Regulation of The Metastatic Phenotype by Growth Factors and Oncogenes.

by

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REGULATION OF THE METASTATIC PHENOTYPE BY GROWTH FACTORS AND ONCOGENES

BY

WILLIAM ROMAN TAYLOR

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

ABSTRACT

The metastatic dissemination of tumor cells is the major cause of cancer mortality. This study has focused on the elucidation of mechanisms underlying the metastatic phenotype. We tested the hypothesis that the ras oncoprotein controls malignant progression through a mechanism that involves growth factor regulation of cell motility, oncogene cooperation, and altered gene expression mediated by histone phosphorylation. The first part of this study was aimed at determining the mechanism by which the secreted growth regulators, fibroblast growth factors (FGF) and transforming growth factors (TGF), regulate the metastatic phenotype. We observed that, coincident upon inducing the metastatic phenotype, fibroblast growth factors also stimulated the ability of malignant tumor cells to locomote. Significantly, we found that K-FGF was a potent inducer of cellular motility in fibrosarcoma cells, an activity which had not previously been attributable to this member of the fibroblast growth factor family. Furthermore the rate of motility of fibroblast growth factor transformed cells showed a positive correlation with their metastatic potential which provides evidence that increased cellular motility is required for the metastatic capabilities of growth factor transformed cells. We also observed that ablation of $TGF\beta_1$ synthesis in metastatic cells with antisense oligonucleotides led to reductions in both cellular locomotion and metastatic potential. This strongly suggests that growth factor induced motility is required for their ability to induce malignant behaviour.

The ras oncogene acts downstream of fibroblast growth factors and can induce the metastatic phenotype. However, the induction of metastasis by ras occurs at a low

frequency. Our next series of experiments were aimed at determining the genetic requirements for induction of the metastatic phenotype by ras. We observed that cellular transformation *in vitro* by ras could be significantly enhanced if cells also overexpressed c-myc, and a dominant negative form of the p53 tumor suppressor. In addition, we observed that cells expressing ras alone, or ras in combination with c-myc, or ras in combination with mutant p53 were not capable of metastasizing when injected into mice. However, cells expressing all three genes could efficiently metastasize after injection. These experiments showed that the metastatic phenotype is cooperatively induced by ras, myc and mutant p53, and that there is a requirement for at least three genetic events for induction of metastatic potential with these cells. We next analyzed the mechanism of oncogene cooperation. These studies were focused on the myn gene which encodes a protein essential to the function of myc. We found that the myn gene is overexpressed in the cell lines transformed with combinations of ras, myc and mutant p53, and that myn expression is responsive to an activated ras. Based on these observations we have proposed a model which suggests that oncogene cooperation involving ras + myc includes an effect of ras on myn expression and the ability of myn to inhibit ras transformation when overexpressed.

We have investigated a potential mechanism responsible for altered gene expression in malignant cells which involves altered chromatin structure due to ras increased histone H1 phosphorylation. We found that in cells transformed by combinations of ras, myc and mutant p53, histone H1 is much more highly phosphorylated. H1 phosphorylation may contribute to altered gene expression by altering the chromatin structure of a particular gene allowing more efficient transcription.

Although activated ras has been shown to regulate the $p34^{cdc2}$ histone H1 kinase in *Xenopus*, there is no evidence that ras can regulate H1 kinases in mammalian cells. Our observation of increased H1 phosphorylation is important not only in understanding novel mechanisms of gene regulation during metastatic progression, but also in understanding the pathways which regulate cellular proliferation by oncogenes. $p34^{cdc2}$ is a universal regulator of M-phase and other closely related H1 kinases also regulate cell cycle progression. Therefore our novel observation of a link between ras activation and H1 phosphorylation, suggests a link between chromatin structural properties, gene transcription and malignant potential.

This study has shown that the ability of fibroblast growth factors to stimulate cellular locomotion is required for their induction of the metastatic phenotype. The induction of the metastatic phenotype by the ras protein, which acts downstream of fibroblast growth factors, also requires the alteration of at least two other growth regulatory genes. Cooperative transformation of cells by ras, myc, and mutant p53 involves altered expression of the myn gene, and the alteration of gene transcription in ras-transformed cells may involve altered chromatin structure due to phosphorylation of H1 histone. These studies have contributed to the understanding of the mechanisms of malignant progression. The elucidation of the molecular basis of the metastatic phenotype is required for a rational approach to the problem of cancer dissemination, the major factor in cancer mortality.

This is dedicated to Rose, Bill,

and my best friend and partner in everything,

Charlene

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During these past several years that I have used to carry out my Ph.D. research, I have also been fortunate to witness the end of the Cold War, the dismantling of the Berlin Wall and the beginnings of democracy in Russia and South Africa. I can only hope that the future will bring us more demonstrations of concern for the human condition.

Courage

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ABREVIATIONS

α-MEM	alpha-minimal essential media
γ-IR	gamma radiation
aFGF	acidic fibroblast growth factor
AMF	autocrine motility factor
AUT	acetic acid-urea-triton
bFGF	basic fibroblast growth factor
bfgf	gene encoding basic fibroblast growth factor
bHLH	basic helix loop helix
bp	base pairs
BSA	bovine serum albumin
CBF	cat box binding factor
CDK	cyclin dependant kinase
CHO	chinese hamster ovary cell line
Ci	curie
CMV	cytomegalovirus
cpm	counts per minute
D.M.	defined medium
DAPI	4',6-diamidino-2-phenylindole
dATP	2'-deoxyadenosine 5'-triphosphate
DCS	dialyzed calf serum
dCTP	2'-deoxycytosine 5'-triphosphate

DEPC	diethyl pyrocarbonate
dGTP	2'-deoxyguanosine 5'-triphophate
DMSO	dimethylsulfoxide
dTTP	2'-deoxythymidine 5'-triphosphate
DTA	ethylenediaminetetraacetate
EGF	epidermal growth factor
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
GAP	GTPase activating proteins
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDP	guanosine 5'-diphosphate
GDRF	guanine diphosphate releasing factor
grb2	growth factor receptor bound protein-2
GTP	guanosine 5'-triphosphate
h	hours
HGF	hepatocyte growth factor
HSV	harvey sarcoma virus
K-FGF	Kaposi-fibroblast growth factor
K-fgf	gene encoding Kaposi-fibroblast growth factor
kb	kilobase
kD	kilodalton
KGF	keratinocyte growth factor
MAPK	mitogen activated protein kinase

MCK	muscle specific creatine kinase
min	minute
NP40	nonidet-P40
oligos	oligodeoxynucleotides
PBS	phosphate buffered saline
PCA	perchloric acid
CNA	proliferating cell nuclear antigen
PDGF	platelet derived growth factor
PKA	cyclic AMP-dependant protein kinase
PKG	cyclic GMP-dependant protein kinase
PLC	phospholipase C
PVDF	polyvinylidene difluoride
RGD	Arg-Gly-Asp
RSK	ribosomal S6 kinase
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SOS	son of sevenless homologue
SRF	serum response factor
ssbFGF	signal sequence bFGF
TBP	tata binding protein
TCA	trichloroacetic acid
TGF	transforming growth factor
TIMP	tissue inhibitor of metalloprotease

TPA	12-O-tetradecanoylphorbol-13-acetate
uPA	urokinase-type plasminogen activator
v	volume
w	weight

I. INTRODUCTION

1. The Metastatic Phenotype

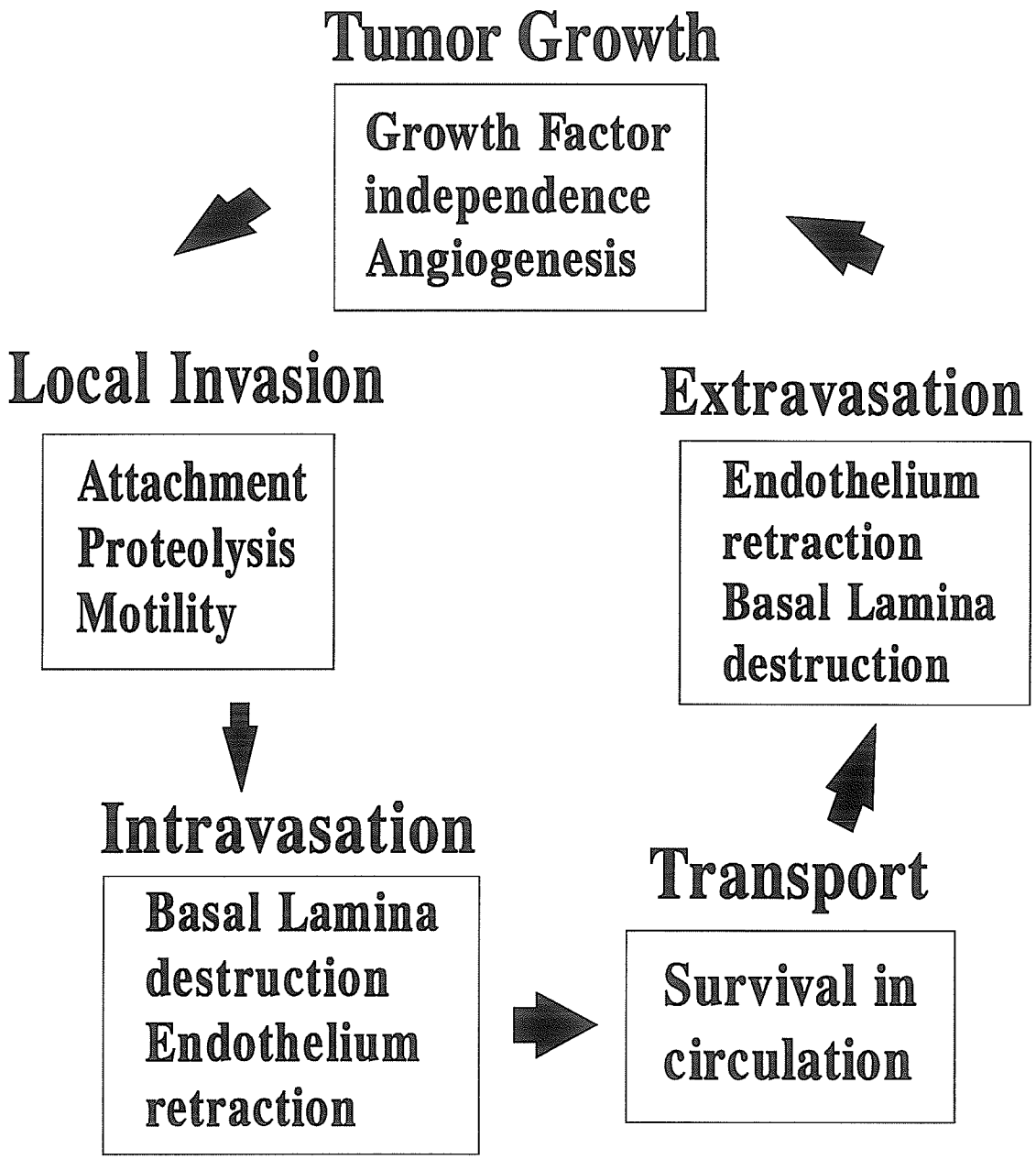
1.1 Overview of the Metastatic Cascade

Malignant tumors spread within an organism by the process of metastasis. This process encompasses multiple steps. It is initiated when tumor cells at the primary tumor site invade the tissue immediately adjacent the tumor. Invasion proceeds until a tumor cell contacts a lymphatic or blood vessel, and then gains entry into the lumen of the vessel. A tumor cell which survives hydrodynamic pressure changes and attack by cells of the immune system within the vessel will be carried to a distant site. The next step in the metastatic cascade involves the attachment of the cell to the endothelium which commonly occurs in a capillary bed, causing the tumor cells to arrest. Next, the cell will exit from the circulation and begin to invade the intercellular spaces of a new tissue. If a tumor cell is capable of growing in this new environment it will form an autonomous tumor completing the cycle of metastasis (figure 1). This process therefore leads to the production of multiple tumors at sites far removed from the primary neoplasm (for a review see Aznavoorian et al, 1993; Barskey, 1988; Liotta et al, 1991).

Invasion of tumor cells within the primary site and eventually within new tissues requires that they cross several barriers. In carcinomas, the first barrier is the subepithelial basement membrane which lies at the base of epithelial tissues and separates these cells from the underlying connective tissue (Aznavoorian et al, 1993). Basement

FIGURE 1.

Overview of the Metastatic Cascade. Metastasis is initiated when tumor cells at the primary tumor site invade the tissue immediately adjacent the tumor. Invasion proceeds until a tumor cell contacts a lymphatic or blood vessel, where it enters the circulation by the process of intravasation. A tumor cell which survives in the circulation will be carried to a distant site, where after extravasating from the vessel it will form a secondary tumor.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Survival in circulation

membranes contain collagen type IV, laminin fibronectin, and a heparan sulfate proteoglycan. Collagen type IV, and laminin are almost exclusively found in basement membranes (Martinez-Hernandez & Amenta, 1983). Highly metastatic cells are able to interact with and degrade these extracellular components, which is likely required for them to traverse the basement membrane (Liotta et al, 1979).

The next barrier is the connective tissue which underlies the epithelium which must be traversed in order for a metastatic cell to come in contact with a lymphatic or blood vessel. This type of connective tissue, also called interstitial tissue has been characterized as a loose connective tissue and contains fibers of collagen I, as well as other extracellular matrix components (Liotta et al, 1991). In contrast to basement membranes, interstitium is inhabited by several types of cells including fibroblasts and macrophages, which may play a role in the invasion of this tissue by tumor cells. Also present in this tissue are many blood vessels and capillaries. When a metastasizing tumor cell comes in contact with one of these vessels it may enter the circulation by a process termed intravasation. The first step in this process is the attachment and dissolution of the subendothelial basement membrane which surrounds blood vessels by the tumor cell (Brown et al, 1990). This step will require cellular functions common to those required to traverse the subepithelial basement membrane as these two structures are very similar in composition. During the course of intravasation, the tumor cell will retract the endothelial cells lining the vessel, allowing it to enter the circulation. Arrested tumor cells exit the circulation and enter a new tissue environment by the process of extravasation which also involves endothelial cell retraction and subendothelial basement membrane dissolution. The metastatic cascade is complete when an autonomous tumor

forms at the secondary site. The complexity of the metastatic cascade requires that metastatic cells acquire many biological and biochemical alterations (Weber, 1983). These alterations will confer on the metastatic cell the ability to invade foreign tissues, survive in the circulation and grow autonomously (Aznavoorian et al, 1993; Barskey, 1988; Liotta et al, 1991).

1.2 Attachment to Extracellular Matrix

Metastasizing tumor cells cross basement membranes and interstitial tissues by attaching to and degrading the extracellular components of these barriers and then locomoting into the resulting space. Attachment is carried out by cell surface receptors whose ligands are components of the extracellular matrix. The ligands include fibronectin, laminin, and vitronectin and the receptors include those of the integrin and cadherin family as well as other cell surface receptors such as the laminin receptor (Aznavoorian et al, 1993). Integrin receptors are heterodimeric molecules composed of one α subunit and one β subunit. There are at least 14 α and 8 β subunits which gives rise to at least 20 different integrins. These adhesion molecules can bind to several different ligands including fibronectin and vitronectin (Hynes, 1987). Some integrins have been implicated in the process of invasion and metastasis. For example, transfection of chinese hamster ovary cells with the $\alpha_5\beta_1$ fibronectin receptor reduces their tumorigenicity and migratory ability (Giancotti & Ruoslahti, 1990). However, decreased adhesion does not always enhance malignant behaviour. For example many integrins bind ligands at a site which contains the amino acid sequence Arg-Gly-Asp (RGD). In

addition, peptides containing RGD compete with ligands for interaction with several types of integrins. Treatment of tumor cells with these competing peptides reduces the ability of tumor cells to metastasize suggesting that integrins are required at some stage to promote metastatic dissemination (Humphries et al, 1986). It is evident that a critical balance between adhesiveness and loss of intercellular contact is required to successfully metastasize.

Cadherins are a family of calcium ion dependent cell surface receptors that mediate cell-cell interaction. They do so by binding to cadherins on adjacent cells through homotypic and heterotypic interactions (Takeichi, 1990). E-cadherin is one type of cadherin which has been implicated in metastatic spread. It has been found that blocking E-cadherin function with antibodies which bind to this receptor can increase the invasiveness of epithelial cells (Behrens et al, 1989). In addition, overexpression of E-cadherin leads to the diminution of metastatic ability in malignant tumor cells (Behrens et al, 1989). Thus, E-cadherin may inhibit tumor cell invasion and metastasis by promoting cell-cell interaction possibly at the primary site of tumor formation.

1.3 Enzymatic Degradation of the Extracellular Matrix

In addition to altered adhesiveness, malignant cells must degrade extracellular matrix components to provide a space for locomotion. Degradation is carried out by enzymes secreted by tumor cells, which degrade proteins and glycoproteins. Positive correlations between aggressiveness and secretion of several types of enzymes has been observed. These include serine, aspartyl, cysteinyl and metal binding proteases

(Aznavoorian et al, 1993).

Urokinase-type plasminogen activator (uPA) is a serine protease which enhances metastatic ability when overexpressed in ras transformed cells (Axelrod et al, 1989). Inhibition of uPA with antibodies results in the reduction of malignant behaviour of B16F10 melanoma cells (Ossowski & Reich, 1983). The role of uPA in metastasis is thought to be related to its ability to cleave and activate plasmin. Plasmin has fibronectinolytic activity and can also cleave interstitial collagenase, a metal binding enzyme, into its active form. Thus, uPA is an example in which a cascade of enzymes may aid in matrix dissolution.

The role of metalloproteases in metastasis has been most extensively studied. These enzymes include the interstitial collagenases, type IV collagenases and the stromelysins (Aznavoorian et al, 1993). Interstitial collagenases degrade type I, III and X collagen, which may be important in the ability of malignant cells to cross interstitial tissues (Templeton et al, 1990). Type IV collagenases degrade type IV, V, VII, IX, and X collagen, as well as fibronectin and elastin (Stetler-Stevenson, 1990). Stromelysins are a group of metalloproteases which have a wider substrate specificity (McDonnell & Matrisian, 1990). They can degrade proteoglycan core protein, laminin, fibronectin, and some types of collagen. There are four known types of stromelysin including stromelysin-1, stromelysin-2 and PUMP-1, also called matrilysin, and stromelysin-3. Metalloprotease activity is regulated in several ways. They are secreted from tumor cells in an inactive zymogen form which must be activated by cleavage. Metalloproteases can also interact with endogenous inhibitors called TIMP-1 and TIMP-2 (tissue inhibitor of metalloprotease). Thus, the activity of these proteases will depend on the rate of

secretion, the rate of activation and the presence of endogenous inhibitors (McDonnell & Matrisian, 1990).

Several studies have suggested a causal role for metalloproteases in tumor cell dissemination. Cells which have acquired metastatic ability by virtue of ras activation exhibited very high levels of stromelysin mRNA and also secreted high levels of this enzyme (Matrisian et al, 1985). The increased level of stromelysin expression was found to be due to the presence of a DNA element present 5' of the promoter of the stromelysin gene. This element was subsequently found to bind the product of the ets oncogene (Wasylyk et al, 1991). It has also been found that ets overexpression leads to increased stromelysin expression (Wasylyk et al, 1991). Thus, induction of the metastatic phenotype by ras presumably is partly due to its ability to utilize the ets transcriptional activator to induce stromelysin expression. However, it is not known how ras can regulate ets activity.

Strong evidence for a causal role of metalloproteases like stromelysin, in metastasis have come from studies of their natural inhibitors. TIMP-1 is a glycoprotein of 28.5 kd which forms a 1:1 complex with interstitial collagenase, activated stromelysin and a 94kd type IV collagenase. TIMP-2 is a 21 kd nonglycosylated secreted protein with 65.6% homology to TIMP-1 at the amino acid level. TIMP-2 can also inhibit all members of the metalloprotease family but unlike TIMP-1 preferentially binds to the 72kd type IV collagenase, rather than the 94kd collagenase (Liotta et al, 1991; Carmichael et al, 1986). Treatment of metastatic cells with TIMP-1 or TIMP-2 reduces their ability to invade and metastasize (Schultz et al, 1988). This suggests that degradation of extracellular matrix molecules by matrix metalloproteases is an important requirement for

the metastatic phenotype.

1.4 Cellular Locomotion in the Metastatic Phenotype

The next step in the invasion response involves active locomotion into the space left behind by matrix dissolution. Several studies suggest that tumor cell motility is required for the metastatic phenotype (Strauli & Weiss, 1977, Taylor et al, 1993). Indeed, metastatic cells show higher rates of motility than normal or benign transformed cells (Orr et al, 1981; Varani et al, 1978). Increased motility can be induced by factors which also induce the metastatic phenotype such as oncogene products and growth factors. For example the ras oncogene can induce the metastatic phenotype in appropriate cells (Greenberg et al, 1989). In addition, ras transformed cells show higher rates of motility and it has been found that the regulated induction of activated ras expression leads to a transient increase in cell motility (Varani et al, 1986; Turley et al, 1991). Similarly, microinjection of ras protein into fibroblast cells causes membrane ruffling, a process that accompanies cellular locomotion (Bar-Sagi & Feramisco, 1986). Cellular locomotion induced by ras was found to be dependent on RHAMM, which is a cell surface receptor for the glycosaminoglycan hyaluronan. The induction of ras leads to an increase in RHAMM expression on the cell surface and increased secretion of hyaluronan (Turley et al, 1991). Hyaluronan then can induce cellular locomotion as a consequence of binding to its cell surface receptor, RHAMM. The mechanism by which hyaluronan can induce cellular locomotion through RHAMM is not well understood.

Several secreted factors have been found to have motility inducing activities.

Scatter factor is secreted from several types of cells and is capable of increasing the motility of cells with scatter factor receptors (Stoker et al, 1987). Scatter factor was subsequently shown to be identical to hepatocyte growth factor (HGF) (Naldini et al, 1991). HGF was also previously shown to induce the proliferation of hepatocytes and was found to be an important factor in liver regeneration (Michalopoulos, 1990). The receptor for scatter factor/HGF is the product of the met gene which was originally described as an oncogene that can induce malignant transformation (Naldini, et al, 1991; Cooper et al, 1984). Thus induction of malignancy by met may be partly due to the constitutive activation of a signal transduction pathway that stimulates cellular locomotion.

TGFβ is a multifunctional secretory growth regulator which can stimulate cellular locomotion, an activity which may be important in the regulation of the metastatic phenotype by this molecule (Postlethwaite et al, 1987; Samuel et al, 1992). Metastatic, highly motile ras-transformed fibroblasts secrete higher levels of $TGF\beta_1$ than normal cells (Schwarz et al, 1990). It has been found that the high rate of motility in these cells is partly due to the presence of high levels of this growth factor which also is capable of stimulating the RHAMM/hyaluronan pathway for cell motility (Samuel et al, 1993).

Fibroblast growth factors are another group of secreted factors which can induce metastasis and have potent motility stimulatory activity (Wright et al, 1993; Burgess & Maciag, 1989). Basic fibroblast growth factor (bFGF) can induce the motility of endothelial and fibroblast cells (Taylor et al, 1993). bFGF molecules exert their biological functions by binding to cell surface receptor tyrosine kinases (Jaye et al, 1992). In the case of TGFβ, the cell surface receptors are serine/threonine kinases (Kingsly, 1994). Thus specific pathways emanating at the cell surface which involve tyrosine,

serine, or threonine phosphorylation can lead to increased motility.

These studies have suggested that metastatic dissemination requires altered cellular motility. During malignant transformation this is brought about by the unregulated expression of factors capable of stimulating locomotion, such as oncogene products and growth factors. Thus, some of the same biochemical changes that induce degradation of the extracellular matrix such as ras activation, can also conspire to induce altered locomotion which will allow the metastasizing tumor cell to migrate into the space created by matrix destruction.

Recent experiments have suggested a mechanism that may explain how cells are able to locomote and how this has the potential to be regulated by cell surface receptors for growth and motility factors (Stossel, 1993). It has been found that at the periphery of the cell in the cytoplasm is a pool of unpolymerized actin, called the cortical actin (Stossel, 1993). Actin is a cytoskeletal protein which in the interior of the cell is polymerized to form part of the cytoskeletal network (Herman, 1993). The polymerization of actin to form filaments which have considerable tensile strength is regulated by a class of actin binding proteins (Hartwig & Kwiatkowski, 1991). It is thought that the strength of filamentous actin is high enough to move the cell membrane (Condeelis, 1993). Thus, the regulated polymerization of part of the cortical actin might produce a cellular projection. Some actin binding proteins are regulated by phospholipids and many cell surface receptors alter membrane phospholipid metabolism when activated. Therefore, metabolism of lipids at cell membrane may lead to the production of a second signal which could ultimately lead to actin polymerization (Lassing et al, 1991; Hartwig & Kwiatkowski, 1991). However, cellular movement is more complicated than the

production of a cytoplasmic projection. In fact at the leading edge of some moving cells a large flat projection called a lamellopodia is observed. As the cell moves, the ventral plane of this structure becomes attached to the substratum via cell surface receptors while attachment is loosened at the trailing edge of the cell (Stossel, 1993). An attempt has been made to explain these complicated alterations on the basis of waves of actin polymerization (Stossel, 1993). However, the most intriguing feature of this model is that it implicates certain types of lipids in motility, which has the potential to explain how growth factors can alter cell movement.

Another cytoskeletal modification that is required by cells for locomtion is focal adhesion disassembly. Focal adhesions are formed at the cell surface and allow the cell to contact the substratum. They contain integrins, span the membrane and contact extracellular matrix molecules. Inside the cell, the integrin is associated with the cytoskeleton by its interaction with a number of proteins found at focal adhesions such as vinculin and talin (Burridge et al, 1988). During motility, focal adhesions are disassembled. Reduction of vinculin expression by treatment with antisense oligonucletides leads to increased locomotion (Rodriguez-Fernandez et al, 1993). Furthermore, transfection of fibroblasts with vinculin suppresses motility which is consistent with a role for focal adhesions in suppressing motility (Rodriguez-Fernandez et al, 1992). Recent studies have suggested how the formation of focal adhesions might be regulated (for a review see Zachary & Rozengurt, 1992). In some cell types, binding of integrins to their ligands leads to the formation of focal adhesions and the activation of cellular protein kinases which phosphorylate proteins found in those structures. One of the tyrosine kinase substrates is the focal adhesion kinase ($p125^{FAK}$) which itself is

activated by phosphorylation. A role for $p125^{FAK}$ in the formation of focal adhesions after integrin engagement is suggested by the effect of treatment with kinase inhibitors. Treatment of fibroblasts with herbimycin A inhibits the integrin induced phosphorylation of $p125^{FAK}$ and inhibits the formation of focal contacts (Burridge et al, 1992). Therefore, $p125^{FAK}$ may induce focal adhesion formation leading to alterations in cellular motility.

Tumor cell migration through tissue parenchyma requires a coordination between destruction of the matrix and motility. A specific model to account for this suggests that invasion of the intercellular spaces of a tissue and the basement membrane occurs by a cyclical process (Liotta, 1986). This process consists of attachment of the cell to the extracellular matrix, followed by secretion of enzymes which degrade the matrix, which is followed by locomotion into the resulting spaces. A repetition of this cycle is thought to allow metastatic cells to invade these tissue components. The first step in this process is attachment to the extracellular matrix mediated by cell surface receptors specific for components of this matrix. It has been suggested that binding to the matrix then triggers the release of enzymes from the tumor cell which can degrade the extracellular matrix. Support for this model comes from studies by Liotta's group which shows that laminin can induce the secretion of type IV collagenase from malignant cells (Turpeenniemi-Hujanen et al, 1986). This should lead to the production of a space into which the tumor must locomote in order to successfully invade. Interestingly, degradation products of collagen are potent regulators of motility in rat hepatoma cells, which suggests that the products of protease action on the extracellular matrix can induce movement into the resulting space (Nabeshima et al, 1986).

1.5 Metastatic Spread and Autonomous Growth

Tumor cell metastasis is dependent on the spread of tumor cells to distant sites via the circulation, where they grow as autonomous tumors. Successful dissemination depends on the ability of the tumor cell to survive in the circulation and grow in a new tissue environment. Tumor cells within the bloodstream are subject to considerable shear forces as they are forced through branching channels in capillary beds. Studies of metastatic cells show that physical deformability is variable and may play an important role in avoiding mechanical cytolysis in the circulation (Gabor and Weiss, 1986). Metastatic cells are also subject to attack by cells of the immune system and in several systems alterations in immune recognition are responsible for alterations in metastatic potential (Miller, 1993).

Tumor growth in a new tissue environment will also require unregulated proliferation and an independence from adjacent normal cells. Tumor cell independence may be aided by the downregulation of gap junction formation which is induced by malignant transformation (Hotz-Wagenblatt and Shalloway, 1993). However, absolute autonomy can only allow limited tumor growth, as tumors require their own source of nutrients and oxygen (Liotta et al, 1991). This requirement is fulfilled by new blood vessels which form by the process of angiogenesis (Folkman and Klagsbrun, 1987). Metastatic tumor deposits are capable of stimulating this process in their host. Angiogenesis is controlled by "angiogenic" factors secreted by the tumor. These factors, such as bFGF, stimulate the proliferation and motility of endothelial cells which then form a vessel leading to the tumor. In addition to bringing required nutrients, angiogenesis

also furnishes the tumor with new routes of dissemination so that the metastatic process can be initiated again.

1.6 Genetic Regulation of the Metastatic Phenotype

The capacity to metastasize is a heritable phenotype, acquired as a result of genetic alterations in oncogenes, tumor suppressor genes, and metastasis regulatory genes (Greenberg et al, 1989). The oncogenes are a diverse group of growth regulatory genes which stimulate the neoplastic growth of cells when they become activated (Bishop, 1987). Diverse types of oncogenes including growth factors, growth factor receptors and intracellular signal transduction molecules can induce the metastatic phenotype when these genes become activated (Egan et al, 1987b). The ras protein is one example of a signal transduction molecule capable of inducing metastatic ability (Thorgeirsson et al, 1985; Egan et al, 1987a). It has also been shown that other oncogenes including the growth factor receptor, v-fms, and the growth factors K-FGF and bFGF can induce the metastatic phenotype (Egan et al, 1987b; Damen et al, 1991; Egan et al, 1990).

In contrast to the oncogenes, tumor suppressors must be inactivated to stimulate tumor progression. The p53 gene encodes a transcription factor whose loss contributes to neoplastic transformation (Finlay et al, 1989) and malignant progression (Taylor et al, 1992). In 1988, Jens Pohl found that cells in which p53 was inactivated were more metastatic than those containing a wild type p53 (Pohl et al, 1988a). The nm23 gene also induces the metastatic phenotype when inactivated, however it's mechanism of action may be different from p53 (Leone et al, 1991). Although the nm23 can act as a transcription

factor which regulates the transcription of the myc oncogene, it also encodes a nucleoside diphosphate kinase (Leone et al, 1991; Postel et al, 1993). The involvement of this enzymatic activity in the regulation of the metastatic phenotype is not known. These studies suggested the possibility that cancer metastasis naturally occurs as a result of the inappropriate activation of oncogenes and loss of tumor suppressor gene function during tumor progression.

2. Fibroblast Growth Factors and the Metastatic Phenotype.

2.1 The Fibroblast Growth Factor Family

The fibroblast growth factor family consists of at least seven members which exhibit diverse biological effects on a wide spectrum of cell types (for a review see Burgess & Maciag, 1989; Gospodarowicz, 1989). Some of the members of this family have been directly implicated in the metastatic phenotype (Damen et al, 1991; Egan et al, 1990). Two of the first discovered and best studied isoforms are the bFGF and the acidic fibroblast growth factor (aFGF). Basic FGF was originally purified from the bovine pituitary on the basis of its ability to stimulate fibroblast proliferation. During the same period, aFGF was purified from brain based on its ability to stimulate endothelial cell proliferation. K-FGF is a more recent member which has been implicated in cellular transformation. The K-FGF gene has been frequently isolated during the selection of transformed foci of NIH3T3 cells transfected with human tumor DNA. For example, K-FGF was isolated after transfection of NIH3T3 cells with DNA from a Kaposi sarcoma

tumor (Delli-Bovi et al, 1987). The identical gene, termed hst was isolated by a different group using a similar method with either gastric cancer DNA or even DNA from normal stomach mucosa (Sakamoto et al, 1986). The ability of the K-FGF genomic sequence cloned from normal human DNA to transform NIH3T3 cells suggests that overexpression and not mutation is the mechanism of activation of this oncogene (Delli-Bovi et al, 1988; Yoshida et al, 1987). Consistent with this, amplification of the hst locus is a common feature of human breast and esophageal cancer (Adnane et al, 1989; Tsuda et al, 1989; Tsuda et al, 1989). Also included in the FGF family are keratinocyte growth factor (KGF), int-2, hst/kFGF, fgf-5, and fgf-6 (Burgess & Maciag, 1989; Gospodarowicz, 1989).

The polysulfated glycosaminoglycan heparin binds with high affinity to several members of the FGF family and potentiates their biological effects. FGF's are distributed in many different tissues and have both cytoplasmic, nuclear, and extracellular localization (Burgess & Maciag, 1989). Recently, an amino acid sequence present in bFGF has been found to be responsible for its nuclear localization, however the role of nuclear bFGF is not clearly understood (Zhan et al, 1992). Extracellular FGF is occasionally found in association with the extracellular matrix. FGF's have been found in basement membranes and subendothelial extracellular matrix. Furthermore, FGF's can be released from these matrices by the action of hydrolytic enzymes such as heparitinase and heparinase (Burgess et al, 1989).

2.2 The Fibroblast Growth Factor Receptor Family

The biological effects of FGF's are mediated, in part, by binding to cell surface receptors specific for these growth factors. Both high and low affinity FGF receptors have been described and both types appear to be necessary for a biological effect (for a review see Jaye et al, 1992; Klagsbrun & Baird, 1991). Low affinity receptors are commonly cell surface heparan sulfate proteoglycans which do not induce intracellular signalling events when they bind to FGF molecules. However, binding to the low affinity receptor appears to be required for the interaction of the growth factor with it's specific high affinity receptor. The high affinity receptors are responsible for intracellular signalling events. Cytoplasmic signalling is initiated by tyrosine phosphorylation of cellular proteins including the high affinity receptors, by a ligand-dependent tyrosine kinase intrinsic to the cytoplasmic domain of these receptors (Jaye et al, 1992). There exist at least four high affinity receptors for FGF's including flg, bek, cek-2,and fgfr-4. These receptors have the potential for a much larger diversity due to extensive splice variation. For example, there are nine splice variants of flg and eleven splice variants of bek. These variants give rise to receptors with very different structures including secreted forms of the receptor lacking the tyrosine kinase domain, and intracellular forms with a kinase domain (Jaye et al, 1992). In the case of transmembrane FGF receptor variants, signal transduction is thought to be initiated by ligand binding, followed by receptor oligomerization and receptor transphosphorylation. Transphosphorylation results in the production of binding sites for additional signalling molecules, such as PLCγ (phospholipase C), which binds to tyrosine phosphate containing peptides in the

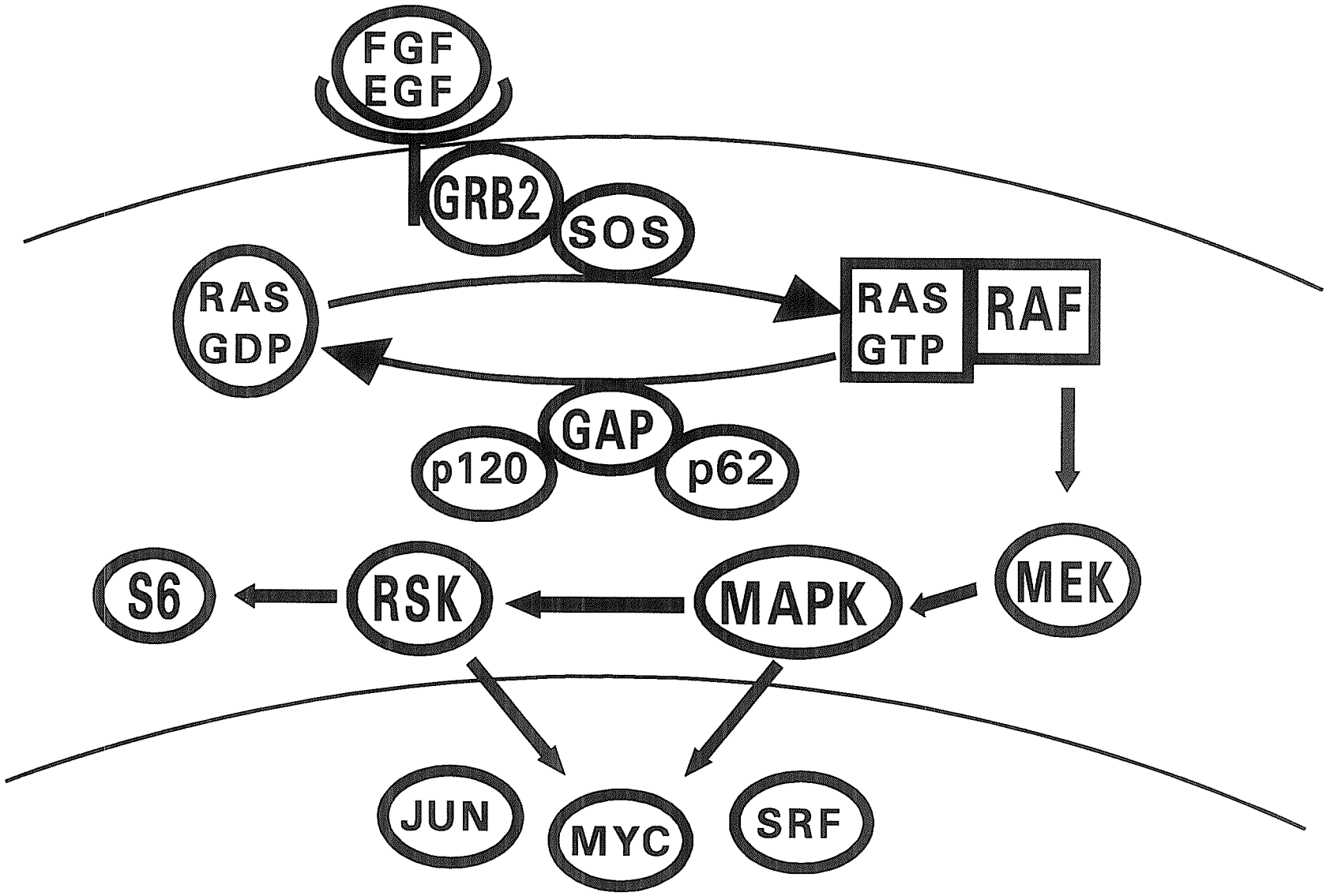
cytoplasmic domain of the FGF receptor (Mohammadi et al, 1991). This binding event may bring PLCγ into proximity with substrates it can use to initiate an intracellular signal. Additional studies in the neuronal PC12 cell have also implicated a sequential requirement for the function of the tyrosine kinase src and the small GTP binding protein ras in FGF signalling (Kremer et al, 1991). Furthermore, the induction of mesoderm in *Xenopus* embryos by bFGF is dependent on the raf cytoplasmic serine threonine kinase and is associated with the activation of the mitogen activated protein kinase (MacNicol et al, 1993; Graves et al, 1994; figure 2). The result of activation of these signalling molecules can include alterations in cellular proliferation or motility depending on the cell type.

2.3 Malignant Transformation by Fibroblast Growth Factors

Fibroblast growth factors are potent inducers of mesenchymal and endothelial growth and motility (Burgess & Maciag, 1989). Some of these factors, such as K-FGF can also lead to cellular transformation (Yoshida et al, 1987). In contrast to K-FGF, bFGF has limited transforming potential, and this has been linked to the absence of a signal sequence. For example, bFGF which naturally is found without a signal sequence can marginally transform cells when very high intracellular levels of growth factor are achieved (Quarto et al, 1989). However, K-FGF which naturally is secreted due to a signal sequence and bFGF fused to a signal sequence from another gene are capable of transforming cells at a much higher frequency (Yoshida et al, 1987; Rogelj et al, 1988). Furthermore, deletion of the signal sequence from K-FGF results in a protein that is no

FIGURE 2.

The ras dependent signal transduction pathway. Growth factors, such as epidermal growth factor (EGF) and fibroblast growth factor (FGF) carry out biological functions by stimulating a ras dependent signal transduction pathway. The pathway is initiated by receptor phosphorylation which induces complex formation between the growth factor receptor, the grb2 (growth factor receptor bound protein-2) adaptor protein and the son of sevenless homologue (SOS). SOS then induces exchange of GDP for GTP on ras. The GTP bound form of ras can bind to the raf protein kinase, which can phosphorylate and activate the MEK kinase. MEK can activate the mitogen activated protein kinase (MAPK) which phosphorylates several early response proteins such as jun, myc, and the serum response factor (SRF). MAPK also activates the ribosomal S6 kinase (RSK) which phosphorylates the ribosomal S6 protein.


FGF
EGF
GRB2
SOS
RAS
GDP
RAS
GTP
RAF
GAP
p120
p62
S6
RSK
MAPK
MEK
JUN
MYC
SRF

longer secreted and does not transform transfected cells. Similar results were obtained when the K-FGF signal sequence was replaced with an endoplasmic reticulum retention signal (Fuller-Pace et al, 1991; Talarico & Basilico, 1991). Consistent with these reports it has been shown that signal sequence bFGF (ssbFGF) and K-FGF but not wild type bFGF can induce the metastatic phenotype in transfected fibroblasts (Damen et al, 1991; Egan et al, 1990). The requirement for secretion is in agreement with the presence of cell surface FGF receptors. Access to these receptors would presumably require secretion, however the role of intracrine stimulation of the same or other receptors by wild type bFGF has not been ruled out. The ability of FGF's to induce the metastatic phenotype has been recently been linked to their induction of increased tumor cell locomotion (Taylor et al, 1993).

Recent studies have shown that fibroblast growth factors are also capable of regulating one aspect of genomic instability. During the process of tumor progression, oncogenes are activated and tumor suppressors inactivated. One of the mechanisms of oncogene activation is through the amplification of DNA sequences containing an oncogene. For example, the N-myc gene is frequently amplified in neuroblastoma (Kohl et al, 1983). The ability of cells to amplify DNA is variable and tumor cells can show very high rates of amplification (Tlsty et al, 1989). High rates of DNA amplification are observed in cell lines overexpressing K-FGF or bFGF suggesting that these growth factors play an important role in the regulation of this process (Huang & Wright, 1994).

3. The Role of Oncogenes in the Metastatic Phenotype

3.1 The ras Oncogene

The ras gene can induce the metastatic phenotype, and is required for several of the biological activities of bFGF including stimulation of motility in endothelial cells (Greenberg et al, 1989, Kremer et al 1991, D. Stacey, personal communication). Mammalian ras genes were first implicated in neoplastic transformation by transfection experiments using DNA from human tumors (Shih & Weinberg, 1982). They were one of the first loci to be experimentally shown to induce the metastatic phenotype (Thorgeirsson et al, 1985). The ras genes were first encountered as part of the genome of the Harvey Sarcoma transforming viruses (HSV)(Coffin et al, 1981). The ras sequences in these viruses were derived from rat DNA sequences that had become integrated into the virus. The transforming capability of these viruses was attributable to these rat sequences. Several years later, the ras gene was also found to be present in the human genome in normal tissue. It was found that DNA sequences derived from the T24 EJ bladder carcinoma were capable of transforming NIH3T3 cells. When the gene responsible for this effect was cloned and sequenced it was found to be homologous to the ras gene sequence in HSV (Parada et al, 1982). The same gene was present in normal human tissue however, it was evident that the T24 ras molecule contained a mutation (Tabin et al, 1982). This was a glycine to valine transition at codon 12 which leads to production of a mutant ras protein with enhanced biological activity.

The three known members of the ras gene family are N-ras, K-ras, and H-ras. H-

ras is found on chromosome 11, N-ras is found on chromosome 1 and K-ras is located on chromosome 12 (for a review see Barbacid, 1987). All ras genes have four exons and three introns. The protein encoded by ras genes is a 21000 dalton phosphoprotein which is localized to the inner cytoplasmic membrane. Membrane localization is accomplished by the covalent attachment of isoprenyl or palmitoyl groups to the CAAX amino acid sequence (X = any amino acid) in the carboxyl terminus of the ras protein. This lipid is inserted into the lipid bilayer of the plasma membrane anchoring ras to this location. p21ras proteins bind to the guanine nucleotides GDP and GTP. p21 ras also has an intrinsic GTPase activity which allows it to convert bound GTP to GDP. It is also known that the GTP bound form of ras is the active form of ras which transmits signals within the cell. Indeed, activating mutations in ras lead to either an impaired GTPase activity or an enhanced rate of release of GDP, or both of these alterations. Impaired GTPase activity will lead to higher levels of the active GTP bound form of ras. The T24 EJ H-ras allele is an example of such a mutant. High levels of GTP bound ras will also be the case when there is an increased rate of GDP release as the intracellular GTP concentration is tenfold higher than GDP (Barbacid, 1987).

Studies with mutated ras genes found that they were capable of transforming fibroblasts in tissue culture. Transformation was characterized by loss of contact inhibition of growth, the ability to grow independent of anchorage to a substratum and the ability to form tumors in animals (Spandidos & Wilke, 1984). It was also found that the ability of ras to transform fibroblasts could be greatly enhanced when an additional growth regulatory gene was also deregulated (Land et al, 1983; for a review of oncogene cooperation see Hunter, 1991). For example, the introduction of mutant ras along with

an overexpressed myc gene led to higher levels of transformation compared to ras alone (Land et al, 1983). Myc was incapable of transforming on its own. Oncogene cooperation in transformation was also observed with other combinations of oncogenes such as src and large T antigen from the SV40 DNA virus. Some combinations could not cooperate leading R. A. Weinberg and colleagues to propose that there were at least two complementation groups for oncogenes (Land et al, 1983). The first group was termed the transforming genes or the cytoplasmic oncogenes and included ras and src. These genes were capable of transforming on their own but at a relatively low frequency. Genes within this group did not cooperate. The second group was termed the nontransforming or nuclear oncogenes and included myc and p53. These genes could not transform fibroblasts on their own, and genes in this group could not cooperate with each other. It was clear that many combinations including one gene from each group led to an enhancement of fibroblast transformation.

The normal function of ras may be involved in integrating signals impinging from the outside of the cell into messages that will direct a biological response. The ras protein is an essential protein required for signal transduction events initiated by tyrosine kinase oncoproteins. Thus, inactivation of ras by microinjection of cells with ras antibodies inhibits the ability of the src tyrosine kinase to induce cellular transformation (Smith et al, 1986). ras is also required for signals emitted by transmembrane receptor tyrosine kinases such as the PDGF (platelet derived growth factor), and bFGF receptors (Mulcahy et al, 1985; Kremer et al, 1991). The molecular basis of this may lie in the interaction of ras with additional signal transduction molecules.

The cellular regulation of ras has been partially elucidated. As mentioned above,

ras proteins bind guanine nucleotides. The rate of ras GTPase activity and the rate of nucleotide exchange on ras is enhanced in the cell by GAP (GTPase activating proteins) proteins and GDRF (Guanine Diphosphate releasing factor) proteins respectively (Boguski & McCormick, 1993). GDRF proteins bind to ras and in so doing displace the GDP bound to ras. Empty ras is then able to bind to GTP and since GTP is in tenfold excess over GDP in the cell the action of GDRF will result in ras then being bound to GTP. Recent experiments suggest that several of these GDRF proteins may be regulated by tyrosine kinases of the receptor and nonreceptor type. For example, it was found that the EGF receptor tyrosine kinase can bind to a heterodimeric complex consisting of mSOS (murine son of sevenless homologue), which is a GDRF for ras and the grb2 protein which acts as a bridge between the receptor and the GDRF (Egan et al, 1993). Due to the localization of the EGF receptor, this multimeric complex is localized to the inner surface of the plasma membrane which allows the mSOS GDRF to have access to ras. This leads to replacement of GDP on ras by GTP leading to ras activation. ras activation has recently been shown to have several important downstream effects. This includes the activation of a protein kinase cascade which results in the sequential activation of at least four protein kinases (Crews & Erikson, 1993; Khosravi-Far, 1993). The first kinase to become activated is the product of the raf oncogene. raf encodes a serine threonine kinase which binds with high affinity to the GTP bound form of ras, but not the GDP-bound form. Binding to ras is thought to alter the conformation of raf leading to the activation of its kinase activity (Zhang et al, 1993; Vojtek et al, 1993). The raf kinase then is capable of phosphorylating the MEK kinase which becomes active and catalyzes the addition of activating phosphates onto its only known substrate, the MAP kinase

(Howe et al, 1992; Crews et al, 1992). Active MAP kinase then phosphorylates and activates transcription factors such as fos and myc which are known to stimulate cellular proliferation (Crews & Erikson, 1993; figure 2).

p120 GAP also may be involved in the tyrosine kinase ras pathway. GAP can bind to ras but unlike GDRF molecules, GAP can activate the intrinsic GTPase activity of ras leading the conversion of bound GTP to GDP (Trahey & McCormick, 1987). Interestingly, GAP can bind efficiently to the phosphotyrosine containing cytoplasmic domains of several tyrosine kinase receptor molecules such as the PDGF receptor (Kazlauskas et al, 1990). Therefore GAP may represent an addition link between tyrosine kinases and ras, however, the details of this are not well understood. There is evidence that GAP may also work downstream of ras carrying signals from ras into the cell. For example, GAP binds to the effector domain of ras (Adari et al, 1988). This domain, when mutated, does not lead to alterations in GTPase activity but abolishes the ability of ras to signal. In addition, GAP binds to two identified cellular proteins, p190 and p62 (Ellis et al, 1990). The genes encoding both of these proteins have been cloned and from sequence comparisons, p190 encodes three regions of homology to a GTPase, a GAP and a transcriptional repressor (Settleman et al, 1992). p62 encodes regions of homology to a protein involved in mRNA processing (Wong et al, 1992). The precise role of these GAP associated proteins in ras signalling is not yet known.

3.2 The myc Oncogene

The c-myc gene is a member of a family of oncogenes which also contains L-

myc, N-myc, s-myc and b-myc (for a recent review see Zimmerman & Alt, 1990; Lüsher, 1990). The myc oncogene was originally discovered as the cellular homologue of the transforming sequence from the MC29 avian retrovirus (Sheiness & Bishop, 1979). It was then found to be present at the junction of a common chromosomal translocation in Burkitt's lymphoma (Chung et al, 1986). This translocation places the coding exons of myc adjacent the immunoglobulin promoter in B cells leading to overexpression of myc and transformation of these cells. The myc family of genes also include N-myc which is amplified in neuroblastomas, L-myc, which is amplified in small cell tumors of the lung, and b-myc, and s-myc which have been less well characterized (Kohl et al, 1983; Nau et al, 1985; Ingvarsson et al, 1988; Sugiyama et al, 1989). As suggested, the main mode by which myc can be activated to transform cells is by overexpression. The c-myc, L-myc, and N-myc genes all consist of 3 exons and 2 introns, where the first exon is noncoding. Expression is regulated in c-myc by three promoters, P1, and P2 which are found in the first exon and Po which is found upstream of the exon 1 (Spencer & Groudine, 1991).

The proteins encoded by the c-myc gene, are nuclear phosphoproteins of 62 and 64000 daltons. The c-myc proteins result from alternative translation initiation at an AUG codon and a CUG codon 15 codons upstream (Hann et al, 1988). The myc protein binds to DNA and contains domains of functional importance with homology to transcriptional activator proteins. These regions include a bHLH domain which consists of a stretch of basic amino acids followed by two amphipathic α-helices separated by a loop. A second region is a leucine zipper motif which consists of a long α-helix with intermittent leucine residues (Landschulz et al, 1988). Both the bHLH and leucine zipper

motifs are used by other proteins as dimerization surfaces. For example, both the fos and jun proteins dimerize at their leucine zipper to form the AP-1 transcriptional regulatory complex (Landschulz et al, 1988). Recently a protein which is capable of dimerizing to myc in such a manner has been identified. This protein termed, max can bind to myc through its own bHLH leucine zipper motif (Blackwood & Eisenman, 1991). These myc+max heterodimers can bind to a specific DNA sequence containing the core hexanucleotide sequence CACGTG and activate transcription of genes which are linked to this sequence (Kretzner et al, 1992; Amin et al, 1993). Two additional proteins termed mad and mxi1 have been recently described to form heterodimers with max which are incapable of stimulating transcription of genes linked to the myc binding element (Zervos et al, 1993; Ayer et al, 1993; figure 3). In addition, max can homodimerize to form a complex that suppresses transcription from myc binding elements (Amin et al, 1993). In addition, the ability of myc to transform cells and to cooperate with ras in transformation is dependent on its ability to heterodimerize with max (Amati et al, 1993). Recent experiments suggest that myn, the murine homologue of max is inducible by an activated ras allele and that high levels of myn can suppress transformation by ras. One mechanism to explain these observations is that high levels of myn which result from ras transformation form homodimers which suppress the transcription of myc target genes. This suggest that the molecular basis of ras+myc cooperation may be the result of the conversion of excess transcriptionally inactive myn homodimers to heterodimers of myc and myn which stimulate the transcription of myc target genes and enhance transformation (Taylor et al, 1994).

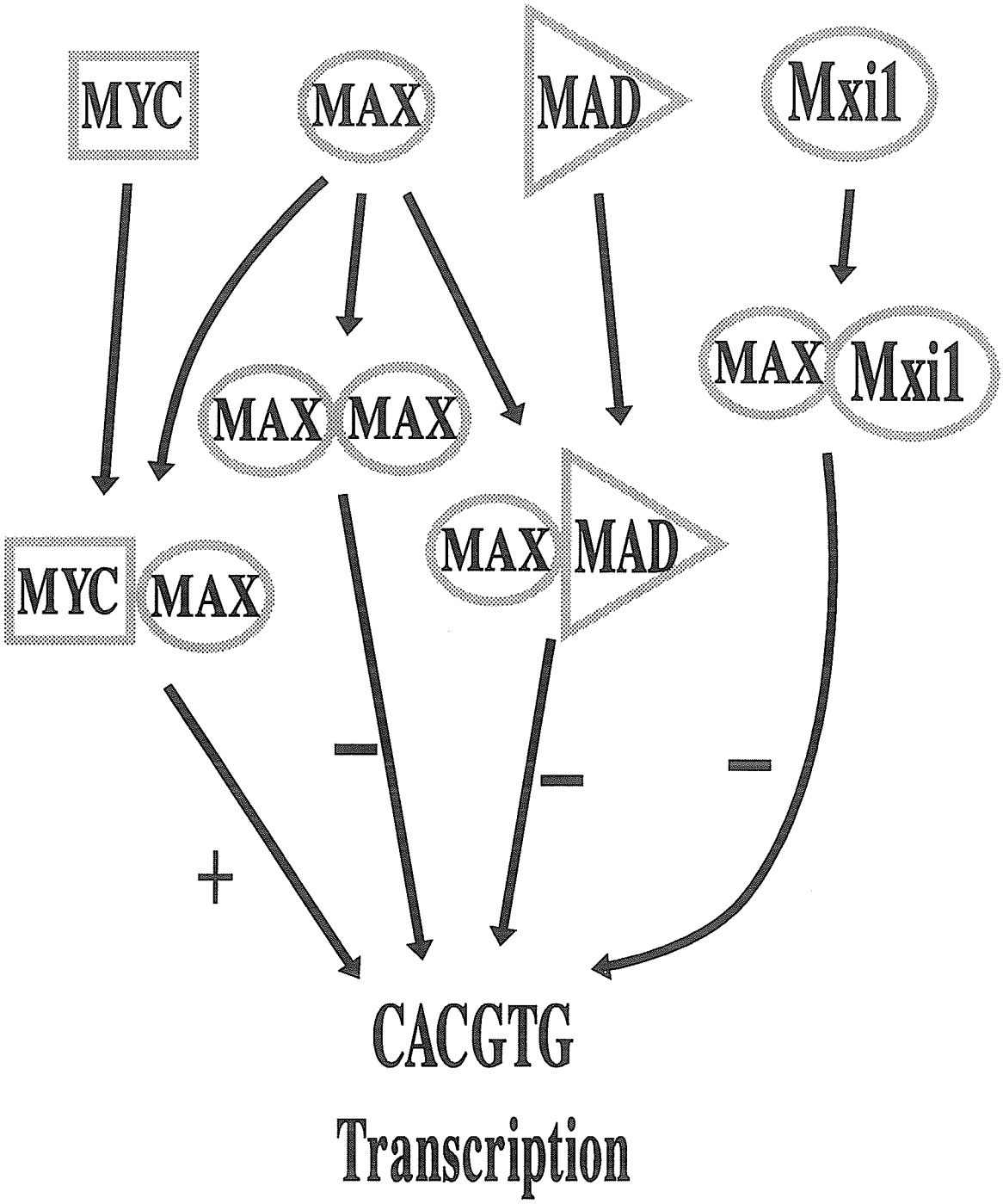
The biological activity of the myc protein has been studied by transfecting the myc

gene into cells *in vitro* and *in vivo*. Myc overexpression following transfection leads to immortalization of senescent rat embryo fibroblasts (Land et al, 1983). This is in contrast to ras which, although able to transform REF's cannot efficiently immortalize them. However, myc does not transform this cell type. In some immortalized cell lines, myc is capable of inducing tumorigenicity, suggesting that the ability to transform by myc is cell type specific (Keath et al, 1984). These studies suggest a role for myc in normal cells, which is to regulate cellular proliferation. This is supported by the expression patterns of the endogenous myc genes in different cells. For example, quiescent cells show very low myc levels, and when stimulated to enter the cell cycle by serum addition show a large transient increase in myc mRNA expression (Müller et al, 1984). Furthermore, microinjection of purified myc protein into cells induces DNA synthesis (Kaczmarek et al, 1985). When certain types of cells are induced to differentiate *in vitro*, the levels of myc mRNA are markedly reduced. For example, stimulation of the promyelocytic cell line HL60 with agents that induce terminal differentiation leads to a decrease in myc expression by both transcriptional and post transcriptional regulation. Decreased myc expression in HL60 cells is observed before the appearance of any markers of differentiation (Sienbenlist et al, 1988; Bentley & Groudine, 1986). Furthermore, enforced myc expression can inhibit the differentiation of 3T3-Li preadipocytes into mature adipocytes when challenged with differentiation inducing agents (Freytag, 1988). These experiments suggest that myc expression is required for cell proliferation, but inhibitory to differentiation. This is consistent with the ability of myc to contribute to neoplastic transformation.

The myc protein has recently been implicated in apoptosis. Apoptosis is the

FIGURE 3.

Intracellular myc binding regulatory proteins. Myc activates transcription from CACGTG elements only when in a heterodimeric complex with max. Max dimeric complexes containing either mad, mxi1 or max itself can bind to the CACGTG element but cannot stimulate transcription from these sequences.


MYC
MAX
MAD
Mxi1
MAX MAX
MAX Mxi1
MYC MAX
MAX MAD
+
CACGTG
Transcription

process of programmed cell death. This process is an important physiological process during mammalian development and it is characterized by nuclear condensation, membrane blebbing, cytoplasmic condensation, and finally, cell fragmentation (Williams & Smith, 1993). It was found that when RAT1 cells, which overexpress c-myc, were cultured in low serum, the cell number did not increase even though cell division was not arrested. This was due to the apoptotic death of these myc overexpression cells (Evan et al, 1992). Induction of cell death by myc contrasts with it's ability to induce cellular proliferation and transformation. This has led to the suggestion that myc regulates both proliferation and cell death, and for a cell to survive high myc levels another alteration that protects from apoptosis must occur. This may be an alteration such as ras activation which mimics the presence of growth serum by inducing a growth signal within the cell. Thus, the inhibition of myc induced apoptosis by a second complimenting oncogene represents one model to explain the previously described phenomenon of oncogene cooperation.

3.3 The p53 Tumor Suppressor.

The p53 protein was first identified by Lane and Crawford in 1979. They described it as a cellular protein that binds to the large T antigen of SV40 tumor virus (Lane & Crawford, 1979). It was also capable of acting as a tumor antigen and was found to have elevated levels in a number of tumor cells (Deleo et al, 1979; Mowat et al, 1985). A direct role for p53 in regulating cellular proliferation was first suggested when the gene encoding p53 was introduced into fibroblasts. These experiments showed

that p53 could confer an indefinite lifespan on senescent rat embryo fibroblasts, and could cooperate with a mutant ras in the transformation of these cells (Jenkins et al, 1984; Parada et al, 1984). These early studies suggested that the p53 protein was a dominantly acting oncoprotein, capable of stimulating tumor progression when overexpressed. However, several observations could not be easily accounted for by this model of p53 function. Most notably, the p53 locus was found to be inactivated during the progression of erythroleukemia in mice. This occurred by the integration of the Friend erythroleukemia virus into both alleles of the p53 gene (Hicks & Mowat, 1988). In addition, a clone of p53, termed 11-4, was isolated and found to be unable to transform cells in cooperation with ras (Finlay et al, 1988). The sequence of 11-4/p53 was compared to several earlier clones capable of transforming cells. It was evident that amino acid substitutions had occurred in clones capable of transforming. Comparison of the genomic 11-4/p53 with a transforming genomic clone termed LTRcGp53 showed that the LTR clone had a valine at position 135 whereas the 11-4 sequence encoded an alanine (Hinds et al, 1989). Replacement of alanine by valine in the 11-4 clone activated this protein for transformation. By analyzing the restriction enzyme digest patterns of normal mouse tissue DNA it was found that valine was the wild type amino acid at this position, strongly suggesting that the 11-4 clone was a wild type p53 (Hinds et al, 1989).

The identification of amino acid alterations in some alleles of p53 suggested that the ability to transform in cooperation with ras is an activity that occurs upon mutation of p53. This led to the idea that wild type p53 may actually act as a tumor suppressor protein consistent with it being disrupted by virus integration. It was also suggested that mutant p53 may be able to interfere with the wild type function of the endogenous p53

protein, allowing mutant p53 to immortalize and transform cells (Lane & Benchimol, 1990). Strong evidence for this hypothesis was available when the wild type form of p53 was found to be capable of suppressing the frequency of transformation by activated ras in combination with the adenoviral transforming protein E1a (Finlay et al, 1989; Eliyahu et al, 1989). These studies strongly support the contention that p53 acts as a tumor suppressor gene which, if inactivated contributes to cancer progression.

It is now known that the p53 gene is one of the most frequently mutated loci in human cancer (for a review see Hollstein et al, 1991). The identification of loss of p53 function in human tumors first came from the study of colorectal cancer tumorigenesis (Baker et al, 1989). It was known for several years that late in the progression of this disease, deletion of allelic markers on chromosome 17 occur, in the vicinity of p53 (Bos et al, 1987). In 1989, Bert Vogelstein and his colleagues analyzed this region in more detail in colorectal tumors (Baker et al, 1989). They found that in 75% of tumors, deletions of one allele of the short arm of chromosome 17 occurred. In both of two tumors that were analyzed, the remaining p53 allele was found to contain a mutation. This led to the hypothesis that somatic mutation in p53 coupled with loss of the remaining normal allele is required for colorectal progression. Mutations in p53 are also found in many other carcinomas including those originating from the lung, breast, esophagus, liver, bladder, ovary, and also in leukemias and some sarcomas (Hollstein et al, 1991)

3.4 The Structure of the p53 Gene and Protein.

There are several important structural features of the p53 gene and protein which

suggest how it may carry out its tumor suppressor function (for a review see Vogelstein & Kinzler, 1992; Montenarh, 1992; Prives & Manfredi, 1993). The p53 gene is found on the short arm of chromosome 17. It contains 11 exons, the first of which is noncoding, and 10 introns. The p53 gene is regulated by two promoters. The P1 promoter is found 150 to 250 bp upstream of exon I, and the P2 promoter is found within the first intron. Upstream of these elements are found binding sites for the NF-1, AP-1, and HLH type transcription factors, which presumably regulate the activity of the p53 promoters.

The p53 protein is a phosphoprotein of 53,000 daltons which contains an amino terminal acidic region approximately 80 residues long and a carboxyl terminal basic region of about 115 residues (Montenarh, 1992). The p53 protein forms many complexes in the cell. For example, proteins encoded by several DNA tumor viruses have evolved to bind the p53 protein and may inactivate it by this mechanism (Schreier & Gruber, 1990; Green, 1989). Inactivation of the tumor suppressor function would allow virally infected cells to proliferate which would allow more efficient viral replication. The large T antigen of the SV40 tumor virus, and the large tumor antigen of lymphotropic papovavirus both bind to p53 but only when the p53 protein is unmutated (Finlay et al, 1988; Symonds et al, 1991). p53 is also bound by the E1b protein of adenovirus 5, and the E6 protein of human papilloma viruses 16, and 18 (Sarnow et al, 1982; Werness et al, 1990). The mutant version of p53 binds to the cellular hsp70 protein, and the wild type p53 protein binds to several cellular proteins including a protein called MDM2 (Finlay et al, 1988; Momand et al, 1992). The normal function of interaction with hsp70 and MDM2 is not clearly understood, however, the MDM2 gene has been found to be

amplified in sarcomas suggesting that the MDM2 protein may inactivate the wild type p53 protein allowing tumor progression to occur (Oliner et al, 1992). p53 is also capable of binding to two cellular protein serine threonine kinases. These are the cdc2 kinase which acts as a master regulator of cell cycle progression and casein kinase II. Both of these kinases can phosphorylate p53 and it is known that at least for casein kinase II, phosphorylation is required for one of the biochemical functions of p53 which will be described below (Milner & Medcalf, 1990; Herrmann et al, 1991).

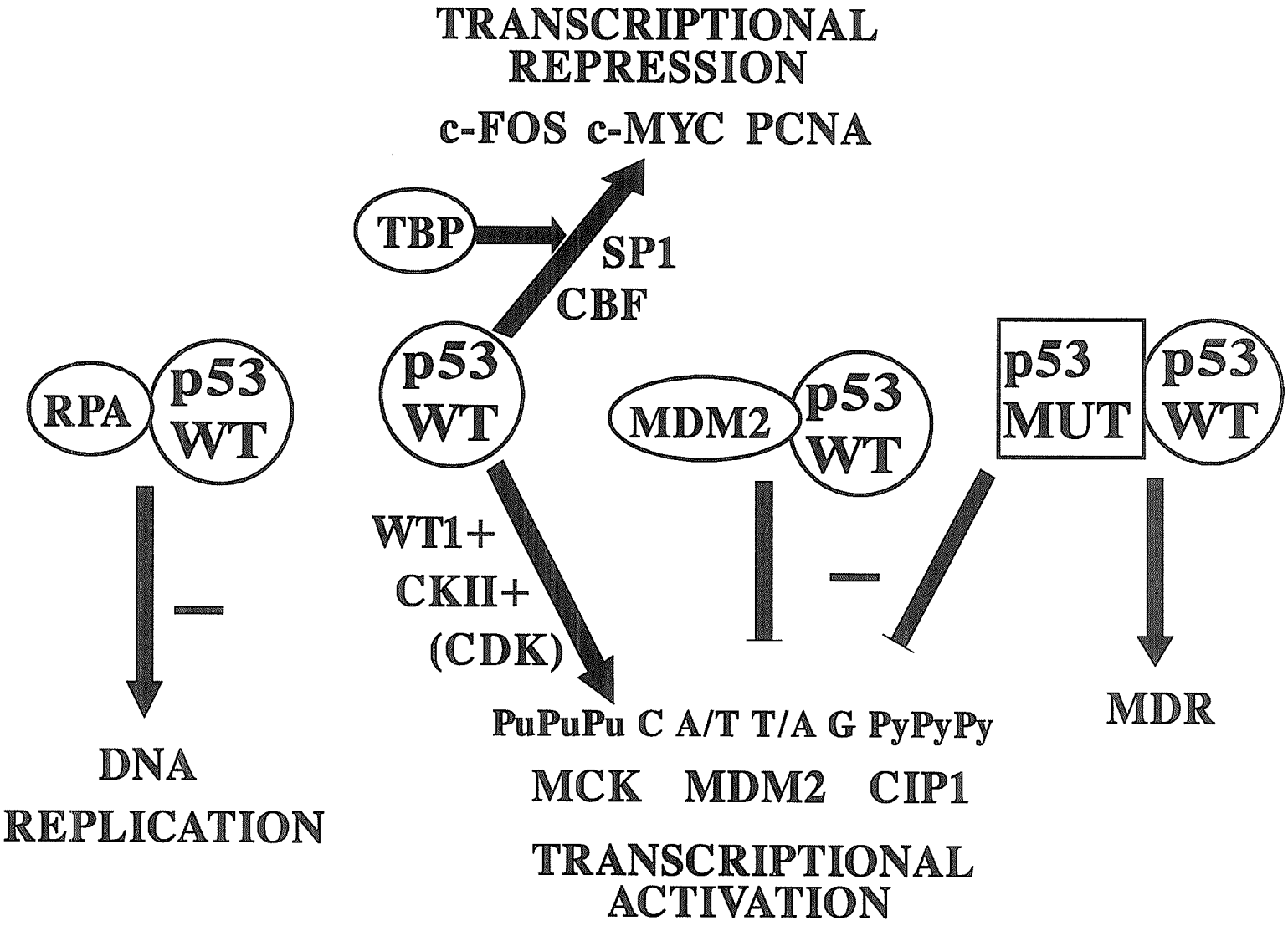
The p53 protein is primarily a nuclear protein however it has been found to be localized in the cytoplasm during G1 and late S phase of the cell cycle of growth stimulated cells (Shaulsky et al, 1990). In these cells, p53 is found in the nucleus at the beginning of S phase. p53 can bind nonspecifically to single stranded and double stranded DNA which would be consistent with a nuclear localization (Steinmeyer & Deppert, 1988). It can also bind specifically to a sequence containing two or three repeats of the sequence TGCCT. Once bound, p53 can activate the transcription of genes linked to this sequence (Kern et al, 1991). Several natural promoters are positively regulated by p53. These include the LTR intracisternal A particle, muscle specific creatine kinase and the IL-6 promoter. p53 can repress the transcription of c-fos, β-actin, c-jun, MHC class I and its own promoter (Montenarh, 1992). It has been recently shown that binding of MDM2 to p53 can inhibit its ability to mediate sequence specific transcriptional activation (Momand et al, 1992). Furthermore, the ability of p53 to induce the expression of mdm2 by binding to a TGCCT element in the mdm2 promoter has suggested that there is a negative feedback loop involving these two proteins (Juven et al, 1993).

Phosphorylation of p53 by casein kinase II is required for p53 to activate transcription and allow it to suppress neoplastic transformation (Hupp et al, 1992; Milne et al, 1992). p53 also binds to another tumor suppressor with transcriptional activation activity. This is the product of the WT-1 gene. Binding of p53 to WT-1 potentiates the ability of p53 to activate transcription but inhibits the ability of WT-1 to activate (Maheswaran et al, 1993). The ability of p53 to repress transcription has been suggested to be due to its ability to bind to the basal transcription factor, TATA binding protein (TBP)(Chen et al, 1993; figure 4).

The regulation of genes by p53 may be involved in its ability to regulate cell proliferation at the level of cell cycle control. It has been found that microinjected antibodies to p53 can inhibit the entry of growth stimulated cells to S phase, suggesting an important role in G1-S phase transition (Mercer et al, 1982). However, p53 does not act as an activator of cell cycle progression when overexpressed consistent with its tumor suppressor function. For example, overexpression of wild type p53 in human glioblastoma tumor cells, prostate cancer cells, or osteosarcoma cells, leads to inhibition of cell cycle progression (Montenarh, 1992). Overexpression of wild type p53 leads to growth arrest in G1 and G2 of the cell cycle (Michalovitz et al, 1990). The regulation of cell cycle progression by p53 may be tied to its state of phosphorylation as it is underphosphorylated in G0/G1, compared to S phase (Bischoff et al, 1990). Thus, the underphosphorylated form of p53 may exert its cell cycle control. The p53 protein has been postulated to have a direct role in the regulation of DNA synthesis. It has been found that p53 can inactivate the DNA replication factor, RP-A by forming a complex with it (Dutta et al, 1993). At present it is not known if p53 requires its DNA synthesis

FIGURE 4.

Regulation of transcription and DNA replication by p53. p53 activates transcription from specific DNA sequence elements found in several promoters such as muscle specific creatine kinase (MCK), the mdm2 gene and the cip1/waf1 gene. It can repress transcription from several genes such as c-fos, c-myc, and proliferating cell nuclear antigen (PCNA) presumably by binding to the TATA binding protein (TBP), the CAT BOX binding factor (CBF) and the SP1 transcription factor. Phosphorylation of p53 by casein kinase II is required for its transcription activation ability, however the function of cdk phosphorylation is not known. p53 forms an intracellular complex with the MDM2 protein which can inhibit its ability to mediate sequence specific transcriptional activation. Binding of mutant p53 to wild type p53 can also inhibit the ability of wild type p53 to activate transcription. p53 also binds to the WT-1 tumor suppressor. Binding of p53 to WT-1 potentiates the ability of p53 to activate transcription but inhibits the ability of WT-1 to activate. p53 can also bind to and inhibit the activity of the DNA replication factor RP-A.


TRANSCRIPTIONAL
REPRESSION
c-FOS c-MYC PCNA
TBP
SP1
CBF
p53
WT
RPA
p53
WT
WT1+
CKII+
(CDK)
MDM2
p53
WT
p53
MUT
p53
WT
MDR
DNA
REPLICATION
PuPuPu C A/T T/A G PyPyPy
MCK MDM2 CIP1
TRANSCRIPTIONAL
ACTIVATION

regulating function to alter cell cycle progression. In contrast, growth regulation by p53 appears to depend on its ability to activate transcription (Pietenpol et al, 1994)

Cell cycle arrest by p53 may also be important in the cellular response to DNA damage (Lane, 1992). It has been found that induction of DNA damage by γ irradiation in mammalian cells leads to an increase in p53 expression (Kastan et al, 1991). This may relate to one proposed function of p53 in the cell. High levels of p53 induce cell cycle arrest and exposure of cells to DNA damaging agents such as γ irradiation, N-(phosphonacetyl)-L- aspartate, or methotrexate, also leads to growth arrest (Kastan et al, 1991; Yin et al, 1992; Livingstone et al, 1992). Furthermore, growth arrest induced by DNA damaging drugs was found not to occur in cells harbouring a mutant p53 (Yin et al, 1992; Livingstone et al, 1992). Thus, high levels of p53, which occur upon DNA damage, may lead to growth arrest. The evolution of such an arrest would allow the DNA repair machinery to repair the damage to the DNA before the cell continues to divide. In the presence of a mutant p53, cells do not arrest, and accumulate DNA damage such as the amplification of discrete regions of DNA (Lane, 1992). Thus, the function of p53 may be to arrest cell growth in G1 or G2, during DNA damage so that the damage can be repaired before the cell continues towards proliferation.

Recent experiments have suggested a mechanism by which p53 might induce growth arrest. The waf1 protein was recently cloned based on its ability to be induced by p53 (El-Diery et al, 1993). Sequencing analysis found that it was identical to the cip1 gene which is a potent inhibitor of the cdk2 cell cycle kinase (Harper et al, 1993). This suggested that the high levels of p53 which occur upon DNA damage leads to high levels of waf1/cip1, which inhibits cell cycle progression by inhibiting the kinase activity of

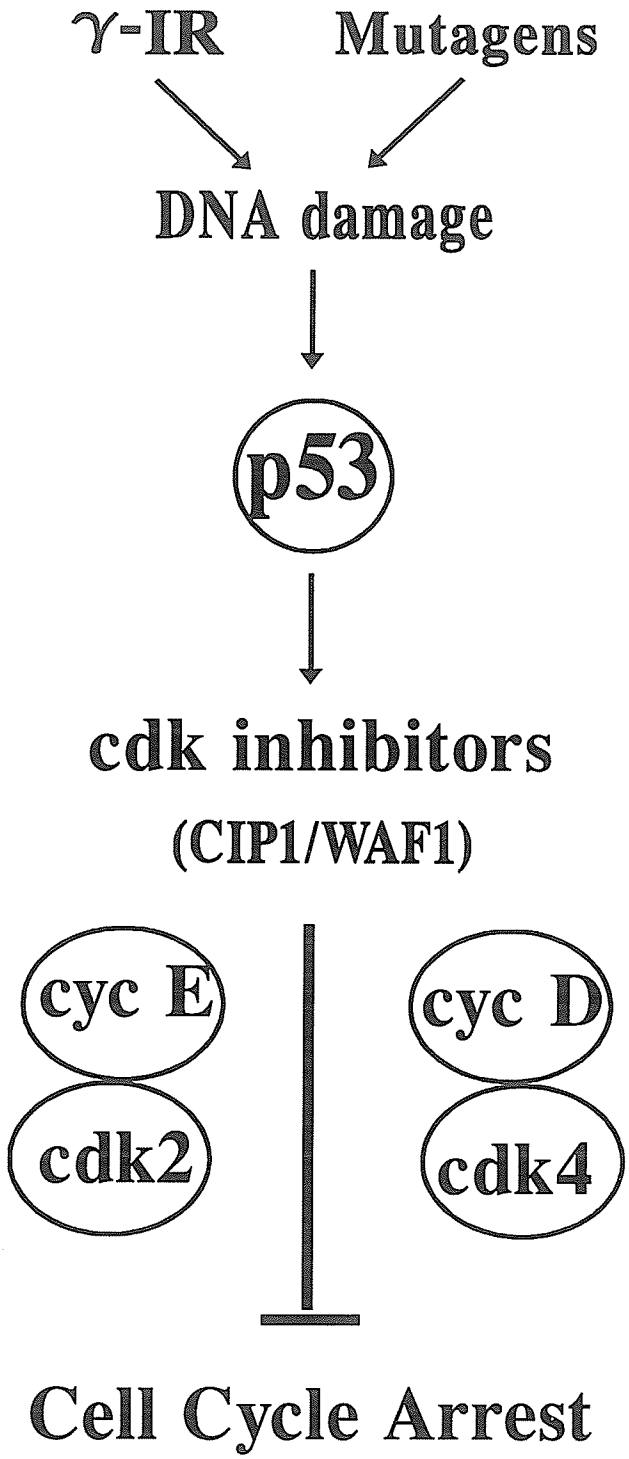
cdk2. Indeed it has been recently found that cdk2 activity is inhibited during DNA damage induced growth arrest in a p53 dependent manner (Dulić et al, 1994). Thus, p53 may regulate cell cycle progression by impacting on the regulatory components of the cell cycle, the cyclin dependent kinases (figure 5).

3.5 Oncogene Cooperation and the Metastatic Phenotype

Cancer mortality is primarily caused by the ability of tumor cells to invade and metastasize (Poste & Fidler, 1980). The finding that oncogenes of diverse functional classes can modulate the metastatic properties of cells, has led to the view that these genes play a role in metastasis because they are involved in the regulation of key cellular activities required to achieve the metastatic cascade (Wright et al, 1990). This is in keeping with observations that oncogenes can influence such diverse cellular functions as motility (Taniguchi et al, 1989), secretion of proteases (Denhardt et al, 1987) and growth autonomy (Egan et al, 1987a, b). Although an individual oncogene may regulate several activities important for metastasis (Wright et al, 1990), tumor progression leading to metastatic ability occurs only after accumulation of several oncogenic events (Bishop, 1987). For example, K-ras is frequently activated in human benign colonic adenomas (Fearon & Vogelstein, 1990). Progression to late adenoma and colon carcinoma is accompanied by loss of function mutations at the p53 and DCC loci (Baker et al, 1989; Fearon et al, 1990). Colon carcinoma development correlates with at least five oncogenic mutations, and it has been suggested that the number rather than the order of events is important in progression (Fearon & Vogelstein, 1990).

FIGURE 5.

A model for the function of p53 in DNA damage induced growth arrest. DNA damage induced by gamma radiation (γ-IR) or mutagenic chemicals stimulates the synthesis of p53 protein which then induces the expression of inhibitors of cyclin dependent kinases (CDK) such as the cip1/waf1 protein. These inhibitors cause cell cycle arrest by inhibiting the kinase activity of cdk's.


γ-IR
Mutagens
DNA damage
p53
cdk inhibitors
(CIP1/WAF1)
cyc E
cyc D
cdk2
cdk4
Cell Cycle Arrest

Efficient ras transformation *in vitro* depends upon other cooperating activities (Ruley, 1987). For example, certain alleles of the p53 gene can cooperate with ras in transformation assays (Parada et al, 1984; Eliyahu et al, 1984). Furthermore, several observations argue that p53 is a tumor suppressor gene (Levine et al, 1991). It is also known that p53 alleles that compliment ras in transformation experiments contain mutations (Hinds et al, 1989). The ability of p53 to form oligomeric complexes (Kraiss et al, 1988), and the demonstration that mutant p53 can convert cotranslated wild type p53 into the mutant conformation may help explain these findings (Milner & Medcalf, 1991). It appears that p53 missense mutants may act in a dominant negative manner to interfere with the tumor suppressor activity of the wild type protein (Rovinski and Benchimol, 1988; Munroe et al., 1990). Cotransfection experiments carried out with ras and myc have shown that these two oncogenes can also cooperate in the transformation of permissive cells (Land et al, 1983; Land et al, 1986), and these findings have been supported by results obtained *in vivo* (Thompson et al, 1989). However, it appears that deregulation of ras and myc alone is suboptimal for transformation. This is suggested by the clonal development of tumors in ras and myc transgenics (Sinn et al, 1987), the finding that a serum factor can elevate the efficiency of transformation by ras and myc (Hsiao et al, 1987), and by observations from our laboratory with 10T½ cells (Egan et al, 1989b). Studies from our laboratory have shown that transfection with myc and ras usually leads to benign transformation, and that malignant clones appear stochastically. These results suggested that another event in addition to ras and myc deregulation is required for complete transformation and tumor progression. Furthermore, cell fusion experiments suggest that the event required for malignant transformation by ras and myc

is the loss of a tumor suppressor. We have recently demonstrated that the introduction of a mutant form of p53 can cooperate with ras and myc to regulate cellular transformation *in vitro* and tumor dissemination *in vivo* (Taylor et al, 1992).

3.6 Mechanisms of Oncogene Cooperation.

There are several models to explain the mechanism of oncogene cooperation. One of these models suggests that different oncogenes can cooperate because they are capable of regulating different cellular characteristics, all of which are required for full transformation (for a review see Hunter, 1991). For example, it has been proposed that the inability of ras to cause the outgrowth of transformed cells when transfected into primary fibroblasts is due to the persistence of a negative growth signal emitted by neighboring cells, which suppresses the growth of the cell containing a mutant ras. Full transformation can be induced by treating ras transfected cells with the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA). Since TPA treatment on its own does not transform these cells, it has been suggested that its role is to circumvent the growth suppressive effects of neighboring cells, while the role of ras is to induce cellular transformation (Balmain et al, 1984). A slightly different model along these lines involves the cooperative induction of erythroid leukemia by a combination of v-ErbB and v-ErbA. In this case, v-ErbB, which is a constitutively active form of the epidermal growth factor receptor, is required to induce a constitutive proliferative signal. The role of v-ErbA is to block the expression of genes which would normally induce erythroid differentiation. Thus, the combination leads to a cell with a great potential to proliferate

which is unable to differentiate (Frykberg et al, 1983). Another type of model suggests that oncogenes can cooperate in cellular transformation because they can cooperatively regulate a common characteristic required for transformation. The best example of this is the cooperative transformation of fibroblasts by the nuclear oncogenes, fos and jun. Fos and jun are capable of dimerizing to form the AP-1 transcription factor which activates genes involved in cellular proliferation. Since the fos/jun heterodimer formation is required for efficient transactivation, the requirement for both is based on their intracellular binding (Schutte et al, 1989).

Previous experiments from our laboratory have shown that ras, myc, and mutant p53 can cooperate in the induction of the metastatic phenotype in fibrosarcoma cells (Taylor et al, 1992). Studies from another group demonstrated that transfection of fibroblasts with myn, the murine homologue of max, increased focus formation in the presence of ras and myc (Prendergast et al, 1991). This suggested a role for myn in the mechanism of ras + myc cooperation. Our recent studies have shown that myn is elevated in a variety of ras transformed cells. We have also found that myn is inducible by ras. This, along with our observation that high levels of myn can inhibit transformation by ras *in vitro* have allowed us to propose a novel mechanism of oncogene cooperation (Taylor et al, 1994). This model suggests that myn homodimer formation suppresses ras transformation by competing for myc binding elements. Competition is relieved by the addition of exogenous myc which can convert myn homodimers to heterodimers of myc and myn which can activate transcription of genes important in neoplastic transformation.

4. The Structure of Chromatin and Malignancy.

4.1 The Structure of Chromatin.

Malignant progression is accompanied by multiple changes in the expression of cellular genes. Some of these changes are likely due to regulatory changes in transcription factors (Pohl et al, 1988b, Wasylyk et al, 1991). However, some transcriptional alterations in malignant cells may be the result of altered chromatin structure. The components of eukaryotic chromatin include the histone proteins, the nonhistone chromatin associated proteins such as transcription factors and polymerase molecules, and the DNA itself. The function of the histones is to compact the DNA allowing it to occupy a very small area. A typical mammalian genome if extended as a linear molecule would measure approximately three meters. It is evident that a considerable amount of compaction must occur for this length of DNA to fit into a nucleus with a typical diameter of a few micrometers. Compaction of DNA must also be dynamic to allow the processes of transcription, replication, and mitotic condensation to occur. This dynamic structural requirement for chromatin is fulfilled by the histone proteins (for a review see van Holde, 1989b).

There are two groups of eukaryotic histones. These are the core histones and the linker histones. The core histones form a structure around which DNA is wound to form the nucleosome. Nucleosomes are further packaged with the aid of the linker histones. The four major types of core histones are H2A, H2B, H3, and H4 and the two major types of linker histone are H1 and H5.

Histones are present in the somatic cells of almost all eukaryotic organisms (van Holde, 1989b). However, some eukaryotes, such as certain dinoflagellates do not contain histones, and H1 has not yet been detected in yeast (Rizzo & Nooden, 1974; Certa et al, 1984). It is possible that an "H1-like" molecule functions in yeast since both *neurospora* and *aspergillus* contain an H1 protein (Goff, 1976; Felden et al, 1976). There is also some variability in histone distribution in higher eukaryotes. For example, although vertebrate somatic cells contain a full complement of histones, histones are lost during sperm development. In mature sperm, histones are replaced by protamines which compact the DNA (Avramova et al, 1980). Despite this variability, histones are highly conserved proteins as shown by the presence of only two amino acid changes in the 102 amino acid sequence of H4 when calf and pea histones are compared (DeLange et al, 1969). The presence of histones in many different types of organisms likely reflects the widespread requirement for a dynamic yet compact chromatin structure.

The histone proteins are very basic proteins containing high levels of positively charged amino acids such as lysine and arginine. It is the interaction of these positively charged residues with the negatively charged sugar phosphate backbone of DNA which allows histone/DNA interaction. A second feature of the histones is their domain structure. Histones contain three domains, a central globular domain which is electrostatically neutral at physiological pH and has a high propensity to form α-helix and β-sheet structures. The globular domain is flanked on both amino and carboxyl termini by the nonglobular tails. It is in these tail regions where the majority of charged amino acids are found, and these tails are believed to mainly exist with a random coil configuration (van Holde, 1989b). Chemical crosslinking studies have implicated the

globular domains in histone/histone interactions. The nonglobular tails are believed to be involved in the interactions of histone with the DNA (Callaway et al, 1985; van Holde, 1989b).

Histones interact ionically with other histones and with the DNA. These interactions allow DNA compaction at several structural levels. The basic structural unit of chromatin is the nucleosome and much of the chromatin is believed to be packaged by the formation of this structure (Noll, 1974; van Holde, 1989a). The nucleosome consists of 146 bp of DNA wrapped around an octameric structure which itself consists of two copies of each of the four core histones, H2A, H2B, H3, and H4. X-ray diffraction of isolated chromatin have demonstrated several additional structural features. At low ionic strength, chromatin exhibits a "beads on a string" appearance when viewed by an electron microscope. The beads correspond to the nucleosomes and the string corresponds to the linker DNA which lies between adjacent nucleosomes (Olins & Olins, 1974). Histone H1 interacts via its globular domain to histones H2A and H3 in the histone octamer and also with approximately 20 base pairs of DNA immediately adjacent the nucleosome (Thomas & Khabaza, 1980; Boulikas et al, 1980). The basic repeating unit of chromatin, consisting of the nucleosome, an H1 molecule, and between 20 and 75 base pairs of linker DNA is called the chromatosome. Packaging of DNA into nucleosomes leads to approximately a fivefold order of compaction. This is not sufficient to allow packaging into a typical eukaryotic nucleus. Further packaging is accomplished by "higher order" chromatin structures. Some aspects of higher order structure have been determined from electron microscopic studies. Such studies suggest that H1 is required for this further level of compaction which may take the form of a "helical winding of a string of

nucleosomes" (Finch et al, 1976; Thoma et al, 1979; McGhee & Felsenfeld, 1983).

Extensive studies have been performed to analyze the structure of chromatin during gene transcription. Several lines of evidence suggest that chromatin exhibits structural alterations during this process. For example, induction of β-globin gene expression is accompanied by an increase in DNAase 1 sensitivity over a large domain surrounding this locus, whereas the untranscribed globin gene is DNAase insensitive. Furthermore, transcription is accompanied by hypomethylation of the DNA in regions 5' of the coding sequence and by the appearance of DNA sites hypersensitive to DNAase 1 (Weintraub & Groudine, 1976; Larsen & Weintraub, 1982). Furthermore, it has been found that when transcribed and nontranscribed regions of DNA are compared by sucrose density gradient centrifugation, the transcribed DNA sediments at a rate similar to unfolded chromatin while the sedimentation of untranscribed chromatin is similar to tightly folded chromatin. This difference can be abolished by reducing the ionic strength, a treatment known to cause the unfolding of chromatin, or by the extraction of histone H1 (Kimura et al, 1983; Smith et al, 1984). Unfolding of transcribed chromatin is also consistent with increased nuclease sensitivity as tight packaging may hinder the accessibility of the DNA to these proteins. These studies suggest that transcribed chromatin exists in an open conformation, and that histone H1 may be involved in the process of unfolding.

4.2 Variation in Chromatin Structure: Histone Subtypes and Modifications

Variation in the chromatin structure of different regions of the genome is thought

to be due in part to variations in the histones. Part of the histone variation within an organism comes from the presence of histone subtypes or variants. Histone variants arise as the product of different genes and they differ in primary structure. Mammals show 5 H2A variants, 3 H2B variants, 4 H3 variants, however H4 exists only as one identifiable protein in mammals. The five major H1 variants are H1a, H1b, H1c, H1d, and H1e. A testis specific H1t has also been found and nonproliferative cells contain H1o. The function of the histone variants have not been clearly elucidated. However, many amino acid changes in the variants occur in the globular domain. Given the apparent importance of the globular domain in histone/histone interactions, chromatin containing different variants may exhibit altered structure at the nucleosomal level (van Holde, 1989b).

Further histone variation arises from post-translational modification. Modifications include acetylation, methylation, glycosylation, ADP-ribosylation, ubiquitination, and phosphorylation. Modification is a dynamic process carried out by enzymes which add a particular group, and coordinately regulated enzymes which remove that group. All five major types of histones are modified by at least some of these mechanisms and there is evidence that such modifications regulate histone function (van Holde, 1989b; Davie & Candido, 1980).

In the case of acetylation, all five major histone types are modified. In addition, there are two types of acetylation reaction which occur (Doenecke & Gallwitz, 1982). The first has been shown to modify H2A, H4 and H1 and involves the addition of an acetyl group to the α-amino group of N-terminal serine residues. The second type of acetylation takes place on all four core histones but not on H1 and involves the addition

of an acetyl group to the ξ-amino group of selected lysine residues within the histone molecule. Covalent addition of acetyl groups to lysines in the N-terminal tail is predicted to reduce the positive charge of this histone domain potentially weakening its interaction with the DNA. Thus, acetylation has been postulated to have a role in gene transcription where altered chromatin structure is required.

Histone methylation results from the enzymatic replacement of an ξ-amino proton of lysine residues with a methyl group. This type of modification has been reported for all five types of histones, however, its function is not well understood (van Holde, 1989b). The role of glycosylation of histones is also not well understood, although it has been observed in the histones of *tetrahymena* (Levy-Wilson et al, 1983).

Histones are also subject to ADP-ribosylation where H1 is most extensively modified. However, the frequency of this modification is very low and it has been estimated that only 0.005 mol of ADP-ribose are found per mole of H1 (van Holde, 1989b). The ribosylation reaction involves the cleavage of nicotinamide adenine dinucleotide to yield nicotinamide and adenosine diphosphate ribose which is linked to an acceptor group on a histone molecule. Glutamate, arginine, and phosphoserine residues have all been reported to act as acceptor groups, as well as the ADP-ribose unit itself. This can lead to the formation of linear and branched chains of ADP-ribose groups linked on one end to a histone. There have also been reports of such a chain, 15 ADP-ribose units long, covalently attached at both ends to a H1 molecule, giving the potential to cross link adjacent chromatin strands (Wong et al, 1983). This may allow the alteration of higher order chromatin structure. ADP-ribosylation may also have a role in DNA repair, as the level of this modification rises abruptly after DNA damage (Kreimeyer et al,

1984).

Ubiquitin is a small protein covalently attached by its C-terminus to the ξ-amino group of lysine 119 in H2A (Goldknopf et al, 1980). Lower amounts of ubiquitinated H2B have also been observed (West & Bonner, 1980). The loss of ubiquitin during metaphase and the preferential association of ubiquitinated H2A and H2B with actively transcribing genes suggest that this modification may aid in chromatin decondensation (Matsui et al, 1979; Varshavsky et al, 1982; Nickel et al, 1989).

Histones are also subject to the enzymatic addition of phosphate groups to certain amino acid residues. The presence of phosphoserine residues in H1 and H3 was originally detected by Kleinsmith in 1966 (Kleinsmith et al, 1966). Subsequently, two types of phosphorylation have been found in histones. The first type, which represents the most frequent type of phosphorylation and affects all major histone types involves addition of a phosphate to the hydroxyl group of serine or threonine residues. The other more minor modification involves phosphorylation via P-N linkages to lysine, histidine and possibly arginine of H1 and H4 (van Holde, 1989b). The addition of phosphate to serine or threonine is catalyzed by protein kinases which include cAMP-dependent kinase (PKA), cGMP-dependent kinase (PKG), Histone kinase type II, growth associated histone kinase, H3 specific kinase, H4-kinase type I and H4-kinase type II (Shlyapnikov et al, 1975; Hashimoto et al, 1976; Langan, 1978; Shoemaker & Chalkley, 1980; Masaracchia et al, 1977). Phosphorylation often occurs in the tail regions of the histone and the sequences surrounding the phosphorylated residue depend on the kinase involved. For example, phosphorylation of H1 by the growth associated kinase $p34^{cdc2}$ occurs at SPKK motifs (Hill et al, 1990). Phosphorylation of tail regions may reflect regulation of

DNA/histone interactions which may alter higher order structure.

Many of the sites of histone phosphorylation have been mapped. Histone H2A is phosphorylated on up to five serine residues, and the number of phosphorylated residues is constitutively high throughout the cell cycle. Thus, it has been suggested that phosphorylation of H2A may have a role in gene transcription. The kinases involved have been found to be both PKA and PKG (Shlyapnikov et al, 1975; Hashimoto et al, 1976). These two kinases also phosphorylate two serine residues in H2B. The phosphorylation of H3 is carried out by PKA and occurs on one serine residue (Martinage et al, 1981). Phosphorylation of H3 is confined to mitotic cells and is lost after cell division presumably by the action of a phosphatase. Thus, phosphorylation of H3 may play a role in chromatin condensation. Histone H4 contains one phosphate which is added in the cytoplasm to the N-terminal serine shortly after H4 synthesis (Ruiz-Carillo et al, 1975).

The regulation of H1 phosphorylation has been extensively studied and shows intriguing cell cycle kinetics. In quiescent chinese hamster ovary (CHO) cells, H1 is found to be unphosphorylated. Cells stimulated to enter G1 phase contain H1 with one phosphate found on a serine residue in the C-terminal nonglobular domain. Progression through S-phase is accompanied by phosphorylation of two additional serines, also in the C-terminus. Entry into mitosis is characterized by a burst of H1 phosphorylation which results in the addition of phosphate to a C-terminal threonine, as well as a serine and a threonine in the N-terminal nonglobular domain (Hohmann et al, 1976; van Holde, 1989b). In other mammalian cells, serine 38 is phosphorylated by PKA however, the cell cycle kinetics of this reaction is not yet known. Serine 38 phosphorylation is under

hormonal control and may be important in the process of transcription (Langan, 1969).

Recently, much work has been done on a number of cell cycle regulated histone H1 kinases. The prototypical member of this group is $p34^{cdc2}$ (Meyerson et al, 1992). This enzyme is activated only during M-phase where it acts as a universal regulator of M phase onset (Pondaven et al, 1990; Draetta, 1990; Nurse, 1990). $p34^{cdc2}$ is primarily responsible for the burst of H1 phosphorylation which occurs during this cell cycle stage (Arion et al, 1988). $p34^{cdc2}$ itself is activated by binding to a mitotic cyclin protein which oscillates in level reaching its highest level at mitosis (Murray et al, 1989). $p34^{cdc2}$ is further activated by dephosphorylation of tyrosine 15 and threonine 16 by the action of the cdc25 phosphatase and by phosphorylation of threonine 161 by CAK, the $p34^{cdc2}$ activating kinase (Millar et al, 1991; Poon et al, 1993; Solomon et al, 1993). Thus, the mechanism responsible for the burst in H1 phosphorylation at M phase appears to involve the activation of $p34^{cdc2}$ by binding to cyclin followed by dephosphorylation. There are additional kinases very similar to $p34^{cdc2}$ which form a family of cyclin dependent kinases or cdk's (Meyerson et al, 1992). Some of these enzymes also are capable of phosphorylating histone H1. For example, cdk2 can phosphorylate histone H1, and this kinase is activated during late G1 of the cell cycle by binding to a cyclin which shows maximal levels at G1 (Elledge et al, 1992). Cdk 4 is a G1 cdk which cannot use H1 as a substrate (Matsushime et al, 1992). Therefore some of the cell cycle regulated phosphorylations which occur on H1 *in vivo* may be due to the action of different cdk's activated at different times in the cell cycle. Extensive studies on cdk's have also shown that these kinases are responsible for regulating the mammalian cell cycle (Nurse, 1990). There appear to be different cdk's governing the different cell cycle transitions, some of

which act as efficient histone H1 kinases. For example, recent studies suggest that cdk4/cyclin D is required for early G1 events, cdk2/cyclin A for G1 to S transition, and cdc2/cyclin B/A for transition through mitosis (Sherr, 1993). The coordinate phosphorylation of histone H1 by the kinases which regulate the cell cycle underscore the importance of this histone modification during cell cycle transit and likely reflects a need to restructure chromatin at defined times in the cell cycle, such as during DNA synthesis and chromosome formation.

The function of H1 phosphorylation has been suggested by its cell cycle kinetics (Lennox & Cohen, 1983; Roth & Allis, 1992). Phosphorylation of histone H1 at cell cycle regulated sites is thought to have effects on both the secondary structure and charge of the region containing the phosphorylated residue which loosen their contact with linker DNA and in so doing regulate the condensation of chromatin (Green et al, 1993). This may lead to a more open chromatin configuration. Therefore, histone phosphorylation during S phase may reflect a need to decondense the chromatin to allow DNA polymerase access to the DNA. However, the function of phosphorylation of H1 during M phase cannot be so easily understood as it is during this phase where maximal chromatin condensation occurs. Thus it has been postulated that phosphorylation of H1 during M phase may be required to allow access to other proteins which can condense the chromatin into mitotic chromosomes (Roth & Allis, 1992). These other proteins may include topoisomerase II which is found in high amount in chromosomes.

4.3 Histone H1 Phosphorylation During Malignant Transformation

Histone phosphorylation may have a further function in the regulation of transcription. It has been recently shown that purified histone H1 is a potent inhibitor of transcription *in vitro* (Laybourn & Kadonaga, 1991). This may be linked to its ability to stabilize higher order structure. It has been speculated that the ability of phosphorylation to weaken the interaction of H1 with DNA may lead to more open chromatin structures, more amenable to transcription (Roth & Allis, 1992). If true, histone H1 phosphorylation may be a target of physiological stimuli which alter gene expression. In this regard, it is intriguing that the ras oncogene, which is known to alter the expression of certain cellular genes, can lead to a reduction in the compaction of chromatin in NIH3T3 cells (Laitinen et al, 1990). In addition, ras has been shown to activate the cell cycle regulated H1 kinase $p34^{cdc2}$ (Barrett et al, 1990; Daar et al, 1991). Our recent experiments have shown that H1 is more highly phosphorylated in a variety of ras-transformed cell lines compared to untransformed control cell lines (Taylor et al, 1994). Our working hypothesis is that ras-induced increases in H1 phosphorylation are important in the ability of ras to regulate gene transcription. The phosphorylation of histone H1, induced by signal transduction pathways which regulate metastasis may be important in the deregulation of genes which are required to fulfil the metastatic cascade.

II. Materials and Methods

1. Cell lines and culture conditions

1.1 Cell lines

NIH3T3 and cell lines derived therefrom, including dC2, NIH-3G, and NIH-3G-20, A1, BNM 35, BNM 46, Ig 60 and Ig 68 have been previously described (Damen et al, 1991; Egan et al, 1990; Rogelj et al, 1988). The NIH-3G line is a derivative of the NIH3T3 line that has been transformed by the K-FGF gene. This cell line secretes higher levels of K-FGF into the medium than NIH3T3 cells. The NIH-3G-20 cell line is also transformed by K-FGF, however, this cell line harbors an amplified K-FGF gene and shows higher levels of secretion of K-FGF compared to the NIH-3G line. 3G-T is a subpopulation of the NIH-3G-20 cell line grown in the absence of selection. The dC2 cell line is a NIH3T3 derived line transfected with the vector used to introduce K-FGF into NIH-3G and NIH-3G-20 which lacks the K-FGF coding sequence (Damen et al, 1991). The BNM 35 and BNM 46 cell lines are clones that have been transfected with the wild type sequence for the basic isoform of FGF. Ig 60 and Ig 68 have been transfected with a plasmid that directs the synthesis of a fusion protein which places the signal sequence of the immunoglobulin locus N-terminal to the wild type bFGF sequence. This signal sequence is expected to direct this protein into the secretory pathway. A1 is a cell line transfected with a backbone vector used to introduce either the wild type bFGF gene or the signal sequence bFGF fusion gene into NIH3T3 cells (Egan et al, 1990; Rogelj et al,

1988). Two additional NIH3T3 derived cell lines were used. These were pEJ1 cells, which contain a constitutively expressed T24 H-ras mutant and the NIH3T3-433 cell line which contains the T24 H-ras oncogene under the transcriptional control of the dexamethasone inducible mouse mammary tumor virus promoter (Huang et al, 1981).

The mouse 10T½ fibroblast cell line was obtained from American Type Culture Collection (ATCC)(Rockville, Maryland) and was used to isolate cell lines expressing combinations of ras, myc and mutant p53. These cell lines were derived by transfection of 10T½ cell line with appropriate plasmids as described below (Stable transfection). The 17 clonal cell lines isolated by this procedure were named R-1, R-2, R-3, R-4, RM-3, RM-4, RM-5, RM-6, RP-1, RP-3, RP-4, RP-6, RMP-1, RMP-2, RMP-4, RMP-5, RMP-6. Cell lines denoted by the letter R were transfected with the T24 H-ras oncogene, whereas RM clones were transfected with ras and myc, RP clones were transfected with ras and p53, and RMP clones were transfected with ras, myc and p53.

The normal human diploid cell strain WI-38 and the human cervical carcinoma cell line Hela were obtained from ATCC. The human Colo 320 HSR line contains an amplified c-myc gene and overexpresses c-myc protein (Hann & Eisenman, 1984). The mouse 10T½ 17-17 cell line was obtained by transfection of ras, myc and the proline 193 mutant form of p53, as described above. Since this line synthesizes mutant p53 protein at a very high rate it was used as a control for mutant p53 expression. The 10T½ derived ras inducible cell line 2H1 contains the T24 H-ras oncogene under the transcriptional control of the zinc inducible metallothionein promoter (Haliotis et al, 1990), and contains one intact ras-metallothionein promoter hybrid gene (Trimble, 1987). Ciras-2, NR4 and Ciras-3 cells are mouse 10T½ derived transformed cell lines containing

a constitutively overexpressed T24 H-ras oncogene (Egan et al, 1987a).

1.2 Culture conditions

Cell lines were grown in plastic tissue culture plates in a humidified atmosphere containing 7% CO_2 in medium supplemented with penicillin G (100 units/ml) (Sigma, St. Louis, MO), and streptomycin sulfate (100 μg/ml) (Sigma, St. Louis, MO). NIH3T3 and the transfected derivatives were grown in α-minimal essential media (α-MEM) (Gibco, Grand Island, New York) containing 10% fetal bovine serum (FBS)(Intergen, Purchase, New York). 10T½ derived cell lines transfected with combinations of ras, myc and mutant p53 were maintained in α-minimal essential medium (α-MEM) (Gibco, Grand Island, N.Y.) containing 10% dialyzed calf serum (DCS)(Gibco). 10T½ cells were grown in α-MEM plus 10% DCS one week prior to transfection with ras, myc and mutant p53 and these transfections were carried out in this medium. The WI-38 cell strain and the Hela cell line were grown in α-MEM containing 10% fetal bovine serum (Intergen, Purchase, N.Y.). The Colo 320 HSR cell line was grown in RPMI medium (Gibco) supplemented with 10% fetal bovine serum. The 10T½ 2H1, Ciras-2, Ciras-3 and NR4 cell lines were grown in 10% fetal bovine serum in F12 medium (Gibco, Grand Island, NY, USA), and in comparative studies, 10T½ cells were switched to this medium.

1.3 Routine culture procedures

1.3.1 Cell removal with trypsin solution.

Medium was aspirated and 1 to 2 ml of trypsin solution (0.3% trypsin [Sigma, St. Louis, MO; Difco Laboratories] and 2 mM ethylenediaminetetraacetate [EDTA] in phosphate buffered saline [PBS]) was added to culture plates. PBS contains 140 mM NaCl, 2.7 mM KCl, 1.6mM KH_2PO_4,and 8.1 mM Na_2HPO_4. Trypsin was allowed to act for 1 to 5 minutes at room temperature and then was inactivated by the addition of two volumes of 10% fetal bovine serum in α-MEM. Cells were then used as required.

1.3.2 Subculture.

Cell lines were subcultured when they reached 75% confluence. Cells were removed with trypsin solution, and an aliquot containing one tenth the number of cells was added to a new plate containing growth medium. For 150mm plates, cells were seeded at approximately $5x10^5$ cells per plate and for 100mm plates at $1x10^5$.

1.3.3 Long term storage of cells.

Cells were removed from plates with trypsin solution, centrifuged at 1000 x g for 5 minutes, and then resuspended in normal growth medium containing 10% FBS and 10% dimethylsulfoxide (DMSO). Cells were resuspended at approximately $5x10^6$ cells/ml and

1 ml was added to a cryotube (Nunc) which was stored at -80°C. For later studies, cells were thawed out rapidly by placing the cryotube in a 37°C waterbath for 3 minutes. The contents of the tube were then added to a culture plate containing growth media. After three h, when the cells had attached to the culture plate, the medium containing DMSO was removed and fresh medium was added to the plate.

1.3.4 Cell counting

Cells that had been removed from plates with trypsin solution were counted with a hemocytometer (American Optic) and at least 150 cells were counted for each determination.

1.4 Dialysis of serum

Calf serum was dialyzed against 10 volumes of 0.8% NaCl for 10 changes in dialysis tubing with a 12,000 to 14,000 molecular weight cut off (BRL Gaithersburg, MD).

1.5 Stable transfection

The plasmids pH06T1, pHmrn17 and pEC53 were used in transfection experiments. The pH06T1 plasmid contains T24 H-ras linked to the aminoglycoside phosphotransferase gene to confer resistance to G418 (Spandidos & Wilke, 1984). The

pHmrn17 plasmid contains T24 H-ras, the second and third human c-myc exons with a SV40 promoter, and the aminoglycoside phosphotransferase gene (Kohl & Ruley, 1987). The pEC53 plasmid contains a complete murine genomic sequence of p53 cloned into the pECE vector (Hicks et al, 1991). The p53 allele in pEC53 was previously cloned from the CB7 erythroleukemia cell line and contains an arginine to proline transition at position 193 (Rovinski et al., 1987; Munroe et al, 1990).

Foreign DNA was introduced into cells using CellPhect kits (Pharmacia, Upsala, Sweden). A total of 2 x 10^5 cells were transfected 18 h after addition to culture plates. A 120 μl aliquot of buffer containing 0.5 M $CaCl_2$ and 0.1 M Hepes was mixed with 120 μl of DNA in distilled water, and incubated for 10 minutes at room temperature. A 240 μl aliquot of buffer containing 0.28 M NaCl, 0.05 M Hepes, and 0.75 mM sodium hydrogen phosphate was added, mixed and incubated for 15 minutes at room temperature. The calcium phosphate-DNA precipitate was added to cells on culture plates, which were then incubated for 6 h at 37°C in a humidified 5% carbon dioxide atmosphere. The culture plates were washed with growth medium and then treated with 1.5 ml of 15% glycerol in isotonic Hepes, pH 7.5 for 3 minutes, and then washed again and replenished with fresh growth medium (α-MEM plus 10% dialyzed calf serum). Transfection of 10T½ cells was carried out with 0.4 μg of pH06T1 or 0.4 μg of pHmrn17 plasmid, and cotransfections were carried out with 2 μg of pEC53 plasmid.

Transfected cultures were used to isolate cell lines for further study. When transformed foci became visible (40 X magnification) they were isolated with cloning cylinders (Freshney, 1983), and expanded as cell lines. Cloned lines were grown in 400 μg of G418 sulfate (Gibco) for one week to eliminate nontransfected cells.

2. Isolation and Sources of DNA Probes and Plasmids

2.1 Sources of plasmids containing cloned DNA.

The pHOMER6, pHO6T1, pSVHmycMoneo, and pHmrn17 plasmids were generous gifts from H. E. Ruley. These four plasmids contain the aminoglycoside phosphotransferase gene to confer resistance to G418. pHOMER6 is the backbone vector containing only this G418 resistance gene. pHO6T1 contains in addition, the T24 H-ras gene, while pSVHmycMoneo contains the human c-myc gene. pHmrn17 contains the G418 resistance gene, T24 H-ras and the human c-myc gene. pCMVMyn was a generous gift from Dr. E. Ziff and contains the mouse myn cDNA driven by the cytomegalovirus promoter. Plasmid pA_{BB} contains a portion of the human 28S gene and was used for the isolation of a 28S probe. The pEC53 plasmid contains a complete murine mutant genomic sequence of p53 cloned into the pECE vector and was obtained from from Dr. M. Mowat. Glyceraldehyde-3-phosphate-dehydrogenase cDNA was obtained from Dr. C.L.J. Parfett, Mutagenesis Section, Environmental Health Directorate, Health and Welfare Canada, Tunney's Pasture, Ottawa, Ontario.

2.2 Propagation and purification of plasmid DNA

2.2.1 Transformation of *E. coli*

To propagate the plasmids used in these studies, they were introduced into the

HB101 strain of *E. coli* by transformation. *E. coli* were made competent for transformation by growing bacteria in 35 ml of LB medium (1% w/v bacto-tryptone, 0.5% w/v bacto-yeast extract and 1% w/v NaCl, pH 7.5) until they obtained an OD_{590} of 0.2 (Sambrook et al, 1989). The cells were collected by centrifugation, resuspended in 15 ml of 50 mM $CaCl_2$ at 4°C and incubated for 30 min on ice. The cells were collected by centrifugation and resuspended in 3 ml of 50 mM $CaCl_2$ at 4°C. Glycerol was added to 10% (v/v) and the competent bacteria were stored at -80°C in cryovials (1 ml per vial). For the transformation of competent bacteria, plasmid was diluted to 100μl with TE (10mM Tris-HCl, pH 8.0, 1 mM EDTA), added to a vial of *E.coli* and incubated for 30 min on ice. The cells were then incubated at 42°C for 2 min. and then transferred to 1 ml LB medium which was then incubated at 37°C for 45 min. The cells were collected by centrifugation, resuspended in 100 μl LB medium, and spread on LB plates containing 50 μg/ml ampicillin. The plates were incubated for 18 to 24 h at 37°C, and individual colonies were picked and used for large scale plasmid preparation.

2.2.2 Large scale plasmid preparation

Large quantities of plasmid were prepared by the methods described by Sambrook et al (1989). A single colony of HB101 *E. coli* transformed with the appropriate plasmid was inoculated into 5 ml of LB medium containing 50 μg/ml ampicillin. After overnight incubation at 37°C the suspension was added to 1 litre of LB medium. The 1 litre culture was incubated at 37°C in a shaking incubator set at 250 rpm until the culture obtained an OD_{550} of 0.4 (approximately 4 h). Five ml of spectinomycin stock solution (54mg/ml)

was added and the culture was incubated overnight at 37°C with shaking. The cells were collected by centrifugation at 4,000 xg for 10 min. and then washed once in 25 ml of STE buffer (0.1 M NaCl, 10 mM Tris-HCl [pH 8.0], 0.1 mM EDTA and 0.5% v/v Triton X-100). The cell pellet was resuspended in 10 ml sucrose lysis buffer (10% w/v sucrose, 50 mM Tris-HCl, pH 8.0). Two ml of fresh lysozyme dissolved at 10mg/ml in water was added. Then, 8.0 ml of 0.25 M EDTA was added and the sample was incubated on ice for 10 min. Four ml of 10% sodium dodecyl sulfate (SDS) was added, stirred once with a glass rod, and then 6 ml of 5 M NaCl was added, stirred again gently with a glass rod and the mixture was incubated for 1 h on ice. The lysed bacteria were then centrifuged for 30 min. at 30,000 rpm at 4°C to remove cellular debris. The supernatant containing the plasmid DNA was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1) and twice with chloroform:isoamyl alcohol (24:1). The plasmid DNA present in the aqueous phase was further purified and concentrated by adding 0.54 volumes of isopropanol and incubating at -20°C for a minimum of 1 h. Precipitated plasmid DNA was collected by centrifugation at 15,000 xg for 30 min. The DNA pellet was carefully washed once with 70% ethanol without recentrifugation and the ethanol was removed by vacuum desiccation. Plasmid was dissolved in 4 ml of 100% w/v CsCl in TE buffer. The dissolved plasmid was added to a Beckman Quick seal centrifuge tube and 0.2 ml of ethidium bromide (10 mg/ml) was carefully layered on top of the plasmid solution. Mineral oil was layered on top of the ethidium bromide to fill the tube and prevent collapse during centrifugation. Tubes were balanced with mineral oil, sealed with a Beckman heat sealer, mixed by inversion and placed in a Beckman Ti70.1 rotor. Samples were centrifuged at 42,000 rpm overnight at 20°C.

After centrifugation, two ethidium stained bands were visible. The bottom band, which corresponds to closed circular plasmid DNA was removed by puncturing the centrifuge tube with a hyperdermic needle. The band was withdrawn from the tube and extracted 4-5 times with an equal volume of water-saturated-butanol to remove the ethidium bromide. The sample was concentrated by extracting twice with 2 to 3 volumes of sec-butyl-alcohol. The CsCl was removed by dialysis against 2 litres of 1 x TE buffer for three changes.

Plasmid concentration was determined by measuring the absorbance of the dialyzed plasmid, diluted with 20 volumes 1 x TE buffer, at 260 nm and 280 nm. DNA typically showed a ratio of OD_{260}/OD_{280} of 1.7 to 1.8. At this ratio, 1 OD_{260} unit corresponds to a DNA concentration of 50 μg/ml (Sambrook et al, 1989). Plasmids were stored at 4°C after the addition of several drops of chloroform:isoamyl alcohol (24:1). The identity of all plasmids were confirmed by restriction enzyme digest analysis after large scale preparation.

2.3 Purification of probes

To isolate the DNA fragments to be used as probes, plasmids were first incubated with the appropriate restriction endonucleases (3 units/μg DNA) at 37°C for 1 h. The myn probe used was a 1.9 kb fragment isolated from pCMVMyn by digestion with EcoR1 and Hind III (Prendergast et al, 1991), the 2.9 kb ras probe was isolated from pEJ by digestion with Sac I (Chang et al, 1982), the c-myc probe used was a 0.4 kb DNA fragment isolated from pSVHMycMoneo by digestion with Pst 1 (Kohl & Ruley, 1987).

A 1.3 kb probe specific for the 28S rRNA was used as an internal control and was isolated from the plasmid pA_{BB} by digestion with Bam H1 (Gonzales et al, 1985). The GAPDH probe was isolated from the plasmid pM-GAP by digestion with Pst 1 (Edwards et al, 1985). After digestion with the appropriate restriction enzyme, the DNA sample was fractionated by electrophoresis in 1% agarose gels containing 2.5 μg/ml ethidium bromide in 1 x TBE buffer (89 mM Tris-borate, 89 mM boric acid and 2 mM EDTA) for 18 h at 30 volts. The DNA fragments were visualized under ultraviolet light and the fragment to be used for probing was cut out with a scalpel. The piece of gel was placed into a dialysis tube to which was added 1 ml of 0.5 x TBE buffer. The dialysis bag was closed, placed in an electrophoresis tank and the DNA fragment electroeluted from the gel piece by passing 100 volts through the tank for 2 to 3 h. When complete, the DNA fragment was released from the inside wall of the dialysis tube by reversing the polarity for 2 min. The solution containing the eluted fragment was then passed over a mini-column-D (Sigma, St. Louis, MO) and eluted with 0.5 ml high salt buffer (1.0 M NaCl, 1.0 mM EDTA, 20 mM Tris-HCl, pH 7.4,). One ml of absolute ethanol was then added and the DNA precipitated by incubating at -20°C for 18 h. The precipitated DNA was collected by centrifugation at 6000 x g for 20 min, and resuspended in 20 to 40 μl TE buffer to give a concentration of approximately 500 ng/μl. Probes were stored at -80°C.

2.4 Preparation of oligodeoxynucleotides

Oligonucleotides were synthesized on a model 380 B DNA synthesizer (ABI, Mississauga, Ontario) using phosphoroamidate chemistry and TETD for the sulphurization

step (Bulletin 58, ABI). The program allowed the synthesis of phosphodiester and phosphorothioate derivatives in the same oligonucleotide sequence. Oligonucleotides were deprotected by heating at 55°C in ammonium hydroxide overnight, and purification columns (Bulletin 59, ABI) were used to remove the trityl groups and to purify the oligonucleotides (Spearman et al, 1994).

3. Labelling of cDNA Probes with ^{32}P-dCTP

cDNA probes isolated as described above were labelled using the hexadeoxyribonucleotide method of Feinberg and Vogelstein (1983), to specific activities between 0.5-1.0 x 10^9 cpm/μg using ^{32}P-dCTP. Reagents required for labelling were obtained from an oligolabelling kit supplied by Pharmacia (Uppsala, Sweden). Approximately 500 ng of the cDNA probe in a volume of 33 μl was boiled for 2-3 min, and then cooled on ice for 2 min. After cooling, 10μl of reagent mix (containing the random hexadeoxyribonucleotide fragments, and dATP, dGTP, and dTTP stored in a concentrated buffer solution of Tris-HCl, pH 7.8, magnesium chloride, and β-mercaptoethanol) was added, followed by the addition of 1 μl of DNA polymerase I ('Klenow' fragment: 1 unit/μl, stored in 50 mM $KHPO_4$, pH 6.5, 10 mM β-mercaptoethanol, and 50% glycerol), and 5 μl ^{32}P-dCTP (specific activity 3000 Ci/mmol). This reaction mixture was incubated at room temperature for 2 to 3 h. and then passed through a spin column of sephadex G-50 (prepared in a microfuge tube and spun in a clinical centrifuge at 500 xg). Additional labelled probe was eluted from the column with 100 μl TE buffer. The incorporation of label into the probe was determined by counting 3

μl of the labelled probe in a liquid scintillation counter (model LS 7800)(Beckman, Irvine, CA) using a ^{32}P scintillation counter program based on Čerenekov radiation (detection from 0 to 2000 KeV)(Čerenekov, 1934). For hybridization, 1 x 10^6 cpm/ml was added to the hybridization buffer.

4. Isolation of Genomic DNA and Southern Blot Analysis.

4.1 Isolation of genomic DNA.

Genomic DNA was isolated from cells with a rapid method (Blin and Stafford, 1976). Cells were harvested from 3-5 subconfluent 150 mm plates with trypsin solution, collected by centrifugation and washed once with PBS. Cell pellets were either used immediately for DNA isolation or were stored at -80°C and DNA was isolated at a later time. For DNA isolation, the pellet was resuspended in 4.5 ml TEN buffer (10mM Tris, 10 mM EDTA, 10mM NaCl pH 8.0) at 4°C and lysed by the addition of 0.5 ml of 10% SDS. After mixing in the SDS, 50 μl of RNAase (stock solution of 10mg/ml) was added and the solution was mixed again. The solution was incubated at 37°C for 2 h after which 50 μl of proteinase K (stock solution of 10mg/ml) was mixed in and the solution was incubated at 55°C for 3 h. The DNA was extracted once with an equal volume of phenol saturated with TE buffer, twice with phenol:chloroform:isoamyl alcohol (25:24:1) and then twice with an equal volume of chloroform:isoamyl alcohol (24:1). The sample was then dialyzed against 4 litres of TE buffer for three changes. DNA was stored at 4°C after the addition of several drops of chloroform:isoamyl alcohol (24:1).

4.2 Southern blotting

Twenty μg of DNA was digested with 3-4 units/μg DNA of the desired restriction endonuclease for 18 h. The sample was reduced to a volume of 40 μl with a speed-vac concentrator (Savant). DNA loading buffer (6 x stock: 0.25% bromophenol blue, 0.25% xylene cyanol, and 30% glycerol) was added to the sample which was loaded onto an agarose gel (0.7% agarose in 1 x TBE buffer) containing 0.5 μg/ml ethidium bromide. The samples were electrophoresed at 30 volts for 18 h in 1 x TBE buffer. Molecular weight markers were loaded at the same time to determine the size of the DNA fragments detected. Ethidium bromide stained DNA was viewed under ultraviolet light after electrophoresis to confirm that digestion was complete, and to ensure that equivalent amounts of DNA were loaded. The distance migrated by the molecular weight markers, visualised in the same manner, was measured. Gels were then incubated in 0.25 M HCl for 15 min. and then washed twice in base (0.5 M NaOH, 1.5 M NaCl) for 15 min. and then neutralized by two 20 min. washes in Tris buffer containing salt (0.5 M Tris-HCl [pH 7.5], 1.5 M NaCl). Gels were blotted onto nitrocellulose or nylon membranes, by capillary blotting using 20 x SSC (1 x SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) for 18 h. Blotting was carried out by placing the gel on a filter paper wick which was in contact with the SSC. A membrane was then placed on top of the gel and 2 pieces of filter paper were placed on top of the membrane. A stack of paper towels was then placed on top of the filter paper, and a weight (approximately 0.5 kg) placed on top of the paper towels to ensure that the SSC was drawn through the gel allowing the DNA to migrate onto the membrane. Once blotted, the membrane was air dried, and

then baked at 80°C for 1 to 2 h.

The blots were prehybridized for 3 to 18 h at 42°C in hybridization solution (50% [v/v] formamide, 0.1% SDS, 1.0 M NaCl, 7.5 x Denhardt's solution [1 x Denhardt's solution contains 20 mg each of Ficoll, polyvinyl pyrrolidone, and bovine serum albumin {BSA} in 100 ml water], 10% dextran sulfate) containing 100 μg/ml boiled salmon sperm DNA. Hybridizations were preformed in the same solution for 18 h with 10^6 counts per minute (cpm)/ml of ^{32}P-labelled probe. After hybridization, the membranes were washed twice in cold wash solution (2 x SSC ,0.1% SDS) at room temperature for 20 min. each, and then twice in hot wash solution (0.2 x SSC, 0.1% SDS) at 57°C for 30 min. each. The blots were sealed in plastic bags and exposed to film (Kodak X-Omat AR film) at-80°C using Cronex Lightning Plus intensifying screens for various times depending on the intensity of the radioactive signal.

5. Isolation of RNA and Northern Blot Analysis

5.1 Isolation of cytoplasmic RNA

RNA was isolated by a rapid method (Gough, 1988). Cells were harvested with trypsin solution from one 150 mm plate at 75% confluence. Cells were washed with PBS and the pellet lysed in 200 μl of lysis buffer (10 mM Tris-Cl pH 7.6, 0.15 M NaCl, 1.5 mM $MgCl_2$ and 0.65% Nonidet-P40 [NP40]). The solution was centrifuged at 4000 xg in a microcentrifuge and the nuclear pellet discarded. The supernatant was added to a suspension containing 200 μl of 7 M urea, 1% SDS, 0.35 M NaCl, 10 mM EDTA and

10 mM Tris-Cl pH 7.6 and 400 μl phenol:chloroform:isoamyl alcohol (25:24:1). This mixture was vortexed and spun at 14000 xg for 10 min. The aqueous phase was extracted once more with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform:isoamyl alcohol (24:1). To further purify and concentrate the RNA, 800 μl of 100% ethanol was added and the solution was incubated at -20°C for 18 h. The RNA pellet was collected by centrifugation at 14000xg for 20 min and resuspended in 20 μl RNA water (0.01% diethyl pyrocarbonate (DEPC) added to deionized water and autoclaved 15 min, at 120°C). The concentration of RNA in a sample was determined by measuring the absorbance at 260 nm and using the formula: 1 OD_{260} = 40μg/ml RNA (Sambrook et al. 1989).

5.2 Northern blotting

Northern blot analysis was performed as previously described (Wright et al, 1987). RNA samples containing 20 μg of cellular RNA in a volume of 4.5 μl was added to 2.0 μl 5 x MOPS buffer (1 x MOPS contains 40 mM morpholinopropanesulfonic acid, pH 7.0, 10 mM sodium acetate and 1 mM EDTA), 3.5 μl formaldehyde and 10 μl formamide. The sample was incubated at 55°C for 15 min. and then 2.0 μl of 5 x gel loading buffer (50% glycerol, 1 mM EDTA, 0.4% bromophenol blue and 0.4% xylene cyanol) was added. The sample was loaded onto a 1% agarose gel (18% formaldehyde, 1 x MOPS, 1% agarose) and separated by electrophoresis in 1 X MOPS buffer at 30 volts for 18 h. The RNA was blotted onto nitrocellulose or nylon membranes in 20 x SSC as described for southern blots. The membranes were incubated at 80°C for 1 h,

prehybridized, hybridized with ^{32}P labelled DNA probes, washed and developed as described for southern blots.

6. Neoplastic Transformation *in vitro*

6.1 Analysis of focus formation

Plates transfected with combinations of ras, myc and mutant p53 as described above were used to isolate clonal lines or to analyze focus formation. To determine focus formation, transfected plates were cultured for 15 days and then cells were stained with a saturated solution of methylene blue in 50% ethanol (Hsiao et al, 1987).

6.2 Suppression of ras-induced focus formation by myn

Two x 10^5 10T½ cells were added to 60mm tissue culture plates and transfected by $CaPO_4$ precipitation using a CellPhect kit as described for stable transfection. The plasmids used for transfection were pHOMER6 (control DNA), pHO6T1 (activated T24 H-ras), and pCMVMyn (murine myn) (Spandidos & Wilke, 1984; Prendergast et al, 1991). Cells were transfected with 0.5 μg of ras plus increasing concentrations of myn (0 to 2.5 μg), and pHOMER6 was used as a control for nonspecific effects of transfected DNA. After transfection, cell cultures were maintained without drug selection for untransfected cells for 14 days. Monolayers were then stained with methylene blue as described above.

7. Cellular Motility

7.1 Invasion into collagen gels

Rat tail collagen containing mostly collagen I was obtained from Celltrix laboratories (Palo Alto, California) Collagen gels were prepared by mixing collagen, distilled water and 5 x concentrated Dulbecco's modified Eagle's medium (GIBCO BRL, Bethesda, MD) in a ratio of 5:3:2. This results in a final collagen concentration of 1.45 mg/ml in an isotonic buffered solution. Two ml of collagen solution was added to each 9.4 cm^2 well of a six-well plate. Plates were incubated for 30 minutes in a 5% CO_2 incubator at 37 °C to permit collagen polymerization. Cell cultures between 50 and 75% confluent were released from monolayers with trypsin solution plus 0.5mM EDTA. Trypsin was inactivated by adding 2 volumes of 10% FBS in α-MEM. Cells were counted and 8 x 10^4 cells were added per 9.4cm^2 well in a volume of 1.5 ml of 10% FBS in α-MEM on top of the polymerized collagen. Collagen invasion was quantitated by determining the number and depth from the surface of each cell in several fields after 24 or 48 h. First, the top surface of the gel was brought into focus and the number of cells in that focal plane was determined. Cells that had invaded below the surface of the gel were brought into focus and the depth of each cell was measured using a fine adjustment knob micrometer. Cells that had invaded less than 10 μm deep were considered to be on the surface of the gel due to the size of the cells examined. The percentage invasion relates the number of cells 10 or more micrometers below the surface to the total number of cells in several fields. The depth of invasion is the average depth to which all invading

cells have migrated (Schor, 1980). Invasion of A1, BNM46 and Ig60 cells were performed after plating $2x10^5$ cells, and only the percent invasion was determined.

7.2 Inhibition of invasion with antisense oligodeoxynucleotides

Antisense oligodeoxynucleotides (oligos) targetted to $TGF\beta_1$ (Spearman et al, 1994) were tested for their ability to inhibit collagen invasion of Ciras-3 cells. Subconfluent Ciras-3 cells were trypsinized, washed twice with PBS and counted. Four x 10^4 cells were added on top of a collagen gel prepared as described above in a volume of 1 ml of defined medium (D.M.)(10μg/ml insulin, 5μg/ml transferrin in α-MEM)(Schwarz et al, 1988). Oligos were added to the well to give a final concentration of 0.5 μM. An equal volume of PBS was added to control plates with no oligos. After 24 h, collagen invasion was determined.

7.3 Time lapse cinemicrography.

Cells (between 50-75% confluent) were suspended from culture with trypsin solution, which then was inactivated by adding 2 volumes of 10% FBS in α-MEM. Cells were counted and 40 000 cells were added ton a 25 cm^2 tissue culture flask. Twenty four h later, the rate of locomotion was determined using time-lapse video microscopy (Turley et al, 1991). Images were taken with a microscope (model IM35; Zeiss) using a 10X objective, every 10 minutes for a duration of 1.5 h. The rate of random locomotion was determined using an image analysis program which quantitates nuclear displacement

(Image I, Universal Imaging Corporation, PA, USA). Tracking was repeated at 48 and 72 h following subculture. Between 60 and 120 cells (30 cells per flask) were tracked for each point. To test the effect of suramin on motility, 80 000 cells were plated onto 25cm^2 flasks. Suramin (obtained from Mobay Chemical Corp [New York, New York]) was added 24 h after plating and cells were tracked 24 h after adding suramin.

8. Assessment of Neoplastic Transformation *in vivo*.

8.1 Tumorigenicity and metastatic potential.

Immunocompetent C3H/HeN syngeneic mice, 9 to 10 weeks old (Charles River, Quebec) were used to analyze the tumorigenic and metastatic properties of 10T½ derived cell lines. Athymic BALB/c nu/nu mice (Life Sciences, St Petersberg, FL) were used for *in vivo* tumor metastasis studies of NIH3T3 derived cell lines. Cells were collected, washed twice with sterile PBS and counted. Tumor latency was determined by injecting 1 x 10^5 cells subcutaneously into the lower midback and recording the time at which a tumor (2 x 2 mm) became detectable by palpation (Egan et al, 1987a). Tumor size was also recorded at 28 days post-injection by multiplying the length at right angles of the cross section of the tumor. To quantitate lung colonizing potential of cell lines, the experimental metastasis assay was used (Egan et al, 1987a; Damen et al, 1989, Taylor et al, 1992). A total of 1 x 10^5 cells were injected into the tail veins of mice, and 21 days later the lungs were stained by injecting Bouins solution (picric acid, formaldehyde, acetic acid [15:5:1]) intratracheally. Pulmonary tumors were then counted with the aid

of a dissecting microscope.

8.2 Inhibition of metastatic dissemination with antisense oligodeoxynucleotides

Antisense oligos targetted to $TGF\beta_1$ were tested for their ability to inhibit metastatic dissemination of Ciras-3 cells. Ciras-3 cells were treated with 0.5 μM antisense or sense oligos, or an equal volume of PBS for 24 h. Cells were trypsinized, counted and 2 x 10^5 cells were injected per animal into the tail veins of syngeneic C3H mice. Twenty one days post-injection, the formation of metastatic tumors was determined.

9. Immunoprecipitation and Immunoblotting Experiments.

9.1 Quantitation of protein concentration.

Protein concentrations were determined using the Bradford method (Bradford, 1976). Protein assay reagent was purchased from Biorad (Biorad Laboratories, Richmond, CA), and used according to the manufacturers instructions. BSA was used as a standard and a linear relationship between absorbance and protein concentration was observed from 0 to 100 μg protein. For immunoblot analysis to detect ras, protein concentration was determined prior to addition of sample loading buffer. For immunoblot analysis to detect myc, protein concentrations were determined by the Bradford method, after removal of SDS by ion pair extraction (Konigsberg & Henderson, 1983). Briefly,

extraction buffer (acetone:acetic acid:triethylamine, 90:5:5) was added at a twenty fold excess to the lysate and the mixture was spun at 735 x g for 15 minutes. The pellet was washed once with acetone, air dried for 5 minutes and resuspended for quantitation with Biorad reagent. Samples treated this way were compared to BSA standards dissolved in SDS lysis buffer and extracted by the same method. Protein concentrations were corrected for protein loss, which was estimated by extraction of cell lysates of known concentration. The level of protein expression detected by immunoblotting or immunoprecipitation was quantitated with a Biorad (Richmond, CA) video densitometer and corrected for amount of protein loaded. For ras, myc, myn, and histone H1 immunoblots, these levels were presented as fold increase relative to the 10T½ control. In the case of p53, the ratio of the rates of synthesis of protein immunoprecipitated by Pab421 to protein immunoprecipitated by Pab246 was determined.

9.2 Electrophoresis

9.2.1 SDS-Polyacrylamide gel electrophoresis

Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in slab gels (Laemmli, 1980). The running gel, which comprised the bottom 3/4 of the gel contained 12.5% acrylamide, 0.1% SDS, and 375mM Tris at pH 8.8. The stacking gel was poured over the polymerized running gel and consisted of 3% acrylamide, 0.1% SDS and 125mM Tris at pH 6.8. Gels were run in a continuous buffer consisting of 193 mM glycine, 1% SDS and 25mM Tris at pH 8.3. Sample loading buffer for SDS-PAGE

(4 x stock: 277mM SDS, 396mM Tris, 402mM dithiothreitol, 40% glycerol, 0.4% bromophenol blue, pH 6.8) was added to the protein sample prior to electrophoresis. Samples were then boiled for 5 min., centrifuged at 10,000xg for 10 min. and loaded onto the gel. Proteins were run towards the anode at 35mA for approximately 5 h.

9.2.2 Acid-urea-triton gel electrophoresis.

Histones were separated using acetic acid-urea-triton (AUT) gels (Panyim & Chalkley, 1969). The stacking gel consisted of 6.7 urea, 7.5% acrylamide (w/v), 375mM potassium acetate at pH 4.0, 0.0004% (w/v) riboflavin, 6mM triton-X 100, 1% thiodyglycol (v/v), and 1% temed (v/v). The running gel consisted of 6.7 M urea, 15% acrylamide (w/v), 0.5% temed (v/v), 0.835M acetic acid, 0.00036% riboflavin (v/v), 6mM triton-x-100, and 1% thiodyglycol (v/v). Sample loading buffer for AUT gels (2 x stock: 8M Urea, 0.75 M Potassium acetate, 30 % w/v sucrose, 0.1% w/v pyonin Y) was added to the histone samples which were loaded onto the gel without boiling or centrifugation. Histones were run towards the cathode in 0.9M acetic acid at 300 volts for 24 h.

9.2.3 Two dimensional gel electrophoresis

Two dimensional electrophoresis was used to separate H1 histones and was carried out with AUT in the first dimension and SDS-PAGE in the second dimension (Lennox & Cohen, 1983). AUT gel slices were soaked in 19:1 v/v buffer O (125mM Tris-Cl, 4%

SDS):14.4 M β-mercaptoethanol for 30 min before being placed on the SDS gel. 1 μg of histone H1 was separated for each cell line and visualized by staining with Coomasie Brilliant Blue or used for immunoblotting as described below.

9.3 Electroblotting

Proteins separated by electrophoresis were transferred to nitrocellulose or PVDF (Polyvinylidene difluoride) membranes by the method of Towbin (Towbin et al, 1979). Gels were placed on the membrane prewet in transfer buffer (25mM Tris, 192mM glycine, 20% [v/v] methanol). PVDF membranes were first wet with 100% methanol for 3 min. and then equilibrated with transfer buffer for 3 min. Two pieces of filter paper were placed on either side of the gel and membrane and all layers were secured in a plastic clamp (Biorad) and immersed in transfer buffer in a Biorad transfer tank. Proteins were transferred to the membrane by passing 40 volts through the tank for 18 h. Membranes were then removed and blocked as described below. Histones were also transferred by electroblotting as previously described (Delcuve & Davie, 1992). After electrophoresis, histones were transferred to PVDF membranes (Biorad Laboratories, Hercules CA) by electroblotting in transfer buffer (25mM CAPS pH 4.0, 20% methanol) for 20 h.

9.4 Immunoprecipitation analysis of p53 protein.

The p53 protein was detected by immunoprecipitating ^{35}S labelled proteins as

described previously (Rovinski et al, 1987). Antibodies used were PAb421, PAb246 and PAb419. PAb421 is a pan-reactive p53 monoclonal antibody whose ability to immunoprecipitate p53 is not affected by many mutations including the proline 193 mutation used in this study (Finlay et al, 1988). PAb246 is a monoclonal antibody that recognizes a conformation-dependent epitope of p53 that is lost upon mutation (Yewdell et al, 1986). For example, a change of alanine to valine at position 135 causes a loss of PAb246 reactivity (Finlay et al, 1988). PAb246 does not recognize the proline 193 mutant form of p53 (Hicks et al, 1991), and is specific for wild type p53 protein. PAb419 is a monoclonal antibody that recognizes the SV40 large T antigen, and was used as a control for nonspecific binding (Harlow et al, 1981). For immunoprecipitation, 1 x 10^6 cells were plated 24 h prior to labelling with ^{35}S-methionine for 3 h. Cells were treated with p53-lysis buffer (1% Nonidet P-40, 150 mM NaCl, 20 mM Tris-HCl [pH 8.0], 50 μg aprotinin [Sigma, St. Louis, MO]), precleared with formalin fixed *Staphylococcus aureus* (Sigma, St. Louis, MO) and incubated with antibody. Antibody complexes were precipitated with protein-A Sepharose (Pharmacia, Uppsala, Sweden), and washed with p53-lysis buffer, and the precipitated proteins were separated by SDS-PAGE. Gels were fixed for 30 minutes (7% acetic acid, 25% methanol), washed in Enhance (NEN, Boston, MA) for 30 minutes, dried and exposed to film.

9.5 Immunoblot analysis of p21-ras protein

The p21 H-ras protein was detected by immunoblotting with the monoclonal antibody Ras10 (Dupont, Boston, MA); an antibody which recognizes mutant and wild

type H-, Ki-, and N-ras proteins. Immunoblots were performed as previously described (Hicks et al, 1991, Taylor et al, 1992). A total of 2.5 x 10^5 cell equivalents were treated with ras-lysis buffer (18 mM Tris [pH 8.0], 177 mM LiCl, 0.7 mM EDTA, 5% NP40, 50 μg aprotinin [Sigma, St. Louis, MO]), separated by SDS-PAGE and transferred to nitrocellulose membranes. Blots were probed with Ras10 and bands were detected using Vectastain kits (Vector Laboratories, Burlingame, CA). Briefly, blots were incubated with biotinylated anti-mouse IgG for 30 minutes, washed in TBST buffer (0.1 M Tris, 0.3 M NaCl, 0.05% Tween-20), incubated with alkaline phosphatase conjugated to avidin for 30 minutes, washed and visualized using the Alkaline Phosphatase Substrate Kit II, following the manufacturer's instructions (Vector Laboratories, Burlingame, CA).

9.6 Immunoblot analysis of p62-myc protein

The p62 c-myc protein was detected by immunoblotting with the monoclonal antibody 9E10 (c-myc Ab-1, Oncogene Science, Manhasset, N.Y.). This antibody recognizes human c-myc protein (Evan et al, 1985). A total of 1 x 10^6 cells were added to culture plates with growth medium 24 h prior to lysis. Cells were treated with lysis buffer (62.5 mM Tris-HCl [pH 6.8], 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.0125% bromphenol blue) and material from 4 x 10^5 cells was separated by SDS-PAGE without additional sample loading buffer. Proteins were transferred to nitrocellulose membranes, which were washed in blocking solution for 30 minutes [5% (wt/vol) nonfat dry milk in TBST buffer]. Blots were incubated with the 9E10 antibody and immunoreactive bands were detected by reacting with alkaline phosphatase.

9.7 Immunoblot analysis of myn protein

The antiserum used was generated against a fusion protein containing 6 histidine amino acids fused to the full length murine myn protein (UBI, Lake Placid NY). For blocking experiments, 5 μg of purified human GST-MAX was added to 5 ml of the diluted antiserum (1:1000) 15 min prior to use. Although raised against the murine myn protein, the antiserum used also cross reacts with the human max protein (Technical data, UBI, Lake Placid NY). Two x 10^6 cells were lysed in 75 μl lysis buffer (18mM Tris [pH 8.0], 177 mM LiCl, 0.7 mM EDTA, 5% Nonidet P-40, 50 μg aprotinin [Sigma, St Louis MO]). Aliquots were removed to determine protein concentration using the standard Biorad protein assay (Biorad Laboratories, Hercules CA). Twenty five μl of 4 x SDS sample loading buffer (277mM SDS, 396mM Tris, 402mM dithiothreitol, 40% glycerol, 0.4% bromphenol blue, pH 6.8) was added and 350 μg of total protein was separated by SDS-PAGE and transferred to PVDF membranes. Membranes were blocked in 10% nonfat dry milk dissolved in TBST buffer (0.1M Tris, 0.3M NaCl, 0.05% Tween-20) and probed with the myn antiserum diluted in TBST buffer containing 0.5% nonfat dry milk. Membranes were probed with a goat antirabbit antibody linked to horseradish peroxidase (Sigma, St Louis MO) and myn was detected using the ECL (enhanced chemiluminescence) detection system (Amersham, Oakville, Ontario, Canada) (Samuel et al, 1993).

9.8 Immunoblot analysis of phosphorylated histone H1.

Highly phosphorylated H1 histone was detected by immunoblotting procedures, using an antiserum raised in rabbits against phosphorylated H1 histone from *tetrahymena*. The antibody reacts with phosphorylated murine H1 histone (Lu et al, 1994). Briefly, histone H1 was isolated by the method described below, and 1 μg was resolved either by SDS-PAGE alone or by two dimensional AUT gels into SDS polyacrylamide gels (Laemmli, 1980; Lennox & Cohen, 1983). H1 histones were then transferred to PVDF membranes as described above. Membranes were blocked in 7.5% nonfat dry milk dissolved in TBST buffer (0.1M Tris, 0.3M NaCl, 0.05% Tween-20) for 2 h and then incubated in antibody to phosphorylated histone, diluted (1:1000) in antibody buffer (TBST plus 0.5% milk), for 1 h at 24 °C. Membranes were washed and then incubated for 1 h in a goat antirabbit antibody linked to horseradish peroxidase (Sigma, St Louis MO) diluted in antibody buffer (1:5000). The membrane was washed and the antigen was detected using the ECL detection system (Amersham, Oakville, Ontario, Canada).

10. Isolation and Analysis of Histones.

10.1 Isolation of histones.

Histones were isolated as described with some modifications (Yasuda et al, 1981). For the isolation of histones, $5x10^5$ cells were plated per 150mm plate, grown in 10% calf serum in α-MEM and harvested 72 h later with trypsin solution. Approximately $4x10^7$

cells in total were used for histone preparation. Harvested cells were washed twice with PBS, and homogenized in a glass homogenizer in 5ml Nuclear Preparation buffer (10mM Tris-Cl pH 7.6, 150mM NaCl, 1.5 mM $MgCl_2$ and 0.65% Nonidet-P40). Nuclei were recovered by centrifugation at 1500 xg for 10 min. All centrifugations were carried out at 4°C. Nuclei were resuspended in 3 ml RSB buffer (10mM Tris-HCl pH 7.5, 3mM $MgCl_2$, 10mM NaCl), and sulfuric acid was added to a final concentration of 4N. Nonhistone proteins were allowed to precipitate for 30 min on ice and the supernatent was collected by centrifugation at 12,000 xg for 20 min. Perchloric acid (PCA) was added to the supernatent to a final concentration of 5% and left on ice for 15 min. The core histone pellet was recovered by centrifugation at 12,000 xg for 20 min and resuspended in distilled water and the H1 histones present in the supernatent were recovered by precipitation with 18%N trichloroacetic acid (TCA) for 30 min followed by centrifugation at 12,000 xg for 20 min. The TCA pellet was washed once with acetone-HCl solution (99.5% acetone v/v, 0.5% HCl v/v) and once with 100% acetone, and then resuspended in water. Protein concentration of histone samples were determined by spectrophotometric determination (at 400nm) of histones precipitated in 16.7% TCA for 15 min. The concentration (μg/ml) of histone = (A_{400} x 120)/0.0093 (Luck et al, 1958). In addition, concentrations were verified using the standard Biorad protein assay (Biorad Laboratories, Hercules CA).

10.2 Metabolic labelling of H1 histone.

Metabolic labelling was carried out as previously described (Yasuda et al, 1981).

Briefly, $2.5x10^6$ cells (per 150mm plate) were grown for 24 h. The monolayers were washed twice with PBS, and then 5 ml of phosphate free α-MEM containing 100μCi/ml orthophosphate ^{32}P were added. Cells were incubated for 2 h, and then removed with trypsin solution and washed twice with PBS. H1 histone was isolated as described above. ^{32}P labelled H1 histones were separated by SDS-PAGE and dried gels were exposed to film.

10.3 Immunofluorescence

Immunofluorescence was carried out as previously described with some modifications (Lu et al, 1994). One x 10^5 cells were plated onto glass coverslips (22x22mm) which had been placed in the bottom of each 9.4 cm^2 well of a six-well plate. After 24 h, the cells were washed twice with PBS, and fixed for 20 min in formaldehyde fixative (2% formaldehyde in PBS), then in anhydrous methanol for 20 min. Fixed cells were washed three times with PBS, and antibody (diluted 1:100 in 10% fetal calf serum in PBS) was added for 1 h at 37°C. Antibody was removed with three washes of PBS and secondary antibody added (FITC [fluorescein isothiocyanate]-labelled goat-antirabbit antibody diluted 1:200 in PBS plus 10% fetal calf serum). After 2 h, the secondary antibody was removed with 5 washes of PBS plus 10% FBS. Cells were mounted in 20μl mounting reagent (50mg p-phenylenediamine, 5 ml 1M Tris pH 7.4, 37.5 ml glycerol) and visualized with an inverted fluorescence microscope. Normal preimmune rabbit serum was used as a negative control and was treated in the manner described above. DAPI [4',6-diamidino-2-phenylindole] staining was carried out with coverslips prepared

as described above. Cells were incubated at room temperature for 5 min in 1 μg/ml of DAPI. Excess stain was washed off by three changes of PBS.

11. Regulated Induction of ras Expression.

To induce ras expression in NIH3T3-433 cells, which contain a T24 H-ras gene linked to the mouse mammary tumor virus promotor, these cells were grown in $2x10^{-6}$M dexamethasone (Sigma, St Louis, Mo.) for 7 days (Huang et al, 1981). Zinc induction of ras in 2H1 cells, which contain a T24 H-ras gene linked to the metallothionein promotor, was accomplished by adding zinc sulphate to a final concentration of 50μM (Haliotis et al, 1990).

12. Flow Cytometry

Cell cycle analysis of all cell lines was determined by flow cytometry (Yasuda et al, 1981). Cells were plated in the same manner as those used to isolate H1 histone. Five x 10^5 cells were plated per 150mm plate, grown in 10% calf serum in α-MEM and harvested 72 h later with trypsin solution. Cells were washed twice with PBS, resuspended in PBS and fixed by the slow addition of 5 parts 95% ethanol. Fixed cells were washed twice in PBS, and treated with 10μg/ml RNAase for two h at 37°C. Cells were stained with ethidium bromide (10 μg/ml) and analyzed with a fluorescence activated cell sorter to determine cell cycle distribution.

13. Arrest of Cells in G1 Phase of the Cell Cycle

Five x 10^5 cells were plated per 150mm plate, and grown for 72 h. Fresh medium (10% calf serum in α-MEM) containing 2mM hydroxyurea (Sigma, St. Louis, MO) was then added to the plates and cells were grown for an additional 24 h (Ashihara & Baserga, 1979). Cells were then harvested and used for flow cytometric analysis or for histone extraction.

14. Induction of DNA Synthesis by K-FGF Conditioned Medium

K-FGF secreted by NIH3T3 cells transfected with the K-fgf gene was determined using a bioassay. Two ml of medium conditioned by 5 x 10^5 cells for 24 h was collected, centrifuged for 10 min at 10,000g, and used in the DNA synthesis assay (Klagsbrun et al, 1977). The induction of DNA synthesis was analyzed in confluent, serum starved 10T½ fibroblasts, prepared as follows: 10T½ cells were resuspended at a concentration of 5 x 10^4 cells/ml in α-MEM plus 10% FBS. An aliquot (0.2 ml) was added to each well of a 96 well plate. Plates were incubated until cells reached confluence (2 days). At this time, the growth medium was replaced with D. M. (Schwarz et al, 1988). After 3 days of incubation in defined medium, conditioned medium was added to the 10T½ cells in a total volume of 0.2 ml along with 0.8 μCi of [methyl-3H]thymidine (Amersham, Oakville, Ontario). Cells were harvested with an automatic cell harvester (Skatron, Lier, Norway) 18 h later and incorporated radioactivity was counted. The level of induction of DNA synthesis was compared to the ability of known quantities of human

recombinant bFGF to induce DNA synthesis under the same conditions (GIBCO BRL).

15. Statistical Analysis

The various properties of the FGF transfected cells or ras, myc, p53 transfected cells were compared with paired t-tests or an analysis of variance. Where significant differences were detected, multiple comparisons were used with the least square means method to determine which data sets were different. Statistical analysis of the correlation between metastasis and motility was accomplished with linear regression analysis.

III. Results

1. Growth Factor Induced Motility and the Metastatic Phenotype

1.1 Invasion of collagen gels

As shown in table 1 NIH3T3 cells transformed by members of the fibroblast growth factor family vary with respect to their malignant potential as judged by the ability to disseminate to the lungs of BALB/c nu/nu mice when injected intravenously (Damen et al, 1991; Egan et al, 1990). For example, analysis of cells transfected with K-fgf revealed that expression of this proto-oncogene could induce both tumorigenic and metastatic characteristics (Damen et al, 1991). However, the transforming and metastatic properties of bFGF appeared to depend upon the presence of a heterologous signal sequence for secretion (Egan et al, 1990; Rogelj et al, 1988). The ability to invade collagen gels is frequently observed with populations of highly malignant cells and is due in part to cell motility (Wright et al, 1993). The properties of the cell lines described under Materials and Methods and summarized in table 1 provided an opportunity to test whether K-fgf gene expression could alter invasive characteristics on a physiologically relevant collagen substratum, and whether bfgf gene expression had to be linked to a signal sequence for secretion to modify the motility characteristics of NIH3T3 fibroblasts. As shown in figure 6a, the untransformed dC2 cell line showed very low levels of invasion with only 2.1% and 3.6% of the cells observed below the surface of the gel after 24h or 48h, respectively. The two K-FGF transformed cell lines, 3G and

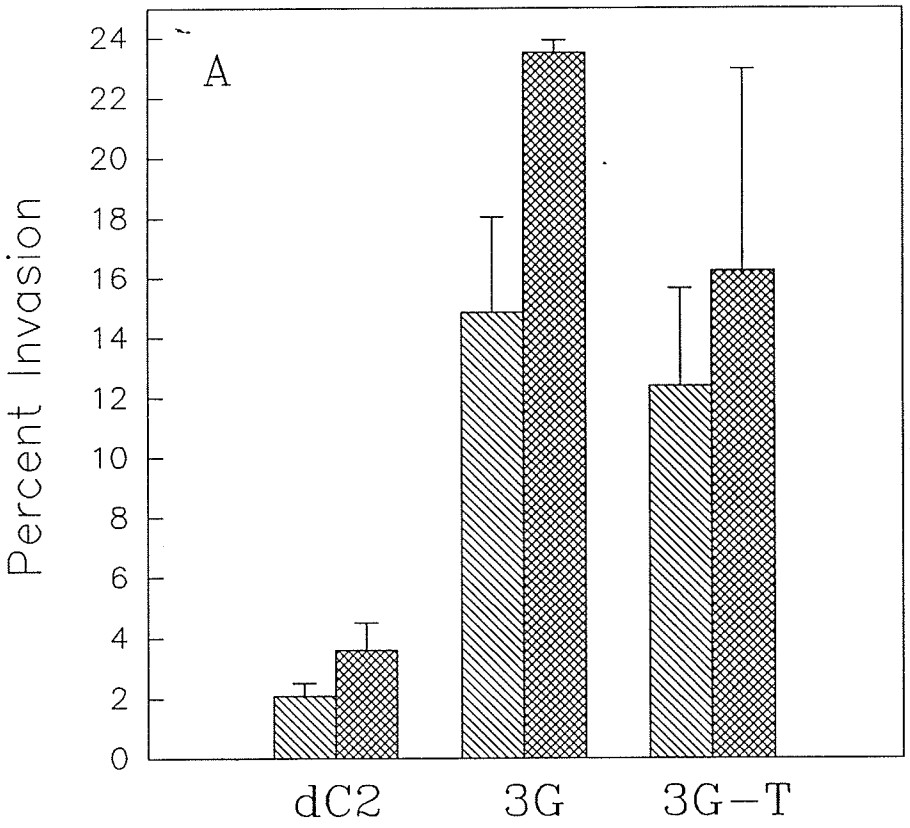
TABLE 1.

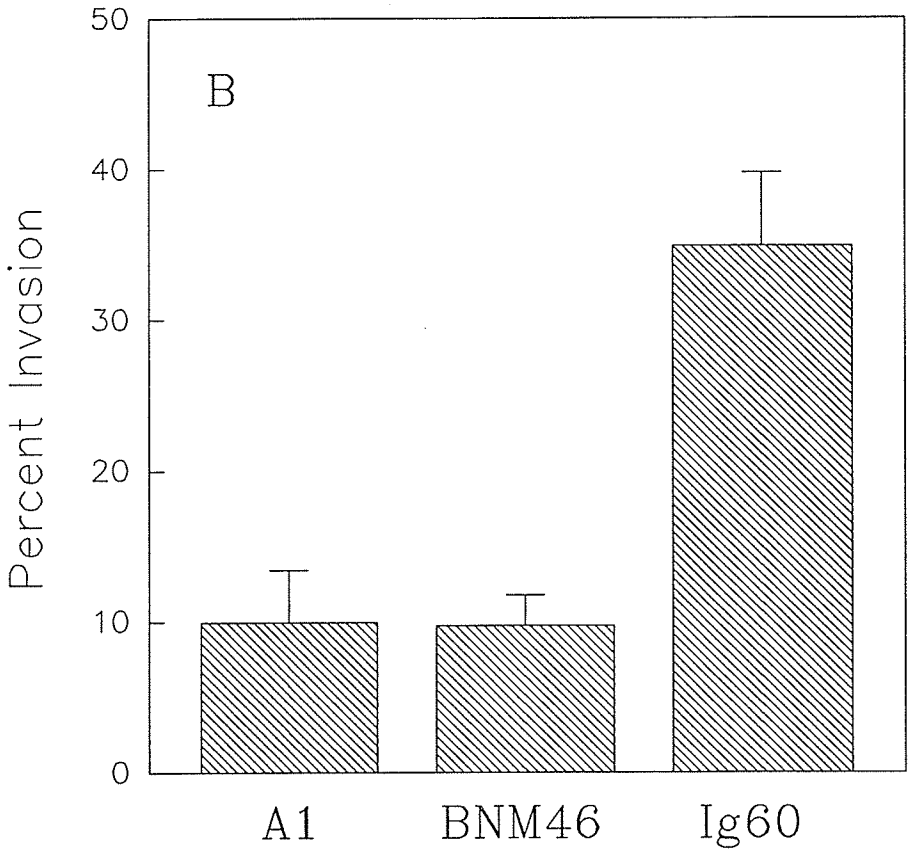
Summary of the Metastatic Potential of NIH3T3 Fibroblasts Transfected with K-fgf or bfgf Sequences. Metastatic potential was determined by the lung colony assay 21 days after injecting cells into the tail veins of BALB/c nu/nu mice as previously described (Damen et al, 1991; Egan et al, 1990). ssbFGF contains the bFGF coding region fused to the immunoglobulin signal sequence for secretion. The growth factor activity produced by bfgf-transfected cell lines was previously determined (Egan et al, 1990; Rogelj et al, 1988). Briefly, intracellular bFGF levels were determined by analysis of the induction of thymidine incorporation into quiescent NIH3T3 cells by cell lysates prepared from bfgf-transfected cells. Basic FGF was not detectable in the medium from cells transfected with the natural form of bFGF (BNM46 and BNM35), consistent with the lack of a signal sequence for secretion in this molecule. Consistent with previous results, bFGF was not detected in the medium of cells transfected with the ssbFGF molecule (Ig60 and Ig68), presumably due to its immediate association with the cell surface where it activates an autocrine pathway for transformation (Rogelj et al, 1988). K-FGF levels secreted into the medium were determined by a similar method using 10T½ cells as the indicator cells, and conditioned medium from dC2, 3G and 3G-T. Previous studies have shown that these K-fgf-transfected fibroblasts secrete K-FGF into the medium and undetectable amounts are found intracellularly (Damen et al, 1991; Damen 1990). Growth factor activity is shown as units (U)/10^4 cells where one unit is the amount of growth factor required to induce half-maximal DNA synthesis as determined using purified bFGF. Background activity produced by control cells was subtracted to give the activities shown.

Cell Line	Gene	Growth Factor Activity ($U/10^4$ cells)	Metastatic Potential (number of lung tumors)	Number of Cells Injected
A1	vector	-	0	$3x10^5$
BNM46	bFGF	2.5	0	$3x10^5$
BNM35	bFGF	4.0	0.2	$3x10^5$
IG60	ssbFGF	0.7	153	$3x10^5$
IG68	ssbFGF	0.6	27	$3x10^5$
DC2	vector	-	0	$1x10^5$
3G	K-FGF	3.6	6.7	$1x10^5$
3G-T	K-FGF	3.0	37.3	$1x10^5$

FIGURE 6.

Invasion of collagen gels by NIH3T3 fibroblasts. (A) The percent of cells below the surface of the gel relative to the total cell number observed in three to four microscopic fields (average of 2 trials) is shown for the control dC2 cell line and the K-fgf transformants 3G, and 3G-T, after 24 h (hatched bar) and after 48 h (cross-hatched bar). (B) The percent of cells below the surface of the gel relative to the total cell number observed is shown for the control A1 cell line, the BNM 46 cell line (contains bFGF sequence without a signal sequence for secretion), and the Ig60 cell line (contains a heterologous signal sequence for secretion) after 24 h (average of 3 trials). Standard errors are shown by the bars.


A
Percent Invasion
0
2
4
6
8
10
12
14
16
18
20
22
24
dC2
3G
3G−T

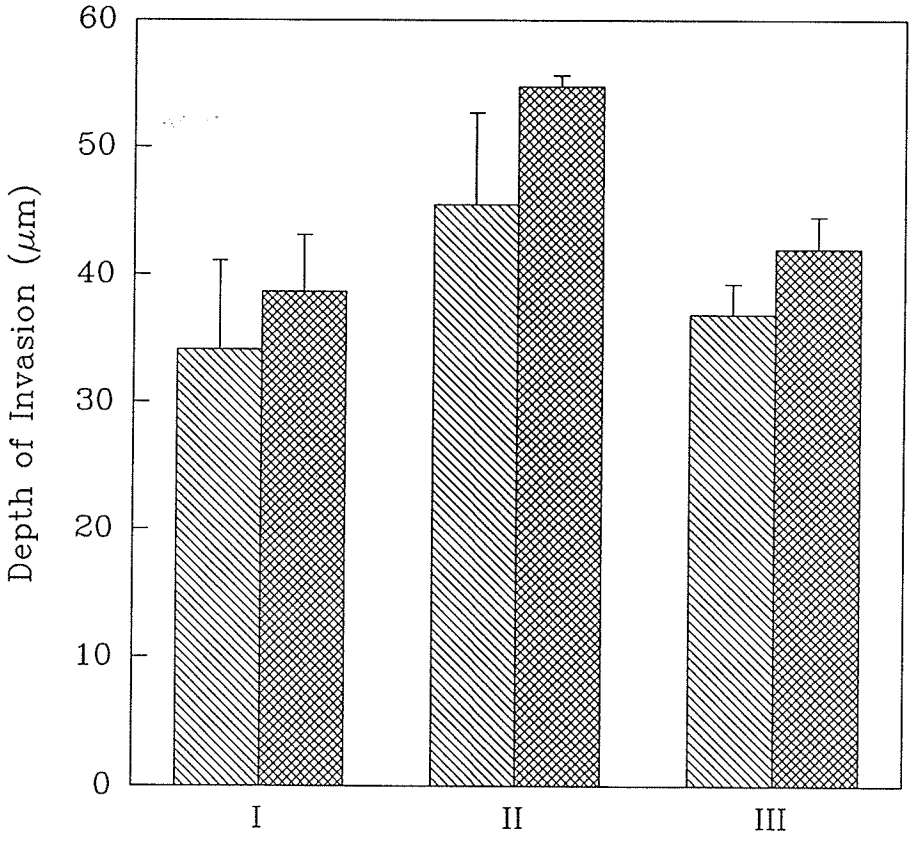

B
Percent Invasion
0
10
20
30
40
50
A1
BNM46
Ig60

3G-T, showed much higher levels of invasion at both 24h and 48 h. The 3G and 3G-T cell lines were approximately 6.5 to 7 times and 4.5 to 6 times, respectively, more aggressive in this assay when compared to the control dC2 cell line. Statistical analysis clearly showed that the results obtained with the 3G and 3G-T cell lines were significantly different from those obtained with dC2 cells ($p=0.0045$ and 0.012 respectively). No statistically significant differences between the two K-fgf-transfected cell lines were observed. We next analyzed the depth to which K-fgf-transfected cells invaded during collagen invasion. We found that the few dC2 cells below the surface of the gel invaded to an average depth of 34.1 ± 7.0um, 24 h after plating and 38.7 ± 4.5um 48 h after plating (figure 7). Invading 3G cells and 3G-T cells showed average depths of invasion of 45.5 ± 7.1um and 36.9 ± 2.4um respectively, at 24 h and 54.7 ± 0.9um and 42.1 ± 2.5um, respectively at 48 h. There was no significant difference in the depth of invasion exhibited by 3G or 3G-T cells compared to dC2 cells (p values of 0.083 and 0.603, respectively).

Examination of the bFGF transformed NIH3T3 cells indicated that both the vector-transfected control A1 line, and the bfgf-transfected BNM46 line (lacking a secretory signal sequence) demonstrated relatively low levels of invasion of 10.0% and 9.7%, respectively after 24 h (figure 6b). In contrast, the Ig60 cell line transfected with bFGF fused to a signal sequence for secretion exhibited 35.0% invasion into the collagen gel. The invasive potential of Ig60 cells was significantly higher than that of both A1 (p value of 0.0005) and BNM46 (p value of 0.0005).

FIGURE 7.

Depth of invasion of K-fgf transfected NIH3T3 fibroblasts. The average depths of invasion (μm) into gels of collagen I was determined at 24 h (hatched bar) and at 48 h (cross-hatched bar) after plating. The depth of invasion of (I) dC2, (II) 3G, and (III) 3G-T cells is shown and standard errors are shown by the bars.


60
50
40
30
20
10
0
Depth of Invasion (μm)
I
II
III

1.2 Motility of K-fgf transfected cells

To test the possibility that a relationship existed between the invasive potential of K-fgf-transfected cells as shown by collagen invasion assays and alterations in rates of cellular locomotion, as determined by nuclear displacement over time, we measured the rates of cellular motility of transfected cells in normal growth medium after 24, 48 and 72 h. The rate of locomotion, or motility, is the average distance moved by all of the cells under observation, for a fixed period of time. Cellular position is defined as the position of the nucleus (Turley et al, 1991). No statistical differences in motility rates were observed 24 h after subculture, but it was clear that 48 and 72 h after subculture the K-fgf-transfected cells exhibited higher rates of locomotion when compared to the control dC2 cell line (figure 8). Statistical analysis showed that 3G and 3G-T cells were significantly more motile than dC2 cells, with p values of 0.0077 and 0.0004 respectively.

1.3 Rates of locomotion of bFGF-transfected cells.

The invasive capacity of transfected cells receiving the bFGF signal sequence for secretion, when compared to that of cells containing the normal bFGF (figure 6b) predicted that targeting the growth factor to the secretory pathway may also be important in the induction of a motile response. Therefore, the motility rates of cells transfected with the bFGF Ig signal sequence fusion gene was compared with cells transfected with the normal bFGF sequence. Figure 9 shows that three of the four bFGF-transfected lines showed higher rates of locomotion when compared to the control A1 cell line and to

FIGURE 8.

Rate of locomotion of K-fgf transfected NIH3T3 fibroblasts. The average rates of random locomotion (μm/min.) for the cell lines dC2 (triangle), 3G (white square) and 3G-T (black square) are shown. The rates at which cells move along the surface of a tissue culture flask (rate of locomotion) were determined by quantitating nuclear displacement as described under Materials and Methods. The rate of locomotion is an average rate of movement exhibited by the individual cells in the population under observation. Rates were determined at 24, 48, and 72 h and standard errors are shown by the bars. Experiments were performed between 2 and 4 times for each condition.

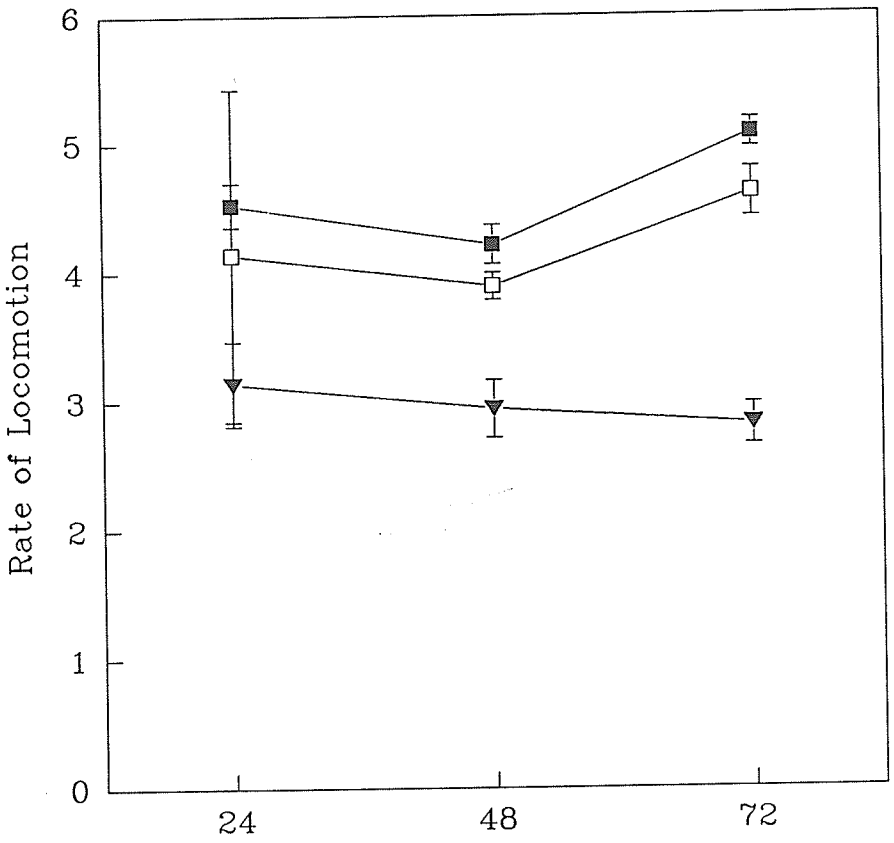
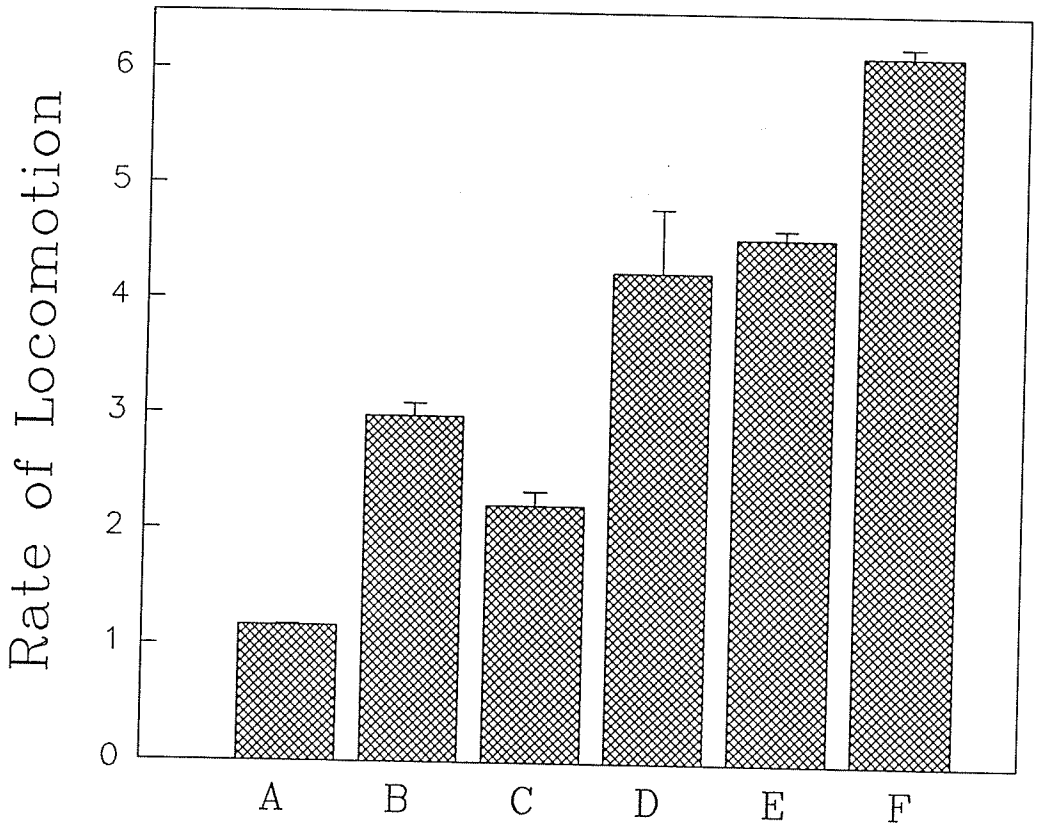

Rate of Locomotion
6
5
4
3
2
1
0
24
48
72

FIGURE 9.

Rate of locomotion of NIH3T3 fibroblasts transfected with bFGF sequences. The average rate of random locomotion (μm/min) for NIH3T3 (A), A1 (B), BNM 46 (C), BNM 35 (D), Ig60 (E), Ig68 (F) is shown. The A1 cell line was transfected with the vector lacking bFGF sequence, whereas the BNM 46 and BNM 35 cell lines were transfected with the intracellular form of bFGF. Ig60 and Ig68 are cell lines which overexpress a bFGF sequence fused to the immunoglobulin signal sequence for secretion. Cells were tracked 72 h after addition. Standard errors are shown by the bars and experiments were performed between two and four times per condition.


Rate of Locomotion
0
1
2
3
4
5
6
A
B
C
D
E
F

untransfected NIH3T3 fibroblasts. However, fusion to a signal sequence did not reproducibly enhance bFGF-induced motility. Thus, Ig68, a cell line containing the bFGF signal sequence fusion gene showed a rate of locomotion of 4.54 um/min, which was similar to BNM35, a cell line containing bFGF lacking a conventional secretion signal sequence and exhibiting a motility rate of 4.24 um/min. Interestingly the most metastatic line, Ig60, showed the highest rate of motility with a rate of 6.13 um/min while the least metastatic lines, A1 and BNM46, exhibited lower levels of motility, with rates of 2.98 and 2.21 respectively. Statistical analysis of the data showed that the BNM35, Ig60, and Ig68 cell lines exhibited motility rates significantly different from that of the control A1 cell line ($p=0.043$, 0.0005 and 0.016, respectively), and as expected no significant differences between A1 and BNM46 cells were found.

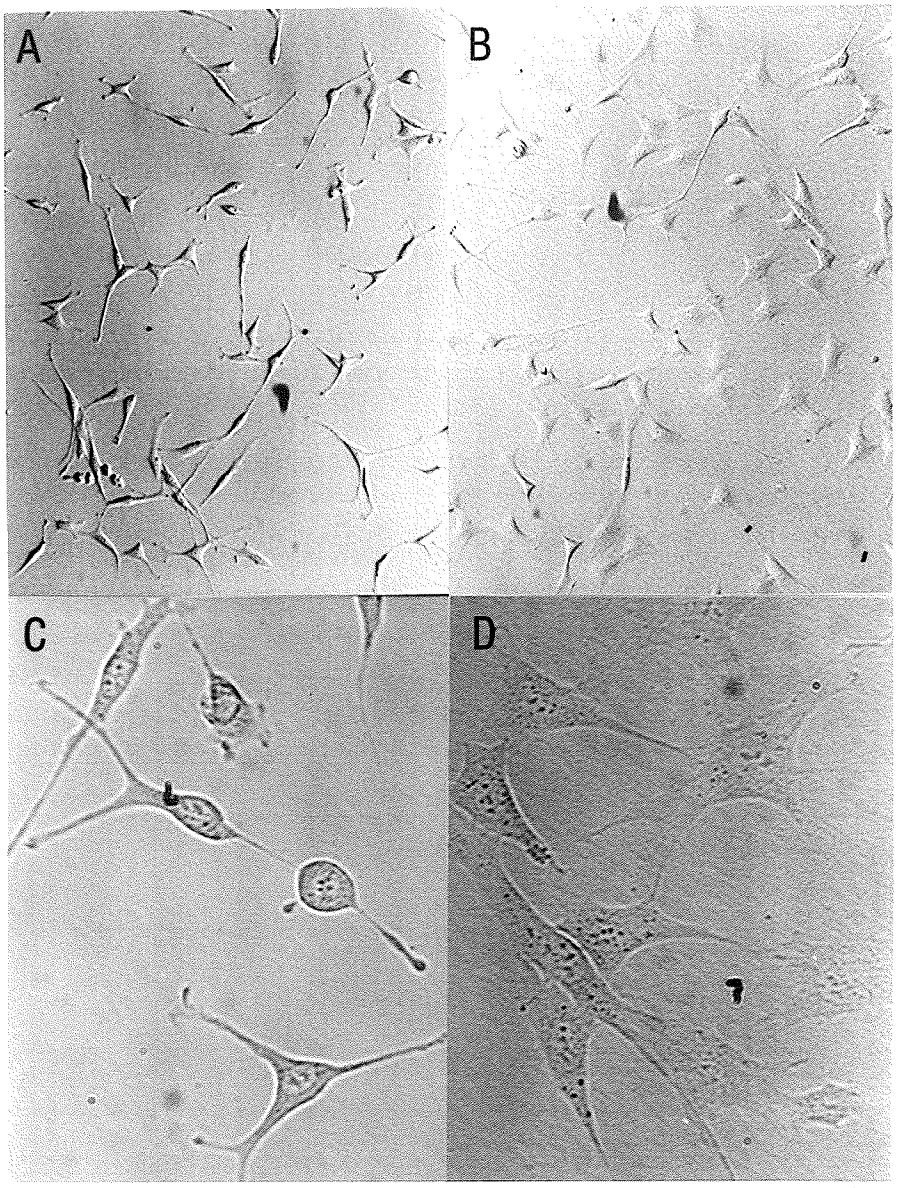
1.4 Effect of suramin on K-FGF induced motility and morphology

Suramin is a polysulfonated urea compound which interferes with interaction of fibroblast growth factors and other heparin binding growth factors with their receptors (LaRocca et al, 1990). Suramin has been previously found to reverse the morphological transformation induced by K-fgf transfection (Moscatelli & Quarto, 1989). This effect is thought to be caused by the ability of suramin to interfere with a transforming loop involving K-FGF and its receptors.

We noticed that when 3G and 3G-T cells are exposed to 250 uM suramin for 24 h, these cells loose their transformed morphology consistent with previous reports with other K-FGF transformed cell lines (figure 10). Suramin treatment had a striking effect

FIGURE 10.

The effect of suramin on the morphology of cells transfected with bFGF fused to a signal sequence for secretion. Cells were treated for 24 h with suramin and then photographed. The Ig60 cell line containing bFGF fused to a heterologous signal sequence is shown in the absence (A & C) and presence (B & D) of 250μM suramin.


A
B
C
D

on the motility of K-fgf-transfected cells. As shown in figure 11a, both 3G and 3G-T cells showed considerable reductions in their motility rates after suramin treatment, whereas the rate of locomotion of dC2 cells was unaffected by the presence of 250uM suramin. The reduction of motility in 3G and 3G-T cells was found to be statistically significant (p values of 0.0033, and 0.00001, respectively). We found that suramin treatment also led to a significant reduction in the rate of locomotion of the ssbFGF-transfected cell line Ig60 (p value = 0.0006, figure 11a)

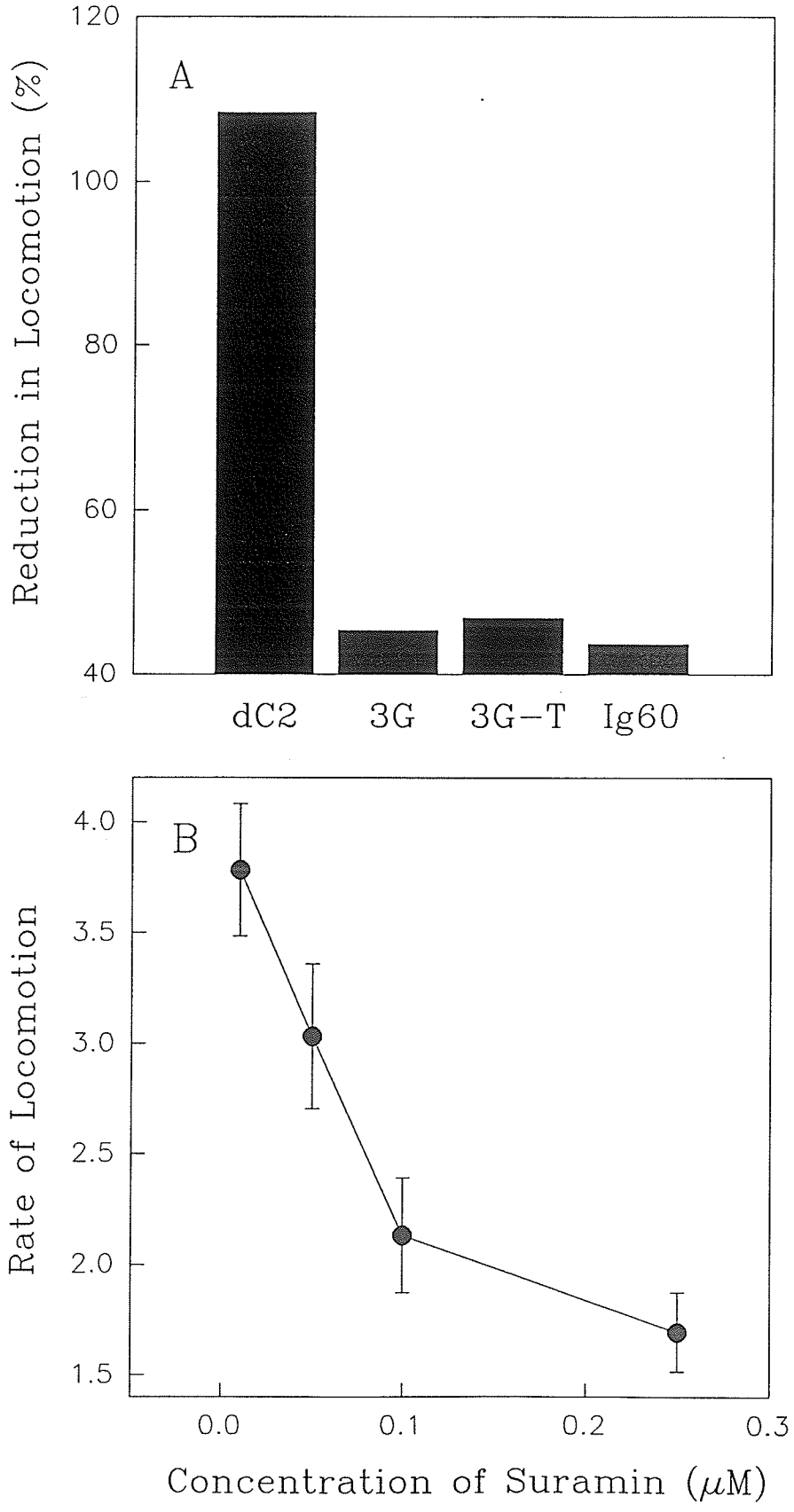
Next, we determined the effect of suramin concentration on the motility of K-fgf-transfected cells. The extent of reduction in motility of 3G-T cells was found to be dependent on the concentration of suramin used (figure 11b). We noted that suramin has a maximal effect on motility at concentrations up to 0.10μM. Concentrations higher than 0.10 μM had little further effect on the motility of these cells.

1.5 Correlation of metastatic ability and rate of locomotion

Our analysis of the motility of bFGF transfectants revealed that increased motility was not restricted to bFGF-fused signal sequence transfectants. One line transfected with the bfgf sequence lacking a signal sequence showed a high rate of motility. This clone, although transfected with an intracellular form of bFGF is capable of forming a small number of lung tumors when injected intravenously (table 1). This is presumably due to the high level of intracellular growth factor produced by this cell line (table 1). Taken together these observations suggested that the rate of motility of these cell lines may be correlated with metastatic potential. When the frequency of metastasis of all the K-fgf-

FIGURE 11.

The effect of suramin on the rate of locomotion of cells transfected with a K-fgf or bfgf sequence fused to a signal sequence for secretion. (A) The reduction in locomotion (%) shows the average rate of random locomotion in the presence of 250 μM suramin as a percentage of the average rate of random locomotion in the absence of suramin. This is shown for dC2, 3G, 3G-T, and Ig60 cells. Cells were treated with suramin for 24 h prior to the determination of the rate of locomotion. Experiments were performed between three and six times and the standard errors for all points were below 12% of their respective average rates of locomotion. (B) The effect of increasing concentrations of suramin on the rate of locomotion of 3G-T cells is shown. Standard errors are indicated by the bars (two or three trials per condition).


A
Reduction in Locomotion (%)
120
100
80
60
40
dC2
3G
3G−T
Ig60
B
Rate of Locomotion
4.0
3.5
3.0
2.5
2.0
1.5
0.0
0.1
0.2
0.3
Concentration of Suramin (μM)

and bfgf-transfected cell line was analyzed as a function of motility rates (figure 12), benign tumor cells exhibited rates between 2.21 and 4.24 μm/min, whereas all metastatic cell lines showed relatively high motility rates between 4.25 and 6.14 μm/min. Statistical analysis demonstrated that a significant correlation existed between cell locomotion and metastatic potential (p value = 0.037).

1.6 Involvement of TGFβ_1 in motility and metastasis

The correlation between motility and metastasis observed with fibroblast growth factor transformed cell lines suggested that part of the ability of growth factors to regulate the metastatic phenotype depends on their ability to regulate motility. We tested this hypothesis by determining the effect of another growth factor, TGFβ_1, on metastasis. We employed the Ciras-3 cell line which is transformed by an activated ras allele, secretes high levels of TGFβ_1, and can efficiently metastasize in syngeneic mice (Schwartz et al, 1990; Egan et al, 1987a). Ciras-3 cells were treated with an antisense oligonucleotide specific for TGFβ_1, capable of reducing the secretion of TGFβ_1 from these cells (table 2)(Spearman et al 1994). Treatment with the antisense but not the control sense oligodeoxynucleotide led to a significant reduction in invasion into gels of collagen I (figure 13). Antisense-mediated reduction in invasion could be abrogated by the addition of exogenous TGFβ_1 suggesting that the effect of the oligodeoxynucleotide is specific for this growth factor. In addition, antisense treated cells showed significantly lower abilities to metastasize *in vivo* compared to control treated cells (figure 14).

FIGURE 12.

The relationship between the rate of locomotion (μm/min.) and metastatic potential of NIH3T3 fibroblasts transfected with the K-fgf proto-oncogene or by bFGF sequences. The average rate of random locomotion of individual cell lines 72 h after plating was analyzed against metastatic potential (evaluated as the number of lung tumors per $1x10^5$ cells injected).

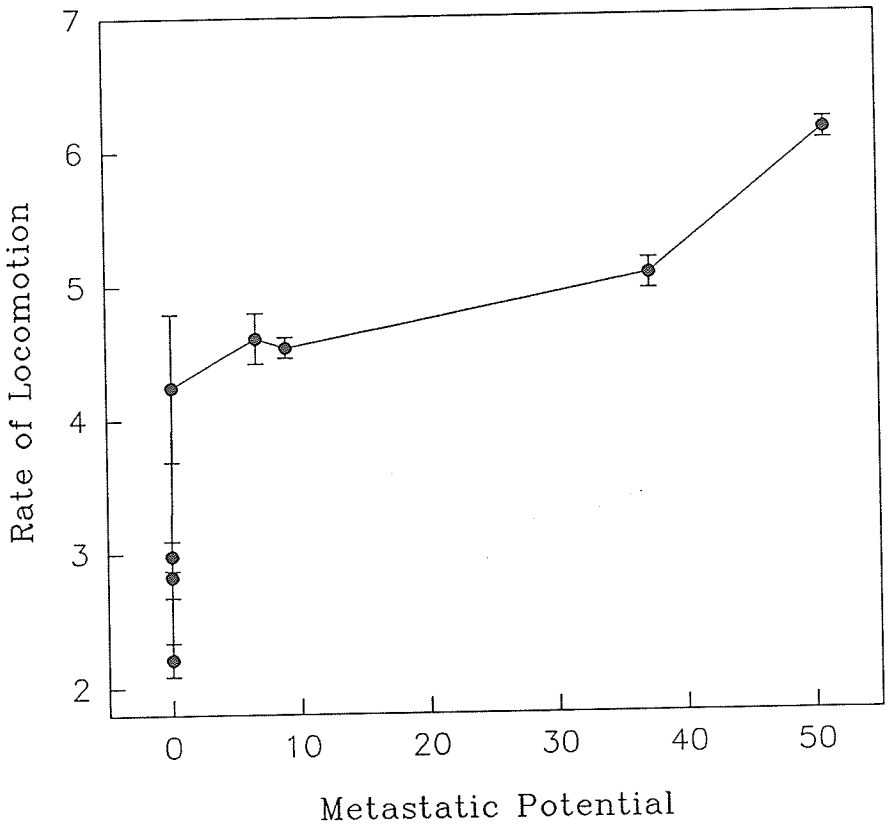

Rate of Locomotion
7
6
5
4
3
2
0
10
20
30
40
50
Metastatic Potential

TABLE 2.

Oligonucleotide sequences. All the oligonucleotides are phosphorothioate derivatized, except the bold face nucleotides which represent unmodified phosphodiester groups. The sense oligonucleotides, which were used as controls, are complimentary to the antisense oligonucleotides with the same phosphorothioate derivatization.

Coding sequence	5'	CGCTCTCCGAGGTGCCGTGGGGCGCCGCCTCCCCCATGCCGCCCTCGG 3'
AS-4	3'	GCGGCGGAGGGGGTA 5'
AS-5-0S0	3'	**CGGAG**GGGGTACG**GCGGGA** 5'

FIGURE 13.

Antisense oligonucleotide effects on the invasion of Ciras-3 cells into collagen I gels. Ciras-3 cells were plated onto collagen gels in D.M. in the presence of oligonucleotide. Percent invasion was determined 24 h later as previously described (Taylor et al., 1993). The following treatments are shown: (1) Control, 0.5 μM sense oligonucleotide [S-5-OSO] and PBS treated, (2) 0.5 μM antisense [AS-5-OSO], (3) 1.0 μM antisense [AS-5-OSO] plus human recombinant TGF-β_1 [10ng/ml], (4) 1.0 μM sense [S-4], and (5) 1.0 μM antisense [AS-4]. Six independent trials were performed. Statistical analysis revealed that there was a significant difference when treatment (2) was compared to either (1) or (3), (p values of 0.026 and 0.038 respectively) however no evidence for a difference between treatments (4) and (5) were observed (p value of 0.45).

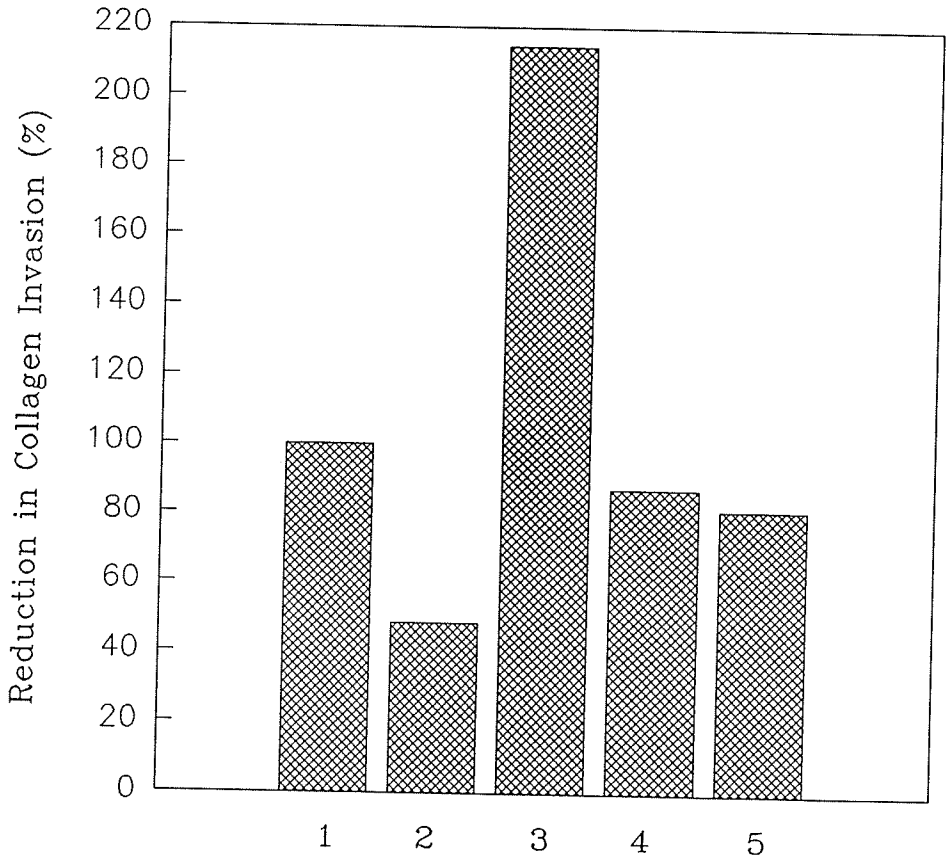
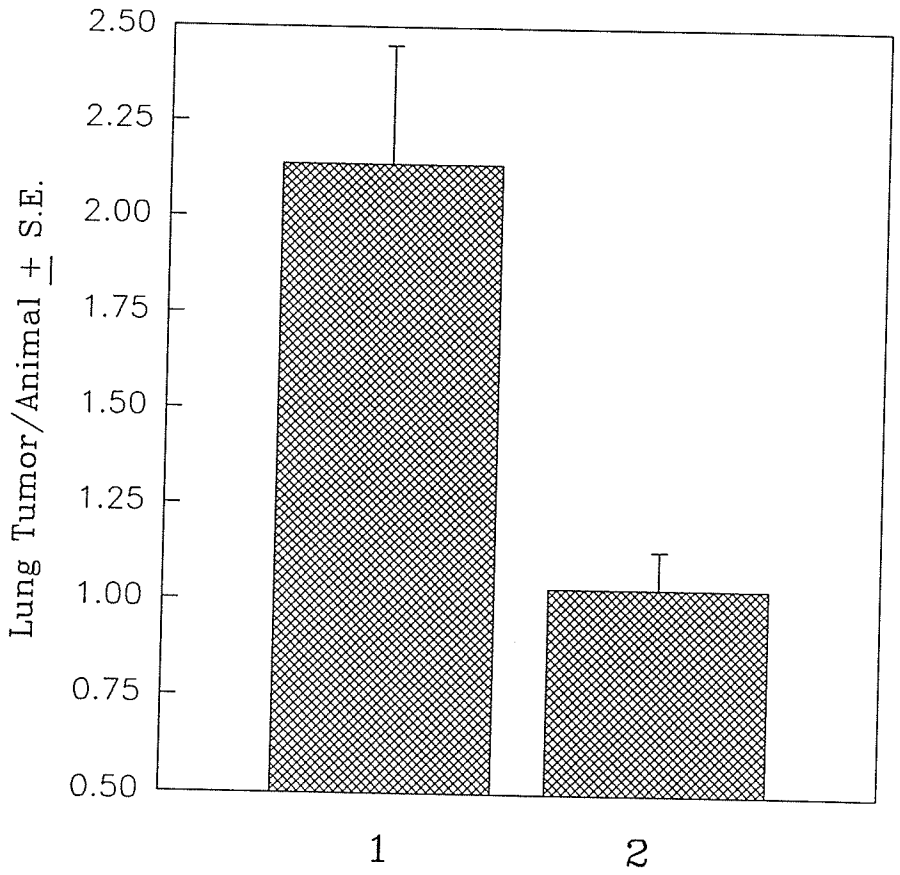

Reduction in Collagen Invasion (%)
220
200
180
160
140
120
100
80
60
40
20
0
1
2
3
4
5

FIGURE 14.

Antisense oligonucleotide effects on tumor dissemination of Ciras-3 cells to the lungs of syngeneic mice. Ciras-3 cells were treated with 0.5 μM oligonucleotide in D.M. for 24 h, removed from culture plates with trypsin solution, and 10^5 cells were injected into the tail veins of syngeneic mice as previously described (Egan et al., 1987a; Damen et al., 1991; Taylor et al., 1992). (1) Control animals received cells treated with sense oligonucleotide (S-5-0S0) or an equal volume of PBS. (2) Antisense treated cells were exposed to AS-5-0S0. At least eight animals/group were used, and the number of lung tumors/animal was determined. The values were 24 tumors/11 animals (an average of 2.2/animal), 31 tumors/8 animals (an average of 3.9/animal), and 44 tumors/9 animals (an average of 4.9/animal) for AS-5-0S0, S-5-0S0 and PBS alone. The value obtained for the antisense AS-5-0S0 oligonucleotides was significantly different than the value obtained in the control experiments ($p = 0.05$).


2.50
2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
Lung Tumor/Animal ± S.E.
1
2

2. Oncogene Cooperation and Metastatic Progression

2.1 Focus formation by cells transfected with combinations of ras, myc and p53.

The ras oncogene, which has been postulated to act downstream of growth factors such as bFGF, can induce the metastatic phenotype, however only inefficiently (Egan et al 1989b; Kremer et al 1991). We tested the ability of myc and p53 to cooperate with ras in the malignant transformation of fibroblasts. Mouse 10T½ cells were transfected with combinations of ras, myc and the proline 193 mutant form of p53 (Munroe et al, 1990; Rovinski et al., 1987), and examined for the ability to produce transformed foci (figure 15), an *in vitro* test for cellular transformation (Hsiao et al, 1987). Transfection with myc or myc/p53 did not lead to focus formation. However, transfection with ras, ras/myc or ras/p53 induced a low level of focus forming ability (table 3). Interestingly, inclusion of the three genes ras, myc and p53 together led to a relatively large increase in the number of foci observed. Although no evidence was obtained for significant differences in the mean number of foci formed among cells transfected with ras, ras/myc or ras/p53, the three gene ras/myc/p53 combination was significantly more efficient than the other gene combinations in the ability to produce transformed foci (p values of 0.006, 0.014 and 0.012 respectively). To determine if the genes had been integrated and expressed in the transfected cells, we derived cell lines from individual foci and tested them further.

FIGURE 15.

Foci formation following oncogene transfections. (1) 10T½ cells were transfected with the plasmid pH06T1 (ras) and (2) 10T½ cells were transfected with the plasmids pHmrn17 and pEC53 (ras/myc/p53).

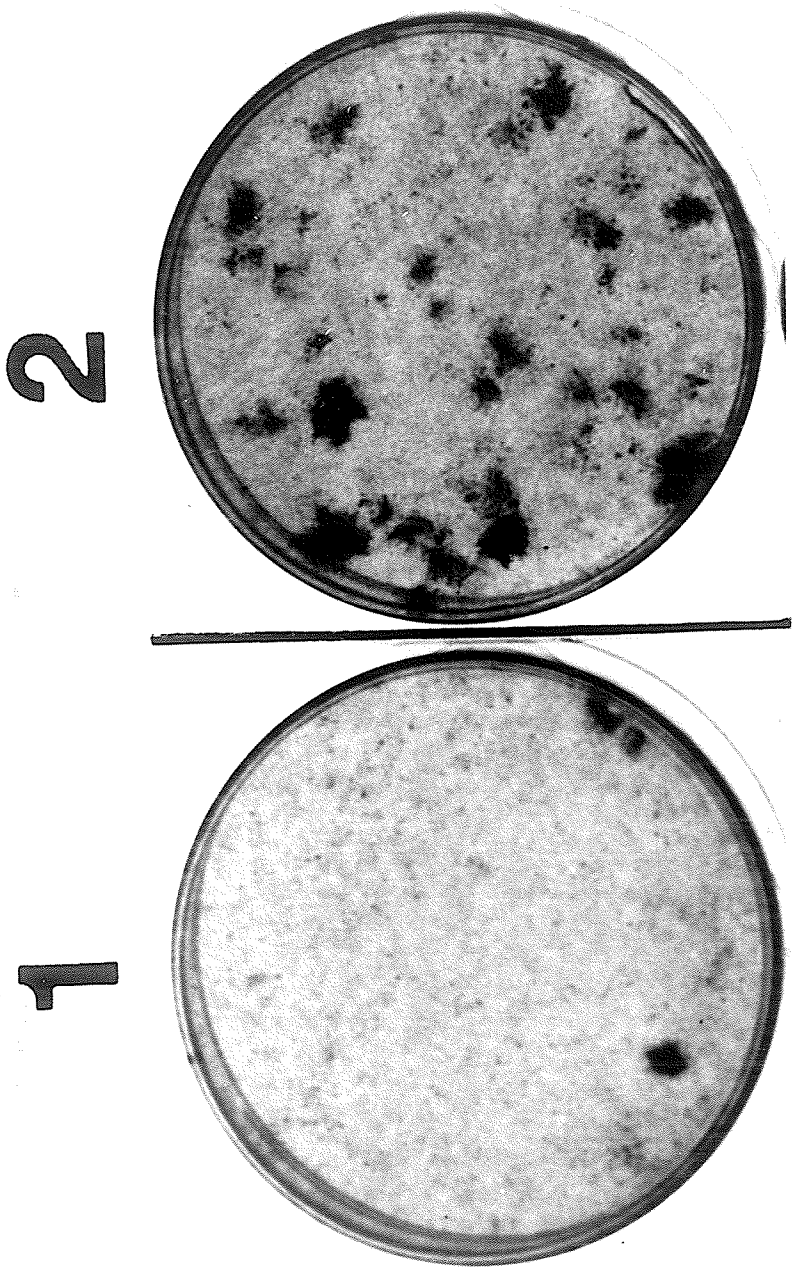

2
1

TABLE 3.

Effects of Oncogene Transfection on Transformed Foci Formation

* Six independent transfection experiments were performed.

Statistical analysis indicated that the ras/myc/p53 combination was significantly different from the ras, ras/myc or ras/p53 combinations with p values of 0.006, 0.014, 0.012 respectively. In an overall F test the p value was 0.021.

Genes	Average No. of Foci ± S.E.*
ras	3.8 ± 1.6
ras/myc	5.7 ± 3.2
ras/p53	5.3 ± 2.2
ras/myc/p53	25.2 ± 6.8

2.2 Expression of ras, myc and p53 proteins.

Seventeen clones were obtained from foci after transfection. Five clones were picked after transfection with the three gene combination of ras/myc/p53, and 4 clones were obtained from each transfection carried out with ras, ras/myc or ras/p53. Cells from isolated foci were cultured in the presence of 400 μg G418 sulfate for 1 week. Cells from all the clones proliferated in the drug supplemented medium indicating that they had incorporated the neo^R gene in the transfection procedure. The incorporation of exogenous DNA was also confirmed by Southern blot analysis (figure 16). Analysis of ras proteins in these clones demonstrated that most of the clones overproduced ras, with levels ranging between approximately one- and sixfold compared to non-transfected 10T½ cells (figure 17). Cells transfected with ras, ras/myc or ras/p53 showed an average increase of ras protein of about twofold, whereas the ras/myc/p53 gene combination exhibited an average elevation of about 4.5 fold (figure 18). It is clear that the differences in ras protein levels observed between the ras/myc/p53 transfectants and the ras, ras/myc or ras/p53 gene combinations are statistically significant (p values of 0.0003, 0.0001 and 0.0008 respectively).

Cells from nine clones transfected with p53 were analyzed for the presence of the mutant form of p53 protein. Lysates containing [^{35}S] methionine-labelled proteins were immunoprecipitated with an antibody capable of binding to mutant and wild type p53 protein (Pab 421) as well as an antibody which recognizes p53 only in the wild type conformation (Pab 246). Expression of the proline 193 mutant form was indicated by an increase in p53 protein precipitated with Pab 421 relative to Pab 246.

FIGURE 16.

Examples of southern blot analysis of cell lines transfected with ras, myc and mutant p53. Genomic DNA was isolated, digested with Hind III, separated by agarose gel electrophoresis and probed with a ^{32}P labelled cDNA fragment specific for the ras gene. Lanes: 1) 10T½, 2) R-1, 3) RM-5, 4) RP-6, 5) RMP-1, 6) RMP-4, 7) RMP-6. Hind III recognized one site in the ras containing transfected plasmids, pHmnr17 and pHO6T1. Due to the size of these plasmids, we expected a band of at least 19kb in cell lines containing pHmnr17 (lanes 3, 5, 6, and 7) and a band of at least 14kb in cell lines containing pHO6T1 (lanes 2 and 4).

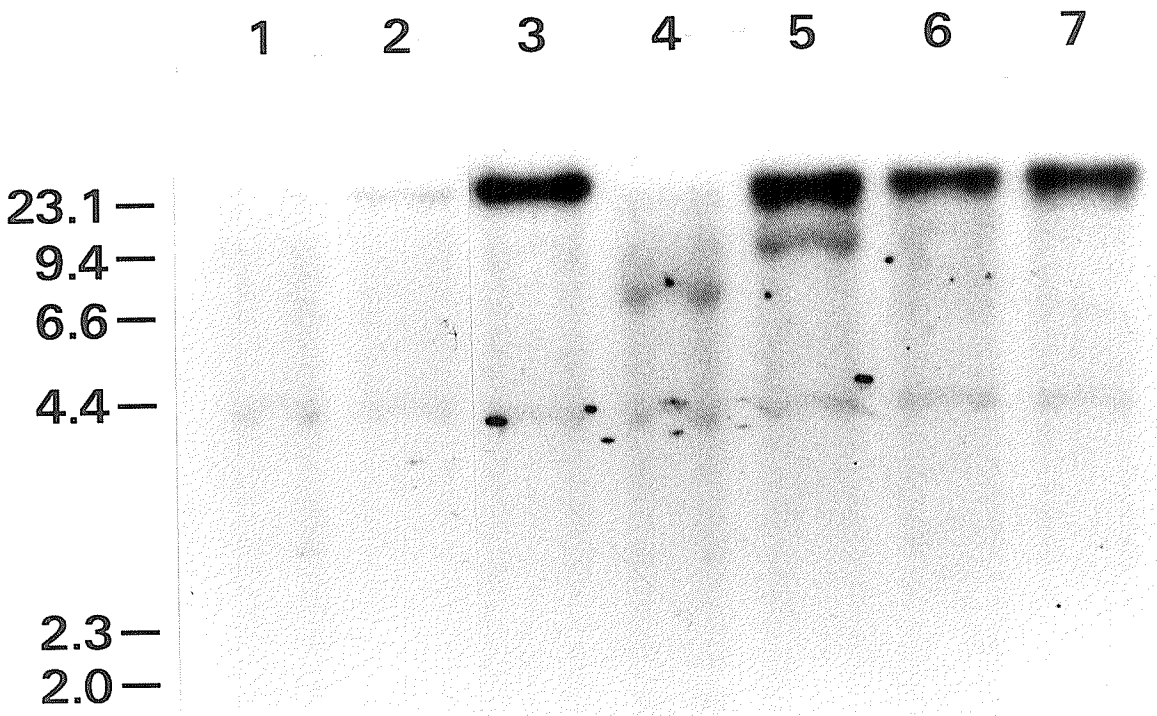

1
2
3
4
5
6
7
23.1
9.4
6.6
4.4
2.3
2.0

FIGURE 17.

Immunoblot analysis of p21-ras levels in focus derived cell lines. The ras oncoprotein expressed from the parental 10T½ line and the 17 selected lines is shown. Cell lines containing ras, myc, and p53 and are denoted RMP-1 to 6, those with ras and myc are denoted RM-3 to 6, those with ras and p53 are denoted RP-1 to 6 and those with only ras are denoted R-1 to 4. The amount of protein loaded for each sample is shown after the names of the cell lines loaded. Lanes: (1) 10T½, 86μg; (2) RMP-1, 50μg; (3) RMP-2, 50μg; (4) RMP-4, 55μg; (5) RMP-5, 58μg; (6) RMP-6, 65μg; (7) RP-1, 70μg; (8) RP-3, 78μg; (9) RP-4, 65μg; (10) RP-6, 38μg; (11) RM-3, 103μg; (12) RM-4, 35μg; (13) RM-5, 35μg; (14) RM-6, 73μg; (15) R-1, 35μg; (16) R-2, 50μg; (17) R-3, 65μg; (18) R-4 58μg.

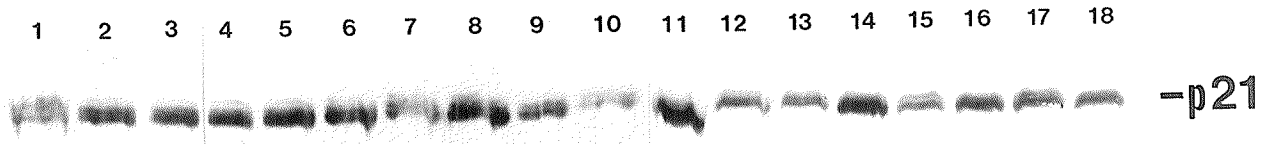
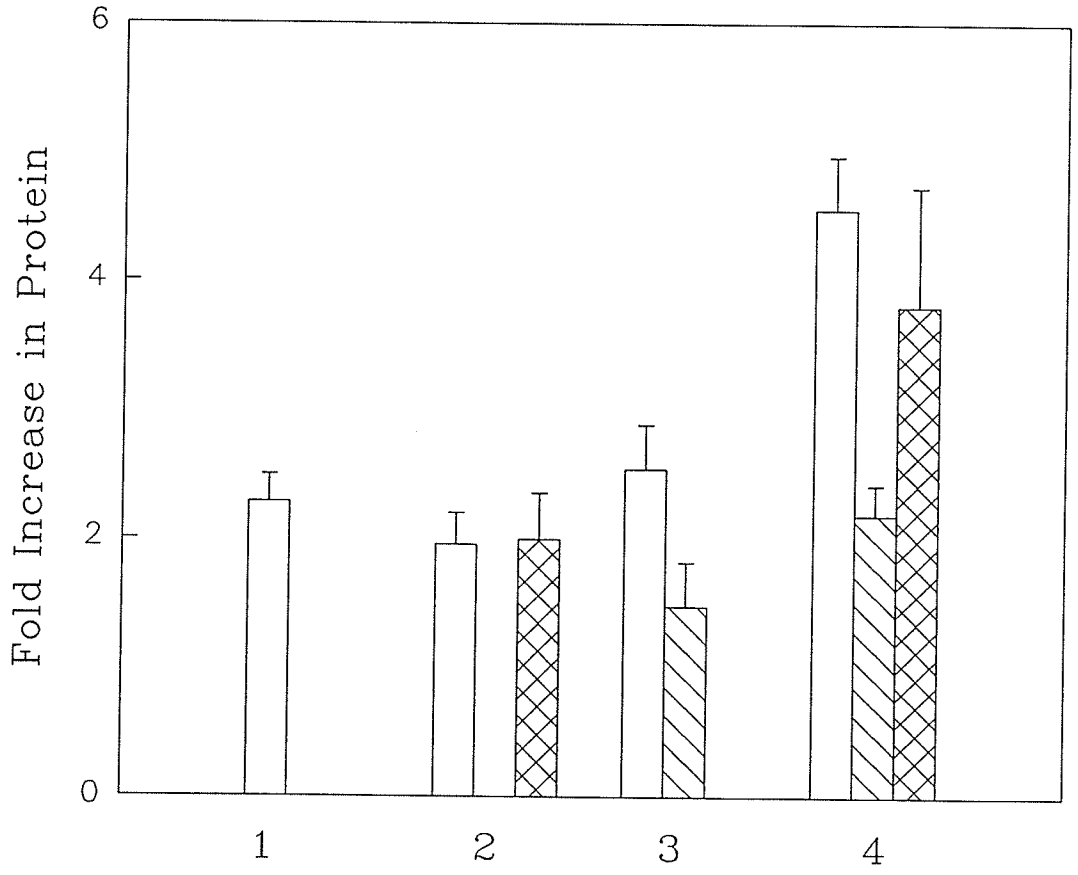

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
-p21

FIGURE 18.

Comparison of the average oncoprotein levels determined in transfected lines. The steady state level of ras (open bars), and myc (cross hatched bars) and the average increase in synthetic rates of mutant p53 (hatched bars) are shown. For ras and myc, these levels are relative to the 10T½ control. For p53, the ratio of the rates of synthesis of protein immunoprecipitated by Pab421 compared with Pab246 is shown. The average fold increase in protein expression ± S.E. for cells transfected with ras(1), ras/myc(2), ras/p53(3), or ras/myc/p53(4) is presented. Statistical analysis of ras protein data indicated significant differences existed between the ras/myc/p53 gene combination and the ras, ras/myc or ras/p53 combinations, with p values of 0.0003, 0.0001 and 0.0008 respectively. Statistical analysis of myc protein levels indicated a trend toward increased myc protein levels in the ras/myc/p53 gene combination compared with the ras/myc combination, but this was not statistically significant at the 95% confidence level (p value of 0.144). Statistical analysis of mutant p53 synthetic rates also did not show significant differences at the 95% confidence level between the ras/myc/p53 gene combination and the ras/p53 combination (p value of 0.270).


Fold Increase in Protein
6
4
2
0
1
2
3
4

Lysates were also immunoprecipitated with an antibody specific to the SV40 large T antigen. Since 10T½ cells do not express the large T antigen, this allowed identification of proteins precipitated by non-specific interactions with the antibody-protein A-Sepharose complex described in the Materials and Methods section. The cell line 17-17 was used as a positive control for the expression of mutant p53 as it had integrated the transfected p53 sequences and expressed high levels of mutant p53 (figure 19). Eight out of nine lines analyzed showed expression of the mutant protein (figure 20). However, no evidence for a statistically significant difference at the 95 % confidence level was observed when the average rates of mutant p53 protein synthesis in cells containing the two-gene combination (ras/p53) were compared with the three-gene combination of ras/myc/p53 ($p = 0.270$)(figure 18).

Cells from nine clones transfected with c-myc were tested for c-myc protein expression. Immunoblots revealed that all lines analyzed exhibited an increase in the 62 kD species of c-myc (figure 21a). That this was the transfected human c-myc was confirmed in immunoblotting experiments with a human c-myc specific monoclonal antibody, which showed that the 62 kD band comigrated with an amplified band in extracts of the human Colo320 HSR cell line (Figure 21b); this cell line contains an amplified c-myc gene, and overproduces c-myc protein (Hann & Eisenman, 1984). Densitometric measurements of immunoblots showed a range of increase in human c-myc approximately one- to 6.5- fold in the clones tested (Figure 18). A statistical analysis of differences in human c-myc protein between the two gene combinations that express this protein indicated that, although there was a trend towards higher levels in the ras/myc/p53 gene combination, this was not significant at the 95 % confidence level ($p = 0.144$).

FIGURE 19.

Characterization of the 17-17 cell line used as a positive control for p53 expression. 10T½ cells were transfected with the CB7 mutant of p53 as described under Materials and Methods and the 17-17 cell lines isolated after transfection. A)Southern blot analysis of 1) 10T½ and 2) 17-17 probed for p53 sequences. B) Immunoprecipitation analysis of p53 expression in 1) 10T½ and 2) 17-17 cells. Separate aliquots of lysate for each cell line were subject to immunoprecipitation with Pab 419 (large T) (a), Pab 421 (pan reactive p53) (b), and Pab 246 (wild type p53) (c).

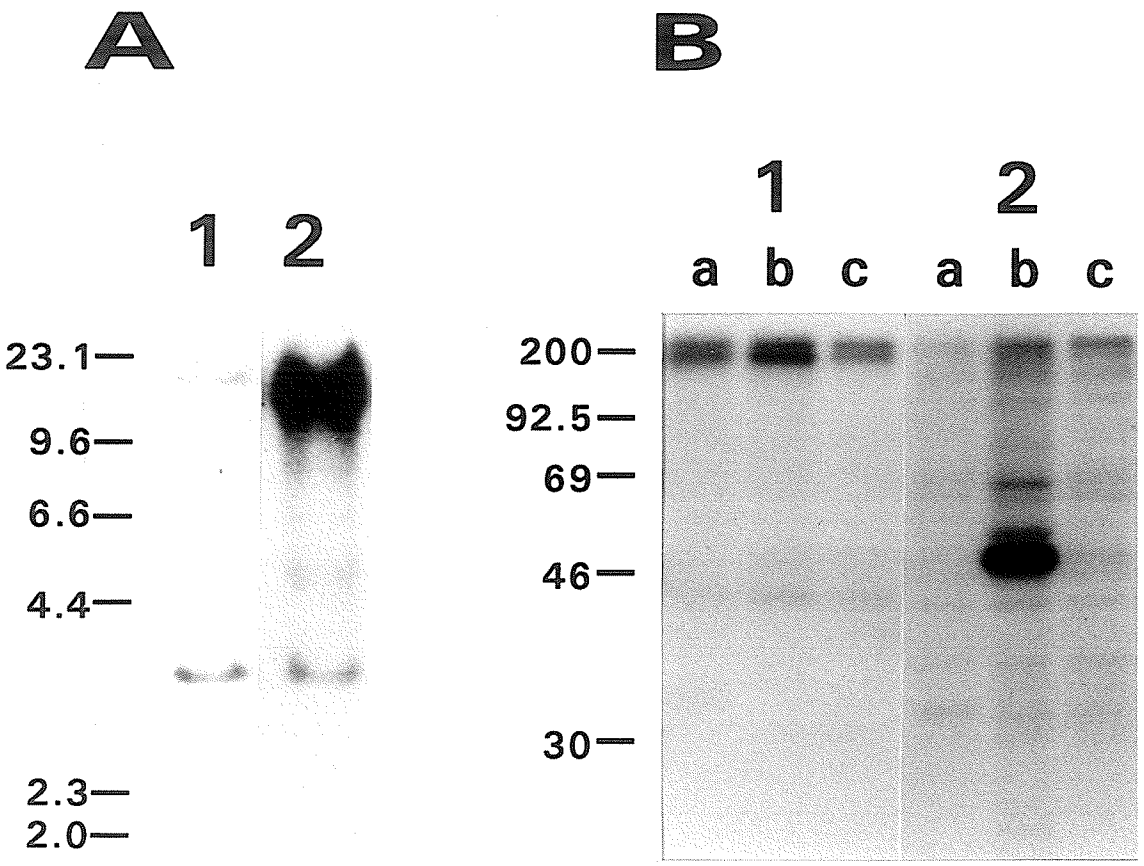

A
1 2
23.1
9.6
6.6
4.4
2.3
2.0
B
1
2
a b c a b c
200
92.5
69
46
30

FIGURE 20.

Immunoprecipitation analysis of p53 protein in focus derived cell lines. Separate aliquots of lysate for each p53 transfected cell line (nine in all) were subject to immunoprecipitation with Pab 419 (large T) (A), Pab 421 (pan reactive p53) (B), and Pab 246 (wild type p53) (C). The lines analyzed were (1) 10T½; (2) 17-17; (3) RMP-1; (4) RMP-2; (5) RMP-4; (6) RMP-5; (7) RMP-6; (8) RP-1; (9) RP-3; (10) RP-4; (11) RP-6. Cell line 17-17 is a positive control, previously shown to express mutant p53 (see figure 19).

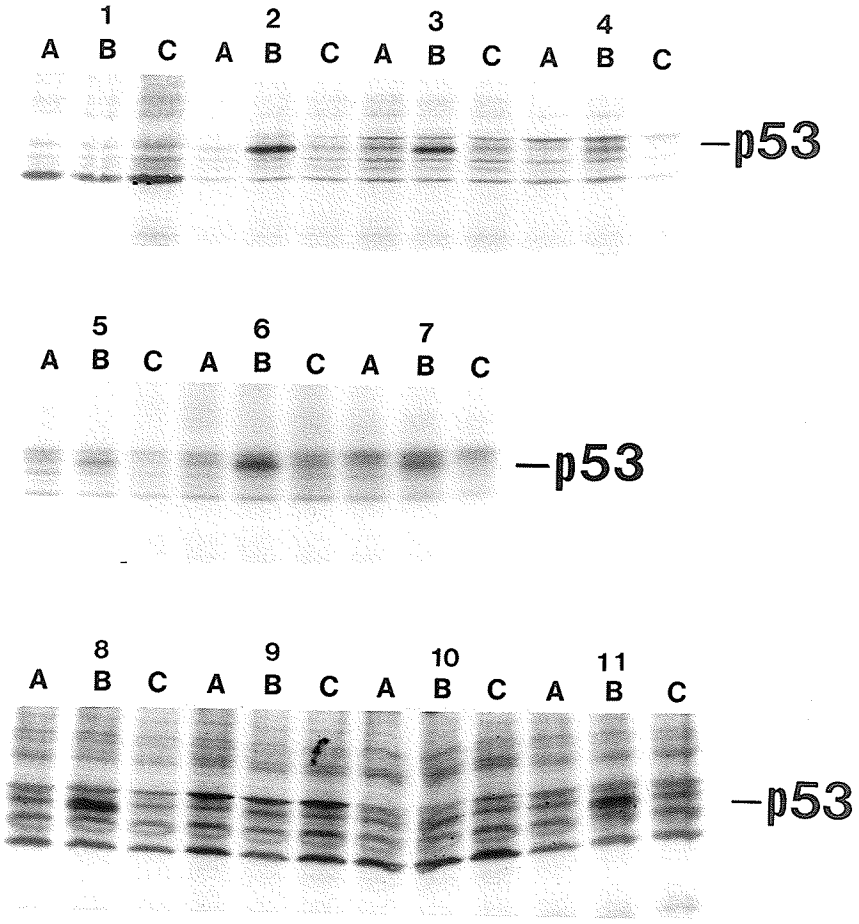
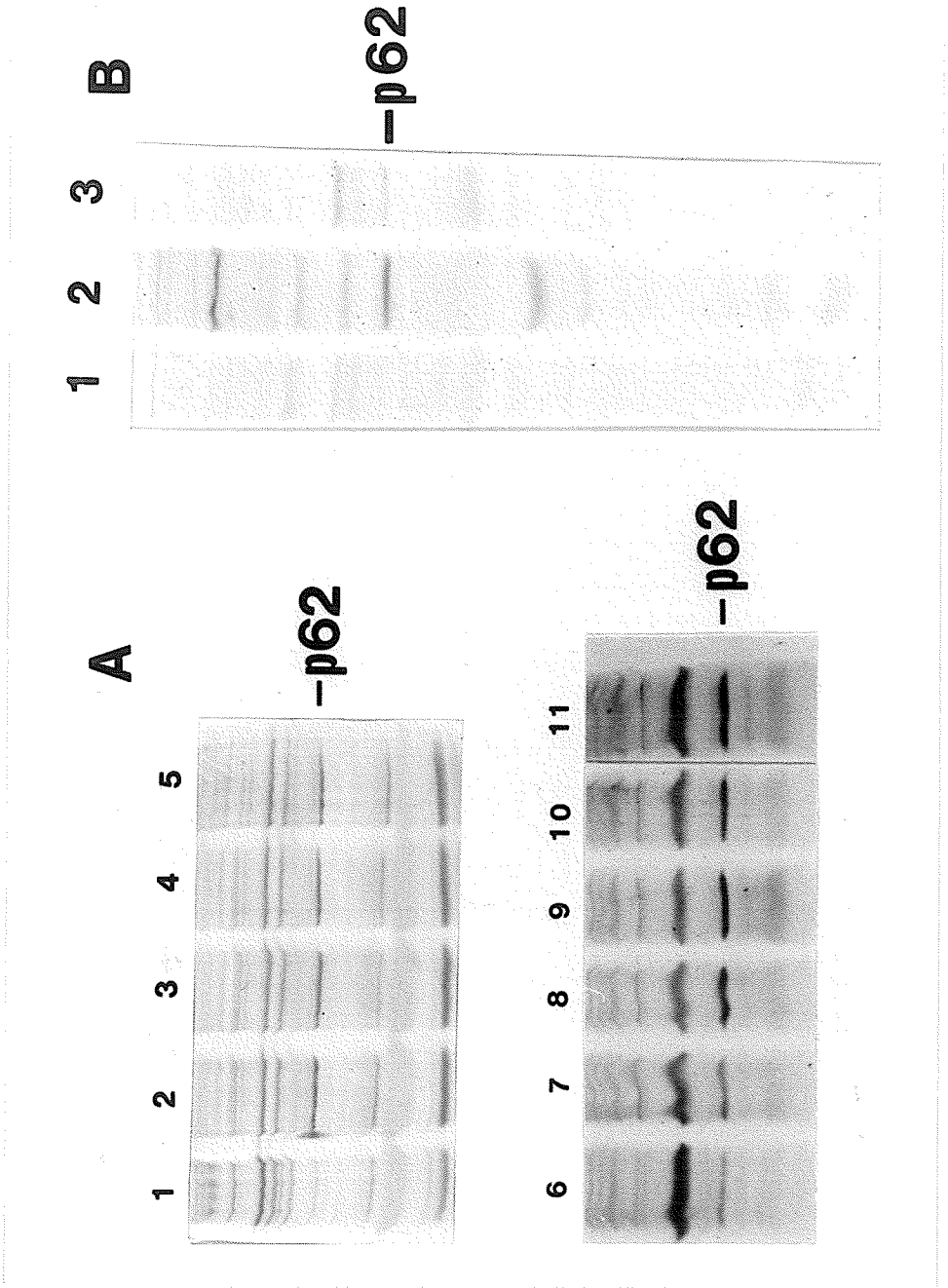

1
2
3
4
A B C A B C A B C A B C
–p53
5
6
7
A B C A B C A B C
–p53
8
9
10
11
A B C A B C A B C A B C
–p53

FIGURE 21.

(A) Immunoblot analysis of p62-c-myc in focus derived cell lines. The myc transfected clones were analyzed for myc protein expression. Amount of protein is shown for each lane. (1) 10T½, 63μg; (2) RM-4, 58μg; 3) RM-6, 63μg; (4) RMP-4, 70μg; (5) RMP-6, 58μg; (6) 10T½, 114μg; (7) RM-3, 136μg; (8) RM-5, 145μg; (9) RMP-1, 119μg; (10) RMP-2, 105μg; (11) RMP-5 136μg. Two gels are shown. Monoclonal antibody 9E10 was used in these studies. (B) Comigration of the transfected myc protein with p62-c-myc in Colo320 cells. Lysates were analyzed by immunoblotting. The amounts of protein per lane is given in brackets below. Lane 1 is the WI-38 cell strain (36 μg), lane (2) is RMP-5 (107 μg) and lane (3) is Colo320 (38 μg). Densitometric measurements showed approximately 2.7- and 1.7-fold higher levels of p62-c-myc in Colo320 and RMP-5 cells respectively, when compared to WI38 cells. The p62 protein band is clearly evident in the Colo320 HSR-positive control and in the transfectant cell line. Monoclonal antibody 9E10 was used in these studies.


B
−p62
1
2
3
A
−p62
1
2
3
4
5
−p62
6
7
8
9
10
11

2.3 Growth and malignant potential of ras/myc/p53 transfected cells.

Cell lines were evaluated for tumor forming ability by injection into syngeneic C3H/HeN mice (Egan et al, 1987a; Damen et al, 1989). The parental 10T½ cell line is nontumorigenic (Egan et al., 1987a), however, as shown in table 4 each transfected cell line tested was found to be capable of forming tumors with a high frequency. Interestingly, tumor latency was significantly shorter (p values ranging between 0.039 and 0.050) with cell lines transfected with ras/myc/p53 (about 8 days) when compared to cell lines transfected with only a single gene or two gene combinations (11 to 12 days). After 28 days all tumors had reached an average size of 2.6 to 4.6 cm^2 (table 4), and no evidence for a statistically significant difference at the 95% confidence level was found for the average size of tumors among the four groups (p values ranging between 0.281 and 0.664).

Intravenous injections of cells from the various gene combination groups demonstrated interesting differences in the ability of oncogene transfected cells to colonize the lungs of syngeneic mice (figure 22). Cells transfected with ras alone, or ras in combination with myc or p53 were poorly metastatic. The four ras clones exhibited an average of 1.6 lung tumors/mouse, and the ras/myc or ras/p53 transfected clones gave an average of 0.7 and 3.1 lung tumors, respectively. However, the ras/myc/p53 gene combination generally produced a large number of tumors, with an average of 36.6 lung tumors/mouse. Statistical analysis provided good evidence for differences in metastatic potential between ras/myc/p53 transfected cell lines and each of the other groups of cell lines transfected with ras, ras/myc/ or ras/p53 (p values of 0.024, 0.014 and 0.035).

TABLE 4.

Effect of Oncogene Transfection on Tumorigenicity

[a] Tumor latency (days) and tumor size (cm^2) are shown as the average $\pm$ S.E. for each group of transfected lines (transfected with ras, or ras/myc, or ras/p53 or ras/myc/p53).

[b] Size was determined 28 days post-injection.

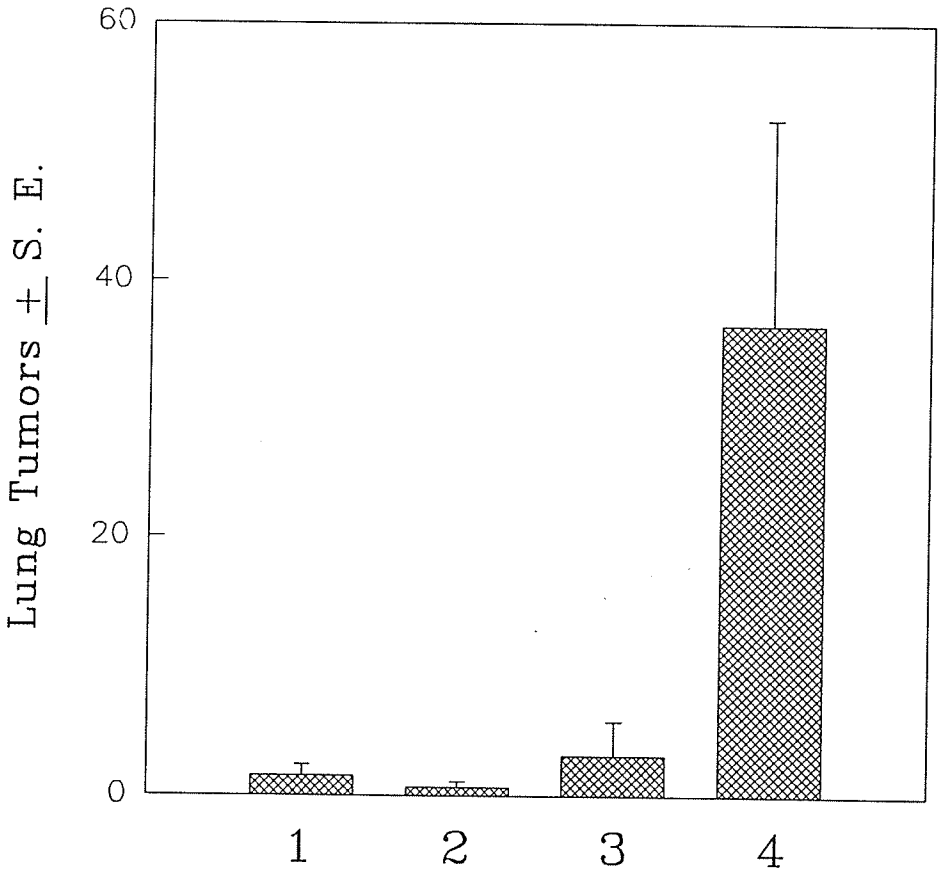
Statistical analysis of tumor latency data indicated differences between the ras/myc/p53 gene combination and the ras, ras/myc and ras/p53 gene combinations with p values of 0.039, 0.041 and 0.050. Statistical analysis of tumor size data did not show significance at the 95% confidence level in tumor sizes obtained with the ras/myc/p53 combination compared with the ras, ras/myc or ras/p53 combinations (p values of 0.281, 0.469, and 0.664, respectively). In addition, when the ras, ras/myc, and ras/p53 clones were treated as a single group and compared with the ras/myc/p53 clones, tumor latency was found to be significantly different (p value of 0.0075), and no significant difference in tumor size was found (p value of 0.608).

NT, not tested.

Gene	Line	Tumor Frequency	Tumor Latency	Average[a] Latency/Group	Tumor Size[b]	Average[a] Size/Group
ras	R-2	5/5	15.0	12.0 ± 1.33	2.66	2.63 ± 0.04
	R-3	5/5	9.0		2.59	
ras / myc	RM-3	3/5	12.7	11.2 ± 0.62	2.26	3.25 ± 0.62
	RM-4	5/5	13.2		2.94	
	RM-5	5/5	11.0		5.06	
	RM-6	5/5	8.4		2.73	
ras / p53	RP-3	5/5	10.8	11.3 ± 0.77	2.76	4.61 ± 0.95
	RP-4	5/5	12.8		5.17	
	RP-6	5/5	10.2		5.90	
ras / myc / p53	RMP-1	5/5	7.8	7.9 ± 0.42	2.30	4.09 ± 0.95
	RMP-4	5/5	8.2		4.43	
	RMP-5	5/5	8.0		5.54	
	RMP-6	5/5	7.4		NT	

FIGURE 22.

Comparison of the average experimental metastatic potential in transfected lines. Five mice were injected per clone and all the transfected lines isolated were analyzed. The four conditions shown are transfection with ras (1), ras/myc (2), ras/p53 (3), or ras/myc/p53 (4). Statistical analyses indicated that the metastatic potential of the ras/myc/p53 transfected cell lines was significantly higher than the potential of the ras, ras/myc and ras/p53 combinations, with p values of 0.024, 0.014 and 0.035, respectively. Furthermore, when ras, ras/myc and ras/p53 clones were treated as a single group and compared with the ras/myc/p53 clones, the differences in metastatic potential between these two groups were found to be statistically significant with a p value of 0.0027.


Lung Tumors ± S. E.
60
40
20
0
1
2
3
4

2.4 Collagen invasion by cells transfected with T24 H-ras, c-myc, and mutant p53

To test if the cooperative induction of metastasis by ras, myc and p53 correlated with increased invasion, we analyzed the ability of cells transfected with these genes to invade into gels of collagen I. Figure 23a shows that there are significant alterations in the motile behaviour of the oncogene transformed cell lines. Cell lines transfected with ras alone or with ras plus mutant p53 showed significantly higher levels of collagen invasion than 10T½ control cells. However, cell lines expressing myc including those transfected with ras+myc or with ras+myc+p53 showed levels of invasion slightly higher than 10T½ cells but significantly lower than non-myc transfected cell lines (figure 23b). Furthermore, collagen invasion did not correlate with metastatic potential as shown by the low invasiveness of highly metastatic ras+myc+p53 transfected cell lines.

2.5 Secretion of gelatinases from ras, myc, p53 transfected cells

Many metastatic cell lines secrete high levels of proteolytic enzymes which aid in their invasion (Denhardt et al, 1987). Several of these enzymes can be assayed by zymography (Samuel et al, 1992). Zymograms were prepared using gelatin as an in situ substrate. We analyzed the gelatinases secreted from the 10T½ cell line and the oncogene transfected derivatives. Figure 24 shows that there are two easily detectable gelatinases secreted from these cells. The major protein comigrates with the 69kD marker and the minor enzyme slightly slower. We found no evidence for an increased level of secretion in the transformed and metastatic cell lines, which is consistent with the lack of

FIGURE 23.

Average percent invasion of oncogene transfected cell lines into gels of collagen I. (A) The average percent invasion 24 h after plating is shown for 1) 10T½ cells, 2) ras transfected, 3) ras + myc transfected, 4) ras + p53 transfected and 5) ras + myc + p53 transfected cell lines. (B) The average percent invasion of non-myc-expressing clones (1) is compared to myc-expressing clones (2). Standard errors are shown by the bars.

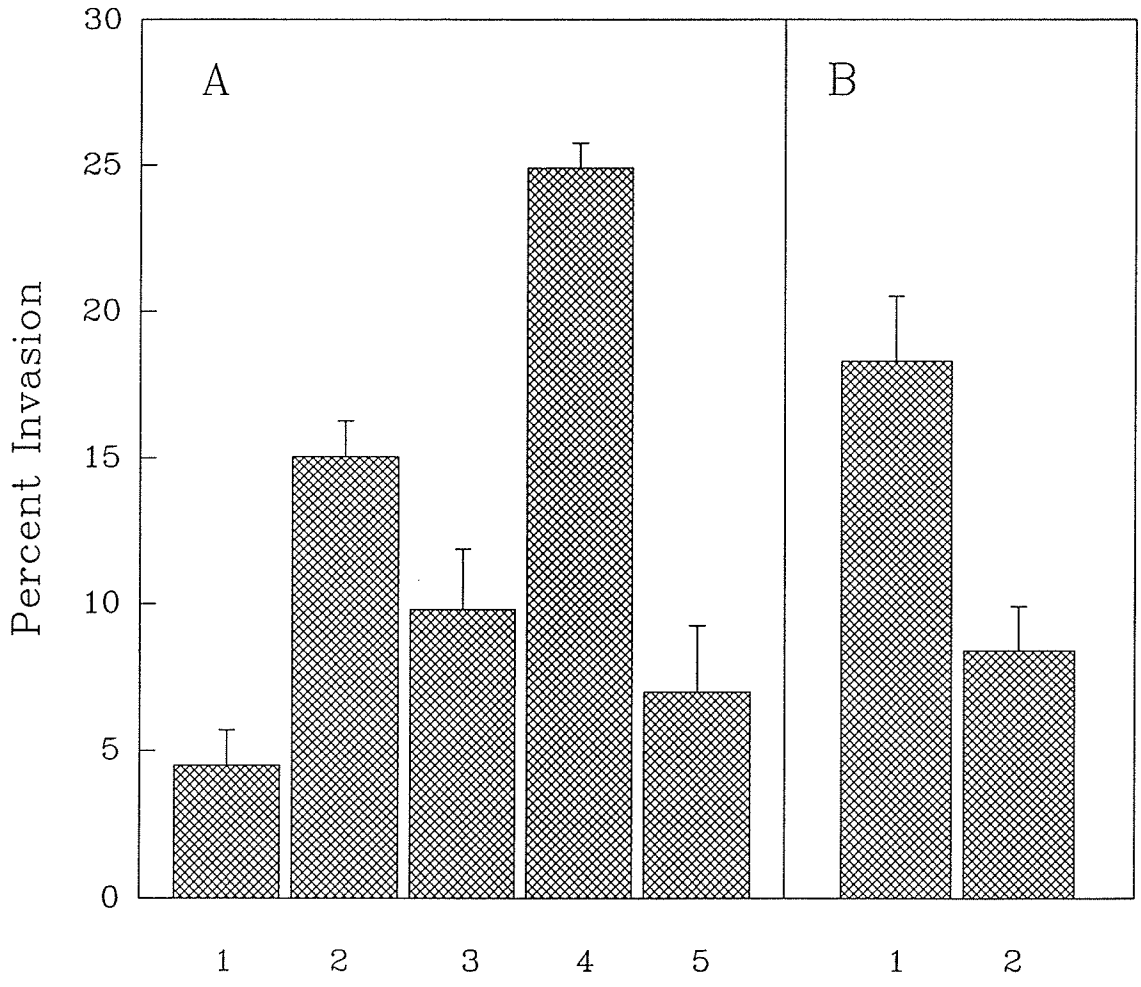
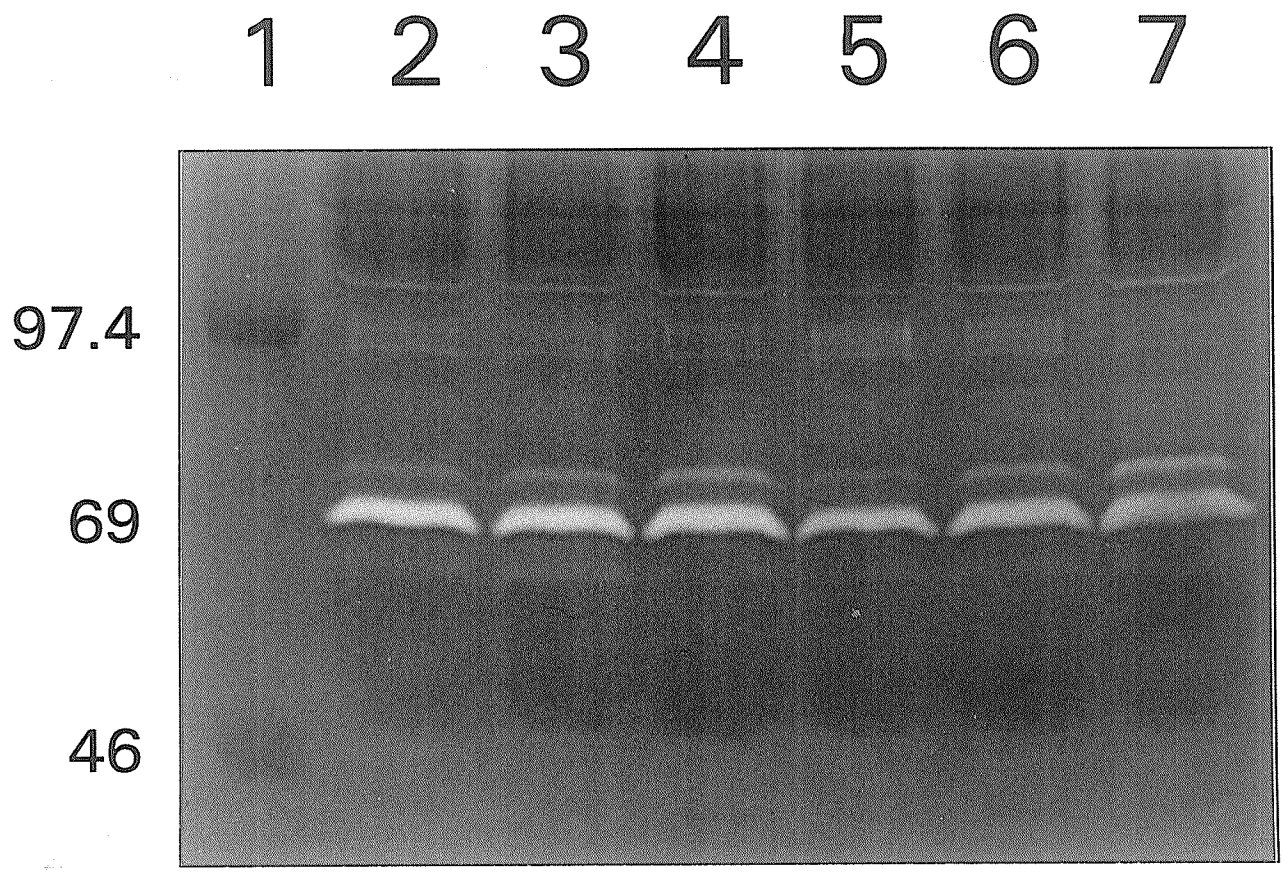

30
25
20
15
10
5
0
Percent Invasion
A
B
1
2
3
4
5
1
2

FIGURE 24.

Zymogram analysis of cells lines transfected with ras, myc and mutant p53. Gelatinases found in the medium conditioned by the following cell lines is shown. Lane 1) Molecular weight markers, 2) 10T½, 3) RM-5, 4) RMP-1, 5) RMP-2, 6) RMP-3, 7)RMP-5.


1 2 3 4 5 6 7
97.4
69
46

correlation observed with the invasion of these cell lines *in vitro* and their metastatic potentials (figure 23).

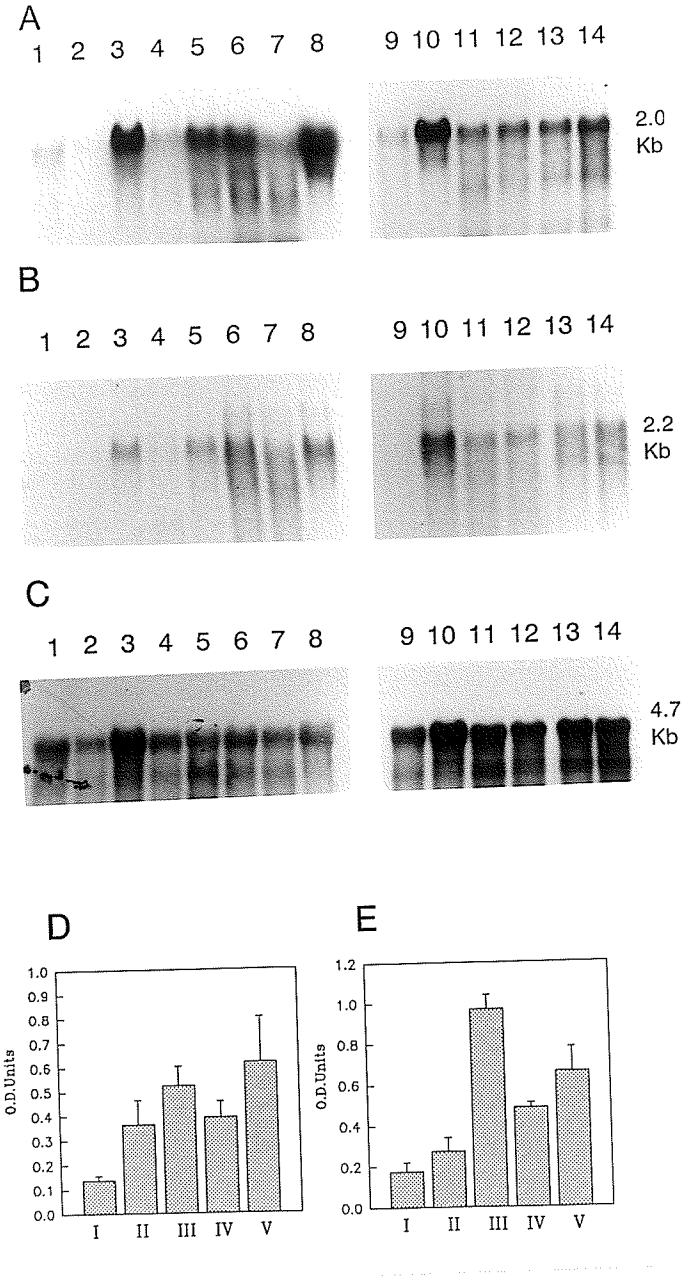
3. Mechanism of Oncogene Cooperation

3.1 Expression of myn in cells transformed by T24 H-ras, c-myc, and mutant p53

In an attempt to gain some insight into a possible mechanism of cooperative induction of the metastatic phenotype by ras, myc and mutant p53 we studied the expression of the myn gene. Northern blots of RNA were prepared from 10T½ cells transformed by ras, ras+myc, ras+p53 and ras+myc+p53. When probed for myn expression we detected a major 2.0 kb RNA species which corresponds to the major product of the myn gene in murine cells (Prendergast et al, 1991). We found low levels of myn expression in control untransfected 10T½ cells, however most of the transformed cells examined showed higher levels of expression than control cells (figure 25a). None of the transformed cell lines showed substantially lower levels of myn than 10T½ control cells, whereas one showed no increase in myn expression, one showed a small elevation of 1.5-fold and 9/11 showed higher levels of myn expression ranging from 2.8 to 6.9 fold. To determine if the level of myn correlated with the presence of either ras, myc, or p53, we plotted the level of myn expression, as determined by densitometry with respect to oncogene combination. We found that ras transfected clones showed an average 2.7 fold higher level of myn expression compared to 10T½ cells (figure 25d). Also, ras+myc transfected clones showed an average 3.8 fold increase, ras+p53

FIGURE 25.

Expression of myn and myc mRNA in cells transformed by ras, myc, and p53. Total cellular RNA was probed for the expression of (A) myn, (B) myc, and (C) 28S rRNA (used as an internal loading control). The following samples were analyzed: (1) 10T½, (2) R-1, (3) R-2, (4) R-3, (5) R-4, (6) RM-4, (7) RM-5, (8) RM-6, (9) 10T½, (10) RM-6, (11) RP-1, (12) RP-3, (13) RMP-5, and (14) RMP-6. The average level of (D) myn, and (E) myc expression corrected for loading (28S rRNA expression) is shown for the four groups of oncogene transformed cells. The following groups of cells are shown: (I)10T½ control cells, (II)ras transformed clones, (III)ras + myc clones, (IV)ras + p53 clones, and (V)ras + myc + p53 clones. Standard errors are shown by the bars. As a group, myn expression is significantly higher in these oncogene transformed cell lines compared to the parental 10T½ cell line (p value of 0.04).


A
1 2 3 4 5 6 7 8
9 10 11 12 13 14
2.0
Kb
B
1 2 3 4 5 6 7 8
9 10 11 12 13 14
2.2
Kb
C
1 2 3 4 5 6 7 8
9 10 11 12 13 14
4.7
Kb
D
O.D.Units
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
I II III IV V
E
O.D.Units
1.2
1.0
0.8
0.6
0.4
0.2
0.0
I II III IV V

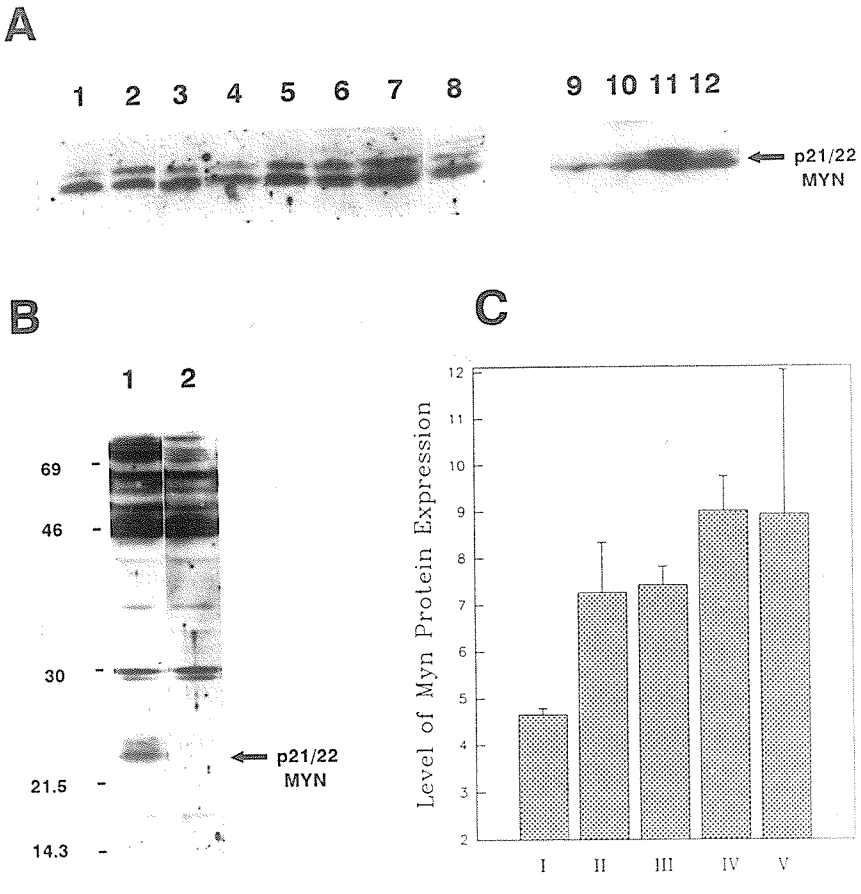
transfected clones, an average 2.9 fold increase, and ras+myc+p53 transfected clones, an average 4.5 fold increase in myn expression. Thus, all four types of transformed cell lines clearly showed increases in myn expression (p value < 0.05). Next, we determined the level of c-myc mRNA expression in these cell lines. The expression level of the 2.2 kb myc mRNA was increased in the transformed cells, and as expected, showed highest levels in myc transfected cells (figures 25b & 25e).

3.2 Synthesis of myn protein in transformed fibroblasts

The increase in myn mRNA expression in ras transformed cells suggested that these cells exhibit higher levels of expression of the myn protein product. Therefore, we analyzed myn expression in ras transformed cells by immunoblotting with a rabbit antiserum generated against a full length murine myn protein. We detected two bands with molecular weights of 21 and 22 kD (figure 26a). This corresponds to the size of the myn product in mouse cells (Blackwood et al, 1992). Preincubation of the antiserum with an excess of human GST-MAX protein, which is specifically recognized by this antiserum led to the disappearance of the 21 and 22 kD bands in immunoblots indicating that these are myn polypeptides (figure 26b). Figure 26c shows that cell lines expressing activated ras, alone, or in combination with myc, mutant p53, or both myc and p53 exhibit higher levels of myn protein compared to 10T½ cells. Although two cell lines showed undetectable levels of myn protein (data not shown), the cell lines in which myn was detected showed significantly higher levels of myn expression compared to 10T½ cells ($p < 0.05$).

FIGURE 26.

Immunoblot analysis of myn in oncogene transformed cell lines. (A) Total cell lysates were analyzed for the level of myn expression by immunoblotting with an anti-myn rabbit antiserum. The cell lines shown are (1)10T½, (2)R-1, (3)RM-4, (4)RM-5, (5)RP-1, (6)RP-5, (7)RMP-1, (8)RMP-4, (9)10T½, (10)10T½ [grown to confluence], (11)R-1, (12)RP-1. Lanes 1 to 8 are from a different experiment than lanes 9 to 12. (B) Parallel blots of identical cell lysates (prepared from the R-4 cell line) were probed with anti-myn serum in the absence (1) or presence (2) of excess competing antigen as described in Materials and Methods. (C) The average level of myn protein expression is shown for the four groups of oncogene transformed cells. The following groups of cells are shown: (I)10T½ control cells, (II)ras transformed clones, (III) ras + myc clones, (IV) ras + p53 clones, and (V) ras + myc + p53 clones. myn expression is significantly higher in these oncogene transformed cell lines compared to the parental 10T½ cell line (p value of 0.03).


A
1 2 3 4 5 6 7 8
9 10 11 12
p21/22
MYN
B
1 2
69
46
30
21.5
14.3
p21/22
MYN
C
Level of Myn Protein Expression
12
11
10
9
8
7
6
5
4
3
2
I
II
III
IV
V

3.3 Induction of myn expression by increased ras levels

The observed increase in myn expression in all groups of transfectants suggested that myc, p53 or both of these genes in combination do not directly alter myn expression (figure 25d). In contrast, since all transformed cell lines examined contained an activated ras, this suggested that myn expression may be responsive to the presence of an activated ras gene. To test this hypothesis, we employed two cell lines, both of which harbor an inducible ras gene. The first cell line was derived from 10T½ cells by transfection with the T24 H-ras mutant linked to the zinc inducible metallothionein promoter (Haliotis et al, 1990; Trimble, 1987). Figure 27b shows that treatment of these cells with zinc sulphate for 48 h led to a 5.6 fold induction of ras expression. In addition, we detected a 2.6 fold increase in myn mRNA expression 48 h after addition of zinc sulphate to the medium (figure 27a). Zinc sulphate treatment of the nontransformed control 10T½ cell line or the transformed 10T½ Ciras-3 cell line, which constitutively expresses an activated ras gene (Egan et al, 1987a; Craig et al, 1990), did not lead to an increase in either ras or myn (figures 27a & 27b). However, in support of a relationship between ras and myn expression, the 10T½ Ciras-3 cell line exhibited an elevation in myn mRNA of approximately 3.4 fold when compared to the parental 10T½ line. Interesting results were also obtained with a second ras inducible cell line, NIH3T3-433, which was derived from NIH3T3 cells, and contains the T24-H-ras gene linked to the dexamethasone inducible mouse mammary tumor virus promoter (Huang et al, 1981). Treatment of this cell line for 7 days with dexamethasone led to a 2.0 fold increase in the level of myn expression, as determined by northern blot analysis (figure 28). This cell line

FIGURE 27.

Induction of myn expression by activated ras. The expression of ras and myn in cell lines grown in the presence (lanes 2, 4 and 6) or absence (lanes 1, 3, and 5) of 50μM zinc sulphate: (A) myn mRNA, (B) ras mRNA and (C) 28S rRNA (used as an internal loading control). Cell lines analyzed were 10T½ control cells (lanes 1 and 2), 2H1 cells containing a zinc inducible ras gene (lanes 3 and 4), and 10T½ Ciras-3 cells containing a constitutively overexpressed activated ras gene (lanes 5 and 6). (D) The average level of myn expression was determined after induction of ras in both dexamethasone (NIH3T3-433) and zinc inducible (10T½-2H1) cell lines. This has been corrected for the expression of myn in control cell lines (pEJ1 and 10T½) treated in the identical manner. myn expression in the absence (I) and presence (II) of inducing agent is shown. Standard errors are shown by the bars. myn expression was significantly higher after induction of ras expression compared to control cells (p value of 0.04). There were no significant differences in either myn or ras mRNA levels when 10T½ Ciras-3 cells grown in the presence or absence of zinc sulphate were compared. However, the ras transfected 10T½ Ciras-3 cells exhibited an elevation in myn message of 3.4 fold, when compared to parental 10T½ cells.

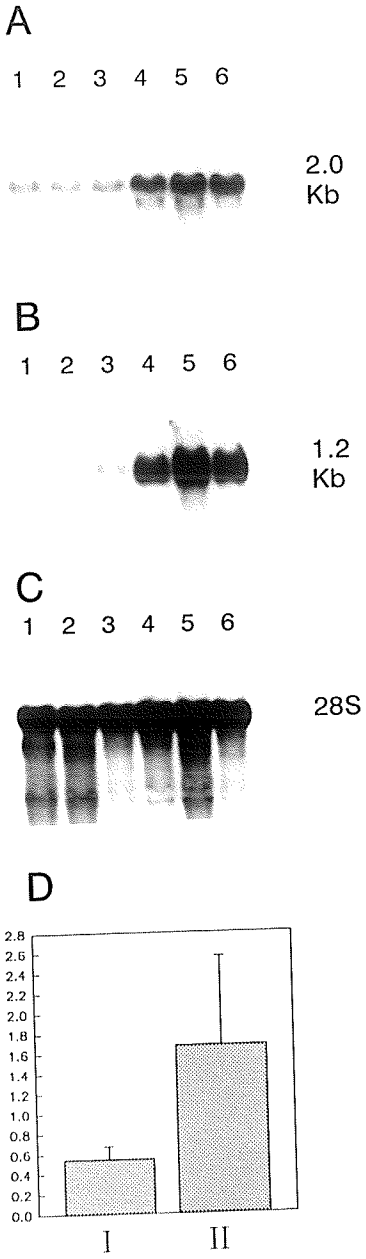
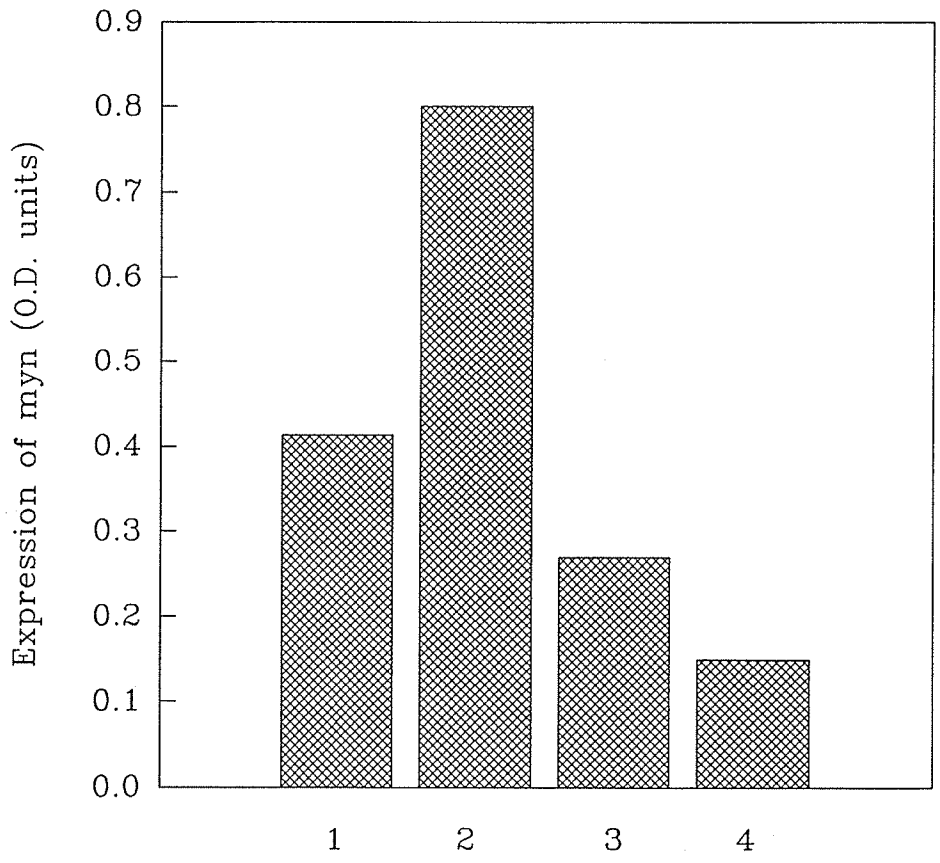

A
1 2 3 4 5 6
2.0
Kb
B
1 2 3 4 5 6
1.2
Kb
C
1 2 3 4 5 6
28S
D
O.D. UNITS
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
I
II

FIGURE 28.

Effect of induction of ras expression on myn in NIH3T3-433 cells. Desitometric scan of a northern blot for myn mRNA in the presence (2 and 4) and absence (1 and 3) of dexamethasone for the cell lines NIH3T3-433 (1 and 2) and the pEJ cell line (3 and 4) is shown. The NIH3T3-433 contains a T24-Ha-ras gene driven by the dexamethasone-inducible MMTV promoter, and the pEJ cell line is a NIH3T3 derived cell line which contains a constitutively expressed T24-Ha-ras gene.


Expression of myn (O.D. units)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
1
2
3
4

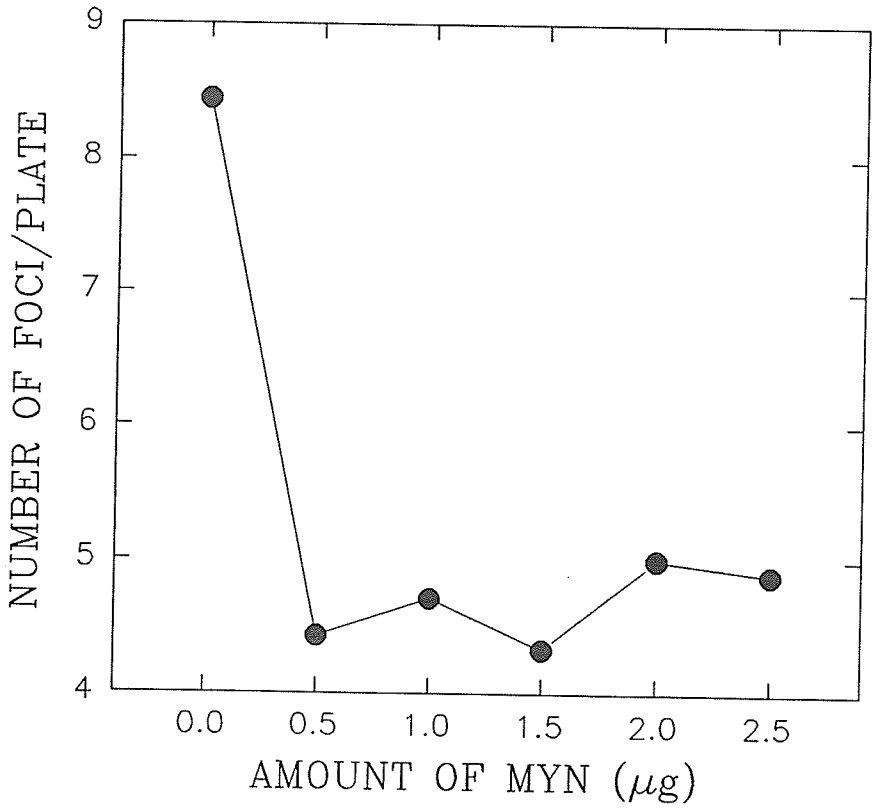
significantly increases its p21 ras expression under these conditions (Huang et al, 1981). No change in myn was observed in a control NIH3T3 cell line, pEJ1, transformed by a constitutive T24-H-ras gene, when grown in the presence of dexamethasone (figure 28). When we combined the results using both zinc and dexamethasone inducible cell lines, we found, after ras induction, an average 2.8 fold increase in myn expression, which was significantly different than what was observed with control cells (figure 27c, p value $<$ 0.05).

3.4 Suppression of ras transformation by myn overexpression

The overexpression of myn in transformed 10T½ cells, particularly those transformed by ras, in addition to the induction of myn expression by ras suggested that myn may be involved in transformation by ras. One way to test this is to determine the effect of high levels of myn on ras transformation. Therefore, we tested the ability of myn to cooperate with ras in the transformation of 10T½ cells. We found that cotransfection with ras and increasing amounts of myn led to a suppression of focus formation. Figure 29 shows that the addition of 0.5 μg of a plasmid containing myn driven by a cytomegalovirus (CMV) promoter led to a 45% decrease in focus formation compared to transfection with ras alone. A similar level of suppression was observed with higher doses of myn, suggesting that this effect is saturated at low concentrations of myn. The reduction in focus formation was found to be statistically significant (p value $<$ 0.05).

FIGURE 29.

Effect of myn on ras induced focus formation. 10T½ cells were transfected with ras, alone, or in the presence of increasing amounts of myn expression plasmid. pHOMER6 was added to control for nonspecific effects of transfected DNA. The average number of transformed foci determined by methylene blue staining are shown. Cotransfection of 10T½ cells with 2.5 μg of myn expression plasmid led to a significant decrease in ras induced focus formation (p value of 0.03).


NUMBER OF FOCI/PLATE
9
8
7
6
5
4
0.0
0.5
1.0
1.5
2.0
2.5
AMOUNT OF MYN (μg)

3.5 Increased expression of the glyceraldehyde-3-phosphate dehydrogenase gene in cells transfected with ras, myc, and p53.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a glycolytic enzyme found to exhibit altered expression in other transformed cell lines (Schek et al, 1988). We analyzed the expression of this gene to determine if genes other than myn may be altered in their expression during cooperative transformation by ras, myc, and p53. Northern blots were prepared from cells transfected with combinations of ras, myc, and p53 and were probed for expression of the GAPDH gene. We observed a RNA species of 1.4 kB in all cell lines (figure 30a). In addition, this mRNA was substantially overexpressed in the various transformed cell lines compared to the parental control 10T½ cell line (figure 31) when 28S rRNA was used as a loading control. To test the accuracy of both the loading control and the densitometric analysis used, a northern blot of serial dilutions of RNA was probed for 28S rRNA. Densitometric scanning of this blot was linear up 40 μg of RNA (figure 32).

FIGURE 30.

Expression of GAPDH mRNA and 28S rRNA in oncogene transformed murine fibrosarcoma cell lines. (A) Northern analysis of GAPDH expression is shown. Lanes: 1) 10T½, 2) R-1, 3) R-2, 4) R-3, 5) R-4, 6) RM-4, 7) RM-5, 8) RM-6, 9) 10T½, 10) RM-6, 11) RP-1, 12) RP-3, 13) RMP-5, 14) RMP-6. Cell lines are named for the expressed oncoprotein, where R denotes ras, M denotes myc and P denotes p53. (B) Northern analysis of 28S rRNA for the same cell lines.

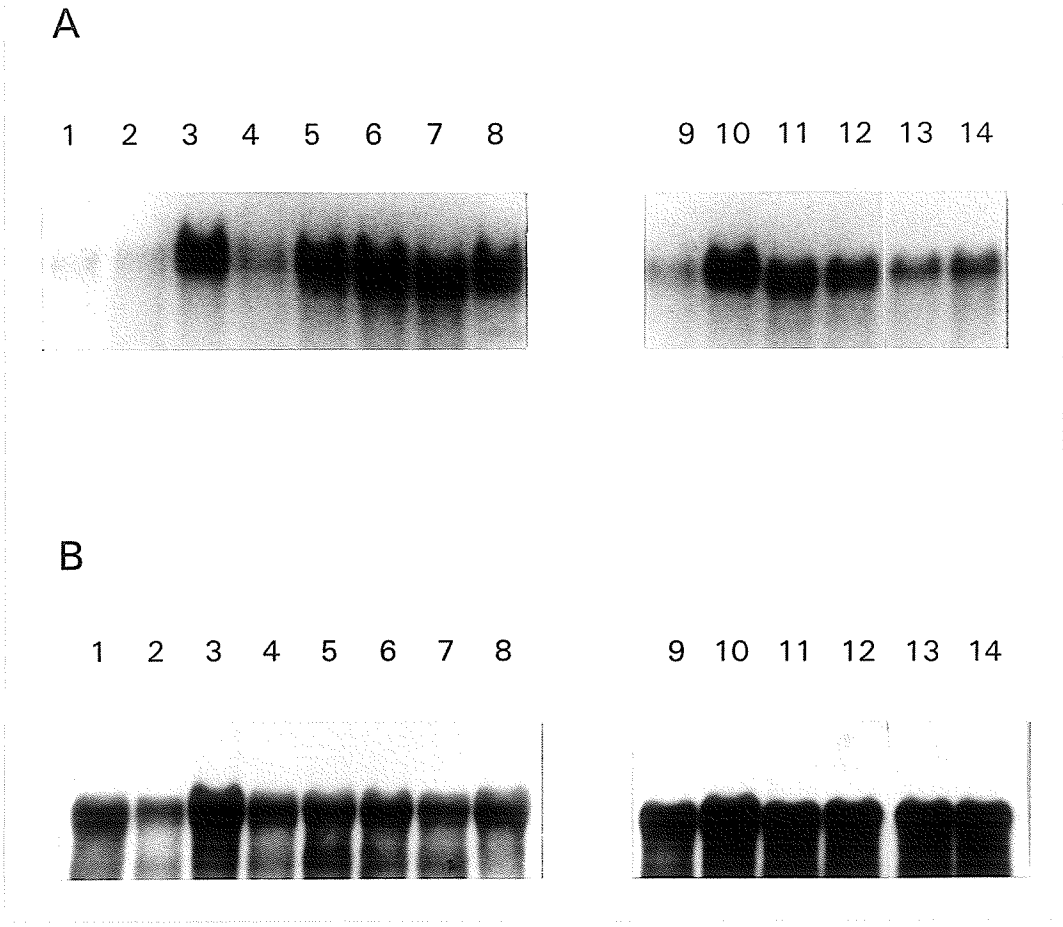

A
1 2 3 4 5 6 7 8
9 10 11 12 13 14
B
1 2 3 4 5 6 7 8
9 10 11 12 13 14

FIGURE 31.

Summary of the relative levels of GAPDH and 28S expression in the following cell lines is shown: 1) 10T½, 2) R-1, 3) R-2, 4) R-3, 5) R-4, 6) RM-4, 7) RM-5, 8) RM-6, 9) RP-1, 10) RP-3, 11) RMP-5, 12) RMP-6. The level of expression of GAPDH is shown by the black bar, whereas that of 28S is shown by the white bar.

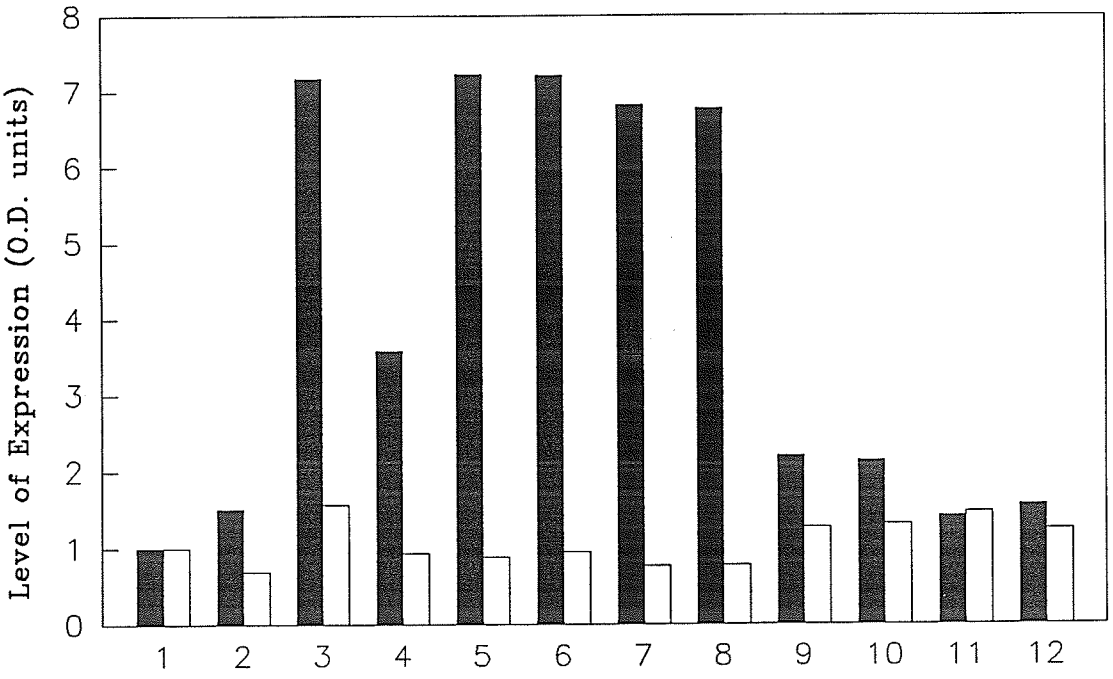
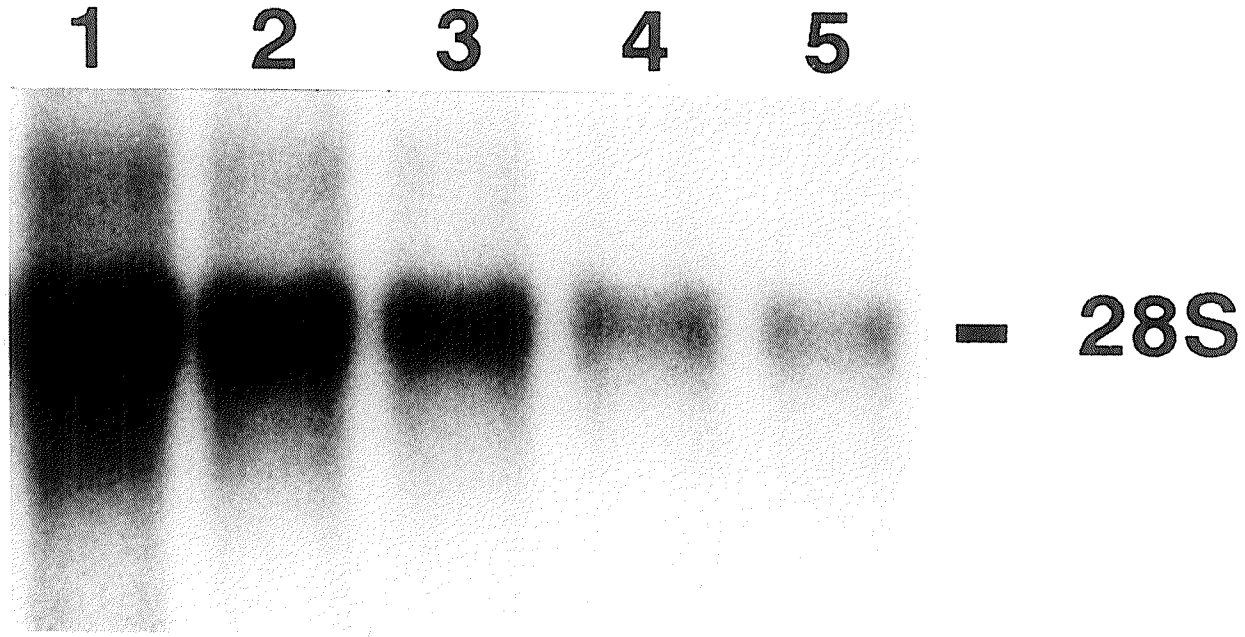

Level of Expression (O.D. units)
0
1
2
3
4
5
6
7
8
1
2
3
4
5
6
7
8
9
10
11
12

FIGURE 32.

Expression of 28S rRNA is proportional to the amount of RNA loaded. Northern analysis was carried out with 1:2 dilutions of total RNA from the 1AI cell line, starting with 40μg (lane 1). When the intensities were analyzed by densitometry, an average ratio of 0.51 was found for lane 2/lane 1, lane 3/lane 2, lane 4/lane 3, and lane 5/lane 4, with a standard error of 0.03.


1
2
3
4
5
28S

4. The Role of Chromatin Structure in Gene Expression During Malignant Progression

4.1 Phosphorylation of H1 histones in normal and ras-transformed fibroblasts

The analysis of both the myn and the gapdh genes showed that alterations in gene expression occur during malignant transformation by ras, myc, and mutant p53. One mechanism to explain these alterations is that the chromatin in these cells is altered. We investigated the phosphorylation of H1 histones, a modification important in the regulation of chromatin structure (Green et al, 1993), to determine its involvement in the regulation of gene transcription during malignant transformation. We determined if ^{32}P could be incorporated into H1 histones from the immortalized murine fibroblast 10T½. We also analyzed two transformed derivatives of these cells which have been transfected with an activated Ha-ras allele. H1 histones were isolated from cells metabolically labelled with ^{32}P orthophosphate and separated by SDS-PAGE. As shown in figure 33, we found that 10T½ H1 histone does incorporate ^{32}P consistent with it containing phosphorylated amino acids. However, we found that the nonmetastatic transformed cell line NR3 (Egan et al, 1987a) contained 3.3 fold higher levels of radioactive H1 histones, and the metastatic Ciras-2 (Egan et al, 1987a) cell line contained 5.1 fold higher levels of radioactivity suggesting that the H1 histones in these cell lines are phosphorylated to a much higher extent than the normal parental 10T½ cell line (figure 34). These differences were not due to loading of the different samples as determined by Coomassie blue staining of the gel (data not shown). A smaller ^{32}P labelled band was also observed. The incorporation of ^{32}P into this band was also higher in ras transfected cells compared to control cells.

FIGURE 33.

Incorporation of ^{32}P into H1 histones in ras-transformed and normal fibroblasts. H1 histones were extracted from cells metabolically labelled with ^{32}P orthophosphate, separated by SDS-PAGE, and dried gels were exposed to film. Two μg of histone were loaded per lane. Lane 1: 10T½, lane 2: NR4, lane 3: Ciras-3. Both NR4 and Ciras-3 were derived from the 10T½ cell line by transfection with T24 Ha-ras, and while the NR4 line is not metastatic, the Ciras-3 line is highly metastatic as determined by intravenous injection of tumor cells.

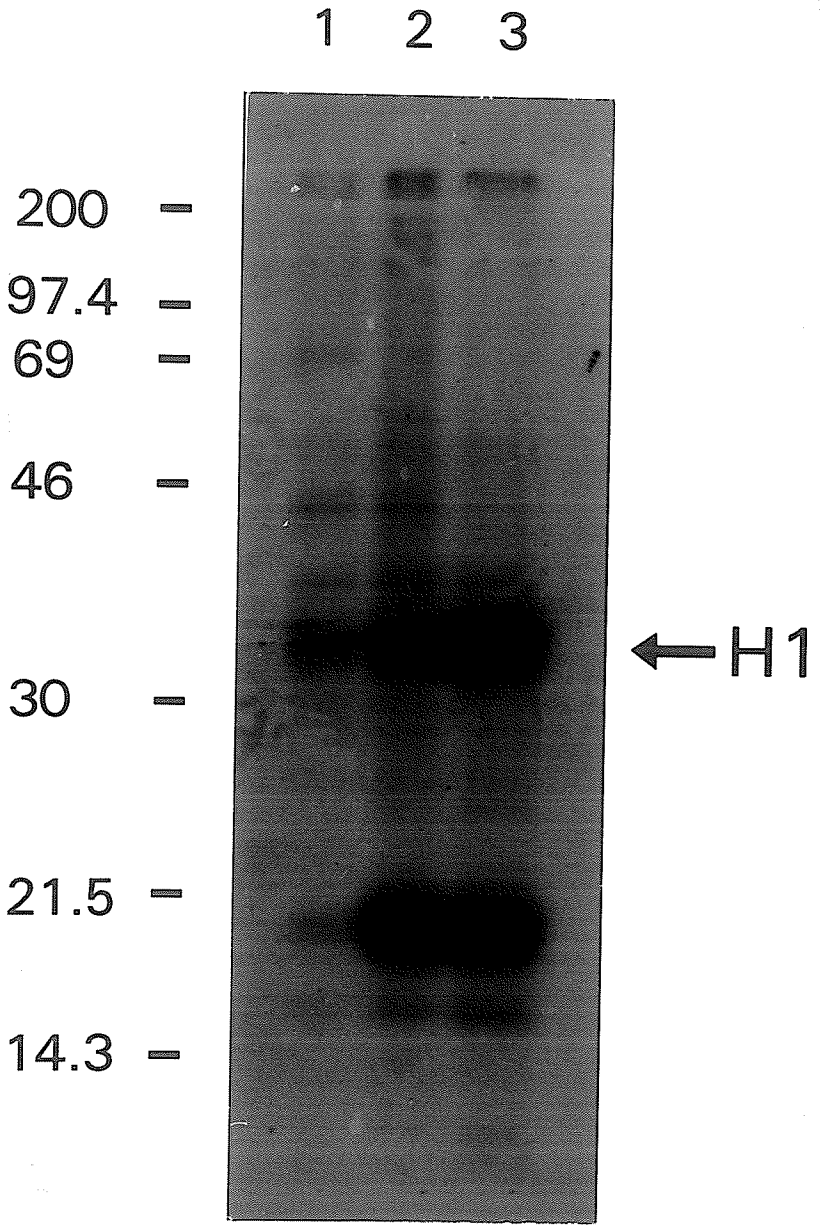
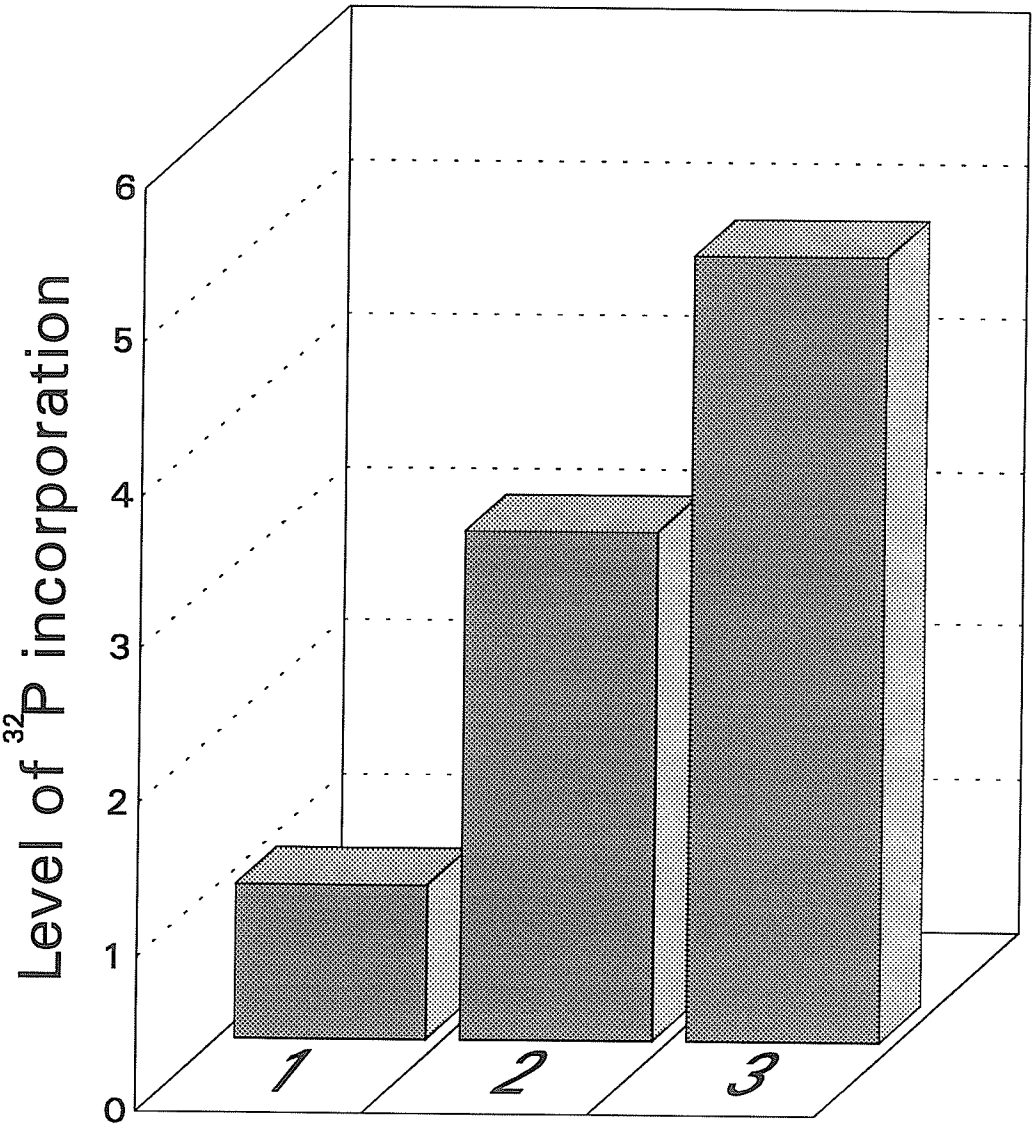

1
2
3
200
97.4
69
46
30
21.5
14.3
H1

FIGURE 34.

Level of ^{32}P incorporation into H1 histones of normal and ras transformed fibroblasts. Autoradiograms of H1 histone metabolically labelled with ^{32}P were scanned with a video densitometer. The amount of labelling is expressed as optical density (O. D.) units and is shown for 1) 10T½ cells, 2) NR4 cells, and 3) Ciras-3 cells. The results from one experiment are shown. These results are typical of the incorporation of ^{32}P into H1 histone observed with other ras transformed cell lines.


Level of ^{32}P incorporation
0
1
2
3
4
5
6
1
2
3

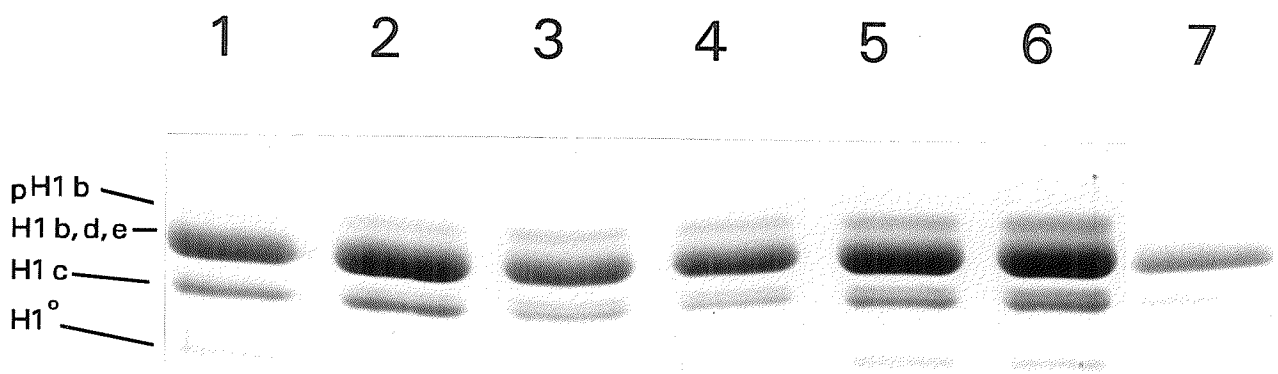
4.2 Phosphorylation of H1 histones in cell lines transformed with combinations of ras, myc and mutant p53

Metabolic labelling experiments described above suggest that H1 histone is more highly phosphorylated in transformed cells compared to normal cells. However, equivocal conclusions using labelling experiments are hindered by possible changes in phosphate metabolism in transformed cells. Thus, we decided to assay H1 histone phosphorylation by staining the H1 protein with Coomassie blue and also by using an antibody which recognizes hyperphosphorylated histone H1. Perchloric acid-extracted H1 histones were separated by SDS-PAGE and stained with Coomassie blue. As shown in figure 35, four bands were visible. The largest protein migrated with an apparent molecular weight of 30.5 kD while smaller bands had apparent molecular weights of 29.5, and 28.5 kD. Comparison of these bands with those previously published suggested that the 30.5 kD band corresponds to a mixture of H1 subtype b, d, and e, and possibly some phosphorylated forms of subtype d and e. The 29.5 kD band corresponds to subtype H1c and the 28.5 kD band corresponds to subtype H1° (Lennox & Cohen, 1983).

We also detected a band which migrates slightly slower than the 30.5 kD band. This 31 kD band most likely contains phosphorylated forms of H1b (Lennox & Cohen, 1983). To confirm that this band contains phosphorylated protein we treated the H1 histones from the RP-4 cell line with alkaline phosphatase. As shown in figure 35, this band disappeared after phosphatase treatment suggesting that it is a phosphorylated H1 species (compare lanes 4 and 7). Inspection of the Coomassie stained gel in figure 35

FIGURE 35.

SDS-PAGE analysis of H1 histones from fibroblasts transfected with ras, myc, and mutant p53. H1 histones were extracted and separated by SDS-PAGE. Gels were stained with Coomassie blue. Two μg of histone were loaded per lane. Lane 1) 10T½, 2) R-2, 3) RM-5, 4) RP-4, 5) RP-6, 6) RMP-6, 7) RP-4 treated with alkaline phosphatase.


1
2
3
4
5
6
7
pH1 b
H1 b, d, e
H1 c
H1°

shows that this band is clearly visible in most of the transformed cell lines however it cannot be seen in the 10T½ cell line consistent with 10T½ showing much lower levels of ^{32}P incorporation into H1 histones. Coomassie stained SDS-PAGE gels of core histones isolated from these cell lines showed no obvious difference in the relative levels of the four core histones (figure 36).

4.3 Detection of phosphorylated H1 histones with a specific antibody

We used an antiserum that can recognize phosphorylated H1 histone but not unphosphorylated H1 (Lu et al, 1994). H1 histones from cells transfected with ras, myc, and p53 were separated by SDS-PAGE and probed with the antiserum. As shown in figure 37a, we detected a low amount of phosphorylated H1 histones in the parental 10T½ cell line. However, cells transformed by combinations of ras, myc, and p53 contained between 4 and 20 fold higher levels of this modified histone (figure 38). Loading of the immunoblot was verified by staining with india ink (figure 37b). Comparison of the migration of the antibody reactive band with all of the H1 histones stained with Coomassie blue or india ink showed that it migrated at a position similar to the phosphorylated band we detected by Coomassie blue staining. To verify that the antibody recognized phosphorylated H1 histone, we performed immunoblot analysis with histones dephosphorylated with alkaline phoshatase. Figure 37a shows that antibody reactivity decreases substantially upon phosphatase treatment (compare lanes 4 and 7).

To further analyze which H1 histone subtype was overphosphorylated in ras-transformed cells we separated H1 histones by two dimensional gel electrophoresis.

FIGURE 36.

Synthesis of core histones in cells transfected with combinations of ras, myc and mutant p53. Core histones were isolated by extraction with sulfuric acid, and then separated by SDS-PAGE. Lanes: 1) 10T½, 2) R-2, 3) RM-5, 4) RP-4, 5) RP-6, 6) RMP-6. Histones were visualized by staining with Coomassie blue.

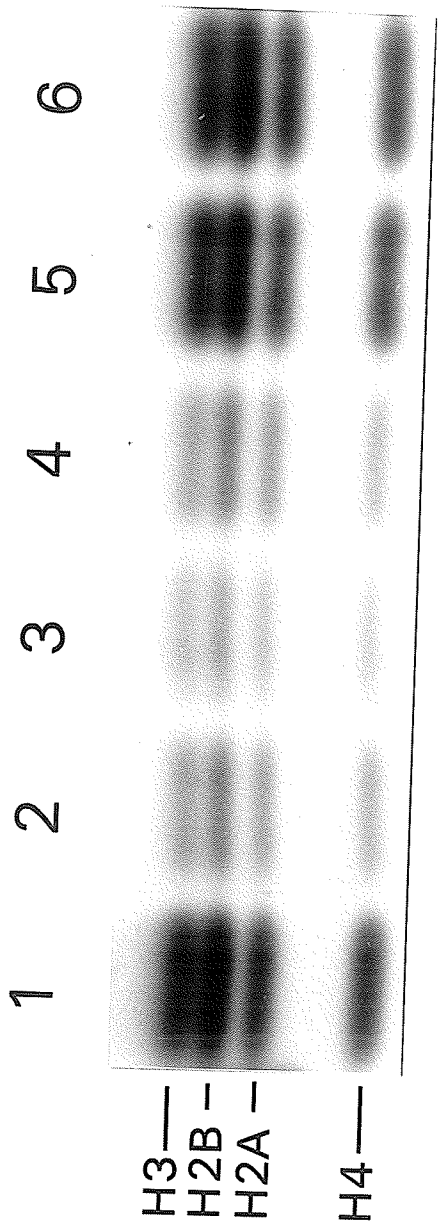

1
2
3
4
5
6
H3
H2B
H2A
H4

FIGURE 37.

Immunoblot analysis of hyperphosporylated H1 histones from fibroblasts transfected with ras, myc, and mutant p53. H1 histones were extracted from normal and transformed cell lines, separated by SDS-PAGE and transferred to membranes. Two μg of histone were loaded per lane. (A) Membranes were probed with an antibody specific for hyperphosphorylated H1 histone. Lane 1) 10T½, 2) R-2, 3) RM-5, 4) RP-4, 5) RP-6, 6) RMP-6, 7) RP-4 treated with alkaline phosphatase. (B) India ink staining of proteins transferred to the membrane. Equal loading of protein on the membrane was confirmed by staining the membrane with india ink prior to blocking. The same membrane as was probed in figure 37a is shown, and the lanes are as above.

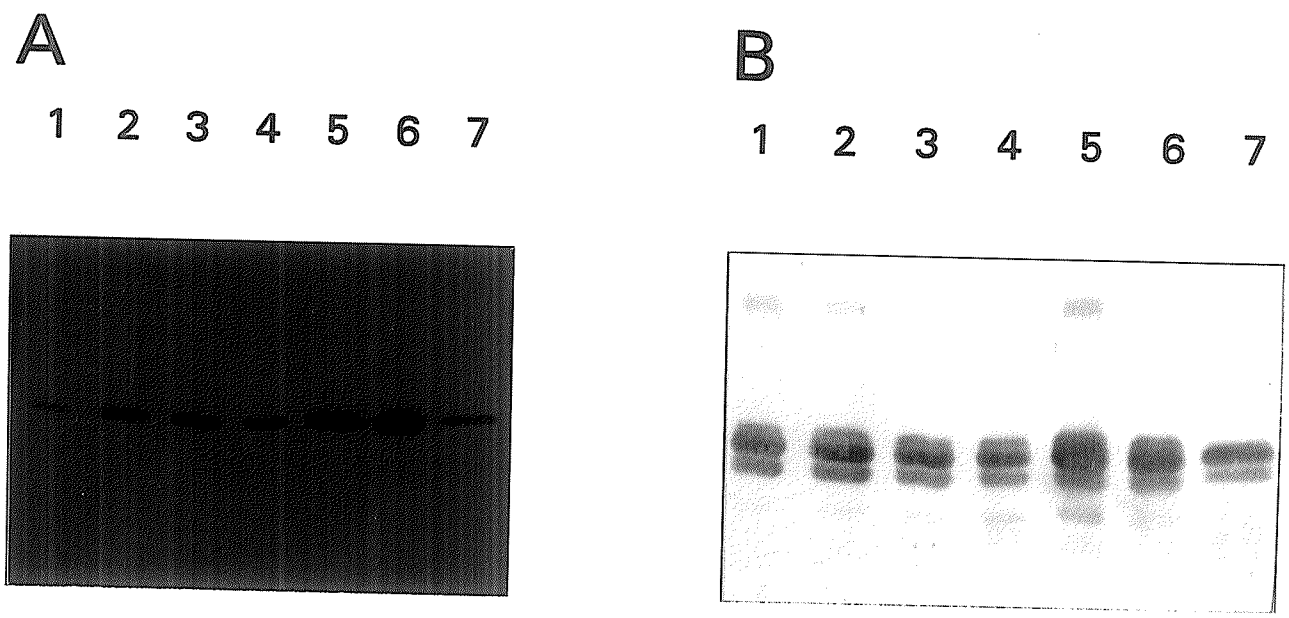
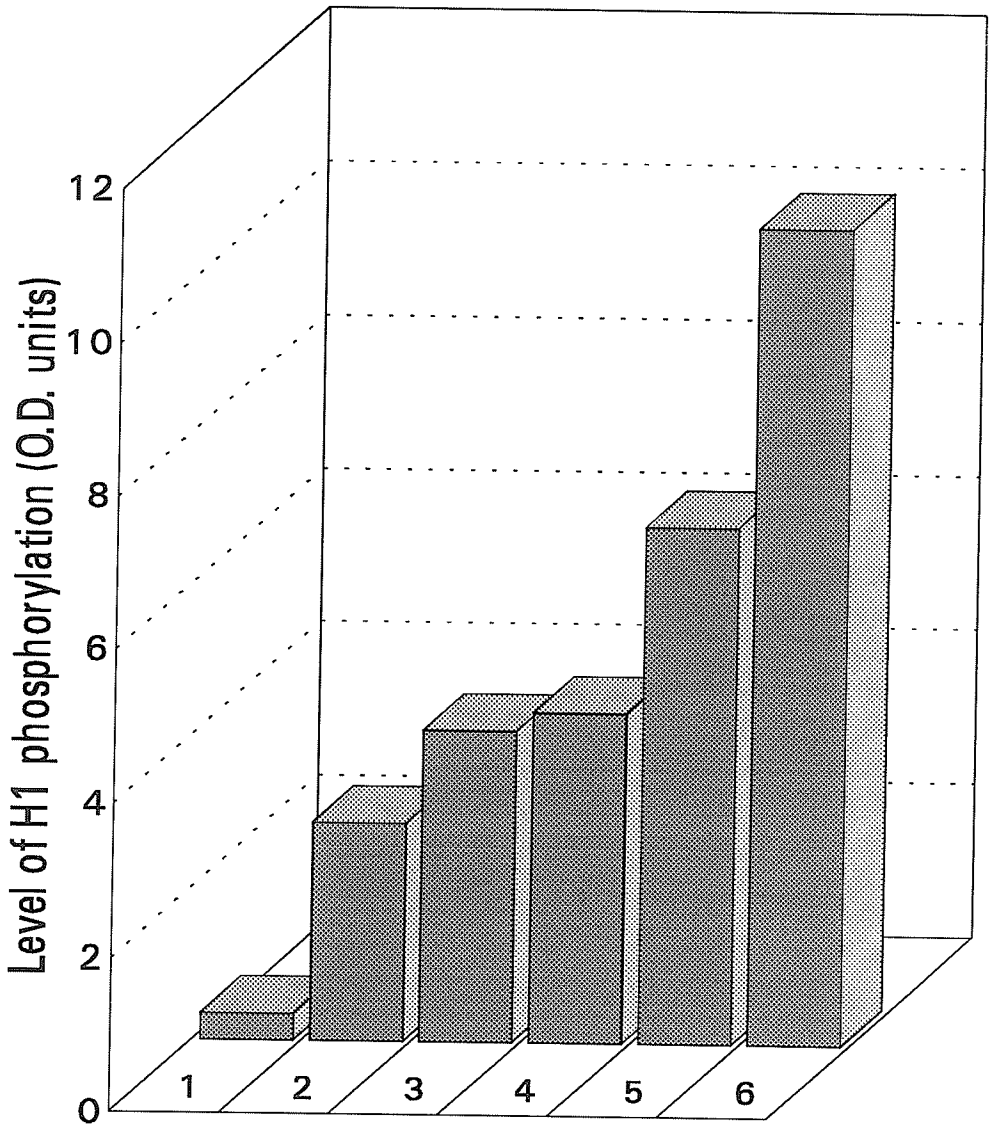

A
1 2 3 4 5 6 7
B
1 2 3 4 5 6 7

FIGURE 38.

Level of phosphorylation of H1 histones in cells transfected with combinations of ras, myc and mutant p53. Western blots were scanned with a video densitometer to determine the level of phosphorylation (O.D. units). The cell lines shown are: 1) 10T½, 2) R-2, 3) RM-5, 4) RP-4, 5) RP-6, 6) RMP-6.


Level of H1 phosphorylation (O.D. units)
0
2
4
6
8
10
12
1
2
3
4
5
6

Histones were first separated by acid-urea-triton polyacrylamide gel electrophoresis. As shown in figure 39, H1 histones from the parental 10T½ cell line resolves into three bands. However, the transformed cell lines contained up to two additional bands which migrated slower than the bands detected in 10T½ cells, suggesting that they contained additional negative charges consistent with the presence of additional phosphate groups. To produce two dimensional H1 histone maps, acid-urea-triton (AUT) gels were electrophoresed into SDS-PAGE gels. As shown in figure 40, several spots were distinguishable which corresponded to subtypes H1 b, c, d, e, and some of their phosphorylated derivatives as determined by comparison to previously published maps (Lennox & Cohen, 1983). When histones from the parental 10T½ cells, and the ras, myc, p53 transformed fibroblasts were compared, the spot corresponding to phosphorylated H1b was found to be significantly more abundant in transformed cells compared to 10T½ cells (figure 41). When a two dimensional map was transblotted to a membrane and probed with the antiserum specific for phosphorylated H1, we detected one spot (figure 42). This spot comigrated with the spot found by Coomassie staining to be more abundant in transformed cell lines. The intensity of the signal generated by immunoblotting was much higher in transformed cells suggesting that H1b is more highly phosphorylated in transformed cells.

4.4 Cell cycle analysis

Previous studies have shown that the level of phosphorylation of H1 histones varies considerably during the cell cycle. In G1 phase, H1 generally contains

FIGURE 39.

Analysis of H1 histones from cells transfected with combinations of ras, myc and mutant p53 by AUT polyacrylamide gel electrophoresis. H1 histones were purified by extraction in perchloric acid, and separated in AUT gels. Lanes 1) 10T½, 2) R-2, 3) RM-5, 4) RP-4, 5) RP-6, 6) RMP-6.

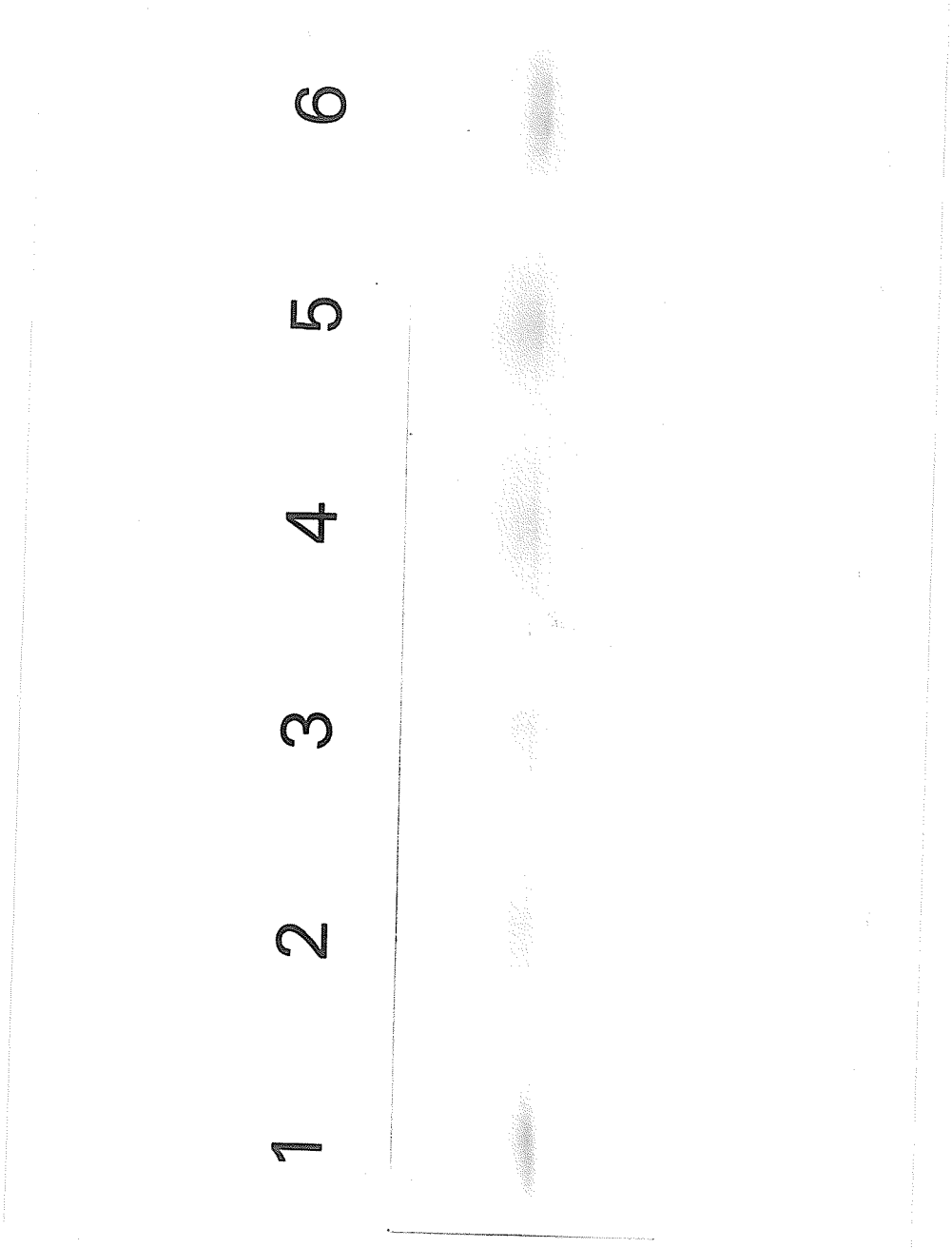

6
5
4
3
2
1

FIGURE 40.

Two dimensional gel analysis of H1 histone from a ras-transformed fibrosarcoma cell line. H1 histones were purified from Ciras-3 cells by extraction in perchloric acid, and then separated first by AUT polyacrylamide gel electrophoresis, and then by SDS-PAGE. The histones were visualized by staining with Coomassie blue. Histone subtypes were identified by comparison with two dimensional maps previously published (Lennox & Cohen, 1983).

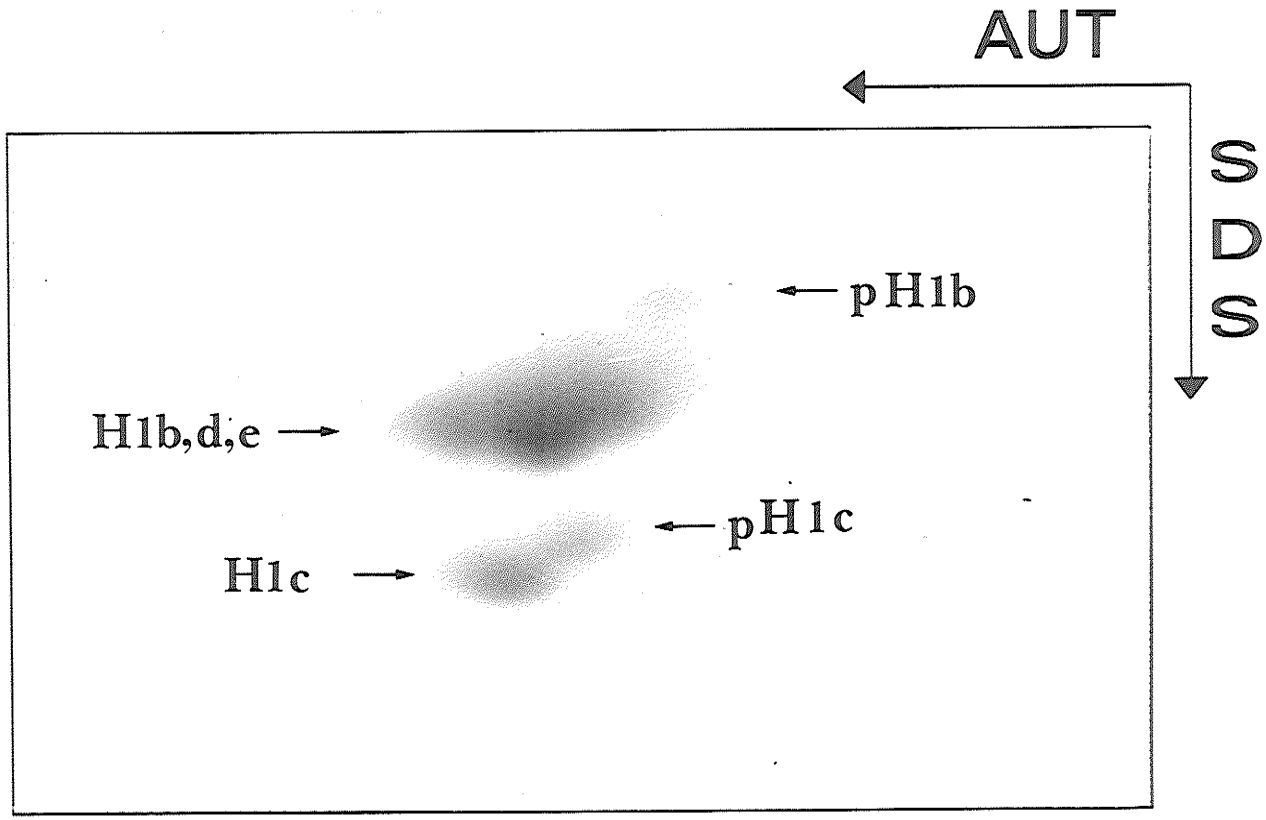

AUT
SDS
pH1b
H1b,d,e
pH1c
H1c

FIGURE 41.

Two dimensional gel electrophoresis of H1 histones from transformed fibroblasts. (A) H1 histones were extracted, separated by AUT gels (first dimension) and then by SDS-PAGE (second dimension). Proteins were visualized by staining with Coomassie blue. Lane 1) 10T½, 2) R-2, 3) RM-5, 4) RP-4, 5) RP-6, 6) RMP-6.

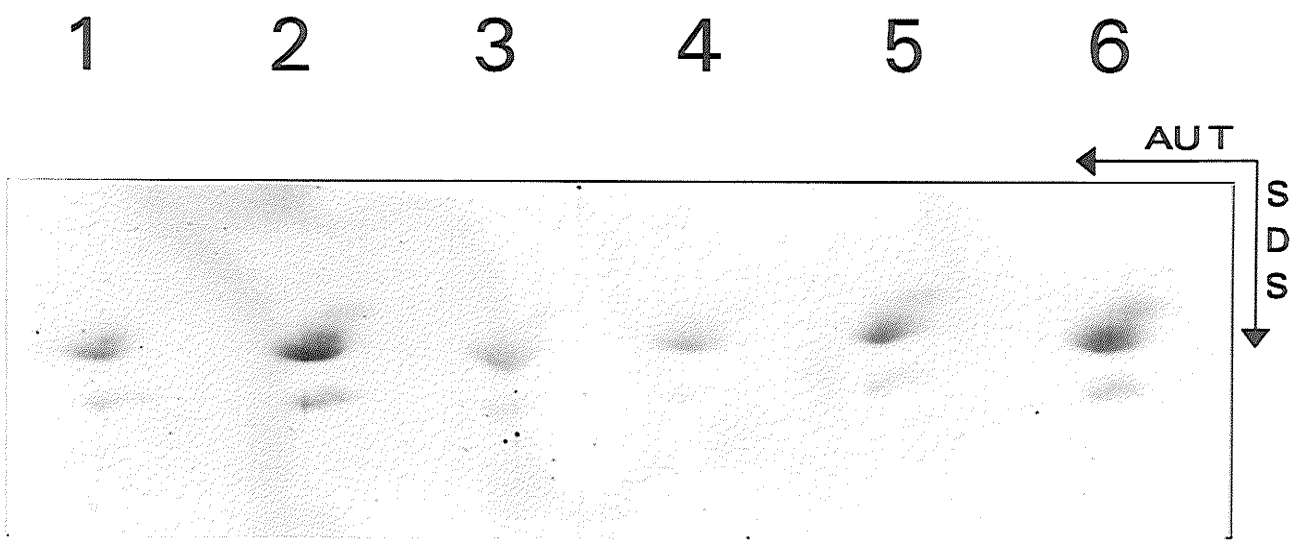

1
2
3
4
5
6
AUT
SDS

FIGURE 42.

Immunoblot analysis of H1 histones separated by two dimensional gel electrophoresis. (A) H1 histones were separated by two dimensional electrophoresis, transferred to membranes and probed with an antibody specific for hyperphosphorylated H1 histone. Lane 1) 10T½, 2) R-2, 3) RM-5, 4) RP-4, 5) RP-6, 6) RMP-6. (B) India ink stain of the same membrane before probing to determine protein loading.


A
1
2
3
4
5
6
B
1
2
3
4
5
6

approximately 1 phosphorylated amino acid per molecule. However, the level of phosphorylation rises to approximately 6 residues per molecule when cells enter M phase. H1 becomes dephosphorylated as cells exit M phase (Hohmann et al, 1976). We reasoned that one explanation for the altered levels of phosphorylation observed in the transformed cell lines may be that they exhibit a different cell cycle distribution compared to control cells. We analyzed cell cycle distribution by flow cytometry of cells stained with ethidium bromide (Yasuda et al, 1981). Figures 43 and table 5 show that there are minor differences in the cell cycle distributions of some of the transformed cells compared to 10T½ cells. For example, most transformed cell lines showed slightly higher levels of cells outside of G1 where the lowest phosphorylation occurs. Cell line RMP-6 showed 25% higher levels of cells in M phase compared to 10T½ cells. In contrast, the cell line R-2 showed higher levels of G1 cells compared to 10T½ cells. Statistical analysis of the cell cycle distribution of 10T½ and R-2 cells showed that there was no evidence for a difference in their cell cycle distributions (figure 44).

4.5 Phosphorylation of H1 histones in G1 phase of the cell cycle

Flow cytometry suggested that the increase in H1 histone phosphorylation found in a ras-transformed cell line was not secondary to changes in its cell cycle distribution relative to the control 10T½ cell line (figures 37, 44). However, these experiments do not indicate at which point in the cell cycle the increase in phosphorylation occurs. In order for H1 phosphorylation to have a impact on gene transcription, we hypothesized that the increase in phosphorylation would have to occur during those cell cycle phases

FIGURE 43.

Cell cycle histogram of fibroblasts transfected with ras, myc, and mutant p53. Cells were fixed, stained with ethidium bromide and their DNA content was determined by flow cytometry. G1: first gap of interphase, S: DNA synthesis phase, G2: second gap of interphase, M: mitosis. A) 10T½, B) R-2, C) RP-4, D) RP-6, E) RMP-6.

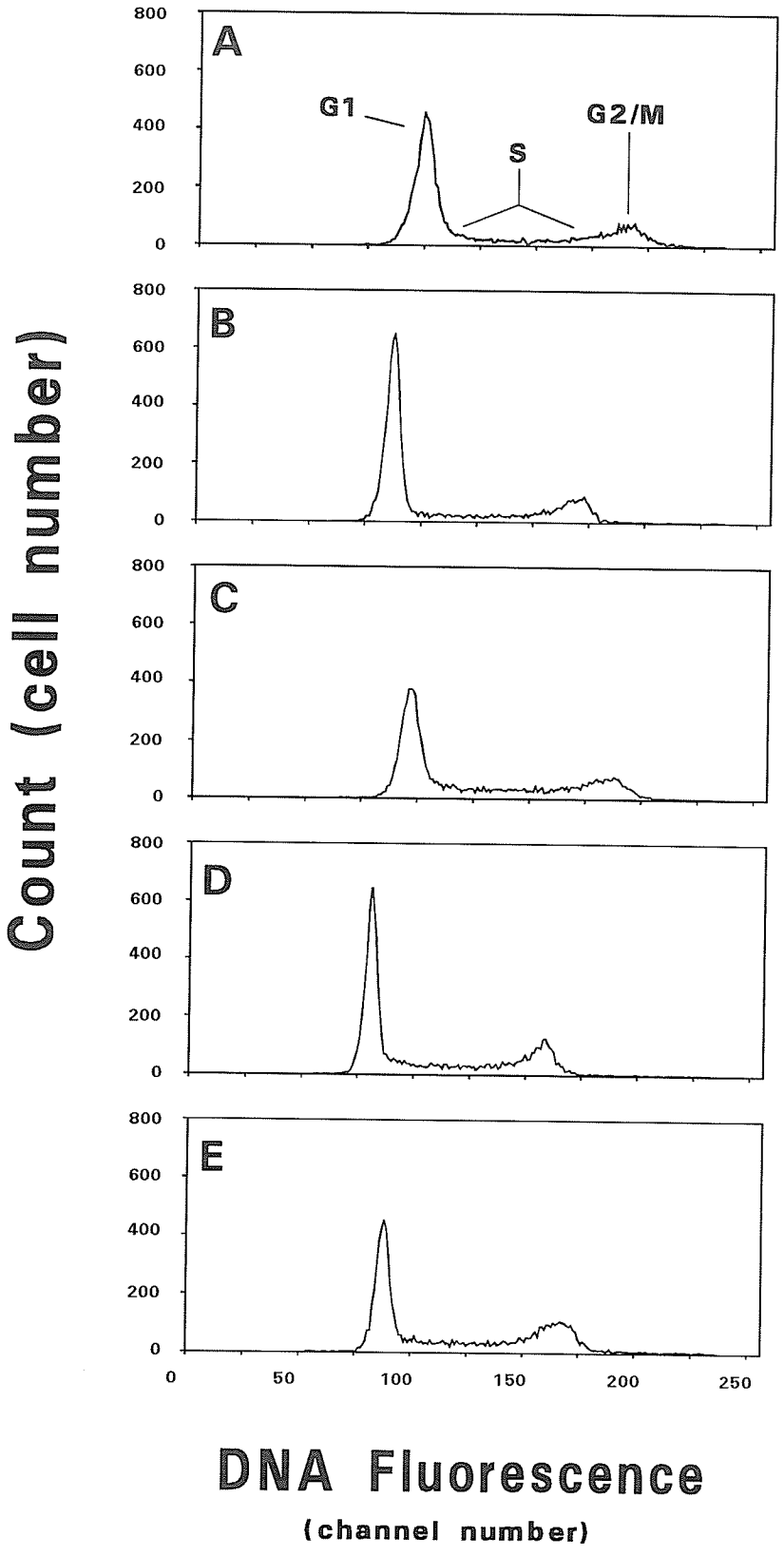

A
G1
S
G2/M
B
C
D
E
800
600
400
200
0
0
50
100
150
200
250
Count (cell number)
DNA Fluorescence
(channel number)

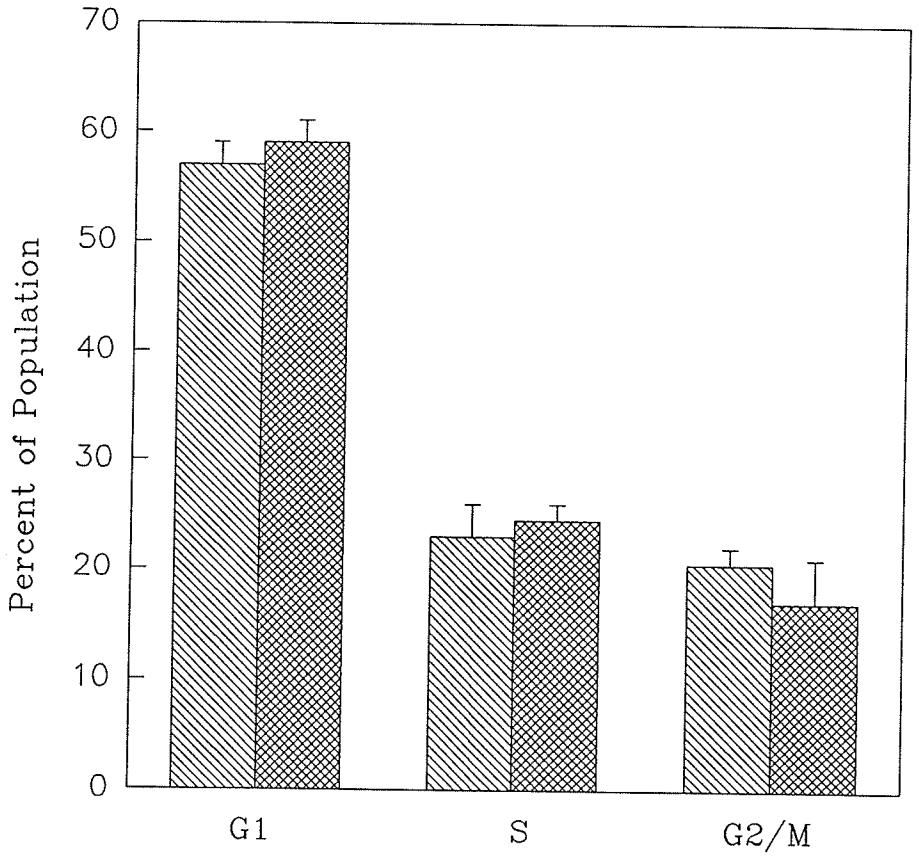
TABLE 5.

Cell cycle distribution of fibroblasts transfected with ras, myc, and mutant p53. The percentage of cells in each of three identifiable cell cycle phase is shown. Results from one experiment are shown.

Cell Line	Cell Cycle Phase		
	G1	S	G2/M
10T1/2	55	26	19
R-2	61	26	13
RP-4	49	31	19
RP-6	50	33	17
RMP-6	45	31	24

FIGURE 44.

Cell cycle distribution of normal and ras-transformed fibroblasts. The cell cycle distribution of 10T½ cells (hatched bar) and R-2 cells (cross hatched bar) is shown. The percent of cells in each of the three cell cycle phases, determined from two independent experiments is shown. Standard errors are indicated by the bars. No evidence for a statistically significant difference between 10T½ and R-2 cells was found when G1, S, or G2/M phase cells were compared (all p values > 0.3).


70
60
50
40
30
20
10
0
Percent of Population
G1
S
G2/M

where gene transcription is known to occur at a high rate, namely G1 (Marks et al, 1973). To test this hypothesis, we treated cells with the DNA synthesis inhibitor, hydroxyurea, which is capable of arresting cells in G1 phase of the cell cycle. (Ashihara & Baserga, 1979). Figure 45 shows that when both 10T½ and R-2 are treated with hydroxyurea, their distribution changes so that fewer cells are in G2/M phase and more cells are in G1 phase. This is consistent with a G1 arrest. Next, H1 histone was extracted from 10T½, R-2, and Ciras-2 cells after hydroxyurea-induced G1 arrest. Immunoblot analysis showed that G1 arrested ras-transformed cell lines contained higher levels of H1 phosphorylation compared to G1 arrested 10T½ control cells (figure 46).

4.6 Subcellular localization of phosphorylated H1 histones

The increase in H1 histone phosphorylation in ras transformed cells suggested that this histone is regulated by phosphorylation differently during the process of transformation. Therefore, we investigated whether or not the subcellular localization of phosphorylated H1 was also changed during malignant transformation. Cells growing on coverslips were fixed and phosphorylated H1 histone was localized by immunofluorescence using the specific antiserum described above. Figure 47 shows that the exposure of cells to the control preimmune serum led to faint cytoplasmic staining. Exposure to the specific antiserum resulted in marked nuclear staining. Comparison of 10T½ control cells with several ras transformed cell lines indicated that this nuclear distribution of phosphorylated H1 does not change upon transformation (figure 48). One exception to the nuclear staining observed with normal and ras transformed cells was

FIGURE 45.

Effect of hydroxyurea treatment on the cell cycle distribution of normal and ras-transformed cell lines. Cells were fixed with ethanol, stained with ethidium bromide and their cell cycle distributions determined by flow cytometry. 10T½ (A & B) and R-2 (C & D) cell lines were grown for 24 h in the absence (A & C) or presence (B & D) of 2mM hydroxyurea.

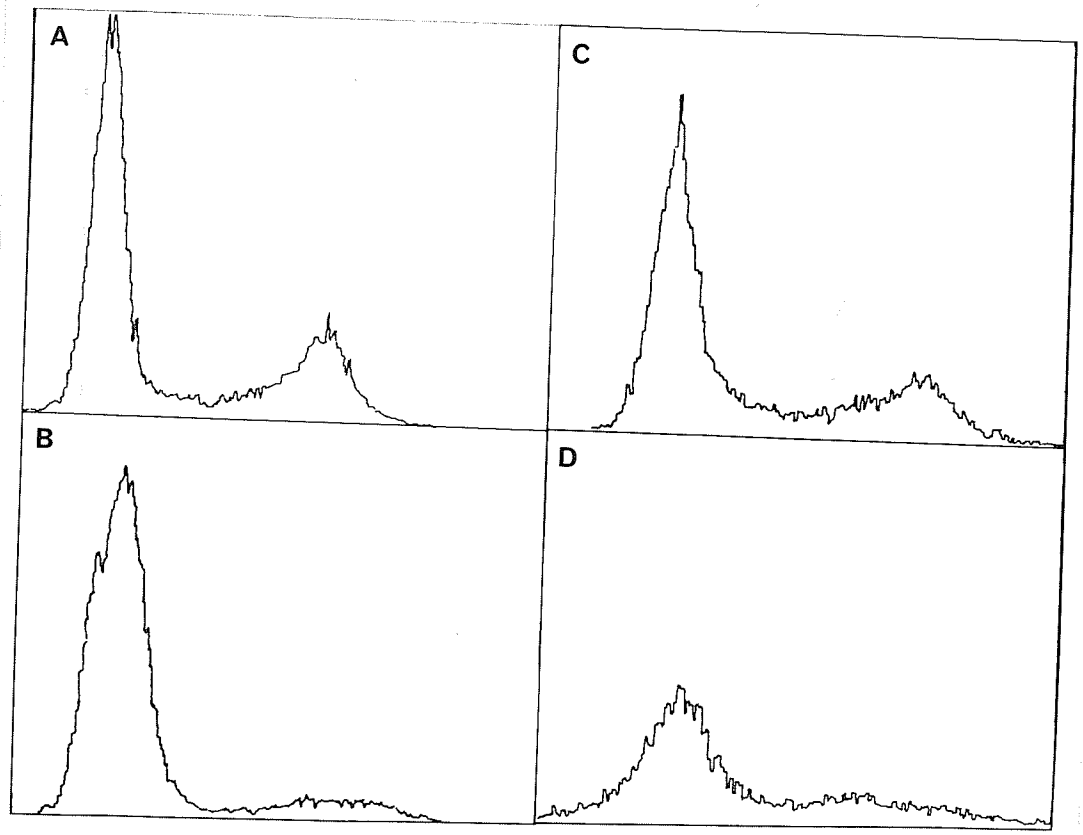

A
C
B
D

FIGURE 46.

Immunoblot analysis of phosphorylated H1 histones in normal and ras-transformed cells treated with hydroxyurea. (A) Cells were treated with 2mM hydroxyurea for 24 h, after which H1 histone was extracted and analyzed by immunoblotting with an antiserum specific for phosphorylated H1 histones. The amount of protein [μg] loaded in each lane is shown. Lanes 1) 10T½ [1.5μg], 2) 10T½ [2.0μg], 3) Ciras-2 [2.0μg], 4) R-2 [1.5μg]. (B) India ink staining of the same membrane as was probed above to confirm equal loading.

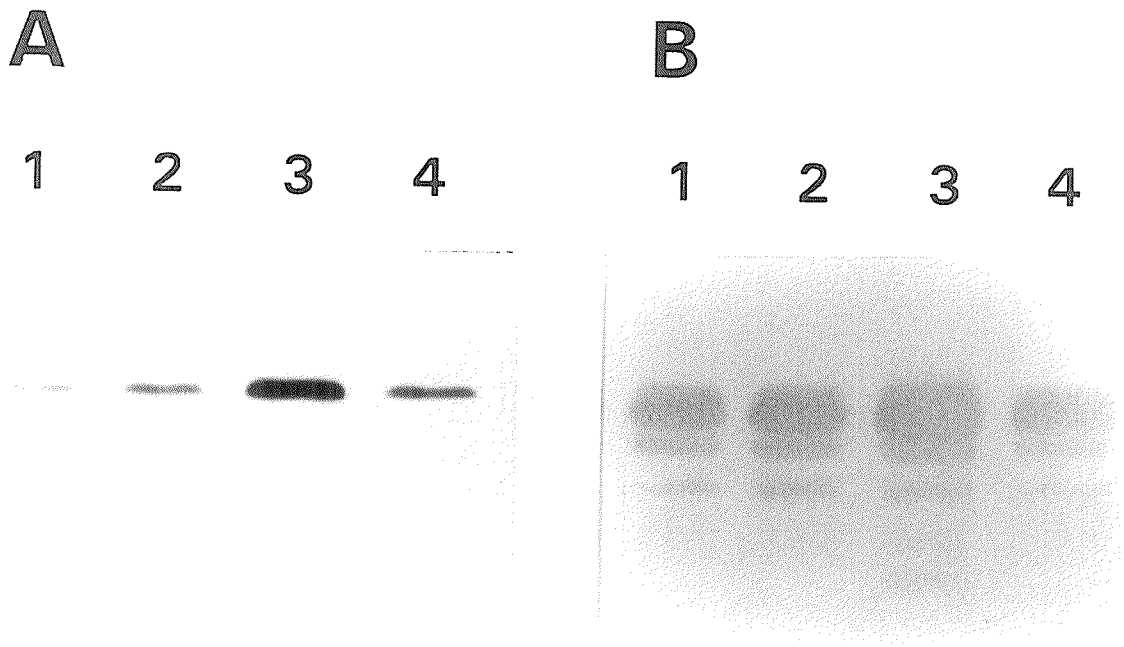
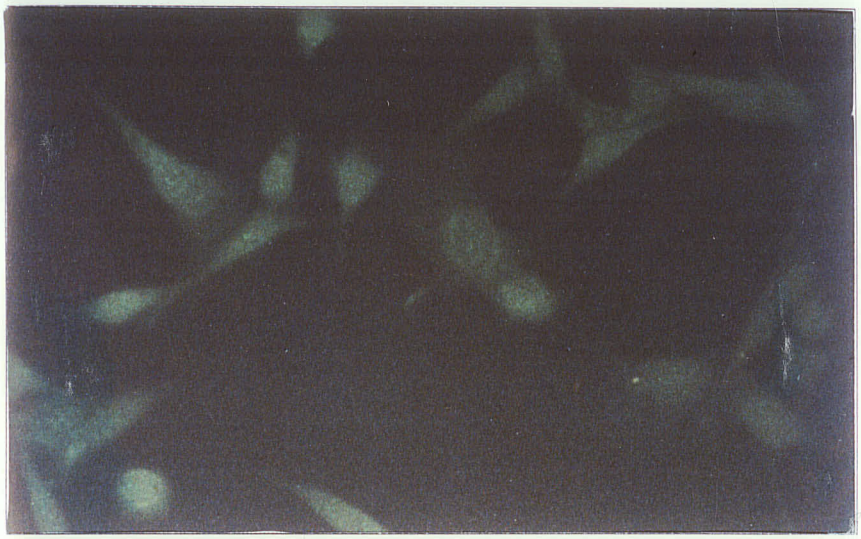

A
1
2
3
4
B
1
2
3
4

FIGURE 47.

Specific localization of phosphorylated H1 histone in fibrosarcoma cells. RP-4 cells were grown on separate coverslips, fixed, and incubated with either preimmune rabbit serum, or specific rabbit antiserum raised against phosphorylated H1 histone. Cells were then incubated with an anti-rabbit IgG antiserum raised in goats and linked to FITC. Cells were then photographed under a fluorescent microscope.

pre-immune



anti-H1

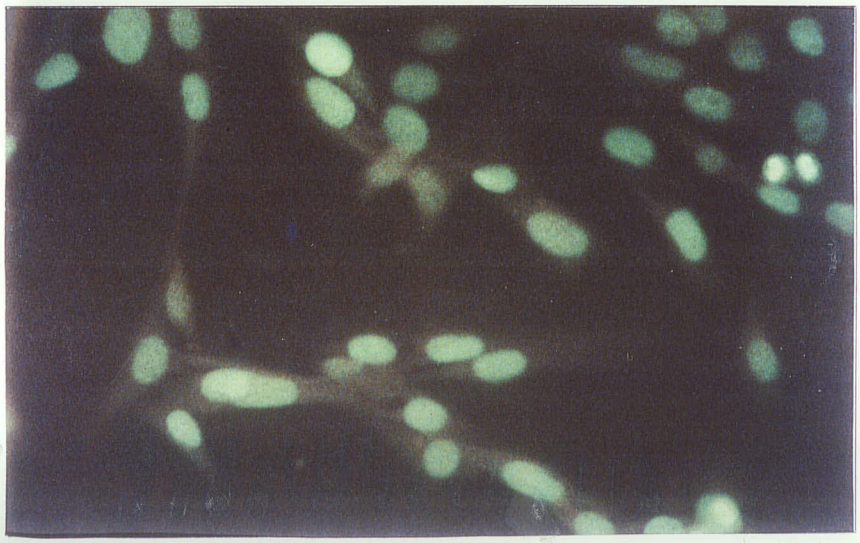
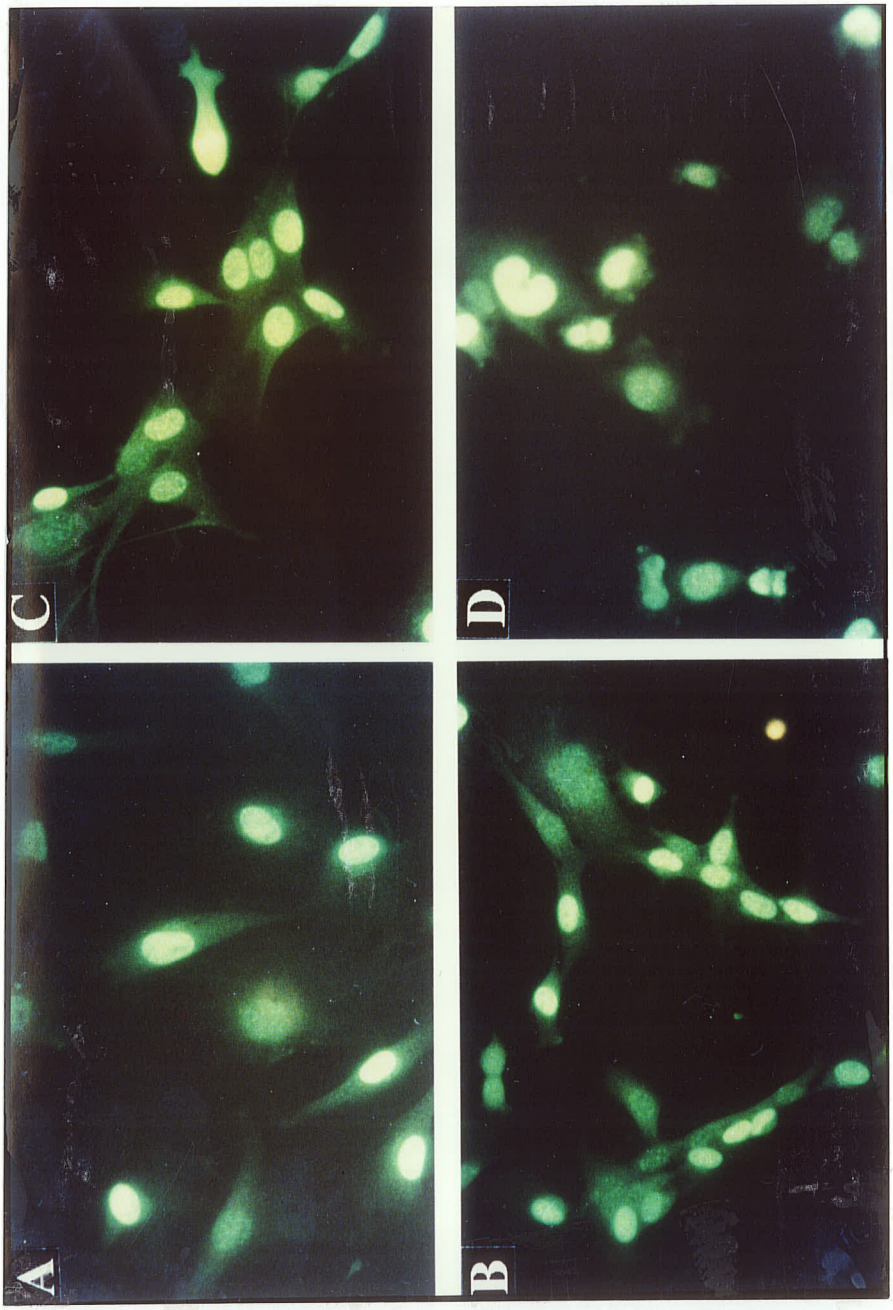


FIGURE 48.

Subcellular localization of hyperphosphorylated H1 histone in normal and transformed fibroblasts. Cells were fixed on the surface of a coverslip and stained by indirect immunofluorescence for the presence of hyperphosphorylated H1 histone. A) 10T½, B) R-2, C) RP-6, D) RMP-6 cells.


A
B
C
D

evident in cells undergoing mitosis. Mitotic cells stained very highly for phosphorylated H1 and a considerable amount of this reactivity was found in the cytoplasm (figure 48c). Inspection of high power photomicrographs revealed an additional aspect of H1 localization. Phosphorylated H1 stains the nucleus in a punctate pattern (figure 49). These small points of concentrated fluorescence were seen in normal 10T½ cells and in ras transformed cells.

Immunofluorescence revealed one striking difference between normal and ras transformed cells. Whereas all of the 10½ cells contained nuclei that were either round or oval, a considerable number of the transformed cells contained multilobed nuclei (figure 50). To determine if this represented an altered nuclear morphology, or an altered histone distribution, we stained cells with the fluorescent nuclear dye, DAPI (Lu et al, 1994). DAPI staining revealed that most of the cells contained a round or oval nucleus (figure 51). However, some of the transformed cell lines and very few of the untransformed 10T½ cells contained a multilobed nucleus (figures 52). Nuclei with two or more observable lobes were counted. Figure 53 shows that although very few 10T½ cells contained aberrant nuclei, considerably more were observed in cells transfected with either ras alone, or ras in combination with myc or mutant p53. Metastatic cells transfected with ras, myc, and mutant p53 showed the highest level of multilobed nuclei.

FIGURE 49.

Punctate nuclear distribution of hyperphosphorylated H1 histone in normal and transformed fibroblasts. High power magnification of phosphorylated H1 immunofluorescence is shown. A) 10T½, B) RMP-6 cells.

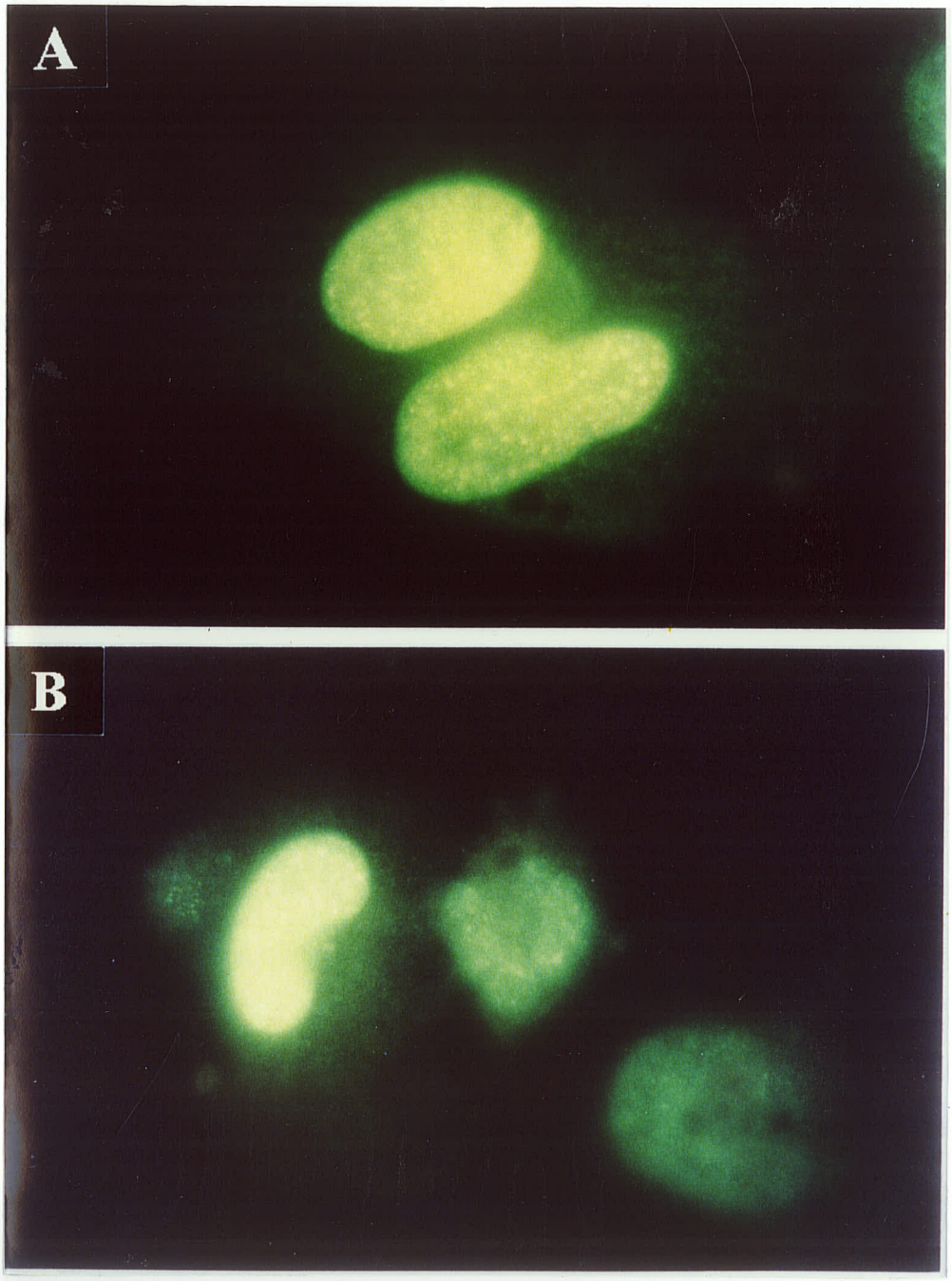

A
B

FIGURE 50.

Multilobed localization of phosphorylated H1 histone in metastatic, ras, myc and mutant p53 transfected fibrosarcoma cells. RMP-6 cells grown on coverslips were fixed and analyzed by indirect immunofluorescence using a primary antibody specific for phosphorylated H1 histone. Cells from five separate fields are shown.

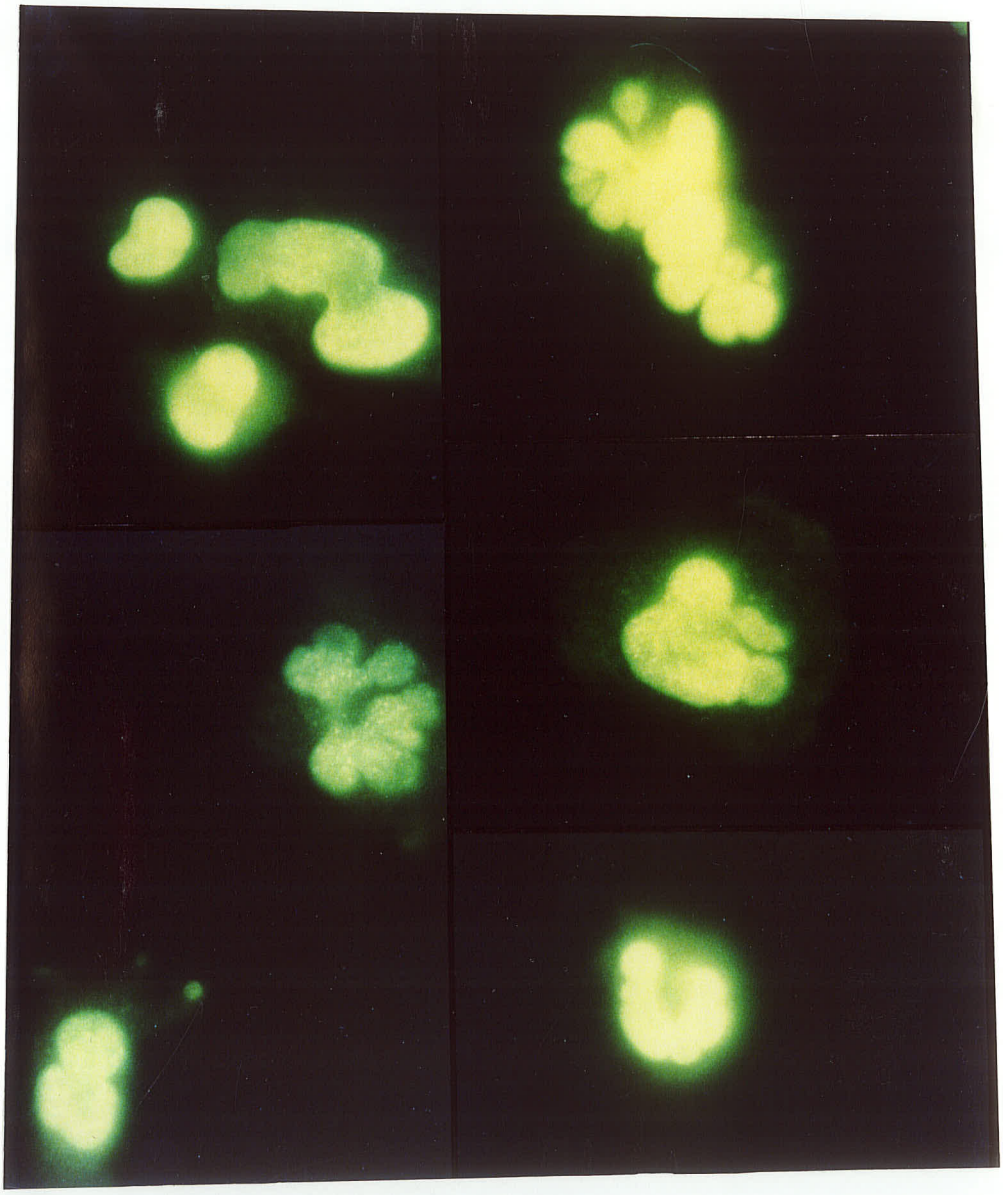


FIGURE 51.

Nuclear morphology of fibroblasts transformed by combinations of ras, myc and mutant p53. Cells were grown on coverslips, fixed, and stained with the DNA stain, DAPI, and photographed. The following cell lines are shown: A) 10T½, B) R-2, C) RM-5, D) RP-6, E) RMP-6.

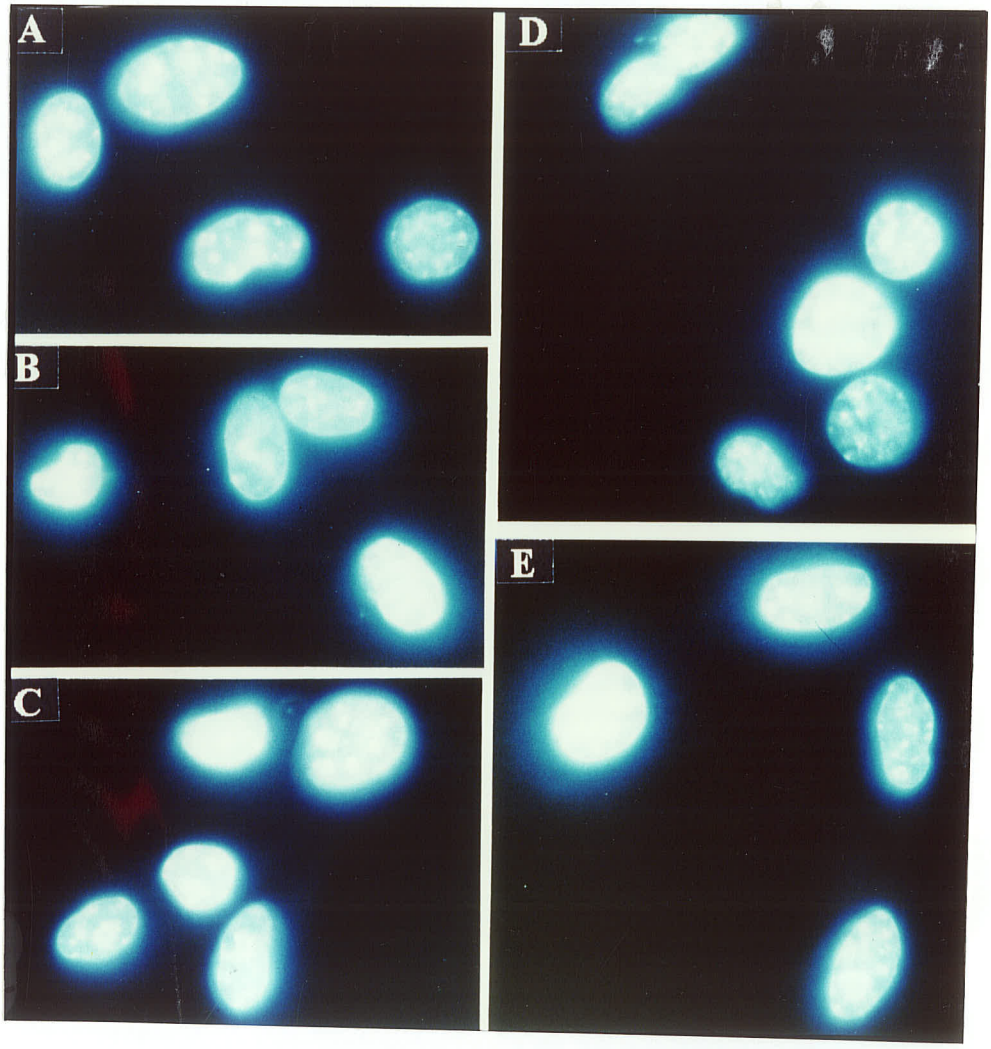

A
B
C
D
E

FIGURE 52.

Altered nuclear morphology associated with oncogenic transformation. Cells were fixed on the surface of a coverslip and stained with the fluorescent DNA dye DAPI. Eight separate fields of the metastatic RMP-6 cell line are shown.

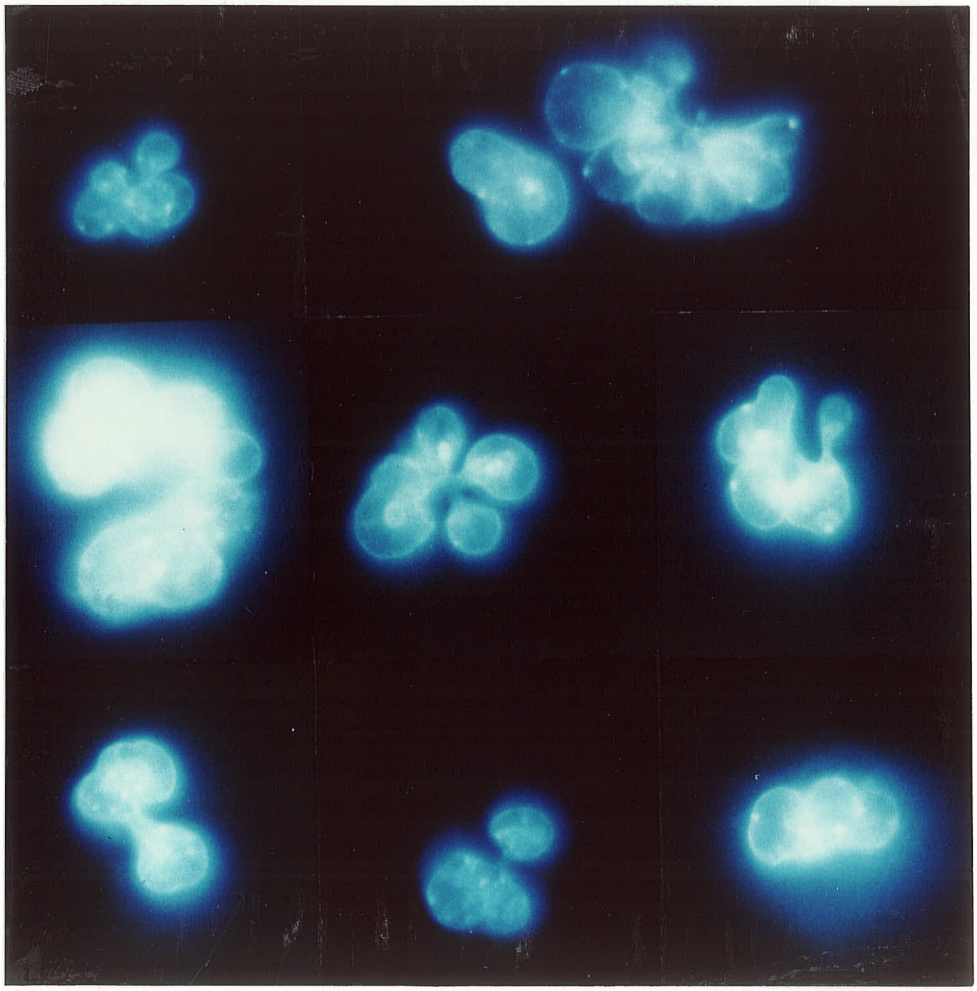
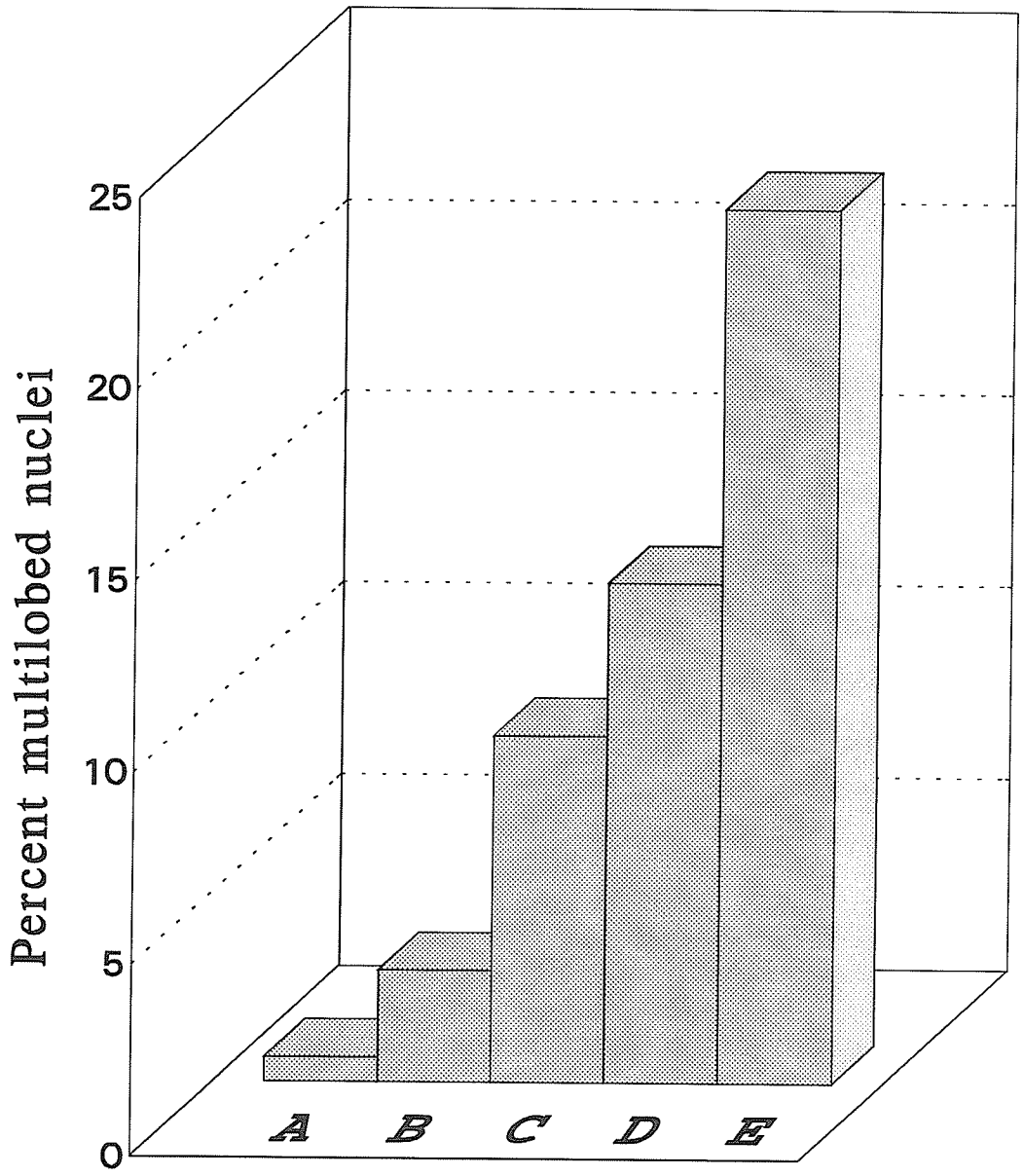


FIGURE 53.

Occurrence of multilobed nuclei in cells transformed by combinations of ras, myc and mutant p53. Cells were grown on the surface of coverslips, fixed and stained with DAPI. Nuclei containing at least two distinct lobes were counted and at least 200 cells were examined for each condition. The following cell lines are shown: A) 10T½, B) R-2, C) RM-5, D) RP-6, E) RMP-6.


Percent multilobed nuclei
25
20
15
10
5
0
A
B
C
D
E

IV. DISCUSSION

1. Growth Factor Induced Motility in the Metastatic Phenotype

Fibroblast growth factors can induce the metastatic phenotype in transfected cell lines (Damen et al, 1991; Egan et al, 1990). This is likely linked to the biological activities of this group of growth factors. Fibroblast growth factors have several physiological roles including the control of some aspects of mammalian ontogeny. K-FGF is expressed in early stem cells and peri-implantation mouse embryos but not in post-implantation embryos or adult tissues to a great extent. bFGF expression is found after implantation in several tissues (Hebert et al, 1990). The importance of FGF's in development has been directly shown by the ablation of FGF signalling using dominant negative FGF receptor mutants (Amaya et al, 1991). Introduction of such a mutant receptor into embryo stem cells results in abnormal development characterized by deficiencies in the formation of lateral mesoderm.

In addition to development, FGF's have physiological roles in wounding where they may be responsible for the induction of an angiogenic response. Angiogenesis is a complex process characterized by increased growth and motility of endothelial cells (Burgess & Maciag, 1989). The contribution of fibroblast growth factors to tumor formation and malignancy also may rely on their ability to stimulate angiogenesis. The blood supply to a growing tumor is partially maintained by neovascularization triggered by growth factors secreted by the tumor, including growth factors of the fibroblast growth factor family. In addition, the stimulation of growth and motility of cells by fibroblast

growth factors may be important in dissemination of tumor cells to distant sites.

bFGF is a chemotactic factor (Terranova et al, 1985) and intracellular bFGF levels correlate with the motility of endothelial cells (Tsuboi et al, 1990). However, although K-FGF has been shown to regulate the metastatic phenotype (Damen et al, 1991) its ability to regulate motility has not been previously examined. Therefore, we determined the importance of motility in the metastatic dissemination of FGF-transfected cells. We found that both K-FGF transfected cells and cells transfected with bFGF fused to a signal sequence for secretion showed significantly higher rates of collagen invasion than control cell lines (figure 6). These results suggest that metastatic cell lines transformed by FGF's have increased ability to invade into the physiologically relevant collagen I substratum. This extracellular matrix molecule is abundant in the loose connective tissue of most organs but not in the basement membrane where collagen IV is more predominant (Liotta et al, 1986). Increased invasion into gels of collagen I may be indicative of an ability of metastasizing cells to move within the intercellular spaces of primary or secondary organs once the cells have traversed the requisite basement membrane barriers.

In addition to increased collagen invasion, K-fgf and some bfgf transfected cell lines showed increased rates of locomotion, which could be reversed by suramin treatment (figures 8,9,11). This is consistent with the increase in locomotion being induced by the FGF produced by these cells. In the case of bFGF transfected cells, increases in motility did not correlate strictly with the presence of a heterologous signal sequence. These results are consistent with previous reports showing that elevations in intracellular bFGF levels can modify motility properties (Tsuboi et al, 1990). The results also resemble findings obtained with aFGF, in which no differences in motility rates were observed

between cells transfected with an aFGF sequence lacking a signal sequence for secretion and cells transfected with an aFGF sequence fused to a conventional secretory signal sequence (Jouanneau et al, 1991). Previous studies have also shown that native bFGF without a signal sequence can partially transform NIH3T3 cells when high intracellular levels are achieved (Quarto et al, 1989). These results support models of cell locomotion in which stimulation of the process can occur through both autocrine and intracrine pathways (Wright et al, 1993), and are consistent with other studies of growth-stimulating factors known to be secreted from the cell, where it has been reported that at least part of their mechanism of action involves an interaction with intracellular components (Bejceck et al, 1989; Huang & Huang, 1988; Dunbar et al, 1989).

Our analysis of the relationship between metastatic potential and the rate of motility of NIH3T3 cells transfected with K-fgf or bfgf strongly supports the concept that metastasis induced by these factors is linked to their ability to regulate cellular motility (Taylor et al, 1993). We found that there was a statistically significant correlation between motility and metastatic potential. Furthermore, there appears to be a biphasic relationship between these two parameters (figure 12). We interpret this as suggesting that motility is required for metastatic spread since all metastatic cells show high rates of motility above approximately 4.25 um/min. However, it is apparent that increased motility, as seen with some clones, is not sufficient to induce the metastatic phenotype. This is expected from the complexity of the metastatic cascade and also points out the importance of other biological alterations in the induction of the metastatic phenotype.

The studies described above suggest that increased cellular motility and collagen invasion independently play an important role in the metastatic phenotype of fibroblast

growth factor transformed cells. To test the generality of this observation, we examined the importance of motility in the metastatic phenotype induced by another growth factor, $TGF\beta_1$. Previous studies have demonstrated that the highly malignant cell line, Ciras-3 secretes high levels of $TGF\beta_1$ which acts as a stimulator of proliferation in this cell line (Schwarz et al, 1988; Schwarz et al, 1990). It has been postulated that the ability of this cell line to metastasize may be regulated by an autocrine loop involving $TGF\beta_1$ and its cell surface receptors. We found that treatment of Ciras-3 cells with antisense oligonucleotides, which can reduce $TGF\beta_1$ secretion (Spearman et al, 1994), also leads to significant reductions in both collagen invasion and metastatic potential (figure 13, 14). These studies show that overexpression of $TGF\beta_1$ is required for high rates of motility and metastatic ability in this cell line, and further emphasizes the relationship between motility and malignant transformation facilitated by overexpression of growth factors.

2. Oncogene Cooperation and Metastatic Progression

The signal transduction pathway utilized by FGF appears to rely on the activity of the ras proto-oncogene (Kremer et al, 1991). Furthermore, induction of motility by bFGF in endothelial cells is dependent on ras activity (D. Stacy, personal communication). In addition, the metastatic phenotype can also be induced by expression of constitutively active forms of ras (Greenberg et al, 1989). However, the induction of metastasis by ras occurs at a low frequency. The next series of experiments were aimed at determining the genetic requirements for induction of the metastatic phenotype by ras. These studies indicate that elevated expression of activated ras in combination with myc

expression and the presence of a mutant form of p53 produces a significantly more malignant condition than when these genes are tested separately or in combinations involving only two genes. This conclusion is based upon observations obtained in transformation foci experiments, and from results obtained in *in vivo* studies of tumor latency and metastatic potential. These findings are in general agreement with investigations of human tumors, which have shown that multiple oncogenic events are involved in mechanisms of tumor progression (Weston et al, 1989; Baker et al, 1989; Fearon et al, 1990), and that there is a direct relationship between the number of oncogenic alterations and malignant potential (Fearon & Vogelstein, 1990).

The results of a study with rat embryo fibroblasts by Peacock et al (1990) showed an interesting synergistic effect between pairs of immortalizing genes in transformation focus forming experiments. They demonstrated that ras-dependent transformation *in vitro* was more efficient if the HPV-16 E7 gene and either c-myc or a mutant form of p53 were included in the transfection experiments. The number of transformed foci observed was larger than the sum of the foci detected when each of the immortalizing genes was tested separately. Similarly, findings of synergism between E7, p53 and ras in the transformation of primary rat kidney cells have been reported by Crook et al. (1991). The results we obtained in focus forming experiments are consistent with the observations by Peacock et al. (1990) and by Crook et al. (1991). We found a low level of focus forming ability in mouse 10T½ cells transfected with ras alone (Table 3), and were unable to observe focus formation in experiments with cells transfected with either myc or myc/p53. However, when cells were transfected with ras, myc, and p53 together, focus forming ability was markedly elevated from an average of less than 4 with ras alone

to about 25 with the three gene combination (figure 15). These results are in keeping with the idea that the action of the three oncogenes in the transformation of 10T½ cells occurs along separate but interactive pathways. In addition, we extended the observations obtained in the *in vitro* transformation assay to include an analysis of the malignant properties of oncogene transfected cells *in vivo*. The results of these studies are interesting and also support the idea of synergistic interactions between ras, myc and p53 in malignant transformation. For example, ras/myc or ras/p53 transfectants showed an average of less than one and about three metastases per syngeneic mouse, respectively, whereas the ras/myc/p53 gene combination produced cell lines capable of forming an average of about 37 metastases per mouse (figure 22). Transfection with ras alone produced cell lines exhibiting low metastatic ability (fewer than 2 lung tumors per mouse). These results, and previous observations that myc and p53 are not capable of inducing metastasis in non-tumorigenic fibroblasts when tested separately (Egan et al, 1987b), provide strong support for the concept of synergistic interactions between deregulated ras, myc and p53 genes in mechanisms of malignant transformation. Such interactions are of potential clinical significance.

An analysis of protein expression in the oncogene transfected lines showed several interesting correlations which suggest potential mechanisms of oncogene cooperation. For example, the level of ras protein was significantly elevated in ras/myc/p53-transfected cell lines when compared to the less malignant single or double oncogene transfected lines (figure 18). This observation indicates that an important contribution to the increased malignant characteristics exhibited by ras/myc/p53-transfected lines may be the relatively high levels of activated ras protein. Previous studies have demonstrated that ras can play

a direct role in regulating the metastatic properties of mouse 10T½ cells, and that cells with higher levels of ras are more malignant (Egan et al, 1987a, Wright et al, 1990). There are several genes that appear to be regulated by ras expression and have also been implicated in mechanisms of metastasis and invasion, including genes coding for proteases (Denhardt et al, 1987), matrix proteins (Gingras et al, 1990), and genes regulating growth factor responses (Schwartz et al, 1988; 1990). In addition, previous studies have demonstrated that ras mutations that are capable of cellular transformation have the potential to induce the full metastatic phenotype, supporting the concept that both transformation and metastasis are induced through the p21-GTP model of ras action (Egan et al, 1989a). The results obtained in the present study are in agreement with these ideas. However, it is also clear from this study and from many others, that the ability of ras to transform or induce metastatic progression is also dependent upon the occurrence of other cellular alterations (Wright et al, 1990).

Some cell lines are not susceptible to ras-induced transformation or metastasis. For example, the rat PC12 pheochromocytoma line differentiates into neuron-like cells with activated ras protein (Noda et al, 1985), and a high level of expression of ras in the rat embryo fibroblast REF 52 line leads to growth arrest primarily at the G_1/S or G_2/M boundary of the cell cycle (Hirakawa & Ruley, 1988; Hicks et al, 1991). However, a recent study has shown that overexpression of ras is possible in these cells in the presence of mutant forms of p53 (Hicks et al, 1991). Since wild type p53 acts as a tumor suppressor that is involved in negative growth regulation (Levine et al, 1991), the physical interaction of mutant forms of p53 protein with wild type proteins would substantially decrease endogenous levels of wild type p53 activity in a dominant negative

fashion (Hicks et al, 1991). According to this model the mutant form of p53 in the three gene transfection experiments is necessary to overcome cell cycle arrest that would normally occur in the presence of high levels of ras. However, in the mouse 10T½ cells system used in the present study, transfection of ras and p53 together did not produce cell lines that contained significantly higher ras protein, or cell lines that were significantly more malignant than lines obtained following transfection of ras alone (figures 18 and 22). This observation demonstrates that, in addition to the presence of a mutant form of p53, elevated expression of ras protein and increased malignancy in these cells also requires modification in the expression of at least one other activity. This finding indicates the importance of the myc gene in the ras/myc/p53 transfectants, which together with mutant p53 permits expression of higher levels of ras. Kohl and Ruley (1987) showed that very high levels of myc expression enabled low efficiency ras transformation of REF52 fibroblasts. Although an average increase in myc protein of about twofold was observed when ras/myc/p53 cell lines were compared to the less malignant ras/myc combination in the present study (figure 18), statistical analysis did not indicate that these differences were significant at the 95% confidence level, suggesting that a basal level of myc protein is sufficient for ras-mediated metastatic conversion, when combined with the presence of a mutant form of p53 protein.

Previous studies have implicated both myc and p53 in DNA replication and cell cycle progression (Waitz & Loidl, 1991; Michalovitz et al, 1990), and there are several potentially interesting regulatory similarities between these two proteins. For example, they have both been shown to cooperate with ras in the transformation of permissive cell lines (Eliyahu et al, 1984; Land et al, 1986; Ruley, 1987), and changes in expression of

p53 and myc genes have been found to correlate in some malignant lines, implying common regulatory control of these genes (Chenevix-Trench et al, 1990). Interestingly, some recent work suggests that overexpression of myc in co-transfection assays can influence the activity of the p53 promoter (Reisman et al, 1993). Furthermore, myc expression appears to be under the control of the tumor suppressor Rb gene (Pietenpol et al, 1990), and there are numerous reports that some DNA tumor viruses have evolved proteins that bind and inactivate the functions of both p53 and Rb (Bichoff et al, 1990; Wrede et al, 1991). Indeed, mutations within Rb and p53 genes are commonly found in many human malignancies, and loss of function of these tumor suppressor genes appears to be a critical event in malignant development (Knudson, 1993). In addition, both Rb and p53 proteins exist in several different phosphorylation states that appear to be important in the regulation of cell proliferation (Sturzbecher et al, 1990; Mihara et al, 1989). Although there are several possible explanations for the oncogene synergism described in this report, we would like to suggest that alterations in p53 and myc observed in the ras/myc/p53 cell lines play a critical role in allowing the overexpression of ras protein and the accompanying changes in ras-regulated gene expressions associated with malignant conversion (e.g. Denhardt et al, 1987; Schwarz et al, 1988, 1990; Gingras et al, 1990; Craig et al, 1990). The studies showing cell cycle regulatory connections between p53 and Rb, and the observation that myc expression is controlled through an Rb dependent pathway, suggest that alterations in myc and p53 may induce key changes that include both of these tumor suppressor dependent pathways of cell cycle regulation which are important in mechanisms of malignant transformation involving ras.

To gain insight into the biological mechanism responsible for metastatic conversion

by ras, myc, and mutant p53 we tested the importance of increased motility in cells transfected with these genes. We found that cells transformed by ras alone or by ras + p53 showed higher rates of invasion than control 10T½ cells, consistent with ras acting downstream of fibroblast growth factors. However, in ras transformed cells also containing the myc oncogene there was a significant decrease in invasive potential *in vitro*. This observation was also extended to the ras+myc+p53 transformants which show very high metastatic potentials *in vivo*. These observations suggest that motility *in vitro* does not always correlate with malignant behaviour and also suggests that myc can inhibit cellular motility. This is consistent with our observation that although increased motility was required for FGF induced malignancy, other alterations were likely to contribute to this complex phenotype.

3. Mechanism of Oncogene Cooperation Involving the myn Gene

The cooperative induction of metastasis by ras, myc, and mutant p53 is associated with elevated levels of ras suggesting that myc and p53 may cooperate to allow the cell to tolerate this alteration. Previous studies showed that the product of the max gene could also cooperate with myc in the transformation of cells by ras (Prendergast et al, 1991). The max protein can regulate myc function and this suggested the possibility that p53 may cooperate with myc by altering the level of max (Amati et al, 1993). Our next series of experiments were aimed at determining if max had a role in the cooperative induction of the metastatic phenotype. Our results showed that p53 was not capable of regulating max levels however several observations suggested that max plays an important role in

oncogene cooperation.

Cellular transformation by myc requires domains which allow it to activate transcription from specific DNA sequences (Zimmerman & Alt, 1990). The activation of transcription by myc, in turn, appears to be dependent upon its presence in a heterodimeric complex with the max protein (Amati et al, 1993). Given that max homodimerizes to form a protein complex which can bind to the myc binding element yet is unable to activate transcription, the transcriptional activity of myc target genes and myc induced transformation will likely be determined by the relative levels of both myc and max capable of dimerizing (Amin et al, 1993; Kretzner et al, 1992). The ability of myc to cooperate with ras in the transformation of fibroblasts also requires domains of myc which allow dimerization with max (Amati et al, 1993). This suggests that ras+myc cooperation might be influenced by the level of max expression, as max can influence the transcriptional activity of myc (Amati et al, 1993; Amin et al, 1993; Kretzner et al, 1992).

We found that myn is significantly overexpressed in cells transformed by various combinations of ras, myc, and p53 (Taylor et al, 1994). Similar increases in myn mRNA and protein levels were detected in cell lines transformed by ras alone, or in combination with myc, p53 or both myc and p53. Some variability in myn expression among the different clones was observed, as is expected if the regulation of this gene is modified by a variety of factors that are subject to clonal variation (figures 25,26). In total, the results suggest that myn is involved in the process of transformation and may have a specific function during tumor progression. This function may involve the ability to regulate c-myc which has been shown to be intimately linked to transformation by the ras

oncogene (Amati et al, 1993). As myn has other dimerization partners, including mad, mxi-1, L-myc and N-myc, it is likely that the regulation of c-myc function is not the only way in which myn is involved in cellular transformation (Blackwood & Eisenman, 1991; Ayer et al, 1993; Zervos et al, 1993; Wenzel et al, 1991).

One mechanism that could account for the increased levels of myn in ras transformed cells is that ras itself can regulate the production of myn. To test this, we employed a cell line which contains an inducible ras gene. We found that the induction of ras expression was associated with an increase in myn mRNA levels (figure 27, 28). As a test of the effect of increased myn on ras transformation, we cotransfected 10T½ cells with ras and myn and found that myn was capable of suppressing ras induced focus formation (figure 29). It has been estimated that nearly all of the myc protein present in the cell is in complex with myn (Blackwood et al, 1992). It has been suggested that myc is limiting for the formation of these heterodimers and therefore, fluctuations in myc levels are primarily important in regulating the transcriptional activity from myc binding elements (Blackwood et al, 1992). It might be argued that since myc is limiting for the formation of myc+myn heterodimers, a further increase in myn will be of little functional significance. However, we believe that this is not the case as we observed that myn overexpression is capable of suppressing ras induced focus formation. One possible explanation for these observations is linked to the ability of myn to form homodimers capable of suppressing transcription of myc target genes (Amin et al, 1993). Thus, ras overexpression may lead to the formation of myn homodimers which suppress the transcription of myc target genes and in so doing suppress ras transformation. Suppression of ras induced focus formation by increased myn is consistent with myn

being present in excess, and myc limiting during ras transformation. In contrast, if myn were limiting, the addition of exogenous myn would be expected to stimulate ras induced focus formation. This is due to the fact that exogenous myn would be expected to bind to the excess myc in the cell, forming heterodimers, which have been shown to be required for cooperative transformation with ras (Amati et al, 1993).

If myn homodimer formation is responsible for suppression of ras transformation, we can predict the effect of adding exogenous myc which is present during ras+myc cotransfection experiments. It is evident that an increase in myc expression will lead to the conversion of inhibitory myn homodimers to heterodimers of myc and myn, which activate transcription and lead to a stimulation of transformation (Craig et al, 1990).

4. The Role of Chromatin in Gene Expression and Malignancy

The link between the state of chromatin condensation and malignancy involves the regulation of transcription of genes involved in tumor cell phenotypes. Our studies on the level of myn in ras-transformed cells had demonstrated the importance of altered gene expression during malignant progression. We have also found that the gene for glyceraldehyde-3-phosphate dehydrogenase is upregulated in these cells lines suggesting that there may be many genes which exhibit altered patterns of expression in malignant cells. Other groups have found that the expression of metalloproteases, which degrade extracellular matrix proteins and facilitate invasion, is frequently elevated in malignant cells (McDonnell & Matrisian, 1990). One mechanism to explain altered expression is that these genes have acquired an altered chromatin structure. In fact, it has been found

that ras-transformed cells exhibit a more decondensed chromatin structure (Laitinen et al, 1990). Decondensation of chromatin may be induced by phosphorylation of the linker H1 histone (Roth & Allis, 1992). Furthermore, a well known H1 kinase, $p34^{cdc2}$ is activated by a constitutively active ras mutant (Daar et al, 1991; Barrett et al, 1990). Thus, during ras-induced malignancy, H1 kinases may become activated, leading to a more decondensed chromatin structure allowing alterations in gene expression.

We observed that in cells transformed by combinations of ras, myc and mutant p53, H1 histone exhibits between a 2 and 10 fold increase in its phosphorylation. This was observed by metabolically labelling H1 histones with ^{32}P and by using an antibody specific for hyperphosphorylated H1 (figures 33, 37). Two dimensional electrophoresis followed by immunoblotting with the same antibody suggests that the H1b subtype is more highly phosphorylated in these transformed cells (figure 42). This is consistent with ras activation leading to increased H1 phosphorylation and may explain the increase in chromatin condensation previously observed in ras-transformed cells. Furthermore, the increase in H1 phosphorylation may also be important in the increased transcription of genes observed in malignant ras-transformed cells.

Immunofluorescence analysis has shown that hyperphosphorylated H1 is localized in a punctate pattern in the nucleus of most cells. This distribution is very similar in normal untransformed cells and in transformed and metastatic cells. However, in mitotic cells, fluorescence is also detected in the cytoplasm, suggesting that during this stage of the cell cycle phosphorylated H1 is no longer associated with the DNA (figures 47,48,49). Furthermore, mitotic cells stain much more highly than interphase cells. This is consistent with previous reports of a burst of H1 phosphorylation which occurs during

M phase (Hohmann et al, 1976). These observations suggested that the increase in phosphorylation in transformed cells may be due to altered cell cycle distribution. This possibility has been eliminated by the determination of cell cycle distribution using flow cytometry. Comparison of the R-2 cell line with the 10T½ parental cell line showed that there was no statistical evidence for a difference in their cell cycle distributions. (figure 44). On the basis of the known cell cycle fluctuations in H1 phosphorylation, no difference in the level of phosphorylation between 10T½ and R-2 would be expected. However, the R-2 cell line showed a 4 fold increase in H1 phosphorylation. Thus, induction of increased H1 phosphorylation by ras is not secondary to alterations in the cell cycle distribution. Other cell lines, such as the metastatic RMP-6 cell line exhibited increased numbers of cells in M phase and fewer G1 cells. This alteration likely contributes to its extremely high levels of H1 phosphorylation, which is 20 fold higher than 10T½ cells and 4 fold higher than the R-2 cell line.

Although these cell cycle studies suggested that the observed increase in H1 phosphorylation is not only a result of altered cell cycle distribution, they do not indicate during which stage of the cell cycle the increase occurs. However, it is evident from the analysis of G1 arrested cells that at least part of the increase is observed in cells at this stage of the cell cycle (figures 45, 46). This is consistent with a role for H1 phosphorylation in the regulation of gene transcription, as this process is actively carried out in G1 (Marks et al, 1973).

Analysis of the subcellular localization of phosphorylated H1 revealed a striking difference between normal and malignant cells. We observed that some of the malignant cells showed an altered pattern of nuclear staining typified by multilobed structures (figure

50). To determine if this was due to altered subnuclear localization of phosphorylated H1 or to altered nuclear morphology we stained cells with the DNA dye, DAPI. This revealed that the nucleus of malignant cells had an altered morphology (figures 52, 53). The mechanism responsible for this is not known, however, some yeast cell cycle mutants show altered nuclear morphology due to entry into mitosis before the completion of DNA synthesis, a phenotype called mitotic catastrophe (Lundgren et al, 1991). This phenotype can also be induced in mammalian cells by the overexpression of the cell cycle regulatory protein kinase cdc2 (Heald et al, 1993). The malignant cells analyzed in the present study contain several oncogenes with demonstrated effects on the mammalian cell cycle. Therefore, the altered nuclear morphology may be due to aberrant mitotic events, possibly as a result of activation of cell cycle H1 kinases.

It was previously suggested that $p34^{cdc2}$ might be a candidate kinase responsible for the ras induced phosphorylation of H1. However, $p34^{cdc2}$ is only active during M phase at a time when genes are not actively transcribed (Nurse, 1990). More likely candidates for an H1 kinase that can alter transcription include members of the $p34^{cdc2}$ which are active during the G1-S transition. These include cdk2 and cdk3 which are good H1 kinases (van den Heuvel & Harlow, 1993). However, it is not known if these kinases are regulated by ras. Cdk4 is a G1 cdk whose regulatory cyclin subunits are growth factor inducible (Matsushime et al, 1992; 1991). It is not yet known if these growth factor responsive cyclins are ras responsive, however a link between the ras pathway of yeast and the yeast G1 cyclins has been observed (Hubler et al, 1993). If mammalian G1 cyclins are also found to be ras-responsive, the induction of H1 phosphorylation by cdk4 would be expected to be indirect, as this kinase cannot

phosphorylate H1 but can regulate cdk2 by competing for binding to an inhibitor of cdk2 called Kip1 (Polyak et al, 1994). It has been suggested that Kip1 acts as a link between the early G1 activation of cdk4 by growth factors and the late G1 activation of the cdk2 histone H1 kinase. The ras oncogene may short circuit the growth factor pathway leading to cdk4 activation and downstream activation of cdk2. The effect of this would be a rise in H1 phosphorylation required to decondense genes involved in metastatic dissemination.

The observed increase in H1 phosphorylation in cells transformed with combinations of ras, myc, and mutant p53 might explain previous results which show that chromatin from ras transformed cell lines is more sensitive to digestion with nucleases (Laitinen et al, 1990). Sensitivity to nucleases was attributed to a partial decondensation of the chromatin. Histone H1 is required for higher order chromatin packaging and phosphorylation of H1 might inhibit its ability to condense chromatin (Roth & Allis, 1992). Therefore, the basis for the previously described chromatin decondensation may involve a ras-induced increase in H1 phosphorylation. H1 histone is also a potent inhibitor of gene transcription, an activity that might also depend on its ability to condense chromatin (Laybourn & Kadonaga, 1991). This suggests that decondensation of chromatin caused by modification of the linker H1 histones might facilitate transcriptional alteration of gene expression. This is consistent with the observation that many genes show altered transcription in malignant ras-transformed cells, including genes encoding proteins required for metastatic dissemination (McDonnell & Matrisian, 1990). Therefore, the ras-induced increase in H1 phosphorylation may, through a decondensation of chromatin, lead to alterations in transcription of genes required to fulfil the metastatic cascade.

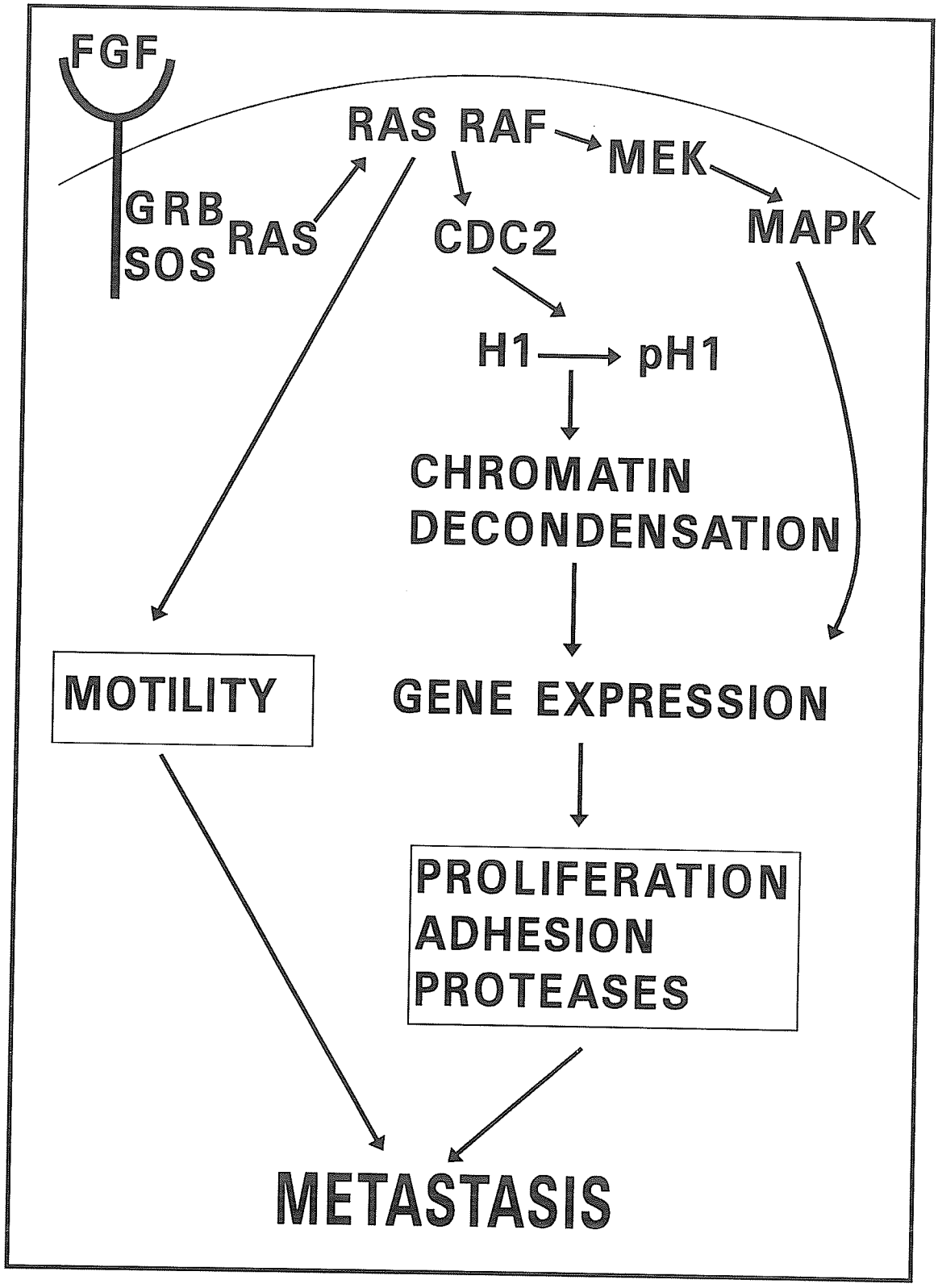
V. CONCLUSIONS

The studies described herein describe several aspects of the induction of the metastatic phenotype by growth factors and oncogenes (figure 54). Fibroblast growth factors including K-FGF and secreted bFGF can stimulate malignancy when overexpressed (Damen et al, 1991; Egan et al, 1990). bFGF has been found to stimulate cellular locomotion, a process important during the metastatic cascade (Taylor et al, 1993; Wright et al, 1993). We found that K-FGF was also capable of regulating cellular locomotion and that the induction of locomotion by fibroblast growth factors is important in their ability to regulate the metastatic phenotype. The importance of growth factor induced locomotion in the metastatic phenotype was further shown by the ability of antisense oligonucleotides to $TGF\beta_1$ to inhibit cellular locomotion and metastatic dissemination.

Fibroblast growth factors induce a biological response by stimulating signal transduction pathways, one of which depends on the ras proto-oncogene (Kremer et al, 1991, figure 54). We found that consistent with its position downstream of FGF's, ras was capable of stimulating cellular locomotion. However, ras was not efficient at inducing the metastatic phenotype and we found that alterations in myc and mutant p53 can cooperate with ras to produce malignant cells. Although cellular locomotion is correlated with malignancy, our studies suggest that it is not sufficient on its own and other alterations may be required. This is consistent with the complexity of the metastatic cascade and we predict that in some circumstances, other factors such as immune recognition will be as important as cellular motility in allowing a cell to successfully

FIGURE 54.

Induction of the metastatic phenotype by growth factors and oncogenes. Fibroblast growth factors can affect cellular motility by stimulating a ras-dependent signal transduction pathway. Ras also stimulates motility, and both FGF and ras can induce the metastatic phenotype. Ras stimulates a kinase cascade including raf, mek, and mapk which regulate gene transcription. Part of the mechanism of altered gene transcription in metastatic ras-transformed cells may involve alterations in chromatin structure which result from a ras-induced increase in H1 histone phosphorylation. Increased motility together with altered expression of genes which regulate cellular proliferation, adhesion and proteolytic destruction of the extracellular matrix, conspire to allow metastatic dissemination.


FGF
RAS
RAF
MEK
GRB
SOS
RAS
CDC2
MAPK
H1
pH1
CHROMATIN
DECONDENSATION
MOTILITY
GENE EXPRESSION
PROLIFERATION
ADHESION
PROTEASES
METASTASIS

complete the metastatic cascade (Miller, 1993). For example, we observed that myc was capable of inhibiting cellular locomotion even in cells that were highly malignant. Thus, cellular locomotion *in vitro* does not always correlate with metastatic dissemination despite its importance in growth factor induced malignancy.

The cooperative induction of metastasis by ras, myc and p53 led us to analyze the molecular mechanism responsible for this observation. We found that the myn gene was overexpressed in cells containing a mutant ras allele. We also found that myn was capable of suppressing ras transformation, suggesting an integral role for myn in ras transformation. On the basis of these studies we have proposed that the general inability of ras to transform fibroblasts on its own is due to the induction of myn, which forms homodimers capable of suppressing ras transformation. This suppression is alleviated by exogenous myc, which converts the inhibitory myn homodimers to heterodimers of myn and myc. These heterodimers no longer suppress transformation and likely contribute to focus formation by activating the transcription of genes responsible for this process.

In addition to the myn gene, we observed that the gene encoding GAPDH was overexpressed in transformed and metastatic ras-transformed fibrosarcoma cells. We have begun to investigate the role of H1 histone phosphorylation in altering chromatin structure and facilitating altered gene transcription in metastatic cells. Our studies have shown that H1 histone shows much higher levels of phosphorylation in ras-transformed cells. This may explain an earlier observation that ras-transformed cells show more decondensed chromatin (Laitinen et al, 1990). H1-dependent chromatin decondensation may facilitate gene transcription by rendering a particular promotor more accessible to transcription factors. These studies have also shown that malignant cells show a striking

alteration in their nuclear morphology typified by multiple lobes. This is reminiscent of a phenomenon called mitotic catastrophe which can be induced in mammalian cells by the acute, inappropriate activation of cell cycle regulatory protein kinases (Heald et al, 1993). Some of these cyclin dependent kinases have been shown to be major physiological H1 kinases. Therefore, if cdk's are the target of ras, they might lead not only to increased H1 phosphorylation, but also to the occasional mitotic catastrophe. The elucidation of the ras responsive H1 kinase will await future experimentation.

The molecular biology of the metastatic cascade involves many different cellular aspects, due to the complexity of the process of metastasis. Our observations describing the importance of growth factor induced motility, oncogene cooperation, altered gene expression and histone modification contribute to the understanding of the metastatic phenotype. The alleviation of cancer suffering should be possible by interfering with the mechanisms that lead to the widespread dissemination of tumor cells. Rational approaches to stop metastatic spread will depend on a thorough understanding of these mechanisms.

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