A Sex Chromosomal Restriction-Fragment-Length Marker Linked to Melanoma-Determining *Tu* Loci in Xiphophorus

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ABSTRACT

In Xiphophorus, the causative genetic information for melanoma formation has been assigned by classical genetics to chromosomal loci, which are located on the sex chromosomes. In our attempts to molecularly clone these melanoma-determining loci, named *Tu*, we have looked for restriction-fragment-length markers (RFLMs) linked to the *Tu* loci. These RFLMs should be useful in obtaining a physical map of a *Tu* locus, which will aid in the cloning of the corresponding sequences. DNA samples from various Xiphophorus strains and hybrids including those bearing different *Tu* wild-type, deletion and translocation chromosomes, were screened for the presence of random RFLMs using homologous or heterologous sequences as hybridization probes. We find an *Eco*RI restriction fragment which shows limited crosshybridization to the *v-erb B* gene—but not representing the authentic *c-erb B* gene of Xiphophorus—to be polymorphic with respect to different sex chromosomes. Linkage analysis revealed that a 5-kb fragment is linked to the *Tu-Sd* locus on the *X* chromosome, a 7-kb fragment is linked to the *Tu-Sr* locus on the *Y* chromosome, both of *Xiphophorus maculatus*, and that a 12-kb fragment is linked to the *Tu-Li* locus on the *X* chromosome of *Xiphophorus variatus*. Using different chromosomal mutants this RFLM has been mapped to a frequent deletion/translocation breakpoint of the *X* chromosome, less than 0.3 cM apart from the *Tu* locus.

IN Xiphophorus, certain hybrid genotypes develop spontaneous malignant melanoma. Melanoma formation has been attributed by classical genetic findings to the overexpression of a cellular gene, termed *Tu*. In nontumurous fish *Tu* was proposed to be negatively controlled by cellular regulatory genes (more recently termed antioncogenes by some authors) (for review, see ANDERS *et al.* 1984). In a typical crossing experiment a female *Xiphophorus maculatus* (platyfish) containing a specific *Tu* locus and its corresponding regulatory gene, which are both located on different chromosomes, is crossed with a male *Xiphophorus helleri* (swordtail), which is thought not to contain this particular *Tu* locus and its corresponding regulatory gene. Backcrossing of the *Tu*-containing hybrids to *X. helleri* results, in effect, in the progressive replacement of regulatory gene bearing chromosomes originating from the *X. maculatus* by chromosomes of *X. helleri*. This stepwise elimination of regulatory genes allows increased expression of *Tu*, resulting in the development of malignant melanoma in the hybrids.

A lot of the information on the genetic basis for melanoma formation was gained thanks to the fact that in most cases the melanoma-determining locus *Tu*, besides often being linked to a pterinophore locus, is genetically associated with a locus for melanophore pigmentation patterns. These patterns are formed by a type of very large pigment cells resembling those cells which also were found in the melanoma of the tumorous hybrids. This pigment cell type has been referred to as macromelanophore (GORDON 1958) or more recently "transformed" (tr)-melanophore (ANDERS *et al.* 1979). Various tr-melanophore patterns, differing with respect to the region of the body surface where the pigment cells are located, have been found in the wild-type populations of Xiphophorus. The different patterns are determined by dominant acting genetic loci, the majority of which are located on the sex-determining chromosomes (see KALLMAN 1975). All of them harbor the melanoma-determining *Tu* locus (ANDERS *et al.* 1984).

The molecular nature of the color genes, the oncogene *Tu* and its corresponding regulatory genes is unknown. To obtain information on the molecular genetics of melanoma formation, cloning of the melanoma-determining loci is required. So far, any approach in the Xiphophorus system to analyze the molecular genetics of the regulation of tumor expression failed due to the lack of molecular markers for the loci involved in oncogenesis. Our approach is based on (1) the availability of several mutants in Xiphophorus affecting the process of tumor formation as well as the wild-type pigmentation patterns; (2) a DNA transfection bioassay for *Tu* (for details on the assay, see VIELKIND *et al.* 1982); and (3) subtraction-cloning strategies utilizing *Tu*-wild-type

and deletion genotypes for enrichment of *Tu*-containing DNA. The precondition for cloning of *Tu* is a physical characterization of the *Tu* locus and the different mutant loci. Recently, for the human and the mouse genome, randomly selected DNA fragments that detect restriction-fragment-length polymorphisms (RFLPs) for distinct sections of chromosomes have established the exact location of several genes, which—like the Xiphophorus *Tu* gene—so far had been detected only by their phenotype. These RFLPs have made it possible to construct physical maps of the loci in question (ORKIN 1986; CAVANEE 1986). This information, together with the use of chromosome specific gene libraries, led to the successful cloning of such genes, *e.g.*, the gene for retinoblastoma (FRIEND *et al.* 1986), Duchenne muscular dystrophy (MONACO *et al.* 1986) and chronic granulomatous disease (ROYER-POKORA *et al.* 1986). In this paper the first restriction fragment length marker linked to the *Tu* locus is reported and a tentative genetic map of the *Tu*-containing section of the *X* chromosome of *X. maculatus* is presented.

MATERIALS AND METHODS

Fish: All strains derived from natural populations of Xiphophorus that were employed in this study have been maintained as closed stocks and have been randomly inbred for at least 30 generations. Interspecific F_1 hybrids were produced by artificial insemination. Introgressive hybridization was used for the incorporation of a particular chromosome into the genetic background of another species. Backcross hybrids, which have been backcrossed for more than 10 generations to the same parental strain (*hell*, see below), are referred to as BC_n. The mutants used have either arisen spontaneously or were induced by X-irradiation (ANDERS, ANDERS and KLINKE 1973). Founder fish were kindly provided by A. and F. ANDERS (Giessen).

Fish from the following natural populations were used:

1. *X. helleri* from Rio Lancetilla, Belize (*hell*). In all populations of this species, no sex chromosomes have been found—sex is determined polygenically, with male and female factors apparently scattered throughout the chromosomes (KOSSWIG 1939; DZWILLO and ZANDER 1967). However, an autosome pair is homologous to the sex chromosomes of other *Xiphophorus* species, permitting normal meiosis and fertile hybrids in interspecific crosses (KOSSWIG 1939). A strain was used which does not exhibit any pterinophore or tr-melanophore pattern.

2. *Xiphophorus variatus* from Rio Panuco, Mexico (*var* RP). These fish show male heterogamety. The *X* chromosome carries the pterinophore locus *Ye* (yellow) and the tr-melanophore locus *Li* (lineatus); the *Y* chromosome carries the pterinophore locus *Or* (orange) and the tr-melanophore locus *Pu* (punctatus).

3. *X. maculatus* from Rio Jamapa, Mexico (*mac* RJ). This stock demonstrates male heterogamety. The *X* chromosome carries the pterinophore locus *Dr* (dorsal red) and the tr-melanophore locus *Sd* (spotted dorsal); the *Y* chromosome carries the pterinophore locus *Ar* (anal red) and the tr-melanophore locus *Sr* (striped).

4. *X. maculatus* from Rio Usumacinta, Mexico (*mac* RU). In the natural population of *mac* RU, in addition to the *X* chromosome a second female determining sex chromosome, designated *W*, has been found. Due to its strong, dominant female factor, heterogametic females (*WY*) and homogametic males (*YY*) can be obtained. Such a strain was used with both sex chromosomes being devoid of phenotypically expressed colour genes.

Until more precise information is available, the tr-melanophore pattern loci will be formally equated with the melanoma determining loci (for evidence see ANDERS *et al.* 1984) and will be referred to as *Tu-Sd*, *Tu-Li*, etc. The sex chromosomes will be referred to, based upon their wild-type origin, as $X^{Dr\ Tu\text{-}Sd}_{mac\ RJ}$, etc., autosomes as A_{hell}, etc.

The following mutant sex chromosomes were used in this study:

$X^{Dr}_{mac\ RJ}$: Fish carrying this chromosome do not express the Tu-Sd phenotype, and cytogenetic evidence for a terminal deletion of that locus from the *X* chromosome has been presented (AHUJA, LEPPER and ANDERS 1979).

$X^{Dr\ Ar\ Tu\text{-}Sr}_{mac\ RJ}$: This chromosome is described as an *X*/*Y* translocation of *mac* RJ (AHUJA, LEPPER and ANDERS 1979).

$X^{Dr\ Tu\text{-}Li}_{mac\ RJ}$: This chromosome is reported to have occurred as the result of a cross-over between the $X^{Dr\ Tu\text{-}Sd}_{mac\ RJ}$ and the $X^{Ye\ Tu\text{-}Li}_{var\ RP}$ chromosomes (ANDERS, ANDERS and KLINKE 1973) in a *mac* RJ/*var* RP hybrid female.

$X^{Dr\ Ar}_{mac\ RJ}$: This chromosome has been recently obtained following a deletion of the *Tu-Sr* locus from the $X^{Dr\ Ar\ Tu\text{-}Sr}_{mac\ RJ}$ chromosome (A. ANDERS, personal communication).

$A^{Tu\text{-}Sd}_{hell}$: Fish carrying this chromosome express the Tu-Sd phenotype but not the Dr phenotype. This chromosome arose spontaneously in the hybrid offspring of a *mac* RJ/*hell* F_1 hybrid; it is described as a translocation of the *Tu-Sd* locus from the $X^{Dr\ Tu\text{-}Sd}_{mac\ RJ}$ wild-type chromosome to an autosome of *hell* (ANDERS, ANDERS and KLINKE 1973).

DNA probes: All probes were separated from vector sequences after appropriate restriction enzyme digestion by low melting point agarose gel electrophoresis and further purification through NACS columns (BRL, Eggenstein, FRG). The following fragments were used: (1) 600 bp *Bam*HI fragment D of pAE II (VENNSTRÖM *et al.* 1980) representing the central part of the cytoplasmic domain of the v-*erb* B gene of avian erythroblastosis virus; (2) 5800-bp and 5100-bp *Eco*RI fragments from clone λ6.1 which was isolated from a genomic library of *X. maculatus* due to cross-hybridization to the v-*erb* B probe. This fragment represents a gene showing limited similarity to the kinase domain of the c-*erb* B gene of vertebrates but it is different from the Xiphophorus *erb* B gene (our unpublished data); (3) 4500 bp *Eco*RI fragment from clone λ44, isolated as above. This fragment contains sequences from the kinase domain of the Xiphophorus *erb* B gene (our unpublished data).

RFL analyses: High molecular weight DNA (>70 kb) from pooled brain, liver, kidney, gills and gonad of single fish was prepared according to the method of BLIN and STAFFORD (1976). Aliquots (10 μg) of the appropriate DNA were digested to completion with restriction enzymes, run on 0.8% agarose gels, and transferred to a nylon hybridization membrane (Gene Screen plus; NEN, Dreieich, FRG) by the alkaline transfer procedure (REED and MANN 1985). The filters were hybridized with approximately $5–10 \times 10^6$ cpm of ^{32}P-nick-translated probe (specific activity usually $3–6 \times 10^8$ cpm/μg DNA). The hybridization was performed at 42° in a buffer containing 5 × standard saline citrate (SSC) and 40% formamide in the case of nonhomologous probes and 50% formamide in the case of a homologous probe. Subsequent washings were performed

TABLE 1

***RFLM-1 Eco*RI restriction-fragment length in parental and hybrid genotypes carrying different sex chromosomes**

Genotype	*n*	Sex chromosomes							*RFLM-1* *Eco*RI band (kb)		
		None	$X_{mac\ RJ}$	$Y_{mac\ RJ}$	$X_{var\ RP}$	$Y_{var\ RP}$	$W_{mac\ RU}$	$Y_{mac\ RU}$	5.0	7.0	12
hell	51	×							−	−	−
mac RU	31							×	−	−	−
mac RU	1						×	×	−	−	−
mac RJ	19		×	×					+	+	−
mac RJ	12		×						+	−	−
var RP	4				×				−	−	+
var RP	3				×	×			−	−	+
mac RJ/ *hell*, F_1	1		×						+	−	−
mac RJ/ *hell*, F_1	3			×					−	+	−
mac RJ/ *hell*, BC_3	5		×						+	−	−
mac RJ/ *hell*, BC_4	19		×						+	−	−
mac RJ/ *hell*, BC_5	3		×						+	−	−
mac RJ/ *hell*, BC_n	1			×					−	+	−
var RP/ *hell*, BC_n	4				×				−	−	+
Σ	157										

n = number of animals tested.

in 1 × SSC at 60° in the case of nonhomologous probes and in 0.1 × SSC at 68° in the case of homologous probes.

RESULTS

Polymorphism of restriction-fragment lengths in different species and populations of Xiphophorus: Using a series of different 6-base and 4-base cutting restriction enzymes and different homologous or heterologous sequences as hybridization probes (mostly oncogenes) in Southern blotting experiments, in general a considerable polymorphism of the hybridizing restriction fragments was detected between the different species, populations, and the hybrid genotypes of Xiphophorus. Only the *Eco*RI restriction pattern obtained with the viral *erb* B probe proved to be useful for further analysis. Several bands (21, 15, 7.5, 5.8 and 2.1 kb) were invariant in all the species, populations and their hybrids investigated so far. A 4.4-kb and a 3.5-kb fragment was found to be highly polymorphic within the different populations. An additional fragment showed interstrain polymorphism depending upon the presence of the different sex chromosomes. Fish carrying the wild-type $X^{Dr\ Tu\text{-}Sd}_{mac\ RJ}$ chromosome exhibit a 5-kb band, fish with a wild-type $Y^{Ar\ Tu\text{-}Sr}_{mac\ RJ}$ chromosome a 7-kb band, while fish carrying the wild-type $X^{Ye\ Tu\text{-}Li}_{var\ RP}$ chromosome have a 12-kb band (Figure 1). *hell* (apparently sex chromosome free) and *mac* RU (carrying the $W_{mac\ RU}$ and the $Y_{mac\ RU}$, both without a phenotypically recognizable *Tu* locus did not exhibit any of these additional bands (Table 1, Figure 1a). Analysis of different fish being homogametic or heterogametic (*XX*, *XY*, *YY*, *WY*) exhibited the expected homozygosity or heterozygosity of that fragment (Table 1,

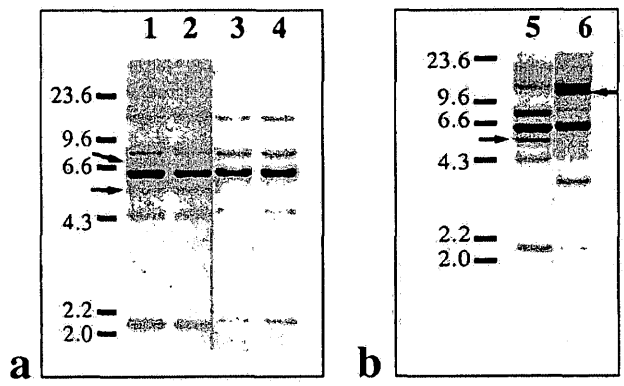

FIGURE 1.—*Eco*RI digest of DNA from fish carrying different wild-type or mutant sex chromosomes, hybridized to the *v-erb* B probe. (a) Lane 1: *mac* RJ, male, carrying one $X^{Dr\ Tu\text{-}Sd}_{mac\ RJ}$ and one $Y^{Ar\ Tu\text{-}Sr}_{mac\ RJ}$ chromosome. Lane 2: *mac* RJ, female homozygous for the $X^{Dr\ Tu\text{-}Sd}_{mac\ RJ}$ chromosome. Lane 3: *mac* RU, male, homozygons for the $Y_{mac\ RU}$ chromosome. Lane 4: *mac* RU, female, carrying one $Y_{mac\ RU}$ and one $W_{mac\ RU}$ chromosome. Arrows: sex chromosome-specific 7-kb (present in lane 1, absent in lane 2) and 5-kb fragments. Note lack of such bands in lane 3 and 4. (b) Lane 5: fish carrying the $W_{mac\ RU}$ and the $X^{Dr\ Tu\text{-}Li}_{mac\ RJ}$ translocation chromosome, exhibiting the $X_{mac\ RJ}$ specific 5-kb fragment (arrow). Lane 6: *var* RP, female, homozygous for the $X^{Ye\ Tu\text{-}Li}_{var\ RP}$ chromosome exhibiting the $X_{var\ RP}$-specific 12-kb fragment (arrow).

TABLE 2

***RFLM-1 Eco*RI restriction-fragment length in chromosome mutants**

Genotype	*n*	Sex chromosome	*RFLM-1* *Eco*RI band (kb)
mac RJ	25	$X^{Dr\ Tu\text{-}Li}_{mac\ RJ}$	5.0
mac RJ	4	$X^{Dr}_{mac\ RJ}$	5.0
mac RJ	4	$X^{Dr}_{mac\ RJ}$ $Y^{Ar\ Tu\text{-}Sr}_{mac\ RJ}$	5.0 7.0
mac RJ	8	$X^{Dr}_{mac\ RJ}$ $X^{Dr\ Ar\ Tu\text{-}Sr}_{mac\ RJ}$	5.0 7.0
mac RJ/ *hell*, BC_3	4	$X^{Dr}_{mac\ RJ}$	5.0
mac RJ/ *hell*, BC_4	4	$X^{Dr}_{mac\ RJ}$	5.0
mac RJ/ *hell*, BC_n	15	$A^{Tu\text{-}Sd}_{hell}$	5.0
mac RJ/ *hell*, BC_n	1	$X^{Dr\ Ar}_{mac\ RJ}$	—
	Σ 65		

n = number of animals tested.

Figure 1a). As the presence of this restriction fragment is diagnostic for the presence of the *X* and *Y* chromosomes of *mac* RJ and the *X* chromosome of *var* RP, it is concluded that it represents a genomic locus located on these chromosomes. Due to the fragment length it is a marker for each of the three sex chromosomes, and the locus was therefore designated *RFLM-1*.

In *Hin*dIII digests, a corresponding RFLM was also detected. However, due to the high molecular weight of the corresponding fragments, it was not so readily accessible by conventional gel electrophoresis as the *Eco*RI pattern.

Linkage analysis of *RFLM-1* and *Tu* loci: In order to allow a detailed analysis of the linkage of *RFLM-1* to the *Tu* loci on the different wild-type and mutant chromosomes from *mac* and *var*, all these chromosomes were introduced by introgressive crossing (at least seven backcross generations) into the genetic background of *hell*, which does not exhibit *RFLM-1*. An analysis of parental *mac* RJ and *hell*, their F_1 hybrids, and of backcross generation animals revealed the expected co-segregation of the 5-kb band and the $X^{Dr\ Tu\text{-}Sd}_{mac\ RJ}$ chromosome (Table 1). Backcross hybrids carrying either the wild-type $X^{Dr\ Tu\text{-}Sd}_{mac\ RJ}$ chromosome, the corresponding *Tu-Sd* deletion chromosome ($X^{Dr}_{mac\ RJ}$) or the *Tu-Sd* translocation chromosome ($A^{Tu\text{-}Sd}_{hell}$) exhibited the $X_{mac\ RJ}$ specific 5-kb band of *RFLM-1* (Table 2, Figure 2). This indicates that *RFLM-1* should be located in a region of the $X^{Dr\ Tu\text{-}Sd}_{mac\ RJ}$ chromosome which was not lost in the deletion of *Tu-Sd*, but was part of the fragment which was translocated with *Tu-Sd* onto a A_{hell} chromosome. Fish carrying the $X^{Dr\ Tu\text{-}Li}_{mac\ RJ}$ recombinant chromosome exhibited only the $X_{mac\ RJ}$ specific 5-kb band, indicating that in this case the *Tu-Li* containing part of the $X_{var\ RP}$ chromosome distal from *RFLM-1* was translocated to the $X_{mac\ RJ}$ chromosome (Table 2,

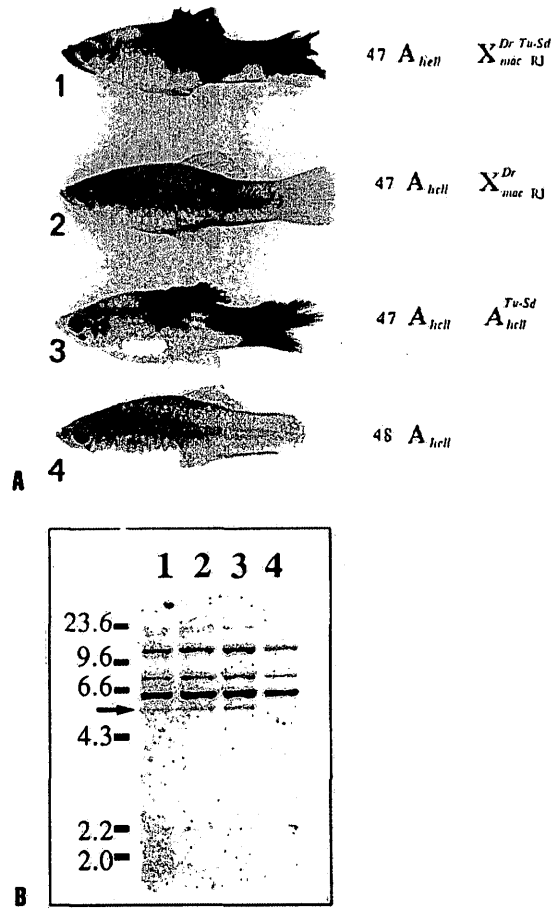

FIGURE 2.—Linkage analysis of *RFLM-1* and *Tu* loci. (A) Genotypes used for the RFLM-analysis shown in (B). (1) Backcross hybrid carrying the wild-type $X^{Dr\ Tu\text{-}Sd}_{mac\ RJ}$ chromosome, (2) backcross hybrid carrying the *Tu-Sd* deletion $X^{Dr}_{mac\ RJ}$ chromosome, (3) backcross hybrid carrying the translocation $A^{Tu\text{-}Sd}_{hell}$ chromosome, (4) *hell*, representing the common genetic background of genotypes 1–3. Fish of *hell* were used as the recurrent parent in the backcrossings. On the right the chromosome complement of the fish is indicated. (B) *Eco*RI digest of DNA from fish as shown in (a), hybridized to the v-*erb* B probe. Note presence of the 5-kb fragment in genotypes carrying the $X_{mac\ RJ}$ chromosome (1) or parts of it (2, 3) which is not detected in the sample from the fish representing the common genetic background (4).

Figure 1b). Fish carrying the $X^{Dr\ Ar\ Tu\text{-}Sr}_{mac\ RJ}$ chromosome exhibited the 7-kb *RFLM-1* band, indicating that the *X/Y* translocation occurred proximal of the *RFLM-1* and the *Tu-Sr* locus on the $Y_{mac\ RJ}$ chromosome (Table 2). In DNA from a fish with the corresponding deletion chromosome ($X^{Dr\ Ar}_{mac\ RJ}$) the *RFLM-1* band was not detected, indicating that this sequence was part of the deleted fragment.

In order to characterize the *RFLM-1* sequence, *Eco*RI digested DNA of different genotypes was hybridized to a probe derived from the Xiphophorus *erb* B gene (*Xerb* B). Under conditions of high stringencies only the invariant 5.8-kb band was seen in all samples irrespective of the presence or absence of the *mac* RJ *X* or *Y* chromosome. In *Hin*dIII digests

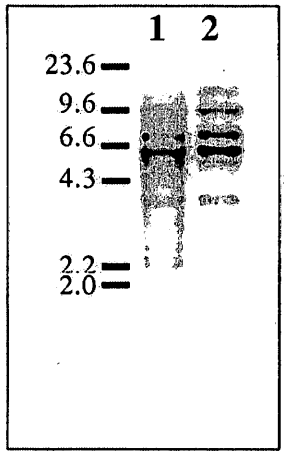

FIGURE 3.—*Eco*RI digest of DNA from progressively growing malignant melanoma (lane 1) (from fish as shown in Figure 2A, fish 1) and of DNA from the liver (lane 2) of the same fish was hybridized to the v-*erb* B probe. Equal amounts of DNA were loaded on each lane of the gel. Note uniformity in intensity and fragment length of the banding pattern.

the *Xerb* B probe detects a fragment which is polymorphic between *mac* RJ (1.7 kb) and *hell* (1.2 kb). Backcross hybrids carrying the $X_{mac\ RJ}$-chromosome with the genetic background of *hell* exhibit only the *hell*-specific 1.2-kb band indicating that the *Xerb* B gene most obviously is not located on the $X_{mac\ RJ}$-chromosome and that this gene is different from *RFLM-1*. Two other probes derived from a gene which also shows cross-hybridization to the viral *erb* B-probe but is different from the *Xerb* B gene, hybridized to a 5.8-kb *Eco*RI fragment or to a 15-kb fragment the presence of which did not correlate with the presence or absence of the $X_{mac\ RJ}$ chromosome. This indicates that the *RFLM-1* sequence represents a different gene which is a member of a multigene family of *erb* B-related genes of growth factor receptors in Xiphophorus.

To investigate whether rearrangement or amplification of *erb* B related sequences occurs during tumor formation, equal amounts of DNAs from progressively growing malignant melanotic melanoma and from nontumorous organs of the same fish (*mac* RJ/*hell* BC_5 carrying the $X_{mac\ RJ}^{Dr\ Tu\text{-}Sd}$ chromosome) were hybridized to the v-*erb* B probe. In all cases the restriction-fragment lengths and the intensity of the hybridizing bands did not differ significantly in the tumor DNA and in the DNA from the nontumorous organs (Figure 3).

DISCUSSION

In this paper, experimental evidence is presented that a v-*erb* B related sequence of Xiphophorus, designated *RFLM-1*, is linked to several melanoma inducing loci on sex chromosomes. This linkage was established through analysis of a total of 221 fish

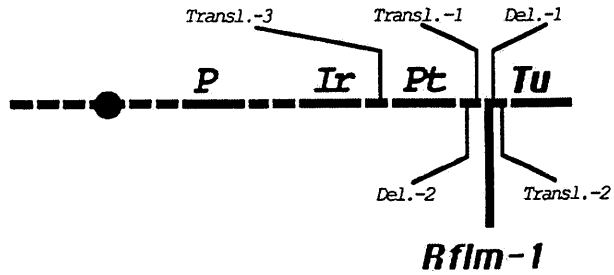

FIGURE 4.—Preliminary schematic map of a *Tu* locus containing sex-chromosome. *P*: locus for onset of sexual maturation, *Ir*: locus for eye coloration, *Pt*: locus for pterinophore pattern (*e.g.*, *Dr*), *Tu*: *Tu* locus (*e.g.*, *Tu-Sd*), Del.-1: chromosomal breakpoint of the *Tu-Sd* deletion, Del.-2: chromosomal breakpoint of the *Tu-Sr* deletion, Transl.-1: chromosomal breakpoint of the *Tu-Sd* translocation, Transl.-2: chromosomal breakpoint of the *Tu-Li* translocation, Transl.-3: chromosomal breakpoint of the *Tu-Sr* translocation.

which were tested for the presence or absence of *RFLM-1* and a phenotypically expressed *Tu* locus located on three different sex chromosomes of *X. maculatus* and *X. variatus*. All the hybrids tested showed the expected co-segregation of *RFLM-1* and the *Tu* locus in question, indicating that the *RFLM-1* sequence might be closely linked to *Tu*. Analyses of several mutants have further placed the *RFLM-1* sequence distal from the sex chromosomal pterinophore locus (*Pt*) and proximal to the *Tu* locus. This suggests a linear arrangement of *Pt-RFLM-1-Tu*. Interestingly, this places *RFLM-1* into a region which has been described as a frequent chromosomal breakpoint for several deletions, translocations and *X/Y* crossovers (ANDERS, ANDERS and KLINKE 1973; KALLMAN 1975; ANDERS *et al.* 1984). In combining the data from crossing analyses with those on the molecular marker described in this paper, a tentative map of the sex chromosomal region harboring *Tu* can be established (Figure 4). This map will be improved by the detection of further RFLMs for that region. Further segregation analysis—so far 63 hybrids were tested, all showing the expected cosegregation of *Tu* and *RFLM-1*—may lead to the detection of recombinants and to establishment of recombination frequencies between *Tu* and *RFLM-1*. In our crossings the observed recombination frequency between *Tu* and *Pt* is less than 0.3%, suggesting the *RFLM-1* and *Tu* are less apart than approximately 0.3 cM on the chromosome.

RFLM-1 seems to be present on some sex chromosomes of different species of Xiphophorus, while other sex chromosomes do not exhibit this molecular marker. The failure to detect *RFLM-1* sequences in *X. helleri* and in fish carrying the *Y* and *W* chromosomes of *X. maculatus* from Rio Usumacinta, could possibly be explained if the corresponding band in these samples is hidden by a comigrating nonpolymorphic fragment, or if the polymorphism is repre-

sented in the low resolution, high molecular weight fragments. From analyses using restriction enzymes other than *Eco*RI, no indication for either of these explanations can be drawn. Final clarification of this issue will have to await the availability of the cloned *RFLM-1* sequences from Xiphophorus as a homologous hybridization probe. If the *RFLM-1* sequences are indeed chromosome-specific, this would raise some interesting implications regarding the evolution of sex determining chromosomes in this genus. It has been reasoned that in Xiphophorus the different sex chromosomes are still in a relatively early stage of evolution and that they do not differ markedly from each other (MITTWOCH 1974). Evidence supporting this comes from the fertility of X-irradiation induced sex-inverted *XY* females and of normal *YY* males (ANDERS *et al.* 1970; see also KALLMAN 1984) and from the similar linear arrangement of phenotypically recognizable loci on different sex chromosomes of the whole genus (ANDERS, ANDERS and KLINKE 1973; KALLMAN 1984). The presence of *RFLM-1* on *X*—as well as on *Y*—chromosomes is in good agreement with these data. It has been hypothesized (KALLMAN 1984) that the occurrence of natural female heterogamety in some populations of *X. maculatus*, due to the presence of a strong female determining chromosome leading to *WY* females and *YY* males, has evolved by simple gene mutation or chromosome rearrangement from an *XY* sex determination mechanism with the ancestral *X* chromosome becoming the *W* chromosome. The lack of *RFLM-1* sequences in the genome of *WY* females and *YY* males of *mac* RU would suggest that sex chromosomes in this population have evolved from an independent line as compared to the *X* and *Y* chromosomes of *mac* RJ carrying the *RFLM-1*. This would also justify regarding the male determining chromosome in these fish as independent from the *Y* chromosome. This chromosome should therefore be referred to as *Z*, as already suggested by several investigators.

For further analyses it will be of importance to find out if the RFLM described here is a real RFLP marker because utilization of cloned RFLP sequences in Southern blot hybridizations of pulse field gel electrophoresis-separated Xiphophorus DNA should enable us to clone the sequences which represent the sex chromosomal *Tu* locus and its flanking regions. The concept of RFLPs requires that the *X*-specific 5-kb band and the *Y*-specific 7-kb band mark codominant alleles at one locus. As the closely linked *Tu* and *Pt* loci on the sex chromosomes are allelic (ANDERS, ANDERS and KLINKE 1973; KALLMAN, 1984), it seems reasonable to assume this also for *RFLM-1*. The question if *RFLM-1* is a single copy sequence will only be answered by molecular cloning. At least, it was shown to be different from the actual c-*erb* B gene of Xiphophorus (*Xerb* B). What the relation of *RFLM-1* to the other weaker hybridizing bands might be is unclear at the moment. In the case of *src*-related sequences in Xiphophorus using a viral *src*-probe on Southern blots, also a number of weakly and strongly hybridizing bands is discovered. Cloning experiments have revealed that all of these represent real single copy genes of a multi-gene family (SCHARTL *et al.* 1988). Thus, it seems reasonable to assume that also the *RFLM-1* sequence represents a single copy gene which is a member of a family of *erb* B related genes that code for growth factor receptors. Interestingly, it was shown recently that a gene which cross-hybridizes to the v-*erb* B probe but is different from the X-*erb* B gene shows a very high expression in melanoma cells of Xiphophorus (MÄUELER, RAULF and SCHARTL 1988). At present we are not able to decide if this gene is represented by the *RFLM-1* sequence and if *RFLM-1* might be not only a molecular marker for the *Tu* locus but is even part of it.

It has been shown that the c-*src* gene of Xiphophorus shows a tumor-specific expression in the melanoma cells (SCHARTL *et al.* 1982, 1985, 1988; MÄUELER, RAULF and SCHARTL 1988). The X-*src* gene, however, is not located on the *Tu*-carrying sex chromosomes (SCHARTL *et al.* 1988). This tempts one to speculate that deregulation of the *Tu* locus also leads to enhanced expression of several other proto-oncogenes not structurally related to *Tu*. As the crossing and mutant analyses in the past have clearly revealed that *Tu* is the primary genetic factor responsible for melanoma formation, activation of those proto-oncogenes might then be a further step in the multistep process of tumor formation. This would be in good agreement with several findings using in vitro systems that more than one proto-oncogene has to be activated in order to induce the full neoplastic phenotype (for review see LAND, PARADA and WEINBERG 1983; KLEIN and KLEIN 1985; VERMA 1986). In this context it is important to realize that in the Xiphophorus-melanoma system, due to a strict hereditary etiology of the tumor, no structural alteration of the proto-oncogenes including the *Tu* locus are involved in the activation of the proto-oncogenes. Solely the impairment of negative-acting regulatory genes (termed antioncogenes by some authors) is the causative event leading to neoplastic transformation. A similar situation has been found for human retinoblastoma (KNUDSON 1985; FRIEND *et al.* 1986). It is presently unknown how many human tumors which were not found to contain any detectable alteration of a known proto-oncogene arose due to an etiology like retinoblastoma. For those cases the Xiphophorus-melanoma system offers a model system for the analysis of oncogene regulation and deregulation and of possible interactions between different oncogenes.

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