PARTIAL NUCLEOTIDE SEQUENCE, MAPPING AND RELATIVE EXPRESSION OF A *XIPHOPHORUS* FISH *NF1* GENE

THESIS

Presented to the Graduate College of

Southwest Texas State University

in Partial Fulfillment of

the Requirements

For the Degree of Master of Science

By

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San Marcos, Texas

May, 2001

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to Dr. Ronald Walter for the criticism and guidance he extended to me. I appreciate the opportunity to work and learn in his lab. It has been a challenging and rewarding experience.

I would like to thank both Dr. Joseph Koke and Dr. James Irvin for their contributions to and criticisms of this manuscript. I appreciate their patience and guidance.

I am very grateful to Kevin Kelnar for the hours of technical assistance, advice and motivation. I am especially appreciative for the assistance and friendship of Doug Rains and Christina Daniels. I am extraordinarily grateful to Dr. Steve Kazianis for his consistent willingness to assist and direct the writing of this manuscript. I also must include Ramiro Garcia, Patrick DeGuelle, Autumn Sansom, Vesselina Ivanova, Anthony Pedroza, and Jarah Meador without whom this experience would never have been so much fun.

I also wish to thank my husband, Owen Wheeler for the understanding and encouragement which he never failed to provide. I also want to express the deep appreciation and respect I have for my parents, Bill and Cindy Kennedy. Their support and advice through the years has been a source of strength that is incomparable.

This research was supported in part by grants from the National Center for Research Resources (R24-RR12253), NCI (P01-CA75137), and the Roy F. and Joan C. Mitte Foundation.

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Introduction

Neurofibromatosis, also called von Recklinghausen syndrome, is a genetic disease affecting approximately 1 in 3500 people of all ethnic groups (Shen *et al.*, 1996). This disorder exhibits an autosomal dominant pattern of inheritance and complete penetrance (Riccardi, 1981). There is a high degree of variability in phenotypic expression of neurofibromatosis, even within predisposed families (Shen *et al.*, 1996). Three characteristics that clinically define neurofibromatosis are the presence of multiple neurofibromas, café au lait spots, and lisch nodules of the iris (Riccardi, 1981). Neurofibromas are benign cutaneous tumors that occur in tissue derived from neural crest and carry an increased risk for malignancy (Riccardi, 1981). Café au lait spots are pigmented birthmarks that contain melanocytes harboring macromelanosomes, but have no clinical significance other than serving as a diagnostic indicator (Gutmann and Collins, 1993). The diagnostic criteria for neurofibromatosis includes the presence of six or more café au lait macules in postpubescent individuals that are 15 mm or greater in diameter (Stumpf *et al.*, 1988). Lisch nodules are small lesions on the iris that appear during childhood and are not associated with morbidity (Gutmann and Collins, 1993). Although the three characteristic features occur in over 90% of all neurofibromatosis patients by puberty, the number of lesions is extremely variable (Shen *et al.*, 1996).

Other features present in a minority of cases include learning disabilities, seizures, macrocephaly, short stature, and scoliosis (Riccardi, 1981).

NF1 Gene

Neurofibromatosis arises as the result of alterations within the *NF1* gene. Human *NF1* spans over 350 kb of genomic DNA (Wallace *et al.*, 1990). The gene maps to chromosomal region 17q11.2 (Wallace *et al.*, 1990). The *Fugu NF1* gene spans only 27 kb, which is 13 times smaller than the human counterpart (Kehrer-Sawatzki *et al.*, 1998). The complete genomic structure of the human *NF1* gene has not been elucidated because of unsuccessful amplification across several introns (Li, *et al.*, 1995). In humans, there are two exceptionally large introns. Intron 1 spans approximately 120 kb and intron 27b spans almost 50 kb of human genomic DNA (Li *et* al., 1995).

Three genes (EVI2A, EVI2B, and OMGP) are embedded within intron 27b and are transcribed in the opposite orientation to *NF1* (Shen *et al.*, 1996). It is not clear whether these genes play a role in the variability of *NF1* expression. EVI2A and EVI2B are the human homologues of murine Evi-2A (Cawthon *et al.*, 1990) and Evi-2B (Cawthon *et al.*, 1991) respectively. Evi-2A is a putative oncogene and is involved in retrovirus induced murine myeloid leukemia (Buchberg *et al.*, 1990). Evi-2B lies in the midst of a cluster of viral integration sites identified in retrovirus induced myeloid tumors and may thus also function as an oncogene in these tumors (Cawthon *et al.*, 1991). EVI2A is highly expressed in brain, bone marrow, and peripheral blood while EVI2B is expressed in bone marrow and peripheral blood but not in brain (Shen *et al.*, 1996). OMGP encodes the oligodendrocyte-myelin glycoprotein, which is expressed only in

oligodendrocytes of the central nervous system (Viskochil *et al.*, 1991). OMGP may function as a cell adhesion molecule in the myelin of the central nervous system (Mikol *et al.*, 1990).

The finding of three genes within an intron of a fourth is unique and the complexity of this region suggests unusual modes of transcriptional regulation. It is not known whether it is possible to simultaneously transcribe nested genes in opposite orientations. Therefore, activation of an embedded gene might inhibit transcription of the NF1 gene or vice versa (Cawthon *et al.*, 1990). Multiple ATTTA pentamers are present in the 3' noncoding sequences of all three *NF1* embedded genes (Viskochil *et al.*, 1991) and this motif is postulated to promote rapid cytoplasmic degradation of mRNA (Brawerman, 1989). It is also logical to speculate these embedded genes might be responsible for specific NF1 phenotypes. Evi-2A and Evi-2b are the murine homologues of EVI2A and EVI2B and are both involved in induction of murine myelogenous leukemia (Buchberg *et al.*, 1990); therefore, higher frequencies of juvenile-chronic myelogenous leukemia found among patients with neurofibromatosis may be related to mutations that disrupt EVI2A or EVI2B (Clark and Hutter, 1982). Learning disabilities may be correlated with mutations on the OMGP gene because of its known role in oligodendrocytes as an externally located cell adhesion molecule active in mediating proper cell motility and differentiation during brain development (Mikol *et al.*, 1990).

NF1 Transcript

NF1 encodes an mRNA transcript of approximately 13 kb and encompasses 59 exons (Wallace *et al.*, 1990). *NF1* is widely expressed in many tissues. PCR analysis of RNA expression demonstrates an amplified fragment in reverse transcribed RNA from human brain, neuroblastoma, kidney, melanoma and neurofibroma (Wallace *et al.*, 1990). A 13 kb transcript can be detected in murine kidney, brain, and melanoma cells, but not in mouse skin, spleen, thymus or liver (Buchberg *et al.*, 1990). Exons 21-27a show homology to the catalytic domain of the mammalian GTPase activating protein (GAP) (Li *et al.*, 1995), and *NF1* is therefore classified as a member of the GAP gene family. GAPs are cytosolic proteins that catalyze the conversion of active GTP-bound *ras* to the inactive GDP-bound form (Trahey and McCormick, 1987). The 13 kb transcript is referred to as the original transcript and is called *NF1-GRD I* (Gap Related Domain I).

Four alternatively spliced *NF1* transcripts have been identified (Figure 1-1). 5'ALT1, a truncated form of *NF1*, is about 2.9 kb in length, having an open reading frame predicting a 551 amino acid protein and shares the same 547 amino-terminal residues with the original *NF1* product (Suzuki *et al.*, 1992). 5'ALT2 contains a 30 bp addition between exons 9b and 10 and is highly expressed in the central nervous system (Danglot *et al.*, 1995). 3'ALT shows a 54 bp addition between exons 48 and 49 and is expressed in adult and fetal cardiac tissue unlike the original *NF1* transcript (Gutmann *et al.*, 1993). GRDII has a 63 bp addition (exon 23a) of largely basic amino acids in the GAP related domain (GRD), resulting in obliteration of GTPase activity (Shen *et al.*, 1996). *NF1-GRDII* is expressed in adult differentiated brain tissue, whereas *NF1-GRD I* is predominantly expressed in fetal brain tissue (Shen *et al.*, 1996).

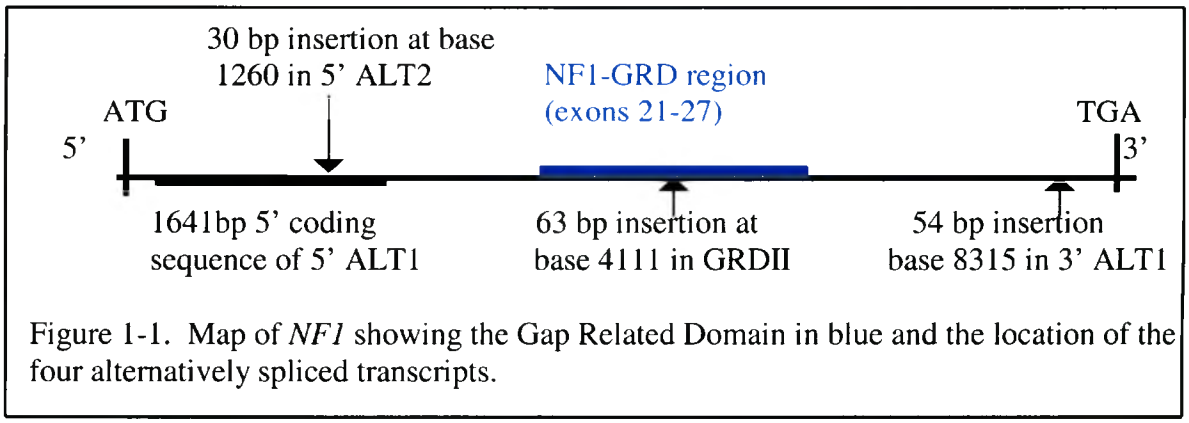


Figure 1-1. Map of *NF1* showing the Gap Related Domain in blue and the location of the four alternatively spliced transcripts.

NF1 Protein

The *NF1* gene encodes neurofibromin, which is a 2818 amino acid protein (Marchuk *et al.*, 1991). The protein does not show a membrane-spanning region (Marchuk *et* al., 1991) and is predicted to be cytosolic by discriminant analysis (Klein *et al.*, 1985). Six potential cAMP-dependent protein kinase phosphorylation sites and a single potential tyrosine phosphorylation site are present (Marchuk *et al.*, 1991). The neurofibromin protein can be divided into three domains; an amino-terminus of unknown function, a GAP-related middle domain, and a carboxy-terminus related to the yeast IRA2 and IRA1 (homologues of mammalian GAP) gene products (Marchuk *et al.*, 1991). Unfortunately, the function of the C-terminal domain is not known even in the yeast (Marchuk *et al.*, 1991).

Antibodies recognizing the neurofibromin protein have been used to show expression in kidney, spleen, lung, muscle, skin, and highest levels in human brain tissue (Gutmann *et al.*, 1991). In rats, neurofibromin antibodies indicate the highest levels of the neurofibromin protein occurs in brain, spinal cord, sciatic nerve, and adrenal glands; while lower levels are found in liver, spleen, pancreas, and cardiac tissue (Daston *et al.*,

1992). *NF1* expression was not detected in rat skeletal muscle, lung, kidney, or skin using the same *NF1* antibody (Daston *et al.*, 1992).

NF1 and *ras*

In humans, mutations within the *NF1* gene result in loss of function of the neurofibromin protein and overexpression of p21ras setting the stage for development of benign neurofibroma tumors (Basu *et al.*, 1992). The *ras* genes are required in normal mitogenic signaling and are mutationally activated in many different types of tumors (DeClue *et al.*, 1991). Ligand-bound receptor tyrosine kinases phosphorylate tyrosines that are high affinity binding sites for SH2 and SH3 domains (Figure 1-2) (Clapham, 1997). These domains mediate interactions between the tyrosine-phosphorylated receptor and adaptor proteins resulting in Ras-dependent MAP kinase activation (Clapham, 1997). MAP kinases phosphorylate and activate nuclear transcription factors that in turn activate genes controlling the cell cycle (Clapham, 1997). When Ras is bound to GTP it is active and able to initiate the MAP kinase cascade leading to cell proliferation or differentiation (Shen *et al.*, 1996). When Ras is bound to GDP it is biologically inactive (Shen *et al.*, 1996).

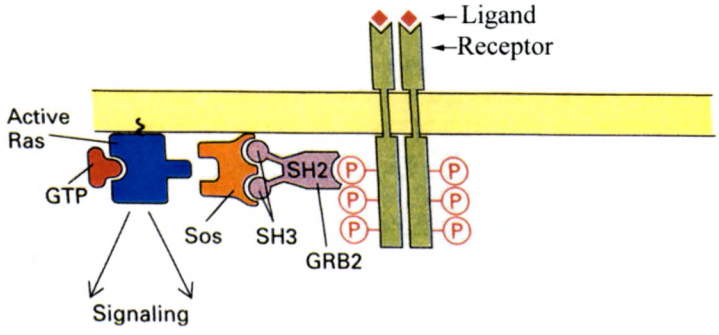


Figure 1-2. Activation of Ras following binding of ligand to receptor.

Three closely related *ras* genes (H-*ras*, Ki-*ras*, and N-*ras*) encode 21 kDa proteins (therefore called p21ras) with intrinsic GTPase activity (Bourne *et al.*, 1990). Biochemical analyses have shown that the GAP related domain of human neurofibromin (exons 21-27a) has the ability to accelerate the conversion of the active GTP bound p21ras to the inactive form of GDP bound p21ras and thus downregulate *ras* activity (Figure 1-3) (Ballester *et al.*, 1990).

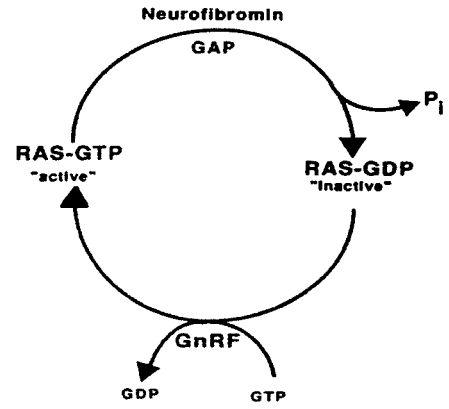


Figure 1-3. Neurofibromin is a member of the GAP family and catalyzes the intrinsic GTPase activity of Ras. Ras is inactive when bound to GDP and active when bound to GTP.

GAP proteins have two SH2 domains and a SH3 domain in their amino terminal ends and domains that bind *ras* in the c-termini (Koch *et al*, 1991). Neurofibromin has several potential sites for phosphorylation but lacks the SH2 and SH3 domains that direct interactions with phosphotyrosine proteins involved in signal transduction (Marchuk *et al.*, 1991). This suggests that neurofibromin is not interchangeable with other GAP family members and implies that *NF1* activity is probably not directly modulated through tyrosine phosphorylation by activated growth factor receptors (Koch, 1991). These differences between GAP and *NF1* may allow the cell to regulate p21ras in a more elaborate way, by sensing incoming signals as well as providing a constitutive down-

modulation of p21ras function (DeClue *et al.*, 1991). *NF1* may not only be an upstream regulatory protein for *ras*, but may also function as a downstream effector (Marchuk *et al.*, 1991). *NF1-GRD* interactions with effector and oncogenic mutants of *ras* have suggested that *NF1* may not only regulate *ras* activity by hydrolyzing GTP to GDP but also interacts with *ras* through its effector domain (Martin *et al.*, 1990).

Activating mutations in *ras* lead to aberrant signaling for cell proliferation and are involved in the genesis of many human malignant tumors (Bos, 1989). Germline and somatic *NF1* mutations lead to neurofibromatosis by inactivating the gene, rendering it incapable of negatively regulating the p21ras oncogene (Viskochil *et al.*, 1993). Malignancies in neurofibromatosis are believed to follow the two-hit model proposed for stepwise inactivation of a tumor suppressor gene, in which one allele is constitutively inactivated while the other allele becomes inactivated at the somatic level (Shannon *et al.*, 1994). The strongest evidence that directly implicates the *NF1* tumor suppressor gene in carcinogenesis comes from studies of neurofibrosarcomas in NF1 patients (DeClue *et al.*, 1992). In cell lines derived from these malignant tumors, levels of neurofibromin were diminished, whereas ras^{GTP} levels were elevated and growth promoting (Basu *et al.*, 1992).

Xiphophorus as a Model

The structural conservation of the *NF1* gene among vertebrates is poorly understood since full-length loci have only been characterized in two vertebrates; human (Li *et al.*, 1995) and the pufferfish, *Fugu rubripes* (Kehrer-Sawatzki *et al.*, 1998). The fish used in this study are swordtails and platyfish of the genus *Xiphophorus*.

Xiphophorus fishes are represented by 22 species within the family Poeciliidae (Rosen, 1979). The *Xiphophorus* fish genetic model is well suited for studies into vertebrate genome evolution. It is estimated that fishes diverged from a common tetrapod ancestor about 450 million years ago (Morizot, 1990). The *Xiphophorus* genome appears to retain many primitive vertebrate patterns of genome organization and genetic fine structure (Walter and Morizot, 1996). It is composed of 24 pairs of acrocentric chromosomes (Ohno, 1970) and contains about 20% the DNA found in mammalian cells (Hinegardner and Rosen, 1972). Most of what is known concerning the genetic mechanisms giving rise to complex phenotypes comes from studies on organisms separated by more than a billion years of evolutionary divergence (*E. coli*, yeast, mammals). Studies of fish genomes may provide a valuable median point between yeast and mammals and help fill this extensive evolutionary gap (Walter and Morizot, 1996). The *Xiphophorus* linkage map comprises over 300 AP-PCR/RAPD markers and 103 genes (Morizot *et al.*, 1998). Markers have been assigned to 24 multipoint linkage groups and 10 unassigned linkage groups (Morizot *et al.*, 1998). Among vertebrates, many examples of syntenic group conservation have been maintained over 450 million years of evolution (Walter and Morizot, 1996). Mapping of genes in *Xiphophorus* has provided speculation of the genetic content of a primitive vertebrate genome since conserved gene arrangements were probably derived from the ancestral vertebrate progenitor (Morizot, 1990).

Xiphophorus fishes represent an established model for vertebrate carcinogenesis (Schwab *et al.*, 1978, 1979; Schwab, 1982, Vielkind *et al.*, 1989). Selective breeding of the known 22 species produces fertile offspring, which makes them a valuable tool for genetic study (Rosen, 1979). Interspecies hybrids of these fish exhibit a number of

Table 1-1. A few examples of spontaneous and induced tumor models identified in *Xiphophorus* fishes.

Species Utilized	Tumor Type	Experimental Treatment
X. helleri (×) (*X. maculatus* Jp 163 A (×) *X. helleri*)	Melanoma[5]	Spontaneous in hybrids
X. andersi (×) (*X. maculatus* Jp 163 B (×) *X. andersi*)	Melanoma[3]	MNU induced in hybrids
X. helleri (×) (*X. maculatus* Jp 163 B (×) *X. helleri*)	Melanoma[4]	UVB radiation of hybrids
X. maculatus (×) *X. couchianus*	Retinoblastoma[6]	MNU induced in hybrids
X. variatus (×) *X. helleri*	Neuroblastoma[2]	X-ray induced in hybrids
X. variatus (×) *X. helleri*	Rhabdomyosarcoma[1]	MNU induced in hybrids
X. variatus (×) *X. helleri*	Fibrosarcoma[1]	MNU induced in hybrids
X. cortezi	Melanoma[1]	Spontaneous
X. variatus	Melanoma[1]	Spontaneous, age related

References used for table 1-1 (Schwab *et al.*, 1978[1], 1979[2], Kazianis *et* al., 2001[3]; Nairn *et al.*, 1996[4]; Vielkind *et al.*, 1989[5]; Walter and Kazianis, 2001[6]).

spontaneous and induced malignancies (Table 1-1). For example, *X. maculatus* (×) *X. helleri* backcross hybrids exhibit spontaneous benign and malignant melanomas in classical Mendelian ratios (Gordon, 1927). The process of tumorigenesis is still far from understood due to the complexity of the neoplastic phenotype and the multiplicity of the underlying molecular mechanisms (Schartl, 1995). Model genetic systems, such as *Xiphophorus*, have the advantage that the gene(s) associated with neoplastic transformation are clearly defined by classical genetic mechanisms. It is expected that the study of these tumor models will provide crucial information for understanding the molecular events leading to tumor predisposition (Schartl, 1995). Thus, it is of interest to isolate *Xiphophorus* oncogene and tumor suppressor homologues, such as *NF1*, in order to test their potential roles in *Xiphophorus* interspecies tumorigenesis. Use of the highly inbred and genetically well characterized *Xiphophorus* fish species may facilitate identification of novel factors associated with neurofibroma formation and these factors, once isolated, may be tested for similar association in human carcinogenesis. Our hypothesis is that the *NF1* gene associates with tumors of neuroectodermal origin in *Xiphophorus* fish. Here we present the partial nucleotide sequence, predicted amino acid sequence, gene map assignment, and tissue specific expression patterns of a *NF1* homologue of *Xiphophorus* fishes.

Chapter 2

Materials and Methods

Fish Stocks

Table 2-1. Stocks of *Xiphophorus* fish used in this study and the experimental objective of each. All fishes were provided by the *Xiphophorus* Genetic Stock Center, Department of Chemistry and Biochemistry, Southwest Texas State University. The IACUC animal use approval number is 99-11.

Species Utilized	**Nucleic Acids Isolated**	**Experimental Objective**
X. maculatus Jp 163 A	Genomic DNA RNA→cDNA	Gene Isolation
X. maculatus Jp 163 B	Genomic DNA RNA→cDNA	Mapping R-Q-RT-PCR
X. helleri Sarabia	Genomic DNA	Mapping
X. helleri (×) *X. maculatus* Jp 163 B	Genomic DNA	Mapping
X. helleri (×) (*X. maculatus* Jp 163 B (×) *X. helleri*)	Genomic DNA	Mapping
X. couchianus (×) (*X. maculatus* Jp 163 B (×) *X. couchianus*)	RNA→cDNA	R-Q-RT-PCR

Genomic DNA Isolation

Fish are euthanized by placing them in an ice bath followed by cranial pith. Generally, DNA is isolated from brain, eye, liver, gill, ovary, or testes tissues. Fish tissues are dissected directly into a Dounce homogenizer standing in a dry ice-ethanol bath. Lysis buffer (1% SDS, 10 mM Tris-HCl [pH 7.5], 100 mM EDTA, 0.1 M NaCl, 300 mg Proteinase K) is added at 3 ml per 1.0 gram of tissue. The tissue is homogenized on wet ice (3 strokes) and then poured into a 15 ml polypropylene tube and incubated at 55°C for one hour. One milliliter of Tris (pH 8.0)-saturated phenol is added and the suspension mixed on a tube rotator for ten minutes, followed by centrifugation in a Beckman tabletop centrifuge (Model TJ-6; Fullerton, CA) at 2000 x g for 10 minutes. The aqueous phase is removed to a new centrifuge tube and extracted once more with phenol. Sevags solution (96 ml choloroform, 4 mL isoamyl alcohol) is used to extract the aqueous phase two additional times. The nucleic acids are precipitated by adding 0.1 volume of 3 M sodium acetate (pH 7.0) and two volumes freezer cold 100% ethanol followed by incubation at -80°C for 30 minutes. Nucleic acids are pelleted by centrifugation in a Beckman tabletop centrifuge at 2000 x g for 30 minutes. The pellet is washed with 70% ethanol, dried in a vacuum centrifuge (Savant SpeedVac; Holbrook, NY) for 10 minutes, and resuspended in 500 µl TE (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]). The DNA is transferred to a 1.5 ml microcentrifuge tube treated with RNase A (1 µg/µl) for 30 minutes. The sample is phenol and Sevags extracted as above then precipitated with cold 100% ethanol at -80°C and pelleted at 9000 x g for 10 minutes. The ethanol is decanted and the pellet washed with 70% ethanol. The nucleic acids are dried in a vacuum centrifuge and resuspended in 300 µl TE. The DNA concentration is

determined using a DU-64 spectrophotometer (Beckman Instruments Inc.; Fullerton, CA). Measurements are taken at A_{260} and A_{280} and calculated using the relation that 1 A_{260} unit equals 50 μg/ml of double stranded DNA (Maniatis *et al.*, 1989). An aliquot of each DNA sample is run on a 1% agarose gel in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA) to determine that it is high molecular weight.

Polymerase Chain Reaction

For each sample, the following are combined in a 0.25 ml tube; 50-500 ng of target DNA (resuspended in sterile, distilled water); 20 μM of each appropriate primer; 5 μl of 10 x PCR buffer (500 mM KCl, 200 mM Tris-HCl [pH 8.4]), 1.5 mM $MgCl_2$, and 2.5 units of native *Thermus aquaticus (Taq)* Polymerase (Gibco BRL; Rockville, MD). Sterile distilled water is added for a final volume of 50 μl. PCR controls are also established; these included DNA blanks, *Taq* Polymerase blanks, and primer blanks. The blanks are treated the same as the other samples except that they lacked DNA, *Taq,* or primers. The blanks control for contamination and primer dimer formation. The sample tubes are placed in a GeneAmp PCR System 2400 Thermal Cycler (Perkin Elmer Biosystems; Foster City, CA) to complete successive rounds of denaturation, primer annealing, and extension. To visualize the amplified product, an aliquot of the sample is fractionated on an ethidium bromide stained 2.0% agarose gel in TAE buffer.

Table 2-2. Primers and conditions for amplification of the *Xiphophorus NF1* gene.

NF1 PCR Primers

Forward	Reverse
B1- 5'-TTC/TATGGAA/GGT-3'	B2- 5'-GAA/GTTC/TGAC/TAC-3'
B3- 5'-AACATA/GTTCCA-3'	B4- 5'-TTA/GAAA/GCAA/GAA-3'
C1- 5'-CAA/GGAA/GAAA/GATA/T/CGG-3'	C2- 5'-TTC/TGAC/TAAA/GATGGC-3'
C3- 5'-TCC/TTCT/CTTT/CTCA/GTG-3'	C4- 5'-GCC/TTGA/GTAA/GAAG/A/TAT-3'
B2x- 5'-GAGTTTGACACGTTGGCAGAG-3'	B3x- 5'-GTTCCACAACAGCTGGTAGAG-3'
777F-5'-GCTCGAGTTCTGGTGACCCT-3'	777R-5'-GCCACAGGTTTGTGCTCTGG-3'

PCR Amplification Parameters

Primer Pair	Template	Denaturation (94°C)	Annealing	Extension (72°C)	Cycle #
B2x/B3x	genomic	45 sec.	60°C- 1 min. 15 sec.	2 min. 15 sec.	35
B2x/B3x	cDNA	30 sec.	60°C- 45 sec.	1 min.	29
777F/777R	cDNA	45 sec.	60°C- 1 min. 30 sec.	2 min.	30
B1/B4	genomic	30 sec.	35°C- 30 sec.	1 min.	30
B2/B3	B1B4 amplicon	30 sec.	35°C- 30 sec.	1 min.	30
C1/C4	cDNA	30 sec.	35°C- 30 sec.	1 min.	30
C2/C3	C1C4 amplicon	30 sec.	35°C- 30 sec.	1 min.	30

DNA Isolation from Agarose Gels

The CONCERT™ gel extraction system (Gibco BRL; Rockville, MD) uses silica resin to capture and purify DNA fragments. The matrix gel extraction protocol is performed as follows; the area of the gel containing the DNA fragment of interest is cut using a clean, sharp blade. The gel slice is weighed and 30 µl of Gel Solubilization Buffer (L1) added for every 10 mg of gel. One microliter of silica resin is added per 10 mg of gel. The sample is vortexed and incubated at 50°C for 15 minutes, mixing every three minutes. The sample is then centrifuged in a microcentrifuge (Savant; Holbrook, NY) at 9000 x g for 30 seconds. The supernatant is removed and discarded, and the sample is subjected to a high salt wash by adding 30 µl of Gel Solubilization Buffer (L1) for every 10 mg of gel. The resin is resuspended by vortexing and centrifuged (9000 x g)

for 30 seconds. The supernatant is removed and discarded. The sample is then subjected to two low salt washes by adding 30 µl of Wash Buffer (L2) for every 10 mg of gel. The resin is suspended by vortexing and centrifuged (9000 x g) for 30 seconds and the supernatant is removed and discarded. The resin is air dried for five minutes and the DNA eluted by adding 40 µl of TE buffer and vortexing the resin. The sample is incubated for 5 minutes at 50°C, vortexing once during incubation. The sample is then centrifuged (9000 x g) for 30 seconds and the supernatant pipeted into a fresh microcentrifuge tube. The DNA fragment is then subcloned into a plasmid vector as described below.

Subcloning PCR Amplimers

Stratagene's (La Jolla, CA) PCR-Script™ Amp cloning kit permits the efficient cloning of PCR fragments. The product is first purified with the StrataPrep PCR purification kit (Stratagene; La Jolla, CA). The purification is performed as follows; one volume of DNA-binding solution is added to the amplification reaction and the mixture transferred to a microspin cup, seated in a 2-ml receptacle tube. The tubes are spun in a microcentrifuge for 30 seconds and the DNA bound to a silica-based fiber matrix. The DNA-binding solution is discarded. Then, 750 µl of 1x PCR wash buffer (10mM Tris [pH 7.5], 100 mM NaCl) is added to the microspin cup and spun for 30 seconds at 9000 x g. The wash buffer is discarded and the microspin cup is spun at 9000 x g for an additional 30 seconds to remove residual wash buffer. The microspin cup is transferred to a fresh 1.5 ml microcentrifuge tube. 50 µl of elution buffer (10 mM Tris [pH 8.5], 10

mM EDTA) is added directly onto the top of the fiber matrix and incubated at room temperature for five minutes, then spun for 30 seconds (9000 x g) to elute the DNA.

The ends of PCR products generated with *Taq* DNA polymerase need to be polished. Ten µl of the purified product, 1 µl of 10 mM dNTP mix (2.5 mM each), 1.3 µl of 10x polishing buffer (200 mM Tris [pH 8.75], 100 mM KCl, 100 mM $(NH_4)2SO_4$, 20mM $MgSO_4$, 1 mg/ml BSA, 1% Triton X-100), and 1 µl of cloned *Pfu* DNA polymerase (0.5 U) is gently mixed and a 20 µl mineral oil overlay is added. The polishing reaction is incubated at 72°C for 30 minutes.

The polished product is then ligated with the pPCR-Script Amp SK(+) vector as follows; 1 µl of the pPCR-Script Amp SK(+) cloning vector (10 ng/µl), 1 µl of PCR-Script 10 x reaction buffer, 0.5 µl of 10 mM rATP, 3.5 µl of the blunt-ended product, 1 µl of *Srf* I restriction enzyme (5 U/µl), 1 µl T4 DNA ligase (4 U/µl), and 2 µl of distilled water. The ligation reaction is mixed and incubated at room temperature for one hour, then heated to 65°C for 10 minutes.

The ligation reaction is transformed into XL10-Gold Kan ultracompetent *E.coli* cells (Stratagene; La Jolla, CA). The transformation is performed as follows; ultracompetent cells are thawed on ice, 40 µl of cells were aliquoted into chilled 15 ml Falcon polypropylene tubes, and 1.6 µl of XL10-Gold β-mercaptoethanol was added. The tube is swirled gently and incubated on ice for 10 minutes, then 2 µl of the ligation reaction is added to the cells and incubated on ice for 30 minutes. The reactions are heat pulsed in a 42°C water bath for 30 seconds and then incubated on ice for 2 minutes. 450 µl of Luria-Bertani (LB) broth, preheated to 42°C, is added and incubated in a 37°C shaker bath (Precision Scientific 360 Orbital Shaker Bath; Chicago, IL) at 240 rpm for

one hour. 10 µl, 50 µl and 100 µl aliquots of the transformation reaction are plated onto LB-ampicillin (0.15 mg/ml) agar plates containing 2% X-gal and 10 mM IPTG (isopropylthio-β-D-galactoside). The plates are incubated overnight at 37°C, and white colonies selected for alkaline lysis.

Alkaline Lysis

To isolate DNA from potential subclones, and to use in DNA sequencing reactions, alkaline lysis protocols are performed as follows. A 3 ml culture of LB broth containing 25 µg/µl ampicillin is inoculated with the desired cell line and grown overnight to stationary phase in a 37°C shaker bath. The cells are pelleted in a microcentrifuge for 30 seconds (9000 x g) and resuspended in 100 µl of sterile glucose solution (50 mM glucose, 25 mM Tris-HCl [pH 8.0] 10 mM EDTA). To this, 200 µl of freshly made 0.2 M NaOH and 1% SDS is added and the mixture incubated on ice for 5 minutes, then 150 µl of potassium acetate solution (5.0 M potassium acetate [pH 5.8]) is added, the tubes are briefly vortexed, and incubated on ice for 5 minutes. The suspension is spun in a microcentrifuge (9000 x g) for 5 minutes and the supernatant transferred to a fresh tube. The nucleic acids are precipitated by the addition of 1 volume 100% ice cold ethanol. After a 10 minute incubation at room temperature, the nucleic acids are spun in a microcentrifuge (9000 x g) for 10 minutes, the alcohol decanted and the pellet washed with 70% ethanol. The nucleic acids are dried under a vacuum and resuspended in 50 µl TE buffer. 2.5 µg of RNase A is added, followed by a 30 minute incubation at 37°C. Then the DNA is extracted once with phenol and once with Sevag's solution. The nucleic acids are precipitated in 2.5 volumes of 100% ice cold ethanol and 3 M sodium

acetate (pH 7.0) and placed at -80°C for ten minutes. Then the DNA is pelleted at 9000 x g for 10 minutes. The pellet is dried under a vacuum and the DNA is resuspended in 50 µl TE buffer.

Nucleotide Sequencing and Sequence Comparisons

Nucleotide sequence determination of the plasmid subclones is carried out utilizing dideoxynucleotide chain termination protocol (Sanger, 1977), using a Sequenase® Version 2.0 DNA sequencing kit (United States Biochemical Corporation; Cleveland, OH). 2-5 µg of alkaline lysis mini-prep DNA is used for each reaction. DNA is denatured with 8 µl of 1 N NaOH, 2 µl of 5 mM EDTA, 1 µl of the appropriate primer, and 19 µl of ddH_2O at 37°C for 30 minutes. 4 µl of 3 M NaOAc and 90 µl of ice cold 100% EtOH are added, and the mixture is placed at -80°C for 10 minutes. DNA is pelleted at 9000 x g for 10 minutes and the pellet is washed with 70% EtOH. The pellet is dried under a vacuum and resuspended in 7 µl of water, 1 µl of the appropriate primer (20 µg/µl), and 2 µl of reaction buffer (200 mM Tris/HCl [pH 7.5], 100 mM $MgCl_2$, 250 mM NaCl). The mixture is incubated at 65°C for 5 minutes, and 24°C for 5 minutes, then placed on ice for 5 minutes. In corresponding tubes, 2.5 µl of each respective dideoxynucleotide mixture (8 µM of the appropriate ddNTP, 80 µM of the other dNTPs, 50 mM NaCl) are aliquoted and placed at 37°C. To the tube containing the annealed primer/DNA sequence mix, 1 µl of 100 mM dithiothreitol (DTT), 2 µl of labeling mix (7.5 µM dGTP, 7.5 µM dCTP, 7.5 µM dTTP, diluted 1:4 with sterile H_2O), 1 µl of [α-^{35}S]dATP (1250 Ci/mmol, NEN Life Science; Boston, MA) and 2 µl of Sequenase®

Version 2.0 T7 Polymerase, diluted 1:9 in enzyme dilution buffer (10 mM Tris/HCl [pH 7.5], 5 mM DTT) are added for chain elongation. This reaction mixture is incubated for 5 minutes at room temperature, then to each of the respective dideoxynucleotide aliquots, 3.5 µl of elongation mixture is added and the tubes are incubated at 37°C for 5 minutes. The reaction is terminated by the addition of 5 µl of stop solution (95% formamide, 20 mM EDTA [pH 8.0], 0.05% bromophenol blue, 0.05% xylene cyanol). The sequencing reactions are denatured at 94°C for 5 minutes and fractionated on 6.0% polyacrylamide (acrylamide:bis-acrylamide 19:1) containing 5 M urea (0.45 mm in thickness) using a Kodak BIOMAX STS electrophoresis unit. Sequencing gels are run at approximately 50 volts/centimeter in 1 x TBE buffer until the xylene cyanol marker dye migrates 20 cm from the origin. After electrophoresis, sequencing gels are transferred onto 3 MM Whatman paper and dried under vacuum pressure for 90 minutes at 80°C using a Savant (Holbrook, NY) gel dryer. The gel is then used to expose X-OMAT AR film (Eastman-Kodak Inc.; Rochester, NY) to visualize the sequence fragments. Reactions are initiated using universal T3, T7, or custom synthesized nucleotide primers (Table 2-2). DNA sequence analysis was performed using the Mac Vector™ 6.5 software package (Eastman Kodak, NY) and the GenBank database and Entrez search retrieval system (National Center for Biotechnology Information, Bethesda, MD).

Table 2-3. Synthetic oligonucleotide primers designed for the *Xiphophorus NF1* gene.

NF1 Sequencing Primers

SP1-	5'-GAACCTCTGAATGT-3'
SPA-	5'-GACCGCTCCAGA-3'
SP22-	5'-TTCTCTAAAGAGGTGGAA-3'
SP26R-	5'-CTGCCCAATCCTCTCCTG-3'

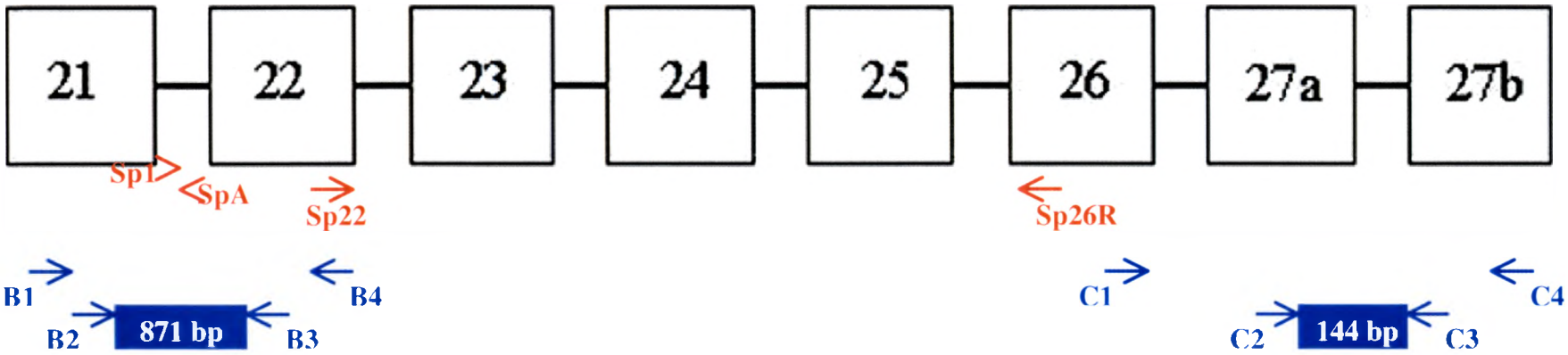


Figure 2-1. Map of *NF1-GRDII* fragment showing the location of primer binding sites. Text in red represents sequencing primers, blue represents PCR primers. The sizes of the PCR amplicons are given.

Determination of *NF1* Genotypes

Polymerase chain reactions are set up as described above. The fragments are visualized on 2% agarose gels. A PCR polymorphism is established between two parental species using primers that cross an intron. This informative polymorphism allows screening of a panel of backcross hybrids. The bands are visualized on an ethidium-bromide stained 2% agarose gel and scored as homozygous (showing the banding pattern of a parental species) or heterozygous (showing the banding pattern of an F_1 hybrid). A 1:1 ratio of homozygous(parental) : heterozygous(recombinant) is expected for Mendelian segregation of unlinked loci. Linkage of two loci is determined by bias of this ratio from 1:1 random segregation as determined by Chi-square analysis. Genotypic data are entered into a spreadsheet and exported into Map Manager QT (version b29) to generate pair wise comparisons (i.e., recombination values and LOD values) among all loci in each data set. The LOD score is the logarithm of the odds that two loci are linked and is a function of the Chi-square test using the null hypothesis of 50% recombinants. A LOD score of 3.0 or higher is considered significant evidence for linkage. MAPMAKER for Apple Macintosh (version 2.0) is used to determine maximum-likelihood gene map orders.

RNA Isolation

Tissues from *X. maculatus* Jp 163 B are flash frozen on a dry ice/ethanol bath as previously described (see Genomic DNA Isolation). All supplies and reagents needed for the RNA isolation are treated with 0.1% diethyl pyrocarbonate (DEPC) solution and autoclaved. Individual tissue samples are homogenized in 1 ml of TRI REAGENT™

(Sigma; St. Louis, MO) per 100 mg of tissue. The homogenized samples are incubated 5 minutes at room temperature to allow complete dissociation of the nucleoprotein complexes, then 0.2 ml chloroform is added per 1 ml of TRI REAGENT™, and the samples vigorously shaken for 15 seconds. The suspension is incubated at room temperature for 10 minutes, followed by centrifugation at 12,000 x g (Beckman Model J2-21 Centrifuge; Fullerton, CA) for 15 minutes at 4°C. The RNA-containing aqueous phase is transferred to a fresh tube and mixed with 0.5 ml isopropanol per 1 ml of TRI REAGENT™ initially used. Following a 10 minute room temperature incubation, the RNA is pelleted at 12,000 x g for 15 minutes at 4°C. The supernatant is decanted and the pellet washed once with 1 ml 70% EtOH per ml of TRI REAGENT™ used. The samples are centrifuged at 7,500 x g for 5 min. at 4°C, and the alcohol is decanted. The RNA pellets are air dried for 15 min., then resuspended in 500 µl H_2O. 40 µl of RNAse free DNase (2U/µl; Ambion; Austin, TX) and 54 µl of 10 x DNase buffer (100 mM Tris-HCl [pH 7.5], 25 mM $MgCl_2$, 1 mM $CaCl_2$) are added and the sample incubated at 37°C for 30 minutes, followed by extraction with phenol and Sevag's solutions. The RNA is precipitated with 1/10 volume 3 M NaOAc and 2 volumes 100% ice cold EtOH at -80°C for 10 minutes, then centrifuged (13,000 x g) for ten minutes and dried under a vacuum. The RNA is resuspended in 100 µl of H_2O and concentrations determined using a DU-64 spectrophotometer (Beckman Instruments Inc.; Fullerton, CA), with a value of 1 A_{260} unit considered equivalent to 40 mg/ml of single stranded RNA (Maniatis, *et al.* 1989).

Reverse Transcription Reaction

The RETROscript™ kit (Ambion, Austin, TX) is used to generate the complementary DNA (cDNA) strand of a desired RNA target sequence. 2 μg of total RNA, 4 μl of dNTP mix (2.5 mM each dNTP), 2 μl of appropriate first strand primers (random decamers [50 μM] or oligo dT_{18} [50 μM]), and nuclease free dH_2O to 16 μl are mixed together. The sample is heated to 80°C for 3 minutes. 2 μl of RT-PCR buffer (100 mM Tris/HCl [pH 8.3], 500 mM KCl, 15 mM $MgCl_2$), 1 μl of placental RNase Inhibitor (10 U/μl), and 1 μl M-MLV Reverse Transcriptase (100 U/μl) are added and the sample is mixed and incubated at 42°C for one hour. The reverse transcriptase is then inactivated at 92°C for 10 minutes.

Relative Quantitative RT-PCR

In RT-PCR, an RNA template is copied into a complementary DNA transcript, which is then amplified using PCR. When the products are analyzed, the amount of product from each reaction should be proportional to the abundance of the RNA transcript in each sample. The abundance of the *NF1* transcript is quantified relative to an endogenous 18S rRNA control.

a) Linear Range. Initial experiments are conducted to ensure that PCR amplification will be within the linear range. The linear range of the reaction is defined as the period of the PCR in which the amplification efficiency is at its maximum and remains constant over a number of cycles. A cocktail (0.2 μg cDNA, 1 x PCR buffer [200 mM Tris-HCl [pH 8.4], 500 mM KCl; Gibco BRL; Rockville, MD], 1 mM $MgCl_2$,

0.25 μM dNTP mix, 0.32 μM of gene specific primers, 5 μl of α^{32}P-dCTP [3000 Ci/mmol, NEN Life Science; Boston, MA], and distilled water to 50 μl) is aliquoted into 10 thin walled PCR tubes. The sample tubes are placed in a GeneAmp PCR system 2400 thermal cycler (Perkin Elmer; Foster City, CA) for rounds of denaturation (94°C-30 sec.), primer annealing (60°C-45 sec.) and extension (72°C-1 min.). Starting at the 15th cycle, one sample is removed every other cycle.

Ten μl of stop solution (95% formamide, 20 mM EDTA [pH 8.0], 0.05% bromophenol blue, 0.05% xylene cyanol) is added. The samples are denatured at 94°C for 5 minutes and fractionated on 6.0% polyacrylamide (acrylamide:bis-acrylamide 19:1) and 5 M urea denaturing gels (0.45 mm in thickness) using a Kodak BIOMAX STS electrophoresis unit. Gels were run at approximately 50 v/cm in 1 x TBE buffer until the xylene cyanol marker dye had migrated 30 cm from the origin. After electrophoresis gels are transferred to 3 MM Whatman paper and dried for 90 minutes at 80°C. The gel is exposed to phosphorimaging screens (Packard Instrument Company; Meriden, CT) for ten minutes and then scanned using the Cyclone storage phosphor system (Packard Instrument Company; Meriden, CT). The scanned image is analyzed using the OptiQuant™ software (Packard Instrument Company; Meriden, CT).

b) Primer : Competimer Ratio. Since the 18S primers compete with the gene specific primers for available resources, it is critical that the 18S control target be amplified from the cDNA at a level similar to the *NF1* target. Experiments are conducted to determine the optimal ratio of 18S primers and competimers. The cocktail is set up as described above with the addition of 4 μl of a primer:competimer mix. The sample tubes

are placed in a GeneAmp PCR system 2400 thermal cycler (Perkin Elmer; Foster City, CA) for rounds of denaturation (94°C-30 sec.), primer annealing (60°C-45 sec.) and extension (72°C- 1 min.) for the previously determined number of cycles corresponding to the linear range. 10 µl of stop solution is added and the samples are denatured at 94°C for 5 minutes. The samples are fractionated on a 6% polyacrylamide gel as described above and quantitated as previously described.

c) R-Q-RT-PCR. Multiplex PCR is performed with the gene specific primers and 4 µl of kit provided 18S rRNA primers and competimers (Ambion; Austin, TX) at the determined optimal ratio. A cocktail is made as described above lacking the cDNA and aliquoted into 10 thin walled PCR tubes. 0.2 µg of tissue specific cDNA is added to each aliquot. The samples are placed in a GeneAmp PCR system 2400 thermal cycler for rounds of denaturation (94°C-30 sec.), primer annealing (60°C-45 sec.), and extension (72°C-1 min.) at the cycle number corresponding to the middle of the linear range. 10 µl of stop solution is added and the samples are denatured at 94°C for 5 minutes. The samples are fractionated on a 6% polyacrylamide gel as described above and quantitated as previously described. Ratios of *NF1* (digital light units [DLU]) to 18S rRNA (DLU) were determined. *NF1* RNA expression was quantitated in relationship to amplification of 18S rRNA for individual tissues.

Chapter 3-Results and Discussion

Isolation and Nucleotide Sequence of a *Xiphophorus NF1-GRDII* Fragment

To isolate the *X. maculatus* Jp 163 A locus homologous to the mammalian *NF1* gene, we designed nested degenerate primer sets (Figure 3-1) based on highly conserved regions of the human (NCBI gene ID#: NM_000267), mouse (I54352) and *Drosophila* (T13947) *NF1* genes (Figure 3-1). We designed nested primer sets to enhance the specificity of our reactions. To design degenerate primers we first looked for regions of high amino acid homology between the human, mouse and *Drosophila* sequences. We observed highest homology in the GAP related domain of the gene (exons 21-27b) and therefore designed primers in this region. Next, we looked for amino acids with low degeneracy.

One primer set (B1 and B4) amplified a region of *Xiphophorus* genomic DNA and was used as a template for the B2 and B3 nested primer pair. This clone is therefore referred to as the pNF1B2B3 clone and represents exons 21 through 22. A second primer set (C1 and C4) amplified a region of cDNA and was used as a template for the primers C2 and C3. This clone is called pNF1C2C3 and represents portions of exons 27a and 27b (Figure 3-2). We then designed specific primers (777F and 777R) based on this known sequence to amplify the intervening exons from cDNA.

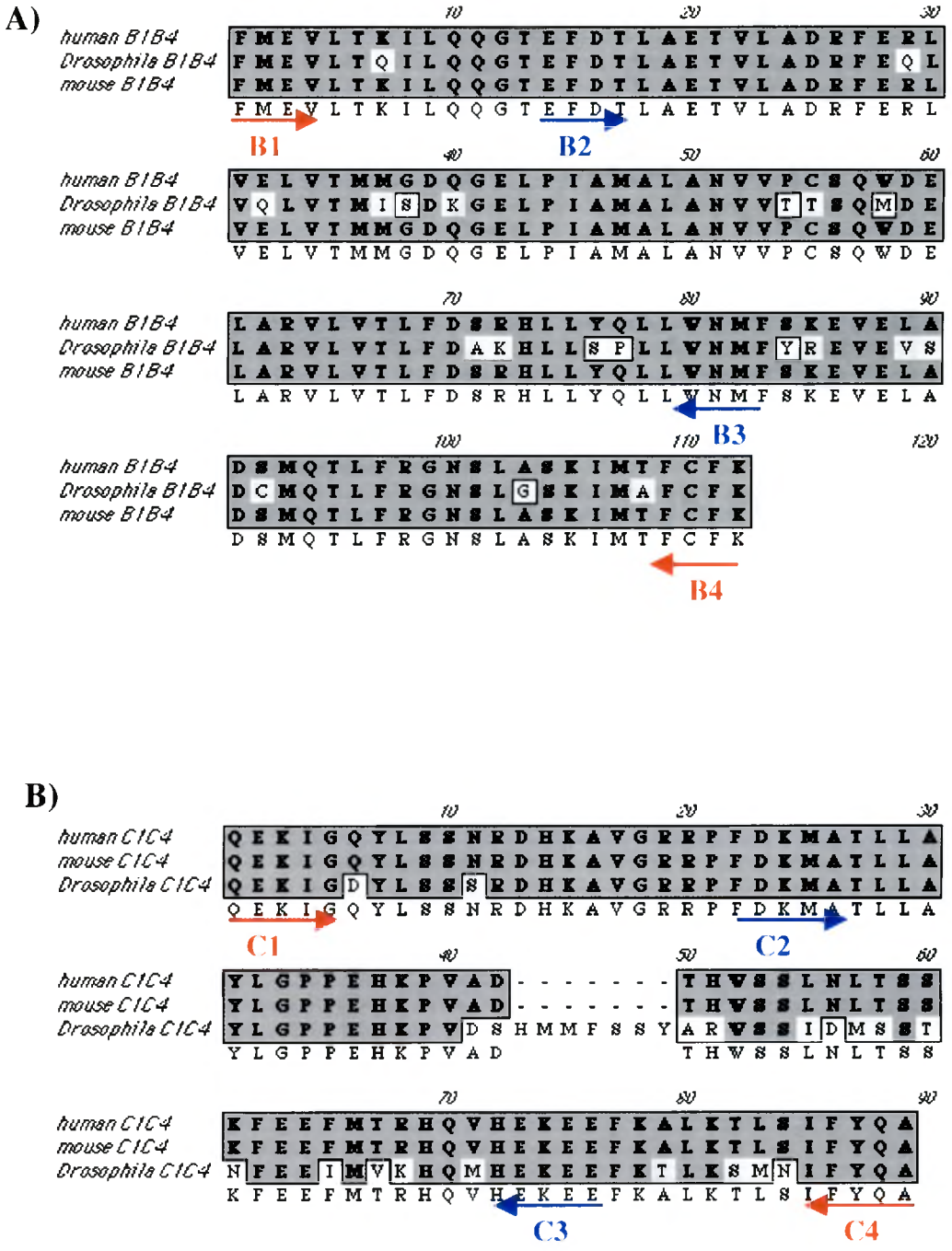


Figure 3-1. Amino acid alignment used to design degenerate primer sets. The outside primer pairs are shown in red and the inside nested pair is shown in blue. Primers were designed based on high conservation between the three species and low amino acid degeneracy.

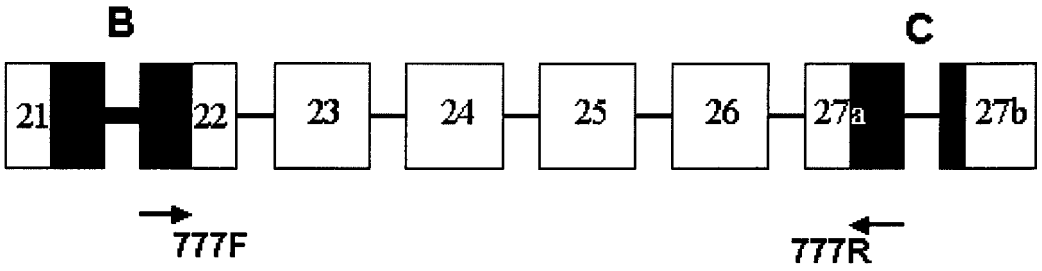


Figure 3-2. Map of exons 21 through 27b of *NF1* showing the pNF1B2B3 clone, the pNF1C2C3 clone, and the 777 primer set.

The primer pair (777F and 777R) amplified a band of the expected size (777 bp). This band was cut out of an agarose gel and the DNA was subcloned (pNF1-777) for nucleotide sequencing. The sequence of the 777 bp amplicon (Figure 3-3) represents eight putative exons of the *Xiphophorus NF1* gene. Translation of the fragment indicates 97% amino acid identity with exons 21 through 27b of the *Fugu NF1* gene and 91% identity with the human *NF1* gene (Table 3-1). The nucleotide identity is extraordinarily high (~79%) with the mammalian *NF1* genes. This high level of nucleotide conservation suggests this gene is under strong selective pressure to provide an essential cellular function. A clustal alignment is shown in figure 3-4.

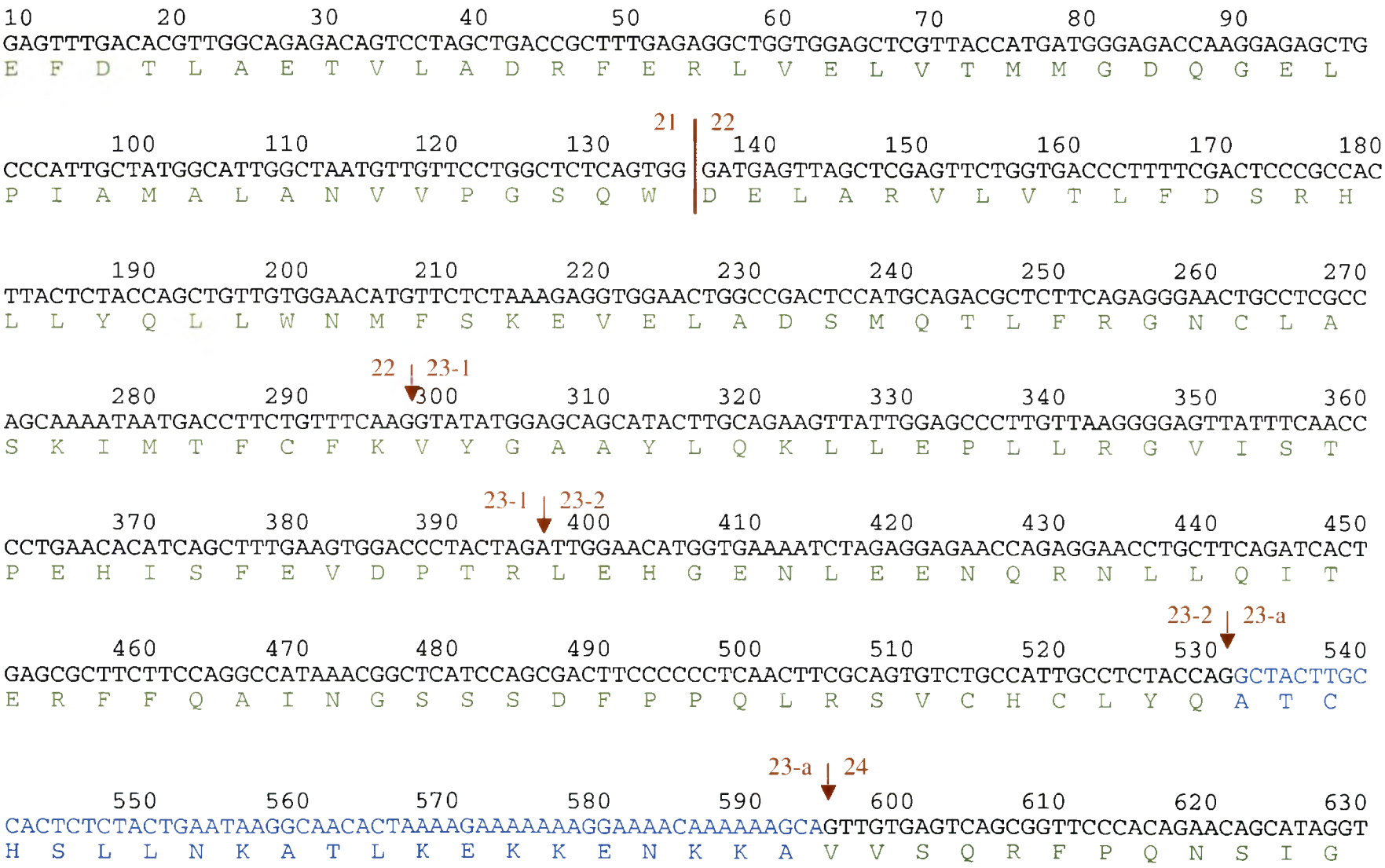
	Fugu	Human	Rat	Mouse	*Drosophila*
Amino Acid Identity (%)	97	91	92	92	69
Nucleotide Identity (%)	50	79	79	80	60

Table 3-1. Nucleotide and amino acid identities of the *Xiphophorus* pNF1-777 clone compared to the *NF1-GRDII* region of other species. The clone shows approximately 92% amino acid identity and 79% nucleotide identity with the mammalian gene.

Exons 21 through 27 of the mammalian *NF1* gene represent the gap related domain (GRDI). This domain is responsible for inactivating p21ras by hydrolyzing ras-GTP to ras-GDP thereby rendering ras inactive and incapable of initiating cell proliferation or differentiation. GRDII is an isoform of wildtype *NF1-GRDI* that has an addition of 21 amino acids (exon 23a) and lacks GTPase capability. The *Xiphophorus NF1* sequence that we isolated shows this addition (represented in blue in Figure 3-3) and is therefore referred to herein as *NF1-GRDII.* The added exon 23a has a stretch of basic amino acids (six lysines out of 21 amino acids) that is thought to alter the protein conformation in such a way that it is not able to hydrolyze ras-GTP to ras-GDP. However, GRDII has a two-fold higher affinity for H-ras-GTP than GRDI (Viskochil *et al.,* 1993).

GRDI is expressed in fetal brain tissue whereas GRDII is expressed in adult differentiated brain tissue (Shen *et al.*, 1996). The *Xiphophorus NF1-GRDII* was isolated from cDNA made from RNA of adult differentiated brain tissue. It is postulated that switching from the GRDI isoform to the GRDII isoform may regulate cell differentiation in the brain tissue and possibly in other neural tissues (Viskochil *et al.*, 1993). If GTP hydrolysis is necessary for signal termination, then the combination of decreased activity with increased binding to ras-GTP suggests that *NF1-GRDII* might form a more stable complex with the activated ras to increase the gain toward differentiation (McCormick, 1989). Nishi *et al.* (1991) observed that neuroblastoma cells induced to differentiate with retinoic acid showed higher expression of *NF1-GRDII* than *NF1-GRDI.*

Figure 3-3. Partial nucleotide sequence of the *X. maculatus* Jp 163 A *NF1* gene. The green letters indicate the corresponding amino acids. The red line represents the intron exon boundary between exons 21 and 22. The red arrows represent putative intron exon boundaries based on mammalian sequence. The sequence in blue denotes the insertion of 21 amino acids that are present in mammalian *NF1-GRDII* and not in mammalian *NF1-GRDI.*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E F D T L A E T V L A D R F E R L V E L V T M M G D Q G E L
21
22
100 110 120 130 140 150 160 170 180
CCCATTGCTATGGCATTGGCTAATGTTGTTCCTGGCTCTCAGTGG GATGAGTTAGCTCGAGTTCTGGTGACCCTTTTCGACTCCCGCCAC
P I A M A L A N V V P G S Q W D E L A R V L V T L F D S R H
190 200 210 220 230 240 250 260 270
TTACTCTACCAGCTGTTGTGGAACATGTTCTCTAAAGAGGTGGAACTGGCCGACTCCATGCAGACGCTCTTCAGAGGGAACTGCCTCGCC
L L Y Q L L W N M F S K E V E L A D S M Q T L F R G N C L A
22
23-1
280 290 300 310 320 330 340 350 360
AGCAAAATAATGACCTTCTGTTTCAAGGTATATGGAGCAGCATACTTGCAGAAGTTATTGGAGCCCTTGTTAAGGGGAGTTATTTCAACC
S K I M T F C F K V Y G A A Y L Q K L L E P L L R G V I S T
23-1
23-2
370 380 390 400 410 420 430 440 450
CCTGAACACATCAGCTTTGAAGTGGACCCTACTAGATTGGAACATGGTGAAAATCTAGAGGAGAACCAGAGGAACCTGCTTCAGATCACT
P E H I S F E V D P T R L E H G E N L E E N Q R N L L Q I T
23-2
23-a
460 470 480 490 500 510 520 530 540
GAGCGCTTCTTCCAGGCCATAAACGGCTCATCCAGCGACTTCCCCCCTCAACTTCGCAGTGTCTGCCATTGCCTCTACCAGGCTACTTGC
E R F F Q A I N G S S S D F P P Q L R S V C H C L Y Q A T C
23-a
24
550 560 570 580 590 600 610 620 630
CACTCTCTACTGAATAAGGCAACACTAAAAGAAAAAAAGGAAAACAAAAAAGCAGTTGTGAGTCAGCGGTTCCCACAGAACAGCATAGGT
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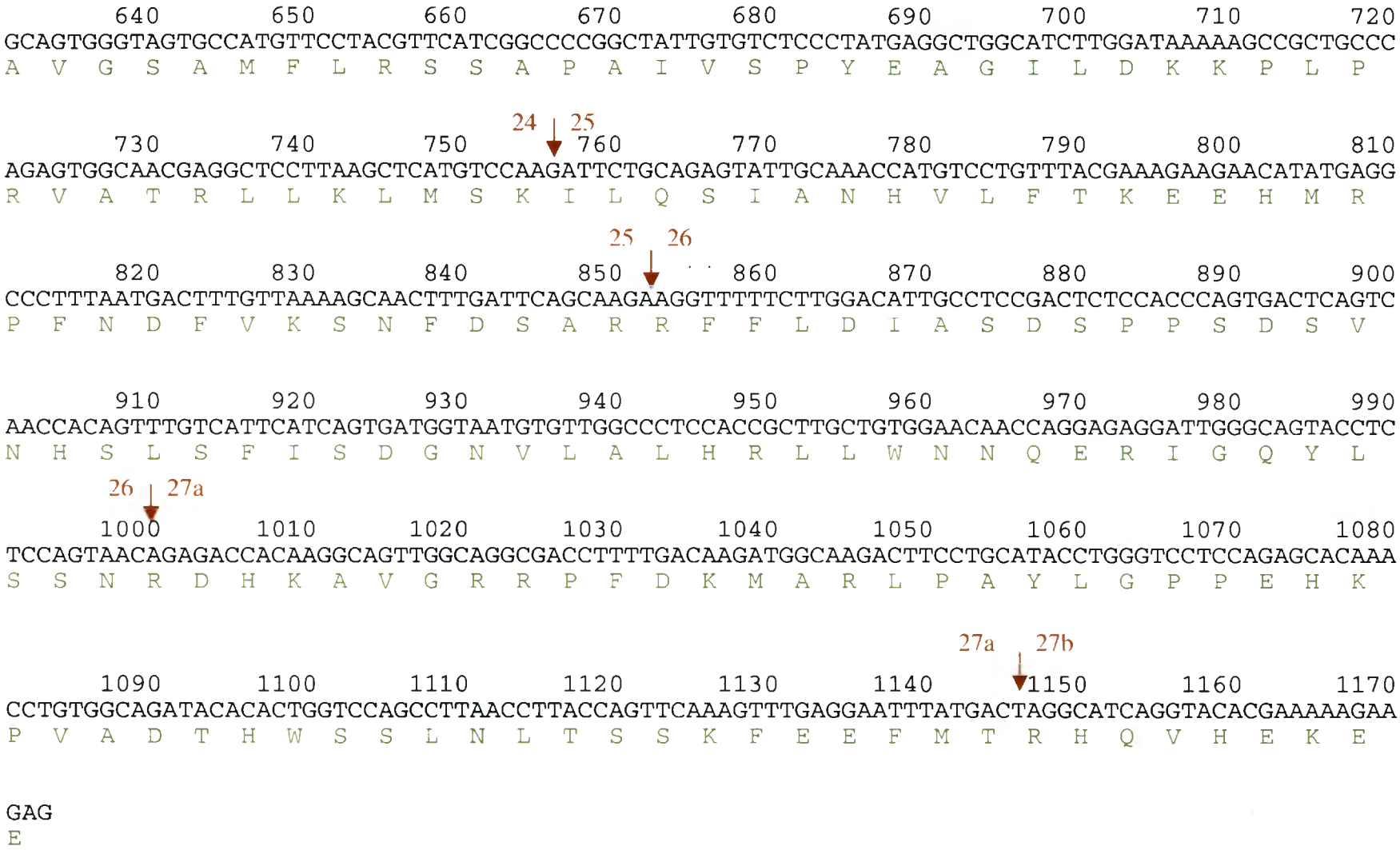
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24 25
730 740 750 760 770 780 790 800 810
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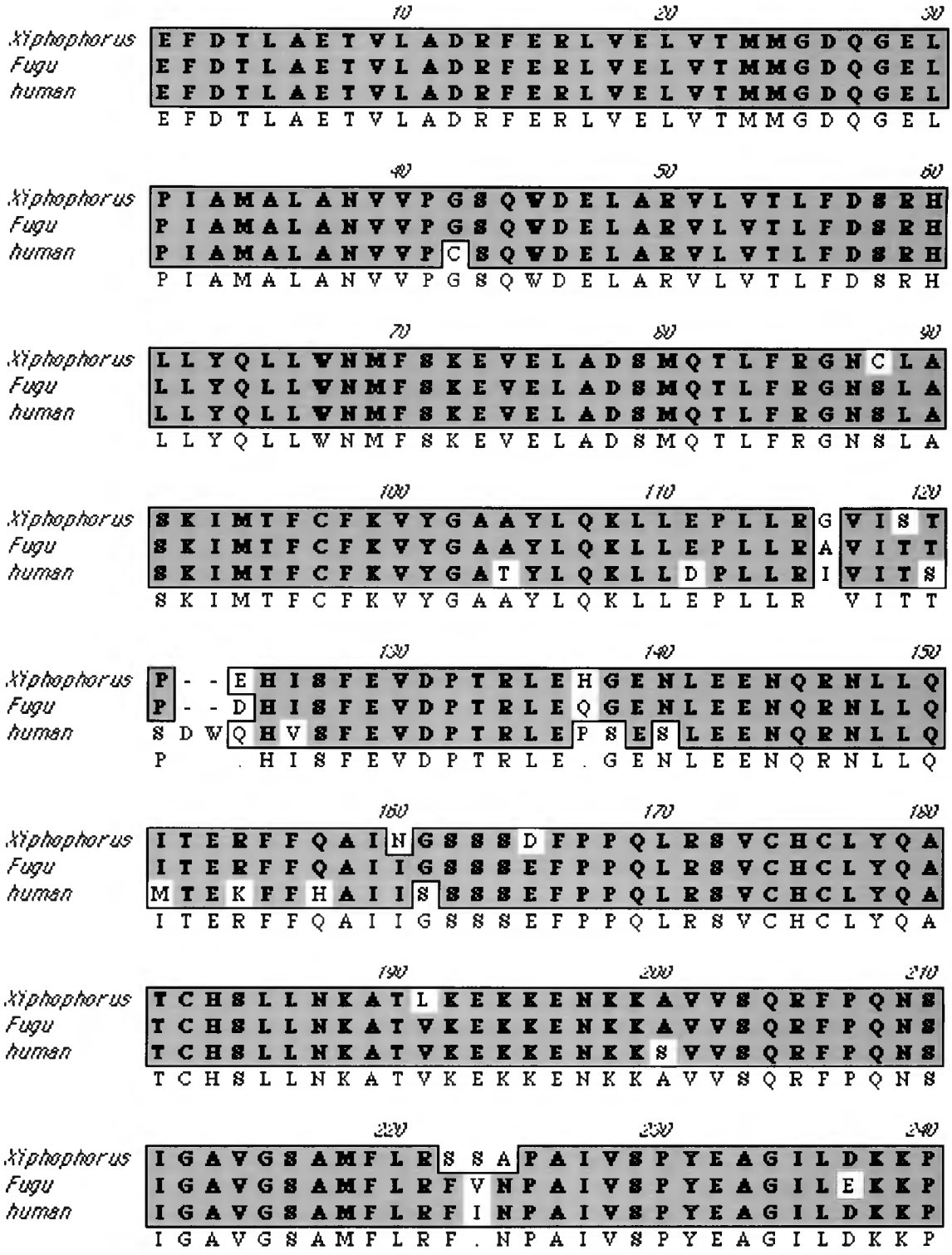
R V A T R L L K L M S K I L Q S I A N H V L F T K E E H M R
25 26
820 830 840 850 860 870 880 890 900
CCCTTTAATGACTTTGTTAAAAGCAACTTTGATTCAGCAAGAAGGTTTTTCTTGGACATTGCCTCCGACTCTCCACCCAGTGACTCAGTC
P F N D F V K S N F D S A R R F F L D I A S D S P P S D S V
910 920 930 940 950 960 970 980 990
AACCACAGTTTGTCATTCATCAGTGATGGTAATGTGTTGGCCCTCCACCGCTTGCTGTGGAACAACCAGGAGAGGATTGGGCAGTACCTC
N H S L S F I S D G N V L A L H R L L W N N Q E R I G Q Y L
26 27a
1000 1010 1020 1030 1040 1050 1060 1070 1080
TCCAGTAACAGAGACCACAAGGCAGTTGGCAGGCGACCTTTTGACAAGATGGCAAGACTTCCTGCATACCTGGGTCCTCCAGAGCACAAA
S S N R D H K A V G R R P F D K M A R L P A Y L G P P E H K
27a 27b
1090 1100 1110 1120 1130 1140 1150 1160 1170
CCTGTGGCAGATACACACTGGTCCAGCCTTAACCTTACCAGTTCAAAGTTTGAGGAATTTATGACTAGGCATCAGGTACACGAAAAAGAA
P V A D T H W S S L N L T S S K F E E F M T R H Q V H E K E
GAG
E


10 20 30
Xiphophorus EFDTLAETVLADRFERLVELVTMMGDQGEL
Fugu EFDTLAETVLADRFERLVELVTMMGDQGEL
human EFDTLAETVLADRFERLVELVTMMGDQGEL
EFDTLAETVLADRFERLVELVTMMGDQGEL
40 50 60
Xiphophorus PIAMALANVVPGSQWDELARVLVTLFDSRH
Fugu PIAMALANVVPGSQWDELARVLVTLFDSRH
human PIAMALANVVPCSQWDELARVLVTLFDSRH
PIAMALANVVPGSQWDELARVLVTLFDSRH
70 80 90
Xiphophorus LLYQLLWNMFSKEVELADSMQTLFRGNCLA
Fugu LLYQLLWNMFSKEVELADSMQTLFRGNSLA
human LLYQLLWNMFSKEVELADSMQTLFRGNSLA
LLYQLLWNMFSKEVELADSMQTLFRGNSLA
100 110 120
Xiphophorus SKIMTFCFKVYGAAYLQKLLEPLLRGVIST
Fugu SKIMTFCFKVYGAAYLQKLLEPLLRAVITT
human SKIMTFCFKVYGATYLQKLLDPLLRIVITS
SKIMTFCFKVYGAAYLQKLLEPLLR VITT
130 140 150
Xiphophorus P--EHISFEVDPTRLEHGENLEENQRNLLQ
Fugu P--DHISFEVDPTRLEQGENLEENQRNLLQ
human SDWQHVSFEVDPTRLEPSESLEENQRNLLQ
P .HISFEVDPTRLE.GENLEENQRNLLQ
160 170 180
Xiphophorus ITERFFQAINGSSSDFPPQLRSVCHCLYQA
Fugu ITERFFQAIIGSSSEFPPQLRSVCHCLYQA
human MTEKFFHAIISSSSEFPPQLRSVCHCLYQA
ITERFFQAIIGSSSEFPPQLRSVCHCLYQA
190 200 210
Xiphophorus TCHSLLNKATLKEKKENKKAVVSQRFPQNS
Fugu TCHSLLNKATVKEKKENKKAVVSQRFPQNS
human TCHSLLNKATVKEKKENKKSVVSQRFPQNS
TCHSLLNKATVKEKKENKKAVVSQRFPQNS
220 230 240
Xiphophorus IGAVGSAMFLRSSAPAIVSPYEAGILDKKP
Fugu IGAVGSAMFLRFVNPAIVSPYEAGILEKKP
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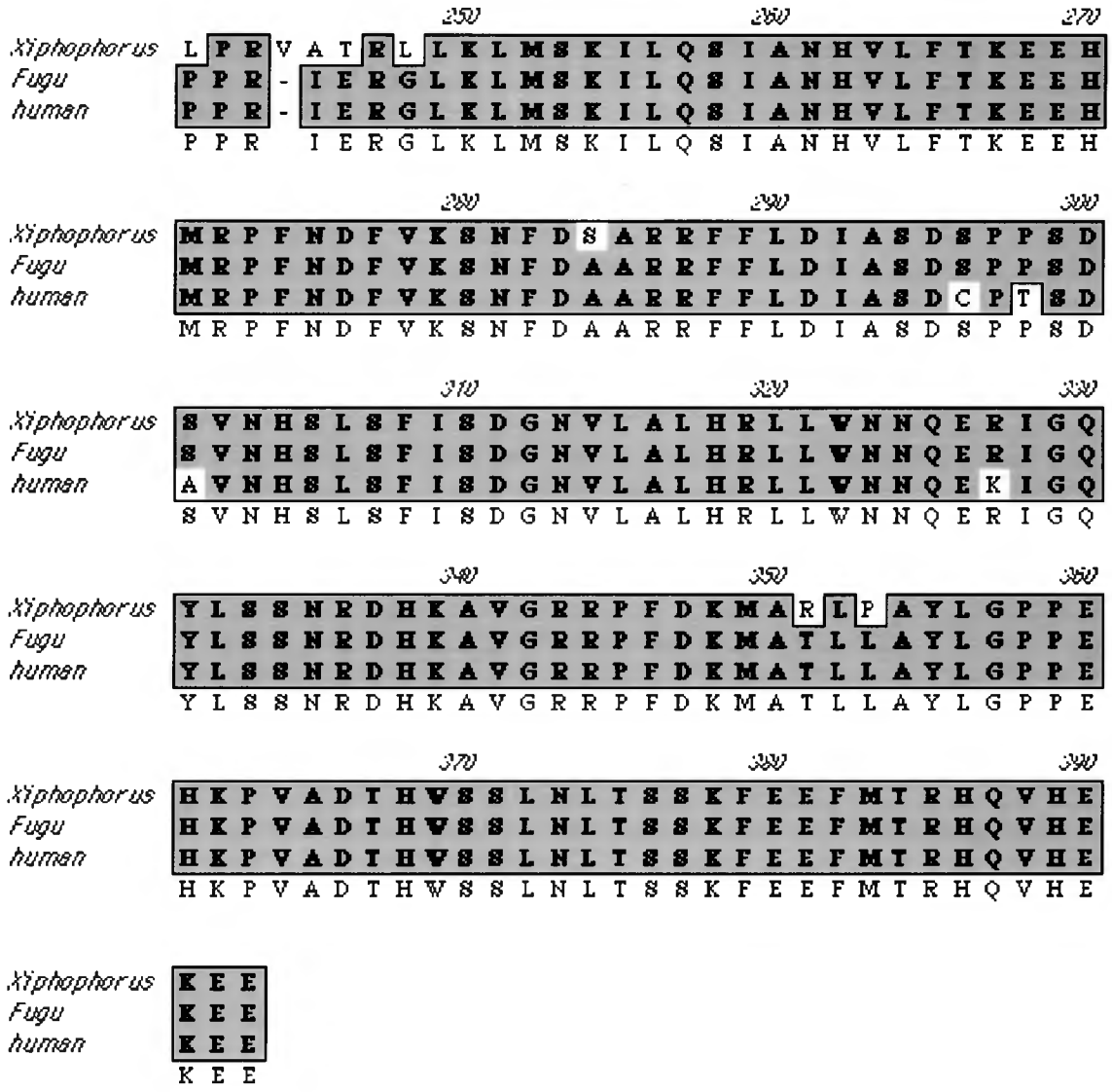


Figure 3-4. Clustal alignment of the *Xiphophorus, Fugu*, and human NF1-*GRDII* region. The shaded gray areas represent homologous regions between the three species. Dark gray represents identical amino acids, light gray represents similar amino acids. Position 1 is equivalent to 1192 in human and 1123 in *Fugu rubripes*.

The only definitive *Xiphophorus* intron/exon boundary identified is between exons 21 and 22, since this sequence was obtained from genomic DNA. Attempts at PCR amplification across intron 27a were unsuccessful, possibly due to the size of this intron. Similarly, attempts to amplify intron 27a in human genomic DNA were unsuccessful (Li *et al.*, 1995). A comparison of intron 21 sizes is presented in Table 3-2. The *Xiphophorus* genome is approximately $1/5^{th}$ as large as the human genome, and the *Fugu* genome is about half the size of the *Xiphophorus* genome. The compact nature of the *Fugu* genome reflects the small size of intergenic and intronic sequences (Kehrer-Sawatzki, *et al.*, 1998). Comparison of the sizes of intron 21 in these species is reflective of the fact that the *Xiphophorus* genome is more compact than the human genome and the *Fugu* genome is more compact than the *Xiphophorus* genome. It also supports the hypothesis that the compact nature of these genomes is a result of smaller introns because the exon sizes of the *NF1* gene are highly conserved in these species.

	Intron 21 Size (kb)
Fugu	0.12
Xiphophorus	0.66
Human	2.20

Table 3-2. A comparison of *Fugu, Xiphophorus*, and human intron 21.

The sequence of intron 21 is given in Figure 3-5. Breathnach *et al.* (1978) observed that introns usually begin with GT and end with AG. At the predicted *Xiphophorus NF1* 5'-donor splice site and 3'-acceptor site, the *X. maculatus* sequence follows this rule. It is necessary to obtain genomic sequence for the remaining introns to precisely confirm putative splice junctions.

```
        10         20         30         40         50         60         70         80         90
GTATGTAGTCCAGCTTTTTTGTATGATCAGTTAAAATATTTGCATAGATACTCGTATTCTGCAGACTGTACTAGCTTTTCAGAAGAAGAA

       100        110        120        130        140        150        160        170        180
ATTTGCATTTGGATGCGTTTGTGTTTTTGACTTTTGGCTTGGTACCAAGGTATTATTACCAATGTGTTCCAAGCAACACAGTTATGTAGT

       190        200        210        220        230        240        250        260        270
TCTGTAGCGAGGCTGTTATAATTGTTGAACCTCTGAATGTTTCTTGGTGTTTTATTTACAGTTAAATCTGAATAATTCTCCATCAAAGCA

       280        290        300        310        320        330        340        350        360
CACTTCAGCTATTATGGTTTAAATAAATGCATTATTTAAATCAGTATTAAATGTCCACTAAGTCACCTTTGCACAAGAACAACGTGTGTT

       370        380        390        400        410        420        430        440        450
TATGCTACATTCATTAACATCACCACATTGCATAAAATAATGTTTCCGTGCTGCGTTGGTGGGATTTTGTGAAATAAATGTGACCAGGTC

       460        470        480        490        500        510        520        530        540
TAAATGCAAATGTTTTTTTAAAATCCCAGCACAATGTCAGTTGTCATGTTTACTTACCCTTGTGAATTCTTTCTGTTGTTTGCTGGATCA

       550        560        570        580        590        600        610        620        630
GAACTAATAATATCAGTTATTTTGGTTAATGGTTATTGTTTATTAAGAGTATTAGTTTCTGGAGCGGTCAGCCTTTGNCAAAGTGTGTTT

       640        650        660
GTGGTCACGACCCGTCTCATCTCTGATTTCCAG
```

Figure 3-5. Sequence of intron 21 from *X. maculatus* Jp 163 A *NF1*.

The protein derived from the *X. maculatus NF1 GRDII* cDNA sequence contains 391 amino acids. It has a predicted molecular weight of 44.5 KD and a theoretical pI of 7.07. Table 3-3 displays the predicted molecular weights, derived pI values and amino acid content of the *X. maculatus, Fugu,* and human *NF1-GRDII* regions. All values are very similar, reflecting the conserved nature of the *NF1* gene in this region. The *Fugu NF1-GRDII* region is composed of 390 amino acids and the human region is composed of 392 amino acids. The calculated molecular weight reflects the number of amino acids present. Of the 391 amino acids of the *X. maculatus NF1-GRDII*, leucine (49) and serine (35) are the most abundant. Tryptophan (4) and cysteine (5) are the least utilized amino acids. Similarly, leucine and serine are the most utilized amino acids and tryptophan and cysteine are the least utilized amino acids in the *Fugu* and human *NF1-GRDII* region. Exons 21 and 22 are the most conserved exons. Exon 21 shows a glycine to cysteine conservative substitution between the fish and human. Exon 22 shows a cysteine to serine conservative substitution between *Xiphophorus* and the other two species. These substitutions are conservative because both are polar amino acids and will not drastically alter the protein conformation. Exon 23-1 is the least conserved between the fish and human showing 5 amino acid differences and an addition of two amino acids (aspartic acid and tryptophan). Exon 24 is the most diverged exon overall showing the addition of a valine and eight amino acid differences between *Xiphophorus, Fugu,* and human.

Table 3-3. The predicted molecular mass (in kilodaltons), derived pI values and amino acid content of the *X. maculatus* Jp 163 A, *Fugu*, and human neurofibromin GRD-II protein fragments. The individual amino acids are grouped as non-polar, polar, acidic, or basic amino acids. The frequency and percent usage of each amino acid is also presented.

		X. maculatus (391 a.a.)		*Fugu* (390 a.a.)		Human (392 a.a.)	
Calculated Molecular Weight:		44.5 KD		44.4 KD		44.8 KD	
Estimated pI:		7.07		6.55		6.59	
Amino Acid Composition:		#	%	#	%	#	%
Non-Polar:	Alanine	28	7.16	28	7.18	26	6.63
	Valine	24	6.14	25	6.41	25	6.38
	Leucine	49	12.53	47	12.05	47	11.99
	Isoleucine	14	3.58	16	4.10	16	4.08
	Proline	20	5.12	20	5.13	19	4.85
	Methionine	11	2.81	11	2.82	12	3.06
	Phenylalanine	23	5.88	24	6.15	24	6.12
	Tryptophan	4	1.02	4	1.03	5	1.28
Polar:	Glycine	15	3.84	15	3.85	12	3.06
	Serine	35	8.95	32	8.21	36	9.18
	Threonine	16	4.09	17	4.36	18	4.59
	Cysteine	5	1.28	4	1.03	6	1.53
	Tyrosine	7	1.79	7	1.79	7	1.79
	Asparagine	19	4.86	19	4.87	18	4.59
	Glutamine	16	4.09	17	4.36	16	4.08
Acidic:	Aspartic Acid	18	4.60	17	4.36	19	4.85
	Glutamic Acid	28	7.16	30	7.69	28	7.14
Basic:	Lysine	21	5.37	21	5.38	23	5.87
	Arginine	24	6.14	23	5.90	21	5.36
	Histidine	14	3.58	13	3.33	14	3.57

Chapter 4-Results and Discussion

Mapping the *Xiphophorus NF1* Gene Homologue

A PCR polymorphism was established between *X. maculatus* Jp 163 A and *X. helleri* Sarabia (Figure 4-1) using primers (B2x and B3x) that cross intron 21. This informative polymorphism allowed screening of a panel of backcross hybrids. Gene mapping in *Xiphophorus* is done by assessing reciprocal first backcross individuals (Figure 4-2) for joint segregation of markers to detect deviations from independent assortment expectations due to genetic linkage (Morizot *et al.*, 1998). A panel consisting of fifty BC_1 hybrid DNAs of *X. helleri* (×) (*X. maculatus* Jp 163 B (×) *X. helleri*) were scored as showing the banding pattern of the *X. helleri* parent (homozygous) or the F_1 parent (heterozygous). Genotypic data were entered on a Microsoft Excel spreadsheet and exported into Map Manager QT to generate recombination values and LOD scores (Table 4-1). Two by two linkage analyses of these markers resulted in localization of the *Xiphophorus NF1* gene to unassigned linkage group U18 (Figure 4-3), cosegregating with three previously mapped AP-PCR/RAPD markers (LOD scores > 4.2).

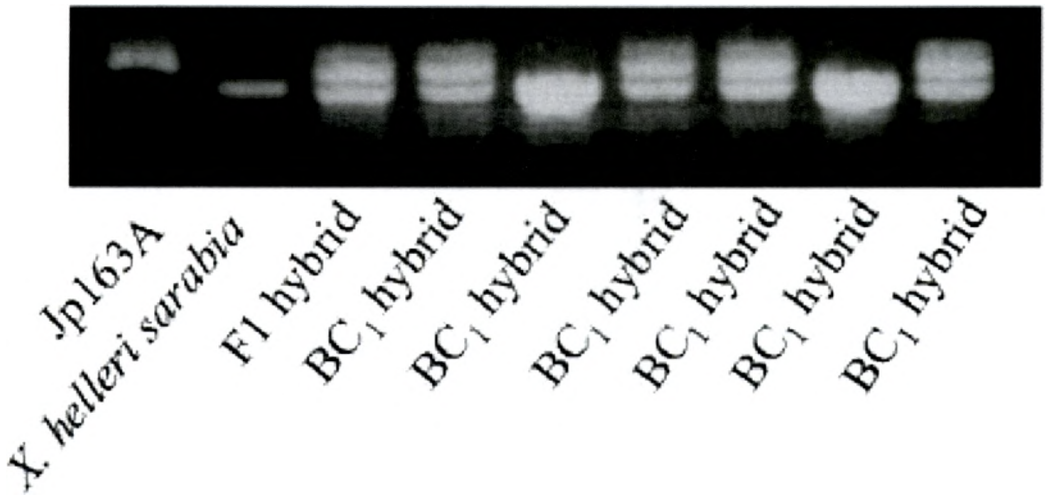


Figure 4-1. Representative example of intron 21 size polymorphism between *X. maculatus* and *X. helleri*. The F_1 hybrid shows a band representative of each parent. The BC_1 hybrids that are heterozygous show the doublet corresponding with the F_1 parent. The BC_1 hybrids that are homozygous show a single band corresponding to the *X. helleri* parent.

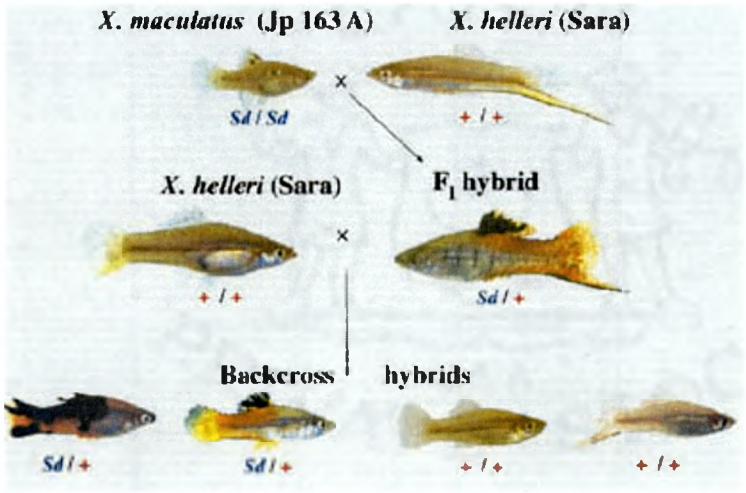


Figure 4-2. *X. maculatus* Jp 163 A is crossed with *X. helleri* Sarabia to yield the F_1 progeny which are then backcrossed to *X. helleri*. Fifty percent of the backcross hybrids produced from this mating have the genetic makeup of the F_1 parent (heterozygous) and fifty percent have the genetic makeup of the *X. helleri* parent (homozygous).

Locus	LG	Recombinants	Parentals	% Recombination	LOD
G6PD	I	13	10	50	-
ES2	II	10	10	50	-
TCIL1	III	6	4	50	-
XDO107(FP10)	IV	5	12	29.4	0.6
ES1	V	12	10	50	-
XDO108(FP11a)	VI	8	9	47	0.0
IDH2	VII	10	12	45.4	0.0
GALT1	VIII	11	6	50	-
PGM	IX	10	13	43.8	0.1
PVALB2	X	10	12	45.5	0.0
CKM	XI	12	11	50	-
GDA	XII	10	12	45.4	0.0
XDO104(FP.7)	XIII	7	10	41.2	0.1
XDO088(ACO1)	XIV	6	7	46.1	0.0
XDO223(RTF2.3)	XV	11	10	50	-
ACTB	XVI	6	8	42.8	0.3
XDO022(CP1.4)	XVII	11	8	50	-
XDOO78(CP1.1)	U16	8	8	50	-
XDO079(CP1.2)	**U18**	**0**	**16**	**0**	**4.8**
XDO084(CP1.4a)	**U18**	**1**	**16**	**5.9**	**3.5**
XDO111(g102a)	**U18**	**0**	**15**	**0**	**4.5**
XDO118(LinF1.3)	**U18**	**0**	**14**	**0**	**4.2**
XDO165(PR3.417)	**U18**	**1**	**17**	**5.6**	**3.7**
XDO102(FP.4)	U19	5	12	29.4	0.6
XDO088(CP1.9)	U20	9	9	50	-
XDO089(CP1.10)	U22	7	8	46.7	0.0
XDO177(R1.8)	U23	7	13	35.0	0.4
XDO154(P7.1b)	U24	5	10	33.3	0.4

Table 4-1. Representative examples of joint segregation analyses showing percent recombination and LOD scores for markers compared with *NF1*. A LOD score greater than three is criteria for linkage and is observed for markers in linkage group U18 (shown in bold). XD denotes anonymous *Xiphophorus* DNA sequence.

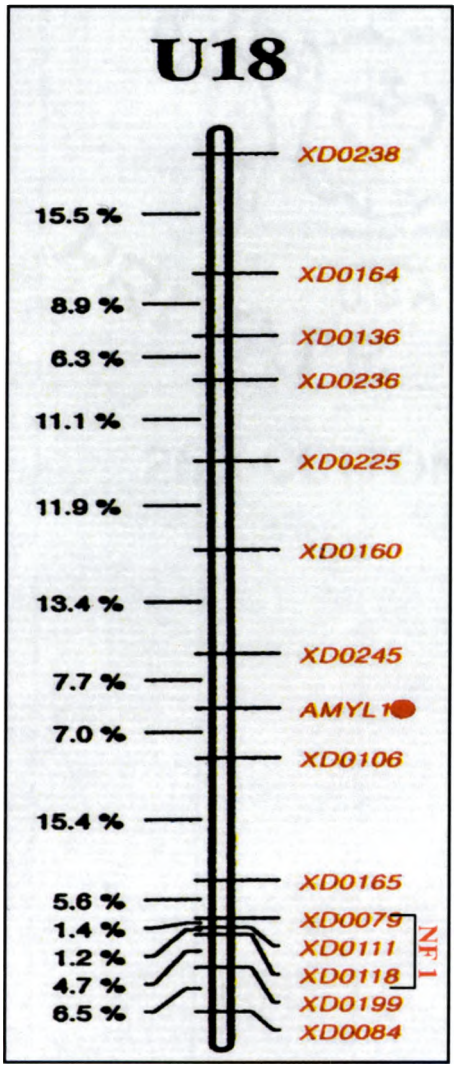


Figure 4-3. Unassigned linkage group U18. *NF1* cosegregates with AP-PCR/RAPD markers XD0079, XD0111, and XD0118 at the distal end of this linkage group. Further linkage data are required to determine the exact map order of these markers because *NF1* exhibits zero percent recombination with all three, using a sample size of fifty.

NF1 has been assigned to the distal end of *Xiphophorus* linkage group U18. *NF1* cosegregates with three previously mapped AP-PCR/RAPD markers and therefore the map order of these markers cannot be elucidated until more linkage data is accumulated. The human *NF1* gene has been assigned to chromosome region 17q11.2, immediately downstream of the *p53* tumor suppressor gene. However, a *Xiphophorus* orthologue of the human *p53* tumor suppressor gene (*TP53*) gene has been mapped to LG XIV (Nairn *et al.*, 1996). Therefore, the syntenic organization of these two tumor suppressor genes does not appear to have been conserved between *Xiphophorus* and mammals. One possible explanation for this is that unassigned linkage group U18 may coalesce with LGXIV, but the accumulating data indicates that U18 will coalesce with LG XIX (Kazianis, personal comm.) Another possible explanation is that *Xiphophorus* express more than one orthologue of the human *p53* tumor suppressor gene that may map to linkage group U18 upstream of *NF1*. Fishes, in general, express many more duplicated genes than do mammals (Walter and Morizot, 1996).

In many malignant tumors from neurofibromatosis patients, mutations and deletions of the *p53* gene have been identified. It is speculated that *NF1* and *p53* cooperate to accelerate tumorigenesis. Evidence for this is seen in the mouse tumor model for neurofibromatosis. The *NF1* and *p53* genes are linked in humans (chromosome 17) and in mice (chromosome 11). Unlike humans, mice that are heterozygous for a mutation in *NF1* do not develop neurofibromas. However, one hundred percent of mice harboring null *NF1* and *p53* alleles in cis synergize to develop soft tissue sarcomas (Vogel *et al*, 1999). This supports a cooperative and causal role for *p53* mutations in neurofibromatosis.

Chapter 5-Results and Discussion

Relative Expression of the *Xiphophorus NF1* Gene

Tissue expression of the *NF1* transcript was determined by relative quantitative RT-PCR. We used PCR primers B2x and B3x to amplify the *Xiphophorus* Jp 163 B *NF1* total cDNA sequence representing putative exons 21 and 22. Expression of the 232 bp amplicon was compared to an internal 18S rRNA control. Initially, the linear range of these primers was determined to be 29 cycles (Figure 5-1). Since the 18S primers compete with the gene specific primers for available resources, it is critical that the 18S rRNA control target be amplified from the cDNA at a level similar to the *NF1* target. The optimal 18S primer:competimer ratio was determined to be 2:29 (Figure 5-3).

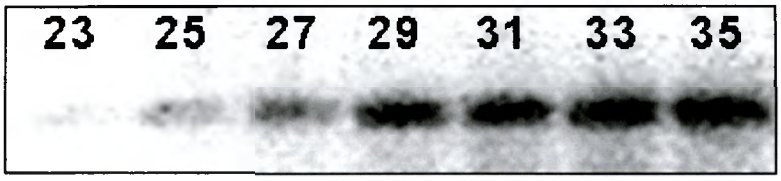


Figure 5-1. Linear Range of the 232 bp amplicon. The amplified product is in the middle of the linear range at cycle 29 and saturates by cycle 33.

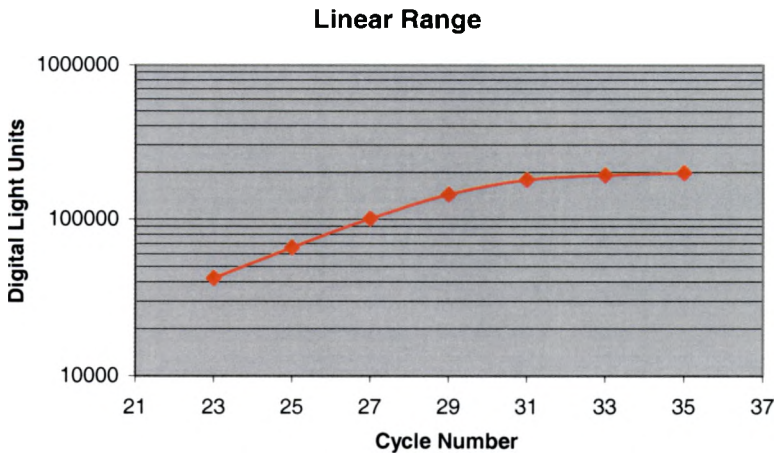


Figure 5-2. Graph of the linear range of the 232 bp amplicon. The product is in linear range between cylces 25 and 31 and saturates by cycle 33.

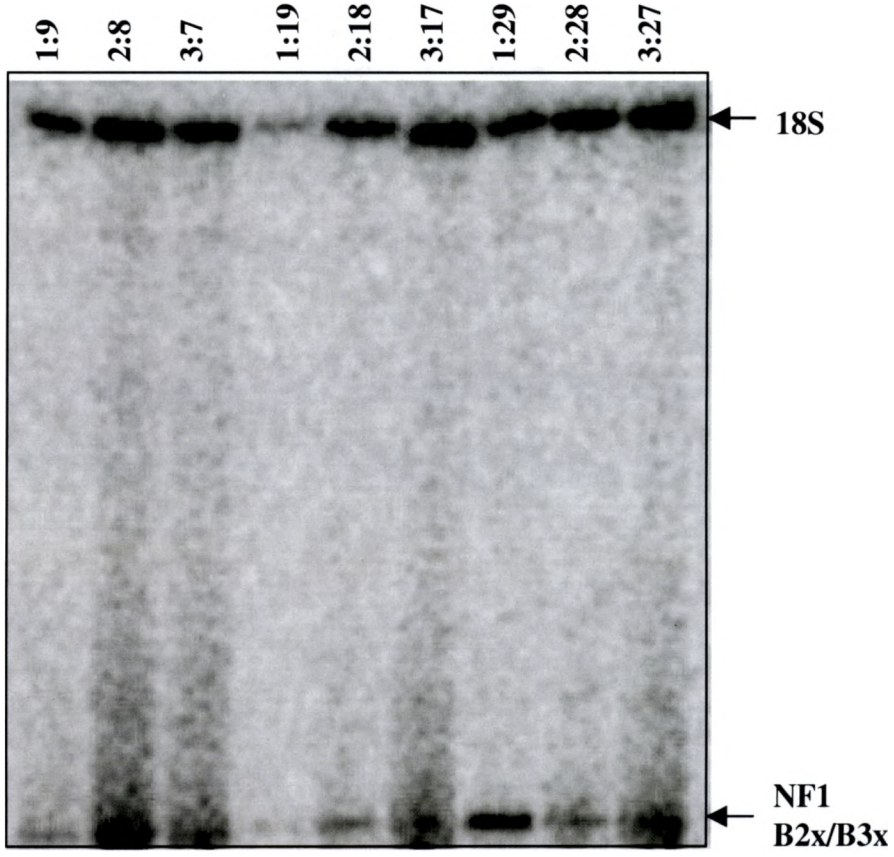


Figure 5-3. 18S Primer:Competimer ratio. The 1:29 ratio shows equal amplification of the 18S rRNA product and the NF1 amplicon. The radioactive intensity of the 18S band is 858261 DLU and the intensity of the NF1 band is 836349 DLU. Therefore, the 1:29 ratio was used for the Q-RT-PCR experiment.

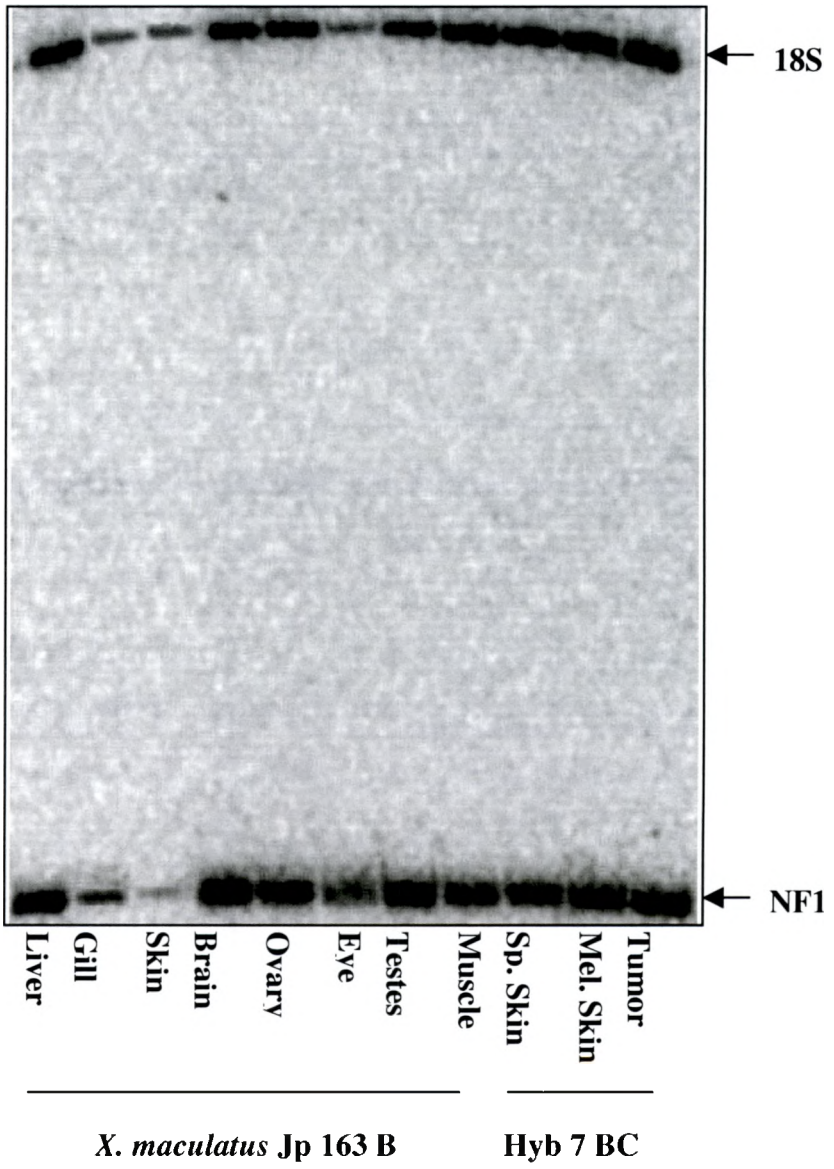


Figure 5-4. Relative Quantitative RT-PCR. To determine the relative expression of *NF1* in each tissue, the total digital light units (DLU) of the *NF1* band is divided by the DLU of the 18S rRNA band. The backcross (*X. couchianus* (×) [*X. maculatus* Jp 163 B (×) *X. couchianus*]) animals exhibited two melanization patterns. The skin tissue from animals exhibiting spotted skin (light pigmentation) was separated from melanized skin (heavy pigmentation). The tumor tissue was taken from animals with melanoma.

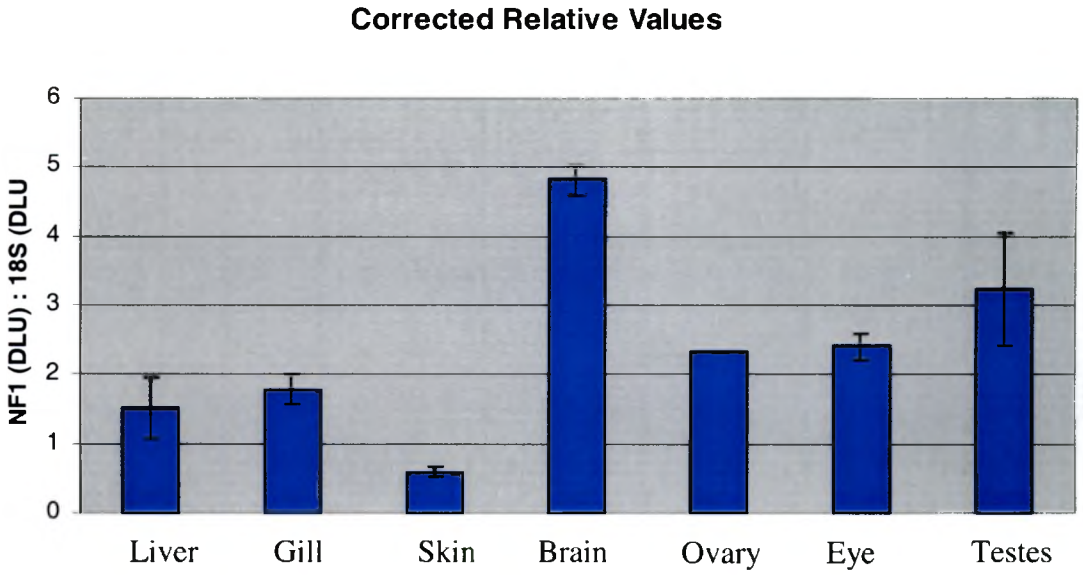


Figure 5-5. Relative quantitative RT-PCR data from *X. maculatus* Jp 163 B tissue. Highest relative expression of *NF1* is shown in the brain tissue of *X. maculatus* Jp 163 B. The graph represents four independent experiments. The error bars indicate standard deviation.

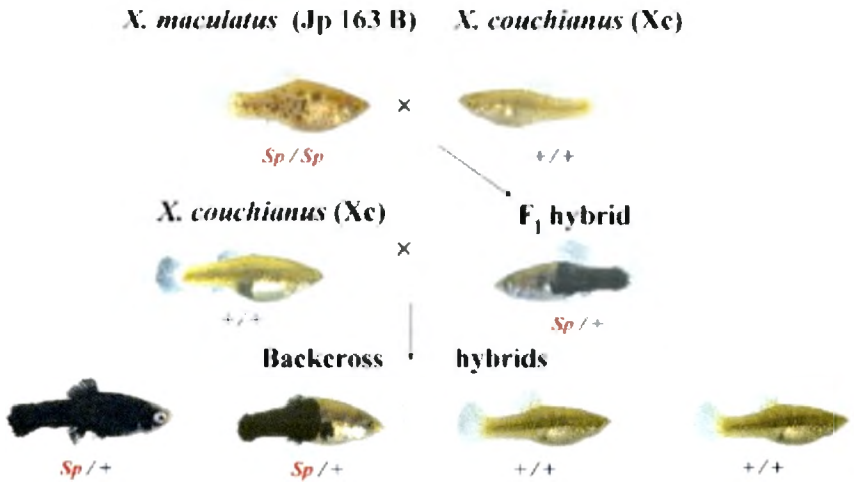
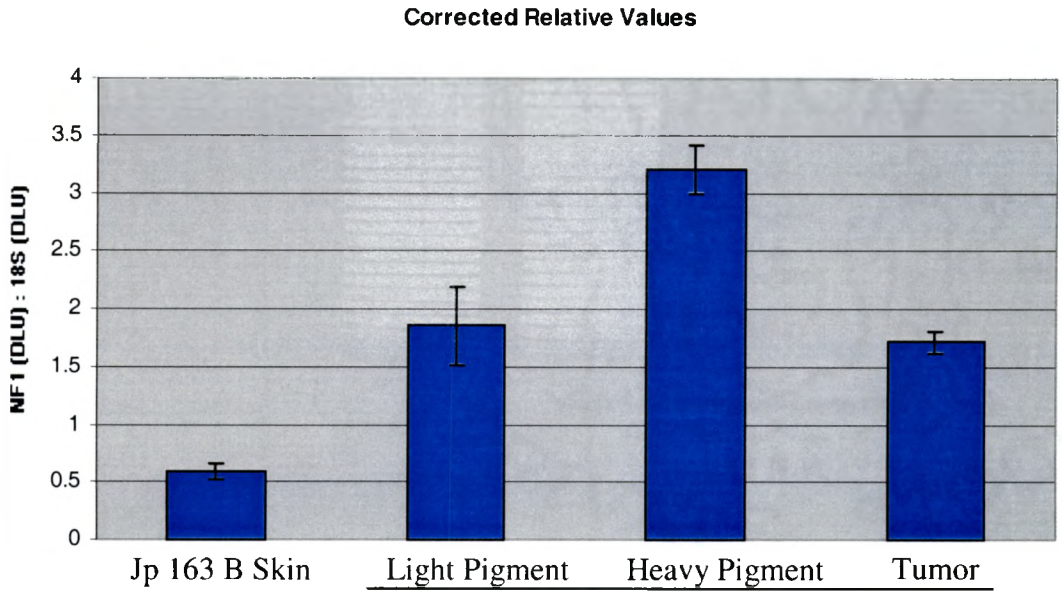


Figure 5-6. *X. couchianus* (×) (*X. maculatus* Jp 163 B (×) *X. couchianus*) backcross. The fish farthest on the left represents heavy pigmentation. The fish immediately adjacent to the heavily pigmented fish represents light pigmentation.



X. couchianus (×) (*X. maculatus* Jp 163 B (×) *X. couchianus*)

Figure 5-7. Relative expression of *X. maculatus* Jp 163 B Skin with hybrid 7 backcross skin. Melanization of 163 B skin is lighter than hybrid 7 spotted skin. Expression of *NF1* increases relative to melanization and is reduced in melanoma tumor tissue.

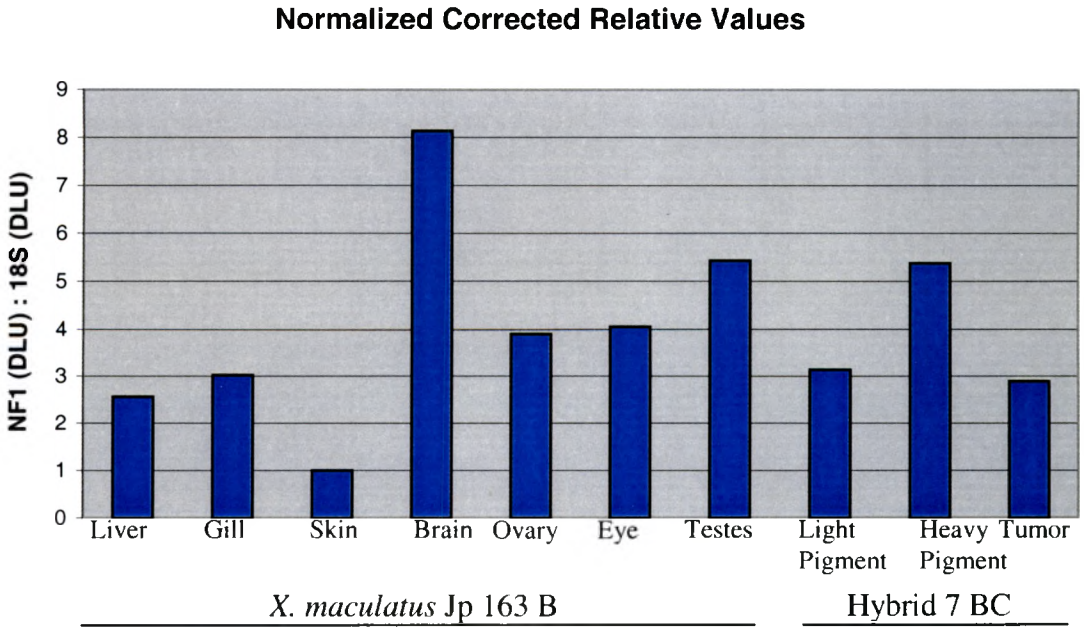


Figure 5-8. Normalized Corrected Relative Values. The relative expression of *NF1* in *X. maculatus* Jp 163 B skin was set as one and the relative expression of *NF1* in the other tissues was normalized to this. Highest expression is observed in brain tissue.

The highest relative expression of the *Xiphophorus* Jp 163 B *NF1* transcript was observed to be in brain tissue and lowest level of expression in the unpigmented skin tissue (Figure 5-4). This is consistent with the findings of Daston *et al.* (1992), who reported the highest levels of rat neurofibromin in brain, lower levels in liver and none in skin. We also looked at expression of *NF1* in the backcross hybrids produced from mating *X. couchianus* (×) (*X. maculatus* Jp 163 B (×) *X. couchianus*). We found highest level of expression in heavily pigmented skin tissue, and lower levels in lightly pigmented skin and melanoma tumor tissue (Figure 5-5). The *NF1* transcript was detected in murine melanocytes (Buchberg *et al.*, 1990). The increase in expression from lightly pigmented tissue to heavily pigmented tissue is consistent with expression of *Xiphophorus NF1* transcript in melanocytes. Analysis of the expression of this transcript in various tissues of adult mouse revealed predominant expression in neural tissues such as brain and spinal cord (Mantani *et al.*, 1994). Analysis of the transcript in various adult *Xiphophorus* tissues also reveals a pattern of higher expression in neural derived tissues such as brain and melanocytes.

Expression of the *NF1* transcript in our study increases with pigmentation, but expression is reduced in melanoma tumor tissue (Figure 5-5). We speculate this finding could implicate *NF1* in melanomagenesis. Data is accumulating that supports a plausible role for *NF1* mutations in human epithelial carcinogenesis. Atit *et al.* (2000) found the *NF1* tumor suppressor gene is implicated in carcinogen-induced pigmentation and papilloma formation in mice. A mutation in a single *NF1* allele (*NF1*+/-) in mice alters the susceptibility of skin to pigmentation and tumors induced by carcinogens. *NF1*+/- keratinocytes and melanocytes showed significant, sustained increases in proliferation

after treatment with tumor promoting carcinogens (phorbol esters) in C57BL/6 mice, noted for resistance to chemical carcinogens.

The reduction of the *NF1* transcript expression observed in the backcross melanoma tissue could be the result of invoked loss of heterozygosity at the *NF1* locus. Andersen *et al.* (1993) reported a large homozygous deletion of *NF1* in two of nine malignant melanoma cell lines leading to loss of detectable mRNA and protein suggesting that *NF1* can function as a tumor suppressor gene in the development or progression of malignant melanoma. Similarly, Johnson *et al.* (1993) found that melanoma and neuroblastoma cell lines established from tumors occurring in patients without neurofibromatosis contain reduced or undetectable levels of neurofibromin with concomitant genetic abnormalities of the *NF1* locus. Therefore, our data indicate that loss of heterozygosity of the *NF1* tumor suppressor gene may play a role in *Xiphophorus* melanomagenesis. One common mechanism of tumor-supressor gene inactivation during tumorigenesis is promoter hypermethylation, being functionally equivalent to a second hit somatic mutation. The methylation status of the *NF1* promoter region was assessed by bisulfite-modified genetic sequencing in neurofibromatosis specific tumors, and tumor specific CpG methylation of six distinct CpG sites in the promoter region of *NF1* was identified (Horan *et al.*, 2000). Further experiments need to be done to determine the mechanism by which the *NF1* transcript is reduced in *Xiphophorus* melanoma and hypermethylation of the promoter region is one area that remains to be examined.

Neurofibromatosis, like melanoma, is believed to be a disorder of neural crest origin and is associated with a number of different malignancies, but a definite association between cutaneous malignant melanoma and neurofibromatosis has not been

established; however, mutations of *NF1* have been identified in patients with malignant melanoma (Duve and Rakoski 1994). A stage dependent model of melanoma carcinogenesis analogous to colorectal cancer remains to be established (Waldmann *et al.*, 1999). Ongoing research in our laboratory will likely contribute to this. *X. maculatus* (×) *X. helleri* hybrids spontaneously develop melanoma in classical Mendelian ratios. Now we will be able to determine the role of *NF1* in melanomagenesis in these fish and provide crucial information for the understanding of the molecular events of tumorigenesis.

Chapter 6

Conclusion

In summary, I have identified a 391 amino acid region of *Xiphophorus* cDNA that corresponds with the Gap Related Domain II (GRDII) region of the mammalian *NF1* gene. The exons are highly conserved, showing 91% amino acid identity and 79% nucleotide identity with the human *NF1* gene and 97% amino acid identity with the *Fugu rubripes NF1* gene. I identified the genomic sequence of intron 21 and determined that it is approximately 1/3 the size of human intron 21 and approximately five times the size of *Fugu* intron 21, reflecting the compact nature of the *Fugu* and *Xiphophorus* genomes relative to the human genome. I mapped the *Xiphophorus NF1* gene to the distal end of unassigned linkage group U18. Unlike the human *NF1* and *p53* genes (both on chromosome 17), *Xiphophorus NF1* and *TP53* genes are probably not located on the same chromosome. I determined highest relative expression of the *NF1* transcript in neural crest derived brain and melanocytes. *NF1* expression is reduced in melanoma tissue derived from *X. couchianus* (×) (*X. maculatus* Jp 163 B (×) *X. couchianus*).

There is an abundance of work to be done with *NF1* in *Xiphophorus*. The remaining genomic sequence remains to be elucidated. We are interested in the presence or absence of the three embedded genes in human intron 27b because *Fugu* intron 27b only contains two of the three embedded genes (Kehrer-Sawatzki *et al.*, 1998). Also, the

exact map order of the distal end of linkage group U18 remains to be determined. Furthermore, it will be interesting to determine the tissue specific expression of the alternate isoforms of *NF1*. More specifically, it is of interest to determine if GRDI is found in fetal brain tissue. It would be interesting to do fluorescent microscopy to determine the cell types expressing *NF1*. We speculate that melanocytes express *Xiphophorus NF1*, however we have not definitively proven this. It remains to be seen if *Xiphophorus NF1-GRDII* possesses the GTPase ability found in mammalian and *Fugu* neurofibromin. The promoter region of *NF1* needs to be studied to determine if hypermethylation plays a role in reduced expression of the *NF1* transcript in melanoma tumor tissue. Eventually, *Xiphophorus* fish may used as a model for studying human neurofibromatosis and the association of *NF1* with melanomagenesis.

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