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MOLECULAR ASPECTS OF MELANOMA FORMATION IN *XIPHOPHORUS*[1]

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INTRODUCTION

Studies over the last years have suggested the involvement of specific cellular genes in the pathogenesis of human, rodent and avian neoplasms. These genes have been identified by their biological activity in transfection assays by transferring the neoplastic phenotype to recipient cells and/or by homology to the transforming genes of known acutely transforming retroviruses. In the transforming (i.e. activated) state these genes are designated oncogenes, while in the non-transforming (i.e. inactive) state they are referred to as proto-oncogenes (for a recent review see Bishop 1986) Conversion of the non-transforming proto-oncogene into a transforming oncogene has been shown to be possible by either qualitative changes and/or quantitative changes of the oncogene product. Most of the known oncogenes can be classified into one of 4 major categories: those, like the *src*, *yes*, *abl*, and *erb* B oncogenes, which code for protein-tyrosine kinases; those whose products have GTP-binding activity, like the *ras*-oncogene family; those with structural homology to growth factors, like the *sis* oncogene product; and those oncogenes whose protein products are localized in the nucleus, like *myc*, *myb* and *fos*. To contribute to an understanding of the function of oncogenes, we are using the *Xiphophorus* fish melanoma

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1) Contains parts of the Ph.D. theses of Winfried Mäueler, Friedrich Raulf and Scott M. Robertson.

system, which provides the opportunity to study in a genetically well defined system oncogenes during embryogenesis and normal cell differentiation, as well as during melanoma formation.

In Xiphophorus, certain hybrid genotypes develop spontaneous malignant melanoma. Melanoma formation has been attributed by classical genetic and cytogenetic findings to the overexpression of a cellular gene (assigned Tu). In non-tumorous fish Tu was proposed to be negatively controlled by cellular regulatory genes (more recently termed anti-oncogenes by some authors) (for review see Anders et al., 1984). In a typical crossing experiment a female Xiphophorus maculatus (platyfish) containing a specific Tu-locus and its corresponding regulatory genes is crossed with a male Xiphophorus helleri (swordtail), which is thought not to contain this particular Tu-locus and its corresponding regulatory genes. Further backcrossing of the Tu-containing hybrids to Xiphophorus helleri results, in effect, in the progressive replacement of regulatory gene bearing chromosomes originating from the Xiphophorus maculatus by chromosomes of Xiphophorus helleri (apparently regulatory gene free). This stepwise elimination of regulatory genes therefore allows increased expression of Tu (i.e. activation of the proto-oncogene), resulting in the development of malignant melanoma.

To date, the molecular nature of Tu and its corresponding regulatory genes is totally unknown. Recently, however, we could show that the degree of expression of Tu is paralleled by the level of enzymatic activity - protein-tyrosine kinase - of the gene product ($pp60^{c\text{-}src}$) of the c-src gene (Schartl et al., 1982, Schartl et al., 1985). This striking observation led us to question whether c-src and perhaps also other known oncogenes are involved in melanoma formation in Xiphophorus. Secondly, we are investigating the molecular basis for the apparent correlation in Tu and c-src expression.

ONCOGENE EXPRESSION IN NON-TRANSFORMED CELLS.

Reasoning that an understanding of the normal cellular functions of proto-oncogenes would give an indication as to the role of the activated oncogenes in tumor cells, we have examined the expression of several oncogenes in non-transformed tissues of adult fish and during normal embryogenesis. RNAs prepared from adult fish organs and from developmentally staged embryos of a tumor-free genotype were analyzed by Northern blot hybridization. The probes used for identifying transcripts were either derived from cloned *Xiphophorus* proto-oncogenes or from the corresponding viral oncogenes. In an attempt to increase the sensitivity of transcript detection we have used, when possible, radioactively labelled *in vitro* RNA transcripts ("Riboprobes") as hybridization probes.

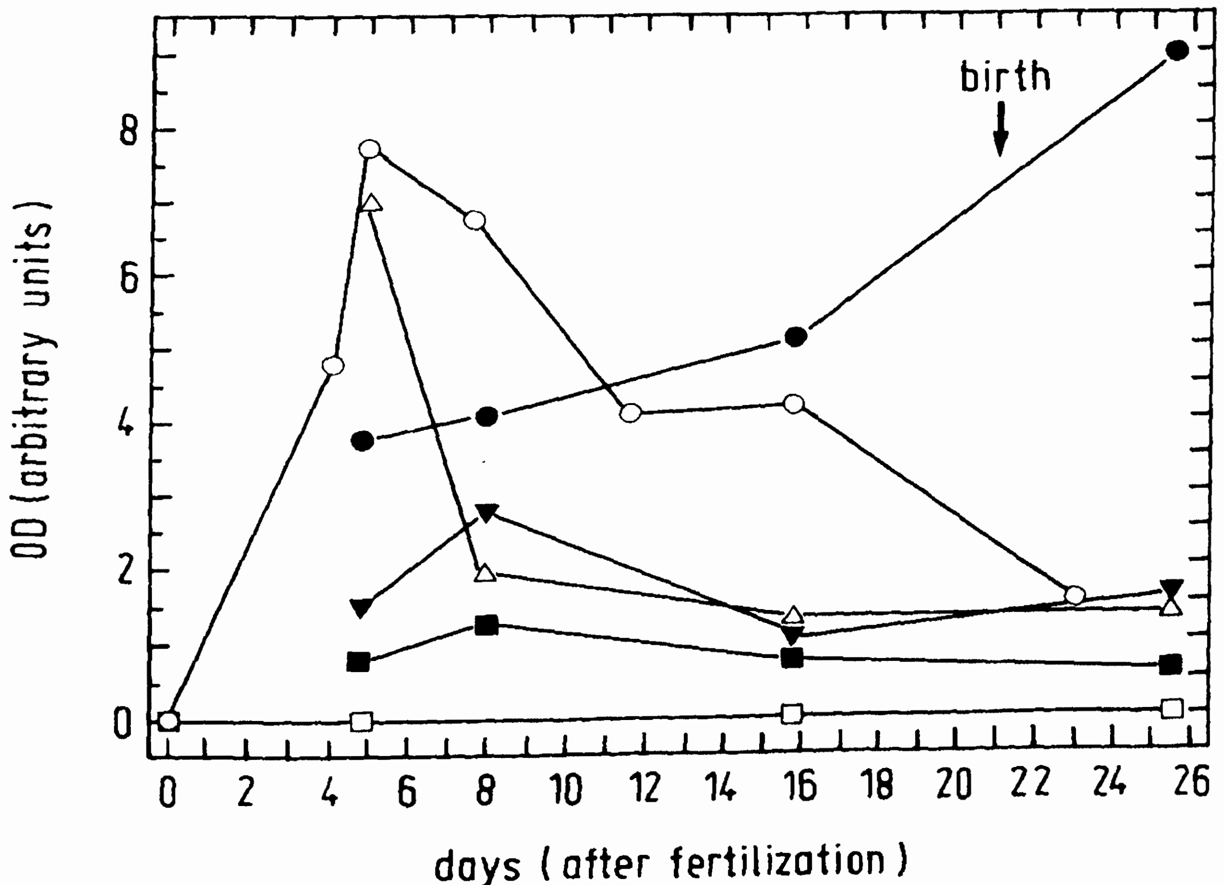

Fig. 1. Semiquantitative normalization of proto-oncogene expression during embryonic and neonatal development of *Xiphophorus helleri*. ○-○ : c-*src*, ●-● : c-*sis*, □-□ : c-*erb* B, ■-■ : c-*ras* 1, ▼-▼ : c-*ras* 2, △-△ : c-*ras* 3.

The pattern of proto-oncogene expression during embryogenesis and in different organs of adult *Xiphophorus* is differential. (Fig. 1 and 2). The c-*erb* B gene shows no expression during embryogenesis and in adults except for barely detectable levels in eyes. A c-*erb* A transcript has so far only been detected in skeletal muscle. Elevated c-*sis* expression was found in 5 day old neonate fish, which is the period of development marked by maturation of the immune system. No c-*sis* expression has been detected in adult fish. c-*myc* expression was detected in adult brain and liver. For c-*src*, no transcripts could be detected during early stages of embryogenesis (cleavage to late neurula). However, during organogenesis stages a considerable elevation of c-*src* expression is apparent, which then later in embryogenesis decreases to the basal level found in neonate and young fish. In adults, the c-*src* gene is preferentially expressed in brain and eyes. For *ras*-related genes three transcripts of different size have been detected, each of which is differentially expressed during embryogenesis with maximal levels of each at different stages of organogenesis. In adults, c-*ras* at varying levels of expression has been found in nearly all organs tested.

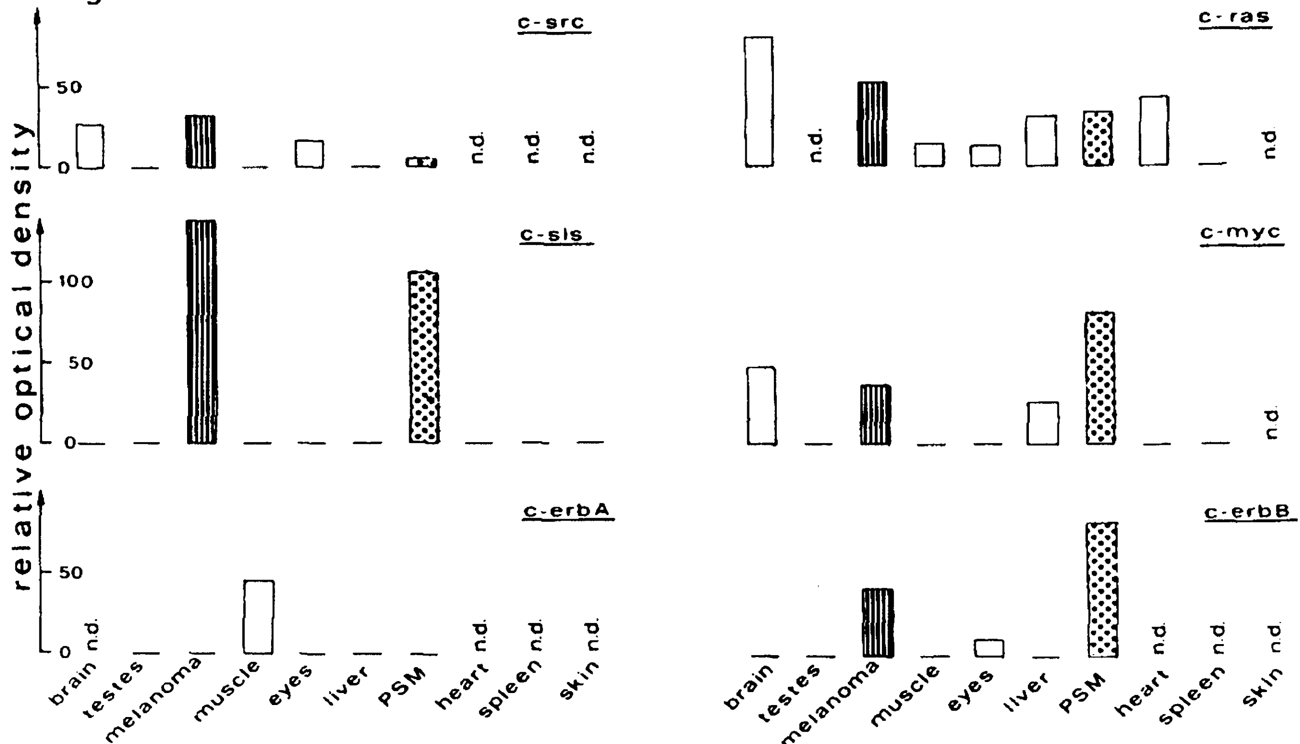

Fig. 2. Expression of c-*src*, c-*sis*, c-*erb A*, c-*erb B*, c-*ras* and c-*myc* in non-tumorous organs, in melanotic melanoma and in a melanoma derived cell line (PSM).

The tissue-type specificity of proto-oncogene expression and the failure to detect proto-oncogene transcripts in the early embryo, when undifferentiated cells undergo rapid cell division, led us to the assumption that the physiological role of these genes is more likely confined to the differentiated state of specific cell types rather than to cell proliferation in general.

ONCOGENE EXPRESSION IN MELANOMA CELLS.

In malignant melanotic melanomas from platyfish/swordtail backcross hybrids carrying a derepressed (i.e. activated) Tu-gene (the Sd gene complex of X.maculatus originating from Rio Jamapa, Mexico - see Anders et al., 1984, for a more detailed description of fish genotypes and specific Tu-gene complex), a variety of oncogenes have been found to be expressed. The same is also true for a melanoma-derived cell line, PSM 1a, established from a Xiphophorus melanoma by Y. Wakamatsu (Wakamatsu et al., 1984). The level of c-ras expression observed in the melanomas does not exceed the levels found in normal organs. The amount of c-src and c-myc m-RNA in melanoma cells, however, was found to be higher than in any non-transformed tissue. The c-src expression data indicate that reported elevated kinase activity of $pp60^{c\text{-}src}$ in Xiphophorus melanoma reported earlier (Schartl et al., 1982, 1985) may be due at least in part to an elevated level of c-src messenger RNA in the tumor cells. The c-sis and the c-erb B genes, which show no or only barely detectable levels of expression in non-tumorous tissues, are both expressed to considerable levels in the tumor and in the tumor derived cell line. These data indicate that probably a variety of cellular proto-oncogenes within the same tumor can undergo de-regulation and that several oncogenes might be involved in the multistep process of tumor formation and tumor progression.

MOLECULAR CLONING OF XIPHOPHORUS PROTO-ONCOGENES.

For studies on gene regulation, the availability of the molecularly cloned gene in question and its flanking regulatory sequences is the experimental precondition. Therefore, we constructed genomic libraries from the platyfish Xiphophorus maculatus using the phage EMBL 4 as

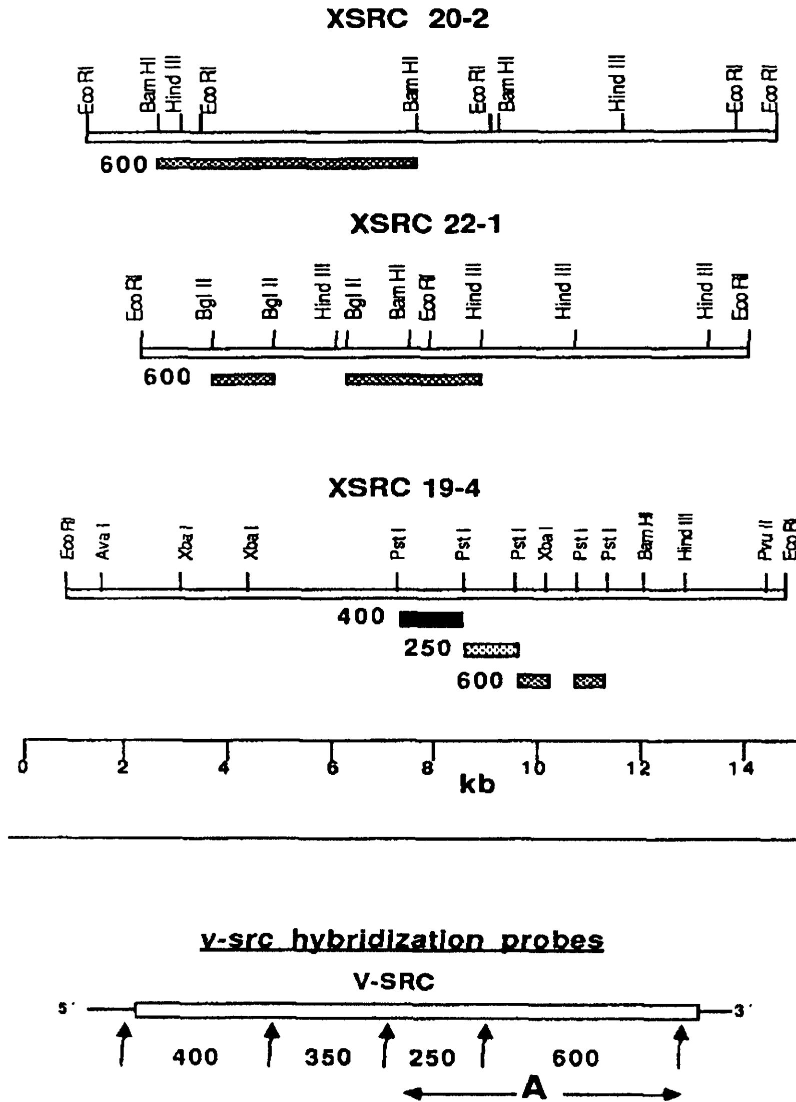

Fig. 3. Restriction maps of three clones with homology to the tyrosine kinase domain of the src gene isolated from a genomic library. A, indicates the tyrosine kinase domain.

cloning vector. To isolate the c-src gene and related members of the tyrosine-kinase gene family, the library was screened with the 600 bp Pst I fragment (F) from the 3'-half of the viral src gene of Rous sarcoma virus (strain Schmidt-Ruppin A), which encompasses most of the tyrosine kinase domain that is conserved to varying degrees between the members of the tyrosine kinase gene family of oncogenes. A total of 26 independent clones were isolated and several have been characterized by

restriction enzyme analysis and the mapping of v-src homologous sequences (Fig. 3). The independent nature of some of these clones was further confirmed by the following set of experiments: If the tyrosine kinase encoding domain of the viral src gene (i.e. Pst I fragment F) is hybridized to a Southern blot of Xiphophorus DNA under conditions of moderate stringency, a species-specific pattern of several bands of differing intensity is detected. (Fig. 4a). Using the same conditions of stringency but the tyrosine kinase encoding domain of another oncogene, namely the viral yes gene (which is closely related by sequence to src), the same bands are detected, but, with a differing pattern of relative intensities (Fig. 4b). Assuming that the relative intensity of a hybridization band under given conditions of strigency can be used as a crude measure of sequence homology, these data indicate that a whole family of genes is present in Xiphophorus that share to different extents homology to the tyrosine kinase domain of the src gene. Using particular fragments from the different genomic clones as hybridization probes under conditions of high stringency, only one or two bands of the v-src pattern is detected by each clone. As a first attempt to find out which members of the tyrosine kinase family of oncogenes are represented by the different Xiphophorus clones, some of the clones were hybridized to most of the known viral tyrosine kinase oncogenes. So far, one clone has proven by cross-hybridization to represent the authentic c-src gene of Xiphophorus, indicating that in Xiphophorus, as in higher vertebrates, only one copy per haploid genomes of that gene appears to be present. This clone was partially sequenced (corresponding to chicken c-src exons 8,9, and 10) and it was found that the tyrosine kinase domain of the Xiphophorus c-src gene shares about 80 % homology on the nucleotide level and about 88 % on the amino acid level with the c-src gene of chicken.

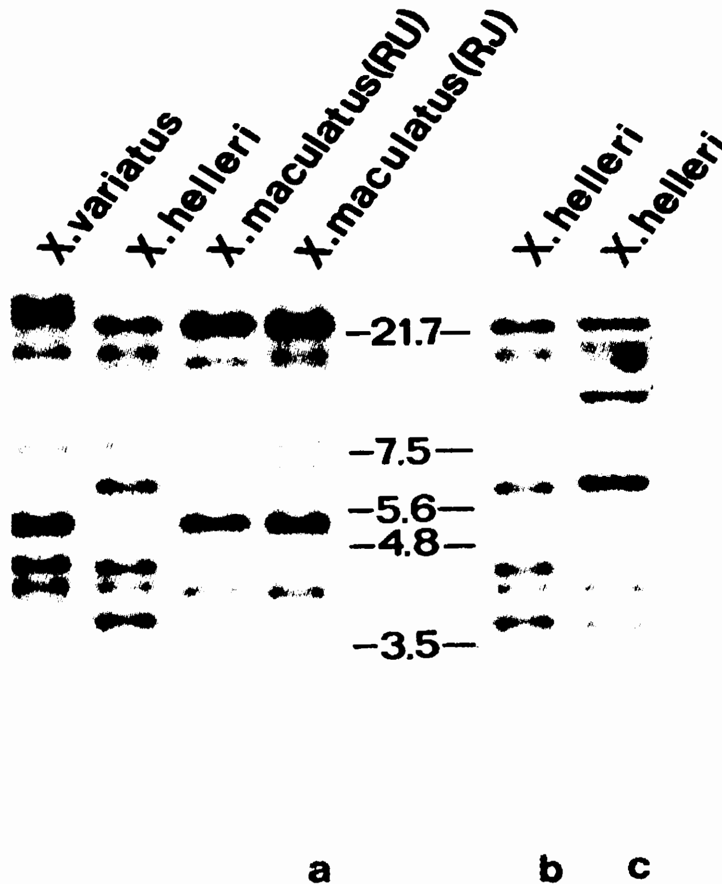

Fig. 4. Restriction fragment pattern of sequences homologous to viral oncogenes. a) Southern blot analysis of Eco RI digested DNA of different species of Xiphophorus, hybridized to v-src (600 bp PstI fragment). b) Eco RI digested DNA of Xiphophorus helleri, hybridized as in a). c) Same filter as in b), however, hybridized to v-yes (1100 bp Pst I fragment) after removing the v-src probe. RU, Rio Usumacinta, RJ, Rio Jamapa population.

METHYLATION OF C-SRC.

It is currently widely accepted that DNA-methylation plays an important role in regulation of gene expression. In earlier studies involving Xiphophorus, the overall methylation of all cytosine residues in genomic DNA was found to be relatively high - approximately 8 % (Herbert, 1983). In a first attempt to define the methylation characteristics of a specific gene of interest, we have used the following approach in analyzing c-src and related sequences. There exist, for particular DNA sequences,

several different restriction enzymes that, although recognizing the same target sequence, differ in their ability to cut that sequence depending upon the methylation state of certain bases within the sequence. For example, Dpn I and Sau 3A both recognize the sequence CATC. However, DpnI will cut only if the adenosine is methylated, whereas Sau 3A cleaves this site regardless of methylation state. Another pair of enzymes, Msp I and Hpa II, recognize the sequence CCGG, with Msp I cleaving regardless of methylation state, whereas Hpa II only recognizes unmethylated DNA. Total genomic DNA of Xiphophorus maculatus was digested with these enzymes and separated on agarose gels. Ethidium bromide staining of the DNA revealed that Xiphophorus DNA is relatively

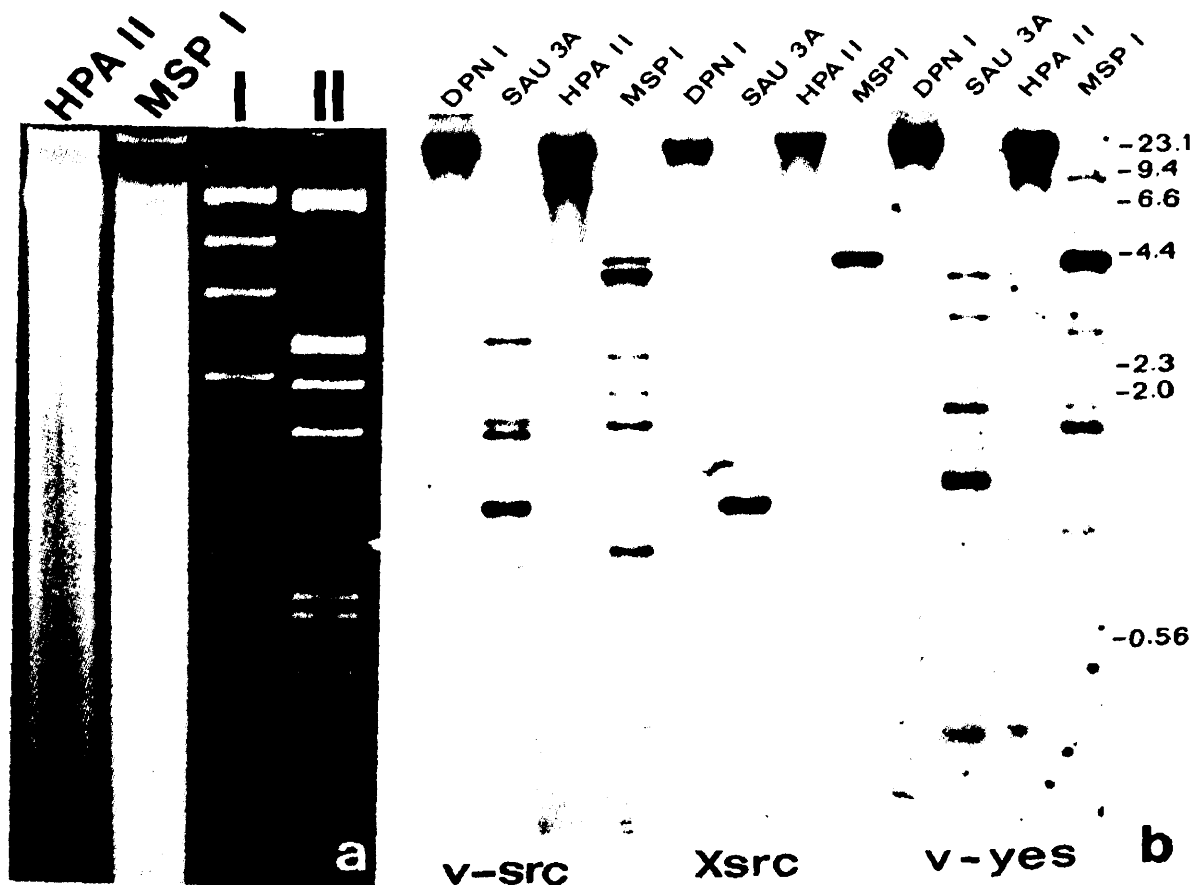

Fig. 5. Methylation of tyrosine kinase genes. a) Ethidium-bromide stained gel of Hpa II and Msp I digested DNA of Xiphophorus; I, II λ molecular weight markers; I, Hind III fragments, II, Hind III/Eco RI fragments. b) Restriction enzyme digested DNA hybridized to the v-src, the Xiphophorus c-src (Xsrc) and the v-yes genes.

resistant to digestion with Hpa II but readily digested with Msp I. (Fig. 5a). This suggests that the DNA contains a high proportion of $C^{m}CGG$ sequences in comparison to CCGG-sequences. Conversely, as the DNA is extremely resistant to Dpn I digestion but not to Sau 3A digestion, the overall methyl-adenosine content in the sequence GATC is low. Using these digested DNA's for Southern blot hybridizations with the viral *yes*, the viral *src* and the *Xiphophorus* c-*src* genes as probes, a similar methylation status is observed for these genes and their related sequences (Fig. 5b). As most sequences detected by the probes were methylated in the cytosine of the recognition sequence CCGG, and as cytosine methylation in CG sequences is thought to influence gene expression (Riggs 1975), the role of methylation/demethylation in the induction of c-*src* (and related members of the tyrosine kinase family of oncogenes) during embryogenesis and in tumor formation will be investigated in the future.

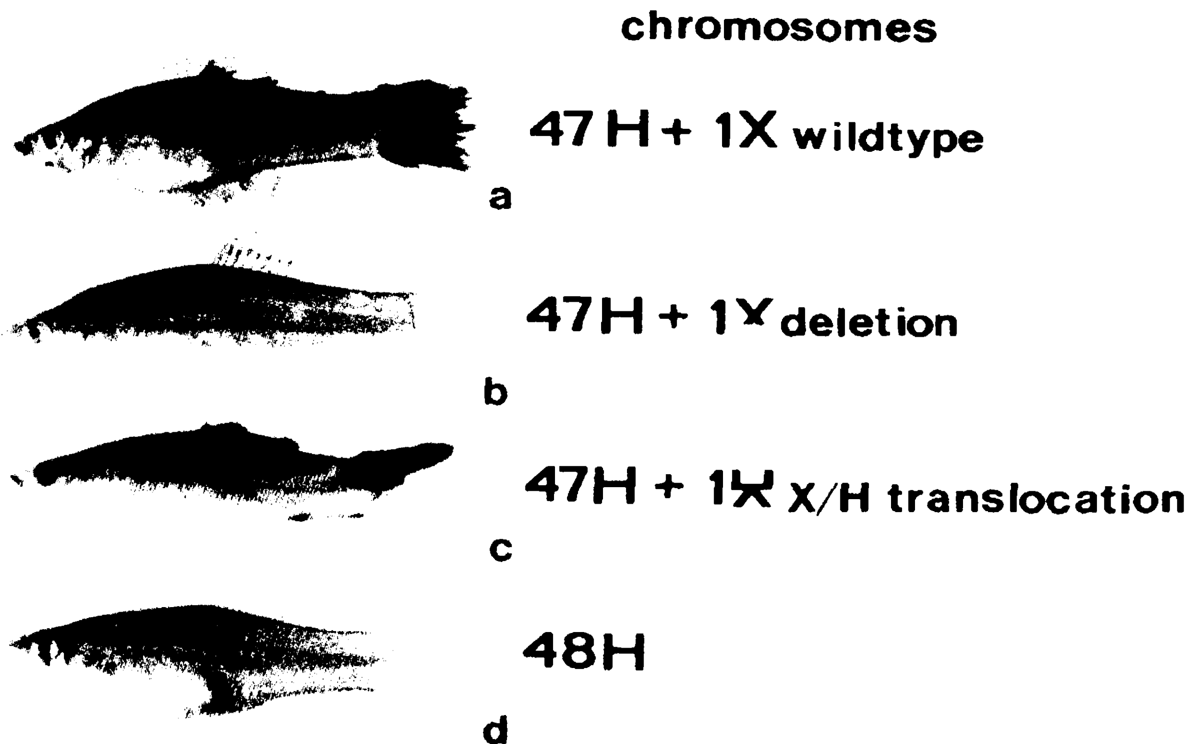

Fig. 6. Genotypes used for the assignment of known sequences to the X-chromosome of *Xiphophorus maculatus*.

ARE TU AND C-SRC ONE AND THE SAME GENE?

The apparent correlation between what is phenotypically recognized as Tu expression (i.e. melanoma severity) and what is molecularly recognized as c-src expression has led to the proposal that these two genes might be one and the same gene or at least closely-linked constituents of a coordinately regulated gene locus. We have approached this question experimentally by utilizing different mutants affecting a particular Tu locus and by analyzing by way of Southern blots the molecular arrangement of c-src and related sequences within these mutants. For the Tu-gene, which in the non-activated state gives rise to the spotted dorsal phenotype (Sd) of *Xiphophorus maculatus* from Rio Jamapa and which maps to the X-chromosome, a deletion and a translocation have been isolated by Anders and coworkers (Anders et al., 1973). It was shown that the deletion comprises the terminal portion of the X-chromosome (Ahuja et al., 1979). Platyfish-swordtail backcross hybrids carrying the Sd-deletion chromosome do not develop spontaneous melanoma as do the corresponding backcross hybrids carrying the Sd-wildtype X-chromosome. The translocation arose in a hybrid fish and was characterized as the fusion of the Sd-locus from *Xiphophorus maculatus* to an autosome of *Xiphophorus helleri*. (Fig. 6).By independently introducing the wildtype X-chromosome and the two mutant chromosomes by way of continuous backcrossing into an identical genetic background (*Xiphophorus helleri*, h III, see Fig. 6) the following analysis can be made: If a restriction fragment of a specific gene as detected by Southern blot hybridization is in the DNA of the fish carrying the wildtype and the translocation chromosome but not in the DNA of fish carrying the deletion chromosome and of fish which represent the genetic background, this restriction fragment can be assigned to the terminal region of the X-chromosome and therefore may make up part of the Tu-locus. If a fragment is detected in the wildtype, the deletion and the translocation DNA but not in the DNA of h III, this fragment would map in the vicinity of the Tu-Sd locus, e.g. to the deletion/translocation breakpoint. If the fragment is present in all DNA samples, it does not map to the X-chromosome at all. The latter was the case for c-src and all other members of the src related tyrosine kinase family as tested by using the v-src gene and the cloned *Xiphophorus* c-src gene and other related

cloned genes as hybridization probes. (Fig. 7). Thus, neither c-src nor any related tyrosine kinase gene is structurally related to Tu or linked to Tu on the same chromosome.

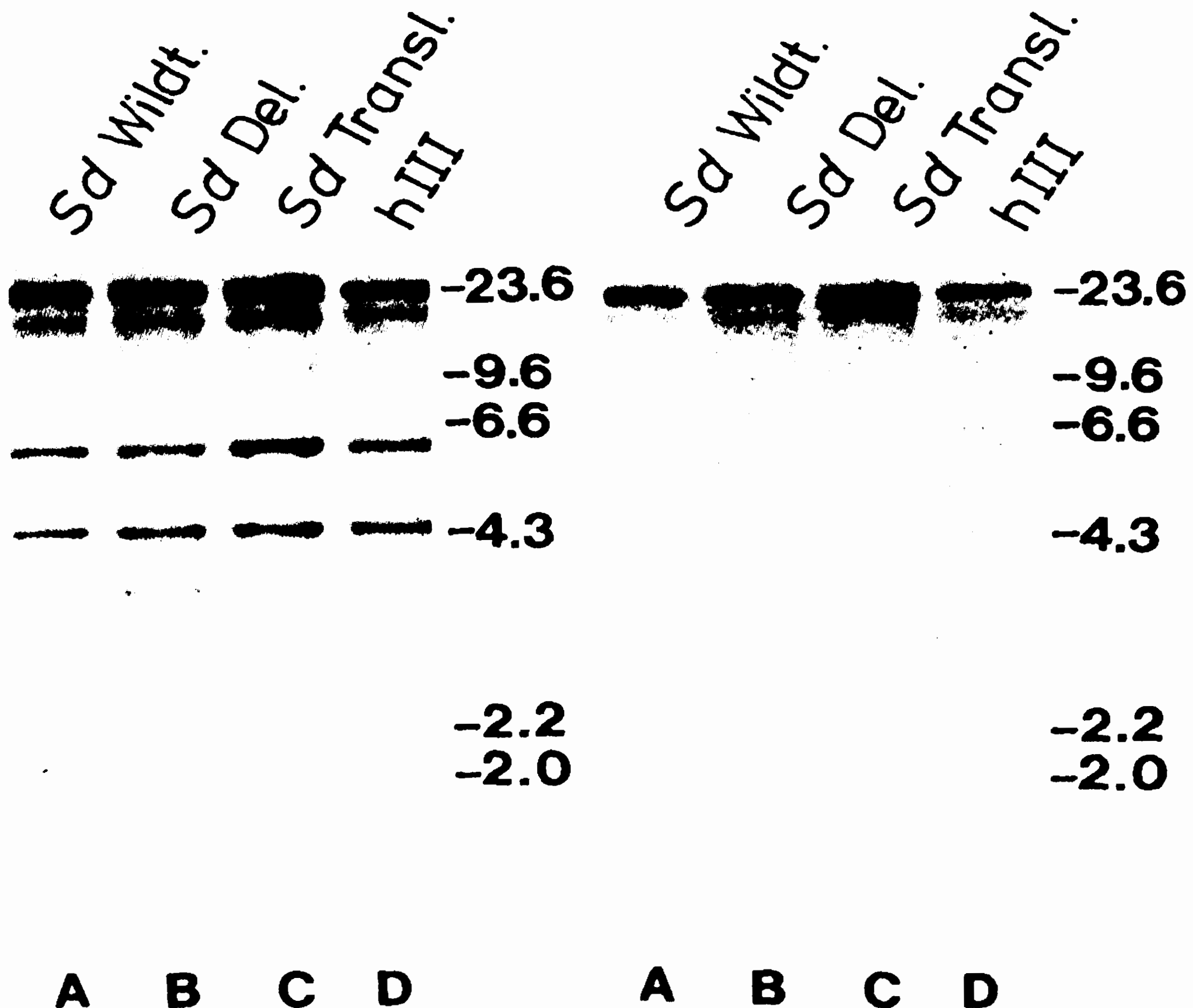

Fig. 7. Southern blots of Eco RI digested DNA from fish containing the wildtype X-chromosome (A), the deletion chromosome (B), or the translocation chromosome (C), and from fish representing the genetic background (D) used for the continuous backcrossing, hybridized to the v-src gene (left) and to the Xiphophorus c-src gene (right).

This suggests that the correlation in expression of Tu and c-src might be due to a common regulatory mechanism, or that the expression of the one gene somehow influences the expression of the other gene.

Using a similar experimental approach we could also show that also no member of the *ras* gene family is located on the X-chromosome of *Xiphophorus maculatus*. However, a sequence that demonstrates homology to the epidermal growth factor receptor (using a v-*erb* B probe) seems to be a molecular marker for the X-chromosome.

FUTURE PROSPECTS

In our attempts to understand the coordinated regulation of oncogenes in melanoma of *Xiphophorus*, one major approach is devoted to the analysis of the regulation of known oncogenes. These experiments include the functional analysis of the flanking regions of cloned *Xiphophorus* oncogenes and studies on the effect of modulated gene expression *in vitro* and *in vivo*. The other major approach is aimed at isolating the *Tu* gene by progressive subdivision and sib-selection of a cosmid library prepared from *Tu* containing fish using a DNA transfection bioassay for *Tu* (for details on the assay see Vielkind et al. 1982). This approach will be aided by isolation of polymorphic restriction fragments associated with the *Tu* locus and by subtraction-cloning strategies utilizing *Tu*-wildtype and deletion genotypes for enrichment of *Tu*-containing DNA.

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