THE UNIVERSITY OF MANITOBA

Molecular Cloning of A Gene from Metastatic Fibrosarcoma Cells Converted by Viral Insertional Mutagenesis

by

EDWARD F. DENG

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MOLECULAR CLONING OF A GENE FROM METASTATIC FIBROSARCOMA CELLS CONVERTED BY VIRAL INSERTIONAL MUTAGENESIS

BY

EDWARD F. DENG

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

MASTER OF SCIENCE

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ABSTRACT

A benign fibroma cell line MRPD was converted into a metastatic fibrosarcoma by infection of Moloney murine leukemia virus (MMLV). The gene which had been integrated by MMLV may be important in malignant conversion. The inverse PCR technique was used to isolate a genomic DNA fragment called 2br37, which is immediately adjacent to the viral integration site. PCR screening of mouse brain cDNA library using the DNA 2br37 fragment as a probe, yielded a PCR clone called 2brC1 which overlapped with 2br37. With this PCR clone as probe, a mouse fibroblast cDNA library was screened. An overlapping cDNA clone SW1 was isolated and sequenced. Open reading frame analysis showed a possible open reading frame in the cDNA clone with a poly (A) signal AATAAA sequence in a AT rich region, but DNA sequencing did not show a potential start codon. The 5' RACE technique was used to purify a SW1 overlapping clone, named RACE5. The combined sequences of RACE5 and SW1 demonstrated a complete open reading frame with 140 amino acids, which would yield a predicted 15 Kd protein. Multiple sequence comparisons of 2br37, 2brC1, SW1 and RACE5 showed that there is a 19 nucleotide fragment deletion in the genomic DNA of clone 2br37. This may be caused by retroviral infection. The significance of this deletion is not clear. This sequence comparison also showed that clone SW1 and RACE 5 have the same open reading frame but differ in their 5' end sequence. This indicates that there may be two mRNA transcripts and the difference at the 5' end may due to differential mRNA splicing events. A sequence homology search of both DNA and the deduced

amino acids did not show any significant homology with any known gene in Genbank. Southern hybridization of the genomic DNA with cDNA SW1 as probe demonstrated the loss of heterozygosity of this gene, named MAG (metastasis associated gene), in the malignant 2br VI cells when compared with normal parental 10T1/2 mouse fibroblast and the benign tumor cell line, MRPD. Decrease mRNA expression of this gene was detected in 2br VI cells with Northern hybridization. Two mRNA transcripts, 1.8 and 3.0 Kb, were found in 10T1/2 and MRPD cells. These two mRNA transcripts are consistent with the cloning results which implied the existence of two transcripts. All these results indicate that the MAG gene may be a regulatory gene associated with metastasis conversion. Disruption of this gene by viral insertion may be critical in the transformation of the benign tumor cell line, MRPD, into the metastatic cell line 2br VI.

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I. Introduction

1. The Metastatic Phenotype

Tumor progression, or invasion and metastasis, is a critical part in the stepwise oncogenic processes. Metastasis represents the end stage of cancer development in patients. The life threatening feature of this process dictates the importance of metastasis in cancer research. However, the progress towards understanding the molecular mechanisms involved in metastasis has lagged behind other developments in the cancer field (Liotta, 1991). Metastasis has been well accepted as a highly selective process, favoring the survival of a subpopulation of metastatic tumor cells that preexist within the heterogeneous primary tumor (Fidler and Kripke, 1977; Kerbel , 1979; Poste and Fidler. 1980; Fidler and Hart 1982; Liotta , 1986; Sobel, 1990). Metastasis occurs via a complex cascade of events or series of sequential steps whereby malignant cells must be able to disseminate from primary tumor, invade the adjacent tissue, penetrate into the lymphatics and blood vessels, survive in circulation and the attack by host immune system, arrest at the selected distant vascular bed, extravasate into the target organ and proliferate as a secondary colony. Because of the complicated process of metastasis, investigators have separated invasion and metastasis into the series of defined sequential steps as mentioned above. Recent studies have demonstrated that there are both stimulatory and inhibitory factors associated with different steps on the route to metastasis, in addition to deregulated growth control. A general theme has emerged that the balance in favor of the inhibition in nonmetastatic cells, is tilted to stimulation in metastatic cells.

2. The Interrelationship Between Tumorigenicity and Metastasis

It has been proposed that tumorigenicity and metastasis are under independent genetic control (Liotta, 1986; Sobel, 1990). This was based on several lines of evidence. Transfection of *ras* oncogene in some cells induce fully tumorigenic but not metastatic phenotype (Muschel, 1985). Secondly, The adenovirus-2 E1A protein can suppress *ras* induced metastasis but not tumorigenicity (Pozzatti, 1986). Furthermore, the cell fusion studies showed that the metastatic, not the tumorigenic, phenotype can be suppressed in mouse metastatic lung carcinoma and rat metastatic mammary carcinoma cells when fused with their corresponding tumorigenic but nonmetastatic cells (Ramshaw et al. 1983 ; Layton and Franks 1986). However, it can be argued that tumorigenicity and metastatic potential have both overlapping and separate features. A cell has to be tumorigenic to become metastatic. In addition to tumorigenicity, other genetic changes are needed for a tumor cell to acquire metastatic ability. This has been elegantly shown in the tumor development model, proposed by Vogelstein's group in the human colorectal carcinoma (Vogelstein, et al. 1988; Fearon, and Vogelstein, 1990). Both the activation of oncogenes and inactivation of tumor suppressor genes were found in a stepwise manner to correlate with clinical stages of cancer progression. The apparent criticism of the role of oncogenes in metastasis is that many oncogenes were defined by their ability to cause uncontrolled growth of cells in vitro and tumor formation in vivo, and not many oncogenes or tumor suppressor genes have been linked to featured processes of metastasis. However, during last decade, it has been established by several laboratories including our own that the ras oncogene can cause metastatic transformation in both aneuploid fibroblast cells and

diploid primary rat embryo cells (Thorgeirsson et al., 1985; Bernstein and Weinberg et al., 1985; Pozzatti et al., 1986; Egan et al., 1987a; Bradley et al., 1986). Other than fibroblast lines, several nonmetastatic tumors were also converted to metastatic progression by *ras* transfection (Collard, et al. 1987; Vousden, et al. 1986). Other oncogenes, such as *raf*, have been shown to cause metastasis as well in certain cells (Brodeur, et al. 1984, ; Yokota, et al. 1986 ; Egan, et al. 1987b ; Greenberg, et al. 1989). A positive correlation of *ras* induced metastasis and proteinase expression, and an inverse relation with a proteinase inhibitor (TIMPs) have been reported (Denhardt, et at. 1987; Greenberg, et al. 1989; Chambers and Tuck 1993; Su, 1993). Other oncogenes were also found to regulate genes involved in invasion (Hennigan, 1994). These compelling results strongly suggested that the accumulated lesions, caused by mutations of oncogenes and tumor suppressor genes formed the basis on which tumor cells acquire their metastatic capacity. Some oncogenes, such as *ras*, may directly regulate cellular genes involved in invasion and metastasis.

3. Oncogenes in Tumorigenesis

3. 1 *Ras* Oncogene Family

Ras has been known to be a guanine nucleotide binding protein with intrinsic GTPase activity. Like all GTP-binding proteins, *Ras* switches between an inactive (GDP-bound) and an active (GTP-bound) form, and is involved in controlling cell growth and differentiation (Barbacid 1987; Boguski and McCormick, 1993; Dickson and Hafen 1994). The biochemical function for *ras* has recently been established as a critical regulator of MAP kinase cascade in which *ras* can activate *raf*, a downstream target with

serine-threonine kinase activity, by recruiting *raf* to the plasma membrane (Stokoe, et al. 1994; Leevers, et al. 1994 ; Hall 1994). The *ras* regulated MAP kinase pathway is by far the best defined mechanism in elucidating how proliferating signals can be transduced from extracellular mitogens to nuclear transcription factors (Marshall, 1995).

However, the involvement of *ras* in metastatic transformation is still not fully understood. Whether *ras* has additional roles in tumor metastasis has been investigated. *Rac* and *Rho* genes, which are members of the *ras* superfamily of small GTP-binding proteins, have been shown to regulate growth factor-induced membrane ruffling and cell motility (Ridley, 1992, Cell ; Takaishi, et al. 1994; Matuoka, et al. 1993), and *Rho* may exert its effects on cytoskeletal reorganization via phosphatidylinositide 3-kinase (Zhang, et al. 1993). Some of the regulatory molecules of the *Rho* gene may cross-talk with the *ras* signalling pathway (Nobes and Hall 1994; Ridley, et al. 1995).

3. 2 *Myc* Oncogene Family

The *c-myc* oncogene was originally found as the cellular homologue of the transforming sequence from the MC29 avian retrovirus (Sheiness & Bishop, 1979). The *c-myc* gene was later found to be one member of a *myc* oncogene superfamily, which also contains *L-myc, N-myc, s-myc* and *b-myc* (for review see Zimmerman & Alt, 1990). The *c-myc, L-myc and N-myc* genes all consist of 3 exons and 2 introns, where the first exon is noncoding. Expression of *c-myc* is regulated by three promoters. P1 and P2 are found in the first exon and Po is found upstream of the exon 1 (Spencer & Groudine, 1991). The *c-myc* proteins are nuclear phosphoproteins which contain domains of functional importance with homology to transcriptional activator proteins. These domains

include a β-HLH domain which consists of a stretch of basic amino acids followed by two amphipathic α-helices separated by a loop. The second region is a leucine zipper motif which consists of a long α-helix with intermittent leucine residues (Landschultz et al, 1988). Both the β-HLH and leucine zipper motifs are used by other proteins as dimerization surfaces. Recently a protein termed *max* was found to bind to *myc* through its own β-HLH leucine zipper motif (Blackwood & Eisenman, 1991). The heterodimers of *myc* and *max* can bind to specific DNA sequence containing the core hexanucleotide sequence CACGTG and activate transcription of genes which contain this sequence (Kretzner et al, 1992; Amin et al, 1993). The ability of *myc* to transform cells and to cooperate with *ras* in transformation is dependent on its ability to heterodimerize with *max* (Amati et al, 1993).

It has long been recognized that a chromosomal translocation t(8;14)(q24;q32), in which the coding exons of *c-myc* were relocated adjacent to the immunoglobulin gene promoter in B cells leading to overexpression of *myc* and cellular transformation is present in the malignant cells of patients with the Burkitt's lymphoma (Chung et al, 1986). *N-myc* was found amplified in neuroblastomas, and *L-myc* was amplified in small cell carcinoma of the lung (Kohl et al, 1983; Nau et al, 1985). In small cell lung carcinoma, it was found that the level of *L-myc*-amplification was correlated with increased invasiveness, and high metastatic ability (Little et al., 1983).

In the human myelocytic leukemia cell line HL60, the *c-myc* gene was significantly amplified (Collins et al., 1988). It has also been demonstrated that *c-myc* and a mutant type of p53 could permit elevated expression of *ras* protein and increased

malignancy (Taylor et al., 1992). The biological function of the *myc* protein has been studied by transfecting the *myc* gene into cells in vitro and in vivo. *Myc* overexpression, like some other nuclear oncogenes, leads to immortalization of senescent rat embryo fibroblasts (Land et al, 1983). This is in contrast to *ras* and some other cytoplasmic oncogenes which , although are able to transform REF's , cannot efficiently immortalize them. Even though *myc* does not transform REF cell, it is shown that *myc* is able to induce tumorigenicity in some already immortalized cell lines, suggesting that the ability to transform cell by *myc* is cell type specific (Keath et al., 1984). It has also been shown that decreased expression of *myc* seems to be a early event leading to differentiation of the HL60 cells (Siebenlist et al, 1988; Bentley & Groudine, 1986).

4. Loss of Heterozygosity and Tumor Suppressor Genes in Tumorigenesis

4. 1 Loss of Heterozygosity (LOH)

During the last decade, tumor suppressor genes have emerged as equally important players as oncogenes in tumor formation and progression. Historically, three lines of evidence -- cell hybrids, familial cancer, and loss of heterozygosity in tumors, firmly established that inactivation of tumor suppressor genes exist widely in human tumors, and are critical in neoplastic transformation (see reviews by Klein, 1987; Sager 1989; Weinberg, 1991; Marshall, 1991). It was the last line of evidence (Cavenee, et al., 1983; Benedict, et al., 1983; Godbout, et al., 1983) which confirmed Knudson's two hit hypothesis in which he postulated that childhood retinoblastoma (RB) is triggered by two successive lesions in the cell genome (Knudson, 1971). This in turn lead to identification

of the first human tumor suppressor gene RB (Friend, 1986; Lee, 1987; and Fung, 1987). The loss of heterozygosity (LOH) in tumors has since been used as an indication of the presence of a tumor suppressor gene. In LOH experiments, DNA markers mapping to the chromosome region of choice are used in Southern blotting, by comparing DNA fragments cut by a specific restriction enzyme from normal and tumor cells. In normal cells, the specific restriction site may have been altered (by somatic or germline mutation) in one copy of the potential tumor suppressor gene, which will be detected by the DNA marker as two fragments (showing heterozygosity) on Southern blotting. In tumor cells, the surviving wild-type allele may often be replaced by a duplicated copy of the homologous chromosome region that carries the mutant allele or loss of the normal allele. This elimination of the wild-type allele may be accomplished by mechanisms like chromosomal nondisjunction, mitotic recombination, gene conversion, or deletion (Cavenee, et al. 1983). Most tumors that lack functional copies of a suppressor gene (like RB) display two identically mutated alleles. This will result in LOH (indicated as a single fragment on Southern blotting). By using this approach, a number of tumor suppressor genes have shown LOH., such as P53 (Baker, et al. 1989), DCC (Fearon, et al., 1990), as well as metastasis suppressor gene nm23 in colon cancer (Wang, et al., 1993).

4. 2 Retinoblastoma Gene (Rb)

The Rb gene located in human chromosome 13q14, was originally isolated from retinoblastoma. Both familial and sporadic forms of retinoblastoma have resulted from the deletion or inactivation of two alleles of the Rb gene (Knudson, 1971). RB inactivation is also found in other sarcomas, small cell carcinoma of the lung and in carcinoma of

the breast, bladder and prostate (Lee, et al. 1988; Horowits, et al. 1990; Bookstein, et al. 1990; Yokota, et al. 1988). The frequent inactivation of RB in various tumors indicated that RB is an important component of the growth control machinery in normal cells. The protein product of Rb is a 110 K nuclear phosphoprotein (Lee, 1987), which has been shown to act as a cell cycle control checkpoint protein acting at the G1 phase. Cells in G0/G1 express RB in a hypophosphorylated form (functional RB), but in hyperphosphorylated form (nonfunctional) in late G1 phase and remains hyperphosphorylated in S, G2 and M phases (DeCaprio, et al. 1989; Buchkovich, et al. 1989; Chen, et al.1989; Mihara, et al. 1989).

Several lines of evidence indicate that phosphorylation causes the inactivation of RB. The oncoproteins E1A, SV40 large T antigen, Human papillomaviruses (HPV-E7) can bind and sequester hypophosphorylated pRB, dissociate RB from its targets, including E2F (Chellappan, et al. 1992). The study on G1 cyclins has demonstrated that cyclin D, cyclin-dependent kinases (CDK) 4 and 6 and cyclin E are able to phosphorylate pRB (Kato, et al.1993; Ewen, et al. 1993; Hinds, 1992). Several cellular targets of RB have recently identified. One of them, the transcription factor E2F, was a essential factor that participates in growth control and DNA synthesis (Chellappan, et al. 1991; Nevins, 1992). When pRB is hypophosphorylated, it is capable of binding to the E2F transcription factor; phosphorylation causes it to release E2F, which in turn activates E2F controlled transcription of genes including *c-myc, B-myb,* cdc2, dihydrofolate reductase, thymidine kinase and the E2F-1 gene itself (Nevins, 1992).

Genes encoding other pRB-binding proteins have been reported over the past

several years (Wang, et al. 1994). Most intriguing of these is the nuclear tyrosine Kinase encoded by the *c-abl* proto-oncogene. Hypophosphorylated pRB can bind directly to the active catalytic domain of the c-abl kinase, blocking its function. Hyperphosphorylated pRB loses this binding ability (Welch, et al.1993; Welch, et al. 1995). It appears that pRB may control cell proliferation by binding multiple effectors such as E2F and c-abl, to modulate simultaneously the activity of a number of downstream pathways. Rb also plays an important role in control of differentiation during development. RB gene knock-out mice are nonviable and show abnormalities of erythropoiesis and neural development (Lee, et al. 1992). Study on muscle differentiation also demonstrated that RB-mediated cell cycle arrest is necessary for induction of a myogenic differentiation pathway (Gu, et al. 1993)

4. 3 The p53 Tumor Suppressor Gene

The protein p53 was originally found in cells transformed in vitro by the tumor virus SV40 (Simian Virus 40)(Linzer, and Levine 1979; Lane, and Crowford 1979). The SV40 large T antigen was found to bind to p53. Because large T antigen was needed to maintain the transformed phenotype, it was thought that p53 would cooperate with large T antigen for transformation. Consistent with this finding, the expression of p53 was elevated in tumor derived or transformed cell lines. The half life of p53 in transformed cells was much longer than that in nontransformed cells (Oren, M., 1981). Still other experiments demonstrated that a variety of genomic and cDNA p53 clones could immortalize cells and cooperate with the ras oncogene to transform primary rat embryo fibroblasts in cell culture(Parada, L. F., et al. 1984; Eliyahu, D., et al. 1984). Because of

these experiments p53 was then classified as an oncogene. However, several observations could not be explained by this view of p53 function. Most notably the p53 locus was found rearranged or inactivated during the progression of erythroleukemia in mice (Mowat, et al. 1985; Hicks, and Mowat, 1988). In addition , a p53 clone termed 11-4 was found to be unable to transform cells in cooperation with ras (Finlay et al, 1988). It was latter found that replacement of alanine by valine at position 135 can render the capability of the p53 clone for transformation. By restriction enzyme polymorphism in the p53 gene, it was shown that normal mouse DNA encodes alanine at position 135 in the p53 protein. It was then clear that all of the transforming p53 clones turned out to be mutant forms of p53 (Hinds, et al. 1989). They derived their transforming potential from their ability to act in a dominant negative fashion by binding to the wild-type p53 protein and abrogate the physiological function of p53. Therefore p53 is instead a tumor suppressor gene (Finlay , et al. 1989).

In tissue culture, transfection of cells with normal p53 genomic or cDNA clones actually does not promote transformation, as does transfection with mutant p53 genes. Rather, the normal p53 gene suppresses transformation of cells in culture by other oncogenes, suppresses growth of previously transformed cell lines, and inhibits the tumorigenic potential of transformed cells in animals (Chen, et al. 1990). The p53 protein is present in virtually all cells of the body at low levels, primarily because of its short half life. Mutations and deletions of the p53 gene have now been implicated in a wide variety of inherited and sporadic forms of human malignancy. P53 mutations were found in over 75% of such tumors (Levine, 1992). Usually, one allele contains a point mutation,

and the other is lost (loss of heterozygosity).

From the above information, it is clear that wild-type p53 may play a role in regulating or monitoring cell proliferation. Supporting evidence comes from the fact that p53 is capable of binding to two cell cycle regulators cdc2 and casein kinase II. Phosphorylation of p53 by casein kinase II is required for p53 to activate transcription and allow it to suppress neoplastic transformation (Hupp, et al.. 1992; Milne et al. 1992). Overexpression of wild type p53 in human glioblastoma, prostate cancer or osteosarcoma cells leads to inhibition of cell cycle progression (Montenarh, 1992), and growth arrest in G1 and G2 of the cell cycle (Michalovits et al., 1990). Cell cycle arrest by p53 may also be important in the cellular response to DNA damage (Lane, 1992). It has been found that induction of DNA damage by γ-irradiation and by drug administration in mammalian cells leads to an increase in p53 expression, which in turn can lead to cell cycle arrest (Kastan, et al., 1991; Yin, et al., 1992). The p53 mediated cell cycle arrest in respond to DNA damage is important because it would allow the DNA repair machinery to correct the error before DNA replication (G1 arrest) or cell division (G2 arrest). A recent study has found that the protein of several target genes of p53, Waf1/cip1/p21, is an inhibitor of cyclin dependent kinases(cdk)(Harper et al. 1993). The model of p53 function has emerged that the high level of p53 which occurs upon DNA damage leads to high levels of WAF1/cip1/p21, a family of proteins which inhibit cell cycle progression by inhibiting the kinase activity of cdk2, 4, 6. This model was further supported by recent results showing that cdk2 activity is inhibited during DNA damage induced growth arrest in a p53 dependent manner (Dulić et. al. 1994). The WAF1/cip1/p21 also binds to the

proliferating-cell nuclear antigen (PCNA) and inhibits *in vitro* PCNA-dependent DNA replication. The CDK and PCNA inhibitory activities of p21 are independent and reside in separate protein domains on p21. The CDK inhibitory activity resides in the N-terminal domains of these proteins whereas the PCNA binding and inhibitory activity reside in the C-terminal domain (Luo, et al., 1995; Chen, et al., 1995).

5. Oncogenes and Tumor Suppressor Genes in Metastatic Transformation

The *ras*, *myc* and mutant p53 oncogenes were demonstrated to interact synergically to increase metastatic potential in a mouse fibrosarcoma cell line by decreasing the latency period of tumor formation and increasing ability to form lung tumor in an *in vivo* experimental metastasis assay (Taylor, et al. 1992). A similar result was observed in mouse prostate reconstitution model system where the progression of carcinomas in the *ras*, *myc* transformed tumor was invariably associated with either complete loss, partial deletion or loss of expression of wild type p53 allele (Thompson, et al. 1995). The interaction between *ras* and mutant p53 in rat embryonic fibroblasts (REF) also resulted in increased metastatic potential in nude mice (Kikuchi, et al. 1995). The *myc* protooncogene was observed to be associated with lymph node metastasis in colorectal cancer and the survival rate tended to be poorer in patients with c-myc overexpression than in those without it (Sato, et al., 1994). The study on human sarcomas showed that tumors in which the expression of a tumor suppressor gene, Rb, was decreased were more aggressive than tumors in which Rb protein was normally expressed. The role of oncogenes and tumor suppressor genes in metastatic conversion is not clear yet. Some

studies showed that ras can induce cell membrane ruffling and motility (Bar-Sagi, et al 1986; Noble, et al., 1993). Another recent study showed that the *ras* oncogene may influence metastasis through activating an integrin-binding phosphoprotein which is associated with tumor metastasis (Guo, et al., 1995).

6. Other Metastasis Related Genes

In search of analogous set of metastasis suppressor genes (in contrast with tumor suppressor), the nm23 gene was first identified in murine melanoma cell lines with a decreased mRNA level as the cells' metastatic potential increased (Steeg, et al. 1988). The association of nm23 with metastatic potential was confirmed later (Leone et al. 1991; Leone, et al. 1993). Two human homologous, nm23-H1 and nm23-H2 have now been identified (Rosengard, et al. 1989; Stahl, et al. 1991). Reduced nm23 expression was associated with poor prognosis of breast cancer and melanoma, hepatocellular and gastric carcinomas. Mutations of nm23 were also detected in several human tumors (MacDonald, et al. 1993; Chang, et al. 1994). The nm23-H2 was shown to be a *c-myc* transcription factor (Postel,et al. 1993). The nm23-H1 was recently found to induce basement membrane formation and growth arrest of breast cancer cells (Howlett et al. 1994).

Proteins involved in invasion are likely to influence metastasis, as has been shown for proteases, especially the matrix metalloprotease gene family and their inhibitors (Liotta et al. 1991). A positive correlation of elevated type IV collagenase with metastatic potential has been well documented (Liotta, et al., 1980; Turpeenniemi-Hujanen, et al., 1985; Garbisa S, et al., 1987). The level of tissue inhibitor of metalloprotease (TIMP), an inhibitor for both stromelysin (also known as transin) and interstitial collagenase

(Galloway et al., 1983; Chin, et al., 1985), has been shown to have an inverse correlation with the invasive potential of murine and human tumor cells (Halaka, et al. 1983; Hicks, et al. 1984; Schultz, et al,1988; Khokha, et al. 1989). TIMP-2 has been shown to complex with type IV collagenase and inhibit activity of the enzyme (Stetler-Stevenson, et al.1989).

Another group of molecules that are involved in cell adhesion are also likely to regulate the invasion process. The receptors for extracellular matrix, such as RHAM, a hyaluronan receptor can mediate tumor cell motility (Turley, et al. 1991,; Hardwick, et al. 1992), and was required in TGF-β1 stimulated locomotion (Wright, Turley, and Greenberg, 1993); The β1 integrin VLA-2, which is a cell surface adhesion receptor, can increase adhesion of human rhabdomyosarcoma cells *in vitro* and enhance metastasis *in vivo* (Chan, et al. 1991). Both positive and negative regulators in angiogenesis are also well correlated with metastasis (see reviews by Fidler and Ellis, 1994; Folkman 1995).

Although the studies on oncogenes and tumor suppressor genes have yielded significant progress in our understanding of tumor formation and progression, the direct link of those genes to metastasis is not clear yet. The knowledge we have is largely based on associated changes. Although we can argue that metastasis is separated in sequential steps for clarity, clinically it is difficult to define a clear line between each step, if there is any. Hence sufficient changes beyond deregulated growth have to occur before the benign tumor cells can take the road to metastasis. Then the immediate question is whether there is a critical change which can cause genetic instability, and in turn to ignite a cascade of changes required in specific steps for metastasis, and how the specific

changes for metastasis can be credited to oncogenes and tumor suppressor genes. The answers are not known yet. To tackle the problem, we have designed an viral insertional mutagenesis approach to search for a gene(s), more directly linked to metastasis. Recently, a group with similar cloning method has successfully identified an invasion inducing gene Tiam-1, a GDP-GTP exchanger for *Rho*-like protein (Habets, et al. 1994).

7. Insertional Mutagenesis

Viral insertional mutagenesis has long been used as a tool to identify genes that might play a role in oncogenesis. Three classical criteria have been used to identify such genes: (i) homology with known viral transforming genes (v-oncogenes); (ii) activated expression in tumor cells (iii) transforming activity in cultured mouse cells (Varmus, 1983). The notion that retroviruses that do not harbor oncogenes but can activate proto-oncogenes has prompted a search for unknown genes with a tumorigenic potential, which led the early findings of *c-myc* gene in avian leukosis virus (ALV) induced avian leukemias and c-myb gene in myeloblastosis-associated virus (MAV) induced avian B cell lymphomas (Varmus, H. E., 1983); *c-erb* B in AVL induced erythroleukemias (Fung, Y.K., et al. 1983); *int*-1 and *int*-2 in mouse mammary tumor virus (MMTV) induced mammary tumors (Nusse and Varmus,1982).

As tumor suppressor genes emerged as equal important factors as oncogene in tumorigenesis, the same notion has been further developed in that the insertional events can disrupt tumor suppressor genes as well. It has been demonstrated that the p53 tumor suppressor gene was inactivated by Friend leukemia virus (FLV) in erythroleukemic cells (Hicks and Mowat, 1988; Ben-David, et al. 1988 and 1990); APC tumor suppressor gene

was disrupted by insertion of retrotransposon long interspersed repetitive element (LINE-1) into the last exon of the gene in colon cancer (Miki, et al. 1992).

This retrovirus insertional mutagenesis method was also used to clone gene(s) which may be directly involved in invasion and metastasis transformation, such as Tiam-1 gene (Habets, et al., 1994). In our previous studies, the mouse 10T1/2 cells have been transformed by H-ras/neo and *v-myc* oncogene in dialysed calf serum. A cell line, designated MRPD (Egan, et al., 1989) was selected in G418 and shown to be a benign fibroma when injected into mice. Dr. Pardeep Bhatia in our institute infected MRPD cells with a competent Moloney murine leukemia virus (provided by Dr. Mowat), carrying a bacterial tyrosine suppressor gene supF in its long terminal repeat as a marker gene (Reik, et al., 1985). One x 10^6 cells were infected with 1 ml of virus with a titre of 10^6 pfu/ml, in the presence of polybrene. Since p-LTR supF is a competent vector, it is expected to infect every cell (Goff, 1987). Infected cells were passaged several times to allow for the viral insertion into both alleles or to allow somatic recombination to eliminate the corresponding allele. Virally infected cells were tested for metastases in an experimental metastasis assay (Fidler and Kripke, 1977; Egan, et al., 1987). Following intravenous injection, only a few lung metastasis were detected and these were then established as in vitro cell lines. Several independent cell lines were cultured in vitro and $5x10^5$ cells from each clone were reinjected into syngeneic mice in the metastasis assay, to test for stable integration and to select cells with better capability to form metastasis (Fidler, 1973). Again, the lung metastases were established as cell lines. To investigate virus integration in metastatic clones, DNA from these lung tumor cell lines were

extracted, and digested with different restriction enzymes and separated on agarose gels, blotted and probed with the 220 bp with SupF gene. The results showed these cell lines had distinct patterns of virus integration, except one common band present in three of these cell lines, which may suggest a common integration site. Since supF gene is not in mammalian cells, it is only carried by the recombinant virus, so the hybridization results confirmed that the viruses have been integrated into the host genome. Because the virus does not contain any oncogenic sequence, a viral integration event may have been crucial in metastasis conversion. To clone the gene from viral integration site, the inverse PCR method (Silver and Keerikatte, 1989) was used by Dr. Bhatia to amplify the DNA sequence immediately adjacent to the integrated virus. By such a method, only one genomic DNA fragment was isolated, designated 2br37. After sequencing, it was found that this clone contains the viral sequence as well as new genomic DNA sequence. The viral sequence was removed by nested PCR with primers specific to nonviral sequence. The PCR products were subcloned into the plasmid pCRII (Invitrogen). The new DNA sequence was compared with those in Genbank, and there was no homologous sequence found. This indicates that it is a sequence from a novel gene. At this point, several questions were raised immediately. Firstly, does the DNA clone isolated from metastatic cells represent an intron or exon sequence of the gene, and is the sequence expressed as mRNA? If it is not expressed, this DNA fragment may be in a regulatory region of an unknown gene associated with metastatic transformation, so that it can show its impact by modifying the function of cis-regulating elements. If it is expressed, does integration of the virus alter expression of the gene it is integrated into, either quantitatively or

qualitatively at the transcriptional or translational level. If the expression of the viral integrated gene was altered in the metastatic cell, it will suggest a link between metastatic transformation and mutation of this gene. Then the question which follows is how can we isolate the cDNA clone of the gene and further test its physiological function? All of these question have to be answered before we can address the potential biological impact of this gene on metastatic transformation.

8. Perspectives

During the studies in the last two decades, it has been established that tumor formation and progression require alteration in both positive (ie.oncogene) and negative (ie.tumor suppressor gene) regulators. Cancer cells must be tumorigenic to grow as a metastatic colony (Fearon and Vogelstein, 1990; Sobel 1990; Liotta, et al., 1991); however, tumorigenic cells are not necessarily invasive and metastatic. The link between the two phenotypes are closely related, but the molecular mechanisms remain to be determined. Part of the reason may due to the possibility that some critical factors are still unknown. In joining the effort to search for these factors, we tried to clone a gene(s) related to transformation from a benign to metastatic tumor. If successful, I believe that our studies will help in understanding molecular mechanisms of metastasis.

II. Materials and Methods

1. Materials

1.1 Cell Lines

The mouse 10T1/2 fibroblast and MRPD which was derived by transformation of 10T1/2 with v-myc and E.J-H-ras oncogenes, were established by Egan et al. (Egan et al., 1987a, 1987b: Egan 1989, Ph.D thesis, University of Manitoba). 2brVI which was established by Dr. Pardeep Bhatia, was derived by infection of the MRPD cell with recombinant Moloney Murine Leukemia virus p-LTR supF (provided by Dr. Mike Mowat) and selecting lung colonies in C3H mice. The p-LTR supF carries a bacterial tyrosine suppressor gene in its long terminal repeat as a selective marker gene(Reik, et al., 1985).

1.2 Genomic DNA Fragment

The genomic DNA fragment flanking retroviral integration site, namely 2br37, was isolated using inverse PCR (Silver and Keerikatte, 1989) and sequenced, by Dr. Pardeep Bhatia (personal communication, unpublished data).

The sequence is as follows:

<u>2BR37 SEQUENCE</u>

ACAGTGAGTCGGACGCGGGAGTCTTGAAACCAGCAGAAGCCGCCTGATCATT

GCCTCTGTAGGCGGGGCGGAGCAACCATCCACTGGGGGAACCTTGCCGGGCC

GTGCACCGCTCTGCGTAGGAGTCGGGTTGGTGGGGCGCATCGAGTACCCTAG

ACGGGCGCAGGGGTGCAGAGTAACACGCACTCGCTCACCTTCCAACCGAGCA

CATCCTT

<u>Complementary sequence of 2br37 sequence</u>

AAGGATGTGCTCGGTTGGAAGGTGAGCGAGTGCGTGTTACTCTGCACCCCTG

CGCCCGTCTAGGGTACTCGATGCGCCCCACCAACCCGACTCCTACGCAGAGC

GGTGCACGGCCCGGCAAGGTTCCCCCAGTGGATGGTTGCTCCGCCCCGCCTA

CAGAGGCAATGATCAGGCGGCTTCTGCTGGTTTCAAGACTCCCGCGTCCGACT

CACTGT

1.3 Primers

Most of the primers used in PCR and sequencing were synthesized on a DNA synthesizer (ABI) by Dr. M. Mowat (Table 1).

Table 1. list of primers used in PCR and Sequencing

Name of primers	Sequences
2br1.	5' TTGAAACCAGCAGAAGCCGCC 3'
2br3.	5' CACGCACTCGCTCACCTTCCA 3'
2br4.	5' GCGGGAGTCTTGAAACCAGCA 3'
2br5.	5' TGTAGGCGGGGCGGAGCGAAC 3'
2br6.	5' TGCCACCGTCTGCGTAGGAGT 3'
2br7.	5' ACCGAGTGTCTTGTGTGTCTGTC 3'
2br.a	5' CGGTTGGAAGGTGAGCGAGTG 3'
2br.b	5' CGCCCCACCAACCCGACTCCT 3'
2br.c	5' TGCTGGTTTCAAGACTCCCGC 3'
2br.d	5' TTCGCTCCGCCCCGCCTACAG 3'
2br.e (C1.A)	5' GACAGACACACAAGACACTCGGT 3'
2br.f (C1.B)	5' AAATAAGGAGCCAGTGACAATAAAT 3'
Lambda gt10.for[1]	5' AGCAAGTTCAGCCTGGTTAAG 3'
Lambda gt10.rev[2]	5' TTATGAGTATTTCTTCCAGGG 3'
Lambda gt11.for[3]	5' GGTGGCGACGACTCCTGGAGCC 3'
Lambda gt11.rev[4]	5' GACACCAGACCAACTGGTAATG 3'

1. *Located 10-30 bp upstream of the EcoRI site of Lambda gt10.*
2. *Located 14-34 bp downstream of the EcoRI site of Lambda gt10.*
3. *Located 16-37 bp upstream of the EcoRI site of Lambda gt11.*
4. *Located 22-44 bp downstream of the EcoRI site of Lambda gt11.*

1.4 Restriction Enzymes

Restriction endonucleases, *Bam HI, Hind III* and *EcoR I* (Pharmacia Biotech. Inc. Quebec) were used (5 u/μg DNA) to digest genomic DNA from mouse 10T1/2, MRPD and 2br VI cells. The genomic DNAs were mixed with 10x restriction reaction buffer (Pharmacia Biotech. Inc.) and each of the restriction endonuclease mentioned above. The reactions were carried out at 37^{0}C overnight.

1.5 cDNA Library

Mouse brain 5'-Stretch Plus cDNA library, was made from mRNA of normal whole brains from an adult BALB/c male mouse, on a Lambda gt10 vector, by oligo(dT) plus random primer. The library can be plated on C600Hfl bacterial strain, with a titer greater than 10^8 pfu/ml (Clontech, Palo Alto, CA).

Swiss 3T3 fibroblast cDNA library was made from mRNA of normal fibroblasts from adult Balb/c mouse on a Lambda gt11 vector by oligo(dT) and ramdom primer. The library can be plated on Y1090$^-$ strain, with a titer greater than 10^8 pfu/ml (Clontech. Palo Alto, CA).

2. Methods

2.1 Cell Culture

Normal Mouse 10T1/2 cells, benign MRPD (Egan,S. thesis), and metastatic 2br7 cells were grown to confluence in α-MEM (Flow Laboratories Inc., Rockville, MD) supplemented by 10 % of fetal bovine serum [(FBS) HyClone Laboratories, Utah], penicillin G (100units/ml) and streptomycin sulfate (100 units/ul), at 37°C in a 5% CO_2 in a humidity controlled incubator. Cells that approached confluence were washed with

PBS buffer to remove dead floating cells, and then lysed directly on the culture plates with cell lysis buffer for genomic DNA or RNA isolation.

2.2 DNA Preparation and Southern Blot Analysis

Genomic DNA was purified from cells by phenol-chloroform extraction (Sambrook, et al. 1989). 10 μg of DNA was digested to completion with 5units/μg of DNA of required restriction endonucleases, fractionated on 0.7% of agarose gels, denatured in 0.5% NaOH and 1.5% NaCl for 30 minutes, neutralized in 0.5 M Tris (pH 8.0) and 1.5 M NaCl, and transferred to NYTRAN membrane (Schleicher & Schuell, Keene, NH) with alkali (0.4M NaOH) blotting method (Amersham, UK). The membrane was then fixed by UV-Cross linker (Strategene, La Jolla, CA), hybridized to radioactive α^{32}P random labelled probe (1x10^8cmp/μg DNA) (Random Labelling Kit, Pharmacia Biotech Inc. Quebec) in 2x Pipes, 50% formamide, 0.5% SDS and 100 μg/ml of fragmented salmon sperm DNA for 18 hours at 42°C . After hybridization, membranes were washed in 2x SSC and 0.5x SDS at 37°C for 60 minutes, then in 0.1xSSC and 0.1% SDS at 65°C for 60 minutes. Autoradiography was performed at -70°C with Kodak MR film (Eastman Kodak Co., Rochester, N.Y.) for 2-7 days. The X-ray film was developed for visualizing the exposed bands.

For reprobing, the membrane was stripped as soon as possible after autoradiography. It was then washed 2 times for 30 minutes each in a 500 ml of 0.1x SSC and 0.5% SDS at 95°C as in the Zeta-probe membrane protocol (Bio Rad Labotoratory. Mississauga).

2.3 RNA Preparation and Northern Hybridization Analysis

mRNA was isolated from cells by the guanidinium isothiocyanate and oligo(dT) cellulose method (Invitrogen corp. San Diego). 2 μg of mRNA (in 11 μl) was mixed with 5 μl 10x MOPS, 8.75 μl formaldehyde and 25 μl of deionized formamide, put at 65°C for 5 minutes, separated on 1% formaldehyde gel (dissolving 3g of agarose in 220ml H_2o, 50 ml Formaldehyde and 40 ml 10x MOPS) at 90V for 8 hours. The RNA gel was directly transferred to NYTRAN membrane by alkali (0.05M NaOH) procedure. The membrane was fix and hybridized to radioactive probe same as in above Southern blotting except the washing temperature was 60°C in stead 65°C.

2.4 Preparation of cDNA Probe

Minipreparation of Plasmids

The bacterial colony containing the desired plasmid was cultured in 3ul of LB broth complemented by 100 ug/ml of ampicillin at 37°C in a shaking incubator for 16 hours. The plasmid was purified by Miniprep methods (Birnboim, H.C. and Doly, J., Nucl.Acids Res. 7, 1513, 1979; Chen, E.Y., and Seeberg, P.H., DNA 4, 165, 1985; Henikoff, S., Methods Enzymol. 155, 1987), plus phenol/Chloroform extraction. The DNA prepared can be used directly for restriction digestion and sequencing reaction.

Isolation of Inserts

The plasmid DNA was cut by required restriction endonuclease and separated on 1% agarose gel. The target DNA insert was purified by Genclean procedure (Bio 101, Vista, CA). The purified insert was used as probe in hybridization in Southern and Northern blotting.

Labelling of probes

The probes were radiolabelled with [a^{32}P]dCTP(Amersham) using a random labelling kit (BRL Life Technologies, Inc. Bethesda, MA). Fifty ng of DNA in 34 ul of water was denatured by boiling for 3 minutes, cooled on ice, added by 10 ul mixture of dATP, dGTP and dTTP as well as buffer. The [a^{32}P]dCTP and DNA polymerase I Klenow fragment was added, was incubated at room temperature for 60 minutes. The labelling mixture was passed through a spin column of sephadex G-50 by centrifuge for 1 minutes at 2000g.

2.5 Screening of cDNA Library

Screening by plating

The first screening was made on 150 mm LB Broth agar plates (90 mm plates for second and third screening) complemented by 10mM $MgSO_4$. The λgt11 phage library dilution was prepared to yield an approximately 30,000 pfu for each 150 mm plate (10,000 pfu for each 90 mm plate), combined with 600 μl of overnight culture of bacteria (200 μl for 90 mm plate) (Bacteria Y1090r^- were grown overnight at 37°C in a shaking incubator in LB Broth with 10mM $MgSO_4$ and 0.2% maltose), mixed in a 37°C incubator (250 rpm/min) for 15 minutes. Melted LB soft top agarose (7 ml) was added with 10 mM $MgSO_4$ to the cell suspension for each 150 mm plate (or 3 ml for each 90 mm plate). The plates were inverted and incubated at 37°C for 8-12 hours. The plates were chilled at 4°C for at least 1 hour. The plaques were transferred to duplicate nitrocellulose membranes (Schleicher & Schuell, Keene, NH), and the phage DNA denatrured on the membrane by alkali procedures (Clontech, Palo Alto, CA). The hybridization was performed the same

way as Southern hybridization. The membranes were washed in 2x SSC + 0.5% SDS at room temperature for 1 hour, in 1x SSC + 0.1% SDS at 65°C for 1 hour, and this washing step was repeated once. Then membranes were dried in room temperature and exposed to X-ray film as described in Southern Blotting.

Screening by PCR (Hamilton, 1991)

Four sets of combination primers, namely Lambda gt10.for/2br.a, Lambda gt10.for/2br1, Lambda gt10.rev/2br.a, and Lambda gt10.rev/2br1 were used to perform PCR directly on diluted mouse brain cDNA library ($5x10^4$ pfu/μl) in 4 separated reactions. The reaction mixture was prepared by 5 μl of PCR buffer (Pharmacia Biotech. Inc. Quebec), 20 mM dNTP, 30 pmol of each primer, 5 μl diluted phage library (phage DNA was pre-boiled for 10 minutes), 2.5 units of Taq DNA polymerase, adding water to a total of 50 μl, with 40 μl of mineral oil on top of reaction mixture. The PCR procedure begin with a hot start by adding the Taq polymerase after the reaction mixture was heated to 94°C. The Cycle was set at 94°C for 45 seconds, 60°C for 45 seconds, 72°C for 1.5 minutes, repeated for 30 times, elongated at 72°C for 7 minutes. The PCR product was then checked on 1% agarose gel and Southern Blotting.

2.6 Subcloning cDNA or PCR Product

cDNA cut from phage vector was seperated on 1% agarose gel and purified by Geneclean Kit (Bio 101, Vista, CA). The DNA 5 μl was then mixed with 2 μl of 10x PCR buffer and 10 mM dATP and 2.5 units of Taq polymerase in a total of 20 μl of reaction volume, incubated at 72°C for 1 hour. The cDNA in this preparation had a 3' adenosine nucleotide overhangs, and it was ready to be subcloned into TA vector as a

PCR product. The standard procedure for ligation of PCR products to TA vector followed the TA cloning manual (Invitrogen, San Diego).

2.7 DNA Sequencing

Sanger's dideoxynucleotide(dd NTP) method (Sanger, and Coulson, 1975) was used for all DNA sequencing experiment.

DNA sample (2 μg) was first denatured by mixing with 2 μl of 2M NaOH and 2mM EDTA at room temperature for 5 minutes. The reaction was neutralized by addition of 3 μl 3 M Sodium Acetate(pH 5.0) and 7 μl of H_2o. The denatured DNA was precipated in absolute ethanol at -70^{0}C for 10 minutes. The sample was centrifuged for 15 minutes at top speed in a microcentrifuge. The pellet was washed with 1 ml of -20^{0}C 70% ethanol and centrifuged again for 15 minutes. The DNA was air dried and dissolved in 10 μl of H_2O. Primer annealing was done by adding 2 μl of 0.5 pmol/μl primer and 2 μl of Annealing buffer (Pharmacia Biotech.) to the DNA sample and incubate at 37^{0}C for 20 minutes. Cool the sample at room temperature for at least 10 minutes. All sequencing reaction steps followed the manufacture's instruction in the T7 Sequencing Kit (Pharmacia Biotech.). ^{35}S-(d)ATP was used as radioactive labelling nucleotide (Amersham Canada Ltd., Oakville, Ontario).

DNA sequencing was performed on a Bio-Rad sequencing aparatus with 8% acrylamide gel. The gel was degased for better resolution of the band. The gel (50ml) was combined with 20 ul TEMED and 75 ul Amonia persulfate before pouring the gel. The samples were loaded on top of the gel and the DNA with different length (bp) were separated on the gel with 2200 voltage for 5-6 hours.

The sequencing gel was dried at 80°C for 1 hour in a gel drier and exposed to a Kodak MR film(Eastman Kodak Co. Rochester, N.Y.). The DNA sequences were read by using a Genereader (Bio-Rad).

2.8 Densitometry

Densitometry analysis was performed on Southern and Northern results. The density of each band was recorded using 2-D scan program on BioRad Densitometer (model 620). The data is then analyzed and depicted as bar graph by using Sigmaplot software.

2.9 5' RACE Cloning

5' AmpliFINDER RACE Kit was used to clone 5' end sequences (Clontech, Palo Alto, CA). 2 μg mRNA from MRPD benign tumor cells and primer 2br5 (see Table 1) were used to make first strand cDNA (for mRNA isolation method refer to 2.3). The protocols in 5' RACE kit were followed exactly for cDNA synthesis, RNA hydrolysis, cDNA purification, and anchor ligation. After the anchor fragment was ligated to the 3' end of the first strand cDNA, PCR amplification was performed (following the 5' RACE Kit protocol exactly), using the anchor primer and a nested primer 2br6 (see Table 1). The PCR products which were flanked by anchor primer (5'-CTGGTTCGGCCCACCTCTGAAGGTTCCAGAATCGAT AG-3') and primer 2br6 were cloned into a TA vector (method see **2.6**). This clone was named clone Race5.

III. Results

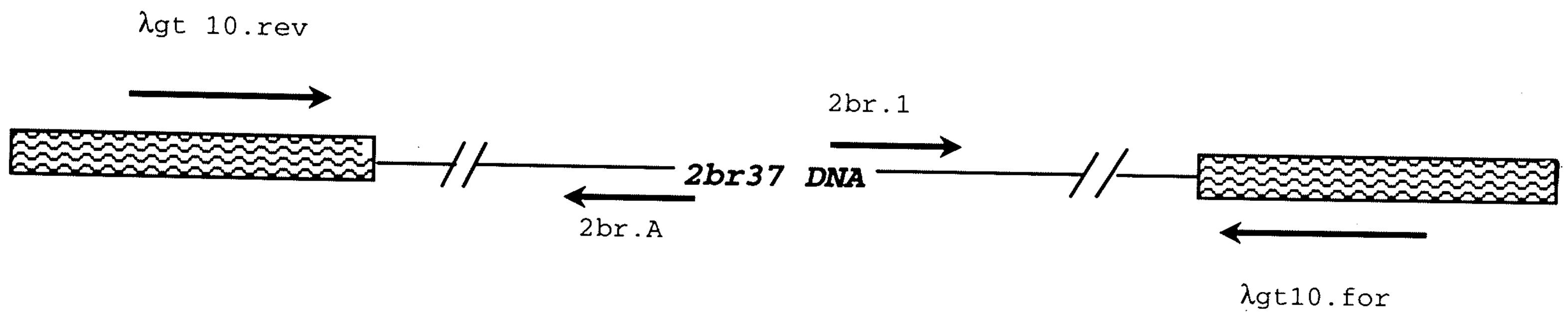
1. PCR Library Screening Analysis

In the preliminary study, using inverse PCR, a genomic DNA fragment was isolated by Dr. Bhatia from the viral insertion site in 2brVI cell line, a metastatic cell line established from lung metastasis (see 1.1). By using this fragment as a probe, we adopted a PCR library screening technique (Hamilton, 1991). In this experiment, we designed two primers, 2br.A and 2br.1 within the genomic DNA fragment, and two primers on the λgt10 vector, forward (λgt10.for.) and reverse (λgt10.rev.). Using four combinations of primers, we screened a mouse cDNA library and looked for overlapping clones because we did not know the orientation of the insert (Fig. 1A).

The brain library was diluted to 2.5×10^5 pfu/μl. Four separate PCR reactions were performed for each set of primer combination, and a total of 1×10^6pfu were screened for each set of primers. The 16 PCR products were separated on 1% agarose gel, blotted and hybridized with the 2br37 probe. Three positive products were detected in 2brA/ gt10.for primed PCR and two were found in 2brA/gt10.rev primed PCR (Fig. 2). The size of these fragments ranged from 400 to 600 bp. These positive PCR clones were subjected to second PCR by using a nested primer 2br.B, as shown (Fig. 1B). The second PCR product was seperated on agarose gel side by side with the original PCR clone and Southern hybridization was performed with 2br37 as probe. The result showed positive signals for both clones and the size of the second clone was as expected (Fig. 3).

Fig. 1 Library PCR Screening Design. A. Four primers were used in four different combination, λ gt10.rev/2br.A, λ gt10.rev/2br.1, λgt10.for/2br.1, and λgt10.for/2br.A. B. Nested primer 2br.B was used for second screening.

A.



B.

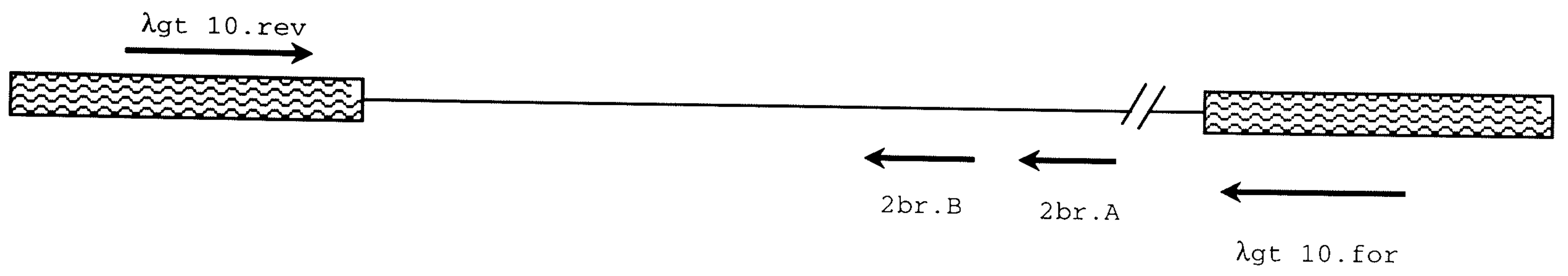
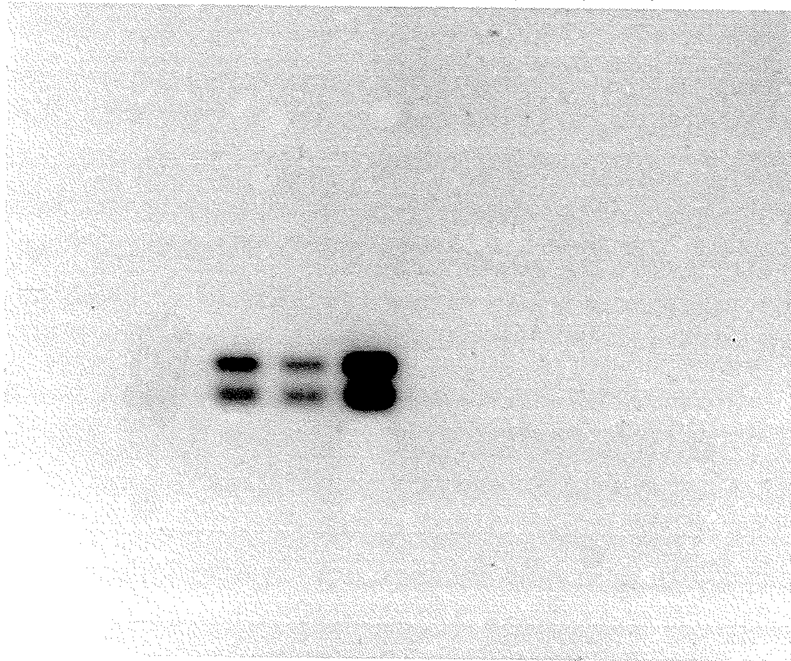


Fig. 2 First screening of mouse brain library yielded 5 positive PCR products after blotting and hybridizing with 2br37. The lanes 1.1, 1.2, 1.3, and 1.4 are PCR products of the primer set λ gt10.for/2br.A (refer to Fig. 1), and three of them show a 0.4 and a 0.5 Kb DNA fragment. The lanes 3.1, 3.2, 3.3 and 3.4 are PCR products of the primer set λgt10.rev/2br.A (refer to Fig. 1). Lane 3.1 and 3.2 show a 0.6 Kb DNA fragment.


1.1
1.2
1.3
1.4
2.1
2.2
2.3
2.4
Kb
0.5
0.4

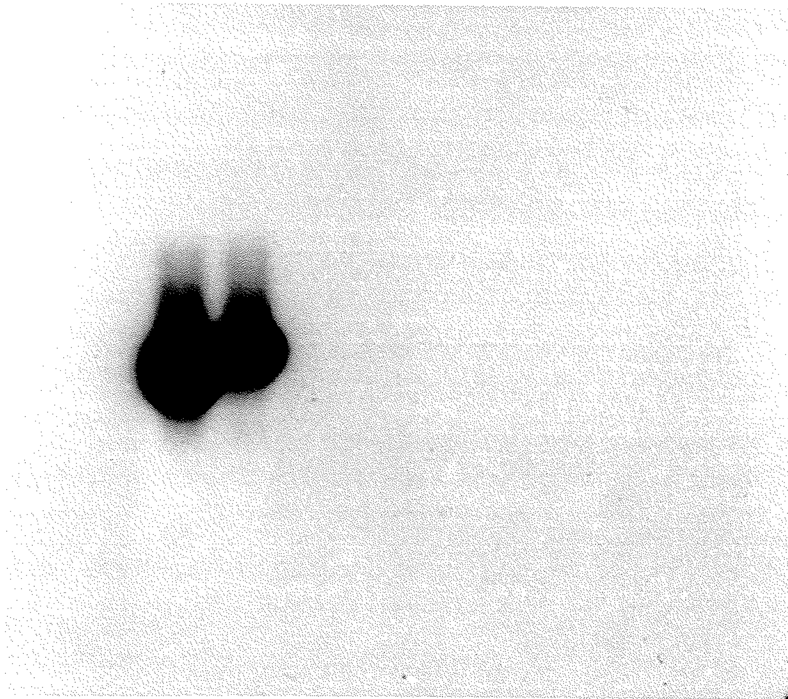
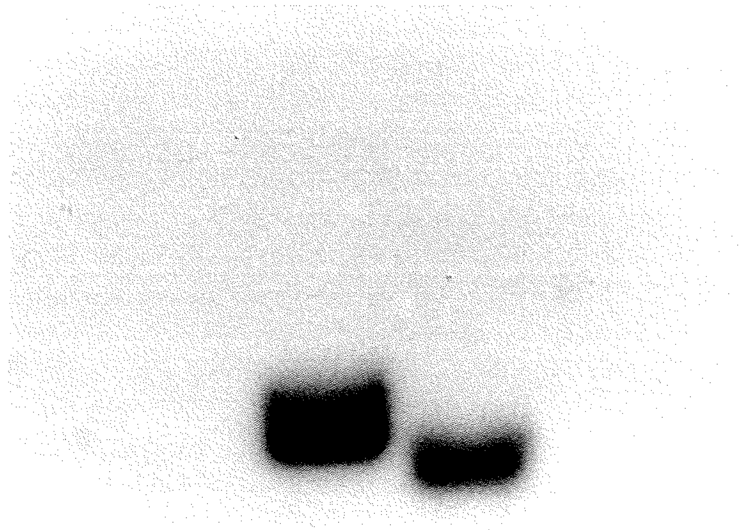

0.6
3.1
3.2
3.3
3.4
4.1
4.2
4.3
4.4

Fig. 3 A second PCR using the nested primer set λgt10.rev/2br.b (Fig. 1) on the first PCR products (Fig. 2, lane 3.1) results in the product named 2brC1 clone (lane 2) which is 41 nucleotides less than the first PCR product (lane 1).

1 2

Kb



0.5

2. Subcloning Analysis

The positive second PCR clone, was then subcloned into a TA cloning vector. The plasmids were purified and digested with the EcoR I restriction enzyme. The fragments were separated on agarose gel, blotted and hybridized with the 2br.37 probe. Three clones were detected and they were similar in size (0.5Kb) (Fig. 4)

3. DNA Sequencing Analysis

The DNA sequence was determined as described in 'Methods' and read on a Bio-Rad Genereader, then compared with the original sequence of 2br.37. The result is shown in Figure 5. The new PCR clone, named, 2brC1 was identical with complementary nucleotides of 2br37, starting from the 75th nucleotide to the 3' end (Fig. 5). There are 6 mismatched nucleotides. In addition, 270 bp of new sequence was found at the 3' end of 2brC1. Sequence analysis revealed a poly-(A) signal sequence in a A-T rich region (Fig. 5).

4. cDNA Library Screening Analysis

By using the PCR clone 2brC1 as a probe, the mouse brain cDNA library and mouse Swiss 3T3 fibroblast cDNA library were screened. Only one positive clone was detected in fibroblast cDNA library after screening 1.5 x 10^6 plaques. The positive clone was plated for the second and third time to isolate the single plaque. Each plate was blotted by duplicate membranes and hybridized with 2brC1, as described in 'Methods'. The clone was confirmed to be true positive after second and third screening (Fig. 6). After subcloning and sequencing, a 650 bp cDNA clone was identified, which overlapped with 2brC1 and had 185 bp of new sequence on the 5' end (Fig.7). This clone was named SW1(short for first cDNA clone from Swiss 3T3 library). The open reading frame analysis of cDNA clone SW1 showed a potential open reading frame, but did not contain a start codon (Fig.12).

Fig. 4 The second PCR product was subcloned into the TA vector. After restriction digestion by EcoRI, the insert was separated on agarose gel, blotted and hybridized again with 2br37. The insert has a size of less than 0.5 Kb (lower band, complete digestion without anchor primer sequence). The upper band may due to the noncomplete digestion with anchor primer sequence in it, because there is one EcoRI site in the cloning site of TA vector. The three different clones were subsequently sequenced and proved to be identical.

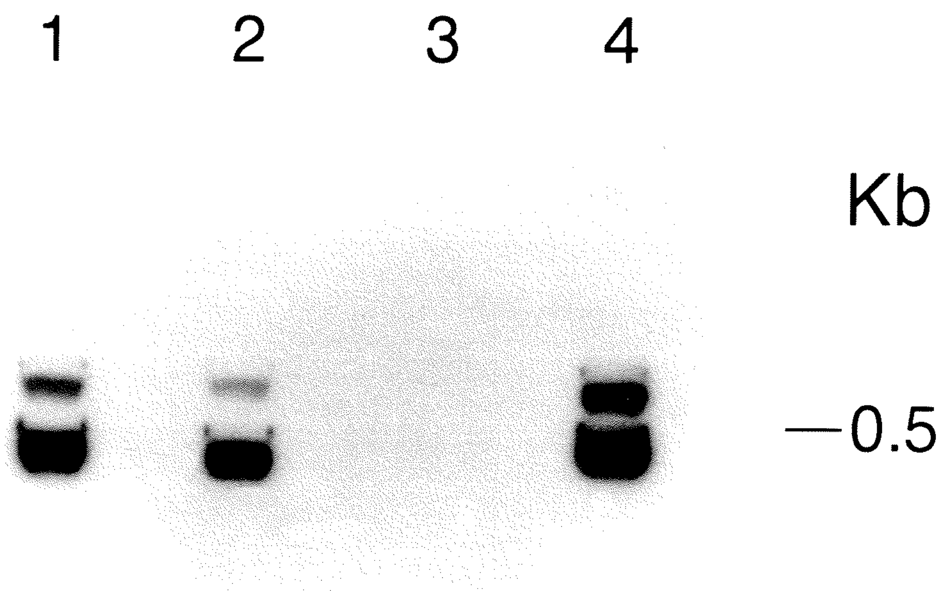

1
2
3
4
Kb
0.5

Fig. 5 Comparison of PCR Clone 2brC1 With Genomic DNA Clone 2br37 Complementary Sequence by Global Alignments Program in NIHMBUG Software (Wilbur, W. J. & Lipman D. J., 1983). 135 Out of 141 Nucleotides at the 5' End of 2brC1 Matched to 2br37 Complementary Sequence.

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Global Alignments of DNA Sequences

ALIGNMENT OF:

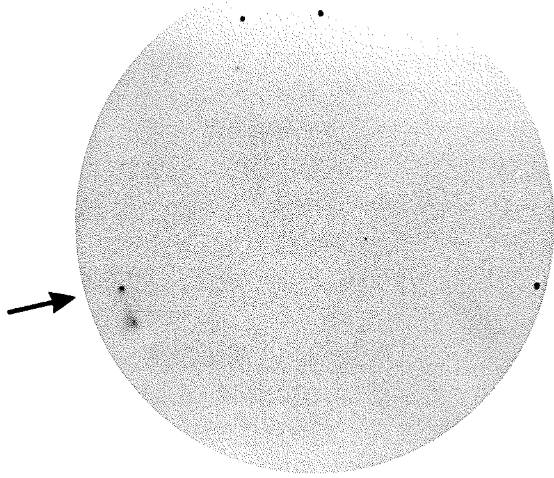
2br37 215 BASES VERSUS *2brc1 467 BASES*

```
2br37    1  AAGGATGTGCTCGGTTGGAAGGTGAGCGAGTGCGTGTTACTCTGCACCCCTGCGCCCGTC

2br37   61  TAGGGTACTCGATGCGCCCCACCAACCCGACTCCTACGCAGAGCGGTGCACGGCCCGGCA
                          ::::::::::::::::::::::::::::  :  :::::::::: ::
2brc1    1                CGCCCCACCAACCCGACTCCTACGCAGACGGTGGCACGGCCCG-CA

2br37  121  AGGTTCCCCCAGTGGATGGTT-GCTCCGCCCCGCCTACAGAGGCAATGATCAGGCGGCTT
            ::::::::::::::::::::: ::::::::::::::::::::::::::::::::::::::
2brc1   46  AGGTTCCCCCAGTGGATGGTTCGCTCCGCCCCGCCTACAGAGGCAATGATCAGGCGGCTT

2br37  180  CTGCTGGTTTCAAGACTCCCGCGTCCGACTCACTGT
            :::::::::::::::::::::::::::::::::::
2brc1  106  CTGCTGGTTTCAAGACTCCCGCGTCCGACTCACTGGGGACCTTGCCAGGCCTTGGCCTTC

2brc1  166  CGAACCTCTTAAGGGTTGGGGGCAGTGGTCTTTAGTGGTGCTCCCTGGCGCTCACATAGA

2brc1  226  CGCAGCCTTGGGTCGGGCTAGTCTCTGGGACACCGGCCAGCCTCCGCCTTCAGAGCTGCG

2brc1  286  CGAGCGTTGTCTATCATGGTACACAGACTGGAATGAGCTTGTTCTTAACCTCAGCTTCAC

2brc1  346  TGTCCTCCTCCCCCACAGAGACTATTCCAGGATGCGCGACAGACACACAAGACACTCGGT

2brc1  406  GAGGGTTAAAGATCTTTAAATAAGGAGCCAGTGACAATAAATAATACTGTACAGGGGGCC
```

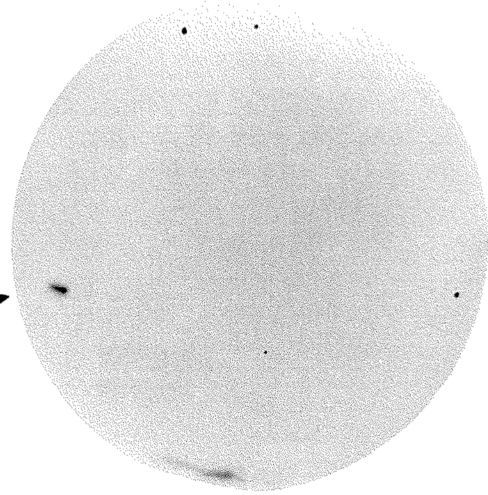
NUMBER OF MATCHED BASES=135

Fig. 6 Screening of Swiss 3T3 fibroblast cDNA library. Plate 1,2 are duplicate membranes of the first screening using 2brC1 as a probe. Only one positive clone was detected on both membranes. Plate 3,4 are duplicate membranes of the second screening of the positive clone. Plate 5,6 are duplicate membranes of the third screening. The second and third screening confirmed the positive clone.

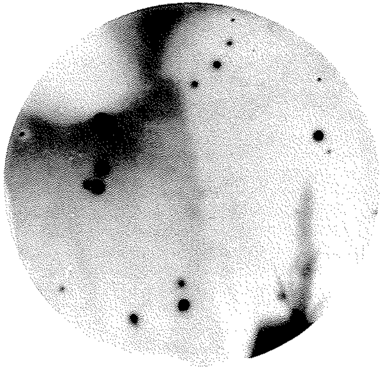
1



2



3



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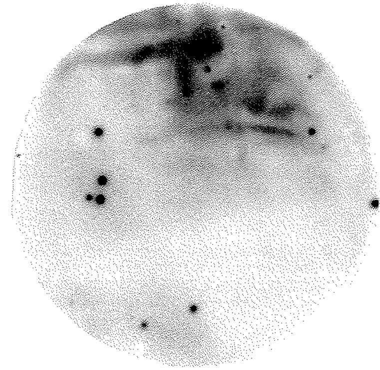
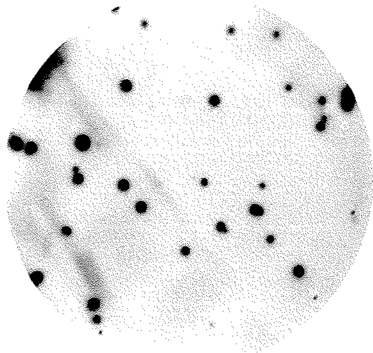


5



6

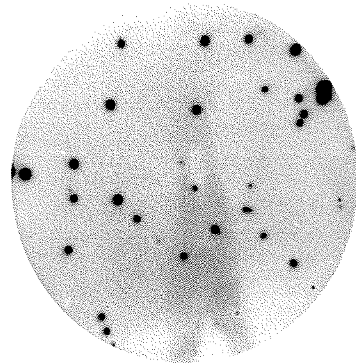


Fig. 7 DNA Sequence Comparison of PCR Clone 2brC1 and cDNA Clone SW1. The nucleotide sequence of SW1 is overlapping with that of 2brC1, starting from the position 186th of SW1. In addition, there are 185 new nucleotides on 5'end. The poly (A) signal is in block letters.

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Global Alignments of DNA Sequences

ALIGNMENT OF:

2brc1 467 BASES VERSUS sw1 651 BASES

```
sw1      1  CGGATGCCCAATGTTTTCGGGAAAGAGAGCAAGAAGAAAGAGCTGGTCAACAACCTGGGA
sw1     61  GAGATCTACCAGAAGACAAGGGCGGCTCCCGGAAGGATGTGCTCGGTTGGAAGGTGAGGC
sw1    121  AAGTGGACGACGGGTGGCGAGTGCGTGTTACTCTGCACCCCTGCGCCCGTCTAGGGTACT

2brc1    1       CGCCCCACCAACCCGACTCCTACGCAGACGGTGGCACGGCCCGCAAGGTTCCCCC
                 :::::::::::::::::::::::::::::::::::::::::::::::::::::::
sw1    181  GCATGCGCCCCACCAACCCGACTCCTACGCAGACGGTGGCACGGCCCGCAAGGTTCCCCC

2brc1   56  AGTGGATGGTTCGCTCCGCCCCGCCTACAGAGGCAATGATCAGGCGGCTTCTGCTGGTTT
            ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
sw1    241  AGTGGATGGTTCGCTCCGCCCCGCCTACAGAGGCAATGATCAGGCGGCTTCTGCTGGTTT

2brc1  116  CAAGACTCCCGCGTCCGACTCACTGGGGACCTTGCCAGGCCTTGGCCTTCCGAACCTCTT
            :::::::::::::::::::::::::: :::::::::::::::::::::::::::::::::
sw1    301  CAAGACTCCCGCGTCCGACTCACTGGAGACCTTGCCAGGCCTTGGCCTTCCGAACCTCTT

2brc1  176  AAGGGTTGGGGGCAGTGGTCTTTAGTGGTGCTCCCTGGCGCTCACATAGACGCAGCCTTG
            ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
sw1    361  AAGGGTTGGGGGCAGTGGTCTTTAGTGGTGCTCCCTGGCGCTCACATAGACGCAGCCTTG

2brc1  236  GGTCGGGCTAGTCTCTGGGACACCGGCCAGCCTCCGCCTTCAGAGCTGCGCGAGCGTTGT
            ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
sw1    421  GGTCGGGCTAGTCTCTGGGACACCGGCCAGCCTCCGCCTTCAGAGCTGCGCGAGCGTTGT

2brc1  296  CTATCATGGTACACAGACTGGAATGAGCTTGTTCTTAACCTCAGCTTCACTGTCCTCCTC
            ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
sw1    481  CTATCATGGTACACAGACTGGAATGAGCTTGTTCTTAACCTCAGCTTCACTGTCCTCCTC

2brc1  356  CCCCACAGAGACTATTCCAGGATGCGCGACAGACACACAAGACACTCGGTGAGGGTTAAA
            ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
sw1    541  CCCCACAGAGACTATTCCAGGATGCGCGACAGACACACAAGACACTCGGTGAGGGTTAAA

2brc1  416  GATCTTTAAATAAGGAGCCAGTGACAATAAATAATACTGTACAGGGGGCCCG
            :::::::::::::::::::::::::::::::::::::::::::::::: ::
sw1    601  GATCTTTAAATAAGGAGCCAGTGACAATAAATAATACTGTACAGGGGCCCG
```

NUMBER OF MATCHED BASES=463

5. Southern Blot Analysis

By using the SW1 cDNA as a probe, genomic DNA from mouse 10T1/2, MRPD and 2BRVI were digested separately by either Bam HI, Hind III, Sca I or Xba I, and Southern blotting was performed. The results (Fig. 8A and 9A) showed that in Bam HI, Hind III and Sca I digested DNA, a single band was detected, but there was a DNA band shift to a larger fragment in the metastatic cell 2br VI compared with that in normal parental 10T1/2 as well as non-infected MRPD cells from which the 2brVI was derived. In Xba I digested DNA, there was no change in restriction fragment size detected. Densitometric analysis of the intensity of different bands demonstrated that the intensity of the larger bands in metastatic 2br37 cells are reduced by approximately half of that in normal 10T1/2 and MRPD cells (Fig. 8B and 9B).

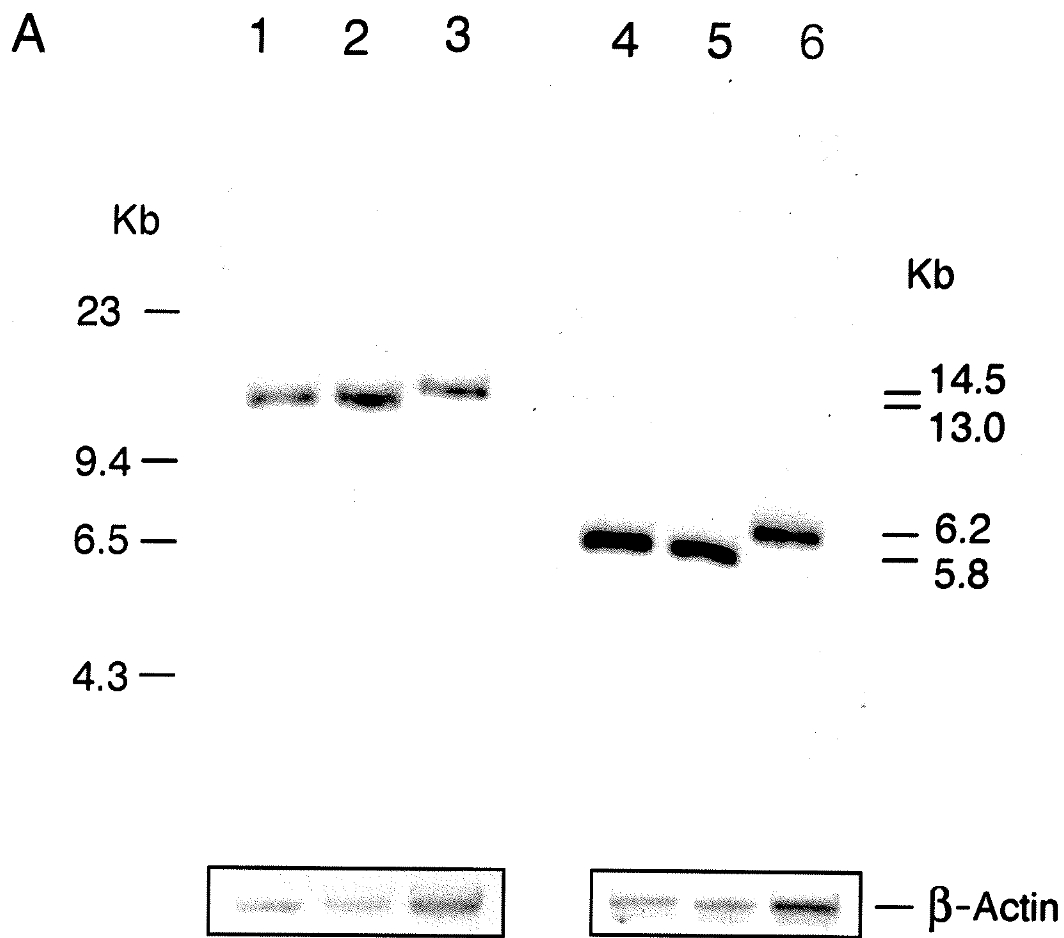
6. 5' RACE Cloning Analysis

Sequence analysis of SW1 still did not show any start codon which would match the Kozak rule (Kozak, M., 1991), which meant that the 5' end of the reading frame was still missing. Since the poly (A) signal sequence is found in a A-T rich region following the stop codon (Fig. 12), it suggested that the clone SW1 is the 3' end of the complete open reading frame of the cDNA. In order to get a complete open reading frame, we tried rapid amplification of 5' cDNA end (5' RACE) since other attempts at library screening failed to yield more new sequences. After cloning and sequencing, a new clone, named RACE 5 was identified. In addition to the overlapping sequences with SW1, Race 5 yielded 139 new nucleotide sequence (Fig. 10).

Fig. 8 **A.** Southern Blotting Analysis of genomic DNA from 10 T1/2 (lane 1, 4), MRPD (lane 2, 5) and 2brVI (lane 3, 6). DNA was digested by BamH (lanes 1, 2, 3) or Hind III (lanes 4, 5, 6). After hybridization with SW1 cDNA, A larger band was detected in 2br VI in both BamH I (14.5Kb) and Hind III (6.2 Kb) digested DNA comparing with the normal band of 13 Kb and 5.8Kb in 10T1/2 and MRPD DNA.

B. Densitometric analysis demonstrated that the intensity of the band in 2brVI is approximately half of that in 10T1/2 or MRPD cells. This indicates that about half of the gene copy was deleted in metastatic 2br VI cells.

* The panels between A and B are DNA loading control with β-actin as probe.


A
1
2
3
4
5
6
Kb
23
9.4
6.5
4.3
Kb
14.5
13.0
6.2
5.8
β-Actin

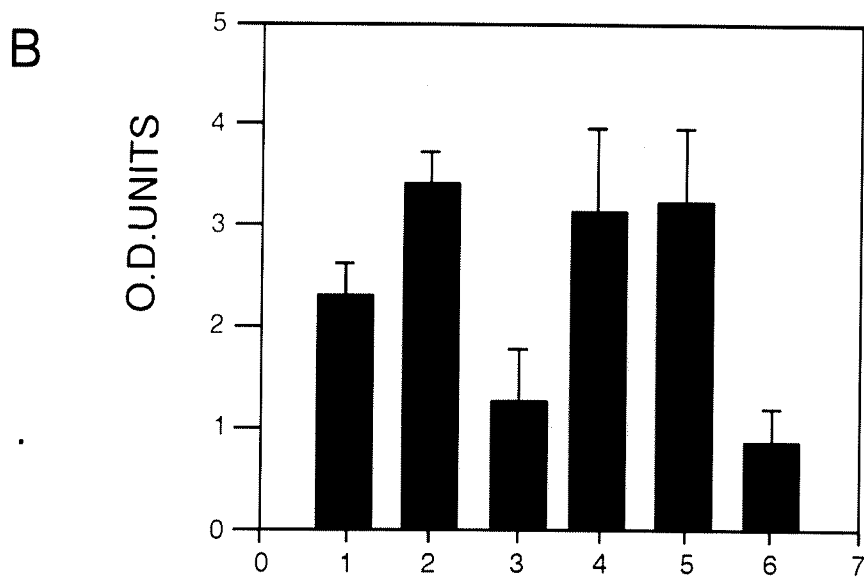
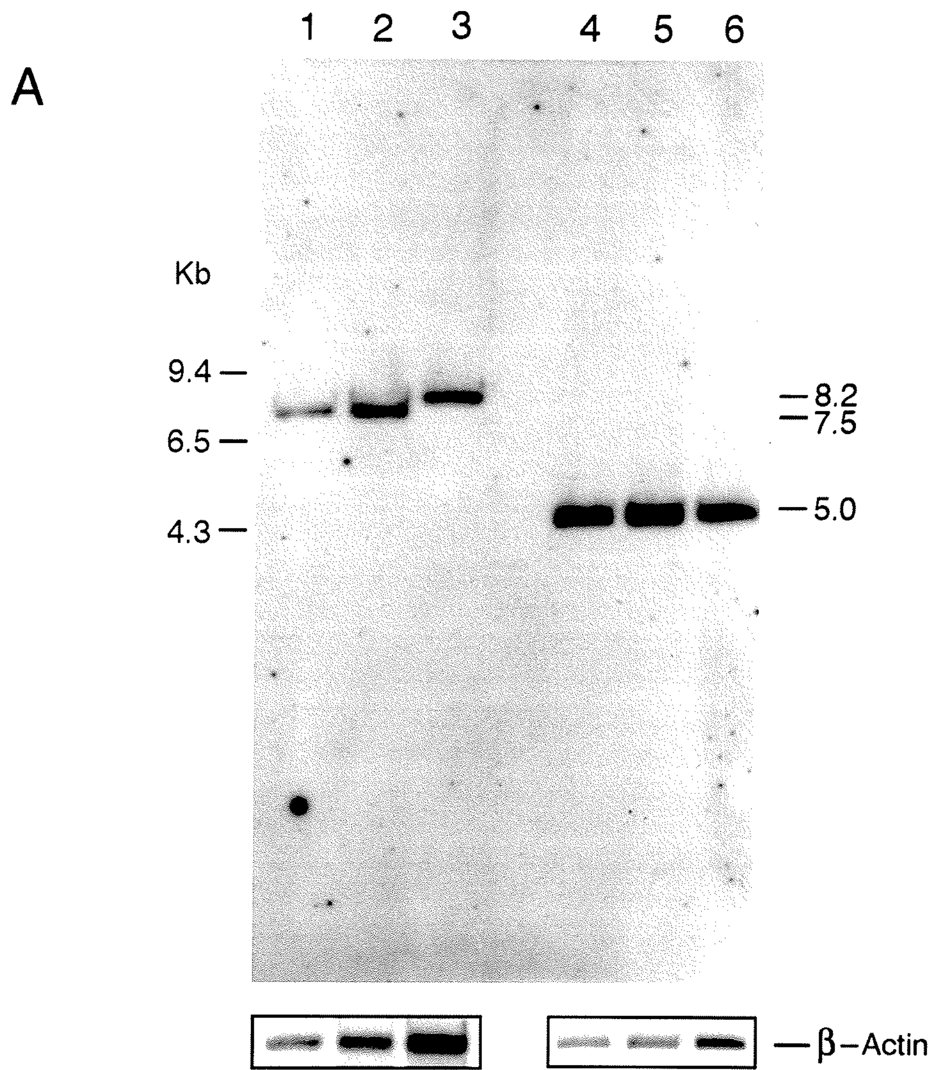

B
O.D.UNITS
0
1
2
3
4
5
6
7

Fig. 9 **A.** Southern Blotting Analysis of genomic DNA from 10 T1/2 (lane 1,4), MRPD (lane 2, 5) and 2brVI (lane 3, 6). Genomic DNA was digested by Sca I (lanes 1, 2, 3) or Xba I (lanes 4, 5, 6). After hybridization with SW1 cDNA, a larger band (8.3 Kb) was detected in 2brVI in Sca I digested DNA comparing with the normal band (7.1 Kb) in 10T1/2 and MRPD DNA. The Xba I digested DNA did not show any change of size (5.0 Kb) among the three cell lines.

B. Densitometric analysis demonstrated that the intensity of the band in 2brVI is approximately half of that in 10T1/2 or MRPD cells. As shown in Fig.8 for other enzymes, about half of the gene copy number was deleted in metastatic 2br VI cells.

* The panels shown between A and B are DNA loading control with β-actin as probe.


A
1
2
3
4
5
6
Kb
9.4
6.5
4.3
8.2
7.5
5.0
β-Actin

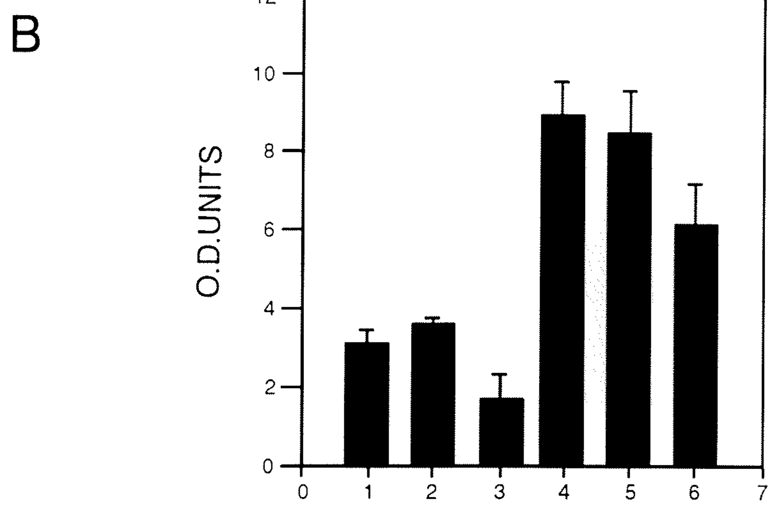

B
O.D.UNITS
12
10
8
6
4
2
0
0
1
2
3
4
5
6
7

Fig. 10 Sequence comparison of the cDNA clone Race 5 isolated by the 5' RACE method (see 2.9) with cDNA clone SW1. The 3' end 150 nucleotides of Race5 matched the 5'end of SW1 sequence starting from position 70. The difference at 5' end may due to differentially spliced mRNA.

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Global Alignments of DNA Sequences

ALIGNMENT OF:

race5 289 BASES **VERSUS** *sw1 651 BASES*

```
race5   1    GATGTCACCTGAATCTAGTAGCGCGCAGCATGGAACGCGAGTCGCGACCCCGGGTCCCCA
race5   61   CGGCGGCGCGCGTCTCCCGGGACACGAGGGTTGGGCGGCGGGATAGGAATTCAACATTCA

sw1     1                                    CGGATGCCCAATGTTTTCGGGAAAGAGAGCAAG

race5   121  AAGAAAGAGCAGGTGGATTTTTTTTTTTTAAGACAAGGGCGGCTCCCGGAAGGATGTGCT
                                              :::::::::::::::::::::::::::::::
sw1     44   TGGTCAACAACCTGGGAGAGATCTACCAGAAGACAAGGGCGGCTCCCGGAAGGATGTGCT

race5   171  CGGTTGGAAGGTGAGGCAAGTGGACGACGGGTGGCGAGTGCGTGTTACTCTGCACCCCTG
             ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
sw1     104  CGGTTGGAAGGTGAGGCAAGTGGACGACGGGTGGCGAGTGCGTGTTACTCTGCACCCCTG

race5   231  CGCCCGTCTAGGGTACTGCATGCGCCCCACCAACCCGACTCCTACGCAGACGGTGGCAC
             :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
sw1     164  CGCCCGTCTAGGGTACTGCATGCGCCCCACCAACCCGACTCCTACGCAGACGGTGGCACG


sw1     224  GCCCGCAAGGTTCCCCCAGTGGATGGTTCGCTCCGCCCCGCCTACAGAGGCAATGATCAG
sw1     284  GCGGCTTCTGCTGGTTTCAAGACTCCCGCGTCCGACTCACTGGAGACCTTGCCAGGCCTT
sw1     344  GGCCTTCCGAACCTCTTAAGGGTTGGGGGCAGTGGTCTTTAGTGGTGCTCCCTGGCGCTC
sw1     404  ACATAGACGCAGCCTTGGGTCGGGCTAGTCTCTGGGACACCGGCCAGCCTCCGCCTTCAG
sw1     464  AGCTGCGCGAGCGTTGTCTATCATGGTACACAGACTGGAATGAGCTTGTTCTTAACCTCA
sw1     524  GCTTCACTGTCCTCCTCCCCCACAGAGACTATTCCAGGATGCGCGACAGACACACAAGAC
sw1     584  ACTCGGTGAGGGTTAAAGATCTTTAAATAAGGAGCCAGTGACAATAAATAATACTGTACA
sw1     644  GGGGCCCG
```

NUMBER OF MATCHED BASES=173

7. Predicted Protein Product of cDNA Clones

Based on the facts that the sequence of three PCR clones (Fig. 4) and three cDNA clones (data not shown) are all identical at 3' end as cDNA clone SW1, I assume that the sequence of 5' race clone would be the same at 3' end as cDNA SW1. Sequence analysis (NIHMBUG) of the 5' RACE clone RACE5 showed the largest predicted open reading frame of 140 amino acids (Fig. 11). The 5' end sequences of SW1 and RACE5 are different (Fig. 10). Sequence analysis on SW1 does not show a possible start codon (Fig.12) whereas the 5' end of RACE5 (Fig. 11) does show a start codon that fits in Kozak rule which states that the minus 3 position of a cDNA sequence is usually a purine and the plus 4 position should be a guanine nucleotide (Kozak, M., 1991). The molecular weight of the deduced protein is about 15 kD(Fig. 13). The other five reading frames have many scattered stop codons which make them less likely to be open reading frame. The estimated molecular weight of the protein and pI and amino acid composition are shown (Fig. 13).

8. Multiple DNA Alignment Analysis

Multiple DNA alignment was made of the genomic DNA fragment 2br37, PCR clone 2brC1, cDNA clone SW1 and 5' RACE clone (Fig. 15). By comparing 2br37 sequence with that of cDNA clone SW1 and RACE5, it was found that a 19 nucleotide fragment was deleted within the genomic clone 2br37. The multiple alignment also detected two point mutation of A to G and C to G, as well as a single T nucleotide deletion in PCR clone 2brC1, based on cDNA clone SW1 sequence. These may due to PCR errors.

Fig. 11 **Predicted open reading frame of metastisis associated gene (MAG) which is derived from integration of the 5' end of cDNA clone RACE5 and SW1 sequence.**

PREDICTED OPEN READING FRAME of RACE5

1 GAT GTC ACC TGA ATC TAG TAG CGC GCA GCA TGG AAC GCG AGT

M E R E S

43 CGC GAC CCC GGG TCC CCA CGG CGG CGC GCG TCT CCC GGG ACA

R P R V P T A A R V S R D T

85 CGA GGG TTG GGC GGC GGG ATA GGA ATT CAA CAT TCA AGG TGG

R V G R R D R N S T F K V G

127 GAT TTT TTT TTT TAA GAC AAG GGC GGC TCC CGG AAG GAT GTG

F F F L R Q G R L P E G C A

169 CTC GGT TGG AAG GTG AGG CAA GTG GAC GAC GGG TGG CGA GTG

R L E G E A S G R R V A S A

211 CGT GTT ACT CTG CAC CCC TGC GCC CGT CTA GGG TAC TGC ATG

C Y S A P L R P S R V L H A

253 CGC CCC ACC AAC CCG ACT CCT ACG CAG ACG GTG GCA CGG CCC

P H Q P D S Y A D G G T A R

295 GCA AGG TTC CCC CAG TGG ATG GTT CGC TCC GCC CCG CCT ACA

K V P P V D G S L R P A Y R

337 GAG GCA ATG ATC AGG CGG CTT CTG CTG GTT TCA AGA CTC CCG

G N D Q A A S A G F K T P A

379 CGT CCG ACT CAC TGG AGA CCT TGC CAG GCC TTG GCC TTC CGA

S D S L E T L P G L G L P N

421 ACC TCT TAA GGG TTG GGG GCA GTG GTC TTT AGT GGT GCT CCC

L L R V G G S G L Z

463 TGG CGC TCA CAT AGA CGC AGC CTT GGG TCG GGC TAG TCT CTG
505 GGA CAC CGG CCA GCC TCC GCC TTC AGA GCT GCG CGA GCG TTG
547 TCT ATC ATG GTA CAC AGA CTG GAA TGA GCT TGT TCT TAA CCT
589 CAG CTT CAC TGT CCT CCT CCC CCA CAG AGA CTA TTC CAG GAT
631 GCG CGA CAG ACA CAC AAG ACA CTC GGT GAG GGT TAA AGA TCT
673 TTA AAT AAG GAG CCA GTG ACA ATA AAT AAT ACT GTA CAG GGG
715 CCC G

Fig. 12 Predicted open reading frame from sw1 sequence. The 5'end of cDNA clone sw1 is different from that of clone RACE5. The amino acids in bold letter show the identical amino acids between the two clones in the same open reading frame. The 5'end of the clone SW1 does not show a possible start codon whereas the 5'end of the clone RACE5 does (Fig. 11).

PREDICTED OPEN READING FRAME OF SW1

```
1      CGG ATG CCC AAT GTT TTC GGG AAA GAG AGC AAG AAG AAA GAG
         G   C   P   M   F   S   G   K   R   A   R   R   K   S
43     CTG GTC AAC AAC CTG GGA GAG ATC TAC CAG AAG ACA AGG GCG
         W   S   T   T   W   E   R   S   T   R   R   Q   G   R
85     GCT CCC GGA AGG ATG TGC TCG GTT GGA AGG TGA GGC AAG TGG
         L   P   E   G   C   A   R   L   E   G   E   A   S   G
127    ACG ACG GGT GGC GAG TGC GTG TTA CTC TGC ACC CCT GCG CCC
         R   R   V   A   S   A   C   Y   S   A   P   L   R   P
169    GTC TAG GGT ACT GCA TGC GCC CCA CCA ACC CGA CTC CTA CGC
         S   R   V   L   H   A   P   H   Q   P   D   S   Y   A
211    AGA CGG TGG CAC GGC CCG CAA GGT TCC CCC AGT GGA TGG TTC
         D   G   G   T   A   R   K   V   P   P   V   D   G   S
253    GCT CCG CCC CGC CTA CAG AGG CAA TGA TCA GGC GGC TTC TGC
         L   R   P   A   Y   R   G   N   D   Q   A   A   S   A
295    TGG TTT CAA GAC TCC CGC GTC CGA CTC ACT GGA GAC CTT GCC
         G   F   K   T   P   A   S   D   S   L   E   T   L   P
337    AGG CCT TGG CCT TCC GAA CCT CTT AAG GGT TGG GGG CAG TGG
         G   L   G   L   P   N   L   L   R   V   G   G   S   G
379    TCT TTA GTG GTG CTC CCT GGC GCT CAC ATA GAC GCA GCC TTG
         L   Z
421    GGT CGG GCT AGT CTC TGG GAC ACC GGC CAG CCT CCG CCT TCA
463    GAG CTG CGC GAG CGT TGT CTA TCA TGG TAC ACA GAC TGG AAT
505    GAG CTT GTT CTT AAC CTC AGC TTC ACT GTC CTC CTC CCC CAC
547    AGA GAC TAT TCC AGG ATG CGC GAC AGA CAC ACA AGA CAC TCG
589    GTG AGG GTT AAA GAT CTT TAA ATA AGG AGC CAG TGA CAA TAA
631    ATA ATA CTG TAC AGG GGC CCG
```

Fig. 13 **Open reading frame analysis of the MAG gene derived from RACE5.**

>from DNA sequence Mag 30 to 449, length 149, 5'end-Ter

MERESRPRVPTAARVSRDTRVGRRDRNSTFKVGFFFLRQGRLPEGCARLEGEASGRRVASACYSAPLRPS

RVLHAPHQPDSYADGGTARKVPPVDGSLRPAYRGNDQAASAGFKTPASDSLETLPGLGLPNLLRVGGSGL*

Frame 3

Range	Nucl. Length	Polypeptide Length	Type of orf
30 to 449	420	140	Met-Ter

The estimated molecular weight of protein Mag

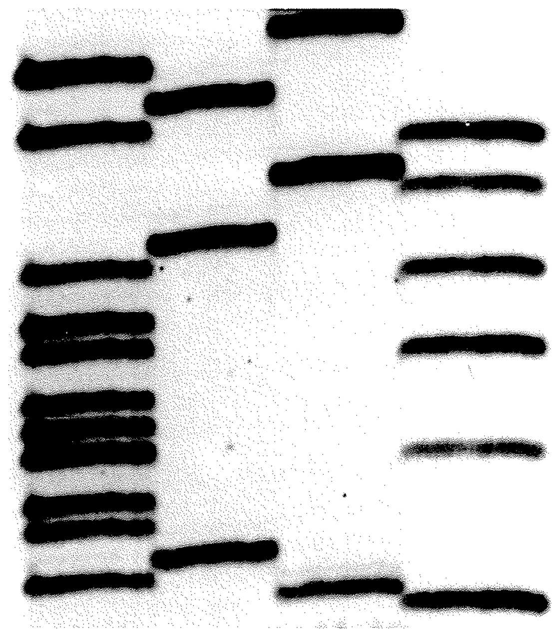
is 14997 daltons(~15Kd) and the estimated pI is 8.78

AMINO ACID COMPOSITION

Ala A: 15(10.7%)	Arg R: 20(14.3%)	Asn N: 3(2.1%)	Asp D: 7(5.0%)
Gln Q: 3(2.1%)	Glu E: 6(4.3%)	Gly G: 16(11.4%)	His H: 2(1.4%)
Iso I: 0(0.0%)	Leu L: 13(9.3%)	Lys K: 3(2.1%)	Met M: 1(0.7%)
Phe F: 5(3.6%)	Pro P: 13(9.3%)	Ser S: 13(9.3%)	Thr T: 6(4.3%)
Cys C: 2(1.4%)	Trp W: 0(0.0%)	Tyr Y: 3(2.1%)	Val V: 9(6.4%)

Fig. 14 A. Polyadenylation signal AATAAA (in block letters) from cDNA clone SW1 sequence. B. DNA sequence analysis shows that an ATG start codon (in block letters) from clone RACE5 which fits the Kozak rule (position -3 is an Adenine and +4 is a guanine nucleotide)

A



B

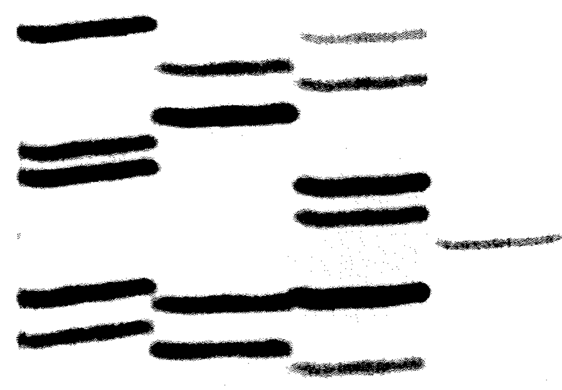


Fig. 15 CLUSTAL W(1.5) Multiple Sequence Alignment. The complete DNA sequence comparison of four clones 2br37, 2brC1, SW1 and Race 5. The star signs idicate identical nucleotides among the alignments. The space between the stars at the bottom line indicate that there is a disagreement on that nucleotide between any two clones.

```
BR37              ------------------------------------------------------------
2BRC1             ------------------------------------------------------------
SW1               ------------------------------------------------------------
RACE5    1        GATGTCACCTGAATCTAGTAGCGCGCAGCATGGAACGCGAGTCGCGACCCCGGGTCCCCAC

2BR37             ------------------------------------------------------------
2BRC1             ------------------------------------------------------------
SW1      1        ------CGGATGCCCAATGTTTTCGGGAAAGAGAGCAAGAAGAAAGAGCTGGTCAACAAC
RACE5    62       GGCGGCGCGCGTCTCCCGGGACACGAGGGTTGGGCGGCGGGATAGGAATTCAACATTCAA

2BR37    1        -------------------------------------AAGGATGTGCTCGGTTGGAAGG
2BRC1             ------------------------------------------------------------
SW1      55       CTGGGAGAGATCTACCAGAAGACAAGGGCGGCTCCCGGAAGGATGTGCTCGGTTGGAAGG
RACE5    123      GGTGGGATTTTTTTTTTTAAGACAAGGGCGGCTCCCGGAAGGATGTGCTCGGTTGGAAGG
                                  ******************************************

2BR37    23       TGAG------------------CGAGTGCGTGTTACTCTGCACCCCTGCGCCCGTCTAG
2BRC1             ------------------------------------------------------------
SW1      115      TGAGGCAAGTGGACGACGGGTGGCGAGTGCGTGTTACTCTGCACCCCTGCGCCCGTCTAG
RACE5    183      TGAGGCAAGTGGACGACGGGTGGCGAGTGCGTGTTACTCTGCACCCCTGCGCCCGTCTAG
                  ************************************************************

2BR37    64       GGTACTCGATGCGCCCCACCAACCCGACTCCTACGCAGAGCGGTGCACGGCCCGGCAAGG
2BRC1    1        -----------CGCCCCACCAACCCGACTCCTACGCAGACGGTGGCACGGCCCG-CAAGG
SW1      175      GGTACTGCATGCGCCCCACCAACCCGACTCCTACGCAGACGGTGGCACGGCCCG-CAAGG
RACE5    243      GGTACTGCATGCGCCCCACCAACCCGACTCCTACGCAGACGGTGGCAC------------
                  ***************************************   *   ********** *****

2BR37    124      TTCCCCCAGTGGATGGTT-GCTCCGCCCCGCCTACAGAGGCAATGATCAGGCGGCTTCTG
2BRC1    49       TTCCCCCAGTGGATGGTTCGCTCCGCCCCGCCTACAGAGGCAATGATCAGGCGGCTTCTG
SW1      234      TTCCCCCAGTGGATGGTTCGCTCCGCCCCGCCTACAGAGGCAATGATCAGGCGGCTTCTG
RACE5             ------------------------------------------------------------
                  ****************** *****************************************

2BR37    183      CTGGTTTCAAGACTCCCGCGTCCGACTCACTGT---------------------------
2BRC1    109      CTGGTTTCAAGACTCCCGCGTCCGACTCACTGGGGACCTTGCCAGGCCTTGGCCTTCCGA
SW1      294      CTGGTTTCAAGACTCCCGCGTCCGACTCACTGGAGACCTTGCCAGGCCTTGGCCTTCCGA
RACE5             ------------------------------------------------------------
                  ********************************  **************************

2BR37             ------------------------------------------------------------
2BRC1    169      ACCTCTTAAGGGTTGGGGGCAGTGGTCTT-AGTGGTGCTCCCTGGCGCTCACATAGACGC
SW1      354      ACCTCTTAAGGGTTGGGGGCAGTGGTCTTTAGTGGTGCTCCCTGGCGCTCACATAGACGC
RACE5             ------------------------------------------------------------
                  ***************************** ******************************

2BR37             ------------------------------------------------------------
2BRC1    228      AGCCTTGGGTCGGGCTAGTCTCTGGGACACCGGCCAGCCTCCGCCTTCAGAGCTGCGCGA
SW1      414      AGCCTTGGGTCGGGCTAGTCTCTGGGACACCGGCCAGCCTCCGCCTTCAGAGCTGCGCGA
RACE5             ------------------------------------------------------------
                  ************************************************************

2BR37             ------------------------------------------------------------
2BRC1    288      GCGTTGTCTATCATGGTACACAGACTGGAATGAGCTTGTTCTTAACCTCAGCTTCACTGT
SW1      474      GCGTTGTCTATCATGGTACACAGACTGGAATGAGCTTGTTCTTAACCTCAGCTTCACTGT
RACE5             ------------------------------------------------------------
                  ************************************************************

2BR37             ------------------------------------------------------------
2BRC1    348      CCTCCTCCCCCACAGAGACTATTCCAGGATGCGCGACAGACACACAAGACACTCGGTGAG
SW1      534      CCTCCTCCCCCACAGAGACTATTCCAGGATGCGCGACAGACACACAAGACACTCGGTGAG
RACE5             ------------------------------------------------------------
                  ************************************************************

2BR37             -----------------------------------------------------------
2BRC1    408      GGTTAAAGATCTTTAAATAAGGAGCCAGTGACAATAAATAATACTGTACAGGGGGCCCG
SW1      594      GGTTAAAGATCTTTAAATAAGGAGCCAGTGACAATAAATAATACTGTACAGGGGCCCG-
RACE5             -----------------------------------------------------------
                  ******************************************************* **
```

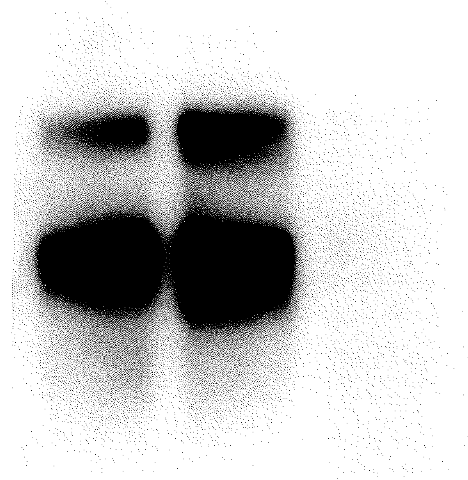
9. Northern Blot Analysis

The mRNAs were isolated from 10T1/2, MRPD and 2br VI cells. Northern hybridizations were performed. A 1.8 Kb and a 3.0 Kb transcript was detected using SW1 as probe in normal 10T1/2 and benign MRPD cells. These transcripts are absent or greatly reduced in metastatic 2br VI. (Fig. 16).

10. Secondary Structure Pattern Analysis of the Predicted MAG Protein

The PepPlot program is used to analyse the protein secondary structure (Chou and Fasman, 1978; Garnier, et al., 1978). Three CF α helices and two CF β sheets have been observed in the protein structure. The protein also shows a hydrophilic nature meaning that it is likely a cytosolic protein (Fig. 17). Amino acid sequence pattern analysis by using ExPASY program indicates several potential modification sites including one glycosylation site, four protein Kinase C (PKC) phosphorylation sites, one Casein kinase II phosphorylation site, one myristylation site and two amidation sites (Fig. 18).

Fig. 16 Northern Blotting analysis using cDNA clone sw1 as a probe shows that transcription is greatly reduced in metastatic 2brVI cells (lane 3) comparing with that in normal 10T1/2 (lane 1) and benign MRPD cells (lane 2). There are two normal transcripts with the size of 3.0 and 1.8 Kb. The bottom panel shows a loading control probed with β-actin.


1 2 3
Kb
3.0
1.8

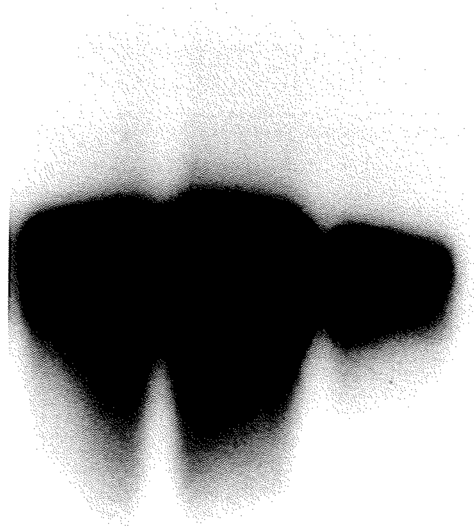

0.8

Fig. 17 **Secondary structure analysis of MAG protein. Bold arrow indicate α helices, dotted arrow indicate β sheet.**

PLOTSTRUCTURE of: magpro.pep ck: 6800

Garnier-Osguthorpe-Robson Prediction
February 20, 1996 19:00

KD Hydrophilicity >=1.3
KD Hydrophobicity >=1.3

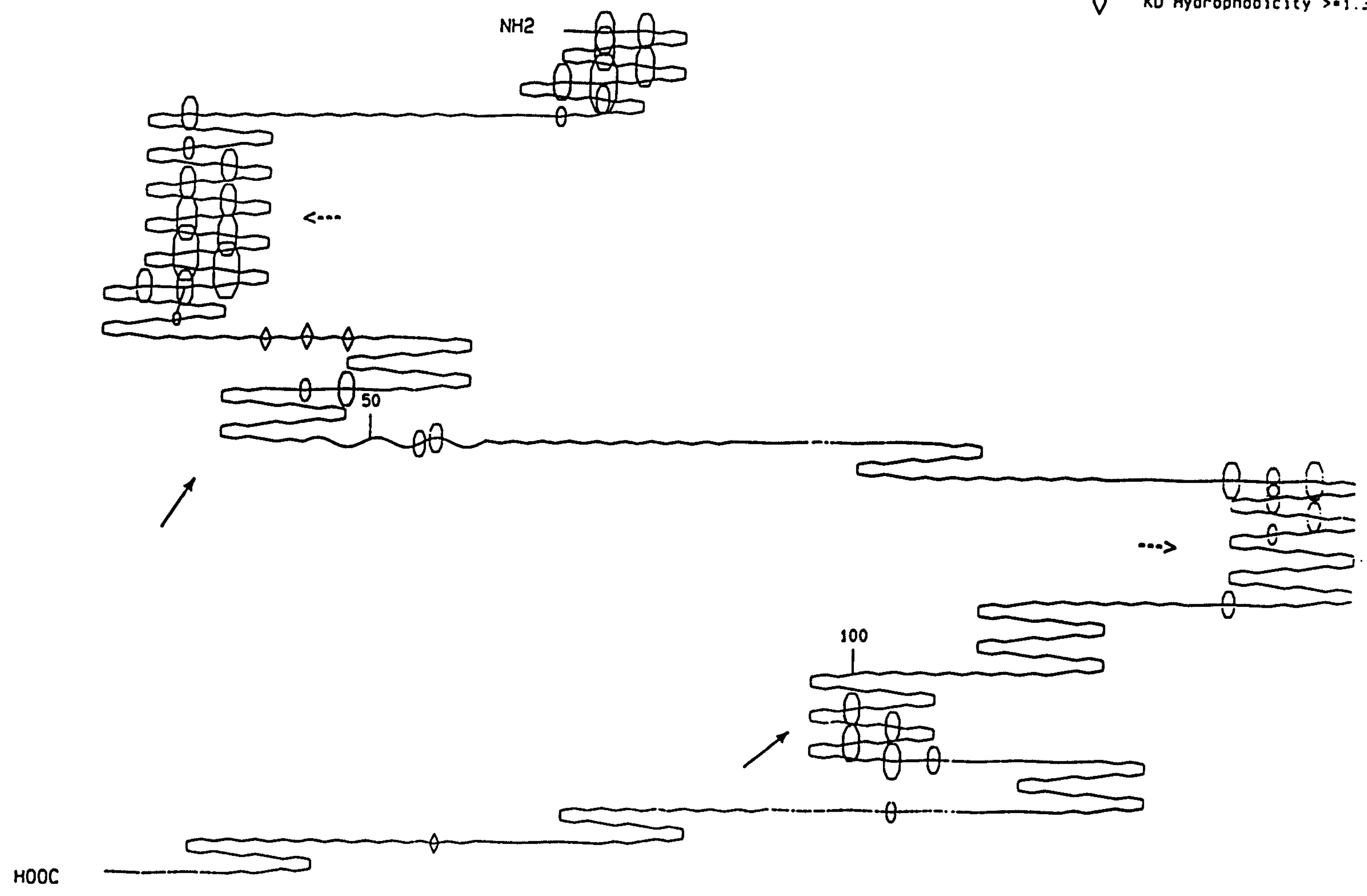


Fig. 18 **Amino acid pattern analysis of MAG protein using ExPASy program**

MERESRPRVP TAARVSRDTR VGRRDRNSTF KVGFFFLRQG RLPEGCARLE GEASGRRVAS
ACYSAPLRPS RVLHAPHQPD SYADGGTARK VPPVDGSLRP AYRGNDQAAS AGFKTPASDS
LETLPGLGLP NLLRVGGSGL

---

1 PDOC00001 PS00001 **ASN_GLYCOSYLATION**
N-glycosylation site

27-30 NSTF

---

2 PDOC00005 PS00005 **PKC_PHOSPHO_SITE**
Protein kinase C phosphorylation site

Number of matches: 4

1	29-31	TFK
2	54-56	SGR
3	87-89	TAR
4	97-99	SLR

---

3 PDOC00006 PS00006 **CK2_PHOSPHO_SITE**
Casein kinase II phosphorylation site

81-84 SYAD

---

4 PDOC00008 PS00008 **MYRISTYL**
N-myristoylation site

104-109 GNDQAA

---

5 PDOC00009 PS00009 **AMIDATION**
Amidation site

Number of matches: 2

1	21-24	VGRR
2	54-57	SGRR

IV. Discussion

1. Metastasis and Viral Insertinal Mutagenesis

Tumorigenesis and metastasis are interrelated processes which are thought to be caused by a variety of genetic mutations. Tumor formation does not necessarily lead to metastasis. The metastatic process therefore is an unique form of tumorigenesis which is characterized by invasion and remote colonization of pre-formed tumor cells (Liotta, L., 1991; Fidler, I. J., 1990). The multistep nature of cancer development in humans is well established (Vogelstein, et al. 1988; Fearon and Vogelstein. 1990). Oncogenes and tumor suppressor genes are the two main gene groups on which our current models of oncogenesis are based. As the biochemical mechanisms of these genes are unravelled, an examination of cancer development is necessary to see whether the multistep biological processes of cancer can be accounted for by these biochemical mechanisms.

The factors that bridge the transformation from benign to malignant tumor, the most challenging and significant part from the clinical point of view, are still not well understood. This has prompted many laboratories to hunt for these factors. A variety of methods have been applied including viral insertional mutagenesis which has been used to identify oncogenes and tumor suppressor genes. It is also an attractive technique to search for metastasis associated genes (MAG). The viral insertion event can either activate adjacent oncogenes (Goff, 1987) or inactivate tumor suppressor genes (Hicks and Mowat, 1988). Viral insertional metagenesis has a variety of effects on tumor formation. As mentioned earlier, retroviruses which do not carry oncogenic sequences can cause

tumors by either activating adjacent oncogenes or disrupting tumor suppressor genes. Retroviruses are reversely transcribed into viral DNA after they infect host cells. These viral DNA sequences exist in three forms (Varmus 1975; Weinberg 1977). The linear duplex DNA is the most abundant viral DNA in infected cells. These linear DNA are in latent form and located in the cytoplasm, which can migrate to nucleus under certain circumstances. The second form is circular viral DNA which located in nucleus of host cell, this viral DNA maintains the infectious property of the retrovirus. The third form is the viral DNA which is covalently integrated into the host genome. It is the integrated viral DNA which was demonstrated to be important in oncogenesis (for review see Temin, 1990 ; Peters, 1990; Kung. 1991).

Tumor induction by the transforming retroviruses is mediated by the constitutively expressed viral oncogenes carried by these viruses (Teich, N. et al. 1983; Bishop, J.M. and Varmus, H. 1983). However, viruses that lack an oncogene also induce tumors. The paradox of tumor induction by viruses that lacked an oncogene was first approached by a genetic analysis of the retrovirus genome. These studies revealed that transcription control sequences rather than coding sequences were the major determinants of the oncogenic potential of the virus (Tsichlis and Coffin, 1980; Lenz, J. et al. 1984). Most viral integrations are random events, the biological impact is dependent on whether or not the integration event can alter the expression level or mutate the protein product of the gene. As demonstrated in the integration of Mammary tumor virus(MMTV) in *int-1* gene (Ooyen, A. V, 1984; Marchetti, A. et al. 1991), the same virus can either integrate into coding region of a gene or integrate into an untranslated region. MMTV can also cause

tumors by integrating into an intron sequence, as in the case of the Int-6 gene (Marchetti, A. et al. 1995). This results in the expression of a truncated chimeric RNA which may act dominantly in tumorigenesis.

In preliminary work, Dr. Pardeep Bhatia in our laboratory converted a benign fibroma to a malignant fibrosarcoma by using recombinant Moloney murine leukemia virus (MMLV). The genomic DNA fragment named 2br37 was isolated by inverse PCR (Silver, J. and Keerikatte., 1989). The new DNA sequence was compared with those in Genbank, and no homologous sequence was found indicating that this is the sequence of a novel gene. At this point, several questions were immediately raised. Firstly, does the DNA clone isolated from metastatic cells represent an intron or exon sequence of the gene and consequently is it expressed as mRNA? If it is not expressed, this DNA fragment may be in a regulatory region of the gene associated with metastatic transformation, so that it shows its impact by modifying the function of cis-regulating elements (Goff, S. P., 1987). If it is expressed, then how was the expression changed quantitatively or qualitatively? Secondly, How was the genomic DNA altered after viral insertion?

To address these questions, Northern blotting was done and after repeated attempts did not show a strong signal in either normal 10T1/2, benign M4R4 and metastatic 2br VI cells. This can be explained by several possible reasons. Firstly, 2br37 is simply an intron sequence, so it is not expressed at all. Secondly, it is expressed, but the genomic DNA probe may not hybridize well in these experiments since it may be too small. Another possibility is that the gene encodes low abundance transcripts, so the Northern

blotting was not sensitive enough to detect the signal. To resolve this quickly, we PCR amplified a mouse brain cDNA library (Hamilton, B. A. et al. 1991; Frohman, M. A., et al. 1988). A 467 base pair PCR clone, named 2brC1, was purified and it showed an overlapping sequence with 2br37 (Fig. 5). This result indicates that 2br37 was expressed as a mRNA in the mouse brain.

Sequence information of this clone did not show a possible open reading frame, but did show an A-T rich region and Polyadenylation signal sequence AATAAA at the 3' end of the clone. The next step was to answer whether the cDNA could be detected in other tissues. A Swiss mouse fibroblast cDNA library was screened and a 651 nucleotide cDNA clone named SW1, was purified and subcloned. The sequence data showed that in addition to the new nucleotides sequence at the 5' end, SW1 overlapped with 2brC1 and 2br37(Fig. 7). This confirmed that the 2br 37 is expressed, therefore it is an exon sequence. Since it was present in both brain tissue and fibroblast cDNA libraries, it is less likely to be a tissue specific gene. Sequence analysis still did not show a start codon at the 5' end.

2. Mutations and Metastasis

It became clear that finding the complete open reading frame was the next important step. A 5'RACE clone was then isolated from benign fibroma mRNA, which extends further in the 5' end of cDNA clone SW1. In spite of the overlapping sequence of SW1 and RACE5, the 5' sequences are different (Fig.10), which may suggest that SW1 and RACE5 may be two differentially spliced mRNA transcripts from the same gene. However since the RACE5 clone does not cover the complete 3' end sequences of

SW1, one question can be asked is whether RACE5 possesses a differentially spliced 3' end sequences as in SW1. Since I have obtained three PCR clones (Fig. 4) and three cDNA clones (data not shown) which had identical sequence at the 3' end as SW1, I assume that the two transcripts detected on Northern blot (Fig. 16) would be differ only in the 5' end. Therefore we can also assume that the 3' end sequence of the clone RACE5 is also identical to SW1. Sequence analysis (NIHMBUG software) of RACE5 demonstrated an open reading frame with an ATG start codon (Fig. 11), which fit perfectly with the Kozak rule (Kozak, 1991). Deduced translation from this open reading frame will give rise to a protein of 140 amino acids, with a predicted molecular weight of 15 Kd (Fig. 13). Multiple Sequence Alignment of 2br37, 2brC1, SW1 and RACE5 revealed a 19 nucleotide fragment missing in the 2br37 sequence (Fig. 15). This sequence is intact in both SW1 and Race 5.

We can also argue that the deletion may have been caused by the manipulation of DNA during the cloning process, because PCR techniques were used in the cloning of 2br37 genomic clone. The PCR technique is a very powerful and sensitive method widely used in molecular biology and clinical diagnosis. Like many other techniques, PCR has its own disadvantages. The major problem is the thermostable Taq DNA polymerase used in this technique. Unlike other DNA polymerase, Taq polymerase does not have 3'-5' exonuclease activity, or proof-reading activity. This imperfection has raised questions about the fidelity of the DNA products in early PCR application. The major defects in PCR products are point mutations, usually A-T to G-C switch. In addition , Taq DNA polymerase can delete or add a nucleotide to its DNA product. However, it has not been

reported that Taq DNA polymerase can encompass or delete as many as 19 nucleotides and continue to synthesize DNA. This can only happen in the presence of DNA secondary structure, which most likely will happen to other polymerases rather than Taq polymerase mediated DNA synthesis. Because DNA secondary structures are easier to form in relatively low temperature, rather than the commonly used 72^0C for DNA synthesis in PCR, it is unlikely that the deletion was caused by the techniques used in cloning 2br37 and is likely due to retroviral insertion and subsequent genomic rearrangements..

3. Loss of Heterozygosity and Metastasis

At this point, we can propose that this result may represent a classical loss-of-function mutation, in which a metastasis suppressor gene was inactivated by deletion. Inactivation by deletion of DNA sequence has long been recognized as a common finding among tumor suppressor genes(for review, see Marshall, 1991; Fearon and Vogelstein. 1990). Although we do not know if the protein is expressed, the low mRNA level means that it is likely not. In addition to the loss of normal protein, the mutated protein might be functional if it were expressed, a gain-of-function that may lead to tumor progression. For example, the deduced mutant protein still has 52 amino acid identical to the normal protein, so it may play a dominant negative role in host cells as in the case of the p53 tumor suppressor gene. When it is mutated, p53 will change its role from tumor suppressor gene to a dominant negative gene with oncogenic potential (Levine, A. 1991; Vogelstein and Kinzler 1992). These synergistic actions are very likely to play a role in metastatic conversion of fibroma (MRPD) to fibrosarcoma (2brVI). The mutated gene therefore may represent a gene that influenced the metastatic conversion of the tumor.

In our experiment, DNAs from normal 10T1/2 fibroblasts , benign MRPD and metastatic 2br VI were digested by restriction enzymes BamH I, Hind III, Sca I and Xba I separately. Southern hybridization were performed by using cDNA clone SW1 as a probe. The results showed that the genomic DNA fragment in 10T1/2 and MRPD was identical but not detected in metastatic 2brVI. Instead, a larger fragment was detected 2br VI (Fig. 8 and 9). Of further interest, the normal allele was not detectable in metastatic 2br VI cells presumably due to viral integration. Thus we detected both a deletion of the normal copy of the gene with a shift in the second copy indicating a mutation of this allele in metastatic 2brVI cells. The result is similar to the case of inactivation of a suppressor gene, rather than activation of an oncogene, because there is no amplification or simple rearrangement of the gene.

The results may be accounted for by four possibilities (Fig. 19). Firstly, the retrovirus was inserted into both alleles of the MAG gene (Fig.19-4), as in the case of a Friend murine leukemia virus involving the p53 tumor suppressor gene (Hicks and Mowat. 1988). Even though it is a rare event , this will switch the restriction site, and the larger band may represent both alleles containing a fusion DNA fragment of cellular and viral DNA. The earlier work done by Dr. Pardeep Bhatia in our laboratory showed that the 2br37 genomic DNA fragment was immediately adjecent to the retroviral sequence (unpublished data), which means the retrovirus integrated into the gene, but did not indicate whether it integrated into one or two copies of the gene. As shown later on the desitometric analysis of the Southern blots, about half of the gene copies were lost in

metastatic 2br VI cell compared with 10t1/2 and MRPD cells, so it is not likely the retrovirus integrated into both alleles of the gene.

The second possibility is that the virus integrated into one of the normal alleles, and the other copy (or copies since MRPD is not a diploid cell) was lost due to nondisjunction, and then reduplication of the mutant copy (Fig. 19-1). This is less likely in the same way as above that the intensity of the larger band is approximately half of the normal band based on densitometry analysis.

Another possibility is that there is only one copy of this MAG gene in normal cells, because the 10T1/2 mouse fibroblast is an aneuploid cell. The viral integration simply changed the restriction site and caused the band shift (Fig. 19-3), or rearrangement of the gene. This rearrangement could either activate or disrupt the adjacent gene, or the MAG gene in our case. According to Knudson's hypothesis, if normal 10T1/2 cells and benign cells only contain one copy of the normal gene, as in familial retinoblastoma, the chance to develop into malignancy will be significantly increased due to somatic mutation which only needs to abolish the remaining copy of the gene (Knudson, 1971). Therefore the normal 10T1/2 cell would be predisposed to tumor formation, or metastasis. But in our previous study, 10T1/2 does not show an increased tendency of spontaneous tumor formation, nor an increased rate of spontaneous metastasis (Egan, 1987, 1988; Bhatia, unpublished data). Also the reduction in DNA by half is not consistent with only a single allele being mutated. So this hypothesis is also less likely.

The next possibility is that virus inserted into one allele of the gene. After viral insertion, the transfected cells were subjected to selection through a metastasis assay. The

cells might have undergone mitotic recombination or regional events such as gene conversion, deletion, which mutated the second copy of the gene (Hansen and Cavenee. 1987), leading to loss of heterozygosity (LOH) for the mutated allele (Fig.19-2) and these cells were selected in the metastasis assay. So it would mimic the sporadic retinoblastoma in Knudson's two hit model, in which two mutational events are required for tumor formation. The hereditary cases would have inherited a germinal mutation that does not in itself cause the tumor but rather predisposes each cell to a further transforming event; whereas the sporadic cases would arise by two somatic mutations and both events would have to occur in the same somatic cells. In the absence of gross chromosomal deletion, other genetic events must be considered as mentioned above. In our experiment, the viral insertion may well be the first somatic mutation which in turn, predisposed the cell to the subsequent genetic change leading to homozygosity. Therefore, this mutation would disrupt the normal gene and predispose the cell to metastasis transformation. In fact, the densitometry analysis of Southern blotting showed that the intensity of the MAG gene has been reduced in metastatic 2brVI cells approximately half of that in normal 10T 1/2 cells (Fig. 8B and 9B). This is in strong support for the last hypothesis. It is also in accord with our cloning results, in which both the deletion in the open reading frame of 2br37 and viral insertion abrogate the normal function of the gene, then the possibility leading to metastatic conversion. The deletion alone may have been sufficient to abolish the physiological function of MAG gene. Even though our experimental results support the above hypothesis, the mechanism of the microdeletion may or may not have been caused by viral insertinal event. This may simply be a second independent event.

4. Gene Expression and Metastasis

Northern blotting indicated that there are two transcripts in normal 10T1/2 and benign MRPD cells (Fig. 16). This Northern result is consistent with our cloning results in which there are two clones that differ at the 5'end (Fig.15). This may be due to alternative RNA splicing, in which the exons of the same gene were differentially recombined to produce different mRNA transcripts, or the two mRNA transcripts may be derived from different promotors of the same gene, similar to that of the *c-myc* oncogene (Reddy, etc. 1988). A homologous gene could also account for the second band. The mechnism responsible for the two transcripts of the MAG gene remains unknown.

The mRNA level in metastatic 2brVI cells was greatly reduced. This may have been caused by the mutation demonstrated in our Southern blot results, or the microdeletion on the gene may have imposed a negative effect on the stability of the mRNA of the MAG gene. Like other tumor suppressor genes or metastatic-associated genes, the mRNA expression is often decreased in cancer cells or the protein products are mutated (for Review see Liotta., et al. 1991; Weinberg, 1991). Similar results has been reported for the human nm23 gene, a candidate metastatic suppressor gene. It was found that a 64 nucleotide fragment in the coding region of the gene was deleted and a mutant protein was expressed in colorectal adenocarcinomas associated with metastasis in lymph nodes, lung or liver. The deletion was not found in colorectal cancer specimens without metastasis (Wang, et al. 1993). Interestingly, an increased expression of nm23 expression was observed in colorectal carcinomas with no relationship between the expression level of nm23 and metastatic activity (Hout, et al. 1991). This result raises the

question of whether genes in the presence of microdeletion in the coding region may still be transcribed and translated into a mutant product, which can not be detected by the traditional Northern blot analysis. If true, this result also implies that a dominant negative effect may be the mechanism in metastatic transformation of those colorectal adenocarcinomas. It may explain why a candidate metastatic suppressor gene can demonstrate decreased expression in some tumors but not the others. Point mutations in the p53 tumor suppressor gene is another good example, where the expression level of p53 obscured our understanding of the suppressor function of the gene for many years. It was found that mutations in p53 and the subsequently loss of normal function dramatically established its role as a tumor suppressor gene rather than a oncogene. Therefore, the demonstration of mutations and especially deletion in a cancer related gene is a relatively more reliable predictor of inactivation or suppressive function of the gene.

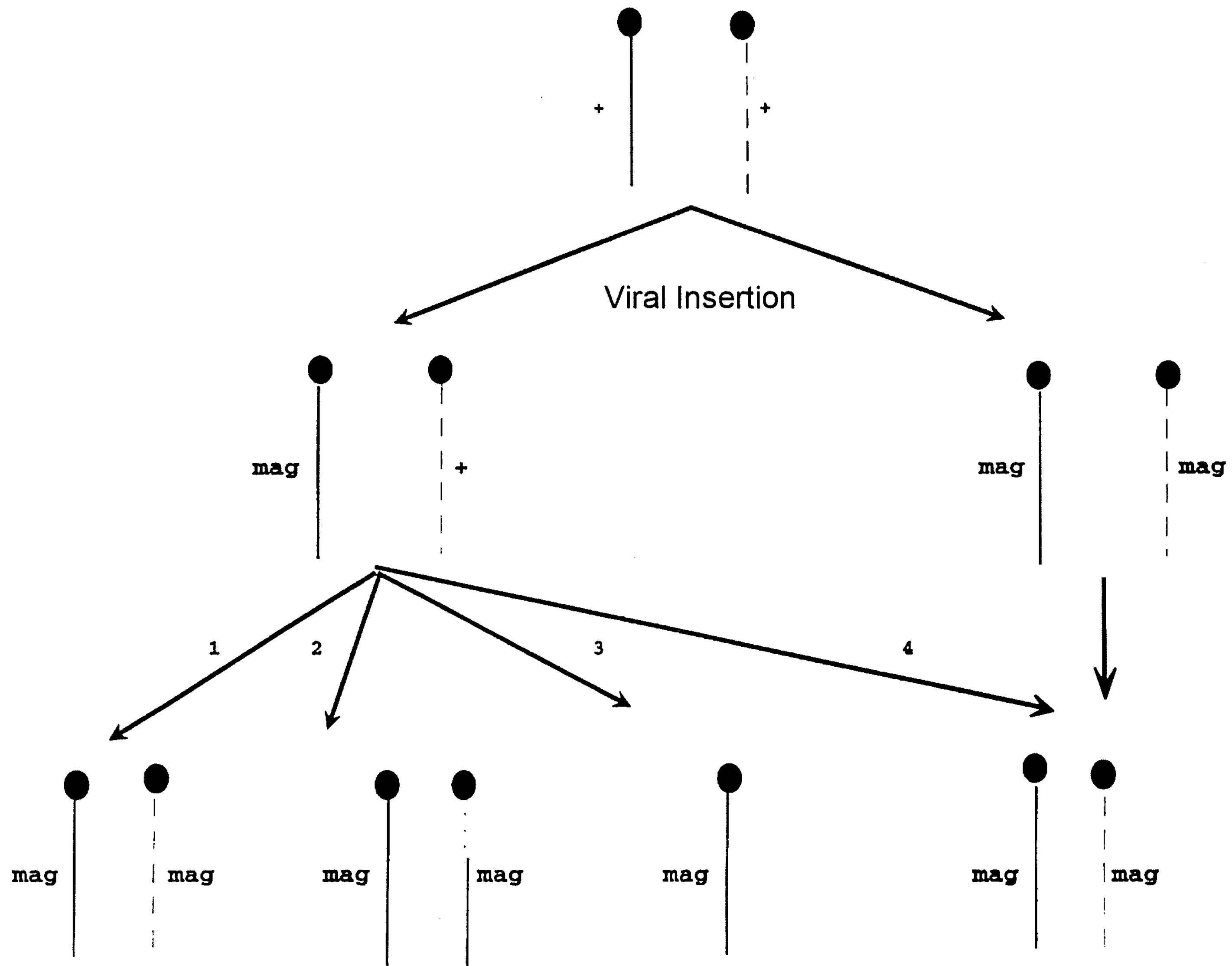
V. Conclusions and Future Prospects

In this study, three cell lines, mouse fibroblast 10T1/2, benign tumor cell MRPD and metastatic 2brVI were used. The 2brVI cell line may have acquired metastatic ability from retroviral integration into MRPD genomic DNA. Loss of heterozygosity has been detected in metastatic 2brVI genomic DNA. Decreased transcription level has been observed in 2brVI cells. The cDNA of the gene, named MAG, which flank the insertion site of retrovirus MMLV was cloned and sequenced, and yielded a predicted 15 kD protein (p15). The cDNA sequence analysis reveals that a 19 nucleotide fragment was deleted in the exon of the 2brVI genomic DNA. The secondary structure of the wild type protein shows that it is hydrophilic meaning it is likely a cytosolic protein. Based on the

observation that the loss of gene copy, a partial deletion of the normal coding sequences, and the decreased transcription of MAG, we can propose that the metastatic transformation of fibroma to fibrosarcoma by viral integration, may be caused by the loss of function of the MAG gene which is adjacent to a viral integration site.

To test this hypothesis, other metastatic tumors can be used in an attempt to detect any change in expression level of the MAG gene. If decreased expression is observed, it will imply that the expression level is important in controlling metastatic conversion. If negative, other mechnisms have to be explored. To test the hypothesis more directly, stable antisense olionucleotides against sequences flanking the start codon or against the deleted fragment of the cDNA can be introduced into benign MRPD cell. If the decreased transcription and subsequently decreased translation can cause metastatic conversion, it will confirm that the expression of the gene is important in controlling the metastatic change meaning a metastasis suppressor gene. If metastatic potential did not change, other possible phenotypic changes should be analyzed to define the function of the gene, such as cell motility and invasion potential. In the same way, normal cDNA of the MAG gene can be expressed in the metastatic tumor 2brVI. If the metastatic potential is decreased, it also confirms that MAG is a metastasis suppressor gene. If no changed is detected in metastatic ability, other functions of the gene should be investigated.

Fig. 19 Hypothetic possibilities of metastatic convertion of 2brVI cell by loss of heterozygosity (LOH) of the MAG gene. 1. MMLV virus integrated into one of the normal allele and the other copy(s) was lost due to nondisjunction and reduplication of the mutant copy. 2. The virus inserted into one allele of the MAG gene, the other allele(s) had undergone mitotic recombination or regional event such as gene conversion, deletion, leading to LOH, and these cells were selected in the metastasis assay. 3. There is only one allele of MAG gene, the viral integration simply rearraged the gene. 4. The virus integrated into both alleles of the MAG gene, leading to LOH.



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VI. References

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