THE UNIVERSITY OF MANITOBA

CYTOGENETICS AND MOLECULAR ASPECTS OF *RAS*-TRANSFORMED 10T½ FIBROBLAST CELL LINES

by

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A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

MASTER OF SCIENCE

DEPARTMENT OF HUMAN GENETICS
UNIVERSITY OF MANITOBA
WINNIPEG, MANITOBA
November, 1993

ISBN 0-315-99011-2

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BY

PING YANG

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

MASTER OF SCIENCE

ACKNOWLEDGEMENTS

First of all, I would like to thank my supervisors, Drs. Arnold Greenberg and Jim Wright for their guiding me and advising me. They are great teachers. Without their patience and understanding, I would not have been able to bring this project to fruition.

I would also like to thank the many colleagues and friends that put up with me in the lab and made my life a lot easier and manageable. Without their sincere encouragement and kind help, this rookie would have been lost in the jungle of medical terms, seminars and experiment procedures. Arthur Chan, in particular, was always there to help in all things and at all hours. Special thanks must be extended to Dr. Maureen Spearman who offered valuable advice and help when I most needed, and to Dr. M. Mowat for his scientific advice and experimental materials.

I cherish the many hours of entertaining and enlightening conversation with lab-mates Francis, Bill, Chen, Aiping, Pardeep, Anne and Myke. Good luck to you all in your future studies.

I am also thankful to the Department of Human Genetics for accepting me. Although I have not spent as much time as I would like at the functions of the Department, the knowledge that I belonged was most comforting and the support I got was tremendous. I am grateful to the Genetics staff and students for their support, friendship and patience

with this absentee student. I am particularly indebted to the following four people: Dr. Phyllis McAlpine who gave my project guidance and direction, the late Dr. M. Ray who gave me valuable lessons in experimental technique and reviewed my karyotyping data, Doctor-to-be Michael Carpenter, who taught me some useful techniques in Southern blot analysis and Shibani Bal who was an excellent friend.

The financial support of NCI of Canada has been valuable and is appreciated.

Finally, but perhaps most importantly, I must thank my parents for their continued moral support throughout these difficult years.

ABSTRACT

CYTOGENETICS AND MOLECULAR ASPECTS OF *RAS*-TRANSFORMED 10T½ FIBROBLAST CELL LINES

The loss of genetic material from specific chromosomal locations in a given tumor type provides evidence of the importance of tumor suppressor genes at these loci in the genesis of the tumor.

In this study, chromosome analysis was performed in a 10T½ mouse fibroblast cell line and in seven 10T½ cell lines transformed by the oncogenes *myc* and /or *ras*. These included two tumorigenic but non-metastatic cell lines M4R4 and MRPD, and the five metastatic cell lines NR4, C1, C2, C3, and 10T½*RAS*. Structural and numerical chromosome aberrations in each cell line were observed. Three kinds of aberrations of chromosome 8 were observed in the metastatic cell lines, but not in the non-metastatic cell lines. These aberrations were: losses of the C-E region on chromosome 8, deletions of a copy of chromosome 8 and inversions of both copies of chromosome 8. The fragment of chromosome 8B-E is homologous with human chromosome 16q. The non-metastatic cell lines had 3-4 copies of chromosome 8.

In order to map the minimal chromosome loss region in chromosome 8, a panel of genes which had been identified on chromosome 8 or human chromosome 16q region was screened against our 10T½ tumor lines by Southern blot hybridization. These genes

included Mt-1, Um, Hp, Ctrb, Tat, Gnb-3, Aprt and Mtv-21. Gene rearrangements were observed at the regions of Um, Ctrb, Aprt and Gnb-3 in C3 or 10T½*ras* cell lines. With the exception of Ctrb, Southern blot analysis did not show any reduction in the intensity of hybridization bands with DNA prepared from metastatic cell lines when compared to DNA obtained from non-metastatic cell lines.

The results of this study indicate that the loss region of chromosome 8E-C may contain a recessive allele important in oncogene complementation that is involved in advanced metastatic procession. This recessive allele may be located between Ctrb and Aprt region. Also the Gnb-3 gene, which encodes the β-subunit of a G-protein, may be important in metastatic progression.

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I. INTRODUCTION

- Metastasis is a complex process that results in tumor growth at different sites from the primary neoplasm. It requires many acquired cellular characteristics, such as increased motility, degradation of extra cellular matrix, and loss of growth control. It appears that the development of normal cells into cancer is mediated by the activation or inactivation of certain critical cell regulator genes which are responsible for controlling the many cellular functions such as proliferation, differentiation, intercellular communication and motility (Foulds, 1975; Greenberg et al., 1989; Wright et al., 1990; Bishop, 1987; Sager, 1989). These genes are frequently referred to as oncogenes and tumor suppressor genes (Klein, 1988; Muschel et al., 1989).

The oncogenes play a dominant role in the induction of tumorigenic metastatic tumor lines. The tumor suppressor genes can prevent the tumorigenic transformation of cells. It has been demonstrated by several laboratories that an activated *ras* oncogene can induce metastatic transformation in mouse fibroblast 10T½ and NIH3T3 cells (Egan et al., 1987; Greig et al., 1985; Muschel et al., 1985), but this induction of the metastatic phenotype by activated *ras* can be suppressed if the adenovirus type 2 E1A oncogene is expressed (Pozzatti etal, 1988). Also certain cell lines are only susceptible to benign transformation by H-*ras* (Muschel et al., 1985). Therefore, some aspects of the *ras*-induced metastatic phenotype are separable from transformation by *ras*.

Transformations of murine 10T½ cells by *ras* and/or *myc* oncogenes were performed in our laboratory. It has been shown that transformation of mouse 10T½ cells with *ras* in fetal bovine serum (FBS) results in the generation of metastatic lines, but all of the lines transformed by *myc* and *ras* in dialyzed calf serum are tumorigenic and non-metastatic. This suggests that either an additional event is required for metastatic transformation beside expression of *myc* and *ras*, or expression of *myc* can inhibit the metastatic phenotype in *ras*-transformed cell lines. To test these possibilities, a retrovirus containing *myc/neo* was used to infect the *ras* metastatic transformed cell line 10T½*ras*. All the clones isolated that exhibited G418 resistance were found to be metastatic and expressed V-*myc* RNA. This experiment demonstrated that the expression of *myc* can not inhibit the *ras* induced metastatic phenotype. Somatic cell hybridization between the non-metastatic *ras/myc* transformed cell line M4R4 and the metastatic line 10T½*RAS* also showed that six out of eight of hybrids were either poorly metastatic or non-metastatic, but they were all tumorigenic. These data suggest that in order for metastatic transformation to take place an additional event is required which cooperates with *ras* to induce metastatic behaviour, and in the absence of this event *myc*-expression can cooperate with *ras* to induce benign transformation.

The expression of P53 and *Rb* proteins was tested by immunoprecipitation and Western blotting in the 10T½ metastatic cells and non-metastatic cells in our laboratory, and little difference in the expression of both tumor suppressor gene products was observed. In addition, expression of the nm23 gene was not correlated with metastatic

or non-metastatic characteristics.

Based on the data above, it appears that the cooperative interaction of dominant oncogenes like *myc* and *ras* is not sufficient on their own for expression of the metastatic phenotype, and an additional unknown recessive event is required for metastatic progression. In order to identify this recessive allele, chromosomal G-banding analysis was performed. Chromosomal loss and inversion involving region 8E-C was observed in the metastatic cell lines but not in non-metastatic cell lines in this study. Therefore, this chromosome 8E-C region, which is homologous with human chromosome 16q, may contain a recessive allele important in oncogene complementation of metastatic progression. Several recent reports of tumors with allelic losses in 16q have suggested that a suppressor gene may exist in this region. To confirm the deletion and map the minimal chromosome loss region, a panel of mouse chromosome 8 or human chromosome 16q genes, which have been cloned were used in Southern hybridization experiments with 10T½ tumorigenic and metastatic cell lines. Gene rearrangements were observed at the region of Um, Ctrb, Aprt and Gnb-3 in C3 and 10T½*ras* cell lines. The result of this study demonstrates that there may be a recessive allele at the mouse chromosome 8E-C region, possibly between Ctrb and Aprt, and that this recessive allele may play a role in cellular transformation and tumor dissemination.

II. LITERATURE REVIEW

II. 1. Cytogenetics in Cancer

The evidence for the presence of recurring chromosome abnormalities in a wide variety of neoplasms is the result of 30 years of chromosome analysis by cytogeneticists. As early as the 1960's and 1970's, the association between certain chromosome defects like translocation and deletion with some types of human cancer was established (Rowley, 1990). The first evidence for chromosome abnormalities was observed in chronic granulocytic leukaemia (CGL) in Philadelphia by Nowell and Hungerford (1960). This small chromosome was named philadelphia or Ph. It gave the first evidence of consistent genetic material-associated changes in a tumor. Based on this evidence, it was assumed that consistent chromosome changes would be found in most malignant cells, but this test was difficult in the 1960s before the development of chromosome banding.

The introduction of chromosome banding techniques in 1970 revolutionized cancer cytogenetics. It became possible to identify each human chromosome and parts of chromosomes precisely. With the use of chromosome banding, the Philadelphia chromosome was shown to involve chromosomes 22 and 9 [t(9;22)(q34;q11)] (Rowley et al., 1973). Other structural chromosome rearrangements seen in tumors are inversions, deletions, insertions and formation of isochromosomes. For example, inv(16)(p13;q22) has been described in acute myelomonocytic leukaemia with abnormal eosinophils.

Iso(17q) consisting of the duplicated long arm of chromosome 17, is present in the acute phase of chronic granulocytic leukaemia. Deletions in the p13 region of chromosome 11 in Wilms' tumor, deletions of the long arm of chromosome 13, del(13)(q14) in patients with a high incidence of retinoblastoma (Francke, 1976), and loss of chromosome 18 in colorectal cells were observed (Fearon et al., 1990). There are at least 70 recurring translocations detected in human malignant cells. Data for consistent chromosome aberrations in leukaemias and lymphomas are shown in Table 1, while data for consistent chromosome aberrations in solid tumors are presented in Table 2.

The importance of karyotypic alterations became evident when the genes involved in some of the chromosome rearrangements were identified, and some of them proved to be oncogenes. For example, the regions around the breakpoints of the Philadelphia translocation and of the translocations associated with Burkitt's lymphoma have been cloned and shown to involve the cellular oncogenes c-*abl* and c-*myc*, respectively (Dalla-Favera et al., 1982; Tanb et al., 1982; Adams et al., 1983; Croce et al., 1983).

The correlation of the chromosome locations of human protooncogenes with recurring chromosome rearrangement is observed in many different human malignant cells. The fact that oncogenes are directly involved in chromosome translocations demonstrates that both translocation and oncogenes are critically involved in human cancer.

Table 1: Consistent Chromosome Aberrations in Leukaemias and Lymphomas

Malignancy	Chromosome aberration
Leukaemias	
Chronic granulocytic leukaemia	t(9;22)(q34;q11)
Acute myeloid leukaemia	
M1[a]	t(9;22)(q34;q11)
M2[b]	t(8;21)(q22;q22)
M3[c]	t(15;17)(q22;q12-21)
M4[d]	inv(16)(p13;q22)
Chronic lymphocytic leukaemia	t(11;14)(q13;q23) trisomy 12
Acute lymphocytic leukaemia	t(9;22)(q34;q32) t(4;11)(q21;q23) t(8;14)(q24;q32)
Lymphomas	
Burkitt's lymphoma	t(8;14)(q24;q32) t(2;8)(p12;q24) t(8;22)(q24;q11)
Small non-cleaved cell lymphoma	t(8;14)(q24;q11)
Follicular small cleaved cell lymphoma	t(14;18)(q32;q32)
Small cell lymphocytic lymphoma	trisomy 12

a: acute myeloblastic leukaemia without maturation. b: acute myeloblastic leukaemia with maturation. c: acute promyelocytic leukaemia. d: acute myelomonocytic leukaemia. (partly taken from Yunis, 1983)

Table 2: Consistent Chromosome Aberrations in Solid Tumors

Malignancy	Chromosomal Aberration
Neuroblastoma	del(1)(p31;p36)
Small cell lung carcinoma	del(3)(p14;p23)
Melanoma	del(6)(q15;q23) and t(6q)
Mixed parotid gland tumor	t(3;8)(q25;q21)
Ewing's sarcoma	t(11;22)(q24;q12)
Meningioma	monosomy 22
Wilms' tumor	del(11)(p13)
Retinoblastoma	del(13)(q14)
Colorectal cancer	del(18)(q21-qter)

(Partly taken from Yunis, 1983; Grosschedl et al., 1985)

Karyotype analysis has been the key to defining the major rearrangements, which are translocations, consistent deletions and inversions. The identification of consistent chromosome deletions is as important as identifying translocations because it provides essential information regarding a deletion of a gene likely to be involved in cancer. Based on the analysis of chromosome deletion, it may be possible to clone a gene which is responsible for malignant development; at least we would have an idea of the chromosomal region where the gene is located. The identified deletions of 13q14 in patients with retinoblastoma (Francke, 1976), and deletions of 18q in colon cancer cells (Mulleris, et al., 1985) by cytogeneticists have led to the cloning of the retinoblastoma gene and DCC (deleted in colorectal carcinomas) gene (Fearon et al., 1990).

II. 2. Oncogenes

Tumor development is a complex and multi-step genetic process. Our present knowledge suggests that aberrations of two major classes of genes can lead to malignancy: abnormal activation (by mutation, rearrangement, amplification, etc.) of the first class of altered genes known as oncogenes which act dominantly in producing the malignant phenotype, and the inactivation (by mutation, deletion,etc.) of the second class of genes known as tumor suppressor genes which oppose tumorigenic transformation, that is, acting as negative controlling elements (Sager, 1989).

As many as 60 distinct oncogenes of viral and cellular origin have been identified.

The known oncogenes all play a role in the regulation of cell division and associated growth or regulatory processes. The oncogenes fall into 5 distinct categories. These are (1) growth factors, e.g. c-*sis* (2) growth factor receptors, e.g. *erbB*, *csf*, *erbA* (3) protein kinases, e.g. *src* (4) signal transducers, e.g. H-*ras* (5) nuclear oncogenes, e.g. *myc, myb*, *fos and jun* (Klein, 1988). The proto-oncogene is the normal allele which may be altered to become an oncogene by a point mutation, chromosomal translocation, amplification, or by insertion of a mobile genetic element such as a retrovirus. Oncogenes can contribute to tumor development either as a result of activation that modifies normal regulatory signals, or due to structure changes that lead to loss of their normal signalling function (Weinberg, 1985).

II. 2.1 *Ras*

The *ras* oncogene is one of the most studied oncogenes because the presence of dominantly acting *ras* oncogenes in human and carcinogen-induced animal tumors (Greenberg et al., 1989). The three proteins encoded by human *ras* genes (H-*ras*, K-*ras*, N-*ras*) have GTPase activity (Barbacid, 1987). These 21-KD *ras* proteins are associated with the inner surface of the plasma membrane. The p21 proteins can bind GTP and GDP with high affinity and hydrolyse GTP with low catalytic efficiency. The function and structure of p21 *ras* are similar to other G proteins (Satoh et al., 1987). They serve as signal transducers by switching from an active GTP-bound form to an inactive GDP-bound form (McCormick et al., 1989; Trahey et al., 1987). The regulation of *ras* activity

is controlled by guanine nucleotide releasing proteins (GNRPs), which facilitate the release of GDP thereby allowing GTP to bind, and by GTPase-activity proteins (GAPs) which accelerate the hydrolysis of GTP (Bollag et al., 1991). Mutation of *ras* genes cause the *ras* protein to be trapped in the GTP-bound form resulting in transformation of permissive cell types. Most experimental evidence supports that *ras* acts via the p21-GTP complex, and its transforming properties are similarly dependent on the generation of this complex (Greenberg et al., 1989).

Experiments demonstrated that *ras* can covert a nonsenescing murine fibroblast line (Radinsky et al., 1987; Thorgeirsson et al., 1985; Egan et al., 1987), primary rat embryo fibroblasts (Pozzatti et al., 1986), nonmetastatic murine mammary carcinomas (Bondy et al., 1985) and nonmetastatic murine lymphomas to metastatic tumors (Gao et al., 1988). The direct correlation between high level of *ras* gene expression, rearrangement and amplification with metastatic efficiency in the experimental metastasis assay were observed in mouse NIH-3T3 cells, mouse 10T½ cells (Egan et al., 1987; Hill et al., 1988), BALB/c-3T3 cells (Radinsky et al., 1987) and a nonmetastatic T lymphoma cell line (Collard et al., 1987). Both expression and mutation studies support the idea that p21 *ras* directly regulates metastatic potential.

However, experiments also demonstrate that many examples of human and murine tumors show no difference in *ras* expression in metastatic and primary tumors. Furthermore, not all recipient cells are susceptible to *ras*-induced transformation or

metastatic induction. *Pc12* rat pheochromocytoma line differentiates into neuron-like cells in the presence of mutated *ras* protein (Noda et al., 1985). Normal human fibroblasts and bronchiolar epithelium are difficult to transform with *ras*. These observations suggest that the ability of *ras* to transform and induce metastasis formation may be regulated by the genetic background of the recipient cell. More experiments show that *ras* expression and activity are regulated by other genes, e.g. *myc* (Land et al., 1986), E1A (Ruley, 1983), and K-*rev* (Kitayama et al., 1989). Furthermore, transfection of *ras* alone, or *myc* and *ras* in primary rat embryo fibroblasts, resulted in *ras* being inserted into chromosome 3 in all lines analyzed. This suggested that the insertional mutagenesis at this locus was essential for the metastatic transformation (Mckenna et al., 1988).

II. 2.2. *Myc*

It is found that translocations of t(8;14)(q24;q32), t(2;8)(p12;q24) and t(8;14)(q24;q11) are present in the malignant cells of patients with the Burkitt's lymphoma, with the first one accounting for 90% of the cases and the last two accounting for 10%. The oncogene c-*myc* is located on band q24 of chromosome 8 while the immunoglobulin heavy (IgH) chain, the kappa and lambda light chains are located on chromosomes 14, 2, and 22 respectively. All the three translocations in Burkitt's lymphoma are translocations of the immunoglobulin gene to the vicinity of the c-*myc* gene, and result in enhanced transcription of the translocated c-*myc* gene. In small cell

lung carcinoma, it was found that *myc*-amplification is correlated with increased invasiveness, high metastatic ability and morphological and enzymatic changes associated with poor prognosis (Little et al., 1983). The HL60, an established myelocytic leukemia line, contains approximately 40-60 *myc* genes per cell (Collins et al., 1988). Co-transfection experiments have shown that the *myc* gene can cooperate with *ras* in the transformation of permissive cells (Lane et al., 1983; 1986; Ruley, 1987). Recently, our laboratory demonstrated that *myc* and a mutant type of p53 could permit elevated expression of *ras* protein and increased malignancy (Taylor et al., 1992).

c-*myc* is one of the nuclear oncogenes. Molecular studies find that the c-*myc* gene consists of three exons separated by two introns. The first exon has an interesting feature: it has two active promoters with transcription initiation sites from which two transcripts are synthesized. The sequence within the first exon of c-*myc* is involved in regulating expression, and removal of this exon in some tumors may be responsible for activation of the oncogene. However, the normal function of this protein is unknown. The structure of c-*myc* is similar to several other nuclear proteins, which are known transcription factors, such as c-*jun*, c-*fos*, E/*CBP* and *myoD* (Candschulz et al., 1988; Lassar et al., 1989; Murre at al., 1989a; 1989b). This structural similarity has led to the suggestion that c-*myc* might be a transcription factor which controls the expression of specific genes (Collum et al., 1990). This hypothesis is supported by the fact that the c-*myc* gene can recognize and bind to a specific sequence in DNA (Blackwell et al., 1990) and that c-*myc* can activate gene expression and it binds directly to enhancers and

putative origins of replication (Kaddurah-Daouk et al., 1987; Ariga et al., 1989; Iguchi-Ariga et al., 1988). On the other hand, high levels of *myc* protein have correlated with reduced expression of specific cellular matrix genes (Bernards et al., 1986; Versteeg et al., 1988), therefore an important aspect of c-*myc* transformation activity is the ability to suppress specific cellular gene expression (Yang et al., 1991).

II. 3. Tumor Suppressor Genes

II. 3.1 Evidence for Tumor Suppressor Genes

The first evidence for tumor suppressing genes stem from pioneering somatic cell hybrid studies of Harris and Klein in 1969 and in 1971 (Harris et al., 1969; Klein et al., 1971). They fused normal mouse fibroblasts with a tumorigenic mouse cell line, the majority of hybrid clones were found to be suppressed for tumorigenicity. The expulsion of certain chromosomes, especially mouse chromosome 4, resulted in the reappearance of the tumorigenic phenotype (Evans et al., 1982; Miller et al., 1983). The suppression of tumorigenicity in these hybrid colones, therefore, implies the existence of dominantly acting tumor suppressor genes contributed by the normal parental cells. The reappearance of tumorigenicity associated with loss of specific chromosomes suggests that loss of chromosomes carrying tumor suppressor gene is responsible for their tumorigenicity. Therefore, suppression of tumorigenicity should depend on chromosome stability.

Extensive series of somatic cell hybridizations have been done between different tumor and normal cells or between two different tumor cells (Klinger et al., 1980; 1983; Stanbridge, 1976; Srinvtson et al., 1986; Weissman et al., 1983). The results of those fusion experiments have shown complete tumor suppression so long as specific chromosomes were not lost.

Human-rodent interspecies cell hybrids have been used with the human chromosome preferentially eliminated so that human chromosome with tumor suppressor activity could be identified, with the added advantage of easier cytogenetic analysis due to the differential staining of human and rodent chromosomes (Friend et al., 1976; Jonasson et al., 1977; Klinger et al., 1978). In these types of hybrids, Stoler and Bouche (1985) showed that the human chromosome 1 loss is associated with reexpression of tumorigenicity of human fibroblast x tumorigenic hamster cells. Klinger (1982) demonstrated the importance of human chromosome 2 in suppressing the tumorigenicity of chinese hamster ovary cells. Other human intraspecies hybrids have suggested tumor suppression activity on chromosome 4, 11, 13, 17 and 20 (Klinger et al., 1978; 1983).

The most stable hybrids are generated by using intraspecies human cell hybrids. The Hela carcinoma cell line (zybalski et al., 1962) fused to various normal cells have been studied most extensively (Stanbridge et al., 1982). The fusion of Hela and normal fibroblasts results in hybrid cells that are non-tumorigenic but continued to behave as transformed cells in culture. Therefore, the transformed and tumorigenic phenotypes

appear to be under separate control (Stanbridge, 1989). Furthermore, it was founded that re-expression of tumorigenicity was associated with the loss of a single copy of the fibroblast chromosome 11. The conclusion that chromosome 11 is involved in control of tumorigenic expression of Hela cells was made indirectly from the cell fusion studies (Kaelbling et al., 1986).

The direct confirmation of the existence of a tumorigenic suppressor on chromosome 11 was to transfer single chromosome 11 from normal human fibroblast into Hela x fibroblast tumorigenic segregant cells by the technique of microcell mediated chromosome transfer (Fournier et al., 1977; Saxon et al., 1986). The microcell hybrids restores the control of tumorigenic expression and results in non-tumorigenic hybrids. In 1987, Weissman et al introduced the human fibroblast t(X;11) chromosome into a Wilms' tumor cell line resulted in suppression of tumorigenicity (Weissman et al., 1987). The microcell hybrids regained their ability to form tumors and to grow in medium containing calf serum after they lost t(X;11) chromosome. In 1989, Stanbridge successfully introduced human chromosome 13 into osteosarcoma cells and suppression of tumorigenicity (Stanbridge, 1989). Microcell transfers have been used to identify human chromosomes that carry putative suppressor genes are shown in Table 3 (Sager, 1989).

Both the somatic cell hybridization and microcell mediated chromosome transfer studies have demonstrated the existence of tumor suppressor genes. Tumorigenicity can be suppressed by fusion of the tumor cell with a different cell which contains the normal

tumor suppressor genes.

II. 3.2. Retinoblastoma

Retinoblastoma is a neoplasm of embryonic retina disease. In 1971, Knudson proposed that there are two forms of retinoblastoma (Knudson, 1971), one is an inherited form, another is spontaneous. Both forms are result in the mutation or inactivation of two genetic loci. The inherited form has a germ line mutation as a primary event, the somatic mutation of a second allele is required for the expression of tumorigenicity. The spontaneous form requires both of allelic mutation somatically. The chromosomal mechanisms of involve in inactivation of both alleles are explained on Figure 1. The Retinoblastoma gene (*RB*) was initially mapped by cytogenetic means. Chromosomal deletion in 13q was detected in 3-5% of retinoblastoma cases (Wilson et al., 1973; Yunis et al, 1978). Moreover, It was found that the retinoblastoma locus is tight linkage to the esterase D gene which facilitated localization in 13q14 (parkes et al., 1980; 1983). Restriction fragment length polymorphism (RFLP) analysis of both spontaneous and inherited retinoblastoma tissues compared to normal tissue demonstrate the transition from heterozygosity to homozygosity around the *RB* locus at several loci on chromosome 13 (Cavenee et al., 1983; 1985). The successful use of RFLP analysis in detecting loss of heterozygosity with retinoblastoma specimens led to widespread use of this method with other cancer.

Table 3: Tumor Suppression by Chromosome Transfer

Tumor cell recipient	Chromosome transferred		Reference
	Suppressive	Nonsuppressive	
Hela (cervical)	11	X	Saxon et al., 1986
Wilms' (renal)	11	X,13	Weissman et al., 1987
SiHa (cervical)	11	N.D.*	Oshimura et al., 1989
A204 (rhabdomyosarcoma)	11	N.D.	Oshimura et al., 1989
HHUA (endomentrial)	1,6,9	11	Oshimura et al., 1989
YCR-1 (renal)	3p	11	Oshimura et al., 1989
SK-N-MY (neuroblastoma)	1	11	Oshimura et al., 1989
COKFu (colon carcinoma)	5,8	N.D.	Tanaka et al., 1991

*: not done
(Partly taken from Sager, 1989; Tanaka et al., 1991)

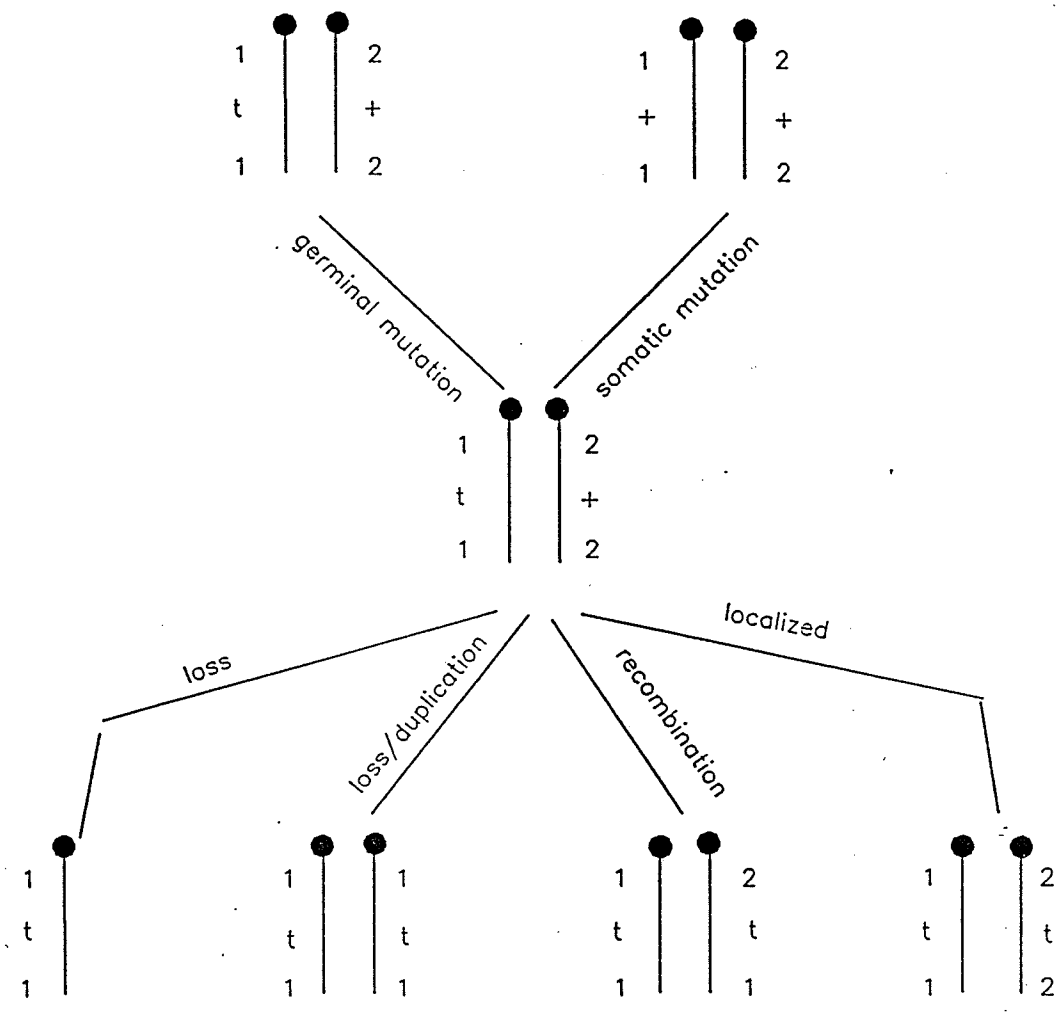


Fig. 1: A model for heritable and sporadic mutations that could lead to a tumor. 1 and 2 are variants at arbitary loci.
(Modified from Cavenee et al., In Genetic Analysis of Tumor Suppression, Ciba Foundation Symposium 142, John Wiley Sons Ltd., UK, 1989).

Subsequent cloning of esterase D provided the possibility for chromosome walking to clone *RB*. The first candidate *RB* cDNA named P4.7R was reported by Friend et al. in 1986. This 4.7R was expressed in normal retinal tissue, but not in any retinoblastoma or osteosarcoma tissues. Subsequent experiments of detected transcripts and ribonuclease protection demonstrated that the P4.7R clone represented the *RB* gene (Goddard et al., 1988; Dunn et al., 1988). The point mutation and small deletions of P4.7R was observed in retinoblastoma tumors. In 1988, Huang and colleagues introduced the gene mediated by expression of retrovirus into human retinoblastoma cell line. The neoplastic phenotype was suppressed by the *RB* gene (Huang et al., 1988).

It was detected that the protein product of *RB* gene is a 110 to 114-KDa mol wt phosphoprotein (Lee et al., 1987). This protein is localized to nucleus and is able to bind with single or double strand DNA. Also the *Rb* protein is able to complex with E1A oncoprotein. The E1A oncoprotein may exert an inhibitory effect on the functioning of *Rb* protein with which it is complexed (Whyte et al., 1988). Since E1A is a transcription regulator it is plausible that the *Rb* protein is involved in modulating the transcription of critical target genes, regulating genes involved in growth regulation or differentiation. Loss of *RB* gene in breast cancer cell lines,osteosarcoma and small-cell carcinoma of the lung suggested that the *RB* gene is involved with malignancies other than retinoblastoma.

So far, RB gene is the most understood tumor suppressor gene. The cloning, identification and understanding of the *RB* gene and its protein function have given us a

very useful model system for understanding the tumor suppressor gene.

II. 3.3. P53

The most cancer-related genetic change known at the gene level is p53 mutation (Vogelstein, 1990). The p53 gene encompasses 16-20 kb of DNA on the short arm of human chromosome 17 at position 17p13.1 (Isobe et al., 1986; McBride et al., 1986; Miller et al., 1986). In mouse, the p53 gene is on chromosome 11 (Czosnek et al., 1984; Rotter et al., 1984). Both genes are composed of eleven exons. The p53 protein comprises three hypothetical domains based upon analysis of the primary sequence (Pennica et al., 1984). Mutations such as allelic loss, rearrangements, deletions and single base substitutions in the evolutionarily conserved codons of the p53 are common in various types of human cancer (Fearon et al., 1987). Most of the base substitution mutations fall within a 600-base pair region which encompasses exons 5 through 8 and mutations outside exons 5 through 8 are rare.

The p53 protein was first detected by immunologic methods in simian virus 40 transformed cells in 1979 (Deleo et al., 1979; Lane et al., 1979). It is a nuclear phosphoprotein expressed at low levels in nontransformed cell lines. The levels of p53 are often elevated (Benchimol et al., 1982; Dippold et al., 1981; Thomas et al., 1983). In particular, in cells transformed by SV40 or adenovirus type5, the levels of p53 are approximately 100-fold higher than in the nontransformed cell, and the half-life of p53

is correspondingly extended, which is from 20 min to 24hr (Oren et al., 1981; Reich et al., 1983). Therefore, the elevation of p53 levels has often been correlated with cellular transformation. It was found that cooperation of p53 with a *ras* oncogene resulted in the transformation of rat embryo fibroblasts (Parada et al., 1984; Eliyahu et al., 1984) and overexpression of P53 resulted in the immortalization of rodent cells (Jenkins et al., 1984; Rovinski et al., 1988). In addition, p53 expression also exerted *in vivo* effects by enhancing the malignancy of cell lines that are only slightly tumorigenic (Wolf et al., 1984; Eliyahu et al., 1985), and by increasing the metastatic capacity of tumor cells (Pohl et al., 1988; Taylor et al., 1992). These results supported the hypothesis that p53 was a dominant growth-activating oncogene.

However, recent analyses indicate that the p53 gene used in these transfection experiments was in fact a mutant, and that the wild-type p53 gene is unable to transform rat embryo fibroblasts in cooperation with *ras* (Eliyahu et al., 1988; Hinds et al., 1989). The wild type p53 can block the ability of Ela and *ras* to contransform primary rodent cells, the rare clones of transformed foci that result from E1A plus *ras* plus wild-type triple transfections all contained the p53 DNA in their genome, but the great majority failed to express the p53 protein (Finlay et al., 1989). Other studies demonstrated that overexpression of the wild type gene can block the growth of osteosarcoma cells and cause a reversible growth arrest (Michalovitz et al., 1990; Mercer et al., 1990). These findings raised the possibility that wild type p53 could actually act as a tumor suppressor or transformation-inhibitory gene. A summary of biological activities of mutant and wt

p53 is presented in Table 4.

In many different types of tumors or transformed cells, p53 expression is elevated when compared to the normal situation. These higher levels appear to result in a mutant p53 protein with a longer half-life (Finlay et al., 1988). Therefore, the p53 mutant proteins are present at much higher concentrations in transformed cells than the wild-type p53 proteins. Mutant p53 proteins can bind to a cellular heat shock protein, hsp70, which is constitutively expressed in these transformed cells (Finlay et al., 1988; Hinds et al., 1989). However most of the mutant p53 proteins bound poorly or not at all to the SV40 large T antigen (Tan et al., 1985). Wild type p53 binds to one of two domains of the SV40 large T antigen. This domain contains a number of activities such as an ATPase, a helicase and binding site for α-DNA polymerase. The murine p53 protein, when bound to T antigen, can block the binding of the DNA ploymerase to T antigen and inhibit the helicase activity (Gannon et al., 1987). Therefore, it is possible that p53 could negatively regulate a cellular protein that is involved in cellular DNA replication in analogy to its interaction with T antigen. Experiments with a p53-GAL4 fusion protein have suggested that p53 might contain a transcription activation domain (Fields et al., 1990; Raycroft et al., 1990). P53 suppresses Rb transcription through inhibition of the basal promoter activity, which indicates that p53 can act as a transcriptional regulator *in vivo* (Shiio et al., 1992).

Table 4: Biological Effects of p53 Overexpression

p53 Introduced	Recipient cells	Effect
Mutant	Primary rat embryo fibroblasts	Immortalization
	Primary rat embryo fibroblasts	Transformation in concert with *ras*
	Adult rat chondrocytes	Immortalization
	Mouse Swiss 3T3 line	Induction of cellular DNA synthesis
	Abelson transformed mouse line L12	Conversion into more tumorigenic state
	Murine bladder carcinoma line	Increase in metastatic capacity
	Rat-1 line	Enhancement of tumorigenicity
Wild type	Primary rat embryo fibroblasts	Suppression of transformation
	Rat-1 line	Reduction in cloning efficiency
	Human glioblastoma line	Growth arrest
	Oncogene-transformed fibroblasts	Growth arrest
	Osteosarcoma	Growth arrest

(Partly taken from Michalovitz et al., 1991)

It has been suggested that mutant p53 acts in a dominant fashion, with the mutant protein having an ability to be dominant over the wild type, perhaps by the formation of nonfunctional multimeric complexes of proteins. The mutant p53 protein may do this by entering into a complex with hsc70 and wild type p53, thereby inactivating a function of the wild type p53 protein. Such complexes have been observed in several studies (Hinds et al., 1989; Finlay et al., 1988; 1989; Herskowitz, 1987; Green, 1989).

II. 3.4. *DCC*

It was reported that the most frequently observed deletions in colorectal cancers were chromosome 17p and 18q, each of which was lost in more than 70% of carcinomas (Grosschedl et al., 1985; Foster et al., 1985; Queen et al., 1984; Gopal et al., 1985). The target of chromosome 17p loss in colorectal tumors is the p53 gene. The common region of deletion on chromosome 18 is 18q2l-qter. The frequent loss of chromosome 18q in colorectal carcinomas would imply that this region might contain a tumor suppressor gene. However, a candidate suppressor gene on this chromosome had not been identified until 1990. In 1990, Fearon et al (1990) first cloned a gene termed *DCC* through a strategy of combining chromosome walking, isolation and sequencing expression, and cDNA cloning. They observed that one allele of the *DCC* gene was deleted in 29 of 41 (71%) colorectal carcinomas. The gene was expressed in almost all normal tissues tested, but *DCC* expression was greatly reduced or absent in 15 of 17 (77%) colorectal carcinoma cell lines. Therefore, they suggested that this gene should

be considered as a candidate colorectal tumor suppressor. The predicted amino acid sequence of *DCC* is highly homologous to the neural cell adhesion molecules and cell surface glycoproteins (Maxam et al., 1980). So the *DCC* gene may play a role in the pathogenesis of human colorectal neoplasia through alteration of the normal cell-cell interactions controlling growth. Confirmation of this hypothesis came from the introduction of whole chromosome 18 into COKFu, a human colon carcinoma cell line, can completely suppress tumorigenicity in athymic nude mice (Tanaka et al., 1991). Therefore, it has been directly demonstrated that the genes on chromosome 18 functions as a tumor suppressors in colon carcinogenesis.

II. 3.5. *Nm23*

The *nm23* gene was identified in the K-1735 melanoma cell line by Steeg etal (1988). It was found that *nm23* RNA levels were highest in a variety of normal tissues, including murine liver, lung and muscle cells and tumors of low metastatic potential (Steeg et al. , 1988). Also, studies of DNA from normal and tumor tissues from the breast, colon, lung and kindney indicated somatic deletion of one allele of the human *nm23*-Hl gene. After transfection of the murine *nm23*-1 cDNA into the highly metastatic K1735 TK murine melanoma line, the transformed cells expressing *nm23*-1 produced 90% fewer metastases in mice than non-transfected control cell lines (Liotta et al. , 1990). These observations suggest that the *nm23* gene is a candidate suppressor gene for metastasis.

Further studies have shown that proteins *nm23* and *Awd* (product of a *D. melanogaster* developmental gene for abnormal wing discs), bear a striking homology. *Awd* mutations affect the development of multiple tissues after embryonic development. Wallet et al (1990) demonstrated that proteins *Gip*17 and *Guk*7.2 are more than 60% identical to the sequence of the *nm23* protein. *Gip*17 and *Guk*7.2 are nucleoside diphosphate (NDP) kinases. NDP kinases are known to participate in three major function: (1) microtubule assembly and disassembly, (2) signal transduction through G proteins (Stryer et al., 1986), and (3) converts deoxyribonucleoside diphosphates to deoxyribonucleoside triphosphates (Wright, 1989). Therefore, *nm23* may have same function as *Awd, Gip17* and *Guk7.2* and contribution to the normal development of tissues, which include cell division, cell-to-cell communication and signal transduction (Wallet et al., 1990; Rosengard et al., 1989).

II. 3.6. Cloned Supressors

To date, approximately eleven putative tumor suppressors have been cloned. Two were cloned from flies (*Drosophila melanogaster*) and fish (*Xiphophorus*), and the rest were cloned from human cells. They are described in Table 5.

II. 4. Human Chromosome 16q and Mouse Chromosome 8

Comparative genetics of man and mouse have proven that human chromosome

Table 5: Cloned Suppressors

Origin	Gene	Action
Flies		
2L 21A	1(2)gl	Cell adhesion molecule-like
Fish		
X	Tu	Tyrosine kinase
Human		
11p13	Wilms' tumor	Unknown, zinc finger motifs suggest transcription factor
13q14	*RB*-1	DNA binding; cell cycle specific phosphorylation SV40-T,E1A,E7 binding
15	Thrombospondin	Suppressor of angiogenesis
16	*CAR*	Cell adhesion regulator
17p13	p53	Dominant-negative alleles sequester wild type product; E1b binding
18q	*DCC*	Cell adhension molecule-like
	K-*rev*-1	Anti-Ki-*ras*
	NTS-1	Anti-Ha-*ras*
	*nm*23	Suppressor of metastasis

(Partly taken from Mikkelsen et al., 1991; Pullman et al., 1992).

16ql2-l6q24 is geneticly homologous with the mouse chromosome 8 region A4-E2 (Searle et al., 1989; Ceci, 1991; Reeders et al., 1991). The homologous region and genes which both have been identified on the chromosomes and have been used as probes for Southern blot hybridization analysis in this study are shown on Figure 2.

Human chromosome 16q deletions and loss of heterozygosity (LOH) have been reported in Wilms' tumor, primitive neuroectodermal tumors, breast, prostate and hepatocellular carcinomas recently (Maw et al., 1992; Thomas et al. , 1991; Sato et al., 1990; Carter et al. , 1990; Tsuda et al., 1990). In 1990, Tsuda et al found that allele loss on chromosome 16q between the HP locus (16q22.1) and the CTRB locus (16q22.3-16q23.2) was associated with progression of human hepatocellular carcinoma (HCC). Among HCC with LOH at one or more loci, 58% of tumors were considered to have total loss of one chromosome or total loss of the long arm. In the other 42% of tumors, allele loss on chromosome 16 was partial. Different patients had different allele loss at one or more loci, e.g., in one case, allele loss was detected at D16Sl31 and APRT; while in another case, allele loss was detected at HBA1, CETP, MT2, D16S4 and TAT. Allele loss on chromosome 16 did not occur in HCC at the early stage; it was detected in 57% of HCC at the more advanced stage. This suggested that allele loss on chromosome 16 was not associated with the formation of HCC, but rather with the progression to an advanced stage and the common deletion region between HP and CTRB loci may contain an unknown tumor suppressor gene (Tsuda et al, 1990).

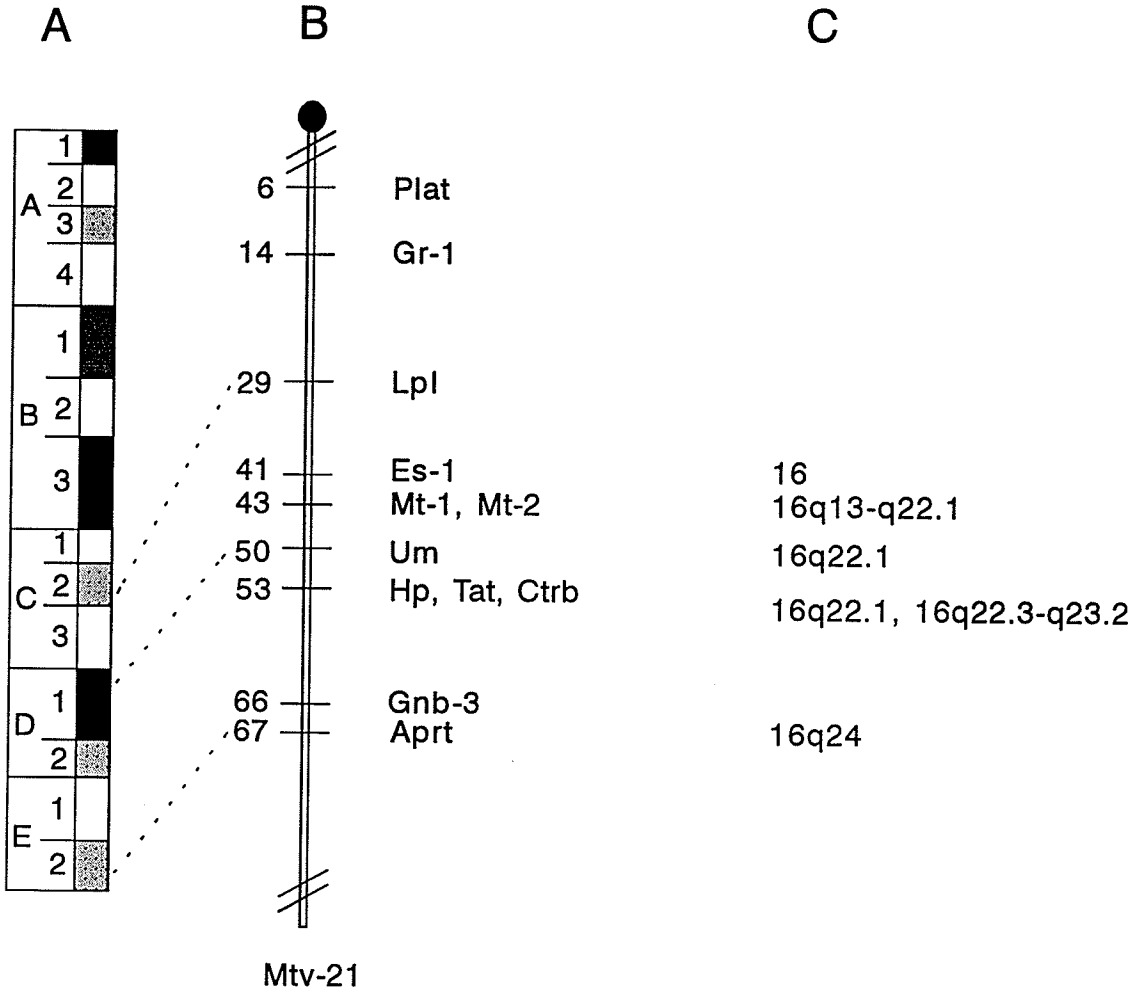


Figure 2.

A: Mouse chromosome 8 banding

B: Several gene loci on mouse chromosome 8. Some of these genes have been used as probes to hybridize with DNA from 10T1/2 Cells by Southern blot hybridization in this study

C: Human Chromosome 16q homologous is presented

(modified from Celi, 1991)

Also in 1990, allelic loss in prostate cancer was examined (Carter et al., 1990). Chromosome 16q and 10q exhibited the highest frequency of LOH where 30% of informative patients exhibited loss. The LOH region on chromosome 16 is located on 16q22-24. It was therefore possible that chromosome 16q and 10q may contain tumor suppressor genes whose inactivation is involved in prostatic tumorigenesis.

In breast cancer, cytogenetic studies have shown that the most frequently lost chromosome were 1, 8, 13, 17, and 16 (Rodgers et al. , 1984). LOH have been identified on lq, 3p, 11p, 13q, 17p and 16q (Chen et al., 1989; Devilee et al., 1989; Lundberg et al., 1987; Ali et al., 1987; Mackay et al., 1988a, 1988b). The LOH site of chromosome 16 is located on 16q24.3 (Dl6S7) (Sato et al. , 1990). 45% of LOH on chromosome 16q was observed in primary breast cancer. Tumors showing loss of chromosome 16q had frequent lymph node metastases (67%), compared to the group of tumors in which chromosome 16q was not lost. Based on the cytogenetic and molecular studies, a three-step hypothesis of breast cancer development has been proposed: (1) loss of chromosome 17p which act at an early stage in primary breast cancer, (2) loss of chromosome 13q, (3) loss of chromosome 16 which might have a significant role in the lymph node metastasis.

More evidence of LOH in chromosome 16q came from the human central nervous system primitive neuroectodermal tumors (PNET). Cytogenetic studies have shown that deletions of 17p, 6q, 16q and 22 in PNET (Griffin et al., 1988; Biegel et al., 1989;

Vagner-capodano et al., 1989; Bigner et al., 1988). In further RFLP analysis, six of the 23 tumors showed loss of markers on 17p, five of 23 tumors showed loss of markers on 6q, and 3 showed loss of markers on 16q (Thomas et al., 1991). The probes used for 16q RFLP analysis are in the region between 16q22.1 and 16q24.

Recently, Maw et al (1992) found that in addition to loss on chromosome llp in Wilms' tumor there was significant loss on chromosome 16q. The allelic loss of chromosome 16q was overlapped between 16q2l and 16q24.1. Therefore, it was suggested that 16q might be the location of a third tumor suppressor gene underlying Wilms' tumorigenesis. It is hypothesized that the potential candidate for the critical Wilms' tumor gene on 16q is uvomorulin. Uvomorulin encodes a cell adhesion molecule which may play a role in preventing tumor invasion in some cancers.

Most recently, a cell adhesion regulator (CAR) cDNA clone has been isolated (Pullman et al., 1992). The CAR gene is located on the long arm of chromosome 16q and encodes a protein of 142 amino acids. Transfection the CAR gene into SW620, a colon cancer-derived metastatic line enhanced two to three fold adhesiveness of SW620 to extracelluar matrix. CAR protein has a cytoplasmic region, and no transmembrane region. CAR is thus unlikely to be a adhesion molecule. It was suggested that the CAR protein may play a possible role in signal transduction and therefore enable the external environment to influence cell shape and behaviour. Loss of CAR activity could cause loss of cell differentiation induction or tumor cell release from extracellular matrix attachments. Therefore, CAR is a candidate tumor suppressor gene.

III. MATERIALS AND METHODS

III. 1. 0. Materials

Cells

All of 10T½ transformed cell lines were established by Egan et al. (Egan et al., 1987a, 1987b; Egan, 1989, Ph.D thesis). *Myc*l.4 and *Myc*l.1 cell lines are G418 resistant clones generated from infected 10T½ fibroblasts by helper free v-*myc/neo* retrovirus. Control G418 resistant cell lines SVX-1 and SVX-2 are generated through infection of 10T½ cells with the neo virus ZIPNE/SVX. MRPD and M4R4 cell lines were devirved from *myc* 1.4 and *myc* 1.1 transfected with v-H-*ras*. Cl, C2, C3, NR3 and NR4 cell lines were generated through infection of 10T½ cells with T-24-H-*ras*. 10T½*RAS* was cloned through infection of SVX2 cells with EJ-H-*ras*. RHMRA, RHMRB and RHMRC are hybrids of non-metastatic M4R4 and metastatic 10T½*ras*. All the cell lines used in this study are listed in Table 6.

Probes

The probes used in this study are listed in Table 7. The metallothionein-1 (Mt-1) in pBX (a pBR 322 derivitive) plasmid was obtained from Dr. Reichard Palmiter (University of Washington, Seattle). The uvomorulin (Um) cDNA in PUC8 was obtained

Table 6: Characteristics of 10T½ and 10T½ Transformed by *ras* and *myc*

Cell Line	Transfected Genes	Transfection Medium	Tumorigenicity Frequency	Experimental Metastasis
10T½	None	0	0	0
SVX1	*neo*	FBS	0/3	0±0
*myc*1.1	*myc*	FBS	0/3	0±0
MRPD	v-*myc*, EJ-H-*ras*	dCS	3/3	0±0
*myc*1.4	v-*myc*	FBS	0/3	0±0
M4R4	v-*myc*, H-*ras*	FBS	4/4	0.2±0.2
NR3	*T24-H-ras*	FBS	6/8	0/5
NR4	*T24-H-ras*	FBS	10/10	3/5
C1	T24-H-*ras*	FBS	5/5	12±5
C2	T24-H-*ras*	FBS	5/5	78±9
C3	*T24-H-ras*	FBS	5/5	85±11
10T½*RAS*	*hyg*, EJ-H-*ras*	FBS	4/4	48±6
Hybrids of M4R4 and 10T½ *ras*				
RHMRA			3/3	2±1
RHMRB			3/3	0.3±0.3
RHMRC			N.D.**	94±34

*: Taken from Egan et al., 1987a; 1987b; Egan, Ph.D thesis, 1989

**: N.D. = Not Done

Table 7: Listing of Loci Mapped, Probes Used

Locus	Gene Name	Probe	Species	Inserts Cutter	Probe Reference*
Mt-1	Metallothionein-1	mMT-1	Mouse	BamH1	1
Um	Uvomorulin	F5H3	Mouse	HindIII/EcoR1	2
Hp	Haptoglobin	hp2alpha	Human	BamH1/HindIII	3
Ctrb	Chymotrypsin B	PBS-2-1	Rat	EcoR1	4
Tat	Tyrosine Amino-transferase	PmCTAT	Mouse	BamH1/EcoR1	5
Gnb-3	β-subunit of Retinal Transducin	pGem3Z	Bovine	EcoR1	6
Aprt	Adenosine Phospho-ribosyltransferase	Puc3.1	Mouse	EcoR1/sph1	7
Mtv-21	Mammary Tumor Virus-21	Mtv21-5'C	Mouse	BamH1/EcoR1	8

*: 1. Glanville et al., 1981; 2. Eistetter et al., 1988; 3. Baumann et al., 1985; 4. Natt et al., 1988; 5. Muller et al., 1985; 6. Danciger et al., 1990; 7. Sikela et al., 1983; 8. Kozak et al., 1987

from Dr. Martin Ringwald (Max-Planck-Institut Fur Immunbiologie, Stubeweg 51, Freiburg, Germany). The haptoglobin (Hp) in pAT153 vector was purchased from the American Type Culture Collection (ATCC, Rockville, Maryland). The chymotrypsin (Ctrb) in Bluescript was obtained from Dr. Christopher Newgard (Southwestern Medical Center, The University of Texas at Dallas). The tyrosine aminotransferase (Tat) in Bluescript M13- was obtained from Dr. Siegfried Ruppert (Institute of Cell and Tumor Biology, German Cancer Research Center, Heidelberg, Germany). The β-subunit of retinal transducin (Gnb-3) in pGEM 3Z vector was obtained from Dr. Arieh Katz (California Institute of Technology, Pasadena). The adenosine phosphoribosyltransferase (Aprt) in pUC19 was obtained from Dr. Peter Stambrook (Medical Center, University of Cincinnati, Cincinnati, Ohio). The mammary tumor virus-21 (Mtv-21) in pSP65 vector was obtained from Dr. Gordon Peters (Imperial Cancer Research Fund Laboratories, London, England). The locations of probes on human chromosome 16 and mouse chromosome 8 are listed in Table 8.

Table 8: Listing of Locations of Probes on Human and Mouse Chromosome

Locus	Location		Reference
	Human	Mouse	
Mt-1	16q21-q22.1	8A4-C3	Searle et al., 1989
Um	16q22.1	8C3-E1	Reeders et al., 1991 Eistetter et al.,1988
Hp	16q22.1	8C3-E1	Reeders et al., 1991 Ceci, 1991
Ctrb	16q22	8C3-E1	Scherer et al., 1989
Tat	16q22.1	8C3-E1	Muller et al., 1985
Gnb-3	N.I.*	8E1-ter	Ceci, 1991 Danciger et al., 1990
Aprt	16q24.2-qter	8E1-ter	Reeders et al., 1991 Searle et al., 1989
Mtv-21	N.I.	8**	Ceci et al., 1991

*: NI=not identified on a human chromosome
**: Mtv-21 has been mapped on mouse chromosome 8, but the exact region is not known

III. 2. 0. Methods

III. 2. 1. Cytogenetic Analysis

Cell Culturing Techniques

Mouse C3H lung or spleen tissue was wrapped in cloth and mechanically minced by sterilized scissors and forceps to achieve a single cell suspension in phosphate buffered saline (PBS) buffer. The cells were washed three times in PBS and then were seeded at $2x10^6$ cells/ml in alpha minimal essential media [(α-MEM) Flow laboratories Inc., Rockville, MD] plus 20% fetal bovine serum (FBS), antibiotics, penicillin G (100 units/ml) and streptomycin sulphate (100μg/ml) and grown for 24h to 48h. Murine 10T½ fibroblasts and the transformed cell lines were maintained in α-MEM supplemented with 10% FBS, antibiotics, penicillin G and streptomycin sulfate and grown for 24h. All of the cultures were incubated at 37°C in a 5% $C0_2$ atmosphere in a humidity controlled incubator. Cell cultures that approached confluence were subcultured, frozen for long-term storage at -70°C in cyrotube vials (Nunc, Kamstrup, Denmark) or harvested for chromosome preparation and genomic DNA isolation.

Chromosome Preparation

In order to increase the mitotic index at the time of harvest, one of two techniques was used. The first technique was to incubate the normal C3H cells and 10T½ cells at 4°C for 12-16h before harvesting. The second was to add 0.05μg/ml of colcemid one hour before harvesting. Then the cells were harvested by treatment with trypsin (0.3% bacto trypsin was prepared in PBS, PH 7.3, consisting of l40mM NaCl, 2.7 mM KC1, 1.6 mM KH_2PO_4, and 8.1 mM Na_2HP0_4) and incubated in 0.075M KC1 for 30 min at 4°C prior to fixation with methanol-acetic acid (3:1 [vol/vol]) for a minimum of 24h. The fixed cells were washed with fixation buffer for two times. The slides were pretreated at 0°C in cold distilled water before use. The fixed cells were dropped onto cold and wet slides. Then they were incubated immediately at 50°C-60°C in slides incubator.

G-banding

Slides were aged for about 3-7 days before G-banding. There are two methods that have been used for G-banding. Trypsin G-banding: the metaphase cells were rinsed in 0.85% saline, then the cells were treated by trypsin solution at approximately 17°C for 2-5 seconds, and finally the cells were rinsed twice in 0.85% saline (Nesbitt et al., 1973). Urea G-banding: cells were rinsed in 0.85% saline, then were treated with 2.5M urea at 22°C for 2-15 seconds, and the urea buffer was washed away with 0.85% saline three times. The slides made by both methods were stained with 5% Giemsa for 10-20

mins at room temperature and analyzed by light microscopy. Up to 100 metaphases of chromosome numbers were counted under microscopy for each cell line. Metaphases were photographed. Chromosome analysis were carried out with the use of the photographs. Up to 50 metaphases for each cell line were examined. Karyotypes were made for eight cell lines of 10T½.

III. 2. 2. Preparation of Probes

Propagation of Plasmids

The competent *E. coli* prepared by using calcium chloride (Sambrook etal, Molecular Cloning a Laboratory Manual, second edition, page 1.82-1.84, Cold Spring Harbor laboratory press, 1989). Each plasmid used in this study was diluted to 100ul with lxTE (tris EDTA) (40ng plasmid/100μl) and mixed with a competent strain of *E. coli* culture 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 for lh in 1 ml LB broth (LB medium consisting of 1% w/v bacto-tryptone, 0.5% w/v bacto-yeast extract and 1% w/v NaCl) (Maniatis et al., 1982). The mixture was spread on LB plates containing 50μg/ml ampicillin. The plates were incubated overnight at 37°C, then the colonies were picked and used to inoculate two tubes of 5 ml LB medium containing ampicillin and then grown overnight at 37°C. Next day, one of the 5 ml of cultured bacteria was supplemented with 0.5 ml dimethylsufoxide and placed in five 1 ml cyotube vials (Nunc, Kamstrup, Denmark), then frozen down at

-70°C for long term storage. Another 5 ml of cultured bacteria was added to 1 liter of LB medium in a 2 liter flask containing 50μg/ml ampicillin. The bacteria were cultured in a shaker at 37°C for 3-4h until the density reached an OD of 0.5 at 550 nm. Then spectinomycin (5ml/liter) was added to the flasks and the bacteria was further cultured on a shaker at 37°C overnight. The bacteria were centrifuged at 4000g for 10 min at 4°C. The plasmids were isolated and purified by nucleobond AX kits (Macherey-Nagel GmbH & Co. Germany). The pellet of bacterial cells are resuspended in a buffer of 50mM Tris/HCL, 10mM EDTA, 400ug RNase A/ml, pH8.0. The suspension is mixed gently with a buffer of 200mM NaOH, 1% SDS. The a buffer of 2.80M KAc is added and mixed by shaking several times and centrifuged at 12,000g at 4°C for 45 minutes. The supernatant is removed and loaded on a nucleobond AX cartridge. The cartridge is washed with two times 10.0ml of a buffer of 100mM Tris/H_3PO_4, 15% ethanol, 1300 mM KCL. The plasmid DNA is eluted with a buffer of 100mM Tris/H_3PO_4, 15% ethanol, 1000mM KCL, pH8.5. The purified plasmid DNA is precipitated with 100% ethanol and dissolved in 1xTE buffer.

Isolation of Inserts

Each of the probes used for Southern blot hybridization were cDNA or genomic DNA inserts which were removed from plasmids. Restriction enzymes used for obtaining each insert are listed on Table 7. The probes were radiolabelled with [α^{32}P]dCTP (Amersham) using a nick-translation kit [Bethesda Research Laboratories (BRL) Life

Technologies, Inc. Bethesda, MA]. This method is described by Rigby et al (1977). 500 ng of probe was diluted to 28ul with distilled H_2O. 5ul of the unlabelled of the dATP, dGTP and dTTP in concentrated buffer were added plus 5ul of DNA polymerase 1/ DNase 1 (100 units) and 7ul [α^{32}P]dCTP (specific activity 3000 Ci/ mol). The mixture was incubated at 16°C for 1 hour and then was passed through a spin column of sephadex G-50 and the labelled probe was eluted with 100ul 1xTE.

III. 2. 3. Southern Blot Analysis

Genomic DNA was isolated from cells by phenol-chloroform extraction (Blin et al., 1976). 10 μg of DNA was digested to completion with 3-4 units/μg DNA of the desired restriction endonuclease, fractionated on 0.8% agarose gels and transferred to the Zeta-probe membrane with Zeta-probe Southern blotting protocol (Bio Rad Laboratory, Mississauga). Filters were hybridized to [α^{32}P] labelled nick-translated probe (Rigby et al., 1977) (lxl0^8 cpm/μg) for 16h in 1 mM EDTA, 0.5 M $NaHPO_4$, pH7.2, 7% SDS, at 65°C. After hybridization, filters were washed at 65°C in 1 mM EDTA, 40 mM $NaHP0_4$, pH7.2, 5% SDS for 5-15 min, followed by 1 mM EDTA, 40 mM $NaHP0_4$, pH7.2, and 1% SDS. Autoradiography was performed at -70°C with X-omat AR film (Eastman Kodak Co. , Rochester, N.Y.) for 3-10 days. The X-ray film was then developed for visualization of the exposed bands.

Densitometric analysis on Southern hybridization band intensities appropriate for

direct comparisons between cell lines, was performed using a Beckman DU-8 scanning spectrophotometer.

For reprobing, the membrane was stripped as soon as possible after autoradiography. It was then washed 2 times for 30 minutes each in a 400ml volume of 0.1xSSC/0.5% SDS at 95°C.

IV. RESULTS

IV. 1. Cytogenetic Analysis

In this study, C3H lung or spleen cells and the 10T½ fibroblast cell lines were examined cytogenetically as controls to the 10T½ and seven 10T½ transformed cell lines. Two of the transformed lines MRPD and M4R4 were tumorigenic but non-metastatic. The other five transformed lines NR4, Cl, C2, C3 and 10T½*ras* were both tumorigenic and metastatic (Egan et al., 1987a; 1987b; Egan, 1989, Ph.D thesis). Karyotypic analyses were performed on the seven transformed cell lines. Changes of chromosome numbers and structure including deletions, translocations, DNA amplifications, inversions and marker chromosomes were observed in 10T½ cells, but these changes were not consistently present in each cell line, with one exception. Abnormal chromosome 8 was found in all metastatic cell lines. The frequency distribution of chromosome numbers in each cell line is presented in Table 9. The

cytogenetic analysis data is summarized in Table 10.

Normal C3H Mouse Chromosomes

The normal mouse chromosome number is n=40. The G-banding is shown in Figure 3, which is the same as observed in previous reports (Nesbitt et al., 1973; Sawyer et al., 1986).

10T½ Fibroblasts

The chromosome numbers were variable among 10T½ cells, with a range of 50-132. Chromosomal deletion, translocation, DNA amplification and marker chromosomes were observed. The number of copies of chromosome 8 was 2-3. A representative karyotype is shown in Figure 4.

Table 9: Frequency Distribution of Chromosomes in 10T½ Lines

Cell Line	Number of Chromosomes*				
	40-60	61-70	71-80	81-90	90>
10T½	12.6%	41.3%	38.3%	3.6%	4.2%
MRPD	11.5%	32.6%	45%	5.2%	5.7%
M4R4	18.2%	35.4%	36%	4.6%	5.8%
NR4	10.6%	42.5%	33.6%	6.2%	7.1%
C1	13.3%	24.5%	49%	6.1%	7.1%
C2	11.4%	29.5%	47.6%	3.8%	7.6%
C3	37.8%	39.5%	11.8%	3.4%	7.6%
10T½*ras*	10.9%	42.5%	36.6%	5%	5%

*: The percentage of cells in a cell line with a given number of chromosomes (grouped into 5 ranges)

Table 10: Karyotypic Analysis of Metastatic and Non-Metastatic 10T½ Lines

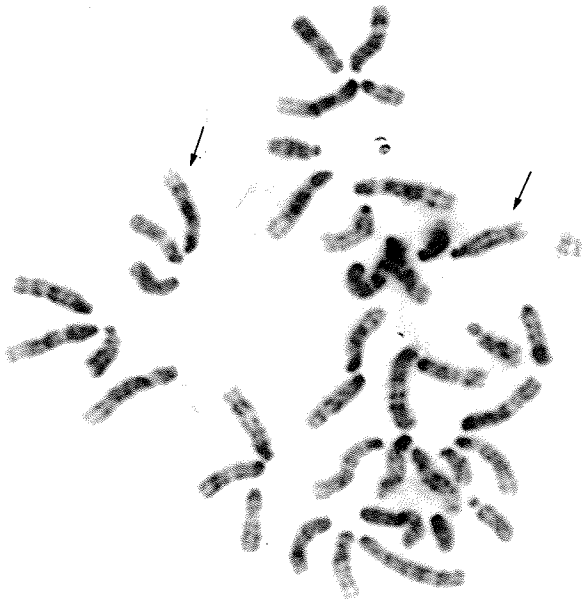
Cell Line	Transfected Genes	Experimental Metastasis[a]	Chromosome Number	Copy of Chromosome 8[b]	Frequency of Abnormal 8[c]
Normal	None	0	40	2	
10T½	None	0	50-132	2-3 35% tris.	
MRPD	v-*myc*, EJ-H-*ras*	0	52-125	2-3 46% tris.	
M4R4	v-*myc*, v-H-*ras*	0.2±0.2	50-164	3-4 52% tris. 48% tetr.	35%inv.(1x8)(B1-B3)
NR4	T-24-H-*ras*	3/5	50-124	2-3 36% tris.	53%inv(8)(D1-E1)
C1	T-24-H-*ras*	12±5	53-131	1-2 62% mon.	26% del.(1x8)[d](E1) 12% del.(2x8)[e](E1)
C2	T-24-H-*ras*	78±9	43-137	0-2 25% null. 51% mon.	14% del.(1x8)(C1) 10% del.(2x8)(C1)
C3	T-24-H-*ras*	85±11	49-107	0-2 24% null. 50% mon.	16% del.(1x8)(C3) 10% del.(2x8)(C3)
10T½*RAS*	T-24-H-*ras*	48±6	51-114	0-2 23% null. 55% mon.	12% del.(1x8)(C3) 10% del.(2x8)(C3)

a: Taken from Egan et al., 1987a; 1987b; Egan, 1989, Ph.D thesis
b: Null= nullisomy; mon= monosomy; tetr= tetrasomy
c: Inv= inversion; del= deletion
d: 1x8: one copy of chromosome 8
e: 2x8: two copies of chromosome 8

Figure 3

a: Representative chromosome spread of normal C3H mouse, 2n=40,XY
b: Karyotype from a
Arrows indicate chromosome 8.

a



b

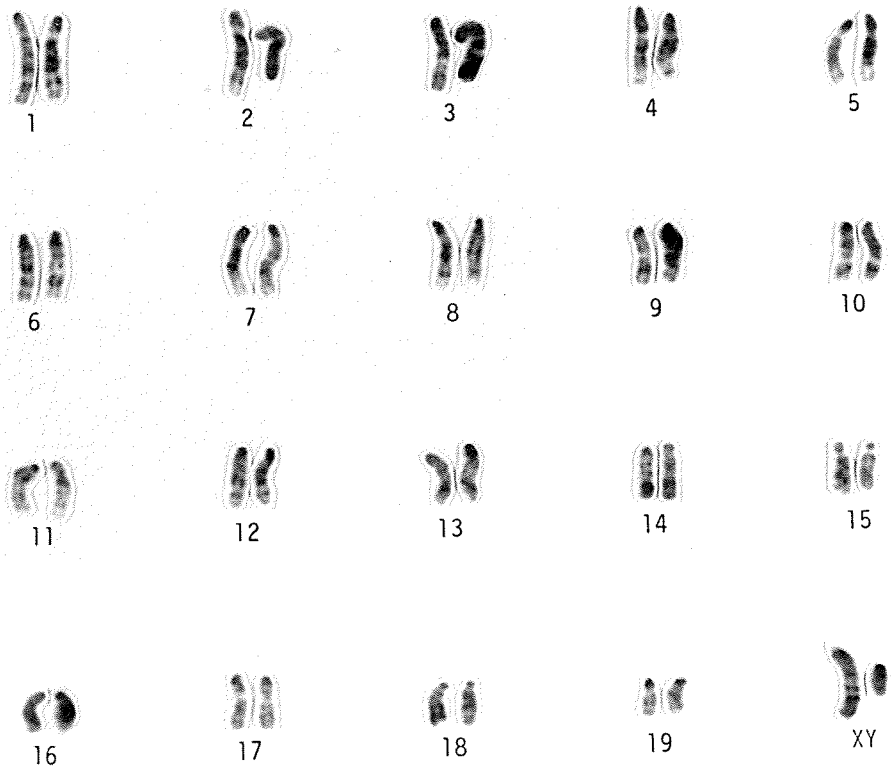
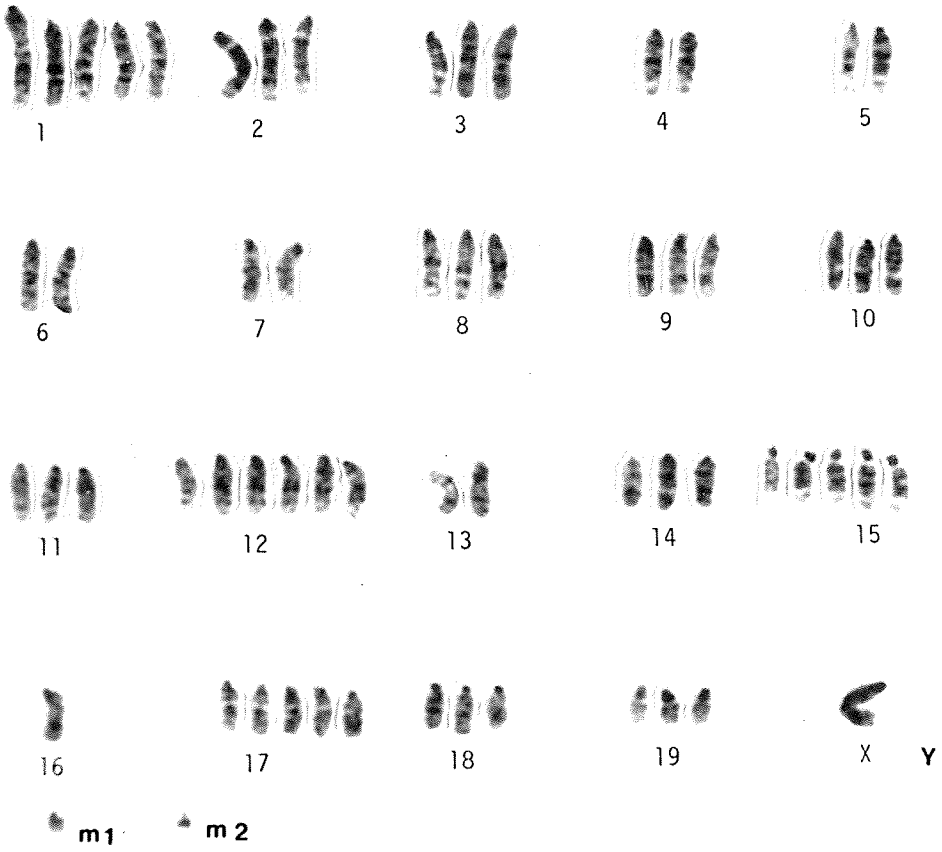


Figure 4

a: Representative chromosome spread of 10T½. 64, X, +1, +1, +1, +2, +3, +8, +9, +10, +11, +12, +12, +12, +12, +14, +15, +15, +15, -16, +17, +17, +17, +18, +19, -Y, +M1, +M2
b: Karyotype from a
Arrows indicate chromosome 8

a

b



MRPD

The chromosome numbers varied between 52-125. Chromosomal aberrations were observed with DNA amplification and marker chromosomes with 2-3 copies of chromosome 8. A representative karyotype of MRPD is shown in Figure 5.

M4R4

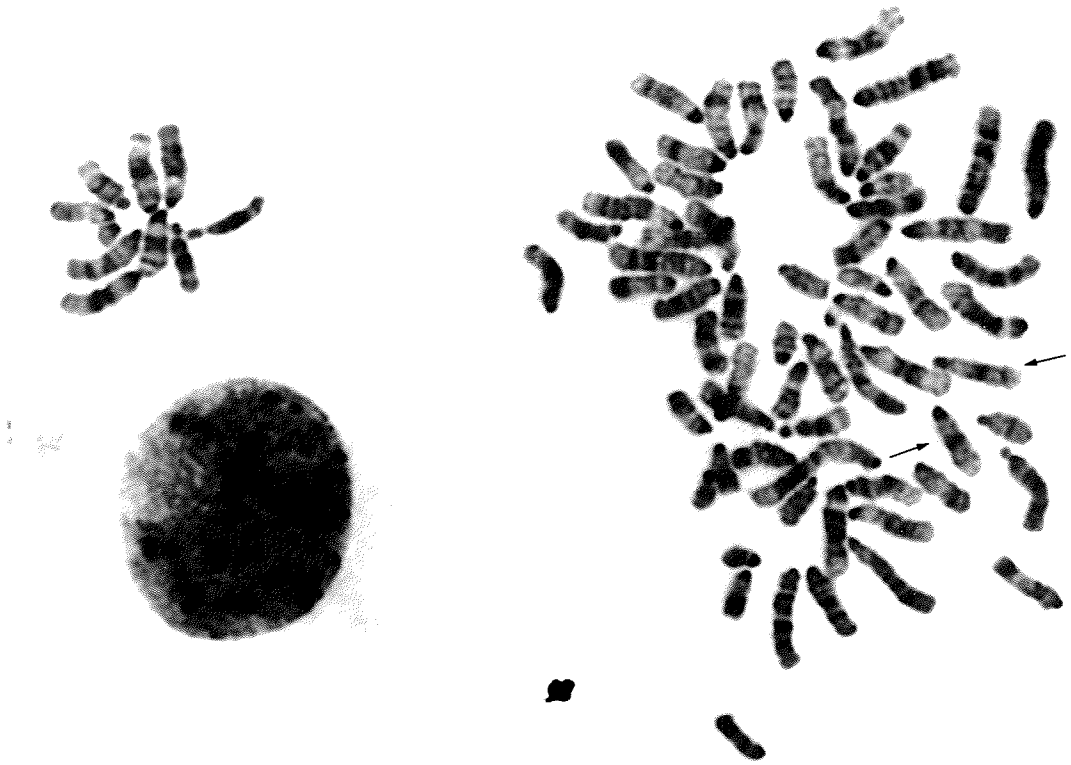
The chromosome numbers varied between 50-164. Chromosomal translocations, amplifications and marker chromosomes were observed. There were 3-4 copies of chromosome 8 with one copy containing an inversion (Bl-B2). A representative karyotype is presented in Figure 6.

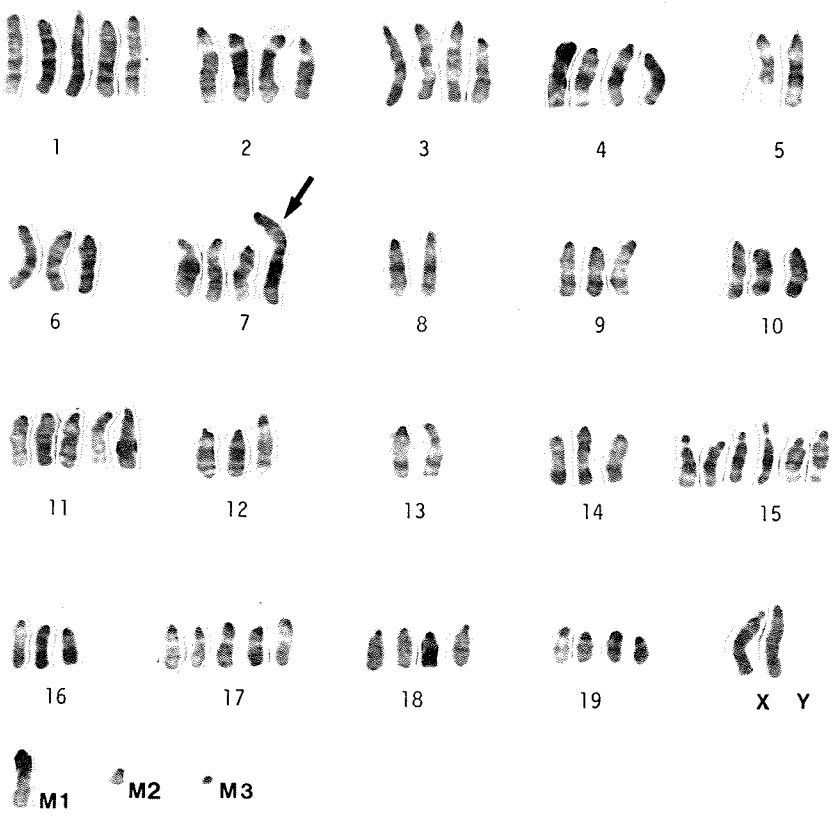
NR4

The chromosome numbers varied between 50-124. There were chromosomal amplifications, deletions, and marker chromosomes. There were 2-3 copies of chromosome 8, all with an inversion (Dl-El). The representative karyotype is presented in Figure 7.

Figure 5

a: Representative chromosome spread of MRPD. 74, XX, +1, +1, +1, +2, +2, +3, +3, +4, +4, +6, +7, +(7;17), +9, +10, +11, 11, +11, +12, +14, +15, +15, +15, +15, +16, +17, +17, +17, +18, +18, +19, +19, +M1, +M2, +M3
b: Karyotype from a
Arrows indicate chromosome 8 and the large straight arrow shows chromosomal translocation


a


b
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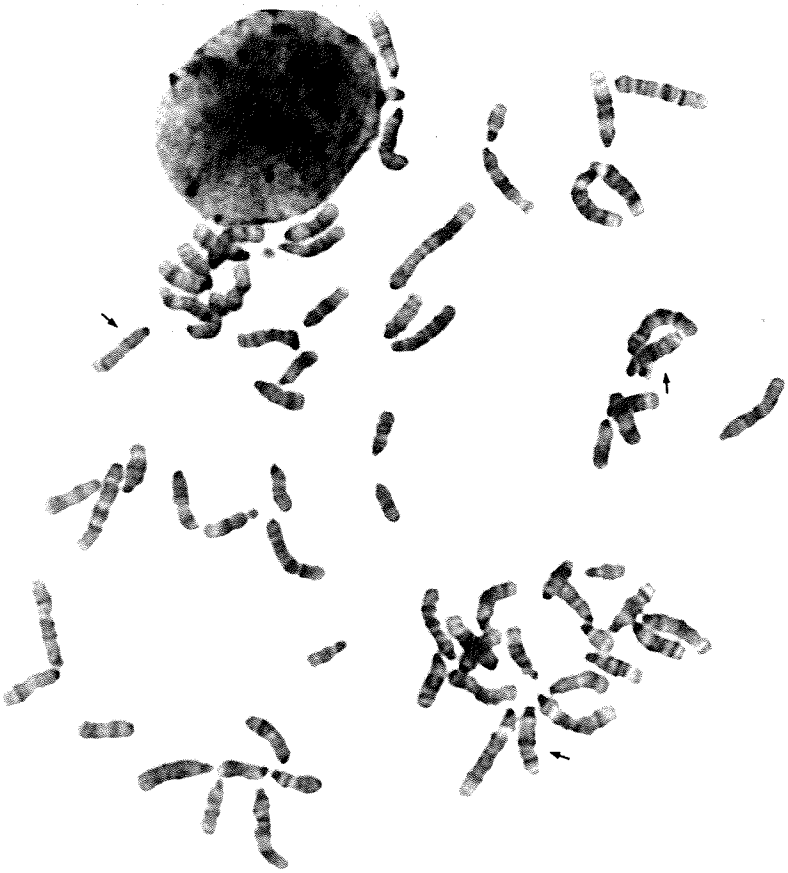
19
X Y
M1
M2
M3

Figure 6

a: Representative chromosome spread of M4R4. 71, XXY, +1, +2, +3, +4, +4, +6, +6, +7,+7, +8, inv(8)(B1-B3), +9, +9, +11, t(11;5), +12, +12, +12, +14, +15, +15, +15, +16, +17, +19, +19, +M1, +M2, +M3, +M4, +M5, +M6
b: Karyotype from a
Arrows show chromosome 8. The large straight arrow indicates translocation. The large curved arrow shows the inversion on chromosome 8.

a



b

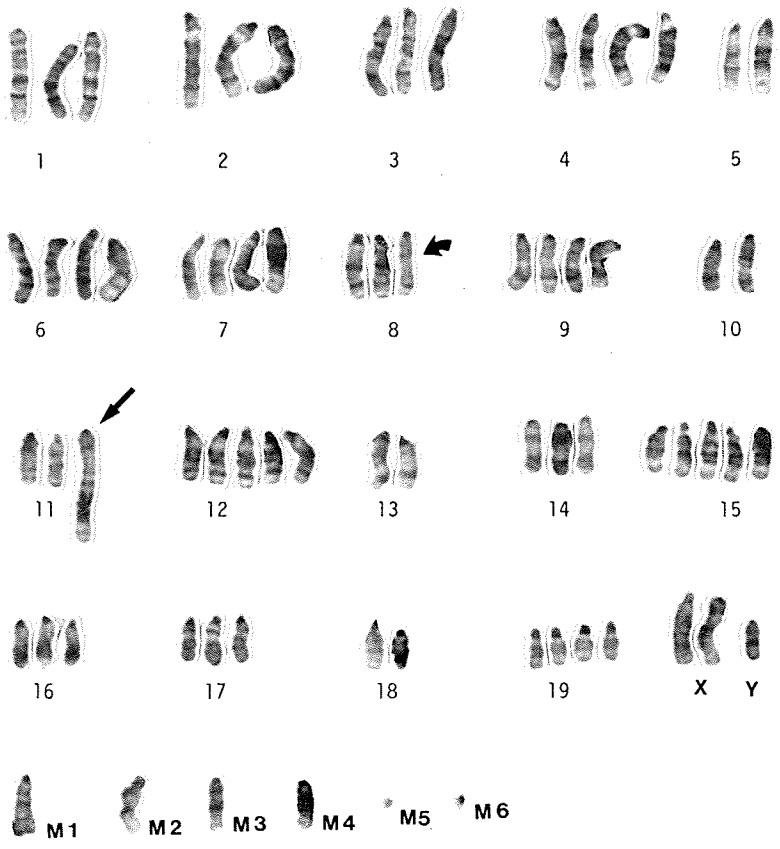
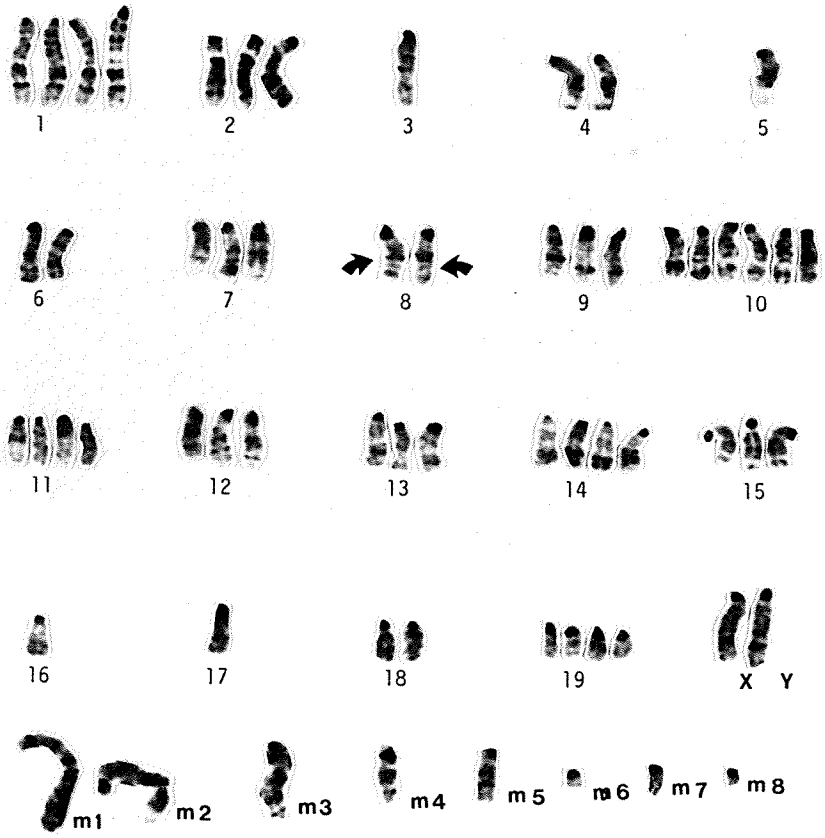


Figure 7

a: Representative chromosome spread of NR4. 62, XX, +1, +1, +2, -3, -5, +7, inv(8)(D1-E1), +9, +10, +10, +10, +10, +11, +11, +12, +13, +14, +14, +15, -16, -17, +19, +19, +M1, +M2, +M3, +M4, +M5, +M6, +M7, +M8.
b: Karyotype from a
Arrows indicate chromosome 8. The large curved arrows shows the inversion region on chromosome 8.

a

b



C1

The chromosome numbers varied between 53-131. Chromosomal aberrations with deletions, amplifications and marker chromosomes were observed. The translocations often involved chromosomes 5 and 11, t(5,11). One copy of chromosome 8 was missing or the fragment (El-E2) was deleted. The representative karyotype is shown in Figure 8.

C2

The chromosome numbers varied between 43-137. Marker chromosomes, translocations, and amplifications were observed. Deletions of 1-2 copies of chromosome 8 were observed and a fragment (El region) was identified. A representative karyotype is shown in Figure 9.

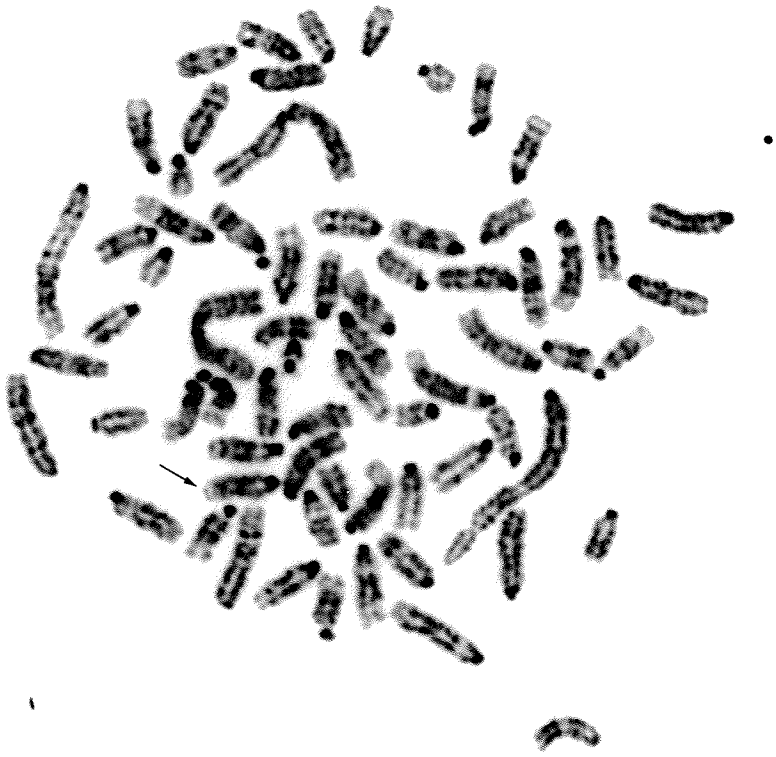
C3

The chromosome numbers varied between 49-107. Chromosomal aberrations of deletions, amplifications and marker chromosomes were observed. Monosomy and nullisomy of chromosome 8 were identified. A representative karyotype of C3 is shown in Figure 10.

Figure 8

a: Representative chromosome spread of C1. 72, XY, +1, +2, +3, +3, +3, +5, +6, -8, +10, +10, +11, +11, +11, +11, +t(11;7), +12, +13, +13, +13, +13, +13, +14, +14, +15, +15, +17, +17, +18, +18, +18, +19, +19, +M1, +M2.
b: Karyotype from a
Arrows shows chromosome 8. The thick short arrow indicates one copy of chromosome 8 deletion. The large long arrows show translocations.

a



b

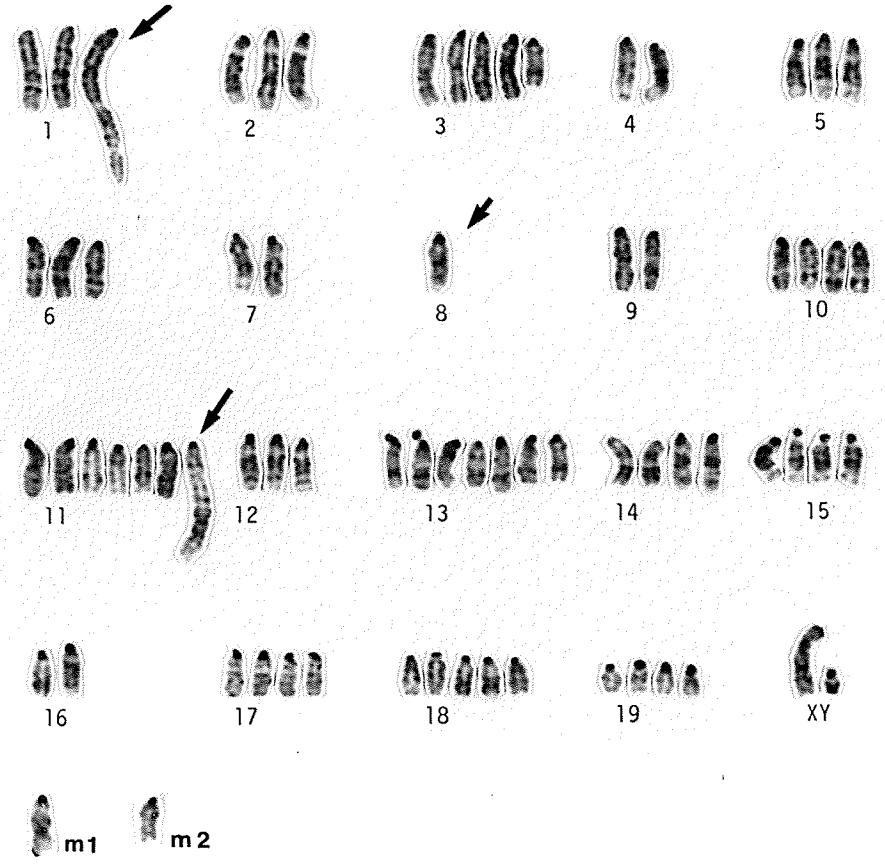
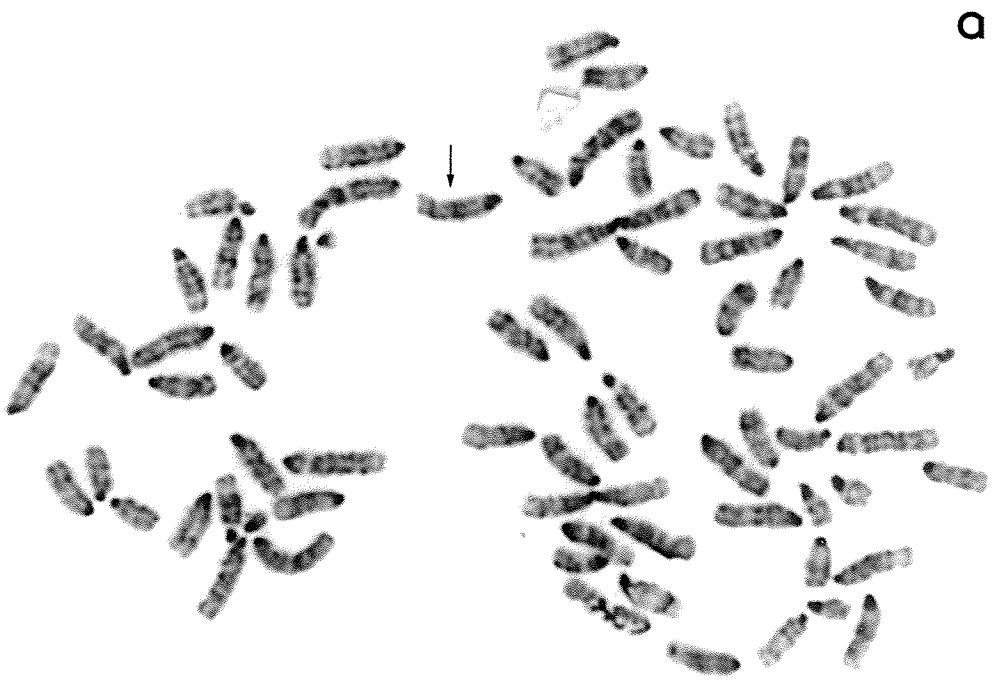
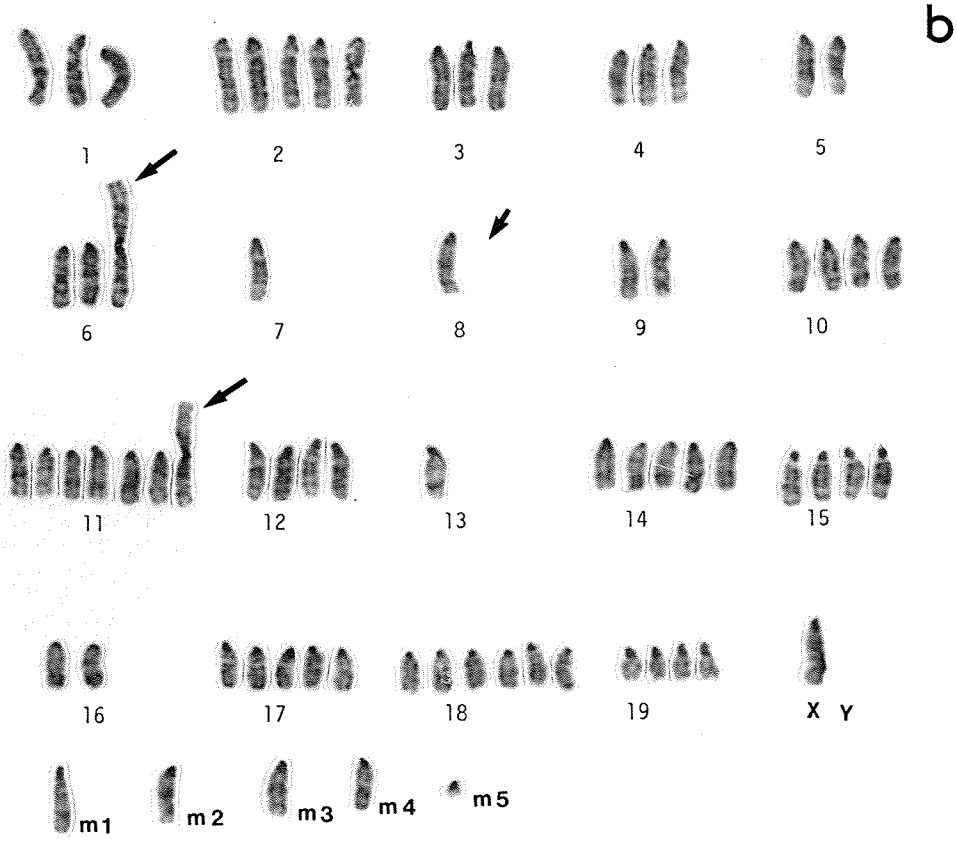


Figure 9

a: Representative chromosome spread of C2. 71, X, +1, +2, +2, +2, +3, +4, +6, t(6;7), -7, -8, +10, +10, +11, +11, +11, +11, +11, +12, +12, -13, +14, +14, +14, +15, +15, +17, +17, +17, +18, +18, +18, +18, +19, +19, -Y, +M1, +M2, +M3, +M4, +M5.
b: Karyotype from a
Arrows indicates chromosome 8. The large short arrow shows one copy of chromosome 8 deletion. Large long arrows indicate translocations.


a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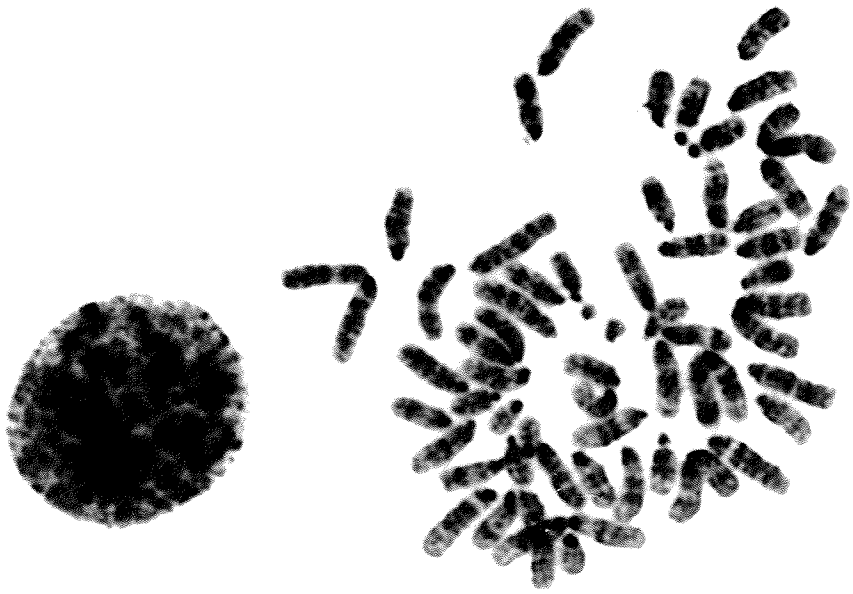
m3
m4
m5

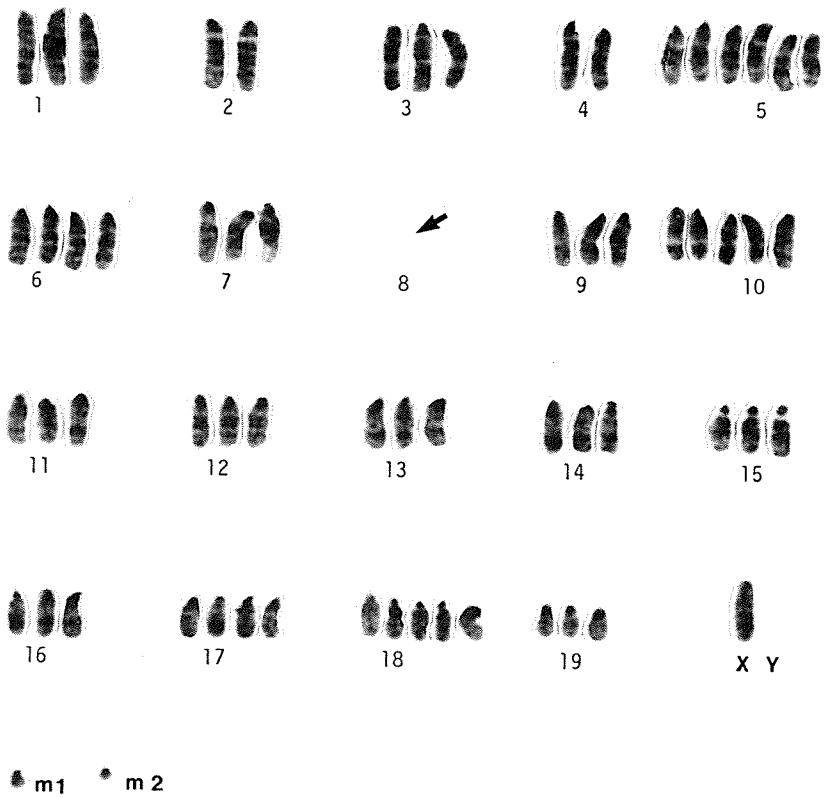
Figure 10

a: Representative chromosome spread of C3. 64, X, +1, +3, +5, +5, +5, +5, +6, +6, -8, -8, +9, +10, +10, +10, +11, +12, +13, +14, +15, +16, +17, +17, +18, +18, +18, +19, -Y, +M1, +M2.
b: Karyotype from a
Arrow indicates nullisomy of chromosome 8.

a



b



10T½*RAS*

The chromosome number varied between 51-114. Deletions, amplifications, and translocations were observed. Monosomy and nullisomy of chromosome 8 were identified. The representative karyotype of 10T½*RAS* is shown in Figure 11.

The cytogenetic data described above demonstrate that there are numerous changes in chromosome structure and numbers in 10T½ cell lines, but most of these changes are not consistently observed in each cell line. The notable exception was chromosome 8, where, fragment inversion and deletion, or whole copy deletion, are observed consistently and only in metastatic cell lines. We therefore hypothesize that chromosome 8 mutation or deletion is associated with metastatic progression.

IV. 2. Southern Blot Analysis

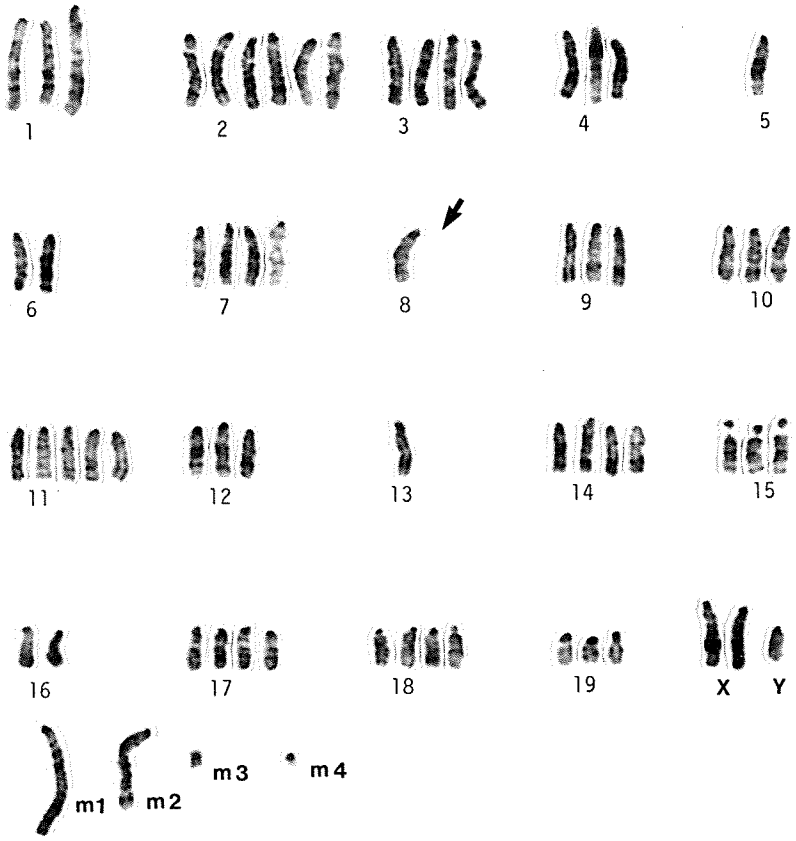
Many studies have demonstrated deletions in specific chromosomal regions that have been suggested to be responsible for development of human cancers. Mutations of specific genes located in specific chromosomal regions and elimination of their normal alleles are considered to be involved in carcinogenesis or tumor progression. From cytogenetic analysis of 10T½ transformed cell lines, alterations involving chromosome 8 have been constantly observed in metastatic cell lines. The next question we posed was whether there are any gene deletions or rearrangements on chromosome 8 in our 10T½

Figure 11

a: Representative chromosome spread of 10T½*ras*. 66, XXY, +1, +2, +2, +2, +2, +3, +3, +4, -5, +7, +7, -8, +9, +10, +11, +11, +11, +12, -13, +14, +14, +15, +17, +17, +18, +18, +19, +M1, +M2, +M3,+M4.
b: Karyotype from a
Arrows indicate chromosome 8. Large short arrow shows one copy deletion of chromosome 8.

a

b



transformed cell lines? If so, are those deletions or rearrangements correlated with transformation, tumorigenesis or metastasis? To examine these questions, further Southern blot hybridizations were performed with polymorphic DNA markers localized on mouse chromosome 8 or the homologous region of human chromosome 16 (Table 8).

Mt-1

Ten micrograms of genomic DNA were digested with the restriction enzyme BglII, and analyzed by Southern blot hybridization which was performed with metallothionein-1 (Mt-1) cDNA. The hybridization bands are shown in Figure 12. All of the cell lines have the same bands and similar intensity except 10T½*RAS* (lane G). In order to determine whether these darker bands are due to gene amplification or due to over-loaded DNA, a control experiment was carried out by hybridizing the same filter with ribonucleotide reductase M2 polypeptide (Rrm2) cDNA which is located on mouse chromosome 12 (Searle et al., 1989). The result of the control is shown in Figure 13. The optical intensity of the bands in Figure 12 (Mt-1) and Figure 13 (M2) was read with a densitometer, and the ratio of the densitometric readings of Mt-1 to that of M2 was calculated for each cell line. Since lane A was the control cell line (10T½), the Mt-1/M2 ratios of the other lanes were compared with that of lane A and the relative ratio computed. Table 11 tabulates the Mt-1 and M2 readings, the Mt-l/M2 ratio and the relative ratio for each cell line. As can be seen, none of the other lanes had a relative ratio of exactly 1.00. Most were greater than 1.00 but two (lane I & J) were less than

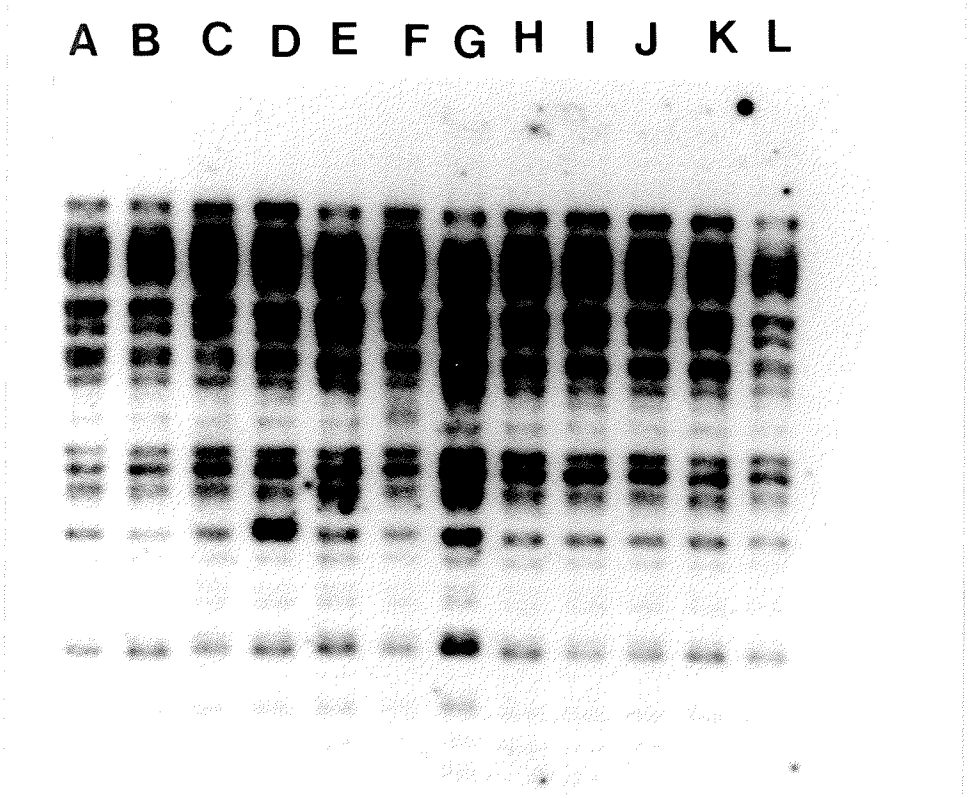


Figure 12

Southern blot analysis of Mt-1. Genomic DNA was digested to completion with the restriction enzyme BglII and run beside DNA size markers (1Kb DNA ladder obtained from BRL). Lane (A) is the 10T½, lane (B) is M4R4, lane (C) is MRPD, lane (D) is C1, lane (E) is C2, lane (F) is C3, lane (G) is 10T½*ras*, lane (H) is NR4, lane (I) is NR3, lane (J) is RHMRA, lane (K) is RHMRB and lane (L) is RHMRC.

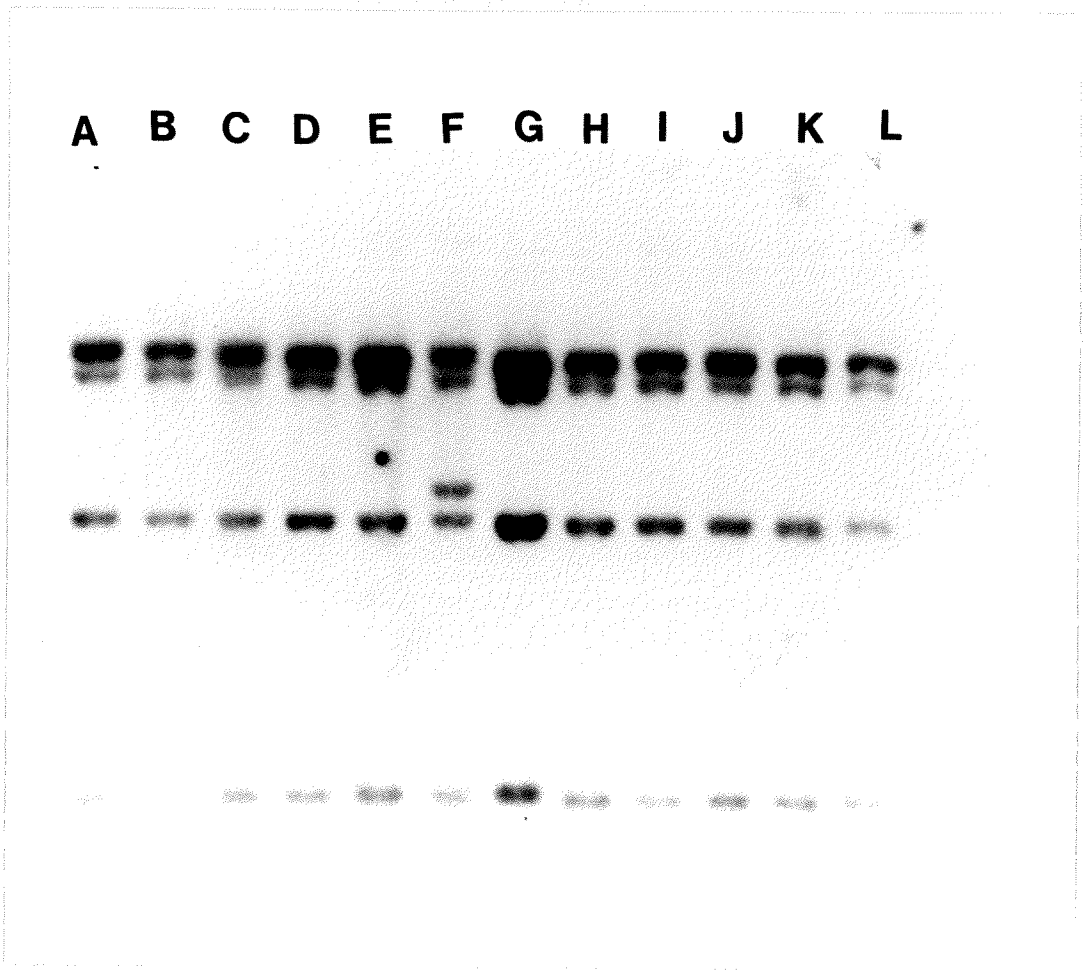


Figure 13

The same blot of figure 12 re-probed with M2 to show the DNA loading in all lanes. 10T½*ras* (lane G) is overloaded.

Table 11: Densitometric Results From Mt-1 or M2 Hybridization Experiments

Probe	Cell lines											
	A	B	C	D	E	F	G	H	I	J	K	L
Mt-1[a]	0.83	1.01	0.90	1.30	1.43	0.81	2.37	1.17	0.84	0.83	0.87	0.63
M2[a]	5.68	4.18	5.43	7.78	7.83	4.43	11.16	7.33	7.16	6.13	5.84	2.71
Mt-1 / M2	0.15	0.24	0.17	0.17	0.18	0.18	0.21	0.16	0.12	0.14	0.15	0.23
R.R.[b]	1.00	1.60	1.13	1.13	1.20	1.20	1.40	1.06	0.80	0.93	1.00	1.53

a: Densitometric readings for each cell lines
b: Relative ratios to control lane A

Lane (A) is the 10T½, lane (B) is M4R4, lane (C) is MRPD, lane (D) is C1, lane (E) is C2, lane (F) is C3, lane (G) is 10T½*ras*, lane (H) is NR4, lane (I) is NR3, lane (J) is RHMRA, lane (K) is RHMRB, and lane (L) is RHMRC.

1.00, but all the deviations were small. The variance is probably due to the fact that the experiments with Mt-1 and M2 were performed separately, and the stochastic nature of these experiments produced variations. It could also be possible that some cell lines had a slightly higher percentage of certain genes. The densitometer also had random fluctuations in its readings. Since the relative ratios are fairly close to 1.00, we conclude that there is neither significant gene deletion nor amplification in the Mt-1 locus of the 10T½ transformed cells as determined by Southern blot hybridization.

Um

Ten micrograms of genomic DNA was digested with restriction enzyme EcoRl, and Southern blot hybridization was carried out with uvomorulin cDNA. The hybridization result is shown in Figure 14. All of the 10T½ cell lines exhibited two bands. Gene amplification was detected in the 10T½*ras* line.

HP

Haptoglobin cDNA was used in hybridization experiments by reprobing the filter used to examine Mt-1 hybridizations (Figure 12). No rearrangements bands were detected by Southern blot hybridization at the Hp locus (Figure 15). Lane G had higher intensity bands. The optical intensity of the bands in Figure 15 was read with a densitometer and compared with the optical intensity data from the control (Figure 13). The results were

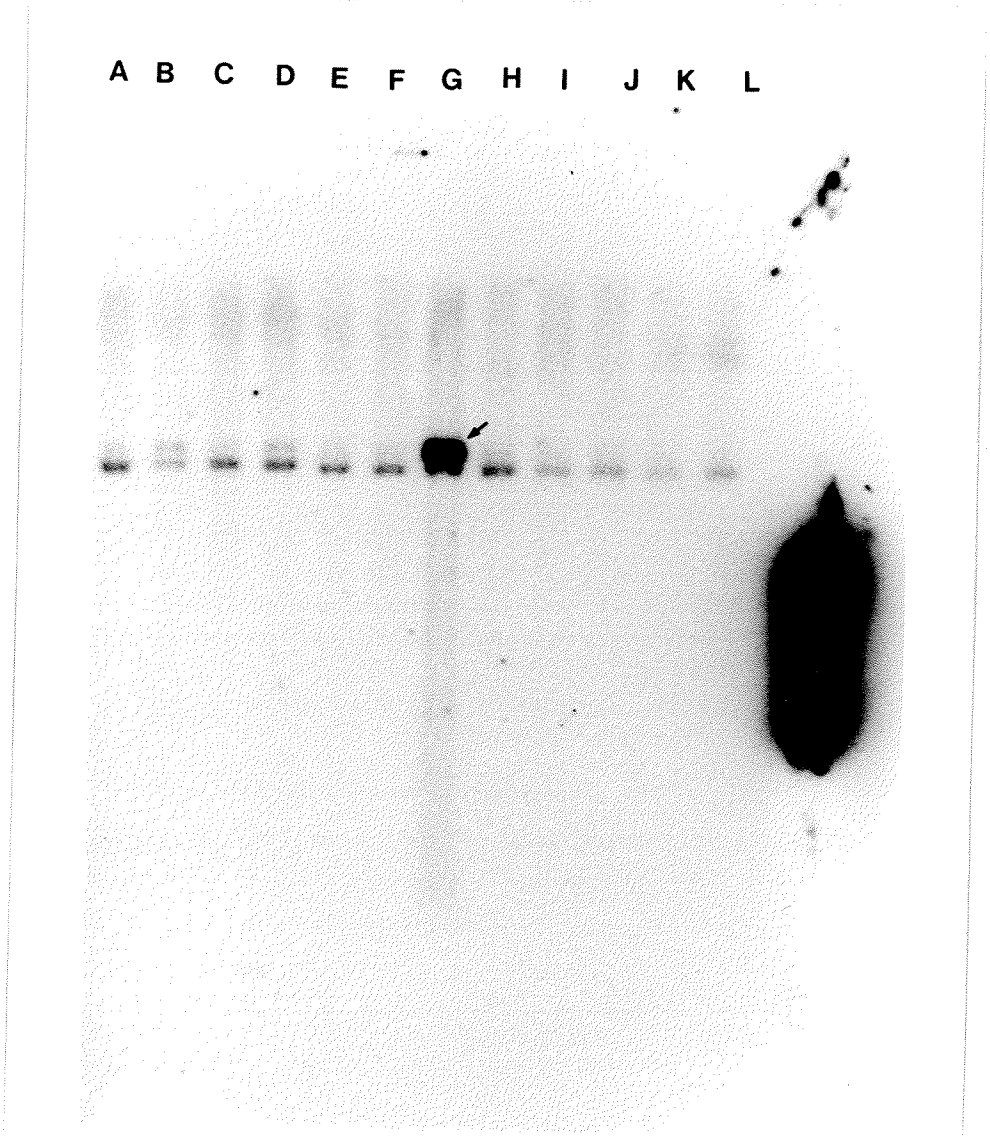


Figure 14

Southern blot analysis of Um. Genomic DNA was digested to completion with the restriction enzyme EcoR1 and run beside DNA size markers (1Kb DNA ladder obtained from BRL). Lane (A) is the 10T½, lane (B) is M4R4, lane (C) is MRPD, lane (D) is C1, lane (E) is C2, lane (F) is C3, lane (G) is 10T½*ras*, lane (H) is NR4, lane (I) is NR3, lane (J) is RHMRA, lane (K) is RHMRB and lane (L) is RHMRC.
Arrow indicates the top band of 10T½*ras* which is amplified.

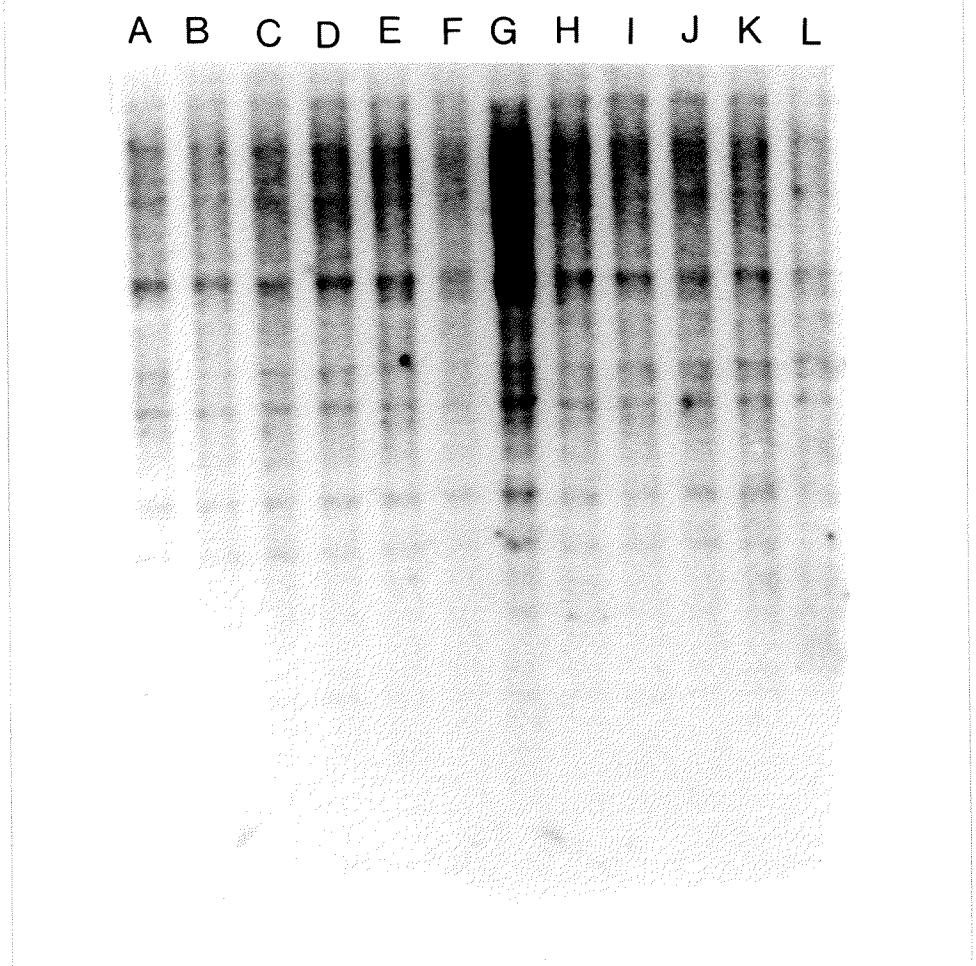


Figure 15

Southern blot analysis of HP. The blot of figure 13 was stripped and re-probed with HP. Lane (A) is the 10T½, lane (B) is M4R4, lane (C) is MRPD, lane (D) is C1, lane (E) is C2, lane (F) is C3, lane (G) is 10T½*ras*, lane (H) is NR4, lane (I) is NR3, lane (J) is RHMRA, lane (K) is RHMRB and lane (L) is RHMRC.

summarized in Table 12. There is no significant difference in the relative ratio. A small difference in relative ratio can be caused by the factors discussed in the Mt-1 section. Therefore, there is no obvious indication of gene deletion or amplification at the Hp locus of the 10T½ transformed cells as determined by Southern blot hybridization.

Ctrb

Chymotrypsin B cDNA was used in hybridizations experiments by reprobing the filter used to examine Mt-1 and Hp cDNA hybridizations. An extra band was observed with DNA from C3 cells (Figure 16, arrow indicated). The other lanes had two bands and showed no rearrangement bands. optical intensity analysis was performed on the two bands of each lane. The densitometric readings of the top and bottom bands of each lane were compared and the ratio calculated (Table 13). Using lane A (which was the 10T½ cell line used as control) as reference, the relative ratios of the other lanes were also calculated. The relative ratio of lane A (l0T½) was, of course, 1.00. With the exception of lane C (MRPD), the relative ratios of all other lanes were less than 1.00. The four metastatic cell lines, C2 (lane E), 10T½*ras* (G), NR4 (lane H), and RHMRC (lane L) had the lowest four ratios.

Tat

Tyrosine aminotransferase cDNA was used in hybridization experiments by reprobing

Table 12: Densitometric Results From Hp or M2 Hybridization Experiments

Probe	Cell lines											
	A	B	C	D	E	F	G	H	I	J	K	L
Hp[a]	2.32	1.95	2.59	3.06	3.21	2.12	6.02	3.16	2.85	2.76	2.90	1.99
M2[a]	5.68	4.18	5.43	7.78	7.83	4.43	11.16	7.33	7.16	6.13	5.84	2.71
Hp/M2 R.R.[b]	0.41	0.47	0.48	0.39	0.41	0.48	0.54	0.43	0.40	0.45	0.50	0.73
	1.00	1.15	1.17	0.95	1.00	1.17	1.32	1.05	0.98	1.10	1.22	1.78

a: Densitometric readings for each cell lines
b: Relative ratios to control lane A

Lane (A) is the 10T½, lane (B) is M4R4, lane (C) is MRPD, lane (D) is C1, lane (E) is C2, lane (F) is C3, lane (G) is 10T½*ras*, lane (H) is NR4, lane (I) is NR3, lane (J) is RHMRA, lane (K) is RHMRB, and lane (L) is RHMRC.

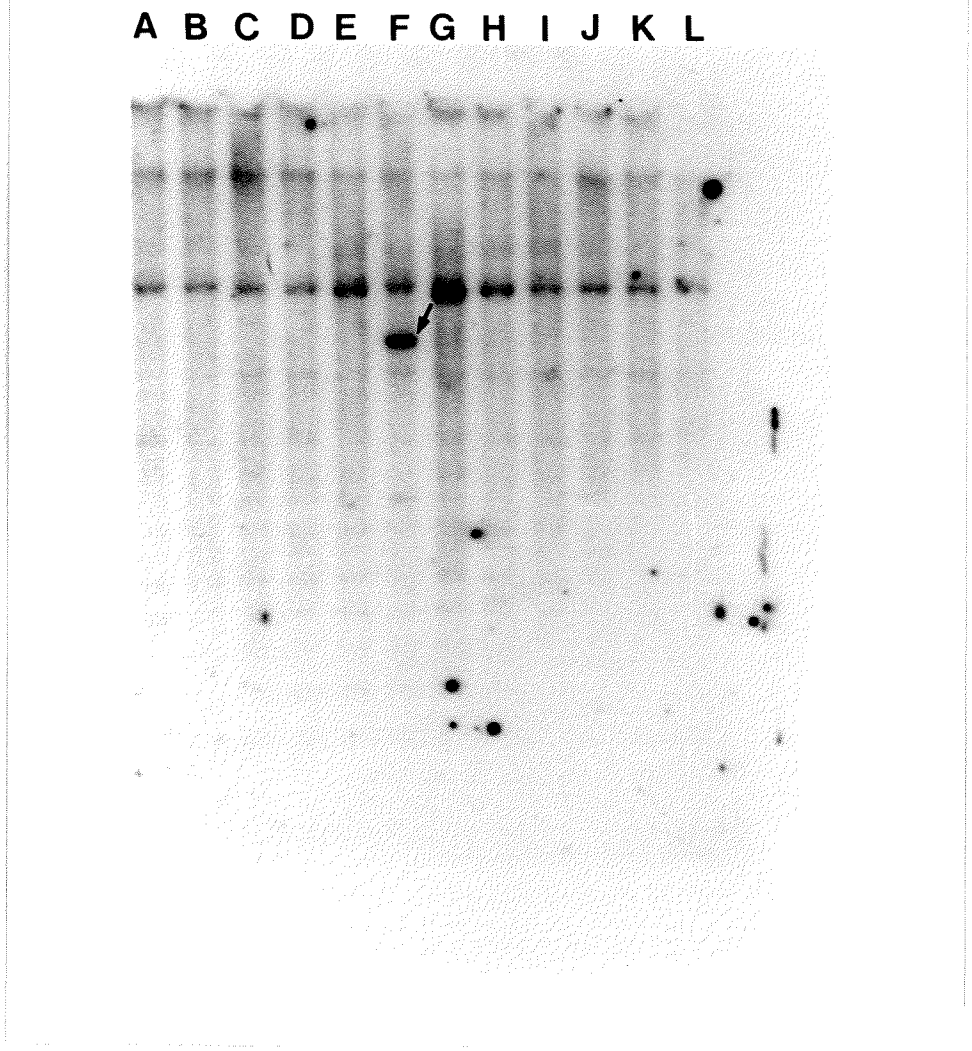


Figure 16

Southern blot analysis of Ctrb. The blot of Figure 13 was stripped and re-probed with Ctrb. Lane (A) is the 10T½, lane (B) is M4R4, lane (C) is MRPD, lane (D) is C1, lane (E) is C2, lane (F) is C3 with a extra band, lane (G) is 10T½*ras*, lane (H) is NR4, lane (I) is NR3, lane (J) is RHMRA, lane (K) is RHMRB and lane (L) is RHMRC.

Table 13: Densitometric Results From top and bottom bands of Ctrb Hybridization Experiments

Band	Cell lines											
	A	B	C	D	E	F	G	H	I	J	K	L
Top[a]	0.89	1.12	1.67	1.05	0.71	0.69	0.46	0.58	0.71	1.08	0.65	0.23
Bot.[a*]	0.57	0.95	0.89	0.75	1.59	1.10	2.89	1.32	1.15	0.87	0.96	0.58
Top / Bot.	1.56	1.17	1.88	1.40	0.45	0.63	0.16	0.44	0.62	1.24	0.68	0.40
R.R.[b]	1.00	0.75	1.21	0.90	0.29	0.40	0.10	0.28	0.40	0.79	0.44	0.26

a: Densitometric readings for each cell lines
b: Relative ratios to control lane A
*: Bot. = bottom

Lane (A) is the 10T½, lane (B) is M4R4, lane (C) is MRPD, lane (D) is C1, lane (E) is C2, lane (F) is C3, lane (G) is 10T½*ras*, lane (H) is NR4, lane (I) is NR3, lane (J) is RHMRA, lane (K) is RHMRB, and lane (L) is RHMRC.

the filter used to examine Mt-1 hybridization (Figure 17). No rearrangement bands were detected by Southern blot hybridization at the Tat locus. The higher intensity bands in lane G seem more not likely to be the result of gene amplification (Table 14).

Gnb-3

Ten microgram of genomic DNA were digested with enzyme HindIII, and Southern blot hybridization was performed with β-subunit of retinal transducin (Gnb-3) cDNA. The results of this hybridization showed one extra band with DNA from C3 cells. The extra band is identified by an arrow in Figure 18. No rearrangement bands were observed with other cell lines at the Gnb-3 locus.

Aprt

The Aprt genomic DNA probe is 3.1 kb in length (Dush et al., 1985, Turker, 1990) and contains five exons and six introns.

Aprt genomic DNA was used in hybridization experiments by reprobing the filter used to examine Mt-1, Tat, Hp, and Ctrb cDNA hybridizations. An extra band was detected with DNA from C3 cells (Figure 19). In order to test whether there is gene amplification in this locus, optical intensity analysis was performed. The intensity

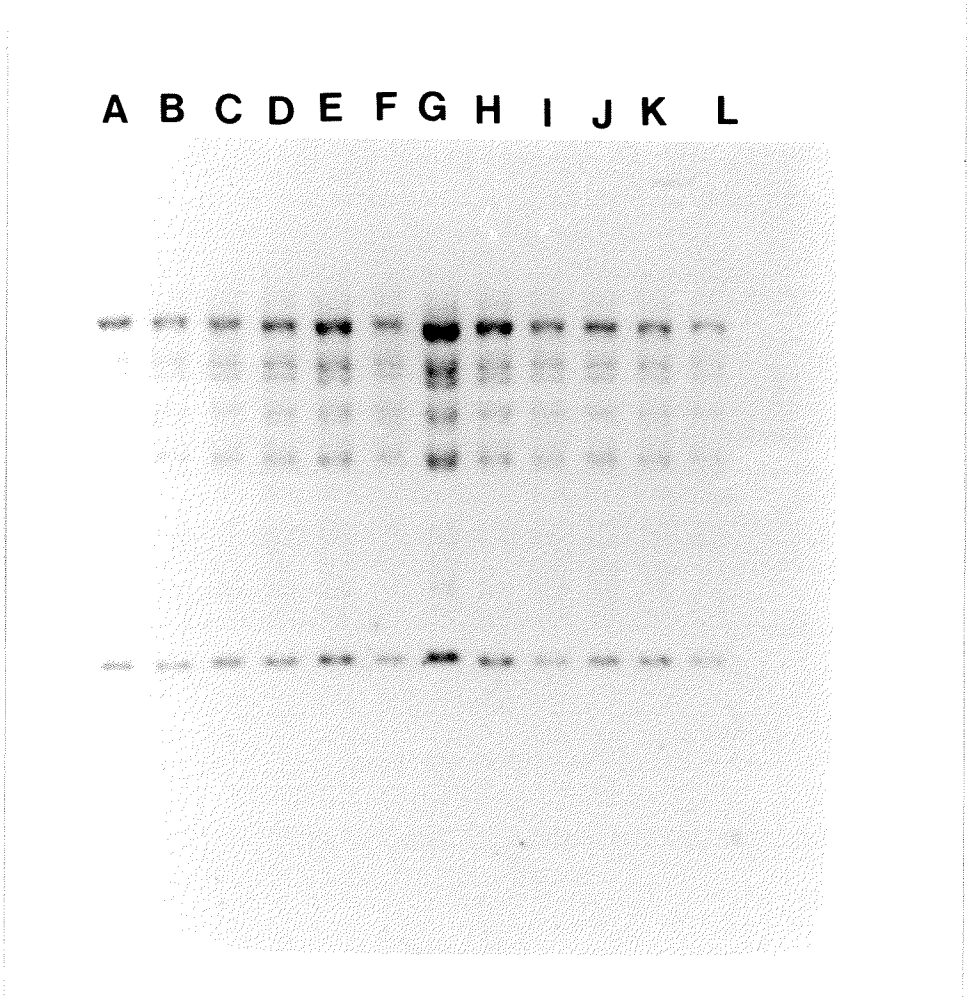


Figure 17

Southern blot analysis of Tat. The blot of Figure 13 was stripped and re-probed with Tat. Lane (A) is the 10T½, lane (B) is M4R4, lane (C) is MRPD, lane (D) is C1, lane (E) is C2, lane (F) is C3 with a extra band, lane (G) is 10T½*ras*, lane (H) is NR4, lane (I) is NR3, lane (J) is RHMRA, lane (K) is RHMRB and lane (L) is RHMRC.

Table 14: Densitometric Results From Tat or M2 Hybridization Experiments

Probe	Cell lines											
	A	B	C	D	E	F	G	H	I	J	K	L
Tat[a]	0.90	0.74	0.82	1.15	1.76	0.82	2.38	1.55	0.92	1.04	0.90	0.53
M2[a]	5.68	4.18	5.43	7.78	7.83	4.43	11.16	7.33	7.16	6.13	5.84	2.71
Tat / M2	0.16	0.18	0.15	0.15	0.22	0.19	0.21	0.20	0.13	0.17	0.15	0.20
R.R.[b]	1.00	1.13	0.94	0.94	1.37	1.20	1.31	1.25	0.81	1.06	0.94	1.25

a: Densitometric readings for each cell lines
b: Relative ratios to control lane A

Lane (A) is the 10T½, lane (B) is M4R4, lane (C) is MRPD, lane (D) is C1, lane (E) is C2, lane (F) is C3, lane (G) is 10T½*ras*, lane (H) is NR4, lane (I) is NR3, lane (J) is RHMRA, lane (K) is RHMRB, and lane (L) is RHMRC.

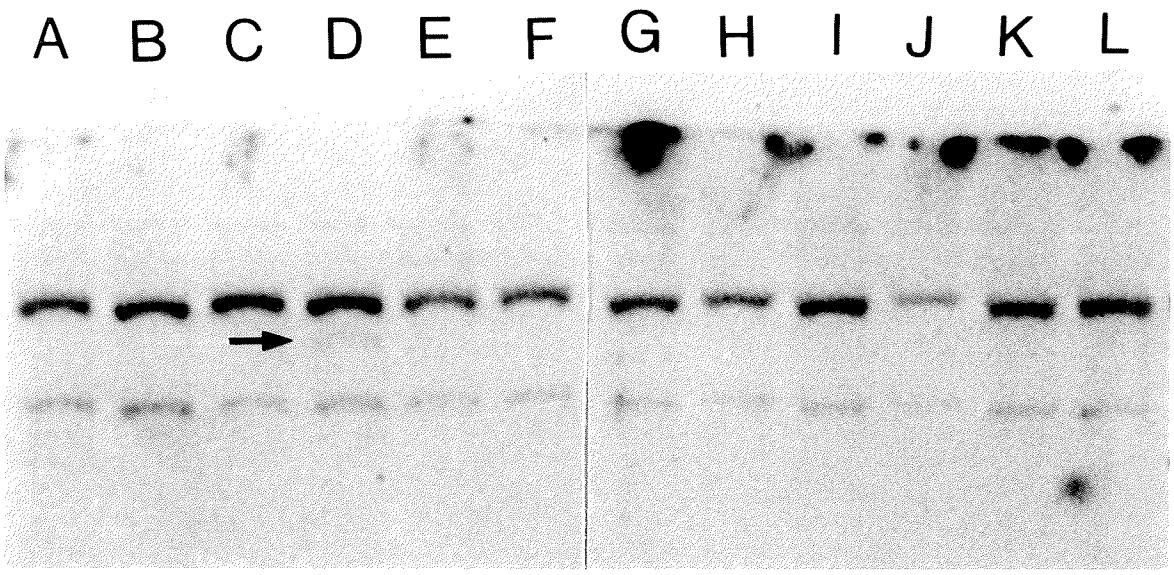


Figure 18

Southern blot analysis of Gnb-3. Genomic DNA was digested to completion with the restriction enzyme HindIII and run beside DNA size markers (1kb DNA ladder). Lane (A,H) are 10T½, lane (B) is C1, lane (C) is C2, lane (D) is C3, lane (E) is NR3, lane (F) is NR4, lane (G) is Svx-1, lane (I) is myc1.4, lane (J) is myc1.1, lane (K) is MRPD, lane (L) is 10T½*ras*,
lane (M) is M4R4, lane (N) is RHMRA, lane (O) is RHMRB and lane (P) is RHMRC. Arrows indicate extra bands.

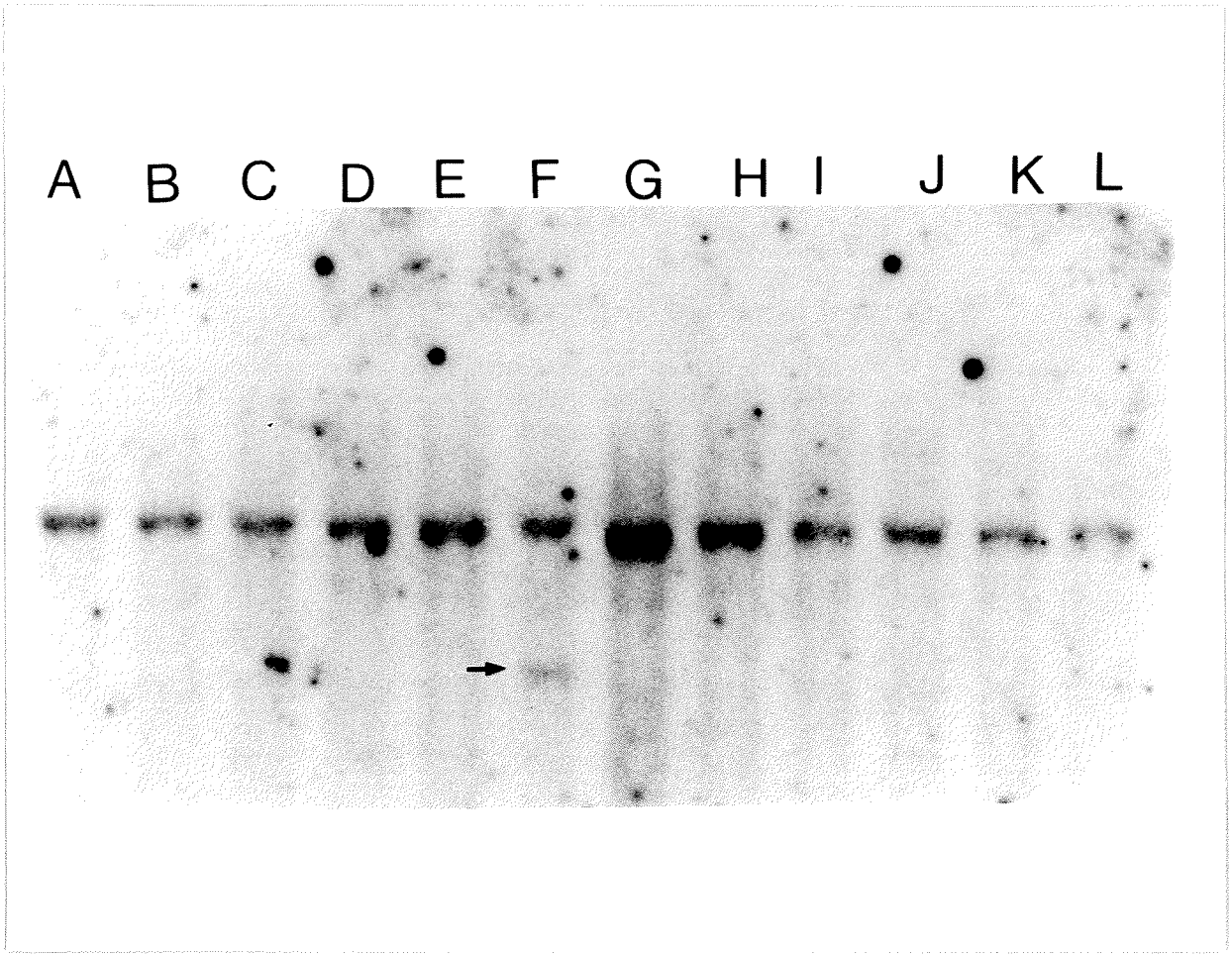


Figure 19

Southern blot analysis of Aprt. The blot of Figure 12 was stripped and re-probed with Aprt. Lane (A) is the 10T½, lane (B) is M4R4, lane (C) is MRPD, lane (D) is C1, lane (E) is C2, lane (F) is C3 with a extra band, lane (G) is 10T½*ras*, lane (H) is NR4, lane (I) is NR3, lane (J) is RHMRA, lane (K) is RHMRB and lane (L) is RHMRC.

readings from Figure 19 were compared to the control readings from Figure 13. The result was presented in Table 15. The small difference in relative ratio may be caused by the factors discussed in the Mt-1 section.

Mtv-21

Ten micrograms of genomic DNA were digested with enzyme EcoRl, and Southern blot hybridization was carried out with Mtv-21 cDNA. The results of hybridization studies showed that every cell line had the same pattern of two bands (Figure 20).

V. Discussion

Chromosomal Anomaly and Tumor Progression

The concept that a chromosomal anomaly is consistently associated with some types of human cancer is a common theme. Chromosomal rearrangement is an inherent feature of tumor cells, and increasing evidence indicates that such an occurrence may play a role in the initiation and progression of neoplasia (Lebeau et al., 1984; Yunis, 1987). Many studies at the cytogenetic and molecular levels have shown that cells from a variety of tumors contain mutations and chromosomal rearrangements, which often involve activation of cellular protooncogenes and inactivation of tumor suppressor genes (Bishop,

Table 15: Densitometric Results From Aprt or M2 Hybridization Experiments

Probe	Cell lines											
	A	B	C	D	E	F	G	H	I	J	K	L
Aprt[a]	2.09	2.09	2.72	3.84	4.87	3.06	5.04	4.80	2.69	2.24	1.58	1.20
M2[a]	5.68	4.18	5.43	7.78	7.83	4.43	11.16	7.33	7.16	6.13	5.84	2.71
Aprt / M2	0.37	0.50	0.50	0.49	0.62	0.69	0.45	0.61	0.38	0.37	0.27	0.44
R.R.[b]	1.00	1.35	1.35	1.32	1.68	1.86	1.22	1.65	1.03	1.00	0.73	1.19

a: Densitometric readings for each cell lines
b: Relative ratios to control lane A

Lane (A) is the 10T½, lane (B) is M4R4, lane (C) is MRPD, lane (D) is C1, lane (E) is C2, lane (F) is C3, lane (G) is 10T½*ras*, lane (H) is NR4, lane (I) is NR3, lane (J) is RHMRA, lane (K) is RHMRB, and lane (L) is RHMRC.

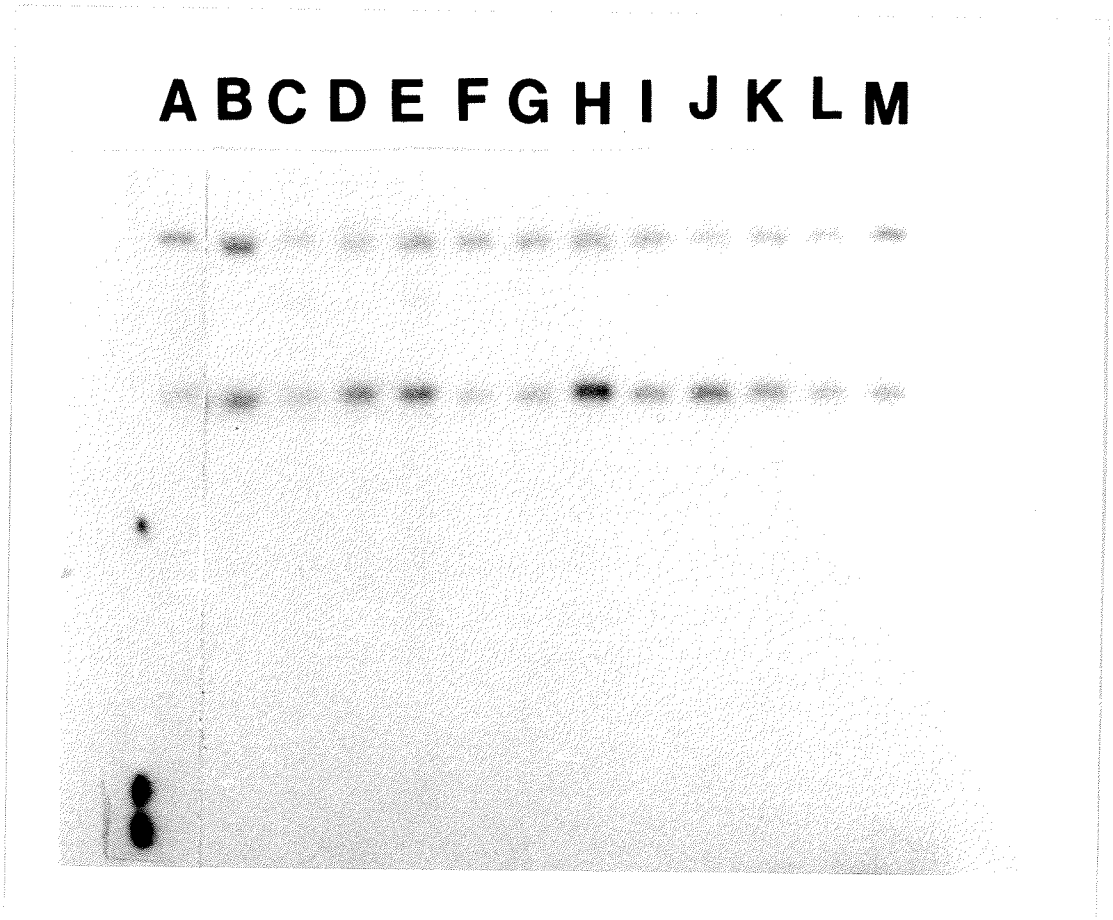


Figure 20

Southern blot analysis of Mtv-21. Genomic DNA was digested to completion with the restriction enzyme EcoR1 and run beside DNA size markers (1Kb DNA ladder obtained from BRL). Lane (A) is C3H lung DNA (obtained from Dr. M. Mowat's Lab. Manitoba Institute of Cell Biology), lane (B) is the 10T½, lane (C) is M4R4, lane (D) is MRPD, lane (E) is C1, lane (F) is C2, lane (G) is C3, lane (H) is 10T½*ras*, lane (I) is NR4, lane (J) is NR3, lane (K) is RHMRA, lane (L) is RHMRB and lane (M) is RHMRC.

1987; Mitelman, 1988). It is likely that the progression from normal to the metastatic phenotype in a tumor is a controlled and ordered process as described by Vogelstein in the case of colon cancer (Fearon et al., 1990). The progression from normal epithelium to metastasis in colon cancer involves a chromosome 5 gene alteration, *ras* gene mutation, chromosome 18 loss, chromosome 17 loss and other chromosome losses. Consistent chromosome loss or chromosome fragment deletion may indicate that the deleted chromosome and fragment may contain recessive genes which are capable of suppressing tumorigenesis. Cytogenetics studies have lead to the identification and cloning of the tumor suppressor genes or the candidate of tumor suppressors in retinoblastoma, Wilms' tumor and colon carcinoma (Friend et al., 1986; Gessler et al., 1990; Fearon et al., 1990).

Tumorigenic and metastatic assays performed with 10T½ murine fibroblast transformed cell lines containing *ras* and/or *myc* indicated that cooperative interaction of *ras* and *myc* is sufficient for benign tumor formation, but is not sufficient on their own for expression of a metastatic phenotype (Egan et al., 1987a; 1987b; Ph.D thesis, 1989). These observations lead us to hypothesize that a recessive event is required for induction of metastatic behaviour. To test the hypothesis, karyotypic analysis was carried out in this study.

The normal mouse chromosome number is 2n=40. Both numeric and structural chromosome aberrations were observed in all of the 10T½ cell lines. The chromosome

numbers in 10T½ cell lines were more than 40, ranging from 43 to 164. Anomalies in chromosome numbers may be caused by nondisjunction, loss of chromosomes and multiploidy. It is most likely that the numeric aberrations in 10T½ cell lines were caused by a combination of these three alterations. With an increased dosage (e.g. extra copies of chromosomes), cells may acquire a growth advantage which allows them to progress towards malignancy (Kaprowski et al., 1985).

The aberrations of chromosomal structure in 10T½ transformed cell lines include translocations (Robertsonian translocations), inversions, deletions (whole copy of a chromosome and fragments), and marker chromosomes.

Translocations have typically affected either the expression or the biochemical function of proto-oncogenes. The breakpoints where portions of two chromosomes are joined together by translocations frequently lie within or adjacent to proto-oncogenes (Bishop, 1987). Several cases of translocations affecting proto-oncogenes are already known from the study of retroviruses (Varmus, 1984; Bishop, 1987), Philadelphia translocation and Burkitt's lymphoma (literature review section). From this study, it was observed that translocations in MRPD, C1, C2 cell lines frequently involve chromosome 7 and 11. *Erb*A (avia erythroblastosis oncogene A) is located on chromosome 11. The H-*ras* and K-*ras* proto-oncogenes are located on chromosome 7 (Searle et al., 1989). The coincidence may directly or indirectly contribute to tumorigenesis. Since in this study we concentrated on determining whether or not tumor suppressor mutations or deletions may

have occurred and not on oncogene activation, the calculation of translocation rates was not done.

Although paracentric inversions were observed in every 10T½ transformed cell line, most of the inversions did not consistently occur on a specific chromosome or specific chromosomal fragment. Only specific inversions of chromosome 8, inv8(Dl;El), in the NR4 cell line have been observed.

Marker chromosomes existed in each 10T½ transformed cell line, but all of the marker chromosomes had a different appearance from each other. They were probably derived from chromosome fragment deletions where the centromere is retained, and the chromosome with the deleted fragment might further translocate to other chromosomes. After deletion and translocation, the resulting chromosomal composition was difficult to identify.

Loss of chromosomes and deleted fragments were observed in each of the 10T½ cell lines. The consistent loss of chromosome 8, monosomic and nullisomic chromosome 8, were observed in the metastatic cell lines Cl, C2, C3, and 10T½*ras*. Loss of whole copies of chromosome(s) may be caused by various factors: (a) nondisjunction: chromosomes that should normally be separated during cell division remain together and are transported in anaphase to one pole. This may occur at mitotic cell division and lead to trisomic and monosomic cells; (b) loss of single chromosomes, presumably due to

anaphase lagging; (c) polyploidization: the whole genome is present more than twice in a cell (Vogel et al., 1979). It seems more likely in 10T½ cells that the chromosome is lost by nondisjunction and anaphase lagging after the polyploidization.

While chromosome fragment deletion was frequently observed in 10T½ transformed cell lines, a chromosome 8C fragment in C2, C3 and 10T½*ras*, or 8E fragment in Cl was consistently deleted. The mechanism of fragment deletion may be caused by chromosomal breakage (Vogel et al., 1979). The breakage produces a shorter chromosome with a centromere and an acentric fragment. This acentric fragment, lacking a centromere, has a high risk of being lost during the subsequent mitosis (Vogel et al., 1979).

Consistent deletions of genetic materials may signal the existence of genetic elements involved in tumorigenesis and metastasis (Bishop, 1987; Literature review section). The metastatic cell lines NR4, Cl, C2, C3 and 10T½*ras* have constant chromosome 8 inversions and deletions in the region of C-E. This C-E region is homologous with human chromosome 16ql2.1-16q24.3 (figure 2). Based on the tumorigenic and metastatic assays performed with 10T½ transformed cell lines (Egan et al., 1987a; 1987b), and the cytogenetic analysis carried out in this study, it is possible that the mouse chromosome 8, especially 8C-E region, may contain a gene or genes, the deletion of which is important to metastatic progression.

From studies of somatic cell hybrids produced from normal mouse fibroblasts and

tumorigenic mouse cell lines, chromosome 4 has been associated with tumor suppression in a number of mouse malignant cell lines (Miller et al., 1983; Evans et al., 1982). Chromosomal analysis in the present study revealed that the non-metastatic cell lines MRPD and M4R4 have four copies of chromosome 4, while the metastatic cell lines NR4, Cl, and C3 have two copies of chromosome 4. The lower copies or lower gene dosage of chromosome 4 may be one of the factors which contribute to metastatic development.

Gene Rearrangement and Metastasis

Mutations of specific genes located in specific chromosomal regions and the elimination of their normal alleles can play an important role in carcinogenesis and tumor progression (Knudson, 1985). Therefore, it is important to map the minimal chromosome loss region and to identify mutations of genes on chromosome 8. Metallothionein-1 (Mt-1) (Glanville et al., 1981), Uvomorulin (Um) (Eistetter et al., 1988), Haptoglobin (Hp) (Ceci et al., 1990), Chymotrypsin B (Ctrb) (Callen et al., 1988), Tyrosine aminotransferase (Tat) (Muller et al., 1985), β-subunit of retinal transducin (Gnb-3) (Danciger et al., 1990), Adenosine phosphoribosyl transferase (Aprt) (Sikela et al., 1983) and Mammary tumor virus-21 (Mtv21) genes (Peters et al., 1986), which are located on chromosome 8 and human chromosome 16q, were collected and used as probes for our 10T½ transformed genomic DNA by Southern blot hybridization.

Loss of heterozygosity (LOH) at the Mt-1 locus was detected in human hepatocellular

carcinoma and human central nervous system primitive neuroectodermal tumors (Tsuda et al., 1990; Thomas et al., 1991). Mt-1 is located on mouse 8A4-C3 and human 16q22.1-q21 (Searle et al., 1989). Southern blot hybridization analysis in the present study demonstrated that there was no gene rearrangement at this locus in any of the 10T½ cell lines.

Um maps in mouse 8C3-El and human 16q22.2, thus the locus is within the region of lose in Wilms' tumors and proximal to the common loss regions in prostate and hepatocellular carcinoma (Maw et al., 1992; Tsuda et al., 1990). Um encodes a cell adhesion molecule. Since cell adhesion molecules may act as tumor suppressors, Maw et al.(1992) suggested that um was a potential candidate gene for the Wilms' tumor suppressor gene by a mechanism that prevented tumor invasion. However, when Southern blot analysis was performed with DNA prepared from 37 Wilms' tumor patients probed with um cDNA, no rearrangement was found (Maw et al., 1992). In the present study, Southern blot hybridization with DNA prepared from 10T½ cell lines probed with um cDNA revealed that um was amplified in the 10T½*ras* metastatic cell line (Figure 14), while no rearrangements were observed in any of the other 10T½ cell lines. Since 10T½*ras* is a high metastatic cell line (Table 10), the amplification of um in 10T½*ras* may indicate that a nearby gene is involved in advanced metastasis.

Hp, Tat and Ctrb map to mouse chromosome 8C3-El (Reeders et al., 1991; Muller et al., 1985; Scherer et al., 1989). LOH at those three loci in hepatocellular carcinomas

and LOH at Hp and Tat in prostate and neuroectodermal tumors has been reported (Tsuda et al., 1990; Carter et al., 1990, Thomas et al., 1991). The 10T½ cell lines did not show gene rearrangements involving the linked Hp and Tat loci (Figures 2, 15, 17), but the Ctrb gene, which is approximately 800 kilobase pairs away from Tat in human chromosome 16 (Tsuda et al., 1990), was rearranged in the C3 cell line (Figure 16). The gene copies were most reduced in the metastatic cell lines of C2, 10T½*ras*, NR4 and RHMRC.

Gnb-3 is located at 8El-ter (Ceci, 1991). Preliminary Southern blot analysis detected that C3, NR3, and NR4 cell lines had rearrangement bands at this locus. Both the rearrangement bands in NR3 and NR4 appeared with lower intensity compared with that in C3 (data is not shown). In order to determine whether these bands in NR3 and NR4 were specific bands or non-specific bands, the Southern blot hybridization was repeated by using the same restriction enzyme (HindIII) to digest the genomic DNA and then probing the Gnb-3 cDNA. The repeated result was shown in Figure 18. There was no rearrangement bands in NR3 and NR4. Therefore, the lower intensity bands in NR3 and NR4 in the first Southern blot analysis may have been caused by the incomplete restriction enzyme digestion of the genomic DNA.

Aprt is located proximal to Gnb-3 at 8El-ter, human 16q24.2-qter (Reeders et al., 1991). southern blot hybridization study with DNA from 10T½ cell lines using Aprt as 19)

In all the Southern blot analysis data, the C3 cell line showed rearrangement bands at Ctrb, Gnb-3 and Aprt loci; while cell lines NR4, C2, 10T½*ras* and RHMRC showed lower dosage at the Ctrb locus. C3 is the most metastatic cell line next to the hybrid line of RHMRC (Table 6), hence it is suggested that the gene rearrangement at Ctrb, Gnb-3 and Aprt loci is associated with advanced metastatic progression. It is hypothesized that the region between the Ctrb locus and the Aprt locus on mouse chromosome 8 or human chromosome 16q22.3-qter region may contain one or more genes involved in advanced metastatic progression. This suggestion is consistent with the reports of human hepatocellular carcinoma (HHC) and breast cancer (Tsuda et al., 1990; Sato et al., 1990). Allele losses on human chromosome 16q between Tat and Aprt occurs at a late stage of HCC progression (Tsuda et al., 1990), and at advanced lymph node metastasis in breast cancer (Sato et al., 1990).

Cytogenetic data showed that the metastatic cell lines NR4, C1, C2, C3 and 10T½*ras* had consistent chromosome 8 deletions and inversions; hence, it would seem logical to expect gene dosages at loci on mouse chromosome 8 to be reduced in these metastatic cell lines. However, Southern blot analysis did not show any reduction in the intensity of hybridization bands with DNA prepared from these cell lines when compared to DNA obtained from non-metastatic cell lines. This inconsistency is probably due to the fact that the missing segments of chromosome 8 were still present elsewhere in the genome in various combinations with other chromosomes through translocation and recombination, hence the genes in the cell were not missing. These new combinations of

segments of chromosome 8 with other chromosomes are very difficult to identify using metaphase G-chromosomal banding techniques. A high resolution technique may be able to identify the combined chromosomes (Sawyer et al, 1986).

Gnb-3, Aprt and metastasis

Since the higher metastatic cell line C3 showed rearrangements at the Aprt locus or the Gnb-3 locus, it raises the question of whether there is a possibility that the Aprt or Gnb-3 genes themselves may contribute to metastatic progression.

The Gnb-3 gene encodes the ß-subunit of retinal rod transducin (Danciger et al., 1989). The gene for the α-subunit of retinal rod transducin maps to mouse chromosome 9 (Danciger et al., 1989). The retinal rod transducin is a member of a family of heterotrimeric signal-coupling proteins known as G proteins which act as intermediaries between activated transmembrane receptors and cellular effectors (Stryer, 1986). Retinal rod transducin can be activated by a signal from retinal rod photoreceptor cells. The activated retinal rod transducin then interacts with cGMP-phosphodiesterase (cGMP-PDE) to degrade cGMP (Farber et al., 1986). A defect in any of the subunits of transducin could result in the loss of transducin's ability to activate cGMP-PDE. Then a deficient cGMP-PDE activity would result in high levels of cGMP (Danciger et al., 1990). cGMP is an intracellular messenger which is required to open Na^+ channels on rod plasma membrane. Higher levels of cGMP can keep the Na^+ channels of the rod plasma

membrane open. The channels through which Na^+ enters are permeable to other cations, including Ca^{2+}. Therefore, maintaining Na^+ channels open results in increasing influx of Ca^{2+}. Protein kinase C (C-kinase) is Ca^{2+}-dependent. It seems likely that C-kinase is normally activated by the cooperative effects of diacylglycerol and an increase in cytosolic Ca^{2+} (Alberts et al, 1989). Evidence shows that C-kinase is involved with activation of many oncogenes (Cantley et al., 1991). For instance, activation of C-kinase can induce dephosphorylation of a critical threonine residue in the *c-jun* onco-protein that allows *c-jun* protein to associate with the TPA response element (Boyle et al., 1991), and thereby activate transcription. Also, c-kinase can activate the *ras* oncogene by preventing its downregulation by *ras* GAP (Downward et al., 1990).

Although a possible relationship between the Gnb-3 gene and activation of oncogenes has been hypothesized, the mechanisms involved are not known completely. However, it is possible that Gnb-3 may play a role in the metastatic progression of cells by regulating cGMP levels, which would significantly affect signal transduction pathways. The result obtained in this study and the relationship between Gnb-3, cGMP levels and oncogene activation observed in other studies (Danciger et al., 1990; Cantley et al., 1991; Boyle et al., 1991; Downward et al., 1990) suggest that Gnb-3 could be considered as a candidate for advanced metastatic suppressor gene function. Further investigations to test this idea may be worthwhile.

Aprt and Tumorigenesis

The Aprt locus encodes the enzyme responsible for adenine salvage in mammalian cells. Several groups have focused extensively on Aprt mutations in Chinese hamster ovary (CHO) cells and found the mutations to include base pair substitution (Drobetsky et al., 1987; Grosovsky et al., 1988; Nalbantoglu et al., 1987), frame shift (Grosovsky et al., 1988), gene deletion (Simon et al., 1983; Adair et al., 1983;), DNA rearrangement (Grosovsky et al., 1986), and insertion (Nalbantoglu et al., 1988). When one copy of chromosome 8 is absent and the other duplicated, it results in the expression of a recessive Aprt mutation in the mouse CAK cells (Eves et al., 1983). The observation of Aprt gene deletion and loss of heterozygosity for syntenic markers in the mouse teratocarcinoma stem cell line suggested that an initial deletion of the Aprt gene and surrounding regions may result in chromosome instability (Cooper et al., 1991; Turker et al., 1989).

Aprt deficiency in humans has been observed, with clinical symptoms (Simmonda et al., 1976). Aprt is a purine salvage enzyme which catalyses the conversion of adenine to 5'-AMP in the presence of 5-phosphoribosyl-pyrophosphate and magnesium. With Aprt deficiency, adenine is not salvaged to AMP and the only metabolic pathway available for adenine is through its oxidation to 8-hydroxyadenine and subsequently to 2,8-dihydroxyadenine in reactions catalyzed by xanthine oxidase. Both of these products are relatively insoluble in urine and thus precipitated, leading to the formation of urinary stones in affected patients (Simmonda et al., 1976).

Up to now, there has been no reports to directly show Aprt can affect tumorigenesis or metastasis. However, evidence mentioned above clearly demonstrate that the Aprt locus and surrounding regions are unstable. Possible instability in the Aprt region, together with our observations on the Aprt rearrangements in tumorigenic and metastatic cell lines C3, NR3, NR4, M4R4 and RHMRA, RHMRB, and RHMRC, especially the higher gene dosage rearrangement in the highly metastatic cell line C3, suggest that the Aprt flanking region is important in metastatic progression.

In a recently published report, Pullman et al.(1992) have isolated a cell adhesion regulator (CAR) gene that maps to the human chromosome 16q region. It is suggested that this CAR gene is a candidate tumor suppressor gene (literature review section) . The precise location of this gene on 16q has not been identified, therefore, it is not clear whether the CAR gene flanks the Gnb-3 or Aprt loci. If it does not, a gene flanking Gnb-3 or Aprt should be a second candidate tumor suppressor on 16q, similar to the two candidate tumor suppressors identified on human chromosome 11 in Wilms' tumor (Maw et al., 1992).

VI. CONCLUSION

In this study, chromosomal abnormalities such as DNA amplification, translocation, deletion, inversion and marker chromosomes are detected in seven of the murine 10T½ cell lines transformed by the oncogenes *ras* and/or *myc*. Inversions of both copies of chromosome 8, deletions of a copy of chromosome 8 and losses of fragments of chromosome 8B-E have been revealed in metastatic cell lines but not in non-metastatic cell lines. This chromosome 8B-E region is homologous with human chromosome 16q. Hence, it is suggested that chromosome 8B-E may contain a gene the loss of which is involved in metastatic progression.

cDNAs of seven genes which have been cloned from mouse chromosome 8 and human chromosome 16q were used as probes in Southern blot hybridization experiments with DNA prepared from 10T½ tumour cell lines. Gene rearrangements were detected at the Ctrb, Gnb-3 and Aprt loci in the C3 cell line. Since the C3 line has a very high metastatic ability, it is possible that a gene which is located between Ctrb and Aprt is important in oncogene complementation for advanced metastatic progression. The mutation of the Gnb-3 gene itself may also contribute to metastatic progression.

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