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ELEVATED EXPRESSION OF THE CELLULAR *SRC* GENE IN TUMORS OF DIFFERING ETIOLOGIES IN *XIPHOPHORUS*

Manfred SCHARTL[1,3], Carl-Rudolf SCHMIDT[1,4], Annerose ANDERS[1] and Angelika BARNEKOW[2]

[1]*Genetisches Institut and* [2]*Institut für Medizinische Virologie der Justus-Liebig-Universität Giessen, D-6300 Giessen, Fed. Rep. of Germany.*

In the fish *Xiphophorus* we have detected elevated levels of pp60$^{c\text{-}src}$ kinase activity in a variety of tumors (n = 34) of neurogenic, epithelial, and mesenchymal origin either of hereditary etiology or induced by carcinogens. This elevation ranged from 2-fold up to 50-fold compared to the corresponding non-tumorous tissue and up to 6-fold compared to the highest activities found in any of the normal organs. The level of elevation parallels the degree of malignancy in melanoma and in tumors of mesenchymal origin. In fish bearing tumors of hereditary etiology kinase activity was also elevated in the non-tumorous brain, while in fish bearing induced tumors, kinase activity was elevated only in the cells of the neoplasia.

The viral oncogenes (v-*oncs*) of the acutely transforming retroviruses, which initiate and maintain the neoplastic phenotype of the host cell and thus are responsible for virus-mediated tumor formation, have been derived from normal cellular genes (Stehelin *et al.*, 1976; for review see Bishop, 1983), which were consequently designated cellular oncogenes (c-*oncs*). Cellular oncogenes are highly conserved in phylogenesis, *e.g.* the c-*src* gene, the cellular homologue of the Rous sarcoma virus (RSV)-transforming gene, appears first during phylogenesis in the sponges and is conserved in all metazoans (Schartl and Barnekow, 1982; Barnekow and Schartl, 1984; Lev *et al.*, 1984). The c-*ras* gene, the cellular homologue of the murine sarcoma virus oncogene, even shows homology to cellular sequences in yeast (Powers *et al.*, 1984; Gallwitz *et al.*, 1983; DeFeo-Jones *et al.*, 1983). These genes have been shown to be transcribed in some tumor cells as well as in distinct normal cells (Eva *et al.*, 1982; Westin *et al.*, 1982). Their normal function can be inferred either from given homologies to already known cellular genes, like that for platelet-derived growth factor homologous to the *sis* oncogene (Waterfield *et al.*, 1983; Doolittle *et al.*, 1983; Johnson *et al.*, 1984) and the gene for the epidermal growth factor receptor homologous to the *erb* B oncogene (Downward *et al.*, 1984), or from studies on the expression of the cellular oncogene itself (for review see Müller and Verma, 1984).

While the tumor-inducing function of the v-*oncs* is beyond question in every case, the neoplastic potential of only a few of the cellular counterparts has been confirmed. The mode of activation of cellular oncogenes in the tumorous condition can be a quantitative and/or a qualitative alteration of oncogene expression. Quantitative alteration of c-*oncs* in human and animal tumors was shown for c-*myc* and oncogenes related to c-*myc*, and in one case for c-*abl*; qualitative effects of the c-*oncs* are reported for the members of the *ras*-family (for review see Cooper and Lane, 1984).

The causal relationship between c-*onc* gene alterations and the neoplastic phenotype of tumor cells, however, remains unclear. This is mainly due to an insufficient knowledge of the biology of the cellular and the viral oncogene in question. However, in the case of the *src*-oncogene, several biological and biochemical properties are well known (for review see Bishop, 1983). The viral as well as the cellular gene product (pp60^{src}) is a 60,000 dalton phosphoprotein with a tyrosine-specific kinase activity. A variety of possible cellular substrates have been identified (Cooper and Hunter, 1983; Sefton *et al.*, 1981). The cellular *src*-gene shows an organ-specific expression with nervous tissues displaying the highest and muscular tissues expressing the lowest level (Gonda *et al.*, 1982; Barnekow and Bauer, 1984; Gessler and Barnekow, 1984; Cotton and Brugge, 1983; Sorge *et al.*, 1984; Schartl and Barnekow, 1982). c-*src* is differentially expressed during vertebrate development, suggesting a function during organogenesis (Schartl and Barnekow, 1984; Barnekow and Bauer, 1984). Its possible involvement in normal cellular differentiation processes has been demonstrated (Barnekow and Bauer, 1984; Cotton and Brugge, 1983; Sorge *et al.*, 1984). While the results of studies on the possible normal function are consistent, the data on the oncogenic potential of c-*src* are equivocal. Attempts to transform mammalian or avian cells with the cloned chicken c-*src* gene have so far been unsuccessful (Parker *et al.*, 1984; Iba *et al.*, 1984). On the other hand, elevated levels of c-*src* m-RNA are reported for a human lymphosarcoma and two chronic myelogenic leukemias (Slamon *et al.*, 1984), and a 4- to 20-fold enhanced activity of the pp60$^{c\text{-}src}$ kinase was found in some human sarcomas and mammary carcinomas (Jacobs and Rübsamen, 1983). We have reported elevated levels of pp60$^{c\text{-}src}$ kinase activity in hereditary melanoma of the fish *Xiphophorus* (Schartl *et al.*, 1982*b*; Barnekow *et al.*, 1982). Since the genetic basis of tumor formation in *Xiphophorus* is fairly well understood (Anders *et al.*, 1984) and a unified concept of the origin of neoplasia in this experimental tumor system has been developed (Ahuja and Anders, 1977), we have extended our studies on c-*src* expression to a variety of tumors of neurogenic, epithelial and mesenchymal origin with different types of etiology in order to study how c-*src* may be involved in neoplastic processes in these fish.

In *Xiphophorus*, it has been shown by means of formal genetics that neoplastic transformation is me-

Abbreviations: RSV, Rous sarcoma virus; TBR, tumor-bearing rabbit; v-*src*, RSV transforming gene; c-src, cellular homologue of the RSV transforming gene; pp60$^{v\text{-}src}$, gene product of v-*src*; pp60$^{c\text{-}src}$, gene product of c-*src*; Ap4A, diadenosine 5′5‴-P^1,P^4-tetraphosphate; *Tu*, tumor gene of *Xiphophorus*; *R*, regulating gene specific to *Tu*.

[3]To whom reprint requests should be sent. Present address: Gentechnologische Arbeitsgruppe "Molekulare Embryologie", Max-Planck-Institut für Biochemie, D-8033 Martinsried b. München, FRG.

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diated by a cellular oncogene, designated tumor gene (*Tu*) (Anders and Anders, 1978). *Tu* belongs to different gene-complexes, some of which are phenotypically marked by pigment cell patterns. The expression of *Tu* is normally suppressed by systems of chromosomally linked and/or non-linked regulating genes (*R*). Complete elimination of the *R*-gene systems leads to expression of *Tu* resulting in tumor formation. The mode by which the *R*-genes are dismantled determines the etiology of the tumor. Crossing-conditioned elimination of *R* results in hereditary tumors, whereas somatic-mutation-conditioned impairment of *R* leads to induced tumors. If additional genes are present which arrest those cells containing a derepressed *Tu* in an early stage of cell differentiation and in which the potential neoplastic information can not be realized, an additional trigger, namely tumor promotion, is needed for tumor formation. Tumor promotion in *Xiphophorus*, therefore, is recognized as a shift in cell differentiation from a precompetent stage to a competent stage, where a genetic composition compatible with cellular transformation realizes its full effect (for review see Anders *et al.*, 1984).

MATERIAL AND METHODS

Experimental animals

The fish used in this study were hybrids between different species of *Xiphophorus* from wild populations and a pure-bred wild-type *Heterandria bimaculata* (*Teleostei:Poeciliidae*). They were all bred in the aquarium of the Genetisches Institut, Giessen, and raised under standard conditions (see Kallman, 1975). Crosses were made using wild-type-strains of *X. maculatus* from Rio Jamapa, Mexico, *X. variatus* from Rio Panuco, Mexico, and *X. helleri*, Rio Lancetilla, Mexico, as well as a mutant strain of *X. maculatus* and the albino strain of *X. helleri* (*a/a*). Four different chromsomes carrying known copies of the *Tu*-gene complexes and all marked by different integumental pigment patterns, namely *Tu-Sd* (Spotted dorsal, from *X. maculatus*), *Tu-Li* (Lineatus, from *X. variatus*), *Tu-Sr* (Striped, from *X. maculatus*), and *Tu-Sr*^{rec} (Striped recombinant, from *X. maculatus*, see Anders *et al.*, 1973), were employed in this study (for detailed description of the crossing procedures, the genotypes and phenotypes, and the rationale underlying the generation of fish strains prone to develop neoplasia of different etiology see Anders *et al.*, 1973, 1981, 1984).

(1) (*X. maculatus,Tu-Sd,* × *X. helleri*) × *X. helleri*, BC_2. Fish of this genotype are either tumor-free (50%) or bear malignant (25%) or benign (25%) melanotic melanoma of hereditary origin depending on the presence of the *Tu-Sd* chromosome. Neoplasia develops in this genotype due to crossing-conditioned elimination of non-linked *R* genes (see Anders and Anders, 1978). These fish will be designated *Tu-Sd*, BC_2 below.

(2) (*X. maculatus, Tu-Sr,* × *X. helleri*) × *X. helleri*, BC_4;(*X. maculatus, Tu-Sr*^{rec} × *X. helleri*) × *X. helleri*, BC_3. Due to the presence of only a single *Tu*-linked *R*-gene, fish of these genotypes are highly susceptible to development of neoplasia following mutagen treatment (Anders and Anders, 1978). They will be designated *Tu-Sr*, BC_4 and *Tu-Sr*^{rec}BC_3, respectively, in the following.

(3) (*X. variatus, Tu-Li,* × *X. helleri, a/a*) × *X. helleri a/a*, BC_{27}. Only those backcross segregants that were homozygous for the albino gene were used in this study. These fish do not develop melanoma spontaneously though they carry a partially derepressed *Tu* due to crossing-conditioned elimination of non-linked *R* gene. An additional carcinogenic trigger, namely tumor promotion by way of testosterone treatment, is needed to induce tumor formation in this genotype (Schartl *et al.*, 1982*a*). They will be designated *Tu-Li*, BC_{27} *a/a* in the following.

Carcinogen treatment

X-ray treatment was performed by irradiating the fish with 1,000 R, 3 times for 45 min at 6-weekly intervals. N-methyl-N-nitrosurea (MNU) and ethylnitrosurea (ENU) were administered by exposing the fish to a 10^{-3}M solution of the carcinogen, 4 times for 1 hr at 2-weekly intervals. Embryos were treated by exposing the pregnant female to the carcinogen. Testosterone treatment was performed by adding an ethanolic solution (1 mg/ml) of 17 β-hydroxy-17-methylandrost-4-en-3-one (17-methyltestosterone, EGA-Chemie, Steinheim, FRG) to the aquarium water. The treatment was carried out continuously over 16 weeks with an effective dosage of 10^{-8}M per day.

Tumor diagnosis

All tumors were classified according to data obtained by gross inspection of growth rate, histopathological analysis, and transmission electron microscopy. For light microscopy all specimens were fixed in Bouin's solution. Excess picric acid was eluted with 70% ethanol. The fixed specimens were dehydrated and embedded in paraffin; 5-μm sections were cut with a Leitz base sledge microtome and stained according to classical histopathological staining methods used for vertebrate tumor diagnosis. For electron microscopy small pieces of tumor tissue were fixed in 3% glutaraldehyde for 3 hr. After washing in phosphate-buffered saline having the osmolality of fish cells, the specimens were post-fixed in 2% osmic acid. The tissue blocks were prestained with 2% uranyl acetate in 20% ethanol at 60°C for 3 hr, then they were dehydrated and embedded in ERL-4206. Ultra-thin sections were cut with a diamond knife using a Reichert ultramicrotome Om U 3 and examined in a Zeiss EM 10 A electron microscope.

Antisera

Antisera from Rous sarcoma virus (RSV) tumor-bearing rabbits (TBR-serum) were prepared by simultaneous injection of RSV strains SR-D and PR-C into newborn rabbits in a modification (Ziemiecki and Friis, 1980) of the procedure described by Brugge and Erikson (1977). A total of 3 different antisera were used in most of the experiments, all with the same result.

Preparation of cell extracts and immunoprecipitation

Tissue samples were lysed and clarified as described previously (Barnekow *et al.*, 1982). Two-tenths mg soluble protein were incubated with 5 μl TBR-serum for at least 60 min at 4°C and thereafter precipitated with protein-A-containing *Staphylococcus aureus*. The *S. aureus*-bound immune complex was washed twice with 1 ml kinase washing buffer (10 mM sodium phosphate, 40 mM NaF, 10 mM EDTA, 0,2% Triton X-100 1 M NaCl) and once with 1 ml H_2O. The immune complexes were then subjected to the kinase assay.

Protein kinase assay

The protein kinase assay was carried out by a modification (Barnekow and Bauer, 1984) of the method of

Collet and Erikson (1978). For quantitation, the radioactive gel bands (IgG heavy chain, 53kd) were cut out and solubilized, and their radioactivity was determined by liquid scintillation counting. Repeated analysis of the same sample revealed figures for the kinase activity with a standard deviation of about 5%. To confirm that the ^{32}P-labelled 53kd band was indeed heavy chain IgG, aliquots of randomly selected samples were run under non-reducing conditions and the majority of the ^{32}P counts were detected in a high-molecular-weight band > 150kd.

Sensitivity of the kinase activity to diadenosine 5',5'''-P^1,P^4-tetraphosphate (Ap4A)

For the Ap4A experiments, various concentrations of Ap4A were added to the washed immunoprecipitates 5 min before the kinase reaction was started by addition of γ-^{32}P-ATP.

Phosphoamino-acid analysis

^{32}P-labelled IgG was cut out of the gel, eluted and processed for phosphoamino-acid analysis (Barnekow and Bauer, 1984), following the method of Hunter and Sefton (1980).

Protein determination

Determination of protein concentration in the supernatant of the centrifuged cell lysates was carried out on trichloroacetic acid-precipitated aliquots according to Lowry *et al.* (1951).

RESULTS

Description of tumors

We have studied a variety of tumor types of different etiologies for the expression of the c-*src* gene by performing the pp60^{src} specific kinase assay. To investigate whether the tumor type, etiology, degree of malignancy or stage of tumor progression correlated in any way with c-*src* expression, we performed a pathomorphological characterization of the tumors which revealed the following:

Fibrosarcomas. All fibrosarcomas employed in this study (Table I, F,G,H,I) originated in the soft tissues of the trunk. They were not encapsulated and showed fast, infiltrative growth. Metastases, however, were not detected. The bulk of the tumor cells were poorly differentiated, as shown by the densely crowded cells in the invasive parts of the tumors, which were more or less arranged in intertwining bundles and large bands, or showed typical whirls after destruction of muscle bundles. These cells were spindle-shaped or oval, and mononuclear with one or two basophilic nucleoli. Only at the periphery of the tumor were well-differentiated cells frequently detected. Tumors F,G and H were classified as malignant; tumor I was very fast-growing and highly malignant.

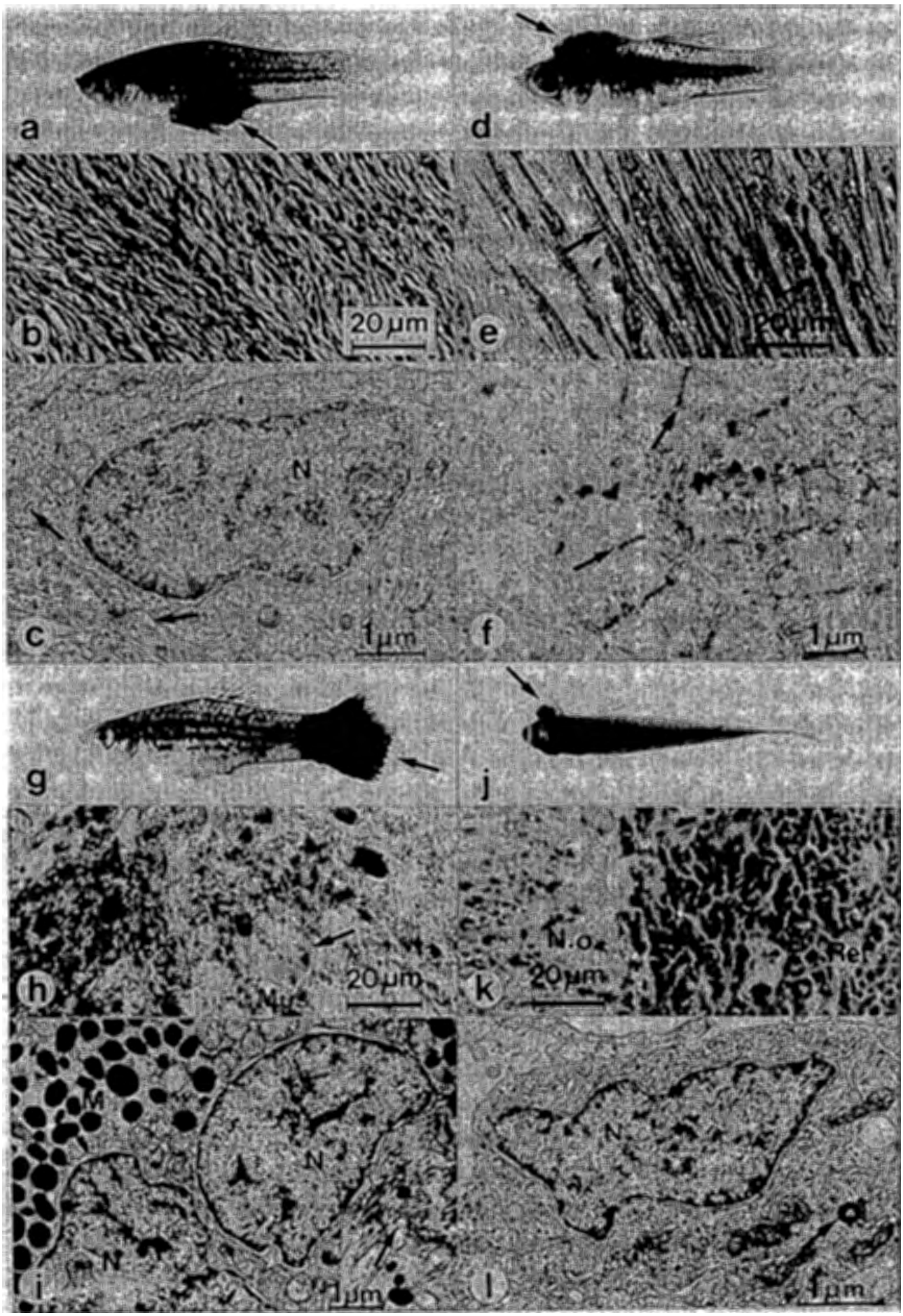
Rhabdomyosarcomas. Rhabdomyosarcomas (Table I, N,O,R) developed from skeletal muscle. The infiltrative growth of these tumors was not limited by a capsule, but metastases were never observed. The tumorous tissue was composed of transformed cells of the striated muscle cell lineage, as shown by actin and myosin bundles and z-bands in TEM-preparations, and histologically by cross-striation in spindle-shaped cells of the tumor (Fig. 1). In addition, polygonal and mono- and multinucleated giant cells contributed to the neoplasms. Nuclei were round or ovoid; some cells, however, showed the elongated nuclei typical of differentiated muscle cells. These tumors were classified as malignant, being composed of cells with different degrees of differentiation.

Melanomas. All melanomas examined in this study (Table I, A,B,C,L,M,Q) were of cutaneous origin. The benign melanomas showed limited two-dimensional growth in the epidermis until a maximal area was covered that was not exceeded further. The malignant melanoma exhibited 3-dimensional growth thus forming exophytic nodules and spreading by infiltration into the subcutaneous organs. Metastases, however, were not observed. The melanotic melanomas were supported by only a few blood vessels, while vascularization was abundant in the amelanotic mela-

TABLE I - PP60$^{c\text{-}src}$ KINASE ACTIVITY IN *XIPHOPHORUS* BEARING TUMORS OF DIFFERENT ETIOLOGIES

Tumor	Etiology	Genotype	Remarks	Factor by which the kinase activity is elevated	
				Tumor	Brain
Melanoma (A)	Hereditary	*Tu-Sd*, BC_2	Benign	2-3[1]	1,5-2
Melanoma (B)	Hereditary	*Tu-Sd*, BC_2	Malignant	4-8[1]	2-3
Melanoma (C)	X-ray, adult	*Tu-Sr*, BC_4	Invasive, malignant	5[1]	No
Squamous-cell carcinoma (D)	X-ray, adult	*Tu-Sr*, BC_4	Invasive	1[1]	No
Epithelioma (E)	X-ray, adult	*Tu-Sr*, BC_4	Benign	2[1]	No
Fibrosarcoma (F)	ENU, adult	*Tu-Sr*, BC_4	Invasive, malignant	13[3]	No
Fibrosarcoma (G)	MNU, adult	*Tu-Sr*, BC_4	Malignant	10[3]	1,4
Fibrosarcoma (H)	MNU, adult	*Tu-Sr*, BC_4	Invasive, malignant	10[3]	NT
Fibrosarcoma (I)	MNU, adult	*Tu-Sr*, BC_4	Invasive, highly malignant	50[3]	No
Retinoblastoma (J)	MNU, adult	*Tu-Sr*, BC_4	Progressive growth	3[2]	No
Retinoblastoma (K)	MNU, adult	*Tu-Sr*^{rec}, BC_3	Progressive growth	3[2]	No
Melanoma (L)	MNU, embr.	*Tu-Sr*, BC_4	Invasive	8[1]	No
Melanoma (M)	MNU, embr.	*Tu-Sr*, BC_4	Invasive	10[1]	No
Rhabdomyosarcoma (N)	MNU, emb.	*Tu-Sr*^{rec}, BC_3		6[3]	NT
Rhabdomyosarcoma (O)	MNU, adult	*Tu-Sr*, BC_4	Highly malignant, invasive	50[3]	No
Mesenchymal tumor (P)	MNU testosterone	*Tu-Sr*, BC_4	Exophytic, slow growing	7[3]	NT
Melanoma amelanotic (Q)	Testosterone	*Tu-Li*, $BC_{27}a/a$	Highly malignant	30[1]	No
Rhabdomyosarcoma (R)	Unknown	*H. bimaculata*	Invasive	20[3]	NT

For comparison non-tumorous organs were used:
[1]Skin (including epidermis, stratum compactum, dermis, vasculary system).-[2]Eye (whole bulbus).-[3]Muscle (including muscle fibers, surrounding connective tissue, vasculary system).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noma. Non-transformed cells of connective tissue origin were also present in all specimens. The melanoma cells—small or large polygonal cells, or spindle-shaped cells—either formed loose associations or were arranged in compact bundles. Poorly differentiated melanoma cells showed no pigment granules or premelanosomes, while terminally differentiated cells were recognized by their high content of densely packed melanosomes. The polymorphic nuclei containing one or two nucleoli showed large nuclear pockets. While the benign melanoma consisted predominantly of terminally differentiated cells, the malignant melanoma was composed of large numbers of poorly differentiated cells. The amelanotic melanoma typically exhibited extremely high malignancy.

Retinoblastomas. Both retinoblastomas (Table I, J,K) were unilateral, thus the contralateral non-tumorous eye could be employed in the kinase assay as a control. Rapid progression of tumor growth resulted within one or two weeks in protrusion of the tumorous eye out of the orbit. The tumors spread by infiltrating the optic nerve. They were supported by numerous blood vessels and lacunae. The bulk of the neoplastic cells were undifferentiated. They were closely aggregated and of round, ovoid or polygonal shape with nuclei (containing one or two nucleoli) surrounded by a narrow rim of cytoplasm. These tumors were of very high malignancy.

Squamous-cell carcinoma. This tumor (Table I, D) developed in the cutis of the posterior part of the body. Infiltrative growth was poor without any sign of metastasis. The tumor tissue was composed of small undifferentiated cells and larger cells showing a higher degree of differentation. Mucinous cells were spread as single cells or agregates locally over the neoplasm. The nuclei were round, oval or lobed with one or two prominent nucleoli. This tumor was classified as moderately malignant.

Epithelioma. The tumor (Table I, F) developed on the dorsocranium as a cauliflower-like heavily-pigmented exophytic mass. Infiltrative growth was not observed. The tumor tissue was composed of differentiated epithelial cells interspersed with numerous pigment cells of red as well as of black cell lineage. The tumor was classified as a benign lesion.

Characterization of the pp60$^{c\text{-}src}$ kinase in fish tumors

In all fish tumors investigated so far we have detected a protein kinase activity that is immunoprecipitable by pp60^{src} specific antisera. To confirm that this enzyme activity was indeed due to pp60$^{c\text{-}src}$ we made use of several unique characteristics of this enzyme. Firstly, in the TBR-serum-precipitated tumor cell extracts, this protein displayed a c-AMP-independent protein kinase activity that was able to phosphorylate the heavy chain of immunoglobulin G (IgG, MW 53,000). Two-dimensional phosphoamino-acid analysis of ^{32}P-labelled IgG of TBR-serum-precipitated cell extracts revealed that only tyrosine residues were phosphorylated. No phosphorylation of serine or threonine was observed (Fig. 2).

Secondly, we determined the effect of the diadenosine nucleotide Ap4A on kinase activity *in vitro*. Ap4A is found in eukaryotic cells and is believed to be associated with the regulation of cell prolilferation (Rapaport and Zamecnik, 1976). Recently, it was shown that the viral and the cellular pp60$^{c\text{-}src}$ kinase exhibit a differential sensitivity to inhibition by Ap4A (Barnekow, 1983). Whereas the pp60$^{v\text{-}src}$ kinase activity can be almost completely inhibited by addition of 100 μm Ap4A, the pp60$^{c\text{-}src}$ kinase activity is relatively insensitive to inhibition by this nucleotide. In this study we found that the kinase activity from all tumor samples investigated was not inhibited in the presence of 100 μm Ap4A.

So far, we have not detected any qualitative difference in the biochemical properties of pp60$^{c\text{-}src}$ from neoplastically transformed cells from the fish tumors or from non-transformed cells of the non-tumorous organs.

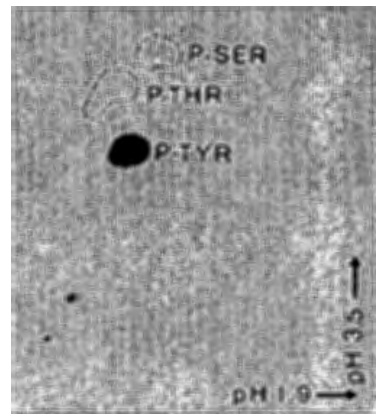

FIGURE 2 – Two-dimensional thin-layer electrophoresis of ^{32}P-labelled heavy chain of TBR-serum-precipitated cell extracts. The ^{32}P-labelled heavy-chain IgG was eluted from the gel and hydrolyzed, and the phosphoamino-acids were separated by electrophoresis at pH 1.9 in the first dimension and at pH 3.5 in the second dimension. P-ser, phosphoserine, P-thr, phosphothreonine, P-tyr, phosphotyrosine, o, origin.

FIGURE 1 – Gross morphology, histopathology and ultrastructure of induced tumors. (*a*) Backcross hybrid (*Tu-Sr*, BC_4) with fibrosarcoma (arrow). The dark pigmentation of the tumor is caused by interspersed, non-transformed melanophores. (*b*) Histological section of fibrosarcoma showing spindle-shaped tumor cells arranged in bundles; H and E stain. (*c*) Electron microscopic view of a tumor cell with typical fibrillous structures (arrows). N; nucleus. (*d*) Backcross hybrid (*Tu-Sr*^{rec}, BC_3 treated 3 × MNU as embryo) with rhabdomyosarcoma (arrow). (*e*) Histological appearance of rhabdomyosarcoma showing mature rhabdomyotubes with prominent cross-striation (arrows). Note: irregular arrangement of tumor cells. H and E stain. (*f*) Ultrastructure of mature rhabdomyotube from rhabdomyosarcoma showing sarcomere organization of myofibrills with z-lines (arrows). Note: irregular orientation of sarcomeres. (*g*) Backcross hybrid (*Tu-Sr*, BC_4; treated 1 × ENU as embryo) with melanotic melanoma (arrow). (*h*) Histological section of melanoma showing invasive growth of the melanoma (Mel) into the surrounding muscles (Mus). Note: degradation of muscle fibres (arrow) and aggregation of melanin granules. H and E stain. (*i*) Ultrastructure of a transformed melanocyte or melanophore with densely packed melanosomes (M). The nucleus (N) shows a deep nuclear clefting or possible multinucleation surrounded by abundant mitochondria. Note: collagen fibres (arrow). (*j*) Backcross hybrid (*Tu-Sr*, BC_4; treated 5 × MNU as adult) with unilateral retinoblastoma (arrow). Note: protrusion of the affected eye. (*k*) Histological section showing small, densely-packed tumor cells (Ret) infiltrating the optic nerve (N.O.). H and E stain. (*l*) Ultrastructure of a tumor cell. Note the irregular-shaped nucleus (N) and the ciliary structure (arrow) in the scanty cytoplasm.

Quantitative determination of pp60$^{c\text{-}src}$ kinase activity in fish bearing tumors of different etiology

All fish tumors investigated in this study displayed a marked kinase activity. The tumors may be divided into 2 groups with respect to these activities:

(1) Tumors of hereditary origin (all melanomas) showing a uniform level of kinase activity. The activity found in the benign tumors (n=9) was always in the range of one-third to one-half of that found in the malignant tumors (n=9) (see Fig. 3, lane 4 and 5).

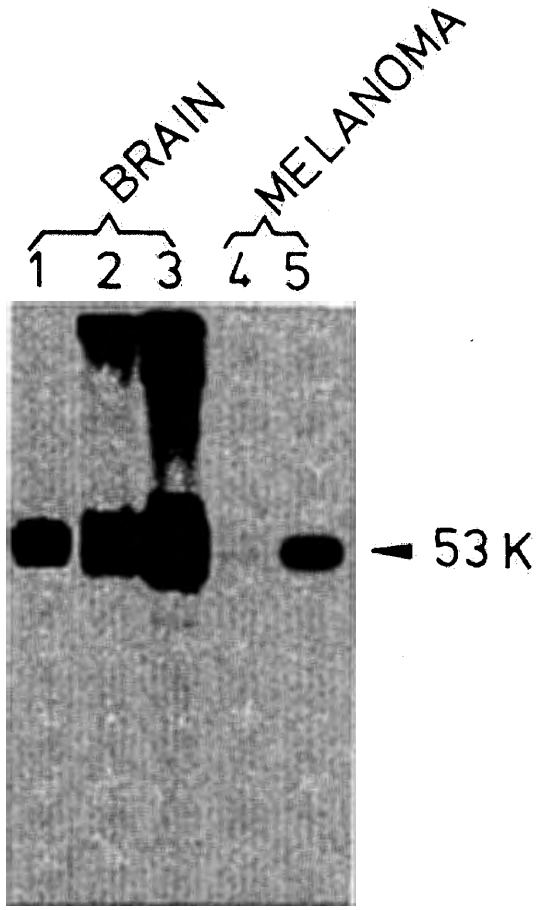

FIGURE 3 – Kinase activity in brain and melanoma of backcross hybrids *Tu-Sd*, BC_2: Tumor-free segregant (1), segregant bearing hereditary benign melanoma (2,4), segregant bearing hereditary malignant melanoma (3,5). 53 kd, IgG heavy chain. Note: elevation of kinase activity in the tumor parallels kinase activities in brain.

Compared to the kinase activity normally observed in non-tumorous organs, the activity in the tumors was about the level of that seen in the non-tumorous brain, rarely exceeding this value by a factor of two in some highly malignant melanomas. Compared to the kinase activity in normal skin even the activity in benign melanoma was elevated 2 to 3 times (Table I).

(2) Induced tumors which were very heterogeneous with respect to their pp60$^{c\text{-}src}$ kinase activity. While some tumors (Table I, D,E) displayed relatively low activities, which were in the range of that found in some normal organs and even lower than in normal brain, others had kinase activities that were up to 6 times higher than in normal brain (Table I, I,O,Q). All induced melanomas (all malignant) showed values of kinase activities that were at least as high as those of malignant melanomas of hereditary origin. In the fish bearing retinoblastoma (J,K) the kinase activity in the tumorous eye was 3 times that of the corresponding non-tumorous eye, and 50% higher than in normal brain. The epithelial tumors (Table I, D,E) had activities that were only slightly above the values of normal skin. In the malignant squamous-cell carcinoma (Table I, D), the invasive compartment of the tumor was represented by an increased kinase activity in the subepidermal muscles (1,450 cpm/mg protein) of the tumor-bearing fish compared to normal muscle (250 cpm/mg protein). In the mesenchymal tumors, kinase activity varied from low levels (Table I, N,P) to very high levels (Table I, I,O). The kinase activity was, however, much higher than in the non-tumorous mesenchymal compartment (muscles plus surrounding connective tissue), which showed only barely detectable activities (Fig. 4). Even in the rhabdomyosarcoma, which showed the lowest kinase activity of all

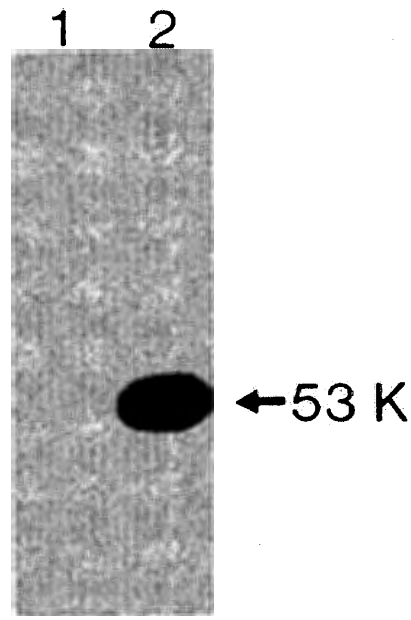

FIGURE 4 – Kinase activity in a fibrosarcoma (2) (Tumor H) and in healthy non-tumorous mesenchymal compartment (1). 53 kd; IgG, heavy chain.

tumors of mesenchymal histogenesis (Table I, N), the activity was 6 times higher than in normal muscle. In a rhabdomyosarcoma (Table I, O) and a fibrosarcoma (Table I, I) the elevation was about 50-fold.

For further analysis of the oncogene function we investigated the pp60$^{c\text{-}src}$ kinase activity in a non-tumorous organ of tumor-bearing fish. Brain was selected for this analysis, because it shows the highest levels of kinase activity of all normal organs (Barnekow *et al.*, 1982); thus quantitative differences between different fish may be more easily observed in this organ. In fish bearing tumors of hereditary origin, the kinase activity in the brains was higher than that seen in brains of the non-tumorous siblings. Moreover, the elevation of kinase activity in the tumorous fish was related to the malignancy of the neoplasm: in fish bearing benign tumors the kinase activity in brain was elevated 50-100% (*n*=6), in fish bearing malignant tumors the kinase activity in brain was elevated once

more to a total value exceeding that of the non-tumorous fish by a mean of about 200% (n=5) (Fig. 4, lane 1,2,3). On the contrary, in fish bearing mutation-conditioned or promoter-triggered tumors the kinase activity in the brain of the tumorous fish was not higher than in non-tumorous siblings (Table I, Fig. 5) in 11/12 cases.

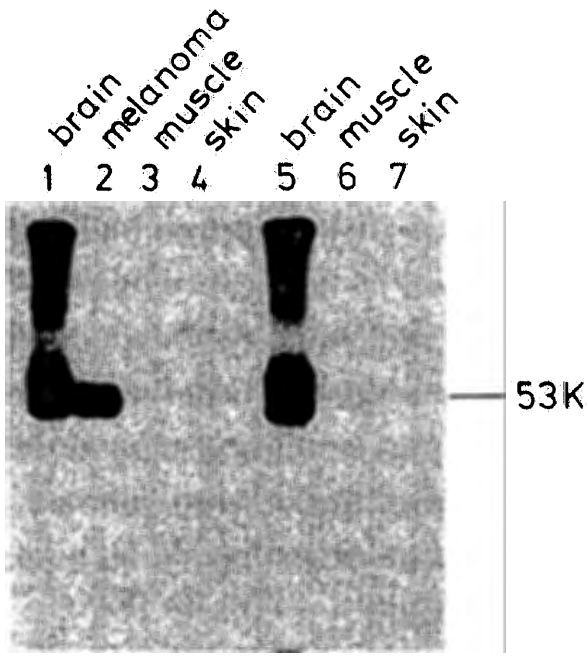

FIGURE 5 – Kinase activity in fish bearing MNU-induced melanoma (1-4) and in untreated controls (5-7). Note: No elevation of kinase activity in the brain of the tumor-bearing fish compared to controls. 53 kd; IgG, heavy chain.

DISCUSSION

We have investigated the activity of the c-*src* gene in tumors of *Xiphophorus* by determining the pp60$^{c\text{-}src}$ kinase activity. Since determination of gene activity at the transcriptional level does not necessarily reflect the amount or activity of a particular gene product, measuring the enzymatic activity of pp60$^{c\text{-}src}$ would provide information regarding the ultimate gene action. However, it has been shown that the level of protein kinase activity directly reflects the amount of pp60$^{c\text{-}src}$ protein (Barnekow *et al.*, 1982) and of c-*src* specific m-RNA (Gessler and Barnekow, 1984). By using the kinase assay we have shown that an *src* gene is expressed in all tumors of *Xiphophorus* investigated so far (n=34). In a variety of fish tumors the occurrence of viruses—either as causative agent [*e.g.* in tumors of the northern pike (Papas *et al.*, 1977) or in flatfish lymphocystis disease (Berthiaume *et al.*, 1984)] or post-transformational as adventitious infection [*e.g.* in stomatopapilloma of the European eel (N. Peters, pers. comm.)]—has been reported. We thus had to confirm that the kinase activity in our experiments was indeed of cellular and not viral origin. We have therefore studied the effect of the nucleotide diadenosine tetraphosphate Ap4A on the kinase activity. While the kinase activity of the viral pp60^{src} can be completely inhibited by Ap4A, the kinase activity of the cellular pp60^{src} is not inhibitable by this agent. We have shown that the kinase activity, which we have detected in the tumors of *Xiphophorus*, was consistently insensitive to Ap4A. In addition, no structures resembling virus-like particles were detected in the routinely screened TEM preparations of the tumors employed in this study.

If the kinase activity thus represents an active c-*src* gene in *Xiphophorus*, the question arises how this kinase activity is related to the tumorous state. Regarding the kinase activity in the neoplasms themselves, no correlation of c-*src* expression with the type of etiology could be seen, because all tumors exhibited kinase activity, and neither could any relation of c-*src* expression to histogenesis of the tumors be demonstrated. A positive correlation between the level of kinase activity and the malignancy of the tumor was clearly seen in all melanomas, either of hereditary origin or induced. In the tumors of mesenchymal origin the same tendency could be seen: high levels of kinase activity coincided with high malignancy. However, in a study on pp60$^{c\text{-}src}$ kinase activity in human tumors (Jacobs and Rübsamen, 1983), in the few cases where growth rates could be assessed, no correlation between malignancy and kinase was detected.

What is the significance of the elevated kinase activity in tumors of *Xiphophorus*? Three possibilities may be discussed: Elevated pp60$^{c\text{-}src}$ kinase activity may be a cause or a consequence of neoplastic transformation, or be unrelated to it.

If the kinase activity measured is unrelated to neoplastic processes, a possible explanation for its elevation in tumors compared to the non-tumorous tissue would then be that a specific cell type having *per se* a high kinase activity is highly represented in the tumor but weakly in normal tissue. The propagation of this cell type in the tumor might be a consequence of hyperplastic growth of a non-tumorous cell, or of neoplastic transformation. Hyperplastic growth leading to a high number of non-transformed cells in the tumor was, however, not observed in any of the histological sections of the tumors employed in this study. High activity in a transformed cell which is also displayed in its non-transformed state, can at least be excluded for the tumors of mesenchymal origin. It was shown that fibroblasts and myoblasts, which are the normal counterparts of the transformed cells leading to fibrosarcomas and myosarcomas, respectively, show only barely detectable activities (data not shown). The corresponding tumors (F,G,H,I,N,O,P,R), however, displayed kinase activities elevated up to 50-fold. This issue will be clarified by *in situ*-hybridization studies.

The kinase activity in cells carrying an active v-*src* gene was shown to be a prerequisite for neoplastic transformation of these cells (see Bishop, 1983). In some *in vitro* systems as little as a 5-fold excess of pp60$^{v\text{-}src}$ over pp60$^{c\text{-}src}$ was sufficient for transformation (Parker *et al.*, 1984), but generally the excess is 50-fold. On the contrary a 10-fold elevation of the pp60$^{c\text{-}src}$ kinase following introduction of additional (but non-homologous) copies of c-*src* did not induce neoplastic transformation (Parker *et al.*, 1984). Thus the ability of c-*src* to induce neoplasia merely through overexpression seems questionable. Determination of kinase activity in solid tumors as reported here must not necessarily reflect the primary events during the process of neoplastic transformation. Elevation of kinase activity up to 50-fold compared to the corresponding non-tumorous tissue and up to 6-fold compared to the highest activities found in any of the normal organs makes this kinase at least a good candidate as one of the primary effectors in neoplastic transformation of some of the tumors. Moreover, though no differences in the biochemical properties of the pp60$^{c\text{-}src}$ from tumors and from non-tumorous tis-

sues have been detected in this study and in a related study on humans (Jacobs and Rübsamen, 1983), additional qualitative changes in the pp60$^{c\text{-}src}$ of the neoplastic cells cannot be excluded. Concordant alteration of the expression and of the structure of an oncogene as a prerequisite for neoplastic transformation has recently been demonstrated for the *ras*-oncogene in an *in vitro* system (Spandidos and Wilkie, 1984).

High kinase activities in the tumors as epiphenomena of tumor growth or as a consequence of the secondary changes involved in tumor progression cannot be ruled out in the induced tumors but seem to be unlikely, if the tumors of hereditary origin are considered. In these fish the elevation found in the tumor was also seen in the non-tumorous brain of the same fish. Thus elevation of kinase seems more likely to be due to a genetic change already present in the germ line of these animals than to epigenetic alterations.

As concluded from phenogenetics, the genetic change underlying neoplastic transformation in *Xiphophorus* is the elimination of *R*-genes, leading to an elevated expression of *Tu* (Anders and Anders, 1978). The expression of *Tu* shows a correlation to the expression of c-*src* (Schartl *et al.*, 1982). This correlation is also seen in the present study. In fish bearing hereditary tumors the kinase activity is elevated in the tumor tissue as well as in the non-tumorous brain. In these fish the genetic event leading to tumor formation, namely elimination of *R*-genes controlling *Tu*, is present in all cells of the organism. In fish bearing tumors of somatic origin, the kinase is elevated only in the tumor itself. In these fish the elimination of the regulating genes has only taken place in the cells giving rise to the tumor.

Two possible causative events leading to elevated kinase activities have to be taken into consideration: 1. A structural change in the c-*src* gene itself may lead to a qualitatively different kinase that exhibits an elevated tyrosine phosphorylating activity. 2. Another gene that controls c-*src* may be affected in such a way that it confers a higher level of expression to the oncogene. This other gene might exert a positive or a negative control on c-*src*. Determination of c-*src* m-RNA levels and structural analysis of the c-*src* gene from genomic libraries from tumors and from different available mutant strains of *Xiphophorus* should help to shed further light on the possible role of c-*src* in tumor formation.

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