MOLECULAR CHARACTERIZATION, TRANSCRIPTIONAL EXPRESSION, AND MAPPING OF A *X. maculatus RAB27* GENE

THESIS

PRESENTED TO THE GRADUATE COUNCIL OF
SOUTHWEST TEXAS STATE UNIVERSITY
IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS

FOR THE DEGREE OF MASTER OF SCIENCE

BY

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SAN MARCOS, TEXAS
AUGUST 2001

Acknowledgements

I am grateful to Dr. Ron Walter for teaching me the science of research through both his guidance and ideas behind the laboratory work for this project. It has been a gratifying experience from which the techniques and principles will follow me in my future.

I would also like to thank Dr. Steve Kazianis, Dr. Jake Kirchner, and Dr. Linette Watkins for contributing and editing this thesis. I am thankful to Dr. Kazianis for his additional information and effort in the gene mapping section.

I am very thankful for the friendships of Doug Rains, Angela Wheeler, Tina Daniels, Patrick DeGuelle, Anthony Pedroza, and Ramiro Garcia, for they all added the needed fun over the years and supplied input on various techniques tailored for this project. I am especially grateful to Angela for giving the extra time for my questions and editorial comments towards this manuscript.

I would like to thank my wife, Dawn Kelnar, for the endless support and push to drive me to the finish-line. I also would like to thank the rest of my family, especially my parents, for the prayers and the opportunity to continue my education.

This research was funded by grants from the National Center for Research Resources (R24-RR12253), NCI (PO1-CA75137), and the Roy F. and Joan C. Mitte Foundation.

Table of Contents

List of Tables

Tables

List of Figures

Chapter 1

Introduction

Rabs represent a family of GTP-binding proteins in the ras superfamily. The ras superfamily of genes encode membrane bound GTP binding proteins that are thought to participate in a regulated activation/deactivation cycle (Valencia et al, 1991, Gutierrez et al., 1989). Upon activation, ras superfamily proteins are involved in signal transduction and cellular vesicle transport pathways. Characteristics of ras superfamily members include highly conserved GTP-binding domains (GTPase activity) necessary for protein activation (Barbacid, 1987) and a conserved C-terminal motif (Gibbs, 1991). *RAB* genes constitute a subfamily of the ras superfamily and play a role in the regulation of vesicle trafficking (Novick and Brennwald, 1993). There are over 50 known mammalian Rab proteins each responsible for docking inter- and intracellular transport vesicles (Pereira-Leal and Seabra, 2000). Detailed herein is the cloning and characterization of a *Xiphophorus* fish gene structurally related to the mammalian ras family of genes and exhibiting sequence homology to the human *RAB27A* and *RAB27B* genes.

***RAB* Genes - Genomic Structure**

The *RAB27*subfamily is comprised of two genes, *RAB27A* and *RAB27B*, that may have arisen via ancestral gene duplication since they share 67% nucleotide identity. The human *RAB27A* and *RAB27B* genes were mapped by radiation hybrid techniques and fluorescent *in situ* hybridization (FISH) analyses to human chromosomes 15q15-21.1

(*RAB27A*) and 18q21.1 (*RAB27B*) (Ramalho et al., 2001, Tolmachova et al., 1999).

RAB27A was initially isolated as a homologue of the rat *ram* p25 (hereafter termed *RAB27A*), while *RAB27B* encodes a human protein previously designated c25KG (hereafter termed *RAB27B*). Human *RAB27A* encompasses 65 kb of genomic DNA containing 2 untranslated exons (1a and 1b) and 5 translated exons (Chen et al., 1997a, Tolmachova et al., 1999). An extremely large intron (33 kb), between untranslated exons 1a and 1b corresponds to nearly half of the human *RAB27A* genomic sequence. The exon/intron sizes for *RAB27A* and *RAB27B* are presented in Table 1-1 and a pictorial representation of the gene structure is displayed in Figure 1-1. Upstream of the 5' start codon is a TATA box on the antisense strand at position –15 (TTTACA) that serves as the *RAB27A* transcriptional start site (Chen et al., 1997a).

Human *RAB27B* encompasses 69 kb of genomic DNA and is comprised of 6 exons. As in *RAB27A,* there are 5 translated exons, however, only a single untranslated exon is found in *RAB27B* located 49 kb upstream of exon 2. The *RAB27B* exon 6 has an exceptionally long (6.4 kb) 3' untranslated region (UTR) of unknown function (Chen et al., 1997a).

	RAB27A		*RAB27B*	
Exon Number	Exon Size (bp)	Intron Size (bp)	Exon Size (bp)	Intron Size (bp)
1	-	-	71	49,000
1a	~230	33,000	-	-
1b	89	3,400	-	-
2	175	4,000	172	1,600
3	86	2,000	86	5,300
4	104	4,500	104	3,500
5	124	14,000	124	1,100
6	2,740	-	6,624	-

Table 1-1. Exon/intron comparison of the human *RAB27A* and *RAB27*B genes.

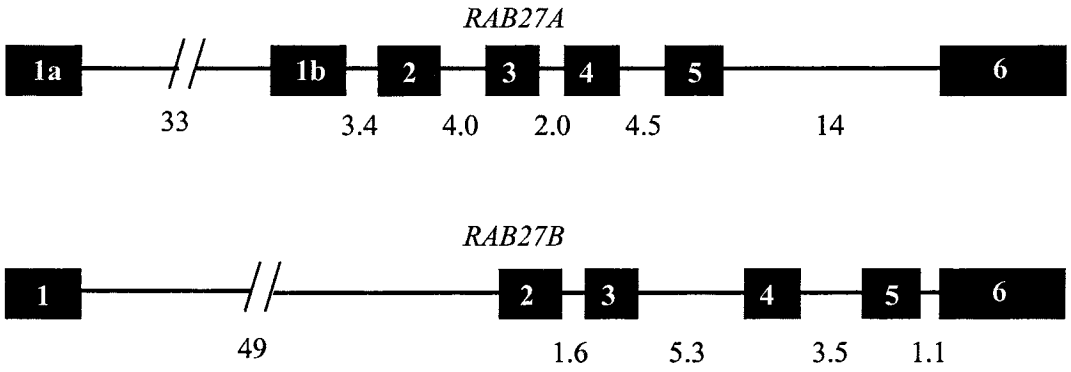


Figure 1-1. Molecular arrangement of human *RAB27A* and *RAB27B* genes. Exon and intron junctions are indicated where black boxes represent exons. Numbers between exons represent the intron sizes (in kb).

Transcription of the *RAB* Genes

The mRNA sequences of *RAB27A* and *RAB27B* were elucidated via cloning of cDNA derived from human melanocytes and melanoma cell lines. *RAB27A* and *RAB27B* mRNAs share 66% identity in their open reading frames (ORFs) but much reduced homology in the 5' and 3' UTRs, especially distal to GTP binding domain IV (Chen et al., 1997a; Figure 1-2).

Northern blot analyses used to determine tissue specific expression of *RAB27A* and *RAB27B* indicate differences in expression among mammalian species (i.e. mouse and human.). For example, *RAB27A* is expressed in human liver but not in mouse liver, at least at the level detectable in northern blots (Chen et al., 1997a).

Using a cloned 3'-RACE (rapid amplification of cDNA ends) fragment of *RAB27A* as a probe, one to three transcripts were detected (1.3, 2.7, and 3.7 kb in size) in many human tissues. The 3.7 kb transcript was present in all human tissues examined,

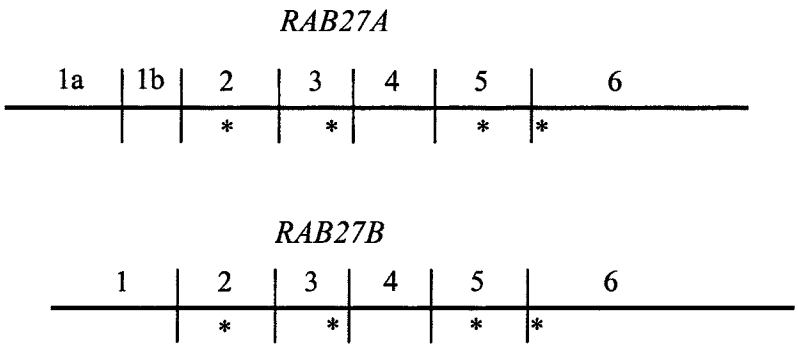


Figure 1-2. Molecular arrangement of the major human *RAB27A* and *RAB27B* transcripts. The asterisks (*) represent GTP-binding domains I-IV.

except brain, and also found expressed in several tumor cell lines (Chen et al., 1997a). The different size transcripts are considered the result of numerous poly-A addition sites located in the 3' UTR (Tolmachova et al., 1999). Chen et al. (1997a) reports the 1.3 kb transcript had lower expression levels (no quantitative data published) compared to the 3.7 kb transcript in most tissues, except for expression in leukocytes where the ratio of 1.3 kb transcript to 3.7 kb transcript appeared higher. Tumor cell line studies indicate *RAB27A* transcript was relatively highly expressed in promyelocytic leukemia HL-60, chronic myelogenous leukemia K-562, Hela, and melanoma (G361) cells (Chen et al., 1997a). In contrast to *RAB27A*, expression of a 1.4 kb *RAB27B* transcript is observed at the highest levels in testes tissue and expressed at very low levels in other tissues examined. The 1.4 kb *RAB27B* message was detected in melanoma, melanocyte and fibroblast cell lines by RT-PCR (Chen et al., 1997a). In addition, using a PCR-based strategy, pigmented melanoma cells were shown to express at least 17 different Rab mRNAs, including *RAB27A* and *RAB27B* (Chen et al., 1997b).

Protein Structure of the Rab27 Sub-Family

Rab27a and Rab27b proteins share 71% amino acid identity, but exhibit relatively low homology to other Rab subfamily proteins (e.g., 41-44% with the Rab3 sub-family; Chen et al., 1997a). Rab proteins contain four highly conserved GTP-binding domains (I to IV) implicated in the activation cycle and a consensus CC or CXC C-terminal sequence (Nuoffer and Balch, 1994, Chen et al., 1997a). Ostermeier and Brunger (1999) report a crystal structure of a Rab protein family member (Rab3a) in the GTP-bound state. This crystal structure supports a hypothetical Rab3a protein domain which may associate with effector proteins. The two areas of effector/Rab interaction include: (1) the nucleotide binding site areas (GDP/GTP); and (2) a Rab complimentarity-determining region (RabCDR), having variable sequence regions among Rab family members that may serve to establish specificity of interaction between each Rab protein and specific downstream effectors (e.g., see t-SNARE inhibitors in the **Protein Shuttle** section, below; Chavrier and Goud, 1999).

Rab27a and Rab27b proteins possess GTP-binding domains, C-terminal motifs, and comparable putative effector domains exhibited in all Rab subfamily proteins, but there are several differences in 27a and 27b that set them apart from other members of the Rab subfamily (Chen et al., 1997a). Rab27 members possess a 10 amino acid insertion between GTP-binding domains I and II that is not observed in other Rabs (Chen et al., 1997a). Also, highly conserved amino acids among all other Rab family members are not present in Rab27a and Rab27b proteins suggesting that Rab27a and 27b may constitute their own subfamily (Chen et al., 1997a). An additional difference of the Rab27 subfamily is in the number of amino acid residues and proteins sizes. Rab27a is a 221

amino acid polypeptide with a molecular weight of 25 kDa in size, while Rab27b is a 218 amino acid polypeptide having a molecular weight of 24.7 kDa (Chen et al., 1997a).

The Rab Activation Cycle

Rab proteins undergo cyclic activation and deactivation between a membrane-bound, active GTP-bound form and a cytosolic, inactive GDP-bound form (Figure 1-3). The GTP-binding domains (I to IV) are implicated in the GTP/GDP conversion during the Rab cycling (Chen et. al., 1997a; Figure 1-2). In the inactive form, a guanine nucleotide exchange factor (GEF) promotes release of GDP from Rab, allowing binding by a new GTP (Gonzalez, 1999). When in its active, GTP-bound form, Rab associates with effector proteins that assist in fusion of a transport vesicle with a target organelle. To return Rab to its inactive and GDP-bound form, a GTPase activating protein (GAP) catalyzes the hydrolysis of GTP to GDP. The result of GAP activity, GTP hydrolysis, commits Rab proteins to a unidirectional activation/inactivation pathway (Bourne et. al., 1990). A crystal structure of the GDP-bound Rab protein has not yet been solved, however, it is speculated that upon GTP hydrolysis, Rab protein conformation is changed rendering them unrecognizable to effector proteins (Gonzalez, 1999). Rab is then shuttled to the donor organelle membrane for a new round of protein transport (see **Protein Shuttle**, below).

Under normal cellular conditions, GTP is more apt to enter the nucleotide binding site of the Rab proteins than GDP, which places the protein in the active form (Bourne et al., 1991). Thus, the amount of membrane-bound active Rab protein relies on: (1) the dissociation of GDP from the inactive GDP-bound Rab by GEFs; $k_{\text{diss GDP}}$; and (2) the hydrolysis of GTP to GDP from the active GTP-bound Rab by GAPs; $k_{\text{cat GTP}}$. This

suggests that levels of active Rab protein can be raised by increasing the GEF activity and/or decreasing GAP activity (Bourne et al., 1990).

Geranylgeranylation of Rab

Rab proteins are distinguished from other Ras superfamily proteins by major structural differences at their C-terminal regions. The C-terminal region of Ras contains a CAAX motif (C = Cysteine, A = any aliphatic residue, X = any amino acid; Barbacid, 1987) which is essential for acylation and binding to a lipid bi-layer (Magee and Hanley, 1988). Ras proteins must be membrane bound to be in an active form and undergo a modification of the C-terminal (Treston and Mulshine, 1989). Gibbs (1991) has shown that a farnesyl transferase attaches a farnesyl pyrophosphate to the –SH group of the cysteine in the C-terminal CAAX region of unmodified Ras protein. This reaction forms a thioether bond and releases pyrophosphate (Gibbs, 1991). After formation of the thioether bond, the last three amino acids are cleaved leaving a cysteine residue at the C-

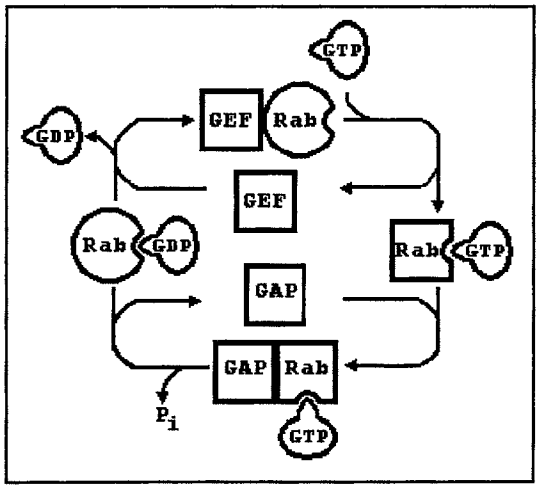


Figure 1-3. Rab protein is inactive when bound with GDP and active when bound with GTP. GEFs displace GDP, which then allow GTP to bind to and activate Rab. GAPs hydrolyze the GTP, which returns Rab to its inactive and GDP-bound state.

terminal (Magee and Hanley, 1988). The C-terminal may then be methylated by a carboxymethyl transferase increasing the hydrophobicity of the C-terminal region and providing a supplementary hydrophobic surface for attachment to membranes (Clarke et al., 1988).

In contrast to the Ras proteins, Rab's terminate in either CC or CXC (C=Cysteine, X=any amino acid). The CC or CXC motif in Rab proteins is essential for isoprenylation (Khosravi-Far et al., 1991). Geranylgeranyl (GG) groups must be attached to the Rab C-terminal to facilitate membrane binding (Seabra et al., 1995). Khosravi-Far et al. (1991) have shown a significant difference in the prenyltransferase that modifies Ras and Rab. Rab geranylgeranyl transferase (Rab GGTase) appears specific for the attachment of GG groups to the –SH group of one or both C-terminal cysteine residues in Rab proteins. Rab GGTase is an α/β heterodimer that exhibits structural homology to farnesyl transferase (Seabra et al., 1995). Also, an accessory protein, Rab escort protein (REP), is required for Rab GGTase activity. REP associates to unprenylated Rabs and transfers them to Rab GGTase for GG group transfer. The REP remains associated with the prenylated Rab and assists its delivery to the donor organelle (Seabra et al., 1992, Seabra et al., 1995). REP and GDP dissociation inhibitors (GDI) share homology in their structure and presumed mechanisms of delivering prenylated Rab proteins to a donor organelle. However, REPs traffic newly synthesized Rab proteins and GDIs aid in Rab recycling (Seabra et al., 1995). Like Ras, Rab proteins undergo C-terminal carboxymethylation (Farnsworth et al., 1991).

Protein Shuttle

Upon activation, prenylation, and membrane attachment, Rab proteins are capable

of vesicle transport (Figure 1-4). Rothman and Sollner (1997) have speculated the role of Rab proteins in the cellular vesicular transport pathway. According to their scheme, proteins are encapsulated in the bi-layer membrane of a donor organelle that contains an active, membrane associated (GTP-bound) Rab on its cytoplasmic surface. The encapsulated proteins bud from the donor organelle and travel through the cytoplasm to a target organelle possibly via associations with cytoskeletal elements and transport motors (Gonzalez, 1999). Specific SNAREs (soluble NSF [N-ethyl maleimide sensitive factor] attachment protein receptors), present on both the transport vesicle (v-SNARE) and the target organelle (t-SNARE), are a complex of membrane-bound proteins that serve as vesicle recognition sites. An inhibitor protein is bound to the t-SNARE that prevents v- and t-SNAREs from association (Pevsner et al., 1994, Chavrier and Goud, 1999). Active GTP-bound Rab proteins interact with t-SNAREs and this interaction serves to displace the inhibitor protein(s). This allows the v- and t-SNAREs to form stable complexes and position the lipid bi-layer of the transport vesicle properly such that it may fuse with that of the target organelle; thus, delivering the protein cargo. At this step, GAPs hydrolyze the Rab-bound GTP to GDP consequently converting Rab to its inactive state. Rab proteins are thus made more soluble by an interaction with GDIs which return the inactive Rab to its donor organelle membrane by an, as yet, unknown mechanism (Seabra, 1995). The inactive, GDP-bound Rab becomes available for a new round of protein transport (Bourne et al., 1990). GDI attachment to Rab blocks conversion from the current inactive GDP-bound form to the active GTP-bound form. These principles illustrate that Rab proteins act as traffic regulators of vesicle transport, but not as essential recognition proteins.

Rab-Associated Diseases

Rab proteins are active when prenylated by geranylgeranyl transferase (GGTase). The absence of Rab27a prenylation in humans leads to a recessive X-linked retinal degeneration disease called choroideremia (CHM). In this disease, degeneration of the retinal pigment epithelium (RPE) and choriocapillaris may lead to complete blindness by middle age, and is predominantly identified in males. In females, irregular degeneration of these cell layers is due to random inactivation of the X chromosomes (Seabra et al., 1993). Retinal degeneration of CHM may be the result of a defective gene on the X chromosome (Xq21) that encodes a Rab GGTase accessory protein, Rab escort protein-1 (REP-1), resulting in an accumulation of unprenylated and inactive Rab27a (Andres et al., 1993, Seabra et al., 1993, and Tolmachova et al., 1999). The degenerative characteristics of the disease can be slowed by expression of a different escort protein, REP-2. REP-2 protein sequence is 75% identical to REP-1 and may partially compensate for REP-1 loss of function (Chen et al., 1997a, Seabra et al., 1995, and Tolmachova et al., 1999).

Another disease associated with Rab dysfunction is termed platelet storage pool deficiency (PSPD). The pathology of PSPD follows that of CHM (Detter et al., 2000). However, a mutated α-subunit inactivates the Rab GGTase leaving Rab27b unprenylated (Andres et al., 1993, Detter et al., 2000). Unprenylated Rab27b is not membrane-bound resulting in unsuccessful protein delivery. This may lead to increased bleeding and a decrease in α- and δ-granules in platelets (Swank et al., 1993).

The *Xiphophorus* Fish Model System

The fish used in these studies are platyfishes and swordtails of the genus

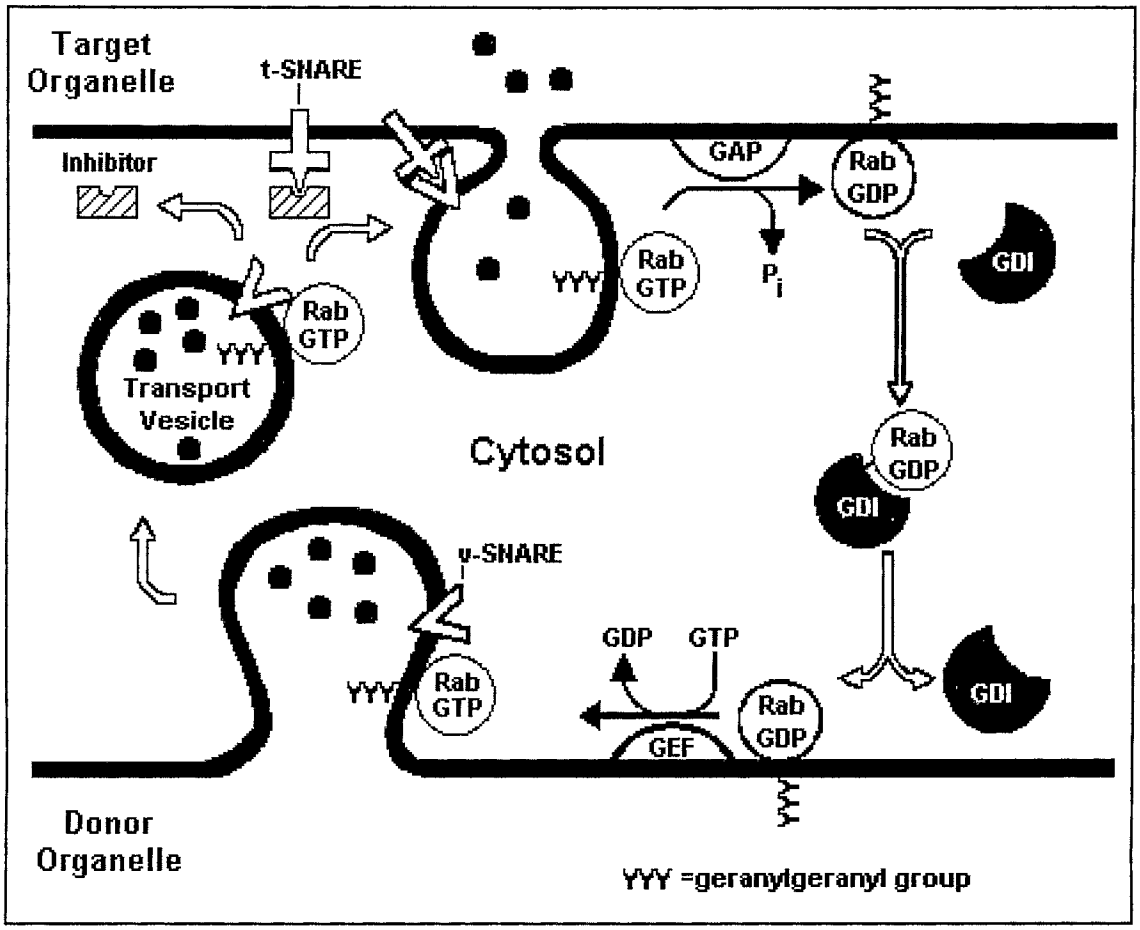


Figure 1-4. Pictoral diagram of the membrane trafficking cycle of Rab proteins. Vesicle encapsulated proteins are transported through the cytosol to the select target organelles. The active, GTP-bound Rab displaces the SNARE inhibitor, which then allows v- and t-SNARE to form a complex. The transport vesicle and target organelle membrane fuse, permitting successful delivery of the proteins. To recycle Rab, a GAP protein hydrolyzes the GTP Rab bound to GTP, placing Rab in an inactive and GDP-bound state. The inactive Rab is then recycled to the donor organelle by GDI proteins, where it can become active again by GEFs and assist in another round of vesicle transport.

Xiphophorus (Poeciliidae: Teleostei). There are at least 22 species of *Xiphophorus* (Rauchenberger et al., 1990). A well developed fish gene map and the ability to perform interspecies hybridization between pedigreed fish lines provides a solid genetic system for studies in many and varied scientific disciplines (Walter & Kazianis, 2001). Fishes are considered to have diverged more than 450 million years ago from the common ancestor leading to tetrapods (Morizot, 1990). The *Xiphophorus* genome contains about 20% the DNA found in human cells (Hinegardner and Rosen, 1972) located on 24 pairs of chromosomes (Ohno, 1970). Studies of fish genes and genomes serve to illuminate complex phenotypes that are conserved among vertebrate organisms but are poorly understood due to multifactorial inheritance patterns and population variability (Walter & Morizot, 1996). To date, over 370 genetic markers (RAPDs, AP-PCR, isozymes, etc.) are assigned to the *Xiphophorus* linkage map (S. Kazianis, personal comm.). These markers are clustered into 24 multipoint linkage groups (Morizot et al., 1998; and unpublished data).

Interspecific genetic backcross (BC_1) hybrids between certain species of *Xiphophorus* yield progeny that either spontaneously develop tumors or are susceptible to UVB or MNU induced tumorigenesis (Gordon, 1927, Vielkind et al., 1989, reviewed in Walter and Kazianis, 2001). Interspecies hybrids of these fish exhibit a number of spontaneous and induced malignancies, including melanomas, neuroblastomas, fibrosarcomas, and retinoblastomas (Schwab *et al.*, 1979, Vielkind *et al.*, 1989, Walter and Kazianis, 2001). The "Gordon-Kosswig" cross, *X. helleri* × (*X. maculatus* Jp 163 A × *X. helleri*) produces backcross hybrids that develop spontaneous melanoma in Mendelian ratios (Gordon, 1927). The study of this tumor model has provided a

paradigm for understanding the molecular events leading to melanoma predisposition (Schartl, 1995). New *Xiphophorus* tumor models exhibiting genetic predisposition to induced (UV or MNU) tumor development offer great potential to identify *Xiphophorus* genes that provide the genetic background necessary for disease onset and progression. Given the strong conservation observed between fish and human genes involved in oncogenesis this model is an important resource for our future ability to provide candidate loci that may be tested for association with human tumor development (Anders, 1991, Walter and Morizot, 1996).

In order to attempt isolation of genes specifically up-regulated in *Xiphophorus* melanoma tissue, we employed differential display technology that amplified subsets of mRNAs expressed in melanoma tissue and normal pigmented fish tissue. Amplified mRNA fragments exhibiting signal suggesting they are upregulated in melanoma tissues were cloned and sequenced for eventual confirmation of their use as molecular markers for melanoma. During the course of these studies we isolated and sequenced a gene that appears to be homologous to the human *RAB27* subfamily.

In humans, *RAB27* sub-family transcripts exhibit high expression in melanocytes, platelets, and a melanoma cell line (Chen et. al., 1997a). Also, both Rab27a and Rab27b proteins were isolated from melanocyte and platelet cells, suggesting an important function in these cells and in diseases related to them (Nagata et al., 1990, Chen et al, 1997a), such as melanoma. The following data presents the nucleotide sequence, predicted amino acid sequence, map assignment, and tissue specific expression patterns of a *Xiphophorus RAB27* homologue.

CHAPTER 2

MATERIALS & METHODS

Fish Stocks

The fish stocks and lines used in the studies presented herein are listed in Table 2-1. Fishes were provided by the *Xiphophorus* Genetic Stock Center, Department of Chemistry and Biochemistry, Southwest Texas State University; San Marcos, TX.

Fish stocks and lines	Nucleic acids used	Experimental use
X. maculatus Jp 163 A	Genomic DNA	Gene mapping
X. helleri (Sara)	Genomic DNA	Gene mapping
X. helleri (Sara) (×) (*X. maculatus* Jp 163 A (×) *X. helleri* (Sara)	Genomic DNA cDNA	Gene mapping Differential display
X. maculatus Jp 163 B	cDNA Genomic DNA	Relative, Quantitative RT-PCR Gene Isolation
X. couchianus × (*X. maculatus* (×) *X. couchianus*)	cDNA	Relative, Quantitative RT-PCR

Table 2-1. *Xiphophorus* fishes used in this research and their experimental purpose.

RNA Isolation

To isolate total RNA from select tissues, fish are anesthetized in ice/water and using sterile instruments, required tissues are dissected into Dounce homogenizer pestles standing in a dry ice/ethanol bath. Each tissue sample is homogenized in TRI REAGENT™ (Sigma; St. Louis, MO) at 1 ml/100 mg of tissue. Samples are incubated at

room temperature (Rt; 24°C) for 5 min., then 0.2 ml of chloroform per ml of TRI REAGENT™ is added. Capped tubes are shaken vigorously for 15 seconds and then incubated at Rt for 10 min. The aqueous layer (containing the RNA) is separated from the organic layer by centrifugation (12,000 × g) for 15 min. at 4°C (Beckman J2-21 Centrifuge; Fullerton, CA) followed by transfer of the aqueous phase to a fresh tube. The aqueous phase is extracted with isopropanol at 0.5 ml/ml of TRI REAGENT™ followed by 10 min. incubation at Rt, after which the RNA is pelleted from the suspension by centrifugation (12,000 × g) for 15 min. at 4°C. The supernatant is decanted and the RNA pellet washed with 70% EtOH followed by brief centrifugation (7,500 × g; 5 min. at 4°C) to ensure the RNA remains pelleted. The supernatant is removed and the RNA pellet air-dried for 15 min., then the pellet is resuspended in 500 µl double distilled (ddH_20) water that had previously been treated with 0.1% diethyl pyrocarbonate (DEPC). The RNA is then cleared of contaminating DNA by digestion with 80 U RNase-free DNase (Ambion; Austin, TX) and 54 µl 10 X DNase buffer (100 mM Tris-HCl [pH 7.5], 25 mM $MgCl_2$, and 1 mM $CaCl_2$). The DNase reaction is incubated at 37°C for 30 min., then the nucleic acids extracted with one volume phenol, and then, Sevag's solution. Organic extractions consist of a brief vortex, centrifugation (13,000 × g for 2 min.), and transfer of the aqueous layer to a fresh tube. RNA is precipitated with the addition of 1/10 volume 3 M NaOAc (pH 5.2) and 2 volumes 100% cold (-20°C) EtOH. The tubes are placed at -80°C for 10 min. and then centrifuged (13,000 × g) for 10 min. The ethanol is decanted and the pellet dried in a vacuum centrifuge (Savant Instruments; Marietta, OH). The RNA pellet is resuspended in 100 µl DEPC water and the amount of RNA determined using a Beckman DU-64 spectrophotometer (Beckman-Coulter; Fullerton, CA) where 1 A_{260}

represents 40 μg/ml RNA (Maniatis et al, 1989).

Polymerase Chain Reaction

Polymerase chain reaction (PCR) is a method for amplifying a target nucleic acid sequence using a heat-stable DNA-polymerase and two oligonucleotide primers. Oligonucleotide primers used must flank the intended target and be specific for amplification of only the target sequence. Thus, it is very much advantageous to have nucleotide sequence information available in order to design appropriate primers for PCR. *In lieu* of the availability of precise sequence information, redundant primers can be made based on genetic database alignment of homologous genes from various organisms, that perform adequately, particularly in gene isolation protocols. To perform standard PCR, 0.2 μl thin-walled PCR tubes are used. Each reaction contains 50-500 ng of target DNA, Polymerase buffer (20 mM Tris-HCl [pH 8.4], 50 mM KCl), 1.5-3.5 mM $MgCl_2$, 62.5 μM of each dNTP, 0.16 μM of each primer (Table2-4), 2.5 units of *Taq* DNA polymerase (Gibco BRL; Grand Island, NY), and sterile distilled water to a final volume of 50 μl. In the studies detailed herein, a Perkin Elmer 2400 GeneAmp PCR system (PE Applied Biosystems; Foster City, CA) was used to perform successive rounds (eg. 35 cycles) of denaturing (94°C), primer annealing (52°C-68°C), and strand elongation (72°C; Table 2-2). Upon amplification, the products of the reaction are visualized after running a 0.8 to 2.0% agarose gel run in tris-acetate EDTA buffer (1 mM Tris-$0Ac^-$, pH 7.0; 0.1 mM EDTA) including ethidium-bromide.

Extra Long Polymerase Chain Reaction

The XL (extra long) PCR reaction is designed to amplify target fragments over 6 kb in length and ranging up to 40 kb, depending on the reaction conditions. The XL-PCR

kit used in these studies (PE Applied Biosystems; Foster City, CA) contained *rTth* polymerase which possesses greater processivity than *Taq* and has 3'-5' proofreading activity. The XL-PCR reaction contains two "cocktail" component mixtures to allow "hot start" PCR. One cocktail contains: XL buffer II, 0.8 mM $Mg(OAc)_2$, 0.3 μM of each primer (Table 2-4), 500 ng of DNA, and sterile ddH_20 for a 20 μl total reaction volume. Upon incubation at 94°C for 5 min to remove nucleic acid secondary structure 30 μl of the second cocktail is added which contains: XL buffer II, 50 μM of each dNTP, r*Tth* DNA polymerase XL (2 U), and sterile ddH_20 at a 30 μl total volume. Samples undergo successive PCR cycles; denaturing, primer annealing, and strand elongation to amplify the desired target sequence (Table 2-2). The products are visualized on an ethidium-bromide stained agarose gel as detailed above.

Reverse Transcription Reaction

To produce complementary DNA sequences (cDNA) allowing PCR amplification of mRNA, the RETROscript first-strand synthesis kit (Ambion; Austin, TX) was used. cDNA products served as template sequences used to amplify target regions for cloning and gene expression studies. In our reverse transcription reactions the following

***RAB27* PCR Conditions**

Primer Pair	Template	Cycle #	Denaturation	Annealing	Elongation	Amplicon Size (bp)
Rab-224F/ Rab-224R	Genomic DNA	35	94°C, 30 sec	66°C, 1 min	72°C, 1 min	224
Rab-ex1bF/ Rab-ex4R	cDNA	33	94°C, 45 sec	53°C, 45 sec	72°C, 1.5 min	419
Rab-ex1bF/ Rab-ex6Rc	Genomic DNA	26	94°C, 30 sec	53°C, 30 sec	68°C, 5 min (cycle 17 +15sec])	496

Table 2-2. Conditions for amplification of the *Xiphophorus RAB27* gene.

components are added to a 0.2 µl tube: 1-2 µg of total RNA, 4µl of dNTP mix (2.5 mM each dNTP), 2 µl first-strand primer (50 µM oligo dT primer or 50 µM random decamers), and nuclease-free water to a final volume of 16 µl. The reaction is briefly mixed, centrifuged, and incubated at 75°C for 3 min. followed by quenching on ice. On ice, the final components are added to each reaction: 2 µl of 10X RT-PCR buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl, 15 mM $MgCl_2$), 1 µl of placental RNase inhibitor (10 U/µl), and 1 µl of M-MLV reverse transcriptase (100 U/µl). The reaction is mixed, briefly centrifuged, then incubated at 42°C for 1 hour. The reverse transcription reaction is stopped by heat inactivation at 92°C for 10 min. and stored at -20°C for later use.

Differential Display

The Delta Differential Display kit (Clontech; Palo Alto, CA) allows coordinated amplification of subsets of RT-PCR fragments in order to attempt to identify specific messages that are expressed differently in two, or more, target tissues. Once these fragments are identified, they must be cloned and confirmed for differential or modulated expression since a known caveat of this technique is the high percentage of false positive amplicons. We wished to develop this technique to attempt identification of transcripts from *Xiphophorus* melanoma tumor tissue that appear substantially up or down regulated compared to transcripts in non-tumor tissue of like source. To perform the

1 cycle		2 cycles		30 cycles		1 hold
94°C, 5 min 40°C, 5 min 68°C, 5 min	⇒	94°C, 30 sec 40°C, 5 min 68°C, 5 min	⇒	94°C, 30 sec 60°C, 30 sec 68°C, 2 min	⇒	68°C, 7 min

Table 2-3. Temperatures and conditions used in the differential display reaction.

differential display reaction, one combines 500 ng of cDNA (oligo d[T] primed), 2 μl of 10X Advantage PCR buffer, 0.2 μl of dNTP mix (5 mM each), 0.2 μl of α^{32}P-dCTP (3000 Ci/mmol), 0.4 μl of 50X Advantage KlenTaq polymerase, and sterile water to 50 μl final volume in a thin walled 0.2 ml reaction tube. The primer sets consist of oligo-dT primers (designated as T primers) that have a two base anchor on their 3' end, and arbitrary primers (designated as P primers) that are designed from common motifs found in mRNAs. Three initial low-stringency PCR cycles are followed by standard PCR conditions (35 cycles; see Table 2-3).

Following PCR, 4 μl of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol FF) is added to the reaction and the amplicons in the mixture denatured at 94°C for 5 min. Two μl of each reaction was applied to a 6.0% polyacrylamide (acrylamide:bis-acrylamide 19:1) and 5 M urea denaturing gel (0.4 mm in diameter). Electrophoretic separation is achieved using a 40 cm Kodak Biomax electrophoresis apparatus run at 80 V/cm until the xylene cyanol marker dye had migrated 25 cm from the loading wells. The gel is transferred to chromatography paper (3MM) and dried under vacuum for 1 hour (Savant; Marietta, OH) then placed in a cassette between two intensifying screens and allowed to expose to Kodak X-OMAT AR film (Eastman Kodak Co.; Rochester, NY). Exposure times were empirically determined.

DNA Purification from Agarose Gels

Two methods were used to extract fractionated bands (DNA fragments or PCR amplimers) from agarose gels. One method employs a matrix gel extraction protocol while the other uses a rapid spin column (CONCERT Gel Extraction System; Gibco

BRL; Grand Island, NY).

Matrix Gel Extraction

The matrix gel extraction system uses a resin which capable of isolating up to 7.5 μg of DNA. A desired DNA fragment is cut out of the agarose gel with a clean glass cover slip and placed in a 1.5 ml microcentrifuge tube. Thirty μl of gel solubilization buffer (L1) and 1 μl of silica resin for every 10 μg of gel is added. The agarose gel plug is then incubated at 50°C for 15 min., vortexing every 3 min. The reaction is centrifuged (9,000 × g) for 1 min. and the supernatant discarded with a pipet. Thirty μl of gel solubilization buffer (L1) is again added for every 10 μg of gel followed by brief vortexing. The tube is centrifuged (9,000 × g) for 1 min., and the supernatant discarded. To the remaining resin, 30 μl of wash buffer (L2) is added for every 10 μg of gel. The resin is resuspended by vortexing, centrifuged (9,000 × g) for 1 min., and the supernatant discarded. This step is repeated and the resin pellet air dried (5 min.). The DNA is eluted from the resin by adding 20 μl TE buffer (10 mM Tris-HCl [pH 8.0], 0.1 mM EDTA). The tube is mixed (vortexed) to resuspend the resin and then placed at 50°C for 10 min. The resin is pelleted by centrifugation (9,000 × g) for 1 min. and the supernatant transferred to a fresh tube. The elution step is repeated once again and the supernatants combined. DNA recovered is ethanol precipitated (2 vols ETOH) after addition of 1/10 volume 3M sodium acetate.

Rapid Gel Extraction

The rapid gel extraction system uses spin cartridges that contain a silica membrane capable of isolating up to 15 μg of DNA, and the cartridges fit tightly into standard 1.5 ml microcentrifuge tubes. DNA fragments cut out of the agarose gels are

solubilized with 30 µl buffer L1 for every 10 mg of gel. The agarose gel plug is incubated at 50°C for 15 min., vortexing every 3 min., and then transferred to a spin cartridge (placed in a wash tube). The cartridge is centrifuged (9,000 × g) for 1 min. and the flow-through is discarded. Five hundred µl of gel solubilization buffer (L1) is added, incubated at Rt for 1 min., and then centrifuged (9,000 × g) for 1 min. (discard the flow-through). To the cartridge, 700 µl of wash buffer (L2; containing ethanol) is added followed by incubation at Rt for 5 min. The cartridge is centrifuged (9,000 × g) for 1 min. and the flow-through is discarded. This step is repeated two additional times. The spin cartridge is placed into a 1.5 ml recovery tube and 50 µl of warm TE buffer (65°C) is added to the center of the silica membrane. The tube is incubated at Rt for 1 min., and then centrifuged (9,000 × g) for 2 min. to elute the DNA.

PCR-Script Subcloning of DNA Fragments

Two host-vector systems were used during the course of these studies to subclone DNA fragments isolated from agarose gels. These two systems are (1) The PCR-Script™ cloning kit (Stratagene; La Jolla, CA) and (2) The TOPO TA cloning® kit (Invitrogen Co; Carlsbad, CA). Brief details of both are provided below.

PCR-Script™ subcloning

For the PCR-script protocol, DNA fragments isolated from an agarose gels are first "polished" to create blunt ends for ligation and subcloning. The following components are combined: 10 µl purified PCR product (i.e. DNA fragment), 1 µl 10 mM dNTP mix (2.5 mM each), 1.3 µl 10X polishing buffer (200 mM Tris-HCl [pH 8.75], 100 mM KCl, 100 mM $(NH_4)2SO_4$, 20 mM $MgSO_4$, 1 mg/ml BSA, and 1% Triton X-100) and 1 µl cloned *Pfu* polymerase (0.5 U/µl). The reaction is mixed gently and incubated at

72°C for 30 min.

Ligation and Transformation

The blunt-ended PCR product is inserted into a pPCR-Script Amp SK(+) cloning vector during the ligation reaction by adding the following components in order: 1 µl of pPCR-Script Amp SK(+) cloning vector, 1 µl of PCR-Script reaction buffer, 0.5 µl of 10 mM rATP, 3 µl of the polished PCR product, 1 µl of *Srf* I restriction enzyme (5 U/µl), 1 µl of T4 DNA ligase (4 U/µl), and 2.5 µl sterile distilled water to 10 µl final volume. The reaction is mixed and incubated at Rt for 1 hour. Following ligation, the reaction is stopped by heat inactivation at 65°C for 10 min. and stored on ice for the transformation reaction. This system ligates target DNA into the *Srf*I restriction endonuclease recognition site, thereby disrupting the ability of this enzyme to cleave vector DNA. A successful cloning event cannot be cleaved by the *Srf*I restriction enzyme, thus over time during the reaction successful cloning events are trapped by ligation and very little material is favored.

Following ligation, the reaction is transformed into competent XL10-Gold Kan *E.coli*. Transformation is performed as follows: 40 µl ultracompetent cells (thawed on ice) are added to a chilled (4°C) 15 ml Falcon polypropylene tube and 1.6 µl XL10-Gold β-mercaptoethanol mix is added. The tube is incubated on ice for 10 min. with gentle swirling every 2 min. Two µl of the ligation reaction is added, mixed by swirling, and then incubated on ice for 30 min. The tube is heat pulsed at 42°C for 30 seconds and placed on ice for 2 min. 450 µl of warm (42°C) Luria-Bertani (LB) broth is added and the reaction is incubated at 37°C for 1 hour with shaking (225 rpm). The transformation reaction is plated (20 and 100 µl) on LB-ampicillin (0.15 mg/ml) agar plates (2% X-gal

and 10 mM IPTG [isopropylthio-β-D-galactoside]) using a sterile cell spreader. The plates are inverted and incubated at 37°C for 17 hours. White colonies are chosen for further analysis.

Subcloning using the TOPO TA cloning Kit

The TOPO TA cloning® kit (Invitrogen Co; Carlsbad, CA) provides a linearized vector with a covalent attachment of Topoisomerase I that can cleave the vector after 5'-CCCT | T, leaving a single deoxythymidine (T) overhang. *Taq* polymerase has a terminal transferase activity that adds a single deoxyadenosine (A) to the 3'-end of many PCR products. This property of *Taq* enables isolated PCR amplimer fragments to bind efficiently with the linearized TOPO TA vector overhang. Four μl PCR fragment (DNA target fragment) isolated from an agarose gel is combined with 1 μl salt solution (1.2 M NaCl, 0.06 M $MgCl_2$), and 1 μl 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 of BSA, and phenol red). The reaction is mixed by pipetting and incubated at Rt between 30 sec and 30 min (depending on the target fragment size). On ice, 2 μl of the TOPO® reaction is added to one tube of One Shot® competent *E. coli*, mixed gently, and incubated on ice for 5 to 30 min. The cells are heat shocked for 30 sec at 42° and then placed on 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 20 mM glucose) is added and the reaction is shaken (200 rpm) at 37°C for 1 hour. Using a sterile cell spreader, the transformation reaction is plated (20 and 100 μl) on pre-warmed LB-ampicillin agar plates. The plates are inverted and incubated at 37°C for 17 hours. White colonies are chosen for further analysis.

Plasmid DNA Isolation by Alkaline Lysis (Minipreparation)

Plasmid DNA for use in confirmation of subcloning, DNA sequencing and labeling via nick translation for use as a probe, is isolated from bacterial cell clones by the alkaline lysis protocol. Cells harboring the plasmid of interest are incubated in 3 ml of LB broth (containing 50 μg/ml of ampicillin) and grown while shaking (200 rpm) in a 37°C water bath for 17 hours. The cells are pelleted from the broth in a 1.5 ml microcentrifuge tube by centrifugation (9,000 × g) for 30 seconds, and the supernatant is discarded. The cells are washed in 200 μl of a sterile glucose solution (50 mM glucose, 25 mM Tris-HCl [pH 8.0], 10 mM EDTA), then lysed by addition of 200 μl of freshly made 0.2 M NaOH and 1% SDS. The lysate is inverted to mix and placed on ice for 5 min. To this, 300 μl of 5M potassium acetate is added, mixed, and incubated on ice for 5 min. to precipitate the protein. Cellular debris is pelleted by centrifugation (13,000 × g) for 2 min. The supernatant is carefully transferred to a fresh tube and the nucleic acids precipitated by the addition of 1 volume cold (-20°C) 100% ethanol. The tube is incubated at Rt for 2 min. and then centrifuged (13,000 × g) for 10 min. The supernatant is decanted and the pellet is air dried for 5 min. to remove residual ethanol. The pellet is resuspended in 50 μl TE buffer, treated with 2.5 μg of RNase A, and incubated at 37°C for 30 min. The plasmid DNA is extracted with 1 volume phenol, one phenol/Sevag's, and then one Sevag's solution. After the final extraction, the DNA is precipitated by adding 1/10 volume 3 M NaOAc (pH 5.2) and 2 volumes of cold (-20°C) 100% ethanol. The tube is incubated at -80°C for 10 min. and the DNA is pelleted by centrifugation (13,000 × g) for 10 min. After decanting the ethanol, the pellet is washed with 70% ethanol and centrifuged (13,000 × g) for 5 min. The wash is decanted and the pellet is

dried in a vacuum centrifuge to remove residual ethanol, and then resuspended in 40 µl of TE buffer.

Nucleotide Sequencing

The Sanger dideoxy DNA sequencing method is utilized to determine the primary nucleotide sequence of selected DNA fragment cloned into plasmid vectors. The Sequenase Version 2.0 DNA sequencing kit (USB; Cleveland, OH) is utilized with the following protocol. Combined in a 1.5 ml microcentrifuge tube are 2-5 µg dsDNA, 8 µl 1 M NaOH, 1.6 µl 5 mM EDTA, 1 µl 20 µM oligonucleotide primer (T3, T7, or custom designed oligonucleotides, see Table 2-4), and sterile distilled water to a final volume of 40 µl. The reaction is incubated at 37°C for 30 min. to denature the dsDNA. The denatured DNA is precipitated with 1/10 volume 3 M NaOAc (pH 5.2) and 90 µl cold (-20 C) 100% ethanol, and then placed at -80°C for 10 min. The DNA is pelleted by centrifugation (13,000 × g) for 10 min., washed with 70% ethanol, and then air dried for 5 min. to remove residual ethanol. The pellet is resuspended in 7 µl ddH_2O, 2 µl reaction buffer (200 mM Tris-HCl [pH 7.5], 100 mM $MgCl_2$, 250 mM NaCl), and 1 µl 20 µM oligonucleotide primer. The reaction is incubated at 65° for 5 min., Rt for 5 min., and then on ice for 5 min. To this, 1 µl 0.1 M dithiothreitol (DTT), 2 µl labeling mix (7.5 µM dGTP, 7.5 µM dTTP, 7.5 µM dCTP; diluted 1:5 in ddH_2O), and 1 µl [α-^{35}S]dATP (1250 Ci/mmol) (NEN Life Science; Boston, MA) is added and the reaction incubated at Rt for 2 min. Two µl Sequenase Version 2.0 DNA T7 polymerase diluted 1:8 in enzyme dilution buffer (10 mM Tris-HCl [pH 7.5], 5 mM DTT) is added and incubated at Rt for 5 min. In separate tubes, 2.5 µl of each termination mix (8 µM appropriate ddNTP, 80 µM all four dNTPs, 50 mM NaCl) is aliquoted and placed at 37°C to warm for 2 min. To

Primer	Primer Sequence
Rab-224F	5'-GCTTGTGGGCACTAAGGCAG-3'
Rab-224R	5'-GCTGGTGGTGGGGCTCCCAT-3'
Rab-ex1bFa	5'-ACATGTGAGGCGAGGAA-3'
Rab-ex1bF	5'-GTGGTGAACGGCTTCTG-3'
Rab-ex1bR	5'-GACTCTCAATCTATCTGCTGG-3'
Rab-int1bF.1	5'-CACATCTCATAACGATGACCT-3'
Rab-int1bR	5'-CTGACTGGGAGCAGCCTGGCC-3'
Rab-ex2F	5'-GCTCCTGGCGCTCGGGCACTC-3'
Rab-ex2R	5'-GGTCGTGAACTTCCTGTTGAA-3'
Rab-int2R.1	5'-AACATGTTTAACATTGGTTCA-3'
Rab-int2R.2	5'-GGCACATATTACATACAGTTG-3'
Rab-int2R.3	5'-CGTTCCTCCAGGCTCTAACTT-3'
Rab-int2Fa	5'-TTACCATCTACAACTCTTTAA-3'
Rab-ex3F	5'-ACACGGGGACCGGTGCTGATG-3'
Rab-ex4F.1	5'-CGCAGCCTCACAACGGCTTTC-3'
Rab-ex4R	5'-CCAGTTCCTGACGTTAAC-3'
Rab-ex4R.1	5'-GCTGATTGGTCAAGTCGAACA-3'
Rab-ex5Fb	5'-AACCCAGATGTGGTGCTTGTG-3'
Rab-ex5R	5'-CGTATCTGTCAGCCATCTCTC-3'
Rab-int5R.1	5'-GGATATCTCATCCAAACTTTG-3'
Rab-ex6Ra	5'-GGTTCAGAGCCAGGGGCCCCA-3'
Rab-ex6Rb	5'-CGTGCTCTGCTCCATCCT-3'
Rab-ex6Rc	5'-TCACCAGGTTCAGCAGGG-3'

Table 2-4. Custom synthesized oligonucleotide primers used for 5'-RACE, PCR, and sequencing the *X. maculatus RAB27* gene.

each termination tube, 3.5 μl of the labeling reaction is added and the tube is incubated at 37°C for 5 min. The reaction is terminated with by addition of 5 μl stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol FF).

Prior to fractionation on denaturing acrylamide gels, sequencing reactions are denatured at 94°C for 5 min. Forty cm long gels with spacers producing 0.4 mm thickness were utilized for sequencing. Sequencing gels were generally 6.0% polyacrylamide (acrylamide:bis-acrylamide 19:1) containing 5 M urea. Gel are generally run at 50 V/cm in 1X TBE buffer with successive runs loaded when the xylene cyanol marker had migrated 20 cm from the loading well. After electrophoresis, the gel is transferred to chromatography paper (3MM) and dried under vacuum for 1 hour at 80 C. The gel is used to expose Kodak X-OMAT AR film to visualize the sequencing fragments.

High Purity Plasmid Purification (Large Scale)

The CONCERT high purity plasmid purification system (Gibco BRL; Rockville, MD) is an anion exchange system for large-scale purification (up to 500 μg) of plasmid DNA. Three ml of LB broth with 50 μg/ml ampicillin is inoculated with a bacterial cell line harboring a plasmid of interest and the culture is shaken (200 rpm) for 17 hours in a 37°C water bath. One ml of this fresh overnight culture is added to 500 ml LB broth containing 50 μg/ml ampicillin and shaken (200 rpm) for 17 hours in a 37 °C water bath. The cells are harvested by centrifugation (10,600 × g) for 10 min. at 4°C, and the supernatant decanted. The pellet is resuspended in 10 ml cell suspension buffer (50 mM Tris-HCl [pH 8.0], 10 mM EDTA) containing RNase. To this, 10 ml of cell lysis solution (200 mM NaOH, 1% SDS [w/v]) is added, the capped tube is inverted 5 times to mix the

lysate and then incubated at Rt for 5 min. Ten ml of neutralization buffer (3.1 M potassium acetate [pH 5.5]) is added and the capped tube inverted 5 times to mix. The cellular debris is pelleted by centrifugation (15,000 × g) for 10 min. at Rt. A separation column is equilibrated by allowing 30 ml equilibration buffer (600 mM NaCl, 100 mM sodium acetate [pH 5.0], 0.15% Triton® X-100 [v/v]) to pass through the column by gravity flow (discard flow-through). The supernatant derived from pelleting of cell debris (above) is layered on the column and allowed to pass by gravity flow (discard flow-through). The column is washed with 60 ml wash buffer (800 mM NaCl, 100 mM sodium acetate [pH 5.0]) and then the column bound DNA is eluted into a fresh collection tube with the addition of 15 ml elution buffer (1.25 M NaCl, 100 mM Tris-HCl [pH 8.5]). To the eluate, 10.5 ml isopropanol is added, mixed, and then nucleic acids are pelleted by centrifugation (15,000 × g) for 30 min. at 4°C. The supernatant is discarded and the pellet is washed with 5 ml 70% ethanol. The tube is centrifuged (15,000 × g) for 5 min. at 4°C and the supernatant is decanted. The pellet is air dried for 10 min. to remove residual ethanol, and the pellet is resuspended in 500 µl TE buffer.

5'-RACE

5'-RACE (rapid amplification of cDNA ends; Frohman et al., 1988) is a method used to clone the 5' ends of cDNAs when sequence information for this region is not available. The technique employs PCR, a primer of known sequence (the 3' primer; Table 2-4) and addition of known sequence to 5' end of the cDNA to supply sites complimentary to a second primer. Generally we employed use of a 5'-RACE kit (GIBCO BRL; Rockville, MD) to perform this step.

First Strand Synthesis

In 5' RACE, in order to maximize the target mRNA conversion to cDNA, a gene specific primer (GSP-1) was used as the first strand primer. The following were combined: 2.5 pmoles gene specific primer, 2.5 μg fish total RNA, and 0.1% DEPC water to 15.5 μl final volume. The reaction is incubated at 70°C for 10 min. and then chilled on ice for 1 min. The following are added in order: 2.5 μl 10X PCR buffer [200mM Tris-HCl (pH 8.4), 500 mM KCl], 2.5 μl 25 mM $MgCl_2$, 1 μl 10 mM dNTP mix, and 2.5 μl 0.1 M DTT. The reaction is mixed gently and placed on ice for 1 min. prior to adding 200 U of SuperScript II reverse transcriptase. The reaction tube is mixed gently and incubated at 42°C for 50 min. The reaction is terminated by heating at 70°C for 15 min. One μl RNase mix (RNase H and T_1) is added and then the tube incubated at 37°C for 30 min. to degrade the RNA strands.

The gene specific primer and unincorporated dNTPs must be removed so as to not interfere with the subsequent tailing reaction. To the first strand synthesis reaction, 120 μl of Rt binding solution (6M NaI) is added and mixed by pipet, transferred to a GlassMAX cartridge, and then centrifuged (13,000 × g) for 20 seconds. The spin cartridge is washed with 400 μl cold (4°C) 1X wash buffer and centrifuged (13,000 × g) for 20 seconds. The wash step is repeated three additional times and the flow-through is discarded after each wash. The spin cartridge is then washed two times with 400 μl cold (4°) 70% EtOH and centrifuged (13,000 × g) after each wash for 20 seconds (the flow-through is discarded). The spin cartridge is centrifuged (13,000 × g) for 1 min. to remove any residual ethanol, and the spin cartridge is placed in a fresh recovery tube. The cDNA is eluted from the column with 50 μl warm, sterilized, distilled water (65°C) and

centrifugation (13,000 x g) for 20 seconds.

TdT Tailing of cDNA

The purified cDNA is "tailed" (on the 3'-end of the DNA strand) to create a binding site for the abridged second (or "anchor") primer (kit supplied). In a fresh tube the following are combined: 6.5 µl 0.1% DEPC water, 5 µl 5X tailing buffer [50 mM Tris-HCl (pH 8.4), 125 mM KCl, 7.5 mM $MgCl_2$], 2.5 µl 2mM dCTP, and 10 µl purified cDNA. The reaction is heated to 94°C for 2.5 min., briefly centrifuged, and then placed on ice for 1 min. To this, 1 µl *TdT* (terminal deoxynucleotidyl transferase) is added, mixed gently, and incubated at 37°C for 10 min. The reaction is terminated by heating to 65°C for 10 min. and then placed on ice.

Primary PCR of dC-tailed cDNA

The tailed cDNA is then amplified using a PCR strategy employing gene specific and secondary or anchored primers. To do this, the following components are added to a 0.2 ml thin-walled PCR tube standing in ice: 31.5 µl sterile, distilled water, 5 µl 10X PCR buffer, 3 µl 25 mM $MgCl_2$, 1 µl 10 mM dNTP mix, 2 µl 10 µM nested primer (GSP-2), 2 µl 10 µM abridged anchor primer, and 5 µl of the dC-tailed cDNA reaction. The reaction is briefly incubated at 94°C before adding 2.5 U of *Taq* polymerase. Previously described PCR conditions are followed for amplification and visualization of the amplimers.

Secondary, Nested PCR

In some cases, where target cDNAs are very rare or when a primer set has cross reactivity with several loci in total genomic DNA or RNA preparations, nested PCR is performed. Nested PCR serves to increase amplification specificity of the target. Two

primers are designed to amplify the target, one primer set being designed to bind within a flanking primer set (i.e. the "nest"). Initial rounds of PCR use the exterior set, then the amplified products from this are subjected to amplification with the nesting (interior) primer set. Double selection of a particular sequence as having to be amplified by two distinct primer sets generally ensures reaction specificity. To perform nested PCR the following are combined in a 0.2 ml thin-walled PCR tube standing on ice: 33.5 µl sterile, distilled water, 5 µl 10X PCR buffer, 3 µl 25 mM $MgCl_2$, 1 µl 10 mM dNTP mix, 1 µl 10 µM nested primer (GSP), 1 µl 10 µM universal amplification primer, and 5 µl from the primary PCR. The reaction is briefly incubated at 94°C and 2.5 U *Taq* polymerase is added. Previously described PCR conditions for amplification are followed, and the PCR products are visualized on an ethidium-bromide stained 1% agarose gel.

Relative, Quantitative RT-PCR

Relative, Quantitative RT-PCR (Q-RTR-PCR) is a technique allowing one to derive relative mRNA levels for genes that are expressed at very low levels. The technique does not allow direct quantification of a single message, such as the ribonuclease protection assay, but is a reliable relative assay allowing multiple mRNA levels to be determined within the same RNA preparation. To determine relative expression of the *Xiphophorus* RAB27 gene we employed Q-RT-PCR utilizing the Ambion's QuantumRNA™ 18S kit (Ambion; Austin, TX). This assay enables the determination of tissue specific expression of a target gene relative to the 18S RNA present in the total RNA preparation. Relative expression of the target gene is analyzed by a multiplex PCR using a gene specific primer set and primers that amplify the internal 18S control. To correct for the relative abundance of target mRNA to 18S RNA, normal

primers for the internal standard are mixed with identical sequence primers that are 3' end blocked and cannot be extended by the *Taq* polymerase (i.e. competimers). The ratio of normal primer to competimers leading to amplification signal equal to the target is used to calculate relative mRNA expression. The three step experimental plan consists of detecting the linear range of amplification of the gene of interest, determining the correct ratio of 18S primers:competimers, and performing the multiplex reaction.

Linear range of amplification

The maximum efficiency of amplification over a range of cycles in PCR defines the linear range of amplification. To determine this linear range, the following reaction components are combined in a 0.2 µl thin-walled PCR tube (on ice): 100 ng cDNA (pooled from all non-tumor tissues for a mean representation), 10X PCR buffer (200 mM Tris-HCl [pH 8.4], 500 mM KCl), 1.5-3.5 mM $MgCl_2$, 62.5 µM each dNTP, 0.16 µM each primer, 0.5 µl α^{32}P-dCTP (3000 Ci/mmol), 2.5 units *Taq* polymerase, and sterile distilled water to 50 µl final volume. PCR amplification is performed as previously described. Starting at the end of cycle 15, a tube is removed after every other cycle and placed on ice. Ten µl of loading dye (95% formamide, 0.025% xylene cyanol, 0.025% bromophenol blue, 18 mM of EDTA, and 0.025% of SDS) is added to each tube, and the samples are denatured at 94°C for 4 min. prior to loading on a 6% polyacrylamide 5M urea gel. The gel was run at 80 V/cm until the xylene cyanol marker migrated 25 cm from the loading wells. The gel is transferred to chromatography paper (3MM) and dried under vacuum at 80 C for 90 min. The dried gel is then exposed to a phosphorimage screen for an empirically determined time. The screen is scanned using a Cyclone storage phosphor system (Packard; Downers Grove, IL) to determine radioactive

intensity of the amplified product, and then analyzed using the OptiQuant™ software (Packard Instrument Company; Meriden, CT). Radioactive intensity of the amplicon, reported as digital light units (DLU), and is used to graph the amplification curve and determine where amplification is linear.

Determination of the 18S primer:competimer ratio

Amplification levels of the 18S internal standard is varied by changing the ratio of 18S primers:competimers. The competimers are 18S primers modified at their 3' ends to block extension by DNA polymerase. A cocktail is setup as described above with the addition of 4 µl primer:competimer mix. The following 18S primer:competimer ratios were tested: 1:9, 1:19, 1:29, and 1:39. Standard PCR conditions are employed in the reaction using the previously determined cycle number corresponding to the middle of the linear range for the target transcript. The reactions are fractionated and exposed to a phosphorimager screen as described above. The best 1:1 intensity ratio of the gene of interest to the 18S band is determined and used in the following multiplex reaction.

Multiplex Relative, Quantitative RT-PCR

Previously determined optimal PCR conditions, cycle number, and 18S primer:competimer ratios are utilized in the multiplex PCR reaction. Four µl of the optimal 18S primer:competimer mixture is added to the PCR cocktail detailed above and aliquoted into 0.2 µl thin-walled PCR tubes containing 100 ng cDNA from a specific tissue sample bringing the final volume to 50 µl. The samples undergo standard PCR conditions using the previously determined conditions. The reactions are fractionated and exposed to a phosphorimager screen as described above. The radioactive intensity of *RAB27* compared to the intensity of the 18S standard yields a relative, quantitative value

of expression. Comparing multiple tissues provides a relative expression pattern of *RAB27* in each tissue

Genomic DNA Isolation

To isolate genomic DNA, fish are anesthetized and tissues dissected as described for RNA isolation (above). To the cell lysate, in a Dounce homogenizer standing in a dry ice/ethanol bath, 3 ml/g tissue of fresh lysis buffer (1% SDS, 10 mM Tris-HCl [pH 7.5], 100 mM EDTA, 0.1 M NaCl, 300 mg Proteinase K) is added. The tissues are homogenized and transferred to a 15 ml polypropylene conical tube, then the tube is incubated at 55°C for 1 hour and inverted every 10 min. The cell lysate is cleared with successive 1 volume extractions of phenol followed by extraction with Sevag's solution. Each extraction mixed for 10 min. on a tube rotator prior to centrifugation (2,000 × g; Beckman Model TJ-6, Beckman Instruments Inc.; Fullerton, CA) for 10 min. and transfer of the aqueous layer to a fresh tube. The nucleic acids are precipitated with 1/10 volume 3 M NaOAc (pH 5.2) and 2 volumes 100% cold (-20°C) EtOH. The tube is placed at -80°C for 10 min. and the nucleic acids pelleted by centrifugation (12,000 × g) for 30 min. The ethanol is decanted, the pellet is washed with 1 ml 70% ethanol, and then centrifuged (12,000 × g) for 5 min. The wash is decanted and the pellet is air dried for 10 min. to remove residual ethanol. The pellet is resuspended in 250 µl TE buffer and transferred to a 1.5 ml microcentrifuge tube. Five µl RNase (10 mg/ml) is added and the tube is incubated at 37°C for 30 min. The DNA is precipitated with 1/10 volume 3 M NaOAc (pH 5.2) and 2 volumes 100% cold (-20°C) EtOH. The tube is placed at -80°C for 10 min., the DNA is pelleted by centrifugation (9,000 × g) for 10 min., and the supernatant is decanted. The pellet is dried under a vacuum for 5 min., and then resuspended in 100

μl TE buffer. DNA amounts were determined with a Beckman DU-64 spectrophotometer (Beckman-Coulter; Fullerton, CA) where 1 A_{260} represents 50 μg/ml of dsDNA (Manitatis et al, 1989). An aliquot of the sample is visualized on an ethidium-bromide stained 0.8% agarose gel to verify it is high molecular weight DNA.

Nick Translation

DNA can be radioactively labeled and used as a probe to screen λ phage libraries or in Southern hybridizations. A DNA fragment required to probe for a specific gene is isolated from restriction endonuclease digestion of a plasmid subclone or PCR amplified and cleaned by isolation from an agarose gel. The probe fragment is then treated lightly with DNase to produce single-stranded nicks, to which DNA polymerase can initiate incorporation of radioactive nucleotides (i.e., nick translation). To perform nick translation the following components are combined in a 1.5 microcentrifuge tube: 0.5-1 μg dsDNA, 73.5 μM each dNTP except dCTP, 4 μl α^{32}P-dCTP (3000 Ci/mmol), 10X nick translation buffer (0.5 M Tris-HCl [pH 7.5], 50 mM $MgCl_2$, 0.1 M β-mercaptoethanol, 0.5 mg/ml BSA), 4 U DNA polymerase I, 3.2 U DNase I, and sterile distilled water to 17 μl final volume. The reaction is incubated at 37°C for 10 min., on ice for 10 min., and then at 14°-15°C for 2-2.5 hours.

Unincorporated dNTPs are separated from the radioactively labeled probe by two passages through Sephadex G-50 columns (Sigma; St. Louis, MO) constructed in a 1 ml syringe (Maniatis et al., 1989). The nick translation reaction is loaded onto the column and centrifuged at low speed (~1600 × g) for 10 min. The flow-through is collected and passed through a second column again. Dilutions of the reaction are used to calculate radioactivity (Chenkov counting) using a Beckman 6000 IC scintillation counter

(Beckman Instruments Inc.; Fullerton, CA). The specific activity generally determined is ~1 × 10^9 cpm/μg DNA.

Bacteriophage λ Library Screening

A λ phage genomic library was constructed using randomly sheared *X. maculatus* Jp 163 A DNA and the λ FIX II phage replacement vector (Stratagene; La Jolla, CA). Recombinant phage in this library have previously been determined (R. Walter, personal com.) to harbor inserts averaging 17 kb.

Plating and Library Lifts

Appropriate dilutions of the λ phage suspension are plated with 200 μl cells (*E. coli,* C600Hfl) derived from a fresh stationary phase culture. The phage suspension and cells are plated in 3 ml of 0.7% agar on top of standard 1.2% LB agar plates and allowed to incubate for 17 hrs. at 37°C for plaque formation.

The plates carrying phage plaques are cooled to 4°C for 2 hours before taking lifts. A nitrocellulose membrane (Stratagene; La Jolla, CA) is placed onto the surface of a plate, numbered and marked with three asymmetrical lines on the bottom of the plate and on the membrane. The membrane is removed after 2 min. with blunt end forceps and placed (plaque side up) in the following solutions: blotting base (0.5 N NaOH and 1.5 M NaCl) for 1.5 min., neutralizer solution (0.5 M Tris-HCl [pH 7.5], 1.5 M NaCl) for 2 min., and then 2X SSC for 1 min. The membranes are placed on chromatography paper (3MM) for 2 hours to air dry and then baked at 80°C for 2 hours to crosslink the DNA to the membrane. The membranes are then sealed in a thermo-stable bag until hybridization.

Plaque Hybridization

Within heat sealable bags, the nitrocellulose membranes are incubated with pre-hybridization solution [35% formamide, 5X Denhardt's (0.02% ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin for a 1X solution), 5X SSC (3.0 M NaCl, 0.3 M sodium citrate [pH 7.0] for a 20X solution], 100 μg/ml of salmon sperm (sheared and denatured), 0.5% SDS, and water to a final volume of 125 ml. The filters are pre-hybridized for 1 hour at 42°C in a shaker bath (200 rpm). Half of the pre-hybridization solution is poured out of the bags and the denatured probe (94 C for 5 min.) is added. The bag is resealed and shaken (220 rpm) at 42°C for 17 hours to allow hybridization to occur.

Washing the membranes

The hybridization solution is decanted and the membranes are washed with increasing stringency (24°C, 37°C, 45°C-50°C, and 65°C) with the following increasingly stringent solutions (6X SSC and 0.1% SDS, 1X SSC and 0.5% SDS, or 0.1X SSC and 1% SDS), until the radioactivity measured (via Geiger counter) is reduced to near background levels. After washing, the membranes are air dried for 2 hours, positioned in a cassette between two intensifying screens and used to exposed to X-ray film at -80°C. After developing the film, the membranes are aligned to the film and the edges of the membranes and the asymmetric marks are traced on the film. A pipet tip is used to plug out positive plaques into microcentrifuge tubes that contains 200 μl SM buffer and 5 μl chloroform. The tube is vortexed, briefly centrifuged, and stored at 4°C for later usage. The protocol is repeated on the phage candidate clones until a particular phage plug is purified to a single phage particle containing a positively hybridizing insert.

Isolation of λ Bacteriophage DNA

DNA is isolated from λ phage using the following protocol. The λ phage clone is plated to confluence (~10^{12} plaque forming units) on multiple LB agar plates incubated for 17 hours at 37°C. Three ml of SM buffer is added to the plates and the plates are shaken slowly at Rt for 4-6 hour. The SM buffer is drained to the edge of the plate and collected in Oak Ridge centrifuge tubes. The plates are washed two more times with 3 ml, then 2 ml of SM buffer and the recovered buffer added to the corresponding initial tube. The phage suspensions are centrifuged (10,100 × g) for 30 min. and the clear lysate decanted into a fresh centrifuge tube. To this, 100 µl DNase I (10mg/ml) is added, the tube is incubated at 37°C for 30 min., and then split into two 30 ml ultra-centrifuge tubes fitting a Beckman SW27 rotor. The tubes are filled and balanced with SM buffer. The phage particles are pelleted in an ultracentrifuge (64,330 × g) for 2 hours at 4°C. The supernatant is decanted and the pellet is dissolved in 1 ml SM buffer and stored overnight at 4°C. The solution is gently pipetted to ensure complete resuspension of the pellet and then transferred to a 15 ml polypropylene conical tube. The centrifuge tube is washed twice with a total of 3 ml SM buffer, and the following is added: 50 µl 0.5 M EDTA, 10 µl Proteinase K (20 mg/ml), and 50 µl 10% (w/v) SDS. The reaction is mixed and incubated at 55°C for 30 min. The phage DNA is then deproteinated with successive 1 volume extractions of phenol, phenol:Sevags, and Sevags solutions. The DNA is precipitated with 1/10 volume 3 M NaOAc (pH 5.2), 2 volumes 100% cold (-20°C) EtOH, at -80°C for 10 min. then pelleted by centrifugation (10,100 × g) for 15 min. at 4°C. The pellet is washed with 1 ml 70% EtOH, centrifuged (10,100 × g) for 10 min., and the wash decanted. The pellet is then air dried for 10 min. to remove residual ethanol

and the DNA resuspended in 500 µl diluted (1:10) TE buffer. Amounts are determined as indicated above (See **DNA Isolation**).

Southern Hybridization

The organization of specific sequences within genomic or recombinant phage DNA can be determined with modifications to the transfer techniques described by Southern (1975). The procedure involves performing a restriction digest on genomic DNA and separating it on an agarose gel. The fractionated DNA is then transferred to a nitrocellulose membrane and screened with a radioactively labeled probe.

To perform Southern hybridization on phage clones isolated by library screening the purified λ phage DNA is cut with multiple restriction enzymes. Five µg λ phage DNA is incubated at 65°C for 10 min. to denature secondary structure and placed on ice. Restriction endonculease having recognition sites flanking the phage vector multiple cloning site (MCS) were added with the appropriate buffer (as specified by the manufacturer) and sterile distilled water to a final volume of 19 µl. The tubes are incubated at optimal temperatures for 15 hours, then 1 µl enzyme is added, and the tubes are incubated for 2 hours. The DNA fragments are separated on an ethidium-bromide stained 0.8% agarose gel at 25 V for 26 hours, photographed, and used in the blotting protocol.

The gel fractionated DNA is transferred to a nitrocellulose membrane for hybridization. The standard ladders and control lanes run with the phage restriction enzyme cleaved samples are removed from the gel. The remaining gel is soaked: 10 min. in 0.2 N HCl and rinsed in ddH_20, then 45 min. in blotting base (0.5 N NaOH) and rinsed in ddH_20, then 30 min. in neutralizing solution (10 mM Tris-HCl [pH 8.0], 1 mM EDTA)

and rinsed in ddH_20, and finally soaked for 5 min. in 20X SSC. The gel is then inverted on the support surface and one corner is cut off for later orientation of the gel. A nitrocellulose membrane is cut 1 mm larger on all sides than the gel, floated on the surface of ddH_20 to become completely wet, and then immersed in the transfer buffer (6X SSC) for 5 min. A corner is cut from the membrane to match the corner cut from the gel and then placed on top of the gel (cut corners are aligned). The position of the wells are marked on the membrane with a pencil. Two pieces of chromatography paper (3MM; wetted in 2X SSC) are placed on top of the nitrocellulose membrane. Absorbent towels (8 cm high), a glass plate, and a weight (~500 g) are stacked on top of the chromatography paper and DNA transfer is allowed to proceed for 18 hours. The absorbent towels and chromatography paper are removed and the membrane is soaked in 6X SSC at Rt for 5 min. The membrane is then air dried for 30 min. and baked at 80°C for 2 hours to cross-link the DNA to the membrane.

Probe hybridization

The membranes are hybridized and washed as stated above (bacteriophage λ library screening). After hybridization, washes are continued until the reduction in radioactivity reaches near background levels as determined with a Geiger counter. The membrane is then sandwiched between intensifying screens and exposed to film at –80°C as detailed above.

Mapping of *RAB27* Genotype

Using a battery of restriction endonucleases having four base recognition sites we established a polymorphism between several *Xiphophorus* parental species. In the case of *RAB27* the polymorphic amplicon crossed an intron. Using this, a panel of backcross

hybrid individuals was screened for the parental or hybrid genotypes using PCR followed by restriction endonuclease cleavage and agarose (2%) gel electrophoresis. Each BC_1 fish was scored as homozygous (displaying the banding pattern of the non-recurrent parental species) or heterozygous (displaying the banding pattern of an F_1 hybrid). These data were entered into the computer with a data set having several hundred markers scored for each BC_1 hybrid individual. Two-by-Two data analyses for each potential marker pair were then conducted using the MapManager (Manly, 1998, Manly and Olson, 1999) software package. Unlinked loci display a parental:recombinant ratio of 1:1 in Mendelian segregation (i.e., unlinked loci are expected to show 50% parental and 50% recombinant progeny). Linkage of two loci is determined by a bias from a 1:1 ratio of random segregation as determined by Chi-square analysis. The scoring data is exported from a spreadsheet into Map Manager to generate recombination values and LOD scores (Morton, 1955) against all known loci. A LOD (logarithm of the odds) score over 3.0 is significant to indicate linkage. The spreadsheet data is also exported into MapMaker/Exp 3.0 (Lander et al., 1987) to generate gene map orders.

Chapter 3-Results and Discussion

X. maculatus RAB27 Gene Isolation and Nucleotide Sequencing

Introduction

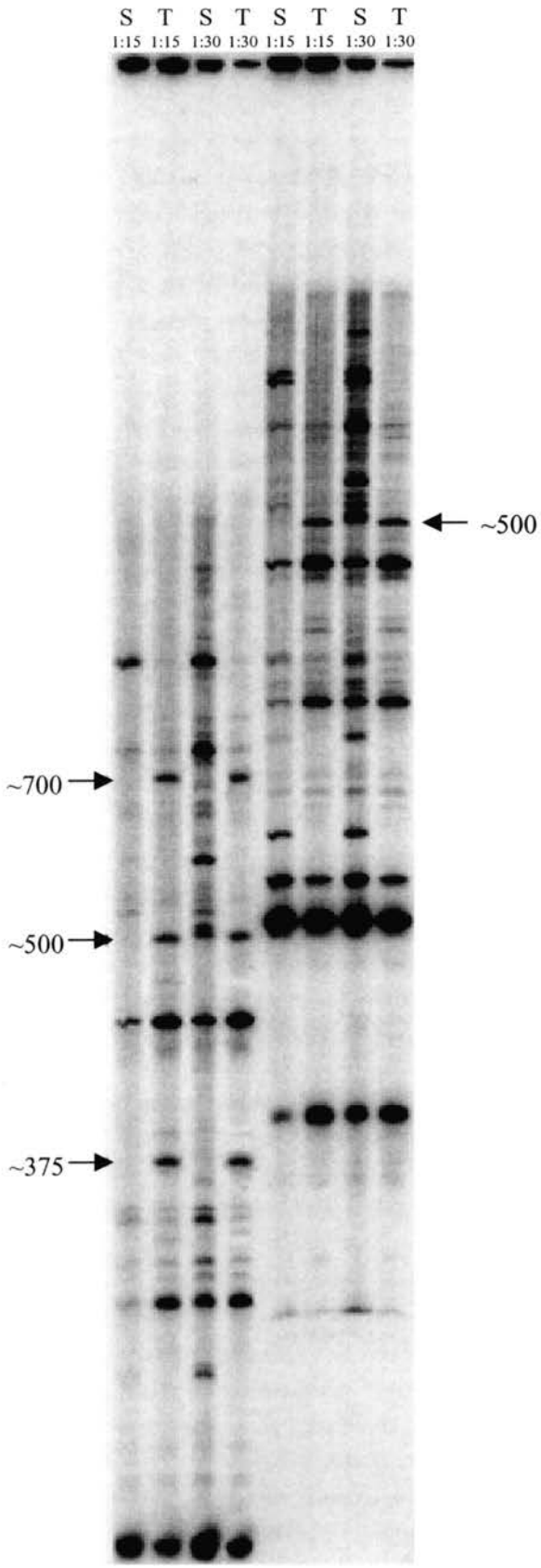
Differential display is a technique allowing one to determine relative transcription levels from divergent RNA sources in order to identify genes having modulated expression among various tissue sources or developmental paths. In our laboratory we are interested in identification of transcripts that exhibit drastic up- or down-regulation in *Xiphophorus* tumor tissue compared to normal (i.e., non-tumor) source tissues. Identification of such transcripts will allow isolation of the genes encoding them and may lead to molecular markers that illuminate the early or late stages of tumor development. Using the *Xiphophorus* genetic system, one may establish polymorphisms for genes exhibiting altered expression and directly assess their concordant inheritance with spontaneous or induced tumorigenesis. With these goals in mind we adapted use of a commercial differential display kit (Delta Display, CloneTech Inc.) to assess transcript analyses with the very small amounts of RNA one may isolate from aquaria fish tumors and other tissues.

Preliminary Results of the Differential Display: Identification of *RAB27*

The Delta Display kit uses oligo-dT primers that have a two base anchor on their 3' end. The anchor causes the forward primer to juxtapose at the correct site (i.e., where the polyA tail and the first 2 mRNA bases join; designated as T oligo-dT or anchored).

The other primers (reverse or toward the 3' end of the transcript; designated as P arbitrary) are designed from common motifs found in mRNAs. With many different P and anchored T primers one can perform an enormous number of independent differential display experiments, each of which will amplify a manageable (e.g., ~50 bands) sub-set of transcripts from the RNA sources (Figure 3-1). Differentially displayed bands are isolated from dried sequencing style gels after autoradiography, re-amplified via PCR, subcloned into suitable plasmid vectors and sequenced. Computer assisted homology searches against public genetic databases allows determination of gene similarity with known sequences. mRNAs from *X. helleri* × (*X. maculatus* Jp 163 B × *X.* helleri) tissues were used as the template in the differential display reaction. In our initial data set we isolated and subcloned 20 bands representing transcripts that showed robust amplification in tumor tissue but were poorly amplified in non-tumor control tissues. These 20 fragments were subjected to nucleotide sequencing and computer analyses. Most of the 20 sequences turned out to exhibit minimal and insignificant homology to known genes and proteins entered into the databases as of February, 2001 (i.e., NLBI's GenBank for Entrez nucleotide level homology searching and Baylor's Blast/Beauty search program for protein translational searching). However, search results from a few subclones revealed homology with known genes. For example, subcloned fragments that were isolated included fish homologues of the mammalian *RAB27* gene family, 50S ribosomal protein, an Ah receptor, and a cytochrome b (Table 3-1). From these studies it became necessary to look at gene candidates that might be expressed in tumors specifically, rather then those suspected to be up-regulated simply due to the growth properties of tumor cells. Thus, we initiated further and detailed characterization of the *Xiphophorus RAB27*

Figure 3-1. Exemplary differential display autoradiograph. The left four lanes represent the first loading, with S representing the non-tumor skin lanes, and T representing the tumor tissue lanes. Approximate amplicon sizes are indicated. The right four lanes correspond to the second run of the same samples.


S T S T S T S T
1:15 1:15 1:30 1:30 1:15 1:15 1:30 1:30
~500
~700
~500
~375

Clone	T3	Results	T7	Results	Category	Amplicon Size (bp)
P2/T2-t2	X	unidentified	X	Unidentified	tumor	400
P2/T2-t2	X	unidentified	X	Unidentified	tumor	400
P3/T4-t2	X	Rab27	X	Rab27	tumor	224
P3/T3-t1	X	unidentified	X	Unidentified	tumor	~425
P3/T3-t3	X	unidentified			tumor	200
P3/T3-t4	X	unidentified	X		tumor	~275
P3/T3-N1			X	Unidentified	normal	200
P4/T4-t1	X	unidentified	X		tumor	~500
P4/T4-t2	X	unidentified	X		tumor	~400
P5/T5-t1	X	unidentified	X	Unidentified	tumor	300
P5/T5-t1			X	cytochrome b	tumor	300
P6/T6-t2	X	unidentified	X	Unidentified	tumor	300
P7/T7-t1			X	Ah receptor	tumor	350
P8/T8-t1	X	unidentified	X	Unidentified	tumor	300
P8/T8-t3	X	cytochrome b	X	cytochrome b	tumor	275
P8/T8-t3	X	unidentified	X	Unidentified	tumor	275
P10/T4-t3	X	Ins. like grwth fctr	X	Unidentified	tumor	-
DFVHHY/T9-3	X	unidentified	X	Unidentified	helicase	-
DFVHHY/T9-4	X	50S ribosomal pro.	X	50S ribosomal pro.	helicase	-

Table 3-1. Differential display clone list including: differential display primers utilized in PCR, MCS sequencing primer used (T3 or T7), results of Blast/Beauty search, tissue source or domain desired, and the resulting amplicon size.

clone (pP3/T4-t2; amplified using the P3 arbitrary and T4 oligo-dT primers).

Isolation of the Genomic *Xiphophorus* Region Harboring the *RAB27* gene

A λ phage genomic library (constructed from *X. maculatus* Jp 163 A DNA) was screened using the subclone isolated from differential display gels and showing high degrees of nucleotide homology to the human *RAB27* as a probe (carried on plasmid pP3/T4-t2). The primary screening led to isolation of 15 plaques that appeared to positively hybridize the pP3/T4-t2 probe. Two of these clones (designated λ G1-1 and λ 8-1.1.1) were considered to be the best candidates for harboring the entire *RAB* gene sequence and were subjected to plaque purification. One of these clones, λ G1-1, was used for isolation of λ DNA. Southern hybridization, using several restriction endonuclease digests of G1-1 phage DNA, was performed with radiolabeled pP3/T4-t2 probe (Figure 3-2). From these results we observed a 2.7 kb *PvuII* generated genomic fragment that strongly hybridized to the probe. This fragment was isolated from the gel and sub-cloned creating plasmid pkGRab-2. Complete nucleotide sequence data of the 2.7 kb region was obtained using custom generated primers to "walk" the insert length. Alignment of the derived nucleotide sequence with other *RAB27* genes available in the databases indicated the 2.7 kb genomic fragment we had cloned from the phage spanned exon 5 and extended into the 3'-UTR. The data also indicated the pkGRab-2 clone did not represent *RAB27* sequence upstream of exon 5. Thus, other methodologies were employed to attempt isolation of the 5' end of the *Xiphophorus RAB27* gene. These methods involved isolation and sequence analysis of a full-length cDNA, employment of 5' RACE, and re-screening of the λ genomic library.

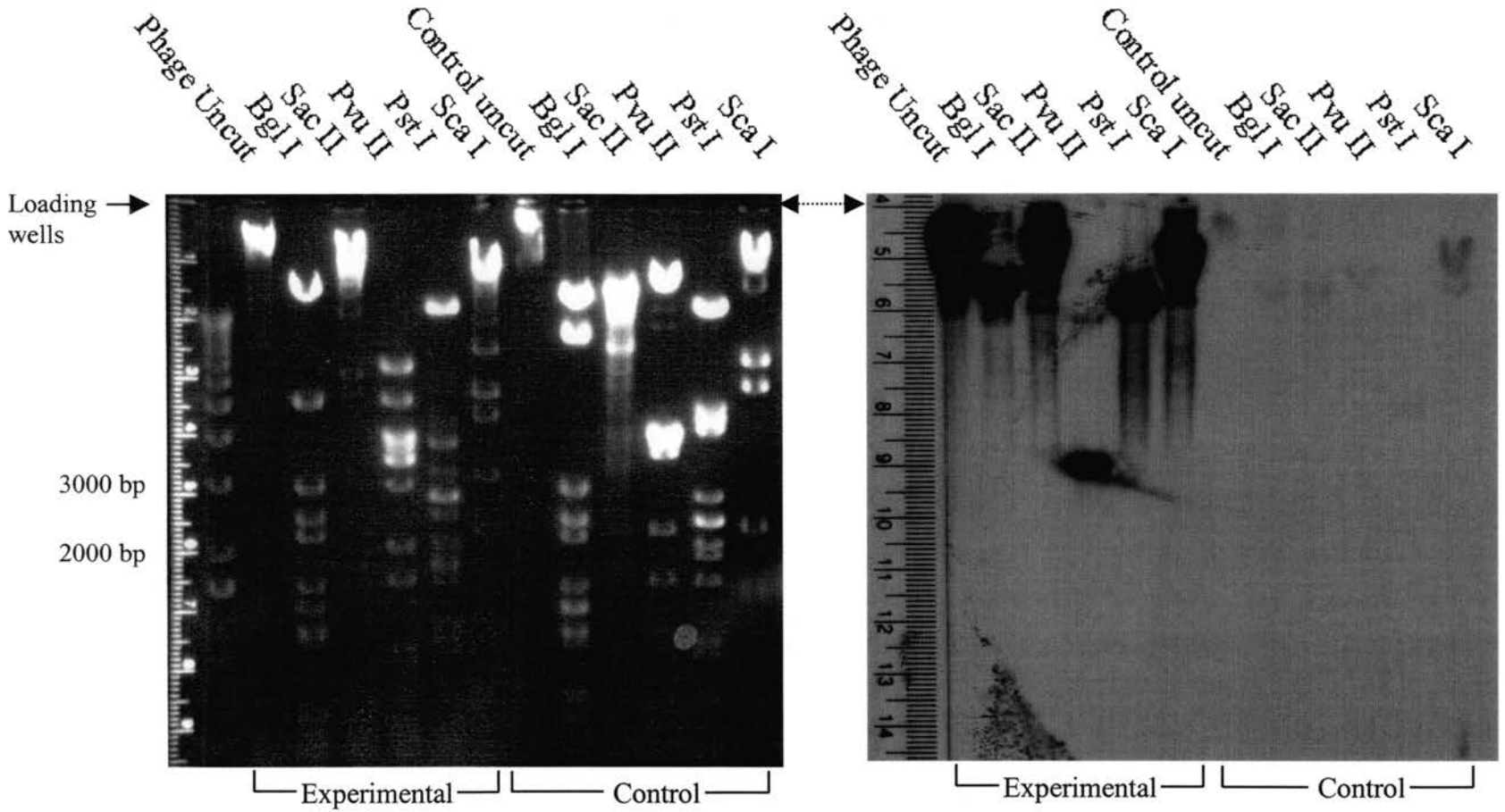


Figure 3-2. G1-1 phage DNA was digested with several endonucleases, and a Southern hybridization was performed using pP3/T4-t2 as the probe. The probe strongly hybridized to a 2.7 kb *PvuII* generated genomic fragment (5 cm from loading wells), not seen in the control lanes, as observed by comparing the autoradiograph (right) with the agarose gel picture (left).

Determination of the *RAB27* cDNA sequence

We had determined through our subcloning efforts that we possessed *RAB27* sequence comprising exon 5 to the 3' UTR of the mRNA. In order to obtain nucleotide sequence data from the 5' end of the gene a 5'-RACE kit was employed. Gene specific primers (Rab-ex6Ra, Rab-ex6Rb, Rab-ex6Rc; Table 2-4) were designed and custom synthesized (IDT Inc., IA) from exon 6 toward the 5' end of the mRNA to be used with the kit primers after the addition of an appropriate 5' end to the cDNA. Upon 5' RACE-RT-PCR, an ~800 bp product (i.e., the expected size) was observed after fractionation of the PCR amplification reaction on an agarose gel. This band was excised and sub-cloned creating plasmid, pkRab-RACE800.4. Nucleotide sequence data from the pkRab-RACE800.4 clone indicated this clone represented exon 1 toward the 3' end including a portion of exon 6 of the mRNA sequence. Analysis of all cDNA sequence data indicates that translation of the *X. maculatus RAB27* transcript would produce a 218 amino acid protein that shared 62-69% amino acid identity with similar mammalian Rab27 proteins (Table 3-2, Figure 3-3).

The *X. maculatus* Rab27 protein sequence contains four GTP-binding domains (amino acid residues 20-22, 74-79, 132-136, and 161-164; Figure 3-3) also observed in human and mouse Rab27a and Rab27b proteins (Figure 3-3). Chen et al. (1997a) reported there is minimal sequence identity between human Rab27a and Rab27b distal to the GTP-binding domain IV. We similarly observe significant reduction of homology between the fish and mammalian genes alignments distal to the homologous GTP domain (i.e., residue #165; Figure 3-3). Also, the fish sequence exhibits the conserved CXC-terminal isoprenylation motif (residues 216-218; Figure 3-3).

	Human *RAB27A*	Mouse *RAB27A*	Human *RAB27B*	Mouse *RAB27B*
Amino Acid Identity %	62	63	69	68
Nucleotide Identity %	51	52	53	50

Table 3-2. Nucleotide and amino acid identity values of the *X. maculatus RAB27* gene compared to the human and mouse *RAB27* genes.

Genomic DNA sequence determination

To generate the above data, we computer assembled the 5' end of the cDNA (isolated via RACE) with the genomic 3' end of the *Xiphophorus RAB27* gene sequence isolated from the λ G1-1 phage. To precisely confirm our sequence and check actual intron/exon splice junctions it is necessary to clone the 5' end of the gene directly from the genome. To do this we used extra-long PCR methods (XL-PCR). XL-PCR was performed with *X. maculatus* Jp 163 B genomic DNA as the template to attempt isolation of the 5' genomic DNA sequence. We used an existing primer (Rab-ex6Rc) for the 3' region and designed a new primer, designated Rab-ex1bF, corresponding to the 5'-end of the cDNA sequence. This primer pair was expected to amplify exons 1 through 6 from the genome. An amplified product ~2400 bp in length was isolated and sub-cloned creating plasmid pkRab-ex1b6.3. Sequence data from this clone showed it represented the expected genomic regions, containing exons 1-6 and all intervening introns.

The *RAB27* 5' Upstream Region

Having the entire coding sequence of the fish *RAB27* led us to analyze the 5' region. In the human *RAB27A* an untranslated exon, 1a, is separated by 33 kb from exon 1b (corresponding to our exon 1). Our current 5' gene sequence ended prior to identification of sequence having similarity to human exon 1a. It was of evolutionary, and perhaps of functional interest, to determine (1) if exon 1a existed in the fish *RAB27* gene, and (2) if the extremely large intron (33 kb) found in the human *RAB27* sequences was also present in the fish. This question could be addressed since our second λ phage isolate, λ 8-1.1.1, harbored the *RAB27* exon 1 and undetermined length of upstream sequence data. To rapidly clone this area, the 5' region from the phage clone was amplified using XL-PCR with one gene specific primer (Rab-ex2R) and the T3 λ phage multiple cloning site primer. A ~1,600 bp product was amplified when these primers were used and this was subcloned creating plasmid pkRab-5'.4. The nucleotide sequence from this clone was determined.

The complete *X. maculatus RAB27* genomic DNA sequence was constructed by combining overlapping nucleotide sequence data from plasmids pkGRab-2, pkRab-ex1b6.3, and pkRab-5'.4. A schematic of the genomic DNA structure and primers used for nucleotide sequencing is presented in Figure 3-4. The *X. maculatus RAB27* DNA sequence with exon/intron boundaries and translated protein is displayed in Figure 3-5. The *X. maculatus RAB27* exon/intron sizes are compared to the human *RAB27A* and *RAB27B* genes in Tables 3-3 and 3-4. The sizes of exons 3, 4, and 5 are conserved in all three genes, but exon organization of the *X. maculatus RAB27* gene is most comparable to the human *RAB27B* gene.

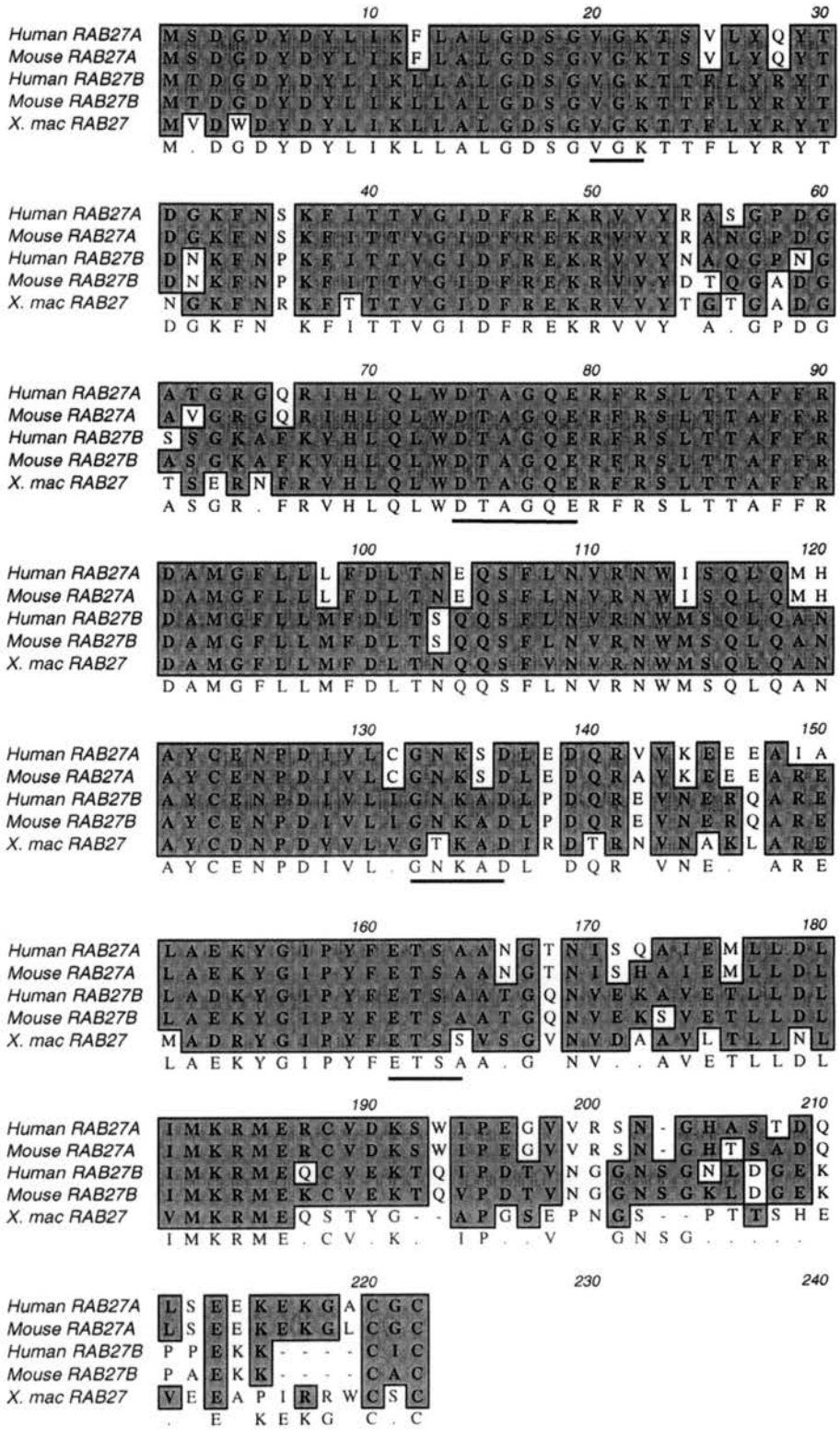
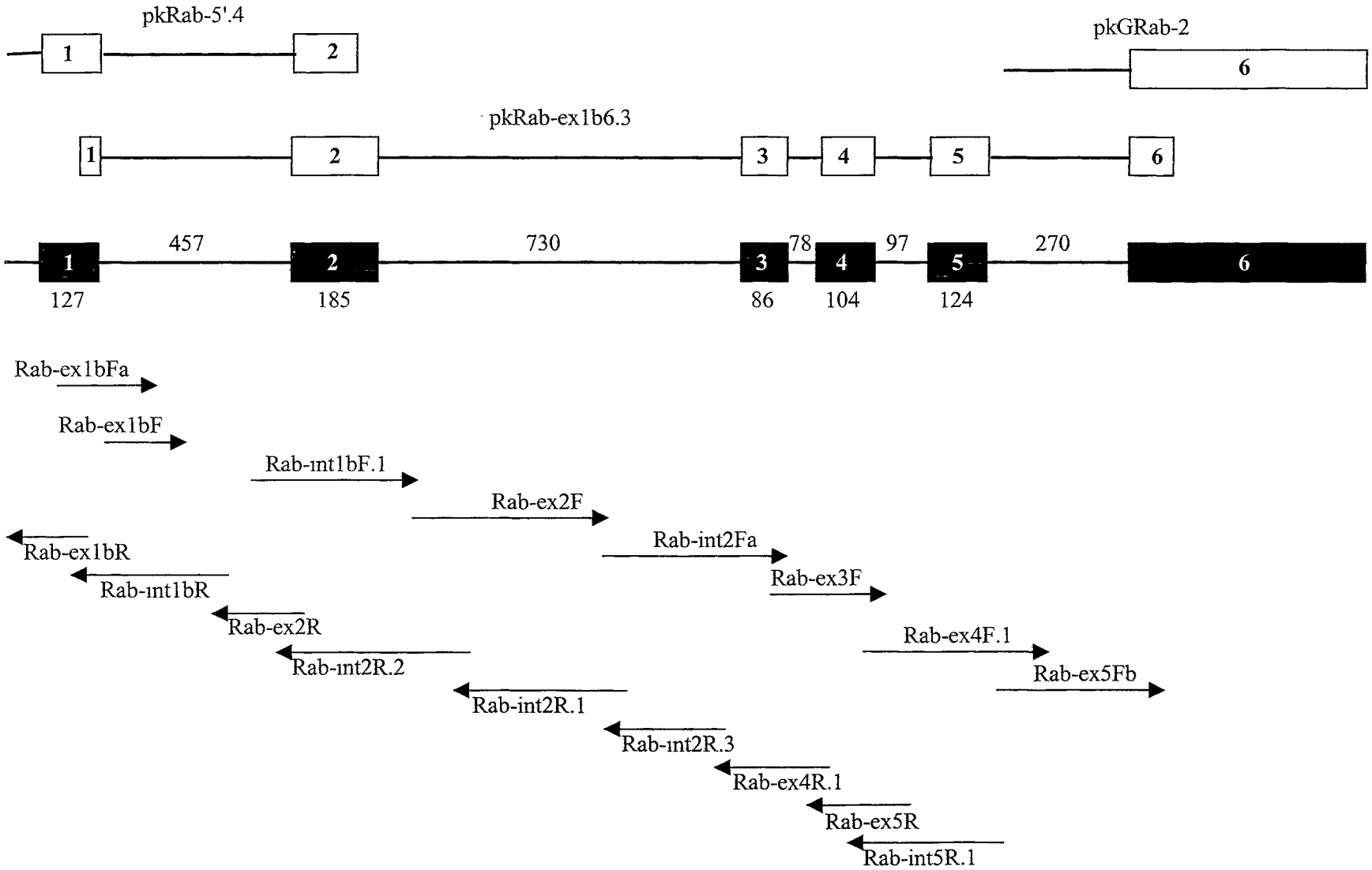


Figure 3-3. ClustalW alignment of the predicted *X. maculatus* Rab27 protein with human and mouse Rab27 proteins. The shaded areas represent conserved amino acids, and GTP-binding domains I-IV are underlined.

Figure 3-4. Pictorial view of the *X. maculatus RAB27* genomic organization determined by overlapping nucleotide sequence data from plasmid subclones pkGRab-2, pkRab-ex1b6.3, and pkRab-5'.4. The black boxes represent exons and the line represents the introns with appropriate sizes indicated. Below the genomic DNA structure is the primers used for nucleotide sequencing (for primer sequences see Table 2-4).


pkRab-5'.4
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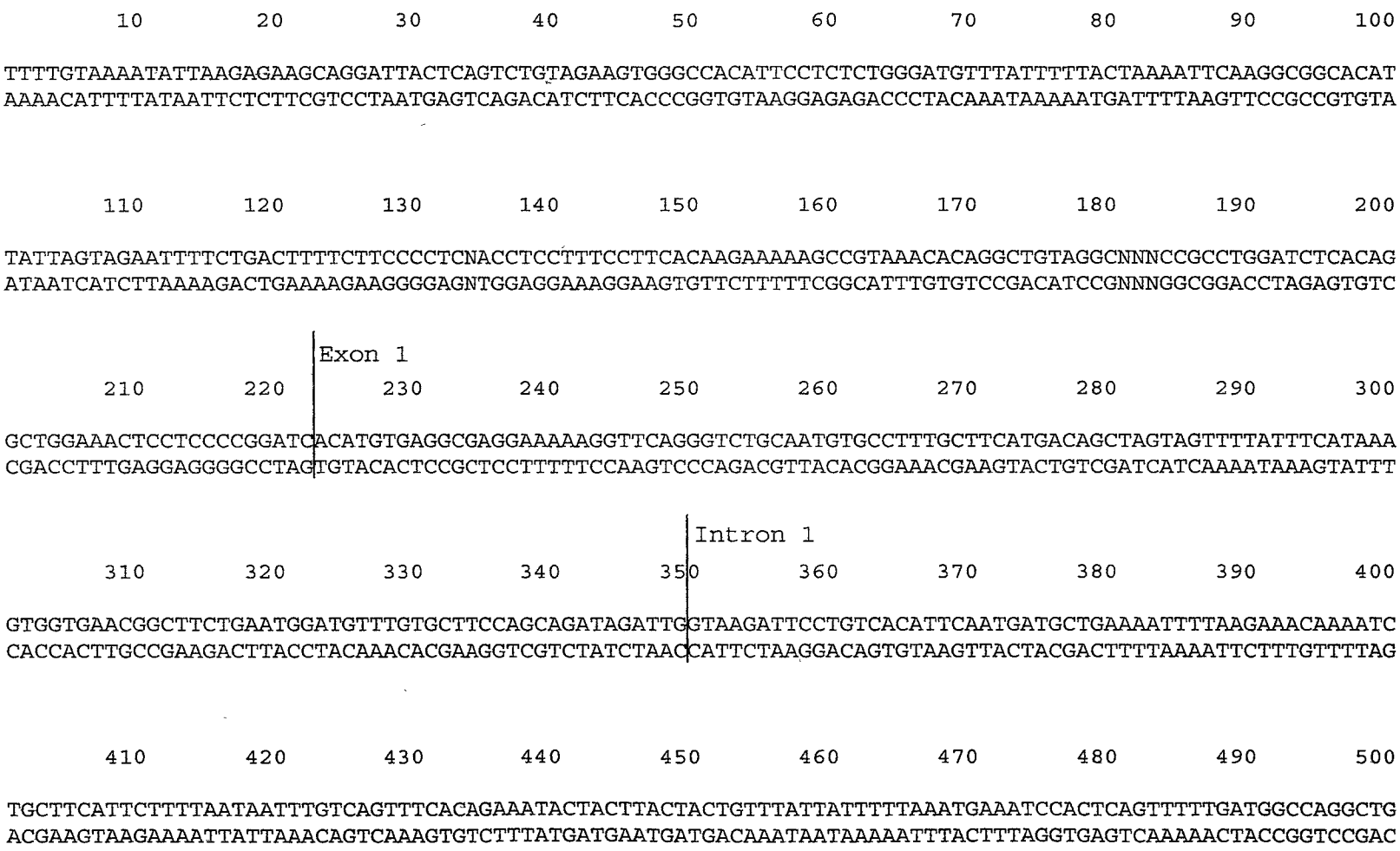
2
pkGRab-2
6
pkRab-ex1b6.3
1
2
3
4
5
6
457
730
78
97
270
1
2
3
4
5
6
127
185
86
104
124
Rab-ex1bFa
Rab-ex1bF
Rab-int1bF.1
Rab-ex2F
Rab-int2Fa
Rab-ex3F
Rab-ex1bR
Rab-int1bR
Rab-ex2R
Rab-int2R.2
Rab-ex4F.1
Rab-ex5Fb
Rab-int2R.1
Rab-int2R.3
Rab-ex4R.1
Rab-ex5R
Rab-int5R.1

Figure 3-5. The *X. maculatus RAB27* genomic DNA sequence with exon/intron boundaries marked. The translated protein amino acid sequence is under the corresponding sequence data with the presumed start and stop codons indicated in bold.


10 20 30 40 50 60 70 80 90 100
TTTTGTAAAATATTAAGAGAAGCAGGATTACTCAGTCTGTAGAAGTGGGCCACATTCCTCTCTGGGATGTTTATTTTTACTAAAATTCAAGGCGGCACAT
AAAACATTTTATAATTCTCTTCGTCCTAATGAGTCAGACATCTTCACCCGGTGTAAGGAGAGACCCTACAAATAAAAATGATTTTAAGTTCCGCCGTGTA
110 120 130 140 150 160 170 180 190 200
TATTAGTAGAATTTTCTGACTTTTCTTCCCCTCNACCTCCTTTCCTTCACAAGAAAAAGCCGTAAACACAGGCTGTAGGCNNNCCGCCTGGATCTCACAG
ATAATCATCTTAAAAGACTGAAAAGAAGGGGAGNTGGAGGAAAGGAAGTGTTCTTTTTCGGCATTTGTGTCCGACATCCGNNNGGCGGACCTAGAGTGTC
Exon 1
210 220 230 240 250 260 270 280 290 300
GCTGGAAACTCCTCCCCGGATCACATGTGAGGCGAGGAAAAAGGTTCAGGGTCTGCAATGTGCCTTTGCTTCATGACAGCTAGTAGTTTTATTTCATAAA
CGACCTTTGAGGAGGGGCCTAGTGTACACTCCGCTCCTTTTTCCAAGTCCCAGACGTTACACGGAAACGAAGTACTGTCGATCATCAAAATAAAGTATTT
Intron 1
310 320 330 340 350 360 370 380 390 400
GTGGTGAACGGCTTCTGAATGGATGTTTGTGCTTCCAGCAGATAGATTGGTAAGATTCCTGTCACATTCAATGATGCTGAAAATTTTAAGAAACAAAATC
CACCACTTGCCGAAGACTTACCTACAAACACGAAGGTCGTCTATCTAACCATTCTAAGGACAGTGTAAGTTACTACGACTTTTAAAATTCTTTGTTTTAG
410 420 430 440 450 460 470 480 490 500
TGCTTCATTCTTTTAATAATTTGTCAGTTTCACAGAAATACTACTTACTACTGTTTATTATTTTTAAATGAAATCCACTCAGTTTTTGATGGCCAGGCTG
ACGAAGTAAGAAAATTATTAAACAGTCAAAGTGTCTTTATGATGAATGATGACAAATAATAAAAATTTACTTTAGGTGAGTCAAAAACTACCGGTCCGAC

```
       510        520        530        540        550        560        570        580        590        600

CTCCCAGTCAGTGCCACATCTCATAACGATGACCTCAGTGGCATAGCTATCTAAGTCAATGAAAGGATTCTTATTTTGCTGAGCGAATTCAGGGAAAACT
GAGGGTCAGTCACGGTGTAGAGTATTGCTACTGGAGTCACCGTATCGATAGATTCAGTTACTTTCCTAAGAATAAAACGACTCGCTTAAGTCCCTTTTGA

       610        620        630        640        650        660        670        680        690        700

CCAGAGAGGCATTTGACTGCTCATGTGGATCCAGCCCTAGTCATGTGCTTGTCCTTCTGTGATCAGGGAGTTATTATGTGCTGTTAAGCCTCTCTGGTGG
GGTCTCTCCGTAAACTGACGAGTACACCTAGGTCGGGATCAGTACACGAACAGGAAGACACTAGTCCCTCAATAATACACGACAATTCGGAGAGACCACC

       710        720        730        740        750        760        770        780        790        800

TCATGCAGAAAGGAAAACAGTAGCTTTGATGGAAGAATTTAACTCACCCTGTCAGGATTTACTGAAAATCGCTCTAATGTGTCTGAACTCCCATGAAAAT
AGTACGTCTTTCCTTTTGTCATCGAAACTACCTTCTTAAATTGAGTGGGACAGTCCTAAATGACTTTTAGCGAGATTACACAGACTTGAGGGTACTTTTA

       Exon 2
       810        820        830        840        850        860        870        880        890        900

CCGCAGAGAGTCGTTCACAGATTCAACCAAGCAGCACGATGGTGGACTGGGACTATGACTATCTGATCAAGCTCCTGGCGCTCGGGACTCGGGCGTGGGA
GGCGTCTCTCAGCAAGTGTCTAAGTTGGTTCGTCGTGCTACCACCTGACCCTGATACTGATAGACTAGTTCGAGGACCGCGAGCCCTGAGCCCGCACCCT
                                    M  V  D  W  D  Y  D  Y  L  I  K  L  L  A  L  G  D  S  G  V  G

                                                                                          Intron 2
       910        920        930        940        950        960        970        980        990       1000

AAGACCACCTTCCTCTACAGGTACACGGACGGCAAGTTCAACAGGAAGTTCACGACCACGGTGGGAATTGACTTCAGGGAAAAGAGAGTGGTGAGTGCTG
TTCTGGTGGAAGGAGATGTCCATGTGCCTGCCGTTCAAGTTGTCCTTCAAGTGCTGGTGCCACCCTTAACTGAAGTCCCTTTTCTCTCACCACTCACGAC
 K  T  T  F  L  Y  R  Y  T  N  G  K  F  N  R  K  F  T  T  T  V  G  I  D  F  R  E  K  R  V
```

1010 1020 1030 1040 1050 1060 1070 1080 1090 1100

GGGAAGATAAAACTAGCTAAAGTCCTTCCATTATATAAACAAGTTCAATCATGGAACAAAAACATGCCAAGATACAGTAAATCATTTGGGTAATTCTAAC
CCCTTCTATTTTGATCGATTTCAGGAAGGTAATATATTTGTTCAAGTTAGTACCTTGTTTTTGTACGGTTCTATGTCATTTAGTAAACCCATTAAGATTG

1110 1120 1130 1140 1150 1160 1170 1180 1190 1200

TACGCTAAAACAAGAAAAGAAGGATCTGATTTGAGTTTTTATTCAGTTTATGTAAAATATCTGATTCAACTGTATGTAATATGTGCCTAGATTCTACATC
ATGCGATTTTGTTCTTTTCTTCCTAGACTAAACTCAAAAATAAGTCAAATACATTTTATAGACTAAGTTGACATACATTATACACGGATCTAAGATGTAG

1210 1220 1230 1240 1250 1260 1270 1280 1290 1300

ATATGTAAAATTGATGTTCTACATCAAAATTTCCATGTTGCTGTCATTTTAGATGCTGTAACATATTACATCTAAAATGAAACAAGAGTTTGACCGTCAG
TATACATTTTAACTACAAGATGTAGTTTTAAAGGTACAACGACAGTAAAATCTACGACATTGTATAATGTAGATTTTACTTTGTTCTCAAACTGGCAGTC

1310 1320 1330 1340 1350 1360 1370 1380 1390 1400

AAAAACAGAACCTCCAGTCAGAGACTTCTCCTGCCCACAGCCATTACCATCTACAACTCTTTAATTAAATGAGTAACAATAACATTTAATTTCCCTTTGG
TTTTTGTCTTGGAGGTCAGTCTCTGAAGAGGACGGGTGTCGGTAATGGTAGATGTTGAGAAATTAATTTACTCATTGTTATTGTAAATTAAAGGGAAACC

1410 1420 1430 1440 1450 1460 1470 1480 1490 1500

ATCAATAAAGTATTTTCAACATTGAACATGGCTGACTAAACCACATCTCCTTGGAGTGAACCAATGTTAAACATGTTGGGGTTTGTTGACTCTAAGGGAC
TAGTTATTTCATAAAAGTTGTAACTTGTACCGACTGATTTGGTGTAGAGGAACCTCACTTGGTTACAATTTGTACAACCCCAAACAACTGAGATTCCCTG

```
      1510       1520       1530       1540       1550       1560       1570       1580       1590       1600

GTTGGTAATTTGGTGAAAGAAATGTAAAATTTGGTGTCACCAGCATAAGGCATATTGGAATTATATAATCTGACCAAAGTGGAGAATAAAGATAATGAAT
CAACCATTAAACCACTTTCTTTACATTTTAAACCACAGTGGTCGTATTCCGTATAACCTTAATATATTAGACTGGTTTCACCTCTTATTTCTATTACTTA

      1610       1620       1630       1640       1650       1660       1670       1680       1690       1700

GAAAGTGGCTCAAAGTTAGAGCCTGGAGGAACGCCCCTGATGACTTTTTTTCCATCCTGATTAAAACAAAGTTGTCCTGGTTTTCCAAGCAAATGACGAT
CTTTCACCGAGTTTCAATCTCGGACCTCCTTGCGGGGACTACTGAAAAAAAGGTAGGACTAATTTTGTTTCAACAGGACCAAAAGGTTCGTTTACTGCTA

                         Exon 3
      1710       1720       1730       1740       1750       1760       1770       1780       1790       1800

GGAGTTCTGGTTTGGTTCAGGTTTACACGGGGACCGGTGCTGATGGGACGAGTGAGAGGAACTTCAGAGTCCACCTCCAGCTCTGGGACACAGCGGGTCA
CCTCAAGACCAAACCAAGTCCAAATGTGCCCCTGGCCACGACTACCCTGCTCACTCTCCTTGAAGTCTCAGGTGGAGGTCGAGACCCTGTGTCGCCCAGT
                         V  Y  T  G  T  G  A  D  G  T  S  E  R  N  F  R  V  H  L  Q  L  W  D  T  A  G  Q

         Intron 3                                                                                Exon 4
      1810       1820       1830       1840       1850       1860       1870       1880       1890       1900

GGAGAGGTAAGTTTGACATTTTTGACATTGACTGTGTTAAGAAGATACTCTTTGCTAGCATGGTTAAATATCCTTCACTCCAAGGTTTCGCAGCCTCACA
CCTCTCCATTCAAACTGTAAAAACTGTAACTGACACAATTCTTCTATGAGAAACGATCGTACCAATTTATAGGAAGTGAGGTTCCAAAGCGTCGGAGTGT
  E  R                                                                                F  R  S  L  T

                                                                                                  Intron 4
      1910       1920       1930       1940       1950       1960       1970       1980       1990       2000

ACGGCTTTCTTCCGAGATGCAATGGGCTTCCTGTTGATGTTCGACTTGACCAATCAGCAAAGTTTTGTTAACGTCAGGAACTGGATGAGTATGTTGTGTC
TGCCGAAAGAAGGCTCTACGTTACCCGAAGGACAACTACAAGCTGAACTGGTTAGTCGTTTCAAAACAATTGCAGTCCTTGACCTACTCATACAACACAG
 T  A  F  F  R  D  A  M  G  F  L  L  M  F  D  L  T  N  Q  Q  S  F  V  N  V  R  N  W  M
```


Exon 5
2010 2020 2030 2040 2050 2060 2070 2080 2090 2100
TTCTCGTTTCCCTCCTGTTGATTCCCAAGCCTGGTTGATTCGCAGTTTCAGGTTGATTGAAGCAGTTTCCCTGGTGTTTATGCAGGTCAGCTTCAGGCCA
AAGAGCAAAGGGAGGACAACTAAGGGTTCGGACCAACTAAGCGTCAAAGTCCAACTAACTTCGTCAAAGGGACCACAAATACGTCCAGTCGAAGTCCGGT
S Q L Q A
2110 2120 2130 2140 2150 2160 2170 2180 2190 2200
ATGCGTACTGCGATAACCCAGATGTGGTGCTTGTGGGCACTAAGGCAGACATCAGAGACACGAGGAACGTTAACGCCAAACTGGCCAGAGAGATGGCTGA
TACGCATGACGCTATTGGGTCTACACCACGAACACCCGTGATTCCGTCTGTAGTCTCTGTGCTCCTTGCAATTGCGGTTTGACCGGTCTCTCTACCGACT
N A Y C D N P D V V L V G T K A D I R D T R N V N A K L A R E M A D
Intron 5
2210 2220 2230 2240 2250 2260 2270 2280 2290 2300
CAGATACGGGTGAGTGGAAACTGATCAGTCTTTATCAGTGAGGAAGATTGGCCTTTTATCTGTTGATAGGATTTTCTTCCTATCCCACTTTTATTATATT
GTCTATGCCCACTCACCTTTGACTAGTCAGAAATAGTCACTCCTTCTAACCGGAAAATAGACAACTATCCTAAAAGAAGGATAGGGTGAAAATAATATAA
R Y G
2310 2320 2330 2340 2350 2360 2370 2380 2390 2400
TAGCAACTGTCGGCTAAATCAGTGTTTTACCCTGTGTTTCAAAGTTTGGATGAGATATCCTTTTAAAGACATTTAAGCAAAATAAAATGTAAAGATAAAA
ATCGTTGACAGCCGATTTAGTCACAAAATGGGACACAAAGTTTCAAACCTACTCTATAGGAAAATTTCTGTAAATTCGTTTTATTTTACATTTCTATTTT
Exon 6
2410 2420 2430 2440 2450 2460 2470 2480 2490 2500
ATATTTGTCTAGATCTCATGAAGTGTTTTGAAGATGATGCATCTTGTACAGTTTTGTGAAATGTTCCTGTTTCCTTCAGCATCCCCTACTTCGAGACGAG
TATAAACAGATCTAGAGTACTTCACAAAACTTCTACTACGTAGAACATGTCAAAACACTTTACAAGGACAAAGGAAGTCGTAGGGGATGAAGCTCTGCTC
I P Y F E T S

```
      2510       2520       2530       2540       2550       2560       2570       2580       2590       2600

TTCTGTGTCGGGAGTGAATGTGGACGCCGCTGTGCTCACCCTGCTGAACCTGGTGATGAAGAGGATGGAGCAGAGCACGTATGGGGCCCCTGGCTCTGAA
AAGACACAGCCCTCACTTACACCTGCGGCGACACGAGTGGGACGACTTGGACCACTACTTCTCCTACCTCGTCTCGTGCATACCCCGGGGACCGAGACTT
  S  V  S  G  V  N  V  D  A  A  V  L  T  L  L  N  L  V  M  K  R  M  E  Q  S  T  Y  G  A  P  G  S  E

      2610       2620       2630       2640       2650       2660       2670       2680       2690       2700

CCCAATGGGAGCCCCACCACCAGCCATGAGGTGGAGGAGGCCCCCATCAGGAGGTGGTGCTCCTGTTAATGTTCCCAGCAGCAGCAGCAACGGCGTAGGA
GGGTTACCCTCGGGGTGGTGGTCGGTACTCCACCTCCTCCGGGGGTAGTCCTCCACCACGAGGACAATTACAAGGGTCGTCGTCGTCGTTGCCGCATCCT
 P  N  G  S  P  T  T  S  H  E  V  E  E  A  P  I  R  R  W  C  S  C  *

      2710       2720       2730       2740       2750       2760       2770       2780       2790       2800

GAAACAGACTTTAAAAAGTGTATAGACAAAGACTTATTGAAATTTCCTATATTTCTTTTGGTTTCTCTTGTTGTGTTAAGAACAGAATAAACTACTTTTG
CTTTGTCTGAAATTTTTCACATATCTGTTTCTGAATAACTTTAAAGGATATAAAGAAAACCAAAGAGAACAACACAATTCTTGTCTTATTTGATGAAAAC

      2810       2820       2830       2840       2850       2860       2870       2880       2890       2900

TCAGTTTCTCCTCCTAGTTTGTTTTCAAAACAAAGAAGCAATAAAGTATATAGTTATGAACTTTAATAAAATAAAATAAAAACAAACCCCTTCATCAGTC
AGTCAAAGAGGAGGATCAAACAAAAGTTTTGTTTCTTCGTTATTTCATATATCAATACTTGAAATTATTTTATTTTATTTTTGTTTGGGGAAGTAGTCAG

      2910       2920       2930       2940       2950       2960       2970       2980       2990       3000

TTACCTGAGAGGCACAAAGTGAATTTATGCAAAGAAACTTAGTTAAGTTACAAAGCACTATTATTCTTATTGATTTCTGTGAGTAATTTTATAATACATC
AATGGACTCTCCGTGTTTCACTTAAATACGTTTCTTTGAATCAATTCAATGTTTCGTGATAATAAGAATAACTAAAGACACTCATTAAAATATTATGTAG
```

```
      3010       3020       3030       3040       3050       3060       3070       3080       3090       3100

CTTGAAAGTATTAATATTTCACTTGTAATAGTATTTTTTTAATCTTTATTGTTTTACATTATTTCTCATCACATAACTCTTGCAAATACATGTCTGTTTG
GAACTTTCATAATTATAAAGTGAACATTATCATAAAAAAATTAGAAATAACAAAATGTAATAAAGAGTAGTGTATTGAGAACGTTTATGTACAGACAAAC

      3110       3120       3130       3140       3150       3160       3170       3180       3190       3200

TTAAAAGGTGCTTCCTTCAACAGGTTAGGATACGTCTAGTCAATACAAGACATGTTCATTACAATTTTTGCACAAAATCATTCTTAGATGATGAGATTTT
AATTTTCCACGAAGGAAGTTGTCCAATCCTATGCAGATCAGTTATGTTCTGTACAAGTAATGTTAAAAACGTGTTTTAGTAAGAATCTACTACTCTAAAA

      3210       3220       3230       3240       3250       3260       3270       3280       3290       3300

AGTCTGTTCAGTTCTGACTATTTCCAGCTCCTTCCAGAACGAGTTGTTTTAGTGCCTCTTGTCACTTTTAATACAAATAAACGGCACAAACAGATGTGCA
TCAGACAAGTCAAGACTGATAAAGGTCGAGGAAGGTCTTGCTCAACAAAATCACGGAGAACAGTGAAAATTATGTTTATTTGCCGTGTTTGTCTACACGT

      3310       3320       3330       3340       3350       3360

ATTACACAGGGGCGGATCCCCCGGGCTGCAGGAATTCGATATCAAGCTTATCGATACCGTCGACCTC
TAATGTGTCCCCGCCTAGGGGGCCCGACGTCCTTAAGCTATAGTTCGAATAGCTATGGCAGCTGGAG
```

Exon (no.)	*X. maculatus RAB27*	Human *RAB27A*	Human *RAB27B*
1	127	-	71
1a	-	~230	-
1b	-	89	-
2	185	175	172
3	86	86	86
4	104	104	104
5	124	124	124
6	888	2740	6624

Table 3-3. Comparable exon organization and sizes (bp) between *X. maculatus* Jp 163 B *RAB27* and human *RAB27A* and *RAB27B*.

Intron (no.)	*X. maculatus RAB27*	Human *RAB27A*	Human *RAB27B*
1	457	-	>30000
1a	-	33000	-
1b	-	3400	-
2	730	4000	1600
3	78	2000	5300
4	97	4500	3500
5	270	14000	11000

Table 3-4. Intron organization and sizes (bp) between *X. maculatus* Jp 163 B *RAB27* and human *RAB27A* and *RAB27B*.

Chapter 4-Results and Discussion

Mapping of *Xiphophorus RAB27* Locus

Introduction

Due to the ability to produce fertile interspecies hybrids, mapping new loci in *Xiphophorus* is a relatively straightforward task. Interspecies hybrid animals (i.e. F_1 hybrids) possess one half of their genetic content from each parent and since these parents are evolutionarily diverged approximately 65 to 80 million years they are polymorphic for many or most loci along the length of every chromosome. Backcrossing the F_1 hybrid to one of the initial parental species (i.e. the recurrent parent) produces backcross hybrid progeny (BC_1 hybrids) carrying, on average, 75% of their genetic information from the recurrent parent and 25% from the non-recurrent parent. Segregation of the non-recurrent parent chromosomes and/or chromosomal regions (due to crossover events) into the BC_1 hybrids allows one to assess the marker inheritance (or genetic association) of each genetic marker with development of a complex phenotype such as tumor development. To do this, one genotypes BC_1 hybrids with a large set of genetic markers, scoring each hybrid as homozygous or heterozygous for the marker at each locus, representing all chromosomes (or linkage groups, LGs). Two-by-two chi square analyses of marker inheritance for each possible marker pair is then performed using computer programs. An excess of parental types among the BC_1 hybrids for any particular marker pair (i.e. deviation from the expected 50:50 ratio for random

segregation) indicates genetic linkage and can be used to assign map position to new loci (Morizot *et al.*, 1998).

Mapping the *Xiphophorus RAB27* locus

We have characterized and sequenced both the *RAB27* cDNA and gene (Chapter 3). Having sequence data for the gene, variable intronic regions allowed us to search for and eventually establish a polymorphism between parental fish stocks that was able to be easily resolved in agarose gels. The parental DNAs were used as target to perform standard PCR of the region spanning *RAB27* intron 5. The amplicon from most parental stocks was of equal size and the species source could not be discerned in agarose gels, however, when the amplified fragments were cleaved with the *RsaI* restriction endonuclease a polymorphism was established. For mapping we used this PCR based *RsaI* polymorphism between parental stocks, *X. maculatus* Jp 163 A and *X. helleri* (Sara; Figure 4-1). The amplified product of *X. maculatus* Jp 163 A contained the *Rsa I* restriction endonuclease site. This polymorphism allowed the screening of BC_1 hybrids produced by crossing the F_1 back to the *X. helleri* parent. A panel of fifty BC_1 hybrid DNAs were scored as exhibiting the banding pattern of either the *X. helleri* (Sarabia) parent (i.e., homozygous) or the F_1 parent (i.e., heterozygous). These genotypic data were entered on a spreadsheet and exported into Map Manager to produce recombination values and LOD scores (Table 4-1). Two by two linkage analyses of these markers resulted in localization of the *X. maculatus RAB27* gene distal to the *PEPS* isozyme marker on a telomeric region of LG XII (Figure 4-2).

Human *RAB27A* was mapped to chromosome 15q15-21.1 between markers WI-3687 and AFM321ZD5 (Tolmachova et al., 1999), and *RAB27B* was mapped

to chromosome 18q21.1 between markers WI-4115 and AFM3577TD5 (Ramalho et al., 2001). Although many areas of the gene maps possess conserved gene order (i.e., are syntenic) over the 450 million years separating fish and humans from a common ancestor, this region of the fish LG XII is not among them. Thus, this mapping assignment, although informative for future works in *Xiphophorus*, does not appear to have any particular evolutionary relevance.

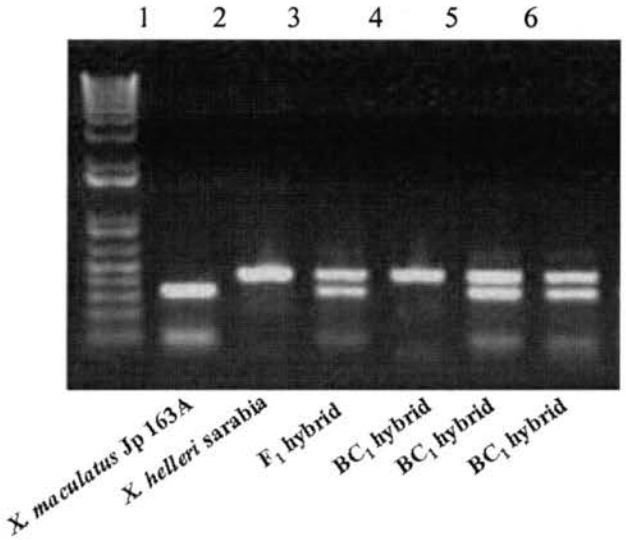


Figure 4-1. An example of the *RAB27* intron 5 PCR-*RsaI* restriction digest polymorphism between *X. maculatus* Jp 163A and *X. helleri* sarabia. The F_1 hybrid contains representative alleles from each parent. The BC_1 hybrids exhibits either the heterozygous F_1 parent (lanes 5 and 6) or the homozygous *X. helleri* parent (lanes 4 and 7).

Marker	LG	Recombinants	Parentals	%Recomination	LOD
XD0120(LinF1.5)	I	16	14	50	0.0
XD0155(P7.2)	II	13	19	40.6	0.2
XD0101(FP.3)	III	18	16	50	0.0
XD0107(FP10)	IV	17	16	50	0.0
P13(CDKN2X)	V	24	19	50	0.0
XD0108(FP11a)	VI	19	15	50	0.0
XD0208(RTF1.3)	VII	13	15	46.4	0.0
XD0227(RTR1.6)	VIII	21	20	50	0.0
XD0158(P7.4)	IX	16	18	47.0	0.0
XD0149(P53P8.6)	X	13	16	44.83	0.1
CKM	XI	24	19	50	0.0
XD0099(FP.2)	**XII**	**7**	**28**	**20.0**	**2.9**
XD0170(R1.2)	**XII**	**7**	**29**	**19.4**	**3.1**
XD0242(RTR2.12)	**XII**	**5**	**25**	**16.7**	**3.2**
T39.11	**XII**	**5**	**25**	**16.7**	**3.2**
PEPS	**XII**	**7**	**33**	**17.5**	**4.0**
XD0104(FP.7)	XIII	20	17	50	0.0
XD0082(CP1.3)	XIV	15	18	45.4	0.1
XD0147(P53P8.4)	XV	13	16	44.8	0.1
XD0179(R1.10)	XVI	19	19	50	0.0
XD0022(CP1.4)	XVII	18	19	48.6	0.3
XD0078(CP1.1)	U16	11	22	33.3	0.8
XD0079(CP1.2)	U18	13	19	40.6	0.2
XD0102(FP.4)	U19	18	19	48.6	0.0
XD0088(CP1.9)	U20	20	16	50	0.0
XD0089(CP1.10)	U22	19	12	50	0.0
XD0177(R1.8)	U23	21	17	50	0.0
XD0154(P7.1b)	U24	9	19	32.1	0.8

Table 4-1. Results from joint segregation (two-by-two) analyses of marker inheritance showing percent recombination for each marker pair and LOD scores for *X. maculatus* Jp 163 B *RAB27* compared with each of the other markers scored in this cross. A LOD score greater than 3.0 is criteria for linkage (P>0.01) and is observed only for markers in linkage group XII (bold rows) indicating this gene locus resides on LG XII.

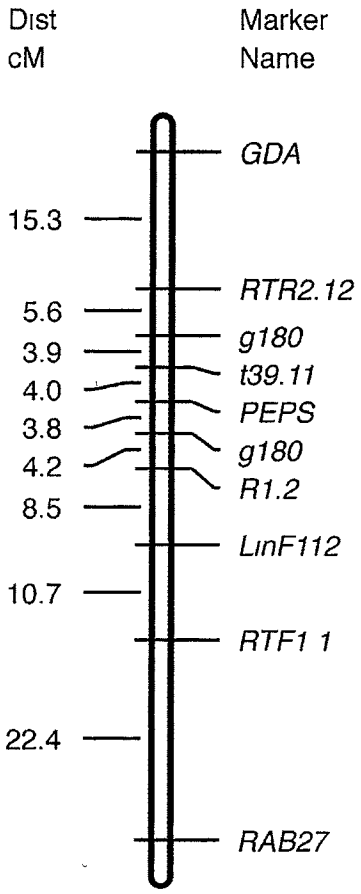


Figure 4-2. Gene order of *Xiphophorus* linkage group XII markers. Further linkage data is required to determine the exact map order and location of *X. maculatus RAB27* on LG XII, however, preliminary assignment to the regions indicated can be made at this time.

Chapter 5-Results and Discussion

Transcriptional Expression of *Xiphophorus RAB27*

Introduction

RAB27A expression varies in multiple human tissues but is highly expressed in liver and testis (Chen et al., 1997a). Furthermore, human muscle exhibits low expression of the *RAB27A* transcript (when compared to liver and testis) and there is no recorded expression in brain. The *RAB27A* transcript is very abundant in human eye tissue and more specifically in tissues associated with the retinal pigment epithelium (RPE) and the choriocapillaris (Seabra et al., 1995). Consistent with this, using an antibody specific for Rab27a, Seabra et al. (1995) reported high levels of Rab27a protein in eye, especially in the RPE and choriocapillaris, while Rab27a protein was low/absent in brain and skeletal muscle. This indicates protein and transcriptional expression patterns are proportional for *RAB27A*, at least in humans. In contrast to *RAB27A*, *RAB27B* transcription exhibits significantly higher expression only in human testis, but is also expressed at low levels in three differentiated cell types including melanocytes, testis, and platelets (Chen et al., 1997a, and Nagata and Nozawa, 1993).

Rab proteins are active when prenylated by Rab geranylgeranyl transferase (Rab GGTase). The absence of Rab27a prenylation in humans leads to an X-linked retinal degeneration disease, choroideremia (CHM). Rab GGTase requires an accessory protein

located on the X chromosome, Rab escort protein-1 (REP-1), to prenylate Rab27a. Without REP-1 there is a degeneration of the retinal pigment epithelium (RPE) and the choriocapillaris. The degenerative characteristics of the disease are slowed by REP-2. The REP-2 protein sequence is 75% identical to REP-1 and may partially compensate for REP-1 loss of function (Chen et. al., 1997a, Seabra et al., 1995, and Tolmachova et al., 1999). Also, it is believed that many Rabs share transportation pathways in a cell. Redundant functionality of Rab27a and Rab27b proteins may supplement the lack of expression/function of one of the genes in most tissues (Ramalho et al., 2001).

Determination of the linear range of amplification

Levels of transcription of the *X. maculatus* Jp 163 B *RAB27* were determined in a set of tissues by relative, quantitative RT-PCR (Q-RT-PCR). The tissue transcript expression level is characterized by comparing the amplification intensity of a *RAB27* amplicon with the amplification intensity of an internal control derived from amplification of 18S rRNA. *X. maculatus* Jp 163 B total RNA from various *Xiphophorus*

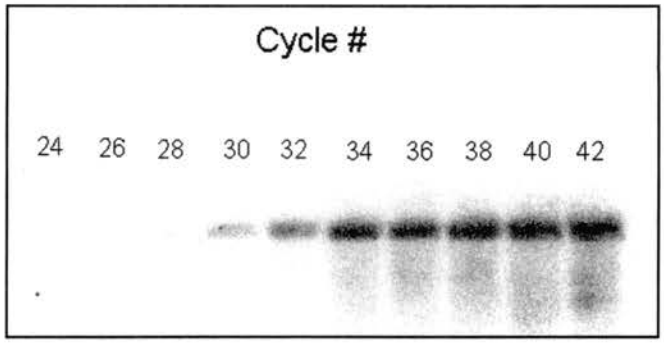


Figure 5-1. Determination of linear range of amplification for the cDNA fragment used in relative Q-RT-PCR. To derive this data, a PCR tube was removed at every 2 cycles, as indicated, and radioactivity of the amplified product was measured using a phosphorimager. The *X. maculatus RAB27* product exhibits linearity between cycles 28 and 34.

tissues was transformed to cDNA via reverse transcription. This cDNA was the target for PCR amplification using primers Rab-ex1bF and Rab-ex4R to amplify 420 bp comprising exons 1 to 4 of *RAB27*. Crossing several introns in this assay assured potential contamination of DNA did not influence to results obtained. Using a mixture of cDNA derived from several tissues, the linear range of amplification for the 420 bp *RAB27* transcript was determined as between cycles 28 to 34 (Figure 5-1, 5-2). In order to maintain linear amplification and maintain product quantity, an endpoint of 33 cycles was used in the PCR experiments aimed at determination of relative transcript levels (see below).

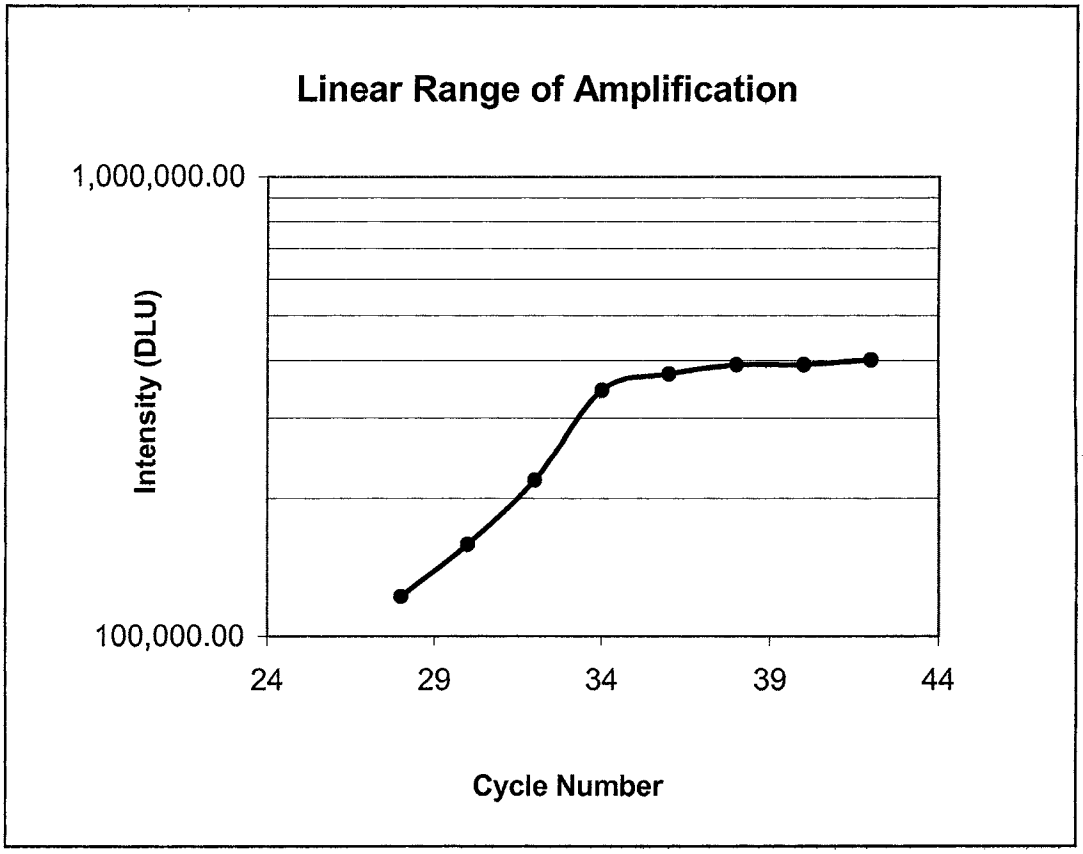


Figure 5-2. The amplification curve for the 420 bp *X. maculatus RAB27* amplicon. The amount of amplified product is linear between cycles 28 and 34.

Determination of the optimal primer:competimer ratio

To quantify relative levels of *X. maculatus RAB27* an internal control (18S rRNA) was used. It is widely held that 18S rRNA levels are relatively constant among various tissues sources and thus should be equally abundant in total RNA preparations derived from them. Thus, determination of the relative abundance of a particular target transcript (in our case *RAB27*) among different tissues can be performed by assessing its level relative to co-isolated 18S RNA. However, since the 18S RNA is extremely abundant in a total RNA preparation, compared to mRNA, the amplification of this internal control must be hampered using mixtures of primers having normal or blocked 3' termini (i.e., competimers). Changing the ratio of 18S primers to competimers in the reaction mixture varies the amplification efficiency of the 18S product. A search for the ratio of 18S primers to competimers that results in amplification nearly equal to the linear range of target gene amplification was initially performed. This analysis (Figure 5-3) indicated that after 33 cycles the optimal 18S primer:competimer ratio, giving amplification equal to the *RAB27* target gene, was 1:19. This ratio of 18S primer:competimer was used in all subsequent experiments.

Relative expression of RAB27 in various tissues

In order to assess relative transcription of the fish *RAB27* gene we performed multiplex (18S and *RAB27* 420 bp co-amplification) Q-RT-PCR reactions using cDNA pools isolated from various tissues. Analysis of these data indicate that overall the *X. maculatus* Jp 163 B *RAB27* transcript exhibits highest expression in eye tissue compared to other tissues tested (Figure 5-4). This expression is consistent with the reported human expression of *RAB27A* and with expression of *RAB27* subfamily genes in pigmented

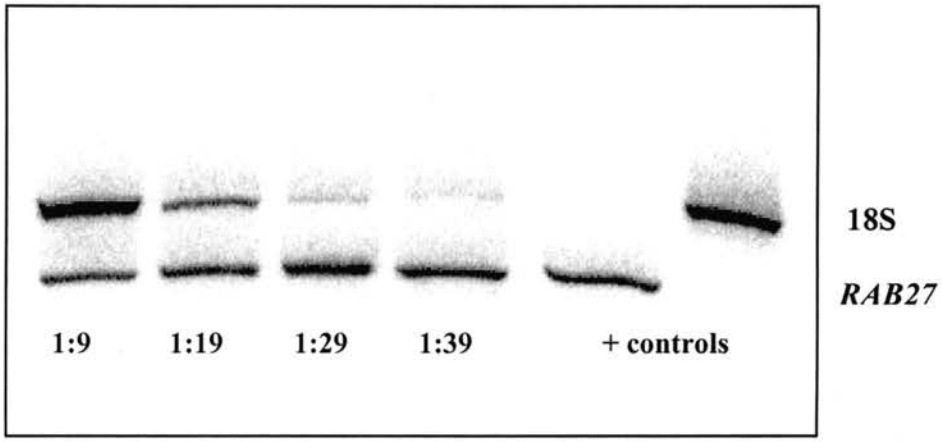


Figure 5-3. Determination of the optimal 18S Primer:Competimer ratio. As shown, a ratio of 1:19 exhibits similar amplification intensities of the 18S rRNA amplicon and the *X. maculatus RAB27* amplicon. Therefore, the 1:19 ratio was used in subsequent relative Q-RT-PCR experiments.

cells. Reasonable *RAB27* expression is also observed in brain, gill, muscle, liver, and testis. These tissues express the *RAB27* transcript at similar levels, at least determined by the relative Q-RT-PCR assay (Figure 5-4).

Relative Q-RT-PCR expression in parental and BC_1 hybrid fishes was also performed. In the *X. couchianus* × (*X. maculatus* Jp 163 B × *X. couchianus*) backcross, the resulting progeny exhibit two melanization patterns based on the degree of black pigment body coverage (Figure 5-5). Thus, we separately assessed *RAB27* expression in animals having spotted skin (light pigmentation) and highly melanized skin tissue (heavy pigmentation).

As shown in figures 5-6 and 5-7, expression of the *X. maculatus RAB27* transcript appears to increase with degree of melanization. Hume et al. (2001) reports that the Rab27a protein in humans is highly expressed in melanocytes and associated with mature melanosomes (i.e. specialized, melanin producing organelles). Data also show Rab27a

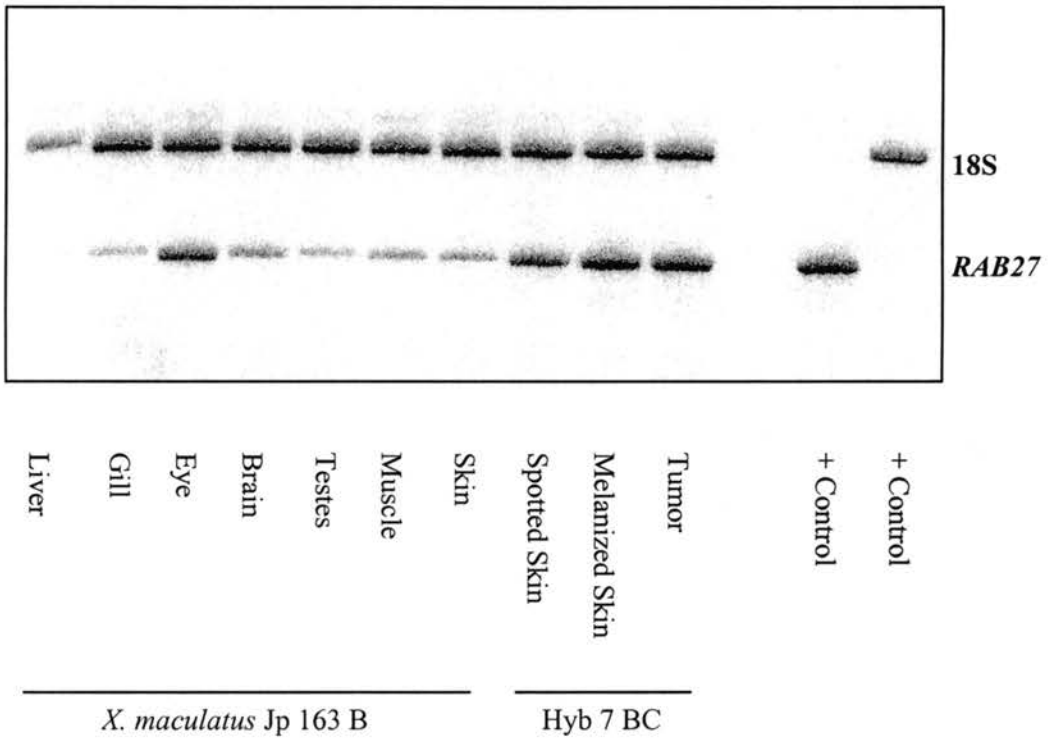


Figure 5-4. Example of a Relative Q- RT-PCR gel showing multiplex analyses of cDNA from various tissues. The total DLUs of the *X. maculatus RAB27* amplicon are divided by the DLUs of the 18S amplicon to normalize the response and provide a direct comparison of expression between tissue source.

and myosinVa co-localize and interact in melanocytes (Chen et al., 1997a, Orlow, 1995). From these reports, a potential function for the Rab27a protein has been forwarded as being involved in establishment or maintenance of the peripheral distribution of melanosomes within the dendritic processes of melanocytes. The movement to, and retention of melanosomes to the tips of melanocyte dendrites is essential for communication with keratinocytes and consequently the production of normal pigmentation patterns. As such, Rab27a may be responsible for recruiting myosinVa to

melanosomes (Hume et al., 2001). A similar role for the fish *RAB27* protein would be consistent with the observed expression patterns (Figures 5-5, 5-6, and 5-7).

X. maculatus RAB27 expression was found at the highest levels in the melanotic tumor tissues taken from *X. couchianus* × (*X. maculatus* Jp 163 B × *X. couchianus*) backcross animals bearing MNU induced melanoma. These results are consistent with the initial isolation of the *RAB27* using differential display methods aimed at observing transcript fragments that were highly expressed in melanoma, but lowly, or not expressed in non-tumor tissue.

In humans, northern analyses of *RAB27A* indicate high expression in two leukemia cell lines, Hela cells and a single melanoma (G361) cell line. In addition, human *RAB27B* expression was detected in melanomas, melanocytes, and fibroblast cells by RT-PCR. Based on the expression pattern of human *RAB27* gene subfamily members and the proposed function of Rab proteins in regulation of vesicular transport, it has been hypothesized that *RAB* gene dysfunction is related to human disorders including Hermansky-Pudlak syndrome (HPS) and the Chediak-Higashi (CHD) disease (Chen et al., 1997a, Chen et al, 1997b). These autosomal recessive disorders exhibit defects in pigment dilution and platelet storage pools, including albinism, decreased platelet dense bodies, and giant or fused, dysfunctional lysosomes (Chen et al., 1997a, Swank et al., 1993). A PCR-based strategy using a pigmented human melanoma cell line as the template resulted in cloning 17 mRNAs having sequence similarity to members of the *RAB* gene family, including *RAB27A* and *RAB27B* (Chen et al., 1997b). Experiments to determine the basis for high expression *RAB27* in *Xiphophorus* melanoma, the functional role played by Rab27, and its possible role as a valuable marker for melanomagenesis

form the basis of future experiments. Data presented detailing the expression of *RAB27* in various non-tumor and tumor tissues establish a baseline upon which one may frame these future studies.

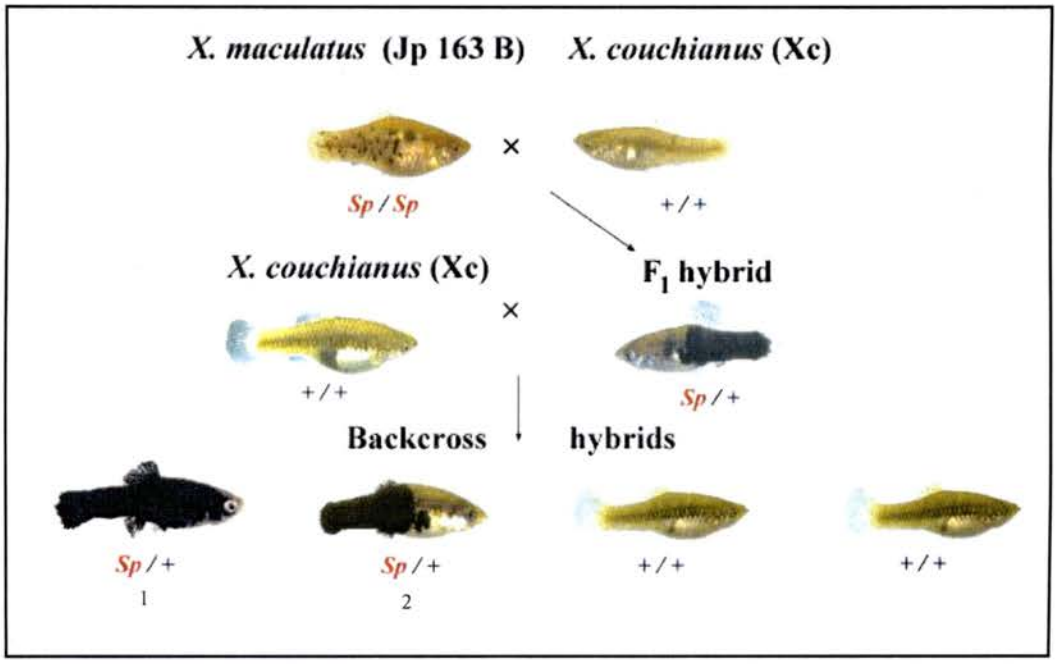


Figure 5-5. The *X. couchianus* × (*X. maculatus* Jp 163 B × *X. couchianus*) backcross. BC_1 progeny from this cross form spontaneous melanoma at under 5% incidence, but exposure to MNU at 6 weeks post-birth results in melanoma induction in up to 28% of the progeny (Walter, R.B., personal comm.). [1]Melanized skin (heavily pigmented). [2]Spotted skin (lightly pigmented).

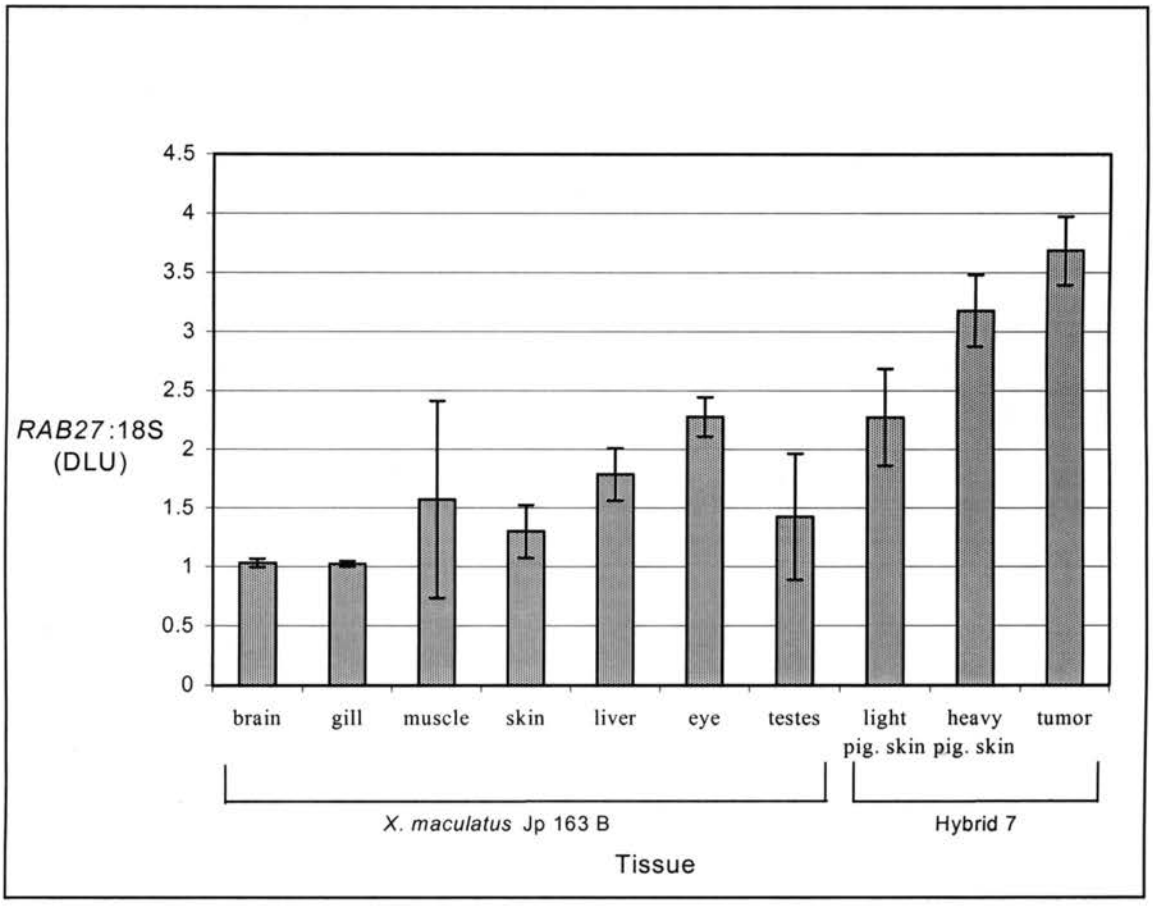


Figure 5-6. Relative, Q-RT-PCR for various tissues from *X. maculatus* Jp 163 B and select tissues from *X. couchianus* × (*X. maculatus* Jp 163 B × *X. couchianus*) BC_1 progeny. The highest relative expression of *X. maculatus RAB27* is in eye tissue. *RAB27* expression in the BC_1 hybrids increases with increasing melanization. Highly melanized tumor tissue exhibited the highest relative expression. Data shown represent three independent repeats of the multiplex experiment using three independent RT reactions. The error bars indicate standard deviation.

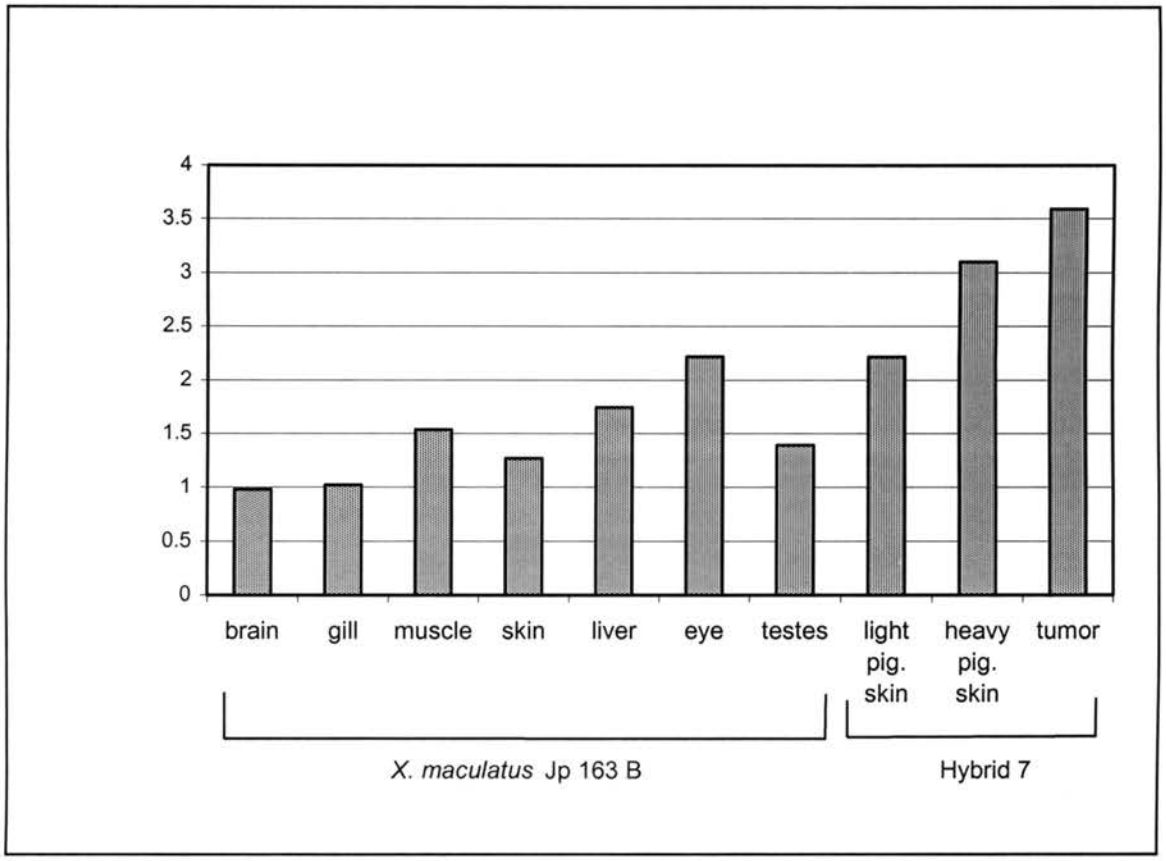


Figure 5-7. Normalized relative, Q-RT-PCR for various tissues from *X. maculatus* Jp 163 B and select tissues from *X. couchianus* × (*X. maculatus* Jp 163 B × *X. couchianus*) BC_1 progeny. Q-RT-PCR data indicate expression of *RAB27* in *X. maculatus* Jp 163 B brain was the lowest and thus brain expression was assigned a value of 1.0. Expression in other tissues was then normalized with the brain expression level.

Chapter 6-Conclusions

A *X. maculatus* Jp 163 B *RAB27* gene and cDNA have been cloned and sequenced. The fish *RAB27* spans >3.3 kb of genomic DNA and is comprised of 6 exons, including the untranslated, exon 1. The presumptive protein sequence, based on the nucleotide sequence assembly, exhibits the four conserved GTP-binding sites and the C-terminal CXC motif as has been previously observed in mammalian Rab proteins. The 218 amino acid fish Rab27 protein shares 62% amino acid identity with human Rab27A and 69% with human Rab27B. The *X. maculatus RAB27* transcript shares 51 and 53% nucleotide identity with the human *RAB27A* and *RAB27B* transcripts, respectively. The *X. maculatus RAB27* gene was mapped to the distal end of *Xiphophorus* LG XII.

Analysis of *RAB27* trancriptional expression in non-tumor fish tissues was performed. These data indicate the highest relative expression of the *X. maculatus RAB27* transcript is in the eye. However, expression of *RAB27* was also observed in several other tissues including; brain, gill, muscle, skin, liver and testes. Use of interspecies backcross hybrids to perform relative Q-RT-PCR indicated *RAB27* expression increases as the degree of melanization increases. Comparison of *RAB27* expression between non-tumor pigmented skin and highly pigmented melanoma tumor tissue show the highest overall expression observed was in melanotic tissue taken from a MNU induced *X. couchianus* × (*X. maculatus* Jp 163 B × *X. couchianus*) BC_1 hybrid.

These studies provide the preliminary data and baseline observations necessary for future studies to be entertained with the *X. maculatus RAB27* gene family. A more precise map position on LG XII of the *RAB27* locus remains to be determined. Also, it may be important to perform 3'-RACE, in order to precisely determine entire transcript size and to test the idea that *RAB*s produce various transcripts resulting from multiple poly-A sites. It would be of interest to determine if *X. maculatus* express only one isoform or contain both *RAB27A* and *RAB27B* genes (i.e. is the hypothetical human *RAB27* duplication a recent evolutionary event?). Furthermore, the work of Chen et al. (1997b) may be pursued using the *Xiphophorus* genetic system to determine the array of *RAB* gene family members that are expressed in melanomas. Ultimately, the use of the *Xiphophorus RAB27* gene as a marker for melanoma development may be evaluated. Such molecular markers are sorely needed to enable definition of the varied pathological states inherent to tumor progression. It is hoped the studies detailed herein will assist investigators to make more rapid progress in answering these and other scientific questions.

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