

COMPARATIVE STUDIES ON THE SRC PROTO-ONCOGENE AND ITS GENE PRODUCT pp60^{c-src} IN NORMAL AND NEOPLASTIC TISSUES OF LOWER VERTEBRATES

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Abstract—1. Oncogenes appear to play an important role in the physiology of transformed as well as of normal cells.

2. The *src* oncogene is by far the most investigated oncogene in birds and mammals with respect to the biochemical characteristics of its tyrosine kinase activity, although the specific function of this enzymatic activity still remains to be uncovered.

3. Systematic studies on the *src* related kinase activity in lower animals are lacking.

4. To contribute to a better understanding of the function of the *c-src* gene, we performed a comparative study on lower chordates.

5. We were able to demonstrate the presence of *c-src* related sequences in Acrania, Cyclostomata, cartilagenous and bony fish.

6. By performing the pp60^{src}-specific immune complex assay we detected a tyrosine specific kinase activity, that shows the same biochemical properties as the pp60^{c-src} from higher vertebrates.

7. The level of kinase activity is regulated in an organ specific manner.

8. In lymphocystis tumors of flat-fish and in stomatopapilloma of freshwater eels a considerable amount of pp60^{c-src} kinase activity was found, which, however, never exceeded the levels found in the normal brain.

INTRODUCTION

Some vertebrates and invertebrates have been shown to contain in their genome a variety of genes, called proto-oncogenes (Barnekow and Scharl, 1984; Scharl and Barnekow, 1982; DeFeo-Jones *et al.*, 1983; Bishop, 1985a; Reymond *et al.*, 1984; Goddard *et al.*, 1986). These genes have raised a great interest because it is known that upon integration into the genome of certain avian and mammalian retroviruses they acquired the ability to rapidly cause tumors and to transform cells of the host organisms *in vitro*. (For review see Bishop, 1985b). Also in some cases of human and rodent tumors of non-viral origin and in tumor derived cell lines, a causal relationship between the activation of a proto-oncogene and the neoplastic state of the tumor cells appears to exist (For review see Bishop, 1985c; Alitalo, 1984). In this context, the following modes of activation have been reported:

overexpression due to dysregulation or gene amplification, and structural alteration of the oncogene product due to point mutation or gross structural changes (Bishop, 1985c).

The oncogenes are classified according to the subcellular localization and to the biochemical properties of their protein products: 1. GTP binding proteins; 2. Nuclear factors; 3. Growth factors and analogous substances; 4. Tyrosine kinases and related proteins. This group of oncogenes is the one which comprises the genes coding for a novel enzyme activity, namely calcium and cyclic AMP independent tyrosine specific protein kinases. The first of these enzymes to be uncovered was the oncogene product of the Rous sarcoma virus (RSV) transforming gene (*v-src*). It is a phosphoprotein of mol. wt 60,000 (pp60^{v-src}) and its kinase activity can be reliably assayed in crude cell extracts. The product of its cellular homologue, the proto-oncogene *c-src*, shows similar biochemical and immunological properties. However, the viral kinase activity is inhibited by the dinucleoside diadenosinetetraphosphate (Ap4A), while the cellular kinase is far more insensitive towards inhibition by that molecule (Barnekow, 1983). Furthermore, pp60^{v-src} may form a complex with two cellular proteins (pp50 and pp89), while pp60^{c-src} is not found in that state. Some minor structural changes might contribute to significant functional differences (see Hunter and Cooper, 1985).

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Abbreviations: TBR-serum, antiserum from Rous sarcoma virus induced tumor bearing rabbits; pp60, phosphoprotein with a mol. wt of 60,000; SR-RSV-D, Rous sarcoma virus strain Schmidt-Ruppin D; PR-RSV-C, Rous sarcoma virus strain Prague C; *src*, gene homologous to the transforming gene of Rous sarcoma virus; Ap4A, diadenosinetetraphosphate; 53 K, heavy chain of immunoglobulin G of TBR-serum.

Besides pp60^{src}, several other viral and cellular oncogene products have been classified as tyrosine kinases (for review see Sefton, 1985). By far the most information on the possible function of tyrosine kinases in normal cells and during cell transformation is available on pp60^{src}. For the viral protein a number of possible substrates mediating the transformed phenotype are currently discussed. (Cooper *et al.*, 1983) For the cellular gene an organ specific expression in adults (Barnekow and Bauer, 1984) and a differential expression during embryonic development was shown. (Gessler and Barnekow, 1984, Scharl and Barnekow, 1984).

The *src* proto-oncogene seems to be present in all metazoans. It has been detected even in the sponges (Scharl and Barnekow, 1982; Barnekow and Scharl, 1984). pp60^{c-src} kinase activity was detected in several human tumors (Barnekow *et al.*, 1986; Jacobs and RübSamen, 1983) and in tumors of laboratory reared animals of the poeciliid fish *Xiphophorus* (Scharl *et al.*, 1985).

We have performed this study on pp60^{c-src} in field collected fish specimens in order to further characterize a tyrosine specific protein kinase in lower vertebrates, thus attempting to contribute to an understanding of its normal function in animals in general, and to search for kinase activity in naturally occurring fish tumors.

MATERIAL AND METHODS

Animals

Living specimens of *Scyliorhinus canicula*, *Scyliorhinus stellaris*, and *Spinachia spinachia* were obtained from the Aquarium of the Biologische Anstalt Helgoland, (FRG). Healthy and tumorous *Pleuronectes platessa* and *Platichthys flesus* were collected in waters 8 sea miles southwest of Helgoland. *Torpedo marmorata* were obtained from the Aquarium of the Station biologique d'Arcachon (France). Healthy and tumorous *Anguilla anguilla* were collected by means of electrofishing in the Wieseck brook near Gießen, FRG (gift of A. Holl, Giessen). *Xiphophorus helleri* (Rio Lancetilla stock) were from a randomly inbred laboratory strain.

Tumor diagnosis

For light microscopy all specimens (Fig. 1) 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 the classical histopathological staining methods. 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 postfixed in 2% osmic acid. The tissue blocks were pre-stained with 2% uranyl acetate in 20% ethanol at 60°C for 3 hr, then dehydrated and embedded in ERL-4206. Ultrathin sections were cut with a diamond knife using a Reichert ultramicrotome Om U3 and examined in a Zeiss EM 10A electron microscope.

Antisera

Antisera from RSV-tumor-bearing rabbits (TBR-sera) were prepared by simultaneous injection of SR-RSV-D and PR-RSV-C (Schmidt-Rupp, Prague) strains of RSV into new-born rabbits in a modification (Ziemiński and Friis, 1980) of the procedure described by Brugge and Erikson (1977).

Preparation of cell extracts and immunoprecipitation

Animals were anesthetized in tricainmethane sulfonate and decapitated. All tissue samples were prepared on ice. Tissue samples were lysed and clarified as described previously (Barnekow and Bauer, 1984). Soluble protein (0.2 mg) was incubated with 5 µl TBR-serum for at least 60 min at 4°C and precipitated with the protein A - containing bacteria *Staphylococcus aureus*. The bacterial bound immunocomplex was washed and the protein kinase assay was carried out by a modification (Barnekow and Bauer, 1984) of the method of Collett and Erikson (1978). The assay is based on the phosphorylation of the heavy chain (53 K) of IgG in the immune complex by the precipitated pp60^{src} after addition of γ(³²P)ATP in the presence of bivalent cations. For the inhibition experiments various amounts of Ap4A were added to the sample prior to the addition of γ(³²P)ATP.

Protein determination

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

Phosphoamino acid analysis

³²P-labelled IgG was cut out of gel, eluted from the gel sample and processed for phosphoamino acid analysis as described recently (Barnekow and Bauer, 1984), following the method by Hunter and Sefton (1980).

Isolation of DNA and hybridization

DNA from whole animals of *Branchiostoma lanceolatum* and *Lampetra planeri* and from the testes of *Scyliorhinus canicula* and of *Xiphophorus helleri* was prepared according to the method of Blin and Stafford (1976). Ten micrograms of either DNA was digested to completion with the restriction enzyme Eco RI, run on 0.8% agarose gels, and transferred to a hybridization membrane (Gene screen plus, NEN, Dreieich, FRG) by the Southern procedure (Southern, 1975). The filters were hybridized to the nick-translated 600 bp *src*-specific Pst I fragment F of clone SRA-2 of SR-RSV (DeLorbe *et al.*, 1980), encompassing the tyrosine kinase domain of the gene. The hybridization was carried out at 43°C in a buffer containing 35% formamide and 5 × SSC in the presence of 6 × 10⁶ cpm ³²P-labelled probe (specific activity 6 × 10⁸ cpm/µg DNA). Subsequent washings were performed in 1 × SSC at 60°C.

RESULTS

To confirm that the different classes of lower vertebrates indeed contain genes related to the *c-src* proto-oncogene of higher vertebrates, genomic DNA from *Branchiostoma lanceolatum*, *Lampetra planeri* and *Scyliorhinus canicula* was hybridized to a *src*-specific probe in a Southern Blot analysis. For comparison, DNA from *Xiphophorus helleri*, was hybridized under the same conditions. The DNA from *X. helleri* has been proven by gene cloning (Robertson and Scharl, unpublished) to contain genes of the *src*-related members of the tyrosine kinase family of proto-oncogenes. The presence of clear hybridization bands in all animals (Fig. 2) points to the presence of *c-src* related genes with a high degree of sequence conservation, even in Acrania, Cyclostomata and Chondrichthyes.

To characterize the *c-src* gene product in lower vertebrates, quantitative analysis of the tyrosine specific pp60^{c-src} kinase activity was performed in various organs of healthy cartilaginous and bony fish

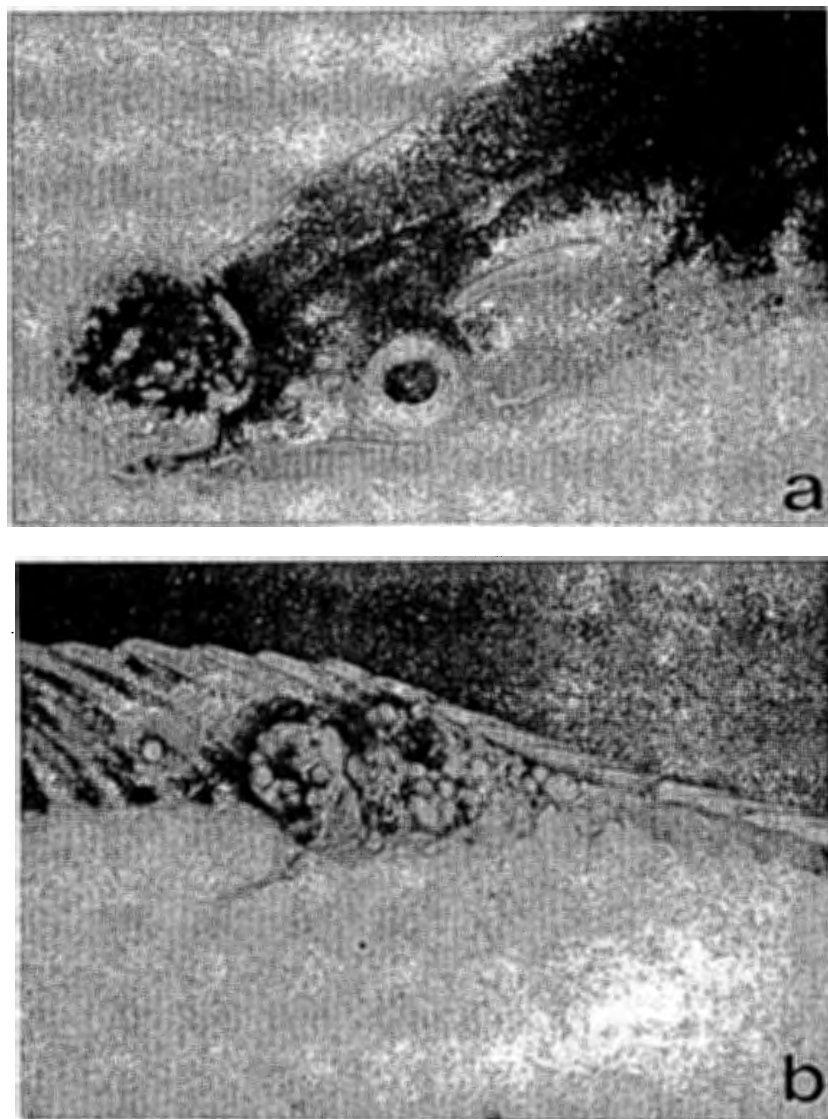


Fig. 1. a: Eel (*Anguilla anguilla*) with stomatopapilloma of the snout. b: Plaice (*Pleuronectes platessa*) with multiple lymphocystis nodules.

by means of the solid phase immune complex kinase reaction in the presence of excess antiserum. A kinase activity was as detected. It was shown that adding further amounts of TBR-serum did not increase the phosphorylation of the pp60^{c-src} immune complex (Fig. 3). As the plateau of the reaction curve is reached after 3–6 min under the conditions used, the reaction time of 5 min in each kinase assay proved to be sufficient to obtain maximal ³²P incorporation into IgG heavy chain. As a control, cell extracts were incubated with pre-immune rabbit serum and in no case was any kinase activity precipitated.

To prove that it is indeed the heavy chain of the pp60^{c-src} antibody from TBR-serum which is phosphorylated in the *in vitro* kinase assays, aliquots of each sample were run under non-reducing conditions and the radioactivity was then detected in the 150 K IgG (data not shown). For quantitation (see below) a total of three different TBR-sera were used throughout the experiments, all with the same results. The SD of the data was always less than 5%. In order to establish that the IgG heavy chain phos-

phorylation was due to a tyrosine specific kinase activity, we performed two-dimensional phosphoamino acid analyses. One example is shown for brain extract from the eel (Fig. 4). The data indicated that there was exclusively tyrosine phosphorylation.

Quantitative determination of kinase activity in different organs confirmed the organ specific expression of *c-src*, which has been intensively studied in higher vertebrates. The *src* gene product is highly expressed in brain, whereas in muscle tissue no significant amounts of kinase activity could be found (Figs 5, 6, 7). Testes, as investigated in the shark, show activities comparable to that found in brain, while liver of cartilaginous, as well as of bony fish, and skin have only moderate to low levels of kinase. Since it has been reasoned that pp60^{c-src} might play a functional role in electrogenic organs (Maness and Fults, 1985), we also quantitated the amount of pp60^{c-src} kinase in extracts from the electric organs of *Torpedo marmorata*. Compared to the brain, the electric organ showed only moderate levels of kinase activity. In the normal skeletal muscle of *Torpedo*,

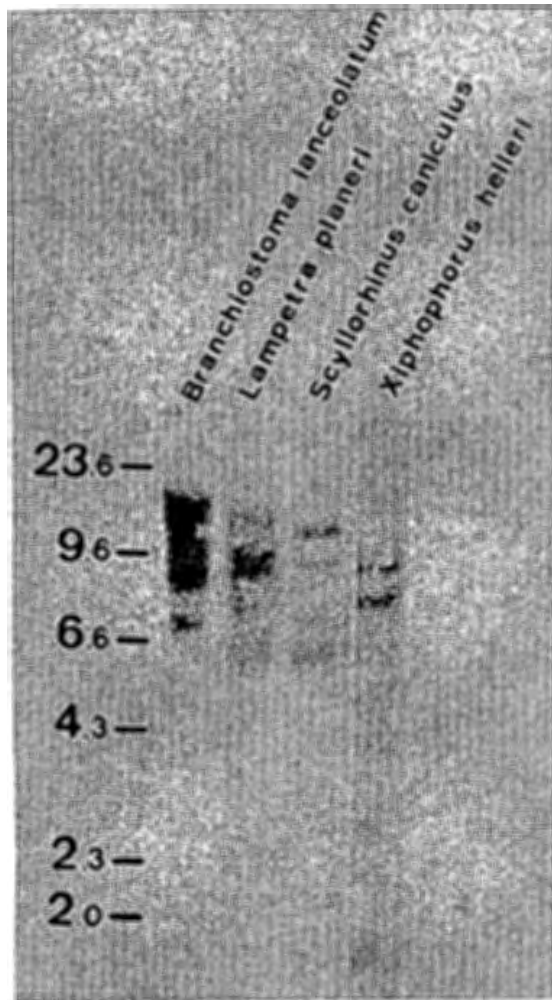


Fig. 2. Southern blot analysis of Eco RI digested DNA of *Branchiostoma lanceolatum*, *Lampetra planeri*, *Scyllorhinus caniculus* and *Xiphophorus helleri* hybridized to a *v-src* specific probe.

like in all other animals, no significant activity could be detected (Fig. 6).

To investigate if the *c-src* gene is expressed in naturally occurring fish tumors, we studied the kinase activity in lymphocystis tumors of flatfish and in stomatopapilloma of the european eel. All tumors employed in this study were diagnosed by light and electron microscopy. Parallel samples from the tumors were used for the biochemical analysis.

In flatfish, ($N = 3$), the tumor extracts display a considerable amount of kinase activity, which in every case was elevated compared to the activity detected in normal skin extracts of the same animal and of healthy control fish. However, the kinase activity in the tumors in no case exceeded that found in normal brain (Fig. 7). To compare the data on naturally occurring fish tumors with our earlier results on *c-src* expression in experimentally induced tumors of laboratory reared *Xiphophorus* hybrids (Schartl *et al.*, 1985), we determined the kinase levels also of brain extracts of all tumorous and non-tumorous flatfish and found uniform levels as in carcinogen treated *Xiphophorus*. Also in healthy and stomatopapilloma bearing eel the brain extracts had

comparable activities. The eel tumors also showed *c-src* expression, the level, however, was not elevated compared to the skin of healthy fish. Interestingly, non-papillomatous areas of skin from tumorous fish show higher levels of kinase compared to papillomatous areas and to the skin of healthy fish (Fig. 5).

In both flatfish and eel tumors the occurrence of viruses, either as causative agent or as adventitious infections, has been reported (Berthiaume *et al.*, 1984; Flügel, 1985; Pfitzner and Schubert, 1969; Peters and Peters, 1970). These viruses, as far as investigated, are not related to retroviruses and thus might not contribute an RSV-*v-src*-like kinase activity. To prove this further, we tested the pp60^{*c-src*} kinase activity in the tumor cell extracts in the presence of diadenosinetetraphosphate (Ap4A), which has an inhibitory effect on the viral kinase activity. Ap4A has no effect on the cellular enzyme in concentrations up to 100 μ M (Barnekow, 1983). Therefore, the use of Ap4A should be a way to distinguish the viral from the cellular form of the enzyme. In all cases tested, the kinase activity was insensitive to inhibition towards Ap4A in concentrations from 1 to 100 μ M (Fig. 8).

DISCUSSION

In this paper we have presented evidence that the *c-src* gene is not only present in all lower vertebrates but also in their primitive chordate ancestors. The high degree of conservation on the DNA level, which has led to the detection of the gene by DNA-DNA cross hybridization, also applies for the protein product. Antibodies raised in RSV tumor bearing rabbits readily detected a pp60^{*c-src*} related protein kinase activity in cartilagenous and bony fish. Also the known biochemical properties of the kinase (tyrosine specific phosphorylation, insensitivity to Ap4A inhibition) and the organ-specificity in expression proved to be conserved in lower chordates. Therefore it seems reasonable to postulate similar biological functions for that enzyme in higher as well as in lower vertebrates. Unfortunately, this biological function has not been elucidated in any experimental system so far. Some authors have reasoned that the function of

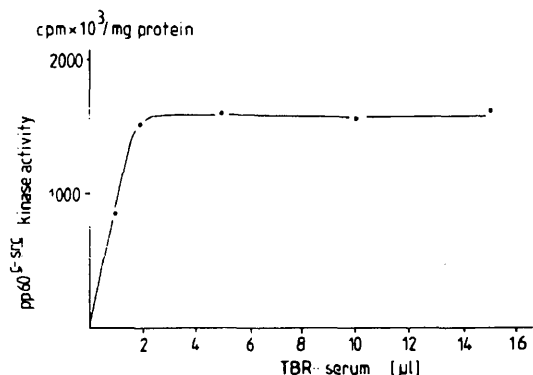


Fig. 3. Titration of the RSV-tumor-bearing rabbit sera used for the immunoprecipitation studies. Equal amounts of protein (220 μ g) of *Xiphophorus* cell extracts were incubated with increasing amounts of RSV-tumor-bearing rabbit serum followed by the kinase assay.

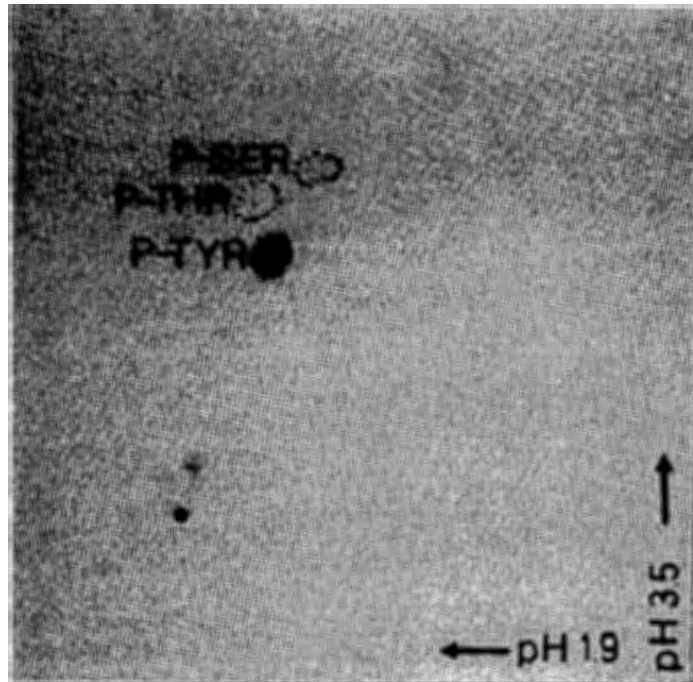


Fig. 4. Identification of phosphoamino acids in phosphorylated immunoglobulin heavy-chain of TBR-IgG after precipitation of brain extract from the freshwater eel and subsequent performance of the kinase assay. The phosphorylated proteins were subjected to acid hydrolysis and the phosphoamino acids were separated by thin-layer electrophoresis at pH 1.9 in the first dimension and at pH 3.5 in the second dimension. P-SER = P-serine; P-THR = P-threonine; P-TYR = P-tyrosine. ● = origin.

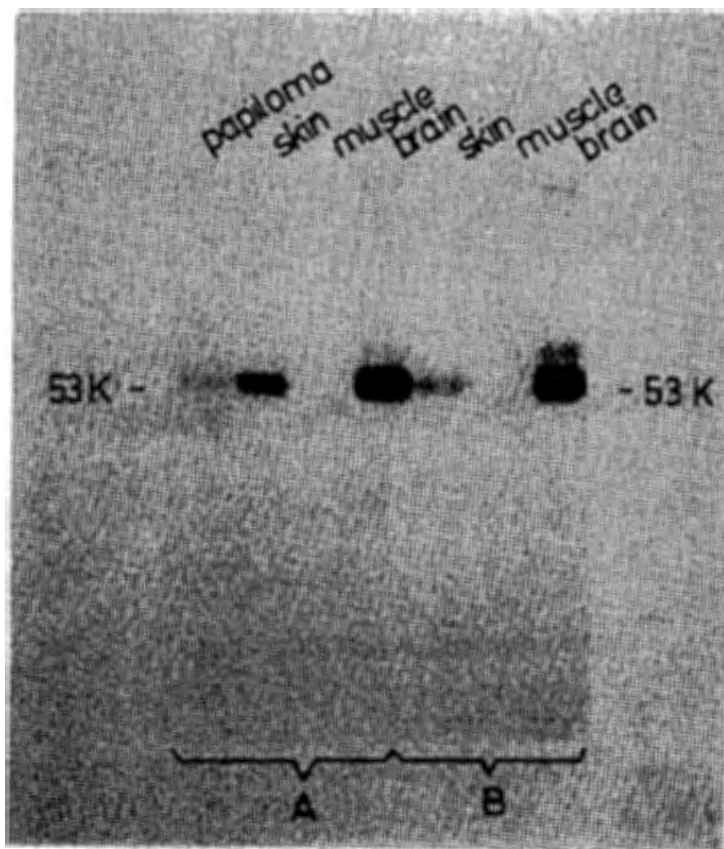


Fig. 5. Expression of the pp60^{src} kinase activity in various tissues of a tumor-bearing freshwater eel (A) and a non-tumorous control fish (B). 53K: IgG heavy chain.

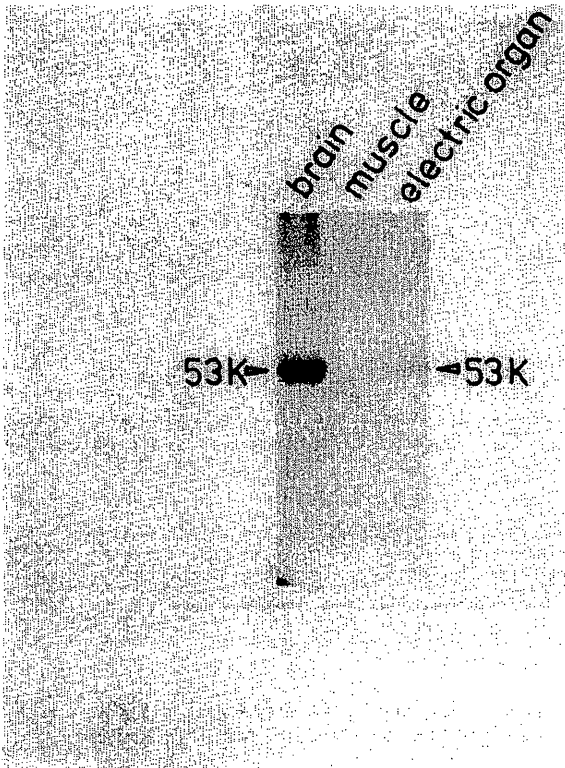


Fig. 6. Immunoprecipitation of protein kinase activity from various tissue extracts of the electric ray (*Torpedo marmorata*). 53 K: IgG heavy chain.

the pp60^{c-src} kinase is somehow connected to biochemical peculiarities of electrogenic tissues (Maness and Fults, 1985), because of the high kinase activities found in brain, retina and other neural tissues. Therefore, high kinase activities could be expected for the electric organ of the electric ray *Torpedo marmorata*. This, however, was not the case. Our failure to detect high levels in that organ might be attributed to the highly specialized function of the organ or to experimental details, e.g. the batch of antibodies used throughout the whole study. On the other hand, heart muscle tissue as well proved to have an intermediate level of kinase activity (Gessler and Barnekow, 1984). It appears that high kinase activities are functionally restricted to derivatives of the neuroectoderm and might play a functional role in the terminal differentiation processes of these cells (Cotton and Brugge, 1983; Levy *et al.*, 1984; Barnekow and Bauer, 1984).

In lymphocystis tumors of flatfish and in stomatopapilloma of the eel we also detected a pp60 kinase activity. However, the level of elevation compared to normal skin was only in the range of 5-fold in flatfish and approx. 2-fold in the eel tumors. This kinase activity was proven not to be of viral origin and thus might not be attributed to the virus particles found as causative agent in lymphocystis tumors (Flügel, 1985) or as adventitious infection in eel tumors (Peters and Peters, 1970). These viruses might, as a pathogenic effect, activate either the c-src gene expression or the protein kinase activity. The

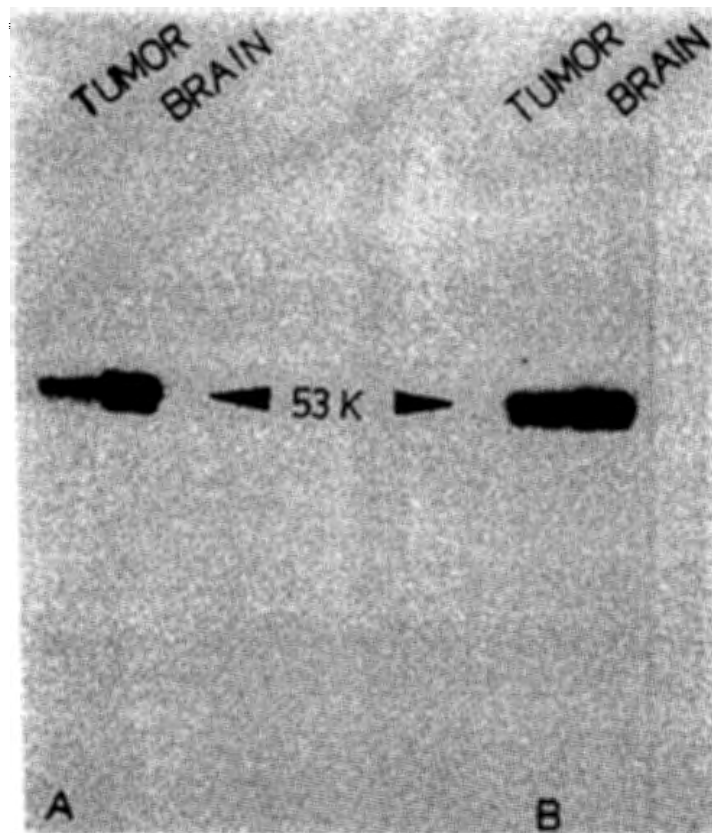


Fig. 7. pp60^{c-src} kinase activity in tumor-bearing flatfish. A: plaice (*Pleuronectes platessa*), B: flounder (*Platichthys flesus*).

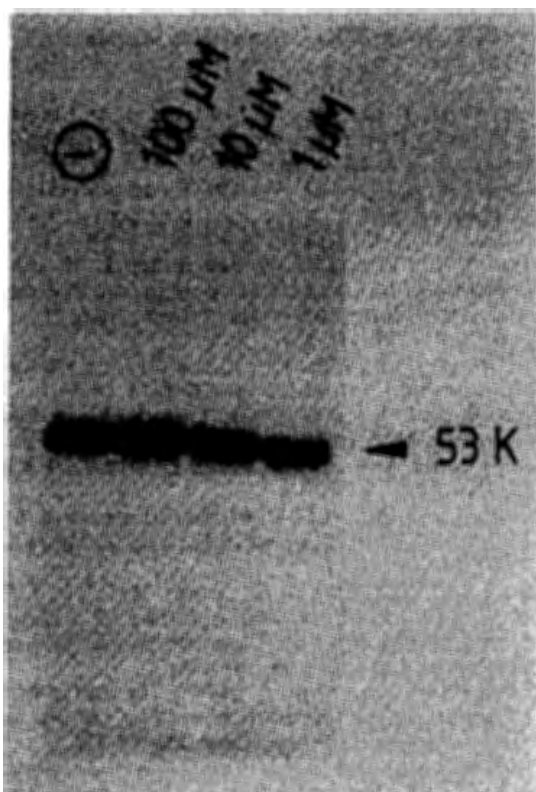


Fig. 8. Effect of different amounts of Ap4A on the phosphorylating activity of pp60^{c-src} precipitated from flatfish tumor. 53 K: IgG heavy chain.

latter has been shown to be the case in Polyoma virus infected cells (Courtneidge, 1985).

The level of elevation in the tumors investigated in this study is comparable to that found in carcinogen induced tumors of epithelial origin in *Xiphophorus* (Schartl *et al.*, 1985). In extended studies on kinase activity in tumorous *Xiphophorus* fish, we have shown previously that in tumors of hereditary origin, pp60^{c-src} kinase activity was elevated not only in the tumor but also in brains of the same fish. In fish bearing tumors of somatic origin, the kinase was only elevated in the tumor itself (Schartl *et al.*, 1985). In this study, no elevation of kinase was detected in the brain of the tumor bearing fish compared to healthy fish. At least for the lymphocystis tumors the somatic origin is obvious, which is in accordance with the kinase findings.

So far, we cannot decide what the significance is of the pp60^{c-src} kinase activity in the tumors of marine and freshwater fish. Two possibilities have to be considered for its involvement in the process of tumor formation. The *c-src* proto-oncogene might be causally involved in the primary events of neoplastic transformation, or the gene might be activated during the secondary processes of tumor growth and tumor progression. A quantitative effect of pp60^{c-src} in the tumor cells seems to be unlikely, as the level of kinase activity found was not considerably elevated above the level of normal organs, especially as compared to neural tissue. However, the effect of proto-oncogene expression in the "wrong" cell at the "wrong" time is not known. On the other hand, it was shown that elevation of pp60^{c-src} kinase activity alone is not sufficient to mediate neoplastic transformation of

cells (Parker *et al.*, 1984; Iba *et al.*, 1984). Another effect of pp60^{c-src} in fish tumors might be a qualitative change of the protein, either due to mutational changes in the primary structure or to new unique amino-terminal tyrosine phosphorylation of the pp60^{c-src} itself, as was shown in a human neuroblastoma cell line (Bolen *et al.* 1985). To answer some of these questions, purification of pp60^{c-src} from normal cells and from fish tumors has to be performed and the expression of the *c-src* gene has to be studied by *in vitro* hybridization experiments.

For stomatopapilloma of the eel and lymphocystis tumors of flatfish, an environmental etiology is discussed (Peters, 1981) and heavy metal ions, especially titanium, manganese and cadmium, are made responsible for the induction of the diseases. These heavy metal ions are present in high concentrations in the North Sea due to increased pollution. If there is a causal relationship between *c-src* expression and tumor formation, this would point to a novel activating mechanism of oncogenes.

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