Correlations of Inheritance and Expression betw
Cellular Homolog of the Rous Sarcoma Virus-transforming Gene in
Xiphophorus

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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, 60,000 phosphoprotein with associated kinase activity (pp60c-src) that reacts with antiserum against viral pp60src. We followed the inheritance of Tu (identified by spots and melanomas) compared to the expression of c-src identified by the pp60c-src-associated protein kinase). By quantitative determination of kinase activity in immunoprecipitated pp60c-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 pp60c-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 pp60c-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, containing fish express more pp60c-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 pp60c-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.

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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.
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; Xiphophorus variatus (Günther) 1866, collected from Rio Jamapa (Mexico) in 1951. Tu-containing gene complexes of X. maculatus were as follows: Sd-Tu ("spotted dorsal," Sd), mutation of CoSb, small black spots in the dorsal fin, X-chromosomal. Sp-Tu ("spotted," Sp), mutation of CoPb, small black spots in the posterior part of the body, X-chromosomal; Sr-Tu ("striped," Sr), mutation of CoPb, 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 NaHPO₄, NaH₂PO₄, 40 mM NaF, 10 mM EDTA, 1% Triton X-100, and 5% trasyol (Bayer, Federal Republic of Germany) as protease inhibitor, using a tight-fitting Dounce homogenizer (10 strokes at 4°). Determined 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 (12) as modified by Barnekow et al. (8). Cell lysesates 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-³²P]ATP (>2000 Ci/mmol; The Radiochemical Centre, Abersham, United Kingdom). As shown in Chart 2, maximum ³²P incorporation occurred between 3 and 6 min. Further incubation did not increase the phosphorylation of the pp60⁺⁺⁺ 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 potassium 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.

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

RESULTS

We have previously reported that a pp60⁺⁺⁺ protein kinase activity in fish is related to the v-src gene product of Rous sarcoma virus in that (a) the phosphoprotein can be immuno-precipitated 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⁺⁺⁺ 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-³²P]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⁺⁺⁺-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 homzygous 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 F₁ hybrids, all carrying the Sr-Tu chromosome, did not show any increase in the expression of Tu. The same was true if F₁ hybrids were mated to X. helleri for all backcross (BC₁) 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⁺⁺⁺-associated activity was determined by liquid scintillation counting. This radioactivity expressed as cpm is taken as a measure for the protein kinase activity.

For a detailed description of the experimental procedures, see the report of Barnekow et al. (8).
kinase revealed no elevated activity in brain extracts of F, and BC, segregants as compared to fish of the parental generation. Muscle cells of all fish in this experiment were negative for pp60c-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, 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, hybrids as compared to the X. maculatus parent. Additional replacement of R-carrying chromosomes by backcrossing the F, hybrid to X. helleri leads to BC, segregants as follows: 50% of the animals that have not inherited the Tu; and 50% that have inherited the Tu. Those Tu-carrying BC, segregants that have still a single R in their genome developed benign melanoma like the F, hybrids did. Those that have inherited no R developed extremely malignant melanoma due to an unrestricted expression of Tu.

Analysis of pp60c-src-associated kinase (Fig. 2) revealed that brain extracts of fish developing benign melanoma (F, as well as BC, 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, segregants that developed malignant melanoma (600 ± cpm/mg protein). In the BC, 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 pp60c-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, hybrids which developed benign melanoma, the activity was 170 ± 10 cpm/mg protein; and in BC, 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 pp60c-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 virus c-src, codes like an associated protein kinase activity (19, 20), called pp60c-src. Normal chicken cells express only about 2% as much of pp60c-src as the Rous sarcoma virus-transformed cells do of the analogous pp60c-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 pp60c-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 pp60c-src and a tyrosine-specific kinase activity to be detected by use of pp60c-src-specific antiserum and that, in Xiphophorus, Tu-determined tumor-bearing fish show a parallel increase of pp60c-src kinase activity in tumor tissue and in brain (8). Hence, it is possible to determine pp60c-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 pp60c-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 pp60c-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 pp60c-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^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^pr^ is mutated; in the Sp-Tu gene complex, the Co^pR^ 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^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^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 carrying pp60^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^src-associated kinase activity. Diagram shows kinase activity of brain cell extracts in parental, F1, and backcross (BC1) 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-32P]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-src-associated kinase activity. Diagram shows kinase activity of brain cell extracts in parental, F₁, and backcross (BC₁) 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-32P]ATP. Kinase activity is expressed as cpm/mg protein rounded off to the nearest significant figure. For the actual mean kinase activities, see text.
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