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## Induction of Proteases and Their Inhibitors in Cell Lines of Different Metastatic Potentials and Activated Oncogenes

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**INDUCTION OF PROTEASES AND THEIR INHIBITORS IN CELL LINES  
OF DIFFERENT METASTATIC POTENTIALS AND ACTIVATED ONCOGENES**

by

**Jianyi Zhang**

A Dissertation Submitted to the Faculty of the  
Graduate School of Loyola University of Chicago  
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1993

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## VITA

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## LIST OF ABBREVIATIONS

BCIP/NBT	5-bromo-4-chloro-3-indoly phosphate/nitroblue tetrazolium
BSA	bovine serum albumin
CAMP	cyclic adenosine monophosphate
CB	cathepsin B
CHX	cycloheximide
CL	cathepsin L
C.M.	conditioned medium
CPI	cysteine proteinase inhibitor
DAG	diacylglycerol
DFP	diisopropyl fluorophosphate
DMEM	Dulbecco's modified Eagle' medium
EDTA	ethylenediamine tetraacetic acid
EGF	epidermal growth factor
FBS	fetal bovine serum
FGF	fibroblast growth factor
GAP	GTPase activating protein
GDP	guanidine diphosphate
GTP	guanidine triphosphate
HI-FBS	heat-inactivated fetal bovine serum
KB	kilobase
MEM	Eagle's minimum essential medium
MEP	major excreted protein
PA	plasminogen activator
PAI-1	plasminogen activator inhibitor type 1

PAI-2	plasminogen activator inhibitor type 2
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PMA	phorbol-12-myristate-13-acetate
R.M.	regular medium
SDS	sodium dodesyl sulfate
S.P.	streptomycin and penicillin
SSC	standard saline citrate
TIMP	tissue inhibitor of metalloproteinases
TIMP-1	tissue inhibitor of metalloproteinases type 1
TIMP-2	tissue inhibitor of metalloproteinases type 2
tPA	tissue type plasminogen activator
uPA	urokinase type plasminogen activator

## CHAPTER 1

### INTRODUCTION

Malignant cells commonly exhibit increased activity of several degradative enzymes that are believed to contribute to their invasive and metastatic potential. Of special interest in this regard are proteases such as cathepsin B and L, and urine-type plasminogen activator (uPA). However, little is known about the pattern of protease gene expressions caused by different oncogene transformation.

Non-transformed NIH 3T3 and NIH 3T3 murine fibroblasts transfected with 4 different ras oncogene constructs have been characterized with respect to their metastatic potential in nude mice. The PC3-P and PC3-M cell lines are human prostate carcinoma cell lines with a different metastatic potential. A375-P & A375-M cells lines are derived from a human melanoma with a different metastatic potential.

Tumor promoting phorbol esters such as phorbol-12-myristate-13-acetate (PMA) are diacylglycerol (DAG) analogs which directly stimulate the phospholipid-dependent protein kinase C (PKC). Studies on the effects of the potent tumor promoter (PMA) have demonstrated that it induces a highly

pleiotropic response in a variety of cell lines, including a more metastatic phenotype, and increased expression of several different proteases and protease inhibitors.

However, it is not known whether the cellular response to phorbol esters differs among tumor cells with different oncogene transformation and metastatic potentials. There were three specific aims of this project:

(1) To explore the mechanism by which oncogenes induce the metastatic phenotype by studying protease gene induction and regulation by ras oncogenes.

(2) To determine how exogenous signal factors regulate protease and protease inhibitor expressions in transformed and metastatic cells.

The results of this project may contribute to the understanding of the complexity of proteases expression and involvement in tumor metastasis.



## Chapter II

### REVIEW OF THE LITERATURE

#### Tumor Metastasis and Proteases

Malignant tumors differ from benign tumors in their ability to spread throughout the body, giving rise to metastases at sites distant from the primary tumor. Metastasis is a complex process involving many steps, each has its own regulatory mechanism (Poste, G. and Fidler, J. J., 1980). However, from a simple point of view, the following types of invasive and degradative processes must occur in order for a secondary tumor to be established. A cell must break away from the primary tumor mass, penetrate the tissues surrounding the tumor, and move through the cellular layers and basement membrane of blood and lymph vessels in order to enter the circulatory system. Tumor cells are then disseminated by the blood and lymphatic circulation. Subsequently, they must extravasate from the circulation and reach a site favorable for proliferation. The result of the process is the formation of a new tumor.

It has been recognized for many years that a positive

correlation exists between cellular invasion and proteinase production, and it has been hypothesized that proteolytic activity may, in fact, be required for invasiveness. According to this theory, cells are invasive by virtue of their ability to secrete (or induce secretion of) proteinases capable of degrading the molecules which compose the barriers they must cross. These barriers include basement membrane, interstitial tissue and extracellular matrices, which are composed primarily of collagen, proteoglycan, elastin and other glycoproteins.

Considerable evidence has amassed suggesting that a number of proteinases capable of degrading the molecular barriers are associated with transformed cells. The best characterized or identified enzymes are urokinase-type plasminogen activator (uPA), type IV collagenases, stromelysin, and cathepsin B-like and cathepsin L-like proteinases.

### **Biochemistry of Plasminogen Activators**

One of the most studied wide-spectrum proteinase is the serine protease plasmin. Plasmin is able to dissolve fibrin clots, digest extracellular matrix proteins, and activate latent enzymes and hormones (Mullins and Rohrllich, 1983). It is formed by the proteolytic cleavage of the abundant proenzyme plasminogen. Plasminogen is a 791 amino

acid zymogen which is activated to the broad-specificity protease plasmin by a single proteolytic cleavage at Arg-560. The reaction is mainly catalyzed by two molecularly different, functionally similar, enzymes called plasminogen activators (PAs), urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (t-PA). Both are widely distributed in body fluids and tissues, but uPA is more abundant in urine while t-PA is more abundant in plasma (Blasi, et al., 1987). They participate in the regulation of several physiological processes by initiating a reaction leading to the formation of active plasmin. t-PA is regarded as an important mediator of the fibrinolytic activity in circulation. u-PA has a role in cellular invasion and tissue involution (Beer et al., 1975), cellular migratory activity (Vassalli, et al., 1976), trophoblast implantation (Strickland et al., 1976), and mammary gland involution (Ossowski et al., 1979), tumor invasion and metastasis (Ossowski and Reich, 1983), and a variety of other physiological processes.

### **Structure and Regulation of uPA**

The primary structure of uPA has been determined both by protein sequencing and by deducing the amino acid sequence from its cloned cDNA. The human uPA is the translated, active and mature product of a 2.5 kb-long mRNA,

(Verde et al., 1984) which is transcribed from a 6.5 kb gene present on the long arm of chromosome 10 (Tripputi et al., 1985). It is organized in 11 exons and 10 introns (Ricchio et al., 1985). Translation of the mRNA yields a prepro-uPA with 431 amino and with a molecular weight 54 kD. Removal of a 20 residue long signal peptide gives rise to the secreted single-chain pro-uPA, which is a glycosylated polypeptide (in human) of about 50 kD (Pennica et al., 1983). Mouse u-PA, however, contains no N-glycosylation site (Belin et al., 1985). It is this pro-uPA that is released from a variety of cultured normal and neoplastic cell types, and appears to be the predominant extracellular form of u-PA in the organism (Dano, et al., 1985). The single-chain uPA is considered a proenzyme form that has little or no proteolytic activity against the natural substrate plasminogen, and no activity against synthetic plasminogen activator substrates. Single-chain uPA is converted into an active two chain form with Mr 50,000 by proteolytic cleavage after lysine-158, resulting in two polypeptide chains held together by one disulfide bond (Guenzler et al., 1982) This conversion can be catalyzed by plasmin (Dano et al., 1985), plasma kallikrein (Ichinose et al., 1986), factor XIIa (Ichinose et al., 1986), trypsin (Eaton et al., 1984), and thrombin (Easton et al., 1984).

The regulation of uPA production has been reported to be associated with various compounds such as growth factors

and the tumor promoter phorbol-12-myristate-13-acetate (PMA) (Degen et al., 1985; Collart et al., 1986; Bell et al., 1990). It is increased by epidermal growth factor (EGF) (Boyd, 1989; Niedbala and Sartorelli, 1989), platelet-derived growth factor (PDGF) (Stoppelli et al., 1986), interferon (INF-gamma) (Collart et al., 1986), transforming growth factor beta (Keski-Oja et al., 1988), vasopressin (Nagamine et al., 1983) and estrogen (Mira-y-Lopez et al., 1983). Glucocorticoids have been shown to decrease uPA production in certain normal and malignant cells (Medcalf et al, 1986, Busso et al., 1987).

### **uPA receptor**

Several types of cells possess specific high affinity ( $K_d = 10^{-10}$  M) plasma membrane binding sites for uPA. Human peripheral blood monocytes (Vassalli et al., 1985), the monocyte-like U937 cells, fibroblasts, and HT1080 fibrosarcoma cells (Testa and Quigley, 1988) all have approximately  $10^5$  such specific receptors per cell. The receptor was shown to be a 55-60 kD glycoprotein (Nielsen et al., 1988). The region of u-PA that binds the receptor is located on the 135-residue-long amino terminal fragment (ATF) of the A-chain of two chain urokinase. More precisely, the first 32 residues of the "growth factor" region of the ATF peptide is required for receptor binding. The ATF

fragment comprises two structural domains, a "growth factor" region with homology to EGF and a kringle domain (Patthy L., 1985). In contrast, the  $M_r$  33,000 KDa form of u-PA, a partial degradation product of the two chain  $M_r$  50,000 enzyme, lacks the ATF and does not interact with the receptor. The 33,000 dalton form, however, retains catalytic activity (Stoppelli et al., 1985). Receptor binding does not require u-PA catalytic activity, and the single-chain zymogen u-PA binds with about the same affinity as does the active  $M_r$  50,000 two-chain enzyme.

The receptor-bound forms of plasminogen activator is enzymatic active, and binding of either the one-chain and two-chain u-PA to the u-PA receptor on cultured human monocytes-macrophage results in the generation of plasmin-mediated pericellular proteolysis (Dano et al., 1985). Thus the presence of a u-PA receptor allows cells to acquire surface-bound plasminogen-activating ability.

### **Roles of uPA in Neoplasia**

The first experiment showing a direct cause-effect relationship between uPA and metastasis is that performed by Ossowski and Reich (Ossowski and Reich, 1983). They found that human HEP3 cells inoculated onto the chorio-allantoic membrane of a chicken embryo were able to metastasize to the lung of the embryo and that this process could be blocked by

specific anti-catalytic antibodies against human uPA.

There are multiple reports of an increase in the synthesis of uPA in cultured cells transformed by oncogenes or oncogenic viruses, on treatment of cultured cells with tumor promoter, and on the assay of transformed tissue in comparison to surrounding normal tissues (reviewed by Dano et al., 1985). However there also are a number of reports showing a lack of correlation or a negative correlation between uPA activity and ability to metastasize (Harvey et al., 1988; Graf et al., 1984; Giraldi et al., 1985). Recently, more direct evidence in support of a role of urokinase in tumor cell metastasis has come from experiments in which the expression of the human urokinase sense and anti-sense genes expressed in murine B16 cells has been shown to increase or decrease the metastatic potential of these cells (Yu and Schultz, 1990). Also, the overexpression of human uPA increases the capacity of ras-transformed murine NIH 3T3 cells to colonize the nude mouse lung after tail-vein injection. (Axelrod et al., 1989).

### **PA inhibitor**

Precise regulation of plasminogen activator (PA) activity constitutes a critical feature of many biological processes. This regulation may occur at various levels, including the synthesis and secretion of PAs, and the

interaction with specific PA inhibitors, the PAIs (Sprengers and Kluft, 1987). The two most important protease inhibitors specific for PAs (PAI-1 and PAI-2) have recently been identified and characterized.

Plasminogen activator inhibitor type 1 (PAI-1) is the major PAI found in plasma (Kruithof et al., 1983; Thorsen and Philips, 1984). It has been shown to be expressed in many body tissues (Quax et al., 1990; Erickson et al., 1990), the conditioned media of fibrosarcoma cells (Andreason et al., 1986), hepatocytes (Sprengers et al., 1985), smooth muscle (Laug, 1987) and melanoma cells (Wagner and Binder, 1986). It is a 50-kDa glycoprotein, belonging to the serpin family (Pannekoek et al., 1986; Ny et al., 1986), and a specific inhibitor of both tissue-type PA and urokinase-type PA (Kruithof et al., 1984; Kruithof et al., 1986).

The biosynthesis of PAI-1 appears to be highly regulated. For example, PAI-1 synthesis can be induced by endotoxin (Culucci et al., 1985), inflammatory mediators (Emeis and Kooistra, 1986; Sawdey et al., 1989), glucocorticoid (Andreasen et al., 1987), insulin (Kooistra et al., 1989) and phorbol ester (Mayer et al., 1988).

Plasminogen activator inhibitor type 2 (PAI-2) has been purified from the human placenta (Kawano et al., 1986) and epidermis (Astedt et al., 1986) It is produced by many cell types including monocyte-macrophage and human



histiocytic lymphoma cells (Kruithof et al., 1986). The cDNA cloning and amino acid sequence of PAI-2 have been determined from human placenta (Ye et al., 1987), human histiocytic lymphoma cells (Schleuning et al., 1987), and human monocyte (Wagner and Binder, 1987). PAI-2 consists of 415 amino acids, corresponding to a  $M_r$  47 kD protein. It exists in two forms, a 47 -kD, pI 5.0, nonglycosylated form and a 60-kd, pI 4.4, glycosylated form, with similar PA inhibition characteristics (Genton, et al., 1987; Wohlwend et al., 1987). Interestingly, in short-term culture, the nonglycosylated form, representing over 90% of synthesized PAI-2, is only found intracellularly, whereas the glycosylated form is secreted (Genton, et al., 1987; Wohlwend et al., 1987).

The regulation of PAI-2 production in macrophage has been characterized in some detail. It is decreased by dexamethasone but increased by the tumor promoter phorbol myristate acetate (Kruithof et al., 1986; Schleuning, et al., 1987), endotoxin (Chapman and Stone, 1985), and muramyl dipeptide (Stephens, et al., 1985). In peripheral blood mononuclear cells lipopolysaccharide increases the release of PAI-2 leading to an anti-fibrinolytic state (Schwartz et al., 1988).

## Structure and Regulation of Cathepsin B

Cathepsin B is one of most characterized cysteine proteinase. Cathepsin B has been purified from many different normal tissues of numerous species. Mature cathepsin B seems to exist in either a single chain form of 39 kDa or a double chain form of 25 and 5 kDa. The proportions of the single and double chain forms varying from species to species. The active site of cathepsin B was found in the light or 5 kDa chain of the double chain form.

The preprocathepsin B cDNAs have been isolated from rat (Segundo et al., 1986), mouse (Chan et al., 1986) and human (Chan et al., 1986). Based on the nucleotide sequence, cathepsin B is synthesized as a preproenzyme of 339 amino acids, which consists of a 17 residue prepeptide sequence at the N-terminus, a 62 residue propeptide sequence, and a 6 residue extension at the carboxyl terminus. The signal peptide of 17 amino acids in preprocathepsin B is cleaved to give procathespin B in the endoplasmic reticulum. The protein is glycolated in the Golgi complex and, in nontransformed cells, the majority is transported to lysosomes through the mannose-6-phosphate signal pathway (Gal et al., 1985). A defect in this pathway in I-cell disease results in the release of lysosomal enzymes and the association of some lysosomal enzymes with small vesicles near the plasma membrane (van Dongen et al., 1985). A

similar process may occur in some transformed cells (Archkar et al., 1989). Within the lysosomal acid compartment, procathepsin B is processed to the single-chain mature form of cathepsin B (Steiner et al., 1984).

Using a human cDNA to the cathepsin B as a hybridization probe, 2.2 and 4.1 kb mRNA species were detected in Northern blots of normal murine lines and murine melanomas (Fong et al., 1986). However, Qian reported that B16 melanoma variants contain 2.2, 4.0 and 5.0 kb mRNA transcripts for cathepsin B, but normal tissues only contain the 2.2 kb transcript (Qian et al., 1989; 1991a; 1991b) and Chambers et al., (1992) have observed a 4.0 kb cathepsin B mRNA in metastatic ras-transformed NIH 3T3 cell but not in non-metastatic variants. The larger transcripts are thought to be due to alternative polyadenylation site selection during the processing of the 3'-end of the cathepsin B mRNA (Qian et al., 1991a; 1991b). Variant messengers for Cathepsin B are also found in normal human tissue and tumors, and these arise as a result of alternative splicing of the 5' and 3' levels of the cathepsin B mRNAs (Gong and Frankfater, personal communication).

### **Enzymology of Cathepsin B**

The pH profile for cathepsin B activity is substrate-dependent. The optimum pH is about 6.0 for synthetic

substrates such as carbobenzyloxy-L-arginine-L-arginine-2-naphthylamide (Z-Arg-Arg-NHMec) (Barret and Kirschke, 1981). At pH 7.4 significant activity can be observed for times up to 12 hours if the assay is performed against large protein substrates that presumably stabilize the enzyme, provide resistance to inactivation by cysteine proteinase inhibitors (CPI) and physiological pH (Rozhin et al., 1987; 1990; Lah et al., 1989). Natural physiological substrates for cathepsin B are still a matter of speculation. Laminin, fibrinogen (Lah et al., 1989), fibronectin (Recklies et al., 1982), trypsinogen (Figarella et al., 1988) are all degraded by tumor and liver cathepsin B at both acid and neutral pH in vitro (Lah et al., 1990; Sloane et al., 1990). Cathepsin B has been shown to be able to degrade proteins of the extracellular matrix including the non-helical regions of basement membrane type IV collagen, in vitro (Morrison et al., 1973; Burleigh et al., 1974; reviewed in Sloane and Honn, 1984).

### **Cathepsin B and Tumor Malignancy**

There is an extensive literature on the elevation of release of cathepsin B from both human and animal tumor cells in vitro (Sloane and Honn, 1984; Recklies, 1987). It is reported that a rise of cathepsin B activity was observed in human malignant breast tumors (Recklies et al., 1980), in

rat metastatic pancreatic cancer (Koppel et al., 1986), in murine B16 melanoma (Sloane et al., 1982), in Lewis lung carcinoma (Sloane et al., 1984), and in a rat sarcoma LW13K2 (Krepela et al., 1989).

Cathepsin B is released into extracellular fluid by breast tumor cells in organ culture in two major forms, a latent and an active form, with  $M_r$  precursor of 40 kD for both forms (Mort et al., 1980). The latent form can be activated with pepsin, resulting in the formation of a 33 kD active form of cathepsin B. A high  $M_r$  (40 kD) active form of cathepsin B was also reported in culture media of spontaneous mammary glands (Racklies et al., 1982) and lactating mammary glands (Recklies and Mort, 1985). The levels of a latent cathepsin B-like activities secreted by B16 melanoma variants parallels the metastatic capabilities of the variants (Qian et al., 1989). In addition, cathepsin B activity has been associated with the plasma membrane fractions of various tumor cell lines (Sloane et al., 1987; Sloane et al., 1986; Rozhin et al., 1990). An immunofluorescence study showed that the distribution of cathepsin B in cultured human colon carcinoma cells appears to be at the cell surface (Maciewixz et al., 1989).

How cathepsin B is released by tumors or how it is associated with the plasma membrane-enriched fractions is not clear. It is suggested that either oversaturation of mannose-6-pathway and/or defects in the mannose-6-pathway

could be involved in these non-lysosomal localization (Goldberg et al., 1983; Mainferme et al., 1985; Achkar et al., 1990; Pracht and Frankfater, unpublished data).

### **Structure of Cathepsin L**

Cathepsin L, another cysteine protease, is one of several acid protease-like cathepsins to be involved in the degradation of polypeptides within the lysosomes (Gottesman, 1978). Full length cDNA clones encoding the entire cathepsin L precursor molecule have been isolated from both mouse (Troen et al., 1987; Portnoy et al., 1986; Joseph et al., 1988) and human cells (Joseph et al., 1988; Gal S. and Gottesman M. M., 1988).

The exact cleavage sites involved in processed forms of lysosomal cathepsin L have been determined by comparing actual amino acid sequences of the mature protein and sequences deduced from the cDNA clones.

Cathepsin L is synthesized as a prepropeptide containing a hydrophobic signal sequence of 17 amino acids which is cleaved to give procathepsin L. This protein is glycosylated in the Golgi complex and, in nontransformed cells, the majority is transported to the lysosomes through the mannose-6-phosphate pathway (Sahagian and Gottesman, 1982). Within the acidic compartment, procathepsin L is processed to the single-chain mature form via cleavage

between Lys 113 and Ile 114 (amino acid numbering starts with Met-1 of the signal sequence). This form can be further processed to the two chain species by cleavage between Ser-290 and Asn-291. The large and small chains produced by this final cleavage remain associated by disulfide linkage(s) in the enzymatically active mature protein (Smith and Gottesman 1989; Ritonja et al., 1988).

Mature cathepsin L has been purified from a number of eukaryotic origins, including human kidney (Ritonja et al., 1988), rat (Towatari and Katunuma, 1988), rabbit (Mason and Taylor, 1984), sheep (Mason, 1986), chicken (Dufour et al., 1987) and human liver (Mason et al., 1985). In addition, the precursor form of the enzyme has been isolated from the cultured supernatant of transformed NIH/3T3 cells, which may secrete abundant amounts of the protein (Gottesman et al., 1978).

### **Enzymology of Cathepsin L**

Mature cathepsin L isolated from the lysosomes exhibits activity over a broad pH range, with maximal activity between pH 4.5-5.5. The mature enzyme retains activity, but is extremely unstable at neutral pH (Mason et al., 1985; Kirschke 1982). In contrast, procathepsin L, the secreted form of the protein, is very stable at neutral pH, but has little or no protease activity in that range

(neutral pH), which suggests that propeptide might be involved in protein stabilization at neutral pH.

Mature cathepsin L is the most active lysosomal protease in vitro against a variety of neutral substrates, including aozcasein (Troen et al., 1987), elastin (Mason et al., 1986) and collagen (Kirschke et al., 1982). No absolute bond specificity of cathepsin L action has been determined, though it may show a preference for peptide bonds near hydrophobic amino acids (Mason et al., 1985).

Cathepsin L can be distinguished from other cathepsins by its very limited activity in hydrolyzing some synthetic substrates. Z-Phe-Arg-NMe has turned out to be the most useful synthetic substrate for assaying cathepsin L activity (Kirschke et al., 1982). It is extremely susceptible to cathepsin L hydrolysis, but much less sensitive to proteolysis by cathepsin B, with Km values differing by as much as 20- to 50-fold for the two enzymes (Mason et al., 1985; Mason et al., 1987; Kirschke et al., 1982).

### **Regulation of Expression of Cathepsin L**

There are several reports on the regulation of cathepsin L synthesis by growth factors such as PDGF and EGF (Scher et al.; 1983, Nielsen-Hamilton et al., 1982), tumor promoters such as phorbol-myristate-acetate (PMA or TPA),



protein kinase A - cAMP (Kane and Gotterman, 1990), and ras oncogene transformation (Gottesman, 1979) which have been shown to increase cathepsin L synthesis in mouse fibroblast cells (Gottesman and Sobel, 1980). Increases of cathepsin L have also been reported to be associated with activation of macrophage (Portnoy et al., 1986), and on treatment of bovine endothelial cells with heparin (Cochran et al., 1988).

### **Cathepsin L and Tumor Malignancy**

A cathepsin L-like protein was first reported as a major excreted protein (MEP) whose extracellular and intracellular abundance was augmented when mouse fibroblasts were transformed by tumor viruses or methylcholanthrene (Gottesman, 1978). In subsequent work the protein was purified and shown to possess the lysosomal marker mannose-6-phosphate (Sahagian and Gottesman, 1982). The sequence of MEP cDNA was determined and its deduced amino acid sequence was found to be identical to a partial protein sequence of cathepsin L (Troen et al., 1987). MEP was found to be the precursor to the lysosomal form of cathepsin L, i.e. procathespin L. Malignant transformation by several different oncogenes increases the rate of synthesis and secretion of procathespin L (Gottesman 1979). A set of 10T1/2 cell lines that had been transfected by the T24-H-ras

oncogene revealed a positive correlation between metastatic potentials and MEP (Denhardt et al., 1987).

### **Cysteine Proteinase Inhibitors (CPI)**

The cystatin superfamily of cysteine proteinase inhibitors (CPI) consists of three families: (1) the stefins, (2) the cystatins and (3) the kininogenes. In terms of inhibition of cathepsin B, the stefins and cystatins appears to be more physiologically important as their  $K_i$ s for inhibition of cathepsin B are much lower than the kininogenes (Barret, 1987).

The stefin family of CPIs have been purified from human liver (Lah et al., 1989) and a human sarcoma (Sloane et al., 1989). They consist of proteins of about 11 kD that have no disulfide bonds or carbohydrate and exist primarily intracellularly. Two type of stefins have been identified: stefins A with acidic isoelectric points and stefins B with neutral isoelectric points.

The cystatin superfamily is another class of natural proteins known to interact strongly with cysteine proteases in vivo (Barrett, 1987). These are reversible, competitive inhibitors. The cystatins may localize to the cell cytoplasm, resulting in inactivating lysosomal proteases that escape out of the lysosomes into other parts of the cell. Many of the cystatins can also be secreted into the

extracellular environment and are found circulating in plasma. These inhibitors may serve as a further safeguard against cysteine proteases which have leaked out of cells, or they might be part of an intricate regulatory mechanism of extracellular protease activity (Kruithof, 1983).

Down-regulation of the synthesis of stefin A occurs during the progression of murine epidermal carcinomas from a pre-malignant to a malignant state (Hawley-Nelson et al., 1988). A reduced amount was found in skin tumors (Jarvinen et al., 1987), and B16a tumors were found to have less stefin A than normal liver cells (Sloane, 1990).

Analyzing the subcellular distribution of cathepsin L suggests that CPI might be involved in regulating of cathepsin L activity in murine B16 amelanotic melanoma cells (Rozhin et al., 1989). The plasma membrane fractions from B16 melanoma subpopulations of "low" and "high" metastatic potential were assayed for activity of cathepsin L and for heat stable endogenous cathepsin L inhibitors. The relative specific activity of cathepsin L was 7-fold greater in the subpopulation of "high" metastatic potentials, whereas cysteine proteinase inhibitory activity was 5-fold less.

### **Tissue Inhibitor of Metalloproteinases (TIMP)**

The TIMPs are stoichiometric inhibitors of many mammalian metalloproteinases including collagenases that

degrade interstitial collagens (type I-III) as well as type IV and V collagens (Liotta et al., 1982; Goldfarb and Liotta, 1986; Torgeirsson et al., 1982). The TIMP family consists of at least two members. TIMP-1, first isolated from rabbit bone, is a glycoprotein with a core polypeptide of  $M_r$  21,000 produced by many mesodermal tissues and is found in a wide variety of human body fluids and cells in culture (Welgus and Stricklin, 1983; Welgus et al., 1985). Determination of the molecular weight of the secreted, glycosylated protein ranged from 25,000 to 36,000 (Cawston, 1986; Drouin et al., 1988). The human TIMP-1 has been isolated (Welgus et al., 1983), cloned and sequenced by several groups (Carmichael et al., 1986; Docherty et al., 1985; Gasson et al., 1985). Mouse cDNA (Edwards et al., 1986), and rabbit (Horowitz et al., 1989). cDNA clones have also been isolated. The encoded proteins of the murine and human TIMPs are 76% identical at the nucleotide level and 73% identical at the amino acid level. Murine TIMP-1 has been mapped to the X-chromosome (Jackson et al., 1987). Human TIMP-1 is identical in amino acid sequence to a human collagenase inhibitor isolated from human fibroblasts (Carmichael et al., 1986) and also inhibits other neutral metalloproteinases with gelatinase, proteoglycanase, and stromelase activities (Heath et al., 1982; Sellers et al., 1979).

The expression of TIMP-1 mRNA in murine fibroblasts

is induced by serum, platelet-derived growth factor, epidermal growth factor, and PMA (Edwards et al., 1986; Gewert et al., 1987; Denhart et al., 1986). A coordinated temporal regulation of metalloproteinases and TIMP have been observed in human fibroblasts which are induced to produce TIMP, and to suppress collagenase and stromelysin, under stimulation of transforming growth factor beta-1 (Machida et al., 1988; Overall et al., 1989).

Natural TIMP-1 has been shown to inhibit tumor cell invasion of the human amnion, which models invasion of tumor cells across the basement membrane and stroma in vitro (Torgeirsson et al., 1982; Mignatti et al., 1986). The most direct evidence of metalloproteinase involvement in cancer metastasis has come from this laboratory which showed that human recombinant TIMP acts as an inhibitor of tumor cell invasion in the in vitro amnion membrane invasion assay and in an in vivo lung colonization assay with B16 F10 melanoma cells in C57BL/6 mice (Schultz et al., 1988).

TIMP-2 is nonglycosylated and has recently been isolated from media conditioned with human melanoma cells (Stetler-Stevenson et al., 1989) or bovine endothelial cells (De Clerck et al., 1989). TIMP-2 shares a 43% amino acid sequence homology with the TIMP-1 and is found as a complex with the 72-kD type IV procollagenase, an enzyme closely linked to the invasive phenotype of both human melanoma and endothelial cells (Goldberg et al., 1989).

## Structure of Mammalian Ras

All the properties of both normal and malignant cells are determined by the subset of those genes expressed in their genome. A number of these genes have been shown to have a role in the process of malignant transformation and have been termed oncogenes. Oncogenes are derived from normal genes (proto-oncogenes or cellular oncogenes) that are highly conserved in evolution and that code for proteins that have important roles in normal cellular processes such as the regulation of gene expression or the transduction of growth signals. Of the 50 to 60 oncogenes so far identified as capable of transforming cells, the ras family of oncogenes are among the most frequently activated in human cancer (reviewed by Bos, 1989).

The ras genes include (at least) three well-characterized genes that have been associated with human cancers: Harvey-ras (H-ras), Kirsten-ras (K-ras), and N-ras (Barbacid, 1987; Bos, 1989). All of the three ras genes code for closely related 21-kD proteins that can bind GDP and GTP, and also have intrinsic GTPase activity which functions to convert Ras-GTP to Ras-GDP. Rates of GTP hydrolysis by wild-type Ras protein, but not oncogenic protein, are vastly elevated in vivo (Trahey and McCormick, 1987). The discrepancies led to the discovery of GAP, a GTPase-activating protein found ubiquitously in cell. The carboxy-

terminal 40K acts catalytically upon wild-type Ras proteins to stimulate their hydrolysis of bound GTP, but fails to stimulate GTP hydrolysis by mutant oncogenic Ras (Marshall et al., 1989). The ras proteins share structural homology with G proteins and play a role in the transduction of growth signals. The transforming potential of ras proteins (p21) is usually associated with a point mutation in the codons encoding amino acids 12, 13, or 61 (Sistonen and Alitalo, 1986). These critical amino acid substitutions all occur in or near the GTP binding domain of the protein, and the transforming potential of altered p21 is associated with the loss of GTPase activity. This view has been recently confirmed by crystallographic studies of the protein (Tong et al., 1989)

Ras proteins are in the cytosol and become associated with the inner side of the plasma membrane for functional activity. Mutations at the cysteine of the COOH-terminal Cys-a-a-X box (where a is an aliphatic amino acid and X any of the common amino acids) generate an inactive cytoplasmic protein by blocking a complex series of post-translational modifications that includes removal of the three terminal amino acids and polyisoprenylation of the new COOH-terminal cysteine (Hancock et al., 1989). The Cys-a-a-X box is a general signal for addition of the 15-carbon farnesyl lipid moiety which increases the hydrophobicity of the protein, but a further signal is required for targeting to specific

membranes (Holtz et al., 1989). Localization of N-ras and H-ras to the plasma membrane requires addition of palmitic acid at another cysteine residue (181 or 184). In cellular Ki-ras, which does not contain an upstream cysteine and is not palmitoylated, a region rich in basic amino acids (residue 175-182) seems to provide this additional signal (Holtz et al., 1989).

### Functions of Mammalian Ras

Genetic analysis of mammalian Ras is not yet possible and biochemical approaches have been used to analyze its function. Although the ras genes cannot be deleted, the proteins can be neutralized by microinjecting antibodies to Ras into cells treated with growth factor (Mulcahy, 1985). The result shows that the ras product is essential during the first 8 hours of the G<sub>1</sub> phase of the cell cycle. After this time cells are committed to complete G<sub>1</sub>, enter the S phase, and complete a cycle.

Although mutationally activated ras genes suffice to transform NIH 3T3 mouse fibroblasts in vitro, a single genetic event is probably not sufficient to cause human cancers. In fact, it is believed that as many as five or six steps are required for human malignancies (Weinberg, 1989). The cooperation of ras genes with other oncogenes has been elegantly shown in the now classic experiments of Land and



coworkers (Land et al., 1983). Rat embryo fibroblasts (REF cells) could be transformed only when at least two different oncogenes were introduced: a myc-like and a ras-like gene.

So far, little progress in identifying the Ras signaling pathway has been made from analyzing changes in second messengers. It is not known whether Ras controls the classical growth factor responses such as phospholipid turnover and protein kinase activation or whether it generates a novel signal.

#### **Structure and Function of Ras in yeast S. cerevisiae.**

Saccharomyces cerevisiae contains two genes, RAS1 and RAS2, which produce ras-like gene products. The RAS1 and RAS2 proteins contain 309 and 322 amino acids, respectively, compared to the mammalian p21 of Ha-ras protein with 189 amino acids. Alignment of the sequence of the mammalian p21 with RAS1 and RAS2 proteins show the yeast Ras contains seven additional amino acids on the NH<sub>2</sub>-terminal end and the rest of the additional amino acids (except for one) are added to the COOH-terminal end of the p21-ras sequence. The first 80 amino acids of mammalian Ha-ras show a greater than 90% identity to the near NH<sub>2</sub>-terminal sequences in the yeast RAS proteins (residues 8 to 88), and the next 80 amino acids show nearly 50% identity (Powers, et al., 1984).

Based on their extensive sequence and structural

homology, yeast and mammalian Ras proteins show an apparent functional homology. Mammalian ras can substitute for yeast RAS genes in S. cerevisiae (Kataoka, et al., 1985). In turn, mutated RAS1 can transform mammalian cells (Bradley, et al., 1986).

Genetic analysis has provided important insights into the function of the ras proteins in this organism. Deletion of both RAS1 and RAS2 genes, for example, is lethal, but cells can be rescued by the gene (BCY1) encoding the regulatory subunit of the cAMP-dependent protein kinase (Toda et al., 1985). This RAS-deficient strain can be stimulated in vitro by addition of recombinant Ras protein of yeast or mammalian source (Broek et al., 1985). There is no doubt that in S. cerevisiae Ras proteins regulate adenylate cyclase activity and cAMP concentrations (Broek et al., 1989). There is still some doubt, for example, whether Ras activates adenylate cyclase directly or through an intermediary cyclase-associated protein (CAP), and there is strong genetic indication that Ras controls another additional signaling pathway distinct from adenylate cyclase but essential for cell viability (Field et al., 1990). The proteins encoded by IRA1 and IRA2 genes both have homology to mammalian GTPase activating protein (GAP). Strains deleted in IRA1 can be rescued by introducing the mammalian GAP gene (Tanaka et al., 1990). These findings suggest that IRA1 and IRA2 encode GAPs involved in the down-regulation of

Ras.

### **Tumor Promoting Phorbol Esters**

Tumor promoting phorbol esters were isolated from the oil of the *Euphorbiacea Croton tiglium L* and structurally identified to be a diacylglycerol analog (Hecker 1968; van Duuren et al., 1965). The most potent phorbol ester is phorbol-12-myristate-13-acetate (abbreviated as PMA or TPA). One of the best known effects of PMA is to enhance transformation of cells to tumor cells (Blumberg et al., 1988). PMA can induce normal cells into the tumor initiation stage of carcinogenesis (Slaga, 1984).

In addition to its tumor promoter property, PMA has been found to have a pleiotropic action on cells causing alterations in the state of differentiation, proliferation, metastatic potentials and the expression of specific genes (O'Brian and Ward, 1989; Weinstein, 1988; Blumberg, 1988).

PMA binds to and directly activates the phospholipid-dependent protein kinase C (PKC), a major protein in the inositol phosphate signal transduction pathway (Nishizuka, 1988; Person et al., 1988). The activation of PKC by PMA mimics the action of the inositol phosphate generated diacylglycerol on PKC (Castagna et al., 1982; Ashendel, 1985). Activated PKC binds to the plasma membrane and through its catalytic phosphorylations on substrate

effectors, which have not yet been identified, induces the described pleiotropic changes (Kiss, 1990). A part of its action is to increase the expression of the early response oncogenes, *c-fos*, *c-myc* and *c-jun* (Abate and Curran, 1990). The c-Fos protein and c-Jun protein form the AP-1 complex which binds to the PMA or TPA response elements (TREs) present in the phorbol ester responsive genes (Abate and Curran, 1990; Chiu et al., 1988; Sassone-Corsi et al., 1988; Lamph et al., 1988).

The mechanism of activation of PKC by phorbol ester is still a open question. The protein kinase C (PKC) family of phospholipid-dependent protein kinases play a key role in signal transduction. The PKC isoenzymes are normally activated by diacylglycerol (DAG), one of the products of the receptor-mediated hydrolysis of inositol phospholipid, and also by certain drugs, including the phorbol ester tumor promoters. PKC isoenzymes consist of a catalytic (carboxyl-terminal half) and a regulatory domain (amino-terminal half) (Nishizuka, 1988). The catalytic domain contains sequences, including an ATP-binding site, that resemble other protein kinases. All PKC isoenzymes contain a so-called pseudosubstrate motif and a tandem repeat of cysteine-rich sequence in their regulatory domain (Nishizuka, 1988). The central motif of the pseudosubstrate region (RKGALR) is identical in sequence and position in PKC isoforms *a*, *b* and *rPKC* (House and Kemp, 1987). It has been suggested that

cysteine-rich regions in PKC are essential for phorbol-ester binding (Ono et al., 1989). It is possible that the phorbol ester as well as DAG are hydrogen-bonded to the sulfhydryl groups in these cysteine-rich regions (Gechwendt, 1991).

PMA is known to induce the expression of genes coding for collagenase (Whitham et al., 1986), stromelysin (Matrisian et al., 1985), urokinase (Degen et al., 1985; Collart et al., 1986; Bell et al., 1990), cathepsin L (Gottesman and Sobel, 1980) and metallothionein (Imbral and Karin, 1987; Angel et al., 1987). These proteins may play a role in invasion and metastasis (Mignatti et al., 1986). Some products of proto-oncogenes, such as the products of sis (Colamonici et al., 1986), myc (Greenber and Ziff, 1984; Kelly et al., 1983), pro-1 (Lerman and Colburn, 1987), fos (Kruijer et al., 1984), and c-Ha-ras (Balmain et al., 1984; Weinstein, 1987) are potential targets for PKC. The most direct evidence for PKC involvement in tumor metastasis is from an experiment that pre-treats B16 melanoma cell sublines with PMA and shows promotion of the metastatic potential of these treated cells in a lung colonization assay (Gopalakrishna and Barsky, 1988).

### CHAPTER III

#### MATERIALS AND METHODS

##### Materials

Zeta-probe filters were from Bio-Rad (Richmond, CA.), a-32P-dCTP and a-32P-UTP (specific activity 3,000 Ci/mmol) were from ICN Biomedicals, Inc., (Irvine, CA). NACS PREPAC CARTRIDGE and the Nick Translation System were purchased from BRL Life Technologies, Inc., Gaithersburg, MD. Kodak X-OMAT AR Diagnostic Film was from Eastman Kodak Co. (Rochester, NY). Intensifying screen (Dupont Cronex Lighting Plus GJ 220004) was purchased from Dupont Co. (Wilmington, DE). Polyclonal antibodies against human PAI-2 and goat anti-mouse antibody were purchased from American Diagnostica Inc., (New York, NY). The BCA protein assay kit was from Pierce Chemical Company, Rockford, Ill.

The cell culture supplies were purchased from either Flow Laboratories (McLean, VA) or Gibco Laboratories (Grand Island, NY).

The substrate for uPA enzymatic activity analysis, S-2251 (H-D-Val-Leu-Lys-p-nitroanilide) was purchased from KabiVitrum, Stockholm, Sweden. The substrate for cathepsin L

enzymatic activity analysis, N-carbobenzoxy-L-Phe-L-Arg-7-aminomethylcoumarin (Z-Phe-Arg-AMC), was from BACHEM Bioscience Inc. (Philadelphia, PA).

The mouse uPA cDNA plasmid was a gift of D. Belin (University of Geneva), the mouse CL cDNA was a gift of L. Joseph and V. P. Sukhatme (University of Chicago), the mouse CB cDNA was a gift of S. J. Chan (University of Chicago), human uPA cDNA was from W. E. Holmes, (Katholieke Universitert Leuven, Belgium), human TIMP cDNA was a gift of D. F. Carmicheal (Synergen Company, Colorado), human PAI-1 cDNA was from D. J. Loskutoff (Scrips Institute, La Jolla, CA), and human PAI-2 cDNA was a gift from J. E. Sadler (Washington University, St. Louis, MO.). *Drosophila actin* was from Dr. Mark Kelly (Loyola University of Chicago), and human glycerol aldehyde phosphate dehydrogenase cDNA containing plasmid was obtained from American Tissue Culture Collection.

Mouse cathepsin B (1,130 base pairs (bp) (Chan, 1986), mouse uPA (1,000 bp) (Belin, 1985), mouse cathepsin L (1,100 bp) (Joseph et al., 1988), human uPA (880 bp) (Holmes et al., 1985), human TIMP (800 bp) (Carmicheal et al., 1988), PAI-1 (3,000 kb) (Loskutoff, 1985), human PAI-2 (1,800 bp) (Sadler, 1987) cDNA fragments were prepared in this laboratories. Enzymes and chemicals were purchased from Bethesda Research Laboratories (BRL, Geithersburg, MD) and Boehringer Mannheim Biochemicals (Indianapolis, IN). All of

these fragments were obtained by digesting the plasmids with the endonuclease suggested by the providers, and the resulting digested patterns are consistent with the published literatures. The endonucleases utilized to digested these plasmids to obtain the fragments for Northern hybridization are discussed below, under Electrophoresis and Northern Blot Analysis.

### Cell Lines

Non-transformed NIH 3T3 and NIH 3T3 murine fibroblasts transfected with 4 different ras oncogene constructs were obtained from M. O. Bradley (Merck Research Institute, West Point, PA), and have been characterized with respect to their metastatic potential in nude mice (Bradley, et al., 1986). Their characteristics are shown on Table 1.

The PC3-P and PC3-M cell lines, which are human prostate carcinoma cell lines, were obtained from Dr. James Kozlowski (Northwestern University, Chicago, Ill.). The PC3-M cells are highly metastatic in the nude mice, while the PC3-P (parental) cells have a significantly lower metastatic potential (Kozlowski, et al., 1984).

A375-P & A375-M cells lines, derived from a human melanoma, were kindly provided by Dr. I.J. Fidler (University of Texas, M. D. Anderson Medical Center, Houston, TX). A375-P cells show a low metastatic potential



in nude mice and A375-M is a high metastatic derivative (Kozlowski et al., 1984). A summary of major properties of above two cell lines are in Table 2.

The PC3-P and M cells were grown in Eagle's minimal RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS) (Flow Laboratory, McLean, VA) and 100 units/ml of penicillin and 100 ug/ml of streptomycin sulfate (Gibco Laboratories).

The A375-P and SM cells were grown in Eagle minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS) (Flow Laboratory, McLean, VA), 1% MEM nonessential amino acids (Gibco Laboratories, Grand Island, NY), 1 mM sodium pyruvate, 1 mM MEM vitamin solution, 100 units/ml of penicillin and 100 ug/ml of streptomycin sulfate (Gibco Laboratories).

For the subculture of these human cells, the cells were detached from the cell culture flask by using 1 mM of ethylenediamine tetracetic acid (EDTA) in phosphate buffer saline (EDTA-PBS), pH 7.4, and were split 1:5.

All cell lines discussed above were incubated at 37°C in 5% CO<sub>2</sub> saturated with water vapor and maintained by changing the medium every 2-3 days. The total number of passages was less than 3 to keep the genetic drift of the cells to minimal levels.

Medium and supplements used in these cell cultures are summarized in Table 3.

**Table 1 Characteristics of ras Oncogenes Transfected**

**NIH 3T3 Fibroblasts**

(Data from Bradley et al., 1986)

CELL LINE	ONCOGENE CONSTRUCT	Ras MUTATION <sup>a</sup>	Average No. of tumor per lung	S.D.
NIH/3T3	Ras Proto-oncogene	Gly <sup>12</sup> , Ala <sup>59</sup> , Gln <sup>61</sup> ,	0	0
EJ- <u>ras</u>	Human Bladder Carcinoma T-24	<u>Val</u> <sup>12</sup> , Ala <sup>59</sup> , Gln <sup>61</sup> ,	9	14
EJ/vHA- <u>ras</u>	Chimeric Human and Viral-oncogene	<u>Val</u> <sup>12</sup> , <u>Thr</u> <sup>59</sup> , Gln <sup>61</sup> ,	72	32
HYB C <sup>Leu</sup>	Chimeric Yeast and Viral-oncogene	Gly <sup>19</sup> , Ala <sup>66</sup> , <u>Leu</u> <sup>68</sup> ,	25 <sup>b</sup>	40
RAS1 <sup>Leu</sup> del	Recombinant Yeast Oncogene	Gly <sup>19</sup> , Ala <sup>66</sup> , <u>Leu</u> <sup>68</sup> ,	>280 <sup>c</sup>	29

<sup>a</sup>Underlined amino acid represents a mutation from normal amino acid of cellular Ras protein at designated position 12, 59 or 61. In yeast sequence position 19, 66 and 68 are equivalent to 12, 59, 61 of mammalian Ras.

<sup>b</sup>Number of injected cells was  $2.9 \times 10^5$  per mice.

<sup>c</sup>Number of injected cells was  $1.8 \times 10^5$  per mice.

TABLE 2 Experimental Metastasis of Human Tumor Cells  
in Nude Mouse

(Data from Kozlowski et al., 1984)

Cell Line	origin	median no. of apparent lung tumor nodules <sup>a</sup>	no. of animals with microscopic metastases in lung <sup>b</sup>
A375-P	Human Melenoma	0 (0-5) <sup>c</sup>	2/5
A375-M	Human Melenoma	69 (0-250)	5/5
PC3-P	Human Prostate Carcinoma	0	1/15
PC3-M	Human Prostate Carcinoma	38 (0-250)	13/15

<sup>a</sup>Number of grossly apparent tumor foci on lung surface were determined with the aid of a dissecting microscope.

<sup>b</sup>Stepwise sections from lungs of each animal were examined for present of microscopic parenchymal metastases.

<sup>c</sup>Number in parentheses, range.

TABLE 3 Culture Medium of Cell Lines

Name	Species	Medium	Supplement	Detachment media
NIH/3T3	mouse	DMEM	10% HI-FBS <sup>a</sup> gentomycin	0.1% trypsin & 1 mM EDTA
EJ- <u>ras</u>	mouse	DMEM	10% HI-FBS gentomycin	0.1% trypsin in 1 mM EDTA
EJ/vHa- <u>ras</u>	mouse	DMEM	10% HI-FBS gentomycin	0.1% trypsin & 1 mM EDTA
HYB C	mouse	DMEM	10% HI-FBS gentomycin	0.1% trypsin & 1 mM EDTA
<u>RAS1</u> <sup>Leu</sup> del	mouse	DMEM	10% HI-FBS gentomycin	0.1% trypsin & 1 mM EDTA
PC3-P	human	RMPI 1640	10% HI-FBS S. P. <sup>b</sup>	1 mM EDTA
PC3-M	human	RMPI 1640	10% HI-FBS S. P.	1 mM EDTA
A375-P	human	MEM	10% HI-FBS S. P.	1 mM EDTA
A375-SM	human	MEM	10%-HI-FBS S. P.	1 mM EDTA

<sup>a</sup>HI-FBS: heat-inactivated fetal bovine serum.

<sup>b</sup>S.P.: streptomycin 100 ug/ml and pencillin 100 units/ml.

## Preparation of mRNA

Northern blots were used to study the correlation between steady-state mRNA levels of the genes and metastatic potentials. The procedure for preparation of glasswares and the solutions were ones described by Maniatis (1984). The cultured cells were washed with cold phosphate-buffer saline (PBS), removed from the flasks with 1 mM EDTA-PBS and pelleted by centrifugation. The pellet was resuspended with 1 ml of 10 mM Tris (pH 7.9), 1.5 mM MgCl<sub>2</sub>, and 0.15 M NaCl. The suspension was mixed with 200 ul of detergent solution containing 10 mM Tris, 1.5 mM MgCl<sub>2</sub>, 1.5 mM MgCl<sub>2</sub>, 0.15 M NaCl and 13% NP-40, and kept in ice for 10 min. The nuclei were pelleted at 10,000 rpm, the supernatant was poured into a 30 ml Corex tube with 1 ml of buffer (10 mM Tris, 0.35 M NaCl, 5 mM EDTA, 1% SDS and 7 M urea), 2 ml phenol and 6 ml chloroform. The pellet was saved at -70°C for later nuclear transcription assays. The initial supernatant was centrifuged again and the upper liquid portion was transferred to a clean tube. After the addition of 2.5 volumes of cold ethanol, the tube kept at -20°C overnight. The RNA precipitate was obtained by centrifuging at 10,000 rpm for 20 min. The concentration of RNA was determined with a UV spectrophotometer at 260 nm (Maniatis, 1982).

## Electrophoresis and Northern Blot Analysis

The poly(A) rich RNA was obtained using poly(dT) resin chromatography (BRL). The resulting RNAs were separated overnight by electrophoresis in 1% agarose, 0.02% formaldehyde gel in MOPS buffer (0.2 M MOPS, 0.05 M sodium acetate, 0.01 M EDTA) at 25 V. The RNAs were transferred to Zeta-probe Membrane (Bio-Rad, CA) by the capillary transfer method in 20 x SSC (1x SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) overnight and immobilized by the UV crosslinking technique (UV Stratalinker 1800, Stratagene, La Jolla, CA). For preparation of the respective cDNA probes, the mouse uPA cDNA containing plasmid was double digested with Hind III/EcoR1; the mouse cathepsin B plasmid was double digested with Hind III/EcoR1; the mouse cathepsin L plasmid with BamH1, the human TIMP plasmid with EcoR1; human PAI-1 plasmid with EcoR1; human PAI-2 plasmid with EcoR1. The DNA fragments were separated in 1% low-melting agarose (Sigma Chemicals Co.) by electrophoresis and the fragments of interest were identified by their expected molecular weight, and isolated from the gel by razor blade.

The fragments were labeled with  $^{32}$ -P-dCTP by a random-primer labeling kit (Boehringer Mannheim Biochemicals). Briefly, DNA fragments were first denatured by heating for 10 min at 95°C and subsequently cooling on ice. The DNA hexanucleotide mixture, 2 U Klenow enzyme and 50 uCi of 32-

p-dCTP were mixed with a buffer solution containing 0.25 mM dATP, dGTP, dTTP, the reaction carried on in 37°C for 1 hr, and unincorporated <sup>32</sup>-P-dCTP separated with a NACS Prepac Cartridge (BRL).

Sometimes labeling was carried out by the nick translation procedure (Nick Translation System, BRL Life Technologies, Inc., Gaithersburg, MD). The plasmid DNA was added to the reaction mixture containing dATP, dGTP, dTTP, and <sup>32</sup>-P-dCTP (ICN Biomedicals, Inc., Irvine, CA) and nick-translated by incubation with 1 U of DNA polymerase I and 100 pg of DNase I at 15°C for 60 min.

The labeled DNA fragments were hybridized in to the filter bound in RNAs in a hybridization solution containing 0.5 M NaH<sub>2</sub>PO<sub>4</sub> pH 7.2, 0.25 M EDTA and 1% bovine serum albumin at 65°C overnight. The filter was washed 2 times for 30 min each with 10 mM EDTA, 40 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.5% bovine serum albumin fragment V (Boehringer Mannheim Biochemicals) and 1% SDS; and two times for 30 min each with 10 mM EDTA, 40 mM NaH<sub>2</sub>PO<sub>4</sub>, 1% SDS. The filter was exposed to X-ray film (Kodak X-OMAT AR Diagnostic Film, Eastman Kodak Co., Rochester, NY) at -70°C with an intensifying screen (Dupont Cronex Lighting Plus GJ 220004, Dupont Co., Wilmington, DE). To estimate the relative amounts of hybridization, the autoradiograms were scanned with a Beckman Densitometry System or other similar densitometer. For rehybridization,

the attached probe was removed by washing the filter at 95°C 2 times for 20 min each in 0.1 x SSC containing 0.5% SDS. The filter was exposed to X-ray film to check the efficiency of the stripping. The stripped filter was then rehybridized as described above.

For the internal standard control, the filter was similarly hybridized with either <sup>32</sup>P-labeled plasmids containing the Drosophila actin cDNA (Fryberg et al., 1983) obtained from Dr. Mark Kelly (Loyola University Medical Center) or a human glyceraldehyde-3-phosphate dehydrogenase (GAPD) (American Tissue Culture Collection), and the labeled filter exposed to X-ray film.

#### **Time Course of Stimulation of Protease and Anti-Protease Genes by Phorbol Ester**

Phorbol ester stimulation of gene expressions of proteases and protease inhibitors were assayed by the slot and Northern blot techniques. In these procedures the cells were grown in T-75 culture flasks (GIBCO, Grand Island, NY) until subconfluent. The culture medium was then removed and 10 ml fresh medium with 10% heat-inactivated fetal bovine serum (HI-FBS) and 20 nM phorbol-12-myristate-13-acetate (PMA, Sigma Chemical Co.) was added. After incubation with PMA for 2, 4, 8, 16, and 32 hr, the cells were harvested in phosphate buffer saline containing 1 mM EDTA (PBS-EDTA) and



centrifuged at 500 x g. The cells without PMA treatment, designated 0 hr, was used as the unstimulated control. The cytoplasmic RNA was extracted (see above). A Zeta-probe filter was soaked in deionized water for 30 min, and the filter was placed in a slot blot apparatus (Bio-Rad). RNAs were denatured by incubating in a solution containing 45% formamide, 0.01 M MOPS (Sigma Chemical Co.), 0.05 M sodium acetate, 10 mM EDTA, 20% formaldehyde, 7.5% glycerol at 95°C for 10 min. The filter was then rinsed in a solution containing 10 mM NaOH and 1 mM EDTA, and hybridized with <sup>32</sup>P-labeled probes by the procedure described previously. Northern blots were performed with RNA samples at a given time points to verify the observations based on slot blots.

### **Run-on Assay for Transcription Rate**

Increases in mRNA steady-state concentration may be due to either an increased rate of synthesis or to a decreased rate of degradation. In order to determine the mechanism for the changes in mRNA levels observed in slot blot procedure, run-on assays were carried out.

The procedure was as described by Nevins, 1987.

Nuclei, prepared as previously described in the section on preparation of mRNA above, were washed in the reaction buffer (20 mM Tris, pH 7.9; 20% glycerol; 140 mM KCl; 10 mM MgCl<sub>2</sub>; 1 mM dithiothreitol; 1 mM each ATP, CTP, GTP). The

nuclei were quickly frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$  in a freezer.

Nuclei were resuspended in reaction buffer containing nucleotide triphosphates, alpha- $^{32}\text{P}$ -UTP (ICN Biomedicals, Inc., Irvine, CA) and creatine kinase (BRL, Gaithersburg, MD). The nuclei were incubated at  $37^{\circ}\text{C}$  for 10 min. The reaction was stopped by the addition of a 1 ml solution containing 10 mM Tris (pH 7.4), 0.5 M  $\text{NaCl}_2$ , 50 mM  $\text{MgCl}_2$  and 2 mM  $\text{CaCl}_2$  to lyse the nuclei. The unincorporated label was removed by a 3 times repeated ethanol precipitation of the labeled product. Labeled RNA was dissolved in 200  $\mu\text{l}$  of 0.02 M Tris-HCl (pH 7.4), 1 mM  $\text{MgCl}_2$  and incubated with 50  $\mu\text{g}/\text{ml}$  DNase (Sigma Chemical Co.) at  $37^{\circ}\text{C}$  for 15 min. DNase was removed by extraction with phenol-chloroform. The RNA product was fragmented by incubating with 0.2 N NaOH on ice for 30 min, and an aliquot was removed for measuring incorporated radioactivities with a Beckman LS 7500 Liquid Scintillation System.

The plasmids containing the cDNAs for the genes of interest were denatured by boiling the DNA in 0.1 N NaOH for 10 min. A nitrocellulose filter (Bio-Rad) was washed with 6 x SSC (1 X SSC = 0.15 M sodium acetate, 0.15 M sodium chloride). The denatured cDNA-containing plasmids were slowly loaded onto the filter. The filter was washed with 6 x SSC, dried, and baked in a vacuum oven at  $80^{\circ}\text{C}$  for 2 hr.

The dried filter was first incubated in prehybridization buffer containing 50 mM HEPES (pH 7.4), 0.3 M NaCl, 10 mM EDTA, 0.2% SDS, 1 mg/ml yeast tRNA (Boehringer Mannheim Biochemicals, Indianapolis, IN), 0.5 mg/ml poly(A), 1% sodium pyrophosphate, 5 x Denhardts't solution ion (1 x Denharht 'solution equal 0.02 % polyvinylpyrilydone, 0.02% bovine serum albumin, 0.02% Ficoll 400 and 0.02% BSA) without albumin for 60 hr and replaced with hybridization buffer containing 50 mM HEPES (pH 7.4), 0.3 M NaCl, 10 mM EDTA, 0.2% SDS, 100 ug/ml yeast tRNA, 100 ug/ml poly(A), 0.1% sodium pyrophosphate, 1 x Denhardt's solution and equal counts of the labeled RNA. The filter was incubated at 65°C for 36-40 hr, washed 3 times with 2 x SSC at 65°C for 30 min, and treated with a mixture of T1 RNase (5 U/ml, Borhringer Mannheim Biochemicals) and pancreatic RNase (2.5 ug/ml, Sigma Chemical Co.) in 2 x SSC for 1 hr at 37°C. After digestion, the fluid was removed, and the filter was washed 2 times with 2 x SSC at room temperature for 30 min. The filter was then exposed to X-ray film (Kodak X-OMAT AR Diagnostica Film, Eastman Kodak Co., Rochester, NY) at -70°C with an intensifying screen (Dupont Cronex Light Plus GH 220004, Dupont Co., Wilmington DE).

### **Enzyme Assays for Cathepsin L**

NIH/3T3 fibroblasts, EJ/vHa-ras and RAS1<sup>Leu</sup>del-

transformed NIH/3T3 fibroblasts were cultured 50,000 cells/well in 24-well flasks (Flow Laboratories). The cells were grown in Dulbecco's modified Eagle's minimal essential media (DMEM) with 10% heat-inactivated FBS and 50 ug/ml Gentamicin until subconfluent (80-90%), washed with PBS and incubated in serum-free media for 24 h, the media collected, and centrifuged at 10,000 rpm in Beckman Microfuge E.

The cells remaining in the wells were lifted with 1 mM EDTA in PBS and counted in a model ZBI Coulter counter, the cells lysed with 1% SDS and 1 mM EDTA in PBS, and dialyzed against PBS for overnight in the cold room with 3 times changes. The resulting preparation were saved in  $-20^{\circ}\text{C}$ . The substrate N-carbobenzoxy-L-Phe-L-Arg-7-aminomethylcoumarin (Z-Phe-Arg-AMC) (BACHEM Bioscience Inc. Philadelphia, PA) was used to assay for cathepsin L (Mason, 1986; Baricos, et al., 1988). Both cathepsin B and L can catalyze the hydrolysis of this substrate. Based on the  $k_{\text{cat}}$  and  $K_{\text{m}}$  already reported for these substrates with the two enzymes from human and rat (Mason, 1986; Baricos, et al., 1988) at the substrate concentration of 5  $\mu\text{M}$ , the cathepsin L/cathepsin B activity ratio for Z-Phe-Arg-AMC was calculated to be 9 to 1. Procathepsin L in the cells was activated by pepsin (3900 units/mg protein, Sigma). The optimal concentration of pepsin for activation of procathepsin L was 1-2.5 mg/ml, which was determined experimentally by varying the pepsin concentration stock

solution (between 0 and 10 mg/ml in 0.2 M sodium acetate, pH 4.2) in activation buffer (30 mM dithiothreitol and 15 mM EDTA, pH 5.2) for 30 min at 37°C. The activity of the activated enzyme was measured by the addition of 0.8 ml of 0.2 M Na citrate/Na phosphate at pH 6.2 containing Z-Phe-Arg-AMC at final concentration of 5  $\mu$ M. The appearance of product 7-aminomethylcoumarin (AMC) was followed in a SLM-Aminco SPF-500 C spectrofluorometer at room temperature at excitation and emission wavelengths of 370 and 450 nm, respectively.

#### **Enzyme Assay for uPA**

Urokinase plasminogen activator activity was measured spectroscopically using a chromogenic substrate for plasmin as previously described by Whur et al., 1980. The plasminogen was pre-treated with diisopropylfluorophosphate (DFP), to inhibit any active plasmin activity present. Specifically, plasminogen was incubated with 25 mM DFP in 50 mM Tris buffer, pH 8.1, at 37°C for 1 hour, and then extensively dialyzing against the same buffer. Plasmin activity was measured in the DFP-treated plasminogen to ensure endogenous activity was undetected by the assay.

Fifty  $\mu$ l of the medium was added to 100  $\mu$ l of 100 mM Tris buffer, pH 8.1, containing 5  $\mu$ g of DFP-treated plasminogen (Sigma Chemical Co.), 15  $\mu$ g of S-2251 (H-D-Val-

Leu-Lys-p-nitroanilide, KabiVitrum, Stockholm, Sweden), and 0.1% Triton X-100. After incubating for 16 hr at 37°C, the concentration of p-nitroaniline, corresponding to substrate hydrolysis by plasmin, was read at 405 nm in a Perkin-Elmer 320 spectrometer.

The background activity was determined by subtracting reaction buffer for conditioned medium, and each subtracting the background from each reading. Each experiment was performed in three separate wells. Plasminogen activator activity was expressed in absorbance per  $10^6$  cells per hour.

### **Antisense Oligodeoxynucleotides Transfection**

The mouse urokinase gene (Degen *et al.*, 1987) and the mouse cathepsin L cDNA (Troen *et al.*, 1988) have been sequenced. The three 21-mer oligonucleotides sense-uPA (5'-ATGAAAGTCTGGCTGGCGAGC-3'), anti-uPA (5'-GCTCGCCAGCCAGACTTTCAT-3'), anti-CL (5'-CAAAAGGAGTAAAAGATTCAT-3') were made. The anti-uPA and anti-CL sequences were in an antisense directions, respectively, against the first 21 nucleotides starting from the 5' start of translation in the mRNAs for uPA and CL, respectively. The sense-uPA was made in the sense direction from the start of translation of the uPA mRNA and was used as a control oligo. In initial experiments NIH/3T3, EJ-vHa-ras- and

RAS1<sup>Leu</sup>del-transformed cells were seeded 50,000 cells per well in a 24 well Petri dish (Flow Lab) and incubated for 24 hr in media containing 10% heated-inactivated FBS (HI-FBS). The medium was changed to media containing an oligonucleotide and incubated for 24 h. Anti-CL and anti-uPA were used at concentration of 10 uM, 50 uM, 100 uM and 200 uM, 0.3 ml/per well. The wells were washed 3 x with serum-free media, 0.5 ml of serum-free media added to each well and conditioned media removed every 12 hr, up to for 48 hr. The conditioned media was centrifuged at 10,000 rpm (Beckman) in 1.5 ml microcentrifuge tubes and saved at -20 °C until assayed.

### **Lung Colonization Assay**

The murine lung colonization assay was performed in a similar manner as previously described (Bradley et al., 1986). The semiconfluent cells were harvested by adding 0.1% trypsin and 1 mM EDTA in PBS and the cells were quickly removed from trypsin in complete medium, centrifuged at 500 x g, and resuspended in PBS (no calcium or magnesium). Cells were counted in a Coulter counter, and diluted to 1,500,000 cells/ml. Cells were maintained on ice prior to injection. Athymic female nude mice (Ncr *nu/nu*) were obtained from NIH-Frederick Facility and housed in the Loyola Medical Center Animal Care Facility in sterile cages for 1 week prior to

use. The mice used in the experiment were 4-5 weeks of age at the time of injection. A 0.2-ml cell suspension (300,000 cells/mouse) was slowly injected into the lateral tail vein with a 26-gauge needle. The injection schedule was alternated between animals in the control group and animals in the experimental group, to minimize experimental variances, especially time. At end of injection, cell viability was determined by the trypan blue dye exclusion test (Phillips, 1973) assay. Viable cells ranged 91-97% under the experimental conditions.

The mice were maintained under sterile barrier conditions and sacrificed 14 days after tail vein injection, the lungs were removed, and the animal were examined for disseminated tumor colonies. Lungs were fixed in Bouin's solution and the number of surface colonies were counted under a dissecting microscope.

### **Polyacrylamide Gel Electrophoresis**

Discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed to verify the induced proteins in Western blots after phorbol ester stimulation of the cell lines. The procedure was that of Laemmli (1970) as detailed by Davis et al. (1986). All electrophoresis equipment and reagents were from Bio-Rad (Richmond, CA). Gels were 16 X 18 X 0.75 cm thick and are composed of a



stacking gel (4-5 cm) of 3% acrylamide and a resolving gel of 11% acrylamide. Electrophoresis was performed at 10 mA for overnight in a running buffer of 0.025 M Tris, 0.192 M glycine (pH 8.3) containing 1% SDS. The lower gel buffer was 0.5 M Tris pH 6.3 containing 1.5 M Tris (pH 8.8) and 0.4% SDS. The stacking gel buffer was 0.5 M Tris, pH 6.3, containing 0.4% SDS. Samples for electrophoresis were diluted 1:1 in sample buffer (10 ml glycerol, 23 ml of 10% SDS, 2 ml of 1% bromphenol blue, 8.3 ml stacking gel buffer and 6.7 ml H<sub>2</sub>O).

### **Western Blot**

Western blot analysis was used to detect increased amount of protein after phorbol ester stimulation. Polyclonal antibodies against human PAI-2 and urokinase were purchased from American Diagnostica Inc. (New York, NY). The cells were grown in 6-well cell culture cluster dish (GIBCO, Grand Island, NY) to subconfluence. Culture media (1 ml) containing 20 nM PMA and 10% HI-FBS were added into the flasks. The media were collected and the attached cells were lysed with 0.2 ml of solution containing 1% SDS and 1 mM EDTA. The lysed cells were mixed with the collected culture media and saved at -20°C until assayed. The protein concentrations were determined by the BCA protein assay method (Pierce Chemical Company, Rockford, Ill.). The

procedure for SDS-polyacrylamide gel electrophoresis was the same as that described as above. Ten mg of the protein sample were loaded into each well. After electrophoresis, the protein were transferred to a nitrocellulose filter (Bio-Rad, Richmond, CA) with an electrophoretic transfer apparatus (Bio-Rad) in a transfer buffer containing 0.05 M Tris, 0.2 M NaCl, 0.05% Tween and 1% bovine serum albumin (TBST buffer) for overnight at 4°C to block any non-specific binding sites, washed for 30 min with TBST twice, and then incubated with mouse anti-human antibody (American Diagnostica Inc. New York, NY) at a concentration of 5 ug/ml for 2 hr. Nonspecifically bound antibody was removed by washing the filter with TBS 3 times. Biotinylated goat anti-mouse IgG (BRL) was next added at a concentration of 1.5 ug/ml in TBST. Bound antibody was detected with avidin conjugated to horse-radish peroxidase (Avidin-HRP) and BCIP/NBT (5-bromo-4-chloro-3-indoylphosphate/nitroblue tetrazolium) as the substrate in carbonate buffer (0.1 N NaHCO<sub>3</sub>, 10 mM MgCl<sub>2</sub>, pH 9.8) was added for color development.

### **Preparation of Cultured Media from Organ Fragments of Nude Mice**

The procedure for the collection of conditioned media from organ culture was the similar to that described by

(Price et al., 1988). Female balc mice (5-6 weeks) were purchased from NCI-Frederick Facility and housed in the Loyola Medical Center Animal Care Facility in sterile cages for 1 week prior to use. The mice were sacrificed by exposure to ether, and their lungs, kidneys and spleens were detached. The organs were rinsed with the medium, and fatty tissue was removed. The organs were cut with a razor blade into 1-2 mm<sup>3</sup> pieces, washed 3 times with cold RMPI 1640 culture medium, and the pieces were incubated in cell culture dishes with 10 ml of RMPI 1640 at 37 C with 1% fetal bovine serum for 6 hours, and replaced with 10 ml of fresh media containing 10 % HI-FBS (Flow Lab). The media were collected after 24 hr, and centrifuged at 500 x g to remove debris.

## CHAPTER IV RESULTS

### 1. Expression of Mouse Urokinase mRNA in NIH 3T3 Fibroblasts and ras Oncogene Transformed NIH 3T3 Cell Lines with Phorbol Ester (PMA) Treatment.

The NIH 3T3 cell lines transformed by the oncogenes EJ-ras, EJ/vHA-ras, HYB C<sup>Leu</sup> and RAS1<sup>Leu</sup>del have been previously described (Bradley et al., 1986). EJ-ras contains an activating Gly to Val mutation at codon 12 and was originally isolated from a human carcinoma (Capon et al., 1983). The EJ/vHA-ras is a chimeric construct (Gibbs et al., 1984), which combines the first 22 amino acid codons of EJ-ras and the remaining COOH-end amino acids from the viral v-Ha-ras gene. EJ/vHA-ras has two activating mutations, at codons 12 and 59. HYB C<sup>Leu</sup> and RAS1<sup>Leu</sup>del are two yeast RAS1 containing sequences used to transform the NIH 3T3 cells (Defoe-Jones et al., 1985). The HYB C<sup>Leu</sup> gene contains the yeast RAS1 sequences 1 to 161 joined to amino acids 154 to 189 from the v-Ha-ras sequence with residue 68 mutated to Leu. Amino acid 68 is structurally analogous to amino acid 61 in the mammalian Ha-ras protein sequence (Table 4). The RAS1<sup>Leu</sup>del contains all yeast RAS1 sequences and was prepared

by deleting amino acid codons 186 through 302 of RAS1 and then ligating to codon 186 the COOH-terminal codons for residues 303 to 309 in the RAS1 sequence which provide the Cys-a-a-X sequence required for farnesylation and membrane association (Hancock, et al., 1989). The sequence also contains the activating Leu mutation at amino acid position 68 (analogous to position 61 in the Ha-ras protein sequence, Table 4). These activated mammalian ras and yeast RAS genes transform NIH 3T3 fibroblasts into cells which are tumorigenic and metastatic in nude mice (Bradley et al., 1986). The ras-transformed cells do not show a correlation of metastatic ability with the amount of activated Ras protein present (Bradley et al., 1986). Of the Ras transformed cells, the EJ/vHa-ras and the RAS1<sup>Leu</sup>del transformations appear most malignant, each giving a 9- to 10-fold greater number of lung colonies than the other activated Ras transformed cell lines (EJ-ras and HYB C<sup>Leu</sup>-ras) when tail vein injected into nude mice (Bradley et al., 1986). The prior results of Bradley on the nude mice assay of these ras-transformed cells are summarized in Table 1.

To study the correlation between mouse urokinase expression and tumor metastatic potentials, the RNAs were isolated from untransformed NIH 3T3 fibroblast and the four different ras oncogene transformed mouse cell lines, under subconfluent conditions. A slot blot analysis was carried out to study relative amount of uPA mRNA among these cell

lines. The results are shown on Figure 1. The highly malignant EJ/vHa-ras-transformed cells show a high constitutive level of uPA mRNA over NIH 3T3 controls and other ras oncogene-transformed cells, (see zero hour time point in Figure 1). However, the highly malignant RAS1<sup>Leu</sup>del-transformed cells show low levels of uPA mRNA, similar to control NIH 3T3 cells.

Tumor cells may change phenotype in the different steps of metastasis. The change, if it occurs, may be related to environmental signal factors near each of the step barriers. A highly metastatic tumor cell in this paradigm, is characterized by its facile ability to change its phenotype in response to environmental signal factors. As it is currently impossible to study how the protease activities are actually regulated in vivo by signal factors, I tried to obtain support for this idea by adding a tumor promoter, the phorbol ester, phorbol-12-myristate-13-acetate (PMA or TPA), into cell culture. While phorbol ester is not a naturally occurring signal factor, it activates protein kinase C (PKC) which is a part of the inositol phosphate signal transduction pathway (O'Brian and Ward, 1988; Weinstein, 1988; Blumer, 1988). This is one of the major pathways by which extracellular signals are transmitted intracellularly, and thus may mimic natural signals that a tumor cell could encounter in the metastatic process.

Figure 1 then also shows the changes in steady-state

Table 4. Alignment of Ras Sequences<sup>a</sup>

	1 <sup>b</sup>	* <sup>d</sup>					
{EJ- <u>ras</u> }	.....MTE	YKLVVVGAvG	VGKSALTIQL	IQNHFVDEYD	PTIEDSYRKQ		43
{EJ/vHa- <u>ras</u> }	.....MTE	YKLVVVGAvG	VGKSALTIQL	IQNHFVDEYD	PTIEDSYRKQ		
{c-Ha- <u>ras</u> }	.....MTE	YKLVVVGAGG	VGKSALTIQL	IQNHFVDEYD	PTIEDSYRKQ		
{Hyb C}	MQGNKStirE	YKiVVVGgGG	VGKSALTIQF	IQsyFVDEYD	PTIEDSYRKQ		
{RAS1-del}	MQGNKStirE	YKiVVVGgGG	VGKSALTIQF	IQsyFVDEYD	PTIEDSYRKQ		
	1 <sup>c</sup>						50
	44		* *				93
{EJ- <u>ras</u> }	VVIDGETCLL	DILDTAGQEE	YSAMRDQYMR	TGEGFLCVFA	INNTKSFEDI		
{EJ/vHa- <u>ras</u> }	VVIDGETCLL	DILDtGQEE	YSAMRDQYMR	TGEGFLCVFA	INNTKSFEDI		
{c-Ha- <u>ras</u> }	VVIDGETCLL	DILDTAGQEE	YSAMRDQYMR	TGEGFLCVFA	INNTKSFEDI		
{Hyb C}	VVIDdkvsiL	DILDTAGLEE	YSAMREQYMR	TGEGFLlVYs	VtsrnsFDEL		
{RAS1-del}	VVIDdkvsiL	DILDTAGLEE	YSAMREQYMR	TGEGFLlVYs	VtsrnsFDEL		
	51						100
	94						142
{EJ- <u>ras</u> }	HQYREQIKRV	KSDDDVPMVL	VGKCDLA.A	RTVESRQAQD	LARSYGIPYI		
{EJ/vHa- <u>ras</u> }	HQYREQIKRV	KSDDDVPMVL	VGKCDLA.A	RTVESRQAQD	LARSYGIPYI		
{c-Ha- <u>ras</u> }	HQYREQIKRV	KSDDDVPMVL	VGKCDLA.A	RTVESRQAQD	LARSYGIPYI		
{Hyb C}	lsYyqQIqRV	KSDSyIPvVv	VGKlDLeNe	RqVsyedglr	LAkqlnaPF1		
{RAS1-del}	lsYyqQIqRV	KSDSyIPvVv	VGKlDLeNe	RqVsyedglr	LAkqlnaPF1		
	101						150
	143						189
{EJ- <u>ras</u> }	ETSAKTRQGV	EDAFYTLVRE	IRQH..KLRK	LNPPDESGPG	CMSCKCVLS*		
{EJ/vHa- <u>ras</u> }	ETSAKTRQGV	EDAFYTLVRE	IRQH..KLRK	LNPPDESGPG	CMSCKCVLS*		
{c-Ha- <u>ras</u> }	ETSAKTRQGV	EDAFYTLVRE	IRQH..KLRK	LNPPDESGPG	CMSCKCVLS*		
{Hyb C}	ETSAKqainV	DDAFYTLVRE	IRQH..KLRK	LNPPDESGPG	CMSCKCVLS*		
{RAS1-del}	ETSAKqainV	DEAFYsLIRl	VRddggKyns	MNrqlDgGcc	iic*.....		
	151						193

aAlignment made by the PILEUP algorithms in Genetics Computer Group Software [Devereux, J., Haeberli, P., Smithies, O. (1984) Nucleic Acids Research 12, 387-395], using default settings. Upper case letters show consensus between three or more sequences. Sequences utilized from the GenBank and EMBL data bases are human c-Ha-ras-1 [accession number J00277] for c-Ha-ras, murine Sarcoma virus (Harvey-strain) H-ras gene [accession number X00740] for v-Ha-ras, and yeast (Saccharomyces cerevisiae) ras-H related gene c-ras-sc-1 [accession number X00527] for RAS1. Cell lines containing the constructs were previously prepared and reported by Bradley et al.(1986).

<sup>b</sup> Numbering based on sequence of EJ-ras.

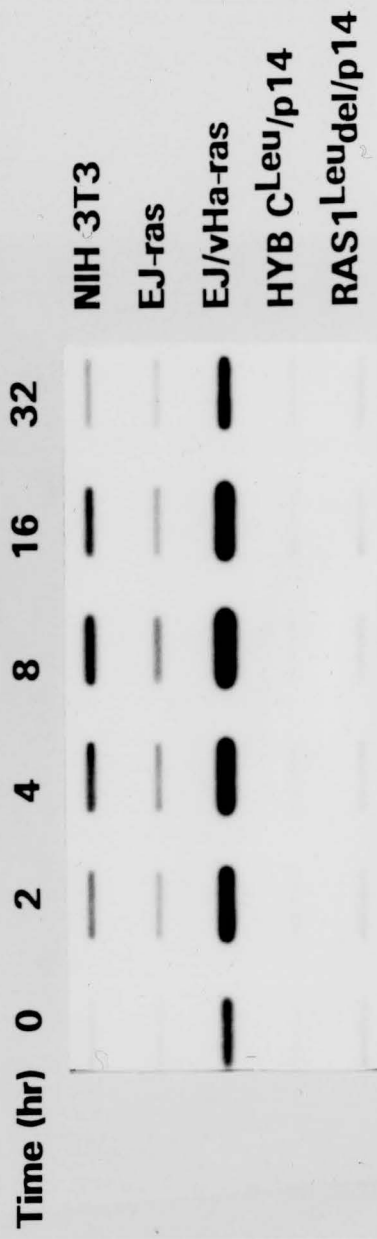
<sup>c</sup> Numbering based on sequence of RAS1<sup>Leu</sup>delprotein.

<sup>d</sup> Asterisk (\*) on top of line designates point of activating mutation in one or more of the sequences. Underlined Cys near COOH-terminal end shows potential farnesylation site.



**Figure 1. Slot Blot Analysis of Mouse Urokinase mRNA Levels from NIH 3T3 Fibroblasts and ras Oncogene Transformed NIH 3T3 Cell Lines.**

The cells were treated with PMA (20 nM) for the designated times, prior to total RNA extraction. The RNAs were immobilized on a Zeta-probe filter and hybridized with <sup>32</sup>P-labeled mouse urokinase cDNA. Washed filter was exposed to X-ray film.



concentrations of uPA mRNA in mouse NIH 3T3 and the ras oncogene-transformed cells treated with 20 nM of PMA, for the time periods indicated. On PMA treatment, increases in uPA mRNA levels occur in the EJ/vHa-ras and EJ-ras transformed cells as well as the untransformed NIH 3T3 cells. In contrast, the normal PMA induction of uPA was suppressed in cells transformed by the RAS1<sup>Leu</sup>del and the HYB C<sup>Leu</sup> yeast ras genes.

The same RNA filter used in Figure 1 was rinsed and rehybridized with a Drosophila actin probe. The filters obtained with mouse urokinase and actin probes were traced with a densitometer. The densitometer trace was normalized to the actin mRNA control. Table 5 shows the average values for uPA mRNA levels from 4 different experiments. In EJ/vHa-ras cells the uPA mRNA levels are 5.4-fold higher than NIH 3T3 cells with a standard deviation 1.7 at 0 hr PMA treatment. The uPA mRNA of other ras transformed cell lines at 0 hr PMA treatment have similar amounts within experimental error of uPA mRNA as NIH 3T3.

With PMA treatment, uPA mRNA showed a 6-fold induction in NIH 3T3 cells and a 2- to 3-fold induction in both EJ-ras and EJ/vHa-ras transformed cells at 8 hours. No induction was observed in the yeast ras transformed lines HYB C<sup>Leu</sup> and the RAS1<sup>Leu</sup>del lines.

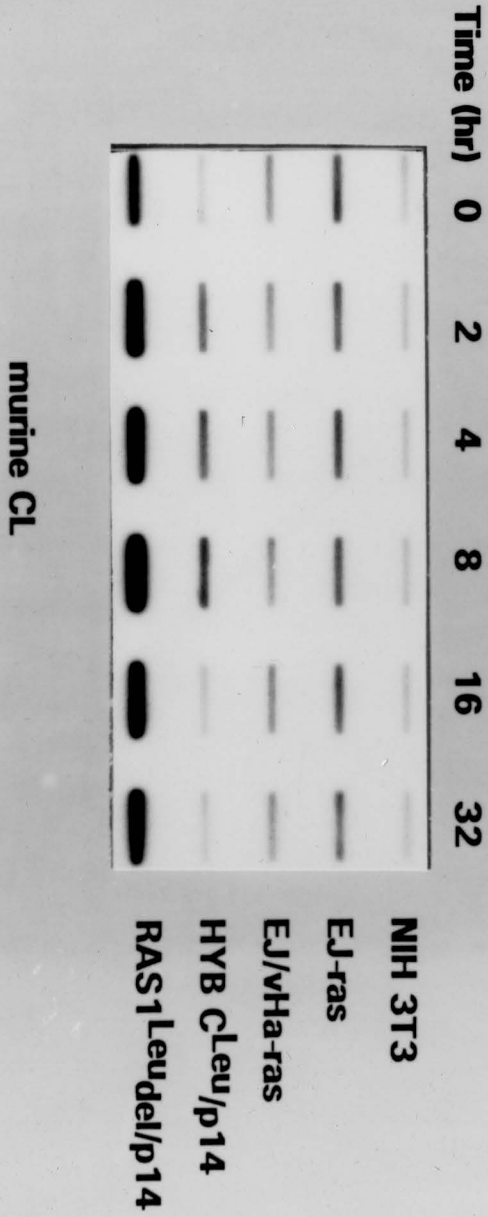
**2. Expression of mouse cathepsin L mRNA in NIH 3T3 Fibroblasts and ras Oncogene Transformed NIH 3T3 Cell Lines with Phorbol Ester (PMA) Treatment.**

As CL was previously reported to be highly expressed in transformed cells (Gottesman, 1988), I applied a similar strategy as above to study the CL mRNA expression in the ras transformed cell lines. Figure 2 shows mRNA levels for CL in NIH 3T3 and ras transformed cell lines treated with PMA for the times indicated. Strikingly, only in the highly malignant RAS1<sup>Leu</sup>del transformed cells are the CL mRNA constitutively expressed at high steady-state concentrations; while in the EJ-ras and EJ/vHa-ras cells the levels are close to one of control NIH 3T3 cells (constitutive levels are observed at the zero time points). In addition, Figure 2 shows that the CL mRNA levels are strongly induced by 20 nM PMA only in the yeast RAS1<sup>Leu</sup>-transformed cells (HYB C<sup>Leu</sup> or RAS1<sup>Leu</sup>del genes cell lines). CL is clearly not induced by 20 nM PMA in the cells transformed by the EJ/vHa-ras, EJ-ras, or in untransformed NIH 3T3 cells.

The same RNA filter used in Figure 2 was rinsed and rehybridized with the Drosophila actin probe. Densitometer traces were normalized to the actin mRNA controls. The experiments were run 4 times, average values of 4 independent experiments with standard deviation are

**Figure 2. Slot Blot Analysis of Mouse Cathepsin L mRNA Levels in NIH 3T3 Fibroblast and ras Oncogenes Transformed NIH 3T3 Cell Lines.**

The cells were treated with PMA (20 nM) for the designated time, the total RNAs were extracted, the RNAs immobilized on a Zeta-probe filter, hybridized with <sup>32</sup>P-labeled mouse cathepsin L cDNA probe, and exposed to X-ray film.



**Table 5. Levels of uPA mRNA among ras Oncogene-transformed Mouse Fibroblasts  
Treated with PMA and Normalized with Actin<sup>a</sup>**

Hours PMA Treatment	0	2	4	8	16	32
NIH/3T3	1.0(0.3) <sup>b</sup>	3.5(0.1)	5.4(1.5)	5.5(2.1)	4.2(1.8)	2.0(0.7)
EJ- <u>ras</u>	1.0(0.1)	1.9(0.3)	3.2(2.1)	2.7(1.2)	2.4(0.6)	1.2(0.7)
EJ/vHA- <u>ras</u>	5.4(1.7)	8.9(0.7)	11.5(1.7)	12.9(2.8)	10.0(4.4)	6.6(2.3)
HYB C <sup>Leu</sup>	0.9(0.3)	1.0(0.2)	1.0(0.2)	1.1(0.1)	1.4(0.4)	0.9(0.3)
RAS1 <sup>Leu</sup> del	1.3(0.4)	1.1(0.1)	1.2(0.4)	1.2(0.2)	1.2(0.3)	1.3(0.3)

<sup>a</sup>mRNAs of the cell lines were determined by slot blots analysis of total RNA (5 ug/per slot). Following autoradiography, the intensities of the slot signals were quantified by densitometry. The results were normalized to actin, which was hybridized and densitometrized with the same filter. The numbers are the average of 4 separate runs and standard deviation is in bracket.

<sup>b</sup>Unit is densitometric value.

**Figure 3. Slot Blot Analysis of Mouse Cathepsin B mRNA Levels in NIH 3T3 Fibroblast and ras Oncogenes Transformed NIH 3T3 Cell Lines.**

The cells were treated with PMA (20 nM) for the designated times, total RNAs were extracted, the RNAs were immobilized on Zeta-probe filter, hybridized with  $^{32}\text{P}$ -labeled mouse cathepsin B cDNA probe, and exposed to X-ray film.



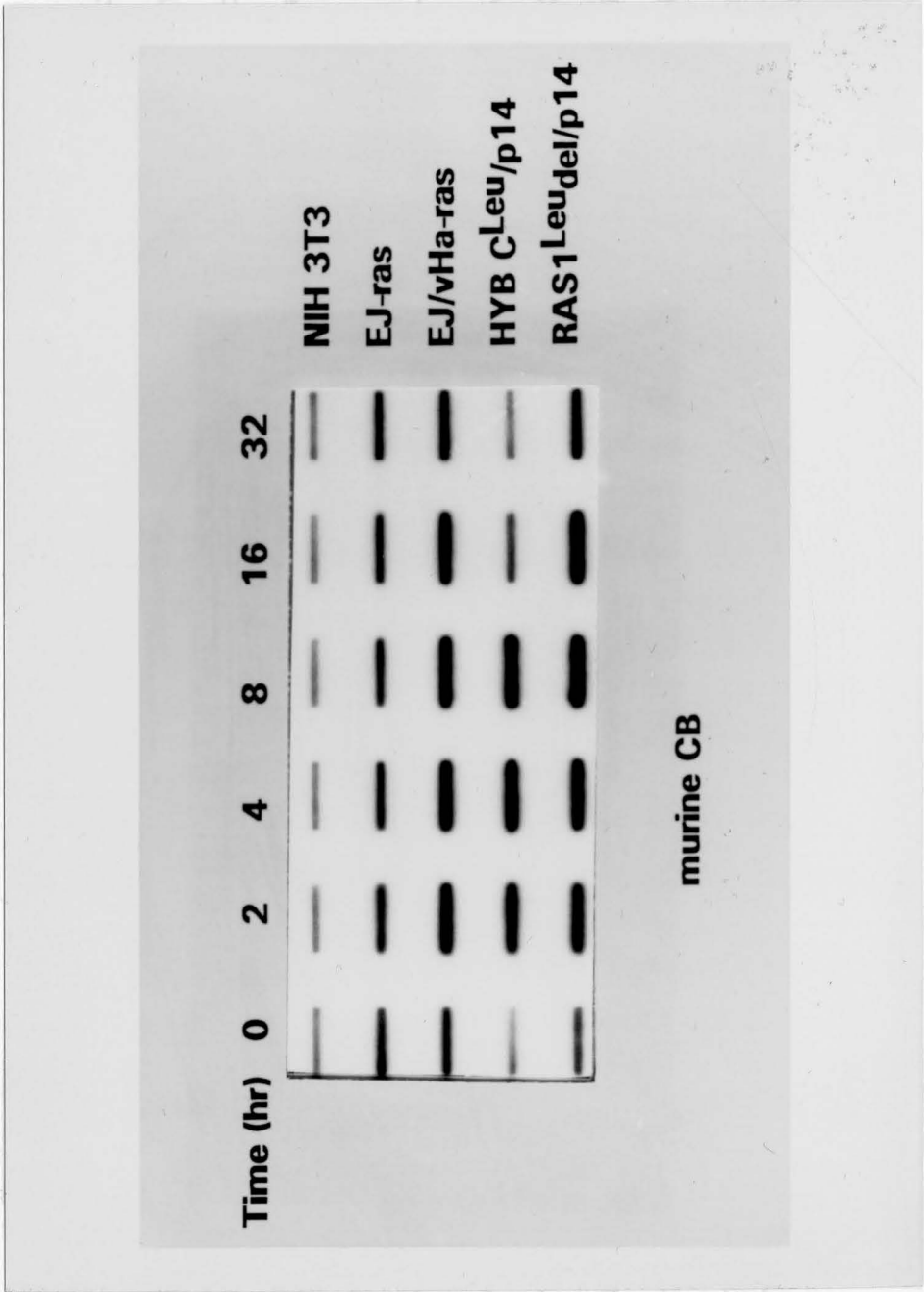


Table 9. Levels of CB mRNA among rat Oncogene-transformed Mouse Fibroblasts

**Table 6. Levels of CL mRNA among ras Oncogene-transformed Mouse Fibroblasts  
Treated with PMA and Normalized with Actin<sup>a</sup>**

Hours PMA Treatment	0	2	4	8	16	32
NIH/3T3	1.4(0.1)	1.4(0.1)	1.5(0.2)	1.2(0.1)	1.3(0.2)	1.1(0.1)
EJ- <u>ras</u>	3.5(0.3)	3.1(0.3)	3.2(0.5)	3.2(0.3)	3.3(0.3)	3.1(0.2)
EJ/vHA- <u>ras</u>	1.8(0.4)	1.6(0.3)	1.5(0.7)	1.5(0.3)	1.6(0.4)	1.8(0.6)
HYB C <sup>Leu</sup>	1.1(0.2)	2.8(0.6)	3.4(1.3)	3.1(1.0)	1.3(0.1)	1.1(0.4)
RAS1 <sup>Leu</sup> del	6.1(1.1)	14.8(4.2)	12.4(4.9)	16.8(1.2)	15.0(2.7)	14.7(4.0)

<sup>a</sup>mRNAs of the cell lines were determined by slot blots analysis of total RNA (5 ug/per slot). Following autoradiography, the intensities of the slot signals were quantified by densitometry. The results were normalized to actin, which was hybridized and densitometrized with the same filter. The numbers are the average of 4 separate runs and standard deviation is in bracket.

<sup>b</sup>Unit is densitometric value.

displayed in Table 6. The data show that CL mRNA levels are 6.1-fold (standard deviation 1.1) greater in RAS1<sup>Leu</sup>del cells than NIH 3T3 cells. The other transformed cell lines show the 1.1 to 3.5-fold higher CL mRNA level than NIH 3T3 cells. The data also show the increase of induction of CL mRNA by PMA was 2.5- to 3.2-fold in HYB C<sup>Leu</sup> and 2- to 3-fold in RAS1<sup>Leu</sup>del at 8 hours. No apparent inductions with PMA were observed in the EJ/vHa-ras, EJ-ras or NIH 3T3 cells.

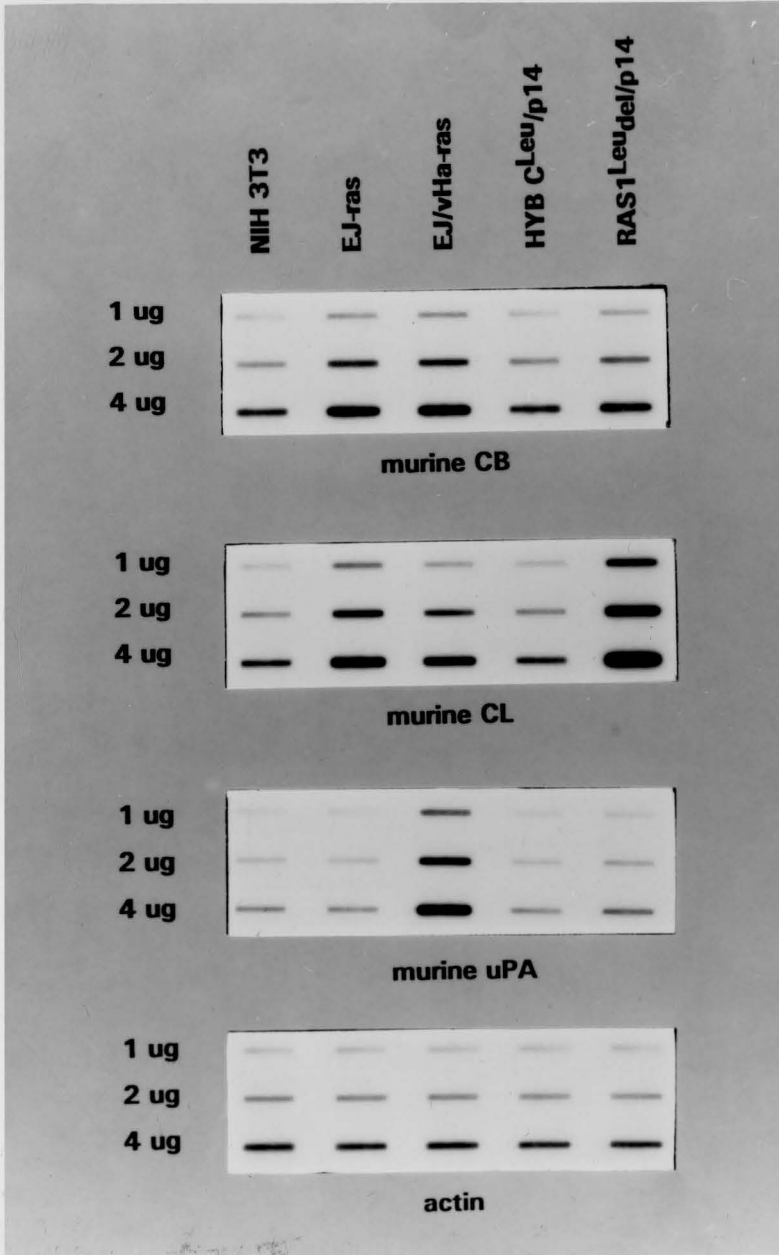
### 3. Expression of Mouse Cathepsin B mRNA in NIH 3T3

#### **Fibroblasts and ras Oncogene Transformed NIH 3T3 Cell Lines with Phorbol Ester (PMA) Treatment.**

Figure 3 shows the changes in CB mRNA steady-state concentrations under conditions of 20 nM PMA stimulation. The changes in CB mRNA are in-between the patterns observed for CL and uPA mRNAs. Constitutively higher levels of CB mRNA are observed in both mammalian-like EJ-ras and EJ/vHa-ras, and in yeast-like RAS1<sup>Leu</sup>del transformed cells. However, neither PMA induction of CB mRNA was observed in the transformed EJ-ras cells, nor is PMA induction observed in the untransformed NIH 3T3 controls. The same RNAs filter was rehybridized with the Drosophila actin probe. Densitometer traces normalized to the actin mRNA controls and averaged over 4 independent experiments (Table 7) show that a 2-fold increase CB mRNA is observed at 4 hours of PMA

**Figure 4. Slot Blot Analysis of Mouse Protease mRNAs Levels from NIH 3T3 Fibroblast and ras Oncogenes Transformed NIH 3T3 Cell Lines after Serial Dilutions.**

Total RNAs were extracted from NIH 3T3 cell and four cell lines transfected with different ras oncogenes without PMA stimulation. The RNAs were serially diluted and immobilized on a Zeta-probe filter and hybridized with <sup>32</sup>P-labeled mouse urokinase, cathepsin L, B and Drosophila cDNA actin probes, and exposed to X-ray film to different times.



**Table 7. Levels of CB mRNA among ras Oncogene-transformed Mouse Fibroblasts  
Treated with PMA and Normalized with Actin<sup>a</sup>**

Hours PMA Treatment	0	2	4	8	16	32
NIH/3T3	2.8(0.5)	3.2(0.3)	3.5(0.6)	3.3(0.3)	2.9(0.3)	2.6(0.5)
EJ- <u>ras</u>	7.8(0.2)	6.8(1.6)	6.6(0.6)	6.5(0.3)	3.3(0.3)	3.1(0.2)
EJ/vHA- <u>ras</u>	5.4(0.3)	12.5(2.0)	12.8(2.4)	9.8(0.7)	8.5(0.8)	5.9(1.0)
HYB C <sup>Leu</sup>	2.4(0.1)	10.3(3.7)	9.9(2.7)	10.2(0.4)	3.6(0.1)	1.1(0.4)
RAS1 <sup>Leu</sup> del	4.2(0.3)	12.8(9.4)	12.1(5.0)	12.3(1.1)	11.0(2.5)	6.3(2.8)

<sup>a</sup>mRNAs of the cell lines were determined by slot blots analysis of total RNA (5 ug/per slot). Following autoradiography, the intensities of the slot signals were quantified by densitometry. The results were normalized to actin, which was hybridized and densitometrized with the same filter. The numbers are the average of 4 separate runs and standard deviation is in bracket.

<sup>b</sup>Unit is densitometric value.

stimulation in cells transformed with mammalian-like EJ/vHA-ras and the increase persisted for up to 8 hours. CB mRNA concentration increased 4-fold at between 2 and 8 hours after PMA stimulation in HYB C<sup>Leu</sup> transformed cells and 3-fold during that same period in RAS1<sup>Leu</sup>del transformed cells.

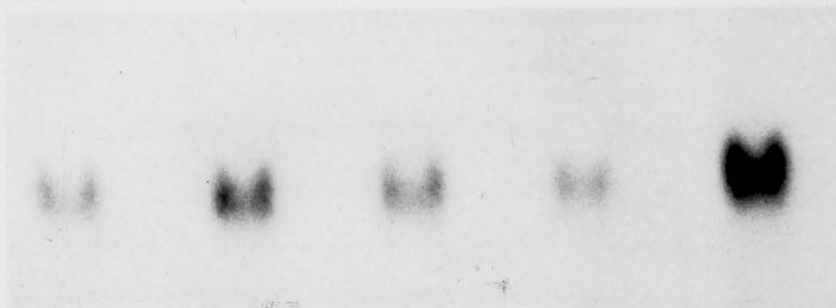
To order to demonstrate that conclusions from the slot blots above are not caused by over-saturating the filter membrane, the RNA samples (without PMA stimulation) were serially diluted from 1 to 4 ug per slot. The filters were hybridized with the cDNA probes under the identical conditions as above. The results are shown in Figure 4. Just as previously described, the highly malignant EJ/vHa-ras-transformed cells show a higher constitutive level of uPA mRNA over NIH 3T3 controls and other ras oncogene-transformed cells. The CL mRNA is constitutively expressed at the highest steady-state concentrations in the highly malignant RAS1<sup>Leu</sup>del transformed cells. Densitometer analysis of the blots confirms the relative concentrations previously described.

To further confirm the results of the slot blots for CL, Northern blots were carried out. The Northern not only compares the concentrations of mRNAs, but also demonstrates the molecular weights of the mRNAs. Cytoplasmic RNAs were isolated from cell cultures without PMA treatment, and the poly(A)<sup>+</sup> mRNAs were obtained with a poly(dT) column. The

**Figure 5. Northern Blot Analysis of Mouse Cathepsin L mRNA Levels from NIH 3T3 Fibroblast and ras Oncogenes Transformed NIH 3T3 Cell Lines.**

Poly(A<sup>+</sup>) mRNAs were extracted from NIH 3T3 cell and four other cell lines transfected with different ras oncogenes. The RNAs were separated by electrophoresis through 1% agarose gel. The RNAs were transferred onto Zeta-probe filter, hybridized with <sup>32</sup>P-labeled mouse cathepsin L cDNA probe (top) or GAPD (bottom), and exposed to X-ray film.



**NIH 3T3****EJ-ras****EJ/vHA-ras****HYB C<sup>Leu</sup>****RAS1<sup>Leu</sup> del**

mRNAs were separated by electrophoresis through a 1% agarose gel. The RNAs were transferred onto a Zeta-Probe filter and hybridized with a  $^{32}\text{P}$ -labeled mouse cathepsin L cDNA probe. The result is displayed on Figure 5. In this figure, only the RAS1<sup>Leu</sup> del transformed cells show highest expression among the other cell lines. Other observations are also consistent with those obtained in the slot blot analyses, described above. The molecular weight of the CL mRNA blotted is 1.8 to 2.0 kilobase (kb), which is consistent with the literature (Troen et al., 1987).

#### **4. Comparison of Transcription Rates of uPA, CB and CL**

**with and without PMA Stimulation in NIH 3T3**

**Fibroblasts and ras Oncogene Transformed NIH 3T3 Cell Lines**

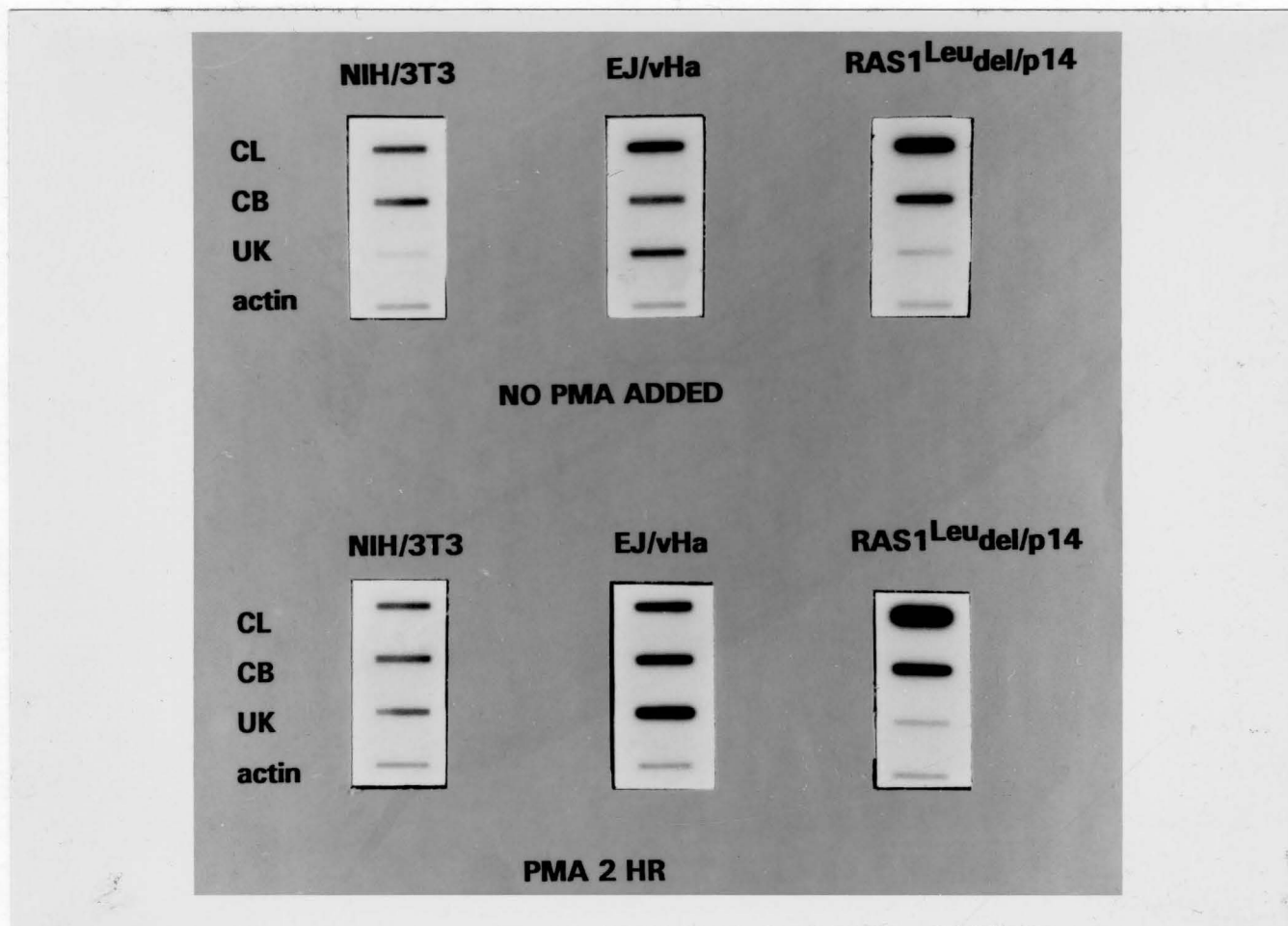
The induction of the protease mRNA levels could be the result of either increases in transcription rates or decreases in mRNA degradation rates. In order to further investigate the mechanism of induction, run-on assays were carried out. The nuclei of the cell were isolated from the cells in culture either with 20 nM PMA for 2 hours or without PMA treatment. The nuclei were incubated with  $^{32}\text{P}$ -UTP. The labeled RNAs were isolated and hybridized to the protease cDNA plasmids immobilized on a nitrocellulose filter. The filters were washed and exposed to X-ray film.

The results are displayed on Figure 6. Of the 3 cell lines studied, the CL gene transcription rate shows a much higher transcription rate and PMA inducibility only in the yeast RAS1<sup>Leu</sup>del transformed cells. PMA stimulation of CL transcription is not observed in the EJ/vHA-ras transformed or untransformed NIH 3T3 fibroblasts. CB transcription rates show a mild increase in both the RAS1<sup>Leu</sup>del transformed cells and the EJ/vHA-ras transformed cells on PMA stimulation, but shows no PMA stimulation in the untransformed NIH 3T3 cells. The transcription rate of the uPA gene is higher in the EJ/vHa-ras transformed cell than the NIH 3T3 cells, uPA gene transcription rate in the RAS1<sup>Leu</sup>del cells is not significantly different from one in NIH 3T3. In addition, PMA shows a significant increase in uPA gene transcription in the untransformed NIH 3T3 cells and in the EJ/vHA-ras transformed cells, but no PMA stimulation of transcription rate is observed in the RAS1<sup>Leu</sup>del-transformed cells.

Results from the densitometer traces of the run-on assays are shown on Table 8. The data shows that the CL gene transcription rate in RAS1<sup>Leu</sup>del cells was 6-fold greater than in NIH 3T3 cells, and PMA causes a further 2-fold increase of CL gene transcription rates was observed in RAS1<sup>Leu</sup>del-transformed cells. CL transcription rate are not PMA stimulated in NIH/3T3 or EJ/vHa-ras-transformed cells. The uPA transcription rate was 7-fold higher in

Figure 6. The Run-on Assays of uPA, CB and CL with and without PMA Stimulation in NIH 3T3 cells, EJ/vHa-ras and RAS1<sup>Leu</sup>del-transformed Cells.

The nuclei were isolated from the cell cultures with PMA treatment (20 nM, 2 hours) or without PMA treatment. Nuclei were incubated with <sup>32</sup>P-UTP, and RNAs were isolated, and hybridized to the cDNAs for uPA, CB and CL immobilized on a nitrocellulose paper. The filters were washed and exposed to X-ray film.



**TABLE 8 Relative mRNA Transcription Rates  
by Run-on Assay<sup>a</sup>**

**A. No PMA treatment**

**CELL LINES**

<b>GENE ASSAYED</b>	<b>NIH/3T3</b>	<b>EJ/vHA-<u>ras</u></b>	<b>RAS1<sup>Leu</sup>DEL</b>
CL	0.89	2.38	5.24
CB	0.86	0.91	1.61
uPA	0.10	0.72	0.13
ACTIN	0.31	0.33	0.30

**B. Treatment with PMA (20 nM) for 2 hours**

**CELL LINES**

<b>GENE ASSAYED</b>	<b>NIH/3T3</b>	<b>EJ/vHA-<u>ras</u></b>	<b>RAS1<sup>Leu</sup>DEL</b>
CL	0.93	2.02	9.61
CB	0.90	1.81	2.70
uPA	0.39	2.75	0.15
ACTIN	0.28	0.35	0.29

<sup>a</sup>Values from desitometer trace of autoradiography.

EJ/vHa-ras-transformed cells than in untransformed NIH 3T3 cell. PMA treatment caused a further 4-fold stimulation of transcription rate in untransformed NIH 3T3 cells and in EJ/vHa-ras-transformed cells. The uPA gene transcription rate was not elevated in the RAS1<sup>Leu</sup>del cell nor was uPA transcription stimulated by PMA in this cell line. The results of the run-on assays mimic the changes observed in steady-state mRNA concentrations in Figures 1, 2 and 3, and demonstrate that the differences observed in the constitutive steady-state mRNA levels and with PMA stimulation for the different ras-transformations are primarily due to increases in transcription rates and not to differences in mRNA stability.

##### **5. Determination of uPA Enzymatic Activities of Conditioned Media from NIH 3T3 Fibroblasts and ras Oncogene Transformed NIH 3T3 Cell Lines Treated with Oligonucleotides**

Based on the observation that uPA mRNA is highly expressed in the cells transformed with the EJ/vHa-ras, while cathepsin L mRNA is highly expressed in the cells transformed with RAS1<sup>Leu</sup>del gene, it would be of interest to test the possibility of different involvements of uPA and CL in a metastatic assay.

Anti-sense oligodeoxynucleotides are short synthetic

nucleotide sequences formulated to be complementary to a specific gene or RNA message. Through the binding of these oligomers to a target sequence in a cell, transcription or translation of the gene can be selectively blocked. A number of studies were completed with oligodeoxynucleotides (reviewed by Rothenberg et al., 1989), which suggests that antisense deoxyoligonucleotide inhibitors may be also able to selectively inhibit the urokinase and CL gene expressions in the ras oncogenes transformed cell lines. If antisense oligonucleotide could inhibit CL and uPA gene expressions, then the cells could be then injected into nude mice to determine the effect of selectively inhibiting uPA and CL gene expressions on experimental metastasis.

Three 21-mer oligonucleotides used in this work were sense-uPA (5'-ATGAAAGTCTGGCTGGCGAGC-3'), anti-uPA (5'-GCTCGCCAGCCAGACTTTCAT-3'), and anti-CL (5'-CAAAAGGAGTAAAAGATTTCAT-3'). The optimum concentration to achieve the maximal inhibition was determined by varying the concentration of the oligo. The secretion of uPA and CL activity into the cell culture medium after removal of these oligonucleotide inhibitors were quantified by chromogenic or fluorogenic substrate assays (discussed in the following paragraphs). A concentration of 10, 20, 50, 100, 200 uM was tested for each of the antisense oligonucleotides, and the optimum concentration to achieve the maximal inhibition was found to be 100 uM for both ant-uPA and anti-CL (Table 9).



This concentration was the one used in the following inhibitory studies with these oligos.

The secretion of uPA activity into the cell culture medium after removal of the oligonucleotide inhibitors was quantified by measuring plasmin activities following the activation of plasminogen to plasmin by the urokinase. Cells were plated into wells and incubated in complete media containing 10% FBS and the different oligos (100  $\mu$ M) for 24 hr as described in methods. The media was removed. The cells were then incubated in serum-free, oligo-free media. This medium was replaced every 12 hrs for upto 48 hrs. The media samples were assayed for secreted uPA enzyme activity.

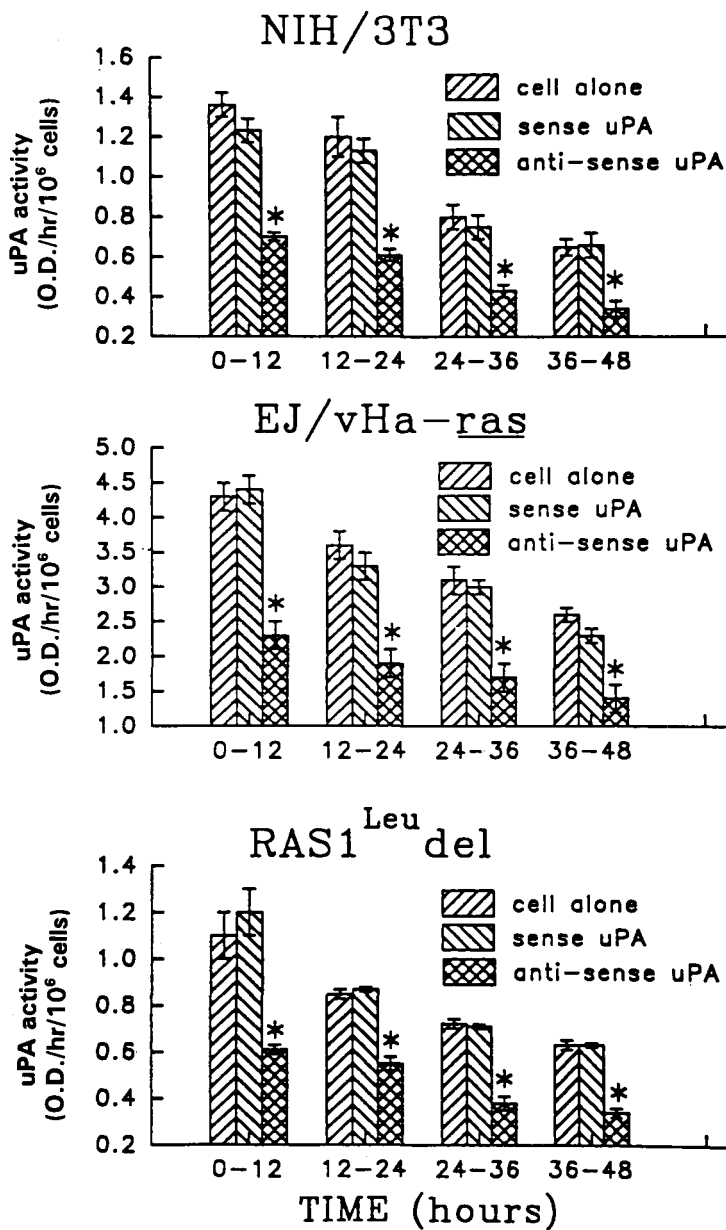
Figure 7 is a demonstration of uPA activities of these cell lines treated with three oligos. EJ/vHa-ras has the highest uPA enzymatic activity of these cell lines. Anti-uPA inhibited secreted activity by approximately 45% in all cell lines. The inhibition lasted for at least 48 hours after removal of the antisense oligo from the media. uPA sense oligo has no obvious effect on the uPA enzymatic activities of these cell lines.

Table 10 gives the numbers plotted in Figure 8 and shows that without the oligonucleotide inhibitor, NIH 3T3 cells displayed a uPA enzymatic activity corresponding to an absorbance 1.36 per hour per  $10^6$  cells for the first 12 hours. This is equal to the activity level of RAS1<sup>Leu</sup>del, whereas EJ/vHA-ras cells showed about a 3-fold higher uPA

**Figure 7. uPA Enzymatic Activities of Conditioned Media from NIH 3T3 Fibroblasts and ras Oncogene Transformed NIH 3T3 Cell Lines on Treatment with Oligodeoxynucleotides.**

Cells were plated into wells and incubated in complete media containing 10% FBS and oligo (100 uM) for 24 hr. The media was removed and the cells were incubated in serum-free, oligo-free media which was removed from cells every 12 hrs up to 48 hrs and assayed for secreted enzyme activity.

Asterisk (\*) on the top of the bars designated specific condition with significant difference from the control group (no oligo treatment).



enzymatic activity. The difference in activity levels are in the direction expected from their respective mRNA levels and gene transcription rates, previously determined. Treatment with sense-uPA oligo caused no apparent change in uPA enzymatic activities in these cell lines studied.

**6. Determination of CL Enzymatic Activities of Conditioned Media from Mouse NIH 3T3 Fibroblast and ras Oncogene Transformed NIH 3T3 Cell Lines Treated with Oligonucleotides.**

CL enzymatic activities were determined against the substrate N-carbobenzoxy-L-Phe-L-Arg-7-aminomethylcoumarin (Z-Phe-Arg-AMC). Total enzymatic activity was first determined both in secreted medium and in membrane associated components. The results are shown in Table 11. As the results show the cells-associated activity is only 11-25 % of total enzymatic activities, the following assays to test inhibitory activities of the oligonucleotide antisense were carried out only on the secreted medium.

The results are displayed in Figure 8. Obviously, RAS1<sup>Leu</sup> del cells has the highest CL enzymatic activity among the cell lines. Anti-CL inhibited secreted activities by about 35% in all cell lines, and its inhibitory effect lasted for 24 hours after removal of the antisense oligo. uPA sense oligo has no noticeable effect on the CL enzymatic

**Table 9. Concentration Dependence of Oligonucleotides  
Inhibitory Effects<sup>a</sup>**

**EJ-vHa-ras Cells**

anti-uPA concentration	uPA activity	% inhibition
no oligo	4.8 <sup>b</sup> (0.9) <sup>c</sup>	
10 uM	4.6 (1.0)	10
20 uM	4.2 (0.9)	13
50 uM	3.7 (0.8)	23
100 uM	2.7 (0.7)	44
200 uM	2.6 (0.5)	45

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**RAS1<sup>Leu</sup>del Cells**

anti-CL concentration	CL activity	% inhibition
no oligo	78.2 <sup>d</sup> (15.2)	
10 uM	75.4 (15.7)	4
20 uM	71.3 (13.8)	9
50 uM	58.6 (11.4)	25
100 uM	50.8 (9.9)	35
200 uM	51.3 (11.1)	34

<sup>a</sup>Enzymatic activities were determined with the first 12 hour serum-free medium after removal of the oligo inhibitors.

<sup>b</sup>uPA enzymatic activities were determined with the chromogenic substrate S-2551. Numbers are the average of 3 separate runs with units of O.D. per 10<sup>6</sup> cells per hour

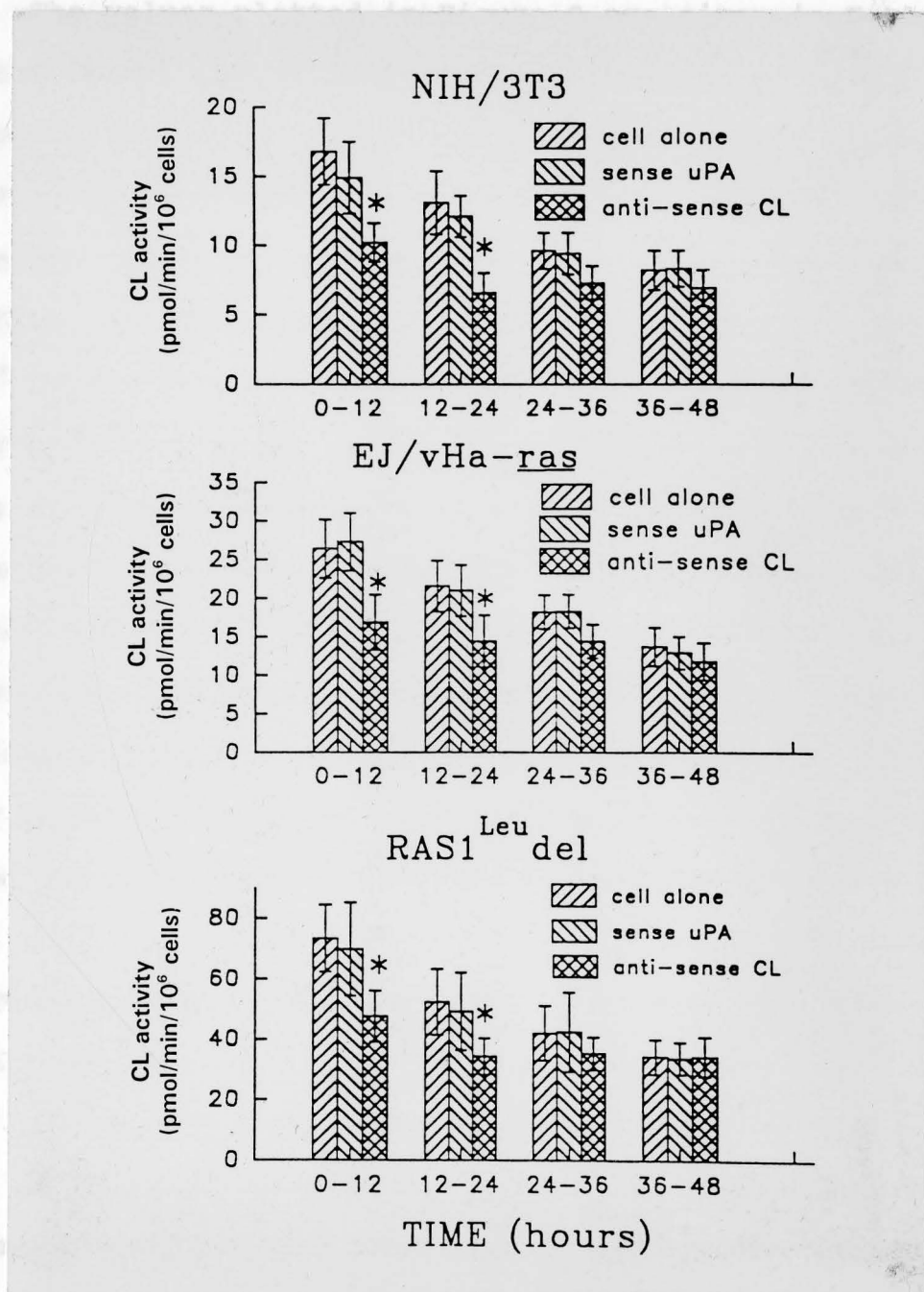
<sup>c</sup>The number in bracket is standard deviation.

<sup>d</sup>CL enzymatic activities were determined by substrate N-carbobenzoxy-L-Phe-L-Arg-7-aminomethylcoumarin (Z-Phe-Arg-AMC) with units of pmol/min/10<sup>6</sup> cells.

**Figure 8. Cathepsin L Enzymatic Activities of Conditioned Media from NIH 3T3 Fibroblasts and ras Oncogene Transformed NIH 3T3 Cell Lines on Treatment with Oligodeoxynucleotides.**

Cells were plated into wells and incubated in complete media containing 10% HI-FBS and oligo (100 uM) for 24 hr. The media was removed and the cells were incubated in serum-free, oligo-free media which was removed from cells every 12 hours up to 48 hours and assayed for the secreted enzyme activity.

Asterisk (\*) on the top of the bars designated specific condition with significant difference from the control group (no oligo treatment).



activities of these cell lines under the experimental conditions.

The values plotted in Figure 8 are given in Table 12 for the anti-CL oligo inhibitory effects on the cell lines. Without oligonucleotide inhibitor, NIH 3T3 cells showed an activity of  $16.8 \text{ pmol/min}/10^6 \text{ cells}$  with a standard deviation 1.4 in the first 12 hrs. EJ/vHA-ras secreted about 1.6-fold more CL enzymatic activity than NIH 3T3, whereas RAS1<sup>Leu</sup>del cells showed a 4.8-fold higher CL activity than NIH 3T3. CL enzymatic activities in all of the these cell lines was not significantly decreased after treatment with the sense-uPA oligos from the activity under non-oligo treated condition. Treatment with anti-CL decreased the CL activity from 33 to 39 % for up to 24 hours. After 24 hours the inhibitory effects of the oligos lessened. At 48 hours after removal of oligo, the inhibitory effect of the anti-CL was not significant.

## **7. Investigation of Tumor Metastatic Potentials by Lung Colonization Assay**

It has been concluded by several investigators that the critical timing for lung colonization in the tail vein assay of metastasis is the first 12 hours following the injection of tumor cells into mice (Fidler, 1978; Humphries et al., 1986; Morikawa et al., 1988). It was observed that



the antisense oligonucleotides have an inhibitory effect on uPA and CL enzymatic activities, and the inhibitory effects of the antisense oligos lasted at least 24 hours after removal of oligonucleotide antisense inhibitors from the cell culture media. Thus, the duration of inhibition falls within the critical time frame for the tail-vein assay of metastasis.

In experiment one, EJ/vHA-ras cells were treated in vitro with the oligonucleotides for 24 hours and the oligo treated tumor cells ( $3 \times 10^5$ ) were injected into the tail veins of 4 - 6 week athymic (Ncr nu/nu) female mice. The mice were sacrificed 14 days later. Lungs were dissected from the mice and the number of surface tumors per lung counted. The results are shown in Table 13. With the sense uPA oligonucleotide treatment (control), injected tumor cells produced an average of 130 tumors per lungs. With anti-CL oligonucleotide treatment, the injected EJ/vHa-ras tumor cells produced an average of 122 tumors per lungs which is not significantly different from the control treated cells, even though CL activity had been decreased 35% by the antisense treatment. With anti-uPA treatment (uPA activity was lowered 45%), the injected tumor cells produced an average of 41 tumors per lung, significantly below the control group ( $p < 0.001$ ).

In experiment two, RAS1<sup>Leu</sup>del cells were treated with the same oligonucleotides. The treated cells were injected

into nude mice and the results are also shown in Table 13. The results show that with the sense uPA oligonucleotide treatment (for control), injected tumor cells produced an average of 94 tumors per lung. With anti-CL oligonucleotide treatment (CL enzymatic activities was lowered about 35%), the cells produced an average of 26 tumors in lung ( $p < 0.01$  with respect to control). With anti-uPA treatment (uPA activities was lowered by 45%), the injected tumor cells produced an average of 81 tumors per lungs, not significantly different from the control group.

The work on RAS1<sup>Leu</sup>del cells were repeated once, and the results are displayed on Table 14. With the sense uPA oligonucleotide treatment (control), the injected tumor cells produced tumors with average 76 per lung. With anti-CL oligonucleotide treatment, the injected RAS1<sup>Leu</sup>del cells produced 29 tumors per lungs ( $p < .05$  by Student' T test;  $p > .05$  by nonparametric Wilcoxon rank sum test). With anti-uPA oligonucleotide treatment, the injected cells produced 65 tumors per lung, not significantly different from the control group.

The results from the nude mice experiments suggest that either high levels of uPA or CL are induced by different ras oncogene transformations, and uPA and CL may substitute for each other in the lung colonization processes.

**Table 10 uPA Secreted Enzymatic Activity of ras-transformed Cell**

**Lines Treated with Sense- and Anti-sense Oligonucleotides**

**NIH/3T3**

Time after removal of oligo	no oligo	sense-uPA (100 uM)	anti-sense uPA (100 uM)	% inhibition of uPA activity
0-12 hr	1.36 <sup>a</sup> (0.06) <sup>b</sup>	1.23 (0.06)	0.70 (0.02)	49
12-24 hr	1.2 (0.1)	1.13 (0.06)	0.61 (0.03)	47
24-36 hr	0.80 (0.06)	0.75 (0.06)	0.43 (0.03)	44
36-48 hr	0.65 (0.04)	0.66 (0.06)	0.34 (0.04)	46

**EJ/vHA-ras**

Time after removal of oligo	no oligo (100 uM)	sense-uPA (100 uM)	anti-sense uPA of uPA	% inhibition activity
0-12 hr	4.3 (0.2)	4.4 (0.2)	2.3 (0.2)	47
12-24 hr	3.6 (0.2)	3.3 (0.2)	1.9 (0.2)	45
24-36 hr	3.1 (0.2)	3.0 (0.1)	1.7 (0.2)	45
36-48 hr	2.6 (0.1)	2.3 (0.1)	1.4 (0.2)	44

Table 10. (continued)

RAS1<sup>Leu</sup>del

Time after removal of oligo	no oligo	sense-uPA (100 uM)	anti-sense uPA (100 uM)	% inhibition of uPA activity
0-12 hr	1.1 (0.1)	1.2 (0.1)	0.61 (0.02)	45
12-24 hr	0.85 (0.02)	0.87 (0.01)	0.55 (0.03)	44
24-36 hr	0.72 (0.02)	0.71 (0.01)	0.38 (0.03)	47
36-48 hr	0.63 (0.02)	0.63 (0.01)	0.34 (0.02)	45

<sup>a</sup>uPA enzymatic activities were determined by chromogenic substrate S-2551.

<sup>b</sup>Numbers are the average of 3 separate runs with units of O.D. per 10<sup>6</sup> cells per hour.

The number in bracket is standard deviation.

**Table 11 CL Enzymatic Activity of ras-transformed Cell Lines<sup>a</sup>**

	Medium	Cells-ass.	Total	Cells-ass. /Medium
NIH/3T3	18.6	3.4	22.0	0.18
	19.5	4.3	23.8	0.22
	20.1	5.2	25.3	0.25
EJ/vHa- <u>ras</u>	28.6	3.1	31.7	0.11
	30.8	4.7	35.5	0.15
	31.5	3.8	36.3	0.12
<u>RAS1</u> <sup>Leu</sup> del	77.4	14.1	91.5	0.18
	80.1	15.7	95.8	0.20
	81.8	15.4	97.2	0.19

<sup>a</sup>CL enzymatic activities were determined by substrate N-carbobenzoxy-L-Phe-L-Arg-7-aminomethylcoumarin (Z-Phe-Arg-AMC) with units of pmol/min/10<sup>6</sup> cells.

**Table 12 CL Enzymatic Activity of ras-transformed Cell**

**Lines Treated with Anti-CL Oligonucleotide**

**NIH/3T3**

Time after removal of oligo	no oligo	sense uPA (100 uM)	anti-CL (100 uM)	% inhibition of CL activity
0-12 hr	16.8 <sup>a</sup> (2.4) <sup>b</sup>	14.9 (2.6)	10.2 (1.4)	39
12-24 hr	13.1 (2.3)	12.1 (1.5)	6.6 (1.4)	34
24-36 hr	9.6 (1.3)	9.4 (1.5)	7.3 (1.2)	23
36-48 hr	8.2 (1.4)	8.3 (1.3)	6.9 (1.3)	15

**EJ/vHA-ras**

Time after removal of oligo	no oligo	sense uPA (100 uM)	anti-CL (100 uM)	% inhibition of CL activity
0-12 hr	26.4 (3.8)	27.2 (3.7)	16.9 (3.6)	36
12-24 hr	21.6 (3.3)	21.0 (3.3)	14.4 (3.4)	33
24-36 hr	18.2 (2.2)	18.3 (2.2)	14.4 (2.2)	20
36-48 hr	13.7 (2.5)	12.9 (2.1)	11.8 (2.4)	14

**Table 12. (continued)**

**RAS1<sup>Leu</sup>del**

Time after removal of oligo	no oligo	sense uPA (100 uM)	anti-CL (100 uM)	%inhibition of CL activity
0-12 hr	73.4 (11.1)	69.8 (15.4)	47.6 (8.5)	35
12-24 hr	52.3 (11.0)	49.1 (12.9)	34.2 (6.1)	34
24-36 hr	42.0 (9.1)	42.4 (13.1)	35.3 (5.4)	15
36-48 hr	34.2 (5.8)	33.7 (5.3)	34.3 (6.5)	8

<sup>a</sup>CL enzymatic activities were determined by substrate N-carbobenzoxy-L-Phe-L-Arg-7-aminomethylcoumarin (Z-Phe-Arg-AMC). Numbers are the average of 3 separate run with units of pmol/min/10<sup>6</sup> cells.

<sup>b</sup>The number in bracket is standard deviation.

**Table 13. Lung Colonization of Nude Mice by ras-transformed NIH 3T3 Cells Selectively Inhibited by Antisense Oligos<sup>a</sup>**

Oligo -----	Number of tumors/Lung -----	Mean $\pm$ S.D. -----
<b>EJ/vHa-<u>ras</u>-transformed NIH 3T3 Cells</b>		
sense-uPA	105, 113, 115, 121, 137 139, 143, 166	130 $\pm$ 20
anti-CL	69, 85, 102, 104, 117 137, 147, 213	122 $\pm$ 45
anti-uPA	27, 29, 32, 42, 45 52, 68	41 $\pm$ 14 <sup>b</sup>
<b><u>Ras1</u><sup>Leu</sup> del-transformed NIH 3T3 Cells</b>		
sense-uPA	48, 63, 78, 87, 129, 159	94 $\pm$ 38
anti-CL	18, 21, 23, 27, 31, 35	26 $\pm$ 6 <sup>c</sup>
anti-uPA	38, 47, 56, 78, 86, 94, 170	81 $\pm$ 44

<sup>a</sup>Oligo treated tumor cells ( $3 \times 10^5$ ) were injected into the tail veins of 4 - 6 week athymic NCr (nu/nu) female mice, and the mice were sacrificed 14 days later. Lungs were dissected from mice and the number of surface tumors per lung counted.

<sup>b</sup>Significantly different from other groups at  $p < 0.001$  by nonparametric Wilcoxon rank sum test.

<sup>c</sup>Significantly different from other groups at  $P < 0.01$  by nonparametric Wilcoxon rank sum test.



**Table 14. Lung Colonization of Nude Mice by RAS1<sup>Leu</sup> del-  
Transformed Cells Selectively Inhibited by Antisense Oligos<sup>a</sup>  
(Experiment 2)**

Oligo	Number of tumors/Lung	Mean $\pm$ S.D.
sense-uPA	10, 37, 82, 85, 112, 130	76 $\pm$ 45
anti-CL	9, 18, 22, 29, 32, 35, 60	29 $\pm$ 16 <sup>b</sup>
anti-uPA	11, 34, 44, 69, 92, 101, 107	65 $\pm$ 37

<sup>a</sup>Oligo treated tumor cells ( $3 \times 10^5$ ) were injected into the tail veins of 4 - 6 week athymic NCr (nu/nu) female mice, and the mice were sacrificed 14 days later. Lungs were dissected from mice and the number of surface tumors per lung counted.

<sup>b</sup>Significantly different from other groups at  $p < 0.05$  by Student' t test. No significantly different from other groups by nonparametric Wilcoxon rank sum test.

## 8. Phorbol Ester Induction of Proteases and Protease Inhibitors in Human Prostate Carcinoma Cell Lines PC3-P and PC3-M

In addition to work done on the mouse NIH 3T3 cell lines, phorbol ester response was also studied in two pairs of related human cell lines of different metastatic potential. The major properties of these cell lines have been described in the Methods Section.

The PC3-M (metastatic) cell line was isolated from the PC3-P (parental) and shows a significantly greater ability in both spontaneous metastasis and lung colonization than the PC3-P cells (see Table 2) (Kozlowski et al., 1984). Figure 9 and 10 show that phorbol ester induces both the uPA mRNA and PAI-2 mRNA levels in PC3-P and PC3-M cells. The mRNA induction follows a time course in which mRNA levels increase between 4 and 16 hours, and then decrease by 32 hours to basal levels. At all time points, uPA and PAI-2 mRNA levels were compared to that of glyceraldehyde phosphate dehydrogenase (GAPD) mRNA which is not stimulated by PMA in these cell lines and therefore serves as a control. In contrast to the results with ras-transformed NIH/3T3 cells, in PC3 cells, the increased ability of the M lines to metastasize may correlate most closely with a decreased induction of PAI-2 mRNA levels when compared with the P lines (parental, low metastatic potential) rather than

with an increase in uPA mRNA levels in the M lines.

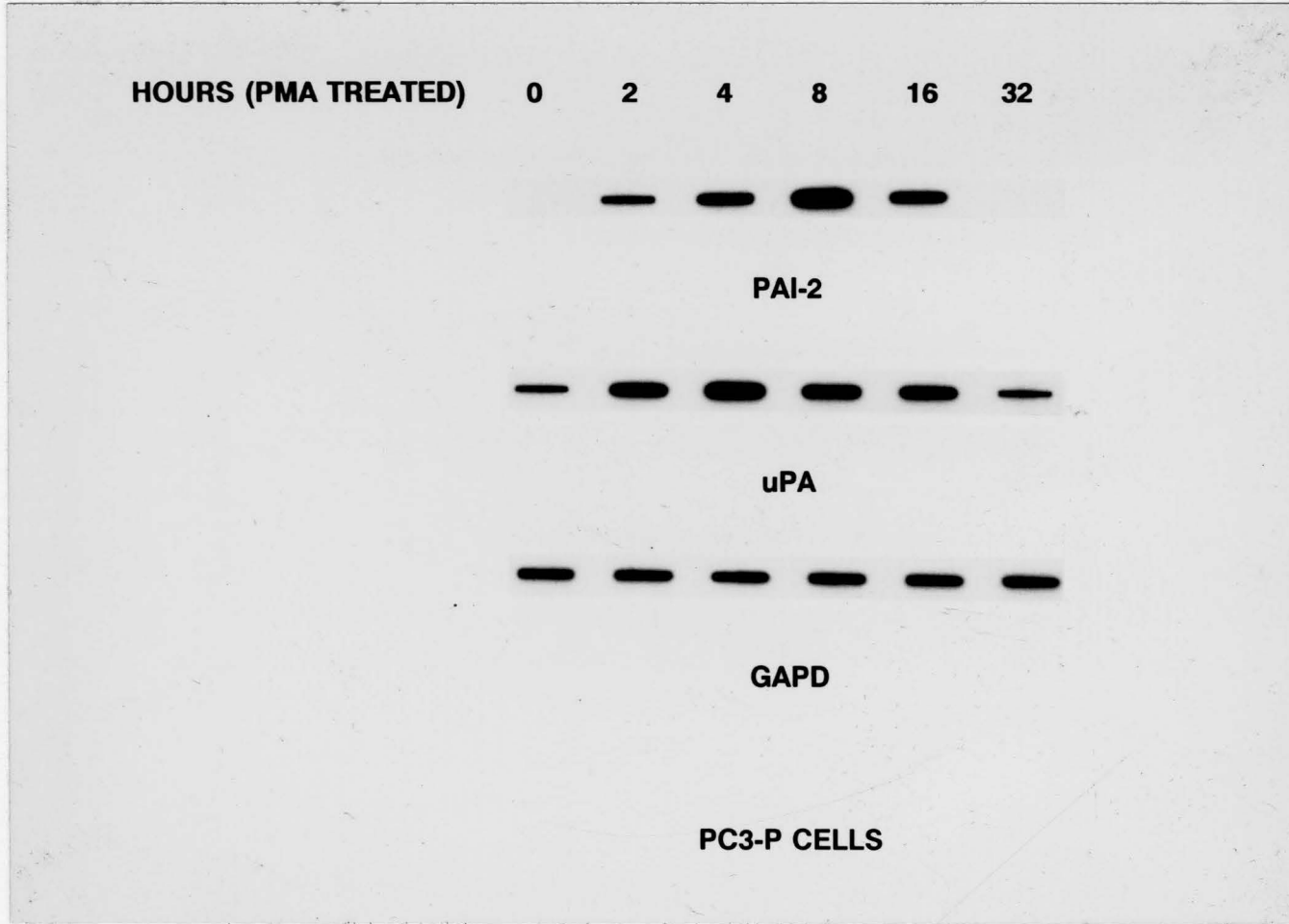
Stimulation of uPA mRNA in PC3-M cells was only 1.2-fold over that of the PMA stimulation of uPA mRNA levels in PC3-P cells. However, on PMA induction, the PAI-2 mRNA reached levels 6-fold higher in PC3-P (low metastatic) than in PC3-M cells (highly metastatic). GAPD was not significantly induced at the identical conditions. Densitometer traces normalized to the GAPD mRNA controls and averaged over 3 independent experiments are shown in Table 15. There is no PAI-2 detected in either PC3 - P or PC3-M prior to stimulation. PAI-2 mRNA was induced by 2 hours in both PC3-P and PC3-M lines, and reached peaks of 3.32 densitometric units (S.D.  $\pm$  0.8) in PC3-P cells and 0.56 densitometric units (S.D.  $\pm$  0.07) in PC3-M cells, respectively by 8 hours. No apparent inductions with PMA were observed in the GAPD mRNA.

To verify the results for PAI-2 from these slot blot analyses, a Northern blot analysis was carried out to study the PAI-2 mRNA levels from PC3-P and M cell lines. The result is displayed in Figure 11. Without PMA stimulation, no PAI-2 was detected in either cell line (lanes 1 and 3). Under PMA stimulation (20 nM) for 8 hours, PAI-2 could be detected in both cell lines, the P line (lane 4) showed significantly higher PAI-2 level than the M lines (lane 2).

PMA induction of mRNA levels can take place through either induction of new protein(s) (transcription factor

**Figure 9. Slot Blot Analysis of Human uPA and PAI-2 mRNA Levels in PC3-P Cells with PMA Treatment.**

The PC3-P cells were treated with PMA (20 nM) for the designated times and total RNAs were extracted. The RNAs were immobilized on a Zeta-probe filter, hybridized with the <sup>32</sup>P-labeled human uPA, PAI-2 and GAPD cDNA probes, and exposed to X-ray film.

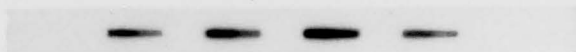


**Figure 10. Slot Blot Analysis of Human uPA and PAI-2 mRNA Levels in PC3-M Cells with PMA Treatment.**

The PC3-M cells were treated with PMA (20 nM) for the designated times and total RNAs were extracted. The RNAs were immobilized on a Zeta-probe filter, hybridized with the <sup>32</sup>P-labeled human uPA, PAI-2 and GAPD cDNA probes, and exposed to X-ray film.

HOURS (PMA TREATED)

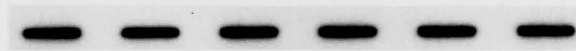
0 2 4 8 16 32



PAI-2



uPA



GAPD

PC3-M CELLS

Table 15. Levels of Human uPA and PAI-2 mRNA

in PC3-P and PC3-M with PMA<sup>a</sup>

		PC3-P					
Hours PMA Treated		0	2	4	8	16	32
uPA/GADP		0.55 <sup>b</sup> (.12)	1.63(.22)	2.34(.4)	1.82(.32)	1.28(.24)	0.52(.14)
PAI-2/GADP		N.O. <sup>b</sup>	0.61(.17)	1.27(.31)	3.32(.76)	1.21(.28)	N.O.
		PC3-M					
Hours PMA Treated		0	2	4	8	16	32
uPA/GADP		0.92(.21)	1.12(.20)	1.61(.35)	2.41(.72)	2.91(.55)	0.85(.19)
PAI-2/GADP		N.O.	0.22(.04)	0.32(.08)	0.56(.07)	0.28(.03)	N.O.

<sup>a</sup>mRNAs of the cell lines were determined by slot blots analysis of total RNA (4 ug/per slot). Following autoradiography, the intensity of the each slot signal was quantified by densitometry. The results were normalized to GADP, which was hybridized and densitometrized with the same filter. The numbers are the average densitometric value of 3 separate runs and standard deviation is in bracket.

<sup>b</sup>Not observable.



**Figure 11. Northern Blot Analysis of Human PAI-2 and TIMP mRNA Levels from PC3-P and PC3-M Cells with PMA Treatment.**

Total RNAs were extracted from human prostate carcinoma PC3-P and PC3-M cell lines with and without PMA (20 nM) treatment. The RNAs were separated by electrophoresis through a 1% agarose gel. The RNAs were transferred onto a Zeta-probe filter, hybridized with <sup>32</sup>P-labeled human PAI-2 and TIMP inserts. The resulting filter was exposed to X-ray film.

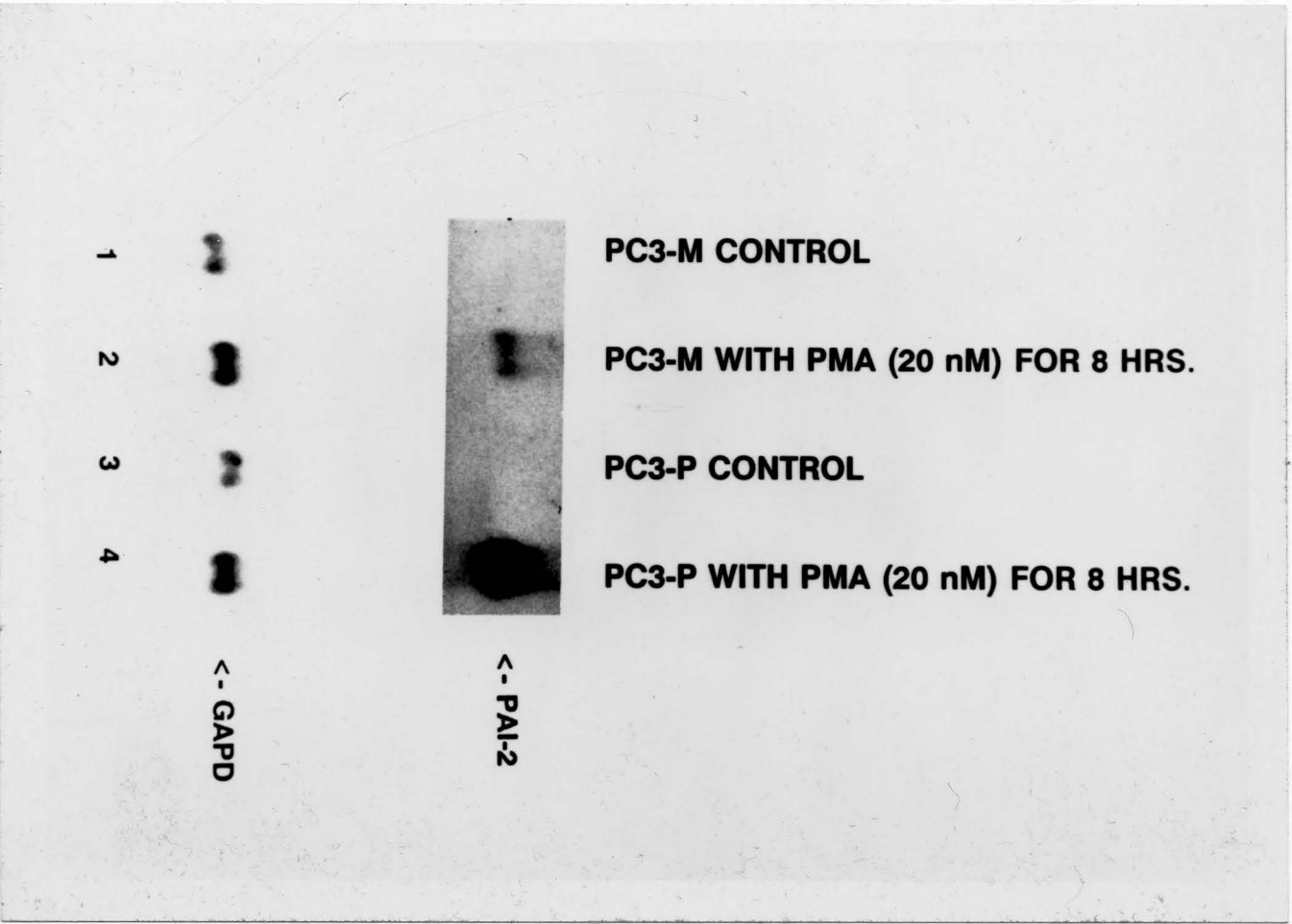
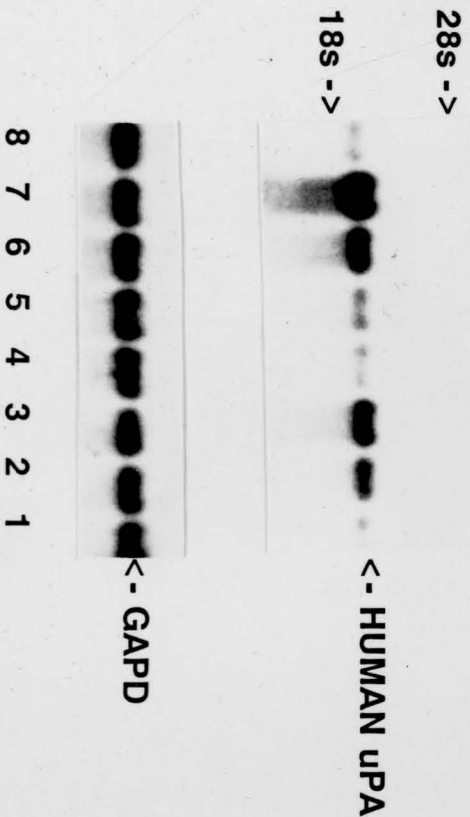


Figure 12. Northern blot analysis of human uPA mRNA levels in PC3-P and M cell lines with PMA as well as cycloheximide

**Figure 12. Northern Blot Analysis of Human uPA mRNA Levels in PC3-P and M Cell Lines with PMA as well as Cycloheximide Treatment.**

Total RNAs were extracted from human prostate carcinoma PC3-P and PC3-M cell lines after incubation with PMA (20 nM) and/or cycloheximide. Lanes 1 and 5: without treatment of PMA and cycloheximide (CHX); lanes 2 and 6: treatment with PMA for 16 hours; lanes 3 and 7: treatment with PMA (20 nM) and CHX (10 mg/ml) for 16 hours; lanes 4 and 8: treatment with CHX (10 mg/ml) for 16 hours. The RNAs were separated by electrophoresis through a 1% agarose gel. The RNAs were transferred onto a Zeta-probe filter, hybridized with the <sup>32</sup>P-labeled human plasmid uPA insert, and exposed to X-ray film.

PC3-M CYCLOHEMIXIDE 16 HRS  
PC3-M PMA+CYCLOHEMIXIDE 16 HRS  
PC3-M PMA 16 HRS  
PC3-M CONTROL  
PC3-P CYCLOHEMIXIDE 16 HRS  
PC3-P PMA+CYCLOHEMIXIDE 16 HRS  
PC3-P PMA 16 HRS  
PC3-P CONTROL



activators) or by a direct stimulation of mRNA synthesis through activation of factors that do not require new protein synthesis. To explore these different alternatives, cycloheximide (10 mg/ml), a protein synthesis inhibitor, was added to the cell culture medium for 16 hours. Its effect on PMA induction was followed by a Northern blot analysis of uPA mRNA, shown in Figure 12. Lanes 1 and 5 contain mRNAs isolated from PC3-P and PC3-M cells respectively without prior PMA and represent basal levels of uPA mRNA. PMA induced uPA mRNA expression in both cell lines (lane 2 and 6), and cycloheximide added to the cell culture medium together with PMA (lanes 3 and 7) enhanced uPA mRNA still further in both cell lines. Cycloheximide (lanes 4 and 8) alone had no inductive effect.

#### **9. Phorbol Ester Induction of Protease Inhibitor in Human Melanoma Cell Lines A375-P and A375-SM**

The A375-P is a human melanoma cell line with low metastatic potential. A375-SM was isolated from the A375-P and it shows a substantially greater ability to metastasize as measured by a lung colonization assay (see Table 2). These cell lines were treated with PMA as described above for PC3-P and PC3-M cells. Figure 13 is the Northern blot analysis of PAI-2 with and without PMA treatment. PAI-2 mRNA could not be detected in either A375-P and A375-SM cell

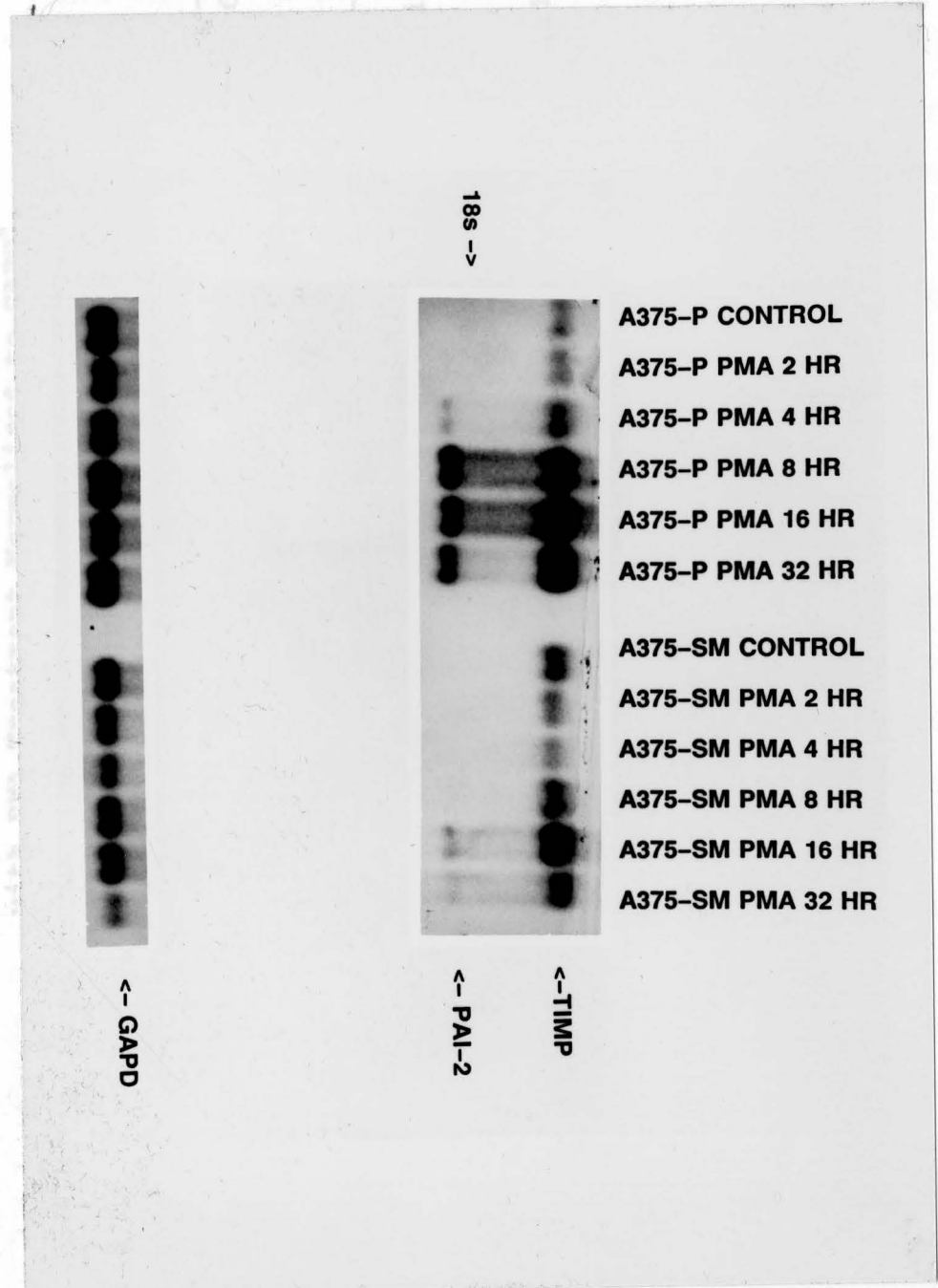
lines two hours after treatment PMA (20 nM), but then increased to high levels in A375-P between 8 and 16 hours (Figure 15). In contrast, PAI-2 mRNA was only barely detectable after 16 hr PMA treatment in A375-SM cells under identical conditions (Figure 13). The values from the densitometer traces normalized to the GAPDH mRNA controls are displayed in Table 16. The amount of PAI-2 produced by A375-P was 0.82 densitometer units at 8 to 16 hours, whereas PAI-2 levels in A375-SM only 0.12 and 0.05 densitometer units at the same time points, respectively. Maximum levels of PAI-2 mRNA were 16-fold higher in the A375-P cells (low metastatic) than in the A375-SM cells (highly metastatic).

In order to follow PMA induction at the protein level, a Western blot analysis of human PAI-2 in the two human melanoma cells A375-P and SM was carried out. Proteins were collected from the cell culture media. Cells were lysed and homogenized and the protein concentration of each sample determined with the BCA assay (Pierce Chemical Company, Rockford, Ill.). Ten mg of protein were loaded into each slot of SDS-PAGE gel and electrophoresed. The protein then were transferred to a nitrocellulose filter (Bio-Rad) and PAI-2 was detected with polyclonal antibodies against human PAI-2 (American Diagnostica Inc., New York, NY). The details of the assay are discussed in the Methods Section. The results are displayed in Figure 14. No PAI-2 was detected in either A375-P or A375-SM cell lines prior to PMA treatment.

**Figure 13. Northern Blot Analysis of Human PAI-2 and TIMP mRNA in A375-P and A375-SM Cells with PMA Treatment.**

Total RNAs were extracted from human melanoma cells A375-P and SM cells with and without PMA (20 nM) treatment. The RNAs were separated by electrophoresis through a 1% agarose gel. The RNAs were transferred to a Zeta-probe filter and hybridized with the <sup>32</sup>P-labeled human PAI-2 and TIMP plasmid inserts. The resulting filter was exposed to X-ray film.

Table 16. Levels of PAI-2 mRNA in A375-P and A375-SM Cells



<- GAPD

<- PAI-2

<- TIMP

18s ->

- A375-P CONTROL
- A375-P PMA 2 HR
- A375-P PMA 4 HR
- A375-P PMA 8 HR
- A375-P PMA 16 HR
- A375-P PMA 32 HR
  
- A375-SM CONTROL
- A375-SM PMA 2 HR
- A375-SM PMA 4 HR
- A375-SM PMA 8 HR
- A375-SM PMA 16 HR
- A375-SM PMA 32 HR



**Table 16. Levels of PAI-2 mRNA in A375-P and SM Cells  
with PMA Treatment Normalized to GAPD<sup>a</sup>**

Hours PMA Treated	0	2	4	8	16	32
A375-P	N.O. <sup>b</sup>	N.O.	0.12	0.82	0.82	0.49
A375-SM	N.O.	N.O.	N.O.	N.O.	0.12	0.05

<sup>a</sup>mRNAs of the cell lines were determined by Northern blots analysis of total RNA (20 ug/well). Following autoradiography, the intensity of the signals was quantified by densitometry. The results were the densitometric value of PAI-2 normalized to the densitometric value of GAPD, which was hybridized with the same filter.

<sup>b</sup>Not observable.

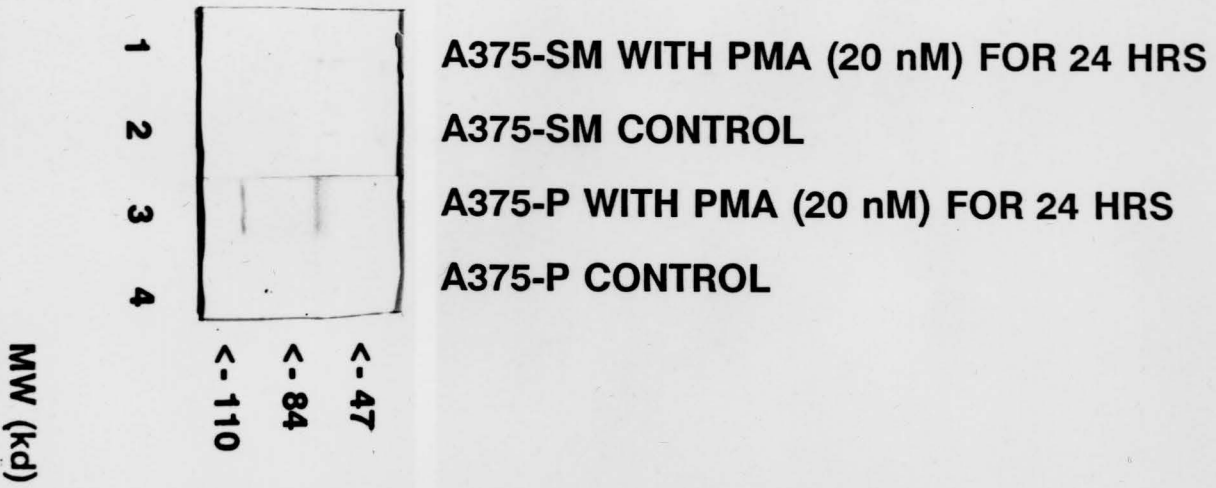
With PMA (20 nM) incubation for 24 hours, both cell lines produced detectable PAI-2 with the P line giving a higher amount than the SM line. Two bands are shown in the Figure 14, a low  $M_r$  band of about 48 kD, and a high  $M_r$  band of 96 kD. The 48 kD band is ostensibly PAI-2 protein (Kruithof et al., 1986). The nature of 96 kD band is not clear. The high molecular weight band, most likely, is a complex formed between PAI-2 and uPA. A similar finding was also reported from other laboratories (Nelson et al., 1986).

#### **10. Induction of PAI-2 in Human Prostate Carcinoma Cell Lines PC3-P and PC3-M with Conditioned Media**

The surprising observation that PMA induction of PAI-2 may more closely correlate with metastatic potential than the uPA protease activity suggests that gene expressions of both protease and protease inhibitors might be altered by growth factors in vivo. As PMA is a compound which does not exist in the living animal, conditioned media containing putative growth factors was collected from mouse organs explants for 24 hours. The details of media collection are discussed in the Methods Section. The conditioned medium was used to replace PMA in treating PC3-P and PC3-M cell lines. PAI-2 gene expression was measured by a Northern blot

**Figure 14. Western Blot Analysis of Human PAI-2 Protein in PC3-P and M Cells with PMA Treatment**

Ten mg of the proteins sample was loaded into each slot of a SDS-PAGE gel, and separated by electrophoresis. The proteins were transferred to a nitrocellulose filter. PAI-2 polyclonal antibody was used as a primary antibody, followed by goat anti-mouse antibody (Sigma Chem. Co.) conjugated with alkaline phosphatase.



analysis. The result is displayed in Figure 15. No PAI-2 mRNA was detected in either PC3-P and M cells cultured in normal medium (lanes 1 and 5) and in conditioned medium from kidney (lanes 3 and 7), and spleen conditioned medium (lanes 4, 8). However, incubation of the cells with conditioned medium from the lung organ culture showed a stimulating effect. Both PC3-P and M produced PAI-2 mRNAs, with higher induction in the P cells than the M cells (lanes 2 and 6).

**Figure 15. Northern Blot Analysis of Human PAI-2 mRNA from PC3-P and M Cells Treated with the Conditioned Medium**

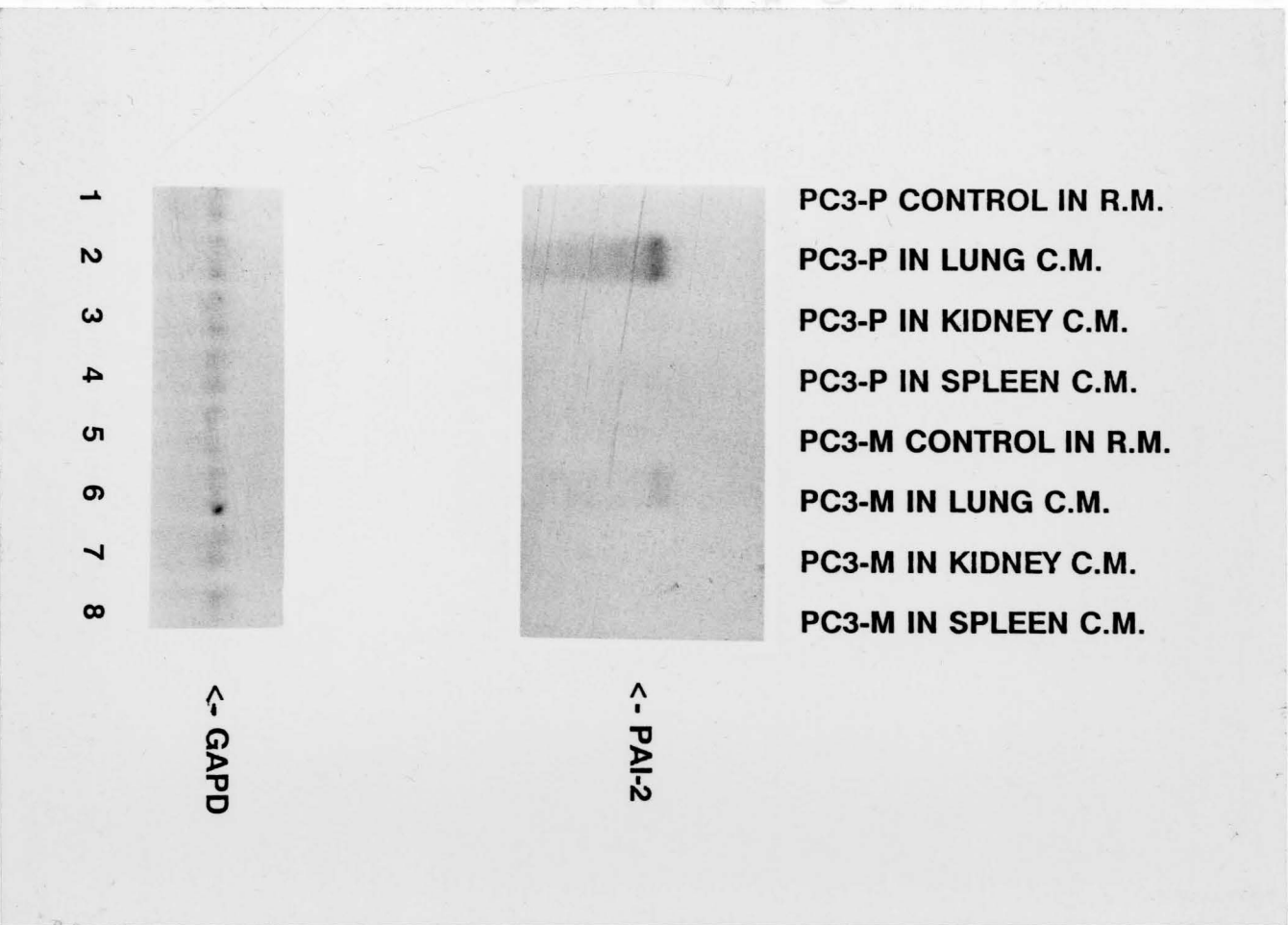
Total RNAs were extracted from human prostate carcinoma PC3-P and M cell lines after incubation with different conditioned media. Lanes 1 and 5: control with normal medium; lanes 2 and 6: treatment with lung conditioned medium for 16 hours; lanes 3 and 7: treatment with kidney conditioned medium for 16 hours; lanes 4 and 8: treatment with spleen conditioned medium for 16 hours. The RNAs were separated by electrophoresis through a 1% agarose gel. The RNAs were transferred onto a Zeta-probe filter, hybridized with the  $^{32}\text{P}$ -labeled human PAI-2 insert, and exposed to X-ray film.

PC3-P CONTROL IN R.M.  
 PC3-P IN LUNG C.M.  
 PC3-P IN KIDNEY C.M.  
 PC3-P IN SPLEEN C.M.  
 PC3-M CONTROL IN R.M.  
 PC3-M IN LUNG C.M.  
 PC3-M IN KIDNEY C.M.  
 PC3-M IN SPLEEN C.M.

<- PAI-2

<- GAPD

1  
2  
3  
4  
5  
6  
7  
8



## Chapter V

### DISCUSSION

#### Constitutively High Expression of Different Proteases in Cell Lines with Different ras-transformations

EJ/vHa-ras cells were produced by Bradley et al., (1986) by transforming NIH 3T3 cells with a chimeric ras construct containing two activating mutations, at codons 12 and 59 (Gibbs, et al., 1984). RAS1<sup>Leu</sup>del cells were similarly developed by Bradley et al. (1986) by transfecting NIH 3T3 cells by an homologous yeast RAS1 sequence activated by the substitution of a Leu at position 68 (analogous to position 61 in the mammalian Ha-ras sequence). Both cell lines were shown to have very high metastatic potentials in a nude mouse lung colonization assay (Bradley et al., 1986). In my study I show that the EJ/vHa-ras cells express a similar constitutive level of CL mRNA as untransformed NIH 3T3 cells, but a 5.4-fold higher constitutive level of the mRNA for the protease uPA with compared with NIH 3T3 cells (Table 5). In contrast, RAS1<sup>Leu</sup>del cells show low levels of constitutive uPA mRNA but a 4.4-fold higher concentration of the mRNA for the protease CL when compared with NIH 3T3



cells (Table 5).

Chromogenic substrate assays were used to study the enzymatic activities of uPA and CL in these cells. The substrate N-carbobenzoxy-L-Phe-L-Arg-7-aminomethylcoumarin (Z-Phe-Arg-AMC) was used to assay for cathepsin L-like activity (Mason, 1986; Baricos et al., 1988). The uPA enzymatic activity was determined with the chromogenic substrate S-2551, which measures the activity of plasmin generated following uPA treatment of plasminogen. The CL-like activity of the RAS1<sup>Leu</sup>del cells was found to be 3.8-fold higher than in its non-transformed parental NIH 3T3 cells, whereas CL activity of the EJ/vHa-ras cells was only 1.6-fold higher than in the NIH 3T3 cells. The highest uPA enzymatic activity was found in EJ/vHa-ras cells, and was 3.2-fold higher than in NIH 3T3 cells. The uPA enzymatic activity of RAS1<sup>Leu</sup>del cells was only 80% of the NIH 3T3 cell line. The enzymatic activities of the ras-transformed cell lines are, thus, proportional to the different mRNA levels found in these cell lines. The results showed that higher constitutive levels of either uPA and CL are associated with cells transformed with the EJ/vHa-ras or RAS1<sup>Leu</sup>del cells, respectively.

The concentrations of CB mRNA has also been measured with the slot blot assay. The result is not as dramatically different as CL and uPA among the different ras transformed cell lines. The highly metastatic EJ/vHA-ras only had a 1.9-

fold increase in CB mRNA over NIH 3T3 cells. The highly metastatic RAS1<sup>Leu</sup>del cell had a 1.5-fold increase in CB mRNA above NIH 3T3 cells. Thus, the contribution of CB may not be a major factor affecting the tumor metastatic potentials of these cell lines.

### **Different Responses of ras-transformed Cell Lines to PMA**

The gene expressions of the proteases are affected in vitro by many hormones, growth factors, and phorbol ester (Sloane et al., 1987; Markus, 1988). There has been very little research comparing the differential expressions of proteases in cell lines derived from same parental cell line and transformed with different oncogenes. It is a part of the hypothesis of this project that the proteolytic activities of tumor cells may be modified in vivo by signal factors, and different oncogene transformation may lead to different responses to the signal factors.

As it is currently impossible to study how the protease activities are actually regulated in vivo by signal factors, phorbol-12-myristate-13-acetate (PMA), a diacylglycerol (DAG) analog, was used to activate the protein kinase C (PKC) in the inositol phosphate signal pathway (see Literature Section). The PKC pathway is one of the major signal transduction pathways used by exogenous

signal factors and the pathway is presumed to be utilized by in vivo signal mechanisms.

Subconfluent cultures of cells were treated with 20 nM PMA and the total RNA was isolated by standard techniques at 0, 2, 4, 8, 16 and 32 hours. The variations of protease mRNA levels at these time points were determined by a slot blot technique. On PMA treatment, increases in uPA mRNA levels occur in the EJ/vHa-ras and EJ-ras transformed cells as well as untransformed NIH 3T3 cells. In contrast, there was no induction of uPA mRNA in cells transformed by the yeast RAS1<sup>Leu</sup> del sequence or the yeast HYB C<sup>Leu</sup> sequence. Thus RAS1<sup>Leu</sup> del transformations appear to suppress the normal PMA induction of uPA mRNA, which is stimulated in EJ-ras and EJ/vHa-ras transformed cells and untransformed NIH 3T3 cells.

Recently, Givol cloned (Givol, et al., 1992) three ras genes with different mutations at codon 12 into the same vector and used these ras oncogenes to transform chick embryo fibroblasts. When the clones were introduced into chick embryo, three types of organ specificity of the metastatic tumors were observed, which were dependent on the amino acid substitution at codon 12. The outcome of this project correlates with this organ-specific metastasis experiment. Every organ has its specific signal factors profile, which may cause a diverse protease and protease inhibitor expression among the cells transformed with

different ras oncogenes.

**Both Constitutively High Protease Levels and PMA Response Are Mainly Due to Changes in Transcription Rate**

In order to determine the mechanism of PMA inducibility of uPA, CL and CB mRNA, a nuclear transcription (run-on) assay was carried out. Without PMA treatment, the CL transcription rate in RAS1<sup>Leu</sup> cells is 6-fold higher than NIH 3T3 cells while the rate in EJ/vHa-ras cells is 2.5-fold higher than NIH 3T3 cells. The uPA transcription rate of EJ/vHa-ras cells is 7-fold higher than NIH 3T3 cells (Table 5). The results on the relative constitutive level of uPA is also confirmed by Dr. Silberman in this lab with CAT assays utilizing part of the 5' promoter region of the uPA gene as an enhancer in a CAT vector with the a heterologous SV40 promoter. In these experiments, CAT activity was 7-fold higher in EJ/vHa-ras cells than NIH 3T3 cells, while CAT activity in RAS1<sup>Leu</sup> transformed cells was equal to that in NIH 3T3 cells (S. Silberman and R. M. Schultz, personal communication). The difference in transcription rates of CL and uPA in these cell lines are consistent with difference observed in these cell lines in their mRNA levels and in their levels of expressed enzymatic activities.

With PMA treatment for 2 hours, uPA transcription

rate increased 4-fold in the NIH 3T3 cells, 3.6-fold in the EJ/vHa-ras cells and only 10% in RAS1<sup>Leu</sup> cells. In contrast, a 2 fold PMA stimulation of CL transcription is observed in RAS1<sup>Leu</sup> cells. No stimulation by PMA of CL transcription is observed in the EJ/vHa-ras or NIH 3T3 cell lines.

The results are summarized in the hypothetical scheme of Figure 16 which displays independent pathways for activated yeast RAS1, activated mammalian-like ras, and the PKC signal transduction pathway through non-activated cellular p21<sup>ras</sup>. PKC may control the activation of p21<sup>ras</sup> by its down-regulation of GAP (Downward et al., 1990). c-Fos and c-Jun are transcription factors activated by the PKC/p21<sup>c-ras</sup> signal transduction pathway (Cai, et al., 1990). The scheme then depicts the results of this work: (i) the high constitutive expression of the uPA gene induced by mammalian/viral EJ/vHa-ras transformation and the PMA inducibility of uPA in mammalian ras-transformed (as well as untransformed) NIH 3T3 cells, (ii) the repression of PMA induction of uPA transcription in cells transformed by activated RAS1 sequences (HYB C<sup>Leu</sup> and RAS1<sup>Leu</sup>del transformed cells), (iii) the high constitutive expressions and PMA inducibility of CL and CB genes in HYB C<sup>Leu</sup> and RAS1<sup>Leu</sup>del transformed cells, and (iv) the lack of a PMA induction of the CL gene in mammalian-like ras and untransformed NIH 3T3 cells.

**Putative Mechanisms for Differential Responses to  
PMA in the ras-transformed Cell Lines**

The dissimilar patterns of protease expressions infer the existence of at least two discrete signal transduction pathways for Ras oncoproteins leading to alternate phenotypes for the transformed state. The observation of multiple pathways may be in agreement with the recent report that more than one type of GAP protein can bind to the effector site in Ras, and both GAPs are present in equal amounts in NIH 3T3 cells (Bollag and McCormick, 1991; Vogel et al., 1988). The increases observed in one or the other pattern for protease expression, depending on the mutated form of the ras oncogene, further shows the presence of alternative compensating protease pathways which may exist for the degradation of extracellular matrix during metastasis even in transformed cells obtained from the same parental cell line.

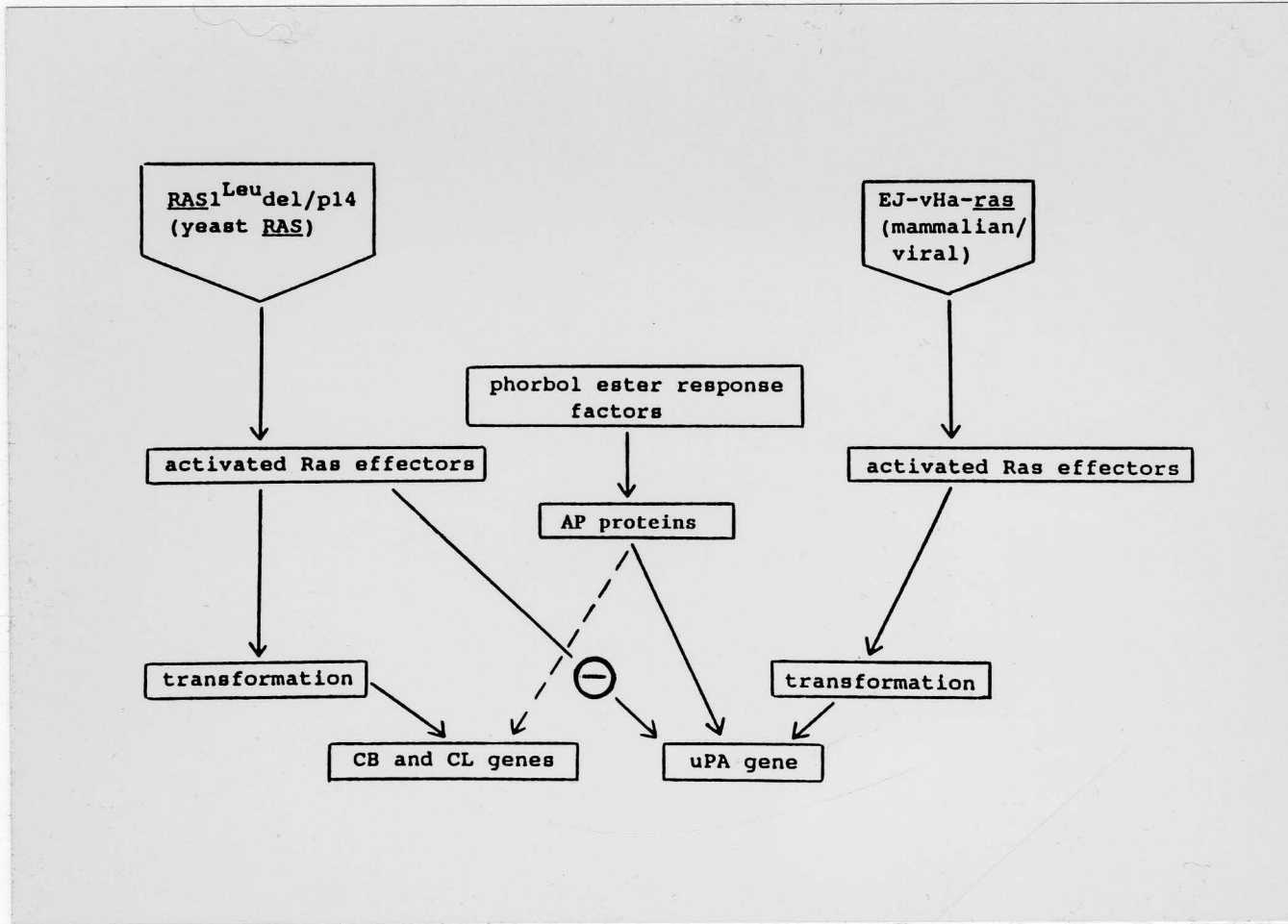
In humans, there are at least two genes encoding proteins which stimulate the GTPase activity (GAP). One of molecular mass 120,000 daltons is referred to as p120<sup>GAP</sup>, and the other NF1<sup>GAP</sup> (p280<sup>GAP</sup>) is encoded by the neurofibromatosis type-1 gene. p120<sup>GAP</sup> and NF1<sup>GAP</sup> proteins bind to the same effector site in Ras, and it is the heterodimer of GAP-RAS<sup>GTP</sup> that is believed to transmit the downstream effector signal from Ras (Martin et al., 1990; Bollag and McCormick, 1990).

EJ/vHa-ras has an activating mutation at codon 12 while RAS1<sup>Leu</sup>del has an activating mutation at codon 68 (equivalent to codon 61 in Ha-ras). The affinity of the p120<sup>GAP</sup> has been showed to increase significantly, when these mutations are present. Accordingly, the affinity of p120<sup>GAP</sup> may be better to RAS1<sup>Leu</sup>del with mutated codon at 68 as compared to its binding affinity to mutated codon 12 in the EJ/vHa-ras. As p120<sup>GAP</sup> and p280<sup>GAP</sup> have been shown to be present in approximately equal concentrations in NIH 3T3 cells (Martin, et al., 1990, Bollag and McCormick, 1990), it may be that changes in amino acid codon from 12 (in EJ/vHa-ras) to codon 68 (in RAS1<sup>Leu</sup>del) changes the relative affinities and thus the type of the GAP effector associated with the respective activated Ras protein. This putative change in the GAP effector could cause the switching of the signal from one biochemical pathway to the other. It is possible that the NF1<sup>GAP</sup>-Ras and the p120<sup>GAP</sup>-Ras complexes activate different pathways for the transformation and generation of the metastatic phenotype.

**Figure 16. Hypothetical Pathways for Normal Ras and Activated Mammalian and Yeast Ras Regulation of Pro-metastatic uPA, CL and CB Protease Genes.**

Suppression of uPA induction by the PKC/p21<sup>c-ras</sup> pathway in yeast RAS1-transformed cells shown by negative symbol. Dotted line indicates the inducibility of CL and CB genes by the PKC/p21<sup>c-ras</sup> pathway only in yeast RAS1 transformed cells.





**Anti-sense Oligonucleotides Can Partially Inhibit  
uPA and CL Enzymatic Activities in A Transient  
Manner**

Advances in molecular biology and synthetic chemistry over the past two decades have stimulated research into the use of antisense oligonucleotides as potential therapeutic and research agents (Rothenberg et al., 1989). Although some limitations, such as short half-lives in vivo and cytotoxicity, prevent the use of unmodified oligonucleotide as therapeutic agents, there are still reports of successful applications to influence the expression of a given gene of interest. For example, Zamecnik et al. (1986) and Goodchild et al. (1988) demonstrated inhibition of HIV replication upon addition of antisense oligonucleotides targeted against viral mRNA. Studies by Wickstrom et al. (1988) and Holt et al. (1988) demonstrated repression of HL60 cell proliferation and c-myc expression following addition of antisense oligonucleotide directed against human c-myc mRNA. Oligonucleotides complementary to c-myc-encoded mRNA was shown to inhibit normal human hematopoiesis in vitro (Geirtz and Calabretta, 1988) and proliferation of human myeloid leukemia cell lines (Anfossi et al., 1989). An antisense oligonucleotide to basic fibroblast growth factor repressed proliferation of human malignant melanomas (Dorothea et al., 1989). No general tenet for prediction of the effectiveness

of a given oligonucleotide has been suggested. The process seems to be system- and sequence-specific. In this work, three 21-mer deoxyoligonucleotides was prepared: sense-uPA (5'-ATGAAAGTCTGGCTGGCGAGC-3'), anti-uPA (5'-GCTCGCCAGCCAGACTTTCAT-3'), and anti-CL (5'-CAAAGGAGTAAAAGATTCAT-3'). Anti-uPA and anti-CL were prepared in an antisense direction (3' -> 5') of the first 21 nucleotides from the 5' start of translation in the mRNAs for murine uPA and CL, respectively. NIH 3T3 cells, and EJ-vHa-ras- and RAS1<sup>Leu</sup>del-transformed cells were incubated with 100 uM of these oligos in the media containing 10% FBS for 24 hours. The oligo containing culture media was removed and replaced with the oligo-free, serum-free medium. The CL and uPA activities produced by the cells were tested in the serum-free medium. The assay became impossible after 48 hour, as majority of the cells became detached from the flasks in the cell culture. Enzymatic activities of uPA and cathepsin L were determined by chromogenic or fluorogenic substrates as described in methods.

In contrast to our expectations, the non-chemically modified oligonucleotides significantly inhibited the respective gene expressions, even if just in a transient manner. Accordingly the synthesis of S-oligos was not required. In both EJ/vHa-ras and RAS1<sup>Leu</sup>del-transformed cell lines, anti-sense uPA selectively inhibited 44-49% of uPA activities up to 48 hrs; and anti-sense cathepsin L

selectively inhibited 33-39% of the CL enzymatic activities up to 24 hrs. The different durations of inhibition may be related to the different abundance of the uPA and CL mRNA inside the cells.

### **Lung Colonization Assay**

To measure the effect of anti-sense oligonucleotides on metastatic potential, nude mouse lung colonization assays were employed. The ras-transformed cells were treated in vitro with the oligonucleotides described above for 24 hours, the oligo-treated cells were injected into tail veins of nude mice, and 2 weeks later the animals were sacrificed and the tumor nodules on the lung counted under a dissecting microscope. This assay measures tumor cell extravasation from the blood stream with invasion of basement membrane and extracellular matrix, and then growth into pulmonary tumors.

If a change in the number of metastatic colonies is evidence for the effect of the oligo on the metastatic process, it is important that the method for determining the number of metastatic colonies be accurate and reproducible. As stated by Fidler (1978), and discussed in the references therein, most lung tumors in mice appear on the surface and can be counted with a dissecting microscope.

There is some variation in the number of metastases between control groups used in different experiments, but

they are not statistically different. The variation probably results from using cells maintained in culture for different lengths of time as well as the use of different batches of mice. As a valid control group was utilized with each experimental group, the dissimilarity in the number of lung colonies between different control groups carried out on different days with different lots of mice, should not affect the conclusion of the project.

**Anti-sense Oligonucleotides Can Selectively Decrease Tumor Metastatic Potential in Particular Cell Lines.**

As noted above, uPA and CL was shown to be more highly expressed in EJ/vHa-ras and RAS1<sup>Leu</sup>del transformed cells, respectively. Expressed enzymatic activities of uPA and cathepsin L were significantly inhibited by the O-oligonucleotide in a transient manner, as measured with fluorogenic substrates. In both EJ/vHa-ras cell line and RAS1<sup>Leu</sup>del cell line, anti-sense oligo inhibited at least 34% of the enzymatic activities up to 24 hrs. The time course of the initial arrest and invasion by tumor cells of an organ in vivo, after tail vein injection, has been evaluated by radiolabeling of a tumor cells with I<sup>125</sup> 2-iodo-deoxy-uridine (Fidler, 1978). The result showed the critical time in lung colonization for cell adhesion and invasion is the first 12 to 24 hours after tail vein injection of the cells.

Both uPA and CL have been implicated as enzymes capable of much extracellular tissue destruction, including the degradation of extracellular basement membrane. The production of high levels of uPA and CL has been shown to correlate with high levels of metastasis. Direct evidence has shown that uPA plays a role in B16 melanoma metastasis in C57BL mice (Yu and Schultz, 1990).

In this project, anti-sense oligo compounds have been shown to be specific inhibitors of the proteolytic enzymes cathepsin L and uPA in vitro. If uPA or CL is crucial to the metastatic process in vivo in these cell lines, then inhibition of the protease activities may lead to a reduction of metastasis in a lung colonization assay. Treatment of the cells with the anti-sense oligo prior to injection of cells into nude mice would be expected to lower the protease activities in the treated cells for at least 24 hours. Accordingly, the cells were injected immediately after ceasing treatment with the anti-sense oligo. The results were expected to give a clear picture as to the importance of the particular protease in metastasis in vivo. The nude mice experiments tests the hypothesis of the selective involvement of proteases in different ras-transformed cells lines.

Experiment 1 utilized EJ/vHa-ras cells. The result (Table 13) shows that the sense-uPA treated cells (control oligo) produced an average of 130 tumors per lung. The anti-

CL treated cells gave an average of 122 tumors per lung, which is not statically different from the control group. The anti-sense uPA-treated cells produced an average of 41 tumors per lung, significantly below the control group ( $P < 0.001$ ).

Experiment 2 used RAS1<sup>Leu</sup>del cells. The results showed that the sense uPA treated cells (control oligo) produced an average of 94 tumors per lung. The anti-uPA treated cells produced an average of 81 tumors per lung, not significantly different from the control group. The anti-CL treated cells produced an average of 26 tumors in lungs which was significantly lower than the control ( $p < 0.01$ ).

The latter work was repeated once more, the number of tumors per lung was more variable than in the preceding experiment (Table 14), but the anti-CL treated cells still caused significantly fewer lungs colonies when compared with the sense-uPA treated cells ( $P < 0.05$  ).

Tumor metastatic potential does not appear to be directly proportional to changes in the protease activity of tumor cells. For example, anti-CL inhibits CL enzymatic activity by 35% in RAS1<sup>Leu</sup>del cells, in which the total tumor lung colonizations is reduced 70% with respect to the controls group.

As CL inhibition in EJ/vHa-ras cells and uPA inhibition in RAS1<sup>Leu</sup>del cells had no effect on lung colonization ability, there may be a threshold amount of uPA

or CL required for promotion of metastasis. CL concentration in EJ/vHa-ras cells and uPA concentration in RAS1<sup>Leu</sup>del cells, respectively, may be too low to affect lung colonization ability one way or another. Therefore, inhibition of their already low respective activities had no affect. On reaching a threshold level, metastatic ability then becomes dependent on their relative amounts.

The results of the lung colonization assay with anti-sense inhibitors clearly show that metastasis can be achieved with aid of alternative proteases. uPA and CL proteases were separately induced by different ras oncogene transformations. uPA and CL may compensate for one another in tumor metastasis.

#### **Direct Evidence for Involvement of CL in Tumor Metastasis**

Although an increase of CL has been reported to be correlated with tumor metastatic potentials (Gottesman, 1988), this correlative evidence by its nature is an indirect type of evidence. The specific inhibition of CL synthesis by anti-CL and corresponding decrease in lung colonization observed in this project is the first direct evidence for a role of CL in tumor metastasis.

A problem in assigning a role to CL in tumor metastasis is that CL is an acidic protease, its optimal pH



for activity is 4. At neutral pH, which is the expected pH of the extracellular space in the tumor invasion process, CL enzymatic activities should be low. In addition, prior evidence indicated CL was unstable at neutral pH, and thus its activity will be predicted to last only a short time (Gottesman, 1987). These properties of CL implied that CL may not be a crucial enzyme in metastasis.

The doubts can be addressed in several ways. First, CL is secreted as a proenzyme, which is stable at neutral pH, and hence may be an excellent molecular vehicle for the transfer of the protease in a cancer cell to the extracellular environment. After secretion, procathepsin L must be activated to its functional form. The mechanism of activation is unknown. Secondly, even the mature form of cathepsin L shows activity at neutral pH, albeit briefly (Mason et al., 1987). In addition, CL could act in an acidic microenvironment in the region of contact between the cell surface and the extracellular matrix or basement membrane.

Secreted procathepsin L passes through the acidic vesicles of the exocytic pathway in cancer cells. It might act in this compartment to process, and perhaps activate, cell surface receptors and secreted growth factors. Increased amounts of cathepsin L in various intracellular compartments, including the lysosomes of cancer cells, could facilitate protein turnover during the growth of these cells (Laumas et al., 1989). Cathepsin L could also be essential

for the activation or inactivation of oncogene or anti-oncogene products or other intermediates in the process of malignant transformation. In addition to a possible direct role of cathepsin L in tumor metastasis, cathepsin L might interfere with antigen processing with the potential ablation of the immune response to tumors (Mouritsen et al., 1991).

### **PMA Induces More PAI-2 in Human Tumor Cell Lines with Less Metastatic Potential**

The activity of uPA can be regulated through control of the amount of protease synthesized, or through the production of its inhibitor(s). Some proteinase inhibitors such as  $\alpha_2$ -macroglobulin,  $\alpha_1$ -proteinase inhibitor, and antithrombin III inhibit a wide spectrum of proteases. As their molecular weights are large and their activities are non-specific in nature, these protease inhibitors are not commonly thought to be of importance in precise control of proteinase activity in tumor metastasis in vivo (Markus, 1988). However, there are at least two plasminogen activator inhibitors (PAIs) that bind PAs specifically, PAI-1 and PAI-2. The pertinent information on these PAIs have been reviewed in the Literature Section.

The PC3-P and PC3-M cell lines are from a human prostate carcinoma. The PC3-P (parental) cell line has a significantly lower metastatic potential in the nude mice

assay (Kozlowski et al., 1984), while the PC3-M cell line is a derivative of the P line with a higher metastatic potential (see Table 2).

A375-P & A375-SM cell lines are derived from a human melanoma. A375-P cells show a low metastatic potential in the nude mice assay, and A375-SM cells show a higher metastatic potential than A375-P cells (see Table 2) (Kozlowski et al., 1984).

Initial efforts to correlate uPA levels and tumor metastatic potential in the PC3 and A375 cell lines failed to detect any meaningful relationships. Attempts have also been made to obtain a purposeful relationship between PAI-2 and tumor metastatic capability. However, no PAI-2 mRNA was observed under basal conditions in these cells. However, on treatment of these cell lines with PMA, both the PC3 and A375 lines show observable increases of the uPA and PAI-2 mRNAs. In particular the less metastatic P lines make more PAI-2 mRNA than the more metastatic M-line. The ability to metastasize may thus correlate better with an increased induction of PAI-2 mRNA levels in the P lines (parental, low metastatic potential) than with an increase in urokinase mRNA levels in the M lines (high metastatic potential). Data showed that stimulation of uPA mRNA in PC3-M cells was only 20% (1.2-fold) over that of the PMA stimulation of urokinase mRNA levels in PC3-P cells. In both the A375-P and M cell lines, no uPA mRNA was observable even with PMA treatment.

However, with PMA induction, both cell lines (PC3-P and A375-P) with lower metastatic potentials produced much more PAI-2 mRNA than those of their partners with high metastatic potentials (PC3-M and A375-SM) (6-fold higher in PC3-P, 16-fold higher in A375-P). In addition, mRNA for a collagenase inhibitor, TIMP, was also much more highly induced in the A375-P than in the A375-SM cells (3-fold higher in A375-P).

Attempts were made to assay PAI-1 with a cDNA probe for PAI-1 mRNA in these cell lines. No hybridization was observed after extensive trials.

Although there are many prior studies on protease inhibitors such as PAI-2 and TIMP, few reports have directly connected PAI-2 with metastatic capacity of tumors until recently. This work is among the first studies correlating the tumor metastatic potentials with the uPA inhibitors levels as opposed to uPA itself. The significance of difference in the inductions of PAI-2 for tumor metastasis is not yet clear, as we have not yet shown that an antisense oligonucleotide for PAI-2 can promote metastasis. However, induction of PAI-2 in vivo in both cell lines is a likely event and may occur through PKC. In addition, our data implicates the importance of signal transduction pathways in inducing both pro- and anti- metastatic promoting gene expressions, and suggests an important role for protease inhibitors in determining the metastatic potential of a cell. The variations in PAI-2 levels may be more important

than variations in urokinase levels in determining the net urokinase activity and consequently the metastatic potential of the cell lines PC3 and A375.

### **Superinduction of uPA by Cycloheximide**

To test whether protein synthesis is required for the effect of PMA on the PAI-2 mRNA level, PC3-P and M cells were incubated for 24 hr with PMA, cycloheximide (CHX), and a combination of the two compounds. Total RNAs were isolated from the cells and analyzed by Northern blotting. Inhibition of protein synthesis by CHX does not inhibit the induction by PMA of uPA mRNA synthesis in these cell lines. Rather the combination of PMA and CHX produces a 2.5-fold higher level of uPA mRNA than PMA alone. Thus, cycloheximide leads to a superinduction of uPA mRNA under PMA stimulation.

Superinduction of gene expression by incubation of cultured cells with CHX as inducer has been described in a variety of systems, such as superinduction of tPA mRNA in isoproterenol-treated granulosa cells (Oikawa and Hsueh, 1989), PAI-2 in the human promyelocytic cell line HL-60 (Antalis and Dickinson, 1992), and the induction of uPA in Hela cells (Novak et al., 1991). The exact mechanism(s) underlying the superinduction by CHX in cells is unknown. There are a few explanations for the marked accumulation of uPA mRNA in the presence of PMA and CHX on the cells

studied. The superinduction is probably not due to a stabilization of uPA mRNA by CHX, as CHX alone did not result in an increase in levels of uPA mRNA. However, in the 3' end of uPA a consensus AUUA sequence is found (Verde et al., 1985), and this sequence has been shown to confer the superinduction effect of CHX. The CHX effect might work on protein(s) induced by PMA which modulate the increases in the mRNA transcription rate normally observed with PMA induction, or increase the uPA mRNA degradation rate. In absence of the protein synthesis inhibitor, the full effect of PMA induction is observed.

**Conditioned Medium Induce More PAI-2 in Human  
Prostate PC3-P Cells Than in PC3-M Cells**

Considerable efforts has been made in this project to study the different response of transformed cells to the PKC activator PMA. As PMA is a compound isolated from a plant and does not exist inside the body of animals, the data based on PMA work is only a hypothesis. The idea needs to be extrapolated to tumor metastasis in the animal. We do not know, however, the identity of the signal factor(s) in vivo. To try to find a signal factor(s) in vivo, a dozen purified growth factors and hormones that are commercially available were utilized as a replacement of PMA in stimulation experiments in cell culture. The factors studied were

platelet-derived growth factor (PDGF), epidermal growth factor (EGF), interleukin 1, interleukin 6, and nerve growth factor (NGF). However, all these factors failed to produce any stimulation of the proteases and inhibitors in the human tumor cell lines PC3-P, PC3-M, A375-P and A375-SM. Finally, the conditioned media from nude mice organ cultures were collected, and used to culture these cell lines.

PC3-P and M cells were cultured with conditioned media collected from lung, kidney and spleen of female nude mice, and the RNA were isolated from these cells after 16 hr. uPA and PAI-2 cDNA were used as a probe in Northern blot analysis of these cell lines. The astonishing result was that treating PC3-P and M with the conditioned medium from lung organ culture induced PAI-2 production in similar pattern to that shown on PMA stimulation. No effect on PAI-2 mRNA induction was observed with any of the other organ conditioned media. uPA mRNA is not significantly altered with incubation of the conditioned media. Furthermore, these cells only metastasized to the lung in nude mice assays of the metastatic potential of these cell lines (Kozlowski et al., 1984a, 1984b). The outcome shows there exist stimulator(s) in lung, which can modify the protease activities in similar fashion as does PMA in these cell lines. Also, the effect is apparently on PAI-2 mRNA levels, not on the uPA mRNA levels.

The nature of the stimulator existing in lung

conditioned medium is unknown. The effect may be caused by a single signal factor, or a combination of growth factor(s) (Beutler and Cerami, 1986; Roberts et al., 1985).

Although similar observation was found with the conditioned medium, the strength of the effector in the conditioned medium was less than PMA (20 nM). This conclusion was obtained from time length of film exposure in Northern blot analysis required to achieve a similar density of hybridization (data not shown). This may be due to both a short half-life and/or the strength of the effector in the conditioned media resulting in a less effective stimulation than PMA in these experiments.

### **General Principle of Proteases Involved in Tumor Metastasis**

The increases observed in one or the other pattern for protease expression, depending on the mutated form of the ras oncogene, further indicates the presence of alternative compensating protease pathways which may exist for the degradation of extracellular matrix during metastasis, even in transformed cells obtained from the same parental cell line. The antisense oligo data show that in one pathway uPA is an important facilitator of experimental metastasis (lung colonization), while in the second pathway CL facilitates experimental metastasis. A role for proteases



in metastasis has long been suggested, but direct evidence for a role of particular proteases has not been clear-cut. In part, the confusion is due to the large amount of correlative evidence from miscellaneous systems with different proteases, which are often contradictory. Direct evidence for a role of a particular protease in metastatic processes only comes from the few experiments in which selective inhibitors of proteases were utilized (Schultz, et al., 1988), and from molecular biology experiments in which particular protease gene expressions can be selectively increased or decreased (Yu and Schultz, 1990). However, an additional factor contributing to the confusion is the possibility that an enzyme activity required for the metastatic process in one tumor cell type may be not required in another. This project show a reciprocal variation for the importance of CL activity and uPA activity in the different ras-transformed NIH 3T3 cells. The data supports the hypothesis that different biochemical pathways exist that generate different malignant phenotypes, even within the same parental cell type that use a different set of proteases to achieve metastasis. In addition, this project obtained the first direct evidence for the importance for CL in metastasis.

Although the complexity of proteases involved in tumor metastasis is now appreciated, the result from this project may contribute to some general principle in this

field. Firstly, there is unlikely to exist a universal protease produced by all cancer cells which can account for the invasive and metastatic properties in cancer. Rather, different cancers appears to employ different protease(s), or combinations of proteases, to achieve the same end. The decision as to which protease or proteases a specific tumor type will use probably depends on the tissue of origin of the cancer and the manner in which transformation is achieved.

Secondly, differences in protease mRNA levels and their activity observed in vitro may, or may not, be reflective of the actual protease activity expressed by those cells in vivo. In vitro studies often fail to show a correlation of protease activity with invasiveness in vivo. Partial reasons for the bewilderment is that spectrum of proteases observed in vitro in a tumor cell may be modified in vivo. Possibly, the majority of observations in vitro acquired up to now do not represent the true protease activities actually involved in in vivo tumor metastasis.

Thirdly, the oncogenic mechanism causing increases of protease activity is unclear. In some cases, increased or decreased transcription of the protease gene may occur. In other cases, gain or loss of activity of specific inhibitors appears to contribute significantly to the activity of the protease.

As it is currently not possible to study how the proteases activities are actually regulated in vivo by signal factors, I studied phorbol-12-myristate-13-acetate (PMA or TPA) which mimics diacylglycerol (DAG) in activating the protein kinase C in the inositol phosphate signal pathway.

To investigate the question related to mechanism of transformation, I used a group of mouse cell lines transformed by different ras oncogenes that show different metastatic potentials in a nude mice assay system. NIH 3T3 cells are non-transformed mouse fibroblasts without tumorigenic properties. The EJ-ras and EJ/vHa-ras cell lines are NIH cells transformed by activated mammalian ras oncogenes. Hyb C<sup>Leu</sup> and RAS1<sup>Leu</sup>del cell lines are NIH 3T3 cells transformed by yeast ras oncogene constructs.

By Northern and slot blots analysis of the RNAs for mouse cathepsin B, L and urokinase, I studied mRNAs expressed both constitutively and with phorbol ester stimulation. These data show: 1) urokinase is at a significantly higher constitutive level in the highly metastatic EJ/vHa-ras line, but not in the highly metastatic RAS1<sup>Leu</sup>del cell line. 2) PMA stimulates urokinase mRNA in untransformed NIH 3T3 and in the mammalian-ras transformed cells, but did not stimulate urokinase mRNA in yeast RAS1<sup>Leu</sup>del transformed cells. 3) CL mRNA is significantly higher in highly metastatic RAS1<sup>Leu</sup>del cell lines as compared

with other cell lines. 4) PMA strongly induce CL mRNA in yeast ras transformed HYB C<sup>Leu</sup> and RAS1<sup>Leu</sup>del lines but PMA does not induce CL mRNA in mammalian ras transformed cell lines EJ/vHa-ras or EJ-ras.

RNA transcription rate (run-on) assays were carried out which shows that the differences in mRNA levels for CL and uPA with the different ras oncogenes are primarily due to differences in transcription rates of the genes rather than to differences in mRNA stability.

Based on the observation that urokinase is at a high level and PMA inducible only in mammalian ras oncogene transformed NIH 3T3 cells while cathepsin L is at a high level and inducible only in the yeast ras transformed NIH 3T3 cells, I explored the possible roles of uPA and CL in metastasis in these cell lines using oligonucleotide anti-sense inhibitors. Three 21-mer oligonucleotides were selected based on published mRNA sequences for uPA and CL, the sequences were designated sense-uPA (control), anti-sense uPA, and anti-sense CL. I showed that the antisense O-oligonucleotide can significantly inhibit CL and uPA expressions in a transient manner. In both the EJ/vHa-ras cell line and the RAS1<sup>Leu</sup>del cell lines, anti-sense uPA inhibited 45% of uPA activity up to 48 hours and anti-sense CL inhibited 35% of the CL enzymatic activities up to 24 hours. Based on these results, we proceeded to nude mice experiments to test the selective involvements of CL and

urokinase in these cells lines.

EJ/vHa-ras cells were treated in vitro with the oligonucleotides and injected into nude mice through their tail vein. The anti-sense uPA significantly decrease the metastatic potential of EJ/vHa-ras transformed cells compared to control ( $p < 0.001$ ). However, no effect of anti-uPA was observed with RAS1<sup>Leu</sup>del cells. RAS1<sup>Leu</sup>del cells treated with anti-sense CL showed a significantly decrease in experimental metastasis from the control group ( $P < 0.01$ ). No effect of anti-CL on experimental metastasis was observed on the EJ/vHa-ras cells. The nude mouse assay with RAS1<sup>Leu</sup>del cells was repeated more once, the anti-sense CL group different significantly from sense-uPA group in this repeat ( $p < 0.05$ ).

The results demonstrated that high levels of uPA and CL proteases are separately induced by different ras oncogene transformations and uPA and CL may compensate for each other in these two different metastatic pathways.

Two pairs of cell lines isolated from human origins were also investigated. PC3-P and M were isolated from human prostate carcinoma (Kozlowski et al., 1984). A375-P and A375-SM were from a human melanoma (Kozlowski et al., 1984). Characterization by Fidler and co-worker showed that PC3-P and A375-P exhibited a low metastatic potential, and PC3-M and A375-SM have a higher metastatic potentials. I found that uPA and its inhibitor, plasminogen activator inhibitor

2 (PAI-2) are both induced with PMA in the P and M lines. However, a more dramatic change of PAI-2 mRNA is observed, whereas uPA mRNA levels do not vary as greatly. It appears that the DAG analog (PMA) promotes the P cells to be less metastatic by inducing more PAI-2 rather than inducing the M line to be more metastatic by increasing uPA.

A Western blot was utilized to confirm the induction of PAI-2 at the protein level. Without PMA treatment, there is no PAI-2 observed by Western blot. After PMA induction, PAI-2 protein is observed with much higher levels in the PC3-P cells than in the PC3-M cells.

Cycloheximide, a protein synthesis inhibitor, was utilized to study uPA induction by PMA. Cycloheximide superinduced the uPA mRNA production triggered by PMA in PC3-P and PC3-M cells.

I explored the differential response of PAI-2 in vivo by using conditioned medium from the organs cultures of nude mouse. PC3-P and M showed the increased mRNA production of PAI-2 under the stimulation of lung conditioned medium. There was a 5-fold greater stimulation of PAI-2 in the PC3-P line than PC3-M line. Conditioned media from other organs have no effect on PAI-2 mRNAs in these cell lines.

Some conclusion can be drawn from this project:

1. Transformation of different ras oncogenes can lead to dissimilar metastatic phenotypes shown by diverse protease expressions. Selective inhibition of uPA and CL

showed that uPA is critical in one phenotype and CL in the other phenotype.

2. Signal factors in vivo may modulate tumor metastatic potential through regulation of the genes for protease inhibitors rather than through regulation of the gene for the proteases. Thus, in the PC3 cell lines, tumor metastatic potential may be primarily modulated through alteration of its PAI-2 activity.

## CHAPTER VII

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The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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