THE MOLECULAR GENETICS OF HUMAN ~~GLIOMAS~~ BRAIN TUMOURS

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ABSTRACT

A series of 13 benign and 27 malignant human gliomas was analysed to define the fundamental molecular genetic abnormalities underlying glioma development. Southern analysis of restriction fragment length polymorphisms was used to assess loss of chromosomal material in tumour DNA, an event compatible with dysfunction of putative tumour suppressor genes. Chromosomes 10, 13q and 17p were selected for study on the basis of previously described cytogenetic abnormalities in gliomas. In addition, the integrity of the retinoblastoma gene on chromosome 13q, as well as the putative tumour suppressor gene p53 on chromosome 17p, was assessed. Tumour DNA was also examined for amplification of the epidermal growth factor receptor (EGFR), N-*myc* and c-*erb*B-2 oncogenes.

The results indicated that specific molecular lesions were associated with increasing grades of malignancy. Thus, loss of genetic material on chromosome 17 was present in both benign and malignant gliomas, whereas loss of loci on chromosomes 10 and 13 was seen only in malignant gliomas. A structural abnormality of the retinoblastoma gene coding region was detected in one glioblastoma. Amplification of the EGFR oncogene was present in 5 malignant gliomas. In contrast to other tumours studied, only glioblastomas contained more than one molecular abnormality in the same tumour. The relevance of these findings to our understanding of the molecular mechanisms underlying tumour

development and to their effect on improving the accuracy of tumour diagnosis are discussed.

In a further set of experiments, the NIH3T3 transfection assay was used to reveal the presence of activated oncogenes in the genome of human cell lines thought to be derived from gliomas. One cell line contained an activated oncogene. This oncogene was identified, by oligonucleotide hybridisation to polymerase chain reaction-amplified DNA, as an N-*ras* gene with a codon 61 mutation. However, it was demonstrated by DNA fingerprinting that the supposed human glioma cell line had been previously contaminated with cells of rhabdomyosarcoma origin.

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ABBREVIATIONS USED IN THE TEXT

A -> C	adenosine to cytosine (mutation)
AEV	Avian erythroblastosis virus
a-FGF	acidic fibroblast growth factor
ALL	acute lymphoblastic leukemia
ALV	Avian leukosis virus
A -> T	adenosine to thymidine (mutation)
ATP	adenosine triphosphate
bp	base pairs
BSA	bovine serum albumin
^{0}C	degrees Celsius
$CaCl_2$	calcium chloride
cDNA	complementary DNA
cm	centimetres
CML	chronic myeloid leukaemia
CNS	central nervous system
CO_2	carbon dioxide
c-*onc*	cellular oncogene
cpm	counts per minute
CSF-1	colony stimulating factor 1
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
del	deletion of chromosome
dGTP	deoxyguanosine triphosphate
DMEM	Dulbecco's modified Eagle's medium
DM's	double minute chromosomes
DNA	deoxyribonucleic acid

DNAase	deoxyribonuclease
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
EDTA	ethylenediamine tetra-acetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ENU	ethylnitrosourea
FAP	familial adenomatous polyposis
FCS	foetal calf serum
gal-C	galactocerebroside C
GBM	glioblastoma multiforme
GFAP	glial fibrillary acidic protein
GGF	glial growth factor
G-protein	GTP-binding protein
GTP	guanosine triphosphate
G-TsF	glioma T-cell suppression factor
HBSS	Hank's buffered salts solution
HCl	hydrochloric acid
HPV	human papilloma virus
HSR's	homogeneously staining regions
Kb	kilobase
Kbp	kilobase pair
KD	kilodalton
LN_2	liquid nitrogen
M	molar
MEN 2a	multiple endocrine neoplasia, type 2a
mg	milligrams

$MgCl_2$	magnesium chloride
ml	millilitres
mM	millimolar
mRNA	messenger RNA
NaCl	sodium chloride
NF1	Von Recklinghausen neurofibromatosis
NF2	bilateral acoustic (central) neurofibromatosis
ng	nanogram
nM	nanomolar
O-2A	oligodendrocyte-type 2 astrocyte
OD	optical density
OH	hydroxyl
p	short arm of chromosome
^{32}P-	phosphate 32 radioisotope
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
Ph^1	Philadelphia chromosome
PNK	polynucleotide kinase
PRF's	peptide regulatory factors
q	long arm of chromosome
RB	retinoblastoma (gene/protein)
REF's	rat embryo fibroblasts
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulphate

SSC	standard saline citrate
t	translocation (of chromosome)
TE	tris-EDTA
TGF-a	transforming growth factor alpha
TGF-B	transforming growth factor beta
tris	tris(hydroxymethyl) amino methane
ug	microgram
ul	microlitre
um	micrometre
UV	ultraviolet
VNTR	variable number tandem repeat
v-onc	viral oncogene
v/v	volume/volume
w/v	weight/volume

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CHAPTER 1

INTRODUCTION AND REVIEW OF THE LITERATURE

1.1 INTRODUCTION

A glioma is a neoplasm arising from the neuro-ectodermally derived neuroglial cells of the central nervous system. As a group, gliomas vary widely in their incidence, cellular composition and, most importantly, in the way they affect their host. In the context of this thesis, the generic term glioma will comprise the group of tumours widely referred to as astrocytoma, oligodendroglioma, ependymoma, choroid plexus papilloma, mixed varieties of the aforementioned tumours and glioblastoma multiforme.

Gliomas have certain attributes in common with neoplasias occurring elsewhere in the body, in that they consist of a mass of disorganised tissue formed by the abnormal and inappropriate proliferation of cells. The abnormal cell proliferation is not inhibited by the mechanisms normally controlling cell division and in most cases appears to be irreversible (Anderson, 1985).

In this introduction, the evidence will be reviewed that the underlying abnormality in the development of many types of neoplasm is a stable genetic event. The classification and biology of gliomas will then be discussed. Finally, the rationale for the work carried out in this thesis, consisting of studies on the molecular genetic alterations occurring in gliomas, will be

presented.

1.2 EVIDENCE THAT NEOPLASIA HAS AN UNDERLYING GENETIC CAUSE

1.2.I. SPECIFIC CHROMOSOMAL DEFECTS IN HUMAN TUMOURS

The first observations linking cytogenetic alterations with the development of malignant tumours were reputedly those of Von Hansemann in 1890, who noted the frequent occurrence of nuclear and mitotic irregularities in carcinoma biopsies. In 1914, Boveri formally hypothesised that the chromosomal abnormalities were responsible for causing the malignant proliferation. During the subsequent fifty years, a wide variety of apparently random chromosomal abnormalities were observed in most histological subtypes of malignant tumour. The inability of workers to associate any consistent pattern of chromosome change with a specific tumour subtype led to the belief that such changes were merely epiphenomena with no bearing on the development of neoplasia (Reviewed in Heim and Mitelmann, 1988). See Footnote.

The first consistent chromosomal abnormality in a human cancer was described in 1960, when a small karyotypic marker, the Philadelphia (Ph^1) chromosome, was noted in patients with chronic myeloid leukemia (CML)(Nowell and

Footnote: the word "tumour" will be used largely in a clinical context, for example, when referring to biopsy material removed from a patient, while the word "neoplasm"

will be used in a scientific context to indicate the nature of the pathological process.

Hungerford, 1960). The Ph^1 chromosome was the only chromosomal abnormality present in over 85% of cases of CML, suggesting that it represented a somatic mutation occurring in a haemopoietic stem cell, which was causally related to the development of the leukemia.

The development of chromosome banding techniques in the 1970's, coupled with the ability to maintain primary cultures of solid tumours, allowed fine mapping of the abnormalities to specific chromosome bands. Careful analysis of the accumulated data revealed that, whilst most abnormalities seen in malignant tumours were apparently of a random (or "background") nature, there was an association between certain chromosome changes and the specific tumour subtype (reviewed by Sandberg and Wake, 1981). Mitelman (1984), attempted to distinguish the so-called "primary" chromosome breaks actively involved in malignant tumour development from the "background" breaks by selecting tumours which had only a single structural chromosomal abnormality. He found that only fifty four unique chromosome changes could be identified in at least two representatives of any one specific histologic tumour type. He thus concluded that a limited number of chromosomal regions existed which impinged upon genes essential to the development of neoplasia. Interestingly, many of the

chromosomal changes seen in solid and haemopoietic tumours occur at known chromosomal fragile sites, consistent with the hypothesis that both inherited and constitutive (non-inherited) fragile sites predispose to the formation of these karyotypic changes (Le Beau, 1986).

Examples of specific (primary) chromosome changes occurring in tumours include a deletion of the short arm of chromosome 1 in disseminated neuroblastomas [del 1p (p31p36)]; monosomy of chromosome 22 in 95% of meningiomas; a translocation between the long arms of chromosomes 8 and 14 in over 90% of cases of Burkitt's lymphoma [t(8;14) (8q24.13 and 14q32.33)]; and a deletion of chromosome 13 in retinoblastoma [del(13)(q14)] (Reviewed in Yunis, 1983 and Sandberg et al., 1988).

To summarise, studies on the cytogenetics of haemopoietic and solid tumours revealed the presence of a restricted number of specific chromosomal changes associated with different types of tumour. It was felt that the areas of the genome affected by these changes may harbour genes involved in the neoplastic process.

1.2.II. HEREDITARY NEOPLASIAS

Six percent of patients who suffer from some form of neoplastic disorder have 3 or more close relatives who are similarly afflicted (Ponder, 1987). The members of these "cancer families" possess a predisposition to a single form or a few specific forms of neoplasm. The predisposition

exceeds that expected by chance alone and at a given age, there is an increased risk that a specific tumour will occur, even allowing for possible contributions of environmental factors. The implication is that abnormal genetic material is inherited and is responsible for conferring the increased risk of developing neoplastic disease. Many of these hereditary neoplastic disorders are inherited in a dominant fashion; the reason for their relative scarcity in the population is probably due to the fact that many affected individuals die before reproductive age (Knudson, 1985; Ponder, 1987).

1.2.II.a. Retinoblastoma

Retinoblastoma is a malignant tumour of the eye that affects approximately 5 cases per 100 000 births worldwide (Knudson, 1989). The majority (60%) of cases have disease in only one eye, and are non-hereditary. 40% of cases have a family history of the disease, and patients with the inherited form frequently develop bilateral tumours, as well as multiple tumours in one eye. The risk of developing retinoblastoma in patients who have inherited the propensity is 100 000 times greater than that of the normal population, and 50% of the offspring of patients harbouring the hereditary form develop the disease, consistent with a dominant mode of inheritance (Knudson, 1989).

Studies on retinoblastomas have provided valuable insights into the possible mechanisms of tumour formation

in both these and other hereditary neoplasias. Using classical cytogenetics, a specific deletion of the long arm of chromosome 13 (13q14) was observed in the constitutional (i.e. non-tumour) genome of 5 cases of hereditary retinoblastoma (Knudson, 1976). Molecular genetic investigations showed that the gene esterase D, which also mapped to chromosome 13q14, was closely linked to the inheritance of the familial retinoblastoma trait. Moreover, the esterase D gene was deleted in the constitutional genome of cases of hereditary retinoblastoma which failed to show a cytogenetic deletion of 13q14, suggesting that these cases nonetheless had submicroscopic abnormalities at band 13q14 (Sparkes et al., 1983). Molecular abnormalities of band 13q14 were also demonstrated in the tumour DNA of cases of non-hereditary retinoblastoma, suggesting that this locus was involved in the development of both familial and sporadic retinoblastomas. The molecular abnormality demonstrated in both hereditary and sporadic retinoblastomas was that of loss of heterozygosity (i.e. loss of one of the two parental alleles) at a locus on chromosome 13q14 in the tumour DNA relative to the patient's constitutional DNA (Godbout et al., 1983; Cavenee et al., 1983; Cavenee and Hansen, 1986). The gene at chromosome 13q14 thought to be involved in the genesis of retinoblastomas has now been cloned and has been termed RB, or RB-1 (Friend et al., 1986; Fung et al., 1987). In many cases, an abnormality of chromosome band 13q14 harbouring

the RB gene takes the form of deletion of one entire chromosome 13 (Benedict et al., 1983a). In other tumours, only band 13q14 may be deleted, with the remainder of chromosome 13 being normal; in a subset of tumours, the abnormality is submicroscopic (Balaban-Malenbaum et al., 1981). Therefore loss of function of the RB gene appears to be a common phenomenon in the development of retinoblastomas, and may even prove to be obligatory.

Based on the differing risk of developing retinoblastoma between hereditary and non-hereditary cases, Knudson had hypothesised that two events were necessary to form tumours: in hereditary cases, one of these events was inherited as a DNA abnormality in the germline, and thus present in every cell in the body, and only one further event was required in order to develop a tumour in a somatic cell. In non-hereditary cases, however, both events were required to occur in the same somatic cell, thus explaining the difference in incidence between the inherited and sporadic forms of the disease (Knudson, 1971). Since both inherited and sporadic retinoblastomas appeared to involve abnormalities of the RB gene, it was thought that patients with the hereditary form had inherited one malfunctioning RB allele, and that normal function was maintained by the remaining normal allele. Loss of the remaining normal RB allele in the tumour DNA (as postulated by Cavenee et al., 1983; see above), would then result in malfunction of the gene at this locus. Thus,

although the abnormal RB allele would be inherited as autosomal dominant, the malignant phenotype would remain recessive until a functional abnormality occurred at the remaining (normal) RB allele.

It is possible therefore, that the RB gene acts normally to inhibit cell growth, and that loss of normal RB function results in impaired growth control, contributing to the formation of neoplasia. Therefore, in the context of an evolving neoplasm, the RB protein can be considered to suppress tumour growth and the RB gene is referred to as a "tumour suppressor" gene. As a tumour suppressor gene, the introduction of a wild-type (normal) RB gene into tumour cells in which both RB alleles are abnormal should result in a re-establishment of normal cellular growth control. Experimental evidence now exists for a degree of suppression of the neoplastic phenotype in retinoblastoma cells following re-introduction of a functioning RB gene (Huang et al., 1988), however, this experiment has yet to be successfully repeated by other groups.

In summary, studies on hereditary and sporadic retinoblastomas have resulted in defining a single gene, RB, which appears to be involved tumourigenesis only when both alleles are abnormal. It is possible that the RB gene normally acts as an inhibitor of cell growth, and is therefore a tumour suppressor gene.

1.2.II.b. Neurofibromatosis

The term neurofibromatosis refers to a genetic and clinically heterogeneous group of conditions of which two forms predominate. Both are transmitted as autosomal dominant defects which cause a predisposition to various neural tumours. The predominant form is classical or Von Recklinghausen neurofibromatosis (NF1), which has a frequency of 1 in 3000, of which only 50% of patients have a definite family history, the remainder probably representing new germline mutations. NF1 has a complex clinical presentation, including multiple pigmented skin lesions; hamartomas in the iris ("Lisch nodules"); multiple neurofibromas widely dispersed throughout the body; and an increased risk for other neoplasias, particularly gliomas of the optic nerve, malignant cerebral astrocytomas, glioblastomas, cerebellar astrocytomas, and neurofibrosarcomas. Acoustic neuromas are rarely found in NF1.

The second major form of neurofibromatosis is bilateral acoustic neurofibromatosis, or NF2, and has an occurrence rate of 1 in 100 000. The features of NF2 include bilateral acoustic neuromas (Schwannomas) of the vestibular branch of the eighth cranial nerve, multiple cranial and spinal meningiomas, nerve root neurofibromas and gliomas of the brainstem and spinal cord. Pigmented skin lesions and neurofibromas may occur, but Lisch nodules are absent. Both NF1 and NF2 are complex syndromes which have several features in common, such as the development of

neural tumours including gliomas, (Reviewed in Cotran et al., 1989).

In spite of the similarities between NF1 and NF2, the underlying inherited abnormal gene appears to differ. The genetic locus associated with development of NF1 has been mapped to the long arm of chromosome 17 (17q11.2) (Collins et al., 1989; Menon et al., 1989; Fountain et al., 1989). It is not known how the inherited abnormality might influence the development of a complex disease such as NF1, nor how an abnormality of a postulated single gene can account for the diverse clinical appearances. It may be that abnormalities of several additional, as yet undefined genes, are necessary to bring about each type of lesion.

The defective gene inherited in NF2 is on chromosome 22 (22q11.1-22q13.1), and thus differs from that inherited in NF1 (Seizinger et al., 1986; Rouleau et al., 1987). Loss of heterozygosity of alleles at the NF2 locus were observed in the bilateral acoustic neuromas and meningiomas of NF2 patients, as well as in sporadic (non-NF2) acoustic neuromas and meningiomas (Seizinger et al., 1987). Therefore it appears that abnormalities of a single gene, or a group of closely linked genes on the long arm of chromosome 22, result in the development of both hereditary and non-hereditary meningiomas and acoustic neuromas (Bolger et al., 1985). Interestingly, alterations of chromosome 22 have been reported in sporadic gliomas using molecular genetic techniques (James et al., 1988), as well

as classical cytogenetics (Bigner et al., 1986). The gene at the NF2 locus therefore has similarities with the retinoblastoma gene (described in Section 1.2.II.a) insofar as loss of an allele, implying loss of function (or possibly quantitatively reduced function) of the gene appears to contribute to tumour formation.

In summary, NF1 and NF2 resemble the retinoblastoma model, in that loss of genetic information appears to be associated with tumour development. However, the complex clinical manifestations of NF1 and NF2 suggest that, in addition to the respective candidate genes on chromosomes 17 and 22, other genes may be involved the genesis of the various tumours seen in these disorders. Both inherited (NF2-associated) and sporadic meningiomas and acoustic neuromas harbour an alteration to chromosome 22, suggesting that abnormalities at this locus are involved in the genesis of these specific tumour types.

1.2.II.c. Familial adenomatous polyposis

Familial adenomatous polyposis (FAP) of the large intestine is inherited as an autosomal dominant trait with a high degree of penetrance. Patients develop multiple colorectal adenomas in adolescence and over 90% of affected individuals later develop colorectal adenocarcinoma.

The gene transmitting the FAP trait has been localised to chromosome 5 (5q21-q22) by the demonstration that patients with the inherited syndrome have lost this

particular part of their genome (Bodmer et al., 1987). Loss of chromosome 5q21-22 also occurs in a high proportion of sporadic (non-FAP) cases of colorectal carcinoma (Solomon et al., 1987; Ashton-Rickardt et al., 1989). Loss of sequences on chromosome 5 was detected in 29% of sporadic adenomas and 35% of carcinomas, suggesting that abnormalities of the gene encoding the FAP trait were associated with early events in the progression of colonic epithelial neoplasia (Vogelstein et al., 1988).

The supposed single gene predisposing to FAP is therefore phenomenologically similar to the retinoblastoma gene, in that allelic loss is associated with the development of neoplasia in both the inherited and sporadic forms of the disease (see Section 1.2.II.a, above).

1.2.II.d. Other familial neoplasias

Multiple endocrine neoplasia, type 2a (MEN 2a) is an inherited cancer syndrome characterised by the development of pheochromocytoma, medullary thyroid carcinoma, (tumours both thought to originate from neural crest-derived cells), and hyperplasia or adenoma of the parathyroid glands. The condition is inherited in an autosomal dominant fashion. Molecular linkage analysis has placed the inherited MEN 2a locus on chromosome 10 (10p11.2-q11.2) (Mathew et al., 1987; Nakamura et al., 1989). Pheochromocytomas and medullary thyroid carcinomas occurring in MEN 2a cases also show loss of genetic material on chromosome 1p (1p33-35).

However, the abnormal 1p locus does not appear to be inherited, and the 1p loss might therefore represent a genetic event occurring during tumour progression, rather than an event predisposing the individual to development of the MEN 2a syndrome (Mathew et al., 1987).

Several other inherited and sporadic neoplasias of neuroectodermal origin show loss of regions on chromosome 1p. The gene for the hereditary cutaneous malignant melanoma-dysplastic naevus syndrome has been mapped to chromosome band 1p36 (Bale et al., 1989). Studies on sporadic malignant melanomas have similarly demonstrated loss of 1p36, as well as loss of chromosome band 1p22 (Dracopoli et al., 1989). In neuroblastomas, loss of chromosome 1p36.1-36.3 has been observed in a high proportion of cases in one series (Fong et al., 1989). Therefore, it appears that the short arm of chromosome 1 may harbour several loci which are involved in the progression of neuroectodermal tumours.

Two other inherited neoplasia syndromes in which neuroectodermal tumours occur are Turcot syndrome, consisting of familial polyposis associated with malignant gliomas or medulloblastomas (Turcot et al., 1959; Baughman et al., 1969); and the Li-Fraumeni syndrome, in which soft tissue sarcoma, breast cancer and several other tumour types, including gliomas, occur in families (Li and Fraumeni, 1982; Li, 1988).

Inherited cancer syndromes are not confined to

humans, nor indeed to mammals. Hybrids between certain species of the bony fish Xiphophorus develop malignant melanoma with a high degree of predictability. An autosomal gene named Diff has been isolated which suppresses melanoma formation in Xiphophorus by inducing terminal differentiation of melanocytes to melanophores, with subsequent cessation of cell division. The Diff gene functions by overriding the effects of another gene which promotes melanocyte division, known as Sp/Sd. Structural abnormalities or loss from the cell of the Diff gene are associated with the development of melanomas, in a manner analogous to the effect of loss of function of the RB gene (see Section 1.2.II.a). Thus the Diff gene product is an example of a tumour suppressor protein acting in trans (i.e. acting on a gene not on the same chromosome), to inhibit the function of a growth promoting gene (Schwab, 1989).

Moving down the evolutionary tree, fruitflies of the species Drosophila melanogaster develop inherited malignant neuroblastomas and tumours of the imaginal disc (Gateff, 1978). These tumours appear to arise when both alleles of the gene l(2)gl are inactivated. It is thought that the l(2)gl gene is normally involved in the development of the early embryo. Therefore this abnormality may represent an example of mechanisms which normally control embryonic cell division and differentiation being altered in such a way as to contribute to the development of neoplasia.

Re-introduction of the l(2)gl gene into l(2)gl-deficient larvae results in normal development without neural tumour formation, analogous to the tumour suppressor effect obtained when the RB gene is re-introduced into retinoblastoma cells (Mechler et al., 1989).

In summary, many forms of hereditary neoplasia exist, and two consistent features emerge in those in which detailed molecular studies have been performed. Firstly, the same genetic locus appears to be involved in the genesis of both the inherited and sporadic forms of a particular histologic type of tumour. Secondly, the development of neoplasia seems to occur as a result of loss of function of genetic material at a locus. It is believed that such loci might harbour tumour suppressor genes. In familial cases, every cell carries one abnormal inherited allele, with functional loss of the remaining allele occurring at a later stage in a cell destined to become neoplastic. In non-hereditary cases, two events are required at the same genetic locus in a single somatic cell in order to produce loss of gene function.

1.2.III. VIRAL AND CELLULAR ONCOGENES

1.2.III.a. Introduction

In addition to tumour suppressor genes, a further category of genes may be invoved in carcinogenesis: these are the dominantly-acting oncogenes, whose existence was

first established following studies on tumour viruses.

The correlation between viral infection in animals and the development of certain forms of neoplasia has been recognised for over seventy years. The molecular mechanism underlying RNA and DNA viral tumourigenesis in animals has recently been elucidated, and appears to be due to the action of specific viral genes. Knowledge of these viral "oncogenes" has advanced our understanding of the genetic mechanisms underlying human neoplasia.

1.2.III.b. RNA tumour viruses

Certain type C RNA oncoviruses (or oncornaviruses) cause a variety of tumour types in animals (For reviews, see: Weiss et al., 1982; Bishop, 1983; Bishop, 1985; Wyke, 1981). These retroviruses have in common a stage in their life cycle at which the viral RNA genome is transcribed into DNA by means of the enzyme reverse transcriptase. The viral DNA genes are subsequently inserted into the host cell genome. Many oncogenic retroviruses were known to have the ability to rapidly induce tumours in the host, which suggested that the tumours resulted from the effects of a carcinogenic viral gene (or "oncogene"). Several different type C retroviral oncogenes have now been defined and those listed here are examples which are relevant to parts of the discussion to follow.

The first retroviral oncoprotein to be identified was the product of the v-src oncogene of the Rous sarcoma

virus, which induces fibrosarcomas in chickens. The v-*src* oncoprotein is localised to the cell membrane and functions as a tyrosine kinase, i.e. it phosphorylates tyrosine residues on proteins (Bishop, 1982). Prior to the identification of v-*src*, all the known protein kinases were serine and threonine kinases, thus v-*src* was the first tyrosine kinase to be identified, although it was not clear how it induced tumours.

V-*abl*, the transforming oncoprotein of the Abelson murine leukemia virus; v-*fms*, the transforming oncoprotein of the McDonough strain of feline sarcoma virus; and v-*erb*B, one of the two transforming proteins of the avian erythroblastosis virus, are all cell membrane-associated tyrosine kinases, similar to v-*src* (Graf and Beug, 1983).

The transforming oncoproteins of the Harvey and Kirsten murine sarcoma viruses are known as v-Ha-*ras* and v-Ki-*ras*, respectively. V-Ha-*ras* and v-Ki-*ras* are attached to the plasma membrane and bind GTP, a property shared by the "G proteins" which are involved in signal transduction following stimulation of the B-adrenergic receptor. (Michell, 1989).

The MC29 virus causes myelocytomas, sarcomas, and hepatic and renal carcinomas in chickens. The oncogene product, v-*myc*, is found in the nucleus, as are the v-*fos* oncoproteins of the FBR and FBJ murine osteosarcoma viruses, and the v-*jun* oncoprotein of the avian sarcoma virus 17 (Maki *et al.*, 1987).

The simian sarcoma virus induces fibrosarcomas in wooley monkeys, and manufactures a secreted oncoprotein, known as v-*sis*.

1.2.III.c. Cellular oncogenes homologous to retroviral oncogenes

Despite the fact that numerous retroviral oncogenes had been isolated, it was not clear that their discovery was directly relevant to tumourigenesis in humans. Huebner and Todaro (1969), had postulated that the mammalian genome contained the dormant genomes of type-C retroviruses integrated into the host cell DNA, and that activation of the dormant retroviral genes by carcinogens or irradiation would result in the formation of tumours. However, when cellular DNA sequences homologous (but not identical) to the retroviral v-*src* oncogene were found in the human genome, investigators anticipated that the viral oncogenes might merely be analogues of a class of cellular genes involved in the development of human neoplasia (Spector, 1978). Soon, human homologues of many other retroviral oncogenes were described and thus it was not necessary to invoke the re-activation of retroviral oncogenes as being responsible for tumourigenesis in humans.

The biochemical functions and subcellular localisation of the human oncoproteins were found to be similar to their viral counterparts (Weiss *et al.*, 1982; Bishop, 1983; Marshall, 1982). For example, the c-*erb*B

cellular oncoprotein resembled the v-*erb*B viral protein in exhibiting tyrosine kinase activity, as well as being localised to the cell membrane (Jansson *et al.*, 1983). The oncoproteins coded by the cellular genes c-Ha-*ras*1 and c-Ki-*ras*2 are homologous to the viral oncoproteins v-Ha-*ras* and v-Ki-*ras*, respectively, and exhibit a similar ability to bind GTP. The c-*myc* cellular oncoprotein is localised to the nucleus, like it's viral counterpart v-*myc* (Colby *et al.*, 1983; Bishop, 1985; Marshall, 1982; Weinberg, 1985). Cellular homologues of the viral oncogenes were also found in cells of the natural host species of the virus, as well as in human cells. For example, both chickens and humans were shown to have a cellular c-*erb*B protein.

Therefore, the theories were proposed that i) the viral oncogenes had been acquired by the virus from the host cell by a process known as transduction and ii) the presence of cellular homologues of viral oncogenes in species separated by vast reaches of evolutionary time (such as *ras* genes in humans and yeast, and c-*erb*B genes in humans and *Drosophila*), suggested that these oncoproteins were indispensible to the cell and that, when transduced by viruses, the normal function was altered to result in tumour formation in the viral host.

1.2.III.d Certain oncogenes encode proteins involved in the control of cell growth

The first indication of how cellular and viral

oncoproteins might alter cell growth to cause neoplasia came with the discovery that v-*sis*, the transforming protein of the simian sarcoma virus (see 1.2.IV.b ix, above), was structurally related to platelet-derived growth factor (PDGF) (Waterfield *et al.*, 1983). Thus, a virus which induced the formation of fibrosarcomas, produced an oncoprotein which was identical to PDGF, the major serum mitogen for mesenchymal cells (the cells which presumably give rise to fibrosarcomas). Subsequently, it was demonstrated that the v-*erb*B oncogene of the avian erythroblastosis virus (AEV), had close structural similarity to part of the human epidermal growth factor receptor (EGFR) (Downward *et al.*, 1984). The implication of the EGFR-v-*erb*B similarity was that the AEV had transduced part of the chicken c-*erb*B cellular oncogene which then functioned as a tumourigenic retroviral oncogene, v-*erb*B. Neil *et al.*, (1984), confirmed that retroviruses, such as the feline leukaemia virus, are indeed capable of transducing mammalian cellular genes. A third discovery showed that the cellular oncoprotein c-*fms*, (the homologue of the transforming oncoprotein v-*fms* of the McDonough strain of feline sarcoma virus), was in fact the cell surface receptor for the monocyte growth factor, colony stimulating factor-1 (CSF-1) (Sherr *et al.*, 1985).

Growth factors such as PDGF, EGF and CSF-1 have a mitogenic effect on certain cells. On binding of the growth factor, specific cell-surface receptors cause a series of

chemical signals to be transmitted to the cell nucleus, ultimately resulting in mitosis. In other words, the growth factors and their specific receptors are involved in the process of cellular growth control. The viral oncoproteins which have similarity to a growth factor and to two growth factor receptors may thus induce tumours by interfering with the normal cellular growth control pathways.

It is now believed that the proteins of other cellular oncogenes may also be involved in the process of normal cellular growth control. Thus the cell membrane associated tyrosine kinases c-abl and c-src, are thought to phosphorylate protein substrates and thereby generate a cascade of signals eminating from the cell membrane. (Hunter, 1987; Yarden and Ullrich, 1988; Ellis et al., 1990). The products of the c-Ha-ras1 and c-Ki-ras2 cellular oncogenes are known to bind GTP, and are thought to facilitate signal transduction from molecules in the cell membrane to the cell interior, in a manner analogous to the G-proteins associated with the B-adrenergic receptor (Barbacid, 1987; McCormick, 1989; Michell, 1989). The cellular oncoproteins c-myc, c-fos and c-jun are localised to the nucleus and are thought to play a role in the control of cell division, since induction of mitosis in quiescent cells by growth factors is accompanied by increased production of all three oncoproteins (Thompson et al., 1985; Kruijer et al., 1984; Quantin and Breathnach, 1988; Kaczmarek, 1986). The c-jun oncoprotein appears to be

identical to the human DNA transcription factor AP-1 and is thought to be involved in the transcription of specific genes following induction of mitosis by growth factors (Bohmann et al., 1987; Lamph et al., 1988). The binding of c-jun to transcriptional regulatory sites on DNA is further enhanced by the formation of a complex with the c-fos protein, thus providing an example of a molecular interaction between two oncoproteins (Vogt and Bos, 1989; Mitchell and Tjian, 1989).

Cellular oncoproteins are thought to be involved in the regulation of cell growth in both the developing embryo as well as in the adult organism. Thus, the c-myc gene is expressed in a stage- and cell-type- specific fashion in epithelial cells of late first trimester human embryos (Pfeifer-Ohlsson et al., 1985). Similarly, the c-fos gene is expressed transiently in promyelocytic cells which have been induced to differentiate into macrophages (Muller et al., 1985).

Several cellular oncogenes have been identified by virtue of their homology to other known cellular oncogenes. These include c-erbB-2 (also known as neu or HER-2), which is similar to the c-erbB oncogene (Semba et al., 1985; King et al., 1985); N-ras, similar to the c-Ha-ras1 and c-Ki-ras2 genes (Hall et al., 1983; Taparowsky et al., 1983); and N-myc, similar to the c-myc oncogene (Schwab et al., 1983).

Despite their resemblance to cellular oncoproteins,

the viral oncoproteins do differ structurally from the proteins they have transduced; this altered structure may underlie the ability of these proteins to induce neoplasia. The cellular c-erbB/EGF receptor oncoprotein consists of three regions: an extracellular EGF binding domain, a short transmembrane region and an intracellular tyrosine kinase domain, the proximal portion of which phosphorylates external protein substrates, whilst the C-terminal portion acts as an autophosphorylation site which phosphorylates tyrosine residues on the EGFR protein itself and which may regulate the function of the receptor (Ullrich et al., 1984). The viral counterpart of the EGFR, v-erbB, has a truncated external domain which is unable to bind EGF and does not have an autophosphorylation site (Downward et al., 1984). The viral (v-erbB) protein therefore resembles the cellular EGFR protein in that the main tyrosine kinase domain is intact, but the regulatory components are missing. As a result of this altered structure, the v-erbB protein appears to have a constitutive tyrosine kinase activity, which is switched on independent of EGF binding (Kris et al, 1985). The ability to phosphorylate substrates independent of binding of the EGF ligand may be the means by which the viral oncoprotein subverts the normal mechanisms of cell growth control to induce neoplasia (Downward et al., 1984; Yarden and Ullrich, 1988).

The MC29 virus v-myc oncoprotein differs from its normal cellular counterpart, c-myc, in that only two exons

of the three exons of c-*myc* have been transduced and these exons are linked to the gene encoding the defective viral *gag* protein resulting in the production of a *gag-myc* fusion protein. The ability of the *gag-myc* viral oncoprotein to transform cells may lie in the fact that the normal regulatory sequences are lost in the viral context (Colby *et al.*, 1983; Cullen *et al.*, 1984).

A further mechanism by which the *myc* gene may be involved in tumourigenesis is illustrated in the chicken B-cell lymphomas induced by the avian leukosis virus (ALV). Unlike the retroviruses discussed so far, the ALV does not transform by means of a viral oncogene. Rather, it integrates into the host genome adjacent to the host cell c-*myc* gene, and induces a high rate of transcription of the c-*myc* gene by means of the powerful transcription enhancing effects of the viral long terminal repeat unit. The resultant inappropriate increase in the level of expression of the structurally normal c-*myc* oncogene in the cell contributes to tumour formation (Neel *et al.*, 1981; Payne *et al.*, 1981).

To summarise:

i) Retroviral oncogenes are derived by transduction of cellular oncogenes.

ii) Cellular oncogenes which are homologous to retroviral oncogenes, as well as those which are not transduced by retroviruses, appear to function in the control of normal cell growth and differentiation.

Therefore perturbations of the normal growth control pathways could result in the formation of tumours.

iii) Functional abnormalities which result in tumourigenesis include structural alterations of the viral oncoprotein, as well as overexpression of a normal cellular oncoprotein.

iv) Viral oncogenes act in a dominant fashion i.e. a single viral oncogene is capable of inducing transformation when introduced into immortalised cell lines, in spite of the presence in the cell of two normal cellular alleles which are homologues of the viral oncogene.

1.2.III.e. DNA tumour viruses

For many years, DNA tumour viruses have been employed to induce experimental brain tumours in several species of neonatal animal. The viruses have been members of two main groups: The papovaviruses (SV40, BK and JC), the human adenoviruses (types 5 and 12) and simian adenovirus type 7 (Sharp, 1980). Examples of neural tumours induced by these DNA viruses include medulloblastomas and gliomas induced in hamsters by the JC virus; ependymomas induced in mice by the SV40 virus; and neuroblastomas induced in rats by human adenovirus type 12 (Tooze, 1980). A subgroup of the papovaviruses, the human papillomaviruses (HPV), are implicated in the development of a wide variety of human epithelial tumours, ranging from benign cutaneous

papillomas (also known as viral warts), to malignant tumours of the uterine cervix (Zur Hausen, 1987).

1.2.III.f. Interaction of DNA tumour virus oncogenes with cellular genes

Unlike the oncogenic retroviruses (discussed in Section 1.2.IV.b-d, above), the papova- and adenoviruses do not contain a transforming oncoprotein with known similarity to cellular oncogene products. Rather, certain of the transforming oncoproteins of the papova and adenoviruses may interfere with the normal function of putative tumour suppressor genes, such as the retinoblastoma gene RB. E1A, the essential transforming protein of the adenovirus, has been shown to form a stable complex with the RB protein (Whyte et al., 1988). Similarly, the transforming protein of the SV40 virus, the large T antigen, complexes with the RB protein (DeCaprio et al., 1988), as does the E7 oncoprotein of the human papilloma virus type-16 (Dyson et al., 1989a). Therefore it is possible that the papova and adenovirus transforming oncoproteins contribute towards tumour formation by binding to the RB protein and interfering with its normal growth inhibitory function.

1.3. MOLECULAR ABNORMALITIES OCCURRING IN HUMAN TUMOURS

1.3.I. INTRODUCTION

The preceding section described oncogene

abnormalities which may be involved in the genesis of animal tumours. The possible contribution of cellular oncogenes to human tumour development was indicated by the demonstration that mRNA transcripts of cellular oncogenes were present in human tumour cell lines (Eva et al., 1982), as well as in solid human tumour samples (Slamon et al., 1984). This section will describe selected molecular abnormalities of oncogenes and putative tumour suppressor genes found in non-glial human tumours and, where applicable, their associated cytogenetic changes. The mechanisms underlying such altered gene function will be discussed, as will the possible effect of these abnormalities on the development of clinically-important tumours. (Reviewed in: Marshall, 1982; Weinberg, 1983; Nishimura and Sekiya, 1987; Seemayer and Cavenee, 1989). Knowledge of the causes and effects of such abnormalities on the development of non-glial tumours may serve to enhance our understanding of similar molecular lesions which may be found in gliomas.

1.3.II. MOLECULAR ALTERATIONS UNDERLYING ABNORMAL ONCOGENE FUNCTION IN HUMAN TUMOURS

1.3.II.a Overexpression of a structurally normal oncoprotein

Squamous cell carcinomas of the lung, head and neck, skin, vulva and cervix were observed to express between 2 and 10 times the amount of EGF receptor, when compared to

normal squamous tissue (Ozanne et al., 1985; Gullick et al., 1986). It was subsequently found that the EGFR/c-erbB gene was frequently present in multiple copy numbers in the genome of the squamous carcinoma cells, a phenomenon known as gene amplification. The gene amplification correlated with, and could account for, the increase in c-erbB mRNA and protein (Merlino et al., 1984; Ullrich et al., 1984). The c-erbB gene was subsequently mapped to chromosome 7 (Spurr et al., 1984). In many cases, amplification involves only the c-erbB gene and no other loci on chromosome 7, and thus appears to be a specific effect involving only the oncogene. In other tumours, increased copy number of c-erbB/EGFR results from the presence of numerical alterations of chromosome 7, such as duplication. In certain cases where there is no increased copy number of the c-erbB gene, an elevated level of the specific mRNA is associated with structural alterations of chromosome 7, such as translocations, deletions and inversions. These cytogenetic alterations may serve to alter transcription of the c-erbB gene, by causing abnormal functioning of the gene promotor (Korc et al., 1986). It is possible that the presence of an increased copy number of the c-erbB gene product, the EGF receptor, may impart a growth advantage to the cell, thus contributing towards tumour development.

A further example of gene amplification is found in solid neuroblastomas and neuroblastoma cell lines which contain cytogenetic abnormalities known as double minute

chromosomes (DM's) and homogeneously staining regions (HSR's) (Reviewed in Wolman and Henderson, 1989). DM's and HSR's had previously been shown to be microscopically visible structures formed as a result of amplification of c-myc genes in human neuroendocrine colonic carcinoma cells (Alitalo et al., 1983). DM's and HSR's also represent the site of amplification of another member of the myc family of oncogenes known as N-myc (Schwab et al., 1983). It is thought that N-myc gene amplification may result in the production of elevated levels of the N-myc nuclear oncoprotein, and thus contributes to malignant progression of neuroblastomas (Schwab et al., 1984).

Oncogene overexpression without underlying gene amplification is seen in the elevated levels of expression of the c-myc nuclear oncoprotein occurring in many Burkitt lymphomas. The elevated c-myc expression can be explained by the chromosomal translocations seen in 100% of Burkitt lymphomas. In 80% of cases, band 8q24, the site of the c-myc gene, is translocated to chromosome 14 at band 14q32, the site of the immunoglobulin heavy chain locus. In the remaining 20% of Burkitt lymphomas, the translocations juxtapose the immunoglobulin light chain loci (on chromosomes 22 and 2) and the c-myc gene. In all these translocations, c-myc gene transcription is abnormally elevated in the B-lymphocyte tumour cells. Elevated levels of the c-myc oncoprotein are produced, which may contribute to the development of the tumours (Croce, 1987).

Interestingly, the juxtaposition of the immunoglobulin and c-*myc* genes appears to be an undesirable byproduct of the effects of the enzyme V-D-J recombinase, which normally functions during the rearrangement of the immunoglobulin genes during B-cell ontogeny (Boehm *et al.*, 1989).

1.3.II.b Expression of a structurally abnormal oncoprotein

Identification of functionally abnormal dominant oncogenes which are not associated with cytogenetic abnormalities or increased gene copy number is made possible by using the DNA transfection technique. DNA transfection involves the introduction of naked tumour cell DNA into an indicator cell, usually NIH3T3 mouse fibroblasts (Graham and Van der Eb, 1973; Shih *et al.*, 1981). The presence in the tumour DNA of certain classes of functionally abnormal ("activated") oncogenes will cause a phenotypic alteration of the indicator cells, resulting in the formation of "transformed" foci of cells.

When DNA from a human bladder carcinoma cell line was transfected into NIH3T3 cells, transformation revealed the presence of a cellular transforming oncogene c-Ha-*ras*1, related to the viral oncogene v-Ha-*ras* (Der *et al.*, 1982; Parada *et al.*, 1982). Further analysis revealed that the only difference between the normal cellular c-Ha-*ras*1 gene and the transforming oncogene was a single nucleotide change ("point mutation") where thymidine was substituted

for guanosine. This nucleotide substitution resulted in the amino acid valine occurring at residue number 12 in the transforming c-Ha-*ras*1 oncoprotein, rather than glycine which occurs at the corresponding position in the normal cellular oncoprotein (Reddy *et al.*, 1982; Tabin *et al.*, 1982). It is thought that the position 12 amino acid functions as part of the GTP binding site, and the presence of valine instead of glycine at residue 12 in the transforming c-Ha-*ras*1 oncoprotein might interfere with the ability of the protein to hydrolyse and release bound GTP. The transforming oncoprotein would therefore remain in an activated state and continue to function abnormally (Weinberg, 1983).

The importance of the position 12 amino acid in the function of the *ras* proteins is reinforced by the finding that the v-Ha-*ras* and v-Ki-*ras* viral oncoproteins differ from their normal cellular counterparts by a similar single amino acid change at position 12. Amino acids 13 and 61, in common with amino acid 12, are involved in GTP binding, and therefore substitutions at these loci may also contribute to cell transformation by interfering with GTP release (Bos, 1989). Point mutations in the cellular Ha-, Ki- and N-*ras* genes causing amino acid substitutions at positions 12, 13 and 61 are found in most types of human tumour examined, with an overall incidence of 5-10%. Certain types of tumour possess a high incidence of *ras* mutations, such as pancreatic adenocarcinomas in which over

80% show abnormalities of c-Ki-*ras*2. Abnormal *ras* family oncogene function can also occur by overexpression of the normal protein and c-Ki-*ras*2 and c-Ha-*ras*1 amplification (without structural alterations of the gene) has been documented in human bladder tumours (Nishimura and Sekiya, 1987; Chang *et al.*, 1982).

The association between cytogenetic alterations and abnormal cellular oncogene function is further illustrated by the example of the Philadelphia chromosome (Ph^1) and the c-*abl* oncogene. Ph^1 chromosome was first noted as a chromosome fragment seen in the cells of 90% of cases of chronic myeloid leukemia (CML)(see Section 1.2.I.; Nowell and Hungerford, 1960). Analysis showed that the Ph^1 chromomsome was in fact a shortened chromosome 22 which had undergone a reciprocal translocation with chromosome 9. The translocation resulted in the relocation of the c-*abl* cellular oncogene on chromosome 9q to lie within a 5.6Kb region on chromosome 22 known as the breakpoint cluster region, or *bcr*. In patients harbouring the Ph1 translocation, an abnormal 8Kb *bcr-abl* mRNA transcript is observed, which is larger than the normal c-*abl* transcripts of 6 and 7Kb. In addition, a 210KD *bcr-abl* fusion protein is produced from the transcript, and this large fusion protein has enhanced tyrosine kinase activity, when compared to the normal 145KD c-*abl* protein. The abnormal tyrosine kinase activity in the fusion protein may contribute to cellular transformation, and the development

of CML (Shtivelman et al., 1985; Stam et al., 1987). Additional evidence that bcr-abl fusion may contribute to tumourigenesis is provided by the finding that the identical bcr-abl juxtaposition occurs at the molecular level even in Ph^1 chromosome negative CML (Dreazen et al., 1988). Furthermore, Ph^1 positive cases of acute lymphocytic leukemia (ALL) also produce a novel bcr-abl protein of 190KD in size, suggesting a role for the fusion protein in tumours other than CML (Fainstein, et al., 1987).

1.3.III LOSS OF GENETIC INFORMATION IN HUMAN TUMOURS

The phenomenon of loss of genetic material being associated with the development of certain familial neoplasias has been described in Section 1.2.II above.

A mechanism analogous to that in retinoblastoma involving dysfunction of both alleles of a tumour suppressor gene may operate in the development of Wilm's tumour. Like retinoblastoma, Wilm's tumour occurs in both hereditary and sporadic forms. A putative tumour suppressor gene locus at chromosome 11p13 is implicated in the development of Wilm's tumour, and both cytogenetic alterations as well as submicroscopic molecular abnormalities can be indicative of pathological changes at this locus (Heim and Mitelman, 1988; Glaser et al., 1989). Further evidence that chromosome 11p13 harbours a tumour suppressor gene is provided by the knowledge that introduction of an intact chromosome 11 by microcell

transfer into a Wilm's tumour cell line results in suppression of tumourigenicity (Weissman et al., 1987).

It is probable that loss of function of putative tumour suppressor loci plays a part in the development of many other types of human familial and sporadic tumours. At present, many of these loci are poorly defined, and in some cases, only the chromosome harbouring a putative tumour suppressor locus is known. However, since the demonstration that fusion of malignant cells with normal cells resulted in loss of the malignant phenotype (Harris et al., 1969), it has been demonstrated that chromosomes 1, 2, 9, 10, 11, 13, and 17 may harbour genes whose products inhibit cell growth (Klinger, 1982; Harris, 1988). Studies on other types of familial and sporadic tumours indicate that, in addition to the above chromosomes, chromosomes 3, 18 and 22 might also carry tumour suppressor genes (Reviewed in Ponder, 1989).

In the familial retinoblastoma diathesis (see Section 1.2.II.a, above), loss of function of both alleles coding for the RB gene is required for tumour formation. As mentioned in Section 1.2.III.f, the transforming oncoproteins of several different DNA tumour viruses appear to complex with the RB protein. Thus the normal function of RB in the cell is thought to be impaired, even though the RB gene and protein themselves do not harbour any abnormalities. The ultimate effect of the viral oncogene binding to RB might be similar to the loss of

function resulting from RB gene deletion: that is, a reduction in the level of the normal RB protein in the cell.

1.3.IV. SUMMARY: ABNORMALITIES OF TWO CLASSES OF GENES OCCUR IN HUMAN TUMOURS.

Functional abnormalities of oncogenes and tumour suppressor genes are thought to result in altered cell growth control, one of the fundamental properties of tumours (Cooper & Whyte, 1989). Furthermore, the presence in the same cell of two or more abnormal oncogenes from different functional groups (e.g. the nuclear oncoprotein v-*myc* and the activated cell membrane-associated oncoprotein *ras*), may have a cumulative effect, resulting in the evolution of a more malignant phenotype (Reviewed in Weinberg, 1989). Nuclear oncogenes such as v-*myc* confer the property of immortalisation (i.e. an ability to divide indefinitely) upon primary cells such as rat embryo fibroblasts, but the immortalised cells are not tumourigenic. Cells bearing an activated nuclear oncogene only become tumourigenic following the additional introduction of an activated cytoplasmic oncogene (such as a *ras* family gene), suggesting that at least 2 oncogene-related steps are required to make primary cells capable of forming tumours (Land *et al.*, 1983).

In certain experimental systems, interfering with the normal function of a tumour suppressor gene product can

have the same functional effect on cell transformation as the presence of an abnormal nuclear oncoprotein. Similarly, abnormalities of both oncogenes and tumour suppressor genes occurring in the same cell may result in increased tumourigenicity, and this cumulative effect may be operative in the process of progression of a tumour from a more benign to a more malignant phenotype (Klein and Klein, 1985; Weinberg, 1989).

The view that neoplasia might occur as a result of abnormalities of growth control can be further understood when it is appreciated that cell division frequently ceases when cells differentiate fully. It has been hypothesised that abnormalities of oncogenes and tumour suppressor genes induce a maintainance of cell division associated with a block in cell differentiation pathways. Re-introduction of a normal chromosome (for example chromosome 11 in the case of the Wilm's tumour cell line mentioned above), re-establishes a differentiation pathway, leading to cell growth arrest. Further studies involving chromosome re-introduction have raised the possibility that the newly introduced gene or genes which lead to the re-establishment of a differentiation pathway may differ from the oncogenes or tumour suppressor genes whose abnormalities initially led to the differentiation block (Sachs, 1989).

Abnormalities of oncogenes and putative tumour suppressor genes occur consistently in certain human

tumours, both at the molecular and cytogenetic levels, suggesting that many of these changes are non-random and thus reflect genetic lesions which may contribute to tumour formation. It would appear from the evidence that abnormalities of oncogenes and tumour suppressor genes may underlie the formation of many different types of human tumour.

1.3.V. THE CONTRIBUTION OF NON-NEOPLASTIC CELLS TO TUMOUR DEVELOPMENT: AUTOCRINE AND PARACRINE EFFECTS AND ANGIOGENESIS.

Neoplastic cells bearing a molecular genetic abnormality cannot form a clinically significant tumour without an adequate blood supply. Many different types of tumour cells secrete peptides which promote the proliferation of endothelial cells. Endothelial cells will ultimately form new capillaries and supply the growing tumour cell mass with blood. Examples of angiogenic factors secreted by cultured tumour cells include acidic and basic fibroblast growth factor, platelet-derived growth factor, angiogenin, and transforming growth factors alpha and beta (Reviewed in Folkman and Klagsbrun, 1987). One study has demonstrated that a minority of oncogene-immortalised cells are able to induce angiogenesis when the cells are hyperplastic, prior to their having formed tumours. This implies that oncogenic transformation causes the release of angiogenic factors in a subset of the transformed cells,

presumably by altering angiogenic factor gene transcription in the transformed cells. Therefore it has been suggested that, despite the presence in all of the immortalised cells of the same molecular lesion, only a subset are capable of producing angiogenic factors and therefore have the potential to form clinically significant tumours (Folkman et al., 1989). When viewed histologically, most tumours consist of a complex admixture of tumour cells, vascular elements, stromal cells and inflammatory cells. It is now believed that these various component cells are all capable of producing peptide regulatory factors (PRF's) which may stimulate or inhibit growth of other neoplastic and non-neoplastic cells comprising the tumour mass. The ability to influence the growth of associated cells at close range is termed a paracrine effect, while the ability of a particular cell type to influence its own growth is known as an autocrine effect.

A particular secreted PRF can have both autocrine and paracrine effects in the intimate tumour environment. For example, transforming growth factor beta (TGF-B) inhibits the growth of normal and premalignant keratinocytes, but stimulates endothelial cell and fibroblast proliferation. Pre-malignant keratinocytes harbouring an oncogenic mutated ras gene secrete TGF-B and are sensitive to its growth inhibitory effects. The development of malignancy results in the transformed keratinocytes losing their ability to be inhibited by TGF-B, but as they continue to produce TGF-B,

angiogenesis and stromal fibroblast proliferation are stimulated in the developing malignant tumour (Sporn, 1989; Balmain, 1989; Reiss and Sartorelli, 1987).

Autocrine and paracrine effects of PRF's may intersect with molecular genetic abnormalities in tumour cells, as in the case of amplification of the EGF receptor. Normal keratinocytes produce EGFR's and are responsive to the mitogenic effects of EGF. In squamous cell carcinomas, a malignant tumour derived from keratinocyte precursors, the EGFR is frequently amplified and overexpressed. Thus a cell bearing an increased number of intact EGF receptors on its surface might show increased responsiveness to EGF, a mitogen normally present in serum. In addition, the EGFR also binds transforming growth factor alpha (TGF-a), a PRF secreted by many transformed cells (Stoscheck and King, 1986).

Therefore, a highly complex ecosystem exists in a tumour, in which the molecular genetic abnormalities present in tumour cells are merely one determinant of the biology of the tumour in the host. The PRF's thus constitute epigenetic determinants of tumour cell growth, but the type and amount of PRF secreted, and the response of the tumour cell to a PRF may in turn be influenced by genetic abnormalities (Sporn and Roberts, 1985 and 1988; Rozengurt, 1983; Scott Goustin et al., 1986).

1.3.VI USEFULNESS OF MOLECULAR GENETIC STUDIES IN

DIAGNOSIS AND PROGNOSIS

Molecular genetic studies have enhanced our understanding of tumour biology and improved our capacity for accurate diagnosis, and possibly prognosis (Reviewed in Cline, 1989).

1.3.VI.a. Molecular techniques and tumour diagnosis

The creation of abnormal bcr-abl fusion genes and proteins in CML and ALL have been described above (Section 1.3.II.b). Molecular techniques are capable of identifying the presence of the critical bcr-abl fusion genes and mRNA transcripts, even in patients without a cytogenetically visible Ph^1 chromosome. In addition to being much less costly and simpler to perform than cytogenetic techniques, when coupled with specific DNA amplification, the molecular techniques can detect the neoplasia-associated bcr-abl abnormality in as few as 1 cell in 10^5, thereby enhancing the capacity for detection of residual disease following therapy (Morgan et al., 1989).

The role of abnormal immunoglobulin heavy and light chain gene rearrangements causing transcriptional deregulation of the c-myc gene in Burkitt lymphomas has been described above (Section 1.3.II.a). Rearrangements of the T-cell receptor gene may be implicated in the genesis of T-cell lymphomas in an analogous manner (Baer et al., 1987; Kimura et al., 1989). DNA-based molecular techniques capable of detecting oncogene and immunoglobulin and T-cell

receptor gene rearrangements are useful in studying tumour cell lineage, in establishing the stage of differentiation of tumour cells, in establishing whether a lymphocytic proliferation is clonal (and therefore neoplastic rather than reactive) and in detecting residual neoplastic disease following therapy (Cline, 1989).

Molecular techniques have contributed to our understanding of tumour biology in the cases of Ewing's sarcoma and peripheral neuroepithelioma, two childhood tumours formerly thought to be histogenetically unrelated. Recent work has demonstrated that they both share the same cytogenetic rearrangement, a translocation of chromosomes 11 and 22 [t(11;22)(q24;q12)] (Whang-Peng et al., 1984). In addition, both tumours have identical patterns of oncogene mRNA expression (McKeon et al., 1988). This suggests that both Ewings sarcoma and peripheral neuroepithelioma may arise from a common stem cell, or that the two types of neoplasm may share a common molecular basis for malignant transformation. It is now believed that Ewing's sarcoma is a less well differentiated form of peripheral neuroepithelioma. Therefore, molecular studies have caused us to revise our assumptions about the histogenesis of these two tumour types and has resulted in certain centres now treating both types of tumour identically. However, use of the identical treatment protocol has indicated that neuroepitheliomas may be more treatment resistant than Ewing's sarcoma (Vecchio et al., 1989), making it desirable

to distinguish between these histologically and cytogenetically identical tumours. The recent observation that Ewing's sarcoma expresses dbl oncogene mRNA, whereas peripheral neuroepithelioma does not, may allow workers, by means of molecular techniques, to further refine treatment protocols for these closely related but not identical tumours (Vecchio et al., 1989).

1.3.VI.b. The contribution of molecular genetic techniques to tumour prognosis.

The c-erbB-2 oncogene codes for a protein closely related to, but distinct from, the EGF receptor. In the case of adenocarcinoma of the breast, amplification of the c-erbB-2 gene was shown to correlate with reduced time to relapse and reduced overall survival (Slamon et al., 1987). The level of c-erbB-2 amplification was shown to be an equally strong prognosticating parameter as the extent of lymph node involvement. Further studies using antibodies to the c-erbB-2 protein on tissue sections demonstrated that c-erbB-2 gene amplification correlated with overexpression of the protein (Venter et al., 1987).

In human neuroblastomas, N-myc oncogene amplification appears to correlate with advanced disease stage at diagnosis, being infrequent in stage 1 or 2 disease (Brodeur et al., 1984). In addition, the presence of N-myc amplification is associated with rapid progression of the neuroblastoma, regardless of the stage of disease. Stage IV

neuroblastomas frequently possess high levels of N-*myc* amplification and carry the worst prognosis; patients have metastases to the bone and other sites. In contrast, in the special category of stage IV-S disease, widespread metastases are present but do not include the bone marrow, N-*myc* amplification is not observed, and the prognosis is far better than the usual stage IV disease. (Seeger *et al.*, 1985). It is thought that the presence of N-*myc* gene amplification may result in the production of elevated levels of the N-*myc* nuclear oncoprotein in the cell, and thus contribute to malignant progression of neuroblastomas (Schwab *et al.*, 1984).

The c-*erb*B-2 and N-*myc* oncogenes may thus increase and improve our ability to prognosticate in certain forms of breast adenocarcinoma and neuroblastoma, respectively.

1.3.VI.c. The contribution of molecular genetic studies to our understanding of tumour progression.

The development of colorectal adenocarcinoma is thought to involve progression from the benign states of mucosal epithelial hyperplasia and adenomatous polyp formation to formation of pre-invasive, invasive and metastatic carcinoma. The molecular changes underlying the hyperplasia- adenoma- carcinoma progression in both hereditary and sporadic forms of the disease have now been examined. Mutations of the c-Ki-*ras*2 and N-*ras* oncogenes were present in approximately 50% of adenomas greater than

1cm diameter and in carcinomas, suggesting that the presence of ras mutations might provide the cell with the ability to progress to a more malignant state, but not act as a determinant of that malignant state. Similarly, molecular abnormalities of sequences linked to the gene for familial adenomatous polyposis on chromosome 5q were lost in approximately equal numbers of adenomas and carcinomas in sporadic cases, implicating abnormalities at this locus in early neoplasia (Vogelstein et al., 1988). In contrast, a specific region on chromosome 18q was lost more commonly in advanced adenomas (47%) than in early adenomas (12%), and in 73% of carcinomas. Similarly, sequences on chromosome 17p, some of which specifically involved the putative tumour suppressor gene p53, were abnormal in a low percentage of early and advanced adenomas (6% and 24%, respectively), but frequently lost in carcinomas (75% of cases). This suggests that abnormalities at specific genetic loci on chromosomes 18q and 17p may be implicated in the progression of colonic adenomas to carcinomas (Fearon et al., 1987; Baker et al., 1989).

Therefore, molecular analysis of abnormalities of oncogene and putative tumour suppressor loci in neoplasia can provide insights into the genetic mechanisms which might underlie tumour progression. In addition, molecular analyses may provide novel methods of tumour diagnosis and prognosis, which are superior to and more sensitive than non-nucleic acid based techniques.

1.4 THE BIOLOGY OF MALIGNANT GLIOMAS

1.4.I. Incidence

Population-based studies indicate that the incidence of gliomas in the Caucasian population in Britain is 4-5 people per 100 000. The annual age-adjusted mortality for all patients with gliomas is 2.5 per 100 000.

Several differences in the incidence and site of gliomas of similar histological appearances exist between adults and children, possibly indicating fundamental dissimilarities in the origin and biological behaviour of the tumours. For example, in adults, 55% of gliomas are the highly malignant variety known as glioblastoma multiforme, while this tumour type accounts for 10-15% of the gliomas in childhood. In adults, approximately 70% of gliomas are found above the tentorium cerebelli, while in children, 70% are found below. In children, so-called primitive neurectodermal tumours are much commoner than in adults. Gliomas also occur in the spinal cord, but at approximately one sixth the rate of intracranial tumours (Reviewed in Russel and Rubinstein, 1989; Green et al., 1976; Leetsma, 1980).

1.4.II. Pathogenesis and histogenesis of gliomas

The incidence of gliomas in certain genetically inherited cancer syndromes such as neurofibromatosis, and in Turcot's and Li-Fraumeni syndromes has been described

(see Sections 1.2.II.b and d, above). Several cases are on record of gliomas occurring as the only neoplasm in families (Chemke et al., 1985). The occurrence of such familial gliomas is consistent with inheritance of a defective gene or a number of defective genes.

The cause of spontaneously occurring human gliomas is unknown. There is a relationship in some cases to a previous history of exposure to radiation, especially in children (Shapiro et al., 1989) and exposure to certain chemicals used in the rubber and plastics industries (Preston-Martin et al., 1989), consistent with , but not proof of, the presence of sporadic genetic mutations.

The possible involvement of tumour suppressor genes in the genesis of human and experimental gliomas is suggested by two phenomena. In the case of human tumours, specific cytogenetic abnormalities are found in gliomas, certain of which are consistent with representing the loss of tumour suppressor loci; these abnormalities will be further described in Section 1.4.V.e. Secondly, selected DNA tumour viruses which bind and presumably inactivate the RB protein (thought to have tumour suppressor function) induce gliomas in experimental animals (see Section 1.2.II.a).

Studies on animal models have demonstrated that administration of ethylnitrosourea (ENU) to pregnant rats results in the development of gliomas in a high percentage of the offspring (Jones et al., 1973). Molecular analysis

has revealed that one of the genes affected by ENU is the rat cellular oncogene neu (homologous to human c-erbB-2), which undergoes a single point mutation sufficient to induce cellular transforming capacity (Schechter et al., 1985). Therefore the chemical carcinogen ENU may contribute to the development of rat gliomas by altering the function of an oncoprotein.

It would appear that cells forming the ENU-induced tumours arise from the subependymal plate (Lantos and Pilkington, 1979), an area of high cell turnover in the mammalian foetus which is presumed to give rise to the glial cells. The subependymal plate is also thought to continue as a site of cell division in the adult primate CNS (Reviewed in Russel and Rubinstein, 1989). Many types of tumour occur in tissues exhibiting a high degree of cell division. It is thought that a genetic mutation occurring in a cell will, as a result of DNA replication, be passed on to the daughter cells, thereby conserving the mutation. Therefore cell division is a prerequisite for the development of a clone of cells all bearing the same genetic mutation.

It is possible that environmental carcinogens may be involved in the development of human gliomas by means of inducing mutations in cellular oncogenes, as is the case in the ENU-induced rat glioma model. In addition, human gliomas may arise in areas of continued precursor cell turnover, such as the subependymal plate (Russel and

Rubinstein, 1989).

Neural tumours, including human gliomas and experimental animal gliomas, display marked cellular heterogeneity (Russel and Rubinstein, 1989). The presence of heterogeneous cell types in experimental neural tumours can possibly be explained by the observation that multipotential progenitor cells exist in the developing rat brain. One type of progenitor cell in the developing rat cerebral cortex appears to be capable of giving rise to both neurons and glial cells (Price and Thurlow, 1988). Another type of stem cell, known as the O-2A progenitor cell, is present in neonatal rat optic nerve and is capable of giving rise to both astrocytes and oligodendrocytes (Raff et al., 1983). It is possible that molecular genetic alterations occurring in a rat progenitor cell which gives rise to both astrocytes and oligodendrocytes could give rise to tumours such as mixed oligodendroglioma-astrocytomas. Thus, a carcinogenic event occurring when a cell is still capable of multiple differentiation pathways may result in a tumour composed of more than one neoplastic cell type. Similar stem cells may be present in the human subependymal plate, and give rise to gliomas.

It is not known why certain types of neural tumour, including gliomas, occur with different frequencies in children when compared to adults. The high number of primitive neuroectodermal tumours found in children when

compared to adults may be explained by the fact that neuroblasts, the cells thought to give rise to primitive neuroectodermal tumours, occur with much greater frequency in neonates than in adults (Reviewed in Russel and Rubinstein, 1989). The best documented example of a difference in adult versus perinatal neural progenitor cells is that seen in the rat optic nerve, where the adult O-2A bipotential progenitor cell (which gives rise to astrocytes and oligodendrocytes) differs markedly, in terms of several biological parameters, from the perinatal O-2A progenitor (Wolswijk and Noble, 1989). The occurrence of different progenitor cell populations in adults when compared to neonates may help explain the different spectrum of tumours observed in these two groups (Dehner, 1986; Becker and Hinton, 1983; Gilles et al., 1983).

1.4.III. Classification and prognosis

The primary reason for having a pathological classification is to be able to subdivide the tumours into biologically-meaningful categories. The tumours forming a single category will then have a similar prognosis and respond in a similar manner to the same form of treatment. Thus different forms of therapy can then be compared with the aim of defining the most effective treatment for each type of tumour, one of the fundamental purposes of oncological practise.

In the case of gliomas, current classification

systems are an amalgam of the so-called embryogenic system proposed by Bailey and Cushing in 1926, which was based on the cytologic similarities between tumour cells and developing neuroepithelial cells; and the grading system proposed by Kernohan in 1949 and modified by Rubinstein in 1972, which draws attention to the degree to which tumour cells deviate in appearance from a mature glial cell, with the most "de-differentated" cells being the most malignant. Tumour classification is based primarily on various morphological parameters seen on histological preparations, coupled with the analysis in some cases of the expression of gene products such as intermediate filament proteins. The resemblance of the predominant tumour cell type to a developing glial cell or a mature glial cell is used to classify the type of tumour. Thus, a glioma composed predominantly of cells resembling fibrillary astrocytes which may or may not express glial fibrillary acidic protein is called an astrocytoma. The tumour is then graded according to how closely the tumour cells resemble an idealised "normal" adult astrocyte: tumours composed of cells resembling mature astrocytes are designated low-grade or benign tumours, while those showing minimal resemblance to mature astrocytes are designated high grade, or malignant tumours (Reviewed in: Russel and Rubinstein, 1989; Green et al., 1976; McComb and Bigner, 1984).

Use of the above systems of classification results in recognition of the following main types of glioma which are

the subject of analysis in this thesis:

1) Astrocytic tumours (20-30% of adult glial tumours): astrocytoma grades I-II (benign), grades III and IV (malignant);
2) Oligodendroglial tumours (5% of adult glial tumours): oligodendroglioma grades I-II (benign), grade III (malignant);
3) Ependymal cell tumours (6% of adult glial tumours): ependymoma (benign and malignant or anaplastic grades); choroid plexus papilloma;
4) Mixed tumours e.g. oligo-astrocytomas (benign and malignant grades);
5) Glioblastoma multiforme (55% of adult glial tumours).

The prognosis for each histological type and grade of tumour is generally consistent for tumours arising in a specific anatomical site and in patients of a similar age. Thus grade I and II cerebral astrocytomas in adults may be present for 5-10 years and affect the patient only by causing a localised neurological deficit. However, in at least 50% of low grade astrocytomas, there are small foci of malignant cells and 15% of astrocytomas presenting initially as low grade (I and II) tumours evolve into highly malignant tumours within 5 years. Therefore, prognosticating in the case of such astrocytomas can be problematic.

Oligodendrogliomas show a less marked capacity for malignant change than cerebral astrocytomas, but are even more difficult to assess in terms of potential for malignant progression (Mackenzie et al., 1988). The average survival of patients with oligodendroglioma is 5 years.

In general, benign ependymomas and choroid plexus papillomas infrequently evolve into more malignant forms. The average survival of patients with benign intracerebral ependymomas is 30-40 months, but this varies widely, and the survival is impossible to predict from the histological appearances (Mork and Loken, 1977). Choroid plexus papillomas usually have a good prognosis if surgically accessible.

Glioblastoma multiforme is a highly malignant tumour. The median survival in patients treated with surgery and radiotherapy is 11 months (McComb and Bigner, 1984). Several workers draw no distinction between grade IV astrocytomas and glioblastoma multiforme, but others cite differences in the duration of symptoms prior to diagnosis and in survival following treatment (Burger et al., 1985), therefore the distinction between the two entities will be made in this thesis. Glioblastoma multiforme may arise from the progressive development of malignancy in a pre-existing low-grade glioma, or, more frequently, it may arise de-novo.

Obviously, the anatomical site of a tumour in the central nervous system can determine the ultimate fate of

the patient regardless of the cytological malignancy of the tumour cells. The majority of patients who succumb from intracranial gliomas do so because of the effects of a space-occupying lesion. Metastases are extremely infrequent. When metastases occur, it is usually in cases of glioblastoma following surgical intervention, presumably as a result of allowing tumour cells access to the venous system. However, rare cases of metastases not preceded by surgery have been recorded (Leifer et al., 1989). It is therefore possible that malignant gliomas possess the ability to metastasise, in common with many other types of malignant tumour, but the frequency of metastasis in gliomas is low owing to the patients' succumbing relatively early in the life of the tumour from the effects of a space occupying lesion.

1.4.IV. Problems encountered when using morphologically based systems of tumour classification

In general, the morphological approach to classifying tumours correlates with biological behaviour, as long as the tumours being compared arise from similar sites in patients of a similar age. Thus low grade astrocytomas in the frontal lobes of adults generally take longer to kill the patient by progressive tumour growth than high grade astrocytomas in the same site. However, the anatomical site and the age of the patient can have a major effect on the behaviour of a tumour. Thus, the juvenile (or "pilocytic")

astrocytoma generally seen in the cerebellum of children usually has an excellent prognosis, with infrequent recurrences following surgical removal, even after periods of more than 20 years; this contrasts with the overall prognosis discussed previously for benign cerebral astrocytomas in adults. Furthermore, a glioblastoma multiforme in a 30 year old generally has a better prognosis than a similar tumour in a septuagenarian. Even a tumour of a particular histological type and grade occurring in the same anatomical site in patients of similar age may differ markedly in behavior in certain patients. Glioblastoma multiforme usually has a median survival time of 10 months (following surgery and radiotherapy), but several cases are on record of patients surviving over 20 years. Conversely, oligodendrogliomas of low grade may have a median survival time of 5 years, however this tumour is notorious for having an unpredictable ability to rapidly assume a highly malignant course (Reviewed in Russel and Rubinstein, 1989).

It is obvious that the glioma classification systems currently in use are all subject to limitations, in that they do not infallibly predict the outcome of a particular tumour in a particular site, nor do they attempt to address the question of why histologically similar tumours in different anatomical sites may behave in a dissimilar manner. Therefore it may be that the morphologically based classification systems fail to identify the fundamental

parameters which define the biological behaviour of any particular tumour. Even if biologically important morphological parameters exist, the heterogeneity of gliomas makes identification of such parameters extremely difficult.

Morphologically based classification systems fail to identify the genetic lesions underlying the development of neoplasia, which may be important determinants of tumour prognosis (Gould, 1986).

The limitations of classifying tumours solely on a morphological basis are further demonstrated by a recent study on antigen expression in early glioma cultures which indicated that a minority of cultures expressed the astrocytic marker GFAP, while the majority expressed fibronectin. Furthermore, the expression of GFAP or fibronectin did not correlate in any way with the supposed cell lineage of the tumour, nor did it correlate with the histological grade of the tumour (Kennedy et al., 1987). A further study on tumour cell antigen expression in cell culture indicated that in many primary cultures derived from high grade gliomas, the transformed cells may not even be of glial origin (Franks and Burrow, 1986).

The biology of gliomas has been studied using several non-morphological approaches, for example cell, tissue and organ culture, flow cytometry, in-vitro chemosensitivity and assessment of tumour cell antigen expression (Reviewed in: Russel and Rubinstein, 1989; McComb and Bigner, 1984).

None of these methods have improved our capacity for accurate prognosis in the case of individual gliomas.

1.4.V. Oncogene abnormalities in human gliomas

It is possible that molecular genetic studies may help elucidate the critical genetic lesions which determine the biological behaviour of gliomas and define categories of tumours which will respond homogeneously to a particular treatment modality. Molecular genetic studies which have been performed thus far on gliomas will now be reviewed.

1.4.V.a. Abnormalities of membrane-associated oncogenes

Amplification of the EGFR gene and associated overexpression of its protein has been observed in up to 40% of malignant gliomas, usually in glioblastomas (Libermann et al., 1984; Libermann et al., 1985). A futher study detected amplification in only 4 out of 23 glioblastomas (17%), but in 3 of these cases, there was selective amplification of the transmembrane and cytoplasmic domains only, with no amplification of the external (ligand binding) domain. A truncated EGFR protein was demonstrated in one of these 3 tumours, reminiscent of the truncated protein coded for by the v-erbB oncogene which has lost the external EGF-binding domain and possesses an elevated level of tyrosine kinase activity. Therefore, in the group of glioblastomas described in this study, one of the mechanisms contributing to cell

transformation could be the overexpression of EGF receptors which may have constitutive tyrosine kinase activity independent of ligand binding (Malden et al., 1988).

The c-ros-1 oncoprotein, the human homologue of the v-ros oncoprotein of the UR2 retrovirus, may encode a growth factor receptor, as it has structural similarities to the EGFR protein and also has tyrosine kinase activity. One study detected high levels of c-ros-1 mRNA transcripts in glioblastoma cell lines in the absence of gene amplification (Birchmeier et al., 1987). Further studies are needed to define whether overexpression of c-ros-1 has any role in glial neoplasia.

Both glioblastoma cells and hyperplastic endothelial cells in the same tumour express mRNA encoding the PDGF A-chain and PDGF B-chain/c-sis products, as well as PDGF B-type receptor. Therefore, autocrine and paracrine loops based on PDGF may contribute to glioblastoma growth by promoting the division of neoplastic glial cells, as well as by enhancing angiogenesis by inducing proliferation of endothelial cells (Nister et al., 1984; Hermansson et al., 1988).

1.4.V.b. Abnormalities of oncoproteins thought to function in the nucleus

Amplification of the N-myc oncogene in gliomas is infrequent, being present in less than 5% of malignant gliomas (Garson et al., 1985; Bigner et al., 1988b).

Similarly, only one recorded case exists of c-*myc* amplification (associated with gene rearrangement) in a human glioblastoma (Trent *et al.*, 1986).

The *gli* oncogene was cloned from amplified sequences in a malignant human glioma cell line which possessed double minute chromosomes (Kinzler *et al.*, 1987). *Gli* amplification was then demonstrated in a low percentage (less than 5%) of human glioblastomas (Bigner *et al.*, 1988). The predicted *gli* protein possesses a zinc finger DNA binding domain and shows homology to the *Kruppel* family of zinc finger proteins which play an important role in the development of *Drosophila*. The *gli* protein may similarly play a role in the control of normal mammalian developmental processes, and therefore functional abnormalities may contribute to tumour formation (Kinzler *et al.*, 1988). *Gli* amplification has also been described in a rhabdomyosarcoma and an osteosarcoma, both of which were morphologically extremely undifferentiated, suggesting that abnormalities of *gli* function may interfere with early cell differentiation processes (Roberts *et al.*, 1989).

1.4.V.c. Expression of polypeptides which may contribute to glioma growth

Tumour growth involves proliferation of both tumour cells as well as stromal elements. As discussed previously (Section 1.3.V,) neoplastic cells can affect the proliferation of stromal cells, and vice versa. In many

cases, the mutual neoplastic cell and non-neoplastic cell interactions are mediated by peptide regulatory factors (PRF's). In addition to the possible involvement of autocrine/ paracrine loops based, for example on the PDGF and EGF systems (see 1.4.v.a, above), several other PRF's may contribute to tumour development, and these will be described here.

Glial growth factor (GGF) was purified originally from the bovine pituitary gland, and is mitogenic for rat astrocytes and Schwann cells in vitro (Brockes et al., 1980). A high degree of GGF-like activity is present in human Schwann cell tumours, suggesting a possible autocrine effect (Brockes et al., 1986).

Several glioma cell lines have been shown to secrete the peptide acidic fibroblast growth factor (a-FGF, which has been shown to be identical to endothelial cell growth factor, or ECGF). The glioma cells also express receptors for a-FGF, and are mitogenically stimulated by administration of exogenous a-FGF (Libermann et al., 1987). It is possible that a-FGF is involved in stimulating tumour angiogenesis, as well as in enhancing the proliferation of neoplastic and reactive glial cells in the tumour, in a similar manner to PDGF (see section 1.4.V.a, above).

1.4.V.d. Attempts to correlate oncogene abnormalities with glioma biology.

To date, only one large series of 64 malignant

gliomas has been studied in an attempt to correlate amplification of the EGFR, N-*myc* and *gli* oncogenes with tumour biology. No correlation was found between amplification of these oncogenes with patient age, tumour prognosis, tumour morphology, endothelial hyperplasia or with the presence of necrosis (Bigner *et al.*, 1988). While this lack of correlation was surprising, it may indicate the fact that amplification of these particular oncogenes is not a crucial determinant of glioma behavior at the stage of clinical presentation, as this may represent a relatively late stage of glioma growth. Gene amplification may thus enhance cell growth at a specific stage in the develoment of neoplasia, and subsequently other genetic or epigenetic abnormalities may be required for tumour progression and determine the clinical behaviour of a neoplasm. Obviously, the possibility exists that oncogene amplification merely represents an epiphenomenon, not instrumental in determining the behaviour or the prognosis of gliomas.

The effects on tumour development of oncogene abnormalities in glioma cells, coupled with peptide regulatory factor-mediated cell-cell interactions, are extremely complex and will require much painstaking effort to understand.

1.4.V.e. The impaired cell-mediated immunity observed in glioma patients may be due to peptides

secreted by the glioma cells

Patients harbouring a malignant glioma have been shown to possess impaired cell mediated immunity. Tumour infiltrating lymphocytes isolated directly from malignant gliomas have a depressed mitogenic response to interleukin-2 (Kuppner et al., 1989). Glioblastoma cells are known to produce a peptide, originally named glioblastoma-derived T-cell suppressor factor (G-TsF), which mediates the mitogenic inhibition of T-lymphocytes. Recently, G-TsF was shown to be identical to transforming growth factor beta-2 (TGF B-2), a peptide regulatory factor known to have immunosuppressive effects (DeMartin et al., 1987). TGF B-2 (like TGF-B, also known as TGF-B-1; see Section 1.3.V, above), is also known to promote angiogenesis. Thus TGF B-2 could affect patient survival not only by inhibiting normal tumour-infiltrating lymphocyte function, but also by promoting angiogenesis.

1.4.V.f. Cytogenetic abnormalities found in gliomas.

The majority of cytogenetic studies have been performed on adult malignant gliomas, and in these tumours, the most frequent abnormalities observed are: gains of chromosome 7 (the site of the EGFR gene, which is frequently amplified in malignant gliomas); losses of chromosomes 10, 22, and Y; the presence of double minute chromosomes (thought to represent amplified genes); deletions and translocations of chromosome 9p; and

structural alterations of chromsosmes 1, 6p, 11, 13, 15 and 16 (Bigner et al., 1986; Rey et al., 1987a; Bigner et al., 1988). Interestingly, although fewer studies have been performed on benign gliomas, many of the cytogenetic abnormalities observed in cells cultured from benign gliomas in one study of 8 paediatric and 14 adult cases were similar to those reported in malignant tumours, with polysomy of chromosome 7, and losses of chromosomes 10, 22, and Y predominating (Rey et al., 1987b). Cytogenetic analysis of a further group of 9 paediatric glial neoplasias reported polysomy of chromosome 7, structural abnormalities of chromosome 1p and an isochromosome 17q as the most common alterations (Griffin et al., 1988).

Based on the preceding discussion, it is conceivable that cytogenetic alterations may correlate with molecular and functional abnormalities of oncogenes and loci thought to harbour tumour suppressor genes. However, not all oncogene and tumour suppressor gene abnormalities manifest themselves as cytogenetically visible changes, therefore a more accurate indication of the extent of abnormalities of these loci can be obtained by employing molecular analysis.

1.5 THE WORK OF THIS THESIS

In the preceding discussion, I have outlined how knowledge of molecular abnormalities of oncogenes and tumour suppressor genes may enhance our understanding of tumour biology.

The work done for this thesis will attempt to detect additional molecular abnormalities occurring in human gliomas, with the aim of:

i) Furthering our knowledge of the molecular mechanisms underlying glioma development;

ii) Assessing whether any correlations exist between specific molecular abnormalities and the histological category of glioma;

iii) Assessing whether any molecular abnormalities correlate with the predicted biological behaviour of the tumour, as assessed by histological grading.

It is hoped that knowledge of any such molecular abnormalities may ultimately improve our ability to classify gliomas into categories whose members will behave in an homogeneous manner with respect to prognosis and treatment.

Attempts to identify dominantly-transforming oncogenes which may contribute to glial tumourigenesis will be based on the DNA transfection assay. In addition, several oncogenes will be examined by the technique of Southern analysis for evidence of amplification.

An assessment of the extent of abnormalities of putative tumour suppressor loci will be largely based on previously-reported cytogenetic analyses, and will consist of using Southern analysis to detect allele loss in somatic cells.

CHAPTER 2

MATERIALS AND METHODS

2.1. INTRODUCTION

This section will describe the techniques which are common to several experiments described in the results chapters. Techniques peculiar to one results chapter will be described in the methods section of the appropriate chapter.

The formulae of the stock solutions used are listed at the back of the sections on molecular biology and cell culture techniques. The molecular techniques used are essentially as described in Maniatis *et al.* (1982).

2.2. DNA EXTRACTION

2.2.I. DNA extraction from peripheral blood lymphocytes

2.2.I.a. Cell lysis

Blood samples from patients undergoing glioma biopsy had been obtained previously by other workers in the laboratory, mixed with EDTA to prevent coagulation, and stored at -70^{0}C until used. Frozen blood samples were thawed on ice. Five ml of blood was removed and placed into a 50ml polypropylene tube. Forty five ml of Triton-sucrose buffer (0.32M sucrose, 10mM Tris-Cl pH7.6, 5mM $MgCl_2$ and 1% Triton X-100) was added to the blood and the mixture placed on ice for 5 minutes to lyse the cell membranes. The lysate was then centrifuged at 134g for 15 minutes at 4^{0}C in a

Sorvall Technospin-R centrifuge (Du Pont, UK), in order to precipitate the nuclei. The supernatant was then carefully decanted. The pelleted nuclei were lysed and the DNA liberated by adding 10ml of TNE lysis buffer (0.1M NaCl, 10mM Tris-Cl pH 8.0, 1mM EDTA, 1% (w/v) SDS), followed by gentle mixing.

2.2.I.b. Handling and disposal of human blood, tissues and cultured cells

All procedures involving the handling of human blood, solid tumours and cells were performed in a class II laminar flow cabinet which had an external exhaust system. In order to minimise the possibility of contamination by human pathogens, all buffers used in the preparation of human DNA, prior to the addition of TNE lysis buffer, were discarded into a 5% Chloros solution and left to stand for 6 hours in the laminar flow hood before disposal. All manipulation of these solutions were performed using disposable plastics which were autoclaved prior to discarding.

2.2.I.c. Proteinase K digestion

Proteinase K (Sigma, UK), was added to the DNA lysate to a final concentration of 100ug/ml. The lysate was then placed in a water bath at 55^{0}C for 1 hour, with gentle mixing every 15 minutes.

2.2.I.d. Phenol/chloroform extraction

A mixture of phenol and choloroform was used to remove protein from the DNA samples. Phenol (BRL-Gibco, UK), was saturated with 10mM Tris-Cl pH 7.6, 1mM EDTA (as described in Maniatis et al. (1982)), and added to an equal volume of chloroform (Applied Biosystems, UK). Ten ml of the phenol/chloroform mixture was added to the 10 ml of DNA lysate and mixed for 15 minutes on a rotating wheel at room temperature. The aqueous phase (containing the DNA) was separated from the organic phase (containing the phenol/chloroform and protein) by centrifugation at 1118 **g** at 20^0C for 20 minutes in a Sorvall Technospin R centrifuge (DuPont, UK). The aqueous (upper) phase was carefully removed by using a wide-mouthed pipette and placed into another 50ml polypropylene tube. A further 10 ml of phenol/chloroform solution was added and the mixing and centrifugation steps repeated. The aqueous phase was removed once more, and 10 ml of chloroform was added. Following mixing and centrifugal separation of the phases, the chloroform phase was removed from below the aqueous phase by gentle pipetting. The DNA in the aqueous phase was now ready for precipitation.

2.2.I.e. Precipitation of DNA

The DNA was precipitated by the addition of 25ml of 100% ethanol to the DNA lysate. After gentle mixing, the DNA could be seen as a white gelatinous precipitate. The

DNA was removed from the ethanol-lysis buffer mixture by spooling it onto a glass Pasteur pipette. Removal of DNA from the precipitation mixture by spooling is a useful method of separating any remaining RNA from the DNA. The RNA molecules, by virtue of their smaller size when compared to the high molecular weight DNA molecules, do not get "spooled" onto the Pasteur pipette, and are thus left behind in the precipitation mixture. The DNA attached to the Pasteur pipette was then placed in an Eppendorf microcentrifuge tube containing 400ul of TE (TE consists of 10mM Tris-HCl pH7.6, 1mM EDTA), and was gradually dissolved in the TE.

2.2.I.f. Quantitation of DNA concentration by spectrophotometry

Serial dilutions of the DNA suspended in TE were subjected to ultraviolet (UV) absorbtion spectrophotometry. Readings were taken at an optical density (OD) of 260nm and an OD of 280nm on a DU-50 spectrophotometer (Beckman RIIC Ltd, UK). The OD_{260} : OD_{280} ratio is an indication of the purity of the DNA preparation (with respect to protein contamination). A value of OD_{260} : OD_{280} less than 1.8 indicates a pure preparation. An OD_{260} of 1.0 is equivalent to a double stranded DNA concentration of 50ug/ml.

2.2.II. DNA extraction from solid tumour samples

2.2.II.a. Sources of tumour biopsies

Glioma biopsies were derived from patients at the National Hospitals for Nervous Diseases, Queen Square and Maida Vale, London and the Italian Hospital, London. Biopsies had previously been collected at surgery and frozen immediately in liquid nitrogen. A portion of the biopsy had been used to establish explant cultures, while the remainder was used to provide a routine histopathological diagnosis, which was made by consultant neuropathologists at the above hospitals.

Twenty percent of the frozen tumour samples were analysed at random prior to DNA extraction to confirm that at least 80% of the biopsy consisted of tumour cells. Five micron frozen sections were cut on a cryostat (Bright, UK), stained with haematoxylin and eosin (Sigma, UK) and viewed under a microscope.

2.2.II.b. Preparation of a nuclear pellet from solid tumours

A fragment of solid tumour biopsy of approximately 0.5ml in volume was homogenised on ice in a glass homogeniser in 10ml of PBSA (phosphate buffered salts, calcium and magnesium free, obtained from Flow Laboratories, UK). Homogenisation resulted in the release of the intact cell nuclei. The nuclei were pelleted by centrifugation at 402 g at 4^0C for 15 minutes in a Sorvall Technospin R centrifuge (DuPont, UK). The supernatant was carefully decanted into a Chloros solution

for later disposal, and the nuclear pellet was lysed by the addition of 10ml of TNE lysis buffer, as described in Section 2.2.I.a, above. The DNA lysate was then processed in an identical manner to that described in Sections 2.2.I.c to f. The preparation of a nuclear pellet prior to adding lysis buffer is a useful technique when extracting DNA from solid tumours, as it reduces the amounts of RNA and protein in the lysate.

2.2.III. Extraction of DNA from cultured cells

2.2.III.a. Preparation of a cell lysate

Cultured cells were washed twice in ice-cold PBSA to remove excess medium, and the majority of the PBSA discarded. The cells were then scraped off into the remaining PBSA (approximately 0.5ml) by using a disposable plastic cell scraper (Costar, Northumbrian Biologicals, UK), and the cell suspension transferred to a 50 ml polypropylene tube. TNE lysis buffer (10ml) was added and the DNA was purified exactly as described in Sections 2.2.I.c to f.

2.3. DIGESTION OF DNA WITH RESTRICTION ENDONUCLEASES

All restriction enzymes were obtained from Boehringer Corporation (London) Ltd, UK, except for HinfI, which was obtained from Amersham International Plc, UK.

DNA was digested in a total volume of 50ul of the following mixture:

DNA (10ug) in TE	25ul
10X restriction buffer	5ul
Spermidine (30mM)	2ul
Bovine serum albumin (1mg/ml)	5ul
Beta-mercaptoethanol (140mM)	5ul
Restriction enzyme (50 units)	
Water	to 50ul total

The "10X" restriction buffer was a buffer providing the specific ionic concentration for a particular enzyme supplied by the manufacturer of the restriction enzyme.

Digestion was allowed to proceed for at least 4 hours with an incubation temperature of 37^0C for all enzymes, except for TaqI which required an incubation temperature of 65^0C.

Following a 4 hour digestion period, the DNA restriction fragments were suitable for immediate separation by agarose gel electrophoresis.

2.4. AGAROSE GEL ELECTROPHORESIS

Gels were made by dissolving the requisite amount of agarose (varying from 0.8% to 1.8% w/v of agarose (Gibco-BRL Ltd, UK)) in 400ml of TAE buffer (see Section 2.7 for composition of TAE buffer), by boiling in a microwave oven. The mixture was then allowed to cool to 60^0C and poured into a 20x20cm gel casting tray, which was provided with the gel tank apparatus (International Biotechnologies Inc., New Haven, CT, USA). A comb was

placed in one end of the gel and the gel was allowed to set.

The gel was then submerged in a horizontal electrophoresis tank containing TAE buffer. Samples of restriction enzyme-digested DNA (50ul) were mixed with 10ul of loading buffer and loaded into the wells formed by the comb. The samples were then subjected to electrophoresis for approximately 18 hours at 30 volts DC (constant voltage), using a Hoefer PS500X power pack (Hoefer Scientific Instruments, San Francisco, CA, USA). After electrophoresis, the DNA was visualised by staining the gel in a 1ug/ml ethidium bromide solution for 10 minutes followed by illumination on a long wave UV light box. Photographs of the gel were taken using Polaroid type 57 film, with the gel lying alongside a 20 cm ruler to facilitate accurate sizing of DNA fragments after hybridisation.

Standard DNA markers of known molecular weight were co-electrophoresed alongside the blood, tumour and cell DNA samples, in order to assess the size of the test sample bands. Lambda phage DNA digested to completion with HindIII was used for this purpose. The Lambda-HindIII markers (Gibco-BRL Ltd, UK) yield fragments of the following sizes: 23130, 9416, 6682, 4361, 2322, 2027, 564 and 125 base pairs.

2.5. SOUTHERN BLOTTING OF DNA

Southern blotting was used to transfer DNA fragments separated by agarose gel electrophoresis onto nylon membrane. Once immobilised on the nylon membrane, the DNA could then be probed with labelled DNA probes, in order to identify complementary sequences of interest.

Following photography of the ethidium-stained gel, the gel was soaked in 1 litre of denaturation buffer (1.5M NaCl, 0.5M NaOH) for 30 minutes. Denaturation buffer separates the 2 strands of the DNA duplex and allows them to bind to the nylon membrane, as the membrane will only bind single-stranded nucleic acids. The gel was neutralised by soaking it in 1 litre of neutralising buffer (1M Tris-HCl, pH8.0, 1.5M NaCl) for 30 minutes. The gel was then inverted and placed on a piece of filter paper soaked in 10x SSC (see Section 2.7), which was lying on a gel casting tray. The ends of the filter paper were in contact with a reservoir of 10x SSC, to act as a wick for the DNA transfer. A piece of nylon membrane (Hybond-N, Amersham, UK), previously cut to the same size as the gel, was placed on top of the gel. On top of this were placed two pieces of filter paper, cut to the same size as the gel, followed by a pile of tissue paper at least 5cm thick and a 1Kg weight placed on top of the tissues. Transfer of DNA takes place by capillary action, and was allowed to proceed for at least 12 hours. Following transfer, the Hybond-N membrane was removed, allowed to air dry for 10 minutes and placed in a vacuum oven (Gallenkamp, UK) at 80^{0}C for 2 hours in

order to firmly fix the DNA to the membrane.

2.6. HYBRIDISATION OF DNA IMMOBILISED ON NYLON FILTERS

2.6.I. Preparation of stock solutions used for DNA hybridisation

Denhardt's solution (see Section 2.8 for formula) was prepared as a 50x concentrated stock, filtered through 0.2um filters (Nalgene, UK), decanted into 50ml aliquots and stored at -20^{0}C. The formamide obtained from Fluka Chemicals Ltd, (UK), was of sufficient purity to be used without deionisation. A stock solution of carrier DNA was prepared by dissolving 250mg of calf thymus DNA (Sigma, UK) in 50ml of TE by gentle agitation overnight. The DNA was sheared by means of sonication at full power for 10 minutes in a Soniprep 150 sonicator (MSE, UK). The DNA was aliquoted and stored at -20^{0}C until used. The remainder of the stock solutions used in the hybridisation process did not require any special preparatory techniques.

2.6.II. Radioactive labelling of DNA probes for hybridisation

The DNA probes used to identify specific parts of the human genome were radioactively labelled by means of the "random primer" reaction, first described by Feinberg and Vogelstein (1983). The reagents used for the probe labelling reaction were all present in a random primer kit obtained from Boehringer Corporation (London) Ltd, UK, and

were used according to the manufacturers instructions. Briefly, 100ng of probe DNA (usually between 1 and 5ul) was denatured by heating to 95^0C for 5 minutes followed by immediate cooling on ice. The probe DNA was then mixed with the following solutions to give a final volume of 40ul:

Distilled water (to give a final volume of 40ul)	= 18ul minus the volume of the probe DNA
2ul each of: dATP	
dGTP	
dTTP	= 6ul
Random primer/ buffer mixture	= 4ul
Alpha-^{32}P-dCTP	= 10ul
Klenow enzyme (DNA polymerase)	= 2ul
TOTAL:	40ul

The reaction was incubated at 37^0C for 30-45 minutes in a water bath, and the labelled probe separated from the unincorporated nucleotides by means of spun column chromatography using Worthington "Mini-spin" columns (Lorne Laboratories, Twyford, UK), according to the manufacturers instructions. The separation centrifugation spins were performed at 1483 g for 4 minutes at 20^0C, using an HB-4 swing bucket rotor in a Sorval RC5C centrifuge (DuPont, UK). A 1ul aliquot of probe was removed for estimation of radioactive label incorporation by means of liquid scintillation counting using an LS 1801 scintillation

counter (Beckman RIIC Ltd, UK). Levels of radioactivity in excess of $3x10^6$ cpm/ul of probe reaction mixture were routinely obtained.

The handling and disposal of radioactive materials was performed according to the strict safety regulations adhered to by the Ludwig Institute.

2.6.III. Hybridisation of ^{32}P-labelled DNA probes to nylon filters

Prior to hybridisation, a prehybridisation step was necessary to prevent radioactive probe DNA binding randomly to the nylon membrane. Prehybridisation was carried out as follows:

The nylon membrane removed from the Southern transfer assembly was soaked in 3x SSC for 5 minutes and then added to a disposable plastic bag to which 0.5ml of prehybridisation solution per square cm of membrane was added. The prehybridisation solution was made up as follows:

Formamide	5ml
SSC (20x stock solution)	1.5ml
Denhardt's (50x stock)	0.5ml
Sodium phosphate pH6.5 (0.5M)	0.4ml
Glycine (10% solution)	1ml
Calf Thymus DNA (5mg/ml)	0.4ml
H_2O	1.2ml
TOTAL:	10ml

The calf thymus DNA was denatured prior to adding to the prehybridisation solution by heating to 95^0C for 10 minutes, followed by instant cooling on ice for 3 minutes. The bag was sealed following careful expulsion of all air bubbles and placed in a 42^0C incubator (Leec, UK) on a R100 rotary shaker (Luckham, UK), for at least 1 hour.

The prehybridisation solution was discarded and replaced with hybridisation solution containing radioactive probe. The hybridisation solution was made up as follows:

Formamide	5ml
SSC (20x stock solution)	1.5ml
Denhardt's (50x stock)	0.5ml
Sodium phosphate pH6.5 (0.5M)	0.4ml
Calf Thymus DNA (5mg/ml) (denatured)	0.4ml
H_2O	2.2ml
TOTAL:	10ml

In addition, a volume of labelled probe DNA equivalent to 10^6 cpm was added to each ml of hybridisation buffer, following denaturation by heating to 95^0C for 10 minutes and immediate cooling on ice for 3 minutes. The plastic bag was then sealed and placed in the 42^0C incubator for 18 hours on a rotary shaker.

2.6.IV. Washing of nylon filters following hybridisation

After hybridisation, the membranes were subjected to two washes of 10 minutes each in 2x SSC at room temperature

with gentle shaking on a rotary shaker. The final high stringency washes were in 0.1x SSC/0.1% SDS at 60^{0}C in a shaking water bath (Gallenkamp, UK, model BKS 350).

2.6.V. Autoradiography

Following the high stringency washes, the filters were blotted dry on 3MM filter paper (Whatman, UK), covered with polythene and exposed to Kodak XAR-5 film at -70^{0}C, using a single intensifying screen. The initial exposure time was for 18 hours. Longer exposures were then performed if necessary. The maximum exposure required for a satisfactory result was 5 days.

2.6.VI. Removal of the radioactive probe from the nylon membrane

If the same Southern blot membrane was required for re-probing with a different labelled DNA probe, the previous probe had to be stripped from the blot. Removal of the probe was achieved by immersing the membrane in 250 ml of a solution containing 50% formamide, 10mM Tris-HCl pH7.6 and 1mM EDTA, at 65^{0}C for 45 minutes with vigorous agitation every 15 minutes. The membranes were then ready for rehybridisation.

2.7. FORMULATION OF THE STOCK SOLUTIONS USED FOR MOLECULAR BIOLOGY

Unless otherwise stated in the text, all reagents

were obtained from BDH Ltd, Dagenham, Essex, UK.

TE	10mM Tris-Base 1mM EDTA pH adjusted to required value (pH 7.6 or 8.0 with concentrated HCl)
TNE	100mM sodium chloride 10mM Tris-Base pH 7.6 1mM EDTA
SSC (20x)	3M sodium chloride 0.3M trisodium citrate pH7.0
TAE (50x)	2M Tris-acetate (Tris-Base plus glacial acetic acid) 50mM EDTA
Electrophoresis sample buffer (6x)	0.25% (w/v) bromophenol blue 0.25% (w/v) xylene cyanol 30% (v/v) glycerol in sterile water
Denhardt's solution (50x)	1% (w/v) Ficoll 1% (w/v) polyvinylpyrrolidone 1% (w/v) bovine serum albumin in sterile water
Triton-sucrose	0.32M sucrose (109.5g/litre)

buffer	10mM Tris-Cl pH 7.6 5mM $MgCl_2$ 1% (v/v) Triton X-100

2.8. PREPARATION OF THE STOCK SOLUTIONS USED FOR MOLECULAR BIOLOGY

1M Tris	121g Tris base was dissolved in 800ml H_2O. The pH was adjusted to 7.6 or 8.0 by adding concentrated HCl, and the volume made up to 1 litre. The solution was sterilised by autoclaving.
0.5M EDTA	186.1g disodium EDTA was added to 800ml of H_2O and stirred vigorously while the pH was adjusted to 8.0 with NaOH pellets. The volume was made up to 1 litre and the solution sterilised by autoclaving.
5M NaCl	292.2 grams of NaCl was dissolved in one litre of water. The solution was sterilised by autoclaving.
1M $MgCl_2$	203.3g of $MgCl_2$ was dissolved in 1 litre of water and the solution sterilised by autoclaving.
3M Sodium	408.1g of sodium acetate.$3H_2O$ was dissolved

acetate (pH5.2) in 800ml of H_2O. The pH was adjusted to 5.2 with glacial acetic acid. The volume was then made up to 1 litre, and the solution sterilised by autoclaving.

10% SDS 100g of electrophoresis grade sodium dodecyl sulphate was dissolved in 1 litre of water by heating to 68^0C.

SSC (20x) 175.3g of NaCl and 88.2g of sodium citrate were dissolved in 1 litre of water. The solution was sterilised by autoclaving.

SSPE (20X) 174g of NaCl, 27.6g of NaH_2PO_4 and 7.4g of EDTA was dissolved in 800ml of water. The pH was adjusted to 7.4 with NaOH. The volume was then made up to 1 litre and the solution sterilised by autoclaving.

Ethidium bromide (10mg/ml) 1g of ethidium bromide was dissolved in 100ml of water

2.9. CELL CULTURE

2.9.I. Cell culture

Cells stored in freezing vials in liquid nitrogen were thawed rapidly in a water bath at 37^0C and plated out

onto 80cm^2 tissue culture flasks (Nunc: Gibco-BRL, UK). Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; 30ml per 80cm^2 flask) supplemented with 10% (v/v) heat inactivated foetal calf serum (FCS), 2mM glutamine and 25ug/ml gentamicin. The culture flasks were placed in an incubator at 37^{0}C which contained a humidified atmosphere of 95% air and 5% CO_2. Two thirds of the medium was changed every 3 days. All cell culture procedures were performed in a class II laminar flow cabinet (TUV GMBH, West Germany) with an external exhaust. The status of cell growth was visualised using an inverted phase contrast microscope (Nikon, UK).

2.9.II. Passaging of cultured cells

The cells were passaged when they reached confluency (the time taken for different cultures to reach confluency varied from 4 days to 10 days). Medium was aspirated off the cell layer and the cells were washed with 3ml of Hank's Balanced Salt Solution (HBSS) containing trypsin (0.083%) and EDTA (0.02%, w/v). The solution was aspirated off immediately, replaced with a further 3ml of the same solution and the flask was left at room temperature for 1 minute. When trypsinisation was complete, the activity of the trypsin was inhibited by the addition of 10 ml of DMEM/10% FCS, the cells were then uniformly suspended in this solution and the suspension divided equally into 2 or 3 fresh 80cm^2 flasks. The volume of medium in the flasks

was then made up to 30ml total.

2.9.III. Storage of cultured cells

Cells were stored in liquid nitrogen to provide backup stocks. Cells were generally frozen when in the exponential phase of growth (i.e. before they reached confluence). The cells were suspended by trypsinisation as described above, resuspended in 10ml DMEM/10%FCS, pelleted by centrifugation at 134 g for 5 minutes and the pellet was resuspended in DMEM/10%FCS with 10% dimethylsulphoxide at a density of 1×10^6 cells/ml. The suspension was frozen in 1ml aliquots in cryotubes. Freezing was carried out gradually as follows: -20^0C for 1 hour, -70^0C overnight, after which the cells were placed in the liquid nitrogen (LN_2) storage tank.

2.9.IV. Reagents used for cell culture

DMEM — Dulbecco's Modified Eagle's Medium, containing 1000mg/l glucose (Gibco-BRL, UK).

Gentamicin — 50mg/ml stock solution (Gibco-BRL Ltd, UK).

FCS — Foetal Calf Serum (Imperial Laboratories, UK). Heat inactivated at 56^0C for 45 minutes and then aliquoted into 50ml units and stored at -40^0C.

EDTA	Ethylenediamine tetraacetic acid (sodium salt) (Sigma, UK). Dissolved in Ca++ and Mg++-free HBSS to give a final concentration of 0.54mM (0.02%w/v), filter sterilised and stored at 4^0C.
Glutamine	100mM L-glutamine (Sigma, UK) dissolved in deionised water (Imperial Laboratories, UK), filter sterilised and stored at -20^0C.
Trypsin	Stock solution of 30 000 U/ml bovine pancreas trypsin (Sigma, UK), dissolved in Ca++ and Mg++-free DMEM, filter sterilised and stored in 1 ml aliquots at -20^0C.
PBS	Phosphate buffered saline (Flow Labs., UK).

CHAPTER 3

THE SEARCH FOR DOMINANTLY-TRANSFORMING ONCOGENES IN THE DNA OF GLIOMA CELLS USING DNA TRANSFECTION

3.1. INTRODUCTION

It is possible to detect the presence of certain classes of functionally abnormal ("activated") oncogenes in the DNA of solid tumours and tumour-derived cell lines by means of the DNA transfection assay. One method of DNA transfection involves the introduction of purified tumour cell DNA into an indicator cell (usually NIH3T3 mouse fibroblasts) by means of calcium phosphate precipitation (Graham and Van der Eb, 1973; Shih et al., 1979; Shih et al., 1981). The presence in the tumour DNA of an activated oncogene will result in an alteration of the indicator cell morphology; the indicator cell is then described as exhibiting a "transformed" phenotype. Extraction of DNA from the newly-transformed indicator cells and subsequent re-transfection into untransformed indicator cells will further dilute the amount of human DNA present in the mouse NIH3T3 cell genome. It has been shown that cell transformation occurring after this secondary transfection will be due to the introduction of a single human gene. It is possible then to establish the identity of the transfected activated oncogene by means of DNA hybridisation to known oncogenes, or, if a novel oncogene has been transfected, the new gene can be sequenced.

The NIH3T3 transfection assay has been used to

identify a number of transforming genes occurring in tumours, among them activated *ras* family genes (Tabin *et al.*, 1982), mutated *neu* genes (the rat homologue of human c-*erb*B-2) (Shih *et al.*, 1981), and activated *mas* and *raf* genes (Nishimura and Sekiya, 1987).

This chapter describes a series of NIH3T3 transfection experiments utilising DNA extracted from a series of human neural tumour cell lines. The single transforming oncogene thus identified was characterised, which necessitated further experiments designed to establish the exact nature of the tumour cell lines used.

3.2. MATERIALS AND METHODS

3.2.I. Cell lines

The cell lines used as the source of DNA for this experiment had been derived from explant cultures of biopsy material by workers at the Institute of Neurology, Queen Square, London. The cell lines and the histological diagnosis of the tumour of origin are listed in Table 3.1. Two cell lines were derived from benign gliomas (IN 223 and IN 821), five were derived from malignant gliomas (IN 157, IN 293, IN 235, IN 353 and IN 321) and one was derived from a chordoma (IN 502). The chordoma-derived cell line was included in this experiment as it appeared to be the only chordoma-derived cell line in existence, and therefore worth analysing at a molecular level.

3.2.II. Cell culture

Vials of tumour cells stored in liquid nitrogen were thawed by partial immersion in water at 37^0C and the cells plated into sterile tissue culture flasks in DMEM/10% FCS, in an atmosphere containing 7.5% CO_2 (v/v). Subsequent passaging was performed as described in Section 2.7.II. Sufficient cells were grown to produce at least 150 ug of DNA, at which point any remaining cells were frozen down in order to preserve stocks for future use. NIH3T3 cells, (obtained from Dr. C.S. Cooper, Institute of Cancer Research, London SW3), were cultured in 10ml of DMEM/10% FCS, and incubated at 37 ^{0}C in 7.5% CO_2.

3.2.III. DNA extraction

DNA was extracted from cultured human tumour cells, primary and secondary transfectants and normal human placenta as described in Section 2.2.

3.2.IV. DNA transfection

The transfection procedure used was a modification of that first described by Graham and Van der Eb (1973), and was essentially similar to the protocols used by Wigler et al., (1978) and Andersson et al., (1979).

24 hours before transfection, NIH3T3 recipient (indicator) cells were plated from the same cell pool onto 10cm sterile plates at a concentration of 5 x 10^5 cells per

plate.

60ug of tumour cell DNA was ethanol precipitated and redissolved in 1.31ml of sterile 1mM Tris-HCl pH 7.9, to which 0.19ml of 2M $CaCl_2$ was added. After mixing and then cooling for 20 minutes at 4^0C, the DNA-$CaCl_2$ solution was added dropwise to 1.5mls of HBS solution (HBS consists of 50mM 4-(hydroxyethyl)-1-piperazine sulphonic acid, pH7.15, 1.5mM sodium phosphate, 280mM NaCl) cooled to 4^0C, with constant gentle agitation. The mixture was kept at 20^0C until a fine precipitate formed (which took approximately 20 minutes). One ml of this solution (containing 20ug of the DNA precipitate) was then added to each of three 10cm dishes of NIH3T3 cells.Therefore a total of 3 dishes of NIH3T3 cells were transfected with the DNA derived from each tumour cell line.

The negative transfection control consisted of DNA derived from a thymoma ("Thymoma 4") induced by methyl methane sulphate in AKR mice, which had previously been shown to consistently fail to give rise to any transformed cells using the NIH3T3 transfection assay (Dr. C.S. Cooper, personal communication). The positive transfection control consisted of DNA extracted from MB 486a mouse bladder carcinoma cells. The MB 486a cell line was transformed <u>in vitro</u> by dimethylbenzanthracene and contains an activated oncogene known to transform NIH3T3 cells following transfection (personal communication from Dr. C.S. Cooper, Institute of Cancer Research, who also provided the Thymoma

4 and MB 486a DNA samples). The Thymoma 4 and MB 486a DNA samples were handled in an identical manner to neural tumour DNA samples, with 3 dishes plated with 20ug of DNA each.

After 24 hours, the medium was replaced by fresh DMEM/10% FCS. After 48 hours, the medium was replaced by fresh DMEM containing 5% FCS, in order to inhibit the growth of any spontaneously-occurring foci of NIH3T3 cells which have been reported when these cells are cultured in 10% FCS (Dr.C.S.Cooper, personal communication). The cells were refed with DMEM/5% FCS every 48 hours for 2-3 weeks, at which point the plate of cells was examined microscopically for evidence of morphologically transformed foci.

3.2.V. Propagation of transformed foci

Foci of NIH3T3 cells which showed progressive enlargement and a fusiform cellular morphology with lack of contact inhibition between cells were regarded as possible transformants. Cells making up a transformed focus were removed from the background of untransformed cells by isolating them using a sterile plastic ring of 4mm internal diameter coated with sterile silicone grease. The transformed cells lying within the ring were removed following trypsinisation and subcultured into sterile 25cm^2 flasks containing DMEM/5% FCS. On reaching confluence, approximately 50% of the cells were frozen in liquid

nitrogen to act as a backup stock. Cells were then passaged upon reaching confluence until sufficient cells had been produced to yield 150 ug DNA.

3.2.VI. Southern analysis of total genomic DNA from transformed foci

Southern analysis was performed essentially as described in Sections 2.2 to 2.6. Two Southern blots were made, one using total genomic DNA digested with the restriction enzyme EcoRI, the other with SacI digested DNA; (both enzymes were obtained from Boehringer, UK). Ten ug of DNA was subjected to electrophoresis on 0.8% agarose gels and transferred to Hybond-N filters (Amersham, UK). In order to assess the molecular weight of the samples, 4ug of HindIII-digested lambda phage DNA was run in an adjacent lane. Filters were hybridised to radiolabelled probes for the c-Ha-*ras*1 and N-*ras* oncogenes. The probe used to identify c-Ha-*ras*1 sequences was the 2.9Kb SacI human genomic c-Ha-*ras*1 fragment (Reddy *et al.*, 1982). N-*ras* sequences were identified with the 1.4Kb PvuII genomic N-*ras* insert (Hall *et al.*, 1983). (Both probes were a kind gift of Dr. C.J. Marshall, Institute of Cancer Research.) Following hybridisation, blots were washed at high stringency (60^{0}C), as described in Section 2.6.IV.

3.2.VII. Extraction of DNA from paraffin blocks

Extraction of DNA from archival paraffin wax-embedded

primary tumour tissue was performed using the method described by Dubeau et al., (1986), as modified by Dr. M.R. Stratton (personal communication).

20 serial sections of 25 microns thickness were cut from each paraffin block and placed in a 5ml plastic Bijou vial containing 1 ml of lysis buffer (comprising 10mM Tris-HCl pH 8.0, 100mM NaCl, 1mM EDTA, 1% (w/v) SDS and 500 ug/ml proteinase K). The lysate was then incubated for 18 hours at 48^{0}C in order to release the DNA from the cells, remove any DNA-bound proteins and inactivate DNAase enzymes. After 18 hours, the vial was vortexed briefly, an additional 0.5 ml of the above solution was added, and the incubation continued for a further 24 hours at the same temperature. The lysate/paraffin wax mixture was then transferred to a 15ml polypropylene tube. Non-nucleic acid contaminants in the lysate were extracted by the addition of an equal volume of buffered phenol followed by gentle agitation on a tumbling wheel for 20 minutes and a subsequent 5 second centrifuge spin to separate the aqueous and non-aqueous phases. The supernatant (aqueous phase) was then extracted twice with a 50/50 mixture of phenol chloroform, followed by a single chloroform extraction, as described above. During the extraction procedures, only the aqueous phase was removed from the tube, whilst the congealed paraffin wax was discarded with the organic solvent.

The aqueous phase was then divided into 400 ul

aliquots. The DNA was precipitated by the addition of 3M sodium acetate to a final concentration of 0.3M, followed by addition of 2 volumes of absolute ethanol, and left overnight at -20^{0}C in order to allow precipitation of any small fragments of DNA (100-200 base pairs). The precipitated DNA was then compacted by centrifugation at 12 300 g at 4^{0}C. After removal of the supernatant, the DNA pellet was resuspended in 100ul of TE, pH 7.6 by gentle agitation.

A total of 25 ug of DNA was extracted using the above method from each of the 3 formalin-fixed paraffin-embedded primary tumour samples.

3.2.VIII. DNA amplification using the polymerase chain reaction

DNA samples were subjected to the polymerase chain reaction (PCR), as described originally by Saiki et al., (1985) with appropriate modifications for use with Taq polymerase, the thermostable DNA polymerase from the bacillus Thermus aquaticus (Saiki et al., 1988).

1ug of genomic DNA from each of the transformed cell foci or paraffin block tumour samples was placed in 50ul of a mixture which contained 6.7mM Tris HCl pH8.8, 1.66mM ammonium sulphate, 0.67mM $MgCl_2$, 0.67uM EDTA, 0.37mM each of dATP, dCTP, dGTP and dTTP (Pharmacia, UK), 1uM each of 2 N-ras codon 61 primers (see Figure 3.1 and below), 10mM 2-mercapto ethanol, 5.8mg/ml BSA and 5 units of Taq DNA

polymerase (Perkin Elmer Cetus, UK). All the reagents except the Taq polymerase were heated to 93^0C for 7 minutes using a programmable heating block (Techne, UK). (This was necessary to necessary to denature the template DNA fully and this step is also thought to destroy any proteases which may degrade the Taq polymerase.) The mixture was then placed at 43^0C for 5 minutes which allowed annealing of the primers to the template. Subsequently, the reaction tube was vortexed briefly and 1ul (5 units) of Taq polymerase added, followed by 100ul of paraffin oil (Sigma, UK), (the latter designed to inhibit evaporation of the reaction mixture). The reaction was then subjected to 35 cycles of primed DNA extension for 2 minutes at 71^0C, denaturation for 1 minute at 93^0C and annealing for 2 minutes at 48^0C. After completion of the 35th extension step, the reaction products were allowed to anneal at 48^0C for 5 minutes. The reaction products were then subjected to Southern analysis by means of electrophoresis on 1.8% agarose gels followed by transfer of DNA to Hybond-N membranes as described in section 2.5, except that the denaturing and renaturing steps were performed for only 10 minutes each.

The sequences of the specific oligonucleotide primers used to amplify the portion of the N-ras gene surrounding the codon 61 region are given in Figure 3.1 (Farr et al., 1988). The primer RS60 (the "sense" primer) anneals initially to the non-coding strand of the DNA, whilst the "anti-sense" primer RS61 anneals initially to the coding

strand, with each primer serving to define the outermost limits of the segment of the N-ras gene to be amplified. Each primer was 20 nucleotides long. The primers were a gift of Dr. C.J. Marshall, (Institute of Cancer Research).

3.2.IX. Hybridisation of oligonucleotide probes to PCR-amplified DNA

In order to identify possible mutations in codon 61 of the N-ras gene, Southern blots of DNA amplified by the PCR were probed sequentially with oligonucleotides encoding all the possible mutations at codon 61 (see Table 3.2). Hybridisation and washing conditions of high stringency were used which would allow a ^{32}P-labelled oligonucleotide to remain annealed to the amplified DNA only if the oligonucleotide had the identical complementary sequence to the amplified DNA segment (Wood et al., 1985). Therefore, if the oligonucleotide remained on the blot following washing, as the sequence of the oligonucleotide is known, the exact alteration in the amplified gene coding sequence could be identified.

In order to allow hybridisation of mutation-specific oligonucleotides, blots were prehybridised in 3M tetramethylammonium chloride (Aldrich Chemical Company, UK), 50mM Tris-HCL pH 7.5, 2mM EDTA, 0.3% (w/v) SDS, 100ug/ml calf thymus DNA and 5x Denhardt's solution for 30 minutes at 55^{0}C. After 30 minutes, the prehybridisation solution was discarded and the blots hybridised in an

identical solution containing the labelled ^{32}P-oligonucleotide probe for 60 minutes at 55^{0}C. The blots were then rinsed twice in 2xSSPE (2xSSPE contains 0.36M NaCl, 0.02M sodium phosphate pH 7.7 and 0.2mM EDTA) and 0.1% (w/v) SDS for 5 minutes at room temperature on each occasion. The final wash was at 59.5^{0}C for 30 minutes in 3M tetramethylammonium chloride, 50mM Tris-HCl pH 7.5, 2mM EDTA and 0.3% (w/v) SDS. Autoradiography was performed by exposing the blots to X-ray film at -70^{0}C.

Oligonucleotide probes were end-labelled by addition of gamma-^{32}P-ATP to the 5'-OH group of the oligonucleotide by means of the enzyme T4 polynucleotide kinase (T4 PNK, BRL/Gibco, UK). The labelling mixture contained 20pmol of oligonucleotide, 70mM Tris-HCl pH 7.6, 10mM $MgCl_2$, 5mM DTT, 10 units T4 PNK, 1uM ^{32}P-gamma-ATP (200uCi, Amersham, UK) in a 40ul volume and was incubated for 30 minutes at 37^{0}C. The reaction was terminated by adding 60 ul of 25mM EDTA and the unincorporated oligonucleotides removed by passing the reaction mixture down Nensorb columns (DuPont-NEN, Dreieich, West Germany) and collecting 600-900 ul of the first radioactive peak eluted from the columns, which represents the majority of the labelled oligonucleotides.

Following hybridisation, removal of the probe was performed by the method described in Section 2.6.VI.

3.2.X. DNA fingerprinting

In order to accurately identify the origin of the

tumour cell lines, DNA was subjected to genetic fingerprinting using Southern analysis (Jeffreys et al., 1985). 10ug of DNA digested with the restriction endonuclease HinfI (Amersham, UK) was separated by electrophoresis through 20cm-long 0.8% agarose gels, then transferred to Hybond-N membranes and hybridised to ^{32}P-labelled probes, as described in section 2.6.

The probes used were the locus-specific fingerprinting probes g3 and MS1 (Wong. Z. et al., 1987) (obtained from Cellmark Diagnostics, courtesy of Prof. A.J. Jeffreys). Both probes were labelled by the random-primer method described in Section 2.6.II, and the final washes following hybridisation were carried out at 65^{0}C.

3.3 RESULTS

3.3.I. A transforming gene is detected in tumour cell DNA

3.3.I.a. Primary transfection

The NIH3T3 cells were thought to be transformed if they exhibited a fusiform morphology, were no longer contact inhibited and were more refractile when compared with the surrounding NIH3T3 cells outside the colony (Shih et al., 1979). Transformation was demonstrated in NIH3T3 cells transfected with DNA derived from the IN 157 cell line, thought to be derived from a malignant oligodendroglioma (Kennedy et al., 1987). A single transformed colony of fibroblasts was visible in each of 2

out of 3 plates of cells transfected with IN 157 DNA (see Figure 3.2). It was felt that the transformed colonies exhibited a morphological appearance characteristically induced by an activated ras oncogene (Dr. C.S. Cooper, personal communication). The two foci produced following IN 157 DNA transfection were termed primary transformed foci 2a and 2b. No other transformed foci were seen in DNA derived from any of the other glioma cell lines. DNA derived from the MB 486a cells (which was included as a positive control to demonstrate the efficacy of the transfection technique), induced the formation of a single focus of cells bearing transformed morphology. There were no colonies visible in cells transfected with the negative control DNA derived from Thymoma 4, which had consistently failed to give rise to colonies in NIH3T3 cell transfection experiments (see Table 3.1, for summary of results).

3.3.I.b. Secondary transfection

Following a single round of transfection, it is possible that NIH3T3 cell transformation may be due to the introduction of multiple human genes, which would make identification of any single transforming gene impossible. Therefore, a second round of transfection was carried out using DNA extracted from transformed cells cultured from primary transfection foci 2a and 2b (derived from the IN 157 tumour cells), in order to further dilute the amount of human DNA introduced into the mouse indicator cells. It has

been shown statistically that a transformed focus (i.e. secondary transfectants) arising from the uptake of human DNA derived from primary transfectants is likely to be due to the presence of a single human gene (Shih et al., 1979).

DNA (60ug) extracted from each of the primary foci (2a and 2b) was transfected into NIH3T3 cells exactly as described for the primary transfection protocol, with the identical positive and negative controls being used. DNA derived from the primary transfectants produced a total of 12 new foci with a transformed cellular morphology, 10 foci from primary transfectant 2a, and 2 foci from primary transfectant 2b. Four of the secondary foci from primary transfectant 2a and a single secondary focus from transfectant 2b, chosen at random, were cloned and cultured for further DNA analysis and named 2a.2-2a.5 and 2b.2, respectively. DNA from the MB 486a cell line used as a positive control produced a single focus with a transformed morphology, while the Thymoma 4 DNA used as a negative control failed to produce any foci. The absence of transformed foci in the negative controls in both the primary and secondary transfection experiments indicates that no sustainable spontaneous transformation events occurred in the NIH3T3 cells when cultured in the presence of 5% FCS.

Transfection of NIH3T3 cells with DNA derived from the IN 157 human glioma-derived cell line therefore resulted in the establishment of primary and secondary

transformed foci, consistent with the presence of a dominantly-transforming human oncogene.

3.3.II. Initial characterisation of the transforming DNA

The commonest activated genes detected by the NIH3T3 transfection assay are members of the ras gene family, c-Ha-ras1, c-Ki-ras2 and N-ras (Reviewed in Barbacid, 1987 and Bos, 1989). In order to detect whether the gene transfected from IN 157 cells was a member of the ras gene family, Southern blots of genomic DNA prepared from primary and secondary transformed foci were hybridised to human ras gene probes.

3.3.II.a. Hybridisation with the c-Ha-ras1 probe

A Southern blot made with 10ug of DNA digested with EcoRI was hybridised to the radiolabelled 2.9Kb SacI human c-Ha-ras1 genomic probe (Reddy et al., 1982). Following a high stringency wash, the c-Ha-ras1 probe failed to detect any homologous sequences in the EcoRI-digested DNA derived from the primary and secondary transformed foci, suggesting that the human c-Ha-ras1 gene was not responsible for transformation of the mouse NIH3T3 cell foci. Both the normal human DNA and the IN 157 tumour cell DNA revealed the presence of a band of 23Kb, the expected size for human c-Ha-ras1, as anticipated, since the c-Ha-ras1 gene is present in total genomic DNA (Figure 3.3 B).

3.3.II.b. Hybridisation with the N-ras probe

The EcoRI Southern blot was then re-probed with the 1.4Kb PvuII human N-ras genomic fragment (Hall et al., 1983) after the c-Ha-ras1 probe had been removed. Both the primary and secondary transfectants revealed the presence of a single 7.5Kb band, the expected size for the N-ras gene when digested with EcoRI. As expected, DNA from untransformed mouse NIH3T3 cells failed to reveal the presence of the human N-ras gene, (which is an indication that the probe used is specific for human, as opposed to mouse, N-ras sequences). The presence of human N-ras genomic sequences in cells derived from 2 out of 2 primary transformed foci and all 5 of the secondary foci suggested that the transformed phenotype was associated with the presence of the N-ras gene. Since only one transfected human gene is likely to be present in the mouse NIH3T3 cells following 2 rounds of transfection, it was therefore possible that the exogenously-derived N-ras gene was responsible for conferring the transformed phenotype.

The lack of growth of transformed foci following transfection with either the Thymoma 4 negative control DNA or with DNA derived from the human gliomas other than IN 157 was further evidence that, under the experimental conditions described, NIH3T3 cell transformation was brought about specifically by the introduction of the N-ras gene from the IN 157 tumour cells.

Previous authors (Shih et al., 1981; Tabin et al.,

1982) have shown that transformation of NIH3T3 cells by human oncogenes occur when the oncogene is abnormally activated. The result of the experiment described above suggest that the N-*ras* gene derived from the IN 157 cells caused transformation when introduced into the NIH3T3 cells because the gene was in some way functionally abnormal, and therefore it was decided to define the abnormality.

3.3.III. Defining the molecular abnormality in the transfected N-ras gene

3.3.III.a. Amplification of part of the N-ras gene known to be frequently mutated

In human tumours, activation of *ras* family oncogenes has usually been due to mutations in codons 12, 13 or 61 of the *ras* protein (Bos, 1989). Accordingly, attempts to define any molecular abnormality which rendered the transfected IN 157 N-*ras* gene oncogenic were directed initially at the DNA sequence of codon 61. The decision to examine codon 61 first, before examining codons 12 and 13, was made purely fortuitously.

A segment of genomic DNA surrounding codon 61 was amplified by the PCR method (described in Section 3.2.VIII). In order to assess whether the DNA segment amplified was of the predicted size of 112 base pairs (Farr *et al.*, 1988), the distance between the 5′ ends of the two primers, the PCR products were analysed by gel electrophoresis. Figure 3.4 shows the PCR amplification

products after electrophoresis on a 1.8% agarose gel. The DNA was revealed by ethidium bromide flourescence induced by long wave ultraviolet illumination. The expected 112bp amplified band is present in all the samples. In addition, several bands of higher molecular weight were present in DNA derived from normal placenta, NIH3T3 cells and transformed foci 2a and 2b2. The size of the bands varied from sample to sample. It was not certain whether these additional bands represented amplification of DNA fragments following adherence of primers to non-N-ras sequences (so-called "non-specific priming"), or whether they represented the formation of concatamers (Gibbs and Chamberlain, 1989). However, since the expected 112bp N-ras codon 61 fragment appeared to be the dominant amplification product, it was decided to proceed with Southern blotting and oligonucleotide probing, in order to establish the nucleotide sequence of the codon 61 fragment. If the adventitious bands were not identical to N-ras codon 61 sequences (i.e not concatamers of the 112bp amplification product), the oligonucleotide probe would not hybridise to the non-identical sequences under the high stringency conditions used (Wood et al., 1985).

3.3.III.b. Identification of the specific nucleotide mutation at codon 61 of N-ras

An oligonucleotide probe complementary to the normal ("wild type") codon 61 of N-ras (see Table 3.2), hybridised

to PCR-amplified N-ras sequences derived from normal placenta and the HT1080 human fibrosarcoma cell line (which has one normal N-ras allele and one mutated allele)(Hall et al., 1983; Patterson et al., 1987). In addition the wild type probe also hybridised to DNA derived from the IN 157 tumour cell line (Figure 3.5, panel A) which indicates that IN 157 cells have at least one allele encoding a normal N-ras codon 61. In contrast, the same probe to wild type human N-ras codon 61 failed to hybridise to either DNA from untransfected mouse NIH3T3 cells (as expected) or to DNA from the primary and secondary transformed foci, suggesting that the N-ras gene demonstrated in the transformed foci (see Section 3.3.II above) did not harbour a wild type codon 61 sequence. Thus the transforming N-ras gene was highly likely to have a mutation at codon 61.

In order to determine the exact nucleotide mutation at N-ras codon 61, the wild type probe was removed from the Southern blot and the blot re-probed sequentially with six oligonucleotide probes corresponding to all of the possible mutations at codon 61 (The sequences of the six oligonucleotide probes are given in Table 3.2).

The blots were probed initially with a mixture of two oligonucleotides encoding the A->C and A->T mutations at position three of codon 61, both of which encode the amino acid histidine. As seen on Figure 3.5, panel D, the position 3 mutation oligonucleotides hybridised to the primary and secondary transfectants, as well as to the IN

157 tumour cell line DNA, which had been the source of the DNA used to successfully transform the NIH3T3 cells. Therefore the IN 157 tumour cell line contained a codon 61 mutation in the N-ras oncogene, and this activated oncogene was capable of transforming NIH3T3 cells in a DNA transfection assay. As discussed, an oligonucleotide encoding the wild type codon 61 sequence also hybridised to the IN 157 DNA, indicating that these cells were heterozygous at the N-ras locus, with one normal allele and one allele encoding the codon 61 histidine mutation. As anticipated, the histidine-61 oligonucleotides did not hybridise to the normal placental DNA or to the HT1080 fibrosarcoma DNA, as the latter has a position one codon 61 mutation.

Hybridisation with one of the remaining probes (corresponding to a mutation at position 1 of the codon which would result in substitution of the wild type glutamine for a lysine residue), indicated that neither the human N-ras genes present in the IN 157 tumour cell line nor the IN 157-derived DNA present in the NIH3T3 transformants possessed a codon 61 lysine mutation (Figure 3.5, panel B). As expected, the lysine-61 probe did not hybridise to normal human placental DNA (which encodes glutamine at position 61). The lysine-61 probe did however hybridise to amplified sequences from the HT1080 human fibrosarcoma cell line which is known to be heterozygous for the N-ras gene, harbouring one normal allele and one

allele encoding a mutated codon 61 which encodes lysine (Patterson et al., 1987; Brown et al., 1984; Bos et al., 1984). The HT1080 DNA was included in this experiment to act as a known positive control for the PCR and oligonucleotide hybridisation steps, and was a gift of Dr. C.J. Marshall, Institute of Cancer Research.

Oligonucleotide probes corresponding to the remaining mutation at position 1 and to the 3 possible mutations at position 2 of N-ras codon 61 failed to hybridise to any of the amplified DNA samples on the blot, indicating that the mutation was not at position 1 or 2. Figure 3.5, panel C, demonstrates the failure of one of the position 2 probes (which would identify a locus encoding arginine, instead of the normal glutamine) to hybridise to any of the DNA sequences.

Since a mutation had been identified at codon 61 of the N-ras gene, it was decided not to examine codons 12 and 13 for evidence of mutations.

None of the oligonucleotide probes used hybridised to the adventitious bands which were generated by the PCR reaction, therefore the presence of these bands did not interfere with the interpretation of the results.

3.3.IV. Examination of archival paraffin-embedded tumour tissue for evidence of the N-ras mutation

The presence of an N-ras mutation in the IN 157 tumour cell line was of interest, as it was thought to

represent the first identification of a ras gene mutation in a human glioma. However, it was conceivable that the IN 157 N-ras mutation could have arisen during the long period that the IN 157 cells had been in culture. Alternatively, it was possible that the N-ras mutation could have occurred during the process of transfection, as rare examples do exist of transfection-associated activation of non-ras oncogenes (Martin-Zanca et al., 1986). Since the results of the above experiments were potentially significant for the understanding of the molecular pathology of human gliomas, it was felt desirable to confirm that the N-ras codon 61 mutation was present in the cells of the primary oligodendroglioma which gave rise to the IN 157 cell line.

As with many cultured cell lines, none of the original tumour material remained. Therefore, the only possible source of DNA for analysis of the N-ras gene would be that contained in formalin-fixed tumour specimens which had been embedded in paraffin wax and used for histopathological diagnosis. The average length of the DNA molecules extracted from formalin fixed, wax embedded tissue is only 500 base pairs, and is thus unsuitable for routine Southern analysis, where high molecular weight DNA, of the order of 30-50Kb, is required. However, it is likely that at least some of the low molecular weight DNA molecules extracted from the paraffin-embedded tumour block would be contiguous over the 112 base pair region encompassing codon 61 of the N-ras gene. By using the PCR

technique, this fragment could be amplified and thus analysed further.

The IN 157 cell line was reputedly derived from a patient who was diagnosed as having a malignant oligodendroglioma, grade III-IV, in 1981. The patient subsequently had two recurrences within the next 18 months, both of which necessitated operation, and the recurrent tumours were given the same diagnosis. The three resultant samples of archival paraffin-embedded tumour material were obtained from the neuropathology department at Maida Vale Hospital, London, and were numbered as specimens 83/81, 78/82 and 259/82. The IN 157 cell line was reputed to have been derived from a portion of tumour number 259/82. DNA was extracted from the paraffin-embedded tumours as described in Section 3.2.VII, amplified by the PCR method, and subjected to Southern analysis exactly as described for the DNA derived from the transformed foci.

When the Southern blot of the amplified DNA from the 3 original tumours was probed with the same oligonucleotide specific for the N-ras codon 61 histidine mutation, there was no hybridisation to the tumour sample DNA (see lanes labelled 83/81, 78/82 and 259/82 in Figure 3.6, panel B). Hybridisation did occur to the DNA from the transformed foci 2a and 2a.5, as well as to DNA from the cell line TE671, which was used as a control, since it is known to harbour the identical mutation at codon 61 as the transformed foci and the IN 157 tumour cells (Figure 3.6,

panel B).

The absence of hybridisation of the oligonucleotide encoding the histidine mutation to the tumour-derived DNA was most unexpected. The possibility of DNA degradation, which may have occurred during formalin fixation or wax embedding, was excluded, since subsequent hybridisation of the same blot with the codon 61 wild type probe revealed strong hybridisation (Figure 3.6, panel A). The codon 61 wild type probe also hybridised to DNA from the HT1080 human fibrosarcoma cell line which, as described previously (section 3.3.III), is heterozygous for the N-ras gene, possessing one normal allele and one allele mutated at codon 61. The wild type probe also hybridised to DNA from the TE671 cell line, indicating that TE 671 is also heterozygous for the N-ras gene.

The lack of a demonstrable N-ras codon 61 histidine mutation in DNA derived from all 3 of the original tumour specimens, including specimen number 259/82, the reported source of the cell line IN 157, was a cause for concern. While it was theoretically possible that the N-ras mutation identified in the IN 157 cell line could have occurred as a tissue culture or transfection artefact, this was considered unlikely, due to lack of a reported precedent. The other possibility was that the IN 157 cell line had been contaminated by an unidentified cell line, and the contaminating cells possessed the N-ras mutation which had been identified.

A candidate cell line which may have contaminated the IN 157 cells was suggested by consideration of the following facts (see Section 3.4.III for a full discussion of these points, as well as for the appropriate references):

1) The TE671 cells that were used as a control in these experiments were shown to have the same N-*ras* mutation as the IN 157 cells, namely a point mutation at position three of codon 61, which results in histidine being substituted for glutamine.

2) The codon 61 histidine N-*ras* mutation occurs infrequently, and a thorough literature search revealed that the only other cell line in which it had been reported was the rhabdomyosarcoma line RD.

3) Recently, TE671 cells have been shown, by DNA fingerprinting, to be genetically identical to the rhabdomyosarcoma line RD (Stratton *et al.*, 1989a), hereafter referred to as TE671/RD.

4) Both the TE671/RD cells and the IN 157 cells had previously been cultured simultaneously in the same laboratory, prior to transfer of the IN 157 cells to our laboratory.

5) Both IN 157 cells and TE671/RD cells fail to stain for either GFAP or galactocerebroside C (gal-C).

Therefore, it was possible that the IN 157 cells had been contaminated by TE671/RD cells, and the original oligodendroglioma cells were therefore no longer present,

having been replaced by the TE671/RD rhabdomyosarcoma cells.

3.3.V. Examination of the IN 157 and TE671/RD cell lines by DNA fingerprinting

The so-called "fingerprinting" probes detect DNA restriction fragments which vary in size from individual to individual, but which are inherited in a stable Mendelian fashion, and are therefore highly conserved over many cell divisions in the progeny arising from one cell (Boukamp et al., 1988). The ability to detect such variable but highly conserved restriction fragments has proved extremely useful in cell lineage analysis (Jeffreys et al., 1985). It was therefore decided to use DNA fingerprinting in order to confirm the postulated identity of the IN 157 and TE671/RD cell lines.

Southern blots of HinfI-digested DNA were probed initially with the probe MS1 and, following subsequent removal of the MS1 probe, with the probe g3.

Figure 3.7 shows that the IN 157 cells and the RD cells have an identical DNA fingerprint as revealed by the MS1 and g3 probes. Two groups of IN 157 cells were analysed: IN 157 cells cultured and frozen down in another laboratory, prior to transfer of cell stocks to our laboratory (IN 157(E)) and IN 157 cells derived from the same laboratory, but which had been grown for a prolonged period in our laboratory, and which were used in the

transfection experiments described (IN 157(L)). In order to avoid any possibility of contamination between the two IN 157 lines (E and L) occurring in our laboratory prior to the fingerprint analysis, the IN 157 (E) cells were not cultured, rather, DNA was extracted directly from the frozen cell stocks.

The MS1 probe revealed that both IN 157 (E) and (L) cells possessed 2 alleles of identical size (Figure 3.7, panel A). The g3 probe revealed a single allele, which again was of an identical size. The g3 probe may have identified a second allele less than 1.5Kb in length, but the existence of background hybridisation below 2Kb when using the g3 probe made the identification of any allele of 1.5Kb or smaller impossible. DNA from the RD and TE671 cell lines exhibited bands of an identical size to those present in the two IN 157 cell lines. These results indicate that the IN 157 (E and L) cell lines are of the same genetic origin as the TE671/RD rhabdomyosarcoma cell line.

The degree of polymorphism of the alleles recognised by the MS1 and g3 probes is further demonstrated by the controls depicted in Figure 3.7. DNA in the lanes N-P and N-O was derived from 2 unrelated individuals, and both the MS1 probe (panel A) and g3 probes (panel B) identify 2 separate alleles in the genome of each individual which differ in sizes from the alleles of the unrelated individual in the adjacent lane. The DNA sample labelled N-TR is that of the father of the dizygotic twins, TR-1 and

TR-2. The lower allele revealed by both the MS1 and g3 probes is the same size in the father as well as the offspring, indicating that the lower allele was contributed by the father. The upper allele in lanes TR-1 and TR-2 differs in size from the upper allele of the father (lane N-TR), indicating that the upper allele in the twins DNA samples is inherited from the mother, whose blood was not available for analysis (seen most clearly in panel A). The lack of any visible upper (maternal) allele in the DNA of twin TR-1 when probed with the probe g3 (panel B) is thought to be indicative of a genetic polymorphism occurring in the maternal allele.

The evidence that the IN 157 cells are identical to the TE671/RD cells is thus compelling, when taking into account the identity demonstrated at the DNA fingerprinting level, the existence of the same rare N-ras codon 61 mutation and the lack of expression of GFAP or gal-C.

3.3.VI. DNA fingerprinting of cell lines other than IN 157

The existence of one case of cell line cross-contamination raised the question of whether more such cases may exist in the collection of glioma-derived cell lines present in our laboratory, since many of these lines had been obtained from the same laboratory as the IN 157 line. In order to address the possibility of contamination, samples of DNA from 3 of the cell lines (IN

223, IN 502 and IN 821), which was surplus to the requirements of the transfection experiment, were analysed by fingerprinting. The 3 cell lines chosen were all known to express the neuroectoderm-specific antigen A4. Cell lines expressing the A4 antigen made up a minority (10-15%) of the glioma cell line stocks in our laboratory (Kennedy, et al., 1987). In addition, it was decided to analyse DNA from frozen stocks of the 3 cell lines which were not the same batch of frozen cells as those used to generate DNA for the transfection. It was felt that the analysis of several different batches of cells from what was reputedly the same cell line, but which had been cultured and frozen down at different times (in some cases, separated by over a year), might bring to light a higher number of contamination events than the analysis of a DNA from a single batch of cells. A selection of other cell lines in the laboratory collection, (which had not been used in the transfection experiment), were also analysed for the presence of possible contamination events.

The results of the DNA fingerprinting analysis indicated that contamination events were widespread (Figure 3.8; the suffix "p" following the cell line number indicates the passage number of the frozen cell stocks used to generate the DNA, for example "301p10" indicates cell line IN 301, passage number 10). All 3 of the A4 antigen positive cell lines whose DNA was used in the transfection experiment appear to be genetically identical when probed

with the fingerprinting probes MS1 and g3. Thus, IN 223p3, IN 502p9 and IN 821p3, all of which were used as sources of transfection DNA, show alleles migrating to similar positions (Figure 3.8). The presence of a single allele revealed by probing IN 502p9 with MS1 is could possibly be due to loss of one of the copies of chromosome 1, the site of the MS1 probe. A similar loss of one copy of chromosome 1 could account for the very feint upper MS1 allele in the IN 301p10 DNA; in this case, the majority of the cells in the culture could be derived from a cell which has lost the copy of chromosome 1 contributing the upper allele, while a minority of the remaining cells still have 2 copies of chromosome 1 and thus both MS1 alleles. The phenomenon of loss of a part of or the whole of chromosome 1 has been previously described in neoplasias of neuroectodermal origin (see Section 1.2.II.d.).

The 3 cell lines used for the transfection experiment, as well as the cell lines IN 500 and IN 301, were genetically identical to an extremely rapidly growing glioma-derived cell line known as U251. It is possible that U251, which has been grown for many years in neuropathology laboratories, could have been the cell line which contaminated the other cell lines, but further analysis on several laboratories stocks of U251 would be necessary in order to establish this.

There was also evidence of contamination of different passages of what was thought to be the same cell line. For

example, IN 502p9 was genetically different from IN 502p7 (Figure 3.8). Unlike IN 223p3, DNA from IN 223p7 did not even hybridise to the probes MS1 and g3 (Figure 3.8). Since, in the hybridisation conditions used, MS1 and g3 are specific for human DNA, this suggests that some time after the IN 223 cell line was frozen down at passage 3, it became contaminated by a non-human cell line.

The cell line which contaminated the three cell lines (IN 223p3, IN 502p9 and IN 821p3) was not IN 157/RD. This is demonstrated in Figure 3.9, which reveals the differences between the allele sizes of IN 301p10 (which had previously been demonstrated to be genetically identical to IN 223p3, IN 502p9 and IN 821p3, see Figure 3.8) and the IN 157/RD cell line. One cell line which could possibly have contaminated the glioma cell lines is the HeLa line, which is ubiquitous in cell culture laboratories. Figure 3.9 demonstrates that HeLa cells and their derivatives HeLa J, 2B and 2g3 are genetically dissimilar to both IN 157/RD and to the cell lines IN 301, IN 821 and IN 500, and therefore HeLa cells were not involved in the contamination events described here.

3.4. DISCUSSION

3.4.I. Identification of a transforming oncogene in tumour cell DNA

The efficacy of DNA transfection as a method for identifying dominantly acting activated oncogenes was

demonstrated in this experiment. An activated N-ras oncogene present in the genome of the IN 157 tumour cell line effectively transformed NIH3T3 cells. Furthermore, the exact nature of the activating point mutation in the N-ras oncogene was identified by means of probing PCR-amplified DNA with oligonucleotide probes specific for defined N-ras mutations.

The presence of a mutated N-ras oncogene in tumour cells originally believed to have been derived from a malignant oligodendroglioma was of interest. N-ras mutations had previously been reported in a number of human tumours, such as acute myeloid leukemia (Bos et al., 1985), the myelodysplastic syndrome, in which the presence of an N-ras mutation appears to correlate with the progression to acute leukaemia (Hirai et al., 1987) and in the human neuroblastoma cell line SK-N-SH (Taparowsky et al., 1983). There were no reports of N-ras mutations occurring in gliomas, and therefore it was thought that the mutation present in IN 157 was the first to be discovered. The contribution to the cell transformation process of a mutated N-ras oncogene which erroneously substitutes histidine for glycine at codon 61 is thought to reside in the inability of the mutant oncoprotein to hydrolyse and release bound GTP (Section 1.3.II.b; Barbacid, 1987).

Mutagenic agents known to cause point mutations in oncogenes clearly induce gliomas in experimental animals (Section 1.4.II). It is not known what role they may play

in the genesis of human gliomas and it was hoped that the discovery of an N-ras mutation in an oligodendroglioma might illuminate some of the mechanisms underlying the genensis of these tumours.

3.4.II. Failure to detect the N-ras mutation in the original tumour tissue

The usefulness of the PCR technique was demonstrated by the ability to perform accurate molecular analysis on DNA extracted from solid tumour tissue which had undergone routine formalin fixation and paraffin embedding over 6 years before the DNA was removed.

Examination of three solid malignant oligodendroglial tumours removed at different times from the same patient, including the tumour from which the IN 157 cell line containing the mutant N-ras gene was supposedly derived, failed to reveal the presence of the N-ras mutation, although the normal codon 61 was demonstrated. It was felt that the most likely explanation would be that the cells currently growing under the guise of the IN 157 line were, in fact, another cell line which harboured the N-ras mutation, and which had contaminated the original IN 157 oligodendroglial cells.

3.4.III. A candidate contaminating cell line

The N-ras histidine-61 mutation identified in the IN 157 tumour cell line occurs in a minority of cases of

mutation involving codon 61. For example, in one series of 6 N-ras codon 61 mutations found in human tumour cells, only one mutation coded for histidine in place of the normal glycine, the others encoded arginine (2 cases), lysine (2 cases) and leucine (1 case) (Nishimura and Sekiya, 1987). One other human cell line which possesses the histidine mutation at codon 61 is the rhabdomyosarcoma line RD, originally isolated in 1969 (McAllister et al., 1969; Bos et al., 1984).

Several lines of evidence existed which, when taken together, suggested that the TE671/RD rhabdomyosarcoma cell line may have contaminated the original IN 157 cell line:

a) Both lines possessed the identical rare N-ras mutation

b) The IN 157 cells also failed to stain with antibodies to the oligodendroglial marker galactocerebroside-C (gal-C), or with antibodies to glial fibrillary acidic protein (GFAP), which is expressed by normal astrocytes as well as by neoplastic astrocytes and oligodendrocytes (Kennedy et al., 1987). Similarly, the TE671/RD cells did not express detectable gal-C or GFAP (Stratton et al., 1989a).

c) Recently, the TE671 cell line, which was originally thought to have been derived from a medulloblastoma, was demonstrated to be identical to the RD rhabdomyosarcoma cell line (and therefore possessed the same N-ras mutation), indicating that the RD cell line had

contaminated and outgrown the original TE671 cells (Stratton et al., 1989a). Both the TE671/RD cell line and the IN 157 cell line had, at one stage, been cultured simultaneously in the same laboratory (not our own). It was therefore possible that the original IN 157 cell line had been contaminated by the cell line then known as TE671, but which, as subsequently proved, was actually the rhabdomyosarcoma cell line, RD. It remained to be proven conclusively that the IN 157 cell line was, in fact, identical to the RD cell line.

3.4.IV. DNA fingerprinting established the true identity of the IN 157 cell line

It was felt that the most definite way to exclude or confirm the genetic identity of the IN 157 and TE671/RD cell lines was by means of DNA fingerprinting, using the probes MS1 and g3. Both MS1 and g3 are locus-specific fingerprinting probes which will identify a single tandem repeat sequence in the human genome (Wong, Z. et al., 1987). The large variation throughout the population in the number of repeat sequences lying adjacent to each other at the MS1 and g3 loci means that the tandem repeat units will vary accordingly in length and therefore also in terms of the relative distance of migration through a gel. The phenomenon of variability in size of the fragments of tandem repeat units is termed "polymorphism". The majority of individuals are heterozygous, i.e. exhibit two different

sized bands for each locus-specific probe used (Jeffreys, 1987). The high degree of polymorphism in the population leads to the finding that the bands revealed following Southern analysis of DNA from unrelated individuals are extrememly unlikely to be of an identical molecular weight. Theoretically, when only one of the locus-specific probes are used, the chances of both alleles from two unrelated heterozygous individuals being of identical length (and therefore migrating the identical distance through the gel) are 1 in 5000. When two different locus specific probes are used, as in the experiments described here, the chances of both pairs of alleles being of identical molecular weight in unrelated individuals becomes 1 in 5000^2, or 1 in $2.5x10^7$ (Wong, Z. et al., 1987). As a corollary, if the allele sizes revealed by the MS1 and g3 probes in a series of cell lines are identical, then it is extremely likely that the cell lines were derived from the same patient, and are thus the same cell line, or derivatives of the same cell line.

DNA fingerprinting indicated that, in the genome of the IN 157 and TE671/RD cells, the 2 alleles discernable with the MS1 probe and the single allele revealed by the g3 probe appeared to be of an identical size, and are therefore derived from the same cell line. The fingerprinting result, when taken in conjunction with the immunocytochemical staining properties and the fact that both IN 157 and TE671/RD possess the same rare N-ras

mutation, strongly indicate that the IN 157 cell line had been contaminated with the TE671/RD cell line. Therefore the source of the N-ras mutation was a rhabdomyosarcoma cell line, and not an oligodendroglioma cell line.

Neither the RD line nor the TE671 line had ever been cultured in our laboratory. However, both the TE671 (RD) cells and IN 157 (E and L) cells had been cultured in the same laboratory prior to transfer of the IN 157 (E and L) cells to our laboratory. Therefore, since the IN 157 (E) cells which were shown to be genetically identical to the TE671/RD cells had not been cultured at any stage in our laboratory, it is likely that the original IN 157 oligodendroglioma cell line became contaminated with TE671/RD cells prior to reaching our laboratory.

3.4.V. DNA fingerprinting of several cell lines revealed the presence of widespread contamination

Two of the 7 cell lines thought to be derived from gliomas and used as a source of DNA for the transfection experiment, were shown, with a high degree of certainty, to be genetically identical to each other, and to the commonly-used cell line U251. In addition, the IN 502p9 cells, thought to be derived from a chordoma, were also genetically identical to U251, raising doubts about whether the IN 502p9 cells were actually chordoma cells. Since line IN 502p9 differed genetically from IN 502p7, it was felt that the contamination event had occurred after the 7th

passage. It is not known whether IN 502p7 cells are in fact chordoma cells. The other putative glial cell lines, IN 293, IN 235, IN 353 and IN 231 were not tested for possible contamination. Therefore, cross contamination of A4 antigen positive cell lines appeared to be a common event, although these cell lines accounted for only 10-15% of the cell lines in our laboratory.

The failure of one of the cell lines tested to hybridise to the human-specific fingerprinting probes (IN 223p7) is another indication of the frequency of such contamination events.

3.4.VI. Implications of cell line contamination for future work

Cell line contamination was found to be a frequent phenomenon in the cell lines described here, and therefore it may be necessary to:

1) Verify that a transforming gene found in a cell line is also present in the original tumour.

2) In the case of cell lines which have been in culture for long periods, or which have been handled by inexperienced or inexpert operators, the necessity of establishing the identity of the cell lines by means of DNA fingerprinting must be considered.

TABLE 3.1

Cell Line/DNA	Diagnosis	Colony Count 1° Transfectants	Colony Count 2° Transfectants
MB 486a	Pos. transfection control	1	1#
Thymoma 4	Neg. transfection control	0	0#
IN 223	Mixed oligo-astrocytoma	0	-
IN 157	Malignant oligo.	2	10 and 2
IN 293	Glioblastoma	0	-
IN 235	Astro. Grade 4	0	-
IN 353	Malignant glioma	0	-
IN 502	Chordoma	0	-
IN 231	Astro. Grade 3	0	-
IN 821	Astro. Grade 2	0	-

Key
#: Identical MB 486a and Thymoma 4 DNA samples were used in both 1° and 2° transfection experiments.

Table 3.2. Sequences of the 20-mer N-ras probes

The nucleotide sequences of the codon 61 oligonucleotide probes are depicted. Codon 61 is underlined in the wild type (wt) probe sequence. The only variation between the probes is in the sequence of codon 61, which is listed for each probe, along with the amino acid (given on the right hand column) encoded by that particular codon sequence.

Abbreviations: p1-p3, nucleotide numbers 1-3 of the codon triplet.

TABLE 3.2

Sequences of the 20-mer N-ras probes

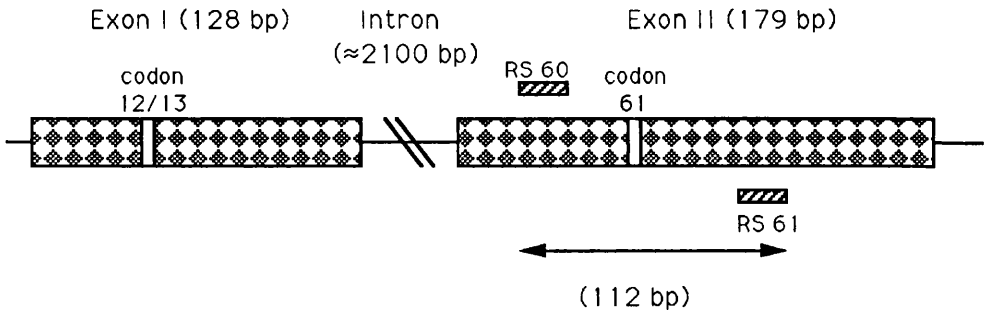
N61 wt	ACA GCT GGA CAA GAA GAG TA	Glutamine
N61 p1	AAA	Lysine
	GAA	Glutamate
N61 p2	CCA	Proline
	CGA	Arginine
	CTA	Leucine
N61 p3	CAC/T	Histidine

Figure 3.1. N-ras codon 61 primers

Schematic diagram of the N-ras gene showing the positions of the two PCR primers, RS60 and RS61, relative to codon 61, with their respective sequences.

FIGURE 3.1

N-ras codon 61 primers



Primer Sequences

RS 60	GTT ATA GAT GGT GAA ACC TG	(sense)
RS 61 (anti-sense)	ATA CAC AGA GGA AGC CTT CG	

Figure 3.2. NIH3T3 fibroblasts transformed by human tumour DNA

Photomicrograph of cultured mouse NIH3T3 fibroblasts, showing a transformed colony (darker cells growing in a swirling pattern, "T") on a background of untransformed fibroblasts (lighter cells, "U").

Fig. 3-2

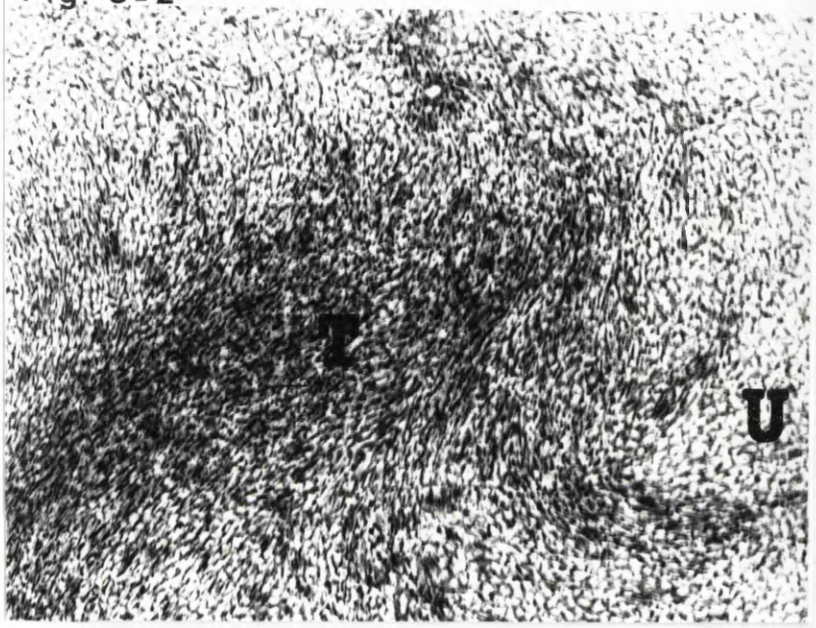


Figure 3.3. Human N-ras genes in transfected mouse NIH3T3 cells

Southern blot of EcoRI-digested DNA hybridised to a 1.4Kb human genomic N-ras probe (PvuII fragment)(panel A), followed by a 2.9Kb human genomic c-Ha-ras1 probe (SacI fragment) (panel B).

DNA samples (10ug per lane) were derived from normal human placenta ("normal"), IN 157 tumour cells, mouse NIH3T3 fibroblasts, primary transfectants 2a and 2b and secondary transfectants 2a.3, 2a.5 and 2b.2.

The 7.5Kb human N-ras sequences are present in all samples except the mouse NIH3T3 cells. The 23Kb human c-Ha-ras1 sequences are only present in the normal and IN 157 DNA and not in any of the transfectants.

The molecular weight was assessed by comparing the relative mobility of the DNA samples to that of HindIII-digested lambda phage DNA fragments.

Fig. 3-3

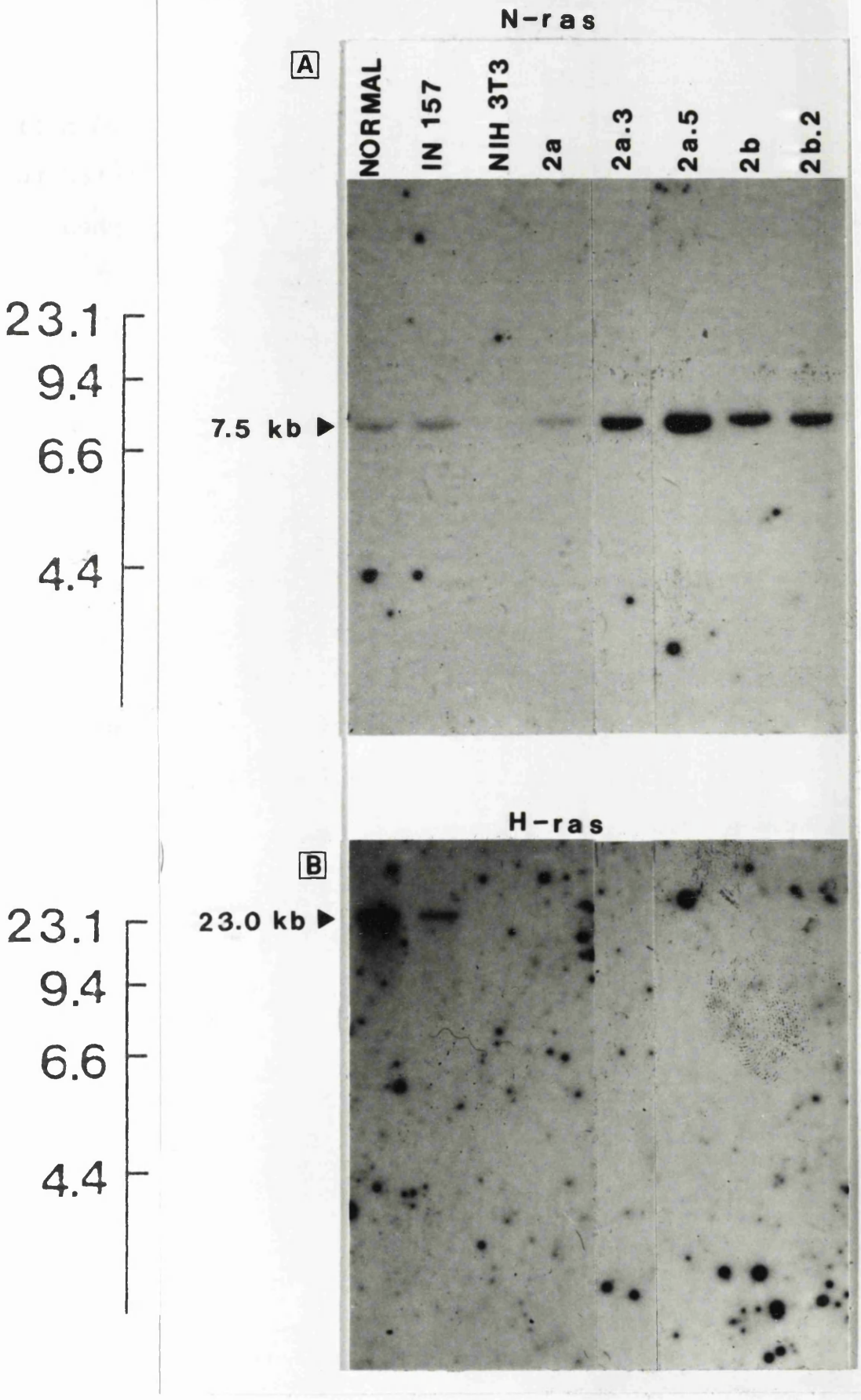

N-ras
A
NORMAL
IN 157
NIH 3T3
2a
2a.3
2a.5
2b
2b.2
23.1
9.4
6.6
4.4
7.5 kb
H-ras
B
23.1
9.4
6.6
4.4
23.0 kb

Figure 3.4. PCR amplification products

DNA sequences amplified by PCR using N-ras codon 61 priners (5ul of amplification mixture), were subjected to electrophoresis on a 1.8% agarose gel and photographed following staining of DNA by ethidium bromide.

The DNA samples used were as listed in the legend for Figure 3.3. In addition, DNA from the HT1080 human fibrosarcoma cell line was used ("HT1080").

All the lanes show the expected 112bp amplified fragment. In addition, adventitious amplified bands, larger than 112bp, are present in the lanes labelled Normal, NIH3T3, 2a and 2b.2.

The 125bp DNA fragment in the lane labelled MW (arrow) is derived from HindIII-digested lambda phage DNA.

Fig. 3-4

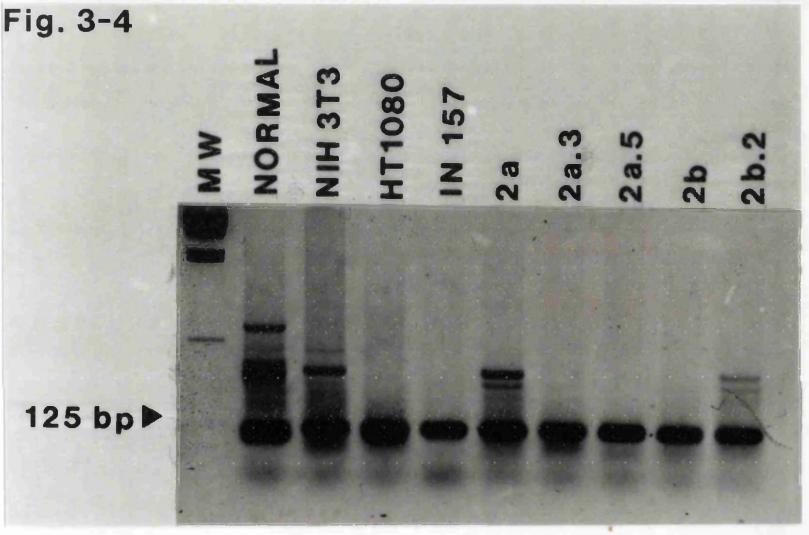


Figure 3.5. Hybridisation of PCR-amplified N-ras codon 61 sequences to oligonucleotide probes

Southern blot of 112bp PCR-amplified N-ras codon 61 fragments hybridised to oligonucleotide probes corresponding to wild type and mutated codon 61 sequences. The DNA samples are as listed in the legend for Figure 3.4.

N-ras codon 61 wild type sequences (encoding glutamine; panel A), are present in normal human DNA (Normal), HT1080 DNA and IN 157 DNA (the IN 157 and HT1080 cells have one normal and one mutated N-ras allele each). Mutated codon 61 sequences encoding histidine (panel D) are present in the IN 157 cell line and in the primary and secondary transfectants 2a, 2b, 2a.3, 2a.5 and 2b.2. Mutated sequences encoding lysine (panel B) are present only in the HT1080 fibrosarcoma cell line. None of the DNA samples possess a codon 61 arginine mutation (panel C).

Fig. 3-5

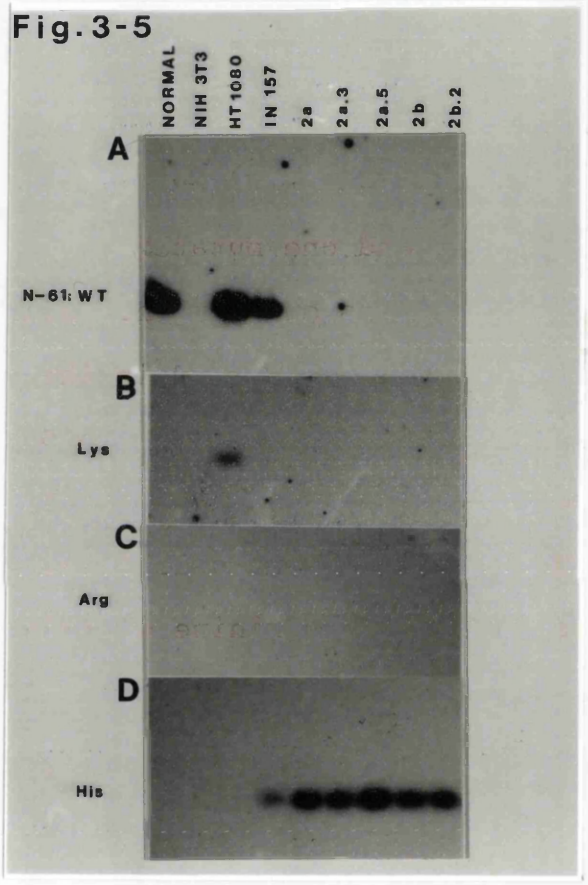

NORMAL
NIH 3T3
HT1080
IN 157
2a
2a.3
2a.5
2b
2b.2
A
N-61: WT
B
Lys
C
Arg
D
His

Figure 3.6. Oligonucleotide probing of DNA derived from transfectants and paraffin-embedded tumour tissue

Southern blot of 112bp PCR-amplified N-ras codon 61 fragments hybridised to oligonucleotide probes corresponding to the wild type and histidine-encoding mutated sequence. DNA samples were derived from mouse NIH3T3 fibroblasts, HT1080 fibrosarcoma cells, TE671/RD rhabdomyosarcoma cells (lane TE671) transfectants 2a and 2a.5 and paraffin-embedded tumour samples (lanes 83/81, 78/82 and 259/82).

N-ras codon 61 wild type sequences (encoding glutamine; panel A), are present in HT1080 and TE671/RD DNA and in DNA derived from the paraffin-embedded samples. Mutated codon 61 sequences encoding histidine (panel B) are present in the TE671/RD DNA and in the transfectants 2a and 2a.5, but not in DNA derived from the paraffin-embedded tumour samples (lanes 83/81, 78/82 and 259/82).

Fig. 3-6

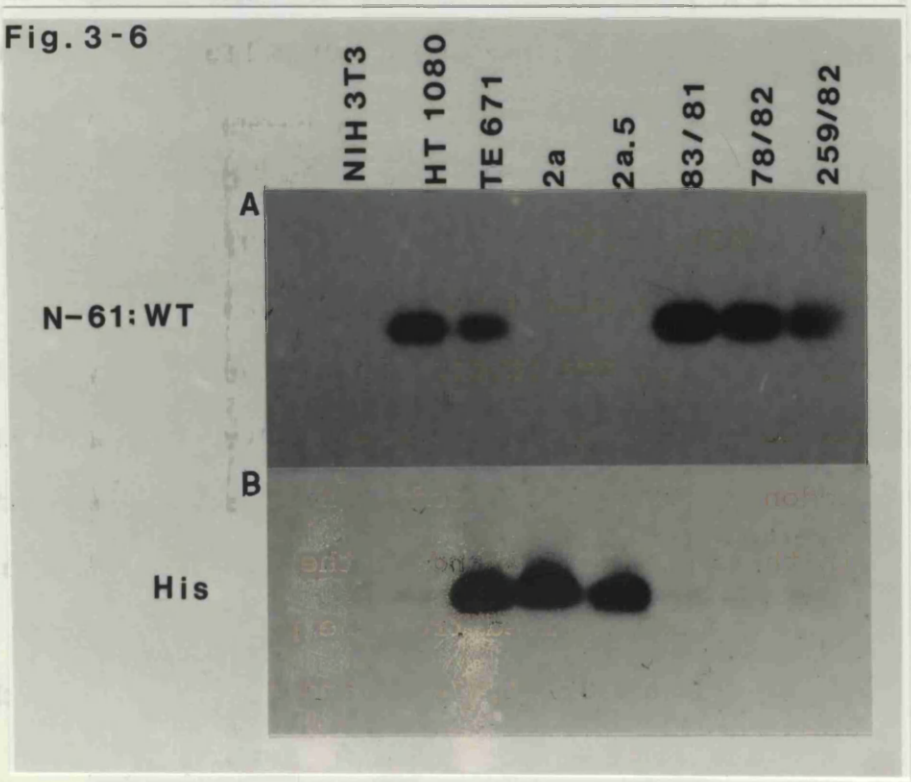


Figure 3.7. DNA fingerprinting of IN 157 and contaminating cell lines

Southern blot of HinfI-digested DNA hybridised to the locus-specific fingerprinting probes MS1 (panel A), followed by g3 (panel B).

DNA samples (10ug per lane) derived from early and late passage cultures of IN 157 tumour cells (lanes IN 157E and L, respectively), TE671/RD and RD cells. Control DNA samples were derived from peripheral blood lymphocytes of unrelated individuals N-P, N-O and N-TR. Controls TR-1 and TR-2 are dizygotic twins fathered by N-TR.

Identical migration of alleles is seen in lanes IN 157 E and L, TE671 and RD, suggesting that these cell lines are derived from the same cells. The individuals N-P, N-O and N-TR have different sized alleles. The dizygotic twins TR-1 and TR-2 have inherited the lower allele from their father, N-TR.

The molecular weight was assessed by comparing the relative mobility of the DNA samples to that of HindIII-digested lambda phage DNA fragments.

Fig.3-7

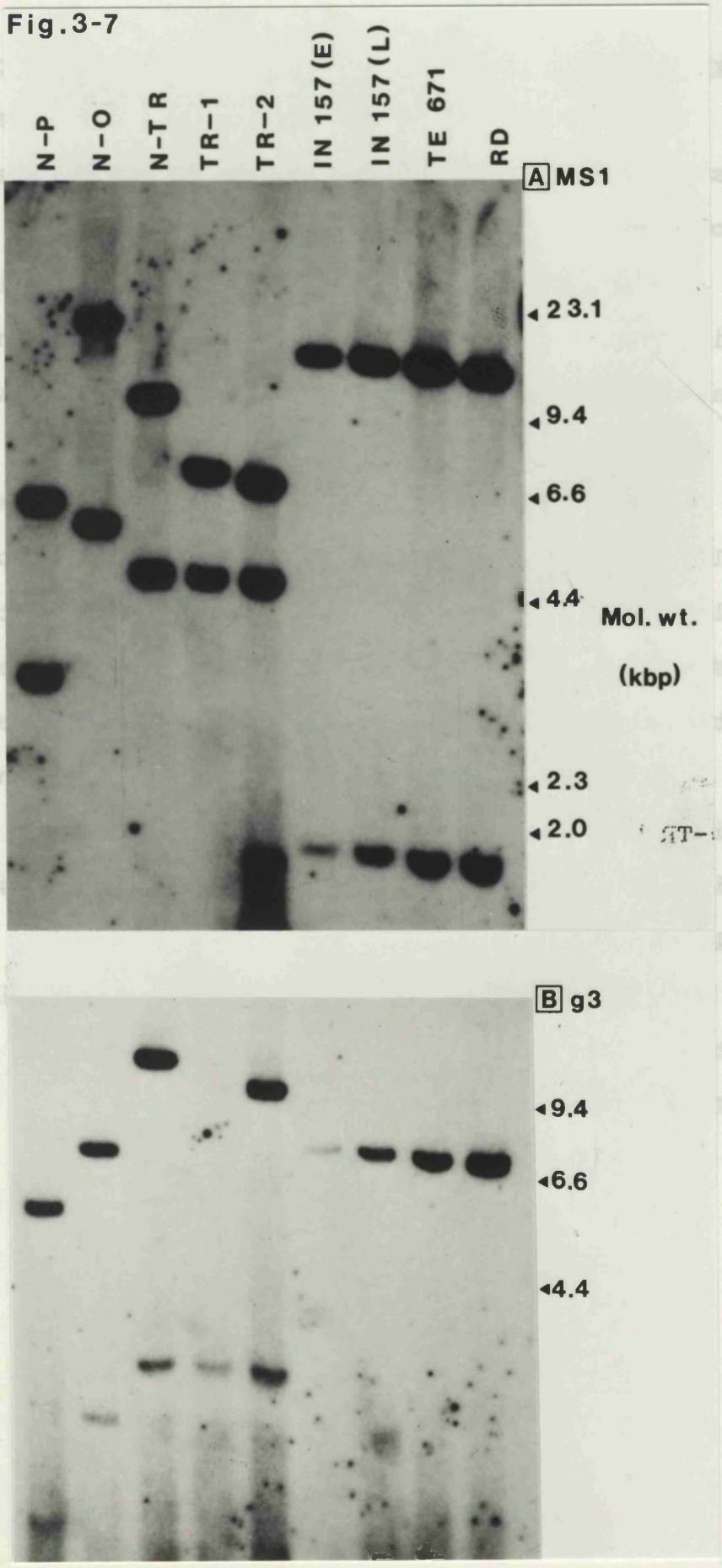

N-P
N-O
N-TR
TR-1
TR-2
IN 157 (E)
IN 157 (L)
TE 671
RD
A MS1
23.1
9.4
6.6
4.4
Mol. wt.
(kbp)
2.3
2.0
B g3
9.4
6.6
4.4

Figure 3.8. DNA fingerprinting of glioma-derived cell lines

Southern blot of HinfI-digested DNA hybridised to the locus-specific fingerprinting probes MS1 (top panel), followed by g3 (bottom panel).

DNA samples (10ug per lane) derived from cells initially cultured from a chordoma (lane 502p7) and gliomas (remaining lanes, except for the single lane containing HeLa cell DNA).

Identical migration of alleles is seen in lanes 301p10, 500p11, U251, 821p3, 223p3, 502p9 and 500p8. No alleles are visible in lane 223p7, suggesting that these cells are not of human origin. Although the alleles identified by the probe MS1 appear to be of an identical size in HeLa cells and 502p7 cells, these cell lines are not identical, as the alleles identified by probe g3 differ in size. The 301p10 and 502p9 cells are homozygous for the MS1 locus, but heterozygous for the g3 locus.

The molecular weight was assessed by comparing the relative mobility of the DNA samples to that of HindIII-digested lambda phage DNA fragments.

Fig. 3-8

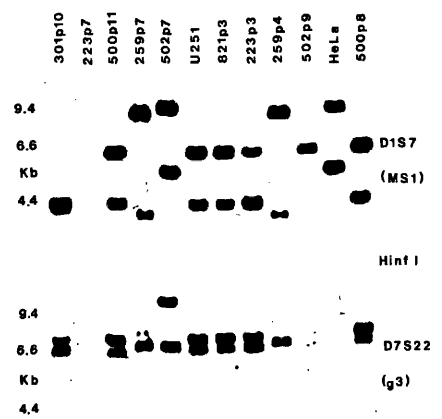

301p10
223p7
500p11
259p7
502p7
U251
821p3
223p3
259p4
502p9
HeLa
500p8
9.4
6.6
Kb
4.4
D1S7
(MS1)
Hinf I
9.4
6.6
Kb
4.4
D7S22
(g3)

Figure 3.9. DNA fingerprinting of glioma-derived cell lines

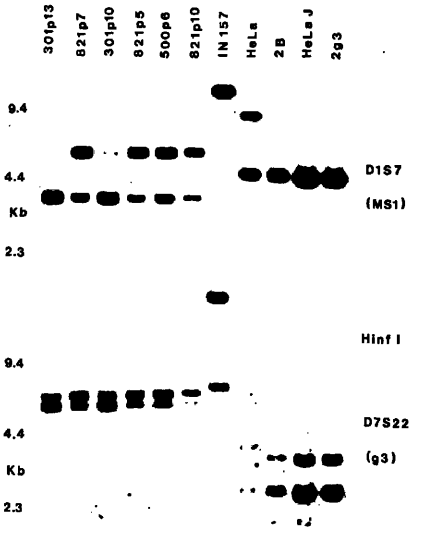
Southern blot of HinfI-digested DNA hybridised to the locus-specific fingerprinting probes MS1 (top panel), followed by g3 (bottom panel).

DNA samples derived from cells initially cultured from gliomas, including IN 157 cells. The control samples consist of DNA derived from HeLa cells and HeLa derived cell lines 2B, HeLaJ and 2g3.

Identical migration of alleles is seen in lanes 301p13, 821p7, 301p10, 821p5, 500p6 and 821p10, but these alleles differ in size from those of IN 157 cells. The 301p13 cells are homozygous for the MS1 locus, but heterozygous for the g3 locus. The HeLa-derived cell lines 2B, HeLaJ and 2g3 have all lost the large allele identified in the parent HeLa cells by the probe MS1.

The molecular weight was assessed by comparing the relative mobility of the DNA samples to that of HindIII-digested lambda phage DNA fragments.

Fig. 3-9


301p13
821p7
301p10
821p5
500p6
821p10
IN 157
HeLa
2B
HeLa J
2g3
9.4
4.4
Kb
2.3
D1S7
(MS1)
Hinf I
9.4
4.4
Kb
2.3
D7S22
(g3)

CHAPTER 4

THE SEARCH FOR ABNORMALITIES OF THE RETINOBLASTOMA GENE IN GLIOMAS

4.1 INTRODUCTION

The retinoblastoma gene RB is one of the best characterised of the tumour suppressor genes and is frequently found to be structurally altered in familial and sporadic retinoblastomas (Lee et al., 1987a). The abnormal RB protein function induced by alterations to the gene is thought to result in loss of an inhibitory effect on cell growth and seems to be an important mechanism underlying the development of retinoblastomas (Reviewed in Section 1.2.II.a; Benedict et al., 1983b).

4.1.I. Function of the RB protein

The RB protein has been localised to the nucleus and is expressed in all mammalian cell types examined to date, unless the gene is mutated (Lee et al., 1987a). The apparently ubiquitous expression of the RB protein suggests that it may play an important role in many cell types including retinoblastoma cells. The relative level of the RB protein appears to be constant throughout the cell cycle. The apparent paradox of why a protein thought to inhibit cell growth was present at a similar level in both actively dividing and quiescent cells was resolved by discovery that the phosphorylation state of the RB protein

varies. The RB protein is minimally phosphorylated in the G_0 and G_1 phases of the cell cycle, but becomes phosphorylated at multiple sites when cells enter the S phase, and remains phosphorylated during the G_2/M phases (Mihara et al., 1989). It appears that only the minimally phosphorylated form of the RB protein is capable of inhibiting cell growth, and that phosphorylation inactivates this inhibitory effect, allowing the cells to divide (Cheng et al., 1989; Buchkovich et al., 1989). The cell cycle-dependent RB phosphorylation and dephosphorylation is thought to be mediated by an as yet undefined group of protein kinases and phosphatases (Cooper and Whyte, 1989).

4.1.II. Possible mechanisms resulting in loss of function of both retinoblastoma gene alleles

A predisposition towards a cell becoming neoplastic as a result of malfunction of the RB gene requires the cell to have two abnormal RB alleles. In the hereditary form of retinoblastoma, a single mutated allele of the RB gene, situated on chromosome 13 band q14, is inherited in the germline and therefore every cell in the body will have one abnormal RB allele. In the sporadic form of the disease, the mutation rendering the first allele abnormal occurs randomly in a somatic cell (see Section 1.2.II.a). Studies on familial and sporadic retinoblastomas have demonstrated several types of mutation in the allele which is affected

first, including deletions of fragments of the coding sequence (Reissman et al., 1989; Friend, et al., 1986; Bookstein, et al., 1988), point mutations in the coding sequence (Dunn et al., 1988) and point mutations affecting splice acceptor sites, leading to lack of incorporation of an exon (Horowitz et al., 1989).

The most common molecular abnormality causing loss of function of the second allele is loss of the entire remaining normal copy of chromosome 13, usually brought about by non-disjunction of homologous chromosome pairs at mitosis (Cavenee and Hansen, 1986). However, loss of function of the remaining normal allele of the RB gene can also result from mitotic recombination involving the RB locus, or from additional mutations in the gene not related to abnormal mitotic events (Hansen et al.,1985). It is believed that, regardless of whether the entire chromosome 13 or only the portion encoding the RB gene is lost in a tumour cell, the effect of such an event is compatible with removal of the only remaining functional RB allele, and thus serves to unmask the presence of the other already mutated allele, with resultant loss of function of the RB protein (Hansen et al., 1985).

4.1.III. Evidence suggesting that retinoblastoma gene abnormalities may be found in gliomas

4.1.III.a. Cytogenetic abnormalities

The RB gene is on chromosome 13 band q14. Several

studies using cytogenetics have documented abnormalities of chromosome 13 in malignant gliomas, although these abnormalities are less common than those involving chromosomes 10 or 7 (described in Section 1.4.V.e). For example, one study on malignant gliomas showed that only 4 out of 32 tumours possessed a microscopically visible loss of part of or the whole of chromosome 13, a figure which contrasted with loss of chromosome 10 in 19 out of the same 32 tumours (Bigner et al., 1988a). The abnormalities described have included loss of one or both copies of chromosome 13 from the cell, as well as structural abnormalities of 13q, such as translocations (Bigner et al., 1986a; Rey et al., 1987; Bigner et al., 1988a). Therefore, structural cytogenetic abnormalities of chromosome 13 may involve the RB locus and cause abnormalities of the gene, while loss of the whole chromosome would result in loss of the entire RB locus from the cell (see Section 4.1.II).

4.1.III.b. Occurrence of gliomas in patients harbouring a germline retinoblastoma gene abnormality

Patients with familial retinoblastoma are thought to have one abnormal RB allele not only in the photoreceptor cells of the retina, but also in every other cell in the body (see Section 1.2.II.a). It is therefore possible that loss of function of the remaining RB allele in certain somatic cell types outside the retina could result in the

development of neoplasia in those cells. Several studies on patients who have inherited a germline RB mutation indicate that these patients have a high incidence of development of a second, non-ocular malignancy (8.4% at 18 years in the study of Draper et al., (1986)), suggesting that abnormalities of the RB gene may indeed lead to the formation of neoplasia in non-ocular cells. The majority of non-ocular second neoplasms in hereditary retinoblastoma cases are sarcomas, but several reports exist of these patients developing benign and malignant gliomas (Roarty et al., 1988; Draper et al., 1986; Abramson et al., 1984; Meadows et al., 1985). Many of the gliomas (as well as other non-ocular second neoplasms) which develop in these patients occur in the field of radiation administered previously as therapy for the retinoblastoma. It is possible, although not proven, that the radiation may damage the remaining normal RB allele in the cells later destined to form gliomas (Draper et al., 1986; Abramson et al., 1984).

Circumstantial evidence thus exists that abnormalities of the RB gene may contribute to the development of gliomas in patients known to have inherited an abnormal RB allele. In glioma patients who have not inherited an abnormal RB allele, the occurrence in a single glioma precursor cell of abnormalities of both RB alleles may contribute towards the development of neoplasia.

4.1.III.c. The oncogenes of DNA tumour viruses bind to the retinoblastoma gene product.

Certain DNA tumour viruses are capable of inducing gliomas following intracerebral inoculation in neonatal animals. The viruses are members of 2 apparently unrelated groups: The papovaviruses and the adenoviruses. Papovaviruses which induce experimental gliomas include SV40 which induces ependymomas, choroid plexus papillomas and astrocytomas in neonatal hamsters (Johnson, 1982; Palmiter and Brinster, 1985), the human JC virus which induces malignant astrocytomas and glioblastomas in owl monkeys (Miller et al., 1984) and hamsters (Walker et al., 1973) and the human BK virus which promotes the formation of choroid plexus papillomas in hamsters (Johnson, 1982). Adenoviruses which induce malignant gliomas in newborn hamsters include human adenoviruses types 5 and 12 (Tooze, 1980) and simian adenovirus type 7 (Ohtaki and Kato, 1989).

The glioma-inducing papova- and adenoviruses appear to induce tumour formation by means of the function of specific viral oncogenes. The major transforming proteins of the types 5 and 12 human adenoviruses and the simian adenovirus type 7 are known as the E1A proteins, and are all similar in structure (Kimelman et al., 1985). Furthermore, the major transforming proteins of the papovaviruses SV40, BK and JC are structurally similar, and are known as the large T proteins (Frisque, 1983). The adenovirus E1A transforming proteins do not, however,

structurally resemble the papovavirus large T proteins.

Although the adenovirus E1A and papovavirus large T oncoproteins do not resemble each other structurally, they may act to transform cells in a functionally similar manner. Thus, both the adenovirus E1A transforming protein (Whyte et al., 1988) and the SV40 papovavirus large T transforming protein (DeCaprio et al., 1988) have been shown to form a stable complex with the RB protein. The binding of the viral transforming proteins to the RB protein is thought to interfere with the postulated growth inhibitory function of RB, and the loss of normal RB protein function may contribute towards the development of neoplasia (Green, 1989; Dyson et al., 1989b).

The demonstration that oncoproteins from two groups of structurally-dissimilar DNA tumour viruses all have a similar function, namely the binding of the RB protein, suggests that RB binding might be a critical event in the generation of the tumours induced by these viruses. Since the viruses described here all induce gliomas, and since the viral oncoproteins have in common the ability to bind the RB protein, it follows that the induction of gliomas by the oncogenic papova- and adenoviruses may involve the functional inactivation of the RB protein.

In summary, three lines of evidence suggest that the RB gene may be involved in the genesis of gliomas. Cytogenetic alterations affecting chromosome 13, on which the RB gene is situated, have been described in human

malignant gliomas. In addition, patients known to have inherited one abnormal RB allele have been observed to develop gliomas with a higher frequency than anticipated, suggesting that somatic inactivation of the remaining RB allele (for example, by radiotherapy), may play a part in the development of these tumours. Finally, the oncogenic DNA viruses may induce gliomas in animals partially as a result of functional inactivation of the RB gene. It is therefore possible that abnormalities of the RB gene and protein may play a role in the development of human gliomas.

4.1.IV. The experimental approaches used to identify abnormalities of the RB gene

This chapter describes two different but complementary experimental approaches aimed at revealing structural abnormalities of the RB gene in a group of low grade and malignant gliomas. It was assumed that the mechanisms responsible for creating functional abnormalities of the RB gene in gliomas would be similar to those described for retinoblastomas (Section 4.1.II). The detection of events thought to render the first RB allele abnormal, such as point mutations or deletions of a small part of the coding region, can be technically extremely difficult, given the large size of the RB gene which extends for 200Kb (T'Ang et al., 1989). In contrast, loss of function of the second RB allele, which is thought to be

the only remaining functional copy of the gene, frequently involves loss of part of or the entire copy of chromosome 13 from the cell, and this is easy to detect using molecular genetic techniques (Hansen et al., 1985).

4.1.IV.a. Detection of loss of retinoblastoma gene alleles

Loss of an RB gene allele is identified by subjecting normal DNA (derived from peripheral blood leukocytes) and tumour DNA from the same patient to Southern analysis using a probe known to detect a locus within the RB gene (Cavenee et al., 1983; Wiggs et al., 1988). The probe chosen for this analysis is known to reveal two alleles in the normal (blood) DNA in most individuals and therefore the blood DNA will reveal 2 different sized bands, one from each parent (Wiggs et al., 1988). If one of the alleles of the RB gene has been lost in the tumour, this will be reflected by the absence of one of the parental bands in the tumour DNA relative to the normal DNA. As discussed previously, the absence of one RB allele may lead to abnormalities in RB protein function, provided that the remaining allele is also defective.

4.1.IV.b. Detection of structural abnormalities in the retinoblastoma gene

The second approach used is designed to detect structural abnormalities in the RB gene, such as deletions

of relatively large regions of the coding region. The technique involves Southern analysis of genomic DNA probed with a cDNA probe which identifies the coding region of the RB gene. The limitations of this second approach are such that only homozygous structural abnormalities are detectable, i.e. those abnormalities present in a single remaining allele, or the identical abnormality occurring in both alleles (Fung et al., 1987).

4.2. MATERIALS AND METHODS

4.2.I. Blood and tumour samples

Thirty six glioma samples were available for study. A portion of the tumour sample had been frozen immediately after biopsy and stored in liquid nitrogen until use. The remainder of the tumour was used to establish primary cultures of tumour cells, and to provide a routine histopathalogical diagnosis. Twenty ml of whole blood had been obtained from each patient, mixed briefly in a tube containing EDTA in order to prevent clotting, and subsequently stored at -70^{0}C until use.

The thirty six tumour samples included 12 benign and 24 malignant gliomas, of which 17 were diagnosed as glioblastoma multiforme (see Appendix I for full list of patient details and diagnoses). The diagnoses were made by consultant neuropathologists at the National Hospitals for Nervous Diseases, Queen Square and Maida Vale, London. None of the patients were known to harbour an hereditary

retinoblastoma diathesis.

4.2.II. Cell culture

The cell line IN 1434, which had been grown as an explant from primary tumour number 19, was cultured in DMEM supplemented with 10% FCS, in an atmosphere of 7.5% CO_2, as described in Section 2.7.

4.2.III. DNA extraction

DNA was extracted from solid glioma samples, peripheral blood samples and cultured cells as described in Section 2.2.

4.2.IV. Southern analysis to detect loss of heterozygosity at the retinoblastoma gene locus

To determine whether loss of RB alleles occurred in gliomas, 36 paired blood and solid tumour DNA samples were subjected to Southern analysis essentially as described in Sections 2.3 to 2.6. DNA (10ug) was digested with the restriction enzyme RsaI (Boehringer, UK), separated by electrophoresis on 20cm long 1.7% agarose gels, and transferred onto nylon membranes (Hybond-N, Amersham, UK). The blots were then probed at high stringency with the probe p68RS2.0, a 2Kb probe to the variable number tandem repeat (VNTR) region within the RB gene (Wiggs et al., 1988). Autoradiography was performed as described in Section 2.6.V, using pre-flashed XAR-5 film (Kodak, UK) at

-70^{0}C. Loss of heterozygosity was confirmed by means of scanning densitometry using a soft laser densitometer (Joyce Loebl, Gateshead, UK).

4.2.V. Southern analysis for detection of homozygous mutations in the retinoblastoma gene coding region.

10ug of DNA extracted from tumours was digested with the restriction enzyme HindIII (Boehringer, UK), separated on 10cm long 1% agarose gels and transferred to Hybond-N membranes as described in Sections 2.3 to 2.5. The blots were then probed sequentially at high stringency with the 3.9Kb EcoRI-EcoRI RB cDNA fragment, and following removal of the 3.8Kb probe, with the 0.9Kb EcoRI-EcoRI cDNA fragment (Fung et al., 1987). Autoradiography was performed as described in Section 2.6.V, using pre-flashed Xar-5 film (Kodak).

4.2.VI. Determination of the number of retinoblastoma gene alleles

To assess the number of RB alleles contributing to the RB gene HindIII banding pattern, the HindIII-digested Southern blots were re-probed with the 1.9Kb EcoRI-EcoRI fragment of the human c-erbA2 probe, which identifies a single copy sequence on chromosome 3 (Middleton et al., 1986; Weinberger et al., 1986). The number of alleles in the tumour DNA was assessed by means of scanning

densitometry.

4.2.VII. DNA fingerprinting

Paired blood and tumour samples were subjected to DNA fingerprinting essentially as described in Section 3.2.X, except that DNA samples were digested with the restriction enzyme Taq1 (Boehringer, UK) at 65^{0}C. The Southern blots were probed at high stringency using the locus-specific probes MS1 and g3 (Cellmark Diagnostics, courtesy Prof. A.J. Jeffreys), which identify loci on chromosomes 1 and 7, respectively (Wong, et al., 1987).

4.3. RESULTS

4.3.I. Loss of heterozygosity of a locus on chromosome 13 occurs in glioblastomas

The probe p68RS2.0 hybridises to a variable number tandem repeat (VNTR) region within the RB gene (see Figure 4.1) and detects a restriction fragment length polymorphism which has a high incidence of heterozygosity in the normal population (Wiggs, et al, 1988). Four of the 12 benign tumours analysed and 12 of the 24 malignant tumours, including 9 glioblastomas, were heterozygous for the p68RS2.0 VNTR locus.

Three of nine glioblastomas from heterozygous patients revealed loss of an allele in the tumour DNA relative to the normal DNA, using the p68RS2.0 probe (Figure 4.2 panel A, tumours 16, 38 and 29). In addition, there was loss of

both normal alleles in another glioblastoma (tumour 19), with the appearance of two new RsaI fragments, both larger than the original allele sizes (Figure 4.2 panel A, tumour 19 lanes B and T). Thus the tumours of 4 out of 9 heterozygous patients with glioblastomas revealed abnormalities of a locus on chromosome 13 situated within the RB gene, an incidence of 44%. The residual bands observed in the tumour DNA at the site of the affected allele were most probably due to the presence in the tumour homogenate of normal inflammatory and stromal cells which did not contain the deletion, a phenomenon which has been described previously (Fujimoto et al., 1989). Loss of heterozygosity was confirmed by scanning densitometry (Figure 4.3).

In order to eliminate the possibility that the appearance of the two new VNTR fragments in tumour number 19 were due to an artefact resulting from a mismatch of one patient's blood with another patient's tumour, the paired samples were subjected to DNA "fingerprinting" on Taq1-digested Southern blots using the locus-specific probes MS1 and g3 (Wong, et al., 1987). Alleles from both blood and tumour samples migrated to identical positions, indicating with a high degree of probability that they were derived from the same patient (Figure 4.4).

The two new RsaI fragments seen in DNA derived from tumour 19 were also present in cells derived from the tumour (Figure 4.2A tumour 19 lanes T and C), indicating

that the genetic lesion which had given rise to the formation of the two novel RsaI fragments in the solid tumour had been retained by the majority of the tumour cells in culture. Scanning densitometry revealed that the largest of these new bands is present at a density compatible with the presence of a single allele, consistent with the frequently observed allele loss seen in glioblastoma patients. However, the smaller of the novel bands in the tumour and cultured tumour cells has only one tenth this density (Figure 4.2A tumour 19 lane C and Figure 3.5, bands T1 and T1').

In order to confirm the abnormalities of the p68RS2.0 locus described in the glioblastomas, and to improve the separation of the alleles in tumours 16 and 29, the Southern analysis was repeated exactly as described previously (Section 4.2.IV) using the same DNA stocks derived from the blood and solid tumour samples, except that 2.5ug of DNA was loaded in each lane following RsaI digestion. There was insufficient DNA remaining from the cells cultured from tumour 19, and therefore a further batch of cells had to be grown up to provide DNA to repeat the experiment. As shown in Figure 4.2B, the results showing loss of heterozygosity were essentially identical to those already described and the objective of obtaining clearer separation of the alleles in samples 16 and 29 had been achieved. In the DNA derived from the new batch of tumour 19 cultured cells (Figure 4.2B, 19 lane C), there is

a difference in the intensity of the novel upper RsaI fragment relative to the lower RsaI fragment when compared to the relative intensities of the same fragments seen in the cell DNA used for the previous experiment (Figure 4.2A, 19 lane C).

4.3.II. Homozygous abnormalities are present in the coding region of the retinoblastoma gene in a glioblastoma

To search for homozygous abnormalities of RB, Southern blots of HindIII-digested DNA derived from tumour samples were probed with 2 RB cDNA probes. The first probe used was the 3.8kb EcoR1 RB cDNA fragment (Fung, et al., 1987). On normal HindIII digested DNA, this probe detects bands of 9.4, 7.4, 6.2, 5.3 (two fragments), 4.5 and 2.1kb in length (see Figure 4.1). The 4.5kb band is difficult to detect for technical reasons and therefore no emphasis will be placed on it in this study.

The 3.8kb RB probe revealed a homozygous genomic abnormality involving loss of the 9.4 and 6.2Kb HindIII bands in tumour 19, but in no other tumour samples (Figure 4.6A). In order to verify the abnormality seen in tumour 19, the experiment was repeated by analysing a further Southern blot in an identical manner on which DNA from the cultured tumour cells as well as the blood and tumour DNA had been immobilised. When compared to the normal 3.8Kb cDNA banding pattern, the loss of the 9.4Kb and 6.2Kb

HindIII bands in DNA from tumour number 19 was confirmed (Figure 4.7A). The residual bands of this size on the autoradiograph were most probably due to the presence in the tumour homogenate of normal inflammatory and stromal cells which did not contain the deletion (Fujimoto et al., 1989). This hypothesis was supported by the observation that cells cultured from this tumour, (which presumably did not contain any inflammatory cells or normal stromal cells), failed to reveal any 9.4 and 6.2Kb bands. In addition to the loss of the 9.4 and 6.Kb bands, tumour and cell DNA also contained a novel 5.1Kb band (Figure 4.7A, arrow)

After autoradiography, the 3.8Kb RB probe was removed and the blots were re probed with the 0.9Kb EcoRI RB cDNA fragment, which gives bands of the following sizes on HindIII digested DNA: 14.5, 5.8, 1.5 and 1.2Kb (see Figure 4.1). No abnormality was seen in tumour 19 or any other tumour when this 0.9Kb fragment was used (Figure 4.6B), although the results obtained by using the 0.9Kb fragment are often difficult to interpret, owing to the presence of background bands generated by cross-hybridisation of a GC rich region in the probe to sequences outside the RB gene (Fung et al., 1987).

To assess the allelic dosage contributing to the RB banding pattern, the relative intensities of the autoradiographic bands produced by the RB gene were compared to those produced by the the human c-erbA2 gene

which is present as a single copy in the genome (described in Section 4.2.VI). The intensities of the autoradiographic bands were measured by means of scanning densitometry. The density of the 7.4Kb RB band relative to that of the c-erbA2 band in the tumour and cultured cells was approximately half that of the ratio derived from scanning the same bands of the normal DNA samples, consistent with the loss of one allele. The loss of the 9.4 and 6.2 Kb RB bands in the tumour and cultured tumour cell DNA was also confirmed by the scanning densitometric analysis (Figures 4.7 and 4.8).

4.4. DISCUSSION

4.4.I. Detection of an abnormality in the coding region of the retinoblastoma gene in a glioblastoma

This series of experiments has revealed a structural abnormality of the coding region of the RB gene in a glioblastoma removed from a 70 year old man. The data on tumour 19 is consistent with the loss of one of the two alleles of the RB locus, (which would give rise to the reduced density ratio of the 7.4Kb band described in Section 4.3.II), accompanied by an internal deletion in the coding sequence of the remaining allele. The deletion most probably extends from 3′ to the 7.4Kb HindIII genomic fragment (still present in the tumour), to include the distal 5.3Kb and 9.4Kb fragments, and extends to involve the proximal portion of the 6.2Kb fragment, thereby

creating the new HindIII fragment seen measuring approximately 5.1Kb in length (see Figure 4.1 for illustration of relative position of HindIII bands).

The VNTR region identified by p68RS2.0 maps to the large intron present 3' to the 7.4Kb genomic HindIII fragment (as depicted in Figure 4.1). Tumour 19 appears to have a breakpoint within this VNTR fragment in the remaining allele giving rise to two new fragments, with only the larger of these capable of hybridising strongly to the probe in the DNA derived from the solid tumour. It has been postulated that tandem repeats such as this VNTR may act as recombination "hot spots" (T'Ang, et al., 1989; Wiggs, et al., 1988; Lehrman et al., 1987; Wahls et al., 1990). The observed appearence of two novel VNTR fragments of differing autoradiographic intensity may thus be the result of a complex recombination event involving this region within the remaining RB allele in this tumour. Two preparations of cells from tumour 19 which had been cultured for a different number of passages prior to DNA extraction appeared to show a difference in the relative intensity of hybridisation of the novel RsaI fragments to the VNTR probe (Figures 4.2A and 4.2B, 19 band C) The difference in intensity of hybridisation of the novel bands to the VNTR probe is consistent with further recombination events at the VNTR locus occurring in culture.

Further verification of the presence of a deletion in the RB coding region was obtained by the demonstration that

the cells cultured from tumour 19 produced a truncated RB mRNA transcript, as seen on northern analysis (Dr T.E.W. Riley and Mr.R.L Ludwig, personal communication; data not shown).

4.4.II. Loss of heterozygosity of the p68RS2.0 locus occurred in a total of four glioblastomas

In addition to tumour number 19, 3 of the glioblastomas studied displayed loss of heterozygosity of the chromosome 13 locus, p68RS2.0. The loss of heterozygosity at the RB locus was more frequent than the incidences of loss of heterozygosity of the loci on chromosome 1p identified by the probe MS1 (5.4%) and chromosome 7p identified by the probe g3 (0%), suggesting that the abnormalities in the RB gene were not merely a reflection of "background" or "random" cytogenetic abberations in the tumour (Appendix 1). The frequency of chromosome 13 allelic loss detected by molecular techniques (44%), is significantly higher than the incidence of chromosome 13 monosomy reported using classical cytogenetics (13%), on a cohort of malignant gliomas (Bigner et al., 1988a).

The loss of heterozygosity observed in these tumours could result from a loss specifically of one copy of the RB gene, from loss of a portion of chromosome 13, or from loss of an entire copy of the chromosome. It is not possible, by only probing a single locus on the chromosome, to detect

what mechanism underlies the loss of heterozygosity, nor is it possible to exclude the loss of other genes on chromosome 13 which may have a role to play in the development of neoplasia. Loss of heterozygosity involving the RB locus, such as the losses detected in these experiments, is thought to indicate loss of the normal RB allele, with the result that the cell would then contain a single mutated RB allele, leading to loss of normal RB protein function (see Section 4.1.IV). Therefore in tumours 16, 29 and 38, the loss of heterozygosity of the RB VNTR locus could lead to loss of normal function of the RB protein in the affected tumour cells if the remaining allele is abnormal. It is therefore necessary to demonstrate structural alterations of the remaining RB allele in these tumours. Such structural abnormalities of the remaining allele could not be detected by probing genomic DNA with a cDNA probe, as attempted here. Since lesions in the RB gene as small as single point mutations can lead to dysfunction of the protein (Horowitz et al., 1989), and the method of cDNA probing used in these experiments would not be capable of detecting such minute abnormalities, it is necessary in future studies to supplement the techniques used here with methods capable of detecting lesions as small as single point mutations (Dunn et al., 1988).

Loss of heterozygosity was detected in 4 glioblastomas out of the 9 glioblastomas in which such a

loss could potentially be observed. In 1 of the 4 tumours (number 19), there is a structural abnormality of the remaining RB allele. It is possible that subtle structural abnormalities may be present in the remaining RB allele of the 3 other glioblastomas. If such abnormalities were demonstrated, then it would mean that up to 44% of the glioblastomas in this series contain abnormalities of the RB gene. Owing to the fact that the no cytogenetic studies had been performed on the tumours analysed here, it was not possible to ascertain whether the material lost from chromosome 13 in the 3 GBM's was in fact a selective loss of the RB gene region. If the 3 GBM's did possess 2 copies of chromosome 13, then the observed homozygosity at the RB gene VNTR locus would indicate that there was selective loss of RB, which would strongly implicate RB abnormalities in the genesis of these 3 tumours. The fact that the rate of chromosome 13 allele loss detected by molecular techniques is much higher than the observed chromosome 13 monosomy detected cytogenetically, (44% as opposed to 13%; Bigner et al., 1988a), is consistent with the interpretation that the homozygosity observed at this locus represents abnormalities of the RB gene. Future studies on these 3 GBM's should include methods capable of detecting subtle abnormalities of the RB gene, in order to confirm whether the remaining RB allele is abnormal.

No abnormalities were seen in benign gliomas, however this may reflect the fact that the techniques used here do

not detect genetic alterations in tumour homogenates in which only a small percentage of the cells are actually tumour cells, as is the case in many benign tumours (Vogelstein et al., 1988). This is because any evidence of a molecular abnormality in the DNA of a tumour cell may not be detectable autoradiographically against the much stronger radioactive signal eminating from the DNA derived from the non-neoplastic cells (Fujimoto et al., 1989).

4.4.III. Abnormalities of the retinoblastoma gene occur in several non-glial tumour types

RB gene alterations have been found in a number of different types of human tumour other than the glioblastoma described in this chapter. These have included adenocarcinoma of the breast (Lee, et al., 1988; T'Ang, et al., 1988; Varley et al., 1989), small cell lung cancer (Harbour, et al., 1988), and in a bladder carcinoma cell line (Horowitz, et al., 1989). Abnormalities of both RB alleles have also been detected in osteosarcomas and other sarcomas arising in patients with the hereditary retinoblastoma diathesis (Hansen et al., 1985), as well as in sporadic soft tissue malignancies (Reissman, et al., 1989; Stratton, et al., 1989b).

4.4.IV. Summary: abnormalities of the retinoblastoma gene may contribute towards the genesis of glioblastomas

A structural abnormality of the RB gene has been detected in one glioblastoma using molecular genetic techniques, and three more glioblastomas have been identified which possess loss of an allele on chromosome 13, a finding commonly seen in tumours with functional abnormalities of the RB protein.

Loss of the normal function of RB, a putative tumour suppressor gene, may play a role in the development of the malignant phenotype of the tumours described here, which are similar to retinoblastomas in their origin from neuroectoderm. Indeed, cells cultured from one of the glioblastomas (tumour 19) contained both a deletion and an alteration of the RB gene identical to that of the parent tumour, suggesting that the cultured cells were clonal in origin, and that the abnormality in this gene may have contributed to the selective growth of this cell population in tissue culture, as well as in the original highly malignant tumour.

Figure 4.1. Structure of the RB gene and cDNA probes

Schematic diagram showing the structure of the RB gene and the HindIII fragments detected by the cDNA probes. Symbols II and * refer to exon allocations described by T'Ang et al., (1989). Exons 5,9 and 24 are too small to be detected using the Southern analysis described in this study. The lowest dark line indicates the deletion described in tumour number 19. "VNTR" = variable number tandem repeat.

FIG. 4-1

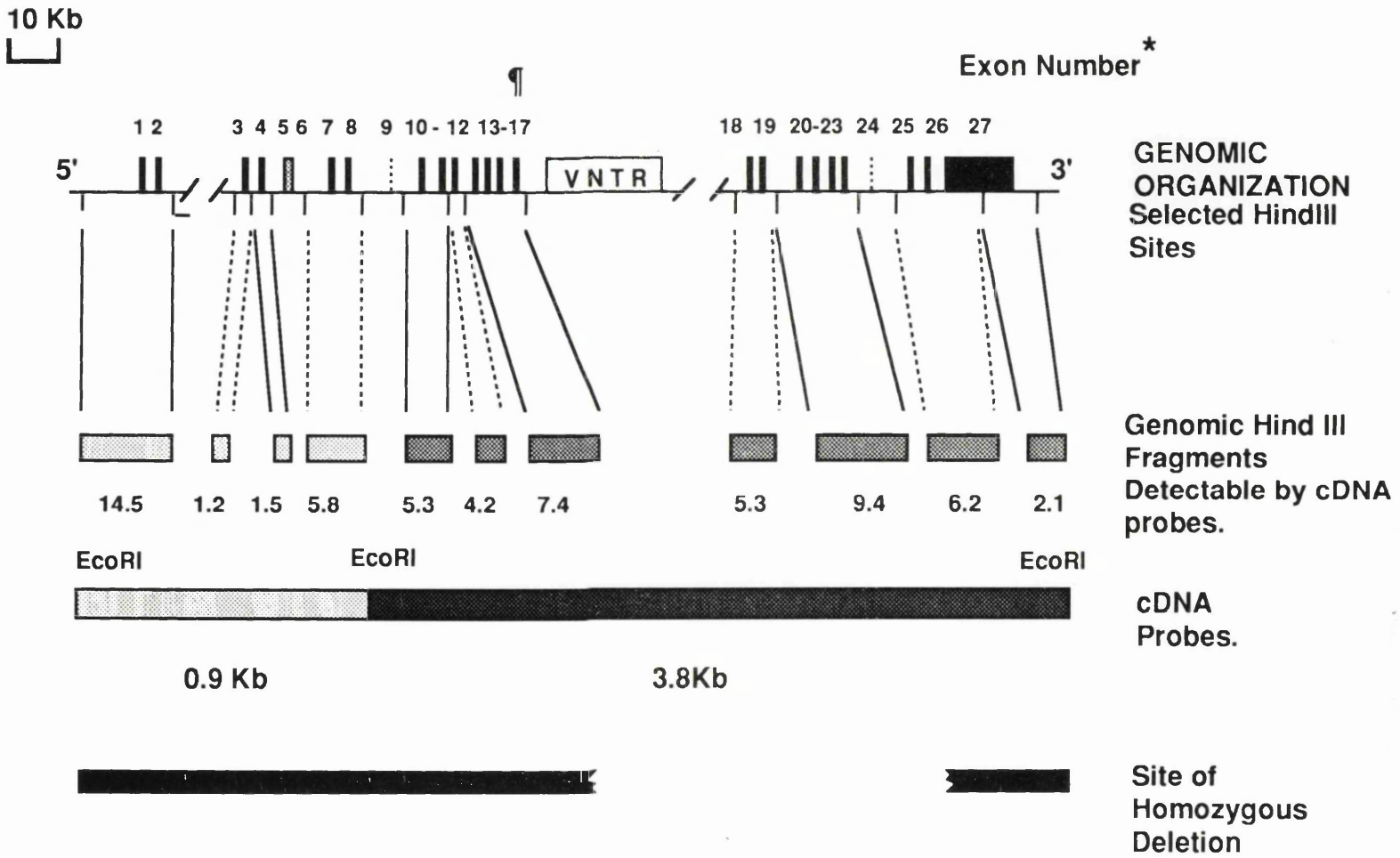

10 Kb
Exon Number*
¶
1 2
3 4 5 6 7 8
9 10 - 12 13-17
18 19 20-23 24 25 26 27
5'
VNTR
3'
GENOMIC ORGANIZATION
Selected HindIII Sites
14.5
1.2
1.5
5.8
5.3
4.2
7.4
5.3
9.4
6.2
2.1
Genomic Hind III Fragments Detectable by cDNA probes.
EcoRI
EcoRI
EcoRI
cDNA Probes.
0.9 Kb
3.8Kb
Site of Homozygous Deletion

Figure 4.2. Loss of chromosome 13 alleles defined by a locus within the RB gene in glioblastomas

Southern analysis of RsaI digested DNA probed with the chromosome 13 VNTR probe p68RS2.0. Panel A: 10ug DNA per lane; panel B: 2.5ug DNA per lane.

The numbers along the top identify each patient's paired blood (B), and tumour (T), DNA samples. Number 19 also shows DNA from cultured tumour cells (C).

Loss of heterozygosity can be seen in tumour numbers 16 (T, top allele), 38 (T, bottom allele) and 29 (T, top allele). Loss of both normal alleles can be seen in number 19, where the arrows indicate the novel bands generated by the breakpoint lying within the VNTR segment. Patient 13 is heterozygous for the p68RS2.0 locus (i.e. possesses 2 alleles of differing molecular weight) and has not lost an allele in the tumour. Patients 11 and 5 are both homozygous at this locus (i.e. possess an allele of similar size in both parents), and therefore loss of an allele in the tumour DNA cannot be detected in these two patients.

Kbp = molecular weight based on migration of HindIII digested Lambda phage DNA.

FIG. 4-2

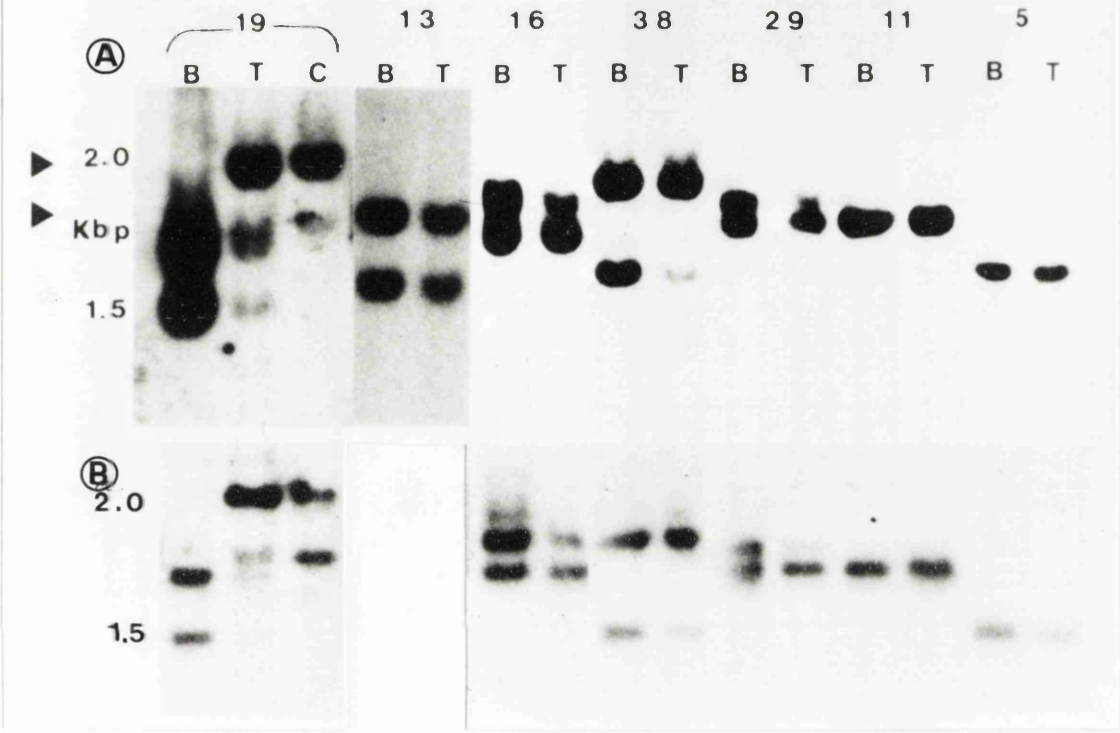


FIG. 4-3

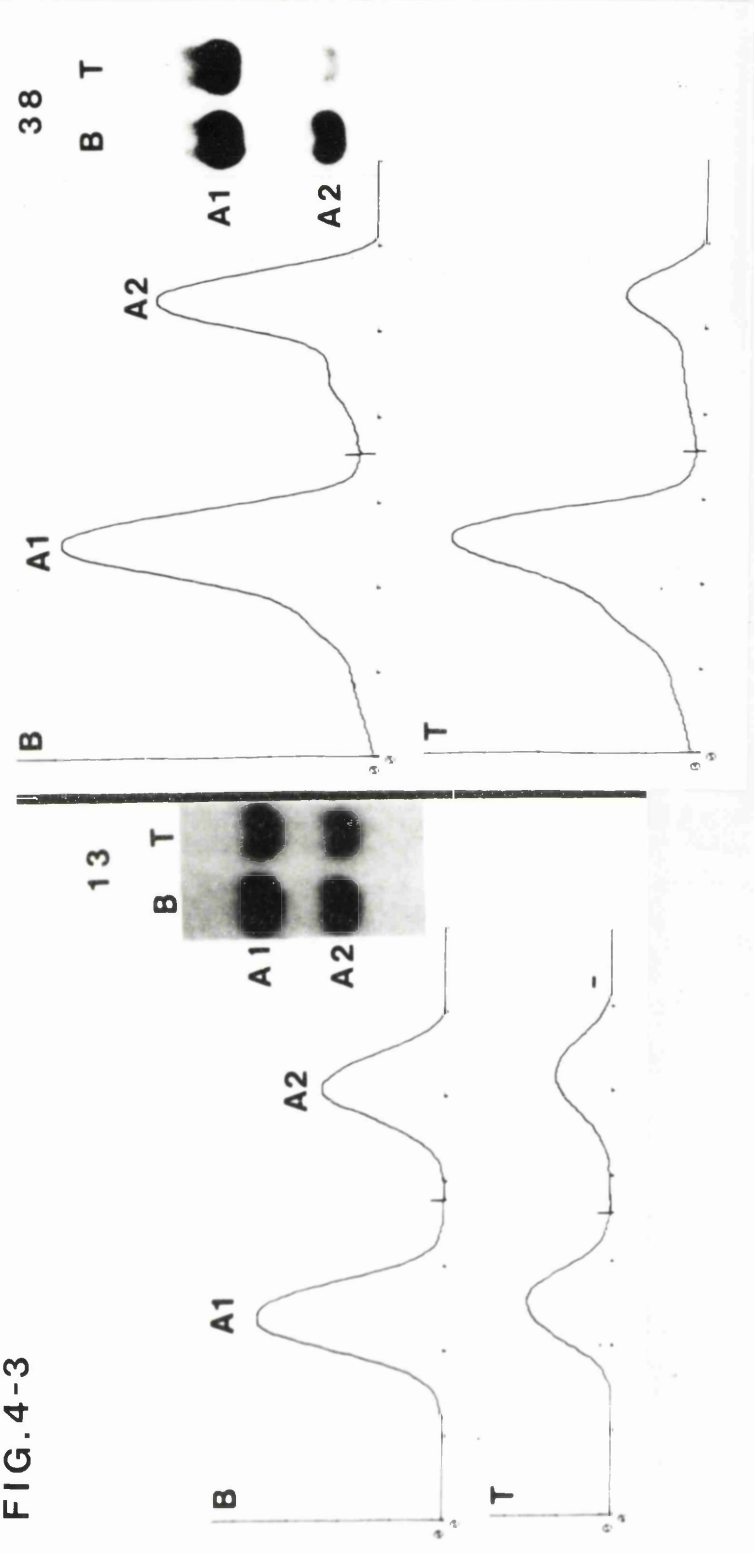


FIG. 4-4

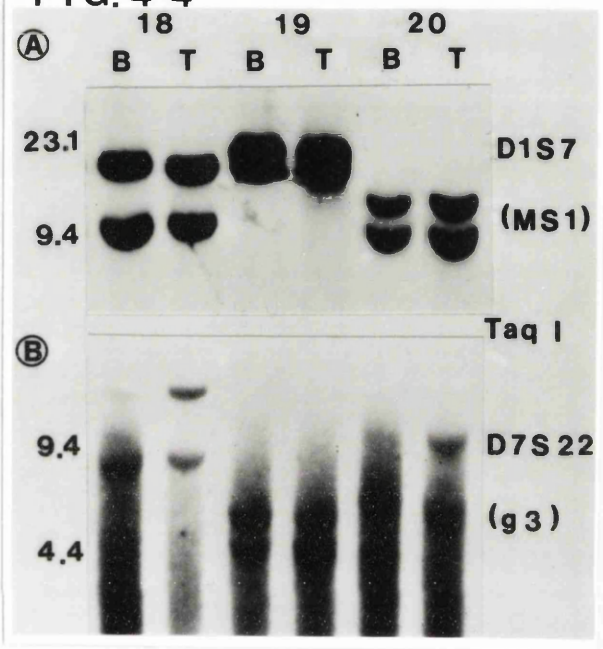
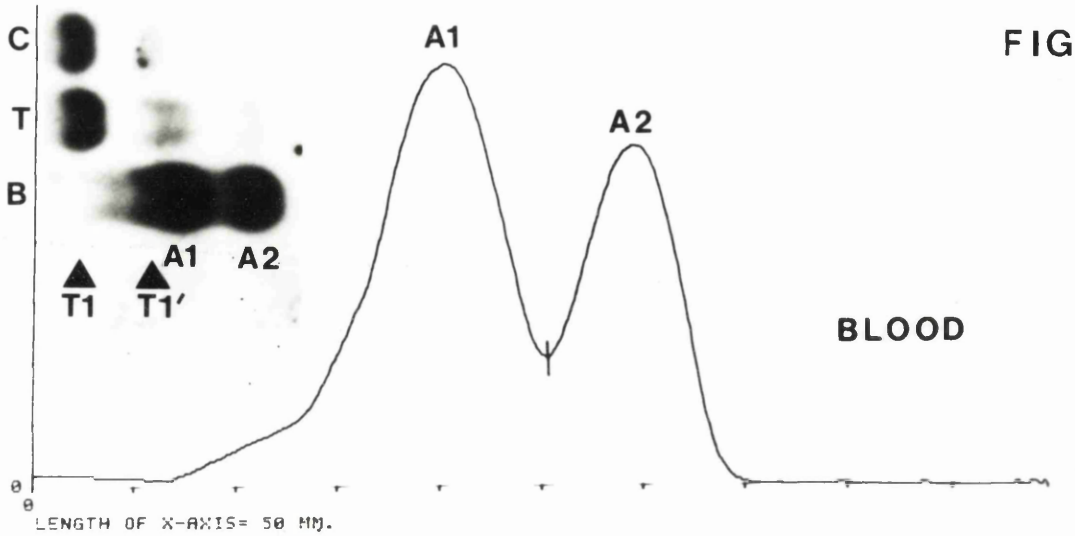


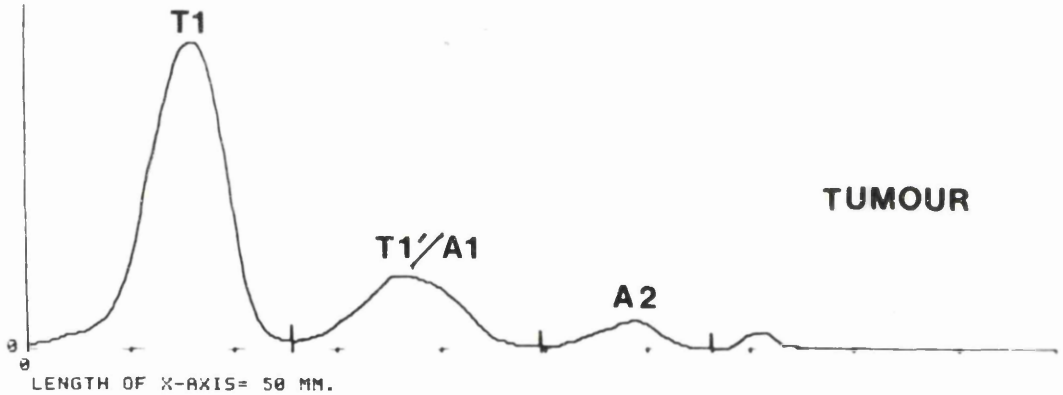
FIG.4-5



LENGTH OF X-AXIS= 50 MM.

TOT. INTEGRAL= 44315

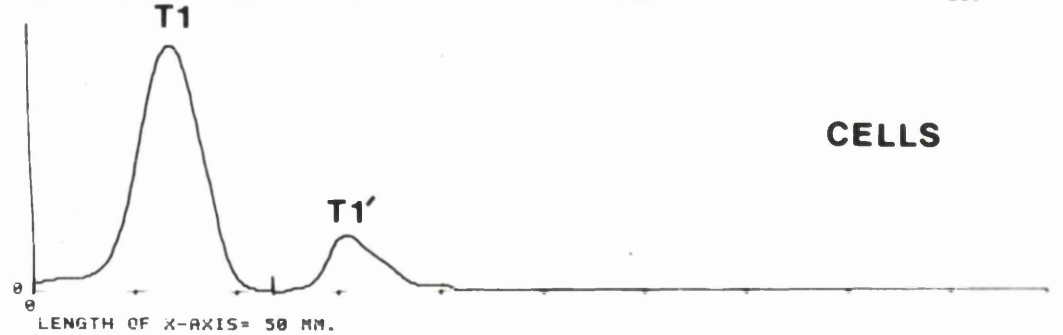
PEAK	POSITION	HEIGHT	REL%	INTEGRAL	PEAK	POSITION	HEIGHT	REL%	INTEGRAL
1	20.65	175	[illegible]	28424	2	29.95	141	35.95	15891



LENGTH OF X-AXIS= 50 MM.

TOT. INTEGRAL= 15218

PEAK	POSITION	HEIGHT	REL%	INTEGRAL	PEAK	POSITION	HEIGHT	REL%	INTEGRAL
1	8.2	122	71.15	10828	2	18.35	29	21.94	3324
3	29.6	12	5.43	827	4	35.7	7	1.57	239



LENGTH OF X-AXIS= 50 MM.

TOT. INTEGRAL= 9196

PEAK	POSITION	HEIGHT	REL%	INTEGRAL	PEAK	POSITION	HEIGHT	REL%	INTEGRAL
1	7.1	102	84.16	7740	2	15.65	24	15.92	1456

FIG. 4-6

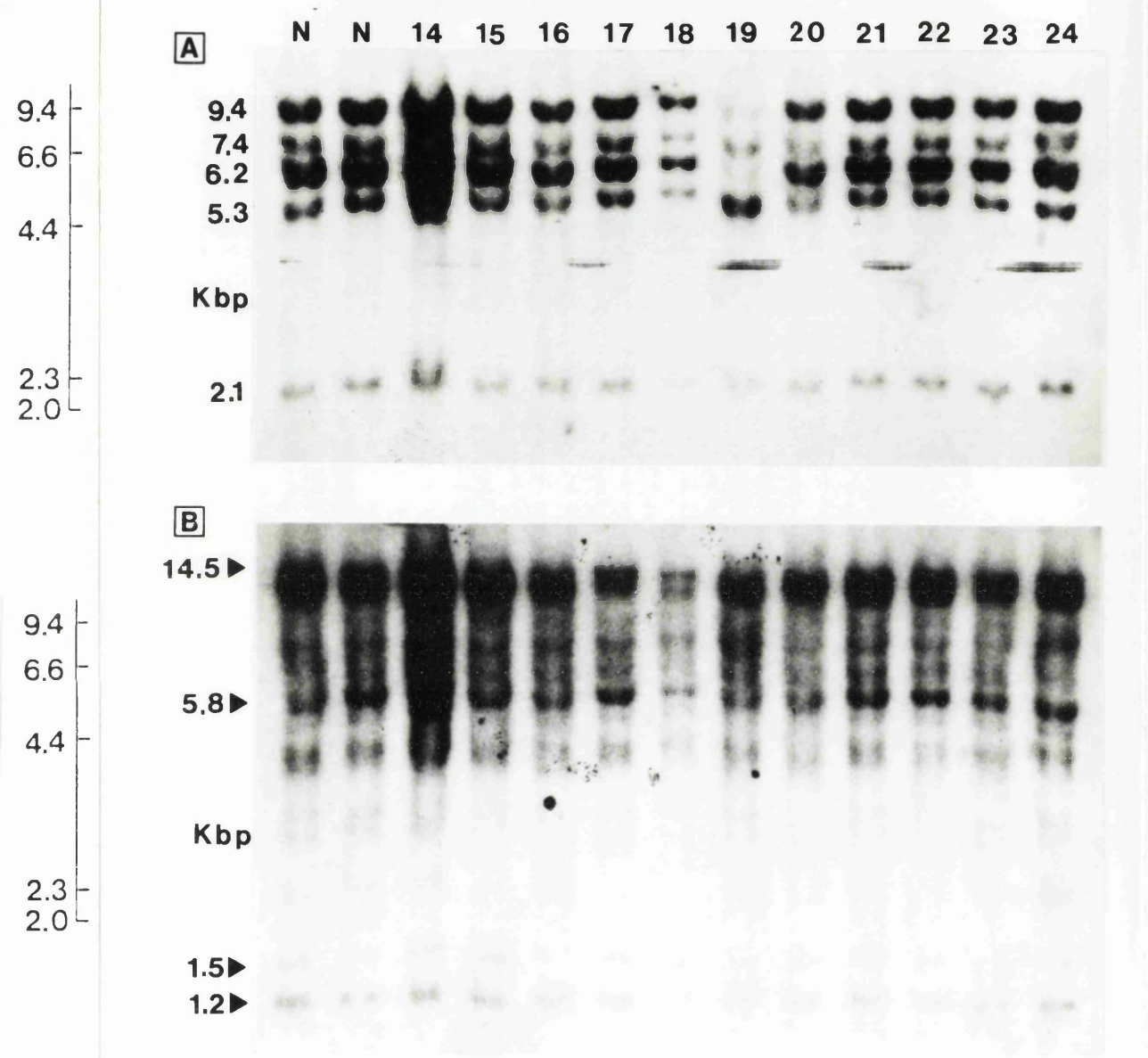

A
N N 14 15 16 17 18 19 20 21 22 23 24
9.4
7.4
6.2
5.3
Kbp
2.1
9.4
6.6
4.4
2.3
2.0
B
14.5▶
5.8▶
Kbp
1.5▶
1.2▶
9.4
6.6
4.4
2.3
2.0

FIG. 4-7

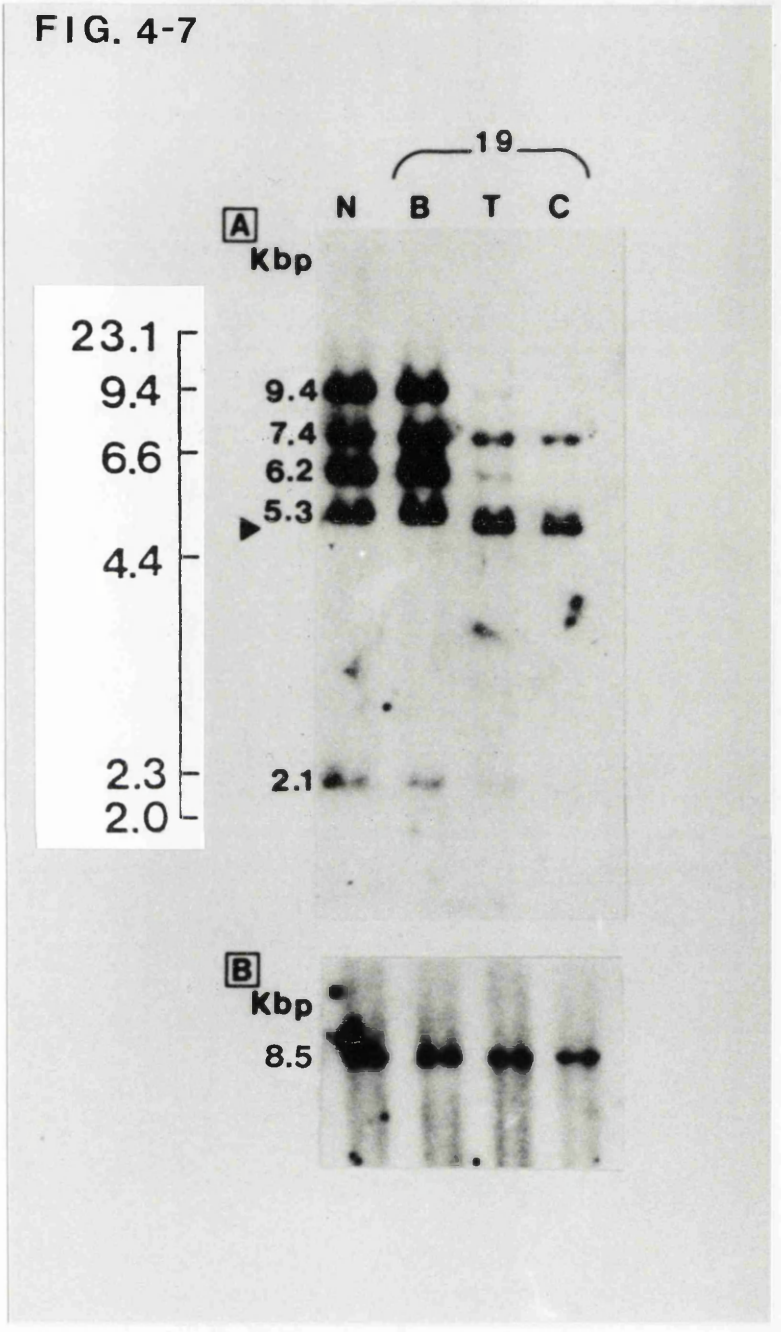
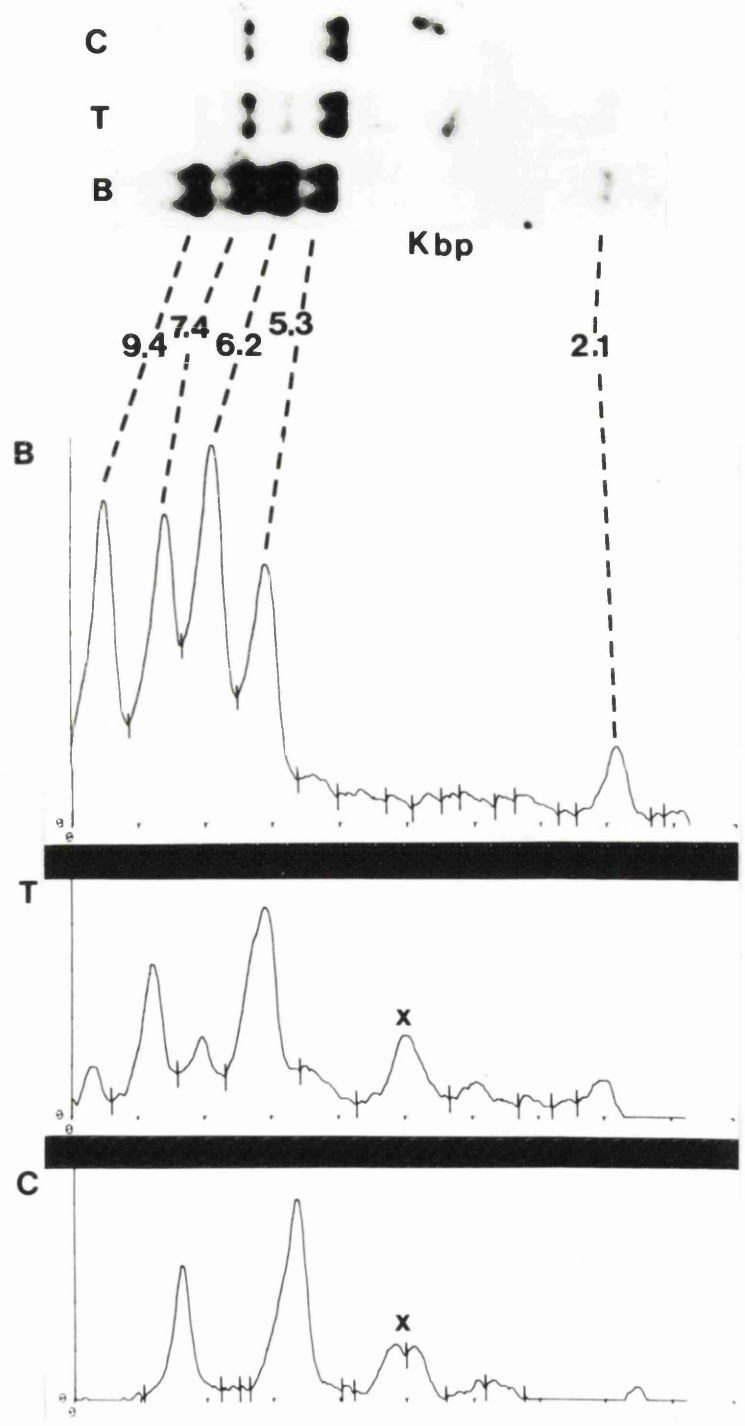


FIG. 4-8



CHAPTER 5

MOLECULAR ANALYSIS OF LOCI ON CHROMOSOMES 10 AND 17 IN GLIOMAS

5.1. INTRODUCTION

Analysis by means of classical cytogenetics as well as by molecular techniques has revealed that losses of specific parts of the genome occur frequently in hereditary and acquired neoplasias (Reviewed in Heim & Mitelman, 1988; Section 1.2.II). It is only in the case of the retinoblastoma gene product that loss of specific genetic material in tumours has conclusively been shown to result in abnormal function of a protein (Sections 1.2.II.a and 4.1.II). However, it is believed that many of the genetic losses seen in tumours which do not affect the RB locus may nevertheless result in altered function of as yet undefined genes whose products may normally act to inhibit cell growth (Knudson, 1985; Ponder, 1987).

In the past, classical cytogenetic techniques have been employed to detect loss of genetic material in solid tumours, despite the fact that these techniques are laborious, difficult to perform and generally require the setting up of cell cultures from the biopsy specimen. Molecular techniques have many advantages when compared to classical cytogenetics, as they can detect loss of genetic material in frozen tumour samples from which no cultures have been obtained, and are also capable of detecting

losses of minute portions of a chromosome which would have been invisible microscopically (Cavenee et al., 1983).

This chapter describes the use of molecular techniques to detect loss of genetic material on chromosomes 10 and 17 in solid glioma tumour samples. The rationale for studying these two chromosomes is given below.

5.1.I. Loss of chromosome 10 occurs in cultured glioma cells

One of the most commonly observed cytogenetic changes seen in the relatively few cases of adult primary glioma cell cultures studied, is a loss of one of the copies of chromosome 10 (Bigner et al., 1986a). In the series described by Bigner et al., (1988a), 19 out of 32 malignant gliomas (59%) revealed a loss of chromosome 10, while the series reported by Rey et al., (1987a) showed a loss in 4 out of 13 malignant tumours (31%). In contrast, no loss of chromosome 10 was seen in a study of 18 adult benign glioma primary cultures, although secondary cultures derived from one of the astrocytomas and one oligodendroglioma in this series did show loss of chromosome 10 (Rey et al., 1987b).

The frequent loss of chromosome 10 in cells cultured from malignant gliomas may represent an event of biological importance. Further analysis therefore seemed appropriate in order to ascertain whether chromosome 10 loss occurred

in primary gliomas, and to determine whether any detectable loss correlated with the degree of malignancy of the primary tumour.

5.1.II. Abnormalities of loci on chromosome 17, including the p53 gene, may be involved in the genesis of gliomas

5.1.II.a. Loss of chromosome 17 has been reported in malignant gliomas

Cytogenetic studies have described loss of one copy of chromosome 17 in malignant gliomas, although the occurrence is infrequent and is not seen in all the reported series (Bigner et al., 1986a). In the study of Bigner et al., (1988a), loss of chromosome 17 was seen in 5 out of 32 adult malignant gliomas of various types (16%), whereas no such loss was seen in 13 malignant astrocytomas studied by Rey et al., (1987a). In contrast to the findings reported in occasional malignant tumours, no loss of chromosome 17 was detected in a series of 17 adult benign gliomas studied by Rey et al., 1987b).

Therefore, chromosome 17 loss has been detected infrequently in sporadically-occurring malignant gliomas using classical cytogenetics (albeit in cells cultured from the solid tumour explants). However, the number of gliomas harbouring a deletion of part of chromosome 17 may be larger than that reported in the series described above, owing to the relative insensitivity of classical

cytogenetics when compared to molecular techniques as a means of detecting deletions of small portions of chromosomes (Cavenee et al., 1983).

5.1.II.b. Possible involvement of the von Recklinghausen neurofibromatosis locus in the development of gliomas

As described in Section 1.2.II.b, the inherited genetic locus associated with the development of von Recklinghausen neurofibromatosis (NF1) maps to the long arm of chromosome 17 (17q11.2). Since patients with NF1 have an increased risk of developing a wide variety of benign and malignant glial neoplasias (reviewed in Section 1.2.II.b), it is possible that abnormalities of the NF1 locus on chromosome 17 may contribute towards the development of these gliomas. It is therefore conceivable that abnormalities of the NF1 locus on chromosome 17 may also play a part in the development of sporadic gliomas found in patients not known to harbour an inherited predisposition to NF1, analogous to the involvement of an abnormal RB gene in both familial and sporadic retinoblastomas (Section 1.2.II.a).

5.1.II.c. The function of the p53 gene may be altered by abnormalities of chromosome 17

The p53 gene, situated on chromosome 17p, was originally identified as a cellular gene whose product

binds tightly to the SV40 large T viral oncoprotein (Lane and Crawford, 1979). Further studies indicated that the p53 protein was capable of functioning as an oncoprotein in that it co-operated with a mutated c-Ha-*ras*1 oncogene to transform primary rat embryo fibroblasts which had been transfected with both genes (Parada *et al.*, 1984). It is now known that the p53 gene used in the early transfection studies, such as that of Parada *et al.*, (1984), was in fact mutated in such a way as to alter its function and render it oncogenic. Recent studies have indicated that the normal (non-mutated) p53 protein does not co-operate with an activated *ras* oncogene to transform primary rat fibroblasts (Tuck and Crawford, 1989) and furthermore, normal p53 is capable of inhibiting cell transformation (Finlay *et al.*, 1989). Therefore p53 may be another example of a tumour suppressor gene.

Studies on sporadic colorectal adenocarcinomas have shown that up to 75% of these tumours possess an allelic deletion of the short arm of chromosome 17 (Vogelstein *et al.*, 1988), and that the region most commonly deleted contains the p53 putative tumour suppressor gene (Baker *et al.*, 1989; Section 1.3.VI.c). The association of familial polyposis coli with malignant gliomas in patients with Turcot's syndrome has been described in Section 1.2.II.d. One could postulate that the development of these 2 tumour types is related to the inheritance of a single abnormal

gene. It is possible that the inherited gene lies on chromosome 17 and may in fact be an abnormal p53 allele.

The p53 protein may also be involved in the development of experimental gliomas induced by DNA tumour viruses. The ability of certain transforming oncogenes of papovaviruses, capable of inducing neural tumours, to bind to the RB protein, and thereby possibly alter its normal function, was described in Sections 1.2.II.e and 4.1.III.c. Similarly, the SV40 large T oncoprotein is capable of binding to the p53 protein (Lane and Crawford, 1979), as is the E1B oncoprotein of human adenovirus 12 (Sarnow et al., 1982), the JC virus large T oncoprotein (Major et al., 1987) and the BK virus large T oncoprotein (Bollag et al., 1989). Interestingly, it has recently been shown that the E6 protein of human papilloma virus 16 is also capable of binding to the p53 protein, indicating that perturbations of p53 may also be operative in the genesis of virally-induced tumours in humans (K. Vousden, personal communication; unpublished results). Therefore one of the mechanisms which might contribute to the development of DNA tumour virus-induced experimental gliomas is the ability of certain of the transforming oncoproteins to bind to the p53 protein and interfere with its function. Thus it is possible that abnormalities of p53 function may also play a role in the development of human gliomas.

Therefore several lines of evidence suggest that abnormalities of loci on chromosome 17 and on some occasions the p53 gene in particular, may be involved in the genesis of gliomas in experimental animals as well as in humans.

5.1.III. The experimental approach used to examine loci on chromosomes 10 and 17

The losses of chromosomes 10 and 17 observed in glioma cells may result in the unmasking of an abnormal, as yet undefined, tumour suppressor allele (or a group of tumour suppressor alleles) on the remaining copy of the chromosome, in a manner similar to that thought to underlie the expression of an abnormal RB gene (Cavenee et al., 1989; Section 4.1.I). The mechanisms underlying the loss of a whole chromosome or part thereof are believed to be similar to those underlying the loss of the remaining normal allele in tumours in which the RB gene appears to be affected (Reviewed in Section 4.1.I; Cavenee et al., 1983). In order to examine gliomas for evidence of loss of all or part of chromosomes 10 and 17, restriction fragment length polymorphism (RFLP) analysis was used to search for evidence of loss of constitutional heterozygosity at loci on these chromosomes (Cavenee et al., 1983), similar to the method employed previously to search for abnormalities of chromosome 13 (Section 4.1.IV.a).

Structural abnormalities of the p53 gene were sought by examining genomic tumour DNA for evidence of deletions or rearrangements of the p53 gene. It was felt that the identification of gliomas which showed a loss of one allele of chromosome 17 associated with a structural alteration of the remaining allele might indicate that abnormalities of p53 protein function occur when both alleles are abnormal, similar to the situation with the RB protein (Section 4.1.II).

5.2. MATERIALS AND METHODS

5.2.I. Blood and tumour samples

Paired blood and glioma biopsy samples were obtained from 41 adult patients. DNA from 36 of the samples analysed and from cells cultured from tumour number 19 had previously been examined for evidence of abnormalities of the RB gene as described in Chapter 4. The 40 gliomas analysed included 13 benign and 27 malignant tumours, of which 26 were diagnosed as glioblastoma multiforme. In addition, DNA from a single cerebellar haemangioblastoma was analysed. None of the patients were known to be members of a family harbouring an inherited predisposition towards the development of neoplasia. The full list of patient details and diagnoses is given in Appendix I.

5.2.II. DNA extraction

DNA was extracted from the additional 5 tumours and matched peripheral blood samples not previously analysed, as described in Section 2.2.

5.2.III. Southern analysis to detect loss of loci on chromosomes 10 and 17.

DNA (10ug) from each sample was subjected to restriction enzyme digestion, electrophoresis and Southern transfer onto nylon membranes (Hybond-N, Amersham) as described in Sections 2.3 to 2.5. The chromosome 10 and 17 RFLP probes used to detect allelic loss are listed in Table 5.I, which indicates the restriction enzymes used and the sizes of the alleles generated by each enzyme. In order to obtain the highest possible degree of heterozygosity for the D10S4 and D10S1 chromosome 10 loci, it was necessary to examine two different Southern blots for each locus, with each blot made using DNA digested by each of the two different appropriate restriction enzymes. DNA was separated on 10cm long 1% agarose gels in all cases other than the RsaI gel (used to examine the D17S31 locus) and the TaqI gel (used to examine the D10S5, D17S30 and D17S28 loci), for which 20cm long 1% agarose gels were used. Membranes were hybridised and washed at high stringency, as described in Section 2.6, and autoradiography performed using Kodak XAR-5 film at -70^{0}C. In some instances, it was necessary to allow rehybridisation of a filter to a

series of different probes, and therefore radioactivity was removed as described in Section 2.6.VI, prior to rehybridisation.

In order to assess the significance of loss of loci on chromosomes 10 and 17, the TaqI Southern blots were rehybridised to probes detecting loci on chromosomes 1 and 7. The rate of loss of loci on chromosomes 10 and 17 was then compared to the rate of loss of the chromosomes 1 and 7 loci. The highly polymorphic probes MS1 and g3 were used, which detect the loci D1S7 (on chromosome 1) and D7S22 (on chromosome 7), respectively (Wong et al., 1987).

5.2.IV. Southern analysis to examine tumour DNA for evidence of structural abnormalities of the p53 gene

The integrity of the p53 putative tumour suppressor gene was assessed by probing Southern blots of 10ug of tumour DNA digested with EcoRI, HindIII and BamHI with the full-length human p53 cDNA probe, essentially as described in Sections 2.3 to 2.6. The p53 probe was a gift of Dr. J. Jenkins, Marie Curie Memorial Foundation, Oxted, Surrey, U.K., and consisted of a fragment amplified by the polymerase chain reaction using a previously isolated human cDNA clone (Zakut-Houri et al., 1985) as a template.

In order to confirm the integrity of the tumour DNA, the p53 probe was removed and the blots probed with the

alpha-1-1 collagen probe (Solomon et al., 1984), which detects a single copy sequence on chromosome 17.

5.3. RESULTS

Loss of heterozygosity at a particular chromosome locus indicates loss of one of the parental alleles (Cavenee et al., 1983). It is possible that as yet undefined loci on chromosomes 10 and 17, which are altered in tumours, may harbour tumour suppressor genes (Knudson, 1985; Ponder, 1987). The study described here is based on the hypothesis that the mechanism resulting in dysfunction of putative tumour suppressor genes on chromosomes 10 and 17 is similar to that ultimately leading to dysfunction of the RB gene, namely loss of part of, or the whole of, the chromosome harbouring the normal allele (Section 4.1.II). It is also possible that functional abnormalities of such putative tumour suppressor genes may be induced by a mechanism which differs from the RB model, such that deletion of only one allele may lead to a reduction in the quantity, and thus the effect, of the protein produced by the remaining normal allele. Loss of an allele resulting in such "dosage effects" would also be detected by the methods employed in this study.

The results of the chromosome deletion analysis are presented in Appendix 2 and summarised in Table 5.2 and are described below.

The cerebellar haemangioblastoma (tumour number 26) did not show allelic loss at loci on chromosomes 1,7 or 10, gave an uninterpretable result when examined at the D17S28 chromosome 17 locus (owing to an autoradiographic artefact), and will not be considered further in these results.

Although the gliomas studied in this series of experiments have sample numbers ranging from 1 to 42, only 40 of the tumours studied were gliomas, as the tumour numbered 26 was an haemangioblastoma, and the tumour originally numbered 8 was found to be taken from the incorrect patient, as described in Appendix 5.1.

5.3.I. Loss of heterozygosity at loci on chromosome 10 was found only in malignant gliomas

Loss of heterozygosity at loci on chromosome 10 was seen in 14 out of 27 malignant tumours (52%), and this figure included a loss in 12 out of 19 glioblastomas (GBM's) (63%). There was no evidence of allele loss in the 13 benign gliomas. All of the 40 tumours showed heterozygosity for one or more of the chromosome 10 loci examined (Table 5.2).

Examples of chromosome 10 allele loss are shown in figures 5.1 to 5.4 and are described fully in the figure legends, although several representative instances will be discussed here. Figure 5.1 (panel A) clearly shows loss of the larger allele (A1) of the D10S4 locus in the tumour DNA

relative to the blood DNA of sample number 29. In tumour number 11 (Figure 5.1, panel A), there is loss of the smaller (A2) D10S4 allele. The residual band observed in the tumour DNA at the site of the A2 allele is most probably due to the presence in the tumour homogenate of normal inflammatory and stromal cells which did not contain the deletion, a phenomenon which has been described previously (Fujimoto et al., 1989).

Figure 5.2 demonstrates the concurrent loss in tumour number 19 of an allele at several loci on chromosome 10 (the loci PLAU, D10S1, D10S5 and S10S21, shown in panels C,D,E and F, respectively). These results indicate that the portion of chromosome 10 lost in tumour 19 extends at least from band q21 to the terminal part of the long arm of chromosome 10. A further finding of interest is that DNA derived from cells cultured from tumour 19 also possess the chromosome 10 loss (Figure 5.2, panel E lane C). Panels A and B of Figure 5.2 demonstrate the histological appearances of two portions of the tumour 19 biopsy, showing two classical features of a glioblastoma, namely necrosis (panel A), and endothelial hyperplasia (panel B, arrow).

The value of examining each blood and tumour DNA sample with probes to more than one locus is demonstrated in Figure 5.3. In panel B, DNA from the blood of patient number 39 is homozygous for the D10S5 locus, and therefore loss of heterozygosity cannot be ascertained in

the tumour. However, when the same Southern blot was re-probed to detect the D10S1 locus, the DNA revealed heterozygosity and showed loss of allele number A1 (the larger allele), thus demonstrating loss of part of chromosome 10 in the tumour DNA. Also in Figure 5.3 A, patient number 40 is heterozygous for the D10S1 locus, but the A2 (smaller) allele in the tumour DNA is obscured by an autoradiographic artefact. However, in panel B, analysis of the DNA with the probe to the D10S5 locus reveals loss of the A2 D10S5 allele in tumour 40.

5.3.II. Loss of heterozygosity at loci on chromosome 17 was found in both benign and malignant gliomas

Loss of heterozygosity for loci on chromosome 17 was observed in 1 out of a total of 10 heterozygous benign gliomas (10%) (Appendix 2 and Table 5.2). A similar percentage of malignant tumours had lost a chromosome 17 allele (3 out of a total of 25 heterozygous tumours, or 12%). Examples of chromosome 17 allele loss are demonstrated in Figure 5.5, where tumour 21 reveals losses of the larger of the two D17S30 alleles and the smaller (A2) D17S31 allele, whilst tumour 27 has lost the smaller D17S30 and D17S31 alleles.

5.3.III. Loss of heterozygosity at a locus on chromosome 1 was an infrequent event

In the gliomas described in this chapter, there was

no allele loss at the locus D1S7 in any of the 25 malignant tumours which were heterozygous for this marker, while two out of 12 (17%) heterozygous benign gliomas showed a loss at this locus when examined using molecular techniques (Table 5.2 and Appendix 2). Figure 5.7 panel A illustrates loss of the smaller chromosome 1 allele revealed by the probe to the D1S7 locus in tumour number 1 (arrow).

No allele loss was observed at the chromosome 7 locus D7S22 in 11 heterozygous benign tumours, nor in 23 heterozygous malignant tumours out of the total of 27 malignant tumours examined (Table 5.2, Appendix 2 and Figure 5.7 panel B).

5.3.IV. No structural abnormalities of the p53 gene were detected

None of the tumours showed evidence of structural abnormalities of the p53 gene, using the 3 different restriction enzymes described (Figure 5.6 panels A,C, and D show examples of analysis of the coding region of the p53 gene).

5.4. DISCUSSION

5.4.I. Losses of loci on chromosomes 17 and 10 appear to be associated with different stages of tumour progression

Allelic loss at loci on chromosome 17 were present in

both benign and malignant gliomas, while loss at loci on chromosome 10 were seen only in malignant gliomas, including GBM's. Only GBM's showed evidence of abnormalities of more than one chromosome.

Loss of heterozygosity at one or more of the three loci on chromosome 17 was observed in 1 benign astrocytoma out of a total of 10 benign tumours (10%). In the case of malignant tumours, 3 tumours out of 25 heterozygous cases (i.e. 12%) showed loss of an allele. Thus the incidence of abnormalities of putative TS gene loci on chromosome 17 was similar in both benign and malignant tumours.

In contrast, loss of heterozygosity of loci on chromosome 10 were not seen in any of 13 benign constitutionally heterozygous tumours, whereas 14 out of 27 malignant tumours showed an abnormality, an incidence of 52%. All of the malignant tumours showing loss of chromosome 17 alleles were GBM's, while of the 14 tumours which had lost an allele on chromosome 10, 12 were GBM's and the other 2 were grade III malignant gliomas.

Because of the limitations of the RFLP analysis technique used in this study, the successful demonstration of an allelic loss in a tumour homogenate implies that the majority of the cells in the tumour are members of a single clone possessing the particular genetic abnormality (Cavenee et al., 1983; James et al., 1988). Thus it is possible that the specific molecular genetic alterations detected may be associated with the biological evolution of

the tumour cell clones. The hypothesis that chromosome 10 loss may be biologically important was further supported by the observation that the chromosome 10 loss detected in the tumour 19 biopsy was also seen in cells cultured from the tumour, suggesting that these cells were clonal in origin, and it is also possible that the presence of the chromosome 10 deletion may have contributed towards the growth of these cells in culture.

The lack of detectable chromosome 10 loss in any of the benign tumours contrasts with the loss of chromosome 10 in one benign astrocytoma and one benign oligodendroglioma reported by Rey et al., (1987b). However, in both these tumours, the observation of chromosome 10 loss involved the use of classical cytogenetic techniques on cultured cells which had undergone repeated passage. Therefore it is possible that prolonged in vitro manipulation may have selected out cells possessing karyotypic abnormalities which might not be representative of the majority of the cells in the original benign tumours. Obviously the use of molecular genetic techniques to examine DNA extracted from tumour biopsies will eliminate any possible tissue culture artefacts.

These results described here concur favourably with the data recently obtained in studies on North American patient cohorts (as opposed to the British patients studied here), and are consistent with the hypothesis that loss of material from chromosome 17p may represent an event in the

development of benign glial tumours (El-Azouzi et al., 1989), while loss of chromosome 10 alleles is associated with a transition to a malignant phenotype (James et al., 1988; Fujimoto et al., 1989). In the study of James et al., (1989), the majority of tumours exhibiting loss of material from chromosome 17 appeared to have lost only a portion of the short arm of the chromosome distal to band 17p11.2. Loss of only a small part of a chromosome, while possibly having critical functional effects on the cell, could be extremely difficult or even impossible to detect by means of classical cytogenetic studies, and may account for the paucity of reports of chromosome 17 loss in benign gliomas.

The von Recklinghausen neurofibromatosis locus is on the long arm of chromosome 17 (Section 1.2.II.b), and is therefore not encompassed by the area on the short arm of chromosome 17 preferentially lost in the astrocytomas studied by James et al., (1989). One cannot therefore directly relate the occurrence of glial tumours in NF1 to abnormalities of the area on chromosome 17p described in sporadic astrocytomas. However, it is possible that chromosome 17 could harbour more than one locus involved in the genesis of gliomas. In addition, loss of an entire copy of chromosome 17 could conceivably affect both the NF1 locus and the area of the chromosome most frequently affected in sporadic astrocytomas.

Interestingly, in the studies of James et al., (1989)

and El-Azouzi et al., (1989), loss of loci on chromosome 17 in benign gliomas was confined to benign astrocytomas, and was not observed in non-astrocytic benign gliomas. The single benign tumour in the series described here was an astrocytoma, which is in keeping with their findings, although obviously a much larger number of benign astrocytomas should be analysed before assuming that chromosome 17 loss is confined to tumours derived from this one cell type.

This study failed to identify loss of an allele at the chromosome 7p locus in the tumours examined. Cytogenetic losses of chromosome 7 were not reported in the series of Bigner et al., (1988a) or those of Rey et al., (1987a and 1987b), although duplications of chromosome 7 were frequently seen.

Chromosome 1 is occasionally involved in structural rearrangements detected by classical cytogenetic studies on gliomas, but in the 32 malignant gliomas examined by Bigner et al., (1988a), no loss of chromosome 1 was detected at the microscopic level. The failure of any of the malignant tumours studied here to display loss of a chromosome 1 allele suggests that this particular molecular event is not detectable in the majority of the cells in any of these malignant tumours. Two of the benign tumours did show allelic loss at the chromosome 1 locus, and further studies are needed to determine whether these losses are indicative of either a random rate of "background" chromosome loss, or

of a molecular event which contributes to the development of some benign gliomas. The techniques used in the present study may not reveal loss of heterozygosity present in a minority of cells in a tumour (Vogelstein et al., 1988), and since many malignant gliomas show pronounced microscopic heterogeneity, it is possible that a subset of the cells making up certain malignant gliomas also have loss of alleles on chromosome 1 (James et al., 1988).

5.4.II. Structural abnormalities of the p53 gene were not detected

The p53 gene, situated on chromosome 17p, is believed to function normally to inhibit cell transformation (see Section 5.1.II), and may therefore represent a specific target of abnormalities of chromosome 17 seen here. In contrast to the RB gene paradigm, it is thought that an abnormality of only one of the p53 alleles may result in abnormal function of the gene product; this could therefore be an example of a a mutation in which abnormal gene function results from inactivation of one allele (known as a "dominant negative" mutation). The protein coded for by the abnormal allele may combine with that of the normal allele, resulting in the formation of a dysfunctional heterodimer which can inhibit the normal p53 gene function (Finlay et al., 1989). Certain point mutations of the p53 gene can even have the effect of causing it to function as an oncogene (Parada et al., 1984).

It is possible to have p53 gene abnormalities whilst heterozygosity is maintained in the tumour DNA if the heterozygous RFLP loci lie outside the gene. Similarly, it is possible to have structural alterations of the gene in tumours which are homozygous for the RFLP probes used, and therefore such tumours would fail to show loss of heterozygosity. Thus the p53 gene was examined directly by probing DNA digested by three different restriction enzymes with the full-length p53 cDNA probe. This approach failed to reveal any abnormalities of the p53 gene, although it has been previously proven to be successful in demonstrating p53 abnormalities in osteosarcomas (Masuda et al., 1987).

Recently, point mutations of the p53 gene were detected in a small number of glioblastoma biopsies (Nigro et al., 1989), carcinomas of the lung (Takahashi et al., 1989) and colorectal adenocarcinomas (Baker et al., 1989). Obviously, point mutations would not have been detected by probing genomic DNA with a cDNA probe, as was performed in the experiments described in this chapter. It is possible therefore that any point mutations of the p53 gene which may exist in the tumours analysed here would have gone undetected, and direct sequencing of the amplified gene product will be necessary to reveal such minute abnormalities.

5.4.III. Summary

The data presented here is consistent with the findings of other groups of workers, and suggests that loss of genetic material on chromosome 17 is an event associated with the development of benign astrocytomas, whereas the development of malignancy is frequently associated with loss of a loci on chromosome 10. It is possible that loss of genetic material on these two chromosomes might result in dysfunction of as yet undefined genes, and such dysfunction may enhance tumour cell growth.

The reasons for supposing that abnormalities of the p53 gene might occur in gliomas were outlined in the introduction to this chapter. Recent work has reported the occurrence of point mutations in p53 in glioblastomas, however such point mutations would not have been detectable by the methods employed here to analyse the p53 gene and future studies will need to use techniques capable of detecting point mutations in the gene.

Table 5.1. Restriction fragment length polymorphism probes

Summary of probes used to examine loci on chromosomes 10, 17, 1 and 7. HGM symbol refers to the symbol given to the locus defined by a particular probe by the Human Gene Mapping Workshop (Pearson et al., 1987).

Ref = References: 1, Litt et al., (1987); 2, Pearson et al., (1987); 3, McDermid et al., (1987); 4, Raeymaekers et al., (1988); 5, Sebastio et al., (1985); 6, Nakamura et al., (1987); 7, Carlson et al., (1988); 8, Wong et al., (1987).

TABLE 5.1: RFLP Probes

Chromosome region	HGM symbol	Probe	Enzyme	Allele sizes (Kb)	Ref.
10q21-q23	D10S4	p1-101	TaqI	A1: 7.4 A2: 4.9	1.
			SacI	B1: 4.7 B2: 0.81	
				C1: 3.2 C2: 1.75	
10q21-q23	D10S1	Dry 5-1	TaqI	A1: 6.3 A2: 3.6	2.
			BglII	B1: 7.6 B2: 4.4	
10q21.1	D10S5	p9-12-A	TaqI	A1: 4.55 A2: 3.8	3.
10q24.33	D10S21	CARLP118.2	BamHI	A1: 7.6 A2: 2.3	4.
10q24-qter	PLAU	pHUK-1	BamHI	A1: 7.0 A2: 1.6	5.
17p	D17S30	pYNZ22	TaqI	>10, 2-3Kb	6.
17p13.3	D17S28	pYNH37.3	TaqI	VNTR, 5 2.0-4.0Kb	2.
17p13.1-11.2	D17S31	pMCT35.1	RsaI	A1: 2.9 A2: 2.1	7.
1p35-p33	D1S7	MS1	TaqI	Multiple, 2-20Kb	8.
7p36-qter	D7S22	g3	TaqI	Multiple, 2-20Kb	8.

TABLE 5.2: SUMMARY OF CHROMOSOME DELETIONS IN GLIOMAS

	LOSS OF HETEROZYGOSITY			
TUMOUR TYPE	Chrom.10	Chrom.17	Chrom.1	Chrom.7
BENIGN (13 cases):				
Oligodendroglioma	0/3	0/3	1/2	0/3
Astrocytoma	0/6	1/5	1/6	0/5
Mixed OA	0/2	0/1	0/2	0/1
Ependymoma	0/1	0/1	0/1	0/1
Choroid plexus P.	0/1	0/0	0/1	0/1
Total lost/het.(%)	0/13	1/10(10)	2/12(17)	0/11
MALIGNANT (27 cases):				
Astrocytoma III	0/5	0/4	0/5	0/4
Oligo-Astro III	2/2	0/2	0/2	0/0
Glioma, undefined	0/1	0/1	0/1	0/1
Glioblastoma	12/19	3/18	0/19	0/10
Total lost/het.(%)	14/27(52)	3/25(12)	0/25	0/23

ABBREVIATIONS and NOTES:

Chrom., chromosome number. For chromosomes 10 and 17, the result refers to a summary of the results obtained for each chromosome using the various probes and restriction enzymes listed in Table 5.1.

OA and Oligo-Astro, mixed oligodendroglioma-astrocytoma.

Het., heterozygous.

P., papilloma.

Figure 5.1. Heterozygous deletions of loci on chromosome 10 in gliomas

Southern blots of TaqI-digested DNA hybridised to the D10S4 probe (panel A), SacI-digested DNA hybridised to the D10S4 probe (panel B) and BglII-digested DNA hybridised to the D10S1 probel (panel C).

DNA was derived from the blood (B) and tumour (T) samples shown. Loss of heterozygosity is seen in the following tumours: Panel A: tumour 11 (lower allele, A2), tumour 16 (A2) and tumour 29 (A1). The residual radioactive signal in each case is most probably due to the presence of contaminating non-neoplastic cells in the tumour. In tumour 8X, the blood and tumour samples are from different individuals (see text: Appendix 5.1). Panel B: Loss in tumour 42 (alleles C2 and B2). Panel C: Losses in tumour 28 (4.4Kb allele) and tumour 42 (7.6Kb allele). Patient 15 (panel A) and patient 32 (panel C) both demonstrate homozygosity at the respective chromosome 10 loci.

The molecular weight was assessed by comparing the relative mobility of the DNA samples to that of HindIII-digested lambda phage DNA fragments.

FIG. 5-1

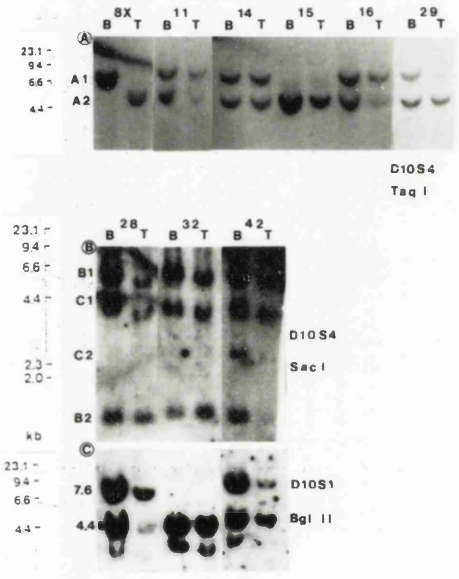

8X
11
14
15
16
29
B T
23.1
9.4
6.6
4.4
A
A1
A2
D10S4
Taq I
28
32
42
2.3
2.0
kb
B
B1
C1
C2
B2
D10S4
Sac I
C
7.6
4.4
D10S1
Bgl II

Figure 5.2. Heterozygous deletions of loci on chromosome 10 in tumour 19

Southern blots of BamHI-digested DNA hybridised to the PLAU (panel C) and D10S21 (panel F) probes and TaqI-digested DNA hybridised to the D10S1 (panel D) and D10S5 (panel E) probes.

DNA was derived from the blood (B), tumour (T) and cultured cell (C) samples shown. Loss of heterozygosity is seen in tumour 19 as follows: Panel C (smaller allele, A2), panel D (A2), panel E (A2; in panel E, cells cultured from tumour 19, labelled C, also reveal loss of allele A2) and panel F (A1 and B2). In panel C, tumour 20 does not show any allele loss.

Panels A and B are photomicrographs of sections of tumour 19, showing areas of necrosis (panel A, N) and endothelial hyperplasia (panel B, arrow), both characteristic of glioblastomas.

The molecular weight was assessed by comparing the relative mobility of the DNA samples to that of HindIII-digested lambda phage DNA fragments.

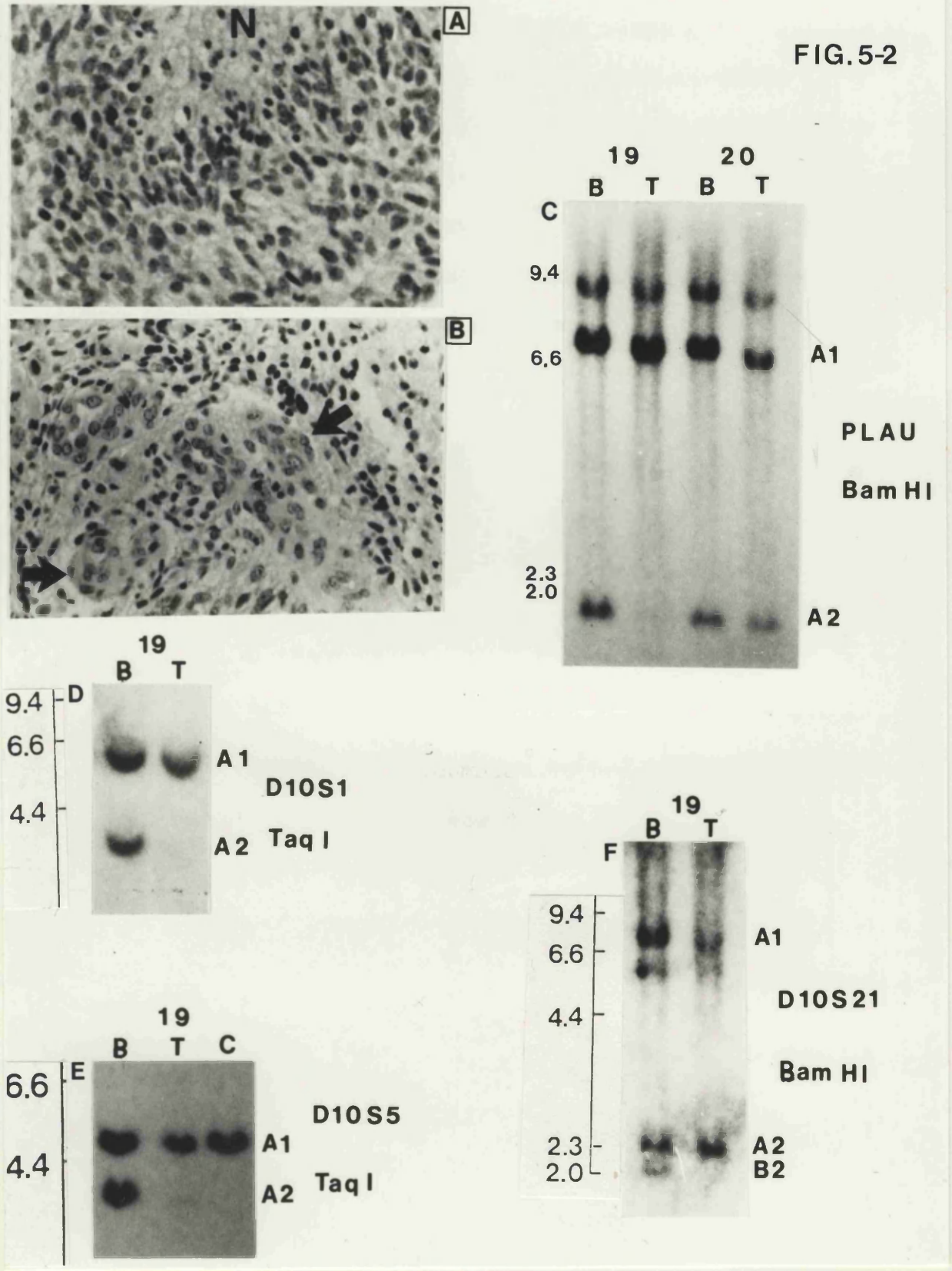

N
A
B
FIG. 5-2
19
20
B
T
B
T
C
9.4
6.6
A1
PLAU
Bam HI
2.3
2.0
A2
19
B
T
D
9.4
6.6
4.4
A1
D10S1
A2
Taq I
19
B
T
C
E
6.6
4.4
D10S5
A1
A2
Taq I
19
B
T
F
9.4
6.6
4.4
A1
D10S21
Bam HI
2.3
2.0
A2
B2

Figure 5.3. Chromosome 10 deletions and confirmation of genetic identity of blood and glioma samples

Southern blot of TaqI-digested DNA hybridised to the D10S1 (panel A), D10S5 (panel B) and MS1 (panel C) probes.

DNA was derived from the blood (B) and tumour (T) samples shown. Loss of heterozygosity is seen in the following tumours: Panel A: tumour 39 (allele A1), tumour 40 (A2, partially obscured by radioactive artefact) and tumour 42 (A2); panel B: Tumour 35 (A2) tumour 40 (A2). Tumours 35, 36 and 37 are homozygous at the D10S1 locus. Panel C shows the same blot probed with the fingerprinting probe, MS1. The identical sizes of the blood and tumour alleles from each patient confirms the genetic identity of the samples.

The molecular weight was assessed by comparing the relative mobility of the DNA samples to that of HindIII-digested lambda phage DNA fragments.

FIG. 5-3


35 36 37 38 39 40 41 42
B T B T B T B T B T B T B T B T
A
6.6
A1
TaqI
4.4
D10S1
A2
B
4.4
A1
A2
D10S5
Kb
C
23.1
9.4
6.6
D1S7
(MS1)

Figure 5.4. Chromosome 10 deletions in glioma samples

Southern blot of BamHI-digested DNA hybridised to the D10S21 (panel A) and PLAU (panel B) probes.

DNA was derived from the blood (B) and tumour (T) samples shown. Loss of heterozygosity is seen in the following tumours: Panel A: tumour 18 (allele A2); panel B: tumour 5 (allele A1). None of the other tumours show allelic loss.

The molecular weight was assessed by comparing the relative mobility of the DNA samples to that of HindIII-digested lambda phage DNA fragments.

Fig. 5-4

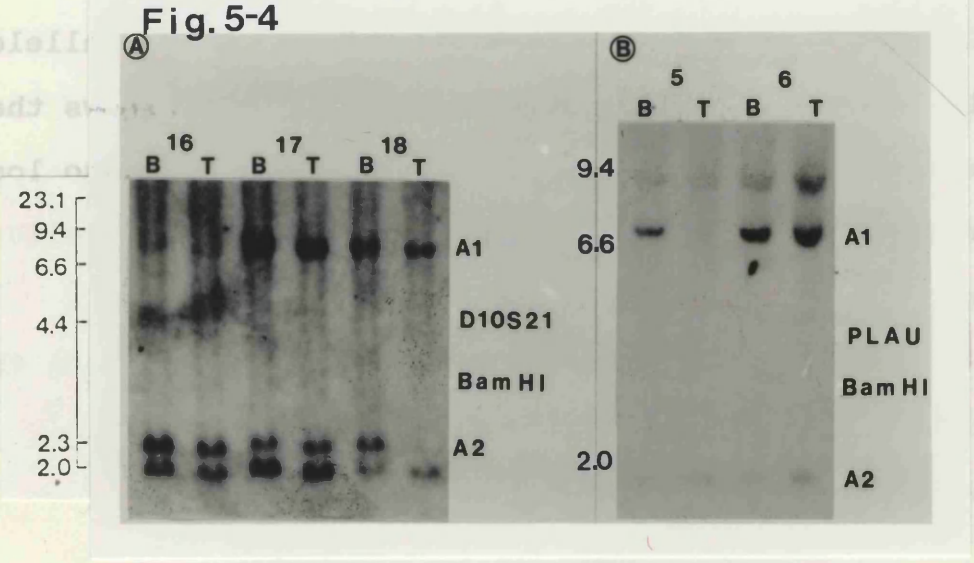

A
16
17
18
B
T
B
T
B
T
23.1
9.4
6.6
4.4
2.3
2.0
A1
D10S21
Bam HI
A2
B
5
6
B
T
B
T
9.4
6.6
2.0
A1
PLAU
Bam HI
A2

Figure 5.5. Chromosome 17 deletions in gliomas

Southern blots of TaqI-digested DNA hybridised to the D17S30 probe (upper panel) and RsaI-digested DNA hybridised to the D17S30 probe (lower panel).

DNA was derived from the blood (B) and tumour (T) samples shown. Loss of heterozygosity is seen in the following tumours: Upper panel: tumour 21 (upper allele) and tumour 27 (lower allele); the lower panel shows that the alleles identified by the D17S31 probe are also lost in tumours 21 and 27. Tumour 24 does not show any allelic loss.

The molecular weight was assessed by comparing the relative mobility of the DNA samples to that of HindIII-digested lambda phage DNA fragments.

Fig. 5-5

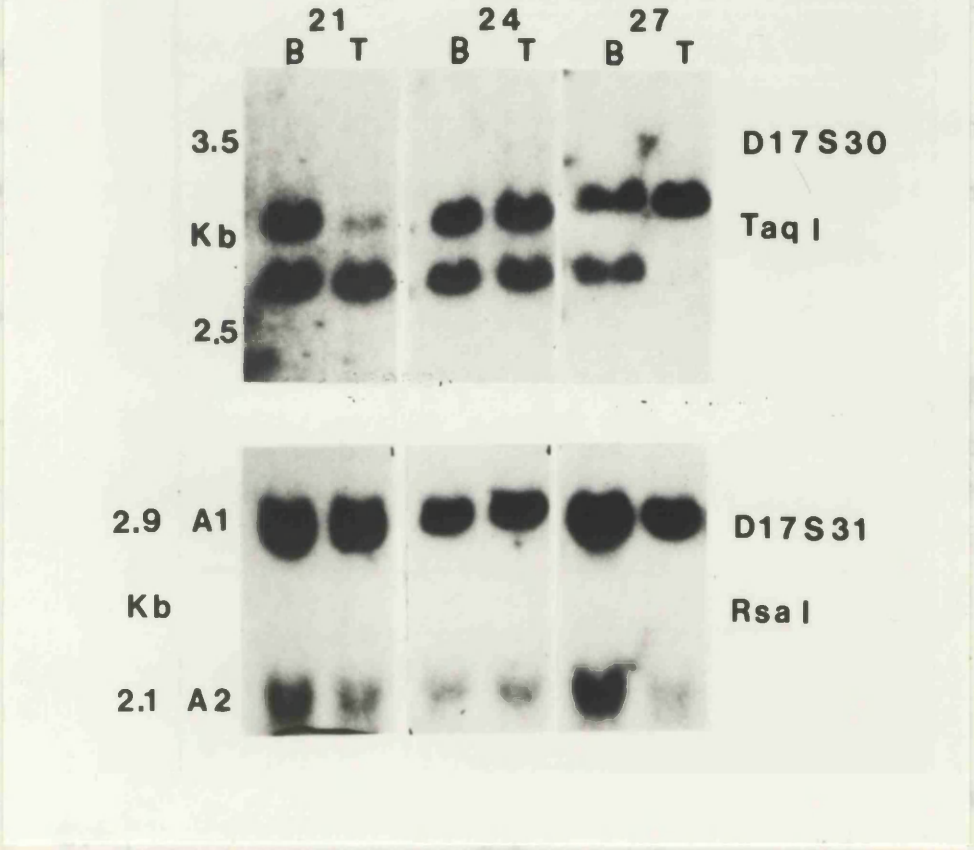


Figure 5.6. Southern analysis of the p53 gene in gliomas

Southern blots of HindIII-digested DNA (panel A), BamHI-digested DNA (panel C) and EcoRI-digested DNA (panel D), hybridised to the human p53 cDNA probe. Panel B shows the HindIII blot rehybridised to the alpha-1-1 collagen probe, in order to confirm the integrity of the DNA.

DNA samples were derived from two normal individuals (lanes labelled N) and from solid tumours (numbered lanes). There is no evidence of any structural alteration of the p53 gene.

The molecular weight was assessed by comparing the relative mobility of the DNA samples to that of HindIII-digested lambda phage DNA fragments.

Fig. 5-6

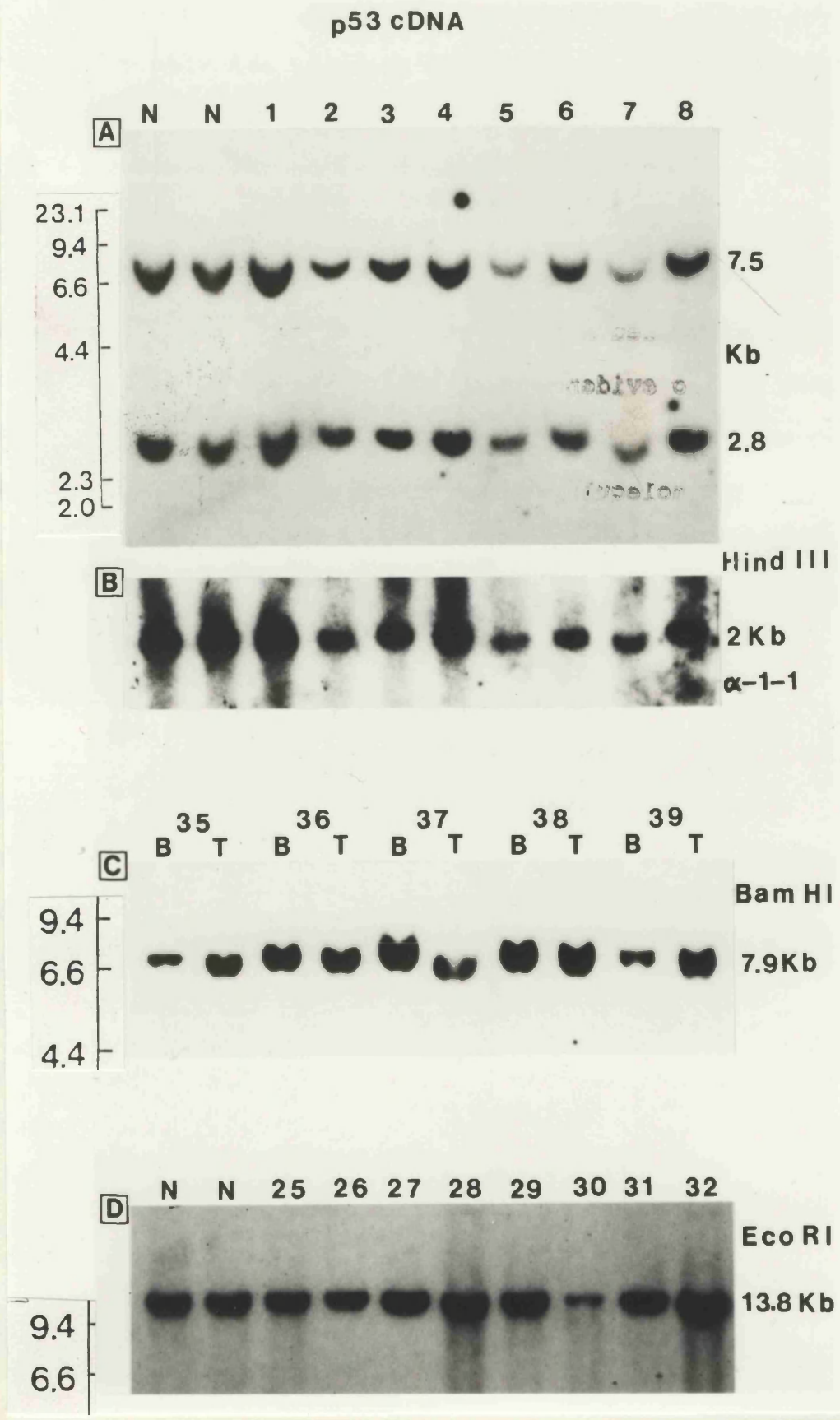

p53 cDNA
N N 1 2 3 4 5 6 7 8
A
23.1
9.4
6.6
4.4
2.3
2.0
7.5
Kb
2.8
Hind III
B
2 Kb
α-1-1
35 36 37 38 39
B T B T B T B T B T
C
Bam HI
9.4
6.6
4.4
7.9Kb
N N 25 26 27 28 29 30 31 32
D
Eco RI
9.4
6.6
13.8 Kb

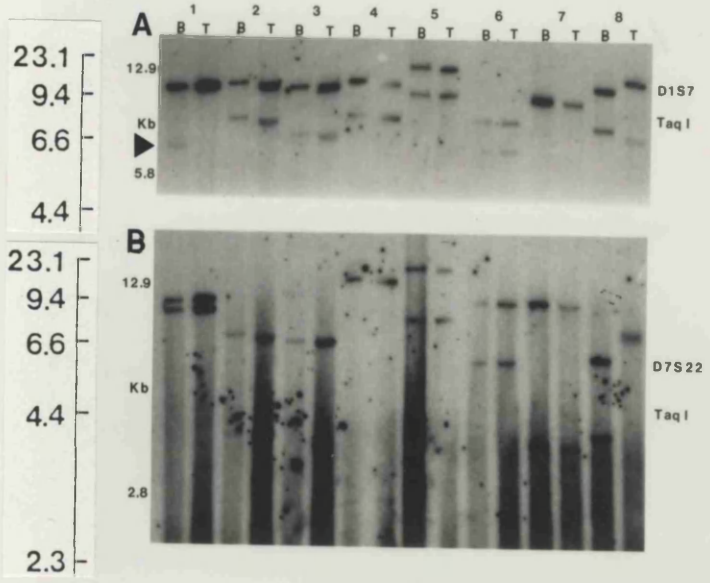
Figure 5.7. Analysis of loci on chromosomes 1 and 7 in gliomas

Southern blot of TaqI-digested DNA hybridised to the D1S7 (panel A) and D7S22 (panel B) probes.

DNA was derived from the blood (B) and tumour (T) samples shown. Loss of heterozygosity of the D1S7 locus on chromosome 1 is seen in tumour 1 (panel A, arrow shows the lower allele lost in tumour 1). None of the other tumours show loss of the chromosome 1 or 7 loci. Patient 7 is homozygous at the D1S7 locus.

The molecular weight was assessed by comparing the relative mobility of the DNA samples to that of HindIII-digested lambda phage DNA fragments.

Fig. 5-7


A
1 2 3 4 5 6 7 8
B T B T B T B T B T B T B T B T
23.1
9.4
6.6
4.4
12.9
Kb
5.8
D1S7
Taq I
B
23.1
9.4
6.6
4.4
2.3
12.9
Kb
2.8
D7S22
Taq I

APPENDIX 5.1

When the DNA samples from tumour number 8 were originally probed with the probe detecting the D10S4 locus, both the blood and tumour DNA revealed homozygosity at this locus, but the alleles identified in each case differed in size (Figure 5.1, panel A, sample 8X). The most likely explanation for such a phenomenon was that the blood sample had been incorrectly matched to a tumour sample from another patient. The lack of genetic identity of the blood and tumour samples was confirmed by the observation that the alleles identified by the MS1 (locus D1S7) and g3 (locus D7S22) fingerprinting probes were of a different size, as shown in Figure 5.7, lanes 8 B and T (see Section 3.4.IV for discussion of the use of these DNA fingerprinting probes). It transpired that the blood DNA from case number 8 had been mismatched to tumour DNA from one of two patients with the same name. The situation was easily rectified by extracting DNA from the tumour derived from the correct patient (now named tumour number 35). Figure 5.3 lane 35 B and T demonstrates that the 2 alleles identified by the fingerprinting probe MS1 (locus D1S7) are of equal size, indicating genetic identity between the blood and the correct tumour sample.

CHAPTER 6

ANALYSIS OF GLIOMAS FOR EVIDENCE OF ONCOGENE AMPLIFICATION

6.1 INTRODUCTION

Elevated levels of a structurally normal oncogene product in the cell can cause functional abnormalities which may contribute towards tumour development. One of the mechanisms underlying overexpression of an oncoprotein is the presence of multiple copies of the specific oncogene, which allows the transcription of inappropriately high amounts of mRNA from the gene. An increase in oncogene copy number can result from chromosome reduplication or from amplification of the gene itself, with the latter phenomenon frequently giving rise to cytogenetically visible structures known as double minute chromosomes and homogeneously staining regions (see Section 1.3.II.a for review).

Malignant gliomas frequently possess cytogenetic abnormalities which may be indicative of the presence of an increased copy number of certain oncogenes (Bigner et al., 1988a).

6.1.I. Cytogenetic evidence indicating that gliomas may possess amplified oncogenes

6.1.I.a. Double minute chromosomes

Double minute chromosomes (DM's) are abnormal, microscopically-visible structures found in the nucleus

of certain tumour cells and are believed to indicate the presence of amplified genes, as has been found in the case of N-myc oncogene amplification (Section 1.3.II.a; Wolman and Henderson, 1989). DM's are a frequent finding in cells cultured from malignant gliomas, having been observed in 18 out of 32 adult malignant gliomas studied by Bigner et al., (1985 and 1988a). In contrast, no DM's were observed in the 4 benign paediatric gliomas examined by Griffin et al., (1988), nor in the 17 adult and 5 paediatric benign gliomas examined by Rey et al., (1987b). No homogeneously staining regions were described in gliomas

The frequent finding of DM's in malignant gliomas cells suggests that these tumours may harbour amplified genes. Not all gene amplification is accompanied by cytogenetic changes such as DM's, and therefore the absence of DM's in benign gliomas does not preclude the occurrence of gene amplification in those tumours (Wolman and Henderson, 1989).

6.1.I.b. Increased copies of chromosome 7

Multiple copies of chromosome 7 are commonly found in malignant gliomas. In one series, 26 out of 32 malignant adult gliomas had gained at least 1 extra copy of chromosome 7, with up to 8 cytogenetically-normal copies of chromosome 7 being present in certain of these tumours (Bigner et al., 1986 and 1988a). Multiple copies

of chromosome 7 are also found in paediatric malignant gliomas, for example 2 out of 3 tumours reported by Griffin et al., (1988). In contrast, chromosome 7 reduplication is a relatively rare phenomenon in benign gliomas, having been observed in only 2 out of 17 adult benign gliomas in one series (Rey et al., 1987b).

6.1.II. The rationale for examining gliomas for evidence of amplification of the EGFR, c-erbB-2 and N-myc oncogenes

Because of the well documented presence in gliomas of DM's and duplications of chromosome 7, it was decided to examine the group of gliomas studied in the previous chapters for evidence of amplification of three specific genes: The EGF receptor, c-erbB-2 and N-myc oncogenes. The rationale for searching for amplification of each of these three oncogenes is given below.

6.1.II.a. The EGF receptor oncogene

The EGFR has been localised to chromosome 7 (Spurr et al., 1984) and therefore duplications of this entire chromosome, such as those observed in gliomas (Section 6.1.I.b), will result in the presence of an abnormally high copy number of the EGFR gene. The increased EGFR gene copy number resulting from chromosome 7 reduplication has been shown to be associated with overexpression of the EGFR mRNA in glioblastoma cell

lines (Henn et al., 1986). Therefore one of the effects on glioma cells of duplication of chromosome 7 might be to cause overexpression of the EGFR protein.

It is thought that an increased copy number of the EGFR gene may also arise from selective reduplication of the gene itself such that more than one copy of the gene is present on each chromosome 7; in other words, it is not necessary to have chromosome 7 reduplication in order to have EGFR amplification (Wolman and Henderson, 1989). Several studies on malignant gliomas have demonstrated that the EGFR gene can be amplified up to 60-fold in glioblastomas, suggesting that selective amplification of the gene itself does occur, as it is unlikely that a 60-fold reduplication of chromosome 7 could exist in a cell (Libermann et al., 1985).

EGFR gene amplification correlates with overexpression of the EGFR mRNA (Wong, A.J. et al., 1987) and protein (Libermann et al., 1985; Malden et al., 1988) in malignant gliomas. The presence of EGFR gene amplification, whether resulting from chromosome 7 reduplication or from selective reduplication of the gene itself, and the resultant overexpression of the protein which functions as a growth factor receptor, might be an event of biological importance in the development of gliomas.

6.1.II.b. The c-erbB-2 oncogene

The c-*erb*B-2 oncogene (also known as *neu* or *HER*-2) has been found to be abnormal in a variety of tumour types. It was originally identified by NIH3T3 transfection as a transforming gene present in the DNA of 4 rat neuro-glioblastomas (hence the acronym "*neu*") which had been induced by the transplacental administration of ethylnitrosourea (ENU) (Shih *et al.*, 1981). All 4 neural tumours, which had been induced independently in different rats, were found to contain the same transforming gene, which was shown to encode a 185 KD cell membrane-associated molecule resembling the EGFR in structure in that it possessed an external domain (thought to bind an as yet undefined ligand), a short transmembrane region and an internal tyrosine kinase domain (Schechter *et al.*, 1984). Comparison of the normal rat *neu* structure with that of the 4 ENU-activated transforming *neu* oncogenes revealed an identical point mutation in all cases, namely a single base substitution which alters a valine residue to a glutamic acid residue at position 664 in the transmembrane region of the protein (Bargmann and Weinberg, 1988). It is thought that substitution of glutamate for valine in the transmembrane region might lead to abnormally elevated tyrosine kinase activity (Bargmann and Weinberg, 1988; Weiner *et al.*, 1989). In addition, it has been postulated that enhanced dimerisation occurs between two altered *neu* molecules, facilitated by the larger size of the glutamate residues

when compared to the valine residues, and that the dimerisation might somehow lead to increased tyrosine kinase activity (Sternberg and Gullick, 1989; 1990).

The human homologue of neu, known as c-erbB-2, was first discovered as an amplified gene in a human breast carcinoma (King et al., 1985) and a salivary gland adenocarcinoma (Semba et al., 1985). The amplified gene, the human equivalent of the rat neu gene, was shown to have a similar structure to the human EGFR and was localised to the long arm of human chromosome 17 at band q21 (Yamamoto et al., 1986; Schechter et al., 1985). The c-erbB-2 oncogene has been found to be amplified in a variety of other adenocarcinomas in addition to breast and salivary gland, such as those of the stomach (Yokota et al., 1985) and colon (Meltzer et al., 1987). No evidence of mutation has been detected in the amplified c-erbB-2 oncogenes in human tumours, and it may be that the mere presence of an abnormal quantity of structurally unaltered c-erbB-2 protein is sufficient to induce cell transformation when overexpressed in NIH3T3 cells (DiFiore et al., 1987). Obviously it is possible that c-erbB-2 structural mutations may yet be detected in human tumours.

The importance of the c-erbB-2 oncogene in molecular oncology lies in the strong correlation observed in breast adenocarcinoma between the degree of c-erbB-2 amplification and protein overexpression and

aggressive biological behaviour of the disease (Section 1.3.IV.b; Venter et al., 1987; Slamon et al., 1987; Varley et al., 1987; Zhou et al., 1987; Berger et al., 1988; Van de Vijver et al., 1988; Gullick et al., 1989), suggesting that elevated levels of the c-erbB-2 protein may play a role in the development and progression of breast adenocarcinomas. However, the association between c-erbB-2 amplification and poor prognosis in breast cancer is not invariably found (Ali et al., 1988), and further studies will be necessary in order to clarify the situation.

In a series of ovarian adenocarcinomas, Slamon et al., (1989), found that overexpression of c-erbB-2, which often occurred as a result of gene amplification, correlated with clinical outcome in a manner analogous to that observed in breast adenocarcinomas.

In summary, the c-erbB-2 oncogene appears to contribute towards the development of tumours in two ways. The first is by a mutation of the transmembrane region, as seen in the case of the neu oncogene (the rat homologue of the human c-erbB-2 gene) which appears to be involved in the genesis of experimental neural tumours. The second is by means of amplification and overexpression of a structurally normal c-erbB-2 molecule which may contribute towards clinically aggressive behaviour in human breast and ovarian adenocarcinomas. Because of the association of the mutated neu/c-erbB-2

oncogene with neural tumours in the rat, it was decided to examine a group of human gliomas for evidence of c-erbB-2 amplification in order to see whether c-erbB-2 amplification mighty play a role in the genesis of human neural tumours and if so, whether amplification correlated with the biological aggressiveness of the tumour.

6.1.II.c. The N-myc oncogene

The prognostic importance of the association between the degree of N-myc amplification and the clinical aggressiveness of neuroblastomas was described in Section 1.3.VI.b. Since gliomas and neuroblastomas are both derived from cells descended from the neural crest, it was possible that N-myc amplification may also occur in gliomas. Two studies have been reported of amplification of the N-myc oncogene in human gliomas, but the incidence of amplification (less than 5%), was surprisingly low when compared to the incidence in neuroblastomas (Garson et al., 1985; Bigner et al., 1988b) and it was therefore decided to extend the number of gliomas assessed for evidence of N-myc amplification to include the group of tumours studied in this thesis.

6.2. MATERIALS AND METHODS

6.2.I. Southern analysis to detect amplification of the EGFR, c-erbB-2 and N-myc oncogenes

Southern analysis was used to examine the same series of 40 gliomas as that examined in Chapter 5, for evidence of oncogene amplification. The Southern blots made from 10ug of EcoRI-digested tumour DNA, which had been previously separated by electrophoresis and blotted (Section 5.2.IV), were sequentially re-probed with probes to the EGFR, c-erbB-2 and N-myc oncogenes.

Amplification of the EGFR gene was assessed with the p64.1 probe, which is a 1838 base pair EcoRI human cDNA fragment (Ullrich et al., 1984). The c-erbB-2 oncogene was examined using the 4.6Kb full-length human c-erbB-2 cDNA probe (Yamamoto et al., 1986). The N-myc oncogene was examined using the 1Kb EcoRI-BamHI human N-myc genomic fragment (Schwab et al., 1983).

The blots were probed and washed at high stringency as described in Section 2.6. Following autoradiography using pre-flashed Kodak XAR-5 film, the radioactivity was removed as described in Section 2.6.VI, prior to hybridisation with the next probe.

6.2.II. Determination of degree of gene amplification

Following hybridisation to the three oncogene probes, the blots were sequentially probed with probes to the alpha-1-I and alpha-2-I collagen genes (Solomon et al., 1984; Sykes and Solomon, 1985, respectively). The blots were hybridised at high stringency and subjected to autoradiograpy on pre-flashed Kodak XAR-5 film, as

described in Section 2.6.V.

Quantification of the strength of the signal obtained by each probe was performed using a soft laser scanning densitometer (Joyce-Loebl, Gateshead, UK).

6.3. RESULTS

The technique employed in this chapter, whereby the density of the autoradiographic signal produced by an oncogene is compared to the signal produced by a single copy gene sequence such as the collagen genes, is suitable for detecting oncogene amplification with certainty when an oncogene is present in at least 3 times the normal copy number (Slamon et al., 1987; Gullick et al., 1989). Southern analysis of genomic DNA is also capable of detecting gene rearrangement, which might indicate the presence of abnormalities of gene structure (Libermann et al., 1985).

The p64.1 probe to the EGF receptor hybridises to the part of the gene encoding most of the external (EGF binding) domain, the transmembrane region and the proximal (5') part of the region coding for the intracytoplasmic domain (Ullrich et al., 1984). The sizes of the EGFR gene normally detected on Southern blots of EcoRI-digested DNA by the p64.1 cDNA probe are as follows: 7.7, 6.9, 5.9, 2.7, 2.4, 2.3, 2.0 and 1.6Kb in length (Libermann et al., 1985).

6.3.I. Amplification of the EGF receptor in malignant gliomas

Amplification of the EGF receptor was present in 5 out of 24 malignant gliomas examined, while none of the 13 benign gliomas showed detectable amplification. Owing to technical difficulties, the result was uninterpretable in 3 of the malignant gliomas analysed. Two of the tumours with EGFR amplification were malignant astrocytomas, while the remaining 3 were GBM's (see Table 6.1 and Appendix 2). Examples of tumours showing EGFR amplification are shown in Figure 6.1 (panel A).

Only tumours displaying greater than a 3-fold increase in the amount of the EGFR gene, as assessed by the methods used in this study, are listed as having amplification. The presence of amplification was detected by scanning densitometry which compared the density of the autoradiographic signal produced by each oncogene probe with that of the single copy collagen genes. The alpha-1-I collagen gene is situated on chromosome 17 (the site of the c-erbB-2 oncogene), while the alpha-2-I collagen gene lies on chromosome 7 (the site of the EGFR gene)(Solomon et al., 1984; Sykes and Solomon, 1985, respectively). Comparison of the strength of the EGFR autoradiographic signal with that produced by the alpha-1-I collagen gene (on chromosome 17) will give an indication of the number of copies of the EGFR gene in the genome independent of the degree of chromosome 7

duplication.

If a tumour possessed loss of one of the alleles of chromosome 17, this would result in a 50% reduction in the density of the autoradiographic signal of the alpha-1-I collagen gene. A 50% reduction in the autoradiographic signal density would have the result that the signal strength of the EGFR gene (on chromosome 7) relative to that of the alpha-1-I collagen gene would appear to be double that of the single copy gene sequence, and the tumour would therefore erroneously be interpreted as having 2-fold amplification of the EGFR oncogene. None of the tumours in which amplification was detected possessed loss of an allele on chromosome 17 as assessed by three chromosome 17 probes (see Chapter 5 and Appendix 2), and therefore the possibility of an error (based on allele loss) in the computation of the degree of EGFR amplification was lessened. Chromosome 17 allele loss may nevertheless be present which was not detected by one of the three RFLP probes used (see chapter 5) and which would result in the erroneous finding of 2-fold EGFR gene amplification in that tumour. Therefore only tumours with greater than a 3-fold increase in the EGFR signal relative to the alpha-1-I collagen signal are considered unequivocally to have EGFR amplification. Reduplication of chromosome 17 is an extremely rare phenomenon in malignant gliomas (Bigner et al., 1988a), and therefore use of the alpha-1-I collagen gene as a

reference for EGFR amplification is unlikely to result in an underestimate of the degree of EGFR amplification.

Comparison of the relative densities of the autoradiographic signals produced by the two collagen probes in tumours 19 and 27 revealed that the density of the alpha-2-I (chromosome 7) signal in each tumour was twice that of the alpha-1-I (chromosome 17) signal. The observed 2-fold increase in the signal density of the chromosome 7 probe (alpha-2-I collagen) is consistent with the presence of 4 copies of chromosome 7 in each of these tumours, a situation which may arise as a result of chromosome 7 duplication. Neither tumour 19 nor tumour 29 had loss of an allele on chromosome 17, so the observed increase in the density of the chromosome 7 probe relative to the chromosome 17 probe was not an artefact explainable by the presence of allele loss on chromosome 17. The formal possibility exists that the alpha-2-I collagen gene itself was reduplicated independently of the rest of the chromosome, however, it is difficult to see what advantage duplication of a collagen gene might confer to the growth of a clone of neoplastic cells. None of the other tumours, in particular those possessing EGFR amplification, showed molecular evidence of chromosome 7 reduplication.

Since the DNA was extracted from an homogenate of tumour cells, this technique will give an indication of the average number of copies of the EGFR gene in the

genome of all the cells in the homogenate (Venter et al., 1987; Slamon et al., 1989).

None of the tumours studies showed evidence of EGFR gene rearrangement.

6.3.II. Absence of N-myc or c-erbB-2 amplification

There was no evidence in any of the tumours of amplification or rearrangement of the N-myc or c-erbB-2 oncogenes (Figure 6.1, panels E and D, respectively).

6.4. DISCUSSION

Previous cytogenetic studies on gliomas have revealed abnormalities known as DM's, which are thought to represent sites of gene amplification. In addition, multiple copies of chromosome 7, the site of the EGFR oncogene, have frequently been observed in malignant gliomas (Section 6.1.I). It was therefore decided to examine the group of 13 benign and 27 malignant gliomas for evidence of amplification of three oncogenes.

Molecular techniques are capable of detecting oncogene amplification in DNA extracted from a solid tumour sample on which no previous cytogenetic data are available (Slamon et al., 1987). In addition, not all amplified genes produce microscopically-visible cytogenetic abnormalities (Wolman and Henderson, 1989), and molecular techniques can reveal the presence of such cytogenetically undetectable oncogene amplification. The techniques described in this

chapter revealed the presence of amplification of the EGFR oncogene in several of the malignant gliomas assayed.

6.4.I. Amplification of the EGF receptor in malignant gliomas

Greater than 3-fold amplification of the EGFR gene was detected in 5 out of 24 malignant gliomas (21%). There was no amplification in any of the 13 benign gliomas examined. The degree of amplification, as determined by densitometry scanning, ranged from 3 to 5-fold (tumours 15 and 33), to 25 to 30-fold (in tumour number 13).

The presence of EGFR amplification in malignant cerebral glioma biopsies has been previously reported. The percentage of malignant gliomas possessing EGFR amplification has varied from 40% and 38% in the studies of Libermann et al., (1985) and Bigner et al., (1988b), respectively, to 18% in the study of Malden et al., (1988). The percentage of EGFR amplification detected in the series of tumours described in this chapter (21%) therefore falls between the highest and lowest percentages detected by previous workers. The vast majority of supratentorial malignant gliomas previously analysed for evidence of EGFR amplification have been glioblastomas (80 out of the total of 97 malignant gliomas assayed by Libermann et al., (1985); Bigner et al., (1988b); Malden et al., (1988)), as were the series of tumours described in this chapter, therefore it is not possible with certainty to assess the

relative frequency of EGFR amplification in glioblastomas versus other types of malignant gliomas. No previous reports exist of EGFR gene amplification occurring in benign gliomas, which is consistent with the lack of EGFR amplification in the benign gliomas reported in this chapter.

No evidence of reduplication of chromosome 7 was seen in any of the tumours harbouring EGFR amplification, therefore it is most likely that the mechanism underlying the presence of increased copy numbers of the EGFR gene in these tumours is selective amplification of the EGFR gene itself, rather than chromosome 7 reduplication. Since cytogenetic studies were not performed on the tumours described here, it is not known whether the EGFR amplification was associated with the presence of DM's in the tumour cells.

It is possible that certain benign gliomas may contain a minority of cells possessing oncogene amplification. The presence of a minority of cells with oncogene amplification would not have been detected by the methods employed in this chapter, which analysed DNA derived from tumour homogenates, owing to dilution of DNA containing the amplified gene by DNA from cells in which the oncogene is not amplified (Bigner et al., 1988b). Since it is possible that sub-clones of cells which have developed EGFR amplification may go on to behave in a more aggressive manner, it is important that future

attempts to examine gliomas for evidence of oncogene overexpression should be performed on tissue sections, using the techniques of immunohistology and in-situ hybridisation, so that the presence of oncogene amplification in a small minority of cells might be detected.

The possible biological effect of EGFR amplification on the growth of tumour cells remains unclear. Since EGFR amplification is frequently associated with overexpression of the EGFR protein (Libermann et al., 1985; Malden et al., 1988), it has been postulated that elevated amounts of the EGF receptor may in some way influence the growth of the tumour cells, possibly by modulating the response of the tumour cells to the growth factors EGF and TGF-a, both of which bind to the EGFR (Ozanne et al., 1985). There are indications that high levels of the EGFR correlate with more aggressive biological behaviour in non-neural malignancies such as breast adenocarcinomas (Sainsbury et al., 1987), and transitional cell carcinomas of the bladder (Neal et al., 1985). However, no correlation was observed between the presence of EGFR amplification and patient survival in the 47 malignant gliomas with EGFR amplification reported by Bigner et al., (1988b). In a recent study on human glioblastoma cell lines EGFR amplification and expression did not correlate with tumourigenicity in nude mice or in soft agar, thereby

leading the authors to conclude that the presence of increased numbers of EGF receptors in tumour cells did not act as a determinant of *in vivo* and *in vitro* tumour growth. They did postulate, however, that the presence of increased EGF receptors may inhibit the ability of the glioma cells to differentiate into more mature cells, and that the less differentiated state is associated with continued cell division, which would allow other molecular events to occur which would determine the growth characteristics of the tumour cells (U *et al.*, 1989). The role of EGFR amplification in the biological behaviour of malignant gliomas therefore remains to be defined.

6.4.II. Lack of amplification of the c-erbB-2 oncogene in gliomas

The absence of c-*erb*B-2 gene amplification in gliomas is of interest because it contrasts with the phenomenon of c-*erb*B-2 gene amplification (with associated overexpression of the c-*erb*B-2 protein) observed in several types of adenocarcinoma (Section 6.1.II.b).

As discussed in Section 6.1.II.b, activation of the *neu* oncogene (the rat homologue of c-*erb*B-2) occurs due to a single point mutation at codon 664, which results in a glutamate for valine substitution in the transmembrane region of the *neu* protein (Bargmann and Weinberg, 1988).

The mutated neu protein is then thought to contribute towards the development of neural tumours in rats. Since amplification of c-erbB-2 did not occur in the human gliomas assayed in this chapter, the possibility was considered that the c-erbB-2 oncogene may nevertheless contribute towards glioma formation by the presence of a point mutation analogous to that found in the mutated rat neu oncogene. Accordingly, the DNA from these tumours was analysed for the presence of possible point mutations at codon 655 (the human equivalent of the mutated rat neu codon 664), which would result in a similar activating amino acid substitution to that encountered in the mutated rat neu protein. The analysis was performed by Dr. N. LeMoine of the ICRF Molecular Oncology Unit, Royal Postgraduate Medical School, London.

No point mutation at codon 655 was detected in any of the tumours (data not shown). Several studies have attempted to define additional areas of neu/c-erbB-2 (other than codons 664 and 655, respectively), which might harbour activating mutations. No further sites of activating mutations were identified, despite the fact that the entire neu/c-erbB-2 cDNA molecule was sequenced in these studies (Bargmann and Weinberg, 1988; Slamon et al., 1989), suggesting that only codon 655 may be a possible target of human c-erbB-2 activation. Therefore the failure to detect mutations at codon 655 in the gliomas studied here suggests that activation of c-erbB-2

by a point mutation did not occur in these tumours.

These results suggest that abnormalities of the c-erbB-2 gene and protein did not play a role in the development of the gliomas studied here.

6.4.III. Absence of N-myc amplification in gliomas

The lack of N-myc amplification in the gliomas studied here concurs with the findings of Bigner et al., (1988b), who reported N-myc amplification in less than 5% of malignant gliomas. These findings contrast with the high incidence of N-myc amplification observed in clinically aggressive neuroblastomas (Section 1.3.VI.b), which, like gliomas, are derived from the neural crest. Therefore amplification of the N-myc oncogene may be a phenomenon which does not occur in all tumours arising in cells derived from the neural crest.

6.4.IV. Summary

Amplification of the EGF receptor gene was detected in 5 out of 24 malignant gliomas, a phenomenon which has been reported previously in tumours of this type, and which may play a role in the development of these tumours.

TABLE 6.1: AMPLIFICATION OF THE EGFR GENE IN GLIOMAS

TUMOUR TYPE	EGFR Amplification
BENIGN (13 cases):	
Oligodendroglioma	0/3
Astrocytoma	0/6
Mixed OA	0/2
Ependymoma	0/1
Choroid plexus P.	0/1
Total	0/13
MALIGNANT (27 cases):	
Astrocytoma III	2/5
Oligo-Astro III	0/2
Glioma, undefined	0/1
Glioblastoma	3/16
Total	5/24 (21%)

ABBREVIATIONS and NOTES:

OA and Oligo-Astro, mixed oligodendroglioma-astrocytoma.

P., papilloma.

EGFR, epidermal growth factor receptor.

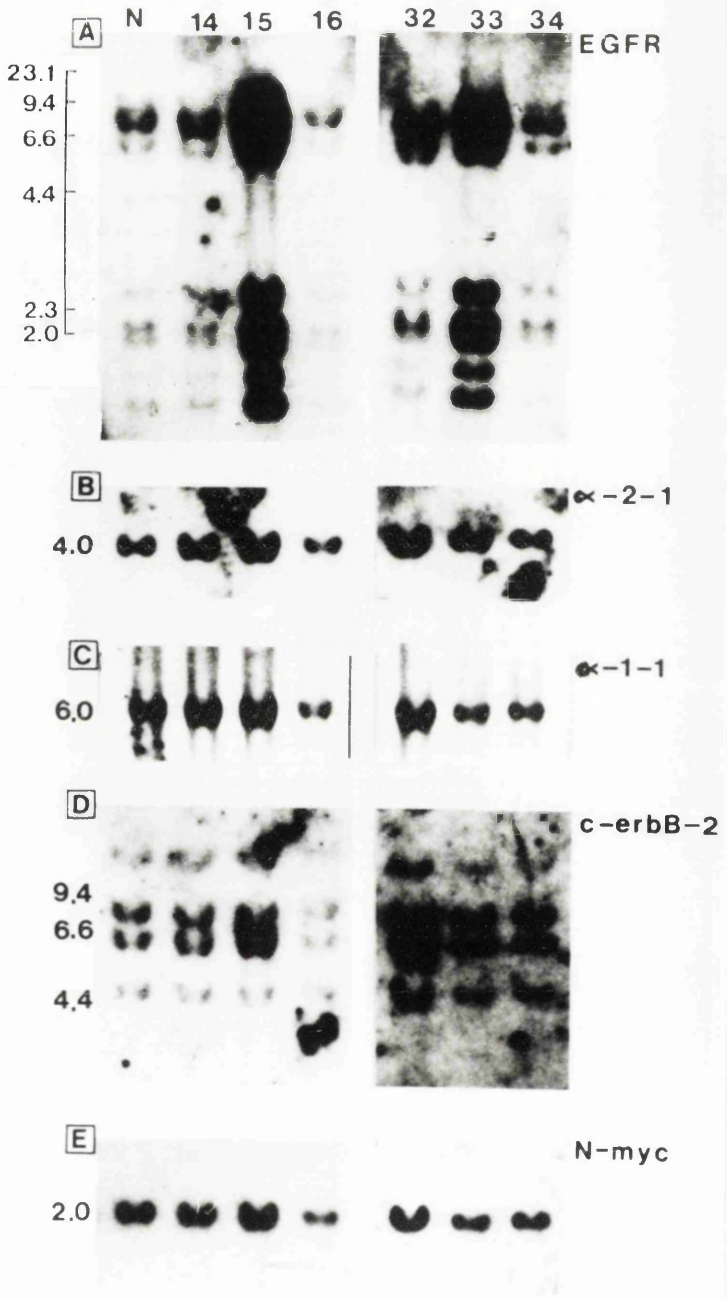
Figure 6.1. Southern analysis of the EGFR, c-erbB-2 and N-myc genes in gliomas

Southern blot of EcoRI-digested DNA hybridised to the human p64.1 EGFR cDNA probe (panel A), the full-length c-erbB-2 cDNA probe (panel D), the pNB-1 N-myc probe (panel E), the alpha-2-1 collagen probe (panel B) and the alpha 1-1 collagen probe (panel C).

DNA samples were derived from a normal individual (lane labelled N) and from solid tumours (numbered lanes). Tumours 15 and 33 show EGFR gene amplification (panel A), relative to the two collagen probes. There is no evidence of amplification of the c-erbB-2 or N-myc genes. None of the three oncogenes show evidence of gene rearrangement.

The molecular weight was assessed by comparing the relative mobility of the DNA samples to that of HindIII-digested lambda phage DNA fragments.

Fig. 6-1


A
N
14
15
16
32
33
34
EGFR
23.1
9.4
6.6
4.4
2.3
2.0
B
4.0
∝-2-1
C
6.0
∝-1-1
D
9.4
6.6
4.4
c-erbB-2
E
2.0
N-myc

CHAPTER 7

GENERAL DISCUSSION

7.1. INTRODUCTION

This thesis has described a series of experiments designed to detect the presence of molecular genetic abnormalities in human gliomas. Recent attempts to define the genetic alterations underlying tumour development have resulted in the identification of two classes of cellular genes, namely oncogenes and tumour suppressor genes. Functional abnormalities of these genes are thought to result in altered cell growth control, one of the fundamental properties of tumours Section 1.3.IV). It is therefore possible that abnormalities of oncogenes and tumour suppressor genes may underlie the development and clinical progression of human gliomas.

A group of 13 benign and 27 malignant gliomas were examined for abnormalities of certain oncogenes, as well as for evidence of alterations to the RB tumour suppressor gene. In addition, evidence of loss of chromosomes 10 and 17 was sought, as it is possible that such allelic loss could result in dysfunction of as yet undefined tumour suppressor genes on these chromosomes. The experiments were performed with the following aims:

i) To assess whether certain molecular abnormalities correlate with the histological grade of

glioma.

ii) To assess whether any molecular abnormalities are found in tumours of a certain histological subtype;

iii) To further knowledge of the molecular mechanisms underlying glioma development.

The results obtained will now be discussed in the light of their implications for understanding tumour biology and for the possible effects on tumour diagnosis.

7.2. CORRELATION OF MOLECULAR ABNORMALITIES WITH TUMOUR GRADE

Deletions of loci on chromosome 17 (thought to be compatible with the development of abnormal function of a putative tumour suppressor gene), were present in both benign and malignant gliomas, while loss of a chromosome 10 allele was seen only in malignant gliomas, including GBM's. Secondly, only GBM's showed evidence of loss of loci on more than one chromosome examined, or loss of a chromosome and amplification of the EGFR oncogene. None of the benign tumours had multiple molecular abnormalities in the same tumour.

Because of the limitations of the RFLP analysis technique used in this study, the successful demonstration of an allelic loss in a tumour homogenate implies that the majority of the cells in the tumour are members of a single clone possessing the particular genetic abnormality. Thus it is possible that the specific molecular genetic

alterations detected may in some way be associated with the biological evolution of the majority, if not all, of the cells present in a tumour (Vogelstein et al., 1988).

Of the malignant tumours, only glioblastomas, the most malignant of the gliomas, revealed multiple molecular abnormalities within the same tumour. Thus, 3 GBM's had a deletion of a putative tumour suppressor locus on chromosome 10q, as well as amplification of the EGFR gene (tumours 5, 13 and 39). A further GBM, (tumour 10), had deletions of alleles on chromosome 10 as well as 17. An additional GBM had loss of an allele on chromosome 10 and an abnormality of the RB gene (tumour 19), while 2 GBM's had loss of an allele on chromosomes 10 and 13 (tumours 16 and 29). The loss of chromosome 13 detected in tumours 16 and 29 may result in dysfunction of the RB gene in these 2 tumours, if the remaining RB allele is abnormal. Therefore, 7 out of a total of 19 GBM's (37%), had multiple abnormalities of the genetic loci assessed in this study.

On the basis of the data presented here, it is possible to associate certain molecular abnormalities with gliomas of increasing grades of malignancy. Thus, benign gliomas (grades I and II) may possess abnormalities of putative tumour suppressor loci on chromosome 17p. The development of malignancy (histological grade III) is frequently associated with loss of genetic material on chromosome 10q. The additional loss of the RB gene or of other chromosomes thought to harbour tumour suppressor

loci, or the presence of amplification of the EGFR gene is associated with the development of an even more malignant malignant phenotype, such as GBM.

Certain molecular abnormalities found in malignant gliomas (such as losses of genetic material on chromosomes 10 and 13), are not detectable in benign solid tumours using the techniques described.

7.3. CORRELATION OF MOLECULAR ABNORMALITIES WITH TUMOURS OF A SPECIFIC HISTOLOGICAL TYPE

Loss of genetic material from chromosome 17 was observed in a single benign astrocytoma, as well as in 3 glioblastomas. As discussed in Section 5.4.I, additional studies have now been published which confirm that loss of chromosome 17 is observed only in gliomas of astrocytic origin and in glioblastomas. The loss of chromosome 17, which is thought to result in dysfunction of a putative tumour suppressor locus on that chromosome (possibly the p53 gene, as discussed in Section 5.1.II.c), therefore appears to be associated with the development of benign neoplasia in astrocytic tumours, and not in tumours thought to arise from oligodendrocytes or ependymal cells. The fact that chromosome 17 loss is detected in malignant astrocytomas suggests that benign cells possessing this particular genetic event give rise to the majority of tumour cells present in a malignant astrocytoma. Therefore, loss of genetic material on chromosome 17, with the implied

dysfunction of a putative tumour suppressor locus on that chromosome, may be a genetic event of biological importance to the tumour cell. Finally, the fact that loss of chromosome 17 is detected in glioblastomas supports the theory that at least some glioblastomas arise from a pre-existing astrocytoma (Russel and Rubinstein, 1989).

7.4. THE POSSIBLE IMPLICATIONS OF THE ABOVE FINDINGS ON OUR UNDERSTANDING OF THE BIOLOGY OF GLIOMAS

7.4.I. The summatory effect of multiple molecular abnormalities in a tumour cell

It is thought that the development of a clinically significant tumour requires the accumulation of multiple genetic abnormalities in a tumour cell, each affecting the control of cell growth (reviewed in Klein and Klein, 1985). In experimental systems, the production of tumourigenic cells from primary (non-immortalised) fibroblasts (Land *et al.*, 1983b) or Schwann cells (Ridley *et al.*, 1988), requires the presence of a nuclear oncogene such as c-*myc*, SV40 large T, or adenovirus E1A, as well as a cytoplasmic oncogene such as an activated *ras* gene. The presence in the same cell of two or more oncogenes from different functional groups, such as the nuclear oncogenes c-*myc* and E1A and the cytoplasmic oncogenes *ras* and the EGFR gene, may have a cumulative effect, resulting in the evolution of a more malignant phenotype (Land *et al.*, 1983a). In addition, interfering with the normal function of a tumour

suppressor gene product can have the same functional effect on cell transformation as the presence of an abnormal nuclear oncoprotein, and therefore abnormalities of both oncogenes and tumour suppressor genes occurring in the same cell may result in increased tumourigenicity. (Weinberg, 1989).

It is possible that the presence in 3 of the GBM's of loss of genetic material on chromosome 10, the site of a putative tumour suppressor gene, as well as amplification of the EGFR, a cytoplasmic oncogene, may be associated in some way with the development of the highly malignant GBM phenotype. Similarly, the association of more than one abnormality thought to affect a tumour suppressor gene in three additional GBM's may have an analogous cumulative effect, resulting in increased tumourigenicity. A large proportion, (37%), of the GBM's (and none of the less malignant tumours), possessed multiple molecular abnormalities; it may be that these observed molecular lesions reflect some of the genetic events which lead to the development of these tumours. Abnormalities of several different oncogenes and/or putative tumour suppressor genes may associate to result in the same malignant phenotypic end point. Further studies are necessary to establish whether this is the case. Other abnormalities which have been described in various neuroectodermal tumours, such as amplification of the gli oncogene in gliomas (Kinzler et al., 1987), and loss of chromosome 22 loci in gliomas and

meningiomas (reviewed in Heim and Mitelman, 1988), may indicate which genetic events to focus these studies on initially.

7.4.II. The progression of a glioma from a benign to a malignant phenotype is associated with the stepwise genetic lesions described in this thesis

A molecular alteration which correlates with the development of a more malignant tumour grade may represent an event which is a determinant of the development of that grade of malignancy. Therefore the presence, in malignant gliomas, of genetic alterations such as loss of material on chromosomes 10 and 13, which are not detectable in benign tumours, is consistent with the interpretation that the development of these specific genetic lesions result in the progression of a tumour to a malignant phenotype. It is possible that the genetic lesions observed in malignant gliomas are simply epiphenomena not associated with the biological behaviour of the tumour. However, the observation in at least one case (tumour 19), that the vast majority of cells cultured from the tumour retained loss of alleles on chromosomes 10 and 13, suggests that the presence of such genetic abnormalities imparted a growth advantage to the cultured cells.

The association of stepwise genetic lesions with increasing grades of glial malignancy may provide the basis for a testable model of the molecular events underlying

glial tumour evolution, similar to the model proposed for the colorectal adenoma-carcinoma progression (Section 1.3.VI.c), in which the progressive loss of genetic material was associated with the development of tumours of an increasing grade of malignancy.

7.5. THE IMPLICATIONS OF THE FINDINGS DESCRIBED ABOVE FOR TUMOUR DIAGNOSIS AND PROGNOSIS

In the majority of cases, the grading of a glioma into a particular benign or malignant category correlates with the prognosis of that tumour (Russel and Rubinstein, 1989). However, there are many examples of the inability of the morphologically-based classification system to predict the clinical outcome of a tumour accurately (see Sections 1.4.III and IV). The identification of molecular abnormalities which prove to be determinants of the biological behaviour of a tumour, may ultimately improve our ability to accurately predict tumour prognosis. The association of specific molecular genetic lesions with differing grades of malignancy may also prove of value in tumour diagnosis.

The choice of treatment given to a particular patient is often based on the histological grading of the tumour. Given the small size of many of the biopsies submitted for diagnosis, coupled with the extreme morphologic heterogeneity present in some gliomas, it may be impossible to identify a minority of malignant cells in an otherwise

benign tumour by means of routine histological techniques. However, since a small proportion of malignant cells may represent a subclone which could ultimately determine the biological behavior of the glioma, it is essential to detect any such subclones. It is possible that malignant subclones, comprised of only a few cells, may be identifiable in the future by the use of gene amplification techniques, which amplify DNA from small numbers of cells, and thereby allow analysis of genetic lesions known to represent specific stages of glial tumour progression.

It is hoped that, by studying sufficiently large numbers of gliomas, the genetic lesions which determine tumour behaviour might be identified. The ability to identify such genetic lesions might enhance and refine the present morphological classification system, giving rise to categories of tumours whose members all have a similar biological behaviour and an identical response to a given therapy.

7.6. ADVANTAGES OF MOLECULAR ANALYSIS OVER THE TECHNIQUES OF CLASSICAL CYTOGENETICS

Loss of an entire chromosome can be visualised relatively easily using cytogenetics, although cytogenetic analysis depends on the availability of cells cultured from fresh tumour samples. However, losses of small regions of the chromosome, up to several hundred kilobases, may not be detectable microscopically using classical cytogenetic

techniques (Heim and Mitelman, 1988). Molecular techniques are superior to those of classical cytogenetics for two reasons. Firstly, molecular analysis of chromosome loss can be performed on stored solid tumour tissue, which has not previously undergone cytogenetic analysis. Secondly, in addition to being capable of detecting loss of an entire chromosome, molecular techniques can also identify the loss of relatively small areas of a chromosome, whose dissappearance would have been undetectable by microscopic analysis (Cavenee et al., 1983).

7.7. DIRECTIONS FOR FUTURE WORK

7.7.I. Extend the study to a large group of gliomas which have follow-up data

The molecular genetic lesions reported here, some of which have been recently verified by other workers, need to be assessed in a much larger group of gliomas. By matching molecular genetic information with comprehensive follow-up data, any possible relationships between the existence of a molecular lesion and tumour biology and prognosis can be detected. The establishment of any such relationships may have a bearing on future diagnostic and thereapeutic practices. As mentioned previously, one of the aims of such studies is to accurately define categories of tumours in which all the members behave in an homogeneous fashion, in order to be able to compare the efficacy of different therapeutic regimens.

7.7.II. Define the putative tumour suppressor genes thought to exist on chromosomes 10 and 17

It is thought, by analogy with the RB gene paradigm, that loss of chromosomes 10 and 17 serves to unmask the presence of an abnormal, as yet undefined tumour suppressor gene on the remaining chromosome, thus resulting in dysfunction of that gene. It is necessary to define the exact area of the chromosome which is lost by fine genetic mapping and to then identify the affected gene by screening tumours with cDNA fragments derived from the same area of the genome. Such molecular studies should be extended to examine other chromosomes which are known to be preferentially lost in gliomas, for example chromosomes 9 and 22 (Bigner et al., 1986; Bigner et al., 1988a), especially since it is thought that these chromosomes also harbour genes which function to inhibit cell growth.

Interestingly, not all genes which are lost during the evolution of tumours towards higher grades of malignancy have an identical function to the RB tumour suppressor gene, which appears to play a role in the control of the cell cycle (Section 4.1.I). As mentioned in section 1.3.VI.c, loss of genetic material on chromosome 18 occurs in over 70% of malignant colonic adenocarcinomas. The gene on chromosome 18 which is affected by such losses has recently been defined, and named DCC (deleted in colonic carcinoma). The putative DCC-encoded protein

resembles the neural cell adhesion molecules, a family of surface glycoproteins thought to play a role in cell-cell interactions and adherence (Fearon et al., 1990). It is thought that loss of function of this protein results in decreased adenocarcinoma cell adhesion and therefore enhances cellular invasion of surrounding tissues and possibly contributes towards the development of metastasis. Loss of chromosome 18 has been reported, albeit infrequently, in malignant gliomas (Bigner et al., 1988a), and it will be interesting to examine gliomas specifically for evidence of loss of the DCC gene.

7.7.III. Analyse the RB and p53 genes for point mutations

In chapter 4, it was mentioned that 3 GBM's exhibited loss of a locus on chromosome 13, an event which would result in dysfunction of the RB gene if the remaining RB allele were abnormal. It is therefore necessary to examine the remaining RB allele in each of these 3 tumours for evidence of subtle abnormalities such as point mutations, in order to establish the existence of RB abnormalities in these tumours. Similarly, future studies on RB gene integrity should be capable of detecting minute structural abnormalities in this gene. Subtle changes in the RB gene can be rapidly detected by using novel techniques such as PCR amplification of cDNA followed by direct sequencing of the amplified product (Horowitz et al., 1989).

In tumours bearing a chromosome 17 loss, the

possibility exists that such a loss gives rise to dysfunction of the p53 gene, situated on the short arm of the chromosome. Abnormalities of the p53 gene need to be sought using methods capable of detecting subtle alterations to the gene, since the majority (86%) of abnormalities of the p53 gene detected in one study consisted of point mutations clustered into 4 areas of the cDNA molecule (Nigro et al., 1989). Rapid analysis of such clustered mutations is theoretically possible by sequencing PCR-amplified fragments.

7.7.IV. Search for transforming oncogenes by means of DNA transfection

The technique of DNA transfection into NIH3T3 indicator cells, as described in Chapter 3, proved to be an effective means of detecting the presence of a dominantly transforming oncogene, albeit in what transpired to be a rhabdomyosarcoma cell line. Therefore the DNA from all 40 of the tumours analysed should ultimately be assessed for evidence of transforming oncogene activity, as the process of oncogene activation may play a role in the development of human gliomas, analogous to that played by the mutated neu gene in experimental rat neural tumours (Section 6.1.II.b).

The type of indicator cell used in a transfection assay could determine the class of activated oncogene detected, as the NIH3T3 cells appear to preferentially

reveal the presence of certain transforming genes, particularly those of the ras family (Section 3.1). Therefore, in order to increase the chances of detecting dominantly transforming oncogenes whose mode of action on the cell may differ from the ras family of genes, other indicator cell systems should be employed in future transfection assays.

One cell type which has been used for preliminary studies is of particular interest. The cells are primary rat embryo fibroblasts (REF's) immortalised with a temperature-sensitive mutant SV40 large T oncogene (Jat and Sharp, 1986). At 33^0C, the so-called permissive temperature (the temperature which allows normal function of the mutant SV40 large T gene), the REF's are immortalised. However, when the cells are grown at 39^0C, the mutant SV40 large T protein degrades, and the cells senesce rapidly (Jat and Sharp, 1989). It is possible that one mechanism by which the SV40 large T gene contributes towards REF immortalisation at 33^0C is by binding to the RB gene (Section 1.2.III.f), and the cells senesce at 39^0C because degradation of the SV40 large T protein at the higher temperature releases bound RB protein to exert its inhibitory effect on cell growth (Vousden and Jat, 1989). Therefore, when these cells are used as recipient cells for tumour cell DNA transfection, any transfected gene product capable of allowing continued cell growth at 39^0C may be exerting its effect by binding the RB protein. In other

words, any human gene capable of taking over the function of the degraded SV40 large T protein at 39^0C may be an RB binding protein, and therefore of importance in the control of the cell cycle. Initial transfection experiments using this cell line as a recipient for human tumour DNA have been carried out, and the results suggest that transfected tumour DNA is capable of allowing continued cell immortalisation at 39^0C (D.J. Venter and P.S. Jat, results not shown).

7.8. CONCLUSION

The results of the work described in this thesis, when combined with the findings of other researchers, suggests that abnormalities of tumour suppressor genes and of loci thought to harbour tumour suppressor genes, may contribute towards the progressive development of a more malignant glioma phenotype. The role of oncogene activation in the development of gliomas is less clear, but the results obtained in the study of colonic neoplasia suggests that *ras* oncogene mutation is an early event in the adenoma-carcinoma sequence, and that dysfunction of successive different tumour suppressor loci correlates best with malignant progression (Section 1.3.VI.c).

Therefore a general pattern may emerge in human tumours, in which oncogene activation initially produces abnormal growth control in a neoplastic cell, but the development of increasing grades of malignancy is primarily

due to the superadded loss of successive levels of growth control resulting from sequential tumour suppressor gene dysfunction. It is felt that the normal state of growth of a cell (when considered in isolation) is one of infinite growth and division, and that the function of growth inhibitory genes, such as tumour suppressor genes, only became necessary with the advent of multicellular organisms, in order to prevent the development of gross tissue disorganisation consequent on uncontrolled cell growth (Harris, 1989). If that is the case, then the future study of clinically-significant neoplasia (uncontrolled cell growth), will primarily involve analysis of tumour suppressor genes. It was Henry Harris who first drew attention to the phenomenon of suppression of malignancy resulting from fusion of a malignant cell with the chromsomes of a normal cell (thought to be due to the action of tumour suppressor genes), and it is fitting that a quote of his should sum up this discussion: "The real puzzle is not why cells multiply, but why cells do not multiply....." (Harris, 1989).

Appendix 1. Summary of patients' clinical data and histopathological diagnoses

Abbreviations:

abn	abnormal	M	male
ang	angioma	malig	malignant
astro	astrocytoma	mod	moderate
BV's	blood vessels	multinuc	multinucleate
Bx	biopsy	nec	necrosis
c.p.	choroid plexus	occ	occasional
CRx	chemotherapy	oligo	oligodendroglioma
EH	endothelial	OP DATE	operation date
	hyperplasia	par	parietal
F	female	pleo	pleomorphic
fro	frontal	post.op.	post operatively
GBM	glioblastoma	PT	patient's initials
	multiforme	R	right
GC's	giant cells	rec	recurrent
infl	infiltrating	RRx	radiotherapy
L	left	temp	temporal
lat	lateral	TUM.	tumour number
lympho	lymphocyte	vasc	vascular

? Alive: patient had not been seen at a follow up clinic within the last 6 months, and the notes did not indicate that he/she was deceased

APPENDIX 1

DIAGNOSIS	PT.	SEX	AGE	SITE	OP. DATE	SUMMARISED HISTOLOGY REPORT	COURSE/TREATMENT
Oligo II	J.I.	M	41	R. frontal	Dec. 1987	Oligo.; mod. EH; some mitoses; few astrocytes	Oligo '82 --RRx; rec '85 --CRx; rec '8
alig. Astro IV	A.S.	M	30	L. temporal	June 1988	Pleo., bizarre nuclei; Mitoses ++; mod. EH.	Cystic Astro II '84; rec 1989 (maligna
Astro II	C.N.	M	26	R. frontal	Jan. 1988	Mod. cell, some multinuc; cysts; mild EH; subpial invasion	RRx post-op.
Malig. Glioma	S.B.	F	37	R. frontal	Nov. 1987	EH++, and other features of malignancy.	Glioma '75 ; rec '84 (oligo II); rec '
GBM	I.W.	F	63	L. frontal	Dec. 1987	Pleo., necrosis, pallisading, mitoses +++, EH +++.	No further Rx.
Astro II	D.H.	M	40	Temporal	Mar. 1988	Astro's; slight pleo.; few mitoses; satellitosis. No necrosis or EH.	RRx post-op.
Oligo II-III	P.D.	M	44	R. frontal	Nov. 1987	Cellular oligo; infl. margins; EH ++; calcification.	RRx post-op.
ee tum. no. 35							
.P. Papilloma	C.S.	M	31	Post. fossa	Mar. 1988	Classic choroid plexus papilloma.	
GBM	C.E.	F	54	R. frontal	Mar. 1988	Small anapl. cells, GC's, gemistocytes ++; EH ++++: ? gliosarcoma	RRx post-op.
alig. O-A III	L.M.	M	51	R. fr.-par.	Jan. 1988	Gemistocytes & oligo's +++; mod pleo.; few mitoses; EH +; nec. +.	RRx post-op.
l. Mixed Glioma	P.F.	M	56	Frontal	Dec. 1987	Infiltrating oligo's & astro's; no obvious malignancy.	Malig. Glioma removed '80 --CRx and RR
GBM	J.M.	M	37	R. frontal	June 1988	Report not available	Tumour 90% removed; RRx post-op.
GBM	J.H.	F	26	L. frontal	Feb. 1989	Malig. pleomorphic cells, some multinuc; mitoses present.	RRx and CRx post-op.
lig. Astro. III	M.R.	M	58	L. temporal	Dec. 1988	Pleomorphic astrocytes; EH +++; necrosis +.	RRx post-op.
GBM	H.H.	M	64	L. frontal	Nov. 1988	Necrotic malignant glioma; EH +++; obliterative vascular changes.	RRx post-op.
Oligo. I-II	C.G.	F	19	L. lat. vent	Oct. 1988	Oligo. with uniform cells, few mitoses; tumour astro's present.	
GBM	M.W.	F	57	L. parietal	Sep. 1988	Pleomorhism ++; multinuc; necrosis & mitoses present; EH ++.	RRx post-op.
GBM	A.W.	M	70	R. frontal	Aug. 1988	Pleo.; EH ++++, necrosis gliosarcoma	No further Rx
igo.-Astro. II	D.P.	M	58		Nov. 1988	Moderately cellular. Some pleomorphism. No EH. Perivascular lympho's.	
Astro II	P.S.	M	43	R. frontal	Aug. 1988	Mod. cellularity; glial fibrils prominent; cysts present.	No further Rx.
tro II (infl.)	A.G.	M	63	L. temporal	Aug. 1988	Small astrocytes, subpial infiltration; neuronal entrapment.	RRx post-op.
Astro. I	J.S.	M	26	Cerebellar	July 1988	Benign astro.; glial fibrils; Rosenthal fibres; haemorrhage & cysts.	RRx post-op.
tro. II; ang. ?	A.R.	M	22	L. temporal	Mar. 1988	Mod. cellular & pleo. glial prolif.; angioma-like vasc. proliferation.	RRx post-op.
ec. Ependymoma	C.L.	M	31	Ventricular	Nov. 1987	Recurrent ependymoma. No malignant progression. No mitoses.	First operation in 1981. No further Rx
emangioblastoma	J.C.	M		Cerebellar	May 1988	Classic haemangioblastoma	
GBM	W.G.	M	14	L. tem.-par.	Nov. 1987	Necrosis & bizarre pleo. cells +++. Abnormal mitoses frequent	
GBM	M.A.	F	55	R. frontal	July 1988	Largely necrotic malignant glioma	
GBM	P.L-L.	M	67	L. tem.-par.	Nov. 1986	Necrosis, pleo. & multinucleate cells; diffuse infiltration; EH +++.	RRx post-op.
GBM	G.P.	M	56	R. frontal	Sep. 1988	Highly malignant & necrotic; pleo. & abn. mitoses +++; EH +++	RRx post-op.
GBM	R.C.	M	64	R. frontal	Sep. 1988	Infiltrative malignant glioma; necrosis & EH ++++. Mitoses rare.	
GBM	G.T.	M	51	L. frontal	Dec. 1988	Pleomorphic, mitoses +++, EH & necrosis +++	RRx and CRx post-op.
lig. astro. III	J.B.	F	39	Frontal	Jan. 1989	Small astro's+++; EH+++; necrosis+, astrocytosis + histios ? prev. RRx	
lig. Astro. III	P.S.	F	42	L. fro.-par.	Feb. 1989	Mod. cellular astrocytoma. Pleo. & bizarre cells, occ. mitoses; EH ++.	
GBM	A.N.	M	53	R. parietal	July 1988	Anaplasia and EH +++. Areas resembling oligodendroglioma.	RRx post-op.
GBM	S.G.	M	61	R. parietal	Sep. 1986	Abnormal mitoses & EH +++, mature collagen & fibroblasts: ? meningeal.	
GBM	M.S.	M	62	R. temporal	Oct. 1986	Mitoses, necrosis & EH++. Perivascular lymphocytes. Meningeal infiltrn	RRx post-op.
GBM	V.L.	M	58	L. temporal	Mar. 1987	Necrosis, pleo. astro's., giant cells. Abn. mitoses +++; EH +.	RRx post-op.
GBM	W.P.	M		R. frontal	Jan. 1987	Pleomorphic with necrosis +++ and EH +++	RRx post-op. Inoperable recurrence Mar
GBM	J.S.	M	56	L. frontal	Jan. 1987	Pleomorphic, mitoses++; necrosis & EH ++++; reactive gliosis & oedema.	RRx post-op.
lig. Astro. III	M.B.	M	32	L. frontal	Oct. 1986	Pleomorphic, giant cells accumulating around BV's and neurons. EH +	No further Rx.
Malig. O-A III	J.W.	F	46	R. parietal	Oct. 1986	Anaplasia, pleomorphism, giant cells, abn. mitoses. Necrosis+ & EH ++	No further Rx.

HISTOLOGY REPORT	COURSE/TREATMENT	FATE
. EH; some mitoses; few astrocytes	Oligo '82 --RRx; rec '85 --CRx; rec '87 (Bx) --CRx; rec'88, (Oligo II)	Alive Aug. 1989, = 20 months.
rre nuclei; Mitoses ++; mod. EH.	Cystic Astro II '84; rec 1989 (malignant).	
some multinuc; cysts; mild EH; subpial invasion	RRx post-op.	Alive Aug. 1989, = 19 months.
ther features of malignancy.	Glioma '75 ; rec '84 (oligo II); rec '85 (malig.) --RRx; rec '87 (Bx).	Died Apr. 1989, = 17 months.
osis, pallisading, mitoses +++, EH +++.	No further Rx.	? Alive Aug. 1989.
ight pleo.; few mitoses; satellitosis. No necrosis or EH.	RRx post-op.	Alive May 1989 , = 14 months.
igo; infl. margins; EH ++; calcification.	RRx post-op.	Alive Aug. 1989, = 21 months.
roid plexus papilloma.	RRx post-op.	Lost to follow-up.
. cells, GC's, gemistocytes ++; EH ++++: ? gliosarcoma	RRx post-op.	? Alive Aug. 1989.
s & oligo's +++; mod pleo.; few mitoses; EH +; nec. +.	Malig. Glioma removed '80 --CRx and RRx; rec '87 (Bx).	? Alive Aug. 1989.
g oligo's & astro's; no obvious malignancy.	Tumour 90% removed; RRx post-op.	Alive Aug. 1989, = 20 months.
available	RRx and CRx post-op.	? Alive Aug. 1989.
morphic cells, some multinuc; mitoses present.	RRx post-op.	Alive Aug. 1989, = 5 months.
astrocytes; EH +++; necrosis +.	RRx post-op.	Alive Aug. 1989, = 8 months.
lignant glioma; EH +++; obliterative vascular changes.		? Alive Aug. 1989.
uniform cells, few mitoses; tumour astro's present.	RRx post-op.	Lost to follow-up (left country).
++; multinuc; necrosis & mitoses present; EH ++.	No further Rx	? Alive Aug. 1989.
+++, necrosis gliosarcoma		? Alive Aug. 1989.
cellular. Some pleomorphism. No EH. Perivascular lympho's.	No further Rx.	? Alive Aug. 1989.
arity; glial fibrils prominent; cysts present.	RRx post-op.	Alive Aug. 1989, = 12 months.
cytes, subpial infiltration; neuronal entrapment.	RRx post-op.	Alive Aug. 1989, = 12 months.
o.; glial fibrils; Rosenthal fibres; haemorrhage & cysts.	RRx post-op.	Alive Aug. 1989, = 13 months.
ar & pleo. glial prolif.; angioma-like vasc. proliferation.	First operation in 1981. No further Rx. following latest op. (1987).	Alive Aug. 1989. = 17 months.
pendymoma. No malignant progression. No mitoses.		? Alive Aug. 1989.
mangioblastoma		
bizarre pleo. cells +++. Abnormal mitoses frequent		? Alive Aug. 1989.
rotic malignant glioma	RRx post-op.	Lost to follow-up (returned to Israel).
leo. & multinucleate cells; diffuse infiltration; EH +++.	RRx post-op.	? Alive Aug. 1989.
gnant & necrotic; pleo. & abn. mitoses +++; EH +++		? Alive Aug. 1989.
e malignant glioma; necrosis & EH ++++. Mitoses rare.	RRx and CRx post-op.	? Alive Aug. 1989.
, mitoses +++, EH & necrosis +++		Alive Aug. 1989.
's+++; EH+++; necrosis+, astrocytosis + histios ? prev. RRx		? Alive Aug. 1989.
ar astrocytoma. Pleo. & bizarre cells, occ. mitoses; EH ++.	RRx post-op.	Alive Aug. 1989.
nd EH +++. Areas resembling oligodendroglioma.		? Alive Aug. 1989.
toses & EH +++, mature collagen & fibroblasts: ? meningeal.	RRx post-op.	
crosis & EH++. Perivascular lymphocytes. Meningeal infiltrn	RRx post-op.	Lost to follow-up (returned to Athens).
leo. astro's., giant cells. Abn. mitoses +++; EH +.	RRx post-op. Inoperable recurrence March 1988 (14 months later)	Died July 1987. = 4 months.
with necrosis +++ and EH +++	RRx post-op.	? Alive Aug. 1989.
, mitoses++; necrosis & EH ++++; reactive gliosis & oedema.	No further Rx.	Lost to follow-up.
, giant cells accumulating around BV's and neurons. EH +	No further Rx.	Died 14/10/86, = 4 days post-op. (seizures)
pleomorphism, giant cells, abn. mitoses. Necrosis+ & EH ++		Died. (Not sure when).

Appendix 2. Summarised results of experiments on tumours

Key:

The numbers 1 and 2 refer to the alleles present on the autoradiograph, with 1 referring to the large allele and 2 to the smaller allele. Thus, if the number 1 appears, it means that only the larger allele is still present in the tumour DNA, and the tumour has therefore lost the smaller allele.

ND, experiment not done for that tumour

U, uninformative result, owing to the patient being homozygous at that locus

RNI, results not interpretable for technical reasons

AMP, amplified

@@@12, both alleles present, but of different sizes in the blood and tumour samples, indicating that the samples were from different patients

HGM, HGM symbol refers to the symbol given to the locus defined by a particular probe by the Human Gene Mapping Workshop (Pearson et al., 1987).

The remainder of the abbreviations are as listed in the legend to Appendix 1.

DIAGNOSIS	SEX	AGE	CHROM. HGM PROBE ENZYME ALLELES	10q21-q23 D10S4 p1-101 TaqI A1,A2	10q21-q23 D10S4 p1-101 SacI B1,B2	10q21-q23 D10S4 p1-101 SacI C1,C2	10q21-q23 D10S1 Dry5-1 TaqI A1,A2	10q21-q23 D10S1 Dry5-1 Bgl-II B1,B2	10q21.1 D10S5 p9-12-a TaqI A1,A2	10q24.33 D10S21 CARLP118.2 BamHI A1,A2	10q24-qter PLAU pHUK-1 BamHI A1,A2	17p D17S30 pYNZ22 TaqI	17p13.3 D17S28 pYNH37.3 TaqI	17p13.1-11.2 D17S31 pMCT35.1 RsaI	13q14 p68RS2.0 RsaI	13q14 cDNA 3.9 HindIII	1p35-p33 DIS7 p\MSI TaqI	7p36-qte D7S22 p\g3 TaqI
Oligo II	M	41		U	12	U	U	U	ND	12	U	ND	U	12	U		1	12
Malig. Astro IV	M	30		12	ND	ND	U	U	U	12	12	12	12	U	U		12	12
Astro II	M	26		12	ND	ND	12	ND	12	12	12	12	U	U	12		12	12
Malig. Glioma	F	37		12	ND	ND	12	ND	U	12	U	12	12	U	U		12	12
GBM	F	63		U	ND	ND	U	ND	U	U	2	12	U	RNI	U		12	12
Astro II	M	40		U	U	12	U	12	12	12	12	12	RNI	U	12		12	12
Oligo II-III	M	44		12	ND	ND	U	U	U	RNI	2	12	RNI	U	12		U	12
See tum. no. 35																	@@@12	@@@12
C.P. Papilloma	M	31		U	ND	ND	12	ND	ND	U	12	ND	RNI	ND	ND		12	12
GBM	F	54		U	U	U	U	U	U	U	2	1	1	U	12		12	12
Malig. O-A III	M	51		1	ND	ND	U	12	12	12	U	12	U	RNI	U		12	RNI
fl. Mixed Glioma	M	56		12	ND	ND	12	ND	U	12	U	12	U	RNI	U		12	RNI
GBM	M	37		2	RNI	RNI	U	12	2	RNI	12	12	RNI	U	12		12	RNI
GBM	F	26		12	ND	ND	U	U	ND	12	U	ND	U	12	U		12	12
alig. Astro. III	M	58		U	U	U	12	U	U	12	12	12	U	12	12		12	12
GBM	M	64		1	ND	ND	U	1	U	U	12	12	U	12	2		12	U
Oligo. I-II	F	19		U	U	U	U	U	U	12	12	U	U	12	U		12	12
GBM	F	57		U	ND	ND	U	ND	1	1	1	12	U	RNI	U		12	12
GBM	M	70		U	ND	ND	1	ND	1	2	1	U	RNI	RNI	1		12	12
ligo.-Astro. II	M	58		U	U	12	U	U	U	U	12	U	RNI	RNI	U		12	12
Astro II	M	43		12	ND	ND	12	ND	12	U	12	2	U	1	12		12	12
stro II (infl.)	M	63		U	U	U	U	12	ND	12	12	ND	RNI	12	U		2	U
Astro. I	M	26		U	RNI	RNI	12	ND	12	U	U	U	RNI	RNI	U		12	12
stro. II; ang. ?	M	22		12	ND	ND	U	U	U	U	U	12	RNI	12	U		12	12
Rec. Ependymoma	M	31		12	ND	ND	U	12	12	U	12	12	RNI	12	U		12	12
aemangioblastoma	M			12	ND	ND	12	ND	ND	12	U	ND	RNI	ND	ND		12	12
GBM	M	14		U	U	U	U	U	U	12	U	1	RNI	1	U		U	U
GBM	F	55		U	12	U	U	1	U	12	12	12	12	12	12		12	12
GBM	M	67		2	ND	ND	U	ND	U	U	U	12	12	RNI	2		12	12
GBM	M	56		U	U	RNI	12	ND	ND	RNI	12	U	RNI	1	U		12	12
GBM	M	64		U	U	U	12	ND	U	U	12	U	12	12	U		U	12
GBM	M	51		U	12	U	U	U	12	U	U	U	U	12	12		12	12
alig. Astro. III	F	39		12	ND	ND	12	ND	12	U	U	U	U	12	12		12	12
alig. Astro. III	F	42		12	ND	ND	12	ND	ND	12	12	U	12	ND	ND		12	12
GBM	M	53		U	U	U	U	U	1	RNI	U	U	12	U	U		12	12
GBM	M	61		U	ND	ND	U	ND	U	1	U	12	12	ND	ND		12	12
GBM	M	62		12	ND	ND	U	12	12	U	12	12	12	12	U		12	12
GBM	M	58		U	U	U	12	12	U	U	12	12	U	12	1		12	12
GBM	M			U	U	U	2	1	U	U	12	12	U	U	12		12	12
GBM	M	56		U	ND	ND	1	ND	1	U	U	12	U	ND	ND		12	12
alig. Astro. III	M	32		12	ND	ND	U	12	U	12	12	U	U	RNI	12		12	12
Malig. O-A III	F	46		1	1	12	1	2	U	12	U	U	12	12	U		12	12

2	10q24-qter PLAU pHUK-1 BamHI A1,A2	17p D17S30 pYNZ22 TaqI	17p13.3 D17S28 pYNH37.3 TaqI	17p13.1-11.2 D17S31 pMCT35.1 RsaI	13q14 p68RS2.0 RsaI	13q14 cDNA 3.9 HindIII	1p35-p33 DIS7 p\MSI TaqI	7p36-qter D7S22 p\g3 TaqI	7p14-p12 EGFR p64.1 EcoRI	17q21 ERBB2 EcoRI	17p13.1 p53 cDNA E,B,H	2p23-pter N-myc pNB-1 EcoRI
M												
1	U	ND	U	12	U		1	12				
2	12	12	12	U	U		12	12				
3	12	12	U	U	12		12	12				
4	U	12	12	U	U		12	12				
5	2	12	U	RNI	U		12	12	AMP 10-15x			
6	12	12	RNI	U	12		12	12				
7	2	12	RNI	U	12		U	12				
8							@@@12	@@@12				
9	12	ND	RNI	ND	ND		12	12				
0	2	1	1	U	12		12	12				
1	U	12	U	RNI	U		12	RNI				
2	U	12	U	RNI	U		12	RNI				
3	12	12	RNI	U	12		12	RNI	AMP 25-30x			
4	U	ND	U	12	U		12	12				
5	12	12	U	12	12		12	12	AMP 3-5x			
6	12	12	U	12	2		12	U				
7	12	U	U	12	U		12	12				
8	1	12	U	RNI	U		12	12				
9	1	U	RNI	RNI	1		12	12				
0	12	U	RNI	RNI	U		12	12				
1	12	2	U	1	12		12	12				
2	12	ND	RNI	12	U		2	U				
3	U	U	RNI	RNI	U		12	12				
4	U	12	RNI	12	U		12	12				
5	12	12	RNI	12	U		12	12				
6	U	ND	RNI	ND	ND		12	12				
7	U	1	RNI	1	U		U	U				
8	12	12	12	12	12		12	12				
9	U	12	12	RNI	2		12	12				
0	12	U	RNI	1	U		12	12	RNI	RNI		RNI
1	12	U	12	12	U		U	12				
2	U	12	U	12	12		12	12				
3	U	U	U	12	12		12	12	AMP 3-5x			
4	12	U	12	ND	ND		12	12				
5	U	U	12	U	U		12	12		RNI		
6	U	12	12	ND	ND		12	12	ND	ND		ND
7	12	12	12	12	U		12	12				
8	12	12	U	12	1		12	12				
9	12	12	U	U	12		12	12	AMP 5-10x			
0	U	12	U	ND	ND		12	12	ND	ND		ND
1	12	U	U	RNI	12		12	12				
2	U	U	12	12	U		12	12				

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