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The Role of Urokinase Plasminogen Activator in Tumor Cell Metastasis

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THE ROLE OF UROKINASE PLASMINOGEN ACTIVATOR

IN TUMOR CELL METASTASIS

by

Heron Yu Cook

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

April

1990

DEDICATION

I wish to dedicate this dissertation to my
family:

My husband, Chyung, who has offered me unselfish
love, support, and understanding throughout the years.

My children, David and Lisa, who have kept me
young, mature, and proud.

My parents, Chan-Jong and Iksoon Yu, who have
believed in me by giving constant encouragement and
support throughout my education.

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VITA

Heron Yu Cook is the daughter of Chan-Jong Yu and Iksoon Kim Yu. She was born on September 3, 1950 in Seoul, Korea.

She obtained B.S. in chemistry at Yonsei University, Seoul, Korea in 1972. In the same year she came to the University of Kentucky, Lexington, KY, where she received a M.S. in Nutrition and Food Science in 1974.

She was employed at the University of Kentucky as a research technician for a year and moved to the Chicago area in the summer of 1975. From 1975 to 1976, she worked at G.D. Searle & Co. as a statistical coordinator in the Department of Statistics. From 1976 to 1980, she raised her children at home. For the next five years, she was employed as a research biochemist in the Section of Genetics at Rush Presbyterian St. Luke's Medical Center studying protein-bound homocysteinuria. She was accepted to the Ph.D. program at Loyola University with a Basic Science Fellowship in 1985 and joined Dr. Schultz's lab in 1987.

VITA (continued)

She is married to Chyung S. Cook, Ph.D. and has two children, David and Lisa. At the completion of her degree, she will pursue a Howard Hughes postdoctoral fellowship in Dr. Vikas Sukhatme's lab in the Department of Molecular Genetics and Cell Biology at the University of Chicago.

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LIST OF ABBREVIATIONS

AS DNA   antisense DNA

ASuPA   antisense urokinase plasminogen activator

BCIP/NBT   5-bromo-4-chloro-3-indoyl phosphate/
                nitroblue tetrazolium

BSA   bovine serum albumin

CAM   chorioallantoic membrane

cAMP   cyclic adenosine monophosphate

ConA   concanavalin A

DFP   diisopropyl fluorophosphate

DMEM   Dulbecco's Modified Eagle's Medium

EDTA   ethylenediamine tetraacetic acid

ELISA   enzyme-linked immunosorbent assay

FBS   fetal bovine serum

HBSP   HEPES-buffered saline phosphate

HEPES   4-(2-hydroxyethyl)-1-piperazineethane-
                sulfonic acid

MEM   Eagle's minimum essential medium

PA   plasminogen activator

PAI   plasminogen activator inhibitor

PBS   phosphate buffered saline, pH 7.4

PMA   phorbol myristate acetate

SDS   sodium dodesyl sulfate

SSPE   saline sodium phosphate EDTA

SFM   serum free medium

SSC   standard saline citrate

TE   Tris-EDTA buffer

TIMP   tissue inhibitor of metalloproteinases

tPA   tissue type plasminogen activator

uPA   urokinase type plasminogen activator

Chapter I

INTRODUCTION

Activation of plasminogen to plasmin by urokinase plasminogen activator may play an essential role in tumor cell invasion and metastasis. Plasmin is capable of digesting the extracellular matrix and basement membrane. It exerts its proteolytic action either directly by degrading glycoproteins, such as laminin, fibronectin and proteoglycans or indirectly by activating procollagenases to collagenases. Collagenases which degrade collagen, a major structural protein of basement membrane, are believed to contribute to the ability of a tumor cell to metastasize. Therefore it has been suggested that urokinase plasminogen activator might be the essential proteolytic enzyme to initiate the cascade of protease activation that results in tumor cell invasion and metastasis.

The goal of this dissertation was to study the role of urokinase plasminogen activator in the metastatic

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process by a direct approach at the gene level. This study was carried out by increasing the production of urokinase activity or inhibiting endogenous urokinase production by means of the urokinase gene modification in tumor cells and testing the metastatic potential of the modified cells.

The results of this study may address the role of the secreted form of urokinase in the later steps of the metastatic process as well as in the full metastatic process.

Chapter II

REVIEW OF THE LITERATURE

Tumor Cell Metastasis

Tumor cell metastasis is a transfer of malignant
tumor cells from the primary site of tumor formation to
distant organs, and is the major cause of cancer deaths.
The metastasis of tumor cells is a complex process which
involves multiple steps.

At the first step of metastasis, primary tumor cells
invade through the extracellular matrix and basement
membrane, spread into adjacent normal tissue, intravasate
through the capillary endothelial walls, and are
eventually released into the circulation. Few tumor cells
survive the transit through the circulation. Those tumor
cells that do survive arrest in the capillary beds of
distant organs and extravasate from the capillaries
through the basement membrane and extracellular matrix
into the surrounding normal tissue. At the final step of
metastasis, the extravasated tumor cells grow and give

rise to secondary tumor deposits.

During the intravasation and extravasation steps, tumor cells must invade a variety of extracellular matrices and basement membranes in order to enter the underlying interstitial stroma. The extracellular matrix is composed of a dense lattice of interstitial collagens and elastin fibers embedded within a ground substance consisting of fibronectin, other glycoproteins, and proteoglycans. The basement membrane is composed of type IV collagen, laminin, other specific glycoproteins, and heparan sulfate proteoglycans.

A three-step theory has been proposed by Liotta et al. (1986) to explain the sequence of tumor cell invasion to the extracellular matrix. The first step is tumor cell attachment to the matrix by cell surface receptors that specifically bind components of the matrix, such as laminin. Laminin forms a bridge between the cell surface laminin receptor and type IV collagen in the basement membrane. The second step of tumor cell invasion is the local degradation of the matrix by proteolytic enzymes. These are produced by tumor cells or host cells that have been induced by tumor cells. The third step is the tumor cell locomotion into the region of matrix modified by the proteolytic enzymes. Thus the degradation of the above

tissue barrier by proteolytic enzymes is a part of the key step of tumor cell invasion and, in turn, metastasis.

Proteases Involved in Tumor Cell Invasion

Due to the complexity of the tissue barriers that tumor cells must cross in the invasion process, the degradation of the matrix may require several proteolytic enzymes. The proteolytic enzymes, which have been implicated in this step are collagenases, particularly type IV, endoglycosidases, cathepsin B-like thiol proteinases, and plasminogen activators (Nicolson, 1982).

A cathepsin B-like proteinase can degrade several components of the extracellular matrix such as denatured collagen, fibronectin and proteoglycan (Sloane and Honn, 1984). In addition, it has been suggested that cathepsin B-like proteinase might have the potential to activate latent type IV collagenase (Sloane et al., 1986). Increased amounts of cathepsin B-like thiol proteinase have been found in solid tumors (Poole et al., 1980; Recklies et al., 1980; Rinderknecht and Renner, 1980; Ryan et al., 1985), transformed cell (Myra-y-Loopez et al., 1985), and tumor cells in culture (Sloan et al., 1981; Sloane et al., 1982; Ryan et al., 1984). However, there

are contradictory results which do not find any correlation between cathepsin B-like activity and metastatic potential (McLaughlin et al., 1983). Others have reported that cathepsin B-like activity is negatively correlated with transformation (Dolbeare et al., 1980; Morgan et al., 1981). Inhibitors of cathepsin B placed in high steady-state concentrations within an animal model for metastasis failed to show any inhibition of metastasis (Ostrawski et al., 1986).

Endoglycosidases cleave heparan sulfate from matrix proteoglycans. Heparan sulfate interacts with matrix macromolecules, such as collagens and fibronectin, and plasma membrane attachment sites. Therefore, endoglycosidases have also been shown to be involved in tumor invasion and metastasis (reviewed by Goldfarb and Liotta, 1986).

Interstitial collagenases hydrolyze collagen types I, II, and III of the extracellular matrix. Several investigators reported a positive correlation between tumor cell secretion of interstitial collagenase by mouse primary tumor cells and lung metastasis (Tarin et al., 1982). Another study showed the invasion of a chick chorioallantoic membrane by human choriocarcinoma cell lines correlated well with the production of collagenase

but did not correlate with secretion of plasminogen activator or cathepsin B (Sekiya et al., 1985).

The major structural protein of basement membrane, type IV collagen, is not degraded by the interstitial collagenase, but by type IV collagenase (Salo et al., 1982). Type IV collagenase is secreted in a latent form and shown to be activated by plasmin in vitro (Werb et al., 1977; Paranjpe et al., 1980; Gross et al., 1982; He et al., 1989). Enhanced levels of type IV collagenase has been shown in malignant tumor cells (Liotta et al., 1986; Goldfarb et al., 1986). Positive correlation between type IV collagenase activity and the pulmonary metastatic potential has been demonstrated in B16 melanoma cell lines (Liotta et al., 1980) and breast cancer cell lines (Nakajima et al., 1986).

Plasminogen activator (PA) is a serine protease which converts the inactive proenzyme plasminogen to plasmin. The plasminogen, is ubiquitous in the blood and tissue spaces. Plasmin can degrade many components of the extracellular matrix in vitro such as proteoglycans (Mochan and Keler, 1984), fibronectin (Liotta et al., 1981; Kramer et al., 1982), and laminin (Liotta et al., 1981; Boyd et al., 1989). Plasmin has also been shown to activate procollagenase to collagenase in vitro, including

type IV collagenase, which degrades type IV collagen (Werb
et al., 1977; Paranjpe et al., 1980; Gross et al., 1982;
He et al. 1989). Therefore, plasminogen activator, via
activation of plasminogen, may have a central role in
initiating cascade reactions that degrade the molecules
composing the barriers which tumor cells must cross during
metastasis. The details of plasminogen activator activity
in tumor cell metastasis will be discussed separately.

Two distinct types of plasminogen activators are
known, tissue-type plasminogen activator and urokinase
type. They are genetically and immunologically distinct
forms. The two proteins have different amino acid
sequences, molecular weights, subunit structures and
catalytic parameters such as affinity to fibrin. Tissue-
type plasminogen activator is released from the
endothelial cells of the blood vessels and urokinase-type
plasminogen activator is synthesized in kidney cells and
released from the renal tubules. Immunological
characterization has shown that most tumor cells produce
urokinase-type plasminogen activator rather than tissue-
type (reviewed by Dano et al., 1985). There are some
exceptions where tissue-type plasminogen activator was
demonstrated in breast tumor cells induced by carcinogens,
in melanoma cells (Dano et al., 1985), and several other

tumor cell lines (Quax et al., 1990). In general though
tissue-type plasminogen activator appears to be involved
in fibrinolytic function of the cell, such as lysis of
fibrin clots, and urokinase-type activity is involved in
cell migration and tumor cell metastasis.

In this report, the term "plasminogen activator" is
used when unable to identify which type of activator is
being studied. The term "urokinase" is used when the
basis of its origin is from the urokinase plasminogen
activator identified by the urokinase plasminogen
activator gene or evaluated by immunological assays.
Tissue-type plasminogen activator is referred to as
"tissue PA".

Biochemistry of Urokinase

The urokinase gene has been isolated and sequenced
from human (Heyneker et al., 1983; Riccio et al., 1985;
Nagai et al., 1985), mouse (Degen et al., 1987), pig
(Nagamine et al., 1985), and chicken (Leslie et al.,
1990). Comparison of the human urokinase gene and the
porcine, murine, or chicken gene has shown an extensive
homology (72-79% nucleotide sequence identity) throughout
the introns and exons (Degen et al., 1987). The four

urokinase genes are also similar in size: human, 6388 base pairs; porcine, 5852 base pairs; murine, 6710 base pairs; and chicken, 8158 base pairs (Degen et al., 1987). The human urokinase gene is located on the long arm of chromosome 10 (Tripputi et al., 1985), while human tissue PA is on chromosome 8 (Blasi et al., 1986). The human urokinase gene consists of 11 exons and 10 introns (Riccio et al., 1985). The mRNA of human urokinase is shown to be 2.5 Kb and the cDNA is composed of 1293 base pairs (Verde et al., 1984). Human urokinase is a glycoprotein (Holmes et al., 1985; Ricco et al., 1985) while mouse urokinase contains no N-glycosylation site (Belin et al., 1985).

Urokinase is synthesized as a single-chain preprourokinase composed of 431 amino acids as shown by its cDNA size and has a molecular weight of 54 Kd (Salerno et al., 1984). It contains a signal leader sequence of 20 amino acids which is removed during protein synthesis (Pennica et al., 1983). Urokinase is secreted as an inactive single-chain prourokinase zymogen of 411 residues. However, some investigators reported that prourokinase purified from certain cell lines demonstrated catalytic activity (Stumpt et al., 1986; Ellis et al., 1987). The single-chain prourokinase is activated by a proteolytic cleavage which removes one amino acid, lysine,

at position 158. Thus the activation generates a two-
chain urokinase molecule (A-chain and B-chain) held
together by one disulfide bond (Gunzler et al., 1982).
The activation is mediated by many compounds such as
plasmin (Dano et al., 1985), plasma kallikrein (Ichinose
et al., 1986), factor XIIa (Ichinose et al., 1986),
trypsin (Eaton et al., 1984), and thrombin (Eaton et al.,
1984). Plasmin appears to be the most efficient activator
(Eaton et al., 1984; Ichinose et al., 1986). At certain
concentrations, thrombin has been found to inactivate
prourokinase by cleaving arg-156 and phe-157 (van
Hinsbergh et al., 1987). The active urokinase can be
further cleaved by plasmin to form a still active 33 Kd
molecule (Stump et al., 1986). Whether the 33 Kd
urokinase has specific function is not known.

Urokinase consists of five distinctive domains,
known as the signal domain, growth factor domain, kringle
domain, connecting domain, and catalytic domain (Verde et
al., 1984). The growth domain is homologous to epidermal
growth factor, alpha transforming growth factor,
coagulation factors IX and X, and prothrombin (Anson et
al., 1984; Fernlund et al., 1982). The homology to growth
factors might suggest a growth promoting activity of
urokinase in certain cell lines (Kirchheimer et al.,

1987). The kringle domain is also present in other
zymogens, such as prothrombin (Magnusson et al., 1976) and
plasminogen (Sottrup-Jensen et al., 1978). The catalytic
domain carries the typical active site of the serine
proteases. Comparison of the amino acid sequence of
tissue PA and urokinase shows that two domains of the
tissue PA are missing in urokinase. The first missing
domain is the finger-like domain which may be responsible
for the high affinity of tissue PA for fibrin (Banyai et
al., 1983) and explains the greater fibrinolytic effect of
tissue PA than of urokinase. The second missing domain in
urokinase is the second kringle domain of tissue PA.

The production of urokinase is affected by several
compounds such as hormones, growth factors, cAMP, and
tumor promoters. It was shown that calcitonin and
vasopressin in porcine kidney cells (Nagamine et al.,
1983) and estrogen in breast carcinoma cells (Mira-y-Lopez
and Ossowski, 1987) induced urokinase mRNA expression.
Glucocorticoid has been shown to depress the expression of
urokinase activity in cultured cells (Busso et al., 1987).
Dexamethasone also decreased the urokinase mRNA levels in
normal and several malignant tumor cells (Medcalf et al.,
1986). Growth factors, such as epidermal growth factor
(EGF) (Boyd, 1989; Niedbala and Sartorelli, 1989),

platelet-derived growth factor (PDGF) (Stoppelli et al.,
1986), and interferon (IFN-gamma) (Collart et al., 1986)
increased the levels of urokinase mRNA in cultured cancer
cells. Transforming growth factor beta gave dual effects,
in that it stimulated urokinase secretion in certain
malignant cell lines (Keski-Oja et al., 1988) and it
decreased the secretion of urokinase in bovine endothelial
cells (Saksela et al., 1987). The transcriptional
increase of urokinase by hormone or growth factor is
mediated through the cAMP pathway (Saksela and Rifkin,
1988). The 5'-nontranscibed regions of murine, porcine,
and human urokinase genes were found to contain
dodecanucleotides homologous to cAMP regulatory sequences
(Degen et al., 1987; Riccio et al., 1985; Nagamine et al.,
1985). The tumor promoter, phorbol myristate acetate
(PMA) also increased urokinase mRNA synthesis in various
carcinoma cells (Kelly et al., 1983; Degen et al., 1985;
Collart et al., 1986), transformed cells (Belin et al.,
1984), and chicken embryo fibroblasts (Bell et al., 1990).
The increase of urokinase mRNA level by PMA stimulation
was parallel to the increase of c-myc transcription
(Stoppelli et al., 1986). The increase occurred within 30
minutes and peaked 2-4 hour after the PMA treatment
(Stoppelli et al., 1986).

The regulation of urokinase activity is also modulated by the urokinase specific cell surface receptor. Another regulatory mechanism for urokinase activity is the secretion of plasminogen activator inhibitors (PAIs). Three of the inhibitors, PAI-1, PAI-2, and protease nexin are reported to regulate plasminogen activator activity by rapid inhibition in vivo (Saksela and Rifkin, 1988).

Urokinase can regulate a variety of physiological events that require extracellular proteolysis, such as fibrin clot lysis, tissue remodeling, and cell migration (reviewed by Dano et al., 1985). Proteolytic degradation of extracellular matrix components starts with activation of plasminogen by plasminogen activator, subsequently the formation of plasmin, and activation of procollagenase to collagenase by plasmin as discussed previously. Involvement of urokinase in cell migration is observed in vitro. It was shown that the continuously migrating, wounded endothelial cells have increased urokinase activity (Pepper et al., 1987). Another physiological process involving urokinase may be angiogenesis. Urokinase has a function in the formation of new blood vessels supplying the newly formed tumor with nutrients required for rapid growth (d'Amore, 1988; Goldfarb et al., 1986). Lastly, urokinase may be involved in ovulation.

Hormones associated with ovulation such as follicle stimulating hormone (FSH) and luteinizing hormone (LH), stimulate plasminogen activator (Ny et al., 1985; Canipari et al., 1987). The regulation of urokinase activity is also shown in pathological processes such as inflammation, pemphigus, cancer, and metastasis (Dano et al, 1985).

Urokinase and Tumor Cell Metastasis

Indirect evidence in support of a role for urokinase in metastasis has been shown by multiple studies and is reviewed by Dano et al. (1985). An increase in urokinase gene expression or an enhanced urokinase synthesis/ secretion have been observed in metastatic variant cell lines such as lung and breast carcinomas (Sappino et al., 1987), mammary adenocarcinomas (Ramshaw et al., 1986; Pereyra-Alfonso et al., 1988; Burtin et al., 1987), Lewis lung carcinoma (Eisenbach et al., 1985), mammary carcinoma (Pauli et al., 1987), human malignant melanoma (Markus et al., 1984), and murine fibrosarcoma (Nagy and Grdina, 1989). Furthermore, many of the solid tumors of lung (Markus et al., 1980), prostate (Kirchheimer et al., 1985; Camiolo et al, 1987; Gaylis et al., 1989), breast (Duffy et al., 1988), stomach (Szczepanski et al., 1982),

digestive tract (Noshino et al., 1988), and colon (de Bruin et al., 1987) showed increased secretion of urokinase as compared to their untransformed counterparts. Treatment of cells with tumor promoters (Degen et al., 1985; Stoppelli et al., 1986), tumor viruses (Unkeless et al., 1973, Skriver et al., 1982), and src oncogene product (Bell et al., 1990) also increased the gene expression or enhanced the secretion of urokinase from cells in vitro.

There have been a number of studies that show a correlation between plasminogen activator production and metastasis in murine B16 melanoma cell lines. Carlsen et al. (1984) isolated clonal tumor cell populations which produced various levels of plasminogen activator activity and determined the metastatic potential of the isolated clones by a lung colonization assay. Their results showed a strong correlation between production of plasminogen activator activity and metastatic potential. Wang et al. (1980) also demonstrated that murine B16-F10 cells, a highly metastatic subline of the B16 cell line, produced a significantly greater amount of plasminogen activator activity than the B16-F1 subline of lower metastatic potential. This result, however, is in contrast to the work of Nicolson et al. (1976) who found that there was essentially no difference in plasminogen activator

activity between B16 variant cell lines.

A recent study showed that transfected mouse L cells expressing the human urokinase gene demonstrated an increased degradation of extracellular matrix protein and invasion of a Matrigel basement membrane. This work indicated that urokinase expression may be sufficient to provide an invasive phenotype in the cell (Cajot et al., 1989).

Immunocytochemical studies have also been used to indicate the importance of urokinase activity in tumor cell metastasis (Pollanen et al., 1987). Skriver et al. (1984) reported an increased urokinase level by means of immunohistochemical staining in areas close to or in contact with normal cells of the surrounding tissue being invaded by the tumor cells.

Since type IV collagen is a major constituent of basement membrane, it has been considered as an important substrate for tumor invasion. Type IV collagenase, which is activated from procollagenase by plasmin, degrades type IV and type V collagen and is therefore believed to have an important role in the invasion of tumor cells through the extracellular matrix and basement membrane (Liotta et al., 1980; Goldfarb and Liotta, 1986; Reich et al., 1988; Schultz et al., 1988).

The human tissue inhibitor metalloproteinase
(TIMP), a glycoprotein with a molecular weight of 28,000,
is an inhibitor of metalloproteinases such as type I
collagenase, type IV collagenase, and stromelysin. TIMP
has been shown to inhibit lung colonization by B16-F10
cells in vivo (Schultz et al., 1988). Similar results
were reported with a synthetic collagenase inhibitor that
reduced the lung lesion in the same system (Reich et al.,
1988). In addition, Khokha et al. (1988) found that
transfection of an antisense RNA, complementary to the
mRNA encoding the collagenase inhibitor (TIMP), changed
the non-metastatic and non-tumorigenic mouse 3T3
fibroblasts, to the metastatic and tumorigenic phenotype
that has the ability to colonize the lungs of nude mice.

Whereas the majority of reports support a positive
correlation between metastatic potential and urokinase
activity, there exist other reports which show a lack of
such a correlation. One report stressed that the only
proteinase secreted by malignant tissue was the cathepsin
B-like thiol proteinase (Rechlies et al., 1980). Others
reported a decreased plasminogen activator activity in the
plasma of tumor-bearing patients compared to normal
subjects (Colombi et al., 1984). Another study reported
that plasminogen activator was enriched in the tumor cell

membranes, however serine inhibitors did not abrogate collagenolytic activity (Zucker et al., 1985).

One of the questions regarding the possible role of urokinase in the metastatic process is whether urokinase activity is required in the early steps such as intravasation or during the later steps such as extravasation. Earlier work of Ossowski and Reich (1983) showed that the injection of an antibody to urokinase into the chick embryo prevented cells, which were placed on the chorioallantoic membrane, from metastasizing to the embryonic lung. However, if the human HEp2 tumor cells were placed directly into a vein, the urokinase antibody had no inhibitory effect on lung colonization (Ossowski, 1988). These results may indicate that urokinase is only required for the intravasation step into the blood vessels, but not for extravasation out of the blood vessels (Ossowski, 1988).

Contrary to this, several other studies reported that an increase in urokinase activity is correlated with increased lung colonization following i.v. injection, urokinase therefore plays a causal role in the extravasation step of the metastatic cascade. Hearing et al. (1988) showed that B16 cells pretreated with antibody to urokinase on their cell surfaces showed decreased

numbers of metastases and that pretreatment of B16-F10 cells with plasmin, which converts single chain urokinase to the more active two chain form of urokinase, increased the numbers of metastases in the lung colonization assay. Very recently, Axelrod et al. (1989) expressed the urokinase gene in H-ras-transformed NIH3T3 cells by transfection of recombinant gene, and found that the increase in urokinase gene expression resulted in a large increase in pulmonary metastasis following tail vein injection in nude mice. Also Brunner et al. (1989) reported that mouse bladder carcinoma cells transfected by Ha-ras cells produced a 3-fold increase in urokinase activity in cell lysates and 3-fold increase in cell surface-associated urokinase activity. This particular clone induced a 6-fold increase in lung colonies.

Urokinase Receptor

A specific cell surface receptor with high affinity for urokinase has been first identified in human blood monocytes (Vassalli et al., 1985) and is present on a variety of normal cells, such as fibroblasts, endothelial cells, granulocytes, spermatozoa, and a number of malignant tumor cells (Testa and Quigley, 1988; Stoppelli

et al., 1986; Vasaalli et al., 1985). It has been
purified from several cell lines (Needham et al., 1987;
Nielsen et al., 1988; Estreicher et al., 1989). The
receptor appears to be a 55-60 Kd glycoprotein (Nielsen et
al., 1988). The number of receptors on cells, such as
monocytes or fibroblasts is about 10^5 sites per cell
(Blasi et al., 1986). The urokinase receptor binds to the
amino terminal fragment (ATF) of the single chain form of
prourokinase or to the two chain form of urokinase
(Stoppelli et al., 1985). More precisely it binds within
the first 32 residues of the growth factor domain in
urokinase (Appellal et al., 1987). Therefore, the binding
of urokinase is completely independent of the catalytic
domain which is located in carboxyl terminal of the
urokinase molecule (Stoppelli et al., 1985; Appella et
al., 1987). The 33 Kd urokinase, generated by plasmin
from the 54 Kd urokinase, does not bind to the receptor
(Needham et al., 1987).

It has been reported by immunofluorescence,
immunoprecipitation, and surface iodination assays that
the same cells have both secreted and membrane associated
urokinase activities (Stoppelli et al., 1986). This
suggests that prourokinase might be initially secreted
into the extracellular space with subsequent binding to

the specific receptor via an autocrine mechanism. When
bound to its specific receptor, urokinase is not
internalized so its enzymatic activity is retained. Mild
acid treatment releases the receptor bound urokinase from
the cell surface (Stoppelli et al., 1986).

The binding of urokinase to its cellular receptor
is shown to be extremely specific. Any other related
proteins, such as epidermal growth factor (EGF), factor IX
and X, tissue PA, plasminogen, and thrombin do not compete
for binding with the amino terminal fragment (ATF) of
urokinase or with high molecular weight urokinase (Blasi
et al., 1986; Blasi, 1988). Urokinase binding also shows
species specificity (Stoppelli et al., 1986; Estreicher et
al., 1989). Murine urokinase binds to murine sperm cells
but not to human cells (Huarte et al., 1987).

The binding of urokinase to its receptor is shown
to be increased by epidermal growth factor (EGF) treatment
of the carcinoma cells (Blasi et al., 1986; Boyd, 1989).
Phorbol myristate acetate (PMA) has been shown to increase
urokinase receptor synthesis in a time and concentration
dependent manner. PMA treatment also caused a decrease in
affinity of the urokinase receptor, suggesting another way
of regulating the interaction between urokinase and its
receptors (Picone et al., 1988).

The function of the urokinase receptor has not been established, however, possible advantages have been suggested. First, since the carboxyl terminal domain remains exposed on the cell surface upon receptor binding, urokinase binding might stimulate localized proteolytic activity (Stoppelli et al., 1985; Pollanen et al., 1990). Secondly, the receptor bound enzyme might be protected from inactivation by inhibitors. It has been shown that plasmin generated from the cell bound plasminogen is less susceptible to inhibitors than its fluid phase counterpart (Plow et al., 1986). Thirdly, the binding of urokinase to the cell surface might concentrate other substrates of the multistep pathway, and subsequently increase the rate of the cascade reaction (Moscatelli and Rifkin, 1988).

A possible role of urokinase binding to the receptor has been shown by several investigators. As stated earlier, urokinase has a growth factor domain in its amino acid sequence (Verde et al., 1984) which might provide the mitogenic activity of urokinase. The binding of urokinase to its receptor seems to be necessary for the growth stimulation of certain cell lines. It was demonstrated that the 54-Kd urokinase which binds to its receptors stimulated the growth and proliferation of human epidermal cells (Kirchheimer et al., 1989) and uterine and

ovarian cancer cells (Takada and Takada, 1989), whereas the 33-Kd urokinase fragment, which lacks the growth factor domain and contains only the active-site domain, did not stimulate cell proliferation (Kirchheimer et al., 1989).

A question regarding the role of urokinase in metastasis concerns its possible location of action. Recent work has shown that many tumor cells contain urokinase receptors on their cell surfaces (Needham et al., 1987; Stoppelli et al., 1986). Likewise, an increase in cell surface urokinase receptor has been correlated with an increased metastatic potential (Boyd et al., 1988). In vivo invasion assays with chick embryo chorioallantoic membrane (CAM) showed that the invasive ability of human tumor cells was augmented by saturating their receptors with exogenous urokinase. The stimulation of urokinase production in the absence of binding to cell receptors did not enhance invasiveness (Ossowski, 1988). Boyd et al. (1988) determined the levels of urokinase and its receptor in colon carcinoma cell lines of different differentiation degrees, and found aggressive metastatic cell lines expressed a higher number of receptors and a higher level of secreted endogenous urokinase activity than more indolent cell lines.

Chapter III

MATERIALS AND METHODS

Cell Culture

The B16-F1 and B16-F10 cells (Fidler, 1975) were kindly provided by Dr. I.J. Fidler, M.D. Anderson Medical Center, University of Texas, Houston, TX. Cells were grown in Eagle's minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Flow Laboratory, McLean, VA), 1% MEM nonessential amino acids (Gibco Laboratories, Grand Island, NY), 1 mM sodium pyruvate, 1% MEM vitamin solution (Gibco Laboratories), 75 units/ml of penicillin and 75 ug/ml of streptomycin sulfate (Gibco Laboratories). Cells were incubated at 37 C in 5% CO_2 saturated with water vapor and were maintained by changing the medium every three days and subculturing at a 1:3 ratio at confluence. For the subculture, cells were detached from the flasks by incubation with 1 mM ethylenediamine tetracetic acid (EDTA) in phosphate buffered saline (PBS) for several

minutes. The total number of viable cells were determined by trypan blue exclusion in a hemocytometer (C.A. Hausser and Son, Philadelphia, PA).

pSV2-uPA Plasmid Construction

The plasmid pSV2-uPA containing the human preprourokinase plasminogen activator coding sequences in the sense direction was made from two plasmids, pSV2-dhfr and pRdzbuktt. pSV2-dhfr was purchased from American Tissue Culture Center, Rockville, Md. and pRdzbuktt was a gift of Dr. Michael E. Reff, Smith Kline & French & Co., King of Prussia, PA. The plasmid pRdzbuktt is a mammalian expression vector containing the full length cDNA for human preprourokinase, Rous sarcoma virus (RSV) long terminal repeats (LTR) promoter, a bovine growth hormone (BGH) polyadenylation signal, the dihydrofolate reductase (dhfr) terminator, the pBR322 origin of replication, and an ampicillin resistant gene (Figure 1). pSV2-dhfr is a mammalian expression vector containing the dihydrofolate reductase (dhfr) gene, a SV40 early and late gene promoters, a SV40 polyadenylation signal, the pBR322 origin of replication, and an ampicillin resistant gene. The strategy of plasmid construction, as shown in Figure 2, was to replace the dihydrofolate reductase (dhfr) gene

Figure 1. Structure of the plasmid pRdzbuktt

The plasmid pRdzbuktt contains the full
length cDNA for human preprourokinase, Rous sarcoma
virus (RSV) long terminal repeats (LTR) promoter, a
bovine growth hormone (BGH) polyadenylation signal,
the dihydrofolate reductase (dhfr) terminator, the
pBR322 origin of replication, and an ampicillin
resistant gene. The human preprourokinase gene can
be retrieved from the plasmid by digestion with
HindIII and SacI. The transcription is clockwise in
the 5' to 3' direction. Restriction enzymes with
unique recognition sites are shown on the map.

Sall 4
Aat2 5 / Border 5, 6
Border 33, 34
Border 86, 87
Nrul 214
Mlul 234
Aat2 306

Sapl 5154
Start 5137
Xmnl 4949

Rous LTR

HInd3 612 / Border 612, 3
Start 621
BstU2 636
Border 680, 1

Border 818, 9
Ncol 877
Nael 885

Beta Lactamase

L
GF

End 4277

K

Pstl 987

Cleavage Site 1085, 6
Cleavage Site 1154, 5

PUC19

B

Rdzbuktt

5267

UK Cassette

Eco473 1418

SP

BspK2 1604

Lac2&1

Origin 3519

End 1916
Sacl 1921
Border 1937, 8

Border 3330, 1

BGH

BGH

Border 2168, 9
Hpal 2173
Border 2179, 0
BstX1 2206

Border 3106, 7
Xhol 3093 / Avel 3093
Border 3084, 5

DHFR Terminator

PflM1 2603

Ndel 2432

Avr2 2608

P=PBR, L=leader, GF=growth factor, K=kringle, B=Between K & SP, SP=protease
BGH=BGH PolyA

Enzymes that do not cut:
Af12 Apal Asu2 Bsml BstE2 Clal Dra3 EcoRV Espl Kpnl Narl Nhel Notl Nsil Rsr2
Sac2 Sfil Smal SnaB1 Spel Spll Stul Tth3l Xbal Xma3

Figure 2. Construction of human preprourokinase

expression vector pSV2-uPA containing SV40 early

gene promoter

The human 1021 base pair preprourokinase cDNA

gene (uPA) was obtained by digestion of plasmid

pRdzbuktt with <u>Sac</u>I and <u>Hind</u>III. Plasmid pSV2-dhfr

was digested with <u>Hind</u>III and <u>Bgl</u>II to remove the

dhfr gene. The uPA sequence was ligated to the

large fragment (4249 base pairs) of the pSV2 plasmid

to make the pSV2-uPA expression plasmid containing

human preprourokinase gene in a sense direction with

respect to the SV40 early gene promoter.

Transcription is clockwise in the 5′ to 3′

direction. The protein coding sequences are shown

in hatched area.
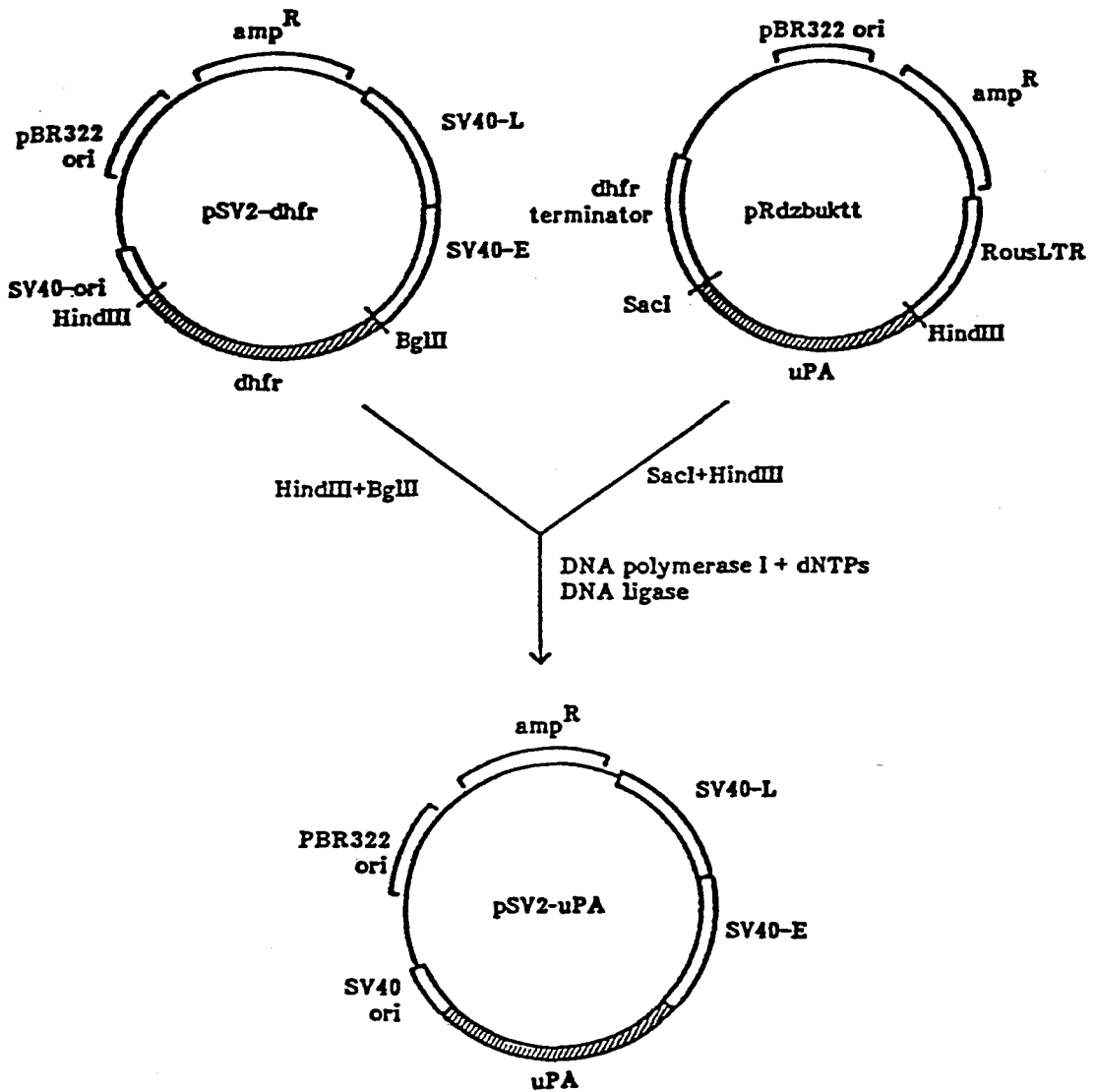
of pSV2-dhfr with the preprourokinase gene of pRdzbuktt in the sense orientation relative to the SV40 early gene promoter. In order to remove the dhfr gene from pSV2-dhfr, the pSV2-dhfr was digested with <u>Hind</u>III and <u>Bgl</u>II. Both cohesive ends were repaired to give blunt ends by incubation with 5 units of T4 DNA polymerase I (Boehringer Mannheim Biochemicals, Indianapolis, IN) and 0.5 mM of the mixture of dATP, dGTP, dCTP, TTP (Sigma Chemical Co.) in an assay buffer provided by the manufacturer, at 37°C for 30 minutes. The repaired DNAs were then treated with 10 units of alkaline phosphatase from calf intestine (Boehringer Mannheim Biochemicals) to prevent self ligation, by incubation for 1 hour at 50 C in the assay buffer. The repaired DNA fragments were separated by electrophoresis in 0.8% agarose gel. The 4249 base pairs of large fragment DNA which contains the entire pSV2-dhfr sequences, except for the dhfr gene, was eluted from agarose gel using the IBI Analytical Electroeluter (International Biotechnologies Inc., New Haven, CT).

To isolate the human preprourokinase cDNA from the plasmid pRdzbuktt, the pRdzbuktt was digested with <u>Hind</u>III and <u>Sac</u>I, blunt ended as described previously for pSV2 vector, and separated by electrophoresis. The small fragment DNA of pRdzbuktt which contains 1021 base pairs

corresponding to the preprourokinase coding sequences was eluted. The pSV2-vector fragment and human preprourokinase fragment, were ligated overnight at room temperature by 2 units of T4 DNA ligase (IBI, New Haven, CT) in the assay buffer provided by the manufacturer. The ligated DNA was then transformed in E-coli strain HB101. Prior to transformation, the E-coli was cultured in SOB medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$), resuspended in RF1 buffer (100mM RbCl, 50 mM $MnCl_2$, 30 mM potassium acetate, 10mM $CaCl_2.2H_2O$ and 15% glycerol), then in RF2 buffer (10 mM MOPS, 10 mM RbCl, 75 mM $CaCl_2.2H_2$, 15% glycerol) to improve transformation efficiency (Hanahan, 1983). Ampicillin resistant clones were selected and grown in LB medium with 50 ug/ml of ampicillin.

Plasmid DNAs of selected clones were screened for the insertion of the preprourokinase gene by restriction endonuclease digestion with BglII and were rescreened for the pSV2-uPA in the sense orientation by restriction endonuclease digestions with BamHI, PstI, and PvuII. The clone with the correct digestion fragments for pSV2-uPA were amplified in the presence of chloroamphenicol (170 ug/ml). The plasmid DNA from the selected clone was prepared in large scale by the lysis of the E-coli cell

wall with 0.2 N NaOH/1% SDS and plasmid DNA extraction with isopropanol. The prepared DNA was purified by centrifugation to equilibrium in cesium chloride-ethium bromide density gradient (Maniatis et al., 1982) for transfection. The plasmid structure and the restriction digestion map of the constructed pSV2-uPA is shown in Figure 3. The final plasmid structure was confirmed by additional restriction endonuclease digestions with BamHI, PvuII, BglI, EcoRI, and PstI and a double digestion with BglII and HpaI.

pCLH3AXBPV-uPA Plasmid Construction

The plasmid pCLH3AXBPV-uPA containing the human preprourokinase plasminogen activator gene in the sense direction was similarly prepared from pRdzbuktt and pCLH3AXBPV as described for pSV2-uPA. The plasmid pCLH3AXBPV, obtained from Dr. Gordon (Integrated Genetics, Framingham, MA) (Reddy et al., 1987), contains the bovine papilloma virus-1 (BPV-1) genome, mouse metallothionein (MT-1) promoter, a SV40 polyadenylation signal, the pML-1 (a derivative of pBR322 origin of replication), and an ampicillin resistant gene. The structure of the plasmid pCLH3AXBPV is shown in Figure 4. The pCLH3AXBPV was linerized by restriction endonuclease digestion with XhoI.

Figure 3.   Structure of the plasmid pSV2-uPA

The plasmid pSV2-uPA contains the full length
cDNA for human preprourokinase in the sense
orientation, SV40 early and late gene promoters, a
SV40 polyadenylation signal, the pBR322 origin of
replication, and an ampicillin resistant gene.   The
SV40 early promoter directs the expression of the
inserted human preprourokinase gene.   The
transcription is clockwise in the 5′ to 3′
direction.   The outside plasmid circle,
corresponding to the inner circle, shows several
restriction enzymes used to verify the constructed
pSV2-uPA structure.
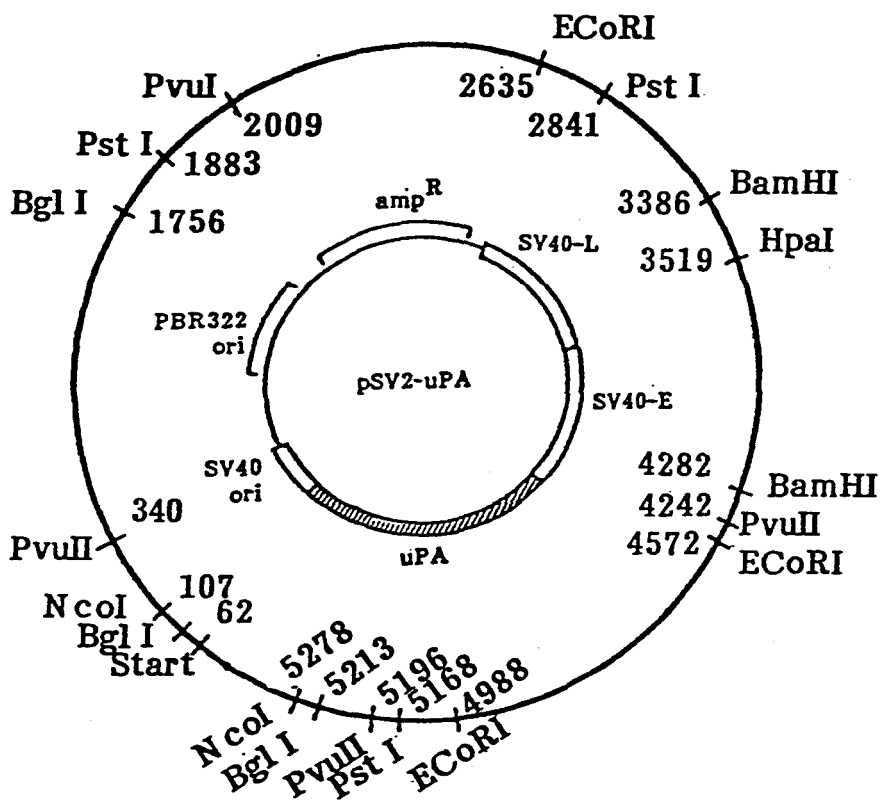
Figure 4. Structure of the plasmid pCLH3AXBPV

The plasmid pCLH3AXBPV contains the bovine papilloma virus-1 (BPV-1) genome, the mouse metallotheionine (MT-1) promoter, a SV40 polyadenylation signal, a derivative of the pBR322 origin of replication (pML-1), and an ampicillin resistant gene. The plasmid was linerized by the restriction endonuclease digestion with XhoI to insert human preprourokinase gene. Several restriction enzymes used to verify the plasmid structure are shown on the map.

MT-1 Promoter — 13286 Xho 1 / SV40 E Poly A

Hpa 1 13421

12586 Kpn 1

BamH I 13529/1

11286 EcoR 1
11260 Hind III

Xba 1 1682

Hind III 2508
Hpa 1 3495
Sma 1 4440

10286 EcoR 1

CLH3AXBPV

9658 Pvu 1

BPV-1

pML-1

7945 Sal 1

EcoR 1 5608

Kpn 1 6950
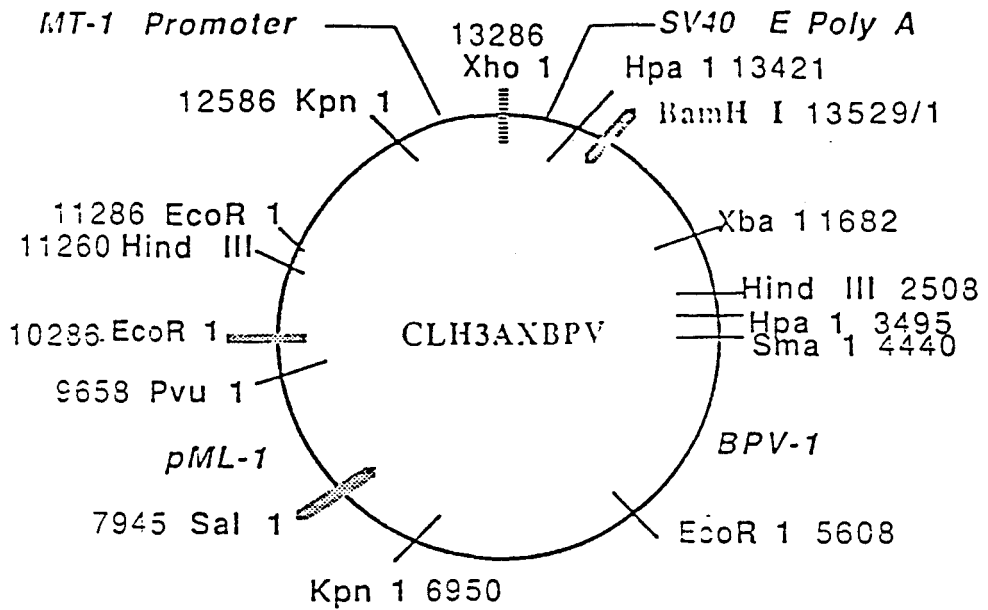
The pRdzbuktt was digested with HindIII and SacI and the human preprourokinase cDNA was separated by electrophoresis and eluted from the agarose gel. The pCLH3AXBPV vector and the human preprourokinase fragment were repaired to blunt ends with T4 DNA polymerase and a mixture of four deoxynucleotide triphosphates and the two ends were ligated. The construction scheme is shown in Figure 5. The ligated DNA was transformed in E-coli and the ampicillin resistant clones were selected. The selected clones were screened for pCLH3AXBPV-uPA by the restriction endonuclease digestions with HpaI, HindIII, and EcoRI. The final plasmid construction was confirmed by the additional restriction digestions with BamHI, PvuII, and a double digestion with PvuI and PstI. The plasmid structure and the restriction digestion map of the constructed pCLH3AXBPV-uPA is shown in Figure 6.

pSV2-ASuPA Antisense Plasmid Construction

The plasmid pSV2-ASuPA containing the 5'end 265-base-pair of preprourokinase sequence in the antisense direction was similarly prepared from pSV2-dhfr and pRdzbuktt as shown in Figure 7. The pSV2-dhfr was digested with HindIII and BglII and the vector fragment of pSV2 was separated by electrophoresis and eluted from the

Figure 5. Construction of human preprourokinase

expression vector pCLH3AXBPV-uPA containing bovine

papilloma virus-1 (BPV-1) and metallothionein-1 (MT-

1) promoter

The human 1021 base pair preprourokinase cDNA

gene (uPA) was obtained by digestion of plasmid

pRdzbuktt with Sac1 and HindIII. The plasmid

pCLH3AXBPV was linerized by the digestion with XhoI.

The uPA sequence was ligated to the linear

pCLH3AXBPV to make the pCLH3AXBPV-uPA expression

plasmid containing human preprourokinase gene in a

sense direction with respect to the MT-1 promoter.

Transcription is clockwise in the 5' to 3'

direction. The hatched area represents the

preprourokinase coding sequences.
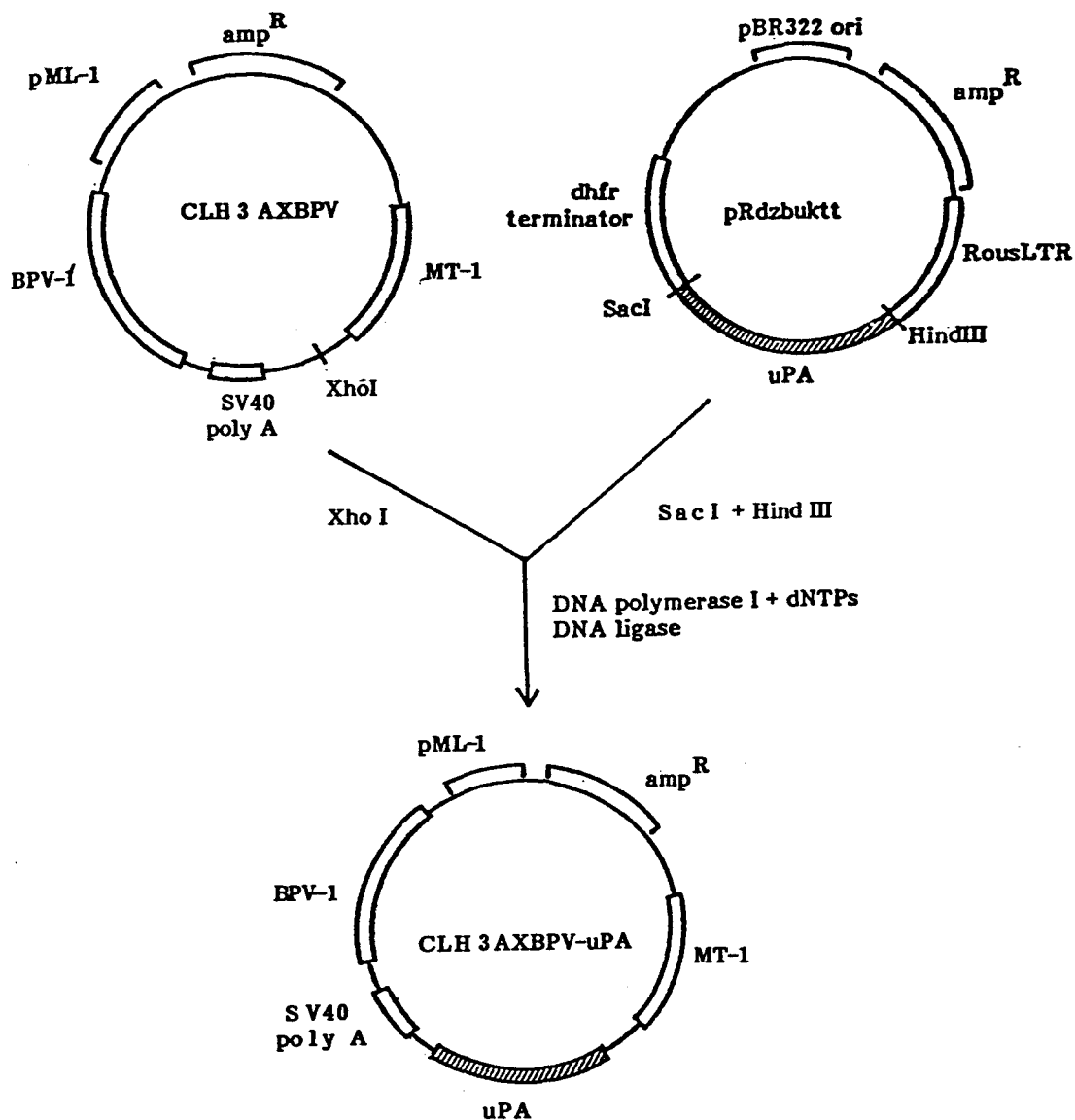
Figure 6.   Structure of the plasmid pCLH3AXBPV-uPA

The structure of plasmid pCLH3AXBPV-uPA is identical with that of pCLH3AXBPV except the full length cDNA for human preprourokinase was inserted in the sense orientation at <u>Xho</u>I site.   The MT-1 promoter directs the expression of inserted human preprourokinase gene.   The transcription is clockwise in the 5' to 3' direction.   The outside plasmid circle, corresponding to the inner circle, shows several restriction enzymes used to verify the constructed pCLH3AXBPV-uPA structure.
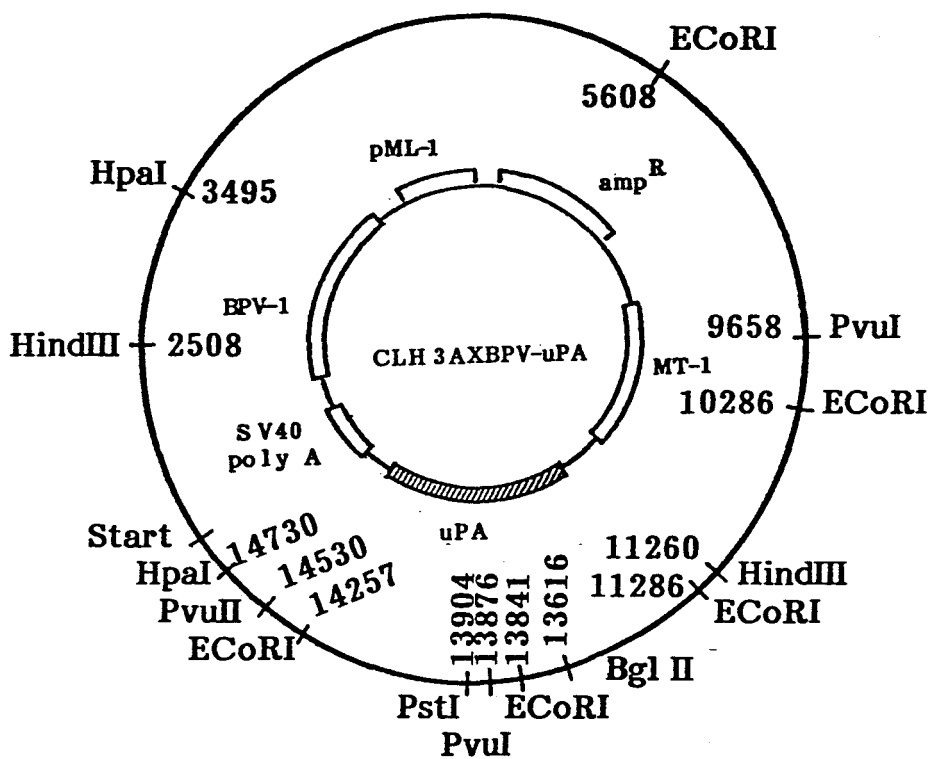
Figure 7. Construction of human urokinase antisense
gene expression vector pSV2-ASuPA.

The human 5'end 265 base pairs of
preprourokinase cDNA was obtained by digestion of
plasmid pRdzbuktt with HindIII and NcoI. Plasmid
pSV2-dhfr was digested with HindIII and BglII and
the large fragment was purified. Two fragments were
ligated at HindIII sites in an antisense direction.
The cohesive ends of ligated DNA, NcoI and BglII
sites, were blunted by T4 DNA polymerase followed by
blunt ligation. Transcription is clockwise in the
5' to 3' direction.
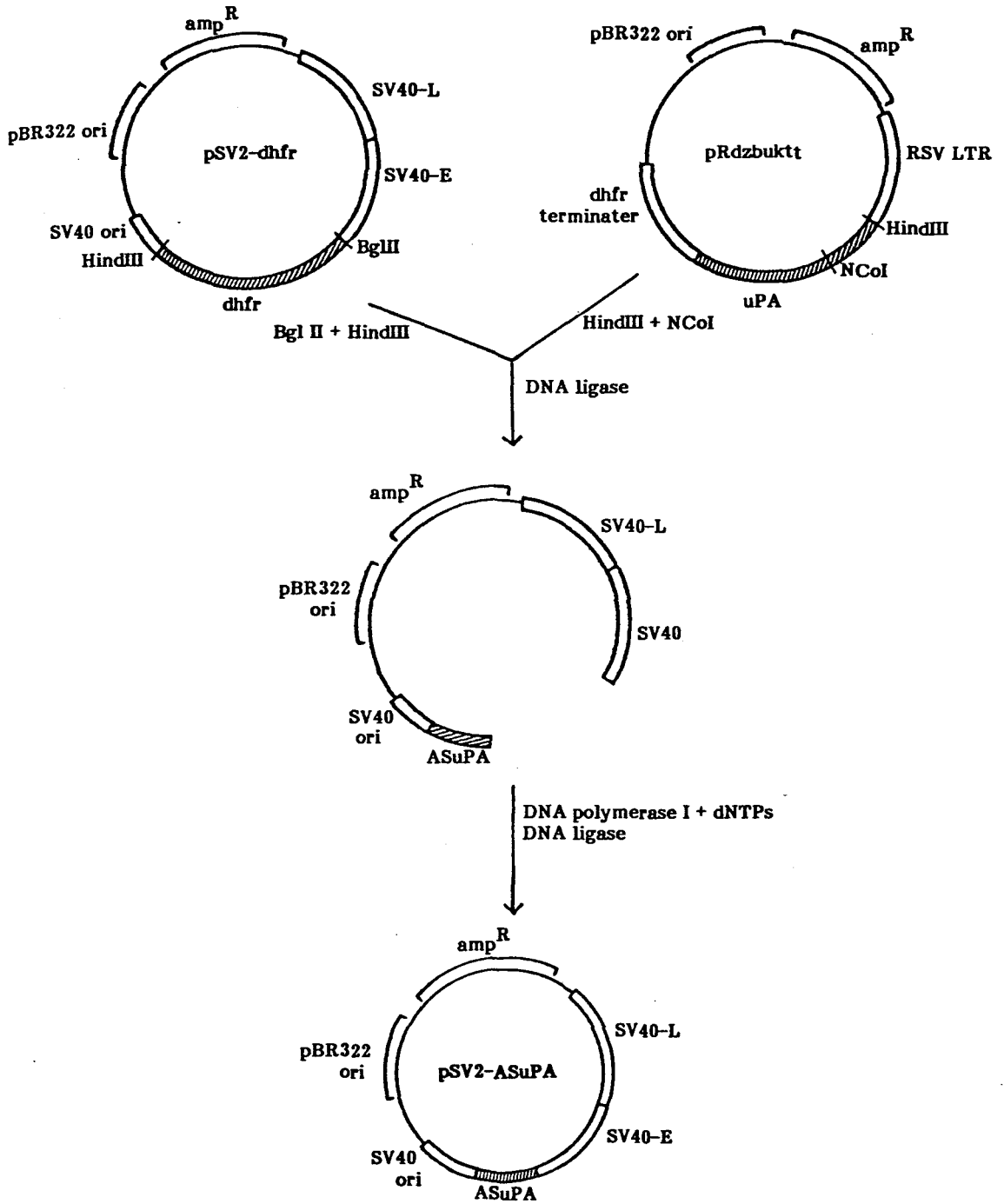
agarose gel. The pRdzbuktt was digested with <u>Hind</u>III and <u>Nco</u>I and 265-base-pair fragment containing the human preprourokinase sequence was purified and eluted from the agarose gel. Two purified fragments were ligated at the <u>Hind</u>III site which resulted in a 265-base-pair fragment attached to pSV2-dhfr digest in an antisense orientation with respect to the SV40 early gene promoter. The cohesive ends of the ligated DNA were blunted with T4 DNA polymerase and a mixture of four deoxynucleoside triphosphates, ligated, and then transformed in HB101. The selected ampicillin resistant clones were analyzed for the correct orientation by restriction endonuclease digestions with <u>Bgl</u>I, <u>Pst</u>I, and <u>Pvu</u>II. The final pSV2-ASuPA plasmid construction was confirmed by additional restriction digestions with <u>Bgl</u>I and <u>Pst</u>I and double digestions with <u>Pvu</u>II-<u>Bam</u>HI, <u>Bam</u>HI-<u>Eco</u>RI, <u>Eco</u>RI-<u>Hpa</u>I and <u>Hpa</u>I-<u>Pvu</u>I. The plasmid structure and the restriction endonuclease digestion map of the constructed pSV2-ASuPA structure is shown in Figure 8.

<u>DNA</u> <u>Transfection</u>

DNA transfection was performed by the calcium-phosphate coprecipitation technique of Graham and van der Eb (1973), with modifications. Twenty-four hours before

Figure 8. Structure of the plasmid pSV2-ASuPA

The plasmid pSV2-ASuPA contains the HindIII
and NcoI digestion fragment of human preprourokinase
in the antisense orientation with respect to the
SV40 early gene promoter, the SV40 early and late
gene promoters, a SV40 polyadenylation signal, the
pBR322 origin of replication, and an ampicillin
resistant gene. The SV40 early promoter directs the
expression of the inserted human preprourokinase
antisense gene. The transcription is clockwise in
the 5' to 3' direction. The outside plasmid circle,
corresponding to the inner circle, shows several
restriction enzymes used to verify the constructed
pSV2-ASuPA structure.
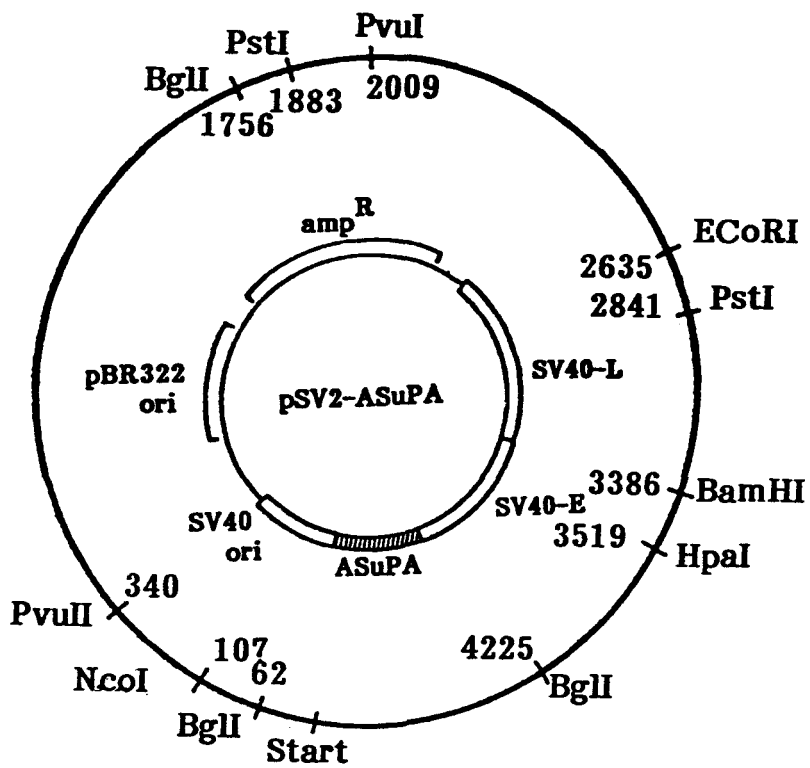
the transfection, approximately 5 x 10^5 cells were seeded

in 100 mm Petri dishes containing Eagle's MEM and 10%

heat-inactivated FBS. The DNA used for transfection was

purified by alkaline lysis extraction followed by CsCl

density gradient centrifugation, and then dialyzed against

TE-buffer (Maniatis et al., 1982). The DNA used for the

transfection was in the form of closed circular plasmid

DNA. B16-F1 cells were cotransfected with 10 ug of

constructed plasmid containing the human preprourokinase

gene (pSV2-uPA, pCLH3AXBPV-uPA or pRdzbuktt) and 1 ug of

pSV2-neo (American Type Culture Collection). B16-F10

cells were cotransfected with 10 ug of pSV2-ASuPA and 1 ug

of pSV2-neo. For the control, 1 ug of pSV2-neo alone was

used. The CaPO$_4$/DNA mixture was prepared by combining DNA

with 2 M CaCl$_2$ in HBSP buffer (1.5 mM Na$_2$HPO$_4$, 10 mM KCl,

280 mM NaCl, 12 mM glucose, 50 mM HEPES, pH 7.05) and was

allowed to form a precipitate for 30 minutes at room

temperature without any disturbance. All the solutions

except DNA were kept sterile prior to transfection. The

CaPO$_4$/DNA precipitate was then slowly added to the cells

in a dropwise manner and the cells were incubated at 37°C

in 5% CO$_2$ saturated with water vapor for 16 hours. At the

end of the transfection period, the cells were shocked

with 15% sterile glycerol in HBSP buffer for 3 minutes at

37°C. The cells were then allowed to recover in MEM supplemented with 10% heat inactivated FBS for 24 hours at 37°C. The cells were subcultured at a 1:3 ratio and transferred to MEM with 10% heat inactivated FBS containing 1.5 mg/ml Geneticin (G-418) (Gibco Laboratories, Grand Island, NY). The cells were provided with fresh MEM with 10% heat inactivated FBS containing Geneticin every three days. Two weeks after the initiation of Geneticin treatment, the transfected cells were monitored every day and about four weeks later the surviving cell colonies were further processed.

Fibrin Overlay Assay for Plasminogen Activator

The transient expression of the urokinase gene of the transfected cells were assayed by the fibrin overlay assay for plasminogen activator with minor modifications (Kenten et al., 1986). Two days after the transfection, the cells were detached and collected from the petri dishes by incubation with 1 mM EDTA in PBS and then washed twice in serum-free MEM. The cell suspension solution (70% MEM supplemented with 10% heat inactivated FBS, 30% Hank's balanced salt buffer (Gibco Laboratories), supplemented with 2.5% low-gelling-temperature agarose) (type VII, A-4018, Sigma Chemical Co.) was prepared and

incubated at 42°C until the agarose was dissolved. To the 9 ml of cell suspension solution, 0.7 units of thrombin (from bovine plasma, T-6759, 276 units/ml, Sigma Chemical Co.) and 5×10^4 cells were added and rapidly mixed by inverting the test tube. Then 1.5 ml of MEM supplemented with 10% heat inactivated FBS containing 30 mg/ml fibrinogen (from bovine blood, F-4753, Sigma Chemical Co.) was rapidly added and the mixture was poured immediately into 100-mm petri dish. The dishes were incubated at 37°C in 5% CO_2 saturated with water vapor for 24 hr. After the incubation, the number of clearing zones which represent the presence of the cells that produce urokinase were counted. As a reference, C127 cells obtained from ATCC were transfected with pCLH3AXBPV-uPA and assayed in the same manner to detect urokinase activity in the transfected cells as documented by Reddy et al. (1987). For the positive control, 10 ul of human urokinase (High molecular weight urokinase, #124, 80,000 IU/mg, from American Diagnostica Inc., New York, NY) solution (3.6 ug/ml) was added dropwise on the agarose fibrin plate.

Immunoblot Detection of Human Urokinase Production by Transfected Cell Colonies

The surviving colonies of transfected cells were

analyzed for the production of human urokinase by a filter immunoblot assay (McCracken and Brown, 1984) with polyclonal antibody to high molecular weight human urokinase (American Diagnostica, New Haven, CN, Antibody #389). This assay was not used quantitatively, but only used to verify the presence of urokinase production in the colonies. Prior to the assay, the medium was removed from the petri dish containing transfected B16-F1 or B16-F10 colonies. The cells were washed twice with serum free medium (SFM), then 2.5 ml of SFM was added to the cell. An autoclaved disc of teflon mesh (Spectrum Medical Industries Inc., Los Angeles, CA) was placed over the cell colonies and a sterile notched nitrocellulose membrane filter disc (Millipore, Bedford, MA) was laid over the teflon mesh. The cell colonies were then incubated for 7 to 8 hours at 37°C. After the incubation, the filter and mesh were removed and the cells were re-fed with MEM containing 10% heat inactivated FBS with Geneticin until colony transfer. The filters were soaked in Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.1% ovalbumin in 150 mM NaCl, 10 mM sodium phosphate, pH 7.4) for 16 hours at 4°C to block any non-specific binding sites, washed with PBS-Tween (PBS containing 0.05% Tween-20), and then incubated with 1:500 diluted rabbit

polyclonal antibody to human urokinase (American

Diagnostica Inc., New York, NY, antibody #389) in PBS-

Tween containing 0.1% ovalbumin for 2 hours at room

temperature. The filters were subsequently washed and

incubated with 1:300 diluted goat antirabbit IgG

conjugated with alkaline phosphatase (Biorad, Richmond,

CA) in PBS-Tween for 1 hour at room temperature. After

the second antibody treatment, filters were again washed

and incubated with BCIP/NBT (5-bromo-4-chloro-3-indoyl

phosphate/nitroblue tetrazolium) solution (Biorad) in

carbonate buffer (0.1 M $NaHCO_3$, 1.0 mM $MgCl_2$, pH 9.8) for

alkaline phosphatase color development. After the filters

were processed, photocopies and transparencies were made.

Quantification of Urokinase Production by ELISA

The transparencies made from the immunoblot filters

were aligned with original culture dishes and twenty

positive colonies, dark purple spots on the filters, were

selected. These were transferred to separate petri

dishes. The cells in each colony were picked up using

yellow Pipetman tips after they were released from the

Petri dishes by scraping under the microscope in a sterile

hood. They were allowed to proliferate in MEM

supplemented with 10% heat inactivated FBS. In order to

select the colonies that produce the highest amount of urokinase, the secretion of urokinase from each colony was quantitated by IMUCLONE Urokinase Plasminogen Activator ELISA Kit (American Diagnostica Inc., New York, NY). This ELISA Kit detects both single and two-chain urokinase (IMMUCLONE uPA ELISA Kit manual, American Diagnostica Inc.). Forty hours prior to ELISA, 2.5×10^4 cells were seeded in 25 mm petri dishes in serum free MEM and incubated at 37°C. A 100 ul aliquot of conditioned medium was removed from each colony and added to the ELISA plate wells coated with murine monoclonal antibody to human urokinase (high and low molecular weight urokinase). The plates were incubated for 90 minutes at 37°C in the buffer provided by the manufacturer. The wells were then washed and incubated with rabbit anti-human urokinase conjugated with horseradish peroxidase at 37°C for 1 hour. After incubation with the second antibody, the substrate was added for peroxidase color development and incubated for 30 minutes at room temperature. After the final incubation, the reaction was stopped with solution provided and absorbances were read at 405 nm in an ELISA microplate spectrometer (Minireader II, Dynatach Labs, Inc., Alexandria, VA). Each colony was tested in triplicate and three separate experiments were performed.

<u>Southern</u> <u>Blot</u> <u>Hybridization</u> <u>Analysis</u> <u>of</u> <u>B16-F1</u> <u>and</u> <u>B16-F1</u> <u>pSV2-uPA</u> <u>transfected</u> <u>cells</u>

The genomic DNAs of B16-F1 cells, pSV2-neo transfected cells, and clone 7 (C7) of pSV2-uPA transfected B16-F1 cells were isolated by standard methods (Davis <u>et</u> <u>al</u>., 1986). The genomic DNAs of B16-F1 and transfected B16-F1 cells and the pSV2-uPA plasmid were digested with <u>Bam</u>HI or <u>Pst</u>I and electrophoresed overnight on a 0.8% agarose gel. The DNAs were Southern blotted to nitrocellulose filter (Trans-Blot Transfer Medium, Bio-Rad) by the capillary transfer method. For the cDNA probe, pSV2-uPA was double digested with <u>Hpa</u>I and <u>Pst</u>I and the DNA fragment containing the preprourokinase sequence was purified by electrophoresis and DNA electroelution. The probe was labeled with ^{32}P-dCTP by nick translation (Nick Translation System, BRL, Life Technologies, Inc., Gaithersburg, MD). Briefly, the probe 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 minutes. The nick-translated CTP was separated from unincorporated ^{32}P-labeled CTP by NICK-Column (Pharmacia Fine Chemicals, Uuppsala, Sweden) which contains Sephadex G-50 DNA Grade.

The nitrocellulose filter was hybridized with ^{32}P-labeled probe in hybridization solution containing 5X Denhardt's (1X Denhardt's = 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA), 1% SDS, 100 ng/ml denatured salmon sperm DNA, 1 M NaCl/10 mM Na_3PO_4 buffer, pH 6.5, at 65°C overnight. The filter was then washed four times for 30 minutes each wash with 0.2X SSC (1X SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) containing 0.5% SDS, at 65°C and 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 autoradiographs were scanned by a Gel Scan densitometer equipped with a Gilford Response UV-VIS Spectrometer (Gilford Systems, Oberline, OH).

Southern Blot Hybridization Analysis of B16-F1 and B16-F1 pSV2-neo and pRdzbuktt transfected cells to detect pSV2-neo incorporation

The genomic DNAs of B16-F1 cells and pSV2-neo transfected cells, pSV2-neo and pRdzbuktt cotransfected cells were similarly isolated. The prepared genomic DNAs and the plasmid pSV2-neo were digested with HindIII or double digested with HindIII and BamHI or HindIII and

PstI, electrophoresed overnight on a 0.8% agarose gel, and
blotted to nitrocellulose filter. For the cDNA probe,
pSV2-neo was digested with HindIII and PstI and the DNA
fragment containing the neo gene was used. The probe was
labeled with ^{32}P-dCTP by nick translation. The
nitrocellulose filter was similarly hybridized with the
probe and exposed to X-ray film.

Southern Blot Hybridization Analysis of B16-F1 and B16-F1
pRdzbuktt transfected cells to detect pRdzbuktt
incorporation

To detect the pRdzbuktt incorporation, the plasmid
pRdzbuktt and the isolated cell DNAs were digested with
HindIII or double digested with HindIII and SacI or
HindIII and PstI and were separated by electrophoresis.
DNAs were Southern blotted to nitrocellulose filter. For
the cDNA probe, pRdzbuktt was double digested with SacI
and HindIII and the fragment containing the human
preprourokinase gene was used for the hybridization. The
probe was labeled with ^{32}P-dCTP by nick translation. The
nitrocellulose filter was similarly hybridized with the
probe and exposed to X-ray film.

Southern Blot Hybridization Analysis of B16-F10 and B16-F10 Antisense Transfected Cells

The genomic DNAs of B16-F10 cells, pSV2-neo transfected cells, and antisense clones 10 (AC10) and 8 (AC8) of pSV2-ASuPA transfected B16-F1 cells were similarly isolated, digested with PstI, and electrophoresed on a 0.8% agarose gel. The DNAs were Southern blotted to nitrocellulose filter. For the cDNA probe, pSV2-ASuPA double digested with HpaI and PstI and the DNA fragment containing the preprourokinase sequence was purified. The probe was labeled with ^{32}P-dCTP by nick translation. The nitrocellulose filter was similarly hybridized with the probe and exposed to X-ray film.

Northern Blot Hybridization Analysis of B16-F1 and B16-F1 Cells Transfected with pSV2-uPA

The cytoplasmic RNAs of B16-F1, B16-F1 pSV2-neo transfected cells, and pSV2-uPA transfected cells (clone 7 and 10) were extracted by a standard method (Ausubel et al., 1988). Briefly, the cells were washed and collected in cold PBS and lysed in ice-cold lysis buffer (50 mM Tris-Cl, pH 8.0, 100 mM NaCl, 5 mM MgCl$_2$, 0.5% Nonidet P-40 in DEPC-treated water). The contaminating proteins

were denatured with proteinase K and the RNA was purified

by phenol extraction. The prepared 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). The RNAs were transferred to

Nytran Nylon Membrane (Schleicher & Schuell, Keene, NH) by

the capillary transfer method, and immobilized by a

covalent bond using the UV crosslinking technique (UV

Stratalinker 1800, Stratagene, La Jolla, CA). For the

cDNA probe, pSV2-uPA was double digested with BamHI and

PstI and the DNA fragment containing preprourokinase

sequence was labeled with ^{32}P-dCTP by nick translation.

The Nytran filter was hybridized with ^{32}P-labeled probe in

hybridization solution containing 50% formamide, 2.5X

Denhardt's reagent, 200 ug/ml of denatured salmon sperm

DNA, 0.5% SDS, and 5X SSPE (1X SSPE = 0.18 M NaCl/10 mM

$NaPO_4$, pH 7.7/1 mM EDTA) at 42°C overnight. The filter

was washed twice in 6X SSPE and 0.5% SDS at room

temperature, twice in 1X SSPE and 0.5% SDS at 37°C, and

once in 1X SSPE and 0.5% SDS at 60°C. The filter was

exposed to X-ray film at -70°C. To rehybridize the blot,

the pSV2-uPA probe was removed by boiling the filter for

20 minutes in 0.01X SSPE/0.5% SDS and rinsing twice in 2X

SSPE. For the internal standard control, the stripped

filter was similarly hybridized with ^{32}P-labeled

Drosophila actin (Fryberg et al., 1983) obtained from Dr.

Mark Kelley (Loyola University Medical Center) and exposed

to X-ray film.

Assay of the Binding of Human Urokinase to the Human PC3-
Met Cell Line and the Murine B16-F1 Cell line

The binding of human urokinase to B16-F1 or PC3-Met

cells (obtained from Dr. James Kozlowski, Northwestern

University, Chicago, IL) was determined by the procedure

of Stoppelli et al. (1985), with modifications. For B16-

F1 cells, 5 x 10^5 cells were seeded in 2 ml of MEM

supplemented with 10% heat-inactivated FBS in 6-well plate

(Elkay Products, Inc., Shrewbury, MA) and incubated at 37°

C in 5% CO_2 for 24 hours prior to the assay. For PC3-Met

cells (Kozlowski et al., 1984), 2.5 x 10^5 cells were

similarly seeded in Dulbecco's modified Eagle's medium

(DMEM) supplemented with 10% heat-inactivated FBS. The

cells were gently washed twice with PBS containing 1 mg/ml

of bovine serum albumin (BSA). In order to unmask

urokinase receptors on the cell surface, the cells were

incubated for 3 minutes at room temperature in 50 mM

glycine-HCl buffer, pH 3.0, containing 0.1 M NaCl, and

quickly neutralized with 0.5 M HEPES buffer, pH 7.5,

containing 0.1 M NaCl (Stoppelli et al., 1986). The acid
treated cells appeared undamaged and bound to the plates.
The cells were then washed twice with serum-free MEM
containing 20 mM HEPES, pH 7.5, and 1 mg/ml of BSA and
incubated for 1 hour at $37^{\circ}C$ in the same buffer containing
4×10^4 cpm of ^{125}I labeled human urokinase (see below) to
which different concentrations of unlabeled human
urokinase (American Diagnostica Inc., high molecular
weight, #124, 80,000 IU/mg) were added. After incubation,
the cells were washed four times with PBS containing 1
mg/ml of BSA and lysed by incubation in 20 mM HEPES
buffer, pH 7.5, with 1% Triton X-100 and 10% glycerol for
30 minutes at room temperature. The lysed cells were
counted in a gamma-ray counter (1191 Gamma Trac, Tm
Analytic, Inc., Elk Grove Village, IL). Nonspecific
binding was determined in the presence of excess (50 nM)
unlabeled human urokinase and subtracted from the each
experimental value. Each experimental concentration was
performed in triplicate and the results were plotted as
the mean cpm $\pm$ standard deviation.

^{125}I Labeling of Human Urokinase

Three Iodo-Beads (Pierce Chemical Co., Rockford,
IL) and 1 mCi of $Na^{125}I$ in NaOH (IMS300, Amersham Corp.,

Arlington Heights, IL) were incubated in 0.05 M $KHPO_4$, 0.2 M NaCl, pH 7.5, at room temperature for 5 minutes with gentle shaking. Human urokinase (High molecular weight urokinase, #124, 80,000 IU/mg from American Diagnostica Inc., New York, NY), 50 ug, was added to the iodination mixture and incubated at room temperature for 20 minutes with gentle shaking. The ^{125}I human urokinase was separated from free $Na^{125}I$ by gel filtration through a 9 ml Bio-Gel P-6 column (100-200 Mesh, Bio-Rad Laboratories) which was presaturated by washing with five column volumes of the above buffer containing 2 mg/ml of BSA. The urokinase ^{125}I fractions were combined and dialyzed extensively against 0.05 M $KHPO_4$, 0.2 M NaCl buffer with 1 mM KI overnight and then against 0.05 M $KHPO_4$, 0.2 M NaCl buffer. The amount of protein was determined by the BIO-RAD Protein Assay Kit (Bio-Rad Laboratories) according to the manufacturer's instruction.

Determination of Glycosylation of Human Urokinase

A Concanavalin A affinity chromatography column was used to determine if human urokinase produced by transfected murine cells was glycosylated. Con A-Sepharose (Pharmacia Fine Chemicals, Uuppsala, Sweden) (1 ml) was packed in a blue Eppendorf pipette tip and washed

with 50 mM Tris buffer, pH 7.5. The resin was further
prepared by washing with buffer containing 100 mM sodium
acetate, 1 mM NaCl, 10 mM $CaCl_2$, 10 mM $MgCl_2$, pH 6.0.
Conditioned medium, prepared by incubating $5x10^5$ cells of
clone 7 of pSV2-uPA transfected B16-F1 in 2 ml of serum
free MEM in a 6-well plate for 40 hours at 37°C, was
collected and applied to the ConA sepharose column. The
column was washed with four column volumes of 50 mM Tris
buffer and then with the same buffer containing 0.5 M
alpha-methyl-D-mannoside (Sigma Chemical Co.) to elute the
glycosylated protein from the resin. Fractions of 400 ul
were collected and assayed for human urokinase by the
IMUCLONE Urokinase Plasminogen Activator ELISA Kit
(American Diagnostica, Inc.) using a monoclonal antibody
to human urokinase, as described above.

**Collection of Conditioned Medium for Quantification of
Urokinase Activity Secreted by Cultured Cells**

The cells were plated in 6-well cell culture plates
at a concentration of 2×10^5 cells per well in MEM
supplemented with 10% heat-inactivated FBS and incubated
overnight at 37°C in a 5% CO_2 atmosphere. The cells were
rinsed twice with serum free medium (SFM) and incubated
for 24 hours in SFM at 37°C in a 5% CO_2 atmosphere. The

cell conditioned media was collected and the cells were
harvested and counted.

Fluorogenic Substrate Assay for Plasminogen Activator Activity

Plasminogen activator activity was measured by
fluorescence spectroscopy as described by O'Donnell-Tormey
and Quigley (1983). Cells to be assayed were collected by
incubation with 0.25% of trypsin (Worthington Biochemical
Corp., Freehold, NJ) in PBS containing 1 mM EDTA for few
seconds. Cells were washed with PBS three times and
resuspended in HEPES buffer (5% dimethyl sulfoxide in 0.05
M HEPES, pH 7.4) at the concentration of 1×10^6 cells/ml.
Cell suspension solution (400 ul) was mixed with 170 ul of
HEPES buffer, 30 ul of fluorogenic substrate for
plasminogen activator, methoxysuccinyl-L-Gly-L-Gly-L-Arg-
7-amino-4-methylcoumarin HCl (Bachem Fine Chemicals, Inc.,
Torrance, Calif.) (final concentration of 10 mM). The
release of 7-amino-4-methylcoumarin (AMC) from the
substrate was measured in a Perkin-Elmer MPF-44B
Fluorescence Spectrophotometer at an excitation wave
length of 380 nm and an emission wave length of 460 nm.
The linear release of AMC was recorded for 5 minutes and
the slope of the linear curve was measured. To calibrate

the instrument, 7-amino-4-methyl coumarine (Sigma Chemical Co.) was used as a standard. For the standard curve, different dilutions of human urokinase (High molecular weight urokinase, #124, from American Diagnostica Inc., New York, NY) were used instead of cells.

Chromogenic Substrate Assay for Plasminogen Activator Activity

Plasminogen activator activity was measured spectroscopically using a chromogenic substrate for plasmin as previously described by Whur et al. (1980). Ten ul of cell conditioned media was added to 100 ul of 100 mM Tris buffer, pH 8.1, containing 5 ug of 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 in 96-well plates. Plasminogen was treated, prior to use, to inhibit any active plasmin activity by incubating plasminogen with diisopropylfluorophosphate (DFP) at 25 mM 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 with DFP-treated plasminogen to ensure plasmin activity was no longer detected by the same assay. The plates were incubated for 16 hours at 37°C in a 5% CO_2 atmosphere. After incubation,

the p-nitroaniline absorbances, corresponding to substrate hydrolysis by plasmin, were read at 405 nm in a spectrometer (Minireader II, Dynatech Labs, Inc., Alexandria, VA).

To test the stability of urokinase gene expression in the transfected cells in a longer period of time, one of the flask of Clone 7 of pSV2-uPA transfected B16-F1 cells were passaged more than 20 times. The conditioned medium was collected from that particular flask and assayed for the plasminogen activator activity by the same method. Determination of the standard curve of plasminogen activator activity and nonspecific activity were included in each assay.

The nonspecific activity was determined in the absence of cell conditioned medium and subtracted from each experimental value. For the background activity, reaction buffer was used instead of plasminogen for the assay. Each experiment was performed in triplicate and five separate experiments were assayed. Plasminogen activator activity was expressed in pg secreted in the conditioned medium per cell per hour.

[125]I-fibrin Plate Assay for Plasminogen Activator Activity

The procedure was similar to that described

previously by Strickland and Beers (1976). Bovine

fibrinogen (Sigma Chemical Co.) was iodinated with $Na^{125}I$

by the same procedure as described above for the ^{125}I

labeling of human urokinase. The specific radioactivity

of the fibrinogen was adjusted to 15,000 cpm/ug of

fibrinogen by the addition of unlabeled fibrinogen. To

each well of a 24-well plate (Gibco Laboratories), 250 ul

of ^{125}I-fibrinogen in 50 mM Tris buffer, pH 8.1 (50,000

cpm per well) was added and dried in a $37^{o}C$ dry oven for

two days. Prior to use, wells were incubated with 500 ul

of MEM supplemented with 5% plasminogen-depleted FBS at 37°

C in a 5% CO_2 atmosphere to convert fibrinogen to fibrin.

Plasminogen-depleted FBS was prepared by repeated

incubation of FBS at $56^{o}C$ for 45 minutes. Wells were

washed three times with 50 mM Tris buffer, pH 8.1 and

incubated for 2 hours with 100 ul of cell conditioned

medium, 75 ug of BSA and 10 ug of DFP treated plasminogen

(Sigma Chemical Co.) in the 300 ul of same buffer at $37^{o}C$

in a 5% CO_2 atmosphere. At the end of the incubation

period, a 100 ul aliquot of supernatant was taken from

each well and counted in a gamma-counter. Nonspecific

activity was determined in the absence of conditioned

medium and subtracted from each experimental value. For

the background activity, reaction buffer was used instead

of plasminogen for the assay. A standard curve was included in every assay. Each experiment was performed in triplicate and five separate experiments were assayed. Plasminogen activator activity was expressed in pg secreted in the conditioned medium per cell per hour.

Murine Lung Colonization Assay

The murine B16-F1 or B16-F10 melanoma lung assay was performed in a similar manner as previously described by Fidler (1978). Transfected B16-F1 or B16-F10 cells were grown to semi-confluency (80-90%) in MEM supplemented with 10% heat inactivated FBS and harvested by the addition of 1 ml of 1 mM EDTA in PBS, pH 7.4 at 37°C for 1 minute. MEM supplemented with 10% heat inactivated FBS was added, centrifuged, and resuspended in PBS. The cells were counted using a Coulter Counter (Coulter Electronics, Inc., Hialeah, FL) and diluted to $5x10^{5}$ cells/ml. The viability of the cells was determined by counting trypan blue exclusion. The cells were maintained on ice prior to injection. A total of 26 female C57BL/6 mice, 6-8 weeks old (Harlan Laboratories, Indianapolis, IN) were equally divided into control and experimental groups. Mice were housed in the Loyola Medical Center Animal Research Facility for one week prior to use. A 0.2 ml aliquot of

cell suspension (100,000 cells per mouse) were slowly injected into the lateral tail vein of each mouse while immobilized in a mouse vice. The injection schedule alternated between mice in the control group and mice in the experimental group to minimize the variance in time. The animals were sacrificed 17 days after the injection of transfected cells by administering an overdose of ether. The lungs were removed and fixed in Bouin's solution (0.75% picric acid, 25% formaldehyde, 5% glacial acetic acid) overnight. The number of surface tumor colonies, easily visible as black or white nodules on the yellow Bouin's background, were counted under a dissecting microscope.

Spontaneous Metastasis Assay

The assay of spontaneous metastasis from a foot pad tumor was carried out by the procedure of Fidler (1986), with minor modifications. Cells were grown to semi-confluency in MEM supplemented with 10% heat inactivated FBS, harvested, and tested for viability, and counted as described above. Female C57BL/6 mice, 6-8 weeks old (Harlan Laboratories) were housed in the Loyola Medical Center Animal Care Facility for one week prior to use. Mice were lightly anesthetized with ether and injected

subcutaneously into a posterior footpad with 10^5 cells of

clone 7 of B16-F1 transfected cells for experimental

animals or pSV2-neo transfected cells for a control. The

cells for injection were suspended in 0.05 ml of PBS. An

1/2 inch 27-gauge needle was inserted into the dorsal

aspect of the foot for injection. Three weeks later,

after allowing the cells to grow into the primary site,

mice were lightly anesthetized with the inhalation

anesthetic, Metofane (Methoxyfurane) (Pitman-Moore, Inc.,

Washington Crossing, NJ) and the tumor-bearing foot was

amputated with a pair of scissors. The wound was

cauterized and drawn together with wound clips (Becton

Dickinson & Co., Parsippany, NJ). Mice were injected with

15 ug of Buprenex (Buprenorphine HCl, Reckitt & Colman

Pharmaceutical, Inc., Norwich, NY) to relieve the pain and

returned to their cages. After an additional 3-4 weeks,

the animals were sacrificed by an overdose of ether. The

lungs were removed and fixed in Bouin's solution

overnight. The number of surface tumor colonies were

counted under a dissecting microscope.

Culture of Lung Colony Tumor Cells

C57BL/6 mice were injected with 100,000 cells of

Clone 7 of pSV2-uPA transfected B16-F1 cells or pSV2-neo

transfected B16-F1 cells. Animals were sacrificed 17 days after injection. The lungs were removed aseptically, cut into small pieces with sterile razor blades, and plated in vitro on petri dishes containing MEM supplemented with 10% heat-inactivated FBS. The medium was changed after the cultures adhered to the petri dishes. The cells were maintained in the same manner as B16-F1 cells when the cultures became confluent.

Cytotoxicity Assay

The cytotoxicity assay was performed to test the immunologic responses in C57BL/6 mice against transfected murine cells which produce human urokinase. The procedure is similar to that described by Hirschberg et al. (1977). Spleens were collected at the time of sacrifice from the mice which were injected with transfected cells. Lymphocytes were harvested from the spleens and served as cytotoxic effectors. The targets cells, B16-F1 transfected with pSV2-neo or clone 7 of pSV2-uPA transfected cells, were labeled with ^{51}Cr. Cytotoxic effectors were mixed with target cells at the ratio of 50:1, 25:1, 12:1 or 6:1 and incubated for 4 hours at 37°C. The mixture was centrifuged and the supernatant was harvested and counted for radioactivity in a gamma counter

and % specific release was calculated. The spleen from the uninjected animal and B16-F1 untransfected cells were used as a control.

Statistical Analysis of Data

Numerical data was expressed as the mean ± standard deviation or the mean ± standard error of several determinations. To compare the data between the control and experimental groups, Student t test and Mann Whitney U-Test (nonparametric rank sum test) were used. P values of < 0.05 were considered to represent significant difference between two groups.

Chapter IV

RESULTS

I. The Production of Human Urokinase in Murine B16-F1
Melanoma Cells and The Study of Its Effect on Tumor
Cell Metastasis

Expression of Human Preprourokinase Gene

To express the human preprourokinase gene in B16-F1
melanoma cells, the plasmid pSV2-uPA which contains human
preprourokinase cDNA in a sense direction with respect to
the SV40 early gene promoter, was prepared. For the
synthesis of pSV2-uPA, the dihydrofolate reductase gene of
the plasmid pSV2-dhfr was replaced by the preprourokinase
gene isolated from the plasmid pRdzbuktt. The detailed
steps involved in subcloning preprourokinase cDNA into
pSV2 vector are summarized in Figure 2. The correct
plasmid which contained the preprourokinase gene in the
sense direction was selected by restriction endonuclease
digestion profile which is shown in Figure 3.

To introduce the human preprourokinase gene into
B16-F1 melanoma cells, transfection technique was used.
Transfection was carried out by using calcium phosphate
precipitation with DNA followed by a glycerol shock.
Cells were cotransfected with plasmids pSV2-uPA and a
selection marker gene pSV2-neo in 10:1 ratio, or
transfected with the pSV2-neo alone for the control. In
the pSV2-neo, SV40 early gene promotes the expression of
an aminoglycoside phosphotransferase from the E. coli
transposable element Tn5. Expression of aminoglycoside
phosphotransferase confers upon mammalian cells resistance
to the toxic aminoglycoside G-418 (Geneticin), a neomycin
analog. Therefore the pSV2-neo recipient mammalian cells
gain the capability to survive in Geneticin upon
successful transfection. To select transfected colonies,
cells were grown in the presence of Geneticin (G-418) for
four to five weeks. The transfection efficiency in the
B16-F1 cell line by the transfection condition described
on Materials and Methods section is shown to be about one
transformant in 5,000 transfected cells when the closed
circular plasmid DNA was used for transfection. The
linerized plasmid DNA gave significantly low transfection
efficiency (data not shown). The efficiency of
transfection was not increased by using a carrier DNA such

as 10 ug/ml of salmon sperm DNA for transfection (data not shown). A change of fresh MEM prior to transfection did not affect transfection efficiency (data not shown). Cells appeared to be damaged and were lifted off from the petri dish if they were shocked longer than 5 minutes. The best transfection results were obtained when the cells underwent at least two cell divisions if thawed from the frozen stock and appeared very healthy before the transfection.

The surviving colonies expressing the neo gene were assayed for the coexpression of the human preprourokinase gene by a filter immunoblot assay as shown in Figure 9. In this assay, all the proteins being secreted from the colonies expressing preprourokinase gene were absorbed to nitrocellulose filter. The filter was treated with polyclonal antibody to human urokinase to select for the positive colonies that produce human urokinase. Figure 9 shows that pSV2-uPA and pSV2-neo transfected cells generated many positive colonies detected by the dark color development. However pSV2-neo transfected control cells show the absence of color development which indicates these cells do not produce human urokinase.

Twenty positive colonies detected for human urokinase secretion by filter immunoblot assay were

**Figure 9.   Filter immunoblot analysis of transfected
B16-F1 cell colonies**

B16-F1 cells were transfected with pSV2-neo
(top) or cotransfected with pSV2-uPA and pSV2-neo
(bottom).   Transfected cells were cultured in MEM
supplemented with 10% heat inactivated FBS
containing Geneticin (G-418).   Five weeks later,
nitrocellulose membrane filters were placed over the
cell colonies to absorb urokinase being secreted.
The filters were treated with rabbit polyclonal
antibody to human urokinase followed by goat anti-
rabbit IgG conjugated with alkaline phosphatase,
which were reacted with NBT/BCIP to give dark purple
color development.   The spots on the filters
correspond to the transfected foci expressing human
urokinase.
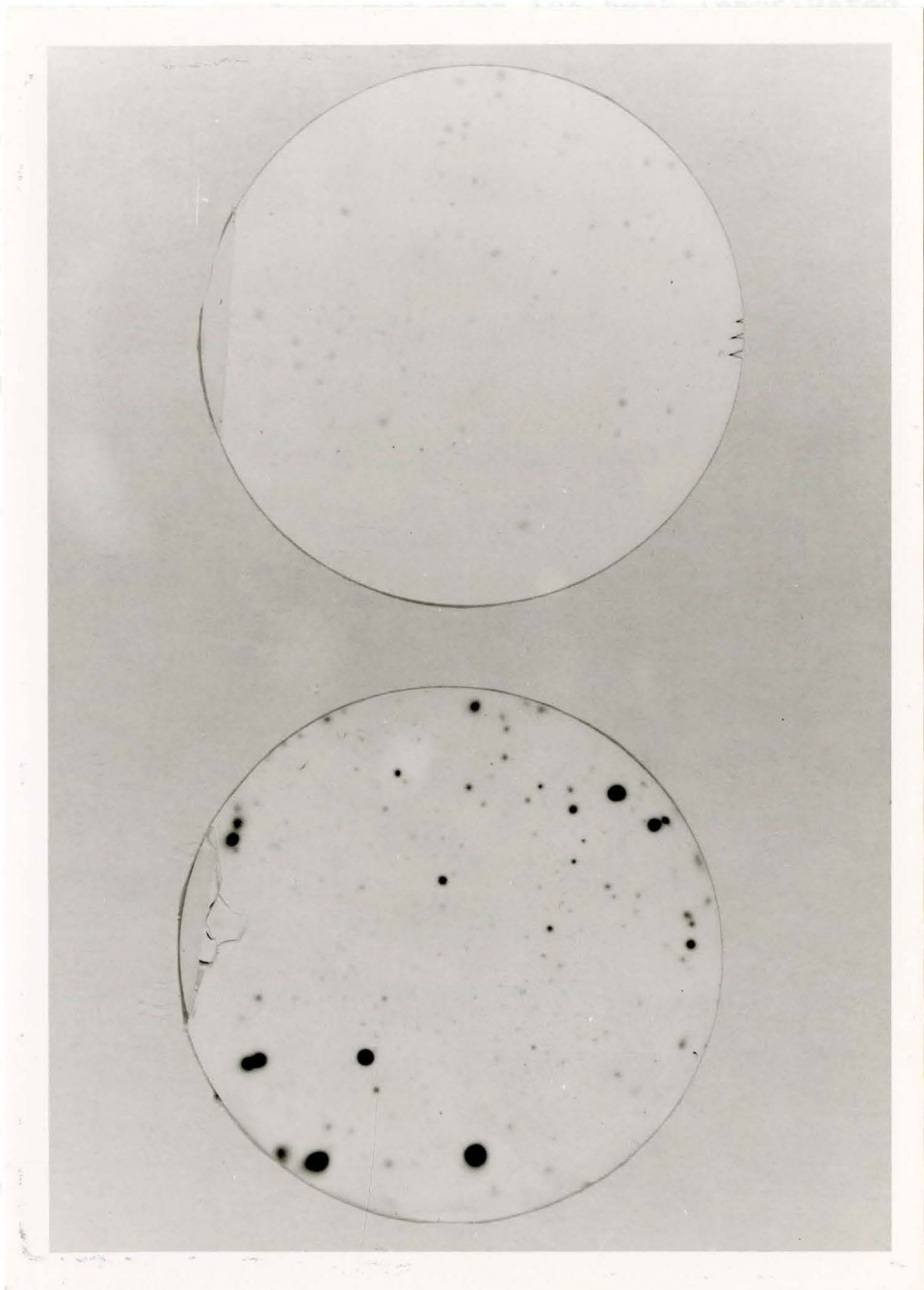
with BamHI or EcoRI, separated by electrophoresis, blotted, and probed with ^{32}P labeled human preprourokinase cDNA.

selected, transferred to separate petri dishes, and
expanded in MEM supplemented with 10% heat inactivated
FBS. Secretion of human urokinase into the cell culture
medium from each colony was then quantitated by a sandwich
type ELISA using a monoclonal antibody against high
molecular weight human urokinase as the primary antibody
and polyclonal antibody against high and low molecular
weight human urokinase as the detecting antibody.
Monoclonal antibody to human urokinase has been reported
to identify the single-chain prourokinase as well as two-
chain urokinase (Sallerno et al., 1984). The ELISA Kit
(American Diagnostica Inc.) was used for the assay to
detect both single and two-chain urokinase. Table 1 shows
that clone 7, 8, 10, and 18 secreted the highest amount of
human urokinase out of 20 positive selected clones. B16-
F1 or B16-F10 untransfected cells give absorption value
equal to zero, as no human urokinase is produced.

The incorporation of human preprourokinase gene to
genomic DNA of transfected cells was demonstrated by a
Southern blot hybridization assay as shown in Figure 10.
The DNAs were prepared from clone 7 of pSV2-uPA
transfected cells and B16-F1 untransfected cells, digested
with BamHI or PstI, separated by electrophoresis, blotted,
and probed with ^{32}P labeled human preprourokinase cDNA.

Table 1. Quantification of human urokinase production by B16-F1 transfected cells with pSV2-uPA detected by ELISA[a]

Clone No	Absorbance (Mean±SD)	Clone No	Absorbance (Mean±SD)
C1	1.79 ± 0.06	C11	2.71 ± 0.15
C2	0.25 ± 0.03	C12	0.14 ± 0.15
C3	1.17 ± 0.01	C13	2.81 ± 0.06
C4	0.72 ± 0.04	C14	0.13 ± 0.02
C5	0.87 ± 0.04	C15	0.34 ± 0.05
C6	0.09 ± 0.03	C16	0.72 ± 0.07
C7	4.24 ± 0.28	C17	0.51 ± 0.02
C8	2.97 ± 0.12	C18	3.22 ± 0.24
C9	0.10 ± 0.03	C19	1.57 ± 0.08
C10	3.43 ± 0.13	C20	1.63 ± 0.05
B16F1	0.08 ± 0.03[b]	B16F10	0.08 ± 0.04[b]

[a]The secretion of urokinase from each colony was quantitated by IMUCLONE uPA ELISA Kit (American Diagnostica Inc.) on conditioned media taken from the cells incubated in the serum free media for 40 hours.

[b]Value equal to zero as no human urokinase is produced by murine B16-F1 cells which were not transfected with the pSV2-uPA plasmid.

Figure 10. Southern blot hybridization analysis of DNA isolated from clone 7 of pSV2-uPA transfected B16-F1 and B16-F1 untransfected cells

DNAs were isolated from clone 7 of transfected B16-F1 cells and untransfected B16-F1 cells. The DNAs (10 ug) were cleaved with PstI or BamHI and electrophoresed through 0.8% agarose gel. A control pSV2-uPA plasmid preparation was similarly digested with PstI or BamHI and different amounts were loaded on the same gel. The DNAs were transferred to nitrocellulose and probed with ^{32}P-labeled HpaI-PstI fragment of pSV2-uPA. Lanes 1-2 contain DNA made from 7 x 10^6 cells of clone 7 of pSV2-uPA transfected cells and lanes 3-4 contain DNA made from 7 x 10^6 untransfected cells. Lanes 5-6 contain DNA made from 3.5 x 10^6 cells of clone 7 and lanes 7-8 from 3.5 x 10^6 untransfected cells. Lanes 9-10, lanes 11-12, lanes 13-14 contain pSV2-uPA DNA of 10, 5, 2 ng respectively. Lanes 1,3,5,7,9,11,13 contain DNA digested with BamHI and lanes 2,4,6,8,10,12,14 contain DNA digested with PstI.
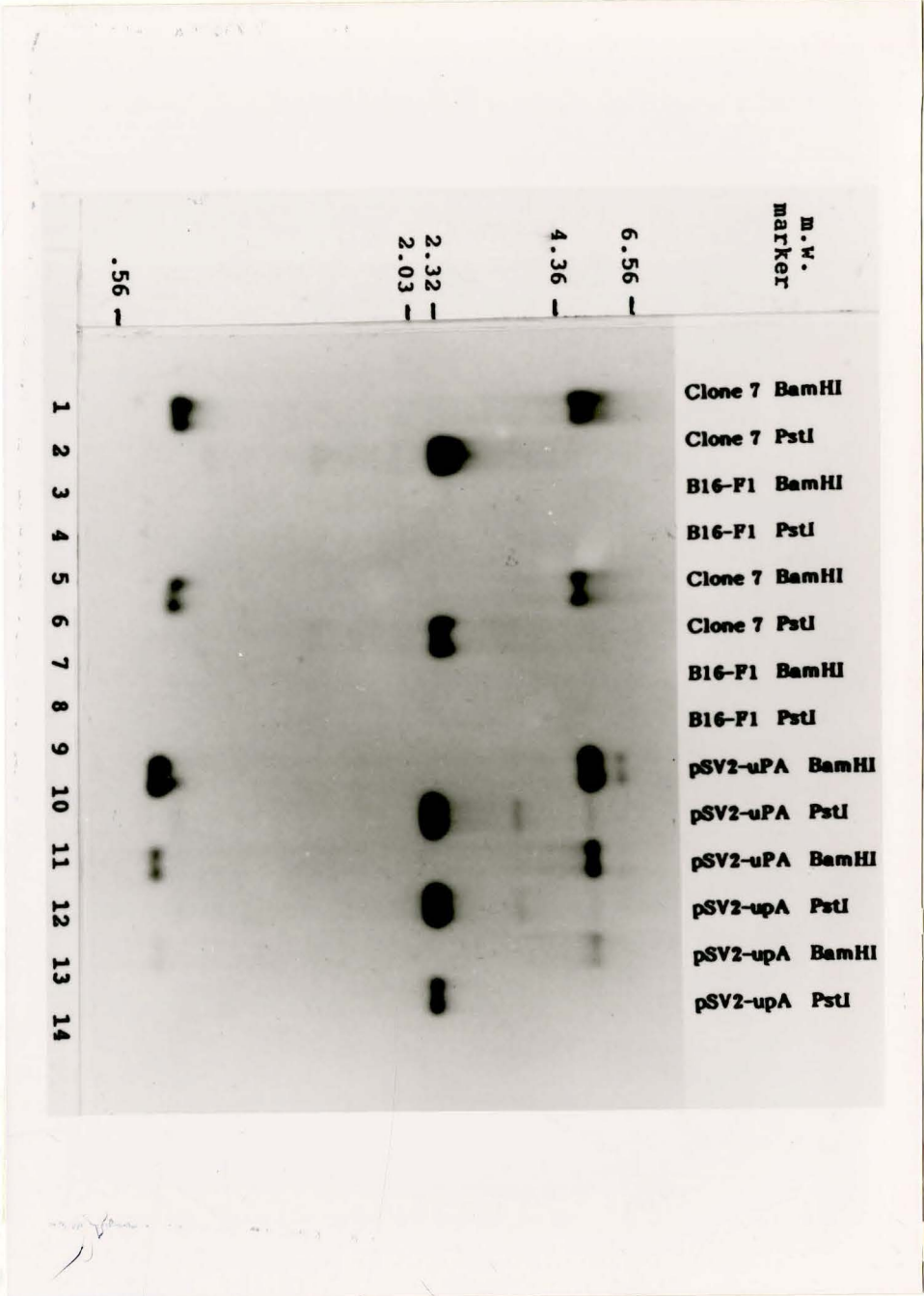
The digestion of pSV2-uPA with BamHI generates two DNA bands with base pairs of 4647 and 896 and the ^{32}P-labeled probe hybridizes with both bands. The digestion of pSV2-uPA with PstI generates three DNA bands with base pairs of 2327, 2258, and 958 and the ^{32}P-labeled probe hybridizes with 2327 and 2258 bands. As shown in Figure 10, clone 7 DNA digested with BamHI (lanes 1 and 5) showed two DNA bands of 4647 and 896 base pairs same as BamHI digested pSV2-uPA (lanes 9, 11, and 13). Again clone 7 DNA digested with PstI (lanes 2 and 6) showed one combined band of 2327 and 2258 base pairs same as PstI digested pSV2-uPA (lanes 10, 12, and 14). Therefore clone 7 demonstrates the restriction endonuclease digested bands identical to those of pSV2-uPA while the untransfected cells (lanes 3, 4, 7, and 8) show the absence of these bands.

The DNA copy numbers of human preprourokinase cDNA in clone 7 were estimated from a densitometer scan of autoradiographs by comparing the amounts of hybridization of a ^{32}P-labeled probe to the genomic DNA of clone 7 cells (lanes 2 and 6) with known amounts of pSV2-uPA plasmids (lanes 10, 12, and 14). Figure 11 demonstrates the densitometer scan of lanes 2 (peak A), 6 (peak B+C), 10 (peak D), 12 (peak E), and 14 (peak F) of Figure 10

Figure 11.   Densitometer scan of autoradiographs of
^{32}P hybridized to the genomic DNA of clone 7 or
pSV2-uPA plasmid DNA

The DNA copy numbers of human preprourokinase
cDNA in clone 7 were estimated from a densitometer
scan of autoradiographs by comparing the amounts of
hybridization of a ^{32}-labeled probe to the genomic
DNA of clone 7 cells with known amounts of pSV2-uPA
plasmids.   Peak A (lane 2 of Figure 10) with peak
area of 12.9 represents DNA prepared from 7 x 10^6
cells, peak B+C (lane 6 of Figure 10) with peak area
of 7.12 represents DNA made from 3.5 X 10^6 cells.
Peaks D (lane 10 of Figure 10), E (lane 12 of Figure
10), and F (lane 14 of figure 10) which show peak
area of 15.0, 8.93, and 2.40 represent 10, 5, and 2
ng of pSV2-uPA DNA respectively.

ANALYSIS =
SAMPLE = 1
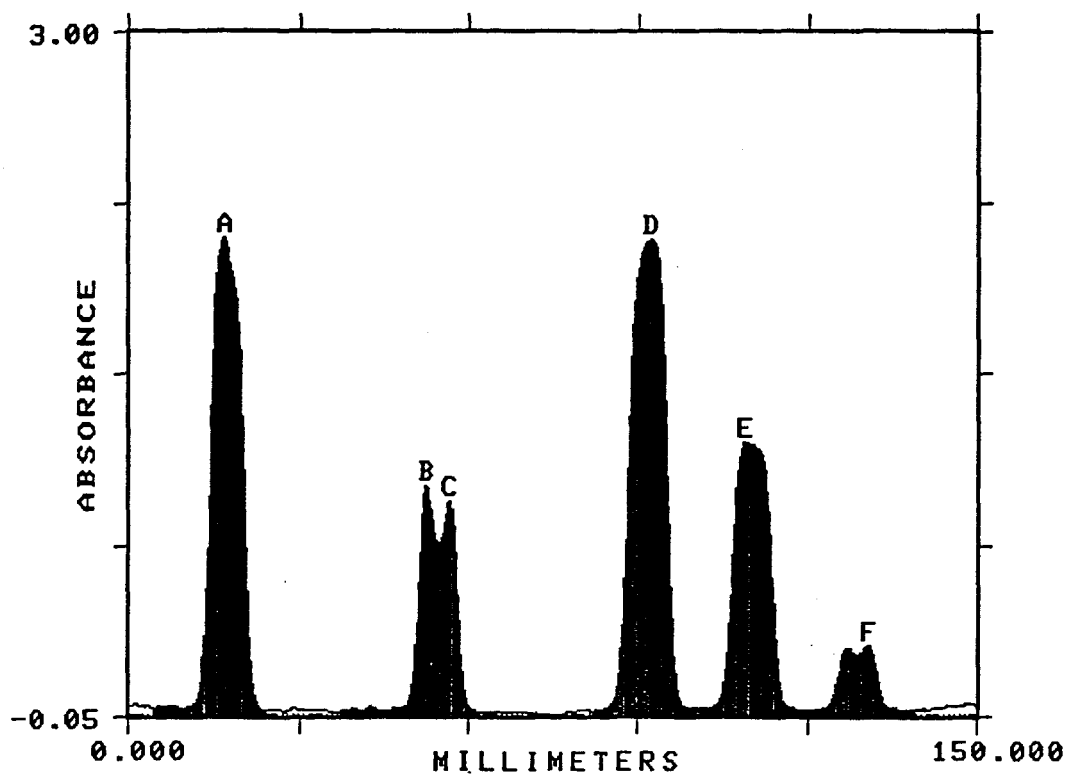
PEAK	LOCATION	PEAK MAX	PEAK AREA	%TOTAL AREA
A	16.76	2.0820	12.9362	27.90
B	52.45	0.9820	3.8075	8.21
C	56.64	0.9151	3.3140	7.15
D	92.33	2.0734	14.9871	32.32
E	108.84	1.1742	8.9285	19.26
F	130.56	0.2684	2.3954	5.17

showing peak areas of 12.9, 7.12, 15.0, 8.93, and 2.40

respectively.   Peak A and peak B+C represent DNAs prepared

from 7×10^6 cells and 3.5×10^6 cells of clone 7.   Peaks

D,E, and F represent 10 ng, 5 ng, and 2 ng of pSV2-uPA

respectively, and accordingly approximately 1×10^9, 5 x

10^8, 2×10^8 copies of pSV2-uPA.   By the comparison of

peak areas of A and B+C with D, E, and F, the copy numbers

of pSV2-uPA in clone 7 lanes were estimated.   The

estimated copy numbers were divided by the clone 7 cell

numbers from which the DNA was prepared to calculate the

copy numbers present per cell of clone 7.   The

calculations by the comparison of A with D, E, or F gave

copy numbers of 131, 123, or 168 respectively and by the

comparison of B+C with D, E, or F gave copy numbers of

149, 156, or 185.   On average, therefore, approximately

150 copies of pSV2-uPA were shown to be incorporated

within the genomic DNA of clone 7.

The expression of human preprourokinase mRNA was

determined by Northern blot hybridization assay as shown

in Figure 12.   The cytoplasmic RNAs from clones 7 and 10

of pSV2-uPA transfected cells, pSV2-neo transfected cells,

and untransfected B16-F1 cells were extracted, separated

by formaldehyde/agarose gel, transferred to filter, and

probed with ^{32}P-labeled human preprourokinase cDNA.   The

Figure 12. Northern blot hybridization analysis of
RNA isolated from clone 7 and clone 10 of pSV2-uPA
transfected cells, pSV2-neo transfected cell, and
B16-F1 untransfected cells

The cytoplasmic RNAs of B16-F1, B16-F1
transfected cells were extracted, separated by The
filter was hybridized with ^{32}P-labeled human
preprourokinase cDNA probe and exposed to X-ray
film. The 2.3 Kb band on the clone 7 and clone 10
lanes demonstrates human urokinase mRNA expression,
while this band is absent in B16-F1 or B16-F1 pSV2-
neo transfected cell RNA. For the internal
standard, ^{32}P-labeled Drosophila actin was used.
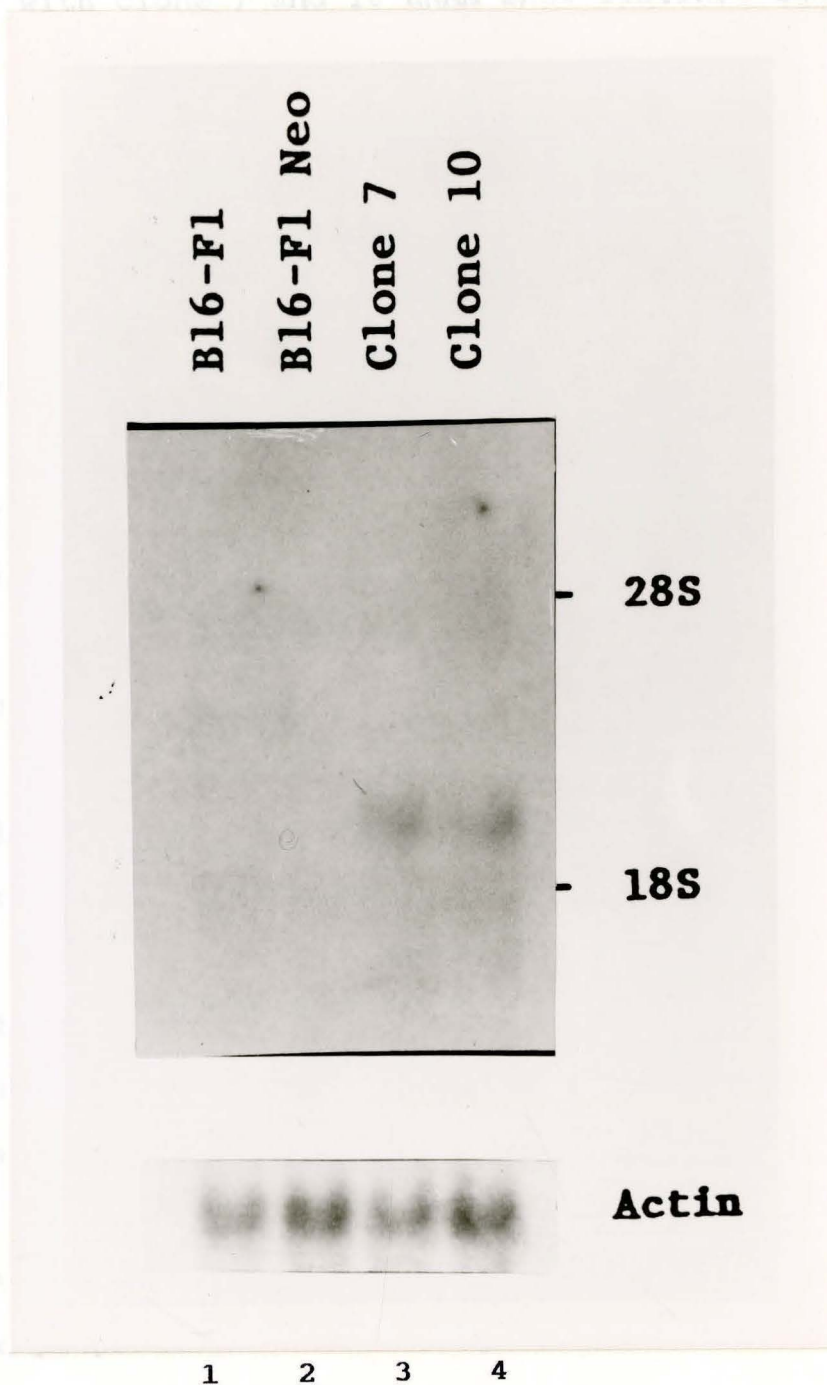
lanes with clone 7 and 10 RNAs show distinct bands which represent a 2.3 Kb mRNA of human preprourokinase gene while the lanes with B16-F1 untransfected cells and pSV2-neo transfected cells show absent of that band. The internal standard of ^{32}P-labeled _Drosophila_ actin shows that similar amounts of RNA were applied to each lane of the gel.

Figure 13 shows a densitometer scan of autoradiographs of a ^{32}P-labeled probe to the RNA of clone 7, 10 or pSV2-neo transfected cells. The densitometer scan of human urokinase RNA of clone 7 (lane 3 of Figure 12, peak A) and clone 10 (lane 4 of Figure 12, peak B) gave the peak area of 1.71 and 1.88 respectively. The densitometer scan of _Drosophila_ actin RNA of lane 3 of Figure 12 (peak D+E) and lane 4 of Figure 12 (peak F+G) gave the peak area of 0.97 and 1.27 respectively. The relative amount of human preprourokinase mRNA expression in clone 7 and 10 was estimated by calculating the ratio of peak areas of human urokinase to actin. The ratio of peak A/peak D+E (1.71/0.97) and peak b/peaks F+G (1.88/1.27) gave the value of 1.76 and 1.48 which indicate human preprourokinase mRNA expression of clone 7 and clone 10 respectively. The ELISA data (Table 1) showed C7 and C10 secreted human urokinase activity with absorbance

Figure 13. Densitometer scan of autoradiographs of
^{32}P hybridized to the RNA of clone 7, 10, or pSV2-
neo transfected cells

The relative amount of RNA of human
preprourokinase in clone 7 and clone 10 were
estimated from a densitometer scan of
autoradiographs by comparing the amounts of
hybridization of a ^{32}P-labeled probe to the internal
standard of _Drosophila_ actin RNA. In the top
figure, peak A (lane 3 of Figure 12) with peak area
of 1.71 represents human urokinase RNA prepared from
clone 7 cells, peak B (lane 4 of Figure 12) with
peak area of 1.88 represents human urokinase RNA
made from clone 10 cells. In the bottom figure,
peaks D+E (lane 3 of Figure 12), F+G (lane 4 of
Figure 12) which show peak area of 0.97 and 1.27
represent _Drosophila_ actin.

ANALYSIS =

SAMPLE = 1
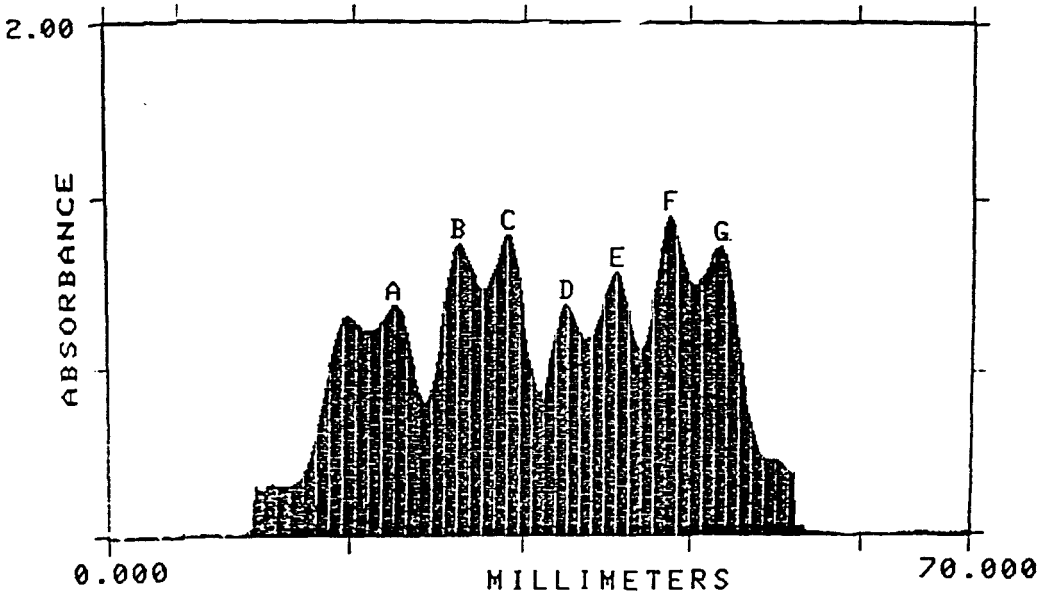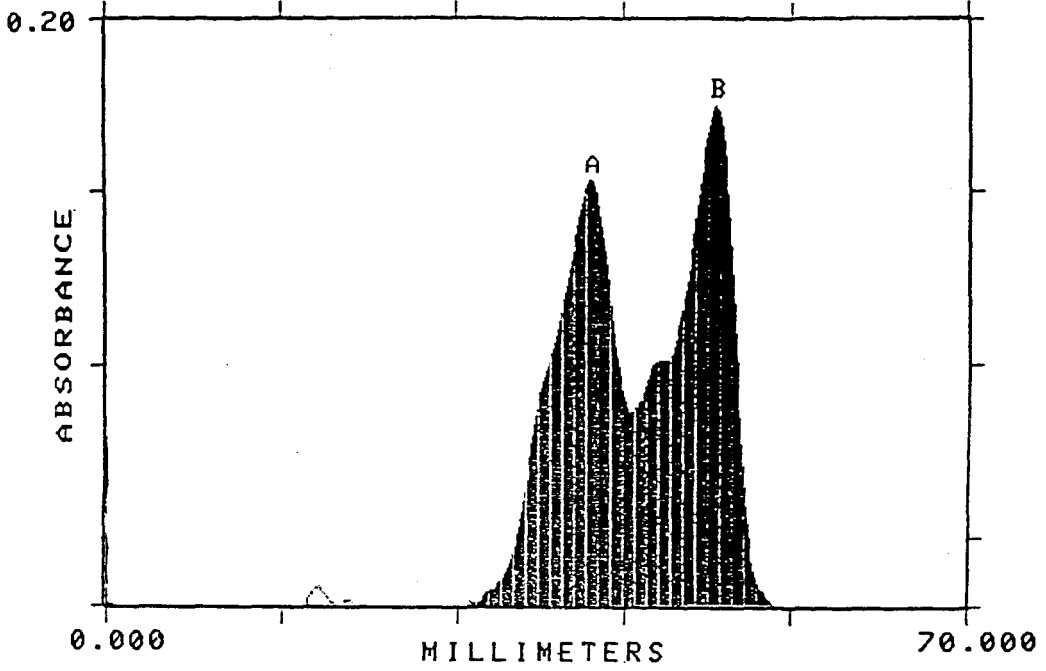
value of 4.24 and 3.43 respectively. The ratio of human
urokinase versus mRNA was calculated to be 2.41
(4.24/1.76) for C7 and 2.32 (3.43/1.48) for C10. The
ratio of mRNA expression and human urokinase secretion
between C7 and C10 is shown to be similar: 1.19
(1.76/1.48) for mRNA and 1.24 (4.24/3.43) for urokinase.

Characterization of Human Urokinase produced by Murine Cells

Human urokinase is a glycoprotein (Holmes et al.,
1985) whereas the murine urokinase lacks glycosylation
sequences and is not glycosylated (Belin et al., 1985).
Since the human urokinase is synthesized by transfected
murine cells, the possible glycosylation of the human
urokinase was tested by Concanavalin A column
chromatography, which tightly binds polysaccharides and
glycoproteins containing alpha-D-mannopyranosyl, alpha-D-
glucopyranosyl and sterically related residues (Narasimha
et al, 1979). Tightly bound glycoproteins can be eluted
by the addition of alpha-methyl-D-mannoside to the buffer
(Aspberg and Porath, 1970). The added alpha-methyl-D-
mannoside will compete for the carbohydrate binding sites
on the ConA, and cause the dissociation of any bound

glycoproteins. The column eluents before and after the addition of _alpha_-methyl-D-mannoside to the elution buffer were collected and assayed for the human urokinase by ELISA with specific monoclonal antibody to human urokinase. The results in Figure 14 show that human urinary urokinase (purchased from American Diagnostica, Inc.) contained a non-binding form of urokinase that was eluted prior to the _alpha_-methyl-D-mannoside wash, and a second peak eluted with _alpha_-methyl-D-mannoside buffer which corresponds to a glycosylated urokinase. The first unglycosylated urokinase peak might be generated from the urokinase from which carbohydrate moiety was degraded during the protein purification procedures or storage. The pSV2-uPA transfected murine B16-F1 clones produced only one human urokinase peak which was eluted with _alpha_-methyl-D-mannoside and coincided with the glycosylated urokinase peak observed in the human urokinase preparation. This result demonstrates that human urokinase produced by the transfected murine B16-F1 cells is glycosylated.

Specific urokinase receptors have been described in several cell lines (Vassalli _et al._, 1985; Stoppelli _et al._, 1985) and receptor binding has been shown to be species specific (Huarte _et al._, 1987; Estreicher _et al._,

Figure 14.   Concanavalin A affinity chromatography
of human urokinase from commercially purified
preparation (A) and from pSV2-uPA transfected B16-F1
conditioned medium (B)

Commercial human urokinase (A) or the serum-
free conditioned medium collected from clone 7 of
pSV2-uPA transfected B16-F1 cells (B) was applied to
the Con A column.   The columns were washed with Tris
buffer and then same buffer containing alpha-methyl-
D-mannoside (arrow).   Each fraction was collected
and assayed for human urokinase by the ELISA using
monoclonal antibody to human urokinase.
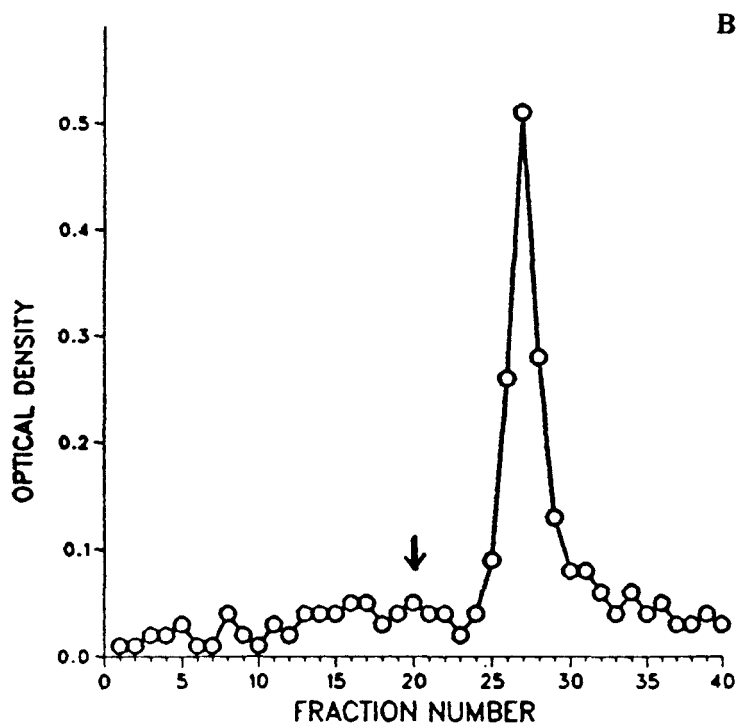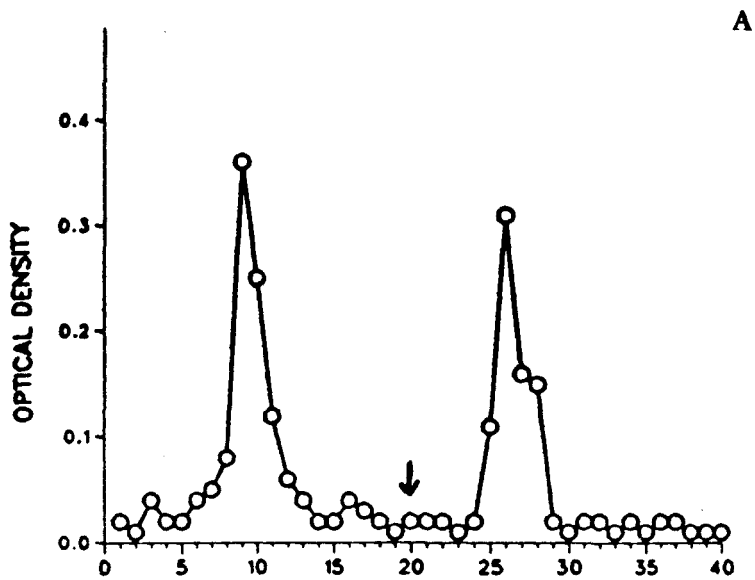
1989). Since human urokinase is produced by the transfected murine cells, the ability of human urokinase to bind to murine cell surface receptors was studied by a competitive binding assay. Cells were treated with mild acid to unmask cell surface receptor sites and assayed for the receptor binding with ^{125}I labeled human urokinase in the presence of different concentrations of unlabeled human urokinase. The results (Figure 15) showed that the human urokinase binds to a human cell line PC-3-Met (Kozlowski et al., 1984), derived from a human prostate carcinoma. In contrast, the human urokinase showed no specific affinity to the cell surface of the murine B16-F1 cells. This result demonstrates that the human urokinase does not bind to the cell surface of murine B16-F1 cells.

Determination of Urokinase Activity

It was of interest to measure the increase in plasminogen activator activity, due to the transfection of pSV2-uPA, over the endogenous murine plasminogen activator activity which is normally produced by B16-F1 cells. The activity was determined with a chromogenic substrate for plasmin which is generated from plasminogen by the urokinase secreted from the transfected cells. Figure 16

Figure 15.   Specific binding of human urokinase to human PC3-Met and murine B16-F1 cells

Binding of human urokinase (uPA) to the human PC3-Met cell surface (○) and the murine B16-F1 cell surface (☐) was determined by receptor binding assay.   Cells were treated with mild acid to unmask the receptors on the cell surface.   PC3-Met and B16-F1 cells were incubated with ^{125}I-labeled human urokinase and different concentration of unlabeled human urokinase.   Cells were washed with PBS/BSA and lysed with detergent.   Lysed cells were counted in a gamma-counter.   Nonspecific binding was determined in the presence of excess (50 nM) unlabeled human urokinase and subtracted from the each experimental value.   Data are expressed as the mean of triplicate determinations and standard deviations of the mean. The human urokinase appears to specifically bind only to the human PC3-Met cells, but not murine B16-F1 cells.
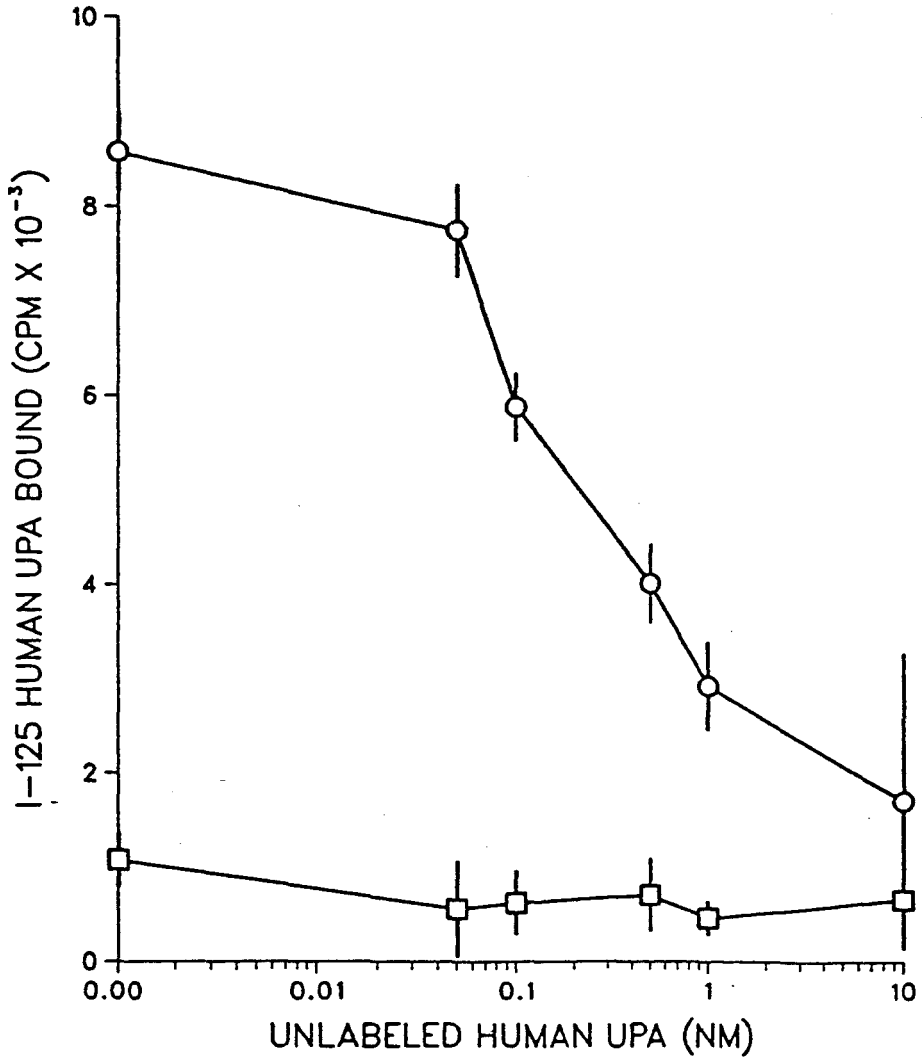
Figure 16. Secreted plasminogen activator activity
of B16-F1 and pSV2-uPA transfected B16-F1 cells as
determined against a chromogenic substrate

B16-F1, B16-F10 or transfected B16-F1 cell
conditioned media were incubated with the plasmin
specific chromogenic substrate S-2251 (H-D-Val-Leu-
Lys-p-nitroanilide) and DFP treated plasminogen.
After the incubation, the p-nitroaniline absorbance,
corresponding to substrate hydrolysis by plasmin
which was activated from plasminogen by urokinase
produced from conditioned media, was measured.
Nonspecific activity was measured in the absence of
cell conditioned media and subtracted from the
experimental value. Plasminogen activator activity
was expressed in pg secreted to conditioned medium
per cell per hour.
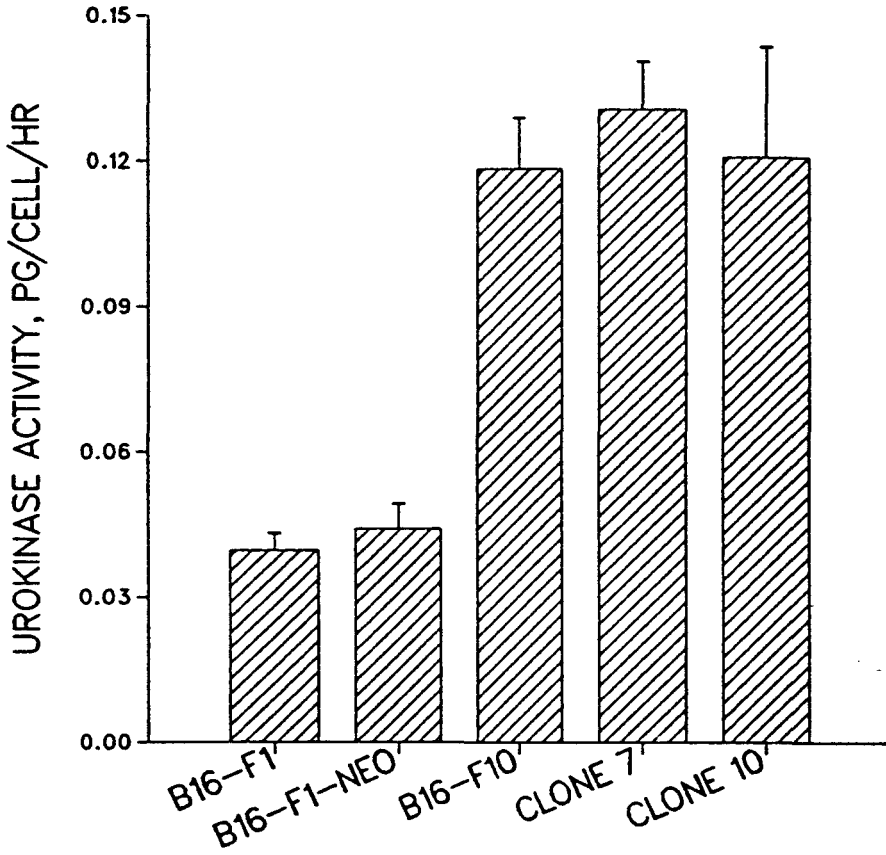
and Table 2 shows an increase in secreted plasminogen
activator activity of 3 to 4-fold in clones 7 and 10 over
cells transfected with pSV2-neo alone or untransfected
B16-F1 cells.   Three to four fold increase in clone 7 and
10 raises the plasminogen activator activity to the level
present in the more highly metastatic B16-F10 cell line.
The result also shows that transfection of pSV2-neo did
not affect urokinase production in B16-F1 cells.   Most of
cells used for the assay were passaged five to seven
times.

The results of the synthetic substrate assay were
confirmed by ^{125}I-fibrin assay for plasmin (Figure 17 and
Table 3).   In this assay, the plasminogen activator
activity was determined by measuring degradation of fibrin
those shown in Figure 16 and Table 2 were obtained by
^{125}I-fibrin plate assay.

From the ELISA data of Table 1, two clones (clones
9 and 12) that produce the least amount of human urokinase
and two clones (clones 8 and 18) that produce the highest
amount of human urokinase (excluding clone 7 and 10) were
selected for further study.   The plasminogen activator
activity was determined with a chromogenic substrate for
plasmin for the clones 8, 9, 12, and 18 and compared to
that of clone 7 or pSV2-neo transfected B16-F1 cells.   The

Table 2. Quantification of secreted plasminogen activator (PA) activity of B16-F1, B16-F10, and transfected B16-F1 cells by chromogenic substrate assay[a]

Cell	Secreted PA activity[b] (Mean ± SD)
B16-F1	3.90 ± 0.36[c,d]
B16-F1 (pSV2-neo)	4.42 ± 0.54[c,e,f]
B16-F10	11.9 ± 1.06[d]
Clone 7 (pSV2-uPA)	13.1 ± 0.98[e,g]
Clone 10 (pSV2-uPA)	12.1 ± 2.29[f,g]

[a]Cell conditioned media collected from B16-F1, B16-F10, and B16-F1 transfected cells with pSV2-neo or pSV2-uPA (clone 7 and clone 10) were incubated for 16 hours with the plasmin specific chromogenic substrate S-2251 (H-D-Val-Leu-Lys-p-nitroanilide) and DFP treated plasminogen. After the incubation, the p-nitroaniline absorbance, corresponding to substrate hydrolysis by plasmin, was measured.

[b]Plasminogen activator activity is expressed in 1×10^{-2} pg secreted to conditioned medium per cell per hour.

[c]Not significantly different ($p = 0.11$)

[d]Significantly different ($p = 0.000$)

[e]Significantly different ($p = 0.000$)
[f]Significantly different ($p = 0.000$)

[g]Not significantly different ($p = 0.25$)

Figure 17. Secreted plasminogen activator activity
of B16-F1 and pSV2-uPA transfected B16-F1 cells as
determined by ^{125}I-fibrin plate assay

Conditioned media from B16-F1, B16-F1 pSV2-neo
transfected, B16-F10, clone 7 and clone 10 of B16-F1
pSV2-uPA transfected cells were incubated with DFP-
treated plasminogen in ^{125}I-fibrin plate. The
ability of plasmin, which was activated from
plasminogen by the urokinase produced from
conditioned media, was measured by the released
soluble ^{125}I-fibrin degradation product.
Nonspecific activity was measured in the absence of
cell conditioned media and subtracted from the
experimental value. Plasminogen activator activity
was expressed in pg secreted to conditioned medium
per cell per hour.
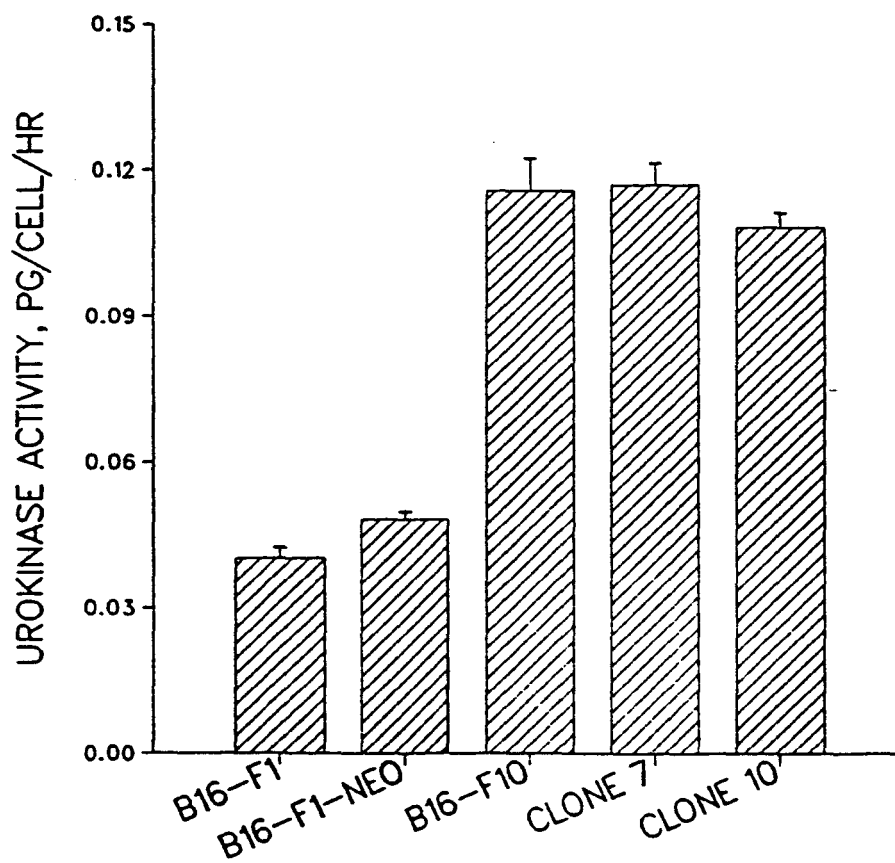
Table 3. Quantification of secreted plasminogen activator (PA) activity of B16-F1, B16-F10, and transfected B16-F1 cells as determined by ^{125}I-fibrin plate assay[a]

Cell	Secreted PA activity[b] (Mean $\pm$ SD)
B16-F1	4.02 ± 0.24[c,d]
B16-F1 (pSV2-neo)	4.81 ± 0.18[c,e,f,]
B16-F10	11.6 ± 0.67[d]
Clone 7 (pSV2-uPA)	11.7 ± 0.45[e,g]
Clone 10 (pSV2-uPA)	10.8 ± 0.39[f,g]

[a]Cell conditioned media collected from B16-F1, B16-F10, and B16-F1 transfected cells with pSV2-neo or pSV2-uPA (clone 7 and clone 10) were incubated with DFP-treated plasminogen in ^{125}I-fibrin plate. The ability of plasmin, which was activated from plasminogen by the plasminogen activator produced from conditioned media, was measured by the released soluble ^{125}I-fibrin degradation product.

[b]Plasminogen activator activity is expressed in 1×10^{-2} pg secreted to conditioned medium per cell per hour.

[c]Not significantly different (p = 0.30)

[d]Significantly different (p = 0.000)

[e]Significantly different (p = 0.000)

[f]Significantly different (p = 0.000)

[g]Not significantly different (p = 0.52)

results in Table 4 show clones 8 and 18 secreted the same amount of plasminogen activator activity as clone 7. Clones 9 and 12 also secreted the same amount of plasminogen activator activity as pSV2-neo transfected cells. The difference in enzyme activity between clones 8 and 18 and clones 9 and 12 indicates that the increased secretion of plasminogen activator activity of clones 7 and 10 is due to the human urokinase expression of the transfected clones.

To test the stability of the urokinase gene expression, the chromogenic assay was performed with the to soluble peptides by plasmin which was activated from plasminogen by the secreted urokinase. Results similar to conditioned medium collected from the C7 passaged more than 20 times in cell culture. The urokinase activity from that particular sample was similar to those of early passaged cells (data not shown).

In vivo Metastasis Assay

This assay assesses the effect of the secreted human urokinase from B16-F1 pSV2-uPA transfected cells on lung colonization, which measures tumor cell extravasation from the blood and then growth into pulmonary tumors.

Table 4. Quantification of secreted plasminogen activator (PA) activity of pSV2-uPA transfected B16-F1 cells (clones 7, 8, 9, 12 and 18) by chromogenic substrate assay[a]

Cell	Secreted PA activity[b] (Mean $\pm$ SD)
Clone 7	6.42 ± 1.61[c,d,e,f]
Clone 8	6.06 ± 0.60[c,d,e]
Clone 9	2.90 ± 0.22[g]
Clone 12	2.89 ± 0.24[h]
Clone 18	6.02 ± 0.75[c,d,f]
B16-F1	2.49 ± 0.59[c]
B16-F1 (pSV2-neo)	2.84 ± 0.46[g,h]

[a]Cell conditioned media collected from 1×10^6 B16-F1 transfected cells with pSV2-neo or pSV2-uPA (clone 7, 8, 9, 12 and 18) for 24 hours in 10 mm petri dishes were incubated for 16 hours with the plasmin specific chromogenic substrate S-2251 and DFP treated plasminogen. After the incubation, the p-nitroaniline absorbance, corresponding to substrate hydrolysis by plasmin, was measured.

[b]Plasminogen activator activity is expressed in 1×10^{-2} pg secreted to conditioned medium per cell per hour.

[c]Clones 7, 8, and 18 are different from B16-F1 at $p = 0.000$.

[d]Clones 7, 8, and 18 are different from B16-F1 (pSV2-neo) at $p = 0.000$.

[e]Not significantly different ($p = 0.35$)

[f]Not significantly different ($p = 0.41$)

[g]Significantly different ($p = 0.009$)

[h]Significantly different ($p = 0.015$)

C57BL/6 mice were tail vein injected with 100,000 tumor cells from clones 7, clone 10, or control cells. The control cells transfected with pSV2-neo only, were made up of a mixture of cells from randomly selected pSV2-neo transfected clones, so as not to bias the controls in any one direction. Cells injected into the animal had been passaged 4 to 5 times in cell culture following the transfection. Thirteen mice each, were used for each control or experimental group, in every experiment. Attention was given to the cell culture conditions, such as confluency of the cells, and number of passages which were kept identical for both control and experimental cells in each experiment. To assess the metastatic potential of transfected cells, the number of lung tumors which appeared on the lung surface were counted under the dissecting microscope. An earlier study compared the results of microscopic examination of lung section to the observation of lung surface tumors and found a close correlation between the two results (Wexler, 1966).

Table 5 compares the number of metastatic tumors formed in the lungs of C57BL/6 mice. The pSV2-neo transfected cell mixture had a similar lung colonization ability to that previously reported for untransfected B16-F1 cells (Fidler, 1983). Thus transfection alone does not

Table 5. Lung Colonization by B16-F1 Murine Melanoma
Cells Transfected with Human Preprourokinase cDNA in a
SV40 Expression Vector[a]

Vector	Number of Tumors	Mean $\pm$ S.E.
Experiment 1		
pSV2-neo (control)	1,1,2,3,3,3,4,4,4,4,9, 11,15	4.92 $\pm$ 1.16[b]
pSV2-neo & pSV2-uPA (Clone 7)	26,55,58,59,60,84,87,92, 99,102,112,137,172	87.9 $\pm$ 10.7[b]
Experiment 2		
pSV2-neo (control)	2,3,7,7,10,10,14,14,16, 21	10.5 $\pm$ 1.88[b]
pSV2-neo & pSV2-uPA (Clone 10)	8,25,27,28,28,30,31,39, 45,58,59,96	39.5 $\pm$ 6.59[b]

[a]Animals were injected i.v. in the tail vein with 100,000
B16-F1 transfected cells. Mice were killed 17 days after
the injection and the number of lung metastatic foci was
counted.

[b]Different at $p < 0.001$ by both the Mann Whitney U-Test
(nonparametric rank sum test) and Student t-test.

appear to significantly increase the metastatic potential of the B16-F1 cells. However, both clones 7 and 10, which were selected for their high secretion of human urokinase, showed a dramatic and significant ($p < 0.001$) increase in lung colonization ability compared to controls.

To confirm our finding that the increased plasminogen activator activity due to the production of urokinase enhanced metastatic potential for clones 7 and 10, clones 8 and 12 were also tested for lung colonization. Clones 8 and 12 were selected for their high and low secretion of human urokinase respectively even though they were transfected with the same vectors, pSV2-uPA and pSV2-neo. The results in Table 6 compare the number of metastatic tumors formed by clones 8 and 12. Clone 8 showed a significant ($p = 0.0019$) increase in lung colonization ability compared to clone 12.

Figure 18 shows typical lungs dissected from control mice which were injected with pSV2-neo transfected B16-F1 cells and experimental mice which received tumor cells from clone 7. Tumors from experimental mice are shown to be mixture of amelanotic and melanotic while tumors from control mice are mainly melanotic. All tumors from the control mice and experimental mice were divided into melanotic or amelanotic groups and the number of

Table 6. Lung Colonization by B16-F1 cells transfected with pSV2-uPA and pSV2-neo (clone 8 and 12)[a]

Vector	Number of Tumors	Mean ± S.E.
pSV2-neo & pSV2-uPA (clone 12)	12,34,46,74,94,98,101, 132,144,151	88.6 ± 14.9[b]
pSV2-neo & pSV2-uPA (Clone 8)	119,128,142,181,185, >300,>300,>300	206.9 ± 28.5[b]

[a]Animals were injected i.v. in the tail vein with 100,000 B16-F1 transfected cells. Mice were killed 17 days after the injection and the number of lung metastatic foci was counted.

[b]Different at p = 0.0019 by Student t-test and p < 0.01 by the Mann Whitney U-Test (nonparametric rank sum test).

Figure 18. Gross appearance of typical lungs
dissected from control (left) and experimental
(right) mice

Control mice were injected with 10^5 B16-F1
cells transfected with pSV2-neo and experimental
mice received 10^5 clone 7 cells transfected with the
pSV2-neo and pSV2-uPA plasmids.
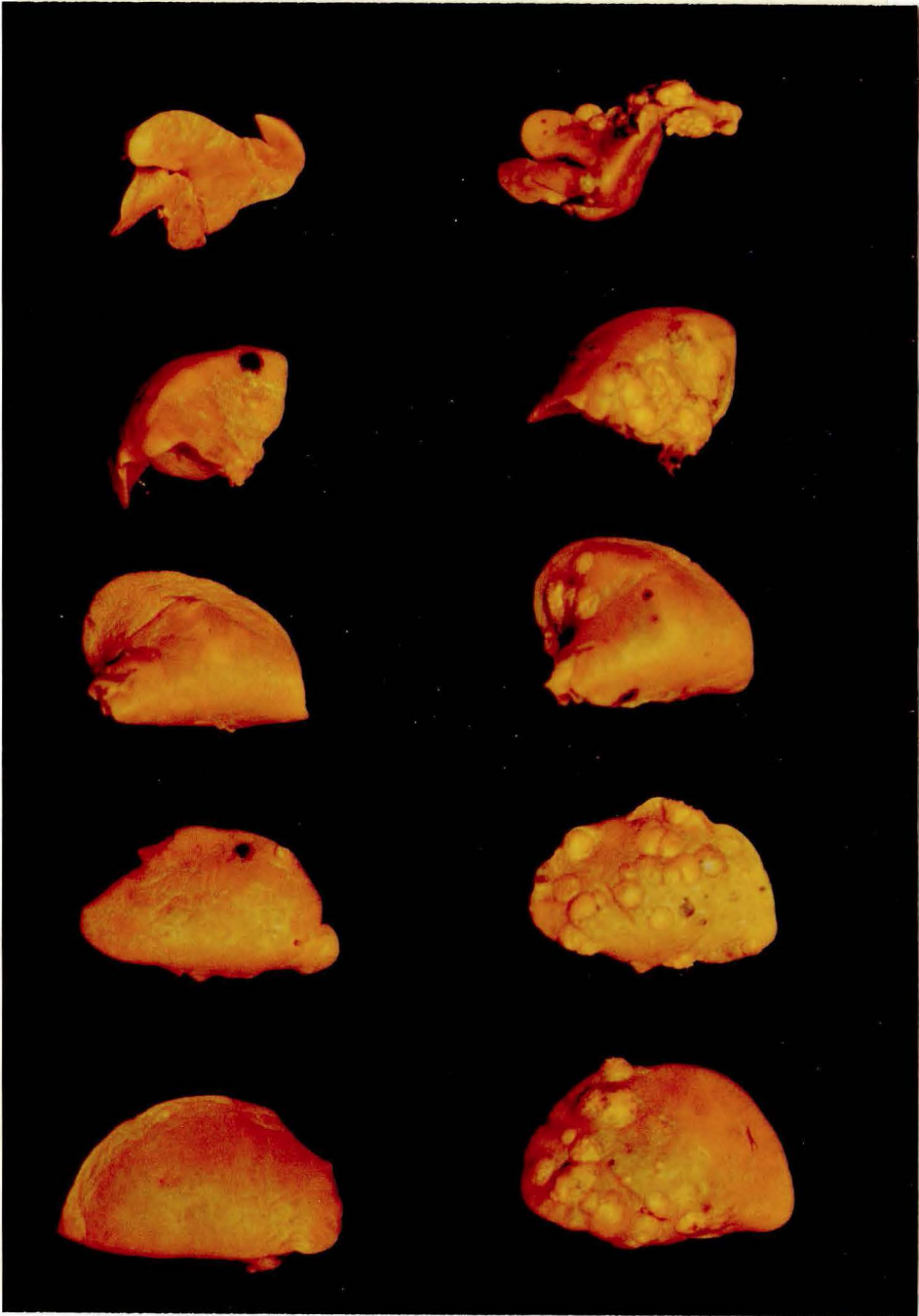
tumors in each group were counted. Table 7 shows the percent of tumors in the two different color groups. It was observed that high urokinase secreting clones (clones 7, 10, and 8) produced predominantly amelanotic tumors while pSV2-neo transfected clones or low urokinase secreting clone (clone 18) produced mainly melanotic tumors.

Cytotoxicity assay was performed to test if murine cells that produce human urokinase might generate any immunologic response in C57BL/6 mice. The ^{51}Cr release result shows no indication of immunological response against the human urokinase produced by transfected murine cells (data not shown). (Assay was carried out by the clinical laboratory staff in Dr. John Clancy's lab., Department of Anatomy).

Spontaneous metastasis from foot pad tumor formed by clone 7 cells was assayed to study the effect of secreted urokinase on the full metastatic process. Table 8 shows the number of pulmonary tumors formed in the spontaneous metastasis experiment from primary tumors grown in the hind leg foot pad of C57BL mice. Clone 7 cells demonstrated an absolute ability to metastasize (all experimental mice showed spontaneous metastasis), while foot pad tumors produced by control B16-F1 pSV2-neo

Table 7. Color distribution of metastatic tumors in murine lung from mice tail-vein injected with B16-F1 transfected cells[a]

| Cells Injected | Percentage of tumors in color category | |
| | Melanotic | Amelanotic |
	(Mean ± S.D.)	
pSV2-neo (control)	97.1 ± 6.98	2.91 ± 6.98
pSV2-neo & pSV2-uPA (C7)	29.4 ± 6.85	70.6 ± 6.81
pSV2-neo (control)	93.1 ± 8.57	6.46 ± 8.80
pSV2-neo & pSV2-uPA (C10)	37.7 ± 8.79	62.5 ± 9.17
pSV2-neo & pSV2-uPA (C12)	91.0 ± 4.84	9.83 ± 4.86
pSV2-neo & pSV2-uPA (C8)	7.66 ± 4.26	92.2 ± 4.49

[a]Color of the lung tumors from control and experimental groups were divided into two groups, melanotic and amelanotic and tumor numbers were counted. Results are expressed in percent ± S.E. of tumor numbers in the different color groups.

Table 8. Spontaneous pulmonary metastasis by B16-F1
Murine Melanoma Cells Transfected with Human
Preprourokinase cDNA in a SV40 Expression Vector[a]

vector	number of colonies	mean ± S.E.
pSV2-neo (control)	0,0,0,0,0,0,0,0,0,2,11	1.18 ± 1.00[b]
pSV2-neo + pSV2-uPA (Clone 7)	1,1,2,2,3,3,3,7,11,23, 26,33	9.58 ± 3.26[b]

[a]Mice were injected subcutaneously into a footpad with 100,000 cells of transfected cells. Three weeks later, tumor-bearing foot was amputated. Seven weeks after the injection, animals were killed and the number of lung metastatic foci were counted.

[b]Different at $p < 0.001$ by the Mann Whitney U-Test (nonparametric rank sum test) and at $p < 0.03$ by the Student t-test.

transfected cells showed a low ability to spontaneously metastasize (only 2 mice out of 11 showed pulmonary tumors).

Demonstration of Human Urokinase Activity in Murine Lung Tumors

To determine the presence of human urokinase produced by the pSV2-uPA transfected cells in murine lung metastatic tumors of clone 7, cells from the lung nodules were plated in vitro and assayed for the human urokinase by ELISA using monoclonal antibody. The ELISA results in Table 9 shows that clone 7 cells passaged through animal secreted similar amount of human urokinase as the original clone 7 cells. The plasminogen activator activity from both cells also was measured by chromogenic substrate assay. Table 10 shows that clone 7 cells, either primary or passaged through animal, secreted similar amounts of plasminogen activator activity. Taken together, these results indicate that the plasminogen activator activity detected in the lung nodules is contributed by the human urokinase from the pSV2-uPA transfected cells and that the phenotype of transfected cells was stable in vivo.

Table 9. Quantification of human urokinase production by B16-F1 transfected cells and the same cells passaged through animal (PTA) by ELISA[a]

Cell	Absorbance (Mean±SD)
B16-F1 (pSV2-neo)	0.01 ± 0.01[b,d,f]
B16-F1 (pSV2-neo) PTA[c]	0.01 ± 0.01[b,d,g]
B16-F1 (pSV2-uPA) C7	0.58 ± 0.05[e,f]
B16-F1 (pSV2-uPA) C7 PTA[c]	0.63 ± 0.06[e,g]

[a]The secretion of urokinase from each colony was quantitated by IMUCLONE uPA ELISA Kit (American Diagnostica Inc.) on conditioned media taken from the cells incubated in the serum free media for 40 hours.

[b]Value equal to zero as no human urokinase is produced by murine B16-F1 cells which was transfected with the pSV2-neo plasmid.

[c]The lung tumors removed from the mice which were injected with the transfected cells were plated in vitro and cultured for the assay.

[d]Not significantly different ($p = 0.49$)

[e]Not significantly different ($p = 0.30$)

[f]Significantly different ($p = 0.000$)

[g]Significantly different ($p = 0.000$)

Table 10. Quantification of secreted plasminogen activator (PA) activity of transfected cells and the cells passaged through animal (PTA) by chromogenic substrate assay[a]

Cell	Secreted PA activity[b] (Mean ± SD)
B16-F1 (pSV2-neo)	$2.49 \pm 0.60^{d,f}$
B16-F1 (pSV2-neo) PTA[c]	$2.84 \pm 0.46^{d,g}$
Clone 7 (pSV2-uPA)	$6.42 \pm 1.61^{d,e,f}$
Clone 7 (pSV2-uPA) PTA[c]	$6.71 \pm 1.73^{d,e,g}$

[a]Cell conditioned media collected from 1×10^6 B16-F1 pSV2-neo, B16-F1 pSV2-neo and pSV2-uPA (clone 7) transfected cells, and the same cells passaged through animal (PTA) for 24 hours in 10 mm petri dishes were incubated for 16 hours with chromogenic substrate S-2251 and DFP treated plasminogen. After the incubation, the p-nitroaniline absorbance, corresponding to substrate hydrolysis by plasmin, was measured.

[b]Plasminogen activator activity is expressed in 1×10^{-2} pg secreted to conditioned medium per cell per hour.

[c]The lung tumors removed from the mice which were injected with the above cells were plated _in vitro_ and cultured for the assay.

[d]Not significantly different (p = 0.29)

[e]Not significantly different (p = 0.67)

[f]Not significantly different (p = 0.90)

[g]Not significantly different (p = 0.85)

II. Inhibition of Urokinase Production in Murine B16-F10 Melanoma Cells and the Study of its Effect on Lung Colonization

Expression of Antisense Human Urokinase Gene

The plasmid pSV2-ASuPA containing 5' end of 265-base-pair of preprourokinase sequence in an antisense direction with respect to SV40 early gene promoter was prepared from the the plasmid pSV2-dhfr and pRdzbuktt as shown in Figure 7. In the synthesis of pSV2-ASuPA, the ligation on HindIII sites of pSV2-dhfr digest and 265 base-pair of human urokinase cDNA automatically brings the pSV2-ASuPA in an antisense orientation. The final pSV2-ASuPA plasmid construction which is shown in Figure 8 was confirmed by restriction endonuclease digestion map.

Murine B16-F10 melanoma cells of high metastatic potential were similarly cotransfected with the plasmids pSV2-ASuPA and pSV2-neo in 10:1 ratio, glycerol shocked, and grown in the presence of Geneticin (G-418). The surviving colonies were assayed for the production of urokinase by a filter immunoblot assay using a polyclonal antibody to human urokinase as shown in Figure 19. It has been shown that polyclonal antibody to human urokinase

Figure 19. Filter immunoblot analysis of transfected B16-F10 cell colonies

B16-F10 cells were transfected with pSV2-neo (top) or cotransfected with pSV2-ASuPA and pSV2-neo (bottom). Transfected cells were cultured in MEM supplemented with 10% heat inactivated FBS containing Geneticin (G-418). Five weeks later, nitrocellulose membrane filters were placed over the cell colonies to absorb urokinase being secreted. The filters were treated with rabbit polyclonal antibody to human urokinase followed by goat anti-rabbit IgG conjugated with alkaline phosphatase, which were reacted with NBT/BCIP to give dark purple color development corresponding to urokinase presence. Fifteen colonies that show light purple color which indicates lower production of urokinase were selected for further study.
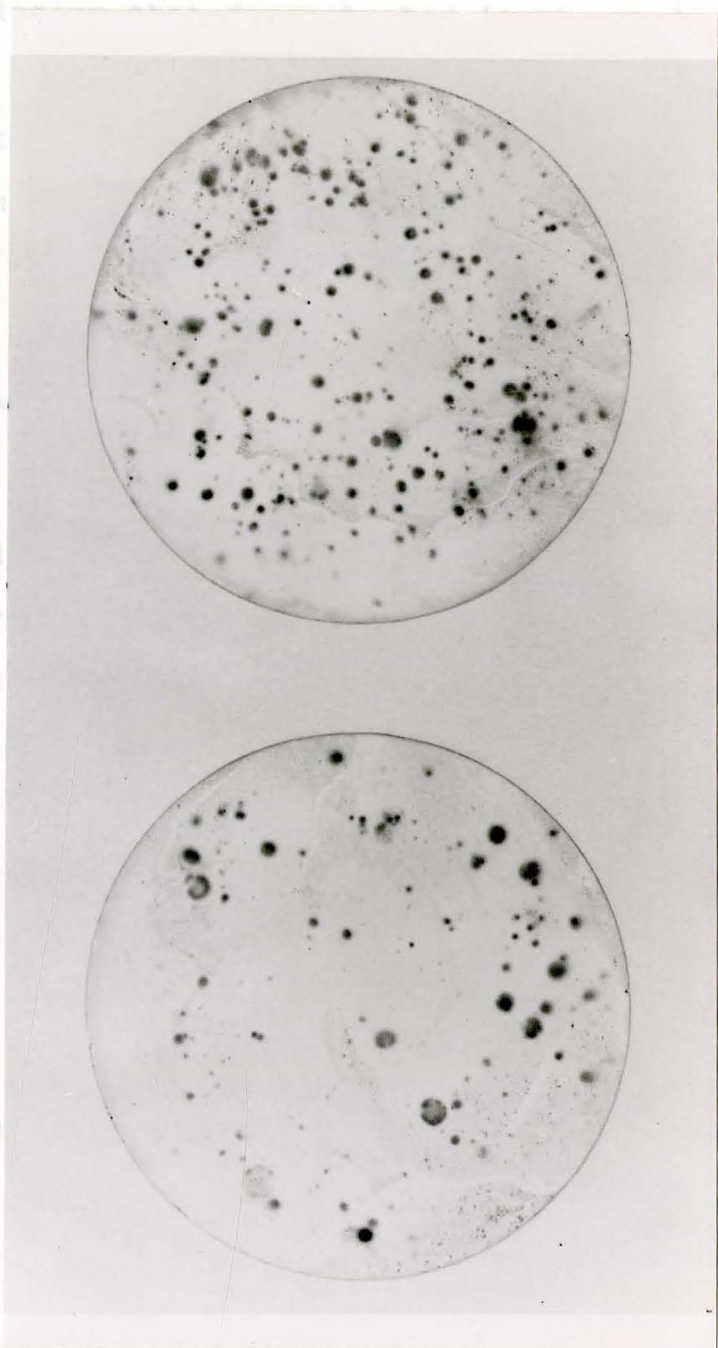
cross reacts with mouse urokinase (Ossowski, 1988).
Fifteen colonies that demonstrated light purple color
indicating decreased amounts of urokinase secretion were
selected, transferred to separate petri dishes, and
expanded in MEM supplemented with 10% heat inactivated
FBS.

Determination of Urokinase Production

The secretion of urokinase activity into the cell
culture medium from the fifteen antisense colonies
selected by filter immunoblot assay were quantitated by a
chromogenic substrate assay following the activation of
plasminogen to plasmin. Table 11 shows that antisense
clones 8, 10, 11, and 12 (AC8, AC10, AC11, and AC12)
secreted lower amounts of urokinase than other clones.
The secretion of urokinase activity of these four
antisense clones was confirmed by ^{125}I-fibrin plate assay
as shown in Figure 20 and Table 12. Of four antisense
clones, clone 10 (AC10) and clone 8 (AC8) secreted
approximately 70% of urokinase when compared to a mixed
population of B16-F10 or pSV2-neo transfected B16-F10
cells.

The incorporations of pSV2-ASuPA and pSV2-neo

Table 11. Quantification of secreted plasminogen
activator activity from B16-F10 transfected cells by
chromogenic substrate assay[a]

Clone No	Absorbance (Mean ± SD)	Clone No	Absorbance (Mean ± SD)
AC1	0.51 ± 0.03	AC9	0.58 ± 0.06
AC2	0.56 ± 0.04	AC10	0.36 ± 0.02
AC3	0.46 ± 0.02	AC11	0.46 ± 0.03
AC4	0.56 ± 0.03	AC12	0.45 ± 0.02
AC5	0.49 ± 0.01	AC13	0.68 ± 0.04
AC6	0.70 ± 0.04	AC14	0.61 ± 0.03
AC7	0.47 ± 0.02	AC15	0.59 ± 0.02
AC8	0.40 ± 0.03		
B16-F10	0.51 ± 0.02	B16-F10Neo	0.52 ± 0.03

[a]B16-F10 or transfected B16-F10 cell conditioned media
were incubated for 16 hours with the plasmin specific
chromogenic substrate S-2251 (H-D-Val-Leu-Lys-p-
nitroanilide) and DFP treated plasminogen. After the
incubation, the p-nitroaniline absorbance, corresponding
to substrate hydrolysis by plasmin, was measured.

Figure 20. Secreted plasminogen activator activity
of B16-F10 and pSV2-ASuPA transfected B16-F1 cells
determined by ^{125}I-fibrin plate assay

Conditioned media from B16-F10, B16-F10
transfected with pSV2-neo, antisense clones 8 (AC8),
10 (AC10), 11 (AC11), and 12 (AC12) of transfected
with pSV2-ASuPA were incubated with DFP-treated
plasminogen in ^{125}I-fibrin plate. The activity of
plasmin, which was activated from plasminogen by the
urokinase produced from conditioned media, was
measured by the released soluble ^{125}I-fibrin
degradation product. Nonspecific activity was
measured in the absence of cell conditioned media
and subtracted from the experimental value.
Plasminogen activator activity was expressed in pg
secreted to conditioned medium per cell per hour.
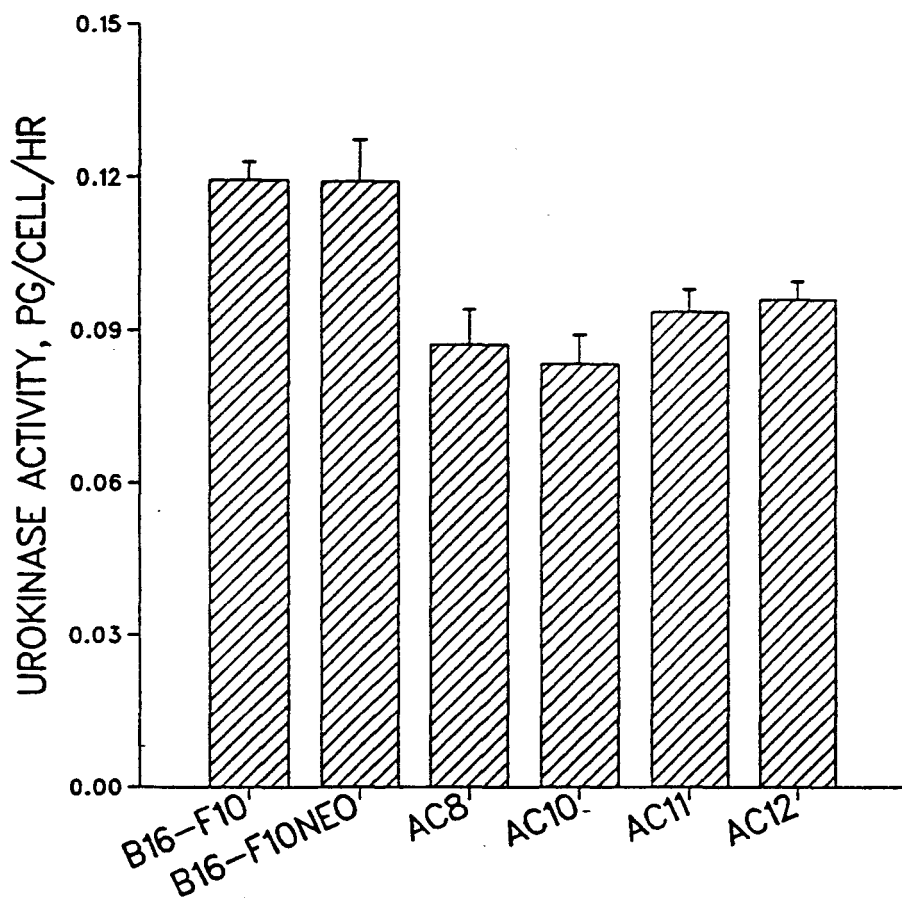
Table 12. Quantification of secreted plasminogen

activator (PA) activity of B16-F10 and transfected B16-F10

cells as determined by ^{125}I-fibrin plate assay[a]

Cell	Secreted PA activity[b] (Mean ± SD)
B16-F10	12.0 ± 0.37[e]
B16-F10 (pSV2-neo)	11.9 ± 0.83[e]
AC8 (pSV2-ASuPA)	8.33 ± 0.59[c,d]
AC10 (pSV2-ASuPA)	8.71 ± 0.71[c,d]
AC11 (pSV2-ASuPA)	9.34 ± 0.46[c,d]
AC12 (pSV2-ASuPA)	9.60 ± 0.37[c,d]

[a]Cell conditioned media collected from B16-F10 and pSV2-neo or pSV2-ASuPA transfected B16-F10 cells were incubated with DFP-treated plasminogen in ^{125}I-fibrin plate. The ability of plasmin, which was activated from plasminogen by the plasminogen activator produced from conditioned media, was measured by the released soluble ^{125}I-fibrin degradation product.

[b]Plasminogen activator activity is expressed in 1×10^{-2} pg secreted to conditioned medium per cell per hour.

[c]AC8, AC10, AC11, and AC12 are significantly different from B16-F10 at p = 0.008, 0.0022, 0.0016, and 0.0014 respectively.

[d]AC8, AC10, AC11, and AC12 are significantly different from B16-F10 (pSV2-neo) at p = 0.0037, 0.0031, 0.0095, and 0.0012 respectively.

[e]Not significantly different (p = 0.95)

plasmids into the B16-F10 cell genomic DNA of antisense

clones 10 and 8 were assayed by Southern blot

hybridization assay as shown in Figure 21. The DNAs were

prepared from the antisense clones 10 and 8 and pSV2-neo

transfected cells. To detect the pSV2-neo and pSV2-ASuPA

incorporation, DNAs were digested with PstI, separated by

electrophoresis, transferred to nitrocellulose filter, and

probed with ^{32}P-labeled HpaI-PstI pSV2-ASuPA fragment.

The digestion of pSV2-ASuPA with PstHI generates four DNA

bands with base pairs of 2418, 1430, 958, and 923 pairs

and ^{32}P-labeled probe hybridizes with all the band. The

digestion of pSV2-neo with PstI generates two DNA bands

with base pairs of 3540 and 958 and the probe hybridizes

with both bands. Figure 21 shows that antisense clones

AC10 and AC8 demonstrate the restriction endonuclease

digested bands identical to those of pSV2-ASuPA plasmid.

The densitomer scan of autoradiograghs (data not shown)

suggests less than 5 copy numbers of pSV2-ASuPA were

present in the transfected antisense clones 10 or 8 cells.

Lung Colonization Assay

To assess the effect of inhibition of urokinase

production on lung colonization, antisense clones 10 and 8

Figure 21. Southern blot hybridization analysis of
DNA isolated from antisense clones 10 (AC10) and 8
(AC8) of pSV2-ASuPA transfected cells, B16-F10, and
B16-F10 pSV2-neo transfected cells

    DNAs were isolated from antisense clone 10
(AC10) and 8 (AC8) of pSV2-ASuPA transfected B16-F10
cells, pSV2-neo transfected cells, or untransfected
B16-F10 cells. The DNAs (10 ug) were cleaved with
PstI and electrophoresed through 0.8% agarose gel.
A control pSV2-ASuPA plasmid preparation was
similarly digested with PstI and different amounts
were loaded onto the same gel. The DNAs were
transferred to nitrocellulose and probed with ^{32}P-
labeled HpaI-PstI fragment of pSV2-ASuPA. Lanes 1
and 2 contain DNA made from 7×10^6, and 3.5×10^6
cells AC10. Lanes 3 and 4 contain DNA made from $7 \times
10^6$ and 3.5×10^6 cells of AC8. Lane 5 contain DNAs
made from 7×10^6 cells of untransfected B16-F10 and
lane 6 from 3.5×10^6 of pSV2-neo transfected cells.
Lanes 7, 8, and 9 contain pSV2-ASuPA DNA of 10, 5, 2
ng respectively.
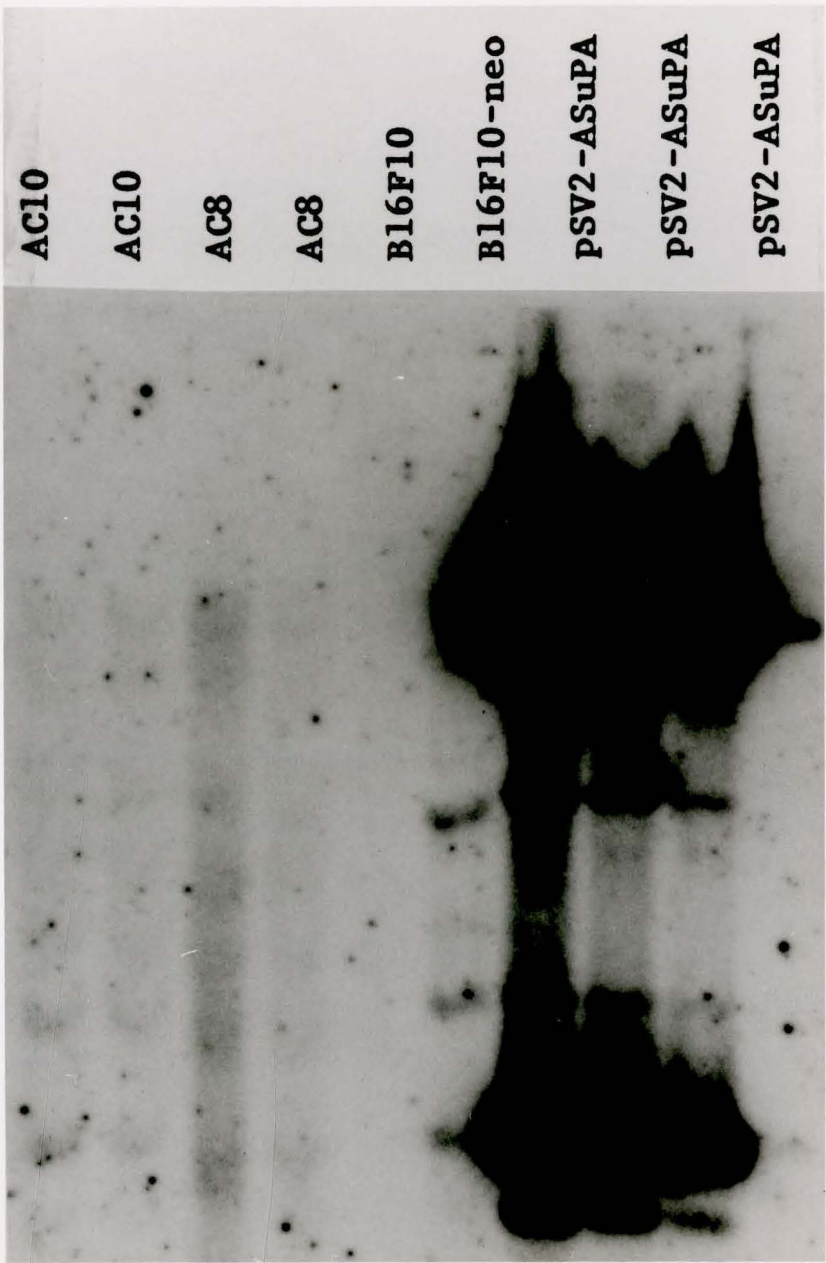
were injected to mice through tail vein. Table 13 shows the results of the lung colonization assay of antisense clones 10 and 8. AC10 and AC8 cells which produced approximately 70% of urokinase compared to B16-F10 cells showed a 40 to 42% decrease in the ability to form lung tumors (p = 0.0016 and 0.0019 respectively by Student's t test and p < 0.005 and p < 0.005 by the Mann Whitney U-test respectively. The pSV2-neo transfected B16-F10 cell mixture showed a similar lung colonization ability compared to untransfected B16-F10 cells (Fidler, 1983). Thus, as shown in B16-F1 cell transfection, transfection alone does not affect metastatic potential of the B16-F10 cells.

Figure 22 shows the gross appearance of typical lungs dissected from control mice (injected with pSV2-neo transfected cells) and experimental mice (injected with pSV2-neo and pSV2-ASuPA transfected cells). It was observed the majority of tumors from the control mice were smaller than that from the experimental mice. Thus, all the tumors from the control and experimental mice were grouped into three different sizes (small, medium, and large) and number of tumors in each size category were counted. Small size tumor indicates less than 0.2 mm, medium size between 0.2 mm and 0.7 mm, and large size more

Table 13. Lung colonization by B16-F10 murine melanoma cells transfected with human preprourokinase antisense gene in a SV40 expression vector[a]

Vector	Number of Tumors	Mean ± S.E.
Experiment 1		
pSV2-neo (control)	32,34,55,56,71,76,80,90 91,92,97,99	72.8 + 6.8[b]
pSV2-neo & pSV2-ASuPA (AC10)	27,28,28,29,33,34,37,40, 45,45,60,67,86	43.0 ± 4.9[b]
Experiment 2		
pSV2-neo (control)	116,136,148,155,189, 192,245,254,275,306,321	212.5 ± 21.5[c]
pSV2-neo & pSV2-ASuPA (AC8)	51,71,73,101,126,135, 150,152,156,166,173	123.1 ± 12.8[c]

[a]Animals were tail vein injected with 1×10^5 B16-F10 transfected cells. Mice were killed 17 days later and number of lung metastatic foci was counted.

[b]Different at p = 0.0016 by Student t-test and p < 0.01 by the Mann Whitney U-test (nonparametric rank sum test).

[c]Different at p = 0.0019 by Student t-test and p < 0.01 by the Mann Whitney U-test.

Figure 22. Gross appearance of typical lungs
dissected from control (left) and experimental
(right) mice

Control mice were injected with 10^5 B16-F10
cells transfected with pSV2-neo and experimental
mice received 10^5 antisense clone 10 (AC10) cells
transfected with the pSV2-neo and pSV2-ASuPA
plasmids.

than 0.7 mm in diameter. Figure 23 and Table 14 shows the size of metastatic tumors in lungs expressed in percent of tumor numbers in the different size categories. It was observed that more than 60% of tumors produced by experimental mice belong to large size category, while B16-F10 pSV2-neo transfected cells produce mainly small and medium size tumors.

Figure 23. Size of metastatic tumors in murine lung from mice tail-vein injected with B16-F10 transfected cells

      Size of the lung tumors from control and experimental mice were determined and divided into three categories, small (<0.2 mm in diameter), medium (0.2 mm-0.7 mm), and large (>0.7 mm). Results are expressed in percent of tumor numbers in the different size category.
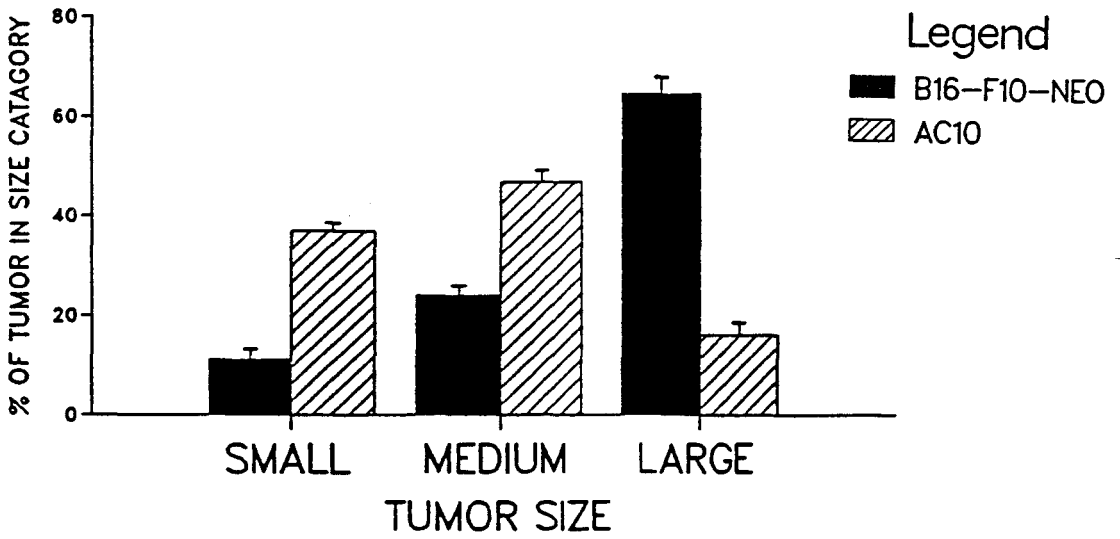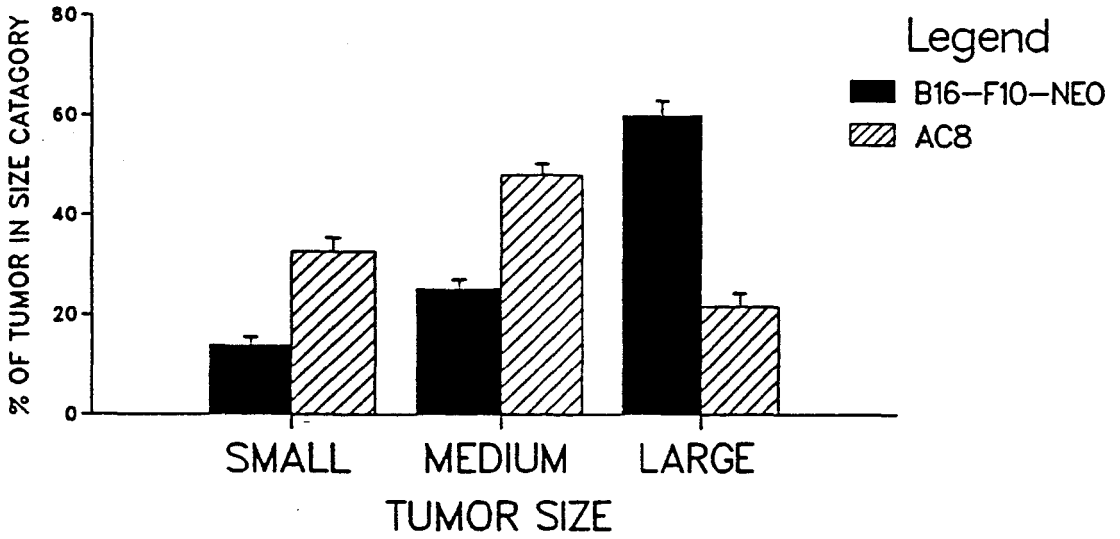
Table 14. Size of metastatic tumors in murine lung from mice tail-vein injected with B16-F10 transfected cells[a]

Cells Injected	Percentage of tumors in size category		
	Small	Medium (Mean ± S.E.)	Large
pSV2-neo (control)	32.8 ± 2.86	48.2 ± 2.37	21.2 ± 2.75
pSV2-neo & pSV2-ASuPA (AC10)	13.9 ± 1.81	25.2 ± 2.07	60.1 ± 3.16
pSV2-neo (control)	37.0 ± 1.86	47.0 ± 2.49	16.1 ± 2.70
pSV2-neo & pSV2-ASuPA (AC8)	11.2 ± 2.27	24.1 ± 2.14	64.7 ± 3.42

[a]Size of the lung tumors from control and experimental groups were measured and divided into three size categories, small (<0.2 mm in diameter), medium (0.2 mm-0.7 mm), and large (>0.7 mm). Results are expressed in percent ± S.E. of tumor numbers in the different size category.

CHAPTER V

DISCUSSION

We have shown in this work direct evidence for the importance of urokinase in tumor cell metastasis by modifying the gene expression in murine B16 melanoma tumor cells. The metastatic potential of the cells transfected with preprourokinase cDNA was dramatically enhanced compared to the control cells _in vivo_. In addition, the cells transfected with antisense cDNA of urokinase gene showed decreased metastatic potential _in vivo_.

Cell Lines Selected for the Study

B16 melanoma cell lines, B16-F1 and B16-F10, which metastasize to form pulmonary tumor nodules (Fidler, 1973) were used in this study. The B16 tumor lines have been established by Fidler (1973) by injecting tumor cells i.v. into syngeneic C57BL/6 mice, isolating, and then culturing the secondary lung tumor nodules in tissue culture. The B16-F1 cell line was passaged once through the selection

process, while B16-F10 cell line was passaged ten times.
The B16-F1 cell line possesses low ability to form
metastatic lung colonies, while the B16-F10 cell line
shows high ability when injected i.v. into syngeneic
C57BL/6 mice (Fidler, 1975). These cell lines have been
used by a number of investigators to determine a
quantitative correlation between the production of
plasminogen activator and their capacity to metastasize.
Wang et al. (1980) reported quantitative differences in
plasminogen activator production between low metastatic
B16-F1 and high metastatic B16-F10 melanoma sublines, the
latter producing more plasminogen activator activity than
the B16-F1 cells. Nicolson et al. (1976) however showed
contradictory results, reporting a lack of correlation in
plasminogen activator activities between variant B16
sublines such as B16-F1, B16-F5, B16-F10, and B16-F13.

Expression of Human Urokinase Gene in B16-F1 Melanoma Cells

The DNA-mediated transfection technique (Graham and
van der Ed, 1973) was used as a means of introducing
foreign genes into the murine melanoma cells. The optimal
transfection conditions as described in the Methods

section were developed specifically for B16 cell lines. The human preprourokinase gene was successfully expressed in B16-F1 cells under the control of an SV40 early gene promoter (pSV2 vector). The expression of the incorporated preprourokinase gene was found to be stable. No loss of human prourokinase expression was observed even after over 20 passages of the transfected cells.

The data in Figure 16 and 17 show that clone 7 and clone 10 of pSV2-uPA transfected cells secrete a three to four fold increase in plasminogen activator activity. The enhanced plasminogen activator activity in clone 7 and clone 10 was shown to be specifically related to <u>human</u> urokinase and not to the endogenous murine urokinase based on the following observations: (i) The clones were initially selected based soly on their high production of human urokinase, by a filter immunoblot assay using a polyclonal antibody to human urokinase (Figure 9), (ii) In a second selection, from those clones selected by the filter immunoblot assay, clones 7 and 10 were shown to produce the highest level of human urokinase by ELISA using a monoclonal antibody to human urokinase (Table 1). Further characterization of the selected cells showed: (i) Urokinase produced by clone 7 was glycosylated as expected for the human urokinase (Figure 16). The murine urokinase

is not a glycoprotein (Belin, et al., 1985), (ii) Southern

blot hybridization analysis (Figure 10) demonstrated that

multiple copies of the human urokinase gene were

incorporated in the genomic DNA of clone 7. The human

urokinase was selectively identified under the blotting

conditions used for the assay. The murine urokinase gene

did not hybridize with the human cDNA probe under the

conditions used, and, (iii) Northern blot hybridization

analysis (Figure 5) demonstrated human urokinase gene

expressions by clone 7 and clone 10.

Glycosylation of Human Urokinase by Murine Cells

The result of concanavalin A affinity

chromatography (Figure 14) demonstrates that the human

urokinase, produced by the transfected murine B16-F1

cells, is glycosylated. The murine urokinase gene lacks a

glycosylation site (Belin et al., 1985) and is therefore

not a glycoprotein. It is not surprising that the human

gene product would be glycosylated since human urokinase

contains a glycosylation site in its amino acid sequence

(Holmes et al., 1985; Ricco et al., 1985). A carbohydrate

side chain, containing at least four glucosamine and two

galactosame residues, was reported at asparagine in

position 144 of the human urokinase B chain (Steffens et
al., 1982).

Lack of Binding of Human Urokinase to Murine Receptor

As depicted in Figure 15, human urokinase does not
bind to cell surface receptor sites on the murine B16-F1
cells. It is not known whether the lack of binding is the
result of the glycosylation of human urokinase, or due to
the other changes in the structure of the urokinase that
prevents the human urokinase from binding to the mouse
receptors. The observation that human urokinase does not
bind to murine cell receptors is in agreement with other
studies that reported a lack of cross-species binding of
urokinase to cell surface receptors. Huate et al. (1987)
reported no significant binding of human prourokinase to
mouse spermatozoa. Most recently, Estreicher et al.
(1989) demonstrated that human, mouse, and porcine
urokinase bind to the corresponding cell receptors of the
same species, while human urokinase failed to bind to
murine MSV-3T3 cells and murine urokinase did not bind to
human HeLa cells.

Experimental Model for in vivo Metastasis Assay

To assay the metastatic potential of the transfected B16-F1 cells expressing the human urokinase gene _in_ _vivo_, the lung colonization and spontaneous metastasis assays were employed. In the lung colonization assay, mice were i.v. injected through the tail vein, and 2-3 weeks later the animals were sacrificed and tumor nodules on the lung surface were counted under a dissecting microscope (Fidler, 1975). This assay measures tumor cell extravasation from the blood stream, invasion of basement membrane and extracellular matrix, and then growth into pulmonary tumors. In the spontaneous metastasis model, tumor cells injected in the foot pad of the mouse, grow into a primary tumor site and metastasize as secondary tumors to the lung (Fidler, 1978). This assay tests the full metastatic process in which the tumor cells intravasate through the capillary endothelial walls, survive in the circulation, extravasate from the capillaries through the basement membrane, and invade the extracellular matrix to establish a new tumor site.

The Effect of Increased Expression of Urokinase on Lung Colonization Potentials

Three of the clones (clones 7, 8, and 10) among the human preprourokinase transfected B16-F1 cells, were

chosen for _in vivo_ metastatic assay. All of them showed a dramatic increase in lung colonization following tail vein inoculation (Table 5 and 6). The ELISA and chromogenic substrate assays for plasminogen activator confirmed the human urokinase activity in the cells from the lung nodules (Table 9 and 10). These results clearly show that the increased lung colonization potential of the clones 7, 8, and 10 is due to the human urokinase produced by the pSV2-uPA transfected cells. Since the cells were solely selected for their ability to secrete human urokinase which does not bind to cell surface receptors, the issue of whether urokinase acts to promote metastasis on the cell surface or at locations away from the surface can be addressed. Our results demonstrate that the increased secretion of human urokinase activity strongly promotes metastasis _in vivo_. These results are in agreement with those recently reported by Cajot _et al_. (1989). These authors transfected the human urokinase gene into mouse L cells and showed a correlation between the modulation of prourokinase gene expression and the degradation of extracellular matrix by _in vitro_ invasion through a basement membrane like material, Matrigel. Their results demonstrated that urokinase binding to the cell surface is not required for the increased degradation and invasion of

the extracellular matrix. These authors did not assay

their cells in an _in vivo_ model for metastasis. Axelrod

_et al_. (1989) also recently demonstrated that their _ras_

transformed murine NIH3T3 cells, transfected with

recombinant human urokinase, produced 30-fold elevation in

urokinase activity and manifested an increased invasion

rate in an _in vitro_ basement membrane assay. They also

showed that the transfected cells had a sixfold increase

in pulmonary metastatic lesions after i.v. injection into

nude mice.

Other authors however support the hypothesis that

cell surface-localized urokinase activity is critical for

tumor cell metastasis. Ossowski (1988) demonstrated that

a saturation of human tumor cell, HeLa, receptors with

exogenous human urokinase enhanced the invasion of the

chick embryo chorioallantoic membrane. This suggests that

urokinase binding to its receptor is important for the

increased invasive potential of the tumor cells. A recent

study by Hearing _et al_. (1988) demonstrated the presence

of urokinase activity on the cell surface of B16 melanoma

cells by immunofluorescence. The authors also reported

that the preincubation of these cells expressing urokinase

with an antibody to urokinase caused a reduction in their

lung colonization ability when injected through the tail

vein of mice. Based on these data, the authors argued that cell surface localized urokinase activity has a critical role in lung colonization. However, the statistical significance of their data does not appear conclusive. In Table 2 of their report, they tested the effect of urokinase specific antibodies on pretreated B16 cells in 12 separate experiments. Out of 12 experiments, the authors reported that three experiments showed no significant difference between the two groups using Student t test at $p \leq 0.05$. However, by a Wilcoxon non-parametric analysis of their experimental data, only two of the twelve experiments with anti-urokinase pretreated cells showed a significant decrease (at $p \leq 0.05$) in lung colonization as compared to the controls. In our study, we show that the human urokinase does not bind to the murine cell surface receptor, yet it clearly promotes metastasis.

The role of the cell surface urokinase receptors may serve to focus the enzyme activity at critical sites on the cell surface. The binding of urokinase to its receptor, however, may also serve different functions than to promote metastasis. For example, it has been suggested that urokinase may bind to cells and act as a cell mitogen to induce cell proliferation (Kirchheimer et al., 1987;

Takada and Takada, 1989; Kirchheimer et al., 1989).

Regardless of the role of the cell surface associated urokinase, the results in this report show that an increase in the unbound, secreted form of human urokinase dramatically increased spontaneous metastasis and lung colonization by the B16-F1 cells. Future investigation that would help define the role of the urokinase receptor may involve cloning the urokinase receptor gene and its subsequent transfection into B16 cells. Such study would help elucidate the effect of urokinase cell surface receptor binding in invasion and metastasis in vivo. The lung colonization data of the clones 7, 8, and 10 in Table 5 and 6 and increased urokinase secretion of these clones infer that the increase in lung colonization by B16-F1 cells is directly related to an increased urokinase activity. A comparison of the plasminogen activator activity of B16-F1 cells, B16-F1 pSV2-uPA transfected cells (clone 7 and clone 10), and B16-F10 cells show that the clone 7 cells produce plasminogen activator activity equal to that of the B16-F10 cells (Table 2 and 3). In addition, the lung colonization potential for B16-F10 cells is similar to that observed for the clone 7 cells (Schultz et al., 1988).

A positive correlation between production of

plasminogen activator and a spontaneous metastatic potential for B16-F1 and B16-F10 cells has been previously reported (Wang et al., 1980). This earlier report, however, while showing a correlative increase in plasminogen activator production with increased spontaneous metastasis, was not based on a known increase in the expression of a single specific gene. Multiple differences have been reported between B16-F1 and B16-F10 cells besides the differences in urokinase production. For example, there are differences in membrane lipid composition (Schroeder and Gardiner, 1984), in the expression of a 72 Kd glycosylated protein by B16-F10 cells and not by B16-F1 (Kimura and Xiang, 1986), and in response of lung colonization to the monoclonal antibodies produced to tumor-associated antigens of B16-F1 or B16-F10 cells (Herd, 1987). In addition, B16-F10 cells showed an increased glycosyltransferase activity as compared to B16-F1 counterparts (Bosmann et al., 1974) and B16-F10 cells have a greater propensity to form in vitro aggregates with artificially disaggregated lung cells than B16-F1 cells (Nicolson and Winkelhake, 1975).

The selection of the clones in this study was based solely on the ability of transfected cells to produce secreted human urokinase. The secreted human urokinase in

the B16-F1 cell line is shown to raise the metastatic potential of B16-F1 cells to that of B16-F10. This finding supports the conclusion that increase in urokinase activity is sufficient to explain the difference in metastatic potential between B16-F10 and B16-F1 cells.

Blood-borne metastasis is a multiple step process which may be subdivided into several steps: (i) detachment of malignant tumor cells from the original tumor mass, (ii) invasion through the extracellular matrix and basement membrane with intravasation through the capillary endothelial walls, (iii) travel of tumor cells through the circulation, (iv) arrest in the capillary beds of the target tissue, (v) extravasation from the capillaries through the basement membrane and extracellular matrix, and (vi) implantation and growth of secondary tumor cells at a new site. The most critical steps in the metastatic process, where proteolytic enzymes have a role, appears to be the intravasation and extravasation steps (Liotta et al., 1982).

The hypothesis that urokinase plays an important role in intravasation comes from the earlier work of Ossowski and Reich (1983) who showed that an antibody to human urokinase only inhibited lung colonization in the chick embryo when human tumor cells were placed on the

chorioallantoic membrane. Ossowski (1988) later reported in this model, a lack of urokinase effect on the extravasation step of metastasis by showing that the urokinase antibody did not inhibit metastasis when human tumor cells were directly injected into a chick embryo vein, as opposed to the cells being placed on the chorioallantoic membrane. These experiments support the argument that urokinase is important in the intravasation step, but not in the extravasation step.

The argument of Ossowski agrees with the current interpretation of the role of fibrin in metastasis. It has been argued that coagulation and the formation of microthrombi might promote the attachment of tumor cells to the capillary walls of the target organ, forming a stationary phase of tumor cells which allows time for the extravasation of tumor cells to occur (Dano et al., 1985; Markus et al., 1984; Harvey et al., 1988). Urokinase, which can promote fibrin degradation through plasmin mediated fibrinolysis, would act against the formation of this protective stationary phase. Subsequently high urokinase activity in the extravasation step should lead to a decrease in lung colonization (Markus et al., 1984). In fact, Markus et al. (1983) compared the secretion of urokinase between the primary and metastatic tumors of

different types and found that urokinase secretion by metastatic tumors were lower than those of primary tumors. Harvey et al. (1988) also made similar observations which led to the proposal that reduced secretion of urokinase of metastatic tumors might provide a distinct advantage to tumor cells destined to initiate metastatic foci, and may contribute to the ability of circulating cancer cells to lodge in the blood vessels of the target organs. This hypothesis is also supported by the study of Gunji and Gorelik (1988) who demonstrated that the presence of fibrin deposition on tumor cells protected them from immunological destruction. However, this hypothesis does not agree with other reports. Iwakawa et al. (1986) demonstrated that an anticoagulant, which suppresses coagulation via inhibition of thrombin (which activates fibrinogen to fibrin) increased, rather than decreased, the number of lung colonies after i.v. injection of tumor cells. Ostrowski et al. (1986) reported similar findings that, mice implanted with mini-isopump infusion of a thrombin inhibitor produced increased B16 melanoma lung colonization.

The results of the lung colonization assay (Table 5 and 6) in this study, clearly shows an increase in urokinase activity by the C7, C10, and C8 clones of the

pSV2-uPA transfected cells which caused a dramatic increase in lung colonization ability. The lung colonization assay by tail vein injection (Table 5 and 6) measures the extravasation step of the metastatic process, as the tumor cells are directly injected into the blood, circumventing the earlier steps of the metastatic process. Our conclusion that urokinase is important in the extravasation step of metastasis is consistent with the recent similar observations by Axelrod et al. (1989), that H-ras-transformed NIH3T3 cells expressing high levels of urokinase activity were more efficient in lung that increased plasminogen activator activity in rat mammary adenocarcinoma cells correlated with their lung colonization ability.

The increase in secreted urokinase activity also provided the clone 7 cells with an ability to spontaneously metastasize from a primary tumor in the foot pad (Table 8). The spontaneous metastasis assay measures the ability of tumor cells to intravasate through the capillary endothelial walls, survive in the circulation, extravasate from the capillaries through the basement membrane, and invade the extracellular matrix to establish a new tumor site. These results are in agreement with earlier work by Wang et al. (1980) who demonstrated that

high plasminogen activator production by B16-F10 subline cells contributed to spontaneous metastasis, whereas B16-F1 cells of low plasminogen activator production, had decreased pulmonary metastases.

The increased spontaneous metastatic ability of clone 7 (Table 8) might be due to (i) the increase in the ability of the clone 7 cells to extravasate, as shown by the colonization assay (Table 5), (ii) the acquisition of colonization. Another report that support this hypothesis is the earlier study by Carlsen et al. (1984) which showed an increased ability to intravasate, or (iii) the increase in ability for both intravasation and extravasation. If urokinase activity was essential only in the extravasation step, then the ability of clone 7 to spontaneously metastasize may be simply due to the increased ability to extravasate. Whether the secreted form of urokinase activity is controlling the intravasation as well as the extravasation steps in the metastatic process remains to be clarified. Models that specifically isolate the intravasation step need to be developed in order to determine precisely how urokinase affects this step in the metastatic process.

The cells secreting lower amounts of human urokinase (pSV2-neo transfected B16-F1 or C12 of pSV2-neo

and pSV2-uPA transfected cells) produce predominantly melanotic tumors as shown in Table 7. In contrast, the lung nodules formed by the cells secreting higher amounts of human urokinase consist of mixtures of amelanotic and melanotic tumors. In all three clones (clones 7, 10, and 8) the lesions predominantly demonstrated the amelanotic phenotype.

Phenotypic change cannot be attributed to the pSV2-neo transfection since all the clones contain the pSV2-neo gene. The overproduction of urokinase may be responsible for the expression of this phenotype since the only difference between these groups is the amount of human urokinase secretion. Therefore high amounts of urokinase secretion in B16-F1 cells might inhibited melanin synthesis/production. The absence of the pigment in amelanotic tumor is reported to be the result of deficiency of tyrosinase activity (Slominski et al., 1987; Bennett et al., 1986) even though some investigators reported that tyrosine activity did not correlate in vitro with melanin synthesis in K1735 SW-1 cells (Price et al., 1988).

A possible link between the high urokinase production and the metastatic potential may be the state of differentiation of the B16 cells. It has been

argued that more malignant cells are less differentiated (Bennett et al., 1986). In the B16 melanoma cell lines, melanin formation may correlated with differentiation. Thus amelanotic cells are less differentiated and also more metastatic. Urokinase production may affect the metastatic potential of a cell by changing its state of differentiation. However B16-F10 cells secrete a similar amount of urokinase as C7 cells but produce melanotic lung nodules.

Several reports have linked melanin synthesis with a reduction in tumor cell proliferation (Gray et al., 1964; Lotan and Lotan, 1980). Other investigators have also suggested a positive correlation between tumor cell malignancy and the amelanotic phenotype in B16 cell lines (Romsdahl and Hsu, 1972; Lotan and Lotan, 1980). Our study confirmed these reports by showing that transfected cells secreting human urokinase undergo a phenotypic shift to cells that are more metastatic and form amelanotic colonies.

The Effect of Inhibition of Urokinase Production in the Lung Colonization Potential

If urokinase is required for tumor cell

colonization (Table 5 and 6), then the inhibition of urokinase activity should lead to a reduction in metastasis. By transfection of B16-F10 cells with an antisense urokinase gene, it may be possible to evaluate the effects of inhibiting urokinase gene expression on the metastatic process. B16-F10 melanoma cells of high metastatic potential were transfected with a plasmid containing human urokinase in the antisense direction (pSV2-ASuPA). The antisense clones 10 (AC10) and 8 (AC8) cells which produced 30% less urokinase activity were selected (Table 11, 12 and Figure 20). A Southern blot hybridization assay showed the incorporation of the antisense plasmid into the genomic DNA of these cells (Figure 21). Tail vein injection by these two clones showed a 40% reduction in lung colonization (Table 13). These results are in agreement with our previous experiments in which pSV2-uPA transfected clones of B16-F1 cells showed increased urokinase production and increased lung colonization. Taken together these two results give supporting evidence that urokinase has an essential role in extravasation process of lung colonization by B16 melanoma cells. This is in contrast to the results reported in the chorioallantoic membrane experiment which concluded that urokinase does not play a role in the

extravasation step (See previous discussion, Ossowski and Reich, 1983).

The human urokinase gene transfected C7 clone showed that approximately 150 copies of the urokinase gene was incorporated in B16-F1 cell DNA genome. After transfection of the antisense gene (pSV2-ASuPA) with the same SV40 expression vector, a small copy number of antisense urokinase genes were shown to be incorporated in the B16-F10 genomic DNA in the most inhibited cell colony. The difficulty of observing high copy number for incorporation of the antisense gene might be explained by the necessity of urokinase expression for cell viability. Cells with higher copies of incorporated antisense urokinase gene might fail to survive, hence escape detection. It has been proposed that urokinase is a growth stimulant in various cell lines by several investigators (Kirchheimer et al., 1987; Kirchheimer et al., 1989; Tanaka and Tanaka, 1989). Earlier study showed the production of plasminogen activator reached maximum during the logarithmic growth of transformed 3T3 cells and decreased as the cells reach the confluence (Jaken and Black, 1979). It was also shown that the production of urokinase correlated with the proliferative state of the melanoma cells (Grimmaldi et al., 1986).

An attempt to demonstrate antisense mRNA expression by Northern blot hybridization was made. However, it was not possible to detect the antisense transcript in the pSV2-ASuPA transfected cells because of low abundance of antisense mRNAs. A more sensitive assay to demonstrate the presence of the antisense transcript would be to use the polymerase chain reaction (PCR) amplification, following reverse transcriptase treatment, to convert mRNA to cDNA (Saiki et al., 1988).

The variation in the numbers of lung colonies between the control groups in Experiment 1 and 2 did not closely coincide, as is shown in Table 13. This may be the result of the use of different lots of animals or the use of cells from different time period. Since the control group was always tested in identical manner to the experimental group for each experiment, the important consideration for interpreting the data is the relative number of metastases for both control and experimental animals in any one set of experiments carried out at the same time and with the same lot of cells and animals. The variation in average numbers of lung colonies in each of the two separate experiments carried out at different time periods should not affect the conclusions of this work.

Table 14 and Figure 22 and 23 show that the

metastatic lung tumors from the mice injected with antisense clones 10 or 8 were larger than those in control mice containing cells transfected with the pSV2-neo plasmid alone. This result suggests that decreased urokinase production inhibits metastasis but not the growth of tumors.

Future studies to assess the role of urokinase in metastasis would be to regulate urokinase activity by means of plasminogen activator inhibitors (PAIs). Several workers have shown that there are at least three immunologically distinct PAIs, including PAI-1, PAI-2, and protease nexin (Loskutoff et al., 1983; Scott et al., 1985; Sprengers and Kluft, 1987). These proteins inactivate both urokinase and tissue-type plasminogen activators by forming covalent complexes with the active site serine residues of the plasminogen activators (Andreasen et al., 1986). PAI-1 is an efficient inhibitor of both urokinase and tissue plasminogen activators. It has been purified (van Mourik et al., 1984) and its cDNA has been sequenced (Ny et al., 1986). PAI-2, which reacts more rapidly with urokinase than with tissue type plasminogen activator, has also been purified (Astedt et al., 1985) and the full length cDNA sequence has been determined (Ye et al., 1987). Future studies could

transfect cloned PAI-1 and PAI-2 genes into metastatic
cells to evaluate their role in metastasis. It could be
predicted that PAI-1 and PAI-2 are metastatic suppresser
genes, in the same way as TIMP appears to be a metastatic
repressor protein (Khokha et al., 1988).

CHAPTER VI

SUMMARY

Metastasis is a process in which tumor cells detach from their primary site(s), invade through the surrounding tissue, and give rise to new lesions at sites distant from the primary tumor(s). Urokinase may have an important role in tumor cell metastasis. It has a proteolytic enzyme activity which can promote degradation of extracellular matrix and basement membrane. In this study, the role of urokinase in tumor cell metastasis was examined by modifying the urokinase gene expression in tumor cells.

A SV40 expression vector containing the human preprourokinase gene (plasmid pSV2-uPA) was constructed and cotransfected with a selection marker gene (pSV2-neo) into murine B16-F1 melanoma cells of low metastatic potential. The expression of the human urokinase gene was demonstrated by a filter immunoblot assay and an ELISA using a monoclonal antibody to human urokinase. Southern

blot hybridization analysis of the clone expressing the highest level of human urokinase showed that the pSV2-uPA plasmid was incorporated in approximately 150 copies. These cells exhibited a 3 to 4-fold increase in secreted urokinase activity. The human urokinase synthesized by the murine cells was found to be glycosylated. Furthermore, it was shown that human urokinase does not bind to the murine B16-F1 cell surface urokinase receptor sites. Thus the human urokinase is simply constitutively synthesized and secreted by the transfected murine melanoma cells.

In the lung colonization assay which measures tumor cell extravasation from the blood and subsequent pulmonary tumor growth, the pSV2-uPA transfected cells showed a 4 to 18-fold increase in the ability to form lung tumors. In the spontaneous metastatic assay, which measures the full metastatic process, the transfected cells showed an absolute ability to metastasize (12 out of 12 mice), while control cells showed a low ability to metastasize (only 2 out of 11 mice). These results clearly show that an increase in secreted urokinase activity promotes both lung colonization and spontaneous metastasis.

In addition, B16-F10 murine melanoma cells of high metastatic ability were cotransfected with a 265 base pair

cDNA containing the leader sequence of the human preprourokinase gene in an antisense direction (plasmid pSV2-ASuPA) and pSV2-neo. Two of the antisense clones secreted approximately 70% of the urokinase activity of control cells. Southern blot hybridization analysis confirmed the incorporation of pSV2-ASuPA into the genomic DNA. In the lung colonization assay, the cells containing antisense urokinase cDNA showed a 40 to 42% decrease in the ability to form lung tumors. These results support the conclusion that urokinase is an important promoter of the extravasation step in tumor cell metastasis. It was also observed that the majority of tumors from the control mice (injected with pSV2-neo transfected cells) were smaller than those of the experimental mice (injected with pSV2-neo and pSV2-ASuPA transfected cells). This result indicates that inhibition of urokinase production may decrease the metastatic potential and correspondingly increase the tumorigenic potential of murine melanoma cells.

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APPENDIX

APPENDIX

Prior to the use of pSV2 expression vector pSV2-uPA (shown in Figure 3), the plasmid pRdzbuktt (shown in Figure 1) which contains Rous sarcoma virus (RSV) long terminal repeats (LTR) promoter was tested for the preprourokinase gene expression. The expression vector containing RSV LTR promoter had been previously used in a variety of murine cell lines other than B16 cell lines (Gorman et al., 1982). B16-F1 cells were cotransfected with pSV2-neo and pRdzbuktt, shocked with glycerol, and grown in the presence of Geneticin as described previously. The DNAs were prepared from the surviving cells of pSV2-neo and pRdzbuktt transfected or pSV2-neo transfected cells. To detect the pSV2-neo incorporation, DNAs were digested with HindIII, or double digested with HindIII/BamHI or HindIII/PstI, separated by electrophoresis, transferred to nitrocellulose filter, and probed with ^{32}P-labeled pSV2-neo fragment. The digestion of pSV2-neo with HindIII generates one single DNA band of 5729 base pairs and ^{32}P-labeled probe hybridizes with the

band. The double digestion of pSV2-neo with HindIII and BamHI generates two DNA bands with base pairs of 3386 and 2346 and the probe hybridizes with the 2346 band. The double digestion of pSV2-neo with HindIII and PstI generates five DNA bands with base pairs of 1883, 1430, 958, 923, and 535 and the probe hybridizes with the 1430 and 923 bands. Figure 24 shows that the cells transfected with pSV2-neo alone or cotransfected with pSV2-neo and pRdzbuktt demonstrate the restriction endonuclease digested bands identical to those of pSV2-neo plasmid.

To detect the pRdzbuktt incorporation, Southern blot hybridization assay was performed using the ^{32}P-labeled SacI and HindIII fragment of pRdzbuktt as a probe. However, in Figure 25, Southern blot hybridization assay failed to show pRdzbuktt incorporation to the genomic DNA of the transfected cells. The production of plasminogen activator by the pRdzbuktt transfected cells was determined by the fluorogenic substrate assay and compared to pSV2-neo transfected or untransfected cells. As shown in Table 15, the cells transfected with pRdzbuktt produced same amounts of plasminogen activator as pSV2-neo transfected or B16-F1 cells. Taking Figure 25 and Table 15 together, the results show that the plasmid pRdzbuktt was not incorporated into the genomic DNA of B16-F1 cells,

Figure 24. Southern blot analysis of DNA isolated
from pSV2-neo and pRdzbuktt transfected cells to
detect the pSV2-neo incorporation

DNAs were isolated from pSV2-neo transfected
or pRdzbuktt transfected B16-F1 cells. The DNAs (10
ug) were cleaved with HindIII or double digested
with HindIII and BamHI or HindIII and PstI and
electrophoresed through 0.8% agarose gel. A control
pSV2-neo plasmid preparation was similarly digested
with the same enzymes. The DNAs were transferred to
nitrocellulose and probed with ^{32}P-labeled PvuII
fragment of pSV2-neo. Lanes 1,2,3 contain DNA made
from the plasmid pSV2-neo, lanes 4,5,6 contain DNA
made from pSV2-neo transfected cells, and lanes
7,8,9 contain DNAs made from the pSV2-neo and
pRdzbuktt transfected cells. DNAs in lanes 1,4,7
were digested with HindIII, DNAs in lanes 2,5,8 were
double digested with HindIII and BamHI, and DNAs in
lanes 3,6,9 were double digested with HindIII and
PstI.

m.w.
marker

pSV2-neo
H H+B H+P

TF-pSV2-neo
H   H+B   H+P

TF-pRdzbuktt
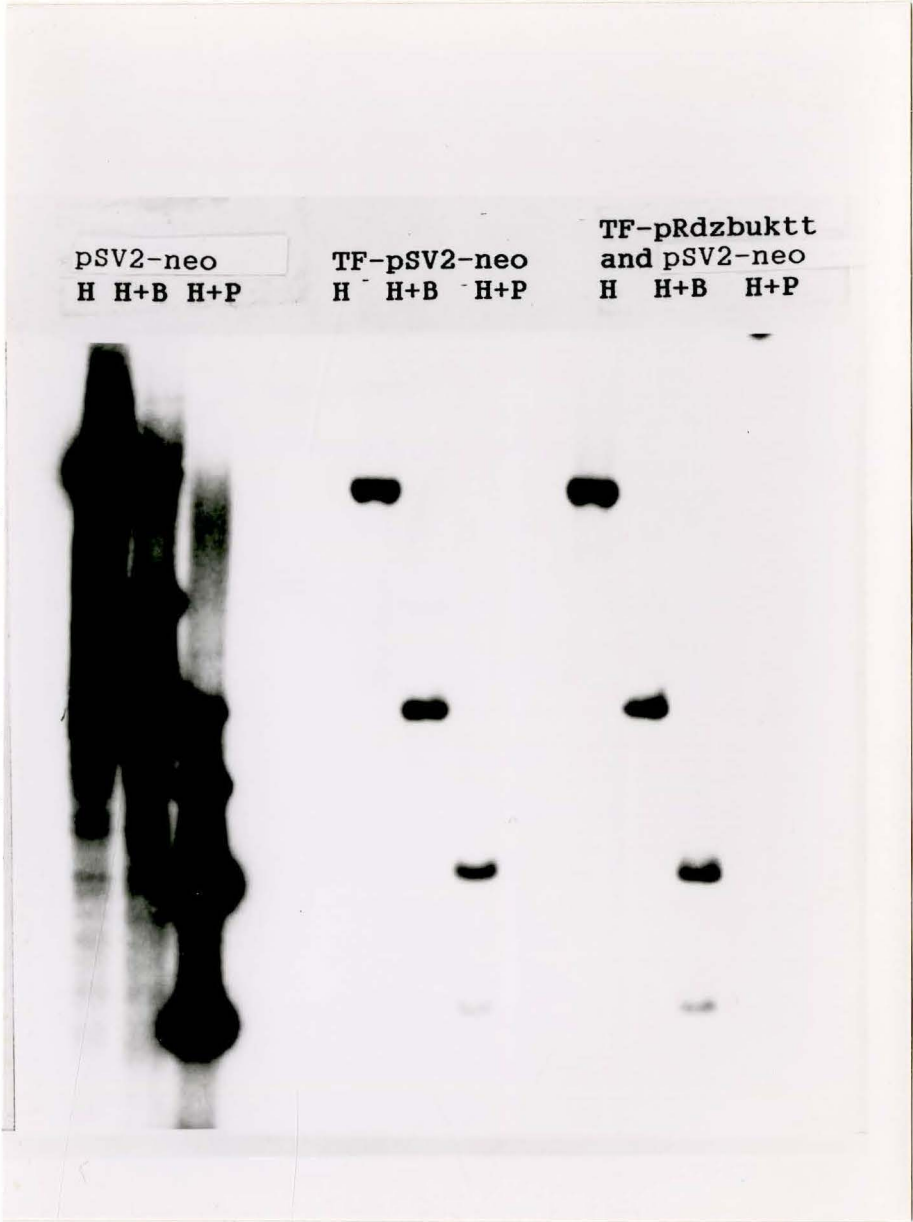and pSV2-neo
H   H+B   H+P

6.56 --

4.36 --

2.32 --

2.03 --

1   2   3   4   5   6   7   8   9

Figure 25. Southern blot analysis of DNA isolated from pSV2-neo and pRdzbuktt transfected cells to detect the pRdzbuktt incorporation

DNAs were isolated from pSV2-neo transfected or pRdzbuktt transfected B16-F1 cells. The DNAs (10 ug) were cleaved with HindIII or double digested with HindIII and SacI or HindIII and PstI and electrophoresed through 0.8% agarose gel. A control pRdzbuktt plasmid preparation was similarly digested with the same enzymes. The DNAs were transferred to nitrocellulose and probed with ^{32}P-labeled HindIII-PstI fragment of pRdzbuktt. Lanes 1,2,3 contain DNA made from the plasmid pRdzbuktt, lanes 4,5,6 contain DNA made from pSV2-neo transfected cells, and lanes 7,8,9 contain DNAs made from the pSV2-neo and pRdzbuktt transfected cells. DNAs in lanes 1,4,7 were digested with HindIII, DNAs in lanes 2,5,8 were double digested with HindIII and SacI, and DNAs in lanes 3,6,9 were double digested with HindIII and PstI.

m.w.
marker

pRdzbuktt
H H+S H+P

TF-pSV2-neo
H H+S H+P

TF-pRdzbuktt
and pSv2-neo
H   H+S   H+P

6.56 --

4.36 --

2.32 --

2.03 --

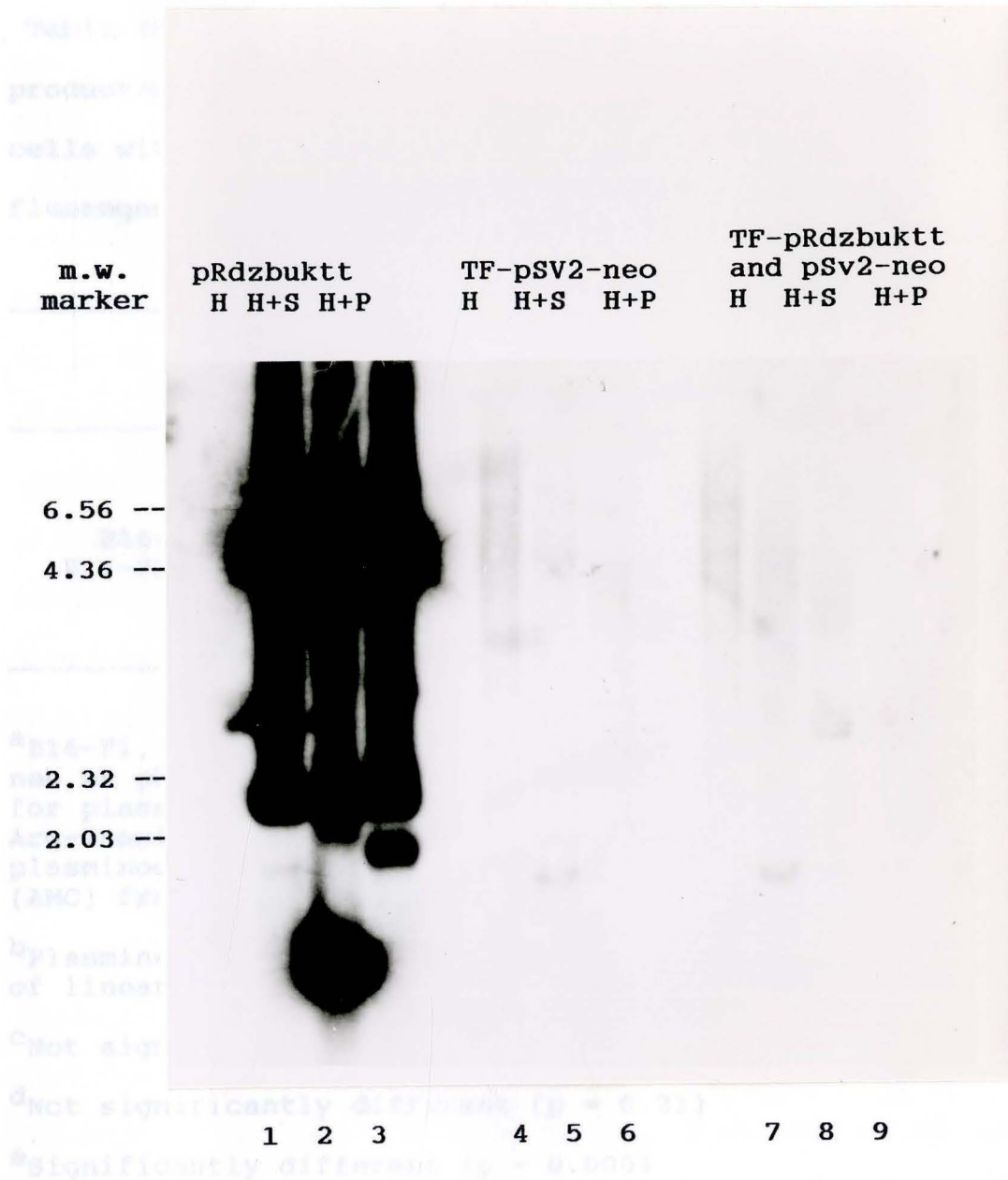1   2   3   4   5   6   7   8   9

Table 15. Quantification of plasminogen activator (PA) production by B16-F1, B16-F10, and transfected B16-F1 cells with pSV2-neo or pRdzbuktt as determined by fluorogenic substrate assay[a]

Cell	PA activity[b] (Mean ± SD)
B16-F1	0.57 ± 0.09[c,d,e]
B16-F1 (pSV2-neo)	0.56 ± 0.13[c]
B16-F1 (pRdzbuktt)	0.51 ± 0.07[d]
B16-F10	1.38 ± 0.10[e]

[a]B16-F1, B16-F10, and B16-F1 transfected cells with pSV2-neo or pRdzbuktt were incubated with fluorogenic substrate for plasminogen activator, methoxysuccinyl-L-Gly-L-Gly-L-Arg-7-amino-4-methylcoumarin HCl. The ability of plasminogen activator to release 7-amino-4-methylcoumarin (AMC) from the substrate was measured.

[b]Plasminogen activator activity is expressed by the slope of linear release of AMC under the assay conditions.

[c]Not significantly different (p = 0.92)

[d]Not significantly different (p = 0.31)

[e]Significantly different (p = 0.000)

which subsequently failed to produce human urokinase.

Another attempt was made to express the human preprourokinase gene by the plasmid, pCLH3AXBPV, which contains bovine papilloma virus (BPV-1) with MT-1 (metallothreonine) promoter (Figure 4). The insertion of human preprourokinase gene into the pCLH3AXBPV is shown in Figure 5 and the final plasmid structure, pCLH3AXBPV-uPA, is shown in Figure 6. The plasmid was similarly transfected to B16-F1 cells and a fibrin overlay assay was performed. The fibrin overlay assay tests the transient expression of urokinase gene prior to its incorporation to the genomic DNA. The optimum condition for the assay was developed for C127 cells (mammary tumor cell line of an RIII mouse obtained from American Tissue Culture Center, No. CRL1616) transfected with pCLH3AXBPV-uPA as described by Reddy et al. (1987). The results in Figure 26 demonstrate the absence of plasminogen activator activity in the fibrin agarose matrix which indicates the cells transfected with plasmid pCLH3AXBPV-uPA did not produce urokinase. In contrast, C127 cells transfected with pCLH3AXBPV-uPA gave positive results with respect to pSV2-neo transfected controls (data not shown).

The efficiency of gene transfer under the control of the viral regulatory sequence might have limitations

Figure 26. Fibrin overlay assay to detect the transient expression of urokinase gene by the transfection of the plasmid pCLH3XBPV-uPA to B16-F1 cells

B16-F1 cells transfected with pCLH3AXBPV-uPA were mixed with agarose in MEM supplemented with 10% heat inactivated FBS containing fibrinogen and thrombin and incubated at 37°C overnight. The ability of plasmin, which was activated from plasminogen by the urokinase, was detected by the clearing zones of fibrin degradations on the agarose matrix. Plasminogen was supplied from the FBS in the reaction mixture and fibrinogen was activated to fibrin by thrombin. The cells transfected with pCLH3AXBPV-uPA shows the absence of clearing zones which indicate that the cells did not produce urokinase (top). A control which contained several drops of urokinase solution showed clearing circles of fibrin degradation (bottom).
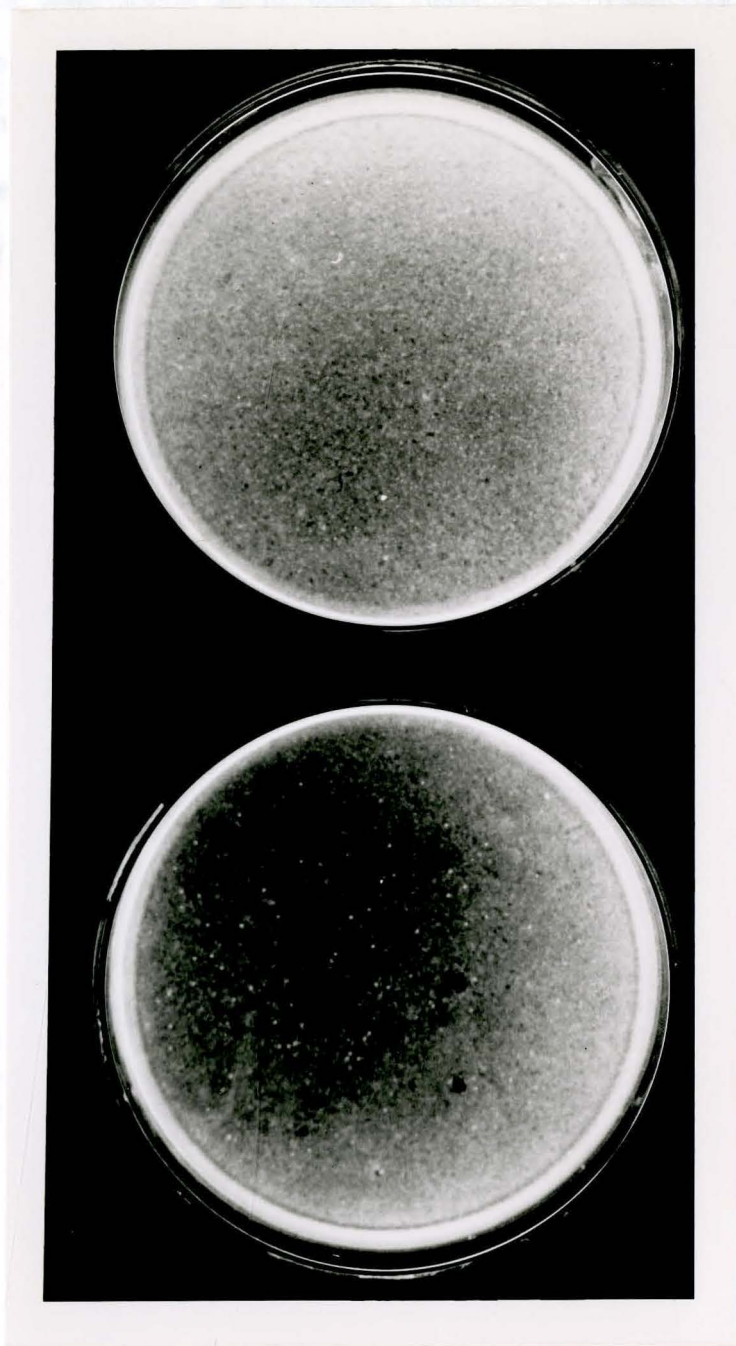
which affect its application to certain cell lines. For example, it has been suggested that the flanking long terminal repeats (LTRs) as well as BPV vectors may interfere with transcriptional regulation of the inserted foreign gene sequences that could lead to a lack of expression in certain cell lines (Howard, 1983).

APPROVAL SHEET

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

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