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## Cathepsin B: NRNA Characterization, Gene Structure and Expression in Rodent Normal Tissues and Malignant Tumors with Different Metastatic Potentials

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CATHEPSIN B: mRNA CHARACTERIZATION, GENE STRUCTURE AND  
EXPRESSION IN RODENT NORMAL TISSUES AND MALIGNANT  
TUMORS WITH DIFFERENT METASTATIC POTENTIALS

by

FANG QIAN

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School of Loyola University of Chicago in Partial  
Fulfillment of the Requirements for the Degree of  
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October

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Finally, but no less sincerely felt, some personal debts to my friends Rebecca Smith, Florence Rozenfeld, Spring Tao and Raymond Carroll: my thanks for their constant support and warm friendship.

## VITA

Fang Qian is the daughter of Jie Qian and Wei Gu, the third oldest child of four children. She was born in Beijing, China and her primary and secondary education were obtained in Beijing and Hefei. She earned her medical degree in 1976 after four year hard study as a top student in Bengbu Medical College in China.

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In 1984, Fang was accepted to the Ph.D. program in the Department of Molecular and Cellular Biochemistry, at Loyola University of Chicago. Her dissertation research involved a collaborative project on the role of cathepsins in tumor metastasis under the guidance of Dr. Allen Frankfater and Dr. Shu J. Chan (U. of Chicago). Her experiments were performed in Dr. Allen Frankfater's lab at Loyola University of Chicago and in Dr. Donald F. Steiner's lab at the University of Chicago. She received a Basic Science Fellowship by Loyola University of Chicago and a Dissertation Fellowship by Arthur J. Schmitt Foundation during her pursuit for her Ph.D. degree. Fang is also a good swimmer and volleyball player.

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## CHAPTER I

### INTRODUCTION

An important characteristic of malignant tumors is their ability to invade normal tissue and to proliferate at distant sites (metastasis). This ability distinguishes benign and malignant tumors. It is the production of metastases which makes cancer such a difficult disease to treat and is ultimately responsible for the death of the patients. The reasons why cancer cells metastasize are currently unknown. It is known, however, that not all malignant cells within a tumor have the capacity to form distant colonies. Clearly therefore, the properties of cancer cells which enable them to form metastases are different from those responsible for tumorigenesis.

The major steps in the invasive and metastatic process include: detachment of cells from the primary tumor; penetration of the extracellular matrix and basement membrane; invasion of the vasculature to enter the blood circulation (intravasation); arrest in the capillaries at the secondary sites (attachment); invasion through the capillary wall (extravasation) and finally, colonization and proliferation

in the distant tissues. Since the extracellular matrix, basement membrane and capillary wall consists primarily of macromolecules such as collagen, proteoglycans, elastin and glycoproteins, it has been proposed that proteolytic enzymes may be responsible for their degradation thereby facilitating tumor cell invasion and, in turn, metastasis (Jones and De Clarck, 1982; Recklies et al, 1982b; Liotta et al, 1983; Mullins et al, 1983; Pauli et al, 1983). Consistent with this hypothesis, numerous studies have reported increased levels of proteolytic enzymes produced by tumor cells. These include neutral metallo-proteinases such as collagenase (Liotta et al, 1982), serine proteinases like tissue plasminogen activator (Moacatelli et al, 1980), and several lysosomal proteinases including cathepsin B (Sloane et al, 1981; Mort et al, 1981; Recklies et al, 1982a and 1982b; Dufek et al, 1984), cathepsin L (Gal and Gottesman, 1986; Troen et al, 1988) and cathepsin D (Capony et al, 1987; Maudelonde et al, 1988). Among these candidate proteinases which may play a role in the invasion and metastasis process, cathepsin B has been extensively investigated.

Many studies have reported a positive correlation between cathepsin B activity and malignant tumors (Pool et al, 1978; Recklies et al, 1980; Sloane et al, 1984a; Koppel et al, 1984; Baici and Knopel, 1986; Keppler, 1988; Krepela et al, 1989 and 1990). Increased secretion of cathepsin B-like enzymes has been observed in ascites fluid from patients with various

kinds of malignant tumors (Pietras and Roberts, 1981; Mort et al, 1981 Dufek et al, 1984) and in conditioned media from cultured human and murine mammary carcinoma (Recklies et al, 1982a; Petrova-Skalkova et al, 1987). The molecular basis for the increased levels of cathepsin B activity found in the tumors remains unknown. Possible factors include mutation in the cathepsin B gene or differences in transcription, translation, and post-translational processing of cathepsin B. Furthermore, the distribution of cathepsin B is often altered in tumor cells, for example, it has been found to be association with the plasma membrane (Pietras and Roberts, 1981; Keren and LeGrue, 1988,) and secreted into the extracellular medium. It is not known whether these phenomena are results of a cellular defect in post-translational processing, or is due to the synthesis in tumor cells of a form of cathepsin B which lacks amino acid sequences that normally target this enzyme to lysosomes.

The goal of this study is to use molecular biological methods to investigate the expressions and structure(s) of cathepsin B gene in normal tissues and in malignant tumors, and to investigate the relationship between cathepsin B expression and metastatic potential. If cathepsin B or any other proteinase could be proven to play a role in the spread of human cancers and its mechanism could be understood, inhibition of this enzyme could open up new therapeutic approaches for the control of malignancy.

In this research I have examined the mRNA levels for cathepsin B in normal tissues (rat and mouse) and in malignant tumors (rat carcinosarcoma and mouse melanoma variants with different metastatic potentials); compared tumor cathepsin B mRNA with normal cathepsin B mRNAs; cloned the three mouse cathepsin B mRNA transcripts in mouse melanoma and determined their sequences; cloned mouse cathepsin B gene and determined its structure, including the transcription start site and putative promotor region; and investigated the possible mechanisms for differential expressions of cathepsin B gene in normal tissues and malignant tumors.

I also examined mRNA levels for other lysosomal cathepsins (D, H, L and S) in normal tissues and malignant tumors to determine whether these lysosomal cathepsins are over-expressed at the same time or whether only specific cathepsin(s) has(have) increased expression in certain tumors.

## CHAPTER II

### REVIEW OF THE RELATED LITERATURE

#### THE CELLULAR PROTEASES

The term proteases have been adopted for proteolytic enzymes by the International Union of Biochemistry (International, 1979). Proteases include two distinct groups: exopeptidases (peptidases) and endopeptidases (proteinases). However, some proteases, such as cathepsin B, cathepsin H and cathepsin D, have both exo- and endo-peptidase activities, for the sake of simplicity those proteases are usually called proteinases since their predominant actions are endopeptidase activities.

Exopeptidases are subclassified as carboxypeptidases, aminopeptidases, dipeptidylpeptidases, peptidyl dipeptidases, dipeptidases and omega peptidases.

The endopeptidases are more commonly called proteinases and five classes are recognized for the purpose of nomenclature (Barrett, 1980b). The proteinases are unique among enzymes in that their classification depends on the

catalytic mechanism of their active centers. These five classes are: serine proteinase, cysteine proteinases, aspartic proteinases, metallo-proteinases and other proteinases. I will briefly review each of the first four classes of the proteinases which are all reported to be involved in tumor metastasis. Next I will introduce lysosomal cathepsins and their correlation with malignant tumors in more detail, emphasizing cathepsin B which is main object of my dissertation.

### Serine Proteinases

Serine proteinases include trypsin, chymotrypsin, elastase, coagulation factors, leukocyte elastase, cathepsin G, chymases, plasminogen activators and so on. They all have a serine in their active sites, are active at the neutral pH and need no cofactors. Based on extensive homologies have been demonstrated for these enzymes it is a reasonable hypothesis that they have all diverged from one primitive enzyme (Hartley, 1970). Serine proteinases are abundant in the body. They are synthesized as inactive precursors that require limited proteolysis to activate them. Serine proteinases are controlled by cascades of activating enzymes and a system of protein proteinase inhibitors. About 10% of human plasma proteins consists of Ser-proteinase inhibitors, the most abundant being  $\alpha_1$ -proteinase inhibitor and  $\alpha_2$ -macroglobulin. Two similarly related Ser-proteinases that have been of particular interest in relation to the growth of

cancer cells are plasmin and plasminogen activator.

### Cysteine Proteinases

The proteinases that depend for activity on an active site cysteine residue (cysteine proteinases) are currently the subject of very active research. In mammalian system, these include lysosomal cysteine proteinases, such as cathepsin B, cathepsin H, cathepsin L, cathepsin S and cathepsin N (Barrett, 1981), and non-lysosomal cysteine proteinases, such as calpains (Murachi et al, 1981). The pH optima for the group of lysosomal cysteine proteinases is about pH 6 with many substrates and they are unstable above pH 7 (Barrett and Kirschke, 1981; Barrett, 1984). The lysosomal cysteine proteinases are synthesized as a large precursor which undergoes proteolytic maturation. Their mature forms are normally confined to the lysosome and play an important function in intracellular protein metabolism. In neoplasms, some lysosomal cysteine proteinases, such as cathepsin B, are found secreted into extracellular environment and their pH tolerance seems increased as well (Barrett, 1980a). Calpains are  $\text{Ca}^{2+}$  activated, cell membrane associated cysteine proteinases (Barth and Elce, 1981; Dayton and Schollmeyer, 1981) and two forms of calpains have been detected (Dayton et al, 1976; Mellgren, 1980). The major distinction between the two forms of the enzymes is that they need different concentrations of  $\text{Ca}^{2+}$  for their maximal activities. However, the two forms have similar Properties: both are active

optimally at pH 7.5, both are inhibited by sulphhydryl modifying reagents and exogenous inhibitors of cysteine proteinases and both appear to consist of two subunits. Calpains have very restricted proteolytic activities and their protein substrates include neurofilament proteins, c-protein, demin, filamin, vimentin and troponins I and T (John et al, 1984).

### Aspartic Proteinases

The aspartic proteinase group has as its major members, pepsin, renin, and cathepsin D. The most studied pepsin is responsible for the digestion of food proteins in the strongly acidic content of the stomach. Cathepsin D is a lysosomal enzyme and makes an important contribution to intracellular proteolysis in the acidic environment of the lysosome. The evolution of renin from the hypothetical cathepsin D-like archetype has taken a quite different turn. It is stable and active at neutral pH and is highly specific for the cleavage of the leu-leu bond that generates angiotensin I from angiotensinogen. This cleavage follows the releases of renin into the blood by kidney in response to low blood pressure.

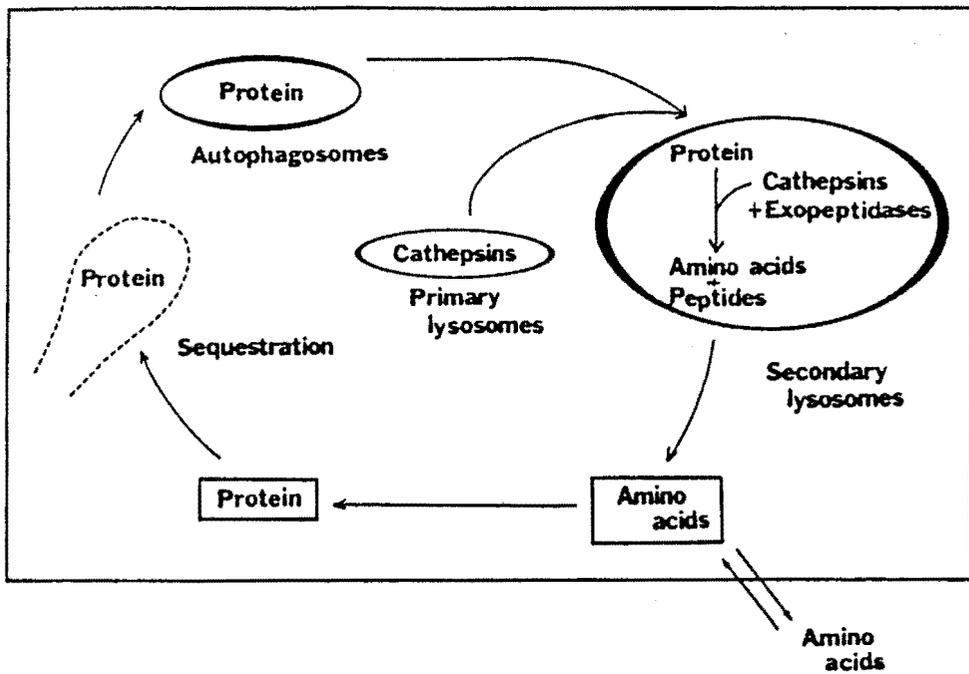
Cathepsin D has been found to be regulated by estrogens in breast cancer (Henry et al, 1990) and correlated with invasive and/or proliferative breast cancer (Rocheffort et al, 1987, Maudelonde, 1988) and endometrial carcinoma (Maudelonde et al, 1990).

### Metallo-Proteinases

Examples of metallo-proteinases include pancreatic carboxyl peptidase, thermolysin and collagenases. Collagenase is a remarkably specific enzyme: it cleaves all three chains of the triple helix of collagen molecule at a single point. It is not known to act on other proteins, except in that it seems to cleave the  $\alpha_2$ -macroglobulin molecule in becoming bound by this inhibitor as part of the mechanism of the inhibition of collagenase (Werb et al, 1974)

The neutral to slightly alkaline pH optimum of the metallo-proteinases, together with their sensitivity to inhibition by thiols, seem to suit them best for an extracellular function. This impression is reinforced by the observation that metallo-proteinases are secreted from cells and not normally stored within them. There have been studies of collagenase in relation to a variety of tumors (Bauer et al, 1977; Dabbous et al, 1977; Ohyama and Hashimoto, 1977).

Figure 1. The Mode of Action of Lysosomal Cathepsins in Intracellular Protein Metabolism



Intracellular proteins are sequestered in autophagosomes, which eventually fuse with lysosomes containing cathepsins capable of degrading proteins to amino acids and small peptides (Katunuma and Kominami, 1987)

## CATHEPSIN B AND OTHER LYSOSOMAL CATHEPSINS

Lysosomal cathepsins consist of two classes of proteinases, cysteine proteinases and aspartic proteinase. Lysosomal cysteine proteinases are thought to be the most active enzymes involved in intracellular protein catabolism (Fig. 1) (Katunuma and Kominami, 1983 and 1987). The main lysosomal aspartic proteinase is cathepsin D, while the lysosomal cysteine proteinases mainly include cathepsin B, cathepsin H, cathepsin L and cathepsin S. Among these, cathepsin B has been the most thoroughly investigated enzyme.

### Cathepsin B

Cathepsin B has been isolated from various mammalian species and tissues, such as rat liver, bovine spleen and liver (Otto, 1971), and human spleen and liver (Barrett, 1973). Using the immunochemical method, cathepsin B has been located within macrophages (Howie et al, 1985), fibroblasts (Mort et al, 1981), neurones (Howie et al, 1985), myocytes (Bird et al, 1985) and the epithelial cells (Howie et al, 1985) of various tissues. Physically important proteins that can be degraded by cathepsin B include cartilage proteoglycan, collagen, contractile proteins of muscle and skin, hemoglobin, immunoglobulin G (Barrett, 1977 and 1980a), glucagon (Aronson and Barrett, 1978), aldolase (Bond and Barrett, 1980), laminin (Lah et al, 1989), elastin (Mason et al, 1986), fibrinogen (Gabrijelcic et al, 1988) and fibronectin (Recklies et al, 1982b). Cathepsin B also functions in the activation of

zymogens to enzymes, such as activation of trypsinogen to trypsin (Figarella et al, 1988) and procollagenase to collagenase (Barrett, 1980a). In addition, cathepsin B has been suggested to have functions in the conversion of some hormones and other biologically important proteins from their precursors, such as albumin from proalbumin (Taber, 1973), renin from prorenin (Takahashi et al, 1982; Taugner et al, 1985; Shinagawa et al, 1990), thyroid hormones from thyroglobulin (Nakagawa et al, 1981a and b), proendorphin to endorphin (Suhar and Marks, 1979) and proinsulin to insulin (Steiner et al, 1974; Kirschke et al, 1980; Docherty et al, 1982; Orci, 1985, 1986 and 1987). Moreover, cathepsin B is considered the enzyme which processes distinct epitopes on myoglobin (Takahashi et al, 1990). At present, the full spectrum of the biological role of cathepsin B is still not quite clear but its very broad tissue distribution suggests it has important functions.

The mature form of cathepsin B has a molecular weight of 26 kd. Its pH optimum is about 6 with many substrates and it becomes unstable above pH 7 (Barrett, 1977). Several synthetic substrates are very specific to cathepsin B, such as carbobenzyloxy-alanine-L-arginine-4-methoxy-B-naphthylamide and carbobenzyloxy-L-arginine-L-arginine-2-naphthylamide. The specific activity of cathepsin B on these substrates is 200 times higher than that of other cathepsins. Fluorogenic and chromogenic synthetic substrates are the most common

substrates used in enzyme assays for determination of the cathepsin B activity (Barrett, 1973 and 1986; Bajkowski and Frankfater, 1975)

Cathepsin B is structural and functional homologous with a plant cysteine proteinase-papain (Bajkowski and Frankfater, 1983a) and has a similar enzymatic mechanism to papain (Bajkowski and Frankfater, 1983b). Cathepsin B from rat, porcine, bovine and human have been found to exist in a single-chain form and in a two-chain form composed of a light and heavy chain. The light and heavy chain most likely result from limited proteolyses in the N-terminal part of the enzyme and are presumed to be connected via a disulfide bridge (Takahashi et al, 1984; San Segundo et al, 1985; Chan et al, 1986). The amino acid sequences of rat (Takio et al, 1983) and human (Ritonja et al, 1985) cathepsin B have been determined. The homology between mammalian cathepsin B sequences with the sequence of papain provides evidence that lysosomal and plant cysteine proteinase have evolved from a common ancestor and cathepsin B is a highly conserved enzyme through evolution.

Biosynthetic studies have indicted that cathepsin B is derived from a larger precursor form, procathepsin B, which in its glycosylated state has a molecular mass of about 40 kd (Steiner et al, 1984a and 1984b; Nishimura and Kato, 1987). Procathepsin B is an inactive form which can be activated in vitro upon limited cleavage by the enzyme pepsin. The

procathepsin B is derived from preprocathepsin B which is synthesized on the rough endoplasmic reticulum. Preprocathepsin B is converted to procathepsin B by a signal peptidase during the process of transportation into the lumen of the rough endoplasmic reticulum. The procathepsin B is transported to the lysosome where it is slowly converted to mature cathepsin B. Procathepsin B may also appear in other compartments such as the secretory granule in insulinomas (Docherty et al, 1984) and secrete into the culture media (Pietras et al, 1981; Recklies et al, 1982a; Archkar et al, 1990).

The preprocathepsin B cDNAs in human (Chan et al, 1986, Fong et al, 1986), rat (Segundo et al, 1986) and mouse (Chan et al, 1986) have been cloned and sequenced. The predicted primary structure of each of them has 339 amino acids, including a 17-residue predominantly hydrophobic sequence at N-terminus, 62-residue N-terminal propeptide extension connected to the 254-residue mature single chain cathepsin B, and a 6-residue carboxyl-terminal extension. The rat and mouse cathepsin B amino acid sequences are strongly homologous, with 90% identity. The human and rat (or mouse) amino sequences are 84% homologous in mature proteins and about 50% homologous in the prepro-regions and C-terminal peptides. Human cathepsin B gene is located in chromosome 8p22 (Tsui et al, 1989).

### Cathepsin L

Cathepsin L is also a glycoprotein with a molecular weight of 27,000-30,000 and consists of two chains linked by disulfide bonds, subunit  $M_r$  values 22,000-25,000 and 5,000-7,000. The pH optimum is 6.0 for the hydrolysis of methylcoumarylamide substrates, but is 3.5 for the degradation of glomerular basement membrane. Cathepsin L is completely inactivated by the active site directed inhibitor, Z-Phe-Phe-CHN<sub>2</sub> (0.56  $\mu$ M) under a condition where cathepsin B is unaffected. Cathepsin L shows greater activity against protein substrates than other lysosomal cysteine proteinases, for example, it is significantly more active in degrading glomerular basement membrane than cathepsin B, elastase, trypsin or bacterial collagenase (Baricos et al, 1988), is at least an order of magnitude faster than cathepsin B in degrading collagen (Mason et al, 1986), and is 100-fold more active than cathepsin B in hydrolysis of a tritiated elastin (Mason et al, 1986). Pro-cathepsin L has a molecular weight of 39,000-42,000 (Smith and Simpson, 1989). The preprocathepsin L cDNAs of mouse (Portnoy et al, 1986, Joseph et al, 1988), rat (Ishidoh et al, 1987b) and human (Mason et al, 1986, Joseph et al, 1988) has been cloned and sequenced. Based on predicted amino acid sequences both rodent and human enzymes show similar organization. However, the length of rodent cathepsin L is 335 amino acids while that of human cathepsin L is 334 amino acids. Each begins with a 17 amino acid long hydrophobic sequence that ends in Ala-X-Ala, a

proposed consensus cleavage site for signal peptides (Perlman and Halvorson, 1983), followed by a 96 amino acid propeptide. The rodent enzyme displays one more carboxyl terminal amino acid than does the human enzyme. It is not known if this residue is present in the mature protein or is removed during proteolytic processing. The gene of rat cathepsin L has also been cloned (Ishidoh et al, 1989b). The gene consists of 8 exons and 7 introns spanning 8.5 kilobase pairs. Human cathepsin L gene is located in chromosome 9q21-q22 (Smith and Simpson, 1989).

### Cathepsin H

Cathepsin H was first isolated from rat liver (Kirschke et al, 1976 and 1979) and subsequently from human liver (Schwartz and Barrett, 1980). It exists as a single chain form with a molecular mass of about 28,000 Dalton and a two-chain form with molecular masses of 22,000 and 6,000 Dalton. It differs from cathepsin B and L in being active both on amino terminal blocked and unblocked synthetic substrates. Cathepsin H has not only endopeptidase activity but also aminopeptidase activity (Kirschke et al, 1976). It has an optimum pH value of 6.5 for most of substrates. Its sensitivity to chemical inhibitors is generally similar to that of cysteine proteinases, but it is unaffected by 1 leupeptin, a concentration which gives complete inhibition of cathepsins B and L. As proteins, cathepsin B and H are easily separated by DEAE-cellulose or concanavalin A-Sepharose while

cathepsin L has a much higher affinity for CM (carboxymethyl)-cellulose at pH 5.0 than the other two enzymes (Schwartz and Barrett, 1980). Little is known about the proteins which cathepsin H can hydrolyze. Kirschke et al (1980) have shown that neither rat nor human cathepsin H has detectable collagenolytic activity. As with the other cysteine proteinases cathepsin H is synthesized as a larger precursor which is posttranslationally processed. This is confirmed by both amino acid sequencing and cDNA cloning. The rat cathepsin H amino acid sequence was determined by Takio et al (1983). Rat preprocathepsin H cDNA (Ishidoh et al, 1987a, Qian et al, 1990) and human cathepsin H cDNA (Fuchs et al, 1988) have been cloned and sequenced later. The deduced amino acid sequences from the nucleotide sequences indicate both rat and human preprocathepsin cathepsin H have 248 amino acids including 21 residues of a signal peptide, 92 residues of propeptide and 220 residues of mature protein. Cathepsin H does not have carboxyl terminal peptide extension as does cathepsin B. The two sequences are highly homologous with 86% identity. Interestingly, the propeptides from the two species are 100% identical. Rat cathepsin H gene has been cloned recently (Ishidoh et al, 1989a). It consists of at least 12 exon spanning in total more than 17.5 kb. The human cathepsin H gene is located in chromosome 15q24-q25 (Cox and Donlon, 1989).

### Cathepsin S

Cathepsin S was originally purified from bovine lymph nodes (Turnsek et al, 1975) and bovine spleen (Locnikar et al, 1981; Kirschke et al, 1986) and then rabbit spleen (Maciewicz and Etherington, 1988). Cathepsin S has been detected in bovine kidney, spleen, lung and lymph nodes by immunochemical methods but not in liver, heart muscle, skeletal muscle, intestine or stomach by the same method (Kirschke et al, 1989). Cathepsin S appears as a single protein with molecular weight about 24,000 under reducing and non-reducing conditions (Locnikar et al, 1981; Kirschke et al, 1986). It has been shown that this enzyme has some similarity to cathepsin L, but is clearly different protein as judged by differences in the hydrolysis of Z-Phe-Arg-NHMec as well as in inhibition by Z-Phe-Phe-CHN<sub>2</sub> (Kirschke et al, 1984 and 1986). Cathepsin S has been found to be able to hydrolyze hemoglobin, albumin, and azo-casein at a similar rate to cathepsin L below pH 7.0. Cathepsin S can also degrade insoluble collagen though at a lower rate to cathepsin L (36%). One property of cathepsin S by which this enzyme differs from all other lysosomal proteinases has to be emphasized: this is the stability of the active enzyme at neutral pH values. Although it has an optimum pH below 7 for most tested proteins, cathepsin S remains stable and degrades proteins at pH 7.5 where cathepsin L is inactive (Kirschke et al, 1989). Mouse cathepsin S cDNA has been cloned and sequenced (Chan et al, unpublished data). The sequencing information indicates cathepsin S is also

synthesized as a larger precursor.

### Cathepsin D

Cathepsin D is a unique aspartic proteinase known to be lysosomal rather than secretory. Unlike cysteine proteinases which have a Cys in their active sites, cathepsin D has two Asp in the active site which are involved in the mechanism of catalysis. The main physiological role of cathepsin D is the break down of tissue proteins. This function makes cathepsin D different from most other aspartic proteases. Like the cysteine cathepsins, cathepsin D is a glycoprotein, has an acid pH optimum and loses activity at or above pH 7. However, its molecular mass (approximately 42 kilodaltons) is much larger than that of cysteine cathepsins. Cathepsin D is not only an endopeptidase but is also a carboxypeptidase. It has been identified and purified from a wide variety of tissues. Cathepsin D is mainly a two-chain protein, although a small fraction of the single-chain species is always present (Shewale et al, 1985). Cathepsin D is thought to make an important contribution to intracellular proteolysis in the acidic environment of the lysosomal interior. The powerful Asp-proteinase inhibitor-pepstatin (Knight and Barrett, 1976) has been shown to cause some slowing of protein break down in perfused liver when carried into the cells in liposomes (Dean, 1976). In normal endometrium cathepsin D is regulated by progesterone. Cathepsin D could be viewed as an indicator of endometrial transformation, suggesting that cathepsin D might

have a role in the biological and morphological modification that occur during the luteal phase. It may play a role in the cyclic destructive autophagy and remodeling of endometrium in the absence of nidation (Maudelonde et al, 1990). Human cathepsin D cDNA has been cloned and sequenced (Faust et al, 1985). Sequencing data reveals that mature cathepsin D is derived from procathepsin D which has a molecular mass of 52 kD while Procathepsin D is in turn derived from preprocathepsin D during its transport into the lumen of ER. The human cathepsin D gene is located in chromosome 11p15.5 (Junien and McBride, 1989).

Summaries of properties and characteristics of lysosomal cathepsins are listed in Table 1 and 2, respectively.

Table 1. Comparison of Properties of Lysosomal Cathepsins

	<u>Cathepsin B</u>	<u>Cathepsin H</u>	<u>Cathepsin L</u>	<u>Cathepsin S</u>	<u>Cathepsin D</u>
Molecular Weight (daltons)	29000	26000	30000	25000	42000
pI	4.9-5.3	6.5-7.5	5.8-6.1	6.3-6.9	3.2-3.5
Optimum pH	6.2	6.5	5.5	6.5	5.6
Catalytic Type	Dipeptidyl carboxypeptidase activity	Amino-peptidase activity			Carboxypeptidase activity
	Moderate endoprotease activity	Variable endoprotease activity	Very High endoprotease activity	Very High endoprotease activity	High endoprotease activity
Suitable Synthetic Substrate	Z-Arg-Arg-NMec	Arg-NMec	Z-Phe-Arg-NMec	Bz-Phe-Val Arg-NHMec	Lys-Pro-Ala-Glu-Phe-(NO <sub>2</sub> )Phe-Leu
Leupeptin Inhibition	High	Low	High	---	No
Pepstain Inhibition	No	No	No	No	High
Z-Phe-Phe-CHN Inhibition	Low	Low	High	Low	No
E-64 Inhibition	High	Low	High	---	No
Leu-CH <sub>3</sub> Cl Inhibition	Low	High	Low	---	No
Monnose 6-Phosphate Marker	Yes	Yes	Yes	Yes	Yes
Glycoprotein	Yes	Yes	Yes	Yes	Yes
Single-Chain Form	Yes	Yes	Yes	Yes	Yes
Two-Chain Form	Yes	Yes	Yes	No	Yes

Table 2. Characteristics of Lysosomal Cathepsins

	B	H	L	S	D
Preproprotein (aa)	339	335	334 (human) 335 (rat)	342	412
Pre-Peptide (aa)	17	21	17	26	20
Pro-Peptide (aa)	62	93	95	100	44
Mature Protein (aa)	254	220	221 (human) 222 (rat)	216	348
Potential Glycosylation Sites (pro+mature)	1+1	2+1	0+2	1+0	0+2
Cysteines (pro+mature)	1+14	1+8	0+11	1+10	0+7
Chromosome loci (human)	8	15	9	?	11

## CORELATION BETWEEN CATHEPSIN ACTIVITY AND MALIGNANT TUMORS

A large body of evidence has accumulated implicating proteolytic enzymes as playing a key role in the process of invasion and metastases of tumors (Strauli et al, 1980; Goldfarb and Liotta, 1986). As mentioned above, among these proteinases, most attention has been focused on the serine proteinase plasminogen activator (Wang et al, 1980; Carlsen et al, 1984), Plasmin (Liotta et al, 1981), the cysteine proteinases cathepsin B (Recklies et al, 1980; Krepela et al, 1990), and L (Vasishta et al 1985; Sheahan et al, 1989), the aspartic proteinase cathepsin D (Rocheffort et al, 1987; Maudelonde et al, 1990) and the metallo-proteinase collagenase type IV (Bauer et al, 1977; Ohyama and Hashimoto, 1977; Turpeenniemi-Hujanen et al, 1985).

Increased cathepsin B activity has been found in a various metastatic tumors by using enzyme assays. For instance, it is elevated in serum of women with genital tract cancers, especially those patients with invasive carcinoma (Pietras et al, 1979), in human malignant breast tumors (Pool et al, 1978, Recklies et al, 1980), in a rabbit V2 carcinoma cells (Baici and Knopfel, 1986), in rat metastatic pancreatic cancer variant (Koppel et al, 1984), in marine B16 melanoma (Sloane et al, 1982) and Lewis lung carcinoma (Sloane et al, 1984b) with high metastatic potential, in a rat sarcoma LW13K2 which produces spontaneous metastases (Krepela et al, 1989), in human primary liver cancer (Dufek et al, 1984), colonic

cancer (Keppler et al, 1988a) and human lung tumors (Krepela, et al, 1990).

Cell culture experiments indicate that cathepsin B may have a cellular detachment function similar to that seen in trypsin in detachment of cells from glass surfaces (Sylven et al, 1968). Considering the known ability of cathepsin B to degrade proteoglycans (Morrison et al, 1973), collagen (Burleigh et al, 1984), fibronectin (Recklies et al, 1982b), laminin (Lah et al, 1989), and to activate trypsinogen to trypsin and procollagenase to collagenase (Barrett, 1980a), the elevated cathepsin B activity in tumor cells suggests it could play a role in degradation of the extracellular matrix and the release of tumor cells. Further studies have shown that increased cathepsin B activity can be found in association with the plasma membrane. Thus, cathepsin B may act at sites of local degradation where the tumor cell membrane and the extracellular matrix come into direct contact. For example, cathepsin B activity has been found in the plasma membrane fractions from the spontaneous BDX rat anaplastic sarcoma (Koppel et al, 1984), from human pancreatic adenocarcinoma cells (Zucker et al, 1985; Zucker, 1988), from mouse melanomas in amounts which correlated with metastatic potential (Sloane et al, 1986), from squamous carcinoma cells of the human ectocervix but not from non-neoplastic cervical cells (Pietras and Roberts, 1981), and in association with the insulin secreting granule of a transplantable rat

insulinoma (Docherty et al, 1984).

Enzymes possessing cathepsin B-like activity have received particular attention because of their extracellular release by tumors (Pietras and Roberts, 1981; Bajkowski et al, 1984). Enzymologically the secreted enzymes are very similar to lysosomal cathepsin B in terms of their reactivity to synthetic substrates and to inhibitors. They differ from the lysosomal cathepsin B, however, in that they have higher molecular weights, different isoelectric points compared with lysosomal cathepsin B and, unlike lysosomal cathepsin B, they are active at pH 7. Two major extracellular high- $M_r$  forms (both have molecular weights around 40 kd) of human cathepsin B have been described, one is latent, another is active. A latent form of cathepsin B has been found in ascitic fluid and in culture medium of ascites cells from cancer patients. The latent enzyme could be rendered active by prior proteinase treatment (Mort et al, 1981a). In addition, human malignant breast tumors in organ culture release a high- $M_r$  form of cathepsin B that was detected by its ability to hydrolyze synthetic substrates for which lysosomal cathepsin B has high affinity (Mort et al, 1980). A similar activity has been characterized in culture media of spontaneous mammary tumors (Recklies et al, 1982a) and lactating mouse mammary gland (Recklies and Mort, 1985a). The active high- $M_r$  cathepsin B cleaves a range of synthetic substrate in a similar manner to that of the lysosomal enzyme (Mort and Recklies, 1986). The

two forms of high-M<sub>r</sub> cathepsin B (active and latent) proteins have also been detected in murine B16a melanoma (Bajkowski et al, 1984; Qian et al, 1989).

Bellelli et al (1990) have found that parenteral administration of papain in mice induced antipapain antibody cross-reacting with cathepsin B and cathepsin H. In such papain-immunized animals the invasion of the abdominal wall tissue (in intraperitoneally transplanted mice) was clearly reduced; tumor growth rates were significantly lowered; fewer pulmonary metastases were found, and the mean survival time increased up to 180%, as compared to the controls. The thiol proteinase inhibitor leupeptin has also been reported to inhibit metastasis in some experimental system (McCarty, 1982).

From the evidence presented above, cathepsin B is a strong candidate for involvement in some steps of the metastatic cascade. However, it is not clear whether the increased cathepsin B activity is a result of an increased synthesis of cathepsin B or a result of a decreased degradation of the enzyme, whether the secreted tumor-associated cathepsin B-like enzymes are high molecular weight precursor forms of cathepsin B, a mutated cathepsin B or a related enzyme from a different gene locus. One study has reported that amino acid composition data and peptide mapping revealed significant differences between the cathepsin B from normal mouse liver and a cathepsin B-like enzyme from an

analogous tumor (Olstein and Liender, 1983).

Also, it should be noted that a lack of correlation between cathepsin B and metastatic tumors has been reported in a rat prostatic adenocarcinomas (Lowe and Issacs, 1984), and in the variants of a methychloranthrene-induced sarcoma (McLaughlin et al, 1983). Krepela et al (1989) have proposed an explanation for the lack of correlation between cathepsin B and metastatic potential of two rat sarcoma variants. They found that the variant with high metastatic potential has lower intracellular level of cathepsin B activity compared to the variant with low metastatic potential. However, in the serum-free medium at pH 6.5 the variant with high metastatic potential released a significantly higher amount of cathepsin B activity into the medium than the one with low metastatic potential. They thus suggested that the two variant cell lines may have different capabilities to regulate their intracellular pH, which could affect the externalization of intracellular vesicles containing cathepsin B. In addition, they proposed that the mildly acidic microenvironment generated and frequently maintained at the surface of tumor cells could not only trigger cathepsin B release but also be favorable for maintaining the activity and the stability of the released enzyme.

Cathepsin L has attracted great attention since a major excreted protein (MEP) was cloned from transformed NIH3T3 cells and it was shown to be cathepsin L (Troen et al 1987;

Joseph et al, 1987 and 1988). MEP has long been observed to be secreted by transformed murine fibroblasts in cell culture. After transformation of murine NIH or BALB 3T3 fibroblasts with K-ras, cellular MEP rose from less than 0.02 to 1% of the total intracellular protein. The MEP was described as a mannose 6-phosphate-containing glycoprotein of  $M_r$  39,000 which binds to purified phosphomannosyl receptor responsible for targeting protein to lysosomes. The MEP accounts for up to 30% of the excreted proteins in transformed fibroblasts. Similar results apply to transformation by Ha-ras, MSV, SV40, and methylcholanthrene (Gottesman, 1978; Gottesman and Sobel, 1980; Gal et al, 1985; Gal and Gottesman, 1986). Secretion of MEP from NIH3T3 cells was also observed upon treatment of cells with a tumor promoter, TPA and PDGF (Gottesman and Sobel, 1980; Docherty et al, 1985; Frick et al, 1985; Rabin et al, 1986). Stimulation of non-transformed 3T3 fibroblasts by plating in serum at low density did not significantly elevate the basal level of MEP secretion. Denhardt and coworkers (1986) examined MEP mRNA expression at different points in the cell-cycle (stimulated by serum) and found less than a two fold change. The definitive identification of this protein as murine cathepsin L was established by isolation and sequencing of the corresponding clone from a human kidney cDNA library (Mason et al, 1986). Increased cathepsin L activity has been found in only a limited number of malignant tumors. Human gastric cancer was found to have increased cathepsin L

activity by two groups (Vasishta et al, 1985; Watanabe et al, 1989). So was human breast tumor (Abecassis et al, 1984). Sheahan et al (1989) examined 29 colorectal cancer patients with Z-Phe-Arg-NHMec as substrates and concluded cathepsin L-like specific activities were significantly elevated ( $p < 0.005$ ) in early stage (Dukes'A) (Dukes, 1932) of colorectal carcinoma while, in medium and late stages (Dukes'B, C and D), it became as low as in normal tissues. Unfortunately Z-Phe-Arg-NHMec is not specific for cathepsin L since it is also hydrolyzed by cathepsin B. Durdey et al (1985) have obtained similar results. However, another group (Keppler et al, 1988a) found the levels of cathepsin L was very low in both extracts of colorectal tumors and normal colonic mucosa and no MEP was detected in the human colon adenocarcinomas checked. Unfortunately, this paper did not identify the stages of the tumors. Gottesman et al have hypothesized that cathepsin L plays a role in malignant transformation; however, up-regulation of the expression of cathepsin L in murine NIH 3T3 fibroblast was not sufficient to induce a transformed phenotype as measured by anchorage or serum independent growth or tumorigenesis in nude mice (Kane et al, 1988). Denhardt et al (1987) have suggested that cathepsin L plays a role in the proteolytic steps of the metastatic cascade since elevated mRNA for cathepsin L in ras transfected fibroblasts correlated with the metastatic capability of the transfected cells. However, not appreciated at that time is the fact that ras

transfection of fibroblasts can also increase cathepsin B expression and procathepsin B secretion. A role for cathepsin L in metastasis would appear a reasonable hypothesis as this cysteine proteinase shows the most potent collagenolytic and elastinolytic activity in vitro of any of the cathepsins (Mason et al, 1986, Kirachke et al, 1982) and it can readily degrade intact basement membranes (Baricos et al, 1988). Furthermore, cathepsin L activity has been detected on cell surface of cultured human colon carcinoma cells (Maciewicz et al, 1989) and murine B16 melanoma cells (Rozhin et al, 1989). Its presence in plasma membrane may lead to the establishment of a local microenvironment in which local degradation of basement membrane could happen. The secreted high molecular weight cathepsin L by malignant tumor was found to be more stable in neutral pH than lysosomal cathepsin L and active as a precursor of cathepsin L (Mason et al, 1987; Nishimura et al, 1988). Maciewicz et al (1989) have found not only mature cathepsin L, but also procathepsin L in culture medium for malignant cells is active against an isolated basement membrane matrix in vitro. Taken together, the stable latent activity of procathepsin L secreted by transformed or malignant tumor cells, the evidence for autoprocessing of procathepsin L to mature cathepsin L in medium, and the significant activity of mature cathepsin L at neutral pH argues for a role of cathepsin L in tumor invasiveness or metastasis. However, normal rat mammary glands in culture

have also been shown to secrete a high molecular weight form of cathepsin L (Recklies and Mort, 1985b).

Cathepsin D, although it is a lysosomal proteinase with a wide tissue distribution, has been shown to be transcriptionally regulated by estrogens in MCF7 cells-a human breast cancer cell line (Westley and May, 1987; Cavailles et al, 1988). It is secreted as a proenzyme which was first described as a 52,000 mol wt protein (Weatley et al, 1980; Capony et al, 1987) before its identification as cathepsin D by molecular cloning and sequencing (Augereau et al, 1988). It was found to be mitogenic in vitro (Vignon et al, 1986), to stimulate DNA synthesis and mitosis in the rat liver (Terayama et al, 1985) and capable of degrading the extracellular matrix (Briozzo et al, 1988), suggesting a potential role in mammary carcinogenesis and metastasis (Rocheffort et al, 1987).

The 52,000 protein was detected in several human primary breast cancers but not in normal breast and endometrium of uterus (Garcia et al, 1984). It is fully active in acidic molecular organelles (endosomes, lysosomes) without much change in its molecular mass, since the autoactivation in acidic pH caused only a slight decrease in mass to 51-kD. Inhibition of the proteolytic activity by pepstatin suggested that the 52-kD protein is an aspartic proteinase. It displayed a maximal activity at pH 5.5 and retained some activity above pH 6, suggesting that this secreted protease

may act extracellularly in vivo (Capony et al, 1987). The cathepsin D level is not significantly regulated in normal mammary epithelial cells. The regulation of cathepsin D by estrogen might be the result of the increase in the estrogen receptor level in the breast cancer cells. This speculation was supported by other findings that Cathepsin D mRNA was not regulated in an estrogen receptor negative breast tumor cell line, BT20, and in two other malignant cell lines HeLa and A431 (Weatley et al, 1987).

Some clinical studies using immunoenzymatic technique suggested cathepsin D expression in breast cancers is associated with prognostic disadvantage. Such as Maudelonde et al (1988) reported that high levels of cathepsin D expression in primary breast cancers are associated with an increased propensity for lymph node metastasis based on a study of 182 breast cancer cytosol. Similarly, Thorpe et al (1989) found that high concentrations of cathepsin D predict poor prognosis in primary breast cancer. The agreement between prospective (Maudelonde et al, 1988) and retrospective studies (Thorpe et al, 1989) of breast cancer patients suggests that cytosolic cathepsin D concentrations may have a prognostic value independent of other prognostic parameters. However, a study of expression of cathepsin D in 94 breast cancers by immunohistochemical technique reported conflicting result, which suggested that cathepsin D was associated with a significant prognostic advantage (Henry et al, 1990). The

study found that cathepsin D positive staining was associated with presence of estrogen receptors. In the estrogen receptor positive breast tumors, cathepsin D staining positive patients had significantly prolonged survival. In a very recently study, it has been claimed that cathepsin D is a good predictor of metastatic breast tumor especially in nude negative patients and in women with aneuploid mammary tumors (Tandon et al, 1990).

The regulation of cathepsin D in human normal endometrium is mostly due to progesterone, not to estradiol, and the cathepsin D level appears to be higher (3-fold) in adenocarcinoma cells than in normal cells. In addition, there is a significant correlation between myometrial invasion and cathepsin D concentrations. The low level of cathepsin D in endometrial cancer appears to be correlated with low myometrial invasion (Maudelonde et al, 1990). Since myometrial invasiveness is considered to be the major prognostic parameter of endometrial carcinoma (Hendrickson et al, 1982), the cellular concentration of cathepsin D may be of interest as an associated biochemical marker of invasion.

Another broad set of explanations for increased secretion of cathepsins including B, L and D in various of tumors involves alterations in protein sorting. Glycosylation abnormalities, which have been extensively described for the cell surface in malignancy (Dennis et al, 1987), could alter sorting of cathepsins, resulting in increased secretion and

altered intracellular distribution. An altered transferase in I cell disease prevents expression of the mannose-6 phosphate marker and results in massive secretion of many lysosomal constituents (Kornfeld, 1986). Some cells, such as J774.2, appear to lack a properly functioning mannose 6 phosphate receptor (215 kD receptor), again resulting marked secretion of multiple lysosomal species (Gabel et al, 1983).

Very few correlation have been observed between malignant tumors and the other two lysosomal cathepsins, H and S. One paper reported that increased cathepsin H activity has been found in human brain neoplasms (Chernaia and Reva, 1989).

Table 3 listed the types of tumors which have been reported to have elevated cathepsin activities or secretions.

**Table 3. Enhanced Activities or Secretions of Cathepsins  
in Different Tumors**

<b>Cathepsin B</b>	<b>↑</b>	<b>Liver Cancer</b> <b>Mammary Carcinoma</b> <b>Melanoma</b> <b>Ovarian Carcinoma</b> <b>Fibrosarcoma</b> <b>Gastric Cancer</b> <b>Lung Cancer</b>
<b>Cathepsin D</b>	<b>↑</b>	<b>Breast Carcinoma</b> <b>Hepatomas</b> <b>Gastric Cancer</b>
<b>Cathepsin L</b>	<b>↑</b>	<b>3T3 Transformed Cells</b>
<b>Cathepsin H</b>	<b>↑</b>	<b>Brain Tumor</b>

## Chapter III

### MATERIALS AND METHODS

#### Materials

Enzymes and chemicals were purchased from Bethesda Research Laboratories (Gaithersburg, MD), New England Biolab (Beverly, MA) and Boehringer Mannheim Biochemicals (Indianapolis, IN). Nitrocellulose filters were from Schleicher and Schnell (Keene, NH).  $\alpha$ -<sup>32</sup>P-dCTP (specific activity 3,000 Ci/mmol), r-<sup>32</sup>P-dATP (specific activity 5,000 ci/mmol) and nick translation kit were from Amersham (Arlington Heights, IL). Protected nucleotide monomers and reagents for DNA synthesis were from Applied Biosystems (Foster City, CA). Mouse cathepsin B (1,200 bp) (Chan et al, 1986) and rat cathepsin S cDNAs (940 bp) (Chan et al, Unpublished data), and rat cathepsin H cDNA (1,400 bp) from rat were prepared in our laboratories. A human cathepsin D cDNA (2,300 bp) (Faust et al, 1985) was a gift from Dr. J. Chirgwin (University of Texas, Health Science Center, San Antonio, TX 78284) and mouse cathepsin L cDNA (850 bp) was a

gift from Dr. V. Sukhatme (Howard Hughes Medical Institute, the University of Chicago, Chicago, IL 60637). Sprague-Dawley rats of both sexes were from Dutchland (Denver, PA). Walker-256 carcinosarcoma, cell line LLC-WRC was obtained from the American Type Culture Collection (Rockville, MD).

The mice used in these studies (C57BL/6, male, 5-7 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME). All animals were housed under identical conditions of photoperiod, feeding regime and temperature for 1 week in the Loyola Animal Facility prior to use. The murine melanoma variants B16-F1, F16-F10, and B16a were obtained from the Division of Cancer Treatment Tumor Depository (National Cancer Institute-Frederick Cancer Research Facility, Frederick, MD) (Filder, 1973). The melanomas were propagated in vivo by subcutaneous injection of the minced tumor into the right axillary region of the host mice.

### Cell Culture

Freshly dispersed tumor cells were adapted for growth in Eagle's Minimum Essential Medium (MEM) containing Hank's salts and supplemented with sodium pyruvate, MEM non-essential amino acids, 75 units/ml penicillin, 75  $\mu$ g/ml streptomycin, 25 mM HEPES and 10% FBS. Semi-confluent cells were subcultured by scraping into 10 ml of incomplete media (MEM), pelleted at 500 x g and resuspended in fresh medium containing 10% FBS. The cells were subcultured approximately once a week for a maximum

three times and the media was changed thrice weekly. secondary cultures were used during log phase of growth and were 70-80% confluent. In a T-75 flask this corresponded to approximately  $3 \times 10^6$  cells with B16-F1,  $6 \times 10^6$  cells with B16-F10, and  $8 \times 10^6$  cells with B16a.

#### Screening of A Rat Liver cDNA Library and DNA Sequencing

A rat liver cDNA library constructed in  $\lambda$ gt11 was a gift from Dr. James Hardwick (Argonne National Laboratory) and was screened with the synthetic 39 bp-long mixed oligonucleotides. The DNA oligonucleotides were synthesized using the phosphoramidite methodology (Beaucage and Caruthers, 1981) on an Applied Biosystems Model 380A synthesizer and were purified by polyacrylamide gel electrophoresis in 7 M urea (Agarwal et al, 1981). After plating the library on E. coli LE392, a total of  $10^6$  plaques were screened. Filters were prehybridized (2 hrs) and hybridized (16 hrs) in a solution containing 2.5 x Dehardt's solution/5 x NaCl/Cit/20 mM sodium phosphate, pH 6.5/0.1% NaDodSO<sub>4</sub>/20% formamide/50  $\mu$ g of sonicated denatured salmon sperm DNA per ml at 37°C. Approximately  $5 \times 10^5$  of radioactive DNA probe per ml was used during hybridization. Hybridized filters were washed at a final temperature of 50°C in a final solution of 2 x NaCl/Ci/0.1% NaDodSO<sub>4</sub>. The filters were autoradiographed at -70°C with Kodak XAR-5 film and DuPont intensifying screens. The positive clones were purified and subcloned into PGEM4Z

M13 mp18, mp19. Sequencing analysis was done using both Maxam-Gilbert chemical method (Maxam and Gilbert, 1977 and 1980) and the dideoxy chain termination method (Sanger et al, 1977).

#### Total RNA and Poly(A)<sup>+</sup> Isolation

Perfused normal tissues, perfused solid tumors and cultured cells (approximately  $5-7 \times 10^7$  cells), frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ , were homogenized in 10 volumes of a solution of 5 M guanidinium thiocyanate, 10 mM EDTA, 50 mM Tris-HCl, PH7.5, 14% (v/v) mercaptoethanol. Homogenates were centrifuged at  $1,000 \times g$  at  $4^{\circ}\text{C}$  for 15 min. in a swinging bucket rotor. The RNA was isolated by centrifugation through a 5.7 M CsCl, 0.1 M EDTA, PH 7.0 cushion at 35,000 rpm at  $20^{\circ}\text{C}$  for 16 h in a SW40 swing bucket rotor. The RNA was then dissolved in 0.2% SDS, extracted with phenol/chloroform and precipitated with ethanol (Chirgwin et al, 1979). The integrity of the RNA was examined by agarose gel electrophoresis after denaturation with glyoxal and dimethylsulfoxide (Maniatis et al, 1982). Poly(A)<sup>+</sup> RNA was then isolated by oligo(dT)-cellulose, chromatography (Maniatis et al, 1982).

#### Specificity and Sensitivity of the cDNA Probes

Since the cysteine cathepsins are structurally related enzymes with significant homology in primary sequences it is

important to establish that each individual cDNA probe hybridizes only to the corresponding cathepsin mRNA. To test this, a preliminary experiment was performed in which five identical dot blots were prepared, 1 ng of cathepsin B, D, L and S cDNAs were spotted onto each blot filter. The identical blots were hybridized with each of five <sup>32</sup>P-labeled cathepsin cDNA probes, respectively, and washed under high stringency condition as described above. The results indicated each probe only hybridized with its corresponding cDNA. No cross reactivity was observed under the condition employed. To test the sensitivity of the cDNA probe, five dot blots were prepared, each blot contained from 0.1 pg to 1.0 mg of one of five cathepsin cDNAs. Each blot was hybridized with each corresponding <sup>32</sup>P-labeled cathepsin cDNA probe and washed as described above. The results indicated that the assay was sufficiently sensitive so as to detect as little as 10 pg of the corresponding unlabeled cathepsin cDNA with overnight autoradiographic exposure.

#### Quantification of mRNA.

The RNA dot blot procedure was used to quantify each specific cathepsin mRNAs. Total RNA from normal tissues and tumors were dissolved in denaturation buffer (Ral et al, 1987) and heated at 65°C for 10 min. The denatured RNA solutions were diluted with 20 x SSC and varying amounts (0.5 µg to 10 µg) were spotted on nitrocellulose filters. After

hybridization (Bell et al, 1981) with the appropriate nick-translated  $^{32}\text{P}$ -labeled cDNA insert (approx.  $5 \times 10^8$  cpm/ $\mu\text{g}$ ), the filters were washed with 0.1% SDS in 0.1 x SSC at room temperature for 1 hr and at 55°C for a second hr. The intensity of the hybridization signal was determined by densitometry (LKB Ultrascan SL Enhanced Laser Densitometer) after autoradiography.

### Northern Blot Analysis

Total RNA or poly(A)<sup>+</sup> RNA was denatured with glyoxal and dimethylsulfoxide and subjected to electrophoresis in a 1.2% agarose gel (Maniatis et al, 1982). The samples were transferred to a nitrocellulose filter (Maniatis et al, 1982) and the filter was hybridized as described above.

### Southern Blot Analysis

High molecular DNA were extracted from both normal mouse liver and B16a melanoma (Maniatis et al, 1982). 20  $\mu\text{g}$  DNA for each sample was digested with restriction enzyme(s) at 37°C over night. The digested DNA was electrophoresed in 0.6% agarose gel. The gel was denatured in a solution of 1.5 M NaCl, 0.5 M NaOH for an hour and neutralized in a solution of 1.5 M NaCl, 0.5 M Tris, PH 7.4 for another hour. The samples were then transferred onto nitrocellulose filter (Maniatis et al, 1982) and the filter was hybridized as described above (Bell et al, 1981).

### Measurements of Metastatic Potential

The metastatic potential of the murine melanoma variants (Fidler, 1973, 1978a and 1978b) were determined using both an intravenous lung colonization assay and a subcutaneous model of spontaneous metastasis as described by Schultz, et al (1988). Monodispersed tumor cells ( $1 \times 10^5$  cells/animal) were obtained from secondary cultures of B16-F1, B16-F10 and B16a melanomas and injected intravenously through the tail vein of syngeneic host mice or subcutaneously in their right axillary region. After 18 days the mice were sacrificed and examined for disseminated tumor colonies. The lungs were removed, washed with PBS, stained with Bouin's fixative and the visible tumor colonies counted.

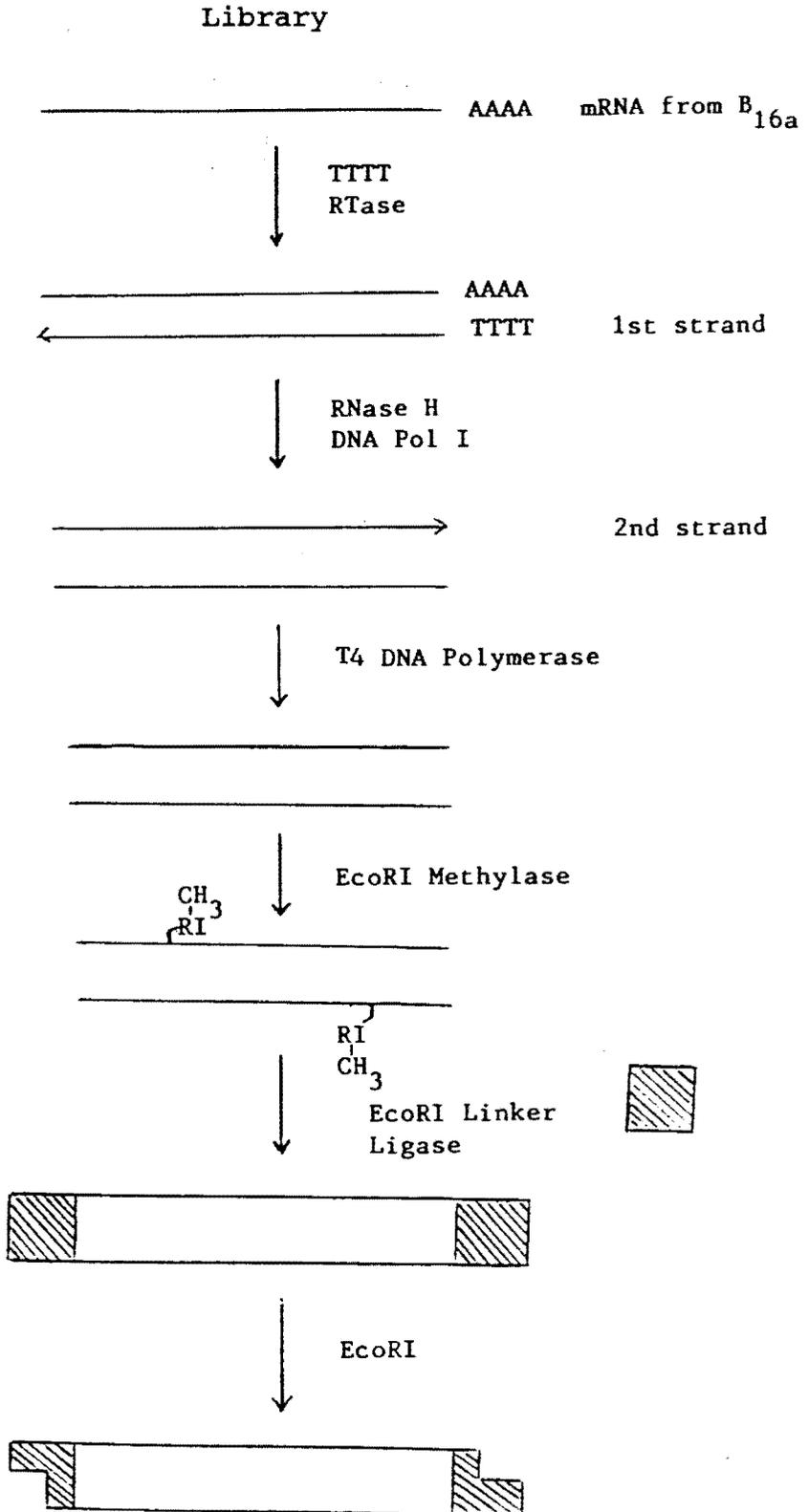
### Construction of a Mouse Melanoma cDNA Library

The mRNA used for construction of the cDNA library was prepared from mouse B16a melanoma (Division of Cancer Treatment Tumor Depository, National Cancer Institute-Frederick Cancer Research Facility, Frederick, MD). cDNA was synthesized following the protocol of Gubler and Hoffman (1983) with minor modification. Poly(A)<sup>+</sup> RNA (5  $\mu$ g) was reverse-transcribed by avian myeloblastosis virus reverse virus transcriptase (Life Science) with oligo dT<sub>(12-18)</sub> as primer. The first-strand heteroduplex was converted to fully double-stranded cDNA by using DNA polymerase I (BRL) and

Figure 2. Scheme for Construction of a B16a Melanoma cDNA  
Library

Poly(A)<sup>+</sup> RNA was reverse-transcribed by avian myeloblastosis virus reverse transcriptase, with oligo dT<sub>(12-18)</sub> as primer. The first-strand heteroduplex was converted to fully double-stranded cDNA by using DNA polymerase I and RNase H. The double stranded cDNA was then methylized with Eco RI methylase followed by Eco RI linker addition. After digestion with Eco RI, the cDNA was inserted into vector Eco RI site.

Figure 2. Scheme for Construction of a B16a Melanoma cDNA



RNase H (BRL). The double stranded cDNA was then methylized with Eco RI methylase followed by Eco RI linker addition (Fig. 2). After digestion with Eco RI and size fractionated through a sepharose CL-4B column the cDNA with the sizes over 600 bp was selected and then inserted into  $\lambda$ gt10 vector (Promega) Eco RI site. The library was plated on E coli 600  $\Delta$ HFI cells. The titer indicated the library has a complexity of 100,000.

### Screening a Melanoma cDNA Library and Sequencing

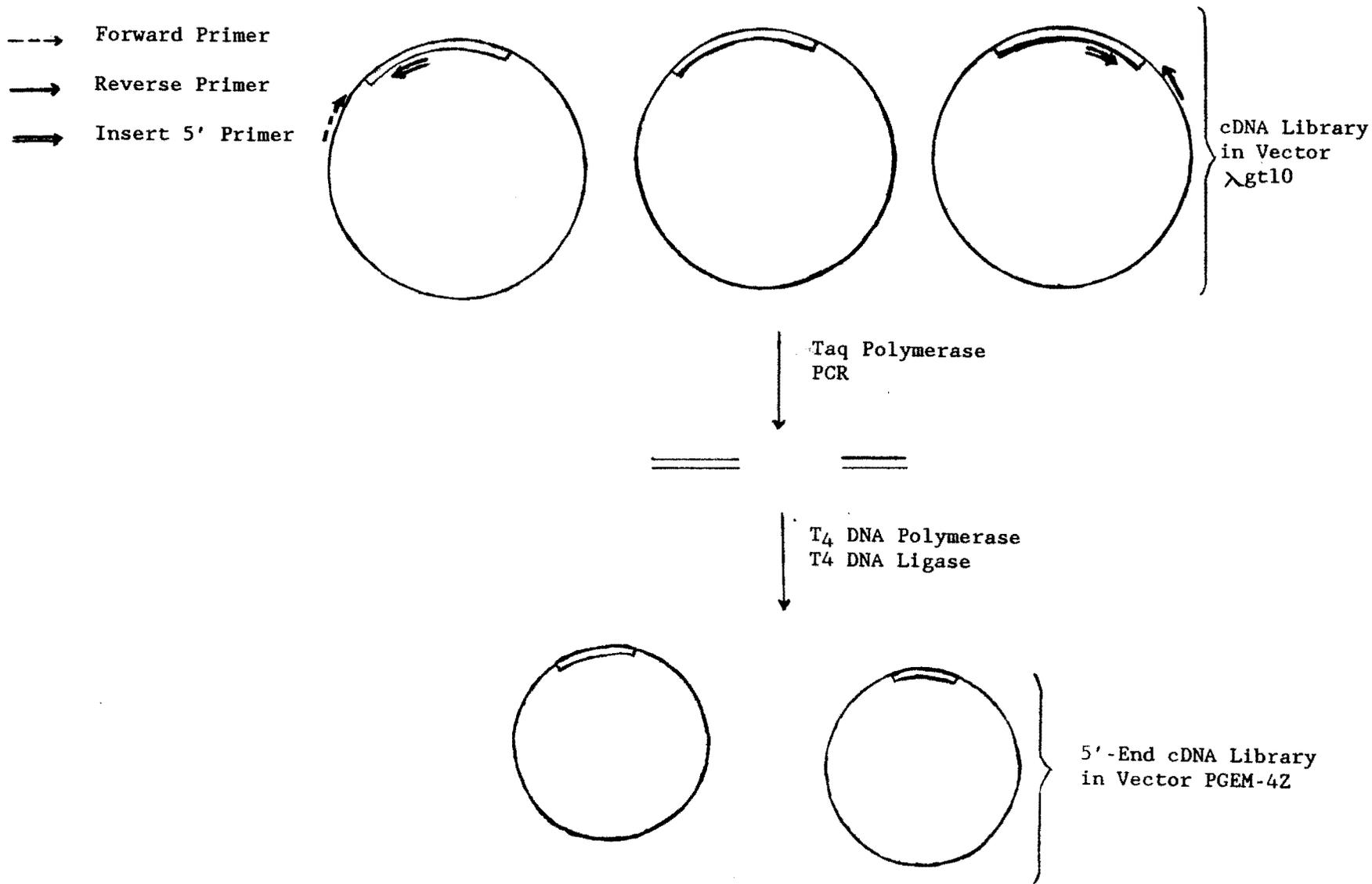
Phage-plaque replicas from melanoma  $\lambda$ gt10 library were prepared by the method of Woo (1979) and nitrocellulose filters (Schleicher and Schuell) replicas were prehybridized and hybridized as the procedure described previously (Qian et al, 1989). DNA probes were labeled with  $\alpha$ -<sup>32</sup>P-dCTP (3,000ci/mmole, Amersham) using either a nick-translation kit (Amersham) or a random priming kit (IBI). The positive clones were then further purified. The purified clones were restriction-mapped and the inserts were subcloned into plasmid vector PGEM-4Z followed by sequencing.

PCR (Polymerase Chain Reaction) was also used in screening the cDNA library. In order to get further 5'-end sequence  $\lambda$ gt10 forward or reverse primer (Biolabs) was used as sense primer while oligonucleotide designed according to known 5'-end cathepsin B sequence were used as antisense primer. The library phage lysate was used as templet

Figure 3. Scheme for Screening the B16a melanoma cDNA Library  
by PCR for 5'-End of cDNA

$\lambda$ gt10 forward or reverse primer was used as sense primer while oligonucleotide designed according to known 5'-end cathepsin B sequence were used as antisense primer. The library phage lysate was used as template directly. PCR was carried by Taq polymerase. The reaction mixtures were extracted with chloroform and the amplified DNAs were polished with T4 DNA polymerase and ligated into the Sma I sites of pGEM-4Z.

Figure 3. Scheme for Screening the B16a melanoma cDNA Library by PCR for 5'-End of cDNA



directly. The reaction solution contained 10 mM Tris-HCl, PH 8.3, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.001% (W/V) gelatin, 1 mM dNTP (dATP, dCTP, dGTP and TTP), 100 pmole each primer, 10<sup>9</sup> phages and 2.5 u Taq polymerase (Perkin Elmer Cetus). The reactions were carried out on 100 µl solution for 30 cycles under the condition of 94°C, 1 min; 55°C, 2 min; 70°C, 3 min. The reaction mixtures were extracted with chloroform and amplified DNAs were subcloned into the Sma I site of pGEM-4Z (Fig. 3). Plasmids with cathepsin B DNA inserts were identified by colony hybridization (Maniatis, 1982) with <sup>32</sup>P-labeled internal oligonucleotide and then sequenced.

The double strand sequencing was carried out with either general primers (Sp6 and T7 primers, promega) or designed oligonucleotides as primers using dideoxy chain-termination method of Sanger et al (1980) with modification essential for double-stranded sequencing as described by Chen and Seeburg (1985). The double strand DNA was first denatured with 0.2 M NaOH and then sequenced with sequenase (USB) and <sup>35</sup>S-dATP.

#### Isolation of Mouse Cathepsin B Gene

Mouse embryo genomic and a NIH3T3 cell genomic libraries were screened by hybridization with <sup>32</sup>P-labeled fragments of the mouse cathepsin B cDNA (Chan et al, 1986). Phages containing fragments of the mouse cathepsin B gene were plaque purified. Phage DNA was prepared and DNA fragments containing

sequences of the gene identified by Southern blotting. Appropriate fragments were isolated and subcloned into plasmid pGEM-4Z. A series of oligonucleotides were designed as primers according to the cDNA sequence and were used for sequencing the gene. The inserts in plasmids were sequenced and the sequences were analyzed with DNASTAR program (DNASTAR, INC).

Despite repeated attempts, a DNA fragment containing sequences of exon 7 to intron 8 could not be cloned by screening the two genomic libraries. I am able to isolate this fragment using a PCR procedure. The PCR reaction of 100  $\mu$ l contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.001% gelatin, 1 mM dNTP, 100 pmole each primer, either 10<sup>9</sup> phages from the genomic libraries or 1  $\mu$ g mouse kidney genomic DNA and 2.5 unit Taq polymerase (Cetus). PCR was carried out for 30 cycles under the conditions of 94°C, 1 min., 55°C, 2 min., 70°C, 3 min. The PCR products were analyzed by electrophoresis in agarose gels. First I tried to use one pair of oligonucleotides corresponding to positions of amino acids 190-196 in exon 7 (sense primer 1) and an oligonucleotide (antisense primer 1) in intron 8. It failed to generate any product when using either phage genomic libraries (Fig.4, lane 6) or mouse kidney genomic DNA (Fig.4, lane 7) as templates. The procedure was thus performed using two pairs of oligonucleotides as primers: one pair corresponded to positions of amino acids 190-196 in exon 7 (sense primer 1)

### Figure 4. Using PCR Procedure to Clone Exon 8 Region

Lane 3, 5 and 7: genomic libraries were used as templates. Lane 1, 4 and 6: mouse kidney genomic DNA was used as templates. The primers for lane 1 and 3 are from exon 8 (sense primer 2) and intron 8 (antisense primer 1). The primers for lane 4 and 5 are from exon 7 (sense primer 1) and exon 8 (antisense primer 2). The primers for lane 6 and 7 are from exon 7 (sense primer 1) and intron 8 (antisense primer 1). Lane 3 is DNA markers ( $\lambda$ HindIII and  $\phi$ x174 HaeIII). The direction of each primer is shown as following:

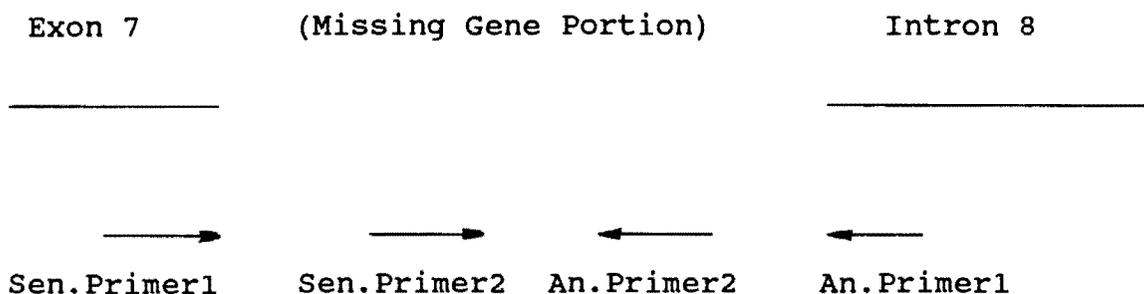
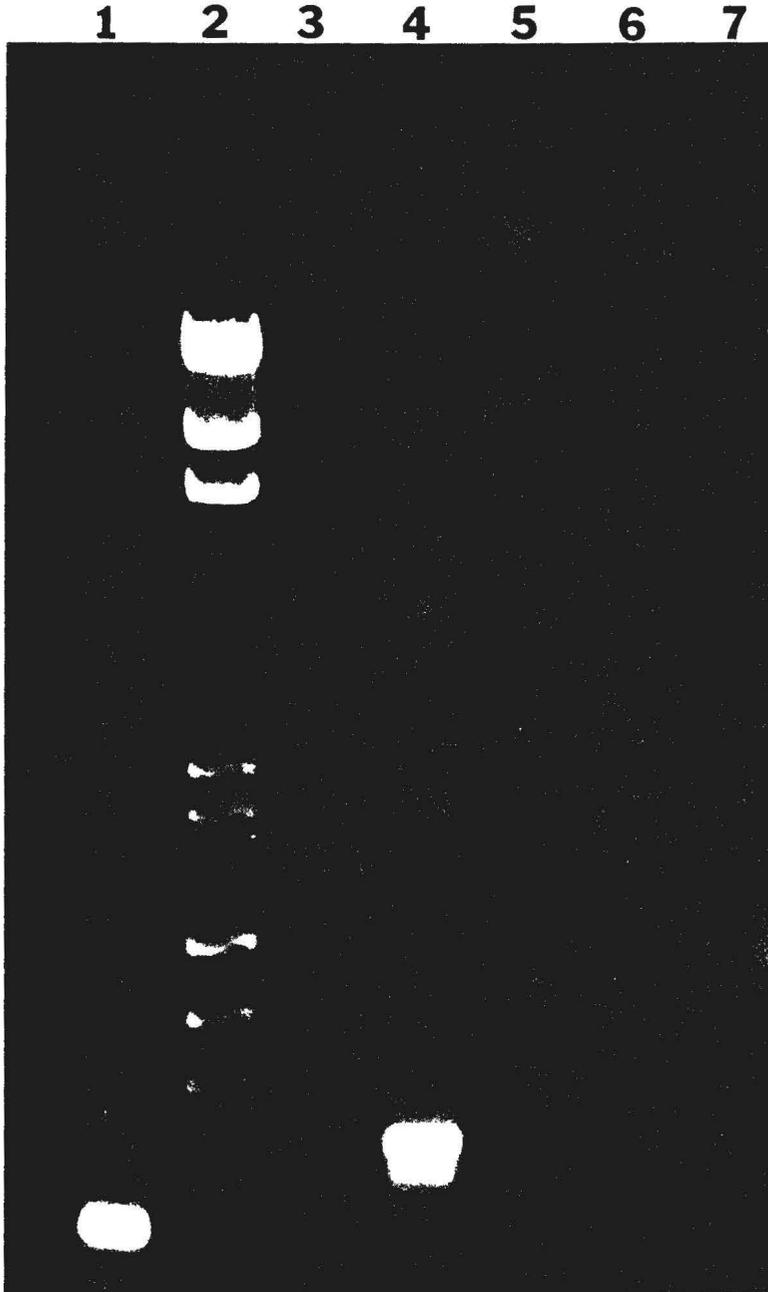


Figure 4. Using PCR Procedure to Clone Exon 8 Region



and amino acids 257-262 (antisense primer 2) in exon 8; the second pair consisted of a primer corresponding to position amino acid 227-232 in exon 8 (sense primer 2) and the antisense primer 1 from intron 8. Both pairs failed to work when using phage genomic libraries as templates (Fig. 4, lane 3 and 5), however, they both worked well when using mouse kidney genomic DNA as templates and produced a 500 bp and a 750 bp band (Fig. 4, lane 1 and 4). So the earlier difficulty encountered may be due to presence of secondary structure which prevents the construction of this fragment into libraries and its synthesis by Taq polymerase when using the first pair of primers. The PCR products were isolated and cloned into the Sma I site of pGEM-4Z. The positive colonies were identified by hybridization with <sup>32</sup>P-labeled appropriate oligonucleotides (Maniatis, 1982). The inserts in the plasmids were sequenced directly using SP6, T7 or designed oligonucleotide primers. PCR was also used to determine the distances between adjacent exons and introns. The templates were either phage DNA, plasmid DNA containing cathepsin B gene sequences (100 ng) or mouse kidney genomic DNA (1 μg).

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

Because our mouse cathepsin B cDNA lacked the region encoded by exon 1, I used RACE methods essentially as described by Frohman et al (1988), with modification. Briefly, the first strand cDNA was prepared from 5 μg mouse

kidney total RNA by using an oligonucleotide primer complimentary to a 5'-end sequence in mouse cathepsin B cDNA and avian myeloblastosis virus reverse transcriptase. The resulting first strand cDNA was tailed with dATP and terminal deoxynucleotide transferase. The second strand cDNA was synthesized with oligo(dT)-adaptor as primer and klenow enzyme. The double strand cDNA was amplified with PCR using the adaptor as sense primer and another internal oligonucleotide as antisense primer. The PCR was carried out for 30 cycles (94°C, 1 min, 60°C, 2 min, 70°C, 2 min). The product was polished with T4 polymerase and subcloned into PGEM-4Z Sma I site followed by analysis and sequencing (Fig. 5).

#### Primer Extension and RNA Protection

The primer extension procedure was as following: 5 µg of total RNA or 2 µg of poly(A) RNA from mouse kidney and 1 pmol of the <sup>32</sup>P-labeled oligonucleotide 5'-GGAGCCGCTCCTGGCCTC-3', complementary to nucleotides 48 to 65 of the mRNA, were hybridized and extended with AMV reverse transcriptase (Life Science) as described by Gil et al (1987). The primer-extended products were separated on a 8% polyacrylamide/8 M urea gel. A sequencing reaction was performed using the same oligonucleotide as above as primer and a 5' upstream fragment of the mouse cathepsin B gene as a template. The sequencing reaction was loaded on the gel at the same time as primer extension reaction was loaded.

RNA protection assay was a modification of the procedure by Little et al (1987). A 241 bp Pst I DNA fragment (from nucleotide -156 to 85 bp) of mouse cathepsin B gene was cloned into pGEM-4Z. This plasmid (pmGB250) was linearized with Hind III and a radioactive antisense RNA probe was synthesized using the Riboprobe Gemini II Core System (Promega), T7 RNA polymerase, and  $\alpha$ -<sup>32</sup>P-UTP. In vitro synthesized RNA was incubated at 45°C overnight with 40  $\mu$ g total RNA in 30  $\mu$ l of hybridization buffer (80% Formamide/40 mM Pipes, pH 6.7/400 mM NaCl/1 mM EDTA). Then 300  $\mu$ l of a solution of 10 mM Tris Cl, pH 7.5/5 mM EDTA/300 mM NaCl/ 40  $\mu$ g/ml RNase A/ 2  $\mu$ g/ml RNase T1 was added and incubated at 30°C for 60 min. The protected fragments were analyzed on a 8% polyacrylamide/8 M urea gel. A sequencing reaction was performed with a 5' upstream fragment of the mouse cathepsin B gene as a template and an antisense oligonucleotide (5'-CCTGCAGCCACCAAGCCA-3') corresponding to nucleotides +86 to +69 from transcription start site as a primer. Since there happen to be a nucleotide C, adjacent to the Pst I site in the vector pGEM-4Z, which is identical to the nucleotide C adjacent to the Pst I site in the gene fragment cloned into pGEM-4Z at position +86 of the mRNA, this primer started from +86 instated of +85 should have the same 5' end as the protected <sup>32</sup>p-cRNA fragment does. The sequence generated by this primer indicates the sequence of the protected mRNA fragments. The sequencing reaction was loaded at the same

time when the RNA protection reactions were loaded.

#### Measurement of mRNA Half Life

NIH3T3 fibroblasts, B16F1 and B16a cells (24 flasks each) were grown in MEM (Eagles's Minimum Essential Medium) supplemented with non-essential amino acids plus Fetal Calf Serum (heat inactivated). Just prior to confluence the medium was replaced and 5  $\mu\text{g/ml}$  Actinomycin D was added. In group of 4 flasks cells were subjected to the treatment for 0, 1, 3, 5, 7 and 12 hours. After each period of the treatment, the medium was discarded and the cells were washed with PBS, trypsinized and suspended in PBS. Then the cells are pelleted by centrifugation 3,000 RPM for 5 min. The supernatant was discarded and 2 ml of PBS was added. The cells were frozen in liquid nitrogen.

The total RNAs were extracted from each group of cells. RNA dot blot and Northern blot were performed by hybridization with mouse cathepsin B cDNA inserts and autoradiographed as described above. The autoradiographies were subjected to densitometer scanning analysis. Relative dot intensity readings were plotted as a function of the time of transcription inhibition by actinomycin D. mRNA half-lives were determined by a least-squares linear regression calculation of the plotted data.

Figure 5. Scheme for Rapid Amplification of 5'-End of cDNA  
(5'-RACE)

The first strand cDNA was prepared from 5  $\mu$ g mouse kidney total RNA by using an oligonucleotide primer complimentary to a 5'-end sequence in mouse cathepsin B cDNA or oligo(dT<sub>12-18</sub>) and avian myeloblastosis virus reverse transcriptase. The resulting first strand cDNA was tailed with dATP and terminal deoxynucleotide transferase. The second strand cDNA was synthesized with oligo(dT)-adaptor as primer and klenow enzyme. The double strand cDNA was amplified with PCR using the adaptor as sense primer and another internal oligonucleotide as antisense primer. The PCR was carried out and the PCR product was polished with T4 polymerase and subcloned into PGEM-4Z Sma I site followed by analysis and sequencing.

Figure 5. Scheme for Rapid Amplification of 5'-End of cDNA  
(5'-RACE)

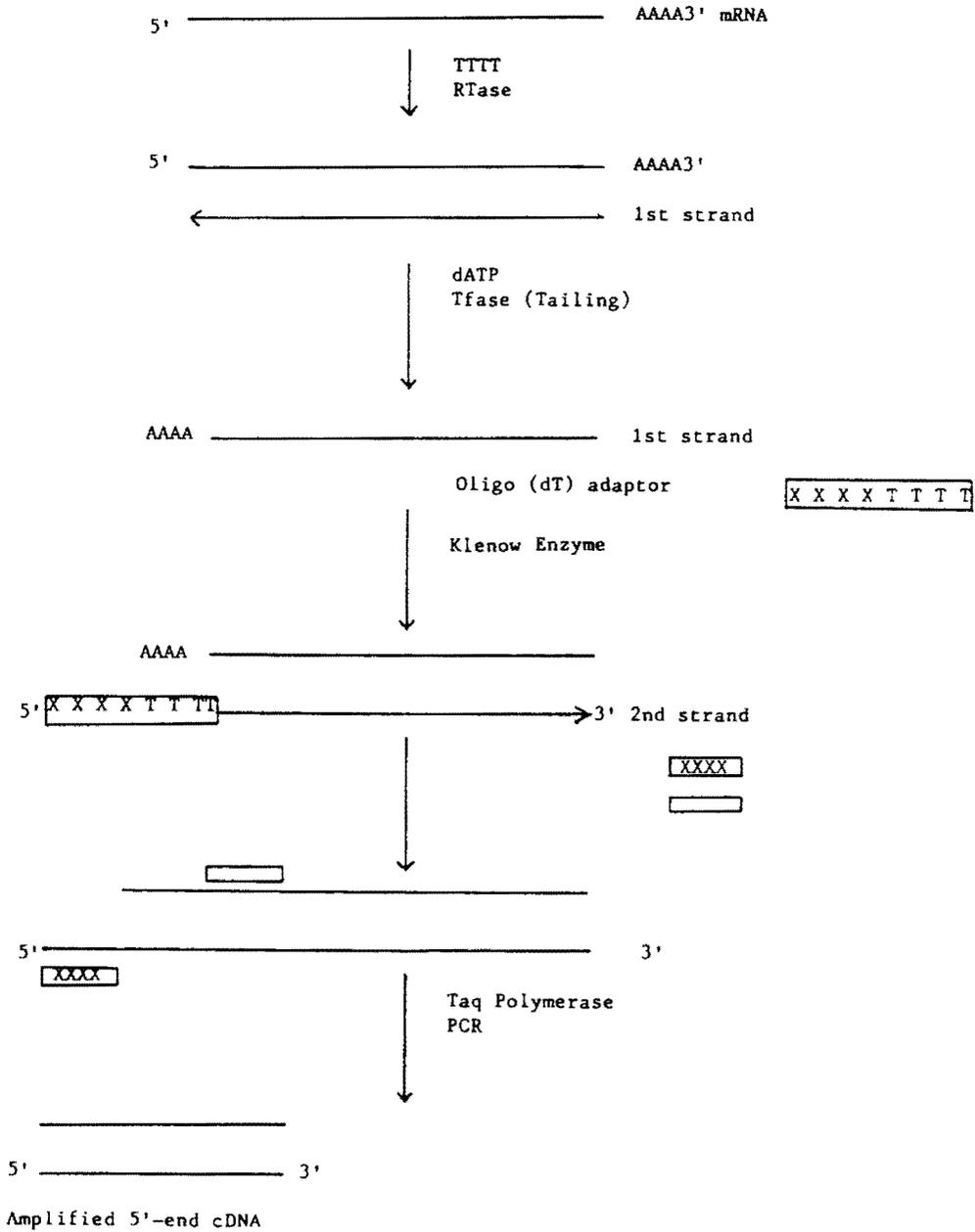
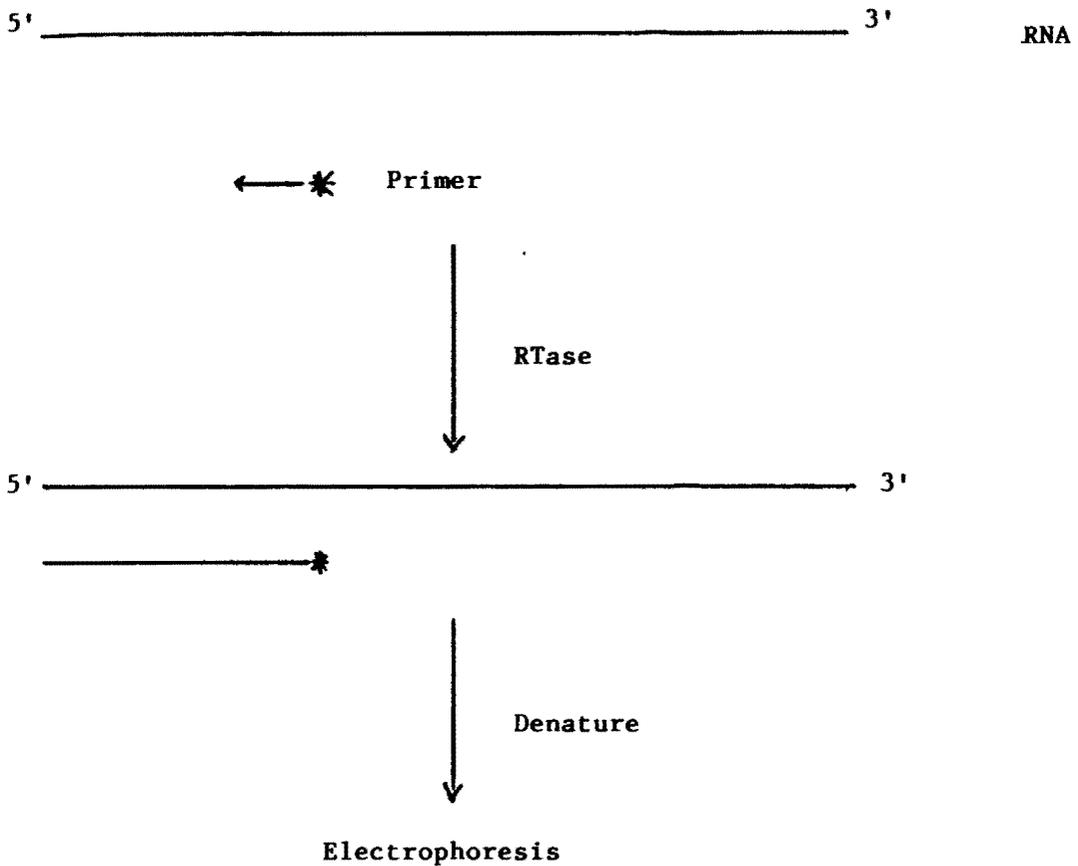


Figure 6. Scheme for Primer Extension Assay

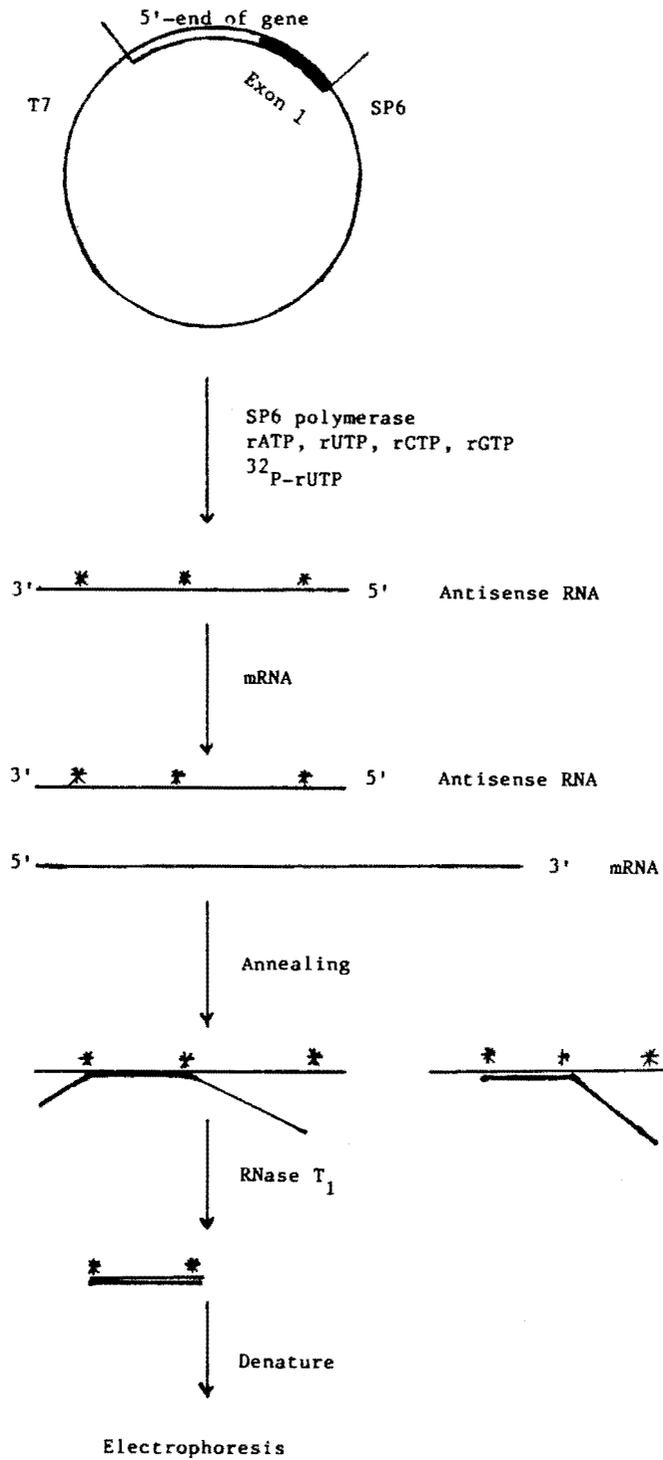


<sup>32</sup>P-labeled oligonucleotide were hybridized with total RNA or poly(A)<sup>+</sup> RNA and extended with AMV reverse transcriptase. The primer-extended products were denatured and separated on a 8% polyacrylamide/8 M urea gel by electrophoresis.

## Figure 7. Scheme for RNA Protection Assay

A DNA fragment containing 5'-end of the gene was cloned into pGEM-4Z. This plasmid was linearized and a radioactive antisense RNA probe was synthesized using the Riboprobe Gemini II Core System (Promega), SP6 or T7 RNA polymerase, and  $\alpha$ - $^{32}\text{P}$ -UTP. In vitro synthesized antisense RNA was incubated with total mouse kidney RNA for annealing. RNase A and RNase T1 was added to digest single strand RNA. The protected fragments were denatured and analyzed on a 8% polyacrylamide/8 M urea gel by electrophoresis. A sequencing reaction was performed with a 5' upstream fragment of the mouse cathepsin B gene as a template and an antisense oligonucleotide (5'-CCTGCAGCCACCAAGCCA-3') corresponding to nucleotides +86 to +69 from transcription start site as a primer which has the same 5' end as the protected  $^{32}\text{P}$ -cRNA does. The sequence generated by this primer indicates the sequence of the protected mRNA fragments. The sequencing reaction was loaded at the same time when the RNA protection reactions were loaded to the gel.

Figure 7. Scheme for RNA Protection Assay



## CHAPTER IV

### RESULTS

#### Cathepsin H Cloning and Sequencing

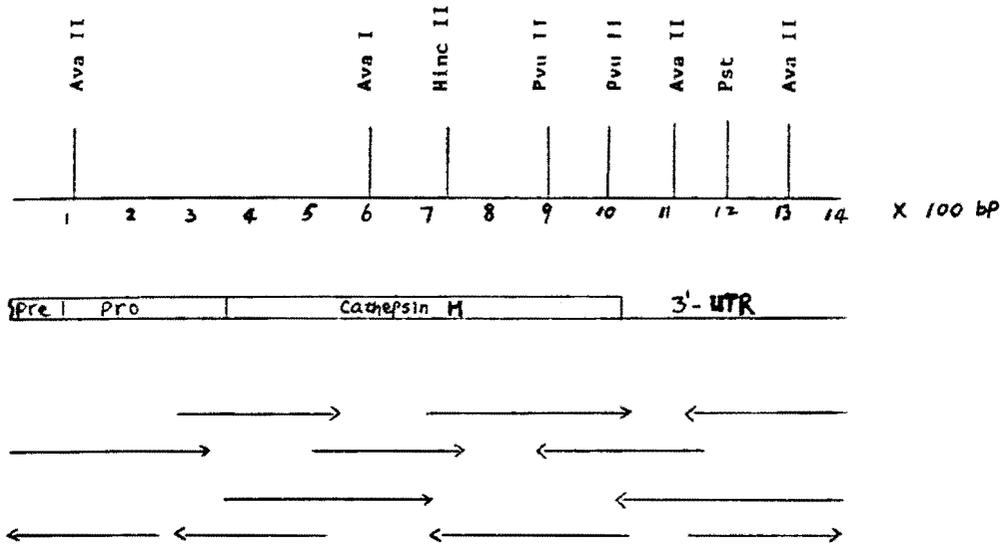
To identify the cathepsin H cDNA mixed chemically synthesized DNA probes were designed on the basis of the amino acid sequence of the mature enzyme. The oligonucleotides were synthesized corresponding to residues F-136 to K-148 of rat cathepsin (Takio et al, 1983). This sequence was chosen due to its low degeneracy. A 20 mer and 27 mer were synthesized as following:

```

                C   A
20 mer: TTTGCCTTTGAGGTCACCGA
27 mer:                CAGTGGCTTCTAAAATACTACATATTT
                        C   G   G           G   C
```

The two strands produce an 8 bp overlap in middle of the sequence. The 20 mer and 27 mer were then annealed and filled in with dNTP and  $\alpha$ -<sup>32</sup>P-dCTP using DNA polymerase (klenow) after first being r-<sup>32</sup>P-labeled with ATP using T4 Polynucleotide kinase. Since they were double labeled, the probes had very high specific activity ( $10^{10}$  cpm/ $\mu$ g). Five clones were

Figure 8. Restriction Map of Rat Cathepsin H cDNA (RHC11)



The open boxes indicate coding region including partial prepeptide (pre), total propeptide (pro) and total mature protein (cathepsin H). 3'-untranslated region was indicated as 3'-UTR. The restriction sites were indicated as shown. Arrow lines show the strategy used to determine the nucleotide sequence.

Isolated after screening  $10^6$  plaques. They partially overlap to each other. The restriction map of the longest clone  $\lambda$ RHC11 (Fig. 8) shows that the insert is 1.4 Kb long. Sequence analysis disclosed the presence of a contiguous open reading frame extending beyond the amino terminus of mature cathepsin H. This indicates that as expected, mature cathepsin H is contained within a larger precursor protein, which includes pre- and pro-peptides at the N-terminus. But unlike cathepsin B, there is no carboxyl-terminal extension in cathepsin H (Fig. 9). Comparison of the sequence and the mixed oligonucleotide probes (39 mer) designed for screening showed a perfect match except for two base pairs. On Northern blot analysis of 10 different rat tissues (Fig. 10, lane 3-13) the CRH11 insert specifically hybridizes to an mRNA of 1.6 kb, which is a little larger than the RCH11 1.4 kb cDNA insert (Fig. 10, Lane 1). A summary of characteristics of rat pro- and mature cathepsin H is shown in Table 4.

#### Expression of Cathepsin H mRNA in 10 Normal Rat Tissues

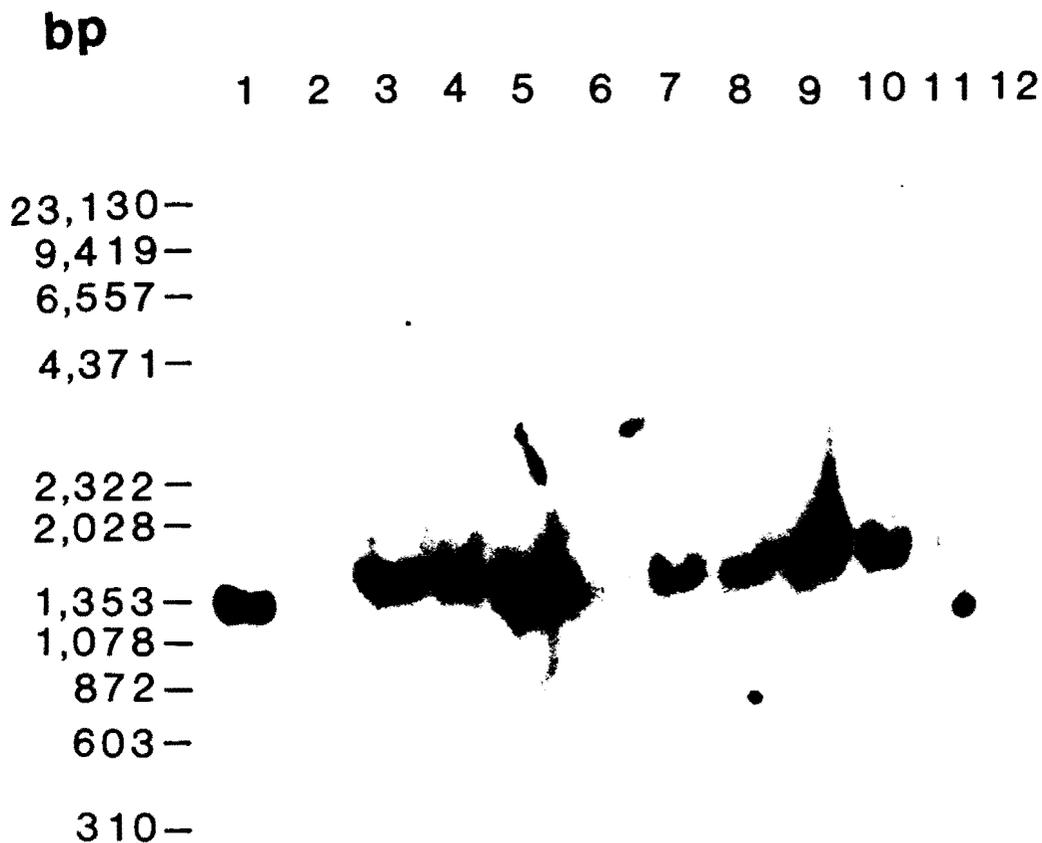
To further quantify cathepsin H mRNA in different tissues, RNA dot blot was performed with RCH11 1.4 bp insert as probe. The dot blot results are shown in Fig. 11a. The experiment was repeated three times. The yeast tRNA was used as a negative control. To rule out the possibility of cross reaction between cathepsin H and other cathepsins, all five cathepsin cDNAs were dotted onto filters in the same doses

Table 4. Summary of Characteristics of RAT Pro- and Mature  
Cathepsin H

	<u>PROCATHEPSIN H</u>	<u>CATHEPSIN H</u>
Amino Acid Residues	313	220
Molecular weight	35,078 daltons	24,001 daltons
Cysteines	9	8
Isoelectric point, pI	8.47	6.57
Potential glycosylation sites	3	1
Predicted secondary structure (CHOU-FASMAN)	51% helix 66% extended 47% turn	51% helix 68% extended 52% turn



Figure 10. Northern Blot of Total RNA from Rat Tissues.  
with Cathepsin H cDNA as Probe



The amount of RNA applied was 20  $\mu$ g per well. Hybridization was performed with a  $^{32}$ P-labeled mouse cathepsin H insert. RNA was isolated from rat liver (lane 3), spleen (lane 4), kidney (lane 5), pancreas (lane 6), heart (lane 7), testis (lane 8), lung (lane 9), uterus (lane 10), brain (lane 11) and thymus (lane 12). Lane 1 is cathepsin H cDNA (RCH11, 5 ng). Sizes of DNA molecular markers are indicated on the left side as base pairs.

(0.1 ng). The results showed only cathepsin H cDNA had a positive signal, indicating that the probe is very specific. There is no any cross hybridization between cathepsin H and other cathepsins. Dot blot analysis showed kidney had highest cathepsin H mRNA level, in contrast, pancreas had very low level of cathepsin H. The distribution of the mRNA was quite uneven in various tissues. There was an over 50 fold variation in cathepsin H mRNA levels among the 10 rat tissues measured by densitometry scanning.

To compare the tissue distribution of cathepsin H mRNA with the other four cathepsin mRNAs, RNA dot blot analyses were also performed with cathepsin B, D, L and S cDNA probes (Fig. 11B-F). The comparison revealed a common feature in distribution of the five cathepsin mRNA: All of them had relatively high mRNA level in the lung and/or kidney while low level in the pancreas. On the other hand, there were marked differences in the distribution of the five cathepsin mRNAs. For instance, spleen contained relatively high level of cathepsin B, D, H and S, but very low level of cathepsin L (almost undetectable). Heart contained considerable level of cathepsin B, D and S, but very low level of cathepsin H and L. In contrast, liver contains considerable cathepsin H and L but very low level of cathepsin B, D and S. Table 5 summarizes the relative levels of the five cathepsin mRNAs.

### Expression of Five Cathepsin mRNAs in Rat Walker-256 Carcinoma

Five cathepsin mRNA expression in Rat Walker-256 carcinoma was examined. Since kidney and/or lung have relatively higher levels of all five cathepsins, they were used as control tissues. RNA dot blot experiments were performed and the densities of dots were scanned by densitometry. The results are shown in Fig. 12. The results revealed among five cathepsins, only cathepsin B had elevated expression compared to the kidney (over 2 fold).

### Expression of Cathepsin mRNAs in Murine Normal Tissues and Melanoma Variants

Agarose gel electrophoresis of total RNA isolated from eight mouse normal tissue and three murine melanoma variants was performed following denaturation with glyoxal and dimethylsulfoxide. The integrity of the 18S and 28S ribosomal RNA indicated that the isolated RNA was largely intact. These RNAs were used in dot blot experiments.

Figure 13 shows the results by densitometry scans obtained after dot blot analysis of total RNA isolated from normal mouse tissues and a yeast tRNA standard which serves as a negative control using cDNAs of cathepsin B, D, H, L and S as probes, respectively. It is evident that the five lysosomal cathepsin mRNAs are very differently distributed among the normal mouse tissues. Kidney shows the highest

Figure 11. RNA Dot Blot Hybridization Analysis with cDNA  
Probes for Cathepsin H, B, D, L and S

The different rat tissues from which total RNA was isolated are indicated on the top of the figure. The RNA doses used varied from 0.05  $\mu$ g to 5.0  $\mu$ g. To verify the specificity of the probes, the cDNA inserts for all five cathepsins were applied to the right hand land of each filter.

Figure 11. RNA Dot Blot Hybridization Analysis with cDNA Probes for Cathepsin H, B, D, L and S

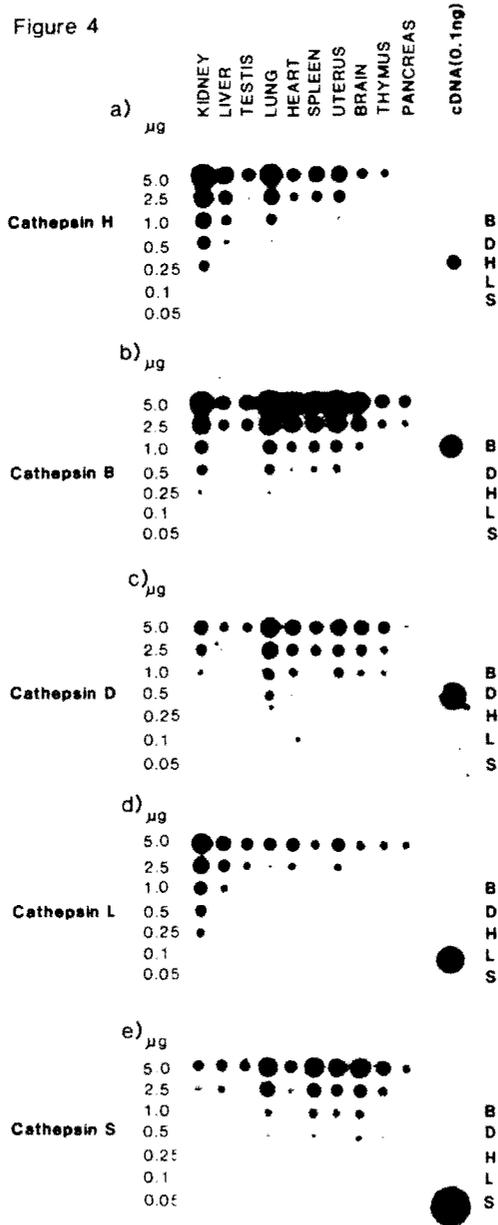


Table 5. Relative Levels of Cathepsin mRNAs in Rat Tissues

Tissue	H	B	D	L	S
Kidney	100 (100)	100	100	100	100
Lung	95 (38)	97	169	44	495
Liver	77 (40)	43	27	57	115
Uterus	73	88	99	47	470
Spleen	67 (34)	83	60	9	500
Heart	27 (6)	72	99	53	41
Testis	23 (4)	44	36	40	90
Brain	12 (2)	75	89	11	480
Thymus	7	35	41	10	134
Pancreas	2	30	10	9	20

mRNA levels of various lysosomal cathepsins were determined by dot blot analysis of total RNA (up to 5  $\mu$ g). Following autoradiography, the intensity of the dot signals was quantified by densitometry and the results were normalized to kidney as 100%. The numbers in brackets are relative levels of immunoreactive cathepsin H (Kominami et al, 1985)

Figure 12. Relative mRNA Levels of Five Cathepsins in Rat  
Lung, Kidney and W-256 Carcinosarcoma

Total RNA was extracted from the tissues and tumor, dot blotted onto nitrocellulose filters and hybridized with specific cDNA probe as indicated in each panel. The intensities of hybridization signals were determined by densitometry and are given in arbitrary units. In each case, the corresponding lung cathepsin mRNA level was set to 10.

Figure 12. Relative mRNA Levels of Five Cathepsins in Rat Lung, Kidney and W-256 Carcinosarcoma

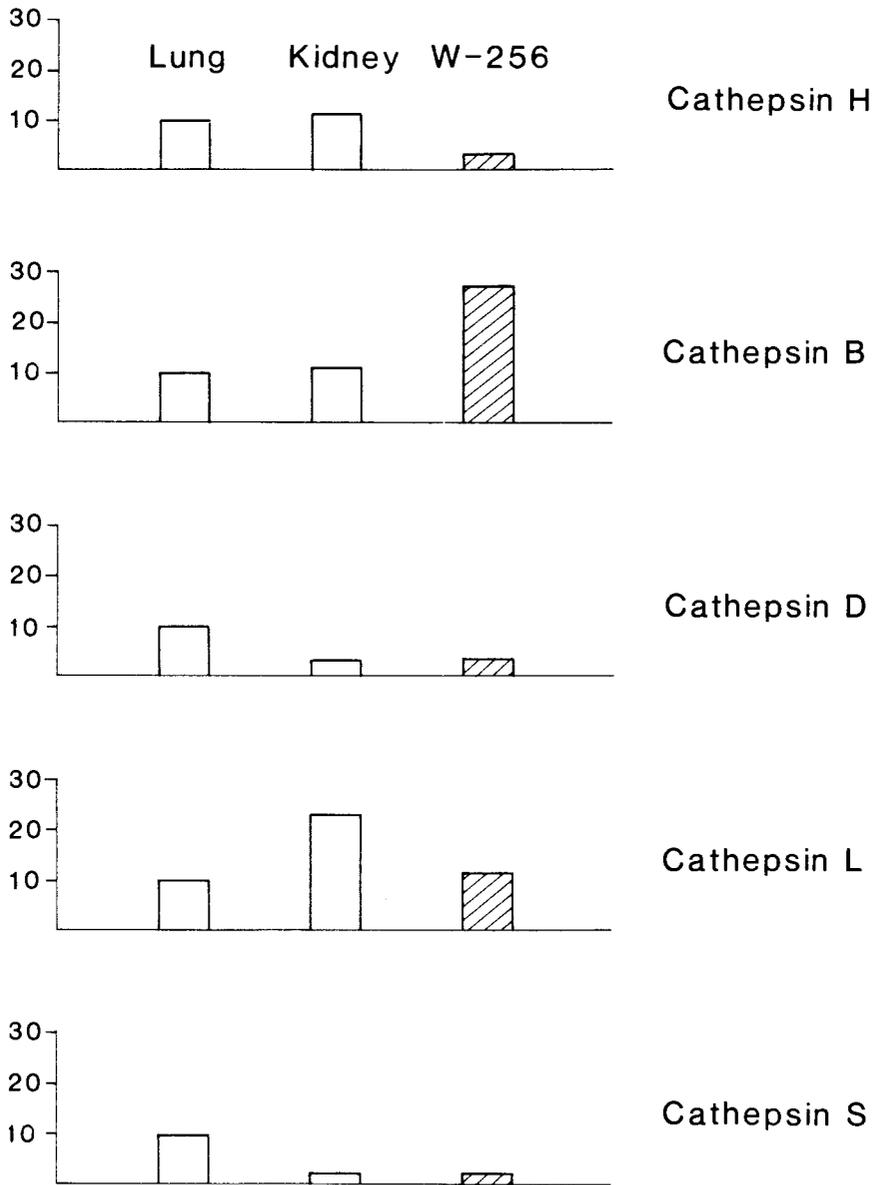
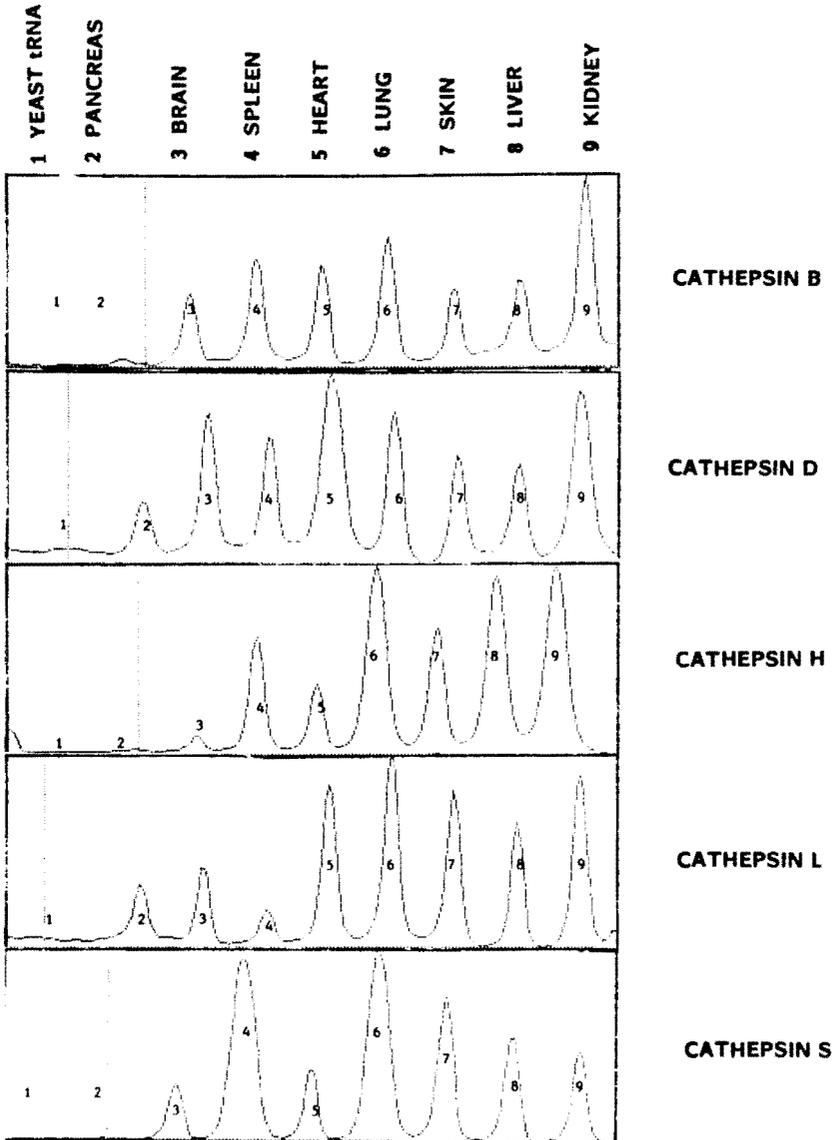


Figure 13. Relative Levels of mRNA for Five Lysosomal  
Cathepsins in Normal Mouse Tissues

Up to 10  $\mu$ g of total RNA isolated from normal tissues was denatured and spotted onto a nitrocellulose filter. After hybridization under high stringency conditions with nick-translated cDNA inserts, the washed filters were autoradiographed. Each panel is result of scanning the dot signals with densitometry. Cathepsin cDNA probes used for hybridization are indicated on right side.

Figure 13. Relative Levels of mRNA for Five Lysosomal  
Cathepsins in Normal Mouse Tissues



expression of cathepsin B and cathepsin H mRNAs, heart has the highest level of cathepsin D mRNA, and lung shows the highest levels of cathepsin L and cathepsin S mRNAs. Pancreas has significant levels of cathepsins D and L mRNAs and barely detectable levels of cathepsin B, H, and S mRNAs.

The metastatic potential of three murine variants (B16a, B16F10 and B16F1) are quite different as shown in Table 6.

Figure 14a shows the dot blot results of cathepsin B mRNA levels in the solid tumors of the three melanoma variants. Cathepsin B mRNA level in B16a was about 6 times higher than B16-F1 and 4 times higher than B16-F10. Because physical inspection of the tumors produced by the three melanomas revealed differences in their texture and appearance, I am concerned that variations in cathepsin B levels could reflect differences in vascularization and infiltration by inflammatory cells such as macrophages. However, Figure 14b reveals that this is not the case, as identical results were also obtained using secondary cultures of the three melanoma variants. The same RNAs were used in Figure 14c and a chicken actin was used as the hybridization probe, the result indicates the amounts of the total RNA on the filter for each variant of the melanomas are the same. When the other four cathepsin (D, H, L and S) cDNAs were used as probes, their expression levels showed no significant differences among these three variants (Fig. 15). Figure 16 shows the densitometry result of cathepsin B expression in three

variants and normal mouse kidney and liver.

A summary of relative mRNA levels of the five cathepsins in the three melanoma variants is shown in Figure 17. The results were obtained from densitometric scans after a similar dot blot analysis of total RNA from secondary cultures of the three melanoma variants. Only cathepsin B mRNA levels are greatly increased in the highly metastatic B16a subcutaneous tumors, whereas the other four lysosomal proteinases show no differences in mRNA expression level among the three melanoma variants.

Table 7 summarizes the relative levels of cathepsin B mRNA in the eight normal mouse tissues and in the three B16 melanoma variants normalized to kidney as 100%. Compared to kidney, which was the tissue possessing the highest level of cathepsin B among the eight normal mouse tissues studied, the cathepsin B mRNA level in B16a was 3.6 fold higher.

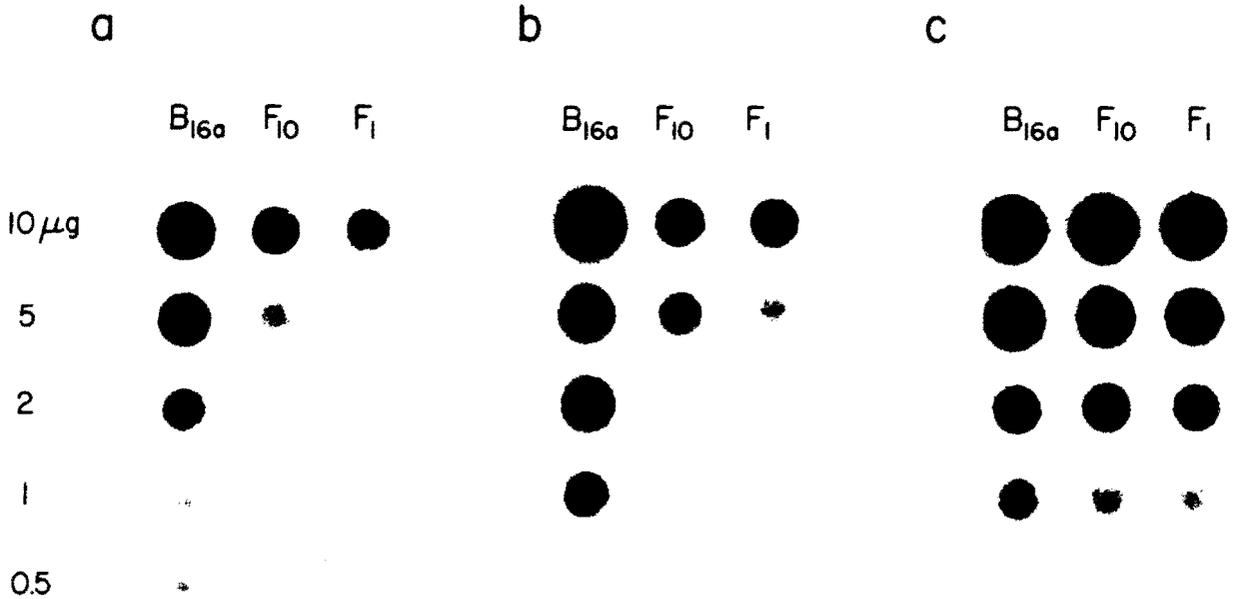
#### Northern Blot Analysis of Cathepsin B mRNA in Normal Mouse tissues and B16 Melanoma Variants

Northern blot analyses of total RNA and poly(A)<sup>+</sup> mRNA from normal mouse tissues revealed a single 2.2 kb band. However the three melanoma variants, in addition to the 2.2 kb transcript, expressed another two mRNA transcripts, 4 kb and 5 kb, which hybridized with the probe under high stringency wash conditions. A typical blot result is shown in Figure 18. The ratios of 5 kb, 4 kb and 2.2 kb transcripts

Table 6. Assay of Metastatic Potential in  
Murine Melanoma Variants

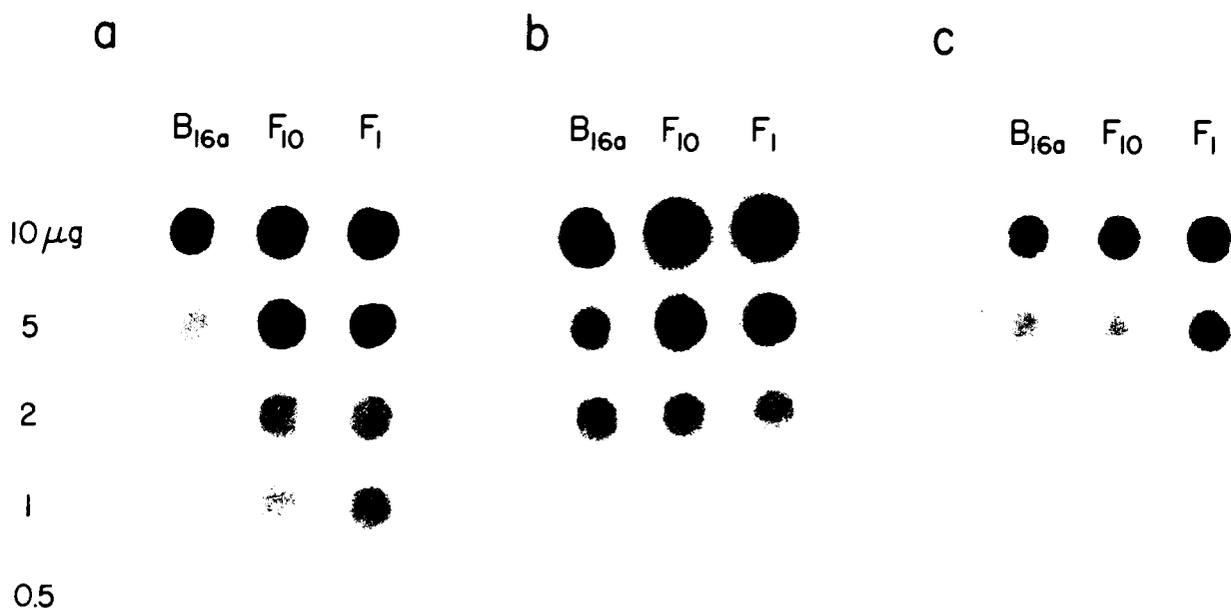
MELANOMA VARIANTS	COLONIES S. C.	COLONIES I. V.
F <sub>1</sub>	0-1	10-30
F <sub>10</sub>	2-3	50-150
B <sub>16a</sub>	100+	300+

Figure 14. RNA Dot Blot Hybridization Analysis of Three B16 Melanoma Variants with Mouse Cathepsin B cDNA



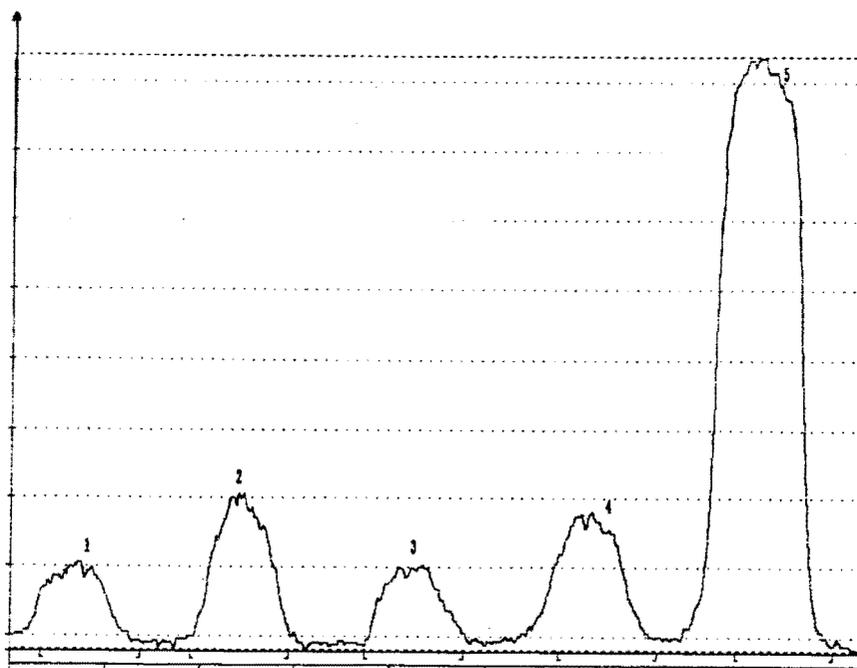
Total RNAs were isolated from solid tumors (panel a) and secondary cultured cells (panel b and c) of three melanoma variants. Panel a and b were hybridized with mouse cathepsin B cDNA as probe. Panel c was hybridized with chicken actin as probe. Dot blot hybridization and autoradiography are described in the Chapter III.

Figure 15. RNA dot Blot Hybridization Analysis of Three B16 Melanoma Variants with Cathepsin D, H and L cDNAs



Total RNAs were isolated from secondary cultured cells of three melanoma variants. Panel a was hybridized with a rat cathepsin H cDNA probe; panel b was hybridized with a human cathepsin D cDNA prober; and panel C was hybridized with a mouse cathepsin L cDNA probe.

Figure 16. Relative Level of mRNA for Cathepsin B in Murine Melanoma Variants and Normal Mouse Kidney and Liver



Total RNAs were isolated from melanomas B16a (peak 5), B16F10 (peak 4), B16F1 (peak 3), and normal kidney (peak 2) and liver (peak 1). Up to 10  $\mu\text{g}$  of total RNA isolated from each tissue was denatured and spotted onto a nitrocellulose filter. After hybridization under high stringency condition with nick-translated mouse cathepsin B cDNA insert, the washed filter was autoradiographed and the film was scanned with densitometer.

Figure 17. Relative mRNA Levels of Five Lysosomal Cathepsins  
in Secondary Cultured Cells from Three Murine  
Melanoma Variants

Total RNA was extracted from the cells, dot-blotted onto nitrocellulose filters and hybridized with specific cDNA probe as indicated in each panel. The intensities of the hybridization signals were determined by densitometry as described in the Chapter III and are given in arbitrary units.

Figure 17. Relative mRNA Levels of Five Lysosomal Cathepsins in Secondary Cultured Cells from Three Murine Melanoma Variants

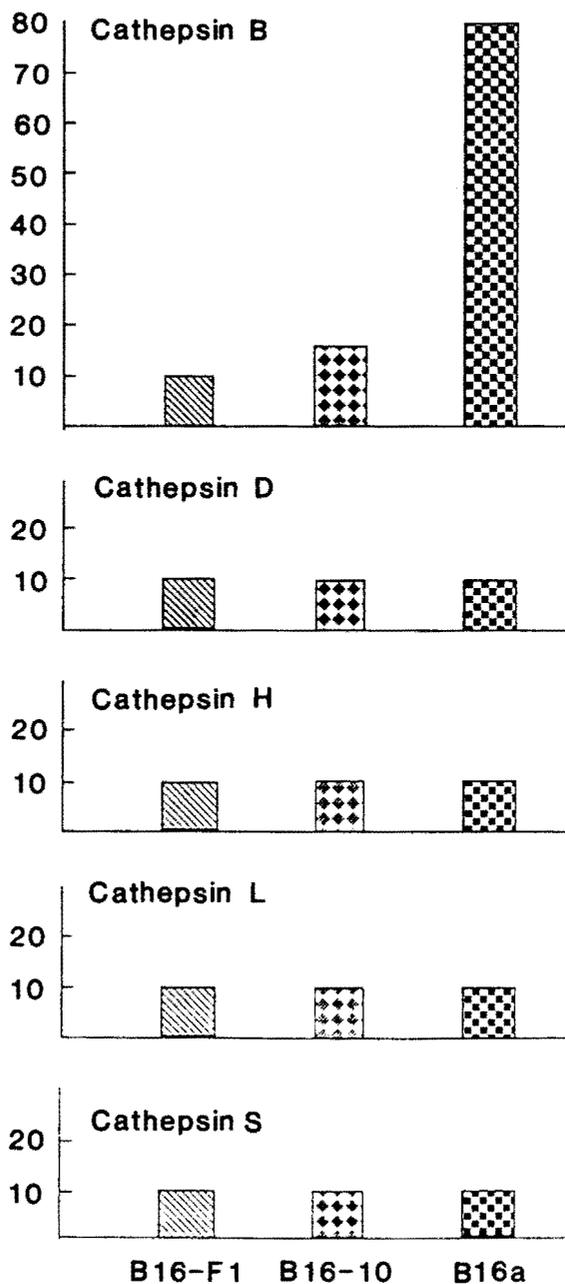
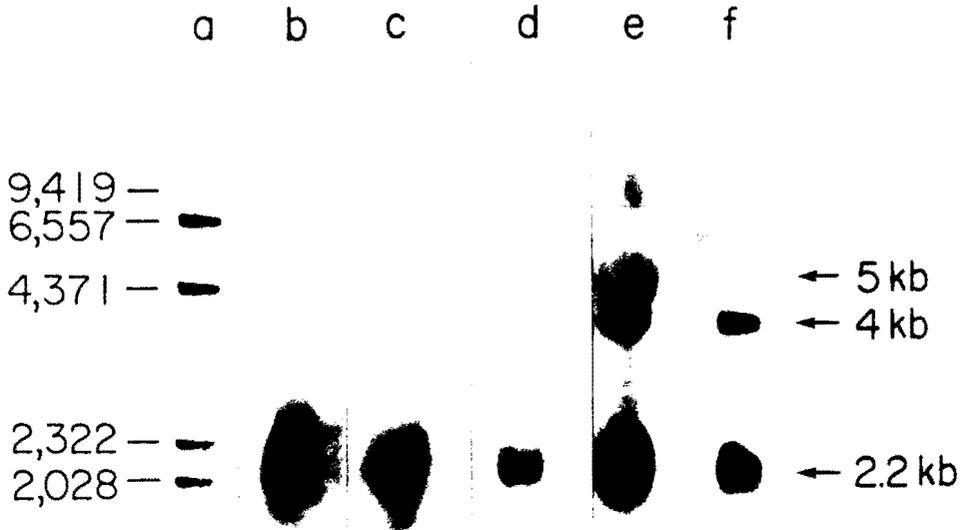


Table 7. Relative Cathepsin mRNA Levels in Normal Murine Tissues and in Solid Tumors Derived from Murine Melanoma Variants

Tissues	Relative Cathepsin B mRNA Levels (100%)
<u>Normal Organs</u>	
Kidney	100
Lung	59
Spleen	50
Liver	48
Heart	44
Skin	38
Brain	37
Pancreas	6
<u>Melanoma Variants</u>	
B16a	362
B16-F10	82
B16-F1	53

mRNA levels of the various lysosomal cathepsins were determined by dot blot analysis of total RNA (up to 10  $\mu$ g). The intensity of the dot signals were quantified by densitometry and the results normalized to kidney as 100%.

Figure 18. Northern Blot of Normal Mouse Tissues and B16a Melanoma with Mouse Cathepsin B cDNA



564 -

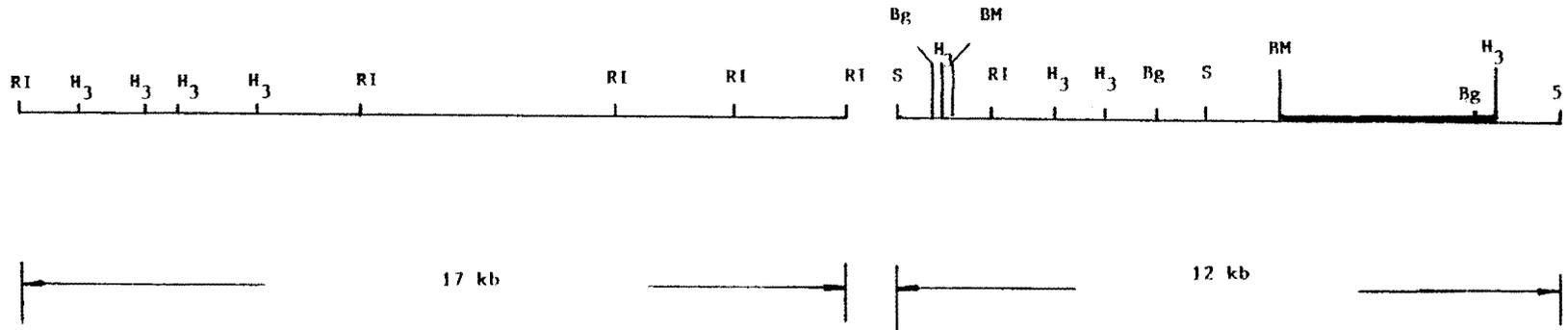
Total RNAs are isolated from murine kidney (lane b, 20  $\mu$ g), spleen (lane c, 40  $\mu$ g), liver (lane d, 20  $\mu$ g), B16a melanoma (lane e, 10  $\mu$ g) and poly(A)<sup>+</sup> mRNA is from B16a melanoma (lane f, 2  $\mu$ g). Hind III fragments of DNA labeled with <sup>32</sup>P and denatured with glyoxal and dimethylsulfoxide were used as standards (lane a). Hybridization was performed with <sup>32</sup>P-labeled mouse cathepsin B cDNA insert.

were approximately 1:10:40. The two larger transcripts were not evident in any of the eight normal tissues surveyed in mice even with prolonged exposure.

#### Molecular Cloning of Multiple Cathepsin B mRNA in B16a Melanoma

A 1,200 bp RI fragment of cDNA which encodes for the mouse preprocathepsin B sequence (Chan et al, 1986) was first used as a probe to screen the melanoma cDNA library. 22 positive clones were detected after screened 50,000 plaques. Analysis and sequence of these clones showed all these clones failed to give additional information beyond the normal mouse cathepsin B cDNA (Chan et al, 1986). Screening a further size fractionated cDNA (above 2.2 kb) library was also unsuccessful. RNA blot showed the three transcripts (2.2, 4.0 and 5.0 kb) could be detected by any portions of known mouse cathepsin B cDNA as probes (data not shown). In order to obtain a specific probe which would help to clone the large sized cathepsin B transcripts, I cloned the mouse cathepsin B gene (Fig. 19) and then performed RNA blots with different portions of the gene as probes. A probe (BH3.8) which detected only 4.0 and 5.0 kb mRNA transcripts on RNA blots was used as a specific probe to screen the cDNA library again (Fig. 20). Six clones were isolated, four of them also hybridized with the 1,200 bp cathepsin B cDNA probe.

Figure 19. The Gene Fragment for Cloning the Larger Transcripts of Mouse Cathepsin B mRNAs



Two mouse cathepsin B gene fragments (17 kb and 12 kb) were digested according to their restriction sites. One fragment (indicated by bold line) which hybridized with only the two larger transcripts (4.0 and 5.0 kb), but not the small one (2.2 kb), was chosen as probe to further screen the melanoma cDNA library. This fragment is 3.8 kb long and has restriction sites-Bam HI and HindIII at its ends, therefore is named BH3.8.

Figure 20. RNA Blot Hybridization Analysis of Mouse B16a  
Melanoma with Gene Fragment as Probe

3  $\mu\text{g}$  of poly(A)<sup>+</sup> RNA per well was denatured, electrophoresed through agarose and transferred onto nitrocellulose filter. Lane 1: filter was hybridized with <sup>32</sup>P-labeled mouse cathepsin B cDNA. Lane 2: filter was hybridized with a <sup>32</sup>P-labeled gene fragment: BH3.8, which is a 3.8 kb Bam HI-Hind III restriction fragment corresponding to a 3'-downstream region of the mouse cathepsin B gene.

Figure 20. RNA Blot Hybridization Analysis of Mouse B16a  
Melanoma with Gene Fragment as Probe

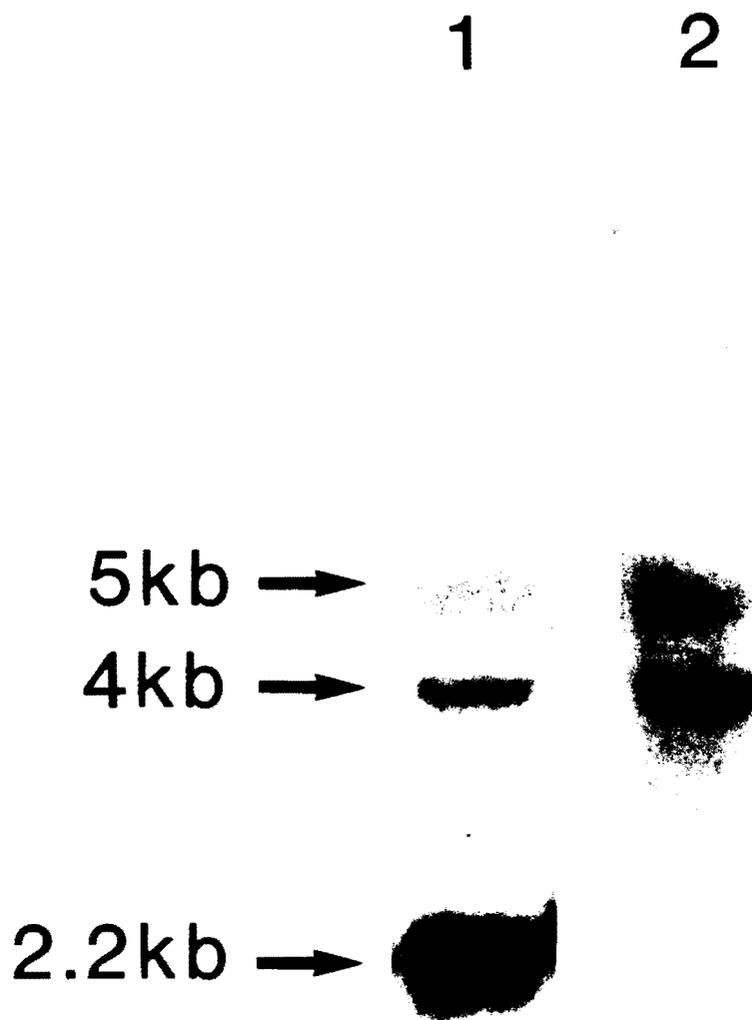
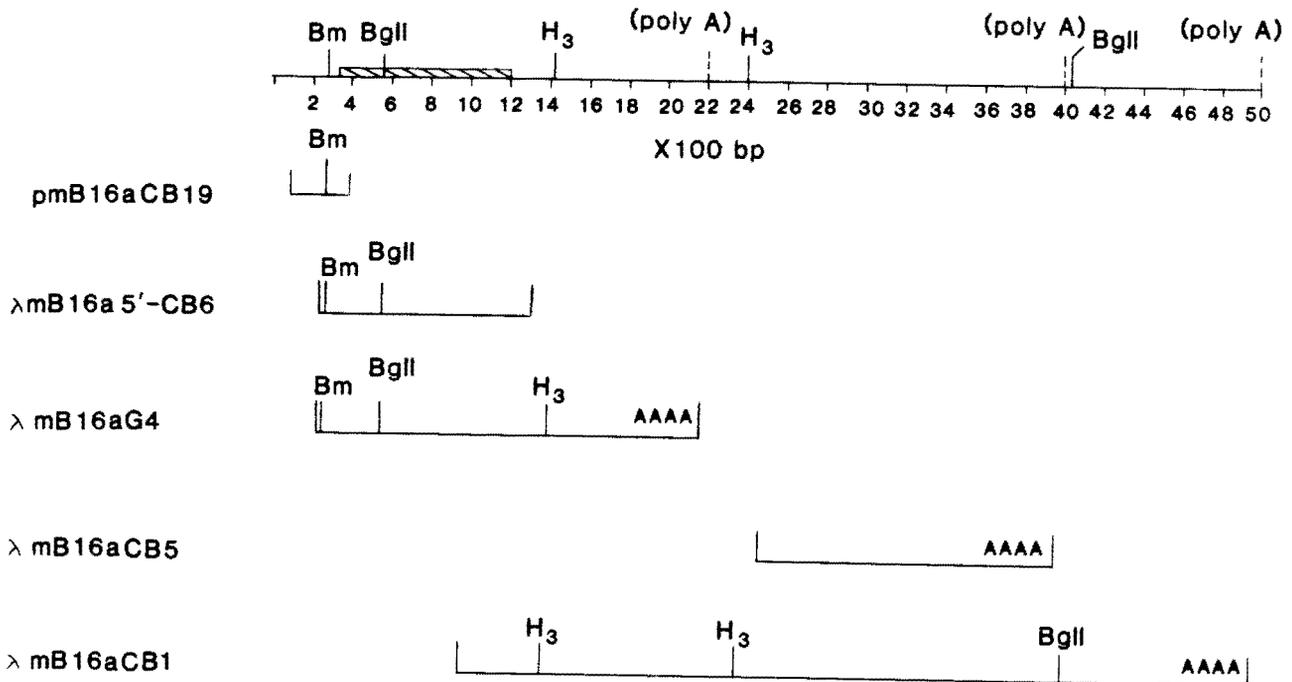


Figure 21. Schematic Map of Mouse B16a Melanoma  
Preprocathepsin B cDNAs



The map was constructed from overlapping clones as shown. The region coding for the cathepsin B precursor is boxed. Restriction sites for Bam HI (Bm), Bgl II (BgII), Hind III (H<sub>3</sub>) as shown. The dotted lines indicate sites where poly(A) tails are added to obtain the 5.0, 4.0 or 2.2 kb transcript. Clones λmB16aG4, λmB16aCB5 and λmB16aCB1, each has a poly(A) tail as indicated by AAAA.

Figure 22. Nucleotide and Predicted Amino Acid Sequences of  
Mouse B16a Melanoma Preprocathepsin B cDNAs

Arrows indicate potential cleavage sites for posttranslational processing. The potential glycosylation sites are underlined. The active site cysteine and histidine residues are circled. The complete 3' untranslated sequences are given for the 2.2, 4.0 and 5.0 kb transcripts. The putative polyadenylation signals are boxed.

Figure 22. Nucleotide and Predicted Amino Acid Sequences of Mouse B16a Melanoma Preprocathepsin B cDNAs

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-125 GGAGGGCGAGGGCGGGGCTACTTAGGAGTGCACGGGAGGGCCAGGCGGCTCGTTGGCTTGGTGGCTGCAGGCCAGGCTGTGGCGCTGGCTCGTGAOTGCAGATCCAGCA

1                               10                               20
Met Trp Trp Ser Leu Ile Leu Leu Ser Cys Leu Leu Ala Leu Thr Ser Ala His Asp Lys Pro Ser Phe His Pro Leu Ser Asp
-6 TCCAGG ATG TGG TGG TCC TTG ATC CTT CTT TCT TGC CTG CTG GCA CTG ACC AGT GCC CAT GAC AAG CCT TCC TTC CAC CCG CTG TCG GAT

30                               40                               50
Asp Leu Ile Asn Tyr Ile Asn Lys Gln Asn Thr Thr Trp Gln Ala Gly Arg Asn Phe Tyr Asn Val Asp Ile Ser Tyr Leu Lys Lys Leu
85 GAC CTG ATT AAC TAT ATC AAC AAA CAG AAT ACA ACA TGG CAG GCT GGA CGC AAC TTC TAC AAT GTT GAC ATA ACC TAT CTG AAG AAG CTG

60                               70                               80
Cys Gly Thr Val Leu Gly Gly Pro Lys Leu Pro Gln Arg Val Ala Phe Gly Gly Asp Ile Asp Leu Thr Pro Glu Thr Phe Asp Ala Arg Glu
175 TGT GGC ACT GTC CTG GGT GCA CCC AAA GGG GTT GCG TTC COT GAG GAC ATA GAT CTA CCT GAA ACC TTT GAT GCA CGG GAA

90                               100                              110
Gln Trp Ser Asn Cys Pro Thr Ile Gly Gln Ile Arg Asp Gln Gly Ser Cys Gly Ser Cys Trp Ala Phe Gly Ala Val Glu Ala Ile Ser
265 CAA TGG TCC AAC TGC CCG ACC ATT GGA CAG ATT AGA GAC CAG GGC TCC TGC GGC TCT CTT TGG GCA GTC GAA GCC ATT TCT

120                              130                              140
Asp Arg Thr Cys Ile His Thr Asn Gly Arg Val Asn Val Glu Val Ser Ala Glu Asp Leu Leu Thr Cys Cys Gly Ile Gln Cys Gly Asp
355 GAC CGA ACC TGC ATT CAC ACC AAT GGC CGA GTC AAC GTG GAG GTG TCT GCT GAA GAC CTG CTT ACT TGC TGT GGT ATC CAG TGT GGG GAC

150                              160                              170
Gly Cys Asn Gly Gly Tyr Pro Ser Gly Ala Trp Ser Phe Trp Thr Lys Lys Gly Leu Val Ser Gly Gly Val Tyr Asn Ser His Val Gly
445 GGC TGT AAT GGT GGC TAT CCC TCT GGA GCA TGG AGC TTC TGG ACA AAA AAA GGC CTG CTT TCA GGT GGA GTC TAC AAT TCT CAT GTA GCG

180                              190                              200
Cys Leu Pro Tyr Thr Ile Pro Pro Cys Glu His His Val Asn Gly Ser Arg Pro Pro Cys Thr Gly Gly Asp Thr Pro Arg Cys Asn
535 TGT TTA CCA TAC ACC ATC CCT CCC TGC GAG CAC CAT GTC AAT GGC TCC COT CCC CCA TGC ACT GGA GAA GGA GAT ACT CCC AGG TGC AAC

210                              220                              230
Lys Ser Cys Glu Ala Gly Tyr Ser Pro Ser Tyr Lys Glu Asp Lys His Phe Gly Tyr Thr Ser Tyr Ser Val Ser Asn Ser Val Lys Glu
625 AAG AGC TGT GAA GCT GGC TAC TCC CCA TCC TAC AAA GAG GAT AAG CAC TTT GGG TAC ACT TCC TAC ACC CTG TCT AAC AGT GTG AAG GAG

240                              250                              260
Ile Met Ala Glu Ile Tyr Lys Asn Gly Pro Val Gln Gly Ala Phe Thr Val Phe Ser Asp Phe Leu Thr Tyr Lys Ser Gly Val Tyr Lys
715 ATC ATG GCA GAA ATC TAC AAA AAT GGC CCA GTG GAG GGT GCC TTC ACT GTG TTT TCT GAC TTC TTG ACT TAC AAA TCA GGA GTA TAC AAG

270                              280                              290
His Glu Ala Gly Asp Met Met Gly Gly His Ala Ile Arg Ile Leu Gly Trp Gly Val Glu Asn Gly Val Pro Tyr Trp Leu Ala Ala Asn
805 CAT GAA GCC GGT GAT ATG ATG GGT GGT GGC CAC GCC ATC CGC ATC CTG GGC TGG GGA GTA GAG AAT GGA GTT CCC TAC TGG CTG GCA GCC AAC

300                              310                              320
Ser Trp Asn Leu Asp Trp Gly Asp Asn Gly Phe Phe Lys Ile Leu Arg Gly Glu Asn His Cys Gly Ile Glu Ser Glu Ile Val Ala Gly
895 TCT TGG AAC CTT GAC TGG GGT GAT AAT GGC TTC TTT AAA ATC CTC AGA GGA GAA AAC CAC TGT GGC ATT GAA TCA GAA ATT GTG GCT GGA

330                              339
Ile Pro Arg Thr Asp Gln Tyr Trp Gly Arg Phe OC
985 ATC CCA CGC ACT GAC CAG TAC TGG GGA AGA TTC TAA TCTGCTTTGACTTTCATGTCCTAGCTCTTGGCCCTTTTTTCAAAATTTAGCGGGCCCTGGCAGGGAATGAGGT

1092 AGACAGGGGAGCTTTTGATTCTTTGACTTTCATCTAACATGCATGAAGCTTTTGGCAAGATTTGGACGACTGGACCAGAGCTGCCTGGCCATCAGAGCTACCCCTCAAACACTGTCTTTACC
1212 TGGCTCTCTCTAGCCCATCCACGACAGTGCATCAGTCTGCATCTAGCCTCTCCCTAGACTAGTGGGTTTGAAGTGCCTGCTTGTGGCTCTGCTGCCATACCTCCACCCCCAT
1332 CCCCATCCAGCATCCGAGGCCAACAGGGCTTTGACTGCAGGACTTCCAAAGAAAGCAATTCACGAGAGGACAAAAGCCACTCTCAGCTGCATCTTGAAGCTGGTACTTCTGGGCTT
1452 GTGGCAGGCAATTCGTCATAGTGGCCACTGCAGTCTCTGAGAAAGCTACCTTTTCCAAAGGCACTGCATCATGCAGACTTGGTAAATGTGGCCAGCTCTCTTGGCTCTGCTCCAGCGG
1572 ATGCTTTTCAATAGGATTTTTATGCTTTGTGTACCTCAACCAACTATGAAGAGCTGTACTGTGTTTTATAGACTCACCCCAATTTGATGGCTTAAAAAAGGCTACATGGTCAATGGTTG
1692 CTGTGTGACCGACAGATAACCAATAAAGTCTAGTCTGAAAAAAAACCTTGTCTTTTGTGTAGTTACACTTAAACCTGTAGTTTAAACAGGAATGTCTGTGCCATATAAACAATTTCT
1812 CCCTCTGCTTGACATTTGCGAGGGCGGCAACCGTTCTGTGCTGCAGTTCGAAGGCTTTTGTCTGTCGCCCTGTTTCTGTCCACCTCAGAAATGGTTCTCAAACCGTACTGTAACTGTG
1932 CACCTCCGGACAGTCTTCCCTGCTTAGAGATAAGGGAGCTCACTGTTTAAATTAACCAAGGCGGGCACTCAGTACATATAGTAAAGGCTTCTTTAGAGCTCTTCTAGAGCTCTTCTGCG
2052 AGTGGGGCCAAAAGCTTCCGACCAATGTTAAGTTTCTGGTACTGATTTTATGATTTTACAAAATATCATAGTGAAGTACAGTTCAAAGGGAACAGGGAGCCTACTCATCAGCG
2172 TTGTCATATGATCTGTACTTCTGCTAAGTGAGAGGGGGGCTGTTTCTCTGGAAGCCCGCTTCCATGTGGCAATCAGAACTATATCAAAGAGCTCTGGATGCTGGCGGAGT
2292 GCTAGAAGTGAATCGTTTGGAGCAACCTGGCTGGGTACTGGGCTTTACTAGTCCAAAGACAGTTCACCGGTCTCAACTACGGCCAAATAGTCCCTCTCAGCTCTCTTTCTGGC
2412 TCACACCATGCCACCTGCAACTCCCTCCCTACCTCCGATAGCCAGTGAAGTGAAGTAAATTAAGAGAAGAGGACCCCTGAACATCTCTGATGGAGCCAGGAAACCGGTTTGTATC
2532 GTATGCTTGGGGTACTCTAGATGTCCTCCATAACAGATTTCTCTGTTGGTGGTTTCTGTGCTCAAGTCAAGAGCTGTCTTCTGCTCATGAAATGGATACTCAGGAGACAGAG
2652 CAAGCCAGTCTCGGAGTGTGGAGCCAGACTGGTCTACAATAGACTTGAAGCTAGGAGCATTTCCTTTCCAGCTGCCACAGGCGCTTTGATGATCTGTGTTTACATCCCTACC
2772 TTTCTACATTAGACTAGATAAGAACCGCTGTGTGTTCTTTGTGTGCTCTCAACCTGAGCCAGTATGATGCCAGGCTTTGAATAGTCGTTTGTAGCCGACTAGGTCATTTAGAT
2892 AGATTTGGGCGATGGCTTCAAACAGGGCTATGACAGACAGCATTCATGCTGCCAGGAACCTATTAGTAAACATGCCAGATCAACTTGGTCAGAAAGTCTTGTCCAGATCATTAGAA
3012 TACAGAACTAGCTTAGCCCTCTCACTTCCATACCACAGAGCTGGGAACTCAACAGCAGGGGAAACCGCTTCAAAGGGCTGTCCAGACAGGATTTGCAAAATACAGAGGAAGTA
3132 CCAAGCAGCATCTTTCTAATAAATATGCTGTATGATGGTGGGCTCTGAGAAGCACACAGATGTGTAAATATATTTAGAGAGTCAAAGCAGGCTCAGGGATGTCTCCATGTGAGATGTT
3252 TGTCCAGCATATTGTCACAGCCCAAGACCTTGGTCCAGTGGCCAGCCAAACCAATAACAGGAGAAAGAGGTGTGTATATGATAGACAGTATGTATAATGTCGACTAAATGTATTT
3372 CTAGATAATGGCTTTATAGAAATGCACCATGTCTAAATATCTCATATAAAAGAAAATCTGAAATTCAGTGGAACTTGGGCTCTATTGCTAAGGAAAATAGTTTCAGGGGEEATG
3492 AGTCTGTCCCTTACCCTTTATATTTATCATGAGTTTATGAGTCTGTCTCATGCATCAGAAAGGAATCGGATCTCTATTATAGATGCTACTAGGCCACTATAGGCCACTATGGGCTTCTG
3612 GAAATTTGACATTTAGCTCTGGAAGAACACTCAGGTCTGAGCCATCTCTCAGCTCAGGTTTGTATGTTAGAGACAGTCTCTTATGATGTCAGGCCCACTCCAACTCAGGAG
3732 GATCTTAAATCAACTCTCCCTCTCCATGCGCTCCCTCCACTCCCATGCTGGGATTAAGAGTTTGTAGTCTCAGAAAGGATGAACTTAAACTCGGTTTCAGGGG
3852 GTGGTATTTGTTCAAGGCTTAGAGCAAGTGAACAAAGTCTCCATGCCCCAGTAACTCAGCAAGGAATCTGTGCTTACAAGTGTGAATGACCACTGAATGCGGAGGCACTCAG
3972 TGAATGAGTCAACATAGCAGGAGGCTTAAGAGGGGAGCAGACTATGCTCTACATGCTCTGTGCTGAGGCTACCCAAAGGTCATATACAGGTTCAAAAATGACAGGTAGCTGAGG
4092 CACTGTTCTCAAAGGGCTCAATGCTAGTCTCCAGAGTTAGGGCTCAGAGACCAAGCTCTGTGGAACAAGTGGTGGCTGTGGCTATAACTGTCATATCAGAAGGCTGAGGCACT
4212 GGGATCAACATAAATCAAGGCTACATAGACTTCCAGGCCAACCTGAGTCAAGTTACAGTGTACAAGTGTGAGATGTCAGATGCCAGGCTCGACTCTGCTCTGTCACGTCTCTCTCT
4332 GCATGCTCTCTCTACTGTAGTGTCAATTTCTCAAGAGGCTCCAAAGGCTAAAGATGGGGCCCCCTCATCTGTACTTCTATCTGCTCTAAGGCTGTACATAAAACATCTCTCTCTCT
4452 TGTAAAGCATGGCCCTCAGTACTTGTCTACAGGCTCAAGAGTGTGGCTAGAGCTCTCCACCGCTCAAATGCTTTCAQATACACAGGTTGTACAGAGGCCACCATGCAATG
4572 AGCCAACTAACCCGCTGATATAAAACCTACCAATATAG - poly A

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Table 8. Difference in Cathepsin B cDNA Sequences derived  
from Murine B16a Melanoma and Macophages

---

B16a Melanoma	Macrophages (Chan et al, 1986)
Gly-143, GGT	Gly-143, GGC
Ser-160, AGC	Asn-160, AAC
Asn-174, AAT	Asp-174, GAT
Val-177, GTA	Ile-177, ATA
Pro-181, CCA	Pro-181, CCG
Pro-197, CCA	Pro-197, CCG
Cyo-198, TGC	Cyo-198, TGT
Gly-200, GGA	Gly-200, GGG
Glu-201, GAA	Glu-201, GAG
Gly-202, GGA	Gly-202, GGG
Pro-205, CCC	Pro-205, CCA
Gly-276, GGG	Gly-276, GGT

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### Restriction Maps and Sequences of Melanoma Cathepsin B cDNAs.

Five representative cathepsin B cDNA clones were mapped with restriction enzymes and sequenced as shown in Figure 21. Among these, two ( $\lambda$ mB16a5'-CB6 and  $\lambda$ mB16aG4) were isolated with the 1.2 kb preprocathepsin B cDNA probe, two ( $\lambda$ mB16aCB5 and  $\lambda$ mB16aCB1) were isolated using the 3' downstream region of the gene, BH3.8 probe and one (pmB16aCB19) was obtained using a PCR approach as described in Chapter III (Materials and Methods). The cDNAs overlap as shown in Figure 21 and the three clones,  $\lambda$ mB16aG4,  $\lambda$ mB16aCB5 and  $\lambda$ mB16aCB1, each has a poly(A) tail over 100 nt in length. However, the adjacent 5' sequences are different, indicating that alternative polyadenylation sites are used. The deduced coding sequence for preprocathepsin B from melanoma clones (Figure 22) was nearly identical to that of mouse macrophage-derived preprocathepsin B cDNA presented by Chan et al (1986), except for the differences noted in Table 8. These nucleotide changes may have been sequencing artifacts in earlier work since I resequenced the macrophage-derived cathepsin B cDNA and found the sequence to be identical to the melanoma-derived cathepsin B cDNA sequence.

Sequence analysis of the 3' downstream regions corresponding to the two large transcripts in clones  $\lambda$ mB16aCB5 and  $\lambda$ mB16aCB1 (Figure 21) failed to detect any contiguous open reading frame beyond the carboxyl terminus of cathepsin B at residue 339. From the results shown in Figures 21 and 22 it

can be determined that the 2.2, 4.0 and 5.0 kb transcripts differ only in the length of their 3' untranslated regions. Moreover, the 3' untranslated sequences of the melanoma-derived preprocathepsin B cDNA were identical and contiguous with the 3' downstream sequence of the cloned mouse cathepsin B gene, thus proving that they were transcribed from a continuous region of the cathepsin B gene and did not arise via alternative splicing. The canonical AATAAA polyadenylation signal was found in the 2.2 kb transcript located 18 nucleotides upstream from the poly(A) tail. Variants of the polyadenylation signal, TATAAA and AATTAA, were observed 12 and 14 nucleotides upstream from the poly(A) sequences in the 4.0 and 5.0 kb transcripts, respectively.

Mapping the 5' End of Cathepsin B mRNAs in normal tissue and B16a melanoma.

The above data demonstrated that the three cathepsin B RNA transcripts found in the B16 melanoma contained alternative 3' untranslated regions. To determine whether these transcripts have the same or different transcription start sites, an RNA protection assay was performed. Figure 23 shows that both B16a Melanoma and murine kidney RNA protected the same size fragment from nuclease digestion and no additional protected fragment is detectable for B16a melanoma compared to kidney. These results indicate that all three cathepsin B mRNA transcripts in B16a melanoma have the

same transcription start site, which is also identical to that for normal tissue derived cathepsin B mRNA. The major protected fragment extends 133 nucleotides upstream from the translational initiation codon for mouse preprocathepsin B.

#### Failure to Detect the large Transcripts of Cathepsin B in Mouse Normal Tissues

A cDNA fragment (Hind III and Bgl II fragment in mB16aCB1 in Fig. 21) unique to the 4.0 and 5.0 kb cathepsin B mRNAs was used as a probe to determine whether small amounts of the larger sized transcripts also occur in normal murine tissues including kidney, which is the normal murine tissue with the highest level of cathepsin B mRNA, liver and spleen. Autoradiography failed to reveal the presence of larger sized cathepsin B mRNA transcripts in any of these normal tissues even with loading large amount of normal tissue RNA (up to 60  $\mu$ g/well) and prolonged exposure of the filter to X-ray film (Figure 24). This result agrees with our earlier failure to detect 4.0 and 5.0 kb cathepsin B mRNAs in normal tissues from mice using the 1.2 kb cathepsin B cDNA probe.

Figure 23. Mapping 5' Ends of Cathepsin B mRNAs  
in Kidney and B16a Melanoma

In vitro synthesized  $^{32}\text{P}$ -cRNA corresponding to the 5' end of the mouse cathepsin B gene was hybridized with 40  $\mu\text{g}$  of either total mouse kidney RNA (Lane 1) or B16a melanoma RNA (Lane 2) and digested with RNase A and RNase T1. The protected fragments were analyzed on a 8% polyacrylamide/8 M urea gel. The sequence ladder in lanes G, A, T and C was obtained by using a primer which has the same 5'-end as that of the protected cRNA with cloned 5'-end mouse cathepsin B gene as template. The arrow indicates the major protected fragments for B16a melanoma and mouse kidney.

Figure 23. Mapping 5' Ends of Cathepsin B mRNAs  
in Kidney and B16a Melanoma

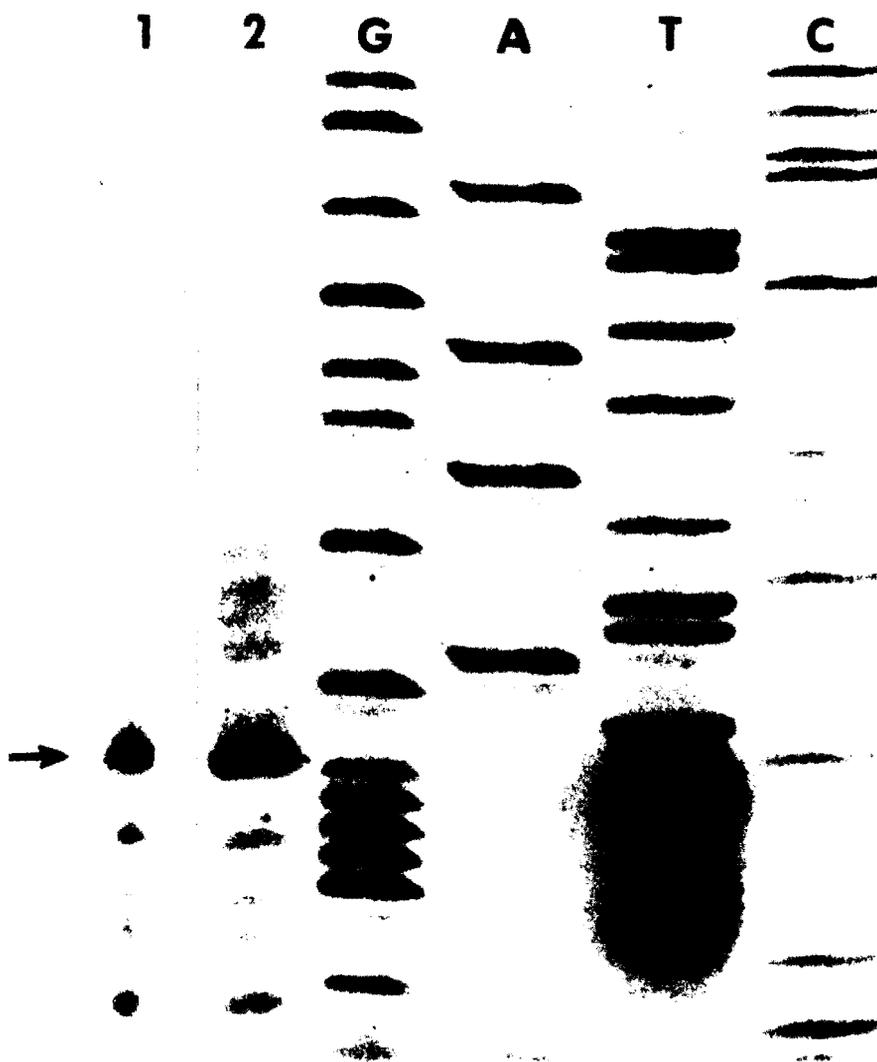
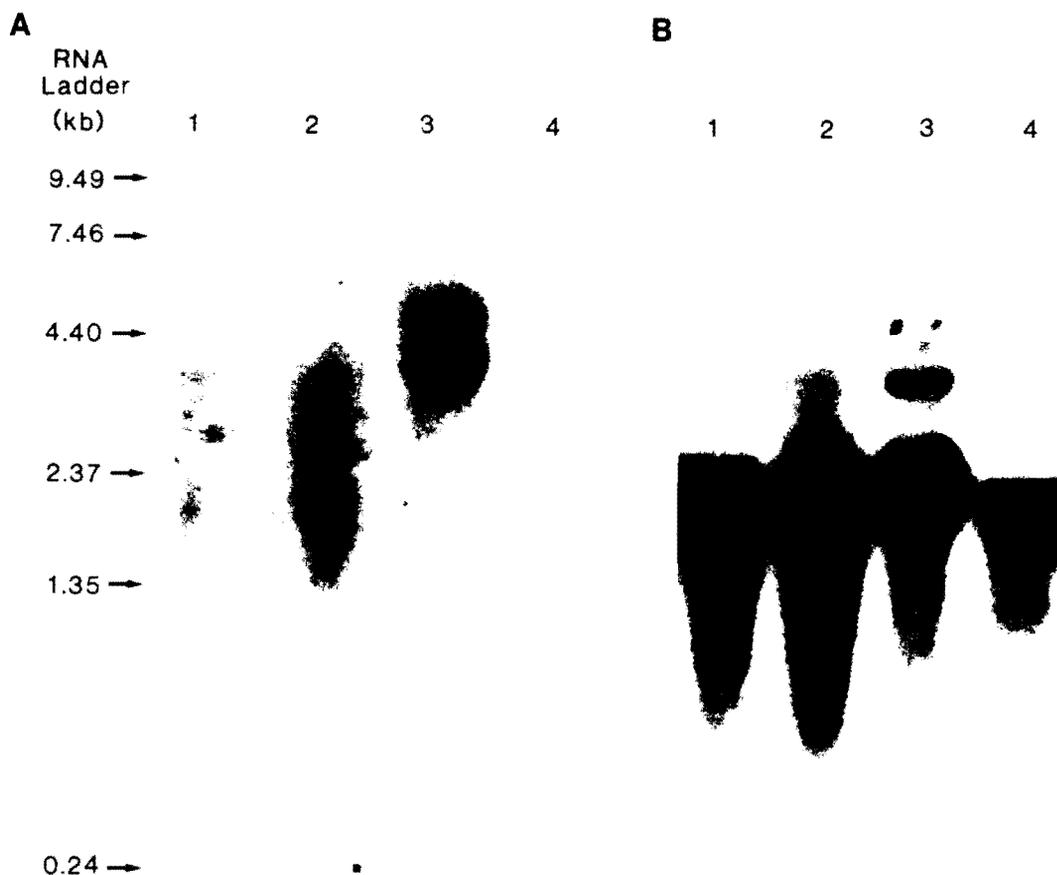


Figure 24. RNA Blot hybridization Analysis with Two Different Mouse Cathepsin B cDNA as Probes



Total RNAs from mouse kidney (lane 1, 30  $\mu$ g), liver (lane 2, 60  $\mu$ g), B16a melanoma (lane 3, 10  $\mu$ g) and spleen (lane 4, 30  $\mu$ g) were denatured, separated by electrophoresis through a 1.2% agarose gel and transferred onto nitrocellulose. Panel A: Blot was hybridized with a 1.6 kb Hind III-Bgl II fragment isolated from  $\lambda$ mB16aCB1 (Figure 21). Panel B: Duplicate blot was hybridized with mouse preprocathepsin B cDNA (Chan et al, 1986). Sizes of RNA ladder molecular markers (BRL) are indicated by the numbers on the left margin.

### Cloning of the Mouse Cathepsin B Gene

Two clones-  $\lambda$ mEGB2 and  $\lambda$ mGB8 (Fig. 25) were identified by screening with mouse cathepsin B cDNA probes. These two clones encode exon 1 through part of exon 7 and part of intron 8 through exon 10, respectively. The rest part of the gene, including the missing portions of exon 7 and intron 8, entire intron 7 and exon 8, was isolated by PCR as described in the Chapter III, the resulting two clones are shown in Fig. 25 as pmGB550 and pmGB700.

The 5'-end of mouse cathepsin B cDNA was cloned using RACE method. The sequence of one of these clones is shown in Fig. 26a. This clone was used as a probe to identify exon 1.

### Transcriptional Start Site and Sequence of 5'-Flanking Region

The nucleotide sequence adjacent to exon 1 is shown in Figure 27. Both RNA protection (Fig. 26c) and primer extension (Fig. 26b) assays generate a major band, indicating the putative principle transcription starting site is 133 nucleotides upstream from the translational start site and eight nucleotides upstream from the 5'-end of the RACE product. The observation of several minor bands in the assays suggests the presence of minor transcription sites. There are three potential Sp1 binding sites in the 5' flanking region. However, there are no TATA and CAAT motifs within 100 nt upstream from the principle transcription start site (Fig. 27). The exon 1 and 5' flanking region is very GC rich.

Figure 25. Restriction Map and Schematic Structure of Mouse Cathepsin B Gene



The exons are indicated by both hatched line boxes (5'- and 3'-end non-coding regions) and solid boxes (coding region). The three parts of the last hatched box indicate the three potential transcription regions. The names of each clones are noted. Restriction sites are indicated: RI (Eco RI), H<sub>3</sub> (Hind III).

## Figure 26. Identification of the Transcription Initiation

## Site of Mouse Cathepsin B Gene

a: Sequence of the 5'-end of cDNA. The clone was obtained with RACE procedure. When compared to the results of primer extension and RNA protection assays, it can be seen that 5'-end of the cDNA is only 8 nucleotides away from the principle transcription start site.

b: Primer Extension. 5  $\mu$ g of mouse kidney total RNA and 1 pmol of the  $^{32}$ P-abeled oligonucleotide complementary to nucleotides 48 to 65 of the mRNA were hybridized and extended with AMV reverse transcriptase. The primer-extended products were separated on a 8% polyacrylamide/8 M urea gel. The right-hand lane shows the primer-extended cDNA. The sequence ladder was obtained by using the same oligonucleotide as a DNA sequencing primer on a 5' upstream fragment of the mouse cathepsin B gene as a template.

c: RNA Protection. A radioactive antisense RNA probe corresponding to a 5'-end fragment of the mouse cathepsin gene was hybridized with mouse kidney total RNA and digested with RNase A and RNase T1. The reaction was analyzed on a 8% polyacrylamide/8 M urea gel. The left-hand lane shows the protected fragment. The sequence ladder was obtained by using an primer which has the same 5'-end as that of  $^{32}$ P-labeled cRNA and a template which is the 5' upstream fragment of the mouse cathepsin B gene.

The arrows indicate the major transcription start site.

Figure 26. Identification of the Transcription Initiation Site of Mouse Cathepsin B Gene

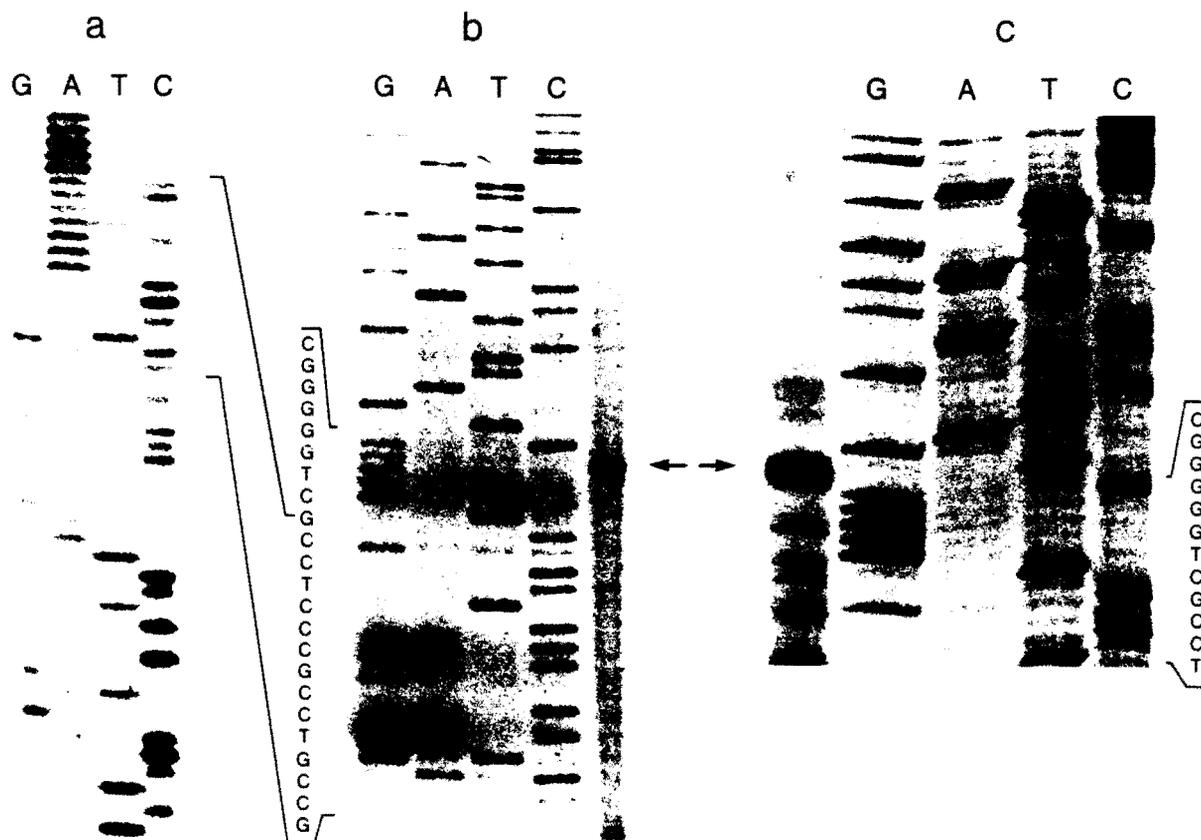


Figure 27. Nucleotide Sequences adjacent the 1st Exon

TTGTAAGTAAGAGCTCTACTTCTGGGCTACAATCCCAGCCCAGTTCTAG  
 TTTTTGGTTGCTTCCCTTGGTTTGAGTATTTCTACGCCATTATAGTCTGA  
 GCTTCAGAATATAAGGCCAGGAGGCTCCTCCAGACATTTCCAGGTCCCGA  
 GCCAGCGGGGCCTTCCCAGGAATGCATGTCACGAAGATGTTGTATCTTC  
 CCAGTGCAGGGTACCTGCAGAGGTCCCTGGAGAGCATCTCTGTGCGAAAT  
 GGAAAGAGAGCAGACAAACCACCAGTTTCTTTTGGGTTTAACACAGCTTG  
 CAGAGTCCCGGGATGAAGGGGGAGGGGCCGGCAGGGGGCGGGGGCGCGGTC  
 AAGCATCACGTGACGAATCA<sup>1</sup>CCCCCAGCGGAGGGCGGACGGCGCGGGTA  
CTTAGGAGTGCACGGGAGAGGCCAGGAGCGGCTCCGTTTGGCTTGGTGGC  
TGCAGGCCCAGGCTGTCGGCTGCGCTCGGTGAGTTTGCCCGGGGACAGT  
 GGCTCCATGGCGGAAGTCACTTTGGCTGGGGTCGGGGGAGCGGGCTGAGC  
 ACCGGTGGTGGAGACA

Exon 1 is boxed. 1 indicates putative transcription start site. Arrowhead indicates the end of cDNA obtained by RACE method. The regions representing potential binding sites for the transcriptional factor Sp1 are underlined.

### Exon-Intron Organization of Mouse Cathepsin B Gene

The ten exons of the mouse cathepsin B gene span about 20 kb. The exon sizes are all about 100 bp long (from 86 to 147 bp) except for exon 10. The intron sizes vary strikingly ranging from 150 bp to about 4,750 bp. The boundary sequences of the exon-intron junctions conform to the GT...AG rule (Breathnach and Chambon, 1981).

Exon 1 encodes most of 5'-untranslated region of the mRNA, exon 2 encodes additional 30 nucleotides of the 5'-UTR (untranslated region), the entire 17 amino acid signal peptide and the first 25 amino acids (aa18-42) of the 62 amino acid propeptide. Exon 3 encodes 29 amino acids (aa43-71) in the propeptide. Exon 4 encodes the remaining 8 amino acids (aa72-79) of the propeptide and the first 30 amino acids (aa80-109) of the mature enzyme. Exons 4-10 encode most of the mature protein (aa110-307). Exon 10 is the largest exon but encodes only 32 amino acids (aa308-339) of the mature protein followed by the translation stop codon and the entire 3'-untranslated sequence. In comparing exon 10 to cathepsin B cDNA sequences cloned from the mouse B16a melanoma, it is evident that the three polyadenylation sites, which are alternatively used in transcription of the cathepsin B mRNAs in the B16 melanoma, are all located in this exon. Thus, the size of exon 10 is either 901, 2,512 or 3,691 bp depending on which polyadenylation site is used. Table 9 shows the summary of the gene organization.

Table 9. Exon-Intron Organization of Mouse Cathepsin B Gene

Exon No.	Exon size bp	<u>Sequence at intron-exon junction</u>		Intron size bp	Amino acid interrupted
		3' splice acceptor	5' splice donor		
1	112		--GGTGAgtttgcccgg----	~4750	
2	147	----tcctttctagGTGAG-----	GGCAGgtaggcacgc----	~570	CAG GCT Glu-42-Ala-43
3	86	----gctctttcagGCTGG-----	GGAAGgtgagtgaca----	~450	AGG Arg-71
4	115	----ttttgectagGGTTG-----	GTTGGgtagacctct----	~750	TGG GCA Trp-109-Ala-110
5	119	----attaccgcagGCATT-----	GACGGgtgagtgttg----	~1500	GGC Gly-149
6	86	----tctttttcagCTGTA-----	TGTAGgtgagtgtgt----	234	GGC Gly-178
7	144	----catctgtcagGCTGC-----	CTTTGgtaagctggg----	~750	GGG Gly-226
8	117	----ctttttacagGGTAC-----	ATCAGgtaccagtca----	~2600	GGA Gly-265
9	129	----gacaaatcagGAGTA-----	TAATGgtgagtggca----	~500	GGC Gly-308
10	901 2,512 3,691	----cccatcccagGCTTC--			

### Southern Blot Analysis of Cathepsin B Gene in Murine Liver and B16a Melanoma

The genomic DNA extracted from both tissues were digested with Eco RI, Bam HI and both enzymes, respectively. The blot was hybridized with labeled mouse cathepsin B cDNA. As the souther blot results showed in Figure 28, there is no difference observed in either the patterns or the densities of the DNA bands between normal liver and B16a melanoma. This result suggests that: (1) there is no gross chromosome rearrangement; (2) there is no amplification of cathepsin B gene in B16a melanoma.

### Half Life of Cathepsin B mRNA

After cells were treated with Actinomycin D for various time, both RNA dot blot (Fig. 29) and Northern blot (Fig. 30) analyses were performed to measure half life of cathepsin B mRNA in NIH3T3 fibroblasts, B16a and B16F1 cells. The calculated half-life of cathepsin B mRNA was ~6 hours for NIH3T3 fibroblast, ~25 hours for B16F1 and ~30 hours for B16a melanoma cells. It is clear that half life of cathepsin B mRNA in B16a melanoma cells is longer than that in B16F1, and much longer than that in the fibroblasts (Fig. 30).

Figure 28. Southern Blot of Genomic DNA From Normal Murine  
Liver and B16a Melanoma

The numbers on the left side indicate molecular weights in base pairs. RI refers to Eco RI. Bam refers to Bam HI. 20  $\mu$ g digested DNA was loaded in each well. Hybridization was performed with labeled mouse cathepsin B cDNA insert.

Figure 28. Southern Blot of Genomic DNA From Normal Murine Liver and B16a Melanoma

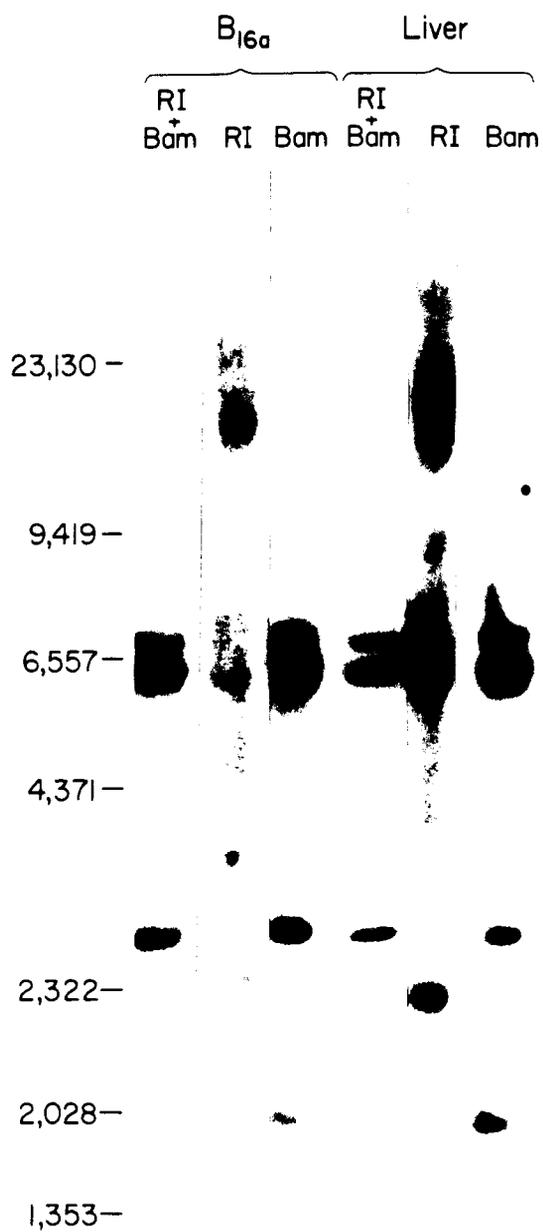


Figure 29. RNA Dot Blot Hybridization Analysis for  
Measurement of Half Life of Cathepsin B mRNA

Total RNAs were extracted from mouse B16a, B16F1 melanoma cells and NIH3T3 fibroblasts which were treated with actinomycin D (5  $\mu$ /ml) for indicated time. For each time point four flask cells were used. The RNAs were denatured and spotted onto nitrocellulose. The blots were hybridized with <sup>32</sup>P-labeled mouse cathepsin B cDNA and autoradiographed.

Figure 29. RNA Dot Blot Hybridization Analysis for  
Measurement of Half Life of Cathepsin B mRNA

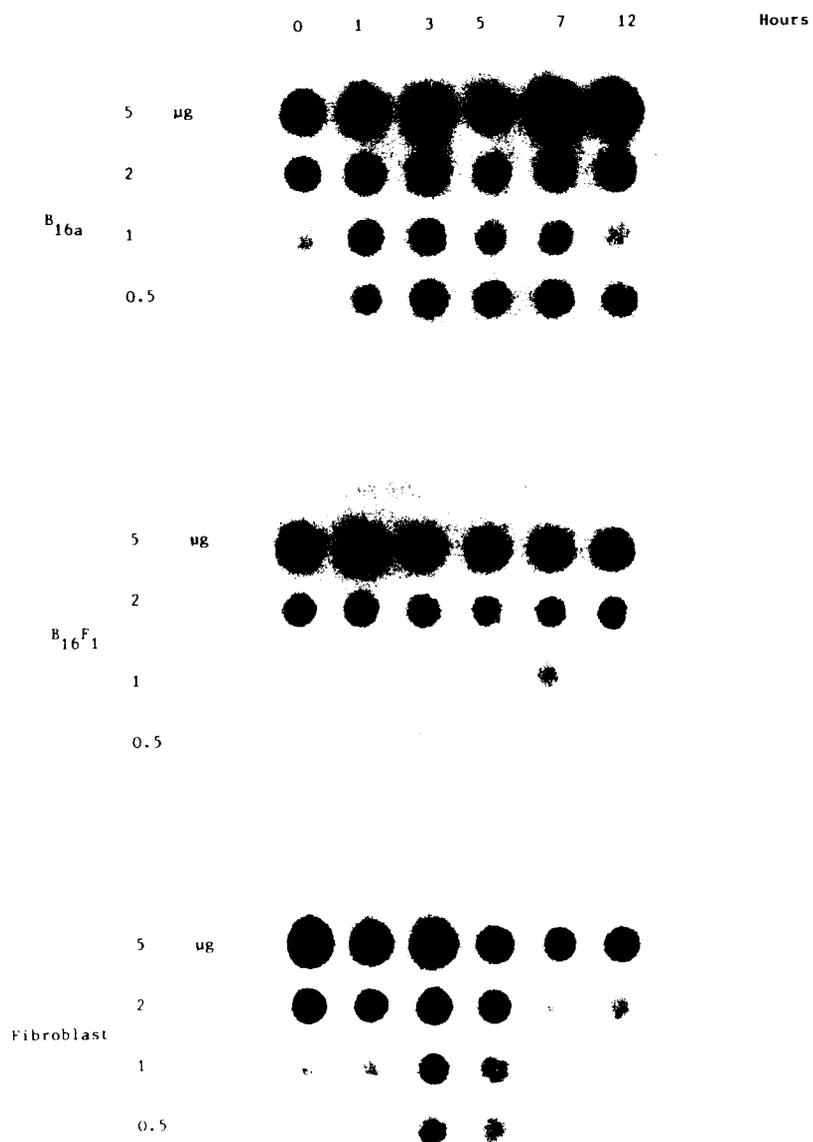


Figure 30. RNA Blot Hybridization Analysis of Mouse B16a  
Melanoma Total RNA

The B16a melanoma cells were treated with actinomycin D (5  $\mu\text{g/ml}$ ) for various length of time from 0 to 12 hours as indicated in each lane. Four dishes were used for each time point. The RNAs extracted from the cells were denatured and separated by electrophoresis through 1.2 % agarose gel. The blot was hybridized with  $^{32}\text{P}$ -labeled mouse cathepsin B cDNA. Arrows indicate cathepsin B mRNA bands with the sizes of 5 kb, 4 kb and 2.2 kb, respectively, from top to bottom.

Figure 30. RNA Blot Hybridization Analysis of Mouse B16a

Melanoma Total RNA

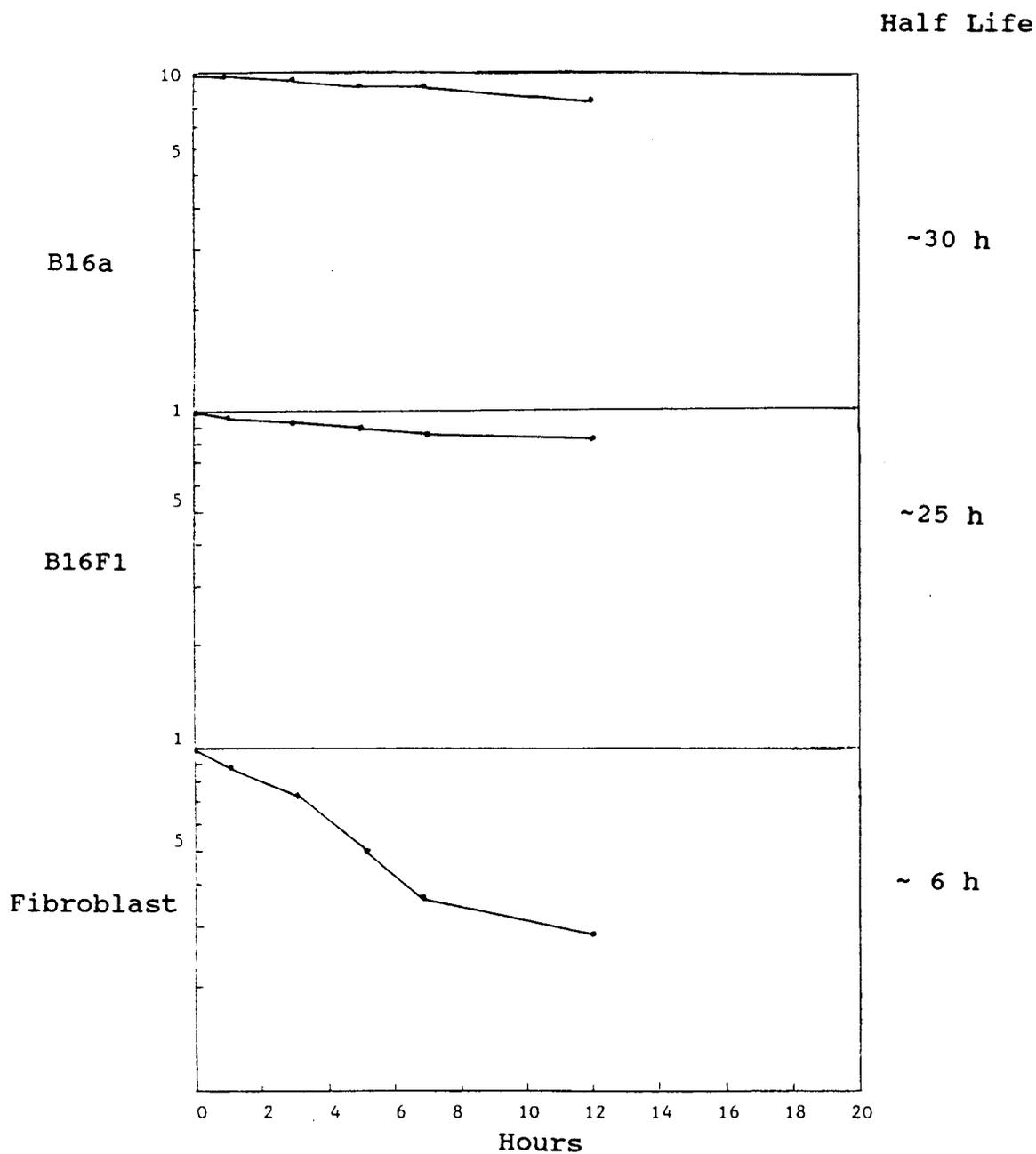
0 3 5 7 12



Figure 31. Half-Life of Cathepsin B mRNA in NIH3T3  
Fibroblasts, B16a and B16F1 Melanoma Cells

Half-life was measured using RNA dot blot results (Figure 29). The autoradiographies were subjected to densitometer scanning analysis. Relative dot intensity readings were plotted as a function of the time of transcription inhibition by actinomycin D. Each time point in the panels was the average of two separated experiments. mRNA half-lives were determined by a least-squares linear regression calculation of the plotted data.

Figure 31. Halfe-Life Of Cathepsin B mRNA in NIH3T3  
Fibroblasts, B16a and B16F1 Melanoma Cells



## CHAPTER V

### DISCUSSION

#### CHARACTERISTICS OF RAT CATHEPSIN H

In order to investigate the expression of the five cathepsins in normal tissues and in malignant tumors, it was necessary to first clone cathepsin H, which is an important member in the cathepsin family and the only cathepsin which had not been cloned at the time when I started this research. The successful cloning of rat cathepsin H cDNA provided not only a cDNA probe, but also better understanding of this enzyme.

While cathepsin B and L are well characterized, much less was known about cathepsin H, especially at the molecular level. Cathepsin H was originally isolated from rat liver (Kirschke et al, 1976), as an approximate 50:50 mixture of a single chain and a two chain form. Both forms are glycosylated and the conversion of the single chain form to the two chain form involves a limited proteolytic cleavage near the carboxyl terminus. The complete amino acid sequence

of the mature enzyme was published in 1983. It contains a total of 220 amino acids (Takio et al, 1983) with a site for glycosyl attachment. The amino acid sequence deduced from the sequence of cloned cDNA indicates that the mature form of cathepsin H is formed from a precursor by limited proteolysis, and that the propeptide additionally contains two putative glycosylation sites. These could play an important role in intracellular protein sorting. The cDNA derived sequence predicts that the two chain form is generated directly by cleavage between residues 177 and 178 without the loss of any intervening residues, which contrasts with cathepsin B where cleavage results in the loss of a dipeptide (San Segundo et al, 1985). Again, unlike cathepsin B, limited proteolytic modification of the C-terminus of cathepsin H does not occur. The deduced amino acid sequence of the mature enzyme coincides completely with that determined by the protein sequencing method. Ishidoh and co-workers (1987) independently cloned rat cathepsin H cDNA almost the same time as I did. My sequence agrees with theirs except for three nucleotides in the 3'-untranslated region. Whether this difference reflects a polymorphism or sequence artifacts remains to be established.

Northern blot analysis of total RNA from ten rat tissues demonstrated the presence of a single, 1.6 kb cathepsin H mRNA. Quantitative study by RNA dot blot disclosed a greater than 50 fold variation in cathepsin H mRNA level between

different tissues. The highest level is observed in kidney while the lowest level is in pancreas. Substantial amounts were also observed in lung, liver, uterus and spleen. In contrast, pancreas, thymus and brain have relatively low concentration of cathepsin H mRNA.

#### CHARACTERISTICS OF EXPRESSION OF FIVE CATHEPSINS IN NORMAL RAT AND MOUSE TISSUES

Although four of the five cathepsins are cysteine proteinases which share some degree of structural and functional homology, no cross-hybridization was observed among the cathepsin cDNAs under the high stringency condition employed in my studies of the expression of five cathepsins in various of tissues. This permitted accurate measurements of relative mRNA levels in different tissues for all five cathepsins. The distribution of five cathepsins shows that they have certain common features: lung has very high levels of mRNAs for all five cathepsins, while pancreas has the lowest mRNA levels for all of them. Kidney has the highest levels of mRNA for cathepsin B, H and L, and intermediate level of cathepsin D mRNA. This is generally in agreement with the reports on the distribution of cathepsin B, H (Kominamal et al, 1985), L (Bando et al, 1986) and D (Whitaker and Rhodes, 1983) in rat tissues by immunochemical assays. On the other hand, the mRNA levels for the five enzymes were distributed in a non-parallel fashion among many of the normal

rat tissues. For example, heart contains significant levels of cathepsin B, D and L but low levels of cathepsin H and S; spleen has high levels of cathepsin S, intermediate level of cathepsin B, D and H whereas low level of cathepsin L. The uneven distribution is also reflected by the fact that there is a 50-fold difference in the cathepsin H mRNA level, 25-fold difference in cathepsin S mRNA level and about 10-fold difference in cathepsin B, D and H mRNA levels across different tissues.

The broad tissue distribution of the five cathepsins agrees with the hypothesis that cathepsins play an important function in intracellular protein degradation. The high level of cathepsins in kidney is entirely consistent with its role in the clearance and degradation of small proteins (Kim et al, 1989; Maack et al, 1979). The ability of alveolar macrophages to degrade abnormal or foreign proteins may similarly account for the presence of high levels of cathepsins in the lung. Similarly, the low levels of cathepsins in the pancreas agree with the finding that this tissue does not contribute significantly to the degradation of circulating plasma proteins (Carew et al, 1982). Most striking is the uneven distribution of cathepsin mRNAs among most of the tissues studied, indicating that their expressions are individually regulated. Consequently, one would expect from such results that the proteolytic enzyme composition of lysosomes varies from organ to organ, presumably reflecting the specialized

physiologies and underlying functional differences between tissues (San Segundo et al, 1986). However, how the lysosomal proteinase content reflects function is not clear, particularly as the roles of individual lysosomal proteinases in protein catabolism and cellular function in vivo are essentially unknown.

#### ENHANCED EXPRESSION OF CATHEPSIN B IN RAT W-256 CARCINOSARCOMA AND IN MOUSE B16 MELANOMAS

A great many reports have indicated elevated cathepsin B (Keren and LeGrue, 1988), D (Maguchi et al, 1988, Maudelonde, 1988) and L (Gal and Gottesman, 1986, Troen et al, 1988) or cathepsin B-like enzymes (Dufek et al, 1984) in different tumor cells. Indeed, elevated proteinase expression has been thought to be a marker of the tumorigenic and/or malignant state (Koppel et al, 1984; Sloane et al, 1981). Most of these experiments used enzymatic or immunochemical methods to detect and quantitate these enzymes. However, using activity measurements to estimate enzyme levels suffer from severe uncertainties concerning the specificity of the substrates. Immunochemical methods may be compromised by differences in the immunoreactivities of precursor and mature forms of the lysosomal enzymes and by the cross-reactivity between closely related cysteine proteinases (Wiederanders and Kirschke, 1986). Consequently, I have employed the cDNAs for five cathepsins to examine their expression in a Walker rat

carcinoma (LLC-WRC 256) and mouse B16 melanomas. I have proven that the DNA probes used in this method is very specific for each cathepsin under high stringency condition. The key word here is "high". Cross reaction among different cathepsins could occur if otherwise low stringency condition is used since the structures of cysteine cathepsins are closely related.

The Walker-256 rat carcinosarcoma arose in a pregnant albino rat in 1928 in the laboratory of John Walker at Johns Hopkins University School of Medicine. Since then it has been one of the most frequently used tumors in cancer research (Rosenoer et al, 1966). Mouse B16 melanomas are a type of skin cancer which can have a high degree of malignancy. The three variants of B16 melanomas were developed from same origin and B16a, B16F10 and B16F1 have high, intermediate and low metastatic potentials (Filder, 1973; Qian et al, 1989).

The measurement of the five cathepsin mRNA levels in Walker-256 carcinosarcoma and B16 melanomas gave consistent results, which revealed that mRNA levels for cathepsins D, H, L and S in these tumors all appeared to fall within the range observed for normal tissues. In contrast, the mRNA levels for cathepsin B in W-256 and B16a tumors are three fold higher than those seen in kidney and lung, the two tissues with the highest expression levels of cathepsin B among the ten normal tissues studied. Considering melanoma is a skin cancer, if we use skin as control B16a is 10 fold, B16F10 is 2.5 fold and

B16F1 is 1.5 fold as high as mouse skin as a result of comparison of their cathepsin B levels. However, a more appropriate control would have been melanocytes if they were available.

These results confirmed the reports of enhanced cathepsin B activities observed in a variety of tumors such as murine fibrosarcomas (Keren and LeGrue, 1988), human liver cancer (Dufek et al, 1984), human ovarian carcinoma (Mort et al, 1981), human genital tract cancer (Pietras and Roberts, 1981), rat pancreatic carcinoma (Koppel et al, 1984), and human (Petrova-Skalkova et al, 1987) and mouse breast carcinoma (Recklies et al, 1982). In addition, my present results are in agreement with the report for human colorectal adenocarcinoma (Kepper et al, 1988), which indicated high levels of cathepsin B and cathepsin B-like proteinase while low levels of cathepsin H and L. The reason for a specific elevation of cathepsin B expression in these tumor cells remains to be established.

#### CORRELATION BETWEEN CATHEPSIN B AND METASTATIC POTENTIAL

The differences among the three melanoma variants are in their abilities to colonize in the lung following intravenous (tail vein) or subcutaneous injection. Assays of metastatic potential based on lung colonization after the intravenous injection indicated that B16-F10 is more metastatic than B16-F1. In contrast, study of metastasis from solid tumors

implanted at a subcutaneous site gave somewhat different results in which neither the B16-F1 or the B16-F10 melanoma cells produced significant numbers of secondary tumors. Similar discrepancies between the two assays of metastatic potential have been observed previously (Gehlsen et al, 1986) and may be attributed to the fact that they measure different steps in the metastatic process. Thus, the subcutaneous assay measures detachment of cells from the primary subcutaneous tumor, invasion of the vasculature (intravasation), arrest in the capillaries in the lung (attachment), invasion through the capillary wall (extravasation), colonization and proliferation. The intravenous assay measures only attachment, extravasation, colonization and proliferation. However, in both assays of metastasis, the B16a cells showed a much higher metastatic activity than either the B16-F1 or B16-F10 cells. Of the 5 cathepsins studied, only the mRNA level of cathepsin B was elevated in the B16a melanoma and the mRNA levels in the three melanoma variants correlated positively with their metastatic potential, especially when measured by subcutaneous injection assay. The ratio of lung colonies formed after subcutaneous injection of B16-F1, B16-F10 and B16a tumor cells was 1:1.5:30 and the corresponding ratio of cathepsin B mRNA levels in B16-F1, B16-F10 and B16a was 1:1.5:7. Moreover, these relative mRNA levels also agree with the levels of latent cathepsin B secreted by the tumor variants (Qian et al, 1989). These results are consistent

with the enzymatic study by Sloane et al (1981) who found that there was a relationship between cathepsin B activity and metastatic potential of B16 melanomas. In contrast, level of cathepsin D and other hydrolytic enzymes showed no relationship with metastatic potential in these cells. The finding that cathepsin B mRNA and the secreted enzyme levels correlated well with metastatic potential based on the subcutaneous assay, but less well when based on the intravenous assay suggested that cathepsin B may have a role in cell detachment (Sylven, 1968) and/or intravasation. This could explain the inability of inhibitors of cathepsin B to block the metastasis of B16-F10 melanoma cells following intravenous injection (Ostrowski et al, 1986). This result is in agreement with the recent report by Sheahan et al (1989) who observed significantly higher cathepsin B activities in colorectal cancer patients with Dukes' A tumors (tumors confined to the bowel wall) than in patients with more advanced tumors (Duke' B, C, or D tumors) and suggested that cathepsin B may play an important role in the early progression of human colorectal carcinoma.

#### ALTERNATIVE TERMINATION OF TRANSCRIPTION

A significant new finding in this present work is the detection in all three B16 murine melanoma variants of two larger cathepsin B mRNA transcripts (4 kb and 5 kb) which were not detected in any of the normal tissues studied. Cloning

and sequencing results revealed that the 4.0 and 5.0 kb transcripts have unusually long 3'-untranslated sequences. The alternative utilizations of polyadenylation signals explain the differences between the three cathepsin B mRNA transcripts (2.0, 4.0, 5.0 kb).

The vast majority of mRNAs in eukaryotic cells are polyadenylated (poly A) at their 3'-ends. Although this modification was observed a number of years ago, the mechanism by which it occurs is only poorly understood. Early experiments (Nevins and Darnell, 1978) with adenovirus late transcription unit revealed approximately equal molar transcription proceeding and following polyadenylic acid addition sites, suggesting that a large pre-mRNA is specifically cleaved to create a 3' end which subsequently undergoes poly A addition. Similar studies with cellular genes yielded similar results (Hofer and Darnell, 1981).

The nucleotide sequence 5'-AAUAAA-3', or a closely related variant of it, has been found approximately 10 to 30 nucleotides upstream of the 3'-ends of all poly A mRNAs studied (Berget, 1984; Proudfoot and Brownlee, 1976). Deletion or point mutation in this signal prevents polyadenylation (Fitzgerald and Shenk, 1981, Higgs et al, 1983). In addition, a sequence located downstream or 3' to the AAUAAA motif was identified by deletion analysis and shown to modulate the efficiency of polyadenylation (Simonsen et al, 1983; Conway et al, 1985; McDevitt et al, 1984). However,

this sequence is less well conserved.

Site-specific polyadenylation clearly requires more than poly(A) polymerase, since this enzyme, when purified, lacks sequence specificity (Edmonds and Winters, 1976). Other proteins have been identified to be involved in site-specific polyadenylation including nuclear RNP particles (Moore and Sharp, 1984 and 1985; Hashimoto and Steity, 1986), a 64 kd nuclear protein (Wilusz and Shenk, 1988) and a cleavage and adenylation factor, CAF (Christofori and Keller, 1988).

There is now evidence that the selection of a poly (A) site can be a regulatory event controlling gene expression when multiple poly (A) sites exist within a single transcription unit. This possibility has been demonstrated in several case, such as the adenovirus late poly (A) sites (Nevins and Wilson, 1981), the immunoglobulin  $\mu$  poly (A) sites (Mather et al, 1984; Yuan et al, 1984), and the calcitonin and CGRP poly(A) sites (Amara et al, 1984). Each of these results suggests the presence of either tissue-specific factors (calcitonin/CGRP) or factors appearing during a developmental pathway (adenovirus and immunoglobulin) that are responsible for selective poly (A) site utilization.

In the case of cathepsin B mRNAs, no mutation or deletion of the AATAAA sequence or modification in the downstream sequences of the AATAAA modify are detected in any of the transcripts. Clearly, the cause of multiple cathepsin B transcripts is not due to mutation or deletion.

Alternative utilization of polyadenylation sites resulting in divergent 3'-untranslated regions has been reported for other tumor mRNAs as well, such as the amylin mRNAs in a human insulinoma (Sanke et al, 1988) and  $\beta_2$ -macroglobulin mRNAs in a mouse teratocarcinoma (Parnes et al, 1983). However this observation is not limited to tumor cells. Multiple mRNA species with distinct 3' untranslated regions have been also found in transcription products in normal cells, including dihydrofolate reductase (DHFR) in mouse liver and cultured mouse cells (Setzer et al, 1980),  $\beta$ -subunit of alcohol dehydrogenase in human liver (Heden et al, 1986) and  $\alpha$ -amylase in both mouse liver and salivary gland tissues (Tosi et al, 1981). The mechanisms for such a phenomenon are unclear. In the present case, since the multiple cathepsin B mRNA transcripts were detected only in the melanomas but not in normal tissues, it is conceivable that the appearance of two novel transcripts is related to the development of the tumor. It is possible during the tumor development process, certain factor or factors appear, which alter the utilization of potential polyadenylation sites. A tumor-type poly(A) polymerase has previously been identified (Rose et al, 1976) and detected in the sera of rats bearing tumors and in some cancer patients, but absent in healthy individuals, cancer patients in remission for several months and hypertensive patients or patients with certain inflammatory diseases (Stetler et al, 1981). The tumor-type

poly(A) polymerase is present even prior to the formation of preneoplastic nodules in the sera of rat fed with hepatocarcinogens (Stetler et al, 1984). Anti-tumor-type poly(A) antibodies can inhibit polyadenylation of Adenovirus L3 pre-mRNA and surprisingly, inhibit the cleavage reaction when it was coupled or uncoupled with polyadenylation (Terns et al, 1989; Jacob et al, 1989). It is possible the tumor-type poly(A) polymerase in concert with other factor(s) play an role in the formation of additional cathepsin B mRNA transcripts in B16 melanomas. The detection of the tumor-type poly(A) polymerase and alternatively polyadenylated mRNAs together might be an unique tumor marker. Further studies are required to validate this possibility.

Study of short lived mRNAs such as c-fos, c-myc and lymphokin mRNAs indicated a major determinant of the rapid turnover of these mRNAs is the multiple copies of AU-rich motifs (consensus sequence UAUUUUAU) common to the 3'UTRs of these mRNAs (Caput et al, 1986; Wilson and Treisman, 1988; Jones and Cole, 1987). Transfer of these segments to the 3'UTR of stable mRNA such as globin mRNA dramatically reduces its stability (Shaw et al, 1986). However, there is no any UAUUUUAU motif in the 3'UTRs of 2.2 and 4.0 kb transcripts and only one copy in the 3'UTR of the 5.0 kb transcript.

Other studies demonstrated Polyadenylation and 3'UTR have significant effects in translation efficiency. The addition of long poly(A) tail increased the rate of lysozyme mRNA

translation in oocytes by 20-fold and chymosin mRNA translation by 10-fold (Drummond et al, 1985). Sequences in the 3'UTR can also influence translation efficiency in vivo. The most striking case is that of the human  $\beta$ -interferon (IFN) mRNA, which is translated very inefficiently in *Xenopus* oocytes. Exchange of the UTR segments between different genes showed that this inhibitory effect lies mainly in the 3' UTR, with only a minor effect of the 5'UTR (Kruys et al, 1987). Some other studies demonstrated that a correlation between 3'-UTR sequences and stability of the mRNA (Caput et al, 1986; Jones and Cole, 1987). The significance of the unusually long 3'UTR in the 4.0 and 5.0 kb cathepsin B mRNAs remains unknown. The possibility of their effects on the stability of the mRNAs or translation of the enzyme needs further investigation.

High molecular weight forms of cathepsin B have been reported in rat islets (Docherty et al, 1983), rat insulinoma (Docherty et al, 1984) and different human and mouse tumors (Recklies et al, 1982a and 1982b; Mort et al, 1984, Dujack et al, 1984). Present data have excluded the possibility that the large molecular forms of cathepsin B are from alternative splicing of cathepsin B mRNA or coding mutation of the gene. Considering the fact that these large molecular forms of cathepsin B can be cleaved in vitro by pepsin to generate a smaller, enzymatically active species (Docherty et al, 1983; Mort et al, 1983), these larger forms are most likely precursor forms of mature cathepsin B resulting from altered

intracellular post-translational processing.

In any event, the differences in length of the three 3'-untranslated regions, 806 bp, 2,417 bp, and 3,596 bp, respectively, are sufficient to account for the size differences observed between the 2.2 kb, 4.0 kb and 5.0 kb cathepsin B transcripts on Northern blots. RNA protection experiments using B16a tumor RNA and mouse kidney RNA indicate that the cathepsin B transcripts in both kidney and B16a melanoma have the same start sites. Thus, the cathepsin B gene promoter seems normal in the B16a tumor and the increased cathepsin B mRNA level is more likely due to increased mRNA stability, up-regulation of an enhancer element in the gene, or the unmasking of a cryptic enhancer.

#### MOUSE CATHEPSIN B GENE STRUCTURE

The mouse cathepsin B gene is comprised of 10 exons and 9 introns that span about 20 kb of DNA. Unlike some other genes, such as insulin receptor (Seino et al, 1989), whose exons encode well defined functional units, mouse cathepsin B gene structure does not correspond well to the units of function. This characteristic was observed for rat cathepsin H (Ishidoh et al, 1989a), L (Ishidoh et al, 1989) and other cysteine proteinase genes such as aleurain, CP1, CP2 and CANP (Whitter et al, 1987; Pears et al, 1987; Emori et al, 1986) as well. An intron-exon junction is located immediately after the active site cysteine in the mouse cathepsin B gene. It

seems a common characteristics for cysteine proteinases that an intron-exon splicing site is always near the active site cysteine, within the highly conserved region (Ishidoh et al, 1987a and 1987b; Whitter et al, 1987; Pears et al, 1987; and Emori et al, 1986). In Figure 32, the gene organizations of rat cathepsin B and mouse cathepsin B are compared with that of rat cathepsin H (Ishidoh et al, 1989a) and L (ishidoh et al, 1989b). Although I did not clone rat cathepsin B gene, it can be assumed that rat cathepsin B has the same gene organization as mouse cathepsin B because they have highly homologous sequences. It is clear that cathepsin B, cathepsin H and cathepsin L sequences have a relatively high degree of identity in their active site cysteine regions and carboxyl-terminal regions. Eight identical amino acid sequences are found in the vicinity of active site cysteine in the three cathepsins. The exon-intron organizations of these genes are partially conserved in evolution, two intron-exon junctions are found in the same positions for cathepsin B and cathepsin H and for cathepsin H and L, respectively, while one intron-exon junctions are found in the same positions for cathepsin B and L. The similarities suggest that they are from a common ancestral gene. While the difference between the genes could be interpreted in terms of models involved intron loss or insertion during evolution, studies in other systems suggest that intron loss is more likely (Gilbert et al, 1986).

Comparison of cathepsin B, H and L genes with other cysteine

Figure 32. Alignment of the Amino Acids Encoded by Mouse  
Cathepsin B, Rat Cathepsin B, L and H

Single-letter amino acid code is used. Identical residues are boxed. Dashes indicate gaps introduced to generate this alignment. The positions at which introns interrupt the sequences are denoted by arrowheads. The arrows indicate the potential proteolytic cleavage sites. Circles indicate the active site cysteines and histidines.

Figure 32. Alignment of the Amino Acids Encoded by Mouse  
Cathepsin B, Rat Cathepsin B, L and H

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B-r
B-m M W W S L I L L S C L L A L T S A H D K P S F H P L S D D L I
H-r M W T A L P L L C A G A W L L S A G A T A E - - - L T V N A I
L-r M T P L L L L A V L C L G T A L A T P K F D Q T F N A Q W H H

B-r
B-m N Y I N K Q N T T W Q A G R N F Y N V D I S Y L K K L C G T V
H-r E K F H F T S W M K Q H Q K T Y S S R E Y S H R - - L - Q V F
L-r W K S T H R R L Y G T N E E E W R R A V W E K N M R M I Q L H

B-r
B-m L G G P - - K L P G R V A F G E D I D L P E T - F - D - A R E
H-r A N N W R K I Q A H N Q R N H T F K M G L N Q F S D M S F A E
L-r - N G E Y S - - - - N G K - H G F T M E M N A F G D M T N E E

B-r
B-m - - Q - - - W S - - - N C - - - - - - - - - - - - - - - -
H-r I K H K Y L W S E P Q N C S A T K S N Y L R G T G P Y P S S M
L-r F R Q I V N G Y R H Q K H K K G R L F Q E P L M L Q I P K T V

B-r
B-m - - - - - P T I G Q I R D Q G S C G S C W A F G A V E A I S
H-r D W R K K G N V V S P V K N Q G A C G S C W A F F S T T G A L E
L-r D W R E K G C - V T P V K N Q G Q C G S C W A F S A S G C L E

B-r
B-m D R T C I H T N G R V N V E V S A E D L L T C C G I Q C G D G
H-r S A V A I - A S G K M M T L A E Q Q - L V D C A Q N F N N H G
L-r G Q M F L K T - G K L I S L S E Q N - L V D C S H D Q G N Q G

B-r
B-m C N G G Y P S G A W S F W T K K G L V S G G V Y N S H V G C L
H-r C Q G G L P S Q A F E Y I L Y N K G I M G E - D - - - S Y - -
L-r C N G G L M D F A F Q Y I - - - K E N - G G L D S E E S Y - -

B-r
B-m P Y T I P P C E H H V N G S R P P C T G E G D T P R C N K S C
H-r P Y - I - - - - - - - - - G K N - - - - G Q - - - - C - K F -
L-r P Y - - - - - E - - - - - A K D - - - - G S - - - - - C - K Y -

B-r
B-m E A G Y S P S Y K E D K H F G Y T S Y S V S N S V K E I M A E
H-r N P E K A V A F V K N V V N I T L N D E A A M V E A V A L - -
L-r R A E Y A V A N D T G F V D I P Q Q - E K A L M K A V A T V G

B-r
B-m I Y K N G P V E G A F T V F S D F L T Y K S G V Y K H E A G D
H-r - Y - N - P V S F A F E V T E D F M M Y K S G V Y S S N S C H
L-r P I S V A M D - - A S H P - S - L Q F Y S S G I Y Y E P N C S

B-r
B-m - - - M M G G H A I R I L G W G V E N G V P - - - - Y W L A
H-r K T P D K V N H A V L A V G Y G E Q N G L L - - - - Y W I V
L-r S K - D - L D H G V L V V G Y G Y E - G T D S N K D K Y W L V

B-r
B-m A N S W N L D W G D N G F F K I L R G E N - H C G I E S E I V
H-r K N S W G S N W G N N G Y F L I E R G K N M - C G L A A C A S
L-r K N S W G K E W G M D G Y I K I A K D R N N H C G L A T A A S

B-r
B-m A G I P R T D Q Y W G R F
H-r Y P I P Q V
L-r Y P I - - V N

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proteinase genes such as aleurain, CP1, CP2 and papain (Michel et al, 1970; Husain and Lowe, 1970) shows that the gene sequences and organizations of cathepsin H, L are more closely related to those of the plant cysteine proteinases than that of cathepsin B, suggesting in the evolution process cathepsin B gene diverged earlier than cathepsin H and L from their common ancestral gene.

In comparison of the GC contents of exons in mouse cathepsin B with that in rat cathepsin H and L (Table 10) indicates a similarity: all first exons of the three lysosomal cysteine proteinase genes have high GC contents (cathepsin B: 73%, cathepsin H: 69%, cathepsin L: 61%)

The 5'-upstream sequence of cathepsin B has something in common with that of cathepsin L: both sequences are GC rich, both have several potential Sp1 binding sites, and both lack TATA motif. Thus, both genes have the features of "house keeping" genes which are constitutively expressed for normal cellular functions. Other housekeeping genes which have been cloned include adenosine deaminase gene (Valerio et al, 1985), dihydrofolate reductase gene (Yang et al, 1984) and insulin receptor gene (Araki et al, 1987; Seino et al, 1989) et al. There are also some differences between the putative promoters of the cathepsin B and L genes. The putative promoter of the cathepsin B gene is far away (4.75 kb) from the second exon while that of cathepsin L is very close (0.7 kb) to the second exon. The putative promoter of cathepsin B does not have CAAT

Table 10. Comparison of GC Contents of Exons in Mouse  
Cathepsin B, Rat Cathepsin H and Rat Cathepsin L

Exon	Cathepsin B (%)	Cathepsin H (%)	Cathepsin L (%)
1	73	69	61
2	59	34	50
3	51	49	51
4	53	42	49
5	47	51	48
6	48	58	49
7	54	55	43
8	45	55	38
9	55	46	
10	46	44	
11		44	
12		50	

The data for cathepsin H is from reference (Ishidoh et al, 1989a). The data for cathepsin L is from reference (Ishidoh et al, 1989b).

box and AP-2 binding sites while that of cathepsin L dose. It has been reported the tissue distributions of cathepsin B and cathepsin L were different in normal tissues (Kominamal et al, 1985; Bando et al, 1986; Qian et al, 1989). In addition, differential increases in the expression and secretion of cathepsin B and cathepsin L are among in different tumor cell variants (Qian et al, 1989; Keppler et al, 1988b) and transformed fibroblasts (Troen et al, 1987; Gottesman and Sobel, 1980). The differences in their putative promotor regions may account, in part, for the differential expression of these two cathepsin genes in normal and tumor tissues.

Analysis of the cathepsin B gene and the cathepsin B mRNA from B16a melanoma reveals that three potential polyadenylation sites are present in exon 10 of the gene. This information suggests that the three cathepsin B mRNA transcripts are from the same gene and very likely result from alternative usage of polyadenylation sites.

The cathepsin B gene information generated in this work will prove useful in the study of the mRNA transcription, translation, stability and regulation of gene expression of this enzyme in normal cells and in malignant cells.

#### POSSIBLE MECHANISM FOR ELEVATED CATHEPSIN B mRNA IN B16a MELANOMA CELLS

The mechanism of the selective elevation of cathepsin B

mRNA levels in the tumor cells can not be finally concluded in this study. However, a number of possible explanations can be eliminated. These are: amplification of cathepsin B gene in the melanoma cells, gross chromosome rearrangement leading to an altered gene expression, and mutation within the exons of the gene. The extended half-life of cathepsin B mRNA in the melanoma cells indicates that the stabilization of cathepsin B mRNA in the melanoma cells can account, at least in part, for the elevated cathepsin B mRNA levels in the tumor cells. It can not account, however, for the entire differences in mRNA levels among the three melanoma variants as the half life of cathepsin B mRNA in B16a (high metastatic potential) is only a slightly longer than that of B16F1 (low metastatic potential).

Half-lives of turnover for eukaryotic mRNAs and proteins range from a few minutes for highly regulated gene products such as oncogenes and rate-limiting enzymes, to more than 100 hours for very stable species. The average half-time of turnover for mRNA in eukaryotic cells is about 10-20 h. A mathematical model described by Hargrove et al (1989) shows that relative rates of mRNA transcription and translation and comparative stabilities of mRNAs and proteins contribute equally to the relative concentrations of different gene products in cells. Because transcription of many genes occurs at a significant basal rate in vertebrate tissues, a change in mRNA stability can be just as effective as a change in

transcription in changing the rate of protein synthesis. The utility of controlling mRNA stability owes partly to the very large amplification that occurs in protein synthesis; typically more than  $10^4$  mol of protein are synthesized per mole of mRNA (Palmiter, 1975). This result can be understood in terms of translational yield, which is defined as the number of molecules of a protein synthesized per molecule of specific mRNA per minute, multiplied by the mean life of the mRNA (mean life = half-life divided by 0.693) (Hargrove and Schmidt, 1989). Thus, it is energetically favorable to increase protein synthesis by making better use of existing mRNA or to decrease protein synthesis by degrading the relatively small amount of mRNA in the transcribed pool. Clearly, the increased cathepsin B mRNA stability in B16a melanoma cells could result in the increased protein synthesis and contribute to the increased cathepsin B activity found in the tumor.

The unusually long 3'UTR sequences in the larger cathepsin B transcripts in the B16 melanomas appear to contribute to the increased stability of the mRNAs as we can see that the 4.0 kb transcript is very stable in the Northern blot analysis (Fig. 30). However, this may not be the only cause since the 2.0 kb transcript in the melanoma cells is also quite stable as shown in Fig. 30. Other possibilities include the presence of a factor or factors in the melanoma cells, which is responsible for the stabilizing the cathepsin

B mRNA in the melanoma cells. This hypothesis need to be investigated further.

Although preliminary evidence has shown cathepsin B mRNA expression in the B16 melanoma cells is regulated, at least in part, at the posttranscriptional level, it is possible that transcriptional regulation also occurs. The evidence for this is that the large differences in cathepsin B mRNA levels between the B16a melanoma variants despite similar half lives for their cathepsin B mRNAs. With the mouse cathepsin gene and its 5'-upstream region now available, it should be possible to determine how transcription of the cathepsin B gene is regulated in the tumor cells and in normal cells.

## CHAPTER VI

### CONCLUSIONS

I. The relative mRNAs of five lysosomal cathepsins (B, D, H, L and S) have been measured in rat and mouse tissues including kidney, lung, liver, spleen, thymus, uterus, testes, skin, brain and pancreas. The differential distribution of these cathepsins in the tissues suggest each cathepsin has its specific functions and may be individually regulated.

II. Cathepsin B mRNA level is specifically elevated in rat Walker-carcinosarcoma and mouse B16 melanomas. In contrast, cathepsin H, D, L and S mRNA levels are not elevated in the same tumors. The elevated cathepsin B mRNA level is in agreement with the increased enzyme activity in the melanoma cells and correlated well with the metastatic potential of the B16 melanoma variants.

III. The selectively elevated cathepsin B mRNA is derived from tumor cells and not from contaminating

reticuloendothelial cells, such as macrophages.

IV. The B16 melanomas express cathepsin B mRNA transcripts with the sizes of 2.2, 4.0 and 5.0 kb. The two larger transcripts were not detected in normal murine tissues. The cloned cDNAs from a B16a melanoma library indicate all three mRNA transcripts encode for the same protein sequence, which is also the same as preprocathepsin B from normal murine tissue. The two larger transcripts have unusually long 3'-untranslated sequences which may result from modified termination of transcription of cathepsin B in the melanomas.

V. Mouse cathepsin B gene consists of 10 exons spanning about 20 kb. The first exon and the putative promotor region are very GC rich and five Sp1 binding sites are present in the 5'-flanking region which, however, has no TATA box. These characteristics suggest cathepsin B gene is a "housekeeping" gene and may be regulated, at least in part, by Sp1 transcriptional factors.

VI. No gene amplification or gross chromosomal rearrangements were found for cathepsin B gene in the melanoma cells. Neither were there any mutations within the coding region of the gene.

VII. Cathepsin B mRNAs appear to have longer half-lives

in the mouse melanoma cells than mouse fibroblasts. The increased stability of cathepsin B mRNA in B16 melanoma cells may account in part for its increased level in the mouse melanomas.

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