THE UNIVERSITY OF MANITOBA

BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF ASPECTS OF TUMOR CELL PROGRESSION

by

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Voor mijn Vader en Moeder, van harte bedankt

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Remember Success is meeting the new challenge beyond every acheivement

ABSTRACT

Tumor progression to the metastatic phenotype is defined as a dynamic process through which tumor cells gradually acquire new advantageous growth characteristics. Two schools of thought exist as to how tumor cells obtain the necessary biochemical characteristics needed to develop the metastatic phenotype: (i) through clonal evolution and genetic instability (increased spontaneous point mutations and gene amplification) or (ii) through the pleiotrophic action of a small number of key oncogene products. The work presented in this thesis was undertaken to evaluate specific hypothesis which attempt to explain aspects of tumor cell progression.

The hypothesis that genetic instability underlies the ability of tumor cell populations to produce metastatic variants was examined using a mutator (thy-49) and two amplificator mutants (YMP1 and YMP7). These lines and their respective normal counterparts were used to generate 10 parallel clones which were injected intravenously into nu/nu mice. No significant differences were observed in the rate of metastatic variants produced for each mutant as compared to the normal cells.

Amplification of several proto-oncogenes has been implicated as an advanced step in the emergence of highly malignant tumors. The role of amplification of a specific proto-oncogene was examined at the molecular level to determine whether the number of gene copies

was important in the process of tumor progression to the metastatic phenotype. The k-fgf proto-oncogene was linked to the dihydrofolate reductase gene, transfected into NIH3T3 mouse fibroblasts and amplification selected for in the presence of methotrexate. Cells containing amplification of k-fgf and dhfr were isolated. It was observed that cell lines containing increased k-fgf gene copies, elevated k-fgf message and protein levels were both tumorigenic and highly metastatic. Therefore, gene amplification of k-fgf can operate as a mechanism in converting non-tumorigenic cells to the full metastatic phenotype.

The process of metastasis requires cells to acquire motility characteristics which provide the ability to infiltrate into distant organs by passing through the basement membrane and endothelium, evading the host's immune system. Several investigations have shown changes in various cell surface properties including changes in the carbohydrate moieties that correlate with this process. Glycosylation inhibitor and lectin binding studies were done on highly metastatic and low metastatic clones of a 10T½ ras transformed mouse fibroblast line, NR4. An inverse correlation observed between soybean agglutinin binding and increasing metastatic potential lead to the identification of two specific glycoproteins, gp80 and gp48 on the surface of low metastatic cells that were missing from the surface of the highly metastatic cells.

In summary, from these studies, it was observed that (a) the rate of generation of metastatic variants is not increased in mutants with increased rates of spontaneous mutation or gene amplification,

(b) the concentration of the kFGF protein is critical in the determination of the potential of cells containing this amplified gene to be able to metastasize and (c) changes in the carbohydrate moieties of specific cell surface glycoproteins are important for the ability of ras transformed murine fibroblasts to aquire the ability to spread to a secondary site.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

∝-MEM	-	alpha minimal essential medium
BHK	-	Baby hamster kidney
bp	-	base pairs
BSA	-	bovine serum albumin
CAT	-	chloramphenicol acetyl transferase
CDP	-	cytosine 5'-diphosphate
Ci	-	Curie
CHO	-	Chinese hamster ovary
Con A	-	Concanavalin A
cpm	-	counts per minute
CTP	-	cytosine 5'-triphosphate
dA	-	2'-deoxyadenosine
dATP	-	2'-deoxyadenosine 5'-triphosphate
dC	-	2'-deoxycytosine
dCDP	-	2'-deoxycytosine 5'-diphosphate
dCTP	-	2'-deoxycytosine 5'-triphosphate
dG	-	deoxyguanosine
dGTP	-	2'-deoxyguanosine 5'-triphophate
dhfr	-	dihydrfolate reductase
dFCS	-	dialized fetal calf serum
dpm	-	disintigrations per minute
dTTP	-	2'-deoxythymidine 5'-triphosphate
ER	-	endoplasmic reticulum
FITC	-	flourescein isothiocyanante

FCS	-	fetal calf serum
fuc	-	fucose
G	-	guanine
GA	-	golgi apparatus
gal	-	galactose
Gal-T	-	galactosyl transferase
GalNAc	-	N-acetyl galactosamine
glc	-	glucose
GlcNAc	-	N-acetyl glucosamine
GlcNAc-T	-	N-acetylglucosylaminytransferase
gp	-	glycoprotein of molecular weight (kD)
GTP	-	guanosine 5'-triphosphate
H	-	hypoxanthine
HBV	-	hepatisis B virus
HPLC	-	high pressure liquid chromatography
HPGRT	-	hypoxanthine guanine phosphoribosyltransferase
IPTG	-	isopropyl β-D-thiogalactoside
kb	-	kilobase
kD	-	kilodalton
k-fgf	-	Kaposi-fibroblast growth factor
min	-	minute
MOPS	-	morpholinopropanesulfonic acid
MTX	-	methotrexate
NeuAc	-	N-acetylneuraminic acid
NP40	-	nonidet P-40
PBS	-	phosphate buffered saline

PCA	-	perchloric acid
Ph^1	-	philadelphia chromosome
PMSF	-	phenylmethylsulfonylflouride
PNA	-	peanut agglutinin
RPE	-	relative plating efficiency
SBA	-	soybean agglutinin
SDS	-	sodium dodecyl sulfate
TBE	-	Tris-borate EDTA
TCA	-	trichloroacetic acid
TE	-	Tris-EDTA
v	-	volume
w	-	weight
WGA	-	wheat germ agglutinin

INTRODUCTION

The process of metastasis is the most life threatening aspect of cancer. It is the ability of cells to travel to a secondary site that results in the inability to ensure the survival of a patient. A better understanding of the mechanisms underlying metastasis as well as the biological characteristics of metastatic cells should eventually lead to better treatment procedures. The work presented in this thesis was undertaken to evaluate specific hypothesis which attempt to explain aspects of tumor cell progression.

The hypothesis that genetic instability underlies the ability of a tumor cell population to produce metastatic variants was examined using mutator (thy-49) and amplificator (YMP1 and YMP7) mutants. To determine the role of genetic instability in tumor progression, the rate at which these mutants produced metastatic variants was calculated following cloning and examination of experimental lung metastases.

The role of amplification of a specific proto-oncogene was examined at the molecular level to determine whether the number of gene copies was important in the process of tumor progression to the metastatic phenotype. This was examined using the proto-oncogene k-fgf, which is frequently found amplified in a number of human tumors. The k-fgf proto-oncogene was linked to the dihydrofolate reductase gene, transfected into NIH3T3 mouse fibroblasts and amplification was selected in the presence of methotrexate. Cells containing amplification of k-fgf and dhfr were then isolated, and

the ability of these cells to produce tumors in nu/nu mice examined to determine the importance of this kind of mechanism in the dissemination of metastatic disease.

The carbohydrate structures on cell surface glycoproteins were examined on a metastatic 10T½ mouse fibroblast line to test the hypothesis that alterations in cell surface glycoproteins play a role in metastatic progression. With the use of glycosylation inhibitors and lectin binding studies, changes in cell surface oligosaccarides and important glycoproteins likely associated with the metastatic phenotype were identified.

In summary, the objectives of this study were the following: (i) determine the role of genetic instability in generating metastatic variants in a tumor cell population, (ii) examine the ability of an amplified proto-oncogene in determining the tumorigenic and metastatic potential of a normal murine fibroblast line and (iii) identify carbohydrate changes and cell surface glycoproteins with altered oligosaccharide structure in a metastatic murine fibroblast line.

HISTORICAL

Cancer is viewed as a fundemental disorder of cellular growth control: a disorder that arises in rare cells through changes in genes or their expression. The discovery 140 years ago, by the German microscopist Johannes Mueller, that cancer is made up of cells (as reviewed by Cairns, 1981) initiated the search for changes which would help pinpoint the specific differences between normal and cancer cells. Cancer cells differ from the other large categories of diseases since cancer can be caused by infection, occurs more often with advancing age, and sometimes depends on inherited mutation (as reviewed by Cairns, 1981). Cancer cells appear to have a growth advantage over surrounding cells which are usually inherited by daughter cells as a stable genetic trait. Rapid advances in biological technology, particularly in cell and molecular biology, has in the past decade resulted in identification of many of the genes affected in cancer cells and has provided some information about the nature of the changes.

Most neoplasms can be divided into three major groups: benign tumors that are noninvasive and nonmetastatic, invasive tumors that are nonmetastatic, and metastatic tumors (Franks, 1986). Benign tumors, like normal cells, are well differentiated, grow slowly and do not spread to distant sites. They may arise in any tissue, proliferate locally and cause damage by local pressure or obstruction. Malignant tumors on the other hand, are undifferentiated and

consist of a large percentage of dividing cells. Many of these contain abnormal chromosomes, exhibit varying degrees of anaplasia and more importantly, have the specific capacity to invade surrounding stroma and vessels and relocate at ectopic sites (Nicolson and Milas, 1984). Histologically, cancer cells can often be recognized as derivatives of certain cell lineages on the basis of their initial location, retention of differentiated functions, morphology, or intercellular arrangments (Pitot, 1978). However, the expansion of cell number, the invasion of surrounding stroma and vessels, the relocation at a secondary site (metastasis), and the atypical appearance of cells indicate fundemental alterations in control of cell growth, shape and function. Clinically, cancers usually behave as an irreversible disorder and the vast majority of them advance to more malignant states.

1. TUMOR CELL PROGRESSION

The process of tumor progression refers to the later stages of tumor cell evolution. Tumors do not come into being with all their characteristics already developed. Tumor progression is defined as a dynamic process whereby there is gradual acquisition of new characteristics as the tumor developes (Foulds, 1954). Tumor cell populations can thus be seen in constant flux, with new subpopulations continually replacing older ones by virtue of the display of one or another novel, advantageous phenotype. The general trend of tumor progression is to go from bad to worse and there is movement

towards a more aggressive, invasive phenotype (Liotta and Hart, 1982). Such change has formal similarities with models in which species change progressively through the processe of evolution. According to such thinking, genetic and phenotypic variability in tumor cell populations makes possible the appearance of variants that display phenotypes conferring advantageous traits such as increased growth rates, decreased dependency on growth factor stimulation, increased tolerance to anoxia, increased ability to evade immunological defences, and increased invasive or metastatic ability (Liotta and Hart, 1982).

1.1 Tumor Cell Heterogenity for Metastatsis

Not all cells in a tumor are identical. There is a range in the tumor cell population of cells expressing many different characteristics. Cells in a tumor may show variations involving growth rate, morphology, marker enzymes, hormone receptors and invasiveness and metastasic abilities (Heppner, 1983). This diversity is a consequence of tumor progression which is accepted by most pathologists, biologists and clinical oncologists. Experimental evidence with transplantable rodent tumors developed the idea that not only are metastases derived from pre-existing subpopulations in the primary tumor but also, metastatis is an inefficient process (Klein, 1955). The majority of tumor cells released into the circulation do not give rise to secondary tumors. The process of metastatic spread appears to depend on an interplay between host factors and intrinsic characteristics of the tumor cells. Only a small proportion of cells

in a primary neoplasm can enter the circulation (Willis, 1972). Only a few cells will survive in the circulation, evade the immune system, the action of natural killer (NK) cells (Hanna, 1982), arrest in the capillary bed, and undergo extravasation (Fidler, 1970). The fact that only a few circulating tumor cells survive to form metastases raises the question of whether their survival is random or nonrandom. The possiblility that cells with high metastatic potential can be isolated from a heterogenous parental tumor population by selection procedures was first suggested by Kosh (1939) using in vivo enrichment of metastatic subpopulations in rodents. Subsequently, investigations of a large number of tumor systems have shown that harvesting of cells with metastatic abilities during succesive in vivo selections, yields cell populations with a greater metastatic potential than cells from the original population (Fidler 1973, Fidler et al. 1976, Tao and Burger 1977, Suzuki et al. 1978, Brunson and Nicolson 1978, Briles and Kornfeld 1978, Fidler 1978, Talmadge 1983, Egan et al. 1987, Gingras et al. 1990). Since this change is relatively stable and heritable, it is believed that production of metastatic variants is the result of selection of mutations and not adaptation. The process of metastases remains inefficient because of the random destruction and elimination of circulating tumor cells. However, there is a selective aspect of the process since metastatic variants can be isolated from heterogeneous populations by selection techniques (Egan et al. 1987b, Buckley and Carlsen 1988, Laferte' and Dennis 1988, Gingras et al. 1990).

The first direct experimental evidence that malignant neoplasms

do in fact contain subpopulation of cells with differing metastatic capabilities was demonstrated by Fidler and Kripke (1977) using the B16 mouse melanoma system. The idea behind the classical fluctuation test devised by Luria and Delbruck (1943), which distinguishs between selection and adaptation in the origin of bacterial mutants, was used. In short, Fidler and Kripke (1977) divided a suspension of B16 melanoma into two aliquots: one portion was injected intravenously into syngenic mice, the other portion was used to produce clones, which were then injected into groups of syngenic mice. If the tumor had been populated by cells of uniform metastatic potential, then the cloned sublines would produce approximately the same number of metastases in different animals. However, the cloned sublines differed markedly in their metastatic potential. There was also considerable variation in the number and sites of extrapulmonary metastases among the clones. From these experiments it was concluded that the parent tumor was heterogenous and that the highly metastatic tumor cell variants pre-existed in the parental population. Similar heterogenity in metastatic potential has now been demonstrated with many different murine tumors (Suzuki and Withers 1978, Nicolson et al. 1978, Brunson and Nicolson 1978, Kripe et al. 1978, Talmadge et al. 1979, Fidler and Kripke 1980, Fidler and Hart 1982, Poste 1982, Heppner and Miller 1983, Damen et al. 1989).

1.2 Mechanisms of Cellular Diversity in Malignant Neoplasms

Considerable biochemical, cytogenic, molecular genetic and immunological evidence now indicates that most neoplasms arise from a single altered clone (Nowell 1976, Fialkow 1979, Arnold et al. 1983, Nowell 1986). The biological events of tumor porgression result in the sequential selection of variant subpopulations within this clone. It has also been hypothesized that clonal evolution of the neoplasm might result from enhanced genetic instability, which increases the probability of further genetic alteration and their subsequent selection (Cairns 1975, Klien 1979, Sager 1985). Mutants that have a selective growth advantage expand to become predominant subpopulations within the neoplasm. The continued presence of multiple subpopulations within the tumor provides the basis for tumor cell heterogeneity.

The heterogenous nature of tumors and their ability to evolve with time was studied extensively by Foulds (1975). He observed that tumors changed gradually and independently and eventually gained autonomy from host controls. Foulds concluded that tumor evolution was the result of permanent, irreversible changes that occurred individually in each tumor. To explain this evolution and diversity Nowell suggested that the transition from normal to transformed cell carried with it the acquisition of inherited genetic instability (Nowell 1976, 1986). This genetic instability, he suggested, allowed transformed cells to mutate at a higher rate than normal cells so that new variants were being produced continuously. Many of these

variants would be eliminated by metabolic or immunological mechanisms but some of these variants would have selective growth advantages, and these clones would grow to dominate the tumor population. According to Nowell (1976, 1986) sequential selection over time would lead to the emergence of sublines which would be increasingly abnormal both genetically and biologically.

Genetic alterations occuring in progressing tumors could range from point mutations to gross abberations such as loss or gain of complete chromosomes. Most of the support for the hypothesis that neoplasms display enhanced genetic instability comes from cytogenetic and genetic data (Sandberg 1980, Nowell 1982, as described in Otto et al. 1989). Malignant solid human tumors commonly show aneuploidy and other chromosomal abberations. A classic example of chromosomal alterations associated with tumor progression is chronic granulocytic leukemia, where the Philadelphia (Ph^1) chromosome was discovered (as described by Rowley, 1980). Advanced disease is characterized by the original 9;22 chromosomal translocation that produces the Ph^1 chromosome and additional cytogenetic changes. In addition, a small segment of the population with inherited chromosomal fragility syndromes, Blooms syndrome and xeroderma pigmentosum for example, (where there is a defect in DNA repair or some other aspect of DNA synthesis) exhibit a high frequency incidence of neoplasia (Hecht and McCaw 1977, German 1983, Langlois et al. 1989).

The mechanisms by which cells could become genetically unstable include both an increased frequency of spontaneous point mutation, through mutations in enzymes controlling the substrates for DNA

synthesis, DNA synthesis itself, and DNA repair (mutants of DNA polymerase or ribonucleotide reductase for example, Weinberg et al. 1981, Liu et al. 1983, Wright et al. 1989, Wright 1989) and increased gene amplification (Schimke et al. 1986, Stark et al. 1989, Wright et al. 1990a). Nowells hypothesis predicts that increasing evolution and progression towards malignancy will be accompanied by increased genetic instability of the malignant cells (Nowell 1982, 1986). To test this experimentally many studies have determined the rate at which metastatic variants, when compared to their nonmetastatic counterparts, exhibit point mutations, (as measured by resistance to drugs such as ouabain and 6-thioguanine), using the Luria-Delbruck fluctuation analysis (Cifone and Fidler 1981, Elmore et al. 1983, Kendal and Frost 1986, Seshardi et al. 1987, Kaden et al. 1989, Tagger et al. 1989). The overall result of these investigation is unclear. Some investigators have shown that metastatic cells exhibit a higher frequency of spontaneous point mutations (Cifone and Fidler 1981, Seshardi et al. 1987) while other have not (Elmore et al. 1983, Yammashina and Heppner 1985, Kendal et al. 1987, Chambers et al. 1988, Tagger et al. 1989, Damen et al. 1989). In studies examining the frequency or rate of drug resistance due to gene amplification, some have shown that metastatic cells amplifiy at an increased rate over their nonmetastatic counterparts (Cillo et al. 1987, Gitelman et al. 1987, Otto et al. 1989, Tlsty et al. 1989). Other studies, however, have not seen this correlation (Chambers et al. 1988, Damen et al. 1989). Although genotypic alterations appear to be extremely important in modifying the behaviour and stability of tumor cells,

the frequency or rate of their appearence is often inconsistent with the hypothesis that genetic instability is required for progression to the metastatic phenotype and therefore the problem remains an important unresolved question in tumor biology.

2. MOLECULAR BASIS OF CANCER

In the past decade, the identity and nature of gene alterations in cancer cells, has been elucidated because of the potent techniques of molecular biology. The results of such studies have produced some unifying conclusions about the molecular basis of cancer. For example, eukaryotic genomes contain a substantial list of genes that may participate in neoplasia following mutation (Bishop 1987, Greenberg et al. 1989, Egan et al. 1990, Wright et al. 1990b). Most of these genes, often refered to as proto-oncogenes, have been shown either to control or be involved in, normal cell growth and differentiation. Alterations such as point mutations, insertions, deletions, gene amplifications and chromosomal translocations, convert these genes into active oncogenes by affecting either their expression or the nature of their gene product (Bishop 1987). Many of these cancer-inducing alterations behave in a dominant manner (Reddy et al 1988), while others appear recessive (Sager 1989). In summary, combinations of alterations in oncogenes and supressor genes (anti-oncogenes) are believed to be required to convert a normal cell into a highly invasive metastatic tumor (Vogelstein et al. 1989, Baker et al. 1989, Stanbridge 1990, Fearon et al 1990).

2.1 Discovery of Oncogenes and Proto-oncogenes

An oncogene can be defined as a viral or cellular gene that can induce one or more characteristics of neoplastic transformation when introduced, (either alone or in combination with another gene) into the appropriate cell type (Bishop, 1987). Normal cellular genes that can be converted to active oncogenes by mutation are called proto-oncogenes. This definition of oncogenes applys to dominantly acting genes. Tumor supressor genes are also involved in neoplastic transformation but only when they fail to function (Huang et al. 1988, Sager 1989). Attempts to identify cellular oncogenes have yielded information about the activation of these genes in the neoplastic cell. Many have been identified in neoplasms where viral insertional mutagenesis, gene translocation, and gene amplification appear to be the mechanism behind the activation and resulting transformation (Varmus 1984, Bishop 1987, Wright et al. 1990a). Other oncogenes, mostly those activated by mutations (such as H-ras, Reddy et al. 1982) have been indentified by gene transfer methods, or by structural or functional similarities to known proto-oncogenes (Varmus, 1984).

It was known that particular avian and murine viruses, which contained only the viral genes required for replication, could cause a variety of diseases including neoplasms such as lymphomas and erythroleukemias (Teich et al. 1982, 1985). Close examination of viral integration in bursal lymphomas for example, demonstrated that

in a large majority of the lymphomas the proviral DNA had intergrated next to the c-myc locus (Hayward et al. 1981). The c-myc gene was first identified as the homolog of v-myc, an oncogene identified in several strains of avian retroviruses which could cause acute myeloid leukemias (Roussel et al. 1979, Shieness and Bishop 1979, Sheiness et al. 1980). The interpretation of this and other similar findings was that infection by a virus is random, but the rare cell that aquires a provirus in the c-myc locus (where the expression of the proto-oncogene is changed) has some acquired growth advantage. This in cooperation with other addititonal mutations was believed to contribute to neoplastic growth (Fung et al. 1981, Payne et al. 1981, Payne et al. 1982).

Genomic rearrangments is a common site in the karoytypes of human cancer cells; for example the long arm of chromosome 8 can be translocated to chromosome 14, 2 or 22 (Dalla-Favera et al. 1982, Taub et al. 1982, 1984, Battey et al. 1983, Groffen et al. 1984). The myc locus also maps to the same position on chromosome 8, and closer examination revealed that deregulation of myc through another mechanism, translocation, was important in neoplastic growth (Leder et al. 1984). Mechanistically, translocations seem to be important in the activation of oncogenes because they usually result in removing the gene form its normal regulatory regions, thus leaving it under the controls of a new chromsomal milieu.

Another type of chromosomal abnormality seen in tumors is related to gene amplification and generally involves huge DNA segments spanning many genes (Stark 1986). The availability of v-onc

probes showed that various tumors contained onc gene amplifications. Amplification of proto-oncogenes in human tumors for example, that are recurrent abnormalities in particular types of tumors, are myc, erb, hst, int2 and neu (Yamamoto et al. 1986, Wong et al. 1987, Yoshida et al. 1988, Zhou et al. 1988, Yokota et al. 1986, 1988, Slamon et al. 1987, van de Vijver et al. 1987, 1988). In particular neu amplification in breast carcinoma reflects tumor progression towards the more advanced malignant state (Slamon et al. 1987, Slamon et al. 1989, Marx 1989, Yarden and Weinberg 1989). In general amplified genes give rise to more mRNA and more onco-protein. The increase in gene product is usually proportional to the magnitude of amplification and the phenotypic effects have thus been attributed to the abundance of gene product.

Although many other cellular genes have been implicated in oncogenesis by virtue of genomic rearrangments, insertion mutations, translocations and gene amplification, the real functional tests for the isolation of oncogenes were based on the transfer of a gene in the form of naked DNA from a transformed to a nontransformed cell. Nontransformed cells that became transformed following transfection of transformed cellular DNA were analyzed and recipient genes identified. Many of these genes were found to be related to already known retroviral oncogenes implicated in the transformed phenotype (Hill and Hillova 1972, Shih et al. 1979, Reddy et al. 1982). Many cellular oncogenes, including ras, were identified using this approach and it is still being used (Der et al. 1982, Parada et al. 1982, Santos et al. 1982, Varmus et al. 1984, 1987, Barbacid 1987).

Following the discovery of oncogenes and the mechanism of their activation subsequent experiments were performed to determine the normal functions of these genes and how changes in their normal functions where related to neoplastic progression.

2.2 Biochemical Properties of Oncoproteins

Based on information derived from the biochemical properties of proteins encoded by oncogenes, and a combination of procedures which have determined the size, cellular location, and chemical modifications of these proteins, most of the known oncogenes and proto-oncogenes can be grouped into four functional families: protein kinases, growth factors, signal transducers, and transcription factors.

Proir to the discovery of oncogenes it was thought that normal protein phosphorylation occured only on serine and threonine residues (Hunter and Cooper, 1985). In anaylzing oncogenes that where shown to have phophorylation activity it became apparent that many were tyrosine kinases (Eckhart et al. 1979, Smith et al. 1979, Hunter and Setton 1980, Witte et al. 1980). Recently, some serine/threonine kinases which are oncogenes have also been identified (Maxwell and Arlinghaus 1985, Schultz et al. 1988). Some of the tyrosine kinases were found to be transmembrane proteins (erb and fms) while others showed membrane affiliation without transmembraneous or extracellular domains (for example, abl, fps/fes, src), (Downward et al. 1984, Sherr et al. 1985, Konopka and Witte 1985, Young and Martin 1984, Hamaguchi and Hanafusa, 1987). The family of protein tyrosine

kinases have been shown to contain highly conserved catalytic domains, and many of the transmembrane proteins are receptors for secreted polypeptide growth factors (Hanks et al. 1988). Much effort is being directed towards the identification and determination of the functional significance of protein tyrosine kinases (Radke and Martin 1979, Hunter and Cooper 1985, Hunter 1987). Many proteins have been identified to contain phosphotyrosine residues following transfection of a oncogenic tyrosine kinase, but the physiological relevence of these are largely unknown (Setton et al. 1980, Lipsich et al. 1982, Morrison et al. 1988).

Unregulated production of growth factors or production of mutant growth factors, affects cells by either autocrine stimulation, or by a paracrine or endocrine mechanism (Sporn and Roberts 1985). Since the halmark of a neoplastic cell is its uncontrolled growth, one might suspect that many oncogenes would be growth factors and growth factor receptors which are responsible for controlling normal growth. Many oncogenes have been identified which are aberant growth factor receptors containing tyrosine phosporylation properties, (as discussed above) but relatively few polypeptide growth factors have been shown to be oncogenic. One example is the v-sis oncogene which encodes the β chain of the platlet derived growth factor PDGF (Doolittle et al. 1983, Waterfield et al. 1983). Another recently implicated oncogenic growth factor is kFGF, which is not normally expressed in adult tissue, and has been shown to be expressed following amplification in a number of malignant human tumors (Ali et al. 1988, Theillet et al. 1989). K-fgf also transforms cells

following transfection into rodent fibroblasts (Delli-Bovi et al. 1988, Wellstein et al. 1990). Other growth factors have been implicated as oncoproteins but the mechanisms by which these factors contribute to transformation is still largely a matter of debate (Rosenthal et al. 1986, Stern et al. 1987, Rogelji et al. 1988).

The most common oncogene isolated by the gene transfer method, ras, which has been shown to be mutated in many different human neoplasms, is believed to fuction normally as a signal transducer (Barbacid, 1987, Greenberg et al. 1989, Wright et al. 1990b). Biochemical studies have shown that the ras protein has the capacity to bind GTP, and belongs to the family of G proteins known to transduce signals from transmembrane receptors for catecholamines to activate adenylate cyclase. This process stimulates production of cAMP which activates the cAMP-dependent kinase (Hurley et al. 1984). Although ras exhibits GTPase activity, and is located at the plasma membrane, the ras protein in mammalian cells has yet to be placed directly in any known signal pathway. Another important signal transduction pathway belived to be involved in tumorigenicity, is the phosphatidylinositol pathway (Berridge and Irvine, 1984). Some oncogenic protein kinases have been associated with the regulation of this pathway, and it has been claimed that others augment its activity (Courtneidge and Heber 1987, Kaplan et al. 1987, Lacal et al. 1987, Irving and Exton 1987) .

The oncogenes that have been shown to exert their effects due to biological activities within the nucleas were initially defined as nuclear oncogenes. Attribution of the biological events responsible

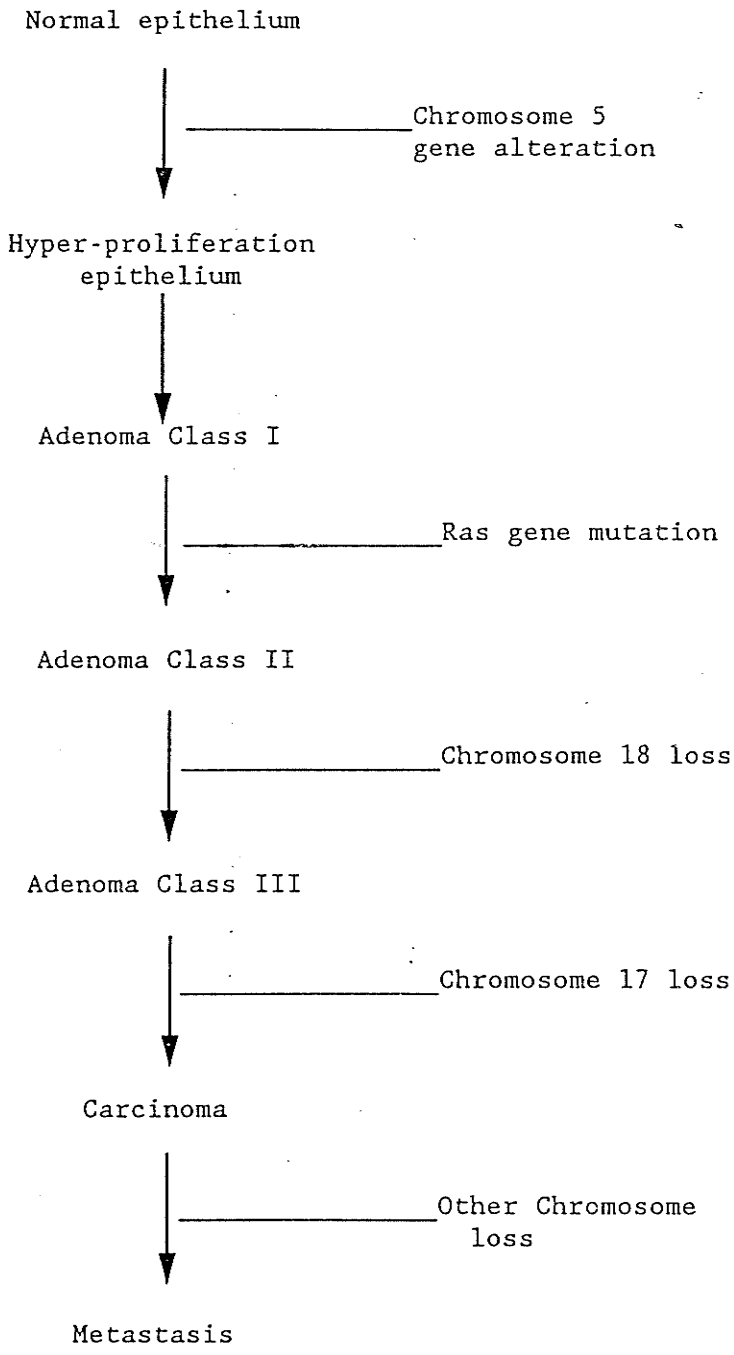
for oncogenic activity was difficult to analyze until the rapid advances in the study of eukaryotic transcription factors. A crucial stage in this area was the discovery of DNA binding domains in oncogenes such as c-jun and c-fos (Sambucetti and Curran 1986, Bohmann et al. 1987, Renz et al 1987). The similarity of these domains to the yeast transcriptional factors is sheading light on the oncogenic activity of these proteins (Struhl 1987, Vogt et al. 1987). Evidence also suggests that many of the nuclear oncogenes are important activators of gene expression (Setoyama et al. 1986, Lillie et al. 1986, Landschulz et al. 1988, Evans 1988).

It is becoming increasingly clear that there are multiple ways in which genes relevant to oncogenesis may be discovered, and, that there are various molecular mechanisms by which such genes may be activated to participate in oncogenic conversion. In most cases it is not possible to be specific either about the number or kinds of oncogenes required to convert a particular cell into a cancer cell. The biochemical events that are crucial for transformation are also not well understood. Recent work in Vogelstein's laboratory however, is beginning to show that there are specific steps and specific oncogenes involved as seen in colon cancer (Fearon et al. 1990). Figure 1 points out the general steps worked out by Vogelstein in the progression of colon cancer (Marx 1989). With more work in this area other cancers may also fit into such a scheme.

Figure 1

Sequence of events that characterize the various stages of colon carcinoma.

adapted from J.Marx, Research News section of Science 246:1386, 1989.


Normal epithelium
Chromosome 5 gene alteration
Hyper-proliferation epithelium
Adenoma Class I
Ras gene mutation
Adenoma Class II
Chromosome 18 loss
Adenoma Class III
Chromosome 17 loss
Carcinoma
Other Chromosome loss
Metastasis

2.3 Oncogenes and Metastasis

An even bigger and more life-threatening question is how oncogenes could be involved in tumor progression to the metastaticphenotype. Some studies have shown that one or two oncogenes, depending on the recipient cell type, can establish a metastatic cell. Metastases, however is a complex multistep process, requiring many changes beyond loss of growth control (Liotta 1986, Greenberg et al. 1989, Weston et al. 1989, Baker et al. 1989, Marx 1989). Since metastasis is a relatively frequent event (most tumors eventually progress to become metastatic), it can be argued that many properties of the metastatic cell may be regulated through the concerted action of a small number of key gene products, most probably oncoproteins (Greenberg et al. 1989, Wright et al. 1990b).

The spread of tumor cells throughout the body, which often occurs as a late step in tumor progression, represents a complex process of multiple distinct steps. A three-step hypothesis of invasion has been proposed to explain the sequence of biochemical events during tumor cell invasion through the extracellular matrix (Liotta 1986). The first step requires tumor attachment to the extracellular matrix. This has been shown to be mediated through specific glycoproteins such as laminin and fibronectin (Hynes 1976, Goldberg 1979, Kramer et al. 1980, Rao et al. 1982, Terranova et al. 1982, Roa et al. 1983, Couchman et al. 1983, Malinoff et al. 1983). After attachment, the tumor cell must secrete hydrolytic enzymes to degrade the matrix. These include such things as serine and cysteine

proteinases (Jones and DeClark 1980, Liotta and Hart 1982, Varani et al. 1985, Turpeenniemi-Hujanen 1985, Trygvason 1987, Denhardt et al. 1987, Reich et al. 1988). The third step is tumor cell locomotion which may be influenced by chemotatic factors (Varani et al. 1980, Liotta et al. 1988). This describes only the process of extravasation. The process of metastasis also requires that the malignant cell survive in the blood or lymphatic systems and be able to intravasate and grow at an ectopic site. Each of the steps of tumor invasion requires many changes to occur and it is not clear whether all these processes are induced by the pleiotrophic action of a few key genes or whether several, functionally distinct genes need to participate.

There has been some success in the pusuit of genes that confer the ability to metastasize on cells already capable of abnormal proliferation (Vilette et al. 1987, Egan et al. 1987, Dotto et al. 1988, Sadowski et al. 1988, Gao et al. 1988, Pohl et al. 1988, Phillips et al. 1990). In some situations, mutations of the c-ras proto-oncogene are able to induce many of the steps required for metastasis (Pulciani et al. 1985, Muschel et al. 1985, Bondy et al. 1985, Collard et al. 1987, Egan et al. 1987, Waghorn et al. 1987, Radinsky et al. 1987, Egan et al. 1989). It has also been hypothesized, because of the evidence of a ras responsive transcription element (RRE), that ras can be directly involved in maintaining the metastatic phenotype through the action of its gene product (Wasylyk et al. 1987, Imler et al. 1988). Ras-regulatable genes may include transformation-related proteases such as collagenase and transin

(Imler et al. 1988). Also c-jun and c-fos have been shown to form a transcriptional regulating element, AP1, which can regulate transcription of the ras RRE (Rauscher et al. 1988). A mutated ras however, has not been found in every metastatic tumor. A link between metastases and oncogenes has also been found for other tyrosine kinases (Egan et al. 1989). The idea that some oncogenes may regulate secondary cellular functions, such that the process of metastases could be the result of a few select cooperating oncogenes, is an appealing one. There are many examples of tumor cells that display alterations in two or more proto-oncogenes, for example, and these genes may embody independent steps in tumor progression (Land et al. 1983a,b, Land et al. 1986, Cleveland et al. 1986, Bishop 1987, Sinn et al. 1987, Davenport et al. 1988, Schutte et al. 1989, Marx 1989, Greenhalgh et al. 1990).

Amplification of several proto-oncogenes has been implicated as an advanced step in the emergence of highly malignant tumors (Yamamoto et al. 1986, Wong et al. 1987, Slamon et al. 1987, Yokota et al. 1986, 1988, van de Vijver et al. 1987, 1988, Yoshida et al. 1988, Zhou et al. 1988,). Much of the evidence has come from studies with N-myc in breast and small cell lung cancer (Little et al. 1983, Escot et al. 1986, Varley et al. 1987, Johnson et al. 1987, Kiefer et al. 1987, Mariani-Constantini et al. 1988). In general there appears to be a higher incidence of oncogene amplification in established human tumor lines than in primary human tumors of the same histology. It is unclear however, whether amplification is important in the intiation of tumor progression. Evidence suggests that the type of

amplifications which have been identified, provide the cell with some kind of selective growth advantage signifying a more important role in tumor progression.

Recently the identification of tumor supressor genes has shown correlations with their inactivation and the ability of the tumor to metastasize (Huang et al. 1988, Steeg et al. 1988, Dear et al. 1988, Sager 1989, Stanbridge 1990, Wright et al. 1990b). Experimental evidence is pointing to the idea that these genes regulate the products of other genes involved in cell proliferation or cell differentiation (Canvance et al. 1986, Friend et al. 1988, Baker et al. 1989). Funtional loss or alteration of such suppressor genes may therefore prevent differentiation or promote cell proliferation (Nowell 1988). It is possible that similar supressor gene elements may also regulate the expression of genes that are involved in cellular invasion, adhesion, and some may function exclusively as metastatic supressor genes (Noda et al. 1983, Schalkin et al. 1988, Khoka et al. 1989, Kitayama et al. 1989). In conclusion, oncogenes and suppressor genes represent essential elements in tumor progression but only provide, at this point, an incomplete description of it. More work is required in the area of oncogene cooperation and establishment of their secondary actions before the genetic basis of metastases can be understood.

3. CELL SURFACE GLYCOPROTEINS AND MALIGNANCY

There is considerable evidence indicating that a large number of glycoproteins are altered in primary cancerous and metastatic tissues. The cell surface is concerned with many normal physiological properties related to neoplastic transformation and metastatic spread (Dennis and Laferte' 1987). These include cell shape, growth, division, differentiation, cellular recognition, communication, adhesiveness, migration, contact inhibition of growth and immunogenicity (Nicholson 1976, Yamada and Poussegur 1978, Atkinson and Bramwell 1981, Hakomori and Kannagi 1983, Hakomori 1983, Fukuda 1985). Poste and Nicolson (1980) have obtained direct evidence that tumor cell surface components could be transferred by polyethylene glycol mediated fusion of membrane vesicles from metastatic cells to less metastatic cells thereby enhancing the latter's metastatic phenotype. Although the nature and function of the relevant membrane constituents are mostly unknown, a number of membrane glycoproteins have been identified. In general many of these glycoproteins have been shown to differ in either quanity or type of glycosylation when high and low metastatic variants are compared (Kerbel et al. 1982, Altevogt et al. 1983, Barnett and Eccles 1984, Dennis 1986, Collard et al. 1986, Dennis and Laferte 1987, Lang et al. 1988, Buckley and Carlsen 1988, Laferte' and Dennis 1988, Bolscher et al. 1988, Dennis and Laferte' 1989, Dennis et al. 1989B, Heffernan et al. 1989, Penno et al. 1989). Detection of these differences in glycosylation has

been done through the use of lectins (which are plant proteins that specifically bind to defined carbohydrate structures), (Lis and Sharon, 1986, Kerbel et al. 1982, Altevogt et al. 1983, Monsigny et al. 1983, Grant and Peters 1984, Lang et al. 1988), monoclonal antibodies (MAbs, which are monoclonal antibodies produced against malignant cell glycoconjugates) (Magnani and Ginsberg 1983, Hakomori 1984, Feizi 1985, Sulitzeanu 1985, Rittenhouse et al. 1985), nuclear magnetic resonance (NMR) (Vliegenthart et al. 1983, Sweeley and Nunez 1985) and fast-atom-bombardment-mass-spectrometry (FAM-MS) (Dell et al. 1983, Reinhold and Carr 1983) which are spectroscopic methods that have been used for structural characterization of glycoconjugates. Most of the changes observed in metastatic cells by these methods are due to altered carbohydrate structures of glycoproteins, and it is still unclear whether these changes are epiphenomenal or an integral part of the process of malignancy.

3.1 Glycoproteins

Glycoprotein are widely distributed macromolecules which contain variable amounts of carbohydrate covalently attached to protein backbones through glycopeptide linkages (Sharon 1975, Lennarz 1980). They function as enzymes, hormones, receptors, lubricants, transport proteins, clotting proteins, structural proteins, immunoprotective proteins and food reserves (Spiro 1973, Sharon and Lis 1982). They are usually found on the cell surface or within the cell membrane with their carbohydrate moieties facing the exterior environment (Hughes 1973, Jarnfelt et al. 1978). Although the functions of many

glycoproteins are known the role the carbohydrate character plays is mostly unclear (West 1986). The major functions that the carbohydrate portions are involved in are; a) the ability to act as a sorting signal in directing glycoproteins to specific cellular organelles and tissues, b) protection of glycoproteins from proteolytic degradation and c) antigenic determinants of glycoproteins (Olden et al. 1982, Reisfeld and Cheresh 1987).

The structures of the major monosaccharides found in glycoproteins can be subdivided into four groups: hexoses, hexosamines, pentoses and sialic acids (Sharon 1975). The two major monosaccharides involved in glycopeptide linkages are N-acetylgalactosamine (GalNAc) and N-acetylglucosamine (GlcNAc). The major glycopeptide linkage found in glycoproteins are; a) the O-glycosidic linkage at serine and threonine, most commonly with a GalNAc, and b) the N-glycosidic linkage at asparagine, most commonly with a GlcNAc (Spiro 1973, Sharon and Lis 1982). Cell surface glycoproteins primarily contain N-linked carbohydrates, however, both O- and N- linked carbohydrates have been found on a number of glycoproteins (Sharon and Lis 1982).

3.2 Glycoprotein synthesis

Glycoprotein synthesis occurs in two major overlapping phases: synthesis of the protein backbone and synthesis of the carbohydrate moieties. The first phase employs the nucleic acid template method of normal protein synthesis and takes place on ribosomes bound to the cytoplasmic organelle, the endoplasmic reticulum (ER). Glycosylation

takes place on and within the ER, and within the golgi apparatus (GA) (Hirschberg and Snider 1987). The addition of O-linked carbohydrate chains to the protein core is relatively simple and proceeds by the sequential addition of monosaccharides from their nucleotide sugar derivatives, UDP-GalNAc, CMP-NeuAc, catalyzed by the appropriate glycosyltranferases (Leloir 1971, Beyer et al. 1981, Sadler 1984). The addition of N-linked carbohydrate chains is more complicated and initially involves synthesis of a precursor carbohydrate chain, $Glc_3Man_9GlcNAc_2$, (where mannose is man and glucose is glc) on a lipid carrier, dolichol-phosphate (Parodi and Leloir 1979, Schachter and Rosseman 1980, Snider 1984, Kornfield and Kornfield 1985).

The process of both O- and N-linked charbohydrate synthesis uses nucleotide sugar derivatives and glycosyltransferases. The precursor carbohydrate chain in N-linked synthesis is usually transferred in a single step by an oligosaccharyltransferase to the amino acid sequence Asn-X-Ser/Thr/Cys (where asn is asparagine, ser is serine, thr is threonine, and cys is cystein) on the nascent polypeptide chain (Das and Heath 1980). X can be any amino acid other than proline (pro) and aspartic acid (asp)(Bause 1983). Lipid-mediated assembly and transfer of the carbohydrate chain is a cotranslational event that takes place on or within the ER (Parodi and Leloir, 1979). After attachment to the polypeptide, the precursor chain, Glc_3Man_9-$GlcNAc_2$-Asn, is processed in the ER and GA by removal of the three Glc residues via glucosidases and four Man residues (by mannosidase I) resulting in $Man_5GlcNAc_2$-Asn (Kornfeld and Kornfeld 1985, Snider 1984). GlcNAc is subsequently added by a GlcNAc-transferase I to a

peripheral Man residue to from $GlcNAcMan_5GlcNAc_2$-Asn. This is further processed by removal of the two remaining peripheral Man residues by mannosidases II. This structure $GlcNacMan_3GlcNAc_2$-Asn is acted upon by GlcNAc-transferases IV and V which adds GlcNAc residues and initiates different branching patterns (Schachter et al. 1982, Schachter 1986). Addition of GlcNAc by GlcNAc-transferases III to the Man-3 residue linked β(1-4) to $GlcNAc_2$-Asn creates the bisected GlcNAc, which is found in many hybrid-type chains. Terminal glycosylation occurs in the GA and involves addition of perpheral monosaccharides, primarily N-acetylneuraminic acid (NeuAc) and fucose (Fuc) to hybrid chains (Dunphy and Rothman 1983). For example, NeuAc is added from CMP-NeuAc by sialyltranferases primarily to β-galactose (Gal) residues but also to GalNAc, GlcNAc and other NeuAc residues (Schachter and Roseman 1980, Beyer et al. 1981). Processing and terminal glycosylation leads to the branching and heterogenity characteristic of many glycoproteins (Schachter 1986).

3.3 Glycoproteins in malignant cells

For many years investigators have identified, isolated, and characterized glycoproteins that they have claimed are specific to, or associated with malignancy (Yamada and Pouysegur 1978, Warren and Buck 1980, Atkinson and Hakimi 1980, Bhavanandan and Davidson 1982, Turner 1982, Dennis and Laferte' 1987, Lang et al. 1988). Few, if any, of these glycoproteins are truely tumor specific, most are normal consistuents of adult tissue; others appear in embryonic tissue or diseased tissue that is nonmetastatic (Smith and Kelleher

1980, Wagener and Breuer 1982, Barroso and Alpert 1983, Nozawa et al. 1983, Fishamn 1987). Glycoproteins such as fibronectin and laminin, for example, have been associated with malignancy (Chen et al. 1979, McCarthy et al. 1985, Yamada et al. 1985). Fibronectin is an adhesive glycoprotein that has been shown to be decreased in amount in transformed cells and correlated with metastatic ability (Carter 1982). Laminin, another adhesive glycoprotein, is expressed early in development, is found in basement membranes, and is produced by tumor cells (Lievo et al. 1980, Timpl et al. 1983, Palm and Furcht 1983). The attachment of metastatic cells to laminin through receptors may be involved in the metastatic spread of cancer (Terranova et al. 1983, Rao et al. 1983, Malinoff and Wicha 1983). The most prominant and consistant findings in tumorigenic and metastatic cells is an increase in the size of Asn-linked oligosaccharides (Warren et al. 1978, Atkinson and Hakimi 1980, Warren and Buck 1980). Most of these have resulted from changes in terminal transferase activity, most notibly increased sialylation due to sialyltransferase, and increases in the activity of other transferases like Gal-T and GlnNAc-T (Takasaki et al. 1980, Glick 1974).

The most characteristic change associated with malignancy is the enrichment of higher molecular weight glycopeptides. Evidence for an increased number of sialic acid residues was provided by the observations that neuraminidase treatment decreased the size of malignant cell glycoproteins to that of glycoproteins found in normal cells, and transformed cells were found to have increased sialyltransferase activity (Warren et al. 1972, Warren and Buck 1980).

Decreased lectin binding was also found to be the result of increased sialylation (Altevolt et al. 1983, Collard et al. 1986, Nabi and Raz 1987). An inverse relationship was found between lectin receptor sites and invasive and metastatic potential (Collard et al. 1986, Lang et al. 1988). Decreased lectin binding was shown to be the result of masking by sialic acid residues as neuraminidase treatment lead to increased binding (Collard et al. 1986). Yogeeswaran and Salk (1981) for example, demonstrated that metastatic potential in a variety of murine tumor cells was correlated positively with the total sialic acid content, particularly the degree of sialylation of Gal and GalNAc residues which can bind specific lectins. Not all metastatic cells, which show a decrease in lectin binding, also exhibit an increase in sialic acid residues. A change in the specificity or in the relative activities of sialyltransferase is also postulated to be important (Altevogt et al. 1983). For example, the attachment variants of the metastatic murine lymphoma, Esb, were found to have unchanged levels of total sialic acid. Lectin binding studies however, suggested that the distribution of sialic acid on the O- and N- linked structures was altered, and this could account for changes observed in attachment and metastatic properties (Altevogt et al. 1983).

Other studies which have focused on the changes that occur in the carbohydrate moieties of glycoproteins involved in metastatses, have pointed out the importance of other transferases, especially GlcNAc and Gal transferase (Dennis et al. 1987, Dennis et al. 1989b, Penno et al. 1989). This is related to the recent findings that

increased β(1-6) branching of complex-type Asn-linked oligosaccharides on cell surface glycoproteins are important in highly metastatic murine tumor cell lines (Dennis et al. 1987, Dennis and Laferte' 1989). Mutants that are deficient in β(1-6)GlcNAc transferase V activity were found to have decreased β(1-6) branching and decreased metastatic potential (Dennis et al. 1987). Also, induction of β(1-6) branching, accompanied by an increase in β(1-6)GlcNAc transferase V activity, in a nonmetastatic murine mammary carcinoma correlated positivley with the acquistion of the metastatic potential (Dennis et al. 1989b). Multiple glycosytranferases within a given study have been demonstrated to have altered activity in a number of human cancers. Increased activity levels of three glycosyltransferases, NeuAc-T, Gal-T and Fuc-T for example, have been found in ovarian epithelial adenocarcinoma (Chatterjee et al. 1979). Gal-T consistantly exhibited elevated activity in the sera of ovarian cancer patients (Chatterjee 1980). Acute myeloid leukemia patients exhibited decreased serum Fuc-T and sometimes decreased GalNAc-T (Kuhns et al. 1980). In general, the results of many investigators suggest that alterations of glycosyltransferases exist in tissue fluids and solid tumors derived from cancer patients, and in malignant cells grown in culture (Ronquist et al. 1982, Capel et al. 1982, Davey et al. 1984).

The relationship of oncogenes to malignant cell glycoproteins has been investigated in some studies. Rousell et al. (1984) studied the v-fms oncogene and with a deletion mutant (which due to incomplete processing of complex carbohydrate chains could not be detected

on the cell surface and could not transform cells) concluded that cell surface expression of wild-type v-fms-coded glycoproteins was required for transformation. Collard et al. (1985) studied the effects of the presence of human ras-oncogenes in NIH3T3 cells. A positive correlation was found between the presence of the oncogene, the tumorigenicity in nude mice, and the expression of a cancer-related glycopeptide enriched in sialic acid residues. Dennis et al. (1989) has shown that transfection of H-ras and v-fps increases β(1-6) branching that correlates with metastatic potential accompanied by an increase in β1-6GlcNAc transferase V activity. Bolscher et al. (1988) has also examined the effects of transfected ras on glycosylation and shown that in NIH3T3 cells, ras induces branching and increases the content of sialic acid in a reporter virus VSV G-protein. Carbohydrate changes are important in the acquistion of the metastatic phenotype, although their function is still unclear. Through studies with oncogenes the molecular basis of these changes may start to become apparent.

In summary, major structural alterations in malignant cells appear to be associated with the carbohydrate moieties. For glycoproteins these alterations are mostly increased sialylation and increased oligosaccharide branching. Recent studies have provided evidence that the metabolic basis of malignant cell glycoprotein alterations primarily involves biosynthetic pathways and the activation of specific glycosyltransferases. However, little is known about what role oncogenes play in bringing about such metabolic changes. Furthermore, it is still not clear whether the glycocon-

jugate alterations brought about by metabolic changes are merely associated with, or an integral part of, the malignant process.

Another piece of evidence which suggests that tumor cell suface oligosaccharides play a significant role in metastasis comes from studies using glycosylation inhibitors. Treatment with either tunicamycin, an inhibitor of the synthesis of an intermediate in Asn-linked protein glycosylation, swainsonine, a potent inhibitor of Golgi α-mannosidase II or castanospermine, an inhibitor of glucosidase I, has been reported to inhibit spontaneous and experimental metastasis in a variety of metastatic cell lines (Irimura et al. 1981, Olden et al. 1985, Mareel et al. 1985, Humphries et al. 1986a, Humphries et al. 1986b, Dennis et al. 1986, Newton et al. 1989, Dennis et al. 1989a). A transient loss of sialylated antennae in complex type oligosaccharides, and a stimulated lectin-resistance phenotype can be induced in wild type cells grown in the presence of swainsonine (Dennis et al. 1986). Both castanospermine and swainsonine prevent formation of complex carbohydrate structures, and produce high mannose or hybrid-type oligosaccharides, respectively (Elbein et al. 1981, Tulsiani et al. 1982, Saul et al. 1983, Pan et al. 1983). Other glycosylation inhibitors include 1-deoxynojirimycin, an inhibitor of glucosidase I and 1-deoxymannojirimycin a mannosidase I inhibitor (Elbein 1984, Fuhrmann et al. 1984). The effect of these on the malignant potential of metastatic cell lines has not been examined.

Whereas neoplastic transformation has been associated with a variety of structural changes in cell surface carbohydrates, it has

been difficult to establish that a specific permutation is relevant to the metastatic process and not just an associated change. The appraoch of modifying a single characteristic using inhibitors of specific enzymes of the processing pathway, may lead to identification of specific oligosaccharide structural features crucial for successful completion of the metastatic cascade.

MATERIALS AND METHODS

1. CELL LINES AND CULTURE CONDITIONS

1.1 Souces of cell lines

The Chinese hamster ovary (CHO) cell line, thy-49, was obtained from Dr. Mark Meuth (Imperial Cancer Research Fund, Clare Hall, Hertfordshire, London, England). The isolation and characterization of this mutant has been previously described (Meuth et al. 1979, Trudel et al. 1984). The baby hamster kidney cell lines, BHK, YMP1 and YMP7, were obtained from Dr. George Stark (Imperial Cancer Research Fund, Lincoln's Inn Fields, London, England). Isolation and characterization of these mutants has also been described (Giulotto et al. 1987, Rolfe et al. 1988). The wildtype CHO K1 dhfr- line was a gift from Dr. R. Johnson (University of Alberta, Calgary, Canada) its isolation and characterization has been described by Gasser et al. (1982). Mouse fibroblast, NIH3T3 cells, were purchased from the American Type Culture Collection (ATCC, Rockville, Maryland).

The 10T½ derived mouse cell lines, NR_4 and Ciras 1 were established in the laboratory as described by Egan et al. (1987). These cell lines were clones selected for G418 resistance (NR4) or foci formation (Ciras 1) following transfection of the T24-H-ras gene and the drug selection marker neomycin into mouse fibroblasts, 10T½. Subsequent analysis of these cell lines showed that the lines were tumorigenic but not very metastatic (Egan et al. 1987).

The NR4dGC2 and NR4dGC1 deoxyguanosine resistant cell lines were cloned from a population of wild type NR_4 cells selected in a step wise procedure for the ability to proliferate in alpha minimal essential media [(α-MEM) Flow Laboratories Inc., Rockville, MD] supplemented with increasing concentrations of deoxyguanosine. Cells were cultured in the presence of increasing concentrations of deoxyguanosine (dG) as follows, 100 μM, 200 μM, 300 μM, 400 μM, 600 μM, 800 μM and 1 mM, until a stable line resistant to 1 mM was established. After achieving this level of resistance, NR4dGC2 cells were routinely grown in the absence of drug, and remained stable for dG resistance for >2 years. Metastatic potential however, is not a particularly stable phenotype (Fidler and Kripke 1980). For this reason the NR4dGC2 cell was recloned to isolate the lines dGC2RevC which is a low metastatic subclone of NR4dGC2 and dGC2M5 which was cloned in low percentage (0.33%) agar and is a high metastatic subclone of NR4dGC2. The dGC2M5 clone was also recloned to produce another low metastatic line dGC2M5.9. The metastatic potential of all clones was reestablished and described in the results.

1.2 Culture Conditions

All cell lines were routinely maintained at 37°C on the surface of plasitic tissue culture plates (Lux Scientific Corp., Newbury Park, CA) in α-MEM supplemented with antibiotics, penicillin G (100 units/ml), and steptomycin sulfate (100 μg/ml) and 10% volume/volume (v/v) fetal calf serum [(FCS) GIBCO, Grand Island, NY]. The formulation of α-MEM has been published by Stanners et al. (1971),

and the medium did not contain ribonucleosides or deoxyribonucleosides. Cultures were incubated at 37°C in a 5% CO_2 atmosphere in a humidity controlled incubator. The thy-49 cells were also supplemented with 10 μM thymidine unless otherwise indicated.

When experiments were carried out in the presence of 10% dialized fetal calf serum (dFCS), FCS was dialized in dialysis bags (Spectra/pore exclusion size 8000 daltons) against 50 x v/v 137 mM NaCl overnight.

1.3 Routine Culture Procedures

1.3.1 Cell removal with trypsin solution

Sterile 0.3% bacto trypsin was prepared in phosphate buffered saline (PBS, pH 7.3) consisting of 140 mM NaCl, 2.7 mM KCl, 1.6 mM KH_2PO_4, and 8.1 mM Na_2HPO_4, and stored at 4°C. To remove cells from the surface of tissue culture plates, the growth medium was aspirated and the plates washed once with PBS. One to two mL of trypsin was then added to the plates, and after either 30 seconds for NR_4 and other less tightly adhering cells, or 5 minutes (min) for more tightly adhering cells like NIH3T3 cells, 2-3 ml of medium containing 10% FCS was added to the plates to neutralize the effects of the trypsin and to remove the cells. The cell suspension was then centrifuged, resuspended in growth medium, and dispensed as required.

1.3.2 Subculture

Cell cultures that appraoched confluence were subcultered. The

cells were detached from the surface of tissue culture plates with the aid of trypsin solution as described above. The density of the cell suspensions was determined and an aliquot of 1 x 10^5 cells was transferred to a fresh 100 mm plate containing 10 ml of fresh growth medium.

1.3.3 Long-term storage of cells

For long-term storage, all cells were suspended between 10^6-10^7 cells in 1 ml α-MEM plus 10% FCS plus 10% dimethylsufoxide. The cell suspensions were placed in cyrotube vials (Nunc, Kamstrup, Denmark) and frozen slowly to -76°C. To recover cells, the vials were rapidly thawed in a 37°C water bath and the suspension placed in 3 ml of growth medium in a sterile Falcon plastic tube (Becton Dickonson, Rutherford, NJ) pippeted up and down, and then centrifuged at 500 xg for 5 min. in order to pellet the cells. The cell pellet was then resuspended in normal growth medium and dispensed appropriately onto the surface of tissue culture plates.

1.3.4 Cell counting

Routinely aliquots of cells removed with trypsin solution as described above were diluted with PBS (usually 100 ul of cell suspension was added to 40 ml PBS) and counted with the aid of a Coulter Particle Counter (Coulter Electronics Ltd., Florida) to determine cell densities. For tumoriginicity and experimental metastatsis assays, cell suspensions were counted using a hemocytometer, averaging at least 4 seperate counts.

2. Protein Determination

Protein content of samples were determined using the Bio-Rad Protein Assay (Bio Rad, Bulletin 82-0275, Richmond, CA) using bovine serum albumin (BSA) as a standard. A linear relationship between absorbance and protein concentration was observed from 0 to 100 μg protein.

3. Measurement of Cell Growth in the Presence of Deoxyguanosine

To determine the exponential growth rates of NR_4, NR4dGC2 and NR4dGC1 cells in culture, in the presence and absence of dG, $5x10^4$ cells were seeded on duplicate 60 mm plates, one containing 300 μM dG, and incubated in α-MEM plus 10% dFCS. After an overnight incubation at 37°C, cells from two of the plates in drug and two of the plates out of drug were independently harvested with trypsin solution and the total number of cells on each plate was determined. These cell numbers were considered as the number of cells at time zero to ensure that the cells were in log phase of growth, minimizing distortion caused by the lag period following plating. At various times following time zero, two plates of cells (both in and out of drug) were again removed with trypsin solution and counted using the Coulter Particle Counter. The results were plotted as the log of cell number versus time of incubation.

4. Determination of Growth Rate

To determine the relative degree of resistance to dG, cells were tested for their abilities to grow in the presence of increasing concentrations of drug. To measure cell growth, cells were seeded at densities of 10^5 cells per 60 mm tissue culture plate in normal growth medium in the presence or absence of drug. The plates were incubated at 37°C for 72 hours (hr), after which the medium was removed by aspiration, and either 1.5 ml of 1 M NaOH was added to each plate or cells were trypsinized and counted. Where NaOH was used the plates were then scraped with a rubber policeman to remove cellular material and the suspension incubated at 50°C for 15 min. The extracts were diluted to an appropriate volume and the absorbance read at 260 nm as a measurement of nucleic acid content (Swyryd et al. 1974, Kempe et al. 1976). For both conditions (counting or examination of nucleic acid content) each experimental point was performed in triplicate and the average value was expressed as a precentage over growth in the absence of drug as 100%.

5. Deoxyguanosine Killing Curves

To determine the colony forming ability of cells in the presence of drug, exponentially growing cells were harvested and counted as described above. A pre-determined number of cells ranging from 2×10^2 to 10^6 were added to 100 mm culture plates with 10 ml of α-MEM plus 10% dFCS. Increasing concentrations of drug were also added to the plates of cells. After an incubation period of 10-14 days at 37°C the medium was removed from the plates and cells were

stained with a filtered 50% solution of ethanol saturated with methylene blue (Sigma Chemical Co., St Louis, Mo) at room temperature for about 15 min. Colonies consisting of more than 50 cells were counted under a dissecting microscope. The plating efficiency, or the number of cells added to the plates divided by the number of colonies observed, was then determined for cells both in and out of drug. The plating efficiency for NR4, NR4dGC2, NR4dGC1, dGC2rev and dGC2M5 were consistantly between 0.65 and 0.75 in the absence of drug. The effect of drug on the growth of cells was then determined by the relative plating efficiency (RPE). The RPE is defined as the plating efficiency in the presence of drug divided by the plating efficiency in the absence of drug (Hards and Wright, 1981). Results were plotted as the log of RPE versus drug concentration.

6. Frequency of Corformycin-Resistant Colonies

The frequency of drug resistant colonies for the BHK cell lines were determined by adding an average of four samples of 10^5 cells onto 100 mm plates and treating immediately with selective medium containing 100 μM azaserine, 100 μM uridine and 20 μM adenine as described by Giulotto et al. (1987). Various concentrations of corformycin were then added. The medium was changed weekly, and the plates fixed and stained after 3 weeks. Colonies were counted under a dissecting microscope. In some cases the number of resistant colonies could not be determined because the plates were confluent (conf.).

7. Deoxyribonucleotide Pool Analysis

Deoxyribonucleoside triphosphates were extracted from cell monolayers, in the presence of 10% trichloroacetic acid (TCA). The nucleotides were extracted using the Froen-amine method as described by Khym (1975). Briefly 10^7 cells were removed from plates, washed with PBS, and 100 μl of TCA was added to the cell pellet. This suspension was left on ice for 20 minutes after which the suspension was spun at 4°C and the pellet kept for DNA determination using the Burton method (Burton 1968). The supernatant was neutralized with the addition of 1 volume of Tri-n-octylamine plus freon (22:78 v/v). Following vortexing the suspension was spun at room temperature, the supernatant removed, and the pH tested to ensure it was at a neutral pH. If not, the procedure was repeated. The ribonucleotides of the cell extracts were then degraded by the periodate oxidation procedure developed by Garrett and Santi (1979). To neutralized extracts, 100 mg/ml of fresh sodium periodate was added followed by a 5% solution of 4 M methylamine phosphate, pH 7.4. This mixture was incubated at 37°C for 1 hr at which time the reaction was stopped by the addition of a 5% solution of 1 M rhamnose. Extracts were further concentrated and cleared up by passing through QMA Sep-pak cartridges (Waters, Mississauga, Ont.) and nucleotides were eluted with 1 M HCl neutralizing with 10 M KOH.

Nucleoside triphospates were separated by high performance liquid chromatography (HPLC) at room temperature using a Whatmann Partisal 10-SAX anion exchange column (4.6 x 250 mm) at a flow rate of 2ml/min (Waters). Isocratic elution was accomplished with 0.4 M ammonium

phosphate buffer pH 3.4 containing 2.5% acetonitrile. Nucleotides were identified by comparing the retention times to that of known standards. Quanitation was carried out by measuring the absorbance of periodate treated extracts at 254 nm using the highest sensitivity (AUFS 0.005) followed by integration of the peaks. These were compared to standards of a known concentration.

Results were presented as picomoles of nucleotide per mg DNA using the Burton (1968) assay for DNA determination on the pellet following Freon-amine extraction. Briefly 200 μl of 0.5M perchloric acid (PCA) was added to the DNA pellet and incubated at 80°C for 10 min. The mixture was then centrifiged, and the supernatant transfered to a new microfuge tube. Another 200 μl of 0.5 M PCA was added to the pellet, this procedure repeated, and the supernatants combined. Several aliqots of the hydrolysate (ie. 20, 30, 40 μl) were then removed and 0.5 M PCA was added to a final volume of 200 μl. To this mixture 400 μl of reagent was added: Soln A- 33.5% v/v Glacial acetic acid, 0.5% v/v H_2SO_4, and 0.5% diphenylamine, mixed 200 to 1 with Soln B- 1.6% v/v acetaldehyde. The above procedure was carried out with different concentrations of salmon sperm DNA and a
standard curve was constructed. The above mixtures were incubated overnight at room temperature in the dark. The next day the absorbance was determined at 600 nm, on a LKB biochrom ultrospec 4050 spectrometer, using glacial actetic acid to dilute the samples.

8. In Vitro Ribonucleotide Reductase Assay

Enzyme activity was measured in sonicated cell extracts (10^7 cells) using ^{14}C-cytosine 5'-diphosphate (CDP) as subtrate, according to the procedure of Lewis and Wright (1978). The reaction mixture contained in a final volume of 50 μl: 6 mM DTT, 4 mM $MgCl_2$, 4 mM ATP, 5 mM NaF, 100 mM Hepes buffer (pH 7.5), 50 μM ^{14}C-CDP [0.09 mCurie (mCi)], an appropriate amount of protein, and increasing concentrations of either deoxyguanosine triphosphate (dGTP) or deoxyadenosine triphosphate (dATP). Reactions were carried out for 30 min. at 37°C, and terminated by boiling for 4 min. The deoxycytidine phosphates were then converted to deoxycytidine by addition of 1 mg of Crotalus atrox venom (Cory and Whiteford, 1972) prepared on 0.1 M Hepes, N-(2-Hydroxyethyl)piperazine-N-(2-ethanesulfonic acid), (pH 8.0), plus 10 mM MgCl2 (20 mg venom per ml). Following incubation for 1 hr at 37°C the reaction was terminated by boiling for 4 min and 0.5 ml distilled water was added to each assay tube. The tubes were then centrifuged to remove the heat precipitated material, and the supernatant loaded onto a 5 x 80 mm column of Dowex-1-borate (Steeper and Stuart 1970, Cory and Whitford 1972), to separate the nucleosides from the deoxyribonucleosides. The deoxycytosine was eluted from the column, since cytosine remains bound through it's cis-diol to the borate ions on the column, into scintillation vials with 5 ml distilled water. Results were expressed as nmoles dCDP formed/mg protein/hr.

9. Growth in semisolid medium

Growth in soft agar was determined with a 0.5% Bacto-Agar (Difco Laboratories, Detroit, Mich.)-10% FCS-α-MEM base layer and a 0.33% agar-10% FCS-α-MEM growth layer (Egan et al. 1987). Colonies larger than approximately 50 cells were scored after 20 days.

10. Tumorigenicity and Experimental Metastasis Assay

Tumor growth rates were determined as previously described (Greenberg et al. 1987). Briefly, cells were removed from culture plates, and washed two times with sterile PBS. For tumor growth rate studies 0.1 ml of 1-3 x 10^6 cells/ml from each cell line were injected subcutaneously into the lower midback of 9 to 10 week old female mice. BALB/c nu/nu (Life Sciences, St Petersberg, FL) mice were used for the CHO cell line, CD-1 nu/nu (Charles River, Montreal, Canada) mice for the BHK cell lines, and C3H-HeJ syngeneic mice for the 10T½ derived cell lines. Tumor size was calculated by multiplying the dimensions a right angles of the cross section of the tumor using a maximum radius. The latency, or time at which a subcutaneous tumor first appeared after injection, size 2 x 2 mm, was recorded and the average reported. For the 3T3 lines the frequency of the number of mice that recieved subcutanoues tumors as well as the latency when they first appeared was determined following injection of 1 x 10^5 cells in BALB/c nu/nu mice.

For the experimental metastasis assay 2 x 10^5 cells for the BHK

cell lines, 4 x 10^5 cells for the CHO lines, 1-3 x 10^5 cells for the 10T½ derived cell lines and 1 and 5 x 10^5 for the 3T3 lines, were injected in a volume of 0.2 ml into the tail veins of the appropriate mice. Mice were sacrificed after 3-4 weeks by ether anasthesia and Bouins solution (picric acid, formaldehyde, acetic acid [15:5:1]) injected intratracheally. Lungs were then removed and examined for the presence of metastatic foci.

11. Rate of Metastasis

The rate of metastatic variants produced by clonal populations was determined as previously described (Hill et al. 1984). Cells were cloned by limiting dilution into 96-well tissue culture plates (Lux Scientific Corp.). Briefly, exponentially growing cells were removed from culture plates and counted. They were then diluted in growth medium to a concentration of approximately 5 cells/ml and dispensed in 200 μl aliquots into each well of a 96-well tissue culture plate. The BHK cell lines were maintained in 10% FCS plus α-MEM. The thy-49 cells were, for these experiments, kept in α-MEM plus 10% dFCS supplemented with either 1 μM or 100 μM thymidine. A well containing a single clone was grown to approximately 500 cells, then trypsinized and transfered to a 60 mm plate in the appropriate medium.

The clones were allowed to grow until they reached a total popultion size of 2 ± 0.3 x 10^6 for the thy-49 cells, and 1 ± 0.3 x 10^6 for the BHK cell lines. The total number of cells was quanitated

using a hemocytometer. Ten clonal populations for each cell line, thy-49 in 1 μM thymidine, thy-49 in 100 μM thymidine, BHK, YMP1, YMP7, were then injected into the tail vein of 5 mice each. A total of 4×10^5 cells per mouse in BALB/c nu/nu for the thy-49 cells, and a total of 2×10^5 cells per mouse in CD-1 nu/nu for the BHK cell lines. After 21 days for the CHO cells and 28 days for the BHK cell lines the mice were killed and the lungs examined for metastases as previously described.

The results of the number of lung metastases for the parallel clones were analyzed using the fluctuation test of Luria and Delbruck (1943). The equation determines the rate of generation of variants, in this case metastatic variants, per cell per generation. The equation uses the mean number of variant cells per parallel culture, r, present at the time of selection;

$$r = aN \ln(NCa)$$

where N is the final number of cells per parallel culture and C is the number of parallel clones. The rate a, can be determined using the Newton-Raphson iteration as shown in tabular form by Capizzi and Jameson (1973).

12. SOUTHERN AND NORTHERN BLOT ANALYSIS

12.1 DNA Isolation and Southern Blot Analysis

Genomic DNA was isolated from cells according to the procedure of Blin and Stafford (1976). Cells were harvested from 3-5 sub-confluent 150 mm plates, centrifuged, washed once with PBS and

resuspended in an ice-cold solution of 10mM Tris-HCl (pH 8.0) plus 1mM EDTA (TE) at a concentration of approximately 10^8 cells/ml. To the suspension, 10 volumes of DNA isolation buffer consisting of 0.1M EDTA, 0.15 M NaCl, 0.5% sarcosyl and 100 μg/ml proteinase K was added. Following incubation at 50°C for 3 hr, the DNA was extracted 3 times with an equal volume of phenol. The DNA was then dialyzed overnight against 4 liters of buffer containing 25 mM Tris-HCl (pH 8.0), 10mM EDTA, and 10mM NaCl, allowing room in the dialysis tubing for the sample to increase about 3-fold in volume. The sample was then treated with 100 μg/ml of DNAse free RNAse (Sigma Chemical Co.) at 37°C for 3 hr, extracted twice with an equal volume of phenol/chloroform (note that chloroform in all cases means chloroform:isoamyl alcohol, 24:1) and then once with an equal volume of chloroform. The DNA sample was then concentrated with 1-3 volumes of sec-butyl-alcohol and dialyzed extensively against TE. A couple of drops of chloroform was added to the final solution of DNA for preservation and stored at 4°C.

The concentration of DNA was determined by diluting usually 50 μl of DNA in 950 μl TE and measuring the absorbance at both 260 and 280 nm. Only those preperations having OD260/OD280 ratios of 1.75-1.80 were used. Using the formula: 1 OD_{260}=50 μg/ml DNA, when the 260/280 ratio is 1.80 (Maniatis et al. 1982), allowed calculation of the DNA concentration.

For southern blots, 20 μg of DNA was digested to completion with 3-4 units/μg DNA of the desired resriction endonuclease for 3 hr to overnight. The sample was then dried down to a volume of 40 μl

using a speed-vac concentrator (Savant). To the sample was added 1/5 the volume of 6x gel loading buffer containing 0.25% bromophenol blue, 0.25% xylene cyanol, and 30% glycerol, and the sample loaded onto a 0.7% agarose, Tris, borate, EDTA (TBE) gel containing 0.5 μg/ml ethidium bromide. The gel was electrophoresed overnight at 30 volts in the same buffer (89 mM Tris-borate, 89 mM boric acid and 2 mM EDTA). Molecular weight markers were always loaded onto seperate lanes in solutions containing the same salt concentration. Gels were viewed under ultraviolet light to evaluate the digestion, to ensure that equivalent amounts of DNA were loaded, and to measure the distance between the well and each band of the molecular weight markers, so the size of subsequent bands could be determined. Gels were then treated so that they could be transferred to either nitrocellulose or nylon membranes. The gel was first treated with 0.25 M HCl for 15 min. followed by 0.5 M NaOH plus 1.5 M NaCl twice for 15 min. and finally with 0.5 M Tris-HCl (pH 7.5) plus 1.5 M NaCl twice for 20 min. The gel was blotted overnight in 20 x SSC (1 x SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) buffer onto either nitrocellulose or nylon membrane, by placing the membrane next to the gel on top of which 2 pieces of filter paper were placed, and a stack of paper towels with a weight to ensure the drawing up of the salt solution and DNA through to the blotting membrane. The next day the blotting membrane was removed from the gel, rinsed in 2-6 x SSC, air dried, and then baked at 80°C for 1 to 2 hrs. The blots were prehybridized for 3 hr to overnight at 42°C in 50% (v/v) foramide, 0.1% sodium dodecyl sulfate (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 BSA in 100 ml water), 10% dextran sulfate all preheated to 60°C and 100 μg/ml boiled salmon sperm. Hybridizations were preformed in the same solution for 16 hr with 10^6 counts per minute (cpm)/ml of ^{32}P-labeled probe. The blots were washed twice in 2 x SSC and 0.1% SDS at room temperature for 20 min. each, and then twice with 0.2-0.5 x SSC, and 0.1% SDS at 57°C for 30 min. each. If nylon membrane was used the blots were bagged wet otherwise they were blotted dry and autoradiographed at -70°C using Kodak X-Omat AR film and Cronex Lightning Plus intensifying screens for a period of 24 hr upto 7 days.

12.2 RNA Isolation and Northern Blot Analysis

Total cellular RNA was extracted from logrithmically growing cells using the guanidinium isothiocyanate/CsCl method (Chirqwin et al. 1979). Cells were harvested from 6-8, 150 mm culture plates, washed once with PBS and centrifuged. The pellet of approximately 10^8 cells was then resuspended in 2 ml of 4 M guanadinium thiocyanate, 25 mM sodium citrate (pH 7.5), 0.5% N-laurysarosine, 0.1% antifoam A and 0.007% β-mercaptoethanol. The suspension was then pipetted with a syringe approximately 20 times to disperse the DNA. The cell lysate was then diluted up to 9 ml with the guanidinium isothiocyanate solution and layered on top of 3.0 ml of 5.7 M CsCl and 0.1 M EDTA (pH 7.0) in a Beckman SW41 centrifuge tube. The suspension was then centrifuged at 20°C in a SW41 Ti Beckman rotor at 25,000 rpm for 18 hr. The top guanidinium isothiocynate layer and

the CsCl, including the band of DNA, was removed with a pasteur pipette, and the tube was cut slighly above the pellet to avoid excess salt contamination. The sides of the centrifuge tube and the pellet were washed once with the guanidinium isothiocyanate solution and then twice with 70% ethanol plus 0.1% SDS. The RNA pellet was resuspended in 500 μl sterile water, transferred to a microfuge tube and precipitated overnight at -20°C by the addition of 10% (v/v) 3 M sodium acetate (pH 5.5) and 2 volumes of absolute ethanol. The sample was then centrifuged (10-15 min. in a benchtop microcentrifuge at 4°C) and the pellet containing total cellular RNA resuspended in 100-150 μl of sterile water and stored at -70°C.

The concentration of RNA in a sample was determined by measuring the absorbance of the sample at 260 nm and using the formula: 1 OD260 = 40μg/ml RNA (Maniatis et al. 1982). The RNA was prepared for Northern blot analysis as follows: to 4.5 μl RNA sample containing 20 μg of cellular RNA was added 2.0 μl 5 x MOPS buffer (1 x MOPS is, 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 then incubated at 55°C for 15 min. after which 2.0 μl of 5 x gel loading buffer containing 50% glycerol, 1 mM EDTA, 0.4% bromophenol blue and 0.4% xylene cyanol was added. The sample was then loaded onto a 1% formaldehyde-agarose gel containing 18% foraldehyde and 1 x MOPS and electrophoresed overnight in MOPS buffer at 30 volts. The gel was then blotted onto nitrocellulose or nylon paper using 20 x SSC as previousy described for Southern blots. The membranes were again baked at 80°C for 1 hr and the blots prehybridized, hybridized,

washed and developed as outlined for Southern blots, except that prehybridization and hybridization solutions contained 750 μl/ml denatured salmon sperm DNA to allow more efficient blocking.

Where mRNA was used to screen clones for the expression of a transfected gene a rapid method for mRNA isolation was used (Gough 1988) and Nothern blot analysis was performed as described above. Briefly, one 150 mm plate of 80% subconfluent cells were harvested, washed, and the pellet lysed in 200 μl of 10 mM Tris-Cl pH 7.6, 0.15 M NaCl, 1.5 mM MgCl2 and 0.65% Nonidet-P40 (NP40). This was centrifuged at 4000 xg in a microcentrifuge. 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 (1:1). This mixture was vortexed and spun at 14000 xg for 10 min. The resulting top clear supernatant was precipitated in 800 μl of 100% ethanol overnight at -20°C.

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

cDNA probes between the size of 100 bp-2000 bp were labelled, using the hexanucleotide method of Feinberg and Volgelstein (1983), to specific activities between 0.5-1.0 x 10^9 cpm/ug using ^{32}P-dCTP. Approximately 500 ng of an appropriate cDNA probe in a volume of 25 μl was boiled for 5 min, and then chilled. Labelling of the single stranded cDNA probe was then performed using the hexanucleotide kit supplied by Amersham (Oakville Ont.), by adding 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), 15 μl of multiprime

buffer solution (containing random hexanucleotides in a concentrated buffer solution of Tris-HCl, pH 7.8, $MgCl_2$, and β-mercaptoethanol), 2.0 μl each of dTTP (2'-deoxythymidine 5'-triphosphate), dATP and dGTP in the same buffer, and 5 μl ^{32}P-dCTP (specific activity 3000 Ci/mmol) to the probe. Following incubation at room temperature for a minimum of 3 hr, the reaction mixture was passed through a spin column of sephadex G-50 (prepared in a microfuge tube and spun in a clinical centrifuge at 500 xg) and the labelled probe was eluted with 100 μl TE. The incorporation of label into the probe was determined by counting 1 μl of the effluent using a ^{32}P Cerenekov scintillation counter program. An appropriate volume was then added to the hybridization mixture to achieve 1 x 10^6 cpm/ml. For probes larger than 2.0 kb (kilobases), the dihydrofolate reductase (dhfr) probe for instance, labeling was done by the Nick Translation System as described by Bethesda Reasearch Laboratories (BRL) Life Technologies, Inc in the manufacture's specifications (Bethesda, MA). Again 500 ng of probe was diluted up to 28 μl with distilled water. To this 5 μl of the appropriate deoxynucleotide triphosphates in concentrated buffer was added plus 5 μl of DNA PolymeraseI/DNase I (100 units) and 7 μl ^{32}P-CTP (specific activity 3000 Ci/mmol). The mixture was incubated at 12°C for 60 minutes and then treated as described above.

13. ISOATION AND SOURCES OF cDNA PROBES AND PLASMIDS

13.1 Sources of cDNA Probes and Plasmids

Ribonucleotide reductase probes for the M1 cDNA (clone 65) and M2 cDNA (clone 10) were obtained from Dr. L. Thelander of the Karolinska Institute, Stockholm (Thelander and Berg, 1986). The T24-H-ras blots were probed with a v-ras probe purchased from Oncogene Science (Mineola, New York). The hepatitis B virus (HBV) cDNA was cut out from the pER-dATP^r plasmid kindly provided by Caras and Martin (1988). The mammalian lac inducible plasmid system, pCMVlacI and pSVLacOCAT, was a gift from Dr. J. Figge (Cell 1988). The Kaposi-fibroblast growth factor (k-fgf) cDNA [pG3(B)-SacI] used as a probe as well as the genomic k-fgf gene pG6.6, was obtained from Dr. C. Basilico and has been described by Delli Bovi et al. (Cell, 1987). The dhfr-containing amplification plasmid containing the dhfr mini-gene, MG4, in the pGEM3 vector was a generous gift from Dr. R. Johnston (personnal communication). The probes required for experiments were obtained from plasmid preparations cut with the appropriate restriction endonucleases and purified as described below.

13.2 Large Scale Plasmid Preparation

Large scale preparations of plasmid DNA were obtained according to the procedures of Manniatis et al. (1982). A single colony of HB101 E. coli (gift from Dr. P. Loewen, University of Manitoba, Winnipeg, Canada) transformed with the appropriate plasmid was inoculated into 5 ml LB medium (pH7.5), (LB medium consists of 1% w/v

bacto-tryptone, 0.5% w/v bacto-yeast extract and 1% w/v NaCl) containing 50 μg/ml ampicillin and incubated at 37°C overnight. The entire suspension was then inoculated into 1 litre of LB medium, and incubated until an OD_{550} value of 0.4 was reached, at which time 5 ml of a 54 mg/ml solution of spectinomycin was added and the cells were reincubated overnight while being shaken at 250 rpm. The cell suspension was then centrifuged at 4,000 xg for 10 min. and the supernatent discarded while the pellet was resuspended in 25 ml of a ice-cold solution of 0.1 M NaCl, 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA and 0.5% v/v Triton X-100 (STE) and recentrifuged. The pellet was then resuspended in 10 ml of 50 mM Tris-HCl buffer (pH 8.0) containing 10% w (weight)/v sucrose. To the preparation 20 mg of lysozyme was added in a volume of 2.0 ml followed by addition of 8.0 ml of 0.25 M EDTA. The sample was incubated on ice for 10 min. and then 4 ml of 10% SDS was added and mixed quickly. Immediately afterwards, 6 ml of 5 M NaCl was added and the sample set on ice for 1 hr. The sample was then centrifuged for 30 min. at 30,000 rpm at 4°C to remove high molecular weight DNA and bacterial debris. The supernatant was saved and extracted twice with phenol/chloroform and then twice with chloroform. To the aqueous layer was added 0.54 volumes of isopropanol, and the sample mixed and incubated at -20°C for a minimum of 1 hr. The sample was then thawed and centrifuged at 15,000 xg for 30 min. The supernatant was discarded, the pellet washed once with 70% ethanol, dried under vacuum and resuspended in 4 ml TE. For each ml of the DNA solution 1 g of solid CsCl was added and dissolved. The sample was then transferred to a Beckman Quick

seal centrifuge tube and 0.2 ml of a 10 mg/ml ethidium bromide solution was layed on top. The tube was filled to the rim with mineral oil, balanced, and then sealed using a Beckman heat sealer. The tube was then inverted and quickly placed in a Beckman Ti70.1 rotor and centrifuged at 42,000 rpm overnight at 20°C. Two bands were visible under ultraviolet light, the lower band consisting of closed circular plasmid DNA was removed by puncturing the bottom side of the tube with a hyperdermic needle. The ethidium bromide was removed from the plasmid DNA by extracting the sample 4 to 5 times with an equal volume of TE/CsCl saturated butanol. The sample was then extensively dialyzed against 3 two litre changes of TE. The concentration of plasmid DNA in the prepartation was determined by measuring the absorbance of the sample at 260 nm as previously described. The plasmid preparation was stored at 4°C with a couple of drops of chloroform.

13.3 Purification of Probes

To isolate the appropriate piece of DNA required as a probe the plasmids were first cut with the appropriate restriction endonucleases (BRL) at 37°C for 1 hr using 3 units/μg DNA. The C10 plasmid harboring the cDNA sequence encoding the M2 subunit of ribonucleotide reductase was digested with Sal 1 and Pst 1 to yield the appropriate 1487 bp band corresponding to M2 cDNA. The D65 plasmid harboring the cDNA sequence encoding the M1 subunit of ribonucleotide reductase was digested with Nco 1 to yield the appropriate 2000 bp band corresponding to M1 cDNA. The pG3(B)-Sac I plasmid containing the cDNA

sequence for the k-fgf gene was digested with Eco R1 and Sac1 to yeild the 600 bp band corresponding to k-fgf cDNA. The MG4 amplification plasmid (R. Johnson, personnal communication) was cut with Hind III and Pst I to yield the 4.2 kb murine genomic dhfr gene which also was used as a probe. The 618 bp HBV probe used to detect transfected mutant M1 was cut from the pER-dATP^r plasmid using Kpn I and Sac II digestions.

Following digestion the required bands where purified away from the rest of the plasmid on 1% agarose gels in 1 x TBE. After running the samples overnight at 30 volts in TBE running buffer the appropriate band was removed from the gel by placing the gel on a ultraviolet light and cutting out the band. The piece of gel containing the band was then placed in dialysis tubing containing, approximately 1 ml of 0.5 x TBE, and the insert was then electroeluted from the gel by passing a current of 100 volts for 2-3 hours across the sample. Once all of the insert had come out of the gel the current was reversed, and the dialysis bag containing the insert was electrophoresed in the opposite direction for 60 seconds to remove any insert that may have stuck to the dialysis bag during elution. The plasmid insert now in 0.5 X TBE buffer was passed through a mini-column-D (Sigma Chemical Co.) and eluted with 0.5 ml high salt buffer (1.0 M NaCl, 1.0 mM EDTA, 20 mM Tris-HCl, pH 7.4,). The DNA was then precipitated by the addition of 2 volumes of ethanol and overnight incubation at -20°C. The sample was centrifuged, washed once with 70% ethanol, and resuspended in TE buffer in a concentration of approximately 500 ng/ul for subsequent labelling.

14. CONSTRUCTION AND MANIPULATION OF PLASMIDS

14.1 Isolation and Ligation of K-*fgf* and the DHFR Plasmid

The dhfr amplification plasmid consists of the MG4 dhfr minigene (Gasser *et al*. 1982) in a pGEM3 vector (Promega, Madison, WI), in the multicloning region at the Hind III and Pst I site. The genomic k-*fgf* gene in the pG6.6 plasmid (Delli Bovi *et al*.1987) was also cloned into a pGEM3 vector in the multicloning region at the Sal I site. To construct a k-*fgf* amplification plasmid the k-*fgf* gene was isolated and purified from the pG6.6 plasmid and inserted into the dhfr plasmid at the Sal I site of the multicloning region. More specifically 10 μg of the pG6.6 plasmid was cut with 8 units/μg Sal I, for 3 hr. The resulting sample was then electrophoresed on a 1% TBE agarose mini-gel at 100 volts for 3 hr to seperate the genomic 6.6kb k-*fgf* gene from the rest of the pGEM3 vector. The 6.6 kb fragment was cut out of the gel and the insert purified using a Geneclean kit (Bio 101 Inc. La Jolla, CA). The resulting insert was resuspended in TE buffer at a concentration of approximately 0.5 μg/ul. The vector, dhfr plasmid was also cut with 8 units/ug Sal I in the presence of TA buffer (TA buffer contains 33 mM Tris-acetate, pH7.9, 66 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM 2-mercaptoethanol, and 50 micrograms per ml BSA) for 2 hr. The vector was then dephosphorylated using HK phosphatase by first adding 5 mM CaCl to the cut vector and then 1 unit/μg HK phosphatase. The vector was incubated for 1 hr at 30°C and the HK phosphatase was deactivated by incubation of the vector at 65°C for 30 min.. The vector and cut k-*fgf* gene

were then mixed in a ratio of 2:1, vector to insert, in a final concentration of 50 μg/ml total DNA in a total of 20 μl. As a control, vector alone was also diluted to 50 μl/ml in a total of 20μl TE buffer. Ligase, 1 x ligase buffer (5 x ligase buffer is 0.25 M Tris-HCl, pH 7.6, 50 mM $MgCl_2$, 5 mM ATP, 5 mM DTT and 25% polyethlyene glycol-8000) and 10 mM dATP, final concentration, were added to the vector and vector, insert mixture and the samples were incubated at 12°C overnight. The next day the mixture was used to transform HB101 E. coli as described below.

14.2 Construction of the LacM1 Plasmid

To construct an inducible mutant M1 gene we used the pSVlacOCat plasmid (Figge et al. 1988) replacing the chloramphenicol acetyl transferase (CAT) gene with mutant M1. First, the mutant M1 gene was cut out of the plasmid pER-dATP^r obtained from Caras and Martin (1988), at the sfi I sites. The resulting sample was then electrophoresed on a 1% TBE agarose mini-gel at 100 volts for 3 hr and the 3.5 kb fragment representing the mutant M1 gene was cut out of the gel. This insert was subsequently purified using a Geneclean kit (Bio 101) and resuspended in TE at a concerntration of 0.1 ug/ul. The 3' ends of the insert (1 μg) were then subjected to 15 units terminal transferase for 30 min. at 37°C in the presence of 20 μM dGTP such that a tail of dG's was added. The tailed insert was then cut with Hind III, 2 units/ug for 1 hr at 37°C , after which the insert was purified with a Geneclean kit. At the same time the vector pSVlacO-Cat was cut with Bam HI, 2 units/ug for 1 hr at 37°C, and the

resulting linear plasmid subjected to terminal transferase in the presense of 100 μM dCTP. The tailed vector was then partially cut with Hind III, 1 unit/ug for 15 min. at 37°C. This mixture was run on a 1% TBE agarose mini-gel to purify the vector fragment containing the lac operator. The Geneclean kit was used to purify this fragment, and it was resupended in TE at a concentration of 0.1 ug/ul. The resulting purified fragments, vector and insert, were mixed in a molar ratio of 1:1, in a final concentration of 50 μg/ml total DNA in a total of 20 μl. Ligase, 1 x ligase buffer and 10 mM dATP, final concentration, were added to the mixture and the sample incubated at 12°C overnight. The next day the mixture was used to transform HB101 E. coli as described below.

14.3 Transformation of HB101 E. coli.

To find recombinant plasmids the above ligated solution was used to transform HB101 E. coli as follows; HB101 E. coli were first made competent by growing from a single clone of HB101 E. coli, 35 ml of cells in fresh LB broth until the OD_{590} = 0.2 (Maniatis et al. 1982). The cells were then pelleted and resuspended in 15 ml ice cold 50 mM $CaCl_2$. Following incubation for 30 minutes on ice the cells were again pelleted and gently resuspended in 3 ml ice cold 50 mM $CaCl_2$. The suspension was then aliquoted into sterile eppendorf tubes each containing 10% glycerol and stored at -70°C until required. The ligated plasmids were diluted to 100μl with TE and mixed with the competent E. coli and set on ice for 30 min. The E. coli cells were

then heat shocked at 42°C for 2 min. and incubated at 37°C in 1 ml LB broth. The mixture was centrifuged, resuspended in 100 μl LB broth, and spread on LB plates containing 50 μg/ml ampicillin. The plates were incubated overnight at 37°C, after which colonies were picked and used to inoculate 5 ml LB medium containing ampicillin, such that a mini plasmid preparation could be carried out to identify recombinant plasmids.

14.4 Mini Plasmid Preparation

From 5 ml of each transformed E. coli colony grown overnight at 37°C (as described above), 0.2 ml were set aside. The remaining 4.8 ml were centrifuged and the pellet resuspended in 90 μl 50 mM glucose, 10 mM EDTA, 25 mM Tris-Cl pH 8.0 plus 10 μl of 10 mg/ml lysozyme (modification of Maniatis et al. 1982). The suspension was set on ice for 10 min, after which 200 μl of 0.2 N NaOH and 1% SDS was added and the solution mixed thoroughly. The mixture was incubated on ice for a further 5 min. and then 150 μl of 3 M sodium acetate was added. The mixture was again incubated on ice for 10 minutes after which the preparation was centrifuged and the pellet discarded. To the supernatant, 1 ml of absolute ethanol was added to precipitate plasmid DNA. The mixture was placed at -20°C for 30 min. and then centrifuged at 4°C in a microcentrifuge for 15 min. The resulting pellet was washed with 70% ethanol and resupened in 20 μl of TE. To confirm recombinant plasmids had been constructed, 5 μl of the mini plasmid preparation was digested with an appropriate restriction endonuclease, as well as vector and insert as controls,

and run on a 1% agarose gel containing ethidium bromide so that the resulting bands could be analyzed and a picture taken.

14.5 CAT Assays

The Lac inducible plasmid system consisted of the Lac operator next to the CAT gene, pSVlacOCAT, and the Lac repressor, pCMVlacI. Together with a plasmid bearing a drug resistant marker these three plasmids must be transfected into cells in a particular ratio to ensure sufficient repressor is present to bind the operator in the absence of isopropyl β-D-thiogalactoside (IPTG) (Barkley and Bougeois, 1980). Too much repressor would not allow full induction of the Lac operator in the presence of IPTG. To determine the ratio of the plasmids that would permit functioning in a particular cell line, transient transfections were performed and the level of CAT activity was determined (Sliegh 1986). Transient transfection was performed as described under 'transfection' using the calcium phosphate method with the exception of the drug marker plasmid. All the cells (7×10^6) used in the transfection were harvested 60 hr following the glycerol shock. This was done for various molar ratios of pSVLacOCAT to pCMVlacI plasmid in the presence and absence of 15 mM IPTG.

Cells were harvested, washed, resuspended in 50 μl of PBS and lysed by freeze thawing. Following heat inactivation of the protein supension at 65°C for 5 min. the mixture was spun and the supernatant retained to determine the amount of CAT activity. First, the amount of protein in the supernatant was determined using the Bio-Rad

determination as described previously (page 39). From the supernatant 30 μg of protein was diluted to 60 μl with 1 M Tris-Cl pH 7.4. To this was added 20 μl of 8 mM cholamphenicol and 20 μl of 1 μCi C^{14}acetylCoA plus 0.5 mM cold acetylCoA. This mixture was incubated for 4 hr at 37°C after which 100 μl of cold ethyl acetate was added. The mixture was vortexed, spun and the top 90 μl removed and placed in a scintilation vial. This procedure was repeated with another 100 μl cold ethly acetate. To the scintilation vial 5 ml of scintiverse II (Fisher Scientific, Nepean, Ont) was added and the vials examined for radioactivity. Results are shown as the cpm/ug/hr.

14.6 Transfection into Mouse Cell Lines

When a positive recombinant plasmid was identified, the 200 μl of HB101 E. coli set aside (page 61) was used to inoculate 5 ml LB medium plus ampicillin so that a large scale plasmid preparation could be done for subsequent uses, such as in transfection experiments. Two different methodologies were used to transfect mouse cell lines: either Lipofection (Felgner et al. 1987, as described in the BRL manufacture's specifications) which was used with the k-fgf, dhfr plasmid), or calcium phosphate transfection (Graham and Van der Eb 1973, as described in the BRL manufactures specifications) which was used in all other cases.

Cells were prepared for Lipofection transfection experiments by plating 2 x 10^5 cells/60mm plate in α-MEM plus 10% FCS and incubating these overnight at 37°C. The next day 10 μg of k-fgf, dhfr plasmid DNA was mixed with 1 μg PY3 marker DNA and the solution diluted to

100 μl in sterile water. This was mixed with the lipofection reagent as described by BRL also diluted 60μl/100μl in distilled water, and the solution incubated at room temperature for 15 min. In the meantime, the plates containing the NIH3T3 cells were washed with PBS and given 6.0 ml of growth medium without serum. The lipofection, DNA mixture was then added to the plates containing growth medium with no FCS and the cells were incubated overnight at 37°C. The next day the plates were again washed, and new growth medium containing 10% FCS was added. Two days later the medium was changed and 0.2 mg/ml hygromycin was added to the plates. Approximately 10-14 days later drug resistant colonies appeared, and these were picked with a pasteur pipette and transferred to 60 mm plates, where the clones were allowed to grow up. These were then analyzed by Southern and Northern blot analysis to confirm integration and expression of foreign DNA.

For calcium phosphate tranfection experiments, cells were plated at 2 x 10^5/60mm plate and were incubated overnight in α-MEM plus 10% FCS. The following day plasmids to be transfected, LacM1, pCMVlacI, and PY3 were mixed in a 7:1:0.6 molar ratio in approximately 17 ug DNA. This mixture was diluted to 120 μl with sterile water and 120 μl of solution A, as described by BRL in the manufactures specifications. Following mixing, the solution was incubated at room temperature for 10 min. with 240 μl of buffer B (as described by BRL) and the solution vortexed and the precipitate incubated at room temperature for 20 min. The calcium phosphate-DNA precipitate was to added the the cells (without removing the medium) and the cells

incubated for 6-12 hr at 37°C. The precipitate was removed and the cells were washed twice with PBS. The cells were then glycerol shocked, to enhance uptake of the precipitate by adding 1.0 ml of 15% glycerol in isotonic Hepes buffer for 2 min. Following washing, growth medium was added to the cells. Two days later the medium was changed and 0.2 mg/ml hygromycin was added to the plates. Resistant colonies were picked using a pasteur pipette 10-14 days later (Thompson et al. 1971).

15. Western Blot Analysis

To determine the amount of protein produced by transfected cell lines, 5 x 10^6 cells were first pelleted, then resuspended in 10 mM Tris-Cl (pH 8.0) and sonicated. Centrifugation at 5000 xg for 5 min. removed cellular debris leaving cytosolic protein. The concentration of protein was determined using an aliquot of the solution in the Bio-Rad protein assay described previously (page 39). A determined amount of protein was prepared for SDS polyacrilamide gel electrophoresis by addition of sample loading buffer containing 3% (w/v) SDS, 10% glycerol, 5% β-mercaptoethanol, 0.05% (w/v) bromophenol blue and 0.6 M Tris-Cl (pH 6.8). The sample was denatured by boiling for 5 min. followed by chilling and centrifugation. The supernatant was then loaded onto a 10% SDS polyacrylamide gel (Laemmli, 1970) composed of 1% SDS, 10% polyacrylamide and 0.4 M Tris-Cl (pH 8.8). Following electrophoresis on a vertical slab gel (Protean, Bio-Rad Laboratories), in running buffer containing 25 mM Tris, 200 mM Glycine and 0.1% (w/v) SDS (pH 7.5), for approximately 1-4 hr at a

constant current of 30 mAmp, the gel was transferred to nitrocellulose membranes by the method of Towbin et al. (1979). Briefly, the gel was removed from the glass plates and equilibrated in the transfer running buffer of 25 mM Tris and 200 mM glycine for approximately 1 hr. Pre-wet (in distilled water) nitrocellulose was then laid on top of the gel between two pieces of Whatman filter paper and sandwiched together in a cassette which was placed in a transfer tank with the membrane closest to the positive electrode. Proteins were transferred for 4-18 hr at 60 volts. After transfer, the nitrocellulose filter was blocked in TBS-Tween (50 mM Tris-Cl, pH 7.6, 150 mM NaCl, and 0.5% v/v Tween 20) plus 1% w/v BSA for 1 hr. The filter was then incubated in the TBS-Tween overnight containing the first antibody in the cold at 4°C. Both the M1 monoclonal and the k-fgf polyclonal antibodies were used in a 1:200 dilution. The following day the filter was washed three times for 30 min. each in TBS-Tween after which the second antibody conjugated to alkaline phosphatase was incubated with the filter for 1.5-3 hr. After washing three times the filter was placed in 100 ml of 10 mM $NaHCO_3$ and 0.1 mM $MgCl_2$. To this 1 ml of nitro blue tetrazolium (0.03 g/ml in 70% dimethylformamide) and 1 ml of bromochloroindolyl phosphate (0.015 g/ml dissolved in 70% dimethylformamide) was added. The filter was allowed to develope for 10-30 min. at which time developing was stopped by placing the filter in water.

16. LECTIN CELL BINDING ASSAYS

16.1 ^{3}H-Concanavalin A Binding Assay

A predetermined number of cells were plated out either on to 60 mm plates or 6-well plates for experiments containing drug, and grown to approximately 70% confluence. The growth medium was changed 20 hr prior to the binding assay to α-MEM without FCS (as described by Spearman et al. 1987). Before addition of the lectin, cells were precooled on ice for 5 min and then washed twice with 2 ml ice cold Concanavalin A (Con A) binding buffer (0.85% NaCl, 0.01 M NaN_3, 0.001 M $CaCl_2$, 0.001 M $MnCl_2$, and 0.001 $MgCl_2$, pH 7.0). The cells were then covered with an additional 2ml cold binding buffer and left on ice for a further 40 min. To start the binding assay 0.5 ml of ice cold buffer supplemented with either various concentration of cold Con A or the standard concentration of 100 μg/ml Con A (Sigma Chemical Co.) plus [^{3}H]-acetyl-N-acetylated Con A [43.5 Ci/mmol (New England Nuclear, Mississauga, Ont.)] was added to a final activity of 4 nCi/μg. The cells were then incubated either for a set time, 20 min., or set concentrations of Con A, 100 μg/ul. Where the effect of a drug was examined, the cells were grown in drug and the binding assay was done for 20 min. with 100 μg/ml cold Con A. Following completion of the binding assay the lectin solution was aspirated and the cells washed 4-5 times with 2.0 ml cold binding buffer. The cells were subsequently solubilized in 1.0 ml of 10% triton X-100 overnight at 37°C. Cells were removed from plates using a rubber policeman and the plates were rinsed once with distilled water. The

cells were then counted in 5.0 ml Scintiverse II (Fisher Scientific). Non-specific binding was measured in the presence of 0.2 M α-methlymannoside. Results were calculated by first subtracting non-specific binding and then expressing the counts as μg Con A/mg protein. The effect of glycosylation inhibitors on ^{3}H-Con A binding was examined by the above described procedure, growing the cells in increasing concentrations of drug and doing the binding assay for 20 min. using 100 μg/ml Con A.

16.2 Soybean and Wheat Germ Agglutinin Binding Assay

The procedure followed is similar to the one described above. Wheat germ agglutinin (WGA) and Soybean agglutinin (SBA) were purchased from the Sigma Chemical Co. and labelled with ^{125}I following the protocall described by Tanner and Anstee (1976). Ten mg of WGA or SBA was dissolved in 1.0 ml of binding buffer (8.0 g/L NaCl, 0.2 g/L KCl, 0.2 g/L KH_2PO_4, 1.15 g/L Na_2HPO_4, 0.13 g/L $CaCl_2$ and 0.2 g/L $MgCl_2$, pH 7.0) with 250 mM of the appropriate sugar inhibitor to protect the active site. The lectin solution was then iodinated by addition to 1 mCi of ^{125}I and 200 μl of a 1 mg/ml solution of choramine T. This mixture was incubated at room temperature for 1 min. and then 100 μl of 2.4 mg/ml Na metabisulfate and 0.2 ml of 10 mg/ml potassium iodide were added. Labelled lectin was then passed through a Biogel P10 or P6 (Sigma Chemical Co.) coloumn eluting with binding buffer. When required for a binding assay labelled lectin was added such that there were approximately 100,000 cpm/0.5 ml. Except for the binding buffer, the binding assay

was performed as previously described for Con A. Non-specific binding was measured in the presence of 0.2 M N-acetylglucosamine (GlcNAc) in the case of WGA, and 0.2 M N-acetylgalactosamine (GalNAc) in the case of SBA.

16.3 FITC Soybean Agglutinin Binding Assay

Cells required to examine the amount of fluorescein isothiocyanate (FITC)-SBA bound were grown on 150 mm tissue culture plates to a density of 70%. The growth medium was then removed 20 hr before the actual assay and replaced with medium without serum. Cells were removed from tissue culture plates using 2 mM EDTA instead of trypsin solution and the EDTA was neutralized using α-MEM containing 2% BSA. The cells were then pelleted and washed twice with SBA binding buffer to remove any residual BSA. Cells were counted using a hemocytometer and resuspended at a density of 5×10^5 cells per 500 μl of binding buffer. To this mixture 250 μg of FITC-SBA was added and in the control, 250 μg of FITC-SBA plus 0.2 M GalNAc was used. This was incubated on ice for 30 min. with regular mixing of the solution. The cells were then washed 3 times with SBA binding buffer, resuspended in 1.0 ml binding buffer and analyzed using a Coulter Epics V multiparameter sensor system (Blosmanis et al. 1987). In this analysis 10^3 cells were counted and the mean flourecent intensity determined. Results are shown as the mean flourescence intensity minus the mean flourescence intensity of cells in the presence of 0.2 M GalNAc.

17. Effect of Glycosylation Inhibitors on the Metastatic Potential of Mouse Cells

To examine if glycosylation inhibitors had any effect on the metastatic potential of the 10T½ cell line dGC2M5 these cells were grown for 48 hr on 150 mm tissue culture plates in regular growth medium containing the concentration of drug that was shown to have a maximal effect on Con A binding with these cells: 2.9 μM swainsonine, 0.05 mM castanospermine and 1.0 mM deoxymannorjirimycin. The cells where then harvested using very mild trypsin treatment, washed once with PBS and resuspened in a concentration of 0.5-1.5 x 10^6 cells/ml. Between 1 and 3 x 10^5 cells were injected intravenously in a volume of 0.2 ml into syngenic C3H/HeJ mice. Three weeks later mice were sacrificed and the number of lung tumors determined as previously described (page xx). Results are shown as a percent inhibition of lung metastases as compared to cells grown without drug and examined at the same time.

18. SBA Precipitation of ^{125}I-Labelled Glycoproteins and SDS-PAGE

To identify the lectin binding glycoprotein, twenty 150 mm plates of the wild type NR4 and mutant dGC2M5 were grown to approximately 80% confluence. Cells were harvested following 18 hr incubation in serum free α-MEM using 2 mM EDTA and neutralizing with α-MEM plus 2% BSA. Cells were washed and resuspended in SBA binding buffer as previously described (Lang et al. 1988). An aliquot of cells was sonicated and the amount of protein was determined. The

suspension was then diluted to contain 20 mg protein/ml. This mixture was iodinated by the lactoperoxidase/H_2O_2 method (Ledbetter et al. 1981). Briefly 1 mCi/ml of ^{125}I was added to the cell suspension followed by 30 μl of a 166units/ml lactoperoxidase stock (Sigma Chemical Co.) and 10 μl each of 0.33 mM, 1 mM, 3 mM and 9 mM H_2O_2. Between each H_2O_2 addition the cells were left at room temperature for 5 min. After the last addition the suspension was washed 3 times with SBA binding buffer. The final pellet was resuspended in 1 ml of SBA binding buffer. To half of the cells, 500 μl, 100 μg of biotin-conjugated SBA (Sigma Chemical Co.) was added and the cells were incubated on ice for 45 min. After washing the cells coated with lectin, and those without, they were solubilized in lysis buffer [SBA binding buffer containing 1% NP40 and 1 mM phenylmethylsulfonylfluoride (PMSF)] sonicated and incubated on ice for a further 15 min. Nuclei and insoluble material were removed by centrifugation for 10 min. at 3500 rpm in a microcentrifuge. Lysates were diluted to 0.2% NP40 with PBS, and 1mM PMSF followed by addition of 1 unit avidin conjugated to agarose beads (Sigma Chemical Co.). The controls contained only cell extract and avidin agarose with no lectin added. These suspensions were then incubated for 1 hr at 4°C by over-end rotation. The agarose complex was washed five times with PBS containing 0.2% NP40. Bound glycoproteins were eluted with 0.25 M GalNAc for 30 min. at 40°C. The resulting eluant was subjected to SDS-PAGE using a 10% gel as described in Western blot analysis (page 65) using ^{14}C BRL standards. The gels were subsequently silver stained as described below, dried, and exposed at

-70°C for 3 days to Kodak X-Omat AR fild and Cronex Lightning Plus intensifying screens.

18.1 Silver staining of SDS-PAGE gels

Gels were first fixed in 7.5% acetic acid for >2 hours and then soaked in 50% methanol for >1 hr (modification of Morrissey, 1981). Following rinsing of the gel 3-4 times with distilled water the gel were incubated for 30 min. in 100 ml of 5 μg/ml DTT, then 30 min in 100 ml of 0.1% $AgNO_3$. The gels were again washed 2 times with water and rinsed 2 times in 50ml developer (3% Na_2CO_3 plus 100 μl formaldehyde/200 ml distilled water). Gels were developed in 100 ml developer for approximatley 10 min. Developing was stopped by removing the developer and adding 1% acetic acid. Gels were subsequently neutralized in distilled water for 30 min.

19. Preparation of Conditioned Medium from kFGF Producing Cells

Conditioned medium was prepared from exponentially growing cells. The cells were harvested and counted. An aliquot of 5 x 10^5 cells was plated in a 60 mm plate in medium containing 10% FCS. After 3 hr the growth medium was removed and the cells were washed twice with PBS. Two ml of defined medium, α-MEM plus 10 μg/ml insulin, 5 μg/ml transferrin and 10 μg/ml heparin, was then added to the cells, and the cells were incubated at 37°C for 20 hr. Following the 20 hr incubation the medium was removed and labelled as conditioned medium.

20. Measurement of HGPRT Enzyme Activity In vivo

HGPRTase activity was determined in vivo as previously described (Ullman et al. 1979b). Briefly 10^5 cells were grown in 6 well plates in the absence of label overnight. The following day 10 μM ^{3}H-hypoxanthine was added and the cells were harvested at time 0, 30 min., 60 min. and 90 min. The cells were then washed twice with PBS, resuspended in 50 μl of distilled water and sonicated. The entire cell lysate was placed on DEAE-Whatman filter discs and washed 6 times with 3 liters of water. Each disc was counted in 5 ml Aquasol-II (New England Nuclear).

21. TCA precipitated DNA

Cells were seeded at 10^5 cells/well in 6 well plates and incubated at 37°C overnight. Incorporation of ^{3}H-deoxyadenosine, ^{3}H-deoxycytosine, ^{3}H-deoxyguanosine, ^{3}H-guanosine, and ^{3}H-hypoxanthine into DNA was measured following addition of 0.3 μM of each nucleotide (Nicander and Reichard 1985). As a control, uptake was also measured for 1 μCi/ml of ^{3}H-thymidine. Following incubation of the cells in each labelled nucleotide for 2.5 hr at 37°C, the cells were washed 3 times with PBS and 1 ml of 0.3 M NaOH was added. This mixture was incubated for 18 hr at 37°C. The following day 3 ml of 10% ice cold TCA was added to each plate and the cells were incubated at 0°C for 30 min. The TCA precipitated DNA was then passed through GF/A Whatman glass filter discs pretreated with 10% TCA and the discs washed with 95% ethanol. Discs were then counted in 5 ml Aquasol-II

(New England Nuclear). Results are shown as disintigations per minute (dpm) for each nucleotide/dpm of thymidine incorporated.

RESULTS

1. GENETIC INSTABILITY IN TUMOR PROGRESSION

1.1.a. Characterization of the mutator and amplificator mutants

The phenotypes of the spontanteous mutation rate and amplificator mutants used in this study were examined before testing the hypothesis that cells with increased rates of mutation (point mutation and gene amplification) have an increased rate of production of metastatic variants. To test that the thy-49 cell line isolated by Meuth's laboratory (Meuth et al. 1979) exhibited the mutator phenotype, due to a mutant CTP synthetase, deoxyribonucleoside triphosphate pools under specific culture conditions were examined. The cells were maintained in α-MEM, 10% FCS, and 10 μM thymidine until twenty four hours before harvesting. Cells were then washed and the medium was replaced with α-MEM, 10% dFCS and either 1 μM or 100 μM thymidine. Analysis of the deoxyribonucleoside triphosphate pools by HPLC (see Figure 2) confirmed that under these culture conditions deoxyribonucleotide pools had changed in keeping with the changes in mutation rates reported by Meuth et al. (1979). Table 1 shows that when the mutant thy-49 cells were grown in 1 μM of thymidine the dTTP pool was lower, and the dCTP pool much higher, than cells grown in 100 μM thymidine. The dATP pool remained constant. The dCTP/dTTP ratio for the cells grown in 1 μM thymidine was approximately 15, whereas the dCTP/dTTP ratio for the cells

Figure 2

Actual HPLC tracings of deoxyribonucleotides isolated from cell extracts following elution from a Whatmann Partisal 10-SAX anion exchange column with 0.4 M ammonium phosphate buffer. Peak (1) is dCTP, peak (2) is dTTP, peak (3) is dATP, while peak (4) is dGTP. Intergration of peaks was obtained from a Hewlett-Packard integrator.

A) Standard deoxyribonucleotides were at a concentration of 18.7 nM

B) 50 μl of Thy-49 cell extract from 10^7 cells grown in 1μM thymidine

C) 50 μl of Thy-49 cell extract from 10^7 cells grown in 100μM thymidine

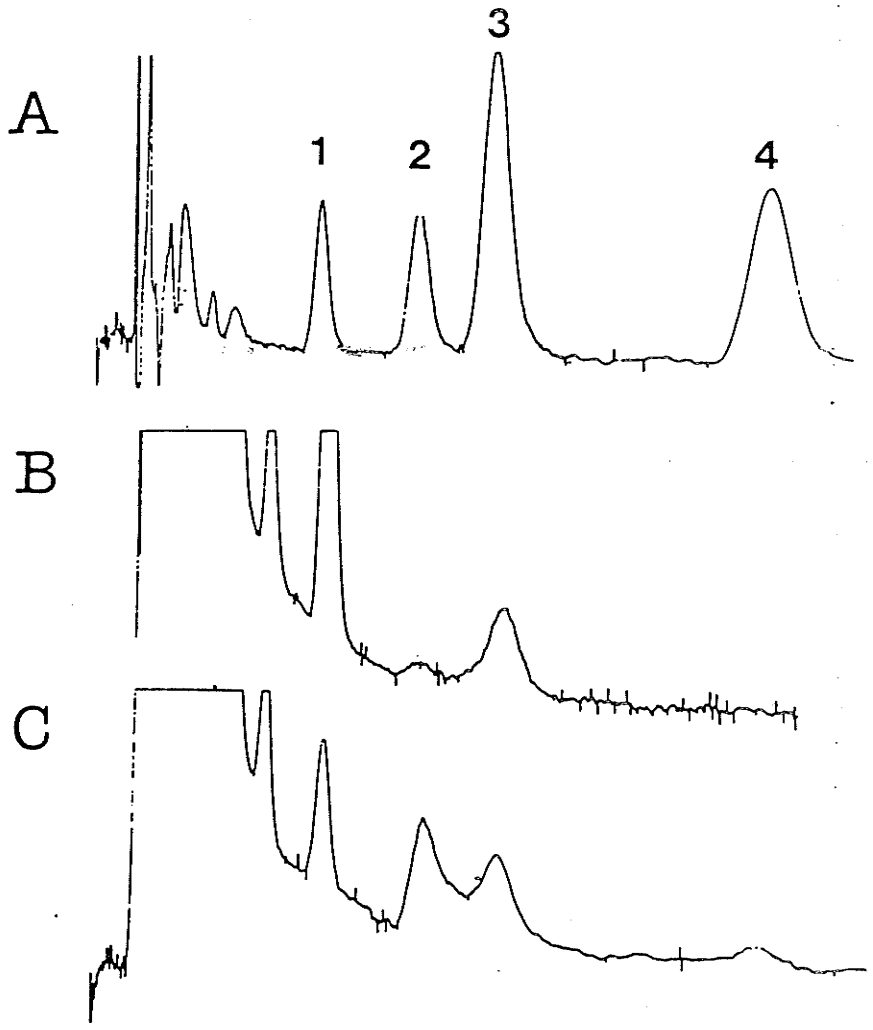

A
1
2
3
4
B
C
Time

Table 1.

Deoxyribonucleoside triphosphate pool concentrations in the mutant thy-49 CHO cells grown in either 1 μM or 100 μM thymidine. The concentration for dGTP was very low and difficult to detect. It was thus ommitted from the analysis.

Thymidine concentration μM	pmoles/μg of DNA (mean ± SE) dCTP	dTTP	dATP
1.0	14.89 ± 2.64 (4)	<1.00 (5)	1.32 ± 0.19 (5)
100	1.61 ± 0.76 (5)	8.04 ± 0.58 (5)	1.00 ± 0.22 (5)

the number of determinations is shown in brackets

grown in 100 μM thymidine was much lower, (0.2). Meuth's laboratory has shown that it is this change in deoxyribonucleotide pools that affects the fidelity of DNA replication and the rate at which point mutations occur (Phear et al., 1987).

The amplification rates of mutant lines isolated by Stark's laboratory (Giullotto et al. 1987) were analyzed by examining the number of resistant colonies per 10^5 cells in varing concentrations of corformycin as described by Giulotto et al. (1987). Table 2 shows that the wildtype BHK cell line formed few corformycin resistant colonies whereas the mutants, YMP1 and YMP7 produced significantly more colonies and grew better in the same concentration of the drug. Although the mutation(s) that gives these cells their amplificator phenotype has not yet been identified, they have been characterized using various drugs in which resistance has been shown to be the result of gene amplification. Corformycin for example, inhibits AMP deaminase and gene amplification of the AMP deaminase gene has been shown to be responsible for corformycin resistance (Debatisse et al., 1986). These results confirm that the YMP mutants are more resistant than wild type cells to corformycin in agreement with the results of Giullotto et al. (1987) and Rolfe et al. (1988) and the view that they have maintained their amplificator phenotype.

1.1.b. Tumorigenicity and Metastatic characterisics of the Mutator and Amplificator mutants

The thy-49 CHO cell line and the BHK cell lines used in this

Table 2.

Frequency of corformycin resistant colonies/10^5 cells for the BHK wild type and YMP1 and YMP7 mutants. This is a typical example of resistance to corformycin for these cell lines (done in triplicate) as observed in three independent experiments.

Concentration of Corformycin μg/ml	Resistant colonies per 10^5 cells (mean[a]) BHK	YMP1	YMP7
0.8	21.9	confl	>350
1.0	3.0	>200	100.3
1.2	1.2	98.9	29.9

where confl, indicates confluent.

a) This is a typical example of resistance to corformycin for these cell lines (done in triplicate) as observed in three independent experiments.

study had not previously been tested for tumorigenic or metastatic potential. Before analyzing the effect of genetic instability on the the ability of these cells to generate metastatic variants, these characteristics were first determined. Examination of anchorage independent growth (characteristic of tumor cells) was determined by the ability of these cells to grow in low percentage agar. As shown in Table 3 all lines were found to grow well in low percentage agar with an average of approximately 10% of the plated cells producing colonies. The ability of these lines to form tumors in mice was examined by injection of 2 x 10^6 cells subcutaneously into the midback of nu/nu mice. The latency or time at which a tumor of minimum size (2 x 2mm) was first observed, as well as the number of mice in which a subcutaneous tumor formed, was also recorded. The growth rate of the tumor was measured for 4-5 weeks. Both the thy-49 CHO and the BHK cell lines were found to be highly tumorigenic in nude mice. Their tumor growth rates can be seen in Figure 3.

The metastatic potential of thy-49, the BHK cell line and the mutant clones YMP1 and YMP7 was determined following injection of 4 x10^5 cells of the CHO line and 2 x 10^5 cells of the BHK cell lines intravenously into nu/nu mice and examination of the resulting lung tumors. Low passage (passage 1 - passage 5) cells were used and in both cases, the thy-49 CHO cells were found to have a low metastatic potential (Table 3), while the BHK cell lines were found to have a much higher metastatic potential. Since the thy-49 cell line could not be maintained in medium containing 1 μM thymidine for long periods of time, the metastatic potential of the population was

Table 3.

Tumorigenicity and metastatic characteristics of the CHO mutator line, thy-49, and BHK wild type cell line and amplificator mutants, YMP1 and YMP7.

Cell line	Tumorigenicity		Exptl. metastasis		Anchorage independent colonies/ 10^3 cells (mean[e]±SE)
	frequency of mice with tumors	latency (days mean±SE)	frequency of mice with tumors	No. (mean±SE)	
Thy-49[a]	7/7	11.6 ± 1.1[b]	7/8	2.75 ± 1.0[c]	65.2 ± 7.3
BHK	6/7	9.9 ± 1.6[b]	5/5	129.0 ± 41.3[d]	88.3 ± 8.0
YMP1	7/7	4.6 ± 0.2[b]	5/5	96.8 ± 39.5[d]	105.0 ± 7.0
YMP7	7/7	10.7 ± 0.8[b]	5/7	15.2 ± 9.0[d]	109.0 ± 13.6

a -cells were grown in medium containing 10μM thymidine

b -$2x10^5$ cells in 0.1ml were injected subcutaneously into 7 mice each

c -$4x10^5$ cells in 0.2ml were injected intravenously into 8 mice each

d -$2x10^5$ cells in 0.2ml were injected intravenously into 5-7 mice each

e -the mean is the average number determined from 2 independent experiments consisting of five plates/experiment.

Figure 3

Growth rates of tumors in mice following injection of the following cell lines:

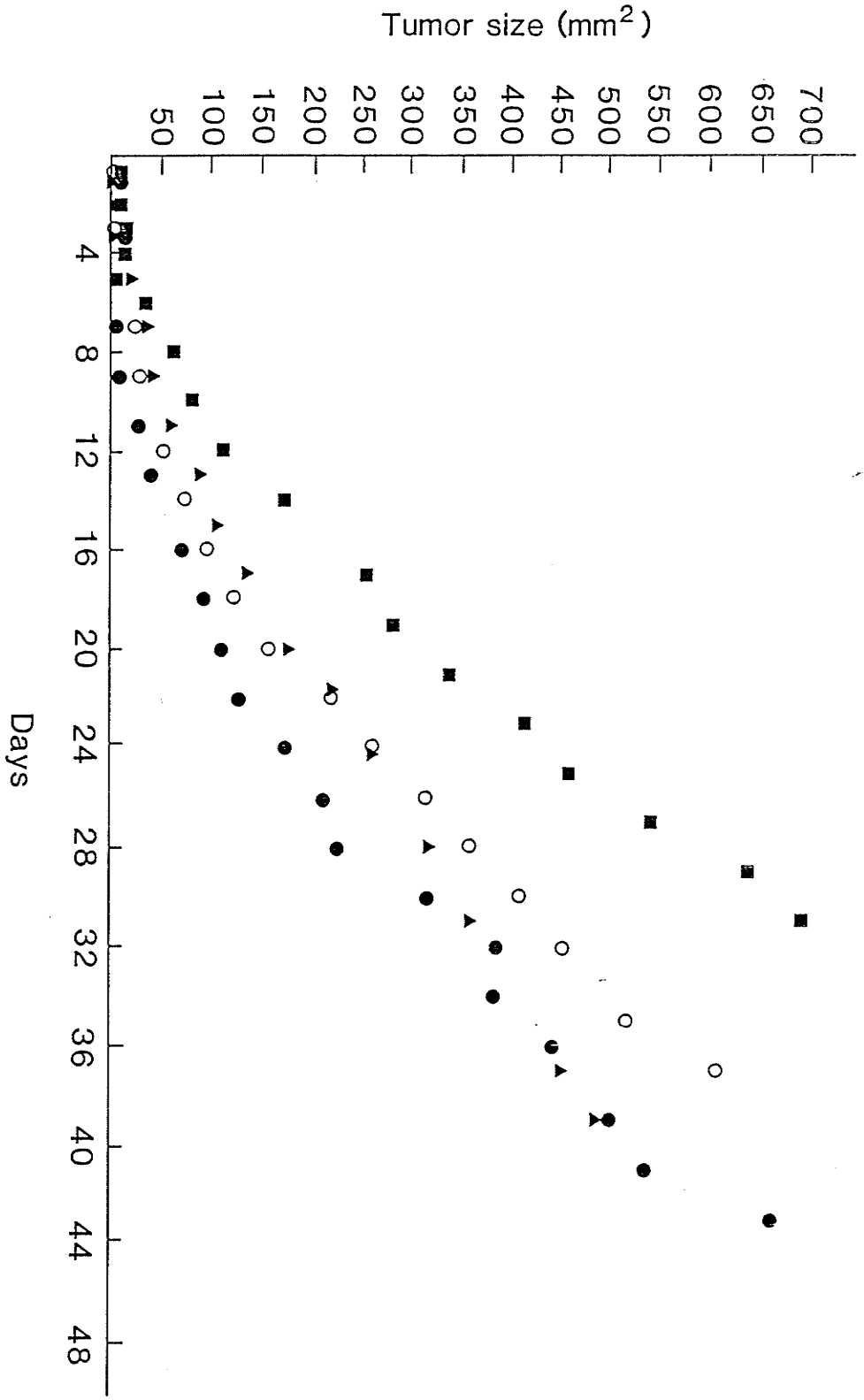
○ thy-49

● BHK wildtype

■ YMP1

▲ YMP7

2×10^5 cells from each cell line was injected subcutaneously into the lower midback of either BALB/c or CD-1 nu/nu mice and tumor growth was analysed for 5 weeks as described in Materials and Methods.


Tumor size (mm²)
700
650
600
550
500
450
400
350
300
250
200
150
100
50
4
8
12
16
20
24
28
32
36
40
44
48
Days

determined for cells grown in regular serum and 10 μM thymidine. The wild type line of thy-49, which is auxotrophic for proline (pro-), was also examined for experimental metastasis and no differences were found in the number of lung tumors between these two lines (data not shown). In summary, the cell lines used for this study were found to be similar in their tumorigenic characteristics, both for the mutant and the wild type lines, and, both were metastatic so that the rate of generation of metastatic variants could be determined.

1.1.c. Rate of Metastasis

The hypothesis proposed by Nowell (1976), suggests that genetic instability may be the cause of tumor progression leading to the metastatic phenotype. To more directly test this theory, the rates at which the BHK and CHO mutants formed metastatic variants were examined using the technique described by Hill et al.(1894). This technique allows the evaluation of the rate at which a cell from a clone can generate metastatic variants. If genetic instability is the cause of tumor progression, then cells with an increased rate of spontaneous point mutation or increased rate of gene amplification should produce metastatic variants at a higher rate than their normal counterparts.

To determine the rate of metastasis, parallel clonal isolates must first be grown to a defined cell number. A critical population size needs to be attained before there is significant probability that variants will occur in that population. Also, if one hypothesi-

zes that the spontaneous mutation rate or rate of gene amplification is important in generating metastatic variants, the critical population must be large enough to accomodate these phenomena. Typically for the wild type situation, the critical population size should be one in which a small number of variants is observed. If there is an increase in the rate of generation of metastatic variants in the mutant line it can be easily detected. By definition, using Poisson statistics, the critical population size is the clone size at which the probability of at least one variant per clone, in a set of parallel clones, is equal to one half (Harris et al., 1982). Ten clones for each culture condition were examined with the thy-49 cell line. Each of the clones were grown to final critical size of 2 x 10^6 cells and 4 x 10^5 cells were injected into five mice. Figure 4 shows the number of lung tumors in each of the five mice injected plotted in rank order. The rate of generation of metastatic variants was calculated from these numbers. No significant difference in the rate of generation of metastatic variants was observed for this mutant line grown under the two different culture conditions (Table 4).

The rate of generation of drug resistant amplification mutants was found by Giuotto et al. (1987) to be approximately 5 x 10^{-5} mutations/cell/generation for the BHK cell line. Higher rates were shown for the mutants, YMP1 and YMP7. To test the rate of generation of metastatic variants between these lines, the cells were cloned and grown to a critical population size of 1 x 10^6 cells, ensuring the occurence of gene amplification. For each cell line ten parallel

Figure 4

The number of lung tumors per 4×10^5 cells injected per mouse for each of ten clones shown in increasing rank order. The filled circles represent the mutator line thy-49 clones grown in 100 μM thymidine, the open circles represent clones grown in 1 μM thymidine.

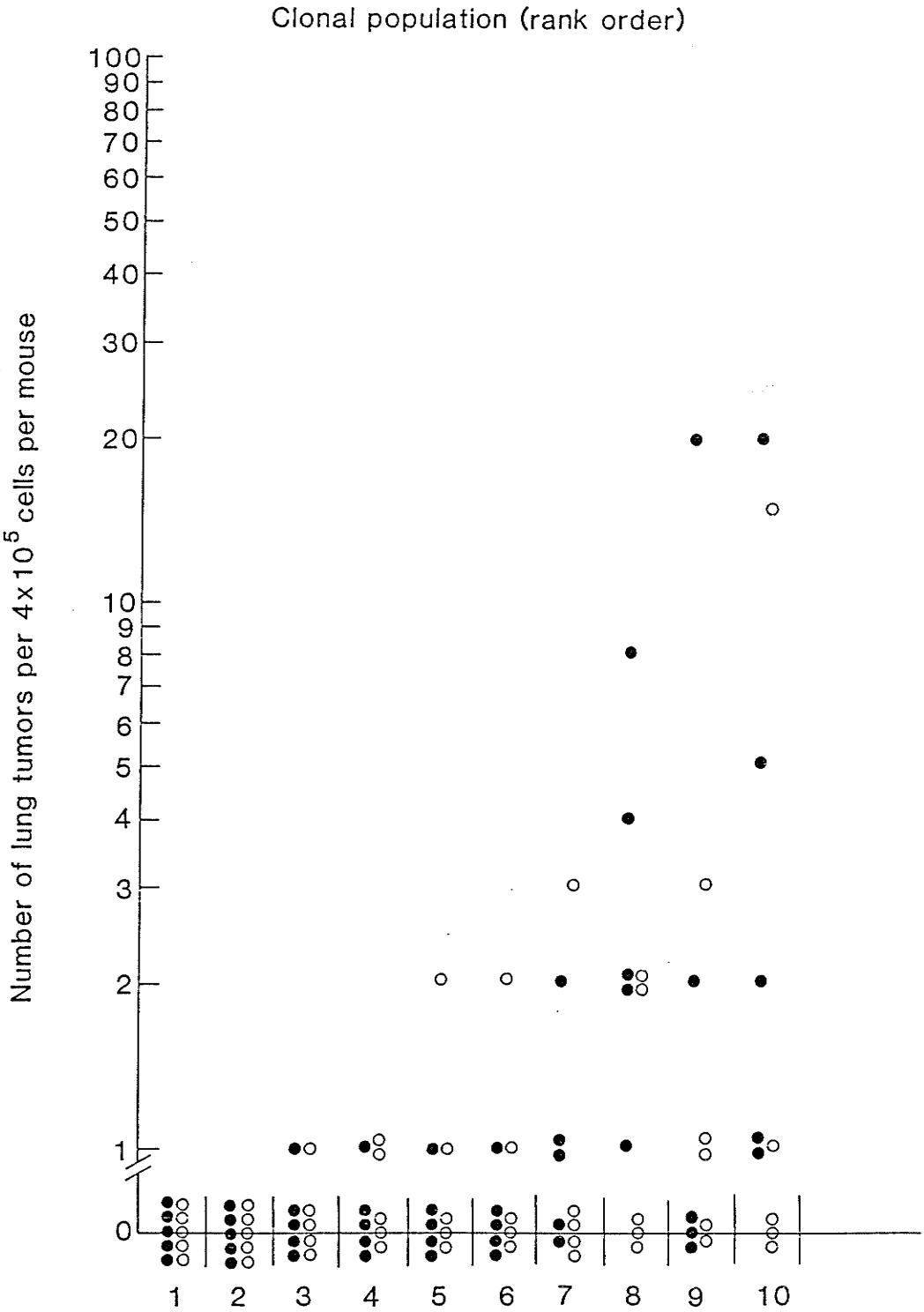

Clonal population (rank order)
Number of lung tumors per 4×10^5 cells per mouse
100
90
80
70
60
50
40
30
20
10
9
8
7
6
5
4
3
2
1
0
1
2
3
4
5
6
7
8
9
10

Table 4.

Rate of metastatic formation in the mutator, thy-49 cell line, the BHK wild type cell line and the amplificator mutants, YMP1 and YMP7

Cell line	Mean No. of lung Metastases/clone ± SE	Rate of metastasis x 10^{-6} /cell/generation
thy-49 100μM	7.30 ± 3.24	1.07 ± 0.33[a]
thy-49 1μM	3.60 ± 1.37	0.67 ± 0.22
BHK	55.2 ± 20.2	10.4 ± 3.30
YMP1	37.6 ± 14.3	8.2 ± 2.70
YMP7	19.1 ± 17.6	4.3 ± 3.00

a- the error was determined by calculating for the mean plus and minus the standard error of the rate, using the Luria and Delbruck equation $r = aN \ln(CaN)$, and subsequently determining a range of values for the rate, the largest difference between the range and the mean value becoming the error.

clones were again prepared from low passage BHK, YMP1 and YMP7 clones. Injection of 2 x 10^5 cells per mouse, into five mice, shown in Figure 5, demonstrated no significant difference between the wild type and mutant cell lines in the rate of generation of lung metastases (Table 4). The variation in lung tumors between parallel clones for the wild type BHK cell line observed in Figure 5, is high for this type of analysis. However, 50% of the BHK wild type clones showed a low number of lung metastases and with the mutants YMP1 and YMP7 more than 50% of the clones showed low lung metastases. The critical population size used ensured that the process of gene amplification would take place in the generation of clones. In summary, Table 4, shows that the mutant cell lines have no increase in the rate at which they produce lung metastases and even show as light decrease. All the rates shown for metastasis are typical of those found for other lines such as the KHT fibrosarcoma and B16F10 melanoma cell lines (Young and Hill 1986, Harris et al. 1987).

1.2.a. Construction of the Inducible M1 Vector

To further examine the effect of spontaneous mutation rates in other cell lines the only cloned gene encoding a dominant mutator phenotype, (the mutant M1 subunit of ribonucleotide reductase), was used to determine whether cells following stable transfection would exhibit a mutator phenotype and whether transfected lines exhibited an increase the rate at which they produced lung metastases. To do this an inducible expression vector and a tumorigenic but poorly

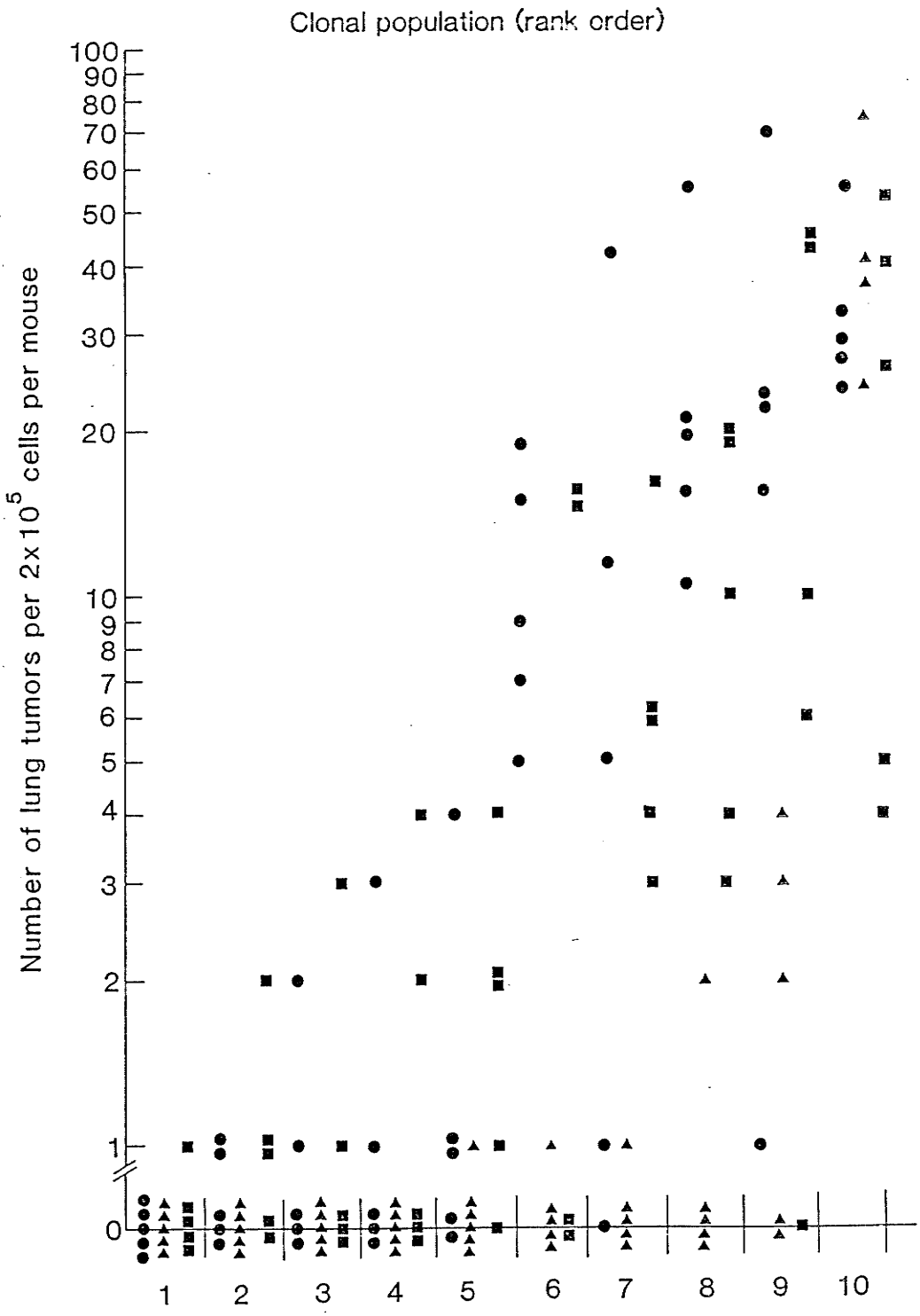
Figure 5

The number of lung tumors per 2 x 10^5 cells injected per mouse for each of ten clones shown in increasing rank order.

● BHK cell line

■ YMP1 amplificator mutant

▲ YMP7 amplificator mutant


Clonal population (rank order)
Number of lung tumors per 2×10^5 cells per mouse
100
90
80
70
60
50
40
30
20
10
9
8
7
6
5
4
3
2
1
0
1
2
3
4
5
6
7
8
9
10

metastatic cell line were needed. The inducible vector system chosen was a recently constructed and characterized Lac inducible plasmid (Figge et al., 1988). This inducible plasmid system consists of two plasmids; the Lac operator containing a SV40 early promoter and enhancer element linked to the CAT gene and the lacI gene linked to the CMV IE94 promoter, which produces, the lac repressor protein. This system has been shown by Livingstons group (Figge et al. 1988) to produce maximum (60-fold) CAT activity after 4 days of exposure to IPTG (15mM) following stable transfection in COS monkey kidney cells. Cells were transfected in a specific 16:1:1.2 molar ratio with the LacI plasmid, LacOCAT, and a drug marker plasmid, respectively, to ensure proper induction of the Lac operator by IPTG.

In constructing a Lac inducible mutator phenotype with the mutant M1 gene, the CAT gene in the Lac operator plasmid was removed, and then replaced with the mutant M1 gene (as described in the Materials and Methods section of this thesis, page 60). The recombinant plasmid was identified by restriction enzyme mapping, using the restriction enzymes Pvu I, Kpn, Bam HI, and Hind III, (Figure 6). From the resulting bands pLacOM1 was characterized (Figure 7). The cell line into which we chose to transfect this system was one isolated and characterized in this laboratory, Ciras 1. Ciras 1 was isolated following transfection of the T24-H-ras gene into the normal mouse 10T fibroblast line (Egan et al., 1987). Elevated ras expression in this line has been shown to render these cells highly tumorigenic, but poorly metastatic.

Figure 6

Photograph of an ethidium bromide stained 1% agarose gel of restriction enyzme digests of the parent plasmids, (1): pLacOCat and (2): pERdATP^r and the recombinant plasmid (3): pLacOM1 with the restriction enzymes (a): Pvu I, (b): Kpn I, (c): Bam HI, and (d): Hind III. The DNA size markers were Hind III digested lambda-DNA obtained from BRL (Bethesda, MA).

a b c d

1 2 3 1 2 3 1 2 3 1 2 3

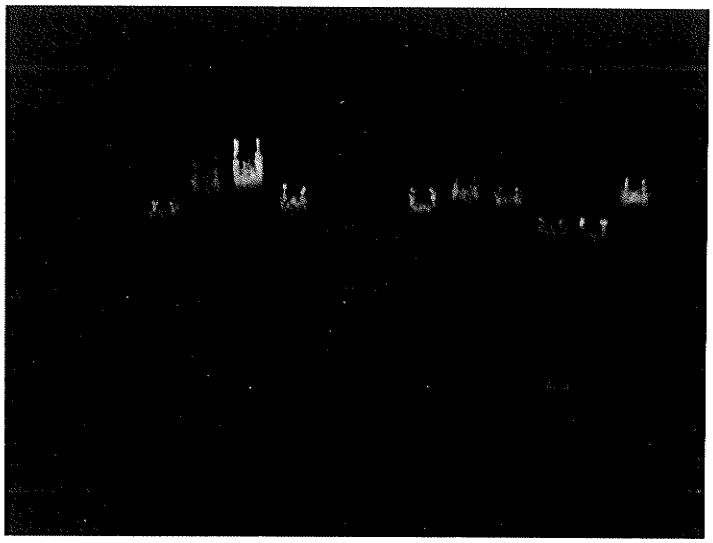
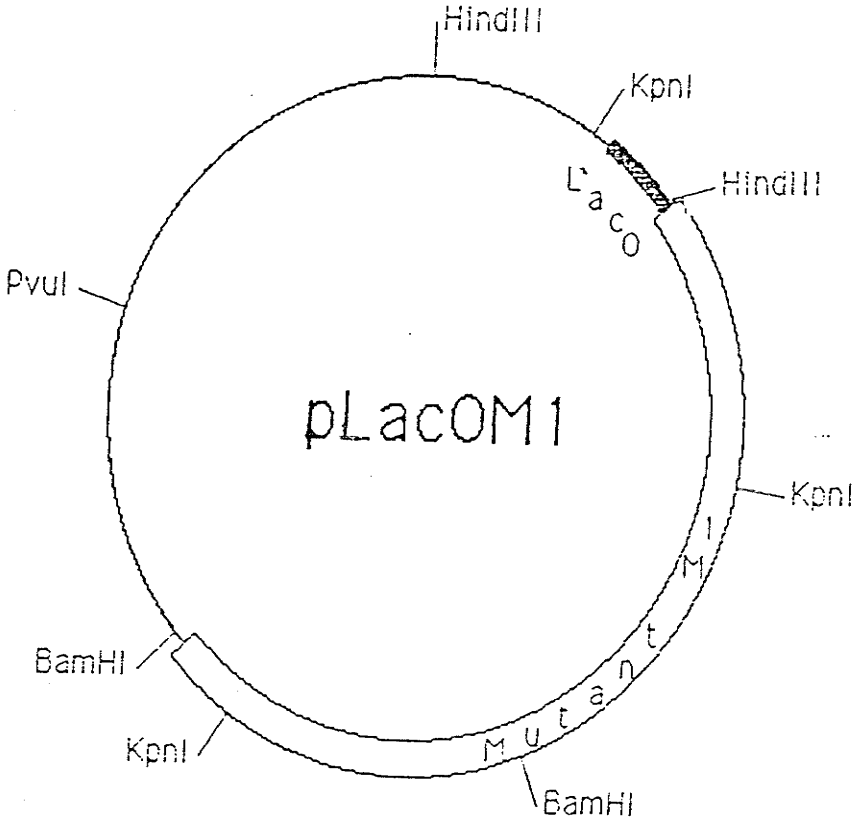


Figure 7

Plasmid map of pLacOMI based on band sizes of the restriction enzyme digests seen in Figure 6. The total size of the LacOM1 plasmid is approximately 7.6 kb.


HindIII
KpnI
HindIII
LacO
PvuI
pLacOM1
KpnI
lacI
Mutant
BamHI
KpnI
BamHI

1.2.b. Characterization and Isolation of transfected, mutator M1 mouse cells

To determine if the lac repressor could be expressed in the Ciras 1 mouse fibroblasts, and the ratio of repressor to operator required for efficient induction, transient transfections were first carried out using pLacOCAT and pCMVLacI. CAT assays were performed on crude protein extracts of transiently transfected Ciras 1 cells. Table 5 shows that a molar ratio of 7:1 operator to repressor was the ratio of choice. This ratio gave the best induction of the operator in the presence of the allolactose analogue, IPTG, and the best repression of CAT activity in its absence. The Ciras 1 cells were then transfected with the constructed LacOM1 plasmid, the LacI plasmid and the drug marker plasmid, PY3, in a 7:1:0.5 ratio, using the calcium phosphate method as described in the Materials and Methods, page 64. Transfected cells were grown in 200 μg/ml hygromycin for 2 weeks after which 40 drug resistant colonies were isolated. Each clone was grown in and out of 15 mM IPTG so that RNA could be isolated. These RNA preparations were screened by Northern blot analysis for the transfected mutant M1 messenger RNA, using the HBV probe which only picks up transfected mutant M1 because of its HBV poly A sequences (Caras and Martin 1988). Figure 8 shows that from this initial screen 3 clones, 2 of which are shown (clones D2 and D4), were observed to contain the 3.6 kb mutant M1 message. These 3 clones, however, were all constitutively produced message for mutant M1 even in the absence of the IPTG inducer. The two positive clones, D2 and D4, were examined further.

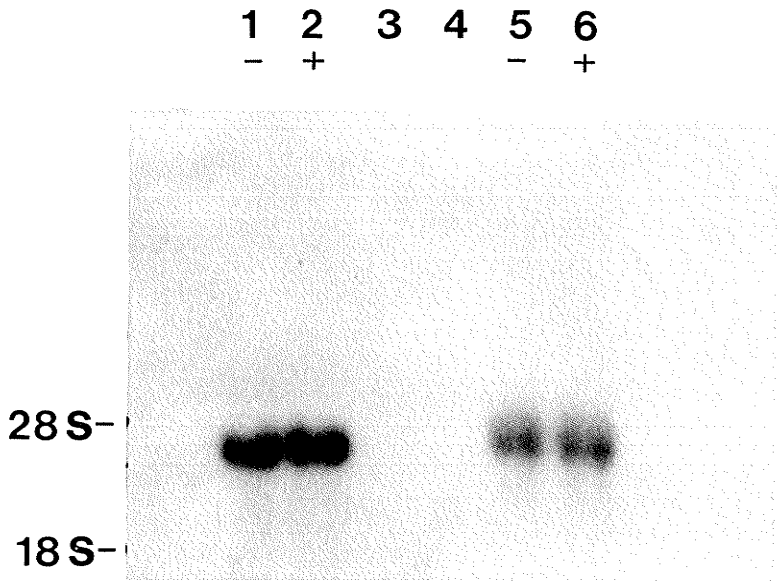
Table 5

CAT activity/μg pLacOCat transfected transiently into Ciras 1 cells in the presence and absence of 15 mM IPTG.

TRANSIENT TRANSFECION	15mM IPTG	CPM (C^{14}) PER μg CAT PLASMID TRANFECTED
LACO CAT		15178
1 LACO CAT+	-	3968
1 LAC R	+	7366
1 LACO CAT+	-	2012
7 LAC R	+	18398
1 LACO CAT+	-	980
14 LAC R	+	4388

Figure 8

Northern blot analysis of mutant M1 mRNA in a series of transfected clones isolated in the presence (+) and absence (-) of 15 mM IPTG. Lanes 1 and 2 are the transfected clone D2; lanes 2 and 4, clone D3; and lanes 5 and 6, clone D4. The autoradiograms were exposed overnight at -70°C with intensifying screens.


1 2 3 4 5 6
− + − +
28S
18S

Southern blot analysis shows that the 2 positive clones, D2 and D4, contained transfected mutant M1 gene (Figure 9). Lane 1 shows the endogenous M1 bands, all others are the result of transfection. Re-evaluation of the RNA showed that transfected M1 message was apparent, (3.6 kb lanes 1 and 2), and reprobing of this same filter showed that the messenger RNA for the endogenous M1, 3.2 kb, was stronger than that of transfected M1, (Figure 10). To be able to observe the mutator phenotype approximately a 3 fold elevation in M1 mutant messages is required (Caras, personnal cummunication). To ensure this was not an abnormal occurance in the mRNA, M1 protein was also assayed using Western blot analysis. Figure 11 indicates that no increase in M1 protein was observed between the transfected and untransfected Ciras 1 lines. As described by Caras and Martin, (1988) the mutator M1 phenotype confers deoxyguanosine (dG) and thymidine resistance to the cells. Examination of the resistance of the transfected mouse cells to dG, Figure 12, showed no difference in their resistance compared to normal untransfected cells.

Before trying this same protocol in other cell lines the repressor was analysed to determine why the positive transfected clones were not inducible as expected. Nothern blot analysis of the filters in which these clones were first detected, were reprobed for the Lac repressor mRNA. Figure 13 shows that these two positive clones, lanes 1,2,5 and 6 produce large amounts of repressor message as indicated by the arrowhead. Western blot analysis using a polyclonal antibody for the Lac repressor protein, provided by Dr. S.Bain (Princeton University, New Jersey, USA.), also showed that the

Figure 9

Southern blot analysis of transfected and endogenous M1. Genomic DNA was digested to completion with the restriction enzyme EcoRI and run beside DNA size markers (Hind III digested lambda-DNA obtained from BRL). Lane (1) is the wildtype Ciras 1 line, lane (2) is a transfected line that gave a negative result in the Northern blot screening test, lanes (3) and (4) shows the 2 clones that gave a positive result in the Northern blot screening test (D2 and D4). Transfected bands can be seen in lanes 3 and 4.

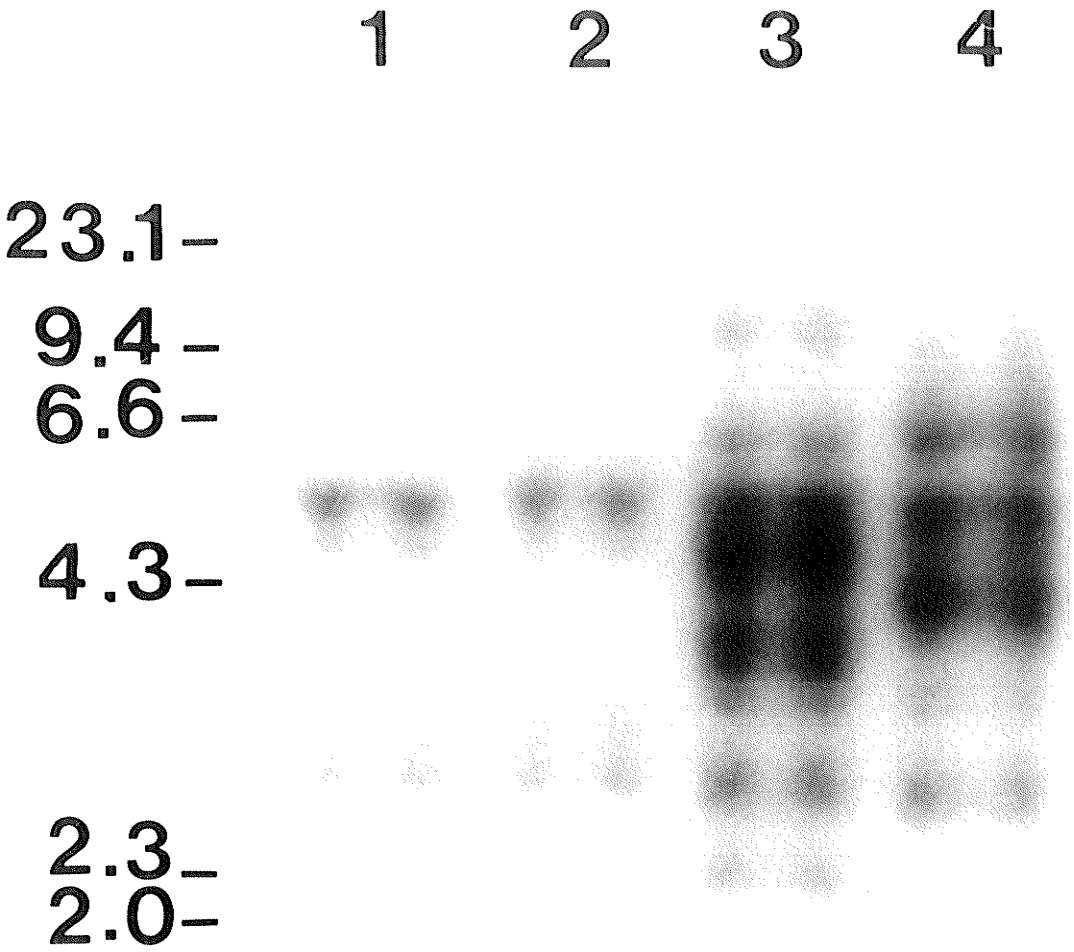

1
2
3
4
23.1
9.4
6.6
4.3
2.3
2.0

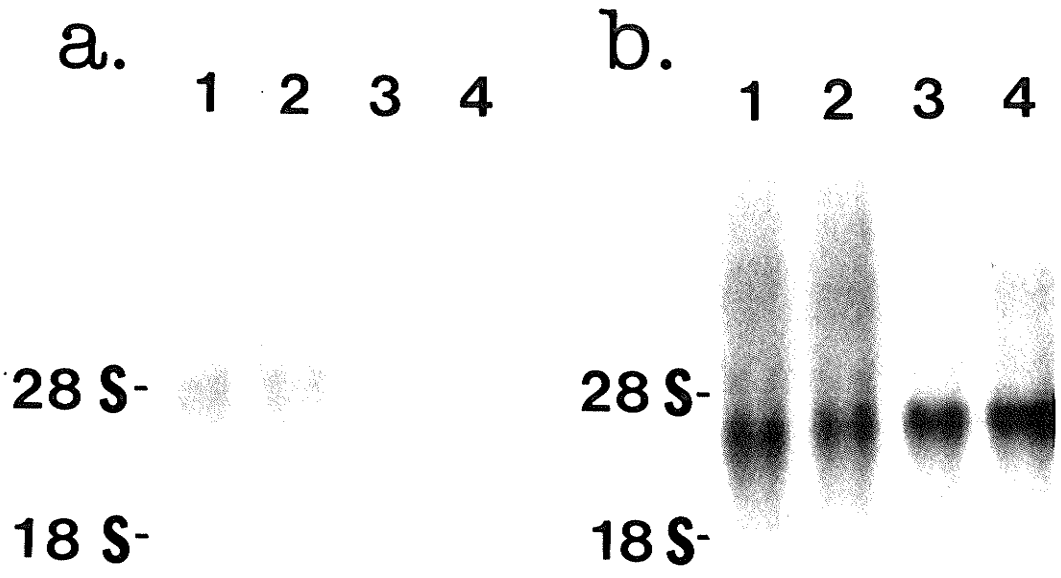
Figure 10

Northern blot analysis of M1 mRNA. Lane (4) is the wildtype Ciras 1 line, lane (3) is a transfected M1 negative control, lanes (1) and (2) are the transfected M1 positives (D2 and D4).

Blot a: was probed for the HBV sequences contained in the transfected mutant M1 gene. The 3.6 kb mutant M1 message was observed

Blot b: is the same blot re-probed for M1; this shows the normal 3.2 kb message as well as the transfected mutant 3.6 kb message.

Blot c: is the same filter re-probed with GAPDH to show that RNA loading in all lanes was the same.


a.
1 2 3 4
28 S-
18 S-
b.
1 2 3 4
28 S-
18 S-

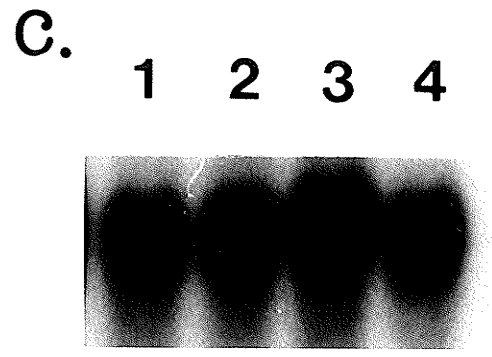

c.
1 2 3 4

Figure 11

Photograph of a Western blot analysis of M1 protein. Lanes (1) and (2) show the amount of M1 protein in the transfected lines expressing low levels of mutant M1 (D2 and D4). Lane (3) is the transfected nonexpressing control and lane (4) shows the amount of M1 protein in the wild type Ciras 1 cell line. One μg/ml of the M1 monoclonal antibody (purchased from Inro Biomedtek, Umea, Sweden) was incubated with the nitorcellulose filter at 4°C overnight. The protein standards run were BRL (Bethesda, MA) standards with molecular weights ranging from 200 kD to 43 kD.

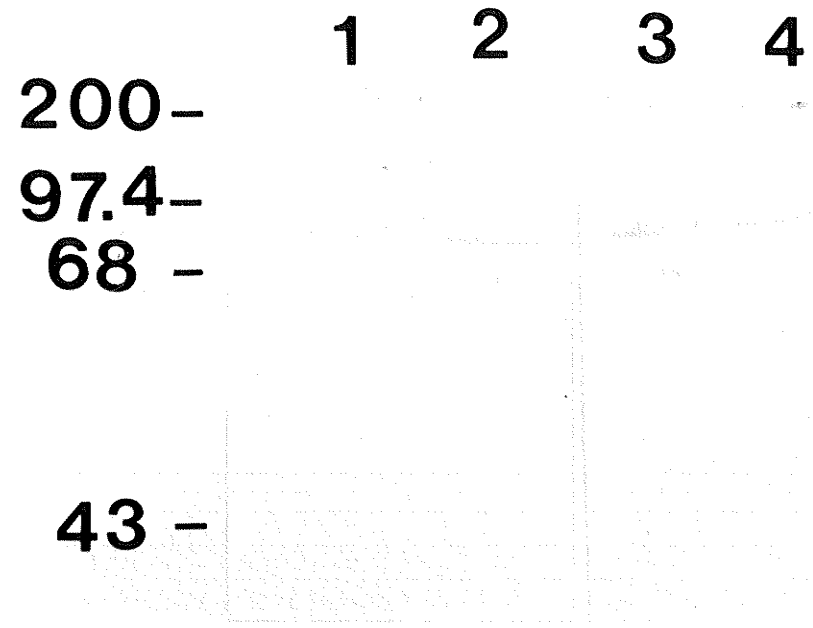

1 2 3 4
200-
97.4-
68 -
43 -

Figure 12

Growth inhibition curve measured after 72 hours in increasing concentrations of deoxyguanosine for the wild type Ciras 1 line ○ , the negative transfected control △ , and the positive expressing transfected M1 clones D2 □ and D4 ◇ . (see Materials and Methods, Determination of Growth Rate).

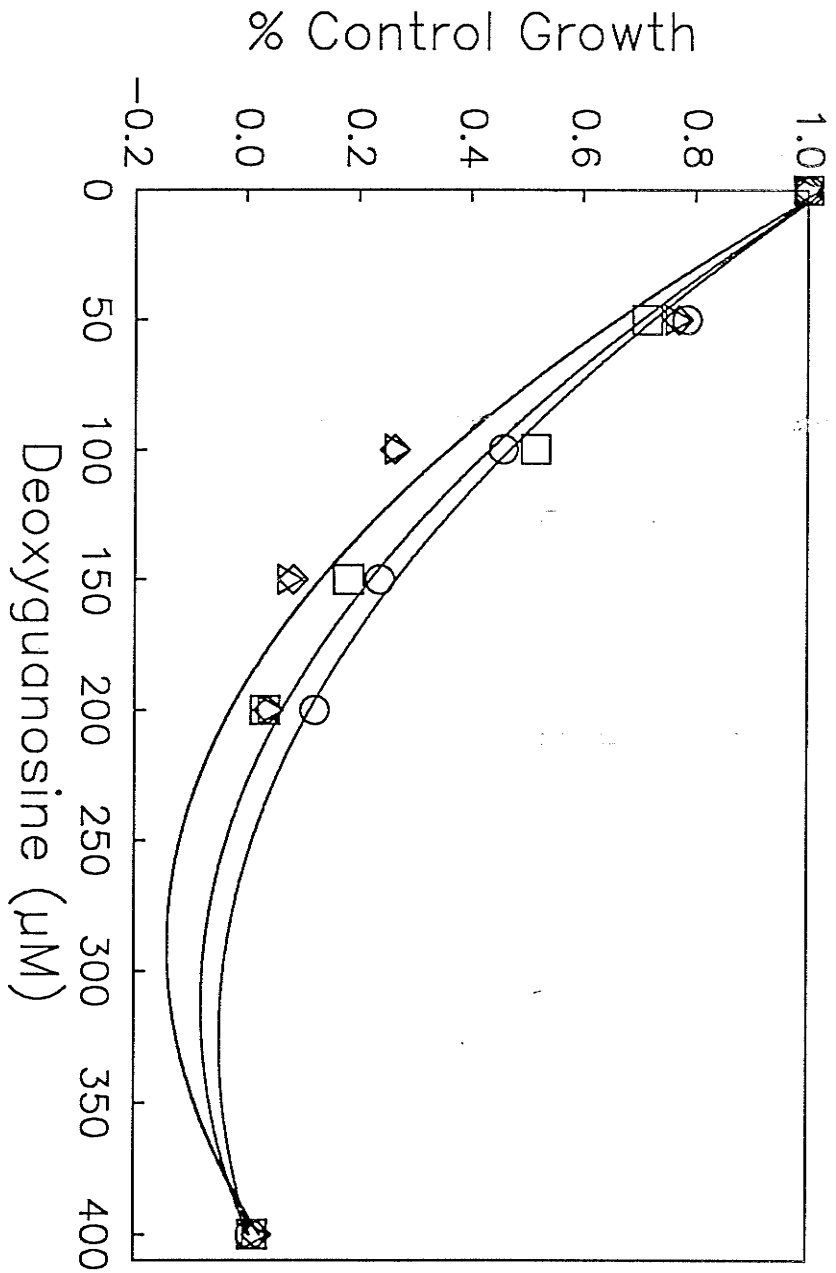

% Control Growth
−0.2
0.0
0.2
0.4
0.6
0.8
1.0
0
50
100
150
200
250
300
350
400
Deoxyguanosine (μM)

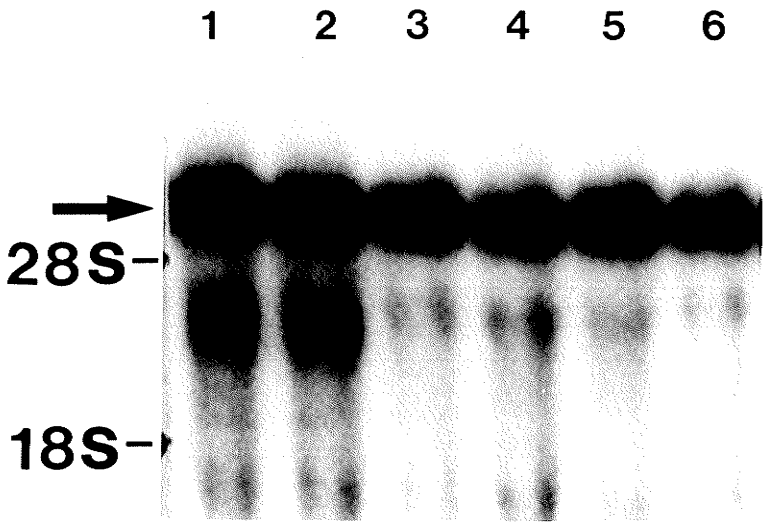
Figure 13

Nothern blot analysis of Lac repressor mRNA in a series of transfected Ciras 1 cell lines. All clones shown were transfected with both pCMVLacI and pLacOM1. Lanes 1,2,5 and 6 show the positive transfected mutant M1 clones;

D2- lane (1), + IPTG and lane (2), - IPTG and

D4- lane (5), + IPTG and lane (6), - IPTG,

(see Figure 8).


1
2
3
4
5
6
28S
18S

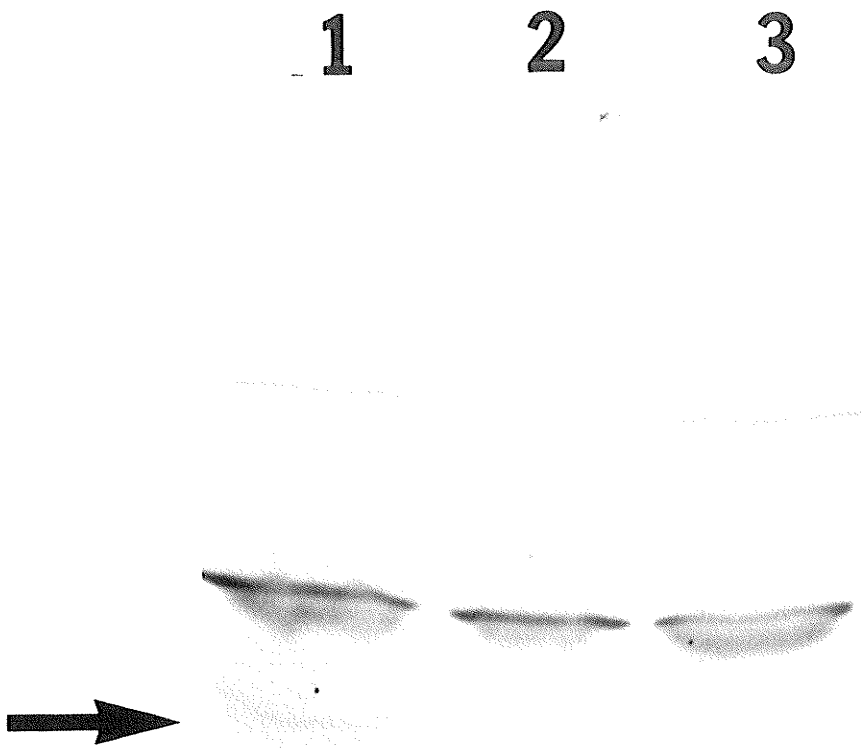
two previously analyzed positive clones produced the 38/36 kD (kilodalton) doublet indicating the Lac repressor protein (Figure 14). There seems to be no explanation why the repressor, although made, is not inhibiting the transfected mutant M1 message. Despite this, CAT activity was checked for the proper ratios of repressor to operator in both NIH3T3 and COS monkey cells (data not shown). The pLacOM1 was then transfected into both these lines. Transfection into mouse NIH3T3 cells again produced 2 clones with constitutively expressed, low mutant M1 message (data not shown). In the COS cells no positive clones were identified. To the best of my knowledge there have been no further publications indicating that the Lac inducible system works following stable transfection in any other cell lines nor, has there been any publications on the expression of the mutant M1 gene in any other cell line but CHO.

2. GENE AMPLIFICATION AND TUMOR PROGRESSION

The mechanisms involved in the amplification of genes has not yet been described. Can amplification of a specific gene, however, play a direct role in tumor progression? Previous evidence has shown that some oncogenes are typically amplified and subsequently activated in some human tumors. Amplification of these genes has also been correlated with the metastatic aggressiveness of the tumor (Kiefer et al. 1987, Varley et al. 1987, Yamamato et al. 1986). Recently amplification of the proto-oncogene k-fgf or HST growth

Figure 14

Photograph of a Western blot analysis of lac repressor protein. Lanes (1) and (2) show the amount of Lac repressor protein in the expressing mutant M1 clones, D2 and D4. Lane (3) shows the wild type Ciras 1 cell line. The arrowhead indicates the 38/36 kD doublet of the Lac repressor protein. The other bands are the result of non-specific binding to the polyclonal Lac R antibody obtained from S. Bain [Princeton University, Princteton, NJ (used at a 1:200 dilution)] also seen by Figge's laboratory (Brown et al. 1987)


1
2
3

factor (Delli-Bovi et al. 1988), has been observed in many human tumors including breast carcinoma (Zhou et al. 1988, Yoshida et al.1988). This member of the fibroblast growth factor family is not normally expressed in adult tissue (Basilico et al. 1989).

To examine whether this proto-oncogene is activated by amplification, and if the amount of amplification is a determinant in the level of tumor progression, a dhfr plus k-fgf expression vector was constructed and transfected into normal mouse fibroblasts.

2.1 Construction and Isolation of a K-fgf Amplified Cell Line

To test the question: does amplified k-fgf proto-oncogene expression correlate with tumorigenic and metastatic potential, amplification of this gene in normal mouse fibroblasts was carried out. To do this the k-fgf proto-oncogene was placed in an amplification plasmid containing an amplifiable gene, dihydrofolate reductase (dhfr). Resistance to the drug methotrexate (MTX) selects for amplification of the dhfr gene (Shimke et al. 1978, Alt et al. 1978). Dhfr, has previously been used in conjunction with other genes in plasmid vectors to select amplified linked genes (McCormick et al.1984, Kaufman et al. 1985, Wurm et al. 1986). Ideally, amplification of a gene using linkage to the dhfr gene is done in a cell line that lacks the endogenous dhfr gene. Resistance to MTX must in this case must be the result of amplification of the transfected dhfr gene. The only cell type at this time that is dhfr-, is the CHO dhfr- line selected by Urlaub and Chasin (1980), but since this line is naturally tumorigenic and it was not applicable. More importantly,

the CHO dhfr- line was also tested for kFGF responsiveness, through stimulation by bFGF which has been shown to use the same receptor as kFGF (Manusukhani et al. 1990). Figure 15 shows that CHO cells do not respond to bFGF stimulation whereas NIH3T3 cells do. The biological response of a cell to amplification and overexpression of a growth factor would require the ability of the cell to respond to the factor, through autocrine stimulation. Therefore, the question of amplification of the k-fgf proto-oncogene and its effects on tumorigenicity and metastasis was carried out with NIH3T3 cells.

The first step was to construct a k-fgf, dhfr, amplification plasmid. The procedure can be found in the Materials and Methods section of this thesis (page 58). The recombinant plasmid, k-fgf + dhfr, was identified by restriction enzyme mapping using the enzymes Eco RI, Bam I, Kpn I, and Sac I, following digestion to completion (Figure 16A). The following enzyme restriction digests were also examined in conjuntion with Bam HI: Pst I, Xho I, Xba I, and HindIII, (Figure 16B). A restriction enzyme map, Figure 17, was constructed based on the sizes of the resulting bands. Following identification of the k-fgf + dhfr plasmid or pKFD, lipofection was done in normal mouse fibroblasts, NIH3T3, in a 5:1 molar ratio of pKFD to the drug marker plasmid, PY3 (see Materials and Methods, page 64). The transfected cells were grown in the presence of 200 μg/ml hygromycin for 2 weeks after which time 30 drug resistant colonies were picked as described in the Material and Methods, (page 64). As a control NIH3T3 cells were also co-transfected with only the dhfr plasmid and the drug marker, PY3. The pKFD transfected drug resistant clones

Figure 15

The relative stimulation of DNA synthesis as measured by ^{3}H-thymidine incorporation in the presence or absence of bFGF (10 ng/ml). Lane (1), CHO K1 dhfr- cells, absence of bFGF; lane (2), CHO K1 dhfr- cells, presence of bFGF; lane (3), NIH3T3 cells, absence of bFGF; lane (4), NIH3T3 cells, presence of bFGF. Relative stimulation is based on the average of five determinations/cell line in both the absence and presence of bFGF.

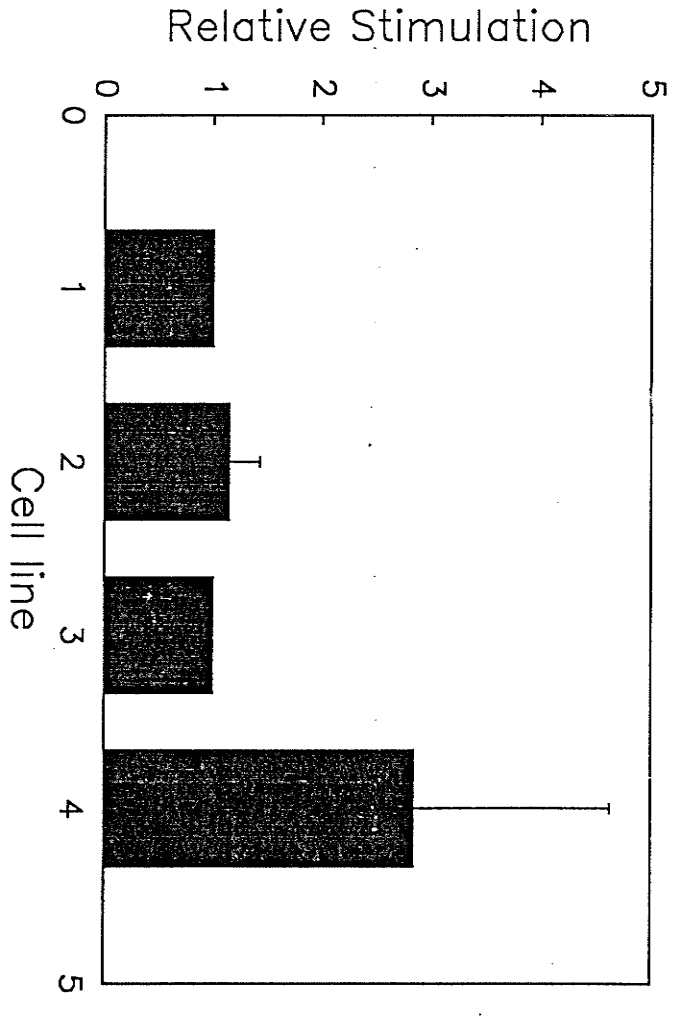

Relative Stimulation
0
1
2
3
4
5
Cell line
0
1
2
3
4
5

Figure 16

A-Photograph of an ethidium bromide stained 1% agarose gel of restriction enzyme digests of the parent plasmids (1) pLacoCat, (2) pMG4 and the recombinant plasmid (3) pKFD, with the enzymes:

(a) Eco RI,

(b) Bam HI,

(c) Kpn I and

(d) Sac I.

The DNA size markers are a Hind III digest of lamba-DNA obtained from BRL (Bethesda, MA).

B-Photograph of an ethidium bromide stained 1% agarose gel of restriction enzyme digest of the recombinant plasmid pKFD, using the enzymes:

(a) Pst I

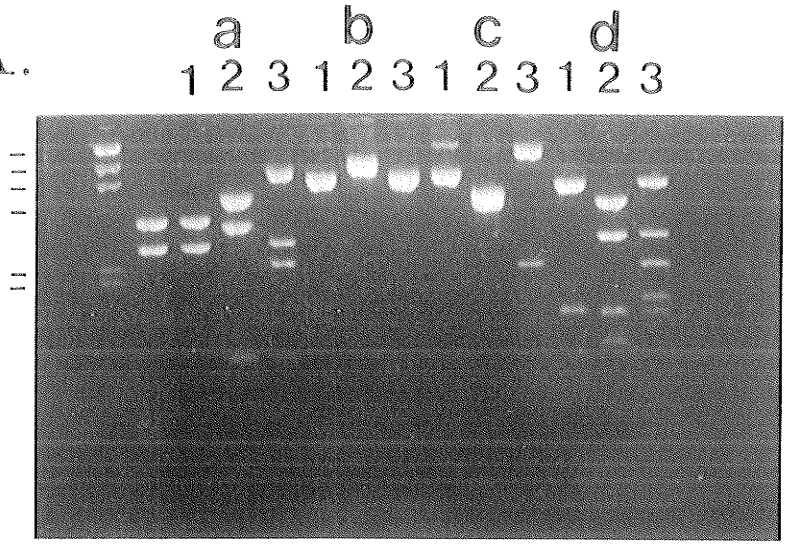
(b) Xho I

(c) Xba I

(d) Hind III

either alone (1), or in conjunction with Bam HI (2). The DNA size markers are a Hind III digest of lambda-DNA from BRL (Bethesda, MA).

A.



B.

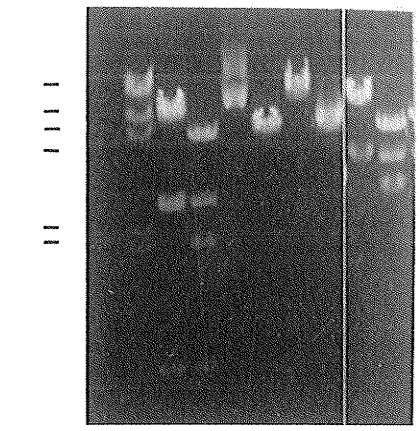
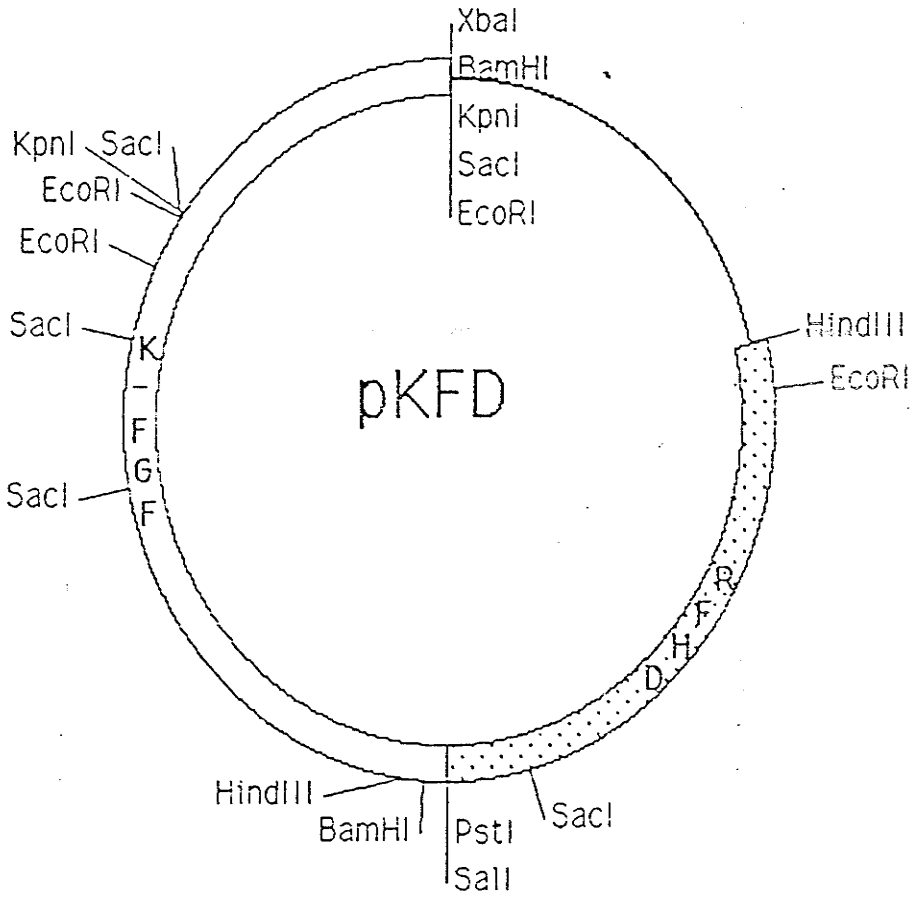


Figure 17

Plasmid map of pKFD based on the band sizes of the restriction enzyme digests shown in Figure 16. The total size of the pKFD plasmid is approximately 13.8 kb.


XbaI
BamHI
KpnI
SacI
EcoRI
KpnI
SacI
EcoRI
EcoRI
SacI
K
-
F
G
F
SacI
pKFD
HindIII
EcoRI
R
F
H
D
HindIII
BamHI
PstI
SalI
SacI

were grown and tested for the acquistion of the pKFD plasmid using Southern analysis of DNA digested to completion with the restiction enzyme Pst I. Figure 18 shows some of the clones tested and probed for k-fgf sequences. As can be seen there is one endogenous k-fgf band denoted by an arrowhead, all others are transfected sequences. After this initial screening, all positive clones that had transfected sequences were kept and frozen away. A small precentage of these clones, 4 out of 17, appeared to have a transformed morphology, such that they were spindle shaped and refractile. All clones were subjected to 5 nM MTX in which there appeared to be no inhibition of growth. Following one passage in 5 nM MTX the cells were next plated at high density in 10 nM MTX. At this concentration the majority of the clones appeared to grow slowly, except the apparent transformed clones. All clones were maintained at this concentration for a period of approximately 3 weeks until their growth rate appeared normal, (which is an approximate doubling time of 18 hours). The clones were then subjected to 20 nM MTX until the growth rates were again normal, approximately 4-8 weeks depending on the clone. This procedure was repeated with 40 nM MTX. When more than half the clones grew well in 40 nM MTX, all were harvested for DNA and Southern blot analysis was done. Figure 19 shows southerns (of DNA digested to completion with Xba I) performed with DNA obtained with some of the clones using the dhfr probe. There are four endogenous dhfr bands when DNA is digested to completion with the restriction enzyme Xba I, as indicated by the arrowheads. In every case, except one, it is apparent that the endogenous dhfr genes have amplified due

Figure 18

Southern blot analysis of a series of clones, isolated following transfection of pKFD. Twenty μg of DNA was digested to completion with the restriction enzyme Pst I and the blot probed with the cDNA for k-fgf. Lane (1) shows the wildtype NIH3T3 line. The arrowhead denotes the endogenous k-fgf gene.

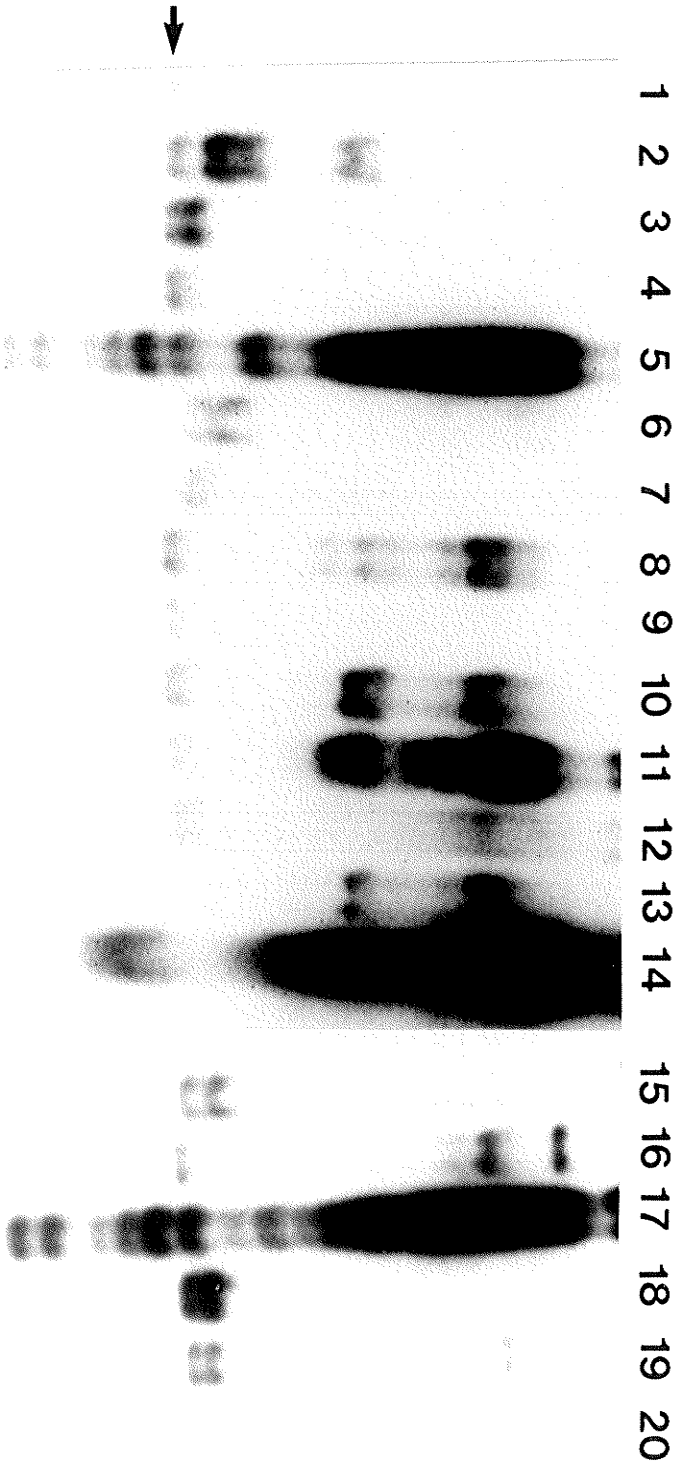

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Figure 19

Southern blot analysis of positive pKFD transfected clones for dhfr sequences. Twenty μg of DNA was digested to completion with the restriction enzyme Xba I. Lane (1) is the wild type NIH3T3 cell line. The remaining lanes are of a series of positive transfected clones in the absence: (-), and in the presence: (+), of growth in MTX as indicated below in nM concentrations:

lane 2 -) 3T3.26
+) 3T3.26-40

lane 3 -) 3T3.29
+) 3T3.29-40

lane 4 -) 3T3.3A
+) 3T3.3A-20

lane 5 -) 3T3.3G
+) 3T3.3G-40

lane 6 -) 3T3.4B
+) 3T3.4B-20

lane 7 -) 3T3.4C
+) 3T3.4C-20

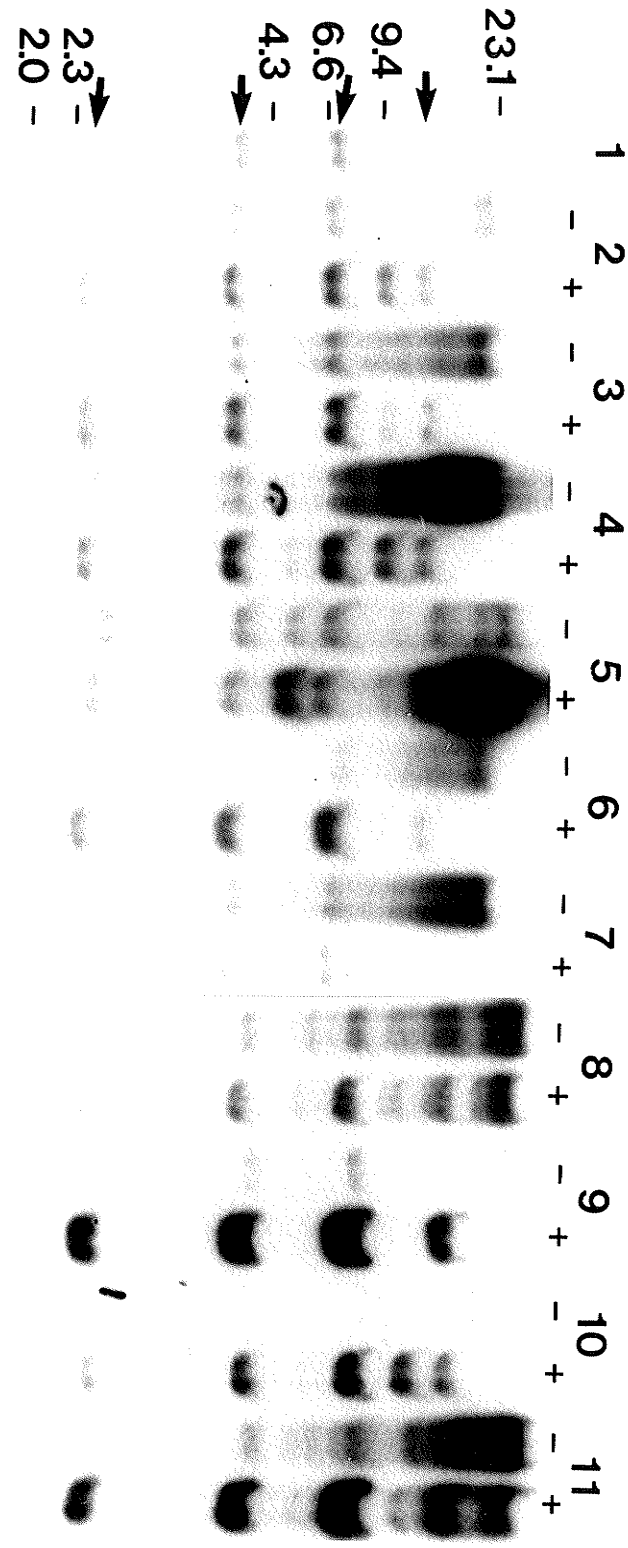
lane 8 -) 3T3.210
+) 3T3.210-40

lane 9 -) 3T3.212
+) 3T3.212-80

lane 10 -) 3T3.1A
+) 3T3.1A-20

lane 11 -) 3T3.1D
+) 3T3.1D-40

The four endogenous dhfr sequences are indicated by arrowheads.


23.1
9.4
6.6
4.3
2.3
2.0
1
2
3
4
5
6
7
8
9
10
11
- + - + - + - + - + - + - + - + - + - + - +

to selection with MTX. The one clone that showed the transfected dhfr amplified, clone 3T3.3G in lane 5, was one that appeared to have a transformed morphology (all clones with a transformed morphology seemed unaffected by the presence of 10 nM MTX and were very easily stepped up in increasing concentrations of MTX). As a control clone 3T3.29, lane 3, was also kept and stepped-up further. Southern analysis was performed on this clone, 3T3.29, to detect k-fgf amplfication. Figure 20 showed this clone had lost the transfected k-fgf sequences. From the control transfection where NIH3T3 cells were transfected with the dhfr plasmid alone, not containing the the k-fgf gene, two clones dB7 and dC2 were isolated and frozen away. Southern analysis of these clones showed they had incorporated and amplified the dhfr transfected plasmid following selection in MTX (Figure 21).

The control cells, dB7 and dC2, were extremely difficult to step-up in MTX. Clone 3T3.29 was also difficult to step-up. Ultimately the 3T3.29 clone was stepped up to grow in 80 nM MTX and the dB7 and dC2 clones stepped up to grow in 40 and 10 nM MTX respectively. The 3T3.3G line was relatively easy to step-up in MTX and lines were isolated that grew in 10, 20, 40, 60, 80 and 120 nM MTX. Southern analysis using the restriction enzyme Xba I, was done with 2 μg of DNA, for both dhfr and k-fgf in all these final clones (Figure 22A and 22B). Compared to the transfected 3T3.3G clone densitometric analysis showed that the 3T3.3G-80 clone had approximately a 15 fold amplification of the k-fgf gene, and an approximately 8 fold amplification of the dhfr gene.

Figure 20

Southern blot analysis of k-fgf sequences in the wild type line NIH3T3, lane (1) and the transfected clone 3T3.29, lane (2) grown in the absence (-), and presence (+) of 40 nM of MTX. Twenty μg of DNA was digested to completion with the enzyme Xba I.

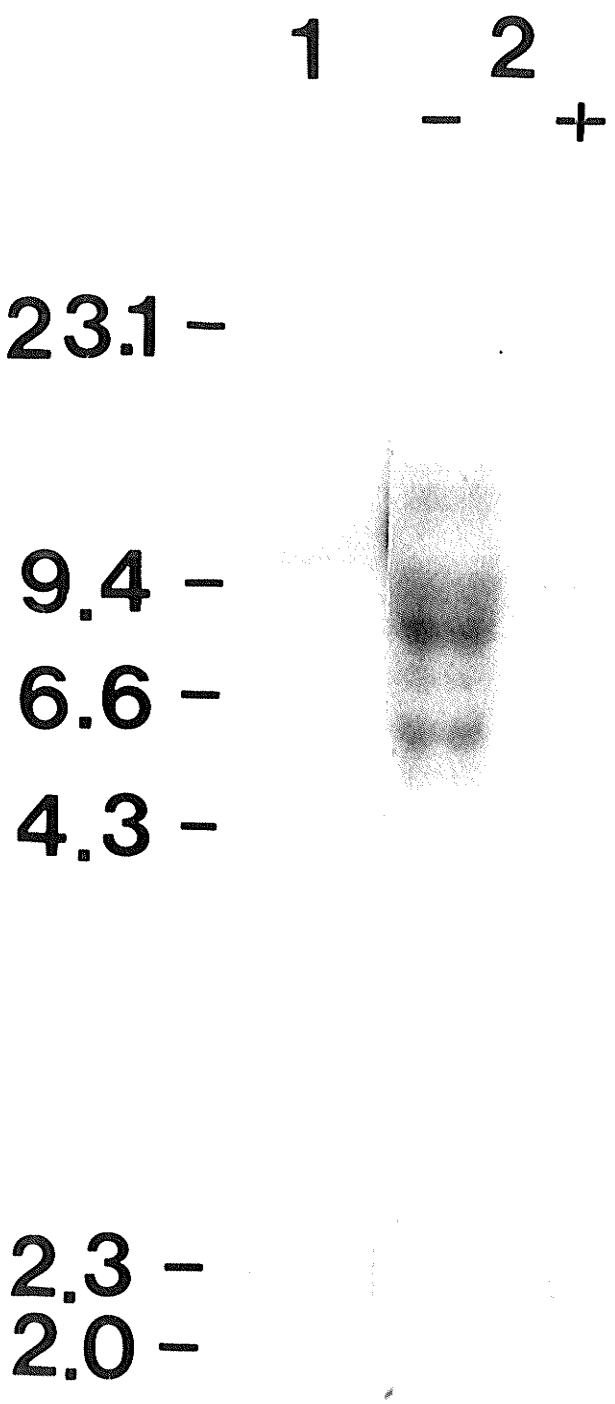

1
2
−
+
23.1 −
9.4 −
6.6 −
4.3 −
2.3 −
2.0 −

Figure 21

Southern blot analysis of dhfr sequences for the wild type line NIH3T3, lane (1), and the control clones: dB7, lane (2) in the absence (-) and presence (+) of 40 nM MTX, and dC2, lane (3) in the absence (-) and presence (+) of 10 nM MTX. In all cases twenty μg of DNA was digested to completion using the enzyme Xba I.

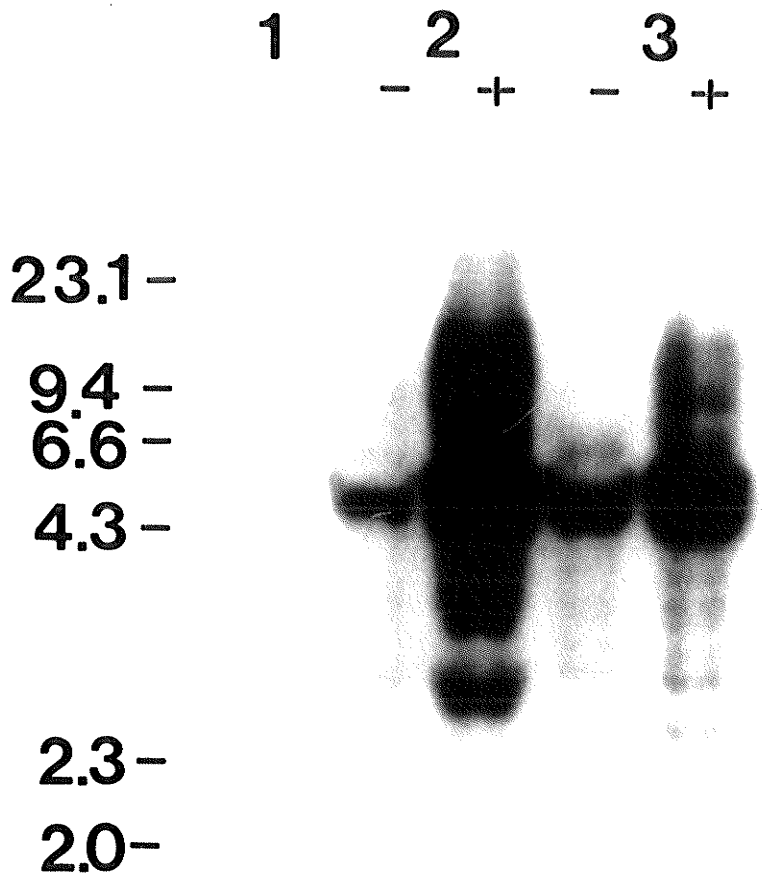
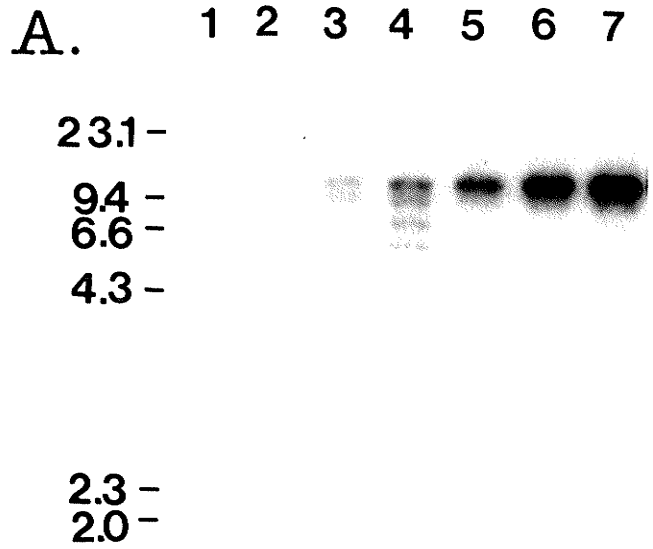
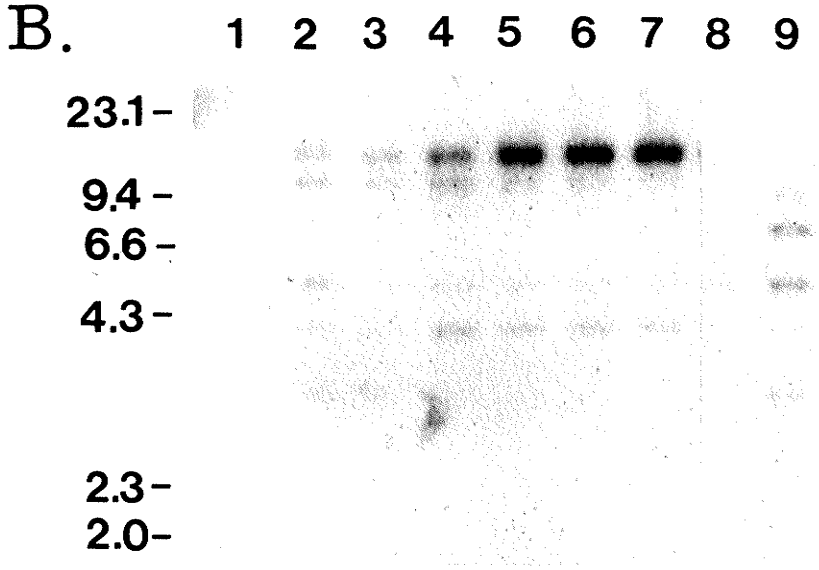

1
2
3
−
+
−
+
23.1
9.4
6.6
4.3
2.3
2.0

Figure 22

A-Southern blot analysis of k-fgf sequences following digestion of 2 μg of DNA to completion with Xba 1. Lane (1) is the wild type NIH3T3 line, and the transfected clones:
lane (2), 3T3.3G; lane (3), 3T3.3G-10; lane (4), 3T3.3G-20; lane (5), 3T3.3G-40; lane (6), 3T3.3G-60; lane (7), 3T3.3G-80;

B-Southern blot analysis of dhfr sequences following digestion of 2 μg of DNA to completion with Xba I. Lane (1) is the wild type NIH3T3 line, and the transfected clones:
lane (2), 3T3.3G; lane (3), 3T3.3G-10; lane (4), 3T3.3G-20; lane (5), 3T3.3G-40; lane (6), 3T3.3G-60; lane (7), 3T3.3G-80; lane (8), 3T3.29; lane (9), 3T3.29-80.


A.
1 2 3 4 5 6 7
23.1 -
9.4 -
6.6 -
4.3 -
2.3 -
2.0 -


B.
1 2 3 4 5 6 7 8 9
23.1 -
9.4 -
6.6 -
4.3 -
2.3 -
2.0 -

2.2 Characterization of the K-fgf Amplified Clones

The next step in characterizing the k-fgf amplified clones, 3T3.3G, 3T3.3G-10 to -120, was to examine the amount of both k-fgf and dhfr messenger RNA by Northern blot analysis. RNA was isolated from clone 3T3.3G, 3T3.3G-10 to -120, 3T3.29-80, and dB7-40. As shown in Figure 23A only the 3T3.3G clones produced k-fgf messenger RNA. Although gene amplification was shown to be progressive for these clones the level of mRNA was found to rise quickly in the amplified, MTX resistant lines, and then slowly decrease. Clone 3T3.3G was observed to have a very low level of k-fgf mRNA which was only apparent after overexposure of above described blot (Figure 23A, 1°-3°). To detect if differences were due to different loading equivalent amounts of mRNA were loaded onto the gels and blotted onto the nylon filters probed with k-fgf and dhfr, blots were re-probed with glyceraldehyde-3 phosphate dehydrogenase (GAPDH) cDNA. As shown in Figure 23C, it was evident that approximately equal amounts of mRNA were loaded and blotted onto the filters. Figure 23B, shows that all clones expressed mRNA for dhfr. Two different size species of dhfr mRNA were apparent. The larger of the two was only observed in the 3T3.3G clones and was due to the transfected dhfr which produced both the 5 kb and 2 kb dhfr messages shown. Endogenous dhfr message is also 2 kb. As can be seen in Figure 23B both controls, 3T3.29-80 (lane 10), and dB7-40 (lane 11), produced a higher dhfr mRNA than wild type 3T3 cells (lane 9) in which the dhfr message observed was bearly detectable for 20 μg of total RNA. The k-fgf transfectants, 3T3.3G, 3T3.3G-10 to -120 all produced both dhfr

Figure 23

Northern blot analysis* of:

a- k-fgf message, 1°, 2°, and 3° are an overexposure of lanes 1, 2 and 3.

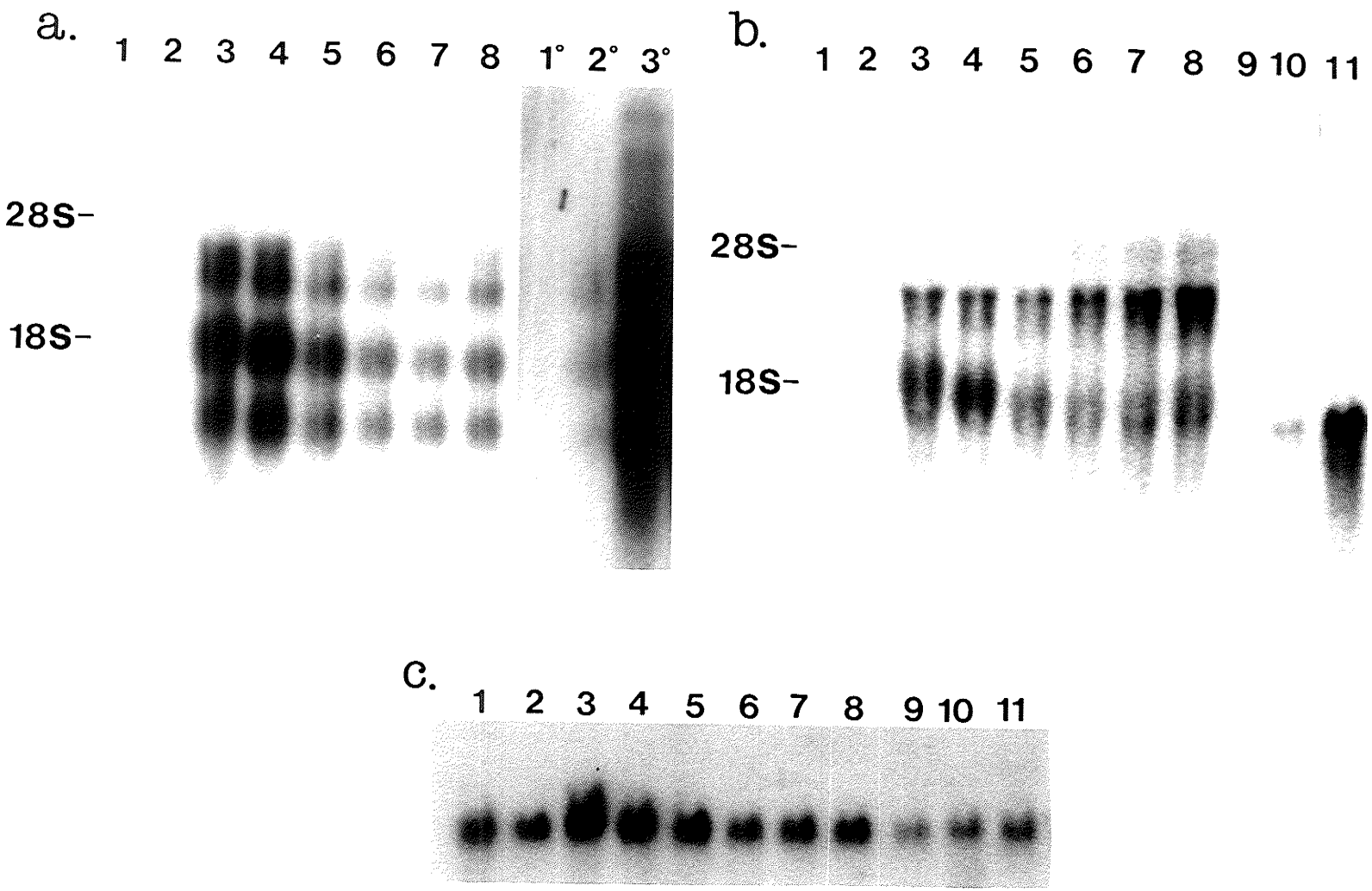
b- dhfr message

c- GAPDH as a control to demonstrate approximately equal loading in all lanes

Lanes (1) and (9) indicate the wild type line NIH3T3. The transfected cell lines are as follows:
lane (2), 3T3.3G; lane (3), 3T3.3G-10; lane (4), 3T3.3G-20; lane (5), 3T3.3G-40; lane (6), 3T3.3G-60; lane (7), 3T3.3G-80; lane (8), 3T3.3G-120; lane (10), 3T3.29-80; lane (11), dB7-40.

Lanes 1°, 2° and 3° are an overexposure of lanes 1, 2 and 3 following developing of the blotted filter for 5 days as opposed to 24 hours.

*-RNA was isolated from each cell line 20 hours after changing the growth media.


a.
1 2 3 4 5 6 7 8 1° 2° 3°
28S-
18S-
b.
1 2 3 4 5 6 7 8 9 10 11
28S-
18S-
c.
1 2 3 4 5 6 7 8 9 10 11

messages. The tranfected 5 kb dhfr message showed a progressive increase in the stepped up clones, like that observed at the gene level.

The kFGF protein levels were analyzed by Western blot analysis of conditioned medium produced from clones 3T3.3G, -20 and -80 and NIH3T3, to determine if there was a correlation with the message levels observed. Conditioned medium was prepared from four plates of each line, as described in the Materials and Methods, and concentrated through a centricon 10 microconcentrator (Amicon, Danvers, MA). Upon concentration to a final volume of 100 μl/sample, 30 μl of each sample was loaded on a 10% SDS-PAGE gel. Figure 24 shows that kFGF protein is clearly present in the medium of both 3T3.3G-20, and 3T3.3G-80, whereas 3T3.3G has much less kFGF protein. In conditioned medium from wild type 3T3 cells kFGF protein could not be detected.

2.3 Gene Amplification of K-fgf and Tumor Progression

To examine what effect amplification of the k-fgf gene and subsequent overexpression of the k-fgf protein had on the tumorigenic and metastatic characteristics of NIH3T3 cells the following lines, 3T3, 3T3.3G, 3T3.3G-10, 3T3.3G-20, 3T3.3G-80, 3T3.29-80, dB7-40 and dC2-10, were injected at a concentration of 1×10^5 cells both subcutaneously and intraveneously into BALB/c nu/nu mice. The results in Table 6 show that non of the controls were tumorigenic or metastatic. However, all of the k-fgf transfected lines were both highly tumorigenic and metastatic. The 3T3.3G clone which only expressed low amount of both k-fgf mRNA and protein showed, in two

Figure 24

A photograph of a Western blot showing k-fgf protein isolated from identical amounts of conditioned medium from: lane (1), 3T3.3G-80; lane (2), 3T3.3G-20; lane (3), 3T3.3G; lane (4), NIH3T3 wild type. The k-fgf polyclonal antibody, 682, was a gift from Dr. C. Basilico (Quarto et al. 1989), and it was used at a 1:200 dilution.

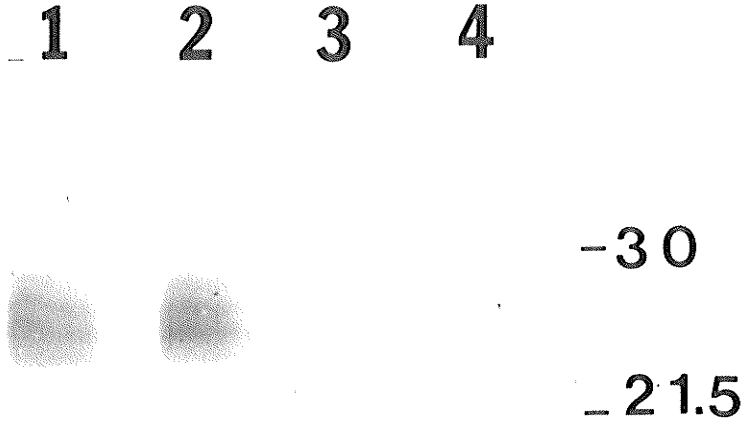

1
2
3
4
30
21.5

Table 6

Tumorigenicity and metastatic characteristics of the wild type NIH3T3 cell line and the transfected clones 3T3.3G, 3T3.3G-10, 3T3.3G-20, 3T3.3G-80, 3T3.29-80, dB7-40, and dC2-10.

Cell Line	Tumorigenicity		Experimental Metastasis		
	Freq. of mice with tumors*	Latency (days mean ± SE)	Freq. of mice with tumors	No. of Lung tumors* (mean ± SE)	Mean ± std error of the log (#tumors + 1)
NIH3T3	0/5	0	0/5	0	ND
3T3.3G	10/10	6.5 ± 0.8	9/10	6.7 ± 3.3	0.696 ± 0.186**
3T3.3G-10	ND	ND	10/10	31.6 ± 8.21	1.305 ± 0.186
3T3.3G-20	10/10	5.7 ± 0.9	9/10	37.3 ± 22.2	1.196 ± 0.186
3T3.3G-80	10/10	12.3 ± 1.0	10/10	31.4 ± 15.2	1.228 ± 0.186
3T3.3G-120	ND	ND	8/9	34.6 ± 11.6	1.166 ± 0.196
3T3.29-80	0/5	0	0/5	0	ND
dB7-40	0/5	0	0/5	0	ND
dC2-10	0/5	0	0/5	0	ND

* -10^5 cells [grown for greater than 2 months in the continuous presence of drug (MTX)] were injected subcutaneously or intravenously into nu/nu mice after changing growth medium 20 hours prior to injection.

** - An analysis of variance (ANOVA) was performed and t-t-tests done to examine differences in experimental metastasis between cell lines. From the mean ± standard error of the log (#tumors + 1) it was determined that there is no significant difference between the clones 3T3.3G-10, -20, -80, and -120. There is a significant difference at the 5% level between the clone 3T3.3G and all the drug selected clones listed above. The average fold difference as analysed by the analysis of variance was determined to be 3.5 times.

separate experiments of 5 mice each, an average of 6.5 lung metastasis per mouse per 10^5 cells. The other clones expressing a much greater amount of both k-fgf mRNA (20 times 3T3.3G for clone 3T3.3G-20) and protein showed on the average 5 times the amount of lung tumors per mouse per 10^5 cells. Examination of the experimental metastasis data using an analysis of variance (ANOVA) and t-t-tests showed that at the 5% level there is a significant difference between the clone 3T3.3G and all the drug selected clones. The average fold difference observed as determined by this analysis was 3.5 times. Although the quantity of lung tumors per mouse between the high expressing clones does not vary, the metastatic potential of these lines is shown to correlate with expression of kFGF to a maximum point.

3. CELL SURFACE GLYCOPROTEINS AND MALIGNANCY

3.1 Isolation and Characterization of Deoxyguanosine Resistant Mouse Cell Lines

To test the relationship between altered mutation rates and tumor progression further, we decided to attempt to isolate cell lines altered in ribonucleotide reductase activity by selecting for resistance to dG. It has been shown that dG resistant cell lines altered in ribonucleotide reductase exhibit modified spontaneous mutation rates (Weinberg et al. 1981). However as will be shown, our selections for dG resistance did not lead to the isolation of

variant lines altered in ribonucleotide reductase. Interestingly the dG resistant cell lines were observed to exhibit elevated metastatic characteristics when compared to the wild type cells, and we were therefore interested in characterizing them further.

3.1.1 Characterization of deoxygaunosine resistance

A 10T mouse cell line, NR4, which was tumorigenic but not metastatic (Egan et al. 1987) was selected for resistant to dG as described in Materials and Methods. Two clones NR4dGC1 and NR4dGC2 both resistant to 1 mM dG were characterized for dG resistance in three ways. First these clones were grown for 72 hr in increasing concentration of dG, after which their percentage growth, compared to cells grown in the absence drug, was calculated. Figure 25 shows that after 72 hr, growth of the wild type line NR4 was reduced to 20% in 200 μM dG, while the resistant clones only showed a 3% reduction of growth. Even at the highest concentration of dG, 1 mM, the resistant clones only showed a 13% decrease in growth. The actual growth rates of NR4, NR4dGC1 and NR4dGC2 were then examined in the presence and absence of 300 μM dG. As can be seen in Figure 26, the growth rate of both dG resistant clones were unaffected in 300 μM dG while the wild type NR4 line showed no growth in the presence of the drug. The relative plating efficiency for NR4, NR4dGC1, NR4dGC2 and clones derived from NR4dGC2, dGC2M5 and dGC2M5.9, (the isolation of these is described in the Materials and Methods) was determined from colony forming experiments. The results are as shown in Figure 27. Again it was obvious that the dG resistant clones were

Figure 25

Growth inhibition measured after 72 hours for increasing concentrations of deoxyguanosine in the mutants NR4dGC1 ▲, NR4dGC2 ■, and the wildtype line NR4 ●, (as described in the Materials and Methods, Determination of Growth Rate).

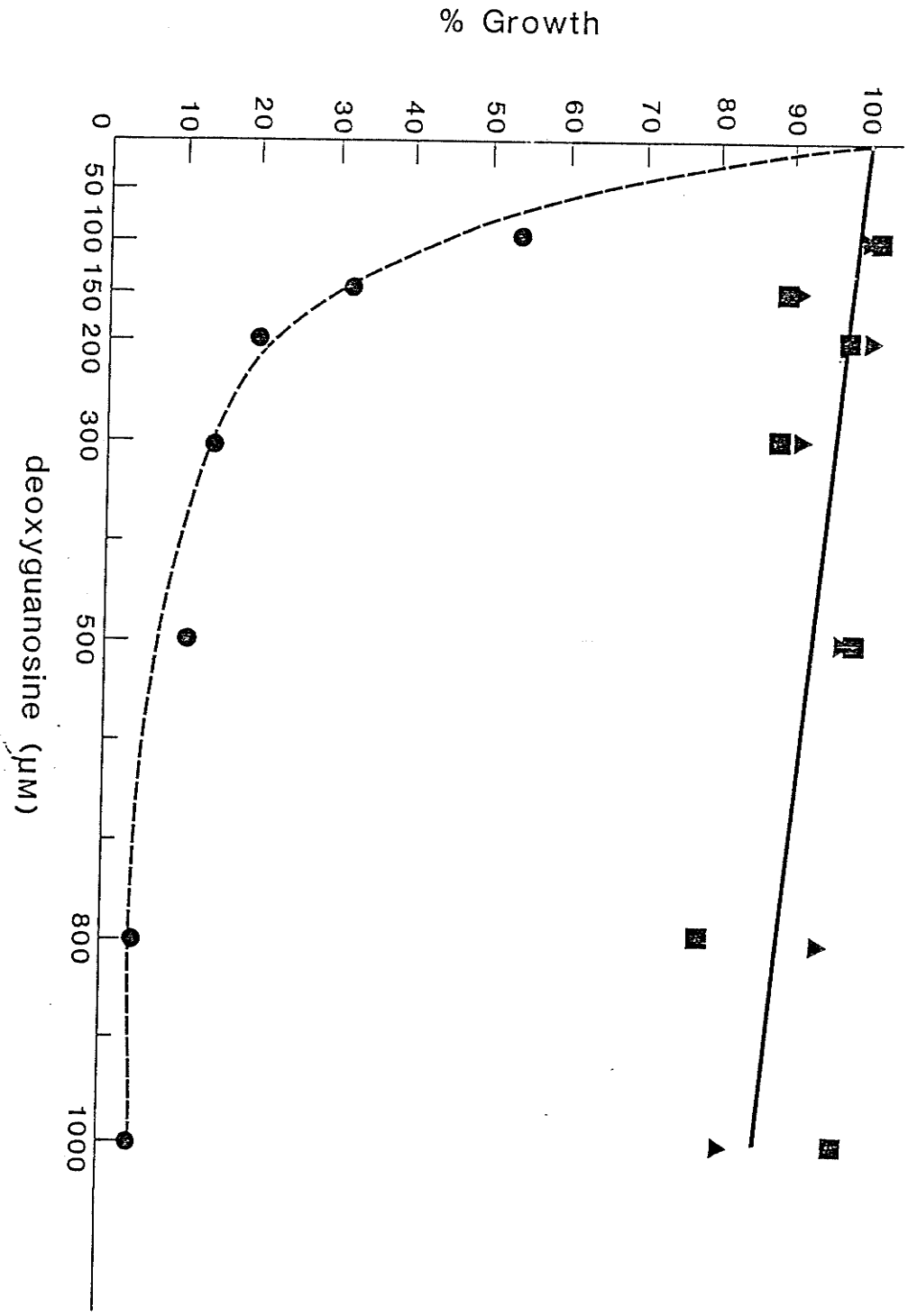

% Growth
100
90
80
70
60
50
40
30
20
10
0
50 100 150 200 300 500 800 1000
deoxyguanosine (μM)
NR4
dGC1
dGC2

Figure 26

Growth rates of 3 x 10^4 cells in the presence (filled symbols) and absence (open symbols) of 300 μM deoxyguanosine for the mutants NR4dGC1 △, NR4dGC2 □, and the wildtype NR4 ○.

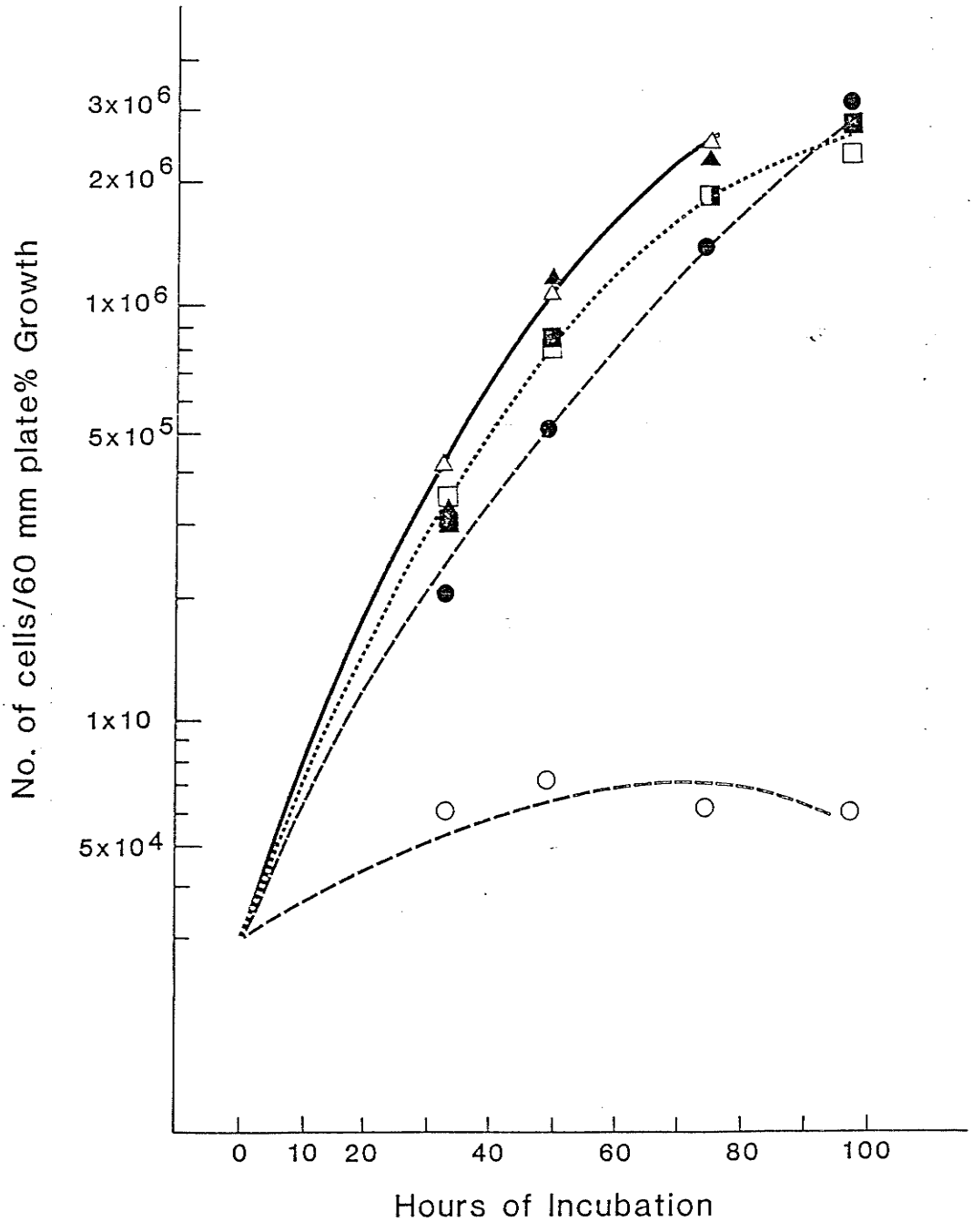
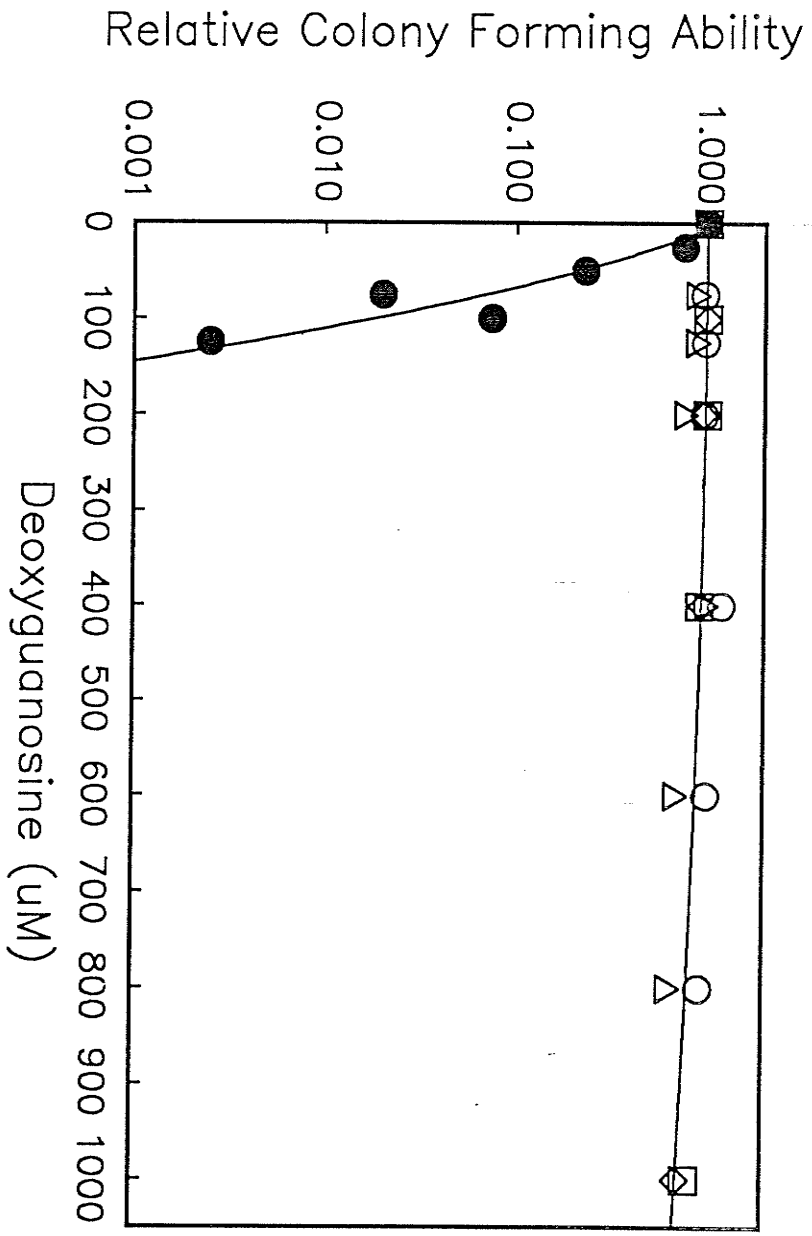

No. of cells/60 mm plate% Growth
3x10^6
2x10^6
1x10^6
5x10^5
1x10
5x10^4
0
10
20
40
60
80
100
Hours of Incubation

Figure 27

Relative colony-forming ability of the wildtype NR4 ● , and the mutants NR4dGC1 ○ , NR4dGC2 △ , dGC2M5 □ , dGC2M5.9 ◇ , in the presence of increasing concentration of deoxyguanosine.


Relative Colony Forming Ability
0.001
0.010
0.100
1.000
0
100
200
300
400
500
600
700
800
900
1000
Deoxyguanosine (uM)

significantly resistant in concentrations of drug as high as 1 mM deoxyguanosine while the wild type NR4 line was considerably more sensitive, showing only 10% survival in 100 μM deoxyguanosine.

3.1.2 Examination of a possible mechanism for deoxyguanosine resistance

To characterize the mechanism behind this resistance to dG, the cells were first examined for a mutant M1 subunit of ribonucleotide reductase since isolation of such mutants has been shown to occur through acquisition of dG resistance (Weinberg et al. 1981). This alteration in ribonucleotide reductase activity was also accompanied by changes in deoxyribonucleotide pool sizes. Mutants have been shown to exhibit a mutator phenotype due to changes in the deoxyribonucleotide pools such that the fidelity of DNA replication is compromised (Weinberg et al. 1981, Phear et al.. 1987). To determine if the NR4dGC1 and NR4dGC2 mutants had deoxyribonucleotide pool imbalances, the deoxynucleotide concentrations were measured using HPLC analysis. Table 7 shows the results of this analysis. The dTTP pools in both mutants showed a significant increase over that observed for wildtype cells, while the dCTP, dATP and dGTP pools remain unchanged. Other mutant M1 cells have shown a general increase in all the deoxynucleotide pools (Weinberg et al. 1981). The dGuo-200-1 ribonucleotide reductase M1 mutant characterized by Martin's group (Eriksson et al. 1981) for example, showed reduced binding affinity for dATP at the allosteric activity site and, this resulted in resistance of the enzyme to normal feedback regulation by

Table 7

Deoxyribonucleoside triphophate pool concentrations in the wild type NR4 cell line and the mutant lines NR4dGC1 and NR4dGC2

Cell Line	Deoxyribonucleotide conc. in pmoles/μg DNA (mean ± SE)			
	dCTP	dTTP	dATP	dGTP
NR4	5.08 ± 1.11 (4)	2.95 ± 0.52 (2)	2.00 ± 0.26 (2)	0.82 ± 0.16 (4)
NR4dGC1	4.34 (1)	15.05 ± 2.16 (2)	2.34 ± 0.35 (2)	1.30 ± 0.30 (2)
NR4dGC2	4.93 ± 0.45 (4)	4.95 ± 0.71 (2)	1.26 ± 1.12 (2)	0.92 ± 0.28 (4)

The number shown in brackets is the number of determinations

dATP. To examine our dG mutant NR4dGC2 for this same defect the activity of ribonucleotide reductase was examined in the presence of both dATP and dGTP. Table 8 shows that in the mutant cell line NR4dGC2 and wild type cells NR4, dATP and dGTP inhibited the enzyme activity to approximately the same level at all concentrations tested. In total, these experiments indicated that the dG mutant NR4dGC2 do not exhibit the same characteristics as those isolated by Martin's group (Erikkson et al. 1981). The enzyme, ribonuceotide reductase, was then examined at the molecular level by Southern blot analysis. Ribonucleotide reductase consists of 2 protein subunits, M1 and M2, and there are two genes encoding these subunit (Wright 1989). Figure 28 shows two resistiction enzyme digests, Eco R1 and Hind III, of DNA isolated from NR4, NR4dGC1 and NR4dGC2. No obvious rearrangments in the ribonucleotide reductase genes or amplifications were apparent when compared to the wild type situation. Analysis of both M1 and M2 messenger RNA levels also showed no difference when compared to the wild type NR4 cell line (Figure 29).

3.1.2.1 Tumorigenic and metastatic character of the deoxyguanosine resistant clones

At the same time these lines were being characterized for their resistance to dG they were examined for their tumorigenic and metastatic potential. The ability of these lines to grow in low percentage agar (a characteristic of transformed cells), as well as their ability to grow subcutaneously and at a secondary site following injection into the tail vein of syngenic mice was examined.

Figure 28

Southern blot analysis of ribonucleotide reductase M1 or M2. Twenty μg of DNA digested to completion with both Eco R1, Blots 1 and 2 and Hind III, Blots 3 and 4. The wild type NR4 line in each blot is lane (a), lane (b) is the mutant line NR4dGC1 and lane (c) is the mutant line NR4dGC2.

1. M1
a b c
2. M2
a b c
3. M1
a b c
4. M2
a b c
23.1–
9.4 –
6.6 –
4.3–
2.3–
2.0–

Table 8.

Ribonucleotide reductase activity measured in the presence of dATP and dGTP for the wild type cell line NR4 and the mutant NR4dGC2.

Percent dCDP production		
[dGTP]μM	NR_4	NR4dGC2
0	100	100
50	23.9	24.8
100	15.0	12.3
200	17.3	8.9
400	3.1	0
[dATP]μM	NR4	NR4dGC2
0	100	100
25	39.2	38.2
50	40.9	29.8

100% activity in nmoles dCDP/mg/hr for the wild type NR4 cell line and the mutant line NR4dGC2 was found to be 0.429 ± 0.057, and 0.401 ± 0.039 respectively.

Figure 29

Nothern blot analysis of mRNA for M1 (Blot 1) and M2 (Blot 2). Lanes (a) and (d) show the wild type NR4 in each blot, lanes (b) and (e) the mutant line NR4dGC1, and lanes (c) and (f) the mutant line NR4dGC2. Control actin probes to show approximately equal loading are presented below each blot.

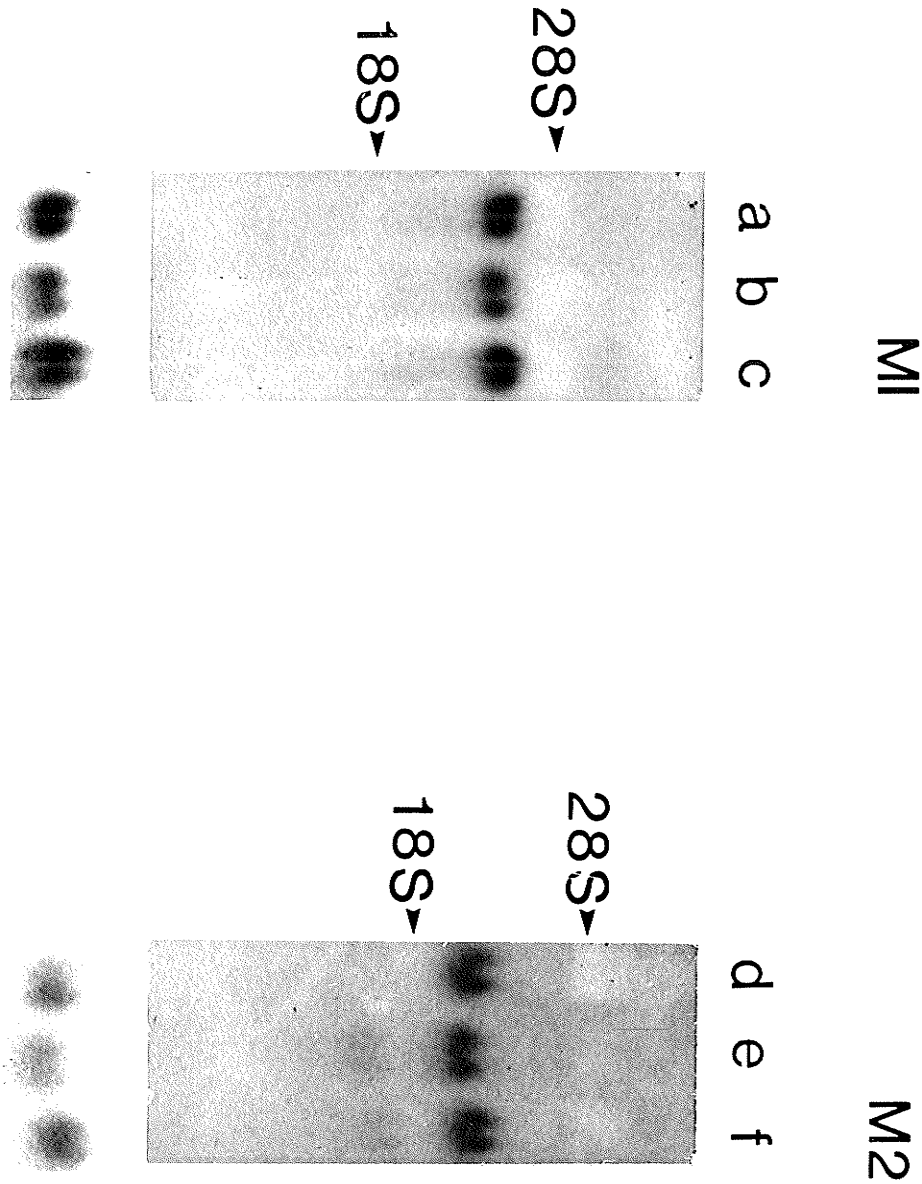

28S
18S
a b c
MI
28S
18S
d e f
M2

Table 9 shows that the NR4dGC2 clone had acquired both the ability to grow in low percentage agar and at a secondary site (experimental metastasis). A significant difference at the 5 % level was observed using an analysis of variance (ANOVA) and t-t-tests between the NR4dGC2 cell line and the wild type line NR4. The NR4dGC1 line had the same ability as the wild type NR4 line to grow at a secondary site, but had acquired the ability to grow in low percentage agar. The NR4 wild type line is a T24-H-*ras* transfected 10T clone (Egan *et al*. 1987), and *ras* is a GTP binding protein (Greenberg *et al*. 1989, Wright *et al*. 1990b). The possibility that the increase in tumorigenicity and metastasis as well as the resistance to dG was due to overexpression of this transfected gene in the NR4dGC2 clone, was examined by both Southern and Northern analysis. Figures 30 and 31 show that there has been no changes in either the gene copy number or the level mRNA expression of the *ras* gene.

Although these dG resistant lines did not appear to contain the mutator phenotype as described by Martin's group (Weinberg *et al*.1981), the NR4dGC2 line had acquired a very high metastatic phenotype. Two questions arose: a) what was the biochemical lesion that had resulted in this dG resistance and b) if this isn't related to the increase in metastatic potential, what other biochemical properties have changed in the NR4dGC2 line to allow it to become so highly metastatic? Both questions were subsequently addressed.

When dG is given exogenously to cells it enters by a nucleoside transport protein mechanism (Ullman *et al*. 1979) and can be converted to dGMP by deoxycytidine kinase activity (Gudas *et al*. 1978). This

Table 9

Metastatic and tumorigenic characteristics of the wild type cell line NR4 and the mutant lines, NR4dGC1 and NR4dGC2.

Cell Line	Tumorigenicity[a]		Experimental Metastasis[a]			Frequency (mean ± SE) of Cloning in soft agar		
	Freq.	Latency (days, mean ± SE)	Freq.	No. of tumors (mean ± SE)	mean ± std error of the log (#tumors + 1)	10^3	10^4	10^5
NR4	7/7	7.9 ± 1.1	11/14	3.0 ± 0.5	0.532 ± 0.090	0	0	0.7 ±0.7
NR4dGC1	5/7	12.2 ± 1.9	9/10	4.6 ± 1.2	0.636 ± 0.106	242 ± 6	>500	
NR4dGC2	6/7	12.5 ± 1.3	10/10	98.1 ± 13.5	1.879 ± 0.106[b]	125 ± 12	300 ± 16	>500

a-3 x 10^5 cells/0.2 ml were injected subcutaneously and intravenously into all the cell lines shown.

b-An analysis of variance (ANOVA) on the mean number of metastatic lung tumors ± standard error of the log (#tumors +1) for all cell lines showed that only the line NR4dGC2 differred significantly at the 5% level from the cell lines NR4 and NR4dGC1 which did not differ from each other.

Figure 30

Southern blot analysis of ras. Twenty μg of DNA was digested to completion with BamHI for the wild type cell lines, 10T , lane (a) and NR4, lane (b) and mutant lines NR4dGC1, lane (c) and NR4dGC2, lane (d).

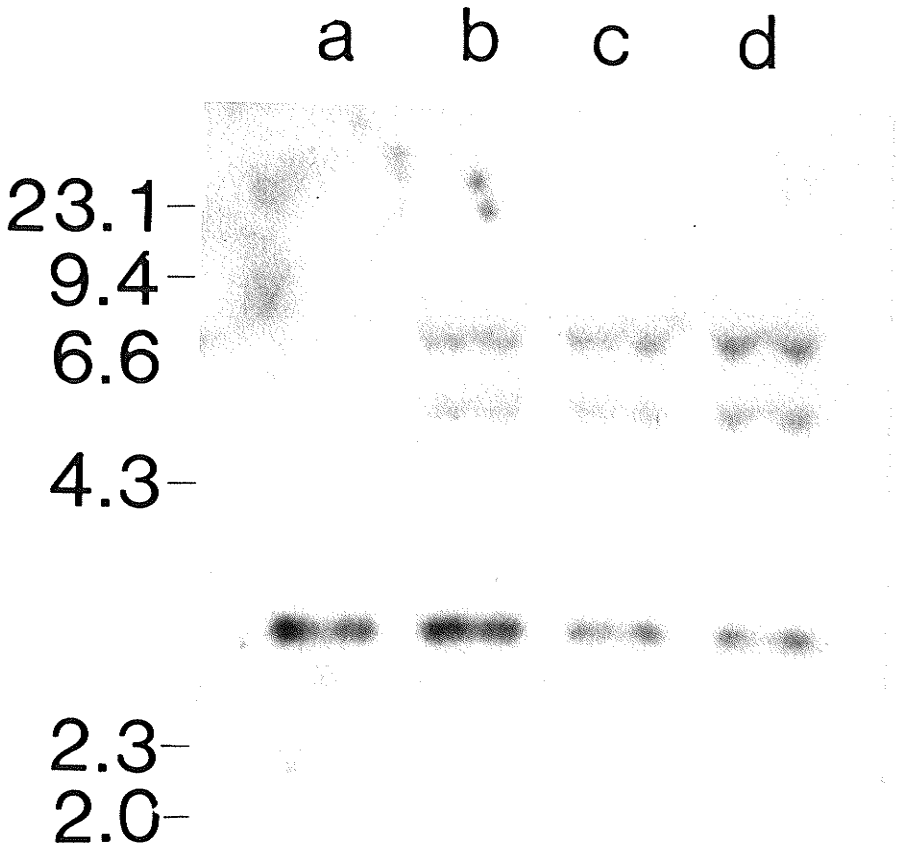
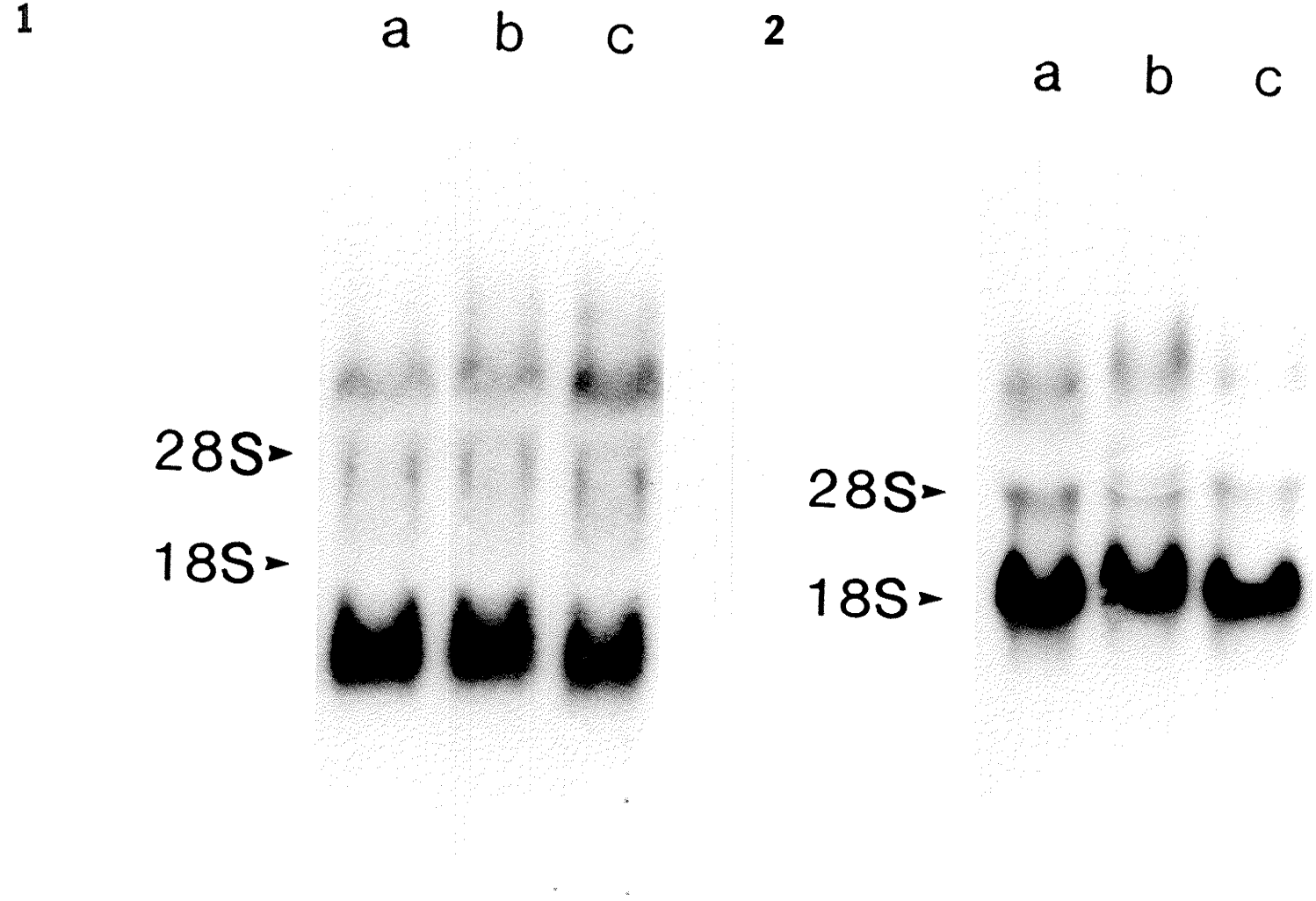

a
b
c
d
23.1
9.4
6.6
4.3
2.3
2.0

Figure 31

Northern blot analysis of (1): ras and (2): the same filter re-probed with actin as a control to determine RNA loading in all lanes.

Lane (a) is the wild type line NR4; lane (b), NR4dGC1; and lane (c), NR4dGC2.


1
a
b
c
28S
18S
2
a
b
c
28S
18S

leads to the formation of dGTP (Ullman and Martin 1984), followed by incorporation into DNA via ribonucleotide reductase (Reichard 1988). Alternatively exogenous dG can be converted to guanosine by a purine nucleoside phosphorylase (Ullman et al. 1979) and quickly converted to GMP by the enzyme hypoxanthine-guanine phosphoribosyltransferase (Sidi and Mitchell 1984). Typically, dG resistant mutants have been placed into the following four biochemical groups (as reviewed by Ullman 1989) exhibiting:

a) Nucleoside transport deficiency

b) Deoxycytidine kinase deficiency

c) Purine nucleoside phosphorylase and Hypoxanthine-Guanine phosphoribosyltranferase deficiency

d) Ribonucleotide reductase alterations

It had already been determined that the NR4dGC2 line was not resistant to dG because of an altered ribonucleotide reductase. To test which of the three remaining alterations might be the cause for resistance in NR4dGC2, cells were incubated with 0.3 μM radiolabelled dG, guanosine (G), hypoxanthine (H), deoxycytosine (dC) and deoxyadenosine (dA) and incorporation of these compounds into DNA was measured. As shown in Table 10, the mutant NR4dGC2 line incorporated the same amount of dC and dA into DNA suggesting that it was not a transport mutant. The amount of G incorporated however, was 6.5 fold less in the mutant; the amount of dG incorporated was 5 fold less, and most importantly the amount of H incorporated into DNA in themutant line was 47 fold less than that for the wild type NR4 line. In a previous attempt to analyze the mutation rates of this mutant

Table 10.

Total counts of TCA precipitated DNA per total counts of ^{3}H-thymidine for the wild type NR4 cell line and mutant NR4dGC2 cell line grown in the presence of 0.3 μM ^{3}H-labelled dC, dA, G, dG and H.

Nucleotide	dpm/dpm Thymidine incorporated NR4	NR4dGC2	Fold decrease
dC	0.52	0.44	-
dA	74.9	86.2	-
G	14.1	2.2	6.5
dG	5.3	1.1	4.8
H	18.8	0.4	47

*- the specific activity of the ^{3}H-thymidine used was 35 Ci/mmole

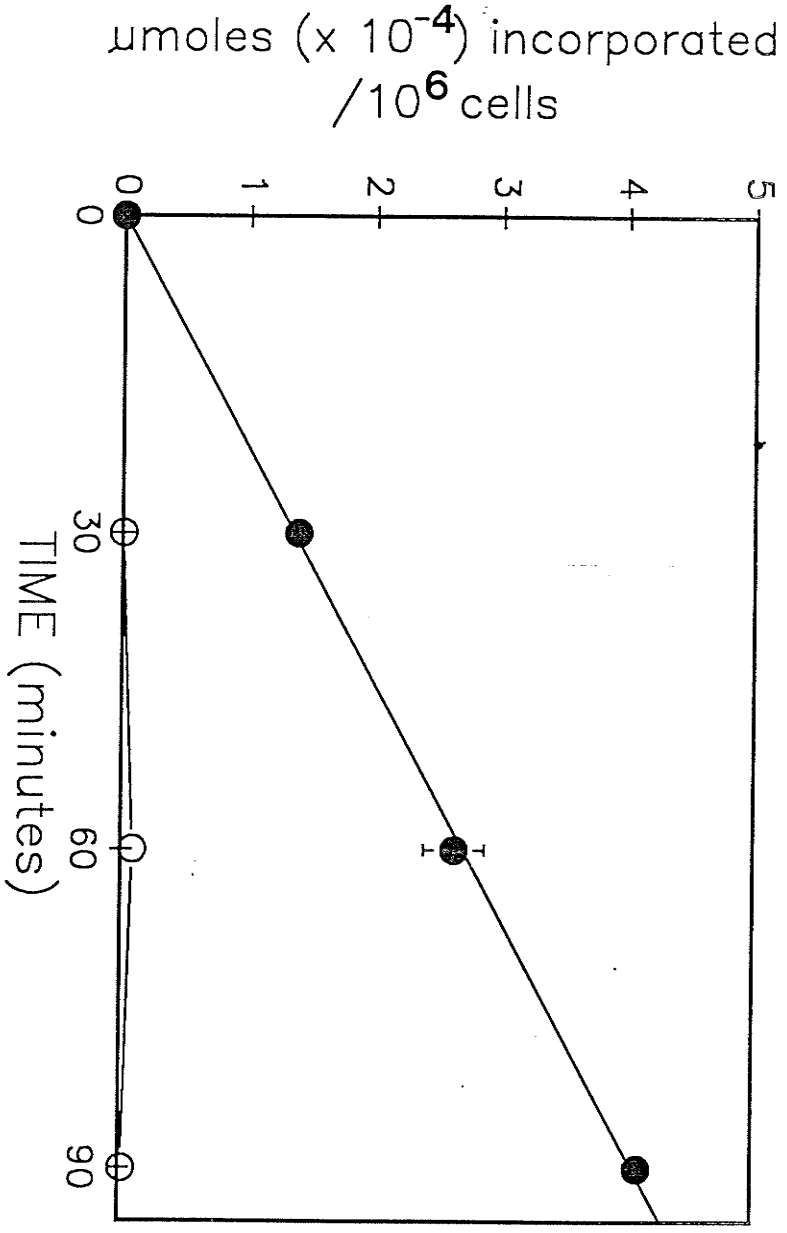
line it was found that it was also cross resistant to 6-thioguanine (data not shown). Resistance to 6-thioguanine often results from mutation in the enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT) (Morrow et al. 1989). An in vivo assay was used to test the activity of the enzyme HGPRT, as described by Ullman et al.(1979b). Figure 32 shows that the binding of labelled anionic metabolites of hypoxanthine to the anion DEAE exchange discs is linear with time in the wild type NR4 cell line. No significant HPGRTase activity was found in the highly metastatic mutant NR4dGC2. This does not however, explain why the NR4dGC2 line acquired such a high metastatic potential.

3.2 Characterization of a Glycoprotein Associated with the Altered Metastatic Phenotype

One very important characteristic required for cells that acquire the potential to metastasize is the ability to become motile and travel to secondary site (Liotta and Hart, 1982). Treatment with tunicimycin, for example, which removes N-linked carbohydrate structures from cell surface glycoproteins renders a previously metastatic cell, non metastatic (Poste 1982). Many studies have also shown, through lectin binding experiments, that the carbohydrate character on metastatic cells can be very different from that of their nonmetastatic counterparts (Altevogt et al. 1983, Lang et al. 1988, Kerbel et al. 1982, Barnett and Eccles 1984). To examine the possibility that there may be some carbohydrate changes involved in the acquistion of the metastatic phenotype in the dG resistant clone

Figure 32

In vivo HPGRTase activity measured following incorporation of ^{3}H-hypoxanthine into the wild type line NR4, ●, and the mutant dGC2M5, ○, for increasing amounts of time. Cell lysates were placed on DEAE-Whatmann filter discs, washed and counted as described in Materials and Methods.


µmoles (x 10⁻⁴) incorporated
/10⁶ cells
0
1
2
3
4
5
0
30
60
90
TIME (minutes)

NR4dGC2, lectin binding studies were carried out. Since the clone NR4dGC2 had been carried in culture for over 1 year, this line was recloned in low percentage agar. This new clone designated dGC2M5 was retested for dG resistance (Figure 27) and its metastatic and tumorigenic characteristics (Table 11). At the same time, NR4dGC2 and the new clone dGC2M5 were both recloned, using limiting dilution (cells were harvested, diluted to a concentration of 5 cells/ml and aliquoted, 200 μl/well in 96 well plates) to isolate clones within the population that were revertants for the metastatic phenotype. These clones, dGC2REVC and dGC2M5.9 were also recharacterized for their metastatic and tumorigenic potential (Table 11). There was a significant difference at the 5 % level using an analysis of variance (ANOVA) and t-t-tests between the lines NR4dGC2 and dGC2M5 versus NR4, dGC2RevC, and dGC2M5.9 but not between lines in these two groups. The dGC2M5.9 clone was also checked for dG sensitivity to determine if this resistance was maintained (Figure 27).

3.2.1 Inhibition of metastasis by glycosylation inhibitors

To examine if carbohydrate structure on the surface of these dG resistant cells was important in determining metastatic ability, the effects of the glycosylation inhibitors, castanospermine, swainsonine and deoxymannojirimycin (Elbein, 1984) on the metastatic properties of dGC2M5 was determined. The highly metastatic clone dGC2M5 and the wild type NR4 line were first tested for the concentrations of these drugs required to have an effect on the glycoprotein character of the cell without inhibiting growth in cell culture (Figure 33 a,b,c).

Table 11

Tumorigenic and metastatic characteristics of the wild type NR4 cell line and the mutant clones NR4dGC2, dGC2REVC, dGC2M5, dGC2M5.9.

CELL LINE	EXPERIMENTAL METASTASIS			FREQUENCY (Mean ± S.E) OF CLONING IN SOFT AGAR		
	FREQ.	no. of lung tumors (mean ± SE)	mean ± std error of the log(# tumors + 1)	10^3	10^4	10^5
	/3 x 10^5 cells					
NR4	11/14	3.0 ± 0.5	0.531 ± 0.150*	0 ± 0	0 ± 0	8.1 ± 0.5
NR4dGC2	12/13	92.9 ± 20.5	1.773 ± 0.150**	125 ± 12	300 ± 16	>500
dGC2REVC	7/10	7.8 ± 3.6	0.517 ± 0.177*	36 ± 4	>250	>500
dGC2M5	5/5	>250	ND	108 ± 7	>500	
dGC2M.9	5/5	15.0 ± 8.0	0.941 ± 0.251*		N.D	
	/1 x 10^5 cells		x3 of 1 x 10^5			
dGC2M5	9/10	50.2 ± 8.7	1.889 ± 0.177**			

*, ** - An analysis of variance (ANOVA) was performed and t-t-tests done to examine differences in experimental metastasis between cell lines. From the mean ± standard error of the log (number of tumors + 1) it was determined that there is no significant difference between the clones indicated *, or between the clones indicated **. There is a significant difference at the 5% level between the clones * versus **.

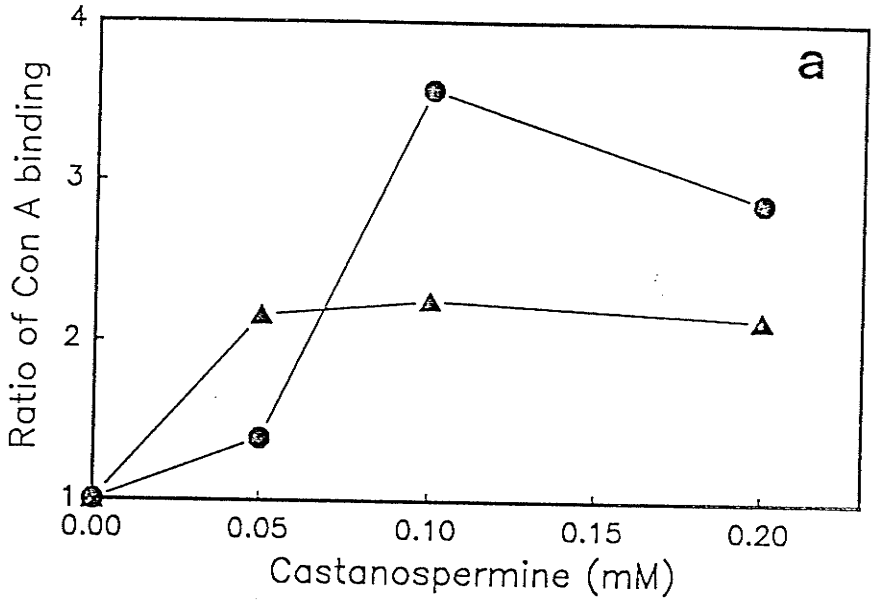
Figure 33

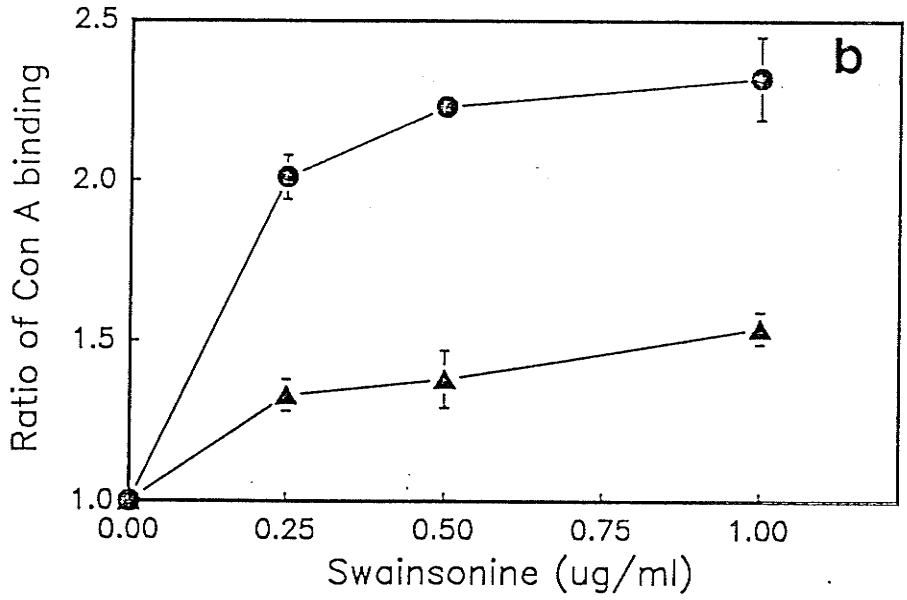
The ratio of Con A binding in the presence and absence of drug for the wild type NR4 cell line, ● ; and the mutant cells dGC2M5, ▲ . Binding was carried out in the presence of 100 μg/ml labelled Con A, and the ratio of Con A bound/mg protein is presented for:

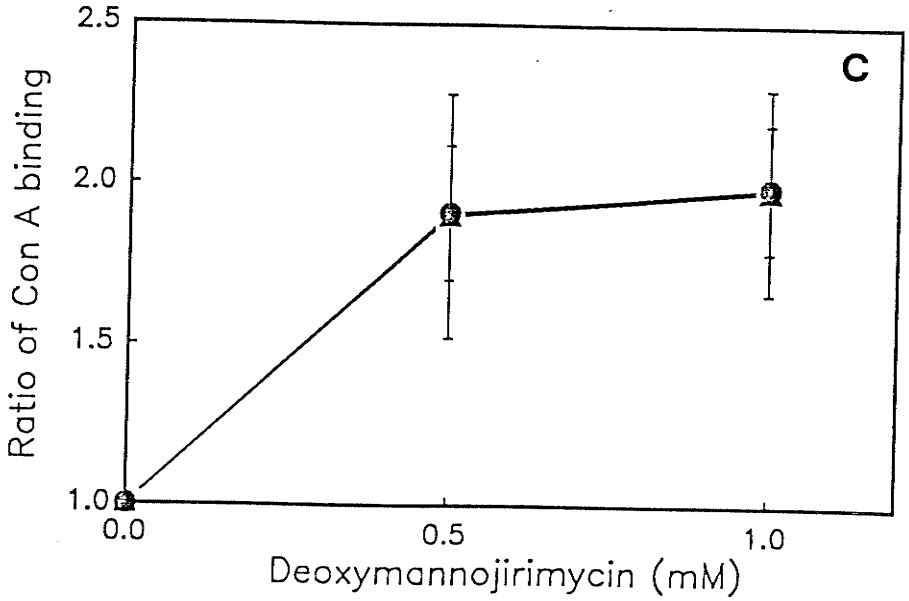
a-castanospermine.

b-swainsonine.

c-deoxymannojirimycin.


a
Ratio of Con A binding
4
3
2
1
0.00
0.05
0.10
0.15
0.20
Castanospermine (mM)


b
Ratio of Con A binding
2.5
2.0
1.5
1.0
0.00
0.25
0.50
0.75
1.00
Swainsonine (ug/ml)


c
Ratio of Con A binding
2.5
2.0
1.5
1.0
0.0
0.5
1.0
Deoxymannojirimycin (mM)

These figures show that since the binding of Con A [which binds to high mannose residues (Sharon and Lis 1986)] in drug over the binding in the absence of drug is greater than one, the synthesis of complex carbohydrates is being inhibited and more high mannose residues are apparent on the cell surface. From the ratio of Con A binding, the concentration where these drugs had a maximum affect on the cells, could be determined. To ensure maximum effectiveness without inhibition of growth, these concentrations, 0.05 mM castanospermine, 1 μg/ml swainsonine and 1.0 mM deoxymannojirimycin, were used in the examination of the effect of inhibition on the metastatic phenotype of dGC2M5. The dGC2M5 clone was incubated in the presence of these concentrations of drug for 48 hours before injection into syngenic mice. Note that trypan blue dye exclusion was done on cells before injection to ensure that more than 80% of the cells were viable and that 2×10^5 viable cells were injected into each mouse. The results presented in Tables 12A and 12B show that swainsonine and deoxymannojirimycin both had a dramatic effect on decreasing the metastatic phenotype of dGC2M5 cells when compared to cells not treated with drug. This was statistically significant as shown for an example experiment in Table 12B. Both these drugs inhibited the metastatic capacity of dGC2M5 by 40% whereas castanospermine had no effect.

3.2.2 Lectin binding characteristics of the metastatic deoxyguanosine clone

Lectin binding studies were performed with Con A, WGA and SBA to determine if any obvious differences in surface carbohydrate

Table 12a

The percent inhibition of the mean number of lung metastases in dGC2M5 cells grown in the presence of the glycosylation inhibitors: castanospermine, swainsonine, and deoxymannojirimycin. The results were calculated from the average number of lung tumors obtained in three seperate experiments.

DRUG	Percent Inhibition of Metastases Formation
0.05 mM Castanospermine	no change
6.0 μM Swainsonine	34 ± 17
1.0 mM Deoxymannojirimycin	39 ± 11

On average the number of lung tumors in mice injected with 2×10^5 cells/mouse for the dGC2M5 cell line in the absence of drug was approximately 100. In the presence of swainsonine and deoxymannojirimycin the number of lung tumors/mouse under the same conditions was approximately 65.

Table 12b

A typical experiment as indicated in table 12a

Drug	Experimental metastasis mean ± std error of the log (#tumors + 1) - drug	+ drug
Castanospermine	1.654 ± 0.336	1.508 ± 0.336[a]
Swansonine	1.468 ± 0.178	0.814 ± 0.178[b]
Deoxymannojirimycin	1.654 ± 0.340	0.941 ± 0.340[c]

An analysis of variance (ANOVA) was performed and t-t-tests done to examine differences in experimental metastasis between each line in the absence and presence of drug from the mean ± standard error of the log(number of tumors +1).

a- showed there was no significant difference in the number of lung metastsis for cells grown in the presence of castanospermine

b- showed there was a significant difference in the number of lung metastasis at the 5% level for cells grown in the presence of swainsonine.

c- showed there was a significant difference in the number of lung metastasis at the 20 % level for cells grwon in the presence of deoxymannojirimycin.

structures could be detected between the cell lines, NR4, dGC2M5 and dGC2REVC. This was done using lectins that have been used to show differences in cell surface structures with other metastatic cell lines (Altevogt et al. 1983, Barnett and Eccles 1984, Collard et al.1986, Lang et al. 1988, Laferte' and Dennis 1988, Buckley and Carlsen 1988). The three Figures 34, 35, and 36, show the dose response curves for the binding of Con A, WGA, and SBA to the wild type NR4 line and the dG resistant clones dGC2REVC and dGC2M5. Taking into consideration both the time and concentration graphs for each lectin, no correlation between metastasis and Con A or WGA binding was observed. The SBA curves, on the other hand, showed that the wild type non metastatic line NR4 bound high amounts of SBA whereas the highly metastatic clone dGC2M5 did not. In Figure 37 which summarizes these findings, a four fold increase in SBA binding (in 20 min) was observed with NR4 cells compared to the highly metastatic line dGC2M5. The dGCREVC line was found to have moderate binding to SBA, 2 times that of NR4 cells. A clone of dGC2M5, dGC2M5.9, which was tested in the metastatsis assay and found to be poorly metastatic, also showed moderate SBA binding (2 times that of NR4 cells).

To seek confirmation of the observation of this apparent inverse correlation of the binding of SBA lectin with the metastatic potential of the dG resistant clones, a second type of lectin binding experiment was carried out on these lines. SBA conjugated to FITC, a flourescent label, was used in conjuntion with FACS analysis. Unlike the previous binding experiments, cells were first harvested

Figure 34

The concentration of labelled Con A bound/mg protein presented for:

a-increasing concentrations of Con A (μg/ml) bound/ 20 minute assay.

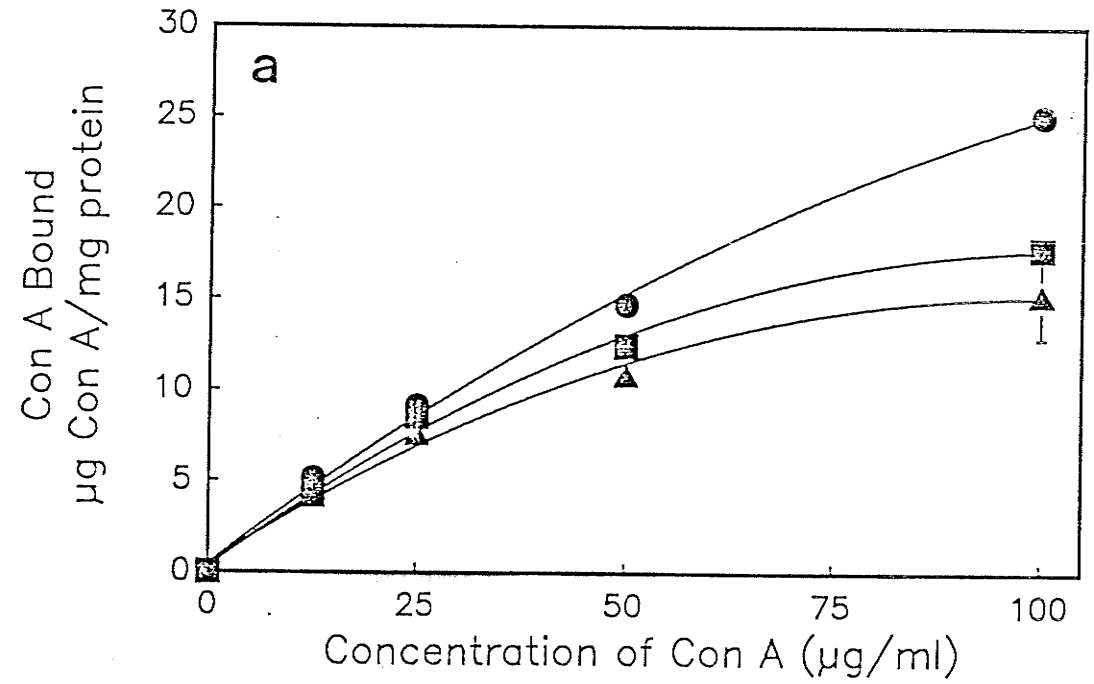
b-100 μg/ml Con A for increasing amounts of time (min).

In the following cell lines:

● -NR4

■ -dGC2RevC

▲ -dGC2M5


a
Con A Bound
µg Con A/mg protein
30
25
20
15
10
5
0
0
25
50
75
100
Concentration of Con A (µg/ml)

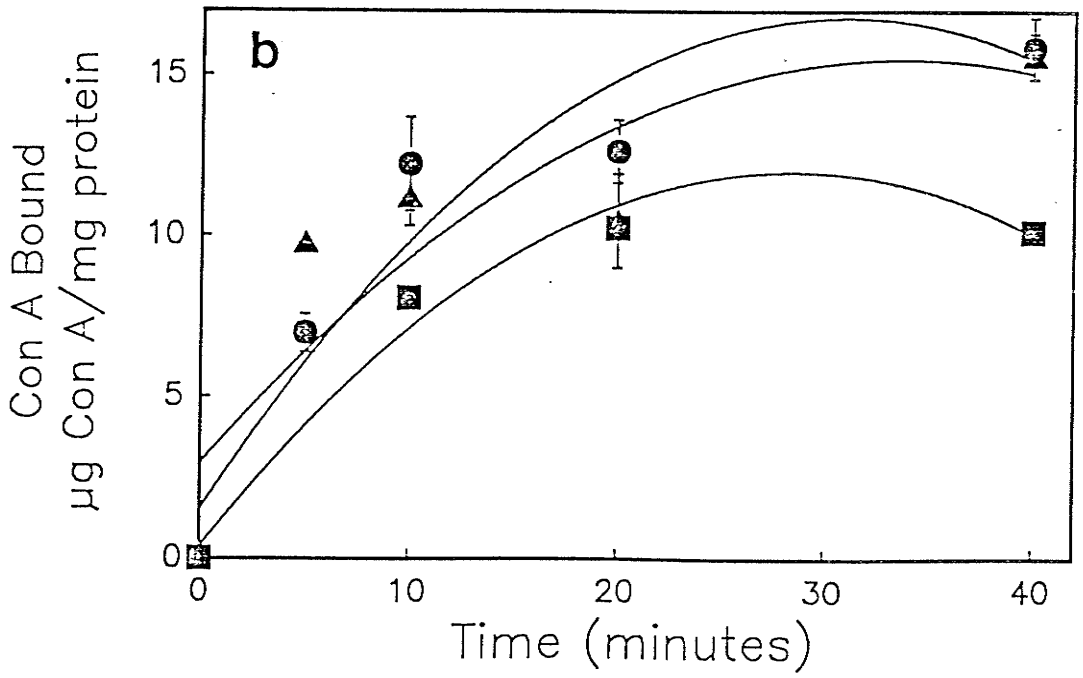

b
Con A Bound
µg Con A/mg protein
15
10
5
0
0
10
20
30
40
Time (minutes)

Figure 35

The concentration of labelled WGA bound/mg protein is presented for:

a-increasing concentration of WGA (μg/ml) bound/ 20 minute assay.

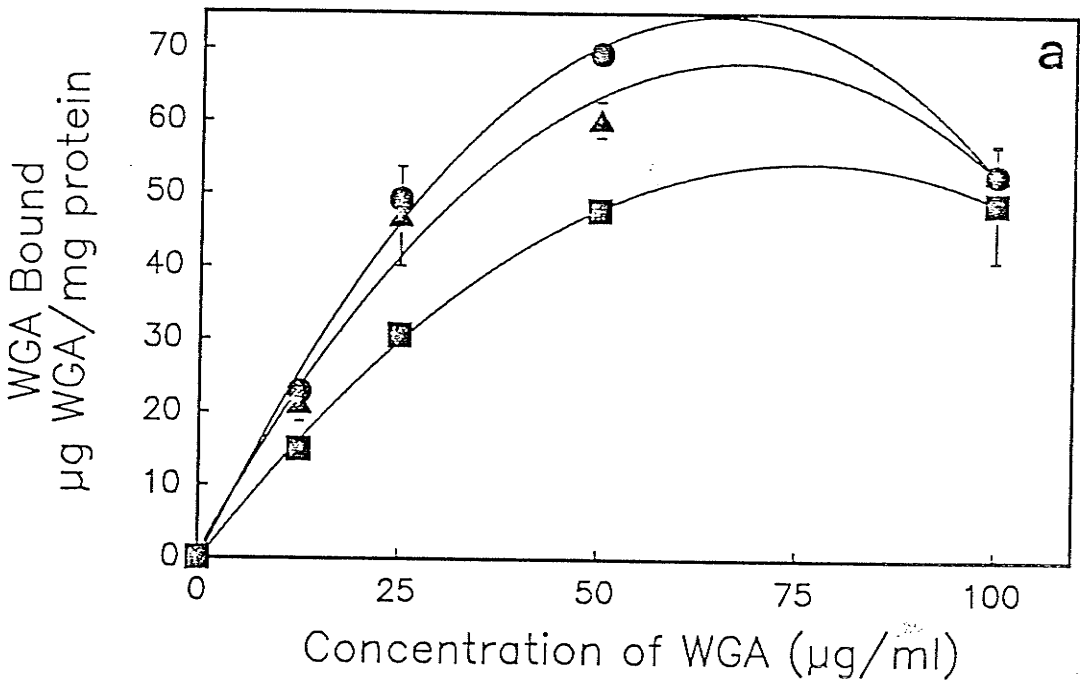
b-100 μg/ml of WGA for increasing amounts of time (min).

In the following cell lines:

● -NR4

■ -dGC2RevC

▲ -dGC2M5


a
WGA Bound
µg WGA/mg protein
70
60
50
40
30
20
10
0
0
25
50
75
100
Concentration of WGA (µg/ml)

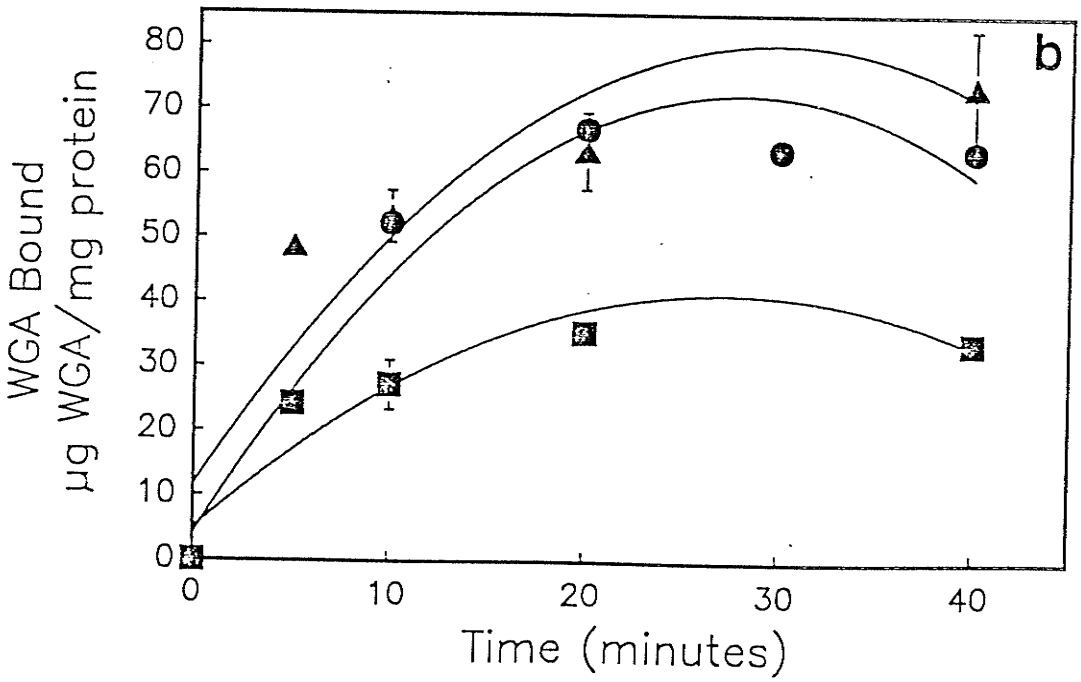

b
WGA Bound
µg WGA/mg protein
80
70
60
50
40
30
20
10
0
0
10
20
30
40
Time (minutes)

Figure 36

The concentration of labelled SBA bound/mg protein is presented for:

a-increasing concentrations of SBA (μg/ml) bound/ 20 minute assay.

b-100 μg/ml SBA for increasing amounts of time (min).

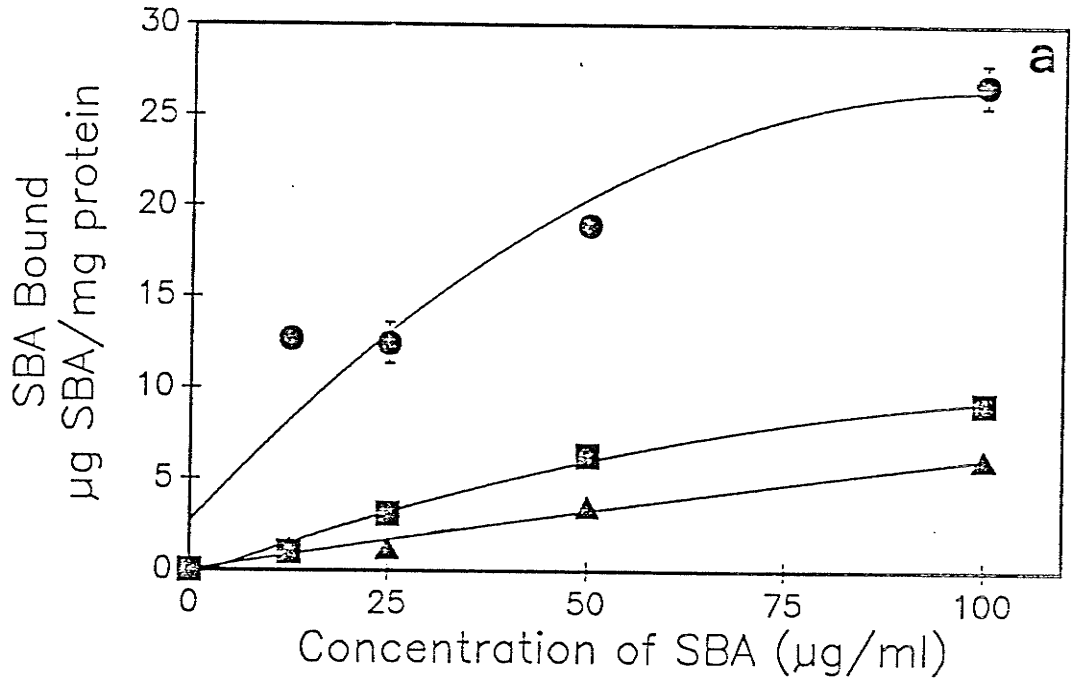
In the following cell lines:

● -NR4

■ -dGC2RevC

▲ -dGC2M5

◆ -dGC2M5.9


a
SBA Bound
µg SBA/mg protein
30
25
20
15
10
5
0
0
25
50
75
100
Concentration of SBA (µg/ml)

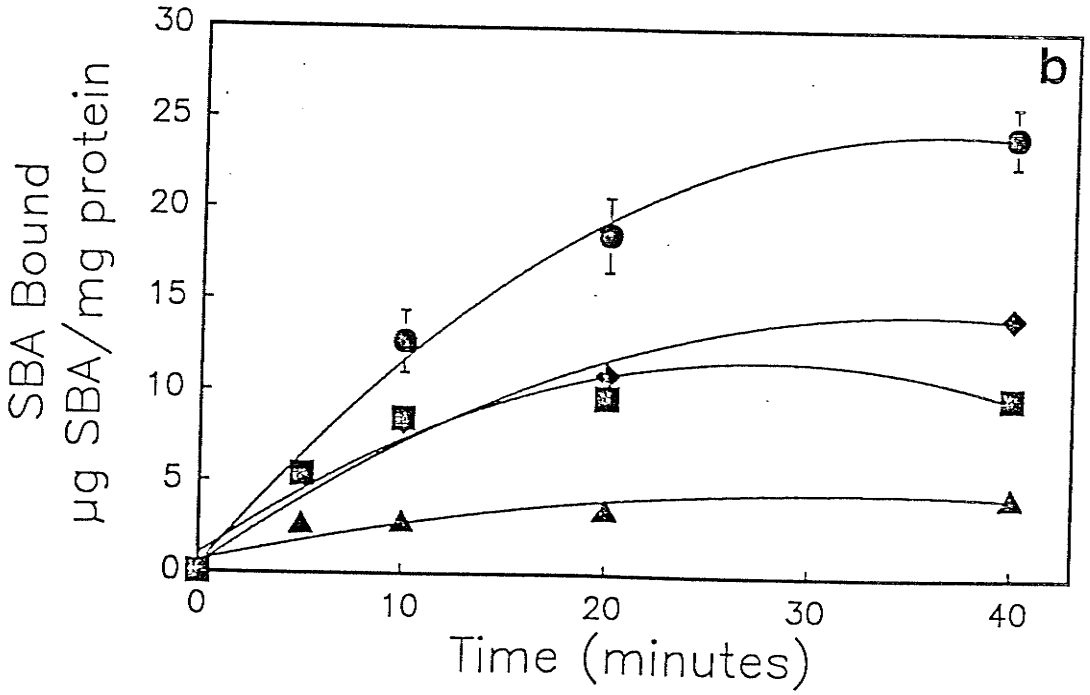
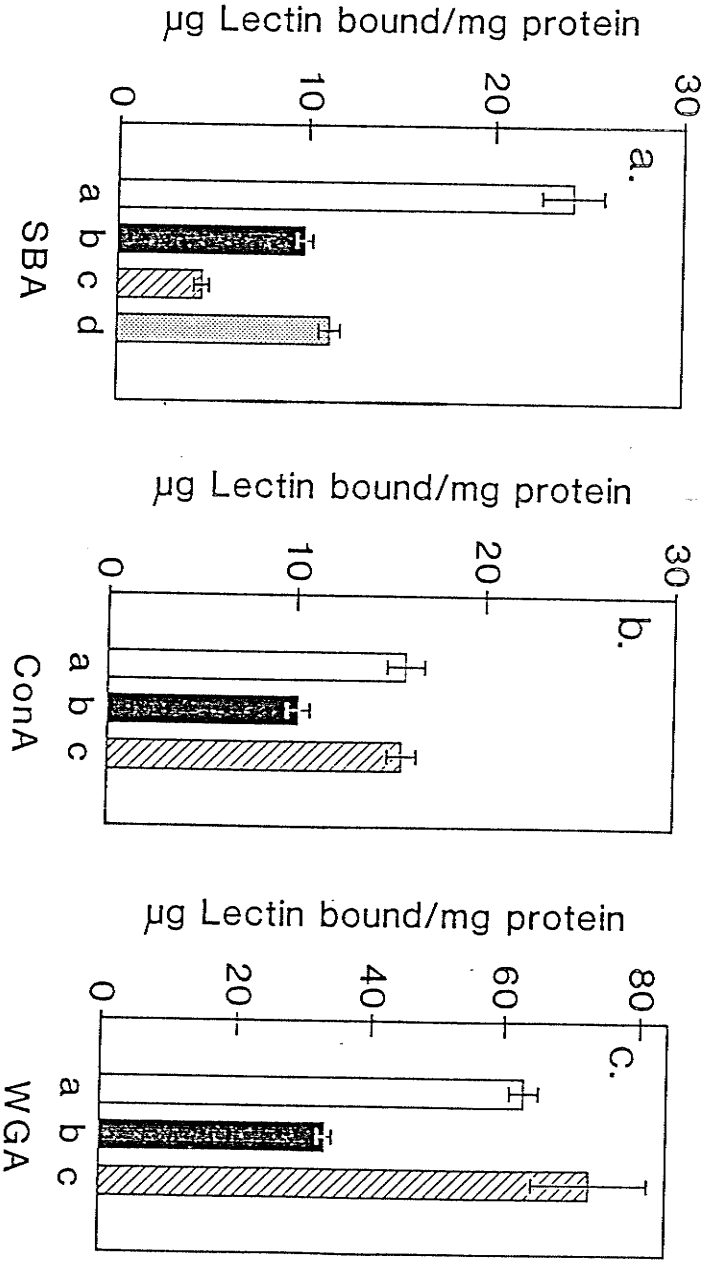

b
SBA Bound
µg SBA/mg protein
30
25
20
15
10
5
0
0
10
20
30
40
Time (minutes)

Figure 37

The amount of lectin bound for 100 μg/ml of (a) SBA, (b) Con A, and (c) WGA in 20 min. for the following cell lines:
lane (a), the wild type NR4; lane (b), dGC2M5; lane (c), dGC2REVC; and lane (d), dGC2M5.9.


µg Lectin bound/mg protein
0
10
20
30
a.
SBA
a b c d
µg Lectin bound/mg protein
0
10
20
30
b.
ConA
a b c
µg Lectin bound/mg protein
0
20
40
60
80
c.
WGA
a b c

using 0.2 mM EDTA and then incubated in suspension on ice with FITC-SBA (described in the Materials and Methods). The results are shown as the mean flourescence minus the mean flourescence of the cells in the presence of 0.2 M GalNAc, per mean flourescence of the wild type NR4 line, for the average of 3 seperate experiments. This was plotted against the cell lines arranged in order of increasing metastatic potential (Figure 38). As seen in Figure 38 as the cells became more metastatic their affinity for the lectin, SBA, decreased.

3.2.3 Identification of a SBA binding glycoprotein absent in the metastatic deoxyguanosine dGC2M5 clone

Identification of this negative correlation between metastatic ability and the ability of the cells to bind SBA, led to the question of whether there was a general loss of overall carbohydrate structure or if this was the result of the loss of a specific glycoprotein. Previous studies in lymphocytes had shown that SBA binding was lost in highly metastatic cells due to carbohydrate changes on a few select glycoproteins (Lang et al. 1988). To identify potential glycoproteins that bind SBA on the wild type line but not on the dGC2M5 line, a lectin precipitation technique was used in which whole cells were first labeled with ^{125}I such that all cell surface glycoproteins were labelled (Lang et al. 1988). The whole cells were then incubated in the presence of the lectin, SBA, bound to biotin and the cells were solubilized. Subsequent incubation with avidin bound to agarose beads allowed precipitation of the complex of

Figure 38

Ratio of FITC labelled SBA binding with the mutant dG resistant clones compared to the binding with the wild type NR4 line, presented in increasing order of metastatic potential.

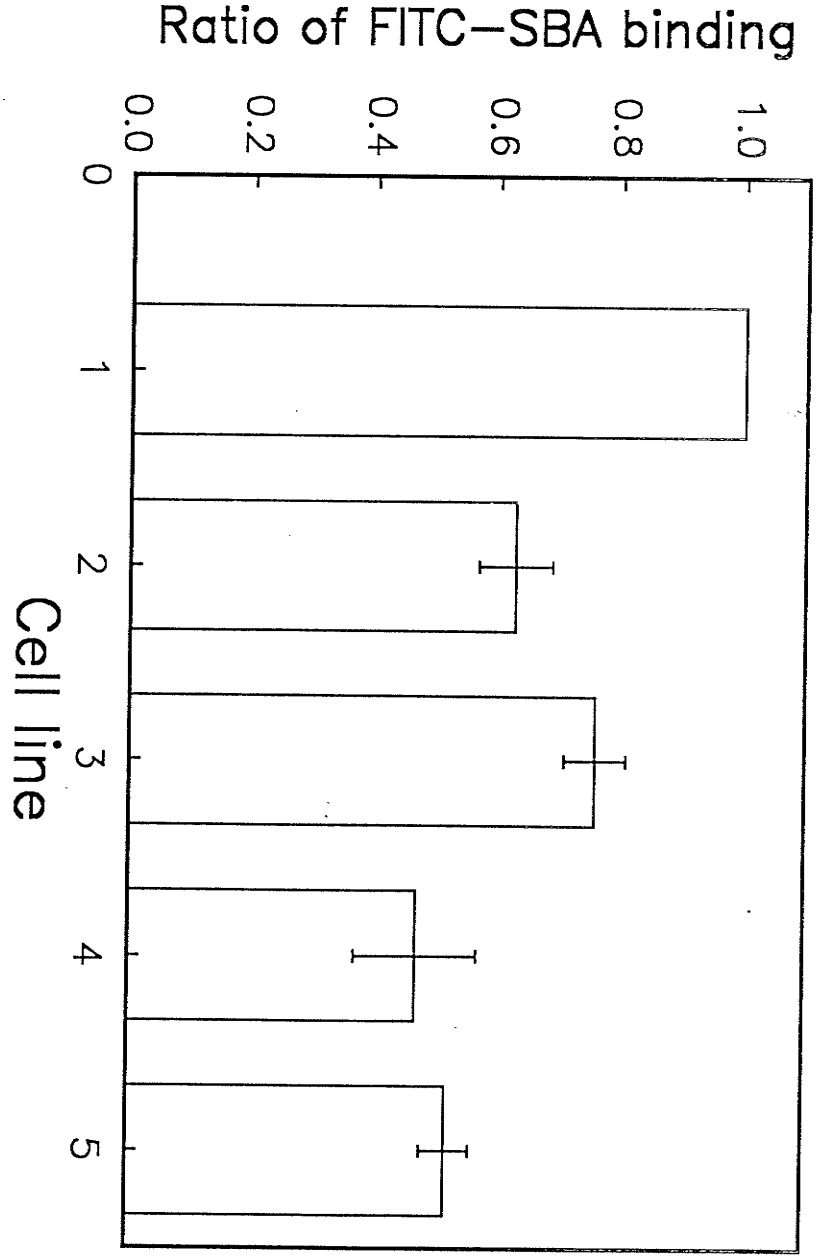
1-NR4

2-dGC2RevC

3-dGC2M5.9

4-dGC2

5-dGC2M5

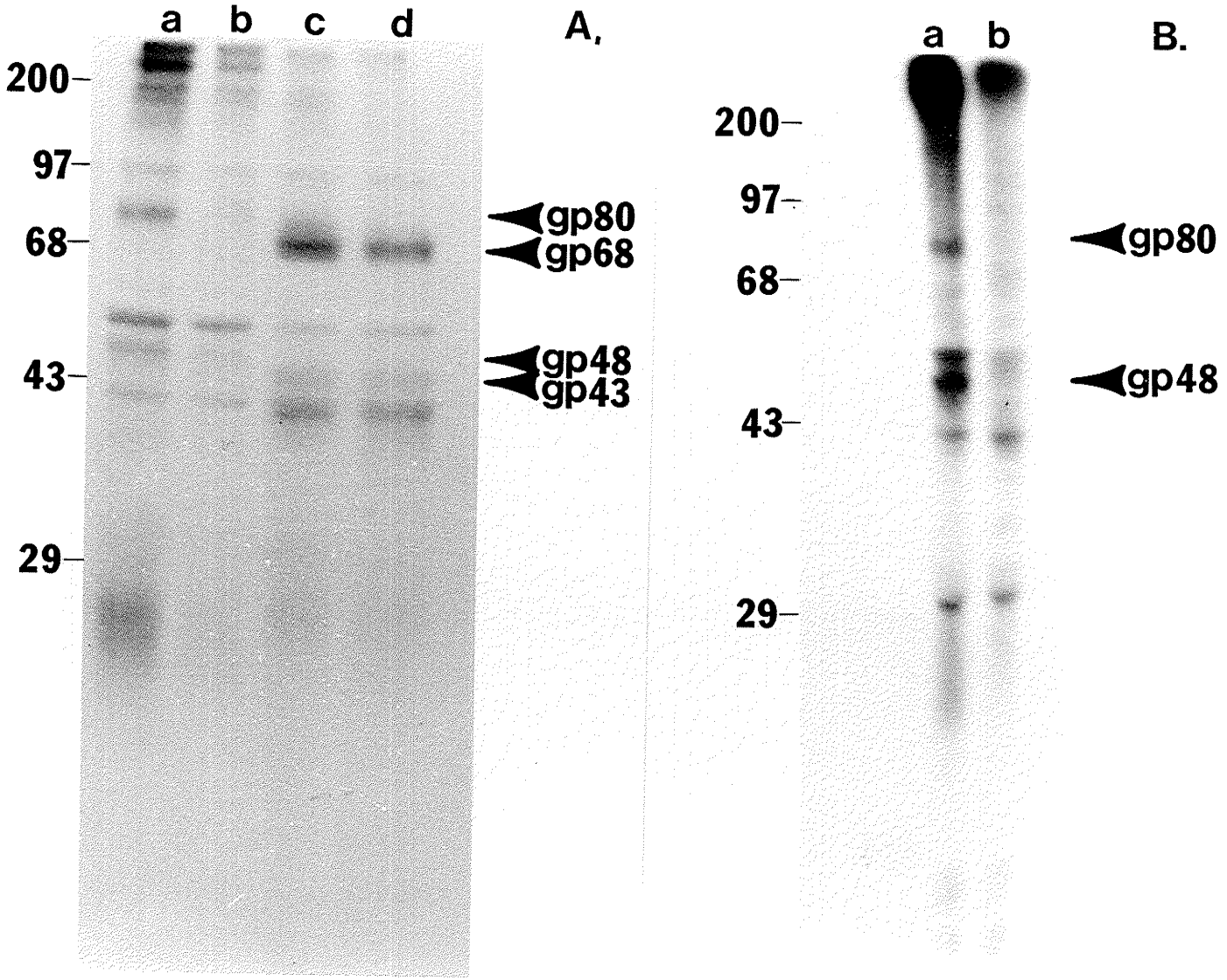

Ratio of FITC–SBA binding
0.0
0.2
0.4
0.6
0.8
1.0
Cell line
0
1
2
3
4
5

glycoprotein-SBA-biotin-avidin-agarose. The glycoprotein was then specifically eluted from the SBA using the sugar inhibitor GalNAc. The subsequent eluants were then run on a 10% SDS-PAGE gel and, following silver staining to control for the protein loaded, the gel was dried and an autoradiogram obtained as shown in Figure 39A. The agarose beads were boiled following elution with GalNAc, in 100 μl of 0.2% NP40 buffer plus sample buffer and also run on a 10% SDS-PAGE gel (Figure 39B). The control lanes, Figure 39A, are the result of avidin nonspecific binding to labeled proteins (lanes b and d). Two bands, of molecular weight 80,000 daltons (gp80) and 48,000 daltons (gp48), indicated by the arrowheads were clearly present on the wild type NR4 cell line (lane a), and not on the dGC2M5 line (lane c). The gp48 band appears to bind tightly to SBA-biotin as it was not eluted as efficiently with GalNAc, but showed up clearly once the beads were boiled (Figure 39B). Equal intensity bands were observed in the dGC2.M5 lane slightly below the p80 and p48 observed in the NR4 line, gp68 and gp43. These proteins do not specifically bind SBA since they also appeared in the control lane (lane d), where only avidin-agarose was used. These results suggest that the gp68 and p43 observed on the dGC2.M5 cells, which do not bind SBA, may be the same proteins as the gp80 and gp48 seen on the NR4 line following some kind of specific carbohydrate alteration (making them smaller and no longer able to specifically bind SBA). Alternatively, the glyco-proteins gp80 and gp48 may not be expressed in the metastatic dGC2M5 line and gp68 and gp43 may also be unique proteins to this line.

Figure 39

A-SBA lectin precipitation of 10mg each of ^{125}I-labelled cell extracts run on a 10% SDS-PAGE gel for the wildtype NR4 line, lane (a) and the mutant dGC2M5, lane (c) following eluation of labelled protein from SBA with 0.25 M GalNAc at 40°C for 30 min. Lanes (b) and (d) are controls of both lines where the cells were incubated in avidin-agarose without previous addition of SBA -biotin, see Materials and Method.

B-Following elution from SBA-biotin-avidin-agarose with GalNAc, as described in Figure 39A, the remianing SBA precipitable material was boiled in 0.2% NP40 plus sample buffer and run on a 10% SDS-PAGE. The wild type NR4 line is lane (a), and the mutant dGC2M5 lane (b).


a
b
c
d
A.
200
97
68
43
29
gp80
gp68
gp48
gp43
a
b
B.
200
97
68
43
29
gp80
gp48

DISCUSSION

1. Genetic Instability and Tumor Progression

Tumor progression involves the generation of cells with increasingly malignant properties. This results in a tumor which is more aggressive and difficult to treat. It is crucial in the treatment of cancer to understand the mechanism by which metastatic cells evolve. It has been known for some time that most neoplasms arise from a single altered clone (Nowell 1976, Fialkow 1979, Arnold et al. 1983). In the dynamic process of tumor progression there is gradual acquisition of new characteristics in this clone, such that a new subpopulation with an advantageous phenotype arises (Foulds 1954). Eventually through mutation and selection, this clone gains autonomy from host controls and the ability to metastasize. To explain tumor cell evolution and diversity, Nowell suggested this transition from normal to transformed cells involves inherited genetic instability (Nowell 1976, 1986). Thus unstable transformed cells could mutate at a higher rate and therefore continously produce new variants. Although some of these variants would be eliminated due to lethal mutations, or by metabolic and immunological mechanisms, some would have a selective advantage and proliferate to dominate the population. An additional prediction of Nowell's hypothesis was that increased genetic instability of tumor cells would be associated with increased malignant potential.

Cytogenetic data supported the idea of increasing genetic

alterations in tumor cell evolution, and this prompted many investigators to test the mutation rates in metastatic cell lines compared to their normal counterparts. This was done for both spontaneous point mutations as well as gene amplification rates using the Luria and Delbruck (1943) fluctuation analysis. This analysis determines the rate at which variants originate spontaneously and randomly with heritable transmission. To test for spontaneous point mutation rates metastatic cells were examined at independent loci for drug resistance using drugs in which resistance is typically due to point mutation (usually 6-thioguanine and oubain). Some investigators found an increase in the rate of spontaneous point mutations in highly metastatic lines (Cifone and Fidler 1981, Seshardi et al. 1987), while others did not (Elmore et al. 1983, Yamashina et al. 1985, Kendal and Frost 1986, Kaden et al. 1989, Tagger et al. 1989). Examination of rates of gene amplification (again using drugs in which resistance had been shown to be the result of gene amplification) showed, in some studies, an increased rate in highly tumorgenic and metastatic cells (Cillo et al. 1987, Tlsty et al. 1989). Chambers et al (1988) however, found no correlation in the rate of gene amplification and metastatic potential using a cell line that is temperature sensitive for malignant transformation. Because of the inconsistancies revealed by these investigations, and the importance of understanding the mechanisms behind tumor progression we decided to examine this question from a different point of view.

Since Nowells hypothesis predicts that genetic instability is required for tumor progression, we used a modification of the Luria

and Delbruck (1943) fluctuation analysis to test the hypothesis, by determining the rates of generation of metastatic variants within mutator and amplificator mutant populations. If the hypothesis predicts that metastatic cells would have increased rates of spontaneous point mutation and gene amplification, then tumorigenic cell lines that have increased rates of spontaneous point mutation and gene amplification should also exhibit an increased rate of generation of metastatic variants. The idea of determining the rate at which metastatic variants are produced within a tumor cell population came from the "dynamic heterogeneity" model of metastasis originally described by Hill et al. (1984). In short, these investigators showed that when various metastatic cell lines were grown to a small defined size the frequency of cells in the clone capable of forming experimental metastasis was very small (Hill et al. 1984, Chambers et al. 1984, Young and Hill 1986, Harris et al. 1987). When the clones were grown to a larger population size a substantially larger number of cells produced metastases. Analysis of these results using the approach of Luria and Delbruck (1943) indicated that the generation of the variants was a stochastic process for which a rate could be determined.

Mutants exhibiting an increase in the rate of spontaneous point mutations have existed for several years and have been collectively named mutator mutants. These are typically mutants of DNA repair, DNA synthesis or other pathways involved with supplying the precursors for DNA synthesis (Meuth et al. 1979, Weinberg et al. 1981, Liu et al. 1983, Wright et al. 1989, Wright 1989). The best charac-

terized mutator mutant is the thy-49 line described by Trudel et al. (1984). This mutant has been shown to exert its effects on the fidelity of DNA synthesis through a mutant CTP synthetase gene. Gross deoxyribonucleotide pool changes involving dCTP and dTTP were observed in this mutant line depending on the amount of thymidine in which the cells were grown. Since the ability to change the mutation rates of this line using different growth medium existed, we decided it would be an ideal line in which to examine metastatic rates. We hypothesized that if altered mutation rates played a role in generating metastatic variants, cell populations exhibiting increased spontaneous mutation rates should also exhibit increased metastatic rates in the Luria and Delbruck fluctuation experiments.

To date only four amplificator mutants with increased rates of gene amplification have been described in detail (Giulotto et al. 1987). The mechanism behind the ability of these lines to amplify at increased rates is still unknown. However, these mutant lines provided us with a novel approach for investigating the hypothesis that alterations in amplification rates can influence the rate of generation of metastatic variants. Again this hypothesis was tested by the Luria and Delbruck (1943) fluctuation experimental approach. Two of these mutants, YMP1 and YMP7 as well as the wild type BHK cell line were obtained to examine the rate at which the mutants compared to the wild type produced metastatic variants.

Once the mutator thy-49 cell line and the amplificator mutants YMP1 and YMP7 were obtained important phenotypic features of these lines were first examined. Deoxyribonucleotide pool analysis, shown

in section 1.1 of the Results, revealed the same shift in dCTP and dTTP pools as described by Trudel et al. (1984). The amplificator mutants showed increased resistance to corformycin over that of the wild type line, in keeping with the veiw that the amplificator phenotype was also functioning as described by Giulotto et al. (1987). To be applicable for our study both the mutator and amplificator mutants had to be capable of producing metastatic variants. Neither of these lines had previously been tested for tumorigenic or metastasic potential, nor had they been isolated from tumor lines. Analysis of their tumorigenic and metastatic potential, section 1.2 of the Results, revealed that all lines were tumorigenic in nu/nu mice as well as metastatic.

To determine the rate at which the mutator and amplificator mutants produced metastatic variants compared to their respective wild types, ten parallel clones were isolated for each line and each clone was injected into five mice. The number of resulting lung tumors was used to calculate the rate of production of metastatic variants as described by Hill et al. (1984). Contrary to what one would predict according to Nowell's hypothesis, the rate of generation of metastatic variants was not increased in these mutator and amplificator mutants. The rates were in the order of 10^{-6} lung colonies per cell per generation, and in the range of 4-10 x 10^{-6} lung colonies per cell per generation for the thy-49 line and the BHK cell lines, respectively.

Our results suggest that elevated rates of spontaneous point mutation and gene amplification are not sufficient on their own to

promote tumor progression in the cell lines analyzed. This questions the overall significance of previous observations which correlated increased rates of drug resistance with metastatic ability and the generality of the hypothesis which states that metastatic variants arise from populations exhibiting an unstable genetic backround. With the discovery of the pleiotrophic action of oncogenes (as described in Egan et al. 1989) it is more likely that specific mutations in oncogenes or groups of oncogenes are required for tumor progression to the metastatic phenotype. It is hard to imagine that tumor progression would occur in a non-specific fashion, depending on non-specific random mutations. It is more likely that tumor progression to the metastatic phenotype is a controlled and ordered process as described by Vogelstein in the case of colon cancer [Figure 1, (Fearon et al. 1990)]. Genetic instability may in some cases be the result of the process of tumor progression rather than the cause. Our results indicate that more information about specific pathways of tumor progression is required. Determination of connections between specific stages and types of tumors will aid in resolving these pathways.

An attempt was also made to analyze another mutator mutant, the mutant M1 subunit of ribonucleotide reductase, for which the mutant gene has been cloned (Caras and Martin 1988). The idea was to construct an inducible mutator expression plasmid that could be transfected and expressed in a variety of cell lines. This would provide a system in which the effect of an induced mutator phenotype on the metastatic potential could be examined in any normal,

tumorigenic or metastatic cell line. An inducible Lac plasmid was chosen because of its specificity of induction (Figge et al 1988).

The enzyme ribonucleotide reductase consists of two protein subunits, M1 and M2 (in mammalian cells), and is responsible for supplying the precursor deoxyribonucleotides required for DNA synthesis (Wright 1989, Wright et al. 1989). The M1 subunit contains the allosteric effector sites of the enzyme, which are responsible for directing the specificity of each ribonucleotide as well as the overall activity of the enzyme. Excess dATP for example, inhibits the overall function of ribonucleotide reductase (Wright 1989). The dG resistant cell line, dGuo-200-1, containing a mutant M1 subunit (as described by Martin's laboratory; Erikkson et al. 1981) was shown to be resistant to feedback inhibition by dATP. This resulted in abnormal concentrations of all the deoxyribonucleotide pools and an increase in the mutation rate in this cell line (Weinberg et al. 1981). The mutant M1 gene was subsequently cloned, and transfection into CHO cells was shown to confer the mutator phenotype in these cells (Caras and Martin 1988).

The Lac operator is a prokaroytic operator which is normally not expressed due to repression by the Lac repressor protein (Miller and Reznikoff 1980). When lactose is the only sugar source, the repressor becomes unbound from the operator because of the presence of allolactose (Miller and Reznikoff 1980). The repressor can also be removed from the operator using the allolactose analoge IPTG (Barkley and Bougeois 1980). The Lac inducible system was constructed to operate in eukaroytic cells by Hu and Davidson (1987) and Brown et

al. (1987). Since the process of metastasis is the result of many genes, many of which are controlled by inducible and noninducible regulatory elements (Greenberg et al. 1989, Egan et al. 1990, Wright et al. 1990b), this system was used to ensure that no secondary effects occured due to induction. A LacOMI mutator plasmid was constructed, characterized and transfected into Ciras 1, ras transformed 10T½ mouse fibroblasts, as described in section 1.2 of the results. Screening of transfected clones showed that the mutator M1 message was not inducible but constitutive. Also the level of expression of the mutator M1 was not high enough to exert its dominant effects.

Two possible explanations exist for the lack of success of this study. First: the Lac system may not be constitutive because the repressor may not have the signal sequences required to enter the mammalian nucleus. Thus it remains in the cytoplasm where it cannot inhibit the Lac operator. This may not have been apparent in the original COS cell transfection experiments (Figge et al. 1988), because COS cells are SV40 transformed monkey kidney cells which have a tendency to replicate SV40 promoters (Gluzman Y. 1981). The LacOCAT plasmid transfected by Figge et al. (1988) might have replicated numerous times in the cytoplasm, appearing to be a stabily transfected line, but in fact was not. This would also explain why such a seemingly unique and important system for induction of transfected genes has not, to the best of my knowledge, been reported to work by any other research group. Also, it is possible that either the promoter is not allowing good expression of the mutant M1 gene or

most cells can not tolerate overexpression of mutant M1 and the resultant mutator phenotype. The original question, however, may still be addressed using a different expression system and the basic techniques described above. Other mutator and amplificator genes must first be identified and cloned to further examine their effects on the generation of metastatic variants using a molecular biological approach.

2. Gene Amplification and Tumor Progression

Amplification of localized discrete chromosomal segments is essentially an aberrant process which frequently occurs during tumorigenesis (as reviewed in Alitalo and Schwab 1986, Stark 1986, and Nishimura and Sekiya 1987). In several malignancies, molecular hybridization techniques have identified amplification of cellular oncogenes. Despite the relative frequency of gene amplification, little is known about the mechanism. It is generally believed that genes are often amplified when cells are subjected to selective pressures, as is the case with genes conferring drug resistance (Schimke 1984). Furthermore, frequent amplification of oncogenes are consistently observed in various human malignancies (Alitalo and Schwab 1986). Examples include amplification of c-myc in cell lines derived from small-cell lung cancer (Little et al. 1983), promyelocytic leukemia (Dalla-Favera et al. 1982), colon carcinoma (Alitalo et

al. 1983) and breast carcinoma (Kozbar and Croce 1984); N-myc in neuroblastoma (Schwab et al. 1983) and retinoblastoma (Lee et al. 1984); c-abl in leukemia (Collins and Groudine 1983); c-myb in colon carcinoma (Alitalo et al. 1984), and leukemia (Pelicci et al. 1984); c-erb-B2 (or neu) and int-2 in breast tumors (reviewed in Ali and Callahan 1989); c-erb-B in brain tumors (Liberman et al. 1985, Bigner et al. 1987); and more recently k-fgf in human breast carcinomas (Ali et al. 1989, Theillet et al. 1989), melanoma (Adelaide et al. 1988) and stomach cancer (Yoshida et al. 1988).

The concept that amplification of cellular genes might confer a growth advantage for proliferation of tumor cells and, therefore be related to disease prognosis, is a popular theme in cancer research. To date most of the research associating the amount of amplification with the aggressive nature of the disease has shown a positive correlation (Johnson et al. 1987, Varley et al. 1987, Slamon et al. 1987, Libereau et al. 1988, Slamon et al. 1989, Marx 1989a). Not much has been done at the molecular level in experimental models, however, to test the idea that increasing amplification of a particular proto-oncogene leads to a more aggresive phenotype. Two studies have been done using linkage to the dhfr gene to amplify both the myc proto-oncogene (Wurm et al. 1986) and the neu proto-oncogene (Hung et al. 1989). Results from the c-myc amplification experiments showed that overexpression was cytotoxic in the transfected dhfr- CHO cells. Overexpression of neu in NIH3T3 cells did not change the morphology of cells. Injection of the NIH3T3 cells containing

amplified neu sequences, subcutaneously into nude mice, did not result in tumor formation until 3 months later. Subsequent isolation of cells from the tumor showed that a small number of the amplified genes were actually mutated, and these cells exhibited the transformed morphology. These investigators suggest that converting a proto-oncogene into an activated oncogene might be through a two-step mechanism, amplification and then mutation (Hung et al. 1989).

We previously examined the hypothesis that increased rates of gene amplification would lead to an increase in the rate of production of metastatic variants, and showed that in the cell system examined this was not the case (results section 1.1.c). Since there are many examples of oncogene amplification in tumor cells, we developed an experimental model system to investigate amplification of a specific oncogene and to determine if increasing oncogene amplification was accompanied by increasing metastatic ability in transfected cell lines. Review of the recent literature involving gene amplification in malignant human cancers identified a gene encoding a growth factor, k-fgf, which has significant homology to basic and acidic FGF growth factors (Yoshida et al. 1988, Miyagawa et al. 1988, Adelaide et al. 1988, Ali et al. 1988, Theillet et al. 1989). Several investigations have shown that the gene encoding k-fgf is amplified in many human tumors including breast carcinoma, melanoma and stomach cancer (Adelaide et al. 1988, Yoshida et al. 1988, Ali et al. 1989, Theillet et al. 1989). Autocrine stimulation by growth factors is believed to be an important mechanism by which oncogenes transform cells to a neoplastic state (Weinberg, 1989).

Many tumor cells release potently mitogenic growth factors which may contribute to the malignant state (reviewed in Sporn and Roberts, 1985). The only evidence to date of a growth factor acting as an oncogene comes from the work on the sis oncogene product and the β chain of PDGF (Doolittle et al. 1983, Waterfield et al. 1983). Recently, transfection of the k-fgf proto-oncogene has shown that it too is an oncogene (Delli-Bovi and Basilico 1987, Delli-Bovi et al. 1988, Quarto et al. 1989). Under the control of strong viral promoters k-fgf has been shown to transform NIH3T3 cells much more effectively than its homologe b-fgf. The reason for its effectiveness is believed to be based soley on the fact that k-fgf contains a signal sequence, which b-fgf does not have (as described in Quarto et al. 1989). When a signal sequence is constructed in front of the b-fgf gene it also has shown much greater transforming potential (Rogelji et al. 1988).

Normally k-fgf is not expressed in adult tissue (as reviewed by Basilico et al. 1989). Expression may be activated by amplification through rearrangment of controlling regulatory sequences surrounding this gene. Although amplification of k-fgf has been associated with many human tumors, the level of mRNA for k-fgf has only been examined in one (Ali et al. 1988). The general hypothesis for proto-oncogenes where amplification has been found in human tumors is: the more amplification and subsequent expression, the more aggressive the resulting tumor. Transfection studies with k-fgf have shown that transformed cells expressing k-fgf also produce subcutaneous tumors in nu/nu mice. However, the metastatic potential of these trans-

formed NIH3T3 cells was not examined. Our study was two fold. First the ability of k-fgf to convert cells to the full metastatic phenotype was determined. Subsequently, if k-fgf transfected cells were metastatic, then we could determine how much expression and amplification is required for k-fgf to promote tumor progression and the metastatic phenotype. The proto-oncogene was obtained and amplified on its own promoter using linkage to the dhfr gene. The dhfr gene has been used in conjunction with other genes to select for amplification and this technique has met with reasonable success (McCormick et al. 1984, Kaufman et al. 1985, Wurm et al, 1986).

The proto-k-fgf gene isolated by Delli-Bovi and Basilico (1987) was obtained, as well as a plasmid containing the dhfr gene on its own promoter. A recombinant plasmid containing both genes was then contructed as described in section 14.1 of the Materials and Methods. Section 2.1 of the results show construction and characterization of the recombinant pKFD plasmid. The pKFD plasmid was then transfected into normal NIH3T3 mouse fibroblasts with a drug marker, PY3, to identify cells which had stably intergrated these plasmids into the genome. Southern blot analysis of drug resistant clones showed that 17 had integrated the pKFD plasmid. Cells containing amplified sequences of k-fgf were isolated following growth of the pKFD intergrated clones in the presence of increasing concentrations of MTX. Resistance to MTX would be the result of either amplification of the endogenous dhfr gene or the transfected dhfr gene in which case the k-fgf gene should also be amplified. In most cases amplification of the endogenous dhfr was observed. We assumed that since amplifica-

tion of either the endogenous or transfected dhfr gene would allow the cells to grow in MTX, the frequency of amplification of either gene would be approximately 50%. This was not observed, as only one out of 17 clones showed amplification of transfected dhfr gene. The frequency at which the dhfr gene is amplified, of course, also depends on the expression of the transfected gene. This one clone out of 17 exhibited a transformed morphology such that the cells appeared more spindle shaped and refractile than the wild type NIH3T3 cells.

Before examining the effect of gene amplification of k-fgf on tumorigenic and metastatic potential, we constructed a series of amplified clones with varying gene copy numbers, and tested to make sure this was reflected in both the mRNA levels produced as well as the amount of kFGF protein secreted. Sections 2.1 and 2.2 of the results shows that gene amplification of both k-fgf and dhfr genes was progressive for each concentration of MTX, but mRNA levels for k-fgf were not. Each step-up of MTX concentration produced a 1-3 fold amplification in the k-fgf and dhfr genes to a maximum in the range of 8-15 fold. Analysis of mRNA levels for the dhfr gene also showed a progressive increase, however k-fgf message was observed to increase approximately 20 fold over that of transfected message after only 1 step-up in the presence of MTX. Subsequent steps showed a high k-fgf message, which gradually declined in cell lines subjected to increasing concentrations of MTX. K-fgf protein secreted by three of these clones, 3T3.3G, 3T3.3G-20 and 3T3.3G-80, also reflected what was observed at the level of mRNA. We expected a progessive increase

in dhfr message with increasing concentration of MTX which would explain the growth and apparent resistance of cells to MTX. The large increase in k-fgf message at the initial selection step was not expected.

Recent studies have shown that expression of the k-fgf proto-oncogene is strictly controlled in embroyonal carcinoma (EC) cells (Rizzino et al. 1989). These investigators showed that induced differentiation of EC using retinoic acid caused a dramatic decrease in the expression of k-fgf. Also Curatola and Basilico (1990) have found 3' regulatory elements in k-fgf which specifically function in EC cells. K-fgf mRNA has never been found in any normal human or mouse tissue, but is expressed in mouse blastocytes, EC cells and mouse embryonal cells (as described in Curatola and Basilico; 1990). Thus, expression of the k-fgf proto-oncogene appears to be restricted to specific cells and stages of development. Such complicated regulation of expression could be the result of many gene regulatory elements as described for most developmentally regulated genes (as reviewed for embryonic pattern formation in Drosphila; Ingham 1988). Our results suggest that in the first step of amplification in the 3T3.3G clone, some of these regulatory elements may have been affected thus resulting in a large increase in the message observed. The subsequent decline of message may be due to some kind of down-regulation mechanism. We know for instance that the murine FGF receptor, which binds both bFGF and kFGF, is down regulated when bFGF or kFGF expression levels become too high (Mansukhani et al. 1990). Amplification of the k-fgf gene in tumor

cells may be a mechanism by which expression of this proto-oncogene is activated through dissociation from regulatory elements. Our results support this type of hypothesis as expression was low in cells until the gene was amplified, at which time this apparent dissociation could occur.

The effect of increasing concentrations of k-fgf observed between the wild type, NIH3T3 line, the transfected, low expressing clone 3T3.3G and the high expressing clones 3T3.3G-10, -20, and -80, on the tumorgenic and metastatic potenial of these line was examined both by subcutaneous and intravenous injection of 10^5 cells. As shown in section 2.3 of the Results, all the k-fgf transfected clones were both tumorigenic and metastatic. Subcutaneous injection showed that the latency of both the 3T3.3G clone and the 3T3.3G-20 clone were the same (within error), while in the 3T3.3G-80 clone tumors took twice as long to develop. Our data suggests that the amount of kFGF expressed in the 3T3.3G clone was the maximum (or more than the maximum) required to cause formation of subcutaneous tumors. Since the FGF receptor is down regulated after a certain concentration of kFGF expression (Mansukhani et al. 1990), the 3T3.3G-80 clone may have by-passed this critical kFGF concentration, down regulated its FGF receptors, reduced the responsivenes to autocrine stimulation, and therefore exhibited an extended tumor latency. Examination of the metastatic potential exhibited by these clones shows that the 3T3.3G clone is not as metastatic as the other clones, 3T3.3G-10, -20, -80 and -120. The level of expression of 3T3.3G is 5 times lower compared to the other drug resistant clones, and this suggests that

the level of kFGF protein expression determines the metastatic potential in these clones. Amplification and overexpression of k-fgf shown in the higher expressing clones, has lead to an increase in the metastatic potential but only to a maximum. The amount of k-fgf expression is lower in the 3T3.3G-80 and 3T3.3G-120 clones than the 3T3.3G-10 and 3T3.3G-20 clones but the metastatic potential was apparently not affected. This suggests that the amount of k-fgf message required to produce the most lung metastases is a concentration between that observed for the 3T3.3G clone and the 3T3.3G-80 and -120 clones. If the FGF receptor is down regulated this does not appear to have any appreciable affect on the metastatic phenotype.

Two other growth factors which have been described to fuction as oncogenes are PDGF (Waterfield et al. 1983, Keating and Williams 1988) and bFGF (Rogelji et al. 1988, Quarto et al. 1989). For both these growth factors there is evidence for intracellular binding and activation of their receptors (Keating and Williams 1988, Quarto et al. 1989). Studies with bFGF transfected cells have failed to find substantial amounts of the growth factor in the medium, and most was found within the cell memebrane. Although a slight reduction in tumorigenic ability has been observed following addition of PDGF antibodies to PDGF transformed lines (Johnsson et al. 1985), this could not be accomplished with bFGF transformed cells (Quarto et al. 1989). Analysis of secreted bFGF growth factor could not be demonstrated following transfection into cells (Rogelji et al. 1988, Quarto et al. 1989) unless a signal peptide sequence was first fused to the b-fgf gene. In contrast to these studies, k-fgf transformed

cells have been shown to secrete most of the kFGF protein made by the cells and very little remains within the cell (Quarto et al. 1989). Also it has been shown that kFGF functions by an autocrine mechanism stimulating the FGF receptor following secretion, and this can be reversibly inhibited by the polyanionic compound suramin (Wellstein et al. 1989). We initially tried to examine the amount of kFGF in cell extracts in the transfected 3T3.3G cells but could not detect any difference in the transfected lines compared to the wild type NIH3T3 cells. The conditioned medium was then examined and it was here that large amounts of kFGF protein was found but only in the transfected lines.

In summary these experiments are the first to show that amplification and subsequent overexpression of the amplified proto-oncogene k-fgf, is directly related to the metatatic potential of the resulting cell line. Also our results suggest that amplification of k-fgf may be a mechanism for the abnormal activation of expression of this usually non-expressed gene in adult cells. Interestingly, the tumorigenic potential was observed to be less in a high expressing clone, 3T3.3G-80, (than 3T3.3G) which could be the result of down regulation of the FGF receptor. Our hypothesis is that the amount of kFGF protein expressed in the 3T3.3G clone is sufficient to transform wild type NIH3T3 cells to the tumorigenic state, but down regulation of the receptor reduces autocrine stimulation such that the latency of tumor formation is extended in the 3T3.3G-80 clone. This kind of phenomenon may not have previously been observed in PDGF and bFGF transformed cells because of the ability of these growth factors to

function intracellularly unlike kFGF, where the kFGF protein is immediately secreted. The data also suggests that, for the experimental metastatic potential observed, the amount of kFGF expressed by the 3T3.3G clone population is not enough to produce the maximum amount of lung metastases, as is the case with all the other higher expressing clones. The number of experimental metastases is also not affected by the down regulation of the receptor, thus resembling an all or none response, unlike that observed for the tumorigenic potential. This hypothesis can be examined further. Receptor binding studies with these clones for example, using labeled kFGF, could determine if the FGF receptor is down regulated as speculated above. Also, the polyanionic compound, suramin, could be used to block autocrine stimulation of secreted kFGF (Wellstein et al. 1989). The effect of this on the tumorigenic and metastatic potential could then be examined.

3. CELL SURFACE GLYCOPORTEINS AND MALIGNANCY

3.1 Isolation and Characterization of a Metastatic Deoxyguanosine Resistant Mouse Cell Line.

Initially a low metastatic T24-H-ras transfected mouse 10T½ fibroblast line, NR4 was grown in the presence of increasing concentration of deoxyguanosine to isolate a mutant line resistant to dG and altered in the M1 subunit of ribonuceotide reductase. As

discussed in section 1 (page 198), dG resistant mutants containing alterations to the M1 subunit of the enzyme ribonucleotide reductase have been described by Weinberg et al. (1981), and shown to exhibit a mutator phenotype. It was our intent to also isolate a mutator mutant in a 10T½ tumorigenic line, and examine what effect this might have on the rate at which these cells generated metastatic variants (as discussed in discussion, section 1). Following selection in a step wise fashion in dG two resistant NR4 cell lines, NR4dGC1 and NR4dGC2, were isolated and characterized. The ability of these lines compared to the wild type to both grow and form colonies in increasing concentration of dG is shown section 3.1.1 of the results. These studies indicated that NR4dGC1 and NR4dGC2 were resistant to 1.0 mM dG. The drug resistant lines were next examined for the biochemical lesion accounting for their drug resistance. Analysis of ribonucleotide reductase indicated that these lines were not M1 mutants like the cells described by Weinberg et al. (1981). In fact, further investigations indicated that the mutant NR4dGC2 did not incorporate labelled hypoxanthine into DNA and was also cross-resistant to 6-thioguanine. Other ways in which cells can become resistant to dG include mutations in the enzyme Hypoxanthine-Guanine Phosphoribosyltransferase (Ullman 1989). Analysis of HGPRT activity showed no detectable activity in the mutant NR4dGC2 cells whereas in the wild type, NR4 line, HGPRT activity was linear with time.

At the same time as the biochemical lesion in these mutants were being analysed we also examined their tumorigenic and metastatic potential. Section 3.1.2.1 of the results shows that one of the

mutants, NR4dGC2, had acquired a significant increase in metastatic potential compared to the wild type NR4 cell line. Once we had determined that this line was not altered in the M1 subunit of ribonucleotide reductase, we were left with the question, what was it about these cells that allowed them to be become metastatic? Knowing these cells were ras transformed we first analyzed the expression of ras to ensure it had not changed. Increased expression of ras has been shown to correlate with metastatic potential (Egan et al. 1987). No change was observed in this dG resistant clone in ras expression. An important characteristic required for cells that acquire the potential to metastasize is the ability to become motile and travel to a secondary site (Liotta and Hart, 1982). Based on studies of many investigators, which have found changes in the carbohydrate structures of cell surface glycoproteins associated with metastasis, we decided to examine the NR4dGC2 line for possible unique changes in its cell surface glycoproteins, that might be associated with its metastatic potential.

3.2 Characterization of a Glycoprotein Associated with the Metastatic Phenotype.

The process of metastasis requires that tumor cells become motile, for the ability to infiltrate into distant organs through the basement membrane and endothelium, evading the host's immune system. Several investigations have shown changes in various cell surface properties including alterations in glycoprotein carbohydrate

moieties that correlate with this process (reviewed by Dennis and Laferte 1987, and Alhadeff 1989). Before studies were initiated to analyze the charbohydrate structures of cell surface glycoproteins in the NR4dGC2 line compared to NR4, the NR4dGC2 line was cloned to isolate lines exhibiting either higher or lower metastatic characteristics. To determine if alterations had occurred in the carbohydrate moieties of surface glycoproteins in the metastatic clone dGC2M5, a study was initiated using glycosylation inhibitors (Spearman et al. 1987). The idea being, if there were changes in the carbohydrate moieties important to the process of metastasis, then inhibition of the synthesis of normal carbohydrate structures on the dGC2M5 clone should also inhibit the formation of lung metastases.

Previous investigations with swainsonine and castonospermine, inhibitors of glycosylation enzymes, have reported inhibition of experimental metastasis in some metastatic cell lines (Humphries et al. 1986a, Humphries et al. 1986b, Dennis et al. 1986, Dennis et al 1989a, Newton et al. 1989). We examined the effects on the metastatic potential of dGC2M5 cells of swainsonine, castanospermine, and deoxymannojirimycin, (another glycosyation inhibitor not previously tested for its effects on metastatic cells). Section 3.2.1 of the Results shows that both swansonine and deoxymannojirimycin reduced the metastatic potential of the highly metastatic dG resistant clone dGC2M5 by 40%, whereas castanospermine had no effect. This suggested that the type of carbohydrate change which is important in the metastatic potential of these cells is specific, and involves processing by mannosidase II (inhibited by swansonine) and man-

nosidase I (inhibited by dexymannorjirimycin) but not glucosidase I (inhibited by castanospermine) (Spearman et al 1987).

To investigate further the nature of the carbohydrate change associated with metastasis of the dGC2M5 line, lectin binding studies were performed using lectins in which changes of affinity have been associated with metastasis in other cell lines (Kerbel et al. 1982, Altevogt et al. 1983, Barnett and Eccles 1984, Dennis 1986, Buckley and Carlsen 1988, Lang et al. 1988). Con A, WGA and SBA were examined, but only with SBA a lack of binding was found to correlate with metastatic potential. A low metastatic clone of dGC2M5 was generated, dGC2M5.9, and was found to exhibit increased binding with SBA as expected from our previous results. To analyze whether this inverse relationship with SBA binding and metastasis was the result of a general carbohydrate change or was specific to a few isolated glycoproteins, a SBA precipitation technique was used with cell extracts of dGC2M5 and the wild type NR4 cell line. Section 3.2.3 of the Results show that two glycoproteins were identified on NR4 cells that bound specifically to SBA, and they were not present on dGC2M5 cells. Our results are consistant with those found in other studies. For example, Lang et al. (1988) also identified a glycoprotein gp210, in the murine lymphoma line, ESb-MP, which had lost its ability to bind SBA in a highly metastatic clone called, ESb. This glycoprotein maintained its ability to bind peanut aggultinin (PNA) and Con A, and the investigators suggested that this was due to the result of a structural alteration in some of the oligosaccharide side-chains of the gp210 molecule. The observation of changes in SBA binding is not

unique to this murine lymphoma line. Other studies have also shown differences in SBA binding when comparing high and low metastatic lines. Collard et al. (1986) for example also found that highly metastatic variants of T cell hybridomas lost reactivity for SBA. Also, Altevogt et al. (1983) found decreased affinity for SBA in a highly metastatic MDAY-D2/MD40 line. Two other studies found the reverse correlation with SBA reactivity and metastasis (Barnett and Eccles 1984, Buckley and Carlsen 1988, Carlsen et al. 1990). In general it appears SBA reactivity is important, and the changes observed in metastatic lines depends on the cell system being investigated.

Other studies have identified specific glycoproteins which have been altered in highly metastatic lines compared to low metastatic lines. Laferte and Dennis (1988) for example, have shown that two glycoproteins called P2A and P2B, are responsible for most of the glycosylated membrane sialoglycoprotiens isolated in a lymporecticular tumor line. These glycoproteins ranging in size from 80,000 to 160,000 can be detected by WGA and L-PHA, both of which exhibit increased binding in highly malignant clones of this MDAY-D2 tumor cell line. Analysis of the P2B glycoprotein in particular has shown that, although changes occur in the branching of oligosaccharides in this glycoprotein, comparable levels of mRNA were found in both metastatic and nonmetastatic cell lines (Heffernan et al. 1989). Binding studies of P2B stripped of sialic acid residues showed that it could bind extracellular matrix proteins, collogen type I, fibronectin and laminin (Larferte and Dennis 1988). The hypothesis

is that sialylation may contribute to the poorly adhesive and motile phenotype of the malignant cells.

Another recent study has shown that invasiveness of murine adrenal carcinoma cells depends on the galactosylation status of a 90-110 kD cell surface glycoprotein (Penno et al. 1989). Again, this study suggests changes in the carbohydrate moiety of a specific glycoprotein is important for some unidentified fuction. Irimura et al. (1988) has also shown expression of a 900,000 molecular weight sialoglycoprotein associated with human colon carcinoma cells. Nabi and Raz (1987) have shown with a metastatic melanoma line B16, the acqusition of sialylated PNA reactivity on a 90,000 - 130,000 molecular weight glycoprotein, which is associated with increased metastatic potential. Our study shows two proteins, molecular weight 80 kD (gp80) and 48 kD (gp48), that bind to SBA when the cells are nonmetastatic. Also in the metastatic line dGC2M5 we have observed the appearance of two cell surface proteins which appear following non-specific binding, gp68 and gp43. The decrease in SBA binding that we see in the dGC2M5 metastatic cells may be the result of loss of gp80 and gp48. The loss of gp80 and gp48 could be due to an alteration of carbohydrate structure, such that the glycoproteins no longer bind SBA, and are either not precipitated or are precipitated nonspecifically, in which case they would be observed most likely at a different molecular weight (Wright 1979, Parfett et al. 1983, Altevogt et al. 1983, Dennis and Laferte' 1987). Changes in the protein structure so that the type and amount of carbohydrate glycosylation is different could also account for the loss of gp80

and gp48 (and SBA binding), and the appearance of gp68 and gp43. Alternatively, the expression of the proteins, gp80 and gp48, may have been lost when the cells acquired the ability to metastasize. The appearance of gp68 and gp43 may be the result of novel proteins in which expression has increased.

The glycoproteins which we have identified are an example of the same general type of glycoproteins (P2B) observed by Dennis group in MDAY-D2 cells; that is they appear to be important in the metastatic ability in these ras transformed murine fibrosarcomas. Isolation of these glycoproteins and analysis of their amino acid structure (to see if they are related to other well known cell surface proteins) as well as their charbohydrate character, may aid in attempts to understanding glycoprotein function and its relationship to the metastatic process. Alternatively, antibodies to other known metastasis associated glycoproteins may be used to examine if there is a relationship to the glycoproteins observed in this study. Although the function of carbohydrate changes is not understood, structural changes may play significant roles in the ability of cells to bind extracelluar matrix components such as fibronectin (Lafarte' and Dennis 1988), or possibly inhibit receptor function. Oligosaccharide processing and expression of embryonic carbohydrate antigens which have been shown to be regulated during embryogenesis may influence cell motility, proliferation and differentiation (Hakomori and Kannagi 1983). These same parameters on the surface of tumor cells may also directly affect the metastatic potential. The results of lectin binding studies indicate that carbohydrate changes

correlating with metastasis may be specific to cell type. However, the underlying mechanisms responsible for these observations are not known. Since changes in terminal carbohydrates have been reported to be associated with metastasis in different tumor systems, structure/-function studies of cell surface carbohydrates are obviously important, and should lead to a better understanding of the complex metastatic process.

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