Oncogenes and Melanoma Formation in Xiphophorus (Teleostei: Poeciliidae)

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ABSTRACT

In Xiphophorus melanoma formation has been attributed by classical genetic findings to the overexpression of a cellular oncogene (*Tu*) due to elimination of the corresponding regulatory gene locus in hybrids. We have attempted to elucidate this phenomenon on the molecular biological level. Studies on the structure and expression of known proto-oncogenes revealed that several of these genes, especially the c-*src* gene of Xiphophorus, may act as effectors in establishing the neoplastic phenotype of the melanoma cells. However, these genes appear more to participate in secondary steps of tumorigenesis. Another gene, being termed *Xmrk*, which represents obviously a so far unknown proto-oncogene but with a considerably high similarity to the epidermal growth-factor-receptor gene, was mapped to the *Tu*-containing region of the chromosome. This gene shows features with respect to its structure and expression that seem to justify it to be regarded as a candidate for a gene involved in the primary processes leading to neoplastic transformation of pigment cells in Xiphophorus.

INTRODUCTION

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 non-tumorous fish *Tu* was proposed to be negatively controlled by cellular regulatory genes (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 (*R*), 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 *Xiphophorus helleri* then results, in effect, in the progressive replacement of regulatory gene bearing chromosomes originating from *Xiphophorus maculatus* by chromosomes of *Xiphophorus helleri*. This stepwise elimination of regulatory genes allows increased expression of *Tu*, resulting in the development of malignant melanoma in the hybrids.

Most of the information on the genetic basis for melanoma formation has been 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 are also found in the melanoma of the tumor-bearing hybrid fish. This pigment cell type has been referred to as a macromelanophore (Gordon 1959) or more recently "transformed" (tr)-melanophore (Anders et al. 1979). Various macromelanophore patterns, differing with respect to the region of the body surface where the pigment cells are located, have been found in the wildtype populations of Xiphophorus. The different patterns are determined by codominant acting genetic loci, the majority of which are located on the sex-determining chromosomes (see Kallman 1975). All of them harbour the melanoma determining *Tu*-locus (Anders et al. 1984).

In contrast to very clearcut models concerning the formal genetics of tumor formation in Xiphophorus (see Anders et al. 1984), the molecular processes underlying this tumorigenesis have remained obscure over a long period of time and are still largely not clearly understood. To improve further our knowledge in this field, we are following two approaches:

1.) We are studying those genes of Xiphophorus which share sequence similarity and therefore probable functional homology to a group of genes in higher vertebrates known as cellular oncogenes. These genes are of special interest because studies over the last few years have suggested them to be involved in the pathogenesis of human, rodent and avian neoplasms. One group of these genes has been identified by their biological activity in transfection assays by transferring the neoplastic phenotype to recipient cells and/or by homology to the tumor genes of known acutely transforming retroviruses. In the transforming state these genes are designated oncogenes, while in the non-transforming state they are referred to as proto-oncogenes (for a review see Bishop 1985). Conversion of the non-transforming proto-oncogene into a transforming oncogene (sometimes referred to as activation) has been shown to be possible by either qualitative changes and/or quantitative changes of the oncogene product. Besides the "classical" oncogenes, which act in a dominant fashion, a totally different second class of genes is also thought to play a crucial role in the process of tumor formation, namely recessive oncogenes, sometimes also termed "anti-oncogenes" (Knudson 1985).

2.) The second approach we are undertaking is aimed at directly isolating and molecularly characterizing the genomic loci of *Tu* and *R* . These loci should contain the genetic information which is causally responsible for the neoplastic transformation of a normal pigment cell to a tumor cell.

Proto-oncogene Sequences in the Genome of Xiphophorus

Using specific probes derived from the viral *src*, *yes*, *erb*B, *erb*A, ras^{Ha}, *myb*, *fos*, *sis*, *fms*, *abl*, *mil*, *fes* and *fgr* transforming oncogenes, as well as from the trout c-*myc* gene (Van Beneden et al. 1986), the human c-*hck*, and the mouse c-*lck* and *int*-1 genes, we could demonstrate corresponding sequences in the genome of Xiphophorus by Southern blot hybridizations (Fig. 1, see Mäueler et al. 1988 a,b; Schartl 1988; Hannig et al. 1991, Raulf et al., submitted). With most of the probes a rather complex array of hybridizing restriction fragments of differing intensities was detected. This indicates that these proto-oncogene sequences in Xiphophorus represent different members of multi-gene families similar to the situation in higher vertebrates for the same genes. For some of them a considerable restriction fragment length polymorphism within different Xiphophorus genotypes has been found. As this situation precludes in most cases the use of the heterologous probes for genetic analysis, we have set out to clone several of the Xiphophorus proto-oncogenes, which were deemed to be of interest for a molecular understanding of melanoma formation. These homologous sequences are also the tools for any further analysis of the respective gene's function.

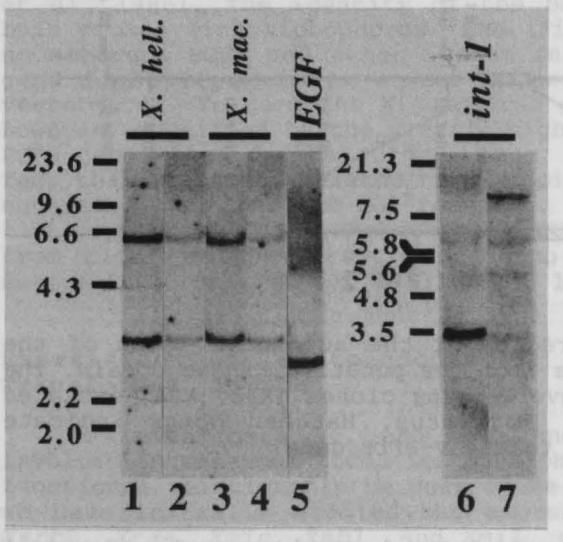

Fig.1: Southern blot analysis of restriction enzyme digested (lane 1-6 EcoRI, lane 7 HindIII) DNA from *X. hell* (lane 1,2) and *X .mac* (lane 3-7) probed with the extracellular domains of the human EGFR-gene (lane 1,3) and the rat c-*neu*-gene (lane 2,4), see text; b) the murine EGF-gene (lane 5), see text; c) the murine *int*-1 gene (lane 6,7, probe 2.4 kb EcoRI/HindIII fragment from pSP65 *int*-1, containing the cDNA of murine *int*-1, Rijsewijk et al. 1986).
Hybr. stringency: 35% formamide, 42°C, 5xSSC; wash. conditions 68°C, 1 x SSC.

From the multigene family of the *src*-related tyrosine-specific protein kinases we have cloned genomic sequences from *X. maculatus* which represent the fish homologues of the c-*src*, c-*yes* and c-*lck* genes of higher vertebrates, and a full-length cDNA from the

Xiphophorus c-*src* gene (*Xsrc*) (Raulf et al., submitted). Sequence analysis revealed that within the highly conserved tyrosine kinase domain the *Xsrc* gene and the Xiphophorus c-*yes* gene (*Xyes*) share 70-80% identity on the nucleic acid and 90% identity on the amino acid level with the corresponding human genes (Raulf et al., submitted). Outside the kinase domain this value drops to as low as 30% within the 80-90 amino acids at the amino-terminus of the protein. However, the value for the evolutionary rate of the kinase domain region of the gene is still approximately 1.4 percent amino acid changes per 100 million years. This value is extremely low and is lower only in the most highly conserved genes like the histone genes (0.1 - 0.9). The high degree of structural conservation combined with the estimated evolutionary age of the *src* proto-oncogene of approximately 1.5 x 10^9 years (deduced from the fact that the *src* gene appears first during phylogenesis in sponges; Schartl and Barnekow 1982; Barnekow and Schartl 1984) points to a very basic physiological function for the c-*src* gene product in all metazoans. Interestingly, the fish *Xsrc* and *Xyes* genes show a common intron/exon arrangement and identical exon size when compared to each other and to the homologous mammalian genes as well as to all members of the vertebrate *src*/tyrosine kinase gene family that have been characterized to date (Hannig et al. 1991; Raulf et al., submitted).

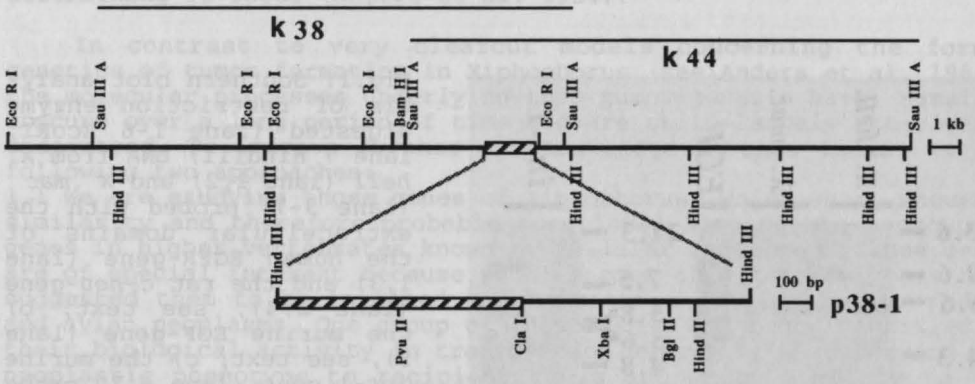

Fig.2: Restriction map of a region of the autosomal locus of the *Xerb*B gene containing two exons from the putative kinase domain. The region is represented by two overlapping clones (k38, k44) isolated from a genomic library of *X. maculatus*. Hatched boxes indicate fragments which crosshybridize to the v-*erb*B gene.

The proto-oncogene c-*erb* B, which has been shown to be identical with the gene for the epidermal growth factor receptor (EGFR; Downward et al. 1984) in birds and mammals, is also a member of a family of closely related genes. They all code for transmembrane receptors with a cytoplasmic tyrosine kinase domain. If Xiphophorus DNA is hybridized in a Southern blot experiment to a probe derived from the central part of the highly conserved tyrosine kinase domain

of the viral *erb*B gene, multiple bands of differing intensities are obtained, indicating also in Xiphophorus a multigene family of EGFR-related genes (Schartl 1988). Genomic sequences were cloned from *Xiphophorus maculatus* that correspond to those giving the strongest hybridization signals to the v-*erb*B probe in Southern blot experiments. They therefore should represent the Xiphophorus EGFR - gene and are termed *Xerb*B. The clones (Fig. 2) represent at least three non-overlapping genomic regions, each containing coding exons from the kinase domain. This indicates that the genomic locus of the *Xerb*B gene spans at least 60 kb. Other clones isolated were identified as being structurally closely related to the *Xerb*B gene but deriving from other genomic loci. From higher vertebrates it is known that the c-*neu* gene (or HER-2) shows the highest sequence relationship to the EGFR-gene. We were therefore interested to see if also an independent c-*neu* gene is present in the Xiphophorus genome, and if one of the *Xerb*B-related clones could represent that gene. DNA from Xiphophorus was therefore hybridized on the one hand to a probe derived from the extracellular ligand binding domain of the human EGFR gene (1.85 kb EcoRI fragment of clone pHER-AG4-1, representing the extracellular and transmembrane domain of the human EGFR, (Ullrich et al. 1984) and on the other hand to a probe from the corresponding region of the c-*neu* gene (420 bp BamHI fragment from clone neu c(t)3.4, representing the part of the extracellular domain between the two cysteine-rich clusters of the rat c-*neu*, Bergmann et al. 1986). This region is less conserved between the two genes than the kinase domain and the probes used are therefore specific for the respective gene in higher vertebrates (see Bergmann et al. 1986). The identity of the banding patterns obtained with both probes with Xiphophorus DNA (Fig. 1) suggests that there are no separate EGFR and c-*neu* genes in the fish, and that the *Xerb*B gene might represent a common ancestor for both genes of higher vertebrates. To term the Xiphophorus gene the EGFR-gene of fish is, however, justified by the clearly higher similarity to the mammalian EGFR gene than to the c-*neu* gene. This is also supported by the fact that sequences related to the epidermal growth factor, i.e. the natural ligand for the EGF-receptor, are also detected in Southern blot experiments with Xiphophorus DNA (probe: 400 bp *PstI* fragment from clone pm EGF-26F12, containing the sequence coding for the murine EGF; Gray et al. 1983) (Fig. 1).

Expression of Proto-oncogenes in Embryonic, Adult and Transformed Tissue.

To investigate if, and how, known proto-oncogenes might be involved in melanoma formation in Xiphophorus and if there exists a functional relationship between these genes and the activity of *Tu*, we have initially studied the expression of *Xsrc*, *Xmyc* , *Xerb*A, *Xerb*B, *Xras*, *Xsis*, *Xabl* and *Xmil* on the RNA level in tumors of adult fish and in a tumor derived cell line (PSM). Reasoning that an understanding of normal cellular functions of proto-oncogenes would give an indication of the role of the activated oncogenes in tumor cells, we have also examined the expression of these genes in non-transformed tissues of adult fish and during normal embryogenesis.

For *Xsrc* two transcripts (*Xsrc* 1, 3.7 kb; *Xsrc* 2, 3.4 kb) were detected during embryogenesis. In unfertilized mature ovae (stage 0, stages according to Tavolga 1949) and in early stages of embryogenesis, e.g. late blastula (0.5-1 days after fertilization, stage 3-4), approximately similar amounts of both *Xsrc* 1 and *Xsrc* 2 were found, presumably as maternal transcripts. During late organogenesis (5-7 days after fertilization, stage 14-17) a considerable elevation of the *Xsrc* 2 transcript was apparent, forming a peak around stage 17, while the *Xsrc* 1 transcript decreased in comparison to early stages. Later in embryogenesis, in neonates and in young fish both *Xsrc* transcripts are present at basal levels (Raulf et al. 1989). This corroborates earlier findings at the enzyme activity level (Schartl and Barnekow 1984), and also strengthens the hypothesis that c-*src* may play an important role in differentiation processes within neuroectodermal cells. The peak of *Xsrc* 2 expression correlates with the appearance of stellate epineural and cutaneous melanophores, and, among other events, predominantly with the development of the mesencephalon. In situ hybridization studies revealed that the expression of the *Xsrc* gene during organogenesis is restricted to the developing central nervous system and to the sensory layer of the developing retina (Raulf et al. 1989). An interesting discrepancy between mRNA and kinase activity data was established for the unfertilized egg. Our data revealed that in the unfertilized egg and the following early stages of development a readily detectable amount of *Xsrc* mRNA is present. However, no or only a minimal amount of protein kinase activity could be detected then (Schartl and Barnekow 1984). This would indicate that the bulk of the maternal *Xsrc* message does not give rise to an enzymatically active protein.

In non tumorous adult fish *Xsrc* was found to be preferentially expressed in neural tissues. The transcripts were localized by in situ hybridization to distinct regions of the brain (e.g. the granule cell layer of the cerebellum, and the ganglion cell layer of the mesencephalon) and to the bipolar neurons of the inner nuclear layer of the retina (Raulf et al. 1989). All these cells are terminally differentiated and non-proliferating, and the expression of the c-*src* gene in these cells points to a physiological function for that gene in the maintenance of a particular neuronal phenotype, probably as part of a complex cascade of intra- or intercellular signal transduction.

Interestingly the highly proliferating melanoma cells also express considerable amounts of the *Xsrc* 2 transcript, giving rise to elevated enzymatic activities of the protein product $pp60^{c\text{-}src}$. To investigate the relevance of this expression for the neoplastic phenotype we studied how growth autonomy as a unique feature of transformed cells (see Kaplan et al. 1982) relates to the expression status of the *Xsrc* gene. Serum deprivation <u>in vitro</u>, which is readily tolerated by the melanoma cells (PSM cell line) but which is fatal for embryonal cells (A2 cell line), leads to a 2-3fold accumulation of *Xsrc* 2 mRNA in PSM cells. In A2 cells the *Xsrc* 1 transcript, however, disappears, and reappears only as late as 48-72 h after restimulation by serum being the major source for growth stimulating factors in vitro. The different response of melanoma

cells and non-neoplastic cells with respect to *Xsrc* expression indicates that this gene has some relevance for the maintenance of the neoplastic phenotype (Mäueler et al. 1988a).

Using a probe derived from the c-*myc* gene of the rainbow trout (Van Beneden et al. 1986) we could detect a major transcript of 1.9 kb. This transcript is highly abundant in PSM cells and to a lower extent in the A2 cells. Because *Xmyc* shows only low levels of expression in cells from melanoma biopsies which do not exceed the levels found in normal organs (see fig. 3), we are not able at the moment to distinguish whether the *Xmyc* expression is a special feature of the tumor cells or of proliferating cells in general (most obvious with in-vitro cultivated cells). During early embryogenesis a major transcript of 1.2 kb is differentially expressed.

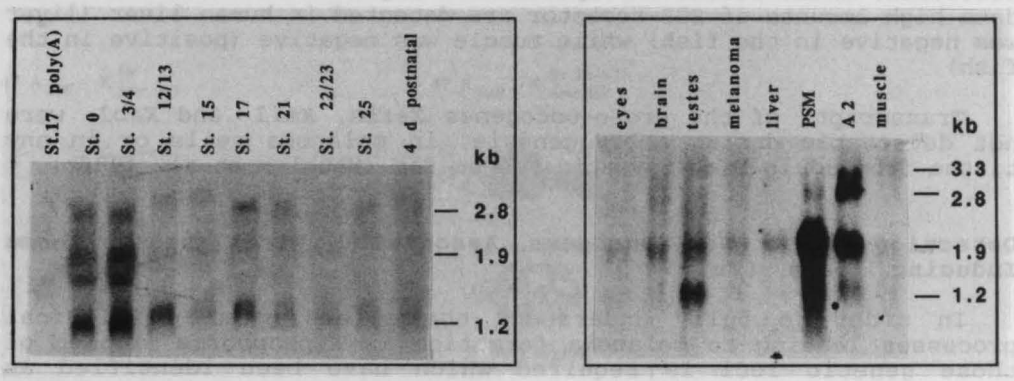

Fig.3: Northern-blot analysis of *Xmyc* expression during embryogenesis, in non-tumorous organs and melanoma of adult fish, and in the A2 and PSM cell lines. For developmental expression 20 µg of total RNA and for expression in adult organs and cell lines 10 µg of poly(A)$^+$ RNA for each sample were analyzed. Probe: 1.5 kb Eco RI/Pst I fragment from clone C 181 of the trout c-*myc* gene.

The *Xsis* gene shows two mature transcripts of 3.4 and 2.5 kb. The gene is differentially expressed during embryogenesis with levels slightly elevating during late organogenesis. High levels of *Xsis* transcripts were detected in the embryonal epitheloid cell line (A2). Normal adult organs and melanomas displayed only barely detectable levels of *Xsis* mRNAs (Mäueler et al. 1988b).

For transcripts from genes of the *ras* gene family a rather complex pattern of differential expression during embryogenesis and in adult organs was found. Although all three *ras* transcripts (3.4

kb, 3 kb, 1.7 kb) were detected in melanoma, we found so far noindications for a primary involvement of *ras*-genes in the process of tumorigenesis (Mäueler et al., 1988b).

Expression of the *Xerb*B gene was not detected during embryogenesis. In adults this gene is preferentially expressed in the head nephros with two transcripts of 5.0 and 3.2 kb, while most other normal organs and tumor cells contain low amounts of these transcripts (Mäueler et al. 1988b). The head nephros of fish is a composite organ which consists of kidney tubules and lymphoid tissue and functions as a part of the immune system. Future studies utilizing in situ hybridization should help to clarify any function of the *Xerb*B gene in the cell differentiation processes that occur in this organ. Results obtained with immunohistochemical methods screening for the EGF-receptor in several human tissues (Gusterson et al . 1984) are in several instances consistent with our RNA data. In accordance with our data human thymic epithelium and kidney tubules were found to contain high amounts of EGF-receptor molecules, while testes and spleen were negative. In contrast to our data high amounts of EGF-receptor are detected in human liver (liver was negative in the fish) while muscle was negative (positive in the fish).

Transcripts of the proto-oncogenes *Xerb*A, *Xmil* and *Xabl* were not detectable during embryogenesis, in melanoma cells or in any tissue from adult fish investigated so far (Mäueler et al, 1988b).

Detection of a New Oncogene Associated with the Melanoma Inducing Locus (*Tu*)

In order to fully understand the molecular and biological processes leading to melanoma formation in Xiphophorus cloning of those genetic loci is required which have been identified as encoding the genetic information for the primary event in neoplastic transformation. As we could show, expression of the *Xsrc* gene might have some relevance for the neoplastic phenotype of the melanoma cells. The question has been raised if the *Xsrc* gene might be closely linked to or even be an integral part of the *Tu*-locus. To investigate this, the following experiment was performed: The X-chromosome of *X. maculatus* containing the spotted dorsal allele of the *Tu*-locus (*Tu-Sd*) was introduced by introgressive breeding into the genetic background of *X. helleri*. A Southern blot experiment was performed with DNA from these fish, using a probe which detects specifically the *X. maculatus* allele of the *Xsrc* gene in *X. maculatus/X. helleri* hybrids. This probe did not hybridize at all to the DNA from the backcross hybrid carrying the *X. maculatus* X-chromosome with the *Tu-Sd* locus. Thus, the *Xsrc* gene is clearly not located on the X-chromosome and is not structurally associated with the *Tu*-locus. Similarly, the *Xerb*B gene is also found to be located on the autosomes and therefore unlinked (Schartl 1988). Preliminary experimental evidence suggests that also the other proto-oncogenes which have been found to be expressed in melanoma cells do not map to the *Tu*-locus. In addition, although a lot of correlations of different biochemical phenotypes and the *Tu*-locus encoded melanoma phenotype have been established in the past (see

Zechel et al. 1991), so far no gene products from the *Tu*-locus or from the *R*-locus have been identified. Therefore cloning of the *Tu*-locus and the *R*-locus by conventional methods of recombinant DNA technology is not possible. As an alternative we have initiated a cloning strategy similar to the "reverse genetics" approach which has led to the successful cloning of several human genetic loci which were also assayable only by their phenotype. Reverse genetics comprises the following experimental steps: 1) Precise localization of the gene on the chromosome, 2) identification and characterization of molecular marker sequences (so-called restriction fragment length polymorphisms, RFLPs) which map close to the locus in question, 3) cloning of the locus by preparative pulsed field gel electrophoresis and chromosome walking/jumping techniques, and 4) identification of the gene.

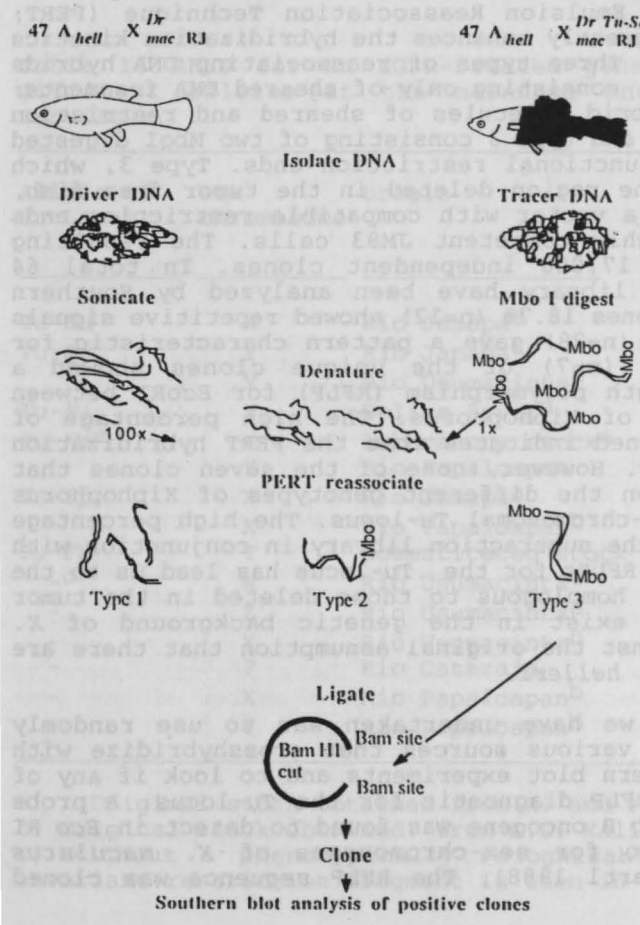

Fig. 4: Experimental strategy for subtraction cloning of a X-chromonsomal deletion using PERT conditions for reassociation (see text)

In the Xiphophorus melanoma system the chromosomal location of *Tu* is well known, the locus maps to the distal part of the respective sex-chromosomes (see Anders et al. 1984). The chromosomal location of *R* is less well-defined. It has been mapped to linkage group V where the locus is loosely linked (20 - 30 centiMorgans) to an esterase locus (Morziot and Siciliano 1983, Ahuja et al. 1980). Therefore we have concentrated on applying this approach to the *Tu*-locus. The most critical step of the reverse genetics approach is to find diagnostic RFLPs for the desired gene.

In a first approach we have attempted to directly clone marker sequences for the *Tu*-locus performing a subtraction hybridization method (Kunkel et al. 1985; Shilo et al. 1987). DNA was prepared from tumorous backcross hybrid fish carrying the *Tu-Sd* locus and from fish which are tumor free due to a deletion of the terminal part of the X-chromosome (i.e. including the *Tu*-locus) but which have the identical genetic background as the tumor bearing fish (see fig 4). The *Tu-Sd* containing DNA was digested with the restriction enzyme MboI and mixed with a hundred-fold excess of sonicated DNA prepared from the tumor free fish. The two DNA species were thermally denatured and reassociated under the specific conditions of the **P**henol **E**mulsion **R**eassociation **T**echnique (PERT; Kohne et al. 1977) which greatly enhances the hybridization kinetics of single copy sequences. Three types of reassociating DNA hybrids could be expected: Type 1, consisting only of sheared DNA fragments; type 2, consisting of hybrid molecules of sheared and restriction enzyme digested fragments and type 3 consisting of two MboI digested fragments reforming the functional restriction ends. Type 3, which should be enriched for the region deleted in the tumor free fish, were directly ligated to a vector with compatible restriction ends and transformed into highly competent JM83 cells. The resulting library contained about 17,000 independent clones. In total 64 single clones from this library have been analyzed by Southern blotting. Out of these clones 18.7% (n=12) showed repetitive signals on Southern blots, 48.3% (n=28) gave a pattern characteristic for unique sequences. 10.9% (n=7) of the unique clones showed a restriction fragment length polymorphism (RFLP) for EcoRI between the different genotypes of Xiphophorus. The high percentage of single copy sequences cloned indicates that the PERT hybridization has worked satisfactorily. However, none of the seven clones that detect EcoRI-RFLPs between the different genotypes of Xiphophorus is diagnostic for the sex-chromosomal *Tu*-locus. The high percentage of single copy clones in the subtraction library in conjunction with the absence of diagnostic RFLPs for the *Tu*-locus has lead us to the conclusion that sequences homologous to those deleted in the tumor free fish probably also exist in the genetic background of *X. helleri*. This argues against the original assumption that there are no *Tu*-like sequences in *X. helleri*.

The second approach we have undertaken was to use randomly selected DNA probes from various sources that crosshybridize with Xiphophorus DNA in Southern blot experiments and to look if any of these probes detects an RFLP diagnostic for the *Tu*-locus. A probe derived from the viral *erb* B oncogene was found to detect in *Eco* RI digests an RFLP specific for sex-chromosomes of *X. maculatus* carrying a *Tu*-locus (Schartl 1988). The RFLP sequence was cloned

from a subgenomic library of male *X. maculatus* (Adam et al. 1988) and used for further analysis. It was found that this sequence in *X. maculatus* detects a 5.0 kb restriction fragment if an X-chromosomal *Tu*-locus is present in the genome and a 6.5 kb fragment in the presence of a Y-chromosomal *Tu*-locus. *X. maculatus* fish having sex-chromosomes without a *Tu*-locus did not show any of these bands (Table 1). In *X. variatus* a 12 kb band is indicative of the presence of an X-chromosomal *Tu*-locus and in *X. montezumae* preliminary evidence suggests that a fragment of 10 kb indicates the presence of a *Tu*-locus. In addition this probe detects in all fish an invariant band of 7.0 kb regardless of the presence or absence of a *Tu*-locus. As the presence of the polymorphic restriction fragment is diagnostic for *Tu* containing sex chromosomes, it is concluded that it represents a genomic locus located on these chromosomes. Linkage analyses using different wildtype, deletion and translocation chromosomes with or without particular *Tu*-loci revealed that this sequence is located less than 0.1 centiMorgan proximal to the *Tu*-locus. This means that the RFLP-sequence may be located within 100-200 kb of the *Tu*-locus, or, alternatively, may even be a structural constituent of *Tu*.

Table 1: RFLP for an EGFR-related gene (*Xmrk*) associated with different alleles at the melanoma oncogene locus (*Tu*) of *X. maculatus*.

Allele of *Tu*-Locus	sex chromosome	origin	Restriction fragment length (kb)	numbers of fish analyzed
Tu-Sd	X	Rio Jamapa[a]	5.0	83
Tu-Sr	Y	Rio Jamapa[a]	6.5	42
	Y	Rio Usumacinta[b]	6.5	2
Tu-Ni	Y	Belize River[a]	6.5	4
Tu-Ni[e]	Y	laboratory stock[a]	6.5	4
	Y	Rio Papaloapan	6.5	1
Tu-Sp	X	Rio Jamapa[a]	5.0	4
	X	Rio Papaloapan[b]	5.0	2
Tu-Fu	Y	domesticated stock	6.5	3
-[c]	W	Rio Usumacinta[a]	-[d]	31
-	Z	Rio Usumacinta[a]	-	1
-	Y	Rio Usumacinta[b]	-	1
-	?	Rio Catazaja	-	2
-	X	Rio Papaloapan[b]	-	2
-	Y	Rio Papaloapan[b]	-	2

a) Original stock obtained from A. and F. Anders, Giessen.
b) Original stock obtained from K.D. Kallman, New York.
c) Without a phenotypically recognizable *Tu*-locus. d) Only the invariant restriction fragment is seen in Southern blots.

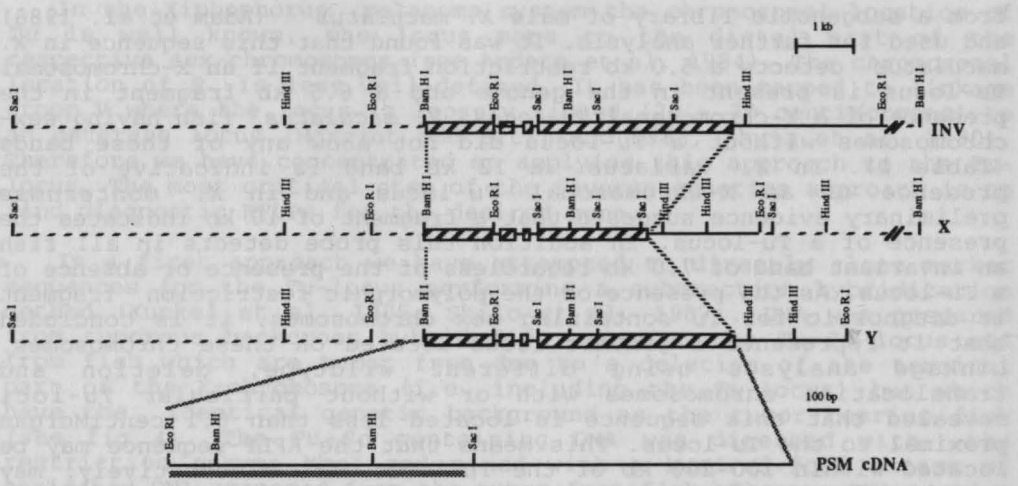

Fig. 5: Restriction map of corresponding regions of the invariant (INV), the X and Y chromosomal *Xmrk* loci and a partial cDNA (lower) from the PSM melanoma cell line. Hatched boxes indicate fragments from the genomic loci that contain coding sequence. The bar below the cDNA map marks the region which shows high similarity to the kinase domain of the human EGFR.

Further cloning revealed that the X-chromosomal, the Y-chromosomal and the invariant band represent three independent genomic loci. They all harbour a gene which is differentially expressed during embryogenesis. As this expression is independent from the presence of a *Tu*-complex and the sex-chromosomal gene loci we conclude that it is the gene from the invariant locus which gives rise to these transcripts. In adult fish expression could only be detected in RNA from melanoma biopsis and from an established melanoma cell line (PSM). The relevance of this gene expression for the neoplastic phenotype was demonstrated as outlined above for the expression of the *Xsrc* gene. It was found that in the PSM cell line serum deprivation leads to an up to 10fold accumulation of the 5.0 kb transcript. If serum is added again to the cultures, the level of this messenger returns quickly to normal within 2 to 4 hours (Mäueler et al. 1988a). A cDNA for this gene has been cloned from PSM cells and sequenced. The gene shows a considerable structural similarity to the EGFR genes of higher vertebrates. It is, however, different from the putative EGFR gene of Xiphophorus, the *Xerb*B, which is encoded by another genomic locus. Therefore this gene is supposed to represent a new oncogene which is a member of the gene familiy of the EGFR-related receptor kinases. Because of this structural feature and of its melanoma-specific expression in tumor bearing fish we have tentatively designated this gene *Xmrk* for **X**iphophorus **m**elanoma **r**eceptor **k**inase.

CONCLUSION

The molecular biological data which we have obtained to date include several implications for our current understanding of the process of melanoma formation in Xiphophorus. Firstly, the studies on the structure and expression of known proto-oncogenes have revealed that several of these genes, especially *Xsrc*, may act as effectors in establishing the neoplastic phenotype of the melanoma cells. They are, however, only candidates for genes participating in secondary steps of tumorigenesis. It is suggested that they become activated through the direct or indirect action of the *Tu*-locus with the effector genes possibly participating in a complicated network of regulatory relationships involving an oncogene cascade. Secondly, if we consider the *Xmrk* gene to be a constituent of the *Tu*-locus, the invariant locus is a proto-oncogene which serves a developmental function during normal embryogenesis as a growth factor receptor. During evolution of the genus Xiphophorus a copy of the gene might have been translocated to the sex chromosome into the region of the macromelanophore-locus. At its new position the gene came under the control of the pigment cell pattern genes, and - deregulated in the hybrid fish - displays its transforming function. The structural similarity of *Xmrk* to growth factor receptors provokes further speculations on this function for the proliferation control of the pigment cell.

To verify these considerations functional assays for the respective genes are being established. This includes the modulation of gene expression in-vitro and the production of transgenic fish. In particular, fish, which normally would not develop melanoma but which carry a *Xsrc* or *Xmrk* transgene (or both) under transcriptional control of a strong inducible promoter, should give insights in the functions of such genes for the process of melanoma formation in Xiphophorus.

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REFERENCES

Adam D., Wittbrodt J., Telling A. and Schartl M. (1988) Nucl.Acid Res. 16: 72-102.

Ahuja M.R., Schwab M. and Anders F. (1980) J. Hered. 71: 403-407.

Anders F., Diehl H., Schwab M. and Anders A. (1979) In: Pigment Cell (ed. S.N. Klaus), 4: Karger, Basel, pp. 142-149.

Anders F., Schartl M., Barnekow A. and Anders A. (1984) Adv. Cancer Res. 42: 191-274.

Bargmann C., Hung M.-C. and Weinberg R. (1986) Nature 319: 226-230.

Barnekow A. and Schartl M. (1984) Mol. Cell. Biol. 4: 1179-1181.

Bishop J.M. (1985) Trends Genet. 1: 245-249.
Downward J., Yarden Y., Mayes E., Scrace G., Totty N., Stockwell P., Ullrich A., Schlessinger J. and Waterfield M.D. (1984) Nature 307: 521-527.
Gordon M. (1959) In: Pigment Cell Biology. (ed. M. Gordon), Acad. Press, New York, pp. 215-239.
Gray A., Dull T.J. and Ullrich A. (1984) Nature 303: 722-725.
Gusterson B., Cowley G., Smith J.A. and Ozanne B. (1984) Cell Bio. Rep. 8: 649-658.
Hannig G., Ottilie S. and Schartl M. (1991) Oncogene 6: 361-369.
Kallman K.D. (1975) In: Handbook of Genetics (ed. R.D. King), . 4: Plenum Press, New York, pp. 81-132.
Kaplan P.L., Anderson M. and Ozanne B. (1982) Proc. Natl. Acad. Sci. U.S.A. 79: 485-489.
Knudson A.G. (1985) Cancer Res. 45: 1437-1443.
Kohne D.E., Levison S.A. and Byers J.M. (1977) Biochemistry 16: 5329-5341.
Kunkel L.M., Monaco A.P., Middlesworth W., Ochs H.D. and Latt S.A. (1985) Proc. Natl. Acad. Sci. U.S.A. 82: 4778-4782.
Mäueler W., Barnekow A., Eigenbrodt E., Raulf F., Falk H.F., Telling A. and Schartl M. (1988a) Oncogene 2: 365-378.
Mäueler W., Raulf F. and Schartl M. (1988b) Oncogene 2: 421-430.
Morizot D.C. and Siciliano M.J. (1983) J. Natl. Cancer Inst. 71: 809-813.
Raulf F., Mäueler W., Robertson S.M. and Schartl M (1989) Oncogene Res. 5: 39-47.
Raulf F., Robertson S.M., Mäueler W. aand Schartl M. (1991) submitted for publication.
Rijsewijk F.A.M., van Lohnitzen M., van Ooyen A. and Nusse R. (1986) Nucl.Acid.Res. 14: 693-702.
Schartl M. (1988) Genetics 119: 93-103.
Schartl M. and Barnekow A. (1982) Differentiation 23: 109-114.
Schartl M. and Barnekow A. (1984) Dev. Biol. 105: 415-422.
Shiloh H., Rose E., Coletti-Feener C., Korf B., Kunkel L.M. and Latt S.A. (1987) Gene 51: 53-59.
Tavolga W.N. (1949) Bull. Am. Mus. Nat. Hist. 94: 161-230.
Ullrich A., Coussens, L., Hayflick, J.S., Dull, T.J., Gray, A., Tam A.W., Lee J., Yarden A., Libermann T.A., Schlessinger J., Downward J., Mayes E.L.V., Whittle N., Waterfield M.D. and Seeburg P.H. (1984) Nature 309: 418-425.
Van Beneden R.J., Watson D.K., Chen T.T., Lautenberger J.A. and Papas T.S. (1986) Proc. Natl. Acad. Sci. USA 83: 3698-370.
Zechel Ch., Schleenbecker U., Anders A. and Anders F. (1991) This issue pp. 93-110.