A Sex Chromosomai 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 EcoRI 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 nontumorous 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 F1 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 Bcn. 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 Lancelula, 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 1985; DWIRL and ZANDUS 1967). However, an autosomal pair is homologous to the sex chromosomes of other Xiphophorus species, permitting normal meiosis and fertile hybrids in interspecific crosses (Kosswig 1985). A strain was used which does not exhibit any pterinophore locus.

2. Xiphophorus variatus from Rio Panuco, Mexico (var RP). These fish show male heterogamy. 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 Jampa, Mexico (mac RP). This stock demonstrates male heterogamy. 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 Usuracinta, 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 Xmac RY, etc., autosomes as Xmac, etc.

The following mutant sex chromosomes were used in this study:

- Xmac RY: 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).
- Xmac RY Tu-Sd: This chromosome is described as an X′Tu-Sd translocation of mac RY (AHUJA, LEPPER and ANDERS 1979).
- Xmac RY Tu-Li: This chromosome is reported to have occurred as the result of a cross-over between the Xmac RY and the Xmac RY Tu-Li chromosomes (ANDERS, ANDERS and KLINKE 1973) in a mac RY var RP hybrid female.
- Xmac RY Tu-Li: This chromosome has been recently obtained following a deletion of the Tu-Sr locus from the Xmac RY chromosome (A. ANDERS, personal communication).
- Xmac RY Tu-Sd: 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 RY hell F1 hybrid; it is described as a translocation of the Tu-Sd locus from the Xmac Tu-Sd wild-type chromosome to an autosomal RY 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 BamHI fragment D of pAE II (VENNSTROM et al. 1980) representing the central part of the cytoplasmic domain of the v-erb B gene of avian erythroblastosis virus; (2) 5000- and 5100-bp EcoRI fragments from clone X6.1 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 v-erb B gene (our unpublished data); (3) 4500 bp EcoRI fragment from clone X44, isolated as above. This fragment contains sequences from the kinase domain of the Xiphophorus v-erb B gene (our unpublished data).

RFL analyses: High molecular weight DNA (>70 kb) from pooled brain, liver, kidney, gonad 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). The following fragments were used: (1) 600 bp BamHI fragment D of pAE II (VENNSTROM et al. 1980) representing the central part of the cytoplasmic domain of the v-erb B gene of avian erythroblastosis virus; (2) 5000- and 5100-bp EcoRI fragments from clone X6.1 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 v-erb B gene (our unpublished data); (3) 4500 bp EcoRI fragment from clone X44, isolated as above. This fragment contains sequences from the kinase domain of the Xiphophorus v-erb B gene (our unpublished data).
TABLE 1

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<th>Genotype</th>
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n = number of animals tested.

in 1 × SSC at 60°C in the case of nonhomologous probes and in 0.1 × SSC at 68°C 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 EcoRI 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_mac_RJ 3d chromosome exhibit a 5-kb band, fish with a wild-type Y_mac_RJ 3d chromosome a 7-kb band, while fish carrying the wild-type X_var_RU 3d chromosome have a 12-kb band (Figure 1). hell (apparently sex chromosome... ...e) and mac RU (carrying the W_max RU and the i_max RU), both without a phenotypically rec-
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 HindIII 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 EcoRI 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 F1 hybrids, and of backcross generation animals revealed the expected co-segregation of the 5-kb band and the X(mac)RJ chromosome (Table 1). Backcross hybrids carrying either the wild-type X(mac)RJ chromosome, the corresponding Tu-Sd deletion chromosome (X(mac)RJ) or the Tu-Sd translocation chromosome (A(mac)RJ) 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(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(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)RJ chromosome distal from RFLM-1 was translocated to the X(mac)RJ chromosome (Table 2, Figure 2).

In order to characterize the RFLM-1 sequence, EcoRI 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 HindIII digests
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 Xmac 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 Xmac 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 EcoRI fragment or to a 15-kb fragment the presence of which did not correlate with the presence or absence of the Xmac 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 BC2 carrying the Xmac Rj56 chromosome) were hybridized to the v-erb B probe. In all cases the restriction-fraction 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 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 co-migrating nonpolymorphic fragment, or if the polymorphism is repre-
presented in the low resolution, high molecular weight fragments. From analyses using restriction enzymes other than EcoRI, 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 homogeneous 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 normal 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 (Mäuler, Rauf 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; Mäuler, Rauf 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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LITERATURE CITED


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