus forming an interspecific hybrid chromosome. A total of 11 translocation events of this type have been observed (see Table 1). Our studies on such a *Sd-Tu* translocation substrain revealed a quasi-purebred greyish green strain of the swordtail that has acquired both the 4.9-kb fragment and the capacity to develop melanoma spontaneously (Fig. 2, lane d). Since, on the one hand, the reddish deletion animals described above have retained the 4.9-kb fragment, and, on the other hand, the greyish green translocation animals have gained this fragment, we conclude that the breaking points of both structural mutation events were different, one proximal to the 4.9-kb fragment and the other distal. The retranslocation of the *Sd-Tu* chromosome fragment (lane d) to the *Dr*-deletion chromosome (lane b) did not affect the 4.9-kb band but restored the capacity to develop melanoma spontaneously (lane c) on the *Dr*-mediated reddish skin.

b) The 6.7-kb Fragment. To analyze the Y-chromosomal 6.7-kb Southern fragment we used BC hybrids carrying a *Dr, Ar Sr-Tu* X/Y translocation chromosome (Fig. 2, lane f) that originated from a *Dr*-deletion X chromosome on which both the *Sd-Tu* complex and the 4.9-kb band were lost and both the *Ar Sr-Tu* and the linked 6.7-kb fragment were gained. Individuals containing this X/Y translocation exhibit phenotypically both the pterinophore patterns "dorsal red" (*Dr*) and "anal red" (*Ar*) and a phenotypically unchanged *Sr-Tu* complex. Following treatment with carcinogens, melanomas develop that are phenotypically the same as those of the BC hybrids carrying the unchanged *Ar Sr-Tu* Y chromosome (lane e). Since the 4.9-kb fragment was not found in these animals, one can assume that the breakpoint of the X chromosome in the X/Y crossover (lane f) was different from that of the *Dr*-deletion X chromosome (lane b) which retained this fragment. On the other hand, the presence of the 6.7-kb fragment indicates its location between the pterinophore locus *Ar* and the *Sr-Tu* complex. A more precise determination of the site of the 6.7-kb fragment comes from a *Sr-Tu* deletion that occurred on

the *Dr, Ar Sr-Tu* X/Y translocation chromosome just mentioned (lane f). The resulting *Dr, Ar,* chromosome (lane g) shows neither the 4.9-kb nor the 6.7-kb band, thus indicating that both fragments each must be normally located between to the pterinophore loci and the melanophore loci. The *Dr, Ar* animals lack the capacity to develop melanoma, possibly because of the loss of the both the *Sd-Tu* and *Sr-Tu* chromosome fragments, including their closely linked x-*erb*B Southern fragments.

c) The 11.5-kb Fragment. This fragment is specific to the *Ye Li-Tu* X chromosome of *X. variatus* (Fig. 1, lane c; Fig. 2, lane h). In the course of a *Li-Tu* translocation onto the *Dr*-deletion X chromosome of *X. maculatus* (lane b) this fragment became detached from *Li-Tu* (break point was proximal to *Li-Tu*) resulting in the *Dr, Li-Tu* hybrid chromosome, which exhibits the *X. maculatus*-specific 4.9-kb fragment but lacks the *X. variatus*-specific 11.5-kb fragment (lane i). The pattern of the melanophores of the BC animals carrying this *X. variatus*/*X. maculatus* hybrid chromosome in their *X. helleri* background genome resemble neither the *Li-Tu* pattern of *X. variatus* nor the *Sd-Tu* pattern of *X. maculatus*. These animals spontaneously develop melanoma of very high malignancy that spreads over and invades the entire body of the fish, indicating that their *Tu* complex is much more out of control than that of any other genotype [14]. The three *Eco*RI restriction fragments comprising 4.9, 6.7, and 11.5 kb are therefore located between the respective pterinophore loci (*Dr, Ar,* or *Ye*) and the remaining parts of the *Tu* complexes (*Sd, Sr,* or *Li*) that are located at the end of the sex chromosomes where the genetic information for melanoma formation (and other types of neoplasia) is encoded.

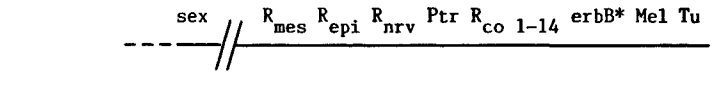

Fig. 3. Preliminary map of the tumor determining region (*Tu* complex) of the sex chromosomes of *X. maculatus* and *X. variatus* based upon 88 deletions, translocations, duplications, and 19 compartment-specific mutations

3. Preliminary Map of the Tumor-Determining Region

A preliminary map of the chromosome region of the *Tu* complex was first proposed 15 years ago [14] and has since that time been subject to several improvements. Based on our earlier observations and the present phenogenetic, cytogenetic, and molecular linkage data we propose one more improvement (Fig. 3):

The tumor-determining region is proximally linked to the sex-determining region. Its constituents are probably located in one Giemsa band which can be lost in total, even homozygously, without creating any detectable disadvantages for the fish [33, 34]. This region is, therefore, considered accessory. Carcinogenesis studies which assigned a large variety of neoplasms other than melanoma to the same *Tu* complex region [17, 35, 36] led to the assumption of a group of tissue-specific regulatory genes (mesenchymal, epidermal, nervous; arbitrary order) adjacent to the sex-determining region. These genes (*R-mes, R-epi, R-nrv*) are followed by at least 14 compartment-specific regulatory genes (*R-co 1–14*) that control the differentiating activity of both the pterinophore (*Ptr*) and melanophore (*Mel*) locus (*Dr Sd, Ar Sr,* etc.). The critical restriction fragments (in Fig. 3 indicated as *erb*B*) are probably very closely linked to both *Ptr* and *Mel*, i.e., they are narrowly intercalated between *Ptr* and *Mel.*

The signal for pigment cell transformation comes certainly from the very end of the chromosome and might possibly be composed of both *erb*B* and *Mel*, which together might represent what was designated arbitrarily as the Mendelian gene *Tu.* In any case, the breaking point data indicate that *erb*B* is not identical with the Mendelian gene *Tu* although it might be involved in its function. Never-

theless we will retain the arbitrary Mendelian symbol *Tu* until we have more information about the biological nature of the entire *Tu* complex.

4. Cloning of the Xiphophorine *erb*B Restriction Fragments

Special information on the nature of these three *Tu* complex-linked fragments comes from studies in which the fragments were molecularly cloned and characterized. As a basis for gene comparison the autosomal *Tu* complex-nonlinked 5.5-kb fragment that is constantly present in all xiphophorine fish irrespective of whether the fish are susceptible to melanoma or insusceptible (see Figs. 1, 2) was also studied. Two λ gt 10 phage libraries were prepared with *Eco*RI digested genomic DNA from *X. maculatus* from Rio Jamapa. By screening the libraries we succeeded in isolation of v-*erb*B homologous clones which contain *Eco*RI inserts representing either the X- and Y-specific 4.9-kb and 6.7-kb fragments (λ x-erb 4.9 gt and λ x-erb 6.7 gt) that are critical for melanoma appearance or the 5.5-kb fragment (λ x-erb 5.5 gt) that appears to be autosomal and, therefore, independent from melanoma appearance.

5. Homology of the Xiphophorine *erb*B Restriction Fragments

Southern blot analysis of restricted DNA from λ x-erb 4.9 gt showed that the v-*erb*B homologous sequences were enclosed in a 0.8-kb *Eco*RI/*Sac*I fragment. Hybridization of this fragment against genomic DNA from *Tu* complex-carrying fish revealed, as shown in Fig. 4 (lanes b–f), that this xiphophorine DNA fragment detects not only the X-chromosomal *Tu* complex-linked 4.9-kb fragment of *X. maculatus* from which it was isolated but also the Y-chromosomal 6.7-kb fragment of the same species and the X-chromosomal 11.5-kb fragment from *X. variatus*. The three fragments are, therefore, highly homologous. No fragment of these lengths could be detected in

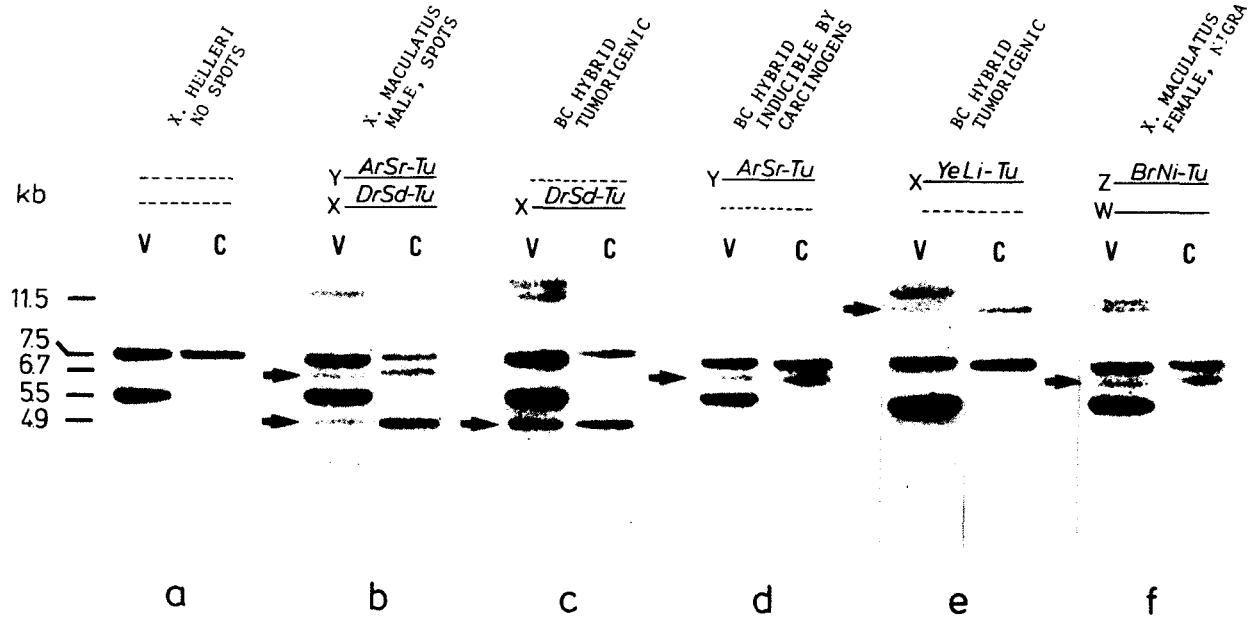

Fig. 4. Hybridization of the cloned 4.9-kb fragment (as indicated in Figs. 1, 2) and hybridization of the v-*erb*B probe against EcoRI digested genomic DNA from purebred and hybrid xiphophorine fish. Note that the 4.9-kb probe detects not only the X-chromosomal 4.9-kb fragment of *X. maculatus* from which it was isolated but also the Y-chromosomal 6.7-kb fragment, the 11.5-kb fragment of the X of *X. variatus*, and an autosomal 7.5-kb fragment. Filters were probed with the v-*erb*B specific fragment under stringent conditions (*V*) and with the 4.9-kb specific fragment under highly stringent conditions (*C*). (For symbols see Table 1)

the genome of *X. helleri* (lane a) or in the genome of the *Tu* complex-lacking purebred and hybrid genotypes (not shown).

As is also shown in Fig. 4, the 0.8-kb *Eco*RI/*Sac*I fragment containing sequences of the 4.9-kb fragment detects not only the other sex-chromosomal *Tu* complex-linked fragments but also an autosomal fragment comprising 7.5 kb which is present in all genotypes irrespective of whether they contain the *Tu* complex (lanes b–f) or not (lane a). This finding is important because earlier and recent carcinogenesis studies suggest that all individuals of *Xiphophorus* contain at least one copy of an autosomal *Tu* complex [3]. Since all deletions of the sex-chromosomal *Tu* complexes are non-lethal in both the heterozygous and the homozygous state, one can conclude that they are accessory for the fish. This is, however, not to say that *Tu* complexes may not be at all essential to the fish. One could, for instance, assume that additional *Tu* complexes present in the autosomes may compensate the loss of the sex-chromosome-linked *Tu* complex according to a gene dosage compensation mechanism, which warrants normal functions. The 7.5-kb fragment could be the indicator of such an indispensable *Tu* complex which is now molecularly approachable. The Southern data obtained with the 4.9-kb fragment and with the v-*erb*B probe under conditions of varied stringency (not shown) suggest that the 7.5-kb band actually consists of two *Eco*RI fragments, the one being closely related to v-*erb*B, and the other being homologous to the 4.9-kb fragment, but more distantly related to v-*erb*B.

Hybridization of the 0.8-kb *Eco*RI/*Sac*I insert of λ x-erb 6.7 gt against genomic *Eco*RI-digested DNA revealed a banding pattern identical to that obtained with the 0.8-kb *Eco*RI/*Sac*I insert of λ x-erb 4.9 gt, indicating more and stronger evidence for a high homology between the X-chromosomal *Tu* complex-linked 4.9-kb fragment and the Y-chromosomal *Tu* complex-linked 6.7-kb fragment.

The restriction map of the λ x-erb 5.5 gt clone showed no similarity to that of the λ x-erb 4.9 gt and λ x-erb 6.7 gt clones which, if compared separately, were very similar. The sequences of λ x-erb 5.5 gt which are homologous to v-*erb*B were enclosed in a 0.8-kb *Xba*I/*Hin*dIII fragment which, when hybridized against *Eco*RI-digested genomic DNA (not shown), detected one single band of 5.5 kb in all individuals of all genotypes, confirming that the insert of this clone represents the always present 5.5-kb fragment, and that the restriction fragment length is always identical.

For further analysis the v-*erb*B homologous regions from λ x-erb 4.9 gt, λ x-erb 6.7 gt and λ x-erb 5.5 gt subcloned in pUC 19 (p x-erb 4.9 gt and p x-erb 6.7 gt, both containing the 0.8-kb *Eco*RI/*Sac*I insert, and p x-erb 5.5 gt, containing the 0.8-kb *Xba*I/*Hin*dIII insert) were further subcloned for sequencing (dideoxy chain termination method).

6. Nucleotide Sequences of Parts of the Xiphophorine *erb*B Restriction Fragments

a) The 4.9-kb Fragment. The nucleotide sequence of the 0.8-kb *Eco*RI/*Sac*I insert of p x-erb 4.9 gt that represents part of the *Tu* complex-linked 4.9-kb fragment of x-*erb*B (see Figs. 1, 2) is shown in Fig. 5. We identified two regions, separated by 88 nucleotides, which share an overall homology of 76% with the nucleotide sequence of v-*erb*B (see [37]). The degree of homology between the partial sequence of the 4.9-kb fragment and the human c-*erb*B1 (the EGF receptor gene; see [38]) is 81% in the first region (nucleotides 70–225) and reaches 76% in the second region (nucleotides 314–391). The homology between the deduced xiphophorine amino acid sequences and that of v-*erb*B was 85% for the first region and 88% for the second region. The degree of homology between the predicted amino acid sequences of the respective regions of human c-*erb*B1 and the xiphophorine 4.9-kb fragment was 81%

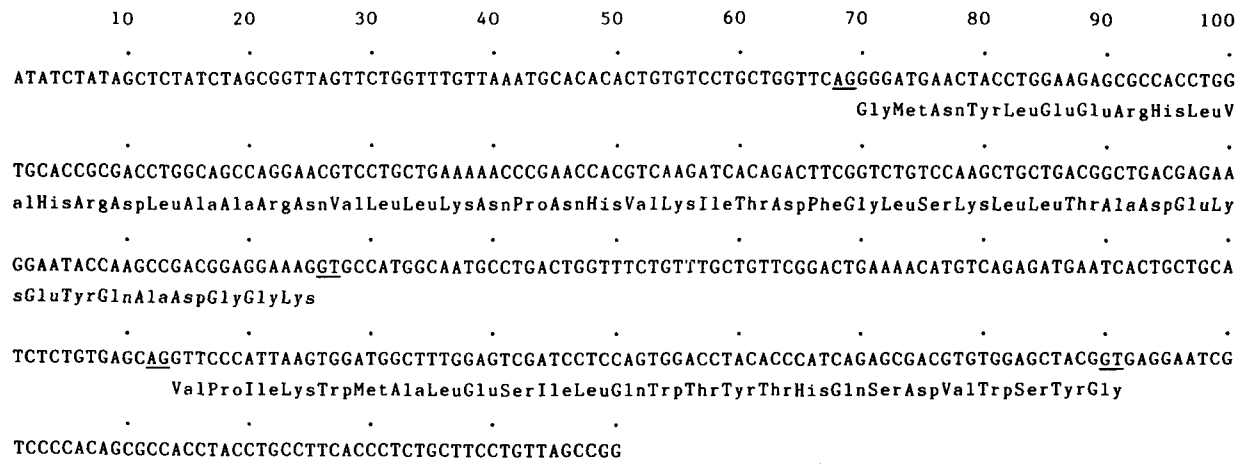

Fig. 5. Nucleotide sequence and the deduced amino acid sequence of the p x-erb 4.9 gt insert. The sequence contains the exons C and D of the X-chromosomal xiphophorine EGF receptor gene. The exons are separated by an intron comprising 88 nucleotides. Nomenclature of the exons is according to that of the human c-*erb*B-2

for the first region and 88% for the second region. Alignment of the deduced amino acid sequences showed that the first region corresponds to the putative exon C of the human c-*erb*B2 (77% homology), and the second region to exon D (85% homology) [39–41]. Since each region is flanked by AG and GT dinucleotides that border the exons of eukaryotic genes, and since the regions show high homology to v-*erb*B and human c-*erb*B on the amino acid level, we suggest that they represent two exons of a xiphophorine gene related to the human EGF receptor gene. In analogy to the human c-*erb*B2 the exons will be referred to as exons C and D.

b) The 6.7-kb Fragment. Sequencing of the 0.8-kb *Eco*RI/*Sac*I insert of p x-erb 6.7 gt (not shown) revealed two putative coding regions that are identical to those of the p x-erb 4.9 gt insert (exons C and D, according to the human c-*erb*B2 gene). The comparison of the putative exons and introns of both inserts revealed two single-nucleotide substitutions in the region of the introns. We consider the genes corresponding to the X- and Y-chromosomal *Tu* complex-linked 4.9-kb and 6.7-kb fragments as two alleles of a xiphophorine gene related to the human EGF receptor gene. These xiphophorine alleles were designated as *x-egfrB-1* (corresponding to the X-chromosomal 4.9-kb fragment) and *x-egfrB-2* (corresponding to the Y-chromosomal 6.7-kb fragment).

c) The 5.5-kb Fragment. The nucleotide sequence of the 0.8-kb *Xba*I/*Hin*dIII insert of p x-erb 5.5 gt that represents part of the *Tu* complex-independent 5.5-kb fragment (see Figs. 1, 2) contains, as shown in Fig. 6, also two putative coding regions; these are separated by 120 nucleotides, which share an overall 82% nucleotide sequence identity with v-*erb*B and 84% with the human c-*erb*B1. The homology between the deduced amino acid sequences of these two regions and the predicted amino acid sequence of v-*erb*B was 91%. Alignment of the amino acid sequences deduced from human c-*erb*B1 and the *Tu*-nonlinked xiphophorine 5.5-kb fragment nucleotide sequence showed that they share 90% homology. The two putative coding regions are flanked by the splicing consensus sequences AG and GT. In contrast to the sequenced coding regions of the X-chromosomal *x-egfrB-1* and *x-egfrB-2* that correspond to exons C and D of the putative human c-*erb*B2, the coding regions of the 5.5-kb fragment correspond to the putative exons B and C of the human c-*erb*B2.

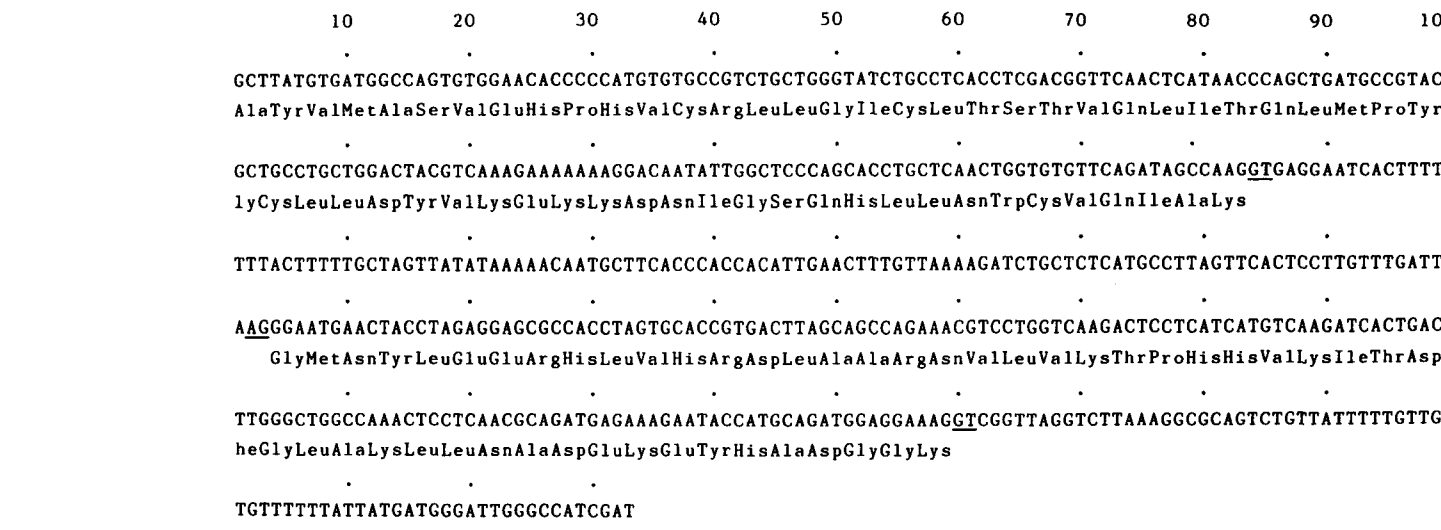

Fig. 6. Nucleotide sequence and the deduced amino acid sequence of the p x-erb 5.5 gt insert. The sequence contains the exons B and C of the autosomal xiphophorine EGF receptor gene. The exons are separated by an intron comprising 120 nucleotides. Nomenclature of the exons is according to that of the human c-*erb*B-2

These data suggest that the sequence of the xiphophorine *Tu*-nonlinked 5.5-kb fragment contains two exons (defined as exons B and C) that also represent part of a xiphophorine gene related to the human EGF receptor gene. This fish gene was designated as *x-egfr*A. Computer-mediated sequence analysis showed that the putative exon C of *x-egfr*A is homologous to the corresponding sequence of several members of the *src* tyrosine kinase family, whereas the sequence of exon B showed no significant homology. The most striking homology was observed with the tyrosine kinase domain encoding sequence of the human EGF receptor gene (c-*erb*B1; [38]).

The homologies between the *Tu* complex-linked 4.9-kb or 6.7-kb fragment and the *Tu* complex-nonlinked 5.5-kb fragment concern the region of the putative exon C and reach a degree of 89% on the amino acid level. Based on our cytogenetic and molecular data we presume that the 4.9-kb and 6.7-kb fragments (and probably the 11.5-kb fragment) and the 5.5-kb fragment are parts of two different types of xiphophorine genes (*x-egfr*A and *x-egfr*B) encoding two slightly different types of EGF receptors, x-EGFR-A and x-EGFR-B. The existence of two different types of EGF receptor genes in *Xiphophorus* (*x-egfr*A and B), one of which (*x-egfr*B) could be involved in the switch from the normal to the neoplastic state while the other is of minor importance in this context, requires discussion concerning structure and function of the receptor domains encoded: One may ask whether both the *x-egfr*A and B encode a growth factor receptor with an extracellular, transmembrane, and cytoplasmic domain. This question arises since it is known from the human EGF receptor that it consists of three domains, and that lack of them is important for receptor regulation [38, 42–46]. Especially it is of interest to determine whether the xiphophorine EGF receptor genes *x-egfr*A and *x-egfr*B encode an extracellular receptor domain capable of binding EGF or other growth factors, and whether the growth factor binding leads to receptor activation. Differences in kinase activity and activation of the receptor by different growth factors could result in a different type of response of the two types of xiphophorine EGF receptors in question to the humoral signals mediating stimulation or inhibition of cell proliferation. Those cells exhibiting the growth factor receptor x-EGFR-B may respond to internal and external signals inducing cell prolif-

eration and changes in a series of cellular regulatory processes, which together could mediate the switch from the normal to the neoplastically transformed phenotype. In this context we want to mention the positive correlation between the presence of the sex-chromosomal *Tu*-linked *x-erb*B genes and the turnover of phosphoinositides, that was discovered very recently in *Xiphophorus* [47–49].

7. Expression of the Xiphophorine EGF Receptor Genes

RNA dot-blot and Northern blot analysis with a probe specific for exons C and D of x-*egfr*B (*Eco*RI/*Rsa*I fragment excised from p x-erb 4.9 gt) showed expression of the respective genes in testes and embryonic tissue of individuals without accessory *Tu* complexes and enhanced expression of x-*egfr*B in melanoma tissue and in a melanoma cell line (see Figs. 7, 8). These data indicate that the sex-chromosomal x-*egfr*B genes are not only structurally but also functionally related to the melanoma-determining accessory *Tu* complexes. On the other hand, it became obvious that x-*egfr*B genes, namely those genes that are probably linked to the indispensable *Tu* complex (disclosed by the ubiquitous 7.5-kb fragment), fulfill an essential function in normal proliferating tissue. These data suggest that the gene products of the accessory x-*egfr*B and the indispensable x-*egfr*B show differences in structure (e.g., amino acid substitutions) and/or function (e.g., regulation, expression) which in turn might "activate the oncogene potential" of the x-*egfr*B and thereby induce the switch from the normal to the neoplastic transformed state of a cell.

Northern blot analyses with a probe specific for exons B and C of x-*egfr*A (*Hin*dIII/*Cla*I fragment excised from p x-erb 5.5 gt) showed that overexpression of a xiphophorine EGF receptor gene can be specified in melanoma with this probe under stringent (Fig. 7), but not under highly stringent conditions. This indicates that x-*egfr*A genes are neither

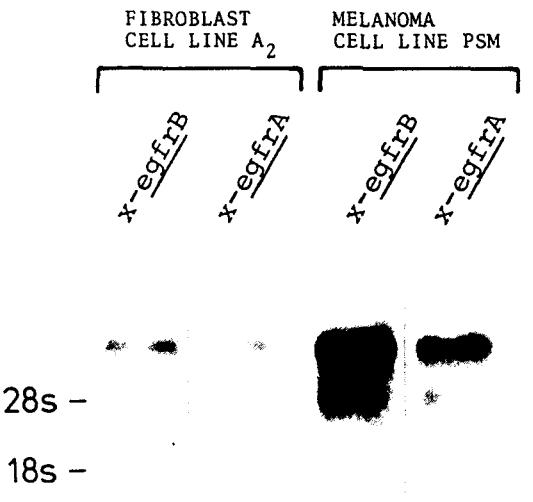

Fig. 7. Expression of xiphophorine EGF receptor genes (Northern blot analysis). Hybridization of probes specific for the x-*egfr*B and the x-*egfr*A against 20 µg total RNA are shown (washing conditions 1 × SSC/1% SDS, 60° C). The hybridization was detected by autoradiography with exposure times of 50 h for the fibroblast cell line RNA and 20 h for the melanoma cell line RNA. Xiphophorine ribosomal RNA of 18 S and 28 S served as internal size markers

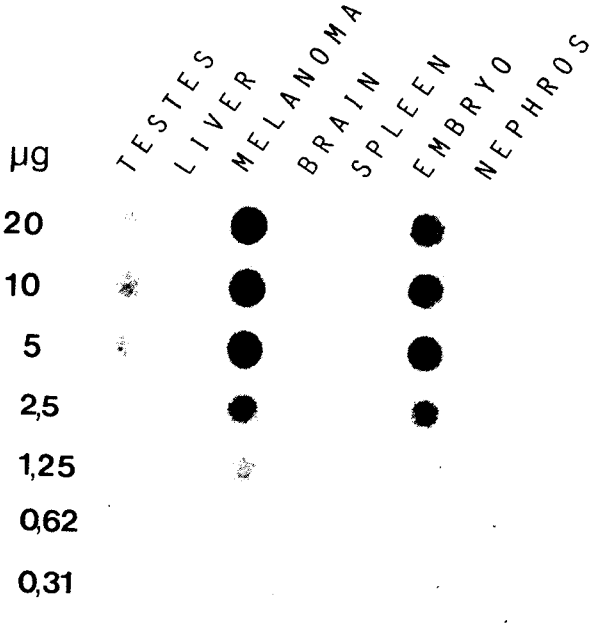

Fig. 8. Expression of the accessory EGF receptor gene x-*egfr*B in normal and transformed tissues (dot-blot analysis). Hybridization of a probe specific for x-*egfr*B against varying amounts of total RNA from different tissues; RNA from whole embryos (stages 17–22, according to [66]) was used. The conditions for the dot-blot analysis were the same as for the Northern blot analysis with the x-*egfr*B specific probe (see Fig. 7).

structurally nor functionally related to the melanoma-determining loci. Expression studies performed with *non*-transformed tissue revealed expression of x-*egfr*A in a fibroblast cell line [22, 28] in eyes, brain and melanoma as well as in a melanoma cell line [22, 28, 30, 31] and a very high amount of x-*egfr*A transcripts in the head nephros [30, 31].

In conclusion, at least two types of xiphophorine EGF receptor genes exist, one of which (x-*egfr*B) is structurally and functionally related to the melanoma-determining loci and therefore could be considered as an oncogene probably critical for the switch from the normal to the neoplastic state of a cell, while the other one (x-*egfr*A) is of minor importance in this context.

II. Oncogenes that Might Be Considered "Critical" for the Late Events in the Manifestation of the Tumor Phenotype

We shall concentrate on genes that might be considered as candidates probably involved in stimulation or repression of proliferation and differentiation of Tr melanocytes.

Not only transforming genes are involved in the causation of spontaneously developing (crossing-conditioned) and induced melanoma. Much more important are the regulatory genes (oncostatic genes) that normally keep the transforming genes and the proliferation genes under negative control [24]. It appears that in the hybridization or in the treatment with the carcinogens some of the *R* genes are lost or impaired, thus permitting an *S* gene-stimulated overexpression of the spotting *Tu* complex that results in the formation of melanoma. It is important to note that a stimulating effect on melanoma formation can also be achieved by tumor promoters such as steroid hormones [15, 36, 50, 51]. These observations led us to the assumption that hormones and hormone receptors, respectively, might be related to the *R* and *S* genes. Since it is known that (a) the members of the steroid/thyroid hormone receptor superfamily act as transcription factors [52, 53], that (b) v-*erb*A is not a direct-acting oncogene but induces the fully transformed phenotype in transformed cells by blocking differentiation [54, 55], and that (c) c-*erb*A encodes a thyroid hormone receptor [56, 57], we started our molecular search for *R* and *S* genes by studies on xiphophorine x-*erb*A genes.

1. Inheritance of Southern Restriction Fragments of the Xiphophorine *erb*A Oncogene

We shall concentrate in particular on certain v-*erb*A homologous DNA fragments which correspond to x-*erb*A genes that probably represent xiphophorine hormone receptor genes (x-*th-r* genes) encoding a receptor which binds thyroid hormone or retinoic acid.

Figure 9 shows a different distribution of several *Eco*RI fragments of x-*erb*A in various purebred and hybrid genotypes: Two fragments comprising 9 and 12 kb are constantly present in all individuals of all populations of *Xiphophorus* tested. Four fragments comprising 2.9, 5.0, 7.5, and 16 kb are restricted to populations of *X. maculatus*. *X. variatus* shows bands of 4.9, 12.0, 9.0 (accessory) and 16 kb. All populations of *X. helleri* tested so far, show species-specific bands of 10 and 14 kb. In addition, it shows species-specific but individually distributed bands comprising 5.2, 5.3, 5.6, and 5.7 kb; at least one, but no more than two of the 5.2-, 5.3-, 5.6-, and 5.7-kb fragments are present in one individual, whereby all combinations of fragments are possible. Southern blot analyses with a probe specific for v-*erb*A revealed a species- and population-specific RFLP for *Hin*dIII-digested genomic DNA (data not shown). Until now, none of the v-*erb*A homologous fragments could be assigned to the *Tu* complex or any *R* or *S* gene. This is not to say that x-*erb*A genes and *R* and *S* genes are not structurally and/or functionally related. Besides the differentiation gene *Diff*, which is molec-

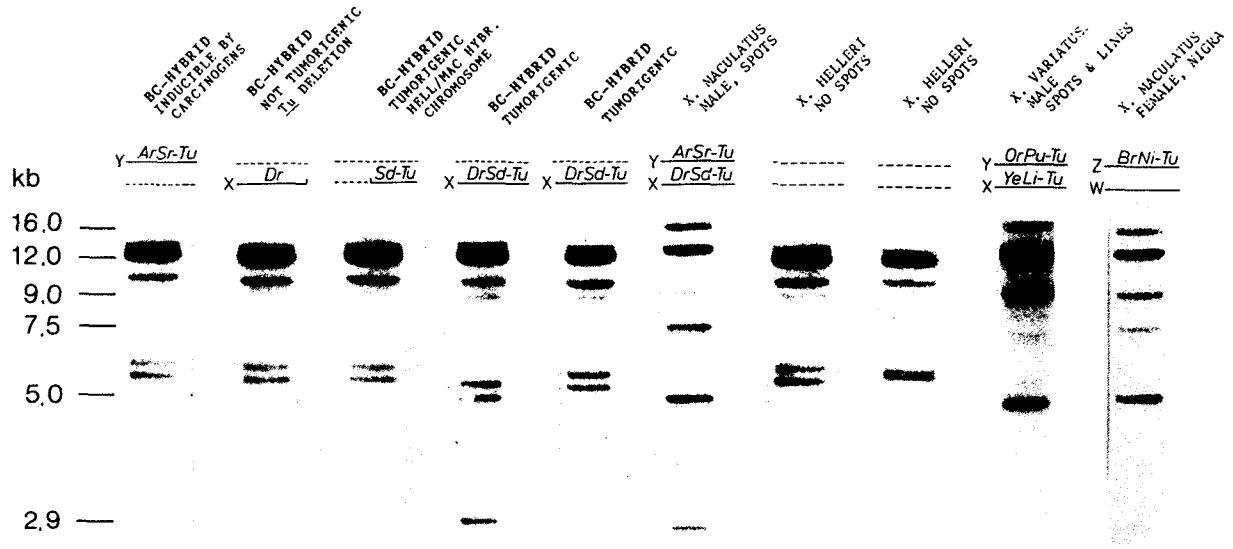

Fig. 9. Detection of an individual- and population-specific RFLP of xiphophorine v-*erb*A homologous sequences (Southern blot analysis). *X,Y,W,Z,* sex chromosomes; *dashes* indicate chromosomes of the recurrent parent *X. helleri* that are homologous to the sex chromosomes of *X. maculatus* and *X. variatus*. For gene symbols and phenotype of the respective animals see Table 1

ularly linked to a locus for esterase 1 [13, 58] and correlated to the appearance of Q base (a highly modified guanine) in certain tRNA species [13, 21], no *Tu*-nonlinked *R* or *S* gene has so far been described to be related to any known molecular or biochemical marker. We wonder whether x-*erb*A-genes themselves might be such markers.

2. Cloning and Sequencing of Xiphophorine v-*erb*A Homologous Restriction Fragments

In order to study x-*erb*A-genes we cloned and characterized four different, distinctly related v-*erb*A homologous restriction fragments, one of which appears to be specific for *X. variatus* [28]. Southern blot and Northern blot analyses confirmed that the cloned sequences are fish specific and represent parts of functional genes.

Two clones representing the v-*erb*A homologous region of the *X. maculatus*-specific 7.5-kb and the ubiquitous 12-kb *Eco*RI fragment [28] were sequenced. Both clones, p x-*erb*A90–3 and p x-*erb* A12–113, contained a stretch of 100 nucleotides exhibiting 75% homology to the v-*erb*A. Alignment of the deduced amino acid sequences of the v-*erb*A homologous regions of the two xiphophorine clones and those deduced from the viral *erb*A [59], the chicken c-*erb*A [56] the human c-*erb*A [57], a human v-*erb*A related sequence representing an open reading frame with hepatitis B virus DNA integration (60) as well as the amino acid sequences predicted for the human retinoic acid receptor h RAR [61, 62], estrogen receptor h ER [63], progesteron receptor h PR [64], and glucocorticoid receptor h GR [65], revealed that both clones contain a sequence probably encoding the first part of the DNA-binding domain (domain C) of two slightly different types of xiphophorine hormone receptors (Fig. 10). The partial sequence of the receptor x-TH-R-1, predicted from the partial sequence of x-*th-r*-1 (clone p x-erbA12–113) shows the most striking homology to the h RAR, while that of x-TH-R-2, deduced from the partial sequence of x-*th-r*-2 (clone p x-erbA90–3) appears to be most homologous to the h T_3R (thyroid hormone receptor; see Fig. 10).

The homologies between the two xiphophorine sequences concern the re-

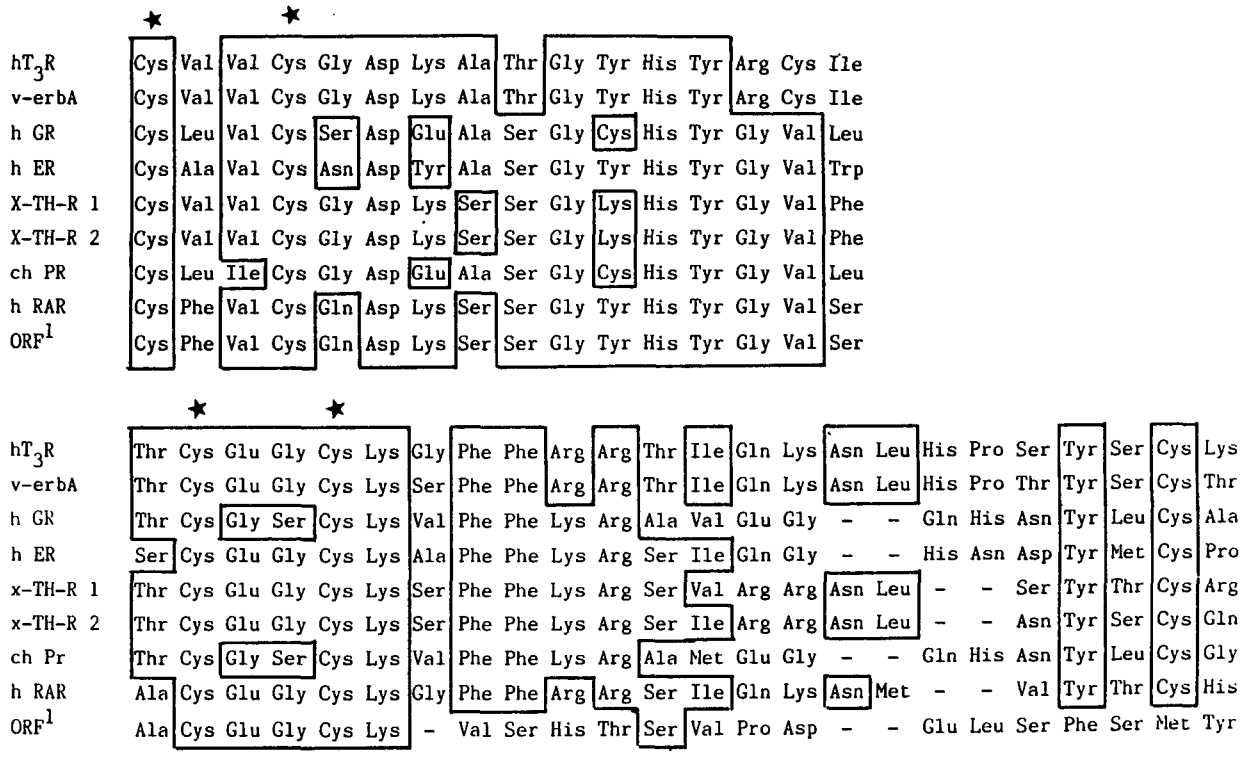

Fig. 10. Comparison between the partial amino acid sequences deduced from the putative xiphophorine hormone receptor genes encoding x-TH-R-1 and x-TH-R-2 and the corresponding sequences of the human thyroid hormone receptor (*hT_3R*), glucocorticoid receptor (*h GR*), estrogen receptor (*h ER*), retinoic acid receptor (*h RAR*), and the chicken progesterone receptor (*ch PR*), as well as those deduced from the viral *erb*A and a human v-*erb*A homologous open reading frame with hepatitis B virus DNA integration (*ORF^1*). Homologous amino acids are *boxed; asterisks* indicate the conserved cysteine residues of the first DNA binding finger of the hormone receptors

gion of the putative exon described above (75% homology to v-*erb*A) as well as the region located upstream to the first mentioned sequence (Fig. 11) and reach about 85% homology on the DNA level. The upstream region shows an open reading frame, which probably could represent a sequence encoding a part of the hypervariable domain A/B of a xiphophorine hormone receptor. Since the hypervariable region A/B is not conserved in the receptors of the steroid/thyroid hormone receptor superfamily [52, 53], we cannot determine whether the upstream region identified in the two xiphophorine sequences represents an exon.

Expression studies showed that x-*erb*A-genes are expressed in a fibroblast and in a melanoma cell line (data not shown). The amount of mRNA, as well as the species of mRNA detected was different in the normal and transformed cells [28].

Since it is known that receptors of the steroid/thyroid hormone receptor superfamily specifically stimulate or repress transcription of distinct genes [53], we wonder whether the x-*erb*A genes x-*th-r*-1 and -2 might be involved in the regulation of the proliferation of Tr melanocytes.

The results obtained in Southern blot, Northern blot, and sequencing analyses indicate that x-*erb*A-genes are differentially organized in the genome of different populations of *Xiphophorus* and that these genes probably encode a variety of different hormone receptors related to the steroid/thyroid hormone receptor superfamily. Further experiments will show whether x-*erb*A genes are involved in the manifestation of the tumor phenotype.

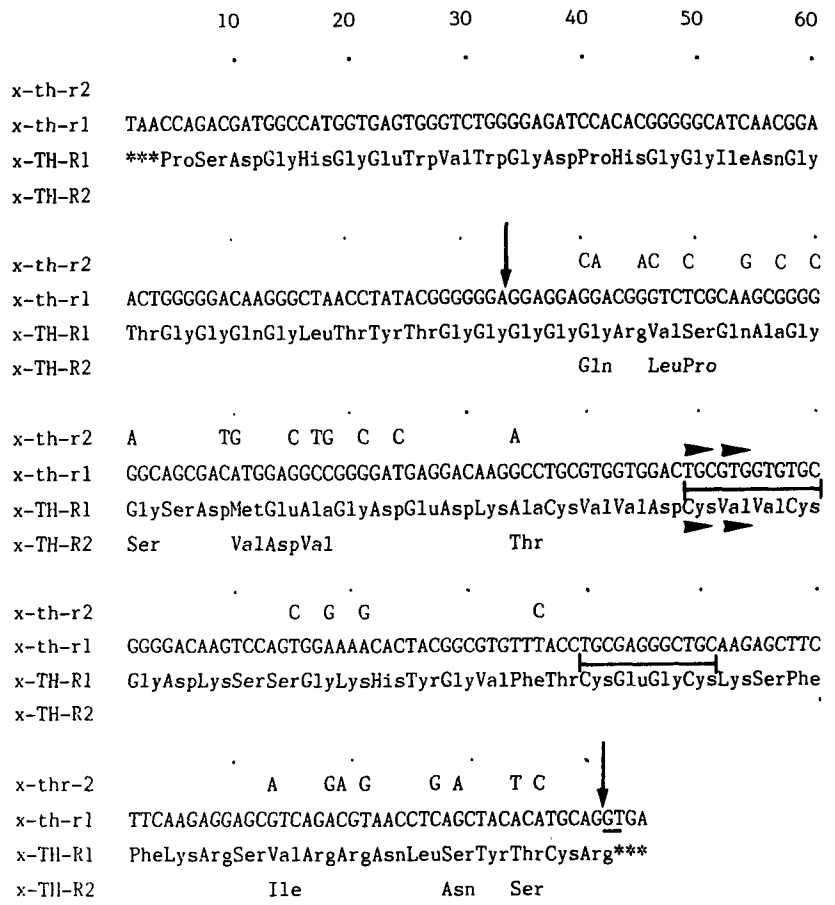

Fig. 11. Partial nucleotide and predicted amino acid sequence of the x-*erb*A clone p x-erb A12-113 (represents part of x-*th-r*-2), and comparison to the x-*erb*A clone p x-erbA90-3 (represents part of x-*th-r*-2). Nucleotides and amino acids of p x-erbA90-3 that are not identical to those of p x-erbA12-113 are shown. *Asterisks* indicate stop codons; *arrows* mark the beginning and the end of the compared region; triangles indicate the beginning of the region homologous to the DNA binding region of steroid and thyroid hormone receptors. The Cys residues corresponding to those conserved in the first DNA binding finger of known hormone receptors are *boxed* (see Fig. 10). The dinucleotide GT that possibly represents a splicing donor site is *underlined*

C. Summary and Conclusions

Southern blot analyses of the xiphophorine genome with probes specific for 15 viral and cellular oncogenes revealed that only three v-*erb*B related *Eco*RI fragments comprising 4.9 kb of a certain X, 11.5 kb of another X, and 6.7 kb of both a Y and a Z chromosome are inherited in parallel with the *Tu* complex and melanoma formation. They are accessory in the genome, and are highly homologous with each other and with an ubiquitous autosomal 7.5-kb fragment. The latter one is probably linked to the indispensable *Tu* complex that is postulated to be present in all individuals of *Xiphophorus* irrespective of whether they possess or lack the capacity to form melanoma in interspecific hybrids. Three restriction fragments, the X-chromosomal 4.9-kb, the Y-chromosomal 6.7-kb and the ubiquitous *Tu*-nonlinked 5.5-kb *Eco*RI fragments were cloned and sequenced. The X- and the Y-chromosomal fragments show perfect identity in the regions of the putative exons C and D of the EGF receptor gene and minor but significant differences to the putative exon C (exon D not identified) of the *Tu*-nonlinked fragment of 5.5 kb, indicating that at least two different types of x-*erb*B genes coding for slightly different EGF-receptors exist in the fish. Northern blot

analyses revealed expression of the *Tu*-linked x-*erb*B genes (x-*gfr*B genes) in both transformed and nontransformed tissue, suggesting their essential role in regulation of normal cell proliferation and in carcinogenesis. We conclude that the indispensable x-*egfr*B genes remain unchanged and strictly regulated, while the sex chromosomal accessory x-*egfr*B genes possibly undergo dramatic changes in structure and/or function (e.g., unscheduled expression, ectopic expression, point mutations, truncation) leading to activation of the oncogenic potential of these genes, which in turn could induce several cellular events involved in the switch from the normal to the transformed state of the cell.

In contrast, none of the x-*erb*A restriction fragments could be assigned to the *Tu*-complex or to any regulatory gene (R or S). These results, however, do not exclude the existence of a structural and/or functional relation between x-*erb*A genes and *R* and *S* genes. We therefore analyzed x-*erb*A genes by cloning, sequencing, and expression studies. The data revealed the existence of at least two types of xiphophorine *erb*A genes (x-*th-r* genes) coding for slightly different hormone receptors that are presumably related to the human thyroid hormone and retinoic acid receptor, respectively. It appears that these genes could be involved in the effect of tumor promoters.

We suppose that in analogy to the *erb* A and *erb*B of the avian erythroblastosis virus, xiphophorine *erb*A and *erb*B genes might somehow act in a synergistic way, whereby the x-*erb*B genes are probably involved in the process of cell transformation while the x-*erb*A genes are possibly responsive for regulation of Tr melanophore differentiation.

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