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GENETIC FACTORS IN TUMOUR FORMATION: THE MELANOMA-INDUCING GENE OF XIPHOPHORUS

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Genes and cancer

Much of the progress in our understanding of the molecular origins of cancer stems from studies on specific genes, the oncogenes, which appear to be responsible for specifying many of the malignant features of tumor cells. Dominant acting oncogenes arise in large part by an activation process from pre-existing proto-oncogenes, which themselves appear to play essential roles in normal cellular physiology, and are often involved with the regulation of normal cell proliferation and differentiation. Recessive oncogenes contribute to the neoplastic phenotype of a cell due to inactivation of both normal alleles, which in their turn are thought to be critically involved in negative control of cell growth, therefore also designated tumor suppressor genes (see Friend et al., 1988). Although the number of known oncogenes increases steadily, and structural and functional analyses have proceeded to extremely high levels, generally the final proof that these genes are causally involved in the process of tumor formation is extremely difficult - if at all - to obtain. The situation is much better in those cases in which genetically defined loci have been found to be responsible for tumor induction. For the human hereditary retinoblastoma and for the Drosophila *lethal* (2) *giant larvae* tumor the causative recessive alleles have been identified and characterized (Friend et al., 1986, Huang et al., 1988, Mechler et al., 1985, Jacob et al., 1987). In addition, gene losses of possible tumor suppressor loci have been observed in a variety of human tumors (see Ponder 1988, for recent compilation and discussion) pointing also to causative recessive oncogenes. For dominant acting oncogenes the melanoma system of the teleost fish Xiphophorus offers a unique experimental situation where - dissimilar to all other vertebrate systems - the loci responsible for tumor formation have been clearly defined by Mendelian genetics.

Genetics of melanoma formation in Xiphophorus

In Xiphophorus, some individuals exhibit spot patterns, composed of large, intensily black pigment cells. These cells have been termed macromelanophores while the normal sized black pigment cells that make up the uniform greyish body coloration have been designated micromelanophores (Gordon 1927). Already more than 60 years ago it was discovered that certain hybrids of the macromelanophore pattern carrying platyfish (*Xiphophorus maculatus*) and of the unspotted swordtail (*X. helleri)* develop spontaneously malignant melanoma (Gordon 1927, Häussler 1928, Kosswig 1928). Shortly thereafter it was recognized that occurence of tumors in hybrids is due to a single locus (the macromelanophore locus) of *X. maculatus* that "interacted" with the *X. helleri* genome (Gordon 1931, Kosswig 1929). This interaction was later on defined as the presence of intensifying genes and/or the absence of repressing genes in the hybrid genome, which act specifically on the macromelanophore locus (Gordon 1958, Atz 1962, Kosswig 1965, Zander 1969, Kallmann 1970).

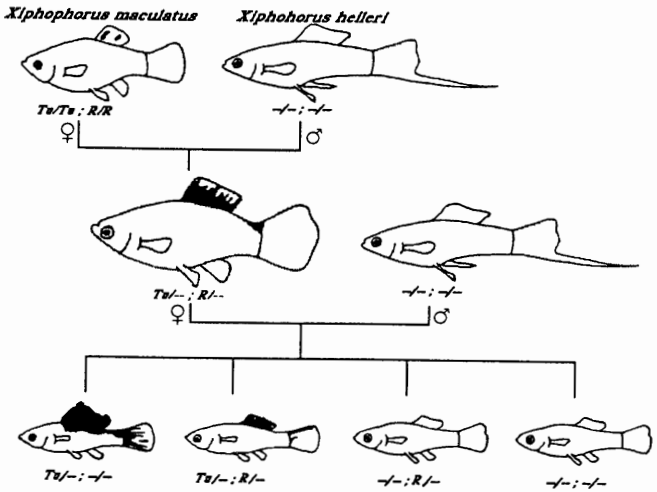

Figure 1: Genetics underlying melanoma formation in Xiphophorus hybrids. For details see text. *Tu*: melanoma oncogene, *R*: tumor suppressor gene.

In a typical crossing experiment (see Fig. 1) a female *X. maculatus* which carries the X-chromosomal macromelanophore locus *Sd* (Spotted dorsal, small spots in the dorsal fin) is mated to *X. helleri* , which does not carry the corresponding locus and exhibits the

uniform wildtype pigmentation. The F1 hybrid shows enhancement of the *Sd* phenotype. Backcrossing of the F1 hybrid to *X. helleri* results in offspring that segregate into 50 % which have not inherited the *Sd*-locus and are phenotypically like the *X. helleri* parental strain and 50 % which carry the macromelanophore locus and develop melanoma. The severity of melanoma ranges from very benign in some individuals (phenotype like the F1 hybrids) to highly malignant in others. Highly malignant melanomas of such fish grow invasive and exophytic and are fatal to the individuum. Based on a variety of such classical crossing experiments a genetic model has been developed to explain tumor formation in Xiphophorus (Ahuja and Anders, 1976). The macromelanophore locus was formally equated to a melanoma oncogene locus, whose critical constituent was designated "tumor-gene" (*Tu*). Melanoma formation then was attributed to the uncontrolled activity of *Tu*. In non-tumorous fish *Tu* activity was proposed to be negatively controlled by cellular regulatory genes or tumor suppressor genes (*R*-genes, corresponding to the modifying genes mentioned above). The major *R*-gene has also been termed *melanoma severity* (*MelSev*, Morizot and Siciliano 1983), because its presence was made directly responsible for the benign phenotype of melanoma in a certain fraction of the hybrids, or *Diff* (Vielkind 1976) because it was hypothesized that it directly controls the terminal differentiation of the macromelanophore. For the crossing experiment outlined above this means that *X. maculatus* contains the *Tu-Sd* locus on the X-chromosome and the corresponding major *R* on an autosome, while *X. helleri* is proposed not to contain this particular *Tu*-locus and its corresponding *R*. Backcrossing of the *Tu*-containing hybrids to *X. helleri* results, in effect, in the progressive replacement of *R*-bearing chromosomes from *X. maculatus* by *R*-free chromosomes of *X. helleri.* This stepwise elimination of regulatory genes is thought to allow expression of the *Tu* phenotype, leading to benign melanoma if one functional allele of *R* is still present (see F_1 hybrid and backcross hybrid D in Fig. 1) or malignant melanoma (see backcross hybrid E in Fig. 1) if *R* is absent (for review see Anders et al., 1984).

Reintroduction of *R* by crossing malignant melanoma bearing hybrids to parental *X. maculatus* was shown to lead to a reversion of the malignant phenotype resulting in totally tumor-free fish in the succeeding backcross generations using again *X. maculatus* as the recurrent parent (Anders et al., 1984). This demonstrates that the melanoma oncogene *Tu* itself remains structurally unaltered during the process of activation via hybridization and that the concerted action of *R* and *Tu* results in non-proliferating macromelanophore spots. A variety of different spot patterns have been identified besides *Tu-Sd* in feral fish and the corresponding loci are designated accordingly *Tu-N* (Nigra), *Tu-Sr* (Striped) etc.. They are located either on the platyfish X- or Y-chromosome.

Reverse genetic approaches towards isolation of the dominant melanoma inducing gene

In order to understand the molecular basis of hereditary melanoma isolation and characterization of the genes involved was attempted. We first concentrated on the dominant acting *Tu* because in the past a large variety of different alleles from natural populations (see Kallman 1975) and several spontaneous and X-ray induced mutants had been isolated and characterized (Anders et al., 1973). *Tu* is far better characterized in terms of classical genetics than *R*, from which so far only the allele from the Rio Jamapa platyfish has been studied. To that point no candidate gene product of *Tu* had been characterized precluding cloning by conventional recombinant DNA technology. We therefore applied a strategy that has been termed "reverse genetics" (Orkin 1986) to isolate the melanoma inducing gene of *Xiphophorus maculatus*. This strategy included the following steps: 1.) Determination of the chromosomal location of *Tu*. 2.) Identification and cloning of a molecular marker sequence for the *Tu*-locus, which is apparent due to a restriction fragment length polymorphism (RFLP). 3.) Cloning of the *Tu*-containing region by chromosome walking or jumping. 4.) Identification and isolation of a candidate gene. 5.) Verification that the candidate gene is indeed responsible for the *Tu*-phenotype, namely melanoma induction in the hybrids, thereby proving that the cloned gene is actually the sought *Tu* gene.

After the chromosomal localization of *Tu* had been clearly defined by recombination and mutation analyses as to reside within the distal portion of the sex-chromosomes, the most critical step was to identify a molecular marker sequence. One of several approaches (see Schartl et al., 1990) was to use heterologous oncogene/proto-oncogene probes for Southern hybridizations under conditions of low stringency. The rationale for this was that most oncogenes/proto-oncogenes of higher vertebrates fall into one of several classes of multigene families. The members of such gene families share highly conserved regions, e.g. kinase domains, DNA-binding domains etc.. A molecular probe of such a conserved region detects not only all members of the gene family of the same organism under conditions of reduced stringency in Southern hybridization, but also from distantly related species, e.g. fish (Mäueler et al., 1988a,b; Hannig et al., 1990). As a lot of sequences are identified in such experiments with a single probe, these are very informative with respect to the detection of restriction fragment length polymorphisms (RFLP), that can be used as molecular markers. In addition, it appeared not totally illusionistic to expect that the sought dominant melanoma oncogene of Xiphophorus may be a member of one of the known

oncogene/proto-oncogene multigene families.

From all probes tested, the viral *erb* B (v-*erb* B) probe was most informative. It is derived from the B oncogene of avian erythoblastosis virus and represents a truncated and oncogenically activated version of the avian epidermal growth factor receptor (EGFR) gene. The v-*erb* B probe that encompasses most of the higly conserved kinase domain, detects in *Eco*RI digests besides other strongly hybridizing bands two weaker bands that were only detected in the DNA of fish carrying a sex-chromosomal *Tu*-locus, one of 6.5 kb cosegregating with Y-chromosomal *Tu*-loci and one of 5 kb, cosegregating with X-chromosomal *Tu* -loci (Schartl 1988). In linkage analysis employing more than 500 individual fish no recombinant between this RFLP and the *Tu*-locus was found (Schartl 1988, 1990, Wittbrodt et al., 1989, Zechel 1988) indicating that this sequence is either intimately linked to *Tu* or even an integral part of the locus. The 5 kb band was cloned and found to detect besides the Y- chromosomal 6.5 kb band a third hybridizing sequence of 7 kb which was invariably present in DNA of all fish irrespective of the presence or absence of a *Tu*-locus (Adam et al., 1988). With this genomic sequence as a probe a corresponding full lenght c-DNA (Wittbrodt et al., 1989) was cloned from Xiphophorus melanoma cells and found to encode a typical growth factor receptor protein with an extracellular ligand binding domain, a transmembrane domain and an intracellular catalytic domain, that contains all eleven structural motifs diagnostic for the protein kinase activity of receptor tyrosine kinases (RTK). The gene was designated X*mrk* for Xiphophorus melanoma receptor kinase. It is closest related to the EGFR of higher vertebrates, but it does not represent the fish homologue of this gene (Wittbrodt et al., 1989). X*mrk* is a bona fide new member of the RTK gene family, whose physiological ligand is unknown to date.

Structure and genomic organization of X*mrk*

In the Xiphophorus genome three different copies of the X*mrk* gene coexist, which were identified due to their restriction fragment length polymorphism in the region encoding the kinase domain. The three copies represent independent genetic loci located on the X- and Y-chromosomes (Wittbrodt et al., 1989; Schartl 1990).

All three copies of X*mrk* share as far as analysed an identical exon/intron arrangement and show a sequence identity of more than 99% including the noncoding intron sequences. Each locus comprises approximately 25 kb (see fig. 2). X*mrk* shares an identical exon/intron arrangement and exon sizes with EGFR, HER 2/*neu* and ERB B 3 (Adam *et al.*, 1990) .

All fish, regardless of the presence or absence of the *Tu*-locus, contain one copy of X*mrk* (recognized by the invariably present 7 kb *Eco*RI fragment, therefore called INV) on each sex-chromosome. It obviously represents a typical proto-oncogene (Wittbrodt et al., 1989; Adam et al., 1990; Schartl 1990). The remaining two copies - named X and Y according to their sex-chromosomal location- are associated with the presence of macromelanophore spot patterns that can give rise to melanoma in the appropriate crossings (Schartl 1990) and are regarded as oncogenic versions of the INV copy.

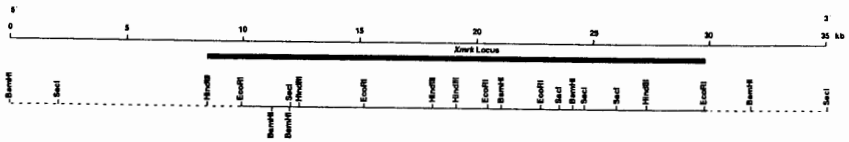

Figure 2: Restriction map of the whole X*mrk*-locus (as examplified for the INV-copy). Solid lines indicate cloned regions, the black bar marks the transcribed part of the locus, the grey bar indicates the promoter region.

Gross structural differences of the X*mrk* copies are restricted to their 3´ parts. X and Y differ from INV by larger deletions and point mutations. Most striking is a deletion of 1344 bp in the X-locus which comprises an entire exon and large surrounding intron regions leading to an internal deletion in the carboxy-terminus of the putative X-locus protein. The loss of sequence is due to a recombination of homologous sequences at the borders of the deletion (Adam *et al.*, 1990). A second large deletion of 581 bp in the 3´ untranslated region of X and Y removes the regular polyadenylation consensus of INV and gives rise to transcripts of X and Y extending to the 3´ adjacent polyA-site. However, the total length of the "trailer" differs only by 102 bases because of the new termination site.

All three copies of X*mrk* show a strong sequence conservation. The kinase domain does not show a single mutation which could lead to an altered protein, such mutations are restricted to the carboxy-terminus of the X*mrk* receptor tyrosine kinase genes. From 122 sequence differences between the proto-oncogene and the two oncogenes found in a total of 18 kb of genomic sequence (exons and introns), 10 lead to amino acid exchanges, only three of which are nonconservative (Adam *et al.*, 1990).

It is not clear at present if the observed sequence differences, or possible mutations in the so far not analysed extracellular, transmembrane and juxtamembrane domains in the Y- and X-locus do contribute to the process of neoplastic transformation. Loss of the exon within the carboxyterminus may be responsible for the higher malignancy of melanomas caused

by X-chromosomal copies of X*mrk* compared to melanomas caused by the Y-locus (Wittbrodt *et al.*, 1989, Adam *et al.*, 1990). Anyway, the point mutations found and also the large deletions in the X- and Y-copies of X*mrk* are not effective as long as the genes are under control of the *R*-locus. Therefore such differences are not responsible for bringing about the appearance of melanoma in the hybrids.

Evaluation of the 122 sequence differences found in all three X*mrk* loci with respect to phylogeny strongly supports the idea of a gene duplication event which created a new copy of the INV gene. This copy was translocated 2cM apart on the Y-chromosome during this duplication process and at a later stage transferred also to the X-chromosome by homologous recombination (Adam *et al.*, 1990; see fig. 3).

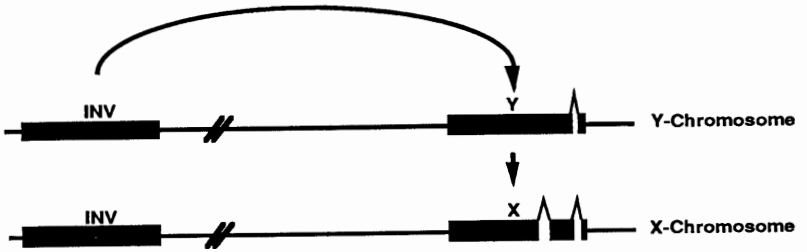

Figure 3: Model for the generation of the oncogenic X*mrk* copies (Y,X) by gene duplication of INV and sex-chromosomal crossing-over (indicated by arrows). Large deletions are indicated by gaps.

Verification that the additional X- or Y-chromosomal copies of X*mrk* are actually the critical, i.e. melanoma inducing constituent of the *Tu*-locus, came from analyses of "loss of function" mutants of *Tu*. Such mutants arise spontaneously with a very low frequency ($<10^{-5}$) in broods of melanoma-bearing hybrids and are characterized by a loss of the ability to develop spontaneous melanoma. One such mutant was found to carry an insertion within one exon of the additional X-chromosomal X*mrk* locus (Wittbrodt *et al.*, 1989), resulting in the inability to develop hereditary melanoma. This showed that the X*mrk* gene is necessary for tumorigenesis and therefore is the critical constituent of the *Tu*-locus.

Transcriptional activation of the X*mrk* oncogene

The X*mrk* genes give rise to two transcripts of different size, one of 5.8 kb and one of 4.7 kb. The shorter transcripts are product of the oncogenic X- and Y-chromosomal X*mrk* copies while the 5.8 kb mRNA is transcribed from the proto-oncogenic INV copy

(Wittbrodt *et al.*, 1989; Adam *et al.*, 1990).

The proto-oncogene transcript is highly abundant as maternal RNA in unfertilized eggs and is differentially expressed during organogenesis. In adult non-tumorous fish, expression of the INV gene is restricted to low levels of transcripts in skin, fins and gills. Low levels of the 5.8 kb transcript are also found in melanoma (Adam *et al.*, 1990).This expression is not influenced by presence or absence of the *R*-locus, as the 5.8 kb transcript is found at a similar level in melanomas of differing malignancy.

In contrast, expression of the X- and Y-copy of *Xmrk* is depending on the absence of the *R* -locus. Expression of the 4.7 kb transcript is limited to melanoma, it cannot be detected in any other tissue. The degree of *Xmrk* oncogene expression and the malignancy of the examined melanomas are definitely correlated: the amount of the 4.7 kb transcript is low in benign and very high in malignant melanomas (Wittbrodt *et al.*, 1989; Adam *et al.*, 1990). Overexpression of the oncogenic X*mrk* copies seems to be a prerequisite for tumor formation after loss of the *R*-locus.

With respect to the size difference of 1.1 kb of the INV transcript on the one, and the X- and Y-transcript on the other side, the question arose whether this might be due to a difference in 3´ end formation.The before mentioned deletion of the regular polyadenylation signal in the oncogenic copies of X*mrk* leads to transcripts with altered 3´ ends. However, the total length of the oncogenic transcripts stays basically the same as compared to the proto-oncogene.Therefore, the size difference does not result from the 3´ end. However, differences in the *5'* region were found to account for the observed difference in transcript length. Both oncogenic copies of X*mrk* use a transcription start site located about 1.1 kb 3´ to that used by the proto-oncogene. This is due to the presence of two different promoters in the different X*mrk* genes (Adam *et al.*, 1990). The promoter of the oncogenic X*mrk* loci is obviously only active in the melanoma cells of the hybrid fish but appears inactive in the purebred parental fish.

Based on this results, it is conceivable to argue that the *R*-locus is somehow involved in transcriptional control of the promoter used by the oncogenic X- and Y-copy of the X*mrk* gene. Loss of the *R*-locus then would lead to uncontrolled expression of X and Y resulting in melanoma formation.

Cooperation of multiple oncogenes?

It is generally accepted that cancer is a multistep process. This is reflected on the molecular level by the fact that in a variety of experimental situations activation of a single oncogene is not sufficient to induce and maintain the neoplastic phenotype of a cell.

Moreover a second or even more activated oncogenes are required (for review see Weinberg 1989). On the first sight it might appear that the situation is more simple in cases where activation of one genetic locus, like *Tu,* is the only event needed for tumor formation. Like with the retrovirus oncogenes expression of the X- or Y-copy of X*mrk* should be the single event necessary and sufficient for melanoma induction. However, overexpression of X*mrk* in the pigment cell lineage leads to a very complex system of alterations, not all of which may be simply explained as physiological consequences of an enhanced receptor tyrosine kinase activity, but may be regarded as secondary activation steps that are the prerequisite to obtain the full neoplastic phenotype of the melanoma.

All three members of the gene family of *src*-related cytoplasmic tyrosine kinases of Xiphophorus, namely X*src*, X*yes* and X*fyn* that have been cloned and analysed so far are highly expressed in melanoma (Raulf et al., 1989, Hannig et al., 1990, Mäueler 1988 a,b). The transcript levels of X*yes* and X*fyn* are even higher as in embryonal cells and in adult brain, the latter being the organ of preferential expression of these genes in non tumorous fish. The situation is even more intruiging with X*src*. Its expression in melanoma cells is specific for the transformed state because non-transformed pigment cells were found not to contain detectable amounts of X*src* transcripts (Raulf et al., 1989). The enzymatic activity of the X*src* encoded protein, $pp60^{Xsrc}$, is strongly enhanced in tumors as compared to any other normal organ, and correlates positivily with the degree of malignancy of the melanoma (Schartl et al., 1982, 1985). The X*src* gene is not structurally associated with the *Tu*-locus and consequently with X*mrk* (Robertson 1989, Schartl 1988), therefore it obviously represents a "secondarily" activated oncogene. If this holds also true for X*yes* and X*fyn* remains to be clarified. It will be also of interest to know why a certain subset of the *src*-family genes which are either suspected or shown to exert their normal function that is totally uncoupled from proliferation in neural tissue (Barnekow et al., 1990) is activated in the highly proliferative melanoma cells, which according to their embryological origin are also derived from the neuroectoderm.

Other observations that possibly will turn out to mark important steps in the generation of the full neoplastic phenotype are: 1.) increased phospholipid turnover (Smith et al., 1990), that might be indicative of an active second messenger system involved in signal transduction of the proliferation response of a receptor tyrosine kinase activation, as has been proposed for the EGFR and might be conceivable also for X*mrk*. 2.) growth of melanoma cells in low serum, accompanied by an accumulation of X*mrk* transcripts (Mäueler et al., 1988 b), possibly related to an autocrine stimulatory loop in proliferation control of Xiphophorus melanoma cells.

Modulation of the neoplastic phenotype

The degree of malignancy of melanoma in *Xiphophorus* is not only controlled genetically by the action of regulatory genes like *R* but can also be influenced by a variety of epigenetic factors. The first hint that one of these factors might be sexual steroids came from the observation that in certain genotypes melanoma formation starts earlier in males and leads to a higher malignancy (Siciliano et al., 1971) and that in another genotype only mature males develop melanoma (Anders et al., 1984).

To investigate whether and how steroids influence the process of melanoma formation and progression more than 3.000 fish were treated (Schartl and Schartl, 1988, Schartl et al., 1982). Besides the expected typical androgenic effects, e.g. induction of male secondary sex characters, treatment with testosterone led to different effects on melanoma formation depending on the genotype and the developmental stage of the tumor of the fish:

1.) Induction of melanoma occured in those genotypes that are predisposed to tumor development according to the deregulation of *Tu*, but that usually do not develop melanoma due to a delay or a total inhibition of pigment cell differentiation. Those tumors were fast growing and highly malignant. 2.) Enhancement of melanoma growth was observed in such genotypes that bear melanoma consisting predominantly of poorly differentiated precursors of pigment cells. This led to a dosage dependent mortality from neoplasia. 3.) Suppression of tumor growth and tumor regression occured in those genotypes that bear melanoma consisting predominantly of not yet completely differentiated pigment cells. The hormone effect was most obvious by reduction of the area covered by transformed pigment cells.

All three effects can be explained with the aid of a differentiation model for melanoma formation. Observations of Gordon (1959) that the melanoma cell is an incompletely differentiated pigment cell and further studies (Anders et al., 1980, Vielkind & Vielkind, 1982) led to the notion that benign melanoma consist mainly of cells in advanced stages of differentiation which have lost their capacity to divide whereas malignant tumors mainly consist of poorly differentiated pigment cells which are still capable to divide (Anders et al., 1979). Based on this model the effects observed could be the results of a promotion of pigment cell differentiation by testosterone. 1) In such genotypes which are predisposed to tumor development but do not develop melanoma due to a delay or a total inhibition of pigment cell differentiation prior to the stage where neoplastic transformation can occur a promotion of pigment cell differentiation would result in supply of stem cells which can be neoplastically transformed. They give rise to fast growing malignant melanoma. 2) In such genotypes that bear melanoma consisting predominantly of poorly differentiated precursor

genotypes that bear melanoma consisting predominantly of poorly differentiated precursor cells a promotion of cell differentiation would result in an increased number of neoplastically transformed pigment cells which are still capable to divide. This leads to the enhancement of tumor growth. 3) In such genotypes that bear relatively benign melanoma consisting predominantly of highly differentiated pigment cells a promotion of cell differentiation decreases the number of cells capable to divide. This would result in a suppression of tumor growth. Removal of terminally differentiated macromelanophores by macrophages then leads to regression of the melanoma.

To show if the effects observed after treatment with testosterone are specific for androgens various other substances were tested. Application of dihydrotestosterone, 17-methyl-testosterone and methyl-androstanolone led to the same effects as described above. Simultaneous application of antiandrogen, cyproterone-acetate, and testosterone reduced the phenotypic effects. Estrogen and diethylstilbestrol acted as antagonists to the androgenic substances with respect to their effect on melanoma induction. Application of cortisone, prednisone and progesterone had no effect (Schartl and Schartl, 1988). These data indicated that the observed modulation of the melanoma phenotype is specific for androgens.

Whether the hormone effects are due to a direct interaction of testosterone with the pigment cell via an androgen receptor could not be clarified to date because of the extremely high instability of this receptor in *Xiphophorus* (unpublished data).

Another substance that was found to modulate the malignancy of melanoma in Xiphophorus is dinitrochlorphenol (DNCB). Application of this compound led to tumor regression (Scholz 1977). DNCB is known to stimulate the immune system and thus may enhance an immune response against the melanoma cells. The same mechanism may explain the observation that raising tumor-bearing backcross hybrids under hyperthermic conditions also suppresses melanoma formation (Perlmutter & Potter, 1988). The high temperature in the environment most likely induces some kind of "artificial fever" in the poikilothermic animals.

Besides steroids and the immune system many more factors may exist that modulate the neoplastic phenotype. Due to the fact that the genetic factors determining malignancy are defined and lead to tumors of high pathophysiological uniformity, melanoma formation in Xiphophorus provides a unique system for studies on epigenetic modulatory factors and their mechanism of action. This hopefully will also offer new perspectives for therapeutic approaches.

Implications and perspectives

The classical model to explain spontaneous melanoma formation in platyfish/swordtail hybrids employing the sex-chromosomal dominant acting oncogene locus *Tu*, which we have shown to encode a copy of the X*mrk* gene, and the autosomal tumor suppressor locus *R*, has been extended to explain also a variety of other phenomenon and experimental observations such as the formation of carcinogen- and X-ray-induced tumors of all histiotypes in Xiphophorus hybrids, and the occurence of macromelanophore spot patterns in several other Xiphophorus species besides *X. maculatus*, some of which predispose to spontaneous melanoma formation in hybrids while others do not. With the availability of the X*mrk* gene these problems can be approached experimentally.

The extended model to explain induction of tumors of all etiologies besides heredity, which was even stretched to a unified concept for the origins of cancers in all multicellular organisms ranging from plants to man (Anders 1990), was faced with the problem that carcinogen treatment led to tumor induction also in hybrids that did not contain a sex-chromosomal *Tu*-locus associated with the macromelanophore locus. It was therefore proposed that *Tu* is present in the genome of Xiphophorus in multiple copies, spread over all chromosomes. The macromelanophore locus associated *Tu*-copy was termed "associate *Tu*", because it is obviously lacking in a variety of genotypes, e.g. the unspotted swordtails, without any negative effect, while the autosomal copies were termed "indispensable" and proposed to encode the information for neoplastic transformation realized in most of the carcinogen-induced tumors and a so far undefined important physiological function. Because the indispensable copies of *Tu* were not easily recognizable by macromelanophore patterns, their existance was only hypothetical. The genomic organization of X*mrk* clearly is not in accordance with these considerations on associate and indispensable *Tu* copies. X*mrk* is only present on the sex-chromosomes (Schartl 1990) and if activated oncogenes responsible for tumor induction after carcinogen treatment could not be mapped to the sex-chromosome (Schwab et al., 1978), they are definitely distinct from X*mrk* and consequently not encoded by *Tu*. Further evidence for this issue may be obtained from studies on X*mrk* expression in tumors of different etiology and also from molecular analysis of the factors responsible for tumor formation following carcinogen treatment.

With respect to the phenotypic diversity of macromelanophore patterns in the feral Xiphophorus populations it was reasoned that *Tu* itself specifies the phenotype of the macromelanophore. Pattern information was proposed to be encoded in a series of closely linked "compartment" genes. The potential for melanoma induction was thought to depend

on the major *R* -gene being closely linked to the *Tu*-locus in those cases, where a macromelanophore locus does not have a malignant potential, or being located on an autosome, thus being separated from *Tu* in backcross hybrids, analogous to the situation described for "Spotted dorsal" of the Rio Jamapa platyfish. Again studies on the genomic organization of X*mrk* led to a different view (Schartl 1990). The genes determining the phenotype of the macromelanophore are different from X*mrk* although closely linked and those macromelanophore loci, which do not predispose for melanoma formation simply do not contain the additional, oncogenic copy of X*mrk*. In fish with those loci only the proto-oncogene INV of X*mrk* is present. In the melanoma predisposing macromelanophore loci the linkage of the oncogenic X*mrk* copy to the pigmentation gene(s) is so tight, that it appears possible to identify those genes by chromosome walking or jumping using X*mrk* as a starting point.

The identity of the X*mrk* gene product as a putative novel growth factor receptor with a tyrosine kinase activity implicates further questions the answers to which should help to understand how overexpression of the gene mediates the initiation and maintenance of the neoplastic phenotype of pigment cells. It will be important to find those cellular substrates for the Xmrk kinase that transduce the mitogenic signal exerted by the Xmrk ligand to the nucleus as well as to identify the genes that are activated following Xmrk stimulation. Isolation and characterization of the ligand will not only help to elucidate the normal, physiological function of X*mrk* but also help to investigate if the melanoma cells constitute an autocrine growth stimulatory loop sensu Todaro and/or if the oncogenic X*mrk* loci encode an "activated" mutant protein, that is constitutively active.

To obtain information how the oncogenic activity of the X- and Y-copies of X*mrk* is suppressed in the parental purebred fish isolation and characterization of the *R* encoded gene(s) is required. The finding that transcriptional control may be the mechanism through which *R* regulates X*mrk* emphasises the importance of identifying the factors that control transcription of the oncogenic X*mrk* copies. An alternative approach will be to use also for cloning of *R* encoded gene(s) the methodology of reverse genetics.

Melanomas in feral Xiphophorus populations are extremely rare (Borowsky 1973, Kallman 1971), however, their existance gives additional significance to the reasoning that the duplicated X*mrk* genes that reside closely to the macromelanophore locus, are potentially injurious. It will be important to exploit also from an evolutionary and social behavioural genetic point of view how such a potential deleterious gene has been maintained in the natural populations of most species of Xiphophorus.

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