Homology of Melanoma-Inducing Loci in the Genus *Xiphophorus*

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ABSTRACT

Several species of the genus *Xiphophorus* are polymorphic for specific pigment patterns. Some of these give rise to malignant melanoma following the appropriate crossings. For one of these pattern loci from the platyfish *Xiphophorus maculatus* the melanoma-inducing gene has been cloned and found to encode a novel receptor tyrosine kinase, designated X*mrk*. Using molecular probes from this gene in Southern blot analyses on single fish DNA preparations from 600 specimens of different populations of various species of the genus *Xiphophorus* and their hybrids, either with or without melanoma-predisposing pattern, it was shown that all individuals contain the X*mrk* gene as a proto-oncogene. It is located on the sex chromosome. All fish that carry a melanoma-predisposing locus which has been identified by Mendelian genetics contain an additional copy of X*mrk*, closely linked to a specific melanophore pattern locus on the sex chromosome. The melanoma-inducing loci of the different species and populations are homologous. The additional copy of X*mrk* obviously arose by a gene-duplication event, thereby acquiring the oncogenic potential. The homology of the melanoma-inducing loci points to a similar mechanism of tumor suppression in all feral fish populations of the different species of the genus *Xiphophorus*.

IN *Xiphophorus* two types of black pigment cells can be distinguished: one, micromelanophores, small pigment cells, which contribute to the uniform grayish body coloration of all individuals of the genus and in some species form polymorphic dark spot patterns and two, macromelanophores, large, intensely black pigment cells that compose certain bold markings (GORDON 1927; KALLMANN 1975). Nine of the 18 species recognized to date (ROSEN 1979; MEYER and SCHARTL 1979; LECHNER and RADDA 1988; SCHARTL and SCHRÖDER 1988) are polymorphic for such macromelanophore patterns (M pattern). These M patterns have attracted a lot of attention because for several of them it has been found that they develop into tumorous lesions following the appropriate inter-populational or interspecific hybridizations (GORDON 1927; KOSSWIG 1928; HÄUSSLER 1928; ATZ 1962; ZANDER 1969; ANDERS, ANDERS and KLINKE 1973; KALLMANN 1975). Detailed studies on the M-pattern genes of the platyfish (*X. maculatus*), in particular on *Spotted dorsal* (*Sd*) in fish of the Rio Jamapa population, have led to the definition of a dominant acting melanoma-inducing gene (*Tu*) as an integral part of the *Sd* locus being referred to as *Tu-Sd*. A model has been proposed by which the transforming activity of *Tu-Sd* in wild-type platyfish is suppressed by antioncogenic loci (*R*), which are nonlinked to *Tu-Sd*. Following crossing-conditioned elimination of *R*-bearing chromosomes of the Rio Jamapa platyfish by backcrossing, *e.g.*, to *X. helleri*, the *Tu-Sd* oncogene can exert its transforming function in the pigment cell lineage. This leads to development of malignant melanomas in the hybrid fish (see AHUJA and ANDERS 1976; ANDERS *et al.* 1984). Based on this melanoma formation, *Xiphophorus* became a unique genetic system in experimental oncology, allowing the dissection of the multiple factors involved in establishing the neoplastic phenotype of the transformed cells.

Using reverse genetic approaches, identification of a marker sequence for the *Tu-Sd* locus (SCHARTL 1988) led to molecular cloning of a gene which is a structural constituent of *Tu-Sd* (WITTBRODT *et al.* 1989). This gene, designated X*mrk*, encodes a novel putative cell surface growth factor receptor with an intracellular tyrosine kinase domain and belongs to the family of epidermal growth factor receptor-related genes. Disruption of X*mrk* in an insertion mutant leads to loss of the malignant phenotype, namely no development of melanoma in hybrids. This showed that X*mrk* activity is essential for melanoma formation and is the critical functional constituent of the *Tu* locus.

In addition to the *Tu-Sd*-encoded sequence, a second nontumorigenic copy of X*mrk*, designated INV-X*mrk*, has been cloned from the platyfish, which due to its differential expression during normal embryonic development is supposed to represent the corresponding proto-oncogene (ADAM, MÄUELER and SCHARTL 1990). Its physiological function is currently unknown.

The question of whether the polymorphic M-pattern loci of the different *Xiphophorus* species are ho-

mologous, *i.e.*, identical by descent and if they all contain an activated oncogene, is of major importance for our understanding of the evolution of the melanoma-inducing gene and its tumorigenic function. The problem has been approached by detailed formal genetic experiments (KALLMAN and ATZ 1966) but could not be solved totally. The authors could show that some M-pattern genes occupy homologous regions on the sex chromosomes but others were found clearly on nonhomologous chromosomes. While some pattern following the appropriate crossings give rise to extremely malignant melanomas of high frequencies, others lead only to a mild melanosis in any crossing, and a third group is not enhanced at all but tends to be reduced following hybridization (ATZ 1962; ZANDER 1969; ANDERS and KLINKE 1965). In addition, the problem of homology of the M-pattern loci implicates other important so far unsolved questions. (1) Do the M-pattern loci represent the wild-type situation or are they due to a mutational event that has become fixed during evolution of the genus? This point is especially intriguing because in all populations polymorphic for M pattern, a certain number of individuals (sometimes in preponderance) can be found which do not show M pattern at all. Some pattern occur with high frequency while others are extremely rare, *e.g.*, the N^2-pattern gene of *X. maculatus* has been found only twice among thousands of fish inspected (K. D. KALLMAN, personal communication). (2) Does the M-pattern locus contain one or more genes which determine on the one hand the physiological properties of the macromelanophore and on the other hand the pattern information directing the development of macromelanophores to distinct compartments of the body? Several phenotypically similar M patterns have been found even in different species, while, *e.g.*, in *X. maculatus* up to five different M-patterns may be found in the same population (GORDON and GORDON 1957).

With the availability of molecular probes from the X*mrk* gene of the *Tu-Sd* locus from the Rio Jamapa platyfish these questions on gene homology and genomic arrangements can now be approached on the molecular genetic level.

MATERIALS AND METHODS

Animals: Fish used in this study were either from closely inbred strains maintained under standard conditions (KALLMAN 1975) in the aquarium of the gene center or collected from their original habitats in Mexico during a collection trip in the spring of 1989. Founder fish for our strains were obtained from A. and F. ANDERS (Genetics Institute, University of Giessen, Federal Republic of Germany), from K. D. KALLMAN (Osborne Laboratories, New York Aquarium, Brooklyn, New York) or from nonscientific institutions. All strains employed in this study were derived from at least one brother-sister mating to warrant absence of polymorphy for the sex chromosomal loci analyzed.

DNA probes: Probes used for Southern analysis were: (1) pXX21: 814-bp *Eco*RI/*Sst*I fragment from the *X* chromosomal X*mrk* gene of *X. maculatus* (Rio Jamapa) (ADAM *et al.* 1988). (2) p17-2: cDNA fragment encompassing the tyrosine kinase and carboxy-terminal domains of a *Y* chromosomal X*mrk* (WITTBRODT *et al.* 1989). (3) p3-2: cDNA fragment encompassing the extracellular, transmembrane and iuxtamembrane domains of a *Y* chromosomal X*mrk* (WITTBRODT *et al.* 1989). (4) X*src* 724: 900-bp fragment containing the entire 3′-untranslated region of the *Xiphophorus* c-*src* cDNA (F. RAULF, unpublished results).

Isolation of DNA and Southern analysis: For field sampling fish were killed by cervical dislocation and the pooled organs (brain, gills, liver, spleen, kidney and testes) were immediately lysed in a sample-storage buffer and gently homogenized with a pestle. The buffer contained 0.5 M EDTA, pH 8, 0.2 M NaCl, 1% SDS, and 1 drop/ml of freshly prepared proteinase K solution (10 mg/ml). In this buffer the DNA can be stored as a crude lysate without further purification for more than 4 weeks without any sign of degradation. This buffer was found to be superior to others based on saturated CsCl solutions or guanidine thiocyanate. In the laboratory the samples were then diluted with 2 volumes H_2O, extracted with phenol/chloroform and the DNA was dissolved following ethanol precipitation in 10 mM Tris/1 mM EDTA (pH 7.6). Generally the average molecular mass of the DNA was above 40 kb. DNA from laboratory fish was prepared essentially as described elsewhere (SCHARTL 1988). The DNA samples were subjected to Southern analysis as described for homologous probes (SCHARTL 1988) except that the probes were labeled by random oligonucleotide priming in the presence of [^{32}P] dCTP (FEINBERG and VOGELSTEIN 1984).

RESULTS

Presence of sequences related to the X*mrk* proto-oncogene of *X. maculatus* in other *Xiphophorus* species: To investigate if the X*mrk* gene is present in other *Xiphophorus* species, *Eco*RI-digested genomic DNA from 16 species of 23 different populations was hybridized to the *X. maculatus* X*mrk*-probes (pXX21, p17-2). Under conditions of high stringency, in *X. gordoni*, *X. couchianus* and *X. meyeri* a 10-kb fragment was detected, while all individuals of all other species exhibited a 7.0-kb fragment corresponding exactly in size to the fragment detected with the same probes as the INV-X*mrk* proto-oncogene of *X. maculatus* (Table 1). It is therefore concluded that the X*mrk* gene is present in all *Xiphophorus* species, although expression studies will have to be performed to confirm that these sequences are functional as proto-oncogenes as in *X. maculatus*. The fragment length polymorphism observed in *X. gordoni*, *X. couchianus* and *X. meyeri vs.* all other species is in line with the taxonomic status of these three species as comprising the phylogenetically oldest species group distinct from all other species of the genus (ROSEN 1979; SCHARTL and SCHRÖDER 1988). Using pXX21 which comprises 0.8 kb of the total 25 kb locus of X*mrk* (ADAM, MÄUERLER and SCHARTL 1990) on *Sac*I-, *Bam*HI- or *Hin*dIII-digested DNA, always single bands were detected (data not

TABLE 1

Presence of sequences related to the INV-X*mrk* gene of *X. maculatus* in other *Xiphophorus* species

Species	Population	*n*	*Eco*RI-RFLP
X. gordoni	Laguna St. Tecla	3	10.0
X. meyeri	Musquiz	2	10.0
X. couchianus	La Huasteca	2	10.0
X. birchmanni	Rio Chilcoaloya	2	7.0
X. cortezi	Rio Axtla	6	7.0
X. montezumae	Cascadas de Tamasopo	12	7.0
	Rio Salto	10	7.0
	Nasciemento	5	7.0
	Ojo Frio	11	7.0
X. milleri	Laguna Catemaco	4	7.0
X. variatus	Rio Panuco	3	7.0
	Rio Coy	4	7.0
X. evelynae	Necaxa	7	7.0
X. xiphidium	Rio Purification	18	7.0
	Rio Soto la Marina	6	7.0
	Santa Engracia	2	7.0
X. pygmaeus	Rio Huchihuayan	2	7.0
X. nigrensis	Rio Choy	2	7.0
X. andersi	Rio Atoyac	4	7.0
X. clemenciae	Rio Sarabia	1	7.0
X. helleri	Rio Lancetilla	21	7.0
	Rio San Juan	3	7.0
	Rio Agua fria	3	7.0
X. signum	Rio Chaymaic	2	7.0
		Σ 135	

shown) supporting that INV-X*mrk* is a single copy gene in *Xiphophorus*.

Sequences homologous to the *Tu-Sd* encoded X*mrk* oncogene of the Rio Jamapa platyfish in other platyfish populations: In the Rio Jamapa platyfish it was shown that the *X* chromosomal *Tu-Sd* M-pattern locus, which gives rise to malignant melanoma following the appropriate crossings, encodes an oncogenic version of the X*mrk* gene (WITTBRODT *et al.* 1989). A genomic X*mrk* probe (pXX21) detects in *Eco*RI digests the *Tu-Sd*-X*mrk* as a 5-kb fragment in Southern analysis, additional to the INV-X*mrk* proto-oncogene which is represented by the 7-kb fragment (see above). This restriction fragment length polymorphism (RFLP) is due to a different genomic organization in the 3′ region of the INV-X*mrk*-locus (WITTBRODT *et al.* 1989; ADAM, MÄUELER and SCHARTL 1990). Similarly a 6.5-kb *Eco*RI RFLP for the *Y* chromosomal *Tu-Sd* of the Rio Jamapa platyfish was observed.

To determine whether a homologous situation is found in other platyfish populations with different M-pattern loci, Southern blot analysis was performed using pXX21 as an X*mrk*-specific probe under conditions of high stringency (Table 2). In 73 individuals without any M pattern only the 7-kb fragment of the INV-X*mrk* proto-oncogene was observed. However, all 191 animals with M-pattern loci, including Rio Jamapa platyfish, showed either a 5- or 6.5-kb additional X*mrk* fragment, depending on the sex chromosome analyzed. In all individuals from natural populations *X* chromosomal M-pattern loci were represented by the 5-kb X*mrk* fragment, while *Y* chromosomal loci were represented by a 6.5-kb fragment, as in the Rio Jamapa platyfish. Only in the two domesticated stocks was this situation reversed. This may simply be explained by a crossing over between the sex chromosomes during the intensive breeding and selection procedures of the domesticated stocks. Sex chromosomal crossing over occurs in *X. maculatus* at a frequency of 0.2–0.3% (KALLMAN 1965). In summary, these data suggest that the X*mrk* oncogene which is part of *Tu-Sd* is also present at the other M-pattern loci of platyfish, namely *Sr* (*Striped*)—as already shown in the Rio Jamapa platyfish (WITTBRODT *et al.* 1989), *Sp* (*Spotted*), *N* (*Nigra*), *Sb* (*Spotted belly*) and *Fu* (*Fuliginosus*). To find out if the additional X*mrk* fragment detected in M-pattern carrying fish represents indeed an additional X*mrk* gene or a polymorphic allele of the INV-X*mrk* proto-oncogene locus, the X*mrk* gene dosage was determined. For this purpose Southern blot analysis was performed using a probe (p3-2) from the 5′ portion of the cDNA. In *Eco*RI-digested DNA this probe was found to detect a single fragment of 8.4 kb for all loci or X*mrk* copies in *X. maculatus* and *X. helleri* (data not shown). When exactly the same amount of DNA was loaded on the gel, samples from fish with one or more *Tu* loci gave a more intensively hybridizing band than those from fish which carry only the INV-X*mrk* (Figure 1). This indicates that the *Tu*-associated X*mrk* sequences represent true additional copies of the gene in the genome of such fish.

Localization of the X*mrk* proto-oncogene and the X*mrk* oncogene on the sex chromosome of *X. maculatus*: To define linkage between sex chromosomal loci, backcross hybrids were analyzed for segregation. Such intercrosses and backcrosses have been shown previously to segregate in agreement with Mendelian expectation using 41 protein-coding loci of 6 linkage groups, and they gave no evidence for segregation distortion or unusual crossover frequencies (MORIZOT and SICILIANO 1984). This we could also demonstrate for the sex chromosomal melanoma oncogene loci. In 47 broods of backcross offspring from *X. maculatus* (Rio Jamapa, *Tu-Sd*) and *X. helleri* using *X. helleri* as the recurrent parent (BC_4 to BC_7) 1410 fish exhibited the *Tu-Sd* phenotype, while 1424 were absolutely tumor-free, demonstrating highly significant (χ^2 = 0.069) a 1:1 segregation and confirming that X*mrk*-

TABLE 2

X*mrk* RFLPs (*Eco*RI) for different *Tu* alleles

Allele of *Tu* locus	Pterinophore locus	Sex chromosome	Origin	Restriction fragment length (kb)	No. of fish analyzed
Tu-Sd	*Dr*	X	Rio Jamapa[a]	5.0	88
Tu-Sr	*Ar*	Y	Rio Jamapa[a]	6.5	53
		Y	Rio Usumacinta[b]	6.5	4
		Y	Rio Coatzecoalcos	6.5	2
Tu-N^1	*Br*	*Y*	Belize River[a]	6.5	4
Tu-N^2	*Br*	*Y*	Rio Coatzecoalcos[a]	6.5	4
	Ar	*Y*	Rio Papaloapan	6.5	3
Tu-Sp^1		*X*	Rio Jamapa[a]	5.0	4
Tu-Sp^4	*Dr*	*Y*	Rio Usumacinta[b]	6.5	3
Tu-Sp^9		*Y*	Rio Coatzecoalcos[b]	6.5	2
Tu-Sp^{10}	*Dr*	*X*	Rio Papaloapan[b]	5.0	8
Tu-Fu	*By*[c]	*X*	Domesticated stock	6.5	3
Tu-Sb[d]	*Rt*	*Y*	Domesticated stock	5.0	13
		W	Rio Usumacinta[a]	—[e]	32
		Y	Rio Usumacinta[a]	—	3
	Iy	*Y*	Rio Usumacinta[b]	—	1
		W	Rio Coatzecoalcos[b]	—	4
		W	Lake Catazaja	—	4
	Cpy	*Y*	Lake Catazaja	—	2
	Dr	*X*	Rio Coatzecoalcos	—	1
		Y	Rio Coatzecoalcos[b]	—	3
	Iy	*X*	Rio Papaloapan[b]	—	7
	Ar	*Y*	Rio Papaloapan[b]	—	3
		X	Rio Papaloapan	—	4
		ND[f]	Rio Tonala	—	5
		ND	Rio Tulija	—	4
					Σ 264

[a] Original stock obtained from A. and F. ANDERS, Giessen.
[b] Original stock obtained from K. D. KALLMAN, New York.
[c] *By*, body yellow, yellow coloration of body and unpaired fins, red in fertile males.
[d] According to KALLMAN (1975), the *Sb* gene described by GORDON (1946) is presumably a type B allele of *Sp* in the Rio Papaloapan population.
[e] Only the invariant restriction fragment is seen in Southern blots.
[f] ND, not determined.

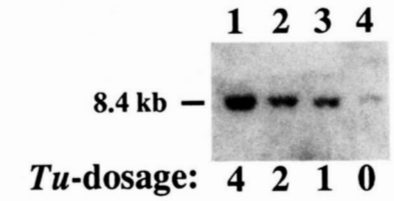

FIGURE 1.—Gene dosage analyses of X*mrk* in platyfish carrying differing numbers of *Tu* loci. Equal amounts of *Eco*RI-digested DNA from *X. maculatus* carrying (*1*) four *Tu* loci ($X^{Tu\text{-}Sd\ Tu\text{-}Sr}/X^{Tu\text{-}Sd\ Tu\text{-}Sr}$) (mutant, unpublished), (*2*) two *Tu* loci ($X^{Tu\text{-}Sd}/X^{Tu\text{-}Sd}$) (Rio Jamapa), (*3*) one *Tu* locus ($W/Y^{Tu\text{-}Sr}$) (laboratory stock, origin of $Y^{Tu\text{-}Sr}$: Rio Jamapa, origin of W: Rio Usumacinta), and (*4*) no *Tu* loci (*W/Y*) (Lake Catazaja) was hybridized to p3-2.

mediated melanoma formation is due to a single Mendelian locus.

The order of known loci (*P* = sexual maturation, *Ir* = iris pigmentation, *Pt* = pterinophore pattern, *Tu*) on the *X. maculatus* sex chromosomes has been determined as cen-*P*-(*Ir-Pt*)-*Tu*-ter (KALLMAN 1970, 1975) with a distance of *P* and the pigmentary pattern loci of approximately 1 cM (GORDON 1937). In 3163 backcross hybrids of 62 broods (BC_2 to BC_7, crossing as above) we obtained no recombinant for the *Tu-Sd* and the *Pt-Dr* loci (parental class 1, *Pt-Dr Tu-Sd*: 1735 individuals, parental class 2, wild type: 1428 individuals). This indicates a very close linkage of less than 0.03 cM for both phenotypic markers, consistent with earlier notions that crossing over between these loci is an extremely rare event (see KALLMAN and ATZ 1966; KALLMANN 1975).

The *Eco*RI polymorphism observed for the INV-X*mrk* gene in *X. couchianus, X. gordoni* and *X. meyeri* allowed chromosomal assignment of the INV-X*mrk* gene of *X. maculatus*. In the Rio Jamapa platyfish it was shown that a 5-kb *Eco*RI fragment represents the *Tu-Sd*-X*mrk* gene on the *X* chromosome (WITTBRODT

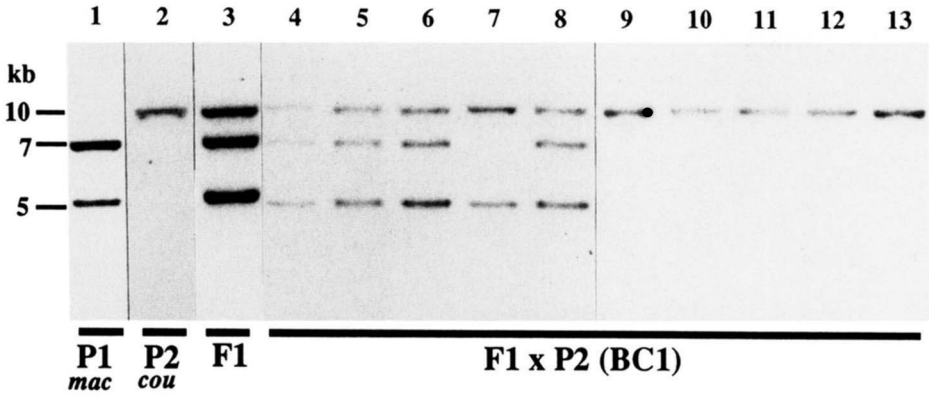

FIGURE 2.—Linkage analysis of INV-X*mrk* and the oncogenic *X* chromosomal X*mrk* locus. *Eco*RI-digested DNA of (*1*) *X. maculatus*, Rio Jamapa, female, $X^{Tu\text{-}Sd}/X^{Tu\text{-}Sd}$, (*2*) *X. couchianus*, male, no sex chromosomal *Tu* locus, (*3*) *mac/couch* F_1-hybrid, (4–13) backcross segregants, was hybridized in Southern blot analyses to the p17-2 probe of X*mrk*. Note cosegregation of the *X. maculatus* 7-kb INV fragment and the *X* chromosomal 5-kb band. Because *X. couchianus* was used as the recurrent parent for backcrossing, all segregants carry the 10-kb *X. couchianus*-specific fragment. Specimen 7 is a recombinant, obviously due to interspecies sex chromosomal crossing over.

TABLE 3

Linkage analysis of the INV-X*mrk* and *Tu-Sd*-X*mrk* genes of *X. maculatus* in *X. maculatus/X. couchianus* backcross hybrids

			N
Parental class 1	INV-X*mrk* 7.0	*Tu-Sd*-X*mrk* 5.0	27
Parental class 2	—	—	20
Recombinant class 1	INV-X*mrk* 7.0	—	0
Recombinant class 2	—	*Tu-Sd*-X*mrk* 5.0	1
Total			48

As *X. couchianus* was used as the recurrent parent in the backcrosses all animals exhibit the *X. couchianus* specific 10-kb X*mrk* fragment.

et al. 1989). The INV-X*mrk* proto-oncogene in platyfish is represented by a 7-kb fragment, while in *X. couchianus* it is represented by a 10-kb fragment. No additional X*mrk* fragment is detected in *X. couchianus*. F_1 hybrids of female platyfish with *X. couchianus* revealed as expected the 10-, 7- and 5-kb fragments in Southern blot analysis. Backcross hybrids using *X. couchianus* as the recurrent parent showed cosegregation of the *X. maculatus* 7-kb INV-X*mrk* fragment with the *X* chromosomal 5-kb *Tu-Sd*-X*mrk* fragment (Figure 2, Table 3). This shows that the INV-X*mrk* gene is also located on the sex-chromosome. The recombination fraction was 2%, therefore both copies of the gene reside on the *X* chromosome within a distance of approximately 2 cM. It can, however, not be excluded that the one "recombinant" observed results from a deletion, gene conversion event, etc. since close flanking markers were not available for this analysis. In such case the distance of both copies would be even closer.

Association of additional X*mrk* copies in other species with M-pattern loci predisposing for melanoma formation: To investigate if–as in *X. maculatus*–melanoma formation may be due to additional X*mrk* copies specifically associated with M-pattern loci also in other species, the genomic structure of X*mrk* in *X. xiphidium* was analyzed. This species is–as is *X. maculatus*–polymorphic for M patterns. A strain derived from the Rio Purification population which exhibits the *Y* chromosomal "flecked" ($Tu\text{-}Fl^1$) M pattern (see ANDERS and KLINKE 1965), one from the Rio Soto la Marina and one from Santa Engracia, both without detectable M pattern, were employed in this study. To assure that the two M-pattern-free strains do not contain cryptic M-pattern loci with low penetrance or without phenotypic expression, five males from each of the three strains were hybridized to *X. helleri*. While all F_1 offspring from the M-pattern-free strains (n = 82 from the Rio Soto la Marina males, n = 167 from the Santa Engracia males) did not exhibit a single macromelanophore spot, half of the offspring from $Y^{Tu\text{-}Fl^1}$ males showed strong enhancement of the M pattern. Backcrossing of such hybrids using *X. helleri* as the recurrent parent yielded the expected segregation (53 with $Tu\text{-}Fl^1$ expression, 60 without M pattern) where the phenotype of the $Tu\text{-}Fl^1$ carrying fish varied from enhanced expression as compared to the wild type to severe malignant melanoma (Figure 3).

For the X*mrk* gene of *X. xiphidium* a *Bam*HI RFLP was found. An additional 10.5-kb fragment was invariably detected in DNA of male fish of the Rio Purification which carry the $Tu\text{-}Fl^1$ locus. In the F_1 and backcross hybrids with *X. helleri* this *Bam*HI fragment cosegregated with $Tu\text{-}Fl^1$ without detectable recombination (Table 4).

A similar result was obtained for the "lined" M pattern of *X. variatus* (Rio Panuco), encoded by the *X* chromosomal *Tu-Li* locus. This pattern is also enhanced following crossing and backcrossing with *X. helleri* (ANDERS and KLINKE 1965); however, highly malignant melanomas develop only in fish homozygous for *Tu-Li*. A *Hin*dIII RFLP was found, giving an additional 12-kb fragment that cosegregates with *Tu-Li* with no recombinants found (Table 4).

For the *Tu-Sc* (*Spotted caudal*) M-pattern locus of *X. cortezi* (Rio Axtla), which is strongly enhanced upon hybridization with *X. helleri*, an additional X*mrk* *Hin*dIII fragment of 6.5 kb was detected (Table 4).

Three M patterns have been described which so far have not been found to be enhanced in any of the

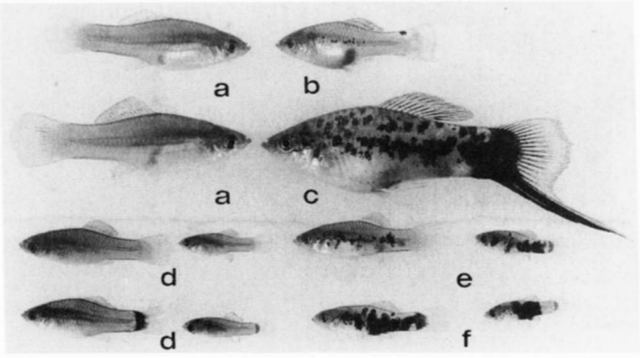

FIGURE 3.—Crossing scheme of *X. helleri* and *X. xiphidium, Tu-Fl*^{1} (a) female of purebred *X. helleri*; (b) *X. xiphidium*, male, exhibiting the Flecked^{1} pattern as dark black spots at the midlateral line of the body; (c) F_1-hybrid, male with strongly enhanced Flecked^{1} pattern, forming two-dimensional growing benign melanoma; (d) tumor-free backcross segregants; (e) backcross segregants with benign melanoma (phenotype comparable to (c)); and (f) backcross segregants with malignant, fatal melanoma.

numerous intra- and interspecific crosses, namely "*Punctatus*" (*Pu*) of *X. variatus*, "*Atromaculatus*" (*At*) of *X. cortezi* and "*Dabbed*" (*Db*) of *X. helleri* (see ATZ 1962; ZANDER 1969). These loci obviously do not predispose for melanoma formation. To investigate if such loci are also associated with an additional X*mrk* fragment, DNA samples of *X. helleri* fish with or without *Db*^{2} were digested with 19 different restriction enzymes and probed with the X*mrk* gene in Southern blot analyses. In no case an indication for an additional X*mrk* gene as in the case of the melanoma-predisposing loci of *X. maculatus, X. xiphidium, X. variatus* and *X. cortezi* was found, neither as an additional polymorphic restriction fragment nor by twice intense bands in the *Db*^{2}-containing fish DNA relative to the *Db*^{2}-less fish DNA (Figure 4). Both phenomena would be indicative of higher X*mrk* gene dosage in *Db*^{2}-containing fish. Using those restriction enzymes which most frequently detect X*mrk* RFLPs in *Xiphophorus*, a similar result was obtained for *At* of *X cortezi* from the Rio Axtla population and *Pu*^{1} of *X. variatus* from Rio Panuco (data not shown).

DISCUSSION

In the Rio Jamapa platyfish X*mrk*, a new growth factor receptor gene of the multigene family of receptor tyrosine kinases has been detected due to its involvement in melanoma formation. The gene was found to be present in two forms: (1) as proto-oncogene, which obviously serves a physiological, so far unknown function, and (2) as an activated oncogene instrumental in induction of melanoma in hybrid fish. In this study sequences hybridizing to the platyfish X*mrk* probe under conditions of high stringency, which exclude cross-hybridization to other closely related members of the gene family, were found in all species of the genus investigated. This indicates that

TABLE 4

Presence of oncogenic X*mrk* fragments in other *Xiphophorus* species

			n
	Tu-Fl^{1}	X*mrk Bam*HI 10.5	
X. xiphidium	+	+	4
	−	−	12
xiph/hellF_1	+	+	9
	−	−	7
xiph/hell/hellBC_1	+	+	28
	−	−	27
	Tu-Li	X*mrk Hin*dIII 12.0	
X. variatus	+	+	7
	−	−	6
var/hellBC_n	+	+	24
	−	−	20
	Tu-Sc	X*mrk Hin*dIII 6.5	
X. cortezi	+	+	8
	−	−	5
			157

the X*mrk* proto-oncogene is present in all *Xiphophorus* fish, further strengthening its supposed physiological function, and that these genes in the different species are homologous. In all platyfish from other populations as well as in fish from other *Xiphophorus* species which contain, as in the Rio Jamapa platyfish, M-pattern loci that predispose to melanoma formation in hybrids additional X*mrk* hybridizing sequences were found. Fish without such M-pattern loci contain only the proto-oncogenic INV-X*mrk* locus. It is therefore suggestive that these additional X*mrk* copies also represent sequences which, as in the Rio Jamapa platyfish, are activated oncogenes that induce melanoma formation in the hybrid genome. They are in the different populations and species considered to be homologous at least by sequence. Further information will be obtained from cloning of these genes and from studies on their expression. The apparent inactivity of such potentially oncogenic X*mrk* copies in the purebred feral fish poses the question of whether a similar mechanism of tumor suppression as in the Rio Jamapa platyfish acts in the other populations and species.

The simultaneous presence of X*mrk* as proto-oncogene and as activated oncogene in the genome of some fish is intriguing with respect to the generation of this situation. The linkage analysis described showed that both forms of X*mrk* reside on the sex-chromosome within quite some distance. As there are no data in *Xiphophorus* relating to genetic versus molecular distance, it is impossible to determine at present how far both copies are molecularly. Both copies are identical with respect to exon/intron arrangement and intron sizes, therefore ruling out the possibility that one copy is a functional processed pseudogene (ADAM, MÄUELER and SCHARTL 1990). Obviously a gene duplication event once generated a second copy of the X*mrk* proto-oncogene which was translocated

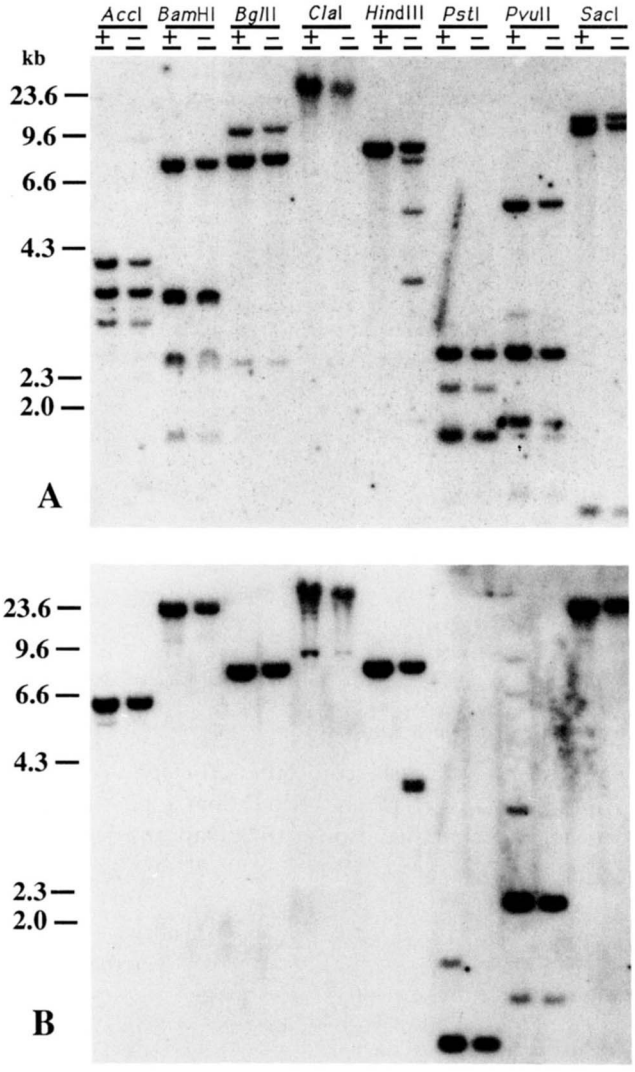

FIGURE 4.—X*mrk* dosage analysis by Southern hybridization of DNA of *X. helleri* (Rio Lancetilla) without (−) or homozygous (+) for Db^2 with (A) the p17-2 X*mrk* probe. For this experiment DNA from ten individuals was pooled and roughly equal amounts were digested with 19 different restriction enzymes. Representative examples are shown. Similar results were obtained for *Xba*I, *Xho*I, *Alu*I, *Ava*I, *Hae*III, *Hin*dII, *Hin*fI, *Msp*I, *Hpa*II, *Rsa*I, *Sau*IIIA and *Taq*I (data not shown). The additional bands in *Hin*dIII-digested DNA of the Db^2-less fish are due to an intrastrain polymorphism (data not shown). (B) For estimation of the DNA amounts loaded, the filter was stripped and rehybridized to the X*src* 724 probe specifically detecting the *Xiphophorus* c-*src* gene, which is a bona fide single copy gene in all vertebrates. The additional bands in *Acc*I-, *Hin*dIII- and *Pst*I-digested DNA of the Db^2-containing fish are due to an intrastrain polymorphism (data not shown).

into the preexisting macromelanophore locus and thereby acquired its oncogenic potential. The preexistence of the macromelanophore locus is supported by the finding that those M-pattern loci which do not give rise to melanoma following hybridization do not contain an additional X*mrk* copy.

Such considerations have several implications for our understanding of the evolution, structure and function of the M-pattern loci. First, at present it is impossible to decide whether the potential oncogenic X*mrk* copies of the different populations and species, which are homologous by sequence, have arisen due to a single gene duplication event early in phylogenesis of the genus, or if such gene duplications have occurred independently several times during speciation. Within different species the simultaneous occurrence of fish with tumorigenic M pattern and with nontumorigenic M pattern in one species as found in *X. variatus* and *X. cortezi* could be explained by such a polyphyletic origin of activated X*mrk* copies in the tumorigenic M-pattern loci. Within one species, *X. maculatus*, the data obtained so far point to a single gene duplication event. It was unequivocally apparent from sequence analyses (ADAM, MÄUELER and SCHARTL 1990) that in the Rio Jamapa platyfish, where different oncogenic X*mrk* copies are present in the *Y* chromosomal "*Striped*" and in the *X* chromosomal "*Spotted-dorsal*" M-pattern loci, a gene duplication of the *Y* chromosomal INV-locus generated the *Y* chromosomal oncogenic "*striped*" X*mrk* copy. The *X* chromosomal "*Spotted-dorsal*" X*mrk* copy is derived not from the *X* chromosomal INV-X*mrk* allele by a second gene duplication, but from the *Y* chromosomal "*Striped*" associated X*mrk* copy, obviously by sex chromosomal crossover. The constancy of sex chromosomal X*mrk* polymorphic fragment sizes (see Table 2) in feral platyfish (6.5 kb for *Y* chromosomal, 5.0 kb for *X* chromosomal loci) points to the assumption that this gene duplication was a single event in the evolutionary history of *X. maculatus*.

Second, the M-pattern loci which give rise to melanomatous enhancement in the hybrid consist of at least two genes, one, the oncogenic (additional) X*mrk* copy and two, of one (or more) genes which determine the specific phenotype of the macromelanophore and the pigmentation pattern. These genes are very intimately linked because so far in more than 500 fish analyzed, no recombination between the oncogenic X*mrk* and the M-pattern locus was detected (SCHARTL 1988; ADAM *et al.* 1988; WITTBRODT *et al.* 1989; this study).

The compartment where the macromelanophores appear and their density are within a given genotype very stable, indicating that the pattern information is also very closely linked to the macromelanophore-determining gene(s). However, in rare instances genetic changes which result in variant patterns have been reported following X-irradiation (ANDERS, ANDERS and KLINKE 1973) or spontaneously (KALLMAN and SCHREIBMAN 1971). Further molecular studies are needed to clarify whether structural genes or regulatory elements of the macromelanophore gene(s) are responsible for the pattern information. This pattern information is obviously independent from the additional, oncogenic X*mrk* copy. Different patterns

are associated with the *X* or the *Y* chromosomal X*mrk* oncogene and one pattern–as shown for "*spotted*" of *X. maculatus*–can be associated with the one or the other.

Last, the complexity of M patterns in the genus *Xiphophorus* and their frequent, although not general, association with the capacity to develop into melanoma of varying degrees of malignancy after hybridization has prompted several authors to postulate a complex genetic system involved in these phenomena (ZANDER 1969; KALLMAN and ATZ 1966). However, the most widely accepted general model of melanoma formation in *Xiphophorus* is very simplistic (for review see ANDERS and ANDERS 1978; ANDERS 1983). Entirely all M-pattern loci of all *Xiphophorus* fish have been formally equated with the melanoma oncogene *Tu* which was thought to determine also the macromelanophore phenotype. Pattern information was postulated to be encoded in a series of closely linked "compartment regulatory genes." Differences in the capacity to develop melanoma in hybrids were explained by the melanoma suppressor locus being located on other chromosomes, which are eliminated after backcrossing, or by the melanoma suppressor locus being linked to *Tu*. Nonlinkage would consequently allow spontaneous melanoma formation as in the case of *Tu-Sd* of *X. maculatus* or *Tu-Fl*^{1} of *X. xiphidium* (AHUJA and ANDERS 1977). Linkage of *Tu* and the suppressor and therefore the inability to develop spontaneously melanoma was postulated for the pattern "*Lined*" (*Li*), "*Striped*' (*Sr*) and "*Dabbed*" (*Db*) (AHUJA and ANDERS 1977), ignoring the fact that *Tu-Li* and *Tu-Sr* are strongly enhanced in some hybrids and even generate melanoma under certain conditions (ZANDER 1969, and our unpublished data). The data presented here show that the macromelanophore-determining gene(s) are definitely distinct from the melanoma-inducing X*mrk* gene which is encoded by the *Tu* locus (WITTBRODT *et al.* 1989). They are preexisting. Loci such as *Db*^{2}, *Pu*^{1} and *At*, which have no oncogenic potential, do not contain X*mrk* and may therefore represent the "wild-type pattern" as a phylogenetic old situation while *Sd, Li, Sr, Fl*^{1} and *Sc* contain X*mrk* and are tumorigenic. It has also been argued that the "tumor gene" *Tu* is present in addition to the M-pattern loci-associated copies in a multiplicity of so-called "indispensable" *Tu* copies spread all over the autosomes of *Xiphophorus* (ANDERS 1983). The data presented here show that X*mrk* exists only in two copies, proto-oncogenic and oncogenic, both of which reside on the sex chromosome.

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