Differences in Transcription and Promoters of Xmrk-1 and Xmrk-2 Genes Suggest a Role for Xmrk-2 in Pigment Pattern Development in the Platyfish, Xiphophorus maculatus

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Abstract
Pigment (macromelanophore) patterns in the platyfish Xiphophorus maculatus are due to a complex pigmentedary locus; for example, the spotted-dorsal (Sd) fin pattern is due to the Sd locus. In interspecific backcross hybrids with the swordtail X. helleri, the Sd pattern changes into benign or malignant dorsal fin melanoma as a result of hemi- or homozygous loss of a platyfish regulatory (R) gene, the tumor suppressor gene Diff, that appears to play a role in the final differentiation of macromelanophores. Closely linked to the pigmentedary locus is an epidermal growth factor receptor-like gene, Xmrk-2, that has arisen by duplication from the linked Xmrk-1. The transcriptional expression of the Xmrk genes was determined in various tissues including Sd pigment patterns and melanomas of various growth potential using reverse transcription-polymerase chain reaction. While Xmrk-1 expression was found in all tissues examined, Xmrk-2 expression correlated with pigment cell growth. Xmrk-2 was highly expressed in the dorsal fin exhibiting the Sd pattern but drastically reduced in a platyfish mutant which has lost the capacity to form these pigment cells in the dorsal fin. Most interestingly, Xmrk-2 expression increased with the proliferative capacity of the melanomas but declined once melanoma growth ceased. We conclude that Xmrk-2 plays a role in the formation of the pigment pattern cell type, perhaps in proliferation of precursor cells, which, in melanoma, are kept in a proliferative state due to loss of Diff. The acquisition of a different promoter by Xmrk-2 during or since its duplication from the ancestral Xmrk-1 is presumably responsible for Xmrk-2 pigment cell specificity. Interestingly, this promoter sequence is found approximately 10 times in the haploid genome.

Introduction
Several species of small tropical fish of the genus Xiphophorus produce pigment patterns composed of black spots or stripes on the fins or flank. These patterns have long interested researchers because, although expression is relatively invariant in a given population or species, drastic changes occur in intra- and interspecific hybrids. These changes range from greatly reduced to greatly enhanced pattern expression and, in certain cases, to melanoma formation. In general, the change in pattern expression depends on the type of pattern and on the foreign gene pool into which the pattern is introduced (for details, see Refs. 1–3).

The platyfish species X. maculatus exhibits a number of patterns which include the Sp, Sb, N, Sd, and Sr patterns. These terms reflect the localization and arrangement of a distinct pigment cell, the macromelanophore, in the adult fish (4). These patterns are coded by a sex-linked, complex macromelanophore or pigmentary locus named according to the pattern, i.e., Sp, Sb, etc. (5). The term tumor gene, Tu, is also found in the literature (6, 7) and is more or less synonymous with the macromelanophore locus. Each pattern is enhanced to a different degree by introgressive hybridization with the swordtail, X. helleri, which does not have the corresponding pigmentedary patterns, and ranges from severe melanoma formation of the Sp pattern to only enhancement of the Sr pattern (8).

The inheritance of the Sd melanoma in backcross hybrids best documents the genetic principle of melanoma formation. The melanoma is not the result of a de novo mutation in a protooncogene but is explained by the Mendelian segregation of the platyfish dominant-acting pigmentary locus and recessive autosomal genes, which in the literature (6, 7) are sometimes referred to as regulatory (R) genes. Inheritance of the Sd locus with hemizygous loss of a particular autosomal R gene results in benign melanomas consisting of moderately differentiated cells of low proliferative capacity, whereas homozygous loss results in malignant melanoma consisting of poorly differentiated cells of high proliferative capacity (9). This observation suggests that this R gene acts as a tumor suppressor gene and has a role in the final differentiation of the macromelanophore. The gene, therefore, has been termed differentiation gene, Diff (10).

Recently, we and others (11, 12) have independently found an epidermal growth factor receptor-like gene which maps to the platyfish pigmentary locus. This gene appears to

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3 The abbreviations used are: Sp, spot-sided; Sb, spotted-belly; N, nigra; Sd, spotted-dorsal; Sr, striped-side; Tu, tumor gene; kb, kilobase(s); RT-PCR, reverse transcription-polymerase chain reaction; bp, base pair(s); cDNA, complementary DNA; RACE, rapid amplification of cDNA ends; RFLP, restriction fragment length polymorphism.

4 Unpublished data.
have arisen by duplication from an ancestral gene, which in the platyfish is also located on the sex chromosomes; the ancestral gene is presumed to be present in all Xiphophorus (13). Based on genomic restriction maps, the complete sequence of the cytoplasmic and partial sequence of the extracellular domain, it appears that the two genes are greater than 97% identical (14). The term Xmrk has sometimes been used for both genes (14), but to avoid confusion and to reflect their evolutionary relationship, we will refer to the ancestral gene as Xmrk-1 and the duplicate gene as Xmrk-2. The Xmrk-1 transcript of the swordtail has been reported to be 5.8 kb and, because of the apparent overexpression of a 4.7-kb transcript in the melanoma of (platyfish/swordtail/swordtail backcross hybrids, the platyfish Xmrk-2 has been interpreted as the melanoma-inducing gene (14). However, it is not clear if this transcriptional overexpression stemmed from the platyfish Xmrk-2 gene alone or from both the platyfish Xmrk-1 and Xmrk-2 genes.

There is also no report that Xmrk-2 is expressed in any other tissue than melanoma. Here we report in greater detail the transcriptional expression of the Xmrk-2 allele that is linked to the platyfish Sd pigmented locus in various tissues including Sd pigment patterns and melanomas of varying growth potential. Because this allele has a small deletion (14), its transcript can be easily distinguished from that of Xmrk-1, whereas the growth of Sd dorsal fin melanoma can be more easily followed than that of melanomas which appear on the flank of the fish. Our studies provide evidence that Xmrk-1 is ubiquitously expressed, but Xmrk-2 expression is connected to pigment cell growth. We also show that the two genes have different promoters, which likely explains their differential expression.

Results
Differential Transcription of Xmrk-2 in Various Tissues. To obtain insight into the function of the Xmrk-2 gene, we performed RT-PCR experiments on total RNA isolated from various tissues including dorsal fins that contained normal Sd pigment pattern cells, the corresponding dorsal fin melanomas of varying growth potential, and dorsal fins that lacked Sd pigment pattern cells due to a mutation in the Sd locus. Experiments were repeated at least three times. We have used RT-PCR instead of Northern blot analysis for several reasons. There is a limited amount of tissue in these small fish, and primers to conserved sequences in the cytoplasmic domain of the ancestral and the duplicate Xmrk gene allow the amplification of transcript sequences of both genes in a single amplification reaction. This permits a semiquantitative evaluation of the transcriptional levels of the two genes because the initial ratio of the targets remains constant throughout the amplification (15). RT-PCR yields a 514-bp fragment for the Xmrk-1 and a 410-bp fragment for the Xmrk-2 mRNA. We have sequenced these fragments (data not shown) and confirmed the mRNA sequence as predicted from the corresponding genomic sequences (16).

Fig. 1a shows comparatively the levels of the Xmrk transcripts in various tissues from a platyfish which is homozygous for the Sd locus and produces a normal Sd pattern. Whereas Xmrk-1 is expressed in all tissues examined, the transcription of Xmrk-2 is tissue specific. The highest level of Xmrk-2 mRNA, exceeding the level of the Xmrk-1 mRNA, is observed in the dorsal fin which contains the Sd pattern. Xmrk-2 is transcribed weakly in both gills and eyes; the lowest expression is found in the caudal fin and in skin lacking Sd pigment pattern cells. In the platyfish Sd mutant that lacks Sd pigment pattern cells, the same pattern of transcriptional expression is observed with the exception of the dorsal fin (data not shown). In this tissue, the expression of Xmrk-2 is drastically reduced as compared to that of Xmrk-1, just the opposite to what is observed in the dorsal fin of the platyfish exhibiting the Sd pattern. Fig. 1, b and c, show the results of RT-PCR from RNA of various tissues from a backcross hybrid carrying a benign (Fig. 1b) and a malignant (Fig. 1c) melanoma. As in the Sd platyfish, Xmrk-1 is expressed in all tissues and in each case at a higher level than Xmrk-2 with the exception of the melanoma. In both the benign and the malignant types, Xmrk-2 is more highly expressed than in the Sd fin of the platyfish, whereas Xmrk-1 in the benign tissue shows some transcription, but in the malignant melanoma tissue, expression of Xmrk-1 can hardly be detected.

The correlation of Xmrk-2 expression with pigment cell formation and melanoma growth becomes more apparent by comparing the results shown in Fig. 1d in which the expression of both genes in a Sd fin is compared with the growth front of a melanoma invading the muscle tissue, a benign melanoma, a malignant melanoma, and fin tissue from a backcross fish which did not inherit the Sd locus, and consequently does not develop Sd pigment cells or a melanoma. Another result which strengthens this correlation is seen in the case of a regressive melanoma; the transcriptional level of Xmrk-2 has declined, and the expression of Xmrk-1 reappeared, reflecting a normalization of the growth of the pigment cells. It is also important to note that the relative level of expression of both genes in the tissues lacking Sd pigment cells appears to be the same in both the melanoma-producing fish and the platyfish parent (Fig. 1a). Because they do not carry Xmrk-2, only Xmrk-1 expression is found in the swordtail parent and in those backcross fish which did not inherit the Sd locus (data not shown).

Determination of the 5' End of the Xmrk-1 and Xmrk-2 Transcripts. To characterize and clone the 5' ends of both the Xmrk-1 and Xmrk-2 transcripts, we performed a 5' RACE using primer mrk9 to generate first strand cDNA. Since, as described above, RT-PCR demonstrated that only the Xmrk-1 gene was transcribed in the liver, total RNA from this tissue (from a homozygous Sd/female) was used as the source of the Xmrk-1 cDNA. Similarly, since the Xmrk-2 gene is almost exclusively transcribed in backcross malignant melanomas, this tissue was used as the source of the Xmrk-2 cDNA. In both cases, subsequent PCR amplification, using the gene-specific primer (mrk8) nested to the mrk9 primer and the 5' anchor primer supplied in the 5' RACE kit, generated a product slightly greater than 300 bp. These fragments were directly sequenced, and five clones of each were also sequenced. This analysis revealed that the longest Xmrk-1 product extended 94 nucleotides, and the longest Xmrk-2 product extended 61 nucleotides upstream of the ATG codon (Fig. 2a, filled arrow). Shorter 5' RACE products were also found but extended within 10 nucleotides of the longest products. We assume that the longest products begin at the transcriptional start sites. Comparison of the nucleotide sequence of the cDNAs of both genes revealed that the Xmrk-1 cDNA had an extra 32-bp sequence (Fig. 2a, bold lettering) extending upstream from the putative start of the Xmrk-2 transcript. The remaining 5' untranslated and coding sequences are nearly identical for both Xmrk gene transcripts. There were changes at eight
nucleotide positions, but this results in only two amino acid differences, at residues 5 and 23, in the putative signal peptide (Fig. 2a).

**Characterization of the 5' Regions: Differences in the Promoter and First Intron of Xmrk-1 and Xmrk-2 Genes.**

The differential expression of the Xmrk-1 and Xmrk-2 genes suggests different transcriptional control elements. To identify the 5' promoter regions and at the same time to verify the 5' cDNA sequences and the putative transcriptional start sites, we screened genomic libraries from *X. maculatus* homozygous for the Sd locus with a 5' cDNA fragment corresponding to nucleotides −45 to +1150 from a Xmrk cDNA clone isolated from a cDNA library of the PSM (platyfish-swordtail melanoma) cell line (17). Several genomic clones were isolated, and restriction mapping revealed that only F506 extended into the 5' direction. This clone, however, did not sequence with primer mk10, which covers nucleotides −29 to −10 in the 5' untranslated region of the transcripts of both genes, suggesting that this clone did not contain the 5' end of either gene and that the 10 kb which mapped upstream from the hybridizing fragment represented intron sequences. Rescreening of the libraries with the 500-bp Sst/HindIII fragment from the 5' end of the F506 insert, resulted in the isolation of a total of 14 clones. Two clones that appeared to extend farthest into the 5' regions of either gene were further analyzed. Clone

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**Fig. 1.** Competitive RT-PCR analyses using the same template primers for the Xmrk-1 (upper) and Xmrk-2 (lower) transcripts allowing a comparison between the transcriptional level of both genes. Xmrk expression in various tissues from (a) an *X. maculatus* Sd homozygous female; (b and c) an *X. maculatus* X. helena backcross hybrids carrying a benign (b) and a malignant (c) Sd melanoma; and (d) in fins with and without Sd pigment cells compared to expression in a malignant, regressive and invading Sd melanoma.
JAS2 was found to contain the presumptive first exon of Xmrk-2 because it contained a sequence identical to the nucleotide sequence obtained in the 5' RACE of the melanoma-derived Xmrk-2 cDNA, corresponding to positions -61 to +90, whereas clone DF6 contained the presumptive first exon of Xmrk-1 because it contained a sequence identical to the nucleotide sequence of the liver Xmrk-1 cDNA from position -94 to +90 (Fig. 2a). These data verify the 5' RACE-derived sequences for both genes. Further sequence analysis into the 5' ends of both genes and comparisons of both nucleotide sequences revealed extensive differences between the 5' regions of the Xmrk-1 and Xmrk-2 genes (Fig. 2a). The putative promoter of the Xmrk-2 gene contains a TATA-box at position -91 and a CAAT-like element at -143. In addition, a CCGCCC sequence, which may represent a potential Sp1 transcription factor binding site, is found 16 nucleotides upstream from the TATA-box in a position that exactly corresponds to one found in the c-myc promoter (18). The location of these elements suggests that the Xmrk-2 cDNA indeed begins at or very near the authentic transcriptional start site. It should be noted that the 32 nucleotides of the Xmrk-1 transcript not found in the Xmrk-2 transcript are also not found in the Xmrk-2 genomic DNA. The genomic sequence for the Xmrk-1 shows that the putative promoter of this gene does not share any similarity to the promoter of the Xmrk-2. It lacks a TATA-box, CAAT-
box, and a CCGCCC sequence. However, a sequence GGAGGAGGA is repeated three times within a GGA motif at positions -210 to -151. Similar GGA repeats are found in the promoters of the human EGFRI (19, 20) and rat neu genes (21), and a complementary sequence (TCCTCGCTCC) is an essential component of the upstream enhancer of the human EGFRI promoter (22, 23).

The comparison of the genomic to the 5’ RACE sequences of Xmrk-1 and Xmrk-2 revealed that the first exons extend to position +90. At this point, genomic DNAs of both genes contain a consensus sequence for a 5’ splice site. Sequence analysis of the genomic clone F506, which contained 10 kb of putative intron sequence, towards the 5’ end of the gene with primer mrk8 (nucleotides 196 to 177) revealed that this clone contained a consensus sequence for a 3’ splice site, followed by a sequence identical to that from -91 to at least +180 of the Xmrk-2 cDNA. Alignment of the restriction map of this clone with that of clone JA52 indicated that exons 1 and 2 of the Xmrk-2 gene are separated by an intron of approximately 1.2 kb. The comparison of these clones with the Xmrk-1 DF6 clone indicates that the first intron of the Xmrk-1 gene differs in structure from that of Xmrk-2. The 500 bp SstI/HindIII fragment from the first intron of the Xmrk-2 gene maps 1.4 kb downstream of the first exon of Xmrk-2, but this fragment maps 6.5 kb downstream from the first exon of Xmrk-1 (Fig. 2b). Since the hybridization pattern of the cloned fragments is consistent with Southern blot analyses of genomic DNA, this difference is not due to a cloning artifact but suggests that the Xmrk-2 gene has acquired a deletion or some other rearrangement of the first intron at the time of or since its duplication from Xmrk-1.

The Xmrk-2 Promoter Is Repeated in the Genome of Platfish and Swordtails. During the process of verification of the differences in the 5’ flanking regions found in both Xmrk genes, we detected, on Southern blots from EcoRI-digested platfish DNA probed with a fragment covering nucleotides -338 to +34 of Xmrk-2 (probe PE-1, promoter and part of exon 1; Fig. 2a), not only the expected 5.3 kb EcoRI fragment from Xmrk-2, but weaker signals at 6.5 and 4.3 kb and a very strong hybridization signal at 2.0 kb. Densitometer tracing revealed that the latter fragment is repeated approximately 10 times in the haploid genome of the platfish Jp163A strain. We also detected a strong hybridization signal at 2.0 kb on Southern blots with EcoRI-digested DNA from the swordtail, suggesting that these sequences are also present at about the same copy number in this species.

To identify the basis for the hybridization to this repeated sequence, we obtained several clones that contained the 2.0-kb sequence from the platfish. Two clones were partially sequenced and found to be nearly identical to each other and contained a sequence virtually identical to a sequence of the Xmrk-2 gene from -339 of the promoter to the transcriptional start site at -60. There was no sequence similarity to the Xmrk genes 3’ of this site. It is likely significant that this site is also the site of divergence of the Xmrk-1 and Xmrk-2 genes; they share no similarity upstream of this site, i.e., they have different promoters, but are nearly identical in coding sequence downstream from this site. Thus, the Xmrk-2 gene appears to be a chimera between the repeated sequence and Xmrk-1 and has most likely originated by nonhomologous recombination between a copy of the repeated sequence and the 5’ untranslated region of Xmrk-1. This suggests that a repeat sequence may also be linked to the Xmrk genes and the pigmentary locus on the sex chromosome. Therefore, we searched for RFLPs between the platfish and swordtail that could be used for linkage analysis. While we could find no RFLPs that could be used to determine if a single copy of the repeat sequence is sex-linked in the platfish, we found PvuII RFLPs that could be used to determine if the repeated sequence is inherited as a linkage group. In the platfish, fragments hybridizing intensely to probe PE-1 were seen at approximately 2.8 and 1.6 kb (Fig. 3, Lane 1), whereas in the swordtail there were many more fragments, indicating that the repeat sequence is much more polymorphic in this species, and, although there are fragments near to 2.8 and 1.6 kb, they are not hybridizing strongly as in the platfish (Fig. 3, Lane 2). Therefore, we examined by Southern blot analysis the segregation of the platfish PvuII fragments in (platfish/swordtail/swordtail backcross hybrids that segregate 1:1 into fish which do (Fig. 3, Lanes 3–5) and do not (Fig. 3, Lanes 6–8) inherit the platfish X chromosome carrying the Sd locus, Xmrk-1, and Xmrk-2 genes. It can be seen that the platfish 2.8 and 1.6-kb fragments appear in both segregation groups, showing that the repeated sequence is not inherited as a single linkage group on the X chromosome. Furthermore, the relative hybridization intensity of these fragments is often much reduced and is quite variable among the backcross animals. This distribution and variability suggest that the repeated sequences are distributed on several autosomes. However, since the platfish and swordtail are only polymorphic for the signal strength of these fragments, it cannot be excluded that one or a few copies may be located on the sex chromosomes of the platfish.

Discussion
The macromelanophore (pigmentary) locus of platfish codes for population- and species-specific macromelano-
phore (pigment cell) patterns. Introduction of this locus into a foreign gene pool by intra- or interspecific crossing can result in modification of the pattern phenotype, at its ex- tremest leading to melanoma. The Xmrk-2 gene, a member of the EGFR gene family, maps to the pigmentedary locus and is believed to have originated by duplication from a linked ancestral gene, Xmrk-1. Since all platyfish which produce the sex-linked pigment patterns carry Xmrk-2 (13), 4 this gene may have a function in the formation of the macromelanophore and/or its pattern. Although some evidence for relatively high transcription of Xmrk-2 in melanomas has been provided (14), greater insight into the function of this gene might be obtained by a comparison of Xmrk-2 expression in the pigment cell patterns of normal platyfish to that in various melanomas in hybrids as well as in normal tissues of both the platyfish and the hybrids.

To examine the transcription of the Xmrk-2 gene and to distinguish it from the transcription of the Xmrk-1 gene, we used genotypes that carried a Xmrk-2 allele with a small deletion in the coding sequence of the cytoplasmic domain compared to Xmrk-1. Using conserved primers in RT-PCR covering this region, we could not only readily distinguish the transcripts of both genes but also determine their relative levels of transcription in a variety of tissues of normal and tumor-bearing fish. This analysis showed that Xmrk-1 is ubiquitously expressed, but the expression of Xmrk-2, at least in part, can be correlated with the presence of macromelanophores. In the normal platyfish, Xmrk-2 is expressed in several, but not all, tissues and, most significantly, it is expressed at its highest level in the dorsal fin exhibiting the 5d macromelanophore pattern. Expression of Xmrk-2 is very low in the dorsal fin of a 5d mutant that lacks macromelanophores. This data, together with the observation that another mutant that lacks these cells has an inser- tional nonsense mutation in Xmrk-2 (14), provides strong evidence that this gene plays a significant role in the normal development of these pigment cells. This is further supported by the very high expression of Xmrk-2 in the melano- nomas of the backcross hybrids. This elevated expression is limited to the melanomas; in the other tissues, Xmrk-2 expression is nearly at the same level as in the normal platyfish. The observation that fast-growing malignant mel- anomas have a high level, while the melanomas which ceased proliferative growth have a lower level of Xmrk-2 expression, may provide a clue as to the function of the gene. We have shown previously (3, 9, 10) that the prolif- erative capacity of melanomas correlates with the number of less differentiated precursor cells. Melanomas that grow slowly or stop proliferating contain more differentiated cells and few, if any, premature cells. Thus, there is a relationship between proliferative capacity of the melanoma paralleled by the degree of content of immature cells and high expres- sion of Xmrk-2. We suggest the gene might function to support proliferation of immature pigment cells.

In this context, it is worthwhile to recall that two types of melanophores are found in the platyfish. The smaller mi- cromelanophores, which are present in all members of the genus Xiphophorus, are not coded by the sex-linked pig- mentary locus and are arranged evenly in the skin (8). They differentiate from their neural crest-derived precursors shortly after those cells have migrated into the skin of the embryo. In contrast, the much larger macromelanophores that compose the patterns are also derived from neural crest cells but differentiate much later, typically not until well after birth (3, 4). Since the spots of the patterns are com- posed of dense clusters of macromelanophores, it is likely that a proliferation of precursor cells precedes the differen- tiation of the macromelanophores. A signal for proliferation of this cell type could be provided by a growth factor/growth factor receptor interaction. We suggest that the tem- poral and spatial expression of the Xmrk-2 gene product and its as yet unidentified ligand may interact to determine the macromelanophore patterns.

Based on this hypothesis, mutations in Xmrk-2 may im- pair proliferation and thus lead to the absence of macromelanophore patterns. As previously mentioned, it has been observed (14) that a mutant lacking macromelanopho- nes had a mutated Xmrk-2 gene. While Southern blot analysis of genomic DNA using probes covering the Xmrk-2 gene has not revealed any gross structural changes of Xmrk-2 in our null mutant, more subtle mutations may have occurred. The very low expression of Xmrk-2 in the dorsal fin could be due to mutations in the promoter sequences. We have sequenced the promoter and exon 1 but did not find any changes from the wild-type. However, because the levels of Xmrk-2 transcription in other tissues such as gills and eyes is not diminished, the low level of transcription in the dorsal fin could be due to a mutation in a tissue- or cell type-specific transcriptional control element; therefore, changes in additional upstream or down- stream regions cannot be excluded. Alternatively, pigment cell precursors that express Xmrk-2 may fail to proliferate due to a mutation in the Xmrk-2 gene that inactivates the Xmrk-2 product. Finally, it is also possible that a tightly linked gene that controls either Xmrk-2 activity or the num- ber of pigment cell precursors has been mutated. In this regard, it has previously been proposed that the pigmentedary locus may encompass several genes, one required for the determination of the macromelanophore cell type and one controlling the localization of the cells, i.e., whether the cells are formed in the dorsal fin or at certain sites on the flank of the fish, and still another controlling melanoma susceptibility in the hybrids (2, 3). Further study of the Xmrk-2 and flanking DNA sequences in this mutant is required.

The Xmrk-1 and Xmrk-2 genes are differentially ex- pressed; after duplication, the two genes must have di- verged in the sequence controlling developmental expres- sion. Indeed, the Xmrk-2 gene does not have the same 5' flanking sequences as Xmrk-1. The Xmrk-2 promoter con- tains typical motifs of tissue-specific expressed genes, such as the TATA- and CAAT-box, while these are not found in the 5' region of Xmrk-1. The putative Xmrk-1 promoter, however, contains a GGA repeat similar to that found in some other EGFR family genes (19–23). We have also observed differences in the first introns of the two genes, which raises the possibility that additional differences could affect transcription of these genes. For example, enhancers have been found in the first intron in certain other genes including EGFR (24, 25). Whether such sequences exist in the intron of either gene still needs to be determined.

While we have shown that the Xmrk-1 and Xmrk-2 genes have different promoters, we also have shown that the 5' ends of the transcripts of these two genes are essentially identical. However, the Xmrk-2 transcript is shorter by 32 nucleotides that are found at the start of the Xmrk-1 trans- script. This sequence is also not found in the Xmrk-2 genomic DNA. Since the cDNAs are consistent with the genomic clones, we believe that the differences between
the 5' regions of the Xmrk-1 and Xmrk-2 genes in X. maculatus are genuine.

Recently, a sequence covering the promoter and extending into the second exon of an allele of the X. maculatus Xmrk-2 gene linked to the Sr locus has been published (26). Although the position of the first intron was not indicated, the sequence does not differ significantly from the sequence we report for the Sd-linked Xmrk-2 gene. The few nucleotide differences are probably not significant and may represent allelic variation. The authors also published a sequence of the 5' end of a Xmrk-1 cDNA (26) cloned from a cell line of a different species, X. xiphidium. Interestingly, the sequence that covers the 5' untranslated region and the signal peptide is different from the sequence we determined for both the Xmrk genes in X. maculatus. Presuming that Xmrk-1 is identical in both species, the authors concluded that the Xmrk-2 gene of X. maculatus must contain a unique sequence. However, they unexpectedly found that this sequence could be amplified by PCR, even from DNA of platyfish and swordtails that carry only Xmrk-1. Our data can easily explain this finding. This “unique” Xmrk-2 sequence is in fact found in both the Xmrk-1 and Xmrk-2 genes in X. maculatus. Furthermore, using RT-PCR to amplify the 5' end of Xmrk-1 from swordtail mRNA, we were able to show that the swordtail Xmrk-1 sequence is nearly identical to Xmrk-1 and Xmrk-2 of the platyfish X. maculatus. Thus, the primers used in the PCR experiments of Adam et al. (26) would amplify Xmrk-1 in both species. Whether their Xmrk-1 sequence of X. xiphidium is species specific or may represent an artifact of the cell line will need to be clarified.

Adam et al. (26) had postulated that Xmrk-2 was created by nonhomologous recombination of the ancestral Xmrk and another locus but also proposed a second recombination event with yet another locus to account for the 5' sequence they believed to be unique to Xmrk-2. As we have already shown, this sequence is also found in Xmrk-1, so only a single, nonhomologous recombination between the 5' untranslated region of the ancestral Xmrk gene and a promoter of a second gene is required to account for the creation of the Xmrk-2 gene. We have also determined that a DNA sequence that contains sequence nearly identical to the Xmrk-2 promoter is present in about 10 copies in the Xiphophorus haploid genome. Linkage analysis shows that copies of this sequence are distributed on several autosomes, although we were unable to exclude linkage of one or a few copies to the pigmentation locus on the X chromosome. Preliminary evidence indicates that this DNA contains a transcription unit because we have detected transcripts of about 3.8 and 2 kb using this DNA to probe Northern blots of melanophores and epithelial cell lines. The significance of this transcription and the multiple copies of this gene has for pigment pattern and melanophore formation is not clear. However, the level of transcription does not seem to be elevated in melanophores. Therefore, it appears that the Xmrk-2 gene must have some different transcriptional control elements that impart greater tissue specificity.

In summary, the Xmrk-2 gene represents a duplicated version of its ancestral gene, Xmrk-1. Gene duplications are important in evolution because the function of the original gene can be maintained while the redundant genetic material can undergo changes and assume new functions, leading to new phenotypic characteristics. Indeed, the Xmrk-2 gene differs from the ancestral gene by its promoter, and there are also differences in the first intron; whether there are further differences will have to be elucidated. The different promoter likely contributes to the tissue-limited transcription, which appears to be associated with macromelanophore development. Therefore, the Xmrk duplication may be one factor in the evolution of pigment patterns in the platyfish, X. maculatus. However, if this Xmrk-2 gene is introduced into a foreign gene pool, especially one that contains only the ancestral gene, one could expect improper regulation of Xmrk-2. This may explain the modification of pigment patterns that occurs in hybrids of which the melanoma formation in platyfish-swordtail backcross hybrids is just one example. In the specific case of the Sd melanoma, the loss of the tumor suppressor gene Diff may be involved in the deregulation of the Xmrk-2 gene. We know very little about the molecular function of Diff, but it appears to have a role in the final differentiation of the macromelanophore. Perhaps it acts directly as a negative regulator of Xmrk-2 transcription to slow cell proliferation and promote differentiation or to induce other genes which have this effect.

Materials and Methods
Fish. Female platyfish, X. maculatus, of the strain Jp163A, which are homozygous for the Sd pigmented locus and the linked Xmrk-2 allele recognized by a 5.0 kb EcoRI RFLP (14), were used for obtaining various tissues and for breeding with the swordtail X. helleri(−/−/−; strain III originated from Genetics Institute, Giessen, Germany) to generate F1 hybrids. Tissues were also obtained from swordtails; the same tissues and melanomas were taken from backcross hybrids; which result from a cross between an F1 hybrid and swordtail and which segregate 1:1 into melanoma-carrying (Sd,Xmrk-2/−/−) and melanoma-free (−/−/−) fish (3). In addition, a platyfish was used which carried a mutation that mapped to the Sd pigmented locus and resulted in loss of the Sd pigment cells.

DNA Isolation and Southern Blot Analysis. DNA from gills of individual platyfish, swordtails and backcross hybrids was extracted as described (27). Plasmid and phage DNA extractions and Southern blot anlyses were done according to standard methods.

RNA Isolation. Total cellular RNA was extracted from brain, liver, eye, gills, skin, dorsal fins, dorsal fin melanoma, and melanoma-invaded muscle tissue of individual fish by a modification of the guanidine isothiocyanate-acid phenol method (28) using a kit from BIO 101. The instructions of the manufacturer were followed except that tissue samples were sheared through an 18 gauge needle in lysis buffer that contained 90 μl of β-mercaptoethanol/50 ml, and centrifugation of the lysate-phenol-chloroform mixture was done at 12,700 × g for 30 min.

RT-PCR. First strand cDNA synthesis was performed on approximately 500 ng of total RNA with 200 units Superscript reverse transcriptase (Gibco BRL), 100 pmol random hexanucleotides, 0.1 unit human placental RNase-inhibitor, 500 μM each deoxynucleoside triphosphate, 50 mM KCl, 20 mM Tris-Cl (pH 8.3), and 2.5 mM MgCl2, incubated at 45°C for 60 min and then heated to 95°C for 10 min. One-tenth of the cDNA mixture was used for PCR amplification with 25 μM each of the mraA and mrkB primer, 200 μM each deoxynucleoside triphosphate, 50 mM KCl, 20 mM Tris-Cl (pH 8.3), 2 mM MgCl2, 2.5 μg/ml bovine serum albumin, and 0.3 unit Taq DNA polymerase/10 μl reaction. The reaction mixture was placed in glass capillary tubes
(Idaho Technology), heated to 94°C for 20 s, and then cycled 40 times through denaturation for less than 1 s at 94°C, annealing for less than 1 s at 60°C, extension for 60 s at 72°C, followed by a final extension for 5 min at 72°C (1605 Air Thermo-Cycler; Idaho Technology). Five μl of the amplification products were fractionated by electrophoresis on a 1.6% agarose gel and visualized after staining with ethidium bromide and UV transillumination.

5’ RACE. To obtain cDNAs of the 5’ end of Xmrk-1 and Xmrk-2 transcripts, 5’ RACE was done using a kit from Gibco BRL according to the manufacturer’s protocol. Single-stranded cDNA was synthesized from total RNA from the liver of a X. maculatus (5d, Xmrk-2/5d, Xmrk-2) or from a malignant melanoma from a first backcross hybrid (5d, Xmrk-2/-) with primer mrk9. After tailing with dCTP, the cDNA was amplified by PCR using the 5’ anchor primer supplied in the kit and a nested primer, mrk8. PCR was done as described above except that the annealing temperature was 55°C.

One-tenth of the amplified samples was separated in a 1.6% agarose gel and stained with ethidium bromide; gel slices containing fragments of approximately 300 bp were excised and crushed in 100 μl 10 mM Tris-HCl (pH 8.0)-0.1 mM EDTA. One μl of these solutions was used as template in a second PCR amplification using the 5’ RACE adapter primer and primer mrk8. The products of these amplifications were used for direct cycle sequencing and also directionally cloned into pKS+ (Stratagene) after digestion with Sall and PsiI.

DNA Sequencing. DNA sequencing was done using the double-stranded DNA cycle sequencing system from Gibco BRL according to their protocol. 5’ RACE products were directly sequenced using primers mrk10, mrk8, and mrkd. Cloned 5’ RACE products and genomic clones were sequenced with primers mrk10, mrk11, and vector-specific M13 forward and reverse primers.

Isolation of 5'-specific Xmrk-1 and Xmrk-2 Genomic Clones. Genomic clones containing the 5’ end of the Xmrk genes were isolated from X. maculatus (strain 163A; 5d, Xmrk-2/5d, Xmrk-2) genomic λ FIX/DASH (Stratagene) libraries and a Agt10 sublibrary by screening with a 32P-oligobaloeled 500-bp Set1/HindIII fragment contained in the first intron of the Xmrk-2 gene.

Primers. The mrk-specific primers (including their length and corresponding nucleotide position in Xmrk cDNA) used for PCR, 5’ RACE, and sequencing were as follows:

- mkA 5'-GCTGAGCTCGATGACGCAGC-3' (20-mer, 2985 to 3003)
- mkB 5'-GGACAGAATGATCAGTCCGC-3' (20-mer, complement of 3498 to 3479)
- mk8 5'-GTGATCCAGTCTTCCCAG-3' (20-mer, complement of 196 to 177)
- mk9 5'-TCGGTACGATGACGATCAC-3' (21-mer, complement of 277 to 257)
- mk10 5'-GGACGACGTTTCTAAACCAGC-3' (20-mer, -29 to -10)
- mk11 5'-TCCCGGTAGAAACACTAGC-3' (19-mer, complement of -26 to -7)
- mkd 5'-GTACCATCCTCCTATTG-3' (19-mer, complement of 158 to 140)

In this paper, nucleotide positions have been numbered where 0 corresponds to the A of the initiator methionine codon and residues preceding it are represented by negative numbers. For primers mrk10, mk11, and mkd see Fig. 2a; for primers mrkA, mkkB, mkrB, and mkr9 refer to the published Xmrk cDNA sequence (14) form the PSM cell line (17), but note the different numbering system. Also note that we have found the nucleotide triplet GGC at position 1552-1554 (encoding a glycine at residue 490 of the mature putative Xmrk protein) which is missing from the published Xmrk sequence.

References


