# MOLECULAR CHARACTERIZATION AND RELATIVE EXPRESSION

.

OF THE Xiphophorus maculatus APURINIC / APYRIMIDINIC

# ENDONUCLEASE GENE.

# THESIS

# Presented to the Graduate Council of Texas State University – San Marcos In Partial Fulfillment of the Requirements

For the Degree of Master of SCIENCE

By

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# CHAPTER 1 INTRODUCTION

#### AP Endonuclease and the Base Excision Repair (BER) Pathway

Approximately 10,000 spontaneous depurinations are believed to occur daily in a human cell (Grösch et al., 1998). In addition to spontaneous reactions such as hydrolytic depurinations, DNA glycosylases act to remove altered or incorrect bases. If unrepaired the resulting abasic site caused by N-glycosylase activity may inhibit the progress of DNA replication and cause mutations (Demple et al., 1991). The base excision repair (BER) pathway is largely responsible for ameliorating base damages and restoring genetic integrity (Intano et al., 2002). The initiation of short-patch BER (< 1 nucleotide residue) involves two major classes of repair enzymes: DNA glycosylases and apurinic / apyrimidinic (AP) endonucleases. DNA glycosylases are damage specific enzymes that hydrolyze the N-glycosidic bond between a damaged base and the deoxyribose moiety, resulting in the creation of an AP site (Wilson et al., 1996). For example, uracil-DNA-Nglycosylase (UNG) acts to remove misincorporated uracil (G:U mismatch) resulting from a cellular cytosine deamination event (Walter et al., 2001). Upon creation of an abasic site, AP endonuclease hydrolyzes the phosphodiester backbone of DNA 5' to an abasic site generating a 3'-terminal-3'-hydroxyl nucleotide and an abasic 5'-terminal

deoxyribose-5-phosphate (Demple *et al.*, 1991). The abasic 5' deoxyribose-5-phosphate is released by hydrolytic action of 5'-deoxyribophosphodiesterase (dRPase) (Wilson *et al.*, 1996). The exposed 3' hydroxyl serves as an ideal substrate for DNA polymerase  $\beta$ ( $\beta$ -pol) to incorporate a single dNMP residue at the abasic site (Srivastava *et al*, 1999), and the phosphate backbone is sealed by DNA ligase I. The sequential actions of these enzymes serve to repair the genomic DNA to its original integrity.

#### AP Endonuclease – Genomic Structure

Akiyama *et al.* (1992) cloned a human AP endonuclease (HAP1) gene and showed that its mRNA has a total length of 2572 bp from the transcription start site to the polyadenylation (poly-A) sequence. The gene consists of five exons and four introns (Figure 1-1), and codes for 318 amino acids (Figure 1-2). Figure 1-3 shows the structural motifs associated with various regions of the amino acid sequence, and Figure 1-4 represents the 3-dimensional orientation of AP endonuclease bound to abasic DNA. The



**Figure 1-1**. Gene structure of human AP endonuclease. Grey areas represent coding region. Black areas represent 5' and 3' untranslated regions.

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490 500 510 520 530 5 GAATTIGACTCGITTGIGCIGGIAACAGCATAIGIACCTAAIGCAGGCCGAGGICIGG			K 37 PCG S 43 ICT S	L GAC. D O TACC Y	F AAC K GG(	A TRAN 38 GGAAC E TRAN Intro 44 TATAG I	E ISLA G G ISLA n4 GCG G G G G G I	L Q TION ACAG Y S IIION AGGA( E E	E OF 39( TGGC OF 0F 45( GGAC E	L HUM ) CGTG V HUM ) (CAT H	GGC G AII GA1 D	CDS 400 CTG0 L CDS 46: CAG0 Q	TA CTT L [A] SAAC E	] ICO S ] GGC( G	H CGC P. 4 CGG R	10 CAG Q 70 GTG V	TGC C ATT	W CCA P GTG	420 CTC L> 480 GCT A>
GAATTIGACTCGTTTGTGCTGGTAACAGCATATGTACCTAATGCAGGCCGAGGTCTGG		N DCT P STT	K 37 ICG S 43 ICT S	L GAC D 0 TACC Y	F AAAC K G G	A _TRAN 38 GGAAC E _TRAN Intro 44 CAT2G I I	E ISLA G G ISLA <b>n4</b> G GCG G ISLA	L Q TION ACAG Y S IIION AGGAG E E FION	OF 39( TGG( OF 450 GGA( E OF	L HUM ) CGTG V HUM ) CAT HUM	GGC G AII GAI D AII	CDS 400 CTGC L CDS 46: CAGC Q CDS	(A) (A) (A) (A) (A) (A)		н СGC Р. 4 СGC 8 4	10 CAG Q 70 GTG V	TGC C ATT	W CCA P GTG V	420 CTC L> 480 GCT A>
REDGEVILLUTZVUGUUUUUU	E E SCTC A LAAC K	N CCT P GTT	K 37 ICG S 43 ICT S	L GAC D TACC Y	F AAAC K GGC G	A TRAN 38 GGAAC E TRAN Intro 44 ATZC I TRAN	E ISLA G G ISLA N G G G ISLA N 0	L Q TION ACAG Y S IIION AGGAG E E FION	OF 39( TGG( OF 45( GGAG E OF	L HUM CGTG V HUM HUM HUM	GGC G AII GAI D AII	CDS 400 CTGC CDS 46: CAGC Q CDS	TTT L [A] SAAC E [A]		H CGC P. 4 <sup>1</sup> CGG R	10 CAG Q 70 GTG V	TGC C ATT	W CCA P GTG V	420 CTC L> 480 GCT A>
TT TO T A T A T A T A T A T A T A A A A	E E E E E E E E E E E E E E E E E E E	N CCTY P STITY V	K 377 CCG S 433 ICT S 49 GAC	L GAC, D O TACC Y O TACC Y	F AAAC K GGC G	A _TRAN 38 GGAAC E _TRAN Intro 44 YATAG I _TRAN 50 GTGC	E ISLA G G ISLA ISLA G G G S S LA ISLA ISLA ISLA ISLA ISLA ISLA IS	L Q FION ACAG' Y S IION AGGAGE E FION	E OF 39( TGGC G OF 45( GGAC E OF 51( AGCA	L HUM ) CGTG V HUM ) KCAT H HUM	AN GGC G AN GAI D AN	CDS 400 CTG0 CDS 46: CQ CDS 520 CCTS	TA TA CTT L (A) GAAC E [A]		H CGC P. 4 CGG R 5 SGC	10 CAG Q 70 GTG V 30 CGA		W CCA P GTG V	420 CTC L> 480 GCT A> 540 GTA
TRANSLATION OF HUMAN CDS [A]	E E CTC A AAAAAAAAAAAAAAAAAAAAAAAAAAAAAA	N CCT P JTT V V	K 37 ICG S 43 ICT S 49 GAC D	L GAC. D TACC Y O TACC S	F AAAC K G G ITTI F	A _TRAN 38 GGAAC E _TRAN Intro 44 YAT2C I _TRAN 50 YGTGC V	E : ISLA G : ISLA G : ISLA G I ISLA G I ISLA C I ISLA C I ISLA C I I I C I I C I I I C I I I C I I I C I I I I	L Q FION ACAG Y S IION AGGAG E E FION FAAC.	E OF 39( TGGC OF 45( 3GAC E 0F 51( AGCA A	L MUH CGTG C C MUH HUM HUH HUM Y	GGC G AN GAI D AN GTA V	CDS 400 CTG0 CDS 46: CAG0 Q CDS 520 CCTS 520 CCTS	A CTTY [A] [A] SAA( E [A] ) MAT( N	ICC S G G G G G G G G G G G G G G G G G G	H 4 CGCC R 4 CGGG R 5 SGCC G	10 CAG Q 70 GTG GTG 30 CGA R	TGC C ATT I GGT	W CCA P GTG V CTG L	5> 4200 CTC L> 4800 GCT A> 5400 GTA V>

**Figure 1.2**. Nucleotide sequence of the coding region of human AP endonuclease. Amino acid sequence is listed under each codon, and intron positions within the coding region are labeled.

560 570 580 590 550 600 CGACTGGAGTACCGGCAGCGCTGGGATGAAGCCTTTVCGCAAGTTVCTGAAGGGCCTGGCT RLEYRQRWDEAFRKFLKGLA> TRANSLATION OF HUMAN CDS [A] > 610 620 63C 640 650 660 TCCCGAAAGCCCCTTGTGCTGTGGGAGACCTCAATGTGGCACATGAAGAAATTGACCTT S R K P L V L C G D L N V A H E E I D L> TRANSLATION OF HUMAN CDS [A]\_\_\_\_\_ 670 680 690 700 710 720 CGCAACCCCAAGGGGAACAAAAAGAATGCTCGCTPCACGCCACAAGAGCGCCAAGGCTTC RNPKGNKKNAGFTPQERQGF> \_\_\_\_\_TRANSLATION OF HUMAN CDS [A]\_\_\_\_\_> 730 750 760 770 780 74C GGGGAATTACTGCAGGCTGTGCCACTGGCTGACAGCTTTAGGCACCTCTACCCCAACACA GELLOAVPLADSFRHLYPNT> TRANSLATION OF HUMAN CDS [A] 790 800 810 820 830 840 PYAYTFW TYMMNARSKN VGW> TRANSLATION OF HUMAN CDS [A]\_\_\_\_\_ 850 860 870 880 890 900 CGCCTTGATTACTTTTTGTTGTCCCACTCTCTGTTACCTGCATTGTGTGACAGCAAGATC RLDYFLLSHSLLPALCDSKI> \_\_\_\_\_\_TRANSLATION OF HUMAN CDS [A]\_\_\_\_\_> 910 920 930 940 CGTTCCAAGGCCCTCGGCAGTGATCACTGTCCTATCACCCTATACCTAG R S K A L G S D H C P I T L Y L X> \_\_\_\_\_TRANSLATION OF HUMAN CDS [A]\_\_\_\_\_>

Figure 1-2. Continued.

5' untranslated region (UTR) consists of exon I (264 base pairs; bp), intron I (182 bp) and the first 67 bp of exon II. Exons II – V contain the mRNA coding regions (Figure 1-2) of HAP1. Exon II contains 120 bp of coding region, including the translation initiation (ATG) codon. Exon III (188 bp) and exon IV (193 bp) are separated by introns II (210 bp) and III (566 bp) respectively. Exon V is 776 bp in length and contains 54% of the



**Figure 1-3**. Structural motifs of human AP endonuclease (amino acids 44 - 318) as determined by X-ray crystallography (Beernink *et al.*, 2001). Located within the protein are 5  $\beta$  hairpins, 10  $\alpha$  helices, 33  $\beta$  turns, 2  $\gamma$  turns, and 12 strands. The amino acid sequence is provided and the various structural motifs associated with various regions of the protein are shown above the sequence.

human AP endonuclease coding region and the entire 3' non-coding region. With the exception of exon V the coding exons are relatively short (around 200 bp or smaller). This may occur in order to maintain gene stability by limiting the probability of gene arrangements resulting from recombination events (Robson *et al.*, 1992). Robson *et al.* (1992) performed *in situ* hybridization using a biotin-labeled HAP1 probe to localize the gene location to human chromosome 14q 11.2-12.

#### **AP Endonuclease – Function**

It is estimated that tens of thousands of AP sites are produced per cell per day, primarily by spontaneous base hydrolysis and cellular metabolites produced with oxygen radicals (Lindahl, 1993). If left unrepaired theses AP sites may lead to mutations and genetic instability (Loeb and Preston, 1986). AP endonucleases act to cleave the AP site producing a suitable substrate for the incorporation of an undamaged nucleotide by DNA polymerase  $\beta$  (pol- $\beta$ ). AP endonuclease is electrostatically oriented to the DNA by divalent Mg<sup>2+</sup> and penetrates the DNA helix from both the major and minor grooves, stabilizing an extrahelical conformation for target abasic nucleotides and excluding normal DNA nucleotides (Mol *et al*, 2000). As shown in Figure 1-5, AP endonuclease may cleave at a number of locations adjacent to the abasic site. Hydrolytic cleavage at site A (major cleavage site) yields a 5'-terminal residue of deoxyribose 5-phosphate and a 3'-terminal residue of deoxyadenosine. Though the 3' hydroxyl is a suitable substrate for polymerase initiated repair, the deoxyribose 5-phosphate cannot be removed DNA pol- $\beta$ . In order to generate the necessary one-nucleotide gap, deoxyribophosphodiesterase (dRpase) cleaves



**Figure 1-4**. 3-dimensional ribbon diagram of AP endonuclease protein as determined by X-ray crystallography (Mol et al., 2000). The protein is oriented adjacent to an abasic site in the DNA by a magnesium (Mg<sup>2+)</sup> cofactor. The light blue regions represent  $\alpha$ -helices, red arrows represent strands, yellow regions represent hairpin turns, green regions represent beta turns, and the green sphere represents the Mg<sup>2+</sup>.



**Figure 1-5**. Possible cleavage sites of AP endonuclease. Product represents cleavage at site A, resulting in a 3' deoxyadenosine and 5' deoxyribose-5-phosphate.

3' to the 5'-terminal deoxyribose. This yields a suitable one-gap substrate for DNA pol- $\beta$ . Cleavage at site C occurs via a  $\beta$ -elimination mechanism to yield an unsaturated aldehyde at the 3'-terminus and a 5'-terminal residue of deoxythymidine (N) 5'-phosphate (in this example). This product is not a suitable substrate for DNA pol- $\beta$ , and processing of this lesion requires diesterase activity to cleave 5' to the base free unsaturated aldehyde and generate the proper polymerase substrate. According to Winters et al. (1994), human AP endonuclease performs this function. Cleavage at sites B and D (Figure 1-5) represent a small percent of DNA cleavages. As with cleavage at site C, the products formed by cleavage at these sites must be further processed before serving as a suitable substrate for DNA pol- $\beta$  (Doetsch and Cunningham, 1990). In addition to the repair activity, AP endonuclease has a dual function in mammalian cells. According to Grösch *et al.* (1998) AP endonuclease regulates the redox state of various transcription factors, such as activator protein-1 and nuclear factor- $\kappa\beta$ . For example, the reduction of activator protein-1 results in enhanced promoter binding activity. Additionally, AP endonuclease has been shown to be involved in calcium mediated transcriptional repression of the parathyroid hormone gene (Wilson *et al.*, 1996). AP endonuclease can directly regulate genes by binding to negative calcium response elements that are present in the promoter region of the parathyroid hormone gene (Grösch *et al.*, 1998). Upon increase of intracellular calcium levels, the activity of this gene becomes down-regulated due to the binding of the AP endonuclease protein to the corresponding regulatory region.

#### The Xiphophorus Fish Model System

For many years fish have been used as experimental models of human disease. Attributes of fish which make them ideal for biomedical studies include: ease of producing large numbers, accessibility to inbred stocks and standard strains, the capacity to create genetic crosses using phenotypically diverse species, and genetic and biochemical processes similar to rodents and humans (Walter *et al.*, 2001). In particular, *Xiphophorus* species have been used over 70 years for cancer research in order to determine the genetics underlying vulnerability to tumor development (Kazianis *et al.*, 2001; Schwab *et al.*, 1979; Walter *et al.*, 2001; Walter and Kazianis, 2001). Interspecies *Xiphophorus* hybrids provide genetically controlled models of tumor formation and offer the potential to analyze the contributions of specific genes to spontaneous and induced tumor development in different, but comparable genetic backgrounds (Nairn *et al.*; 2001). Figure 1-7 represents the most extensively studied *Xiphophorus* hybrid model. In this cross the *X. maculatus* (southern platyfish) parent is pigmented and exhibits the spotted dorsal (*Sd*) pigment pattern. The *X. helleri* (green swordtail) parent is non-pigmented (+). Mating of these two parents results in an  $F_1$  hybrid exhibiting the enhanced *Sd* pattern. Generally, the parental species and F1 hybrids derived from mating them are not susceptible to tumorigenic induction. However,



**Figure 1-6**. The classical Gordon-Kosswig (hybrid 5) cross. The loci derived from *X*. *maculatus* are colored red, while the loci derived from the swordtail *X*. *helleri* are colored blue. Nodular melanoma typically occurs in animals showing the phenotypes similar to the leftmost backcross hybrid (lower left). These fish have inherited the *Xmrk-2* oncogene, but not the *DIFF* tumor suppressor from the *X*. *maculatus* parent.

backcrossing the F<sub>1</sub> hybrid with the *X. helleri* parent results in dramatic enhancement of the *Sd* pattern in half of the pigmented backcross hybrids (25% of the total progeny).
These heavily pigmented progeny develop malignant melanomas from macromelanophore pigment cells originating from the *Sd* pigment pattern in the dorsal fin (Anders, 1991;Nairn et al., 1991; Walter et al., 2001; Walter and Kazianis, 2001).
Genetic analysis coupled with functional and descriptive biochemical studies will help assess the role of BER genes, in particular AP endonuclease, in fish melanomagenesis, and contribute to an overall understanding of melanoma development in vertebrates (Nairn et al.; 2001).

The goal of the project described herein is to: 1) clone and sequence the AP endonuclease gene in *Xiphophorus maculatus*, 2) map the *Xiphophorus* AP endonuclease locus, and 3) measure the transcriptional expression of AP endonuclease in *X. maculatus*. All materials and methods used are described and the results from these techniques are presented and discussed in the following chapters.

# **CHAPTER 2**

# **MATERIALS & METHODS**

#### Fish Stocks

*Xiphophorus maculatus* (Jp 163 A) were provided by the *Xiphophorus* Genetic Stock Center, Department of Chemistry and Biochemistry, Texas State University, San Marcos, TX. Genomic DNA, mRNA and cDNA were used for characterization and relative quantitation of transcriptional expression of the AP Endonuclease gene in *X. maculatus* Jp 163 A.

#### **RNA Isolation**

To isolate total RNA from the healthy tissues of *X. maculatus* Jp 163 A (Figure 2-1), the fish were anaesthetized in an ice water bath. Using sterile dissection instruments, the desired tissues (brain, eye, liver, gill, testes, skin and muscle) were removed from the fish and placed into individual homogenizing pestles standing in a dry ice/ethanol bath or placed in RNA Later<sup>TM</sup> (Ambion; Austin, TX). Each tissue was homogenized in 1 ml TRI REAGENT<sup>TM</sup> / 100 mg of tissue. The homogenized samples incubated at 25°C for 5 min. to ensure complete dissociation of nucleoprotein complexes. To the homogenate 0.2 ml of chloroform was added per ml of TRI REAGENT<sup>TM</sup>. The samples were capped and shaken vigorously for 15 sec., then allowed to stand at room temperature (Rt) for 15 min. This suspension was then centrifuged at 12,000 x g for 15 min. at 4°C (Beckman J2-21 Centrifuge; Fullerton, CA). The aqueous phase (containing RNA) was separated from



**Figure 2-1**. Picture of *X. maculatus* (Jp 163 A). Genomic DNA and mRNA (isolated from the brain, eyes, liver, gill, testes, skin and muscle) were used for characterization and relative, quantitative transcriptional expression of AP endonuclease.

the organic phase (containing protein) and interphase (containing DNA). To the aqueous phase, 0.5 ml of isopropanol per ml of TRI REAGENT<sup> $^{\text{M}}$ </sup> was added and the suspension allowed to incubate at Rt for 10 min. After centrifugation at 12,000 x g for 10 min. at 4°C the RNA precipitate was pelleted in the bottom of the tube. The supernatant was decanted and the RNA pellet was washed by adding 1 ml of 75% ethanol. After vortexing, the sample was centrifuged at 7500 x g for 5 min. at 4°C and the supernatant was decanted. The resulting RNA pellet was air-dried for 10 min. at Rt and then resulting 60 µl nuclease-free water (double distilled water (ddH<sub>2</sub>O) treated with 1%

diethyl-pyrocarbonate (DEPC)). The solution was allowed to incubate for 15 min. in a  $55^{\circ}$ C water bath during resuspension. Contaminating DNA was removed by adding 6.7 µl 10x DNAse buffer and 1 µl DNAse I enzyme (Ambion; Austin, TX). This reaction was allowed to incubate at 37°C for 1 hour, with occasional shaking. To each sample 1/10 volume (6.7 µl) DNAse Inactivating Reagent was added (Ambion; Austin TX) and then mixed gently and centrifuged at 10,000 x g for 1 min. at Rt. The aqueous layer (containing RNA) of each sample was removed, placed in a fresh tube and stored at - 80°C until use.

#### **DNA Isolation**

DNA from fish of interest was isolated from select tissues (brain, eye, liver, gill, testes, skin and muscle) using the PUREGENE<sup>®</sup> DNA Purification Kit (Gentra Systems; Minneapolis, MN). Tissues were dissected from fish and placed in individual 1.5 ml microcentrifuge tubes containing 600  $\mu$ l Cell Lysis Solution (proprietary, kit included) and incubated on ice for 30 min. then these samples were homogenized using sterilized microcentrifuge homogenizing pestles. The homogenized samples were incubated at 65°C for 60 min. then 6  $\mu$ l (20 mg/ml) Proteinase K solution was added and allowed to incubate overnight at 55°C. After overnight incubation 3  $\mu$ l *E. coli* RNAse A (2 units/ $\mu$ l) was added to each sample followed by 37°C incubation for 60 min. The samples were then cooled at 4 °C for 30 min. and 200  $\mu$ l Protein Precipitation Solution added followed by vortexing at high speed for 40 sec. The suspensions were centrifuged at 13,000 x g for 15 min. to precipitate the protein. The supernatant (containing DNA) was decanted

into a fresh 1.5 ml microcentrifuge tube containing 600  $\mu$ l 100% isopropanol, mixed by gentle inversion and the white threads of DNA pelleted by centrifugation (13,000 x g for 3 min.). The supernatant was removed and the DNA washed with 600  $\mu$ l 70% ethanol. Each sample was centrifuged at 10,000 x g for 1 min. and the supernatant removed. The DNA pellet was air-dried at 37°C for 15 min, then 200  $\mu$ l TE (Tris-Cl, 0.5M EDTA, pH 8.0) was added and the RNA resuspended at 55°C for 1 hour. Each tube was gently mixed periodically to aid in resuspension of the DNA. DNA samples were stored at 20°C until use.

#### **Nucleic Acid Quantitation**

Prior to using nucleic acids, total RNA/DNA concentrations were determined using the RiboGreen (RNA) and PicoGreen (DNA) nucleic acid quantitation kits (Molecular Probes; Eugene, OR). These methods utilize fluorescent probes specific for the nucleic acid of choice. Samples were compared to standard curves constructed using  $\lambda$  phage DNA (100 µg/ml) or *E. coli* 16s and 23s rRNA (100 µg/ml). A *FL<sub>x</sub>800* 96-well fluorescence microplate reader (Bio-Tek Instruments; Winooski, Vt) was used with KC4 analysis software to detect fluorescence and quantitate (ng/µl) the nucleic acids.

#### Polymerase Chain Reaction

Polymerase chain reaction (PCR) was performed using two sequence-specific, directional oligonucleotide primers and a heat stable DNA polymerase to amplify a specific DNA target sequence. *Xiphophorus maculatus* nucleotide sequence was used to design individual primers used in each reaction. These primers flank a desired target within the sequence of the AP endonuclease gene. PCR reactions contain 5.0 µl 10 x Buffer (100mM Tris-HCl, pH 8.3; 500 mM KCl; 15 mM MgCl<sub>2</sub>, Invitrogen; Carlsbad, CA), 25 mM MgCl<sub>2</sub>, 6.25 µM dNTP's, 20 mM of each primer (Integrated DNA Technologies; Coralville, IA), 2.5 units *Taq* DNA polymerase (Invitrogen; Carlsbad, CA), 20-500 ng genomic DNA, and ddH<sub>2</sub>0 to a volume of 50 µl (Table 2-1). Each reaction was placed in a 2.0 mL thin-walled PCR tube and PCR performed using the Perkin Elmer GeneAmp PCR system 2400 (PE Applied Biosystems; Foster City, CA). 35 to 40 successive PCR cycles of denaturation (95°C, 15 sec.), annealing (60°C, 15 sec.), and

PCR Component	Quantity / Concentration
DNA	20 – 500 ng
10 x Buffer	5 μl
MgCl <sub>2</sub>	25 mM
dNTP	6.25 mM
Primers	20 µM each primer
Taq polymerase	2.5 units
ddH <sub>2</sub> O	To a final volume of 50 µL

**Table 2-1**. Components of a typical PCR mixture.

extension (72°C, 15 sec.) were performed for amplification of the target sequence. Upon completion of PCR, each reaction was visualized by running the PCR products on 2% agarose gels in 1 x TAE (tris-acetate EDTA) buffer (1 mM Tris-Acetate, pH 7.0; 0.1 mM EDTA) with ethidium bromide.

#### **Reverse-Transcription Reaction**

The RETROscript<sup>™</sup> first strand synthesis kit for RT-PCR (Ambion; Austin, TX) was used to produce DNA sequences complementary to mRNA templates. These complementary DNA (cDNA) sequences were used as templates for amplification in PCR, gene expression studies and to generate inserts for cloning into plasmid vectors. The following were added to a 2 ml thin-walled PCR tube: 1-2 µg total Xiphophorus RNA, 4  $\mu$ l of dNTP mix (2.3 mM each dNTP), 2  $\mu$ l first-strand primers (50  $\mu$ M random decamers or 50 µM oligo dT primers), and DEPC nuclease-free water to a final volume of 16 µl. The tube was capped, mixed briefly, and incubated at 75°C for 3 min. After 3 min., the reaction was briefly centrifuged, then placed on ice. Once on ice, the following components were added: 10 µl 10x PCR Buffer (100mM Tris-HCl, pH 8.3; 500 mM KCl; 15 mM MgCl<sub>2</sub>), 1 µl RNAse Inhibitor (10 units/µl), 5 µl PCR primers (mixture with 5 µM of each primer), 1 µl M-MLV Reverse Transcriptase, and 1 µl Taq DNA polymerase. The capped tube was briefly mixed and incubated at 42°C for one hour. The reverse-transcription reaction was terminated by heat inactivation at 92°C for 10 min., and the products stored at -20°C until use

#### 5' Rapid Amplification of cDNA Ends (RACE)

5' RACE is a technique that facilitates the isolation and characterization of 5' ends from low-copy messages. The 5' RACE System, Version 2.0 (Invitrogen; Carlsbad, CA) was used to clone and characterize the 5' end of the X. maculatus AP endonuclease. Figure 2-2 provides a basic overview of the 5' RACE procedure. First strand synthesis was primed using antisense sequence-specific oligonucleotide primers. This was done by adding 2 µg RNA, 20 nM primer (AP1,R2 from Figure 3-1) and ddH<sub>2</sub>O to a final volume of 12  $\mu$ l in a thin walled PCR tube. This solution was incubated at 75°C for 3 min., then cooled on ice for 1 min. The following components were added to this mixture: 10 mM dNTPs, 20 mM MgCl<sub>2</sub>, 2 µl 10 x PCR buffer (200 mM Tris-HCl [pH 8.4], 500 mM KCl), 0.1 M DTT, 1 µl SuperScript<sup>™</sup> II reverse transcriptase, and ddH<sub>2</sub>O to a final volume of 20 µl. This mixture was incubated at 42°C for 1 hour followed by incubation at 94°C for 10 min. This reaction was designed to convert target mRNA molecules (from brain and testes) to cDNA. Addition of 1  $\mu$ l E. coli RNAse Mix (2 units/ $\mu$ l) to this mixture was used to degrade the RNA present. Newly synthesized cDNA was purified from unincorporated dNTPs, primers or proteins by adding 120 µl binding solution (6 M NaI) and centrifugation (13,000 x g for 20 sec) in a GlassMax DNA isolation spin cartridge (placed in a fresh tube). The eluent was discarded and 0.4 ml ice cold 1x wash buffer (400 mM NaCl) was added to the spin cartridge. This was centrifuged at 13,000 x g for 20 sec. and the eluent was discarded. This washing step was repeated three times, and then the spin cartridge was washed twice with 400 µl cold 70% ethanol. After removing the final 70% ethanol wash from the tube the cartridge was centrifuged at  $13,000 \times g$  for

1 min. The spin cartridge was transferred to a fresh tube, and 50  $\mu$ l ddH<sub>2</sub>O (65°C) was added. This was centrifuged at 13,000 x g for 20 sec. to elute the cDNA. Terminal transferase was used to add homopolymeric tails (dC) by adding 5  $\mu$ l 5 x tailing buffer (50 mM Tris-HCl [pH8.4], 125 mM KCl, 7.5 mM MgCl<sub>2</sub>), 2 mM dCTP, 10  $\mu$ l purified cDNA sample, and



**Figure 2-2**. Overview of the 5' RACE procedure. Primer AP1, R2 anneals to the target mRNA in order to synthesize cDNA. RNAse degrades the mRNA template, the cDNA was purified and isolated, and terminal transferase adds a poly-C tail to the 3' end of the cDNA. PCR was performed using an Abridged Anchor Primer to synthesize dsDNA. The dsDNA was reamplified using a Universal Adapter Primer and AP1, R2.

DEPC-treated water to a final volume of 24  $\mu$ l. This mixture was incubated for 3 min. at 94°C, and chilled on ice for 1 min. Terminal deoxynucleotidyl transferase (TdT, 15 units/ $\mu$ l) was added and this mixture was incubated for 10 min. at 37°C. The TdT was inactivated by incubation at 65°C. PCR of this dC-tailed cDNA product was achieved by adding 8  $\mu$ l 10x PCR buffer, 20 mM MgCl<sub>2</sub>, 10 mM dNTP, 10  $\mu$ M universal abridged primer (UAP, 5'-CUACUACUACUAGGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG – 3'), 20  $\mu$ M primer (AP1,R1), 0.5  $\mu$ l *Taq* DNA polymerase (5 units/ $\mu$ l) and ddH<sub>2</sub>O to a final volume of 45  $\mu$ l. This reaction was amplified according to the conditions detailed in Table 2-3.

Temperature	Time	
94°C	1 min.	
94°C	15 sec.	
55°C	30 sec.	35 cycles
72°C	1 min.	
72°C	7 min.	

**Table 2-2**. 5' RACE and 3' RACE temperature profile. (Exception: initial incubation period for 3' RACE is at 94°C for 3 min.)

#### 3' Rapid Amplification of cDNA Ends (RACE)

3' Rapid Amplification of cDNA Ends (RACE) is a technique that facilitates the isolation and characterization of 3' ends from low-copy messages. The 3' RACE System (Invitrogen; Carlsbad, CA) was used to characterize the 3' end of AP endonuclease in X. maculatus. Figure 2-3 provides a basic overview of the 3' RACE procedure. Since the 3' RACE system uses the poly-A tail region of mRNA as an initial priming site, amplification products may be synthesized. First strand synthesis was achieved by combining 2 µg RNA (from brain and testes) and DEPC-treated water to a final volume of 11 µl in a clean microcentrifuge tube. One µl of the Adapter Primer (AP) solution (10  $\mu$ M) was added to this mixture and the tube was gently mixed. The mixture was incubated at 70°C for 10 min. followed by chilling on ice. To this mixture the following components were added: 2 µl 10 x PCR buffer, 2 µl (25 mM) MgCl<sub>2</sub>, 1 µl (10 mM) dNTP mix and 2  $\mu$ l (0.1 M) DTT. This solution was gently mixed and equilibrated to 42°C. One µl SuperScript<sup>™</sup> II reverse transcriptase was added and this mixture was incubated at 42°C for 50 min. followed by incubation at 70°C for 15 min. (reaction termination). The mixture was placed on ice and 1 µl E. coli RNAse H (2 units/µl) was added. This mixture was incubated at 37°C for 20 min. followed by chilling on ice. PCR of the cDNA was performed by adding the following components to a fresh tube: 5 µl 10 x PCR buffer, 3µl (25mM) MgCl<sub>2</sub>, 1 µl (10 mM) dNTP mix, 1 µl (10µM) primer AP1,F1 (see Table 3-1), 1 µl (10 µM) UAP, 0.5 µl (5 units/µl) Taq polymerase, 2 µl from the cDNA synthesis reaction, and DEPC H<sub>2</sub>O to a final volume of 50 µl. This mixture was

cycled according to the conditions in Table 2-3. The PCR products were then cloned into *E. coli* and sequenced.



**Figure 2-3**. Overview of 3' RACE procedure. A poly-T Adapter Primer binds to the poly-A tail of the mRNA and synthesizes cDNA. RNAse H degrades the RNA template and a Universal Adapter Primer (UAP) and primer AP1, F1 were used to PCR amplify the cDNA.

#### **Agarose Gel Purification**

In some cases DNA amplified by PCR was isolated after electrophoresis in agarose gels. DNA was purified from agarose gel using the S.N.A.P.<sup>™</sup> Gel Purification Kit (Invitrogen; Carlsbad, CA). Using a sterile glass slides, the DNA band was excised from the agarose gel. The excised gel plug was added to a fresh 1.5 ml tube and weighed. 2.5 X the gel plug volume of 6.6 M sodium iodide was added and the tube mixed by vortexing. The gel plug sample was incubated at 50°C until the agarose was completely dissolved. The tube was placed at Rt and 1.5 volumes of Binding Buffer (7 M guanidine HCl) added, mixed, and allowed to incubate for 1 min. The mixture was loaded onto a S.N.A.P.<sup>™</sup> purification column placed inside a collection vial and centrifuged at 3000 x g for 30 sec. at Rt. The flow-through was loaded back onto the purification vial and centrifuged (3000 x g) at Rt. This loading/centrifugation step was repeated one more time, where upon the flow-through was discarded. 400  $\mu$ l of 1x Final Wash (400 mM NaCl) was added to the column, then centrifuged (3,000 x g) for 30 sec. at Rt, and this wash was repeated once more. The eluent was decanted and the column was centrifuged at 13,000 x g for 1 min. to dry the column resin. The column was transferred to a clean 1.5 ml microcentrifuge tube and 50 µl of hot (65°C) sterile water was added directly to the column followed by 1 min. incubation at Rt. The column/tube was centrifuged (13,000 x g) for 1 min. at Rt to elute the DNA from the column into the microcentrifuge tube. The DNA was stored at -20°C until further analysis.

#### **PCR-Script Subcloning of DNA Fragments**

The TOPO-TA Cloning<sup>®</sup> (Version L) kit (Invitrogen; Carlsbad, CA) was used to directly insert Tag polymerase-amplified PCR products into the pCR<sup>®</sup>2.1-TOPO<sup>®</sup> plasmid vector. Provided was linearized plasmid pCR<sup>®</sup>2.1-TOPO<sup>®</sup> vector with Topoisomerase I covalently bound to the vector ends. Topoisomerase (from Vaccinia virus) binds to dsDNA and cleaves the phosphodiester backbone after 5'-CCCTT, resulting in a single 3' deoxythymidine (T) overhang (Figure 2-4). Due to nontemplatedependent terminal transferase activity of *Taq* polymerase, a single deoxyadenosine (A) is often added to the 3' ends of PCR products. This single 3' deoxyadenosine overhang (PCR products) ligates efficiently with the single 3' deoxythymidine overhang of the vector in the presence of topoisomerase. The following TOPO cloning reaction components were added to a microcentrifuge tube: 4 µL PCR product (isolated from agarose gel), 1 µl salt solution (1.2 M NaCl, 0.06 M MgCl<sub>2</sub>), and 1 µl pCR<sup>®</sup>2.1-TOPO<sup>®</sup> vector (10 ng/µl plasmid DNA in: 50% glycerol, 50 mM Tris-HCl [pH 7.4], 1 mM EDTA, 1 mM DTT, 0.1% Triton X-100, 100 µg/ml BSA, and phenol red). The reaction was mixed gently and incubated at Rt from 30 sec. to 30 min., depending on the size of the PCR product. After incubation, 2 µl of the TOPO<sup>®</sup> cloning reaction was added to a vial of One Shot<sup>®</sup> chemically competent *E. coli* and incubated on ice for 30 min. These cells were heat-shocked for 30 sec. at 42°C and immediately transferred to ice. 250 µl of Rt SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl<sub>2</sub>, 10 mM MgSO<sub>4</sub> and 10 mM glucose) was added, and the cells were shaken (200 rpm) at 37°C for1 hour. After one hour 20  $\mu$ l and 100  $\mu$ l of the transformed cells were

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**Figure 2-4.** Illustration of the action of Topoisomerase I. Topoisomerase selectively binds to dsDNA and cleaves the phosphodiester backbone after 5'-CCCTT in one strand. The energy released by phosphodiester bond cleavage is conserved by formation of a covalent bond between the 3' phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of Topoisomerase I. The phosphor-tyrosyl bond between the DNA (vector) and Topoisomerase can be attacked by the 5' hydroxyl of the PCR product, resulting in efficient ligation of the PCR product.

spread onto warm LB(10 g/L Tryptone, 5 g/L yeast extract, and 5 g/L NaCl)-ampicillin (50 mg/ml) agar plates using a sterilized cell spreader. The plates were inverted and

incubated at 37°C for 18 hours. Colonies (white) containing the PCR product were

selected for further studies.

## **Plasmid DNA Isolation**

Plasmid DNA was isolated from E. coli cells using the QIAprep Spin miniprep kit protocol (Qiagen; Valencia, CA). This protocol is based on alkaline lysis of bacterial cells followed by adsorption of DNA onto silica in the presence of high salt. Cultures harboring the plasmid of interest were incubated in 3 ml of LB broth containing 50 µg/ml ampicillin and incubated while shaking in a 37°C water bath for 18 hours. After incubation the cells were pelleted from the LB broth by centrifugation at 9,000 x g for 30 sec. The supernatant was discarded and the cells were resuspended in 250 µl Buffer P1 (50 mM Tris [pH 8.0], 10 mM EDTA, 1 µg/µl RNAse). 250 µl Buffer P2 (200mM NaOH, 1% SDS) is added and the suspension is inverted gently 4 to 6 times. To this suspension, 350 µl N3 (proprietary, kit included) was added and the tube was inverted immediately 4 to 6 times to prevent localized precipitation. The mixture was centrifuged at  $13,000 \ge 10$  min. to remove cellular debris. The supernatant was applied to a spin column (containing a silica gel membrane for selective adsorption of plasmid DNA) in a 2 ml collection tube and centrifuged (13,000 x g) for 30 sec. The eluent was discarded and 500 µl Buffer PB (proprietary, kit included) was added to the column and centrifuged (13,000 x g) for 1 min. The flow through was discarded and the spin column was washed by adding 750 µl Buffer PE (proprietary, kit included) and centrifuging (13,000 x g) for 1 min. The flow-through was discarded and the spin column was centrifuged an additional 1 min. to remove any residual wash buffer. The spin column was place in a clean 1.5 ml microcentrifuge tube and 50 µl warm (55°C) Buffer EB (10 mM Tris-Cl [pH 8.5]) was added to elute the plasmid DNA. Cell stocks were made by storing the cells in a 35% glycerol solution at -80°C. Plasmid DNA was stored at -80°C until use.

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#### One-Step RT-PCR

One-step, reverse-transcription PCR (RT-PCR) is a technique which allows one to determine relative mRNA levels *in vitro*. This technique does not directly measure absolute mRNA levels, but is a consistently reliable relative assay allowing multiple



**Figure 2-5**. Overview of one-step, reverse-transcription PCR (RT-PCR). The reverse primer TM-APE1-Ex2Ra binds to the target mRNA and synthesizes cDNA. The forward primer TM-APE1-Ex1Fs is annealed to the cDNA and synthesizes the complimentary strand during the initial PCR cycle. Subsequent cycles use both forward (TM-APE1-Ex1Fs) and reverse (TM-APE1-Ex2Ra) primers to PCR amplify the target molecules.

mRNA levels to be determined from the same RNA preparation. To determine the relative expression of AP Endonuclease in *Xiphophorus*, one-step RT-PCR was

employed utilizing the TaqMan<sup>®</sup> One-step RT-PCR kit (ABI; Foster City, CA) and the ABI Prism 7700 Sequence Detector (ABI; Foster City, CA). Using the TaqMan<sup>®</sup> reagents, the ABI Prism 7700 was used to perform a reverse transcription (RT) reaction, converting mRNA to cDNA (Figure 2-5). Sequence specific oligonucleotide probes use select cDNA templates as a substrate for amplification. The ABI Prism 7700 determines





the relative abundance of the target template by analyzing the cycle-to-cycle change in

fluorescence signal as a result of the amplification of template during PCR. The 5' to 3'

nuclease activity of AmpliTaq Gold<sup>™</sup> polymerase allows direct detection of PCR products by the release of a fluorescent reporter during the PCR on the ABI Prism 7700 (Figure 2-6). To perform one-step RT-PCR the following were combined in a single well of a 96-well optical plate: 10 ng total mRNA, 12.5 µl 2 x Master Mix, 0.625 µl 40 x Mustiscribe Reverse Transcriptase, 300 nM of each primer, 5 µM probe and doubledistilled water (ddH2O) to 25µL final volume. The reaction was incubated/cycled in the ABI Prism 7700 according to the conditions detailed in Table 2-4.

Real-Time RT-PCR step	Temperature	Time	
Reverse transcription	48	30 min.	
Taq polymerase activation	95	10 min.	
Denaturation	95	15 sec.	
Annealing / extension	55	30 sec.	- $+$ 40 cycles

Table 2-3. Temperature / time profile for a one-step real-time RT-PCR mixture.

#### **CHAPTER 3**

#### **RESULTS AND DISCUSSION**

#### X. maculatus AP Endonuclease Gene Isolation and Nucleotide Sequencing

#### **Cloning of AP Endonuclease**

To clone the Xiphophorus AP endonuclease gene, nucleotide sequences of various species were obtained from the NCBI Blast database. This sequence database incorporates DNA and protein sequences from available public sources, primarily through direct submission of sequence data from individual laboratories and from largescale sequencing projects (Benson et al., 1998). Nucleotide sequences were downloaded from the NCBI database into the MacVector<sup>™</sup> software program (Oxford Molecular Group, v 7.0, 2000). Nucleotide alignments were made by comparing these sequences to the sequences using a ClustalW Alignment feature of MacVector<sup>™</sup>, and degenerate primers were designed using Primer Express<sup>™</sup> (Applied Biosystems, v 1.5, 1995) software by the alignment of highly conserved regions among Homo sapiens, Bos taurus and Rattus norvegicus genes. These primers (Tm-APE1-Ex1Fs and Tm-APE1-Ex2Ra, Table 3-1) were used to perform PCR on cDNA amplified from RNA isolated from muscle tissues of X. maculatus. A resulting 123 bp amplicon (Figure 3-1) was cloned into a plasmid vector, which was transformed into E. coli using the TOPO-TA Cloning Kit (Invitrogen; Carlsbad, CA) as described in Chapter 2. The resulting plasmid (pAPE)

was isolated and the nucleotide sequence determined (Davis Sequencing, LLC; Davis, CA). The insert in pAPE was compared and aligned with known AP endonuclease sequences from various other species. Alignments exhibited homology with *Rattus norvegicus, Bos taurus,* and *Homo sapiens* (NCBI accession numbers NM\_024148, NP\_788782, and D13370 respectively). Additionally, a computer translation of the cloned 123 bp sequence showed homology with the AP endonuclease amino acid sequence of these same species. Table 3-2 shows the nucleotide identity and amino acid similarity values of AP Endonuclease in *X. maculatus* compared to zebrafish, mouse, rat, bull and human.

Oligonucleotide Primer	Primer Sequence	Use
APE1,F1	TGGGTNAARGARGARGCNCC	PCR
APE1,F2	GARGARGARCAYGAYCARGA	PCR
APE1,F3	AARGGNAAYAARAARAAYGC	PCR
APE1,R1	TCYTGRTCRTGYTCYTCYTC	PCR
APE1,R2	GCRTTYTTYTTRTTNCCYTT	PCR
APE1,R3	TTCATCATRATNGTCCARAA	PCR
AP1 PMF <sub>2</sub>	AGAGGCAAGAAGACAGATGATGGGTC	PCR

**Table 3-1**. Synthetic oligonucleotide primers used in PCR, 5' RACE, 3' RACE and onestep real-time RT-PCR. N represent the presence of any nucleotide (A,T,G or C). R represents a purine (A or G) and Y represents a pyrimidine (C or T).

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Oligonucleotide Primer	Primer Sequence	Use
AP1 PMR <sub>1</sub>	TAGAAGTTTGGGAACTCGGCTGTG	PCR
AP1 PMR <sub>2</sub>	TGAGCCCGTCTACGTTCCAG	PCR
TM-APE1-ex1 Fs	AAGATCACCTCCTGGAACGTAGAC	Real-time PCR
TM-APE1-ex2 Ra	GGCTTTCTCTGAACACTTTGTCTCT	Real-time PCR
TM-APE1-ex3 Ra	GGGACGTAGGCCGTCACA	PCR
AP1, F1	GCAAAACCTGGGACGTGGAC	3' RACE
AP1, R2	GAGCTCGCTCAGGTAAGCTC	5' RACE

Table 3-1. (continued).

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TM-APE1-ex1Fs 5'-<u>AAGATCACCTCCTGGAACGTAGAC</u>GGGCTC Intron 3 AGGGCCTGGGTGAAAAAAGAACGGACTGGAT T GGGTGCGTGAGGAGGCGCCAGATGTTCTGTGC CTCCA<u>AGAGACAAAGTGTTCAGAGAAAGCC</u>-3' TM-APE1-ex2Ra

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**Figure 3-1**. 123 bp amplicon obtained using primers TM-APE1-Ex1Fs and TM-APE1-Ex2Ra.

#### 5' RACE:

cDNA sequence information was obtained from *X. maculatus* Jp 163 A (brain and testes) using the 5' RACE system for Rapid Amplification of cDNA Ends, Version 2.0 protocol (Invitrogen; Carlsbad, CA). Amplification by PCR using primer AP1,R2 and Abridged Adapter Primer (AAP) produced a ~650 bp product. This product was isolated and cloned into *E. coli* using the TOPO-TA cloning kit as previously described. DNA from plasmid pAPE was isolated, purified and sequenced. Comparison of the sequence of the clones with 5' sequences of other species (using a Clustal alignment) revealed nucleotide identity, suggesting the presence of the AP Endonuclease gene.

#### <u>**3' RACE:**</u>

The 3' ends of *X. maculatus* AP endonuclease coding region were obtained using the 3' RACE System for Rapid Amplification of cDNA Ends protocol (Invitrogen; Carlsbad, CA). Amplification using primer AP1, F1 and a Universal Adapter Primer (UAP) resulted in a ~ 450 bp product. This product was isolated and cloned using the TOPO-TA cloning kit. The plasmid DNA was isolated, purified and sequenced as described in Chapter 2. Comparison of the cloned sequence with AP endonuclease sequences of other species revealed nucleotide homology at the 3' end of the gene. This suggests that the product isolated from the 3' RACE was derived from the AP endonuclease gene.

#### **Coding Sequence Determination**

After obtaining nucleotide sequences from 5' and 3' RACE, gene-specific primers were used to amplify and clone the entire coding sequence of AP endonuclease. Nucleotide sequencing revealed a 972 bp coding region. Clustal alignments of this 972 bp region revealed the possible locations of the 3 introns based on comparison with rat, bull and human AP endonuclease gene structures (NCBI accession numbers NM\_024148, ,and D13370 respectively). PCR was performed on genomic DNA using primers designed around these introns. Primers AP1 PM F2 and AP1 PMR2 were used to clone the genomic sequence flanking and spanning intron 2. Primers TM-APE1-Ex1Fs and TM-APE1-Ex2Ra were used to amplify and clone the genomic sequence surrounding and spanning introns 3 and 4. These products were cloned into *E. coli* and plasmid DNA was isolated, purified and sequenced. Comparison of the genomic DNA sequence with the *X. maculatus* cDNA sequence and genomic DNA sequences from rat, bull and human



**Figure 3-2**. Gene structure of *Xiphophorus maculatus* AP endonuclease. Exons and their sizes are labeled in blue and introns are labeled in black. Positions of the start and stop sequences are labeled in red.

Intron 2 ~... 3.1 50 ar. 1... СC A IGAA GAGA AGCAAGAAGACAGA IGA TO XITY TO, IY ATROGGAGAA IGA IGGACCODOT M K K 3 K K T D D G S A D ( E N D A C S> PRANSLATION OF XIPHOPHORUS CLS (A) 8. 110 120 36 100 170 150 130 140 160 180 -20 200 210 210 **Intron 3** mtron 3 200 200 201 220 230 240 AACUTACIAO RECTURCISCICTURCISCARAA GRACO LA CITORICITICACIACIACI N V D G L R A U V K K N G ' D T T T \_\_\_ "RANSLATION OF XIPHOPHORUS CDS A)\_\_\_ 290 290 300 250 260 3° C ANGULAGATGTPLIPGTGCCTUCAAGAGACAAAGIGUTCAUAGAAAGINOTTCCTCCCCAG APTVICICETACSEKALPAQ> \_\_\_\_\_\_CRANSLATION OF XIPHOPHURUS (DS (A) \_\_\_\_\_\_> 3.0 357 320 330 3.41. 360 ATVACGIVCANGLICGACTACICCCACAAATALIVKNGCTGCGTCCAANGACAANGAGGGC LISMPEYPHKYMAASNDKEG FRANSLATION OF XIPHOPPORUS CDS [A] 370 380 400 390 410 625 TACASTGETGFAGUEATIGETGEAAGACTGAACGTCACGAAGGTCACGAAGGTCACGAAGGTCACGAAGATTAFTCACG Y S C 7 7. M L C R 7 F P L K V T V S 7 55 TRANSLATIOL OF X PHOPHORUS CDS [A]\_\_\_\_\_ Intron 4 4 440 450 460 11.1 480 400 500 510 520 530 542 GITCUCAAACTICTACCTTGTGACGUTCTACGTCCCGAACGCGAGCAGAGCCCTCGTCDG M P K L L P C D G L R P E R E Q R P R P> TEANSLAILON OF ALPHOPHERUS CDS [A] 59C 580 560 570 550 600 62( 53) 643 65C 66C 810 CGACATCCAGAAGUCCCT9GIGUTGIDOGGOGATCTCAACGTUGOUCACCAGGAGATCGA R H P E A P G A V R R S Q R R A P G E R> TRANSLATION OF XIPHC PHORUS CDS [A]

**Figure 3-3**. Nucleotide sequence of the coding region of AP endonuclease in *Xiphophorus maculatus*. Amino acid sequence is listed under each codon, and intron positions within the coding region are labeled.

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				RAN	SLA	TICI	J OF	XI	PHC	PHC	DRUS	5 C	DS	[A	]				
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# Figure 3-3 (continued).

suggested that the cloned regions were the introns 2, 3 and 4 of AP endonuclease. Figure 3-2 represents the *Xiphophorus* gene structure and figure 3-3 shows the coding sequence for AP endonuclease in *X. maculatus*. Once the coding sequence was determined, the amino acid sequence of the *X. maculatus* AP endonuclease gene was compared with the

.

Zebrafish APE protein rat ap endo mouse ap endo human ap end bull ap endo Xiphophorus protein translation	10     20     30       M P K R A K K	
Zebratish APE protein rat ap endo mouse ap endo human ap end bull ap endo Xiphophorus protein translation	40       50       60         K K E K K G K E P E A P I L Y E D P P E K L T S K D G R A A       K D G R A A G E G P V L Y E D P P D Q K T S A S G K S A         K K T E K E A A G E G P V L Y E D P P D Q K T S P S G K S A         K K N D K E A A G E G P A L Y E D P P D Q K T S P S G K S A         K K N E K E A V G E G A V L Y E D P P D Q K T S P S G K S A         K K N E K E A V G E G A V L Y E D P P D Q K T S P S G K S A         K K N K K K K K K K E P E A P V L Y E D P P D Q K T S S G K S A         K K K K K K E A G E G P V L Y E D P P D Q K T S S G K S A	
Zebrafish APE protein rat ap endo mouse ap endo human ap end bull ap endo Xiphophorus protein translation	70     80     90       N MKITSWNVDGLRAWVKKNGLDWVRKEDPD     TLKICSWNVDGLRAWIKKKGLDWVREEAPD       TLKICSWNVDGLRAWIKKKGLDWVKEEAPD       TLKICSWNVDGLRAWIKKKGLDWVKEEAPD       TLKICSWNVDGLRAWIKKKGLDWVKEEAPD       TLKICSWNVDGLRAWIKKKGLDWVKEEAPD       TLKICSWNVDGLRAWIKKKGLDWVKEEAPD       TLKICSWNVDGLRAWIKKKGLDWVKEEAPD       TLKICSWNVDGLRAWIKKKGLDWVKEEAPD       NMKITSWNVDGLRAWIKKKGLDWVKEEAPD       NMKITSWNVDGLRAWIKKKOLDWVKEEAPD       NMKITSWNVDGLRAWIKKKOLDWVKEEAPD	
Zebrafish APE protein rat ap endo mouse ap endo human ap end bull ap endo Xiphophorus protein translation	100       110       120         ILCLQETKCAEKALPADITAMPEYPHKYWA         ILCLQETKCSENKLPAELQELPGLTHQYWS         ILCLQETKCSENKLPAELQELPGLSHQYWS         ILCLQETKCSENKLPAELQELPGLSHQYWS         ILCLQETKCSENKLPAELQELPGLSHQYWS         ILCLQETKCSENKLPAELQELPGLSHQYWS         ILCLQETKCSENKLPVELQELSGLSHQYWS         ILCLQETKCSENKLPVELQELSGLSHQYWS         VLCLQETKCSENKLPAELQELPGLSHQYWS         ILCLQETKCSEKALPAQIITSMPEYPHKYWA         ILCLQETKCSENKLPAELQELPGL.HQYWS	
Zebrafish APE protein rat ap endo mouse ap endo human ap end bull ap endo Xiphophorus protein translation	130 THO THO 140 150 150 140 150 150 150 150 150 150 150 15	

**Figure 3-4**. Clustal W alignment of the AP endonuclease amino acid sequences of 6 species. Grey areas represent amino acid homology/similarity among species. *Xiphophorus* AP endonuclease amino acid sequence exhibits the greatest homology with Zebrafish (84% identity, 90% similarity) and exhibits relatively lower homology with human and bull (66% identity and 77% similarity).

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Zebrafish APE protein	H	DI	< E	G	R	V	1	Τ	A E	F	Р	D	FF	L	V	T	A	Y I	Y P	N	A	S	R	G	E V	
rat ap endo	H	DO	D E	G	R	V	1	V.	AE	F	E	SI	FT	L	V	T		Y	7 P	N	A	G	R	G	LV	
, mouse ao endo	H	DO	) E	G	R	V.	1	V	AE	F	E	\$ 1	FV	L	V	T		Y	Y P	N	A	G	R	G	LV	
human an end	H	D	Ē	G	R	V	1	V	AE	F	D	s I	FV	L	v	T		Y	7 1	N		G	R	G	L V	
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Figure 3-4. (continued)

amino acid sequences of five other species: zebrafish, rat, mouse, human and bull (Figure 3-2) using MacVector software and a Clustal protein alignment. Table 3-2 shows the percent identity and percent similarity calculated using this alignment. As expected, *X. maculatus* is most homologous with zebrafish than any of the other species compared.

	% Identity	% Similarity
Human AP Endonuclease (NCBI accession # P28352)	66	77
Bull AP Endonuclease (NCBI accession # NP_788782)	66	77
Rat AP Endonuclease (NCBI accession # NP_077062)	67	79
Mouse AP Endonuclease (NCBI accession # AAH52401)	68	79
Zebrafish AP Endonuclease (NCBI accession # P28352)	84	90
Plasmodium AP Endonuclease (NCBI accession # NP_473183)	14	23

**Table 3-2**. Amino acid identity and similarity values of the *X. maculatus* AP endonuclease protein compared to human, bull, rat, mouse, zebrafish and plasmodium. The functional similarity is determined on the basis of amino acid functional group similarities (P=A=G=S=T, Q=N=E=B=D=Z, H=K=R, L=I=V=M, and F=Y=W).

## **CHAPTER 4**

# **RESULTS AND DISCUSSION**

## Mapping of the Xiphophorus AP Endonuclease Locus

*Xiphophorus* fishes have the ability to produce fertile interspecies  $F_1$  hybrids. These hybrids derive one half of their genetic content from each parental species and are thus are polymorphic for many or most loci along the length of their chromosomes. Backcrossing these interspecies hybrid F1 animals to one parent (recurrent) species results in backcross hybrid progeny (BC<sub>1</sub>) carrying, on average, 75% of their genetic information from the recurrent parent and 25% from the non-recurrent parent (as shown in Figure 1-7). Segregation of recurrent and non-recurrent chromosomes into each BC<sub>1</sub> hybrid allows one to assess the inheritance patterns of each genetic marker with the development of a complex phenotype, such as tumorigenesis. To conduct this analysis, one must first genotype  $BC_1$  hybrid cohorts with a large set of genetic markers and score each hybrid as either homozygous or heterozygous for the marker at each locus. Two-bytwo chi square analysis is then performed for each possible marker using Mapmaker (for Macintosh) v2.0 software. An excess of parental types among the  $BC_1$  hybrids, or deviation from the expected 50:50 ratio for random segregation, suggests genetic linkage and can be used to assign map positions to new loci (Morizot et al., 1998). The (X. maculatus (x) X. helleri) (x) X. helleri (Figure 1-7) parental DNAs were used as a target

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to perform standard PCR using primers AP1,PMF2 and AP1,PMR2 (as described in Chapter 2). The amplicons from the parental stocks were of different sizes, thus the species source could be discerned in a standard agarose (2%) gel (Figure 4-1). This allows for the scoring of each BC<sub>1</sub> hybrid as a homozygote or heterozygote. A panel of fifty BC<sub>1</sub> hybrid DNAs was scored as either exhibiting the banding pattern of the *X*. *helleri* (homozygous) parent or the pattern



**Figure 4-1**. Image of agarose (2%) gel exhibiting the banding patterns for Hybrid 5 ([*X.maculatus* (x) *X. helleri*] (x) *X. helleri*) parents,  $F_1$  and  $BC_1$  hybrids (lanes 5-12). Backcross hybrids in lanes 6, 9, 10, 11, and 12 exhibit the banding pattern of the F1 parent, while backcross hybrids in lanes 5, 7, and 8 exhibit the banding pattern of the *X. helleri* parent. Scoring data from a panel of 50 backcross hybrids were used to determine genetic linkage and assign AP endonuclease in *Xiphophorus* to chromosome 1.

observed in the  $F_1$  hybrid (heterozygous) parent. These data were entered into the Mapmaker v2.0 software to determine genetic linkage and assign the map position of AP endonuclease in *Xiphophorus* to chromosome I (Figure 4-2).



**Figure 4-2**. Map position of AP Endonuclease within chromosome I in *Xiphophorus*. Distance (cM) between loci is located on the left side of the figure. One centimorgan is the distance between two loci which have a 1% chance of being separated during recombination in a single generation.

## **CHAPTER 5**

#### **RESULTS AND DISCUSSION**

# Transcriptional Expression of AP Endonuclease in X. maculatus

#### **Characterization of RNA Expression**

In order to characterize the expression patterns of AP endonuclease in X. maculatus tissues, RNA was isolated from brain, eye, liver, gill, testes, skin and muscle. RNA samples from the various tissue sources were standardized to equal concentrations  $(10 \text{ ng/}\mu\text{l})$  and 10 ng of each was used in real-time PCR. An ABI Prism 7700 real-time PCR machine was used to analyze the expression of AP endonuclease in each of these samples. Expression was compared to endogenous transcriptional controls gene targets GAPDH, β-Actin, Xiphophorus-derived calibrator (A2 cell line) and dilution of plasmid pAPE containing inserts specific to AP endonuclease. The starting AP endonuclease mRNA copy number was determined in 10 ng of calibrator RNA by comparing it to a plasmid dilution standard of known concentration. Once established, this numerical value was used to calculate the starting copy number and a relative expression value of the experimental samples by normalizing the experimental data to the signal detected using calibrator RNA. Triplicate runs were performed for each mRNA target, and five total experiments were run. Thus, each experimental measure of relative expression represents an average derived from a total of 15 experimental values. The primers and probes (and nucleotide sequences) used for each assay are listed in Table 5-1. The

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relative position of the primers and probe used for AP endonuclease expression analyses are shown in Figure 5-1.

Oligonucleotide Primer /	Sequence
Probe	
TM-APE1-ex1 Fs	AAGATCACCTCCTGGAACGTAGAC
TM-APE1-ex2 Ra	GGCTTTCTCTGAACACTTTGTCTCT
Tmpr-AP1-Ex2Fs	56FAM-CGCCAGATGTTCTGTGCCTCCAAGA-36TAMT
TM-GAPDH-ex2Fs	CCTTCATCGACCTGCAGTACAT
TM-GAPDH-ex4Ra	GACGTATTTGGCGCCAGCTT
TMPr-GAPDH-ex3,4 Fs	56-FAM-CATCTCTGTTTTCCAGTGTATGAAGCCTGCT-36TAMT
TM-actin-ex2Fs	CAAAGCCAACAGGGAGAAGATG
TM-actin-ex3Ra	CGATACCAGTGGTACGACCAGAA
TMPr-actin-ex3Fs	56FAM-TTGCCATCCAGGCCGTGCTG-36TAMT

Table 5-1. Synthetic oligonucleotide primers / probes used in one-step real-time RT-PCR. 



**Figure 5-1**. 123 bp amplicon using primers TM-APE1-ex1 Fs and TM-APE1-ex2 Ra. The position of the probe (Tmpr-AP1-Ex2Fs) relative to these primers is shown in red.

Primer optimization was performed for each primer set to ensure maximum amplification was achieved. Real-time PCR was performed using 50 nM, 300 nM and 900 nM primer concentrations. Primer concentrations that corresponded to the greatest change in fluorescence detection (i.e., amplification of target molecules) were considered to be optimal and were used for subsequent analyses. In addition, the reaction efficiency of real-time reactions was determined for samples containing plasmid pAPE DNA and *X. maculatus* RNA. Reaction efficiency was determined by comparing the slope of a standard curve generated by plotting starting copy number of pAPE DNA samples versus threshold cycle (Ct; initial detection of change in fluorescence) for multiple assays

(Figure 5-2). Similar slopes between runs suggest similar reaction efficiencies and allows comparison of data generated from separate experiments.



**Figure 5-2**. Graph of starting quantity vs. threshold cycle for a one-step real-time RT-PCR reaction. Dilutions of plasmid pAPE containing inserts specific to AP endonuclease were used to generate a standard curve. Comparison of the data from various tissues in *Xiphophorus* to the standard curve allows for determination of the starting copy number of transcripts present in 10 ng total mRNA derived from each tissue.

#### Analyses of RNA Expression

Transcriptional expression of AP endonuclease in X. maculatus tissues revealed

elevated expression in brain, eye gill and testes (relative to liver, skin and muscle).

Although expression of AP endonuclease in liver, skin and muscle is observed, the

expression levels are less than 25% of that observed in gill. Figure 5-3 illustrates the

expression of AP endonuclease in 7 healthy tissues relative to the A2 calibrator cDNA. Figure 5-4 depicts the starting copy number of AP endonuclease transcripts present in 10 ng RNA from each of 7 tissues. Dilutions of plasmid pAPE containing inserts specific to AP endonuclease were used to generate a standard curve, and comparison of the expression data from seven healthy tissues in *Xiphophorus* to the standard curve allows for determination of the starting copy number of transcripts present in 10 ng total mRNA derived from each tissue. In addition, real-time analyses were performed using various tissues (gill, muscle, melanized skin, melanized tailfin and melanoma tumor tissue) of



**Figure 5-3.** Expression of AP Endonuclease in seven tissues from healthy X. *maculatus* (Jp 163 A) relative to A2 calibrator (A2 = 1). Expression in brain, eye, gill, and testes is more than threefold higher than in liver, skin and muscle

interspecies backcross hybrid 1 ([X. maculatus (Jp 163 B) (x) X. helleri] (x) X. helleri); Figure 5-5) to assess the expression of AP endonuclease in melanized tissues and melanoma. The results suggest AP endonuclease expression is relatively low in gill, muscle, melanized skin, and melanized tailfin (expression ~ threefold lower than melanoma tumor tissues). However, expression in tumor tissues is noticeably increased (Figure 5-6) relative to the 4 other tissues analyzed. Starting copy number (Figure 5-7)



**Expression Levels of AP1 in Healthy Tissues** 

Figure 5-4. Starting copy number (per 10 ng) of AP endonuclease transcripts in healthy tissues of X. maculatus. Starting copy number was determined by comparing expression data for seven X. maculatus tissues to a series of dilutions of plasmid pAPE containing inserts specific to AP endonuclease.

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**Figure 5-5**. Interspecies hybrid 1 ([X, maculatus (x) X. helleri] (x) X. helleri) backcross. The loci derived from X. maculatus are colored red, while the loci derived from the swordtail X. helleri are colored blue. Backcross hybrids were used to assess AP endonuclease expression in melanized tissues and melanoma.



**Figure 5-6**. Expression of AP endonuclease in various tissues of BC<sub>1</sub> hybrids of *Xiphophorus* tumor bearing fish relative to A2 calibrator (A2 = 1). As expected, expression of AP endonuclease in melanoma tumor tissue is higher (~ threefold) than expression in other tissues.

of AP endonuclease transcripts present in 10 ng cDNA derived from gill, muscle, melanized skin, melanized tailfin and melanoma tumor tissue was determined by comparison with dilutions of plasmid pAPE.



Expression of AP1 in Xiphophorus Tumor Fish

**Figure 5-7**. Starting copy number (per 10 ng) of AP endonuclease transcripts in various tissues of *Xiphophorus* Hybrid 1 tumor fish. Starting copy number was determined by comparing expression data for five *X. maculatus* tissues (gill, muscle, melanized skin, melanized tailfin, and melanoma tumor tissue) to a series of dilutions of plasmid pAPE containing inserts specific to AP endonuclease.

#### **CHAPTER 6**

#### CONCLUSIONS

In this study, an effort was made to characterize the AP endonuclease gene in *Xiphophorus maculatus*. The *X. maculatus* AP endonuclease gene was cloned and the nucleotide sequence of the coding region was determined. The *X. maculatus* locus spans 1.3 kb of genomic DNA and contains 3 introns. Analysis of the computer-determined 322 amino acid AP endonuclease protein revealed 66% identity and 77% similarity with human AP endonuclease and 84% identity with zebrafish AP endonuclease. A panel of fifty backcross hybrids from (*X. maculatus* (x) *X. helleri*) (x) *X. helleri* were used in mapping studies of AP endonuclease in *Xiphophorus*. Linkage analysis suggests AP endonuclease maps to linkage group I in *Xiphophorus*.

Analysis of AP endonuclease transcription was performed in healthy tissues (brain, eye, liver, gill, testes, skin and muscle) of *X. maculatus*. Expression observed in eye, testes, brain and gill was over threefold higher than expression in liver, skin, and muscle. Elevated expression is expected in these tissues since DNA repair proteins present in the gills counter the effects of DNA-damaging agents present in the environmental water. Due to the counter-current blood flow of fish, it is expected that organs derived from neural tissue (i.e. brain and eyes) exhibit a larger capacity for DNA repair. Elevated expression of AP endonuclease in the testes is consistent with published data from mice (Intano *et al.*, 2002). Use of interspecies backcross hybrids in one-step real-time RT-PCR assays indicated AP endonuclease expression in gill, muscle, melanized skin, and melanized tailfin was more than threefold lower than expression in tumors. Due to increased BER activity in melanoma tumor tissues, these results are expected

These studies provide preliminary data and baseline observations necessary for future studies of AP endonuclease in *X. maculatus*. It may be important to perform additional 5' and 3' RACE reactions in order to fully characterize the 5' and 3' untranslated regions of the AP endonuclease transcript. Additionally, AP endonuclease protein expression studies would relate the transcriptional expression of AP endonuclease to the protein expression. Furthermore, protein and transcriptional expression studies for other genes involved in the BER pathway may provide insight into genetics underlying vulnerability to tumor development.

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#### VITA

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