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Analysis of an Esterase Linked to a Locus Involved in the Regulation of the Melanoma Oncogene and Isolation of Polymorphic Marker Sequences in *Xiphophorus*

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Melanoma formation in Xiphophorus *hybrids is mediated by a growth factor receptor tyrosine kinase oncogene encoded by the* Tu *locus. In the wild-type parental fish no tumors occur due to the activity of a locus that regulates the activity of the melanoma oncogene. Molecular identification of this regulatory locus* (R) *requires a precise physical map of the chromosomal region. Therefore we studied esterase isozymes in* Xiphophorus, *two of which have been previously reported to be linked to locus* R. *We confirm that* ES1 *is a distant marker for* R *(approx. 30cM), and contrary to earlier studies, we show that this isozyme is present in all species of the genus and at similar activity levels in all organs tested.* ES4, *which has also been reported to be linked to* R, *was found to be a misclassification of liver* ES1. *In an attempt to identify markers that bridge the large distance between* ES1 *and* R, *we have generated DNA probes which are highly polymorphic. They will be useful in finding landmarks on a physical map of the* R*-containing chromosomal region.*

KEY WORDS: *Xiphophorus;* melanoma; oncogene regulation; esterase; molecular marker sequences.

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INTRODUCTION

Melanoma formation in *Xiphophorus* provides a unique system to study the relevance of genetic factors in tumorigenesis and offers the possibility to elucidate the molecular mechanism underlying melanoma induction as well as tumor suppression.

In *Xiphophorus,* pigmentation is composed of two melanophoric types: micromelanophores, responsible mainly for the uniform gray body coloration, and macromelanophores, which form characteristic spot patterns (Gordon, 1927). Several species of *Xiphophorus* are polymorphic for such macromelanophoric patterns. In hybrid crosses, e.g., spotted *X. maculatus* with unspotted *X. helleri,* the F_1 offspring show enhanced macromelanophore pigmentation diagnosed as benign melanoma. Further crossing of the F_1 hybrids with *X. helleri* leads to backcross segregants that develop malignant melanoma due to an uncontrolled proliferation of cells of the macromelanophore lineage (Gordon, 1927; Häussler, 1928; Kosswig, 1927). The phenomenon was explained by an "abnormal interaction" in the hybrid genome of a single sex-linked genetic locus of *X. maculatus* (Gordon, 1958; Atz, 1962; Kosswig, 1965; Anders, 1967; Zander, 1969), the macromelanophore locus. That locus was later designated *Tu* (Anders and Anders, 1978). The "abnormal interaction" was further defined as the presence of intensifying genes and/or the absence of repressive genes in the hybrid genome which act specifically on the macromelanophore locus ["modifier genes" (Gordon, 1958; Atz, 1962; Kosswig, 1965; Zander, 1969; Kallman, 1970)]. The current model proposes that *Tu* is normally under the control of one major autosomal repressor gene [regulating gene, *R* (Ahuja and Anders, 1976)] acting as a tumor suppressor. In the unspotted *X. helleri,* this suppressor gene is thought to be absent. Thus, the *Tu* locus could become activated in hybrid fish in which the suppressor gene has been diluted out by one or more backcrosses with *X. helleri.* Reintroduction of *R* by repeated backcrossing of those fish to *X. maculatus* leads again to the benign phenotype of nonproliferative macromelanophore spots, indicating that the *Tu* locus remains structurally unchanged during the process of hybridization-conditioned oncogenic activation (Anders and Anders, 1978).

Recently, the melanoma-inducing gene from the *Tu* locus of *X. maculatus* was cloned. It encodes a novel receptor tyrosine kinase which is closely related to the EGF receptor (Wittbrodt *et al.,* 1989). This gene, designated *Xmrk,* is present on the sex chromosome of all *Xiphophorus* fish as a protooncogene. Only those fish that carry a macromelanophore locus, which gives rise to melanoma following the appropriate crossings, have an additional oncogenic copy of *Xmrk* closely associated with this macromelanophore locus (Schartl, 1990). The oncogenic version of *Xmrk* is under

different transcriptional control and, so far, was found to be active only in melanoma of the hybrids lacking the *R* locus (Adam *et al.,* 1991).

Of major importance for our understanding as to how suppression of melanoma formation is mediated in the purebred parental fish and how *Xmrk* is activated in the hybrids will be the identification and molecular characterization of those genes encoded by the *R* locus. So far, no gene product derived from *R* is known, therefore precluding cloning of the corresponding genes by conventional recombinant DNA technology. An alternative strategy will be to employ "reverse genetics" (Orkin, 1987), including the following steps: (1) determination of the chromosomal localization of the *R* locus, (2) identification of molecular marker sequences closely linked to *R,* (3) cloning of the *R*-containing region by chromosome walking or jumping, (4) identification of the candidate gene(s), and (5) functional analysis of the melanoma-suppressing capacity of such a gene(s).

Most important for the realization of this approach was the initial observation by Siciliano *et al.* (1976) that a distinct esterase isozyme locus (*ES-1*) cosegregates with a locus that determines the benign phenotype of melanoma in backcross hybrids. This was later confirmed by Ahuja *et al.* (1980), leading to the definition of the modifying locus as *R* [synonymous to *Diff* (Vielkind 1976; Ahuja *et al.,* 1980); *MelSev* (Morizot and Siciliano, 1983a)]. A multipoint linkage group was established with the following order: *ES1—ES4—MDH2—R* and designated linkage group V of *Xiphophorus* (Morizot and Siciliano, 1983a). Unfortunately, severe discrepancies complicate further analyses. Such discrepancies are (1) recombination estimates ranging from 36% (Morizot and Siciliano, 1983a) to 10% (Ahuja *et al.,* 1980), (2) the absence of *ES1* in *X. helleri* (Ahuja *et al.,* 1977, 1980) versus presence of *ES1* (Siciliano *et al.,* 1976; Morizot and Siciliano, 1983a), and (3) the presence of *ES1* in *X. maculatus* liver (Ahuja *et al.,* 1977, 1980) versus its absence (Leslie and Pontier, 1980), leading to the definition of *ES4* as a new linked marker for *R* (Morizot and Siciliano, 1983a). We therefore undertook a systematic study of esterase isozymes in *Xiphophorus* with respect to their biochemical classification and as genetic markers.

Of further relevance is the identification of molecular probes that detect polymorphic loci in *X. maculatus* and *X. helleri.* Such probes with a high polymorphism information content (PIC) will then be useful for finding marker sequences for *R* and establishing a long-range map of the *R*-containing region.

MATERIALS AND METHODS

Experimental animals used in this study were maintained under standard conditions (Kallman, 1975) in the aquarium of the gene center. Purebred stocks were maintained as closely inbred lines. For geographical origins see

Table II. The taxonomy used follows the recent revision of the Panuco basin species by Rauchenberger *et al.* (1990). A previously undescribed mutant, which occurred spontaneously in our stock of *X. maculatus* (Rio Jamapa), was also included. This mutant carries an X chromosome that contains the *Tu-Sr* locus beside the *Tu-Sd* locus, obviously due to an unequal crossover between the wild-type X chromosome (*Tu-Sd*) and the wild-type Y chromosome (*Tu-Sr*). The mutant chromosome is referred to as $X^{Tu\text{-}SdTu\text{-}Sr}$. F_1 hybrids between *X. maculatus* (Rio Jamapa) and *X. helleri* (Rio Lancetilla, stock without Db^2) were produced by artificial insemination. For segregation analyses, backcross hybrids using *X. helleri* as the recurrent parent were analyzed.

Sample Preparation and Electrophoresis of Esterase Isozymes

Fishes were anesthetized and all samples were prepared for protein extraction immediately at 4°C. Eye, brain, gill, fin, liver, and skeletal muscle were homogenized in 4 parts (w/v) of double-distilled water and centrifuged at 12,000 rpm for 1 hr at 4°C. The resulting supernatant was mixed with 1 part (v/v) 0.1 *M* Tris–HCl (*p*H 8.0), 0.01 *M* β-mercaptoethanol, 99% glycerine, and bromphenol blue and subjected either to 7.5% polyacrylamide gels (Williams and Reisfeld, 1964) or to 13% starch gels.

Polyacrylamide electrophoresis was performed for 15–20 hr at 80 V, 60 mA, and 4°C, using 0.08 *M* diethylbarbituric acid, 0.027 *M* Tris–HCl (*p*H 8.0) as gel and running buffer.

Horizontal starch electrophoresis (Siciliano and Shaw, 1976, modified), using hydrolyzed starch from Sigma (No. S-4501) and 0.5 *M* Tris–HCl (*p*H 8.0), 0.2 *M* EDTA, 0.65 *M* borate buffer (1:10 diluted for the gel, undiluted as running buffer), was carried out for 3 hr at 300 V, 60 mA, and 4°C. Before staining, the starch gel was sliced into three parts with a nylon twine.

Because of the shorter separation time, more convenient handling, and different staining possibilities with one gel, starch gel electrophoresis was preferred. The electrophoretic pattern of esterases was identical in the starch gel and polyacrylamide gel electrophoresis (PAGE) (Fig. 1).

Staining was performed essentially as described previously (Shaw and Prasad, 1970) using α-naphthylpropionate, α-naphthylacetate, or α-naphthylbutyrate as substrate (100 mg in 3 ml of acetone, 100 mg fast blue RR salt). Esterase staining was completed after 30–60 min. For inhibitory studies the gels were incubated at room temperature for 45 min in 100 ml of 0.1 *M* Tris–HCl buffer (*p*H 7.0), containing one of the following inhibitors: 10^{-4} *M* diisopropylfluorophosphate (DFP), 10^{-4} *M* eserine sulfate, or 10^{-3} *M* parachloromercuribenzoic acid (PCMB). For a control, one slice of the starch gel was processed similarly but without inhibitor.

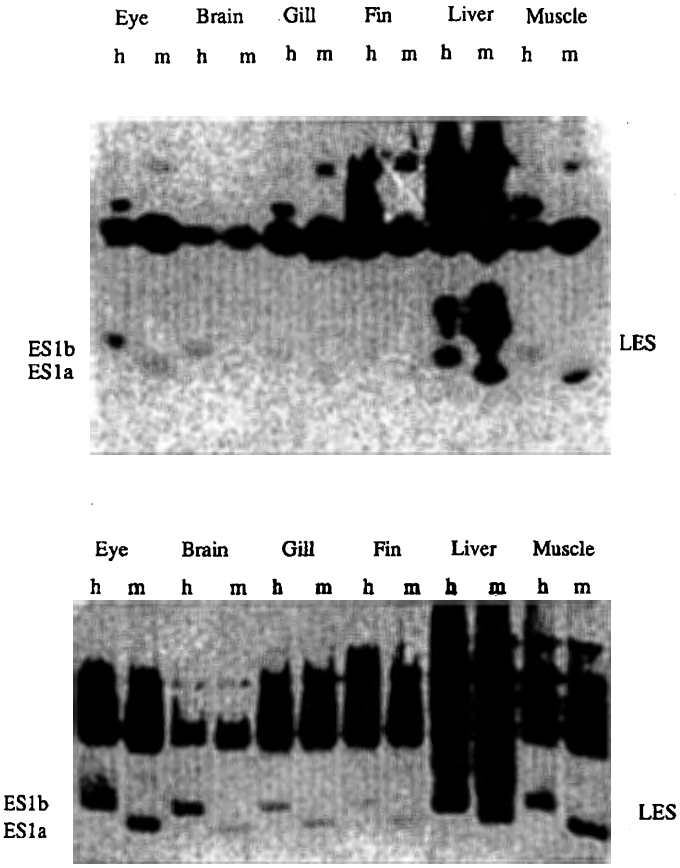

Fig. 1. (a) Separation of esterases from different organs and tissues of *X. helleri* (h) and *X. maculatus* (m) in a 7.5% polyacrylamide gel using α-naphthylpropionate as substrate. (b) Separation of esterases from different organs and tissues of *X. helleri* (h) and *X. maculatus* (m) in a 13% starch gel using α-naphthylpropionate as substrate.

Preparation of PERT Libraries

In order to prepare a genomic library highly enriched for single-copy sequences, a subtractive hybridization was performed essentially as described (Kunkel *et al.,* 1985; Shilo *et al.,* 1987). *Mbo*I-digested genomic DNA from *X. maculatus*/*X. helleri* backcross hybrids was denatured and hybridized in phenol emulsion to a 200-fold excess of randomly sheared denatured *X. helleri* DNA. Reassociated DNA fragments bearing two functional *Mbo*I sites could be ligated to *Bam*HI-cut pUC 19 vector DNA. This DNA was used to transform highly competent *Escherichia coli* MC1061 bacteria. The plasmid inserts from single colonies were prepared and used for RFLP analysis.

Enrichment of Genomic Library for HTF-Island-Containing Clones

In order to isolate probes with a high polymorphic information content (PIC) that can be used directly as starting points for chromosome jumping, a genomic library of *X. maculatus* was screened for clones containing CpG islands.

Total genomic DNA of *X. maculatus* was digested with *Hpa*II to completion. The fraction containing the tiny fragments (500–1800 bp) was isolated after fractionation in a low-melting point agarose gel, concentrated over NACS columns, and then labeled after random priming (Feinberg and Vogelstein, 1983). A *X. maculatus* genomic library was plated at a low density (5000 plaques/150-mm dish), and plaques were lifted to gene screen membranes (DuPont) and hybridized under high stringency conditions (see below for conditions for homologous probes) with the labeled HTF fraction. DNA was prepared from positively hybridizing clones and the *Eco*RI-released inserts were used as probes in RFLP analysis.

Analysis of DNA Polymorphisms

Genomic DNA from *X. helleri* and *X. maculatus* was isolated from pooled organs (brain, liver, gill, testis) (Olson *et al.,* 1979, modified), digested with either *Eco*RI, *Taq*I *Pst*I, *Hin*dII, or *Hin*dIII, separated by agarose gel electrophoresis, and transferred to charged nylon membranes (Southern, 1975; Reed and Mann, 1985). DNA fragments, used as hybridization probes after being separated from vector sequences, were prepared by random primed labeling (Feinberg and Vogelstein, 1983). Hybridization of homologous probes was carried out for 16–20 hr at 65°C in 7% sodium dodecyl sulfate (SDS), 0.001 *M* EDTA, 0.5 *M* sodium phosphate, and 100 μg/ml denatured calf thymus DNA. Heterologous probes were hybridized for 16–20 hr at 42°C in 5× Denhardt's solution, 1% sodium pyrophosphate, 0.05 *M* Tris–HCl (*p*H 7.5), 5% SDS, 1× SSC, 35% formamide, and 100 μg sonicated and denatured calf thymus DNA/ml. For homologous probes, washing was performed for 10 min at room temperature and twice for 30 min at 68°C in 0.1× SSC/1% SDS. For heterologous probes filters were washed in 1× SSC/1% SDS for 10 min at room temperature, followed by two washing steps for 30 min at 55°C.

RESULTS

Detection and Characterization of *Xiphophorus* Esterases

In *X. helleri* and *X. maculatus* in accordance with earlier studies (Ahuja *et al.,* 1977; Leslie and Pontier, 1980), more than five esterases can be detected. No

differences in electrophoretic patterns in starch or polyacrylamide gels were observed. Since we were interested only in polymorphic esterases, noninformative isozymes were omitted from further analyses.

The electrophoretic pattern of esterases was identical in all organs and tissues investigated (eye, brain, gill, fin, muscle), with the exception of the liver, where at least two additional liver specific isozymes were found. Electrophoretic variation between *X. helleri* and *X. maculatus* (the *X. maculatus* allele is designated a; the corresponding *X. helleri* allele is b) was detected for the fast-migrating *ES1,* which is present in all organs and tissues investigated, and for the liver-specific *LES1. X. maculatus* carries an allele for *ES1* (designated $ES1^a$; R_f value, 0.60) which codes for an isozyme that migrates faster than the corresponding one of *X. helleri* ($ES1^b$; R_f value, 0.55). For liver-specific polymorphic isozymes the faster-migrating enzyme ($LES1^b$; R_f value, 0.52) is encoded by *X. helleri;* the slower one ($LES1^a$; R_f value, 0.48), by *X. maculatus* (Figs. 1 and 2). Because of similar electrophoretic mobilities between *ES1* and *LES1,* both cannot be separated from each other in all cases without further classification.

For characterization of these polymorphic isozymes different substrates and inhibitors were used (Table I). Esterolytic activity was estimated visually. *ES1* prefers α-naphthylpropionate and is inhibited only by 10^{-4} *M* DFP. Therefore *ES1* can be classified as carboxylesterase. *LES1* does not show any preference for any of the substrates. Inhibition of *LES1* depends on the substrate used: no inhibition with any drug was observed using α-naphthylpropionate. However, moderate inhibition occurred with α-naphthylacetate as substrate, with either DFP or eserine sulfate as inhibitor.

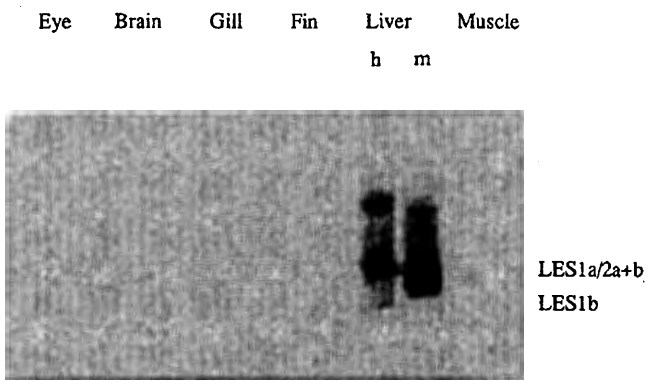

Fig. 2. Separation of esterases from different organs and tissues of *X. helleri* (h) and *X. maculatus* (m) in a 13% starch gel using α-naphthylpropionate as substrate and DFP as inhibitor. Using this electrophoretic system $LES1^a$ is comigrating with $LES2^a$ in *X. maculatus* so that these two alleles cannot be separated as is possible in *X. helleri.*

Table I. Characterization of Fast-Migrating Esterases in *Xiphophorus*

	Esterase 1 isozyme			
	ES 1[a]	*ES 1*[b]	*LES*[a]	*LES*[b]
Substrate				
α-Naphthylacetate	++	++	+++	+++
α-Naphthylbutyrate	+	+	+++	+++
α-Naphthylpropionate	+++	+++	+++	+++
Inhibitor				
Diisopropylfluorophosphate, 10^{-4} *M*	++	++	−/++*	−/++*
Eserine sulfate, 10^{-4} *M*	−	−	−/++*	−/++*
Parachloromercuribenzoic acid, 10^{-3} *M*	−	−	−	−

ES1[a] is the fastest-migrating esterase isozyme in *Xiphophorus maculatus*, *ES1*[b] is the equivalent in *X. helleri*. *LES*[a] represents the liver-specific esterase isozyme which migrates slower than *ES1*[a] in *X. maculatus*; *LES*[b] is the equivalent in *X. helleri* migrating faster than the corresponding *LES*[a]. The relative intensities of the stained bands on gels were scored as follows: +++, highest activity; ++, moderate activity; +, low activity. Enzyme inhibition was scored as a range from no inhibition (−) to total inhibition (+++).
*Depends on substrate: inhibition (++) with α-naphthylacetate; no inhibition (−) with α-naphthylpropionate.

Table II. Alleles of *Esterase 1* in Different Species and Populations of *Xiphophorus*

Species	*n*	*Esterase 1* allele		
		ES1[a]	*ES1*[b]	*ES1*[c]
X. maculatus	160	+	−	−
X. helleri (Rio Lancetilla)	7	−	+	−
X. helleri (Laguna Catemaco)	37	−	+	−
X. helleri	22	−	+	−
X. xiphidium (Rio Soto la Marina)	4	+	−	−
X. variatus (Rio Panuco)	4	+	−	−
X. evelynae (Necaxa)	4	+	−	−
X. nezahualcoyotl (Nasciemento, Los Otates)	7	−	−	+
X. signum (Rio Chaimajc)	10	+	−	−
X. cortezi (Rio Axtla)	4	+	−	−
X. gordoni (Laguna St. Tecla)	2	+	−	−
X. montezumae (Cascadas de Tamasopo)	6	+	−	−
X. andersi (Rio Atoyac)	2	−	+	−
Total	269			

ES1[c]: The electrophoretic mobility of *ES1*[c] is intermediate between *ES1*[a] and *ES1*[b]; however, for historical reasons it is proposed to keep this nomenclature.
X. maculatus is from different populations: Rio Jamapa, Rio Papaloapan, Rio Usumacinta, Rio Coatzecoalcos, Belize River, and Lago Izabal.
The third group of *X. helleri* is from different populations: not specified, Rio San Juan, and Rio Aguafria.

Table III. Linkage Analysis of *R* (Recognized by Benign Melanoma Phenotype) and *ES1*

Genotype	Type of melanoma	*n*	*ES1* allele		Remarks
			ES1^a	*ES1*^{b}	
X. mac./X. hell./X. hell. ($X^{TuSdTuSr}$)BC_1	Benign	23	+	+	Parental
		9	−	+	Recombinant
	Malignant	18	−	+	Parental
		5	+	+	Recombinant
	Malignant	18	−	+	Parental
		5	+	+	Recombinant
X. mac/X. hell./X. hell. (X^{TuSd})BC_1	Benign	9	+	+	Parental
		8	−	+	Recombinant
	Malignant	18	−	+	Parental
		7	+	+	Recombinant
	Total	68			Parental
		29 (=29.9%; SD 8.4)			Recombinant

These differences make it possible to distinguish clearly between *ES1* and liver-specific esterases.

Analysis for the presence of *ES1* in other *Xiphophorus* species is of interest with regard to syntenic loci. If linkage group V represents a syntenic loci group and other species carry *ES1* as well, there will be some evidence for the presence of *R* alleles in other species, too. To detect different *ES1* alleles in other *Xiphophorus* species, fish from 19 populations of 11 species were analyzed (Table II). In all *X. helleri* populations investigated and in *X. andersi,* the slow-migrating allele is found. The fast-migrating *ES1*^{a} allele form from *X. maculatus* is present in all other species investigated with the exception of *X. nezahualcoyotl,* whose *ES1*^{c} migrates with an intermediate mobility between *ES1*^{a} and *ES1*^{b}.

Linkage Analyses of *ES1* and *LES1* with *R* in Backcross Hybrids

To establish linkage between the polymorphic isozyme *ES1* and the *R* locus, 97 backcross hybrids of *X. maculatus* × *X. helleri* × *X. helleri* (BC_1) from two different *X. maculatus* genotypes ($X^{Tu\text{-}Sd}$, $X^{Tu\text{-}SdTu\text{-}Sr}$) were analyzed. For these experiments, fin was used generally, in addition, sometimes, to eye and liver tissue. The results of the recombination analyses are shown in Table III. Among 97 backcross hybrids, 29 recombinants were detected, corresponding to a recombination frequency of 29.9%. There is a remarkably high number of recombinants in the X^{Sd}-carrying backcross hybrids bearing benign mela-

Table IV. Polymorphic Restriction Fragments in *X. maculatus* and *X. helleri* Detected by Hybridization with Different Molecular Probes

Probe designation (fragment)	Restriction enzyme(s)	Size of polymorphic fragment (kilobasepairs)		Remarks
		X. hell	*X. mac*	
Human EGF (0.4-kb *Pst*I)	*Hin*dII	1.1	0.9	Gray *et al.* (1983)
XerbB p 38-I (1.6-kb *Hin*dIII)	*Hin*dIII	1.3	1.6	*EGF receptor,* autosomal
XerbB rel. (Klon 44) (4.6-kb *Eco*RI)	*Taq*I	3	3.9/3.1	
XerbB rel. (Klon 6.1) (5.1-kb *Eco*RI)	*Eco*RI	16	12.5	
	*Hin*dIII	14	11	
Xsrc 71 (1.32-kb *Bam*HI)	*Taq*I	4/3.7/1/0.7	4.4	*src* gene, autosomal
	*Hin*dII	3.8	2.5	
Xhomeo 2-3 (0.53 kb)	*Pst*I	1.4	3.9	"Homeobox"-containing gene
HTF 2a (4-kb *Eco*RI)	*Taq*I	4.1	2.3	
	*Pst*I	4.5/1.7	3/1.2	
	*Hin*dII	6.8	1.9	
	*Hin*dIII	11	4.5/1.9	
HTF 2b (13.5-kb *Eco*RI)	*Taq*I	3.2	2.5	
HTF 7 (16.5-kb *Eco*RI)	*Hin*dIII	2.1	1.6	
HTF 9b (4.5-kb *Eco*RI)	*Eco*RI	3	3.4	
	*Pst*I	4.8	5.7	
	*Hin*dII	2	3	
	*Hin*dIII	5.5	3.2	
HTF 14 (13-kb *Eco*RI)	*Taq*I	0.8	0.7	
	*Pst*I	1.4	1.3	
HTF 16b (12-kb *Eco*RI)	*Hin*dII	3.4	4	
HTF 17a (1.2-kb *Eco*RI)	*Eco*R1	2	1.1	
	*Taq*I	3.2/2.7	7.3/4/3	
	*Pst*I	5	6	
	*Hin*dIII	7.8/2.4	5.5	

Table IV. (continued)

HTF 19a (2.3-kb *Eco*RI)	*Eco*RI	10	5.8	
	*Hin*dII	2.4	2.1	
Human c-abl (1.8-kb *Eco*RI)	*Taq*I	6.3	3.5	Shtivelman *et al.* (1985)
	*Hin*dIII	8	6.8	
Human esterase D (1.2-kb *Xho*I)	*Hin*dII	3.9	1.1	Squire *et al.* (1986)
	*Hin*dIII	4.4/3	1.4	
Human ras (0.8-kb *Eco*RI/*Pst*I)	*Pst*I	1.9	1.3	Ellis *et al.* (1980)
	*Hin*dII	4.2/3.5/2.9	5.2/1.4	
	*Hin*dIII	2.2/1.6	3.9/1.5	
Human fos (1.6-kb *Bam*HI/*Pvu*II)	*Pst*I	3.9	4.4	Curran *et al.* (1982)
	*Hin*dII	5	5.8	
	*Hin*dIII	2.3	2.9	
Wilms candidate (0.25-kb *Sac*I)	*Taq*I	2.5	3.4	Gessler *et al.* (1990)
	*Pst*I	6.5	7.5	
Retinoblastoma 1 (0.9-kb *Eco*RI)	*Hin*dII	16	23	Friend *et al.* (1986)
TGFα-probe (1.1-kb *Cla*I/*Sac*II)	*Hin*dIII	0.9	1.2	
Rat neu (0.42-kb *Bam*HI)	*Eco*RI	6.5/2.6	6/3.1	Bargmann *et al.* (1986)
	*Pst*I	4.8	0.9	
Carp crystallin (0.6-kb *Pst*I)	*Eco*RI	11/6.2	7.3/5.2	Chang and Chang (1987)
	*Hin*dIII	6.4/4.5	6/4.8	
v-fms (0.4-kb *Pst*I)	*Eco*RI	9	5.8	Donner *et al.* (1982)
	*Hin*dIII	6/5.5/1.8/1.5	5/4.3	
PERT p57 I (0.5-kb *Hin*d III)	*Eco*RI	10	8	
PERT p57 II (1.3-kb *Eco*RI/*Hin*dIII)	*Eco*RI	10/7/5	4.5	

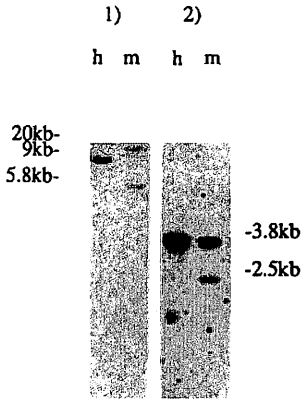

Fig. 3. Southern blot analysis of *X. helleri* (h) and *X. maculatus* (m) DNA with different single-copy probes for detection of polymorphic fragments: (1) hybridization with *v-fms* (0.4-kb *Pst*I fragment) reveals an *Eco*RI polymorphism; (2) hybridization with *Xsrc*71 (1.32-kb *Bam*HI fragment), a *Hin*dII polymorphism.

noma, nearly 50%, whereas all other backcross hybrids show lower frequencies. No linkage between *LES1* and *R* was detected.

Detection of Restriction Fragment Length Polymorphisms in *X. maculatus*

In order to obtain PIC probes for future linkage analysis, different single-copy gene fragments and *Hpa*II tiny fragments (HTF) were tested for polymorphisms between *X. helleri* and *X. maculatus*. Of the 48 probes tested, 17 revealed informative polymorphic loci in DNA from *X. helleri* and *X. maculatus* after digestion with five different restriction enzymes (see Materials and Methods) as shown in Fig. 3 and Table IV.

To establish autosomal versus gonosomal location of sequences detected with PIC probes, backcross hybrids can be employed that carry a *X. maculatus* sex chromosome in the genetic background of *X. helleri*. In such analyses for the *XEGFR* gene (PIC probe *XerbBp38-1*), only the *X. helleri*-specific fragment was obtained in Southern blot hybridizations (Fig. 4), indicating autosomal location. Similarily *Xsrc* has been located to autosomes (Robertson and Schartl, unpublished).

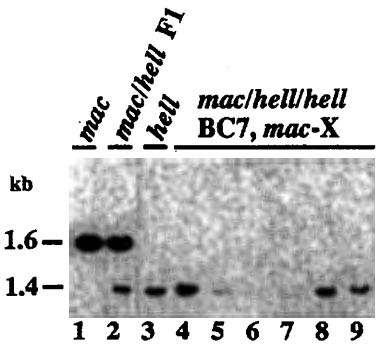

Fig. 4. Determination of autosomal localization of the *Xiphophorus EGF-receptor* gene by Southern blot analyses. *Hin*dIII-digested DNA of *X. maculatus* (X^{Sd}/X^{Sd}; 1), *X. maculatus* × *X. helleri* F_1 hybrid ($X^{Sd}/-$; 2), *X. helleri* (Rio Lancetilla; 3), and backcross hybrids (BC_7 $X.^{Sd}/-$; 4–9) was hybridized to *p38-1*. Additional *X. maculatus* ($n = 6$), *X. helleri* ($n = 2$), F_1 ($n = 3$), and backcross hybrids ($n = 4$) were analyzed and rendered the same bands (data not shown). The lower intensity of the *X. helleri*-specific fragment is due to the high intron content (approximately 50%) of the *X. maculatus*-derived *p38-1* probe.

DISCUSSION

Reverse genetic approaches toward isolation of the *R* locus of *Xiphophorus* depend largely on the availability of markers for the locus in question. Our

data have confirmed that a fast-migrating esterase isozyme (*ES1*) defines a linkage group for *R* that can be used as a basis for breeding experiments and further detailed mapping of *R*.

Our linkage data do not differ dramatically from earlier examinations. The average recombination frequency approaches approximately 30%. Morizot and Siciliano (1983a) have reported 36% linkage for *ES1* with *R*, whereas the values from Ahuja *et al.* (1980) are about 10% recombination. Thus, a loose linkage between $ES1^a$ and *R* there can be assumed. An explanation for differences in the recombination frequencies may be the difficulties in determining the right genotype of melanoma-bearing fish by the tumor phenotype (benign, *R;* malignant, no *R*). This could also be the reason for the high recombination frequency of nearly 50% in benign melanoma-bearing hybrids with the $X^{Tu\text{-}Sd}$ chromosome, whereas linkage for *ES1* and *R* in the same group of the *SdSr* genotype is approximately 30%.

Using different substrates and inhibitors in PAGE as well as starch gel electrophoresis and comparing different organs of *X. maculatus* as well as *X. helleri,* we were able to characterize the *ES1* marker isozyme further and to resolve the discrepancies that exist in the literature. First, *ES1* is definitely present in *X. helleri* and *X. maculatus* as well as in all species of the genus. This is in agreement with data of Morizot and Siciliano (1983a). The fact that Ahuja *et al.* (1977) did not detect *ES1* in *X. helleri* may be easily explained by the disc electrophoresis system used in small columns (Williams and Reisfeld, 1964), where the slow-migrating $ES1^b$ allele of *X. helleri* comigrates with the nonpolymorphic *ES2* (data not shown) and is obscured by the strong activity of this enzyme. Second, the carboxylesterase *ES1* is found invariably in both electrophoretic systems in all organs tested, including liver. This is in contrast to a report by Leslie and Pontier (1980), who claimed the absence of *ES1* activity in liver. We cannot explain this discrepancy. However, the strong activity of *LES1* may complicate detection of the almost comigrating weakly active *ES1* esterases. In addition, Metcalf *et al.* (1972) show the difficulties in exact classification of esterases because of overlapping substrate specificity in different species or with different substrates. One example is the dependence of inhibition with DFP and eserine sulfate from the substrate used for liver-specific polymorphic *LES1.*

The inability of earlier authors to detect ES1 in liver evoked a further complication. Without further specification, Morizot and Siciliano (1983a) described a polymorphic fast-migrating esterase in liver which they suspected, due to the reported absence of ES1, to be another esterase. This was named ES4. We postulate that this ES4 is in fact ES1. Low recombination of ES1 and "ES4" can be easily explained by sampling error in determination of severity of melanoma. This could then help to explain for the mapping

discrepancy found by these authors in linkage group V (Morizot and Siciliano, 1983a).

We detected a liver-specific esterase of the *X. helleri* allele (*LES1*^{b}) which migrates faster than that of *X. maculatus* (*LES1*^{a}), in addition to the *ES1*^{b} and the *ES1*^{a}, which themselves migrate faster than *LES1*. There are difficulties in unambiguous detection of *LES1* because this liver-specific esterase overlaps in mobility with *ES1* and *ES2*, both of which are present in the liver. But by specific inhibition (see Table II) we could distinguish *LES1* from other esterases. For the polymorphic *LES1*, no linkage with *R* could be detected. LES1 is not identical to ES4 from Morizot and Siciliano (1983a) because of its different allelic mobility and different responsiveness to eserine sulfate.

Since the linkage of *ES1*^{a} and *R* is not very tight, this precludes the use of ES sequences for cloning procedures. Extended studies over many years have failed to provide isozyme markers that are less distantly spaced (Morizot and Siciliano, 1983b, 1984). Therefore we are looking for molecular markers which are located closer to the *R* locus. Up to now we have found several PIC probes with different single-copy gene fragments and *Hpa*II tiny fragments (see Table IV). Such PIC probes have to be tested for linkage with *R* by monitoring the severity of the respective melanoma in backcross hybrids. With the help of *ES1*^{a}-positive benign backcross hybrids of *X. maculatus* × *X. helleri*, a mapping of linkage group V will be possible. The PIC probes will also be useful in establishing a detailed linkage map of *Xiphophorus*. In combination with known biochemical genetic markers, this will provide basic information for genetic and molecular studies.

Recently, using a heterologous probe, an *erbB*-like sequence has been mapped to linkage group VI of *Xiphophorus* (Harless *et al.*, 1990). The genes of *Xiphophorus* showing the highest similarity to the heterologous probe are *Xmrk* and the *Xiphophorus EGF receptor* (Wittbrodt *et al.*, 1989; Schartl, 1990). *Xmrk* is located on the X and Y chromosomes (Wittbrodt *et al.*, 1989; Schartl, 1990). In this study we have located the *XEGFR* to the autosomes, therefore suggesting that the *erbB*-like sequence of linkage group VI is in fact the fish *EGF receptor*.

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