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DIFFERENT EXPRESSION PATTERNS OF ONCOGENES AND PROTO-ONCOGENES IN HEREDITARY AND CARCINOGEN-INDUCED TUMORS OF XIPHOPHORUS

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Hereditary melanoma in Xiphophorus hybrids carrying the melanoma-inducing *Tu-Sd* locus is caused by transcriptional activation of the *Xmrk* gene that resides at the *Tu-Sd* locus and encodes a novel member of receptor tyrosine kinases (RTK). In this study, a total of 27 hereditary melanomas from various hybrid genotypes harbouring 7 different *Tu* alleles were also found to over-express the corresponding *Xmrk* alleles. The level of over-expression correlated with the degree of malignancy of the melanoma. In addition, *Xsrc* expression was high in many malignant melanomas. Expression patterns and levels of the Xiphophorus EGF-receptor gene (*Xerb B*), the c-*myc* (*Xmyc*), and the PDGF (*Xsis*) gene(s) were not intriguing. Transcription of the *ras* gene(s) may be correlated to secondary events of melanoma progression. Expression patterns of *Xfms*, the Xiphophorus CSF-1 receptor homologue, can be explained by different contents of infiltrating macrophages in the tumors. In carcinogen-induced tumors including one melanoma no significant expression of the *Xmrk* oncogene could be detected. *Xsrc* expression, however, was strikingly high. This indicates that activation of oncogenes other than *Xmrk* is instrumental in tumorigenesis of neoplasia of non-hereditary origin.

The Xiphophorus melanoma is uniquely suited to study primary events leading to neoplastic transformation of pigment cells as well as secondary and later steps in tumor formation and tumor progression responsible either for the maintenance of the neoplastic phenotype or for tumor malignancy. In addition to spontaneous (hereditary) melanoma formation, certain hybrids of Xiphophorus develop neoplasia of epithelial, mesenchymal and neuronal origin after appropriate treatment with carcinogens (*e.g.*, MNU) (Anders *et al.*, 1984; Schwab *et al.*, 1978) offering the opportunity to study the processes responsible for the malignant phenotype on a comparative level in neoplasia of different etiology.

Spontaneous melanoma formation in certain hybrids of Xiphophorus has been attributed to the unscheduled activity of a cellular oncogene locus denominated *Tu* (Anders *et al.*, 1984). We have cloned the melanoma-inducing gene from one specific allele (*Sd*) of the *Tu* locus (Wittbrodt *et al.*, 1989). It encodes a novel trans-membrane receptor tyrosine kinase belonging to the EGF-receptor-multigene family (see Ullrich and Schlessinger, 1990) that is activated in melanoma (Wittbrodt *et al.*, 1992). This gene is designated *Xmrk*. It is present in one copy as a constituent of the various X- or Y-chromosomal *Tu* alleles of *Xiphophorus maculatus*. Another copy of this gene does not map to the *Tu* locus and is present in all individuals of Xiphophorus. It obviously represents the corresponding *Xmrk* proto-oncogene (*INV*) and is expressed as a single transcript of 5.8 kb at low levels in some epithelial tissues. Our preliminary studies indicated that expression of a certain X-chromosomal *Xmrk* oncogene allele encoded by the *Tu-Sd* locus gives rise to a smaller transcript (4.7 kb) than does the proto-oncogene. Its abundance is high in malignant melanoma from pooled biopsy material. Three other X- and Y-chromosomal alleles of the *Xmrk* oncogene have transcripts of similar size (Wittbrodt *et al.*, 1989). It could be demonstrated that over-expression of the oncogenic *Xmrk* allele from the *Tu-Sd* locus is due to transcriptional activation (Adam *et al.*, 1991, 1993). This is the primary step leading to neoplastic transformation of pigment cells in Xiphophorus hybrids.

Nothing is known about the primary molecular processes leading to chemically or physically induced neoplasia of Xiphophorus hybrids. Several studies, mostly based on classical genetic analyses, led to the general hypothesis that activation of the *Tu* gene is also the primary cause of neoplastic transformation in carcinogen-induced neoplasia (Anders, 1989; Anders *et al.*, 1984; Schwab *et al.*, 1978). However, there is no molecular evidence for this until now. Little data are available comparing induced neoplasia with hereditary tumors at the molecular level. A few spontaneously developing non-melanoma tumors of Xiphophorus hybrids were shown to express the proto-oncogene *Xmrk* but not the oncogene. In genotypes with a de-regulated *Tu* gene that develop melanoma following treatment with steroids the *Xmrk* oncogene transcript was detected (Zechel *et al.*, 1992). Unfortunately, no carcinogen-induced somatic tumor was analyzed in this study. In an earlier study we showed that most of the carcinogen-induced neoplasia analyzed so far display similarly high or up to 5-fold higher levels of $pp60^{c\text{-}src}$-tyrosine-kinase activity (the gene product of the cellular *src* gene of Xiphophorus) as compared with spontaneously developing malignant melanoma. The activity of this tyrosine kinase was positively correlated with the malignancy but not with the etiology of the neoplasia (Schartl *et al.*, 1985). The significance of the elevated tyrosine-kinase activities in all chemically and/or physically induced malignant neoplasia remains unclear, and was considered rather as a secondary phenomenon in tumor formation than as a primary step of neoplastic transformation (Schartl *et al.*, 1985). In a study on different neoplastic lesions in Xiphophorus, it could be demonstrated that the features of intermediary metabolism depend on the tumor compartments (*e.g.*, nodular or invasive areas), as well as the histiotype and the etiology of the tumor samples in a similar manner as it was observed for many mammalian and human neoplasia (Mäueler *et al.*, 1987). Likewise these phenomena were explained as late secondary adaptions of the neoplastic cells during tumor progression.

The availability of molecular probes from the *Tu*-encoded *Xmrk* gene offers tools for a detailed analysis, comparing oncogene expression in single neoplastic lesions of spontaneously developing melanoma as well as single induced neoplasia of different origin in Xiphophorus. To verify that indeed the primary step leading to the formation of hereditary melanoma is over-expression of the oncogenic sex-chromosomal *Xmrk* alleles, we analyzed *Xmrk* expression in 27 single spontaneous

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hereditary melanomas caused by different X- and Y-chromosomal *Tu* loci. To investigate whether over-expression of X*mrk* is also involved in the transformation processes of tumors induced by chemical carcinogens, 3 single neoplastic lesions including one melanoma induced by MNU were examined. To analyze a possible contribution of other receptor tyrosine-kinase genes closely related to X*mrk*, the expression of X*erb* B (the Xiphophorus homologue of the human *EGF* receptor gene; Schartl *et al.*, 1993) and of X*fms* (homologue of the human *CSF-1* receptor gene) was studied in the same neoplastic lesions. Other proto-oncogenes (X*src*, X*ras*, X*myc*, X*sis*) of possible significance for the neoplastic phenotype were also included.

MATERIAL AND METHODS

Experimental animals

The fish used in this study were bred under standard conditions (Kallman, 1975) in the aquarium of the Gene Center at the Max-Planck Institute for Biochemistry. Back-cross hybrids (BC) of different genotypes of *X. maculatus* (*X. mac.*) bearing various sex-chromosomal X*mrk* loci (distinguishable by specific small macro-melanophore spots on the body) using *X. helleri* (*X. hell.*) as the recurrent parent were analyzed. 1, *Sd* +/+: spotted dorsal, X-chromosomal X*mrk* locus; 2, *Sd a*/+, *a:* albino locus; *Sd* carrying hybrids heterozygous for *a* develop melanotic melanoma; 3, *Sd a/a, Sd*-hybrids homozygous for *a* develop amelanotic melanoma; 4, *SdT:* autosomal translocation of the X-chromosomal X*mrk* locus. Of the offspring of all these back-crosses, 25% spontaneously develop benign and 25% malignant melanoma. When compared with malignant *Sd*-melanoma the *Sd a*/+-melanoma were more malignant, while the *Sd a/a* and *SdT* melanomas are more benign; 5, *DrLi:* dorsal red lineatus, mutation of the X-chromosomal X*mrk*-allele Li; 6, N^2 nigra extended, a mutation of the Y-chromosomal X*mrk*-locus nigra; 7, *Sb:* spotted belly, Y-chromosomal X*mrk*-locus; 8, *ArSr'*: anal red, striped, Y-chromosomal X*mrk* allele. The founder fish for this strain had been X-irradiated by A. Anders (at least 20 back-cross generations ago). Of the back-cross hybrids, 1 to 5% develop malignant melanoma spontaneously; 9, *Sr"*: mutation of *Sr;* 10, *Li,* lineatus, X-chromosomal locus from *X. variatus.* These hybrids were used for carcinogen treatment; up to 20% develop, besides benign melanoma, neoplasia of epithelial, mesenchymal and neural origin. The induced neoplasia were highly malignant and killed the fish within 2 to 4 months (for a detailed description of the crossing procedures, the genotypes and the phenotypes, see Anders and Anders, 1978; Anders *et al.*, 1973, 1984; Schartl *et al.*, 1985). Tumors were surgically removed, immediately frozen and stored in liquid nitrogen until preparation of RNA.

Cell lines

Cell lines were cultured under the conditions described (Mäueler *et al.*, 1988*a*). After reaching confluence, cells were harvested and used for preparation of RNA.

Treatment of fish and tumor diagnosis

N-methyl-*N*-nitrosurea (MNU) was administrated by exposing tumor-free *X. var. Li* × *X. hell.* back-cross hybrids to a 10^{-3} M solution of the carcinogen 4 times for 1 hr at 2-week intervals. All tumors were classified according to data obtained by gross inspection of localization and growth rate and by histopathological analysis. For light microscopy, all specimens were fixed in Bouin's solution. Excess picric acid was eluted in 70% ethanol. The fixed specimens were dehydrated and embedded in paraffin. Sections (5 μm) were cut with a Leitz base sledge microtome and stained according to classical histopathological staining methods used for vertebrate tumor diagnosis.

Hybridization probes and labelling

All probes used for hybridization were separated from vector sequences and highly GC-rich sequences of the insert after appropriate restriction-enzyme digestion and low-melting-point agarose-gel electrophoresis. Probes used for nick-translation were further purified through NACS columns (BRL, Eggenstein, Germany). The following heterologous probes were used: (i) 2 internal 400-bp PstI fragments of the v-*fms*-gene 5' of the kinase domain (ATCC, Rockville, MD); (ii) 700-bp BglII/PstI fragment D of pHB-II (Ellis *et al.*, 1980) of the v-*ras* gene of Harvey murine sarcoma virus; (iii) the 1.5-kb EcoRI/PstI fragment of the trout c-*myc* clone C-181 (T*myc*), containing sequences homologous to exon 2 and 3 of chicken c-*myc* (VanBeneden *et al.*, 1986). The Xiphophorus-specific probes used were: (iv) X*mrk* cDNA clone 3-2 (Wittbrodt *et al.*, 1989) encompassing the extracellular and transmembrane domains of X*mrk;* (v) 1.6-kb genomic X*erb* B clone p38-1 (Schartl *et al.*, 1993) containing 2 exons; the coding region of these clones share 82% homology to the v-*erb* B gene and 83% homology to the human *HER*-1 gene; (vi) 300-bp BamHI/BglII X*sis* fragment (gift from U. Schlehenbecker); (vii) 1.3-kb BamHI fragment of the X*src* cDNA (clone 726) containing exons 1–11 (data not shown). Nick-translations of the *fms, ras* and T*myc* probes were performed as described (Sambrook *et al.*, 1988), using a kit from Amersham Buchler (Braunschweig, Germany). All other probes were labelled by random priming according to the protocol of Feinberg and Vogelstein (1984). Labelling was done using ^{32}P-labelled nucleotides. Random-primed probes on the one hand and nick-translated probes on the other hand were labelled to comparable specific activities.

Northern blot analysis

Total cellular RNA was isolated by the LiCl procedure (Le Meur *et al.*, 1981) using ultraturrax N8 (Janke and Kunkel, Staufen, Germany) for homogenization. Total RNA (20 μg) was denatured with formamide/formaldehyde and electrophoresed in 1.2% agarose gels containing 2.2 M formaldehyde (Lehrach *et al.*, 1977). For size calibration, an RNA-ladder (BRL, Bethesda) was included. RNA was electroblotted to Gene screen or Hybond N membranes according to the protocol of the suppliers (Amersham Buchler). For exact quantitation of the RNA amount present on the hybridization membrane, each filter was stained with methylene blue (Khandjian, 1986) and the relative amounts of RNA were determined densitometrically. Filters were hybridized with 10^7 dpm/ml of the labelled probes. The same filters were used for the different probes after stripping off the hybridization signals. Hybridization with homologous probes was carried out with 50% formamide, with heterologous probes with 40% formamide, both at 42°C. Membranes were washed at different temperatures in SSC solutions containing 1% SDS (2 washes for 5 min at room temperature, followed by 2 washes for 45 min at the final washing temperature) and then exposed to Kodak X-OMAT AR 15 X-ray films between 2 intensifying screens. The exact washing conditions are indicated in the figure legends. Exposure times were selected to correct for differences in the specific activities of the probes.

RESULTS

The tumors used in this study were of different etiology, namely hereditary or carcinogen-induced. All were melanomas and fibrosarcomas. All melanomas examined were of cutaneous origin (Fig. 1*a,b*) and could be classified as benign or malignant, melanotic or amelanotic, as described for Xi-

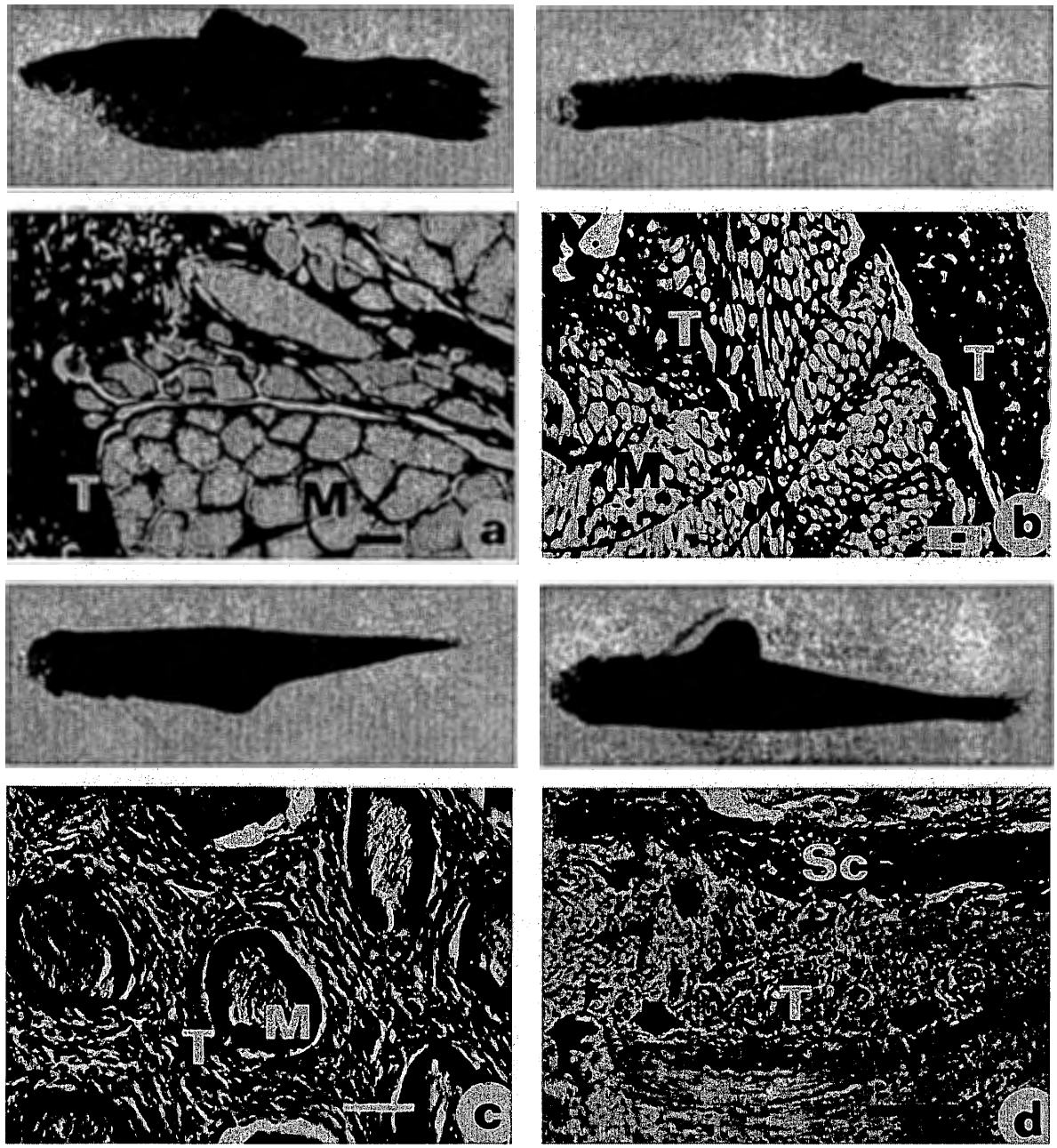

FIGURE 1 – Habitus and histological overview of the tumors. *(a)* Spontaneous hereditary melanoma in the dorsal fin, the dorsal and ventral posterior part of the trunk and tail fin of a back-cross hybrid *X.mac* Sd/*X.hell.* BC_1. Transversal section showing the melanoma invading the muscles (HE stain). *(b)* Induced melanoma in the upper posterior part of the trunk of a back-cross hybrid *X.var.Li/X.hell.* BC_n. Transversal section displaying the melanoma invading the muscles (HE stain). *(c)* Induced fibrosarcoma on the left body side underneath the dorsal fin of a back-cross hybrid *X.var. Li/X.hell.* BC_n. Transversal section demonstrating the spindle-shaped tumor cells invading between the muscle bundles (SA stain). *(d)* Induced pigmented fibrosarcoma on the right side of the body behind the pectoral fin of a back-cross hybrid *X.var. Li/X.hell.* BC_n. Parasagital section illustrating the tumor cells destroying the skeletal muscles, dispersed pigment cells (arrows) and melanin sedimentation (arrow heads). The interspersed pigment cells result in a dark coloring of the exophytic compartment of the tumor (HE stain). M, muscle; Sc, stratum compactum; T, tumor; bars represent 50 μm.

phophorus hybrids (Vielkind *et al.*, 1971; Riehl *et al.*, 1985), of either early or late onset (Wakamatsu *et al.*, 1984). All tumors were well vascularized. The fibrosarcomas (Fig. 1*c,d*) originated in the soft tissue of the trunk. They showed fast, nodular and infiltrative growth. In exophytic tumors the transformed cells penetrated the stratum compactum. Most tumor cells were poorly differentiated and organized in bundles or showed typical whirls after destruction of muscle bundles. Vascularization was only poorly developed. All fibrosarcomas were classified as malignant.

Expression in spontaneously developing melanoma caused by the X-chromosomal Tu-Sd *X*mrk *locus*

In fish of this genotype the benign or malignant state of the melanoma is genetically controlled by the presence or absence of *R* (regulatory gene, controlling expression of *Tu*), thus making it possible to establish correlations of expression levels with tumor malignancy. All melanoma analyzed in this study contained, in addition to the 5.8-kb transcript encoded by the proto-oncogenic *INV* locus (Fig. 2; *Xmrk*, 16) the major 4.7-kb transcript encoded by the oncogenic *Tu-Sd* locus of *Xmrk*. The amount of the *INV* transcript was low. There was some minor variation in expression which followed the expression pattern of the oncogene transcript. The oncogene transcript was present in all single or pooled malignant melanomas at very high levels. The absolute amounts varied considerably between different melanomas; comparison of transcript abundance in *Sd* melanomas of different malignancy (Fig. 2, 1 to 13; Fig. 3, 1 to 3) revealed a positive correlation with the malignant phenotype of the melanoma. A melanoma cell line (PSM) contained the highest amounts of transcripts. In RNA of the immortalized embryonic cell line, A2, only transcripts of the *INV* locus were detectable at low levels.

A single 5.0-kb *Xerb* B transcript was detected in most of the melanoma mRNA at very low levels (Fig. 2). No obvious correlation between expression levels of *Xerb* B and tumor malignancy was observed.

For *Xfms* a single transcript of approximately 6.0 kb was observed in most of the malignant melanoma of the *Tu-Sd* genotype (Fig. 2). The level of expression ranges from relatively high to only barely detectable, indicating no obvious correlation of the expression of *Xfms* with tumor malignancy or expression of the *Xmrk* gene. Whereas in several malignant lesions a clear expression was found, no expression of *Xfms* was detectable in the melanoma cell line and the embryonal A2 cells.

Hybridization with the *Xsrc* cDNA probe revealed a major (*Xsrc*1, 3.7 kb) and a minor *Xsrc* transcript (*Xsrc*2, 3.4 kb) (Fig. 2). The level of expression of *Xsrc*1 in most malignant melanomas was high. However, the correlation with malignancy is less obvious than for *Xmrk*. The *Xsrc*2 transcript was detected in 7 out of 13 melanomas. Interestingly, the melanoma cell line PSM contained only the *Xsrc*2 transcript, and the embryo-derived cell line A2 only the *Xsrc*1 transcript.

Three *Xras*-transcripts (*Xras*1, 3.4 kb; *Xras*2, 3.0 kb; *Xras*3, 1.8 kb; Fig. 2) can be detected in Xiphophorus. *Xras*1 and 3 were present at barely detectable levels in the *Tu-Sd* melanoma. The major transcript *Xras*2 was found in 5 of 13 melanomas in relatively high amounts. Hybridization with the *Xsis* probe revealed very low amounts of a 3.4-kb *Xsis* transcript. Only the non-transformed A2 cells contained slightly higher amounts of the 3.4-kb and a second *Xsis*-transcript (data not shown). With few exceptions, only basal expression of *Xmyc* was seen in the melanomas. Multiple transcripts were, however, readily detectable in both cell lines (Fig. 2).

Expression in spontaneously developing melanoma caused by other sex-chromosomal Xmrk *loci*

To investigate the role of *Xmrk* in spontaneously developing melanoma caused by *Xmrk* alleles encoded by other *Tu* loci than *Tu-Sd* (marked by other pigment cell patterns and by different pathological features of the tumors), we analyzed RNA of single or pooled melanoma of such fish. Hybrids containing either the X-chromosomal *Tu-DrLi* or the Y-chromosomal *Tu-Sr''*, *Tu-Sb*, or *Tu-N*^{2} oncogenic alleles of *Xmrk* spontaneously develop highly malignant, progressively growing melanotic melanoma with a frequency of approximately 15% during senescence. These melanoma develop from superficially spreading non-invasive melanoma which occur in all fish that carry the corresponding *Xmrk* alleles. They are classified as late-onset hereditary melanomas. This is in contrast to *Tu-Sd* melanomas, which develop early in life.

RNA from different malignant melanomas of such hybrids contained, in addition to a low level of the 5.8-kb *INV-Xmrk* proto-oncogene transcript, relatively high amounts of the 4.7-kb transcript (Fig. 3). As in *Sd* melanomas, the low level of the *INV* transcript showed some variation that followed the pattern of the oncogene transcript. For the oncogene transcript, in contrast to the expression from the *Sd* locus, transcript levels of the *DrLi* locus were found to be more variable between different malignant melanomas. RNA of the highly malignant melanomas caused by Y-chromosomal oncogenic loci (*Sr''*, *Sb*, *N*^{2}) uniformly contained amounts of the 4.7-kb transcript similar to or higher than RNA from melanomas caused by the *Sd* locus. RNA of 2 of these melanomas (9, 12) contained in addition a *Xmrk* transcript of very large size. One genotype was included that carries a *Tu* allele of low penetrance (*Tu-ArSr'*). In melanomas of such fish, the corresponding *Xmrk* allele was also found to be over-expressed (Fig. 4).

Using the same RNAs for hybridization, very low expression of *Xerb* B (Fig. 3) and of *Xmyc* was observed. In all melanomas the *Xfms* transcript was detected (Fig. 3). Again, as observed for the *Tu-Sd* melanomas, the levels of transcript ranged from relatively high to only barely detectable.

Hybridization with the *Xsrc* cDNA revealed both *Xsrc* transcripts at variable, but sometimes very high levels (Fig. 3). *Xsrc*2 transcripts were detected only in those melanomas showing the highest levels of *Xsrc*1 transcripts. For *Xras* only the major *Xras*2 transcripts were detected at varying levels. Expression of *Xsis* was observed in RNA of melanomas caused by Y-chromosomal *Xmrk* loci, but at relatively low levels (data not shown).

Expression in chemically induced neoplasms of Xiphophorus

To investigate whether the activity of the different sex-chromosomal *Xmrk* loci is restricted to spontaneously developing (hereditary) melanomas, RNA of chemically induced malignant neoplastic lesions of Xiphophorus was analyzed. In 2 fibrosarcomas (Fig. 4*a*), no transcripts of *Xmrk* were detected. RNA of an induced melanoma contained low amounts of a very large transcript (approximately 9 kb). Such large-size transcripts were also observed in several hereditary melanomas (Fig. 2, 9 to 11; Fig. 3, 9 to 13) and may represent a hnRNA. The mature transcripts from the proto-oncogene (5.8 kb) and the oncogene (4.7 kb) were not detected in induced tumors.

No transcripts of *Xerb* B or *Xfms* were observed. With the *Xsrc* cDNA probe (Fig. 4*b*) very high amounts of *Xsrc*1 were found in both fibrosarcomas, while the melanoma RNA contains only low amounts. *Xsrc* 2 was barely detectable. *Xras* 2 transcripts were found to be present in all 3 induced neoplasms at relatively low levels and comparable to that observed in RNA of most of the hereditary melanomas. *Xras* 1 and 3 transcripts were again not observable. The level of *Xsis* expression was low, but slightly higher than observed in hereditary melanomas (data not shown). Multiple *Xmyc* transcripts were found in the induced neoplasms at low levels. Both fibrosarcomas contain several small transcripts (approx. 1.6 kb) which were not present in the induced melanomas or in most of the spontaneously developing melanomas.

DISCUSSION

In this study we have shown that 27 hereditary malignant melanomas originating from 7 different X- and Y-chromosomal alleles of *Tu* over-express the 4.7-kb *Xmrk* transcript. The abundance of this transcript correlated with the malignancy of the melanoma. For the *Tu-Sd* allele of *Xmrk*, we have

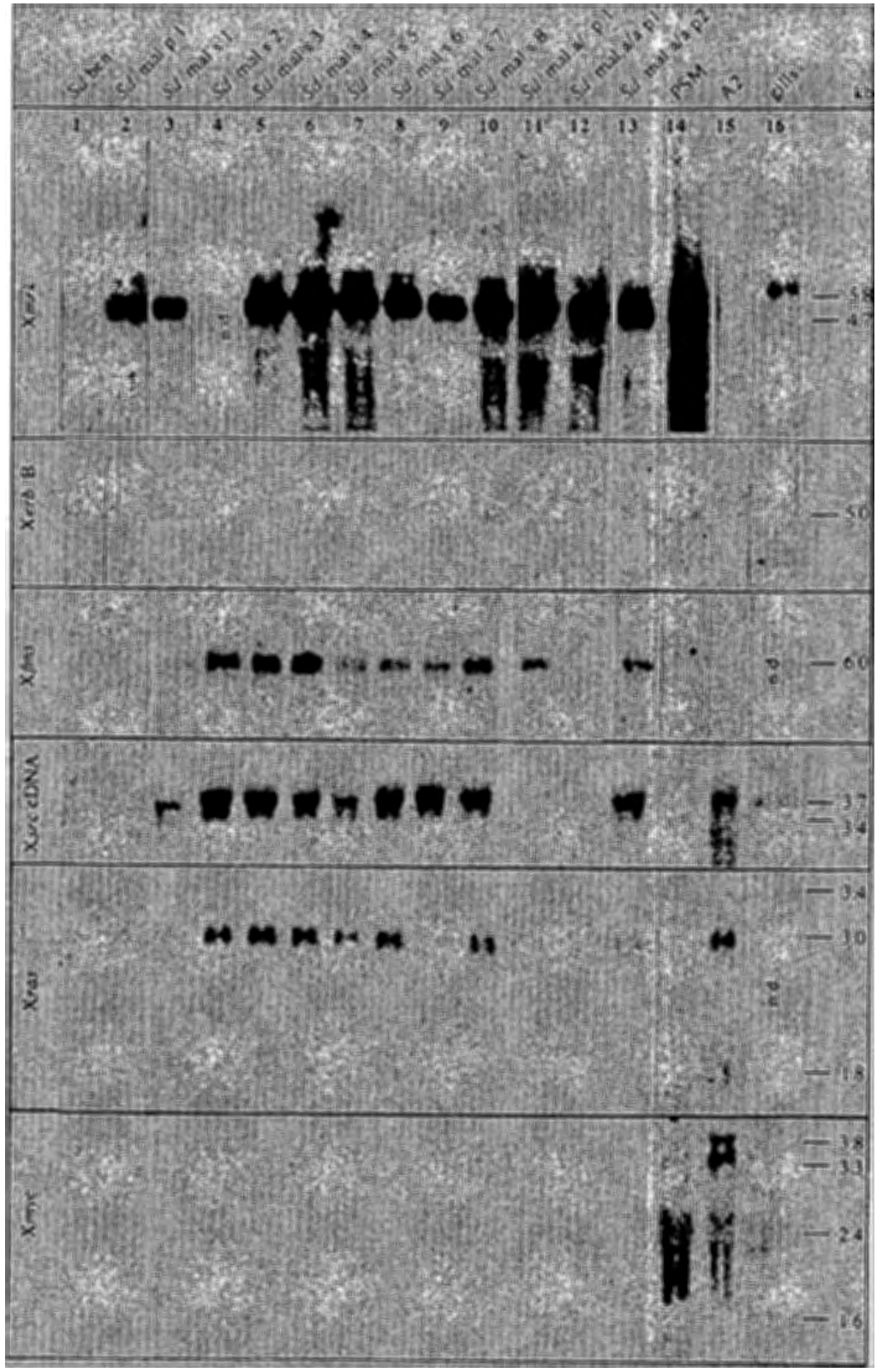

FIGURE 2 – Expression of X*mrk*, X*erb* B, X*fms*, X*src*, X*ras* and X*myc* in RNA of single and pooled *Tu-Sd* melanoma of Xiphophorus; 20 µg of total RNA of each sample were hybridized with the X*mrk*, X*erb* B v-*fms*, v-*ras* and T*myc*-probe as described in "Material and Methods". Lane 1, benign (ben) melanotic melanoma; lanes 2 to 10, highly malignant (mal) melanotic melanoma; lane 11, highly malignant melanotic melanoma from fish heterozygous for *a* (albino); lanes 12 and 13, malignant amelanotic melanoma from fish homozygous for *a* (albino); lane 14, PSM melanoma cell line; lane 15, A2 embryonal cell line; lane 16, *X. maculatus* gills; p, pooled biopsy material; s, individual melanoma; n.d., not done. Final washings were: for X*mrk*, 55°C, 0.5 × SSC (1, 2, 11); 60°C, 0.1 × SSC (3–10, 12–14); for X*erb* B, 50°C, 1 × SSC; for v-*fms*, 55°C, 0.5 × SSC; for X*src* cDNA, 50°C, 1 × SSC; for X*ras*, 50°C, 2 × SSC; for X*myc*, 45°C, 2 × SSC (14, 15); 50°C, 2 × SSC (1–12, 16).

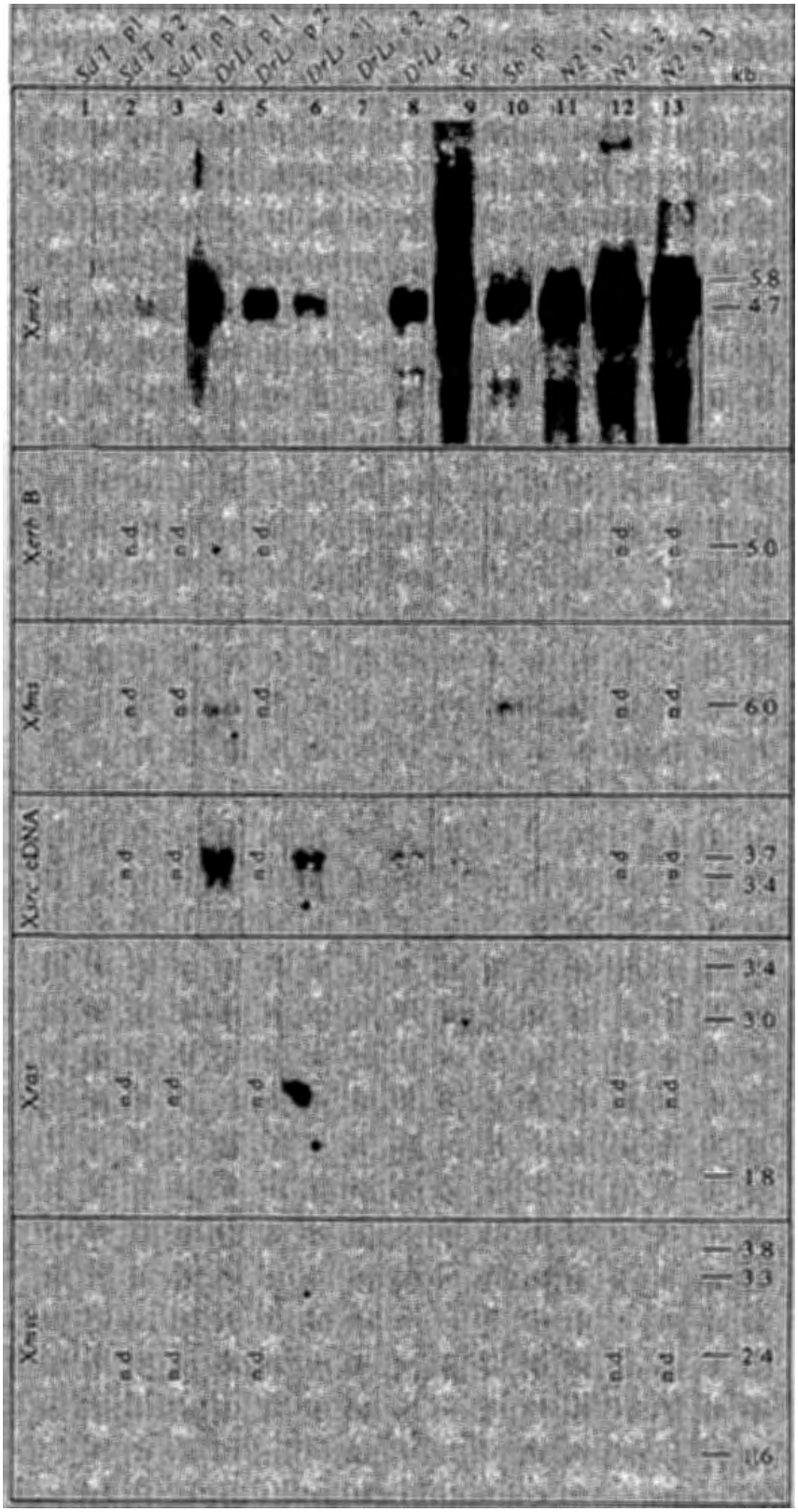

FIGURE 3 – Expression of *Xmrk*, *Xerb* B, *Xfms*, *Xsrc*, *Xras* and *Xmyc* in RNA of single and pooled neoplasia of Xiphophorus caused by different *Tu* alleles; 20 μg of total RNA of each sample were hybridized as indicated in Figure 2. Lanes 1 to 3, benign melanotic *SdT* melanoma; lanes 4 to 8, malignant melanotic *DrLi* melanoma; lane 9, highly malignant melanotic *Sr*" melanoma; lane 10, highly malignant melanotic *Sb* melanoma; lanes 11 to 13, highly malignant melanotic N^2 melanoma. p, pooled biopsy material; s, individual melanoma; n.d., not done. Final washes were: for *Xmrk*, 50°C, 1 × SSC (5–8); 55°C, 0.5 × SSC (2, 10, 13); 60°C, 0.5 × SSC (1, 9, 12); 60°C, 0.1 × SSC (3, 4, 11); for *Xerb* B, 50°C, 1 × SSC; for *Xfms*, 55°C, 0.5 × SSC; for *Xsrc* cDNA, 50°C, 1 × SSC; for *Xras*, 50°C, 2 × SSC; for *Xmyc*, 45°C, 2 × SSC (2–8) 50°C, 2 × SSC (1, 9–11).

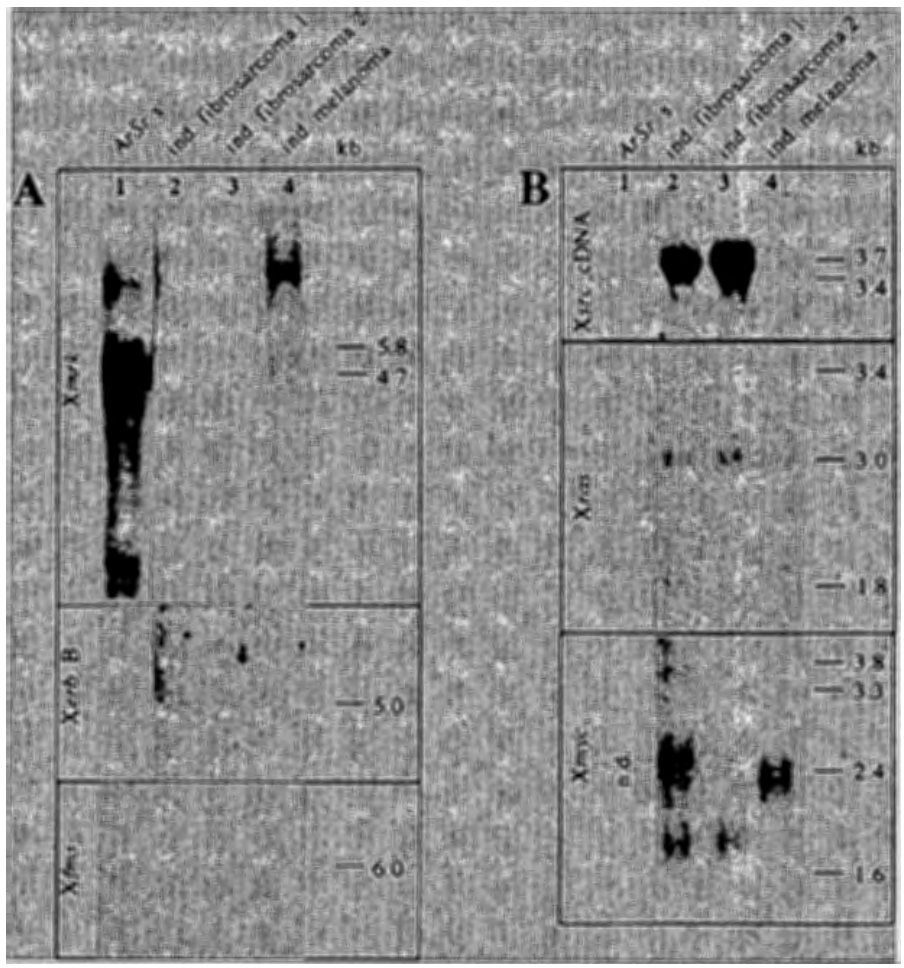

FIGURE 4 – Expression of *(a) Xmrk, Xerb* B, *Xfms* and *(b) Xsrc, Xras, Xmyc* in RNA of single neoplasm of Xiphophorus; 20 μg of total RNA of each sample were hybridized as indicated in Figure 2. Lane 1, spontaneously developing highly malignant melanotic *Sr* melanoma; lanes 2–4, highly malignant chemically induced individual neoplasm; n.d., not done. Final washes were: for *Xmrk*, 60°C, 0.1 × SSC (1); 60°C, 0.5 × SSC (2–4); for *Xerb* B, 50°C, 1 × SSC; for *Xfms*, 55°C, 0.5 × SSC; for *Xsrc* cDNA, 50°C 1 × SSC (2–4); all others 50°C, 1 × SSC.

previously shown that its transcription is controlled by a 5′ upstream region different from that of the corresponding proto-oncogene (Adam *et al.*, 1991). The new promoter was acquired during evolution by non-homologous recombination from a hitherto uncharacterized locus (Adam *et al.*, 1993). Over-expression of other *Xmrk* alleles from other *Tu* loci in malignant melanoma of the corresponding hybrids, as shown in this study, indicates similar genomic organization and mode of oncogene activation. The acquisition of the oncogene promoter would thus date back to an early stage in the evolution of these fish, since some of the different *Xmrk* alleles investigated are derived from feral populations that have been zoogeographically separated for a long time.

The level of over-expression in malignant melanomas was found to be variable to some degree. In general, a positive correlation between over-expression of *Xmrk* and degree of malignancy could be established. Moreover, since undissected melanomas were used in this study, it is possible that the minor variations are due to differences in distinct tumor compartments (*e.g.*, well-vascularized exophytic- and/or rapidly progressing invasive compartments) of the individual melanoma. As shown earlier (Mäueler *et al.*, 1987), such compartments are characterized by differences in intermediary metabolism.

In contrast to the quantitative differences of the *Xmrk* oncogene transcripts in benign and in highly malignant melanomas, the expression of the proto-oncogene remains more or less on a basal level. In some tumors with very high oncogene-transcript levels, a 2- to 3-fold increase in the proto-oncogene mRNA is apparent. This may be explained by a general activation of transcription in these progressive tumors. A similar phenomenon has been observed for expression of the proto-oncogenes *Xsrc, Xras* and *Xmyc*. This is consistent with earlier findings *in vitro* (Mäueler *et al.*, 1988*a*).

Some of the hereditary melanomas contain additional high-molecular-weight transcripts of *Xmrk*. These melanomas were classified as extremely malignant, and displayed the highest amounts of *Xmrk* transcripts. It is possible that these high-molecular-weight transcripts are precursor RNA that is incompletely spliced, probably due to a high transcription rate of the gene. Additional transcripts of abnormal size of an over-expressed human *EGF-R* gene were also observed in glioblastomas (Libermann *et al.*, 1985), as well as in cells derived from squamous-cell carcinomas (Yamamoto *et al.*, 1986) and mammary-carcinoma cells (King *et al.*, 1985; Filmus *et al.*, 1985). Our data, however, do not support the hypothesis of Zechel *et al.* (1992) that such a large *Xmrk*-oncogene transcript is

mRNA specific for the *Tu-Li* locus of *X. variatus*, because we also detect it besides the mature message in melanomas of other genotypes.

In contrast to the situation in hereditary melanomas, mature transcripts of the *INV* or of the oncogenic *Xmrk* locus were not detected in any of the induced tumors. Only the induced melanomas contained a probably unspliced high-molecular-weight *Xmrk* transcript. Earlier hypotheses assumed that one and the same locus, namely *Tu*, was responsible for the development of hereditary melanomas as well as for the neoplastic transformation of cells after treatment with chemical carcinogens (Anders, 1989; Anders *et al.*, 1984; Schwab *et al.*, 1978), or even for tumors of every possible etiology (Zechel *et al.*, 1992). Our data clearly indicate that all hereditary melanomas are caused by over-expression of oncogene alleles of *Xmrk*. Hence, chemical induction of neoplasia including melanoma in Xiphophorus is mediated by activation of other as yet unidentified proto-oncogenes. In view of the clear dichotomy of melanomagenesis in Xiphophorus on the molecular level, it is tempting to discuss whether such dichotomy is also seen in human hereditary tumors and their spontaneous counterparts. We are not aware of any molecular data on this problem. However, there are some clues that point in this direction. For example, in human spontaneous melanoma a plethora of different activated genes, including *ras* genes, growth-factor genes, and p53 have been found (Albino, 1992). At least 3 different chromosomal regions have been implicated in the formation of familial melanoma (for review, see Travis, 1992) none of which relates obviously to the activated genes found in spontaneous melanoma. Similarly, for familial Wilms tumor 3 different loci have been identified, each of which can provide as a tumor-suppressor gene the predisposition to develop this cancer (Francke, 1990). There might be multiple ways on the molecular level to create tumor phenotypes that are otherwise indistinguishable.

In order to study the function of genes closely related to *Xmrk* in hereditary melanomas and induced neoplasia, we measured the expression of *Xerb* B and of *Xfms*. The expression of the *Xerb* B gene was found to be very low in all tumors analyzed. A correlation with tumor malignancy or expression of *Xmrk* was not apparent. Obviously, expression of *Xerb* B is of subordinate importance for these tumors, and is not co-regulated with *Xmrk*. Such a co-regulation of closely related members of a gene family, however, has been observed for *Xyes*, *Xfyn* and *Xsrc* (Hannig *et al.*, 1991). Most of the hereditary melanomas contained relatively high amounts of the *Xfms* transcript. The level of expression is not correlated with the malignancy of the melanoma or with the expression of *Xmrk*. In addition, none of the induced highly malignant neoplasms contained detectable amounts of *Xfms* transcripts. In mammals, *CSF-1 R* is one of the factors playing an important role during maturation and growth activation of macrophages (Sariban *et al.*, 1985; Sherr, 1988; Sherr *et al.*, 1985). Macrophages are present at places that give rise to infection. Necrotic compartments of fish melanomas are often infected by fungi or bacteria which produce an inflammatory response. Therefore an explanation for the variability of *Xfms* expression in the melanomas could be the presence of different amounts of macrophages expressing the *Xfms* gene. This observation is confirmed by the finding that the melanoma cell line PSM does not express the *Xfms* gene. The expression data on *Xmyc*, *Xras* and *Xsis* were not of special interest, they were rather low, and did not correlate with tumor malignancy or etiological origin. Altered expression of these genes, therefore, does not appear to be a relevant factor in tumorigenesis in Xiphophorus.

Expression studies with the Xiphophorus-specific *src* cDNA probe revealed 2 different transcripts. Both transcripts were also detected in normal brain and eyes, thus none is specific for tumor cells (Mäueler *et al.*, 1988*b*; Raulf *et al.*, 1989). The level of expression in most of the neoplasms is higher than in any normal tissue. Earlier experiments revealed approximately 10-fold increased $pp60^{c\text{-}src}$ activity encoded by the *Xsrc*2 transcript of the PSM cells in comparison with that from the *Xsrc*1 transcript of A2 cells (Mäueler *et al.*, 1988*a*). Elevated $pp60^{c\text{-}src}$ kinase activity has been observed in correlation with malignancy in hereditary melanoma and in carcinogen-induced tumors of mesenchymal origin (Schartl *et al.*, 1985). Our RNA expression data are in perfect agreement with these earlier finding. The *Xsrc* gene has been shown to be localized on an autosome and is therefore independent of the sex-chromosomal *Tu* locus encoding *Xmrk*. As the latter is the primary melanoma-inducing gene, activation of *Xsrc* is a downstream event in melanomagenesis. Its association with malignancy of hereditary melanomas and also the activation in several carcinogen-induced tumors of different histiotypes, including fibrosarcoma and melanoma, lead to the assumption that *Xsrc* is an important and necessary factor in tumor progression.

In summary, we have demonstrated that there exist at least 2 molecular mechanisms leading to malignant melanomas in Xiphophorus: *Xmrk* over-expression leading to hereditary melanomas, and the activation of unknown proto-oncogenes causing induced melanomas. The *Xsrc* gene appears as an important factor downstream of the primary event, possibly necessary for tumor progression. Further experiments, screening a much larger population of different induced neoplasms of different histiotypes, for transcripts of as many proto-oncogenes as possible, are needed to identify more of the factors involved in the multistep process of carcinogenesis, and to distinguish phenomena that are common to different cancer cells *in vivo* from those that are specific to the histiotype.

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