STUDY OF THE *CDKN2D* (*P19*) LOCUS IN *XIPHOPHORUS*

THESIS

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CHAPTER 1

INTRODUCTION

The Cyclin Dependent Kinases (CDKs)

In eukaryotes, two fully functional daughter cells are formed after four distinct phases of the cell cycle. The cell cycle (Figure 1-1) consists G_1-S-G_2-M. The G_1 stage stands for "GAP 1", the stage where a cell prepares for chromosomal replication. The S stage stands for "Synthesis", when DNA replication occurs. G_2 "GAP 2" is hallmarked by reorganization of the intercellular components in preparation for mitosis. The M stage "Mitosis", is when the duplicated chromosomes separate and migrate to cell poles prior to cytoplasmic (cytokinesis) division. A terminally differentiated cell may leave the cell cycle at G_1, either temporarily or permanently, entering a phase termed G_0 (G zero) where it is often described as "quiescent". Cancer cells may be considered to have regressed to a more embryonic state where they do not enter the G_0 stage and hence repeat the cell cycle indefinitely. Overall, three groups of proteins are known to exert control on the cell cycle (STEIN 1999). These groups of proteins include;

1. Cyclins: these are further classified into G_1 cyclins, S-phase cyclins and M-phase cyclins. Their activity varies with different stages of the cell cycle.

2. Cyclin-dependent kinases (CDKs): these are similarly classified into G_1 CDKs, S-phase CDKs and M-phase CDKs. Cells maintain a fairly stable level of CDKs, but each must bind the appropriate cyclin (whose activity levels fluctuate) in order to be activated. As kinases, they add phosphate groups to a variety of protein substrates that regulate cell cycle processes.
3. The anaphase-promoting complex (APC) and other proteolytic enzymes: these enzymes promote separation of sister chromatids and promote the degradation of mitotic (M-phase) cyclins.

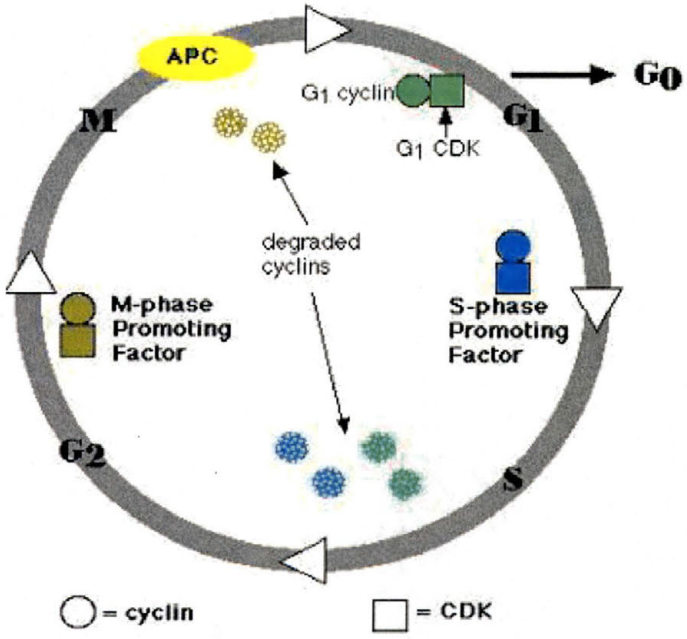


Figure 1-1.The eukaryotic cell cycle, which consists of G_1, S, G_2 and M phase, is controlled by cyclins, cyclin-dependent kinases (CDKs), anaphase promoting complex, (APC) and other proteolytic enzymes. At the start of the cycle, association with a cyclin subunit triggers CDK activity. CDK activity drives the cell through S phase, G_2 phase and the finish is accomplished by proteolytic enzyme, APC, which destroys the cyclin molecules. S-phase promoting factor (SPF) prepares the cell to enter S phase and replicate its DNA. M-phase promoting factor (the complex of mitotic cyclins with M-phase CDK) initiates assembly of the mitotic spindle, breakdown of the nuclear envelope and condensation of the chromosomes. CDK and APC are antagonistic proteins: APC destroys CDK activity by degrading the cyclin, and the cyclin-CDK dimers inactivate APC by phosphorylating one of its subunits.

Cyclin dependent kinases (CDKs) control transitions in the cell cycle and their regulation requires inhibition by checkpoint pathway cascades initiated by serine/threonine kinases (DREXLER 1998; MACLACHLAN 1995). The cell cycle progresses in a sequence, which consists of formation, activation and subsequent inactivation of kinases. These enzymes have two main domains, a catalytic domain (cyclin-dependent kinase, CDK) and a regulatory domain (cyclin). The catalytic activity of CDK is regulated at three different levels: binding of cyclin and activation of the CDK, phosphorylation of the CDK, and inhibition by regulatory proteins called cyclin dependent kinase inhibitors (CDIs).

CDIs are classified into two classes based on their sequence similarity and specificity of action (STEIN 1999).

1. The cip-kip family: are known as universal CDIs and include p21, p27 and p57. The members indifferently inhibit all of the G_1 kinases (CDK2, CDK3, CDK4 and CDK6)
2. The CDKN2 (INK4) family: are known as specific CDIs and include p15, p16, p18 and p19. Their inhibitory activity is restricted to CDK4 and CDK6.

Within the studied mammalian taxa, the cyclin-dependent kinase inhibitor 2 (*CDKN2*) gene family consists of four members, namely *CDKN2A* (also known as *P16*), *CDKN2B* (*P15*), *CDKN2C* (*P18*) and *CDKN2D* (also known as *P19*). In humans, it has been established that *CDKN2* gene family members function as tumor suppressors (DREXLER 1998). The protein p16 and other members of its family (p15, p18 and p19) inhibit the cyclin dependent kinases CDK4 and CDK6, which in turn control the activity

of the retinoblastoma protein (pRb) (Figure 1-2). pRb, in turn regulates proliferation by regulating proteins responsible for the progression into S phase of the cell cycle. All members of the *CDKN2* gene family are known to have a role in specific types of cancers. As examples, expression of p15 is suggested to be a prognostic indicator in myeloid malignancies (TEOFILI 1999); p16 is known to play a role in melanoma while both p18 and p19 are known to act as tumor suppressors in some forms of testicular cancer (THULLBERG et al. 2000).

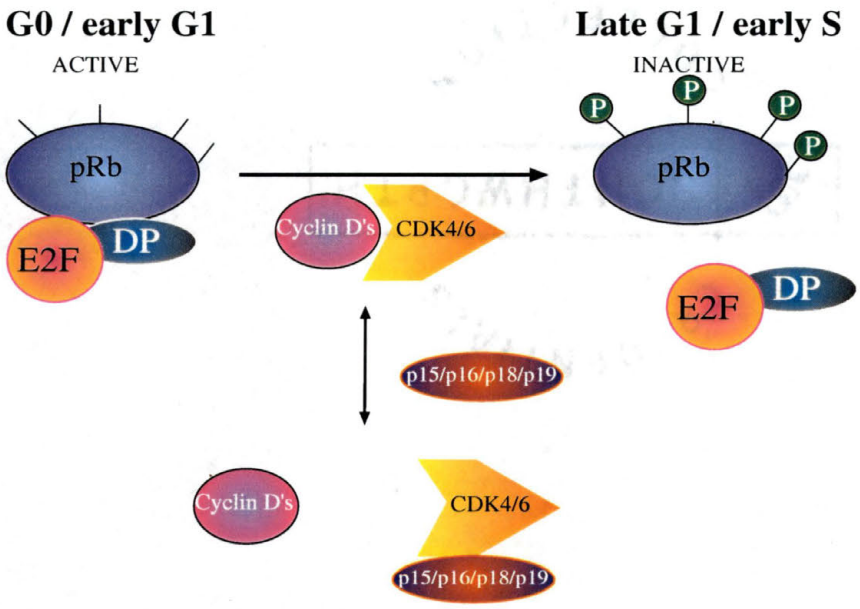


Figure 1-2. Model for the effects of different CDKs in cancer development. p15, p16, p18 and p19 proteins inhibit the cyclin dependent kinases CDK4 and CDK6, which in turn control the activity of retinoblastoma protein (pRb). CDK4 and CDK6, which associate with the D-type cyclins to control progression through the G1 phase of the cell cycle by phosphorylation of the tumor suppressor protein, pRb. Phosphorylation of pRb releases the transcription factor (E2F family) and their dimerization partners (DP family proteins). The E2F/DP protein families control cell cycle progression by acting predominantly as either activators or repressors of transcription.

The *CDKN2D* (*P19*) gene

The human p19 protein consists of 165 amino acids and belongs to INK4 family of CDK4 and CDK6 inhibitors. p19 is made up of five 32-amino acid ankyrin-like repeats, which are believed to play a role in protein-protein interactions (KALUS et al. 1997). This protein selectively inhibits the kinase activities of CDK4 and CDK6, and does not show activity in the inhibition of cyclin E-CDK2, cyclin A-CDK2 or cyclin B-CDC2 (HIRAI et al. 1995).

During the cell cycle, p19 (*CDKN2D*) exhibits a periodic expression, which peaks as cells enter S phase. When expressed constitutively, p19 inhibits cyclin D-dependent kinase activity *in vivo* and promotes G_1 phase arrest (HIRAI et al. 1995). It has also been reported that p19 transcription is upregulated during S and G_2 phase and shows some expression in mouse embryonic stages (ORTEGA 2002). Following mitosis, the p19 protein and p27Kip1 inhibitors are known to function together in maintaining neurons in a potentially reversible, "quiescent" differentiated condition. When p19 and p27Kip1 cell cycle inhibitors are absent after mitosis, ectopic proliferation of neurons takes place in all parts of the brain, including cells of the hippocampus, cortex, hypothalamus, pons and brainstem that are normally dormant (ORTEGA 2002; ZINDY et al. 1999). p19 alterations have been observed and are believed to be the probable contributor to of osteosarcoma in some patients (RUAS and PETERS 1998). *CDKN2D* (p19) activity is observed in the process of spermatogenesis (ZINDY et al. 2000). Studies of p19 expression by Zindy *et al.* have also shown elevated protein levels in testis, spleen, thymus and brain (ZINDY 1997).

Xiphophorus

The poeciliid fish genus *Xiphophorus* has been used as an animal model for more than six decades to generate genetic data for the study of melanoma and other cancers (WALTER and KAZIANIS 2001). Many of the 23 known *Xiphophorus* species are polymorphic for sex-linked pigment patterns. Several authors (for review see Walter and Kazianis, 2001) have proposed that inter-species hybrid fish exhibit melanoma tumors due to a high degree of phenotypic enhancement and underlying hyper-proliferative properties of melanocytes. In some *Xiphophorus* interspecies hybrids, first generation inter-species backcross hybrid fish (BC_1) may develop melanomas spontaneously. Thus, environmental contributions to neoplasia are minimized and the tumor develops due to the select inheritance of certain allele combinations (KAZIANIS et al. 1999). Other *Xiphophorus* hybrid models have been developed that require chemical or physical treatments to induce specific types of cancer including; melanoma, fibrosarcoma, retinoblastoma and sarcoma (KAZIANIS et al. 2001a; KAZIANIS et al. 2001b).

Xiphophorus Melanoma Model

A genetic model has been developed (Figure 1-3) to explain pigment pattern enhancement and melanoma formation in hybrid fish produced by crossing the platyfish *X. maculatus* with the swordtail *X. helleri* (WALTER and KAZIANIS 2001). According to this two gene model the sex-chromosomal spotted dorsal (*Sd*) macromelanophore locus and an autosomal regulatory factor termed *DIFF* (ANDERS 1967; SICILIANO et al. 1976; VIELKIND 1976), normally act antagonistically to keep melanocyte proliferation in check. This model proposes that *DIFF* hypothetically modulates the phenotypic expression of

the pigment pattern, acting as a tumor suppressor gene. The *DIFF* gene was mapped to *Xiphophorus* linkage group V (LGV) (SICILIANO et al. 1976) and this MAP assignment was confirmed by independent studies (AHUJA et al. 1980; FORNZLER et al. 1991; MORIZOT and SICILIANO 1983). When *X. helleri* and *X. maculatus* are crossed and F_1 hybrids obtained, the spotted dorsal (*Sd*) macromelanophore pigment pattern derived from the later species is phenotypically enhanced in the F_1 hybrids. Pigmented hybrids have inherited one copy of the *Xmrk-2* oncogene from *X. maculatus,* which is linked to the *Sd* locus. *X. helleri* appears to lack both the *Xmrk-2* oncogene and the *Sd* pigment pattern loci. Melanotic tissues within F_1 hybrids and first generation backcross (BC_1) hybrids exhibit over expression of the *Xmrk-2* oncogene (KAZIANIS et al. 1999).

CDKN2 gene family in *Xiphophorus*

Kazianis *et al.* have cloned, sequenced and partially characterized the transcriptional expression of a *CDKN2* gene from *X. helleri* and *X. maculatus*. This gene, termed *CDKN2X* shows a high degree of amino acid sequence similarity to members of the mammalian *CDKN2* gene family. Comparative sequence analysis shows that *Xiphophorus CDKN2X* is similarly related to all four mammalian *CDKN2* gene family members, and may represent a descendant of an ancestral prototypic *CDKN2* gene (KAZIANIS et al. 1999) *CDKN2X* was mapped to a *Xiphophorus* autosomal region on linkage group V (LG V) known to contain the hypothetical *DIFF* gene that acts as a tumor suppressor of melanoma formation in *X. helleri/X. maculatus* backcross hybrids (AHUJA et al. 1980; ANDERS 1967; KAZIANIS et al. 1998; SICILIANO et al. 1976). Thus, *CDKN2X* is a candidate for the tumor suppressor *DIFF* gene.

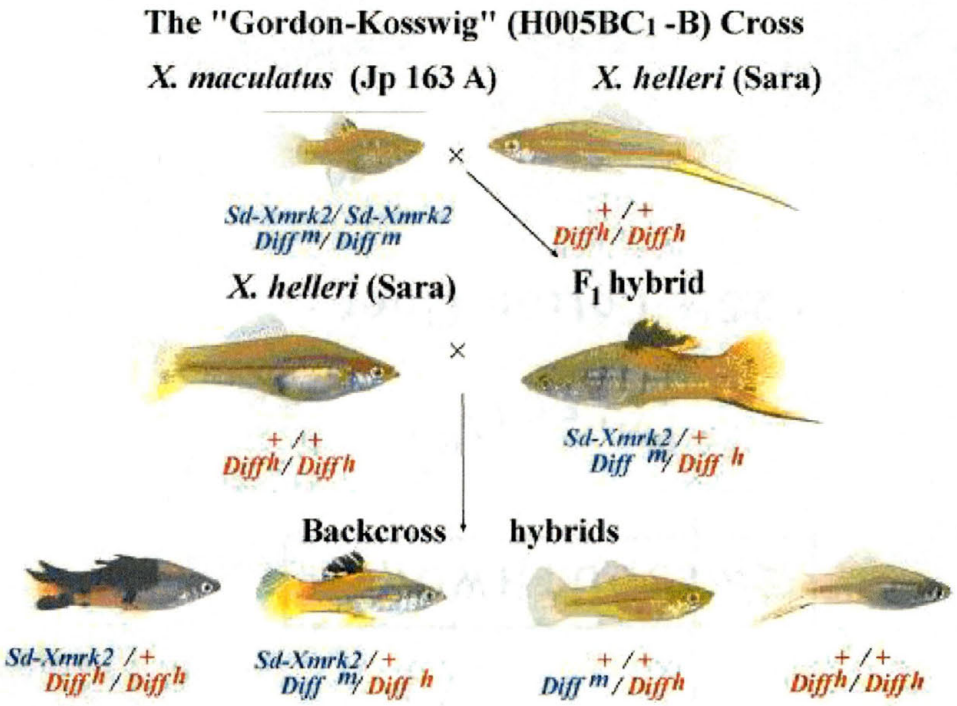


Figure 1-3. Generalized depiction of the Gordon-Kosswig Hybrid Cross. For each fish, the upper line represents the pertinent loci from the sex chromosome (*Sd-Xmrk2*). While the bottom line represents the *DIFF* locus from an autosome (LG V). The loci derived from the platyfish, *X. maculatus* are colored blue, while the corresponding genetic constitution of the swordtail, *X. helleri* is red. Nodular melanoma typically occurs in animals showing phenotypes similar to the one on the lowest left. These fish have inherited the *Xmrk-2* oncogene, but not the *DIFF* tumor suppressor from the *X. maculatus* parent.

The Gordon-Kosswig cross is also augmented by other similar crosses. As an example, *X. maculatus* strain Jp 163 A can be replaced by Jp 163 B, a different substrain, and phenotypic enhancement of the macromelanophore pattern *Sp* is observed (Figure 1-4). Using select *Xiphophorus* interspecies backcrosses such as this, researches (KAZIANIS et al. 1998; NAIRN et al. 1996) have shown that first generation backcross hybrids that develop melanoma were predominantly (>80%) homozygous for *CDKN2X* alleles derived from *X. helleri*, and consequently lacked the corresponding allele from *X.*

maculatus. RT-PCR analyses suggest elevated expression of *CDKN2X* in melanomas derived from this cross. In addition, it was shown that the *X. maculatus CDKN2X* allele produces higher RNA transcript levels than the alleles derived from the swordtail (KAZIANIS 1999). These results led to a hypothesis that the *CDKN2X* locus is effectively trying to slow the proliferation of melanocytes in *X. maculatus* by acting as antagonistic to overexpression of the *Xmrk-2* oncogene. In hybrid fish, *CDKN2X* alleles from *X. helleri* are unable to accomplish this antagonistic action on *Xmrk-2* and thus tumors develop.

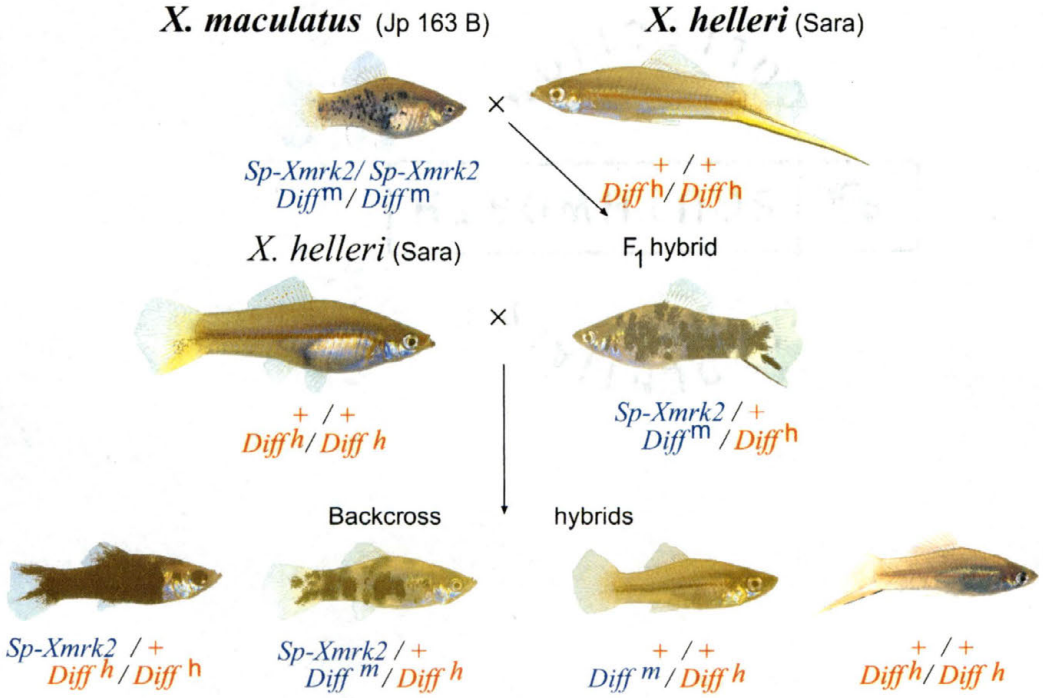


Figure 1-4. Generalized depiction of the a hybrid cross between *X. maculatus* Jp 163 B and *X. helleri*. For each fish, the upper line represents the pertinent loci from the sex chromosome (*Sp-Xmrk2*). While the bottom line represents the *DIFF* locus from an autosome (LG V). The loci derived from the platyfish, *X. maculatus* are colored blue, while the corresponding genetic constitution of the swordtail, *X. helleri* is red. Nodular melanoma typically occurs in animals showing phenotypes similar to the one on the lowest left.

While the above-mentioned experiments served to establish a mechanistic hypothesis for spontaneous interspecies cross melanoma development, there are numerous facts that indicate further work is necessary to understand the etiology of melanoma in *Xiphophorus*. First of all, a good proportion (~20%) of backcross hybrids that develop melanoma are heterozygotes, inheriting *CDKN2X* allele from *X. maculatus*. The development of these melanomas cannot be explained by the low expression of *X. helleri CDKN2X* alleles. In addition, inclusion of the chemical carcinogen *N*-methyl-*N*-nitrosourea (MNU) to backcross hybrid cohorts results in a dramatic increase of melanoma incidence. Genetic analysis of these fish revealed a lack of association of *CDKN2X* genotypes with melanoma development (KAZIANIS et al. 2001b). Clearly, further work needs to be done to define the role of *CDKN2X* and genes that are closely related to it.

Thullberg *et al.* (THULLBERG et al. 2000) have reported that p15, p16, p18 and p19 proteins all possess the ability to stop the growth of cells in G_1 phase and further interact with CDK4 or CDK6. The expression patterns displayed by human *CDKN2* derived proteins vary from cell and tissue types. Thus, redundant and non-overlapping properties are present simultaneously in p15, p16, p18 and p19 proteins, which indicate they have distinct regulatory function in control of the cell cycle and cell differentiation. Based on such results, examination of carcinogenesis in *Xiphophorus* should include study of this entire gene family.

Gilley *et al.* (GILLEY and FRIED 2001) have studied a homologue of the human *CDKN2A/ARF/CDKN2B* suppressor loci in puffer fish, *Fugu rubripes*, and identified two *CDKN2* loci using degenerate PCR and hybridization analyses. They also clone one

gene to be homologous to human *CDKN2D*. Orthology for the other gene isolated could not be established since the *Fugu* sequences were equally related to both *CDKN2A* and *CDKN2B*. They named this locus *CDKN2AB*. Based on the research by Gilley *et al.* we assumed that fishes have at least 2 *CDKN2* loci. The role of *CDKN2D* in cell cycle regulation and melanoma in *Xiphophorus* has yet to be studied. Using degenerate primers designed specifically to amplify *CDKN2* gene family members (but not *CDKN2X*); we cloned and characterized the *CDKN2D* gene. Results of these studies are reported herein.

CHAPTER 2

MATERIALS AND METHODS

Fish Stocks

Poeciliid fishes of the genus *Xiphophorus* were used in this study are listed in Table 2-1. These specimens were obtained from the *Xiphophorus* Genetic Stock Center, Southwest Texas State University, San Marcos, TX.

Species	Strain or Genetic Cross
X. maculatus	*X. maculatus* Jp 163 A
X. helleri	*X. helleri* Sarabia
X. variatus	*X. variatus* Zarco
X. nezahualalcoyotl	*X. nezahulalcoyotl* Ocampo
F_1 Hybrids, H001F_1 1	*X. maculatus* Jp 163 B x *X. helleri* (Sarabia)
BC_1, Hybrid 5, H001	*X. helleri* Sarabia x (*X. maculatus* Jp 163 B x *X. helleri* Sarabia)

Table 2-1. *Xiphophorus* fishes used in this research

RNA Isolation

Brain and testes tissues were extracted from five *X. maculatus*, Jp 163 A fish by established dissection methods and either quick-frozen or placed in RNA Later™ (Ambion, Austin, TX). RNA was extracted using the TRIzol Reagent protocol (Life

(Ambion, Austin, TX). RNA was extracted using the TRIzol Reagent protocol (Life Technologies; Gaithersburg, MD), a modification of the original method developed by Chomczynski and Sacchi (CHOMCZYNSKI and SACCHI 1987).

The RNA isolation procedure involved the following steps:

1. Homogenization: The extracted tissues were homogenized using autoclaved pestles in 250 µl of TRIzol reagent (a monophasic solution of phenol and guanidine isothiocynate). The homogenate was incubated at 25°C to allow complete dissociation of nucleoprotein complexes.
2. Phase Separation: 50 µl of chloroform was added to the homogenate and shaken vigorously by hand for 15 seconds. The mixture was incubated for 3 minutes at room temperature then centrifuged for 15 minutes at 10,000 rpm at 4°C. Centrifugation separates the mixture into a lower phase of phenol-chloroform containing the protein; an interphase consists of DNA, and an aqueous phase consisting of RNA. The aqueous phase was transferred to a fresh microcentrifuge tube
3. RNA Precipitation: 125 µl of the aqueous phase was incubated for 10 minutes at room temperature and centrifuged for 10 minutes at 10,000 rpm at 4°C. The supernatant was carefully discarded by pipetting to obtain clear RNA pellets in a clear gel form at the bottom of the tube.
4. RNA Wash: 250 µl of 75% ethanol (made with 0.1% Diethyl pyrocarbonate (DEPC) treated water; an inhibitor of RNases) (SAMBROOK et al. 1989) was added to the RNA pellets and the mixture was centrifuged for 5 minutes at 10,000 rpm at 4°C. The supernatant was carefully discarded and the RNA pellets were dried at

37°C in an incubator for 10-15 minutes until no detectable traces of ethanol were found.

5. Resuspension: The dried pellets were suspended in 50 µl of autoclaved DEPC treated water by incubation at 55°C for 1 hour.

Isolation of Genomic DNA

The DNAs of interest were isolated from selected tissues using a PUREGENE® DNA Purification Kit (Gentra Systems; Minneapolis, MN). Sixty µl of chilled Cell Lysis solution was added to the tissues and homogenized using autoclaved pestles. Three µl of Proteinase K solution (20 mg/ml) was added to lysate the homogenate and incubated overnight at 55°C. The cell lysate was cooled to room temperature and 3 µl RNase A solution was added. The solution was mixed thoroughly by inverting the tube about 25 times with incubation at 37°C for 60 minutes. The RNase A-treated cell lysate was cooled at 4°C and 200 µl protein precipitation solution was added to it. The mixture was vortexed at high speed for 20 seconds and centrifuged at 13000 X g for 7 minutes, the protein precipitated to form a tight pellet. The supernatant containing nucleic acid was separated into a 1.5 ml microcentrifuge tube containing 600 µl of 100% isopropanol and the solution was mixed by inverting 50 times, so that the nucleic acid formed visible clumps. Six hundred µl of 70% ethanol was added and the tube was inverted several times to wash the pellet. Again, the mixture was centrifuged for 3 minutes at 13000 X g to form a white DNA pellet. The ethanol was removed out and the tube was allowed to dry in at 37°C for 30 minutes. Two hundred µl DNA hydration solution was added to re-

suspend the pellet and the solution incubated at 65°C for 1 hour. This was later agitated overnight on a rotating platform at 4°C.

Quantitation

Quantitation of the isolated RNA and DNA was performed in solution using RiboGreen® and PicoGreen®, respectively (Molecular Probes; Eugene, OR) (SAMBROOK et al. 1989) which are ultra-sensitive fluorescent nucleic acid stains. A *FL$_X$ 800* microplate fluorescence 96 well microplate reader (Bio-Tek Instruments; Winooski, VT) was used. This instrument uses KC4™ analysis software for PC .

Polymerase Chain Reaction (PCR)

Polymerase chain reaction is accomplished using *Taq* DNA-polymerase and oligonucleotide primers (MULLIS et al. 1986). Oligonucleotide primers flank the expected target and amplify the sequence of interest. These oligonucleotides can be designed using *a-priori* DNA sequence information, or can be made using data derived from related species. In the later case, one can make "degenerate" primers that have differing bases at any given nucleotide position.

PCR was typically performed in 0.5 ml centrifuge tubes. The composition of a typical PCR mixture is shown in Table 2-2. Thirty-five PCR cycles were performed in a PCR express (Hybaid, Ashford, UK) thermocycler at sub-ambient and ambient conditions shown in Table 2-3.

PCR Component	Final quantity or Concentration
DNA	5-250 ng
10X PCR Buffer (Tris-HCl-pH 8.4, 200 mM, KCl 500 mM)	1X
$MgCl_2$	25 mM
dNTP	6.25 μM
Taq Polymerase (Invitrogen; Carlsbad, CA)	1.25 units
Primer	20 μM of each
Sterile Distilled Water	To a final volume of 25 μl

Table 2-2. Composition of Standard PCR mixture

PCR Step	Temperature (°C)
Denaturation	94
Primer Annealing	50-65
Elongation	72

Table 2-3. PCR Temperature Profile

Amplified products were visualized after electrophoresis on a 1-2% agarose gels run in tris-actetate EDTA buffer (1 mM Tris-OAc^-, pH 7.0; 0.1 mM EDTA) with ethidium bromide included (MANIATIS et al. 1982).

RACE

To obtain the full sequence of the *Xiphophorus CDKN2D*, a procedure called RACE (Rapid Amplification of cDNA Ends) was used (FROHMAN et al. 1988). Briefly, nucleic acid sequences from a messenger RNA template between a fixed internal site and either the 3' or the 5' end can be characterized by PCR amplification. Depending upon which end is amplified; there are two types of RACE, 3' and 5'.

3' and 5' RACE reactions were carried out using First Choice TM RLM RACE™ Kit (Ambion, Austin, TX). For these RACE reactions RNA extracted from various tissues, in particular brain and testes, was used since *CDKN2D* is expressed well in these tissues (THULLBERG et al. 2000).

RLM Race kit differs from the classic RACE kits because it uses Ligase Mediated Rapid Amplification of cDNA Ends to amplify cDNA only from full-length, capped mRNA, usually producing a single band after PCR (Ambion, Austin, TX).

3' RACE

The 3' RACE procedure required first strand cDNA to be synthesized from total RNA or poly (A)-selected RNA from *X. maculatus* Jp 163 A (brain and testis), using the 3'RACE adapter (kit provided). Nested PCR was carriedout, using primer P19F3/3'RACE outer (kit provided) and P19F4/3'RACE inner (kit provided). Mouse β-actin was used as a control. All custom synthesized oligonucleotide primers used in this study are provided in Table 3-1. The procedure involved the following steps:

Reverse Transcription

Two µl RNA (1µg total RNA or 50 ng poly (A) RNA was added to 4 µl dNTP mix, 2 µl 3'RACE adapter, 2 µl 10XRT buffer (kit provided), 1 µl RNase inhibitor (kit provided), 1 µl M-MLV Reverse Transcriptase and 8 µl nuclease-free water. The components were mixed gently, centrifuged for a short time, and then incubated at 42°C for 1 hour. The mixture was then stored at -20°C before proceeding to the PCR step.

PCR for 3'RLM-RACE

The PCR reaction was "hot-started" at 94°C with 1 µl RT reaction mixture, 5 µl 10X PCR buffer (kit provided), 4 µl dNTP mix, 2 µl P19F3 (10 µM) (Table 3-1), 2 µl 3'RACE Outer Primer, 34 µl water and 1.25 U thermostable DNA Polymerase. The PCR was carried out at conditions shown in Table 2-4. Amplified DNA fragments were resolved in 2% agarose gels then cut out of the gel and isolated using a Gene Clean Spin Kit (Bio 101; Carlsbad, CA).

TYPE	TEMPERATURE (°C)	TIME	# OF CYCLES
Denature	94	3 min.	1
Denature	94	30 sec.	35
Annealing	57	30 sec.	
Extension	72	30 sec.	
Extension	72	7 min.	1

Table 2-4. PCR Conditions for 3'RACE amplification

5'RACE

Total RNA was treated with calf intestinal phosphatase (CIP) to remove 5'-phosphates from molecules such as ribosomal RNA, fragmented mRNA, tRNA and contaminating genomic DNA. The cap structure found on intact 5'ends of mRNA is not be affected by CIP. The RNA was then treated with Tobacco Acid Pyrophosphatase (TAP) to remove the cap structure from full-length mRNA, leaving a 5'-monophosphate. A 45 bp RNA adapter oligonucleotide was ligated to the RNA population using T4 RNA ligase. The adapter could not ligate to dephosphorylated RNA because those molecules lack the 5'-phosphate necessary for ligation. During the ligation reaction, the majority of the full-length decapped mRNAs acquire the adapter sequence at their 5' ends.

RNA Processing (Small-scale reaction)

Calf Intestinal Phosphatase (CIP) treatment

One μg total RNA was gently mixed with 2 μl 10 X CIP buffer, 2 μl Calf Intestinal Phosphatase (CIP) and 20 μl Nuclease-free water. This mixture was centrifuged for a short time and incubated at 37 °C for one hour.

Terminate CIP reaction and phenol: chloroform extract

To the CIP treated mixture, 15 μl ammonium acetate solution, 115 μl dH_2O and 150 μl acid phenol: chloroform was added. The resulting mixture was centrifuged for 5 minutes at room temperature at top speed in a microfuge (>10,000 X g). The supernatant aqueous phase (top layer) was separated, by transferring to a new tube. One hundred fifty μl chloroform was added to the

mixture and vortexed thoroughly. The mixture was then centrifuged for 5 minutes at room temperature at top speed in a microfuge (>10,000 X g). Again, the supernatant aqueous phase (top layer) was separated, by transferring to a fresh tube. One hundred fifty µl isopropanol was added followed by mixing and centrifugation, as above. The resulting mixture was chilled on ice for 10 minutes and centrifuged again at >10,000 X g for 20 minutes. The pellets obtained were washed with 0.5 ml cold 70% ethanol and centrifuged for an additional 5 minutes. The ethanol was carefully removed and the pellets allowed to air dry. 10 µl of 1 X TAP Buffer (kit provided) was prepared and the sample was resuspended in 4µl of 1 X TAP Buffer.

Tobacco Acid Pyrophosphatase (TAP)

Four µl CIP treated RNA and 1 µl Tobacco Acid Pyrophosphatase were mixed gently and incubated at 37 °C for one hour.

5'RACE Adapter Ligation

Five µl CIP/TAP-treated RNA, 1 µl 5' RACE adapter, 1 µl 10X RNA Ligase Buffer (kit provided) and 2 µl T4 RNA Ligase (2.5 U/µl) were gently mixed in 1 µl water and then incubated at 37°C for one hour.

Reverse Transcription

Two µl ligated RNA, 4 µl dNTP Mix, 2 µl random decamers, 2 µl 10X RT Buffer, 1 µl RNase Inhibitor and 1 µl M-MLV reverse Transcriptase were gently mixed in 8 µl Nuclease-free water and incubated at 42°C for one hour.

Nested PCR for 5' RLM-RACE

Outer 5' RLM-RACE PCR

One µl RT reaction, 5 µl 10X PCR Buffer, 4 µl dNTP Mix, 2 µl P19 R1 (10 µM) and 2µl 5'RACE Outer Primer was added to 50 µl Nuclease-free Water and 1.25 U thermostable DNA polymerase. PCR was performed using the steps shown in the Table 2-5.

TYPE	TEMPERATURE (°C)	TIME	# OF CYCLES
Denature	94	3 min.	1
Denature	94	30 sec.	35
Annealing	57	30 sec.	
Extension	72	30 sec.	
Extension	72	7 min.	1

Table 2-5. Reaction Condition for Nested PCR using Outer 5' RLM-RACE

Inner 5'RLM-RACE PCR

Two µl Outer PCR, 5 µl 10X PCR Buffer, 4 µl dNTP Mix, 2 µl P19R2 (10 µM) and 2 µl 5'RACE Inner Primer was added to 50 µl nuclease-free water and 1.25 U thermostable DNA polymerase. PCR was performed using the steps shown in the Table 2-6.

TYPE	TEMPERATURE (°C)	TIME	# OF CYCLES
Denature	94	3 min.	1
Denature	94	30 sec.	35
Annealing	57	30 sec.	
Extension	72	30 sec.	
Extension	72	7 min.	1

Table 2-6. Reaction Condition for Nested PCR using Inner 5' RLM-RACE

Expand Long Template PCR

Expand long template PCR system (Roche Applied Science; Indianapolis, IN) consists of unique enzyme mix containing thermostable *Taq* DNA polymerase and *Tgo* DNA polymerase, a thermostable polymerase with proof-reading activity. The polymerase mixture is designed to give a high yield of PCR product from genomic DNA. Due to the inherent 3'-5' exonuclease or proofreading activity of *Tgo* DNA polymerase, the fidelity of DNA synthesis with Expand Long Template PCR (Table 2-8) System is a 3-fold higher than *Taq* DNA polymerase. The Expand Long Template Enzyme mix contains the Expand Long Template buffer (Table 2-7) and $MgCl_2$ (17.5 mM).

COMPONENT	CONCENTRATION (mM)
Tris-HCl, pH 7.5 (25°C)	20
KCl	100
EDTA	1
Nonidet P40	0.5%
Tween 20 (v/v)	0.5%
Glycerol (v/v)	50%

Table 2-7. Content of Expand Long Template Buffer

TYPE	TEMPERATURE (°C)	TIME	# OF CYCLES
Initial Denaturation	92	2 min.	1
Denaturation	92	10 sec.	10
Annealing	55	30 sec.	
Elongation	68	2 min.	
Denaturation	92	10 sec.	20
Annealing	55	30sec.	
Elongation	68	2 min.	
Extension	68	7 min.	1

Table 2-8. PCR Parameters for Expand Long PCR

THERMOSCRIPT™ RT-PCR System

The ThermoScript RT-PCR System (Life Technologies; Gaithersburg, MD) consists of an avian RNase H-minus reverse transcriptase, engineered to have high thermal stability and to produce high yields of cDNA, than AMV RT. First step cDNA synthesis is performed using total RNA primed with random-hexamer primers, at 25°C for 10 minutes followed by incubation at 65°C for 50 minutes. The reaction is terminated by incubation at 85°C for 5 minutes, adding 1 µl RNase H and incubating at 37°C for 20 minutes. cDNA is stored at –20°C until use.

Purification of DNA from agarose

A Gene Clean Spin Kit (Bio 101; Carlsbad, CA) was used for rapid isolation of double stranded DNA from agarose gels. DNA bands appearing on ethidium bromide

gels under UV are cut from the gel using glass cover slips and placed into microfuge tubes. Gel slices were melted, by adding 400 µl of Gene Clean spin glassmilk and heating at 55°C for 5 minutes, followed by thorough mixing to prevent the matrices from settling. The suspension was filtered using Gene Clean Spin Filter by centrifugation at 14,000 X g rpm and the liquid phase was discarded. To the filter 500 µl of Gene Clean spin new wash (ethanol was added before using) was added on Spin filter and this was centrifuged for 30 seconds at 13,000 X g rpm and the filtrate discarded. The Spin filter was again centrifuged for 2 minutes to dry the pellet and the filter was transfered to a new catch tube. Twenty µl Gene Clean spin elution solution was added to Spin filter and gently pipetted to resuspend Glassmilk. The DNA was eluted from Spin filter by centrifugation for 30 seconds at 13,000 X g rpm. The DNA suspension was collected and quantified using PicoGreen on a fluorometer (see **Quantitation**).

TOPO TA cloning

The cloning of PCR amplified DNA fragments was performed by using TOPO TA Cloning® version K (Invitrogen Corporation; Carlsbad, CA). The reaction was performed by gently mixing 200-300 ng of purified DNA, 1 µl dilute salt solution and 1 µl TOPO (pCR®2.1-TOPO®) vector and brought to a final volume of 6 µl. The reaction mixture was incubated for 5 minutes at room temperature then placed on ice.

One Shot Chemical Transformation

Two µl of TOPO cloning reaction was gently mixed with "One Shot" Chemically Competent *E. coli* in a vial and the mixture was incubated on ice for 5 minutes. The cells were heat shocked for 30 seconds at 42°C and 250 µl SOC medium was added without

shaking. The tube was then shaken at 200 rpm at 37°C for 1 hour. Ten to fifty µl of the resulting mixture was spread on agar plates and was incubated overnight at 37°C. Specific colonies were selected and restreaked for purification. These suspected colonies with desired cloned DNA were cultured in 5 ml 1X LB medium and incubated overnight at 37°C in a water-bath, while shaking at 200 rpm.

1X LB medium

The 1X LB (Luria Broth) medium was prepared by mixing 10 gm of LB mix in 500 ml of deionized H_2O and 825 µl 1M NaOH (pH to7). For overnight cultures, test tubes contained 5 ml 1X LB.

Agar Plates

Agar plates were made by mixing 500 ml 1X LB medium and 7.5 gm of agar. This mixture was autoclaved then cooled to 45°C. Six hundred µl ampicillin (of a 50mg/ml stock in H_2O) and 600 µl X-gal (60mg/ml in Dimethly Formamide) was then added to the agar solution. The molten agar solution was poured on plates and was allowed to solidify. The agar plates were then stored at 4°C until use.

Purification of plasmid DNA

Plasmids were purified using QIAprep Spin Miniprep Kit (Qiagen; Valencia, CA). Overnight liquid cultures were placed in microfuge tubes and centrifuged at 10,000 rpm, so that cells pelleted at the bottom of the tube. The LB medium was extracted from the centrifuge tube leaving behind only the cell pellet. Pellet were re-suspended in 250 µl of

Buffer P1 (50 mM Tris pH 8, 10 mM EDTA, 1μg/μl RNase), and vortexed. Two hundred fifty μl of Buffer P2 (kit provided) was added and the tubes were gently inverted 4-6 times to mix. To this 350 μl of Buffer N3 (kit provided) was added to each tube and they were inverted gently. The solutions were centrifuged for 10 minutes. The supernatants were applied to the QIAprep column by pipeting. The QIAprep spin column was washed by adding 0.5 ml of Buffer PB (kit provided) and centrifuged for 1 minute and the flow-through discarded. QIAprep spin column was washed with 0.75 ml of Buffer PE (kit provided) and centrifuged for 1 minute. The flow-through discarded and the QIAprep column was centrifuged for an additional 1 minute to remove the residual wash buffer. The QIAprep column was placed in a clean 1.5 ml centrifuge tube, 50 μl of H_2O was added, allowed it to stand for a minute and then centrifuged for 1 minute. The purified plasmids were quantitated on the fluorometer using PicoGreen™. Later, to check whether the plasmid had the correct insert, a restriction digest was performed with appropriate endonucleases to determine banding patterns on 1.5% agarose gels.

Partial Inverse Polymerase Chain Reaction (PI-PCR)

Partial inverse PCR (IP-PCR) (PANG 1997) was used to obtain the flanking sequences of the fragment from genomic DNA. PI-PCR is a modification of inverse polymerase chain reaction (IPCR) (OCHMAN et al. 1988) where a partial digest of genomic DNA is re-ligated and used as a template for IP-PCR. A series of restriction digests with *EcoR*I were performed and used as templates for PCR. The digests of genomic DNA were carried out at 5 ng DNA for 1 hour. The reaction was stopped by heating at 65°C for 20 minutes. T4 DNA Ligase was then added for ligation at 22°C for 4 hours. The ligation

reaction was stopped by heating at 65°C for 20 minutes. Using primers P19F10 and P19R9, IPCR was carried out on genomic *X. maculatus* Jp 163 A DNA.

Real Time PCR

For PCR analysis, ABI PRISM® 7700 (Foster City, CA) Sequence Detection System, which is a fully integrated system for real-time PCR was used. The system includes a built-in thermal cycler, lasers to induce fluorescence, CCD (charge-coupled device) detector, real-time sequence detection software, and TaqMan® reagents for the fluorogenic 5' nuclease assay. The basic advantage of this procedure lies in the high precision of the cycle-by-cycle increase in the amount of PCR product.

The ABI PRISM 7700® Spectral Calibrator Kit was used to establish pure dye spectra. The kit contains a passive internal reference used to normalize non-PCR related fluoresence fluctuations. Normalizing with a passive internal reference helps to eliminate errors due to incorrect pipetting and sample evaporation.

Gene expression levels for *CDKN2D* gene was compared by a relative standard curve method. In this type of assay, a reference sample is used as a comparison for all other samples assayed. The values obtained for each sample are normalized based on the value from the reference sample. The normalized values are further converted into relative values based on a calibrator. RNA derived from a *Xiphophorus* cultured cell line, A2, was used as a calibrator (KUHN et al. 1979). The calibrator RNA is chosen from one of the normalized values (and assign that value one unit) so that the other normalized values are either multiples or fractions of the calibrator. This method allows direct comparison of samples assayed in different experimental conditions.

CHAPTER 3

RESULTS AND DISCUSSION

Xiphophorus CDKN2D Gene Isolation and Nucleotide Sequencing

Cloning of *CDKN2D*

In order to clone the *CDKN2D* gene, amino acid sequence information from all published cdkn2 polypeptides was downloaded into the MacVector® software package (IBI; New Haven, CT). Amino acid alignments were prepared using BlockMaker software and, based on the amino acid conservation, degenerate primers were designed using the CODEHOP (ROSE et al. 1998) program. The primers were specifically designed so they would not amplify *CDKN2X,* because of its similarity to *CDKN2D.* These degenerate primers were used in touchdown PCR (DON et al. 1991) to amplify cDNA derived from a mix of three *X. maculatus* tissues (liver, brain and muscle). A 140 bp amplicon (Figure 3-1) was obtained using primers CDKN2degF1 and CDKN2degR1 and then a hemi-nest PCR was performed using primer CDKN2degF1 and CDKN2degR2 (Table 3-1). Amplified DNA was cloned into the vector and the resulting plasmids from 8 clones obtained, isolated, and processed for nucleotide sequencing as described in Chapter 2 (Davis Sequencing, LLC; Davis, CA). Based on the nucleotide sequences, homology searches were performed using the NCBI blastN (nucleotides) and blastX (amino acids) programs. These analyses revealed that 4 of 8 clones were identical

and showed very and exhibited high homology to the *Fugu CDKN2D* locus. Figure 3-2 depicts the amino acid sequence conservation derived from 4 of the 8 cloned the *Xiphophorus* amplification products and other vertebrate taxa.

Oligonucleotide	**Primer Sequence**
CDKN2degF1	cggcaggaccgccatncargtnatga
CDKN2degR1	cgtgcagcaccttcagggtrtcnarraa
CDKN2degR2	ccaggaagcccgcckngcngcrtc
P19F1	atgatgatggggaactcc
P19F2	gggaactccaaagtagcaag
P19F3	gggaactccaaagtagcaaggttgt
P19F4	aggttgttgctggaaaaaggagc
P19F5	atggtcctgagtcagatgga
P19F6	acgacttgtggagacttgacgacg
P19F7	gagacttgacgacgcttggtatct
P19F8	cacacatactgctggaagc
P19F9	ttttggttcttgggattcgacttt
P19F10	gcatatcagtttatttttcttcgt
P19R1	tcatggacaggcgctatg
P19R2	gctatgccgtgtttgtcctgg
P19R3	ctccttttccagcaacaac
P19R4	ttaactgtgaatgtgagcaaaaagt

P19R5	taactgtgaatgtgagcaaaaagtg
P19R6	ctttatgtcagtaagaactcaactcat
P19R7	tgctgccgccgtcaaagc
P19R9	catctgactcaggaccattatactc
P19R10	tattccgtccgttgagatac

Table 3-1. Synthetic oligonucleotide primers used for PCR, 5' and 3'-RACE and sequencing the *X. maculatus CDKN2D* gene

CGGCAGGACCGCCATTCAAGTCATGATGATGGGGA

ACTCCAAAGTAGCAAGGTTGTTGCTGGAAAAAGGA

GCTGAGCCCAACGTCCAGGACAAACACGGCATAGC

GCCTGTCCATGACGCCGCGCGGGCGGGCTTCCTGG

Figure 3-1. 140 bp DNA sequence cloned from *X. maculatus* (primer positions are underlined).

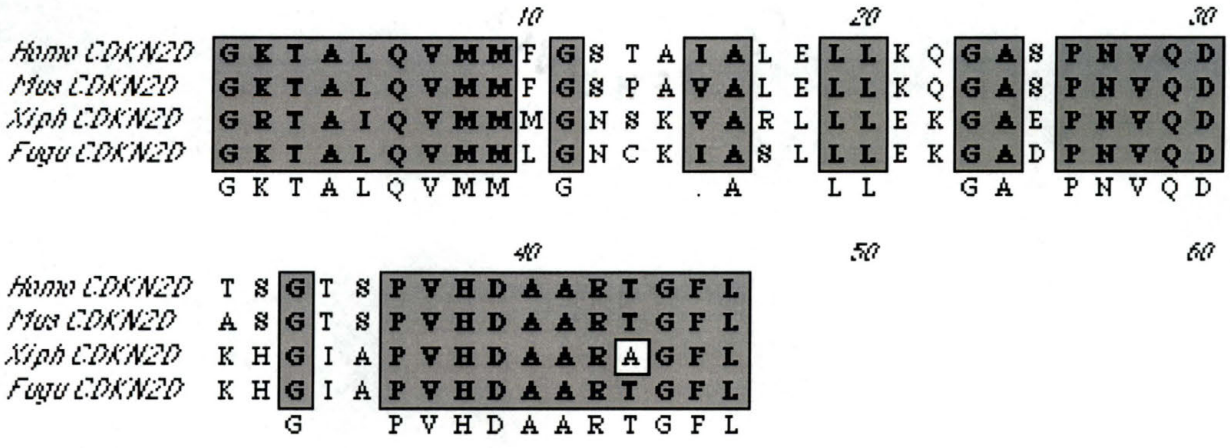


Figure 3-2. Conservation of amino acid sequences derived from human, *Mus musculus*, *Xiphophorus* and *Fugu*. Shaded areas represent amino acid identity or similarity.

3' RACE:

cDNA sequence information was obtained from *X. maculatus* Jp 163 A (testis and brain) using the 3' RACE protocol (Ambion; Austin, TX). Amplification by nested PCR, using primer P19F3/3'RACE Outer (kit provided) and P19F4/3'RACE Inner (kit provided) resulted in a 372 bp product. The isolated DNA was purified from agarose and was cloned using TOPO-TA vector. The plasmid DNA was purified and the clones sequenced. Of six clones, sequences from 5 clones indicated *CDKN2D* 3'DNA and had been isolated. This was concluded based on homology searches using NCBI blastN (nucleotide), which revealed homology to the 3' end of the *Fugu CDKN2D* sequence.

5'RACE

The 5' ends of *X. maculatus* Jp 163 A *CDKN2D* were obtained from cDNA using the 5'RACE protocol (Ambion; Austin, TX). Amplification by nested PCR, using primer P19 R1/5'RACE Outer (kit provided) and P19 R2/5'RACE Inner (kit provided) resulted in a ~350-500 bp products. These bands were isolated and cloned using TOPO-TA vector and sequenced as previously described. Of 5 clones sequenced, one contained extra sequence of 80 bp (total 450 bp) upstream of the translation start site and the others contained 370 bp upstream from the ATG. Due to this extra sequence two transcription patterns can be proposed for *CDKN2D* in *Xiphophorus* as shown in Figure 3-8.

Full Length cDNA Confirmation

After obtaining sequences from 5' and 3' RACE, full-length cDNA was amplified, sequenced and confirmed for *Xiphophorus CDKN2D*. A product size of 742 bp was

amplified from cDNA using primers P19F7/R5 (Table 3-1) followed by hemi nest with primers P19 F5/R5 for 4 different species of *Xiphophorus*; *X. maculatus* (Jp 163 A), *X. helleri* (Sarabia), *X. nezahualcoyotl* (Ocampo) and *X. variatus* (Zarco). Figure 3-3 shows *CDKN2D*-derived protein alignment of these 4 species of *Xiphophorus*. As expected *CDKN2D* sequences are well conserved among the 4 species. Table 3-2 shows nucleotide and amino acid identity and similarity values of *X. maculatus CDKN2D* compared to human, mouse and *Fugu* genes. *X. maculatus CDKN2D* shares 83% identity to *Fugu CDKN2D,* followed by human and mouse *CDKN2D.* The gene shares only 37% identity to *CDKN2X* and cumulatively these data gives us confidence that the cloned gene is a *Xiphophorus CDKN2D* gene. Also, Figure 3-4 shows the aligned nucleotide sequences of *CDKN2D* in *Xiphophorus maculatus*, *Fugu rubripes* and *Homo sapiens*. On the nucleotide level *X. maculatus CDKN2D* shares 51% similarity to human *CDKN2D* and *Fugu CDKN2D*.

Figure 3-5 shows the conserved amino acids of all known *CDKN2* gene family members. The translated sequence of *Xiphophorus CDKN2D* is aligned to those of human (*CDKN2A*, *CDKN2B*, *CDKN2C*, *CDKN2D*), mouse (*CDKN2A*, *CDKN2B*, *CDKN2C*, *CDKN2D*), rat (*CDKN2A*, *CDKN2B*, *CDKN2C*), *Fugu* (*CDKNA/B*, *CDKN2D*), monodelphis (*CDKN2A*), hampster (*CDKN2A*) and chicken (*CDKN2B*). Many regions within the amino acid alignment show 100% identity among all known *CDKN2* gene family members. The structural properties of CDKN2 proteins have been discussed in several studies (KALUS et al. 1997; LUH et al. 1997; VENKATARAMANI et al. 1998). These studies concur that ankyrin structural repeats comprising CDKN2 proteins create β-strand, α-helix/ β-turn/ α-helix extended β-strand motifs and that these motifs

associate with each other through β-sheet and helical bundle interactions. Several residues are likely to be important for such structures and predicted to be conserved in the fish *CDKN2D*. Examples of this are the β-turns which consistently show central glycine residues positioned at 29, 62, 96, 129 and 172 amino acids (Figure 3-5) are conserved among known CDKN2 proteins including *Xiphophorus CDKN2D* protein.

	% Identity	**% Similarity**
Fugu CDKN2D	83	92
Human CDKN2D	51	70
Mouse CDKN2D	54	68
Human CDKN2C	43	64
Mouse CDKN2C	41	62
Human CDKN2A	39	57
Mouse CDKN2A	40	56
Mouse CDKN2B	37	53
Human CDKN2B	36	52
X. mac CDKN2X (A/B)	37	51
Fugu CDKN2A/B	35	50

Table 3-2. Nucleotide identity and amino acid similarity values of the *X. maculatus CDKN2D* gene compared to human, mouse and fugu *CDKN2* genes. The functional similarity is determined on the basis of amino acid characteristics like polar, non- polar, acidic or basic. For example leucine, isoleucine, valine are all non-polar amino acids and they are considered as functionally similar.

Figure 3-6 represents the phylogenetic tree of the *CDKN2* gene family, which was done using the Neighbor-Joining Method, which keeps track of nodes on a tree rather than taxa or clusters of taxa. The tree is constructed by linking the least-distant pair of nodes in a modified distance matrix. At each stage in the process two terminal nodes are replaced by one new node, leading to reduced tree size. *CDKN2D* (p19) of *X. maculatus* is homologous to human, mouse and *Fugu CDKN2D* whereas *CDKN2A/B (CDKN2X,* p13*)* shows only orthology to human and mouse *CDKN2A/B* members.

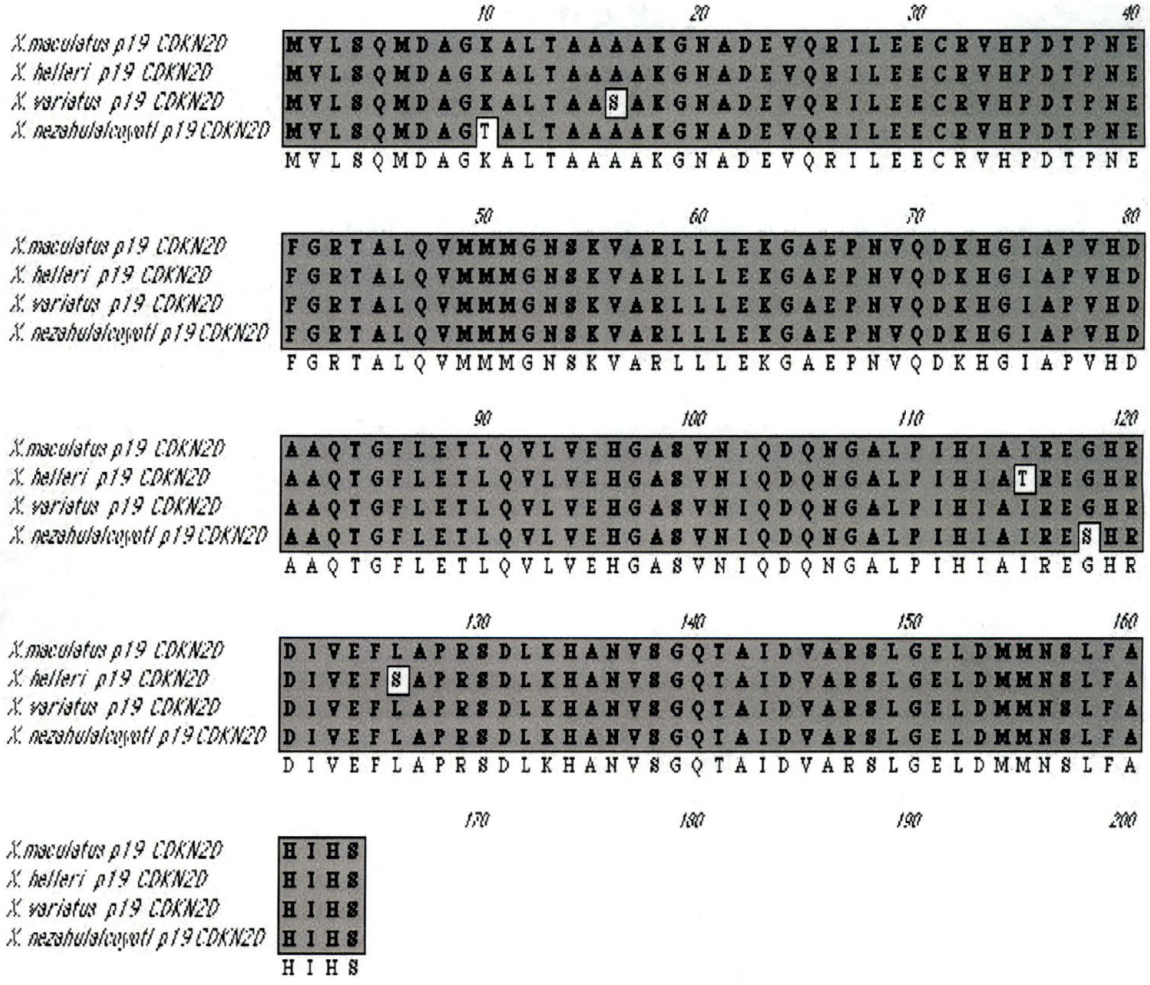
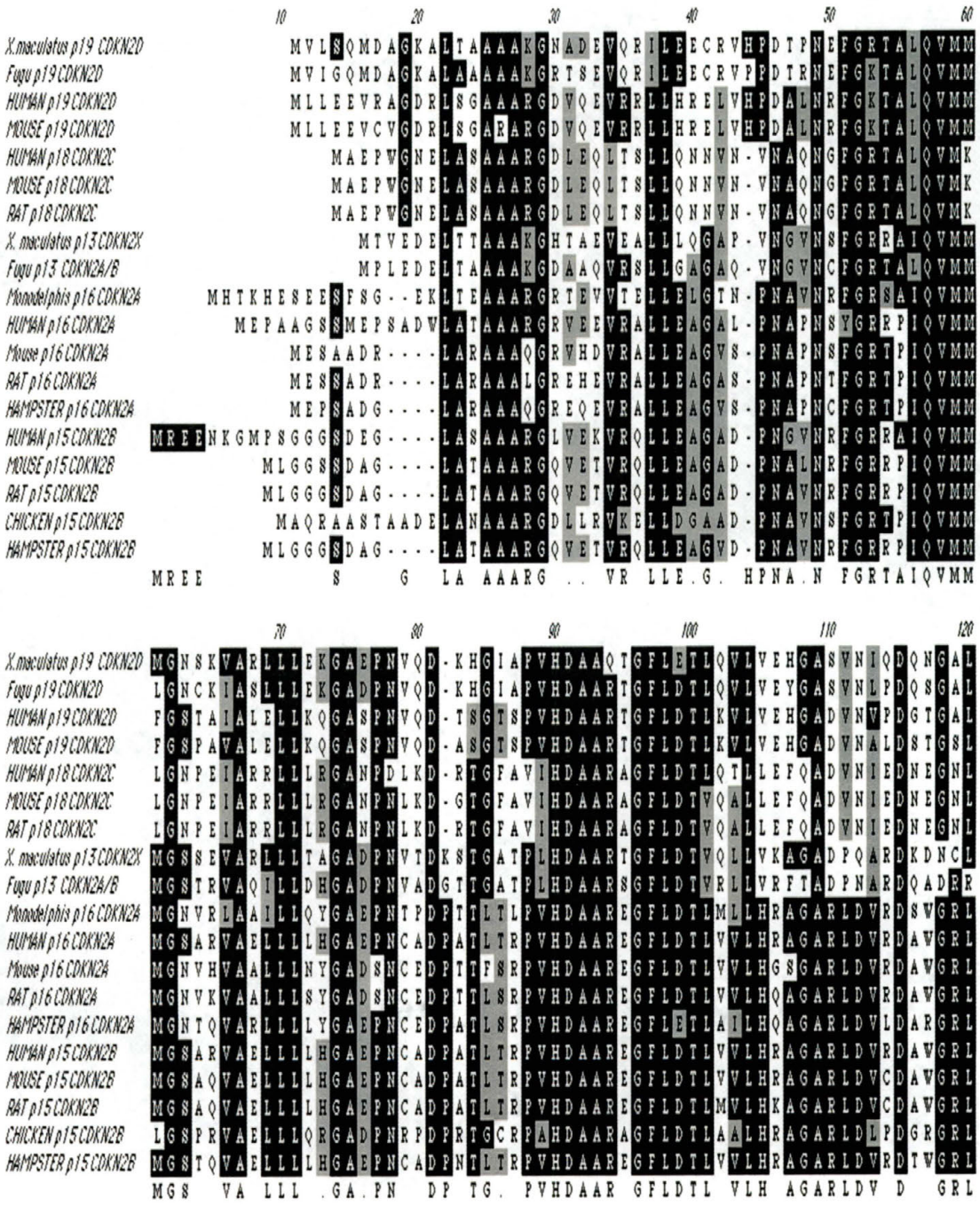


Figure 3-3. Clustal W alignment of the *X. maculatus*, *X. helleri*, *X. variatus* and *X.nezahualcoyotl CDKN2D* amino acid sequences. The shaded areas represent identical amino acids.

```
X. maculatus p19    1 ATGGTCCTGAGTCAGATGGACGCGGGGAAAGCTTTGACGGCGGCAGCAGC  50
Fugu p19 CDKN2D    1 ATGGTCATTGGGCAGATGGATGCCGGTAAAGCGTTGGCGGCGGCGGCAGC  50
Human p19 CDKN2D   1 ATGCTGCTGGAGGAGGTTCGCGCCGGCGACCGGCTGAGTGGGGCGGCGGC  50
                      *** *  *     ** *    ** **  *     **   * *** ** **

X. maculatus p19   51 CAAAGGGAATGCCGATGAGGTGCAGAGGATCCTGGAGGAATGCAGAGTGC 100
Fugu p19 CDKN2D    51 CAAAGGACGGACGAGCGAGGTGCAGAGGATCCTGGAGGAATGCCGAGTGC 100
Human p19 CDKN2D   51 CCGGGGCGACGTGCAGGAGGTGCGCCGCCTTCTGCACCGCGAGCTGGTGC 100
                      *   **          *******   *  * *** *          ****

X. maculatus p19   101 ATCCCGATACTCCCAACGAGTTCGGCCGGACCGCGCTGCAGGTGATGATG 150
Fugu p19 CDKN2D    101 CTCCCGATACTCGCAACGAATTTGGCAAGACTGCACTGCAGGTGATGATG 150
Human p19 CDKN2D   101 ATCCCGACGCCCTCAACCGCTTCGGCAAGACGGCGCTGCAGGTCATGATG 150
                      ******  * * ****   ** ***  *** ** ******** ******

X. maculatus p19   151 ATGGGGAACTCCAAAGTAGCAAGGTTGTTGCTGGAAAAAGGAGCTGAGCC 200
Fugu p19 CDKN2D    151 CTGGGAAACTGCAAGATCGCCAGTTTACTGCTGGAGAAGGGGGCGGACCC 200
Human p19 CDKN2D   151 TTTGGCAGCACCGCCATCGCCCTGGAGCTGCTGAAGCAAGGTGCCAGCCC 200
                      * ** * *  *    * **        ***** *  * ** **    **

X. maculatus p19   201 CAACGTCCAGGACAAACACGGCATAGCGCCTGTCCATGATGCAGCACAGA 250
Fugu p19 CDKN2D    201 CAACGTGCAGGACAAGCACGGCATAGCGCCCGTGCATGACGCCGCTCGGA 250
Human p19 CDKN2D   201 CAATGTCCAGGACACCTCCGGTACCAGTCCAGTCCATGACGCAGCCCGCA 250
                      *** ** *******    *** *     ** ** ***** ** ** *  *

X. maculatus p19   251 CGGGGTTCCTTGAGACCCTGCAGGTCCTGGTGGAGCACGGGGCTTCAGTG 300
Fugu p19 CDKN2D    251 CCGGCTTCCTGGACACTCTGCAGGTTCTGGTGGAGTACGGCGCCTCGGTA 300
Human p19 CDKN2D   251 CTGGATTCCTGGACACCCTGAAGGTCCTAGTGGAGCACGGGGCTGATGTC 300
                      * ** ***** ** ** *** **** ** ****** **** **    **

X. maculatus p19   301 AACATCCAGGACCAGAACGGCGCCCTCCCCATCCACATCGCCATACGAGA 350
Fugu p19 CDKN2D    301 AACCTCCCGGATCAGAGCGGCGCCTTGCCGATCCACATCGCCATCCGGGA 350
Human p19 CDKN2D   301 AACGTGCCTGATGGCACCGGGGCACTTCCAATCCATCTGGCAGTTCAAGA 350
                      *** * *  **    * *** **  * ** *****  * **  * *  **

X. maculatus p19   351 AGGCCACCGGGATATCGTGGAGTTCTTGGCTCCACGATCCGACCTGAAAC 400
Fugu p19 CDKN2D    351 AGGCCACAGGGACGTGGTCGAGTTCCTGGCGCCGCGCTCCGACCTGAAAC 400
Human p19 CDKN2D   351 GGGTCACACTGCTGTGGTCAGCTTTCTGGCAGCTGAATCTGATCTCCATC 400
                      ** ***   *   * **    **  ****  *    ** ** **  * *

X. maculatus p19   401 ATGCCAATGTCAGTGGTCAAACAGCAATAGACGTTGCCCGATCTCTGGGT 450
Fugu p19 CDKN2D    401 ACGCCAACAAGAGCGGGCAGACGGCGGCGGACGTGGCCCGGGCCTCCCGG 450
Human p19 CDKN2D   401 GCAGGGACGCCAGGGGTCTCACACCCTTGGAGCTGGCACTGCAGAGAGGG 450
                            *    ** ** *  **  *    **  * ** *         *

X. maculatus p19   451 GAGCTGGATATGATGAACTCACTTTTTGCTCACATTCACAGTTAGTAAGA 500
Fugu p19 CDKN2D    451 GTCCCCGACATGATGGACTTGCTCTTCTCCCACGTGCACCGCTAG      495
Human p19 CDKN2D   451 GCTCAGGACCTCGTGGACATCCTGCAGGGCCACATGGTGGCCCCGCTGTG 500
                      *  *  **  *  ** **   **       *** *         *

X. maculatus p19   501 ATGGTAAAAAA 511
Fugu p19 CDKN2D    496             495
Human p19 CDKN2D   501 ATCTGGGGT   509
```

Figure 3-4. Nucleotide alignment between *Xiphophorus maculatus*, *Fugu rubripes* and *Homo sapiens* (human) nucleotide *CDKN2D* cDNA sequences.


X.maculatus p19 CDKN2D
Fugu p19 CDKN2D
HUMAN p19 CDKN2D
MOUSE p19 CDKN2D
HUMAN p18 CDKN2C
MOUSE p18 CDKN2C
RAT p18 CDKN2C
X. maculatus p13 CDKN2X
Fugu p13 CDKN2A/B
Monodelphis p16 CDKN2A
HUMAN p16 CDKN2A
Mouse p16 CDKN2A
RAT p16 CDKN2A
HAMPSTER p16 CDKN2A
HUMAN p15 CDKN2B
MOUSE p15 CDKN2B
RAT p15 CDKN2B
CHICKEN p15 CDKN2B
HAMPSTER p15 CDKN2B
MREE S G LA AAARG .. VR LLE.G. HPNA.N FGRTAIQVMM
X.maculatus p19 CDKN2D
Fugu p19 CDKN2D
HUMAN p19 CDKN2D
MOUSE p19 CDKN2D
HUMAN p18 CDKN2C
MOUSE p18 CDKN2C
RAT p18 CDKN2C
X. maculatus p13 CDKN2X
Fugu p13 CDKN2A/B
Monodelphis p16 CDKN2A
HUMAN p16 CDKN2A
Mouse p16 CDKN2A
RAT p16 CDKN2A
HAMPSTER p16 CDKN2A
HUMAN p15 CDKN2B
MOUSE p15 CDKN2B
RAT p15 CDKN2B
CHICKEN p15 CDKN2B
HAMPSTER p15 CDKN2B
MGS VA LLL .GA.PN DP TG. PVHDAAR GFLDTL VLH AGARLDV D GRL

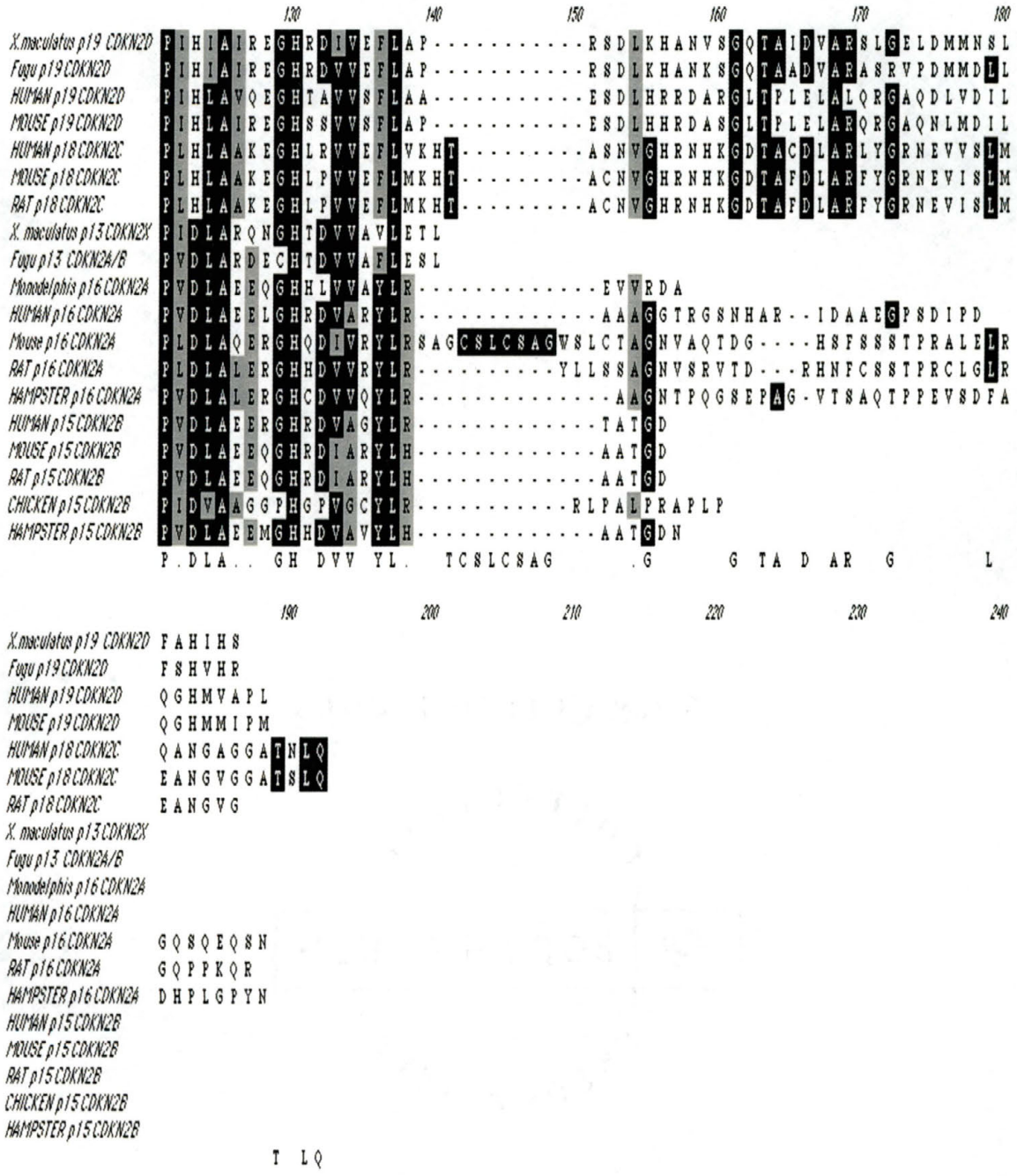


Figure 3-5. Clustal W alignment of *X. maculatus CDKN2D* protein with human, mouse, rat, fugu, chicken and hamster *CDKN2* gene family proteins. The darkly shaded areas represent conserved amino acids, while the lighter shading represents functional similarity between amino acids. The functional similarity is determined on the basis of amino acid characteristics like polar, non- polar, acidic or basic. For example leucine, isoleucine, valine are all non-polar amino acids and they are considered as functionally similar.

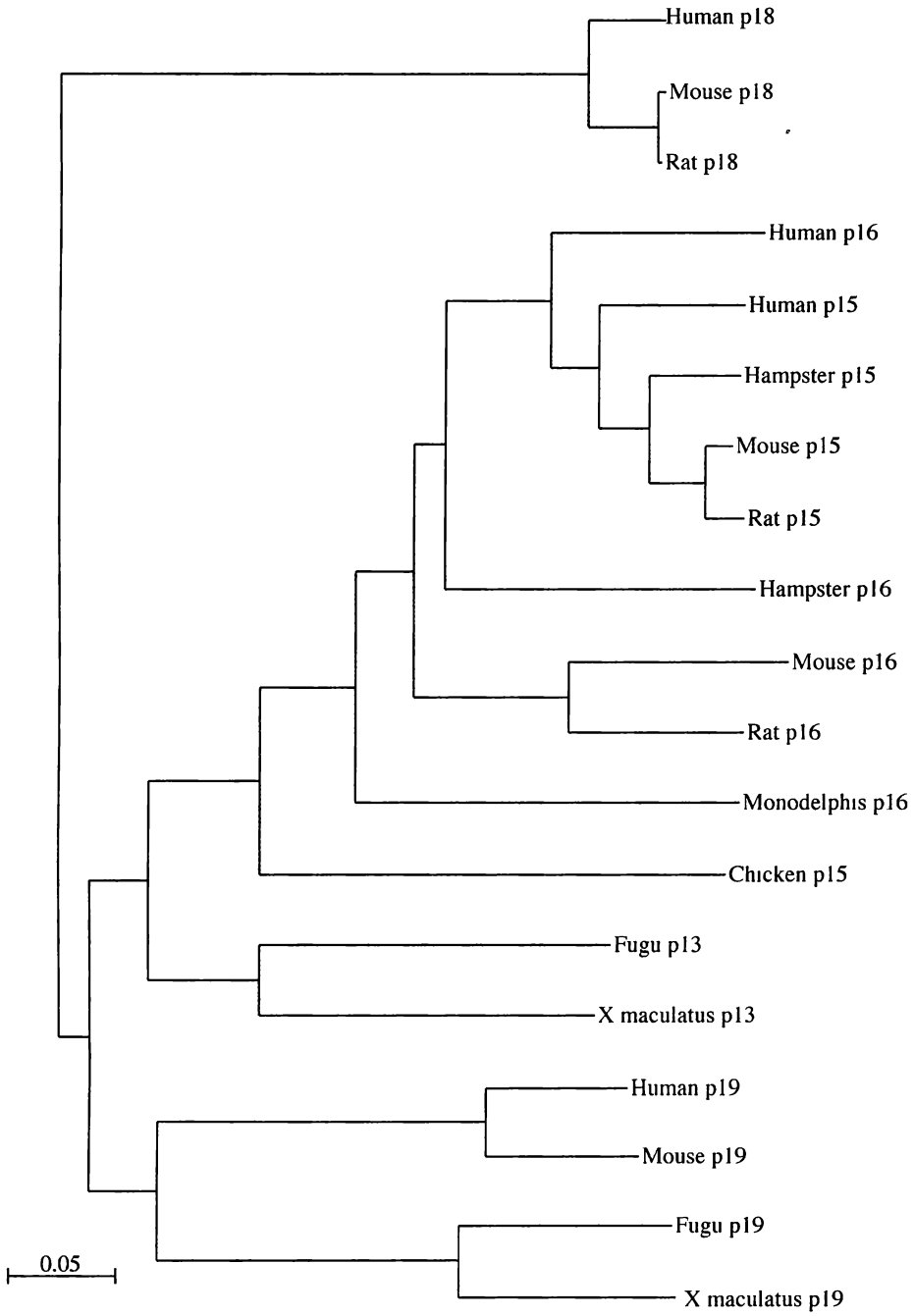


Figure 3-6. Neighbor-joining phylogenetic tree showing all known completely sequenced *CDKN2* gene family members. A phylogenetic tree was generated from a PileUp alignment of known *CDKN2* gene family members using Clustal X (1.8) program employing the neighboring-joining method (see text).

Exon / Intron Organization

Based on the sequences derived from 5' and 3' RACE, a full-length sequence of *Xiphophorus CDKN2D* gene was amplified from genomic DNA. The exon/intron boundaries were defined by alternative amplification procedures using an Expand long-PCR kit (Roche Applied Science; Indianapolis, IN). Using primers P19 F5 and P19 R1 (Table 3-1) and genomic DNA from *X. maculatus* and *X. helleri*, 7 clones containing an intron were obtained. The sequences of these clones indicate an intron size of 1381 bp.

Isolation of sequences upstream of exon 1

Partial inverse PCR (PANG 1997) was used to obtain the flanking sequences of the fragment from genomic DNA. Using primers P19F10 and P19R9 (Table 3-1), a genomic DNA product of ~1011 bp was amplified from *X. maculatus* JP 163 A partially cut with *EcoR*I restriction endonuclease. The nucleotide sequence of this fragment was determined. Homology searches on NCBI blastN programs for the sequence upstream of exon 1 revealed no homology to promoter regions of any of the *CDKN2* gene family members including the *Xiphophorus CDKN2X* promoter region.

The complete *X. maculatus CDKN2D* genomic DNA sequence was constructed by combining overlapping nucleotide sequence data. The genomic organization of the *CDKN2D* gene in *X. maculatus* is shown in Figure 3-7. The gene in consists of 4 exons and 3 introns. The *X. maculatus CDKN2D* DNA sequence with exon/intron boundaries (exons in bold), primer positions and translated amino acid is displayed in Figure 3-9.

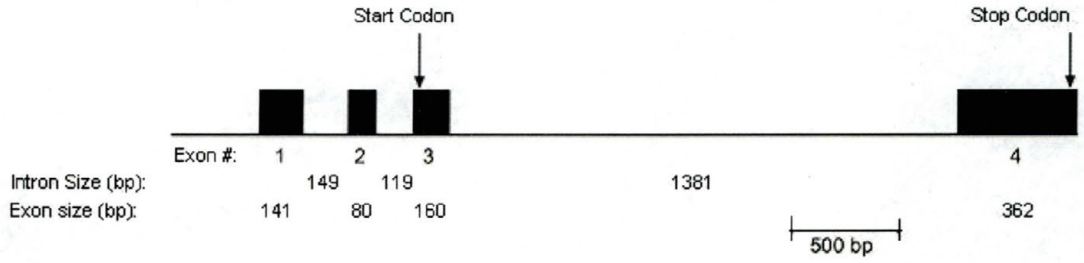


Figure 3-7. Genomic organization of the *CDKN2D* gene in *X. maculatus.* Exon and intron junctions are indicated where black boxes represent exons.

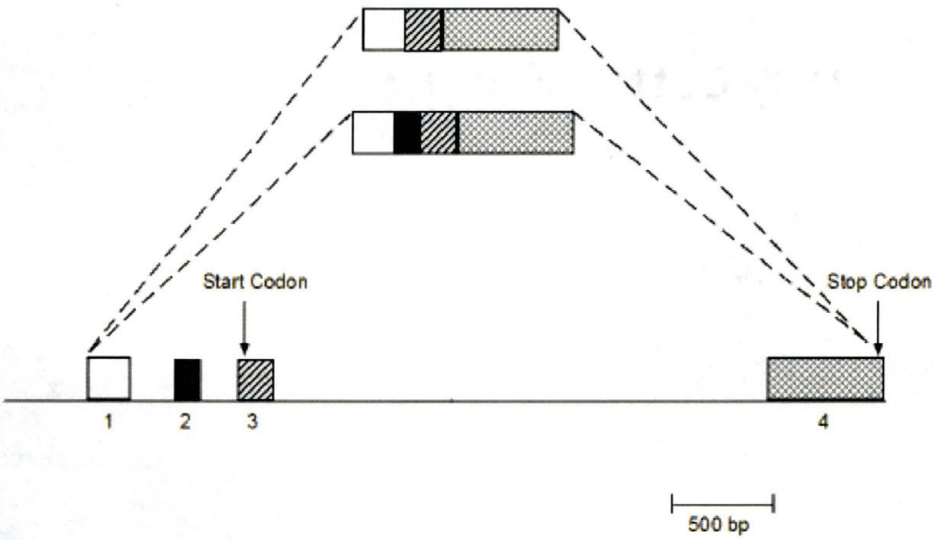


Figure 3-8. Alternative transcripts observed for *CDKN2D* in *X. maculatus*. Exon 2 is 5' to the start of translation and is not always present in *CDKN2D*-derived transcripts. This conclusion is based on 5' RACE experiments which delineated alternate transcriptional patterns for *CDKN2D*. Exon 2 is comprised of 80 bp.

Figure 3-9. Annotated Sequence of *X. maculatus* (Jp 163 A) *CDKN2D*

50
GAATTCAAAGCATCCCATGTACACACATACTGCTGGAGTACAAGCAAAGG

100
ATGGAAATCTGAATGAACTGCCCTTTGAAGACGCCAACGTTTACAAATCG

150
CCCGCTTTATTCGCTTCTAGCTCTGATTGGTCAGATGTTGATGTTGCCAC

200
TGCGAGAGCGTAGTAACAGAGTCATAGCTCCTCCTGTTTTTCCATATATG

250
GACGAACAACGCCTACTGGGAGAGTTGTGATTGGCCAGCGTATTCTCCTT

300
TCCAATGTTTGTAAATTTTAGACTAGGGAGACGCCGCAGTTTGGATCTCA

350
GCGCGCAAATGTATTTTTTAAAGGTTGATAAACTTTTCTCGTTTGAATTG

>P19F7
>P19F6
400
TATTTAAAAATAACTGGGCACTAGAAGCAACGACTTGACGACGCTTGGTA

450
TCTCAACGGACGGAATAAGGTAGGCACAGAAACCTTTATGGTTGATAAAC

500
CGTTAGCTCGGATGCTACTTGTTATTAACATTCAGTCTATATGAATTATC

550
TCTGTCGAGATGGCAAATATTTCCCTTCACCAGTGCGGTTTAACCTTATA

600
TTTTGTTTAAACACCCCAGCGTTGCAGCTGTACGTAACAACCCAGTAATA

650
TGGCGTCAGCGGAGAACTACAAGTTACGTTAGCCGGATGCTGTAGTAAGG

700
TTAGCTAGGGGTAGCCGACATTATCATCCCGGCTTTTCGAAATAACCTGT

Figure 3-9 Annotated Sequence of *X. maculatus* (Jp 163 A) *CDKN2D* (continued)

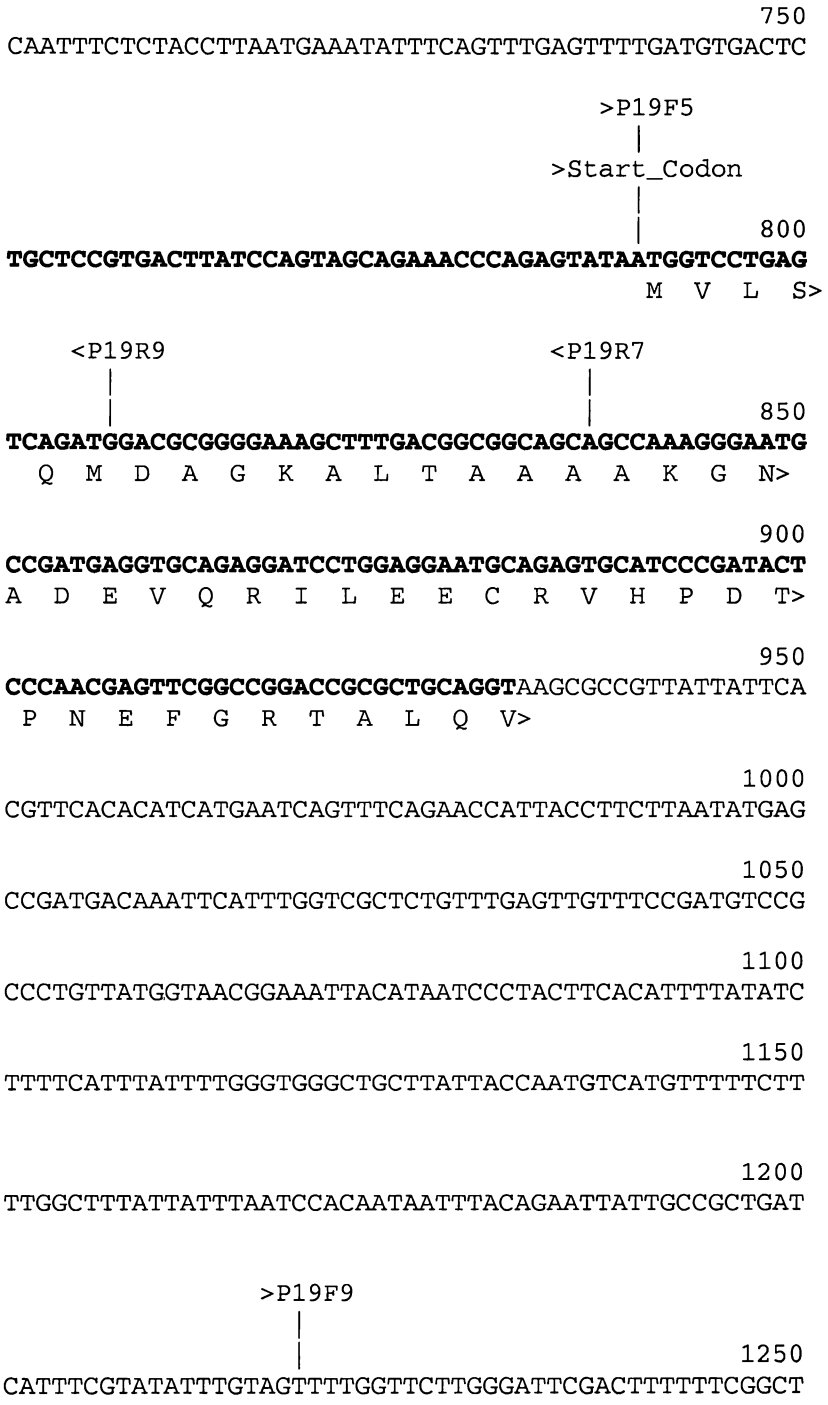


Figure 3-9. Annotated Sequence of *X. maculatus* (Jp 163 A) *CDKN2D* (continued)

```
                                                        >P19F10
                                                          |
                                                         1300
ACGAAAAGACGTCGTCTGTTGTTGGATACCGTGTAATTAAATGATGGCAT

                                                         1350
ATCAGTTTATTTTTCTTCGTAGCAGGGACGTACATAGAATTCAAAAGCAT

      >P19F8
        |
        |                                                1400
CCCATGTACACACATACTGCTGGAAGCTGTATTTATGTCCAAAATACCCA

                                                         1450
AAAAAAAATCATATTTTCCCTACATGCTGGACTCCTGATGCTCTGAAACA

                                                         1500
TCCTGTGCAAACATCTTTAATTCCTACCTTGAAAAAGTCTGTAACTACAA

                                                         1550
ATGAAAACTACAAAGGCATGACATGGTAAATCCATATAAGGCATAGTTGT

                                                         1600
AGTCTATTGCAAAATATGTTTTTGACTCCCAAAAAGAAATAGTTTTTGCC

                                                         1650
ATACCTGTAAATTCGGTTTGACCAGGTAGATTTCAATGTAATTATTTTCA

                                                         1700
GAAAATAAAATGTGACACCTGTATTTGAGCATAATTATCAGTTTATTTTT

                                                         1750
GCAACTCATTACAATTCAGAAGTGCAACTTAAACATACTAATGAGCAAAG

                                                         1800
GTATAAATACATTATTATTAAATTAGTTGCTTGAAGTTTTGAAATGGATT

                                                         1850
GATTTAAAAGGGGACAAAATGGAAACACGTTGGTACGAATCGGGTTTACA

                                                         1900
AATTTACATAGAAGTGAAATTATTTGGAGCTTTCAGGATCTTTTTCTTTA

                                                         1950
TTGTTAGTAGTAGTAGTAGTAGTAGTAATATTTCAGCTAAGGTATGTGGA
```

Figure 3-9 Annotated Sequence of *X. maculatus* (Jp 163 A) *CDKN2D* (continued)

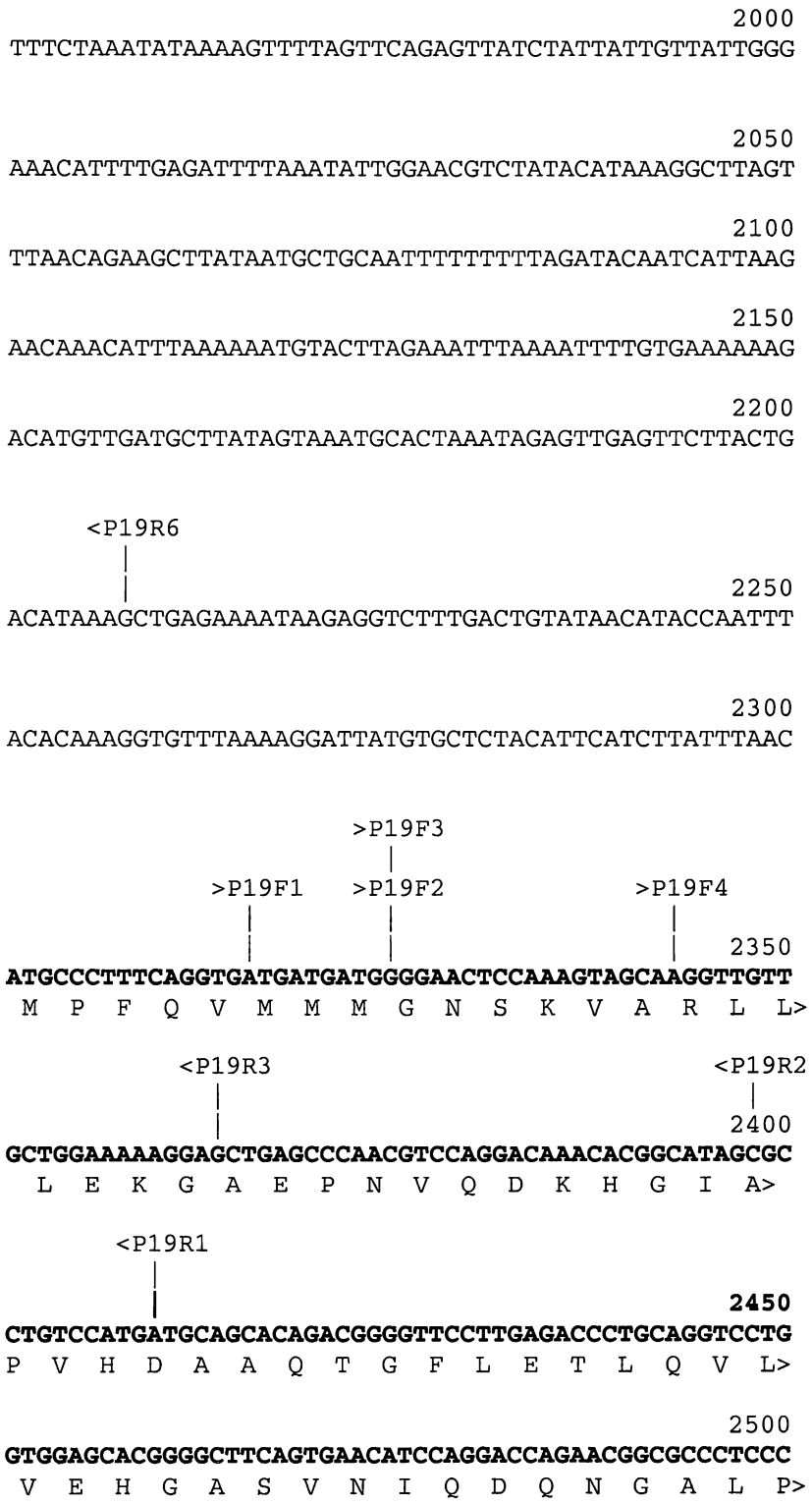
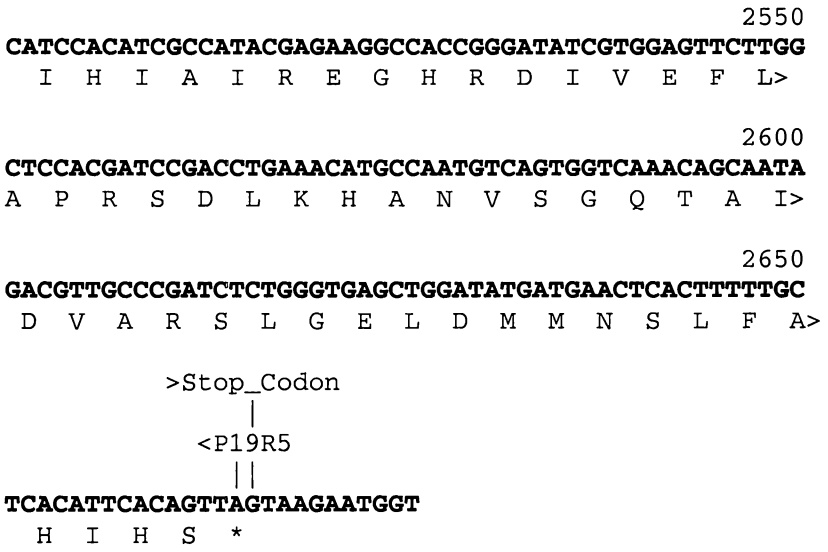


Figure 3-9. Annotated Sequence of *X. maculatus* (Jp 163 A) *CDKN2D* (continued)



CHAPTER 4

RESULTS AND DISCUSSION

Mapping of *Xiphophorus CDKN2D* Locus

Xiphophorus fishes have the ability to produce fertile interspecies hybrids (F_1 hybrids). The interspecies F_1 hybrids produced derive one half of their genetic content from each parent. It can be confirmed from literature (COURTENAY JR. and MEFFE 1989) that *Xiphophorus* species diverged in evolution approximately 65 to 80 million years ago and hence the interspecies hybrids are polymorphic for many or most of the loci along the length of any chromosome. For this reason backcross hybrids between species are used for gene mapping in *Xiphophorus*.

The hybrid progeny obtained by backcrossing an F_1 hybrid to one of the initial parental species, carry on average, 75% of their genetic information from the recurrent parent and 25% from the non-recurrent parent. Inheritance of each genetic marker with development of complex phenotypes, such as tumor development, can be assessed by segregation of the non-recurrent parent chromosomes and/or chromosomal regions into the backcross hybrids.

Mapping of a genotype in BC_1 (Backcross) hybrids is performed with a large set of genetic markers and then scoring each BC_1 hybrid as homozygous or heterozygous for each marker at each locus. Two-by-two chi square analyses of marker inheritance for each possible marker pair is then performed using computer programs (Mapmaker, Map

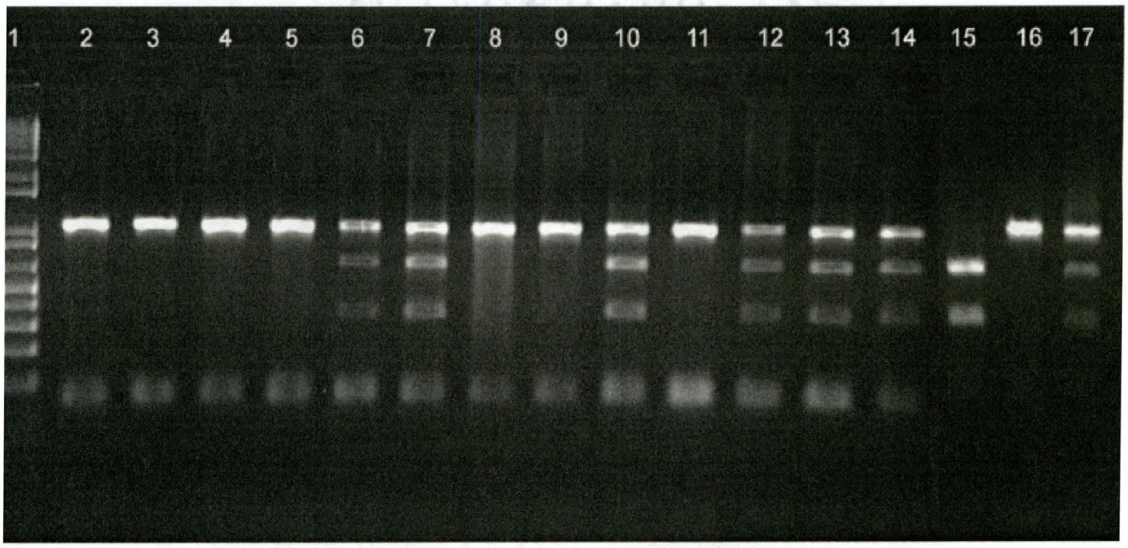
Manager). Genetic linkage is established by observing an excess of parental types among the BC_1 hybrids for any particular marker pair (i.e. deviation from the expected 50:50 ratio for random segregation). This information is used to assign a map position to a new locus (MORIZOT et al. 1998).

Linkage Mapping

BC_1 hybrids derived from *X. maculatus* x *X. helleri* (backcrossed to *X. helleri*) were used to create a linkage map consisting of 403 mapped polymorphic markers (Kazianis et al., unpublished). PCR amplification of *CDKN2D* was performed on DNA samples obtained from 91 BC_1 hybrids using primers P19F10 and P19R6 (Table 3-1). The products of PCR amplification were subsequently subjected to restriction digestion with *Nde*II (Roche Applied Science; Indianapolis, IN), which resulted in a 912 bp product for *X. helleri* and 590 bp and 322 bp products for *X. maculatus* (Figure 4-1).

Maximum–likelihood map orders were established after analysis of genotypic data in Mapmaker (version 3.0 b). For each linkage group, "framework" loci and map orders were determined with orders between them considered >1000 X less likely. Other markers were positioned on the map if alternative positions were 100 X less likely. In cases of tight linkage or insufficient data, loci were placed on the maps using Map Manager QT (MANLY 1999), which was predominantly used to order markers within maps by minimization of double crossovers and map length. Graphic map files were generated using Mapmaker for Macintosh version 2.0 (obtained from S. Tingey, DuPont Co., Wilmington, DE).

A model based on the inheritance of two loci genetically explains the segregation of eavily and lightly pigmented individuals in the first backcross BC_1 (Figure 1-3). hough *CDKN2D* is mapped to the same region as *CDKN2X* (Figure 4-2), these two enes are far apart from each other with a recombinant value of 12.4. The area near *IDH2/CDKN2D* does not show strongest association with the *DIFF* phenotypes, but thi trong association is seen in case of *CDKN2X* (KAZIANIS et al. 1998). This observation trongly suggests that *CDKN2D* might not act as a tumor suppressor of melanoma ormation.



igure 4-1. An example of *CDKN2D* intron 3 PCR-*NdeII* restriction digest olymorphism between *X. maculatus* Jp 163 A (lane15) and *X. helleri* sarabia (lane 16). he F1 hybrid (lane 17) contains representative all from each parent. The BC_1 hybrids xhibit either the heterozygous F_1 parent (lanes 6, 7, 10, 12, 13, 14) or the homozygous *elleri* parent (lanes 2, 3, 4, 5, 8, 9, 11).

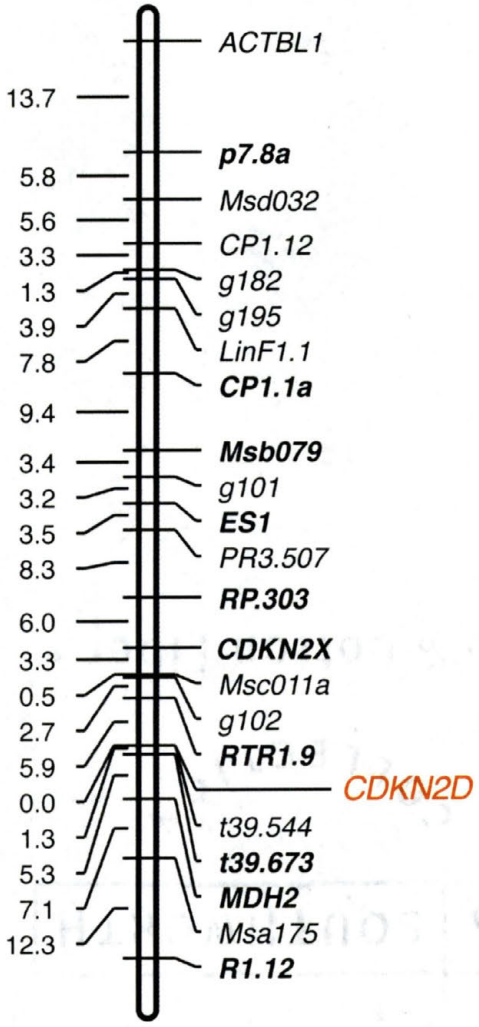


Figure 4-2. Map position of *CDKN2D* within linkage group V in *Xiphophorus*.

CHAPTER 5

RESULTS AND DISCUSSION

Studies of *CDKN2D* Expression in *Xiphophorus*

RNA expression characterization

RNA was derived from brain, eye, liver, gill, testes and skin of *X. maculatus* (Jp 163 A), two F_1 hybrids and 8 BC_1 hybrids. An ABI-7700 Real-time PCR machine was utilized to assay RNA expression from *CDKN2D*, *CDKN2AB* (*CDKN2X*) as well as for endogenous controls *GAPDH* and *β-ACTIN*. All primer sets were first tested to establish conditions that generate single, robust amplicons using cDNA. In addition, primer/probe combinations were optimized for conditions leading to linear amplification. Dilutions of plasmids, containing inserts specific to *CDKN2AB* (*CDKN2X*) and *CDKN2D*, were also tested and copy number capability was established. The starting copy number was determined in 10 ng of calibrator RNA by comparing it to a plasmid dilution standard. Once this numerical value was attached to the calibrator, calculation of the relative expression of the experimental samples was done by simply normalizing experimental points to calibrator. To insure accuracy, each assay was repeated in 2 sets of triplicates thus each datapoint represents an averaged value based on 6 experimental points. Taq-Man primers and probes (in parentheses) for each assay are indicated, as follows: *CDKN2D*: TM-P19-ex3Fs/TM-P19-ex4Ra (TM-P19-ex3, 4Ra), *CDKN2AB*: cdkn2xS1/cdkn2xAS3 (maccdkn2x), *GAPDH*: TM-GAPDH-ex2Fs/TM-GAPDH-ex4Ra

(TMPr-GAPDH-ex3, 4Fs) and *β-ACTIN*: TM-actin-ex2Fs/TM-actin-ex3Ra (TMPr-actin-ex3Fs). Sequences are provided in Table 5-1. All samples were also checked for consistency by applying each assay to RNA derived from a *Xiphophorus* cell line termed A2 (KUHN et al. 1979).

Oligonucleotide	Sequence
TM-P19-ex3Fs	tggaggaatgcagagtgcat
TM-P19-ex4Ra	cctggacgttgggctcag
TM-P19-ex3, 4Ra	56FAM-atcatcatcacctgcagcgc-36TAMT
cdkn2xS1	gacgaagagcaatacaggtgatga
cdkn2xAS3	gcttttgtccgtaacgtttgga
maccdkn2x	56FAM-agccgagccacctccgagctg-36TAMT
TM-GAPDH-ex2Fs	ccttcatcgacctgcagtacat
TM-GAPDH-ex4Ra	gacgtatttggcgccagcttt
TMPr-GAPDH-ex3, 4Fs	56FAM-catctctgttttccagtgtatgaagcctgct-36TAMT
TM-actin-ex2Fs	caaagccaacagggagaagatg
TM-actin-ex3Ra	cgataccagtggtacgaccagaa
TMPr-actin-ex3Fs	5HEX-ttgccatccaggccgtgctg-36TAMT

Table 5-1. Sequences of the primers and probes used in Real-Time PCR experiments

Primer optimization was performed by setting up reactions differing in primer concentrations and then testing each using one-step Real-Time PCR methods. This was done by adding both reverse transcription reaction and *Taq* polymerase, and carrying out RT-PCR in the same well. Once the primer concentration was known, the efficiency of

PCR on plasmid DNA and on cellular RNA was checked. This helped to determine starting copy number for the A2 calibrator.

Analyses of RNA expression

Expression studies of *CDKN2D* in *X. maculatus* revealed elevated expression in testes, as has been reported in other vertebrates (THULLBERG et al. 2000). Although expression of *CDKN2D* was also observed in other tissues (brain, eye, liver, skin), the expression levels were less than 15% of that observed in testes. A composite showing the expression levels of *CDKN2D*, *CDKN2X*, *GAPDH* and *β-ACTIN* for the different normal tissue is illustrated in Figure 5-1. In all the tissues, *CDKN2D*, *CDKN2X*, *GAPDH* and *β-ACTIN* show increasing levels of expression with the exception of brain and testes, where *CDKN2D* levels were higher than *CDKN2X*.

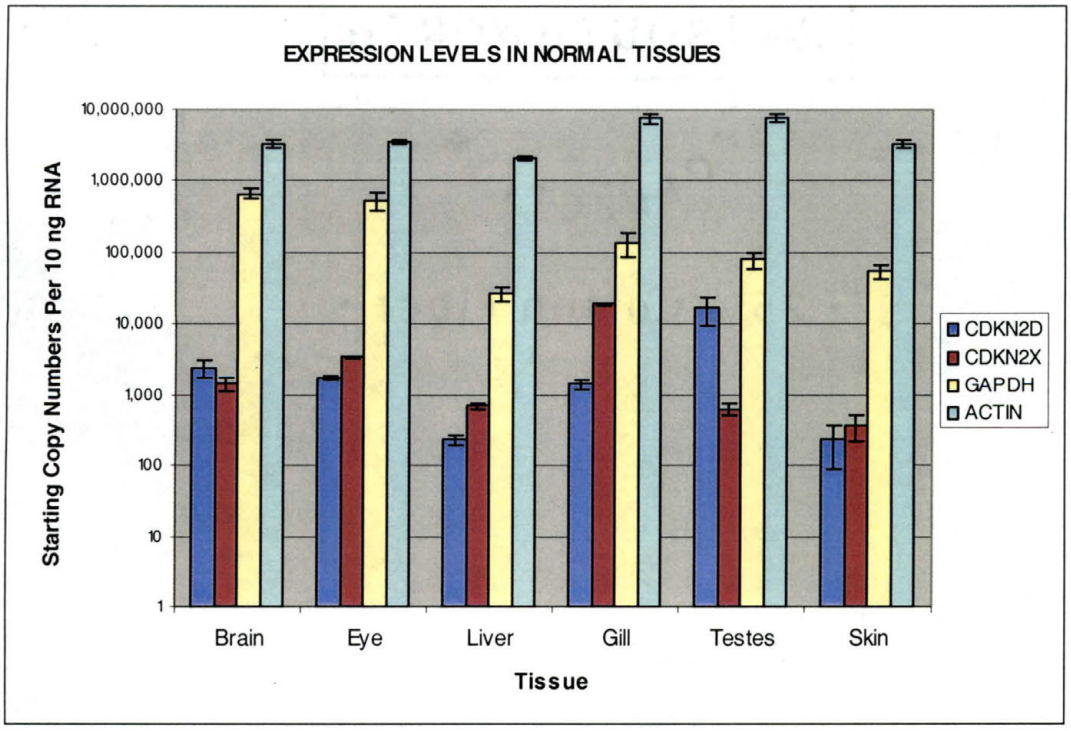


Figure 5-1. Relative expression levels of *CDKN2D*, *CDKN2X*, *GAPDH* and *β-ACTIN* for the different normal tissue in *X. maculatus*.

The normalized expression levels (*GAPDH* = 1) of *CDKN2D* and *CDKN2X* in normal tissues are shown in Figure 5-2.

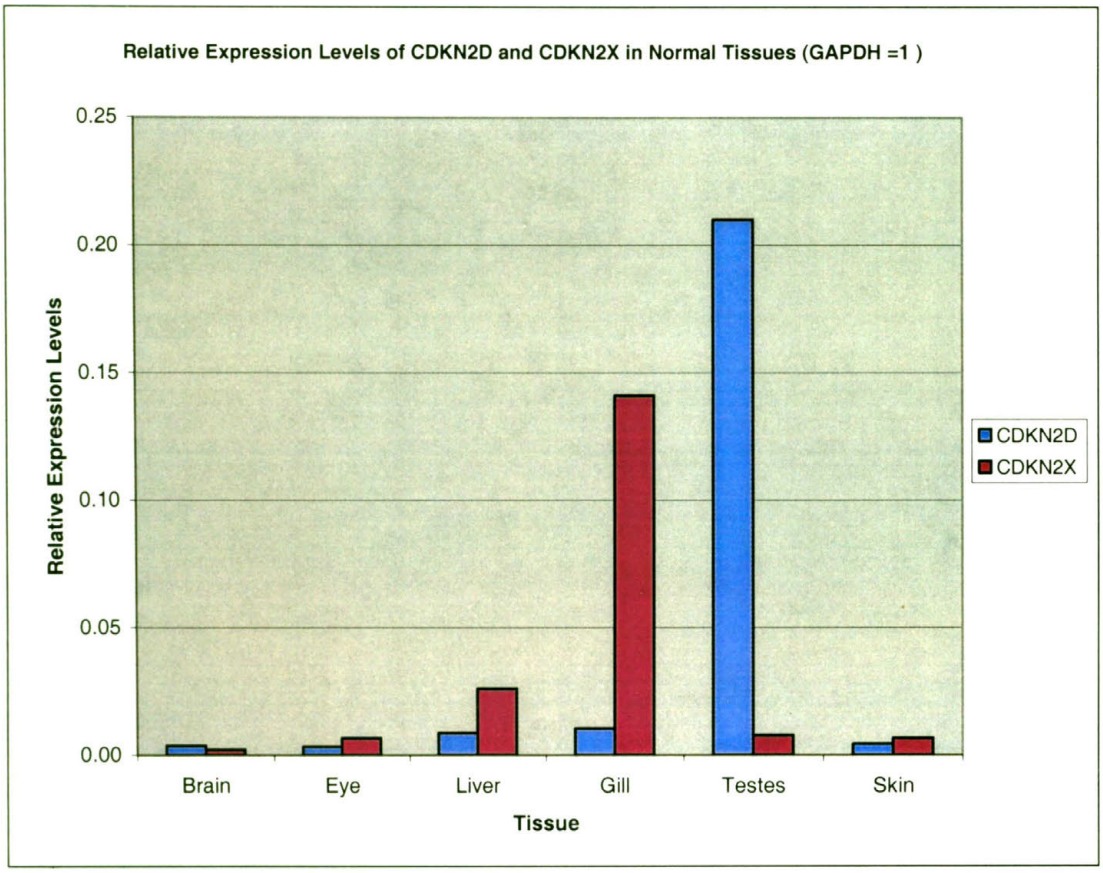


Figure 5-2. Normalized expression levels (*GAPDH* = 1) of *CDKN2D* and *CDKN2X* in tissues samples from *X. maculatus*.

In brain, the *CDKN2D* level is marginally higher than that of *CDKN2X* while in testes, it is higher. Among other tissues studied, the *CDKN2X* level is marginally higher than *CDKN2D* in eye, skin and liver. However, the *CDKN2X* level was higher in gill as illustrated in Figure 5-2.

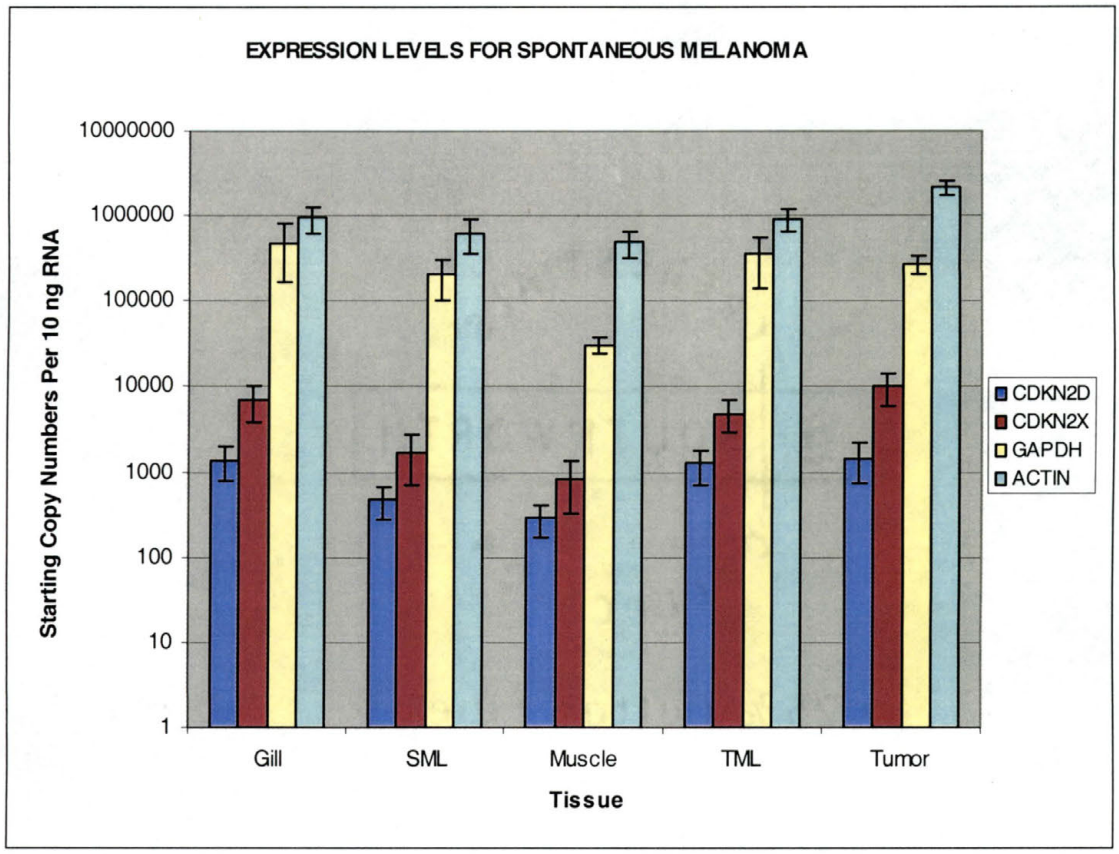


Figure 5-3. Expression levels of *CDKN2D*, *CDKN2X*, *GAPDH* and *β-ACTIN* in different tissues of *Xiphophorus* BC_1 hybrids (Hybrid 1).

In all the melanized tissues, the expression levels of *CDKN2D*, *CDKN2X*, *GAPDH* and *β-ACTIN* show similar relative pattern of expression as shown in Figure 5-3. *CDKN2D* expression was the highest and almost equal in gill, melanized tailfin (TML) and tumor tissues and lowest in melanized skin (SML) and muscle. The *CDKN2X* levels were the highest in tumor and lowest in muscle tissue. *GAPDH* expressed most heavily in gill and the least in muscle while *β-ACTIN* expressed most in tumor tissue and the least in muscle.

The relative expression levels (*GAPDH* = 1) of *CDKN2D* and *CDKN2X* in gill, melanized skin, muscle, melanized tailfin and tumor tissues are shown in Figure 5-4. In all the melanized tissues *CDKN2X* expression is higher than that in *CDKN2D*.

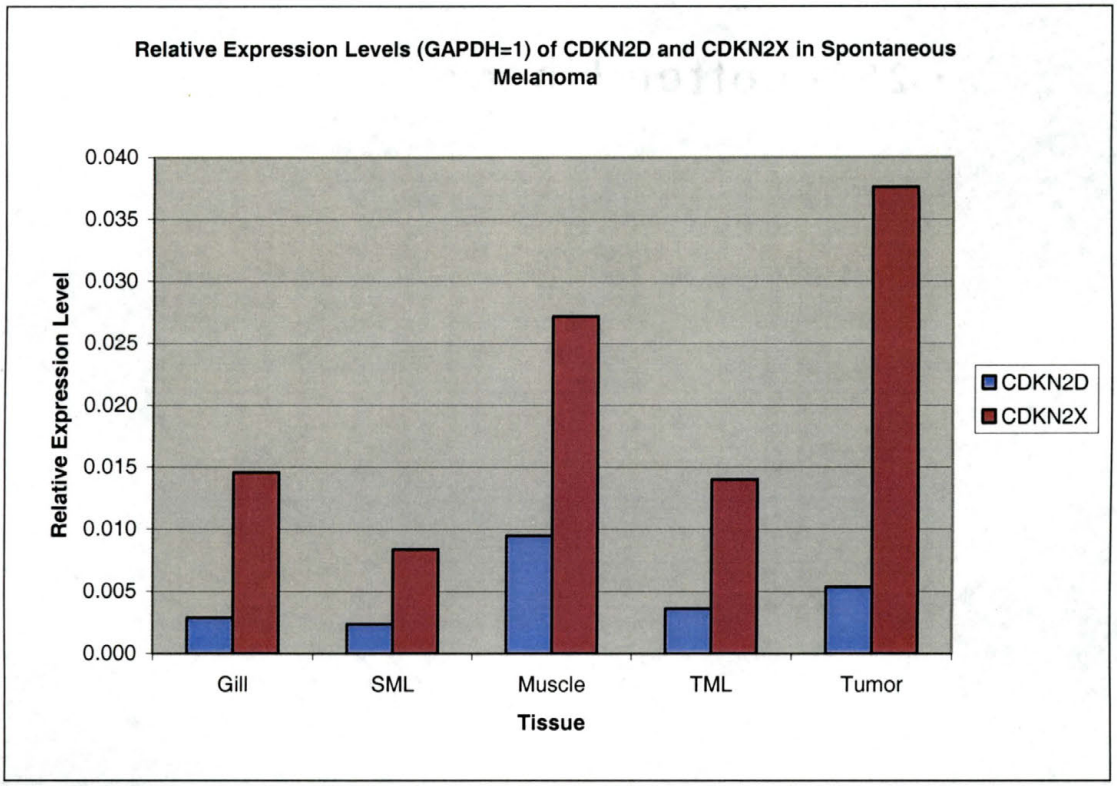


Figure 5-4. Expression levels (*GAPDH* = 1) of *CDKN2D* and *CDKN2X* for different tissues in *Xiphophorus* BC_1 hybrids.

CHAPTER 6

CONCLUSIONS

In this study, an effort was made to characterize the *CDKN2D* gene in *Xiphophorus* and its relationship with the human *CDKN2D*. The *X. maculatus* Jp 163 A *CDKN2D* gene and cDNA were cloned and nucleotide sequence determined. The *Xiphophorus CDKN2D* locus spans 2.6 kb of genomic DNA and is comprised of 4 exons and 3 introns. The start codon was found to be in exon 3. The molecular weight of $p19^{CDKN2D}$ was determined to be 17.7 kD. A 277 bp product was also cloned upstream of exon 1. Complete characterization of the 165 amino acid *X. maculatus CDKN2D* protein revealed 51% identity with human *CDKN2D* and 84% identity with *Fugu*. Linkage analysis showed that the *X. maculatus CDKN2D* gene mapped to *Xiphophorus* LG V.

Analysis of *CDKN2* transcriptional expression in normal fish tissues, namely brain, gill, skin, testes, liver and eye was performed. These data indicate the relative expression of the *X. maculatus CDKN2D* transcript is highest in testes and lowest in eye; while that of *CDKN2X* was found highest in gill and lowest in brain. Use of interspecies backcross hybrids to perform relative RT-PCR indicated low *CDKN2D* expression in melanoma. Comparison of *CDKN2D* expression between non-tumor pigmented skin and highly pigmented melanoma tumor tissue show overall low expression in melanotic tissue in BC_1 hybrids.

It was observed that *Xiphophorus* harbors at least two *CDKN2* loci within LG V, *CDKN2X (CDKN2AB)* and *CDKN2D.* Though the 2 loci are mapped to the same linkage group they show differing RNA-expression patterns in normal tissues. Unlike *CDKN2X*, *CDKN2D* does not show highly elevated expression in melanoma for hybrid fish. *CDKN2X* is homologous to members of mammalian *CDKNA* and *CDKNB*, although orthology cannot be established.

Although previous studies have shown elevated expression levels for *CDKN2X* in spontaneous melanoma in *Xiphophorus*, our investigation has empirically pointed to the existence of higher expression levels of *GAPDH* and *β-ACTIN* in comparison with *CDKN2X.* Verification of this data will provide scope for further studies.

Orthology between *Xiphophorus* and mammalian gene family members can be established for *CDKN2D.* Literature surveys indicated an absence of exhaustive study on this gene family in fish. The data generated by this study reveal that there are at least 2 loci for *CDKN2* genes in fish. Further work can focus on establishing potential homologues of other gene family members in fish, for example *CDKN2C.*

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