INVESTIGATION INTO

CLASS II P-GLYCOPROTEIN

MESSENGER RNA DECAY

IN NORMAL LIVER AND LIVER TUMOURS

by

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Abstract

Class II P-glycoprotein (Pgp2) is a plasma membrane protein associated with multidrug resistance. This protein and its corresponding mRNA are over expressed in many tumours, including liver tumours. Pgp2 has also been found to be overexpressed in all models of rat and mouse liver carcinogenesis. Over expression of Pgp2 in liver tumours is predominantly due to an increase in stability of Pgp2 mRNA. The main objective of this thesis was to compare the degradation pathway of rat Pgp2 mRNA in normal liver and liver tumours, with the goal of understanding why Pgp2 is more stable in liver tumours compared to normal liver. Considerable effort was spent developing the Yeast Poly (A) tailing RT-PCR method in the hope of identifying Pgp2 mRNA degradation product(s) *in vivo*. This study concluded that although *in-vitro* transcribed RNA can be detected, the Yeast Poly (A) tailing RT-PCR method is not suitable for detecting *in vivo* mRNA degradation product(s). This study has introduced key issues related to Pgp2 mRNA that were previously unknown. Primer extension identified that endonucleolytic pathway is at least one mechanism for degradation of Pgp2 mRNA *in vivo*. However, the presence of an endonucleolytic cleaved product in normal liver and liver tumour suggests that endonucleolytic decay is unlikely to contribute to increased stability of Pgp2 mRNA in liver tumours. In summary, the work presented in this thesis does not provide answers as to why Pgp2 mRNA is more stable in liver tumours in comparison with normal liver. It does, however, enhance our knowledge regarding the molecular mechanisms occurring during the processing of Pgp2 mRNA in liver tumours and normal liver.

Table of Contents

CHAPTER 3- USING YEAST POLY(A)- TAILING METHOD TO SEARCH FOR POTENTIAL RAT PGP2 mRNA DEGRADATION PRODUCTS IN NORMAL LIVER AND LIVER TUMOURS

CHAPTER 4 – USING PRIMER EXTENSION EXPERIMENTS TO DETERMINE THE NATURE OF NUCLEOLYTIC (EXONUCLEOLYTIC OR ENDONUCLEOLYTIC) CLEAVAGE

CHAPTER 5 – GENERAL DISCUSSION

List of Tables

List of Figures

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Chapter 1

INTRODUCTION

1.0 Multidrug Resistance and P-glycoprotein

1.0.1 Overview of Mechanisms Related to Multidrug Resistance

Chemotherapy is the major form of treatment for many types of human cancers and involves the administration of various anticancer drugs that target various mechanisms related to the growth and development of cancer cells. Alkylating agents are drugs which act to covalently bond to intracellular macromolecules causing cross-linking of DNA strands thereby increasing cytotoxicity in cells. Antimetabolites are drugs that interfere with normal cellular function through inhibition of the formation of normal nucleotides or prevent normal cell division from occurring. Natural products which have been found to interfere with topoisomerase I and topoisomerase II, bind directly with DNA, and interfere with microtubules. Other drugs that are used in chemotherapy include cisplatin and carboplatin, which bind to DNA and produce interstrand crosslinks and adducts; hydroxyurea, which inhibits DNA synthesis; and L-asparaginase, that causes the degradation of the amino acid L-asparagine required for the viability of tumour cells (Tannock and Hill, 1998). The success of these agents is often hindered by the fact that majorities of cancers are resistant to chemotherapy or develop resistance towards the chemotherapeutic drugs during treatment (Chen and Simon, 2000). After exposure to only a few agents cancer cells can become resistant to many drugs of diverse mechanisms and structures. This phenomenon is termed multidrug resistance (MDR) (Chen and Simon, 2000).

There are many different mechanisms in which drug resistance can occur (summarized in Table 1.1.). One mechanism involves the impairment of drug influx. Drug

uptake can occur through passive diffusion, facilitated diffusion, and active transport. All three mechanisms allow for drug entry into cells down a concentration gradient, and active transport can also lead to transport against a concentration gradient. The development of drug resistance involves impairment of unidirectional drug influx, reduced binding affinity of the transport carrier for drug, reduced number of transport sites, and slower carrier mobility (Tannock and Hill, 1998).

Table 1.1. General mechanisms associated with multidrug resistance (modified from Tannock and Hill, 1998)

Mechansim	Drugs
Decreased Influx	Alkylating agents, antimetabolites, cisplatin
Increased efflux by increased expression of ATP binding cassette transport proteins or decreased expression of topoisomerase II	Natural products
Increase or decrease in levels of target enzymes	Antimetabolites, natural products
Alterations in target enzymes	Antimetabolites, Natural products
Inactivation by glutathione	Alkylating agents, natural products, cisplatin
Increased DNA repair	Alkylating agents, natural products, cisplatin,
Deceased ability to undergo apoptosis	Alkylating agents, natural products, cisplatin,

DNA topoisomerases have also been linked with the development of multidrug resistance. Topoisomerases are enzymes that catalyze topologic changes of DNA structure required for replication and recombination of DNA and for transcription of RNA. These enzymes also play a role in chromosome structure, condensation/decondensation, and segregation. Type I topoisomerases facilitate DNA strand unwinding by passing a single stranded DNA molecule through a strand break in the complementary strand. Type II topoisomerases aid in untwisting of the DNA molecule by catalyzing the passage of double-stranded DNA molecules through double- strand breaks. Topoisomerases serve as targets for chemotherapeutic agents, which seem to stabilize the DNA-enzyme complex, leading to an increase in DNA strand cleavage, thereby increasing DNA damage and ultimately cell death. Down regulation of both types of topoisomerases, and the production of mutant type I topoisomerase have been reported in drug resistant cells (Tannock and Hill, 1998).

Glutathione-s-transferase (GST), has also been reported to play a role in the development of multidrug resistance. GST is a widespread enzyme involved in the detoxification and protection of normal tissue. Reduced forms of glutathione can inactivate peroxides and free radicals produced by some drugs. They can also bind to positively charged electrophilic molecules rendering them less noxious and easily excretable. Drugs conjugated to reduced forms of glutathione are expelled from cells by the GS-X pump, which has a broad specificity for several anticancer drugs. This function is in part reliant on the multidrug resistance protein (MRP) (Borst and Elferink, 2002), therefore drug resistance due to an increase in expression of MRP most likely depends on GSH-mediated conjugation of anticancer drugs (Tannock and Hill, 1998).

DNA repair mechanisms also are involved in drug resistance by enhanced removal of DNA adducts and/or crosslinks from resistant strains. Alkylating agents produce DNA lesions that may be repaired by three mechanisms: damage reversal; nucleotide excision repair; and recombination or complementation. O^6-alkylguanine DNA alkyltransferase (AGAT) and 3-methyl-adenine DNA glycosylase are two enzymes involved in DNA repair, and both have been reported to be increased in tumour cells resistant to chemotherapeutic agents (Tannock and Hill, 1998).

MDR has also been shown to be associated with an important biological process termed apoptosis. Apoptosis is a process where intracellular signals trigger sequential events leading to cellular death. This complex pathway is regulated by many proteins. Mutation of important genes in tumour cells such as *p53*, responsible for the stimulation of apoptosis, may inhibit apoptosis. There is also evidence that some tumours exhibit an increase in *bcl-2*,

a gene that inhibits apoptosis, and a decrease in *bax*, a stimulator of apoptosis. Decreased apoptosis in tumour cells may then lead to drug resistance (Tannock and Hill, 1998)

1.0.2 ABC Transporters and Multidrug Resistance

The best identified actions of multidrug resistance are related to active transport proteins that belong to the ATP-binding cassette (ABC) superfamily (Ruth et al., 2000). This superfamily consists of membrane transporters in yeast, bacteria, parasites, nematodes, plants, and mammals. In mammals, this family includes transporters for bile acids, acylated fatty acids, chloride ions, peptides, and other substrates (Julien and Gros, 2000). ABC transporters play a crucial role in protecting vital bodily structures such as the brain, the testis, the cerebrospinal fluid, and the fetus against the uptake of toxic agents, like food derivatives and drugs into the body (Borst and Elferink, 2002). They have also been associated with many human diseases, including cancer (Gottesman and Ambudkar, 2001). There are four plasma membrane proteins belonging to the ABC superfamily associated with multidrug resistance; multi-drug resistance protein (Cole et al., 1992), lung-resistance protein (Tannock and Hill, 1998), breast cancer resistance protein (Doyle et al., 1998), and P-glycoprotein (Ling, 1974) (to be discussed in detail in section 1.0.3).

Multidrug-resistance Protein (MRP) is a glutathione-X conjugate pump and was first discovered in human lung cancer cells exhibiting multidrug resistance (Cole et al., 1992). The discovery of MRP has led to the characterization of 6 related proteins, all involved in the transport of anticancer compounds (Gottesman, 2002). The MRPs identified thus far differ in substrate specificity, tissue distribution, and intracellular location (Borst and Elferink, 2002). All of the MRPs have been associated with MDR, however, there is yet to be direct

evidence implicating MRPs as the root cause of the development of MDR phenotype in human cancers.

A second protein that is associated with resistance to a wide spectrum of drugs is called lung-resistance protein (LRP). This protein is expressed on intracellular organelles known as vaults, which are multisubunit structures involved in nucleocytoplasmic transport. It has been hypothesized that LRP is involved in causing drug resistance by pumping the drugs into the vaults, which are then exported from the cell (Tannock and Hill, 1998).

Breast cancer resistant protein (BCRP) is a plasma membrane protein located in the apical membranes of placental syncytiotrophoblasts, the epithelial lining of the small intestine and colon, hepatocytes, and the ducts and lobules of the mammary gland (Borst and Elferink, 2002), and has also been associated with multidrug resistance. The highest concentration of BCRP RNA has been found in the placenta suggesting that this protein has an important defense function by preventing entry of the drug to the fetus. Clinically, BCRP has been identified to be overproduced in MCF7 breast cancer cells and has shown to have high mitoxantrone resistance. Possible inhibitors of BCRP include fumitremorgin C and its analogs (Borst and Elferink, 2002).

1.0.3 P-glycoprotein

The first discovered and the only ABC transporter to date shown to be linked with development of clinical MDR is P-glycoprotein (Juliano and Ling, 1976). P-glycoprotein (Pgp) is synthesized in the endoplasmic reticulum, and modified in the golgi apparatus yielding a glycosylated 170 kDa transmembrane protein. Pgp is found on the luminal surface of epithelial cells in various regions of the body such as the large and small intestine, the biliary canalicular membranes of hepatocytes, the apical surface of epithelial cells of the

proximal cells of the kidney, the blood tissue barriers of the testis and brain, the liver, and the epithelia of the choroid plexus, (Loo and Clarke, 1999). It has been proposed that Pgp functions in several different aspects of normal physiology, such as protection from xenobiotics, resistance to apoptosis, intracellular tracking of sterols, and migration of dendritic cells into lymphatic vessels (Luker et al., 2000). As well as offering protection, Pgp has been associated with the transport of molecules across cell membranes (Brown, Thorgeirsson, and Silverman, 1993). These molecules include chemotherapeutic drugs, calcium channel blockers, steroid hormones, immunosuppressive agents, and peptide antibiotics (Hrycyna et al., 1998). Pgp is a 1280 amino acid polypeptide containing two tandem repeats of 610 amino acids, joined by a linker region of 60 amino acids (Loo and Clarke, 1999). It is comprised of two homologous halves, with each half spanning the plasma membrane bilayer six times (Hrycyna et al., 1998). Each halve of Pgp includes a hydrophobic domain with six transmembrane segments and a nucleotide binding ABC domain (Ruth et al. 2000). This domain arrangement is characteristic of the ABC superfamily (Figure 1.1.) (Loo and Clarke, 1999).

Two classes of Pgp genes exist in humans, class I (Pgp1) and class III (Pgp3). Class I (Pgp1) is capable of transporting many chemotherapeutic drugs and is associated with multidrug resistance (Lee, Bradley & Ling, 1998). Class III (Pgp3), which is not associated with multidrug resistance, is known to be a phospholipid translocase abundant in the liver (Romsicki and Sharom, 2001). There are three rodent isoforms, class I, class II, and class III. Class I and II are associated with the development of multidrug resistance, and class III, found to be most abundant in the liver is necessary for transport of phospholipids (Lee, Bradley & Ling, 1998). The MDR gene that codes for Pgp in rats is highly conserved with

the MDR gene that codes for Pgp in humans (Table 1.1). For example, rat Pgp2 is 79.1 % homologous to human Pgp1, and 71.5% homologous to human Pgp3 (Silverman, et al., 1991).

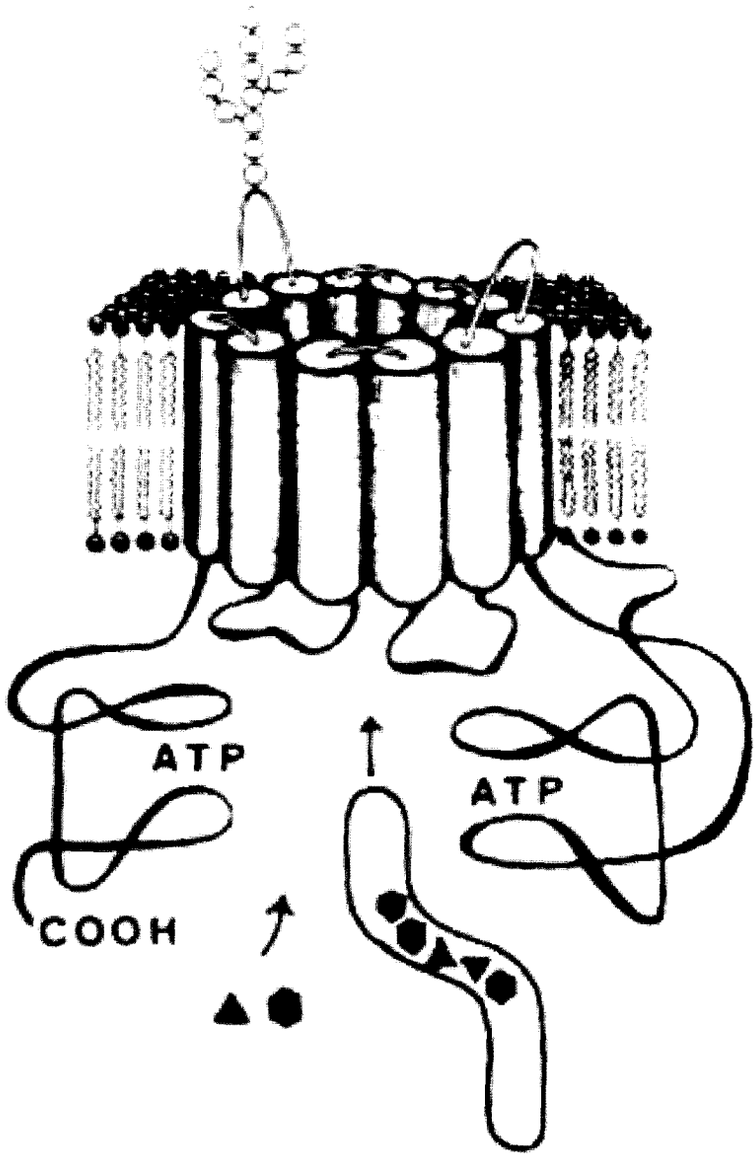


Figure 1.1. Basic structure of P-glycoprotein (modified from Tannock and Hill, 1998).

Table 1.2. Summary of human and rodent P-glycoprotein gene homology. (modified from Silverman et al., 1991).

Genes	Human Pgp1	Human Pgp3
	% Homology	% Homology
Rat Pgp2	79.1	71.5
Hamster Pgp2	75.7	47.7
Hamster Pgp1	83.5	64.2
Mouse Pgp3	71.1	86.1
Mouse Pgp2	78.7	70.6
Mouse Pgp1	82.2	71.6

Multidrug resistance conferred by overexpression of P-glycoprotein (Pgp) is one of the best-characterized forms of resistance mediated by transport molecules (Luker et al., 1997). When overexpressed, Pgp functions to confer multidrug resistance (Loo & Clarke, 1999) by acting as an ATP-dependant efflux pump that transports drugs with cytotoxic activity out of cells before they reach intracellular target (Taguchi et al., 1997). These drugs include many natural product derived anticancer agents such as doxorubicin, daunorubicin, vinblastin, vincristine, and taxol (Gottesman, 2002). The binding of these drugs results in activation of one ATP-binding domain. Hydrolysis of ATP results in a conformational change in Pgp, which then releases the drug into the extracellular space. Hydrolysis of a second ATP molecule is required to restore the protein to its original state (Gottesman, 2002).

P-glycoprotein has observed to be overexpressed in many human cancers, including cancers of the gastrointestinal tract, cancers of the hematopoietic system, cancers of the genitourinary system, and childhood cancers (Goldstein et al., 1989). One of the first studies reporting increased Pgp expression in clinical tumour samples involved two women with ovarian cancer being treated by chemotherapeutic agents (Bell et al., 1985). There is also considerable evidence of highly expressed Pgp in renal (Fojo et al., 1987) and colon tumours (Weinstein et al., 1991). In other cases, an increase in Pgp expression is seen only after exposure to chemotherapeutic drugs or during relapse (Chan et al., 1996). The majority

of clinical efforts to overcome multidrug resistance related to Pgp expression aim to inhibit Pgp's activity. These clinical trials have had limited success (Gottesman, 2002). Much effort has also been put forth to reverse MDR via inhibition of Pgp expression at the DNA and RNA level (Lee, 2003). For instance, ecteinascidin 743, an anti-tumour agent, has shown promising pre-clinical results in inhibiting Pgp activation (Jin et al., 2000). Also more recently, transcription has been targeted by modulation of the nuclear receptor SXR (Synold, Dussault, and Forman, 2001). Strategies have also been suggested involving the use of antisense and transcriptional decoy (Marthinet et al., 2000) and anti-MDR1 mRNA hammerhead ribozymes (Wang et al., 1999). In the treatment of human cancers, Pgp expression has been associated with chemotherapy failure and decreased survival, therefore its regulation is of great clinical importance.

1.0.4 Regulation of Pgp Expression

Regulation of Pgp expression is of great importance in cancer research as its overexpression has been implicated as the main reason for the development of multidrug resistance. These observations have provoked profound studies related to the regulation of Pgp expression. It has been shown that anticancer drugs (Fardel et al., 1997; Schrenk et al., 1996; Zhou and Kuo, 1998; Furuya et al., 1997), carcinogens (Fardel et al., 1996; Nakatsukasa et al., 1993), UV irradiation (Zhen, Shengkan, and Scotto, 2000; Uchiumi et al., 1993), extracellular matrix proteins (Tatsuta et al., 1994), heat shock (Chin et al., 1990), transcription factors (Thottassery et al., 1999; Bargou et al, 1997; Chin et al., 1992), and reactive oxygen species (Ziemann et al., 1999) participate in overexpression of Pgp in human tissues and animal models. Pgp 1 and Pgp2 have been shown to be overexpressed in all models of rat and mouse liver carcinogenesis (Lee, Bradley, and Ling, 1998; Kren, Trembley

and Steer, 1996). An increase in Pgp2 gene expression has been observed during liver regeneration (Nakatsukasa et al., 1993; Teeter et al., 1993; Marino, Gottesman, and Pastan, 1989; Thorgeirsson et al., 1987), during establishment of primary hepatocytes (Lee, Bradley and Ling, 1995; Hirsch-Ernst et al., 1995; Schuetz et al., 1995), in tissues treated with cyclohexamide (Lee, 2001), and in uteral tissues during pregnancy (Croop et al., 1989; Kuo et al., 1995). Interestingly, mRNA stability has been demonstrated in the regeneration of rat liver (Kren et al., 1996) and in liver tumours (Lee, Bradley, and Ling, 1998). There has also been evidence of post-transcriptional control of Pgp in the uterus (Kuo et al., 1995), intestine (Chianale et al., 1995) and in rat tissues subjected to cyclohexamide (Lee, 2001). It has also been determined that DNA cross linking agents can suppress MDR1 gene expression at two independent steps, one at the level of mRNA transcription, and the other at the post transcriptional level (Maitra et al., 2001) More recently, it has been suggested that Pgp expression is regulated at two specific steps in human leukemic cells. These steps include mRNA stabilization and translational initiation (Yague et al., 2003). However, the distinct mechanisms of how regulation occurs at these two steps, in either case, has yet to be determined. Overexpression of Pgp has also been demonstrated in many rat liver carcinogenesis models (Lee et al., 1998; Kuo et al., 1995, Nakatsukasa et al., 1993; Teeter et al., 1993; Teeter et al., 1990). In a transplantable rat liver tumour line, Pgp mRNAs exhibited an increase in stabilization, in comparison to normal liver Pgp mRNA. This has been confirmed through the analysis of mRNA half-lives of the three Pgp. Pgp1, Pgp2 and Pgp3 have a half-life of approximately 1-2 hours in normal liver. In liver tumours, all three Pgp mRNA demonstrated a half-life of over 12 hours (Table 1.2) (Lee et al., 1998). This

indicates that Pgp mRNA in liver tumours is more stable in comparison to normal liver, which accounts for an increase in Pgp mRNA overexpression in liver tumours.

Table 1.3. P-glycoprotein mRNA half lives (t ½) (h) in liver and transplantable liver tumours (modified from Lee et al., 1998)

Genes	Liver	Transplantable Liver Tumours
Pgp1	< 1	> 12
Pgp2	< 1	> 12
Pgp3	1.8	> 12

Mounting evidence suggesting mRNA stability is at least one major mechanism resulting in the overexpression of Pgp (Lee et al., 1998). This warrents further investigation into the molecular mechanisms responsible for the increase in stability demonstrated.

1.1 Messenger RNA Stability

The importance of regulating mRNA stability is shown by the extent of physiological and clinical events that are affected by change in stability (Dodson and Shapiro, 2002). These include development, cell cycle, viral infections, reproduction, and response to cellular environment changes (Dodson and Shapiro, 2002). Messenger RNA stability is very important in relation to protein expression. The length of time mRNA is intact in the cytoplasm directly determines the amount of protein translated (Ross, 1995). Simply, the more stable mRNA transcripts are, the more protein is expressed. In retrospect, if the mRNA transcript is destabilized, less protein production occurs. The molecular mechanisms for activating mRNA degradation or stabilization, particularly in the mammalian system, are not well understood. It also appears that the mechanisms involved and the decay processes that exist in prokaryotic and eukaryotic systems are not conserved, leading to more complication regarding the issue.

1.1.1 Messenger RNA Stability in Prokaryotes

Prokaryotic mRNA has been observed to be very unstable, and decay rates vary extensively (Coburn and Mackie, 1999). There are many factors and pathways involved in the degradation and stabililty of prokaryote mRNA, however the exact mechanisms are not known, and the main nucleases involved only have loose primary sequences or secondary structure specificity (Grunberg-Manago, 1999). mRNA stability is also reliant on several external factors such as growth conditions, environmental signals, and efficiency of translation. It is known that decay is always initiated by endoribonucleolytic cleavage by RNase E or RNAse III. RNase E has been found to cleave substrates nonspecifically 5' to AU dinucleotides in single stranded segments (Mackie, 1998). RNase III cleaves double stranded RNA molecules either with a single strand nick or a double strand break. Endonucleolytic cleavage is followed by exonucleolytic decay at the 3' end. There have been two enzymes responsible for exonucleolytic decay including Polynucleotide Phosphorylase (PNPase) and RNase II. PNPase catalyzes phosphorolysis of mRNA liberating nucleoside diphosphates, while RNase II catalyzes hydrolysis of mRNA releasing 5' monophosphates (Grunberg-Manago, 1999). A multienzyme complex, commonly known as the degradosome has also been identified to play a role in the decay process. It appears that the degradosome is comprised of RNase E, PNPase, a glycolytic enzyme, and different enzymes using ATP as a cofactor. The functional role of the degradosome is unclear, although it is obviously involved in the decay process of bacterial mRNA transcripts (Grunberg-Manago, 1999). The final major enzyme facilitating mRNA decay processes in bacteria is Poly(A) Polymerase. This enzyme adds adenine residues to the 3' hydroxyl termini of a transcript using ATP as a substrate and releasing pyrophosphate. Unlike in

eukaryotic systems where poly (A) tails are used as stability factors for mRNA transcripts, polyadenylation facilitates exonucleolytic decay by providing a single stranded tail for PNPase to attack (Grunberg-Manago, 1999).

1.1.2 Messenger RNA Stability in Lower Eukaryotes (Yeast)

The majority of knowledge obtained to date regarding eukaryotic mRNA stability and degradation mechanisms arises from studies in the yeast *Saccharomyces cerevisiae*. There are three modes of mRNA decay described in yeast: (i) deadenylation dependant decapping, followed by 5'-3' decay; (ii) deadenylation independent from decapping followed by 3'-5' decay; and (iii) endonucleolytic cleavage. The major mechanism of mRNA decay is thought to involve the removal of the 3'poly (A) tail, followed by the removal of the 5' cap. Deadenylation followed by decapping is the mechanism for deadenylation dependent mRNA decay and subjects the mRNA structure to 5'-3'and/or 3'-5' exonuclease activity (Brewer, 2002; Dodson and Shapiro, 2002; Tucker and Parker, 2000) (summarized in Figure 1.2.). Deadenylation in yeast seems to require the use of two agents with exonuclease activity. The first contributor is composed of two proteins Ccr4P/Caf1P, and the second deadenylase is the Pan2p/Pan3p (PAN) exonuclease. The proposed mechanism for deadenylation in yeast involves the PAN subunit that may be responsible for initial trimming of the poly (A) tail, and further deadenylation by the Ccr4P/Caf1P complex. This is followed by removal of the 7-methyl guanosine cap. The removal of the 5' cap in yeast involves the decapping enzyme Dcp1p, and many other protein factors. Decapping is the key step in yeast mRNA degradation as it precedes and permits the decay of the mRNA transcript body by exposing it to a 5'-3' exonuclease (Tucker and Parker, 2000).

Additionally, yeast transcripts can also be degraded in a 3'-5' direction following deadenylation. This is catalyzed by a complex of 3'-5' exonucleases termed the exosome. It is apparent that this degradation pathway is slower than 5'-3' decay, however it is still crucial to development as mutations that inhibit both 5'-3' decay and 3'-5' decay are lethal. This suggests that both 5'-3' and 3'-5' deadenylation dependant degradation are the two significant pathways for mRNA turnover in yeast (Tucker & Parker, 2000).

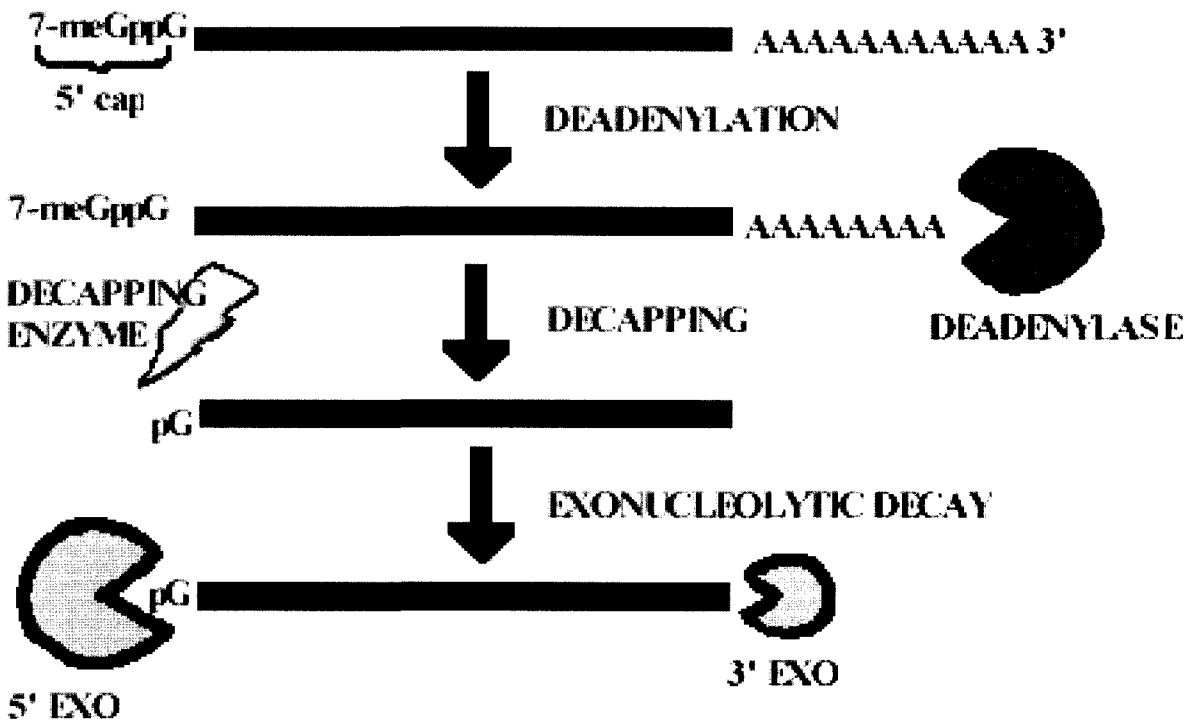


Figure 1.2. Deadenylation dependant mRNA decay mechanism in yeast (modified from Wilusz et al., 2001)

Endonucleolytic cleavage has also been identified to play a role in the stabilization and destabilization in yeast mRNA transcripts (Brewer, 2002; Dodson and Shapiro, 2002). This pathway is reliant on an endoribonuclease to cut the mRNA body internally. This event is followed by exonuclease activity. Endoribonuclease pathway in yeast involves an unfolded protein in the endoplasmic reticulum. The unfolded protein IreI has both protein kinase and

endoribonucleic domains. It has been shown to excise introns and degrade RNA in yeast during periods of cell stress (Tirasophon et al., 2000).

Another method of mRNA decay in lower eukaryotes is termed nonsense-mediated decay or RNA surveillance. This process targets mRNA transcripts containing error, and is therefore involved in the regulation of mRNA. Error can include: nonsense mutations or early termination codons and improperly spliced introns. This pathway has been studied extensively in yeast, and occurs through decapping independent of deadenylation (Dodson and Shapiro, 2002), followed by 5'-3' degradation in the cytoplasm (Brewer, 2002). The same decapping enzyme used in deadenylation dependant decay, is also used for nonsense-mediated decay. Four additional factors have been identified and deemed crucial for nonsense-mediated decay in yeast. These included Upf1, Upf2, Upf3, and Hrp1. It is hypothesized that these factors interact to form a 'surveillance' complex, and associate with release factors at translation termination. The complex then scans the downstream portion of the transcript for a premature termination signal. Hrp1 interacts with the premature termination signal and is thought to act as a marker for targeted nonsense mediated decay (Wilusz et al., 2001).

1.3 Messenger RNA decay in Higher Eukaryotes

1.3.1 Mode of mRNA decay

The mode of messenger RNA decay in vertebrates is currently an unresolved issue. To date, deadenylation dependant decay has been shown to be an important mode of mRNA decay in mammalian cells. The postulated mechanism for this process involves the enzyme poly (A) ribonuclease (PARN), first purified from the thymus of a calf (Brewer, 2002). The

binding of PARN to the 7-methyl guanosine cap activates the processing of the poly(A) tail and suggests a link between decapping and deadenylation. A decapping enzyme has also been identified in mammalian systems. DcpS is an enzyme thought to decap an mRNA transcript that has previously undergone deadenylation and extensive 3'-5' exonuclease degradation (Brewer, 2002). The exact deadenylation-dependant mRNA decay method has not been fully characterized in vertebrates and research into the exact mechanism is still ongoing (Dodson and Shapiro, 2002).

Unlike in lower eukaryotes, there has been no evidence thus far suggesting that 5'-3' exonuclease decay participates in mRNA regulation in vertebrates. There has been however, substantial evidence indicating that endonucleolytic activity also plays an active role in the decay process of vertebrate mRNA (summarized in Figure 1.3.). mRNA control can be obtained by regulation of endoribonuclease activity, controlling the accessibility of the cleavage site, or regulating both levels of control (Dodson and Shapiro, 2002). Endoribonucleic activity seems to be triggered in response to cellular crisis or infection that is characterized by the presence of double stranded RNA (dsRNA). There are two pathways identified in higher eukaryotes which demonstrate that an endoribonuclease can be activated in response to dsRNA. The first involves the synthesis of a 2'-5' linked oligoadenylate (2-5A) in response to viral infection. This in turn activates RNAse L that cleaves the RNA strand at UU or UA residues (Starck et al., 1998). The second mechanism involves interference RNA that targets specific endoribonucleaic cleavage in response to dsRNA. The activated endoribonuclease cleaves the dsRNA into 21-23 nucleotide fragments (Sharp, 2001).

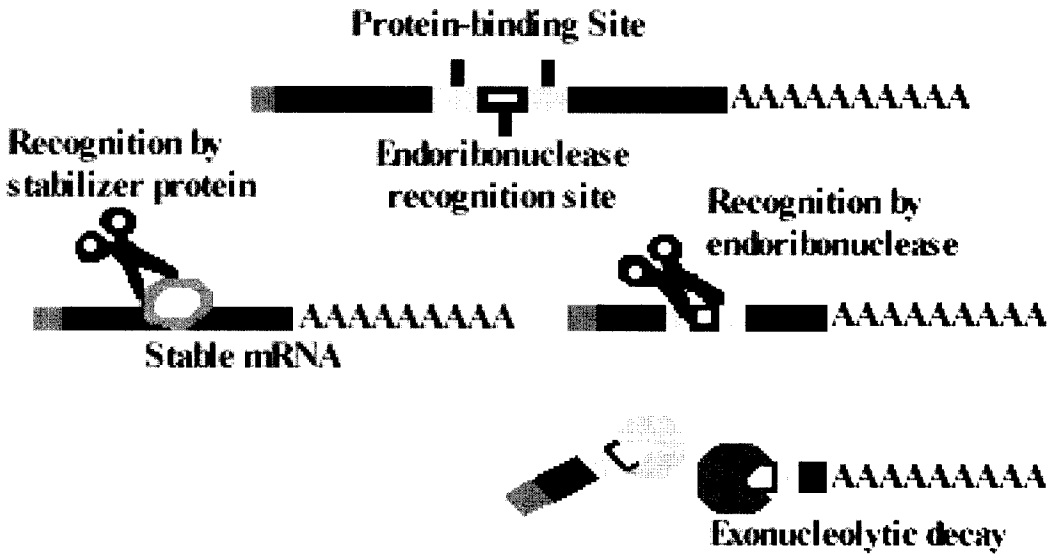


Figure 1.3. General mechanism of endoribonucleic decay in vertebrates (modified from Wilusz et al., 2001)

Cleavage site regulation is also important in controlling mRNA degradation. As later described in detail, mRNA contains *cis*-elements (section 3.0.2.). These elements can act as cleavage sites for specific endoribonuclease activity. Cleavage by an endoribonuclease produces RNA fragments with unprotected 5' and 3' ends. This in turn makes the products very susceptible to exonuclease activity. Protection of the cleavage sites can occur through RNA-binding proteins. For example, control of *c-myc* mRNA has been shown to be partially dependent on an approximately 75 kDa protein that is normally bound to the *c-myc* coding region determinant (Bernstein et al., 1992). This protein acts to protect the mRNA from a 39 kDa endoribonuclease that cleaves the coding region stability determinant in a very specific manner (Lee et al., 1998). This has also been demonstrated in TfR (transferrin receptor) mRNA. It has been shown that the iron response element binding protein (IREBP) increases in response to low levels of iron. The binding of this protein to iron response elements in the 3' UTR of transferrin receptor mRNA protects it from endoribonucleic attack (Dodson and Shaprio, 2002).

In some cases, it has been demonstrated that mRNA processing involves both endoribonucleic degradation and deadenylation-dependant degradation. *c-myc* and *α-globin* are examples where both mechanisms are used to regulate mRNA. Both mRNAs contain *cis* elements that bind to proteins that protect the mRNA strand from endoribonucleic cleavage. Both are also destabilized by deadenylation-dependant mechanisms (Dodson and Shapiro, 2002).

Finally, endoribonucleolytic decay mechanisms for mRNA have been identified in *Xenopus*, chickens, yeast, and mammals. The endonucleases themselves are different in not only the sequences they target, but the structure as well. Endoribonucleases have been identified that cleave A rich segments (Tfr and *c-myc*), U rich segments (p27kip1), C rich segments (α-globin), G rich segments (Igf II and PGK), AU rich segments (apolipoprotein II and gro-α), and ACU rich segments (Hep B, Xlhbox2B, and 9E3) (Dodson & Shapiro, 2002). Most endoribonuclease cleavage sites identified are single stranded, although some have shown to be dependant on secondary RNA structure (IGF II and apolipoprotein). There have been very few mRNA decay intermediates identified *in-vivo* due to the instability of degradation products. To date very few endoribonucleases have been purified and cloned (summarized in Table 1.4.). The ones that have been characterized range in size from 13 to 120 kDa, and are found in a variety of locations such as the cytosol, nucleus, and polysomes (Dodson and Shapiro, 2002).

Nonsense mediated decay has also been identified in higher eukaryotes as a mechanism for mRNA regulation. The exact mechanism is still unclear, although this process appears to be associated with first round translation (Dodson and Shapiro, 2002) and requires specific nuclear events that differ from nonsense mediated decay in yeast (Brewer,

2002). For example, nonsense mediated decay in mammalian cells requires essential components not encoded in the yeast genome (Brewer, 2002). In mammalian cells a premature termination codon is identified if it is located at least 50 – 55 nucleotides from the exon-exon junction at the 3' UTR. It is thought that this junction could lead to the association of Upf3, Upf2, and Upf1. It is not clear if these factors associate sequentially, or how they target mRNA for nonsense mediated decay in vertebrates (Brewer, 2002; Dodson and Shapiro, 2002).

1.3.2 *cis*-Elements in mRNA Decay/Stability

Various mechanisms related to the regulation of unstable mRNA transcripts in vertebrates are also known. Unstable mRNA transcripts such as those belonging to cytokines, growth factors, and transcription factors are regulated by the presence of *cis* destabilizing elements that bind to *trans*-acting factors. This is thought to accelerate or slow entry into the deadenylation dependant decay pathway (Gao et al., 2001; Shyu et al., 1989). There are two elements identified which play this role. The first is the AU-rich element, which is found in a number of mRNAs, the second is Major Coding Region Determinants found in *c-fos* mRNA.

AU-rich elements (AREs) are *cis* elements (Dodson and Shapiro, 2002), and have been grouped into three main classes associated with the mammalian system. Class I AREs represent one or more AUUUA pentamers within a U rich region; class II AREs contain tandem repeats of the AUUUA sequence; and class III are U-rich and are not associated with the AUUUA motif (Brewer, 2002). mRNA transcripts containing class I and III AREs have been shown to encode oncogenes. mRNAs containing class II AREs often encode cytokines and chemokines (Brewer, 2002). All AREs are located in the 3' UTR (Brewer, 2002).

Depending on the cellular environment, different AREs can have different effects on mRNA transcripts (Chen and Shyu, 1995; Peng et al., 1998). There are also many ARE binding proteins that have been identified (Wilusz et al., 2001) which are regulated by various factors such as stress activated kinases, membrane receptors, and members of the steroid receptor family (Sela-Brown et al., 2000; Sheflin et al., 2001). Levels of AREs are also variable during physiological processes such as development, cell senescence, and stress (Wang et al., 2001). The variability surrounding AREs and their ability to be stabilizing or destabilizing agents, seems to be related to the interaction with trans-acting factors (Dodson and Shapiro, 2002).

1.3.3 Specific Examples of mRNA Stability/Decay

Two mammalian mRNA transcripts have been identified to require specific mechanisms related to stability and decay processes. Mammalian *c-fos* mRNA is one example. *c-fos* mRNA has an ARE present in the 3' UTR. In addition to the ARE, it also has a second element called mCRD1 (major coding region instability element) that is involved in degradation regulation. The mCRD1 is a purine-rich segment upstream from the poly (A) tail. The distance between the poly (A) tail and the mCRD1 segment is important as there are five proteins that bind to this region. These proteins include UNr (a purine-rich RNA-binding protein), poly (A)-binding interacting protein, PABP (poly (A) binding protein), NSAIPI, and AUF1 (Grosset et al., 2000). The interaction between these proteins, the coding region determinant and deadenylation mechanisms appear to control the stability of *c-fos* mRNA in a seemingly complicated mechanism (Dodson and Shapiro, 2002).

Mammalian c-myc mRNA is another example of an mRNA transcript whose stability and decay is very specific in nature. Two stability determinants have been identified in c-myc

mRNA. One is present in 3'UTR, and the other is located within the coding region (Bernstein, et al. 1992). The coding region stability determinant has also shown to have instability determinant properties, and act as a site for endonucleolytic cleavage. As previously stated, it has been demonstrated that *c-myc* mRNA can be degraded via two separate mechanisms. One mechanism involves an exonuclease that contributes to the deadenylation dependant 3'-5' degradation process. The second mechanism involves an endoribonuclease that cleaves the coding region stability determinant in predominantly A-rich segments (Lee et al., 1998). As also previously described, the ability of the endoribonuclease to cleave c-myc mRNA is dependant on the presence of a coding redion determinant binding protein. The combination of degradation mechanisms and stability/instability determinants present in *c-myc* RNA make it an interesting example of how complicated the study of mammalian mRNA stability and decay can be.

1.4 Objectives

One method for studying mRNA decay involves the analysis of mRNA degradation intermediates. Degradation intermediates have been identified for several mammalian mRNAs, including *c-myc* (Ioannidis et al., 1996) and albumin mRNA in mouse liver (Tharun and Sirdeshmukh, 1995). It is thought that in some instances the presence of degradation products is a regulated event (Tharun and Sideshmukh, 1995), and in some cases has lead to the purification and characterization of an endoribonuclease (Lee et al., 1998). Another method for studying mRNA decay mechanisms involves the use of polysomes or extracts in *in vitro* reactions. This method allows better detection of mRNA degradation products as

well as the enzymes and other molecules involved in the degradation process (Brewer and Ross, 1990).

In an effort to understand the mechanism for overexpression of Pgp2 mRNA in rat liver tumours, measurements of the half life of Pgp2 mRNA in both normal liver and liver tumour were determined. As indicated above (Table 1.2) it was determined that the half life for Pgp2 mRNA in normal liver was less than 1.8 hours, while the half life of Pgp2 mRNA in liver tumours extended to over twelve hours (Lee, Bradley, and Ling, 1998). The mechanism responsible for the increased mRNA stability demonstrated in rat liver tumours is unknown. This study aims at comparing the *in vivo* degradation pathway of Pgp2 mRNA in normal liver and liver tumour in rats, in an effort to determine what causes Pgp2 mRNA to be more stable in liver tumour. One approach to determine the *in vivo* degradation pathway of Pgp2 mRNA is through identification of Pgp2 mRNA degradation intermediates in liver tumour and normal liver. If similar Pgp2 mRNA degradation products are found in both normal liver and liver tumours, this would suggest similar degradation pathways. This would still be an interesting finding because little is known about Pgp2 mRNA decay pathway. Furthermore, this will provide some insight into the general decay process of mRNA in an animal system. Presence of Pgp2 mRNA decay product(s) in one tissue and not the other, would suggest differential decay pathways. Results obtained from this portion of the study could potentially explain the underlying mechanisms for increased Pgp2 mRNA stability in liver tumours. Advances in the knowledge of how Pgp mRNA is stabilized and degraded can lead to development of therapeutic strategies against Pgp over expression observed in many cancers.

The focus of this research is to detect and analyze rat Pgp2 mRNA degradation products in normal liver and liver tumour, in an effort to compare the *in vivo* degradation

pathway of Pgp2 mRNA in both tissue samples. The major objectives of this study have been divided into three chapters. Chapter 2 describes the establishment and optimization of the Yeast Poly (A) RT-PCR protocol to detect possible degradation intermediates. Chapter 3 describes the use of the Yeast Poly (A) RT-PCR method to detect possible Pgp2 mRNA degradation intermediates in total RNA isolated from liver tumour and normal liver. Chapter 4 describes the use of Primer Extension analysis to determine the nature of cleavage of detected degradation product(s). A general discussion related to the ability of this study to meet the set objectives, and possible directions for future research related to Pgp2 mRNA stability is provided in Chapter 5.

Table 1.4. A comprehensive list of endonucleases identified and their properties (modified from Dodson & Shapiro, 2002).

mRNA	Cleavage Site	Name	Size (kDa)	Location	Activator	Binding Protein	Reference
Undefined[1]	c-myc 3'-UTR	G3BP	120	Cytoplasm	PO4	Not identified	Gallonzi et al., 1998
Albumin[2]	APryUGA, 3'-UTR	PMR-1	60	Polysomes	Estrogen	Not identified	Cunningham et al., 2001
A-Globin	CCCUCCUUGGACC > 24 nt. 3'-UTR	ErEN	Not known	Non-polysomal	Erythroid-enriched	αCP	Wang et al., 2000
Undefined[1]	AU-rich, ssRNA RNAse E substrates	ARD1	13.3	Polysomes	Not identifed	Not identifed	Wennborg et al., 1995; Claverie-Martin et al., 1997
p27kip1	UUCGGUUUGUUUUUU, 5'-UTR	Endonuclease in HUR binding site	Not known	Not known	Not identified	HUR	Zhao et al., 2000
Viral RNA	AA and AU ssRNA	RNase L	40, 80	ER	INF, 2-5A	Not identifed	Gallonzi et al., 1998
Ire1	CUGCAG, AAAACUA, AGUGAA	IRE1	110	ER	Unfolded Protein	Not identifed	Tirasophon et al., 2000

[1] endogenous substrate unknown
[2] isolated from Xenopus laevis

Chapter 2

Establishing and Optimizing the Yeast Poly(A) Tailing RT-PCR Method to Detect RNA Molecules *In-vitro*

This chapter describes the establishment and optimization of the Yeast Poly (A) tailing RT-PCR protocol used for detection of putative Pgp2 mRNA degradation intermediates. The sensitivity of the Yeast Poly (A) tailing RT-PCR assay is determined by running Poly(A)-tailed RT-PCR products of *in-vitro* transcribed RNA, on 6% denaturing polyacrylamide/7M urea gel and on 1.5% agarose gel. Methodology, results and discussion related to these topics are included.

2.0 Methods

2.0.1 *In-vitro* transcription of pGEM4Z-Pgp2 Plasmids

Plasmid constructs designated pGEM4Z-Pgp2A, pGEM4Z-Pgp2B1, pGEM4Z-Pgp2C, PGEM4Z-Pgp2D, and pGEM4Z-Pgp2E1 contain different segments of Pgp2 cDNA were used (Silverman et al., 1991; Fig.2.1.). All plasmids were linearized with the restriction enzyme *Hind*III to prepare for *in-vitro* transcription (see Figure 2.2. for detail of pGEM4Z plasmid). Plasmid DNA (15 μg) was added to 10 μL of *Hind*III, 3 μL of 10X Reaction buffer, and double distilled water (ddH_2O) to make the reaction mixture up to 30 μL. The mixture was incubated at 37° C for 3 hours. 2 μL of linearized plasmid was then loaded and run on an ethidium bromide stained 1% agarose gel. The gel was visualized and a photograph was taken using ChemiImager™ System (Alpha Innotech Corporation, San Leandro, CA).

Once linearized 170 μL of ddH_2O was added to make the volume up to 200 μL. The standard phenol chloroform extraction was performed as follows: 100 μL of phenol and 100

μL of chloroform:isoamylalcohol ($CHCl_3$:IAA) (49:1) were added to the linearized plasmid solution. The solution was vortexed and then centrifuged at 12,000 rpm for 5 minutes. The aqueous layer was transferred to a new microcentrifuge tube and one volume of $CHCl_3$:IAA was added to the new tube. The solution was vortexed and centrifuged for an additional five minutes to remove any excess phenol. The top layer was

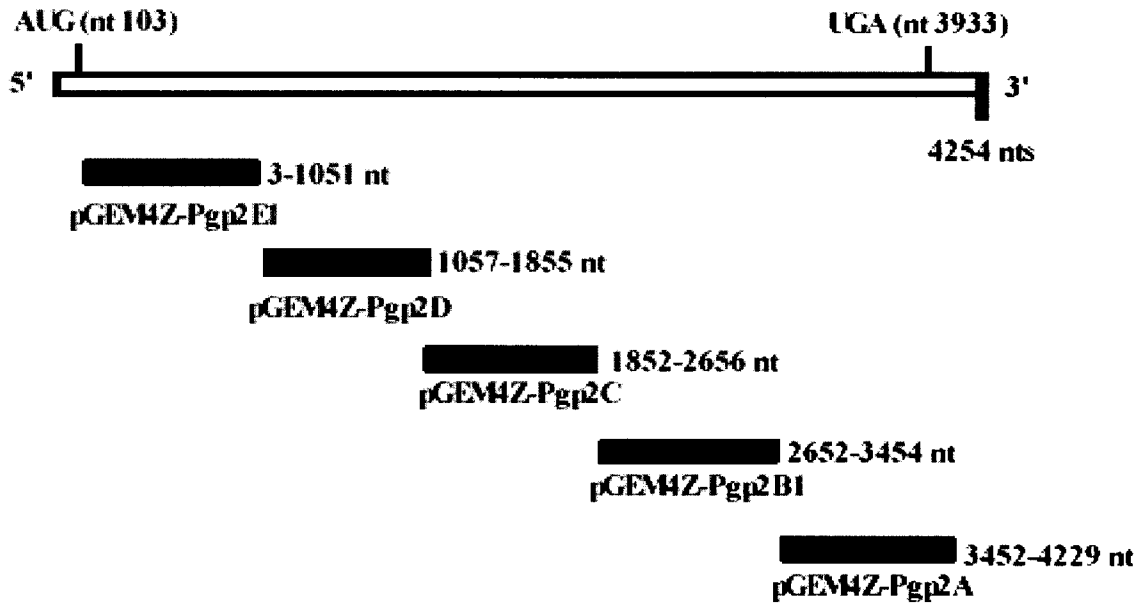


Figure 2.1. Pgp2 cDNA constructs used for making *in-vitro* RNA controls in Yeast Poly(A) tailing RT-PCR method. (Constructs were designed based on cloning of Pgp2 mRNA accomplished by Silverman et al., 1991)

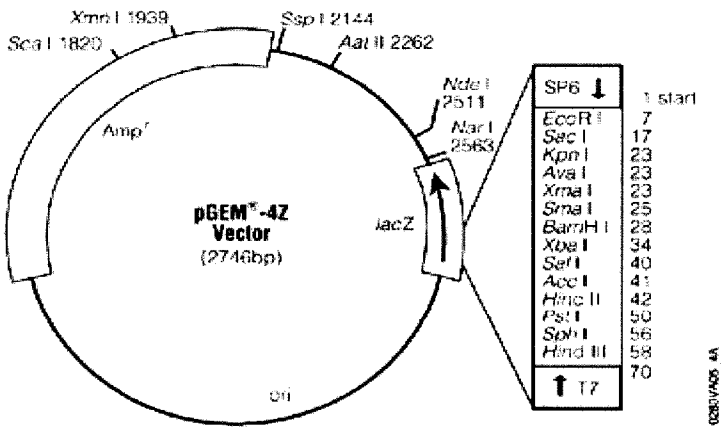


Figure 2.2. pGEM4Z cloning vector used for the construction of Pgp2 cDNA constructs (Promega, Madison, WI).

again transferred to a new tube and the Standard Ethanol Precipitation was performed as follows: two volumes of 100% ethanol and 1/10th of the volume of 3 M sodium acetate (NaOAc) pH 5.2 were added and the solution was mixed thoroughly. The mixture was then stored at -20° C for 20 minutes to precipitate the plasmid DNA. The solution was removed and centrifuged for ten minutes at 12,000 rpm. The supernatant was removed and the DNA pellet was washed by gently adding 200 µL of 70% cold ethanol. The sample was then centrifuged for five minutes and the supernatant was aspirated. The DNA pellet was then allowed to air dry for 15 minutes and was resuspended in 10 µL of ddH_2O.

RNA was synthesized using MEGAscript® SP6 Kit (Ambion, Inc., Austin, Texas). The *in- vitro* transcribed RNA will be used as a control for verification of the Yeast Poly (A) tailing RT-PCR method. Each *in-vitro* transcription reaction entailed: 4 µL of linearized plasmid DNA; 2 uL of 10 X reaction buffer; 2 µL of each 50 mM ATP, CTP, UTP, and GTP; 2 µL of SP6 RNA polymerase; and 4 µL of diethylpyrocarbonate (DEPC) H_2O. The mixture was incubated at 37° C for 2 hours followed by an addition of 1 µL of DNase I. The reaction was incubated again at 37° C for 15 minutes. DEPC H_2O was then added to make the reaction mixture up to 200 µL and the Standard Phenol/Chloroform Extraction, Standard Ethanol Precipitation was performed. The pellet was resuspended in 50 µL of DEPC H_2O, and the solution was added to a ProbeQuant^{TM} G-50 Micro Column (Amersham Pharmacia Biotech). The column was centrifuged for 2 minutes at 3,000 rpm and the RNA sample was collected in a new microcentrifuge tube. The quality of RNA was checked by running 3 µL of column sample with 3 µL cocktail mix [10 X MOPS (N-morpholino propanesulfonic acid, EDTA, NaOAc, ddH_2O, DEPC H_2O), formaldehyde, formamide, ethidium bromide, and

DEPC H_2O and 2μL loading dye on a 1.3% formaldehyde gel. The gel was visualized and photographed using ChemiImager™ System.

2.0.2 Poly(A)-Tailing, Reverse Transcription of *In-vitro* transcribed Pgp 2E1 RNA

In-vitro transcribed Pgp2E1 RNA was determined to be of good quality RNA sample (Figure 2.4., lane 5) and therefore was used to test the sensitivity of this assay. Pgp2E1 RNA (100 ng) was incubated at 65°C for 5 minutes, and then placed on ice. To the sample the following reagents were added: 2 μL of 5X reaction buffer; 1 μL of 10 mM ATP; 0.5 μL RNasin (40 U/μL) (Promega, Madison, WI); 0.5 μL 100 mM DL-Dithiothreitol (DTT); 1 μL Yeast Poly(A) Polymerase (600 U/μL) (USB Corp., Cleveland, OH); and 4 μL DEPC H_2O. The solution was incubated at 30°C for 10 minutes and then incubated at 65°C for an additional 10 minutes. The sample then underwent a reverse transcription reaction where the 10 μL of Pgp2E1 tailed-RNA was again incubated at 65°C for five minutes and then placed on ice. The following reagents were then added: 2 μL of 10mM deoxyribonucleoside triphosphates (dNTPs); 4 μL of 5X AMV Buffer; 1 μL of OligodT_{18}-XhoI primer 5'AACCCGGCTCGAGAGCGGCCGCTTTTTTTTTTTTTTTT 3' (0.5 μg/μL); 1 μL of 100 mM DTT; 1 μL RNasin (20 U/μL); and 1 μL of AMV reverse transcriptase (5U/μL) (Roche Applied Science, Basel, Switzerland). The solution was incubated at 37°C for 20 minutes, followed by incubation at 50°C for 15 minutes. The enzymes were heat activated by incubation at 65°C for 10 minutes. As a control to test if Poly (A) tailing of Pgp2E1 in-vitro transcribed RNA was successful, reverse transcription was carried out on non-tailed RNA samples as well.

2.0.3 Dephosphorylation and Phosphorylation of Forward Primers

It was necessary to 5' label forward primers with γ^{32}P-ATP (50μCi) (Amersham Biosciences Corp, Piscataway, NJ) prior to use for PCR of tailed and nontailed RT samples for analysis on 6% polyacrylamide/7 M urea gel. The primers first needed to be dephoshorylated. Each dephosphorylation reaction included: 10 μg of forward primer, 10 μL of Calf Intestinal Alkaline Phophatase (CIAP) (1 U/μL) (Roche Applied Science, Basel, Switzerland); 10 μL of 10 X dephosphorylation buffer; and ddH_2O to make the final reaction volume up to 100 μL. The reaction mixture was incubated at 37°C for 30 minutes and 100 μL of ddH_2O was added. The standard phenol/chloroform extraction and the standard ethanol precipitation were performed and the DNA pellet was resuspended in 20 μL of ddH_2O.

The phosphorylation reaction of dephosphorylated primers included the following reagents: 10 μL of dephosphorylated primer; 5μL of 5 X phosphorylation buffer; 5 μL of γ^{32}P-ATP; 3 μL (10 U/μL) of T4 Polynucleotide kinase (New England Biolabs, Beverly, MA); and ddH_2O to make the final reaction volume to 25 μL. The reaction mixture was incubated at 37°C for one hour and ddH_2O was added to make the volume up to 50 μL. The sample was added to a ProbeQuant™ G-50 Micro Column and centrifuged for 2 minutes at 3,000 rpm. The sample was collected in a new microcentrifuge tube and ddH_2O was added to make the final volume to 200 μL. The standard phenol/chloroform extraction was performed, followed by the standard ethanol -precipitation. The DNA pellet was resuspended in 200 μL of ddH_2O. 1μL of sample was used for scintillation counting.

2.0.4 Amplification of Poly (A) Tailed Pgp2E1 cDNA through Polymerase Chain Reaction

In order to test the sensitivity of this technique on both a 6% polyacrylamide/7 M urea gel and a 1.5 % agaorse gel, two different PCR reactions were set up. The reaction intended for polyacrylamide gel analysis included: 2 μL of tailed-RT Pgp2E1 cDNA template ranging in concentration from 0 ng/μL to 400ng/μL; 3.5 μL of 10X PCR Buffer (New England Biolabs); 3.5 μL of 2.5 mM dNTPs; 1 μL 50 mM $MgCl_2$; 5 μL (100 ng/μL) of γ^{32}P-ATP 5' labeled EF1 forward primer; 1 μL of reverse OligodT_{18}-XhoI (100 ng/μL); 1 μL (5 U/μL) of Taq Polymerase (Invitrogen Life Technologies, Carlsbad, CA); and ddH_2O to make the final reaction volume up to 35 μL. The reaction intended for anlaysis on a 1.5% ethidium bromide stained agaorse gel involved the substitution of the labeled EF1 forward primer for unlabeled EF1 forward primer (100 ng/μL). PCR parameters were set at 94°C for 30 sec; 50°C for 30 sec; and 72°C for 45 seconds for 30 cycles on a Minicycler™ Peltier Thermal Cycler (MJ Research, Inc., Reno, NV). Unlabeled products were ran on a 1.5% ethidium bromide stained agarose gel with 0.5 X TBE buffer and were visualized using ChemiImager™ System.

Labeled products of the first PCR experiment were run for 3 hours on a 6% polyacrylamide/7M urea gel with 1 X TBE (tris base, boric acid, EDTA) buffer. The gel was dried and exposed for 24 hours. The image was scanned using Cyclone phosphorimager, and visualized using the OpiQuant software (Hewlett Packard, Palo Alto, CA).

2.1 Results

2.1.1 Digestion of pGEM4Z-Pgp2 Plasmids

pGEM4Z-Pgp2 plasmids are double stranded circular DNA molecules derived from bacteria. The circular structure of the intact plasmid molecule appears as two bands after gel electrophoresis. The major band (approximately 1800 bp) is the plasmid DNA, whereas the top band represents nicked plasmid DNA. All plasmids underwent a restriction digest with *Hind*III (Figure 2.3.) to prepare for *in-vitro* transcription. One band is detected in all digested plasmid samples except for pGEM4Z-Pgp2B1 (lane 5). pGEM4Z-Pgp2B1 plasmid exhibits two faint bands due to incomplete digestion.

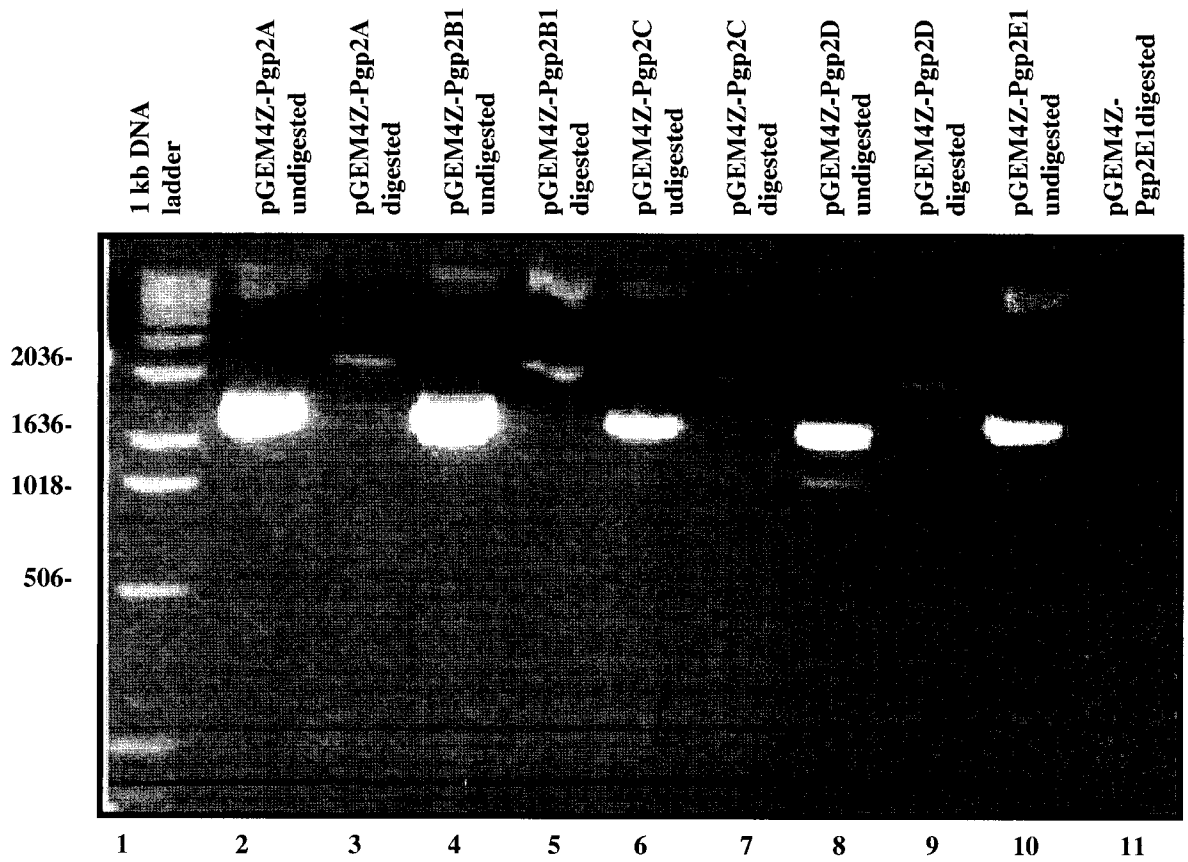


Figure 2.3. pGEM4Z Pgp2 plasmids linearized with restriction enzyme with Hind III in preparation for *in-vitro* transcription on 1% agarose gel. Even numbered lanes exhibit intact pGEM4Z Pgp2 plasminds. Odd numbered lanes show plasmid samples that underwent restriction digest with Hind III.

2.1.2 *In-vitro* transcription of digested pGEM4Z-Pgp2 plasmids

A digest (1-3 μL) sample of each *in-vitro* transcribed Pgp2 RNA was ran on a 1.3 % formaldehyde gel to visualize the RNA (Figure 2.4.). A major species of *in-vitro* transcribed RNA was detected for all samples corresponding to the approximate expected length (see Figure 2.2.; most intense band in Figure 2.4.) except for Pgp2A (lane 1). *In-vitro* transcription of Pgp2A plasmid has resulted in 3 distinct bands. This has been observed in the hands of other experimenters in the lab. *In-vitro* transcribed Pgp2C, and Pgp2D also exhibit a secondary RNA species (lanes 3 and 4). *In-vitro* transcribed Pgp2B1 (lane 2) and Pgp2E1 (lane 5) both exhibit only one species of RNA (lanes 2 and 5). Pgp2E1 RNA was chosen for further studies as it exhibited one intense distinct major species.(see Methods 2.0.2 and 2.0.4).

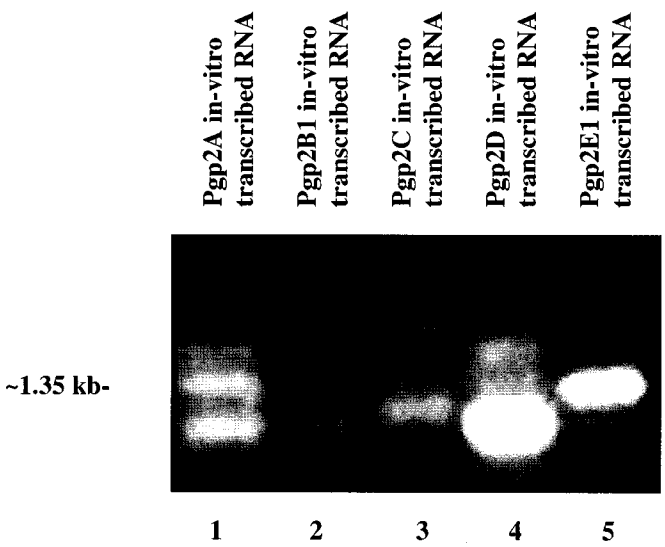


Figure 2.4. In-vitro transcribed RNA on a 1.3% formaldehyde gel. Linearized plasmid samples were subjected to in-vitro transcription using MEGAscript® SP6 Kit (Ambion)

2.1.3 Visualization of unlabeled Poly(A) tailed RT-PCR products on a 1.5% agaorse gel

Concentrations of poly(A) tailed *in-vitro* transcribed Pgp2E1 RNA products from 0 ng/μL to 400 ng/μL were amplified by PCR and were ran on a 1.5% ethidium bromide stained agarose gel to determine the sensitivity of this method (Figure 2.5.). Detection of Poly(A) tailed Pgp2E1 *in-vitro* transcribed RNA (most intense band with a size approximate to 1018 bp) was possible from 400 ng/μL to 0.4 ng/μL (lanes 7 to 11). A less distinct band was present at approximately 506 bp and was detected from 400 ng/μL to 1 ng/μL. Two bands shown at the bottom of the gel in Figure 2.4. are primers.

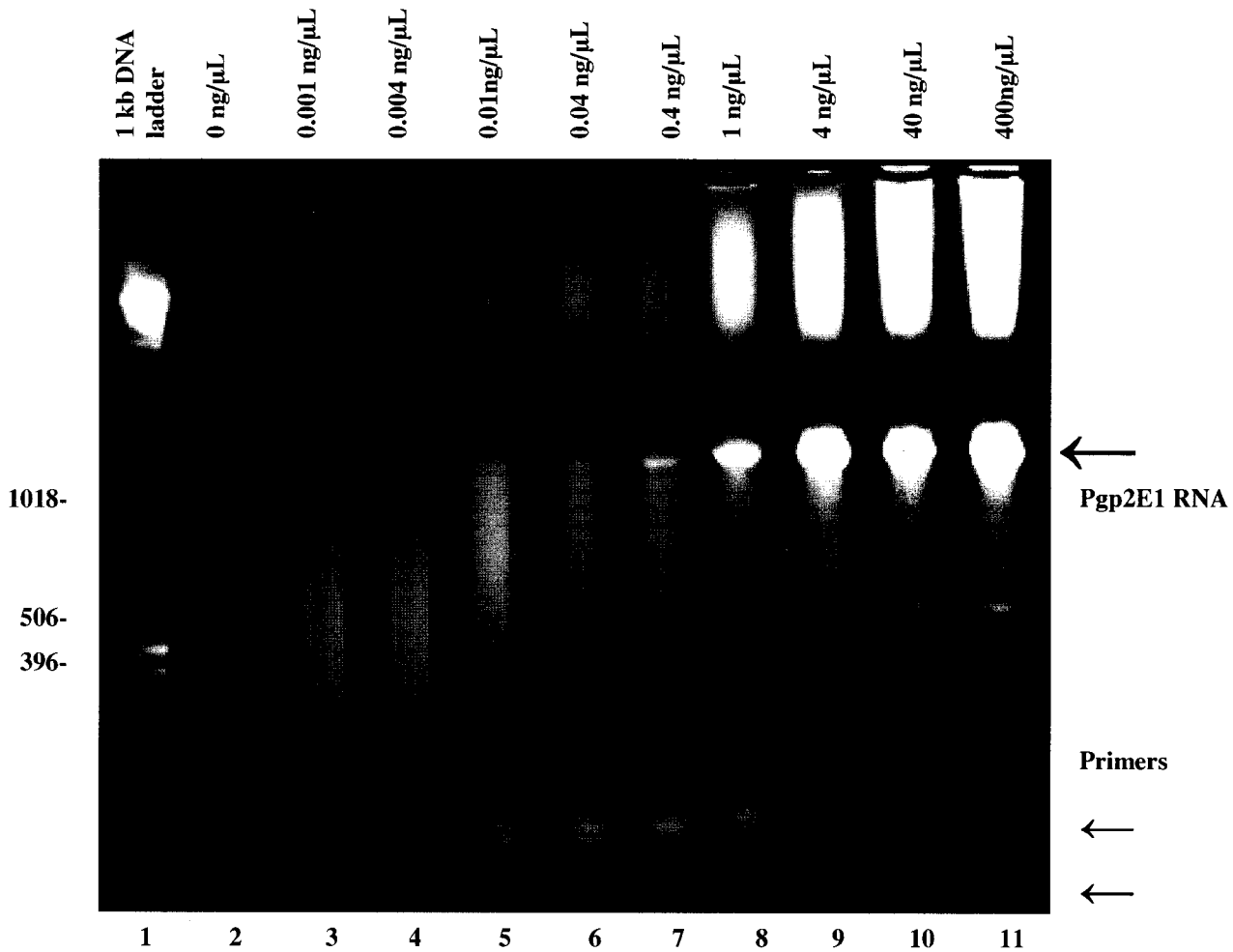


Figure 2.5. Concentrations of *in-vitro* transcribed Pgp2E1 RNA from on a 1.5% agarose gel. Concentrations of poly (A) tailed *in-vitro* transcribed Pgp2E1 RNA products from 400 ng/μL to 0 ng/μL were amplified by PCR and visualized to determine the sensitivity of the Yeast Poly (A) Tailing RT-PCR method.

2.1.4 Visualization of γ^{32}P-ATP labeled Poly(A) tailed RT-PCR products on a 6% denaturing polyacrylamide/urea gel.

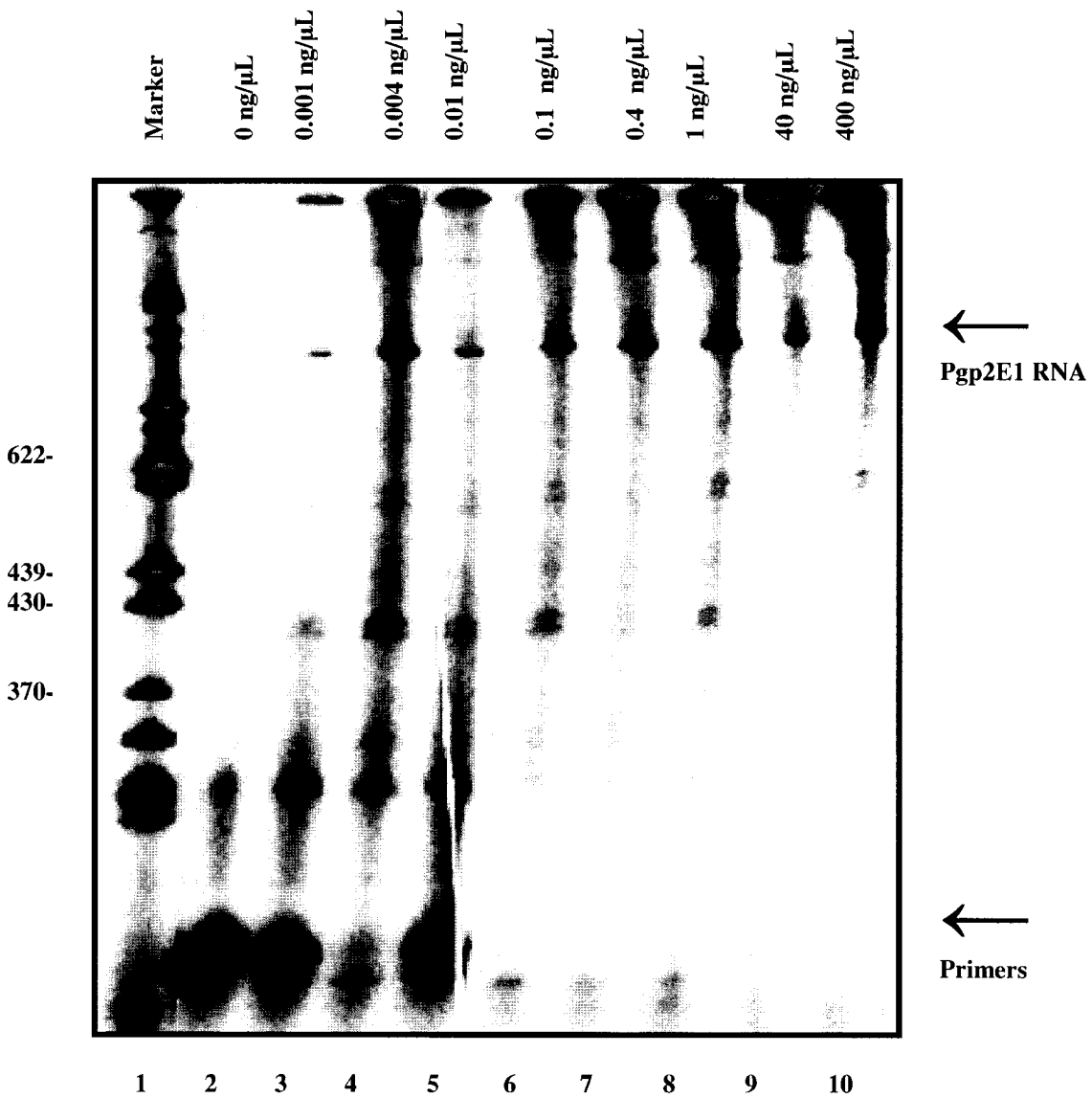


Figure 2.6. Concentrations of *in-vitro* transcribed Pgp2E1 RNA on a 6% denaturing polyacrylamide/7M urea gel. Concentrations of poly (A) tailed *in-vitro* transcribed Pgp2E1 RNA products from 400 ng/µL to 0 ng/µL were amplified by PCR and visualized to determine the sensitivity of the Yeast Poly (A) Tailing RT-PCR method. The marker used is plasmid pBR322 cut with restriction enzyme HaeIII.

To determine whether the Yeast Poly(A) tailing RT-PCR method would be more sensitive, experiments were also carried out using 5' γ^{32}P-ATP labeled Pgp2E1 forward primer. Concentrations of poly(A) tailed *in-vitro* transcribed Pgp2E1 RNA products from

400 ng/μL to 0 ng/μL were amplified by PCR and were run on a 6 % denaturing polyacrylamide/7M urea gel to determine the sensitivity of this method (Figure 2.6.). Detection of Poly (A) tailed Pgp2E1 *in-vitro* transcribed RNA (most intense band with a size approximate to 1048 bp) was possible from 400 ng/μL to 0.001 ng/μL (lanes 3-10). Several less distinct bands were also detected at approximately 600 bp and 400 bp in the same samples. Large smears present at the bottom of the gel are primers.

2.2 Discussion

All pGEM4Z-Pgp2 plasmids were successfully linearized after the restriction digest with Hind III except for pGEM-Pgp2B1, as only one distinct band was exhibited when a small amount of sample was run on a 1% agarose gel (Figure 2.3.). pGEM-Pgp2B1 exhibited partial digestion as one faint band was apparent. In other experiments, complete linearization of pGEM-Pgp2B1 has been observed. This sample was still used for *in-vitro* transcription as some linearized plasmid sample is present.

In-vitro transcription was performed on all pGEM4Z-Pgp2 plasmids. Small samples of the newly synthesized RNA were loaded and run on a 1.3% formaldehyde gel to confirm successful transcription (Figure 2.4.). Ideally *in-vitro* transcribed RNA should only exhibit one band as seen in Pgp2E1 and Pgp2B1 RNA (Figure 2.4.). *In-vitro* transcribed Pgp2C, and Pgp2D RNA display two bands. *In-vitro* transcribed Pgp2A RNA exhibits 3 bands. It is not clear why *in-vitro* transcribed RNA from these plasmids exhibits more than one species. One reason could be related to the presence of a premature termination sequence. If a premature termination sequence is present in the sequence being transcribed, which is recognizable by SP6 RNA polymerase, early termination of transcription would occur. This would explain why two or three different samples of *in-vitro* transcribed RNA are present. However, there

is no premature stop signal present in any of the plasmid sequences. Therefore, it is possible that the structure of the plasmid at certain points may promote dropping off of the SP6 RNA polymerase, yielding the possibility of an incomplete transcript being present. A more likely explanation involves transcription of incomplete digested plasmid which was not observable on an agarose gel. This would lead to the presence of a larger species of RNA, which is evident in this case. In any case, this has not hindered analysis of liver and tumour samples for Pgp2 mRNA degradation products, as the *in-vitro* transcribed RNA is only acting as a control to test if the Yeast Poly (A) RT PCR method is valid, and does not participate in the actual detection of putative decay products.

The Yeast Poly(A) tailing RT-PCR method was able to amplify concentrations of *in-vitro* transcribed Pgp2E1 RNA from 400 ng/μL to 0.4 ng/μL using unlabelled Pgp2E1 forward primer (Figure 2.5.). The sensitivity of this method was improved when using 5' γ^{32}P-ATP labeled Pgp2E1 forward primer and run on a 6% denaturing polyacrylamide/urea gel (Figure 2.6.). In this case, *in-vitro* transcribed Pgp2E1 mRNA could be detected down to 0.001 ng/μL. These results indicate that the Yeast Poly (A) tailing RT-PCR method is highly sensitive in detecting low concentrations of RNA *in-vitro*. It is imperative to bear in mind that the concentration of putative Pgp2 mRNA degradation products *in vivo* is not known, therefore, it is not possible to determine if this method is suitable for *in vivo* applications. One can speculate however, that there is potential for this method to be successful as 0.4 ng/μL and 0.001 ng/μL are very low concentrations. Upon visualization of the 1.5% agarose gel (Figure 2.5.), it was noticed that a less distinct band was visible with a size of approximately 506 bp. Several less distinct bands were also present on the 6% polyacrylamide/7M urea gel (Figure 2.6.) with sizes approximately 500 bp and 400 bp. It is

possible that these bands were amplified and represent regions of *in-vitro* transcribed Pgp2 mRNA rich in adenine residues where the Oligo dT_{18}-XhoI primer could nonspecifically hybridize. It is possible that such nonspecific nature of the Oligo dT_{18}-XhoI primer could pose a problem for *in vivo* applications (Chapter 3); however it will depend on the concentration of tailed Pgp2 mRNA degradation products. If the concentration of successfully tailed Pgp2 mRNA degradation products is high enough, they should be successfully amplified. Due to the sensitivity of this method, observed here *in-vitro* it was determined that this method will be used to detect putative Pgp2 mRNA degradation products in normal liver and liver tumour tissue samples.

Chapter 3

Using Yeast Poly(A) Tailing Method to Search for Putative Rat Pgp2 mRNA Degradation Products in Normal Liver and Liver Tumours

This chapter describes experiments performed to detect putative Pgp2 mRNA degradation intermediates in total RNA from normal liver and liver tumours from three different animals using the Yeast Poly (A) tailing RT-PCR method. All experimental procedures, results, and a discussion related to the use of Yeast Poly (A) tailing RT-PCR method are included. This chapter assesses the efficiency of the Yeast Poly (A) tailing RT-PCR method in detecting Pgp2 mRNA degradation intermediates. This could potentially have implications for detecting any mRNA degradation intermediates in cells or tissues.

3.0 Methods

3.0.1 Poly(A)-Tailing, Reverse Transcription of *In-vitro* transcribed Pgp2A, Pgp2B1, Pgp2C, Pgp2D and Pgp2E1 RNA controls and Total RNA isolated from Normal Liver and Liver Tumour Tissue Samples

All tissue samples previously collected from animals, were subjected to *in vivo* transcriptional inhibition, and were used in experiments described in this thesis (Lee et al., 1998). Briefly, tissue samples of liver and tumours were collected from rats, which had received an intravenous bolus of 50 μg per 100 g body weight of α-amanitin and an intraperitoneal dose of 150 μg per 100 g body weight of actinomycin D. Actinomycin D and α-amanitin are transcriptional inhibitors, therefore halting the process in which mRNA is synthesized from a DNA template. Tissue samples obtained from three different animals at time points 0, 1, 3, 6, and 12 hours post-injection with transcriptional inhibitors were subjected to total RNA isolation (Lee et al., 1998). Total RNA samples at 0 time point were analyzed for the possible presence of Pgp2 mRNA degradation products. Figure 3.1. is a diagrammatic scheme of the Yeast Poly(A) tailing RT-PCR method. These samples were

named Liver 0.1, 0.2, 0.3 and Tumour 0.1, 0.2, 0.3. *In-vitro* transcribed Pgp2 RNA (100 ng) and 10 μg of total RNA from each tissue sample was used for the Poly(A) Tailing method (see Methods 2.0.2). All samples then underwent a reverse transcription reaction (see Methods 2.0.2) As a control to test if Poly(A) tailing was successful, reverse transcription was carried out on non-tailed *in-vitro* transcribed RNA samples. To test for the presence of possible degradation products, reverse transcription was also carried out on nontailed total RNA isolated from all six tissue samples.

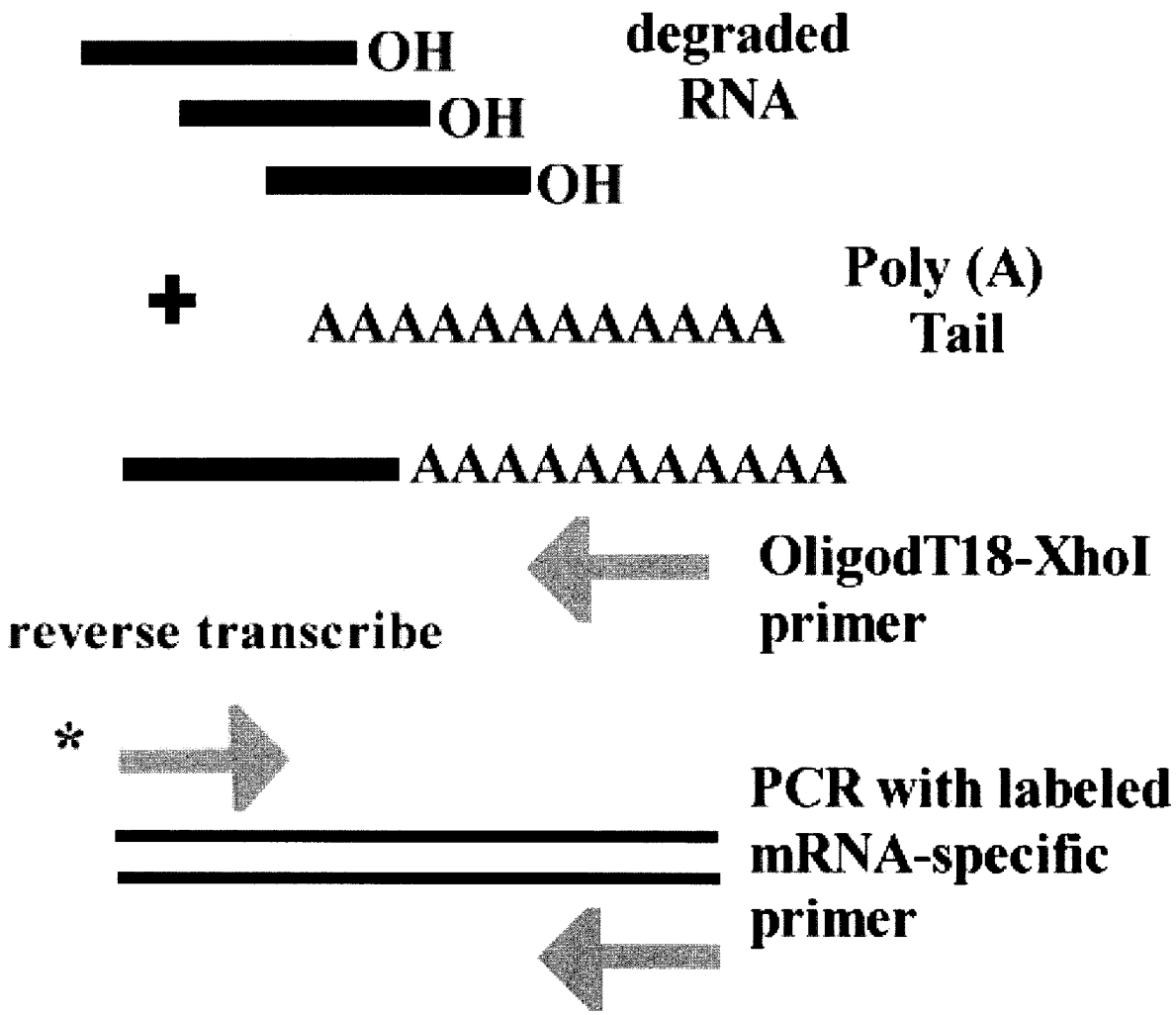


Figure 3.1. Summary of the Yeast Poly(A) tail RT-PCR method used to search for putative Pgp2 mRNA degradation intermediates.

3.0.2 Amplification and Purification of Putative Pgp2 mRNA Degradation Products

PCR was used to amplify possible degradation products of Pgp2 mRNA in normal liver and liver tumour. It was initially thought that identification of possible degradation products would only be possible through analysis of 5' labeled γ^{32}P-ATP PCR products on a 6% polyacrylamide/7M urea gel. However, due to the apparent sensitivity of the technique (see Discussion 2.2), some analysis on cold PCR products were carried out on a 1.5% ethidium bromide stained agarose gel. Five general PCR reactions were set up to analyze the five segments of Pgp2 mRNA represented by pGEM4Z plasmid constructs (Figure 3.2.). Each general reaction included tailed and nontailed samples of the in-vitro transcribed RNA sample corresponding to the region of study (i.e. Pgp2E1), normal liver (0.1, 0.2, 0.3), and liver tumour (0.1, 0.2, 0.3). To each individual reaction the following was added: 3.5 μL of 10X PCR Buffer; 3.5 μL of 2.5 mM dNTPs; 1 μL 50 mM $MgCl_2$; 1 μL of reverse Oligo dT_{18}-XhoI primer (100 ng/μL); 1 μL of Taq Polymerase (5 U/μL); and ddH_2O to make the final reaction volume up to 35 μL. For PCR reactions involving Pgp2A, Pgp2B1, and Pgp2E1 as the region of study, 5 μL of γ^{32}P-ATP 5' labeled 2A, 2B1 and 2E1 forward primer respectively, were added to each individual reaction. For PCR reactions involving Pgp2C and Pgp2D as the region of study, 5 μL (100ng/μL) of unlabeled 2C and 2D Forward Primers respectively were added to each individual reaction (see Table 3.1 for primer sequences). PCR parameters entailed: 94°C for 30 seconds; 50°C for 30 seconds; and 72°C for 45 seconds for 30 cycles on a Minicycler™ Peltier Thermal Cycler. The PCR products utilizing the labeled primers (2A, 2B1, and 2E1) were loaded and ran for 3 hours on a 6% polyacrylamide/7M urea gel with 1 X TBE buffer. The gel was dried and exposed for 24 hours. Autoradiography was carried

out using Cyclone phosphorimager using the OpiQuant software. PCR products utilizing unlabeled forward primers (2D and 2C) were ran on a 1.5% ethidium bromide stained agarose gel with 0.5 X TBE buffer and were visualized using ChemiImager™ System.

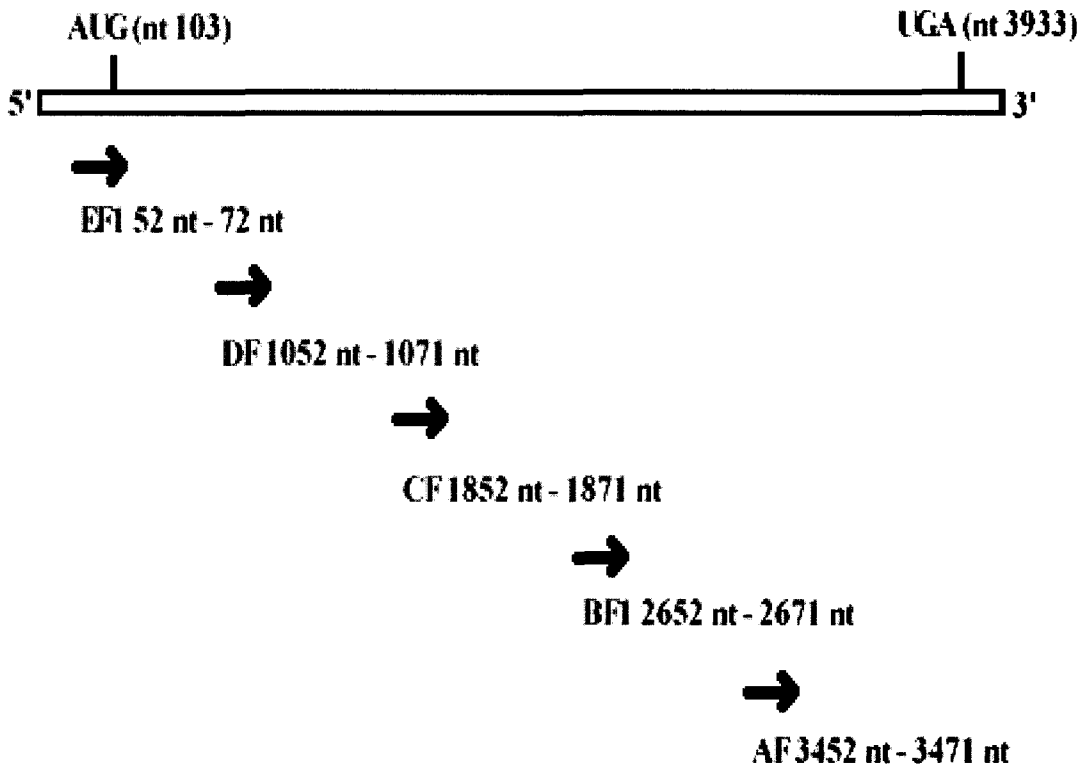


Figure 3.2. Location of forward primers used in the polymerase chain reaction to amplify possible Pgp2 mRNA degradation products.

Table 3.1 Forward primer sequences used in PCR reactions

Primer	**Sequence (5' – 3') ***
2A forward primer	**CTCGGATCC**AGGAGCCCATCCTGTTTGAC
2B1 forward primer	**CTCGGATCC**CTTAGTCTATGGCTGGCAGC
2C forward primer	**CTCGGATCC**ATAGCTCACCGCTTGTCTAC
2D forward primer	**CTCGGATCC**CCTCCTTGGTCCTCTAAAT
2E1 forward primer	**CTCGGATCC**ACATTCTTGGCGGACTTCGCGA

* Bold print represents BamHI restriction site.

Tissues exhibiting differences between tailed and nontailed samples, indicating potential Pgp2 mRNA degradation product(s) underwent another PCR reaction with unlabeled forward primer and were ran on a 1.5% ethidium bromide stained agarose gel with 0.5 X TBE buffer. The gel was visualized using ChemiImager™ System. The potential product(s) was then excised from the gel using a sharp scalpel under UV light, and gel purified using QIAEX II Gel Extraction Kit (QIAGEN, Mississauga, ON). Two μL of the purified sample then was used for 6 PCR reactions and included the following reagents: 3.5 μL of 10X PCR Buffer; 3.5 μL of 2.5 mM dNTPs; 1 μL 50 mM $MgCl_2$; 5 μL (100 ng/μL) of EF1 forward primer; 1 μL of reverse Oligo dT_{18}-XhoI (100 ng/μL) primer ; 1 μL of Taq Polymerase (5 U/μL); and ddH_2O to make the final reaction volume up to 35 μL. The PCR products were pooled together totaling a final volume of 210 μL. Two volumes of 100% ethanol and $1/10^{th}$ the volume of 3 M NaOAc pH 5.2 was added and the solution was mixed thoroughly and precipitated at -20°C for 24 hrs. The solution was removed and centrifuged for ten minutes at 12,000 rpm. The supernatant was removed and the DNA pellet was washed by gently adding 200 μL of 70% cold ethanol. The sample was then centrifuged for 5 minutes and the supernatant was aspirated. The DNA pellet was resuspended in 7 μL of ddH_2O and was loaded on a 1.5% ethidium bromide stained agarose gel. Again the potential product(s) were excised from the gel and purified. The purified sample was then used to perform ligation and transformation experiments in an attempt to sequence the potential degradation product(s).

3.0.3 Preparation of Competent Cells

DH5-α competent cells required for transformation, were then prepared via the

following methodology: 10 μL of frozen stock DH5-α cells were added to 3 mL Luria-Bertani (LB) broth in a sterile 15 mL tube and incubated at 37°C overnight with agitation. Ten μL of this culture was transferred to a flask containing 50 mL LB broth and was again incubated at 37°C with agitation until the OD reached 0.3 (2 hours) at 600 nm. The culture was then centrifuged for 10 minutes at 2,500 rpm and the supernatant was removed. The pellet was resuspended in 3.5 mL of cold 50 mM $CaCl_2$ and the volume was increased to 25 mL. The cells were then placed on ice for 30 minutes and then centrifuged at 2,500 rpm for 10 minutes. The supernatant was again removed and the competent cells were resuspended in 3.5 μL of cold 50 mM $CaCl_2$.

3.0.4 Ligation of Putative Pgp2 mRNA degradation products.

To prepare for the ligation of the potential product(s) it was necessary to perform a restriction digest on both the purified product and pGEM7Zf(+) cloning vector (Fig. 3.1.). All of the gel purified product (20 μL) was added to the restriction digest, which included the following reagents: 6 μL of *Bam*HI; 6 μL of *Xho*I; 3.6 μL of 10X double digest buffer; and 4 μL of dd H2O. The solution was incubated at 37°C for 3 hours. ddH_2O was added to make a final colume of 100 μL, and standard ethanol precipitation was performed. The pellet was resuspended in 10.5 μL of ddH_2O. The resuspended product was added to 1 μL of digested pGEM7Zf(+) vector (Figure 3.3.) and the following reagents were added: 3 μL of T4 DNA Ligase (400 U/μL) (New England Biolabs); and 1.45 μL of 10 X Ligase Buffer (New

England Biolabs). The reaction mixture was incubated at 4°C for 24 hours.

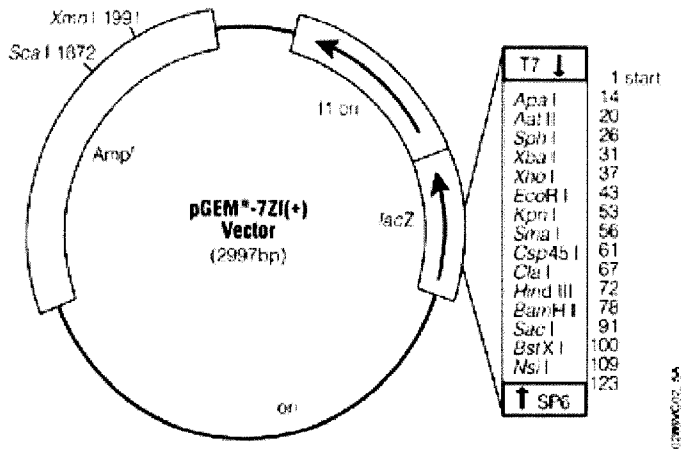


Figure 3.3. pGEM7Zf(+) cloning vector used in ligation reaction (Promega, Madison, WI).

3.0.5 Transformation and Plating

In a prechilled sterile tube all of the ligation mix was added to 200 μL of DH5-α competent cells. The mixture was stored on ice for 30 minutes and then heat shocked at 42°C for 2 minutes. One μL of LB Broth was added and the reaction was incubated at 37°C for 30 minutes with agitation. 2μL of isopropyl b-D-thiogalactoside (IPTG) and 50 μL of 5-bromo-4-chloro-3- indolyl- beta- D- galactopyranoside (X-GAL) were added and the mixture was poured on a pre-warmed agar plate. The plate was incubated at 37°C with the lid partially off in order for the solution to dry for 20 minutes and then inverted. After 24 hours 10 white colonies were picked with a sterile pipette tip, and then placed in a sterile tube containing 3 mL LB broth and 1.5 μL 50 mg/mL ampicilin. The cultures were grown overnight at 37°C with agitation.

To determine if any of the white bacterial colonies positively contained an insert that might be a possible degradation product it was necessary to purify the plasmid DNA from the rest of the culture. The QIAprep Spin Miniprep Kit (QIAGEN) was used for this procedure and yielded a 50 μL sample of purified plasmid DNA.

3 μL of purified plasmid sample then underwent a restriction digest with 1 μL of XhoI, 1μL of BamHI, 1 μL of 10X Double Digest Buffer, and 4 μL ddH_2O. The reaction mixture was incubated for 1 hour at 37°C and then ran on a 1.5% ethidium bromide stained agarose gel. The gel was visualized using ChemiImager™ System.

3.0.6 Dideoxy Sequencing of Putative Degradation Product(s)

Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemical, Cleveland, OH) was used to sequence purified plasmid samples that contain an insert. The protocol is as follows: 5 μL of purified plasmid sample for clones J1, J2, J7, and J10 was added to 15 μL of ddH_2O. 2 μL of 2 M NaOH, 2 mM EDTA mix was added and the solution was incubated at 80°C for 5 minutes in order to hydrolyze any RNA that may be present. ddH_2O was added to increase the volume of the solution to 100 μL. Standard ethanol precipitation was performed and the pellet was resuspended in 7 μL of ddH_2O. To the DNA, 2 μL of sequencing buffer and 10 ng of 7Z-T7 primer was added. The solution was mixed and incubated at 37°C for 15 minutes. The following was then added to the DNA solution: 1μL of 100 mM DTT; 2 μL of diluted dGTP labeling mix (1:20 with ddH_2O); 1 μL of 1 mM $MnCl_2$; 1 μL ^{35}S-dATP (10μCi/μL) (Amersham Biosciences Corp.), and 2 μL Sequenase™ (1:10 dilution with TE). The mixture was left at room temperature for 3 minutes. 3.5 μL of labeling reaction mix was then added to four separate microcentrifuge tubes, each containing 2.5 μL of a different dideoxy triphosphate. The solutions were incubated at 37°C for 5 minutes and 4 μL of Stop Solution was added. The sequencing reaction for Clones J6 and J9 required the use of all purified plasmid sample (45 μL) as the concentration of these plasmid samples were lower than the others. The amount of reagents used for these two samples doubled in all respects.

Prior to loading on an 8% polyacrylamide/ 7 M urea sequencing gel, the solutions were incubated at 80°C for 2 minutes, and then quickly placed on ice. The sequencing gel was also pre-loaded with Formamide Loading Solution to ensure the lanes were not leaking. The gel was ran for 30 minutes at 60 W constant current to pre-warm it. After the samples were loaded, the gel was ran at 60 W constant current until the blue dye of the stop solution reached the very bottom of the gel (approximately 1.5 hours). The gel was then dried and exposed for 48 hours. The image was scanned using Cyclone phosphorimager and visualized using OpiQuant software.

Results 3.1

3.1.4 Detection of degradation products in Liver and Tumour tissue samples using Yeast Poly(A) Polymerase RT-PCR method.

The detection of putative Pgp2 mRNA degradation intermediates in total RNA from normal liver and liver tumour from three different animals involved using the Yeast Poly (A) tailing RT-PCR method. In order to detect potential degradation products it was necessary to include nontailed-RT-PCR products, as presence of bands in tailed samples that were absent in nontailed samples would indicate the existence of potential decay products. The analysis of normal liver and liver tumour tissue samples for putative Pgp2 mRNA decay products involved the use of five different forward primers (see Figure 3.2.) ensuring that the entire Pgp2 mRNA sequence was analyzed. Samples of tailed and nontailed *in-vitro* transcribed RNA corresponding to the region under study were also included as verification that the Yeast Poly (A) tailing RT-PCR method was employable.

Analysis of liver tissue samples with forward primer 2A (Figure 3.4) was carried out

on an ethidium bromide stained 1.5% agarose gel. Several bands are noticed in both the tailed and nontailed samples (lanes 5, 7, 8, and 9), however, there is no significant differences present between tailed and nontailed samples. There were PCR products present/detected in tailed samples that correspond to *in-vitro* transcribed Pgp2A. This sample exhibits two distinct bands, one of approximately 1 kb, while Pgp2A nontailed sample exhibits several. The 1 kg fragment likely corresponds to the Pgp2A RNA size. Differences are noted between tailed and nontailed *in-vitro* transcribed Pgp2A RNA, indicating that the Yeast Poly (A) tailing RT-PCR method was successfully applied.

Analysis of tumour samples with forward primer 2A was carried out on a 6% denaturing polyacrylamide/urea gel (Figure 3.5.). Again, there are no differences between tailed and nontailed tumour samples detected. Similar results were obtained with *in-vitro* transcribed Pgp2A RNA. The tailed sample exhibits two distinct bands (lane 2), while Pgp2A nontailed sample exhibits several (lane 3). As with normal liver samples (Figure 3.4.), no distinct PCR products were seen in tailed tumour samples versus nontailed samples (Figure 3.5., lanes 4-9).

Analysis of liver and tumour samples with forward primer 2B1 was carried out on a 6% denaturing polyacrylamide/urea gel and results are shown in Figure 3.6. As seen in Figure 3.6. there are no differences between tailed and nontailed normal liver and liver tumour tissue samples present (lanes 4-15). *In-vitro* transcribed Pgp2B1 tailed sample exhibits one band of approximately 1 kb, while Pgp2B1 nontailed sample exhibits several. The presence of tailed in-vitro transcribed PCR products (lane 2) indicate that the Yeast Poly (A) tailing RT-PCR method was successful.

Analysis of liver samples (Figure 3.7.) and tumour samples (Figure 3.8.) with forward primer 2C were carried out on ethidium bromide stained 1.5% agarose gels. There are no significant differences between tailed and nontailed tissue samples in both normal liver (see Figure 3.7., lanes 4-9) and liver tumour (see Figure 3.8., lanes 4-9). In tailed samples of both normal liver (lanes 4, 6, and 8) and liver tumour (lanes 4, 6, and 8), a small smear was observed near the well. Curiously, this smear was also present in tailed *in-vitro* transcribed RNA (lane 2). Due to its presence in all tailed samples, this smear was not further investigated as a putative degradation product. *In-vitro* transcribed Pgp2C tailed sample exhibits one distinct band approximately 1 kb in size, while Pgp2C nontailed sample exhibits several (Figure 3.7. and 3.8., lanes 2 and 3), again indicating that the Yeast Poly (A) tailing RT-PCR method was again successful in detecting *in-vitro* transcribed RNA.

Analysis of liver and tumour samples with forward primer 2D was also carried out on a 1.5% ethidium bromide stained agarose gel (Figure 3.9.). There were no differences between tailed and nontailed tissue samples present (lanes). *In-vitro* transcribed Pgp2D tailed sample exhibits one distinct band (lane), while Pgp2D nontailed sample exhibits several (lane). Once again, the differences present in tailed and nontailed *in-vitro* transcribed Pgp2D RNA samples indicate that the Yeast Poly (A) tailing RT-PCR method was successfully employed.

PCR products of normal liver and liver tumour samples with 2E1 forward primer were both analyzed on a 6% denaturing polyacrylamide/urea gel (Figure 3.10. and 3.11. respectively). Distinct differences are present between the tailed samples of both tissue samples (Figure 3.10. and 3.11., lanes 4-9). In tailed liver samples (Figure 3.10., lanes 4, 6, and 8) a smear is present from approximately 500 bp to approximately 181 bp in all three

tissue samples. This smear is not present in nontailed samples (lanes 5, 7, and 9). In tailed liver tumour samples a very intense smear is present from approxiamtely 439 bp to approximately 181 bp in two tissue samples (Figure 3.11., lanes 6 and 8). *In-vitro* transcribed PgpE1 tailed sample exhibits one very intense distinct band approximately 1 kb in size (lane 2), and four faint bands, while Pgp2E1 nontailed sample exhibits several bands (lane 3). The distinct band of approximately 1 kb likely corresponds to *in-vitro* transcribed RNA indicating that the Yeast Poly(A)tailing method was used successfully in this experiment.

Liver and tumour samples with 2E1 forward primer were then ran on an ethidium bromide stained 1.5% agarose gel (Figure 3.12. and 3.13. respectively) to determine if further analysis of these potential Pgp2 mRNA degradation products could proceed without the use of radioactive material. Consistent with analysis on the 6% denaturing polyacrylamide/urea gel (Figure 3.10.), tailed normal liver samples exhibit a smear around the 500 bp size in all three tissue samples (lanes 4, 6, and 8). Nontailed samples do not have a smear present, but one distinct band at approximately 1000 bp (lanes 5, 7, and 9). Tailed tumour samples also exhibit the same smear (lanes 6 and 8) when ran on a 1.5 % agarose gel (Figure 3.13.) which is consistant with results obtained from 6% denaturing polyacrylamide/urea gel analysis (Figure 3.11.). Nontailed samples have 2 bands at approximately 1000 and 506 bp that are present in Tumour 0.1 and Tumour 0.2 (lanes 5 and 7).

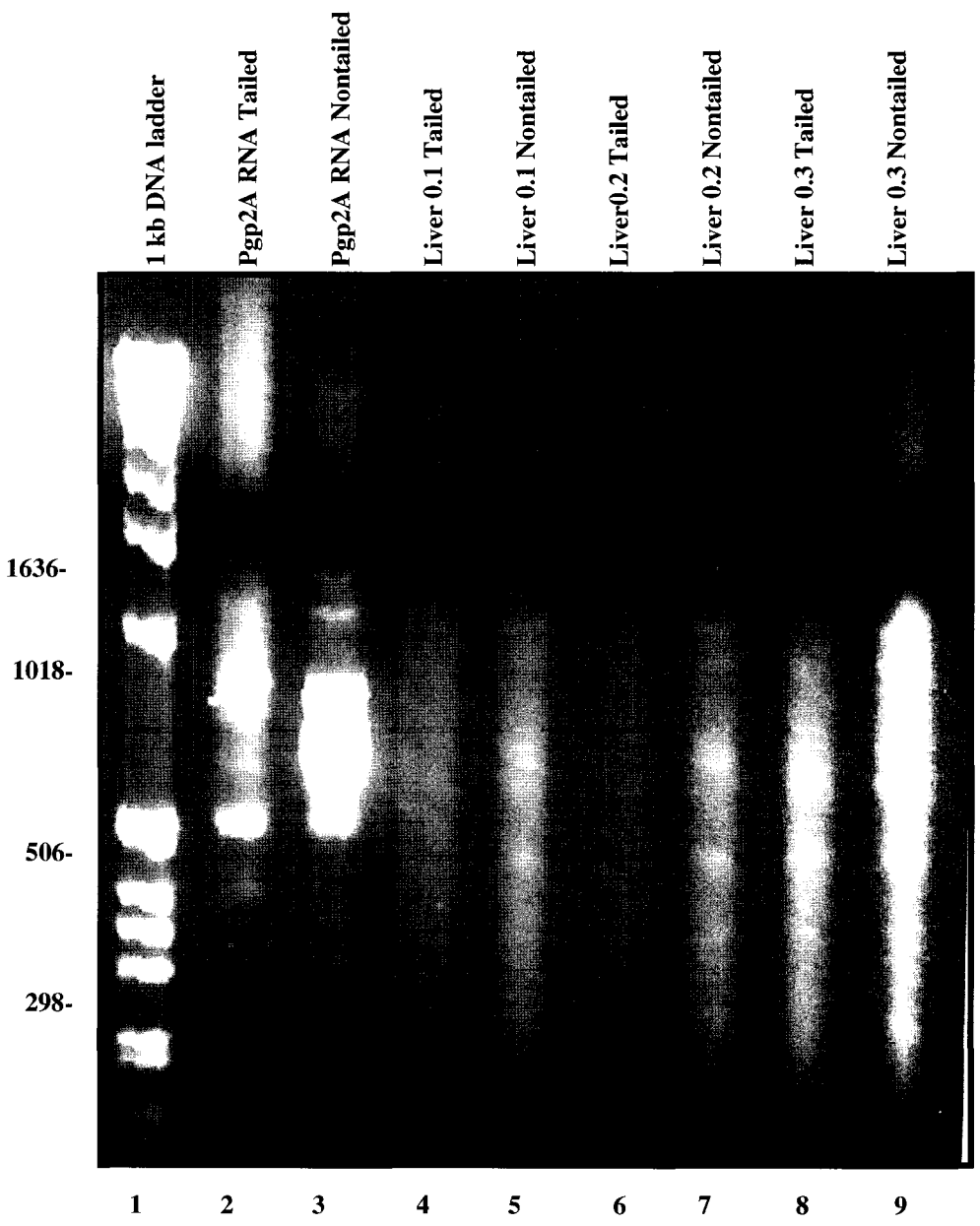


Figure 3.4. Liver samples analyzed with Pgp2A forward primer on a 1.5% agarose gel. Prior to amplification with Pgp2A forward primer, liver samples were either tailed and reverse transcribed (even lanes), or reverse transcribed (odd lanes) to search for putative Pgp2 mRNA degradation intermediates. Pgp2A cDNA control is 799 base pairs (bp) long.

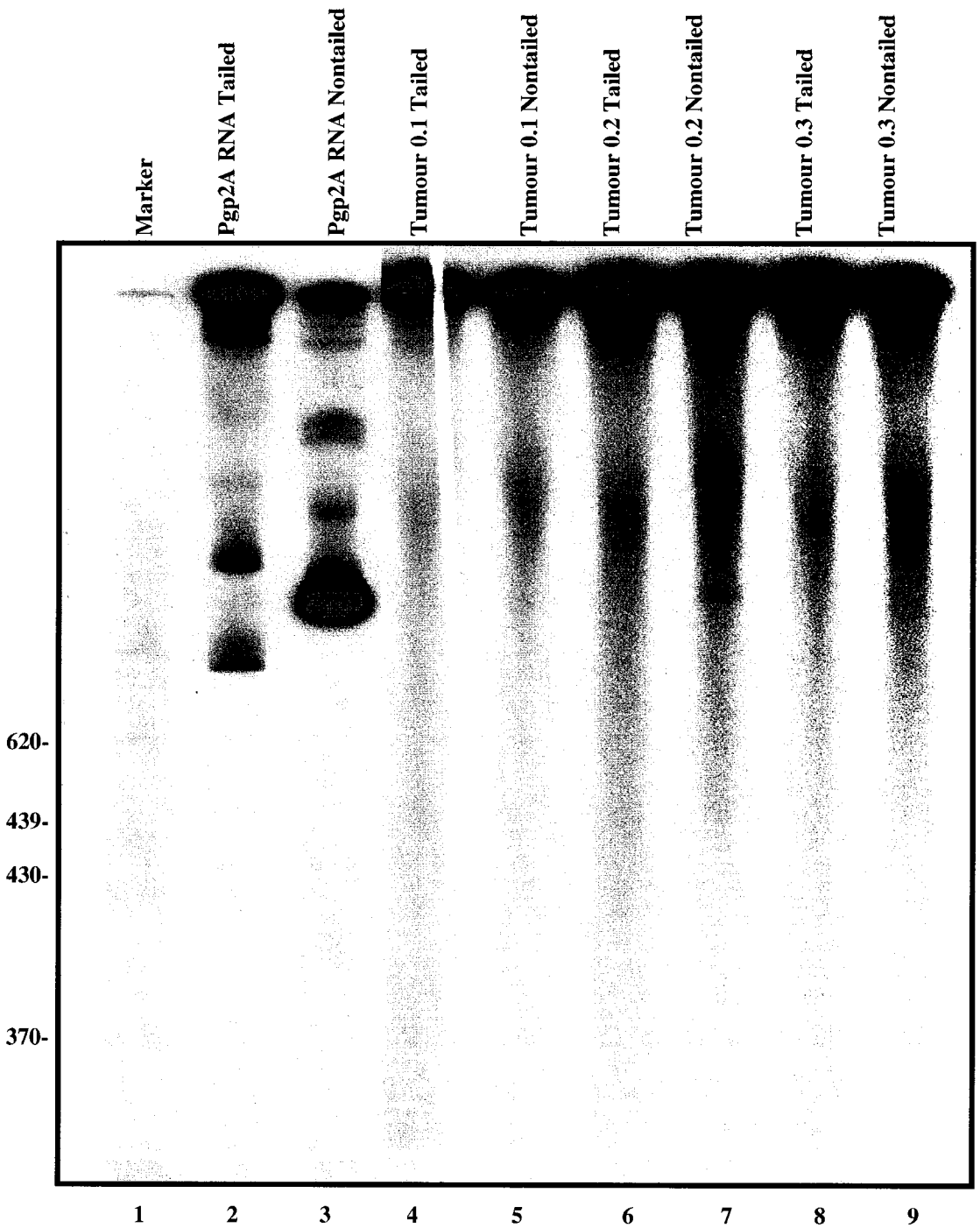


Figure 3.5. Tumour samples analyzed with Pgp2A forward primer on a denaturing 6% polyacrylamde/7M urea gel. Prior to amplification with Pgp2A forward primer, liver samples were either tailed and reverse transcribed (even lanes), or reverse transcribed (odd lanes) to search for putative Pgp2 mRNA degradation intermediates. Pgp2A cDNA control is 799 bp long. The marker used was pBR322 cut with the restriction enzyme Hae II.

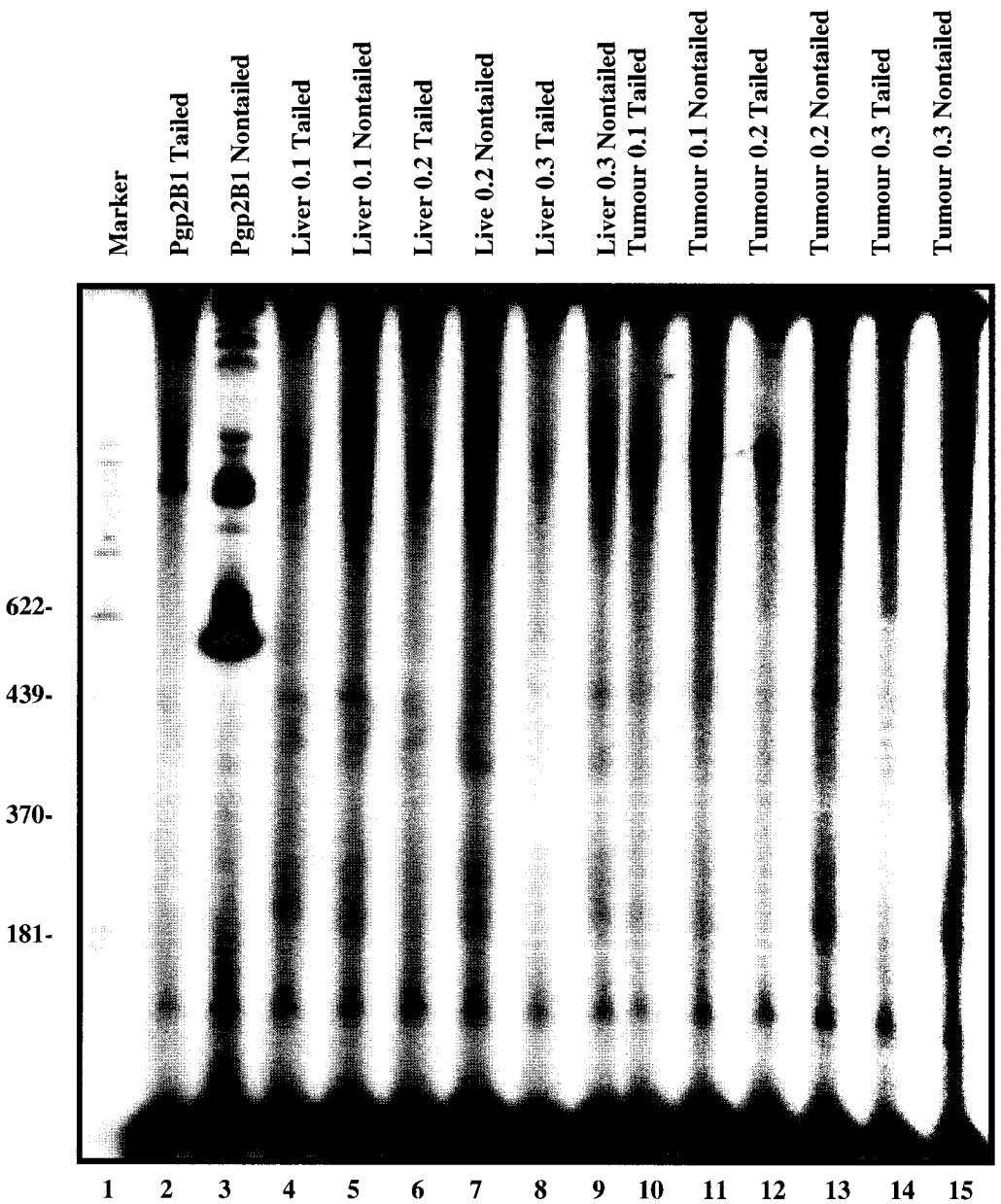


Figure 3.6. Liver and tumour samples analyzed with Pgp2B1 forward primer on a 6% denaturing polyacrylamde/7M urea gel. Prior to amplification with Pgp2B1 forward primer, samples were either tailed and reverse transcribed (even lanes), or reverse transcribed (odd lanes) to search for putative Pgp2 mRNA degradation intermediates. Pgp2B1 cDNA control is 800 bp long. The marker used was pBR322 cut with the restriction enzyme Hae II.

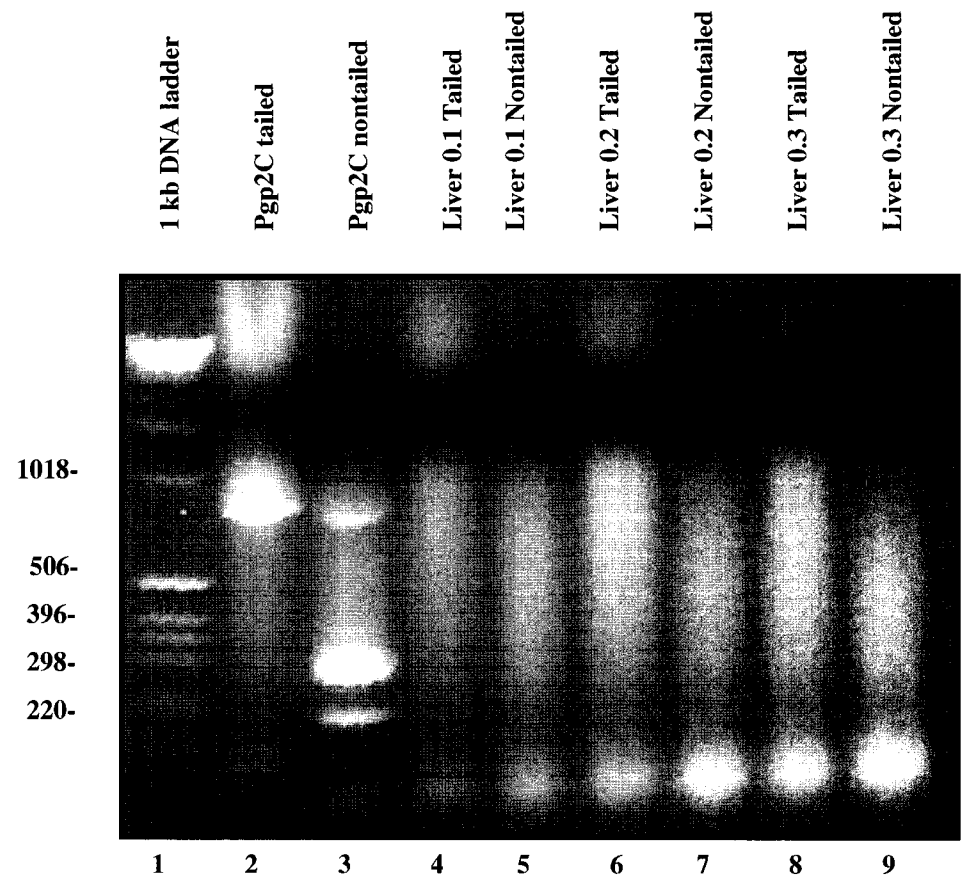


Figure 3.7. Liver samples analyzed with Pgp2C forward primer on a 1.5% agarose gel. Prior to amplification with Pgp2C forward primer, samples were either tailed and reverse transcribed (even lanes), or reverse transcribed (odd lanes) to search for putative Pgp2 mRNA degradation intermediates. Pgp2C cDNA control is 800 bp long.

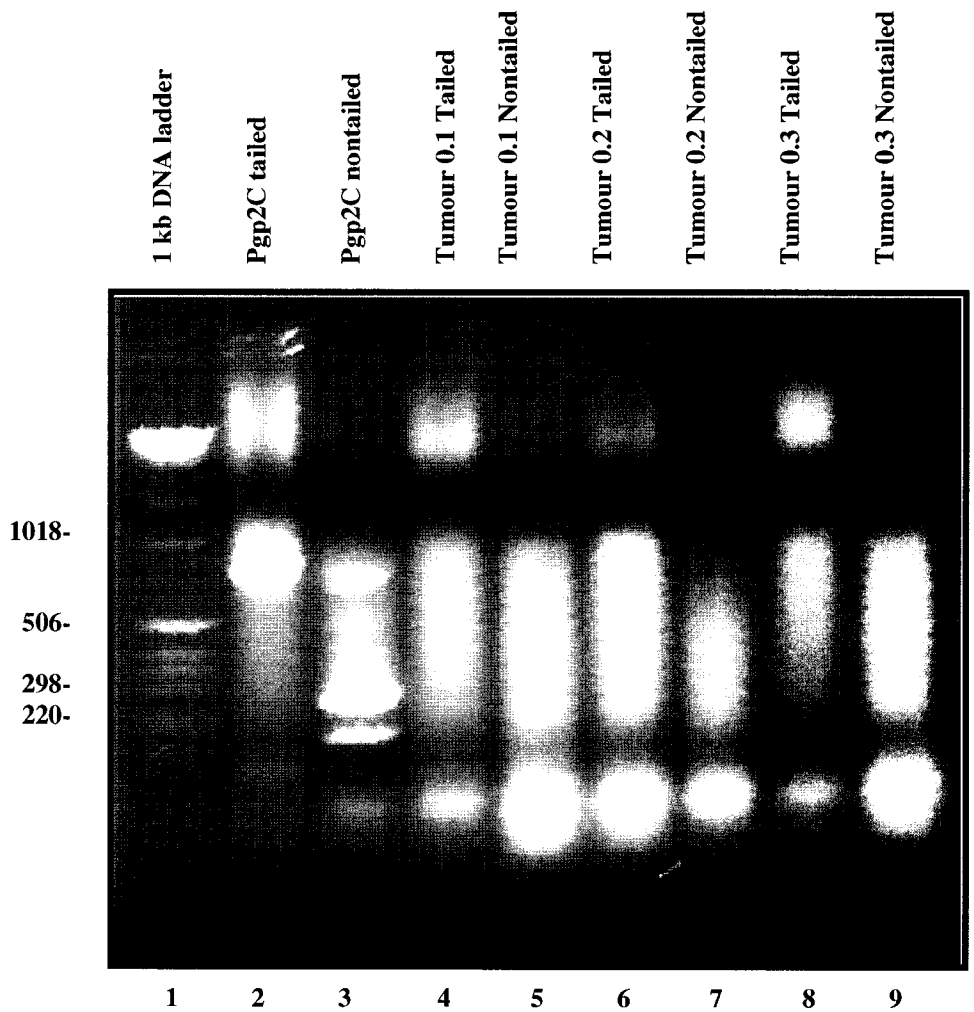


Figure 3.8. Tumour samples analyzed with 2C forward primer on a 1.5% agarose gel. Prior to amplification with Pgp2C forward primer, samples were either tailed and reverse transcribed (even lanes), or reverse transcribed (odd lanes) to search for putative Pgp2 mRNA degradation intermediates. Pgp2C cDNA control is 800 bp long.

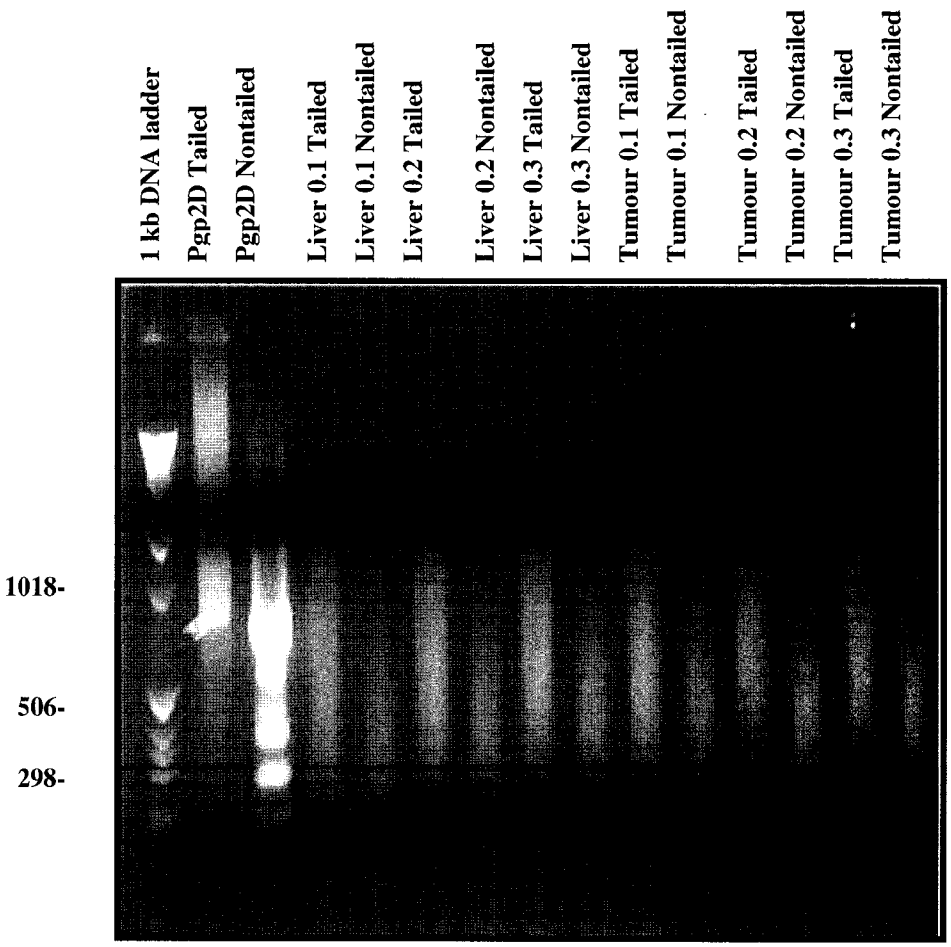


Figure 3.9. Liver and tumour samples analyzed with Pgp2D forward primer on a 1.5% agarose gel. Prior to amplification with Pgp2D forward primer, samples were either tailed and reverse transcribed (even lanes), or reverse transcribed (odd lanes) to search for putative Pgp2 mRNA degradation intermediates. Pgp2D control is 800 bp long.

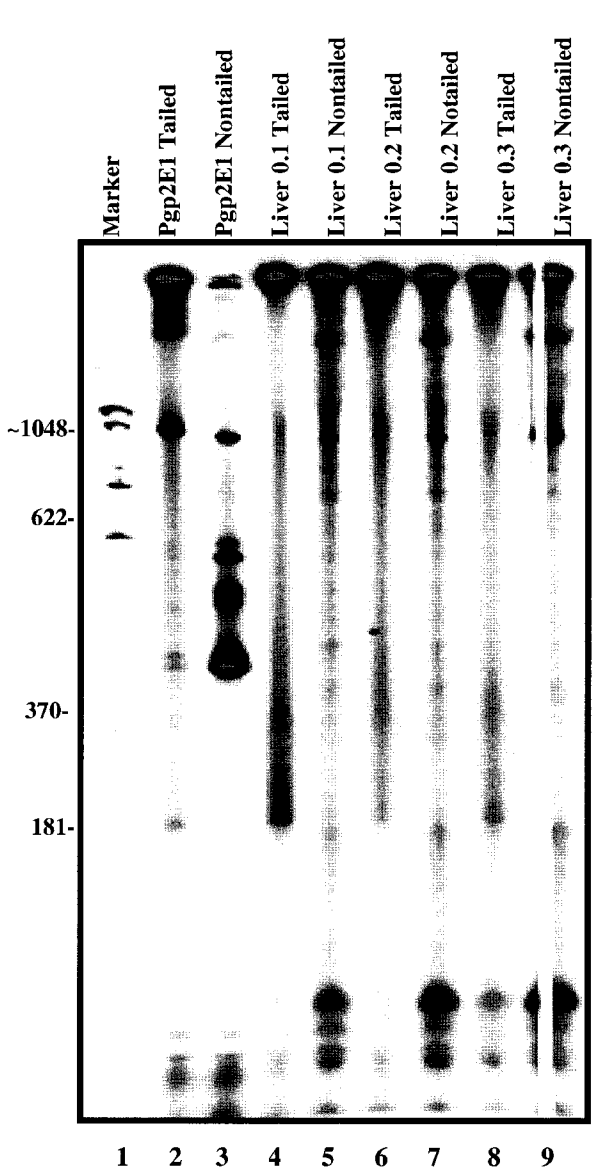


Figure 3.10. Liver tissue samples analyzed with Pgp2E1 forward primer on a 6% denaturing polyacrylamide gel. Prior to amplification with Pgp2E1 forward primer, samples were either tailed and reverse transcribed (even lanes), or reverse transcribed (odd lanes) to search for putative Pgp2 mRNA degradation intermediates.Pgp2E1 control is 1048 bp long. The marker used was pBR322 cut with restriction enzyme Hae II.

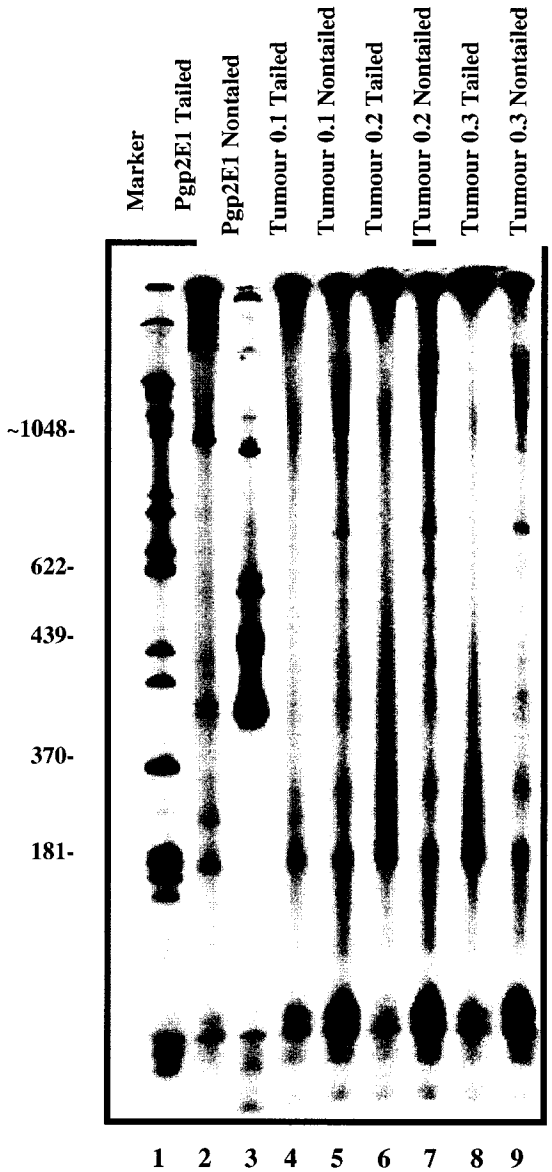


Figure 3.11. Tumour tissue samples analyzed with Pgp2E1 forward primer on a 6% denaturing polyacrylamide gel. Prior to amplification with Pgp2E1 forward primer, samples were either tailed and reverse transcribed (even lanes), or reverse transcribed (odd lanes) to search for putative Pgp2 mRNA degradation intermediates.Pgp2E1 control is 1048 bp long. The marker used was pBR322 cut with restriction enzyme Hae II.

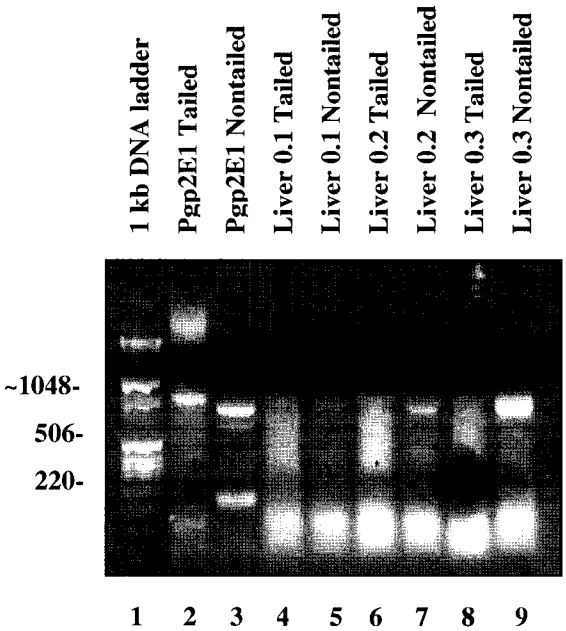


Figure 3.12. Liver tissue samples analyzed with 2E1 forward primer on a 1.5% agarose gel. The same Poly (A) tailed and nontailed samples from Figure 3.10. were analyzed to determine if further analysis could be accomplished without the use of radioactive material.

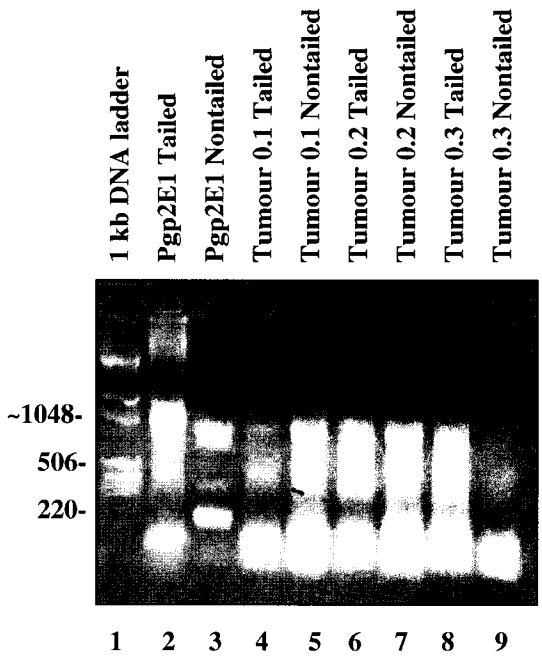


Figure 3.13. Tumour tissue samples analyzed with 2E1 forward primer on a 1.5% agarose gel. The same Poly (A) tailed and nontailed samples from Figure 3.10. were analyzed to determine if further analysis could be accomplished without the use of radioactive material.

3.1.5 Gel Purification and Secondary PCR of Liver samples with 2E1 Forward Primer.

To confirm specificity of the smear observed in normal liver and liver tumour tailed samples, the smear observed in liver samples analyzed with Pgp2E1 forward primer were excised for the gel and purified (see Methods 3.0.2). Secondary PCR was also performed using the purified products as a template. The secondary PCR products were ran on a 1.5% agarose gel to check if the putative degradation products were successfully purified and amplified (Figure 3.14.). All purified secondary PCR liver tissue products exhibit a smear from approximately 600 bp to 250 bp, suggesting that the smear is specific. Putative degradation product(s) present in Liver 0.1 were used in further experiments to determine if the PCR product(s) belong to Pgp2 mRNA.

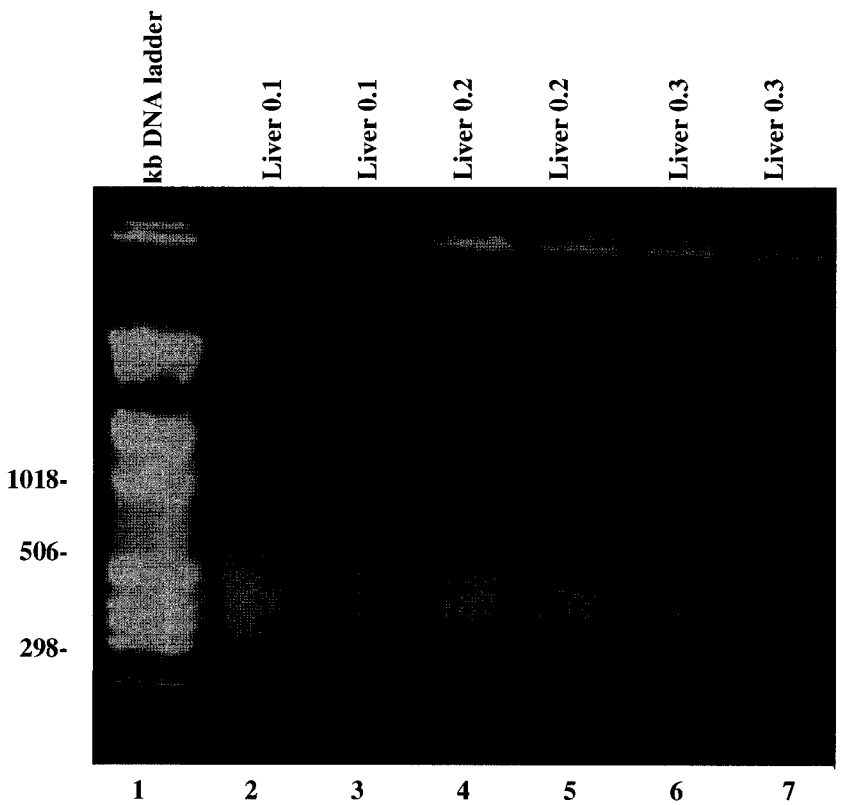


Figure 3.14. Gel purified, secondary PCR products from tailed liver samples on a 1.5% agarose gel. Smears identified in Figure 3.12. were excised and purified. Purified samples underwent secondary PCR with the same primers to determine the specificity of the smears.

3.1.6 Preparation of pGEM7Zf(+) Plasmid Vector for Ligation with Putative Degradation Products.

Plasmid vector pGEM7Zf(+) underwent a linearization reaction with restriction enzymes *Bam*HI and *Xho*I (Figure 3. 15.). The circular structure of the intact plasmid molecule appears as two bands after gel electrophoresis. The major band (approximately 2036 bp) is the plasmid DNA, whereas the top band represents residual chromosomal and nicked plasmid DNA. Complete digestion of the pGEM7Zf(+) plasmid is noted as only 1 band is present in the sample that was digested with *Bam*HI and *Xho*I.

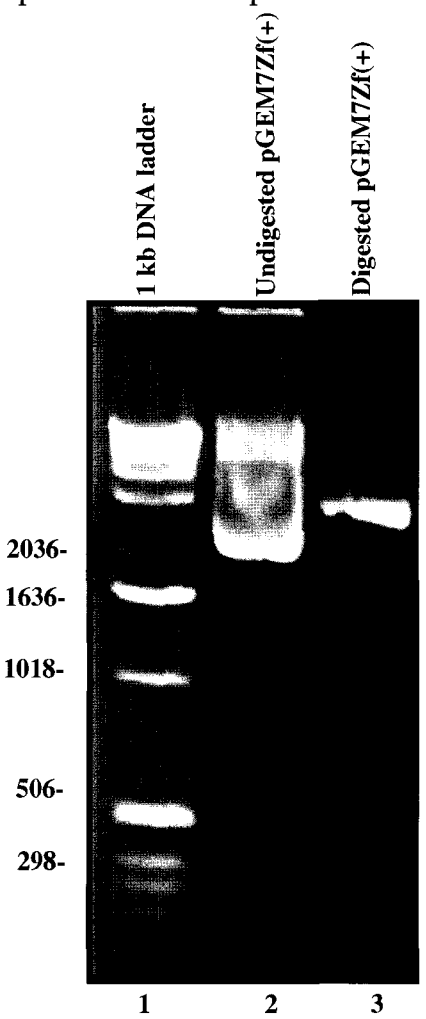


Figure 3.15. pGEM7Zf(+) plasmid linearized with restriction enzymes *Bam*HI and *Xho*I in preparation for ligation reaction on a 1.5% agarose gel. Lane 2 exhibits undigested plasmid, and lane 3 shows digested plasmid sample.

PCR products (see Results section 3.1.2) which are putative Pgp2 mRNA degradation intermediates were purified and digested with *Bam*HI and *Xho*I, and then ligated into linearized pGEM7Zf(+) plasmid (see Method 3.0.4). The ligated reaction mixture was then transformed into DH5-α competent cells (See Method 3.0.5).

3.1.4. Selection of Bacterial Colonies Positive for Insert and Miniprep of Positive Colonies.

White and blue colonies were present on agar plates plated with transformed DH5-α competent cells. Colonies that are positive for the inserted putative degradation product(s) are coloured white. Colonies that do not contain an insert are coloured blue. Ten white colonies were picked and grown in liquid medium overnight (See Methods 3.0.5). The ten plasmid DNA samples were then isolated and subjected to a restriction digest with BamHI and XhoI. Digested plasmid samples were ran on an ethidium bromide stained 1.5% agarose gel (Figures 3.16. and 3.17.) to check for the presence of an insert (See Methods 3.0.5). Figure 3.16. shows results of clones J1 to J5 after restriction digest with BamHI and XhoI. Clone J1 exhibits 2 distinct bands after digestion with BamHI and XhoI (lane 2). The top band represents linearized plasmid DNA, and the second band is approximately 500 bp in size. Clone J2 exhibits 3 bands after digestion (lane 3); the top band again representing plasmid DNA, and two additional bands approximately 506 bp and 220 bp in length. Clones J3, J4, and J5 exhibit only plasmid DNA after the restriction digest (lanes 4, 5, and 6).

Figure 3.17. exhibits clones J6 to J10 after a restriction digest with BamHI and XhoI. Clones J6, J7, J9, and J10 all exhibit two bands (lanes 2, 3, 5, and 6), the top band representing linearized plasmid DNA, and the second band approximately 506 bp in size . Clone J8 exhibits plasmid DNA only. Clones J1, J2, J6, J7, J9, and J10, deemed to contain

inserts were subjected to sequence analysis (see Methods 3.0.5) Samples containing only plasmid DNA after digestion were disregarded and not used in further experiments.

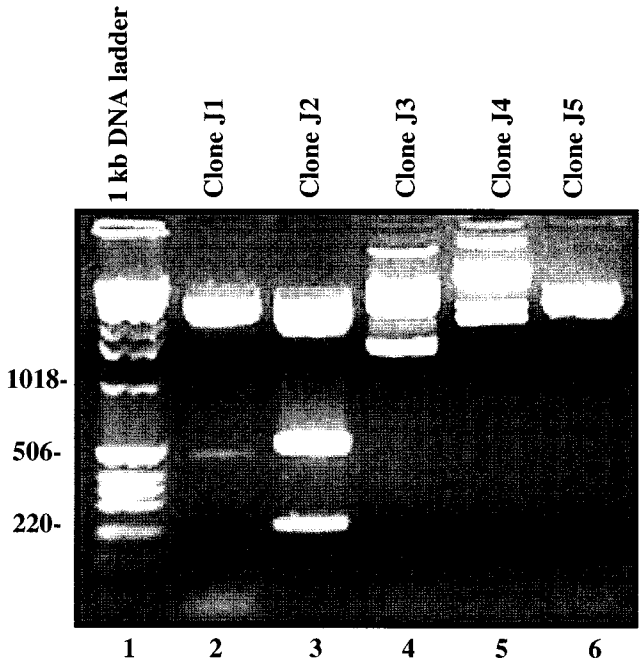


Figure 3.16. Plasmid clones J1-J5 on a 1.5% agarose gel. Clones (lanes 2-6) underwent restriction digest with enzymes BamHI and XhoI to check for the presence of an insert.

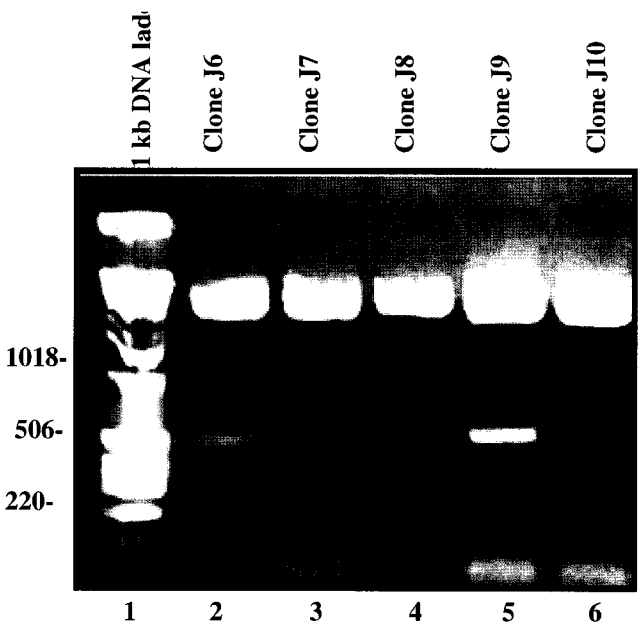


Figure 3.17. Plasmid clones J6-J10 on a 1.5% agarose gel. Clones (lanes 2-6) underwent restriction digest with enzymes BamHI and XhoI to check for the presence of an insert.

3.1.5 Dideoxy Sequencing and Blast Search of Potential Degradation Product(s)

Clones J1, J2, J6, J7, J9, and J10 were manually sequenced (see Methods 3.0.4) to determine if they contained an insert that was a possible Pgp2 mRNA degradation product (See Figure 3.18.). All clones contained the multiple cloning site sequence from the vector pGEM7Zf(+). Sequences for clones J1, J6, J9, and J10 revealed an identical sequence that was different from the vector sequence. This sequence was subjected to a nucleotide BLAST search on the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/) to determine if they were Pgp2 mRNA degradation products. The nucleotide search provided no significant matches. The sequence determined for clone J2 also contained vector sequence and a potential inserted sequence that was unique. This sequence was also subjected to a nucleotide BLAST search that yielded no significant results. This was also the case for clone J7 that again contained the vector sequence plus a potential insert. In conclusion, no Pgp2 mRNA degradation products were detected.

Table 3.2 Sequences of clones identified to contain an insert

Clone	Sequence *
J1	5'... GCCGGGGTCGCGAAGTCCGTTAAGATGGGATCCAAGCTTATCGATTTCGAACCCG GGGTACCGAATTC**CTCGAG**C...3'
J2	5'... TCAGCATGCTCCTCTAGA**CTCGAG**CCGGGGTATATAGCCTTTGAACAACGGTAGC TAGTTTCTAGGCATTCTTTGTCCCTTCCTAACCTCTTGTGCTCGCTGAGACAATTGCCAG TGTCGCAGTCTAAGG...3'
J6	5'GCATGCTCCTCTAGA**CTCGAG**CCGGGGTCGCGAAGTCCGCCAAGATGGGATCCAAGC TTATCGATTTCGAACCCGGGGTACCGAATTCCTCGAGGATT...AGAGCTCCCAACGCGTT GGATGCATAGCTTGAGTA...3'
J7	5'CCTCTAGA**CTCGAG**GAATTCGGTTGACCCCGGGTTCGAAAATCCGATAAGCTTGGATC AAGCTTATCGATTCGACC....3'
J9	5'GCATGCTCCTCTAGA**CTCGAG**CCGGGGTCGCGAAGTCCGCCAAGATGGGATCCAAGC TTATCGATTTCGAACCCGGGGTACCGAATTCCTCGAGGATT...AGAGCTCCCAACGCGTT GGATGCATAGCTTGAGTA...3'
J10	5'CCTCTAGA**CTCGAG**GAATTCGGTTGACCCCGGGTTCGAAAATCCGATAAGCTTGGATC AAGCTTATCGATTCGACC....3'

* XhoI restriction site

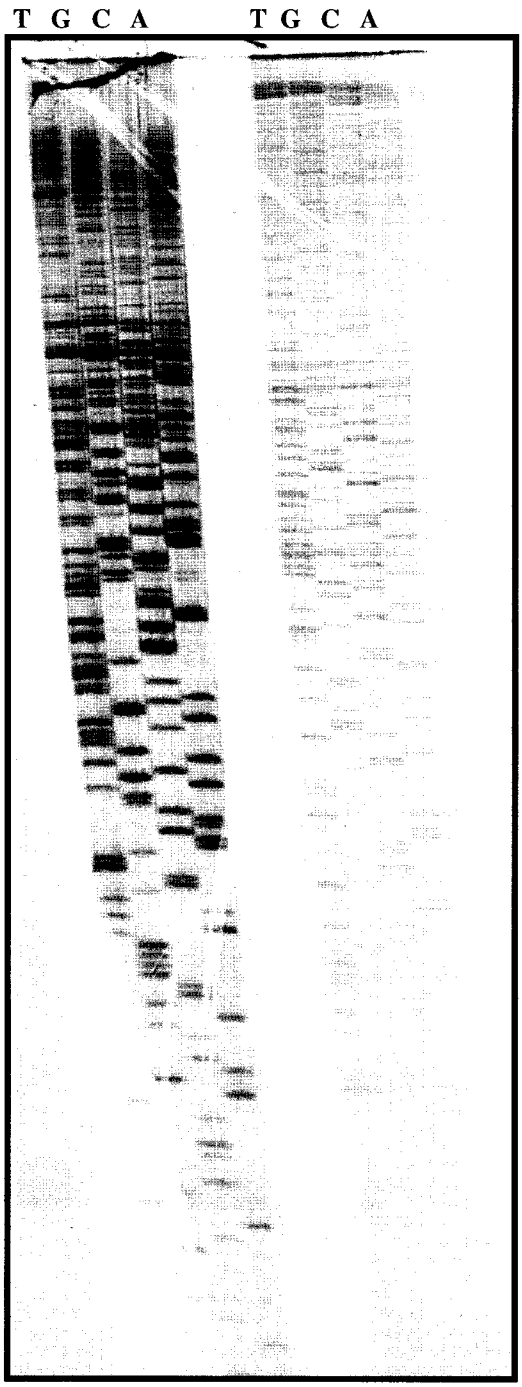


Figure 3.18. An example of nucleotide sequences derived from dideoxy sequencing method (see Methods 3.0.5). The Nucleotide sequence of clones J2 (left) and J1 (right) on an 8% denaturing polyacrylamide/urea gel.

Discussion 3.2

The main objective of this chapter was to use the Yeast Poly (A) tailing RT-PCR method to detect Pgp2 mRNA degradation products *in vivo*. Results using *in-vitro* trasnscribed RNA (Pgp2A-2E1) have shown that RNA can be tailed and amplified using this method. Analysis of tailed and nontailed normal liver and liver tumour samples amplified using five specific forward primers that covered the entire length of Pgp2 mRNA was carried out on both denaturing 6% polyacrylamide gels and 1.5% agarose gels. Analysis using forward primers 2A, 2B1, 2C, and 2D yielded no detection of putative degradation intermediates. This is consistent with data retrieved using ligation-mediated PCR (Lee et al., unpublished results) Analysis of normal liver and liver tumour tissue samples with 2E1 forward primer revealed a large smear present in tailed samples, that was absent in nontailed samples (Figure 3.10. and figure 3.11.). This also was consistent with results obtained from ligand-mediated PCR (Lee et al., unpublished data). The presence of the smear indicated the presence of putative Pgp2 mRNA decay intermediate(s), and required further investigation to confirm this.

In summary, although it appeared it appeared that the Yeast Poly (A) tailing RT-PCR method was successful in detecting *in-vitro* RNA, detection of specific tailed RNA *in vivo* posed a problem. To recap, a smear was present in tailed samples and absent in nontailed samples. This was detected in both normal liver and liver tumours analyzed with EF1 forward primer only. These results were reproducible and PCR products could be reamplified using the same primers (Figure 3.14.). It was also possible to clone amplified fragments into pGEM7Zf+ vector (Figure 3.16. and figure 3.17.). This strongly suggested that the insert contained both BamHI and XhoI restriction sites, consistent with a putative

Pgp2 mRNA decay product amplified with 2E1 forward primer containing a BamHI site, and an Oligo dT_{18}-XhoI reverse primer containing an XhoI site. Surprisingly, sequence analysis revealed that inserts in all positive clones were not that of Pgp2 mRNA. To understand this phenomenon, a few points must be considered. Firstly, the specificity of the Oligo dT_{18}-XhoI primer must be taken under consideration. This primer is nonspecific as it consists of a stretch of T bases. Due to its nonspecific nature, the OligodT_{18}-XhoI primer can hybridize to any stretch of A bases.

A second point that needs consideration relates to the samples used. The samples used to search for Pgp2 mRNA decay intermediates were total RNA samples isolated from liver and tumour. This means that not only did the samples contain possible degraded Pgp2 mRNA, but also various other RNAs. One would think that forward primer 2E1 would be specific for Pgp2 mRNA. However, based on the amplification and sequencing of products that are not Pgp2 mRNA, it is possible that the Pgp2E1 primer is not specific for only Pgp2 mRNA. A nucleotide BLAST search was performed on the 2E1 forward primer sequence, however no significant matches were made other than with Pgp2 mRNA. This result is not surprising though, as the sequences determined contained no significant match after a nucleotide BLAST search either. The sequence of 2E1 forward primer was not detected in sequence analysis, however, manual sequencing only enables reading of the sequence up to a certain point before it becomes illegible. The specificity of 2E1 forward primer is questionable, and can not definitively be ruled out as a possible explanation as to why this method failed in detecting Pgp2 mRNA decay intermediates *in vivo*.

The success of this experiment was also concentration dependant. As previously stated, the concentration of Pgp2 mRNA decay products is unknown. It is possible that

putative Pgp2 mRNA decay products were successfully poly (A) tailed and amplified, however, the concentration of successfully amplified decay products had to have been lower than the fragments that were detected. It is also possible that decay product(s) were not poly (A) tailed due to low abundance, however this is unlikely as a decay intermediate was identified using ligation-mediated PCR, which is less sensitive than the Yeast Poly (A) Tailing RT-PCR method in detecting *in-vitro* RNA (Lee et al., unpublished results). It is likely that the nonspecific nature of the Oligo dT_{18}-XhoI primer, coupled with probable low concentration of decay intermediates and a possible conserved Pgp2E1 forward primer region within another gene were the root causes as to why Pgp2 mRNA decay intermediates were not detected.

As previously stated, all determined sequences did not have a significant match when a nucleotide BLAST search was performed. All one can say regarding the fragments cloned, is that they are located in similar tissue samples as Pgp2 mRNA. Another interesting feature pertaining to the sequencing results, is that one would expect to have a string of T bases at the 5' end of the sequence. This result however, is not present. It is likely that an XhoI restriction site was present upstream from the region where the Oligo dT_{18}-XhoI primer hybridized to. This would explain the presence of the XhoI restriction site in all sequences determined, and a lack of T bases (See Table 3.2).

Detection of decay intermediates *in vivo* has previously been reported. Albumin mRNA degradation products have been identified *in vivo* using ligation-mediated PCR (Hanson and Schoenberg, 2001). This method involves the annealing of a specific primer to the hydroxyl terminus of decay products, followed by amplification using two specific primers. This method has also been used to successfully identify a Pgp2 mRNA decay

intermediate (Lee et al., unpublished results; See Chapter 4 and 5 for discussion). Taken together, it is concluded that primer specificity is very critical for specific amplification of degradation products. This study failed to detect Pgp2 mRNA decay products *in vivo*.

Chapter 4

Using Primer Extension Method to determine the Nature of Nucleolytic (Exonucleolytic or Endonucleolytic) Cleavage

As previously mentioned (Chapter 3, Discussion 3.2) an mRNA degradation product belonging to Pgp2 was identified using ligation-mediated PCR. This method was first described in the identification of albumin mRNA decay intermediates *in vivo* (Hanson and Schoenberg, 2001). The ligation-mediated PCR protocol is useful in detection of mRNA decay intermediates that possess 3' hydroxyl termini. A DNA ligation primer that has a 5'-phosphate and a 3'- amino group is ligated to the decay intermediate using RNA ligase. Reverse transcription is performed with a complementary primer, and the cDNA is amplified using specific primers (Hanson and Schoenberg, 2001). A Pgp2 mRNA decay intermediate was identified using this method and amplified with Pgp2E1 forward primer. This product was confirmed to be a Pgp2 mRNA decay product through sequence analysis (Lee et al., unpublished results). It was then necessary to determine if the product was the result of endonucleolytic cleavage.

Primer extension is an accurate assay used to map *in vivo* endonuclease cleavage sites. This experiment is able to identify unique cleavage sites that will appear as bands that correlate with the exact position where the polymerase runs off the template. Cleavage sites are distinct from polymerase pausing sites, which are identified in the primer extension performed on an *in vitro* control transcript. A DNA sequencing ladder is also included in the analysis to enable precise identification of the cleavage sites. The methodology for this experiment was derived from a detailed review of experiments related to the characterization of mRNA endoribonucleases (Schoenberg & Cunningham, 1999).

This chapter describes the use of Primer Extension method to determine the nature of cleavage of putative Pgp2 mRNA degradation product(s). Results and discussion related to the nature of decay of 5' end Pgp2 mRNA degradation products in normal liver and liver

tumour are included. A discussion regarding possible mechanisms for increased Pgp2 mRNA stability is also included.

Methods 4.0

4.0.1 Primer Extension

Based on results from ligation-mediated PCR, primer RP 5'CGTGAATGACGCTGGGGAGCTCAACACC3' was designed 50 nucleotides downstream from the detected degradation product by ligation-mediated PCR (Figure 4.1).

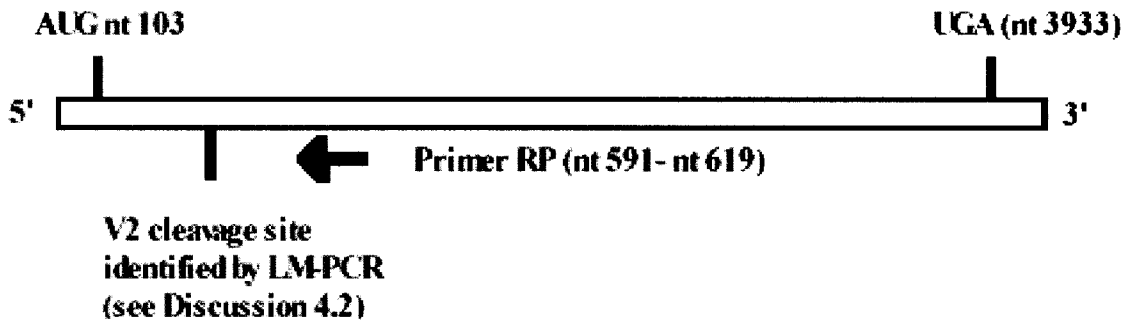


Figure 4.1 Location of Primer RP in relation to previously identified Pgp2 degradation product (Lee et al., unpublished data) for use in primer extension experiment. V2 cleavage site is located at 541 nt.

RP primer was 5' labeled with γ^{32}P-ATP and then used for PCR reaction described below (see Methods 2.0.3). Standard ethanol precipitation (see Methods 2.0.1) was performed on 3 μL of 5' γ^{32}P-ATP labeled primer with 10 μg of total RNA samples extracted from liver 0.1, 0.2 and tumour 0.1, 0.2. As a control, the same amount of 5' labeled primer with 20 ng of *in-vitro* transcribed Pgp2E1 RNA also underwent standard ethanol precipitation. The RNA pellets were resuspended in 10 μL of annealing buffer (50 mM Tris-HCl; pH 8.7; 0.54 M KCl; and 1 mM EDTA), and incubated at 65°C for 10 minutes, 54°C for 90 minutes and slowly cooled to room temperature. 30 μL of Reverse Transcription

mixture (10 mM dATP, dCTP, dGTP, dTTP; 0.25 M Tris-HCl, pH 8.7; 65 mM $MgCl_2$; 0.1 M DTT; MMLV reverse transcriptase; ddH_2O) was added to each sample and the mixture was incubated for 1.5 hours at 42°C. The reaction was stopped by an addition of 260 μL of NET buffer (0.3 M NaAc; 10 mM Tris-HCl, pH 8.0; 1 mM EDTA) and 600 μL of cold 100% ethanol. The samples were precipitated at -20°C for 30 minutes and centrifuged for 10 minutes at 12,000 rpm. The pellets were washed with 200 μL of 70% cold ethanol and centrifuged for an additional 5 minutes. The supernatant was removed and the pellets were allowed to air dry for 15 minutes. The pellets were resuspended in 4 μL of formamide loading buffer, boiled for 5 minutes and placed on ice prior to loading on an 8% polyacrylamide/7 M urea sequencing gel.

Dideoxy Sequencing (see Methods 3.0.4) was carried out on pGEM4Z-Pgp2E1 plasmid with γ^{32}P-ATP 5' labeled primer extension primer with the following changes: After standard ethanol precipitation the pellet was resuspended in 5 μL of ddH2O and ^{35}S-dATP was not used as a labeled primer was utilized.

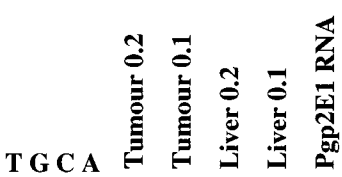
Samples from the Primer Extension and Dideoxy Sequencing experiments were ran at 60 W constant current on a pre-ran 8% polyacrylamide/7 M urea sequencing gel for approximately 1.5 hours. The gel was dried and exposed as previously described (see Methods 3.0.4).

Results 4.1

Primer extension is an accurate assay for mapping endonucleolytic cleavage sites. The presence of bands potentially indicates cleavage sites where the polymerase runs off the

template. Cleavage sites are distinct from polymerase pausing sites, which are identified in the primer extension performed on *in vitro* transcribed Pgp2E1 RNA transcript. The cleavage site positions were determined relative to a DNA sequencing ladder prepared from pGEM4Z-Pgp2E1 plasmid.

This experiment was performed based on results from section 3.1.4, and due to the successful detection and sequencing of a Pgp2 mRNA degradation product present in both liver and tumour tissue samples using ligation-mediated PCR with Pgp2E1 forward primer (Lee et al., unpublished data). The primer extension experiment revealed 5 cleavage sites, one of them consistent with the ligation-mediated PCR detected degradation product (See Figure 4.2. and 4.3. lanes 5, 6, 7, and 8). For simplicity, the cleavage site related to the detected decay intermediate identified by ligand-mediated PCR, is termed V2. All cleavage sites detected were present in both liver and tumour tissue samples (see Figure 4.2., lanes 5, 6, 7, and 8).



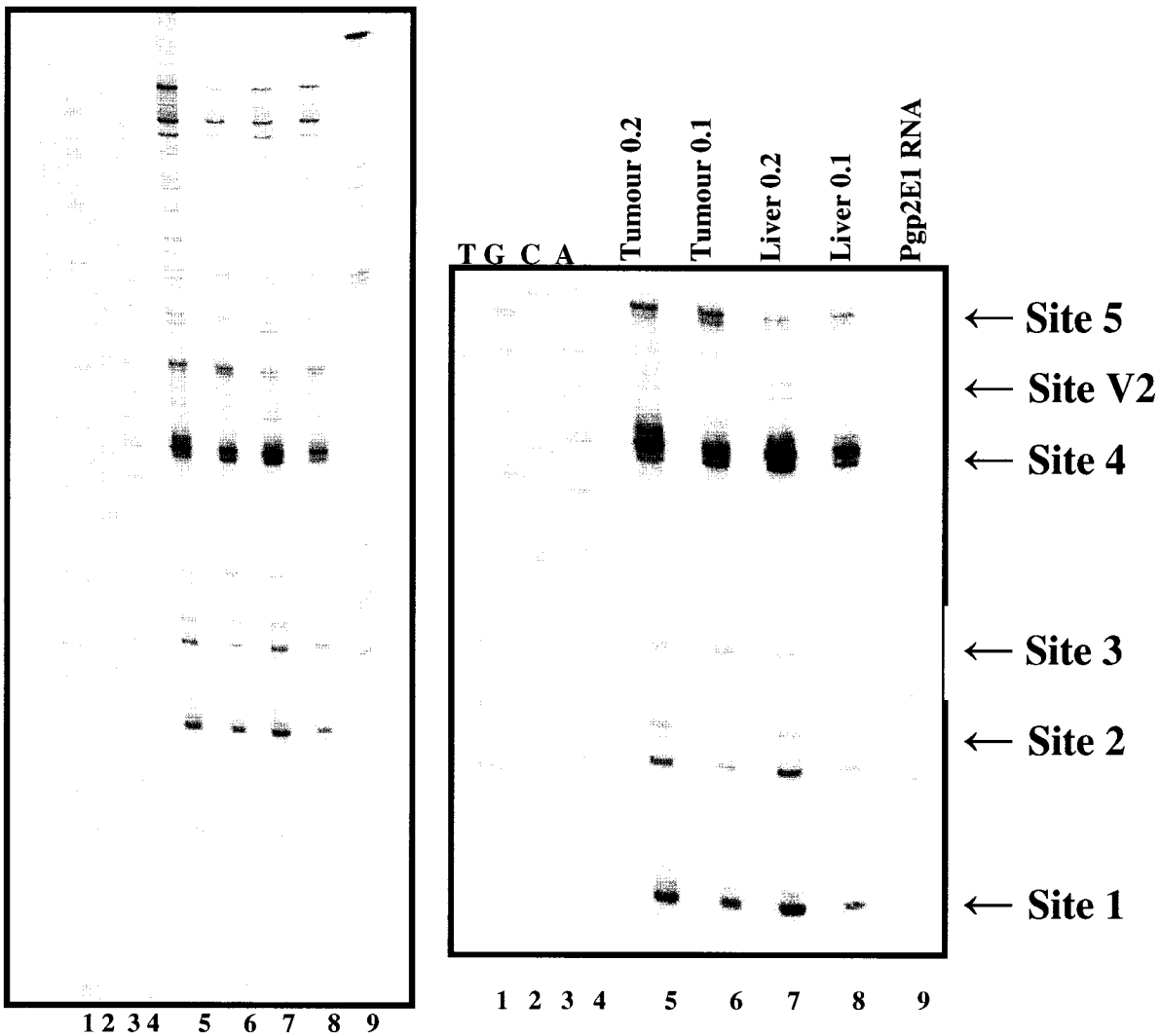


Figure 4.2. Primer extension analysis of liver and tumour tissue samples. Left picture details entire length of experiment. Right picture details important information pertaining to endonucleolytic cleavage sites present in both liver and tumour.

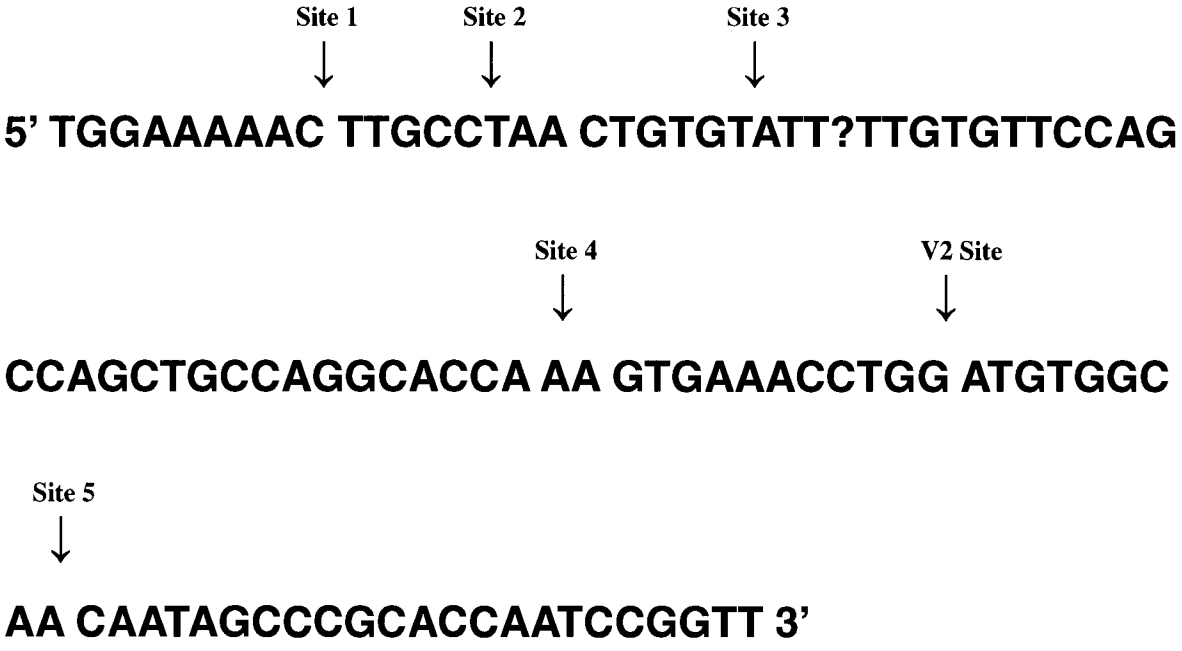


Figure 4.3. Locations of identified cleavage sites in Pgp2 mRNA sequence from primer extension analysis (see figure 4.2.).

Discussion 4.2

Based on findings from primer extension analysis it can be concluded that the Pgp2 mRNA degradation product previously identified by ligation-mediated PCR is the result of endonucleolytic cleavage. Aside from the V2 cleavage site, five major cleavage sites were also identified and were present in both liver and tumour. These sites appear very intense in comparison to the V2 cleavage site, indicating that the V2 cleavage site is a minor site. Why then was it not possible to detect degradation products that parallel these major sites? A possible explanation as to why this was not possible requires investigation into the general process of mRNA decay and into the process of how ligation-mediated PCR detects decay products. mRNA degradation products can be of two natures in regards to their 3' end; the 3' terminus can either have a phosphate or hydroxyl group. As previously described, ligation-mediated PCR employs the use of a specific primer, which is ligated to hydroxyl

termini of decay intermediates (Hanson and Schoenberg, 2001). Therefore, this method, as well as the Yeast Poly(A) tailing RT-PCR method can only detect decay intermediates with hydroxyl termini. It is possible that the major cleavage sites demonstrated in primer extension analysis represent products with phosphate termini, which could not be detected using ligation-mediated PCR or Poly (A) tailing method. Presently, there is no method available to detect mRNA decay products with 3' phosphate termini *in vivo*. However, such possibility would require further investigation.

Identification of the decay intermediate by ligation-mediated PCR in both liver and tumour, and the identification of the exact same cleavage sites by primer extension analysis indicate that the decay pathway of Pgp2 mRNA is the same in both liver and tumour. This result is significant as it demonstrates that increased mRNA stability exhibited in tumours (Lee et al., 1998) is not likely related to endonucleolytic decay of Pgp2 mRNA between normal liver and liver tumour. This suggests that one should examine possible differences in deadenylation and 3'-5' decay.

In conclusion, this experiment identified that endonucleolytic cleavage plays a role in the decay process of Pgp2 mRNA *in vivo*. The mode whereby Pgp2 mRNA is degraded has not been previously described. Identification of endonucleolytic cleavage in Pgp2 mRNA adds to the list of mammalian mRNAs that are degraded by endonucleolytic pathway. Based on the current findings, it is not possible to determine if the cleavage sites are the result of one or more enzymes. Future research related to this finding can pertain to purification and characterization of the endoribonuclease(s) responsible for cleavage of Pgp2 mRNA.

Chapter 5

General Discussion

This study aimed at comparing the *in vivo* degradation pathway of Pgp2 mRNA, through identification of decay intermediates in liver tumour and normal liver. The rational behind the study also relates to the lack of knowledge surrounding decay mechanisms associated with Pgp mRNA. On a global scale, it is also related to the lack of understanding of mRNA decay processes within mammalian systems, especially in an animal model (Ross, 1995). This study attempted to detect Pgp2 mRNA degradation products using the Yeast Poly (A) Tailing RT-PCR method. At first this method was determined to be suitable due to its apparent sensitivity and specificity demonstrated in *in-vitro* assays (see Chapter 2, Discussion 2.2). It became apparent however, that this method was not suitable for use in detection of mRNA decay intermediates *in vivo* because it lacked specificity (See Chapter 3, Discussion 3.2). A Pgp2 mRNA degradation product was identified through an alternative method (ligation-mediated PCR; Lee, et al. unpublished results) in both normal liver and liver tumours. Primer extension analysis provided insight into the decay pathway by confirming that the decay product identified was the result of endonucleolytic cleavage, and by revealing four major cleavage sites within the 2E1 portion of Pgp2 mRNA. These cleavage sites were identical in both normal liver and liver tumour (See Chapter 4). Therefore, even though this study failed to detect Pgp2 mRNA decay products using the Yeast Poly (A) Tailing RT-PCR method, the main objective has been accomplished. Based on the results of the primer extension analysis, there is no indication of differential endonucleolytic decay pathways present between normal liver and liver tumour. The increased Pgp2 mRNA stability demonstrated in liver tumours in comparison to normal liver

(Lee et al., 1998) could be related to some other decay mechanism. Observations made here suggest that future studies should be directed towards examining possible differences in deadenylation and 3'-5' decay. As discussed previously (see Chapter 1), there are few explanations and mechanisms related to mRNA stabiliy in P-glycoprotein. It is the objective of this chapter to identify possible areas of research that could potentially shed more light on the issue.

Many recent observations suggest that Pgp2 mRNA is post-transcriptionally regulated. It was recently demonstrated that the application of cyclohexamide (a protein synthesis inhibitor) significantly increased Pgp2 mRNA in all normal rat tissues. The increase in mRNA transcripts was mainly through post-transcriptional control as there was no indication of a parallel increase in transcriptional activity (Lee, 2001). It has been suggested that this increase in Pgp2 mRNA transcript levels is due to mRNA stability (Lee, 2001). It has also been suggested that trans-acting factors are most likely involved in Pgp2 mRNA stability (Lee, 2001). There are a few explanations for this suggestion. First, even though a change in mRNA stability has been noticed between normal liver and liver tumour, there has been no obvious change in Pgp2 mRNA structure (Kren et al., 1996; Lee et al., 1998). Also, an AU-rich element at the 3'UTR of Pgp mRNA failed to exhibit destabilizing capability demonstrated by other AU-rich elements (Prokipcak et al., 1999). Therefore, investigation into the presence or absence of RNA binding proteins in normal liver and liver tumour would be appropriate.

The involvement of mRNA decay during aging and development has not been widely explored. It has been recognized that specific changes in gene expression can occur during aging and development (Brewer, 2002). It is widely accepted that mRNA decay processes

contribute to the control of gene expression. In some cases, mRNA stability has been linked to development. For instance, it has been demonstrated in mouse albumin mRNA, that stability is partially dependant on development and aging. This conclusion was based on the identification of significant levels of albumin mRNA in fetal mouse liver and undetectable levels in adult liver. This suggests that mRNA turnover rate is higher in fetal liver and decreases during development (Tharun and Sirdeshmukh, 1995). Opposite to the results determined for albumin mRNA, granulocyte/macrophage colony-stimulating factor (GM-CSF) mRNA levels are four fold lower in activated neonatal mononuclear cells from umbilical cord blood, as compared to adult peripheral blood (Brewer, 2002). It has also been proposed that in young cells there is an equal balance of mRNA degradation and stabilization factors present, where as in older cells degradation factors are dominant (Brewer, 2002). While many details related to age-dependant changes in mRNA are currently unknown, it would be interesting to determine if endonucleolytic decay of Pgp2 mRNA in normal liver changed during development.

This study has introduced key issues related to Pgp2 mRNA that were previously unknown. It revealed that the endonucleolytic decay pathway of Pgp2 mRNA in normal liver and liver tumour appears to be the same. It also provided strong evidence for the involvement of endoribonucleic activity in the Pgp2 mRNA decay process *in vivo*. In conclusion, it narrowed the gap of possible explanations as to why Pgp2 mRNA is more stable in liver tumours than normal liver.

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