MOLECULAR CHARACTERIZATION AND RELATIVE EXPRESSION OF THE *Xiphophorus maculatus* APURINIC / APYRIMIDINIC ENDONUCLEASE GENE.

THESIS

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Ramiro T. Sosa III

TEXAS STATE UNIVERSITY – SAN MARCOS

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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-*N*-glycosylase (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 β (β-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

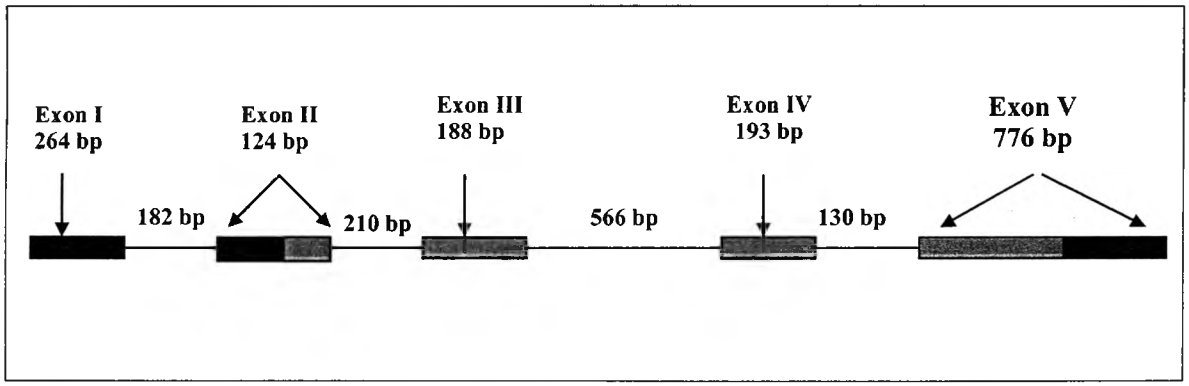

Figure 1-1. Gene structure of human AP endonuclease. Grey areas represent coding region. Black areas represent 5' and 3' untranslated regions.

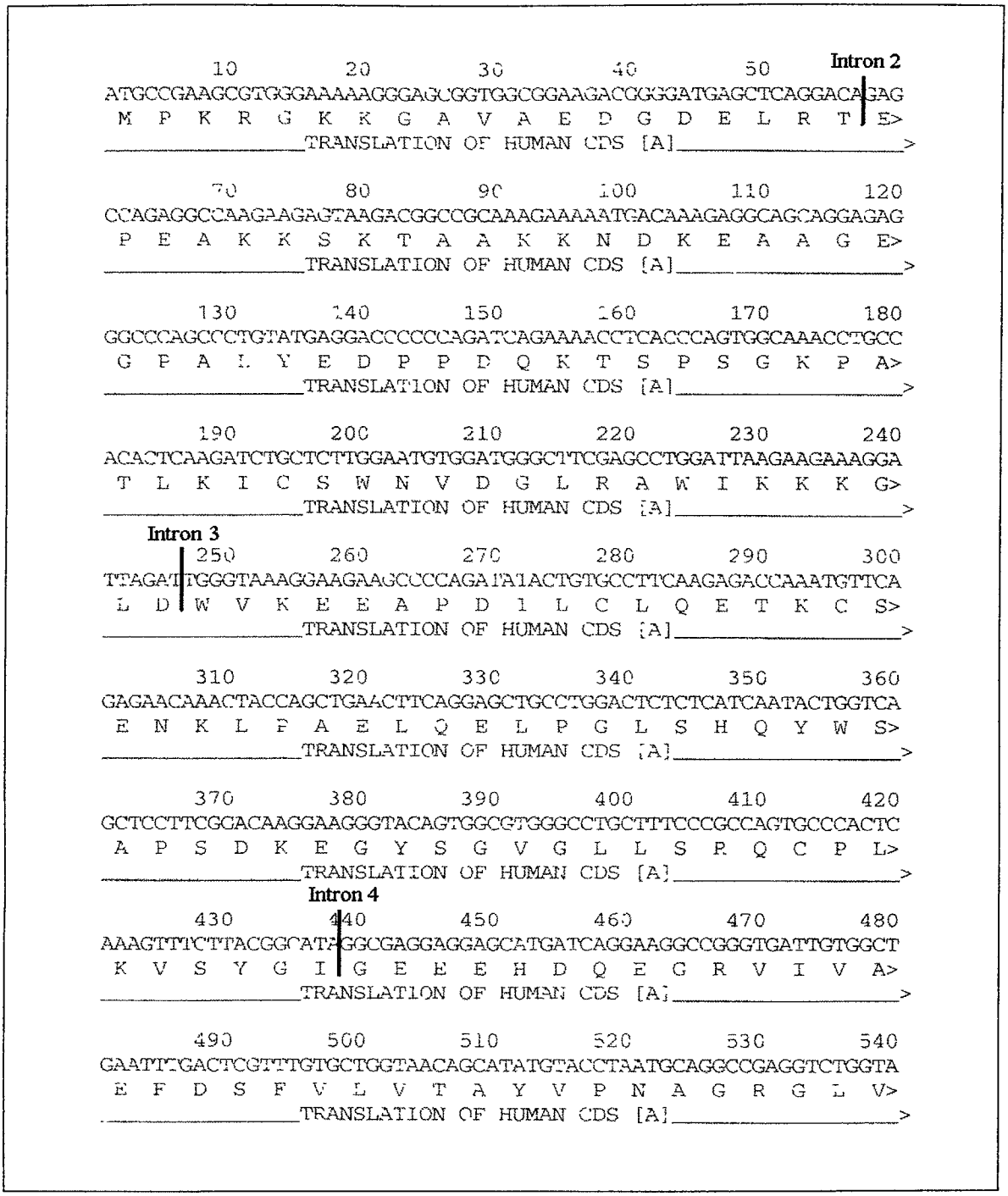

Figure 1.2. Nucleotide sequence of the coding region of human AP endonuclease. Amino acid sequence is listed under each codon, and intron positions within the coding region are labeled.

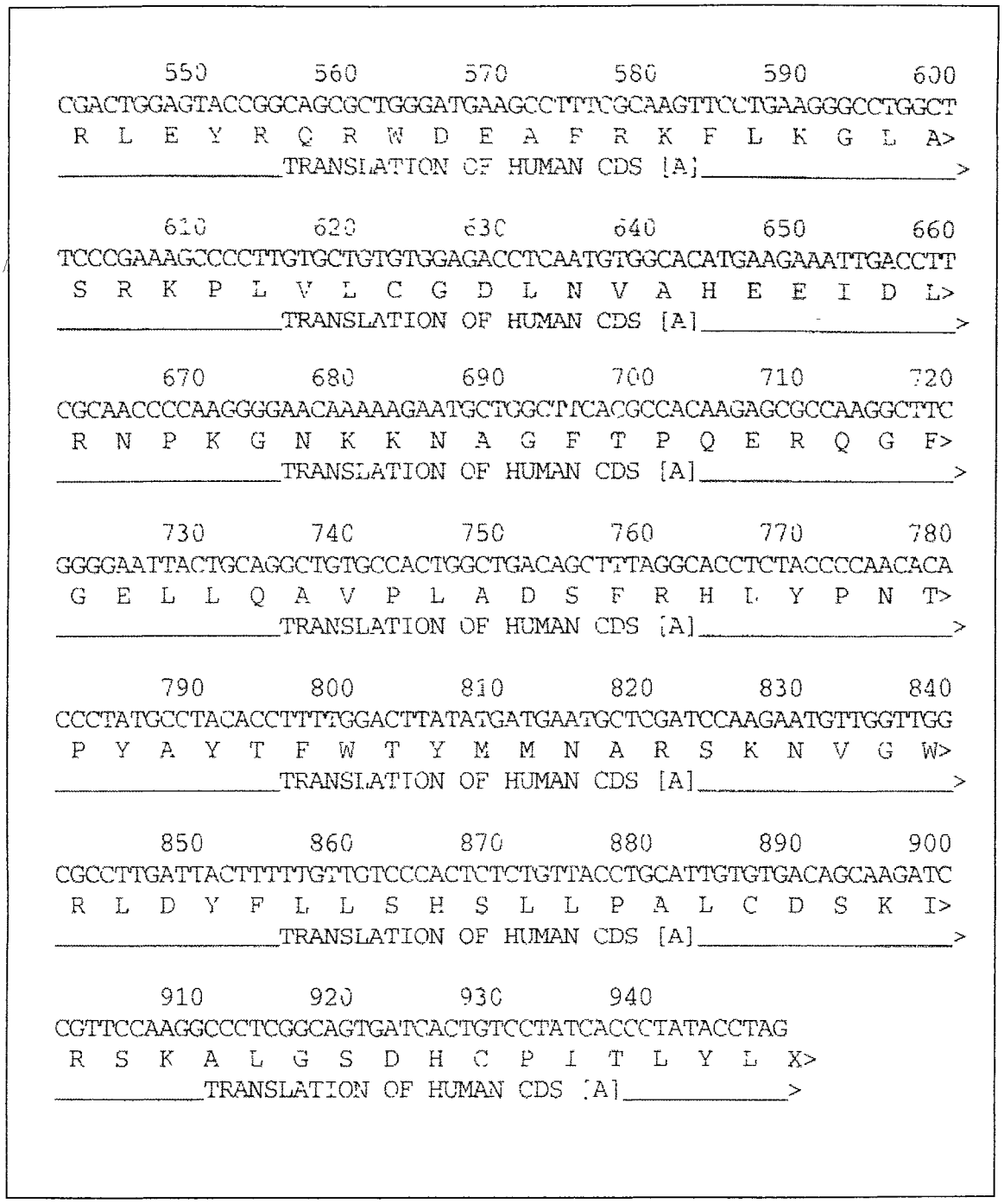

Figure 1-2. Continued.

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

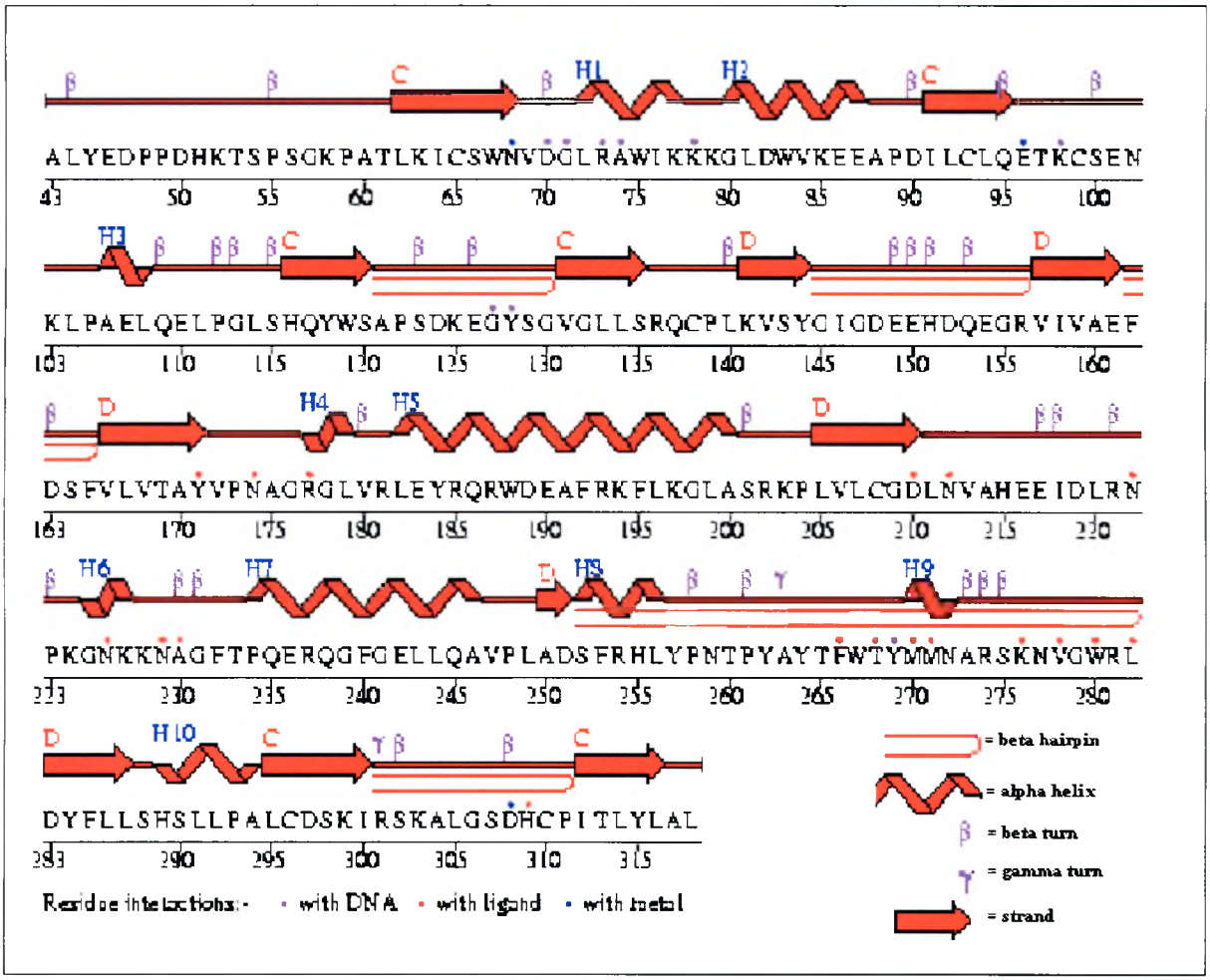

Figure 1-3. Structural motifs of human AP endonuclease (amino acids 44 – 318) as determined by X-ray crystallography (Beernink *et al.*, 2001). Located within the protein are 5 β hairpins, 10 α helices, 33 β turns, 2 γ turns, and 12 strands. The amino acid sequence is provided and the various structural 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 β (pol-β). AP endonuclease is electrostatically oriented to the DNA by divalent Mg^{2+} 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- β. In order to generate the necessary one-nucleotide gap, deoxyribophosphodiesterase (dRpase) cleaves

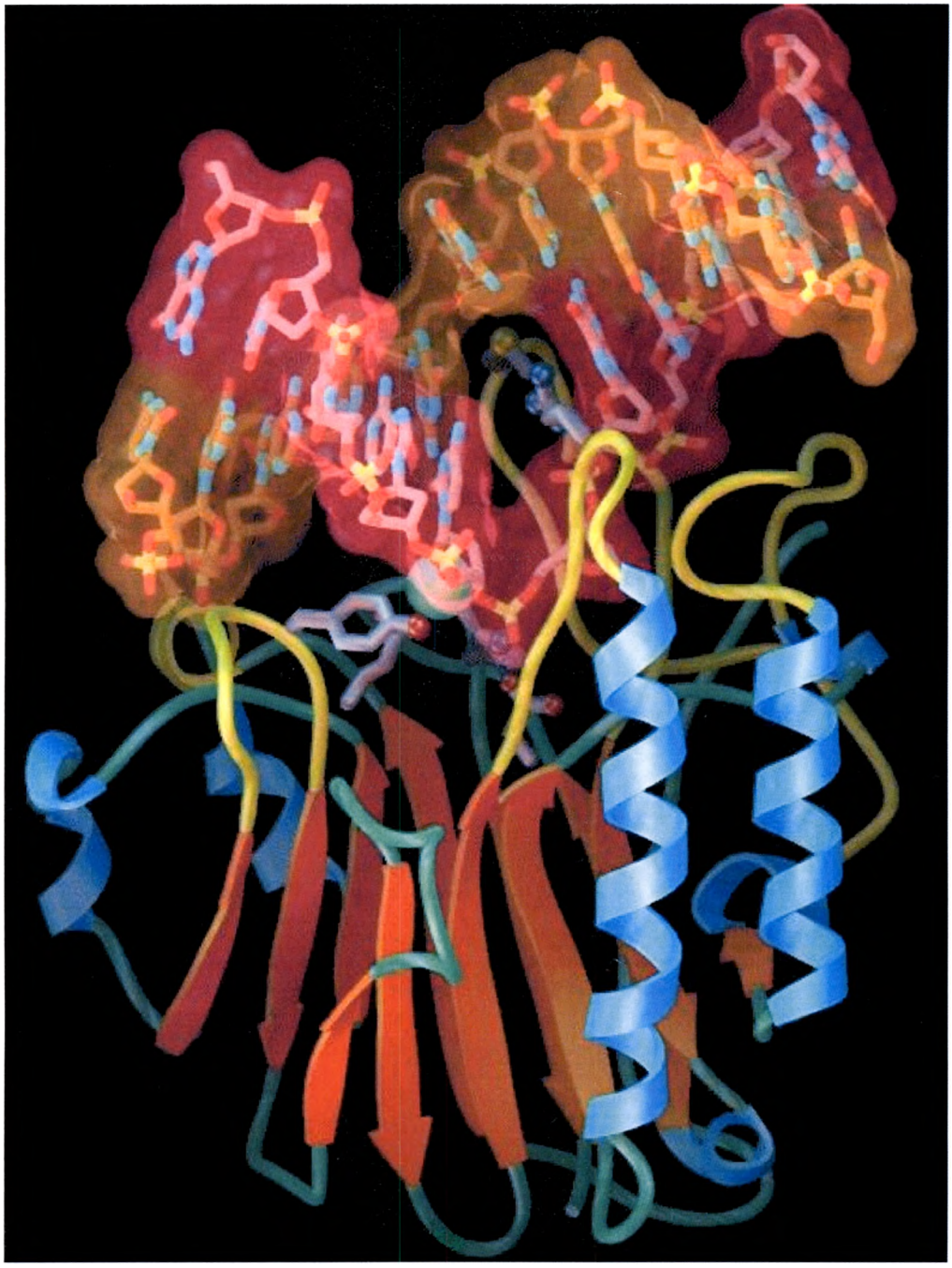

Figure 1-4. 3-dimensional ribbon diagram of AP endonuclease protein as determined by X-ray crystallography (Mol et al., 2000). The protein is oriented adjacent to an abasic site in the DNA by a magnesium (Mg^{2+}) cofactor. The light blue regions represent α-helices, red arrows represent strands, yellow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

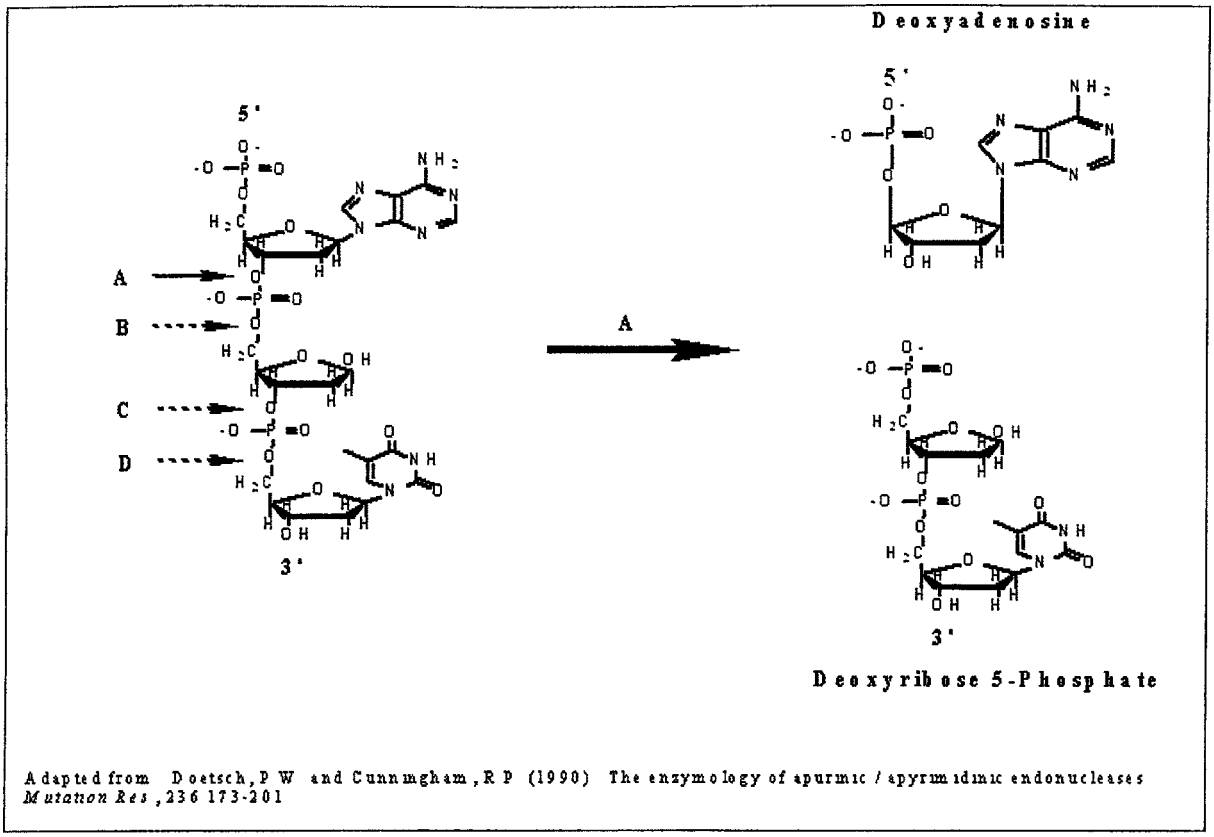

Figure 1-5. Possible cleavage sites of AP endonuclease. Product represents cleavage at site A, resulting in a 3' deoxyadenosine and 5' deoxyribose-5-phosphate.

3' to the 5'-terminal deoxyribose. This yields a suitable one-gap substrate for DNA pol-β. Cleavage at site C occurs via a β-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-β, 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-β (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-κβ. 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,

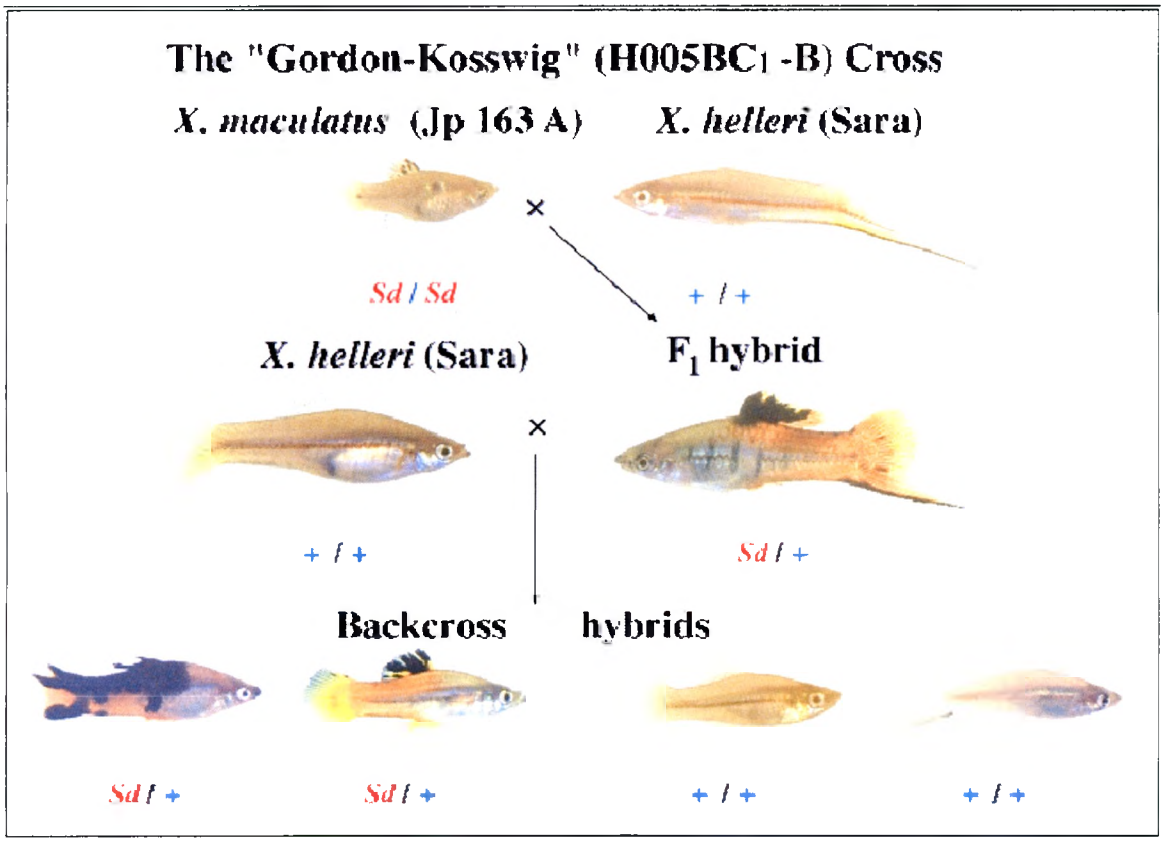

Figure 1-6. The classical Gordon-Kosswig (hybrid 5) cross. The loci derived from *X. maculatus* are colored red, while the loci derived from the swordtail *X. helleri* are colored blue. Nodular melanoma typically occurs in animals showing the phenotypes similar to the leftmost backcross hybrid (lower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

backcrossing the F_1 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™ (Ambion; Austin, TX). Each tissue was homogenized in 1 ml TRI REAGENT™ / 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™. 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™ 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 re-suspended in 60 µl nuclease-free water (double distilled water (ddH_2O) treated with 1%

diethyl-pyrocarbonate (DEPC)). The solution was allowed to incubate for 15 min. in a 55°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® DNA Purification Kit (Gentra Systems; Minneapolis, MN). Tissues were dissected from fish and placed in individual 1.5 ml microcentrifuge tubes containing 600 µl 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 µl (20 mg/ml) Proteinase K solution was added and allowed to incubate overnight at 55°C. After overnight incubation 3 µl *E. coli* RNAse A (2 units/µl) 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 µl 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 µl 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 µl 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 µl 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 λ phage DNA (100 µg/ml) or *E. coli* 16s and 23s rRNA (100 µg/ml). A *FL_X800* 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 $MgCl_2$, Invitrogen; Carlsbad, CA), 25 mM $MgCl_2$, 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_20 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
$MgCl_2$	25 mM
dNTP	6.25 mM
Primers	20 µM each primer
Taq polymerase	2.5 units
ddH_2O	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™ 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 μl of dNTP mix (2.3 mM each dNTP), 2 μl first-strand primers (50 μM 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 $MgCl_2$), 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_2O to a final volume of 12 μl 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 $MgCl_2$, 2 μl 10 x PCR buffer (200 mM Tris-HCl [pH 8.4], 500 mM KCl), 0.1 M DTT, 1 μl SuperScript™ II reverse transcriptase, and ddH_2O 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 μl *E. coli* RNAse Mix (2 units/μl) 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 x g for

1 min. The spin cartridge was transferred to a fresh tube, and 50 µl ddH_2O (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 µl 5 x tailing buffer (50 mM Tris-HCl [pH8.4], 125 mM KCl, 7.5 mM $MgCl_2$), 2 mM dCTP, 10 µl purified cDNA sample, and

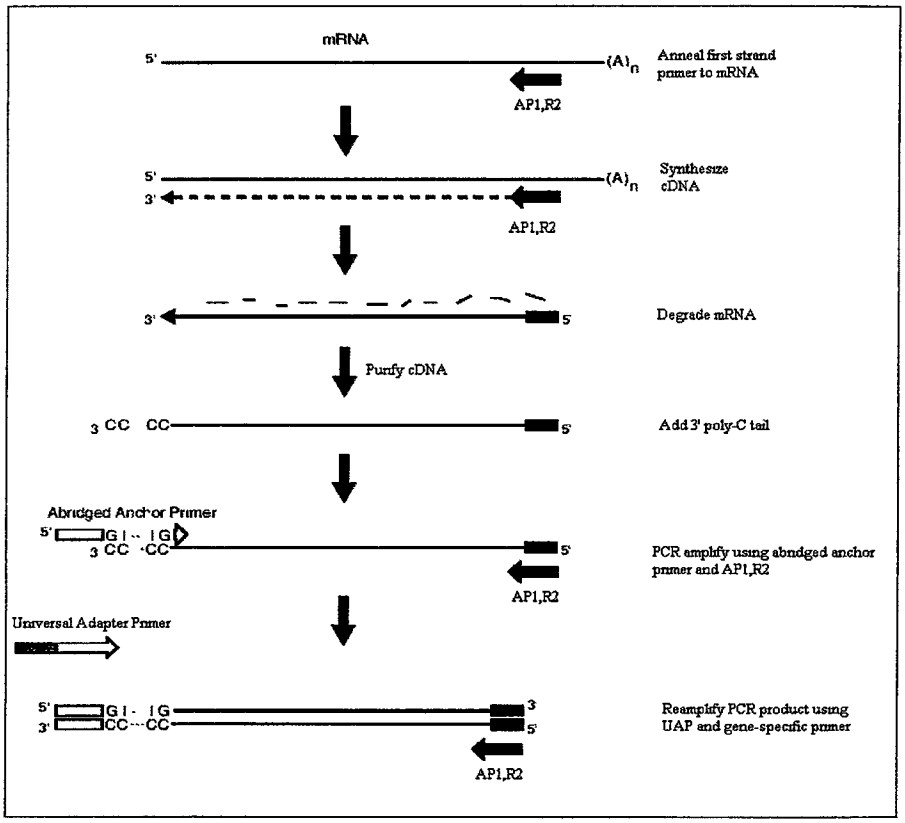

Figure 2-2. Overview of the 5' RACE procedure. Primer AP1, R2 anneals to the target mRNA in order to synthesize cDNA. RNAse degrades the mRNA template, the cDNA was purified and isolated, and terminal transferase adds a poly-C tail to the 3' end of the cDNA. PCR was performed using an Abridged Anchor 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 µl. This mixture was incubated for 3 min. at 94°C, and chilled on ice for 1 min. Terminal deoxynucleotidyl transferase (TdT, 15 units/µl) 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 µl 10x PCR buffer, 20 mM $MgCl_2$, 10 mM dNTP, 10 µM universal abridged primer (UAP, 5'-CUACUACUACUAGGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG – 3'), 20 µM primer (AP1,R1), 0.5 µl *Taq* DNA polymerase (5 units/µl) and ddH_2O to a final volume of 45 µl. 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 μM) 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) $MgCl_2$, 1 μl (10 mM) dNTP mix and 2 μl (0.1 M) DTT. This solution was gently mixed and equilibrated to 42°C. One μl SuperScript™ 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) $MgCl_2$, 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_2O 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.

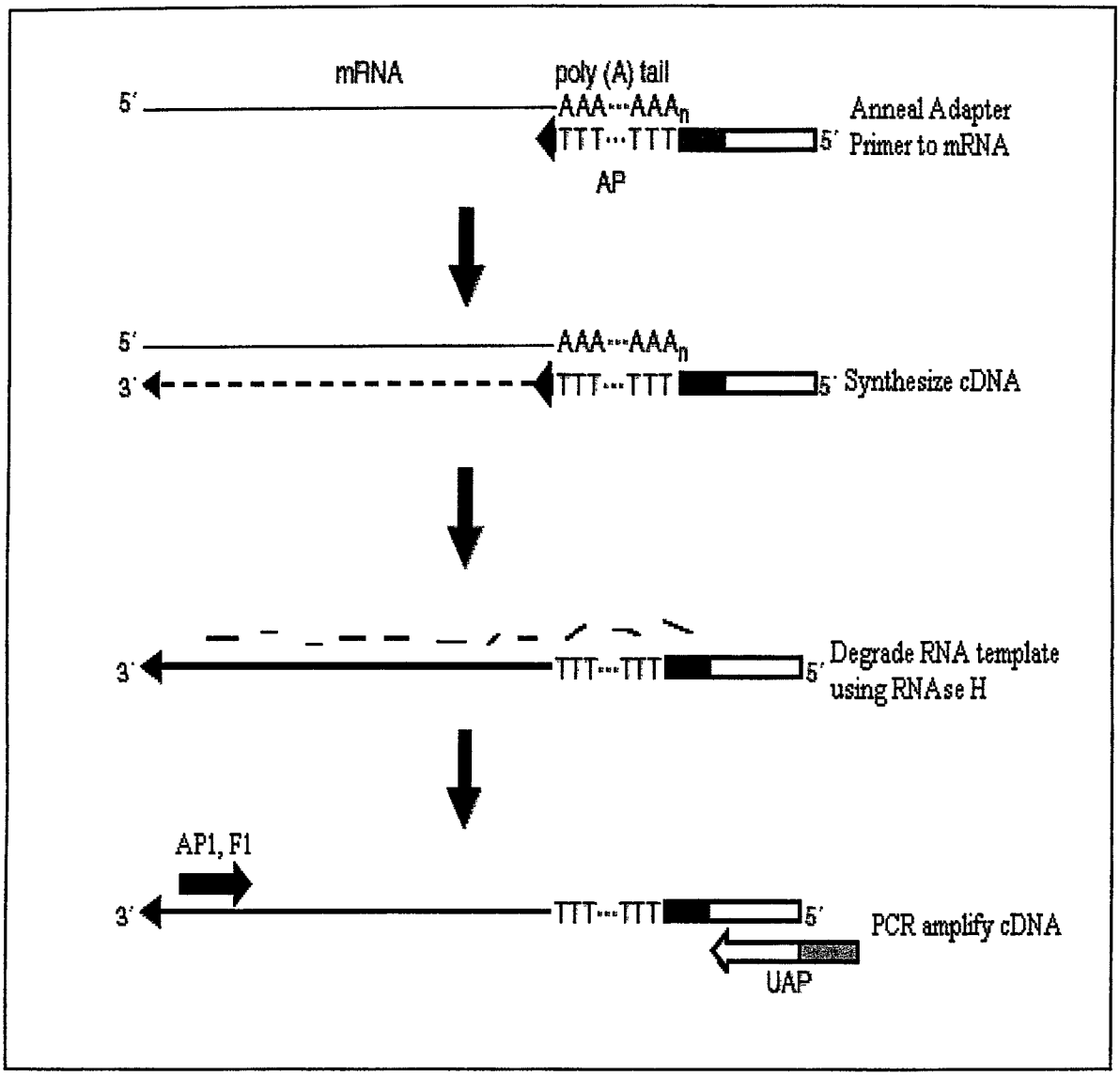

Figure 2-3. Overview of 3' RACE procedure. A poly-T Adapter Primer binds to the poly-A tail of the mRNA and synthesizes cDNA. RNAse H degrades the RNA template and a Universal Adapter Primer (UAP) and primer AP1, F1 were used to PCR amplify the cDNA.

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.™ 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.™ 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 µl 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® (Version L) kit (Invitrogen; Carlsbad, CA) was used to directly insert *Taq* polymerase-amplified PCR products into the pCR®2.1-TOPO® plasmid vector. Provided was linearized plasmid pCR®2.1-TOPO® 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 nontemplate-dependent 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 $MgCl_2$), and 1 μl pCR®2.1-TOPO® 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® cloning reaction was added to a vial of One Shot® 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 $MgCl_2$, 10 mM $MgSO_4$ and 10 mM glucose) was added, and the cells were shaken (200 rpm) at 37°C forl hour. After one hour 20 μl and 100 μl of the transformed cells were

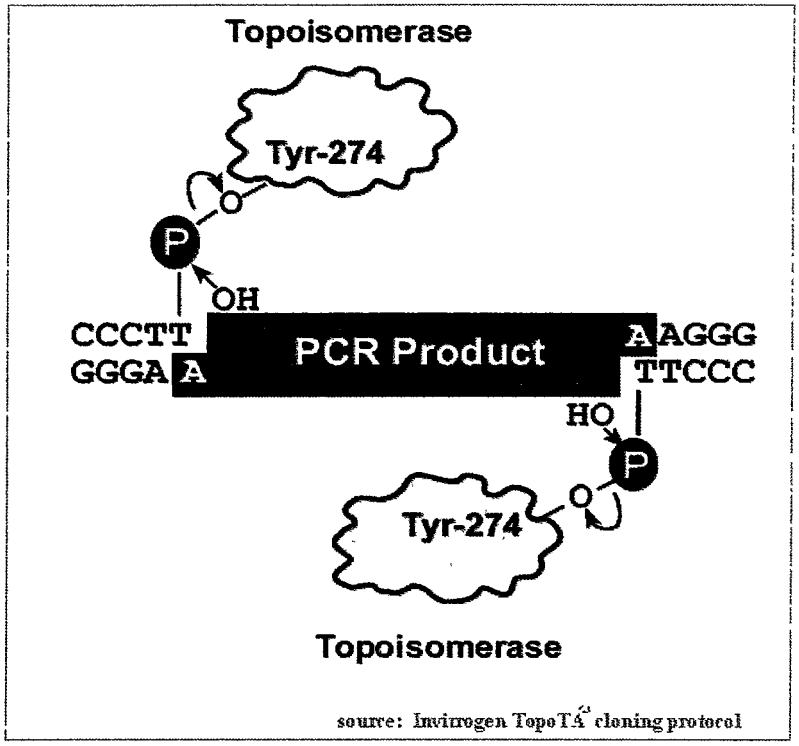

Figure 2-4. Illustration of the action of Topoisomerase I. Topoisomerase selectively binds to dsDNA and cleaves the phosphodiester backbone after 5'-CCCTT in one strand. The energy released by phosphodiester bond cleavage is conserved by formation of a covalent bond between the 3' phosphate of the cleaved 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 x g for 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.

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

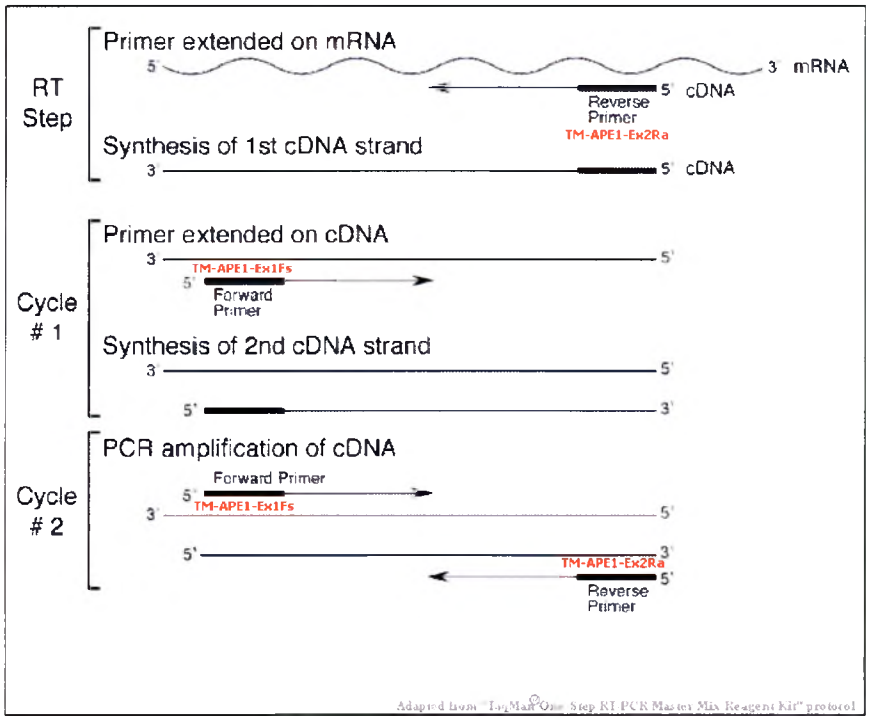

Figure 2-5. Overview of one-step, reverse-transcription PCR (RT-PCR). The reverse primer TM-APE1-Ex2Ra binds to the target mRNA and synthesizes cDNA. The forward primer TM-APE1-Ex1Fs is annealed to the cDNA and synthesizes the complimentary strand during the initial PCR cycle. Subsequent cycles use both 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® One-step RT-PCR kit (ABI; Foster City, CA) and the ABI Prism 7700 Sequence Detector (ABI; Foster City, CA). Using the TaqMan® 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

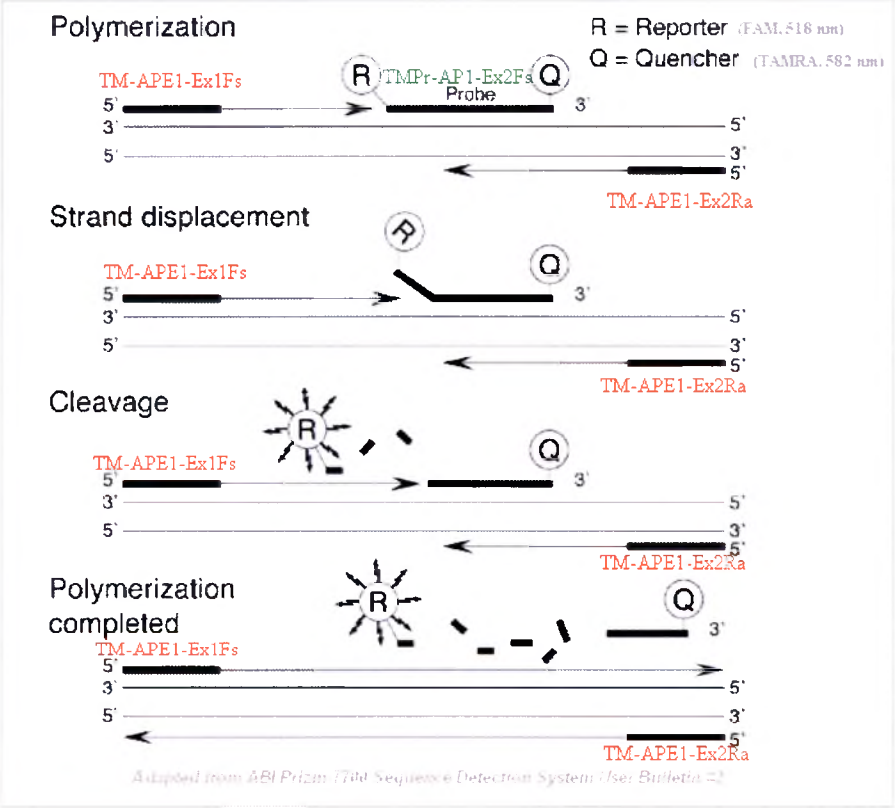

Figure 2-6. Illustration of probe cleavage and fluorescence detection. PCR amplification results in the displacement and cleavage of the fluorescent probe. Cleavage separates the reporter (FAM) from the quencher (TAMRA), and laser excitation results in detection of a fluorescent signal (518 nm) indicative of accumulation of PCR products.

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™ 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 double-distilled 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.	40 cycles
Annealing / extension	55	30 sec.	

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 large-scale sequencing projects (Benson et al., 1998). Nucleotide sequences were downloaded from the NCBI database into the MacVector™ 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™, and degenerate primers were designed using Primer Express™ (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_2	AGAGGCAAGAAGACAGATGATGGGTC	PCR

Table 3-1. Synthetic oligonucleotide primers used in PCR, 5' RACE, 3' RACE and one-step 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).

Oligonucleotide Primer	Primer Sequence	Use
AP1 PMR_1	TAGAAGTTTGGGAACTCGGCTGTG	PCR
AP1 PMR_2	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).

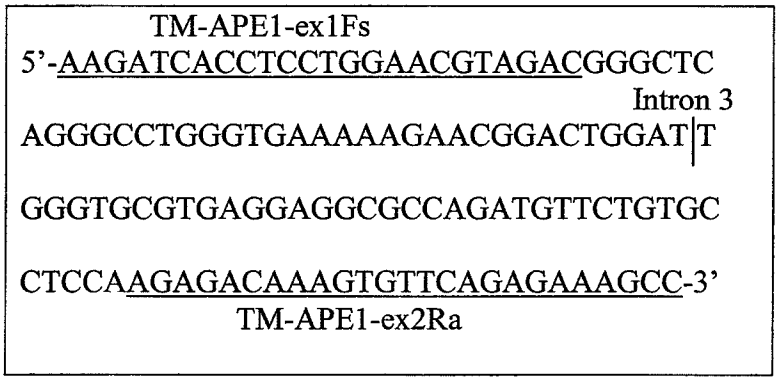

Figure 3-1. 123 bp amplicon obtained using primers TM-APE1-Ex1Fs and TM-APE1-Ex2Ra.

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.

3' RACE:

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

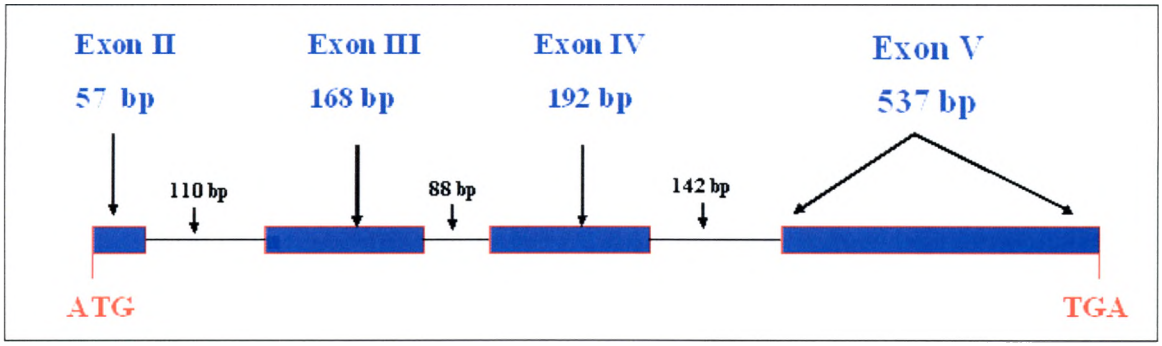

Figure 3-2. Gene structure of *Xiphophorus maculatus* AP endonuclease. Exons and their sizes are labeled in blue and introns are labeled in black. Positions of the start and stop sequences are labeled in red.

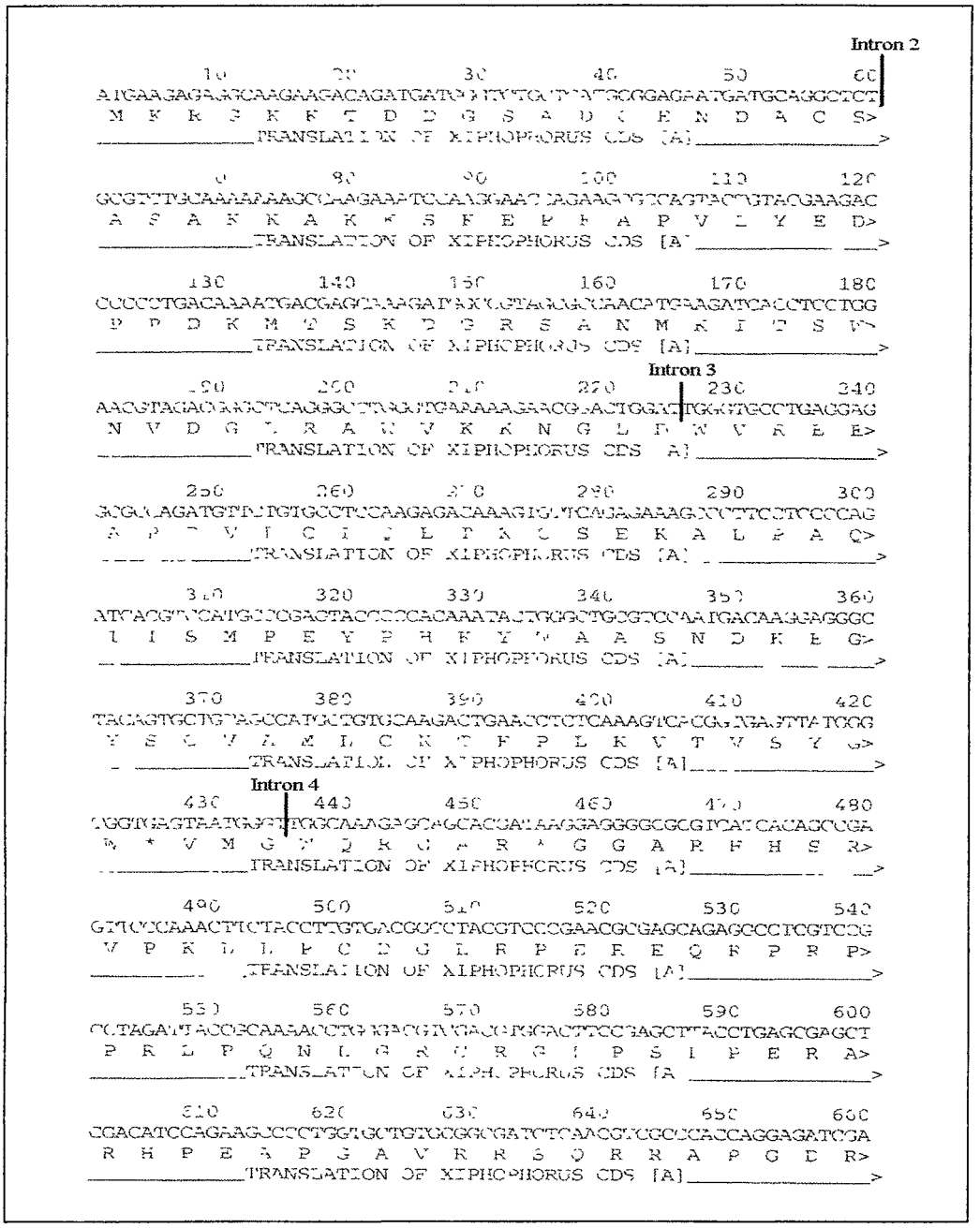

Figure 3-3. Nucleotide sequence of the coding region of AP endonuclease in *Xiphophorus maculatus*. Amino acid sequence is listed under each codon, and intron positions within the coding region are labeled.

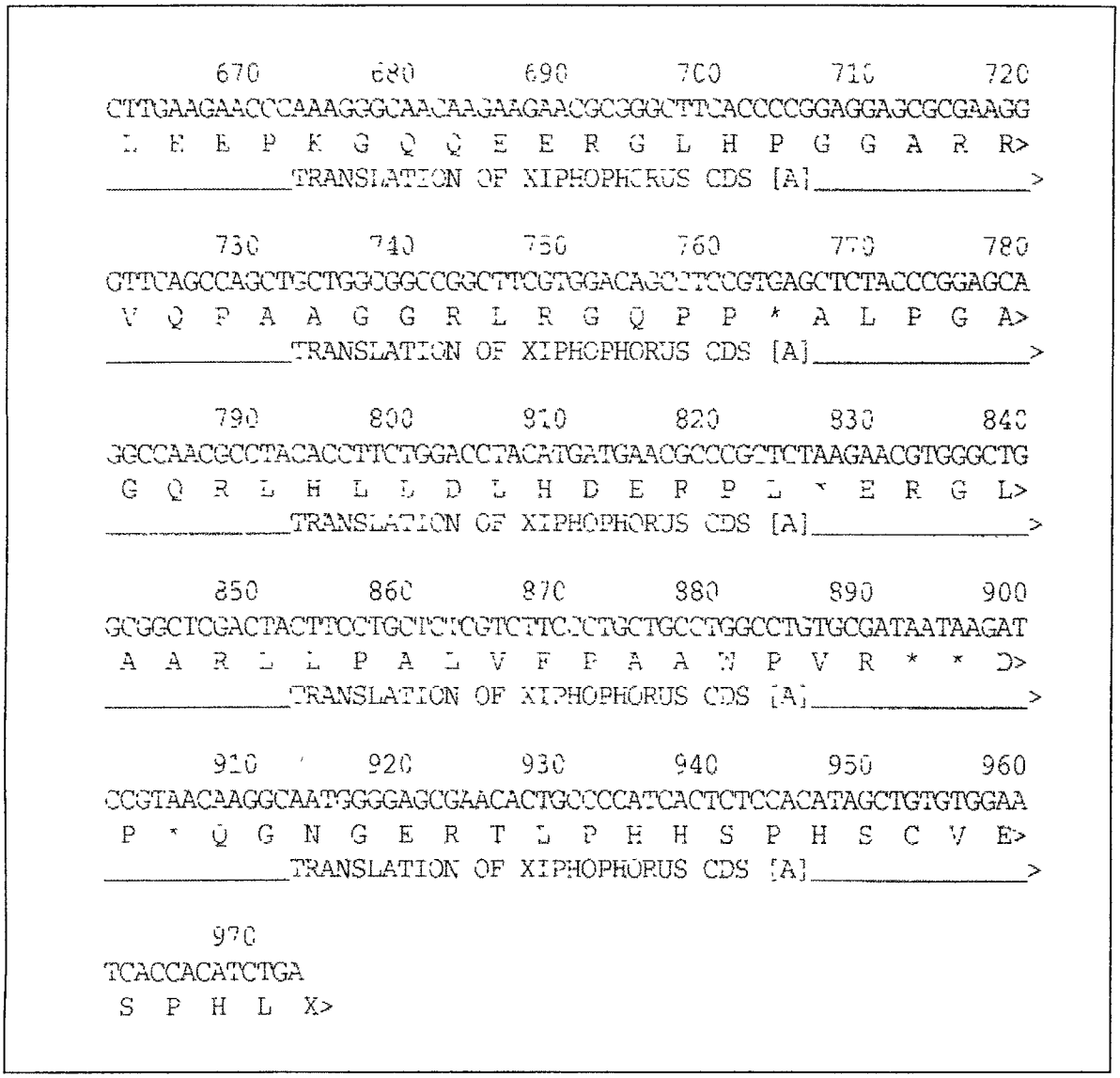

Figure 3-3 (continued).

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

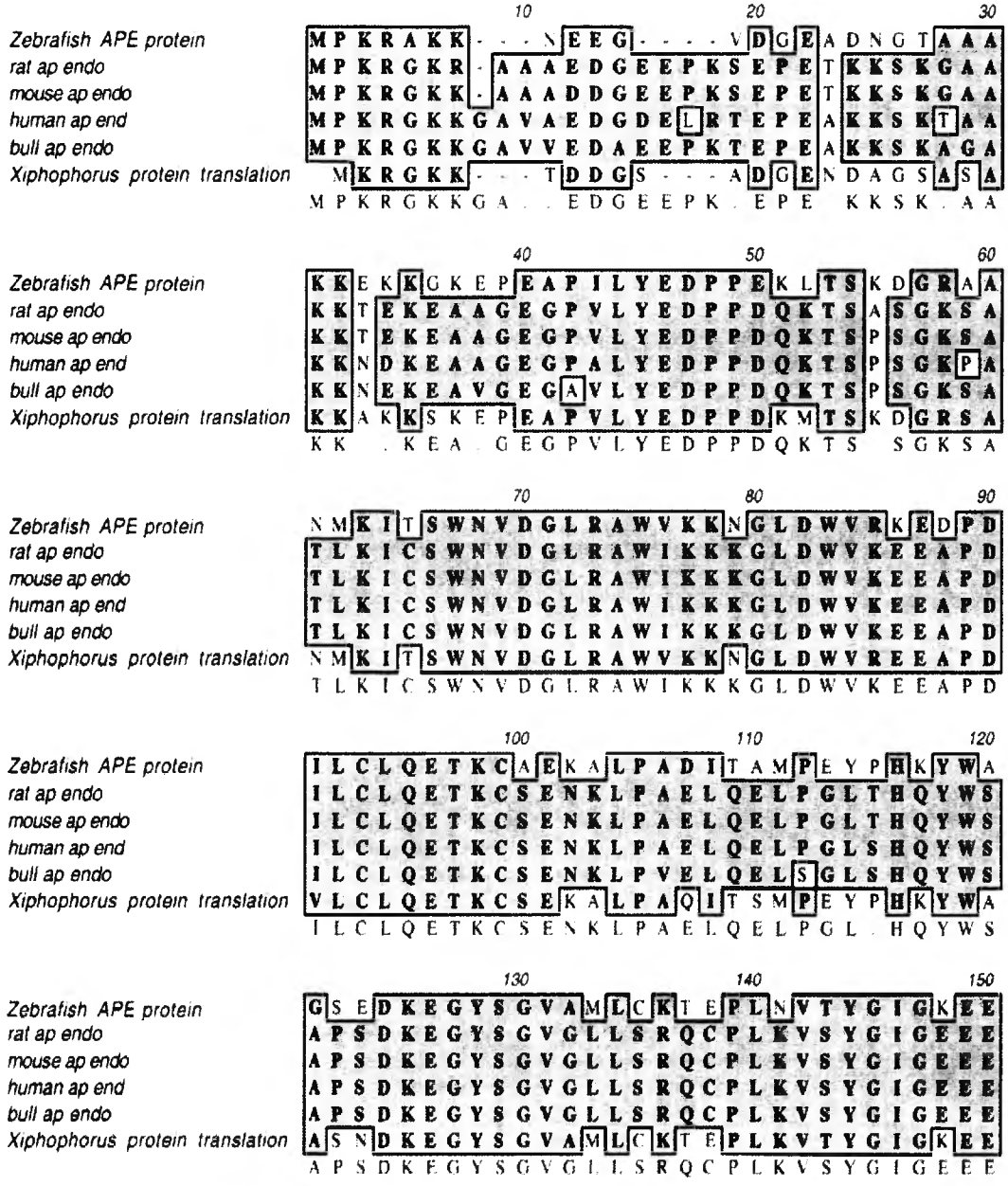

Figure 3-4. Clustal W alignment of the AP endonuclease amino acid sequences of 6 species. Grey areas represent amino acid homology/similarity among species. *Xiphophorus* AP endonuclease amino acid sequence exhibits the greatest homology with Zebrafish (84% identity, 90% similarity) and exhibits relatively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

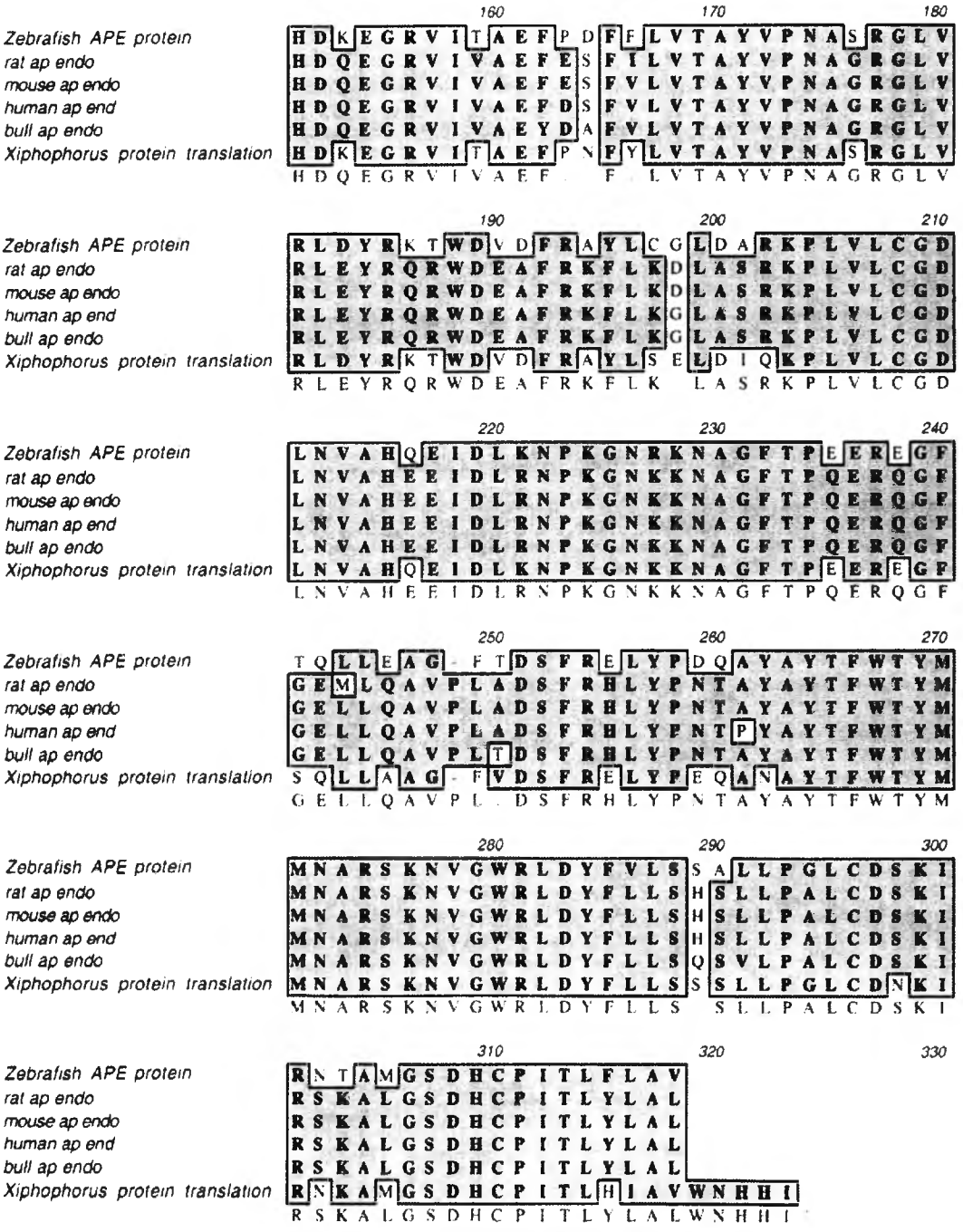

Figure 3-4. (continued)

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_1) 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_1 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-by-two 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

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_1 hybrid as a homozygote or heterozygote. A panel of fifty BC_1 hybrid DNAs was scored as either exhibiting the banding pattern of the *X. helleri* (homozygous) parent or the pattern

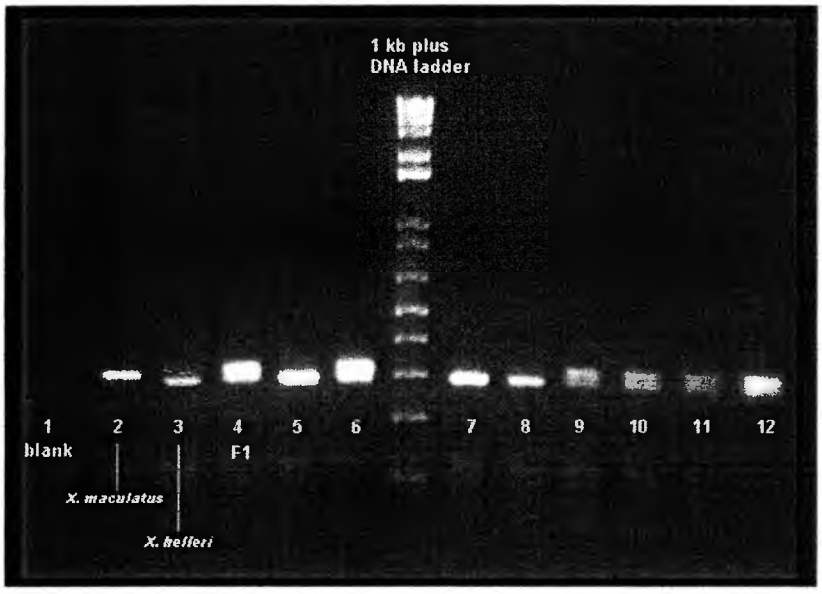

Figure 4-1. Image of agarose (2%) gel exhibiting the banding patterns for Hybrid 5 ([*X.maculatus* (x) *X. helleri*] (x) *X. helleri*) parents, F_1 and BC_1 hybrids (lanes 5-12). Backcross hybrids in lanes 6, 9, 10, 11, and 12 exhibit the banding pattern of the F1 parent, while backcross hybrids in lanes 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).

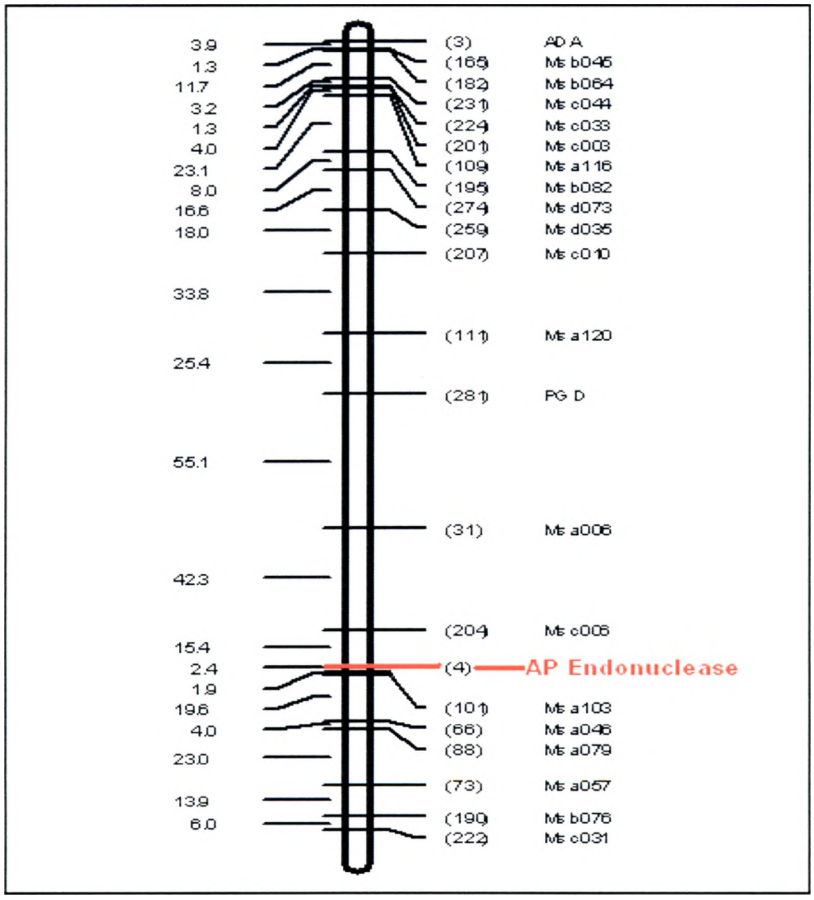

Figure 4-2. Map position of AP Endonuclease within chromosome I in *Xiphophorus*. Distance (cM) between loci is located on the left side of the figure. One centimorgan is the distance between two loci which have a 1% chance of being separated during recombination in a single generation.

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 ng/μl) 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

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

Oligonucleotide Primer / Probe	Sequence
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.

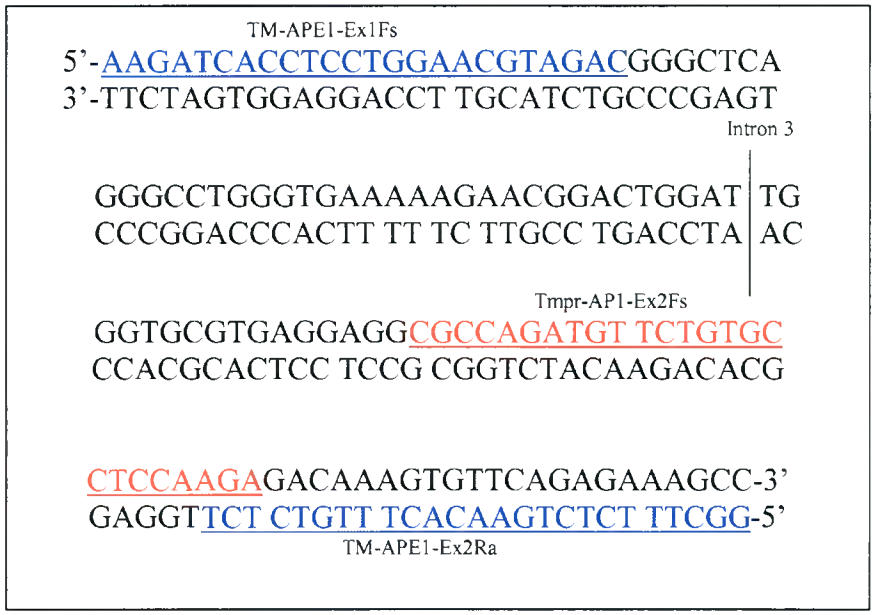

Figure 5-1. 123 bp amplicon using primers TM-APE1-ex1 Fs and TM-APE1-ex2 Ra. The position of the probe (Tmpr-AP1-Ex2Fs) relative to these primers is shown in red.

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.

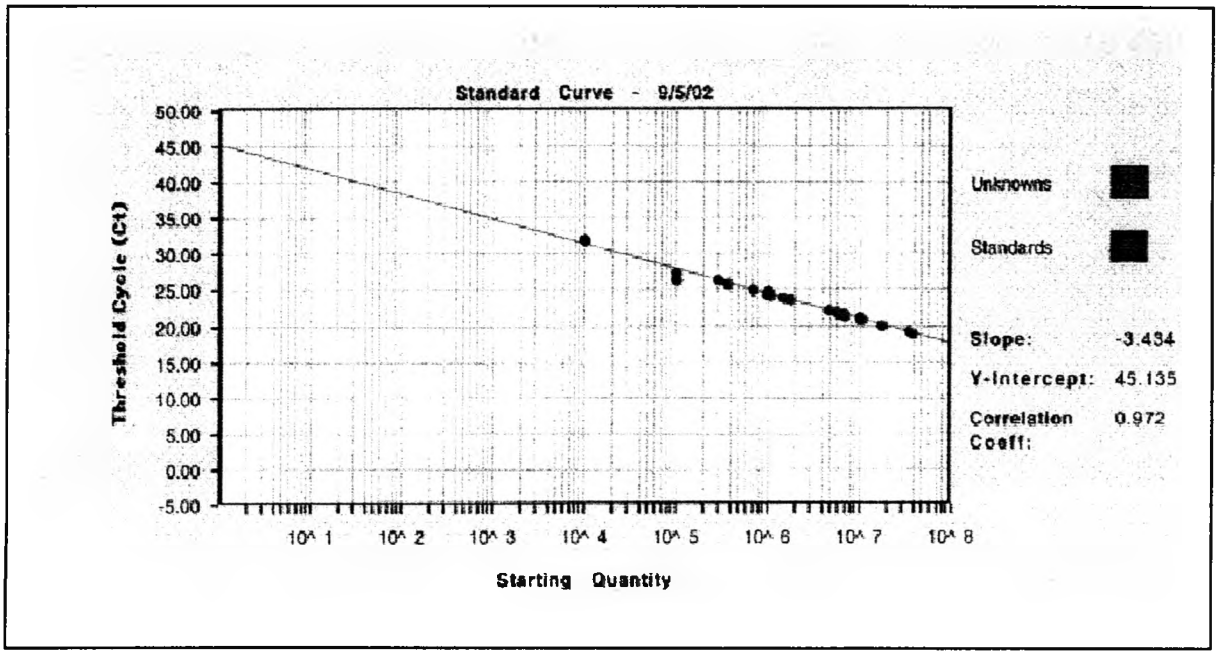

Figure 5-2. Graph of starting quantity vs. threshold cycle for a one-step real-time RT-PCR reaction. Dilutions of plasmid pAPE containing inserts specific to AP endonuclease were used to generate a standard curve. Comparison of the data from various tissues in *Xiphophorus* to the standard curve allows 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

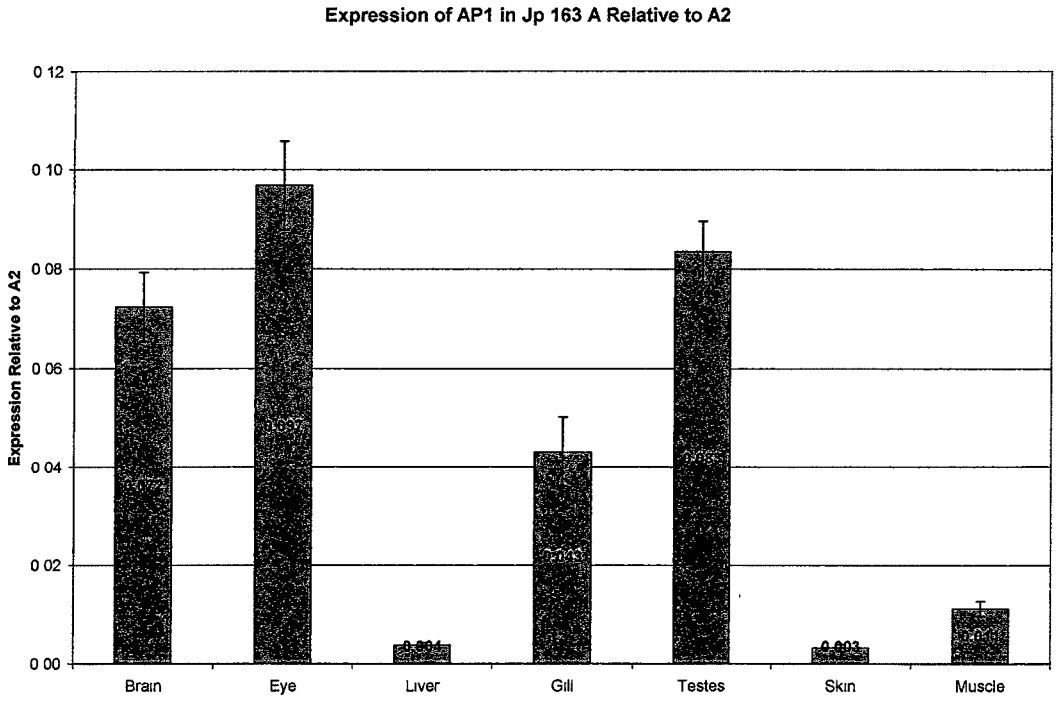

Figure 5-3. Expression of AP Endonuclease in seven tissues from healthy *X. maculatus* (Jp 163 A) relative to A2 calibrator (A2 = 1). Expression in brain, eye, gill, and testes is more than threefold higher than in liver, skin and muscle

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)

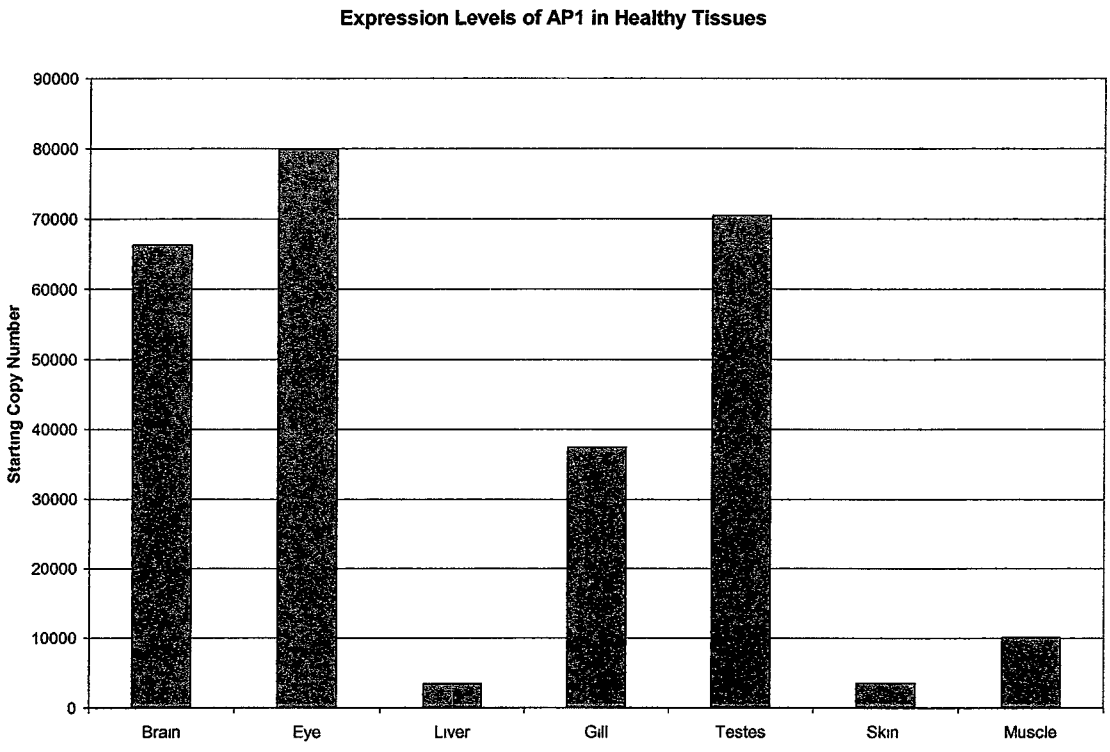

Figure 5-4. Starting copy number (per 10 ng) of AP endonuclease transcripts in healthy tissues of *X. maculatus*. Starting copy number was determined by comparing expression data for seven *X. maculatus* tissues to a series of dilutions of plasmid pAPE containing inserts specific to AP endonuclease.

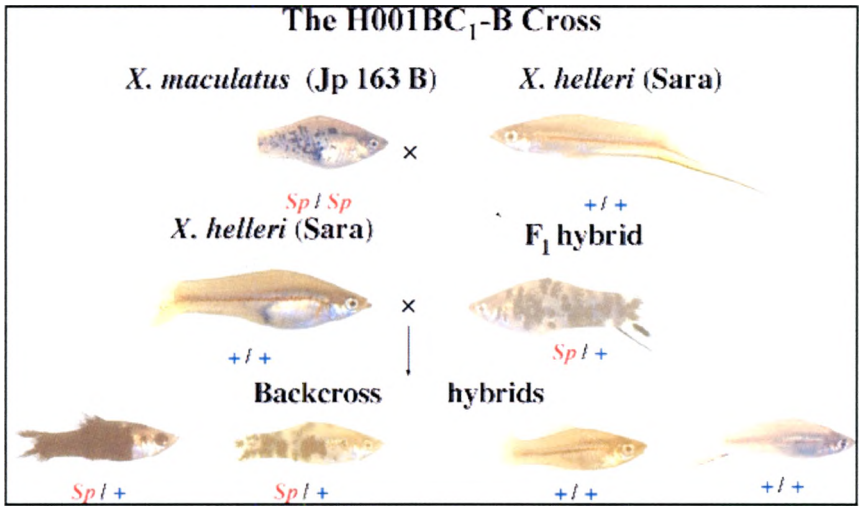

Figure 5-5. Interspecies hybrid 1 ([*X. maculatus* (x) *X. helleri*] (x) *X. helleri*) backcross. The loci derived from *X. maculatus* are colored red, while the loci derived from the swordtail *X. helleri* are colored blue. Backcross hybrids were used to assess AP endonuclease expression in melanized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

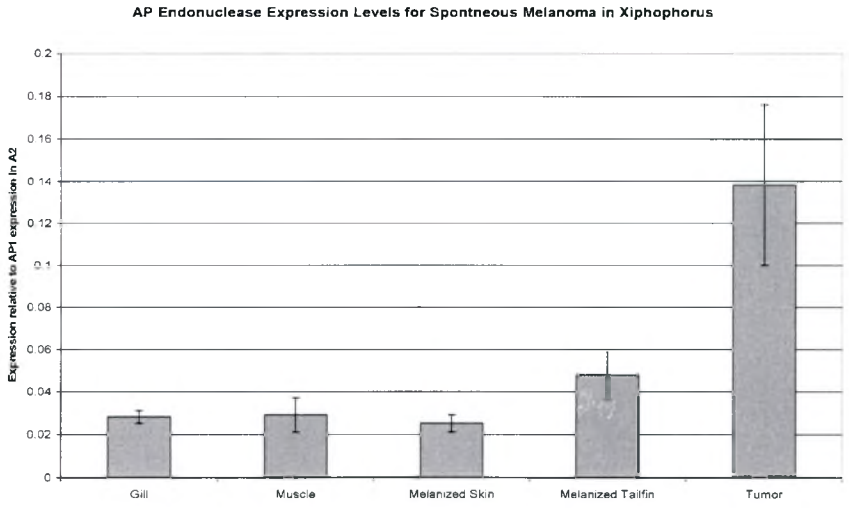

Figure 5-6. Expression of AP endonuclease in various tissues of BC_1 hybrids of *Xiphophorus* tumor bearing fish relative to A2 calibrator (A2 = 1). As expected, expression of AP endonuclease in melanoma tumor tissue is higher (~ threefold) than expression in other tissues.

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.

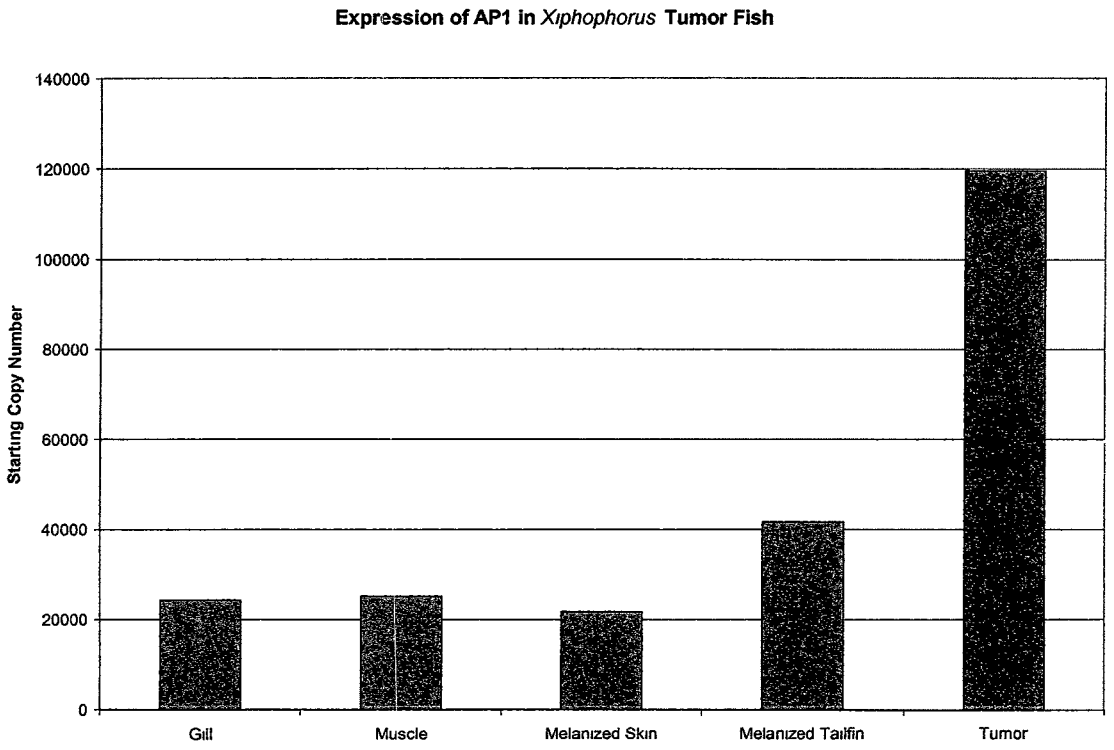

Figure 5-7. Starting copy number (per 10 ng) of AP endonuclease transcripts in various tissues of *Xiphophorus* Hybrid 1 tumor fish. Starting copy number was determined by comparing expression data for five *X. maculatus* tissues (gill, muscle, melanized skin, melanized tailfin, and melanoma tumor tissue)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

Ramiro T. Sosa III was born in Austin, Texas on March 26, 1974, the son of Mr. and Mrs. Ramiro Sosa, Jr. He received his Bachelor of Science degree in Biology from Texas State University – San Marcos (formerly Southwest Texas State University) in December 2001. He began his Master's program in the Department of Chemistry and Biochemistry at Texas State University – San Marcos in January 2001. From January to June 2003 he served with 4th Reconnaissance Bn., 4th MARDIV, United States Marine Corps in Kuwait and Iraq as part of Operation Enduring Freedom and Operation Iraqi Freedom. He returned to the United States in July, 2003 and continued his Master's program in Fall 2003. While at Texas State University – San Marcos he worked as a Teaching Assistant in the Department of Chemistry and Biochemistry and as a Research Assistant in the Molecular Bioscience Research Group under Dr. Ronald B. Walter.

Permanent Address: 10019 Stonemont
La Porte, TX 77571

This thesis was typed by Ramiro T. Sosa III.