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SUPPRESSION OF TUMORIGENICITY OF THE PA-1 HUMAN TERATOCARCINOMA CELL LINE

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Abstract

Teratocarcinomas are tumors that develop spontaneously in the gonads, and usually contain a rapidly dividing undifferentiated stem-cell population. I have used microcell fusion to introduce individually tagged human chromosomes into the PA-1 human teratocarcinoma cell line. Introduction of human chromosome 4 caused a change of cell morphology in culture and suppressed PA-1 tumorigenicity in nude mice, while addition of portions of either chromosome 7 or 12 had no effect on the cell phenotype. The PA-1 cell line regained its tumorigenicity when the tagged chromosome 4 was lost under negative selection. Tumors were noted in the test period after two out of twelve injections of PA-1 harbouring an additional copy of chromosome 4. One tumor derived cell line had lost the complete tagged chromosome while the other had lost portions of the tagged chromosome (4p11-15.3 and q31.1). I concluded that there is a putative tumor suppressor gene on human chromosome 4 whose expression interferes with the tumorigenicity of PA-1 cells, and that this gene maps to the portion of chromosome 4 lost from the tumor-derived cell line. Differential display of mRNAs from PA-1-derived cell lines revealed that at least two genes, the immunoglobulin heavy chain binding protein (BiP gene) and a novel transcript, are over-expressed as a result of the introduction of chromosome 4 into PA-1.

Coincident with the loss of tumorigenicity after introduction of chromosome 4, was an increase in the retinoic acid-induced differentiation of the cell line. Retinoic acid is a potent morphogen and has been implicated in development and differentiation in mammalian systems. Not only did the addition of the tagged chromosome 4 to PA-1 cause an increase

in differentiation potential, it also caused an increase in the induction of both retinoic acid receptor-α and *HOX1F* gene expression. This implies that the increase in retinoid acid-induced differentiation in PA-1-derived cells occurs through a pathway involving retinoic acid receptor-α. Thus, the gene(s) mapping to 4p11.1-15.3 or q31.1 is not only responsible for the suppression of tumorigenicity of PA-1, but it also increases the differentiation potential of the cells.

For Scott and Andrew, without whom nothing matters.

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LIST OF ABBREVIATIONS

DMEM	Dulbecco's modified Eagle medium
FBS	Fetal bovine serum
FISH	Fluorescent *in situ* hybridization
HNPCC	Hereditary non-polyposis colon cancer
HSF	Human skin fibroblasts
MEM	Minimal essential media
RA	All-*trans* retinoic acid
RDA	Representational difference analysis
SDS	Sodium dodecyl sulfate
THC	Tagged human chromosomes
YAC	Yeast artificial chromosome

CHAPTER 1 General Introduction

1.i. Cancer as a Disease

Cells of the body are normally held in a well organized pattern of tissues and organs; in many organs, such as the skin, cells divide in a controlled fashion in order to replace older cells, while in some organs, such as the brain, they rarely divide. The number and type of cells in a particular organ is also regulated through the process of active cell death (apoptosis). Occasionally, a cell may escape from these types of proliferative control. This may occur either through a failure to cease dividing or a circumvention of the apoptosis program (reviewed by Williams, 1991). The cell can then establish itself as a mass of cells, or tumour, which may pose a threat to surrounding tissues and organs. Of even greater concern are those cells which acquire the ability to grow outside the organ of origin. These cells can metastasize and establish themselves in remote areas of the body. As such, metastatic cells endanger the survival and functionality of which ever organs they invade, and thereby the affected individual, by competing for both space and nutrients.

The process by which a cell avoids the normal controls over its growth and proliferation is the subject of extensive research. It is now confirmed that a series of genetic events, involving the disruption of particular genes, is responsible for the transformation of cells to tumorigenic and metastatic phenotypes.

1.ii. Oncogenes and Tumor Suppressor Genes

Two classes of cellular genes, oncogenes and tumor suppressor genes, have been identified as being involved in carcinogenesis. In general, activation of one allele of an oncogene, or inactivation of both alleles of a tumor suppressor gene (Figure 1.i.) contributes to tumor formation; that is, the activated (mutant) allele of oncogenes, and the wild type allele of tumor suppressor genes are dominant. Oncogene-coded proteins have the normal cellular function of promoting cell growth, while tumor suppressor gene products act as components of cellular growth control mechanisms.

Proto-oncogenes can be activated, usually through mutation, to become active oncogenes, which contribute to malignant transformation. Their existence was detected far earlier than that of the tumor suppressor genes. The products of proto-oncogenes normally perform a variety of cellular functions, all leading to cell growth when the appropriate signals are in place. When these signals are overridden, or continually turned on, oncogenesis occurs. Oncoproteins which act as secreted growth factors (*SIS*, Khachigian *et al.*, 1994), growth factor receptors (*ERBA*, Damm, 1993), components of signal transduction pathways (*RAS*, Wood *et al.*, 1992) or transcription factors (*MYC*, Amati *et al.*, 1993) have been identified. The list of known oncogenes is vast, but is being matched quickly by that of the more recently discovered tumor suppressor genes.

Knudson's two hit hypothesis (Knudson, 1971) gave an early, but poorly accepted, indication that tumor suppressor genes exist. He looked at patients who developed a rare

type of childhood cancer of the retina, known as retinoblastoma. This disease occurs in a familial pattern with people of a stricken family being susceptible to developing this type of tumor; there is also a non-familial class of retinoblastoma (Draper *et al.*, 1992). Patients who suffered from this in a non-familial fashion developed the tumors later in life than those who suffered from the familial form. Having examined the occurrence patterns of the familial and non-familial forms, Knudson postulated (Knudson, 1971) that two separate events were responsible for these tumors. This hypothesis is consistent with the idea that a dominant factor is inactivated during the progression of this disease.

A second and more direct piece of evidence for the existence of tumour suppressor genes came from cell biology, more than two decades ago. Whole cell fusion experiments between tumorigenic and non-tumorigenic cell lines can produce hybrids which are non-tumorigenic (Harris, 1971; Stanbridge, 1976; Sager 1985), implying that a dominant factor from the non-tumorigenic cell line can confer its phenotype upon the tumorigenic cell line. Upon the loss of particular chromosomes, the hybrid cells regain their ability to form tumors (Stanbridge *et al.*, 1981). This observation pointed to the loss of a suppressing dominant factor and led investigators to speculate upon the chromosomal location of tumor suppressor genes.

Yet another piece of evidence for the existence of such genes came from cytogenetics. Specific chromosomal abnormalities have been found to be associated with particular types of tumors (Stanbridge 1989). For example, retinoblastoma usually shows chromosomal rearrangements or deletions in a specific region of chromosome 13 (13q14;

Strong *et al.*, 1981). Similarly, many colorectal tumors show deletions in chromosome 5 (5q21; Bulow, 1987). Patients who carry one such abnormal chromosome have been found to have a higher risk for that cancer than the general population. It was hypothesized that chromosomal disruptions could inactivate tumor suppressor genes: if an individual carries one disrupted tumor suppressor allele, only one additional mutation or deletion event on the second allele is required to completely inactivate that gene (Figure 1.i.).

Molecular analysis provided confirmation that the loss of tumor suppressor genes is involved in the progression of some tumors. While normal somatic tissue from an affected individual is often heterozygous for a particular DNA sequence, tumor tissue sometimes shows loss of heterozygosity for the same region (Cavenee *et al.*, 1989). This loss of heterozygosity is usually observed in patients who carry one (germ-line) wild-type allele and one mutant allele of a tumor suppressor gene. When the wild-type copy is lost through a deletion, the loss of heterozygosity can be observed in the resulting tumor tissue. These molecular studies complemented the cytogenetic work. In some cases, tumor samples of a particular type which usually showed chromosomal abnormalities at specific locations had no such rearrangement. When these same tumor samples were examined at the molecular level, loss of heterozygosity was observed, even though the change involved a chromosomal rearrangement which was too small to be detected cytogenetically.

The process which leads to cancer involves a series of events including the activation of oncogenes and inactivation of tumor suppressor genes. Colon cancer, for example involves a well defined pattern of oncogene activation and tumor suppressor gene

inactivation, each event contributing in a step-wise fashion to tumor growth (Figure 1.ii.). This pattern of molecular events has been well documented because of the clinical availability of tumor samples from different stages of colon cancer. This model of monoclonal tumor progression was held without question until quite recently when it was discovered that colonic adenomas did not necessarily evolve from a single cell, but were, instead, polyclonal in origin (Novelli *et al.*, 1996). This central model of tumorigenesis, evidently, will require further investigation in order to be clarified.

Figure 1.i. The process by which inactivation of both alleles of a tumor suppressor gene occurs. A. Two mutations in somatic cells, or B., in an inherited predisposition situation, one somatic and one inherited mutation are required for the inactivation of the tumor suppressor gene. The "+" represents a wild type allele, while the "-" indicates a mutated (inactivated) allele. (Adapted from Recio and Preston, 1993.)

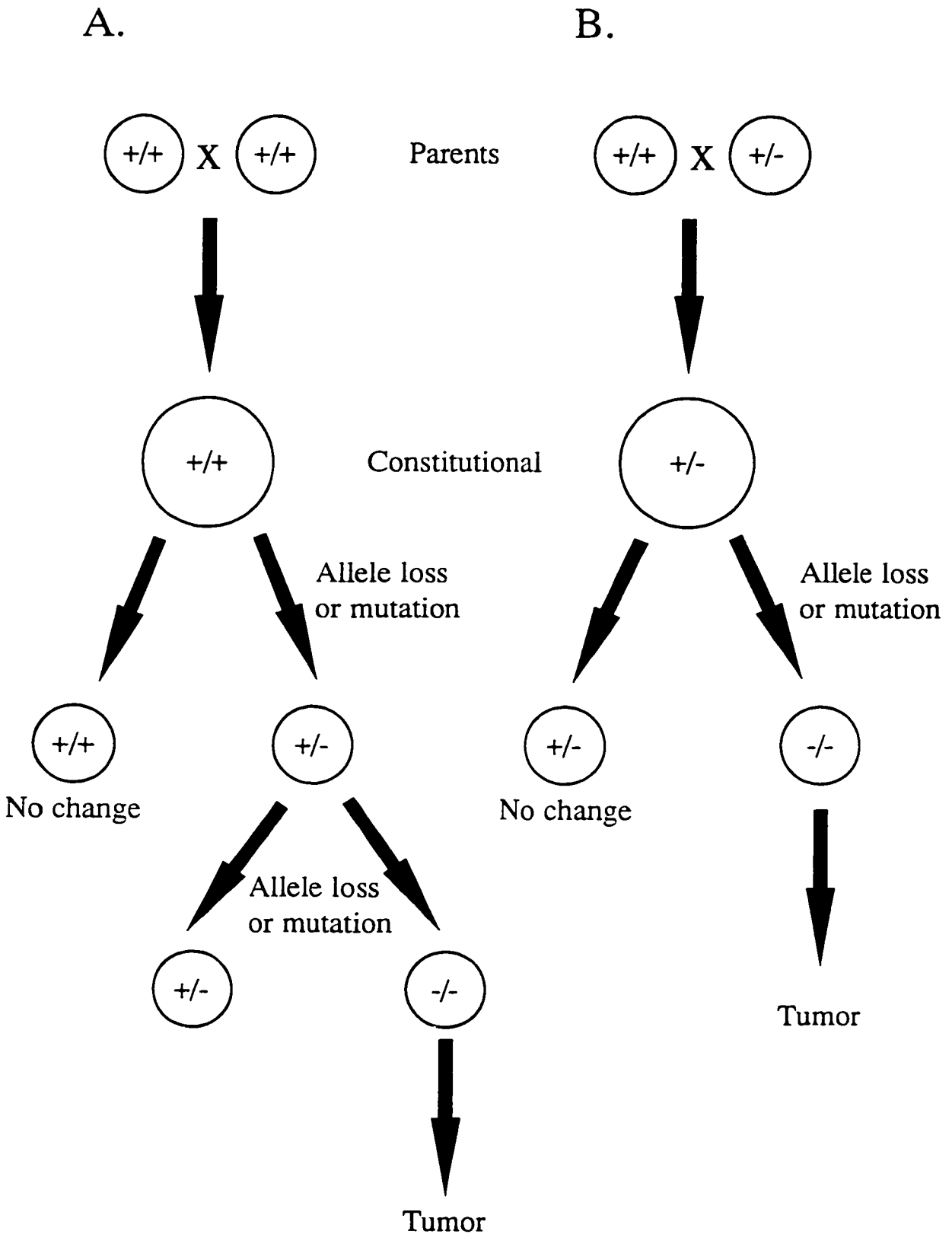
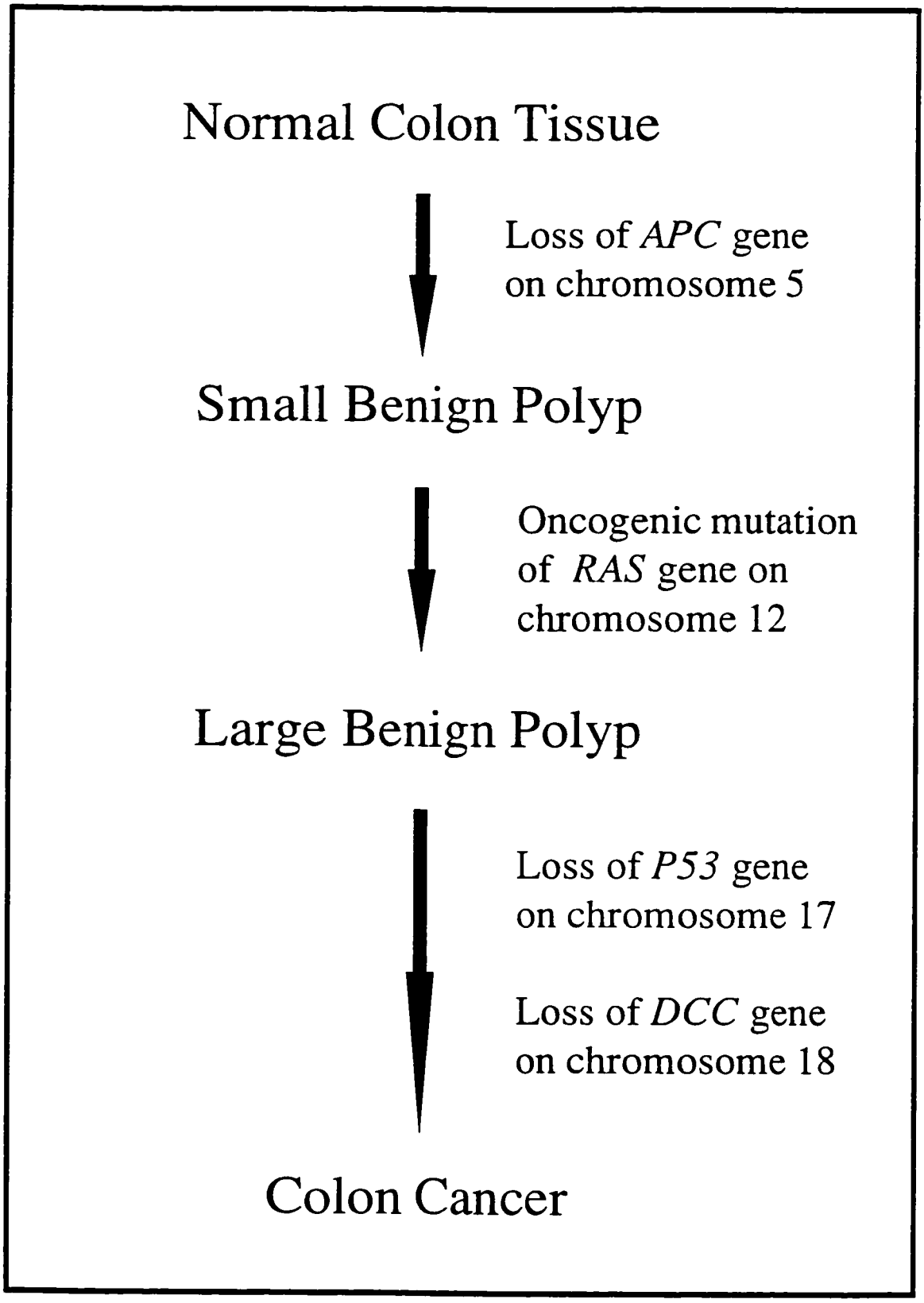

A.
B.
+/+
X
+/+
Parents
+/+
X
+/-
+/+
Constitutional
+/-
Allele loss
or mutation
Allele loss
or mutation
+/+
No change
+/-
+/-
No change
-/-
Allele loss
or mutation
+/-
-/-
Tumor
Tumor

Figure 1.ii. Accumulation of genetic damage leading to colon cancer. Particular genetic changes to a cell occur in the advancement of colon cancer. The inactivation of tumor suppressor genes and activation of the *RAS* oncogene occur in various orders, one of which is depicted. (Based upon Cavanee and White, 1995.)


Normal Colon Tissue
Loss of *APC* gene on chromosome 5
Small Benign Polyp
Oncogenic mutation of *RAS* gene on chromosome 12
Large Benign Polyp
Loss of *P53* gene on chromosome 17
Loss of *DCC* gene on chromosome 18
Colon Cancer

I.iii. Identification of New Tumor Suppressor Genes

Positional cloning is the most frequently used strategy for the cloning of tumor suppressor genes. This technique relies upon cytogenetically observed chromosomal abnormalities and loss of heterozygosity in a given tumor type. Also, genetic linkage analysis is frequently used if families afflicted by a particular type of cancer are available for analysis. Once the chromosomal location of a putative tumor suppressor gene is defined, the laborious task of isolating the gene begins. In many cases, DNA probes from the region of interest are isolated, and a chromosome walk is carried out, using these probes as a starting point and searching for overlapping DNA fragments. Along the way, the isolated DNA sequences are checked for the hallmarks of expressed sequences (such as evolutionary conserved sequences). Other useful techniques for the cloning of closely mapped genes are exon trapping (Auch and Reth, 1990) and exon amplification (Buckler *et al.*, 1991) which rely on the amplification of expressed sequences. Once an expressed sequence is found, DNA samples from many tumors are examined to determine whether this sequence is disrupted. If this is the case, a tumor suppressor gene has probably been identified. Variations of this technique have been successfully used to clone the *RB* (retinoblastoma, Friend *et al.*, 1986), *DCC* (deleted in colorectal cancer, Fearson *et al.*, 1990), *WT1* (Wilm's tumor, Call *et al.*, 1990; Gessler *et al.*, 1990), *NF1* (neurofibromatosis type I, Cawthon *et al.*, 1990; Viskochil *et al.*, 1990; Wallace *et al.*, 1990), and *BRCA1* (breast cancer, Miki *et al.*, 1994) tumor suppressor genes.

Another approach to determining the chromosomal location of tumor suppressor genes is to introduce individual chromosomes or portions of chromosomes into tumorigenic cell lines and subsequently assay for the loss of the tumorigenic phenotype. This approach is generally applicable during the cloning of any gene which confers a dominant phenotype for which the investigator can assay. It has been used successfully for the cloning of the genes encoding TSE1, a tissue-specific extinguisher (Jones *et al.*, 1991), and KAI1 (Dong *et al.*, 1995), a suppressor of prostate metastasis. If the introduced chromosome encodes a tumor suppressor locus which had been inactivated during the development of tumorigenicity, then the cell line will become non-tumorigenic.

Following the identification of the chromosomal location of a tumor suppressor gene, molecular methods such as differential display or subtractive hybridization can be used for the final cloning of the candidate gene (see Chapter 4). Conclusive proof of the existence of a tumor suppressor gene comes only when a candidate gene is reintroduced into the cell line in question and results in the suppression of tumorigenicity.

1.iv. Function of Selected Tumor Suppressor Gene Products

The protein products of the tumor suppressor genes discovered so far have the common feature of retarding cell growth or metastatic potential. The myriad of known tumor suppressor proteins perform this task by a variety of methods. Tumor suppressor proteins that resemble cell adhesion molecules (DCC, Fearson *et al.*, 1990), control the cell cycle (RB, reviewed by Hollingsworth *et al.*, 1993; and P53, reviewed by Shimamura and Fisher, 1996), or affect the activity of the RAS oncoprotein (neurofibromin, Basu *et al.*, 1992; RAP1, Kitayama *et al.*, 1989) have been identified.

One widely studied tumor suppressor gene is the retinoblastoma susceptibility gene, *RB* (Goodrich and Lee, 1993). This phosphoprotein is involved in the regulation of the cell cycle (reviewed by Weinberg, 1995), interacting with a variety of cellular proteins which modulate the cell cycle. While the deletion or mutation of *RB* is characteristic of retinoblastoma, this gene is also sometimes altered in tumors of the breast and prostate, as well as in osteosarcoma (Vogel, 1979). Most frequently, in retinoblastoma, the *RB* gene is inactivated by small deletions or duplications (Dunn *et al.*, 1989) which result in a premature stop codon.

Another tumor suppressor gene involved in regulation of the cell cycle is *P53* (reviewed by Prives and Manfreid, 1993). The P53 protein is a nuclear phosphoprotein which can act as a transcription factor, through its ability to bind DNA with sequence specificity. One particular target of P53 binding is the *P21/WAF1/CIP1* gene (El-Deiry *et*

al., 1993), which encodes a cell cycle regulator. This regulator inhibits DNA replication and, thereby, division of cells. P53 is also thought to enhance the fidelity of the DNA replication process, by acting as a cell cycle "checkpoint" (Kastan *et al.*, 1992). Although *P53* is one of the most widely studied tumor suppressor genes, it is in fact an anomaly, since it is not simple inactivation which contributes to tumorigenesis. Certain mutant forms of the P53 protein can act in a dominant-negative fashion (Dittmer et al., 1993). They are able to bind to and inactivate wild type P53, and in this way promote the tumorigenic phenotype.

The *P53* gene is the most commonly altered gene in human cancers and is associated with the familial Li-Fraumeni syndrome, a rare disease in which sufferers endure a high incidence of a variety of tumors (Malkin *et al.*, 1990; Srivastava *et al.*, 1990), and carry one mutant *P53* allele. In this circumstance, the tumor suppressor gene acts as a cancer predisposition gene, with carriers of one mutant allele being prone to the development of tumors.

The tumor suppressor gene, *VHL*, is involved in von Hippel-Lindau disease, a syndrome characterized by familial kidney cancer. This tumor suppressor gene was identified several years ago (Latif *et al.*, 1993), although its function remained unclear. Recently, however, it became apparent that the protein product of *VHL* plays a key role in transcriptional elongation by negative regulation of the transcription elongation factor, elongin (Duan *et al.*, 1995). The targets of this regulation remain unknown at this time, but it has been speculated that VHL controls the transcription rate of oncogenes such as *MYC* (Krumm and Groudine, 1995).

Other tumor suppressor genes have been identified on the basis of their function. Hereditary non-polyposis colon cancer (HNPCC), which accounts for 10-15% of all colon cancers (Rustgi, 1994), is characterized by DNA instability. Searches for genes which are homologous to those involved in the repair of DNA mismatches in bacteria and yeast, have resulted in the discovery of five human genes which carry out this same function and are mutated in HNPCC patients (Bronner *et al.*, 1994; Fishel *et al.*, 1993; Nicolaides *et al.* 1994; Papadopoulos *et al.*, 1995). Thus, the *MLH-1*, *MSH-2*, *PMS-1*, *PMS-2* and *GTBP* genes are involved in DNA mismatch repair and have been identified as tumor suppressor genes which are involved in this type of cancer.

A recently described tumor suppressor gene function is that of an inhibitor of angiogenesis (O'Reilly *et al.*, 1994). While fitting the description of both a tumor suppressor and a metastasis suppressor, the angiostatin protein inhibits the proliferation of endothelial cells, a crucial step in the vascularization and, therefore, growth of both primary and secondary tumors.

Unlike the tumor suppressor genes which were identified several years ago, the function of some more recently recognized tumor suppressor genes remains unclear. *BRCA1* is such a gene, and is implicated in the etiology of familial breast and ovarian cancers (Miki *et al.*, 1994). It is estimated that *BRCA1* mutations contribute to the development of 60% of familial breast cancers (Easton *et al.*, 1993). A variety of mutations in this gene, which encodes an enormous protein, are found in affected individuals (Futreal *et al.*, 1994). Currently, the only clues regarding the function of the BRCA1 protein is that the gene

contains a granin consensus sequence, indicating that BRCA1 may play a role in the processing of proteins in a secretory pathway (Jensen *et al.*, 1996). What is clear, however, is that *BRCA1* has a growth inhibitory effect on certain cancer cell types, since introduction of this gene causes both retardation of growth and a decrease in tumor forming ability of a breast cancer cell line (Holt *et al.*, 1996).

Another gene, *BRCA2*, which is involved in hereditary breast cancer has been recently cloned (Tavtigian *et al.*, 1996). Mutations in *BRCA2* are responsible for 35% of early-onset hereditary breast cancers (Couch *et al.*, 1996) and usually result in the production of a truncated protein product (Phelan *et al.*, 1996). As is the case for many tumor suppressor proteins, however, the function of BRCA2 remains a mystery. The importance of the *BRCA1* and *BRCA2* genes in the etiology of hereditary breast cancer is without question, however: women carrying a mutation in either of these genes have a 80-90% risk of developing breast cancer (Neuhausen *et al.*, 1996).

A most surprising finding was that RNA can serve a tumor suppressing function. Examples of this phenomenon include the RNA from the 3' untranslated region of α-tropomyosin, which was shown to inhibit the formation of rhabdomyosarcoma; rhabdomyosarcoma cells which had been transfected with the 3'-untranslated region of α-tropomyosin fail to form tumors when implanted in mice (Rastinejad *et al.*, 1993). Another RNA, this time transcribed from the *H19* gene, is able to suppress the tumorigenicity of some embryonal tumor cell lines (Hao *et al.*, 1993). As yet, however, the mode of action of these tumor suppressor RNAs remains a quandary.

I.v. Cancer and Differentiation

There exists a direct relationship between differentiation and tumor suppression. In general, tumor cells are rapidly dividing and undifferentiated while, on the other hand, most normal somatic cells are differentiated and slow growing. In fact Harris (1990) has argued that the circumvention of a normal differentiation pathway, rather than the stimulation of cell division, is fundamental to malignant tumor progression. Of course, this view is not completely consistent with the known role of the *P53* and *RB* tumor suppressor genes as regulators of the cell cycle, as discussed above. None the less, differentiation does play an important role regarding normal cell growth and suppression of the malignant phenotype.

Early experiments pertaining to tumor suppression gave indications that tumor suppression is linked to differentiation. Stanbridge and Ceredig (1981) found that human carcinoma cells fused with human fibroblasts assumed a differentiated morphology similar to that of fibroblasts rather than the undifferentiated morphology characteristic of carcinoma cells. As chromosomes were lost from these hybrid cells, not only did the ability to form tumors return, but the morphology also reverted to that of the carcinoma cells.

Some genes have been observed to evoke their tumor suppressing effect through initiation of a differentiation pathway. In this way, they slow the growth of cells and impart less aggressive growth characteristics upon the cells. Transfection of embryonal tumor cells with an *H19* expression construct resulted in a decrease in growth rate and a change in the *in vitro* morphology of the cells, with the cells becoming flatter in appearance (Hao *et al.*,

1993). The transfected cells also lost their ability to form tumors in nude mice and had a decreased ability to form colonies in soft agar.

Further evidence of a role for differentiation in tumor suppression came from other studies centred upon the phenotype imparted by another RNA, the 3' untranslated region of α-tropomyosin. Initially it was noted that this RNA could induce a differentiation-deficient myogenic cell line to undergo changes which allowed expression of muscle-specific genes (Rastinejad and Blau, 1993). Further studies showed that not only did the 3' untranslated region of α-tropomyosin cause the cells to differentiate, it also inhibited anchorage-independent growth and *in vivo* tumor formation (Rastinejad *et al.*, 1993).

At least one tumor suppressor gene has been cloned on the basis of its ability to confer a differentiated morphology upon a transformed cell line. The *KREV-1/RAP1* gene was identified when a cDNA library from normal human fibroblasts (a well differentiated cell type) was used to transfect a transformed mouse cell line (Kitayama *et al.*, 1989). One colony of the transfected cells exhibited a morphology which differed dramatically from that of the parent cell line: it was flat and epithelial like rather than refractory when viewed by phase-contrast microscopy. The transfected cell line also exhibited low tumor forming ability. The novel cDNA responsible for these phenotypic changes was termed *KREV-1*.

Another, more recently identified tumor suppressor gene has been found to have links to differentiation. As previously discussed, the *BRCA1* gene is involved in the etiology of familial breast and ovarian cancers. Subsequent to the identification of the gene (Miki *et al.*, 1994), it was shown that in mice *BRCA1* is highly expressed in tissues which are undergoing

differentiation, such as the mammary gland during puberty and pregnancy (Marquis *et al.*, 1995). It is also expressed widely in the developing embryo. These findings imply that the product of *BRCA1* is involved in the process of differentiation.

Cultured tumor cells may be induced to differentiate by chemical agents, including retinoids (see Chapter 5). These chemical inducers of differentiation often inhibit the growth of cancerous cells in culture. It is this observed growth inhibition that has led to the hope that retinoids may be useful as a clinical cancer treatment (reviewed by Smith *et al.*, 1992). One retinoid, *N*-(4-hydroxyphenyl)retinamide, both inhibits the metastasis of prostate adenocarcinoma and prevents carcinogen-induced mammary cancer in animals. (Moon *et al.*, 1992; Pollard *et al.*, 1991). 9-*cis*-retinoic acid can inhibit both the development of carcinogen-induced mammary tumors (Anzono *et al.*, 1994) and reduce the growth of xenografted human squamous cell tumors in mice (Shalinsky *et al.*, 1995). Unfortunately, most of the human clinical trials using retinoids which have been completed have focused on patients with advanced tumors (Boccardo *et al.*, 1990; Modiano *et al.*, 1990) and have not been successful. Perhaps less aggressive tumors will prove to be more responsive to induction of differentiation.

The results from several lines of investigation have supported the notion that differentiation and tumor suppression are intertwined phenomena. Thus, it is valuable to identify novel tumor suppressor genes which may play a role in the process of differentiation.

I.vi. Teratocarcinoma and the PA-1 Cell Line

The relationship between uncontrolled growth and differentiation is particularly relevant when one is considering the case of teratocarcinomas. Teratocarcinomas are tumors which are derived from germ cells (Andrews *et al.*, 1987). In humans teratocarcinomas arise in both males and females as a result of germ cell activation (Figure I.iii.). In males, these tumors are usually malignant (Andrews, 1988). In the ovary, on the other hand, these activated cells often fail to undergo malignant transformation and remain as benign tumors termed teratomas (Andrews *et al.*, 1987). Once malignant transformation occurs however, the tumor takes on characteristics of the developing embryo with some cells resembling various somatic tissues. The cells remaining in an undifferentiated state are referred to as embryonal carcinoma cells, the stem cells of teratocarcinoma. When the embryonal carcinoma cells differentiate but retain their malignancy, the tumor is termed an immature teratoma. It contains no true stem cells, but the continued proliferation of the partially differentiated, immature somatic tissues ensures its malignancy.

Although ovarian teratocarcinomas do not frequently occur in a clinical setting, they are of interest from a developmental point of view since they are germ cell in origin. Malignant ovarian teratocarcinomas differ from their counterparts, the benign teratomas, in that they are a rarer form of tumor and usually occur in prepubertal females. They consist of many immature elements and can arise by several mechanisms including failure of meiosis I or II (Figure I.iv.) or fusion of two ova (Ohama *et al.*, 1985).

The human ovarian teratocarcinoma cell line PA-1 was isolated from the ascites fluid of a 12 year-old girl suffering from recurrent malignant teratocarcinoma (Zeuthan *et al.*, 1980). This cell line has the characteristics of an immature teratoma (Giovanella *et al.*, 1974) and is believed to have developed from stem cells prior to the first meiotic division (Figure 1.iv.). Other ovarian teratocarcinomas of this origin have been identified (Hoffner *et al.*, 1992). High passage PA-1 cells are tumorigenic in nude mice upon subcutaneous injection, form embryoid bodies under non-adherence culture conditions, and form colonies in soft agar. Cytogenetically, PA-1 has a stable diploid female karyotype, with a single balanced translocation between chromosomes 15 and 20 (t(15;20); Zeuthan *et al.*, 1980).

Figure 1.iii. The development of germ cell tumors. Teratocarcinomas are composed of embryonal carcinoma cells and immature somatic tissues. Benign teratomas contain immature somatic elements but have lost the ability to metastasize. Immature teratomas contain immature somatic tissues which have retained the ability to metastasize. (Adapted from Andrews *et al.*, 1987.)

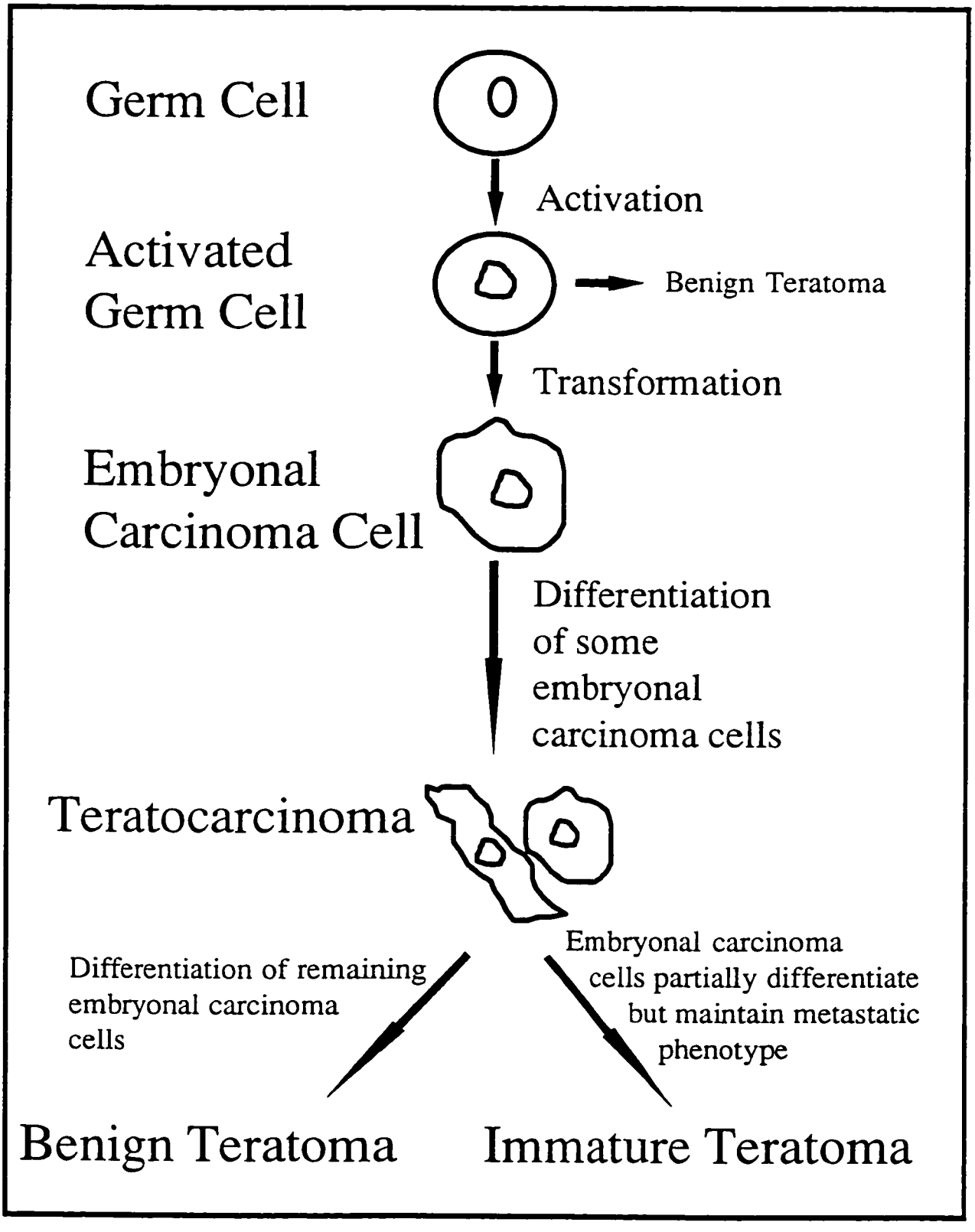
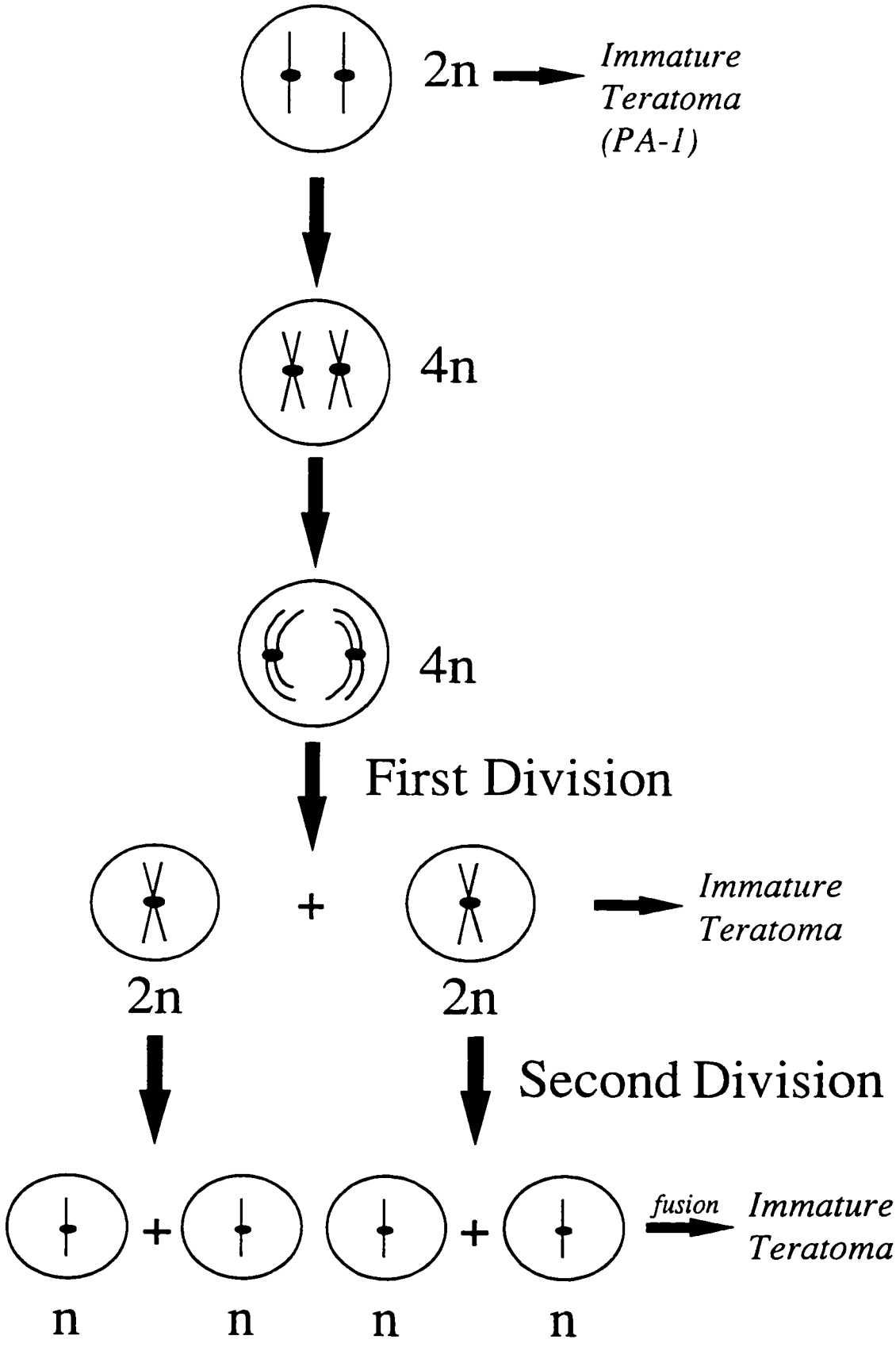

Germ Cell
Activation
Activated Germ Cell
Benign Teratoma
Transformation
Embryonal Carcinoma Cell
Differentiation of some embryonal carcinoma cells
Teratocarcinoma
Differentiation of remaining embryonal carcinoma cells
Embryonal carcinoma cells partially differentiate but maintain metastatic phenotype
Benign Teratoma
Immature Teratoma

Figure 1.iv. Schematic representation of meiosis. Stem cells undergo a duplication of chromatids and are converted from haploid (2n) to tetraploid (4n). Following this, the tetraploid cell undergoes the first meiotic division resulting in two haploid cells. The haploid cells divide further, during a second meiosis, each producing two monoploid (n) cells (Adapted from Klug and Cummings, 1983). Possible origins of immature teratomas, including PA-1, are indicated as failure of the first or second meiosis or fusion of two ova (Hoffner *et al.*, 1992).


2n
Immature Teratoma (PA-1)
4n
4n
First Division
+
Immature Teratoma
2n
2n
Second Division
+
+
fusion
Immature Teratoma
n
n
n
n

I.vii. Rationale and Objectives

The PA-1 human teratocarcinoma cell line represents an interesting opportunity to study the connected fields of tumor suppression and differentiation. It is an ideal cell line to use in this venture for several reasons. First, it is a human cell line; any findings resulting from such a study are directly applicable to the human species without the confounding effect of interspecies differences in gene product functions. Secondly, it is a tumorigenic cell line, making it relevant for the study of cancer. Finally, it is a teratocarcinoma cell line. Since teratocarcinomas are often used as models of differentiation and development, any findings resulting from such a study will have implications in terms of this aspect of biology. As has been discussed above, the phenomena of tumor suppression and differentiation are tightly linked; thus, the PA-1 cell line is a valuable resource which may be used to reveal the identity of genes involved in the complex processes of normal growth and differentiation.

The aim of the studies presented herein was to identify a chromosomal region harbouring a tumor suppressor gene. The inactivation of such a gene is involved in the tumorigenesis of the PA-1 teratocarcinoma cell line. The preliminary portion of the project relied on the introduction of single normal tagged human chromosomes into the PA-1 cell line, with a subsequent assessment of the tumor forming ability of the resulting cell line. Once a chromosomal region which could suppress the tumorigenicity of PA-1 was identified, the goal of isolating candidate tumor suppressor gene cDNAs would be addressed. Finally,

the role of chromosomal locus in both the differentiation of PA-1 and the expression of differentiation-specific genes was explored.

The results described in Chapter 2 have been published (McGowan-Jordan *et al.*, 1994). Experiments to finalize the publication of portions of Chapters 3, 4 and 5 are now being completed (Mario Chevrette, personal communication).

CHAPTER 2 Suppression of Tumorigenicity of PA-1 by Introduction of Tagged Human Chromosomes

2.i. Introduction

Cell fusion experiments gave an early indication that tumor suppressor genes, the wild type of which is dominant, exist. These first experiments were whole cell fusions between tumorigenic and non-tumorigenic cell. The resulting hybrids were non-tumorigenic (Harris, 1971) but were difficult to analyse: in order to determine which chromosome(s) were implicated in the tumor suppression one was forced to karyotype the hybrids, watch for a reversion to the tumorigenic phenotype and repeat the karyotype. Any chromosomes which were consistently lost were associated with tumor suppression (Jonasson *et al.*, 1977; Klinger and Kaelbling, 1986; Stanbridge *et al.*, 1981).

Over several years, whole cell fusions led to the development of the technique of microcell fusion (Fournier and Ruddle, 1977; Ege *et al.*, 1977). This technique has the advantage of allowing the introduction of single chromosomes into a given cell line. It involves the induction of micronucleation in a donor cell line which carries chromosomes encoding a selectable marker. These genes could either be part of the genetic background of the cells undergoing micronucleation, such as hypoxanthine guanine phosphoribosyl transferase (*HGPRT*), or have been introduced to ensure resistance to a specific drug, such as hygromycin B. The micronuclei are physically separated from the donor cells and are then fused chemically to recipient cells. Cells retaining the tagged chromosome, which is introduced into the recipient cells via the micronuclei, can be selected due to the retention of the selectable marker.

Microcell fusion has been used for the isolation of several genes by a rather defined routine. Initially, this technique is used to identify the chromosomal location of genes which act in a dominant fashion to invoke a phenotype for which one can assay. These phenotypes range from the suppression of tumorigenicity (Bérubé *et al.*, 1994; Coleman *et al.*, 1995; Islam *et al.*, 1995; McGowan-Jordan *et al.*, 1994; Tanake *et al.*, 1996), to induction of growth arrest (Speevak and Chevrette, 1996), to cellular senescence (Hensler *et al.*, 1994; Ning *et al.*, 1994; Ogata *et al.*, 1993; Sandhu *et al.*, 1996), to DNA repair (Kaur and Athwal, 1993; Kurimasa *et al.*, 1994), to tissue specific extinguishing of gene expression (Jones *et al.*, 1991; Shapero *et al.*, 1994), and include the complimentation of disease-specific drug sensitivities (Whitney *et al.*, 1995). Following this, sub-chromosomal fragments of DNA can be transferred (Koi *et al.*, 1992; Leach *et al.*, 1989; Whitney *et al.*, 1995) in an effort to clarify the location of the gene which confers the phenotype of interest. Finally, subtractive or differential display techniques are used to clone the gene. Examples of genes which have been identified and cloned using microcell fusion in combination with other techniques are KAI1, a suppressor of prostate cancer metastasis (Dong *et al.*, 1995), and TSE1, an extinguisher of liver-specific gene expression (Jones *et al.*, 1991); however, numerous genes have been localized to specific chromosomal regions using this procedure. Also, the activity of several tumor suppressor genes in the etiology of different cancer types has been supported by microcell fusion experiments in which a chromosome harbouring a putative tumor suppressor gene is introduced into a tumor cell line (Tanaka *et al.*, 1991; Yamada *et*

al., 1995). Once the chromosome is introduced, its involvement in the tumorigenicity is evident by a decrease in the tumor-forming ability of the cell line.

There are several methods of assessing the tumor forming ability of cell lines. A preliminary indication of a change in the tumorigenicity of a cell line may be an observable difference in the morphology of cells *in vitro* following the introduction of chromosomal material or gene transfection (Hao *et al.*, 1993; Kitayama *et al.*, 1989; Stanbridge and Ceredig, 1981). Such a change in cellular morphology may indicate the differentiation of the cell line and thus, a decrease in the counter-related phenotype of tumorigenicity. Another indication that a cell line has reduced ability to form tumors is the inability to grow in an anchorage-independent manner. Growth in soft agar is a means of assessing anchorage-independence. The ability of cells to grow and divide without attachment to a substratum is a hallmark of tumorigenic cells and may mirror the ability of tumorigenic cells to metastasize *in vivo* where cells must be able to survive without the benefit of anchorage to an extracellular matrix.

In an experimental setting, the tumor forming ability of cell lines is often accessed by sub-cutaneous injection into "nude" mice. These nearly hairless mice are athymic due to an autosomal recessive mutation (*nu/nu*) (Pantelouris, 1968). Thus, they elicit a low immunological response due to an absence of both thymus-derived mature T-lymphocytes and a thymus-dependent antibody response. They do not reject and kill the cells which are injected; the cells are then free to form tumors in an environment similar to that of their original host, without the threat of an immunological reaction.

Results from previous experiments have suggested that tumor suppressor genes have been inactivated in PA-1. Early passage PA-1 cells are not tumorigenic in nude mice while late passage cells are tumorigenic and carry an activated *N-RAS* oncogene (Tainsky *et al.*, 1984). Whole cell fusions between early and late passage cells resulted in suppression of the *RAS*-transformed phenotype, suggesting that an active dominant tumor suppressor gene was present in the early passage cells (Krizman *et al.*, 1990). As these whole cell hybrids were cultured, the pattern of chromosome loss and reversion to tumorigenicity was monitored. The pattern suggested the existence of tumor suppressor genes on chromosomes 1, 4 and 11, since tumorigenic cells were often found to have lost one or more of these chromosomes. Two of these chromosomes, 1 and 11, harbour known tumor suppressor genes: *K-REV1/RAP1A* (Rousseau-Merck *et al.*, 1990; Takai *et al.*, 1993) is located on chromosome 1, while chromosome 11 encodes *WT1* (Call *et al.*, 1990) and *KAI1* (Dong *et al.*, 1995). However, no confirmed tumor suppressor gene has yet been mapped to chromosome 4.

The goal of this study was to identify the chromosomal location of a tumor suppressor gene which is involved in the tumorigenic phenotype of the PA-1 cell line. As discussed in Chapter 1, tumor suppressor gene are often localized by the examination of a large number of clinical tumor sample for cytogenetic alterations and loss of heterozygosity. This approach is difficult to employ when dealing with teratocarcinoma since the number of clinical samples is small. Thus, in order to determine the chromosomal location of a putative tumor suppressor gene in PA-1, microcell fusion was used to introduce tagged human chromosomes (THC) into the cell line. Once THC had been introduced into PA1N5 cells,

the phenotype of tumorigenicity was assayed by injection into nude mice since this is the most definitive measure of tumorigenicity.

2.ii. Materials and Methods

2.ii.a. Cell Lines and Culture Conditions

PA-1 (obtained at passage 332 from the American Type Culture Collection, Rockville, MD, USA) and all resulting cell lines were maintained as monolayer cultures at 37°C under 5% CO_2 in minimal essential media (MEM) supplemented with 10% fetal bovine serum (FBS). Electroporation (960μFD, 200V) using a Biorad Gene Pulser apparatus was used to introduce pSV2neo (Southern and Berg, 1982) into PA-1. Several geniticin-resistant (200μg/mL) clones were recovered after plating of the electroporated cells. One clone, PA1N5, with growth properties similar to those of PA-1 was chosen for further experimentation.

For soft agar assays, 5 x 10^4 cells in 3mL of 0.3% agar in media were plated onto a base of 0.6% agar in media. Cultures were fed weekly and foci were counted after two weeks.

For tumorigenicity assays, cells were trypsinized and counted using a Coulter Counter (Coulter Electronics, Inc., Hialeah, FL). Following resuspension in sterile saline, 1 - 1.5 x 10^7 PA1N cells or 1 x 10^7 PAB cells (Figure 2.i.) were injected subcutaneously into *nu/nu* athymic mice (Charles River). Tumor formation was monitored weekly for at least three months or until tumors arose.

B78 mouse melanoma cells (gift from M. Thayer, Oregon Health Sciences University, Portland, OR, USA) were grown in high glucose Dulbecco's modified Eagle

medium (DMEM) supplemented with 10% FBS. B78MC microcell hybrids (Speevak *et al.*, 1995) which carry human chromosomes tagged with the dual-selectable marker, tgCMV/HyTK (provided by S. Lupton, Lupton *et al.*, 1991), were cultured as B78 cells except that hygromycin B (400µg/ml) was added to the media. This marker encodes a fusion gene, the expression of which results in hygromycin B resistance and ganciclovir sensitivity. Thus, by using the respective drug, one may select for either the retention or the segregation of the tagged chromosome.

Human fibroblasts (HSF) which had been established from foreskins (Speevak *et al.*, 1995) were grown in DMEM/Ham's F12 1:1 medium supplemented with 10% FBS.

All cell lines were determined to be free of mycoplasma by Hoechst 33258 staining.

2.ii.b. Microcell-Mediated Chromosome Transfer

The B78MC mouse/human microcell hybrids were used to transfer the tagged human chromosome (THC) into PA1N5, by microcell fusion (Figure 2.i.). All microcell fusions were performed essentially as described by Fournier (1981). The donor B78MC cell lines were grown to near confluence in Corning cell culture flasks (150cm^2, DMEM + 10% FBS, 400µg/mL hygromycin B). For each fusion three or four flasks were used. Micronucleation of the donor cell line was induced by treatment with colcemid (0.06µg/mL) for 24 hours, which traps the cells in mitosis after which the nuclear envelope forms around individual or groups of chromosomes. Following colcemid treatment, the donor cells were trypsinized, added to DMEM + FBS and centrifuged (200xg, 10min) Cells were resuspended in sterile

phosphate buffered saline (137mM NaCl, 2.7mM KCl, 4.3mM Na_2HPO_4, 1.4mM KH_2PO_4). The cells were then layered onto plastic "bullets" which had been sterilized (70% ethanol, at least 2 hours), then prepared for cell attachment by overlaying with equal volumes of 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluene-sulfonate (75mg/mL in 0.9% NaCl, filter sterilized) and concavalin A (15mg/mL in 0.9% NaCl, filter sterilized), left at room temperature for 2 hours then rinsed twice with phosphate buffered saline. Cells were allowed to attach for 15-30 minutes and were then incubated in DMEM + FBS for 2 hours. Following this, the "bullets" were placed in sterile centrifuge tubes containing enucleation media (10µg/mL cytochalasin B in DMEM, 37°C), then centrifuged (34°C, 30min, 32000xg). The supernatant was decanted and the pellet of microcells was resuspended in DMEM (1mL). The suspension from 24 bullets was combined and filtered, first through a 8µm filter, then through a 5µm filter, to remove whole cells. The microcells were then centrifuged (200xg, 10min), and resuspended in MEM (2mL). PA1N5 recipient cells were grown to 80% confluence in tissue culture flasks (25cm^2), rinsed with MEM (5mL). The cells were overlaid with microcell-containing suspension (1mL) then phytohemaglutinin (1mL) and incubated at 37°C. Control flasks were overlaid with MEM, (containing no microcells) and phytohemaglutinin. Once the microcells had attached to the recipient cells (10-15min), the phytohemaglutinin was aspirated, and polyethylene glycol (46-50% w/v in MEM) was added to the flask. Following an incubation of 60 to 90 seconds with gentle rocking, the polyethylene glycol was aspirated and the flask was rinsed rapidly three times with MEM. Cells were maintained in non-selective media for 48 hours where upon the

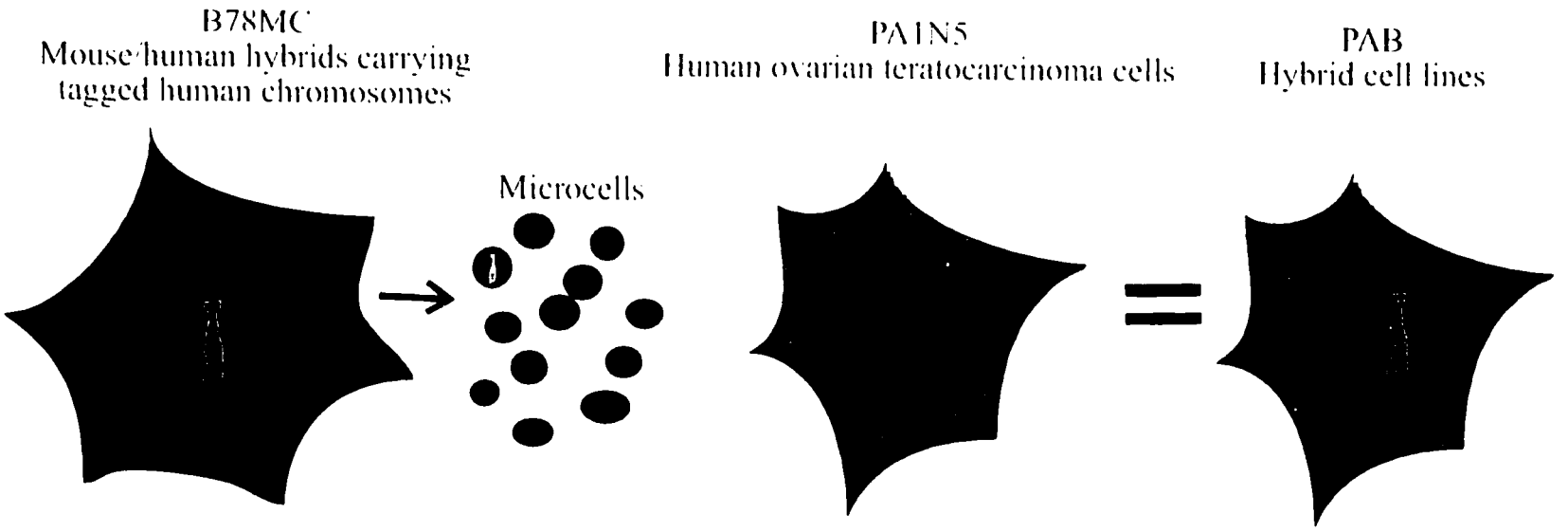
media was changed to selective media containing geniticin (200μg/ml) and hygromycin B (400μg/ml). The fused cells were split at a ratio of 1:15 one day following the fusion. Approximately 3 weeks following the microcell fusion, small colonies visible to the naked eye appeared. These were trypsinized using cloning cylinders. Individual clones were expanded and frozen in liquid nitrogen for long term storage.

Segregants of PAB108, from which the THC had been removed by negative selection, were isolated after three weeks of growth in the presence of ganciclovir (100μM).

2.ii.c. Hygromycin B Resistance

For assessment of hygromycin resistance 2.5 x 10^3 cells were plated in triplicate in MEM + 10% FBS with and without hygromycin B (400μg/ml) in six well plates. After six days, cells were trypsinized and counted using a Coulter Counter.

Figure 2.i. Schematic representation of the microcell fusion process. The production of microcells from the donor cell line, B78MC, and the fusion of microcells to the recipient cell line, PA1N5, is illustrated. The resulting hybrid cell lines were called PAB.


B78MC
Mouse/human hybrids carrying
tagged human chromosomes
Microcells
PA1N5
Human ovarian teratocarcinoma cells
PAB
Hybrid cell lines

2.ii.d. Cytogenetic Analysis

Metaphase chromosome spreads were prepared according to standard procedures. Cells were grown to approximately 80% confluence in flasks ($75cm^2$). Colcemid (0.15mL, 5µg/mL) was added to trap the cells in mitosis. After a two hour incubation the media was collected; the cells were trypsinized, collected in the same tube, and centrifuged (200xg, 10min). The media was aspirated and the cells loosened by tapping the bottom of the tube. The cells were then resuspended in sodium citrate (20mL, 1%, 37°C) and incubated for 20min at 37°C. The cells were centrifuged as above and the supernatant removed except for about 0.5mL which was used to aid in resuspension. While being vortexed, the cells were resuspended in freshly prepared Carnoy's Fixative (10mL, methanol:acetic acid, 3:1, -20°C) which was added drop by drop for the first millilitre. Following a 30min incubation at -20°C, the cells were centrifuged. This fixation procedure was repeated at least one additional time, after which the cells were collected by centrifugation and resuspended in a small volume (1-5mL) of fixative. Cells were then applied dropwise onto glass slides which had been presoaked in ethanol and air dried. Once applied to the slides, the cells were allowed to drain briefly and were then dried onto the slide by placing the slide over a steaming water bath. Slides were aged on a slide warmer, which was kept at 55°C, for a period of one to 14 days.

Metaphase chromosome spreads were G-banded after a brief trypsinization (1% Bacto trypsin in 0.9% NaCl) by sequential treatment in $CaCl_2$ (1%), water, and stain (10%

Leishman's stain, 4% Harleco Giemsa stain, in phosphate buffer, pH6.86) followed by a final water wash.

2.ii.e. Fluorescence In Situ Hybridization.

Fluorescence *In Situ* Hybridization (FISH), as described by Pinkel *et al.* (1988), was used to confirm that human chromosome 4 was introduced into PA1N5 to create the PAB108 cell line. The DNA probe for human chromosome 4, provided by Dr. J. Gray (University of California, San Francisco) was biotinylated and used on metaphase chromosome spreads in the laboratory of Dr. D. Blakey (Health Canada) according to standard procedure. A Nikon Optiphot microscope fitted with a B filter was used for viewing. The fluorescein-labelled probe fluoresces green, while the unhybridized chromosomes are stained with propidium iodide.

2.ii.f. DNA Isolation

DNA was isolated from confluent culture by treating cells with TSM (10mM Tris HCl pH8.0, 150mM NaCl, 2mM $MgCl_2$) + Nonidet P-40 (0.5%), vortexing, incubating on ice (2-3min), then microcentrifuging (12000xg, 10sec) to pellet nuclei. The pellets were then resuspended in nuclei dropping buffer (5mL, 75mM NaCl, 24mM Na_2EDTA pH8.0) containing proteinase K (1mg) and sodium dodecyl sulfate (SDS, 0.25mL, 10%). After shaking on an orbital shaker overnight (37°C), the samples were mixed on an orbital shaker (1-2 hours, room temperature) with distilled water (5mL) and NaCl (5mL, 6M). Following

centrifugation (10min, full speed, Model HN-S Table Top Centrifuge, International Equipment Co.), the supernatant was transferred to a new tube. Ethanol (2 volumes, 99%) was added, allowing for the precipitation of DNA by inverting the tube several times. DNA was retrieved by spooling with a Pasteur pipette, then resuspended overnight in TE (10mM Tris HCl pH8.0, 1mM Na_2EDTA). Finally, DNA was reprecipitated by adding $NH_4C_2H_3O_2$ (0.5 volume, 7.5M) and ethanol (2 volumes, 99%). The spooled DNA was then resuspended in TE^{-4} (10mM Tris HCl pH8.0, 0.1mM Na_2EDTA).

2.ii.g. Alu-PCR FISH.

The single human element in B78MC108 (a mouse/human microcell hybrid containing human chromosome 4 (Speevak *et al.*, 1995)) was amplified using *Alu*-PCR primers 153, 154, 450 and 451 (Dorin *et al.*, 1992). The amplification was carried out using DNA (50ng), P.E. buffer (1X; 10mM Tris pH8.3, 50mM KCl, 1.5mM $MgCl_2$, 0.01% gelatin), dNTPs (200μM), Taq polymerase (1.5U), and appropriate primers (250ng). After an initial denaturation (94°C, 3min), 35 amplification cycles (92°C 45sec, 60°C 1min, 72°C 1min + 6sec each cycle) were completed and followed by an extension period (72°C, 10min). The amplicons were pooled and biotinylated (BioPrime DNA Labelling System), generating the *Alu*B78MC108 probe.

To show that the *Alu*B78MC108 probe is specific for chromosome 4, the probe was hybridized to normal male metaphase chromosome spreads. Human chromosome 4 in metaphase spreads of ganciclovir-resistant PAB108 segregants and tumor derived cell lines

was detected essentially as described by Dorin *et al.* (1992). Slides containing metaphase chromosome spreads were dehydrated in ice-cold ethanol (70% 2min, 80% 2min, 100% 2min), air dried then denatured in preheated denaturation solution (70°C, 70% formamide, 2XSSC, 2min; 1XSSC is 0.15M NaCl and 0.015M sodium citrate). Slides were immediately dehydrated as above and air-dried. Hybridization Mix (2-3µg Cot-1 human DNA, 100ng biotinylated *Alu*-PCR product, 1µg sheared salmon sperm DNA, 58% formamide, 2XSSC, 1mg dextran sulfate; denatured at 75°C 10min, pre-annealed 37°C 30min) was applied to the slide, covered with a glass cover slip, sealed with rubber cement, and incubated overnight (37°C) in a humidified chamber. The slide was then washed (43-45°C, 50% formamide/2XSSC 10min, 2XSSC 10min, 4XSSC/5% Triton X100 5min) with gentle agitation. Slides were stained as outlined in the Oncor Chromosome *in situ* Hybridization System and viewed using a Zeiss Axioskop microscope equipped with 09 filter combination.

2.iii. Results

2.iii.a. Transfection of PA-1 with pSV2neo

To facilitate selection of hybrid teratocarcinoma cells containing single THC, the parental cell line, PA-1, was first transfected with pSV2neo. Several geniticin-resistant clones (PA1N) were tested for growth in soft agar and ability to form tumors upon sub-cutaneous injection in nude mice (Table 2.i.). Clone PA1N5 had the same karyotype (Figure 2.ii.) as has been reported for PA-1 (Zeuthan *et al.*, 1980) carrying 46 chromosomes and only one balanced translocation (t(15p;20q)). It exhibited growth properties similar to the parental PA-1 cells (Table 2.i.), and was chosen for further experiments.

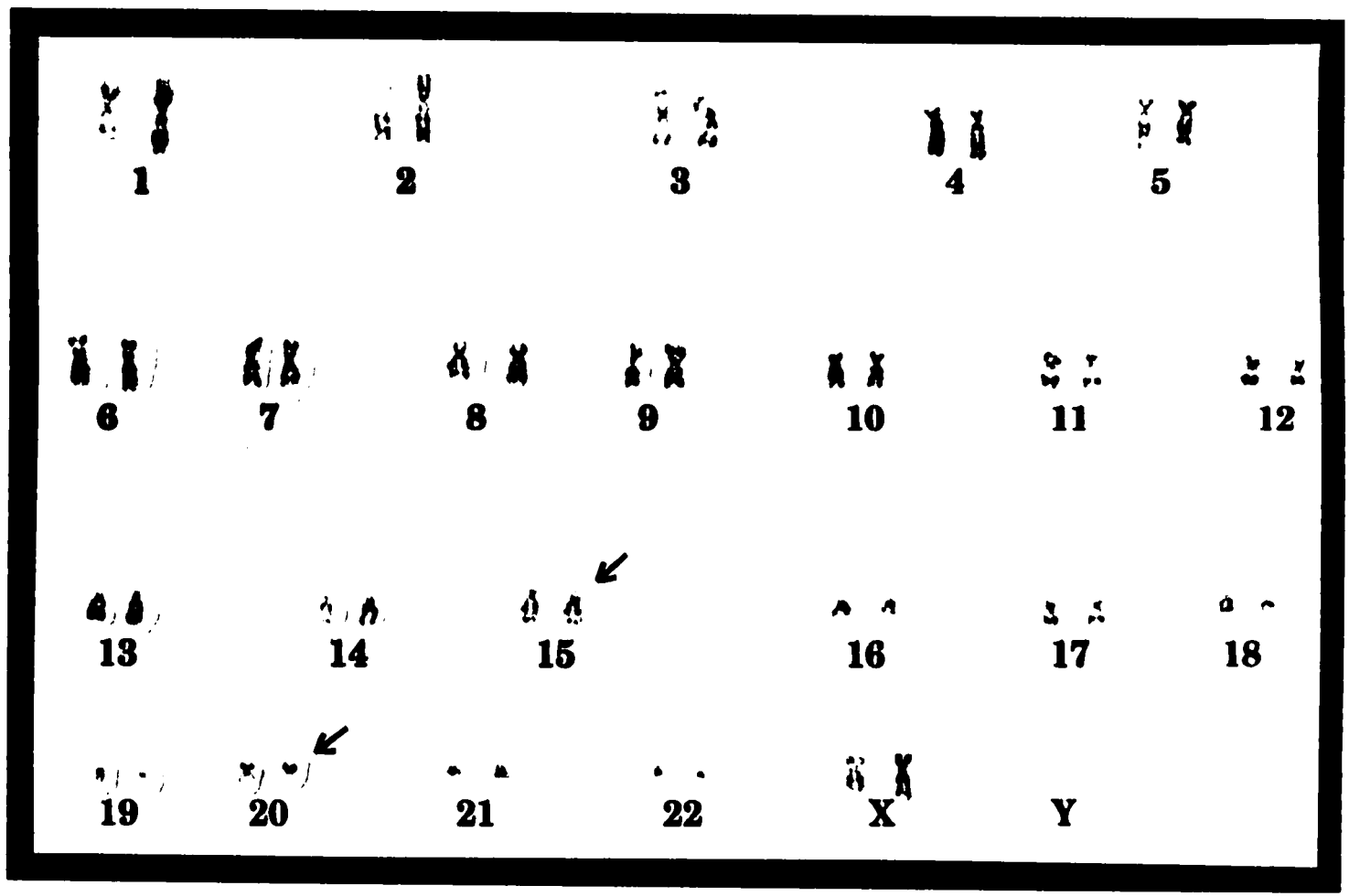
Table 2.i. Growth Properties of PA-1 and PA1N Cell Lines

Cell Line	Geniticin Resistance	% Growth in Soft Agar[a]	Tumorigenicity in Nude Mice[b]
PA-1	-	1.9%	+
PA1N5[c]	+	2.0%	+
PA1N8[c]	+	1.0%	-
PA1N10[c]	+	1.2%	+
PA1N18[c]	+	1.9%	-

[a](The number of foci/60mm^2 dish / number of cells plated) X 100.
[b]Tumors were scored 3 weeks after subcutaneous injection.
[c]Transfection of PA-1 with pSV2neo to create the PA1N cell lines was performed by Marsha Speevak.

Figure 2.ii. Karyotype analysis of PA1N5 showing G-banded chromosomes. The PA1N5 cell line has a normal karyotype, except for one balanced translocation between chromosomes 15 and 20, which is indicated by arrows.

PA1N5



46,XX,t(15p;20q)

2.iii.b. Introduction of THC into PA1N5

As described by Speevak *et al.* (1995), human fibroblasts were established from foreskins. The tgCMV/HyTK plasmid is a dual selectable marker and confers resistance to hygromycin B and sensitivity to ganciclovir (Lupton *et al.*, 1991). This plasmid was introduced into normal human skin fibroblasts to ensure the subsequent retention of the transferred chromosomes in the microcell hybrid line. In the first round of microcell fusions, human chromosomes were transferred into a mouse melanoma cell line, B78, thus generating a mouse/human microcell hybrid panel (B78MC, Speevak *et al.*, 1995). Microcell fusions between B78MC hybrids containing THC and geniticin-resistant PA1N5 were performed (Figure 2.i.). PAB hybrids containing a THC are resistant to both geniticin and hygromycin B, but are sensitive to ganciclovir due to the Herpes thymidine kinase gene encoded by tgCMV/HyTK (Lupton *et al.*, 1991).

Microcell hybrids generated from each fusion were analyzed to confirm the introduction of the THC (Table 2.ii.).

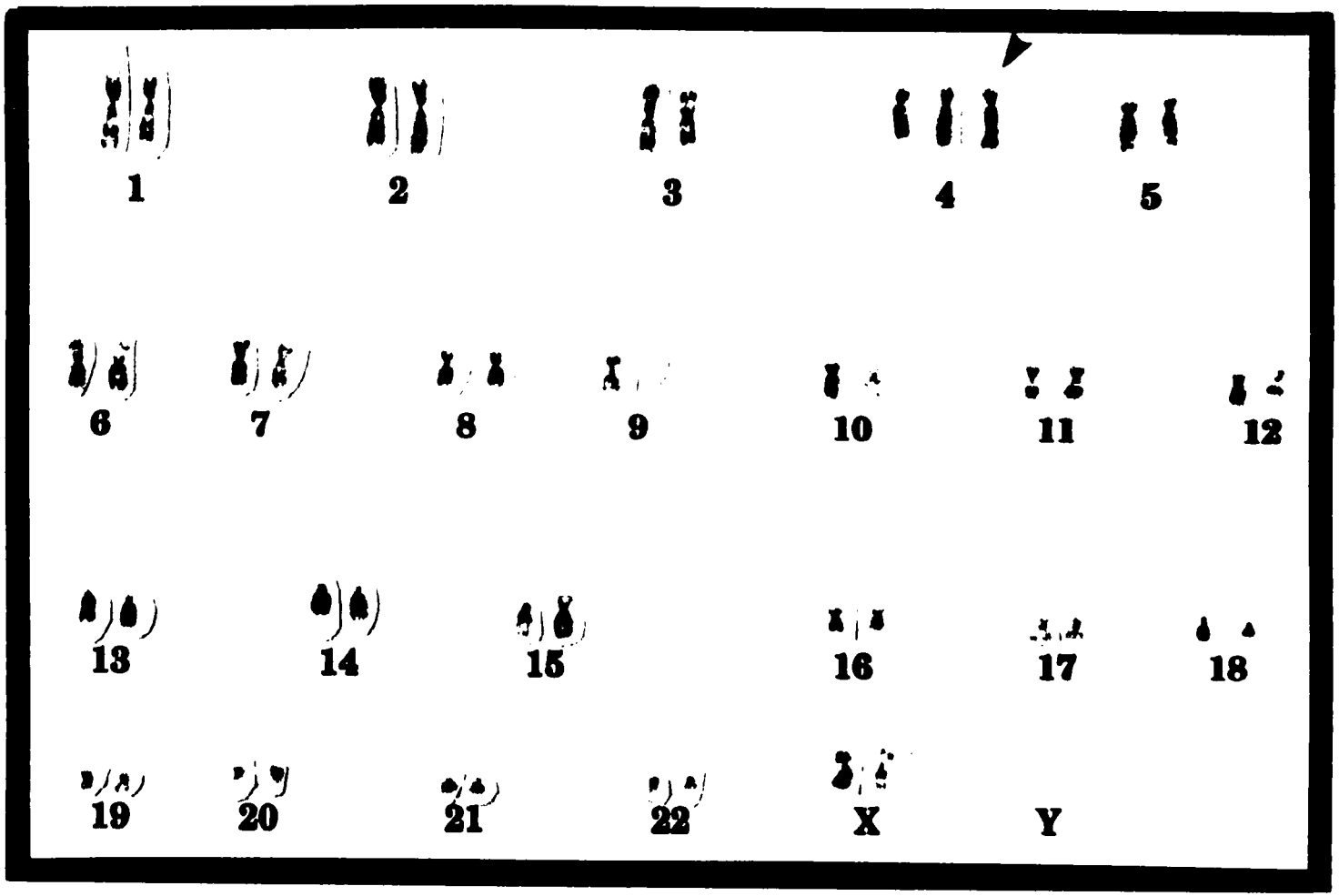
Table 2.ii. Properties of PA1N5-derived microcell hybrids.

Hybrid Name	THC[a]	# Independent Clones Injected	# Tumors/ #Injections[b]	Growth in Hygromycin B (400 µg/ml)
PA1N5	none		9/9	-
PAB9[c]	del (12)	1	2/2	+
PAB63	del (7)	2	4/4	+
PAB108	4	3	2/12	+
PAB129	*	2	6/6	+
PAB108-S	none	2	6/9	-
PAB108-TD1	**			+/-
PAB108-TD2	der (4)			+

[a]THC refers to Tagged Human Chromosomes.
[b]Tumors were scored 21 days after subcutaneous injection of 10^7 cells.
[c]Microcell fusion to create PAB9 was performed by Nathalie Berube.
*Unidentified
**_Alu_-PCR FISH (data not shown) shows that the majority of cells (22 of 35 observed) had lost the third human chromosome 4.

Figure 2.iii. Karyotype analysis of PAB108 showing G-banded chromosomes. The PAB108 cell line has a karyotype identical to that of PA1N5 except for the addition of one copy of chromosome 4, which is indicated by the arrowhead.

PAB108



47,XX,+4,t(15p;20q)

Karyotype analysis revealed that all microcell hybrids carry a supernumerary chromosome: PAB9 contains an extra portion of chromosome 12, since the donor cell line, B78MC9, contains a tagged del (12) chromosome (Speevak *et al.*, 1995); PAB63 contains an extra centromeric portion of chromosome 7 (del (7), data not shown), since B78MC63 contains a tagged chromosome 7 (Speevak *et al.*, 1995); an intact human chromosome 4 was transferred into PAB108 (Figure 2.iii.), and a chromosome determined to be a portion of chromosome 8, 9, or 10 (data not shown) was introduce into PAB129. The cytogenetic identification of the introduced chromosome in PAB108 was confirmed by FISH using a human chromosome 4-specific chromosome "painting" probe (Pinkel *et al.*, 1988), which revealed the presence of a third copy of chromosome 4 (Figure 2.iv.A).

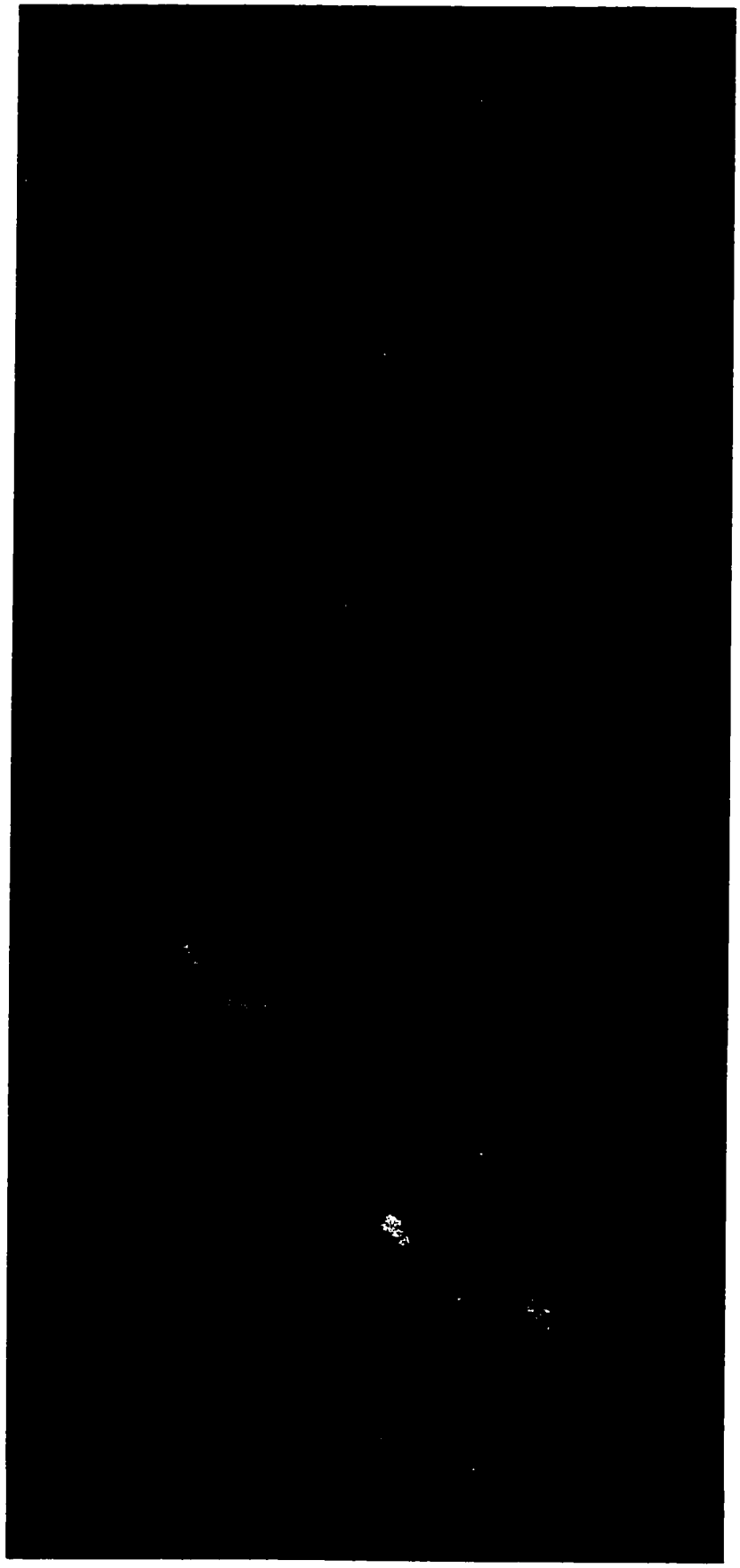
A chromosome 4-specific probe was prepared using *Alu*-PCR and the microcell hybrid B78MC108 which contains only one human chromosome (data not shown). This probe (*Alu*B78MC108) was shown to hybridize to chromosome 4 in a specific manner since it hybridized to only one pair of chromosomes in a spread of normal human cells (Figure 2.iv.C). Ganciclovir was used to segregate the THC from PAB108. These segregants (PAB108-S) are sensitive to hygromycin B (Table 2.ii.) and were shown by *Alu*-PCR FISH (Figure 2.iv.B) to contain only two copies of human chromosome 4.

Figure 2.iv. Analysis of chromosome content in PAB108 and PAB108-S by FISH. A. PAB108 metaphase chromosome spreads were hybridized with a human chromosome 4-specific painting probe. The experiment was performed by K. Huang in the laboratory of Dr. D. Blakey. B. PAB108-S chromosomes were hybridized with *Alu*B78MC108. At least 30 spreads of each cell line were examined. All PAB108 spreads had three hybridizing chromosomes, while all PAB108-S spreads had only two hybridizing chromosomes. C. Human skin fibroblasts were hybridized with *Alu*B78MC108 probe which hybridizes to one pair of human chromosomes and is specific for human chromosome 4.

A.

B.

C.



2.iii.c. Tumorigenicity of PA-1-Derived Microcell Hybrids

The tumorigenicity of several hybrids was tested (Table 2.ii.) by monitoring tumor growth after sub-cutaneous injection of cells into nude mice. The parental cell line PA1N5 formed tumors by 21 days after injection. Microcell hybrid PAB63, containing an extra portion of human chromosome 7 also formed tumors by 21 days post injection as did both PAB9 and PAB129 (Table 2.ii.). In contrast, numerous injections of three independent clones of PAB108 (PAB108-1, PAB108-3, and PAB108-4, isolated from three different flasks following microcell fusion) failed to induce tumors in nude mice, while two PAB108-S clones (PAB108-S25 and PAB108-S33) which had segregated the tagged chromosome 4 upon ganciclovir selection, rapidly formed tumors (Table 2.ii.). These results indicate that there is a tumor suppressor gene on human chromosome 4 which is inactivated in PA-1 cells. It is of interest to note that the putative tumor suppressor gene on chromosome 4 may show some specificity in its action since the mouse melanoma cell line B78MC108 (from which human chromosome 4 was transferred) was tumorigenic when injected into nude mice (M. Speevak, personal communication). However, the tumorigenicity of B78MC108 may be explained since B78 is an extremely aggressive cell line, which is able to for tumors in nude mice in just six days.

2.iii.d. Senescence

Besides its ability to confer a non-tumorigenic phenotype upon PAB108, human chromosome 4 also induced cellular senescence wherein the cells ceased dividing after approximately 25 passages. This phenotype, was also noted in the tumorigenic hybrid, PAB63, which contains a portion of chromosome 7 and senesces at passage 13. Thus, there appear to be at least two activators of the PA-1 cell line senescence phenotype present on chromosomes 4 and 7.

2.iii.e. Tumor Derived Cell Lines

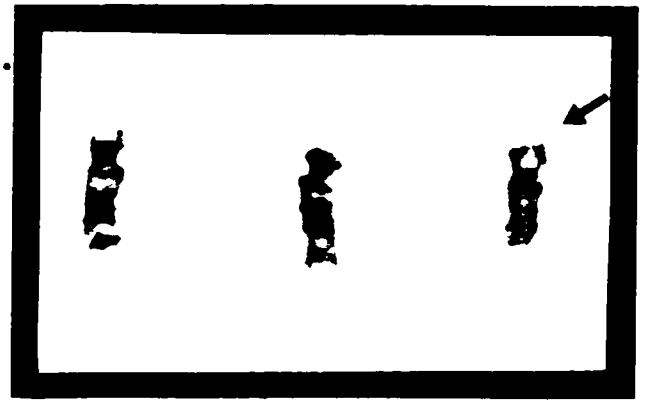
In two instances, injection of PAB108 resulted in tumor formation (Table 2.ii.). Karyotype analysis and FISH were performed and showed that tumor derived cell lines had either lost a copy of chromosome 4 (PAB108-TD1, not shown) or had a rearranged chromosome 4 (PAB108-TD2, Figure 2.v.A). The G-banding pattern showed that the rearranged chromosome 4 present in PAB108-TD2 contained additional material in the short arm, and a deletion of part of the long arm (Figure 2.v.A). Moreover, FISH analysis indicated that the additional material in the short arm was derived from chromosome 4 material, since there was no unhybridized part of the painted chromosome (Figure 2.v.B).

The PAB108-TD1 cell line was sensitive to hygromycin B (Table 2.ii.), indicating that the tagged chromosome 4 had been lost and was responsible for the tumor suppression seen in PAB108. The THC was likely lost from the cells due to the non-selective environment of the mouse. The resistance of PAB108-TD2 to hygromycin B (Table 2.ii.)

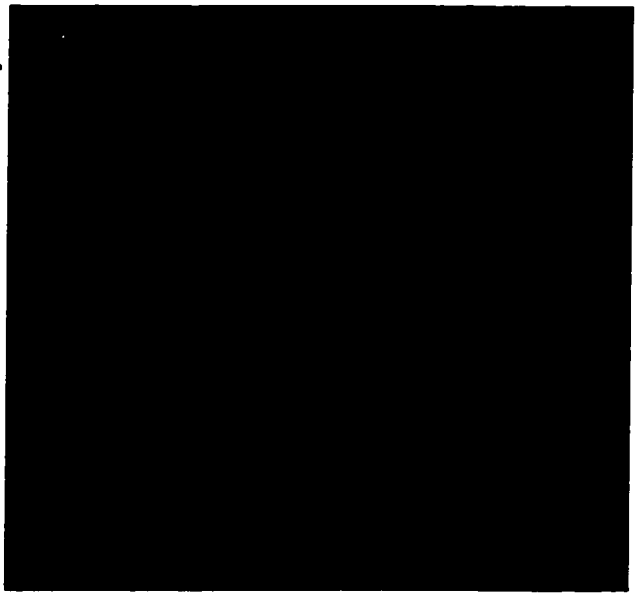
indicates that either an untagged chromosome 4 (originally present in PA1N5) was rearranged or that the tagged chromosome 4 was rearranged without affecting the tgCMV/HyTK tag. In order to differentiate between these two possibilities, a ganciclovir segregant was selected from PAB108-TD2. Karyotype analysis showed that the segregant contains 2 normal copies of chromosome 4 and had lost the rearranged copy (Figure 2.v.C). Thus, I conclude that the rearranged chromosome 4 carries the tgCMV/HyTK tag.

Figure 2.v. Analysis of human chromosome 4 in PAB108-TD2. A. Three G-banded copies of human chromosome 4 from PAB108-TD2 showing two normal copies and one rearranged copy. The arrow indicates the rearranged copy which was present in over 80% of the analyzed spreads. B. FISH analysis of PAB108-TD2. Metaphase chromosome spreads were hybridized with *Alu*B78MC108 which hybridizes to chromosome 4. C. Two G-banded copies of normal chromosome 4 from the PAB108-TD2 segregant, PAB108-TD2-S1.

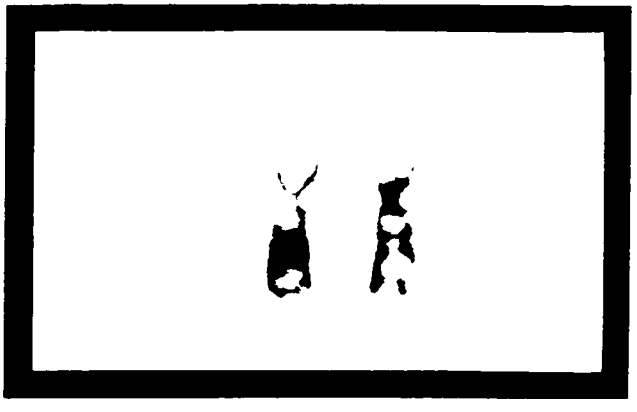
A.



B.



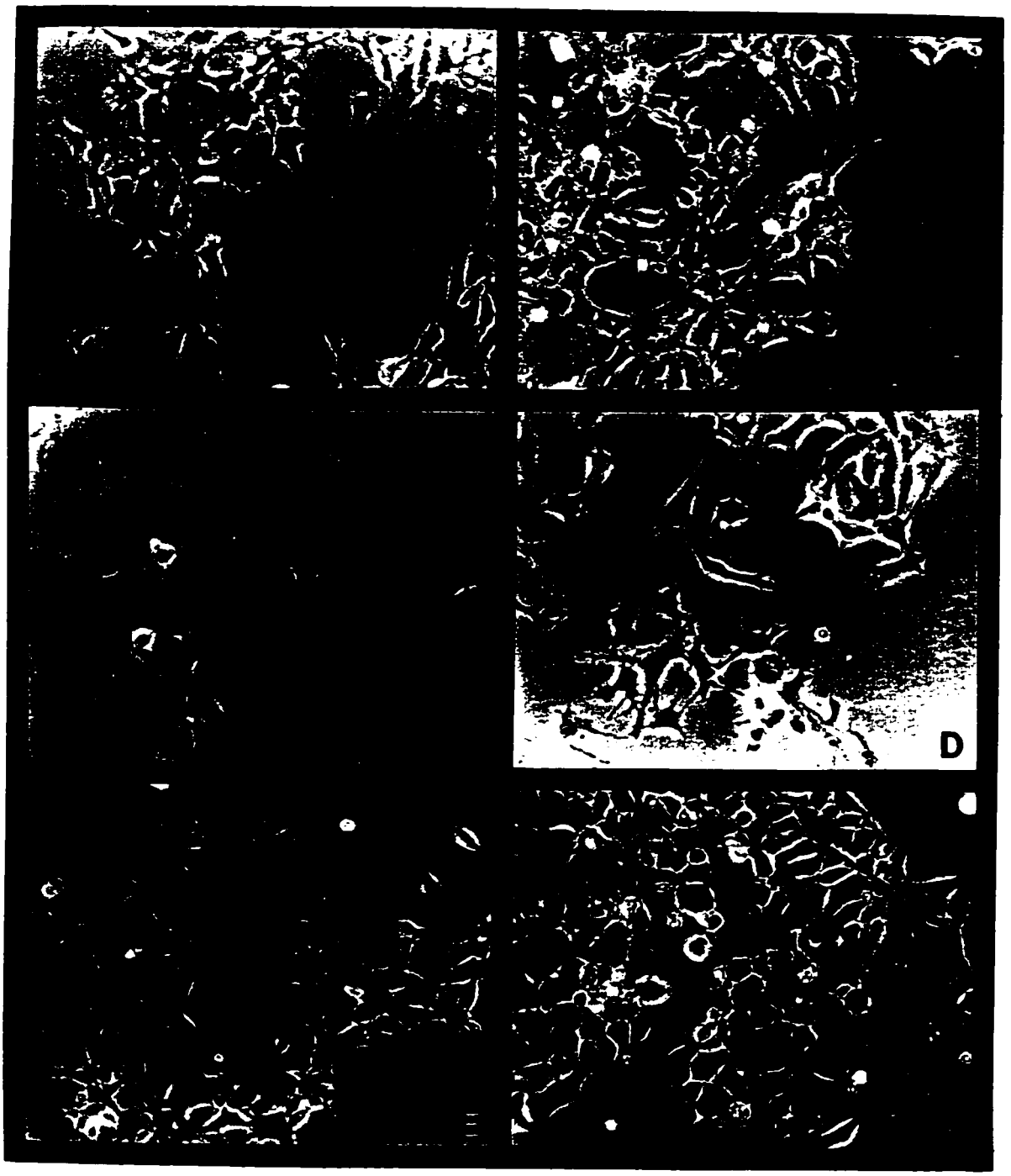
C.



2.iii.f. Morphology

Not only did the introduction of chromosome 4 into PA1N5 cause a suppression of tumorigenicity, it also resulted in a change in cell morphology. Both PA-1 and PA1N5 have an epithelial morphology when grown in tissue culture flasks (Figure 2.vi.) but the addition of either del (7) (Figure 2.vi.B) or del (12) (data not shown) to PA1N5 had no effect on its morphology. However, all three PAB108 clones obtained, each containing a tagged chromosome 4, exhibit a flattened appearance in culture (Figure 2.vi.C). Loss of this chromosome by ganciclovir selection from PAB108 resulted in a cell line, PAB108-S, with a morphology similar to that of PA1N5 (Figure 2.vi.D). The tumor derived cell lines, PAB108-TD1 which lacks the tagged chromosome 4, and PAB108-TD2 which has a rearranged tagged chromosome 4, also reverted to an epithelial morphology (Figure 2.vi.E and F). Although I cannot rule out the possibility that another gene on human chromosome 4 could be responsible for these morphological changes, the fact that they are always associated with the tumorigenic phenotype indicates that they are directly connected with the expression of the tumor suppressor gene.

Figure 2.vi. Morphology of sub-confluent cells in culture. A, PA1N5; B, PAB63; C, PAB108; D, PAB108-S; E, PAB108-TD1; F, PAB108-TD2. All photographs were taken at 100X magnification.


D

2.iv. Discussion

A panel of mouse/human microcell hybrids containing THC was used as a source for the introduction of chromosomes to PA-1. Using these mouse/human hybrids, several THC (del (12), del (7), 4 and an unidentified chromosome) were introduced into a neomycin-resistant derivative of the PA-1 cell line, PA1N5. Once these microcell hybrid cell lines, named PAB, were produced, the tumorigenicity of the hybrids could be determined.

I have shown that introduction of a normal copy of chromosome 4 into PA1N5 resulted in a change in cell morphology and was sufficient to suppress its tumorigenicity while addition of del (7) or del (12) had no effect. Loss of the tagged chromosome 4 by negative selection reverted both the tumorigenic phenotype and the morphology of PAB108 to that of PA1N5, thus providing strong evidence that a tumor suppressor locus is present on this chromosome. Earlier experiments (Krizman *et al.*, 1990) with whole cell hybrids of non-tumorigenic PA-1 and late passage tumorigenic PA-1 lend support to this hypothesis. In these studies, chromosome 4 loss from whole cell hybrids was correlated with reversion of non-tumorigenicity to tumorigenicity.

As in colorectal cancer, the tumorigenicity of PA-1 appears to result from a combination of mutations of oncogenes and inactivation of tumor suppressor genes. Late passage PA-1 is known to have an activated *N-RAS* oncogene (Tainsky *et al.*, 1984) as well as one mutated *P53* allele (Yaginuma and Westphal, 1992). Addition of a normal chromosome 4 to PA1N5 suppressed the effect of these two genetic changes suggesting that

a tumor suppressor gene located on chromosome 4 was inactivated in PA-1. Thus, at least three events contribute to the tumorigenic phenotype of this teratocarcinoma cell line.

Changes in cell morphology, as we have observed when human chromosome 4 was introduced into PA1N5, have been associated previously with reversion to a non-tumorigenic phenotype (Harris and Bramwell, 1987; Harris, 1990; Hao *et al.*, 1993), and may result from the cells entering a differentiation pathway (see Chapter 5). This property was used to clone *KREV-1/RAP1A*, a tumor suppressor gene which suppresses the transforming ability of *K-RAS* in rodent fibroblasts (Kitayama *et al.*, 1989). Although PA-1 has an activated *N-RAS*, the tumor suppressor gene that has been identified here cannot be *KREV-1/RAP1A* which has been mapped to human chromosome 1 (Takai *et al.*, 1993). However, since other members of this family have been mapped on chromosomes 12 and 13 (Rousseau-Merck *et al.*, 1990), it is possible that the new tumor suppressor gene located on chromosome 4 could be a member of the *RAP1A* family.

Currently, there are no proven tumor suppressor genes which map to chromosome 4; however, several lines of evidence suggest that this chromosome may harbour a tumor suppressor gene. Krizman *et al.* (1990) have shown that loss of chromosome 4 from whole cell hybrids of early and late passage PA-1 was associated with reversion to tumorigenicity. Moreover, specific loss of human chromosome 4 has been noted in tumorigenic segregants of whole cell hybrids derived from tumorigenic rodent cells and normal human fibroblasts (Klinger and Shows, 1983) and in hybrids derived from human fibrosarcoma and normal fibroblasts (Benedict *et al.*, 1984). In fact, loss of chromosome 4, implicating tumor

suppressor gene inactivation at this locus, has been reported in a primary human ovarian teratocarcinoma and in a metastatic tumor which was resected 1 year after removal of the primary tumor (Gibas *et al.*, 1993). This finding indicates that loss of a tumor suppressor gene on chromosome 4 may be an early event in the genesis of immature ovarian teratocarcinomas.

Further evidence that chromosome 4 may encode a tumor suppressor gene comes from the finding that several human hepatocellular carcinomas exhibit loss of heterozygosity at chromosome 4q. It has been suggested that inactivation of a tumor suppressor gene at this locus contributed to liver cancers (Buetow *et al.*, 1989). An interstitial deletion of chromosome 4 from q11-q13 has also been detected in the human hepatoma cell line HuH-7 and may have resulted in the loss of tumor suppressor gene activity (Urano *et al.*, 1991). Chromosomal translocation involving human chromosome 4 has also been associated with acute lymphoblastic leukemias (Pui *et al.*, 1990), malignant T cell lymphoma (Laabi *et al.*, 1992) and non-Hodgkin's lymphoma (Dallery *et al.*, 1995). Although such translocations often result in activation of oncogenes (Klein 1983), it is conceivable that they could disrupt a tumor suppressor gene and thus, interfere with its proper transcription.

The addition of human chromosome 4 in cell hybrids has been correlated with cell senescence (Ning *et al.*, 1991), a characteristic of some non-tumorigenic cells. Senescence was also seen in PAB108 cells, indicating that an activator of cellular senescence is present on the copy of chromosome 4 which was introduced into this cell line. Cellular senescence is thought to be related to a lack of telomerase activity (Greider, 1990; Metcalfe *et al.*, 1996),

telomerase being an enzyme which maintains telomere length during cell division. Normal aging cells lose telomerase activity while this activity is regained by many cancer cells (Chadeneau *et al.*, 1995; Sommerfeld et al., 1996). Thus, it is possible that the senescence gene located on chromosome 4 (Ning *et al.*, 1991) has some effect on telomerase activity. What relationship this has with the tumor suppression noted in PAB108 remains unclear at this time.

CHAPTER 3 Mapping of the Tumor Suppressor Gene Candidate Region

3.i. Introduction

Upon injection of the PA-1 cell line containing the normal chromosome 4 into nude mice, tumors arose within the test period in two out of 12 instances. Cytogenetic analysis of tumor derived cell lines revealed that the introduced chromosome 4 was either rearranged (der (4)) or lost from these cells (Chapter 2). This finding further implicated chromosome 4 as having the ability to suppress tumorigenicity in PAB108. But that was not all: the detection of a rearranged chromosome 4 gave a unique opportunity to map the locus responsible for the tumor suppression. By determining which portions of the chromosome had been lost from the rearranged chromosome, I connected a gene in these regions with the phenotype of tumor suppression. This type of analysis has also been used for the mapping of a suppressor locus implicated in mouse sarcoma (Imreh *et al.*, 1994) where portions of human chromosome 3 are lost in a non-random fashion from tumor-derived mouse/human microcell hybrid cell lines.

In recent years mapping of the human and mouse genomes has been substantially simplified by the discovery of a class of polymorphic, repetitive, DNA elements (Weber and May, 1989). These sequences are known as microsatellite repeats and form the basis of recent genetic linkage maps (Dib *et al.*, 1996). These are short tandem repeats of $(AC)_n$ which have been identified along the length of the human genome with the largest gap between the 5264 markers being 11cM. Of this number, 280 reside on chromosome 4. These markers are particularly useful since they are easily detected by amplification using

the polymerase chain reaction. Thus, they may be used in a human background, should the marker be polymorphic in the sample to be analyzed. Alternatively, microsatellite markers may be used in interspecies hybrids to detect the presence of a particular chromosomal region, negating the need to detect polymorphisms.

Sequence tagged sites are another class of markers which are widely used for genomic mapping (Hudson et al., 1995). These landmarks are simply mapped sequences which can be amplified. Through a monumental effort, over 15,000 sequence tagged sites, which cover 94% of the human genome, have been identified.

3.ii. Materials and Methods

3.ii.a. Microcell-Mediated Chromosome Transfer

In order to determine if the der (4) carries the tgCMV/HyTK tag and to facilitate mapping of the rearrangement in this tumor-derived chromosome 4, the tagged chromosome in PAB108-TD2 was transferred into a mouse background by microcell fusion (Figure 3.i.: see Chapter 2.ii. for details of culture conditions and microcell fusion method). This was necessary since a tgCMV/HyTK probe was too short to be used as probe in FISH experiments. PAB108-TD2 was used as the donor cell line while B78 mouse melanoma were the recipient cells. Following the microcell fusion, the resulting microcell hybrids which contain the tagged chromosome, BTD2, were cultured as B78 cells except that hygromycin B (400 µg/ml) was added to the media.

3.ii.b. FISH

A total human genomic probe was used to determine the number of human chromosomes which had been transferred to B78 during the microcell fusion. Hybridization of probe to metaphase chromosome spreads (prepared as described in Chapter 2.ii.) and detection of hybridized probe was carried out using the Oncor Chromosome In Situ Detection System.

3.ii.c. Alu-PCR FISH

To grossly identify the portions of chromosome 4 present in the der (4), the human DNA in the BTD2-3 cell line was amplified using *Alu*-PCR FISH as outlined in Chapter 2.ii.. The amplicons were pooled and biotinylated generating the *Alu*BTD2-3 probe. This probe was hybridized onto normal human chromosome spreads and was detected as described in Chapter 2.ii..

3.ii.d. PCR Mapping

DNA was isolated from confluent cultures as described in Chapter 2.ii.. To more precisely identify the region of chromosome 4 which is missing from the der (4), mapping was carried out using microsatellite repeat primers (MapPairs). Amplifications were achieved as described by Gyapay *et al.* (1994). DNA (40ng) was combined with primers (50pmol of each), P.E. buffer (1X), dNTPs (125μM), and Triton X100 (0.1%) in a volume of 50μL. After denaturation (5min at 96°C), Taq polymerase (1U) was added, the sample was amplified for 35 cycles (94°C 40sec, annealing temperatures varying from 55-67°C, 30sec) and then extended (72°C 2min). Amplified material was resolved on ethidium bromide-stained 2% NuSieve 3:1 agarose gels. B78 mouse melanoma cell line was used as a negative control and B78MC108, a mouse/human microcell hybrid containing an intact human chromosome 4, was used as a positive control for amplification.

3.ii.e. RNA Isolation

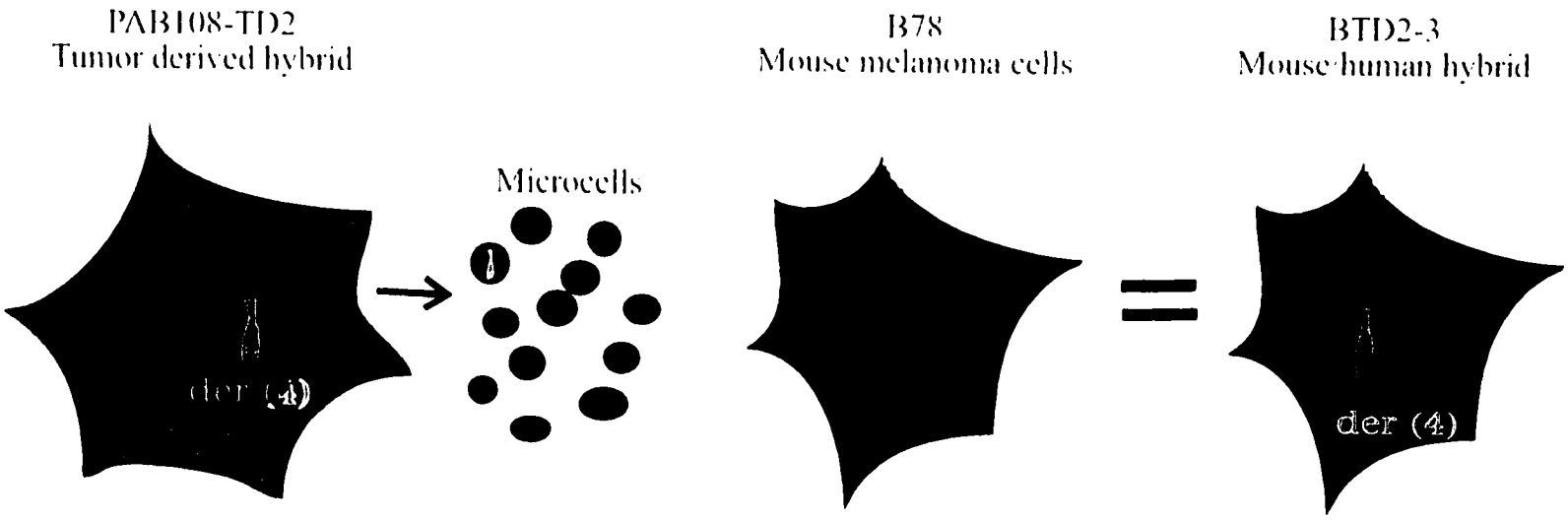
For RNA isolation, cells were grown to approximately 80% confluence in appropriate media before harvesting of total RNA. Cells were lysed with TSM containing Nonidet P-40 (0.5%), and centrifuged briefly to pellet the nuclei. The supernatant was then mixed with TSE+S (10mM Tris HCl pH8.0, 150mM NaCl, 5mM Na_2EDTA, 0.2% SDS) and extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1). RNA was precipitated with NaCl and ethanol, resuspended in TE containing SDS (0.1%) and quantitated. PolyA+ RNA was isolated from total RNA using the PolyATtract mRNA Isolation System (Promega).

3.ii.f. Northern Blots

RNA (15µg total) was prepared in MOPS buffer (1X; 20X MOPS buffer is 0.4M MOPS, 0.1M $NaC_2H_3O_2$, and 10mM Na_2EDTA), formaldehyde (2.2M) and formamide (50%) before being electrophoresed on denaturing formaldehyde gels containing agarose (1.2%), MOPS buffer (1X) and formaldehyde (1M). RNA was then transferred to nylon membranes (Pall Biodyne B) and hybridized with ^{32}P-labelled cDNA probes in hybridization buffer (50% formamide, 0.5M $NaHPO_4$ pH7.2, 1mM Na_2EDTA, 1% bovine serum albumin, 5% SDS) at 42°C for approximately 16 hours. After hybridization, blots were washed twice for 15min each (2XSSC/0.1% SDS, room temperature), then for 30min (0.1XSSC/0.1% SDS, 65°C). Hybridization of the probe on blots was detected using a phosphorimaging screen (Molecular Dynamics) and quantitated using ImageQuantNT software.

The *MAC25* probe was generously provided by Dr. Karen Swisshelm (University of Washington, Seattle WA). Probe (25ng) was labelled using the Stratagene Prime-It II Random Primer Labelling Kit.

Figure 3.i. Schematic representation of the transfer of the tagged chromosome from PAB108-TD2 to B78 by microcell fusion. Microcells are produced from the donor cell line, PAB108-TD2, and fused to the recipient cell line, B78. The resulting cell line containing the der (4) was called BTD2-3.


PAB108-TD2
Tumor derived hybrid
der (4)
Microcells
B78
Mouse melanoma cells
BTD2-3
Mouse-human hybrid
der (4)

3.iii. Results

3.iii.a. FISH

A locus in the region of chromosome 4 present in PAB108 but absent from the der (4), which was isolated from the tumor derived cell line PAB108-TD2, is implicated in the suppression of tumorigenicity of PAB108. Further experiments were therefore performed to map the deletion in the der (4).

The PA1N5 cell line has a normal karyotype except for one balanced translocation as does PA-1 (Tainsky *et al.*, 1988). Thus PAB108, a microcell hybrid created by the introduction of a normal tagged copy of chromosome 4 into PA1N5, has 3 copies of chromosome 4 (Chapter 2); the tumor derived PAB108-TD2 cell line carries a der (4) chromosome along with two copies of chromosome 4 which appeared to be normal cytogenetically (Chapter 2 and McGowan-Jordan *et al.*, 1994).

In a effort to determine whether the der (4) chromosome carries the tgCMV/HyTK tag and to determine which portions of the der (4) were missing, the tagged chromosome was transferred from PAB108-TD2 into a mouse cell line, B78, *via* microcell fusion. The microcell hybrid which was produced was referred to as BTD2. To identify a clone into which only the tagged human chromosome had been transferred, chromosome spreads of BTD2 clones were hybridized with total human genomic probe in FISH experiments. One hybrid, BTD2-3, was found to contain only one human chromosome (Figure 3.ii.A) in a background of mouse chromosomes.

3.iii.b. Alu-PCR FISH

Alu-PCR amplification products of human DNA in BTD2-3 were prepared, biotinilated and used as a FISH probe on normal human chromosome spreads to reveal the human chromosomal content of BTD2-3. The *Alu*BTD2-3 probe hybridized to a pair of human chromosomes corresponding in size and shape to chromosome 4. Further careful inspection of the hybridization revealed that there was an unpainted region on the p-arm of the chromosome and a small unpainted region on the distal region of the q-arm (Figure 3.ii.B). Thus, the der (4), which lacks a large region of chromosome 4p and a small region of 4q, had been transferred into BTD2-3. Since this chromosome, and only this chromosome, was transferable by microcell fusion and conferred hygromycin B resistance upon BTD2-3, it must carry the tgCMV/HyTK tag. This der (4) chromosome originated from a tumor-derived cell line, effectively a sub-clone of PAB108. Therefore, the tumor suppressor gene is located in one of the regions which has been deleted in the der (4).

Interestingly, when PAB108-4 (the cell line which upon injection into a nude mouse gave rise to the PAB108-TD2 cell line) was grown to late passage number in culture, foci appeared. Some of these foci were sub-cloned and one such resulting cell line, 4F1, was analyzed by FISH. Chromosome spreads were hybridized with the *Alu*BTD2-3 probe. These experimental results showed that there are two normal copies and one incomplete copy of chromosome 4 in 4F1 (Figure 3.ii.C). Two of the hybridizing chromosomes show gaps in the hybridization pattern indicating that they are intact copies of chromosome 4. The third chromosome was hybridized completely with this probe which is specific only for the

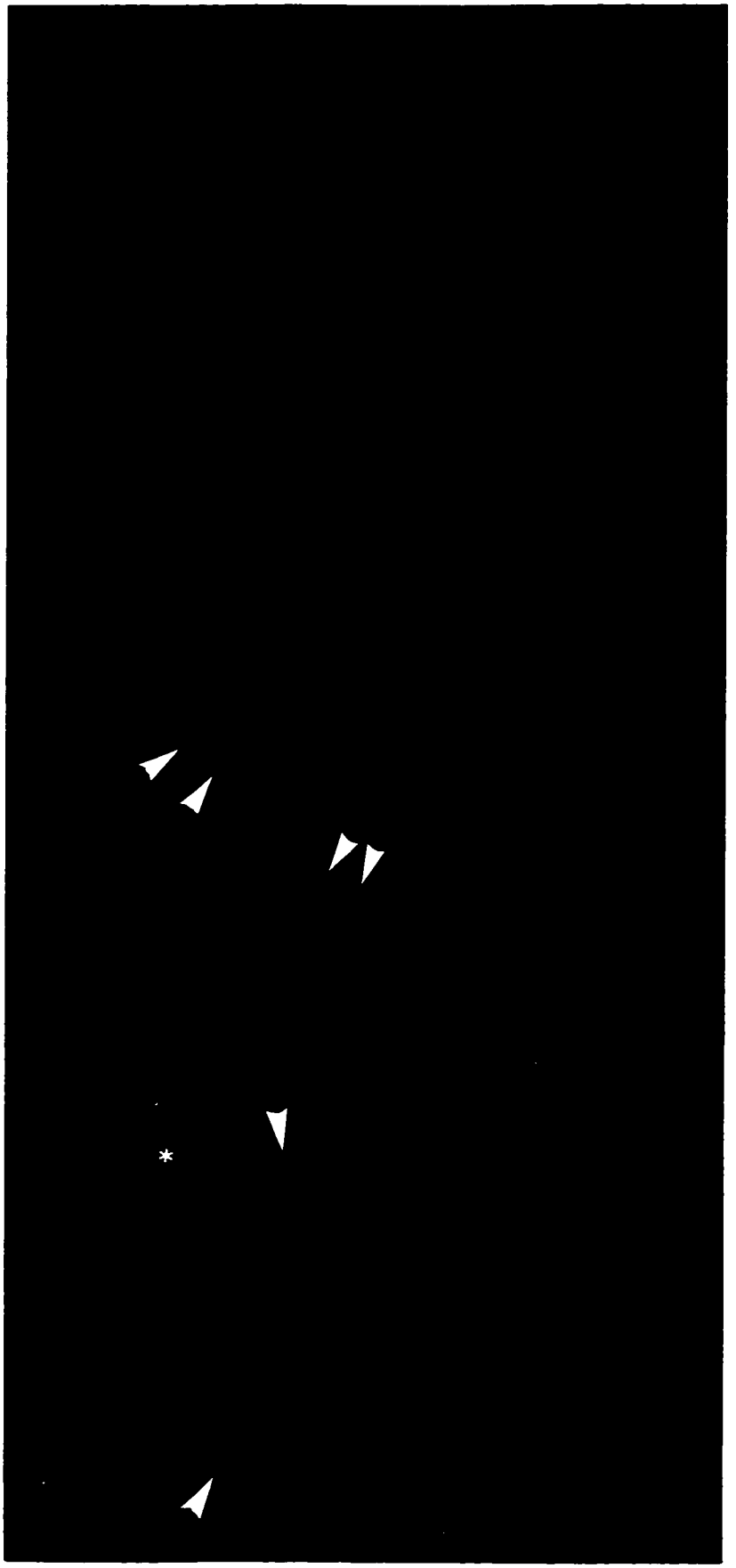
portions of chromosome 4 present in the der (4). Thus, the regions missing from the der (4) are also missing from the chromosome present in 4F1. These data indicate that the PAB108-TD2 cell line likely exits as an expanding sub-population of PAB108-4 cells in culture.

Figure 3.ii. FISH of microcell hybrids containing der (4). A. BTD2-3 metaphase spread with Total Human Genomic probe showing that a single human chromosome was transferred by microcell fusion. B. *Alu*BTD2-3 probe on human skin fibroblast metaphase spread. The arrowheads show that the single human chromosome 4 in BTD2-3 lacks a large region of the short arm and a small region of the long arm. C. *Alu*BTD2-3 probe on 4F1 metaphase spread. The arrowheads show the intact copies of chromosome 4 which are incompletely painted while the asterisk shows the disrupted copy of chromosome 4 which is completely painted.

A.

B.

C.



3.iii.c. PCR Mapping

To more precisely map the der (4) deletion, microsatellite repeats along the length of chromosome 4 (Figure 3.iv.) were PCR amplified and analyzed for their presence or absence in BTD2-3, the mouse/human microcell hybrid containing the der (4), by agarose gel electrophoresis (Figure 3.iii.). In agreement with the FISH experiments, PCR mapping showed that there is a large deletion in the short arm of the der (4), extending from 4p11 to 4p15.3, as well as a small deletion in the long arm at 4q31.1 (Figure 3.iv.).

Figure 3.iii. Representative electrophoretic gels of amplified microsatellite repeats. Microsatellite repeats A. D4S405 (chromosome 4p11) and B. D4S417 (chromosome 4q31.2) were amplified from DNA from cell lines, as described in Materials and Methods, and electrophoresed on ethidium bromide-stained 2% NuSieve 3:1 agarose gels. Gels were photographed under ultra-violet light.

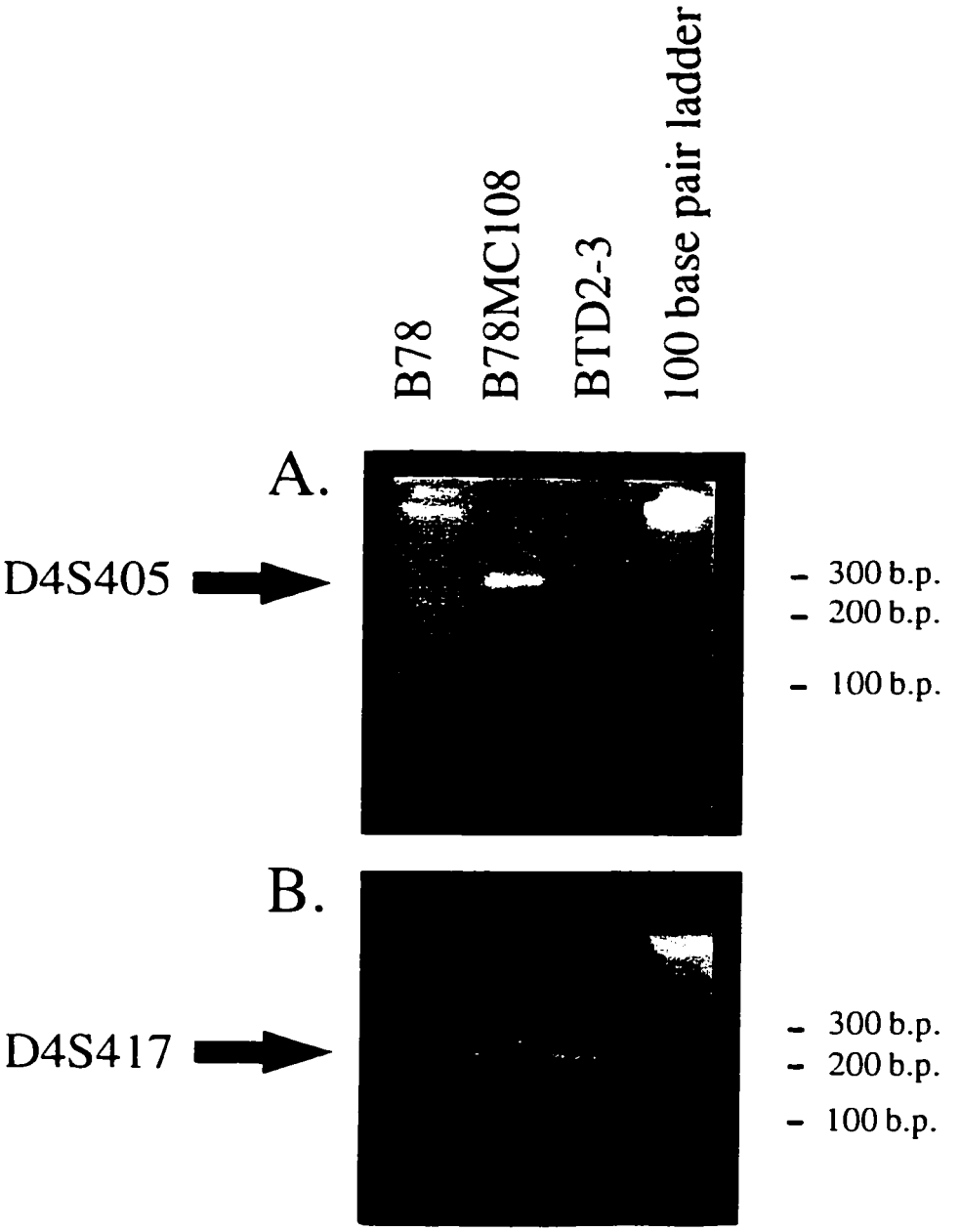
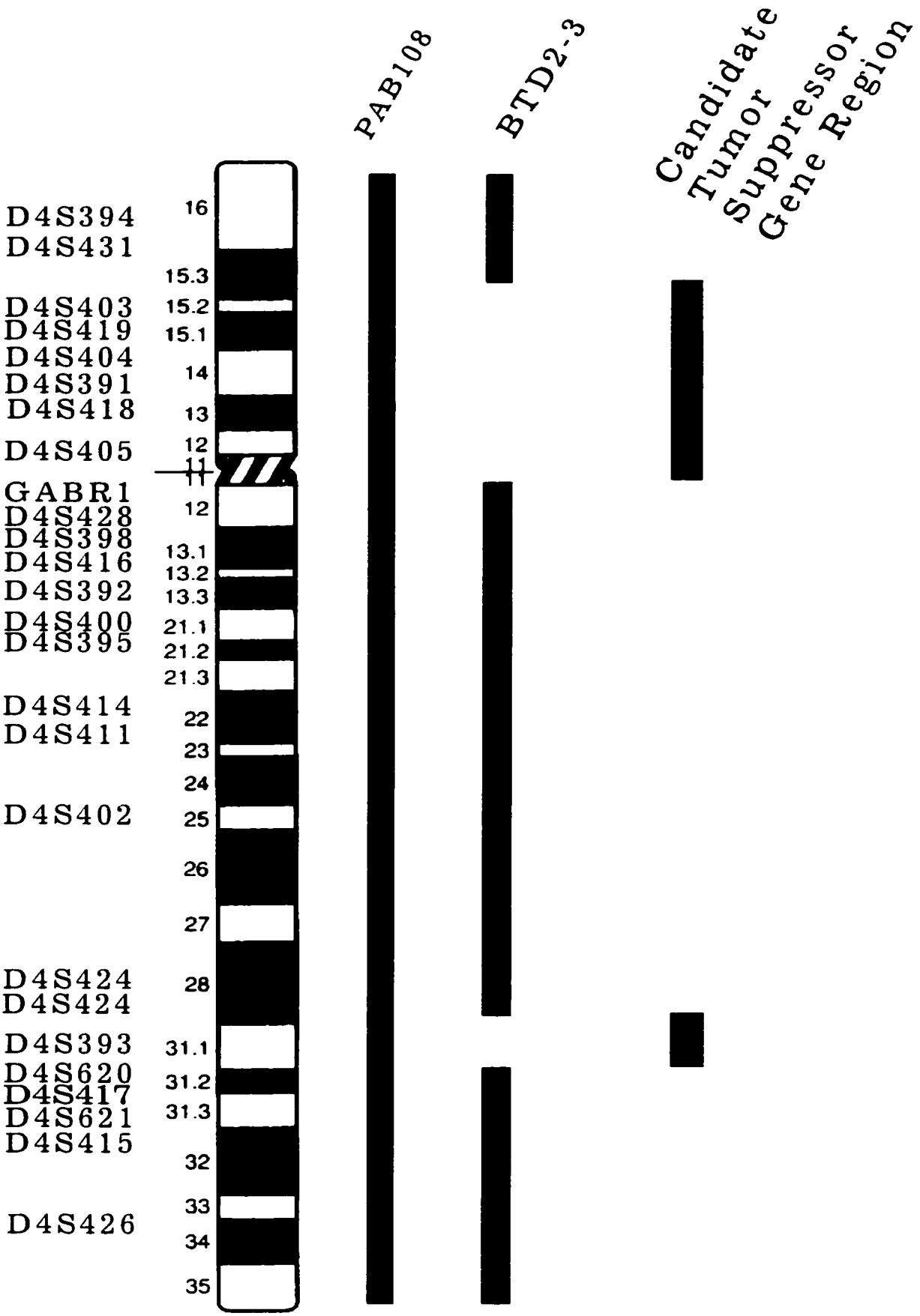

B78
B78MC108
BTD2-3
100 base pair ladder
A.
D4S405
300 b.p.
200 b.p.
100 b.p.
B.
D4S417
300 b.p.
200 b.p.
100 b.p.

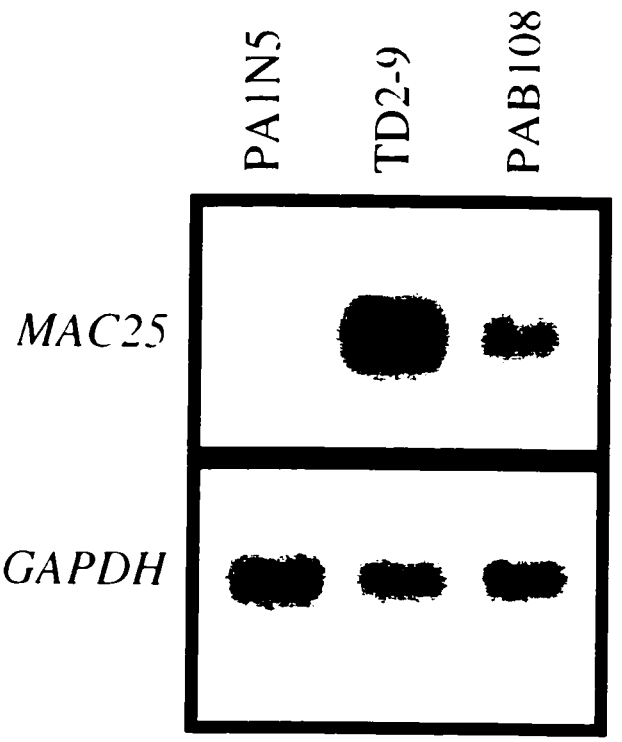
Figure 3.iv. Idiogram of human chromosome 4. Microsatellite repeat primers tested to determine the portions of human chromosome 4 present in BTD2-3 are shown to the left of the idiogram. DNA was amplified using the primers indicated on the left which amplify microsatellite repeats at the specified chromosomal locations. The portions of the tagged normal human chromosome present in PAB108 (as determined from cytogenetic and FISH analysis, Chapter 2) and B78MC108 (purple bar), and BTD2-3 (der (4), blue bars) are indicated. The portions of chromosome 4 missing from the der (4) are indicated by red bars and represents the candidate region for the tumor suppressor gene which is active in PAB108. The position of the *MAC25* gene (Swisshelm *et al.*, 1995) is indicated by an asterisk.


PAB108
BTD2-3
Candidate Tumor Suppressor Gene Region
D4S394
D4S431
D4S403
D4S419
D4S404
D4S391
D4S418
D4S405
GABR1
D4S428
D4S398
D4S416
D4S392
D4S400
D4S395
D4S414
D4S411
D4S402
D4S424
D4S424
D4S393
D4S620
D4S417
D4S621
D4S415
D4S426
16
15.3
15.2
15.1
14
13
12
11
11
12
13.1
13.2
13.3
21.1
21.2
21.3
22
23
24
25
26
27
28
31.1
31.2
31.3
32
33
34
35

3.iii.d. MAC25 Expression

Several genes on chromosome 4 have been considered as candidates. A recently described senescence gene, *MAC25*, maps to chromosome 4q12 (Swisshelm *et al.*, 1995) and is, therefore, not in the candidate region (Figure 3.iv). This gene is expressed in both PAB108 and TD2-9 (a subclone of the PAB108-TD2 cell line) but not in PA1N5 (Figure 3.v.) presumably because this locus is inactivated in PA1N5 but has been reintroduced on the normal chromosome 4 in PAB108. Its activity is maintained in TD2-9 since portions of the normal chromosome 4 including this locus are present on the der (4) (Figure 3.iv.).

Figure 3.v. Northern blot analysis of *MAC25* expression. RNA from PA1N5, TD2-9 and PAB108 was hybridized with *MAC25* probe. Approximately 15μg of total RNA was loaded in each lane. *GAPDH* was used as a control for loading. Probe hybridization was detected using a phosphorimager. Images were reproduced using LogiTech FotoTouch Color and Microsoft PowerPoint software.


PA1N5
TD2-9
PAB108
MAC25
GAPDH

3.iv. Discussion

In order to determine if the der (4) chromosome, which had been isolated from a tumor-derived cell line, carries the tgCMV/HyTK tag, and to determine which portions of this chromosome are missing, the tagged chromosome from PAB108-TD2 was transferred into the B78 mouse cell line. In the resulting microcell hybrid, BTD2-3, it was shown, initially by FISH, that the transferred chromosome was not intact. Thus, the der (4) carries the tag, illustrating that it was the introduced, tagged chromosome in the PAB108 cell line which was altered in the tumor-derived cell line, PAB108-TD2.

The more precise mapping of the rearrangement in the der (4) was carried out in the mouse background. This experimental design was chosen to avoid the encumbrance of using polymorphic markers, which would be necessary in a human background due to the presence of the original copies of chromosome 4. Mapping of the der (4) in the BTD2-3 background showed that the region from 4p11-4p15.3 is missing, as is a small region at 4q31.1. Since this is a tumor-derived chromosome, it is within these deleted regions that the putative tumor suppressor gene is located. Chromosome 4 contains approximately 203Mb of DNA (Morton, 1991); the deleted region which is absent from the short arm of the der (4) is thought to comprise approximately 40Mb of DNA, which would contain between 190 (Wagner *et al.*, 1993) and 1300 (Gilbert, 1992) genes.

Once the region containing the tumor suppressor gene had been defined, it became relevant to investigate the genes which are known to reside in these chromosomal locations.

Genes related to tumorigenesis and differentiation may be relevant to the tumor suppression seen in PAB108. While some promising candidate genes which may be related to tumor suppression reside on chromosome 4, none of these are located in the region deleted from the der (4) chromosome. These include the recently identified senescence gene, *MAC25*, mapping to 4q12 (Swisshelm *et al.*, 1995). This gene is involved in the senescence of mammary epithelial cells and is not expressed in many breast cancer cell lines. Also, its expression is up-regulated in mammary epithelial cells in response to retinoids, implying that it plays a role in the differentiation of these cells (Swisshelm *et al.*, 1995). The *MAC25* gene encodes a protein which is a member of the insulin-like growth factor binding proteins which may have tumor suppressing functions (McGuire *et al.*, 1992). Because PAB108 senesces at higher passage (24-25, see Chapter 2), it was of interest to investigate the expression of this gene. *MAC25* expression was detected in PAB108 cells but not in its parent cell line PA1N5. It was also expressed in TD2-9, which is in agreement with the mapping data which shows that the region of the tagged chromosome encoding *MAC25*, 4q12, is present on the der (4) (Figure 3.iv.).

A putative tumor suppressor gene, the human homolog of the drosophila *FAT* tumor suppressor gene, is mapped at 4q34-q35 (Dunne *et al.*, 1995). The human *FAT* gene was cloned due to its homology with the Drosophila tumor suppressor gene of the same name. Although this gene does have the function of controlling growth in Drosophila (Bryant *et al.*, 1988), such a function is, as yet, unproven in humans. Another chromosome 4 candidate gene is the *MSX1* gene which is related to development and maps to chromosome 4p16.1

(Ivens *et al.*, 1990). This is a candidate gene for human cleft palate since mice lacking functional MSX1 develop cleft palate (Satokata and Maas, 1994), and some Wolf-Hirschhorn syndrome patients (who suffer from facial clefting) have deletions of this locus (Ivens *et al.*, 1990). None of these genes, *MAC25*, *FAT* or *MSX1* map to the candidate regions of 4p11-15.3 or q31.1, and are thus not candidates for the suppression of tumorigenicity in the PAB108 cell line.

A further search of genes which encode proteins related to cell growth and tumor formation revealed that genes encoding two closely related protein tyrosine kinases, TXK (Haire *et al.*, 1994) and TEC (Sato *et al.*, 1994) reside in the candidate tumor suppressor gene region. The *TEC* gene enjoys high expression in patients with myelodysplastic syndrome (Sato *et al.*, 1994), and as such, is not consistent with a gene involved in tumor suppression. Another gene which is potentially involved in tumor formation, *TTF*, is located in the candidate region at band 4p11 (Dallery *et al.*, 1995). This gene has been found as part of a chimeric protein in non-Hodgkin's lymphoma and is a member of *RAS* superfamily. Thus, it appears that this gene may be performing an oncogenic rather than a tumor suppressing role. Since suitable candidate genes for the suppression of PAB108 tumorigenicity were not among the genes known to reside on chromosome 4, further analysis was carried out in an effort to identify additional candidate tumor suppressor genes from the region on chromosome 4 implicated in PA-1 tumorigenicity.

Chapter 4 Identification and Analysis of Candidate Tumor Suppressor Genes

4.i. Introduction

It had been determined that a gene on chromosome 4p11-15.3 or q31.1 is responsible for the tumor suppression in PAB108, but that no known tumor suppressor genes are located in these regions (Chapter 3.iv.). The task remained to isolate candidate genes which are involved in this phenotype. Such genes would be expressed in the suppressed cell line, PAB108, but not in the tumorigenic parental cell line, PA1N5, or the tumor-derived cell line, PAB108-TD2. In order to maximize the odds of successfully cloning the putative tumor suppressor gene, several techniques used for the cloning of differentially expressed genes were considered.

Initially, subtractive cDNA hybridization was considered as the primary method for isolation of candidate tumor suppressor genes. This technique is useful for the isolation of cDNAs expressed in one cell line ("+") but not in another ("-"). Because of the rate of false positives, subtractive hybridization is most useful when the chromosomal location of a gene of interest is known, and when this chromosome is present in an interspecies hybrid cell line. Using this technique to clone candidate tumor suppressor genes, one isolates RNA from a mouse/human hybrid containing the suppressing chromosome (eg. B78MC108, "+") and from a closely related cell line containing a non-suppressing chromosome (eg. BTD2-3, "-"). Following this, an excess of polyA+ RNA from the "-" cell line is biotinylated and hybridized with ^{32}P-labelled cDNA from the "+" cell line. Unhybridized and hybridized polyA+ is removed by the use of streptavidin-coated beads. The result is the purification of

cDNA unique to the "+" cell line, which did not hybridize with the RNA present in the "-" cell line. Additional rounds of hybridization and subtraction are then used to further purify the cDNAs. These labelled cDNAs are then used as probes on a human cDNA library, in order to eliminate all non-human transcripts.

The major draw-back of subtractive hybridization is the large number of false positives. The use of several rounds of subtraction is useful in the elimination of many false positives, however there usually remains an outstanding background against which the true positives must be identified. Other limitations of this technique are the requirement for large amounts of RNA from the "-" cell line and irreproducibility of the technique. Also, in the system used here, I could not be sure that the locus present in PAB108 and B78MC108 but not in PA1N5, PAB108-TD2 and BTD2-3 would be expressed in the B78 mouse background required for the use of subtractive hybridization. In other words, since the functional phenotype of the gene (suppression of tumorigenicity) is not seen in the highly malignant B78 background, there is some question regarding the expression of the candidate tumor suppressor gene in the mouse cells. Because of these multiple limitations, methods other than subtractive hybridization were considered.

Representational difference analysis (RDA) was first described in 1993 (Lisitsyn *et al.*, 1993) as a technique for cloning the differences between genomes. Unlike the other techniques considered for the cloning of the candidate tumor suppressor gene on chromosome 4, it involves the analysis of genomic DNA rather than RNA. The first step in this method is the digestion, at rare restriction sites, of genomic DNA from cell lines

containing ("+") or missing ("-") the gene of interest. This is followed by amplification of the digested DNA and the subsequent ligation of adaptors onto the "+" DNA. The two sets of DNA are hybridized then amplified with one another, a process which favours the amplification of DNA fragments unique to the "+" DNA. Several rounds of such amplification result in the isolation of genomic fragments found only in the "+" cell line (Lisitsyn *et al.*, 1994).

Numerous attempts at using RDA to isolate genomic fragments present in PAB108 but not in PAB108-TD2 resulted in the isolation of fragments which could be amplified reproducibly. However, when tested as probes on Southern blots, these fragments gave smears and thus, revealed themselves as repetitive human elements (data not shown). At this point the RDA technique was abandoned; little mention of its use has appeared in the literature since its introduction (Drew and Bradley, 1995; Schutte *et al.*, 1995; Tsuchiya *et al.*, 1994).

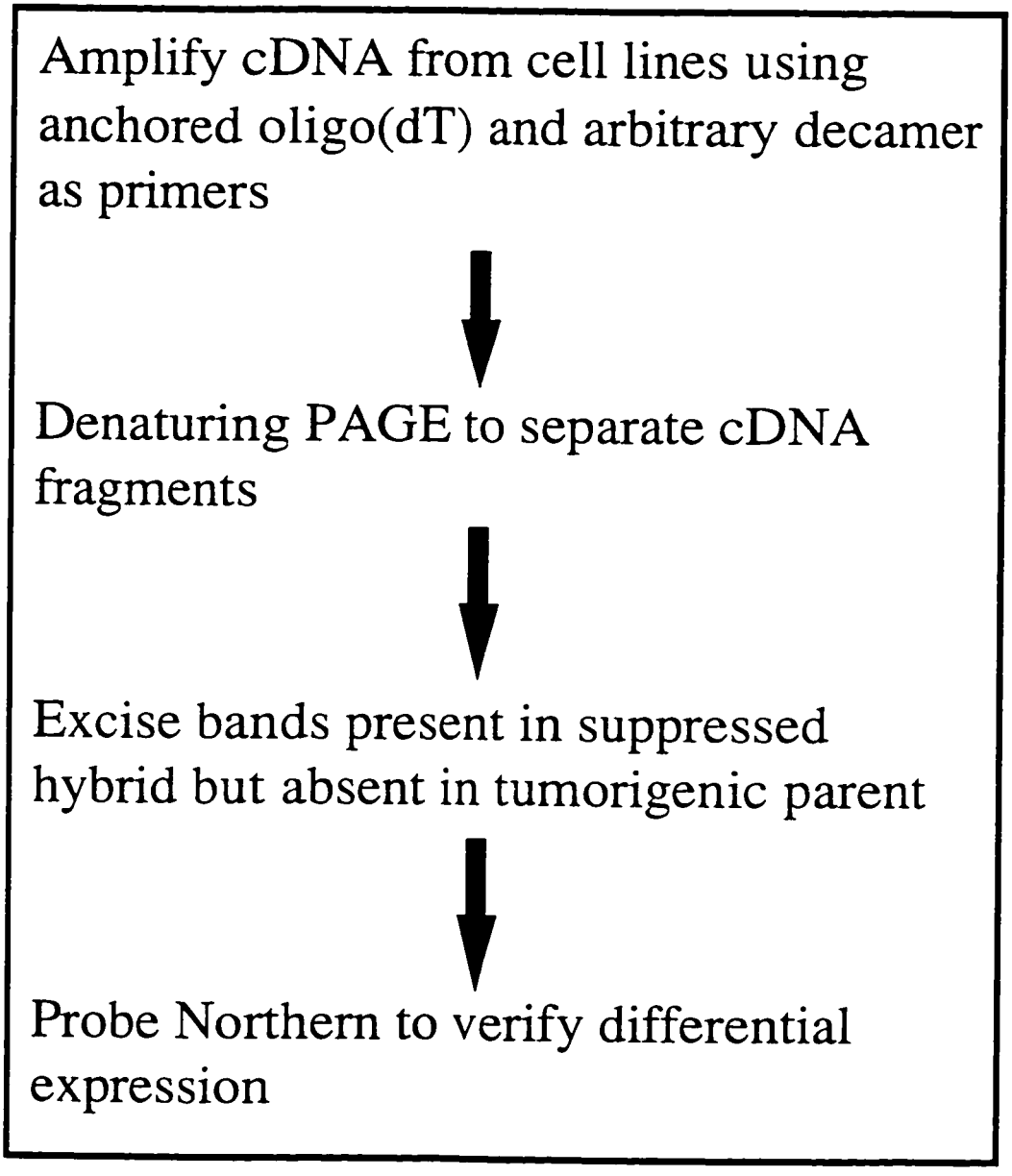
Direct cDNA selection is yet another method which was considered as a technique for isolating cDNAs from a given chromosomal region. While this method is useful for the isolation of cDNAs, it requires that the chromosomal region encoding the gene of interest be identified in advance. This is in contrast to the other differential methods discussed here, which do not rely upon the precise chromosomal location of a gene for the cDNA isolation. On the other hand, cDNA selection is not a differential method but will result in the isolation of all transcripts from a given chromosomal region. Briefly, this technique (Lovett, 1994) involves the hybridization of cDNA from the cell line containing the transcript of interest

with biotinylated genomic DNA, which is usually cloned into yeast artificial chromosomes (YACs). YACs containing human DNA from defined chromosomal regions, including chromosome 4, are available commercially (Research Genetics). After hybridization, cDNAs from the chromosomal region are purified from the population by the use of streptavidin-coated magnetic beads which bind the biotinylated DNA and can be capture by the use of a magnet. These cDNAs can then be subjected to a second round of selection to eliminate some false positives. The resulting cDNA are those expressed from the region of genomic DNA contained in the YACs used for the selection. While this protocol is useful for the generation of transcription maps of genomic regions (Morgan *et al.*, 1992; Rommens *et al.*, 1993), it is generally used for the isolation of transcripts from relatively small regions (400-1000kb), since the result is a large number of small cDNA fragments which must be combined to give full length cDNAs. Since the region termed the candidate tumor suppressor gene region on chromosome 4 (Chapter 3) encompasses approximately 40Mb, direct cDNA selection was determined to be an inappropriate method. It would result in the isolation of a vast number of candidate transcripts from the region of interest (4p11-15.3 and q31.1) which would be difficult to evaluate in terms of their validity as candidate tumor suppressor genes.

The final method considered for the isolation of the putative tumor suppressor gene active in PAB108 was mRNA differential display. This technique was recently developed and optimized (Liang and Pardee, 1992; Liang *et al.*, 1993; Bauer *et al.*, 1993) and has proven useful for both the cloning of genes and the implication of known genes in novel

roles (eg. Liang *et al.*, 1992; Sager *et al.*, 1993; Sun *et al.*, 1994; Wu *et al.*, 1995). It consists of several steps (Figure 4.i.), the first of which is the amplification of cDNA from the "+" and "-" cell lines using arbitrary decamers and degenerate anchored oligo(dT) primers. This results in the amplification of a portion of the 3' ends of the cDNAs from a cell line. Use of numerous decamer-oligo(dT) primer combinations allows one to cover the majority of expressed sequences (Bauer *et al.*, 1993). The amplification patterns from the two cell lines are then compared by electrophoresis of the products on a denaturing polyacrylamide gel. Bands which are reproducibly present in the "+" cell line but not in the "-" cell line are excised and typically tested for true differential expression on Northern blots. They are also subcloned, sequenced and compared to known genes. In general, this method is thought to have the advantages of giving very good representation of expressed sequences and of being time-efficient and reproducible; thus, it was chosen as the method to identify cDNAs involved in the suppression of tumorigenicity in PAB108.

Figure 4.i. Summary of the mRNA differential display method.


Amplify cDNA from cell lines using anchored oligo(dT) and arbitrary decamer as primers
Denaturing PAGE to separate cDNA fragments
Excise bands present in suppressed hybrid but absent in tumorigenic parent
Probe Northern to verify differential expression

4.ii. Materials and Methods

4.ii.a. mRNA Differential Display

RNA was isolated as described in Chapter 3.ii.e. This portion of experimentation was conducted using PA1N5, PAB108 and a subclone of the PAB108-TD2 cell line, TD2-9. Differential display was performed essentially as described by Liang *et al.* (1993). DNA-free RNA from cells was reverse transcribed using RNA (400ng), reverse transcriptase buffer (1X), dithiothreitol (10mM), dNTPs (20μM), $T_{11}MN$ (10μM) primers, RNase inhibitor (10U) and reverse transcriptase (200U) in a volume of 20μL. Fresh cDNA (3μL) was then amplified in the presence of [^{35}S]dATP (10μCi), P.E. buffer (1X), dNTPs (2μM), Taq polymerase (1U), $T_{11}MN$ (1μM), and arbitrary decamers (0.25μM, Bauer *et al.*, 1993) in a 20μL volume. After 40 amplification cycles (94°C for 30sec, 40°C for 2min, 72°C for 30sec) and an extension period (72°C for 5min), the product was combined with formamide loading buffer. A portion (6μL) of the product was denatured (95°C, 5min), incubated on ice, and loaded onto a 6% polyacrylamide gel containing urea (42% w/v) and TBE (1X), which had been polymerized using TEMED and ammonium persulfate and pre-run for at least 15min. The gel was run (1900V, 35mA, 62W, 2 hours) before being overlaid with a piece of Whatmann 3MM paper and dried on a BioRad Gel Drier (80°C, 1 hour). The dry gel was then exposed to autoradiography film. Bands present in PAB108 but not in PA1N5 or TD2-9 were excised by aligning the gel with the autoradiograph. Bands were then eluted from the gel by addition of distilled water (100μL), incubation at room temperature (10min),

then boiling (15min). The paper was remove by spinning the sample in a microcentrifuge for 2min, and transferring the supernatant to a clean tube. The cDNA fragment was then precipitated (60mM $NaC_2H_3O_2$, 100µg glycogen, and 80% ethanol, -80°C, 30min) and microcentrifuged (15000xg, 10min). The pellet was rinsed with cold ethanol (85%) then dried at room temperature, resuspended in distilled water (10µL) and stored at -20°C. This material was either used directly as a probe on Northern blots or subcloned using the TA Cloning Kit (Invitrogen).

4.ii.b. Subcloning of Candidate cDNA Fragments

Many of the cDNA fragments isolated from differential display gels were subcloned using the TA Cloning Kit. An aliquot (4µL) of the material originally isolated from the gel was amplified and then quantitated. Freshly amplified material was then ligated into the pCRII vector, as outlined in the kit manual. Glycerol stocks of bacterial cultures were prepared by mixing overnight cultures (62%) with sterile glycerol (38%), and stored at -80°C.

4.ii.c. Northern Blots

RNA (15µg total or 200ng polyA+) was electrophoresed, hybridized, washed and detected as described in Chapter 3. Probes were prepared using either material which was isolated from the gels or subcloned material. In each case, probes were labelled by amplification in a 20µL volume containing P.E. buffer (1X), dNTPs-dCTP (2.5µM), arbitrary decamer (0.25µM), $T_{11}MN$ (1µM), Taq polymerase (1U), and [α-^{32}P]dCTP (100µCi).

4.ii.d. Sequencing of Candidate cDNA Fragments

Subcloned material was sequenced (Sequenase Version 2.0 sequencing kit, Amersham). [^{35}S]dATP-labelled reaction products were electrophoresed on 6% polyacrylamide gels (Long Ranger) containing urea (42% w/v) and TBE (1.2X). After visualization of bands on autoradiographs, sequences were read manually and compared to sequences in Genbank using the BLAST network service of the National Center for Biotechnology Information (Altschul *et al.*, 1990). The nucleotide sequences of all fragments are reported in Appendix 3.

4.iii. Results

4.iii.a. Candidate cDNAs

In order to isolate candidate cDNAs which may be involved in the suppression of tumorigenicity in PAB108, mRNA differential display was used. Using a total of ten arbitrary decamers and 3 degenerate anchored oligo(dT) primers, 14 candidate cDNA fragments were isolated (Table 4.i., and for example, Figure 4.ii.). These fragments were tested for true differential expression by Northern blot analysis. While most candidates (C, D2, E2, E3, Q3, Q5, F, G, R5, H6, J3, and L) were shown to be false positives, two (D1 and K1) proved to have increased expression in PAB108 over PA1N5 and TD2-9 (Figure 4.iii., and Table 4.i.). Fragment H6 was expressed at equivalent levels in the tumor-derived cell line TD2-9 and in PAB108, and thus, was not a true positive. Fragment D1 is 4.2 times more highly expressed in PAB108 than in PA1N5 and has 6.6 times higher expression in PAB108 relative to TD2-9. D1 has some sequence similarity (see Appendix 3 for sequences of candidate cDNA fragments) to *CENP-E* (Figure 4.iv.A), a gene encoding a protein involved in segregation of chromosomes at mitosis (Liao *et al.*, 1994). The homology between these two sequences covers a 93 base region, 90 of which are identical, near the centre of the 8kb *CENP-E* mRNA (Yen *et al.*, 1992). In fact, two of the three non-identical bases can be explained by the addition of a base in the D1 sequence, while the third mismatch is due to an unidentified base in the D1 sequence (Figure 4.iv.B). There is equivalent homology at the

protein level, confirming that the D1 sequence is homologous to a portion of the *CENP-E* coding region.

Duplicate Northern blots revealed that another fragment, K1, had nearly equivalent expression in PA1N5 and TD2-9 but is 3.5 times more highly expressed in PAB108 than in PA1N5. Sequence comparison of K1 with known sequences showed that it is identical to the 3' end of gene encoding the 78kDa glucose-regulated protein, otherwise known as immunoglobulin heavy chain binding protein (BiP; Ting and Lee, 1988) (Figure 4.v.B). The *BiP* gene is 2.5kb in length; two portions of K1 are nearly identical to two regions of the *BiP* mRNA. This homology is intact in the protein, verifying that K1 is a portion of the *BiP* coding region. The only non-homologous region corresponds to the first 11 bases of K1, which comprise the T_{11} tail and are excluded from the search strategy. The K1 fragment does not cover bases 1968 to 2408 of the BiP sequence while it does cover regions on either side (Figure 4.v.A). The reason for this phenomenon remains unclear but may be due to the apparent duplication in the *BiP* sequence corresponding to bases 131 to 140 of the K1 sequence.

Figure 4.ii. mRNA differential display electrophoretic gel analysis. $T_{11}MA$ and arbitrary decamers were used as primers. PA1N5, the parent cell line. TD2-9, a tumor-derived microcell hybrid containing a tagged der (4) chromosome. PAB108, a non-tumorigenic microcell hybrid containing a tagged normal chromosome 4. Arrows indicate several differentially displayed cDNAs, present in PAB108.

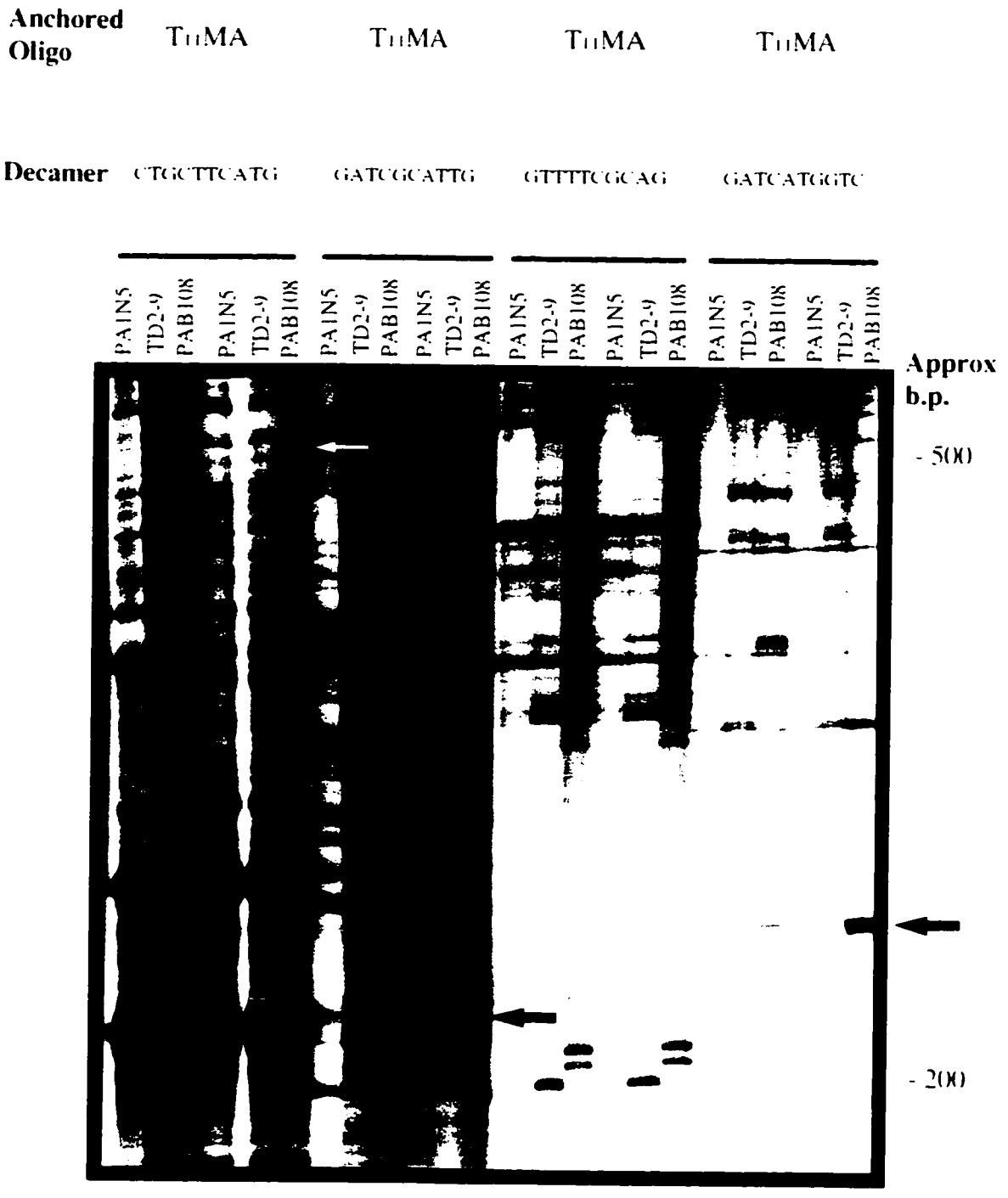

Anchored Oligo
T11MA
T11MA
T11MA
T11MA
Decamer
CTGCTTCATG
GATCGCATTG
GTTTTCGCAG
GATCATGGTC
PA1N5
TD2-9
PAB108
Approx b.p.
- 500
- 200

Table 4.i. Characteristics of cDNA fragments isolated by differential display

Fragment	Oligo dT	Decamer Sequence (5'to 3') (Decamer Reference Number)	Unique Sequences	Size (bp)	Expression PAB108* /PAIN5	Size of Identified RNA (kb)	BLAST Results
C	$T_{11}MC$	TACAACGAGG (DD21)	N.D.	~100	0.5	3.2	N.D.
D	$T_{11}MC$	TCGATACAGG (DD27)	D1 D2	317 325	4.2 1.1-1.4	2.7	small region of homology to CENP-E no significant homology
E	$T_{11}MC$	CTGCTTGATG (DD32)	E2 E3	~150 ~150	N.S. 1.1	N.D. N.D.	N.D.
Q	$T_{11}MG$	TCGATACAGG (DD27)	Q3 Q5	302 323	1.1 1.1-1.3	N.D. N.D.	homology to unknown human cDNA identity to unknown human cDNA
F	$T_{11}MG$	TCGGTCATAG (DD29)	N.D.	~450	< 1	N.D.	N.D.
G	$T_{11}MG$	CTGCTTGATG (DD32)	N.D.	~800	1.4	N.D.	N.D.
R	$T_{11}MG$	CTGCTTGATG (DD32)	R5	173	1.4	N.D.	significant homology to human partial cDNA, isolated from placenta and infant brain
H	$T_{11}MG$	CTGCTTGATG (DD32)	H6	141	2.1	3.1	no significant homology
J	$T_{11}MA$	CTGCTTGATG (DD32)	J3	501	0.6	N.D.	no significant homology
K	$T_{11}MA$	GATCGCATTG (DD42)	K1	210	3.5	3.1	identical to 78 kDa glucose-regulated protein
L	$T_{11}MA$	GATCATGGTC (DD44)	N.D.	~225	N.S.	N.D.	N.D.

*Expression as determined by Northern blot and quantitated by ImageQuantNT software
N.D.: Not Determined
N.S.: No Signal on Northern blot

Figure 4.iii. Northern blot analysis of mRNA differential display-identified cDNA fragments. RNA from PA1N5, TD2-9, and PAB108 was hybridized with cDNA probes indicated on the left. Estimated sizes of mRNA are C: 3.2kb, D1: 2.7kb, K1: 3.1kb. Approximately 15μg of total RNA was loaded in each lane. *GAPDH* was used as a control for loading. Probe hybridization was detected using a phosphorimager and was quantitated using ImageQuantNT software. Images were reproduced using LogiTech FotoTouch Color and Microsoft PowerPoint software.

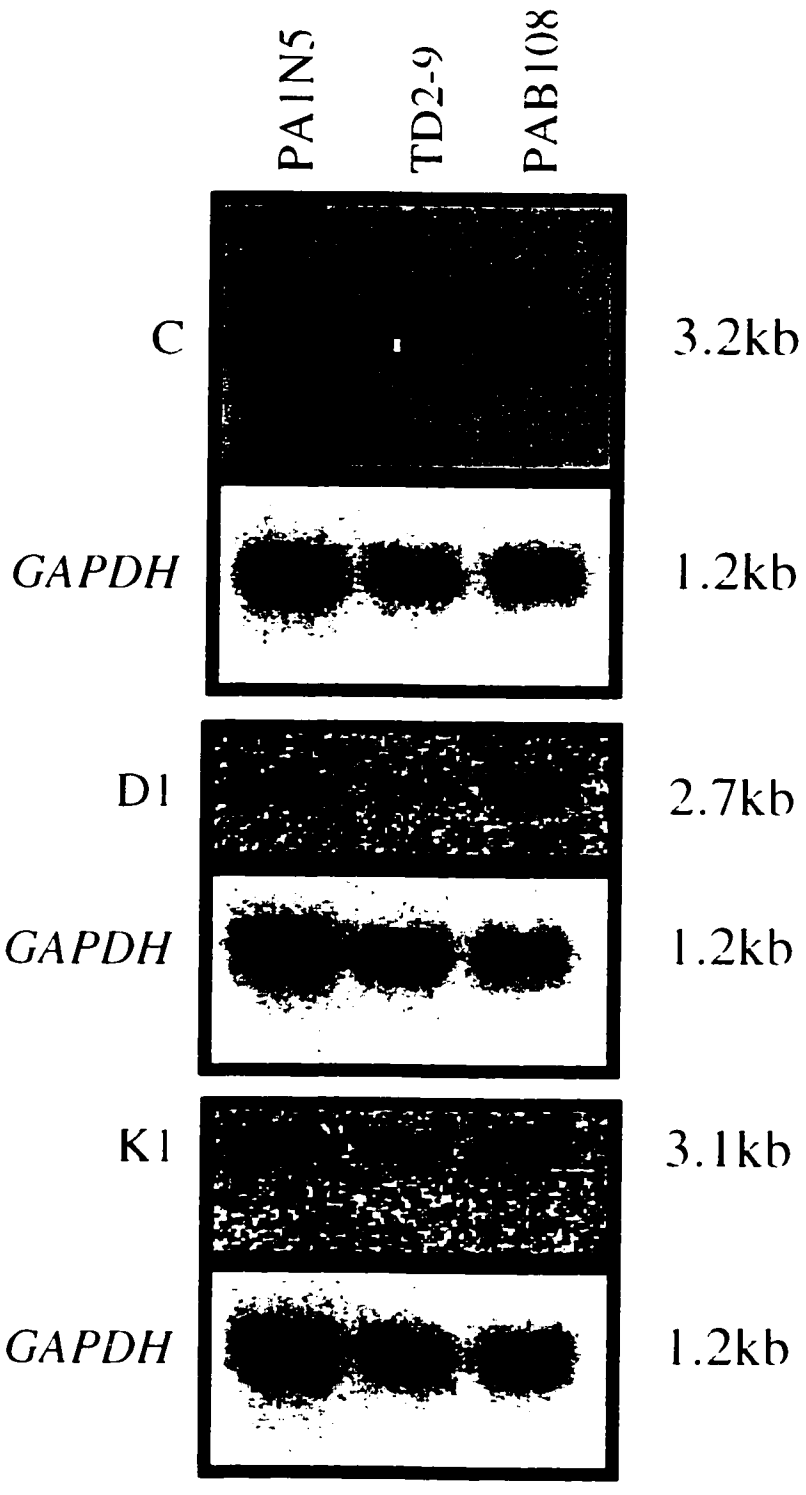
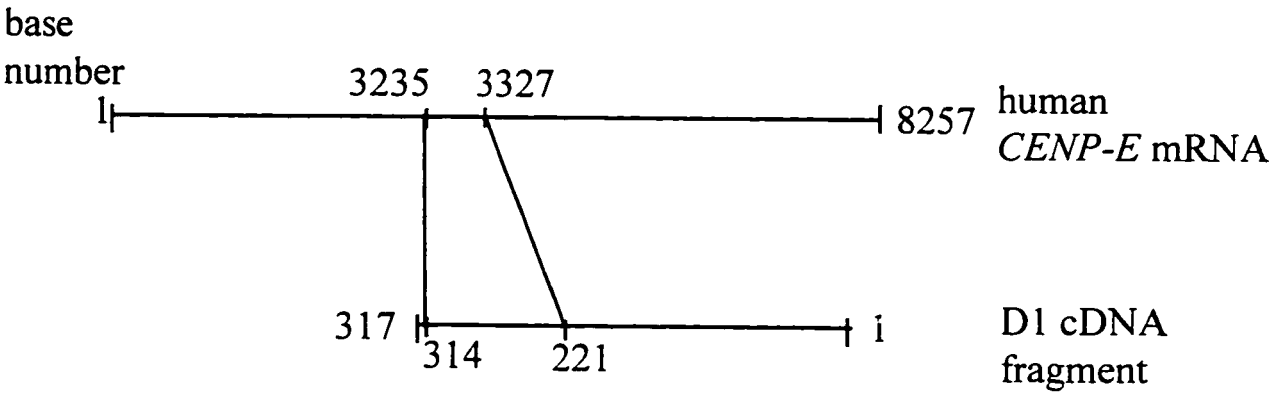

PAIN5
TD2-9
PAB108
C
3.2kb
GAPDH
1.2kb
D1
2.7kb
GAPDH
1.2kb
K1
3.1kb
GAPDH
1.2kb

Figure 4.iv. Region of homology of cDNA fragment D1 with *CENP-E* gene. Numbers refer to base number in the sequence. A. A schematic representation of the homology between the cDNA fragment D1 and a region of the 8kb *CENP-E* mRNA. The homology encompasses bases 3235 to 3327 of *CENP-E* and 221 to 314 of D1. B. Sequence comparison between the D1 cDNA fragment and the *CENP-E* mRNA. Non-identical bases are underlined.

A.



B.

D1 314 ATACAGGAGAAAAATGAACTCCAACAAATGTTAGAGAGTGTTATAGCAGAAAA 262

CENP-E 3235 ATACAGGAGAAAAATGAACTCCAACAAATGTTAGAGAGTGTTATAGCAGAAAA 3286

D1 261 GGAACAATTGAAGACNGACCTAAAGGAAA 232

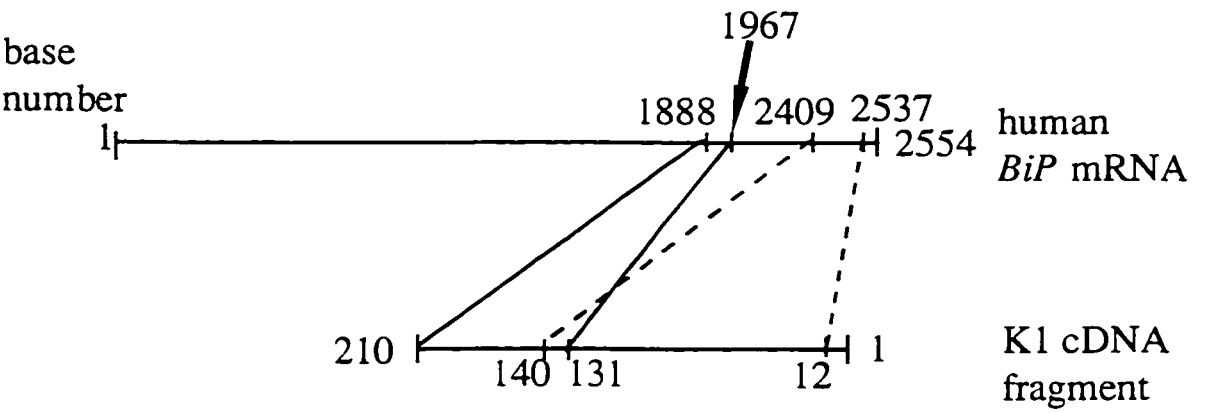
CENP-E 3287 GGAACAATTGAAGACTGACCTAAAGGAAAA 3317

D1 239 AAAGGAAAATATTGAAATG 221

CENP-E 3309 AAAGGAAAATATTGAAATG 3327

Figure 4.v. Region of homology of cDNA fragment K1 with *BiP* gene. Numbers refer to base number in the sequence. A. A schematic representation of the homology between the cDNA fragment K1 and a region of the 2.5kb *BiP* mRNA. The homology encompasses bases 1888 to 2409 of *BiP* and 12 to 210 of K1. B. Sequence comparison between the K1 cDNA fragment and the *BiP* mRNA. Non-identical bases are underlined while the single inserted base is marked with an asterisk.

A.



B.

K1	140	AAAAACCTGGGTTAGGGTGTGTGTTCACCTTCAAAATGTTCTATTTAACAACTGGGTCAT	81
BiP	2409	AAAAACCTGGGTTAGGGTGTGTGTTCACCTTCAAAATGTTCTATTTAACAACTGGGTCAT	2468
K1	80	GTGCATCTGGTGTAGGAAGTTTNNTCTACCATAAGTGACACCAATAAATGTTTGTTATTT	21
BiP	2469	GTGCATCTGGTGTAGGAAGTTTTTTCTACCATAAGTGACACCAATAAATGTTTGTTATTT	2528
K1	20	ACACTGGTC	12
BiP	2529	ACACTGGTC	2537
K1	210	GATCGCATTGATACTAGAAATGAGTTGGAAAGCTATGCCTATTCTCTAAAGAATCAGATT	151
BiP	1888	GAGCGCATTGATACTAGAAATGAGTTGGAAAGCTATGCCTATTCTCTAAAGAATCAGATT	1947
K1	150	GGAGATAAAGAAAA*ACCTGG	131
BiP	1948	GGAGATAAAGAAAAGCTGGG	1967

4.iv. Discussion

Once it was established that the tumorigenicity of PA1N5 was suppressed in PAB108 but not in the tumor derived cell line carrying the der (4) (Chapter 2), I wished to identify the chromosomal location of a gene involved in this suppression. By mapping the deletion present in der (4) (Chapter 3), it has been shown that a locus mapping to chromosome 4p11.1-15.3 or q31.1 is able to suppress the tumorigenicity of PAB108.

To identify cDNAs which are involved in the suppression of PAB108 tumorigenicity, mRNA differential display was used. As has been widely reported in other systems (Liang *et al.*, 1993, Sun *et al.*, 1994), the cDNA fragments isolated reproducibly from sequencing gels included many false positives which were not differentially expressed when tested on Northern blots. However, the expression of two of the fragments was reproducibly higher in PAB108 than in either PA1N5 or TD2-9 on Northern blots (Figure 4.iii.). The novel cDNA fragment D1 was sequenced and was found to have some homology to the human *CENP-E* gene which encodes a kinetochore protein involved in the segregation of chromosomes at mitosis (Liao *et al.*, 1994). The D1 fragment identified a 2.7kb mRNA on Northern blots (Figure 4.iii.) while the *CENP-E* mRNA is over 8kb in length, clearly indicating that D1 is not identifying *CENP-E* expression. The D1 fragment encompasses 317 bases (Appendix 3), 90 of which are identical to bases 3235-3327 of the *CENP-E* mRNA (Figure 4.iv). Thus, the region of homology is not large and may involve a functional domain; no functional information concerning this region is available, aside from the

assertion that it is α-helical (Yen *et al.*, 1992). Since this fragment was also cloned from a tumorigenically suppressed prostate cancer cell line (Nathalie Bérubé, personal communication), it is likely that the gene, of which D1 is part, is truly involved in tumor suppression. Thus, the D1 fragment was amplified in separate experiments from separate suppressed cell lines but was amplified to a lesser extent in tumorigenic cell lines of different origins; contamination of solutions cannot be a contributing factor in the amplification of D1 since the same solutions were used in the amplification of cDNA from both tumorigenic and non-tumorigenic cell lines. The chromosomal location of the gene encompassing this cDNA fragment is as yet unknown. Thus, it remains a possibility that this gene maps to the candidate tumor suppressor gene region on chromosome 4 (Chapter 3). Further analysis is currently underway in an effort to obtain the full length sequence of this novel transcript and to identify the chromosomal location of this gene (Mario Chevrette, personal communication).

The second fragment which is more highly expressed in PAB108 than in PA1N5 or TD2-9, K1, is identical to the 3' end of human mRNA encoding immunoglobulin heavy-chain binding protein (BiP, Figure 4.v.). This protein, also known as 78kDa glucose-regulated protein, a member of the heat-shock protein 70 family, is involved as a molecular chaperone in the folding and assembly of proteins in the endoplasmic reticulum (Ting and Lee, 1988). The *BiP* gene maps to human chromosome 9 (Hendershot *et al.*, 1994); thus, the locus at chromosome 4p11.1-15.3 or 4q31.1 is not *BiP* but must have some downstream effect, either directly or indirectly, on the regulation of BiP transcription. Recently, a

connection between *BiP* expression and tumor progression by a fibrosarcoma cell line has been made (Jamora *et al.*, 1996). In this cell line, suppression of *BiP* gene expression led to an increased sensitivity to cell death *via* apoptosis. Also, regression of tumors formed by cells exhibiting suppressed *BiP* expression was noted. These results indicate that suppression of *BiP* expression can be consequential for control of tumor growth and progression. Evidently, this involvement is at odds with any role which *BiP* over-expression plays in the suppression of PAB108 tumorigenicity.

Chapter 5 Differentiation with Retinoic Acid

5.i. Introduction

The relationship between differentiation and proliferation is well established. In general, the proliferative phenotype is correlated with undifferentiated, rapidly growing cells while differentiated cells tend to be slow growing and quiescent, assuming a flattened appearance under cell culture conditions. Because of the relationship between differentiation and proliferation, interest in the mechanisms of differentiation has increased, especially in relationship to cancer and development.

The retinoids are a group of synthetic and naturally occurring compounds which are related to vitamin A (retinol). Both 9-*cis* and all-*trans* retinoic acid (RA) are natural occurring retinoids with high biological activity. RA is a known morphogen in mammalian systems and is believed to play important roles in embryogenesis (Tabin *et al.*, 1991); it has been widely studied as an inducer of differentiation in *in vitro* models such as teratocarcinoma and embryonal carcinoma cell lines (Abarzua and Sherman, 1990; De Luca, 1991; McBurney, 1993; Rudnicki and McBurney, 1987). The all-*trans* isoform of RA exerts its effect through interaction with retinoic acid receptors (RARs) while the 9-*cis* form binds to both RARs and retinoid X receptors (RXRs). The α, β and γ RARs and RXRs are nuclear receptor belonging to the same family as the steroid/thyroid hormone receptors (Brand *et al.*, 1988, Giguere *et al.*, 1987, Krust *et al.*, 1991, Petkovich, *et al.*, 1987, Mangelsdorf *et al.*, 1990, 1992). Upon binding of RA to receptor protein, the RARs and RXRs heterodimerize. Heterodimerized receptors then interact with retinoid response elements on retinoid-

responsive genes, modulating the transcription of these targets (Bugge *et al.*, 1992, Kliewer, *et al.*, 1992, Leid *et al.*, 1992, Marks *et al.*, 1992, Zhang *et al.*, 1992). The regulation of downstream genes is receptor specific, with each particular RAR regulating the expression of a set of genes (Boylan *et al.*, 1995). Some functional overlapping has been implied, however, by the normal development seen in mice lacking individual *RAR* genes (Lufkin *et al.*, 1993, Luo *et al.*, 1995, Lohnes *et al.*, 1993).

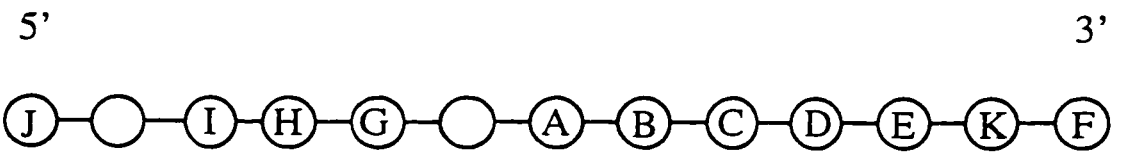
One group of retinoid-responsive genes are the *HOX* genes. These genes encode transcription factors, which bind DNA through the homeodomains which they contain. They are organized into four clusters, 1-4, in vertebrate systems and are located on human chromosomes 7, 17, 12, and 2 respectively. (Boncinelli *et al.*, 1988, 1989, Acampora *et al.*, 1989). RA may exert its control on *HOX* genes through the interaction of activated RAR with retinoid response elements such as that at the 3'end of the *HOX1* cluster (Langston and Gudas, 1992). In human embryonal carcinoma cells, the RA-induced transcriptional activation of these gene clusters occurs in temporal order beginning at the 3' end of the cluster and proceeding to the 5'end (Figure 5.i., Simeone *et al.*, 1991).

Teratocarcinoma cells are *in vitro* models of development and differentiation since they are often multipotent, having the ability to assume characteristics of mesoderm, endoderm and ectoderm. The PA-1 cell line was derived from a human ovarian teratocarcinoma (Zeuthan *et al.*, 1980) and has some potential to differentiate in culture (Tainsky *et al.*, 1988). It has previously been established that *HOX1* genes are RA-inducible in PA-1 (Buettner *et al.* 1991) with early induction beginning at the 3' end of the gene cluster.

In PA-1 this RA-induced *HOX1* gene expression was delayed by *N-RAS* transformation and by the activated *N-RAS* present in late passage cells, supporting the idea that *HOX* gene expression is correlated positively with differentiation and negatively with proliferation.

In this work, I have further investigated the ability of PA-1 to differentiate in the presence of RA. As discussed in the previous chapters and McGowan-Jordan *et al.* (1994), THC were introduced into PA1N5 using microcell fusion and a panel of mouse human hybrids, carrying different dual-selectable tagged human chromosomes (Speevak *et al.* 1995), in order to identify the chromosomal location of a tumor suppressor gene. While several chromosomes were ineffective, chromosome 4 suppressed tumorigenicity of PA-1. In this chapter, I show that a locus mapping to the short arm of chromosome 4, or a small region of the long arm, also enabled the cells to undergo RA-induced differentiation. The expression of *HOX1* and *RAR* genes has also been assessed as a downstream effect of RA-induction.

Figure 5.i. Schematic representation of the human *HOX1* gene cluster. The cluster resides on chromosome 7. Identified genes are shown as letters in circles, whereas postulated but unidentified genes are shown as empty circles. Genes are transcribed in a temporal fashion from right to left. (Adapted from Simeone *et al.*, 1991)


5'
3'
J
I
H
G
A
B
C
D
E
K
F

5.ii. Materials and Methods

5.ii.a. Morphology Studies

For morphology studies, PA1N5, PAB108, PAB108-S and PAB108-TD2 were seeded and allowed to grow overnight as outlined in Chapter 2.ii. before addition of dimethylsulfoxide (DMSO) or all-*trans* RA (10^{-5}M in DMSO). Morphology was assessed after four days, at which time photographs were taken using a phase-contrast microscope. RA was stored at -20°C as a 1mM solution in DMSO.

5.ii.b. Proliferation Assays

For proliferation assays, cells were seeded at a density of 0.2 - 5 x 10^3 cells/cm^2 in appropriate media. Cells were allowed to attach for 24 hours before addition of DMSO or all-*trans* RA (10^{-5}M in DMSO). Cells were trypsinized and counted using a hemocytometer or a Coulter counter.

5.ii.c. RNA Isolation and Northern Blots

For Northern blot analysis, cells were grown to approximately 80% confluence before harvesting of total RNA as described in Chapter 3.ii.e. Some cultures were treated with 10^{-5}M RA for 6 or 72 hours before total RNA isolation. PolyA+ RNA was purified from total RNA using the PolyATtract mRNA Isolation System (Promega).

RNA (15µg total or 200ng polyA+) was electrophoresed on denaturing formaldehyde gels. RNA was then transferred to nylon membranes (Pall Biodyne B) and probed with ^{32}P-labelled cDNA probes. Hybridization of the probe on blots was detected using either autoradiography or a phosphorimaging screen (Molecular Dynamics) and quantitated using a LKB 2222-020 UltroScan XL Laser Densitometer or ImageQuantNT software, respectively.

5.ii.d. cDNA Probes

HOX probes were kindly provided by Dr. E. Boncinelli (Naples, Italy). Murine *RAR* probes were generously provided by Dr. Michael McBurney (University of Ottawa, Canada).

5.iii. Results

5.iii.a. Growth of cells in culture

To determine the ability of various cell lines to undergo retinoic acid-induced differentiation, cells were grown in the presence of 10^{-5}M RA or vehicle (DMSO). While PA1N5, the parent cell line, did not assume a differentiated morphology after RA treatment (Figure 5.ii.A and B), the cell line carrying a normal tagged copy of chromosome 4, PAB108, underwent a dramatic change in appearance (Figure 5.ii.C and D). As has been reported previously for untreated PAB108 cells (McGowan-Jordan *et al.*, 1994), DMSO-treated PAB108 cells are larger than their parental cells (Figure 5.ii.A and C). The morphological difference between these cell lines is even more evident after RA-treatment where PAB108 cells became extremely large and flat in appearance, but remained mononucleated (Figure 5.ii.D). Neither the segregant, PAB108-S, from which the tagged chromosome 4 had been removed by ganciclovir selection, nor the tumor derived cell line harbouring the der (4), differentiated after RA treatment (data not shown and Figure 5.ii.E and F).

Because differentiating cells often experience a decline in proliferation, cell numbers after growth in RA were determined. PA1N5 experienced no drop in its growth potential when grown in the presence of RA; in fact, as has been reported previously (van der Leede *et al.*, 1993), it experienced an increase in growth (Figure 5.iii.). Although the differences were not statistically significant, the growth of PAB108 does not seem to be induced in the presence of RA (Figure 5.iii.).

Figure 5.ii. Effect of RA on morphology of cells in culture. Photographs of PA1N5, PAB108, and PAB108-TD2 were taken using a phase-contrast microscope after treatment for 4 days with DMSO (-) or 10^{-5}M RA (+). All photographs were taken at 100X magnification. Some experiments were performed by Louisa Cale.

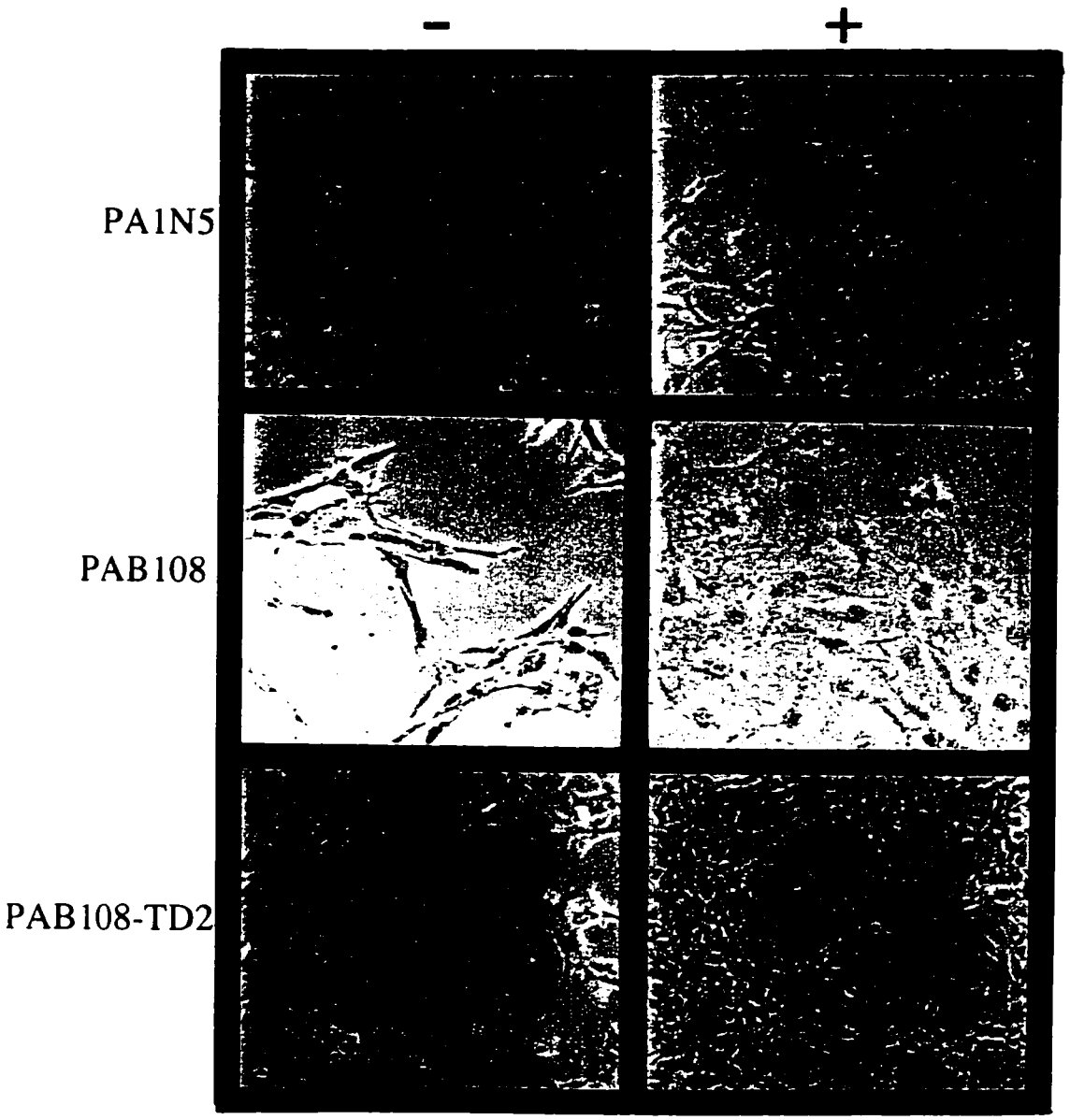
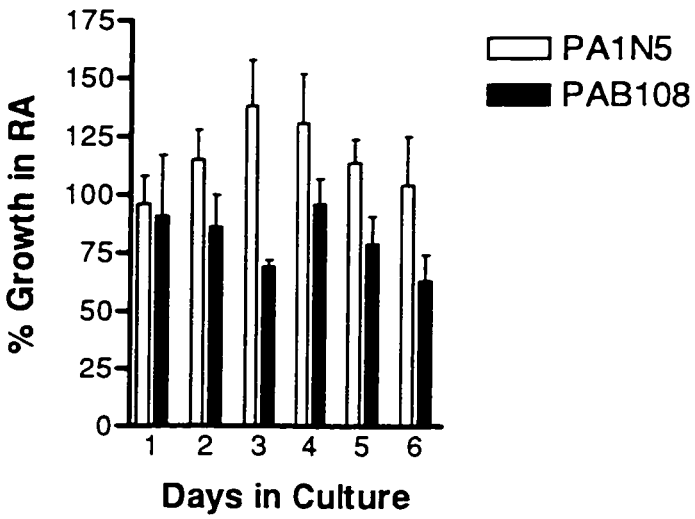

-
+
PA1N5
PAB108
PAB108-TD2

Figure 5.iii. Effect of RA on cell growth. PA1N5 (open bars) and PAB108 (solid bars) were grown as described in Materials and Methods. Values are expressed as percentage of cell number in the presence of 10μM RA relative to controls grown in the presence of vehicle. Error bars represent s.e.m.. Day 1 and 5 results are based on 3 dishes from each treatment. All other results are based on two separate experiments and a total of 5 dishes from each treatment. Some experiments were performed by Louisa Cale.


175
150
125
100
75
50
25
0
% Growth in RA
1
2
3
4
5
6
Days in Culture
PA1N5
PAB108

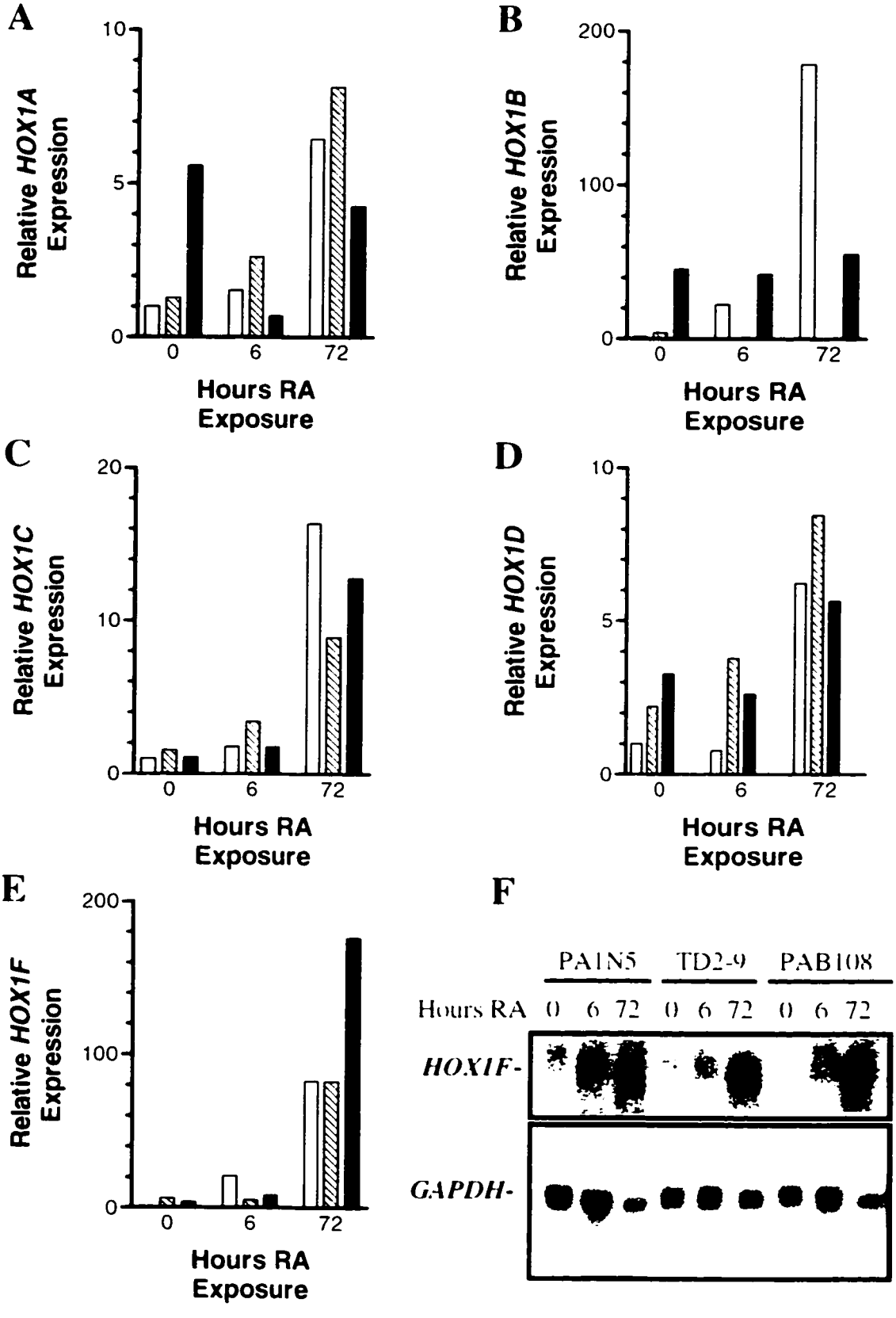
5.iii.b. HOX1 Gene Expression

HOX genes are organized as clusters in mammalian systems and are known to influence the developmental processes. They are retinoic acid responsive genes with the induction of their expression occurring in a temporal fashion according to their position in the gene cluster (Simeone *et al.*, 1991). Buettner *et al.* (1991) have shown that early passage PA-1 cells differ in their ability to undergo RA-induced differentiation and the time of *HOX1* gene induction from those which had undergone *N-RAS* transformation. *N-RAS* transformed cells showed delayed *HOX* gene expression and did not assume a differentiated morphology in response to RA.

In order to investigate the mechanism of the increased ability of PAB108 to differentiate in the presence of RA, the expression of several *HOX1* genes was investigated after RA treatment of PA1N5 and its derivative cell lines. As expected, most of the *HOX1* genes tested were RA-inducible (Figure 5.iv.). Since *HOX1B* is only induced after 4 days of growth in RA (Buettner *et al.*, 1991) it is not surprising that it was undetectable in TD2-9 by 72 hours of RA treatment (Figure 5.iv.B). However, the expression of this same gene was RA-inducible in PA1N5 and was highly but evenly expressed in PAB108 over the time course studied. The expression of *HOX1A*, *1C* and *1D* was induced by RA (Figure 5.iv.A, C, and D). *HOX1A* was highly expressed in PAB108 even in the absence of RA treatment. After 6 and 72 hours of RA treatment *HOX1A* expression in PAB108 resembled that in PA1N5 and PAB108-TD2. Likewise, the expression of both *HOX1C* and *1D* in PAB108 was consistent with levels found in the other cell lines tested at all time points tested (Figure

5.iv.C and D). In contrast to these findings, the induction of *HOX1F* after 72 hours of RA treatment in PAB108 was twice that of either PA1N5 or TD2-9 (Figure 5.iv.E and F).

Figure 5.iv. Expression of *HOX1* genes. Relative expression was determined from Northern blots. RNA was harvested from PA1N5 (open bars), TD2-9 (hatched bars, B and E), PAB108 (solid bars), or PAB108-TD2 (hatched bars, A, C and D) after 0, 6 and 72 hours of 10μM RA treatment. A, C, and D. Approximately 200ng of polyA+ RNA was loaded in each lane before electrophoresis and transfer to nylon membranes. RNA was then hybridized with the probes indicated. Relative probe hybridization was determined using *α-TUBULIN* as a control for loading of polyA+ RNA. Probe hybridization was detected using autoradiography and quantitated by densitometry. B and E. Approximately 15μg of total RNA was loaded per lane. *GAPDH* was used as a loading control. Probe hybridization was detected using a phosphorimager and quantitated by ImageQuantNT software. F. Representative Northern blot revealing expression of *HOX1F*. The images were reproduced using LogiTech FotoTouch Color and Microsoft PowerPoint software.


A
Relative HOX1A Expression
10
5
0
0
6
72
Hours RA Exposure
B
Relative HOX1B Expression
200
100
0
0
6
72
Hours RA Exposure
C
Relative HOX1C Expression
20
10
0
0
6
72
Hours RA Exposure
D
Relative HOX1D Expression
10
5
0
0
6
72
Hours RA Exposure
E
Relative HOX1F Expression
200
100
0
0
6
72
Hours RA Exposure
F
PA1N5
TD2-9
PAB108
Hours RA 0 6 72 0 6 72 0 6 72
HOX1F-
GAPDH-

5.iii.c. RAR Expression

The *RAR*s represent another class of RA-responsive genes, being induced after RA treatment. It is through the RARs that RA exerts its morphogenic effect. It is possible that the RA responsiveness of PAB108 is due to increased expression of these genes. Northern blots were performed and the expression of *RARα* and *RARβ* was determined (Figure 5.v.). As expected both *RAR*s were RA inducible after 6 and 72 hour treatments. This induction was equivalent among the cell lines examined except for a notable over-expression of *RARα* in PAB108 after just 6 hours of RA treatment. This expression was quantitated and was found to constitute an average of 6.2 times over-expression of the upper band and a 3.6 times over-expression of the lower band of *RARα* in PAB108 over that seen in PA1N5 or TD2-9. The over-expression of *RARα* was reproducible. The expression of *RARγ* was also investigated but the mRNA was undetectable even when low stringency washes were used to accommodate the use of a murine probe on human samples (data not shown).

5.iii.d. H6 Expression

One cDNA fragment which had been isolated by mRNA differential display (Chapter 4) was found to be RA-inducible. While the H6 fragment was over-expressed in PAB108 as compared to PA1N5, it was also highly expressed in TD2-9 (Table 4.i.). This expression was low as compared to the expression after treatment with RA (Figure 5.vi.), where the amount of mRNA in both cell lines was over ten-fold higher than basal levels.

Figure 5.v. Northern blot analysis of *RAR* expression. RNA was harvested from PA1N5, TD2-9 and PAB108 after 0, 6 and 72 hours of 10^{-5}M RA treatment. Approximately 15μg of total RNA was loaded in each lane before hybridization with *RARα* or *RARβ* as indicated on the left. Relative probe hybridization was determined using *α-TUBULIN* as a control for loading of total RNA. Approximate sizes of mRNAs are: *RARα*, 3.6 and 2.8kb; *RARβ*, 3.4kb indicated on the right. Probe hybridization was detected using a phosphorimager. The images were reproduced using LogiTech FotoTouch Color and Microsoft PowerPoint software.

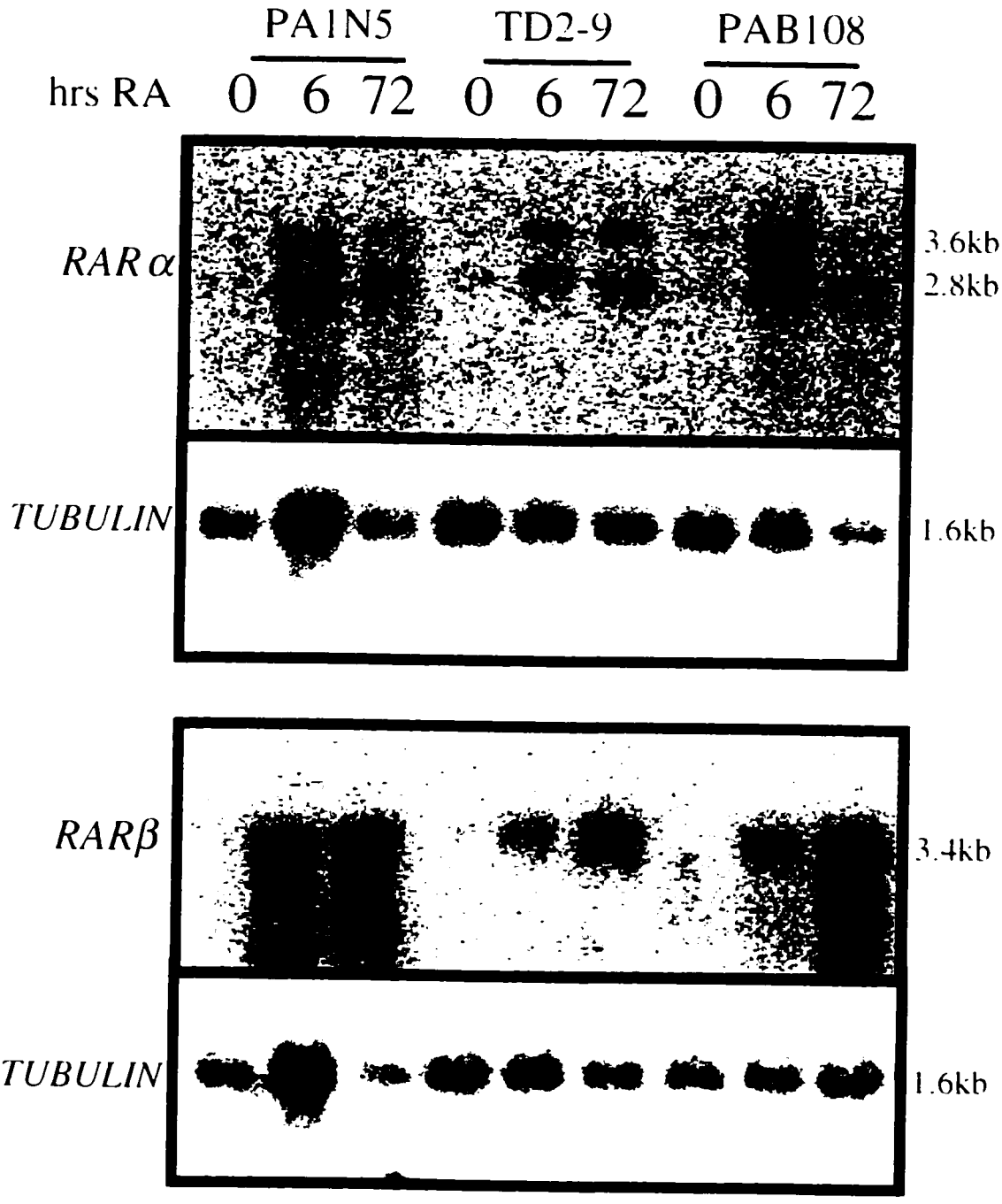
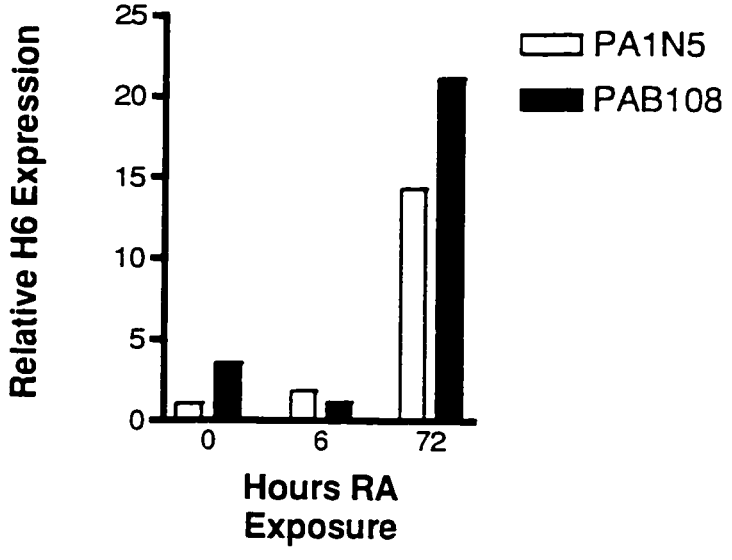

PA1N5
TD2-9
PAB108
hrs RA
0 6 72 0 6 72 0 6 72
RARα
3.6kb
2.8kb
TUBULIN
1.6kb
RARβ
3.4kb
TUBULIN
1.6kb

Figure 5.vi. Expression of cDNA fragment "H6". Relative probe hybridization was determined from duplicate Northern blot analysis. RNA was harvested from PA1N5 and PAB108 after 0, 6 and 72 hours of 10^{-5}M RA treatment. Approximately 200ng of polyA+ RNA was loaded in each lane before hybridization of H6 probe. *α-TUBULIN* was used as a control for loading. Approximate mRNA size is 3kb. Probe hybridization was detected using a phosphorimager and quantitated by ImageQuantNT software.


Relative H6 Expression
25
20
15
10
5
0
0
6
72
Hours RA Exposure
PA1N5
PAB108

5.iv. Discussion

Initially, the aim of this study was to explore the differentiation potential of the human ovarian teratocarcinoma cell line, PA-1, and its derivatives. I used the PA1N5 cell line, the tumorigenically-suppressed microcell hybrid, PAB108, a tumor derived cell line from which portions of the tagged chromosome 4 are missing, PAB108-TD2, and a segregant from which the tagged chromosome has been removed, PAB108-S. When these cell lines were grown in the presence of RA only PAB108, which carries an intact tagged normal chromosome 4, was able to differentiate and assume a large, flattened morphology (Figure 5.ii.). PAB108-TD2 and PAB108-S reverted to the parental phenotype and were unable to undergo the dramatic changes in cell morphology characteristic of PAB108. Thus, a locus on chromosome 4 and which is located in a region which has been lost from the der (4) is responsible for the ability of PAB108 to differentiate with RA treatment. Morphological changes similar to those seen with PAB108 have been observed in early passage PA-1 cells in response to RA (Buettner *et al.*, 1991). Like PAB108, these early passage cells are non-tumorigenic in nude mice. In the same study, *N-RAS*-transformation, whether spontaneous or experimental, caused PA-1 cells to loose their ability to differentiate in culture. Late passage PA-1 cells (those used in this study) contain one activated *N-RAS* allele (Tainsky *et al.*, 1984). Thus it seems that *N-RAS* transformation is one route leading to RA resistance and that a gene on chromosome 4 can reverse this effect.

In an effort to illuminate the pathway by which the differentiation of PAB108, but not the parent cell line, occurs, the expression of several RA-responsive genes was investigated. I determined the expression levels of *HOX1A*, *1B*, *1C*, *1D*, *1E*, and *1F*. The introduction of a normal chromosome 4 into PA1N5 did not affect the expression of the majority of these *HOX* genes. They were equally induced with RA treatment in all cell lines tested (Figure 5.iii). However, the induced expression of *HOX1F* after 72 hours of RA was increased two-fold in PAB108 over levels in either PA1N5 or TD2-9 (Figure 5.iv.E and F). Buettner *et al.* (1991) have found that *HOX1* genes are induced in response to RA in PA-1 cells and that this induction follows a time course according to the position of the gene within the *HOX1* cluster. They found that expression of *HOX1C* and *1D* was delayed by *N-RAS*-transfection and that expression of *HOX1D* in *N-RAS*-transformed cells causes a morphological change in the cells. These data in combination with those shown here indicate that the *HOX1D* expression is sufficient but not necessary for PA-1 cells to differentiate with RA. The locus on chromosome 4 which is responsible for the increased RA-responsiveness of PAB108 is not able to cause earlier induction of *HOX1* genes as is seen in the early passage PA-1 cells. However, this locus causes a quantitative increase in the induction of *HOX1F* after 72 hours of RA treatment. The relationship between *HOX1F* and differentiation has been noted in F9 murine teratocarcinoma cells (Goliger and Gudas, 1992) as these cells show an altered morphology after *HOX 1.6* (the murine equivalent of *HOX1F*) transfection.

*RAR*s are another class of RA-responsive genes, their expression being induced by RA. In this study I have shown that the expression of *RAR*β was unaffected by the addition of a normal chromosome 4 to PA1N5 or the presence of the der (4) in TD2-9 (Figure 5.v.). *RAR*β expression was not detected in untreated cells, and expression was RA-inducible equally among the cell lines tested. *RAR*α expression, on the other hand, was significantly altered by the introduction of chromosome 4 (Figure 5.v.). In PA1N5, *RAR*α was induced with RA treatment as others have reported (Buettner *et al.*, 1991). *RAR*α expression was also RA-inducible in TD2-9, which contains the der (4) chromosome. However, expression in PAB108 was highly induced after only 6 hours treatment with RA. At 72 hours expression levels returned to those seen in PA1N5 and TD2-9. Thus, early, strong induction of *RAR*α correlates with the ability of PAB108 to differentiate in the presence of RA. It is possible that it is this early induction of *RAR*α in PAB108 which causes the RA-induced differentiation since RARs mediate the effect of RA. Cells lacking RARα activity do not differentiate (Pratt *et al.*, 1993) indicating that this receptor plays a key role in the differentiation process. Also, cell lines normally lacking *RAR*α activity can be induced to differentiate in response to RA after reintroduction of a normal *RAR*α gene (Mehta *et al.*, 1996).

That RARα is involved in differentiation and control of proliferation has further evidence. In acute promyelocytic leukemia there exists a characteristic chromosomal translocation involving chromosomes 15 and 17. This translocation results in the fusion of the *RAR*α with that encoding PML (Alcalay *et al.*, 1991, de The *et al.*, 1990) which is

thought to act as a transcription factor. *RARα* is further implicated in cell differentiation since the fusion of these genes results in the production of a mutant *RARα* (Kastner *et al.*, 1992) and at least contributes to the incomplete differentiation of promyelocytes which is characteristic of acute promyelocytic leukemia.

I have shown that a locus at chromosome 4p11.1-15.3 or q31.1 is involved in the RA-induced differentiation of PAB108, a derivative of the PA-1 teratocarcinoma cell line. This locus causes the cell line to undergo morphological changes and to slow in growth in response to RA. These changes are accompanied by a doubling in the RA-induced expression of *HOX1F* and a dramatic early induction of *RARα*.

Chapter 6: Conclusion and Future Studies

6.i. Conclusion

The experimental work presented in this thesis shows that a locus on chromosome 4 mapping to 4p11-15.3 or q31.1 is responsible for the tumor suppression of the PAB108 cell line. These cells were derived from PA-1 following introduction of a neomycin tag with the subsequent introduction of a tagged normal copy of human chromosome 4 (Chapter 2). The fortuitous isolation of a tumor-derived cell line containing a rearranged copy of chromosome 4 allowed me to map the putative tumor suppressor to the chromosomal region mentioned above (Chapter 3).

Differential display was used in an attempt to isolate genes involved in suppression of tumorigenicity of PAB108 (Chapter 4). Two genes were isolated in this manner and found to be over-expressed in PAB108 relative to PA1N5 and TD2-9. The expression of these genes is not only associated with suppression of tumorigenicity but also with the increased ability of PAB108 to differentiate after RA treatment. The *BiP* gene is neither the locus on chromosome 4 responsible for the increase in RA-responsiveness of PAB108, nor the locus responsible for the decreased tumorigenicity of the PAB108 cell line. However, BiP expression must be controlled as a downstream effect of a gene, or genes, mapping to chromosome 4p11-15.3 or q31.1, as is the RA-induced expression of *RARα* and *HOX1F*. Interestingly, *BiP* is known to be expressed during embryogenesis and in teratocarcinoma cells treated with RA (Kim *et al.*, 1990). When viewed along with its over-expression in PAB108 these data suggest that *BiP* plays a role in cellular differentiation.

6.ii. Future Studies

This project, which concerns the identification of a new tumor suppressor gene, sets the stage for a number of future studies. Initially, it will be of interest to obtain full-length clones of the D1 and H6 cDNA fragments which were identified by mRNA differential display. Both D1 and K1 (*BiP*) are more highly expressed in PAB108 than in either PA1N5 or TD2-9, suggesting that the corresponding genes play a role in the suppression of tumorigenicity in PAB108. On the other hand, the expression of the gene corresponding to H6 is induced by retinoic acid treatment, leaving one with the impression that the gene is involved in the retinoid response pathway.

Once the full-length cDNAs are obtained it will be essential to determine the chromosomal location of these genes. This may be done by a variety of strategies. First, FISH may be used wherein the cDNA is used as a probe on normal human chromosome spreads (Edelhoff *et al.*, 1994). The hybridization signal will identify which chromosomal region encodes the gene. This technique, of course, relies on the ability to stain a given chromosome spread both so that the chromosomal identity can be determined karyotypically and so that the FISH signal can be visualized. Minimally, this type of experiment will lead to the identification of a chromosomal group to which the gene maps. Despite the technical challenges of such a venture, experiments such as this do give valuable mapping information.

A second route that may be taken towards the chromosomal mapping of the identified genes is the use of the mouse/human hybrid cell lines containing individual human

chromosomes. An extensive panel of such hybrids is already available in Dr. Chevrette's laboratory (Speevak et al., 1995). It may be used to determine the human chromosome which encodes the gene of interest, either by PCR amplification or Southern blot analysis. This approach would give valuable information, but may only identify the chromosome on which the gene resides rather than the chromosomal band.

Further identification, analysis and characterization of cDNAs which are over-expressed in PAB108 is warranted. The mRNA differential display experiments described in this thesis do not fully cover the genome since it is estimated that a full set of 26 arbitrary decamers is required to complete this task (Bauer *et al.*, 1993). Thus, a continuation of the differential display experiments should be undertaken in an effort to identify further candidate tumor suppressor gene cDNAs, and eventually clone the tumor suppressor gene on chromosome 4.

The project should also be continued in the area of the differentiation potential of PAB108 in the presence of RA. Various aspects of this phenotype may be investigated starting with the exploration of the induction of several other RA-responsive genes, namely the *HOX2*, *HOX3*, *HOX4*, and retinoid X receptor (*RXR*) genes. These studied would clarify further the mechanism of RA-induced differentiation in PAB108 and may further link this phenotype to the chromosome 4-encoded gene.

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Appendix 1 Sources of Reagents

Reagent	Source
Acetic acid	BDH
Acrylamide	Boehringer Mannheim Canada
Agarose	Canadian Life Technologies
Agarose, NuSieve	Mandel Scientific
Alu primers	M. Ekker (Loeb Inst. Ottawa, Ont.)
Ampicillin	Boehringer Mannheim Canada
AmpliTaq, DNA polymerase	Applied Biosystems Canada
Ammonium persulfate	BDH
Amyl alcohol	BDH
Arbitrary decamers	M. Ekker (Loeb Inst. Ottawa, Ont.)
BioPrime DNA Labelling System	Canadian Life Technologies
Bovine serum albumin	Sigma
Bromophenol blue	BDH
Chromosome *in situ* Hybridization System	Oncor
Colcemid	Sigma
Concavalin A type III	Sigma
Cot 1 DNA, human	Canadian Life Technologies
1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-tolune-sulfonate	Sigma
Cytochalasin B	Sigma
Dextran sulfate	Pharmacia
Diethlypyrocarbonate	BDH
Dimethylsulfoxide	BDH
Dithiothreitol	Boehringer Mannheim Canada
DNA, 100 base pair ladder	Pharmacia
DNA, salmon sperm	ICN
dNTPs	Boehringer Mannheim Canada
Dulbecco's modified Eagle medium	Canadian Life Technologies
Dulbecco's modified Eagle medium/Ham's F12 1:1	Professional Diagnostics Inc.
Ethidium bromide	BDH
Ethylenediaminetetraacetic acid, disodium	BDH
Fetal bovine serum	Canadian Life Technologies
Formaldehyde	BDH
Formamide	Canadian Life Technologies
Ganciclovir	Syntex Inc., Palo Alto, CA, USA
Geniticin	Canadian Life Technologies
Glycogen	Beohringer Mannheim Canada
Harleco Giemsa stain	Diagnostic Systems
Hoechst 33258 stain	Sigma
Hygromycin B	Boehringer Mannhein Canada
Leishman's stain	BDH
MapPairs	Research Genetics
Methanol	BDH
Minimal essential media	Canadian Life Technologies

MOPS	USB
Nonidet P-40	Boehringer Mannheim Canada
α-^{32}P-dCTP	Amersham
Phosphate buffer, pH6.86	Fisher
Phytohemaglutinin P, Difco	Canlab
Polyacrylamide, Long Ranger	FMC
Polyethylene glycol	Baker
Proteinase K	Boehringer Mannheim Canada
RNase inhibitor, human placental	Canadian Life Technologies
Retinoic acid, all-*trans*	Sigma
Reverse transcriptase, Superscript	Canadian Life Technologies
^{35}S-dATP	Amersham
Sodium acetate	BDH
Sodium chloride	BDH
Sodium citrate	BDH
Sodium dodecyl sulfate	Canadian Life Technologies
Spermidine	Sigma
Spermine	Sigma
TEMED	BDH
$T_{11}MN$	M. Ekker (Loeb Inst. Ottawa, Ont.)
tRNA, yeast	Boehringer Mannheim Canada
Tris	Canadian Life Technologies
Triton X-100	Boehringer Mannheim Canada
Trypsin	Canadian Life Technologies
Trypsin, Bacto	Canlab
Urea, ultrapure	BDH

Appendix 2 Buffer Recipes

Formamide loading buffer:
- 95% formamide
- 0.09% bromphenol blue (w/v)
- 0.09% xylene cyanol (w/v)

Hybridization buffer:
- 50% formamide
- 0.5M $NaHPO_4$ pH 7.2
- 1mM Na_2EDTA
- 1% bovine serum albumin
- 5% SDS

MOPS buffer (20X, pH7.0):
- 0.4M MOPS
- 0.1M $NaC_2H_3O_2$
- 10mM Na_2EDTA

Nuclei dropping buffer:
- 75mM NaCl
- 24mM Na_2EDTA pH8.0

P.E. buffer (1X):
- 10mM Tris pH8.3
- 50mM KCl
- 1.5mM $MgCl_2$
- 0.01% gelatin

Phosphate buffered saline, (pH~7.3):
- 137mM NaCl
- 2.7mM KCl
- 4.3mM Na_2HPO_4
- 1.4mM KH_2PO_4

SSC (1X):
- 0.15M NaCl
- 0.015M sodium citrate

TBE (10X):

890mM Tris base
890mM boric acid
20mM Na_2EDTA, pH8.0

TE:

10mM Tris HCl pH8.0
1mM Na_2EDTA

TE^{-4}:

10mM Tris HCl pH8.0
0.1mM Na_2EDTA

TSE+S:

10mM Tris HCl pH8.0
150mM NaCl
5mM Na_2EDTA
0.2% SDS

TSM:

10mM Tris HCl pH8.0
150mM NaCl
2mM $MgCl_2$

Appendix 3 Sequence of mRNA Differential Display cDNA Fragments

D1: T_{11} GCCAC TGGTC ATTTT TGCTC TGTAT ATGCC ACAGT GTTGT CTTCT GTCAT GTGAG GGCAG ATACC AAAAA TGTTT TCCTG GATAA ACTTT TTTTT TTTTT TTCCA CCAGA TACAT TTATT GTATA TAAAT TATAC CCCAA TAAAG TTGAT AAAAA TCTGA AACAA ATGAA GACAA AAAGG TAATG CCAAG TATAC AGTGC TAAAT CCTAC CATTT CAATA TTTTC CTTTT AGGTC NGTCT TCAAT TGTTC CTTTT CTGCT ATAAC ACTCT CTAAC ATTTG TTGGA GTTCA TTTTT CTCCT GTATC GA

D2: T_{11} GCCGG TTAAG ATTTT AATGT TTTCT TAATC AAAAT TCTCT GAACT CATTC TCAAT TTACA AGGTT AGAAC TCCTT CAAAG AAAAG ACATA CAGAT ACAGA CTCTA GCTTT AATTA TTATT CTAAA TGTCC ACTAG CCCTG TATCG AAAGT AATTT GCATC TTGAT TCTGT GCTCC ATGAT TTCTC CCAGT AATTT AATAA CTTTT TTAAA GTCCT CACTT TACTA TCCAG ATCGA GATAG GTCTC TCTTG CTTCT TGGTA CTGCC TCTTT ATTTC CTCTC GATCT CCATG ACTAG CATGT TCTGC CTGTA TCGA

Q3: T_{11} GGCCA AGAAA AGGAC AGTGG GATTT ATGAT GTGTT GTTTA AGGCC CAAAA CTTCC CTGGT AACCA CCAGT GTATA GTTTT GCTGC AGGTT GGCAA ACGGC CTAGT TGATT TATTA GCAAG ACTGG CTTGA TCATT ACAAG GGCCT TCCTG AGAGT TTCTC TGGAG CTCTT TCAGA AGCCA TATCT TGGTG ACTCG AGGTG GACTC AGCAC AATCC TCGTG TGTAA GTGCT CACTG GGAGC TCACA TAGGG GAGGG ATGTT TCATA ATACC TGCCT GCATT CTCTT TCTCC T

Q5: T_{11} CNCAA GAACC GCCAA AGTTT TAGTG TTTAA TAATG AATAG CACAA CTGAC CAAGG TCCAA GATGT GAAGA TACCA TGTTC AAGAA ACTGG GGGAA ATCAC TCTAC AAACT AACTA TATAC TAGGT GATAA GAATG CATAC TTTAT AATAT AAAAC CTGTC TACAC ATAGT AGTTA GCTGC AAAAA GCCAT TCGAT CTTCT CTTGG CTTGG AAAAT GCCAG ATTCC AGTAT AAATT ATTAG CAACC TTCAA CTCTT TTGGC GTGGC AGGAT CTTGT TTTCA CTTTC CAGTT CTTCC ACCCC AGCCT GTATC GA

R5: T_{11} CGTAA GTTGT GTTTA CAATC ATCCA TTTAA TCATC TCCAA AAAAA GGATG AATTT ATTAT TTTAA ACTAA CATTC TGTAA GTTAA AGAGA ATAAT CAAGT TGTCT CATGG ACTGG AAGGT GACTT ATTAT TTCCC TTTCA TTTCC AAACC TCATC AAGCA GA

H6: T_{11} GGGAG AGGAG ATTAT TTTTA AAGTC CATTT TCTTC CGAAG AGTGA AAAAG GCCAA GCAAT AAATC CACAG CTGTG CAACC TACCT CAGTG CAAGT CTCCA GGCCA TAATT TGTTA GCCTC CATCA AGCGG

J: T_{11} CTGCT TGATG CACCA GAAAT GTGGG CTAAA GCTGC AGCCA ATGCA TCCAC TGCAC CTTTC TCTTC TATCA GTCTC TGAGC TGATG GTCGG AAAAA ATCAA CAGCA GCATA AGAAA CGGAA GCCAG AGACC TGTTT TATGT GTGGA TTCAT GGCCA TTTCA GTTAC TTCTT CTTGG TCTCA CAGAA AATAA TAGCC CTCCC TTCAG ACCCA CTGTA GACTT GAAGG ACATC TCCAA TAACT GCTGG CCTCT GAGAC CAATG ACACT GGATG GCCAA TGTCA CAGTA GTTGC AGCCT TTTGA GTCAT TTCCA ACAAG GTCAA CCTGT TCATA TCTGG ATTTC ATGTA TTTTT TTGCA ACTTT GTATA CCCAC TGTGG GCAAG TTGCA GAAAA AAGTA AAGTC TGAGG ATTGT CTTCA GAATC AGTTT TGTAG GATTC ATGAA TAATA TCTTC AACTT GTTGA GCGAA ACTTA AATCT AACAT CTGAT CCACT TCATC AAGCA G

K1: T_{11} GACCA GTGTA AATAA CAAAC ATTTA TTGGT GTCAC TTATG GTAGA NNAAA CTTCC TACAC CAGAT GCACA TGACC CAGTT GTTAA ATAGA ACATT TTGAA GGTGA ACACA CACCC TAACC CAGGT TTTTC TTTAT CTCCA ATCTG ATTCT TTAGA GAATA GGCAT AGCTT TCCAA CTCAT TTCTA GTATC AATGC GATC

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EDUCATION:

Ph.D.: Department of Biochemistry, University of Ottawa
Supervisor: Dr. Mario Chevrette
May 1992-present. Expected date of completion is Winter 1996.

M.Sc.: Department of Chemistry and Biochemistry, University of Guelph
Supervisor: Dr. David Josephy
Jan. 1988-May 1990

B.Sc.: Biomedical Toxicology, University of Guelph.
Sept. 1983-April 1987

EXPERIENCE:

i. Scientific

Department of Biochemistry, University of Ottawa. May 1992-present
"Suppression of tumorigenicity of human teratocarcinoma cell line, PA-1, by introduction of human chromosome 4."
Graduate research has provided experience in cell biology, tissue culture and molecular biological methods including fluorescent *in situ* hybridization, Southern blot, Northern blot, polymerase chain reaction, reverse transcriptase polymerase chain reaction, transformations, mRNA differential display, and DNA sequencing.

Brown University, Providence, Rhode Island, Research Assistant. July 1990-Jan. 1992.
"Damage to pulmonary endothelium by polymorphonuclear leukocytes."
Research provided experience with tissue culture and cell biology methods.

Department of Chemistry and Biochemistry, University of Guelph. Jan. 1988-May 1990.

"Peroxidative activation of 3,3'-dichlorobenzidine to a mutagen by hydroperoxidase I in *S. typhimurium*."
Graduate research provided experience in biochemical techniques including Ames assay, bacterial transfections and transductions, spectrophotometry, and polyacrylamide gel electrophoresis of proteins.

Department of Microbiology, University of Guelph, Laboratory Technician. May 1987-Aug. 1987. Research concerning metabolism in *Rhizobium*.

Department of Microbiology, University of Guelph, Laboratory Technician. May 1986-Aug. 1986. Updated experiments and prepared samples for Introductory Microbiology undergraduate laboratories.

ii. Teaching

March-April 1995:
Teaching Assistant for Molecular Biology section of Third Year Biochemistry Laboratory, Department of Biochemistry, University of Ottawa

March-April 1994:
Teaching Assistant for Molecular Biology section of Third Year Biochemistry Laboratory, Department of Biochemistry, University of Ottawa

Jan. 1988-April 1990:
Teaching Assistant for courses in Introductory Organic Chemistry and Introductory Biochemistry, Department of Chemistry and Biochemistry, University of Guelph.

iii. Professional Presentations:

Ottawa Life Sciences Conference Seminar and Poster, Ottawa, Ontario.
Title: Isolation of candidate cDNAs which suppress the tumorigenicity of human teratocarcinoma cell line, PA-1. October 1995.

Foundation for Advanced Cancer Studies Conference Poster, Frederick, Maryland.
Title: A region of human chromosome 4 (4p11-15.3) suppresses tumorigenicity of human teratocarcinoma cell line, PA-1. June 1995.

University of Guelph Department of Chemistry and Biochemistry Seminar.
Title: Tumorigenicity of a human teratocarcinoma cell line is suppressed by the introduction of a normal chromosome 4. June 1993.

Southern Ontario Carcinogenesis Group Spring Meeting Seminar, Toronto, Ontario.

Title: *Salmonella typhimurium* gene products affecting peroxidative activation of aromatic amines. April 1989.

PUBLICATIONS:

i. Book Chapter:

Contribution to: Josephy, P. D. 1997. Carcinogenesis: The genetic targets. In: Molecular Toxicology. (Oxford University Press, New York) pp 309-314.

ii.Research Articles:

McGowan-Jordan, J. J., and Chevrette, M. A locus on chromosome 4p11-15.3 or q31.1 allows a human teratocarcinoma cell line, PA-1, to undergo retinoic acid-induced differentiation. Manuscript in preparation.

Dawicki, D.D., McGowan-Jordan, J., Bullard, S., Pond, S., and Rounds, S. (1995) Extracellular nucleotides stimulate leukocyte adherence to cultured pulmonary artery endothelial cells. *Am. J. Physiol.* **268**, L666-L673.

Speevak, M.D., Berube, N.G., McGowan-Jordan, J.J., Bisson, C., Lupton, S.D. and Chevrette, M. (1995) Construction and analysis of microcell hybrids containing dual selectable tagged human chromosomes. *Cytogenet. Cell Genet.* **69**, 63-65.

McGowan-Jordan, J.J., Speevak, M.D., Blakey, D., and Chevrette, M. (1994) Suppression of tumorigenicity in human teratocarcinoma cell line PA-1 by introduction of chromosome 4, *Cancer Res.* **54**, 2568-2572.

Meharg, J.V., McGowan-Jordan, J., Charles, A., Parmalee, J.T., Cutaia, M.V., and Rounds, S. (1993) Hydrogen peroxide stimulates sodium-potassium pump activity in cultured pulmonary arterial endothelial cells. *Am. J. Physiol.* **265**, L613-L621.

McGowan-Jordan, J.J., and Josephy, P.D. (1990) Hydroperoxidase I catalyses peroxidative activation of 3,3'-dichlorobenzidine in *Salmonella typhimurium*. *Arch. Biochem. Biophys.* **282**, 352-357.

iii.Refereed Abstracts:

Rounds, S. and McGowan-Jordan, J. (1992) Hydrogen peroxide increases ouabain binding to cultured endothelial cells. *Am. Rev. Resp. Dis.* **145**, A838.

Rounds, S., McGowan-Jordan, J. and Bullard, S. (1991) Adenine nucleotides increase granulocyte adherence to cultured endothelial cells. *Am. Rev. Resp. Dis.* **143**, A370.

SCHOLARSHIPS:

Medical Research Council of Canada Studentship:
Held at Dept. of Biochemistry, University of Ottawa, April 1994-present

University of Ottawa Excellence Scholarship:
Held at Dept. of Biochemistry, University of Ottawa, May 1994-present

University of Ottawa Admission Research Scholarship:
Held at Dept. of Biochemistry, University of Ottawa, May 1993-April 1994

University of Ottawa Admission Research Scholarship:
Held at Dept. of Biochemistry, University of Ottawa, May 1992-April 1993

Ontario Graduate Scholarship:
Held at Dept. of Biochemistry, University of Ottawa, May 1992-April 1993

Ontario Graduate Scholarship:
Held at Dept. of Chemistry and Biochemistry, University of Guelph, May 1989-April 1990

University of Guelph Graduate Departmental Scholarship:
Jan. 1988-April 1989

REFERENCES: Available upon request.