Melanoma Loss-of-Function Mutants in Xiphophorus Caused by X*mrk*-Oncogene Deletion and Gene Disruption by a Transposable Element

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ABSTRACT

The overexpression of the X*mrk* oncogene (ONC-X*mrk*) in pigment cells of certain Xiphophorus hybrids has been found to be the primary change that results in the formation of malignant melanoma. Spontaneous mutant stocks have been isolated that have lost the ability to induce tumor formation when crossed with *Xiphophorus helleri*. Two of these loss-of-function mutants were analyzed for genetic defects in ONC-*Xmrk*'s. In the lof-1 mutant a novel transposable element, *TX-1*, has jumped into ONC-X*mrk*, leading to a disruption of the gene and a truncated protein product lacking the carboxyterminal domain of the receptor tyrosine kinase. *TX-1* is obviously an active LTR-containing retrotransposon in Xiphophorus that was not found in other fish species outside the family Poeciliidae. Surprisingly, it does not encode any protein, suggesting the existence of a helper function for this retroelement. In the lof-2 mutant the entire ONC-X*mrk* gene was found to be deleted. These data show that ONC-X*mrk* is indeed the tumor-inducing gene of Xiphophorus and thus the critical constituent of the *tumor* (*Tu*) locus.

MANY individuals of the teleost fish genus Xiphophorus harbor in their genome a tumor locus, *Tu* (Anders 1991), which contains an oncogenic version of the growth factor receptor tyrosine kinase (RTK) gene X*mrk* (Wittbrodt *et al.* 1989). Generally, *Tu* does not exert its transforming function, because its activity is suppressed by another unlinked locus, *R* (Anders 1991). *R* appears to be involved in the transcriptional control of X*mrk*, as overexpression of the RTK gene leads to unrestrained cell proliferation *in vitro* and tumor formation in transgenic animals (Wittbrodt *et al.* 1992; Winkler *et al.* 1994). Earlier concepts, however, had proposed that *R* acts via modification of pigment cell differentiation (see Anders 1991). The X*mrk* oncogene (termed ONC-X*mrk*) is always associated with a locus that determines a specific lineage of melanin-containing pigment cells, the macromelanophores (Weis and Schartl 1998). This locus is referred to as the macromelanophore locus and is designated *Mdl* for macromelanophore determining locus (Weis and Schartl 1998) or *MACR* (Morizot *et al.* 1998). Many different versions of *Mdl* exist, each of which specifies a certain black pigment pattern. *Mdl*-encoded pigmentation is a complex phenomenon, and the resulting pattern phenotype is modulated by a barely understood polygenic system of modifiers. In the platyfish, at least five different "pseudoalleles" of the macromelanophore locus are known. *Mdl* was proposed to consist of a number of closely linked genes, each of which can exist in several allelic states in the various populations from different river systems (see Kallman 1975).

The macromelanophore is also the cell type that gives rise to malignant melanoma. Spontaneous melanoma formation has been observed in very rare instances in older fish where obviously transcriptional repression of ONC-X*mrk* becomes leaky (Schartl *et al.* 1995a). In the laboratory, melanoma can be produced at will by eliminating the *R*-mediated suppression through substitution of *R*-containing chromosomes (*e.g.*, from *Xiphophorus maculatus*) by the *R*-free homologous chromosomes from other species (*e.g.*, *X. helleri*) in introgressive breeding experiments. Genetic mapping and linkage analysis revealed that the Xiphophorus homologue of the tumor suppressor gene *CDKN2* is a candidate gene encoded by the *R* locus (Nairn *et al.* 1996; Kazianis *et al.* 1998). There is, however, another genetic model that is also compatible with the data from the classical crossing experiments, namely that *Tu* activity is due to the presence of intensifying genes contributed by *X. helleri* chromosomes to the hybrid genome (see Schartl and Wellbrock 1998).

The X*mrk* oncogene probably arose from its corresponding proto-oncogene (termed INV-X*mrk*) through a nonhomologous recombination event. This led to a gene duplication of X*mrk*. During this process the duplicated copy was fused in its 5′ region to another sequence and thereby became ONC-X*mrk*. The gene duplication/recombination event generated a new promoter, which

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is responsible for the transcriptional activation of the additional X*mrk* copy in the macromelanophore lineage of the hybrids (Adam *et al.* 1993).

In natural populations of Xiphophorus many fish do not contain ONC-X*mrk*. Thus in wild fish the oncogene is an additional copy and in evolutionary terms can be regarded as a dispensable gene (Ohno 1985). All populations in which alleles of this oncogene have been found so far are polymorphic and often the majority of fish are oncogene free. It has been proposed that the gene has been frequently lost during evolution (Weis and Schartl 1998). We describe two cases, namely a deletion and an insertional disruption by a transposable element of ONC-X*mrk*. In both cases the oncogenic function of the gene is impaired. This provides functional evidence that ONC-X*mrk* is responsible for melanoma formation.

MATERIALS AND METHODS

Fish: All fish were bred and maintained in the aquarium facilities of the Biocenter under standard conditions (Kallman 1975).

The following strains were used.

1. *X. maculatus*, Rio Jamapa, Sd. This stock was derived from the Jp163A strain (*ca.* the 1960s) and is kept as a closed colony, randomly inbred. Females of this stock are homozygous for the X-chromosomal loci Mdl^{Sd}-ONC-X*mrk*.
2. *X. helleri*, Rio Lancetilla, *Db-2*$^{-}$. These fish have neither ONC-X*mrk* nor *Mdl* (Weis and Schartl 1998). They are used for introgressive breeding to separate the Mdl^{Sd}-ONC-X*mrk* from *R.* This stock is also known as the hIII strain.
3. Backcross hybrids of *X. maculatus* (see 1) with *X. helleri* (see 2) using *X. helleri* as the recurrent parent (third backcross generation). The fish carried the wild-type X chromosome of *X. maculatus* with Mdl^{Sd}-ONC-X*mrk* and develop, depending on the presence or absence of *R*, benign or malignant melanoma. They are known as Sd-melanoma fish from the classical Gordon-Kosswig cross (*e.g.*, see Anders 1991; Kazianis *et al.* 1998).
4. Lof-1, backcross hybrids carrying the Sd loss-of-function X chromosome, derived from the wild-type Mdl^{Sd}-ONC-X*mrk* chromosome, in the genetic background of *X. helleri.* Lof-1 fish are hemizygous for the *X. maculatus* sex chromosome. The sex-determining locus on the *X. maculatus* X chromosome overrides the sex-determining mechanism of *X. helleri.* Therefore, the majority of fish with the lof-1 chromosome like the corresponding backcross hybrids with the wild-type Mdl^{Sd}-ONC-X*mrk* X chromosome are females (lof-1, 99%; wt, 90%). The stock is maintained by crossing lof-1 females with purebred *X. helleri* males from the Rio Lancetilla stock (see 2). Lof-1 is a spontaneous mutant that was isolated by A. Anders, Giessen, in the early eighties. This strain is also known as the ArDr strain.
5. Lof-2 is another spontaneous loss-of-function mutant from the Sd locus that occurred in our laboratory in a backcross brood of Sd melanoma fish with *X. helleri* (see 2) as the recurrent parent. Lof-1 and lof-2 are maintained in the same genetic background.
6. Lof-3 is also a spontaneous loss-of-function mutant of the Mdl^{Sd}-ONC-X*mrk* X chromosome, which was isolated by A. and F. Anders. The phenotype in backcross hybrids is identical with that of lof-1 and lof-2. Nothing is known about the molecular basis of the mutation. The mutant chromosome was reintroduced in the genetic background of *X. maculatus* from Rio Jamapa. This platyfish strain has been designated the Dr strain (Anders *et al.* 1973) or Δ-Sd strain (Woolcock *et al.* 1994).
7. For analysis of the phylogenetic distribution of the newly identified transposable element, *TX-1*, DNA from the following fish kept as randomly inbred lines at the Biocenter Würzburg was used (strain designation or geographic origin in parentheses): *X. maculatus* (Rio Jamapa, see 1, Rio Usumacinta), *X. milleri* (Laguna Catemaco), *X. helleri* (Rio Lancetilla, see 2), *X. couchianus* (Apodaca), *X. meyeri* (Musquiz), *X. andersi* (Rio Atoyac), *X. nezahualcoyotl* (Rio El Salto), *X. montezumae* (Cascadas de Tamasopo), *X. variatus* (Ciudad Mante), *X. cortezi* (Rio Axtla), *X. malinche* (Rio Calnali), *Gambusia affinis affinis* (Pena Blanca, Santa Cruz River system, north of Nogales), *Poeciliopsis gracilis* (Rio Jamapa), *Heterandria bimaculata* (Rio Tonto system), *H. formosa* (Fort Lauderdale), *Phallichthyes amates* (aquarium stock), *Poecilia mexicana* (Media Luna), *P. latipinna* (Key Largo), *P. formosa* (Tampico), *Girardinus metallicus* (aquarium stock), and *G. falcatus* (aquarium stock). All these species belong to the family Poeciliidae. As representatives of other families *Fundulus* spp. (Laguna de Labradores), *Oryzias latipes* (Medaka strains HB32C and Carbio, see Hong *et al.* 1998), and *Danio rerio* (zebrafish strain m14) were used. Genomic DNA from Nile Tilapia (*Oreochromis niloticus*) and from Chinese perch (*Siniperca chuatsi*) were a gift from S. Chen (University of Würzburg). Genomic DNA from salmon (*Salmo salar*) was obtained from C. Cunningham (Sars Center for Molecular Marine Biology, Bergen). DNA and tissues from *Battrachocottus baikalensis* (Baikal lake) were kindly provided by S. Kirilchik and M. Grachev (Institute of Limnology, Irkutsk, Russia). Rainbow trout (*Oncorhynchus mykiss*), pike (*Esox lucius*), common carp (*Cyprinus carpio*), european eel (*Anguilla anguilla*), and sturgeon (*Acipenser sturio*) were obtained from a local fish farm.

Southern blot, PCR, and RNA expression analysis: DNA from individual fish was isolated as described (Schartl *et al.* 1995b). RNA was extracted from pooled organs of several fish using the TRIZOL reagent (GIBCO-BRL, Grand Island, NY) according to the supplier's recommendations. Reverse transcription was done with 2 μg total RNA using Superscript II reverse transcriptase (GIBCO-BRL) and random primers (GIBCO-BRL). cDNA from 10 ng (actin) to 300 ng of total RNA was used for PCR. We used 50 pmol of the following gene-specific primers: for X*mrk* Ins4 5′ GCC TCC TGG GAG GAC AGC GAC 3′ and Ins5 5′ AGC GAG CCC TGC ATC CCG CCG 3′ at an annealing temperature of 72°, Ana1 5′ CCC GTC CAG CAG ACC CAG GTT CA 3′ in combination with Ins4 at an annealing temperature of 72°, Lof1A 5′ CCG TTA GAT GGA GAG CTG GTA GC 3′ in combination with Ana1 at an annealing temperature of 72°, and for *actin* Act1 5′ GTA GGT GAT GAA GCC CAG AGC 3′ and Act2 5′ AGG GAG CTC GTA GCT CTT CTC 3′ at an annealing temperature of 66°. PCR bands were quantified using the software from the Easy plus Rev3.16 image analysis system (Herolab).

For Southern analysis 4.5 μg of genomic DNA was digested with restriction enzymes, separated on a 0.8% agarose gel and blotted onto nylon membrane (Hybond N+, Amersham Buchler, Braunschweig, Germany). For analysis of the ONC-X*mrk* locus of the lof-2 mutant, filters were hybridized with several different probes: a 0.7-kb *Bam*HI fragment (probe II) and a 5.3-kb *Eco*RI fragment of the first intron (probe III), the total X*mrk* cDNA (probe I), a PCR product amplified with the primers Ins2/Hg80 (Ins2 5′ AGG GAA TGA ACT ACC

TGG AAG AGC 3′, Hg80 5′ AGG GTG AAG GCA GGT AGG TG 3′) specific for the X*mrk* kinase domain (probe IV), a PCR product amplified with the primers Sw2/Sw1 (Sw2 5′ GCT GAC GGG ATG AAC GC 3′, Sw1 5′ TGA GAA TCC AGT TTC AAC C 3′), specific for the X*mrk* carboxyterminal domain (probe V), and the 3′-end probe of the cosmid 008-G01 (probe VI) containing *X. maculatus* sequences 3′ of the X*mrk* coding region (J.-N. Volff, K. Sweeney, K. Wichert, S. Weis and M. Schartl, unpublished results). For species distribution of the transposable element *TX-1*, the 1.5- and 1.3-kb *Bam*HI fragments from p338 were used.

Hybridization probes were radiolabeled by random priming according to Feinberg and Vogelstein (1983). High-stringency hybridizations were performed at 42° in buffer containing 50% formamide, 5× SSC. Filters were washed at 68° with 0.1× SSC, 1% SDS. Under conditions of low stringency, hybridization was done in 35% formamide and washing was at 50° with 2× SSC, 1% SDS.

A total of 100 ng of lof-2 genomic DNA was used for PCR experiments with the primers Prom3 (5′ AAT GAC TGG GCA GTG CTA AGG 3′) and Prom2 (5′ CCG CTC CTC CGC GCA GAA AC 3′). These primers flank the chromosomal breakpoint of ONC-X*mrk* and generate a product only from the oncogenic copy of X*mrk*, but not from INV-X*mrk*. Amplification was carried out as described (Gutbrod and Schartl 1999) with an annealing temperature of 64° using 1 unit of Taq polymerase (GIBCO-BRL) in a reaction volume of 50 μl.

Cloning and sequencing: DNA from a single fish of the lof-1 mutant was digested to completion with *Eco*RI and separated on a 0.8% low-melting-point agarose gel. The 10–15 kb range was cut out of the gel, eluted, and cloned into the *Eco*RI site of dephosphorylated λEMBL4 arms. Recombinant phage DNA was packaged *in vitro* and the resulting library was screened with the kinase domain probe p17-2 (Wittbrodt *et al.* 1989) of X*mrk*. The entire 15-kb *Eco*RI insert of one of the plaque-purified positive phages was cloned into pBluescript KS+ to generate plasmid p338. Generation of overlapping subclones and sequencing using dideoxy chain termination was essentially done according to standard protocols. The sequence of *TX-1* is deposited in GenBank under accession no. AF130854. The RT-PCR product with primers, Ana1/Ins4 was cloned into pUC 18 using the SureClone ligation kit (Amersham Pharmacia Biotech, Freiburg, Germany) and sequenced. Sequence analysis and similarity searches were done using the program package of NIX at the HGMP (http://menu.hgmp.mrc.ac.uk/menu-bin/Nix/Nix.pl).

RESULTS

Lof-1: insertion of a transposable element: The X*mrk* lof-1 mutant is a spontaneous mutant from a brood of backcross hybrids carrying the wild-type *X. maculatus* X chromosome of the Rio Jamapa strain with the ONC-X*mrk* allele from the *Tu-Sd* locus (= Mdl^{Sd}-ONC-X*mrk*) in the genetic background of *X. helleri* (Rio Lancetilla stock). The mutant has lost the ability to develop spontaneously malignant melanomas (Figure 1). Gross structural analysis of ONC-X*mrk* revealed that the lof-1 mutant has a rearranged fragment for the kinase domain of 12 kb instead of 5 kb in *Eco*RI digests due to an ~7-kb insertion in exon 24 (earlier denominated exon W) of the X*mrk* oncogene (Wittbrodt *et al.* 1989). The rearranged *Eco*RI fragment was isolated from a partial genomic library. Sequence analysis of the borders of the insertion revealed the site of insertion to be between nucleotides (nt) 1652 and 1653 of the X-chromosomal Sd allele of ONC-X*mrk* (numbering according to Adam *et al.* 1991). The inserted DNA thus interrupts the reading frame of the wild-type sequence at the 3′ border of exon 24.

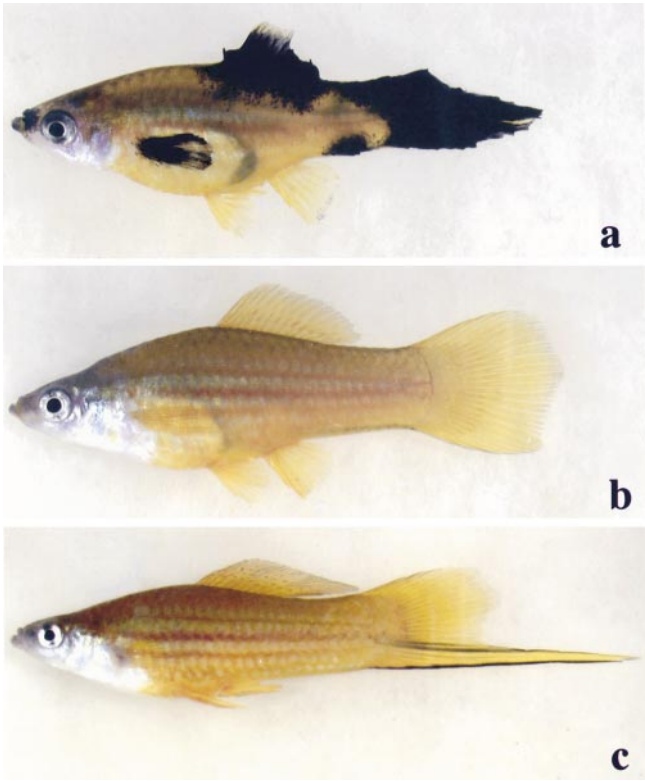


Figure 1.—Backcross hybrids carrying (a) the wild-type Mdl^{Sd}-ONC-X*mrk* chromosome, (b) the mutant lof-1 chromosome, and (c) the mutant lof-2 chromosome.

When the wild-type and lof-1 mutant ONC-X*mrk* sequences were compared, an imperfect 6-bp duplication at the site of insertion was found (5′ border ACA GGT, 3′ border CAA GGT). As this is a known feature of a mutational event generated by the insertion of a transposable element, the structure of the entire interrupting sequence (denominated *TX-1*) was determined (Figure 2). The insert has a total length of 7157 nt. The 5′ and 3′ ends are composed of 2377- and 2313-nt-long terminal direct repeats (96% identity) each of which has almost perfect internal repeats of a 68-nt and a 91-nt sequence. The internal unique sequence lacks an apparent continuous long open reading frame. The longest open reading frame that would translate into a 181-aa protein was found at the 3′ border of the 5′ LTR. When exon-based gene prediction programs were used, no apparent spliced gene structure was predicted. Searching the publicly available databases revealed no significant similarity to known sequences of any organism.

Southern blot analysis of X. *maculatus* DNA using an internal fragment of the insertion as a probe in digests

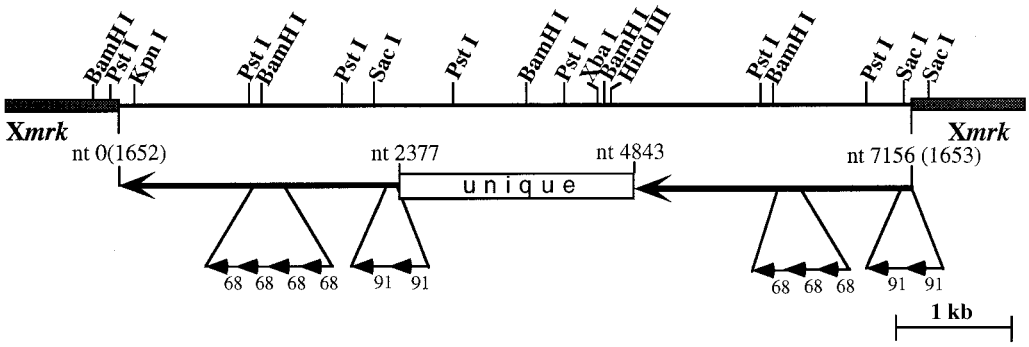


Figure 2.—Restriction map and schematic structure of the lof-1 insertion. Numbering of nucleotides is according to the inserted transposable element *TX-1*; numbers in parentheses indicate the position with respect to the ONC-X*mrk* sequence. Large arrows indicate the LTRs; small arrows show the placement of small internal direct repeats.

with enzymes that cut within the inserted sequence revealed only the expected fragments of the insertion (data not shown). However, the hybridization signal was very strong. In digests with enzymes that do not cut the insertion sequence but only cut the flanking genomic DNA, a multiplicity of fragments ranging in size from 1 kb to >20 kb was obtained. This indicates multiple copies of *TX-1*, certainly >50–100 per genome. Shorter fragments might represent truncated copies. A similar result was obtained for other *X. maculatus* strains as well as for *X. milleri* and *X. helleri* males and females (Figure 3A), *X. couchianus*, *X meyeri*, *X. andersi*, *X. nezahualcoyotl*, *X. montezumae*, *X. variatus*, *X. cortezi*, *X. malinche*, and the *X. maculatus*/*X. helleri* hybrid melanoma cell line PSM (data not shown). *TX-1*-cross-hybridizing sequences were also detected under high-stringency conditions in all members of the family Poeciliidae tested, including *Poecilia formosa*, *Gambusia affinis*, *Girardinus falcatus*, *P. gracilis*, *Heterandria bimaculata*, *Phallichthyes amates* (Figure 3B), *P. mexicana*, *P. latipinna*, *G. metallicus*, and *H. formosa* (data not shown). In contrast, even under low-stringency conditions, no signals were found in other non-poeciliid species. Even in the closely related fishes *Fundulus* spp. (Fundulidae) and medakafish (*Oryzias latipes*, Oryziatidae), which both belong to the same order Cyprinidontiformes, no *TX-1*-related sequences appeared to be present. The same is true for the more distantly related representatives of the main branches of the phylogenetic tree (Nelson 1994) of the bony fishes (Osteichthyes), Nile Tilapia (*Oreochromis nilotica*, Cichlidae), rainbow trout and salmon (*Oncorhynchus mykiss* and *Salmo salar*, Salmonidae), pike (*Esox lucius*, Esocidae), zebrafish (*Danio rerio*, Cyprinidae), common carp (*Cyprinus carpio*, Cyprinidae), european eel (*Anguilla anguilla*, Anguillidae), sturgeon (*Acipenser sturio*, Acipenseridae), human (Figure 3C), Chinese perch (*Siniperca chuatsi*, Serranidae), and *Battrachocottus baikalensis* (Cottidae; not shown). This strongly suggests that *TX-1* is restricted to the family of Poeciliidae.

Lof-2: deletion of ONC-X*mrk*: The lof-2 mutant was detected in a brood of backcross hybrids of the same type as described above for lof-1. No melanoma development was observed among the mutant and its progeny after further backcrossing with *X. helleri* (Figure 1c). The phenotype of lof-2 fish is exactly the same as that of lof-1 fish except that all lof-1 animals are females (like fish with the wild-type chromosome), while lof-2 fish are males. Crossing of lof-1 to lof-2 fish resulted in offspring that all had the mutant phenotype (not shown). This demonstrates that the two mutations do not complement each other. Southern blot analysis with different hybridization probes spanning the whole genomic region of the X*mrk* oncogene from the 5′ flanking sequences downward up to sequences 3′ of the last exon revealed that the whole region is deleted (Figure 4). PCR amplification with the primers prom3/prom2 extending from nt −584 to nt +25 across the promoter region and transcriptional start site failed to produce the expected 609-bp fragment that is obtained from the wild-type X-chromosomal allele of ONC-X*mrk*. In summary these data indicate deletion of the entire oncogene locus.

Expression analysis: To analyze the expression of the X*mrk* proto-oncogene (INV-X*mrk*) the wild-type and mutant ONC-X*mrk* alleles (wt ONC-X*mrk*, lof-1 X*mrk*, lof-2 X*mrk*), an allele-specific RT-PCR was set up (Figure 5a). The wt ONC-X*mrk* allele from the X chromosome of *X. maculatus* differs from INV-X*mrk* by a 1344-bp deletion that includes exon 26 [numbering of exons according to Gutbrod and Schartl (1999); this exon has been denoted Y in earlier articles (Adam *et al.* 1991)].

Primers that flank the deletion (Ins4/5) give an RT-PCR product of 226 bp from the INV-X*mrk* transcript, while the ONC-X*mrk* transcript is amplified as a 128-bp product. In a competitive PCR, a semiquantitative determination of expression levels can be achieved. INV-X*mrk* was found to be expressed at low levels in all healthy organs including skin (number of independent RNA preparations tested: $n = 6$), muscle ($n = 3$), liver ($n = 9$), kidney (head nephros; $n = 3$), testes ($n = 2$), brain ($n = 3$), eye ($n = 14$), and gills ($n = 18$) from parental platyfish (with and without ONC-X*mrk*) and melanoma-bearing as well as healthy backcross hybrids. A similar low expression was seen in benign ($n = 4$) and malignant melanoma ($n = 4$). The wild-type ONC-X*mrk* was highly expressed in malignant melanoma

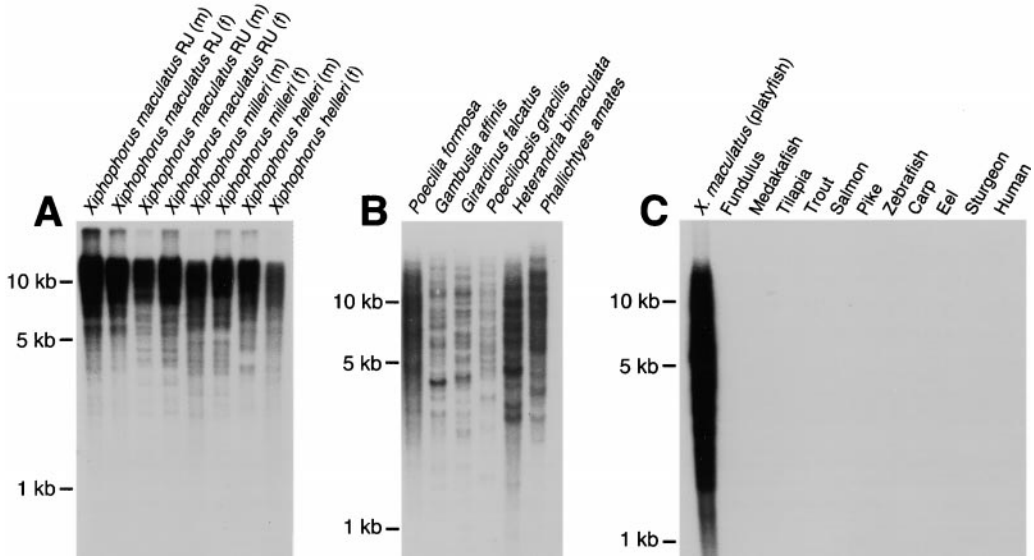


Figure 3.—Southern blot analysis of *TX-1* distribution in (A) the genus Xiphophorus, (B) the family Poeciliidae, and (C) other bony fishes. See materials and methods and results for description of the fishes analyzed. Human DNA was used as a control for unspecific hybridization. Genomic DNAs were cut with *Hin*dIII. Probes are the direct repeats of *TX-1*. Filters A and B were washed at high stringency (0.1× SSC, 68°), filter C at low stringency (2× SSC, 50°). Extreme overexposure of filter C did not reveal any specific signal in fishes outside of the family Poeciliidae.

(~10-fold higher than INV-X*mrk*), but was expressed only at a low level in benign melanoma (at similar levels as INV-X*mrk*). The wild-type ONC-X*mrk* was found to be expressed also in all healthy organs analyzed (except liver) of ONC-X*mrk*-bearing platyfish ($n = 5$) as well as malignant and benign melanoma-bearing backcross hybrid fish ($n = 9$; Figure 5b), but at much lower levels than INV-X*mrk*.

It has been proposed that ONC-X*mrk* expression plays a role in macromelanophore pigment cell pattern formation (Woolcock *et al.* 1994). To investigate this, we prepared RNA of *X. maculatus* (*Mdl*^{Sd}-ONC-X*mrk*) from dorsal fins with and without macromelanophore spots and caudal fins without macromelanophore spots; we also prepared RNA of *X. maculatus* carrying the lof-3 mutant X chromosome from dorsal fins without macromelanophore spots. In all four samples a similar expression level of ONC-X*mrk* was detected (Figure 5c), indicating that expression of the oncogene is not functionally related to formation of macromelanophores.

Expression of the ONC-X*mrk* gene in healthy organs allowed for expression analysis of the mutant alleles from lof-1 and lof-2 (Figure 5d). Consistent with the genomic structure, no expression of ONC-X*mrk* in lof-2 was detected with primers Ins4/5. From the lof-1 mutant allele two transcripts were detected. Amplification with primers Ana1/lof-1A indicated presence of a transcript, which reads through from exon 24 into the 5′ LTR of the insertion sequence. From this mRNA premature termination of the X*mrk* protein will occur due to a stop codon located at 107 bp in the inserted sequence (Figure 6a). A second mRNA that was amplified with primers Ana1/Ins4 is produced. Cloning and sequencing of the PCR product revealed that a cryptic splice donor site (CAG/GTACCT) in exon 24 that is located 16 bp 5′ of the wild-type splice site (CAG/GTACGC) is used. In this transcript the entire inserted DNA is spliced out together with intron 24. However, a deletion of 16 bases is created due to the use of the new splice donor, which leads to a frameshift and termination after 15 codons (Figure 6b). Both transcripts are present at similar levels and both translate into a truncated X*mrk* receptor protein that terminates shortly after the end of the kinase domain and lacks the carboxyterminal domain with the autophosphorylation and substrate binding sites.

DISCUSSION

We have analyzed the molecular basis of two melanoma loss-of-function mutants in Xiphophorus and found that both are due to impairment of the oncogenic copy of X*mrk*. In the lof-2 mutant the whole ONC-X*mrk* gene is missing. ONC-X*mrk* is flanked by the *RY* locus (red and yellow pigment patterns) and *Mdl*, the macromelanophore locus (Gutbrod and Schartl 1999). The deletion of lof-2 obviously does not extend into the *RY* locus, because the phenotype of the pigment pattern Dr is unchanged compared to wild-type fish. Whether the deletion extends into *Mdl* is unclear. No macromelanophores are produced. Thus *Mdl* could be partly or totally missing, but an explanation similar to that for the lof-1 mutant (see below) may be given as well.

In the lof-1 mutant an insertion in ONC-*Xmrk* results in a disruption of the gene leading to aberrant transcripts from which only a truncated protein can be produced. The mutant protein would lack the whole carboxyterminal domain, which contains the binding sites for several important substrate proteins (Wellbrock and Schartl 1999; Wellbrock *et al.* 1999). Thus the truncated receptor should be unable to exert its signaling function, which is required to initiate and maintain

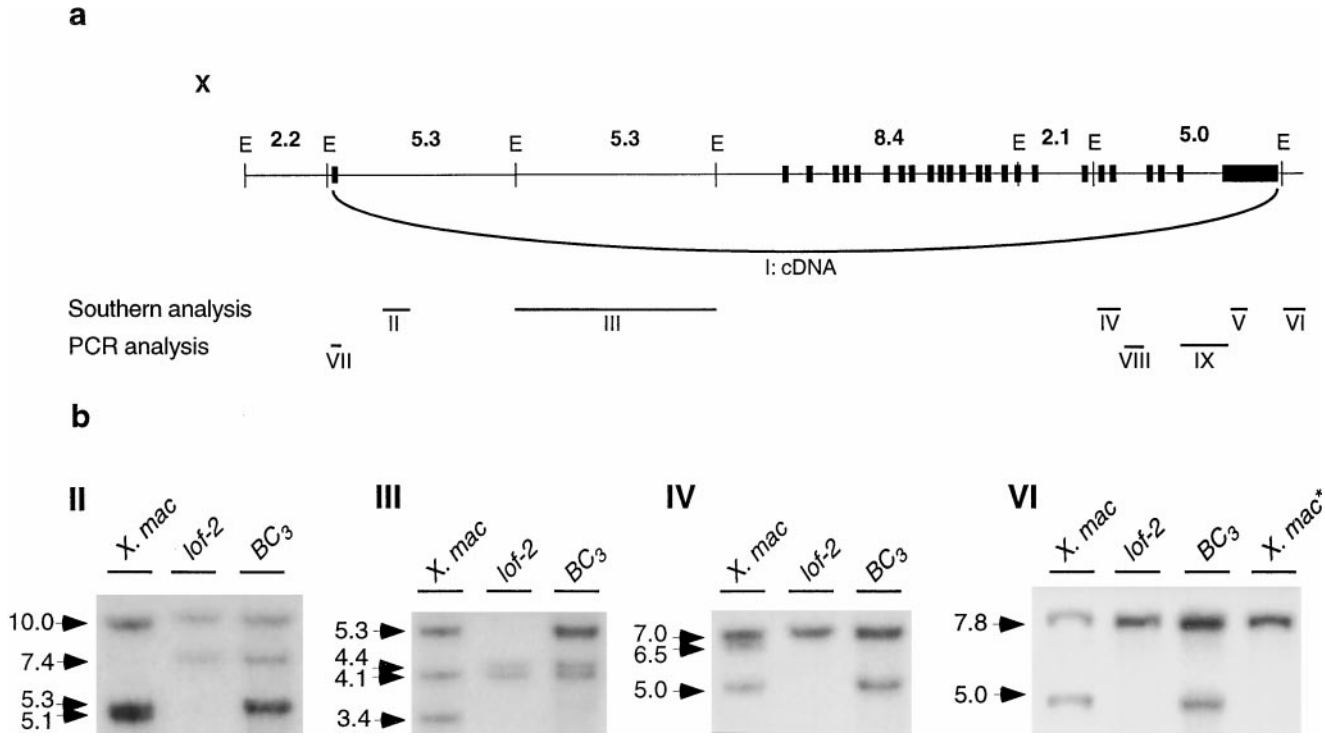


Figure 4.—Analysis of the genomic structure of the ONC-X*mrk* region of the lof-2 mutant. (a) Genomic organization of the X allele of ONC-X*mrk* of wild-type *X. maculatus*. Black boxes represent exons; numbers give the size of the *Eco*RI fragments. Fragments used as hybridization probes on filters with genomic DNA of the lof-2 mutant and wt fish and sequences amplified by PCR are indicated by roman numbers. The PCR products VII, amplified with primers Prom3 and Prom2; VIII, amplified with the primers Xfor/Xrev (Gutbrod and Schartl 1999); and IX, amplified with Ins5/Ins4, are missing in lof-2. (b) Southern blot analysis with probes II, III, IV, and VI. *X. mac* (II–IV), male *X. maculatus* with X- and Y-ONC-X*mrk*; lof-2, lof-2 mutant fish; BC_3, fish of the same pedigree as lof-2, but carrying the wild-type X chromosome with ONC-X*mrk*; *X. mac* (VI), female *X. maculatus* with X-ONC-X*mrk*; *X. mac**, female *X. maculatus* without X*mrk* oncogene. (II) A 0.7-kb *Bam*HI fragment was used as hybridization probe. The 10-kb band of *X. maculatus* represents INV-X*mrk*, whereas the 10-kb band in lof-2 and BC_3 is the INV-X*mrk* allele inherited from the *X. helleri* parent. In these two individuals the *X. maculatus*-derived INV allele on the X chromosome is 7.4 kb in size (J. N. Volff, unpublished results). (III) The 5.3-kb *Eco*RI probe specific for the X allele of ONC-X*mrk* detects its genomic counterpart, a 4.4-kb fragment derived from the *X. helleri* INV-X*mrk* gene, a 4.1-kb *X. maculatus* INV allele, and a 3.4-kb Y-ONC-specific fragment. (IV) The probe covering a part of the X*mrk* kinase domain-encoding sequence detects a 7-kb band representing both *X. maculatus* and *X. helleri* INV-X*mrk* alleles, a 6.5-kb band that is Y-ONC-X*mrk* specific, and a 5-kb fragment that is the X-ONC allele. (VI) A probe from the 3′ end of X*mrk* detects a 7.8-kb band common to *X. maculatus* and *X. helleri* INV-X*mrk* and a 5-kb X-ONC-specific fragment.

the proliferation of the transformed pigment cells. This would explain the phenotype of the lof-1 mutant, which, contrary to the corresponding wild type, does not develop melanoma anymore. The fact that disruption of the X*mrk* oncogene results in a loss of the ability to form melanoma demonstrates that ONC-X*mrk* is indeed the melanoma-inducing gene from the *Tu* locus. As ONC-X*mrk* was isolated by a positional cloning strategy, this proof of its identity appears especially important.

However, another aspect of the lof-1 phenotype is not so easily explained by the molecular data. It was shown that *Mdl*, which specifies the phenotype of the macromelanophore (larger size, lack of distance regulation in pattern formation, etc.), is a separate genetic entity and not identical to the ONC-X*mrk* (Weis and Schartl 1998). Thus it would have been expected that lof-1 fish would still develop some macromelanophores like the parental platyfish. These, however, should not proliferate and grow out to melanoma because of the lack of ONC-X*mrk* function. Surprisingly, not a single macromelanophore has been detected in hundreds of lof-1 fish. A possible explanation would be that the large insertion in ONC-X*mrk* has a remote effect on neighboring genes, *e.g.*, the very closely linked *Mdl*, through changes in chromatin structure. Also, genes associated with transposable elements could be mistakenly identified as genome invaders and become methylated and silenced as well (Henikoff and Matzke 1997). Thus insertion of the *TX-1* sequence may have a negative regulatory effect on the expression of *Mdl*, which has been mapped close to ONC-X*mrk* (Gutbrod and Schartl 1999).

Also in the lof-2 mutant, another locus, which is obviously not affected in its primary structure by the mutation, is affected in its phenotype. Lof-2 fish show a normal expression of the RY locus located 3′ of the deleted

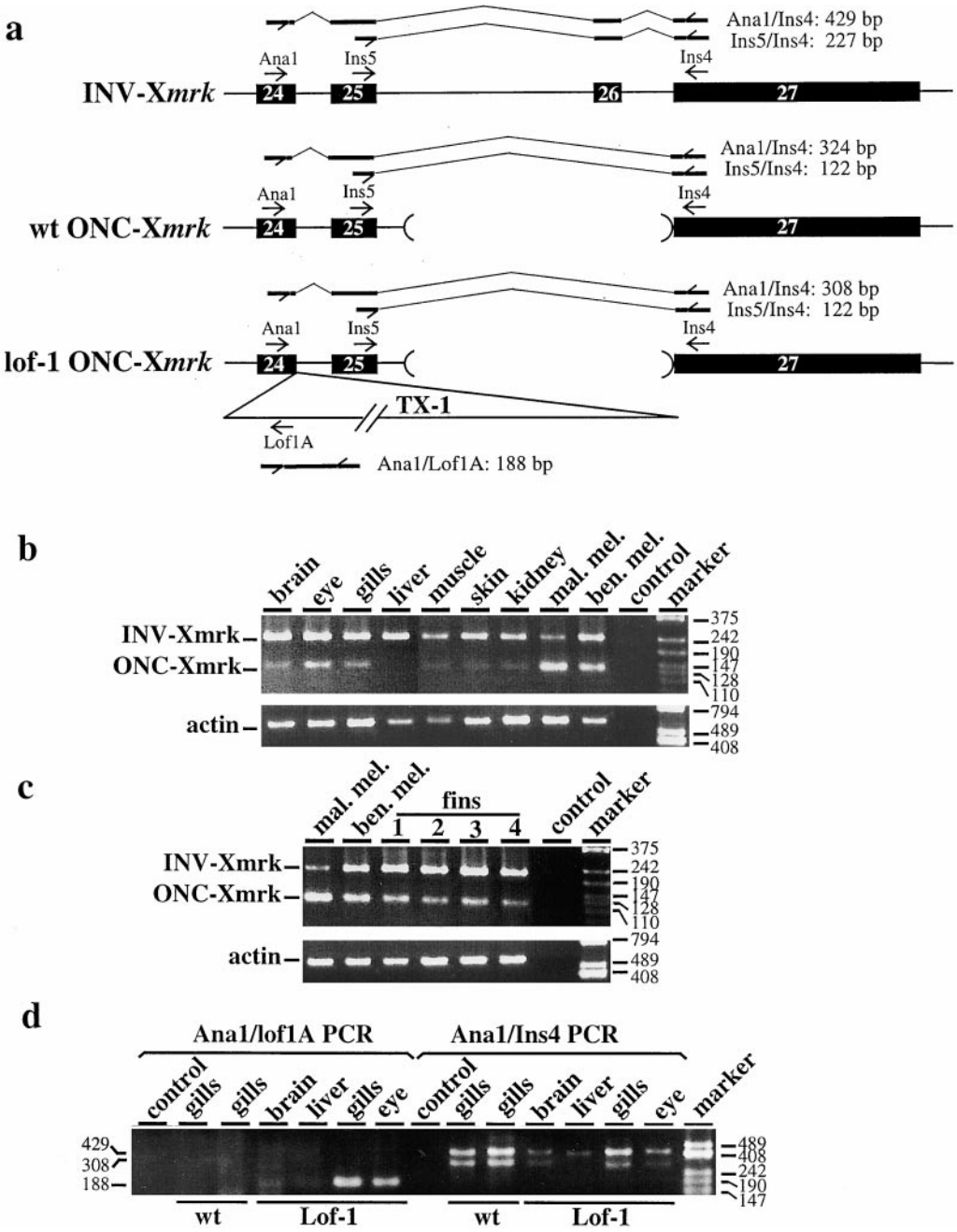
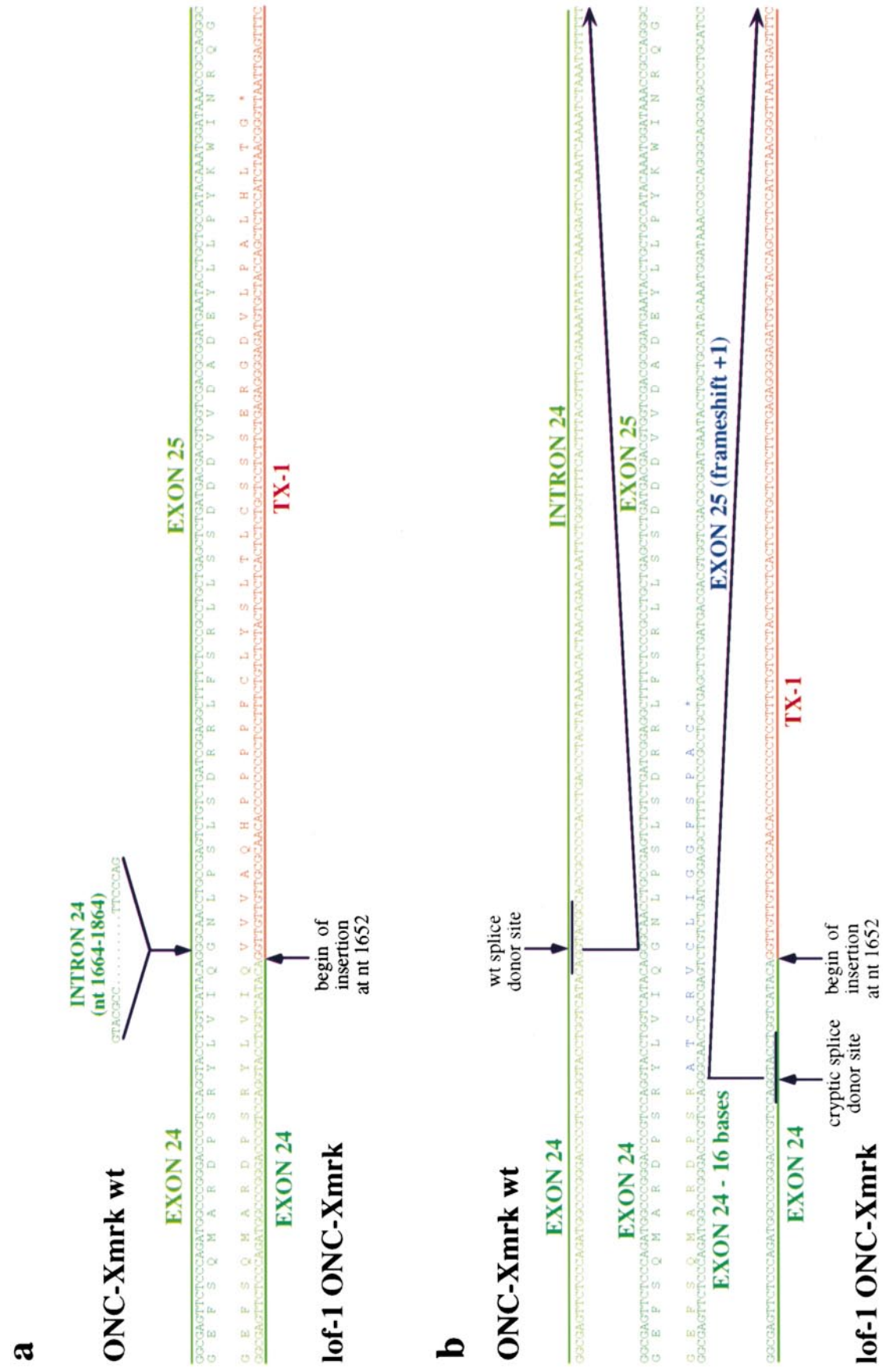


Figure 5.—Expression analysis of the wild-type ONC-X*mrk* gene and the lof-1 mutant. (a) Location of primers and sizes of the corresponding RT-PCR products from the X*mrk* proto-oncogene (INV-X*mrk*), the wild-type X-chromosomal oncogenic copy (wt ONC-X*mrk*) with the deletion including exon 26, and the derived mutant ONC-X*mrk* allele from lof-1 fish (Lof1 ONC-X*mrk*) with insertion of *TX-1*. (b) RT-PCR analysis of normal organs from tumor-bearing backcross hybrids and of benign pigment lesions and malignant melanoma. Primers Ins5 and Ins4 were used, which amplify a 227-bp fragment from the INV-X*mrk* and a 122-bp fragment from the X-chromosomal ONC-X*mrk* transcript. For quantification a PCR for actin with primers Act1 and Act2 (product size 565 bp) was performed from each RT reaction. (c) RT-PCR analysis with primers Ins5 and Ins4 for ONC-X*mrk* expression in macromelanophore spot-containing dorsal fins (1); macromelanophore spot-free dorsal fin areas (2); tail fins (3), which contain no macromelanophores, of wild-type *X. maculatus* from Rio Jamapa with the pattern "spotted dorsal"; and dorsal fins without macromelanophores (4) from the lof-3 mutant. (d) RT-PCR for analysis of expression of the ONC-X*mrk* locus in normal organs from lof-1 fish. With primers Ana1 and Lof1A the transcript that extends into the inserted *TX-1* sequence is specifically amplified (fragment size 188 bp) from the mutant ONC-*Xmrk* locus in lof-1 fish but not from wild-type (wt) fish (melanoma-bearing backcross hybrids with benign or malignant lesions). With primers Ana1/Ins4 a transcript of 308 bp is amplified in which the *TX-1* insertion sequence is spliced out; this transcript has a sequence similar to the wild-type transcript (324 bp). The 429-bp product is amplified by this pair of primers from the INV-X*mrk* transcripts.

ONC-X*mrk*. But in these fish the sex-determining locus that resides behind the *RY* locus further away from ONC-X*mrk* has obviously changed its function from female to male.

Alternatively, ONC-X*mrk*, although not part of *Mdl*, could have some function in *X. maculatus* for macromelanophore pattern formation. In this context the expression of the oncogenic copy of X*mrk* in all normal tissues except liver is somehow enigmatic, because, contrary to expression in the pigment cell lineage, this is not connected to tumorigenesis. Wild-type parental fish do not show any sign of hyperplasia or other proliferative disorders in any of their organs nor do hybrids develop tumors of any other cell type except pigment cells. An explanation may be the low level of expression in normal organs, which is below that of the proto-oncogene and is considerably lower than in the benign pigment lesions. The reasoning that a certain threshold level of


a
ONC-Xmrk wt
INTRON 24
(nt 1664-1864)
EXON 24
EXON 25
TX-1
lof-1 ONC-Xmrk
EXON 24
begin of
insertion
at nt 1652
b
ONC-Xmrk wt
wt splice
donor site
EXON 24
INTRON 24
EXON 25
EXON 24 - 16 bases
EXON 25 (frameshift +1)
TX-1
lof-1 ONC-Xmrk
EXON 24
cryptic splice
donor site
begin of
insertion
at nt 1652

ONC-X*mrk* transcripts is necessary to elicit neoplastic transformation is in accordance with studies on the transforming capacity of ONC-X*mrk* in transgenic fish and in *in vitro* cell systems where high expression of the oncogenic receptor was required (Wittbrodt *et al.* 1992; Winkler *et al.* 1994). Moreover, the malignancy of the tumors, which is determined by the amount of undifferentiated, rapidly dividing cells, correlates positively with the amount of ONC-X*mrk* mRNA (Adam *et al.* 1991; Mäueler *et al.* 1993).

The low-level expression of ONC-X*mrk* in normal organs could point to a normal function of this gene. However, all Xiphophorus populations are polymorphic for ONC-X*mrk*, and many individuals do not have this gene. Using an experimental strategy similar to the one in this study Woolcock *et al.* (1994) found expression of ONC-X*mrk* in dorsal fins with macromelanophore spots, but not in fins of the loss-of-function mutant lof-3, which do not develop macromelanophore spots. This result was interpreted as reflection of a function of ONC-X*mrk* for formation of the "spotted dorsal" pigment pattern. However, this appears unlikely, because macromelanophore patterns are found in fish of several Xiphophorus species in the absence of ONC-X*mrk*, indicating that it is not necessary for development of this cell type (Weis and Schartl 1998). In addition, we have shown that expression of ONC-X*mrk* in the fin tissue is not connected to formation of macromelanophores, as its transcript was detected at similar levels also in fins without spots.

The insertion sequence in lof-1 has several features, which characterize it as a mobile genetic element. We thus propose to name it *TX-1* (for transposable element of Xiphophorus). First, *TX-1* has been observed to move into the X*mrk* oncogene by identification of the first mutant fish. This is a so far unique situation because fish hemizygous for ONC-X*mrk* can be easily bred and because of the dominant phenotype of the X*mrk* oncogene. In the other examples in fish where mutants are due to insertion of sequences with structural similarities to transposable elements, the "no-tail" mutant of the zebrafish (Schulte-Merker *et al.* 1994) and the albino mutants i^1 and i^4 of the medaka (Koga and Hori 1997), the mutations are recessive. As spontaneous mutants they might have been around in the natural fish population or laboratory strain for quite some time before becoming apparent. Thus the creation of the mutant allele and the invasion of the element could not be observed. It should be noted, however, that frequent active excision of the transposable element of the i^4 mutant has been observed (Koga *et al.* 1996). Second, *TX-1* created a 6-bp target sequence duplication at its margins. This is typically seen for all mobile elements but evidently does not occur during insertion events by other types of sequences. Third, *TX-1* has long direct terminal repeats. This would assign *TX-1* to the class of LTR-containing retrotransposons of mobile elements. Fourth, *TX-1* is present in multiple copies in the genome of all poeciliid species tested, but absent from other fish species.

TX-1 has specific features, which distinguish it from most transposable elements analyzed so far. Its terminal repeat is unusually long and may be one of the largest known. The *TX-1* sequence shows no similarity to other retrotransposons, has no long open reading frames that would give rise to proteins known from other mobile DNA elements, and in particular gives no indication of encoding a functional polymerase that would make it capable of transposition. As *TX-1* only recently moved into the X*mrk* gene, either it has lost such sequences during the process of integration or it is dependent on a helper element that contributes the reverse transcriptase function. Elements of the SINE family also do not encode proteins for reverse transcription. *TX-1*, however, has no structural features that are characteristics of SINEs. The overall structure of *TX-1* resembles that of the murine *Etn* elements and human *THE-1*. *THE-1*, however, is 2.3-kb long and has only a 350-bp LTR (Paulson *et al.* 1985). *Etn* elements are almost 6-kb long, have no long open reading frames, and lack sequence similarity to any known retroelements, but have small LTRs (Sonigo *et al.* 1987).

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Figure 6.—Sequence and conceptual translation of the mutant ONC-X*mrk* transcripts of lof-1. (a) "Read through" transcript: cDNA sequence and conceptual translation of the transcript from the wild-type ONC-X*mrk* gene (top) and from the lof-1 mutant locus (bottom). In the mutant, transcription continues into the inserted sequence and translation stops at a TAA codon in *TX-1*. (b) "Splice-out" transcript: genomic structure, cDNA sequence, and conceptual translation of the transcript from the wild-type ONC-X*mrk* (top) and from the lof-1 mutant locus (bottom). Because *TX-1* is inserted into the wild-type splice donor site of the exon 24/intron 24 border, a cryptic splice donor site 16 bases upstream that has a similar sequence is used (CAG/GTACC *vs.* CAG/GTACG). The transcript continues correctly with exon 25, but a frameshift created through the position of the cryptic spice donor site leads to a different amino acid sequence and terminates after 15 residues.

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