Spontaneous Melanoma Formation in Nonhybrid Xiphophorus

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ABSTRACT

Melanoma in hybrids of Xiphophorus is due to the unrestricted activity of a cellular oncogene locus, Tu, encoding the growth factor receptor gene Xmrk. In nonhybrid parental fish, Tu is controlled by a tumor suppressor gene. Thus, its restricted activity leads only to a nonmalignant, species- and population-specific macromelanophore spot pattern. Promoted by enigmatic reports on nonhybrid Xiphophorus with pigmentation abnormalities resembling melanoma, we have studied pigmentation in descendants of wild-caught fish and purebred laboratory stocks derived from wild populations. Whereas most stocks exhibiting macromelanophore patterns never developed pigmentation abnormalities, an exceptional situation for some nonhybrids was found. In X. variatus carrying the macromelanophore pattern "punctatus-2" and in X. cortezi with "spotted caudal," expressivity of the pigmentation gene ranges "lower comet," testosterone-dependent melanoma develops originating from carrying in addition the micromelanophore pattern "anal fin black" or from a few black spots to extreme melanosis and eventually to malignant melanoma. In X. maculatus with the mutant pigmentation gene striped" carrying in addition the micromelanophore pattern "anal fin black" or "lower comet," testosterone-dependent melanoma develop originating from the corresponding micromelanophore pattern. The tumors are highly malignant and express a melanoma-associated antigen. Overexpression of the Xmrk oncogene appears as the underlying molecular mechanism for tumor induction. These findings clearly demonstrate that tumors can also develop in purebred wild-type fish. The classical model for formation of hereditary melanoma in Xiphophorus hybrids does not explain the development of melanoma in the absence of hybridization. However, their existence gives additional support to the reasoning that the Xmrk oncogene associated with the macromelanophore locus is potentially injurious.

INTRODUCTION

The increase in melanoma incidence worldwide has drawn attention not only to the exogenous factors, like UV light, that trigger the disease but also to the genetic factors that may confer a higher risk to the individual to develop melanoma. For functional analyses of such "melanoma genes," platyfish and swordtails of the genus Xiphophorus provide a useful and well-accepted model system (1–3).

Since 1928, it has been known that certain hybrids of Xiphophorus maculatus and Xiphophorus helleri develop melanoma spontaneously (4–8). The tumors originate from certain pigment spot patterns that are made up of a distinct pigment cell type, the macromelanophore. These constitute a separate lineage of chromatophores and can be easily distinguished from the other melanin-containing cells, the micromelanophores, by their larger size (up to 500 μm) (5, 9, 10). Based on Mendelian genetic findings, the formation of melanoma is attributed to a dominant genetic locus, designated Tu. In parental wild fish from natural populations, no tumors will occur due to a locus, R, that suppresses Tu action. The restricted activity of Tu has been proposed to be evident from the occurrence of only nonmalignant macromelanophore spots in nonhybrids. Due to independent assortment, the R-bearing chromosomes can be eliminated in hybrids. This allows expression of the full oncogenic potential of Tu (11). Molecular cloning revealed that the Tu locus includes the Xmrk oncogene (12, 13). Analysis of the corresponding cDNA from melanoma cells identified Xmrk as a novel receptor tyrosine kinase being closely related to, but distinct from, the human epidermal growth factor receptor (13, 14). Studies on the genomic organization disclosed two copies of Xmrk (13, 15–18), a proto-oncogene and an oncogene (19, 20).

The Xmrk oncogene is causative for tumor induction. Its insertional disruption leads to loss of the capacity for melanoma development (13), while in transgenic fish Xmrk overexpression is sufficient for tumor formation (21). The level of oncogene expression correlates with the degree of malignancy of the tumors (13). The Xmrk-encoded receptor protein is highly abundant in melanoma cells and was found to be activated with regard to autophosphorylation constituting an autocrine system with its corresponding, but thus far biochemically uncharacterized, ligand (14, 22). Molecular genetic analyses revealed that the Tu locus-encoded Xmrk oncogene emerged by nonhomologous recombination of the Xmrk proto-oncogene with a previously uncharacterized sequence, D. This event generated an additional copy of Xmrk with a new promoter. Suppression of the new Xmrk promoter by R in parental fish and its deregulation in hybrids explain the genetics of melanoma formation in Xiphophorus (20).

It should be emphasized that all of this information is derived from crossing experiments, molecular cloning, and biochemical analysis of the Xmrk oncogene from the spotted dorsal and striped Tu loci of X. maculatus. Although a structural homology of the Xmrk alleles from different melanoma-inducing loci of a wide variety of genotypes from different species has been shown (19), in most cases the functional homology and the mechanism underlying melanoma formation has not been investigated in detail.

The experimental induction of tumors including melanoma by carcinogens is thus far reported only from hybrids. No tumors, however, were induced after treatment of nonhybrid Xiphophorus (3, 23, 24).

In nonhybrid Xiphophorus, nodular melanotic growth that could possibly represent melanoma has been reported as an occasional enigmatic phenomenon (25–28). The fish were descendants of animals taken from the natural habitats or were from purebred laboratory stocks derived from wild Xiphophorus. However, the etiology and histopathology of these lesions have not been analyzed thus far, and the mechanisms underlying this phenomenon are not understood. Most interestingly, a mutation has been obtained where, for the first time, changes in pigment gene expression were described that could not be attributed to hybridization (29).

In this paper, we demonstrate that the pigmentation abnormalities in nonhybrid Xiphophorus are, indeed, malignant melanoma by all histopathological criteria, including expression of a melanoma-associated antigen. They show overexpression of the Xmrk oncogene and are indistinguishable from the melanoma of hybrids. Environmental or physiological factors may trigger their appearance.

MATERIALS AND METHODS

Nomenclature of Genes and Pigment Patterns. Thus far, no common terminology for genes and pigment patterns in Xiphophorus exists. In this paper, we will use the terms as follows. The macromelanophore locus has been formally equated to the tumor gene locus (3, 18, 30). We will designate the
different macromelanophore genes as independent genes according to the earlier literature (see Refs. 30 and 31) and alleles of these as long as they can be recognized by differences in the phenotype they encode or by molecular features. Sc, therefore, stands for the spotted-caudal gene of X. cortezi, which maps to the Tu locus sensu Anders and is associated with an oncogenic copy of Xmrk. It should be noted, however, that the association of a macromelanophore gene with Tu/Xmrk has been conclusively shown thus far only in a few cases. The current generalization is that any macromelanophore gene that gives rise to hybrid melanoma and to which an additional copy of Xmrk can be assigned represents an analogous situation to Sd of X. maculatus, which has been analyzed in detail. Different alleles of a macromelanophore gene are given superscript numbers. The "punctatus" pattern genes have been abbreviated P1 and P2 in the earlier literature (31, 32); we will use the two-letter code Pu1 and Pu2 because this is the standard for designation of all the other macromelanophore pattern genes and in order to avoid confusion with the P gene of Xiphophorus that controls the onset of sexual maturation (33). The R locus has sometimes been named Dff (for differentiation; Refs. 3 and 18); we use the older term, R, because Dff implies that this locus is instrumental in the control of cell differentiation of the macromelanophore lineage. This is deduced only from correlative observations (34), while the involvement of R in the transcriptional regulation of the Tu encoded Xmrk oncogene is much more evident (16, 20).

Experimental Animals. The fish used in this study were bred under standard conditions (30) either in the aquarium of the Biocenter of Würzburg University or the Department of Biology of New York University.

Details on the genotypes and phenotypes are as follows: In X. variatus, the "punctatus" patterns are encoded by sex-linked genes. They are apparent as a variable number of black spots on the body side of the fish. The "punctatus-1" pattern consists of numerous small spots scattered over the entire body sides. Fish with "punctatus-2" have large, intensely black spots, most numerous along the midlateral line. The Pu2 allele is variable in its expression, from a few spots to a black band along the flanks (31, 32, 35). The "lined" pattern consists of several longitudinal stripes that develop along the midlateral line, above and below (31).

The X. variatus individuals used for histological analysis were all derived from the Arroyo Zarco population of the Rio Sabinas system, Tamaulipas, Mexico (locality map in Ref. 32). They were chosen to represent the range of expression of the Pu2 pattern. All fish were one to five generations removed from the wild, with zero to low inbreeding. Details are given in Table 1. Fish with the Pu2 pattern or without a macromelanophore pattern were from the Rio Panuco system, San Luis Potosi, and from a small ditch approximately 10 km north of Ciudad Mante, Tamaulipas.

All X. cortezi used in this study were from a randomly inbred strain derived from animals collected by Kosswig et al. in 1964 from the Rio Axtla (36). In X. cortezi, the Sc gene leads to the formation of the "spotted caudal" pattern, which typically consists of one or more irregular, elongated patches of black pigmentation commencing close to the base of the middle or lower caudal fin rays and extending posteriorly for roughly one-third of the fin length. Another strain also available for this study derived from the same locality and does not have the Sc pattern.

The striped (Sr) gene of X. maculatus is a mutation of Sr from the Rio Jamapa population, resulting in an enhanced expression of the macromelanophore pattern (37). These fish exhibit macromelanophores already at birth; in adults, macromelanophores are present as a reticulum pattern of the edges of the scales on the whole body. The micromelanophore pattern "anal fin black" (Ab) results in black coloration of parts or the whole male anal fin, which is transformed to a copulatory organ, the gonopodium, in mature males. The relation of Ab to the pattern "black gonopodium" (Gn) of X. milleri and X. variatus, which is similar in gross appearance, is unclear. Further details on Ab will be published elsewhere. The "lower comet" (Lc) tailspot pattern (38) is a stripe of micromelanophores at the ventral edge of the caudal fin that extends from the base to the caudal margin of the fin.

Histology and Antibody Staining. For light microscopy, fish were anesthetized in MS 222, sacrificed, and fixed either in 4% formaldehyde or Bouin's solution. Excess picric acid was eluted in 70% ethanol. After dehydration, the specimens were embedded in paraffin. Five-μm sections were cut with a Leitz base sledge microtome and stained according to classical histological staining methods.

For immunohistochemical analysis, cryotome (Jung Frigocut 2800N) sections (10 μm) were cut from tissue samples, previously dehydrated in 10% sucrese for 24 h, shock-frozen in isopentane (−70°C), and embedded in Tissue Tek (Miles). Endogenous peroxidases were blocked with methanol:3% H2O2:4:1 for 20 min at room temperature. Unspecific binding was prevented by incubation with 1% normal horse serum for 30 min. The slides were incubated with MoAb 21.7 (39) in a humidified chamber at 4°C overnight or for 2 h at room temperature. Specific binding was detected by using the ABC kit (Vectastain; Vector Laboratories) according to the supplier's instructions and using aminothiocarbazol (Sigma Chemical Co.) as substrate, which yields a red product that is easily distinguishable from brown melanin. The slides were counterstained with hematoxyline and embedded in Glycergel (Dako). For control, the irrelevant MoAb R73 (40) directed against a T-cell receptor epitope from rat was used.

Testosterone Treatment. For testosterone treatment, a 0.1% ethanolic solution of 17-methyl testosterone (Sigma) was applied to the tank water daily. The daily dosages ranged from 2–200 μl/l (10−3–10−7 M). For control, fish of the same genotype, sex, and age were treated with ethanol alone.

Southern Blot Analysis. DNA extraction from pooled organs of individual fish and Southern blot analysis were done essentially as described (41). The Xmrk kinase domain (p17–2) and extracellular domain (p3–2) cDNA probes (13) and an oncogene promoter probe (−278/+35) were used under high stringency conditions.

Western Blot Analysis. Tissue samples were homogenized in lysis buffer and Western blot analysis was performed essentially as described (22). Xmrk protein was detected using a polyclonal α-Xmrk rabbit antiserum and a chemoluminescent system (ECL; Amersham).

RESULTS

Melanoma in Nonhybrid Wildtype Fish

Three macromelanophore patterns of X. variatus were examined in detail. Fish carrying the "lined" (Li) and/or the "punctatus-1" (Pu1; Fig. 1a) pattern never developed melanosis or melanoma. This observation is based on more than 500 individuals (juveniles, adults of both sexes, and senescents over 18 months) reared in our laboratories or inspected in their natural habitats. With respect to the ontogeny of the Pu2 pattern, analysis of laboratory raised fish and fish collected over the past 10 years revealed the following. In fish younger than 18 months of age, there are no spontaneous melanomas. Of 11 fish older than 18 months, 5 had melanosis and 3 others had nodular melanomas (see below). These data suggest that the development of melanosis and melanomas in Pu2 fish is age related. The progressive nature of the development of melanomas from this pattern is illustrated by the series of individuals N610→N540→N550P→N610P→N552 that were used for histological analysis defining the different stages of the disease.

Stage I. In fish with stage 1 expression of Pu2 (N610; Fig. 1b), macromelanophores are restricted to the dermis, the meninges, the perivascular connective tissue of the blood vessels, and the peritoneum. No macromelanophores could be detected in the muscles or elsewhere.

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The abbreviations used are: Sc, spotted-caudal; Sr, striped; Pu, punctatus; Li, lined; Lc, lower comet; Ab, anal fin black; MoAb, monoclonal antibody; kb, kilobase(s).

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Stage II. The fish showing stage II expression of \( Pu^2 \) (N540) had an increased number of dermal macromelanophores. Besides the normal range of occurrence, a few macromelanophores are found circling a muscle bundle (Fig. 1, e and f).

Stage III. In fish with pronounced melanosis (N610P, and N550P; Fig. 1c), the flanks are uniformly dark black, but external nodules are not visible. Histological analysis revealed a large number of dermal macromelanophores (that form occasionally several layers). Frequently, macromelanophores are penetrating the stratum compactum of the dermis and invade along the fasciae of the underlying muscles (Fig. 1g). Pathologically, this abnormal pigmentation phenotype can represent the transition of a benign or premalignant lesion to malignant melanoma. It is particularly interesting that the individuals N550P, and N610P, seem to show...
two alternative paths for melanosis, i.e., dermal proliferation and invasion of deeper tissues.

Stage IV. The most extreme expression of Pu2 as in N552 (Fig. 1d) leads to highly malignant melanoma. Typically, three-dimensional growth leads to external nodules consisting of densely packed, heavily pigmented tumor cells. The neoplasia is also highly invasive. In certain regions, the muscle bundles are totally surrounded by irregularly arranged melanoma cells. These cells have polymorphic nuclei, and mitotic cells can frequently be detected. Progressive destruction of muscle tissue (Fig. 1h) is also evident from the swimming disability of older Pu2 melanoma fish. Judging from their histological appearance, these tumors are indistinguishable from the hereditary malignant melanoma of hybrids or from carcinogen-induced melanoma (for a comparison, see also Refs. 42–44).

A similar, although less dramatic situation, is found in X. cortezi. Here, sexually mature fish of at least 9–12 months of age with the “spotted caudal” pattern develop severe melanosis. This phenomenon is observed in approximately 2–5% of fish and is most pronounced in sexually active males of high social rank and old age but is also recognized in old females. The macromelanophore spot increases in size until it covers almost the entire caudal fin. Later, macromelanophores invade the peduncle, leading to black pigmentation of this region. Sometimes exophytic growth occurs around the ventral midline of the peduncle and the caudal fin. The tumors are highly malignant, as seen from the progressive and infiltrative growth. Underlying muscles are destroyed, which is also evident from cachexia of the caudal region and a specific disability of swimming movements. The melanoma are poorly vascularized and frequently show central necrosis (Fig. 2). They progress from stages I to IV.

Hormone-dependent Melanoma in a Nonhybrid Mutant Strain of X. maculatus

Many wild populations of X. maculatus are polymorphic for the micromelanophore tailspot pattern “lower comet” (Lc) and “anal fin black” (Ab). No pigmentation abnormalities of these patterns have been observed in hundreds of wild fish or laboratory stocks of purebred X. maculatus. The combination of Lc and/or Ab with any nonmutant macromelanophore pattern does not influence expression of the micromelanophore pattern. Following hybridization, no premalignant lesions or melanoma develop from these patterns (9, 30). The mutant pattern “striped” leads to an increased macromelanophore pattern (stage I); however, no abnormalities occur except in male fish that carry, in addition, Lc and/or Ab. Such fish develop (if of high social rank and being sexually active) at older age (over 9 months) malignant melanoma that originate from these micromelanophore patterns. To verify that hormones may stimulate the neoplastic growth (see also Refs. 37 and 45), fish were exposed to steroids in the aquarium water. After treatment of young fish (n = 120) with testosterone, macromelanophores appeared in the ventral part of the caudal fin or in the anal fin forming a pattern typical for Lc or Ab. When treatment was ceased after approximately 50 days; after 3–5 months and only in a few cases (<5%), slow growing melanoma without external nodules occurred. When, however, treatment was continued longer than 50 days, all fish developed malignant melanoma initiating from the Lc and Ab pattern. Histologically, the melanoma developed to stage IV. They showed exophytic and invasive growth. The underlying muscles of the peduncle were reduced. In the neighborhood of the nodular growth areas, muscle bundles were destroyed and necrotic (Fig. 3). X. maculatus (n = 10), carrying the Lc or Ab micromelanophore pattern but no macromelanophore pattern, did not develop any premalignant or proliferative malignant pigmentation abnormalities following treatment with androgen, except for a more intense black coloration of the anal fin in Ab fish.

Expression of a Melanoma-associated Antigen in Tumors of Nonhybrid Xiphophorus

Melanoma cells of hybrids express a certain surface antigen that is characterized as a specific marker for this tumor type. This melanoma-associated antigen is also an oncofetal antigen, because it is differentially expressed on embryonal cells but not in any normal organ of adult fish as investigated thus far. MoAb 21–7 detects this antigen and has proven to be a useful and reliable tool for characterizing the malignant state of hereditary melanoma (39). MoAb 21–7 reacted also specifically with the Pu2 nonhybrid melanoma in histological sections. The staining was most abundant in the less pigmented areas of the tumor where lowly differentiated, proliferating cells are more numerous (Fig. 4). Expression of the MoAb 21–7 antigen in the nonhybrid melanotic lesions further characterizes them as highly malignant melanoma that are very much like the corresponding tumors in hybrids.
Association of the Xmrk Oncogene with Nonhybrid Melanoma

Melanoma formation in hybrids is caused by overexpression of the additional, oncogenic copy of Xmrk (for a review, see Ref. 2). We followed the question whether the same oncogene may also be instrumental in neoplastic transformation of pigment cells in the nonhybrid melanoma. Because all nonhybrid melanoma occurred only in fish that carried a macromelanophore pattern, the association of the genetic loci encoding Pu², Sc, and Sr² used in this study was tested by RFLP analysis. Oncogenic copies of Xmrk can be detected as additional bands in Southern blots hybridized with probes from the 3′ region (kinase domain) of Xmrk (12, 19). For Tu-Sc of X. cortezi from several wild populations, additional fragments of 6.5 kb in HindIII and 9.3 kb in BamHI-digested DNA on Southern blots that were hybridized with the kinase domain probe of Xmrk are diagnostic for the presence of the Xmrk oncogene (19). Also, the strain that develops nonhybrid melanoma originating from the Sc pattern has, on the basis of such analysis, an extra copy of Xmrk (Fig. 5). The association of extra EcoRI bands of 10 or 12 kb for the kinase domain in Southern blots of EcoRI digests of DNA from X. variatus with the Pu² pattern, but lack of any additional band for Pu¹ fish, has been shown. This is also found in animals from the population where the fish with nonhybrid melanoma for this study were derived (Refs. 19 and 28; data not shown).

The Sr² mutation is an allele of Sr. An Xmrk oncogene copy from this locus that is apparent by a 6.5-kb EcoRI band has been cloned and functionally analyzed. Using probes for Southern blot analyses that span the entire transcribed region and approximately 300 bp of upstream regulatory sequences revealed that the Xmrk encoded by Sr² has not acquired gross structural changes (data not shown). Following hybridization to X. helleri, highly malignant melanoma originating from the Sr² pattern occur (37). For the micromelanophore patterns Lc and Ab, neither melanoma formation in hybrids with the corresponding X. maculatus genes nor association of the pattern genes with an oncogenic copy of Xmrk was observed (data not shown). In summary, these data show an association of an additional copy of Xmrk with the nonhybrid melanoma.

Expression of the Xmrk Oncoprotein in Pu² Melanoma

To find out if overexpression of the Xmrk oncogene occurs also in nonhybrid melanoma, tumor extracts were subjected to Western blot analyses using an Xmrk-specific antiserum. This was shown to detect the Xmrk receptor tyrosine kinase in melanoma of hybrids and does
dependency of the Sr" melanoma of X. maculatus suggests that the sex and at higher malignancy than females (48). The strict testosterone Sd hybrid melanoma, it was shown that males develop tumors earlier malignancy or even to induce melanoma formation (46, 47). For the development of melanoma in Xiphophorus hybrids. Depending on the genotype, exogenously applied testosterone is able to increase the development of nonhybrid melanoma.

mechanism that causes higher densities of micromelanophores may higher densities recruit macromelanophores from elsewhere. The compartments which normally harbor the micromelanophores at higher densities have been found in Xiphophorus hybrids that are derived from trans and in the positive control (PSM cell line derived from a platyfish/swordtail hybrid melanoma; lane 5, lane 7). Lane 1 contains a protein extract from the corresponding skin and underlying tissue compartment from a X. variatus specimen without Pu for negative control. The role of testosterone in the etiology of nonhybrid melanoma has to be clarified by further experiments.

Certainly, the fact that the nonhybrid melanoma occurs only very late in life points also to environmental factors (e.g., accumulation of carcinogenic mutations) as etiological agents. This has to be followed up because it would give the Xiphophorus melanoma system additional relevance as a model for cancer in higher vertebrates, including humans. Here, carcinogen-induced tumors are in preponderance, while hereditary tumors, which may be most comparable in etiology to the hybrid melanoma of Xiphophorus, comprise only a smaller fraction of all neoplasms. Also, internal factors like hormonal and neurohormonal imbalances could influence formation of nonhybrid melanoma.

For the development of melanoma in hybrids of Xiphophorus, overexpression of the Xmrk oncogene is the primary event and the crucial step in the causation of this tumor. Our finding of overexpression of the same gene in the nonhybrid melanoma indicates that also the same molecular mechanism may play a role in nonhybrid melanoma.

The classical model for melanoma formation in hybrid Xiphophorus (3) that was deduced mainly from experiments using Mendelian genetics does not explain the spontaneous formation of tumors in the absence of hybridization. However, the molecular biological information on the role of the Tw-encoded Xmrk in the induction of hybrid melanoma can be used to find out what leads to hormone state may be an important etiological factor also in the induction of the other cases of nonhybrid melanoma. This is obvious in the Sc melanomas of X. cortezi, where tumors develop more frequently and progress faster in males than in females. In contrast, thus far there are no experimental data on androgen dependency in the cases of Pu2 melanoma in pure X. variatus. They occur in both sexes with approximately equal frequency. A possible explanation is that, in these fish, melanoma occurs very late in life. Females over the age of 18 months may have higher circulating androgen titters than younger females. Such phenomena are known from the development of male secondary sex characters in senescent females in other poeciliid fish, e.g., swordtails. The role of testosterone in the etiology of nonhybrid melanoma has to be clarified by further experiments.

Not cross-react with the closely related epidermal growth factor receptor (22). Due to its low abundance, the corresponding Xmrk proto-oncogene product cannot be detected in melanoma or normal organs. Using this antiserum, the Xmrk protein was detected at high levels in nodular melanoma of all three Pu2 fish analyzed (Fig. 6). The level of expression is comparable to highly malignant hybrid melanoma and the malignant melanoma-derived PSM cell line (14). This demonstrates that the Xmrk oncogene is overexpressed also in the nonhybrid melanoma.

DISCUSSION

We have shown that malignant melanoma in Xiphophorus is not restricted to certain hybrid genotypes but that tumors of the same histiotype can also arise in nonhybrid fish without further genetic manipulation. The high incidence in certain populations establishes that this is a repeatable phenomenon of considerable significance (see also Ref. 28). The data reported here are in accordance and extend earlier enigmatic reports on pigmentation abnormalities resembling melanoma in X. cortezi with Sc (26) and X. variatus with Pu2 (25, 27).

In the Sr" mutant of X. maculatus, the melanomas originated from the compartments where normally micromelanophores occur at higher densities and form the LC and Ab patterns. Thus far, no melanomas have been found in Xiphophorus hybrids that are derived from transformed micromelanophores. The histological analyses of the nonhybrid melanomas in Sr" fish gave no indication that these are micromelanophore tumors. It seems reasonable to suggest that the compartments which normally harbor the micromelanophores at higher densities recruit macromelanophores from elsewhere. The mechanism that causes higher densities of micromelanophores may also act on the macromelanophores and therefore facilitate the initial development of nonhybrid melanoma.

Androgenic steroids have been found to exert a strong influence on the development of melanoma in Xiphophorus hybrids. Depending on the genotype, exogenously applied testosterone is able to increase the malignancy or even to induce melanoma formation (46, 47). For the Sd hybrid melanoma, it was shown that males develop tumors earlier and at higher malignancy than females (48). The strict testosterone dependency of the Sr" melanoma of X. maculatus suggests that the sex hormone state may be an important etiological factor also in the induction of the other cases of nonhybrid melanoma. This is obvious in the Sc melanomas of X. cortezi, where tumors develop more frequently and progress faster in males than in females. In contrast, thus far there are no experimental data on androgen dependency in the cases of Pu2 melanoma in pure X. variatus. They occur in both sexes with approximately equal frequency. A possible explanation is that, in these fish, melanoma occurs very late in life. Females over the age of 18 months may have higher circulating androgen titters than younger females. Such phenomena are known from the development of male secondary sex characters in senescent females in other poeciliid fish, e.g., swordtails. The role of testosterone in the etiology of nonhybrid melanoma has to be clarified by further experiments.
nonhybrid melanoma. Transcriptional activation of the Xmrk oncogene happens in hybrids if control by the tumor suppressor locus R is obsolete due to crossing conditioned elimination of R. It may be a reasonable explanation for the influence of environmental factors like carcinogens (see above) on the induction of nonhybrid melanoma that these may inactivate a component of the transcriptional control machinery for the Xmrk oncogene. The action of androgens may be explained by a hormone responsiveness of the Xmrk promoter.

Given the frequent existence of extra copies of Xmrk in several Xiphophorus species, the question even may be why nonhybrid melanoma is not more common. The existence of nonhybrid melanoma in natural populations further supports the consideration that the Xmrk oncogene is potentially injurious to fish carrying this duplicated and activated copy of a physiologically important proto-oncogene. This oncogene is maintained over long evolutionary distances, as is evident from the existence of homologous Xmrk oncogenes in distant branches of the phylogenetic tree of the genus Xiphophorus (19, 49). It is, therefore, reasonable to assume that it may also confer a certain advantage to its carrier. Whether such advantage lies in the close association of Xmrk with macromelanophore pigmentation patterns remains a scientific challenge for the future.

ACKNOWLEDGMENTS

We thank Renate Kolb for technical assistance with the Southern blot experiments and Georg Schneider, Hugo Schwind, and Petra Weber for breeding of the fish. Prof. Dr. Thomas Häng (Institute of Immunology, University of Würzburg) kindly helped with the production of MoAb 21-7 and supplied R. B. We thank the Government of the United Mexican States for this generous cooperation.

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*Cancer Res* 1995;55:159-165.