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Correlations of Inheritance and Expression betv Cellular Homolog of the Rous Sarcoma Virus-transforming Gene in *Xiphophorus*[1]

Manfred Schartl,[2] Angelika Barnekow, Heinz Bauer, and Fritz Anders

Genetisches Institut der Justus-Liebig-Universität Giessen, Heinrich-Buff-Ring 58-62 [M. S., F. A.], and Institut für Virologie, Fachbereich Humanmedizin, der Justus-Liebig-Universität Giessen, Frankfurter Str. 107 [A. B., H. B.], D-6300 Giessen, Federal Republic of Germany

ABSTRACT

Neoplastic transformation of pigment cells in the teleostean fish *Xiphophorus* is mediated by a cellular oncogene (*Tu*). Normally, *Tu* is suppressed by multiple regulating genes (*R*). Depending on impairment and loss of *R* genes, *Tu* is permitted to express itself phenotypically. In the pigment cell system, different degrees of *Tu* expression lead to small spots of transformed cells or to benign or malignant melanoma. All neoplastic and nonneoplastic cells of all *Xiphophorus* genotypes tested thus far appear to contain the cellular homolog (c-*src*) of the avian sarcoma virus oncogene (v-*src*). The evidence for this stems from the detectability of a M_r 60,000 phosphoprotein with associated kinase activity (pp60$^{c\text{-}src}$) that reacts with antiserum against viral pp60^{src}. We followed the inheritance of *Tu* (identified by spots and melanomas) compared to the expression of c-*src* identified by the pp60$^{c\text{-}src}$-associated protein kinase). By quantitative determination of kinase activity in immunoprecipitated pp60$^{c\text{-}src}$ from fish showing different degrees of *Tu* expression, we have investigated whether there exists a correlation between the expression of c-*src* and *Tu*. In genotypes with the same genetic background, cells from *Tu*-containing fish express more pp60$^{c\text{-}src}$ than do cells from fish lacking *Tu*. In genotypes carrying a *Tu* gene and which show differences in the amount of gene expression due to a different extent of repression by regulating genes, analysis of kinase activity revealed that an increase of *Tu* expression is correlated with an elevated level of pp60$^{c\text{-}src}$-associated kinase activity.

Our findings may indicate that c-*src* activity in *Xiphophorus* is modulated by the *Tu* gene product or that *Tu* and c-*src* are regulated coordinately.

INTRODUCTION

Xiphophorus is a viviparous teleostean fish inhabiting fresh waters in Central America. Fish bred from wild populations are insensitive to carcinogens, while hybrids between populations, local varieties, and species may be sensitive and develop neoplasms at a rate of about 3 to 10%. Certain hybrids develop neoplasia even spontaneously.

In the melanoma system, it was shown by genetic analysis that neoplastic transformation of pigment cells is mediated by a certain chromosomal gene (1, 5). This oncogene was designated *Tu*.[3] Closely linked to *Tu*, genes are located which restrict the action of *Tu* from the pigment cells of a distinct compartment of the body, *e.g.*, the dorsal fin (*Df*), the body side (*Bs*), the anterior part (*Ap*), or the posterior part (*Pp*) of the body (3). These compartment-specific genes (*Co*) work in *cis* position only. Together with the *Co* genes, *Tu* forms a complex locus (Chart 1), representing the "macromelanophore locus" proposed by Gordon (13) and Kosswig (17). If one of the *Co* genes is mutated, pigment cells of its compartment are predisposed to undergo neoplastic transformation if *Tu* becomes active. Mutations affecting the pigment cell system revealed that in *Xiphophorus* there exist several copies of *Tu*, which differ by their compartment-specific restricting genes (3).

In the nonneoplastic cells, the activity of *Tu* is repressed by a variety of regulating genes (*R*), some of which are linked while others are not linked to *Tu*. The *R* genes if linked work in the *cis* position as well as in the *trans* position. In wild-type fish, nearly perfect regulation of *Tu* allows only the appearance of some transformed pigment cells. Because these become rapidly terminally differentiated, they never form a solid tumor but only small black patches. Crossing-conditioned elimination or mutation-induced deletion of *R* genes leads to stepwise derepression of *Tu*, resulting in an increase of *Tu* expression (4). This increase means formation of benign or malignant melanoma.

Thus far, *Tu* has been studied mainly by formal genetic, cytogenetic, and mutagenetic analysis, except for *Tu* transfer experiments, by injection of purified DNA into "*Tu*-free" recipient embryos (25). To study the influence of *Tu* on a biochemically measurable phenomenon, we have undertaken an approach based on the Rous sarcoma virus system.

In a preceding paper (8), we described in *Xiphophorus* a M_r 60,000 phosphoprotein that is related to the transforming protein (pp60^{src}) of Rous sarcoma virus. This cellular pp60$^{c\text{-}src}$ is precipitated by TBR serum, and it is associated with kinase activity that phosphorylates tyrosine residues in the heavy chain of the precipitating immunoglobulins. From these findings, we have concluded that *Xiphophorus*, like all vertebrates tested thus far, contains a gene (c-*src*) which is a homolog of the Rous sarcoma virus-transforming gene (v-*src*). The experiments presented in this paper were performed to find out if the fish endogenous c-*src* activity correlates with inheritance and expression of the fish oncogene *Tu*. Our experiments are based on the assumption that the activity of the pp60$^{c\text{-}src}$-associated

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[2] To whom requests for reprints should be addressed.

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[3] The abbreviations used are: *Tu*, tumor gene; *Co*, compartment-specific gene; *R*, regulating gene; pp60, phosphoprotein with a molecular weight of 60,000; TBR serum, antiserum from Rous sarcoma virus-bearing rabbits.

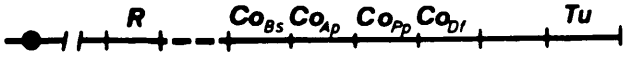

Chart 1. Provisional genetic map of a *Tu*-containing gene complex with linked *R*, based on 56 structural changes, as suggested by Anders and Anders (1); modified. Sequence of *Co* genes chosen arbitrarily. *Co*, compartment-specific genes restricting *Tu* activity in the pigment cells of, *e.g.*, the body side (*Bs*), the anterior part (*Ap*), the posterior part (*Pp*), or the dorsal fin (*Df*); *R*, regulating gene; *Tu*, tumor gene.

phosphokinase monitors the activity of endogenous c-*src* in *Xiphophorus*.

In the preceding paper (8), we have shown that brain and melanoma cell extracts of the same individual display comparable amounts of kinase activity. To exclude that metabolic properties of the tumor cells which are not causally related to the neoplastic state would influence kinase activity, all experiments were performed on brain cell extracts.

MATERIALS AND METHODS

Fish. Animals of 2 species of *Xiphophorus* derived from 3 wild populations were used. They were collected by Myron Gordon and subsequently inbred or bred in closed stocks. All genotypes were raised in our laboratory under standard conditions (15). *Xiphophorus maculatus* (Günther) 1866, collected from Rio Jamapa (Mexico) in 1939; inbred for about 120 generations. *Xiphophorus helleri* Heckel 1848, collected from Rio Lancetilla (Mexico) in 1951. *Tu*-containing gene complexes of *X. maculatus* were as follows: *Sd-Tu* ("spotted dorsal," *Sd*), mutation of Co_{Df}, small black spots in the dorsal fin, X-chromosomal. *Sp-Tu* ("spotted," *Sp*), mutation of Co_{Pp}, small black spots in the posterior part of the body, X-chromosomal; *Sr-Tu* ("striped," *Sr*), mutation of Co_{Bs}, transformed melanophores that form longitudinal stripes at the body side, Y- or X-chromosomal.

Besides these *Tu*-containing gene complexes, all fish are presumed to carry an unknown number of oncogenes with closely linked *R* genes. These oncogenes become active after mutation of *R* but usually show no phenotypic expression as tested thus far (22).

Protein Kinase Assay. Fish tissue (20 to 40 mg, wet weight) was homogenized in 1 ml of phosphate buffer (pH 7.0), containing 10 mM Na_2HPO_4-NaH_2PO_4, 40 mM NaF, 10 mM EDTA, 1% Triton X-100, and 5% trasylol (Bayer, Federal Republic of Germany) as protease inhibitor, using a tight-fitting Dounce homogenizer (10 strokes at 4°). Determination of protein concentration in the supernatant of the centrifuged homogenates was carried out on trichloroacetic acid-precipitated aliquots according to the method of Lowry *et al.* (18). TBR serum was prepared by simultaneous injection into newborn rabbits of the 2 Rous sarcoma virus strains, Schmidt-Ruppin and Prague-C (26), in a modification of the procedure described by Brugge and Erikson (10). The kinase assay was performed according to the method of Collett and Erikson (12) as modified by Barnekow *et al.* (8). Cell lysates were incubated with 5 μl of TBR serum, and the immunoprecipitates from these cell extracts were treated with 10 μl of approximately 0.1 μM [γ-^{32}P]ATP (>2000 Ci/mmol; The Radiochemical Centre, Amersham, United Kingdom). As shown in Chart 2, maximum ^{32}P incorporation occurred between 3 and 6 min. Further incubation did not increase the phosphorylation of the pp60$^{c\text{-}src}$ immunocomplex. Therefore, all kinase assays were performed with an incubation time of 5 min. The reaction was stopped by adding 1 ml quench buffer (100 mM sodium phosphate-10 mM EDTA-40 mM NaF-1 mM ATP, pH 7.0) to the samples. The labeled immune complexes were subjected to gel electrophoresis (11% acrylamide slab gels with a 2.5% acrylamide stacking gel). The labeled heavy chain of IgG was detected by autoradiography. Protein kinase reactions were done on extract samples adjusted to equal protein concentration. As a quantitative method for determination of kinase activity in parallel experiments, the radioactively labeled *M*, 53,000 IgG heavy-chain gel bands were cut out and solubilized, and their radioactivity was determined by liquid scintillation counting. This radioactivity expressed as cpm is taken as a measure for the protein kinase activity.

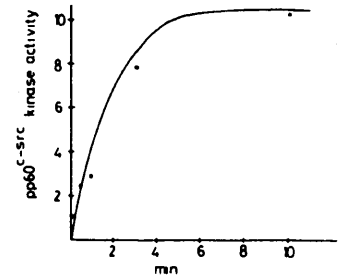

Chart 2. Time-dependent phosphorylation of the pp60$^{c\text{-}src}$ immunocomplex. Equal amounts of fish brain extracts were incubated for 60 min with 5 μl TBR serum. The immunocomplexes were isolated by adding Protein A-containing *Staphylococcus aureus*. The immunoprecipitates were incubated with [γ-^{32}P]ATP for 0.25, 0.5, 1, 3, 5, and 10 min. Quantitative determination of the kinase activity was done as described in "Materials and Methods."

For a detailed description of the experimental procedures, see the report of Barnekow *et al.* (8).

RESULTS

We have previously reported that a pp60$^{c\text{-}src}$ protein kinase activity in fish is related to the v-*src* gene product of Rous sarcoma virus in that (*a*) the phosphoprotein can be immunoprecipitated with TBR serum and (*b*) it exhibits an associated protein kinase activity, which (*c*) phosphorylates IgG heavy chain in tyrosine (8). We were interested in the expression of pp60$^{c\text{-}src}$ protein kinase activity in *Xiphophorus*, as influenced by the *Tu* tumor gene complex. A basal protein kinase activity has been observed, even in fish lacking a phenotypically recognizable *Tu* gene complex, which, however, varied from experiment to experiment depending on the batch and age of [γ-^{32}P]ATP used. In comparison to these, animals expressing a *Tu* gene showed an elevated level of such kinase activity.

Expression of *Tu* Correlating to Levels of Kinase Activity. To investigate whether a correlation between pp60$^{c\text{-}src}$-associated kinase activity and *Tu* expression exists, fish differing only in the extent of regulation of *Tu* were analyzed. For this purpose, 2 different genetic situations concerning the *R* genes were analyzed in a crossing experiment, and the fish were processed for the protein kinase assay. In the wild-type fish of *X. maculatus* that carry the *Tu*-containing gene complex *Sr-Tu*, this *Tu* is regulated by an *R* gene, which is linked to *Tu* (Fig. 1). The appearance at the body side of only some transformed pigment cells, which are terminally differentiated, indicate that *Sr-Tu* is nearly totally repressed (see also Ref. 2). Females homozygous for the *Tu*- and *R*-containing chromosome were crossed with males of *X. helleri* from Rio Lancetilla, lacking this *Tu* and also the corresponding *R*; this genotype was never observed to develop neoplastically transformed pigment cells. The F_1 hybrids, all carrying the *Sr-Tu* chromosome, did not show any increase in the expression of *Tu*. The same was true if F_1 hybrids were mated to *X. helleri* for all backcross (BC_1) segregants carrying the *Sr-Tu* chromosome. Because *R* is linked to *Tu*, it was not eliminated by the crossing procedure, and no melanoma could occur. Analysis of pp60$^{c\text{-}src}$-associated

kinase revealed no elevated activity in brain extracts of F_1 and BC_1 segregants as compared to fish of the parental generation. Muscle cells of all fish in this experiment were negative for pp60$^{c\text{-}src}$-associated kinase.

In wild-type fish of *X. maculatus* that carry the *Tu*-containing gene complex *Sd-Tu*, this *Tu* is regulated by an *R* gene, which is not linked to *Tu* (Fig. 2). These fish show small patches in the dorsal fin that consist of terminally differentiated, transformed pigment cells. Females of this genotype, being homozygous for *Tu* and *R*, were mated to males of *X. helleri* from the Rio Lancetilla stock as described in the previous experiment. All of the resulting F_1 hybrids developed benign melanoma in the dorsal fin. Due to replacement of *R* gene-carrying chromosomes of *X. maculatus* by *R* gene-free chromosomes of *X. helleri*, *Tu* shows an increased expression in F_1 hybrids as compared to the *X. maculatus* parent. Additional replacement of *R*-carrying chromosomes by backcrossing the F_1 hybrid to *X. helleri* leads to BC_1 segregants as follows: 50% of the animals that have not inherited the *Tu*; and 50% that have inherited the *Tu*. Those *Tu*-carrying BC_1 segregants that have still a single *R* in their genome developed benign melanoma like the F_1 hybrids did. Those that have inherited no *R* developed extremely malignant melanoma due to an unrestricted expression of *Tu*.

Analysis of pp60$^{c\text{-}src}$-associated kinase (Fig. 2) revealed that brain extracts of fish developing benign melanoma (F_1 as well as BC_1 segregants) showed higher activity [490 ± (S.D.) or 380 ± 10 cpm/mg protein, respectively] than did the wild-type *X. maculatus* (300 ± 10 cpm/mg protein) that developed only small black patches. The highest kinase activity was found in those BC_1 segregants that developed malignant melanoma (600 ± cpm/mg protein). In the BC_1 segregants that had not inherited the *Sd-Tu*, kinase activity was not increased as compared to wild-type *X. maculatus* or *X. helleri* of the parental generation (all 290 ± 10 cpm/mg protein).

In an analogous crossing experiment in which *X. maculatus* carrying the *Sp-Tu* chromosome which is also regulated by a non-linked *R* like the *Sd-Tu* (2) were crossed to *X. helleri*, an increase in pp60$^{c\text{-}src}$-associated kinase activity was also found in parallel to an elevated expression of *Tu*. In wild-type *X. maculatus*, which develop small patches of terminally differentiated transformed melanophores in the posterior part of the body, the activity was 120 ± 10 cpm/mg protein. In F_1 hybrids which developed benign melanoma, the activity was 170 ± 10 cpm/mg protein; and in BC_1 segregants carrying malignant melanoma, it was 250 ± 10 cpm/mg protein. This experiment showed a result corresponding to that described for the experiment using the *Sd-Tu*-carrying *X. maculatus* for crossing, namely, an increase in pp60$^{c\text{-}src}$-associated kinase activity parallel to an increase in *Tu* expression.

DISCUSSION

The concept of the cellular oncogene or host gene which, having escaped from appropriate regulation, leads to tumor formation was postulated by Huebner and Todaro (14). A variety of such genes have been identified using *in vitro* systems and taking advantage of the fact that such genes can be incorporated into the genomes of retroviruses resulting in the formation of new, rapidly transforming tumor viruses (16). Of these oncogenes, the best characterized is the *src* gene of Rous sarcoma vir
c-*src*, codes like
an associated protein kinase activity (19, 20), called pp60$^{c\text{-}src}$. Normal chicken cells express only about 2% as much of pp60$^{c\text{-}src}$ as the Rous sarcoma virus-transformed cells do of the analogous pp60$^{v\text{-}src}$ (16); the quantitative difference is widely believed to account for the transformed phenotype.

In *Xiphophorus*, an *in vivo* tumor system, the *Tu* gene complex represents by definition an oncogene which has been detected and studied using preponderantly classical genetic methods (1–5). *Tu* is responsible for development of melanomas in these fish, but the degree of expression of this gene is regulated by a genetic element *R* and is restricted to a specific body compartment by a further genetic element *Co*. The molecular nature of the *Tu* gene product is unknown.

In this paper, we present evidence for coordinate expression of *Tu* and of elevated pp60$^{c\text{-}src}$-associated protein kinase activity. Measurements of protein kinase activity were performed using tissue samples of animals from different genotypes, including fish carrying spots of transformed pigment cells, benign or malignant melanomas. We have previously demonstrated in parallel experiments the expression of pp60$^{c\text{-}src}$ and a tyrosine-specific kinase activity to be detected by use of pp60$^{v\text{-}src}$-specific antiserum and that, in *Xiphophorus*, *Tu*-determined tumor-bearing fish show a parallel increase of pp60$^{c\text{-}src}$ kinase activity in tumor tissue and in brain (8). Hence, it is possible to determine pp60$^{c\text{-}src}$-associated protein kinase activity in an nonpathological organ and to relate the activity observed to the *Tu* genotype as ascertained by following the phenotype of pigment-containing cells in the progeny of various matings. The possibility that the differences in kinase activity measured in these fish are due to secondary events of neoplasia [called epiphenomena by Sandberg (21)] appears to be improbable because all these data were derived from nonneoplastic cells.

In all genotypes of *Xiphophorus* tested, we detected a basal pp60$^{c\text{-}src}$-associated kinase activity indicating the presence of the c-*src* gene. This basal kinase activity has been detected in a wide variety of animals ranging from chickens to humans, and now to fish, and may represent a normal cellular function (7, 9, 11, 16, 19, 24). Owing to the conservation of this gene through evolution, one is tempted to assume that the function of c-*src* is essential for cellular metabolism or development of the organism as a whole. In the latter context, it is noteworthy that in previous studies in which kinase activities in different organs and different stages of development of chick embryos were studied, the highest level of expression was consistently found in brain, but in other organs high levels of activity were characteristic of early embryonic development (7, 9).

Elevation of pp60$^{c\text{-}src}$ kinase activity was shown in *Xiphophorus* in parallel with 2 types of genetic modifications in the *Tu* complex: (*a*) introduction of a *Tu* complex that could be assigned to a single chromosome; and (*b*) modified expression of a *Tu* complex owing to altered *R* constellation. The data have been obtained by examining 6 or more fish per genotype and, although the differences in kinase activity associated with *Tu* gene expression were usually only of the order of 2- to 3-fold, the reproducibility among animals of a common genotype was so good that in our opinion the significance of such a correlation between *Tu* expression and level of pp60$^{c\text{-}src}$ expression seems certain.

In fish carrying a nearly totally repressed *Tu*, only some transformed pigment cells appear, which soon become terminally differentiated. Only a few poorly differentiated cells are found in the black spots of these fish (3, 4). In these genotypes, no significant elevation of kinase activity was detected. In fish carrying benign melanomas due to increased *Tu* expression, $pp60^{c\text{-}src}$ kinase activity was elevated 1.5- to 2-fold. In the benign melanoma, some of the cells are undifferentiated, but the majority of the cells is terminally differentiated (3, 4). The highest level of kinase activity (2- to 3-fold) was found in the brains of malignant melanoma-bearing fish that carry a totally derepressed *Tu*. In these malignant tumors, almost all transformed cells are undifferentiated, and only a few cells reach the final stage of terminal differentiation (3, 4).

The experiments showing that increased *Tu* expression owing to modification of the *R* gene is paralleled by increased expression of endogenous c-*src* can be explained by the assumption that the regulating gene (*R*) in repressing *Tu* also directly or indirectly influences c-*src*. In cases where *R* is linked to *Tu*, *Tu* as well as c-*src* always remain relatively repressed, and gene expression is not increased. This was the case in the experiment shown in Fig. 1. In cases where *R* is not linked to *Tu*, the respective genes are separated by means of hybridization. In this case, derepression of *Tu* leads to an increase also in the c-*src* gene expressions as shown in our experiment (see Fig. 2).

The qualitative similarity of the results in the 2 crossing experiments using the *Sd-Tu* gene complex on the one hand and the *Sp-Tu* gene complex on the other hand gives a hint on the role of the *Co* genes. In both crosses, there was the same situation concerning *R* and *Tu*. The fish differed only in their *Co* genes. In the *Sd-Tu* gene complex, the Co_{Df} is mutated; in the *Sp-Tu* gene complex, the Co_{Pp} is mutated. In both experiments after crossing and backcrossing, increased *Tu* expression and increased kinase activity were found. Thus, the *Co* genes seem to have no crucial influence on the expression of *Tu* and c-*src*. This is in accordance with genetic data that clearly indicate that *Co* genes restrict the activity of *Tu* only from specific compartments of the body but cannot regulate *Tu* in other compartments (1–3).

One of the most interesting implications from these data lies in the fact that it is only in fish with a phenotypically expressed *Tu* gene that an elevated $pp60^{c\text{-}src}$-associated protein kinase is observed in brain tissue. The various crosses and genetic configurations shown in Figs. 1 and 2 demonstrate that the 2- to 3-fold elevation in kinase activity correlated well with *Tu* expression. This correlation, whether owing to an altered chromosomal location of *Tu* or to the influence of an *R* gene, suggests that *Tu* must also be expressed in brain tissue, without, however, pathological consequences.

Elevated $pp60^{c\text{-}src}$-associated kinase activity in *Xiphophorus* was always inherited together with a *Tu* gene complex according to Mendelian inheritance and did not become separated in hybrids. One possible way to interpret these data is based on the assumption that in *Xiphophorus* only one copy of c-*src* per haploid genome is present, which is very likely to be the case in the chicken system (23). Basic kinase activity which was found in fish lacking a chromosome that carries a phenotypically recognizable *Tu* gene complex would then show that the c-*src* may be located on chromosomes that do not carry such a *Tu* gene complex. Elevated kinase activity which was detected in fish ca
pronounced by
$pp60^{c\text{-}src}$ or the c-*src* gene itself. If indeed several copies were to be present in the genome of *Xiphophorus*, an alternative explanation for our data would be the possibility that some other copies of c-*src* in *Xiphophorus* may be linked to *Tu* on the same chromosome or may even be part of the same gene complex. Clarification of this issue will, of course, depend on a molecular biological understanding of the *Tu* gene complex and its products.

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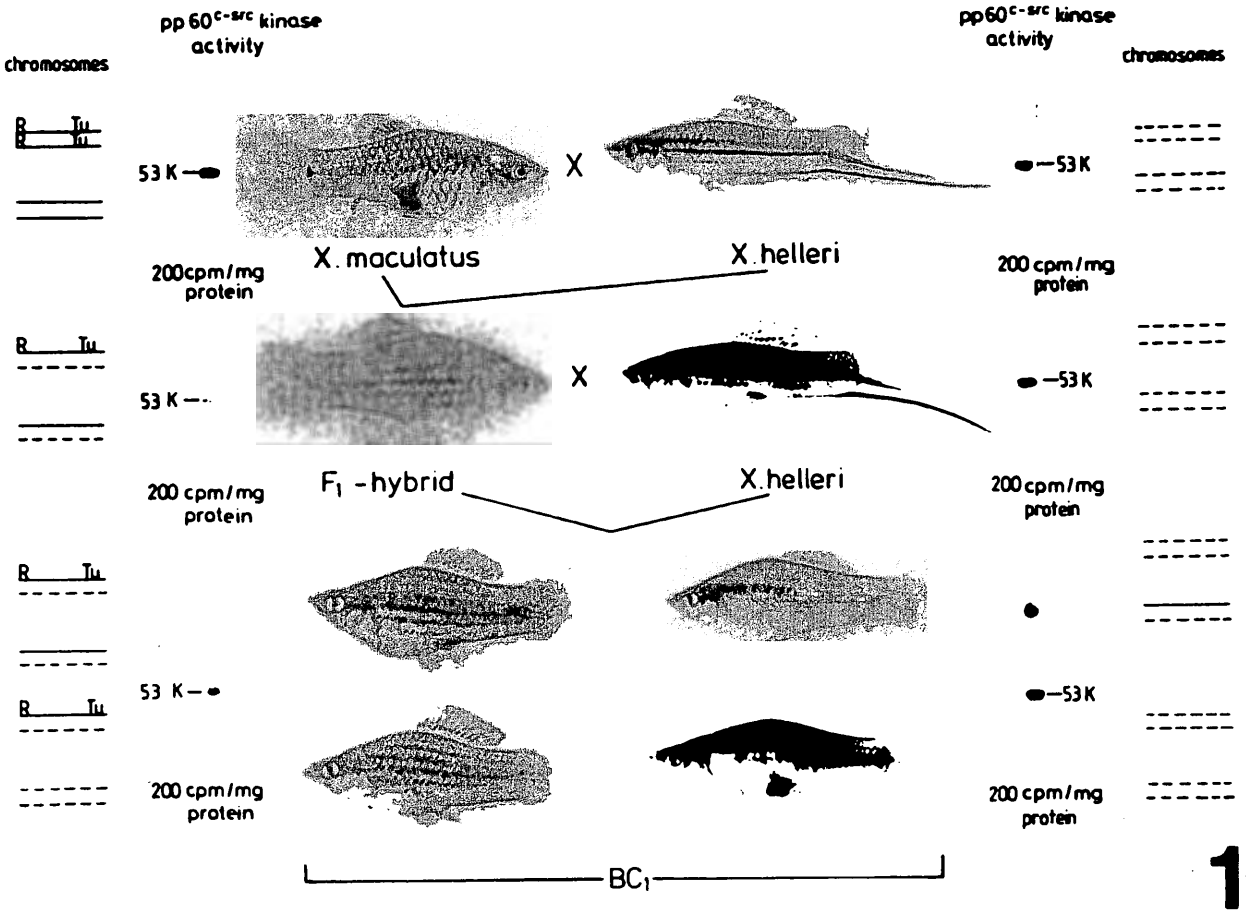

Fig. 1. Inheritance of a *Tu*-containing gene complex (*Sr-Tu*) with linked *R* from *X. maculatus* in a crossing experiment using *X. helleri* as the recurrent parent for backcrossing and pp60$^{c\text{-}src}$-associated kinase activity. Diagram shows kinase activity of brain cell extracts in parental, F_1, and backcross (BC_1) generations (4 to 6 brains per measurement). Only 2 pairs of chromosomes of 24 from each fish are shown. For explanation, see text. ——, chromosomes of *X. maculatus*; - - - -, chromosomes of *X. helleri*; *53 K*, heavy chain of IgG phosphorylated with [γ-^{32}P]ATP. Kinase activity is expressed as cpm rounded off to the nearest significant figure. The range of actual values from 24 fish was: lowest, 173 cpm/mg protein; highest, 221 cpm/mg protein.

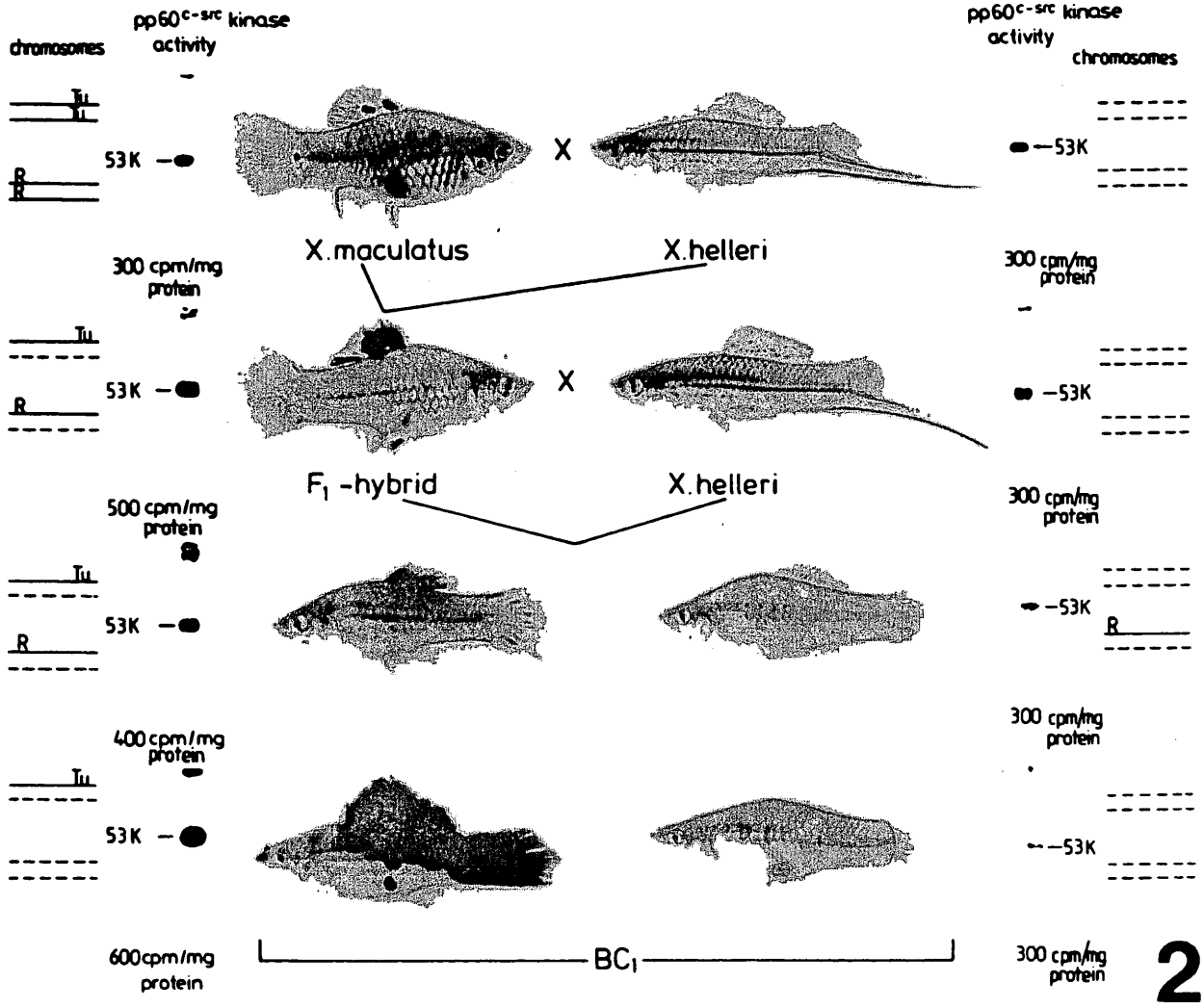

Fig. 2. Inheritance of a *Tu*-containing gene complex (*Sd-Tu*) with nonlinked *R* from *X. maculatus* in a crossing experiment using *X. helleri* as the recurrent parent for backcrossing and pp60$^{c\text{-}src}$-associated kinase activity. Diagram shows kinase activity of brain cell extracts in parental, F_1, and backcross (BC_1) generations (4 to 6 brains per measurement). Only 2 pairs of chromosomes of 24 from each fish are shown. For explanation, see text. ——, chromosomes of *X. maculatus*; - - - -, chromosomes of *X. helleri*; *Tu*, tumor gene, part of the gene complex *Sd-Tu*; *53K*, heavy chain of IgG phosphorylated with [γ-^{32}P]ATP. Kinase activity is expressed as cpm/mg protein rounded off to the nearest significant figure. For the actual mean kinase activities, see text.