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Expression of the xenobiotic transporter P-glycoprotein in the mummichog (Fundulus heteroclitus)

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EXPRESSION OF THE XENOBIOTIC TRANSPORTER P-GLYCOPROTEIN IN THE MUMMICHOG (*FUNDULUS HETEROCLITUS*)

A Dissertation

Presented to

The Faculty of the School of Marine Science

The College of William and Mary in Virginia

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

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by

Peter S. Cooper

1996

APPROVAL SHEET

This dissertation is submitted in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy

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Dedication

I dedicate this work to my loving wife Jeanette and my two beautiful children Pearce and Charlotte with thanks for their patience, support and love throughout.

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ABSTRACT

The mammalian mdr1 P-glycoprotein (Pgp) has been implicated in xenobiotic resistance of drug-resistant cell lines and tumors, and may function in excretion or exclusion of toxic xenobiotics at several sites within the body. Pgp gene family members are expressed in excretory epithelia of several aquatic taxa and may contribute to the survival of pollution-tolerant aquatic animals in contaminated environments. For this reason the expression of Pgp family members was investigated in the livers and liver tumors of creosote-resistant mummichog (*Fundulus heteroclitus*) inhabiting a creosote-contaminated site (Atlantic Wood) in the Elizabeth River, Virginia. Expression of members of the Pgp gene family was detected by immunochemical methods using monoclonal antibody (mAb) C219. An immunoreactive band in the size range of mammalian P-glycoproteins was observed in immunoblots of liver membrane fractions and detergent extracts of mummichog liver from both Atlantic Wood and a reference site. Immunohistochemical staining of mummichog liver demonstrated this antigen on the canalicular surface of hepatocytes in normal liver similar to expression in mammalian and guppy liver. The levels of Pgp were 2-3 fold higher in immunoblots of nontumorous livers of Atlantic Wood mummichog compared with levels in reference site fish. Similar to results reported in mammalian liver neoplasms, Pgp was overexpressed and had aberrant immunohistochemical localization in the majority of mummichog liver neoplasms. Because mAb C219 was expected to recognize all Pgp gene family members expressed in mummichog, molecular techniques were used to characterize the individual Pgp genes expressed in mummichog liver. Reverse transcriptase-polymerase chain reaction (RT-PCR) was used to amplify Pgp gene transcripts. A cDNA fragment (3004 bp) was amplified which was most similar to the recently described mammalian sister gene to P-glycoprotein (spgp). Northern blots showed predominant expression of this gene in mummichog liver. Another cDNA fragment (1713 bp) was amplified from mummichog liver and intestine which was a homolog of the mammalian multidrug-resistance or xenobiotic transporter (mdr1). These results indicate that a xenobiotic transporter may be elevated in the livers and liver tumors of the pollution-tolerant mummichog consistent with the proposed role of these proteins in xenobiotic resistance.

EXPRESSION OF THE XENOBIOTIC TRANSPORTER, P-GLYCOPROTEIN, IN THE MUMMICHOG (*FUNDULUS HETEROCLITUS*)

Chapter I: General Introduction

Multidrug resistance and P-glycoprotein— It has been frequently observed that mammalian cell lines which have been selected for resistance to a single cytotoxic compound simultaneously become cross-resistant to many different cytotoxins (Dano, 1972; Riehm and Biedler, 1972; Ling and Thompson, 1974). A remarkable feature of this multidrug-resistance (MDR) phenotype is that the resistance extends to many compounds which have unrelated modes and sites of activity and are structurally and chemically dissimilar. Although MDR cell lines have many biochemical differences which distinguish them from sensitive cells, a common distinctive characteristic of the MDR cells is high level expression of a large (170 kDa) heavily glycosylated plasma membrane protein (Juliano and Ling, 1976; Kartner et al., 1983). This protein was originally termed P-glycoprotein (Pgp). The prefix P in this case was meant to stand for permeability since expression of Pgp was thought to reduce the permeability of MDR cell lines to cytotoxins (Ling and Thompson, 1974; Juliano and Ling, 1976). Analysis of Pgp cDNA sequences expressed in MDR cell-lines showed that P-glycoproteins had similar deduced amino acid sequences and similar predicted domain structures to a variety of ATP-dependent bacterial transport proteins (Gros et al., 1986; Chen et al., 1986; Gerlach et al, 1986). This suggested that Pgp conferred the MDR phenotype by actively transporting cytotoxins outward across the plasma membrane of drug resistant cells. The role of P-glycoprotein as a xenobiotic efflux pump in MDR cell lines has now been well established (reviewed in van der Bliek and Borst, 1989; Gottesman and Pastan, 1993). Expression of full-length P-glycoprotein cDNAs in sensitive cell lines has been shown to

confer the MDR phenotype (Ueda et al., 1987; Devault and Gros, 1990). This is convincing evidence that high level expression of P-glycoprotein alone is sufficient to confer multidrug-resistance to MDR cell lines.

Resistance to multiple chemotherapeutic drugs is also characteristic of many human neoplasms and is a major barrier to effective treatment of many human cancers (Gottesman et al., 1993). Pgp expression and overexpression have been detected in many different human cancers and in several experimental models of rodent hepatocarcinogenesis (Fairchild et al., 1987; Thorgeirsson et al., 1987; Teeter et al, 1990; Vohm et al., 1990; Bradley et al., 1992). Consistent with the role of Pgp in conferring drug resistance in MDR cell lines, overexpression in human tumors has been associated with resistance to chemotherapy and poor prognosis (Fojo et al., 1987; Goldstein et al., 1989; Weinstein et al., 1991; Arceci, 1993). P-glycoprotein expression may arise in human cancers that recur following chemotherapy (Chan et al.,1990; Miller et al., 1991). Because many of the antineoplastic drugs used in chemotherapy (most notably the *Vinca* alkaloids) have been used as selecting agents for MDR cell lines and are substrates for P-glycoprotein in these cell lines, it has been suggested that chemotherapy may select for resistant tumor cells that overexpress P-glycoprotein (Gottesman, 1993). However the causes and exact role of Pgp expression in the drug-resistance and prognosis of human cancers remains controversial (Kaye, 1992). Pgp overexpression has also been associated with tumor aggressiveness and metastasis (Weinstein et al., 1991; Bradley et al., 1992). Therefore Pgp overexpression may be associated with relapse and poor prognosis in human neoplasms because it serves as a marker of a highly malignant phenotype and may

be independent of any selection for drug resistance (Bradley and Ling, 1994).

The origin of Pgp overexpression in experimentally induced rodent liver tumors is not completely understood. Some evidence from rodent hepatocarcinogenesis models suggests that Pgp expression may be selected for in the process of carcinogenesis (Fairchild et al., 1987; Thorgeirsson et al., 1987). This may occur through selective expansion of xenobiotic resistant preneoplastic cells in the presence of cytotoxic tumor promoters or carcinogens (Farber and Sarma, 1987, Thorgeirsson et al., 1987; Thorgeirsson et al., 1991). Increased Pgp expression was detected at the mRNA level in preneoplastic nodules in one model of rodent hepatocarcinogenesis and was associated with xenobiotic resistance of these lesions (Fairchild et al., 1987, Thorgeirsson et al., 1987). However, Pgp overexpression in liver tumors was also observed without promotion by xenobiotics (Teeter et al, 1990; Vohm et al., 1990; Bradley et al., 1992). This suggests that Pgp overexpression in liver tumors may related to oncogene activation and tumor progression (Bradley et al., 1992; Teeter et al., 1990). Support for this idea comes from *in vitro* studies which found that transfection of rat liver epithelial cells with the oncogene v-H-*ras* induced high level expression of Pgp, and that high level expression of mutant p53 stimulated the promoter of the human *MDR1* gene (Burt et al., 1988; Chin et al., 1992). Both Ras mutations and p53 mutations are common in mammalian neoplasms including hepatocellular carcinoma (Nigro et al., 1989; Hsu et al., 1991; Wiseman et al., 1991). Thus Pgp overexpression may be intrinsic to neoplastic transformation and may be independent of any selection of resistant early preneoplastic lesions by xenobiotics.

In addition to expression in MDR cell lines and tumors, P-glycoproteins are expressed in a variety of normal tissue types. In mammals these include the apical surfaces of many epithelial cells including hepatocytes, bile ductular epithelial cells, the epithelial cells of the proximal convoluted tubule of the kidney, and the cells of the intestinal mucosa (Thiebaut et al., 1987; Croop et al., 1989; Thiebaut et al., 1989; Teeter et al., 1990). Pgps are also expressed in the capillary endothelial cells forming the blood-organ barriers of the testes and brain (Cordon-Cardo et al., 1989; Thiebaut et al., 1989). These sites of expression along with the role of Pgp in conferring drug resistance suggests that Pgp may be involved in defending against toxic compounds either through excretion via the bile or urine or through prevention of uptake in the intestine and blood-organ barriers.

Pgp expression is also found in the adrenal cortex (Theibault et al., 1987; Sugawara et al., 1987) and the pregnant uterus (Arceci et al., 1988). Expression in these locations along with the ability of Pgp to transport certain steroid hormones suggests that Pgp may be involved in steroid secretion or uptake in these organs (Ueda et al., 1992; van Kalken et al., 1993).

These expression results are complicated by the presence two or more P-glycoprotein genes in all organisms that have been studied. Multiple homologs have been identified in vertebrates (Ling et al., 1992) in *Drosophila* (Wu et al., 1991; Gerrard et al., 1993) and the nematode, *Caenorhabditis elegans* (Lincke et al., 1992). Recently a novel type of P-glycoprotein, the sister of P-glycoprotein (spgp), has been identified in the rat, the pig and the winter flounder (Childs et al., 1995). Thus the gene family in vertebrates

contains two distinct categories of Pgp genes; those that code for the traditional highly related mdr P-glycoproteins and those that code for the sister of P-glycoprotein or spgp homologs (Childs et al., 1995). The number of *mdr* loci in vertebrate species ranges from two in fish and primates to five in the pig as determined by Southern blots of genomic DNA (Ling et al., 1992; Childs et al., 1995; Childs and Ling, 1996). In contrast to the *mdr* genes only a single *spgp* locus has been detected in the rat and the pig (Childs et al., 1995).

The *mdr* genes of humans and rodents are best characterized. Humans have two genes termed *MDR1* and *MDR2* - also called *MDR3* (Chen et al., 1986; van der Bliek et al., 1988; Chin et al., 1989). Rodents have three *mdr* genes termed *mdr1a* -also called *mdr3* , *mdr1b* -also called *mdr1*, and *mdr2* in rats and mice (Gros et al., 1986; Gros et al., 1988; Hsu et al., 1989; Devault and Gros, 1990; Silverman et al., 1991; Brown et al., 1993). In the hamster these are known as *pgp1*, *pgp2* and *pgp3* respectively (Endicott et al., 1991). A summary of the rather confusing nomenclature is given in Table 1. These *mdr* genes may be classified based on whether their expression can confer drug resistance to sensitive cell lines when transfected with the full-length cDNAs. Expression of mdr1-type homologs (*MDR1*, *mdr1a*, *mdr1b*, *pgp1* and *pgp2*) have been shown to confer drug resistance to sensitive cells while the mdr2-type homologs (*MDR2*, *mdr2* and *pgp3*) do not (Ueda et al., 1986; Devault and Gros, 1990; Schinckel et al., 1991).

The *mdr* genes of mammals are expressed in a tissue specific manner which suggests specific physiological functions. The mdr1-type P-glycoproteins are expressed

in all of the sites of P-glycoprotein expression including the liver, intestine, kidney, uterus and adrenal medulla whereas mdr2-type isoforms are expressed predominately in the bile canaliculus of the liver (Buschman et al., 1992; Smit et al., 1994). Other sites of expression of the mdr2 P-glycoprotein include skeletal muscle, the heart and spleen (Smit et al., 1994).

The expression patterns of the mdr1 P-glycoproteins along with the specific role of the mdr1 forms in xenobiotic transport in multidrug resistant cell lines suggests that the mdr1 forms are involved in the xenobiotic protective role and in steroid transport. In mice these putative mdr1 functions appear to be partially divided between the mdr1a and mdr1b proteins. Predominant expression of the *mdr1a* gene has been detected in intestine and the capillary endothelial cells forming the barriers of the brain and testes whereas in the adrenal cortex and the uterus *mdr1b* expression is highest (Croop et al., 1989; Teeter et al., 1990; Arceci et al., 1988; Schinkel et al., 1993). However, the sites of expression of the mdr2-type P-glycoprotein and its apparent lack of involvement in multidrug-resistance give little information about the normal physiological function of the mdr2 protein.

Recently strains of mice have been developed which carry homozygous disruptions ("knock-out") of the mdr genes (Smit et al., 1993; Schinkel et al., 1994). Study of these mice has provided important information on the normal physiological role of both the mdr2-type and mdr1-type P-glycoproteins. Observations of *mdr2* (-/-) mice suggested that the physiological requirement for mdr2-type P-glycoprotein in the bile canaliculus is in phosphatidylcholine secretion. The bile of these mice completely lacked

phosphatidylcholine, an important component of normal bile. These mice also developed liver disease which may have been caused by exposure to bile salts which are normally complexed into micelles with phosphatidylcholine (Smit et al., 1993). Another study found that *mdr2* gene product expressed in yeast secretory vesicles functioned as a phosphatidylcholine translocase, flipping the phospholipid in a directed fashion from one leaflet to the other within the lipid bilayer (Ruetz and Gros, 1994). Thus evidence suggests that the mdr2-type P-glycoprotein may function as phosphatidylcholine "flippase" in the canalicular membrane of hepatocytes. Its activity is thought to increase the local concentration of phosphatidylcholine in the outer leaflet of the canalicular membrane where it is extracted into micelles by bile salts (Higgins and Gottesman, 1992; Smit et al., 1993; Ruetz and Gros, 1994).

Strong support for the xenobiotic-protective role of mdr1-type P-glycoproteins was recently provided by study of mice which have a homozygous disruption of the *mdr1a* gene (Schinkel et al., 1994). These mice were phenotypically normal and did not show any defect until challenged with xenobiotics. The *mdr1a* deficient mice were highly sensitive to the toxic effects of a xenobiotic (ivermectin) which is normally tolerated because of the functioning of the blood-brain barrier. These mice also had increased accumulation of ivermectin and of the MDR substrate vinblastine in certain organs as well as decreased elimination of these toxic compounds. Possibly any other physiological requirement for functional *mdr1a* expression was compensated by the observed increased expression of the functional *mdr1b* gene in these mice.

In mammals both *mdr1* and *mdr2* expression may be regulated in part by exposure

to xenobiotics. This regulation may be a component of an inducible response to certain classes of foreign compounds. Exposure to several carcinogens including the cytochrome P4501A (CYP1A) inducer 3-methylcholanthrene (3-MC) has been found to increase Pgp mRNA (*mdr1b* gene transcripts) and Pgp levels in rat liver (Burt and Thorgeirsson, 1988; Gant et al., 1991; Thorgeirsson et al., 1991). Transcripts of the *mdr2* gene as well as the mdr2 P-glycoprotein have also been observed to increase in the livers of monkeys following administration of drugs having a biliary excretion pathway (Gant et al., 1995). Thorgiersson and colleagues have hypothesized that Pgp gene expression is induced in liver by xenobiotics having biliary excretion pathways and may be a part of a coordinated response to xenobiotic exposure which includes induction of xenobiotic metabolizing enzymes such as CYP1A (Thorgeirsson et al., 1991; Gant et al., 1995).

There is as yet no information on the physiological role of the spgp P-glycoprotein. However, northern blots of RNA from rat tissues have demonstrated that expression of this gene is confined to liver where it is expressed along with mdr2 and mdr1 P-glycoproteins in the bile canaliculus (Childs et al., 1996). It can be assumed that its role here is in excretion of an unidentified substance into the bile.

Despite the diversity of functions, the deduced amino acid sequences of all P-glycoproteins are well conserved and predict a highly conserved domain structure for these proteins. P-glycoprotein sequences are similar in sequence and domain structure to a large number of proteins involved in ATP-dependent transmembrane transport. These proteins are members of the ATP binding cassette (ABC) superfamily of transport proteins (reviewed in Higgins et al., 1992 and Childs et al., 1994). Other ABC

transporters include bacterial periplasmic uptake and transport systems (Higgins et al., 1986), bacterial exporters such as the hemolysin B exporter of *E. coli* (Higgins et al., 1986), the pigment transporter products of the *white* and *brown* genes of *Drosophila* (Dreesen et al., 1988), the yeast STE 6 mating factor transporter (McGrath and Varshavsky, 1989), the mammalian multidrug resistance associated transporter, MRP, (Cole et al., 1992) and the mammalian cystic fibrosis transmembrane conductance regulator, CFTR (Riordan et al., 1989). All members of this superfamily have one or two well conserved ATP-binding domains or cassettes of about 200 amino acids (Higgins et al., 1986; Hyde et al., 1990) which surround and include the Walker A and B consensus sequences for nucleotide binding found in the nucleotide binding folds of many proteins (Walker et al., 1982). They all also have one or two hydrophobic membrane spanning domains (Hyde et al., 1990). The ATP binding domain is believed to be involved in coupling the hydrolysis of ATP to the transport of the substrate across the membrane which may involve a conformational change that is transmitted to the membrane spanning domains (Hyde et al., 1990).

P-glycoproteins conform to the general ABC transporter domain structure (Figure 1) and appear to be tandemly duplicated molecules with each half containing a hydrophobic domain made up of six putative transmembrane helices followed by an intracellular ATP- binding domain (Chen et al., 1986; Gros et al., 1986). The sequence of these nucleotide binding domains are well conserved among all P-glycoproteins as well as other ABC superfamily members. The two halves of the protein are connected by a

highly hydrophillic region called the linker region (Chen et al., 1986). The sequence of this region is not well conserved overall but does contain distinctive protein kinase recognition sequences which may confer important differences in regulation to the different classes of homologs within a species (Endicott et al., 1991; Hsu et al., 1989).

Multixenobiotic resistance and P-glycoprotein expression in aquatic organisms— Because of the postulated xenobiotic protective role of mdr1-type P-glycoproteins in mammals, investigators have recently begun to investigate the possible role of Pgp in tolerance and resistance of aquatic organisms to pollutants. It has been observed that aquatic organisms which persist in polluted environments are exposed to many different toxic pollutants. These pollution-tolerant organisms must have physiological and biochemical mechanisms which allow them to cope with a range of toxic compounds which may have differing chemical and biological properties. Kurelec and colleagues have noted that this ability of pollution tolerant aquatic organisms to resist the toxic effect of multiple unrelated toxicants is analogous to the MDR phenotype of mammalian cell lines. This has led to the hypothesis that the activity of P-glycoprotein homologs in the excretory tissues of aquatic organisms is responsible for this multi-xenobiotic resistance (reviewed in Kurelec, 1992). In support of this multixenobiotic-resistance hypothesis, P-glycoprotein-like activities have been detected in excretory tissues of several pollution or toxin tolerant aquatic organisms including marine sponges (Kurelec and Pivcevek, 1992; Kurelec et al., 1992), mollusks (Kurelec and Pivcevec, 1989; Kurelec et al., 1991; Minier et al., 1993; Kurelec et al., 1995; Cornwall et al., 1995; Kurelec et al, 1996) and a marine worm (Toomey and Epel, 1993). All of these reports

have relied on a competition assay for P-glycoprotein activity using the effect of the calcium channel blocker verapamil, a known P-glycoprotein inhibitor, on uptake of potential P-glycoprotein substrates by tissues or membrane vesicles of aquatic organisms. Studies have shown verapamil-sensitive ATP-dependent uptake of carcinogens and the P-glycoprotein substrate vincristine in the presence of verapamil in membrane vesicles made from pollution tolerant marine sponges (Kurelec et al., 1992; Kurelec and Pivcevic, 1992) and from the gills of bivalve mollusks (Kurelec and Pivcivec, 1989; Kurelec et al., 1991). These results are analogous to those obtained in a similar assay with membrane vesicles of MDR cell lines and may identify the activity of P-glycoprotein homologs in these organisms. Using a competitive assay with a fluorescent Pgp substrate Toomey and Epel (1993) have also shown P-glycoprotein-like activity in embryos of the innkeeper worm (*Urechis caupo*). Evidence from this study suggests that this activity may protect the innkeeper worm from toxins present in marine sediments.

Several studies have found that many important environmental contaminants interfere with these P-glycoprotein activities (Kurelec, 1995; Cornwall et al., 1995; Galgani et al., 1996). A range of moderately hydrophobic to very hydrophobic compounds including the important environmental contaminants pentachlorophenol, DDT and its breakdown products, and the PCB mixture Arochlor 1254 inhibited the P-glycoprotein-like activity in the marine mussel, *Mytilus galloprovincialis* (Galgani et al., 1996). However in a similar study using a different species of mussel (*Mytilus californicus*) only the less hydrophobic compounds interfered with Pgp-like activity while the highly hydrophobic compound including Arochlor 1254 and DDT did not

(Cornwall et al., 1995). Another study has found that a water extract of diesel fuel, and organic extracts of polluted river water and sediment pore water interfere with the Pgp-like activity in *M. galloprovincialis* (Kurelec, 1995). While the above results are consistent with some environmental contaminants being substrates for Pgp, active transmembrane transport of these compounds has not yet been demonstrated and mechanisms other than competitive inhibition may be involved.

In addition to Pgp-like activity, investigators have also detected expression of P-glycoprotein or related antigens in the tissues of aquatic organisms. Polyclonal antibodies raised against Pgp from a colchicine resistant Chinese hamster ovary cell line were used to label a 125 kDa glycoprotein band in immunoblots of detergent extracts of two species of marine sponges (Kurelec et al., 1992). Using immunocytochemical staining with this antibody and a monoclonal antibody (mAb C219) which recognizes a conserved epitope present in all mammalian P-glycoproteins, the authors showed that Pgp antigens localized to the plasma membrane of the sponge cells (Kurelec et al., 1992). Toomey and Epel (1992) have also used mAb C219 to detect a 140 kDa protein in immunoblots of egg membrane fractions of the innkeeper worm and found that this antibody labeled a 110 kDa band in immunoblots of adult worm epidermal tissue and intestinal tract. Several studies have used antibodies against mammalian Pgp to examine Pgp expression in marine mollusks. Using the mAb C219, Minier and collaborators (1993) detected two bands at 220 kDa and 240 kDa in immunoblots of detergent extracts of the mussel, *Mytilus edulis* and the oyster *Crassostrea gigas*. Galgani and colleagues (1996) reported both a 145 kDa band and the 220-240 kDa doublet in immunoblots of gill

extracts of *M. galloprovincialis* probed with mAb C219. Kurelec and colleagues have reported a 140 kDa band in immunoblots of gills of *M. galloprovincialis* (Kurelec et al., 1996) and in the marine snail *Monondonta turbinata* (Kurlec et al., 1995) probed with a polyclonal antibody directed against a peptide from a conserved region of human MDR1 P-glycoprotein. Cornwall and colleagues (1995) found that both mAb C219 and a polyclonal antibody directed against mammalian Pgp labeled a 170 kDa band in immunoblots of *M. californicus* but did not report the 220-240 kDa band seen in the other bivalves. Pgp expression has also been detected in aquatic vertebrates. Hemmer and colleagues (Hemmer et al., 1995) used a panel of anti-mammalian Pgp monoclonal (including mAb C219) and polyclonal antibodies in an immunohistochemical study of Pgp expression in the guppy. Expression of Pgp in the guppy was found to be similar to mammals with expression in the apical membrane of the tubular epithelia of the kidney, the brush border membrane of the intestinal epithelium and the canalicular surface of hepatocytes as well as in the corresponding adrenal-like intrarenal tissue.

There is some evidence that, as in mammals, Pgp expression and activity in aquatic animals may be elevated by exposure to toxicants. Increased Pgp-like activity and elevated levels of Pgp related antigens were detected in gills and mantle tissues of marine mollusks inhabiting polluted sites (Minier et al.,1993; Kurelec et al., 1995; Kurelec et al., 1996). Increased Pgp-like activity was measured in a marine snail and a mussel following laboratory exposure to water polluted with fuel oil (Kurelec et al., 1995; Kurelec et al., 1996). In one case increased levels of Pgp antigens were detected in immunoblots of tissue extracts following exposure to contaminants (Kurelec et al., 1995).

These results suggest increased Pgp expression may be an inducible response produced by exposure to certain contaminants. This is similar to induction of Pgp expression in mammalian liver by xenobiotic exposure. It has been suggested that levels of P-glycoprotein expression or activity may be correlated with the degree of tolerance or adaptation to toxic contaminants in a particular species of aquatic organisms (Minier et al., 1996; Kurelec et al., 1995).

Interpretation of data on Pgp expression in aquatic organisms is almost certainly complicated by the presence of multiple P-glycoprotein homologs in individual species of aquatic organisms. As in mammals, these homologs probably have tissue specific expression patterns and distinct substrate specificities. Other than one report in winter flounder (Chan et al., 1992), information on the Pgp genes of environmentally relevant aquatic organisms is absent. Two partial genomic clones have been sequenced for the winter flounder. One of these, *fpgpA,* has now been identified as being a homolog of the recently described mammalian sister of P-glycoprotein (Childs et al., 1995). The relationship of the other winter flounder gene fragment to the mammalian genes has not been determined, but its sequence does not fit clearly into either the *mdr1* or *mdr2* gene categories (Chan et al., 1992). No information has yet been published on tissue specific expression of *mdr* or *spgp* genes in fish although the immunohistochemical study in the guppy shows tissue and subcellular localization of total Pgp antigens that is similar to that of mammals (Hemmer et al., 1995). However, in this study it was not possible to distinguish among the different guppy Pgp isoforms.

Clarification of the role of Pgp expression and activity in resistance and tolerance

of aquatic organisms to pollutants will require molecular and biochemical characterization of the individual members of the P-glycoprotein gene families in aquatic taxa. Initial molecular studies are needed to acquire sequence data. Even modest amounts of sequence data may allow certain functional inferences to be drawn based upon similarities to the sequences of mammalian genes of known function. These data will also allow the construction of gene specific probes which may be used to investigate regulation of expression of the individual genes. These gene specific probes ultimately will be needed to screen cDNA and genomic libraries for full-length cDNA and genomic clones. The cDNA clones may be helpful in identifying substrates and function by allowing *in vitro* expression of the individual gene products (transfection) while the genomic clones may provide insight into regulation of the specific genes.

Scope of the present work— Several previous studies from our laboratory have focused on biochemical changes and adaptations in a small estuarine killifish, the mummichog (*Fundulus heteroclitus*) inhabiting a marsh creek heavily contaminated with creosote from a wood treatment facility (Atlantic Wood Industries) in the Elizabeth River, Portsmouth, Virginia (Figure 2) (Van Veld et al., 1991; Van Veld and Westbrook, 1995; Armknecht, 1996). Mummichog from this site have been reported to have a high prevalence of hepatic neoplasms and putatively preneoplastic lesions (altered foci) as well extrahepatic neoplasms (Vogelbein et al., 1990; Fournie and Vogelbein, 1994). These lesions are presumed to result from chronic exposure to polycyclic aromatic hydrocarbons (PAHs) present in the creosote. The hepatic tumors of these fish have been characterized with respect to expression of the xenobiotic metabolizing enzymes CYP1A

and glutathione S-transferases (GSTs) (Van Veld et al., 1991; Van Veld et al., 1992). These tumors were found to be similar to mammalian hepatic neoplasms and preneoplastic lesions in having reduced expression and activity of CYP1A (Van Veld et al., 1992). In another study no increase in GST activity was observed within grossly visible lesions. However the entire liver of Atlantic Wood mummichog showed elevation of GST expression and activity (Van Veld et al., 1991). This is in contrast to mammalian hepatic neoplasms which have been shown to have elevated levels of GST activity (Farber and Sarma, 1987).

The most remarkable feature of Atlantic Wood mummichog is their resistance to the toxicity of creosote. Williams (1994) found that creosote amended sediments as well as sediments collected from the Atlantic Wood site were significantly more toxic to developing laboratory-spawned embryos of mummichog from an uncontaminated site (Catlett Islands, York River, Virginia, Figure 2) than to laboratory spawned offspring of mummichog from the Atlantic Wood site. This resistance in embryos was shown to be a heritable genetic trait. Atlantic Wood sediment was also observed to be more acutely toxic to adult mummichog collected from an uncontaminated site than to adult Atlantic Wood mummichog (Wolfgang Vogelbein and Peter Van Veld, unpublished observations). Whether this also represents genetic resistance in the adult fish or physiological acclimation is uncertain.

The biochemical mechanism of the resistance or tolerance of Atlantic Wood mummichog to creosote contaminated sediments is an active area of research in our laboratory. Creosote is a complex mixture of toxic xenobiotics which is predominately

PAHs but also contains phenolic compounds and nitrogen and sulfur containing heterocyclic compounds (Mueller et al., 1989). Certain biochemical characteristics of the livers of Atlantic Wood mummichog may be adaptive in the presence of PAH contamination from the creosote. The nontumor-bearing livers of the fish from the Atlantic Wood site had elevated levels and activity of the phase II conjugating enzyme glutathione S-transferase (GST) and suppression of expression and activity of phase I enzyme CYP1A (Van Veld et al., 1991; Van Veld and Westbrook, 1995; Armknecht, 1996). Van Veld and Westbrook (1995) have pointed out that these changes are similar to the xenobiotic resistance phenotype of drug-resistant cell lines and mammalian preneoplastic and neoplastic liver lesions (Farber and Sarma, 1987). As discussed previously, another biochemical change associated with xenobiotic resistance is increased expression of Pgp. Could increased Pgp expression also be contributing to the creosote resistance of these fish? This idea is particularly attractive because of the potential for Pgp to provide a mechanism for elimination of multiple toxic lipophilic substrates as would be required for managing a complex toxic mixture such as creosote. Furthermore, what is the state of Pgp expression in the liver tumors of these fish? Is overexpression of Pgp another characteristic along with reduced CYP1A (Van Veld et al., 1992) which these tumors share with mammalian liver neoplasms? Such biochemical comparisons of cancer in fish and mammals are essential for supporting efforts to use fish as experimental carcinogenesis models and for understanding the biochemical basis of these environmental cancers. In order to begin to address these questions, preliminary work was required to develop methods for identification and quantitation of Pgp in

mummichog tissues and to characterize the nature of specific Pgp homologs expressed in the mummichog. Therefore the work in this dissertation extends our studies of the biochemical properties of the livers and liver tumors of the Atlantic Wood mummichog to include identification and analysis of Pgp expression and the molecular cloning and characterization of mummichog Pgp homologs.

Chapters II and III of this dissertation focus on the development and application of immunological techniques using the anti-mammalian Pgp antibody mAb C219 for detecting P-glycoprotein expression in mummichog liver and liver tumors. Both immunohistochemical and immunoblot techniques were used. The former technique provided useful information on the cellular and subcellular localization of Pgp antigens in mummichog tissues. The latter technique provided confirmation of the specificity of detection of Pgp by the molecular weight of the labeled band and further provided a means of determining relative levels of expression of Pgp between reference site fish and Atlantic Wood mummichog and between normal and tumorous liver of Atlantic Wood mummichog. The immunohistochemical staining results indicated Pgp expression in mummichog liver on the canalicular surface of hepatocytes similar to the localization reported in other vertebrates. Immunohistochemical staining of the more progressed liver tumors (hepatocellular adenomas and hepatocellular carcinomas) demonstrated altered localization and overexpression of Pgp compared to the surrounding normal tissue. This pattern of expression in the mummichog liver tumors is similar to that reported for experimentally induced rodent hepatocellular carcinoma. Immunoblot analysis of Pgp expression in Atlantic Wood mummichog livers indicated elevation in the levels of Pgp

compared to the levels of Pgp in mummichog liver from an uncontaminated site. The causes of this elevation are uncertain but may be due to toxic liver injury, the contribution of overexpression in hidden tumors or induction of expression by one or more components of creosote. This elevation is also consistent with constitutive elevation as a component of the genetic resistance of these fish.

Because the monoclonal antibody (C219) used in the above immunochemical studies recognizes all categories of Pgp (mdr1-type, mdr2-type and spgp), it was necessary to identify the categories of Pgp expressed in mummichog liver in order to better interpret the toxicological relevance of Pgp expression. The method of reverse transcriptase-polymerase chain reaction (RT-PCR) was used to amplify expressed Pgp sequences from mummichog liver RNA. Comparison of the acquired sequences to mammalian sequences was used to categorize the mummichog Pgp homologs. Application of the RT-PCR technique and the analysis of the mummichog Pgp sequences acquired in this way are discussed in Chapters IV and V of this dissertation.

Chapter IV of this dissertation reports the sequence of a fragment of mummichog Pgp cDNA which is the closest homolog to the mammalian spgp P-glycoproteins. Northern blotting results indicated that this mRNA is moderately expressed in mummichog liver but not in intestine or kidney. This liver-specific expression is consistent with that observed for the mammalian *spgp* gene. The mummichog spgp P-glycoprotein likely contributes significantly to the signal seen in immunohistochemical and immunoblot detection of Pgp in mummichog liver. The toxicological relevance of this protein is unknown as its function has not yet been investigated in any animal.

However, the highly conserved sequence between teleosts and mammals and liver-specific expression of this gene indicate an essential function in liver as a canalicular transporter of an unidentified substrate.

Chapter V of this dissertation reports the sequence of a fragment of a mummichog mdr P-glycoprotein. Overlapping fragments of this cDNA were obtained by RT-PCR of mummichog liver and intestinal RNA. While sequence similarities indicated that this product is most similar to the mammalian and *Xenopus* mdr-type P-glycoprotein, overall sequence comparison did not clearly assign this homolog to either the mammalian mdr1 or mdr2 categories. Concerted evolution among the mammalian Pgp genes has apparently greatly diminished sequence differences between the mammalian mdr1 and mdr2 homologs over large portions of the sequence making assignment of the mummichog homolog difficult. However the presence of mdr1-specific protein kinase recognition sequences in the linker region of the deduced amino acid sequence of the mummichog mdr cDNA fragment establish that this is a mummichog mdr1-type P-glycoprotein and is likely to be a xenobiotic transporter. Amplification of this cDNA from both liver and intestine is consistent with the mammalian tissue specific expression of mdr1-type P-glycoproteins. These results indicate that mummichog liver expresses a xenobiotic transporter homolog. Increased expression of this protein could contribute to the survival of mummichog at the Atlantic Wood site. However it is not known at this time which of the Pgp isoforms are elevated in the liver and liver tumors of the Atlantic Wood mummichog.

Further work along several lines of investigation is required to determine if Pgp

expression in Atlantic Wood mummichog has toxicological relevance and contributes to the tolerance or resistance of these fish to their highly contaminated environment. A comparison of Pgp-like activity in liver of Atlantic Wood mummichog and reference site fish is necessary to corroborate expression results. The effect of creosote and its components on this activity also must be investigated. Now that two mummichog partial cDNA sequences are available, gene specific probes may be produced to assess expression of the specific forms and determine which isoform is elevated in the Atlantic Wood mummichog. This will require cloning at least a fragment of the expected mummichog mdr2 homolog. These gene specific probes may also be used to further investigate regulation of expression of the mummichog Pgp homologs in laboratory exposures of mummichog to creosote and its components and to investigate relative levels of Pgp forms in resistant and sensitive fish. The cDNA fragments described in this dissertation may also be used to screen a mummichog liver cDNA library for full-length sequences of mummichog P-glycoprotein mRNAs expressed in liver. The cloning of full-length cDNAs may allow expression of these mummichog proteins in cultured cells and should permit functional characterization of these gene products.

Table 1. Nomenclature and classification of mammalian *mdr* genes. Alternative designations are given in parentheses

Pgp class	human	hamster	mouse/rat	multidrug resistance
mdr1-type	*MDR*1	*pgp*1	*mdr*1a (*mdr*3)	yes
mdr1-type	-	*pgp*2	*mdr*1b (*mdr*1)	yes
mdr2-type	*MDR*2 (*MDR*3)	*pgp*3	*mdr*2	no

Figure 1. Model of P-glycoprotein in the plasma membrane showing the domain structure of the protein. **IN** and **OUT** refer to cytoplasmic and extracellular side of the plasma membrane respectively. The protein is made up of two similar halves connected by a poorly conserved hydrophillic segment (**Linker**). The first extracellular loop of the N-terminal half contains the glycosylation sites (branched structure). Each half of the protein contains a transmembrane domain (**TMD**) made up of six membrane spanning helices. Each half also has an ATP binding cassette (**ABC**) which contains a highly conserved ATP binding fold (circled) within which are the Walker motifs (**A** and **B**) found in a variety of nucleotide binding proteins.

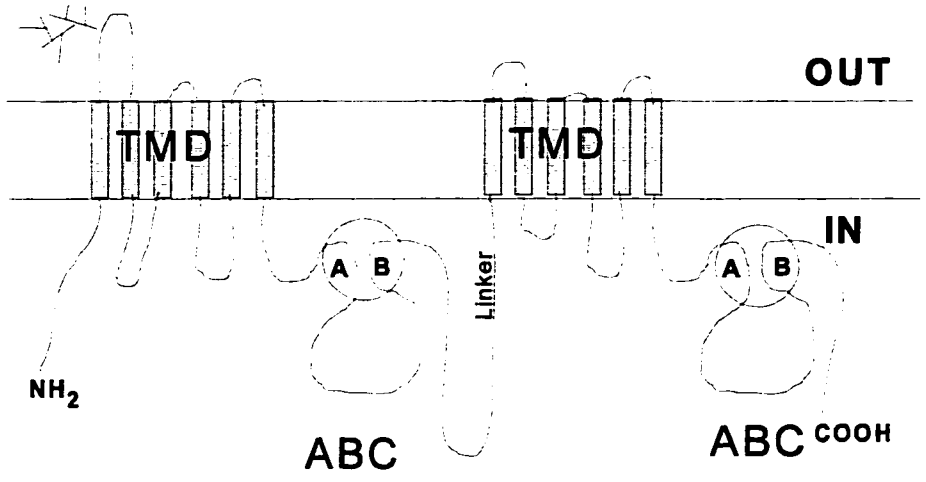
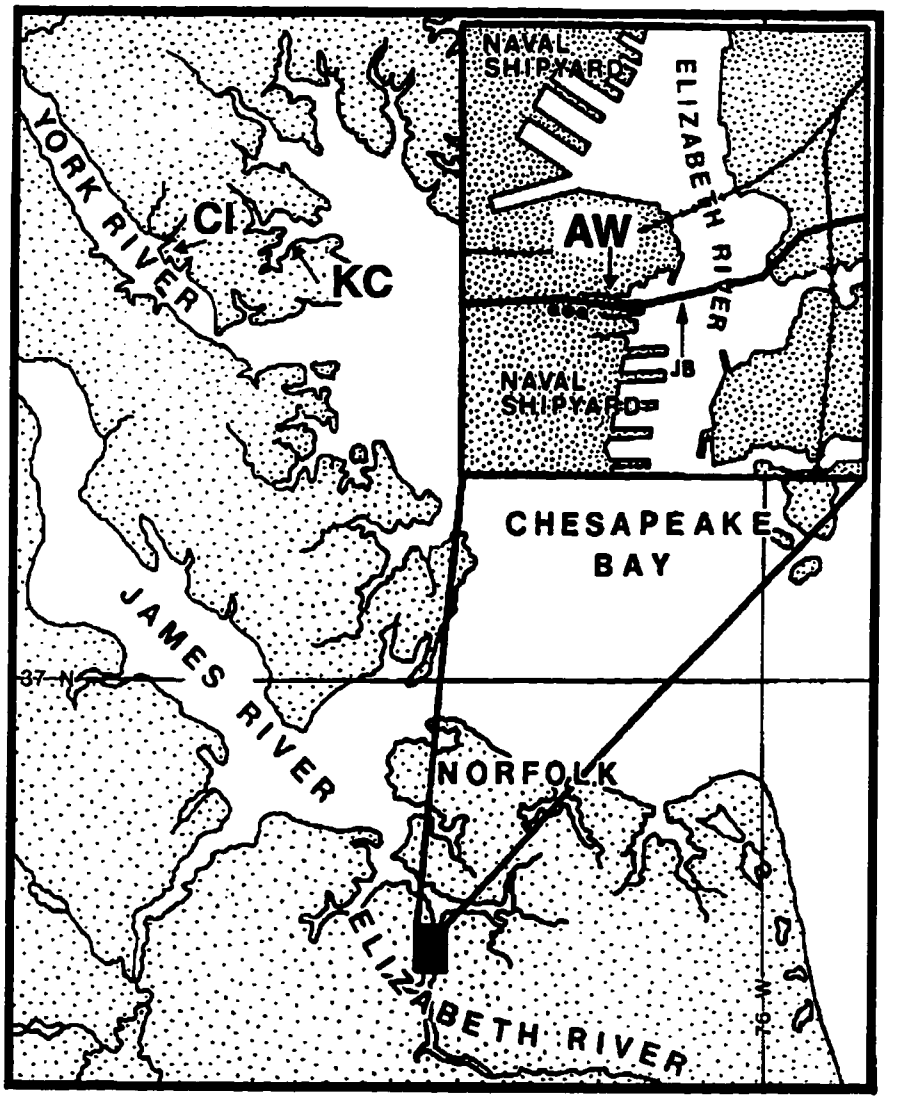


Figure 2. Map of the lower Chesapeake Bay and tributaries showing the locations of the creosote contaminated site (Atlantic Wood, **AW**) and the two uncontaminated reference sites (King Creek, **KC** and Catlett Islands, **CI**) discussed in this work.



Chapter II. Immunohistochemical and Immunoblot Detection of P-Glycoprotein in Normal and Neoplastic Fish Liver

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INTRODUCTION

P-glycoprotein expression in mammals— P-glycoprotein (Pgp) is a membrane transport ATPase that plays a role in efflux of cytotoxic agents from multidrug-resistant (MDR) mammalian cell lines (Kartner et al., 1983; van der Bliek and Borst, 1989, Gottesman and Pastan, 1993). The MDR phenotype arises in cell lines selected for resistance to one cytotoxic agent. These cells are cross-resistant to a variety of other toxicants with differing structures and modes of action. A consistent biochemical change in these cell lines is increased expression of P-glycoprotein (reviewed in van der Bliek and Borst, 1989; Gottesman and Pastan, 1993). Studies of accumulation and efflux of labeled Pgp substrates in MDR cell lines indicate that Pgp expression is associated with decreased accumulation and increased ATP dependent efflux of drugs in these cells (Hamada et al., 1988; Horio et al 1988). Although drug-resistant cell lines often have many biochemical alterations that distinguish them from sensitive cells, transfection of sensitive cells with full length cDNA clones of Pgp transcripts has been found to be sufficient to confer drug resistance (Ueda et al., 1987; Devault and Gros, 1990). This is convincing evidence that overexpression of Pgp alone is sufficient to confer the MDR phenotype.

Since the initial discovery of Pgp in MDR cell lines, normal tissues have been found to express Pgp. The apical surface of many excretory epithelia including those of the intestine, liver and kidney show Pgp expression (Thiebaut et al., 1987; Thiebaut et al., 1989; Bradley et al., 1990). This pattern of expression along with the role of Pgp in efflux of cytotoxins from cell lines suggests that the normal role of Pgp may be the

excretion of endogenous or exogenous toxic compounds or their metabolites. Pgp may therefore represent a line of defense against toxic compounds. Up regulation of Pgp expression could be an adaptive response of organisms to toxicants. In the liver of the rat, Pgp mRNA levels increase following exposure to many xenobiotics including some of the classic P450 inducers (Burt and Thorgiersson, 1988). Thus it is possible that Pgp expression in mammals may be a part of the inducible xenobiotic response pathway that includes the phase I and phase II xenobiotic metabolizing enzymes (Burt and Thorgeirsson, 1988; Thorgeirsson et al., 1991). Although there are many hints that the normal function of Pgp is elimination of xenobiotics or toxic metabolites, the normal substrates for Pgps are unknown, and the relevance of these proteins to toxicology remains uncertain.

In addition to expression in normal tissues, Pgp expression and overexpression are seen in human and animal neoplasia. Overexpression of Pgp occurs in a number of human neoplasms derived from a variety of tissue types (Miller et al., 1987; Fojo et al., 1987; Chan et al., 1990; Goldstein et al., 1990; Weinstein et al., 1991; Gottesman, 1993). In many human tumors Pgp expression is associated with resistance of the tumors to chemotherapy and poor prognosis (Chan et al., 1990; Weinstein et al., 1991; Gottesman et al., 1993). Some cases of overexpression are associated with tumors that recur following chemotherapy (Chan et al., 1990; Miller et al., 1991). Chemotherapy may select for tumor cells that are resistant because of Pgp overexpression (Gottesman, 1993). Pgp overexpression has also been observed in several different laboratory models of rodent hepatocarcinogenesis (Fairchild et al., 1987; Thorgeirsson et al., 1987; Teeter et

al., 1990; Bradley et al., 1992). Evidence from one model suggests that Pgp overexpression occurs early in carcinogenesis and is related to promotion of xenobiotic resistant preneoplastic liver lesions (Fairchild et al., 1987; Thorgeirsson et al., 1987). Other evidence suggests that Pgp overexpression in hepatocellular carcinoma may arise without promotion by xenobiotics (Teeter et al., 1990; Bradley et al., 1992).

P-glycoprotein expression in aquatic animals— Because of the importance of Pgp in conferring MDR, there is interest in the role that Pgp expression may play in tolerance or resistance of aquatic organisms to toxicants. Pgp expression and activity have been detected in tissues of a number of aquatic organisms (Kurelec and Pivcivec, 1991; Kurelec and Pivcivec, 1992; Kurelec et al., 1992; Kurelec 1992; Toomey and Eppel, 1993; Minier et al., 1993). Kurelec and co-workers have hypothesized that certain strains or species of aquatic organisms that are tolerant of pollutants may be so because of the presence and activity of Pgp homologs (reviewed in Kurelec, 1992). They suggest that chemical pollutants or natural toxins may be excluded from these organisms by the externally directed pumping activity of Pgp homologs in epithelial tissues. They have termed such a mechanism "multixenobiotic resistance" (MXR) since, by analogy to the MDR phenotype, such organisms may show cross-resistance to a variety of structurally unrelated toxicants.

Although the above studies suggest a role for Pgp in resistance or tolerance to toxicants, the significance of Pgp expression in aquatic organisms is not clear. Additional studies are needed to establish whether differences in relative levels of Pgp expression or activity are associated with sensitivity of aquatic organisms to toxicants. There is also a

need for the development of methods that would allow measurement of Pgp expression in aquatic organisms.

We are currently investigating expression of Pgp in the liver of the mummichog, *Fundulus heteroclitus*, a small estuarine killifish. Our study site in the Elizabeth River, Virginia is heavily contaminated with polycyclic aromatic hydrocarbons (PAHs) derived from creosote (Vogelbein et al., 1990). High prevalences of hepatic (Vogelbein et al., 1990) and extra-hepatic neoplasms (Fournie and Vogelbein, 1994; Vogelbein and Fournie, 1994) have been reported in mummichog from this site. This situation gives us a unique opportunity to study both the response of Pgp expression to environmental exposure to PAHs in fish liver and to examine alterations in Pgp expression occurring in neoplastic and putative preneoplastic lesions during environmental hepatocarcinogenesis in fish.

The purpose of this Chapter is to describe protocols that we have developed for the immunochemical detection of Pgp expression in fish tissues, primarily liver and liver tumors. These methods should be applicable to other fish tissues and possibly tissues of other aquatic organisms as well. Detailed protocols are given for both immunohistochemical staining and immunoblotting procedures. Whenever possible we use both techniques because of the complementary information that can be derived from each.

MATERIALS REQUIRED

Where manufacturers are not given, materials are readily available from many sources.

Pgp Immunohistochemistry

1. *Staining rack and jars*: (Shandon, Tissue Tek II).

2. *PAP pen*: (Research Products International, Mount Prospect IL, cat. no. 195500).

3. *Ethanol*: 100%, 95% and 70%.

4. *Xylene.*

5. *Elite Avidin-Biotin Complex (ABC) immunohistochemical staining kit, murine*: (Signet Laboratories, Dedham MA, cat. no. 2158).

6. *Normal Horse Serum*: (Sigma, cat. no. H0146).

7. *Bovine Serum Albumin (BSA), Fraction V*: (Sigma, cat. no. A9647).

8. *3,3'-Diaminobenzidine Substrate Tablets*: (Sigma, cat. no. D4168).

9. *Permanent Mounting Medium*: (Preservaslide, EM Science, or equivalent).

10. *Phosphate Buffered Saline* (PBS): (10 mM sodium phosphate, pH 7.4, 150 mM

NaCl). This is prepared as a 10X stock. 14.2 g of anhydrous dibasic sodium phosphate and 87.7 g of NaCl are added to 900 ml of deionized water. The pH is adjusted to 7.4 with 6 M NaOH. The volume is then made up to 1 l with deionized water. The solution is sterilized by filtration (0.2 μm) and stored at room temperature. The 10X stock is diluted with 9 parts deionized water immediately before use.

11. *Blocking Solution*: (10% normal horse serum, 0.02% sodium azide in 1 X PBS). This solution can be divided into aliquots and stored at - 20° C.

12. *3% Hydrogen Peroxide*: This solution must be prepared fresh daily by dilution of 1 part 30% hydrogen peroxide into 9 parts deionized water.

13. *Antibody Buffer*: (1% w/v BSA, 0.02% w/v sodium azide in 1 X PBS). This solution is stored at 4° C. It is stable for at least 2 weeks.

14. *C219 mAb*: (Signet, cat. no. 2158). This reagent is provided lyophilized (100 μg) by the manufacturer. It is diluted with 1 ml of antibody buffer and stored frozen (-70° C) in 20 μl aliquots. For immunohistochemistry, aliquots are diluted immediately before use with 1.3 ml of antibody buffer (1% BSA in PBS).

15. *Mouse Myeloma (UPC 10) Protein (IgG 2a, κ)*: (Sigma, cat. no. M 9144). The

lyophilized protein is diluted to 1.5 μg/ml with antibody buffer and stored in 1 ml aliquots at -20° C.

16. *Harris Hematoxylin*: 5 g of hematoxylin powder (Fisher Scientific, cat. no. H345) is dissolved in 50 ml of 95% ethanol. 100 g of potassium alum is dissolved in 1000 ml of deionized water in a 2 liter Erlenmeyer flask on a heating stirplate. The hematoxylin solution is added to the alum solution, and the mixture is brought to a boil while stirring. 2.5 g of mercuric oxide is carefully added to the hot solution. The solution is heated until a deep purple color is obtained and then is cooled by plunging the flask into cold water. It then is filtered and stored at room temperature. The solution is filtered before each use. The stain is stable for several months at room temperature.

Immunoblotting

1. *Power Homogenizer*: We use an electric drill (Sears Roebuck Co., model 15 315.10042) mounted in a drill press stand (Sears model 25921) to drive a 2 ml Potter-Elvehjem tissue grinder (Wheaton, no. 358003).

2. *Vertical Slab Gel Electrophoresis Apparatus, MiniPROTEAN II*: (Bio-Rad, cat. no. 165-2940).

3. *Electrophoretic Transfer Apparatus, Mini Trans-Blot*: (Bio-Rad, cat. no. 170-

3935).

4. *Electrophoresis Power Supply*: (Bio-Rad, Model 200/2.0, cat. no. 165-4761).

5. *Nitrocellulose Membranes*: (Bio-Rad, cat. no. 162-0147).

6. *40% Acrylamide Stock (37.5:1, Acrylamide:Bis-Acrylamide)*: (Fisher Scientific, cat. no. BP1410-1).

7. *C219 mAb*: (Signet, cat. no. 2158). For immunoblotting, the lyophilized mAb (100 μg) is dissolved in 40 ml immunoblot antibody dilution buffer (see below), divided into two aliquots and stored at - 20° C. Each aliquot can be saved after use, frozen and used again at least ten times.

8. *Goat Anti-Mouse IgG Alkaline Phosphatase Conjugate*: (Bio-Rad, cat. no. 170-6520). The undiluted conjugate is stored at 4° C. Immediately before use the conjugate is diluted 1:3000 with immunoblot antibody dilution buffer.

9. *Lysis Buffer Stock*: 6.06 g tris(hydroxymethyl)aminomethane (Tris) (Sigma, cat. no. T-8524), 8.77 g NaCl, 1.86 g disodium ethylenediaminetetraacetate (EDTA) (Sigma, cat. no. E-4884), 10.0 g sodium deoxycholate (Sigma, cat. no. D-6750), 1.0 g sodium dodecyl sulfate (SDS)(Sigma, cat. no. L-4390) and 10 ml Triton X-

100 (Sigma, cat. no. X-100) are dissolved in 800 ml deionized water. The solution is adjusted to pH 7.4 with 6 M HCl. The volume is made up to 960 ml. The buffer is stored at 4° C. Just before use, the volume of lysis buffer required is prepared in a tube on ice by adding the protease inhibitors from stock solutions.

10. *Protease Inhibitor Stocks*:

N-p-tosyl-L-arginine methyl ester HCl (TAME): (Sigma, cat. no. T 4626). A stock solution of 38 mg/ml in deionized water is prepared fresh before use.

Penylmethylsulfonyl flouride (PMSF): (Sigma, cat. no. P 7626). PMSF is dissolved at a concentration of 10 mg/ml in 100% 2-propanol. The solution is stable for several months at room temperature.

Aprotinin solution (10 units/ml): (Sigma, cat. no. A 6279). This stock is used as is from the producer.

Leupeptin: (Sigma, cat. no. L 2023). Leupeptin is dissolved in deionized water at 5 mg/ml. The solution is divided into 20 µl aliquots and is stored at - 20° C.

11. *1 X Lysis Buffer*: (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1% v/v Triton X-100, 1% w/v sodium deoxycholate, 0.1% w/v SDS, 5 mM EDTA, containing freshly

added 2% v/v aprotinin solution, 380 μg/ml N-tosylamido-L-arginine methyl ester, 100 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin). The following are mixed in a tube on ice immediately before use.

Aprotinin solution (10 U/ml)	0.200 ml
TAME (38 mg/ml)	0.100 ml
PMSF (10 mg/ml in 2-propanol)	0.100 ml
Leupeptin (5 mg/ml)	0.020 ml
Lysis Buffer Stock	9.6 ml

12. *Electrode Buffer*: (25 mM Tris, 192 mM glycine, 0.1% w/v SDS). This is made as a 5X stock containing 15 g Tris base, 72 g glycine and 5 g SDS per liter of solution. The pH of the 5X buffer is near 8.5. The pH should not be adjusted with acid or base as this will increase the electrical conductivity. 5X electrode buffer is diluted with 4 parts water before use.

13. *1 M Tris HCl, pH 6.8*: This buffer is stable for several months at 4 °C.

14. *1.5 M Tris HCl, pH 8.8*: This buffer is stable for several months at 4 °C.

15. *10 % w/v SDS*: This is stored at room temperature.

16. *1 M Dithiothreitol (DTT)*: This is divided into aliquots and stored at -20° C.

17. *10 % w/v Ammonium Persulfate (10% APS)*: 100 mg of APS is added to 1 ml of deionized water. This solution is prepared fresh daily.

18. *N,N,N',N'-Tetramethylethylenediamine(TEMED)*: This is used undiluted from the bottle.

19. *SDS Sample Buffer*: (60 mM Tris-Cl, pH 6.8, 2% w/v SDS, 10% v/v glycerol, 0.025% bromphenol blue, 100 mM dithiothreitol). This is prepared as a stock without the reducing agent dithiothreitol (DTT). The stock is stable at room temperature.

Water	24.5 ml
1.0 M Tris, pH 6.8	3.0 ml
10% SDS	10.0 ml
Glycerol	5.0 ml
0.5% Bromphenol blue	2.5 ml

Just before use, 100 µl of 1 M DTT is added to every 0.9 ml of sample buffer needed for the experiment.

20. *Resolving Gel (5.6 % total acrylamide)*: This is made in a 15 ml disposable screwcap tube. This recipe makes enough for two mini-gels (0.075 cm X 8.0 cm

X 7.3 cm).

Water	5.9 ml
40% Acrylamide stock	1.4 ml
1.5 M Tris, pH 8.8	2.5 ml
10% SDS	0.1 ml

Just before pouring, 100 µl of 10% APS and 8 µl of TEMED are added. The tube is mixed by inversion, and the gels are poured immediately.

21. *Stacking Gel (3% total acrylamide)*: This is made in a 15 ml disposable tube. This recipe makes more than enough for two 1 cm stacking gels for the mini-gels.

Water	2.34 ml
40% Acrylamide stock	0.225 ml
1 M Tris, pH 6.8	0.375 ml
10% SDS	0.030 ml

Just before pouring, 30 µl of 10% APS and 3 µl TEMED are added. The tube is mixed by inversion, and the gels are poured immediately.

22. *Tris Buffered Saline (TBS)*: (20 mM Tris HCl, pH 7.5, 0.5 M NaCl). This buffer is prepared as a 10X stock. 24.2 g of Tris and 292.2 g of NaCl are dissolved in 800 ml of deionized water. The pH is adjusted to 7.5 with 6 M HCl. The volume is made up to 1 l with deionized water. The buffer is sterilized by filtration (0.2 µm) and is stored at room temperature. Before use, the 10X buffer is diluted with 9 volumes of deionized water.

23. *Tris Buffered Saline with Tween 20 (TTBS)*: (1X TBS + 0.05% v/v Tween-20 (Sigma, cat. no. P-9416)). This buffer is usually made up fresh for each run. About 1 l is required.

24. *Transfer Buffer*: (25mM Tris, 192 mM glycine, 20% v/v methanol). This buffer is made up for each run (1 l) and is chilled before use. 14.4 g glycine (Sigma, cat. no. G-7032) and 3.0 g Tris are mixed with 200 ml methanol. The volume is made up to 1 l with deionized water. As with SDS-PAGE electrode buffer, the pH should be around 8.5 and should not be adjusted with acid or base.

25. *Blocking Buffer*: (5% w/v nonfat dry milk, 0.02% sodium azide in TTBS). This buffer is stable for at least two weeks at 4° C.

26. *Immunoblot Antibody Dilution Buffer*: (1% nonfat dry milk, 0.02% sodium azide in TTBS). This buffer is stable for at least two weeks at 4° C.

27. *Alkaline Phosphatase Buffer (AP Buffer)*: (100mM Tris HCl, pH 9.5, 100 mM NaCl, 50mM $MgCl_2$). 12.1 g of Tris and 5.8 g of NaCl are dissolved in 900 ml of deionized water. The pH is adjusted to 9.5 with 6 M HCl before adding 10.0 g of $MgCl_2 \cdot 6H_2O$. The volume is then adjusted to 1 l with deionized water. This buffer is filter sterilized (0.2 μm) and stored at room temperature.

28. *5-Bromo-4-chloro-3-indolyl-phosphate, toluidine salt (BCIP)*: (50 mg/ml in N,N-dimethylformamide, Boehringer Mannheim, cat. number 1383 221). This solution is stored at -20° C.

29. *4-Nitro blue tetrazolium chloride (NBT)*: (100 mg/ml in 70% v/v N,N-dimethylformamide, Boehringer Mannheim, cat. no. 1383 213). This solution is stored at -20° C.

30. *Alkaline Phosphatase Substrate Solution*: 45 µl NBT stock solution and 35 µl BCIP are added to 10 ml of AP buffer. This solution is unstable and light sensitive. It should be prepared immediately before use.

31. *TE*: (10 mM Tris HCl, pH 8.0, 1mM EDTA). This buffer is filter sterilized (0.2 µm) and stored at room temperature.

METHODS

For both immunoblotting and immunohistochemical staining we have used the monoclonal antibody (mAb) C219. This mAb was originally raised against membrane fractions from MDR Chinese hamster ovary cells (Kartner et al., 1985). C219 recognizes a highly conserved linear epitope which is found in all Pgp deduced amino acid sequences including the only published fish sequences (Georges et al., 1990; Chan et al., 1992). This epitope is denaturation resistant which makes the mAb useful both in

immunoblots and in immunohistochemical staining on formalin-fixed paraffin-embedded tissues.

Method I: Pgp Immunohistochemistry

Immunohistochemical staining provides information on Pgp expression at the cellular and subcellular levels. Relative levels of expression in different cell types can be compared on the same slide within the same section. However, variability from slide to slide and from block to block makes this technique difficult to use for quantitative comparisons of expression between individuals. More quantitative information can be obtained from immunoblots (see below).

Several controls are necessary to avoid artifacts in this technique. It is standard practice to include both a positive and a negative control on each slide in the form of a tissue that is known to express Pgp and one that does not. It is also important to incubate sections with an irrelevant antibody to control for nonspecific binding of IgG. For our studies mummichog liver sections serve as both positive and negative tissue controls since these contain cell types that express Pgp (hepatocytes) and cells that apparently do not such as red blood cells. For an antibody control we use a matched isotype mouse myeloma protein (IgG2a, kappa) at the same dilution as C219 (1.5 μg/ml). The irrelevant antibody control can be performed on the adjacent section on the same slide if the sections are circled with a PAP pen (Research Products International). This pen deposits a hydrophobic film on the slide which will retain the antibody solutions on the section and prevent spill-over between sections.

We have developed the immunohistochemical staining protocol for use with

Bouin's fixed paraffin-imbedded tissues. This has allowed us to examine much of the archival material from past histopathological studies when we have routinely fixed specimens in Bouin's fluid. Other fixatives, such as neutral buffered formalin, should give similar results.

Whenever fresh material is used, sub-samples of normal liver and tumors are taken and frozen immediately in liquid nitrogen for analysis by immunoblot (see below). The remaining tissue is fixed and rinsed as follows. Tissues are fixed in Bouin's fluid (Luna, 1968) for 18-24 hrs. The fixed specimens are rinsed in running tap water overnight and are then soaked in several changes of Li_2CO_3 saturated 50% ethanol to remove soluble picrates. Specimens are then stored in 70% ethanol prior to processing.

The Signet ABC reagents in conjunction with mAb C219 are used for detection of antigen. Biotinylated horse anti-mouse IgG is used as a secondary antibody. Bound secondary antibody is detected with an avidin-biotinylated horseradish peroxidase complex (ABC). 3,3' diaminobenzidine (DAB) and hydrogen peroxide are used as chromogenic substrates.

The following procedure begins with 5 μm thick paraffin-embedded tissue sections adhered to slides with gelatin. A section from each specimen is also stained with hematoxylin and eosin (H&E) to allow histopathologic diagnosis of any lesions present. Processing of small fish for histopathological studies has been described by Fournie and colleagues (1996).

Procedure

This procedure is most easily carried out with the aid of a hand staining rack (Shandon, TissueTek II) to hold the various baths. A humidity chamber is required for the antibody incubations. Plastic food storage boxes with tight fitting lids work well for this purpose. The bottom of the box is covered with several layers of wet paper towels or other bibulous paper (Whatman 3MM or similar).

Tissue sections on slides are deparaffinized and rehydrated by the following series of baths: xylene, 2 changes, 5 minutes each; 100% EtOH, 2 changes, 3 minutes each; 95% EtOH, 2 changes, 3 minutes each, deionized water, 2 changes, 2 minutes each followed by a 5 minute bath in deionized water. Then slides are transferred to PBS for 5 minutes. Endogenous peroxidase activity in the sections is then blocked by a bath in 3% hydrogen peroxide for 5 minutes. The slides are returned to the PBS bath for 5 more minutes. Slides are removed from the PBS, and the area surrounding the sections is wiped dry. The section is circled with a PAP pen. This deposits a ring of hydrophobic material around the specimen and serves as a well to retain the antibody solutions on the specimens.

The slides are placed in the humidity chamber and a solution of 10% (w/v) normal horse serum in PBS is applied to each section using a transfer pipet. In this and all subsequent steps, enough solution is applied to completely fill the ring made by the PAP pen and form a bead of solution over the section. The sections are blocked with the horse serum for 1 hour at room temperature. The slides are then held upright and tapped to remove excess blocking solution. The primary antibody solution (C219, 1.5μg/ml in PBS

containing 1% BSA) is applied and the slides are incubated in the humidity chamber overnight (18 hrs.) at 4° C.

The following morning the ABC reagent is prepared according to the manufacturers instructions. This solution must be prepared fresh daily and requires a 30 minute incubation at room temperature before use to insure complete complex formation. The slides are tapped to remove the primary antibody solution and then are rinsed with PBS by gently flooding the surface of the slide several times using a transfer pipet. The biotinylated secondary antibody (biotinylated horse anti-mouse, used at the kit concentration) is then applied to the sections, and the sections are incubated in the humidity chamber at room temperature for 20 minutes. The secondary antibody solution is tapped off, and the slides are rinsed with PBS. The sections are then incubated with the ABC reagent for 20 minutes at room temperature. During this incubation the DAB/H_2O_2 (*Sigma*Fast, Sigma) solution is prepared. After rinsing the slides with PBS, the DAB solution is applied to the sections, and the reaction is allowed to proceed for 3-5 minutes. The reaction is quenched by rinsing the slides with deionized water. The slides are then given a 5 min. tap water rinse, and are counterstained by dipping them for 3-5 seconds in Harris' hematoxylin. The sections are then differentiated in saturated, aqueous $NaHCO_3$ for 2 minutes. The slides are rinsed in tap water and dehydrated through graded alcohols to xylene. The sections are coverslipped using synthetic mounting media (Preservaslide, EM Science).

Method II: Immunoblot analysis of tissue extracts

Immunoblotting provides both a means of quantifying Pgp expression and a check on specificity of detection. Immunoblots have been used frequently to assay expression of Pgp in mammalian organs and tumors (Richert et al., 1988; Lieberman et al., 1989; Fredericks et al., 1991). In these studies crude membrane fractions or microsomes are prepared to generate a Pgp enriched sample. The small size of tissues and especially tumors from small fishes makes this approach of limited use for our application. We routinely use detergent lysates (modified RIPA lysis buffer) of unfractionated organ and tumor samples in immunoblots. This greatly simplifies sample preparation and, in our system, results in sufficient Pgp signal on the blots.

Immunoblots can provide supporting evidence that the signal seen in immunohistochemistry results from Pgp expression and not from an unrelated cross-reactive protein. The expected molecular weight for Pgps is in the vicinity of 170 kDa (Kartner et al., 19832). In addition, known Pgps are heavily glycosylated and are expected to produce a somewhat diffuse band in an immunoblot due to microheterogeneity in glycosylation (Richert et al., 1988). A single band in immunoblots in this molecular weight range and of this characteristic appearance is consistent with specific detection of Pgp.

We use pre-stained, calibrated molecular weight standards for SDS-PAGE (Bio-Rad, cat. no. 161-0309) in our immunoblots. These give visible molecular weight markers on the membrane following transfer. They also provide a check on transfer efficiency, thus eliminating the need to stain the nitrocellulose membrane for total protein following transfer.

Usually a Pgp positive control sample is loaded with each run. We use extracts of the colchicine resistant CH^RC5 cell line (a generous gift from Dr. Victor Ling, Ontario Cancer Institute) for this purpose. Extracts of any convenient mammalian tissue known to have a high level of expression of Pgp should also be useful for this (e.g. adrenal gland).

Densitometric scans of immunoblots can be used to quantify relative levels of expression between individuals with different exposure histories or between tumor and normal tissue. For this purpose it is necessary to load several different amounts of a Pgp standard. This can be prepared from the tissues of interest (e.g. pooled mummichog liver extract) or can be made from any convenient source of Pgp. The different loadings of the Pgp standard are used to form a standard curve of densitometric peak area versus total protein. It is important that standards and samples fall within the linear range of this assay. In our blots using mummichog liver extracts as a standard, this range is usually between 1 and 20 μg of total protein per lane.

The following procedure is used in our laboratory for extraction of Pgp from mummichog liver and liver tumors and subsequent analysis and quantitation of expression by immunoblotting. We use the traditional Laemmli discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system (Laemmli, 1970) followed by 'wet' electrophoretic transfer to nitrocellulose membranes (Towbin et al., 1979). Pgp is detected on the membrane using an indirect technique. The mAb C219 is incubated with the membranes. Bound C219 is detected with alkaline phosphatase conjugated goat anti-mouse IgG followed by reaction with NBT/BCIP.

Procedure

a. Preparation of Tissue Extracts— Fish are anesthestized by placing them in a bath of tricane methanesulfonate (200 mg/l in seawater). Fish are then killed by cervical transection. Samples of liver or other tissues are excised as quickly as possible and either used immediately or snap frozen in liquid nitrogen and stored at -70° C. Homogenization of soft tissues such as liver is accomplished in a teflon-glass homogenizer powered by a electric drill motor. The sample is allowed to just thaw and is placed in the prechilled (on ice) teflon glass homogenizer (Potter-Elvejehm type) containing 5 volumes of cold lysis buffer. The tissue is homogenized by 3 up and down strokes at full speed.

The resulting crude homogenate is transferred to a 1.5 ml polypropylene microcentrifuge tube and is spun at full speed for 5 minutes in a microcentrifuge (Eppendorf, model 5415) in the cold. The homogenate separates into three layers during centrifugation; a floating layer of lipid material, a layer of clear supernatant and a pellet of insoluble tissue debris plus nuclear DNA. The lipid layer is carefully removed with a cotton tipped applicator. The supernatant is then transferred to a fresh tube on ice. The clear lysate can be stored frozen at -70° C at this stage or can be used immediately in immunoblots.

Protein concentrations are determined using a modified Lowry assay that is compatible with the detergents in the lysis buffer (Bio-Rad, *Dc* Protein Assay, cat. no. 500) using BSA as a standard.

b. SDS-PAGE— Samples are resolved on discontinuous SDS-PAGE Laemmli gels using the Bio-Rad Miniprotean II apparatus. We use resolving gel concentration of

5.6% total acrylamide and a 3% stacking gel with the standard buffer. We find it unnecessary to degas the gels if the concentration of ammonium persulfate in the gel recipes is increased to 1%. Gels are prepared from a 40% acrylamide stock solution (acrylamide:bismethylene acrylamide ratio 36.5:1). The electrophoresis apparatus is assembled according to the manufacturer's instructions. The resolving gel is prepared and is poured between the plates using a 10 ml pipet to within 1 cm of the bottom of the gel comb. The gel is then immediately overlain with 0.5 ml of deionized water using a syringe needle. The resolving gel is allowed to polymerize for 30 minutes. The water overlay is poured off, and the residual liquid is blotted out with filter paper. The stacking gel is prepared and poured with the comb in place. After another 30 minutes the combs are removed, and the gel sandwiches are set up in the electrophoresis box according to the manufacturer's instructions.

While the gels are polymerizing, the samples are prepared for electrophoresis. Samples are thawed and kept on ice at all times. Each sample is diluted with Laemmli sample buffer to a final protein concentration of 0.5 to 2 μg/μl. Samples are heat denatured in a 65° C water bath for four minutes and then placed on ice until they are loaded onto the gel. (Note: Higher denaturation temperatures result in polymerization of Pgp.) Usually between 1 and 20 μg of total protein per lane is loaded in a 10 μl volume of sample buffer for mummichog liver samples. Samples are loaded with a Hamilton syringe. Using the small well combs of the minigel apparatus, up to 15 μl of sample can be loaded in each well. Normally a lane of molecular weight standards and a lane containing a Pgp positive control sample are also loaded.

The gels are run at 200 V (constant voltage) at room temperature until the dye front just reaches the bottom of the gel. This takes about 30 minutes.

c. Electrophoretic Transfer to Nitrocellulose Membranes— At the end of the run, the gel sandwiches are disassembled. The somewhat fragile gels are best manipulated while still stuck to the glass plate. Each sandwich is held horizontally with the large plate down, and the small plate is gently pried off using one of the spacers. Usually the gel remains stuck to the large plate. The stacking gel is cut off of each gel and is discarded. This is easily accomplished using one of the gel spacers to gently pull this stacking gel away. The first lane of each gel is marked by cutting off the corner of the gel over the first lane. If two gels are run at the same time they may be identified by cutting different sized pieces from each one.

Each gel is gently floated off of its plate by dipping the plate with the attached gel in a plastic dish containing 100 ml of transfer buffer until the gel slides into the dish. The gel is washed in transfer buffer for 20 minutes at room temperature with gentle agitation.

The blotting materials are prepared while the gels are equilibrating. The nitrocellulose membranes are cut to a size slightly larger than the gels. These are then soaked in transfer buffer. Four sheets (per gel) of chromatography paper (Whatman 3MM) cut to the size of the transfer cassette are also soaked in cold transfer buffer. After 20 minutes the transfer buffer is carefully poured off of the gels and replaced with 100 ml of fresh transfer buffer. The gels are now ready to be placed in the transfer cassette.

The cassette is placed cathode side (black side) down in a baking dish containing a few cm of cold transfer buffer. One of the porous plastic pads provided by the

manufacturer (a rectangular plastic scouring pad also works) is placed in the dish and any bubbles are forced out. The saturated pad is placed on top of the cathode side of the transfer cassette. The gel is then moved onto a stack of two sheets of saturated paper by submerging the papers in the dish containing the gel and floating the gel onto the center of the paper stack. The papers and the gel are then placed on top of the plastic pad in the transfer cassette. All air bubbles are carefully removed from beneath the gel and the blotting paper by rolling a test tube over the surface of the gel. The pre-wetted nitrocellulose membrane is then placed on the gel, and all air bubbles are removed as before. The transfer sandwich is completed by placing two more sheets of buffer-saturated paper on top of the nitrocellulose followed by another saturated plastic pad. The cassette is then placed in the slot in the transblot device. The ice pack is placed next to it, and the buffer chamber is filled with cold transfer buffer. About 1 l of buffer is required for each run. The assembled transfer chamber is placed in a chromatographic refrigerator (4° C). The gels are transferred for 16-18 hours at 34 V (constant voltage). Under these conditions, the apparatus draws about 50 mA of current at the beginning of the run. By the next day the ice in the cooling pack has melted, and the current has increased to about 80 mA.

d. Detection of Immune Complexes— After transfer, the cassettes are disassembled and each membrane is cut to the exact size of its gel. The corresponding corner is cut off to provide unambiguous orientation and the membrane is labelled on the back with a pencil to identify it.

It is possible to stain the membrane for total protein at this point using Ponceau S

to verify transfer efficiency. Membranes are placed directly into Ponceau S solution (0.1% w/v Ponceau S in 5% acetic acid, Sigma, cat. no. P7170) for 10 minutes. This reversibly stains all protein bands on the blot. If molecular weight standards are included, the position of these is marked at this time with a pencil. If no bands appear, it is not worth continuing, since transfer did not occur. A common cause of this is failure to orient the transfer cassette correctly in the transfer apparatus (cathode(black)-gel-nitrocellulose-anode(red)).

An easier alternative to check transfer efficiency and provide size standards is to use pre-stained molecular weight standards as mentioned previously. These are readily visible on the membrane. All but the highest molecular weight standard should completely transfer from the gel. This lane unambiguously shows the orientation of the blot and makes cutting a corner of the blot unnecessary.

After transfer is verified, the membranes are placed in a covered plastic dish containing 100 ml of blocking buffer for 1 hour at room temperature. During all incubation and washing steps, constant gentle agitation is provided by an orbital shaker platform. The membranes are transferred to another plastic dish containing 20 ml of diluted C219 at a concentration of 2.5 μg/ml (1:40 dilution of reconstituted C219) in antibody buffer. Membranes are incubated in the primary Ab for 1 hour at room temperature. (Note: The primary antibody solution can be saved at - 20° C and reused at least 10 times before loss in sensitivity becomes noticeable.) After 1 hr., the membranes are transferred to a container with 100 ml of TTBS and washed for 5 minutes. The wash step is repeated before transferring the membranes to the secondary Ab conjugate

solution. The membranes are incubated for 1 hour at room temperature with 25 ml of freshly diluted goat anti-mouse alkaline phosphatase conjugate (1:3000 in antibody dilution buffer). The membranes are then washed with two changes of TTBS as before, followed by another 5 minute wash in TBS without Tween 20. The membranes are then given a final wash for 2 minutes in AP buffer. The membranes are then placed in a plastic bag containing 10 ml of BCIP/NBT substrate solution. All air bubbles are removed from the membrane surfaces, and the membranes are incubated in the dark until the lowest concentration bands become visible. This usually takes about 15 minutes. The color development reaction is stopped by washing the membrane in deionized water and placing it in TE. Membranes are stored at 4° C in TE.

Blots are then photographed (Polaroid Pos./Neg. film type 665) and are subjected to densitometry if quantitation is desired. We use a Shimadzu 930 TLC scanner set at 550 nm for quantitation.

RESULTS and DISCUSSION

Immunohistochemistry— Immunohistochemical staining has been used to detect Pgp expression in normal mammalian tissues. This technique has shown Pgp expression on the canalicular surface of hepatocytes in normal liver (Thiebaut et al., 1987; Thiebaut et al., 1989; Bradley et al., 1990). We have found that immunohistochemical staining of sections of normal mummichog liver showed specific staining that corresponds to the mammalian pattern of expression with allowances for the differing architecture of fish liver (Hampton et al., 1985). The immunoperoxidase stain clearly showed the structure

of the bile canaliculi within the section (Figure 3A). There was no stain in the cytoplasm or other organelles in normal hepatocytes. No other hepatocellular elements were stained in these preparations. This pattern of subcellular localization is consistent with the hypothesis that P-glycoprotein may have an excretory function in fish liver as has been suggested for mammals (Arias, 1990; Thorgeirsson et al., 1991).

Increased expression of Pgp has been detected by C219 immunohistochemical staining in human neoplasms (Weinstein et al., 1991). Immunohistochemical staining has shown Pgp overexpression in experimentally induced rat hepatocellular carcinoma and advanced preneoplastic lesions (Bradley et al., 1992). Using mummichog from a creosote contaminated site with a high prevalence of hepatic neoplasms, we were able to examine Pgp expression in hepatic tumors of fish. Many tumors showed increased C219 immunoperoxidase staining (overexpression) as well as alterations in the pattern of expression (Figure 3B). Often there was a loss of polarity with the entire plasma membrane staining. Cytoplasmic staining and some paranuclear (Golgi) staining was also evident. The alterations in pattern and degree of immunohistochemical staining observed in these fish liver tumors were similar to those reported for rat liver hyperplastic nodules and hepatocellular carcinoma (Bradley et al., 1992).

In both tumor and normal liver preparations we observed no staining when mouse myeloma protein was used in place of C219 (Figure 3C).

Immunoblots— Immunoblots have been used to identify and quantify Pgp expression in drug resistant mammalian cell lines (Kartner et al., 1985) and in mammalian tumors and normal tissues (Hitchins et al., 1988; Liberman et al., 1989;

Fredericks et al., 1991). Immunoblots have also been used to identify Pgp family members in some aquatic organisms (Kurelec et al., 1992; Toomey and Eppel, 1993; Minier et al., 1993). We have identified a single main immunoreactive band in immunoblots of mummichog liver and tumor extracts (Figure 3D). The mummichog Pgp band migrated at a relative molecular weight of 177 kDa. This is a higher molecular weight than that of the immunoreactive band in extracts of the multidrug-resistant CH^RC5 cell line in our system (165 kDa) but is within the molecular weight range reported for P-glycoproteins. The mummichog liver and liver tumor Pgp bands also had the same characteristic diffuse appearance as the mammalian Pgp. This is consistent with glycosylation of the putative mummichog Pgp. These results help confirm that the antigen labeled in immunohistochemical staining is a Pgp family member.

Densitometric analysis of the immunoblot shown in Figure 3D indicated approximately a three fold increase in expression of Pgp in a mummichog liver adenoma compared to the adjacent normal tissue. These results are consistent with the overexpression seen in immunohistochemical staining of this adenoma and allow a quantitative estimate of overexpression.

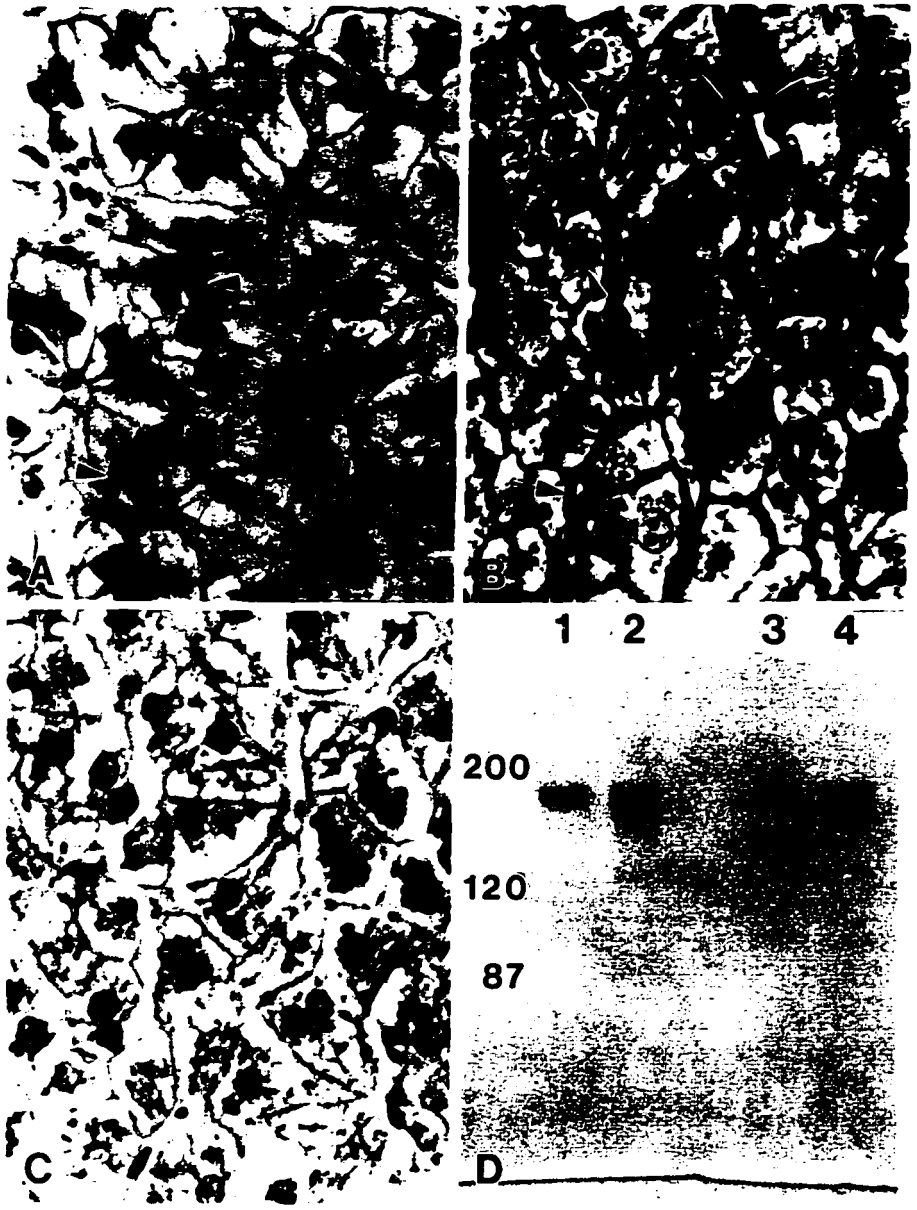
Advantages of Combined Immunochemical Methods— We have found that using immunohistochemical staining and immunoblotting together provides information that neither one alone could give. Immunoblots combined with densitometric analysis have allowed comparison of relative levels of expression between individuals and between discrete proliferative lesions and surrounding non-neoplastic liver.

Immunohistochemical staining has proved less quantitative but has provided

important information on the relative level of expression at the cellular and subcellular levels which is destroyed in whole organ homogenates. In our system the two techniques have supported the assumption that the protein labelled is a P-glycoprotein. Immunoblots show specific staining of a single band of the correct molecular weight and characteristics. Immunohistochemical staining shows that in normal fish liver, as in mammalian liver, Pgp is present only on the canalicular surface of hepatocytes; whereas in hepatic tumors there is often overexpression as well as loss of polar expression of this antigen.

Further Applications— We are continuing to use these techniques in our studies of Pgp expression in the mummichog. We are investigating induction of Pgp in mummichog liver in response to environmental and laboratory exposures to PAHs. We are also studying relationships between relative Pgp expression and hepatic lesion classification in fish from the PAH contaminated site. The highly conserved nature of the C219 epitope and the high specificity demonstrated with mummichog tissues should make the immunochemical techniques described here useful in detecting Pgp expression in many other aquatic organisms.

Figure 3. C219 immunochemical detection of Pgp in liver and hepatic proliferative lesions of mummichog. **A:** Immunochemical localization of Pgp in normal mummichog liver on the bile canalicular surface (*arrowheads*) of hepatocytes. **B:** Immunochemical localization of Pgp in a mummichog liver adenoma showing overexpression, paranuclear (Golgi) staining (*arrowheads*) and degeneration of polar membrane expression including staining of the sinusoidal surface (*arrows*) of hepatocytes. **C:** Matched isotype mAb negative control of section adjacent to that shown in **B. D:** Immunoblot of paired samples of grossly visible lesions (lanes 2 and 4) and adjacent uninvolved liver (lanes 1 and 3); the pair in lanes 1 and 2 are subsamples of the liver and lesion shown in **A** and **B**. Positions of standards are shown with their molecular weight (kDa) at the left.



Chapter III: Increased expression of P-glycoprotein in liver and liver tumors of mummichog (*Fundulus heteroclitus*) inhabiting a creosote-contaminated environment

INTRODUCTION

Chapter II (Cooper et al., 1996) of this dissertation presented methods that use monoclonal antibody (mAb) C219 (Kartner et al., 1985) in immunoblots and immunohistochemical staining to detect and measure relative levels of Pgp expression in mummichog liver and in mummichog liver tumors. Results using these methods indicated that one or more P-glycoprotein homologs are expressed in the bile canalicular surface of hepatocytes in mummichog liver. This expression pattern is consistent with expression reported in mammalian (Thiebaut et al., 1987; Thiebaut et al., 1989; Bradley et al., 1990) and guppy liver (Hemmer et al., 1995). We also have found that some mummichog liver tumors from mummichog inhabiting a heavily creosote-contaminated site (Atlantic Wood) overexpress P-glycoprotein and have altered subcellular localization of this antigen. This is similar to the altered expression of Pgp reported for mammalian hepatic neoplasms (Fairchild et al., 1987; Thorgeirsson et al., 1987; Teeter et al, 1990; Vohm et al., 1990; Bradley et al., 1992) . The conditions at the Atlantic Wood site provide a unique opportunity to study the response of Pgp expression in fish liver to environmental contaminant exposure and to examine changes in Pgp expression during environmental hepatocarcinogenesis in a teleost. In the present Chapter, we have used our immunochemical techniques to survey expression patterns and levels of Pgp antigens in environmentally induced liver tumors and in the livers of mummichog from the Atlantic Wood site.

We have compared levels of Pgp expression in the non tumor-bearing livers of chemically resistant fish from the Atlantic Wood site to levels to expression in sensitive

fish from an uncontaminated environment (King Creek) in order to determine whether the analogy to the xenobiotic resistance phenotype reported for these livers (Van Veld and Westbrook, 1995) extends to increased expression of Pgp. In order to address whether changes in Pgp expression in the livers of resistant fish could result from induction of Pgp expression by exposure to PAHs, we have measured Pgp expression in sensitive fish following exposure to a single carcinogenic PAH (3-methylcholanthrene). We have also extended our studies of the biochemical properties of hepatic proliferative lesions of these fish to include Pgp, a putative marker of the drug-resistance phenotype in mammalian liver tumors (Farber and Sarma, 1987; Fairchild et al., 1987; Thorgeirsson et al., 1987; Thorgeirsson et al., 1991).

Results reported here demonstrate detectable increases of Pgp expression in non-tumor bearing liver of Atlantic Wood mummichog as well as high level overexpression and altered patterns of expression in a majority of hepatic neoplasms examined from these fish. This is the first report of increased Pgp expression in a teleost from a polluted environment and is the first study of Pgp expression in the tumors of a lower vertebrate. These results are similar to what is known about Pgp expression in mammalian liver and liver tumors. This increased Pgp expression may have implications for the tolerance or adaptation of these fish inhabiting a contaminated environment.

MATERIALS AND METHODS

Collection of mummichog livers— Mummichog were collected with minnow traps in October of 1994 and July of 1996 from a creosote contaminated site and a

relatively uncontaminated site. The contaminated site was a marsh creek that collects run-off from a defunct wood treatment facility (Atlantic Wood Products) in the Elizabeth River in Portsmouth, VA. This site has been reported to have very high sediment concentrations of PAHs (2200 mg/Kg dry sediment) (Vogelbein et al., 1990). The uncontaminated site was King Creek, a marsh creek tributary to the Severn River in Gloucester County, VA. Fish from each site were collected within one day of each other and were killed within 48 hr with an overdose of tricaine methane sulfonate (Sigma, St. Louis, MO). Livers were removed, snap frozen in liquid nitrogen and stored at -70° C until use. Only male fish without grossly visible tumors were used in these analyses.

Preparation of liver extracts— Processing of mummichog livers essentially followed the protocols given in Chapter II of this dissertation (Cooper et al., 1996). Mummichog livers were homogenized in 5 to 10 volumes of ice cold lysis buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1% v/v sodium deoxycholate, 0.1% w/v SDS, 5 mM EDTA) which contained freshly added protease inhibitors (2% v/v aprotinin solution (Sigma), 380 µg/ml N-toluenesulfonylamido-L-arginine methyl ester, 100 µg/ml phenyl methyl sulfonyl fluoride, 10 µg/ml leupeptin). Livers were homogenized in a Polytron homogenizer (Brinkman Instruments, Westbury, NY). Homogenates were allowed to stand on ice for 10 minutes and then were clarified by centrifugation in an Eppendorf model 5415 microcentrifuge (10 min, 12000 rpm, 4° C). Protein concentrations of the extracts were determined using the Lowry assay with bovine serum albumin as a standard.

Immunoblotting of mummichog liver extracts with the monoclonal

antibodies C219, JSB1 and C494— The procedure for SDS-PAGE, electrophoretic transfer and immunodetection using mAb C219 essentially were as described in Chapter II (Cooper et al., 1996). Mummichog liver extracts were diluted to a concentration of 0.5 mg/ml with SDS-PAGE sample buffer (60mM Tris-HCl, pH 6.8, 2 % w/v SDS, 10% v/v glycerol, 100 mM dithiothreitol, 0.025% bromphenol blue). Samples were heated to 65° C for 4 min, and were then loaded onto a 5.6% (total) acrylamide SDS-PAGE mini-gel (7.3cm X 8.0 cm X 0.075cm) at 2 μg per lane. Several different amounts of a pooled extract of 5 livers from the uncontaminated site were also loaded onto each gel to serve as a quantitation standard. Usually 0.5 μg, 2 μg, 4 μg and 8 μg of this reference extract were loaded in separate lanes. Samples were electrophoresed under standard conditions (200 V, 30 min). The SDS-Page gels were then transferred electrophoretically (34 V, 18 hr) to nitrocellulose membranes (BioRad, 0.2 μm). The transfer buffer was 25 mM Tris base, 192 mM glycine containing 20% v/v methanol. Following transfer, the nitrocellulose membranes were blocked in Tris buffered saline (TBS) (20mM Tris-HCl, pH 7.5, 0.5M NaCl) containing 5% w/v nonfat dry milk and 0.1%Tween-20. Following blocking the membranes were incubated with mAb C219 (Signet, Dedham, MA) (2.5 μg/ml, in TBS with 1% Tween-20, 1% nonfat dry milk) for 1 hr at room temperature. The membranes were washed twice for 5 min in TBS-Tween 20 and were then incubated for 1 hr at room temperature with goat antimouse IgG-alkaline phosphatase conjugate (BioRad, Richmond, CA) (1:2000 in TBS-Tween 20). The membranes were then washed twice in TBS-Tween 20. After a final wash in TBS, immune complexes were detected on the membrane using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and p-nitro blue

tetrazolium chloride (NBT) as chromogenic substrates for alkaline phosphatase. In addition to mAb C219 two other mammalian monoclonal antibodies, C494 and JSB1 (Signet, Dedham, MA), were also used in immunoblots. The procedure used for detection using these antibodies was similar to that given here for C219 except that these antibodies were used at higher concentrations (10 μg/ml) and were incubated with immunoblot membranes for up to 24 hrs with the goal of increasing sensitivity. Up to 30 μg of total protein was loaded per lane in gels used with these antibodies.

Estimation of relative Pgp content in mummichog liver extracts— The intensity of staining of immunoblots probed with mAb C219 was measured with a Shimadzu CS-930 scanning densitometer set at a wavelength of 550 nm. The densitometric peak area for each Pgp band was normalized by comparing it to a standard curve of peak area versus total protein for varying amounts of a pooled extract of King Creek mummichog liver loaded on each blot. Values of Pgp expression were then expressed relative to this standard extract. Arbitrary units were devised by assigning a value of 100 to the signal obtained from 1 μg of the pooled King Creek liver extract. The specific content of Pgp in each sample was then expressed in terms of these arbitrary units per microgram of protein.

Laboratory exposure of mummichog to 3-methylcholanthrene— Male mummichog collected from King Creek that had been maintained in captivity for several months were used in this portion of the study. Fish were given intraperitoneal (i.p.) injections of either 3-methylcholanthrene (Sigma, St. Louis, MO) dissolved in corn oil (10 mg/ml) or corn oil alone. Injection volumes were adjusted so that each fish received

a dose of 100 mg/Kg body weight or 10 ml corn oil/Kg body weight. The two groups of fish were maintained in separate 10 gallon aquaria provided with flowing water from the York River for 24 hrs before and for 36 hrs following treatment. The fish were fed once daily during this period. Thirty six hrs after injection the fish were killed by overdose with tricaine methane sulfonate and their livers were removed, frozen in liquid nitrogen and stored at -70° C. Detergent extracts of the livers were analyzed for Pgp expression as described above. In addition these extracts were analyzed for relative levels of CYP1A expression as described in the next section using the monoclonal antibody (mAb 1-12-3) produced against the CYP1A protein of the scup (Park et al., 1986). This antibody has been used to measure cytochrome P4501A levels in many fish species including the mummichog (e.g. Van Veld et al., 1992; Van Veld et al., 1990; Gallagher et al., 1995).

Student's t-test was used to test for significant differences among means of expression levels between the contaminated and uncontaminated sites or between 3-methylcholanthrene exposed fish and control fish.

Immunodetection of cytochrome P4501A in extracts of 3-MC treated mummichog liver— Immunodetection of cytochrome P4501A in 3-MC treated liver followed a similar protocol to the one given above for Pgp immunodetection with the substitution of mAb 1-12-3 (Park et al., 1988) as the primary antibody. (This antibody was a generous gift of Dr. John Stegeman, Wood's Hole Oceanographic Institute.) Samples were analyzed by electrophoresis on 10% acrylamide gels instead of 5.6% acrylamide gels because of the lower molecular weight of the CYP1A protein. For CYP1A analysis, 20 μg of total liver protein was loaded per lane of the blot. All other

electrophoresis, transfer and incubation conditions were identical to those for immunoblot detection of Pgp with mAb C219.

Immunohistochemical staining of mummichog liver and liver lesions— Many of the mummichog liver and tumor specimens used in this study were archival specimens used in previously reported histopathological studies (Vogelbein et al., 1990). These specimens were collected in August 1989 and 1990 from the Atlantic Wood site in the Elizabeth River. Additional specimens were collected in 1992 from both Atlantic Wood and King Creek. The fixation and processing of the fish tissues were as described (Vogelbein et al., 1990). Over 90 histologic sections of livers from the creosote contaminated site containing proliferative lesions were stained for Pgp. Immunohistochemical staining of Bouin's fixed paraffin embedded tissue sections followed the protocols presented in Chapter II (Cooper et al., 1996). For immunohistochemical staining with mAb C219, 5 μm thick sections were cut from the paraffin blocks and were adhered to slides with gelatin. A section from each block was also processed for routine staining with hematoxylin and eosin (Luna, 1968). To control for nonspecific binding of the primary and secondary antibodies, histologic sections were also incubated with mouse myeloma protein (Sigma) of the same isotype as mAb C219 (lgG 2a, kappa) and processed as described for mAb C219.

Tissue sections were deparaffinized in xylene and rehydrated through graded alcohols to phosphate buffered saline (PBS). Endogenous peroxidase activity was blocked by a bath in 3% aqueous H_2O_2. After rinsing in PBS, sections were incubated in 10% normal horse serum in PBS to block nonspecific binding. The sections were then

incubated for 18 hrs at 4° C with mAb C219 (Signet, Dedham, MA) (1.5 μg/ml in PBS with 1% bovine serum albumin). Detection of C219 was accomplished using a biotinylated secondary antibody (biotinylated horse anti-mouse) and an avidin-biotinylated horseradish peroxidase complex (Elite Avidin-Biotin Complex Immunohistochemical Staining Kit, Signet Laboratories, Dedham, MA). Immune complexes were stained using 3,3' diaminobenzidine and H_2O_2 (Sigmafast tablets, Sigma, St. Louis, MO) as chromogenic substrates for horseradish peroxidase. Sections were counter stained lightly with Harris' hematoxylin and differentiated in aqueous $NaHCO_3$. Sections were dehydrated through graded alcohols to xylene and mounted in synthetic mounting medium (Preservaslide, EM Science).

RESULTS

Immunoblot detection of Pgp in normal liver— Three different monoclonal antibodies to mammalian Pgps were used in immunoblots to detect P-glycoprotein expression in mummichog liver. Immunoblots of extracts of mummichog liver using the mAb C219 showed a highly immunoreactive band at a size near 170 kDa (Figure 4A). The mAb JSB-1 also reacted with a protein of this molecular weight in mummichog liver extracts although the signal was much weaker (Figure 1B). In the present study no immunoreactive proteins were detected in mummichog liver extracts using the mdr1 specific mAb C494 (Georges et al., 1990) under the same conditions (not shown).

Immunoblot measurement of increased expression of Pgp in livers of mummichog from a creosote contaminated site— The mAb C219 was used to evaluate changes in Pgp expression in mummichog exposed to PAHs. Analysis of mummichog liver extracts from Atlantic Wood and King Creek mummichog collected at two different times indicated a significant increase (Student's t-test, $p<0.05$) in Pgp expression in the livers of Atlantic Wood mummichog (Figure 5, Table 2). The average increase in expression of the Pgp was 3.2 fold and 2.6 fold (Table 2) in the livers of fish from Atlantic Wood in 1996 and 1994 respectively.

Immunoblot assay of Pgp expression following i.p. administration of 3-MC— There was no significant increase in the intensity of staining for Pgp in immunoblots of liver extracts of mummichog given intraperitoneal injections of 3-methylcholanthrene (3-MC) compared to a group of fish injected with corn oil (36 hrs post injection). The relative Pgp contents in the two treatments were 144±30 units/μg protein and 128±32 units/μg protein for the 3-MC and corn oil treatments respectively. However the levels of CYP1A were found to be ten-fold higher in the 3-MC treated fish in immunoblot analysis (Figure 6). This increase in CYP1A established that effective exposure of the fish to the 3-MC had taken place.

Immunohistochemical detection of Pgp expression and overexpression in mummichog hepatic neoplasms and advanced preneoplastic lesions— We used immunohistochemical staining with C219 to examine samples of mummichog liver and liver tumors for Pgp expression. Histologic sections of normal livers of fish from either the creosote contaminated site or from a relatively clean site showed typical specific

immunohistochemical staining of the canalicular surface of hepatocytes (Figure 7). No staining of mummichog liver or liver tumor sections was observed when a matched isotype mouse myeloma protein was used in place of mAb C219 in the immunohistochemical staining protocol (Figure 8).

There was no clear indication of altered expression of Pgp in putative early preneoplastic lesions (altered foci) of Elizabeth River mummichog (not shown) although many of the livers exhibited regional heterogeneity of staining for Pgp. Unlike the early proliferative lesions however, many of the hepatocellular carcinomas and adenomas exhibited obvious alterations in expression of P-glycoprotein (Figure 9). Eight adenomas, sixteen hepatocellular carcinomas and two hepatoblastomas were examined. These lesions have been described previously (Vogelbein et al, 1990; Van Veld et al., 1992; Vogelbein et al., 1996). Many of these lesions had elevated levels of immunohistochemical staining indicating over-expression of Pgp (Figure 9A-F). Of the sixteen hepatocellular carcinomas ten had clear overexpression compared to the surrounding parenchyma while the remainder had reduced or approximately equivalent expression to the surrounding normal tissue. Many of the carcinomas had aberrant localization of the Pgp antigen as well. There were examples of cytoplasmic staining (e.g. Figure 9C&D), paranuclear or "Golgi" staining (e.g. Figure 9G) , and varying degrees of loss of polarity ranging from an increase in size of the region stained surrounding the bile canaliculus (Figure 9B) to staining of the entire plasma membrane (Figure 9E). Six of the eight adenomas had elevated Pgp staining. There was predominant polar staining of the bile canaliculus in all cases (Figure 6 A). Three of

these lesions also had tracts of extreme overexpression, and in these regions there was loss of polarity. High level expression of Pgp was observed in the two hepatoblastomas. One hepatoblastoma had very well developed canalicular staining (Figure 9F) while the second exhibited loss of polar expression. These patterns of expression are consistent with the degree of differentiation of the two lesions at the ultrastructural level (Vogelbein et al., 1996). Overall the retention of polar (canalicular) staining in all three categories of progressed proliferative lesions was correlated with the degree of differentiation of the lesion (morphological resemblance to normal liver).

DISCUSSION

We found elevated expression of one or more P-glycoprotein homologs in the livers and liver tumors of mummichog inhabiting an environment severely contaminated with PAHs. This is believed to be the first evidence of increased expression of Pgp in teleosts from a polluted environment. It also is the first evidence of P-glycoprotein overexpression in the tumors of a lower vertebrate.

Chapter II of this dissertation reported immunoblot and immunohistochemical techniques for detection of Pgp in mummichog liver tumors using mAb C219 (Cooper et al., 1996). Results reported here with mAb C219 and JSB-1 confirm specific identification of Pgp isoforms in immunoblots of mummichog liver. The molecular weight (170 kDa) and characteristic broad appearance of the labeled band in immunoblots indicating a high degree of glycosylation strongly suggest that the C219 and JSB-1 monoclonal antibodies are detecting Pgp homologs expressed in mummichog liver. C219

reacts with a highly conserved epitope (VQEALD) present in all known Pgp deduced amino acid sequences including the only published fish sequences (Georges et al., 1990; Chan et al., 1992). JSB-1 reacts with an unknown epitope present in human and rodent drug-resistance Pgp isoforms (Scheper et al., 1988). This antibody has been reported to react in a specific manner with bile canaliculi of guppy liver in immunohistochemical staining (Hemmer et al., 1995). The failure of the MDR1/pgp1 specific mAb C494 (Georges et al., 1990) to react with mummichog liver extracts in immunoblots is also consistent with the reported absence of immunohistochemical staining of guppy hepatocytes with this antibody (Hemmer et al., 1995).

The specificity of detection in immunoblots using mAb C219 shows that the increased staining seen in immunoblots of Atlantic Wood mummichog livers represents elevated levels of one or more P-glycoprotein isoforms in the livers of these fish. Elevated levels of Pgp related antigens have been reported in gill and mantle tissue of marine mollusks from contaminated sites (Minier, et al., 1993; Kurelec et al., 1995; Kurelec et al., 1996). This is the first report of elevated levels of Pgp in a teleost associated with environmental contaminants. There may be several possible causes for this elevation in the nontumor bearing livers of the Atlantic Wood site: 1) specific Pgp genes may be induced in response to specific components of creosote or their metabolites; 2) Pgp levels may be elevated as a more general response to toxic injury resulting from hepatotoxic contaminant exposure; 3)There may be some contribution to Pgp expression from overexpression in hidden liver tumors; 4) Pgp levels in these fish may be constitutively elevated in the livers of mummichog from this site and are a

component of the observed genetic adaptation of these fish to the acutely toxic effects of creosote. Each of these possibilities is discussed in turn below.

Because Pgp genes are induced in mammalian livers following exposure to xenobiotics including PAHs, it seems likely that Pgp genes may be induced in mummichog liver by exposure to xenobiotics (presumably PAHs) present at the Atlantic Wood site (Thorgeirsson et al., 1988; Gant et al., 1990; Gant et al., 1995). Thorgiersson and colleagues have hypothesized that Pgp gene expression is induced in liver by xenobiotics or their metabolites having biliary excretion pathways (Thorgeirsson et al., 1988; Gant et al., 1995). In fish as well as in mammals biliary excretion is an important route of elimination of PAH metabolites (Varanasi et al., 1989; Schintz et al., 1993). In the present study however we were not able measure increase in Pgp in King Creek mummichog 36 hr following i.p. injection of 3-MC. These conditions did produce a large increase in immunodetectable CYP1A however. While 3-MC has been found to induce expression of Pgp in rat liver, exposures in these cases were for longer times (Thorgeirsson et al, 1988; Gant et al., 1990). Long term exposures to 3-MC may produce induction of Pgp in the mummichog as well. However it is possible that the Pgp genes of fishes may not be responsive to PAHs or their metabolites.

Another explanation for these observations is that the elevated levels of Pgp in mummichog liver from the creosote contaminated site are a result of liver damage in these fish rather than induction of mummichog Pgp genes by PAHs. The livers of mummichog from this site show a high prevalence of pathological changes associated with toxic injury and cell death in addition to the proliferative lesions (Vogelbein et al.,

1990; Wolfgang Vogelbein, personal communication). Levels of Pgp mRNA has been observed to increase in rodent liver under conditions of liver injury such as partial hepatectomy, cholestasis (Thorgeirsson et al., 1987; Marino et al., 1990; Teeter et al., 1991; Schrenk et al., 1993). The Pgp genes in mummichog liver may respond in a similar manner to toxic injury.

Although livers without grossly visible lesions were chosen for this study, it is possible that many of these livers contained neoplasms that were not externally visible. The contribution of Pgp overexpression in small tumors present in Atlantic Wood mummichog liver to the elevated signal seen in immunoblots cannot be ruled out.

Finally, there is also a possibility that elevated levels of hepatic Pgp in Atlantic Wood mummichog are component of the observed genetic resistance of the mummichog at this site to the acute toxicity of creosote (Horton et al., 1993; Williams et al., 1994). Van Veld and Westbrook (1995) have hypothesized that the toxicity resistance in these fish may be related to constitutive alterations in levels and activities of liver enzymes involved in biotransformation and detoxification. These changes are similar to alterations observed multixenobiotic-resistant preneoplastic liver lesions and multidrug-resistant mammalian cell lines and include suppression of phase I oxidative enzymes involved in activation of carcinogens and elevated levels of phase II detoxifying enzymes (such as GSTs) in nontumor-bearing livers of these fish (Van Veld et al., 1991; Van Veld and Westbrook, 1995). Another biochemical alteration seen in chemically resistant cells is increased expression of Pgp. Thus the livers of mummichog from the creosote contaminated site have several of the changes associated with the "resistant hepatocyte"

phenotype seen in chemical carcinogenesis and multidrug resistant cell lines (Farber, 1984a; Farber, 1984b; Farber and Rubin, 1991). These alterations may contribute to the survival of these fish in a PAH contaminated environment. Whether any of these changes in liver biochemistry are inherent or whether they are a result of acclimation (i.e. induction) and other reversible changes is unknown at this point.

Immunohistochemical results reported here confirm localization of Pgp to the canalicular surface of hepatocytes in teleost liver. Similar staining patterns have been reported for histologic sections of guppy liver using this antibody (Hemmer et al., 1995). These staining patterns of teleost livers indicate that the expression of Pgp in fish liver is homologous with the expression pattern of Pgp in mammalian liver with allowances for the differing architecture of fish liver (Hampton et al., 1985). This pattern of expression suggests that Pgp is involved in biliary excretion in teleosts.

We observed overexpression of Pgp in the majority of mummichog hepatic neoplasms examined in this study. Overexpression of Pgp is characteristic of mammalian liver tumors (Goldsmith et al., 1990; Huang et al., 1992; Teeter et al, 1990; Bradley et al., 1992). The altered immunohistochemical staining patterns seen in the mummichog neoplasms here are strikingly similar to those reported for experimentally induced rodent liver neoplasms, human hepatocellular carcinoma and human colon carcinoma (Bradley et al., 1992; Weinstein et al., 1991). These altered patterns include cytoplasmic and paranuclear staining as well as nonpolar plasma membrane expression.

Thus, Pgp overexpression is another biochemical property of mummichog tumors in addition to reduced CYP1A which is similar to biochemical alterations observed in

mammalian liver tumors and that are associated with xenobiotic resistance (Farber 1984a, Farber 1984b; Fairchild et al., 1987; Farber and Rubin, 1991; Van Veld et al., 1992, Van Veld and Westbrook, 1995).

The origin of Pgp overexpression in liver tumors is not well understood. It has been associated with xenobiotic resistance in preneoplastic lesions (Thorgeirsson et al., 1987). Selective promotion of Pgp-expressing lesions in the presence of cytotoxic carcinogens may account for the overexpression observed in hepatocellular carcinoma. However, Pgp overexpression in liver tumors has also been observed without promotion by xenobiotics (Teeter et al, 1990; Bradley et al., 1992). Therefore Pgp overexpression in liver tumors may be a late event related to oncogene activation and tumor progression (Teeter et al., 1990; Bradley et al., 1992; Bradley and Ling, 1994). The environmentally induced liver tumors in mummichog from the Elizabeth River provide an interesting system in which to examine these two alternatives, Pgp expression arising from selection of Pgp overexpressing lesions versus Pgp overexpression as a marker of neoplastic transformation. Mummichog inhabiting the creosote contaminated site are exposed continuously to a mixture of toxic xenobiotics that includes carcinogenic PAHs (Vogelbein et al., 1990). Such conditions seem ideal for the promotion of xenobiotic resistance in early preneoplastic lesions and their eventual progression to resistant tumors. The livers of fish from this site show a variety of microscopic histopathology including several categories of altered foci. Some of these are presumed to be preneoplastic lesions (Vogelbein et al., 1990). If selection of Pgp expression is occurring during carcinogenesis in these fish, then Pgp overexpression should have been apparent in

putative early lesions. This was not the case however. Over 90 different mummichog livers were stained for Pgp expression using mAb C219. Within these livers, only neoplasms (hepatocellular adenomas and hepatocellular carcinomas) showed evidence of overexpression of Pgp. While the majority of these neoplasms overexpressed Pgp, some had reduced or equivalent levels of Pgp expression. Thus Pgp overexpression in these fish tumors is probably a late event which may be independent of selection of xenobiotic resistance in the lesions. This overexpression may be caused by activation of specific oncogenes during tumor progression in mummichog liver.

Consistent with this hypothesis are reports that transfection of rat liver epithelial cells with the oncogene v-H-*ras* induces high level expression of Pgp, and that high level expression of mutant p53 stimulates the promoter of the human *MDR1* gene *in vitro* (Burt et al., 1988; Chin et al., 1992). Both Ras mutations and p53 mutations are common in mammalian neoplasms including hepatocellular carcinoma (Nigro et al., 1989; Hsu et al., 1991; Wiseman et al., 1991). It seems reasonable to assume that these kinds of mutations are also common in hepatocellular carcinoma of teleosts and are responsible for Pgp overexpression in mummichog liver tumors.

Summary— The results reported here show evidence for moderate increases in expression of one or more Pgp homologs in the livers of mummichog from a creosote contaminated site. In addition liver neoplasms of these fish show overexpression of Pgp which is similar to that seen in mammalian hepatocellular carcinoma. The mechanisms responsible for this up regulation of Pgp in these fish livers are unknown. Further work is required to determine if increased expression of Pgp can be induced in laboratory

exposures of these fish to components of creosote particularly individual PAH components. Although no increase in expression of Pgp in mummichog liver was observed following i.p. injection of 3-MC, these results must be viewed as inconclusive since longer times may be required for up regulation of these genes. In tumors high level expression beginning at the neoplasm stage rather than earlier suggests that oncogene activation rather than a role of Pgp expression in chemical resistance of preneoplastic lesions may be responsible for increased expression seen in these tumors.

In the case of both normal mummichog liver and mummichog liver tumors the question of which Pgp family members are increased remains to be answered. Because the antibody used in the present study recognizes an epitope found in all known Pgp deduced amino acid sequences, our expression results include all isoforms expressed in mummichog liver. Further understanding of the relevance of increased Pgp expression to the survival of mummichog in the creosote contaminated environment will require characterization of the Pgp genes expressed in mummichog liver and liver tumors and the development of gene specific probes for these genes. Chapters IV and V of this dissertation report the identification, partial sequence and molecular analysis of two distinct Pgp isoforms expressed in mummichog liver.

Figure 4. Immunoblot detection of Pgp in mummichog liver extracts from the creosote contaminated site (Atlantic Wood) in the Elizabeth River and from a clean reference site (King Creek). **A:** Immunoblot probed with mAb C219 (1.5 μg/ml). Lanes 1-5 Elizabeth River mummichog liver extracts. Lanes 6-10 King Creek mummichog extracts. **B:** Immunoblot probed with mAb JSB-1 (10 μg/ml). Lane 1 Pgp standard, multidrug-resistant $CH^{R}C5$ cell line extract. Lanes 1-5 King Creek mummichog liver extracts, Lanes 6-10 Elizabeth River mummichog liver extracts. Positions of molecular weight standards are shown at the left.

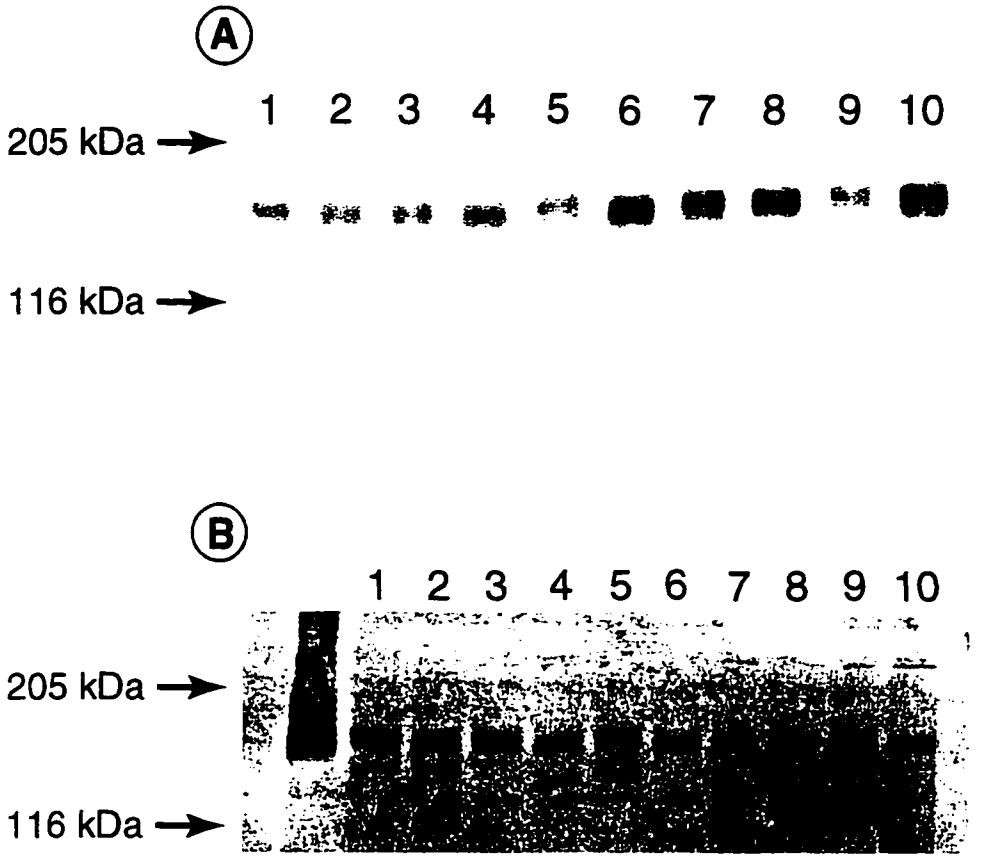
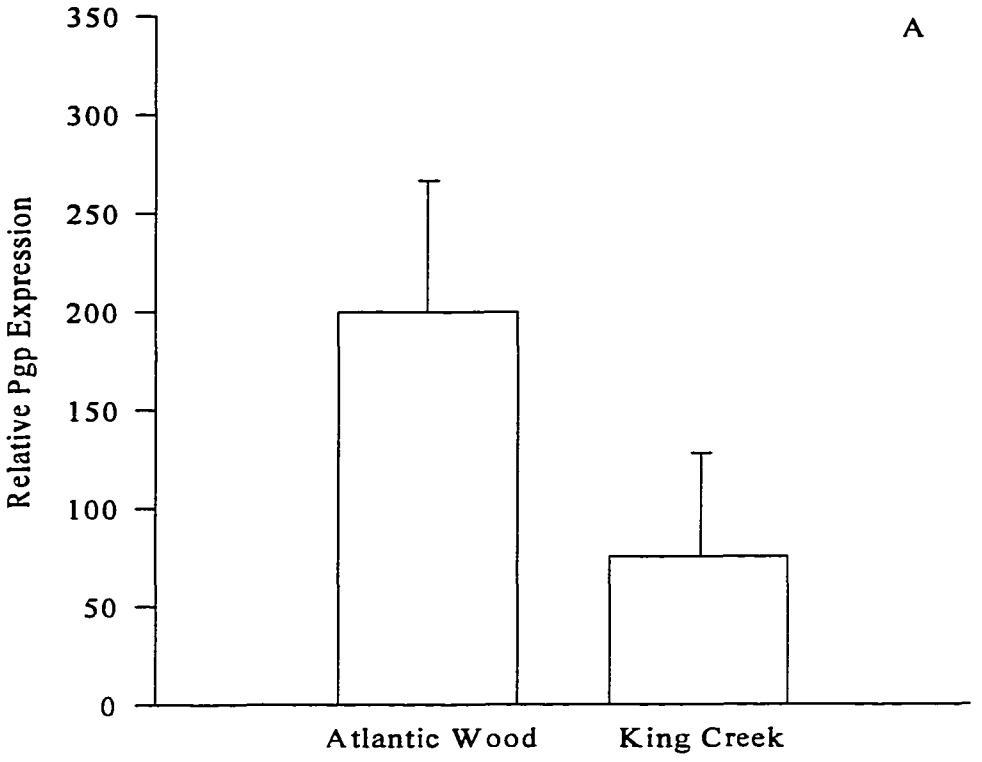


Figure 5. Expression of Pgp in liver extracts of mummichog from the creosote contaminated site (Atlantic Wood) and an uncontaminated reference site (King Creek) determined from immunoblotting of mummichog liver extracts using mAb C219. Five fish from each site were used. Expression is reported in arbitrary units per microgram of liver protein. Units are defined so that 1 μg of a pooled liver extract from reference site mummichog contains 100 units. **A:** Mummichog collected July 1996. **B:** Mummichog collected October 1994. At both times, Elizabeth River mummichog from the creosote contaminated site showed significant elevation of the C219 antigen (Student's t-test, $P<0.05$).



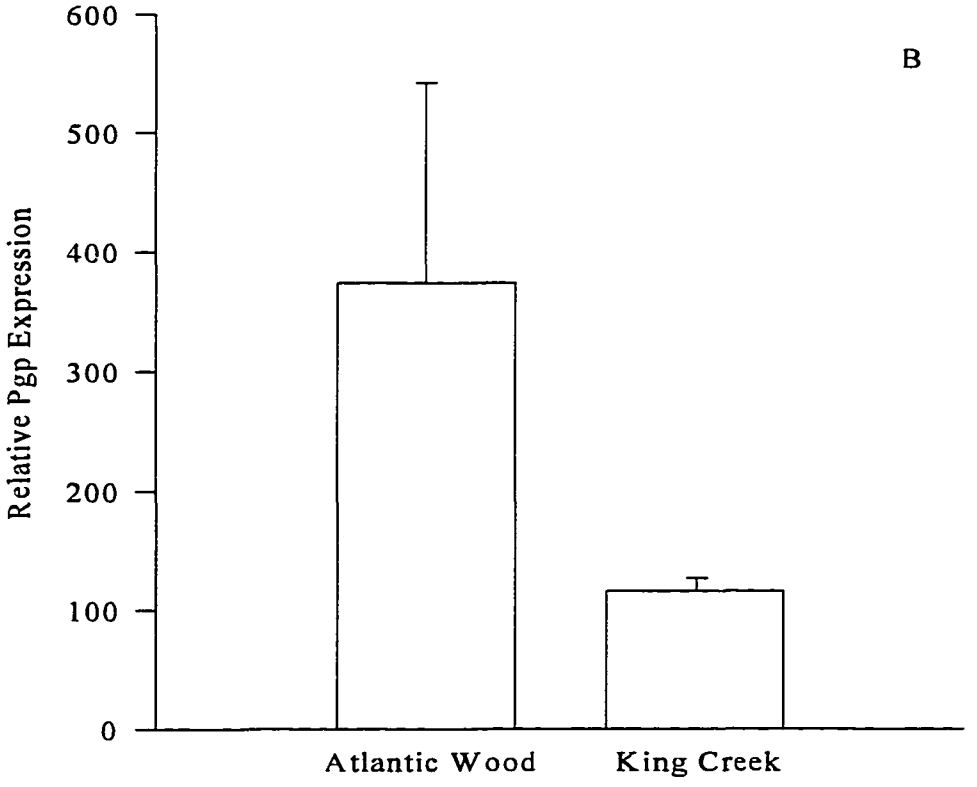


Table 2. Relative content of Pgp antigens in liver extracts of mummichog from the creosote contaminated site (Atlantic Wood) and an uncontaminated reference site (King Creek) determined by densitometric analysis of immunoblots using mAb C219.

	Site		
Date	Atlantic Wood	King Creek	Relative Increase
July 1996	374±167[a]	115±11	3.25
October 1994	199±66[a]	75±52	2.65

Values are arbitrary Pgp units per microgram of liver protein, average of 5 livers from each site ±SD; [a] significantly different from reference site (King Creek) (Student's t-test, $p<0.5$). Relative increase is the ratio of expression in livers of Atlantic Wood fish to expression in King Creek fish.

Figure 6. Immunoblot of liver extracts of mummichog (4 μg/lane) 36 hr following i.p. injections of 3-methylcholanthrene in corn oil (3-MC) or corn oil alone (CO). Upper blot was probed with anti P-glycoprotein antibody (mAb C219). The lower blot was probed with anti scup CYP1A (mAb 1-12-13).

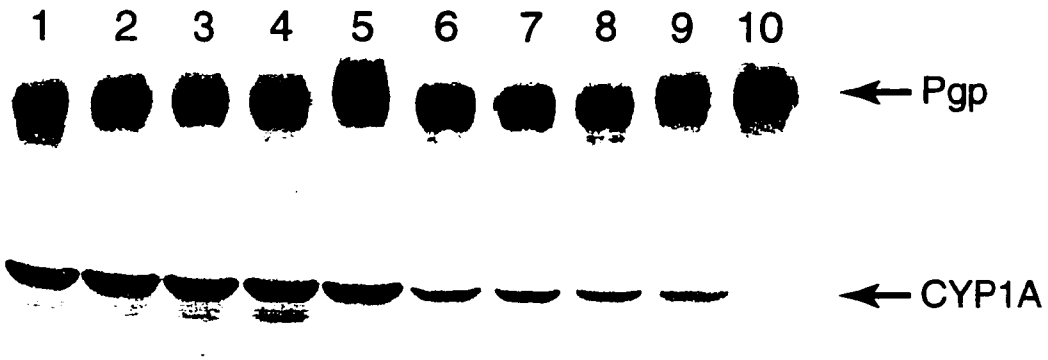
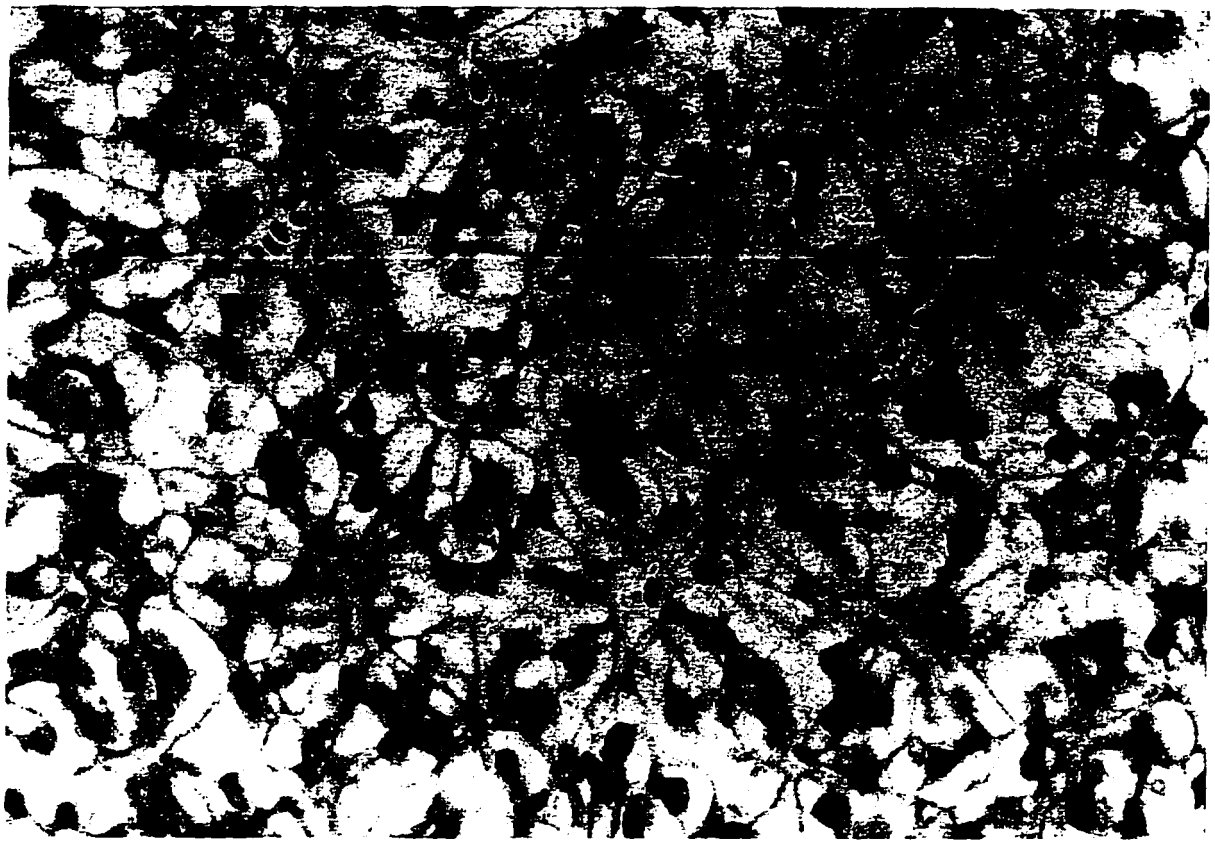


Figure 7. Sections of normal mummichog liver from King Creek. **Top panel:** C219 immunohistochemical staining. Positive immunoperoxidase stain is reddish brown and localizes to the bile canalicular surface of hepatocytes. **Bottom panel:** H&E stained section from the same liver.



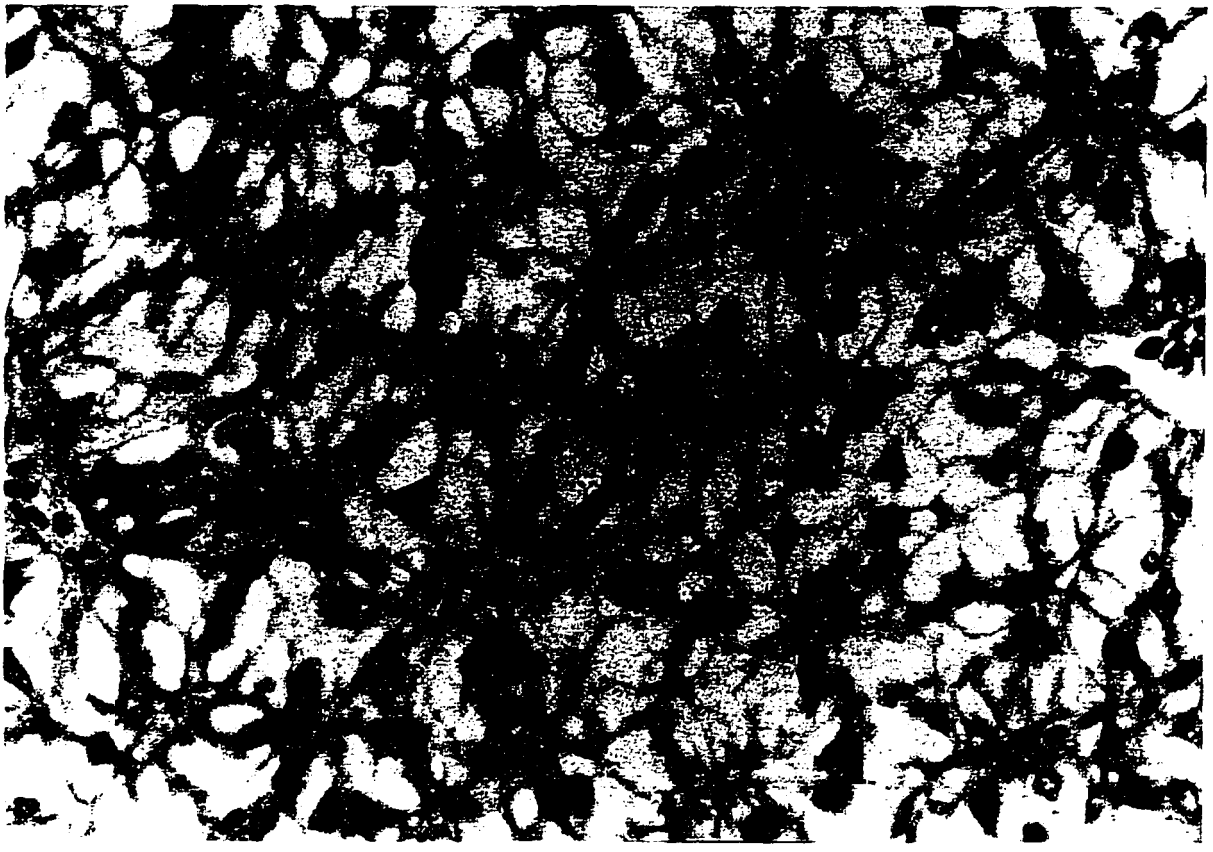


Figure 8. Demonstration of specific immunohistochemical staining with mAb C219. **Top panel:** Immunohistochemical staining using C219 of overexpressing hepatocellular carcinoma with connective tissue component. Note absence of immunoperoxidase stain in connective tissue component (central portion). **Bottom panel:** Immunohistochemical staining of adjacent serial section to the one shown in the top panel using matched isotype mouse myeloma protein (IgG2a, kappa) as primary antibody negative control.

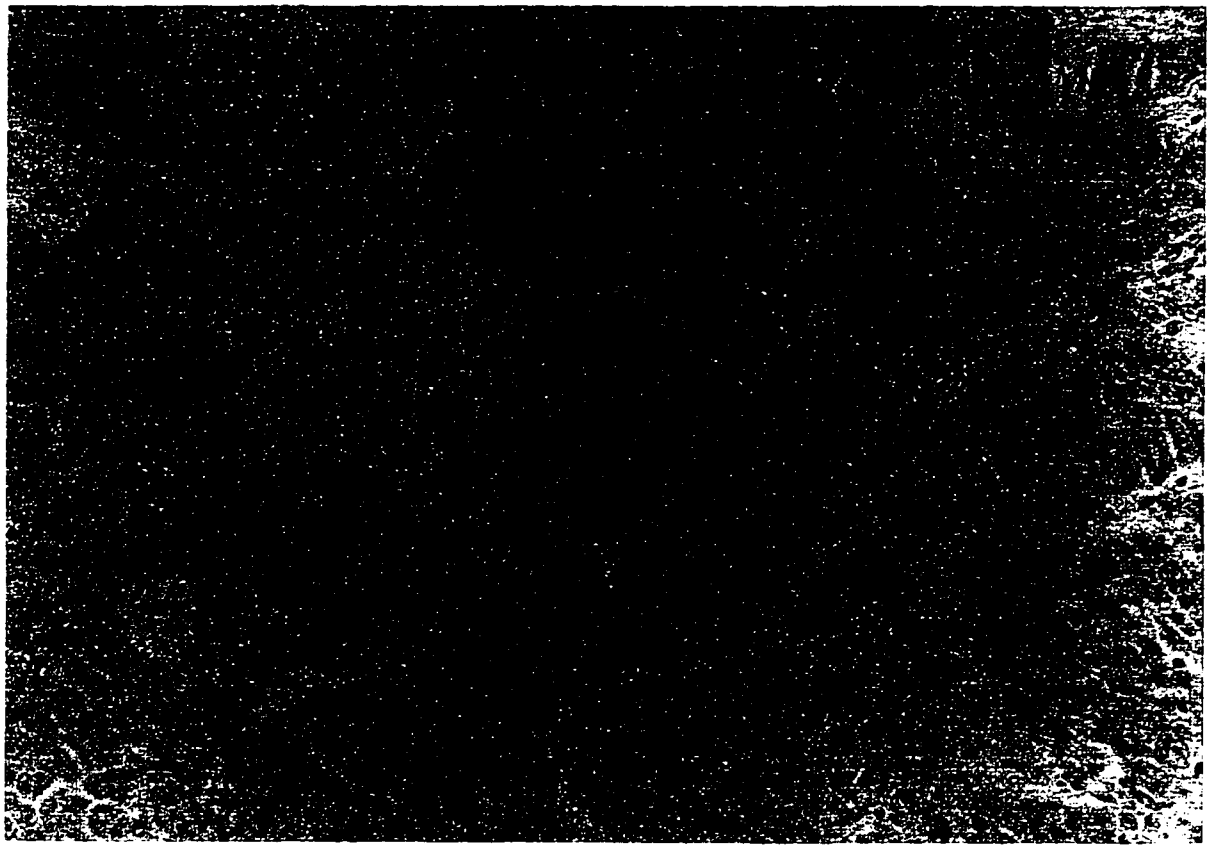
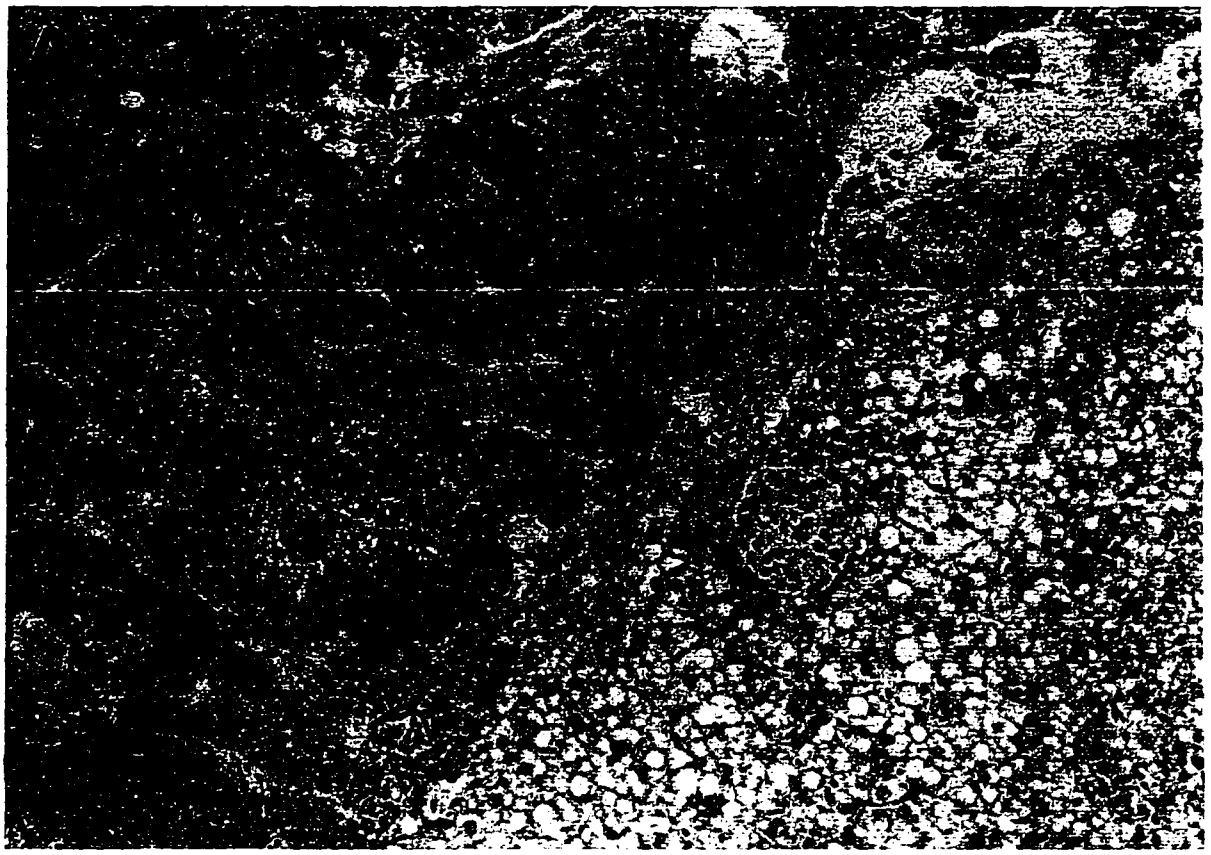
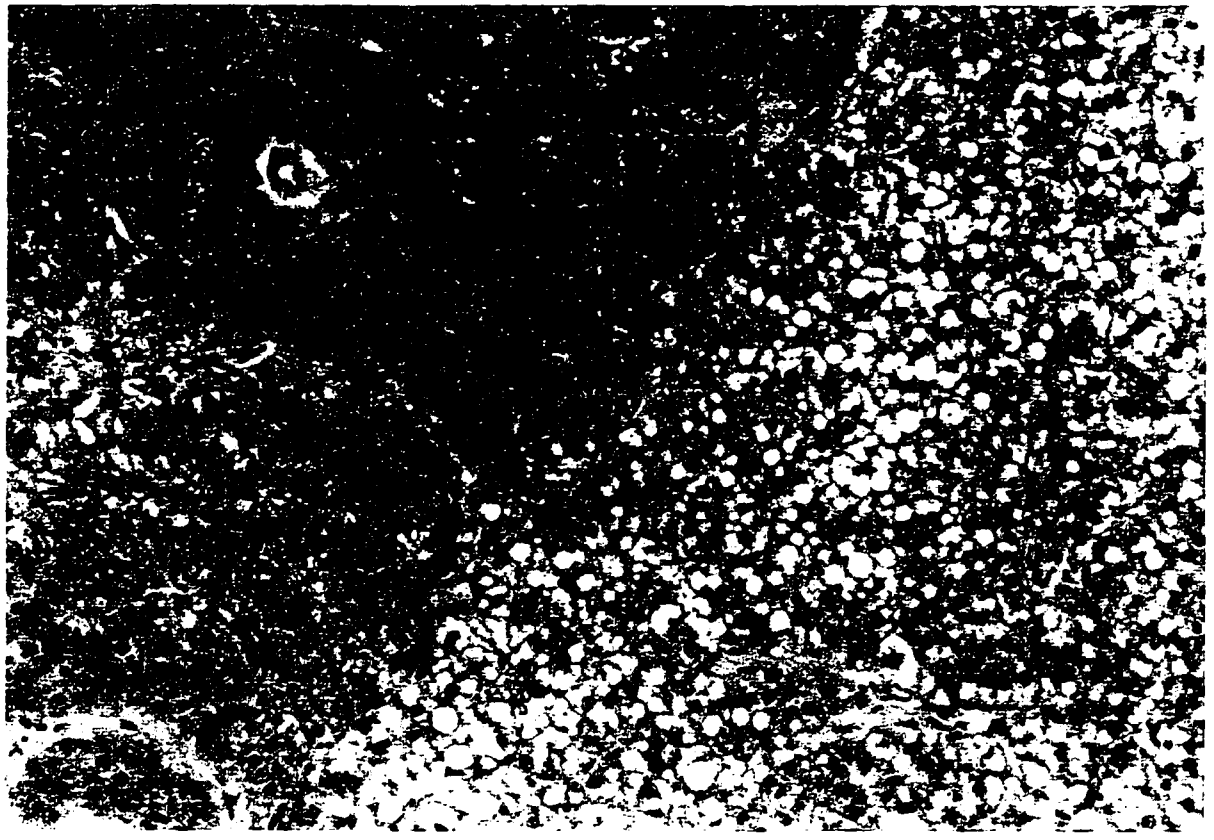
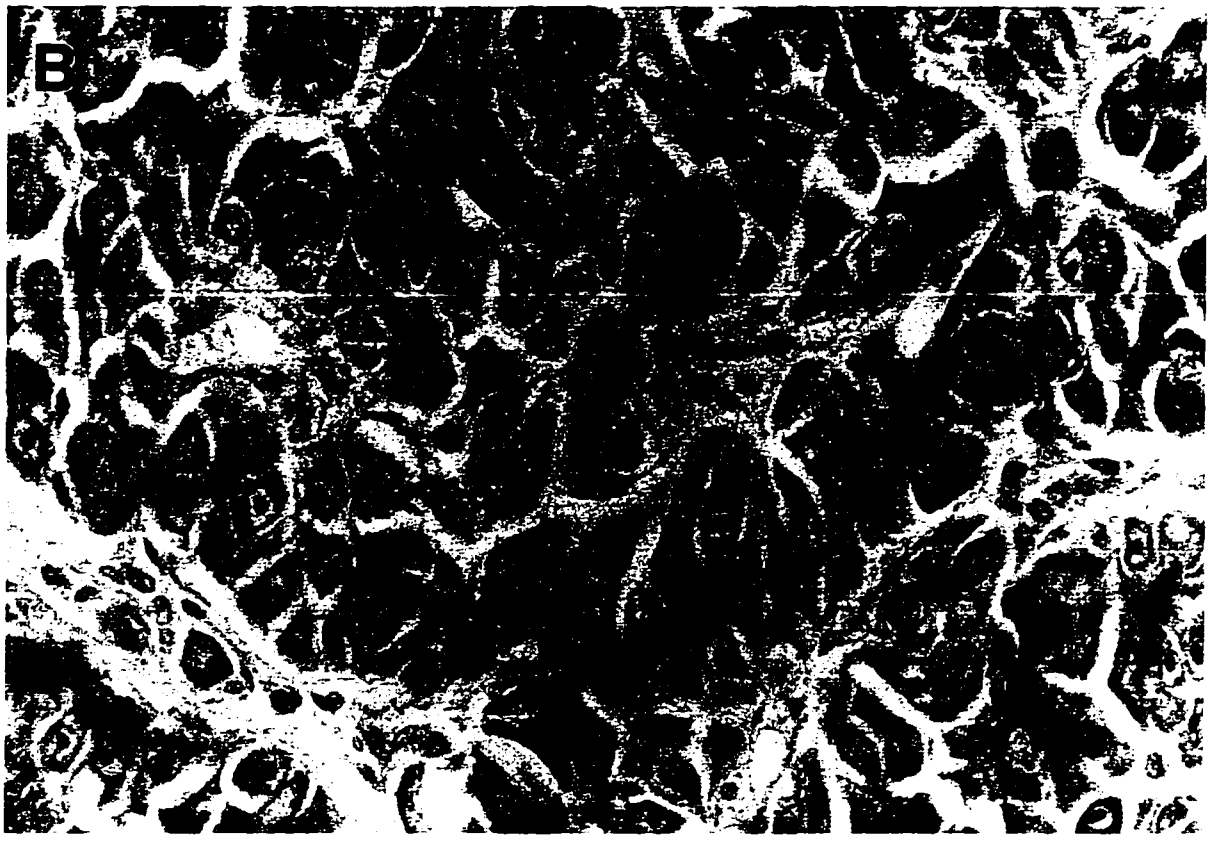
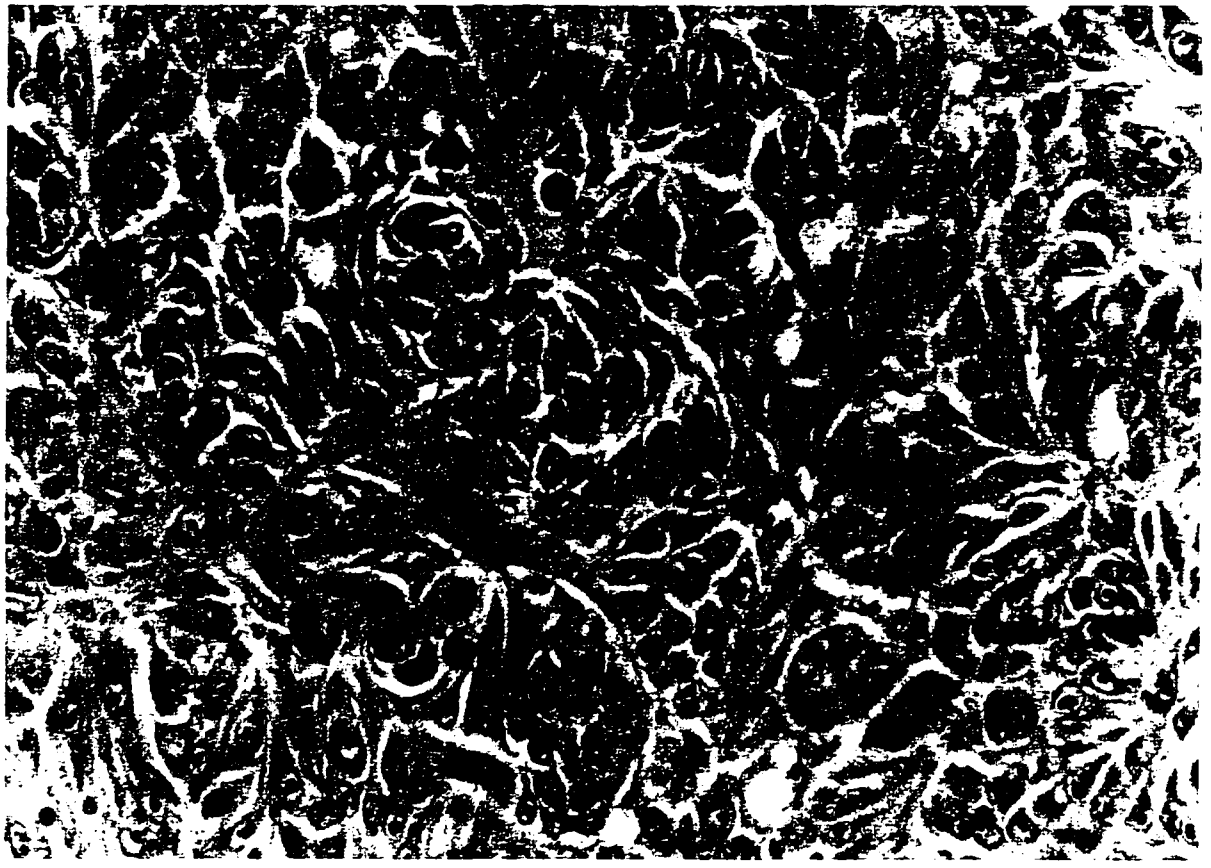


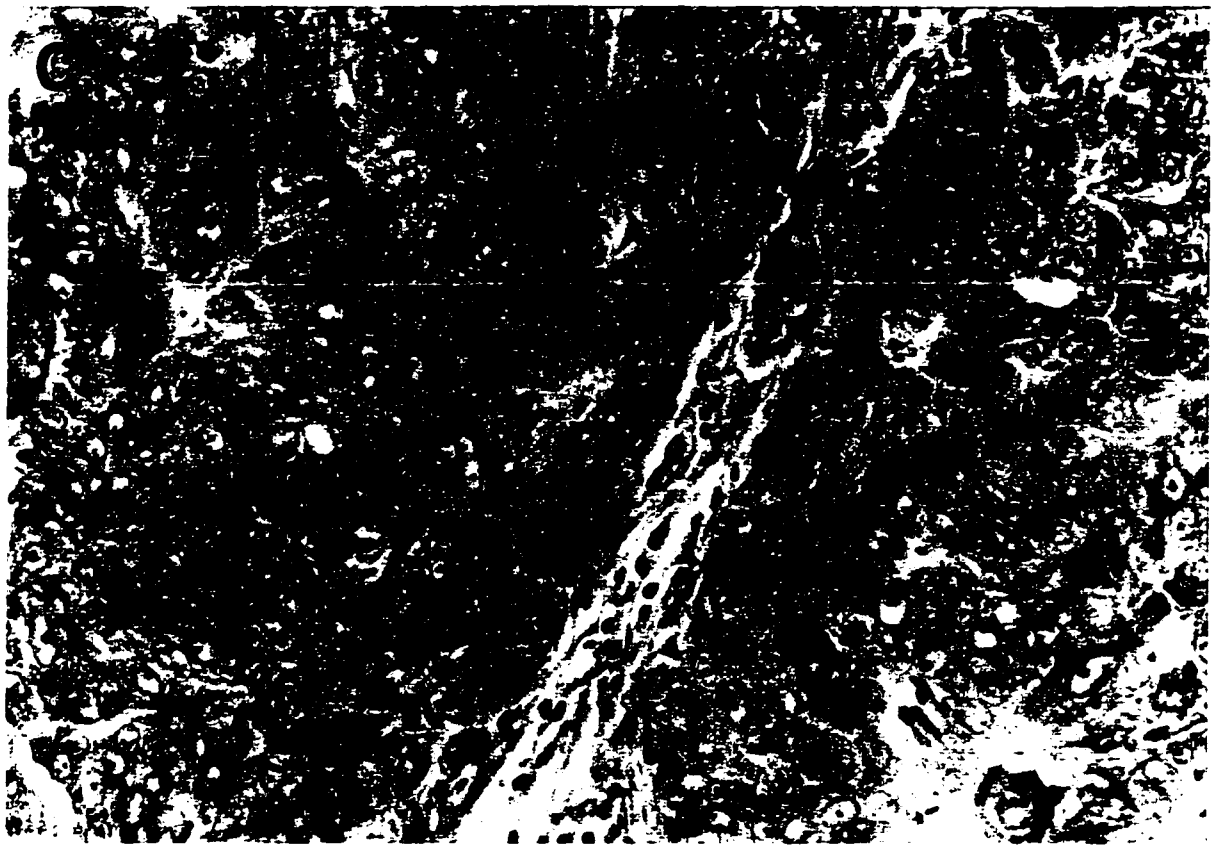
Figure 9. Neoplasms from Elizabeth River mummichog showing various types of C219 immunohistochemical staining. **Top panel:** Immunohistochemical staining with C219. **Bottom panel:** H&E staining. **A:** Hepatocellular adenoma showing overexpression and polar staining for Pgp. Note the well defined border between the over expressing neoplasm and adjacent nonneoplastic liver. **B:** Well differentiated hepatocellular carcinoma showing overexpression of Pgp and predominant polar staining. **C, D & E:** Hepatocellular carcinomas showing overexpression and loss of polar expression for Pgp including cytoplasmic staining (**C & D**) and expression on the entire plasma membrane (**E**). **F:** Hepatoblastoma with hepatocellular carcinoma component showing high level polar expression for Pgp within the hepatoblastoma (top panel, lower left). The hepatocellular carcinoma component shows overexpression and loss of polarity (top panel, extreme upper right). **G:** Hepatocellular carcinoma (lower left) with very large cells showing apparent reduction in staining for Pgp as well as paranuclear staining.

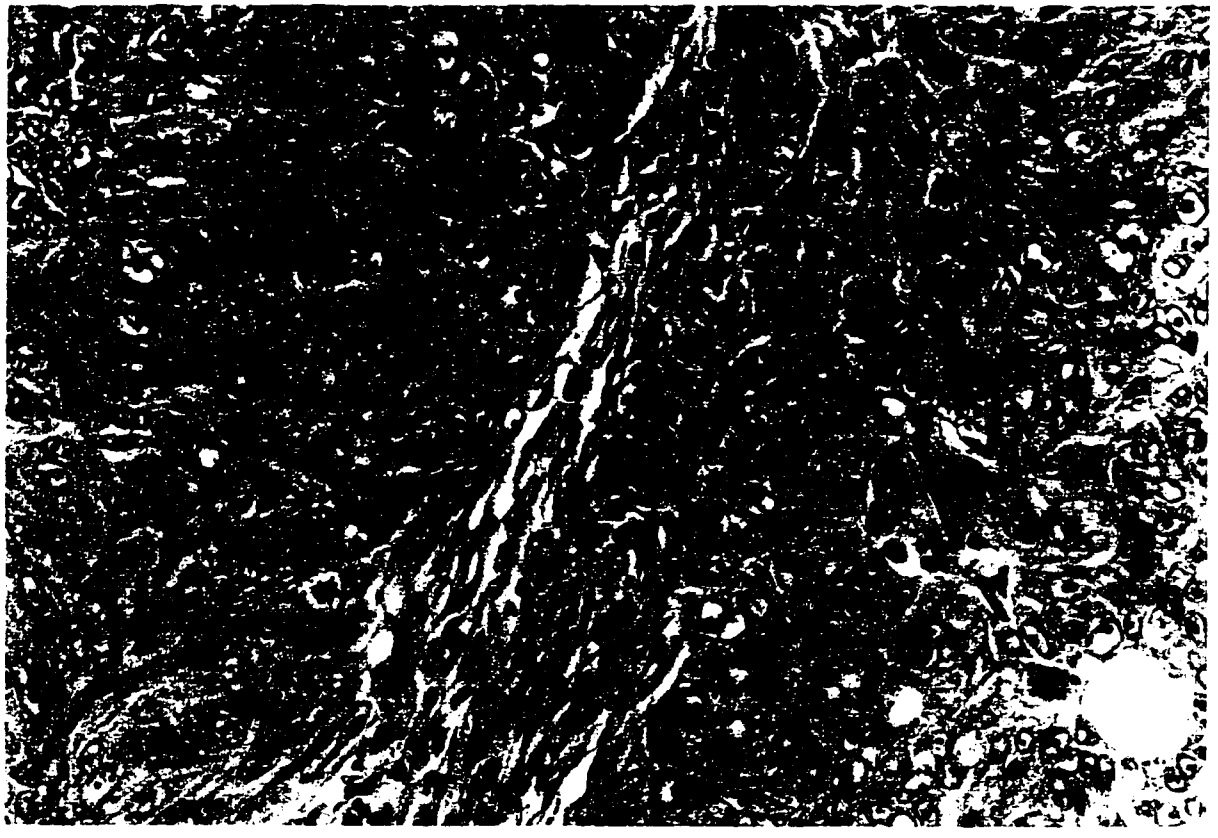


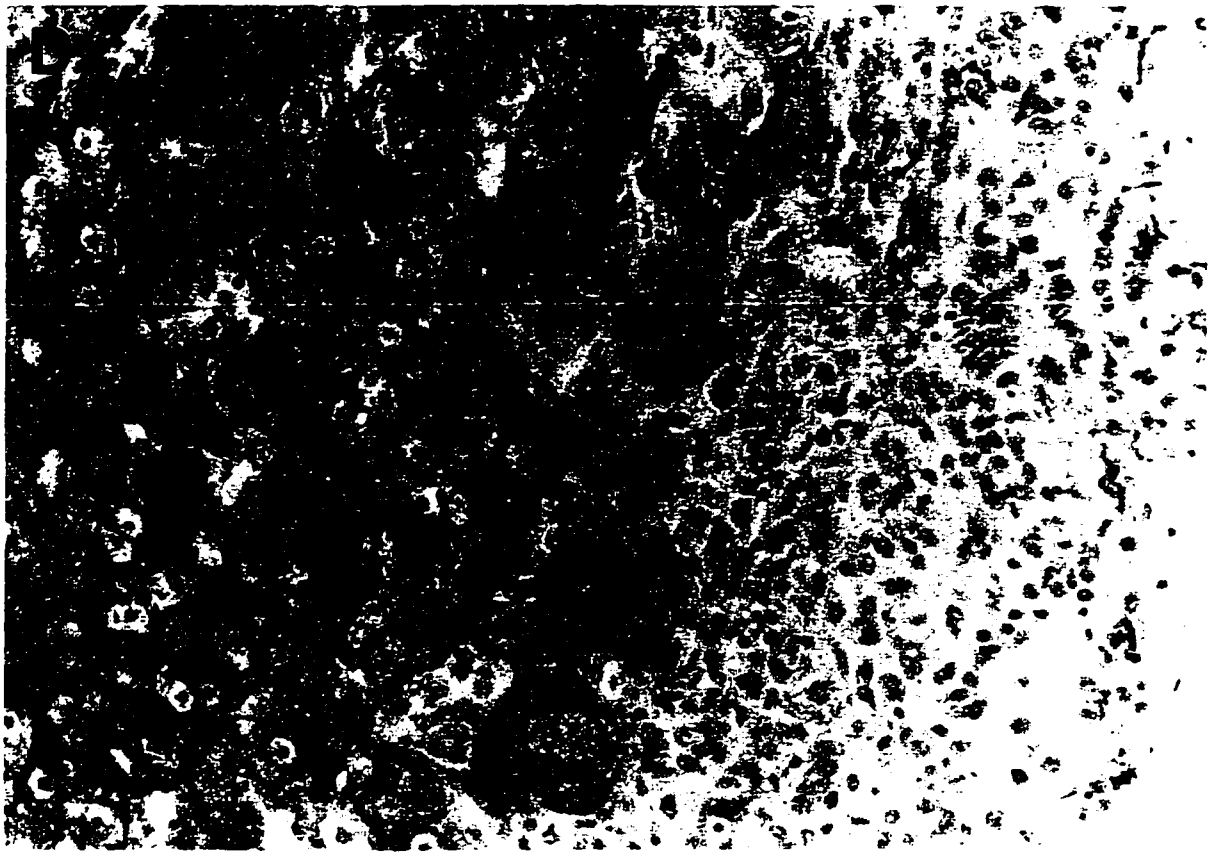


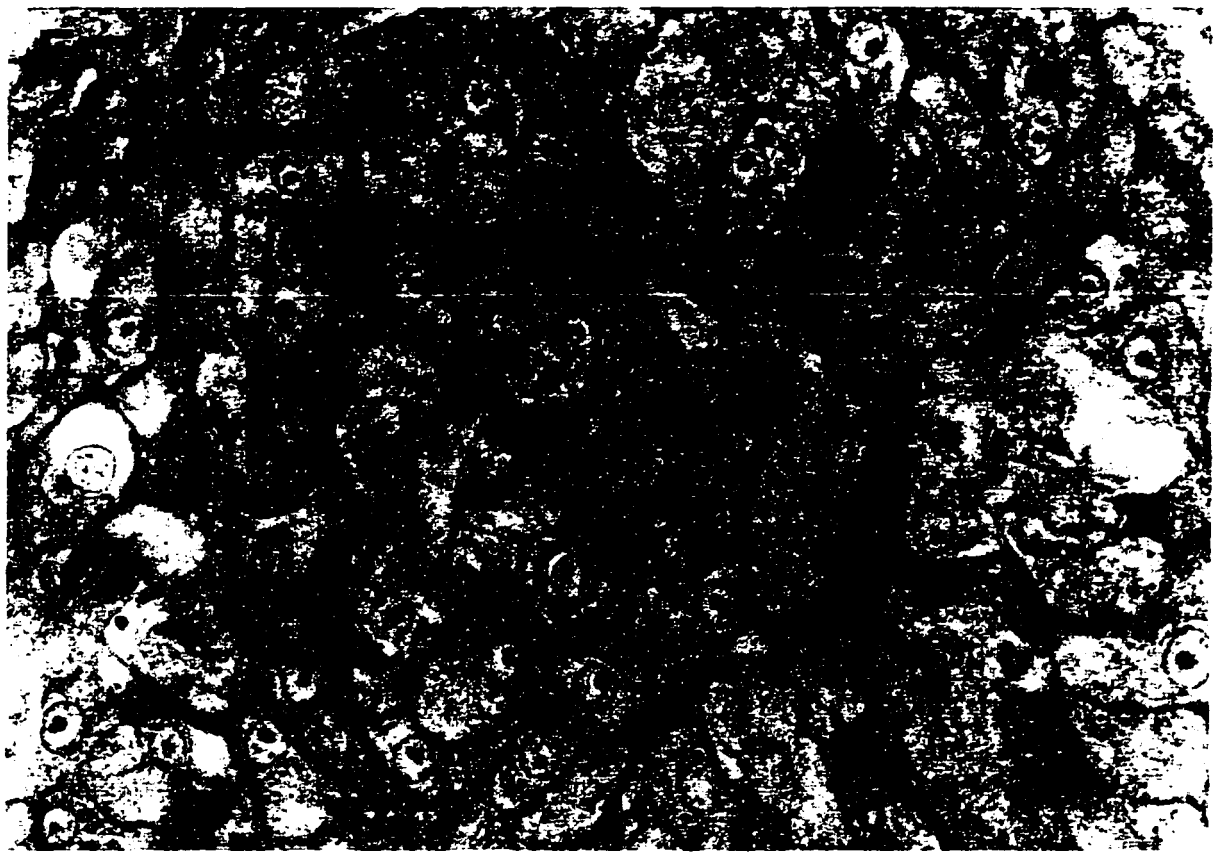


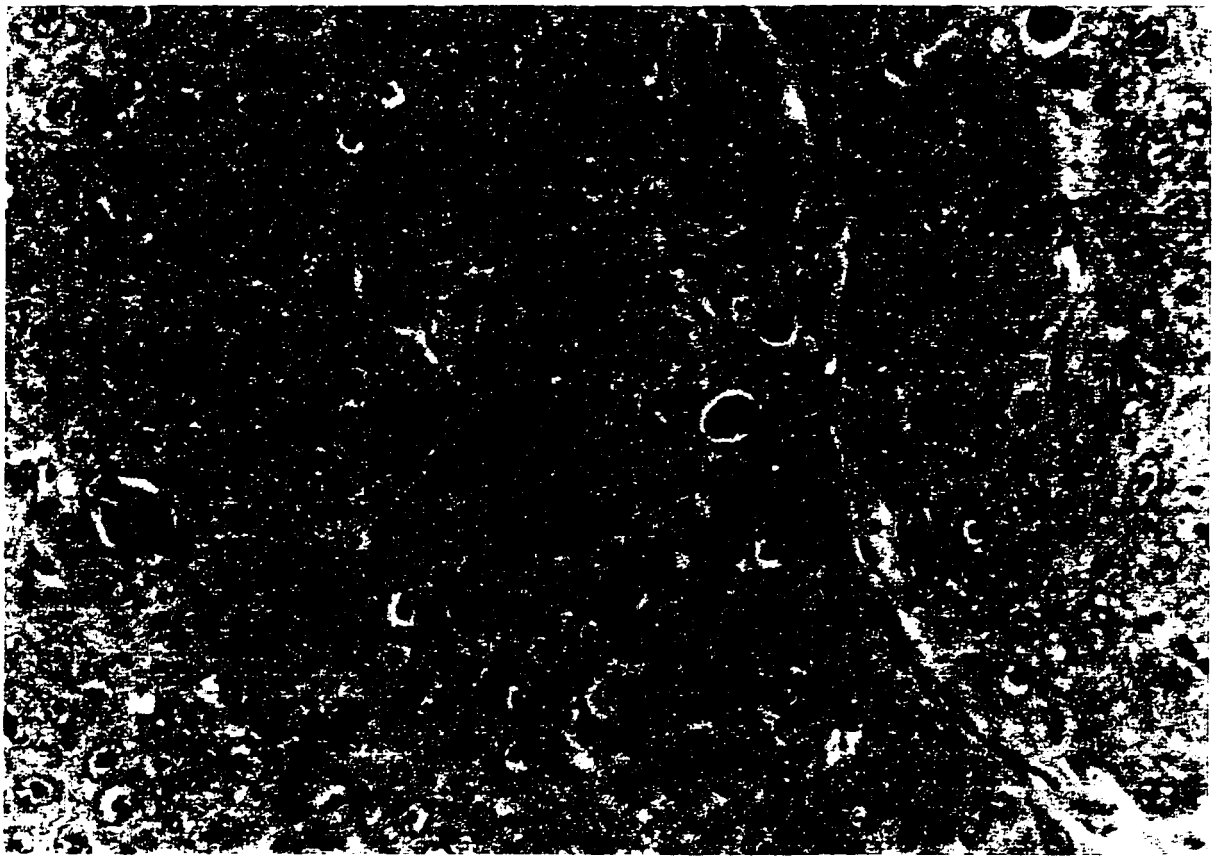


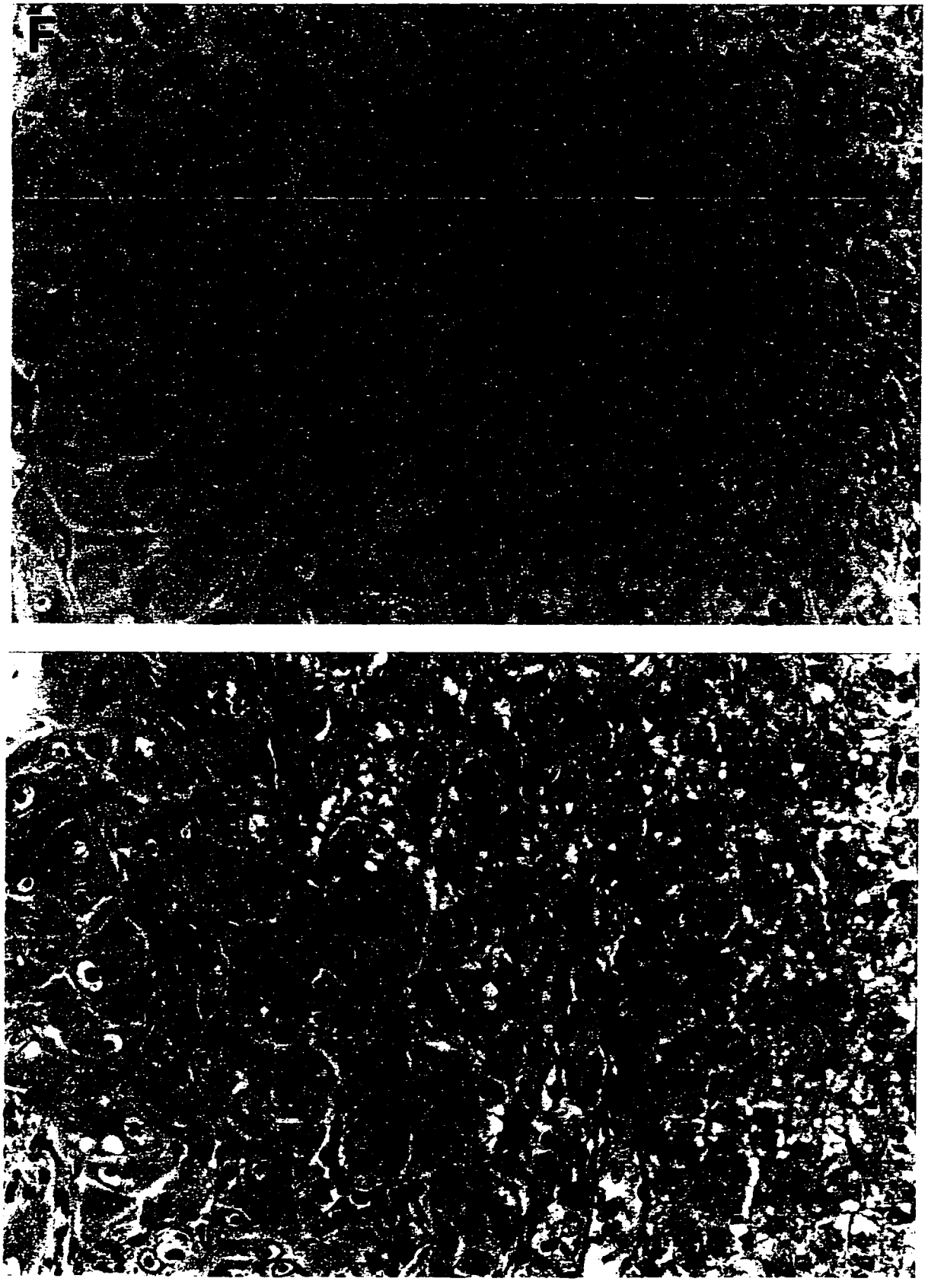


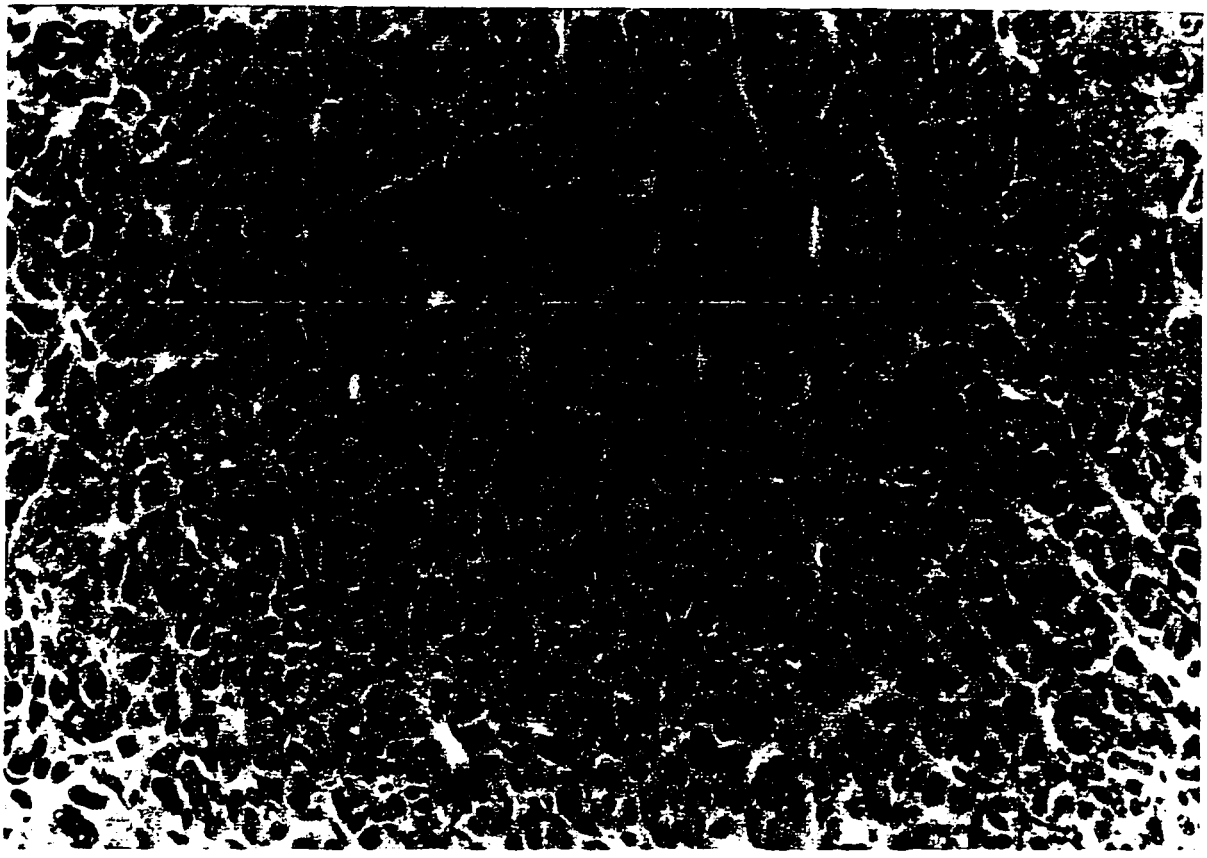


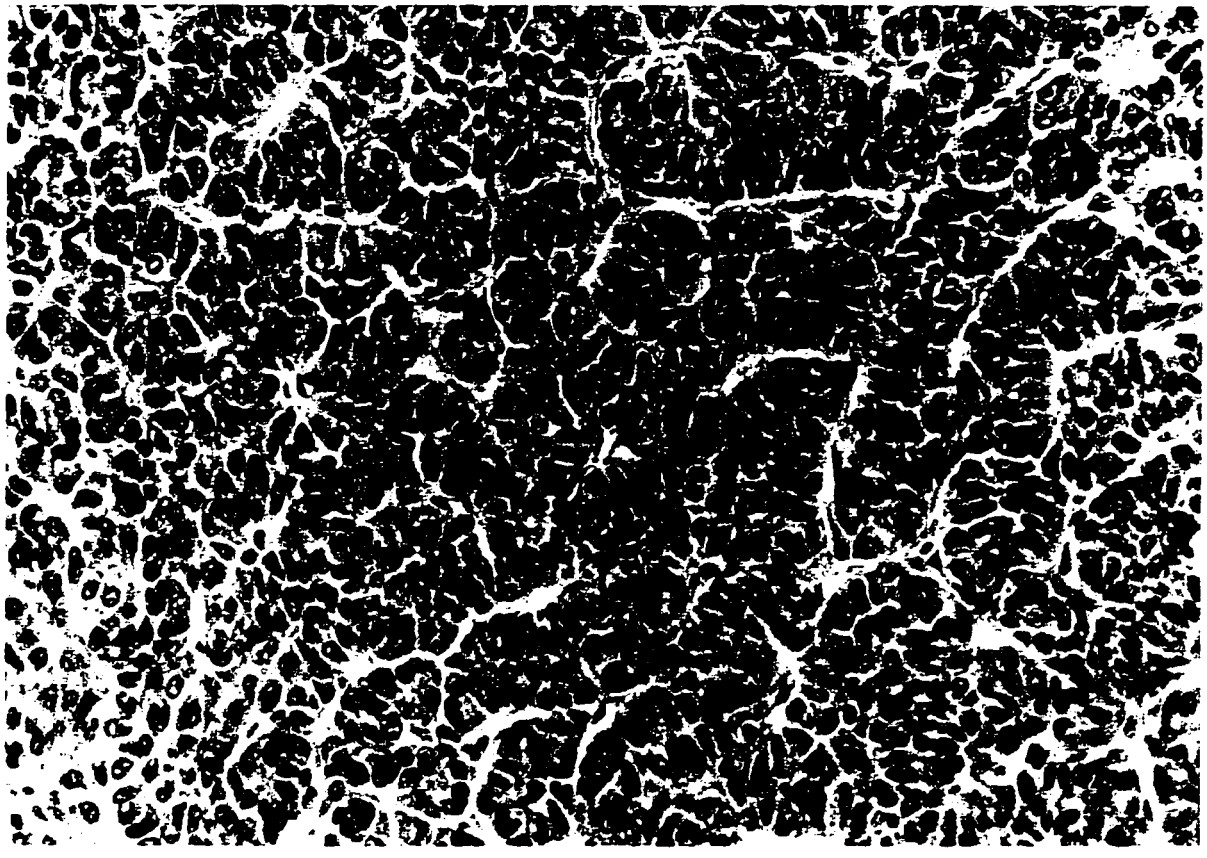












Chapter IV: Expression of a Sister Gene to P-glycoprotein (*spgp*) in the Liver of the mummichog (*Fundulus heteroclitus*)

INTRODUCTION

In Chapters II (Cooper et al., 1996) and III of this dissertation we have used immunohistochemical and immunoblot techniques to examine Pgp expression during environmental hepatocarcinogenesis in mummichog from the creosote-contaminated Atlantic Wood site. The results reported there indicate that a protein or proteins related to P-glycoprotein is elevated in non-tumor-bearing liver of AW mummichog. The elevation in normal liver may be a result of environmental induction by components of creosote or other reversible changes. However it may also be a component of the genetic resistance of these fish. Similar elevation of Pgp-like activity or antigens have been reported in pollution-tolerant mollusks from contaminated sites, and it has been suggested that increased levels of Pgp activity may play a role in the survival of these organisms in contaminated habitats (Minier et al., 1993; Kurelec et al., 1995; Kurelec et al., 1996). We also found that Pgp was overexpressed and had altered patterns of expression in the majority of the progressed environmentally induced liver tumors of the AW mummichog. These results are similar to those reported for experimentally induced rodent liver neoplasms (Teeter et al., 1990; Bradley et al., 1992). The association of increased expression of specific Pgp isoforms (mdr1-type) with xenobiotic resistance in mammalian cell lines suggests that this increased expression of Pgp may contribute to the survival of these fish in the highly contaminated environment.

However, the specific forms of Pgp expressed in the livers and liver tumors of these fish are unknown. The antibody (mAb C219) used in the studies reported in Chapters II and III of this dissertation binds a highly conserved epitope found in the

deduced amino acid sequences of all known Pgp gene family members (Kartner et al., 1983; Georges et al., 1990). Thus multiple Pgp homologs may contribute to the signal seen in immunoblots and immunohistochemical staining of normal mummichog liver and liver tumors. Southern blots of genomic DNA of several fish species indicate that fish have at least two Pgp genes (Ling et al., 1992; Chan et al., 1992). As is the case with mammalian mdr and spgp genes, the teleost homologs of these genes are probably regulated differently from each other, and their gene products have distinct functions. Further understanding of the significance of expression of Pgp in mummichog from the creosote contaminated environment will require characterization of the Pgp gene sequences expressed in mummichog liver and liver tumors.

In the present Chapter we have used reverse transcriptase-polymerase chain reaction (RT-PCR) to amplify Pgp sequences expressed in mummichog liver. Our goals were to amplify, clone and sequence Pgp related sequences expressed mummichog liver, to use these sequences as probes to investigate tissue specific expression of the mummichog gene in northern blots and to relate these sequences to other vertebrate Pgps of known function. These studies are necessary background to investigating the nature of Pgp sequences which are elevated in mummichog from contaminated environments.

We report that we have amplified and cloned overlapping cDNA fragments that are most closely related to the winter flounder *pgpA* gene and the rat and pig spgp P-glycoproteins. This Chapter presents a comparison of the sequences from mummichog liver to that of other vertebrate spgp and Pgp cDNA sequences. Evidence presented here supports the spgp gene category as a distinct and highly conserved branch of the Pgp

multigene family. In the mummichog, as in mammals spgp is most strongly expressed in liver. To our knowledge, this represents the first cDNA sequence of a Pgp homolog from a teleost and is the first report on tissue specific expression of a Pgp gene in a fish.

MATERIALS AND METHODS

Fish collection and tissue preparation— Mummichog were collected in a minnow trap from King Creek a tributary of the Severn River in the Gloucester County Virginia. Fish were sacrificed within 48 hrs of capture by overdose with tricaine methane sulfonate (Sigma). Livers, anterior kidney, brain and intestine were excised and either processed immediately or snap frozen in liquid nitrogen and stored at -70° C until use.

RNA isolation— All aqueous solutions used with RNA were either treated with dethylpyrocarbonate (DEPC) (Sigma, St. Louis, Missouri) or were made with DEPC-treated water (DEPC H_2O) to inhibit any contaminating exogenous ribonucleases (Farrell, 1993).

Total RNA was prepared by a modification method of Chomczynski and Sacchi (1987) using the commercial preparation Tri-Reagent (Chomczynski, 1993) according to the manufacturers instructions (Molecular Research Center, Inc., Cincinnati, Ohio). Frozen organs were quickly broken into small pieces with a hammer and added to 10 volumes of ice cold Tri-Reagent in a Potter-Elvjehm Teflon-glass homogenizer. Homogenization was accomplished using an electric drill motor to drive the Teflon pestle. Seven or more up and down strokes at full speed were used to ensure complete disruption of tissues. Phase separation was achieved with chloroform as described in the

Tri-Reagent protocol. The overlying aqueous phase was collected and total RNA was precipitated from it with isopropanol. The precipitated RNA was colected by centrifugation and washed with 75% ethanol. The pellet was then resuspended in 0.5% SDS, and the concentration of total RNA was estimated by measuring the absorbance of the solution at 260 nm (Farrell, 1993). RNA was then precipitated with sodium acetate and ethanol. Precipitated RNA was stored as a suspension at -80° C until use. The integrity and yield of total RNA was confirmed by agarose/formaldehyde gel electrophoresis using 0.66M formaldehyde concentration in both the gel and the sample buffer (Farrell, 1993). Ethidium bromide was included in the sample buffer so that the RNA bands could be visualized without staining and destaining the entire gel.

A polyA+ enriched RNA fraction was prepared from mummichog liver total RNA using oligo-(dT) cellulose columns (Molecular Research Center) according to standard protocols (Sambrook et al., 1989). The frozen suspension of total RNA was thawed on ice and an aliquot containing between 1 and 1.5 mg of RNA was removed, pelleted in a microcentrifuge, and washed with 75% ethanol. The pellet was then dissolved in 1 ml of binding buffer (0.5 M LiCl, 50 mM disodium citrate, 0.1% SDS) and heated to 70° C for 5 minutes. After cooling on ice for 5 minutes, the entire solution was applied to the equilibrated column. The flow through fraction was collected in a tube on ice and applied onto the column again. The column was then washed with four 0.5 ml portions of binding buffer. A poly A enriched fraction was eluted as a single fraction with 0.6 ml of elution buffer (1mM disodium citrate, 0.1% SDS). This fraction was collected in a pre-chilled microcentrifuge tube on ice. Yield of the poly A+ enriched RNA was estimated

by measuring the absorbance of the solution at 260 nm. This poly A+ fraction was then precipitated with 0.1 volume of sodium acetate, 5 µl of Microcarrier gel (Molecular Research Center) and 2 volumes of ethanol. Precipitated RNA was stored at -80° C until use.

First strand cDNA synthesis— First strand cDNA was synthesized using poly A+ liver RNA and recombinant Moloney murine leukemia virus-reverse transcriptase (MoMLV) (SuperscriptII™ reverse transcriptase, Life Technologies, Bethesda, Maryland). Reverse transcription was primed with either random hexamers (Life Technologies, Bethesda, Maryland) when the resulting cDNA was to be used for amplifying internal segments or an oligo-$(dT)_{17}$ adapter primer (Table 3, custom synthesis, Life Technologies) when amplification of the 3' end of the cDNA was sought (Frohman, 1988). (See the description of 3' RACE given below under *Amplification of the 3' end (S2) of the mummichog spgp cDNA*.) An aliquot of suspended poly A+ RNA (1 µg) was pelleted, washed with 75% ethanol and dried in a SpeedVac (Savant, Farmingdale, New York). The pellet was dissolved in 17.5 µl of DEPC treated water (DEPC H_2O) containing either 125 ng of random hexamers or 500 ng of the oligo-$(dT)_{17}$ adapter primer (Table 3). This primer-RNA mixture was heated to 70° C for five minutes, chilled on ice and used immediately in the first strand synthesis reaction. All first strand reactions took place in 50 µl 1X reverse transcriptase buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3mM $MgCl_2$) containing dithiothreitol (10mM), Rnasin (1 unit/µl) (Promega, Madison, Wisconsin) and the four deoxynucleotide triphosphates (dNTPs, 500 µM each, Life Technologies). Reverse transcriptase (MoMLV, 300 units)

was added to start the reaction which was incubated for 10 minutes at room temperature and then placed in a 42° C water bath for 1 hour. First strand cDNA pool was precipitated with sodium acetate /ethanol and stored overnight at -20° C. On the following day, the cDNA pool was pelleted in a microcentrifuge, washed with 75% ethanol and dissolved in 100 µl of DEPC H_2O. The cDNA pool was then divided into 10 µl aliquots and stored in 0.2 ml PCR tubes at -20° C until use.

General conditions for amplification of first strand cDNA— The polymerase chain reaction (PCR) was used to amplify specific regions of the mummichog spgp first strand cDNA. Oligonucleotide primers were synthesized by Genosys (The Woodlands, Texas) and Life Technologies Inc. (Bethesda, Maryland). *Taq* DNA polymerase was from Boehringer-Mannheim (Indianapolis, Indiana). Sequences of the primers as well as the regions amplified are given in Table 3. Amplifications were performed on Biometra UNO-Thermoblock™ thermocycler (Biometra, Tampa, Florida). All reactions took place in 100 µl 1X PCR buffer (10mM Tris-HCl, pH 8.3, 50 mM KCl) containing $MgCl_2$ (1.5 mM) and the four dNTPs (200 µM each). Primer concentrations were usually 1 µM except where noted for specific amplifications. For each amplification 10 µl of either the random primed or oligo-$(dT)_{17}$ adapter primed cDNA pool was used. *Taq* polymerase (2.5 units) was added to each reaction only after the mixture was denatured for 5 min at 94° C and cooled to 72° C in the thermocycler.

For most amplifications the technique of touchdown PCR (Don et al., 1991) was used. In this technique the annealing temperature during the thermal cycling protocol was initially set higher than the expected annealing temperature of the primers. At each

cycle, the annealing temperature was lowered incrementally through the expected annealing temperature. Several cycles of amplification were then performed using the lowest annealing temperature. This protocol should give a selective advantage to products resulting from primer template pairs with the fewest mismatches (highest Tm). Touchdown PCR has proven useful in reducing the number of nonspecific products in situations where degenerate primers have been used (Don et al., 1991; Roux et al., 1994) as is the case in the present study. The relative sizes of the PCR products were determined by agarose gel electrophoresis. Details of the specific amplification conditions for each fragment (S1, S2, S3 and S4) are given at the end of this section..

Cloning and sequencing of PCR products— PCR products were cloned using the Prime PCR Cloner Kit (5prime- 3prime Inc, Boulder, Colorado). This kit incorporates a column clean-up step of the amplification reaction prior to modification and ligation of the PCR products. For all amplified fragments greater than 1 kb in size a gel purification step was substituted for the for the column clean-up step in the kit protocol. The gel purification step was necessary because of the presence of multiple nonspecific bands in many of the amplification reactions. This step also increased the yield of recombinant colonies per cloning reaction because it concentrated the DNA. The entire amplification reaction (100 µl) was separated by electrophoresis on a 1% agarose gel. Following electrophoresis and staining, the band of interest was cut from the gel. The DNA was separated from the gel matrix using the GeneClean kit (Bio 101, Vista, California). The DNA was eluted from the resulting glass-milk suspension in 15 µl of H_2O. The entire volume was then used in the PCR Cloner protocol according to the

manufacturers instructions. Transformants were selected on Luria Bertani (LB) agar plates containing ampicillin (100 μg/ml), and recombinant colonies were selected by blue/white screening on LB agar plates containing isopropyl β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (Sambrook et al., 1989). Colonies were screened for insert size by agarose gel electrophoresis. Single colonies with the expected insert were grown overnight in LB broth containing ampicillin (100 μg/ml). Plasmids were isolated and prepared for sequencing using the standard alkali lysis protocol (Sambrook et al., 1989).

The nucleotide sequence of the cloned inserts was determined by routine dideoxy chain termination protocol using the Sequenase kit (United States Biochemical, Cleveland, Ohio) following the manufacturers recommendations for sequencing double-stranded templates. α Thio [^{35}S]dATP was used as an internal label. Labeled products were resolved on a 6% acrylamide / 6M urea gel buffered with TBE (Sambrook et al., 1989).

Specific Amplification conditions for spgp cDNA fragments—

1. Amplification of the segment (S1) containing the 3' ATP binding region of mummichog spgp— Degenerate oligonucleotide primers were used to amplify the distal (3')ATP binding region of the mummichog spgp transcript. These primers were designed to match the codons of vertebrate Pgp homologs in the vicinity of the downstream Walker A and B nucleotide binding motifs. The nucleotide inosine was used in the primer sequences in the third position of codons with four base degeneracy to limit the number of different primer molecules (Knoth et al., 1988). The sense strand primer

(Primer A, Table 3) codes for the amino acid sequence Val-Gly-Ser-Ser-Gly-Cys-Gly-Lys (VGSSGCGK) which corresponds to positions 1066-1073 of the hamster Pgp1 amino acid sequence (Endicott et al., 1991). The antisense strand primer (Primer B, Table 3) corresponds to the peptide Ala-Thr-Ser-Ala-Leu-Asp (ATSALD), positions 1199-1204 of the hamster Pgp1 sequence (Endicott et al., 1991). These amino acid positions are identical in all published vertebrate P-glycoprotein deduced amino acid sequences.

The predicted 417 bp fragment, S1, was amplifed from random primed cDNA using primers A and B (4 μM each) and a touchdown cycling protocol. Following denaturation at 94° C, the primers were annealed for 2 min initially at 62° C. The products were then extended at 72° C for 1.5 min followed by denaturation at 94° C for 30 sec. Fifty cycles were carried out with an incremental decrease of 0.4° C in the annealing temperature at each cycle. An additional ten cycles were carried out with the annealing temperature fixed at 42° C. Products were then given a final extension at 72° C for 10 min. The product was cloned and sequenced as described above. The sequence was used to generate a gene specific primer (gsp1) which was used to amplify the 3' end of the mummichog *spgp* cDNA. (See *3* below). Additional gene specific primers were designed which were used in conjuction with another degenerate primer (primer C) to amplify the segment between the two ATP binding regions as described in *2* below.

2. Amplification of the segments (S3 and S4) spanning the second transmembrane domain and the linker domain of mummichog spgp cDNA— Primer C (Table 3) was designed to match the consensus sequence of the upstream ATP binding region of

vertebrate Pgps. This primer codes for the peptide Gly-Gln-Lys-Gln-Arg-Ile-Ala (GQKQRIA) corresponding to positions 533-539 of the hamster Pgp1 deduced amino acid sequence (Endicott et al., 1991). These positions are identical in all published vertebrate Pgp deduced amino acid sequences. Primer C was used initially with a gene specific primer (gsp2, Table 3) designed from the downstream ATP binding region of the mummichog S1 sequence. This primer pair was expected to amplify a segment spanning the two predicted ATP binding regions. These were used in a touch down cycling program with random primed mummichog liver cDNA pool. The cycling program was identical to that used for amplification of the S1 fragment except that the extension time was increased to 3 minutes. Under these conditions a 1.3 kb fragment, S3, was amplified. The sequence of this fragment matched the downstream portion of the expected product. However the gsp2 primer sequence was present on both ends of the amplified fragment. A pair of overlapping gene specific primers (gsp3 and gsp4) were then designed from the sequence of S3 to be used in conjunction with primer C. Gsp3 and gsp4 were used in successive rounds of amplification. In the first round primer C and gsp3 were used in a touchdown cycling protocol. The annealing temperature was decreased from 60° C to 50° C in 50 cycles. Annealing was for 1 min and extension for 2.5 min. The annealing temperature was maintained at 50° C for an additional ten cycles. All other cycling parameters were the same as described above. A fragment of expected size (1.2 kb) was amplified in low yield. This product was gel purified, and the DNA was extracted using the GeneClean kit. A 1 μl aliquot of the eluted DNA was subjected to a second round of amplification using primer C and gsp4. Thirty cycles were performed with the annealing

temperature fixed at 50° C. A 1.2 kb fragment (S4, Table 1) was obtained in good yield.

3. Amplification of the 3' end (S2) of the mummichog spgp cDNA— The 3' end of the mummichog liver spgp cDNA was amplified using the method of rapid amplification of cDNA ends (3' RACE) (Frohman, 1988). This method uses an oligo $(dT)_{17}$ primer with an adapter sequence (Table 1) at its 5' end to prime first strand cDNA synthesis. This primer initiates cDNA synthesis at the complementary poly A tail of the mRNA. It also introduces an artificial sequence, the adapter, at the 5' end of the cDNA. The adapter sequence is used with an upstream gene specific primer to amplify the 3' end of the sequence in PCR. A gene specific primer (gsp1, Table 3) and the adapter primer were used to amplify the mummichog liver cDNA pool that had been primed with the oligo$(dT)_{17}$-adapter primer. A touchdown cycling program was used. The annealing temperature was decreased from 62° C to 48° C over 50 cycles. Annealing was for 2 min and extension was for 3 min. An additional 10 cycles were carried out with the annealing temperature maintained at 45° C. The resulting 1 kb product (S2) was cloned and sequenced as described.

4. Amplification of a mummichog β-actin fragment— Universal actin oligonucleotide primers (Melanie Wilson, Mississippi State Medical Center, unpublished) were used as amplification controls in all cDNA amplifications. These primers gave the expected 660 bp fragment in amplification reactions of liver cDNA pool. This fragment was also cloned and sequenced as described for the mummichog spgp fragments. Its sequence verified that it was a β-actin cDNA fragment. This fragment was used as a control probe in northern blots of mummichog RNA.

Sequence analysis— Sequences were compiled and edited with the aid of of the computer program ESEE (The Eyeball Sequence Editor, version 3, Cabot and Beckenbach, 1989). The PROSITE computer program and data base on the ExPASy World Wide Web Molecular Biology Server (Geneva, Switzerland) was used for identification of putative protein kinase phosphorylation sites in the deduced amino acid sequence (Bairoch et al., 1995). The PCGene computer program package (Inteligenetics, Mountain View, California) was used to locate putative membrane spanning regions using Kyte and Doolitle hydropathy plots (Kyte and Doolittle, 1982) and the method of Eisenberg (Eisenberg et al., 1984). Other features of the sequence were identified by inspection of the multiple sequence alignment and comparison with cDNA and deduced amino acid sequences of human (Chen et al., 1986; van der Bliek et al., 1988) hamster (Endicott et al., 1991) and *Xenopus* (Castillo et al., 1995) mdr isoforms and the rat spgp isoform (unpublished data kindly provided by Sarah Childs). Sequences of Pgp homologs were obtained from the GENBANK sequence database using the World Wide Web server at the National Center for Biotechnology Information (National Institutes of Health, Bethesda, Maryland).

Multiple alignment of the amino acid sequences of Pgp sequences was accomplished with the aid of the computer program CLUSTAL W (Thompson and Higgins, 1994) using the default parameters of the program. Similarity and identity scores on aligned sequences were calculated using the computer program Genedoc (Nicholas and Nicholas, 1996). For amino acid sequences similarity scores were calculated using the BLOSUM62 matrix provided with the program.

Relationships among selected vertebrate and invertebrate P-glycoprotein sequences were assessed by phenetic (overall distance) and cladistic (parsimony) approaches. Both methods relied on the alignment of the deduced amino acid sequences to assign homologous positions. The sequence of the yeast STE6 mating factor transporter (McGrath and Varshavsky, 1989), a member of the ABC superfamily, was also included in the analysis to serve as an out group sequence. The phenetic analysis was done with the aid of the PHYLIP (Version 3.5c) computer program package (Felsenstein, 1993). A pairwise distance matrix of the aligned amino acid sequences was calculated using the PHYLIP Protdist computer program. The PAM/Dayhoff matrix option was used. The resulting distance matrix was used in the PHYLIP implemetation of the neighbor joining method to construct a phylogenetic tree (Saitou and Nei, 1987). For parsimony analysis, the aligned nucleotides of the coding regions of Pgp homologs were input into the computer program PAUP (Swofford, 1993). The nucleotide alignment was created by using amino acid alignment from CLUSTAL W to align the codons of the selected Pgp homologs within the region of overlap with the mummichog sequence. Data were available for the winter flounder sequences and the pig spgp sequence only at the extreme 3' end of this region. The missing portions of these sequences were filled with gaps over the consensus length of the alignment. These were treated as missing data in the analysis. Untranslated regions were not considered in the analysis since no significant alignment could be found among any of the untranslated regions except the mammalian mdr sequences (data not shown). This gave an alignment

with a consensus length of 2394 positions. All nucleotide positions were weighted equally, and uninformative characters were ignored. Most parsimonious trees were searched using the heuristic search option of the PAUP computer program.

Immunoblot detection of P-glycoprotein gene family member expression— Immunoblotting using the monoclonal antibody C219 (Signet Laboratories, Dedham, MA) was used in to detect expression of Pgp gene family members in mummichog liver, kidney, brain, intestine and gill. Immunoblotting and immunohistochemical staining were performed essentially as described in Chapters II and III of this dissertation. In the present study the method was modified to include preparation of a plasma membrane enriched microsomal fraction in order to increase the sensitivity of detection (Fredericks et al., 1991).

Mummichog liver, kidney, brain and gill samples were collected as described above (**Fish collection and tissue processing**). For these, the entire organs were homogenized to make membrane fractions. For intestines, only the epithelial lining was used. This was collected as follows. The entire intestinal tract was removed from the fish. The mesenteries and adhering organs were cut away. Then the intestines were flushed out with ice cold Tris-buffered saline (50 mM Tris-HCl, pH 7.4, 150 mM NaCl) by forcing the solution through from the anterior end using a syringe fitted with a 20 Ga needle. The intestine was cut along its entire length and laid open on a clean microscope slide. A second slide was then used to scrape off the lining of the intestine while the anterior end was held with forceps. The lining was then homogenized immediately.

All mummichog organs were homogenized in 20 volumes of ice cold Tris-

buffered saline (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) containing 5 mM EDTA and freshly added protease inhibitors, N-toluene sulfonamido-L-arginine methyl ester (0.38 mg/ml), aprotinin (2% v/v), phenylmethyl sulfonyl fluoride (100 μg/ml) and leupeptin (10 μg/ml). All inhibitors were from Sigma. Homogenization was accomplished using a Polytron homogenizer (Brinkman Instruments, Westbury, NY).

The plasma membrane enriched microsomal fraction was prepared by differential centrifugation in a Sorvall RS28 centrifuge (DuPont, Wilmington, Delaware) using the F28/13 rotor. Homogenates were centrifuged at 4000 x *g* for ten minutes at 4° C to collect a crude nuclear/mitochondrial pellet. The supernatant was collected and centrifuged at 100,000 x *g* for 1 hr at 4° C. The supernatant was removed, and the microsomal pellet was dissolved in a minimum volume of Tris buffered saline containing 1% v/v Triton X-100, 1% w/v sodium deoxycholate, 0.1% w/v SDS and the protease inhibitors listed above. Protein concentrations were determined by the Lowry assay using bovine serum albumin as a standard.

The solubilized membrane fractions were then analyzed by immunoblotting. Membrane fractions (30 μg total protein) from each organ were separated by electrophoresis on a 5.6% acrylamide SDS-PAGE gel. A detergent extract of the colchicine-resistant cell line $CH^{R}C5$ (Kartner et al., 1993) was also loaded as a positive control. Electrophoresis, electrophoretic transfer to a nitrocellulose membrane, and immunochemical detection conditions were identical to those described in Chapters II and III. The relative molecular weights of immunoreactive bands were determined by comparing their positions with those of prestained protein molecular weight standards

(BioRad, Richmond, California) on the immunoblot.

Northern Blot detection of mummichog *spgp* expression— Expression of mummichog *spgp* sequences was examined in total RNA of several mummichog organs using northern blots. Total RNA was prepared from mummichog intestine, gills, liver, and brain as described above. Total RNA (12 μg) from each organ was electrophoresed on a 1% agarose-formaldehyde gel. Following electrophoresis, RNA was capillary-transferred to positively charged nylon membrane (Boehringer Mannheim) according to standard protocols (Farrell, 1993). After transfer, RNA was crosslinked to the membrane using ultraviolet light (Stratalinker, Stratagene, La Jolla, CA). An antibody-based chemiluminescent labeling and detection system (Genius™ System, Boehringer-Mannheim, Indianapolis, Indiana) was used to prepare digoxigenin labeled cDNA probe and to detect hybridrizing RNA species. The membrane was probed with the digoxigenin (DIG) labeled S4 cDNA fragment or with a DIG labeled mummichog actin fragment. The S4 fragment spans the poorly conserved linker region of the mummichog *spgp* and therefore was expected to serve as a gene specific probe. Labeled pobe was prepared by random priming of the gel-purified (GeneClean) S4 fragment or mummichog actin fragment with digoxigenin-11-dUTP using the Genius System Labeling Kit (Boehringer-Mannheim). The prehybridization and hybridization protocol followed that of Engler-Blum and colleagues (1993). The membrane was prehybridized for 1 hr at 68° C in 0.25M sodium phosphate, pH 7.2, 1mM EDTA, 20% SDS, 0.5% Boehringer-Mannheim blocking reagent. After 1hr, the prehybridization solution was replaced with fresh prehybridization solution that contained an estimated 25 ng/ml of digoxigenin-labeled

probe. The membrane was hybridized overnight at 68° C. On the following day, the membrane was washed three times for 20 min each of 20mM sodium phosphate, pH 7.2, 1mM EDTA, 1% SDS at 65° C. After these washes, DIG-labelled hybrids were detected on the membrane according to the Genius™ System protocol using anti-digoxiginen alkaline phosphatase conjugate. Lumiphos 530 served as a chemiluminescent substrate. Chemiluminescent blots were exposed to X-ray film (Fuji RX) for from 1 to 5 hr.

RESULTS

Amplification of expressed Pgp-related sequences from mummichog liver— A combination of RT-PCR strategies was used to amplify four overlapping segments (S1-S4) of a Pgp gene family member expressed in mummichog liver (Figure 10). Degenerate oligonucleotide primers (primers A and B, Table 3) to the conserved Walker A and B motifs of the ATP binding region of Pgp homologs were used to amplify the corresponding fragment of cDNA from mummichog liver. An amplified product of expected size (S1, 417 bp) was cloned and sequenced. The sequence of this fragment corresponded best to the downstream ATP binding region of vertebrate Pgps. A gene specific primer (gsp1, Table 3) was chosen from this new sequence and used in a 3' RACE protocol with the adapter primer (Table 3) to amplify the region downstream of the S1 fragment and 3' end of the putative Pgp cDNA (Frohman et al., 1989). The sequence of the resulting product (S2, 1007 bp) was most similar to the 3' end of vertebrate Pgp homologs and included a portion of the 3' untranslated region (3' UTR). To amplify sequences upstream of the S1 segment, we used a degenerate oligonucleotide

matching the Walker B motif for the upstream primer (primer C, Table 3) and a gene specific oligonucleotide (gsp2, Table 3) as the downstream primer. A product was amplified (S3, 1327 bp) which overlapped with the S1 region. The upstream sequence of S3 was similar to the transmembrane domain of the 3' half of vertebrate Pgps as expected but did not include the expected Walker B sequence (primer C). Instead it contained the gsp2 primer at the 5' end as well. A nested pair of downstream primers (gsp3 and gsp4, Table 3) was chosen from within the sequence of S3. Using nested amplifications with these gene specific primers and primer C, a fragment was amplified (S4, 1209 bp) which contained the transmembrane domain region and the expected upstream Walker B sequence.

Sequence of mummichog Pgp homolog— The overlapping clones of the amplified fragments yielded the 3004 bp cDNA sequence shown in Figure 11. The fragment continued the reading frame of the C primer. This reading frame terminated in TAA 2295 bp downstream from the primer. There were 700 bp of untranslated sequence beyond the termination codon. The 3' untranslated region did not contain a consensus polyadenylation signal or a poly A tail. This apparently truncated product may have resulted from mispriming of the mRNA during first strand synthesis at an adenosine rich region upstream of the poly A tail. The translation of these overlapping cDNA fragments spanned 765 amino acids. A comparison of the deduced amino acid sequence of this mummichog pgp fragment with that of the full-length hamster pgp1 cDNA indicated that the mumichog sequence spanned 58% of the coding region of the mRNA.

The basic features of P-glycoprotein sequences are present in the mummichog

homolog (Figure 11). The fragment includes the Walker B motif of the upstream ATP binding region, the linker region connecting the two halves of the protein, the membrane spanning domain of the second half of the protein and the downstream ATP binding region including the Walker A and B motifs. Analysis of the mummichog sequence for membrane spanning helices using the Eisenberg (1984) algorhythm indicated the presence of only five membrane spanning segments in the mummichog sequence. The segments correponding to the ninth and tenth transmembrane helices in mammalian Pgp were identified as a single transmembrane segment. Hydropathy plots of the mummichog deduced amino acid sequence and the hamster pgp3 sequence were very similar over this region (Figure 12) suggesting that the spgp P-glycoprotein also has six membrane spanning segments in this region as has been proposed for mammalian mdr P-glycoproteins (Endicott et al., 1991). Therefore transmembrane segments nine and ten (Figure 12) were assigned based on the multiple alignment of vertebrate Pgp sequences (Figure 13B).

The mummichog sequence contained the epitope for the mAb C219 (Val-Gln-Glu-Ala-Leu-Asp, VQEALD, Georges et al., 1990) in both halves of the protein. The calcium and phospholipid dependent protein kinase (protein kinase C) recognition sequence (Kemp and Pearson, 1990) (Thr/Ser)X(Arg/Lys) occured eleven times in the mummichog deduced amino acid sequence (Figure 11). A cluster of three sites was present in the linker region of the protein in the sequence Ser-Tyr-Arg-Ala-Ser-Leu-Arg-Ala-Ser-Ile-Arg (potential phosphorylation sites underlined) in a similar location to the protein kinase C sites found in the linker region of mammalian mdr isoforms (Endicott et

al., 1991). The sequence lacked the consensus cAMP dependent protein kinase (protein kinase A) recognition sequence found in the linker regions of mammalian mdr1-type sequences (Endicott et al., 1991).

Sequence analysis— The only published Pgp sequence data from teleosts are the winter flounder *pgpA* and *pgpB* sequences which comprise 348 and 354 nucleotides of the 3' end of the coding sequence respectively (Chan et al., 1993). For this reason the mummichog sequence was compared to other Pgp homologs over this region initially. A few small gaps were needed for alignment of this portion of the mummichog deduced amino acid sequence and ten other vertebrate P-glycoprotein sequences (Figure 13A). Over this region, 60 of 118 positions (51%) were occupied by identical amino acids and 13 more positions by conservative substitutions in all eleven sequences (62% overall similarity). A pairwise comparison of the mummichog sequence to each of these sequences showed that the mummichog sequence was most similar to the three spgp class sequences (Table 4A). These are the pig spgp sequence (94% similar, 87% identical), the rat spgp sequence (93% similar, 86% identical) and the winter flounder pgpA sequence (88% similar, 80% identical). All spgp-type sequences including the mummichog sequence share a distinctive conserved carboxyl teminal peptide, Thr-Gly-Ala-Pro-Ile-Ser. The similarity of the mummichog sequence to the other Pgp sequences was lower although still high (76-80% similar, 62-68% identical, Table 4A). This indicates that the region of Figure 13A is well conserved in all of these P-glycoproteins.

The mummichog deduced amino acid sequence was aligned with six other vertebrate Pgp sequences over the entire region of overlap (Figure 13B). Significant gaps

were placed in the poorly conserved linker region to optimize the multiple alignment. A pairwise comparison of these sequences showed the mummichog sequence to be most similar to the rat spgp sequence (83% similar, 68% identical, Table 4B). The similarity to the other five vertebrate sequences was significantly lower than this (71-72% similar, 48-50% identical). Both the rat spgp and the mummichog sequence had a longer linker region (78 and 90 amino acids) than the mdr sequences (59-62 amino acids). The alignment also showed that the rat spgp and the mummichog sequence shared the cluster of protein kinase C recognition sites within the linker region in the conserved sequence Ser-Tyr-Arg- X-Ser-Leu-Arg-Ala-Ser-Ile-Arg (where X is Ala in the mummichog sequence and Asp in the rat sequence). Like the mummichog sequence the rat spgp sequence lacked protein kinase A recognition sequences, Arg/Lys-Arg/Lys-X-Ser (Kemp and Pearson, 1990) present in the linker regions of the mdr1 sequences and the *Xenopus* mdr sequence.

Relationships among Pgp sequences— Both phenetic analysis of the deduced amino acid sequences and cladistic analysis of the aligned nucleotides of the cDNAs of a selection of vertebrate and invertebrate Pgp gave nearly identical relationships among the Pgp gene family members (Figure 14). In both cases the rat and mummichog spgp homologs were most closely related to each other and formed a sister group to all other vertebrate P-glycoproteins. Within the vetebrate mdr forms, the mammalian sequences were most closely related to each other while the *Xenopus* sequence was distant from these. The mammalian mdr homologs separated into two groups. This division parallels functional differences between the identified mdr1 and mdr2 P-glycoproteins. The three

Drosophila homologs formed a single clade which was the sister group to all of the vertebrate P-glycoproteins. The *Caenorhabditis elegans* homologs, on the other hand, did not group together. Futhermore, the *C. elegans* mdrC/pgp3 form was quite divergent from all other P-glycoprotein homologs.

The winter flounder pgpA, pgpB and the pig spgp sequences were not included in the above analyses because of the small amount of coding sequence data available for these. When all vetebrate sequences including these were subjected to parsimony analysis, a single most parsimonious tree was obtained (Figure 15A). In this case, the pig spgp sequence was closest to the rat spgp sequence in the spgp clade as expected. Both of the flounder sequences also grouped in the spgp clade. However the placement of the winter flounder pgpB sequence in the spgp clade was unstable when the data were subjected to "bootstrap" resampling and replacement in the PAUP program. Included in the tree in Figure 15 are the levels of bootstrap support for each group in the tree. The pgpB sequence grouped with the spgp sequences in only 48% of the bootstrap replicates (60 of 124, Figure 15A) while in 27% of the replicates (35 of 124, Figure 15A) this sequence was grouped with the vertebrate mdr clade. In contrast the winter flounder pgpA sequence was within the spgp clade in 90% of the replicates (111 of 124, Figure 15A). All other groups within the vertebrate Pgp family members were supported in at least 92% of the replicates. When the winter flounder pgpB sequence was eliminated from the analysis, a single most parsimonious tree was found (Figure 15B) supporting the division of the vertebrate Pgp gene family members into spgp and mdr classes. It also showed the winter flounder pgpA sequence as the closest homolog to the mummichog

spgp sequence.

Detection of expression of Pgp gene family members in mummichog— Both immunoblotting with the monoclonal antibody C219 and northern blotting using DIG-labelled mummichog S4 fragment were used to detect expression of Pgp in mummichog organs. The mAb C219 was expected to recognize all P-glycoproteins while the S2 fragment was expected to function as a gene specific probe for expression of mummichog spgp. The mAb C219 labeled a band at the molecular weight of P-glycoprotein (170 KDa) in immunoblots of mummichog and intestine membrane fractions (Figure 16). Under the same conditions no band in the size range of P-glycoproteins was detected in the mummichog kidney, gill or brain membrane fractions. Several bands of lower molecular weight were present in the CH^RC5, mummichog liver and brain lanes. These may have been cross-reactive proteins or proteolysis products. The mummichog spgp fragment S4 gave a hybridization signal in northern blots of total RNA from mummichog liver (Figure 17). Under the same conditions no signal was seen in RNA from intestine, brain or anterior kidney. Comparison of the intensity of this signal from liver with that of an actin control suggested that the Pgp message is moderately expressed in liver. The molecular size of the band in liver RNA was estimated to be approximately 5.5 Kb.

DISCUSSION

We report the partial sequence of a cDNA for Pgp gene family member expressed in the liver of the mummichog. As far as we know, this is the most complete sequence of a P-glycoprotein cDNA from a teleost reported to date. The sequence of this cDNA was

highly similar to the 3' terminal exons of the winter flounder *pgpA* gene, and the rat and pig spgp cDNA sequences. The mummichog sequence is therefore a transcript of the mummichog *spgp* gene of the newly described sister of P-glycoprotein division of the P-glycoprotein gene family which has recently been identified in three other vertebrate species (Childs et al., 1995).

The basic structure of the P-glycoprotein gene family members is conserved in the mummichog spgp sequence. The fragment reported here contains the upstream Walker B nucleotide binding motif, the linker region connecting the two halves of the gene product, the six putative transmembrane helices, followed by the downstream loop containing the ATP binding motifs. The mummichog sequence is most similar to the other vertebrate Pgp sequences in the ATP binding regions and is most divergent from the other sequences within the linker region.

The nucleotide sequence shows that the mummichog spgp transcript has a relatively long 3' untranslated region. Childs and colleagues (1995) have suggested that a long 3' UTR may be characteristic of *spgp* genes. Our data on the mummichog spgp transcript also support this idea. The 3' RACE product for the mummichog spgp cDNA obtained here has a truncated 3' UTR that is 700 bp long. The message size (5.5 Kb) of the spgp transcript estimated from northern blots of mummichog liver RNA indicates a 3' UTR longer than 1000 bp. This transcript is similar in size to the mRNA of the rat *spgp* gene (Childs et al., 1995). The winter flounder *spgp* gene (*pgpA*) transcript is predicted to have a 3' UTR in excess of 1020 bp (Chan et al, 1992). The function of the untranslated region of mRNA is uncertain but is thought to influence message stability.

The long 3' untranslated regions of the *spgp* transcripts may confer similar stabilities to these transcripts in different species.

Inspection of the alignment of vertebrate P-glycoprotein homologs reveals some features which may be characteristic of spgp proteins. All four representatives have a distinct carboxyl-terminal peptide, Thr-Gly-Ala-Pro-Ile-Ser (TGAPIS). However, the remainder of the sequence in the region of overlap with the flounder and pig sequences is highly conserved with 78-80% of positions conserved between spgp and mdr P-glycoproteins. The alignment of the more complete sequences show some features that are shared only by the rat and by the mummichog spgp sequences. Both of these spgp sequences have a longer linker region compared to other vertebrate Pgp members forcing significant gaps into the multiple alignment. The linker region has been identified as a potential regulatory domain in Pgp family members and contains consensus recognition sequences for both the calcium phospholipid dependent protein kinase (protein kinase C) and the cyclic AMP dependent protein kinase (protein kinase A) in mammalian and *Xenopus* mdr sequences (Endicott et al., 1991; Chambers et al., 1993; Castillo et al., 1995). Both the rat and the mummichog spgp sequences share a cluster of three potential protein kinase C (PKC) phosphorylation sites at corresponding positions the within the linker region. A slightly different cluster of conserved PKC recoginition sequences occurs in the linker region of mammalian and *Xenopus* mdr P-glycoproteins (Endicott et al., 1991; Castillo et al., 1995). These sites on the human MDR1 protein are phosphorylated in intact drug-resistant cell lines and *in vitro* in membrane vesicle prepartions by PKC (Chambers et al., 1992; Chambers et al., 1993). The mammalian

mdr1-type P-glycoproteins and the *Xenopus* homolog also contain protein kinase A recognition sequences in the linker region. However neither the mummichog nor the rat Spgp sequences contain such sites. Phosphorylation seems to be involved in upregulating the pumping activity drug resistance homologs in drug-resistant cell lines (Chambers et al., 1990; Ma et al., 1991). The presence of analogous PKC phosphorylation sites in the spgp homologs suggests that they may also be phosphorylated and regulated by PKC. The distinct but highly conserved nature of the cluster of protein kinase C recognition sequences in the linker region of the two spgp-type P-glycoproteins may indicate some divergence in regulation between the mdr and spgp isoforms.

Previous cladistic analysis of the deduced amino acid sequences of several ABC superfamily members and several P-glycoproteins indicated the existence of the spgp class of P-glycoproteins (Childs et al., 1996). This analysis was performed only over the limited data available for the deduced amino acid sequence of the 3' end of the pig spgp cDNA, a region that contains highly conserved amino acid positions among all P-glycoproteins. The previous analysis did not include any invertebrate Pgp sequences. In order to examine the validity and extent of the spgp category we have peformed cladistic and phenetic analyses of the relationships among P-glycoprotein sequences using a different data set which includes the more complete mummichog spgp cDNA sequence and the rat spgp sequence (kindly provided by S.Childs). We have also included invertebrate P-glycoprotein sequences. Both phenetic analysis of the deduced amino acid sequences and cladistic analysis of the coding sequence of P-glycoproteins support the *spgp* gene class as a distinct and well conserved lineage within the vertebrate P-

glycoprotein gene family. In our analysis the spgp sequences from two teleosts and two mammals form a sister group to the vertebrate mdr sequences which includes the amphibian sequence (*Xenopus* mdr). This suggests that the apparent gene duplication event separating the *spgp* lineage from the *mdr* forms occured in some common ancestor of the vertebrates. However, it is notable that none of the invertebrate sequences nor the amphibian homolog can be assigned to either of the vertebrate P-glycoprotein categories (mdr1 or mdr2) by this method. Furthermore the individual homologs in *Drosophila* and the nematode (*C.elegans*) are less closely related within taxa than are the mammalian mdr and spgp forms. Although this is consistent with independent origins of multiple P-glycoproteins in vertebrate and invertebrate lines, another explanation is the operation of a homogenization mechanism (concerted evolution) within the gene family in various lineages (Arnheim, 1983). The very high relatedness of the mammalian mdr P-glycoproteins has been noted before, and there is evidence of concerted evolution within the *mdr* genes of the hamster (Endicott et al., 1991), the mouse (Hsu et al., 1991) and man (Van der Bliek et al., 1988).

In order to determine the sites of expression of the mummichog *spgp* gene we have used immunoblotting to detect expression of all P-glycoprotein forms in mummichog organs. We also have used a poorly conserved region of the mummichog spgp cDNA as a gene specific probe for mummichog spgp expression in northern blots of RNA from mummichog organs. The work in Chapter II and III of this dissertation as well as another study in the guppy (Hemmer et al., 1995) have found P-glycoprotein expression in teleosts in the bile canalicular membrane of hepatocytes. Pgp expression

was also detected in the apical membrane of the epithelial cells of the kidney tubules and the intestine in the guppy (Hemmer et al., 1995). These are also sites of expression in mammals (Thiebault et al., 1987; Thiebault et al., 1989; Cordon-Cardo et al., 1990; Bradley et al., 1990). Our results here using immunoblots with mAb C219 show evidence of high level expression of P-glycoprotein family members in liver and lower level expression in intestine of the mummichog. However we did not detect P-glycoprotein expression in mummichog kidney. The reason for our inability to detect expression in this organ is unknown. It is possible that the large amount of hematopoetic tissue in the anterior kidney of the mummichog may have diluted the P-glycoprotein signal from the tubules. The hematopoeitic tissue did not react with C219 in immunohistochemical staining of guppy kidney (Hemmer, 1995). Therefore membrane fractions from the whole organ may not contain enough Pgp to detect in immunoblots under our conditions which have been developed for liver. Thus Pgp family members are likely expressed in mummichog liver, intestine and kidney. Since the mummichog spgp P-glycoprotein has the C219 epitope in two places in its sequence, immunochemical results using mAb C219 should detect both spgp and mdr P-glycoproteins. For this reason we have used the S4 fragment of the mummichog spgp cDNA to examine specific expression of *spgp* gene in these organs. S4 spans the less well conserved linker region and should be less likely to cross-hybridize with *mdr* gene transcripts. Northern blots of RNA from brain, kidney, intestine and liver probed with S4 demonstrated expression of the *spgp* gene only in liver. This result is in accord with the liver specific expression reported for the rat *spgp* gene (Childs et al., 1995). Thus results here indicate that

expression of the spgp homolog is responsible for a portion of the signal seen in immunohistochemical staining of histologic sections and immunoblots of teleost liver with C219 in previous studies. The signal seen in other organs of teleosts including kidney and intestine probably results from expression of one or more other homologs.

Restriction of *spgp* expression to the liver in both the rat and the mummichog along with the highly conserved structure and sequence of the gene across such large evolutionary distances suggests that the spgp homolog has an essential role in normal liver physiology of all vertebrates. Immunohistochemical staining of mammalian and teleost liver with C219 has shown Pgp expression mainly on the bile canalicular membrane (Thiebault et al., 1987; Thiebault et al., 1989; Cordon-Cardo et al., 1990; Bradley et al., 1990; Hemmer et al., 1995). The mAb C219 also recognizes the spgp P-glycoprotein; therefore the spgp protein is probably expressed in the canaliculus as well. The sister of P-glycoprotein probably functions in ATP dependent transport of substances into the bile.

In mammalian liver the mdr homologs may have roles in in biliary excretion. Both mdr1 and mdr2 type P-glycoproteins are expressed in mammalian liver (Buschman et al., 1992; Smit et al., 1994; Teeter et al., 1990). Evidence suggests a physiological requirement for expression of mdr2 P-glycoprotein in transport of phosphatidylcholine into bile (Smit et al.,1993; Ruetz and Gros, 1994). The role of the smaller amount of mdr1 is less certain but seems to be that of a xenobiotic transporter (Schinkel et al., 1994). Since the biliary excretion pathway is similar in teleosts and mammals (Honkanen et al., 1985; Sanz et al., 1993), fish are expected to have a requirement for a

phosphatidylcholine translocator (mdr2 function) as well as a xenobiotic transporter (mdr1 function) in addition to the spgp function. Southern blots of genomic DNA of several fish species indicate the presence of two mdr genes (Ling et al., 1992). It seems likely that these will turn out to have homologous roles and expression patterns to the mammalian *mdr1* and *mdr2* genes. One such gene may be the the winter flounder *pgpB* gene. However the relationship of the winter flounder *pgpB* gene to the other vertebrate genes is not resolved here because of the limited amount of coding sequence data which spans a highly conserved region. Perhaps additional sequence will show the winter flouder *pgpB* gene to be a member of the vertebrate *mdr* class.

Measurement of Pgp expression in liver of teleosts with generic Pgp probes such as mAb C219 may be detecting as many as three different proteins (mdr1, mdr2 and spgp). These proteins will have different functions and the expression of each may be regulated differently. Our previous studies have shown elevation of expression of one or more Pgp isoforms in the livers of mummichog and liver tumors of mummichog from the highly contaminated Atlantic Wood site. Understanding the significance of our data on Pgp expression to the survival of mummichog at Atlantic Wood requires knowledge of the specific forms (xenobiotic transporters, phospatidylcholine translocators or spgp) being measured in mummichog liver and liver tumors. Chapter V of this dissertation reports the identification and sequence of a mummichog mdr1 homolog expressed in intestine and liver.

Figure 10. The strategy used to amplify segments of the mummichog *spgp* cDNA. The top of the Figure (dark line) shows a full-length mRNA for a *pgp* gene. The 5' end of the molecule is at the left. The Walker A and B nucleotide binding motifs of the upstream (A and B) and downstream (A' and B') ATP binding regions are shown. The 3' untranslated region (3' UTR) and the poly A tail ($A_{(n)}$) are also shown. The mRNA was reverse transcribed (**RT**) to make first strand cDNA. cDNA was then amplified using the polymerase chain reaction (**PCR**) using primers (boxes with arrows) to specific regions of the cDNA. Primers A,B and C are degenerate primers to conserved regions. Primers gsp1-gsp4 are gene specific primers to the mummichog spgp. Primers and the segments amplified are shown in **Table 1**.

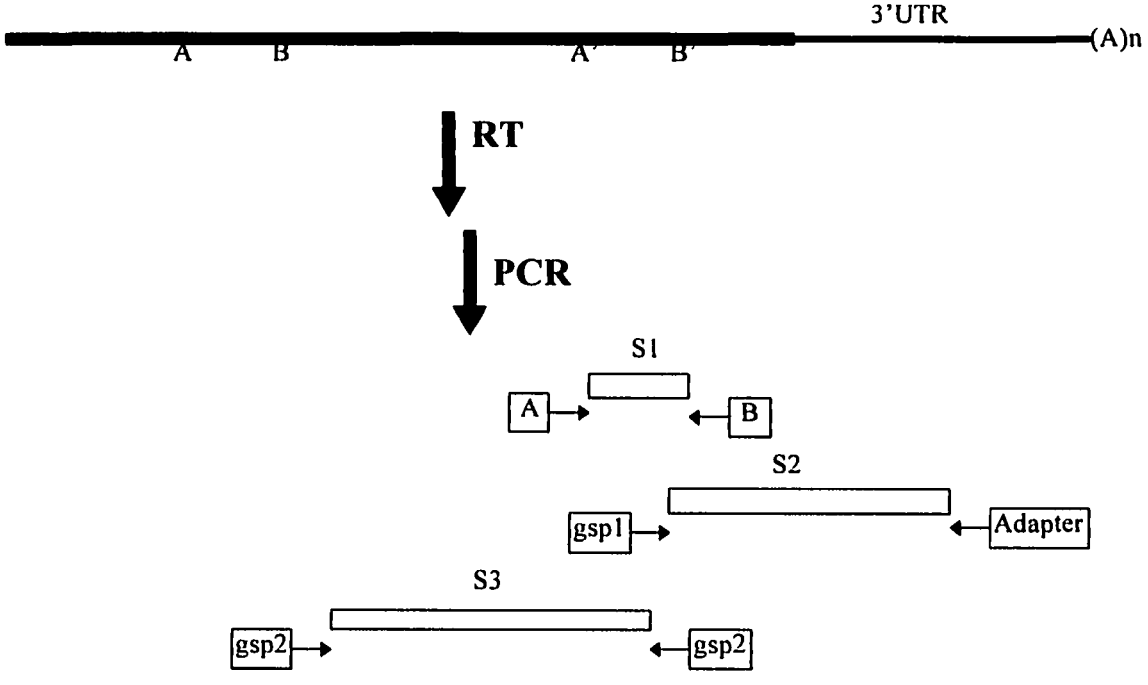
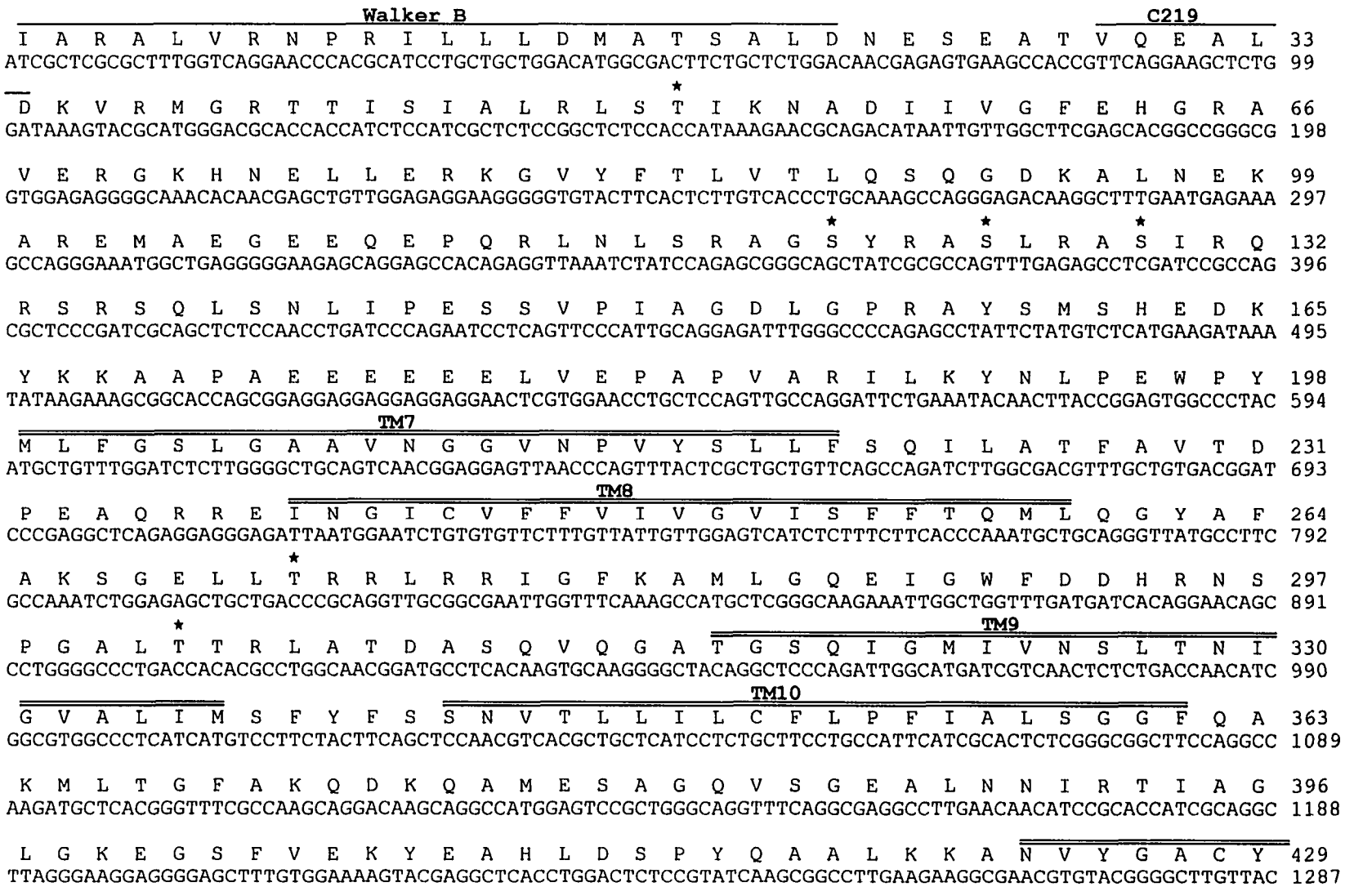


Table 3. Primer pairs used to amplify mummichog spgp cDNA. Peptide sequences are given for degenerate primers (A,B andC). The regions amplified are referenced to conserved features of Pgp sequences; Walker A and Walker B = sequences of the nucleotide binding fold, TMD= transmembrane domain, UTR=untranslated region. The nucleotide binding folds and TMDs occur twice within the sequence and are identified as belonging to either the 5' or 3' half of the molecule. Plus (+) strand primers are complementary to the first strand cDNA. Minus (-) strand primers are complementary to the second strand cDNA. The size of the amplified fragment was determined by sequencing and includes the primer. Key to mixed and unusual base symbols: R=A+G, M=A+C, Y=C+T, H=A+C+T, I=inosine.

Primer Name	Sequence	Strand	Consensus Peptide	Region Amplified	Size of amplified fragment
A	GTIGGIAGYAGYGGITGTGGIAAR	+	VGSSGCGK	3' Walker A - 3' Walker B	417 bp
B	RTCIAGIGCIGAIGTIGC	-	ATSALD		
gsp1	ATTGCCAGGGCGATCATACG	+		3' Walker B - 3' UTR	1007 bp
adapter	TCTGAATTCTCGAGTCGACATCTT	-			
gsp2	CATGCTGATTTCCCGCAAGTTGTC	-/+		3' TMD - 3' Walker A	1327 bp
C	GGICARAARCARMGIATHGC	+	GQKQRIA	5' Walker B - 3' TMD	1209 bp
gsp3	TCCTTCCCTAAGCCTGCGATGG	-			
gsp4	AGCCTGCGATGGTGCGGATGTTG	-			

Figure 11. Nucleotide and deduced amino acid sequence of the mummichog spgp cDNA determined from overlapping RT-PCR amplified fragments. The nucleotide sequence is numbered in 5' to 3' orientation. The sequence does not include the 3' degenerate primer (primer C). Amino acids are aligned with the second nucleotide of each codon. Termination codon (TAA) is indicated by a * in the amino acid sequence. Sequences corresponding to the Walker A and Walker B nucleotide binding motifs are indicated with a line over the sequence. The C219 epitope is also indicated with an overline. The six transmembrane domains are indicated with a double overline and are numbered (TM7-TM12). Potential protein kinase C recognition sequences are marked with a ★.



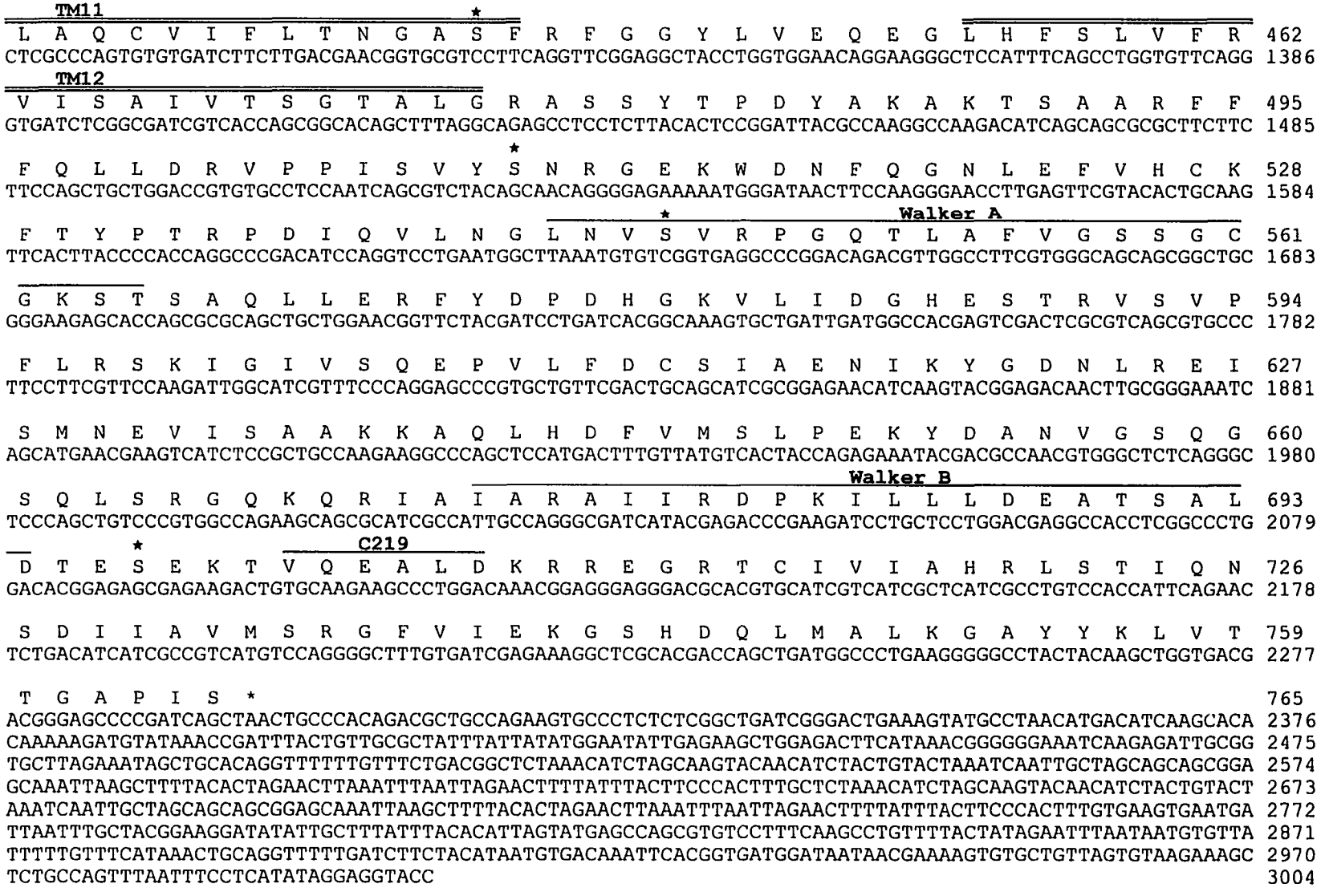
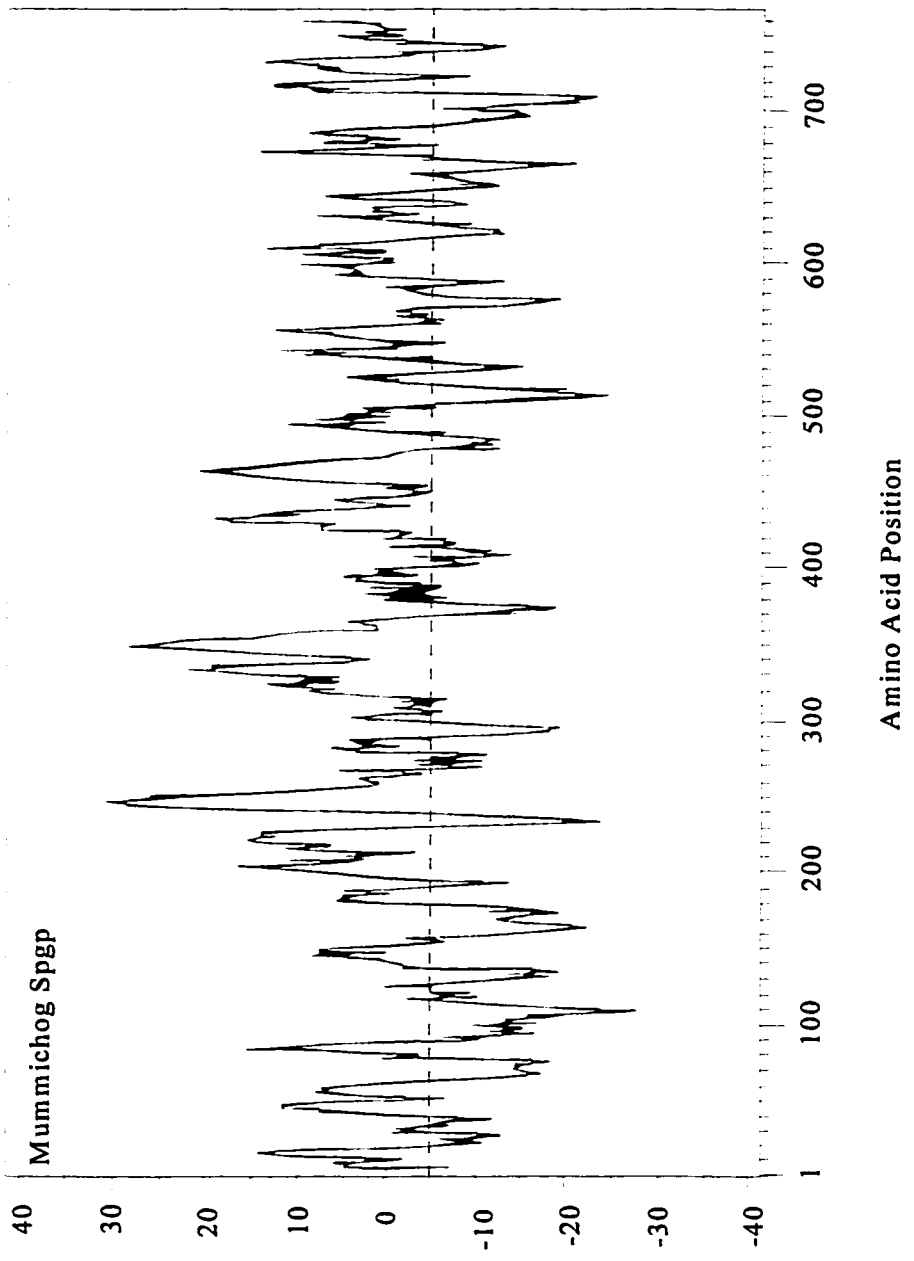


Figure 12. Hydropathy plots (Kyte and Doolittle, 1982) of the mummichog spgp (**A**) deduced amino acid sequence and the corresponding carboxyl terminal half of the hamster pgp3 (**B**) deduced amino acid sequence (Endicott et al., 1991). The plots were created using the SOAP program of the PCGene computer program package (Intelligenetics, Mountain View, California) using a window of nine amino acids.

Figure 12A



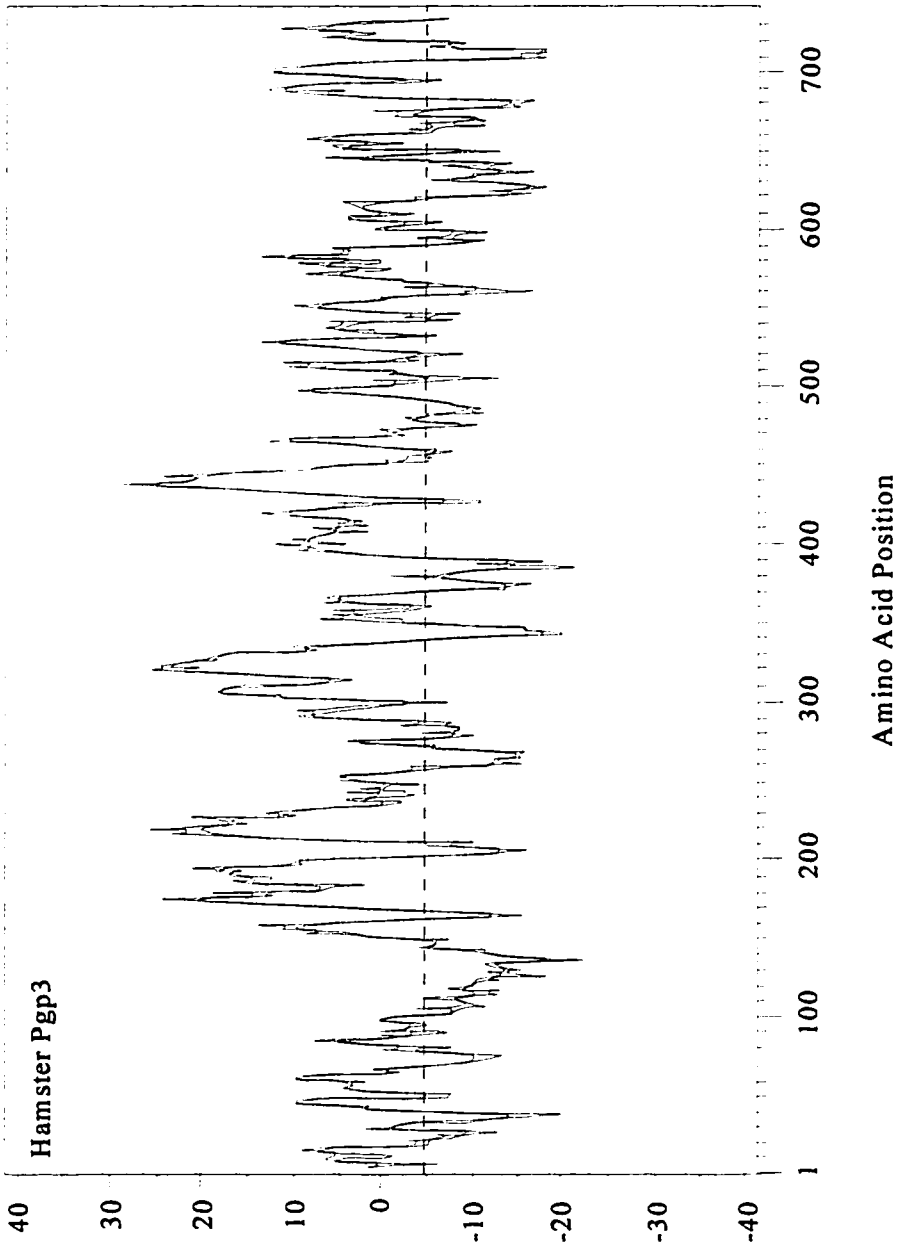


Figure 12B

Figure 13. Multiple alignment of deduced amino acid sequences of selected P-glycoproteins. Multiple alignments were created with the aid of the computer program CLUSTAL W (Thompson and Higgins, 1994). Positions with identical amino acids to mummichog spgp are shown as a dot (.). Gaps inserted into the sequences to optimize the alignment are shown as a dash (-). The positions of the Walker motifs, the linker region, the putative transmembrane segments (TM) and the C219 epitope are indicated. **A:** Multiple alignment of the carboxyl end of P-glycoproteins in the region of the 3' terminal exons of winter flounder sequences. **B:** Multiple alignment of the carboxyl region of more complete P-glycoprotein sequences beginning at the N-terminal Walker B motif. *References*: human MDR1 (Chen et al., 1986); human MDR2 (van der Bliek et al., 1988); hamster pgp1, pgp2 and pgp3 (Endicott et al., 1991); mouse mdr1a (Hsu et al., 1990); mouse mdr2 (Gros et al., 1988); rat mdr1b (Silverman et al., 1991); rat mdr2 (Brown et al., 1993); winter flounder pgpA and pgpB (Chan et al., 1993); pig spgp (Childs et al., 1995); rat spgp (unpublished data kindly provided by S.Childs); *Xenopus* mdr (Castillo et al., 1995).

Figure 13A

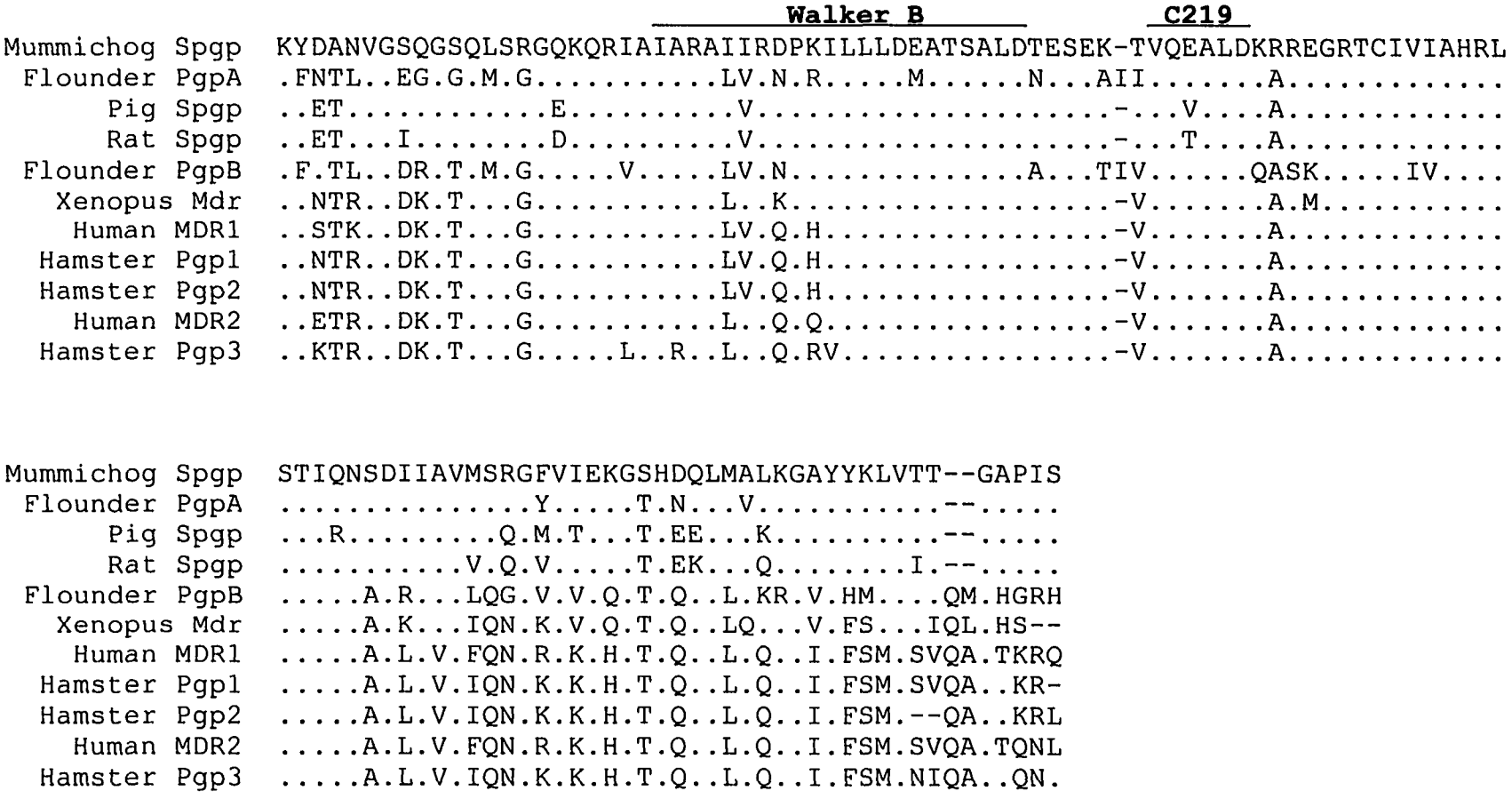


Figure 13B

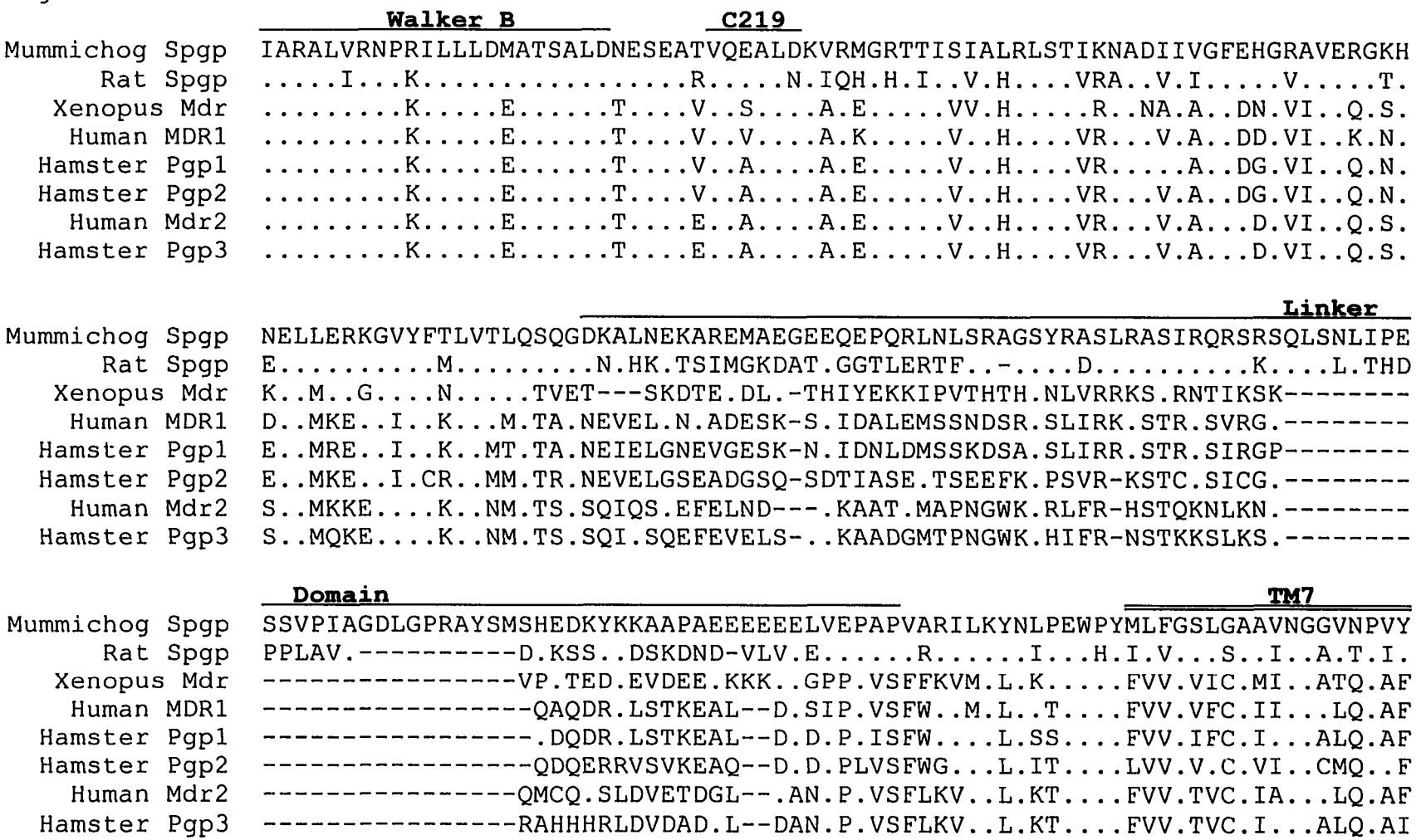


Figure 13B (cont.)

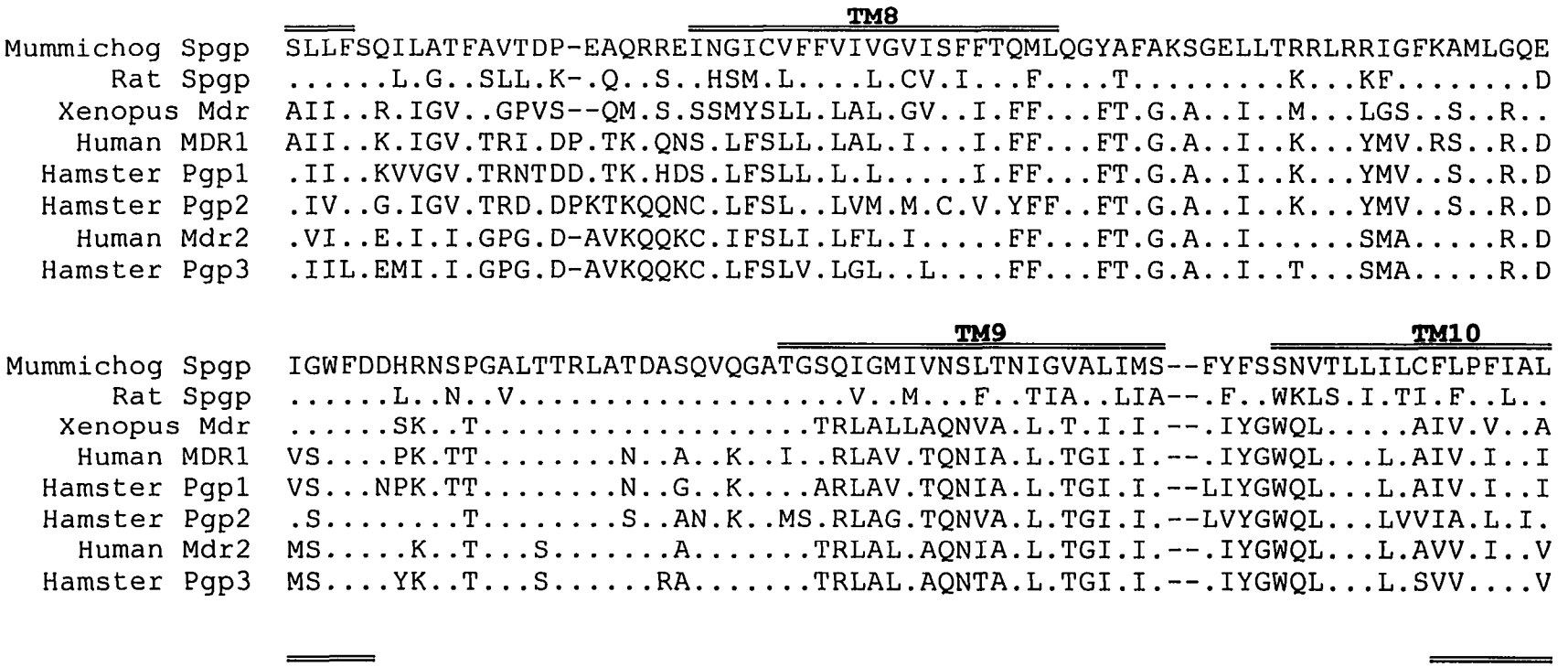


Figure 13B (cont.)

```
                  TM11                                  TM12
Mummichog Spgp  -LAQCVIFLTNGASFR-FGGYLVEQEGLHFSLVFRVISAIVTSGTALGRASSYTPDYAKAKTSAARFFFQLL
      Rat Spgp  AFS.GIA..A.S.AY.-Y....IAY...G..H....V.SVAL.A..V..TF..S.S.....I....-.....
   Xenopus Mdr  G.S.AHHV.CLCWV.SVL.A...VEGLMKLDE..L.S....LGAM...QT..FA...T..MI...H-I.S..
    Human MDR1  SFT.AMMYFSYAGC..-..A...AHKLMS.ED.LL.F..V.FGAM.V.QV..FA.......I...H-IIMII
  Hamster Pgp1  SFT.AMMYFSYA.C..-..A...AR.LMT.EN.LL.F....FGAM.V.QV..FA.......V..SH-IIMII
  Hamster Pgp2  SFT.AMMYFSYA.C..-..A...AHQIMT.EN.ML.F..V.FGAI.A.N...FA.......V..SH-IIRIM
    Human Mdr2  SIS.AFMYFSYAGC..-..A..IVNGHMR.RD.IL.F....FGAV...H...FA.......L...H-L.M.F
  Hamster Pgp3  SIS.AFMYFSYAGC..-..A..IVNGHMR.RD.IL.F....FGAV...H...FA.......L...H-L.S.F

                                                                  WalkerA
Mummichog Spgp  DRVPPISVYSNRGEKWDNFQGNLEFVHCKFTYPTRPDIQVLNGLNVSVRPGQTLAFVGSSGCGKSTSAQLLE
      Rat Spgp  I.K...N...EA.........KID.ID......S..........S...N...................I....
   Xenopus Mdr  E...Q.DS..DQ...PK.CS..VV.KGVN.N.......T..Q..DI..KQ.E...L..........TVS...
    Human MDR1  EKT.L.DS..TE.LMPNTLE..VT.GEVV.N.......P..Q..SLE.KK.....L..........VV....
  Hamster Pgp1  EK..S.DS..TG.L.PNTLE..VK.NEVV.N.......P..Q...LE.KK.....L..........VV....
  Hamster Pgp2  EKI.S.DS..T..L.PNWLE..VK.NEVV.N.......P..Q..SLE.KK.....L..........VV....
    Human Mdr2  E.Q.L.DS..EE.L.P.K.E..IT.NEVV.N....ANVP..Q..SLE.KK.....L..........VV....
  Hamster Pgp3  E.Q.L.DS..GE.LWP.K.E.SVT.NEVV.N....ANMP..Q..SLE.KK.....L..........VV....


Mummichog Spgp  RFYDPDHGKVLIDGHESTRVSVPFLRSKIGIVSQEPVLFDCSIAENIKYGDNLREISMNEVISAAKKAQLHD
      Rat Spgp  ......Q.T.M....D.KK.NIQ....N..............MD.......TK...VERA.A...Q.....
   Xenopus Mdr  .....FE.E..V..LSVRNLNIQWV.AQM.......I......GD..A....N.KVTQE.IET...E.NI.S
    Human MDR1  .....LA....L..K.IK.LN.QW..AHL.......I..........A....S.VV.QE.IVR...E.NI.A
  Hamster Pgp1  .....MA.T.FL..K.VNQLN.QW..AHL.......I..........A....S.VV.QD.IER...E.NI.Q
  Hamster Pgp2  .....MA.T.FL..K.IKQLN.QW..AHL.......I..........A....S.VV.QD.IER...E.NI.Q
    Human Mdr2  .....LA.T..L..Q.AKKLN.QW..AQL.......I..........A....S.VV.QD.IV....A.NI.P
  Hamster Pgp3  .....MA.T..L..Q.AKKLNIQW..AQL..................A....S.VV.QD.IVR...A.NI.P
```

Figure 13B (cont.)

```
                                                  WalkerB                 C219
Mummichog Spgp  FVMSLPEKYDANVGSQGSQLSRGQKQRIAIARAIIRDPKILLLDEATSALDTESEKTVQEALDKRREGRTCI
      Rat Spgp  .........ET...I........D..........V.........................T....A.......
   Xenopus Mdr  .IE..TD..NTR..DK.T...G...........L..K.....................V.......A.M.....
    Human MDR1  .IE...N..STK..DK.T...G...........LV.Q.H...................V.......A.......
  Hamster Pgp1  .IE...D..NTR..DK.T...G...........LV.Q.H...................V.......A.......
  Hamster Pgp2  .IE...D..NTR..DK.T...G...........LV.Q.H...................V.......A.......
    Human Mdr2  .IET..H..ETR..DK.T...G...........L..Q.Q...................V.......A.......
  Hamster Pgp3  .IET..Q..KTR..DK.T...G.....L..R..L..Q.RV.................V.......A.......

Mummichog Spgp  VIAHRLSTIQNSDIIAVMSRGFVIEKGSHDQLMALKGAYYKLVTT--GAPIS
      Rat Spgp  ..................V.Q.V.....T.EK...Q........I.--.....
   Xenopus Mdr  ...........A.K...IQN.K.V.Q.T.Q..LQ...V.FS...IQL.HS--
    Human MDR1  ...........A.L.V.FQN.R.K.H.T.Q..L.Q..I.FSM.SVQA.TKRQ
  Hamster Pgp1  ...........A.L.V.IQN.K.K.H.T.Q..L.Q..I.FSM.SVQA..KR-
  Hamster Pgp2  ...........A.L.V.IQN.K.K.H.T.Q..L.Q..I.FSM.--QA..KR-
    Human Mdr2  ...........A.L.V.FQN.R.K.H.T.Q..L.Q..I.FSM.SVQA.TQNL
  Hamster Pgp3  ...........A.L.V.IQN.K.K.H.T.Q..L.Q..I.FSM.NIQA..QN.
```

Table 4. Pairwise comparison matrices of the deduced amino acid sequences for a selection of Pgp homologs. The three numbers for each entry below the diagonal give the number of positions in the alignment that are identical between the two sequences (top number), identical positions plus conservative substitutions (center number) and the number of positions that line up with a gap (bottom number). The region of the matrix above the diagonal gives these values as percentages. The number along the diagonal gives the number of amino acids in the sequence. Conservative substitution groups from the BLOSUM 62 scoring matrix are (D, N); (E, Q); (S, T); (K, R); (Y, W, F) and (L, I, V, M). **A:** Pairwise matrix of the comparison over the region of the winter flounder sequences. **B:** Pairwise matrix for sequences with complete data over the length of the mummichog spgp sequence. *References*: human MDR1(Chen et al., 1986); human MDR2 (van der Bliek et al., 1988); hamster pgp1, pgp2 and pgp3 (Endicott et al., 1991); mouse mdr1a (Hsu et al., 1990); mouse mdr2 (Gros et al., 1988); rat mdr1b (Silverman et al., 1991); rat mdr2 (Brown et al., 1993); winter flounder pgpA and pgpB (Chan et al., 1993); pig spgp (Childs et al., 1995); rat spgp (unpublished data kindly provided by S.Childs); *Xenopus* mdr (Castillo et al., 1995).

Table 4A

	Mummichog Spgp	Flounder PgpA	Pig Spgp	Rat Spgp	Flounder PgpB	Xenopus Mdr	Human MDR1	Human MDR2	Hamster Pgp1	Hamster Pgp2	Hamster Pgp3
		80%	87%	86%	62%	70%	67%	68%	68%	68%	67%
Mummichog Spgp	115	88%	94%	93%	79%	80%	76%	78%	79%	78%	78%
		0%	0%	0%	2%	3%	1%	1%	2%	3%	1%
	93		77%	77%	68%	69%	66%	66%	68%	68%	66%
Flounder PgpA	103	116	85%	84%	82%	79%	76%	76%	77%	77%	77%
	1		0%	0%	1%	4%	2%	2%	3%	4%	2%
	101	90		91%	62%	68%	67%	67%	68%	68%	65%
Pig Spgp	109	99	115	96%	82%	79%	79%	81%	81%	80%	82%
	0	1		0%	2%	3%	1%	1%	2%	3%	1%
	100	90	105		62%	68%	69%	69%	70%	70%	67%
Rat Spgp	107	98	111	115	81%	78%	77%	79%	79%	79%	81%
	0	1	0		2%	3%	1%	1%	2%	3%	1%
	74	81	74	74		74%	70%	68%	70%	70%	66%
Flounder PgpB	94	97	97	96	118	85%	83%	83%	83%	83%	81%
	3	2	3	3		2%	0%	0%	1%	2%	0%
	83	82	80	80	88		80%	82%	84%	83%	82%
Xenopus MDR	94	94	93	92	101	115	88%	88%	90%	88%	88%
	4	5	4	4	3		1%	1%	0%	3%	1%
	79	79	79	81	83	94		94%	94%	93%	87%
Human MDR1	90	90	93	91	99	104	117	97%	97%	95%	94%
	2	3	2	2	1	2		0%	0%	1%	0%
	80	78	79	81	81	96	110		92%	91%	90%
Human MDR2	92	90	95	93	99	104	114	117	95%	94%	96%
	2	3	2	2	1	2	0		0%	1%	0%
	80	81	80	82	83	98	111	108		97%	90%
Hamster Pgp1	93	92	95	93	99	105	114	112	116	97%	95%
	3	4	3	3	2	1	1	1		2%	0%
	80	81	80	82	83	98	109	107	114		90%
Hamster Pgp2	92	91	94	93	98	103	112	111	114	115	94%
	4	5	4	4	3	4	2	2	3		1%
	79	78	77	79	79	96	102	106	106	106	
Hamster Pgp3	92	91	96	95	96	103	110	113	112	110	117
	2	3	2	2	1	2	0	0	1	2	

Table 4B

	Mummichog	Rat	Xenopus	Human	Human	Hamster	Hamster	Hamster
		68%	50%	48%	50%	49%	48%	49%
Mummichog Spgp	765	83%	71%	71%	71%	71%	71%	71%
		1%	4%	4%	4%	4%	4%	4%
	526		48%	48%	48%	48%	47%	48%
Rat Spgp	639	753	69%	70%	70%	70%	71%	70%
	14		3%	3%	3%	3%	3%	2%
	386	370		66%	66%	67%	63%	65%
Xenopus Mdr	549	528	737	82%	82%	81%	79%	81%
	36	26		1%	1%	1%	1%	1%
	376	371	496		76%	88%	82%	73%
Human MDR1	550	536	616	740	88%	95%	92%	86%
	33	23	11		0%	0%	0%	0%
	386	368	493	568		73%	72%	90%
Human MDR2	552	531	612	654	737	86%	85%	95%
	34	24	12	5		0%	0%	0%
	380	367	498	653	546		84%	73%
Hamster Pgp1	548	534	609	704	640	739	93%	85%
	34	24	10	1	6		0%	0%
	372	363	473	607	535	623		71%
Hamster Pgp2	551	539	593	685	634	692	736	84%
	37	27	13	4	7	3		0%
	378	366	490	548	668	541	530	
Hamster Pgp3	548	531	603	642	707	634	627	739
	32	22	10	3	2	4	5	

Figure 14. Relationships among vertebrate and invertebrate P-glycoproteins. **A:** Results of phenetic (distance) analysis using the PHYLIP computer program package (Felsenstein, 1993) of aligned amino acid sequences. Neighbor-Joining (Saitou and Nei, 1987) was used to assess relationships. Branch lengths are scaled to reflect distances. **B:** The single most parsimonious tree resulting from cladistic analysis on aligned nucleotide sequences using the PAUP computer program (Swofford, 1993). Branch lengths are scaled to the number of steps along each branch. *References*: human MDR1(Chen et al., 1986); human MDR2 (van der Bliek et al., 1988); hamster pgp1, pgp2 and pgp3 (Endicott et al., 1991); mouse mdr1a (Hsu et al., 1990); mouse mdr2 (Gros et al., 1988); rat mdr1b (Silverman et al., 1991); rat mdr2 (Brown et al., 1993); rat spgp (unpublished data kindly provided by S.Childs); *Xenopus* mdr (Castillo et al., 1995); *Caenorhabditis elegans* mdrA/pgp1 and mdr C/pgp3 (Lincke et al., 1992); *Drosophila mdr49* and *mdr65* (Wu et al.,1991) and *mdr49* (Gerrard et al., 1993); yeast STE6 (McGrath and Varsharvsky, 1989).

Figure 14A

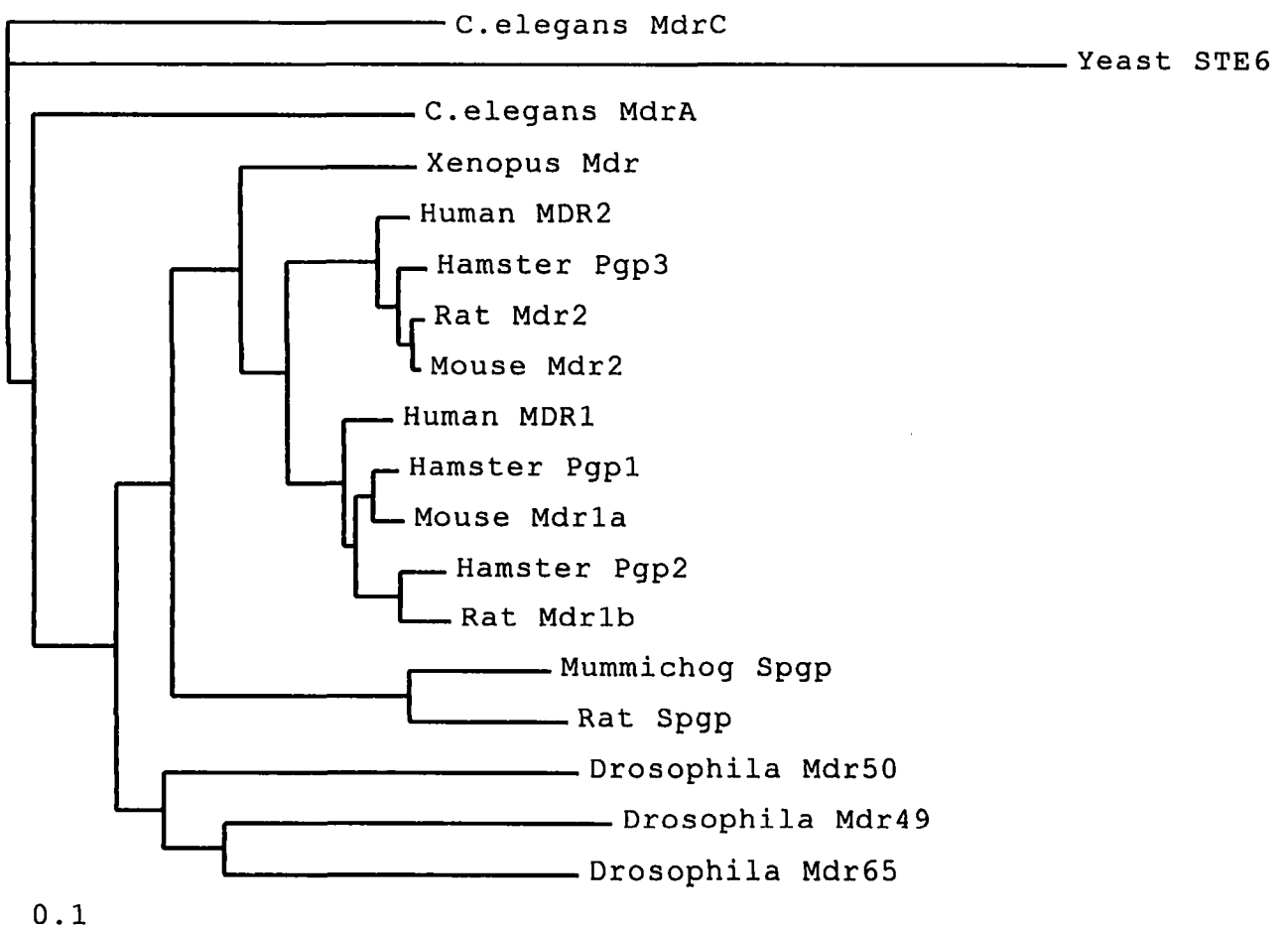


Figure 14B

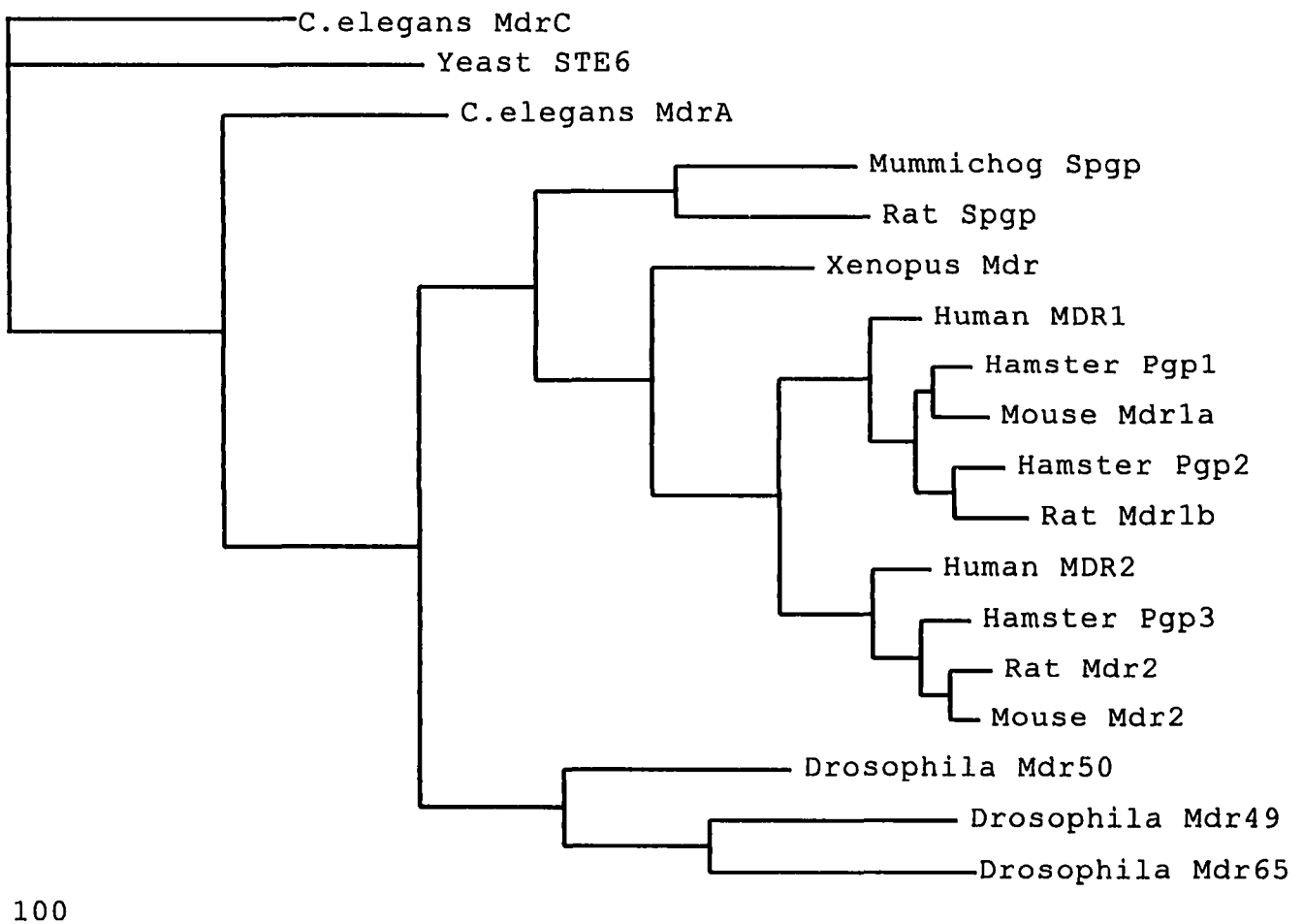
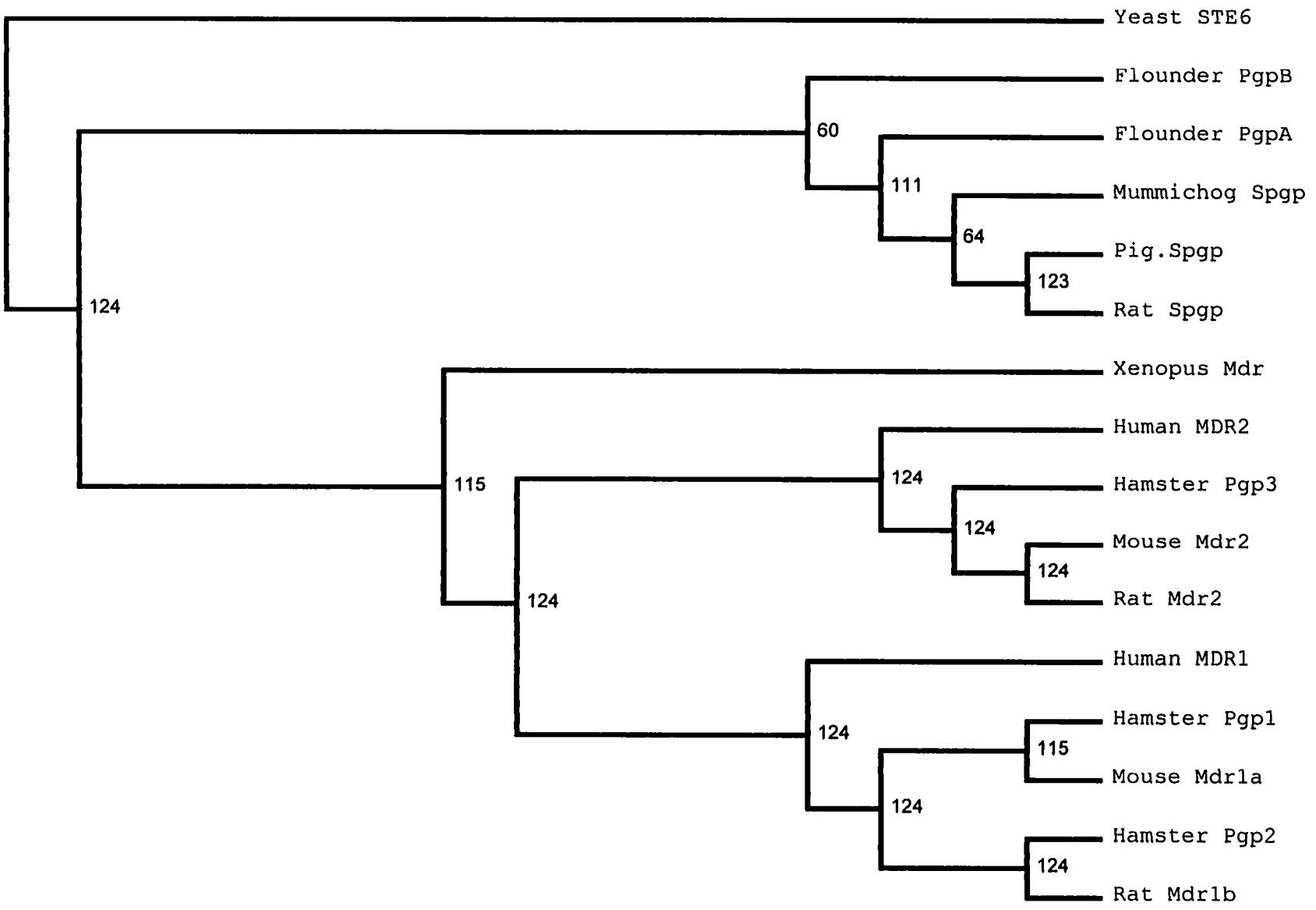


Figure 15. Cladistic analysis of the relationships among the vertebrate P-glycoprotein 3' nucleotide sequences including the sequences with limited data (flounder pgpA and pgpB and pig spgp). Analysis was accomplished with the PAUP computer program (Swofford, 1993). Branch lengths are not shown because of incomplete data. **A:** Single most parsimonious tree obtained with all sequences. Levels of bootstrap support for each node are given. Values are the number of times out of 124 replicates of resampling and replacement that the groups subtended by that node were supported. **B:** Single most parsimonius tree obtained without the winter flounder pgpB sequence. References: winter flounder pgpA and pgpB (Chan et al., 1993), pig spgp (Childs et al., 1995). The references for the other sequences are given in **Figure 14**.



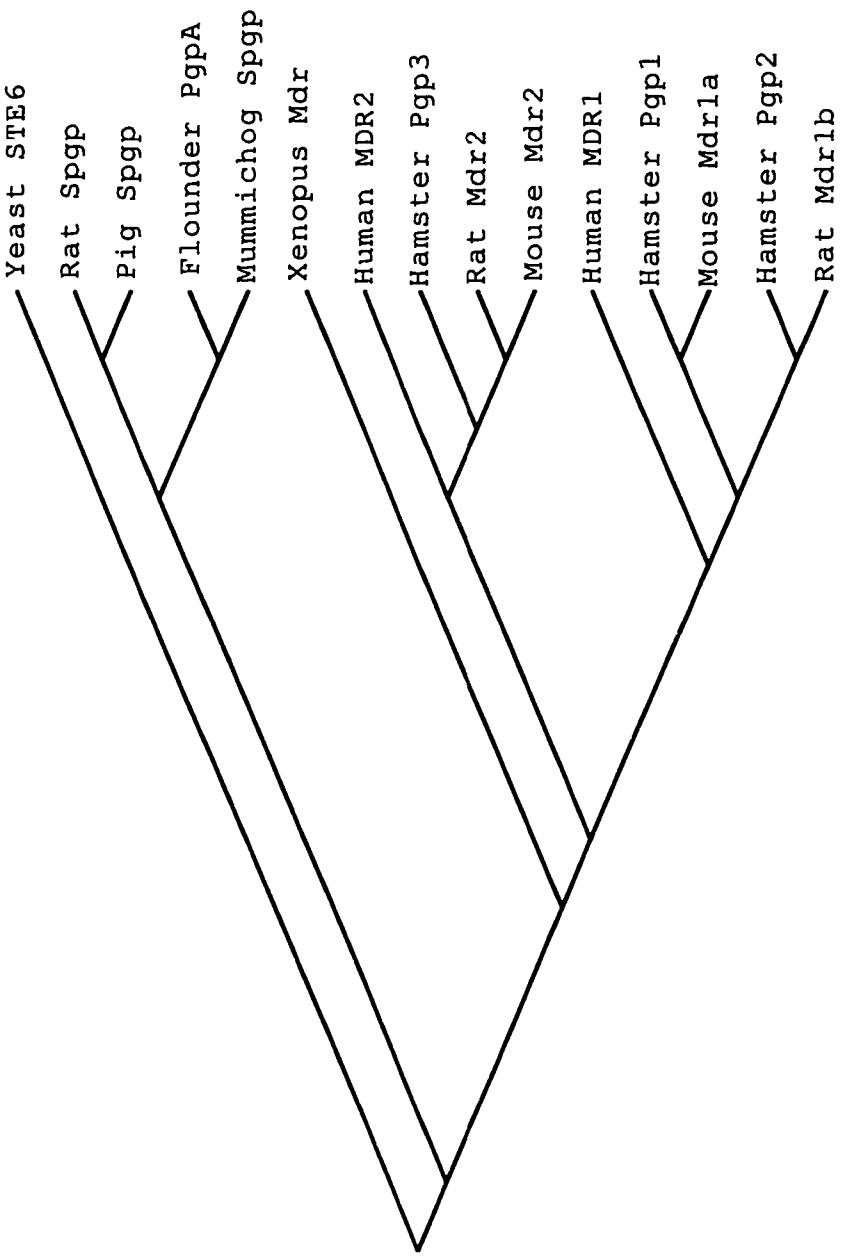


Figure 16. Immunoblot of membrane fractions (30 μg per lane) from mummichog organs probed with mAb C219. **Lane 1:** Extract of cholchicine resistant CH^RC5 cell line. **Lane 2:** Gill. **Lane 3:** Brain. **Lane 4:** Intestine. **Lane 5:** Anterior kidney. Positions of molecular weight standards are shown at the left.

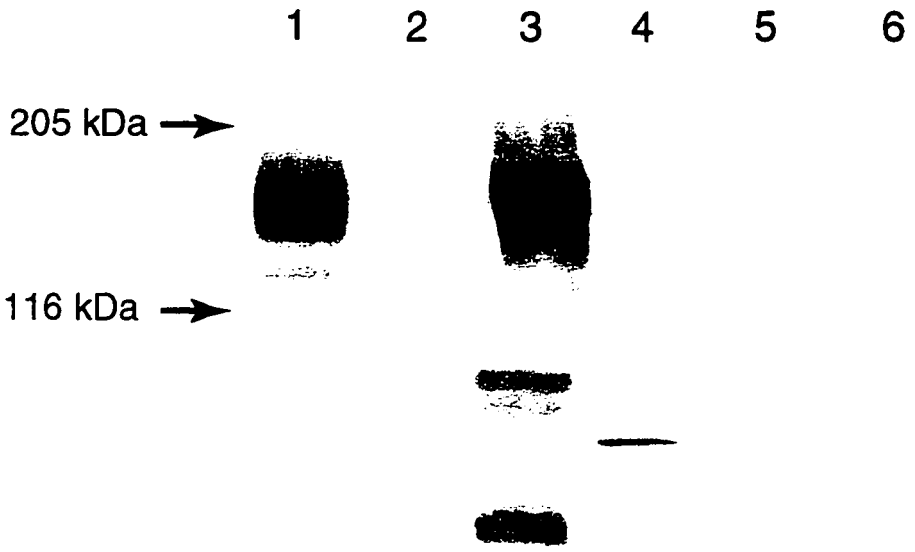
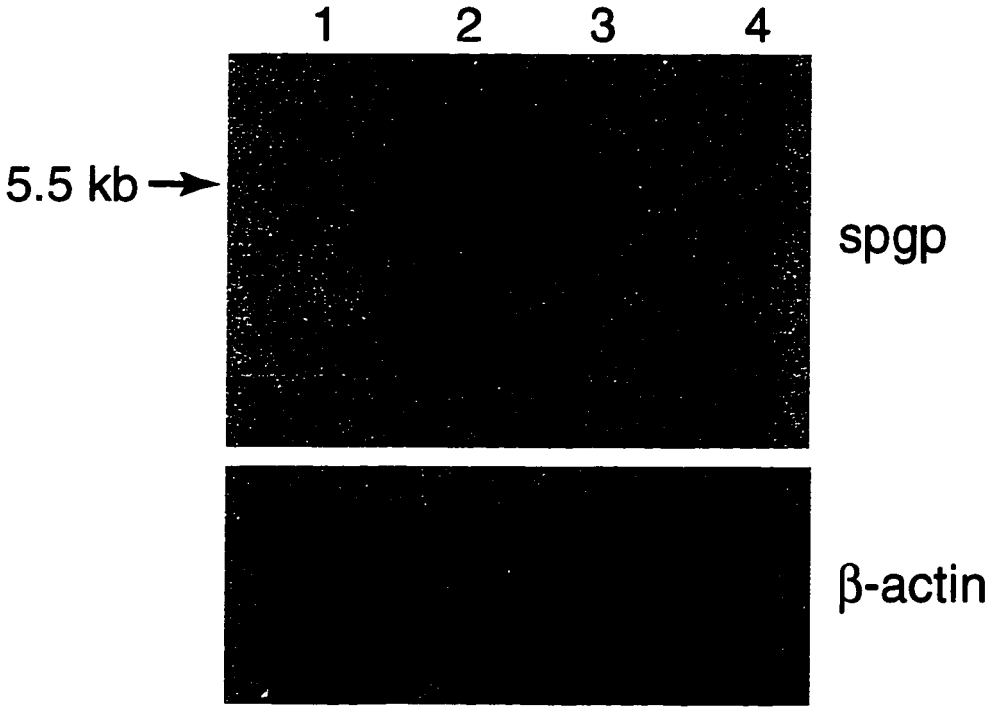


Figure 17. Northern blot of total RNA from mummichog organs probed with DIG-labeled mummichog spgp fragment S4 (upper panel) or mummichog β-actin fragment (lower panel). Hybridizing bands were identified using anti-digoxigenin antibody conjugated with alkaline phosphatase and Lumiphos 530 as a chemiluminescent substrate. **Lane 1:** Anterior kidney. **Lane 2:** Liver. **Lane 3:** Brain. **Lane 4:** Intestine.



Chapter V: Partial sequence of a multidrug resistance transporter (mdr) cDNA from the mummichog (*Fundulus heteroclitus*)

INTRODUCTION

Chapter IV of this dissertation reports on the analysis of cDNA fragments from the liver of the mummichog which were derived from transcripts of the mummichog sister of P-glycoprotein gene. Using one of these amplified fragments as a probe in northern blots, we have shown that, as in mammals (Childs et al., 1995), expression of the *spgp* gene may be restricted to liver in the mummichog. This is the first information on tissue specific expression of Pgp genes in a teleost. These results indicate that some of the signal seen in the immunoblots and immunohistochemical staining of mummichog liver and liver tumors reported in Chapters II and III of this dissertation results from expression of the mummichog *spgp* gene. The toxicological significance of expression of spgp P-glycoprotein homologs is unknown the physiological role of these proteins has not been determined in any animal. Our primary interest in P-glycoprotein expression in mummichog is understanding the role that expression of potential xenobiotic transporter (mdr1-type) P-glycoproteins may play in the resistance or tolerance of mummichog to the toxic components of creosote. Results reported in Chapter III of this dissertation show elevation of one or more Pgp homologs in the livers and liver tumors of mummichog from a creosote contaminated site. The elevation of Pgp in the livers of a chemically resistant fish is interesting because of the paralell to the role of the mdr1 P-glycoproteins in xenobiotic transport in drug-resistant cell lines and tumors. Several other biochemical alterations reported in the livers of these fish are similar to alterations seen in drug-resistant cell lines and tumors (Van Veld and Westbrook, 1995). However understanding the sigificance of elevated hepatic Pgp will require identification and characterization of

the specific Pgp homologs that are elevated in the liver and liver tumors of the Atlantic Wood mummichog because of the diversity of function of the vertebrate P-glycoprotein gene products. An approach to this problem is molecular cloning and sequence analysis of the P-glycoprotien transcripts expressed in mummichog liver.

In the present study we have used the same RT-PCR strategies reported in Chapter IV to amplify overlapping fragments corresponding to a second member of the Pgp gene family from mummichog intestine and liver RNA. We report that we have amplified and cloned cDNA fragments that are most closely related to the vertebrate mdr branch of the P-glycoprotein multigene family. These fragments are probably those of the mummichog mdr1-type P-glycoprotein homolog, a xenobiotic transporter. This is represents the first cDNA sequence of an mdr homolog from a teleost. Gene specific probes derived from these fragments should be useful in investigating expression of mdr genes in teleosts. Increased expression of this homolog in the liver s of Atlntic Wood fish could potentially contribute to the survival of these fish in the creosote-contaminated environment.

MATERIALS AND METHODS

Procedures for collection and processing of mummichog liver and intestinal RNA, synthesis of first strand cDNA, general conditions for amplifications of cDNAs by the polymerase chain rection and cloning and sequencing of PCR products were identical to those detailed in Chapter IV of this dissertation. Procedures specific to the present Chapter are given below.

Southern blot identification of mdr specific amplification products— Because many of the amplification reactions gave multiple products, it was necessary to identify amplified mdr fragments by Southern blotting. A fragment of the human MDR1 cDNA- pHDRV13 (Choi et al., 1988)- was used as a probe in Southern blots of PCR reactions to identify mdr specific fragments. An aliquot (10 µl) of the amplification reaction was separated on a 2% agarose gel. The separated products were capillary transferred to postively charged nylon membranes (Boehringer-Mannheim, Indianapolis, Indiana) according to standard protocols (Sambrook et al., 1989). An antibody-based colorimetric labeling and detection system (Genius™ System, Boehringer-Mannheim, Indianapolis, Indiana) was used to prepare digoxigenin (DIG) labeled cDNA probe and to detect hybridizing species in Southern blots. Labeled pobe was prepared by random priming of the gel-purified (GeneClean) HDR13 fragment with digoxigenin-11-dUTP using the Genius System Labeling Kit (Boehringer-Mannheim). Following transfer and UV cross-linking of the DNA to the membrane, the membrane was prehybridized for 1 hr at 65° C in 5X SSC containing 0.1% sodium N-lauroylsarcosine, 0.1% SDS and 1% Boehringer-Mannheim blocking reagent. After 1hr, the prehybridization solution was replaced with fresh prehybridization solution that contained an estimated 25 ng/ml of DIG-labeled probe. The membrane was hybridized overnight at 50° C. On the following day, the membrane was washed briefly in 2X SSC, 0.1% SDS and then in two changes of 0.5X SSC, 0.1% SDS at 50° C for 15 min each. After these washes, DIG-labeled hybrids were detected on the membrane according to the Genius System protocol using anti-digoxiginen alkaline phosphatase conjugate with p-nitro blue tetrazolium (NBT) and 5-

bromo-4-chloro-indolyl phosphate (BCIP) as colorimetric substrates for the alkaline phosphatase conjugate.

Amplification of the 5' ATP binding region of mummichog mdr cDNA— Degenerate oligonucleotide primers were used to amplify the cDNA sequences corresponding to the 5' ATP binding region of the mummichog mdr transcript from poly A+ selected intestinal RNA. These primers were used in Chapter IV to amplify the downstream ATP binding region of the mummichog spgp cDNA from liver RNA . The sense strand (upstream) primer (primer A, Table 3, Chapter IV this dissertation) matches codons near the conserved Walker A motif which occurs twice in all known Pgp deduced amino acid sequences. The antisense strand (downstream) primer (primer B, Table 3, Chapter IV this dissertation) matches codons near the conserved Walker B motif which also occurs twice in all known P-glycoproteins. A touchdown PCR protocol similar to that given in Chapter IV was used to ampilify this region of the cDNA. Primers A and B (4 μM each) were used with random primed first strand cDNA pool from mummichog intestine. Following denaturation at 94° C, the primers were annealed for 1 min at 60° C. The products were then extended at 72° C for 1.5 min followed by denaturation at 94° C for 30 sec. Fifty cycles were carried out with an incremental decrease of 0.25° C in the annealing temperature at each cycle. The annealing temperature maintained at 45° C for ten more cycles. A final extension was performed at 72° C for 10 min. The resulting product (M1) was cloned and sequenced.

Amplification of segments downstream of the ATP binding fold of the mummichog mdr cDNA— Primer A (4 μM) was used in a 3' RACE protocol (Frohman

et al., 1988) with an adapter primer (Table 3, Chapter IV this dissertation) to amplify first strand cDNA pool from mummichog intestine. The first strand synthesis of this cDNA pool had been primed with an oligo$(dT)_{17}$-adapter primer (Frohman et al., 1988). The touchdown cycling protocol used here was identical to that used above for amplification of the nucleotide binding fold sequences. To increase the yield of specific product a second round of amplification was performed using another sense strand degenerate primer (primer C, Table 3, Chapter IV this dissertation) which is downstream of the primer A sequence. In the second round of amplification 1 µl of the 3'-RACE reaction was used with primer C (8 µM) and the adapter primer (0.5 µM). Annealing was at 54° C for 30 sec followed by extension at 72° C for 30 sec and denaturation at 94° C for 30 sec. No touchdown was used in this amplification. After 40 cycles, products were extended for an additional 10 min at 72° C. A 460 bp product (M2) was obtained which was cloned and sequenced.

Primer C was also used with two gene specific primers in a pair of nested reactions to amplify mdr cDNA fragments from mummichog liver RNA. In the first reaction, primer C was used with a downstream spgp-gene specific primer (primer 111, CT-GAT-GTC-TTG-GCC-TTG-GCG). Primer 111 matches the sequence of the mummichog spgp cDNA (reported in Chapter IV) just downstream of the nucleotides coding for transmembrane segment 12. We observed that RT-PCR amplification of mummichog liver RNA with the mummichog spgp specific primer 111 and the degenerate Walker B primer (primer C) resulted in two products which hybridized to a fragment of the MDR1 cDNA in Southern blots (not shown). Because the mummichog

spgp cDNA is longer than the mdr cDNAs in the region amplified by these two primers, we assumed that the shorter product detected in Southern blots was an mdr product. We therefore used the mdr specific primer BLFA (CTG-GTT-ACC-ATG-CAG-ACC-TTC) that was designed from the sequence of the 460 bp mdr cDNA fragment (M2) from intestine with primer 111 to reamplify an aliquot of PCR products derived from liver cDNA, primer C and primer 111. For primer 111 and primer C annealing was at 45° C for 1 min followed by extension at 72° C for 2 min and denaturation at 94° C for 30 sec. After 40 cycles, products were given a final extension for 10 min at 72° C. An aliquot of this reaction (1 µl) was then subjected to a second round of amplification with primer 111 and BLFA . This mixture underwent 40 cycles of annealing at 58° C for 1 min, followed by extension at 72° C for 1.5 min, and denaturation 94° C for 30 sec. Products were extended for an additional 10 min at 72° C. A 1.2 kb product was obtained which was cloned and sequenced.

Sequence analysis— Sequences were compiled, edited and analyzed essentially as described in Chapter IV. Many features of the sequence were identified by inspection of the multiple sequence alignment and comparison with cDNA and deduced amino acid sequences of human (Chen et al., 1986; van der Bliek et al., 1988) hamster (Endicott et al., 1991) and *Xenopus* (Castillo et al., 1995) mdr isoforms. Sequences of Pgp homologs were obtained from the GENBANK sequence database using the World Wide Web server at the National Center for Biotechnology Information (National Institutes of Health, Bethesda, Maryland).

Multiple alignment of the amino acid sequences of Pgp sequences was

accomplished with the aid of the computer program CLUSTAL W (Thompson and Higgins, 1994) using the default parameters of the program. Similarity and identity scores on aligned sequences were calculated using the computer program Genedoc (Nicholas and Nicholas, 1996). For amino acid sequences, similarity scores were calculated using the BLOSUM62 matrix provided with the program.

Relationships among vertebrate P-glycoprotein sequences were assessed by distance analysis of the deduced amino acid sequences of vertebrate Pgp homologs. The sequence of the MdrA/Pgp1 P-glycoprotein from the nematode, *Caenorhabditis elegans* (Lincke et al., 1992) was included in the analysis to serve as an out-group sequence. As discussed in Chapter IV, this nematode sequence cannot be assigned to either the vertebrate mdr of spgp P-glycoprotein categories. The deduced amino acid sequences were aligned with the aid of CUSTAL W over the length of the mummichog mdr sequence. This gave an alignment with a consensus length of 490 positions including gaps. The distance analysis was done with the aid of the PHYLIP (Version 3.5c) computer program package (Felsenstein, 1993). A pairwise distance matrix of the aligned amino acid sequences was calculated with the PHYLIP Protdist program using the PAM/Dayhoff option. The resulting distance matrix was used in the PHYLIP implemetation of the Neighbor-Joining (Saitou and Nei, 1987) method to construct a gene tree.

RESULTS

Amplification and analysis of the 5' ATP binding region of mummichog mdr cDNA— Degenerate oligonucleotide primers were used in RT-PCR to amplify a cDNA fragment (M1) coding for the ATP binding domain of a P-glycoprotein from mummichog intestinal RNA (Figure 18). The mummichog nucleotide sequence and the deduced amino acid sequence were most similar to the *Xenopus* and hamster P-glycoprotein cDNA sequences corresponding to the 5' or amino (N) terminal ATP binding region. Optimal alignment with the carboxyl-terminal ATP binding domain amino acid sequences required inserting a two position gap in the mummichog sequence (Figure 19). This gap was also required to align the N- and C- terminal ATP binding domains of the other vertebrate mdr-type P-glycoproteins. At the nucleotide level the identity of the mummichog sequence to the 5' vertebrate sequences ranged from 71% to 75% identity while the identity to the 3' sequences was lower, 59-62%. At the amino acid level, the mummichog sequence was most similar to the N-terminal ATP binding domain of the *Xenopus* and hamster mdr sequences (91-94% similar, 78-81% identical) and less similar to the C-terminal ATP binding domain of these proteins (80-83% similar, 58-65% identical, Table 5). These relationships paralleled the relationships between the two ATP binding domains of the *Xenopus* and hamster sequences (Table 5). Furthermore comparison of the two domains *within* the individual *Xenopus* and hamster mdr sequences gave analogous relationships (78-80% similar, 56-64% identical, Table 5).

Amplification and analysis of cDNA sequences downstream of the 5' ATP binding domain of mummichog mdr— We used degenerate primers (primers A and C)

in a nested 3' RACE protocol in an attempt to amplify the 3' end of the mummichog mdr cDNA from mummichog intestinal RNA. Repeated attempts resulted in no specific product of predicted size. However, a 460 bp product was detected in Southern blots of the 3' RACE reactions probed with a fragment of the human MDR1 cDNA (data not shown). The sequence of the 5' end of this product (M2, Figure 18) was identical with 48 bp of the 3' end of the amplified mummichog mdr ATP binding domain sequence (M1) and contains the upstream primer (primer C) and the 3' RACE adapter primer in the expected orientation. However the sequence continued the reading frame of the upstream primer without an in-frame termination codon and therefore did not contain the 3' end of the cDNA. The deduced amino acid sequence of the 5' end of this cDNA fragment was similar to that of the ATP binding domains of vertebrate P-glycoproteins. The amino acid sequence had the highly conserved amino acid positions surrounding the Walker B nucleotide binding motif of ABC transporters and the peptide epitope (Val-Glu-Ala-Ala-Leu-Asp) for the mAb C219 present in all P-glycoproteins (Figure 18). The deduced amino acid sequence of the 3' end of the M2 segment was weakly similar to the linker domain of vertebrate P-glycoproteins. The deduced amino acid sequence of the M2 fragment was 50-53% identical to the corresponding positions in the carboxyl terminal ATP-binding domains and linker domains of the hamster, human and *Xenopus* mdr-type P-glycoproteins. The overlapping sequence of M1 and M2 occurs in a highly conserved region. The nucleotide sequences of the highly related hamster *pgp1* and *pgp2* nucleotide sequences (Endicott et al., 1991) are 100 % identical in this region. For this reason there is a possibility that M1 and M2 were amplified fragments of different P-glycoprotein

genes.

The sequence of M2 was used to design a gene specific primers for 3' RACE of the mummichog mdr cDNA. Repeated 3' RACE attempts yielded no specific product from mummichog intestinal RNA. The major product amplified was an nonspecific 800 bp fragment which contained the adapter primer at both ends. However we did amplify an mdr specific product when we used one of the mummichog mdr primers (PBLFA) in a nested amplification of mummichog liver RNA. The 5' end of the sequence of the resulting 1200 bp product (M3, Figure 18) overlapped with 146 bp of the sequence of M2. The deduced amino acid sequence of the 3' end of M3 aligned with and was similar to the transmembrane domain of vertebrate P-glycoproteins. The deduced amino acid sequence of the mummichog M3 fragment was 53-55% identical and 72-74% similar to the corresponding positions of the linker domain and transmembrane domain of the hamster, human and *Xenopus* mdr-type P-glycoproteins. The similarity to the mummichog spgp sequence was lower (36% identical, 58% similar).

Five membrane spanning helices were identified in the deduced amino acid sequence of the M3 fragment using the method of Eisenberg (1984). The transmembrane helices corresponding to TM9 and TM10 of the human and hamster P-glycoproteins were identified as a single transmembrane helix in our analysis. However because the Kyte and Doolittle hydropathy plots (Kyte and Doolittle, 1982) of the mummichog M3 amino acid sequence and the hamster pgp1 P-glycoprotein (Figure 20) were nearly identical over these positions, the locations of TM9 and TM10 have been assigned by comparison with the hamster pgp1 sequence (Endicott et al., 1991).

The sequences of clones of M2 and M3 were nearly identical (98%) in the 145 bp overlapping portion that corresponds to the poorly conserved linker domain of P-glycoproteins. The nucleotide sequence of the hamster pgp1 and pgp2 cDNAs are only 67% identical in this region while the pgp3 sequence is 43% and 36% identical to pgp1 and pgp2 in this region. Therefore it is likely that M2 and M3 were derived from transcripts of the same gene.

The deduced amino acid sequences of M2 and M3 contained serine and threonine residues in consensus protein kinase recognition sequences (Kemp and Pearson, 1990) for both the calcium and phospholipid dependent protein kinase (protein kinase C) and the cyclic AMP-dependent protein kinase (protein kinase A). The sequence (Thr/Ser)-X-(Arg/Lys) compatible with protein kinase C phosphorylation occured six times in the combined sequences of M2 and M3 (Figure 18). Only one of these locations is within the linker domain and coincides with the pair of potential protein kinase A phosphorylation sites in the sequence Arg-Arg-Lys-Ser-Thr-Arg. This corresponds to the conserved protein kinase A recognition sequence present in the linker domain of human MDR1 and rodent mdr1a P-glycoproteins (Hsu et al., 1989; Endicott et al., 1991).

The deduced amino acid sequences of M2 and M3 are shown combined in the multiple sequence alignment of vertebrate P-glycoproteins in Figure 21. The alignment spans the carboxyl end of the N-terminal ATP binding domain, the complete linker domain and the complete transmembrane domain with the six putative membrane spanning segments (TM7-TM12) of the second half of P-glycoproteins. The mummichog sequence was most similar to the vertebrate mdr-type P-glycoproteins in the alignment

(56-58% identical, 74-77% similar) and less similar to the mummichog spgp sequence (42% identical, 61% similar). The Walker B region of the ATP binding domain and the intracellular loop between TM8 and TM9 were the best conserved regions among all of the sequences. The putative extracellular loop between TM7 and TM8 and the linker domain were regions of reduced similarity among all sequences. Significant gaps were required to align the sequences in the linker region particularly to accommodate the longer and quite divergent linker domain of the mummichog spgp sequence.

Relationships among P-glycoprotein sequences— The pairwise distances between the amino acid sequences corresponding to the combined mummichog M2 and M3 segments of vertebrate P-glycoproteins were analyzed using the Neighbor-Joining method. The resulting phenogram (Figure 22) demonstrated that the mummichog sequence derived from M2 and M3 was more closely related to the vertebrate mdr-type sequences than to the mummichog and rat spgp forms. The mummichog sequence was basal to the remaining vertebrate mdr-type sequences and like the *Xenopus* sequence was not assigned to either the mammalian mdr1 (human MDR1, hamster pgp1 and pgp2) or the mdr2 (human and hamster mdr2) classes by this method.

DISCUSSION

In this study we cloned and sequenced RT-PCR amplified fragments of a P-glycoprotein cDNA from the mummichog liver and intestinal RNA. The sequence of these combined fragments indicates that they are amplified fragments of a vertebrate mdr-type P-glycoprotein. This is the first report of an mdr cDNA sequence from a teleost.

As a part of our on-going studies of P-glycoprotein expression in mummichog liver and liver tumors, we have used several RT-PCR strategies to amplify an mdr-type P-glycoprotein sequence from the mummichog. Although our primary interest is expression in liver we initially amplified the mdr cDNA sequences from mummichog intestine. In Chapter IV of this dissertation we report using degenerate oligonucleotide primers to amplify cDNA sequences corresponding to the ATP binding domain of the carboxyl terminal half of mummichog spgp in RT-PCR of mummichog liver RNA . Contrary to our expectations, we never obtained sequences for the amino-terminal ATP binding domain of spgp or the sequences of any other P-glycoprotein cDNAs from mummichog liver RNA with this technique. For this reason in the present study we applied this technique to mummichog intestinal RNA in to amplify mummichog mdr sequences. This organ was not expected to express *spgp* and in mammals expresses only mdr1-type P-glycoproteins (Croop et al., 1989; Teeter et al., 1990; Childs et al., 1995). Using the degenerate ATP-binding domain primers, we amplified cDNA sequences corresponding to the carboxyl-terminal ATP binding domain of a P-glycoprotein. Repeated attempts to amplify the 3' end of this cDNA from intestinal RNA failed possibly because of competing amplification of an undesired product. We amplified an abortive product which matched the N-terminal ATP-binding domain and linker domain of vertebrate mdr-type P-glycoproteins. This product may have resulted from mispriming of the first strand cDNA by the oligo$(dT)_{17}$ adapter primer in the A-rich codons of the linker region.

Using additional RT-PCR strategies we were able to amplify a cDNA fragment

from mummichog liver cDNA pool that overlapped with the downstream end of the abortive 3' RACE product from intestinal cDNA and aligned with the linker domain and transmembrane domain of the carboxyl terminal half of vertebrate mdr-type P-glycoproteins. Because the sequences of the liver and intestinal cDNA fragments matched in a poorly conserved region of P-glycoproteins, we conclude that these fragments are products of the same mummichog gene which is expressed both in mummichog intestine and mummichog liver.

The deduced amino acid sequence of the mummichog cDNA is very similar to the corresponding positions of vertebrate mdr-type P-glycoproteins and indicates a homologous structure for these proteins. This includes the highly conserved sequence near the ATP binding site, a hydrophillic linker domain and a hydropathy profile indicating the presence of six membrane spanning segments in the transmembrane domain of the protein.

Mammalian mdr-type P-glycoproteins may be divided into mdr1 and mdr2 categories based on sequence similarities. These two classes also have distinct patterns of expression and distinct functions in mammalian physiology. The mammalian mdr2-type P-glycoproteins, which are not involved in multidrug resistance, have been shown to have a role in transport of phosphatidylcholine into the bile (Smit et al., 1993; Ruetz and Gros, 1994). This is consistent with predominant expression of the mdr2 forms in the bile canaliculus of the liver (Buschman et al., 1992; Smit et al., 1994) . The mdr1 forms, which can confer multidrug resistance, may be involved in protection from toxic xenobiotics either by exclusion of toxicants from sensitive organs such as the brain and

testes or by excretion of xenobiotics through the apical membrane of excretory epithelia (Theibaut et al., 1987; Thiebaut et al., 1989; Teeter et al., 1990; Schinkel et al., 1994). The mdr1 forms may also be involved in steroid excretion or uptake in tissues involved in steroid metabolism (Ueda et al., 1992; van Kalken et al., 1993). The mdr gene products of fish have not been previously characterized. Southern blots of teleost genomic DNA indicate that fish, similar to humans, have two mdr-type P-glycoprotein genes (Ling et al., 1992; Chan et al., 1993). The products of these two teleost genes are probably the functional equivalents of the human MDR1 and MDR2 proteins.

In a effort to assign the mummichog mdr sequence to either the mdr1 or mdr2 categories we have analyzed the relationships among the vertebrate P-glycoprotein genes using a distance approach. While this analysis confirms that the mummichog mdr sequence is most closely related to the vertebrate mdr homologs rather than the spgp homologs, it also indicates that the mammalian mdr1 and mdr2 homologs are more similar to each other than either class is to the mummichog homolog. The high similarity of the mammalian mdr sequences has been noted before (van der Bliek et al., 1988; Endicott et al., 1991; Hsu et al., 1991; Chan et al., 1992). There is evidence that a homogenization mechanism, concerted evolution (Arnheim, 1983), has erased many of the intraspecies differences between the *mdr* genes in the hamster (Endicott et al., 1991), the mouse (Hsu et al., 1991) and man (van der Bliek et al., 1988). This has masked the more ancient origin of the mammalian mdr1 and mdr2 gene lineages. The concerted evolution among the mammalian genes within taxa makes the inference of the functional class of the mummichog mdr sequence based on sequence homologies difficult. This is

especially true because the sequential determinants conferring differing functions on the mdr1 and mdr2 P-glycoproteins are unknown.

In spite of this, comparison of the linker domains of the P-glycoprotein deduced amino acid sequences indicates that the mummichog mdr sequence may be an mdr1-type P-glycoprotein. The linker domain is a poorly conserved region that contains sequences that are not conserved between the mammalian mdr1 and mdr2 classes (Hsu et al., 1989). Evidence from analysis of silent and replacement substitutions along the length of the human mdr3 sequence suggested that gene conversion has not affected this domain (Van der Bliek et al., 1988). The linker region has been identified as a regulatory domain in P-glycoproteins and may be the primary site of phosphorylation. The human MDR1 P-glycoprotein is phosphorylated by both protein kinase A (Mellado and Horwitz, 1987) and protein kinase C *in vitro* (Chambers et al., 1992). The linker domain contains conserved recognition sequences for protein kinase C in both mdr1-type and mdr-2 P-glycoproteins (Hsu et al., 1989; Endicott et al., 1991; Chambers et al., 1993). The linker region of mammalian mdr1 P-glycoproteins also has protein kinase A recognition sequences which are lacking in mdr2 P-glycoproteins. An unusual overlapping protein kinase A recognition sequence with two potential phosphorylation sites (Arg/Lys)-(Arg/Lys)-(Arg/Lys)-Ser-(Ser/Thr) is present in the linker regions of the human MDR1 and rodent mdr1a homologs (Hsu et al., 1989; Endicott et al., 1991; Chambers et al., 1993). This sequence is also present in the mummichog mdr linker domain. This is evidence that the mummichog mdr protein is regulated in a similar manner to mammalian mdr1 proteins and that the mummichog mdr protein may have a function similar to the

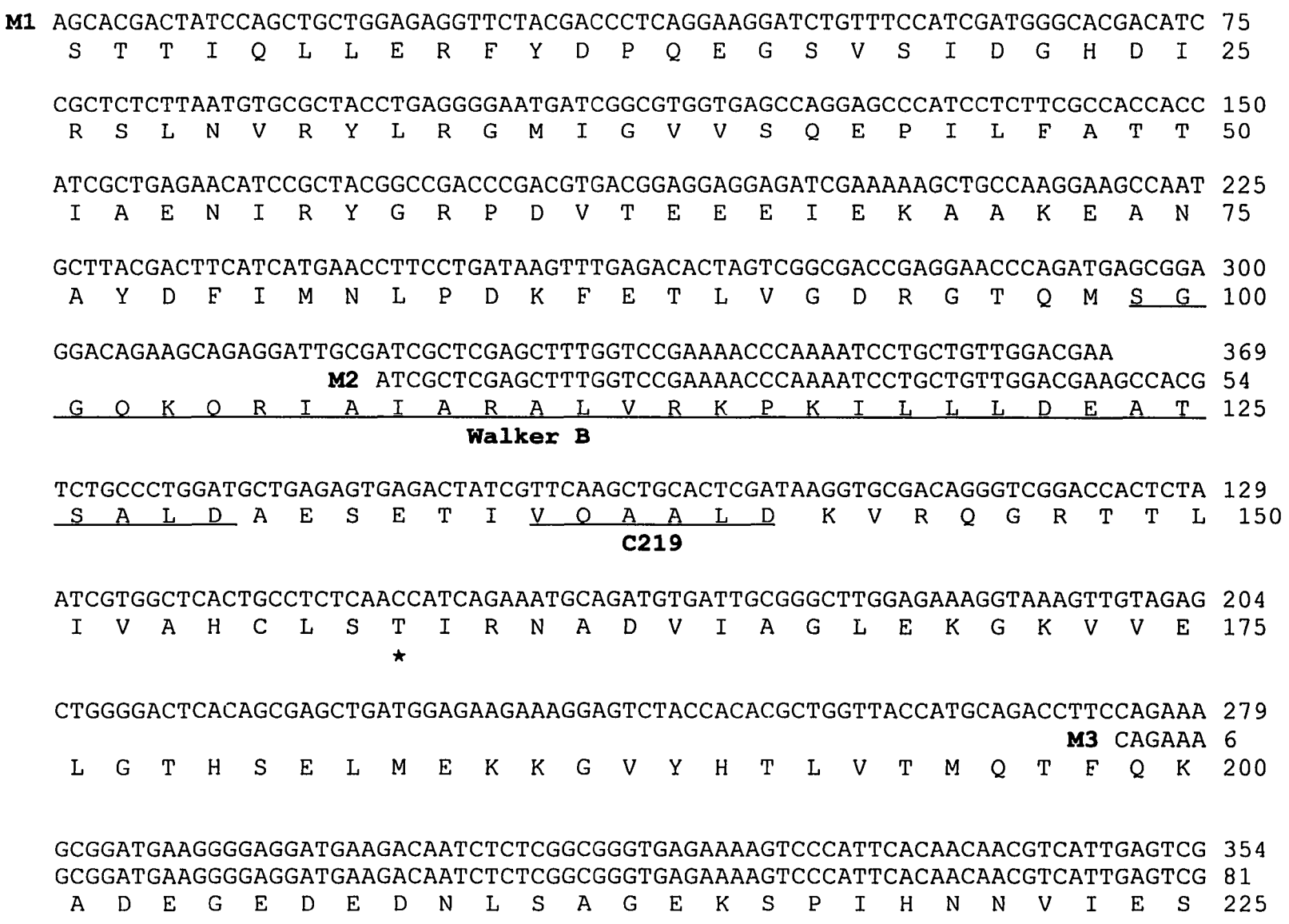
mammalian mdr1 proteins.

In mammals expression of both *mdr1* and *mdr2* class genes has been detected in liver (Theibaut et al., 1987; Teeter et al., 1990; Buschman et al., 1992; Smit et al., 1994). However only *mdr1* expression has been found in mammalian intestine (Croop et al. 1989; Teeter et al., 1990). P-glycoprotein in teleosts has been detected by immunohistochemical staining on the lumenal surfaces of the epithelia of the intestine and kidney and on the canalicular surface of hepatocytes (Hemmer et al., 1995; Chapters II and III, this dissertation) just as in mammals. Although the specific homologs that were expressed in these sites in fish tissues were not identified in these immunohistochemical studies, the results indicate that the tissue specific expression patterns observed in mammals for mdr1 and mdr2 P-glycoproteins are probably conserved in teleosts. Therefore amplification of the mummichog mdr from both liver and intestinal RNA in the present study is further evidence that the cDNA fragments reported here those of an mdr1-type P-glycoprotein with similar tissue specific expression patterns to the mammalian *mdr1* genes.

We have amplified fragments of a mummichog mdr cDNA from mummichog liver. This may be a portion of the cDNA for the mummichog xenobiotic transporter (mdr1). In Chapter IV we identified another member of the P-glycoprotein gene family (spgp) expressed in mummichog liver. We have now identified two of the potential gene products contributing to the signal seen in our immunochemical studies of P-glycoprotein expression during environmental hepatocarcinogenesis in the Atlantic Wood mummichog. Gene specific cDNA probes are now in hand for both the *mdr1* and *spgp*

genes. Future studies using cDNA probes should investigate tissue specific expression patterns of the mummichog *mdr1* gene and should study regulation of expression of both genes by xenobiotics. It is necessary to test whether increased expression of either of these genes is associated with the elevated levels of P-glycoprotein antigens that we have observed in liver and liver tumors of these fish. With a partial sequence of the mummichog xenobiotic transporter now in hand, it should be possible to specifically address the role of this protein in tolerance or resistance of Atlantic Wood mummichog to creosote.

Figure 18. Nucleotide (upper) and deduced amino acid (lower) sequences of the mummichog mdr cDNA fragment determined from the sequence of RT-PCR amplified cDNA products (M1-M3) from mummichog intestine (M1 and M2) and liver (M3). The nucleotide sequence is numbered in 5' to 3' orientation. The upstream degenerate primer sequence is not included. Amino acids are aligned with the second nucleotide of each codon. The conserved Walker B sequence of the nucleotide binding fold of ABC transporters and the C219 epitope are underlined. The six putative transmembrane segments are indicated with a double underline and are numbered TM7-TM12. Potential protein kinase A phosphorylation sites are indicated with a★ and potential protein kinase C phosphorylation site are indicated with a ⋆.



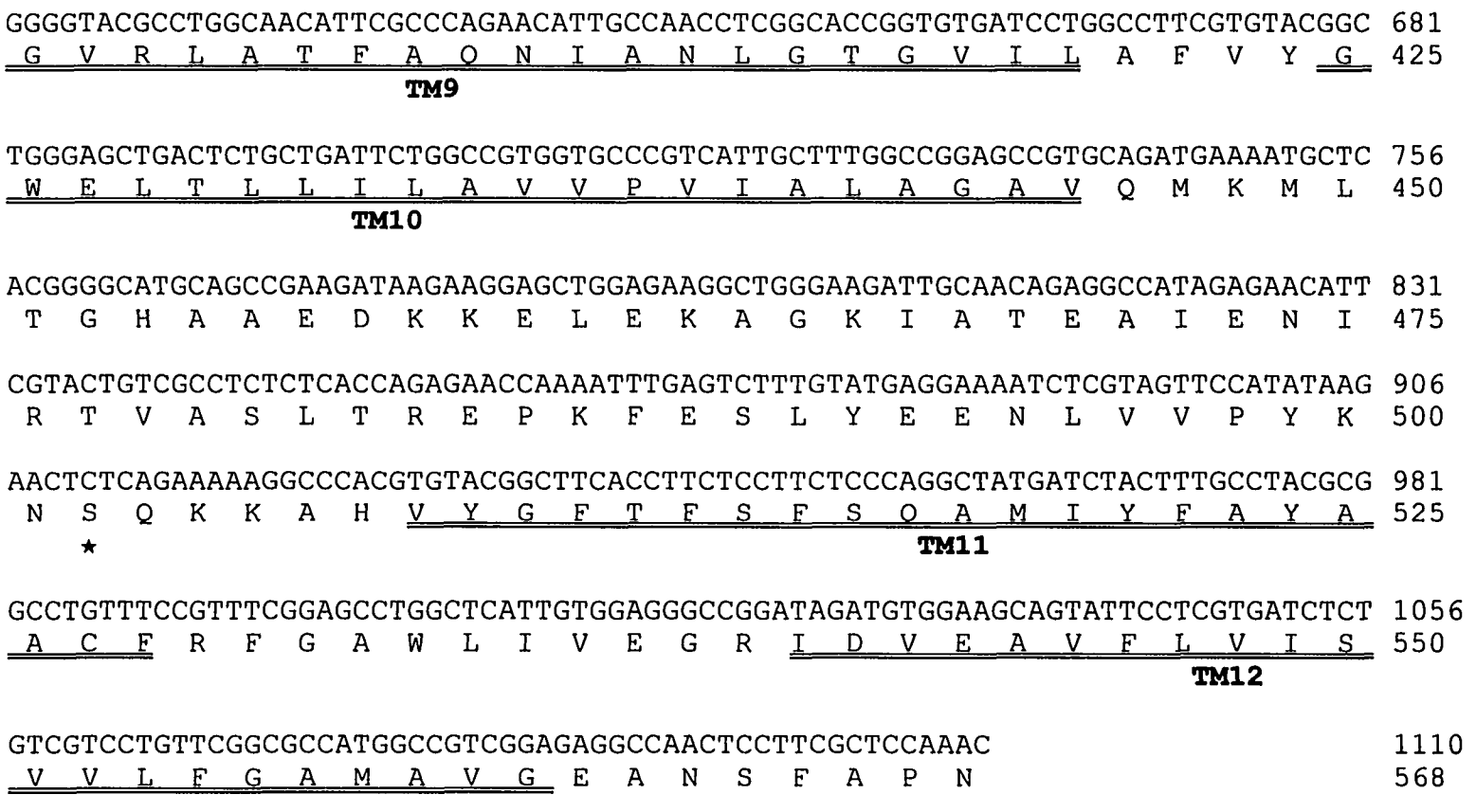


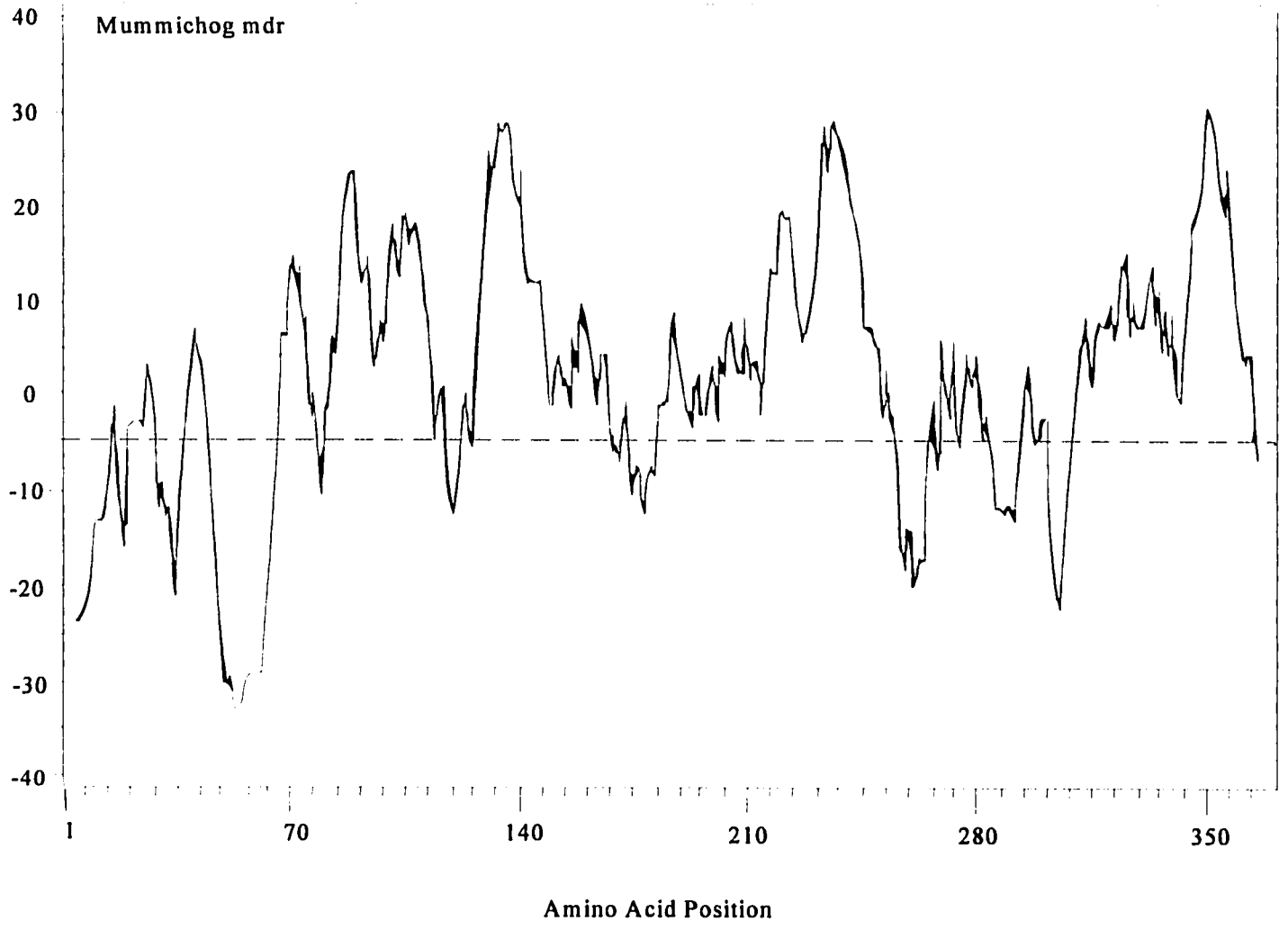
Figure 19. Alignment of the deduced amino acid sequence of the mummichog mdr ATP binding domain with the amino (N) terminal and carboxyl (C) terminal ATP binding domains of *Xenopus* and hamster mdr-type P-glycoproteins. Positions that are identical to the mummichog sequence are indicated with a dot (.). Gaps are indicated with a dash (-). *References*: *Xenopus* mdr (Castillo et al., 1995); Hamster sequences (Endicott et al., 1991).

```
Mummichog Mdr   STTIQLLERFYDPQEGSVSIDGHDIRSLNVRYLRGMIGVVSQEPILFA
Xenopus Mdr     ...V..IQ.....ED.VITL..Q......I....EI..........D
N Hamster Pgp1  ...V...Q.L...T..V.....Q...TI......EI........V...
N Hamster Pgp2  ...V...Q.L...T..V.....Q...TI......EI........V...
N Hamster Pgp3  T..L...Q.L...T..TI....Q...NF......EI........V..S
C Xenopus Mdr   ...VS........F..E.LV..LSV.N..IQWV.AQM.I........D
C Hamster Pgp1  ..VV.........MA.T.FL..KEVNQ...QW..AHL.I........D
C Hamster Pgp2  ..VV.........MA.T.FL..KE.KQ...QW..AHL.I........D
C Hamster Pgp3  ..VV.........MA.T.LL..QEAKK..IQW..AQL.I.....V..D


Mummichog Mdr   TTIAENIRYGRPD--VTEEEIEKAAKEANAYDFIMNLPDKFETLVGDR
Xenopus Mdr     ....D......E.--..K....R.T..........K....L.....E.
N Hamster Pgp1  ..........EN--..MD.....V..........K..H..D....E.
N Hamster Pgp2  ..........EN--..MD.....V..........K..H..D....E.
N Hamster Pgp3  ..........GN--..M...K..V......E...K..Q..D....E.
C Xenopus Mdr   CS.GD..A..DNNRK..Q....T......IHS..ES.T..YN.R...K
C Hamster Pgp1  CS.....A..DNSRV.SQD...R......IHQ..ES....YN.R...K
C Hamster Pgp2  CS.....A..DNSRV.SQD...R......IHQ..ES....YN.R...K
C Hamster Pgp3  CS.....A..DNSRV.SQD..VR...A..IHP..ET..Q.YK.R...K


Mummichog Mdr   GTQMSGGQKQRIAIARALVRKPKILLLDE
Xenopus         ...L..............N........
N Hamster Pgp1  .A.L..............N........
N Hamster Pgp2  .A.L..............N........
N Hamster Pgp3  .A.L..............N........
C Xenopus Mdr   ...L............I..........
C Hamster Pgp1  ...L..............Q.H......
C Hamster Pgp2  ...L..............Q.H......
C Hamster Pgp3  ...L.......L..R...I.Q.RV.....
```

Table 5. Pairwise comparison matrix of the deduced amino acid sequences of the mummichog mdr ATP binding domain and the N and C terminal ATP binding domains of the *Xenopus* and hamster mdr-type P-glycoproteins. The three numbers for each pair below the diagonal give the number of positions that are occupied by identical amino acids in the two sequences (top number), identical plus conservative substitutions (BLOSUM62) (middle number) and the number of positions that align with a gap in the other sequence (bottom number). The region of the matrix above the diagonal gives these values as percentages. The numbers along the diagonal give the number of residues in the region of comparison. *References*: *Xenopus* mdr (Castillo et al., 1995); Hamster (Endicott et al., 1991).

	Mummichog Mdr	N Xenopus Mdr	N Hamster Pgp1	N Hamster Pgp2	N Hamster Pgp3	C Xenopus Mdr	C Hamster Pgp1	C Hamster Pgp2	C Hamster Pgp3
		80%	81%	81%	78%	65%	65%	66%	58%
Mummichog Mdr	123	94%	91%	91%	92%	82%	82%	83%	80%
		0%	0%	0%	0%	1%	1%	1%	1%
	99		82%	82%	78%	64%	64%	64%	59%
N Xenopus Mdr	2	123	95%	95%	93%	80%	80%	80%	78%
	0		0%	0%	0%	1%	1%	1%	1%
	100	101		100%	90%	60%	61%	62%	57%
N Hamster Pgp1	3	117	123	100%	96%	77%	79%	80%	77%
	0	0		0%	0%	1%	1%	1%	1%
	100	101	123		90%	60%	61%	62%	57%
N Hamster Pgp2	4	117	123	123	96%	77%	79%	80%	77%
	0	0	0		0%	1%	1%	1%	1%
	96	97	111	111		58%	58%	59%	56%
N Hamster Pgp3	5	115	119	119	123	78%	80%	81%	78%
	0	0	0	0		1%	1%	1%	1%
	82	80	75	75	73		78%	77%	73%
C Xenopus Mdr	6	100	97	97	98	125	88%	88%	84%
	2	2	2	2	2		0%	0%	0%
	82	80	77	77	73	98		98%	84%
C Hamster Pgp1	7	99	98	98	100	110	125	99%	92%
	2	2	2	2	2	0		0%	0%
	83	81	78	78	74	97	123		85%
C Hamster Pgp2	8	100	99	99	101	111	124	125	93%
	2	2	2	2	2	0	0		0%
	73	74	72	72	70	92	106	107	
C Hamster Pgp3	9	97	96	96	97	106	116	117	125
	2	2	2	2	2	0	0	0	

Figure 20. Hydropathy plots (Kyte and Doolittle, 1982) of the mummichog mdr deduced amino acid sequence for cDNA fragment M3 (**A**) and the corresponding region of the hamster pgp1 (**B**) sequence (Endicott et al., 1991). The plots were created using the SOAP program of the PCGene computer program package (Intelligenetics, Mountain View, California) using a window of nine amino acids.



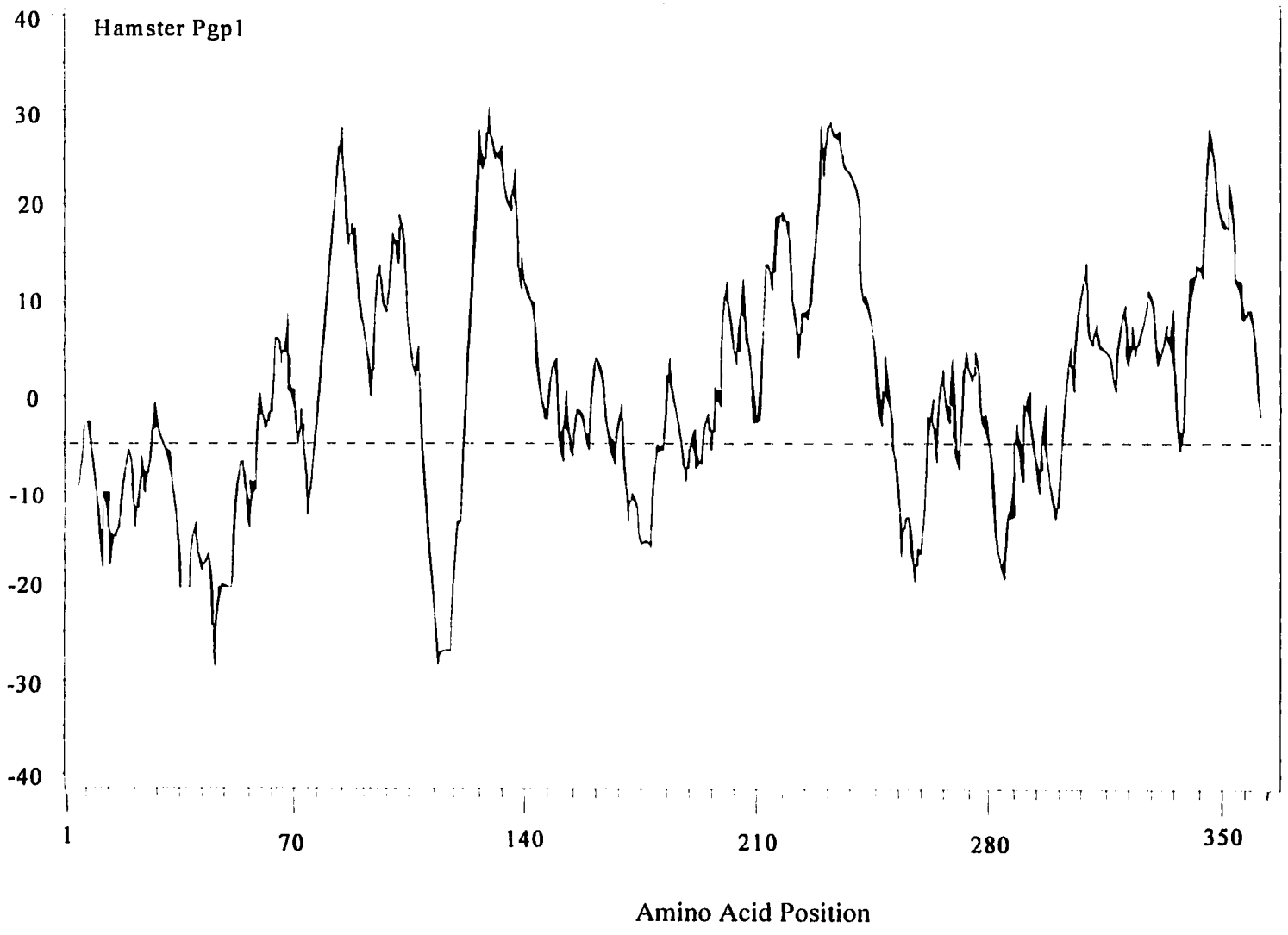
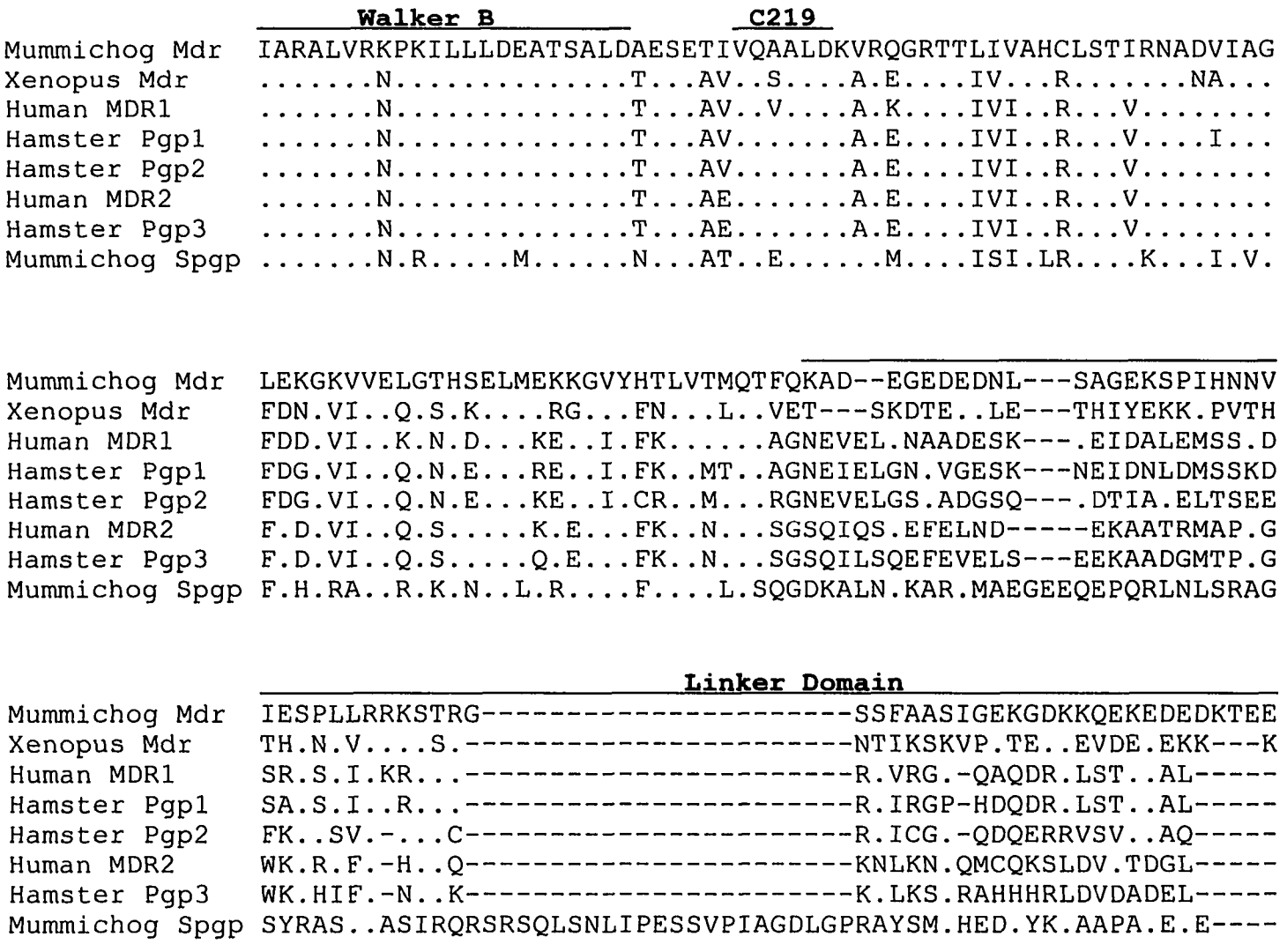
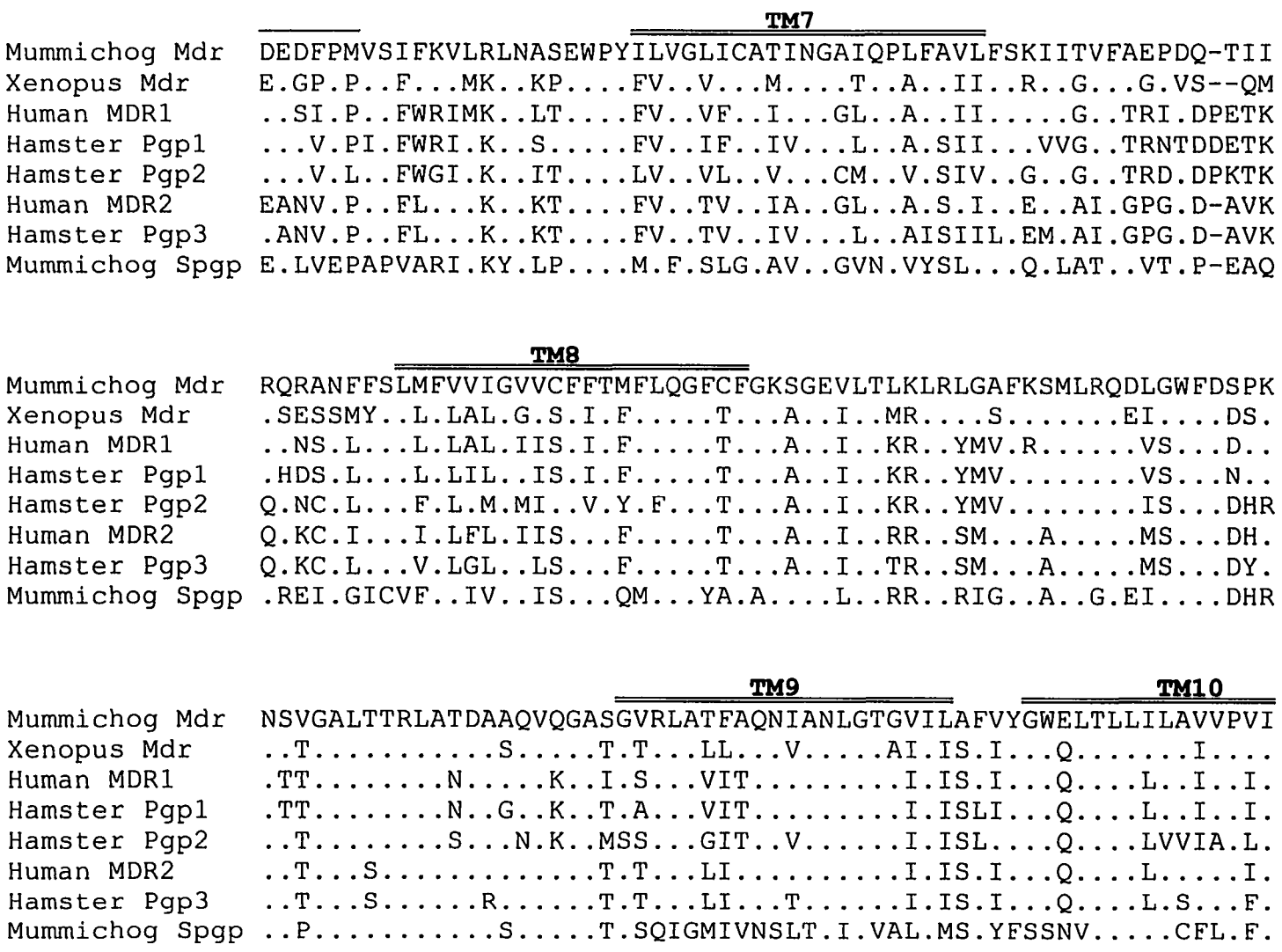


Figure 21. Multiple alignment of deduced amino acid sequences of selected P-glycoproteins over the region of overlap with the mummichog mdr sequence. Multiple alignments were created with the aid of the computer program CLUSTAL W (Thompson and Higgins, 1994). Positions with identical amino acids to mummichog spgp are shown as a dot (.). Gaps inserted into the sequences to optimize the alignment are shown as a dash (-). The positions of the conserved Walker B motif of ABC transporters, the linker region, the putative transmembrane segments (TM7-TM12) and the C219 epitope are indicated. *References*: *Xenopus* mdr (Castillo et al., 1995); human MDR1 (Chen et al., 1986); human MDR2 (van der Bliek et al., 1988); hamster pgp1, pgp2 and pgp3 (Endicott et al., 1991); mummichog spgp (Chapter IV, this dissertation).





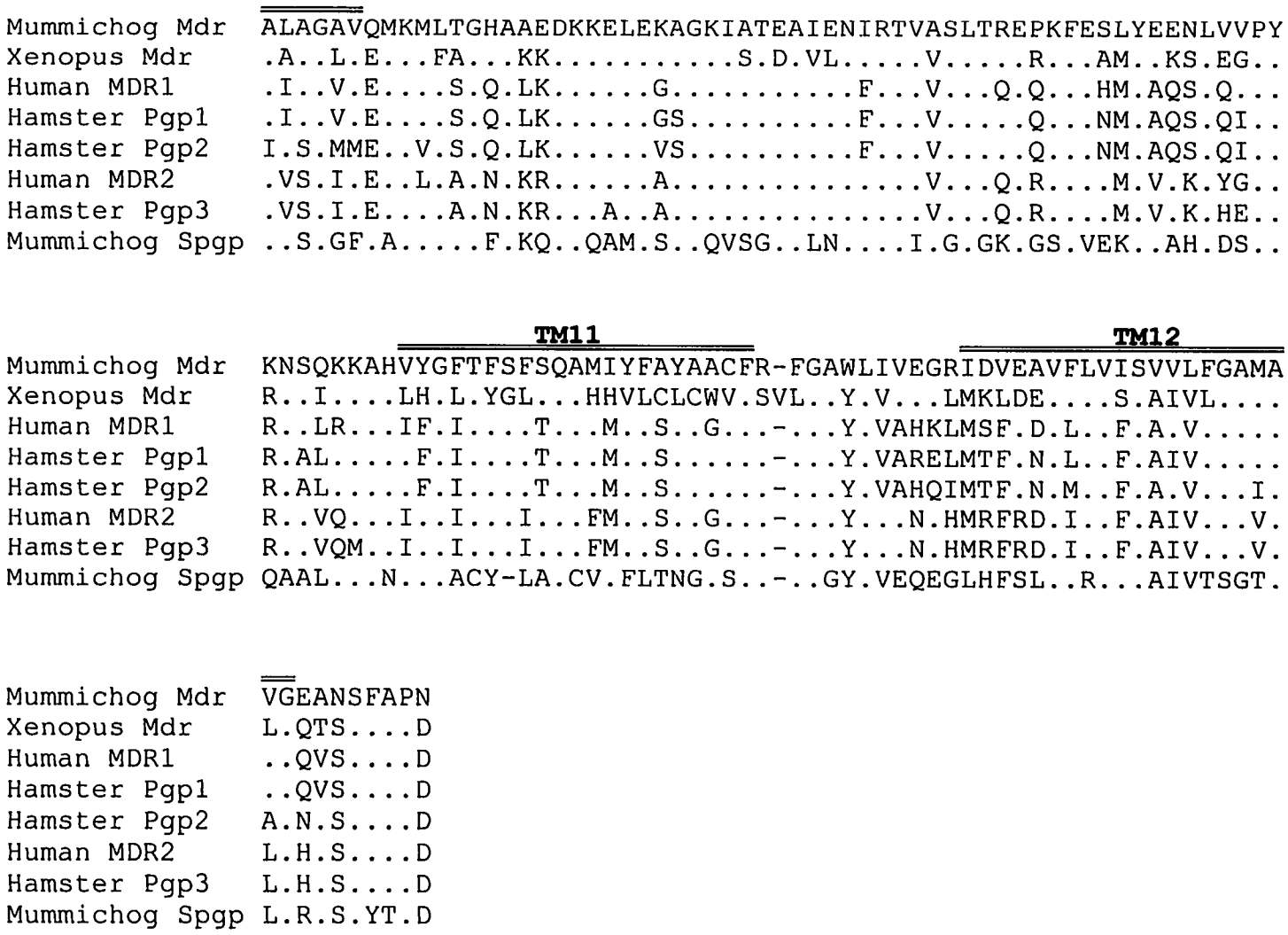
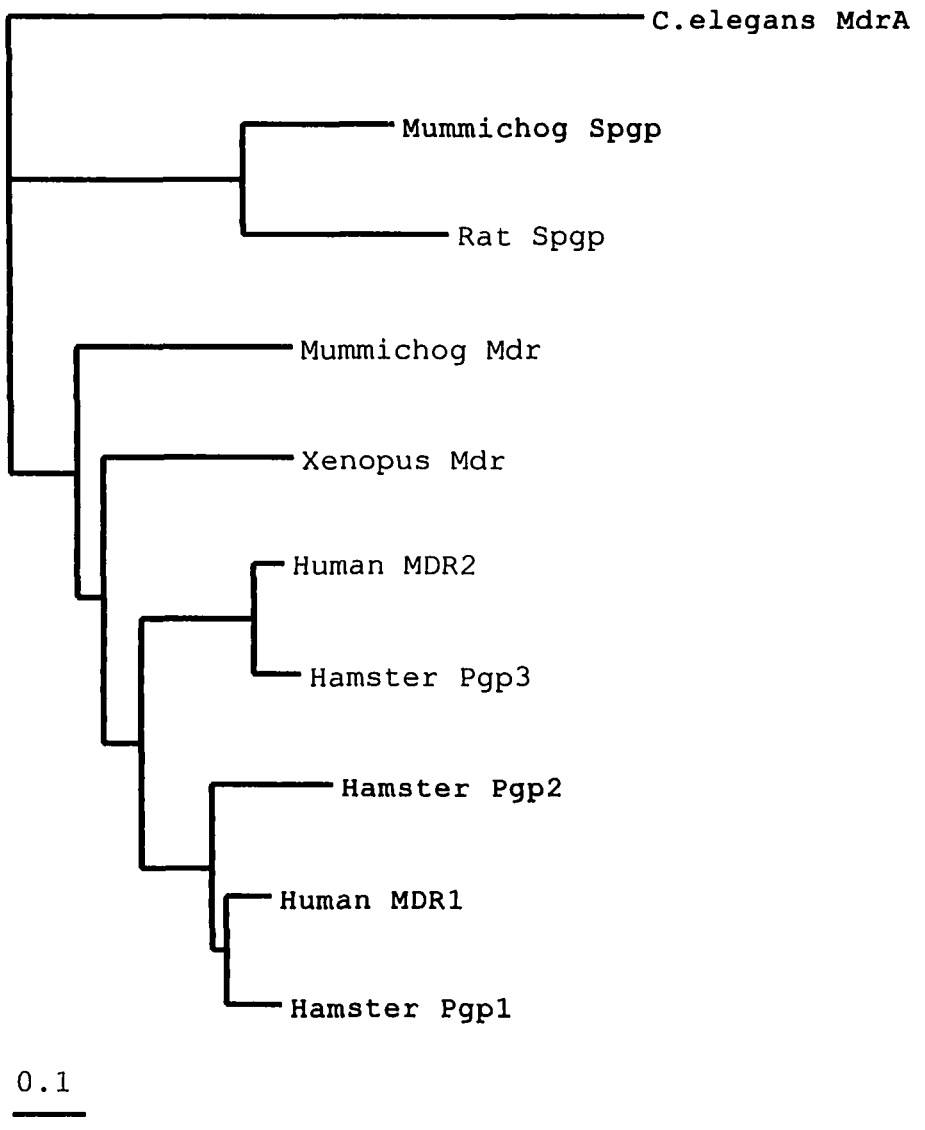


Figure 22. Phenogram of the relationships among P-glycoprotien deduced amino acid sequences. Relationships derived from Neighbor-Joining analysis of the pairwise distances between sequences. Branch lengths are scaled to reflect distances along each branch.The computer program package PHYLIP was used to calculate the distance matrix and construct the phenogram (Felsenstein, 1993).



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