Dissertations   Theses and Dissertations

1993

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

Jianyi Zhang
Loyola University Chicago

Follow this and additional works at: https://ecommons.luc.edu/luc_diss

Part of the Molecular Biology Commons

Recommended Citation

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

in Partial Fulfillment of the

Requirements for the Degree of

Doctor of Philosophy

January

1993

ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Richard Schultz, for his continued encouragement, patience and support during this work.

I would like to gratefully acknowledge Dr. Allen Frankfater, Dr. William H. Simmons, Dr. M. Rita I. Young, Dr. Chester J. Herman for their assistance and also for their critical reading and discussion of this study.

Special thanks go to the Dr. Simone Silberman for her excellent assistance with the nude mice study, and priceless discussion and encouragement.

Most importantly, to my wife, Mei, I express my great appreciation for without her understanding, support and encouragement this dissertation could never have been completed.

VITA

Jianyi Zhang is the third son of Xianwei Zhang and Yifen Sun. He was born on June 1, 1955 in Beijing, China.

He obtained his Bachelor of Medicine from the Department of Medicine, Beijing Medical College, 1982. He worked as a intern in the Department of Nuclear Medicine of the Hospital of Beijing Union Medical College in 1983. He started as a resident in Beijing Cancer Institute in 1984.

He was admitted to Utah State University in October of 1984, and transferred to and accepted at the Ph.D program at Loyola University with a Merit Fellowship in 1985.

He married to Mei Du on July, 1986 and a daughter, Amy Zhang, was born in November, 1987.

He passed the Foreign Medical Graduates Examination in the Medical Science (FMGEMS) and obtained qualification for a resident training in USA on January, 1992. He will pursue a residency program in medicine after his graduation.

TABLE OF CONTENTS

vi

LIST OF FIGURES

LIST OF TABLES

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 <u>ras</u> oncogenes.

(2) To determine how exogenous signal factors regulate protease and protease inhibitor expressions in transformed and metastastic 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 Rohrlich, 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 (Riccio 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
secret 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 procathepsin 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_is
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 came 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 MoCormick, 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_1 phase of the cell cycle. After this time cells are committed to complete G_1, 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_2-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_2-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. cervisiae (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 finding 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-<u>fos</u>, c-<u>myc</u> and c-<u>jun</u> (Abate and Curren, 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 *r*PKC (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., Geithersburg,
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

RPIM 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 $37^{\circ}C$

in 5% CO_2 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[a]	Average No. of tumor per lung	S.D.
NIH/3T3	Ras Proto-oncogene	Gly^{12}, Ala^{59}, Gln^{61},	0	0
EJ-ras	Human Bladder Carcinoma T-24	Val^{12}, Ala^{59}, Gln^{61},	9	14
EJ/vHA-ras	Chimeric Human and Viral-oncogene	Val^{12}, Thr^{59}, Gln^{61},	72	32
HYB C^{Leu}	Chimeric Yeast and Viral-oncogene	Gly^{19}, Ala^{66}, Leu^{68},	25[b]	40
RAS1^{Leu}del	Recombinant Yeast Oncogene	Gly^{19}, Ala^{66}, Leu^{68},	>280[c]	29

[a]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.

[b]Number of injected cells was 2.9 x 10^5 per mice.

[c]Number of injected cells was 1.8 x 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[a]	no. of animals with microscopic metastases in lung[b]
A375-P	Human Melenoma	0 (0-5)[c]	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

[a]Number of grossly apparent tumor foci on lung surface were determined with the aid of a dissecting microscope.

[b]Stepwise sections from lungs of each animal were examined for present of microscopic parenchymal metastases.

[c]Number in parentheses, range.

TABLE 3 Culture Medium of Cell Lines

Name	Species	Medium	Supplement	Detachment media
NIH/3T3	mouse	DMEM	10% HI-FBS[a] gentomycin	0.1% trysin & 1 mM EDTA
EJ-ras	mouse	DMEM	10% HI-FBS gentomycin	0.1% trysin in 1 mM EDTA
EJ/vHa-ras	mouse	DMEM	10% HI-FBS gentomycin	0.1% trysin & 1 mM EDTA
HYB C	mouse	DMEM	10% HI-FBS gentomycin	0.1% trysin & 1 mM EDTA
RAS1^{Leu}del	mouse	DMEM	10% HI-FBS gentomycin	0.1% trysin & 1 mM EDTA
PC3-P	human	RMPI 1640	10% HI-FBS S. P.[b]	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

[a]HI-FBS: heat-inactivated fetal bovine serum.

[b]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 $MgCl_2$, and 0.15 M NaCl. The suspension was mixed with 200 ul of detergent solution containing 10 mM Tris, 1.5 mM $MgCl_2$, 1.5 mM $MgCl_2$, 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 ^{32}-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., Geithersburg, MD). The plasmid DNA was added to the reaction mixture containing dATP, dGTP, dTTP, and ^{32}-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_2PO_4 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_2PO_4, 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_2PO_4, 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 ^{32}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 deioned 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 ^{32}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 $MgCl_2$; 1 mM dithiothreitol; 1 mM each ATP, CTP, GTP). The

nuclei were quickly frozen in liquid nitrogen and stored at -70°C in a freezer.

Nuclei were resuspended in reaction buffer containing nucleotide triphosphates, alpha-^{32}P-UTP (ICN Biomedicals, Inc., Irvine, CA) and creatine kinase (BRL, Geithersburg, MD). The nuclei were incubated at 37°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 NaCl$_2$, 50 mM MgCl$_2$ and 2 mM 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 ul of 0.02 M Tris-HCl (pH 7.4), 1 mM MgCl$_2$ and incubated with 50 ug/ml DNase (Sigma Chemical Co.) at 37°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 Scitilation 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°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 Denhards't solution ion (1 x Denharht 'solution equal 0.02 % polyvinylpyrilidone, 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-<u>ras</u> and <u>RAS1</u>^{Leu}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

^{0}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_{cat}

and K_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 uM, 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 uM.. 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 ul of the medium was added to 100 ul of 100 mM Tris buffer, pH 8.1, containing 5 ug of DFP-treated plasminogen (Sigma Chemical Co.), 15 ug 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
substracting reaction buffer for conditioned medium, and
each subtrasting 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 <u>et al</u>., 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-<u>ras</u>- and

RAS1^{Leu}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 ^{0}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_2O).

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$_3$, 10 mM MgCl$_2$, 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^3 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^{Leu} and $RAS1^{Leu}$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^{Leu} and $RAS1^{Leu}$del are two yeast RAS1 containing sequences used to transform the NIH 3T3 cells (Defoe-Jones et al., 1985). The HYB C^{Leu} 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 $RAS1^{Leu}$del contains all yeast RAS1 sequences and was prepared

54

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^{Leu}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^{Leu}-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-<u>ras</u>-transformed cells show a high

constitutive level of uPA mRNA over NIH 3T3 controls and

other <u>ras</u> oncogene-transformed cells, (see zero hour time

point in Figure 1). However, the highly malignant

<u>RAS</u>1$^{\text{Leu}}$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[a]

```
                   1[b]        *[d]                                        43
{EJ-ras}       .......MTE YKLVVVGAvG VGKSALTIQL IQNHFVDEYD PTIEDSYRKQ
{EJ/vHa-ras}   .......MTE YKLVVVGAvG VGKSALTIQL IQNHFVDEYD PTIEDSYRKQ
{c-Ha-ras}     .......MTE YKLVVVGAGG VGKSALTIQL IQNHFVDEYD PTIEDSYRKQ
{Hyb C}        MQGNKSTirE YKiVVVGgGG VGKSALTIQF IQsyFVDEYD PTIEDSYRKQ
{RAS1-del}     MQGNKSTirE YKiVVVGgGG VGKSALTIQF IQsyFVDEYD PTIEDSYRKQ
               1[c]                                                       50


               44                   * *                                  93
{EJ-ras}       VVIDGETCLL DILDTAGQEE YSAMRDQYMR TGEGFLCVFA INNTKSFEDI
{EJ/vHa-ras}   VVIDGETCLL DILDTtGQEE YSAMRDQYMR TGEGFLCVFA INNTKSFEDI
{c-Ha-ras}     VVIDGETCLL DILDTAGQEE YSAMRDQYMR TGEGFLCVFA INNTKSFEDI
{Hyb C}        VVIDdkvsiL DILDTAGLEE YSAMREQYMR TGEGFLlVYs VtsrnSFDEl
{RAS1-del}     VVIDdkvsiL DILDTAGLEE YSAMREQYMR TGEGFLlVYs VtsrnSFDEl
               51                                                        100


               94                                                        142
{EJ-ras}       HQYREQIKRV KDSDDVPMVL VGNKCDLA.A RTVESRQAQD LARSYGIPYI
{EJ/vHa-ras}   HQYREQIKRV KDSDDVPMVL VGNKCDLA.A RTVESRQAQD LARSYGIPYI
{c-Ha-ras}     HQYREQIKRV KDSDDVPMVL VGNKCDLA.A RTVESRQAQD LARSYGIPYI
{Hyb C}        lsYyqQIqRV KDSDyIPvVv VGNKlDLeNe RqVsyedglr LAkqlnaPFl
{RAS1-del}     lsYyqQIqRV KDSDyIPvVv VGNKlDLeNe RqVsyedglr LAkqlnaPFl
               101                                                       150


               143                                                       189
{EJ-ras}       ETSAKTRQGV EDAFYTLVRE IRQH..KLRK LNPPDESGPG CMSCKCVLS*
{EJ/vHa-ras}   ETSAKTRQGV EDAFYTLVRE IRQH..KLRK LNPPDESGPG CMSCKCVLS*
{c-Ha-ras}     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 alogrithms in Genetics Computer Group Software [Devereux, J., Haeberli, P., Smithies, O. (1984) <u>Nucleic Acids Research 12</u>, 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-<u>ras</u>-1 [accession number J00277] for c-Ha-<u>ras</u>, murine Sarcoma virus (Harvey-strain) H-<u>ras</u> gene [accession number X00740] for v-Ha-<u>ras</u>, and yeast (<u>Saccharomyces cerevisiae</u>) <u>ras</u>-H related gene c-<u>ras</u>-sc-1 [accession number X00527] for <u>RAS</u>1. Cell lines containing the constructs were previously prepared and reported by Bradley et al.(1986).

[b] Numbering based on sequence of EJ-<u>ras</u>.

[c] Numbering based on sequence of <u>RAS</u>1^{Leu}delprotein.

[d]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 <u>ras</u> 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 ^{32}P-labeled mouse urokinase cDNA. Washed filter was exposed to X-ray film.
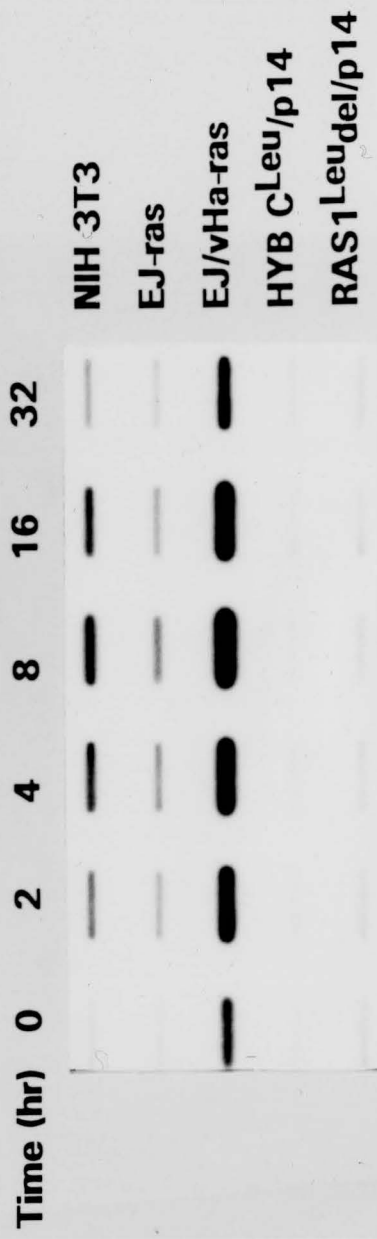
concentrations of uPA mRNA in mouse NIH 3T3 and the ras oncogene-transformed cells treated with 20 n$\underline{M}$ of PMA, for the time periods indicated. On PMA treatment, increases in uPA mRNA levels occur in the EJ/vHa-ras and EJ-ras transformed cells as well as the untransformed NIH 3T3 cells. In contrast, the normal PMA induction of uPA was suppressed in cells transformed by the $\underline{RAS1}^{Leu}$del and the HYB C^{Leu} 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^{Leu} and the $\underline{RAS1}^{Leu}$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 $\underline{RAS1}^{Leu}$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 n$\underline{M}$ PMA only in the yeast $\underline{RAS1}^{Leu}$-transformed cells (HYB C^{Leu} or $\underline{RAS1}^{Leu}$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 ^{32}P-labeled mouse cathepsin L cDNA probe, and exposed to X-ray film.
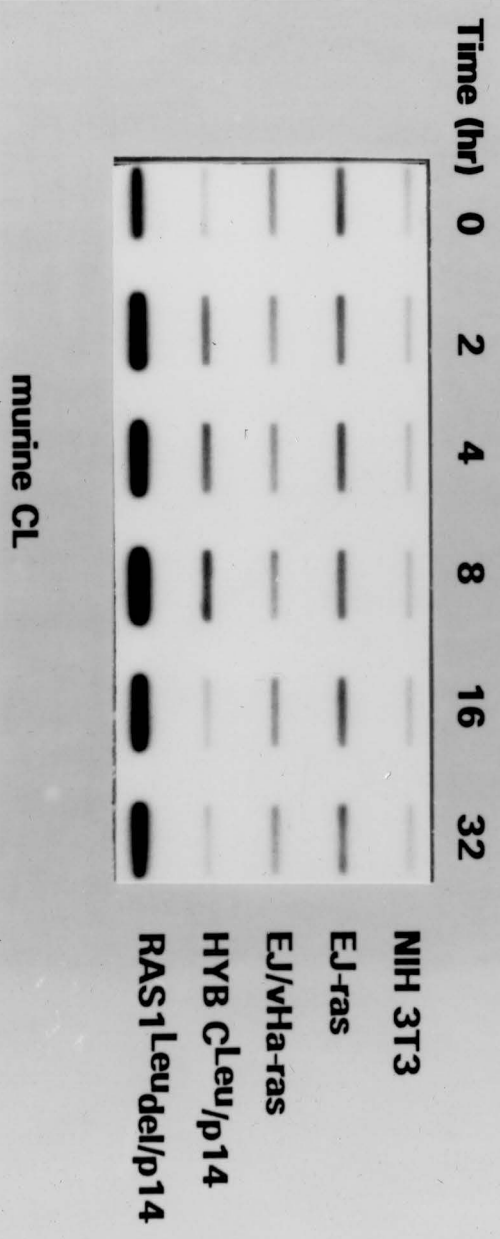
Table 5. Levels of uPA mRNA among ras Oncogene-transformed Mouse Fibroblasts Treated with PMA and Normalized with Actin[a]

Hours PMA Treatment	0	2	4	8	16	32
NIH/3T3	1.0(0.3)[b]	3.5(0.1)	5.4(1.5)	5.5(2.1)	4.2(1.8)	2.0(0.7)
EJ-ras	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-ras	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^{Leu}	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^{Leu}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)

[a]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.

[b]Unit is densitometeric value.

Figure 3. Slot Blot Analysis of Mouse Cathepsin B mRNA
Levels in NIH 3T3 Fibroblast and <u>ras</u> 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}P-
labeled mouse cathepsin B cDNA probe, and exposed to X-ray
film.
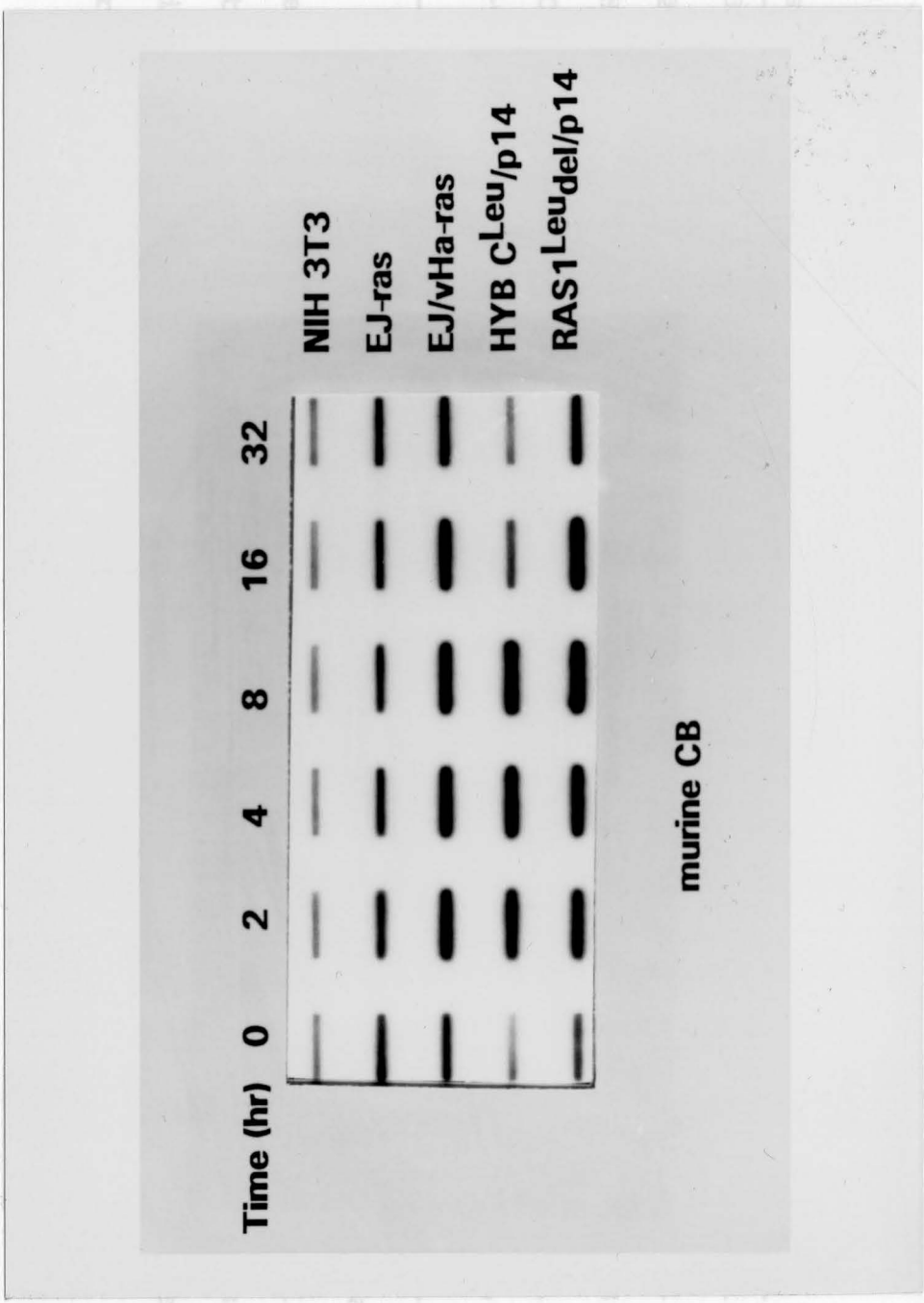
Table 6. Levels of CL mRNA among ras Oncogene-transformed Mouse Fibroblasts Treated with PMA and Normalized with Actin[a]

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-ras	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-ras	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^{Leu}	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^{Leu}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)

[a] 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.

[b] Unit is densitometeric value.

displayed in Table 6. The data show that CL mRNA levels are 6.1-fold (standard deviation 1.1) greater in RAS1^{Leu}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^{Leu} and 2- to 3-fold in RAS1^{Leu}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^{Leu}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 <u>ras</u> Oncogenes Transformed NIH 3T3 Cell Lines after Serial Dilutions.

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

murine CB

murine CL

murine uPA
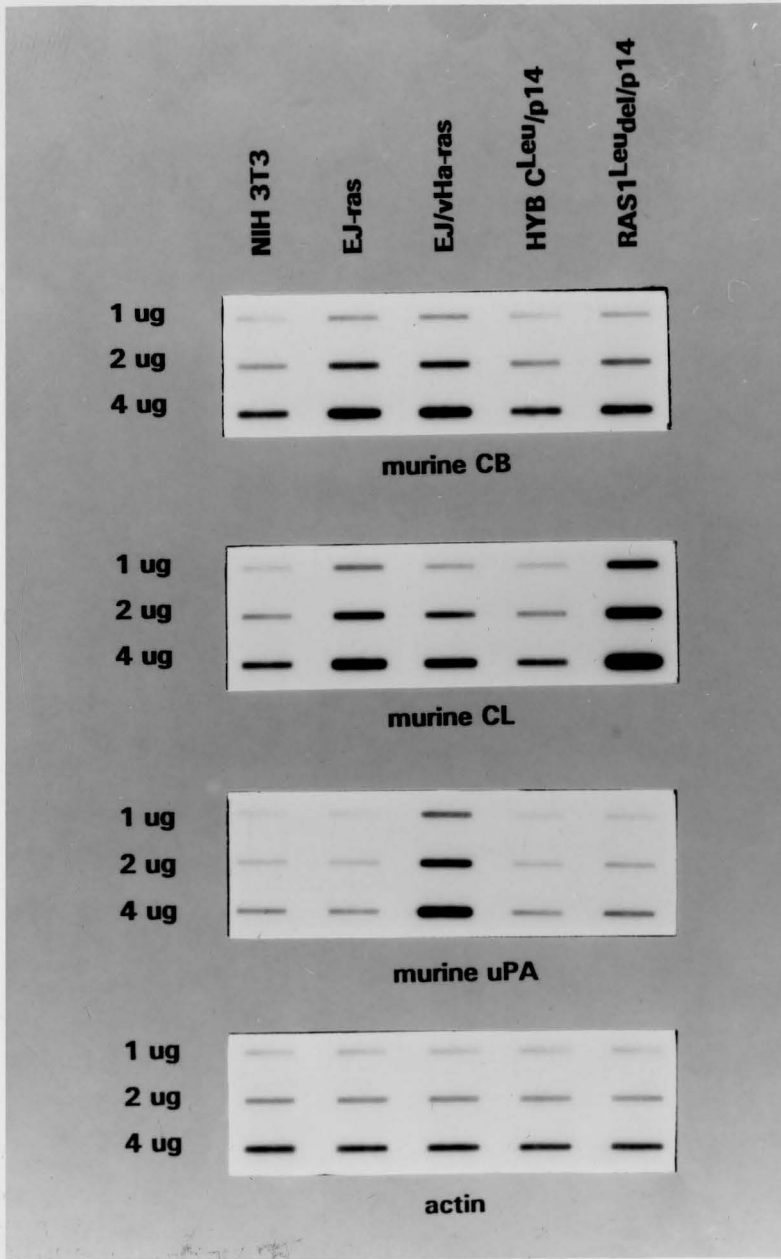
actin

Table 7. Levels of CB mRNA among ras Oncogene-transformed Mouse Fibroblasts

Treated with PMA and Normalized with Actin[a]

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-ras	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-ras	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^{Leu}	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^{Leu}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)

[a]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.

[b]Unit is densitometeric value.

stimulation in cells transformed with

mammalian-likeEJ/vHA-ras and the increase persisted for upto

8 hours. CB mRNA concentration increased 4-fold at between 2

and 8 hours after PMA stimulation in HYB c^{Leu} transformed

cells and 3-fold during that same period in RAS1^{Leu}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^{Leu}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)$^{+}$ 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^+) 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 ^{32}P-labeled mouse cathepsin L
cDNA probe (top) or GAPD (bottom), and exposed to X-ray
film.
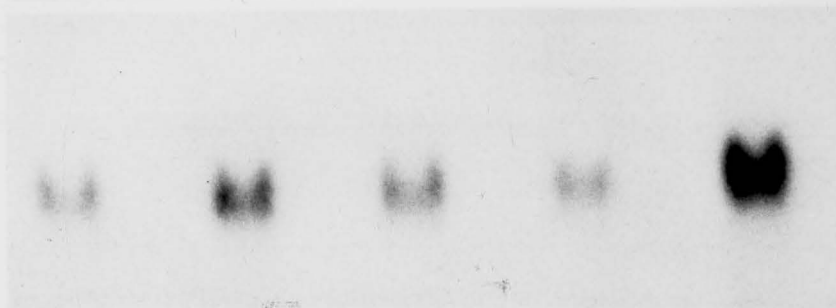
mRNAs were separated by electrophoresis through a 1% agarose gel. The RNAs were transferred onto a Zeta-Probe filter and hybridized with a ^{32}P-labeled mouse cathepsin L cDNA probe. The result is displayed on Figure 5. In this figure, only the RAS1^{Leu}del transformed cells show highest expression 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}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^{Leu}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^{Leu}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^{Leu}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^{Leu}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^{Leu}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^{Leu}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^{Leu}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 ^{32}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.

NIH/3T3   EJ/vHa   RAS1Leudel/p14

CL
CB
UK
actin

NO PMA ADDED

NIH/3T3   EJ/vHa   RAS1Leudel/p14

CL
CB
UK
actin

PMA 2 HR
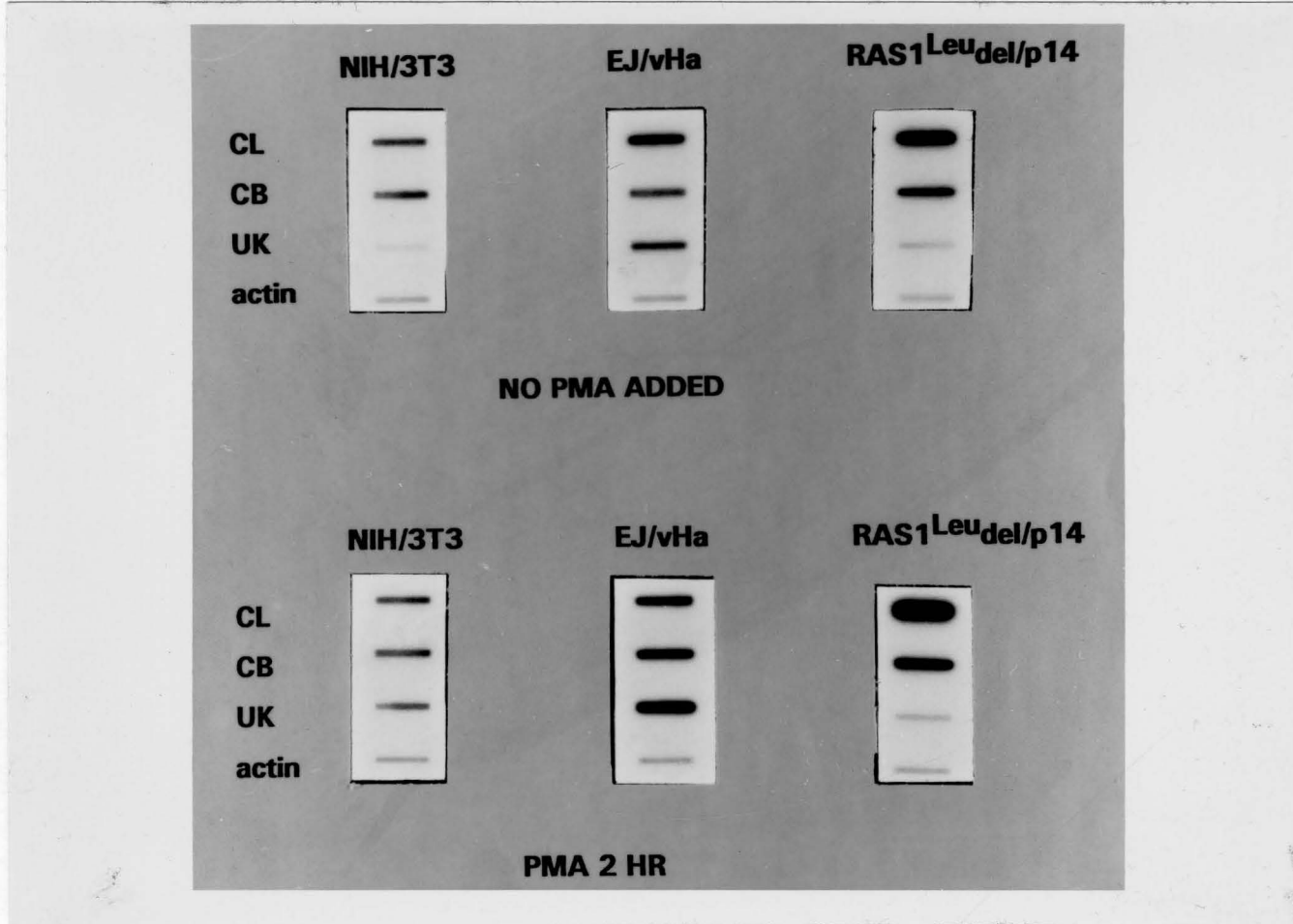
TABLE 8 Relative mRNA Transcription Rates

by Run-on Assay[a]

A. No PMA treatment

CELL LINES

GENE ASSAYED	NIH/3T3	EJ/vHA-ras	RAS1^{Leu}DEL
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

GENE ASSAYED	NIH/3T3	EJ/vHA-ras	RAS1^{Leu}DEL
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

---

[a]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^{Leu}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^{Leu}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'-CAAAAGGAGTAAAAGATTCAT-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 uM) 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^{Leu}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).
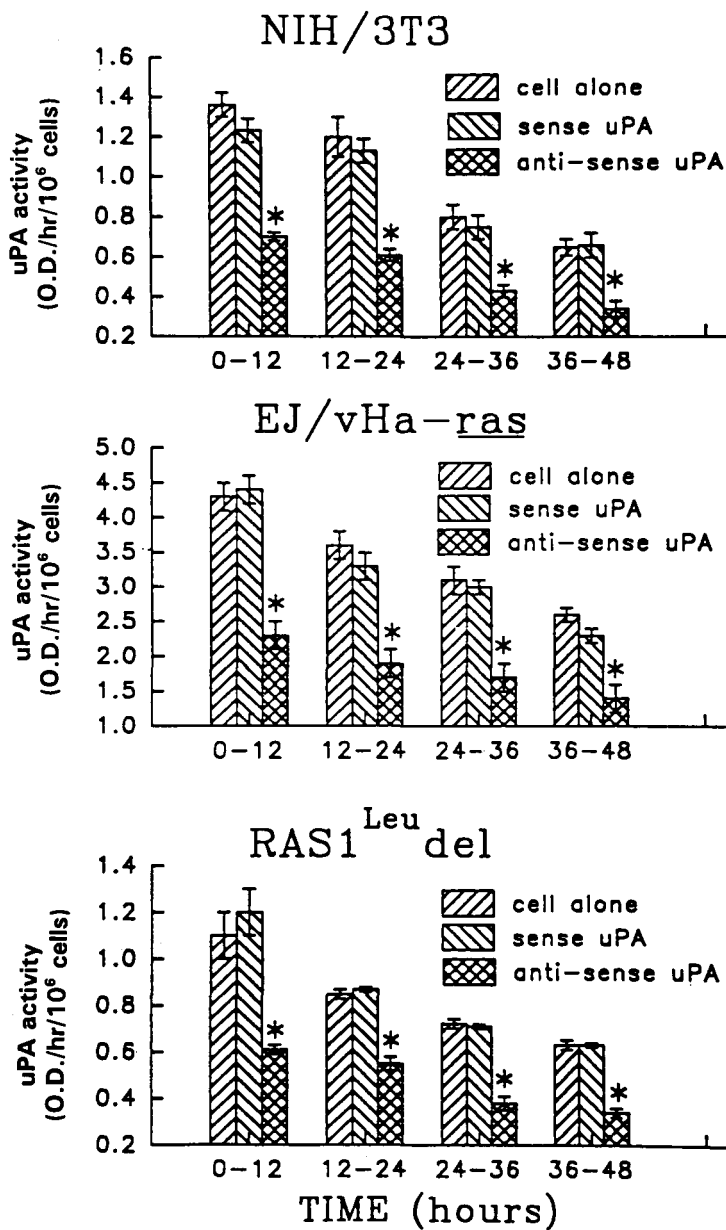
enzymatic activity. The difference in activity levels are in the direction expected from their respective mRNA levels and gene transcription rates, previously determined. Treatment with sense-uPA oligo caused no apparent change in uPA enzymatic activities in these cell lines studied.

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^{Leu}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[a]

EJ-vHa-<u>ras</u> Cells

anti-uPA concentration	uPA activity	% inhibition
no oligo	4.8[b] (0.9)[c]	
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

RAS1^{Leu}del Cells

anti-CL concentration	CL activity	% inhibition
no oligo	78.2[d] (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

[a] Enzymatic activities were determined with the first 12 hour serum-free medium after removal of the oligo inhibitors.

[b] 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 10^6 cells per hour

[c] The number in bracket is standard deviation.

[d] CL enzymatic activities were determined by substrate N-carbobenzoxy-L-Phe-L-Arg-7-aminomethylcoumarin (Z-Phe-Arg-AMC) with units of pmol/min/10^6 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).
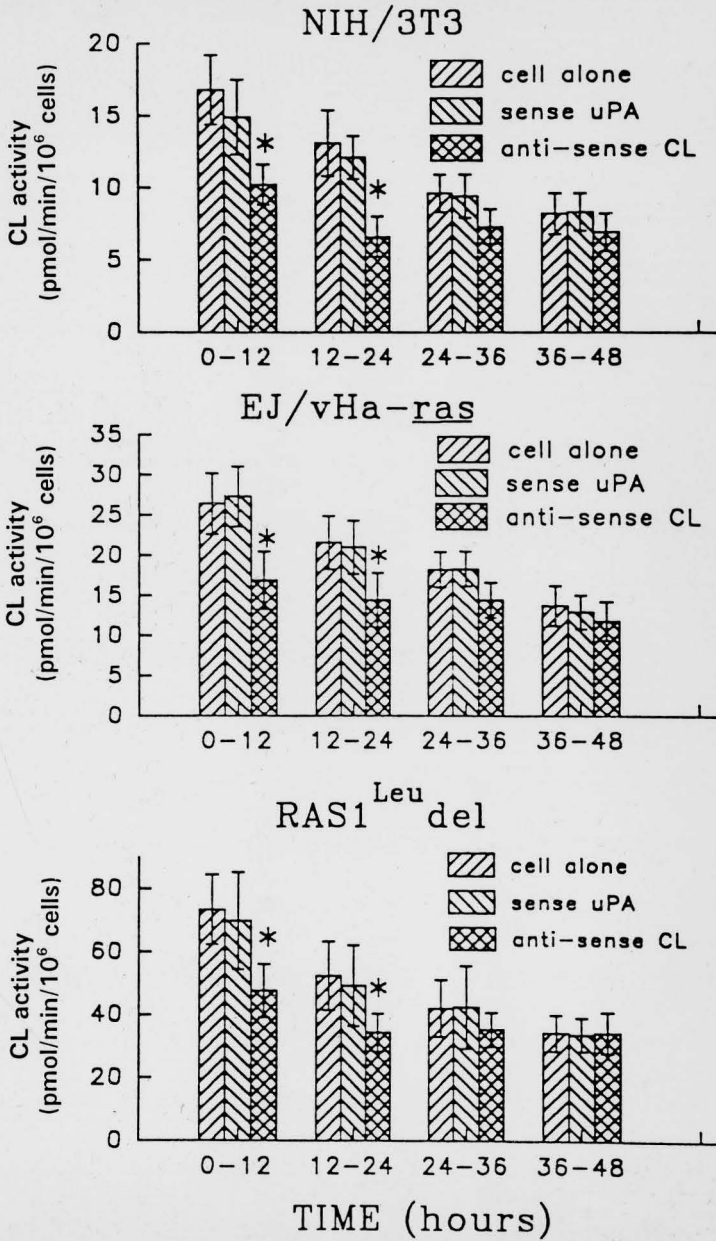
activities of these cell lines under the experimental

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 $pmol/min/10^6$ $cells$ with a standard

deviation 1.4 in the first 12 hrs. EJ/vHA-<u>ras</u> secreted about

1.6-fold more CL enzymatic activity than NIH 3T3, whereas

RAS1^{Leu}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 (3×10^5) 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^{Leu}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 $RAS1^{Leu}$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 $RAS1^{Leu}$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[a] (0.06)[b]	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^{Leu}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

[a]uPA enzymatic activities were determined by chromogenic substrate S-2551.

[b]Numbers are the average of 3 separate runs with units of O.D. per 10^6 cells per hour. The number in bracket is standard deviation.

Table 11 CL Enzymatic Activity of <u>ras</u>-transformed Cell Lines[a]

	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>RAS</u>1^{Leu}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

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

Table 12 CL Enzymatic Activity of <u>ras</u>-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[a] (2.4)[b]	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-<u>ras</u>

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$^{\text{Leu}}$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

[a]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/10^6 cells.

[b]The number in bracket is standard deviation.

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

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

[a]Oligo treated tumor cells (3×10^5) 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 disected from mice and the number of surface tumors per lung counted.

[b]Significantly different from other groups at $p < 0.001$ by nonparametric Wilcoxon rank sum test.

[c]Significantly different from other groups at $P < 0.01$ by nonparametric Wilcoxon rank sum test.

Table 14. Lung Colonization of Nude Mice by $\underline{RAS}1^{Leu}$del-
Transformed Cells Selectively Inhibited by Antisense Oligos[a]

(Experiment 2)

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

---

[a]Oligo treated tumor cells (3×10^5) 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 disected from mice and the number of surface tumors per lung counted.

[b]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). GADP was not significantly induced at the identical conditions. Densitometer traces normalized to the GADP 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 densitometeric units (S.D.$\pm$ 0.8) in PC3-P cells and 0.56 densitometeric 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
^{32}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.
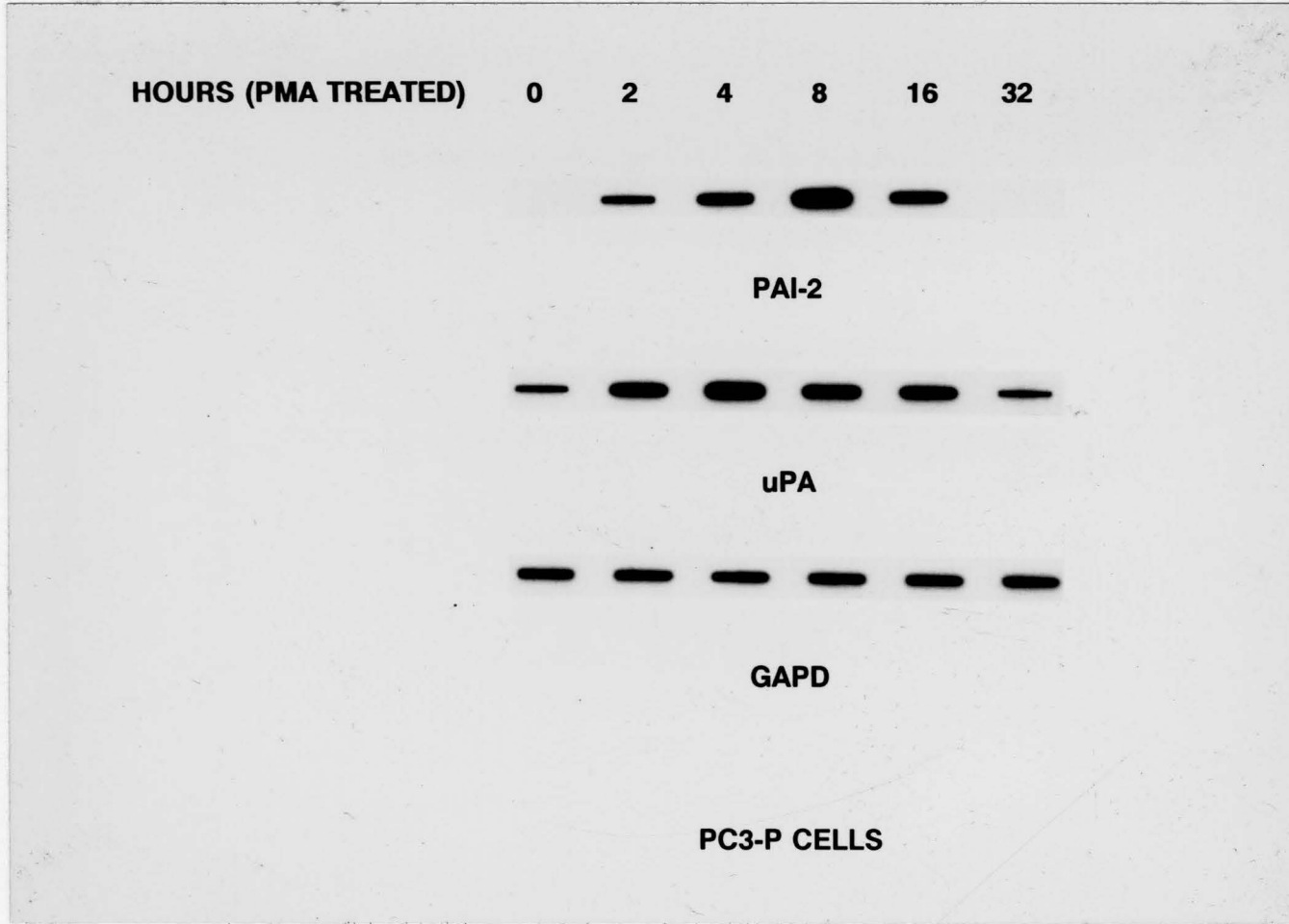
Figure 10. Slot Blot Analysis of Human uPA and PAI-2 mRNA Levels in PC3-M Cells with PMA Treatment.

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 ^{32}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
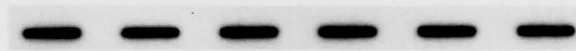
GAPD

PC3-M CELLS

Table 15. Levels of Human uPA and PAI-2 mRNA
in PC3-P and PC3-M with PMA[a]

PC3-P

Hours PMA Treated	0	2	4	8	16	32
uPA/GADP	0.55[b](.12)	1.63(.22)	2.34(.4)	1.82(.32)	1.28(.24)	0.52(.14)
PAI-2/GADP	N.O.[b]	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.

[a]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 densitometeric value of 3 separate runs and standard deviation is in bracket.

[b]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 ^{32}P-labeled human PAI-2 and TIMP inserts. The resulting filter was exposed to X-ray film.

PC3-M CONTROL

PC3-M WITH PMA (20 nM) FOR 8 HRS.

PC3-P CONTROL

PC3-P WITH PMA (20 nM) FOR 8 HRS.

<- GAPD

<- PAI-2

1 2 3 4
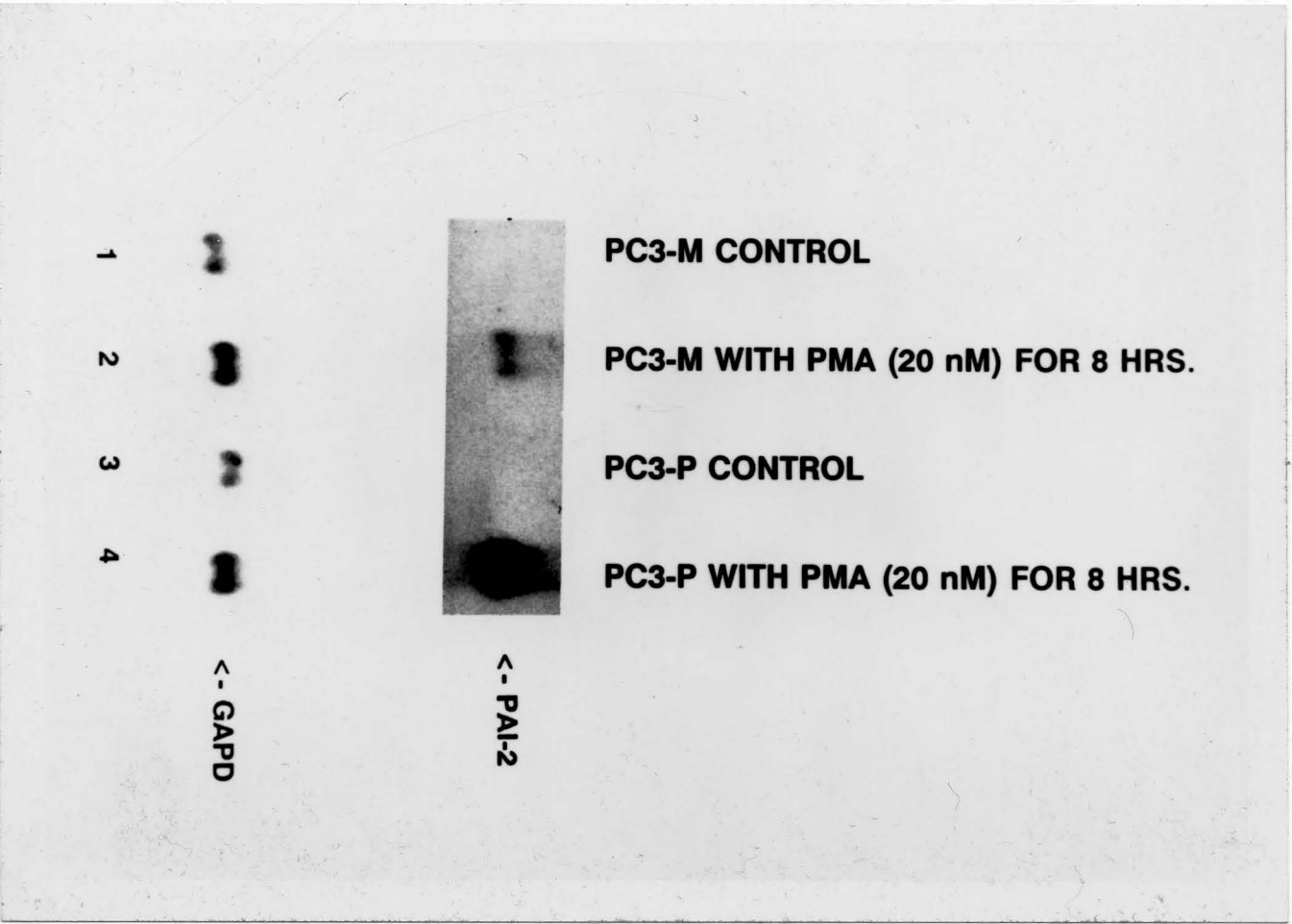
Figure 12. Northern Blot Analysis of Human uPA mRNA Levels in PC3-P and M Cell Lines with PMA as well as Cycloheximide Treatment.

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 ^{32}P-labeled human plasmid uPA insert, and exposed to X-ray film.

28s ->

18s ->

8 7 6 5 4 3 2 1

<- GAPD

<- HUMAN uPA

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
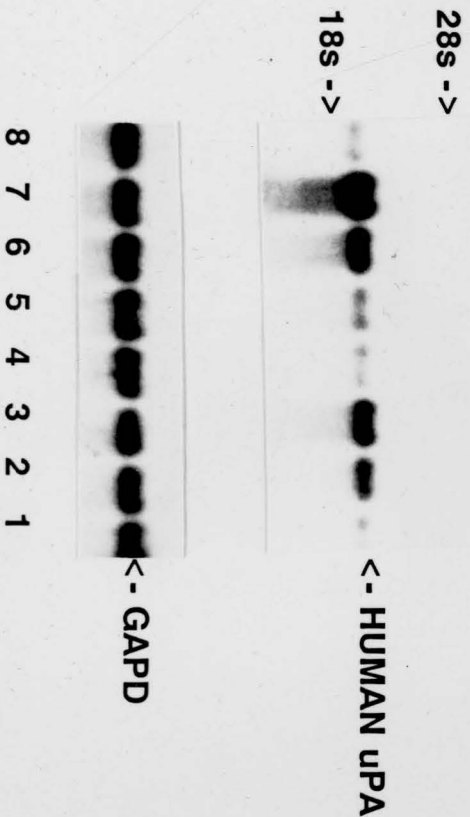
activators) or by a direct stimulation of mRNA synthesis through activation of factors that do not require new protein synthesis. To explore these different alternatives, cycloheximide (10 mg/ml), a protein synthesis inhibitor, was added to the cell culture medium for 16 hours. Its effect on PMA induction 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 GADP 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 ^{32}P-labeled human PAI-2 and
TIMP plasmid inserts. The resulting filter was exposed to X-
ray film.

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

<– GAPD

<– PAI-2

<–TIMP

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

with PMA Treatment Normalized to GAPD[a]

Hours PMA Treated	0	2	4	8	16	32
A375-P	N.O.[b]	N.O.	0.12	0.82	0.82	0.49
A375-SM	N.O.	N.O.	N.O.	N.O.	0.12	0.05

[a]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 densitometeric value of PAI-2 normalized to the densitometeric value of GADP, which was hybridized with the same filter.

[b]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 Mr 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.

A375-SM WITH PMA (20 nM) FOR 24 HRS

A375-SM CONTROL

A375-P WITH PMA (20 nM) FOR 24 HRS

A375-P CONTROL

1

2

3

4

<- 47

<- 84

<- 110

MW (kd)
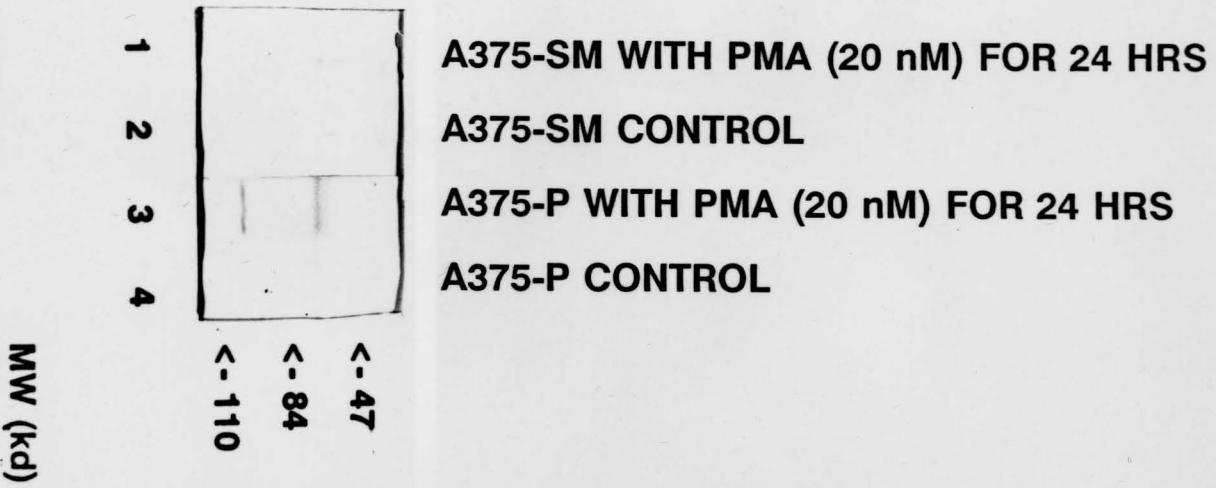
analysis. The result is displayed in Figure 15. No PAI-2 mRNA was detected in either PC3-P and M cells cultured in normal medium (lanes 1 and 5) and in conditioned medium from kidney (lanes 3 and 7), and spleen conditioned medium (lanes 4, 8). However, incubation of the cells with conditioned medium 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}P-labeled human PAI-2 insert, and exposed to X-ray film.

1
2
3
4
5
6
7
8

<- GAPD

<- PAI-2

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.

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^{Leu}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^{Leu}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 $RAS1^{Leu}$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 $RAS1^{Leu}$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 $RAS1^{Leu}$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^{Leu}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^{Leu}del sequence or the yeast HYB C^{Leu} sequence. Thus RAS1^{Leu}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 <u>ras</u> 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 <u>RAS1</u>^{Leu} cells is 6-fold higher than NIH 3T3 cells while the rate in EJ/vHa-<u>ras</u> cells is 2.5-fold higher than NIH 3T3 cells. The uPA transcription rate of EJ/vHa-<u>ras</u> 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-<u>ras</u> cells than NIH 3T3 cells, while CAT activity in <u>RAS1</u>^{Leu} 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^{Leu} cells. In contrast,
a 2 fold PMA stimulation of CL transcription is observed in
RAS1^{Leu} 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^{ras}. PKC may control the activation of p21^{ras} by
its down-regulation of GAP (Downward et al., 1990). c-Fos
and c-Jun are transcription factors activated by the
PKC/p21^{c-ras} 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^{Leu} and RAS1^{Leu}del transformed
cells), (iii) the high constitutive expressions and PMA
inducibility of CL and CB genes in HYB C^{Leu} and RAS1^{Leu}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^{GAP}, and
the other NF1^{GAP} (p280^{GAP}) is encoded by the neurofibromatosis
type-1 gene. p120^{GAP} and NF1^{GAP} proteins bind to the same
effector site in Ras, and it is the heterodimer of GAP-RAS^{GTP}
that is believed to transmit the downstream effector signal
from Ras (Martin et al., 1990; Bollag and McCormick, 1990).

EJ/vHa-<u>ras</u> has an activating mutation at codon 12 while

<u>RAS1</u>^{Leu}del has an activating mutation at codon 68 (equivalent

to codon 61 in Ha-<u>ras</u>). The affinity of the p120^{GAP} has been

showed to increase significantly, when these mutations are

present. Accordingly, the affinity of p120^{GAP} may be better

to <u>RAS1</u>^{Leu}del with mutated codon at 68 as compared to its

binding affinity to mutated codon 12 in the EJ/vHa-<u>ras</u>. As

p120^{GAP} and p280^{GAP} 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-<u>ras</u>) to codon

68 (in <u>RAS1</u>^{Leu}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^{GAP}-Ras and the p120^{GAP}-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^{c-ras} pathway in yeast RAS1-transformed cells shown by negative symbol. Dotted line indicates the inducibility of CL and CB genes by the PKC/p21^{c-ras} pathway only in yeast RAS1 transformed cells.

$RAS1^{Leu}del/p14$
(yeast RAS)

EJ-vHa-ras
(mammalian/
viral)

phorbol ester response factors

activated Ras effectors

activated Ras effectors

AP proteins

transformation

transformation

⊖

CB and CL genes

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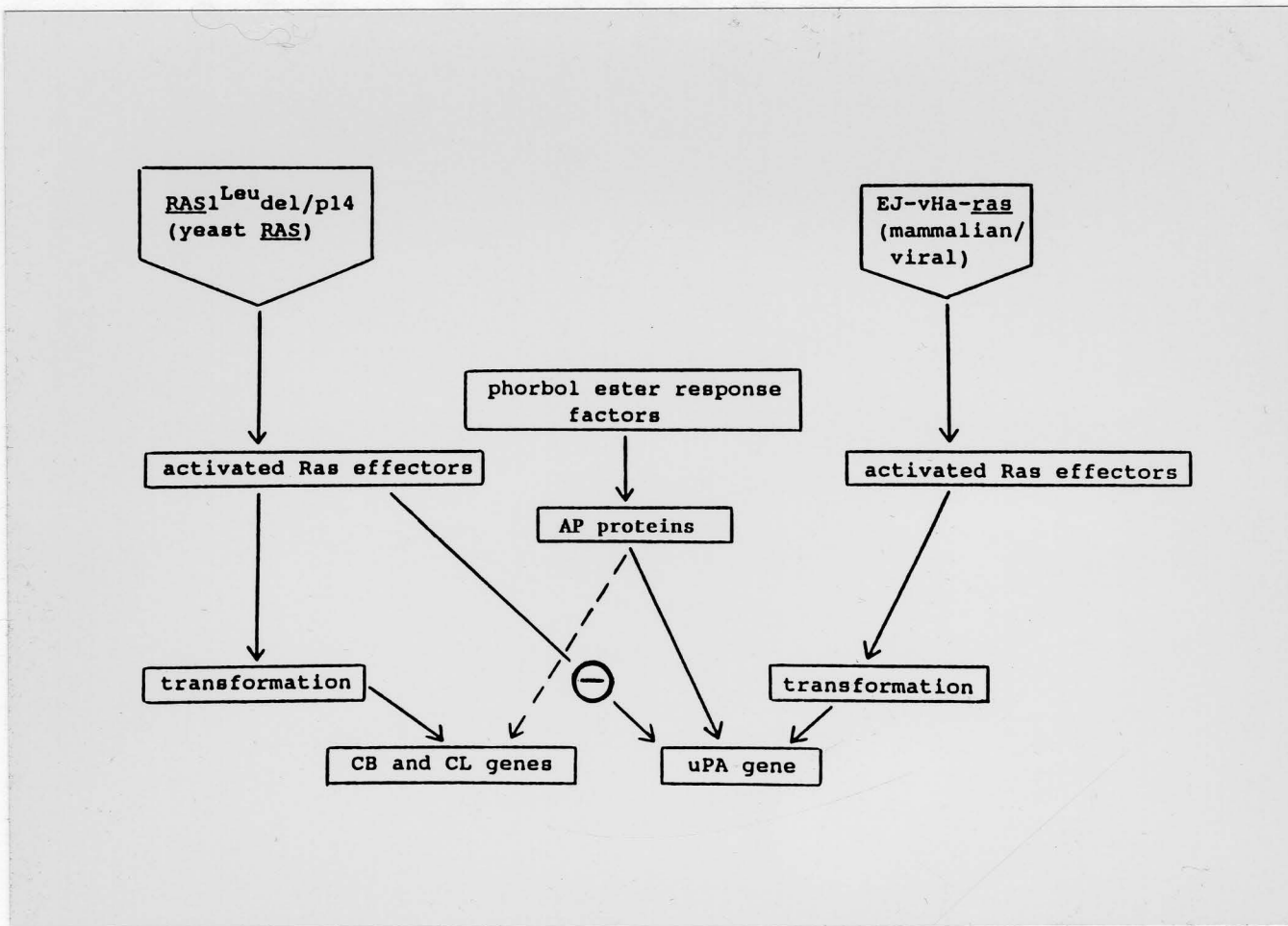
Anti-sense Oligonucleotides Can Partially Inhibit uPA and CL Enzymatic Activities in A Transient Manner

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 oligonucloetides 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-myb-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'-
CAAAAGGAGTAAAAGATTCAT-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-<u>ras</u>- and <u>RAS</u>1^{Leu}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-<u>ras</u> and <u>RAS</u>1^{Leu}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 one 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^{Leu}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^{Leu}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^{125} 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^{Leu}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^{Leu}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^{Leu}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^{Leu}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 interfer 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 a_2 -macroglobulin, a_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 <u>in vivo</u> (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 ex0planations 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^{Leu} and RAS1^{Leu}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^{Leu}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^{Leu}del transformed cells. 3) CL mRNA is significantly higher in highly metastatic RAS1^{Leu}del cell lines as compared

with other cell lines. 4) PMA strongly induce CL mRNA in yeast <u>ras</u> transformed HYB C^{Leu} and <u>RAS</u>1^{Leu}del lines but PMA does not induce CL mRNA in mammalian <u>ras</u> transformed cell lines EJ/vHa-<u>ras</u> or EJ-<u>ras</u>.

RNA transcription rate (run-on) assays were carried out which shows that the differences in mRNA levels for CL and uPA with the different <u>ras</u> 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 <u>ras</u> oncogene transformed NIH 3T3 cells while cathepsin L is at a high level and inducible only in the yeast <u>ras</u> 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-<u>ras</u> cell line and the <u>RAS</u>1^{Leu}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^{Leu}del cells. RAS1^{Leu}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^{Leu}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

REFERENCES

Abate, C., and Curran, T. 1990. Encounters with Fos and Jun on the road to AP-1. Seminars Cancer Biol. 1: 19-26.

Andreasen, P. A., Pyke, C., Riccio, a., Kristesen, P., Neilsine, L.S., Lund, L.R., Blasi, F., and Dano, K. 1987. Plasminogen activator inhibitor type 1 biosynthesis and mRNA level are increased by dexamethasone in human fibrosarcoma cells. Mol. Cell. Biol. 7: 3021-3025.

Andreasen, P. A., Nielsen, L. S., Kristensen, P., Grondahl-Hansen, Skriver, L., and Dano, K. 1986. Plasminogen activator inhibitor from human fibrosarcoma cells bind urokinase-type plasminogen activator, but not its proenzyme. J. Biol. Chem. 261: 7644-7651.

Andreasen, P. A., George B, Lund L. R., Riccio A., and Stacey S. N. 1990. Plasminogen activator inhibitors: hormonally regulated serpins. Mol. Cell. Endocrinol. 68: 1-19.

Anfossi, F., Gewirtz, A. M., and Calabretta, B. 1989. An oligomer complementary to c-myb-encoded mRNA inhibits proliferation of human myeloid leukemia cell lines. Proc. Natl. Acad, Sci., USA 86: 3379-3383.

Angel P., Imagawa M., Chiu R, Stein B. Imbra R. J. 1987. Phorbol ester inducible genes contain a common cis element recognized by a TPA-modulated trans-acting factor. Cell 49: 729-739.

Antalis T. M. and Dickinson J. L. 1992. Control of plasminogen-activator inhibitor type 2 gene expression in the differentiation of monocytic cells. European J. of Biochemistry. 205: 203-209.

Ashendel, C. L. 1985. The phorbol ester receptor: a phospholipid-regulated protein kinase. Biochim Biphys. Acta 822: 219-242.

Astedt, B., Hagerstrand, I., Lecander, I. 1986. Cellular localization in placenta of placental type plasminogen activator inhibitor. Thromb. Haemostasis 56: 63-65.

Axelrod J.H., Reich R, Mishkin R. 1989. Expression of human recombinant plasminogen activator enhances invasion and experimental metastasis of Ha-ras transformed NIH 3T3 cells. Mol. Cell. Biol. 9: 2133-2141.

Ballester, R., Furth M. E., Rosen O. M. 1987. phorbol ester- and protein kinase C-mediated phosphorylation of the cellular kirstein ras gene product. J. Biol. Chem. 262: 2688-2695.

Barbacid, M. 1987. ras genes. Annu. Rev. Biochem. 56: 779-827.

Barrett A. J. 1987. The cystatins: a new class of peptidase inhibitors. Trends Biochem. Sci. 12: 251-256.

Becker, D., Meier, C. B. and Herlyn M. 1989. Proliferation of human malignant melanomas is inhibited by antisense oligodeoxynucleotides targets against basic fibroblast growth factor. EMBO Journal 8: 3685-3691.

Beers, W. H., Stickland, S. and Reich, E. Ovarian. 1975. plasminogen activator: relationship to ovulation and hormone regulation. Cell 6: 387-394.

Belin D.. Vassalli J. D., Combepine C., Godeau F, Nagamine Y., Reich E., Kosher H.P. and Duvoisin R. M. 1985. Cloning, nucleotide sequencing and expression of cDNAs encoding mouse urokinase-type plasminogen activator. Eur. J. Biochem., 148: 225-232.

Bell S. M., Brackenbury R. W, Leslie, N. D. and Degen J. L. 1990. Plasminogen activator gene expression is induced by the src oncogene product and tumor promoters. J. Biol. Chem., 265: 1333-1338.

Beutler B. and Cerami A. 1986. Cachectin and tumour necrosis factor as two sides of the same biological coin. Nature, 320: 584-588.

Birchmeier C., Broek D., Wigler M. 1985. RAS proteins can induce meiosis in Xenopus oocytes. Cell 43: 615-621.

Blasi, F., Vassalli, J-D., and Dano K. 1987. Urokinase-type plasminogen activator: proenzyme, receptor, and inhibitors. Journal of Cell Biol. 104: 801-804.

Blumberg, P. M. 1988. Protein kinase C as the receptor for the phorbol ester tumor promoters: six Rhodes memorial award lecture. Cancer Res. 48: 1-8.

Bollag, G., and McCormick, F. Different regulation of rasGAP and neurofibromatosis gene product activities. Nature, 351: 576-

579.

Bos JL. ras oncogenes in human cancers: a review. Cancer Res. 1989. 49: 4682-9.

Boyd, D., Floret, G., Kim, P., and Brattain, M. 1988. Determination of the level of urokinase and its receptor in human colon carcinoma cell lines. Cancer Res. 48: 3112-3116.

Boyd D. 1989. Examination of the effects of epidermal growth factor of the production of urokinase and the expression of the plasminogen activator receptor in a human colon cancer cell line. Cancer Res. 49: 2427-2432.

Bradley, M. O., Kraynak, A. R., Storer, R. D., and Gibbs, J. B. 1986. Experimental metastasis in nude mice of NIH 3T3 cells containing various ras genes. Proc. Natl. Acad, Sci., USA. 83: 5277-5281.

Broek, D. 1989. Eukaryotic RAS proteins and yeast proteins with which they interact. Current Topics Microbiol.Immun. 147: 155-169.

Busso, D, Belin, C., Failly-Crepin and Vassalli J. D. 1987. Glucocorticoid modulation of plasminogen activators and one of their inhibitors in the human mammary carcinoma cell line MDA-MB-231. Cancer Res., 47: 364-70.

Cai, H., Szeberenyi, J., and Cooper G. M. 1990. Effect of a dominant inhibitory Ha-ras mutation on mitogenic signal transduction in NIH 3T3 cells. Mol and Cellular Biology, 10: 5314-5323.

Capon, D. J., Chen, E. Y., Levinson, A. D., Seeburg, P. H., and Goeddel, D. V. 1983. Complete nucleotide sequences of the T24 human bladder carcinoma oncogene and its normal homologue. Nature, 302: 33-37.

Carmichael, D. F., Sommer, A., Thompson, R. C., Anderson, D. C., Smith, C. G., Welgus, H. G., and Sticklin, G. P. 1986. Primary structure and cDNA cloning of human fibroblast collagenase inhibitor. Proc. Natl. Acad. Sci. USA 83: 2407-2411.

Carol, J. M. 1988. Techniques for using antisense oligodeoxyribonucleotides to study gene expression. Anal. Biochem. 172: 289-295.

Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, V. and Nishizuka, Y. 1982. Direct activation of calcium-activated phospholipid-dependent protein kinase by tumor-promoting phorbol ester. J. Biol. Chem. 257: 7847-7851.

Chan S. J., San Segundo B., McCormick M. B., Steiner D. F. 1986. Nucleotide and predicted amino acid sequences of cloned human and mouse preprocathepsin B cDNAs. Proc. Natl. Acad. Sci., USA. 83: 7721-7725.

Chapman, H. A., Stone, O. L. 1986. A fibrinolytic inhibitor of human alveolar macrophages: induction with endotoxin. Am. Rev. Resp. Dis. 132: 568-575.

Chiu, R., Imagawa, M., Imbra, R.J., Bockovern, J. R., and Karin, M. 1987. Multiple cis- and trans-acting elements mediate the transcriptional response to phorbol ester. Nature 329: 648-651.

Chiu, E., Boyle, W. J., Meek, J., Smeal, J., Hunter, T., and Karin, M. 1988. The c-fos protein interacts with c-jun/AP-1 to stimulate transcription of AP-1 responsive genes. Cell 54: 541-552.

Cochran, D.L., Castellot, J.J., Robinson, J. M., Karnovsky, M. J. 1988. Heparin modulates the secretion of major excreted protein-like molecule by vascular smooth muscle cells. Biochim Biophys Acta 967: 289-295.

Colamonici, R., Trepel, J. B., Vidal, C.A. and Neckers, L. M. 1986. Phorbol ester induces c-sis transcription in stem cell line K-562. Molecular and Cellular Biology 6: 1847-1850.

Collart, M., Belin, D., Vassalli, J. D., Kossodo, S., and Vassalli, P. 1986. Gamma-interferon enhances macrophage transcription of the tumor necrosis factor/cachectin, interleukin 1 and urokinase genes, which are controlled by short-lived regressors. J. Exp. Med., 12: 2113-2118.

Colucci, M., Paramo, J. A. and Collen, D. 1985. Generation in plasma of a fast-acting inhibitor of plasminogen activator in response to endotoxin stimulation. J. Clin. Invest. 75: 818-824.

Dano, K., Andreasen, P.A., Grondahl-Hansen, J., Kristensen, P., Nielsen, L.S., and Skriver, L. 1985. Plasminogen activators, tissue degradation and cancer. Adv. Cancer Res 44: 139-266.

De Clerck, Y. A., Yean, T. D., Ratzkin, B. J. 1989. Purification and characterization of two related but distinct metalloproteinase inhibitors secreted by bovine aortic endothelial cells. J. Biol. Chem. 264: 17445-17453.

Defoe-Jones, D., Tatchell., K., Robinson, L. C., Sigal, I., Vass, W., Lowy, D. R. and Scolnick, E. M. 1985. Mammalian and yeast ras gene products; biological function in their heterologous system. Science, 228: 179-184.

Degen, J. L., Estensen, R. D., Nagamine, Y. and Reich, E. 1985. Induction and expression by tumor promoters. <u>J. Biol. Chem</u>., 260: 12426-12433.

Degen, S.J.F., Heckel, J.L., Reich, E., and Degen, J.L., 1987. The murine urokinase-type plasminogen activator gene. <u>Biochemistry</u> 26: 8270-8279.

Denhardt, D.T., Greenberg A.H., Egan, S.E., Hamilton R. T. and Wright, J. A. 1987. Cysteine proteinase cathepsin L expression correlates closely with the metastatic potential of H-ras-transformed murine fibroblasts. <u>Oncogene</u>, 2: 55-59.

Docherty, A. J. P., Lyons, A., Smith, B. Y., Wright, E. M., Stephens, P.E., Harris, T. J. R., Murphy, G., and Reynolds, J. J. 1985. Sequence of human tissue inhibitor of metalloproteinases and its identify to erythroid-potentiating activity. <u>Nature</u> 318: 66-69.

Downward, J., Riehl, R., Wu, L., and Weinberg, R.A. 1990a. Identification of a nucleotide exchange-promoting activity for p21^{ras}. <u>Proc. Natl. Acad. Sci., USA</u>., 87: 5998-6002.

Downward, J., Graves, J.D., Warne, P.H., Rayter, S., and Cantrell, D.A. 1990b. Stimulation of p21 upon T- cell activation. <u>Nature</u> 346: 719-723.

Dufour, E, Obled, A., Valin, C. and Bechet, D. 1987. Purification and amino acid sequence of chicken liver cathepsin L. <u>Biochemistry</u> 26: 5689-5695.

Eaton, D. L., Scott, R. W., and Baker, J. B. 1984. Purification of human fibroblast urokinase proenzyme and analysis of its regulation by proteases and protease nexin. <u>J. Biol. Chem</u>., 259: 6241-6247.

Edwards, D. R., Waterhouse, P, Holman, M. L., Denhardt, D. T. A growth-responsive gene (16C8) in normal mouse fibroblasts homologous to a human collagenase inhibitor with erythroid-potentiating activity: evidence for inducible and constitutive trnascripts. 1986. <u>Nucleic Acids Res</u>. 14: 8863-8878.

Emeis, J. J., van Hidsberg, W. M., Verheihen, J. H. and Wijingaards, G. 1983. Inhibition of tissue-type plasminogen activator by conditional medium from cultured human and porcine vascular endothelial cells. <u>Biochem. Biophys. Res. Commun</u>. 110: 392-398.

Emeis, J. J., and Kooistra, T. 1986. Interleukin-1 and lipopolysaccharide induce an inhibitor of tissue type plasminogen activator <u>in</u> <u>vivo</u> and in cultured endothelial cells. <u>J. Exp. Med</u>.

163: 1260-1266.

Erickson, L. A., Hekman, C. M., and J. K. Loskutoff. 1985. The primary plasminogen activator inhibitors in endothelial cells, platelets, serum and plasma are immunologically related. Proc. Natl. Acad. Sci. USA. 82: 8710-8714.

Erickson, L.A., Fici, G, J., Lund, J. E. Boyle, T.P., Polites, H. G., and Marotti, K. R. 1990. Development of venous occlusion in mice transgenic for the plasminogen activator inhibitor 1 gene. Nature 346: 74-76.

Estreicher, A., Wohlwend, A., Belin, D., Schleuning, W.D., and Vassalli, J.D. 1989. Characterization of the cellular binding site for the urokinase type plasminogen activator. J. Biol. Chem. 264: 1180-1189.

Fidler, I.J. 1975. BIological behavior of malignant melanoma cells correlated with their survival in vivo. Cancer.Res., 14: 224-265.

Fidler, I. J., 1978. General considerations for studies of experimental cancer metastasis. Methods Cancer Res. 15: 399-439.

Fidler, I.J., 1990. Critical factors in the biology of human cancer metastasis: twenty-eighth GHA Clowes memorial lecture. Cancer Res. 50: 6130-6138

Field, J. 1990. Mutation of the adenyl cyclase gene that block RAS function in Saccharomyces cerevisiae. Science 247: 464-467.

Field, J. 1990. Cloning and characterization of CAP, the S. cereviae gene encoding the 70 kd adenyl cyclase-associated protein. Cell, 61: 319-327.

Gal, S., Willingham, M. C. and Gottesman, M. M. 1985. Processing and lysosomal localization of a glycoprotein whose secretion is transformation stimulated. J. Cell. Biol. 100: 535-544.

Gal, S., and Gottesman, M. M. 1988. Isolation and sequence of a cDNA for human pro-(cathepsin L). Biochem J. 253:303-306.

Gasson, J. C., Golds, D. W., Kaufman, S. E., Westbrook, C. A., Hewick, R. M., Kaufman, R. J., Wong, G. G., Temple, P. A., Leary, A. C., Brown, E. L., Orr, E. C., and Clark, S. C. 1985. Molecular characterization and expression of the gene encoding erythroid-potentiating activity. Nature, 315: 768-771.

Genton, C, Kruithof, E.K.O., Schleuning, W-D. 1987. Phorbol ester induces the biosynthesis of glycosylated and

nonglycosylated plasminogen activator inhibitor 2 in high excess over urokinase-type plasminogen actor to human U-937 lymphoma cells. J. Cell. Biol. 104: 705-712.

Gewirtz, A. M and Calabretta, B. 1988. A c-myb antisense oligodeoxynucleodides inhibits normal human hematopoieses in intro. Science. 242: 1303-1306.

Gibbs, J. B., Ellis, R. W., and Scolnick, E. M. 1984. Autophosphorylation of v-Ha-ras p21 is modulated by amino acid residue 12. Proc. Natl. Acad. Sci. USA 81: 2674-2678.

Giraldi, T., Save, G., Perissin, L., and Zorzet, S. 1985. Proteinase and proteinase inhibition by cytotoxic and antimetastatic drugs in transplantable solid metastasizing tumors in mice. Anticancer Res. 5: 355-359.

Goldberg, G. I., Marmer, B. L., Grant, G. A., Eisen, A. Z., Wilhelm, S. M., and He, C. Human 72-kilodalton type IV collagenase forms a complex with a tissue inhibitor of metalloproteinase designated TIMP-2. Proc. Natl. Acad. Sci. USA. 86: 8207-8211.

Goodchild, J., Agrawal, S., Civeira, M. P., Sarin, P. S., Sun, D. and Zamecnik, P. C. 1988. Inhibition of human immunodeficiency virus replication by antisense oligodeoxynucleotides. Proc. Natl. Acad. Sci. USA 85: 5507-5511.

Gottesman, M. M. 1979. Transformation-dependent secretion of a low molecular weight protein by murine fibroblasts. Proc. Natl. Acad. Sci. USA 75: 2767-2771.

Gottesman, M, M. and Sobel, M. E. 1980. Tumor promoters and Kirsten sarcoma virus increase synthesis of a secreted glycoprotein by regulating levels of translatable mRNA. Cell 19: 449-455.

Graf, F. M., Haemmerli, G., and Strauli, P. 1984. Cathepsin B containing cells in the rabbit mesentery during invasion of V2 carcinoma cells. Histochemistry, 80: 509-515,

Greenberg, M. E., Ziff, E. B. 1984. Stimulation of 3T3 cells induces transcription of the c-fos proto-oncogene. Nature 311: 433-438.

Gross, J.L., Muscatel, D., Gaffe, E.A., and Rifkin, D.B., 1982. Plasminogen activator and collagenase production by cultured capillary endothelial cells. J. Cell Biol. 95: 974-981.

Goldfarb, R. H., and Liotta, L. A. 1986. Proteolytic enzymes in cancer invasion and metastasis. Semin. Thromb. Hemost. 12: 294-307.

Gopalakrishna, R. and Barsky, S. H. 1988. Tumor promoter-induced membrane-bound protein kinase C regulates hematogenous metastasis. <u>Proc. Natl. Acad. Sci</u>. <u>USA</u> 85: 612-616.

Gunji, Y., and Gorelik, E. 1988. Role of fibrin coagulation in protection of murine tumor cells from destruction by cytotoxic cells. <u>Cancer Res</u>. 48: 5216-5221.

Guenzler, W. A., Moscatelli, D, Jaffe, E. A., and Rifkin, D. B. 1982. The primary structure of high molecular mass urokinase from human urine. The complete amino acid sequence of the A chain. <u>Hoppez-Seyler's z. Physiol Chem</u>. 363: 1155-1165.

Hancock J.H., Magee A. I., Childs J. E., Marshall C. 1989. All <u>ras</u> proteins are polyisoproylated but only some are palmitoylated. <u>Cell</u> 516: 1167 - 1177.

Harvey, S. R., Lawrence, D. D., Madeja, J. M., Abbey, S. J., and Markus, G. 1988. <u>Clin. Expl. Metastasis</u> 6: 431-450.

Hawley-Nelson, P., Roop, D. R., Cheng, C.K., Drieg, T. M. and Yuspa, S. H. 1988. Molecular cloning of mouse epidermal systatin A and detection of regulated expression in differentiation and tumorigenesis. <u>Mol. Carcinogenesis</u> 1: 202-211.

Hecker, E. 1968. Cocarcinogenic principles from the seed oil of *Croton tiglium* and from other *Euphorbiaceae*. <u>Cancer Res</u>. 28: 2338-2349.

Heywood, S. M. 1986. tcRNA as a naturally occurring antisense RNA in eukaryotes. <u>Nuc. Res</u>. 14: 6771-6772.

He, C., WIlhelm, S.M., Pentland, A.P., Marmer, B.L., Grant, G.A., Eizen, A.Z., and Goldberg, G.I. 1989. Tissue cooperation in a proteolytic cascade activating human interstitial collagenases. <u>Proc. Natl. Acad. Sci., USA</u>. 86: 2632-2636.

Holmes, W. E., Pennica, D., Blaber, M., Rey, M. W., Guenzler, W. A., Steffens, G. J. and Heynecker, H. L. 1985. Cloning and expression of the gene for prourokinase in <u>Escherichia</u> <u>coli</u>. <u>Biotechnology</u> 3: 923-929.

Holt., J. T., Gopal, T. V., Moulton, A. D., and Nienhius, A. W. Inducible production of c-fos antisense RNA inhibit 3T3 cell proliferation. <u>Proc. Natl. Acad. Sci. USA</u> 83: 4794-4798, 1986.

Holt, J. T., Redner, R. L. and Nienhus, A .W. 1988. An oligomer complementary to c-<u>myc</u> mRNA inhibits proliferation of HL-60 promyelocytic cells and induces differentiation. <u>Mol. Cell. Biol</u>. 8: 963-973.

Holtz, D., Tanaka, R. A., Hartwig, J., McKeon, F. 1989. The CaaX motif of laminin A functions in conjunction with the nuclear localization signal to target assembly to the nuclear envelope. Cell 57: 969-977.

Horowitz, S., Dafni, N, Shapiro, D. L., Wholm, B. A., Notter, R. H. and Quible, D. J. 1989. Hyperoxic exposure alters gene expression in the lung. J. Biol. Chem. 264: 7092-7095.

Huarte, J., Belin, C., Bosco, D., Sappino, A-P., and Vassalli, J-D. 1987. Plasminogen activator and mouse spermatozoa: urokinase synthesis in the male genital tract and binding of the enzyme to the sperm cell surface. J. Cell Biol., 104: 1281-1289.

Humphries, M. J., Matsumoto, K., White, S. L., and Olden, K. 1986. Oligosaccharide modification by sawinsonine treatment inhibits pulmonary colonization by B16-F10 murine melanoma cells. Proc. Natl. Acad. Sci. USA 83: 1752-1756.

Ichinose, A., Fujikawa, K. and Suyyama, T. 1986. The activation of pro-urokinase by plasma kallikrein and its inactivation by thrombin. J. Biol. Chem. 261: 3486-3489.

Imbra, R. J. and Karin, M. 1987. Metallothionin gene expression is regulated by serum factors and activators of proteins kinase C. Mol. and Cell. Biol. 7: 1358-1363.

Iwakawa, A., Gasic, T.B., Viner, E.D., and Gasic, G.J. 1986. Promotion of lung tumor colonization in mice by the synthetic thrombin inhibitor (No. 805) and its reversal by leech salivary gland extracts. Clin. Exp. Metastasis 4:205-220.

Javvien, M., Rinne, A., Hopsu-Harvu, V.K. 1987. Human cystatins in normal and diseased tissues - a review. Acta Histochem. 82: 5-18.

Joseph L. S., Chang L.C., Stamenkovich D, Sukhatme V. 1988. Complete nucleotide and deduced amino acid sequence of murine preprocathepsin L. J. Clin. Invest. 81: 1621-1629.

Kane, S. E. and Gottesman, M. M. 1990. The role of cathepsin L in malignant transformation. Cancer Biology, 1: 127-136.

Kaplan, D. R., Morrison, D. K., Wong, G., McCormick, F. and Williams, I. T. 1990. PDGF beta-receptor stimulates tyrosine phosphorylates of GAP and association of GAP with a signal complex. Cell 61: 125-133.

Kawano, T., Mormoto, K., Uemura, Y. 1986. Urokinase inhibitor in human placenta. Nature. 217: 253-254.

Kelly K., Chochran B.H., Stiles C.D., Leder P. 1983. Cell-

specific regulation of the c-myc gene by lymphocyte mitogens and platelet derived growth factor. <u>Cell</u> 35: 603-610.

Keski-Oja J., Blasi, F., Leof, E. B. and Moses, H. J. 1988. Regulation of the synthesis and activity of urokinase plasminogen activator in A549 human lung carcinoma cells by transforming growth factor-beta. <u>J. Cell. Biol</u>. 106: 451-459.

Kirchheimer, J.C., Christ, G., and Binder, B.R. 1989. Growth stimulation of human epidermal cells by urokinase is restricted to the intact active enzyme. <u>Eur. J. Biochem.</u>, 181: 103-107.

Kirchheimer, J.C., Wojta, J. Christ, G., and Binder, B.R. 1989. Functional inhibition of endogenous kinase decreases cell proliferation in a human melanoma cell line. <u>Proc. Natl. Acad. Sci. USA</u>., 86:5424-5428.

Kiss, Z. 1990. Effects of phorbol ester on phospholipid metabolism. <u>Prog. Lipid Res</u>. 29: 141-166.

Kozlowski, J.M., Hart, I.R., FIdler, I.J., and Hanna, N. 1984a. A human melanoma line heterogeneous with respect to metastatic capacity in athymic nude mice. <u>J. Natl. Cancer Inst</u>. 72: 913-917.

Kozlowski, J.M., FIdler, I.J., Campbell, D., Xu, Z.L., Kaight E., and Hart. I.R. 1984b. Metastatic behavior of human tumor cell lines growth in the nude mouse. <u>Cancer Res</u>. 44: 3522-3529.

Krepela E., Vesely P., Chalouppkva A., Zicha D., Urbanec P., Rasnick D., and Vicar J. 1989. Cathepsin B in cells of two rat sarcoma with different rates of spontaneous metastasis <u>Neoplasms</u>. 36: 529-540.

Kruithof, E.K.O, Ransijin A., Bachmann F. 1983. Inhibition of tissue plasminogen activator by human plasma. <u>Progress in fibrinolysis</u> 6: 365-369.

Kruithof, E. K. O., Tran-Thang, C., Ransijun, A. and Bachmann, F. 1984. Demonstration of a fast-acting inhibitor of plasminogen activators in human plasma. <u>Blood</u> 64: 907-913.

Kruithof, E. K. O., Tran-Thang, C. and Bachmann, F. 1986. The fast-acting inhibitor of tissue-type plasminogen activators in human plasma is also the primary plasma inhibitor of urokinase. <u>Thromb. Haemostasis</u> 55: 65-69.

Kruithof, E.K.O., Vassalli, J. D., Schleuning W. D., Mattaliano, R.J., and Bachmann, F. 1986. Purification and characterization of a plasminogen activator inhibitor from the histiocytic lymphoma cell line U937. <u>J. Biol. Chem</u>. 261: 11207-

11213.

Laemmli, U.K. 1970. cleavage of structural proteins during the assembly of the head of bacteriophage T4. <u>Nature</u> 227: 680.

Lah, T. T., Clifford, J. L., Helmer, K. M., Day, N. A., Moin, K., Honn K. V., Crissman, J. D. and Sloane B. F. 1989. Inhibitory properties of low molecular weight cysteine proteinase inhibitors from human sarcoma. <u>Biochim Biophys</u> a 993: 63-73.

Lah, T.T., Buck, M.R., Honn, K.V. Crissman, J.D. Rao, N.C., Liotta, L.A. and Sloane, B.F. 1989. Degradation of laminin by human tumor cathepsin B. <u>Clin. Exp. Metastasis</u>. 7: 461-468.

Laiho, A. and Keski-O. J. 1989. Growth factors in the regulation of pericellular proteolysis: A review. <u>Cancer Res</u>. 49: 2533-2553.

Lamph, W. W., Wamsley, P., Sassone-Corsi, P., Verma, I., 1988. Induction of proto-oncogene Jun/AP-1 by serum and TPA. <u>Nature</u> 334: 629-631.

Land H, Parada LF, Weinberg RA. 1983. Cellular oncogenes and multistep carcinogen. <u>Science</u> 222: 771-778.

Laug W. E. 1987. Human vascular smooth muscle cells produce plasminogen activator inhibitor 1 and protease nexin (abstract). <u>Thromb. Haemostasis</u> 58: 14.

Laumas, S., Abdel-Ghany, M., Leister, K., Resnick, R., Kandrach, A. and Racker, E. 1989. Decreqsed susceptibility of a 70-kDa protein to cathepsin L after phosphorylation by protein kinase C. <u>Proc. Natl. Acad. Sci. USA</u> 86: 3021-3025.

Davis, L. G., Dibner, M. D., Battey, J. F. 1986. <u>Basic methods in molecular biology</u>. Elsevier. 19-2: 306-310.

Lijnen, J. R., van Hoef, B., Nelles, L., and Collen, D. 1990. Plasminogen activation with single chain urokinase type plasminogen activator. <u>J. Biol. Chem</u>. 265: 5232-5236.

Liotta, L.A., Thorgeirsson, U.P. and Garbisa, S. 1982. Role of collagenases in tumor cell invasion. <u>Cancer Metastasis Rev</u>. 1: 277-288.

Liotta, L.A., Rao, C.N., and Wewer, U.M. 1986. Biochemical interactions of tumor cells with basement membrane. <u>Annu. Reio Biochem</u>. 55:1037-1057.

Liotta, L. A. 1986. Tumor invasion and metastases-role of the extracellular matrix. <u>Cancer Res</u>. 46: 1-7.

Liotta, L. A. 1988. <u>Metastasis, CIBA Foundation Symp</u>. 141: 94-108, John Wiley and Sons (Publ.), Chichester.

Maciewicz, R.A., Wardale, R. J., Etherington, D.J., Paraskeva, C. 1989. Immunodetection of cathepsin B and L present in and secreted from human pre-malignant and malignant colorectal tumor cell lines. <u>Int. J. Cancer</u>, 43: 478-486.

Mainferme, F., Wattiaurx, R., von Figura, K. 1985. Synthesis, transport and processing for cathepsin C in Morris hepatoma 7777 cells and rat hepatocytes. <u>Eur. J. Biochem</u>. 153: 211-216.

Markus, G. 1984. The role of hemostasis and fibrinolysis in the metastatic spread of cancer. <u>Semin. Thromb. Hemostasis</u>, 10: 61-70.

Markus, G., Camiolo, S.M., Kohga, S., Madeja, J.M. and Mittelman, A. 1983. Plasminogen activator secretion of human tumors in short term organ culture: comparison of primary and metastatic colon tumors. <u>Cancer Res</u>., 43: 5517-5525.

Markus, G. 1988. The relevance of plasminogen activators to neoplastic growth: A review of recent literature. <u>Enzyme</u> 40: 158-172.

Martin, G. A., Viskochil, D., Bollag, G., GcCabe, P. C., Crosier, W. J., Haubruck, H., Conroy, L., Clark, R., O'Connell, P., Cawthon, R. W., Innis, M. A., and McCormick, F. The GAP-related domain of the neurofibromatosis type 1 gene product interacts with <u>ras</u>21. <u>Cell</u>, 63: 843-849.

Mason, R. W., Taylor, M.A.J. and Etherington, D. 1984. The purification and properties of cathepsin L from rabbit liver. <u>Biochem J</u>. 217: 209-217.

Mason, R. W., Gal S, Gottesman, M.M. 1987. The identification of the major excreted protein (MEP) from a transformed mouse fibroblast cell line as a catalytically active precursor form of cathepsin L. <u>Biochem. J</u>. 248: 449-454.

Mason, R.W., Johnson, D.A., Barrett, A.J. and Chapman H.A. 1986. Elastinolytic activity of human cathepsin L. <u>Biochem. J</u>. 233: 925-927.

Matrisian, L. M., Glaichenhaus, N., Gesnel, M. C. and Breathnach, R. 1985. Epidermal growth factor and oncogenes induce transcription of the same cellular mRNAs in rat fibroblasts. <u>EMBO J</u>. 4: 1435-1440.

Mayer, M., Lund, L.R., Riccio, A., Skouv, J., Nielsen, L. S., Stacey, S. N., Dano, K., and Andreasen, P. A. 1988.

Plasminogen activator inhibitor type-1 protein mRNA and gene transcription rate are increased by phorbol ester in human rhabdomyosarcoma cells. <u>J. Biol. Chem</u>. 263: 15688-15693.

Medcalf, R. L., Richards, R. I., Crawford, R. J., and Hamiltor J. A. 1986. Suppression of urokinase-type plasminogen activator mRNA levels in human fibrosarcoma cells and synovial fibroblasts by anti-inflammatory glucocorticoid. <u>EMBO J</u>. 5: 2217-2222.

Mignatti, P., Robbins, E., and Rifkin, D.B. 1986. Tumor invasion through the human amniotic membrane: requirement for a proteinase cascade. <u>Cell</u> 47:487-498.

Mira-y-Lopez, R., Reich, E. and Ossowski, L. 1983. Modulation of plasminogen activtor in rodent mammary tumors by hormones and other effectors. <u>Cancer Res</u>., 43: 5467-5477.

Mizuno, T., Chou, M.-Y., and Inouye. A unique mechanism regulating gene expression translational inhibition by a complementary RNA transcript (micRNA). <u>Proc. Natl. Acad. Sci. USA</u> 81: 1966-1970.

Mochan, E., and Keler, T. 1984. Plasmin degradation of cartilage proteoglycan. <u>Biochim. Biophys. Acta</u> 800: 312-315.

Monteasano, R., and Orci, L. 1985. Tumor promoting phorbol esters induce angiogenesis in vitro. <u>Cell</u> 42: 496-477.

Morikawa, K., Walker, S. M., Jessup, J. M. and Fidler, I. J. 1988. <u>in vivo</u> selection of highly metastatic cells from surgical specimens of different primary human colon carcinomas implanted into nude mice. <u>Cancer Res.</u> 48: 1943-1948.

Morrison, R.I.G., Barrett, A.J., and Dingle, J.T., 1973. Cathepsin B1 and D action on human cartilage proteoglycans. <u>Biochim. Biophys. Acta</u> 302: 411-419.

Mouritsen, S., Meldal, M., Ruud-Hansen, J. and Werdelin, D. 1991. T-hepler-cell determinants in protein antigens are preferentially located in cysteine-rich antigen segments resistant to proteolytic cleavage by cathepsin B, L, and D. <u>Scandinavian Journal of Immunology</u> 34: 421-431.

Mulcahy, L.S., Smith, M. R. and Stacey, D. W. 1985. Requirement for ras proto-oncogene function during serum-stimulated growth of NIH 3T3 cells. <u>Nature</u> 313: 241-243.

Mullins, D. E. and Rohrlich, S. T. 1983. The role of proteinases in cellular invasiveness. <u>Biochimica et Biophysica Acta</u> 695: 177-214.

Nagamine, Y., Marius S. and Reich E. 1983. Hormonal regulaion of plasminogen activator mRNA production in porcine kidney cells. Cell, 32; 1181-1190.

Needham, G.K., Sherbet, G.V., Farndon, J.R., and Harris, A.L. 1987. Binding of urokinase to specific receptor sites on human breast cancer membranes. Br. J. Cancer 55: 13-16.

Nelson, N. F., Cieplak, W., Dacus, S. C. and Prager, M. 1986. Characterization of plasminogen activator from two human renal carcinoma cell lines. J. Cell. Physiology. 126: 435-443.

Nielsen-Hamilton, M, Hamilton, R.T. and Adams, G. 1982. Rapid selective stimulation by growth factors of the incorporation by Balb/3T3 cells of [^{35}S] methionine into a glycoprotein and five superinducible proteins. Biochem Biophys Res. Commun. 108: 158-166.

Niedbala, M. J. and Sartorelli, A. C. 1989. Regulation by epidermal growth factor of human squamous cell carcinoma plasminogen activator-mediated proteolysis of extracellular matrix. Cancer Res., 49: 3302-3309.

Nielsen, L. S., Kellerman, G. M., Behrendt, N., Picone, R., Dano, K. and Blasi, F. 1988. The urokinase type of plasminogen activator in cancer of digestive tracts. Thromb. Res., 50: 527-535.

Nishizuka, Y. 1988. The molecular heterogeneity of protein kinase C and its implication for cellular regulation. Nature 334: 661-665.

Novak, U., Cocks B. G. and Hamilton J. A. 1991. A labile repressor acts through the NFkB-like binding sites of the human urokinase gene. Nucleic Acids Res. 19: 3389-3393.

Ny, T., Sawdey, M., Lawrence, D., Mellan, J. L., and Loskutoff, D. J. 1986. Cloning and sequence of cDNA coding for the human beta-migrating endothelial cell type plasminogen activator inhibitor. Proc. Natl. Acad. Sci., USA 83, 6776-80.

Oanrian, and Wa, N. 1989. Biology of the protein kinase C family. Cancer Metast. Rev. 8: 199-214.

Oikawa, M. and Hsueh, A.J.W. 1989. Beta-adrenergic agents stimulate tissue plasminogen activator activity and messenger ribonucleic acid levels in cultured rat granulosa cells. Endocrinology, 125: 2550-2557.

Ono, Y., Fujii. T., Igarashi, K., Kuno, T., Tanaka, C., Kikkawa, U., and Nishizuka, Y. 1989. Phobol ester binding to

protein kinase C requires a cysteine-rich zinc-finger-like sequence. <u>Proc. Natl. Acad. Sci., USA</u> 86, 4868-4871.

Ossowski, L., and Reich, E. 1983. Antibodies to plasminogen activator inhibit human tumor metastasis. <u>Cell</u> 35: 611-619.

Ossowski, L. 1988. Plasminogen activator dependent pathways in the dissemination of human tumor cells in the chick embryo. <u>Cell</u> 52: 321-328.

Ostrowski, L.E., Ahsan, A., Suthar, B.P., Pagast, P., Bain, D.L., Wong, C., Patel, A., and Schultz, R.M. 1986. Selective inhibition of proteolytic enzymes in an <u>in vivo</u> mouse model for experimental metastasis. <u>Cancer Res.</u>, 46:4121-4128.

Pannekoek, H., Veerman, H., Lamber, H., Diergaarde, P., Verweij, C.L., Van Zonneveld, A.J. Van Mourik, J. A. 1986. Endothelial cell plasminogen activator inhibitor (PAI): a new member of the serpin gene family. <u>EMBO J</u>. 5: 2539-2544.

Paranjpe, M., Engel, L., Young, N., and Liotta, L.A., 1980. Activation of human breast carcinoma collagenase through plasminogen activator. <u>Life Sciences</u> 26:1223-1231.

Pennica D., Holmes W. E., Kohr W. J., Harkins R. N., Vehar G. A., Ward C. A., Bennett W. F., Yelverton E., Seeburg P. H., Heyneker H. L., Goeddel D. V., and Collen D. 1983. Cloning and expression of human tissue-type plasminogen activator cDNA in E. coli. <u>Nature</u> 301: 214-221.

Persons, D. A., Wilkinson, W. O., Bell, R. M. and Finn, O. J. 1988. Altered growth regulation and enhanced tumorigenicity of NIH 3T3 fibroblasts transfected with protein kinase C-1 cDNA. <u>Cell</u> 52: 447-458.

Phillips, H.J., chapter 3 in "Tissue Culture:methods and application", Druse, P.F., and Pattersin, M.K., eds., <u>Academic Press</u> (1973).

Price J. E., Naito, S. and Fidler I. J. 1988. Growth in an organ microenvironments as a selective process in metastasis. <u>Clin. Exp. Metastasis</u>. 6: 91-102.

Price, J. E., Aukeraman, S. L., Ananthaswamy, N. H., Mcintyre, B. W., Schackert, G., Schackert, H. K., and Fidler, I. J. 1989. Metastatic potential for cloned murine melanoma cells transfected with c-Ha-ras. <u>Cancer Res</u>. 4274-4281.

Powers, S., Kataoka T., Fassano, O., Goldfarb, M., Strathern, J., Broach, J., and Wigler, M. 1984. Genes in S. cerevisiae encoding proteins with domain homologous to the mammalian ras proteins. <u>Cell</u> 36 607-612.

Pollanen, J., Saksela, O. L., Salonen, E.M., Andreasen, P., Nielsen, L., Dano, K., and Vaheri, A. 1987. Distinct localizations of urokinase-type plasminogen activator and its type I inhibitor under cultured human fibroblasts and sarcoma cells, J. Cell Biol. 104: 1085-1096.

Portnoy DA, Erickson AH, Kochan J, Ravetch JV, Unkeless JC. 1986. Cloning and characterization of a mouse cysteine proteinase. J. Biol. Chem. 261: 14697-14703.

Poste, G. and Fidler, J. 1990. The pathogenesis of cancer metastasis. Nature 283: 139-146.

Quax, P. H., van Leewen R. T., Verspaget H. W. and Verheijen J. H. 1990. Protein and messenger RNA levels of plasminogen activators and inhibitors analyzed in 22 human tumor cell lines. Cancer Res. 50: 1488-1494.

Qian, F., Bajkowski, A. S., Steiner, D. F., Chan, S. J., and Frankfater, A. 1989. Expression of five cathepsin in murine melanomas of varying metastatic potential and normal tissues. Cancer Res. 49: 48705-875.

Qian, F., Frankfater, A., Chan, S. J. and Steiner, D. F. 1991a. The structure of the mouse cathespin B gene and its putative promoter. DNA and Cell Biology 10: 159-168.

Qian, F., Frankfater, A., Steiner, D. F.,Bajkowski, A. S. and Chan, S. J. 1991b. Characterization of multiple cathespin B mRNAs in murine B16a melanoma. Anticancer Res. 11: 1445-1452.

Recklies, A.D. 1987. Biochemical aspects of cellular migration and invasion. Biorheology 23: 93-103.

Riccio, A., Grimaldi, G., Verde, P., Sebastio, G., Boast, S., and Blasi, F. 1985. The human urokinase-plasminogen activator gene and its promoter. Nucleic Acids Res. 12: 2759-2771.

Ritonja, A, Popovic, T, Kotnik, M, Machleidt, W, Turk, V. 1988. Amino acid sequences of the human kidney cathepsin H and L. FEBS Lett. 228: 341-345.

Roberts, A. B., Anzano, M. A., Wakefield, L. M., Roche N. S., Stern, D. F. and Sporn, M. B. 1985. Type beta transforming growth factor: A bifunctional regulator of cellular growth. Proc. Natl. Acad. Sci. USA 82: 119-123.

Rothenberg M., Johnson G., Laughlin C., Green I., Cradock J., Sarver N and Cohen J. 1989. Commentary: oligodeoxynucleotides as anti-sense inhibitors of gene expression: therpeutic implications. J. Nat. Cancer. Res. 81: 1539-1544.

Rozhin, J. Robinson, D., Stevens, M.A., Lah, T.T., Honn K.V., Ryan R.E. and Sloane B.F. 1987. Properties of a plasma membrane-associated cathepsin B-like cysteine proteinase in metastatic B16 melanoma variants. Cancer Res. 47: 6620-6628.

Rozhin J, Gomez A. P., Ziegler G. H., Nelson K. K., Chang Y. S., Fong D, Onoda J. M., Honn K. V. and Sloane B. F. 1990. Correlation of cathepsin B and cysteine proteinase inhibitor activities with metastasis in murine tumor subpopulations.

Rozhin, J., Wade, R.L., Honn K.V. and Sloane B.F. 1989. Membrane-associated cathepsin L: a role in metastasis of melanomas. Biochemical & Biophysical Research Communications. 164: 556-561.

Sahagian, G.G. and Gottesman, M.M. 1982 The predominant secreted protein of transformed murine fibroblasts carries the lysosomal mannose 6-phosphate recognition marker. J. Biol. Chem. 257, 11145-11150.

Saksela, O., and Rifkin, D.B. 1988. Cell-associated plasminogen activate rn: regulation physiology and functions. Annu. Rev. Cell Biol. 4: 93-126.

Sambrook, J., Fritsch E. F. and Maniatis, T. 1989. Molecular Cloning. Cold Spring Harbor Laboratory Press.

Santos, E., and Nebreda, A.R. 1989. Structural and function of properties of Ras proteins. FASEB J. 3: 2151-2163.

Sassone-Corsi, P., La, Wh, W.W., Kamps, M., Verma, I.M., 1988. Fos-associated cellular p39 is related to nuclear transcription factor AP-1. Cell 54:553-560.

Scher, C. D., Dick, R. L., Whipple, A. P. and Locatell K. L. 1983. identification of a BALB/c-3T3 cell protein modulated by platelet-derived growth factor. Mol. Cell Biol. 3: 70-81.

Schleuning, W.-D., Medcalf, R. L., Hession, C., Rothenbuhler, R., Shaw, A., and Kruithof, E. K. O. 1987. Plasminogen activator inhibitor 2: Regulation of gene transcription during phorbol ester-mediated differentiation of U-937 human histiocytic lymphoma cells. Mol. Cell. Biol. 7: 4564-4567.

Schultz, R.M., Silberman, S., Persky, B., Bajkowski, A.S., and Carmichael, D.F. 1988. Inhibition by human recombinant tissue inhibitor of metalloproteinases of human amnion invasion and lung colonization by murine B16-F10 melanoma cells. Cancer Res. 48: 5539-5545.

Schwartz, B. S., Monroe, M. c., and Levin, E. G. 1988.

Increased release of plasminogen activator inhibitor type 2 accompanies the human mononuclear cell tissue factor response to lipopolysaccharide. Blood 71: 734-741.

Sistonen, L., Alitalo, K. 1986. Activation of c-ras oncogenes by mutations and amplification. Ann. Clin. Res. 18:297-303.

Sloane, B. F., and Honn, K. V. 1984. Cysteine proteinases and metastasis. Cancer Metastasis Rev. 3: 249-263.

Sloane, B.F., Rozhin J, Hatfield JS, Crissman JD, Honn KV. 1987. Plasma membrane-associated cysteine proteinases in human and animal tumors. Exp. Cell Biol. 55: 209-224.

Sloane, B.F. 1990. Cathepsin B and cysteins: evidence for a role in cancer progression. Cancer Biology 1: 137-152.

Sloane B.F., Rozhin, J., Ribinson, D. and Honn K.V. 1990. Role of cathespin b and cystatins in tumor growth and progression. Biol. Chem. Hoppz Seyler. 371: 193-198.

Smith, S.M., Gottesman, M. M. 1981. activity and deletion analysis of recombinant human cathepsin L expressed in Escherichia coli. J. Biol. Chem. 264: 20487-20459.

Sprengers, E.D., Princern, H.M.G., Kooistra T. and Van Hinsbergh, V.W.M. 1985. Inhibition of plasminogen activators by conditioned medium of human hepatocytes and hepatoma cell line HepG2. J. Lab. Clin. Med. 105: 751-758.

Sprengers, E. D. and Kluft. 1987. Plasminogen activator inhibitors. Blood 69: 381-387.

Steiner, D. F., Docherty, K. and Carroll, R. 1984. Cathepsin B-related proteases in the insulin secretory granule. J. Biol. Chem. 259: 6041-6044.

Stephens, R. W., Golder, J. P., Fayle, D. R. H., Hume, D. A., Hapel, A., J., Allan, W., Fordham, C. J., an Doe, W. F. Minactivin. 1985. expression in human monocyte and macrophage population. Blood 66: 333-337.

Stetler-Stevenson, W.G., Krutzsch, H. C. and Liotta, L. A. 1989. Tissue inhibitor of metalloproteinase (TIMP-2): A new member of the metalloproteinase inhibitor family. J. Biol. Chem. 264: 17373-17378.

Stoppelli, M. P., Corti, A. Soffienctini, A., Cassani G., Blasi, F. and Assoian R. 1985. differentiation-enhanced binding of the amino-terminal fragment of human urokinase plasminogen activator to a specific receptor on U937 monocytes. Proc. Natl.

<u>Acad. Sci. USA</u> 82: 4939-4943.

Stoppelli, M. P., Verde P., Grimaldi G., Locatelli E. K., and Blasi F. 1986. Increase in urokinase plasminogen activator mRNA synthesis in human carcinoma is a primary effect of the potent tumor promoter, phorbol myristate acetate. <u>J. Cell. Biol</u>. 102: 1235-1241.

Strickland S., Beers W. H. 1976. Studies on the role of plasminogne activator in ovulation. <u>In</u> <u>vitro</u> response of granulosa cells to gonadotropins, cyclic nucleotides, and prostaglandins. <u>J. Biol. Chem</u>., 251: 5694-5702.

Strulovici, B., Daniel-Issakani, S., Oto, E., Nestor, J., Jr.,n,han, H., and Tsou, A.P. 1989. Activation of distinct protein kinase C isozymes by phorbol esters: correlation with induction of interleukin 1-beta gene expression. <u>Biochemistry</u> 28: 3569-3576.

Tanaka, K., Nakafuku, M., Satoh, T., Marshall, M. S., Gibbs, J. B., Matsumoto, K., Kaziro, Y., and Tohe, A. 1990. <u>S. cerevisiae</u> genes IRA1 and IRA2 encode proteins that may be functionally equivalent to mammalian <u>ras</u> GTPase activating protein. <u>Cell</u> 60: 803-807.

Testa J. E. and Quigley J. P. 1988. Protease receptors on cell surfaces: new mechanistic formulas applied on an old problem. <u>J. Cancer</u>, 46: 266-278.

Thorsen, S., Phillips, M. 1984. Isolation of tissue-type plasminogen activator-inhibitor complexes from human plasma. Evidence for a rapid plasminogen activator inhibitor. <u>Biochim. Biophys. Acta</u> 802: 111-118.

Toda T., Uno I., Ishikawa T., Powers S., Kataoka T., Broek D., Cameron S., Broach J., Matsumoto K. and Wigler M. 1985. In yeast, RAS protein are controlling elements of adenyl cyclase. <u>Cell</u> 40: 27-35.

Tong L, De Vos AM, Milburn MV, et al. 1989. Structural differences between ras oncogene protein and the normal protein. <u>Nature</u> 337: 90-3.

Thorgeirsson, U. P., Liotta, L. A., Kalebic, T., Margulies, I. M., Thomas, K., Rios-Candelore, M., and Russo, R. G. 1982. Effect of natural protease inhibitors and a chemoattractive on tumor cell invasion <u>in</u> <u>intro</u>. <u>J. Natl. Cancer Inst</u>. 69: 1049-1054.

Towatari T., Katunuma N. 1988. Amino acid sequence of rat liver cathepsin L. <u>FEBS Lett</u>. 236: 236: 57-61.

Tripputi P., Blasi F., Verde L.A., Cannizzaro B.S., Emanuel B. S., and Croce C. M. 1985. Human urokinase gene is located on the long arm of chromosome 10. Proc. Natl. Acad. Sci. USA 82: 4448-4452.

Troen BR, Gal S, Gottesman MM. 1987. Sequence and expression of the cDNA for MEP (major excreted protein), a transformation-regulated secreted cathepsin. Biochem. J 246, 731-735.

Troen, B.R., Ascherman, D., Atlas, D., and Gottesman, M.M. 1988. NIH 3T3 cells induced by malignant transformation, growth factors and tumor promoters. J. Biol. Chem. 263: 254-261.

van Dongon J. M., Willensin R, Ginns SI, Sips H. J., Tager J.M., Barranger J.A., Reuser A.J.J. 1985. The subcellular localization of soluble and membrane bound lysosomal enzymes in I-cell fibroblasts: a comparative immunocytochemical study. Eur. J. Cell. Biol. 39: 179-189.

van Duuren, B. L., and Orris, L. 1965. The tumor-enhancing principles of Croton tiglium L. Cancer Res. 1871-1875.

Vassalli, J. D., Hamilton, J. and Reich, E. 1976. Macrophage plasminogen activator: modulation of enzyme production by anti-inflammatory steroids, mitotic inhibitors, and cyclic nudleotides. Cell 8: 271-281.

Vassalli, J. D., Dayer, J. M., Wohlwend, A., and Belin, D. 1984. Concomitant secretion of prourokinase and a plasminogen activator-specific inhibitor by cultured human monocytes-macrophages. J. Exp. Med., 159: 1653-1668.

Vassalli J. D., Baccino, D. and Belin D. 1985. A cellular binding site for the Mr 55,000 form of the human plasminogen activator, urokinase. J. Cell. Biol., 100: 86-92.

Verde, P., Stoppelli M. P., Galeffi P., Mocera P. D., and Blasi F. 1984. Identification and primary sequence of an unspliced human urokinase poly(A)$^+$ RNA. Proc. Natl. Acad. Sci. USA, 81: 4727-4731.

Vogel, U. S., Dixon, R.A.F., Schaber, M.D., Diehl, R. E., Marshall, M. S., Scolnick, E. M., Sigal, I. S., and Gibbs, J. B. 1988. Cloning of bovine GAP and its interaction with oncogenic ras p21. Nature 335: 90-93.

Wagner, O. and Binder, B. 1986. Purification of an active plasminogen activator inhibitor immunologically related the endothelial type plasminogen activator from the conditioned media of a human melanoma cell line. J. Biol. Chem. 261: 14474-14481.

Weinberg, R.A. Oncogenes, antioncogenes, and the molecular bases of multistep carcinogenesis. <u>Cancer Res</u>. 1989, 49: 3713-21.

Weinstein, I. B. 1988. The origins of human cancer: molecular mechanisms for carcinogenesis and their implication for cancer prevention and treatment-twenty-seventh G.H.A. Clowes memorial lecture. <u>Cancer Res</u>. 48: 4135-4143.

Welgus, H. G., and Stricklin, G. P. 1983. Human skin fibroblast collagenase inhibitor. <u>J. Biol. Chem</u>. 258: 12259-12264.

Welgus, H. G., Campbell, E. J., Bar-Shavit, Z., Senior, R. M., and Teitelbaum, S.L. 1985. Human alveolar macrophages produce a fibroblast-like collagenase and collagenase inhibitor. <u>J. Clin. Invest</u>. 76: 219-224.

Whitham S.E., Murphey G, Angel P, Rahmsdorf H. J., Smith I. J., Lyons A, Harris T. J. R., Reynolds J. J., Herrlich P and Doherty A. J. P. 1986. Comparison of human stromelysin and collagenase by cloning and sequence analysis. <u>Biochemical Journal</u> 240: 913-916.

Wiman, B., Chmielewska, J. and Ranby M. 1984. Inactivation of tissue plasminogen activator in plasma. Demonstration of a complex with a new rapid inhibitor. <u>J. Biol. Chem</u>. 259: 3644-3647.

Wohlwend, A., Belin, D, Vassalli, J. D. Plasminogen activator-specific inhibitors produced by human monocytes/macrophages. <u>J. Exp. Med</u>. 165: 320-339.

Whur P., Magudia M., and Boston J., Lickwood J., and Williams D. C. 1980. Plasminogen activator in cultured Lewis lung carcinoma cell measured by chromogenic substrate assay. <u>Br. J. Cancer</u>, 42, 305-313.

Yu, H., and Schultz, R. M., 1990. Relationship between secreted urokinase plasminogen activator activity and metastatic potential in murine B16 cells transfected with <u>Cancer Res</u>. 50: 7623-33,

Zhang, K., DeClue, J.E., Vass, W.C., Papageorge, A. G., McCormick, F. and Lowry, D. R. 1990. Suppression of c-ras transformation by GTPase activating protein. <u>Nature</u> 346: 754-756.

APPROVAL SHEET

The dissertation submitted by Jianyi Zhang has been read and approved by the following committee:

Richard M. Schultz, Ph.D.; Director
Professor and Chairman of Cellular and
Molecular Biochemistry
Loyola University of Chicago

Allen Frankfater, Ph.D.
Associate Professor of Cellular and
Molecular Biochemistry
Loyola University of Chicago

William H. Simmons, Ph.D.
Associate Professor of Cellular and
Molecular Biochemistry
Loyola University of Chicago

M. Rita Young, Ph.D.
Career Research Scientist
Veterans Administration and
Associate Professor of Pathology
Loyola University of Chicago

Chester J. Herman, M.D., Ph.D.
Professor of Pathology
Associate Director of Winship Cancer Center
Emory University School of Medicine and
Director of Anatomic Pathology
Grady Memorial Hospital
Atlanta, GA

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.

December 14, 1992
_____
Date

_____
Director's signature