The Role of p53 in Colorectal Carcinogenesis

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Declaration

The work presented in this thesis has been carried out by myself except where specifically indicated in the acknowledgements.

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Dedication

This thesis is dedicated to my wife Shona for her undying support throughout the years it has taken to produce.

Abbreviations

List of abbreviations used in the text:

APC	Adenomatous polyposis coli
APS	Ammonium persulphate
BCIP	5-Bromo-4-cloro-3-indolyl phosphate
CsCl	Caesium chloride
DCC	Deleted in colon cancer
DTT	Dithiothreitol
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetatraacetic acid
FAP	Familial adenomatous polyposis
GS	Gardner's syndrome
HET	Heterozygous
HOM	Homozygous
LMP	Low melting point (agarose)
LIF	Leukaemia inhibitory factor
LOH	Loss of heterozygosity
MCC	Mutated in colorectal cancer
Mn	Manganese
NaCl	Sodium chloride
NBT	Nitroblue tetrazolium
NRS	Normal rabbit serum
PAGE	Poly-acrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMSF	Polymethylsulphonyl fluoride
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
SSC	Saline, sodium citrate buffer
TEMED	N,N,N',N'-Tetramethylethylenediamine
TBE	Tris/Borate/EDTA buffer
TE	Tris/EDTA buffer
TBS	Tris buffered saline

Abstract

Expression of p53 was studied immunohistochemically in a consecutive series of resected human colorectal tumours using monoclonal antibody PAb1801. Expression was detected in 41 of 87 carcinomas (47%) but in only 4 of 46 sporadic adenomas (8.7%) indicating that p53 expression is associated with malignant transition in colorectal tumourigenesis. Immunoblots of total protein extracted from immunohistochemically positive and negative carcinomas confirmed that the immunostaining was indeed marking p53 in these tissues. Immunohistochemistry of a subset of 45 carcinomas using monoclonal antibody PAb240 (mutant p53 specific) showed positivity in 22 (48.9%) and a highly significant correlation with PAb1801 immunostaining suggesting that the p53 expression identified with PAb1801 is due to p53 mutation possibly via protein stabilisation. Expression of p53 did not correlate with Dukes' stage or histopathological markers of poor prognosis but did correlate with the presence of DNA aneuploidy and in particular the presence of multiple divergent clones defined by flow cytometry. This finding agrees with the hypothesis that wild-type p53 has a role in genomic surveillance and loss of this function may result in cell replication in the presence of DNA sequence abnormalities leading to aneuploidy and clonal divergence. DNA aneuploidy is a marker of poor prognosis and p53 expression may therefore mark more aggressive tumours.

Fine mapping of the deletion on chromosome 17p using 4 polymorphisms indicated allele loss in 46 of 79 cases (58%) and that this usually included the p53 gene locus. Loss of any 17p allele and loss of markers adjacent to or within the p53 gene correlated with p53 expression and thus mutation. There

was also a correlation between 17p allele loss and the presence of DNA aneuploidy. These data indicate that 17p allele loss and p53 expression (indicating mutation) are linked suggesting inactivation of both copies of p53 is important in colorectal carcinogenesis. This appears to occur at the phase of malignant transition, possibly by permitting replication of sequence abnormalities and the development of aneuploidy. Analysis of p53 gene sequence in 12 cases defined for 17p allele loss and p53 expression indicated that p53 mutation results in positive p53 immunostaining. Further analysis suggested that not all p53 mutations have the same phenotype some perhaps not requiring deletion of the remaining wild-type gene for expression of the "p53 null" phenotype.

Loss of a marker on chromosome 17q was seen in 13 of 64 (20%) of colorectal cancers. This is a higher frequency than detected previously and correlated in this series with the presence of lymph node metastasis suggesting the presence of a tumour metastasis suppressor gene at this locus.

To investigate the role of germ-line p53 mutation in tumourigenesis, a partial p53 gene containing a single point mutation was inserted into the genomic p53 of pluripotent mouse embryonal stem cells by homologous recombination. Selection for reversion leaving such a mutation in an otherwise unaltered p53 gene is under way. In addition, inactivation of p53 by homologous recombination was achieved and successfully passed into the germ-line. Mice deficient in one (p53+/-) or both (p53-/-) gene alleles were developmentally normal but developed malignant tumours of lymphoid tissue and mesenchyme at high frequency. Homozygous (p53-/-) mice succumbed to malignancy more quickly than heterozygous (p53+/-) litter

mates.

These results indicate a central role for p53 inactivation in human colorectal tumourigenesis probably at the critical phase of malignant transition, possibly by permitting premature replication or replication in the presence of DNA sequence abnormalities and the development of aneuploidy. Although not necessary for normal development in mice, p53 is vital in the prevention of carcinogenesis and p53 deficient mice will permit further study of cancer prevention and therapy. Finally, a possible tumour metastasis suppressor gene locus has been identified on chromosome 17q.

Chapter 1

INTRODUCTION

1.1 General Introduction

Neoplasia can be defined as the proliferation of cells within a tissue outwith the normal growth controls operating in that tissue. Malignant forms of neoplasia (cancer) are distinguished from benign by their ability to invade locally into surrounding normal tissue and to spread widely (metastasise) to distant sites within the body.

As a disease, cancer is an important cause of mortality and morbidity world-wide with 253,000 new diagnoses each year in the UK alone accounting for 162,000 deaths annually. The commonest malignancy in males in the UK is carcinoma of the lung, followed by skin cancers and then carcinoma of the colorectum giving 13,370 new cases each year. In females, colorectal cancer is second only to breast cancer accounting for 13,870 new cases per year (Cancer Research Campaign, 1990). Colorectal cancer causes the deaths of significant numbers of people in the UK and the Western World as a whole despite being potentially curable if diagnosed early. However the incidence of colorectal cancer has not altered materially in the last two decades, nor has the the 37% overall five year survival (Cancer Research Campaign, 1990). Research into the pathogenesis and natural history of this disease is therefore necessary to define the critical events in its development. This knowledge will permit preventative measures to be employed, allow its earlier detection and enable a more rational approach to its treatment to be followed.

Colorectal carcinogenesis is a multi-stage process requiring some or all of a number of specific genetic events of which several have now been described (section 1.5). Some of these events can be inherited as germ-line mutations as in the case of Adenomatous Polyposis Coli gene mutations in Familial Adenomatous Polyposis and Gardner's Syndrome but thus far the genes responsible for Hereditary Non-Polyposis Colorectal Cancer and the familial clustering of "sporadic" colorectal cancer have not been elucidated (section 1.3). Some of the environmental factors associated with colorectal carcinogenesis have been described (section 1.2) but until the critical events are characterized it will not be possible to dissect out the relevant causative factors and remove them from the environment. Some of the critical events are now being identified (section 1.5) and amongst them, p53 abnormalities appear to be the commonest and to act at the critical phase of transition from benign to malignant neoplasm (chapter 2).

This thesis is concerned with the role of p53 in colorectal carcinogenesis. To help understand this role in the context of a complex disease such as colorectal cancer with its multifactorial pathogenesis, the putative environmental and genetic factors will be reviewed initially and the numerous abnormalities of histopathology, karyotype, DNA ploidy and genetic material will be discussed.

1.2 The Role of Environmental Factors in Colorectal Carcinogenesis

1.2.1 Introduction

External factors involved in carcinogenesis fall into 3 categories: oncogenic viruses; electro-magnetic radiation; and chemical carcinogens. Thus far no viruses have been associated with adenocarcinoma of the colorectum. Survivors of the atomic bombs at Hiroshima and Nagasaki in 1945 show an excess of colon cancers (although not rectal cancers) when compared to an unexposed control population after 30 years of follow-up (Kohn et al., 1984). However, the effect is small and unlikely to be important outwith this population. Most external carcinogens affecting the colon and rectum are thus likely to be chemicals.

The epithelium of the colorectum can be exposed to chemical carcinogens via the blood stream or directly by the bowel contents. Most research has focused on the bowel contents: dietary elements, bile, secretions from the gastrointestinal tract and pancreas and their metabolites. Unfortunately, much of the evidence in this field is circumstantial and contradictory. Information of interest comes, however, from epidemiological studies of cancer incidence, case-control studies which suggest some possible dietary factors and more specific studies of dietary fibre, meat, bile acids, faecapentaenes, and calcium. Each of these will be discussed in turn.

1.2.2 Epidemiology

Armstrong and Doll (1975) investigated the correlation of cancer incidence and mortality for individual countries with *per capita* consumption of various

commodities. A positive correlation between colorectal cancer mortality rates and animal protein and fat consumption was demonstrated along with a negative correlation for cereal consumption. A similar study (Liu et al., 1979) again showed a positive correlation of *per capita* consumption of saturated fat and cholesterol with colorectal cancer and a negative correlation with dietary fibre intake. Further analysis revealed cholesterol consumption as having the most significant correlation with colorectal cancer mortality.

Studies of native Japanese emigrating to the USA demonstrated that their relatively low levels of colorectal cancer mortality when living in Japan had, within one generation, risen to equal that of the indigenous white population (Haenszel & Kurihara, 1968). This alteration in colorectal cancer mortality suggests that environmental differences between Japanese and Western life-style and diet rather than genetic differences account for the different incidences in the 2 countries. Furthermore, in Japan itself, since 1945 there has been a dramatic rise in colorectal cancer incidence (Lee et al., 1976) accompanied by increases in fat consumption (Hirayami et al., 1975).

1.2.3 Case-control studies

Population studies assume a uniform consumption across a population and a uniform cancer incidence, ignoring what may be important regional differences. Case control studies of dietary intake in Canada (Jain et al., 1980) and Puerto Rico (Martinez et al., 1979) comparing patients with colorectal cancer to carefully matched controls demonstrated a strong correlation with fat and cholesterol intake (being particularly strong for saturated fat). However, similar studies from the USA (Graham et al., 1978) and Israel (Modan et al., 1975) have failed to show any such correlation.

Further studies in Greece (Manousos et al., 1983) and Australia (Potter & McMichael, 1986) showed that meat intake (which may simply reflect a large source of saturated fat) was strongly related to colorectal cancer risk, a trend enhanced by low dietary fibre. One other notably positive association is with total energy intake (Jain et al., 1980; Potter & McMichael, 1986; Lyon et al., 1987) which may account for some of the discrepancies between studies as dietary fat is a very rich source of energy and not all studies control for total energy intake when assessing dietary fat. Despite this association, obesity does not appear to be a risk factor (Jain et al., 1980).

1.2.4 Dietary fibre

Following a study of rural Africans, Burkitt (1971) proposed that a high intake of unrefined fibre was associated with a lower incidence of colorectal cancer. The high fibre diet of the Africans was associated with a high stool bulk and a more rapid intestinal transit time which Burkitt interpreted as responsible for the lower incidence of colorectal cancer in this population. It was proposed that these factors acted by diluting carcinogens, reducing the time they had to act on the mucosa and preventing bile salt degradation. Further studies have demonstrated a similar correlation in the USA (Graham et al., 1978), Greece (Manousos et al., 1975) and Israel (Modan et al., 1975) although a negative correlation has also been reported (Martinez et al., 1979). A possible cause of this discrepancy is the difference in the protective effects of different varieties of fibre. Consumption of fruit and vegetable fibre is higher in healthy controls than in colorectal cancer patients (Haenszel et al., 1980; Potter & McMichael, 1986; Macquart-Moulin, 1986; La Vecchia et al., 1988) whereas cereal fibre intake is unrelated or positively related to colorectal cancer in the same studies.

Burkitt (1971) proposed that dietary fibre reduced the incidence of colorectal cancer by diluting carcinogens and reducing their time to act on the colonic mucosa. The data showing that fruit and vegetable fibre are protective and cereal fibre is not suggest that this is an over-simplification. It is more likely that certain, undefined, factors consumed along with vegetable fibre are exerting an influence on colorectal cancer risk. Animal studies have suggested that oral fibre can reduce excreted bile acids, especially secondary bile acids (Reddy, 1981) and inhibit the epithelial injury and resulting proliferation induced by fat ingestion (Caderni et al., 1987). Colonic tumours in rats induced by azoxymethane are also significantly reduced in animals fed citrus fibre or wheat bran (Reddy et al., 1981). Dietary fibre can be protective against colorectal cancer but its mechanism of action is far from clear as yet.

In summary, although there are inevitable inconsistencies in the data from these epidemiological and case control studies, it seems that high consumption of saturated fat, cholesterol and meat (whether this is simply a source of fat and cholesterol is not yet clear) along with low intake of unrefined dietary fibre correlate with higher incidence of colorectal cancer. These particular dietary factors are characteristic of the diet found in the Western world and thus laboratory studies are necessary to establish whether they are causative and not simply coincident and to discover their mechanism of action.

1.2.5 Meat consumption

Mice fed a variety of food products including cooked meats have a significantly elevated frequency of nuclear aberration in their colonic mucosa (Bird & Bruce, 1983). Although the particular chemicals have not been isolated, certain carcinogens and mutagens (protein and amino acid pyrolysates) known to be present in cooked meats, mostly heterocyclic amines and polycyclic aromatic hydrocarbons (Felton et al., 1984; Sugimura, 1986) can result in an increase in nuclear aberration frequencies in the colons of laboratory animals (Bird & Bruce, 1984). Further studies have also demonstrated that certain of these products (isolated from ordinarily cooked food) when fed to rats induce colon tumours (Sugimura, 1986). Activation of the *ras* and *raf* oncogenes (presumably by mutation) have been described in tumours induced by the ingestion of 2-amino-3 methylimidaz [4,5-f] quinoline (IQ) and 2-aminodipyrido [1,2-a:3',2'-d] imidazol (Glu-P-2) (Ishikawa et al., 1985), both of which are heterocyclic amines found in cooked food (Sugimara, 1986). These compounds are metabolised by cytochrome P450, are mutagens and induce single strand breaks in double stranded DNA (Yamazoe et al., 1983; Wakata et al., 1985). This evidence, when added to the finding of mutagenic heterocyclic amines in the faeces and urine of human subjects after ingestion of fresh ground beef (Hayatsu et al., 1985) suggests that these compounds may be the colorectal cancer initiators found in meat and responsible for the correlation between meat consumption and colorectal cancer already described (section 1.2.5).

1.2.6 Faecapentaenes

Faecapentaenes are a group of potent faecal mutagens identified at

increased levels in people at higher risk of colon cancer (Reddy et al., 1980). These compounds are isomers of fecapentaene-14 and fecapentaene-12, are bacterially derived polyunsaturated derivatives of glycerol and are more potent mutagens than *N*-methyl-*N*-nitrosurea or formaldehyde (Plummer et al., 1986). The specific mutations caused by faecapentaenes have still to be identified.

1.2.7 Bile acids and cholesterol

Studies into the involvement of fat and cholesterol consumption in colorectal cancer have concentrated on bile acids as they are cholesterol derivatives and they are excreted in response to fat ingestion. High fat diets in both healthy humans and rats result in elevated levels of faecal bile acid excretion (Reddy, 1981) and Hill et al., (1975) showed that patients with colorectal cancer had higher levels of faecal bile acid than healthy matched controls. As long ago as 1964, Fry and Staffeldt demonstrated that in mice, a diet containing sodium deoxycholate resulted in loss of mucosal cells and of goblet cells within 8 days with histological evidence of increased proliferation thereafter. Studies on perfused rat colon have also demonstrated ultrastructural alterations in mucosal cells characteristic of cell injury and exfoliation as well as increased permeability in response to deoxycholate (Goerg et al., 1982). Bile acids and fats instilled into the colons of rats result in a marked increase in [^{3}H] thymidine incorporation into the colon suggestive of active cellular proliferation in response to cell injury and consistent with the action of a tumour promoter (Bull et al., 1983). Short and long term dietary cholic acid also result in an expansion of the proliferating compartment within the rats colonic crypts (Deschner et al., 1981).

Colon tumours in rats can be initiated by a single dose of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). However, subsequent administration of bile acids results in many more animals developing tumours and many more tumours, both adenomas and carcinomas (Narisawa et al., 1974). Suzuki and Bruce (1986) have also been able to show a marked increase in colonic epithelial nuclear aberrations in mice in response to carcinogens along with bile acids when compared to the carcinogen alone. The carcinogens used included substances known to be present in cooked diets including IQ (section 1.2.5).

The action of bile acids on colonic mucosa may be enhanced *in vivo* by metabolism to secondary bile acids by bacterial enzymes in the mammalian gut. Indeed, studies have shown higher levels of certain degradative bacterial enzymes in human subjects taking a 'Western type' diet (Goldin et al., 1980) and in patients suffering from colorectal cancer (Hill et al., 1975). Bile acids, therefore, cause mucosal damage, increased permeability, (perhaps permitting carcinogens to reach crypt stem cells), increased proliferation and tumour promotion after initiation. The evidence of higher bile acid levels in the colons of colorectal cancer patients (Hill et al., 1975) and the increased cellular proliferation seen in colorectal cancer patients (Terpstra et al., 1987) suggest a role for bile acids in human colorectal carcinogenesis.

1.2.8 Calcium

Calcium has been shown to reduce the damage and compensatory proliferation of colonic epithelium induced by bile acid administration either intra-rectally (Wargovich et al., 1983) or orally (Deschner et al., 1981) in rats

and in the perfused colon (Rafter et al.,1986). This protective effect is probably as a result of bile acid precipitation by the calcium, reducing the concentration of free bile acids in solution (Bruce, 1987). However, the promoting effect of fat on carcinogen induced tumour in rats was not altered by oral calcium (Bull et al., 1987). A prospective study of human colorectal cancer risk found a lower calcium consumption in sufferers (Garland et al., 1985) and calcium supplementation in patients from families with hereditary non-polyposis colorectal cancer resulted in reduction of the proliferation of colonic epithelial cells to normal levels (Lipkin & Newmark, 1985).

1.2.9 Summary

The environmental factors involved in human colorectal carcinogenesis are mainly dietary. Evidence from epidemiological studies, case control studies and animal research show that fat (probably via bile acids) and meat consumption along with total energy intake are associated with colorectal cancer and that vegetable fibre and calcium consumption are protective. Cooked meats contain carcinogens capable of activating oncogenes producing initiated cells which may be promoted by the action of bile acids. The bile acids may act along with the initiators by increasing the permeability of the epithelium and permitting access of carcinogens to the crypt stem cells. Calcium may act by modulating free bile acid concentration but the protective influence of certain types of dietary fibre is still unexplained. Demonstration of the specific genetic changes responsible for colorectal cancer might ultimately permit the relevant causative environmental factors to be identified and perhaps removed.

1.3 The Role of Genetic Factors in colorectal carcinogenesis

1.3.1 Introduction

Hereditary factors involved in human colorectal cancer fall into three broad groups: Familial Adenomatous Polyposis (FAP) and its variant Gardner's syndrome (GS); hereditary non-polyposis colorectal cancer (Lynch Syndromes I and II); and the higher incidence of colorectal cancer in first degree relatives of patients. The evidence for the role of each of these factors will be discussed in turn.

1.3.2 Familial Adenomatous Polyposis and Gardner's Syndrome

FAP is a disease characterized by the appearance of multiple adenomas (often thousands) throughout the colon and rectum usually presenting in the 2nd or 3rd decade of life. If left untreated, by prophylactic colectomy, the patient will inevitably develop one or more colorectal carcinomas (Asman & Pierce, 1970). The condition has an estimated frequency of 1 in 8300 births (Reed & Neal, 1955) and is inherited as an autosomal dominant trait (McKusick, 1962). Gardner's Syndrome (Gardner, 1951) is a variant of FAP affecting 1 in 14,000 births (Pierce et al., 1970) also inherited as an autosomal dominant trait (McKusick, 1962) and characterised by multiple osteomas (especially of the jaw, orbit or pelvis), exostoses of the skeleton and soft tissue abnormalities including desmoid tumours, epidermal cysts and lipomas (Garlin & Choudhry, 1960) in addition to the colonic polyposis. More recently the clinical sign of congenital hypertrophic retinal pigment epithelium (CHRPE) has been added to the extracolonic manifestations of GS (Lewis et al., 1984).

The differences between GS and FAP have become less clear as subtle extracolonic changes are often seen in FAP and subsequent genetic linkage analysis demonstrated that the same region (chromosome 5q21-22) was involved in both diseases (Leppert et al., 1987; Bodmer et al., 1987). This finding was consistent with the described cytogenetic abnormalities seen in the region 5q15-22 in patients with GS or FAP and associated mental retardation (Herrera et al., 1986; Hockey et al., 1988).

In 1971, Knudson proposed a theory to explain the incidence of familial and sporadic retinoblastoma. This is a rare childhood tumour which is usually bilateral in familial cases but unilateral in its sporadic form. Knudson suggested that two mutational events were necessary to produce this cancer: in familial cases one was inherited and the other somatic whereas in sporadic cases both were somatic. The gene inactivated by these 2 events has been cloned (*RB1*-Lee et al., 1987) and belongs to a heterogeneous group of genes known as tumour suppressor genes. Both copies of such genes must be inactivated to produce a phenotype and consistent loss of specific alleles in tumours is often taken to indicate the sites of such a genes.

The analogy of Knudson's model for retinoblastoma to FAP and sporadic colorectal cancer is clear and further studies have shown allele loss closely linked to 5q21-22 (Solomon et al., 1987; Vogelstein et al., 1988; Ashton-Rickardt et al., 1989 & 1991) in sporadic colorectal cancer, presumably marking the site of the second mutational event. Allele loss at 5q21.22 can also be detected at a comparable frequency in FAP colorectal cancer but not in FAP adenomas (Solomon et al., 1987; Okamota et al., 1988; Sasaki et al., 1989). Thus, mutation of only one allele (in the germ-line) is sufficient to

produce the phenotype of adenoma formation in the absence of a detectable second event. Adenomatous Polyposis Coli (*APC* - the gene for FAP) differs from *RB1* in this important respect and will be discussed in more detail later in this introduction. Linkage to the locus with colorectal cancer is also seen in families with smaller numbers of colonic polyps, not normally diagnosed as FAP (Leppert et al., 1990).

The FAP phenotype can be modulated, however, by environmental factors. Colonic resection with ileo-rectal anastomosis and regular sigmoidoscopic review of the rectal stump is now the prophylactic treatment of choice in FAP. Regression of the polyps has been described following this procedure (Nicholls et al., 1988) suggesting that a factor normally present in the colonic lumen is necessary to promote the adenoma growth. It is possible that some action of bacterial flora (such as metabolism of bile salts to secondary bile acids - see section 1.2.7) is lost resulting in regression. Therapy with the oral non-steroidal anti-inflammatory drug (NSAID) Sulindac can also result in polyp regression (Waddell et al., 1989).

A number of genes in the region 5q21-22 have now been cloned and two of these, designated *MCC* and *APC*, appear to be involved in colorectal cancer. These will be discussed more fully in section 1.5.4

1.3.3 Hereditary Non-polyposis Colorectal Cancer

This syndrome is divided into 2 sub-groups. Both are characterised by early onset of predominantly proximal colorectal cancer (sporadic colorectal cancer is usually distal), increased frequency of multiple primary cancers, improved 5 year survival when compared to matched controls with sporadic

colorectal cancer and early age of onset. Lynch syndrome II (Cancer family syndrome) is associated with extracolonic malignancies most noticeably endometrial cancer, whereas Lynch syndrome I (hereditary site-specific colon cancer) is not (Lynch et al., 1985a). The incidence of these particular cancers is significantly higher in first degree relatives when compared to non-bloodline relatives which argues strongly for a genetic rather than environmental aetiology.

Biomarker studies were carried out which demonstrated an increase in the development of tetraploidy in dermal fibroblasts cultured *in vitro* from affected individuals and an increase in tritiated thymidine uptake in the distal colonic mucosal crypt compartment (Lynch et al., 1985b) suggesting genetic instability and increased cellular proliferation. Abnormal and elevated patterns of [^{3}H] thymidine uptake are also seen in the normal mucosa of patients with colorectal cancer, FAP and also in unaffected individuals over the age of 60 who are at higher risk of colorectal cancer (Deschner & Lipkin, 1975; Terpstra et al., 1987: Roncucci et al., 1988).

The disease genetics follow a Mendelian dominant inheritance with the penetrance increasing with age (Lynch et al., 1985b). Co-segregation of the disease with the Kidd (J_k) blood group has been demonstrated (Lynch et al., 1985b) although 2 families had to be removed from the analysis to make it significant. The Kidd blood group resides on chromosome 18 as does a gene implicated by other information in colorectal carcinogenesis called *DCC* (see section 1.5.6). No genetic linkage however can be demonstrated between *DCC* markers and HNPCC (MG Dunlop, personal communication). Nor is there any linkage with the *MCC-APC* gene cluster on chromosome 5q21 (Peltomaki et al., 1992)

1.3.4 Inheritance and "Sporadic" colorectal cancer

Studies of the families of patients with apparently non-inherited colorectal cancer has demonstrated an increased risk in first-degree relatives of approximately 3 fold (Macklin, 1960; Lovett, 1976). This risk becomes even higher if the index case has multiple primary tumours of the colon, a past history of previous colon tumours or an early age of onset of colorectal cancer (Lovett, 1976). The pattern of inheritance is difficult to define and can only be described as polygenic.

Later studies investigated a number of kindreds in Utah where the index case had colorectal cancer (Burt et al., 1985; Cannon-Albright et al., 1988). The relatives were screened by flexible procto-sigmoidoscopy for the presence of colorectal adenomas or carcinoma. A statistically significant difference was found in the incidence of adenomas between the first degree relatives and the spouse controls. Analysis of the pedigree suggested that an autosomal dominant pattern of inheritance best fitted the data. These kindreds which do not have polyposis, demonstrate a dominantly inherited susceptibility to develop colorectal adenomas which can be precursors to colorectal carcinoma. Recent data from the CRC group in Edinburgh showed that of 200 index cases of colorectal cancer, approximately 20% have an affected first degree relative (Cancer Research Campaign, Dunlop et al., personal communication) suggesting that many cases of "sporadic" colorectal cancer have an as yet undefined heritable background.

1.3.5 Summary

Inheritance, plays an important part in the genesis of a number of colorectal cancers. Apart from the well defined diseases of FAP and GS, where the susceptibility gene (*APC*) has now been isolated, there are the Lynch syndromes and the adenomatous polyp inheritance which both follow an autosomal dominant pattern along with the complex inheritance pattern in so-called "sporadic" colorectal cancer which in some kindreds at least follows an autosomal dominant mode. Many of these conditions are defined clinically and significant overlap may exist between them which will remain until the responsible genes are identified.

1.4 Histopathology and DNA Ploidy of Colorectal Neoplasia

1.4.1 Histopathology of colorectal carcinomas

Histopathology involves the macro- and microscopic examination of tissue to identify morphological features which will permit a diagnosis to be made giving prognostic information and thus allowing a rational approach to treatment. In the case of colorectal cancer this centres around the differentiation of benign and malignant tumours, the subclassification of adenomas to identify those likely to proceed to malignancy and the subclassification of carcinomas to permit a more accurate estimate of prognosis and thus the most appropriate treatment. Essentially it is an estimate of a tumour's future behaviour based on morphological features sampled (usually) at a single point in time. Considering the limitations inherent in such an exercise, this can be done to a reasonable degree of accuracy and is still the standard method of assessment.

Colorectal tumours are usually assessed by staging (the degree of spread) and grading (histology). In 1932, Dukes proposed a simple, objective method to stage rectal cancer. Stage A were cases with spread limited to the rectal wall, B had spread beyond the rectal wall but did not involve any lymph nodes and Stage C had lymph node metastasis. Stage A had the best prognosis and C the worst. His classification has subsequently been shown to be equally applicable to colon cancer (Grinnell, 1939) and has been modified to include a D stage with distant metastasis. Dukes himself later modified the C stage into C1 (regional lymph nodes only involved) and C2 (nodes at the point of ligation involved) to give better prognostic discrimination (Dukes & Bussey, 1958). The reproducibility and objectivity of

this system means that it is the standard staging system used today.

Grinnell (1938) also attempted to grade colorectal cancer on the basis of the histology. He was able to demonstrate that an assessment of glandular arrangement, invasiveness, loss of nuclear polarity and nature of invasive margin gave information which correlated with prognosis although the relative importance and interdependency of these variables was not assessed. It was not until 1986 (Jass et al.) that multivariate analysis was applied to this problem to identify independent prognostic variables concluding that, for rectal cancer, lymphocytic infiltration, lymph node status and extent of spread (the last two being included in Dukes' staging) gave independent prognostic information. It was also possible to show a correlation of prognosis with tubule configuration (differentiation) and growth pattern (nature of invasive margin). This prompted the proposal of a scoring system (Jass & Morson, 1987) based on extent of invasion, lymph node metastasis, growth pattern and lymphocytic infiltrate. However, inter- and intra- observer agreement for scoring lymphocytic infiltration is poor which limits its use greatly (Dundas et al., 1988).

An assessment based upon Dukes stage and graded (by a single experienced pathologist) for differentiation and growth pattern probably represents the best classification (Dundas et al., 1988) in terms of reproducibility and objectivity. This is the system recommended by the United Kingdom Co-ordinating Commitee on Cancer Research (UKCCCR, 1988) and used in this thesis.

1.4.2 Histopathology of adenomas

There is good, but largely circumstantial, evidence that colorectal cancers develop from pre-existing adenomas. Adenomas have the same distribution within the colon as carcinomas (distal predominance), they occur in the same population as those at risk of colorectal cancer, carcinomas can be found containing morphologically adenomatous tissue for which they probably arose and adenomas with small foci invasion can also be identified. Thus carcinomas can arise from adenomas but the majority of adenomas will not become malignant and it is highly likely that many carcinomas do not have any clearly defined adenomatous intermediate.

The histopathological grading of adenomas depends on size, architecture and cellular morphology. Adenomas greater than 10 mm in diameter, with villous architecture and severe dysplasia tend to be found in the distal colon and rectum and are thus likely to be at higher risk of malignancy (Konishi & Morson, 1982).

1.4.3 DNA ploidy of colorectal tumours

Karyotype analysis of solid tumours is not easy but a number of groups have produced data on chromosome analysis (Sonta & Sandberg, 1978; Reichmann et al., 1981, 1982; Remvikos et al., 1988). In addition to specific chromosome alterations (which will be discussed later in the introduction), aneuploidy was a noticeable finding. DNA aneuploidy simply refers to the fact that cells have a non-diploid DNA content. The processes which bring this about are still not clear but it has been proposed that it results from chromosomal non-disjunction and reduplication. The flow cytometric

estimation of aneuploidy is much simpler, faster, can be applied to archive material (Hedley, 1988) and gives a result very close to that for karyotyping (Petersen & Friedrich, 1986; Remvikos et al., 1988). DNA aneuploidy has been shown to correlate with poor prognosis for colorectal cancer, independent of Dukes' stage (Wolley et al., 1982; Armitage et al., 1985; Quirke et al., 1987; Giaretti et al., 1991) although the reason for this consistent finding is unclear. It is important to sample at multiple sites as heterogeneity of DNA ploidy is seen in approximately 50% of cases (Quirke et al., 1985). DNA aneuploidy is also seen in adenomas at a frequency between 6 and 27% (van den Ingh et al., 1985; Quirke et al., 1986) and tends to be in the larger (> 10 mm) adenomas of tubulovillous or villous architecture which are the subgroups at greater risk of malignant change.

1.5 Molecular Genetic Changes in Colorectal Cancer

1.5.1 Introduction

In 1954, Armitage and Doll carried out an epidemiological study exploring the relationship between mortality and age for a number of common cancers. Analysis of *log* age plotted against *log* death rate for colon cancer gave a straight line with a gradient of approximately 5. This was interpreted as suggesting that 5 discrete events are required for colon carcinogenesis. Interestingly, at younger ages, the death rate is relatively higher with a gradient closer to 4 suggesting that fewer (genetic) events are necessary. This observation could be explained as due to the earlier presentation of familial cancers seen in FAP patients: such patients already possess one germ-line mutation. At the time of writing, Armitage and Doll could have had little idea as to the nature of the 5 "events" they were suggesting but a number of genetic changes have now been described in colorectal cancer and these will be discussed.

1.5.2 Clonal Evolution of Colorectal Tumours

Current theories of carcinogenesis suggest that neoplasms develop from a single cell (Nowell, 1976, 1986). The single cell is altered in some way to permit at least semi-autonomous growth allowing it to proliferate to a greater extent than its normal neighbours. Subsequent (genetic) changes to daughter cells in this clone may be lethal or may confer a growth advantage permitting overgrowth by this new sub-clone. This selection process can continue until an invasive or metastatic clone is produced. The data assessed by Armitage and Doll (1954) suggested that 5 such critical "sub-

cloning" selection events might be required to produce a colorectal cancer. However, it is possible that greater or smaller numbers of genetic events are necessary for colorectal carcinogenesis as an unknown factor affecting the interpretation of the data is the degree of independence of the critical carcinogenic events from each other. If certain of the critical events result in increased genomic instability this would accelerate the rate of acquisition of the other selectable genetic alterations. Later in this thesis evidence will be discussed showing that some critical events (notably p53 inactivation) do indeed influence genomic stability. Long term cultures and transformed cells in culture do have an increased susceptibility to chromosome damage (Parshad et al., 1979) and fibroblasts cultured from patients with a germline p53 mutation immortalise frequently and develop DNA aneuploidy (Bischoff et al., 1990).

The clonal origin of a number of benign and malignant neoplasms has been studied. Using glucose-6-phosphate dehydrogenase mosaicism in black females, Linder and Gartler (1965) were able to demonstrate that leiomyomas of the myometrium were exclusively monoclonal in origin. Using a similar technique, Fialkow et al., (1967) obtained the same result in chronic myelocytic leukaemia and the monoclonality of a number of different lymphoid neoplasms was demonstrated by Arnold et al., (1983) using immunoglobulin gene rearrangements as a marker.

In the case of colorectal neoplasms, monoclonal origin has been demonstrated in both adenomas and carcinomas by the pattern of methylation induced X chromosome inactivation detected using RFLP probes with methylation sensitive restriction enzymes (Fearon et al., 1987). Colonic epithelial dysplasia induced by azoxymethane in chimæric mice has

also been shown to be monoclonal using H2 antigen as a marker (Ponder et al., 1986).

Although the monoclonal origin of neoplasms may hold true for these common sporadic tumours, the rarer, inherited tumours appear different. Benign tumours from GS (Hsu et al., 1983) and neurofibromatosis (Fialkow et al., 1971) both appear polyclonal origin by G-6-PD mosaicism. However, these tumours are in fact all "clonal" for the inherited gene defect in *APC* or *NF1* which is present in all cells of an affected individual including those giving rise to the benign proliferation. The reason why some cells form a tumour while their neighbours do not may involve interaction with environmental factors, the concentration of which may vary locally.

Thus, in general, sporadic colorectal neoplasms arise from a single cell which has undergone alterations to permit its dysregulated growth. Nowell (1976, 1986) proposed that as the neoplastic proliferation proceeds, additional altered cells are produced. Most of these will be eliminated unless they have a growth advantage compared to the parent cell. In this case, it will overgrow the neoplasm and become the predominant cell type, until another similar event occurs to take the neoplasm another stage further along its evolution. Colorectal neoplasia provides a suitable system in which to study this evolution in a human malignancy. The various stages of: normal mucosa; benign adenoma; carcinoma and metastasis can all be identified and separated histopathologically and studied individually to define the events at each stage in the process.

1.5.3 Methylation

One of the earliest detectable alterations found in colorectal neoplasia is that of hypomethylation. Studies using methylation sensitive restriction enzymes to investigate the Southern blot pattern of DNA from adenomas and carcinomas compared to normal colonic mucosa when probed with DNA from diverse genes (mainly these not associated with neoplasia) demonstrated hypomethylation in almost all the neoplasms studied including benign adenomas (Goelz et al., 1985). Measurement of 5-methylcytosine content in paired normal and tumour DNA samples by High Performance Liquid Chromatography (HPLC) confirmed the hypomethylation, which was even more marked in carcinomas compared to adenomas (Feinberg et al., 1988).

DNA methylation is a covalent modification of cytosine occurring at CpG doublets in vertebrate DNA (Bird, 1978, 1986) which is maintained through DNA replication (Wigler et al., 1981). Islands of sequence rich in such CpG motifs (CpG islands) are found in the vicinity of the 5' regions of mammalian ("house-keeping") genes and a reduction in methylation at these sites correlates with active transcription of these genes (Bird et al., 1985; Bird, 1986). Exposure of cells to 5-azacytidine (a cytosine analogue which cannot be methylated at the 5' position) results in hypomethylation, opening of chromatin and transcriptional activation (Groudine et al., 1981). It is tempting, therefore, to assume that hypomethylation of a normal colonic mucosal cell (by an undefined stimulus) results in the activation of genes, or perhaps their exposure to modification by mutagens, leading to proliferation and adenoma formation. However, the evidence is still lacking to show that hypomethylation is a primary event in this process and not simply a

secondary epiphenomenon of cells which are more actively proliferating for other reasons.

1.5.4 Chromosome 5q21

The *APC* gene (implicated in FAP) was mapped to the q21-22 region of chromosome 5 by linkage analysis with restriction fragment length polymorphic (RFLP) probes (Bodmer et al., 1987; Leppert et al., 1987) after the identification of a patient with FAP and mental retardation who had a constitutional deletion of chromosome 5q (Herrera et al., 1986).

The finding that RFLP probes frequently show allele loss in sporadic and FAP colorectal cancer in the region 5q21.22 strongly suggested that *APC* was a tumour suppressor gene in the *RB1* mould (Solomon et al., 1987; Law et al., 1988; Vogelstein et al., 1988; Okamoto et al., 1988; Sasaki et al., 1989; Ashton-Rickardt et al., 1989 & 1991). The further demonstration that returning a normal chromosome 5 to colon cancer cell lines rendered them non-tumourigenic, although not necessarily demonstrating an effect specific for *APC*, added evidence of its probable tumour suppressor function (Tanaka et al., 1993).

However, 5q allele loss is very rare in early FAP adenomas (Solomon et al., 1987; Vogelstein et al., 1988; Sasaki et al., 1989; Miyaki et al., 1990) suggesting that the adenomatous phenotype is expressed when only one allele is mutated (in the germline). Whether this phenomenon is a result of a reduced gene dosage (Bodmer et al., 1987), an interaction of the mutated protein to inactivate the wild type (as is seen with p53, Stürzbecher et al., 1987) or some other mechanisms (Bourne, 1991) remains to be seen.

Expression of the phenotype also seems to depend upon colonic lumenal factors: colectomy and ileo-rectal anastomosis results in regression of adenomas in the rectal stump (Nicholls et al., 1988) as does therapy with the non-steroidal anti-inflammatory drug Sulindac (Waddell et al., 1989).

Recent studies by two independent groups have isolated *APC* (Kinzler et al., 1991a; Joslyn et al., 1991) in a cluster of 6 genes at 5q21. Another gene in the cluster, designated *MCC*, (Kinzler et al., 1991b) also appears to undergo mutation in sporadic colorectal cancer but not FAP (Kinzler et al., 1991a). *APC* was identified from yeast artificial chromosome (YAC) libraries by hybridisation to polymorphic probes shown to flank *APC* by linkage analysis. The gene itself spans approximately 100 kb and has a coding region of 8.5 kb encoding a protein of over 300 kDa. The demonstration that it is mutated in the germline of FAP patients but not in any normal controls and that the mutated allele co-segregates with the disease in FAP and GS kindreds proved that it is the *APC* gene (Joslyn et al., 1991; Nishisho et al., 1991). Germ-line mutation of the murine homologue of *APC* also results in multiple intestinal neoplasia (Su et al., 1992).

The *APC* mutations so far described are either mis-sense mutations (producing stop codons), small (one or two bp) deletions (causing frameshift and termination) or larger deletions removing much of the coding region (Joslyn et al., 1991; Nishisho et al., 1991; Miyoshi et al., 1992a). All tend to cause truncation of the message and therefore a protein with altered or non-existent function. Further analysis suggests that mutations between codons 1250 and 1464 result in a clinical sub-type of FAP characterized by profuse colonic adenomatosis whereas mutations at other sites result in the sparse sub-type (Nagase et al., 1992). It is tempting to assume that it is the

spectrum of different mutations that gives rise to the spectrum of phenotypes seen in FAP, GS and familial colorectal cancer with less marked polyposis. Other types of interaction (environmental or genetic), are suggested, however, by the finding of the same mutation in two unrelated patients, one with extracolonic manifestations (a desmoid tumour) and one without (Nishisha et al., 1991).

The protein has no significant homology with any other protein so far isolated but does have heptad repeat regions known to facilitate protein-protein interaction via extended alpha helices presenting hydrophobic residues along one side (Bourne, 1991). These hydrophobic regions can stabilise by interaction with similar alpha helices to form hetero- or homodimers and a coiled-coil structure. Similar motifs are detected in *MCC* (Bourne, 1991) and possible interaction between these two gene products cannot be ruled out.

APC appears to exist in two forms with an alternately spliced exon 9. The full significance of this is not clear but both forms are expressed together and all tissues so far examined express *APC* mRNA (Groden et al., 1991).

Analysis of the *APC* mutations in sporadic colorectal cancer (Nishisho et al., 1991; Miyoshi et al., 1992b) demonstrates mis-sense mutations causing amino acid substitutions, splice donor and acceptor disruption and stop codons as well as small sequence insertions. However, out of 10 colorectal cancers with a defined *APC* mutation only 2 had a 5q allele loss (Nishisho et al., 1991), suggesting perhaps another departure from the classic tumour suppressor paradigm, exemplified by retinoblastoma. However, subsequent work has suggested inactivation of both copies of *APC* is rather commoner (Miyoshi et al., 1992b). Fine mapping of the deletions in sporadic colorectal

cancers has shown that they are centred around the polymorphic probe L5.71 (Ashton-Rickardt et al., 1991) which is located within *MCC* and not *APC*. Thus, mutation of one copy of *APC* has the phenotype of colonic adenomatosis in FAP. Mutation of *APC* however, can be present without deletion of the wild-type gene in FAP related colorectal adenomas (Miyaki et al., 1990) and in sporadic colorectal cancer the 5q deletions are centred over *MCC* (Ashton-Rickardt et al., 1991). These data are therefore consistent with *APC* mutations acting in a dominant fashion and *MCC* as a tumour suppressor and the target for 5q deletions.

MCC is centromeric to *APC* spanning about 100 kb. Mutations in *MCC* are not found constitutively in FAP or GS patients but can be demonstrated in (a small proportion of) sporadic colorectal cancers although full analysis is still awaited (Kinzler et al., 1991b). It also contains coiled-coil motifs suggesting dimerisations and a possible structural function in the cell (Bourne, 1991). MCC also has an intriguing but short homology to the m3 muscarinic acetylcholine receptor hinting at a signal transduction function, but the significance of this is still unclear.

In summary, two genes, closely linked physically, have been isolated from a region frequently deleted in colorectal cancer and therefore suggestive of a tumour suppressor locus. One, *MCC*, is mutated only in sporadic colorectal cancer (Kinzler et al., 1991a and b) and is at the centre of mapped deletions (Ashton-Rickardt et al., 1991), whereas the other, *APC*, is the heritable genetic lesion in FAP and GS. *APC* is not a "simple" tumour suppressor gene, however, in that a single mutated allele has a phenotype, the phenotype is modulated by unidentified factors; and although deletion of the wild-type gene copy is commonly

seen it may not be the target for such a deletion (Ashton-Rickardt et al., 1991). It is conceivable that *APC* and *MCC* interact at a protein level and that inactivation of either is responsible for the loss of tumour suppressor function (Kinzler et al., 1991). Much more work on these genes will be necessary to define their roles and possible interactions in colorectal cancer.

1.5.5 K-ras Oncogene

Kirsten ras (*K ras*) is a cellular proto-oncogene thought to play a central role in growth control. *K ras* belongs to a family of *ras* genes (along with *H-* and *N ras*) which in turn belong to the GTPase superfamily of conserved proteins involved in "molecular switching" (Bourne et al., 1990). The *c-ras* genes encode 21 kd proteins, $p21^{ras}$, which are located on the inner plasma membrane and bind guanine nucleotides. $p21^{ras}$ is in the active state when GTP bound but inactive when GDP bound and it is the change between these two states which seems to be important for cell proliferation control. Movement into the active (GTP bound) state is enhanced by guanine nucleotide release proteins, GNRP (Wolfman & Macara, 1990) and GTP hydrolysis by $p21^{ras}$ is enhanced by GTPase activating protein, GAP (Trahey & McCormick, 1987). Transformation by $p21^{ras}$ can be achieved either by overexpression or by mutations both acting by increasing the amount of active (GTP bound) protein in the cell (Marshall, 1991). Mutation at codons 12, 59 and 61 greatly reduce the GTPase activity resulting in the predominance of active $p21^{ras}$ (Vogel et al., 1988).

Analysis of *c-ras* gene sequence in human colorectal tumours demonstrated a high frequency (approximately 40% of carcinomas) of *K-ras* mutations (and

fewer *N-ras*) at codons 12, 13 and 61 (Forrester et al., 1987; Bos et al., 1987; Vogelstein et al., 1988). The same mutations are also found at a similar frequency in colorectal adenomas (Vogelstein et al., 1988) and when carcinoma and adenoma can be separated in the same lesion, the same mutation is found in both (Bos et al., 1987; Vogelstein et al., 1988). Mutations of *ras* genes are also more frequent in the larger adenomas which are those more likely to progress to carcinoma (Vogelstein et al., 1988). This suggests that *c-ras* mutation is an event occurring early in colorectal tumourigenesis perhaps during adenoma development.

Thus, activation of *c-ras* by mutation at specific sites which prevent the action of GAP to inactivate $p21^{ras}$ by GTP hydrolysis appears to be responsible for the increased proliferation of the adenomas but not for the transition to malignancy. Experiments in many *in vitro* experimental systems have shown that *ras* cannot fully transform cells when acting alone but requires cooperation with another genetic event (Land et al., 1983). This is likely to be the case in colorectal carcinogenesis although the cooperation may come from tumour suppressor gene inactivation rather than activation of another oncogene.

1.5.6 Chromosome 18q and DCC

Chromosome 18 abnormalities in colorectal cancer were first detected cytogenetically (Muleris et al., 1985) and subsequent studies demonstrated allele loss on 18q (by RFLP analysis) in 52-71% of cases (Law et al., 1988; Vogelstein et al., 1988; Vogelstein et al., 1989; Delattre et al., 1989). These deletions are relatively rare in adenomas (11%) but common in "adenomas" which have foci of invasion (47%) and even more frequent in frank

carcinoma (73%) (Vogelstein et al., 1988). This suggests that 18q allele loss is an event closely concurrent with the evolution of adenoma to carcinoma. Consistent loss of markers at specific loci in tumours is thought to indicate the presence of critical tumour suppressor genes and so fine mapping of these deletions in many tumours was undertaken delimiting the minimum region of overlap to 18q21.qter (Vogelstein et al., 1988). The identification of a marker homozygously deleted in a particular tumour permitted cloning of the gene designated *DCC* (Fearon et al., 1990).

DCC encodes a 750 amino acid protein which is well conserved through evolution and which has significant homology to neural cell adhesion molecules (N-CAM) in that DCC contains 4 immunoglobulin (Ig) like domains and a fibronectin like domain similar to those in N-CAM and other members of the cell adhesion molecule family (Fearon et al., 1990). The introduction of a normal human chromosome 18 into colon carcinoma cell lines renders them non-tumourigenic and reduces their cloning efficiency in soft agar (Tanaka et al., 1991). This further suggests a tumour suppressor gene on chromosome 18. However, the authors did not demonstrate *DCC* inactivation in the parental cell line, nor does this result exclude the effect of any other gene on chromosome 18. However, subsequent studies showed that expression of *DCC* antisense RNA *in vitro* increased growth rate, tumourigenicity in nude mice and decreased cell adhesion (Narayanan et al., 1992) suggesting that *DCC* is indeed a tumour suppressor gene.

The finding that *DCC*, a gene which is inactivated at or about the time of adenoma-carcinoma evolution, should be related to molecules involved in cell-cell interactions is suggestive. One of the hallmarks of carcinomas when compared to adenomas histologically is the reduction or loss of cellular

organisation and architecture and it could be speculated that this is due to the loss (by inactivation of both copies) of a gene, such as *DCC*, which is involved in cell to cell adhesion and interaction.

1.5.7 Chromosome 17p and p53

Cytogenetic studies of the karyotypes of human colorectal cancer demonstrated loss of the short arm of chromosome 17 as a frequent finding (Muleris et al., 1985). Subsequent RFLP analysis confirmed this result with 17p alleles lost in 40-75% of carcinomas (Lothe et al., 1988; Vogelstein et al., 1988; Monpezat et al., 1988; Law et al., 1988; Vogelstein et al., 1989; Delattre et al., 1989). This change is seen rarely in adenomas with foci of invasive carcinoma (24%) and in only 6% of true benign adenomas (Vogelstein et al., 1988) suggesting that this is indeed a late change in the adenoma-carcinoma evolution.

Subsequent mapping of the minimum region of overlap of deletions in a series of colorectal cancers suggested p53 as a possible genetic target for such deletions and the finding that the remaining p53 allele was frequentky mutated seemed to confirm this (Baker et al., 1989). The role of p53 and its involvement in colorectal carcinogenesis will be discussed in more depth in chapter 2.

Deletions of chromosome 17p are frequent findings, especially in the common human malignancies of colorectum (Vogelstein et al., 1988), breast (Mackay et al., 1988) and non small lung cancer (Chiba et al., 1990). No similar correlation between allele loss and p53 mutation has been demonstrated for breast (Varley et al., 1991) or non small cell lung cancer

(Chiba et al., 1990) and for breast cancer, careful deletion mapping has shown a locus distinct from p53 which is also a target for 17p allele loss (Coles et al., 1991). This putative tumour suppressor locus is mapped, provisionally, to a marker MCT 35.1 which is located within the minimum region of overlap demonstrated in colorectal cancer (Baker et al., 1989; Coles et al.,1991). In fact the minimum region of overlap for 17p allele loss in colorectal cancer is so large (approximately 30mb) that it must contain many genes each one of which could have some tumour suppressor function.

1.5.8 Other genetic events in colorectal cancer

C-myc is a proto-oncogene whose gene product (p62-*c-myc*) is normally located in the cell nucleus (Evan et al., 1985). The normal function of *c-myc* is not yet clear but it has been implicated in early development (Müller et al., 1982), control of cell growth (Kelly et al., 1983), patterns of differentiation (Lachman & Skoultchi, 1984) and tissue regeneration (Makino et al., 1984). Elevated levels of *c-myc* protein are associated with malignancy and transformation either as a result of transcriptional activation by adjacent promoter insertion (Hayward et al., 1981), balanced translocation juxtaposing immunoglobulin sequences with *c-myc* as seen in Burkitts' lymphoma (Taub et al., 1982) or gene amplification (Collins & Groudine, 1982).

Overexpression of *c-myc* at the protein and mRNA level is relatively common in colorectal cancer detectable in approximately 70% of cases (Erisman et al., 1985; Rothberg et al., 1985; Sikora et al., 1987). This does not appear to be as a result of gross rearrangement or amplification (Erisman et al., 1985; Sikora et al., 1988) suggesting that it is probably due to transcriptional

activation. Amplification of *c-myc* has been described in certain aggressive subtypes of colorectal cancer (Heerdt et al., 1991) but in view of the fact that the level of amplification is so low (1-3 fold normal) and given the subjectivity of grading tumours, the significance of this result is unclear. Expression of *c-myc* does not correlate with disease recurrence or patient survival (Erisman et al., 1985).

The precise timing of the *c-myc* event resulting in overexpression is not yet clear but a change in the immunohistochemical pattern of staining is observed with tumour evolution. In normal mucosa, the antibody binding is predominantly nuclear becoming more cytoplasmic in adenomas (especially large, dysplastic adenomas) and is predominantly cytoplasmic in all carcinomas (Williams et al., 1990). This suggests that the up-regulation of the protein occurs near the point of adenoma-carcinoma evolution. Cooperation between *myc* and *ras* to fully transform cells *in vitro* is well described (Land et al., 1983) therefore an *in vivo* cooperation in colorectal carcinogenesis is quite possible although, evidence linking the two oncogenes in colorectal cancer is still lacking.

Loss of alleles can be detected for all the arms of all non-acrocentric chromosomes in human colorectal cancer in at least 7% and usually 10+% except in those already described where it is higher (Vogelstein et al., 1989). It is difficult to imagine that these all indicate the sites of tumour suppressor loci. It seems more likely that they either represent telomeric shortening (Hastie et al., 1990) or, more probably, the genomic instability which is seen in transformed cells resulting in the many chromosomal aberrations detected cytogenetically (Muleris et al., 1988). Whatever its role or cause, however, loss of many alleles does correlate with a poorer prognosis (Vogelstein et al.,

1989; Kern et al., 1989).

Colorectal cancer is, therefore, a multistep process involving a number of different molecular genetic steps, some of which have been defined (Fearon & Vogelstein, 1990). Some of these events tend to occur earlier than others, but this proposed order is not exclusive as development of the critical events, in whatever order, appears to lead to carcinogenesis. The most critical event in colorectal tumourigenesis is clearly the development of malignant potential. Whether this is after adenoma growth or in its absence, p53 appears to act at this point and may be central to malignant transformation. The data suggesting this role is discussed below.

Chapter 2

p53

2.1 Summary

The 53 kDalton protein p53 was initially isolated by virtue of its binding to SV40 large T antigen in infected cell lines (Lane & Crawford, 1979; Linzer & Levine, 1979; Rotter, 1983). The detection of p53 in transformed cell lines *in vitro* (De Leo et al., 1979) and *in vivo* (Rotter et al., 1983) resulted in its classification as a tumour antigen. Following the isolation of complementary and genomic clones for p53, it was demonstrated to immortalise both rat embryo fibroblasts (Rovinski & Benchimol, 1988) and adult rat chondrocytes (Jenkins et al., 1984) and to co-operate with *ras* to fully transform rodent embryo cells in culture (Parada et al., 1984; Elijahu et al., 1984). These observations led to the classification of p53 as an oncogene.

However, subsequent studies demonstrated that the p53 gene clones used in these experiments all carried mutations and that true wild type (WT) p53 would suppress transformation in co-transfection assays (Hinds et al., 1989; Finlay et al., 1989). Further work using cell lines derived from tumours has confirmed the negative effect on cellular proliferation of transfected WT p53 (Baker et al., 1990a; Mercer et al., 1990). Thus p53 is currently classified as a tumour suppressor gene product.

In this section, the biochemical and biological properties of p53 and the alterations detected in human neoplasia will be discussed.

2.2 The gene for p53

The gene for p53 has been isolated from a wide variety of vertebrate and invertebrate species covering an evolutionary spectrum including *xenopus laevis* (Soussi et al., 1987), rainbow trout (Caron de Fromentel et al., 1992) chicken (Louis et al., 1988; Soussi et al., 1988), mouse (Oren & Levine 1983; Bienz et al., 1984), rat (Coulier et al., 1985), monkey (Rigaudy & Eckhart 1989) and human (Matlashewski et al 1984) suggesting an important role for p53. However, no p53 homologue has thus been detected in yeast despite extensive screening (JR Jenkins, personal communication).

The human p53 gene is located on the short arm of chromosome 17 in the region p13 (Isobe et al., 1986) and has been further mapped to the D1 sub-region (Baker et al., 1989). The murine p53 gene is on chromosome 11 (Czosneck et al., 1984; Rotter et al., 1984) and the murine genome also contains a processed pseudogene lacking introns on chromosome 14 (Zakut-Houri et al., 1983). Both human and mouse genes consist of 11 exons with a large (6.1-10 kbp) first intron separating the untranslated first exon from exon 2 (Bienz et al., 1984; Lamb & Crawford 1986). The murine protein has a predicted length of 390 amino acids (Zakut-Houri et al., 1983; Pennica et al., 1984) or 387 depending upon which methionine is considered to be the start codon (Soussi et al., 1987). The human protein consists of 393 amino acids and shows 79% amino acid homology with mouse p53 (Harlow et al., 1985). Many of the differences are conservative suggesting a high degree of sequence retention through evolution (Harlow et al., 1985). This hypothesis is strengthened by the finding that p53 from *xenopus laevis* shares 57% primary structure with mouse p53. Further analysis reveals five regions (designated I-V, figure 2.1) in which the homology rises to 91-100%

(Soussi et al., 1987). Such conservation through evolution argues strongly for these regions having an important role in the function of the protein.

Human p53 domain I spans residues 13-19; II, 117-142; III, 171-181; IV, 236-258; and V, 270-288 (Soussi et al., 1987; Levine & Momand, 1990). Interestingly, the binding sites on murine p53 for SV40 large T antigen have been mapped to residues 165-199(A) and 232-285(B) (Jenkins et al., 1988) corresponding to 171-205(A) and 238-291(B) in human p53. These regions span conserved domains III, IV and V suggesting that T antigen may be occupying a site important in the normal (tumour suppressor) function of p53 and could be displacing a normal p53 ligand if such exists.

Analysis of the primary structure of p53 from several species for charge distribution and hydropathic profile provides further evidence of evolutionary conservation (Soussi et al., 1990). The amino terminus region (100 residues) is highly acidic, proline rich and hydrophilic and is predicted to form an α helix; the carboxy terminus is a basic, hydrophilic α helix; and the internal amino acids (containing domains II-IV) consist of few charged residues and are predicted to form a hydrophobic ß pleated sheet. This arrangement would suggest a secondary structure with the amino and carboxy terminals on the external surface and the central residues internal to the folded protein. This may explain why single mis-sense mutations in domains II-IV, which reside in the hydrophobic centre of p53 cause important changes in protein conformation which have signficiant functional sequelae (see Section 2.3).

Northern blot analysis of p53 mRNA detects a transcript of 2.2 kbp in the mouse (Oren et al., 1983) and 2.5-2.8 kbp in the human (Matlashewski et al.,

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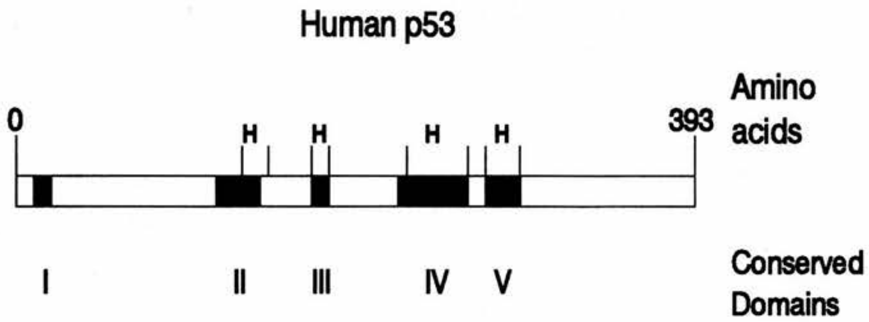
1984; Harlow et al., 1985). The difference in size is largely due to the greater 3' untranslated region in the human gene (Matlashewski et al., 1984) which contains Alu sequences. The gene for p53 does not contain any of the consensus sequences found in most eukaryote promoters such as a CAAT box, a TATA box or G/C rich sequences but promoter activity has been detected in the region 5' to the first exon (Bienz-Tadmor et al., 1985) termed P1. This activity, however, is down-regulated by inclusion of sequences between this region and exon 1. A second promoter site (P2) has been identified downstream of the first exon (Reismann et al., 1988; Reismann & Rotter 1989) but transcript representing *in vivo* activity at this site has not been detected. The relevance of P1 and P2 *in vivo* remains in doubt.

Figure 2.1 Diagram showing the conserved domains of human p53 (I-V, Soussi et al., 1987) and mutation hotspots (H, Hollstein et al., 1991).



2.3 p53 Protein Conformation

The p53 protein exists in different conformations which can be detected by its affinity to a range of monoclonal antibodies. In its wild type or growth suppression form p53 will bind PAb 246 (mouse) (Yewdell et al., 1986), PAb 607 (human and mouse) and PAb 1620 (human and mouse) (Milner et al., 1987). Whereas in its mutant form, p53 will bind PAb 240 (Gannon et al., 1990). The epitopes and binding characteristics are summarised in table 2.1 and figure 2.2. The binding of the PAb 246, PAb 607 and PAb 1620 are denaturation sensitive indicating their dependence on the p53 protein conformation. PAb 240 is denaturation insensitive and binds to an epitope centred on amino acids 213-217 (Stephen & Lane, 1992) which is within the hydrophobic region of the p53 protein predicted to be internal in the wild-type (WT) conformation. Thus, mutation exposes a previously hidden epitope for antibody binding. Mutations at many different sites within p53, especially those within conserved domains II-IV, bring about similar conformational and antibody binding alterations (Gannon et al., 1990). This suggests that mutation within the conserved domains alters the WT conformation and diminishes the tumour suppressor activity of the protein. It cannot be assumed however that all mutants, even if PAb 240 reactive, have the same conformation and biological properties.

In the case of mouse p53, a temperature sensitive mutant exists, which has a valine substituted for alanine at codon 135 (p53 val 135) and behaves like the wild type protein at 32°C but in a mutant fashion at 37°C (Milner & Medcalf, 1991). With this alteration in behaviour there is a conformational change from PAb 246+ at 32°C to PAb 246- at 37°C. These data strongly suggest that it is the conformational change in p53 brought about by

mutation which alters its behaviour from tumour suppressor to non-tumour suppressor and possibly oncogene.

However, different conformations of murine and human p53 translated from WT p53 mRNA in a cell lysate translation system have been detected (Cook & Milner 1990). The mouse protein is either in a PAb 246+ or PAb 246- conformation and both mouse and human protein either PAb 1620+ or PAb 240+ depending upon the batch of reticulocyte lysate used for each experiment. Thus WT p53 can exist in a PAb 246+, PAb 1620+ tumour suppressor conformation or a PAb 246-, PAb 240+ non tumour suppressor conformation. It has also been demonstrated that a change in conformation from PAb 246+ to PAb 246- occurs 1 hour after serum stimulation of cells *in vitro* (Milner & Watson 1990). Thus, it would appear that physiological alterations in WT p53 tertiary structure from PAb 246+, PAb 1620+ to PAb 246-, PAb 240+ (mimicking the alterations seen with p53 mutation) occur in cells stimulated to replicate and this conformational change may be a signal to permit proliferation.

Wild-type p53 can also be induced to change to a "mutant" conformation when co-translated with certain mutant p53 mRNA's. This property appears to depend upon oligomerization between mutant and WT protein and is lost when a truncated mutant lacking the carboxy terminal residues responsible for oligomerization is employed (Milner et al., 1991; Milner & Medcalf 1991). However, a mutant p53 containing a mutation described in the Li-Fraumeni syndrome (Malkin et al., 1990; Srivastava et al., 1990) fails to have any influence upon the co-translated WT protein (Milner & Medcalf 1991). This demonstrates that different mutants of p53 can have different properties.

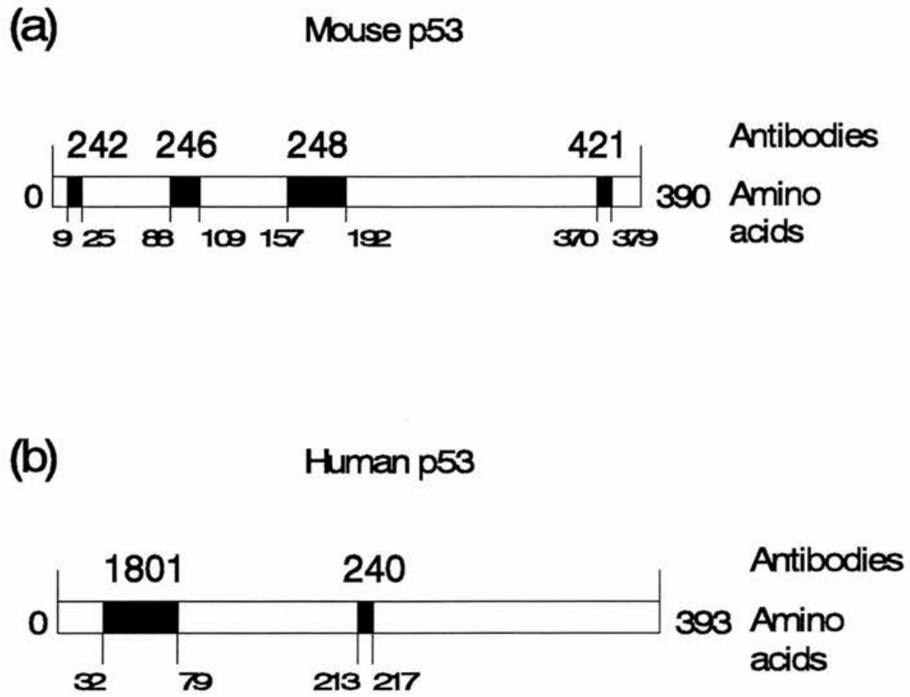
Table 2.1 Summary of p53 antibody binding.

Antibody	Mouse p53		Human p53		Reference
	Wild-type	Mutant	Wild-type	Mutant	
240	-	+	-	+	Gannon et al., 1990
242	+	+	-	-	Yewdell et al., 1986
246	+	-	-	-	Yewdell et al., 1986
248	+	+	-	-	Yewdell et al., 1986
421	+	+	+	+	Harlow et al., 1981
1620	+	-	+	-	Milner et al., 1987
1801	-	-	+	+	Banks et al., 1986

\+ binds antibody

\- does not bind antibody

Figure 2.2 Diagram of mouse (a) and human (b) p53 showing antibody epitopes.



2.4 Subcellular localisation of p53

Analysis of nuclear sub-fractions of normal and transformed cells demonstrated that p53 is found in the chromatin and in the nucleoplasmic and nuclear matrix factions (Deppert & Haug 1986). In *herpes simplex* virus infected cells, p53 co-localises to areas of viral DNA replication within the nucleus (Wilcock & Lane, 1991). This suggests a role for p53 within the nucleus and indeed a nuclear location sequence has been detected which has homology to that described in SV40 large T antigen (Addison et al., 1990). This sequence is highly conserved for human, mouse and rat p53 (Soussi et al., 1990) and when bound to non-nuclear proteins results in their relocation in the nucleus (Dang & Lee 1989; Addison et al., 1990). Mutation of residues within the sequence also result in loss of nuclear localisation (Addison et al., 1990).

The localisation of p53 within the cell is not constant and varies with the cell cycle. In synchronised cells stimulated to grow by the addition of serum, p53 first appears in the cytoplasm coinciding with protein synthesis in a perinuclear location and soon after can be detected in the nucleus during G1 and into early S phase. As S phase proceeds p53 is again found in the cytoplasm (Shaulsky et al., 1990). The temperature sensitive mutant, $p53val^{135}$, which adopts a WT configuration at 32°C and a mutant configuration at 37°C (Milner & Medcalf 1991) is detected within the nucleus at 32°C and within the cytoplasm at 37°C (Gannon & Lane 1991; Ginsberg et al., 1991). At 32°C the cells are growth arrested whereas at 37°C they are actively proliferating. These data suggest that p53 in WT conformation acts within the nucleus at G1 to arrest further replication events and then relocates to the cytoplasm possibly coincident with a conformational change

so allowing replication to proceed.

2.5 Properties and Functions of p53

The major functions of p53 appear to stem from its capacity to bind to other cellular proteins, to itself and to DNA. Deriving from this are its regulatory roles in the cell-cycle and as a transcription factor. The ensuing section attemts to summarise the data supporting these major cellular roles.

p53 was originally isolated by virtue of its ability to bind SV40 large T antigen (Lane & Crawford 1979; Linzer & Levine, 1979; Rotter, 1983). Subsequent studies have implicated p53-protein interactions resulting in p53 inactivation as frequent events in many systems. Complex formation can occur with other p53 molecules (both WT and mutant), viral proteins and other endogenous proteins.

2.5.1 Homologous Complexes

p53 will form high molecular weight, homologous complexes involving interspecies p53 relatively quickly (Kraiss et al., 1988; O'Reilly & Miller 1988). This phenomenon is also detected between WT and mutant p53 from the same species when they are co-translated but not when simply mixed *in vitro* (Milner et al., 1991). In contradistinction to this, p53 from normal cells (presumably of WT) exists in a low molecular weight monomeric form (Kraiss et al., 1988). High molecular weight oligomers of p53 are chemically very stable and require high salt concentration and denaturing detergent to bring about their disaggregation (Kraiss et al., 1992).

The formation of homologous p53 complexes can be shown to be dependent upon the presence of the carboxy terminus of the protein as truncated variants fail to oligomerize (Milner et al., 1991). This process may explain how mutant p53 can act as an "oncogene" in the presence of WT p53. By binding WT p53 into a complex and preventing its normal function, the mutant protein would be acting in a dominant negative fashion by inactivating a tumour suppressor whilst having no independent oncogenic function (Herskovitz et al., 1987).

2.5.2 Heterologous Complexes

Studies have demonstrated that the products of several viruses bind p53 including SV40 large T antigen (Lane & Crawford 1979; Linzer & Levine, 1979; Rotter, 1983), E1b 55kDa protein of adenovirus 5 (Sarnow et al., 1982), the E6 proteins of the human papillomaviruses HPV 16 and HPV 18 (Werness et al., 1990) and the large tumour antigen of lymphotropic papovavirus (Symonds et al., 1991). In the case of SV40 T Ag this complexing will only occur with WT p53 and not mutant p53 (Stürzbecker et al., 1987; Finlay et al., 1988). It has also been shown that p53 forms high molecular weight complexes with the constitutive 70kDa protein of the heat shock protein family (Pinhasi-Kimhi et al., 1986; Hinds et al., 1987; Stürzbecker et al., 1987) and that this association is confined to mutant p53 (Stürzbecker et al., 1987, Finlay et al., 1988; Romano et al., 1989).

WT p53 has a short half life of 6 minutes in spleen tissue (Rogel et al., 1985) and 20-30 minutes in cell lines (Gronostajski et al., 1984). The half life of p53 is greatly extended both in chemically transformed cells, where it is of the order of 5 hours (Reich et al., 1983) and in SV40 transformed cells

where it is about 20 hours (Oren et al., 1981). This extended life span of p53 may be due to complex formation with heterologous proteins such as SV40 T Ag, or adenovirus E1b 55kDa protein (Reihaus et al., 1990) but uncomplexed and stable p53 can be detected in SV40 transformed rodent cells (Deppert et al., 1986). This increase in stability results in much higher levels of p53 protein (Thomas et al., 1983) within transformed cells (regardless of the method of transformation) when compared to their normal counterparts.

The degradation of p53 occurs via a non-lysosomal ATP dependent proteolytic pathway (Gronostajski et al., 1984) and although no specific recognition sequences for degradation have been identified, loss of sequences at the amino terminus result in increased p53 half life (Jenkins et al., 1985; Rovinski et al., 1988). Binding of p53 to the E6 protein of HPV 16 and 18 results in more rapid degradation via an ATP dependent, ubiquitin pathway (Scheffner et al., 1990).

Thus, viruses of diverse types have all evolved strategies for p53 inactivation suggesting a critical role for p53 in preventing viral replication and possibly cellular replication in the presence of such aberrant DNA.

MDM2 is a mammalian protein originally identified as an amplified gene in a transformed mouse cell line (Fakharzadeh et al., 1991). It has been shown to complex with both WT and mutant p53 (Oliner et al., 1992; Momand et al., 1992) and to interfere with its transactivation function (Momand et al., 1992). Amplification of the *MDM2* gene has been detected in a proportion of human sarcomas suggesting that the endogenous protein *MDM2* is inactivating p53 by complexing with it in a fashion analogous to that seen with viral proteins.

2.5.3 Other Protein-Protein Interactions

p53 is associated *in vivo* with two protein kinases for which it acts as a substrate. The first is casein kinase II (CK-II) (Meek et al., 1990) which is ubiquitous in both nucleus and cytoplasm of eukaryotic cells and is stimulated by several mitogens. The second is cdc-2 kinase, a 34 kDa protein which is linked to mitogenic commitment at G1 (Milner et al., 1990; Bischoff et al., 1990; Stürzbecker et al., 1990). The significance of these two kinases which are closely associated with p53 is not yet clear but p53 could be involved in their transport into the nucleus at critical times in the cell cycle.

Phosphorylation of p53 at residues ser 37, ser 310 and/or 312, ser 389 and one or more of serines 7, 9, 12, 18 and 23 has been described in SV40 transformed cells and NIH 3T3 cells. However, two fold higher levels of phosphorylation are detected at serines 312 in the SV40 transformed cells compared to NIH 3T3 raising the possibility that phosphorylation is involved in cell transformation. However mutation of serines 312 to alanine or aspartic acid or loss of serine 312 phosphorylation does not appear to alter the properties of p53 (Meek & Eckhart 1990). Changes in conformation and properties in the temperature sensitive mutant p53val^{135} are not associated with phosphorylation changes suggesting that conformation alterations in WT p53 are not secondary to phosphorylation (Picksely et al., 1992).

2.5.4 DNA Binding

Wild type p53 binds to DNA (Kern et al., 1991a) and this property is greatly diminished by p53 mutation. Further analysis of p53 binding to SV40 DNA

showed this to be sequence specific and also to be diminished in p53 mutants (Bargonetti et al., 1991). More specifically, murine p53 inhibits SV40 origin dependent DNA replication in SV40 transformed monkey cells (Braithwaite et al., 1987) and human WT p53 has similar properties which are again lost with p53 mutation (Friedman et al., 1990). To investigate the specificity of this process more closely the binding of p53 to human genomic clones *in vitro* was tested. Mapping of the p53 binding sites within the clones obtained, revealed 2 copies of the 10 bp sequence 5'-PnPnPnC(A/T)(A/T)GPyPyPy-3' (Kern et al., 1991b). Both sequences, separated by up to 13 bp of random sequence were required for binding. The sequences are symmetrical thus giving four copies in opposing directions suggesting that p53 may bind in a tetrameric structure. Further studies screening oligonucleoticles for p53 binding produced a 20 bp sequence very similar to that described above (Funk et al., 1992). It was also shown that DNA binding is dependent upon the presence of nuclear extracts when *in vitro* translated p53 is employed suggesting that post-translational modification or complex formation with other nuclear proteins is required for this function.

2.5.5 Intracellular Effects

Mutant p53 expression can immortalise both rat embryo fibroblasts (Rovinski & Benchimol, 1988) and adult rat chondrocytes (Jenkins et al., 1984) acting alone and can co-operate with *ras* to fully transform rodent embryo cells in culture (Parada et al., 1984; Eliyahu et al., 1984). However, in similar experiments on rodent cells in culture, WT p53 suppresses transformation when co transfected with activated *ras*, E1b 55 kDa protein and mutant p53 (Hinds et al., 1989; Finlay et al., 1989). Expression of human WT p53 cDNA

will inhibit proliferation of both *Sacchromyces cervisiae* (Bischoff et al., 1992) and *Schizosacchromyces pombe* (Nigro et al., 1992) even though these yeasts have no known p53 homologue of their own. Growth of cells cultured from Friend virus-induced murine erythroleukaemia, human colorectal cancers and human glioblastoma all undergo down-regulation of proliferation when transfected with either murine or human WT p53 usually with arrest at the G1/S boundary (Baker et al., 1990; Mercer et al., 1990; Johnson et al., 1991). Chromosome transfer studies adding a normal human chromosome 17 (containing WT p53) to colorectal cancer cell lines which have lost or inactivated both copies of p53 show a reduction in proliferation (Goyette et al., 1992).

WT p53 is, therefore, classified as a tumour suppressor gene but it is still not clear whether mutant p53 is a true dominant oncogene. A cell line lacking any functional p53 develops enhanced tumourigenicity in immunosuppressed mice when it is transfected with mutant p53 suggesting a true oncogene effect as there can be no dominant negative effect on endogenous WT p53 (Wolf et al., 1984). In other studies, the transfected mutant p53 is under the control of a powerful promoter which results in high levels of expression (Parada et al., 1984; Elijahu et al., 1984). These effects could well be explained by a dominant negative action of the mutant p53 on the endogenous WT p53 complexing with it or altering its conformation. Although mutant p53 may exert its influence via "inactivation" of WT p53, the possibility of a true oncogenic effect over and above this cannot be discounted.

Apoptosis (programmed cell death) is a fundamental biological process involved in tissue remodelling especially during embryogenesis and

restricting the size of immature progenitor cell pools (Duvall & Wyllie 1986). Within a tissue or a neoplasm changes in cell number depend upon the balance between the rate of mitosis and the rate of apoptosis. Thus a neoplasm can only grow if mitotic rate exceeds apoptotic rate. The expression of wild type p53 in myeloid leukaemia cells results in rapid loss of viability which is due to apoptosis (Yanish-Rouach et al., 1991). Stimulation by interleukin 6 results in "rescue" of the cells from apoptosis. Mutant p53 under the same circumstances does not induce apoptosis. Thus a function of WT p53 may be to limit certain cell populations by apoptosis at certain times and then permit their growth when a suitable differentiation or proliferation signal is received. Clearly, loss of cell population control by apoptosis consequent upon loss of WT p53 could lead to population expansion and neoplasia.

Expression of WT p53 in acute phase, chronic myeloid leukaemia cells, which lack endogenous p53 expression, results in a return of some features of differentation seen in chronic phase cells (Feinstein et al., 1992). The mechanism for this effect remains obscure but holding cells in G1 for a greater length of time may be critical.

The amino terminal 73 residues of p53 have been shown to act as transcriptional activators both *in vivo* (Fields & Jang 1990; Raycroft et al., 1990) and *in vitro* (Farmer et al., 1992) and this property is greatly diminished by mutation (Raycroft et al., 1990; Farmer et al., 1992). Further studies have mapped this activation domain to an area between codons 20 and 42 (Unger et al., 1992).

Sequence specific DNA binding and transcriptional activation suggest that

p53 may activate the transcription of genes adjacent to its DNA binding site. The work of Kern et al., 1992 and Funk et al., 1992 confirm this view. Co-transfection of a p53 expression vector and a p53 binding site upstream of a reporter gene resulted in high levels of reporter gene expression. This result has been repeated in a yeast system making it much more likely that p53 is acting directly and not via specific intermediates as no p53 homologue has been detected in yeast (Scharer & Iggo 1992; Kern et al., 1992).

Mutation of p53 results in diminished DNA binding (Kern et al., 1991, 1992) and transcriptional activation (Raycroft et al., 1990; Kern et al., 1992; Farmer et al., 1992). In addition to this mutant/WT p53 complex formation can interfere with WT p53 function (Kern et al., 1992, Farmer et al., 1992).

DNA damage caused by γ irradiation, UV irradiation or chemical mutagens in both cultured cells and normal keratinocytes results in a rise in p53 levels and associated G1 arrest (Maltzman & Czyzyk,, 1984; Kastan et al., 1991; Hall et al., 1993). These effects can be abolished by preventing p53 production using a protein synthesis inhibitor and are not detected in cell lines containing either mutant p53 or no p53 (Kastan et al., 1991). These findings are in complete accord with the other findings of G1 arrest produced by WT p53 expression previously discussed and the rise in p53 levels in G1 seen in normal cells (Reich & Levine, 1984) and suggest that p53 has a role in preventing DNA replication until DNA repair has taken place in G1 following DNA damage. This hypothesis could also explain the large numbers and varied types of tumours developed by patients with a germ-line p53 mutation (Malkin et al., 1990; Srivastava et al., 1990) and in mice with no p53 (Donehower et al., 1992) or over-expressing mutant p53 (Lavigueur et al., 1989) and the instability of cultured fibroblasts from Li-Fraumeni

syndrome patients (Bischoff et al., 1990). Inactivation by complexing or enhanced degradation of p53 by the gene products of viruses is also compatible with this theory as viral infection could well result in G1 arrest by WT p53 and abolition of this effect would be necessary to permit viral replication. Wild type p53, therefore, has a central role in protecting the cell from replicating in the presence of DNA damage. This appears to be brought about by the transcription of as yet, undiscovered genes. Loss or inactivation of p53 results in genetic instability with cellular immortalization and the development of aneuploidy (Bischoff et al., 1990) and neoplastic growth.

2.6 p53 in Carcinogenesis

In keeping with its central role in protecting the genome from replicating in the presence of DNA sequence errors, p53 appears to be the commonest genetic target in human carcinogenesis (Hollstein et al., 1991). Chromosome deletions including the gene for p53 along with p53 mutation, rearrangement and complexing with amplified endogenous or viral proteins have all been described. The evidence for and relevance of these processes will be discussed.

In man, the gene for p53 has been mapped to chromosome 17p13 (Isobe et al., 1986). Loss of genetic material for the short arm of chromosome 17 is a frequent event in colorectal cancer, ranging from 0-75% depending upon the study (Lothe et al., 1988; Vogelstein et al., 1988; Okamato et al., 1988, Law et al., 1988; Monpezat et al., 1988; Vogelstein et al., 1989; Sasaki et al., 1989; Delattre et al., 1989) and possibly the care with which samples were taken to minimize contamination with normal tissue (Vogelstein et al., 1988). In those studies where tissue microdissection was employed, the frequency

of 17p allele loss ranges from 56-75% (Law et al., 1988; Vogelstein et al., 1988; Delattre et al., 1989). Those studies showing less frequent loss of 17p alleles (0-27%) were conducted in Japan and may represent racial genetic differences (Okamato et al., 1988; Sasaki et al., 1989).

Loss of 17p alleles is also a frequent finding in many other common human cancers including small cell and squamous carcinoma of the lung (Yokota et al., 1987; Weston et al., 1989), breast (Devillee et al., 1988; Mackay et al., 1988), bladder (Tsai et al., 1990), glioma (James et al., 1988) and ovary (Eccles et al., 1992).

In order to delineate the minimum region of overlap of chromosome 17p deletion, a large series of colorectal cancers (Baker et al., 1989) and breast cancers (Coles et al., 1990) were studied with numerous DNA probes mapping to 17p. This region, in colorectal cancers lay between probes EW505 and YNZ22.1 spanning 17p12 to17p13.3 and covering approximately 33cM (sex-averaged distance between markers D17S5 and D17S67: Wright et al., 1990). This region includes the p53 gene locus at 17p13.1 (Baker et al., 1989) and as a tumour suppressor gene it was proposed as the target for such deletions. The finding that the remaining p53 gene copy is frequently mutated further suggested that p53 was being inactivated in a fashion similar to *RB1* in retinoblastoma (Cavanee et al., 1983). In a similar study on a much larger series of breast cancers (Coles et al., 1990) the data were incompatible with a single shortest region of overlap of 17p deletion and suggested two loci: one centred on probe MCT35.1 and another on YNZ22.1.

Mutation of the p53 gene also appears to be a common event in human

cancers. They have been demonstrated in cancer of the colorectum (Nigro et al., 1989; Baker et al., 1989, 1990; Rodrigues et al., 1990; Cunningham et al., 1992), lung (Nigro et al., 1989; Iggo et al., 1990; Chiba et al., 1990; Henzel et al., 1992), breast (Nigro et al., 1989; Prosser et al., 1990; Varley et al., 1991; Thompson et al., 1992), bladder (Sidransky et al., 1991), glioblastoma (Nigro et al., 1989), astrocytoma (Fults et al., 1992), stomach (Tamura et al., 1991) and hepatocellular carcinoma (Brassac et al., 1991; Hsu et al., 1991). The majority of the mutations described in these studies can be found in the highly conserved regions of the p53 gene designated II-IV (Soussi et al., 1987) (figure 2.1). In a study of non-small cell lung carcinomas, Chiba et al., 1990, proposed that the distribution of the mutations detected mapped more closely to the SV40 large T antigen binding sites (Jenkins et al., 1988) which has been proposed as a binding site for a putative endogenous ligand.

A review of the current literature shows that the spectrum of mutation varies depending upon the tissue studied (figure 2.3). Tumours of the colorectum and brain most frequently contain G to A and C to T mutations, whereas these are relatively uncommon in lung and hepatocellular carcinoma in Africa in which G to T transversions predominate. This presumably represents exposure to a different spectrum of mutagens in cigarette smoke (Chiba et al., 1990) and aflatoxin B (Brassac et al., 1991; Hsu et al., 1991). The pattern of base pair substitution in breast cancers is also different with G to T transversion being the most common (as in lung cancer) but with C to G mutation also frequent whereas these are very rare in other types of tumour. Thus, each tumour has a different spectrum of base pair substitution presumably reflecting the varied exposure to mutagens. Although the mutations all tend to occur in the conserved regions II-IV, they clearly differ

between tissues and this may have important implications for the behaviour of the different mutant proteins (Halevy et al., 1990).

Mutation of p53 and loss of the remaining (presumably) WT allele tend to occur together in colorectal tumours (Baker et al., 1989; 1990; Cunningham et al., 1992). These events are much commoner in colorectal carcinomas than adenomas. Moreover, where carcinoma and adenoma portions of the same tumour can be analysed separately, mutation and allele loss are present in carcinoma but not adenoma, suggesting that the two events are closely linked and occur at or about the adenoma to carcinoma transition (Baker et al., 1990). A similar close correlation between p53 mutation and 17p allele loss is seen in bladder cancer (Sidransky et al., 1991). p53 would therefore appear to be behaving in a similar way to *RB1* in retinoblastoma (Kundson 1971; Cavanee et al., 1983).

However, similar studies on cancers of the lung and breast have failed to demonstrate a correlation between 17p allele loss and p53 mutation (Chiba et al., 1990; Varley et al., 1990; Thompson et al., 1992). In the case of breast cancer, this may be due to another locus on 17p acting as a target for deletion (Coles et al., 1990). It may also represent the fact that mutated p53 can act as an oncogene even in the presence of a WT copy (possibly in a dominant negative fashion) although not all mutant p53 proteins are equal in their abilities either to co-operate with *ras*, to oligomerize WT p53 or to force it into a mutant conformation when co-translated (Halevy et al., 1990; Milner & Medcalf 1991). The different range of mutations seen in tumours arising from different tissues (figure 2.3) may confer different properties on particular mutants from particular tissues.

Inactivation of p53 by gross gene rearrangement has also been described both in cultured Friend virus enytholeukaemia cells (Mowat et al., 19985) and in human osteoscaroma (Masuda et al., 1987; Miller et al., 1990) although not in any other of the common human malignancies. The functional inactivation of p53 by complexing with viral proteins SV40 T Ag adenovirus E1b protein and large tumour antigen of lymphotropic papovavirus, binding to HPV 16 and 18 E6 protein resulting in enhanced p53 degradation and complexing with amplified levels of *MDM2* have already been discussed (section 2.5.3

The importance of p53 in preventing carcinogenesis can be seen in three systems which have a constitutive, germ-line p53 abnormality: Li-Fraumeni syndrome; transgenic mice over expressing mutant p53; and the gene targeted p53 null mouse. Li-Fraumeni syndrome is a rare dominantly inherited human disease characterised by a spectrum of early onset cancers including breast carcinoma, soft tissue sarcomas, brain tumours, osteosarcoma, leukaemia and adrenocortical carcinoma (Li & Fraumeni 1969). Inheritance of the syndrome is linked directly to germ line p53 gene mutations (Malkin et al., 1990, Srivastava et al., 1990; Borresen et al., 1992). The affected individuals from 6 families studied proved to possess mutations, most closely grouped in conserved domain IV (Soussi et al., 1987) whereas unaffected siblings had WT p53. DNA extracted from 2 tumours from affected individuals and cell lines derived from patients showed loss of the remaining WT allele (Malkin et al., 1991; Srivastava et al., 1992) in a fashion analogous to that seen in familial retinoblastoma (Cavanee et al., 1983).

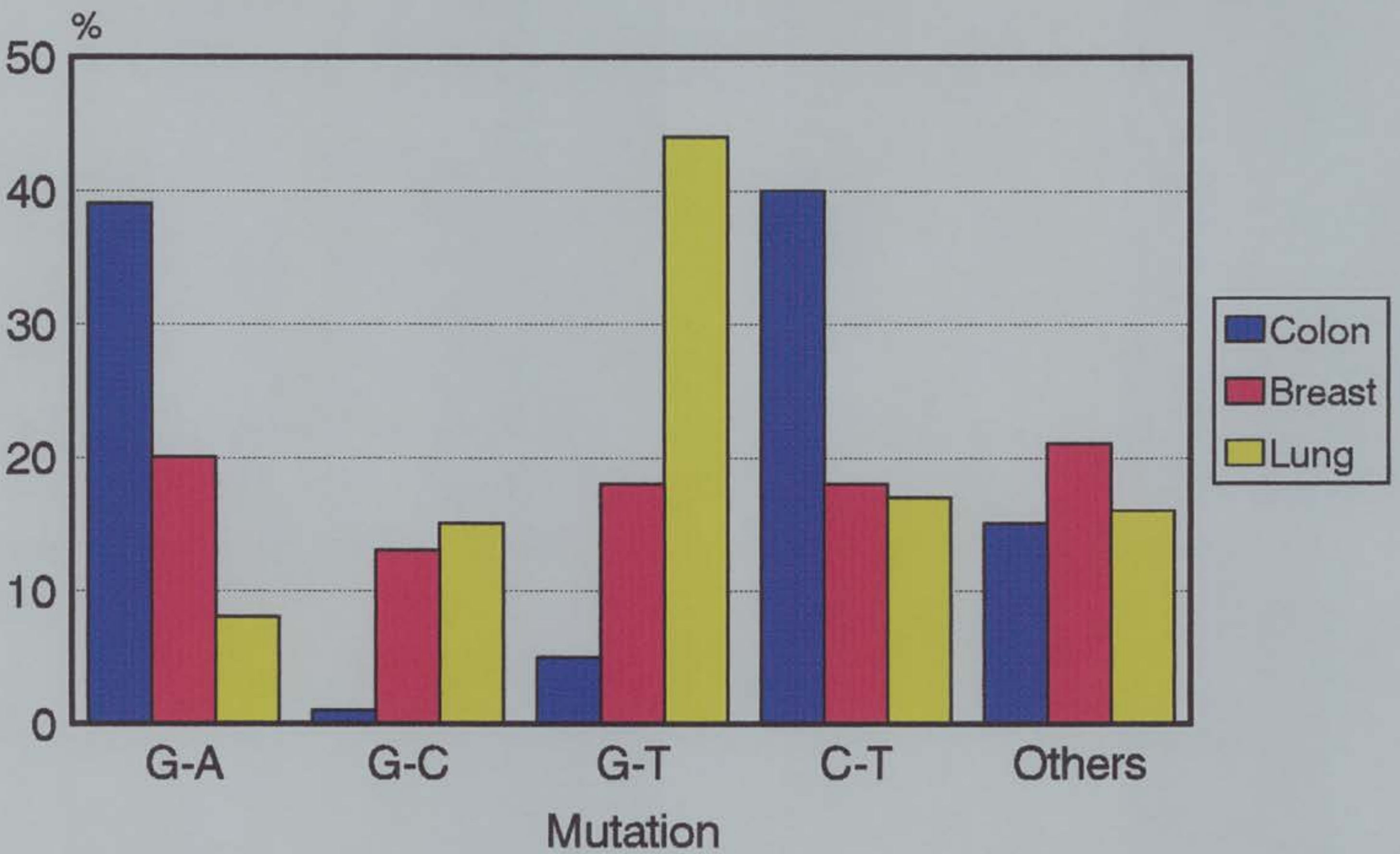
Transgenic mice which over express the p53 val ^{135} develop (in 20% of cases) an excess of lung adenocarcinoma, osteosarcoma and lymphomas (Lavigueur et al., 1989). There is a long latency however and relatively low

penetrance suggesting that many other factors are involved in this process. Over-expression of mutant p53 in this system is likely to be acting in a dominant negative fashion although formal demonstration of WT allele retention in tumours was absent. Mice which have lost both copies of p53, although developmentally normal, develop sarcomas, lymphoma and osteosarcoma at a very early age (Donehower et al., 1992).

1.6.8 Conclusion

p53 has a central role in the well-being of cells by delaying DNA replication until DNA repair has been completed. This process is carried out by transactivation of as yet unidentified genes and its importance can be gauged from the fact that p53 abnormalities are the commonest genetic lesions detected in human cancer. This thesis concerns the study of the role of p53 in colorectal carcinogenesis.

Figure 2.3 Bar chart showing the distribution of p53 mutations in tumours arising in colon, lung and breast. Summarised from review of literature; Baker et al., 1990; Bartek et al., 1990; Chiba et al., 1990; Iggo et al., 1990; Rodrigues et al., 1990; Henszel et al., 1991; Varley et al., 1991; Bodner et al., 1992; Cunningham et al., 1992; Kikuchi-Yanoshita et al., 1992; Mazars et al., 1992; Thompson et al., 1992.


Distribution of p53 mutation by tumour type
%
50
40
30
20
10
0
G-A
G-C
G-T
C-T
Others
Mutation
Colon
Breast
Lung

Chapter 3

p53 Expression

3.1 Introduction

Over-expression of p53 has been demonstrated immunohistochemically in a wide range of common human malignancies including colon (van den Bergh et al., 1989; Rodriguez et al., 1990; Purdie et al., 1991; Scott et al., 1991; Cunningham et al., 1992; Kikuchi-Yanoshita et al., 1992), breast (Cattoretti et al., 1988; Bartek et al., 1990; Ostrowski et al., 1991; Varley et al., 1991; Thompson et al., 1992), lung (Iggo et al., 1990; Bodner et al., 1992), ovary (Eccles et al., 1992), squamous cell carcinoma of larynx (Maestro et al., 1992), gastric carcinoma, malignant melanoma, embryonal carcinoma of the testis, transitional cell carcinoma of the bladder, carcinoma of the uterus and soft tissue sarcoma (Bartek et al., 1991). In the case of breast cancer, a correlation between p53 over-expression and certain immunohistochemical and histopathological indicators of poor prognosis has been demonstrated (Cattoretti et al., 1988), suggesting that p53 over-expression may be associated with enhanced tumour aggression. However, another study (Ostrowski et al., 1991) using clinical outcome as their end-point, could not demonstrate a correlation between p53 over-expression and prognosis. Although the study was relatively small (90 cases as opposed to 200 in the study of Cattoreti et al., 1988) the expected correlation of lymph node status and tumour grade with clinical outcome was clearly demonstrated suggesting that sufficient numbers of patients were included to make the analysis valid.

The reaction product marking antibody binding, in each of these studies was confined to neoplastic cells. In the majority of tumours studied including

colon cancer and non-small cell lung cancer the staining was almost exclusively nuclear (van den Bergh et al., 1989; Rodriguez et al., 1990; Iggo et al., 1990; Bartek et al., 1991) whereas in breast and small cell lung cancer cytoplasmic as well as nuclear staining was detected (Cattoretti et al., 1988; Iggo et al., 1990; Varley et al., 1991). In normal cells, p53 is undetectable by immunohistochemical methods due to its low levels and short half-life (Dippold et al., 1981; Rogel et al., 1985). Mutation of p53 can result in increased protein stability and a greatly extended half-life (Jenkins et al., 1985) permitting its immunohistochemical detection. Mutation, at many sites within the p53 gene, also results in a conformation alteration which changes its immunoreactivity. Wild-type (murine) p53 reacts with PAb246 (Yewdell et al., 1986) and PAb1620 (Milner et al., 1987) whereas mutant p53 fails to bind PAb246 or PAb1620 but reacts with PAb240 (Gannon et al., 1990). Thus, PAb240 binds specifically to mutant p53 (human and murine) and in immunohistochemistry binds the same cells as the conformation independent antibodies PAb421 and PAb1801 (Rodriguez et al., Bartek et al., 1990; 1990, Varley et al., 1991) suggesting that immunohistochemically detectable p53 is mutated.

Wild-type p53 is usually localised in the cell nucleus (Dippold et al., 1981; Rotter et al., 1983; Deppert & Haug, 1986) but a cytoplasmic location has been described in some non-transformed cell lines (Rotter et al., 1983). Localisation of p53 may be modulated during the cell cycle spending short periods in the cytoplasm and cell membrane during mitosis (Milner & Cook, 1986) and accumulating in the nucleus in late G1 (Shaulsky et al., 1990). Mutation of p53 alters its conformation and protein binding characteristics (Gannon et al., 1990) resulting in loss of SV40 large T antigen binding (Tan et al., 1986) and acquisition of hsp 72/73 heat-shock-related protein binding

(Stürzbecher et al., 1987; Hinds et al., 1987). Hsp 72/73 is found in the nucleus and the cytoplasm, thus binding of mutant p53 to cytoplasmic hsp 72/73 may be responsible for its cytoplasmic distribution in some tumours (Cattoretti et al., 1988; Iggo et al., 1990). Studies involving the temperature sensitive mutant p53 val^{135} have demonstrated that at 37°C (when it behaves as a mutant protein) it is located within the cytoplasm but at 32°C (WT) it relocates to the nucleus (Gannon & Lane, 1992; Oren et al., 1992). This process is reversible and appears to depend upon a short lived cytoplasmic anchor protein and not upon hsp 72/73 binding (Gannon & Lane, 1992). Whether these properties are exclusive to p53 val^{135} or pertain to other p53 mutatants remains to be seen but another mutant, p53 cys^{270} does not show this property (Halevy et al., 1990).

Over-expression of p53 has been demonstrated in 55% of sporadic colorectal carcinomas and 10% of sporadic adenomas using PAb421 on cryostat sections (van den Bergh et al., 1988). Higher frequencies of over-expression in FAP associated carcinomas and adenomas were also demonstrated although the numbers studied were rather small. No correlation with degree of adenoma dysplasia was found and no association with prognostic indicators was studied. Rodriguez et al (1990) studied 10 colon cancer cell lines and demonstrated that the 6 which had p53 mutations on sequence analysis were positive immunohistochemically with PAb1801 and PAb240. In another study of p53 expression in primary colorectal carcinomas, Scott et al (1991) demonstrated immunoreactivity for PAb421 in 42% of 52 carcinomas. Staining was confined to neoplastic nuclei and was seen in 70% or more of nuclei in positive cases. In five cases multiple samples were analysed and no intra-tumour heterogeneity was observed. No correlation was demonstrated between positive immunostaining and

markers of tumour aggression or clinical outcome but PAb421 positive cases were more frequently distal to the splenic flexure than proximal and had (in those which were DNA diploid) a higher rate of cell proliferation as assessed by flow cytometry. Fibroblasts cultured from patients with germ-line p53 mutations (Li-Fraumeni syndrome) spontaneously immortalize very readily and develop aneuploidy at high frequency (Bischoff et al., 1990). DNA aneuploidy measured by flow cytometry is a common finding in colorectal cancer which has been shown to correlate with poor prognosis independent of other prognostic indicators (Quirke et al., 1987). Heterogeneity of DNA content within different regions of colorectal cancers is also common indicating the presence of clonal divergence (Quirke et al., 1987).

This chapter concerns the study of a large consecutive series of colorectal carcinomas and polyps for p53 over-expression. Immunohistochemistry using PAb1801 (Banks et al., 1986) was carried out on PLPD fixed, paraffin sections from 86 carcinomas and 62 polyps once it had been established that the results for positivity were identical for material processed in this manner when compared to cryostat sections. Previous studies have been limited to cryostat sections as the PAb1801 epitope is destroyed by formaldehyde fixation. The use of PLPD and paraffin sections permitted a much superior morphologic resolution of immune reaction product. A subset of the carcinoma series was also studied using the mutant p53 specific antibody PAb240 (Gannon et al., 1990). The correlation of staining with well recognised indicators of clinical outcome was investigated. Multiple samples of each tumour were studied for DNA ploidy by flow cytometry to investigate whether p53 over-expression was related to DNA aneuploidy and clonal divergence within a single tumour.

3.2 Results

3.2.1 PAb1801 immunostaining of colorectal carcinomas

Reaction product, marking the binding of PAb1801, was confined to the nuclei of neoplastic cells (figure.3.1). Normal cells, including colonic mucosa, never stained in cytoplasm or nucleus. Rarely pale staining was observed in smooth muscle cells (particularly of blood vessels) at high antibody concentrations but not at the working dilution. Staining within the tumour nucleus (figure.3.2) was granular or reticular in nature sometimes with obvious nucleolar sparing. During mitosis, reaction product was reduced dramatically (figure.3.2), whereas interphase tumour cells never showed cytoplasmic staining. The tumours divided unequivocally into two groups on the basis of PAb1801 immunohistochemistry. Tumours were scored as positive when staining was visible in any nuclei within the tissue section. In such cases, positive nuclear staining of identical incidence and distribution was found even when the concentration of primary antibody was reduced to 1:400 or increased to 1:10.

Similarly cases scored negative remained without staining in any cells even at concentrations as high as 1:10. A subset of 53 carcinomas were stained as cryostat sections at a dilution of 1:100. The distribution of PAb1801 staining within cells and within the tissue was identical in PLPD-fixed and frozen section. No discrepancy in the assignment of PAb1801 positivity between frozen and paraffin sections was found.

In positive tumours the pattern of immunostaining was not uniform. In a small number of tumours almost all the nuclei stained positively, but the more

common pattern was an admixture of tumour acini which showed no staining with those in which most nuclei were stained. The greatest proportion of positive cells was found in acini at the invasive margins of carcinomas. Sometimes the distinction was obvious within a single gland, with strong staining on the outer (invasive) side of a carcinoma and weak, or none, on the inner side (figure.3.3).

In total, 87 carcinomas from 87 patients were analysed with PAb1801 and of these 41 (47%) showed positively stained nuclei. Prognostic indicators (Dukes' stage, degree of tumour differentiation and nature of invasive margin) and DNA aneuploidy, showed no significant correlation with PAb1801 staining (table 3.1). Similarly the proportion of PAb1801 positive tumours was unrelated to their site within the colon or rectum (table 3.1).

Immunoblots of total protein extracted from SV80 cells (known to express high levels of p53) and tissue samples (figure. 3.4) demonstrated that PAb1801 recognises a single 53 kDa protein which could be extracted from those tumours which were immunohistochemically positive but not from negative tumours or normal colonic mucosa. In a series of 5 cases on which both immunohistochemistry and immunoblotting were carried out, the detection of a 53 kDa protein in immunoblots correlated exactly with positive staining in frozen or paraffin sections.

Figure 3.1 PLPD-fixed, paraffin-embedded colorectal carcinoma tissue immunostained using PAb1801 with a light haematoxylin counter-stain. Low power view showing positively stained carcinoma nuclei and adjacent negatively stained normal mucosa (X 40).

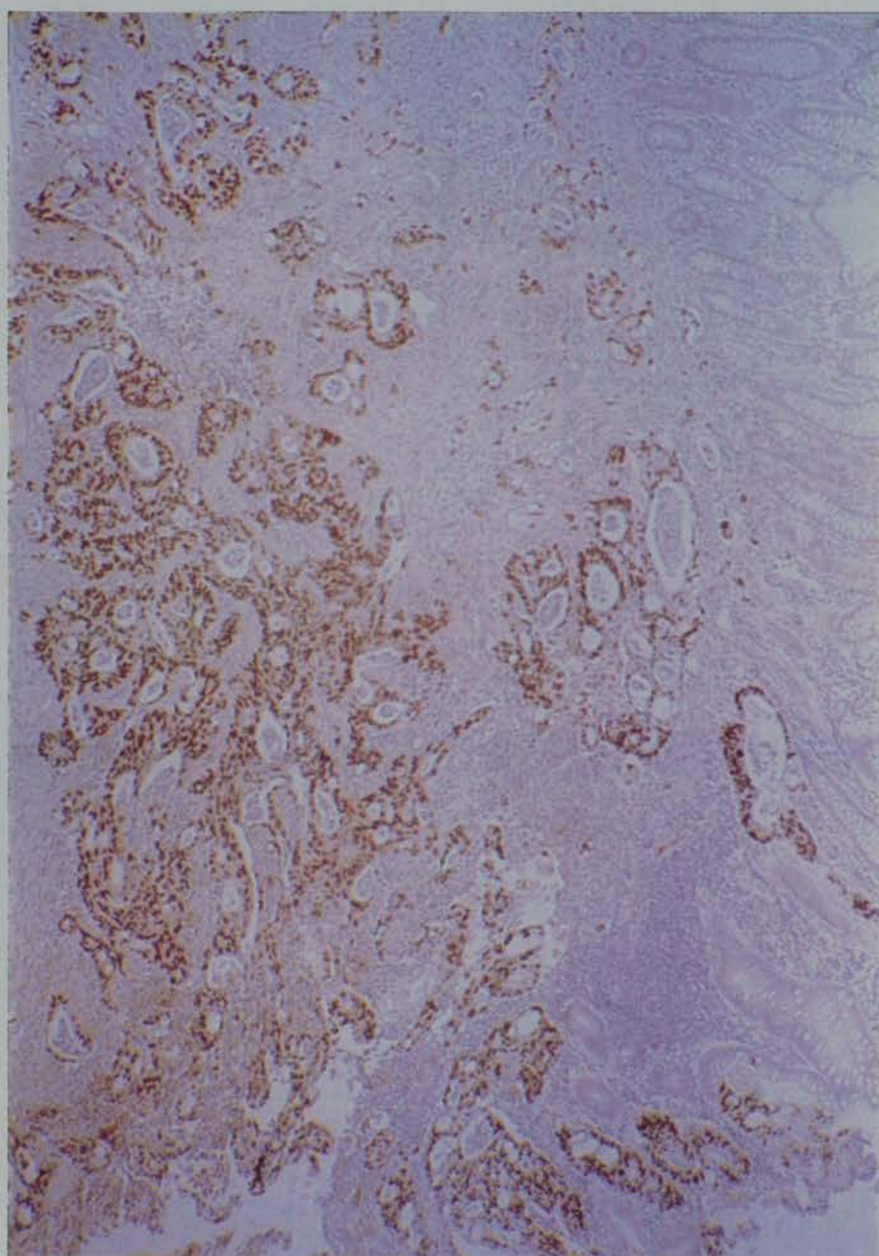


Figure 3.2 PLPD-fixed, paraffin-embedded colorectal carcinoma tissue immunostained using PAb1801 with a light haematoxylin counter-stain. High power view showing granular staining pattern confined to nuclei and several mitotic cells (arrowed) exhibiting reduced intensity of staining compared to other carcinoma nuclei (X 400).

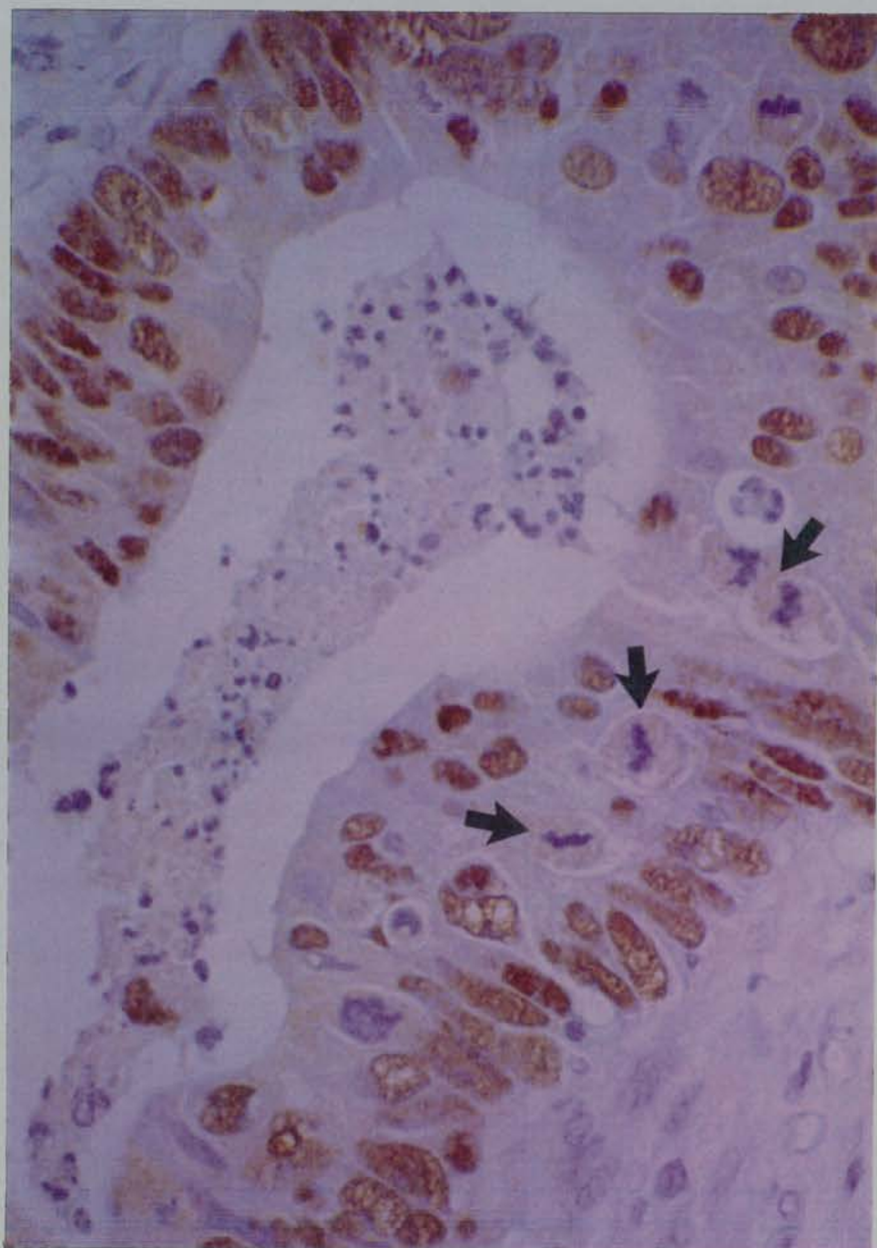


Figure 3.3 PLPD-fixed, paraffin-embedded colorectal carcinoma tissue immunostained using PAb1801 with a light haematoxylin counter-stain. Heterogeneity of staining with more marked staining at the invasive margin of the carcinoma, where it is advancing beneath the adjacent normal mucosa (X 100).

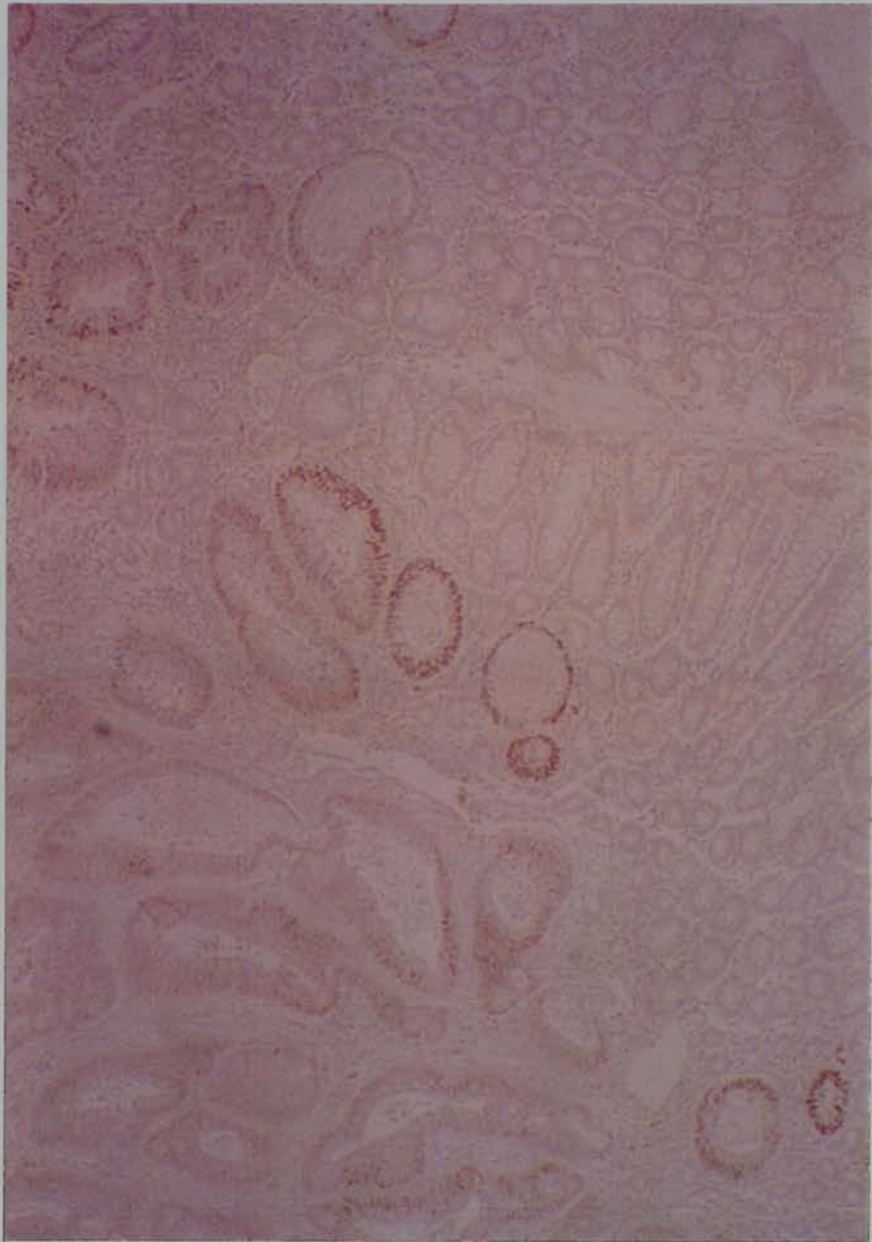


Table 3.1 Correlation of PAb1801 immunostaining with histopathological classification, DNA ploidy, and primary tumour site for carcinomas.

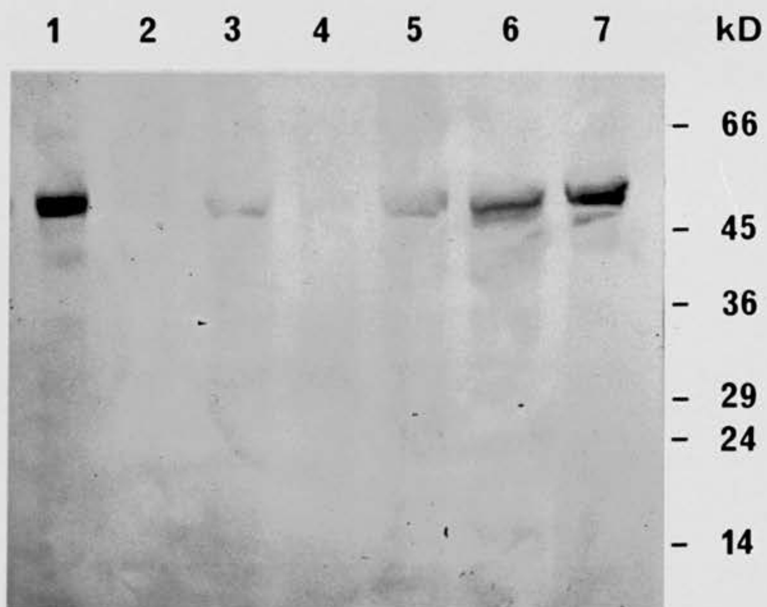
Classification	PAb1801 -	PAb1801 +	p value*
Dukes A Dukes B Dukes C	2 23 21	5 18 18	NS
Margin: Infiltrating : Expanding	40 7	36 5	NS
Differentiation: Other# : Poor	19 26	24 18	NS
DNA Diploid DNA Aneuploid	14 32	6 35	NS
Distal colon Proximal colon	28 15	27 13	NS

* Chi-square test with Yates' correction for continuity.

Well or moderately differentiated.

NS, not significant (p>0.05).

Figure 3.4 Immunoblot of total protein stained using PAb1801. Lane 1: SV80 cells. Lane 2: Normal colonic mucosa. Lane 3: Immunohistochemically positive carcinoma. Lane 4: Immunohistochemically negative carcinoma. Lanes 5 and 6: Immunohistochemically positive carcinomas. Lane 7: SV80 cells. Molecular weights expressed in kiloDaltons.


1
2
3
4
5
6
7
kD
66
45
36
29
24
14

3.2.2 PAb1801 immunostaining of colorectal adenomas

Fifty-five sporadic polyps from 30 patients were studied with PAb1801 comprising 9 metaplastic (hyperplastic) polyps and 46 adenomas. The metaplastic polyps were all negative. Of the 46 adenomas, 4 (8.7%) were positive (table 3.2). The positively staining nuclei were dispersed throughout the adenoma in one case (figure. 3.5) and in the remaining 3 were restricted to only a few glands. All 4 showed either moderate or severe dysplasia, but there is no statistically significant correlation within this series with other morphologic parameters or tumour size (table 3.2). In one large villous adenoma which contained foci of infiltrative carcinoma, however, PAb1801 staining was absent in the adenoma but present in the infiltrative glands only. In addition PLPD-fixed material was available from 9 tubular adenomas (all less than 4 mm in diameter) from 2 patients undergoing colectomy for FAP: all were negative for PAb1801 immunostaining. The difference in incidence of positive staining in adenomas as compared with carcinomas is highly significant (table 3.2).

Table 3.2 Correlation of PAb1801 immunostaining with histopathology of adenomas.

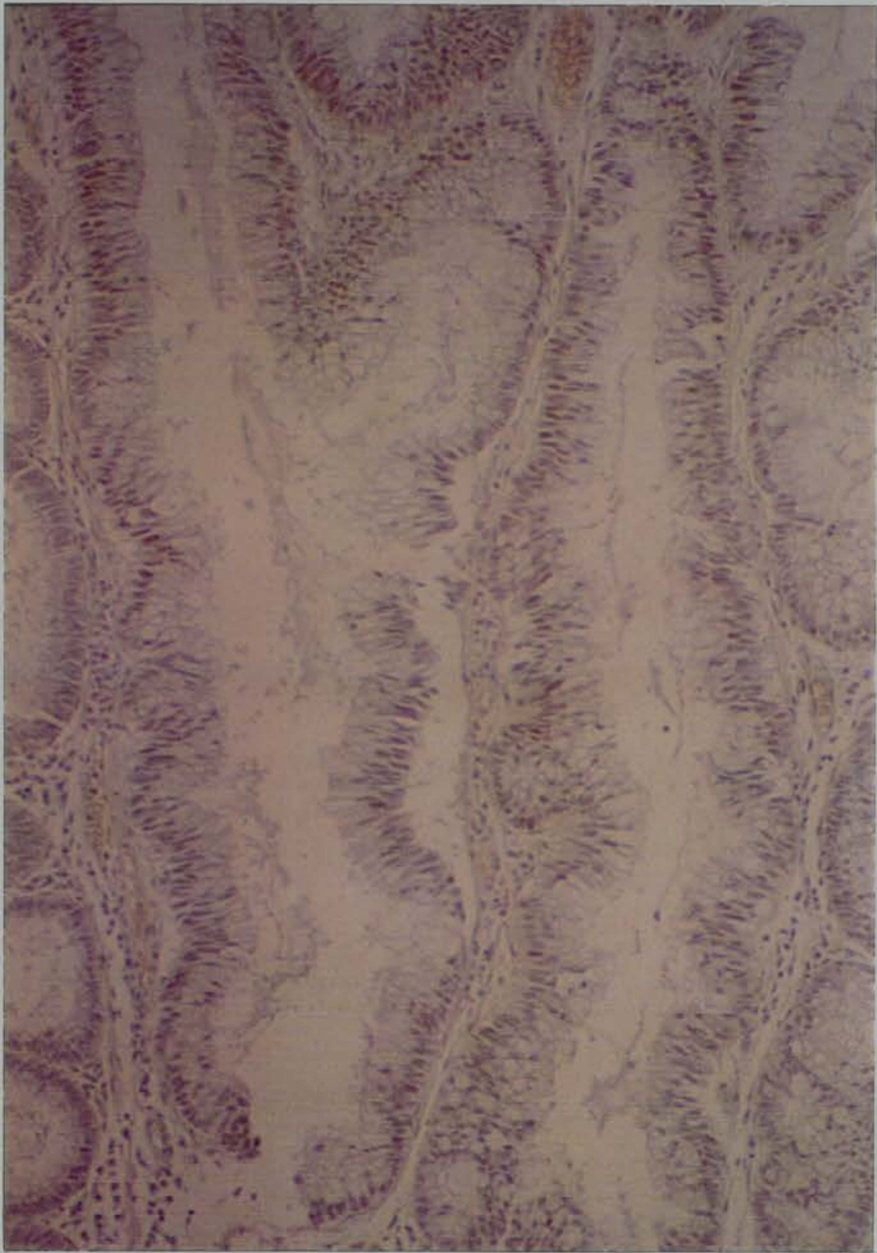
Classification	PAb1801 -	PAb1801 +	p value *
FAP adenomas	9	0	
Metaplastic polyps	9	0	
Tubular adenomas	1	2	
Tubulovillous adenomas	11	2	
Villous adenomas	12	0	NS
Maximum diameter of adenoma #			
> 10mm	29	3	
< 10mm	13	1	NS
Dysplasia: Mild	5	0	
: Moderate	34	2	
: Severe	3	2	NS
Sporadic adenomas	42	4	
Carcinomas	47	40	p<0.001

* Chi-square test with Yates' correction for continuity.

NS, not significant (p>0.05).

Sporadic adenomas only.

Figure 3.5 PLPD-fixed, paraffin-embedded colorectal adenoma tissue immunostained using PAb1801 with a light haematoxylin counter-stain. Positive staining in nuclei dispersed throughout a moderately dysplastic adenoma (X 200).



3.2.3 PAb240 immunostaining of colorectal carcinomas

PAb 240 immunostaining was carried out on a subset of 45 carcinomas from which frozen material of suitable quality was available. The overall pattern and distribution of immunostaining was almost identical to that described for PAb1801. Reaction product was confined to the neoplastic cell nuclei, with a non-uniform distribution within the tumour, although in some cases almost all the tumour nuclei were stained (figure. 3.6)

Of the 45 carcinomas studied, 22 (48.9%) showed positive staining. No significant correlation with prognostic indicators (Dukes' stage, nature of invasive margin and tumour differentiation) or tumour site within colon or rectum was demonstrated (table 3.3) but PAb240 immunostaining did correlate with DNA aneuploidy and this finding prompted further analysis. Comparison of PAb1801and PAb240 immunostaining demonstrated a highly significant correlation ($p<0.001$, table 3.3)

Figure 3.6 Immunostaining of a colorectal carcinoma using PAb240 on acetone-fixed, cryostat section with a light haematoxylin counterstain (X 200).

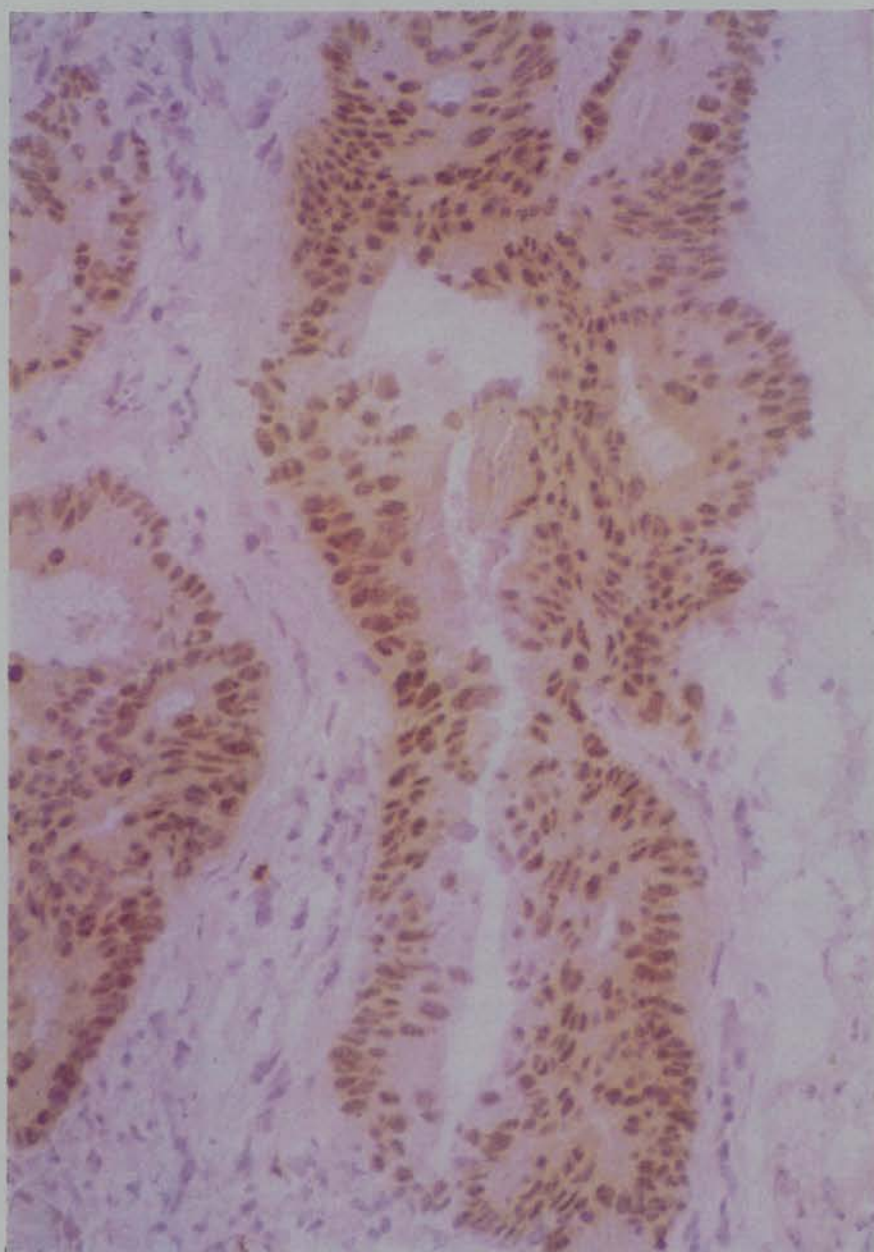


Table 3.3 Correlation of PAb240 immunostaining with histopathological classification, DNA ploidy, primary tumour site and PAb1801 immunostaining for carcinomas.

Classification	PAb240 -	PAb240 +	p value*
Dukes A Dukes B Dukes C	0 9 14	2 8 12	NS
Margin: Infiltrating : Expanding	19 4	20 2	NS
Differentiation: Other# : Poor	11 12	12 10	NS
DNA Diploid DNA Aneuploid	11 12	2 12	p<0.02
Distal colon Proximal colon	14 7	14 8	NS
1801 - 1801 +	22 1	5 17	p<0.001

* Chi-square test with Yates' correction for continuity.

Well or moderately differentiated.

NS, not significant (p>0.05).

3.2.4 p53 expression and DNA ploidy

Comparison of positive p53 immunostaining (positive with PAb240 and/or PAb1801) with DNA aneuploidy also demonstrates a significant correlation (figure 3.7). In a sub-set of 42 cases, the flow cytometry had been carried out on a minimum of 3 areas of each tumour sampled fresh. These cases gave the highest quality of DNA histogram (figure 3.8) permitting accurate identification of different clones within a single tumour. Tumours exhibiting positive immunostaining were significantly more likely to be aneuploid and also to contain multiple neoplastic stem lines (table 3.4).

Figure 3.7 Bar chart showing the correlation of DNA ploidy with p53 immunostaining for colorectal carcinomas. p53 + positive immunostaining with PAb240 and/or PAb1801. p53 - negative immunostaining.

Correlation of DNA ploidy with p53 immunohistochemistry

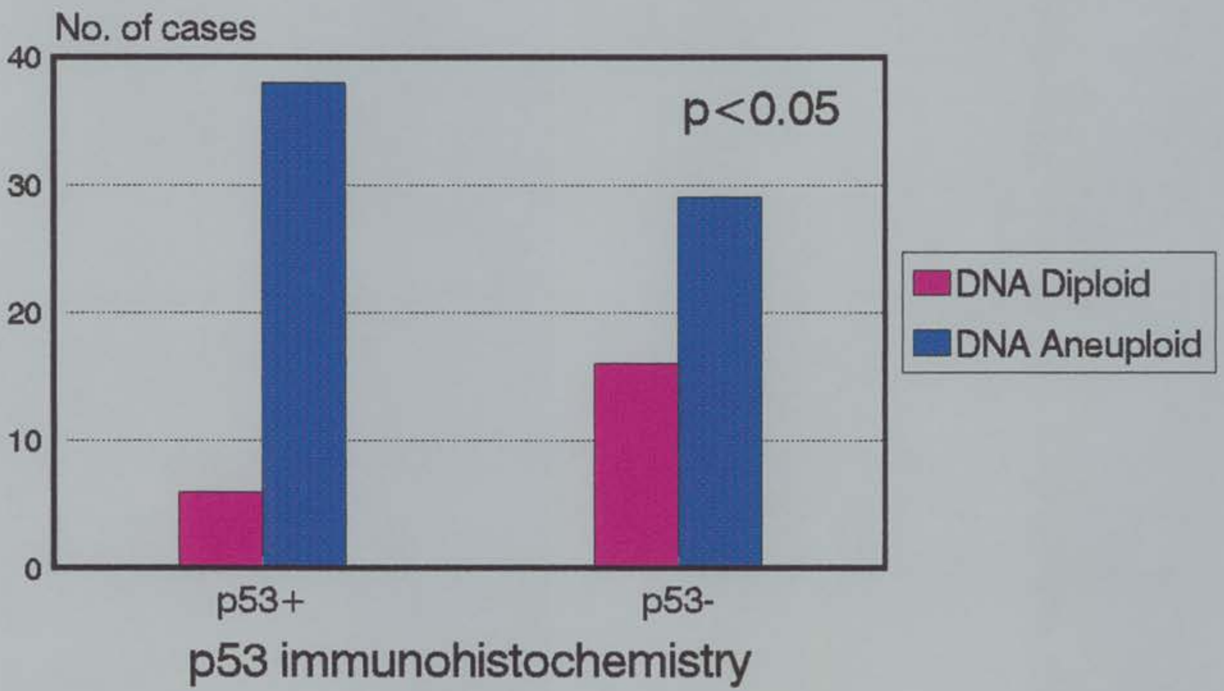
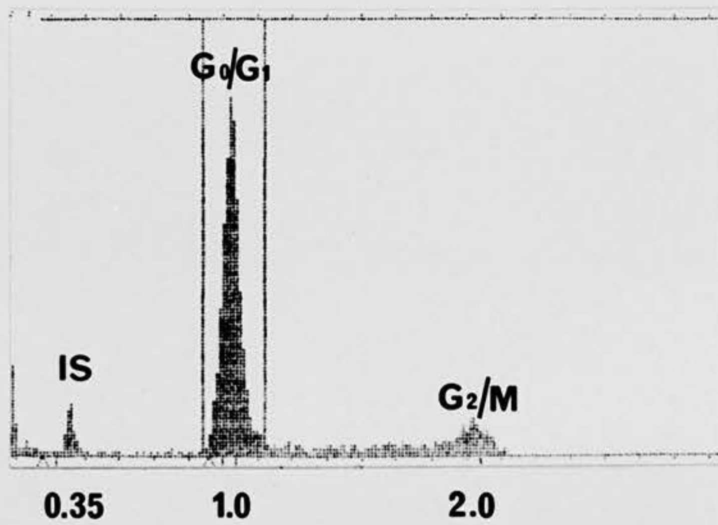


Figure 3.8 DNA histograms obtained from two samples of a single carcinoma. The x axis shows the relative DNA content of each nucleus and the y axis the number of nuclei. The diploid peak (G0/G1) is arbitrarily assigned a DNA index of 1 and an internal standard (IS) of avian erythrocytes is included which has a DNA index of 0.35. **(a)** a diploid area of carcinoma (histological examination of tissue adjacent to that used for flow cytometry confirmed the presence of greater than 50% neoplastic nuclei). **(b)** an aneuploid region of the same carcinoma showing a small diploid peak (D) and a larger aneuploid peak (A) with a DNA index of 1.6. The aneuploid population of cells also has a clearly discernable G2/M peak with a DNA index of 3.2.


G0/G1
IS
G2/M
(a)
0.35
1.0
2.0

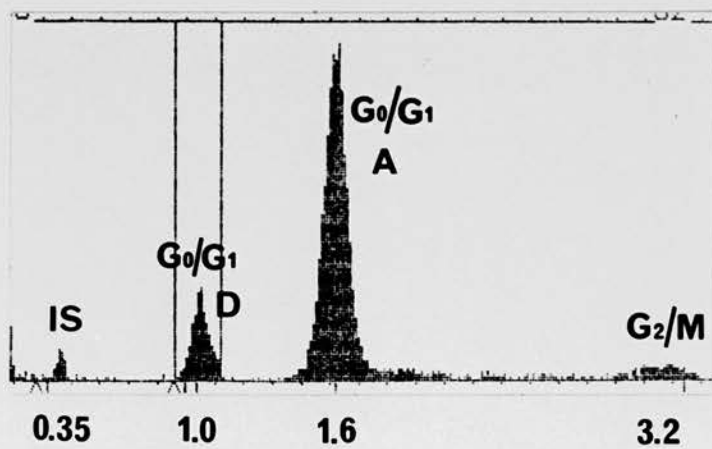

G0/G1
A
G0/G1
D
IS
G2/M
(b)
0.35
1.0
1.6
3.2

Table 3.4 Correlation of p53 immunostaining with DNA ploidy and the number of different clones defined by flow cytometry. Diploid cases had peaks with a DNA index of 1 in all tumour samples. Aneuploid cases were defined as having one clone if all samples had the same non-diploid DNA index and more than one clone if samples had DNA indices differing by more than 0.1.

PAb1801/240 immunostaining	Diploid	Aneuploid 1 stem-line	Aneuploid >1 stem-line	Totals
Positive	4	2	15	21
Negative	10	6	5	21
Totals	14	8	20	42

$p < 0.05$ (Chi-square test with Yates' correction for continuity).

3.3 Discussion

The data demonstrate a strong relationship between p53 expression and malignant transformation in colorectal tumours. In contrast to carcinomas, very few adenomas showed p53 expression and those which did all belonged to the more dysplastic categories in which the risk of transition to invasive carcinoma is higher. In one case of a villous adenoma which had undergone malignant change, p53 expression was restricted to the frankly carcinomatous portion sparing the dysplastic epithelium. In the carcinomas, the advancing edge was the most consistently staining region. Nonetheless, approximately one half of all colorectal carcinomas did not show p53 expression, and comparison of expressing and non-expressing carcinomas revealed no differences in terms of the tumour site or established prognostic indicators such as histologic grade (Jass et al., 1986) and Dukes' stage (Dukes, 1932). The majority of the tumours studied in this consecutive series were (as expected) of Dukes' B and C stages, therefore the lack of correlation with staging is essentially an indicator that p53 expression has no association with tendency to form lymph node metastasis. It appears, therefore, that p53 expression is associated with the transition from adenoma to carcinoma in some colorectal tumours but does not uniquely determine infiltrative activity or relate to other features of carcinoma progression.

The monoclonal antibody PAb1801 is specific for human p53, its epitope being between amino acids 32 and 79 (Banks et al., 1986) at the amino terminal of this protein. Its epitope is distant from the sites of mutation so far described (Nigro et al., 1989; Baker et al., 1990; Rodriguez et al 1990) and it will thus bind wild-type and mutant p53. No immunoreactivity is detected in

any normal tissue in this study, therefore the positively staining cells are likely to contain mutated p53, detectable by virtue of the concomitant increase in half-life (Jenkins et al., 1985). The analysis of a subset of the carcinomas with PAb240, which is specific for the mutant conformation (Gannon et al., 1990) confirms that there is a very high concordance between the two groups ($p<0.001$, table 3.4) and that the intra-tumour and intra-cellular distribution is almost identical. The relationship between mutation and expression is not a simple one and depends at least on the specific mutation (Bodner et al., 1992). In colorectal cancer, more than 90% of the p53 mutations are located within exons 5-9 (Rodrigues et al., 1990; Baker et al., 1992; Cunningham et al., 1992; Kikuchi-Yanoshita et al., 1992) and these are the mutations which tend to produce immunohistochemically detectable p53 (Bodner et al., 1992). Thus immunohistochemically detectable p53 is likely to represent mutated forms of the protein (see also chapter 5).

The p53 detected immunohistochemically in this series was exclusively nuclear in distribution, in agreement with published studies (van den Bergh et al., 1988; Rodriguez et al., 1990, Scott et al., 1991). Studies on breast cancers and small cell lung cancer (Cattoretti et al., 1988; Iggo et al., 1990; Varley et al., 1991) have demonstrated a number of cases with cytoplasmic staining. Whether this is due to complexing of mutant p53 with cytoplasmic hsp 72/73 (Stürzbecher et al., 1987) or whether the mutation affects the nuclear localisation signal (Addison et al., 1991) is not yet clear. From the p53 val^{135} model it might be predicted that mutant protein would be localised to the cytoplasm (Mickalovitz et al., 1990; Gannon & Lane,1992) but this does not appear to be the case (Rodrigues et al., 1991; Cunningham et al., 1992) suggesting that this observation may depend upon the particular

mutation. Differences in the types of mutation detected in different classes of tumour, as a result of exposure to a different set of environmental carcinogens, may reflect this differential intracellular localisation.

Breast carcinomas which express p53 tend to be in poorer prognostic groups (Cattoretti et al., 1988). In this series, p53 immunostaining correlates with DNA aneuploidy which is a recognized independent prognostic variable suggesting that p53 positive tumours may give a poorer prognosis (table 3.3). Clinical follow-up, however, is required to discover if this association is relevant to prognosis.

Within some of the sections showing positive immunostaining there was marked cell-to-cell heterogeneity; a feature described previously (Scott et al., 1991). The mutation of p53 is likely to be clonal (Fearon et al., 1987), therefore this variation in protein level may indicate that mutated p53 is under similar cell cycle control to that demonstrated in WT p53 with levels increasing prior to S phase (Reich and Levine, 1984; Shaulsky et al., 1986). Cell-cycle related changes in protein level are also suggested by the greatly reduced immunostaining in mitotic cells. The distribution of positively stained cells around the advancing margins is a novel feature and may indicate greater replicative activity of invading cells or some form of inductive interaction with stromal tissues.

In 6 cases of the 45 studied with both PAb1801 and PAb240 there was a discrepancy in the result. One case was PAb1801 positive but PAb240 negative probably representing a truncating mutation removing the epitope for PAb240 (amino acids 213-217, Stephen & Lane, 1992) but retaining that of PAb1801; some of these have been described (Baker et al., 1990). The

remaining 5 cases are PAb1801 negative but PAb240 positive probably as a result of mutations which fail to increase the half-life of the protein sufficiently to be detected by PAb1801 or else cause a conformational change at the amino terminal sufficient to prevent PAb1801 binding. Thus, a small number of cases which are negative for p53 immunostaining may contain mutations but use of more than one one antibody will minimise this problem.

Aneuploidy is a common finding in human colorectal tumours which is associated with the malignant phenotype and poor prognosis. Cytogenetic evidence suggests that aneuploidy may arise from non-disjunctive mitosis in tetraploid cells (Nowell, 1976; Reichmann et al., 1981) or from chromosome breakage. Over-expression of p53, (probably representing mutated protein which has lost its normal tumour suppressor function) is linked to the presence of DNA aneuploidy and the presence of multiple divergent clones. The evidence suggests that WT p53 causes cell-cycle arrest in G1 in response to DNA damage to permit repair prior to DNA replication. The loss of this vital function could facilitate endoreduplication and the appearance of aneuploidy with the generation of multiple divergent clones some of which will possess selective growth advantages. This may be the case in colorectal cancer.

The data presented here show that the expression of p53 acts at or about the stage of the acquisition of malignant potential in colorectal tumourigenesis. Although p53 expression does not appear to have any influence on the appearance of lymph node metastesis it does relate to the emergence of aneuploidy and multiple divergent clones consistent with the current hypothesis that p53 has a "genomic surveillance" role to prevent replication prematurely in the cell cycle or in the presence of DNA damage.

When this function is lost, genomic instability will result predisposing to the emergence of clones with a growth advantage and properties such as invasion and metastasis which define malignancy.

Chapter 4

Mapping the Deletion on Chromosome 17

4.1 Introduction

Genetic abnormalities involving chromosome 17 were initially identified in colorectal cancers by cytogenetic techniques which showed loss of chromosome 17 as one of the most frequent abnormalities (Reichmann et al., 1981) . Subsequent studies using RFLP markers have identified loss of 17p alleles in up to 75% of colorectal cancers (Lothe et al., 1988; Vogelstein et al., 1988; Okomato et al., 1988; Law et al 1988; Monpezat et al., 1988; Vogelstein et al., 1989; Sasaki et al., 1989; Delattre et al., 1989). Fine mapping of the region of deletion using a number of RFLP markers for chromosome 17p was carried out on a series of 58 colorectal carcinomas of which only 8 were suitable for detailed analysis (Baker et al., 1989). All the tumours in this series were microdissected to enrich for neoplastic cells by extracting DNA only from areas containing greater than 70% cancer nuclei (Fearon et al., 1987; Vogelstein et al., 1988). This in turn introduced an element of selection bias to the tumour series as certain classes of carcinoma, such as those eliciting a marked desmoplastic response or lymphocytic infiltration would have been excluded.

The common region of deletion identified in this study spanned an area between probes YNZ 22.1 (D17S5) and EW505 (D17S30) which covers 17p12 to 17p13.3 (Baker et al., 1989). This region covers some 33cM of the chromosome arm (sex averaged genetic distance between markers D17S5 and D17S67 [telomeric to D17S30] Wright et al., 1990). A similar study was carried out for osteosarcoma (Toguchida et al., 1989) on 37 tumours. The

region deleted in all cases spanned D17S1 (pHF12-1) to D17S5 (YNZ 22.1) covering approximately 15cM (sex averaged genetic distance, Nakamura et al., 1988).

Loss of 17p alleles is a frequent finding in a number of other common human cancers including breast (Mackay et al., 1988; Devilee et al., 1989), lung (Yokata et al., 1987; Weston et al., 1989), bladder (Tsai et al., 1990), glioma (James et al., 1988), astrocytoma (Fults et al., 1992) and ovary (Eccles et al., 1992). Only in the case of breast cancer has the deletion been mapped to a similar degree to colorectal cancer (Baker et al., 1989). Coles et al (1989) studied a series of 168 breast cancers using RFLP probes on 17p. In 26 of these cases there was less that total 17p arm loss which permitted analysis of the common region of deletion. Two independent, deleted regions on 17p were identified, one centred on MCT35.1 (D17S31) and another near YNZ22.1 (D17S5). Interestingly, both these loci could fall within the region identified in colorectal cancer (Baker et al., 1989) and osteosarcoma (Toguchida et al., 1989).

The gene for p53 is located on chromosome 17p13.1 placing it within the deleted region of colorectal cancer (Baker et al., 1989). The remaining p53 allele in cases with 17p deletion is frequently mutated in colorectal cancer (Baker et al., 1989; Cunningham et al., 1992; Kikuchi-Yanoshita et al., 1992) suggesting that p53 is indeed the target for such deletions in a fashion analogous to *RB1* inactivation in retinoblastoma (Cavanee et al., 1983). However, similar studies in breast and lung cancer have failed to demonstrate any such correlation of p53 mutation with 17p loss (Chiba et al., 1990; Varley et al., 1991; Thompson et al., 1992). A further study on 17p loss in breast cancer showed a strong association with the presence of

lymph node metastasis which was statistically significant at the YNZ22.1 locus but not at the p53 locus. This indicates the presence of two independent and clinically relevant loci on chromosome 17p in human cancer.

The common region of deletion on chromosome 17p so far defined in colorectal cancer is relatively large and encompasses 2 independent regions identified as targets for allele loss in breast cancer (Baker et al., 1989; Coles et al., 1989). The biology of p53 suggests that the mutant protein may inactivate the WT protein by complexing it into high molecular weight oligomers or altering its conformation during translation (Milner et al., 1991; Milner & Medcalf, 1991). Thus concomitant WT allele would appear redundant and without selective growth advantage under such circumstances.

To study the common region of deletion in an independent series of colorectal tumours, a consecutive series of 90 colorectal carcinomas and two large villous adenomas were studied for chromosome 17 allele loss using 5 primary structure polymorphisms (RFLPs). The cases did not undergo any pre-selection in terms of relative quantities of tumour and stroma to avoid bias. Three of the polymorphisms used were located close to, or within the p53 gene to examine this region in depth as a likely target for the deletion. To investigate the association between 17p allele loss and clinical outcome, cases with and without 17p deletions were correlated with established predictors of prognosis in colorectal cancer.

4.2 Results

4.2.1 Deletion mapping

Five RFLPs were used to investigate the loss of alleles on chromosome 17 (table 4.1). Three of them, YNZ22.1, MCT35.1 and THH59 are probes which detect RFLPs by hybridisation to restriction endonuclease digested, genomic DNA after Southern transfer (Southern, 1975). The remaining 2, p53 E4 and p53 I6, are detected by PCR amplification of sequence within the gene for p53 to include primary structure polymorphisms within exon 4 (p53 E4, Banks et al., 1986) and intron 6 (p53 I6, Chumakov, 1989) detected by restriction endonuclease cleavage (figure 4.1). Genomic DNA for Southern blotting was extracted from macroscopically dissected fresh tumour material whereas that for PCR amplification was obtained from microscopic dissection of formalin fixed, paraffin embedded tumour to enrich for neoplastic cells.

Loss of alleles from 17p13 was detected in 47-57% of all tumours analysed whereas only 20% had undergone loss of 17q alleles (table 4.1). The pattern of bands and the physical site of the RFLP are indicated (figure 4.1 a,b). In 44 of the cases there were data from more than one RFLP on 17p13 permitting more detailed analysis of the extent of the deleted region. Of the 44, 17 had retained all alleles at informative sites, whereas 27 (61%) had loss of heterozygosity (LOH) of at least one allele (table 4.2). In 11 cases there was less than total 17p arm loss permitting analysis of the region deleted. A summary of the 11 cases of sub-arm allele loss shows 3 basic patterns (figure 4.1 c). The first 4 cases show retention of heterozygosity telomeric to p53 but loss of alleles within the p53 gene itself. In the next 5,

there is LOH at the telomere with retention of alleles within p53 suggesting a breakpoint between p53 and MCT35.1 or YNZ 22.1 consistent with a lesion involving p53 or a more telomeric locus. The remaining 2 cases show loss of YNZ 22.1 but retention of MCT35.1; no information from within p53 being available. Both of these cases show loss of a chromosome 17q allele raising the possibility that the region spanning MCT35.1 may have become translocated or isolated with loss of the remainder of chromosome 17 including p53. It is perhaps more likely, however, that both copies of p53 have been retained in these cases. The 17q marker loss may well have occurred in the other chromosome 17 from that showing 17p deletion again consistent with p53 locus retention.

Table 4.1 Summary of restriction fragment length polymorphism analysis of chromosome 17. LOH - percentage of informative (heterozygous) cases showing allele loss. Number in parenthesis (n) shows the total number of informative cases.

Probe/poly-morphism	Site	Enzyme	LOH (n)	Reference
YNZ22.1	D17S5	Taq 1	55% (69)	Nakamura et al.,1987
MCT35.1	D17S31	Msp 1	57% (30)	Barker et al., 1987
p53 I 6	p53	Msp 1	56% (18)	Chumakov, 1989
p53 E 4	p53	BstU 1	47% (34)	Matlashewski et al., 1987
THH59	D17S4	Taq 1	20% (64)	Nakamura et al., 1987

Figure 4.1 (a) Examples of RFLP analysis. THH59, MCT35.1 and YNZ22.1 are autoradiographs of DNA blots whereas p53E4 and p53I6 are UV transilluminated, ethidium bromide stained agarose gels. Paired normal (N) and tumour (T) DNA samples are shown with examples of allele retention (left pair) and loss (right pair). **(b)** Idiogram of chromosome 17 with banding pattern showing the mapped positions of the probes used. **(c)** Summary of the pattern of allele loss in the 11 cases with less than total arm loss. Filled circles represent allele loss; open circles, uninformative; and hatched circles, allele retention.

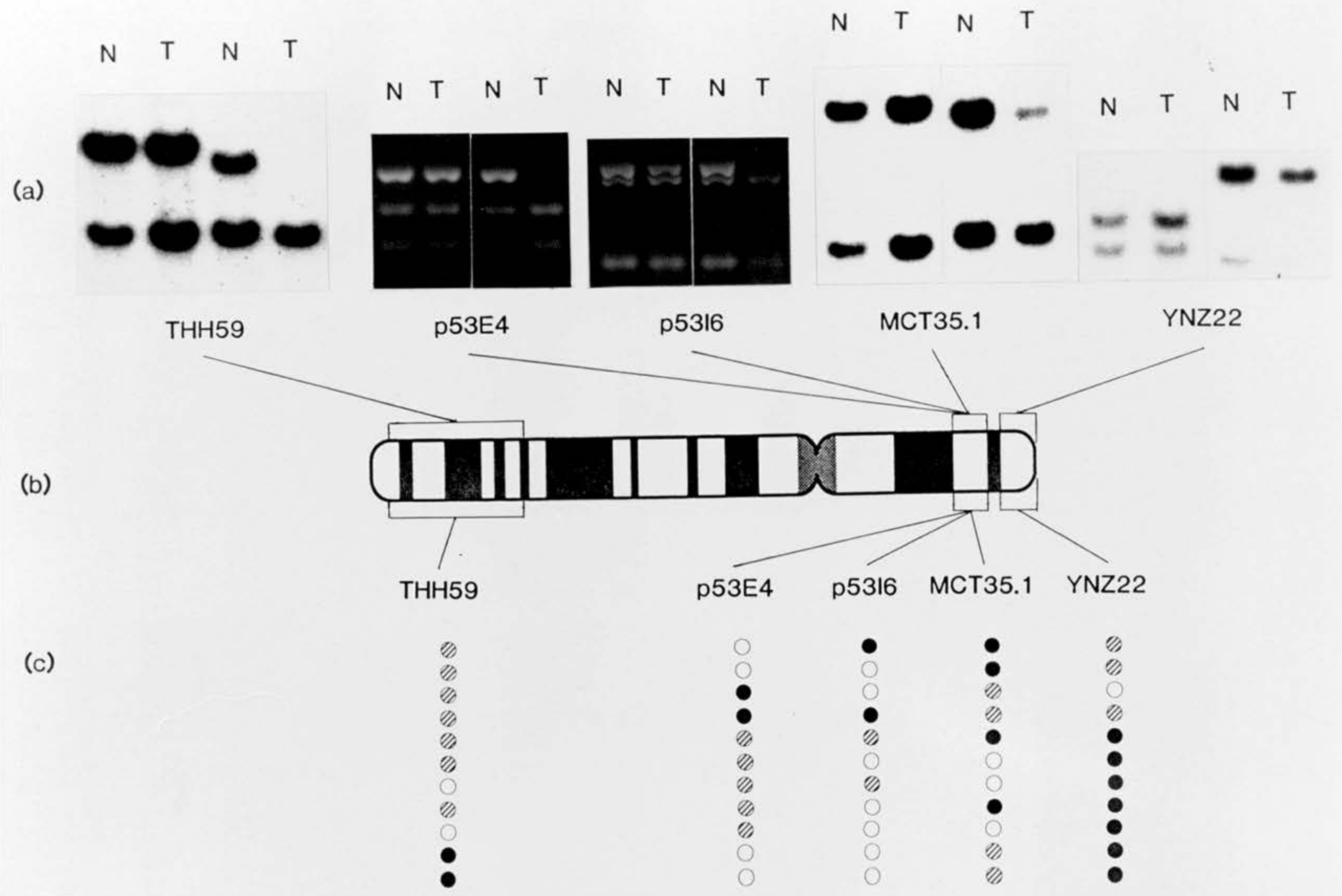

(a)
N T N T
N T N T
N T N T
N T N T
N T N T
THH59
p53E4
p53I6
MCT35.1
YNZ22
(b)
THH59
p53E4
p53I6
MCT35.1
YNZ22
(c)

Table 4.2 Analysis of allele loss on 17p in cases where information from more than one locus is available.

Allele pattern	Number of cases	Number of cases showing loss or retention in 17p
Arm Retention	17	17 (39%)
Arm Loss	16	27 (61%)
Sub-arm Loss	11	
Totals	44	44 (100%)

4.2.2 17p allele loss and p53 expression

Comparison of 17p allele loss with p53 expression detected immunohistochemically by PAb240 or PAb1801 (chapter 3) shows a highly significant correlation (figure 4.2). This correlation is maintained when the allele status is restricted to loci within or adjacent to the p53 gene (table 4.3). Loss of 17p alleles is also significantly associated with the presence of DNA aneuploidy but not with tumour stage, grade or site (table 4.4).

Figure 4.2 Bar chart showing the correlation of chromosome 17p allele loss (at any site) with p53 expression (immunohistochemical positivity with PAb240 or PAb1801). $p < 0.01$, chi-square test with Yates' correction for continuity.

Correlation of 17p allele loss with p53 immunohistochemistry

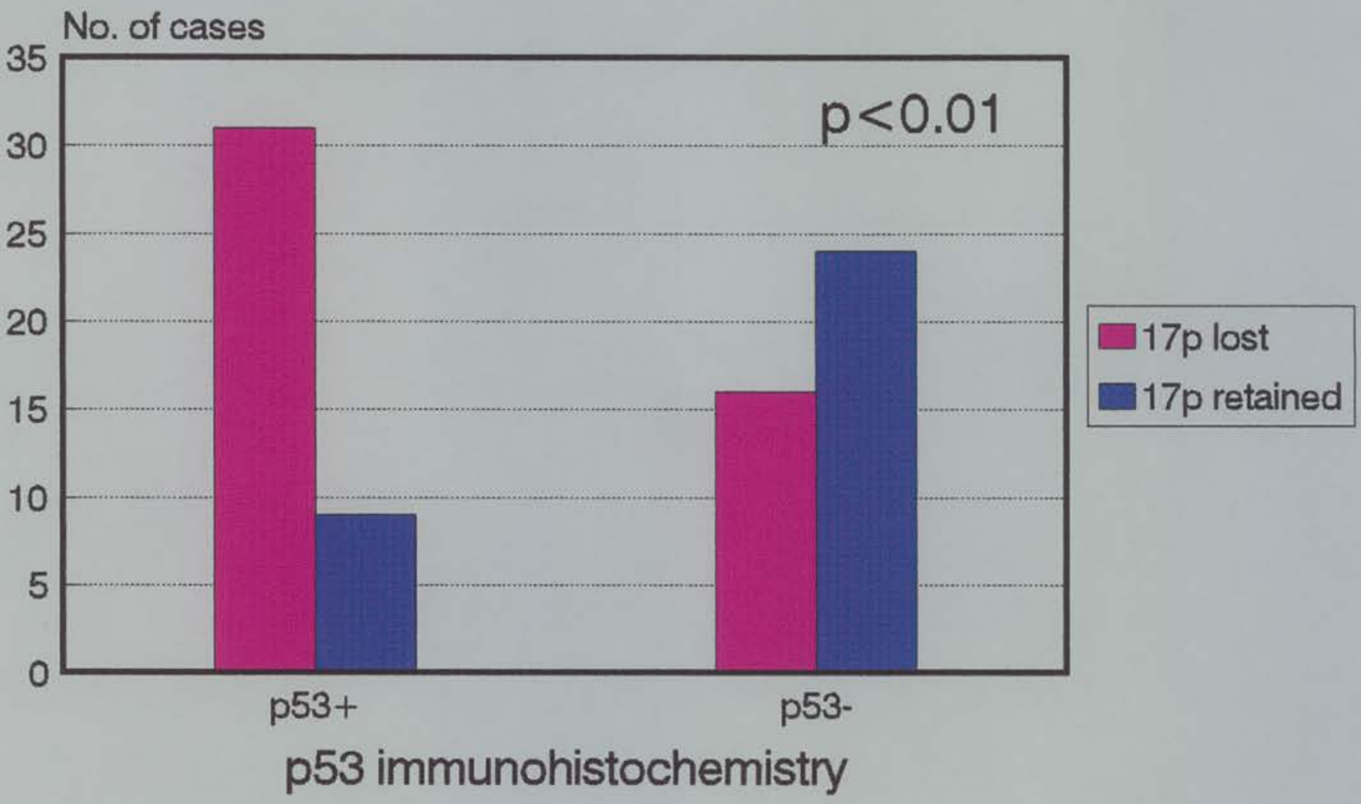


Table 4.3 Correlation of chromosome 17p allele loss at MCT35.1, p53E4 or p53I6 with p53 expression (immunohistochemical positivity with PAb240 or PAb1801).

Immunohistochemistry	17p loss	17p retention	Total
p53 +	19	9	28
p53 -	6	19	25
Totals	25	28	53

$p < 0.01$ chi-square test with Yates' correction for continuity.

Table 4.4 Correlation of chromosome 17p allele loss with tumour stage, histology, site and DNA ploidy.

Classification	17p loss	17p retention	p value*
Dukes A Dukes B Dukes C	4 20 22	3 13 17	 NS
Margin: Infiltrating : Expanding	38 8	29 4	 NS
Differentiation: Other : Poor	25 20	13 20	 NS
DNA Diploid DNA Aneuploid	7 40	13 20	 $p<0.05$
Distal colon Proximal colon	33 13	20 10	 NS
p53 + p53 -	31 16	9 24	 $p<0.01$

* Chi-square test with Yates' correction for continuity.

4.2.3 17q allele loss and markers of tumour aggression

Similar analysis with 17q allele losses revealed a significant correlation with the presence of lymph node metastasis (Dukes' stage C) compared to their absence (Dukes' B) but not with DNA ploidy or tumour histology (table 4.5).

Table 4.5 Correlation of chromosome 17q allele loss with tumour stage, histology, site and DNA ploidy.

Classification	17q loss	17q retention	p value*
Dukes A Dukes B Dukes C	2 2 8	3 29 19	 $p<0.05^*$ $p=0.034^\#$
Margin: Infiltrating : Expanding	9 3	45 6	 NS
Differentiation: Other : Poor	4 8	29 22	 NS
DNA Diploid DNA Aneuploid	3 10	13 37	 NS
Distal colon Proximal colon	11 2	32 16	 NS

* Chi-square test with Yates' correction for continuity.

Fischer's exact test.

4.3 Discussion

Allelic loss in colorectal carcinoma was detected in approximately 55% of cases in this series, which is in accord with previous studies (Vogelstein et al., 1988; Lothe et al., 1988; Monpezat et al., 1988; Okomato et al., 1988; Law et al., 1988; Vogelstein et al., 1989; Sasaki et al., 1989; Delattre et al., 1989). Loss of a 17q marker in 20% is slightly higher than the frequencies of 0-15% previously reported (Lothe et al., 1988; Law et al., 1988; Vogelstein et al., 1989). One particular group consistently detect higher rates of LOH at 17p (and other loci) which they suggest is due to their tissue fractionation technique to select and enrich for neoplastic cells (Vogelstein et al., 1988; 1989). They detect 17p LOH in at least 75% of colorectal cancers whereas only 55% were detected here. However, 17q loss is seen in 20% of this series but only in 15% in studies using tissue fractionation (Vogelstein et al., 1989) suggesting that the different frequency of allele loss in this series is a genuine finding and not simply due to normal tissue contamination masking allele loss.

In the majority of cases showing loss of one allele on 17p there is total arm loss (table 4.2) confirming earlier work (Baker et al., 1989). Eleven cases in this series show less than complete loss of 17p (figure 4.1 c). In 4 of them there is p53 gene loss with retention of more telomeric markers which reinforces the need to use more than one RFLP and not simply rely upon highly informative, sub-telomeric VNTR probes (Cunningham et al., 1992). The next 5 cases have lost alleles telomeric to p53 but retained alleles within p53. This pattern suggests a breakpoint between p53 and the telomere. This could conceivably still involve the p53 gene although gene rearrangements including truncation appear to be rare in colorectal cancer (Masuda et al.,

1987). A Southern blot of digested genomic DNA probed with genomic p53 could distinguish whether this is the case but this pattern suggests retention of both p53 alleles. The final 2 cases (figure 4.1 c) have lost YNZ22.1 but have retained MCT35.1 (a marker close to and telomeric to p53, Baker et al., 1989, Solomon & Ledbetter, 1991) suggesting a chromosome lesion retaining p53.

Therefore, in the majority of cases, the results are consistent with p53 or a gene in the same region as the target for 17p deletions. However, in 7 cases, the pattern is more suggestive that both copies of the p53 gene are retained and there is loss of distinct genetic material from chromosome 17p. One explanation is that there is another tumour suppressor locus which is the target for deletion in a small number of cases. There is evidence for a distinct locus in the case of breast cancer at such a site (Coles et al., 1991; Takita et al., 1992). Another explanation is that in these few cases (7/44, 16%) the loss of alleles is a more "random" event which is conceivable given that allele loss has been detected on every arm of every chromosome so far examined in colorectal cancer with a "background" frequency of 10-20% (Vogelstein et al., 1989).

The allele deletion mapping data, therefore, support the hypothesis that p53 is the tumour suppressor gene target for 17p loss in colorectal cancer. The overexpression of p53 reported previously (Van den Bergh et al., 1988; Purdie et al., 1991; Scott et al., 1991) is closely linked to 17p allele loss in this series (table 4.3). This correlation is maintained when the allele status of the RFLP markers closest to the p53 gene is used (table 4.3) in the analysis. Thus, the 2 events are closely linked, which is in agreement with data presented (chapter 3) for the timing of p53 over expression (at the adenoma

to carcinoma progression) and other work suggesting a similar timing for 17p loss (Vogelstein et al., 1988).

As discussed in chapter 3, overexpression of p53 detected immunohistochemically is most likely to represent mutation of the p53 gene resulting in a product with a greatly enhanced half-life (Jenkins et al., 1985). The concordance of 17p allele loss and p53 expression (and probable mutation) suggests a mechanism of tumour suppressor gene inactivation analogous to that seen in retinoblastoma (Cavanee et al., 1983). Gene sequencing to investigate the relationship of mutation to protein expression in this series is necessary to confirm this finding and is the subject of chapter 5.

Loss of chromosome 17p alleles in colorectal cancer can be shown to predict clinical outcome (Kern et al., 1989). Cases with 17p allele loss are statistically more likely to develop and also to die from metastasis. This trend is independent of Dukes' stage and gives independent prognostic information. No correlation, in this series, with the established prognostic indicators of Dukes' stage (Dukes, 1932) or histopathologic grade (Jass et al., 1986) could be detected but actual clinical follow-up data are required to investigate this fully. 17p allele loss does however correlate with the presence of DNA aneuploidy (table 4.4) as does p53 expression (and thus mutation). This further reinforces the hypothesis that the 17p allele loss is targeted upon p53 as p53 inactivation appears to permit the development of aneuploidy (Bischoff et al., 1991) possibly by permitting premature replication or replication in the presence of damaged DNA.

DNA aneuploidy is also an independent prognostic variable in human

colorectal cancer (Quirke et al., 1988) and the association with 17p loss suggests that 17p loss marks more aggressive tumours. This is consistent with the finding that patients with colorectal cancer showing 17p loss have a poorer prognosis. Clinical follow-up data are required to investigate this trend more fully.

The frequency of allele loss on the long arm of chromosome 17 is 20% which is higher than any previous report (Lothe et al., 1988; Law et al., 1988; Vogelstein et al., 1989). This is likely to represent targeted loss of a further tumour suppressor gene rather than merely "background" loss in view of the association with lymph node metastasis. There are several candidate genes which have been mapped to this region. The nucleoside diphosphate kinases *NM23-1* and *NM23-2* have been localised to chromosome 17q21.3 (Backer et al., 1993). These genes were originally isolated due to their expression in murine melanoma cell lines of low metastatic potential (Steeg et al., 1988). Subsequent studies demonstrated that for primary human breast carcinomas, low *NM23* expression correlated with regional lymph node metastases and decreased disease free survival (Bevilacqua et al., 1989; Henessy et al., 1991). Allelic deletions of *NM23* in colorectal carcinomas with no evidence of of metastasis at the time of resection correlate with the development of metastatic disease (Cohn et al., 1991). However, it is now clear that the *NM23* genes are expressed at elevated levels in colorectal tumours of both high and low metastatic potential and are not mutated (Myerhoff & Markowitz, 1993; Haut et al., 1993). Thus, *NM23* does not appear to be the target for 17q deletions. This study and others (Cohn et al., 1991) do however indicate the presence of a metastasis suppressor gene in the region. Early onset familial breast cancer and familial breast-ovarian cancer are linked to a locus at 17q12-23 (Hall et al., 1990;

Narod et al., 1991). Recently, mutations in the prohibitin gene (which also maps to 17q21) have been identified in sporadic breast cancer suggesting that it may be a tumour suppressor gene involved in breast carcinogenesis (Sato et al., 1992). Further candidate genes on chromosome 17q include the truncated epidermal growth factor receptor (*ERB B2*) oestradiol-17ß dehydrogenase (*EDHB 17*), homeobox 2 (*HOX 2*), retinoic acid receptor a (*RARA*) and *WNT 3* (Solomon & Ledbetter, 1991). Careful mapping of the deletions of 17q in colorectal cancers and analysis of candidate genes for mutations or altered expression are necessary to identify this tumour metastasis suppressor gene.

4.4 Summary

The data presented here clearly show that 17p loss in colorectal cancer involves the gene for p53 (in the majority of cases) and is closely associated with p53 expression (representing mutation). This suggests that inactivation of both copies of p53 is an important step in colorectal carcinogenesis. This hypothesis is further strengthened by the demonstration that 17p loss correlates with DNA aneuploidy (as does p53 expression) consistent with p53 inactivation cuasing genomic instability by permitting inaccurate or premature DNA replication.

DNA aneuploidy is a marker of poor prognosis in colorectal cancer suggesting that 17p loss in this series may also mark more aggressive tumours as has been described previously (Kern et al., 1989). The novel finding that 17q loss is associated with the presence of lymph node metastasis may indicate a tumour metastasis suppressor gene locus and further deletion mapping and close study of candidate genes is necessary to identify the target for such losses.

Chapter 5

p53 Sequence Analysis

5.1 Introduction

Mutations of the p53 gene have been described in the majority of human cancers (reviewed in section 2.6). Germ-line mutation of one copy of p53 described in Li-Fraumeni syndrome, predisposes to the early onset of multiple primary malignancies which show loss of the WT gene copy (Malkin et al., 1990; Srivastava et al., 1990; 1992). Thus, it has been proposed that mutation of one copy of the p53 gene is often followed by deletion (or inactivation by second mutation) of the remaining wild-type copy in a fashion analogous to that seen in retinoblastoma (Cavanee et al., 1983). Such a pattern of inactivation of both p53 gene copies has indeed been described in colorectal cancer (Nigro et al., 1989; Baker et al., 1990; Cunningham et al., 1992), astrocytoma (Fults et al., 1992) and bladder cancer (Sidransky et al., 1991). In all these studies, cases with 17p allele loss but without p53 mutation and p53 mutation without allele loss have been identified. In the case of lung cancer (Chiba et al., 1990) the concordance of 17p allele loss and p53 mutation is less clear cut and for breast cancer there does not appear to be any such correlation (Varley et al., 1991; Thompson et al., 1992).

Overexpression of p53 detected immunohistochemically appears to correlate well with p53 gene mutations in most studies (Bartek et al., 1990; Iggo et al., 1990; Rodrigues et al., 1990; Cunningham et al.,1992; Kikuchi-Yanoshita et al., 1992) employing a variety of antibodies. However, in some studies the concordance is not as close (Borresen et al., 1991; Thompson et al., 1992)

In these cases, breast cancer was under investigation, which has marked heterogeneity of immunostaining making sampling bias a source of error (Varley et al., 1991; Thompson et al., 1992).

Over expression of p53 is closely associated with the transition from adenoma to carcinoma, the appearrance of DNA aneuploidy, clonal divergence and 17p allele loss in colorectal carcinogenesis and appears to have a key role in this process (chapters 3 and 4). To try to identify the mechanisms underlying this over expression, 12 cases were selected for p53 gene sequence analysis. Of the 12, 6 exhibited loss of 17p alleles defined by YNZ22.1 and MCT35.1 and 6 did not. In each sub-set of 6, 3 were immunohistochemically positive with PAb1801 and 3 negative.

5.2 Results

Exons 5-9 were amplified by the polymerase chain reaction (PCR) from genomic p53 extracted from tumour and corresponding normal tissue. Amplified DNA was ligated into a plasmid vector and a preparation of greater than 100 clones sequenced by the dideoxy chain termination method. Base-pair substitutions were detected on sequencing gels in 7 of the 12 cases studied (figures 5.1, 5.2). Six of the mutations were located within conserved regions III-V (Soussi et al., 1987) (table 5.1) of the p53 gene. The concordance between PAb240 and PAb1801 immunostaining was, as expected, very good with only one discrepancy. Case number 89-143 contains a mutation, Arg $\rightarrow$ STOP at codon 213 and is PAb240 positive but PAb1801 negative.

The correlation between the presence or absence of a mutation and the immunostaining was absolute in this small series ($p=0.013$) (table 5.2). In every case, p53 gene analysis from the patient's normal DNA gave wild-type sequence, thus excluding germ-line mutations or PCR artefact as a source of the base-pair substitutions detected in the cancers.

Comparison of p53 gene status and 17p allele status (at the RFLP closest to p53) revealed a tendency for cancers with mutated p53 to have lost the remaining WT gene copy and for heterozygous cancers to have only WT p53 (table 5.3). This pattern does not reach statistical significance but shows the same bias as tables 4.3 and 4.4.

Figure 5.1 Sequence from case 89-143. Sequence from the patient's constitutive DNA (N) and from the tumour DNA (T).showing a CGA->TGA mutation at codon 213. This tumour has lost the remaining WT gene copy; but a faint WT sequence is still present from small quantities of normal cells present within the tumour.

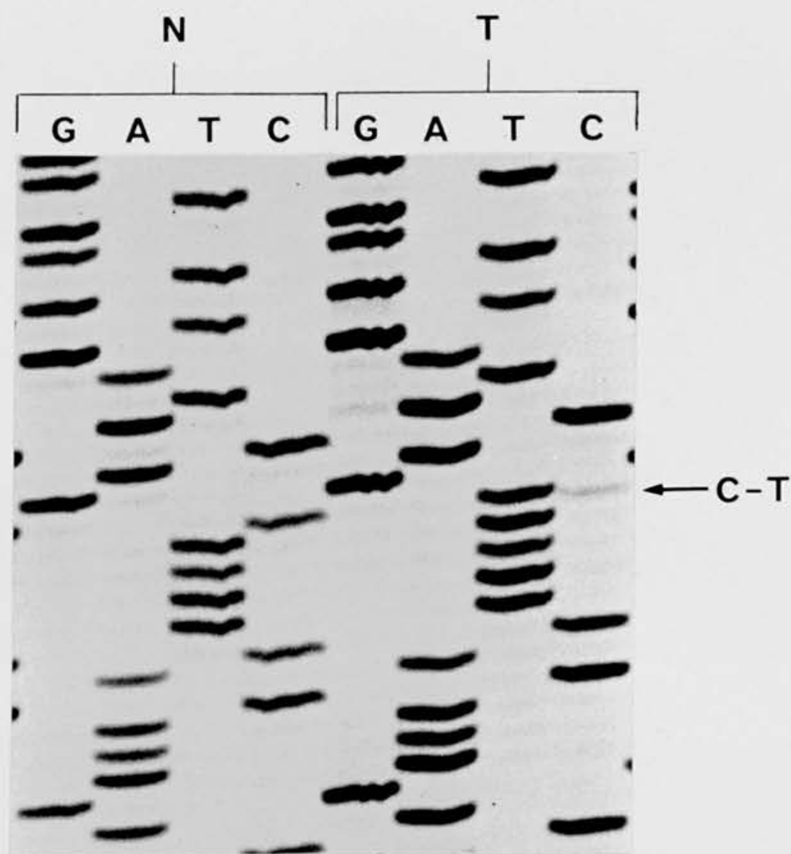

N
T
G
A
T
C
G
A
T
C
C-T

Figure 5.2 Sequence from case 88-878. Sequence from the patient's costitutive DNA (N) and from the tumour DNA (T) showing a CGG->CAG mutation at codon 248.

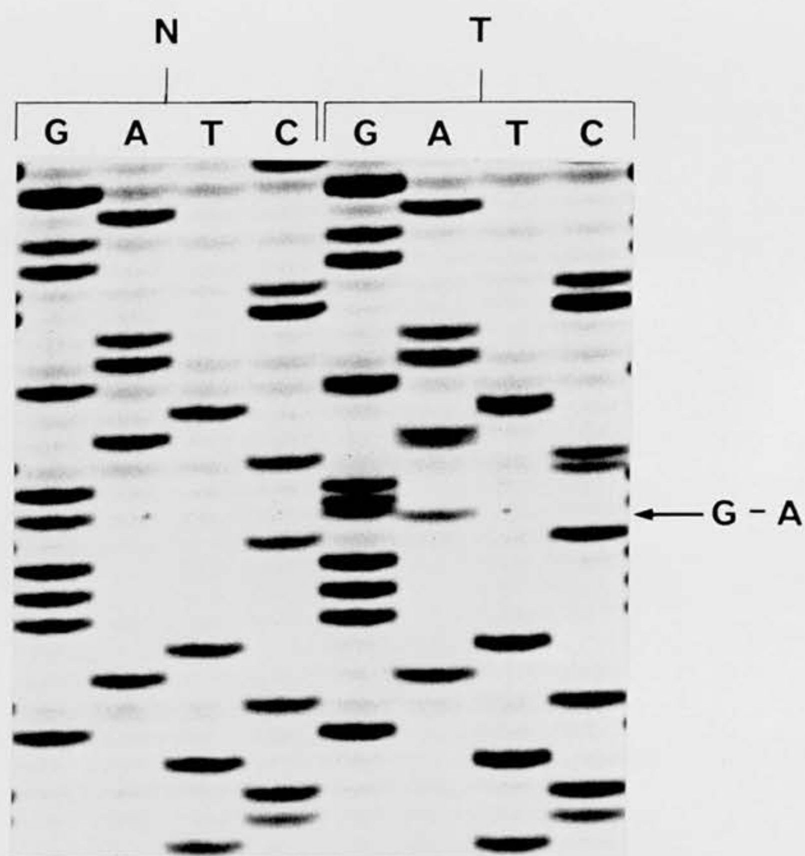

N
T
G
A
T
C
G
A
T
C
G - A

Table 5.1 Summary of results of sequence, 17p deletion and p53 immunohistochemical analysis. Abbreviations: + immunohistochemically positive; - immunohistochemically negative; R allele retained; L allele lost; / non-informative; NA result not available.

Tumour	Exon	Codon	Mutation	Amino acid	PAb1801	PAb240	p53E4	p53I6	MCT35	YNZ22	Region
88-637			WT		-	-	/	L	L	R	
88-696			WT		-	-	R	/	R	R	
88-742	8	278	CCT->TCT	Pro->Ser	+	+	L	L	R	R	V
88-805			WT		-	-	L	/	L	L	
88-878	7	248	CGG->CAG	Arg->Gln	+	+	R	R	/	R	IV
89-022	7	248	CGG->TGG	Arg->Trp	+	+	/	/	L	L	IV
89-037	7	248	CGG->TGG	Arg->Trp	+	+	/	/	R	R	IV
89-143	6	213	CGA->TGA	Arg->STOP	-	+	/	/	L	L	-
89-167	5	180	GAG->TAG	Glu->STOP	+	NA	/	/	L	L	III
89-180	7	245	GGC->AGC	Gly->Ser	+	+	L	L	/	L	IV
89-230			WT		-	-	/	/	R	R	
89-280			WT		-	-	/	NA	R	R	

Table 5.2 Correlation of p53 immunohistochemistry (with PAb 240 and/or PAb1801) with mutation defined by sequencing

Immunohistochemistry	Mutant p53	Wild-type p53	Totals
P53 Positive	7	0	7
p53 Negative	0	5	5
Totals	7	5	12

p = 0.0013 (Fischer's Exact Test)

Figure 5.3 Correlation between p53 mutation defined by sequencing and 17p loss defined at the closest informative locus to p53

17p Status	Mutant p53	Wild Type p53	Totals
LOH	5	2	7
HET	2	3	5
Totals	7	5	12

$p > 0.05$ Not significant.

5.3 Discussion

The mutations detected in this series of colorectal cancers raise a number of issues and will be discussed in terms of their relationship to those described in other studies, p53 immunohistochemistry and 17p allele loss.

5.3.1 p53 mutation

Of the 12 cases studied, 7 had single base-pair substitutions within the coding region of the p53 gene resulting in amino acid substitutions or STOP codons in two cases. Six of the mutations are within conserved regions III-V of p53 (Soussi et al., 1987) and within the 2 SV40 large T antigen binding sites (Jenkins et al., 1988) in agreement with previous studies on colorectal cancer (Baker et al., 1990; Rodrigues et al., 1990; Cunningham et al., 1992) and other common human cancers (Iggo et al., 1990; Chiba et al., 1990; Bartek et al., 1990; Thompson et al., 1992).

The mutations detected here show a similar distribution to those described previously in colorectal cancer (Baker et al., 1990; Rodrigues et al., 1990; Cunningham et al., 1992; Kikuchi-Yanoshita et al., 1992) with G-A and C-T transitions predominating and a hot-spot at codon 248. Deamination of methyl cytosine may be responsible for the C-T transitions (Rideout et al., 1990) and mutagens such as MNNG and NMS are known to cause GC to AT transitions (Miyaki et al., 1983) but their relevance to human colorectal carcinogenesis is unclear. Now that the specific mutations found in human colorectal cancer have been identified, the search for the environmental factors specifically responsible for them can begin (section 1.2).

5.3.2 p53 mutation and immunohistochemistry

In this small series the concordance between the presence of a mutation on p53 gene sequence analysis and immunostaining with PAb240 and PAb1801 is absolute. This confirms the property that PAb240 binds to a conformationally altered p53 brought about by mutations at many sites (Gannon et al., 1990). PAb1801 will bind both WT and mutant p53 (Banks et al., 1986) but the marked positive correlation of PAb1801 immunohistochemistry with PAb240 immunostaining and with the presence of gene mutation is highly suggestive that it is detecting mutated and stabilised p53.

In some studies, discrepancies between p53 overexpression and p53 gene mutation have been encountered (Thompson et al., 1992). This may be due to simple sampling error as already discussed (section 5.1) or to the nature of mutation encountered in certain tissues. In cell lines from small cell and non-small cell lung cancers, mis-sense mutations in exons 5-8 of p53 result in high expression of immunohistochemically detected protein whereas deletions, splicing mutants and mis-sense mutations outside exons 5-8 result in low or absent expression (Bodner et al., 1992). As more than 90% of mutations described in colorectal cancer are base-pair substitutions within exons 5-8 of p53 (Baker et al., 1989;1990; Rodrigues et al., 1990; Cunningham et al., 1992; Kikuchi-Yanoshita et al., 1992) immunohistochemistry should detect the majority of mutations as appears to be the case in this study and others (Rodrigues et al., 1990; Cunningham et al., 1992; Kikuchi-Yanoshita et al., 1992). In other tissues, such as lung cancer, where mutations other than base pair substitutions in exons 5-8 are more common, immunohistochemical detection of p53 may be a less reliable

marker of gene mutation.

Case number 89-143 shows a discrepancy between immunostaining with the two antibodies, being PAb240 positive but PAb1801 negative. This particular mutation, Arg to STOP at codon 213 has been described in two other tumours which were strongly positive for PAb240 but showed weak or negative staining with PAb1801 (Cunningham et al 1992; Bodner et al., 1992). As the epitope for PAb1801, amino acids 35-79 (Banks et al., 1986) is unaltered, this suggests that truncation has resulted in an altered protein conformation which masks this epitope. The epitope for PAb240 has recently been mapped to an area centred on codons 213-217 (Stephen & Lane, 1992). The finding of strongly positve PAb240 immunostaining in cases with STOP codons at position 213 in this study and others (Cunningham et al 1992; Bodner et al., 1992) suggests that the epitope is nearer the amino terminal than codon 213. Discrepancies between antibodies are infrequent but do occur and reinforce the need to use more than one antibody in analysis.

5.3.3 p53 mutation and 17p loss

In 71% of the cases with p53 mutation described here, the remaining WT allele is lost. However, in some cases, one allele is inactivated by mutation or deletion whereas an apparently normal WT allele remains. At least 3 possible explanations exist for this: the second event is yet to occur in the tumour progression; there is a selection advantage in reduction to half gene dosage; or the mutant gene product is inactivating the WT p53 by a dominant negative action. Indeed, some mutations can produce immortalisation and cooperate with *ras* in transformation assays even in the

presence of 2 WT alleles (Eliyahu et al., 1984). However, not all mutations have identical biological properties and some may therefore require WT allele loss to express the full p53 inactivation phenotype whereas others may not (Halevy et al., 1990).

Two of the mutants in this series (89-143 and 89-167) produce STOP codons at amino acids 213 and 180 respectively which would presumably result in a truncated protein product. Both these cases have lost the WT gene copy. The formation of heteroligomers between mutant and WT p53 requires amino acids 345-366 (Milner et al., 1991) and the ability of certain mutant proteins to force a co-translated WT protein into a mutant conformation also depends upon carboxy terminal amino acids (Milner & Medcalf, 1991) absent in these cases. Expression vectors encoding a truncated p53 are unable to immortalise cells containing endogenous WT p53 whereas full length vectors can (Jenkins et al., 1985) suggesting that the carboxy terminal amino acids are required for a dominant negative action. This could explain why there is selective pressure for WT allele loss in cases 89-143 and 89-167 as these truncating mutants would be unable to exert a dominant negative effect by oligomerising or changing the conformation of the WT protein. There may still be a phenotype associated with half gene dosage which would be a factor in these cases even in the presence of the WT p53.

Mutants which have been described in the Li-Fraumeni syndrome (Malkin et al., 1991; Srivastava et al., 1991) are also deficient in their ability to alter the conformation of co-translated WT protein to that of mutant protein (Milner & Medcalf, 1991). Two cases here have mutations described in Li-Fraumeni patients (Malkin et al., 1991): Arg-Trp at codon 248. One case, 89-022, as

might be predicted, has lost the WT allele but the other, 89-037, has not. Case 89-037 is a relatively early Dukes' B stage carcinoma and it may be that WT allele loss has yet to occur in its progression. Retention of both alleles when one has this mutation has been described previously in colorectal cancer (Kikuchi-Yanoshita et al., 1992).

Two cases 88-632 and 88-805 exhibit LOH without p53 overexpression or mutation. In both cases there is definite loss of 17p alleles including the p53 gene locus itself (table 5.1). A possible explanation for this is that mutations are present but are outwith the region sequenced. This appears to be rather rare, and none has been described outwith exons 5-9 in colorectal cancer (Baker et al., 1989; 1990; Rodrigues et al., 1990; Cunningham et al., 1992; Kikuchi-Yanoshita et al., 1992) and in addition, both cases are negative for PAb240 and PAb1801 immunostaining. Such cases are infrequent but make up approximately 6-13% in other series (Baker et al., 1990; Cunningham et al., 1992). A possible explanation is that half gene dosage caused by the allele loss is sufficient to produce a selective growth advantage or that there is a mutation in regulatory sequences outwith the gene itself resulting in low levels of expression.

The correlation between 17p allele loss and p53 expression (tables 4.3 and 4.4) almost certainly represents a correlation between mutation of one p53 allele and loss of the remaining WT allele. This is in agreement with other studies (Baker et al., 1989; 1990; Cunningham et al.,1992; Kikuchi-Yanoshita et al., 1992) suggesting that inactivation of p53 in a two step process is very common in colorectal carcinogenesis. In a minority of cases there is mutation or overexpression of p53 in the absence of detectable allele loss. This could be due to mutation of both alleles (Baker et al., 1990);

certain mutations exerting a dominant negative effect over the WT allele rendering the second (WT deletion) event unnecessary; the second event being yet to happen in the progression of the particular tumour studied; or reduction of gene dosage by half producing a selection advantage. Some cases show loss of 17p alleles without evidence of mutation or overexpression perhaps suggesting an effect of half gene dosage or p53 gene regulatory sequence disruption resulting in low or absent expression.

Disruption of p53 detected by over expression, allele loss or mutation was detected in more than 70% of cancers in this series making it the commonest genetic defect in colorectal cancer.

5.4 Summary

The data presented in chapters 3-5 of this thesis provide information on the role of p53 in colorectal carcinogenesis. Deletions of chromosome 17p usually involve the p53 gene locus and are strongly associated with p53 expression which is due in turn to gene mutation. Thus, both copies of p53 are commonly inactivated in human colorectal cancer.

Particular mutations produce p53 molecules with differing properties and it may be that subsequent inactivation of the WT allele will give no further growth advantage or else the phenotype may not be expressed fully until the WT p53 has been removed. This may explain some of the cases with a mutation which retain both alleles.

Over expression of p53 (representing gene mutation) correlates significantly with malignant rather than benign colorectal neoplasia, the presence of DNA aneuploidy and, in particular, clonal divergence. Thus alterations in p53 occur at the time of adenoma-carcinoma transition and may indeed be responsible for this progression. A possible molecular mechanism for this has recently been suggested (Lane, 1992). Abnormal, inactive p53 has the effect of reducing transcription of genes which cause G1 arrest following DNA damage. Hence, DNA repair may not occur prior to replication and so allowing premature replication and propogation of sequence errors. This in turn would predispose to more rapid proliferation and the appearance of aneuploidy and multiple divergent clones some with the properties of tissue invasion and the ability to metastasize. Thus p53 has a central role to play in human colorectal carcinogenesis.

The correlation of 17p loss and p53 expression with DNA aneuploidy also suggests that detection of these abnormalities may provide useful prognostic information. In the future this may permit a more rational approach to therapy. Identification of the aetiology of the spectrum of mutations identified in colorectal cancer may also permit appropriate preventive measures to be taken, hopefully reducing the incidence of this common human cancer.

Chapter 6

Gene Targeting

6.1 Introduction

Gene targeting is a process by which modifications are introduced into a region of interest in chromosomal DNA by means of recombination with a specifically engineered, homologous, exogenous DNA sequence. This phenomenon depends upon the cells' intrinsic ability to mediate recombination events between homologous DNA sequences. To introduce the subject of gene targeting, the mechanisms of homologous recombination and the features of both extrachromosomal and chromosomal recombination in mammalian cells will be discussed.

6.1.1 Homologous recombination

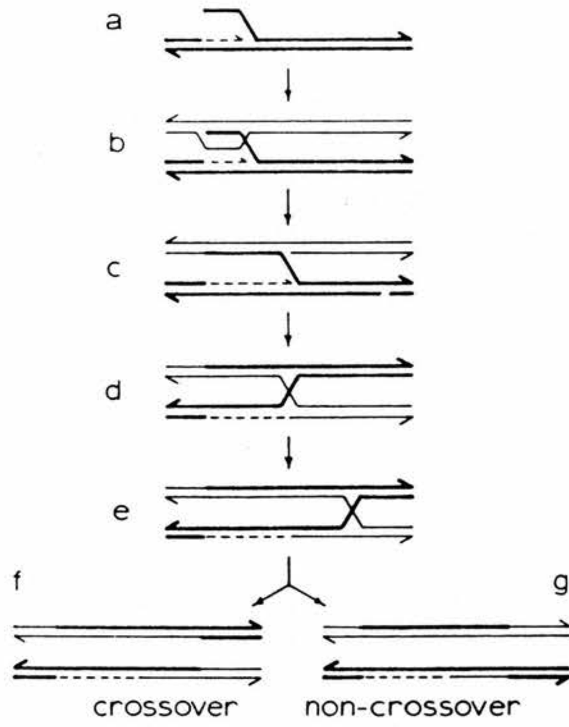
Homologous recombination was initially described in bacteria (Smith, 1989) and yeast (Orr-Weaver et al., 1981) and subsequently demonstrated between 2 non-replicating exogenous plasmid sequences in mammalian, somatic cells (Folger et al., 1982). The precise mechanism of homologous recombination remains obscure but it involves the exchange of genetic information between homologous DNA sequences and can occur during meiosis, mitotic cell growth and DNA repair. Two general models have been proposed to explain homologous recombination in yeast and mammalian cells. The first is based on the Holliday model (Holliday et al., 1964) modified by Meselson and Radding (1975) to explain the observation that heteroduplex DNA usually forms on one chromatid. In this model (figure 6.1a), the 3' end of a single strand nick invades its homologue displacing a

loop and acting as a primer for DNA synthesis. Ligation produces a Holliday junction which can migrate and finally resolve by cutting the crossed strands with or without isomerisation around the crossover.

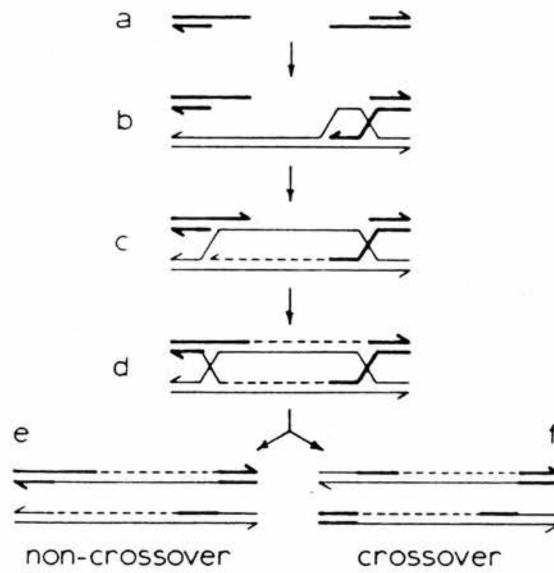
The alternative model was postulated to account for the finding that homologous recombination can be greatly enhanced by the introduction of a double strand break into the DNA and missing sequences can be restored using information from the chromosome (Orr-Weaver et al., 1981). In this model (figure 6.1b), recombination is initiated by a double strand break. One 3' end invades the homologous duplex producing a loop which is enlarged by repair until it reaches and anneals to the remaining strand of the exogenous DNA. Repair of this strand results in two Holliday junctions which can migrate and resolve by crossover cutting.

Figure 6.1 Models of homologous recombination. **(A)** The Meselson-Radding model. Recombination is initiated (a) by a single-strand nick on one of the two interacting duplexes. The 3' end of the nicked strand acts as a primer for DNA synthesis which displaces the strand ahead of it. The displaced single strand invades the other duplex at a homologous site, displacing a D loop (b). The single stranded D loop is degraded, and the invading strand is ligated in place. The region of heteroduplex DNA is expanded (c) by DNA synthesis on the first duplex and by degradation of the second duplex. After the production of the asymmetric heteroduplex DNA stops, either branch migration or isomerization can bring the 5' and 3' single-stranded ends together permitting ligation. The resulting Holliday junction (d) can move along the duplex generating symmetric heteroduplex DNA (e). Resolution can yield either the crossover (f) or the non-crossover (g) configuration. **(B)** The double strand break repair model. A double strand cut is made in one duplex (a), and a gap flanked by 3' single strands is formed by exonuclease action. One 3' end invades a homologous duplex, displacing a D loop (b) which is enlarged by repair synthesis until the other 3' end can anneal to complementary single stranded sequences (c). Repair synthesis from the second 3' end completes the process of gap repair, and branch migration results in 2 Holliday junctions. Resolution of the 2 junctions leads to 4 possible configurations, 2 of which are illustrated. Modified from Szostak et al., 1983.

(A)



(B)



6.1.2 Extrachromosomal homologous recombination in mammalian cells

Although the mechanism of homologous recombination is still not clear, a great deal of work has been carried out on how to increase the efficiency of this process. Much of the early work involved the introduction into mammalian cells of a potential but crippled selectable marker, reconstitution of which required a homologous recombination event. Thus, two copies of the neomycin phosphotransferase gene (*neo*) which confers resistance to G418, containing seperate, non-overlapping, disabling mutations were introduced into mammalian cells by nuclear injection or calcium-phosphate transfection. The survival of cells depended upon homologous recombination to produce a functional *neo* conferring survival in selectable (G418) medium (Kucherlapati et al., 1984; Folger et al., 1985a & b). Such experiments demonstrated that the frequency of recombination is roughly proportional to the length of homology and although detectable homologous recombination is seen with only 25bp of homology (Ayares et al., 1986) this is 2 orders of magnitude less than that seen with 500bp. Linearised DNA is much more efficient than supercoiled DNA (Folger et al., 1982) and homologous recombination is further enhanced by cleavage of the exogenous DNA within the region of homology (Kucherlapati et al., 1984). Homologous recombination is a relatively rapid process, taking place within 1 hour of coinjection of DNA (Folger et al., 1985a & b).

Timing of the introduction of the exogenous DNA relative to the phase of the cell cycle also appears to be important, with homologous recombination peaking during early to mid S-phase (Wong & Capecchi, 1987). The relatively high frequency of homologous recombination events described in

these experiments is not simply due to high concentrations of injected DNA as the process is equally efficient when as few as 5 molecules of each crippled gene are injected at different nuclear sites (Folger et al., 1985a & b). Thus, the kinetics of homologous sequences coming together does not seem to be rate-limiting.

These data demonstrated the feasibility of encouraging homologous recombination between 2 exogenous DNA sequences and paved the way for experiments to target into sequences of cellular DNA.

6.1.3 Chromosomal homologous recombination in mammalian cells

Expression of herpes simplex virus thymidine kinase (HSV*tk*) confers the ability to proliferate in HAT medium and *neo* expression renders cells resistant to G418. Thomas et al (1986) demonstrated that cells with stably integrated, but mutationally inactivated, HSV*tk* or *neo* could be made resistant to HAT medium or G418 respectively by the nuclear injection of another copy of the gene carrying a different mutation. Only homologous recombination between the two sequences could produce a functional gene capable of permitting growth in selection media. This process occurred at high frequency (1 per 10^3 cells receiving DNA) and was again independent of the number of molecules injected or the site of the integrated sequence. Thus, finding the relevant sequences in 3×10^9 bp of cellular DNA does not appear to be a rate limiting step and the site of the original integration does not influence the frequency of homologous recombination (although there may be bias for the original site of integration) suggesting that most of the genome is accessible to the recombination machinery.

The frequency of integrations by non-homologous (illegitimate) recombination exceed by a factor of at least 100 homologous recombination events (Thomas et al., 1986). Therefore, in the absence of a selectable phenotype, specifically targeted homologous recombination events can be difficult to detect. Smithies et al (1985) were the first to demonstrate the insertion of DNA sequences into an endogenous human gene (ß globin) by homologous recombination. The targeting was detected by an elegant screening procedure which involved preparation of DNA from pooled cells, digestion and packaging into a mutant vector phage which would only grow if part of the targeting vector was present in the ligated DNA, and screening by colony hybridisation with appropriate probes from human ß globin outwith the sequences used in the vector. The frequency of the homologous recombination event was between 1 in 300 and 1 in 1100 G418 resistant colonies.

6.1.4 Gene targeting

Pluripotent stem cells isolated from 3½ day mouse embryos can be grown indefinitely in culture (Evans and Kaufman, 1981). These embryonal stem (ES) cells have a normal karyotype, will form teratocarcinomas if injected subcutaneously in syngeneic mice and form germline chimæras if injected into pre-implantation blastocysts which are then returned to pseudo-pregnant female mice (Bradley et al., 1984). More importantly, ES cells can be manipulated *in vitro*, for example by transfection and integration of a *neo* gene and selection in G418, and thereafter will still produce germline chimæras (Robertson et al., 1986; Gossler et al., 1986). To produce an animal lacking a functional gene, a single copy is disrupted in mouse ES cells and these cells injected into a 3½ day blastocyst. The blastocyst is re-

implanted into a pseudo-pregnant female and the resultant chimæras will produce offspring carrying one disrupted gene copy in the constitutive DNA (ie, heterozygous) if the cultured ES cells have successfully colonised the chimæric germ cells. Mating of 2 heterozygotes will result in a conceptus homozygous for the gene disruption and hence lacking any functional copy of the targeted gene.

The gene for hypoxanthine phosphoribosyl transferase (*hprt*) was used as a model system for manipulation of ES cells as it has two useful properties. First it is located on the X chromosome thus is represented in only one copy in male XY ES cells. Second, it is possible to select in culture for its presence in HAT medium or absence in 6-thioguanine (6-TG). ES cells cultured in 6-TG produced spontaneous *hprt-* mutants which produced germ-line chimæras (Hooper et al., 1987). Further experiments on targeting *hprt* in ES cells defined many of the parameters for subsequent targeting strategies in other genes (Thomas and Cappechi, 1987). The targeting vectors fell into 2 categories: "insertion" where the entire exogenous sequence inserts into the endogenous DNA (figure 6.2a); and "replacement" where the homologous endogenous sequence is replaced by the targeting vector (figure 6.2b). Both classes of vector contained the *neo* gene and were designed to recombine near the 8th exon of *hprt* thus disabling it. The number of colonies which were G418 resistant gave the frequency of non-homologous *and* homologous recombination whereas the subset which was also 6-TG resistant gave the frequency of homologous recombination. Both types of vector targeted at roughly the same frequency: 1 in 1000 for the most efficient vectors which were those with the largest regions of homology. Only single copies of the vector were found in each clone of ES cells.

In non-selectable genes, the targeted clones can only be detected by screening many colonies usually by Southern blotting or by the polymerase chain reaction (PCR). This is very labour intensive and two modification to vectors can enrich for homologous recombination over non-homologous recombination. The first modification, only applicable for genes expressed in ES cells, involves cloning the *neo* gene in-frame with the gene sequences in the targeting vector. Following introduction of such a vector, only homologous recombination will juxtapose the targeting vector to the appropriate promoter or enhancer sequences resulting in expression of the fusion protein conferring G418 resistance. This technique can give several hundred-fold enrichment (te Riele et al., 1990). The second strategy is to place one or two counterselectable markers outlying the region of homology in addition to the selectable marker within the homology (fig 6.3). Homologous recombination will result in loss of the conterselectable markers whereas non-homologous recombination would permit retention of one or both and these cells would be selected against. Enrichment of 2000 fold has been demonstrated (Mansour et al., 1988) although other groups have found enrichment of only 2 fold (Mombaerts et al., 1991).

The insertion vector strategy has also been modified, by the addition of a counterselectable marker (usually HSV*tk*), to permit removal of the inserted selection genes and duplicated DNA. Clones are obtained which have the correct insertion event (fig 6.4) and this is confirmed by Southern blotting. The targeted clones are then back-selected in ganciclovir for loss of the HSV*tk* gene. This can occur by homologous recombination between the duplicated regions of the target gene (fig 6.4). The structure will resolve leaving either a reconstituted wild-type gene or one containing exogenous DNA (Hasty et al., 1991; Valancius & Smithies, 1991). Therefore, the target

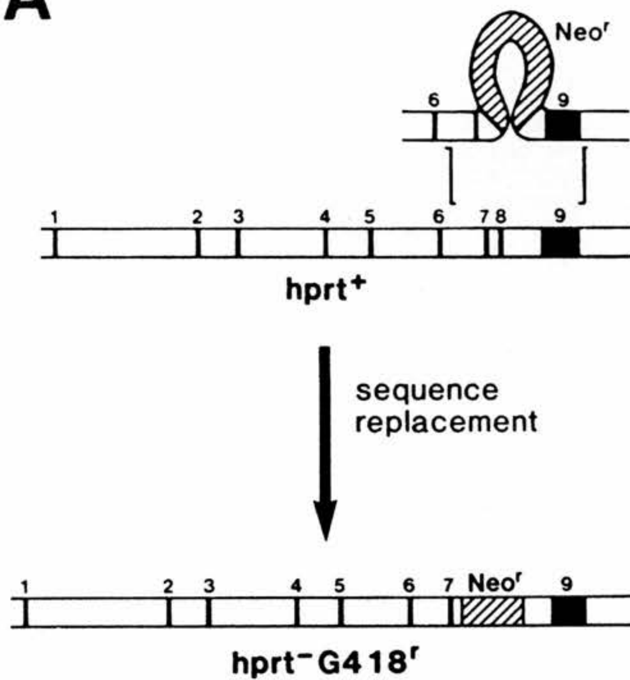
gene (in roughly 50% of cases) has the desired alteration and lacks any exogenous sequence (*neo*, *tk*, enhancer or promoter) which might deregulate neighbouring genes and render the phenotype difficult to interpret. Concerns that gene targeting might not be feasible for genes not transcribed in ES cells are unfounded; work to date suggests that they are as susceptible to homologous recombination as highly expressed genes (Johnson et al., 1989).

Gene targeting in mouse ES cells, formation of chimæras and germ-line transmission of the mutated gene can provide a unique insight into the role of that gene in embryogenesis and development and its function in the whole organism. Disruption of the mouse homeobox gene *hox 1.5* has demonstrated is role in the normal development of neck structures (Chisaka & Capecchi, 1992) and disruption of *wnt-1* results in abnormalities confined to the mid-brain and cerebellum (McMahon & Bradley, 1990). Unexpected effects can also be detected by these techniques such as the role of the proto-oncogene *c-src* in normal bone physiology (Soriano et al., 1991). In addition, gene targeting can provide an animal model of a human genetic disease such as Lesch-Nyhan syndrome (Hooper et al., 1987) and may provide insights into their correction (Thompson et al., 1989).

The normal cellular function of p53 remains obscure (chapter 2) and its possible role in development (suggested by its expression during embryogenesis, Schmid et al., 1991) is also unknown. The human genetic disease, Li-Fraumeni syndrome, is characterised by germ-line p53 gene mutations which co-segregate to affected individuals. The production of a p53 null animal should, therefore, provide much information on the developmental role of p53 (if any) and also on the normal function of p53. An

animal carrying a subtle mis-sense gene mutation of the sort seen in Li-Fraumeni syndrome would create an accurate model of human disease which might provide insight into prevention of tumourigenesis and possible therapy.

Figure 6.2 Disruption of the *hprt* gene by gene targeting. **(A)** Gene replacement. Homologous recombination between vector and endogenous *hprt* sequences results in vector sequences containing *neo* replacing the genomic sequences. **(B)** Sequence insertion. Pairing of the free, homologous ends of the vector with the endogenous gene is followed by recombination at the double strand break and results in the vector being inserted into the endogenous *hprt* gene. This produces a duplication of a portion of the *hprt* gene as well as its disruption. Open boxes represent introns; closed boxes, exons; and the cross-hatched box, *neo*. Modified from Thomas & Cappecchi, 1987.


A
Neo^r
6
9
1
2
3
4
5
6
7
8
9
hprt+
sequence
replacement
1
2
3
4
5
6
7
Neo^r
9
hprt⁻G418^r

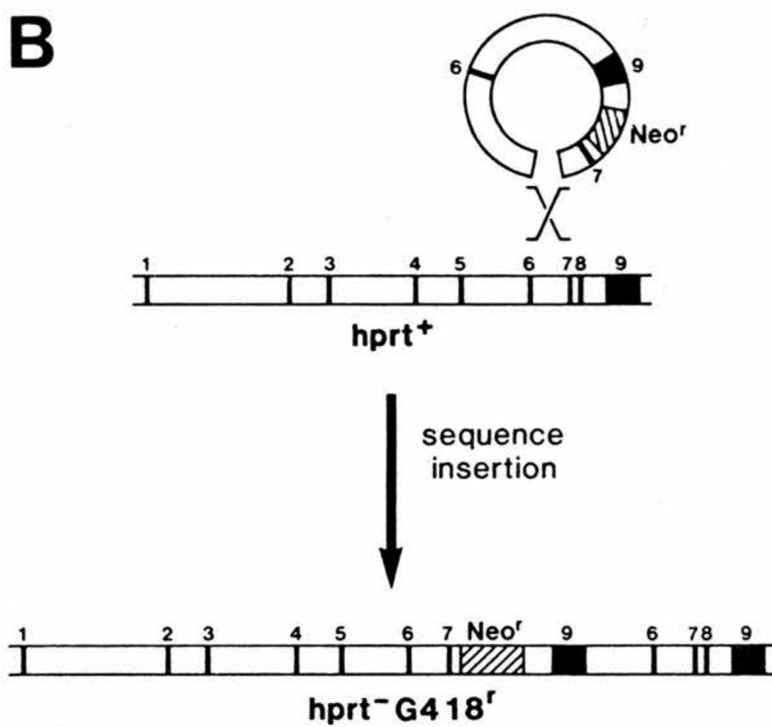
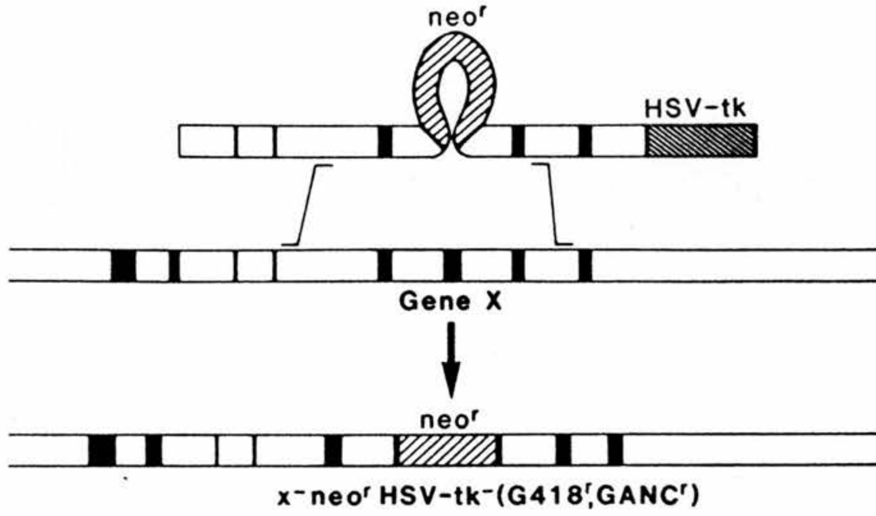

B
6
9
Neo^r
7
1
2
3
4
5
6
7
8
9
hprt+
sequence
insertion
1
2
3
4
5
6
7
Neo^r
9
6
7
8
9
hprt⁻G418^r

Figure 6.3 The positive-negative selection vector. **(a)** Gene targeting. Gene X replacement vector containing a *neo* gene within the region of homology and an HSV*tk* gene outwith the homology. Correct targeting into endogenous gene X results in loss of the HSV*tk* sequences rendering the cells G418 and ganciclovir resistant. **(b)** Random integration results in inclusion of HSV*tk* rendering the cells G418 resistant but ganciclovir sensitive. Thus, inclusion of a counter-selectable marker outwith the region of homology will enrich for homologous over non-homologous integration when cells are selected in medium containing G418 and ganciclovir. Open boxes represent introns; closed boxes, exons; and cross-hatched boxes, *neo and HSVtk*. Modified from Mansour et al., 1988.


a Gene Targeting
neo^r
HSV-tk
Gene X
neo^r
x⁻neo^r HSV-tk⁻(G418^r,GANC^r)

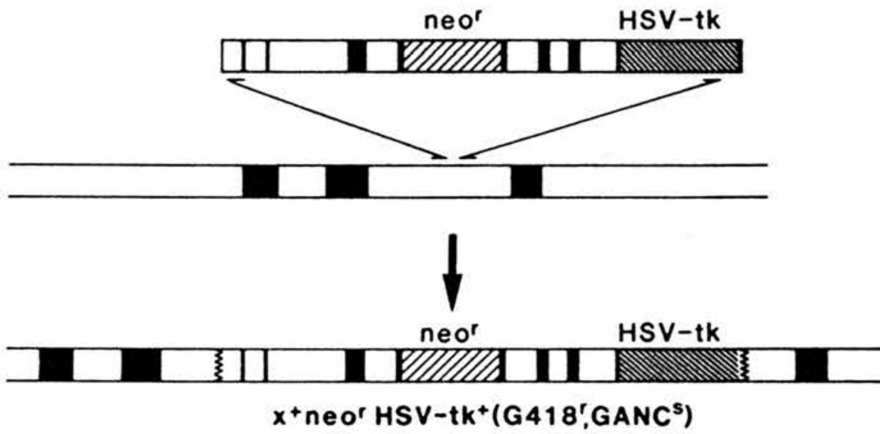
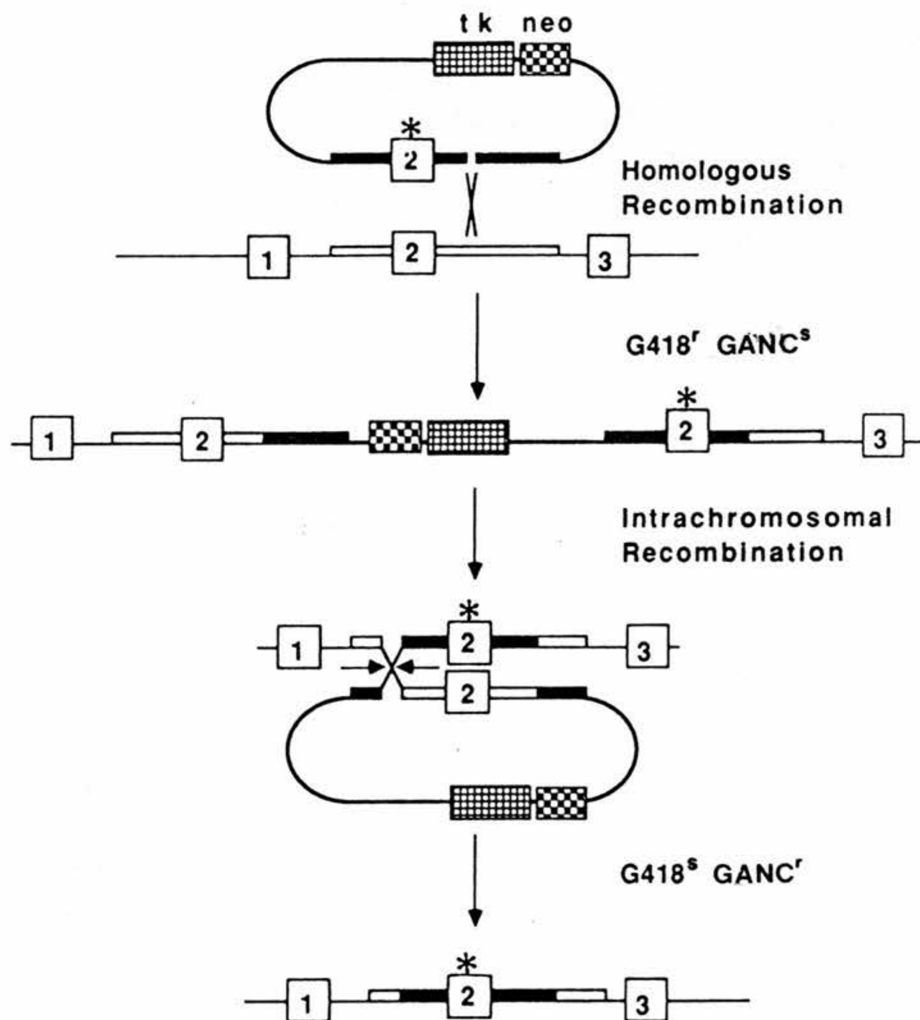

b Random Integration
neo^r
HSV-tk
neo^r
HSV-tk
x⁺neo^r HSV-tk⁺(G418^r,GANC^s)

Figure 6.4 "Hit and Run" gene targeting. The initial step is an insertion event into the target gene of a targeting vector which contains a specific mutation and is linearized within the region of homology with the *neo* and HSV*tk* genes located outwith the homology. This results in duplication of genomic sequences and renders cells G418 resistant but ganciclovir (GANC) sensitive. One, both or neither of the duplicates may contain the desired mutation. G418 colonies are screened for this event, expanded and then selected for intrachromosomal recombination within the duplication by selection in ganciclovir. Filled boxes represent homologous DNA in the vector; open boxes, homologous DNA in endogenous gene; numbered boxes, exons; thick lines, plasmid DNA; thin lines, non-homologous DNA; asterisk, mutation; and the arrows represent the plane of resolution within the Holliday structure. Modified from Hasty et al., 1991.


tk
neo
Homologous
Recombination
1
2
3
G418ʳ GANCˢ
Intrachromosomal
Recombination
G418ˢ GANCʳ

6.2 Targeting p53 by "Hit and Run"

The Li-Fraumeni syndrome is characterised by germ-line transmission of p53 gene mutations which result in a variety of early onset and multiple primary tumours including osteosarcoma and lymphoma (Malkin et al., 1990; Srivastava et al., 1990). Transgenic mice which overexpress mutant p53 develop a range of tumours including: osteosarcoma; lymphoma; adeno-carcinoma of the lung; rhabdomyosarcoma; and skin carcinoma (Lavigueur et al., 1989). In mice over-expressing the transgene containing the Ala-Val mutation at codon 135 the tumours are confined to the osteosarcoma and lymphoma groups and have a latency of 3.5 to 8.5 months. Although there are clear parallels between this mouse model and Li-Fraumeni syndrome, the transgene is over-expressed 40-50 fold making it less like the human situation where over- expression is not seen (Malkin et al., 1990).

The introduction of a single base pair mutation into the p53 gene of mouse ES cells and subsequent production of chimæras and heterozygous offspring would provide a true and accurate model of Li-Fraumeni syndrome, permitting investigation of therapeutic intervention.

The "hit and run" or "in-out" technique (Hasty et al., 1991; Valancius & Smithies, 1991) provides the means of creating a true animal model of this syndrome (section 6.1.4, figure 6.4). An animal bearing a single mutation in an otherwise intact p53 gene would also help explain the mode of action of WT and mutant p53 in tumorigenesis. Comparison of a mutant/WT heterozygote with a mutant/null heterozygote (by mating with the WT/null heterozygote described in chapter 7) would show whether mutant p53 exerts a dominant negative effect by oligomerising (Milner et al., 1991) or altering

the conformation (Milner & Medcalf, 1991) of the WT protein or whether it is simply inactivated by mutation.

Homologous recombination of the insertion type (Thomas & Capecchi, 1987) in some studies occurs at a frequency almost 100 times greater than with replacement vectors (Hasty et al., 1991). For this reason it was decided to attempt to make a mutant p53 gene and a disabled p53 gene in independent experiments using the "Hit and Run" technique as there appeared a higher probability of success than with a replacement strategy.

Two targeting vectors were constructed of the "hit and run" type (Thomas & Capecchi, 1987) each containing a selectable marker (*neo*) and a counterselectable marker (HSV-*tk*). One contained the ala-val mutation at codon 135 which is known to cooperate with *ras* (Findlay et al., 1989), is temperature sensitive (Michalovitz et al., 1990) and causes tumours in transgenic mice (Lavigueur et al., 1989). The second construct was a modification of the first with a 2kbp deletion to render the gene inactive.

6.3 Results

6.3.1 Vector Construction

The p53 gene homology used in this study consisted of the central Hind III fragment of genomic p53 clone Ch53-7 (Bienz et al., 1984) containing exons 2-9. This was inserted into the Hind III site of pGEM PGK-TK (pGEM II vector containing HSV-*tk* under control of the PGK promoter) to create pCPI1.1 (figure 6.5). In order to employ the BamH1 site between exons 6 and 7 of p53 for linearization the Bam H1 site of pSP PGK-NEO had to be destroyed. This was carried out by Bam H1 digestion of pSP PGK-NEO followed by filling in the 5' overhang with the Klenow fragment of DNA polymerase and subsequent blunt end religation. Loss of the Bam H1 site in the resulting plasmid was confirmed by BamH1 digestion. The Bgl II fragment of this modified pSP PGK NEO was excised and ligated into the Bgl II of pCPI 1.1 to make pCPI 2.1 (figure 6.5). pCPI 2.1 was also modified to make a disabling deletion. The 2kbp Xho I fragment of the p53 homology was excised removing the 3' region of intron 1 in addition to the initial 13 codons of exon 2 and the splice acceptor site (figure 6.5). All vectors were confirmed by restriction mapping (figure 6.6). The orientation of the homologous DNA to the rest of the vector was not initially checked as the vector would target with the homology in either orientation. It was assumed initially that the orientation was reversed compared to the actual orientation (subsequently confirmed by further restriction mapping) (figure 6.7) and this influenced some of the screening strategies.

It has been demonstrated that areas of heterology adjacent to the double strand break can be repaired using the target sequences as template

(Valancius & Smithies, 1991). Therefore, care was taken that the 2kbp XhoI deletion was distant from the BamHI linearisation site (2kbp). Unfortunately, the GTG-GCG mutation in exon 6 (codon 135) is close to the Bam HI site increasing the likelihood of correction using the endogenous gene as template. Therefore a screening test had to be developed to detect whether the mis-sense mutation had been incorporated as intended.

Figure 6.5 Plasmid maps showing the construction of pCPI 2.1 and pCPI 2.1 XD. See text for details. Open boxes represent introns; filled boxes, exons; thin line, plasmid DNA; hatched boxes, selectable genes (TK - HSV*tk*, Neo - *neo;* arrows show the direction of transcription); asterisk, mutation val 135. Abbreviations for retriction endonucleases are as follows: R. EcoR1; H, Hind III; X, Xho1; B, BamH1; Bg, Bgl II

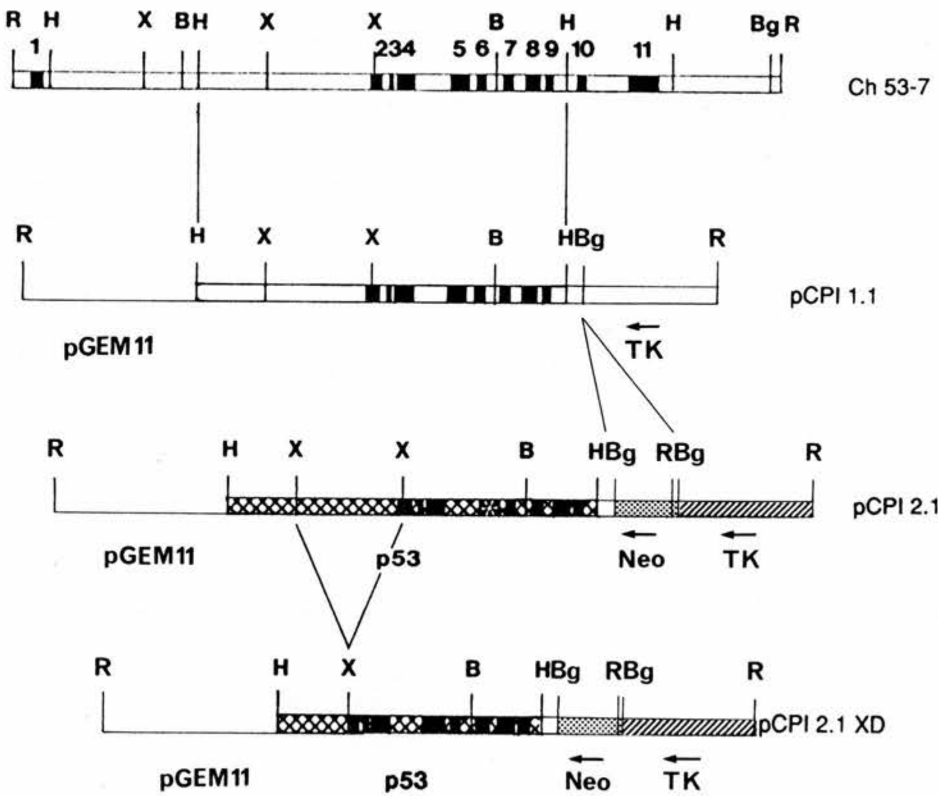

R H X BH X X B H H Bg R
1 234 5 6 7 8 9 10 11
Ch 53-7
R H X X B HBg R
pCPI 1.1
pGEM11
TK
R H X X B HBg RBg R
pCPI 2.1
pGEM11
p53
Neo
TK
R H X B HBg RBg R
pCPI 2.1 XD
pGEM11
p53
Neo
TK
1 kb

Figure 6.6 UV transilluminated, ethidium bromide stained agarose gel cofirming the restriction maps of "hit and run" vectors. **M**, 1 kb ladder molecular weight marker (sizes given in kbp) **1**. pGEM PGK-TK / Hind III. **2**. pCPI 1.1 / Hind III. **3**. pCPI 1.1 / Hind III, BamH I. **4**. pCPI 1.1 / Bgl II. **5**.pCPI 2.1 / Bgl II. **6**. pCPI 2.1 / BamH 1 **7**. pCPI 2.1 / Xho 1. **8**. pCPI 2.1XD / Xho 1.

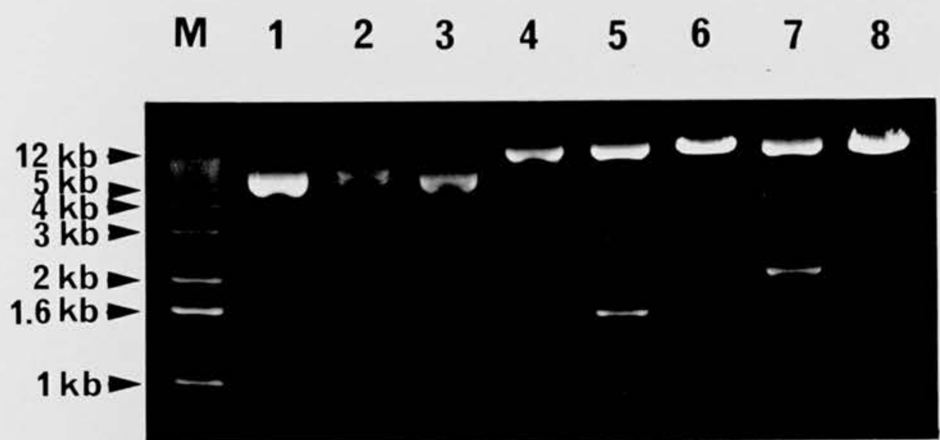
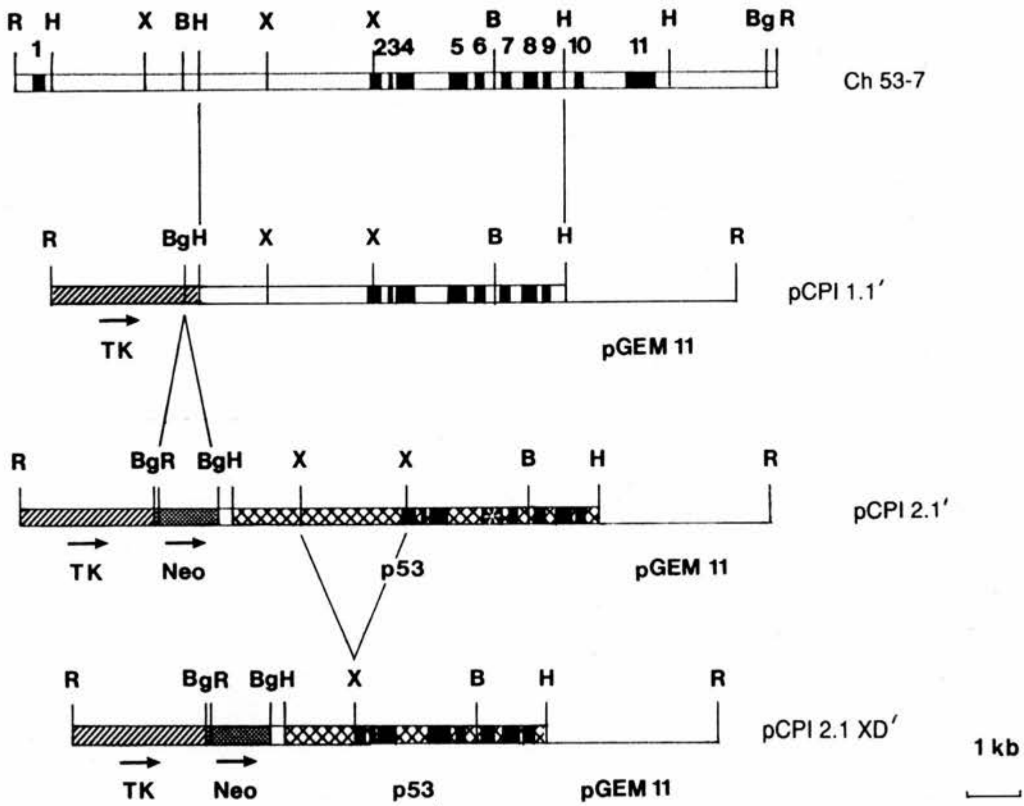

M
1
2
3
4
5
6
7
8
12 kb
5 kb
4 kb
3 kb
2 kb
1.6 kb
1 kb

Figure 6.7 Plasmid maps showing what was initially assumed to be the construction of pCPI 2.1 and pCPI 2.1 XD (designated pCPI 2.1' and pCPI 2.1 XD' respectively). See text for details. Open boxes represent introns; filled boxes, exons; thin line, plasmid DNA; hatched boxes, selectable genes (TK - HSV*tk*, Neo - *neo;* arrows show the direction of transcription); asterisk, mutation val 135. Abbreviations for retriction endonucleases are as follows: R. EcoR1; H, Hind III; X, Xho1; B, BamH1; Bg, Bgl II



R H X BH X X B H H Bg R
1 234 5 6 7 8 9 10 11
Ch 53-7
R BgH X X B H R
pCPI 1.1′
TK
pGEM 11
R BgR BgH X X B H R
pCPI 2.1′
TK Neo p53 pGEM 11
R BgR BgH X B H R
pCPI 2.1 XD′
1 kb
TK Neo p53 pGEM 11

6.3.2 Transfection and Culture

Approximately 50 µg of PCP1 2.1XD and 35 µg of pCPI 2.1 were transfected independently into 10^8 E14 mouse ES cells (Hooper et al., 1987) by electroporation under conditions optimised for single copy transfer. Both vectors were linearised by Bam HI digestion, confirmed by agarose gel electrophoresis, prior to transfection. After 24 hours G418 selection was introduced and this was subsequently withdrawn once the individual colonies had reached a suitable size to be picked.

Of the clones surviving, 254 from pCPI 2.1XD and 245 for pCPI 2.1 were expanded and the DNA harvested for Southern blot and PCR analysis.

6.3.3 Screening of pCPI 2.1 XD clones

The pCPI 2.1XD clones were screened for the "hit" or insertion event by Bgl II digestion, Southern transfer and hybridisation to external probe P1. The predicted recombination events (figure 6.8) and Southern blot pattern are indicated (table 6.1). The external probe, P1, can only show a band shift if the endogenous gene is altered as it cannot hybridise to the randomly integrated vector. Results were obtained from 149 clones and of these 20 showed a pattern consistent with a hit event (fig 6.9a) but 1 showed a larger band shift (fig 6.9a). As P1 is external to homology contained within this vector this 2kbp band shift must represent endogenous gene disruption.

Further analysis was carried out using internal probe P3 with other enzyme digests to confirm that the predicted insertion event had occurred and that "hit and run" had not (fig 6.9b).

Figure 6.8 Scale diagram representing the targeted integration of pCPI 2.1 XD by homologous recombination into genomic p53 followed by intrachromosomal recombination. Open boxes represent introns; filled boxes, exons; thin line, plasmid DNA; hatched boxes, selectable genes (TK - HSV*tk*, Neo - *neo;* arrows show the direction of transcription); cross-hatched boxes, homologous DNA within the vector; P1, external probe for Southern blots; P3, internal probe for Southern blots. Abbreviations for retriction endonucleases are as follows: R. EcoR1; H, Hind III; X, Xho1; B, BamH1; Bg, Bgl II.

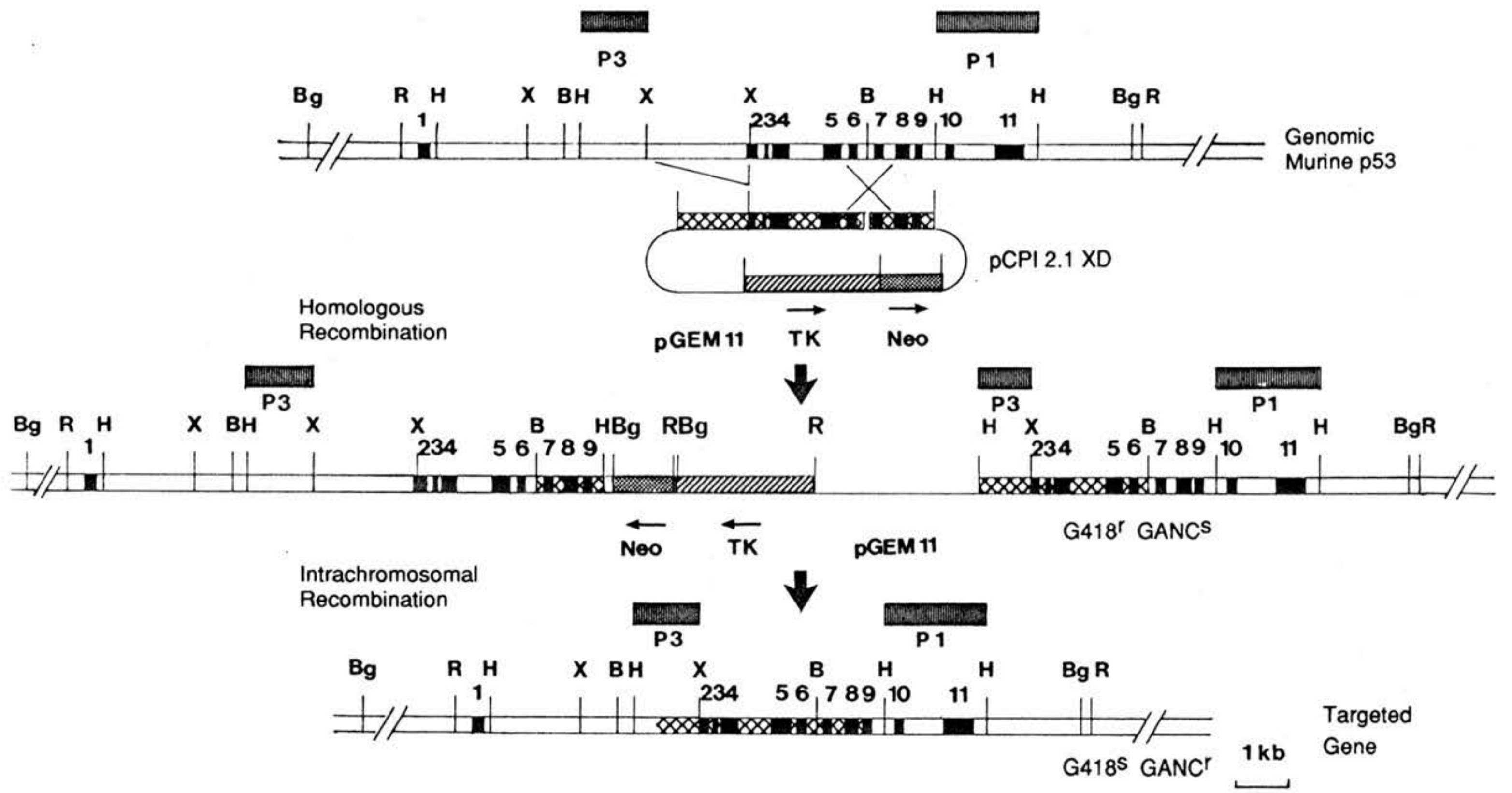
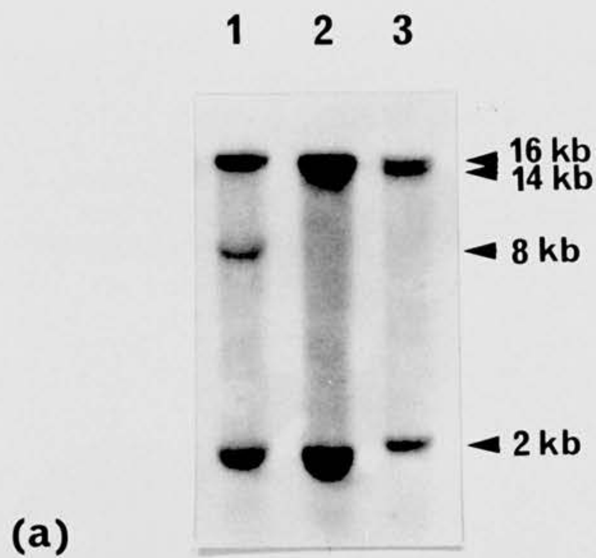

P3
P1
Bg
R H
1
X BH
X
X
234
B
5 6 7 8 9
H
10
11
H
Bg R
Genomic
Murine p53
pCPI 2.1 XD
Homologous
Recombination
pGEM 11
TK
Neo
P3
P3
P1
HBg
RBg
R
G418r GANCs
Neo
TK
pGEM 11
Intrachromosomal
Recombination
Targeted
Gene
G418s GANCr
1 kb

Table 6.1 Summary of predicted Southern blot band sizes for pCPI 2.1 XD clones. Probes P1 and P3 defined in figure 6.8. All sizes are in kbp.

Probe	**P1**	**P3**		
Enzyme	Bgl II	Bgl II	Hind III	BamH 1
Wild-type	16	16	7	6
"Hit" event	16/14	16/14/13	7/7/4.5	6/6/12
"Hit and Run"	16/14	16/14	7/4.5	6/4

Figure 6.9 Autoradiographs of Southern blots of genomic DNA extracted from pCPI 2.1 XD transfected ES cell clones probed with external probe P1. **(a)** Lane 1: clone 82 showing an aberrant pattern with an 8kbp band. Lane 2: clone 83 showing the wild-type pattern. Lane 3: clone 84 showing a pattern consistent with insertion .**(b)** Further Southern blot analysis of clone 84 probed with internal probe P3. Lane 1: BamH 1. Lane 2: Hind III. Lane 3: Bgl II (the 13kbp band has failed to resolve from the 14kbp band). All sizes in kbp.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2
3
16 kb
14 kb
8 kb
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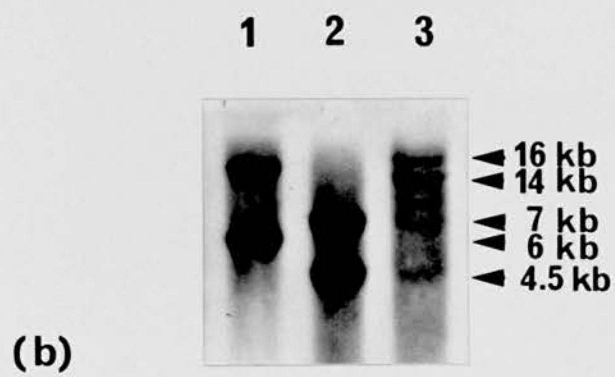
(a)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16 kb
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4.5 kb
(b)

6.3.4 Screening of pCPI 2.1 clones

These clones were screened in an identical fashion to pCPI 2.1 XD by Bgl II digestion and hybridisation to probe P1. However, the vector was initially thought to be pCPI 2.1' (homology in reverse orientation, figure 6.7) which would have produced a diagnostic band shift from 16kbp to 11kbp (figure 6.10). With the actual vector used, pCPI 2.1, both the endogenous gene and the targeted gene give a 16kbp band (figures 6.11 and 6.12a). Screening by Bgl II digestion but probing with DNA from the region 5' to the homology used in the vector construction (such as P4, chapter 7) would give the true frequency of the insertion event. However, not all inserted vectors would have retained the mutation and so a PCR strategy was developed to screen for insertion at the correct site by the targeting vector and retention of the mutation. A PCR primer was designed which had a single base pair mismatch at the 3' end which would create a new Dra III site if the GTG mutation were present (figure 6.13) in exon 5. The 3' PCR primer was located initially within exon 7, across the insertion site of the vector into the endogenous gene (figure 6.11). Thus, amplification would always take place for the endogenous gene (at least) but would not digest with Dra III unless the mutation were both present and integrated at the correct site.

Initial attempts at amplification were unsuccessful due to preferential amplification of the pseudogene which shares a high degree of homology with the cDNA of the true gene (Zakut-Houri et al., 1983). To circumvent this problem the 7th intron was partially sequenced and another PCR primer designed at its 5' end (figure 6.14). Sequences using these 2 primers were amplified from each of the 245 clones, digested with Dra III and the fragments resolved by electrophoresis through a 4% agarose gel (figure

6.12b). DNA from an OLA/129 mouse was used as a negative control and pCPI 2.1 itself as a positive control (thus also confirming the presence of the mutation in the targeting vector). Of the 245 tested, 10 showed a pattern consistent with the presence of the mutation and integration (figure 6.12b). However, 4 of these also showed extra, smaller fragments perhaps suggestive of loss of sequence at the integration site.

The relative frequencies of correct insertion of pCPI 2.1 XD and pCPI 2.1 into the genome are given in table 6.2.

Figure 6.10 Scale diagram representing the targeted integration of pCPI 2.1' by homologous recombination into genomic p53 followed by intrachromosomal recombination. Open boxes represent introns; filled boxes, exons; thin line, plasmid DNA; hatched boxes, selectable genes (TK - HSV*tk*, Neo - *neo;* arrows show the direction of transcription); cross-hatched boxes, homologous DNA within the vector; asterisk, ala-val mutation codon 135; small arrows, position of PCR primers MM 5.5 and MM 7.7 which amplify accross the site of integration; P1, external probe for Southern blots; P3, internal probe for Southern blots. Abbreviations for retriction endonucleases are as follows: R. EcoR1; H, Hind III; X, Xho1; B, BamH1; Bg, Bgl II

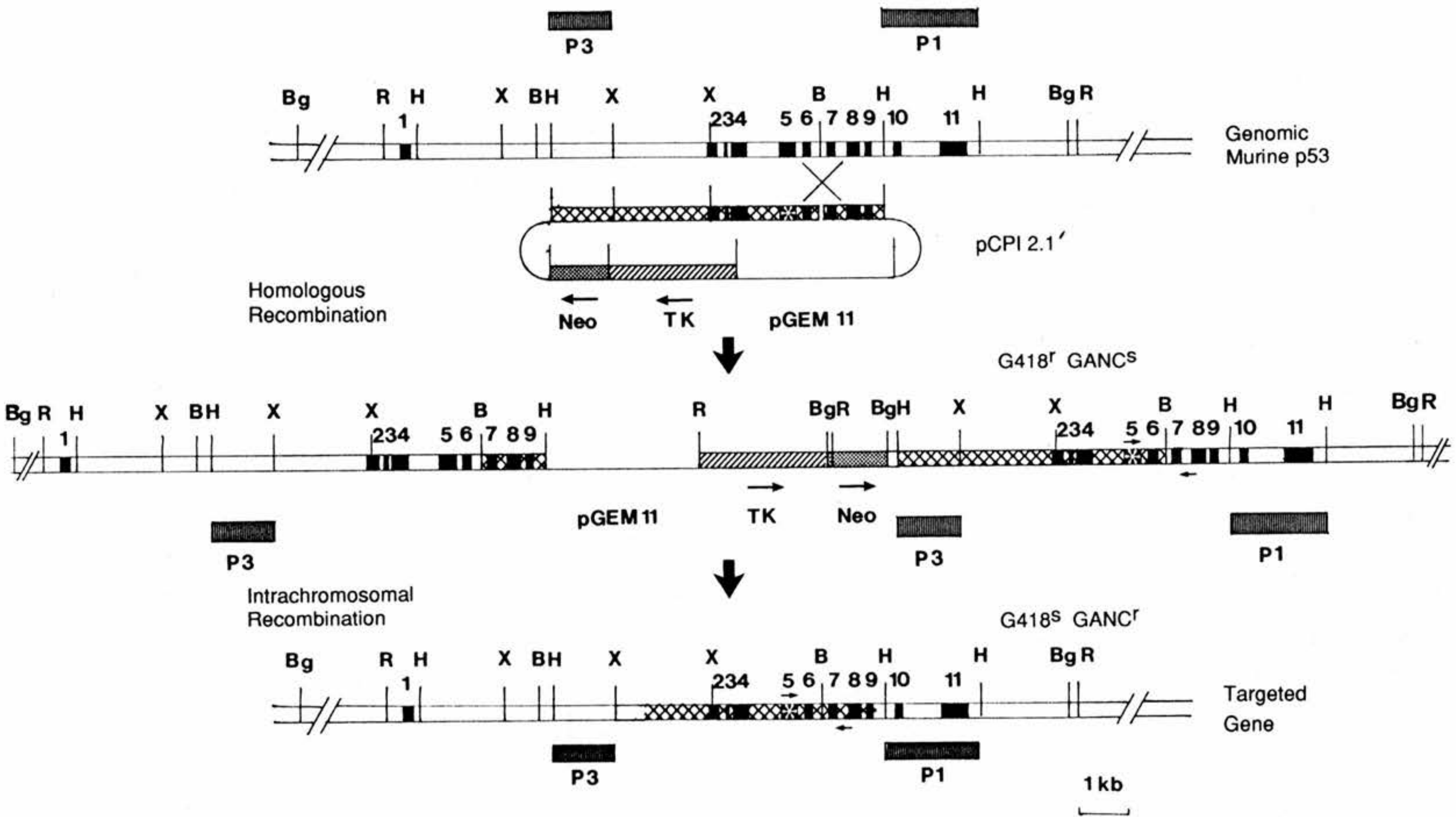

Genomic
Murine p53
pCPI 2.1′
Homologous
Recombination
Neo
TK
pGEM 11
G418^r GANC^S
pGEM 11
Intrachromosomal
Recombination
G418^S GANC^r
Targeted
Gene
P1
P3
1 kb

Figure 6.11 Scale diagram representing the targeted integration of pCPI 2.1 by homologous recombination into genomic p53 followed by intrachromosomal recombination. Open boxes represent introns; filled boxes, exons; thin line, plasmid DNA; hatched boxes, selectable genes (TK - HSV*tk*, Neo - *neo;* arrows show the direction of transcription); cross-hatched boxes, homologous DNA within the vector; asterisk, ala-val mutation codon 135; small arrows, position of PCR primers MM 5.5 and MM 7.7 which amplify accross the site of integration; P1, external probe for Southern blots; P3, internal probe for Southern blots. Abbreviations for retriction endonucleases are as follows: R. EcoR1; H, Hind III; X, Xho1; B, BamH1; Bg, Bgl II

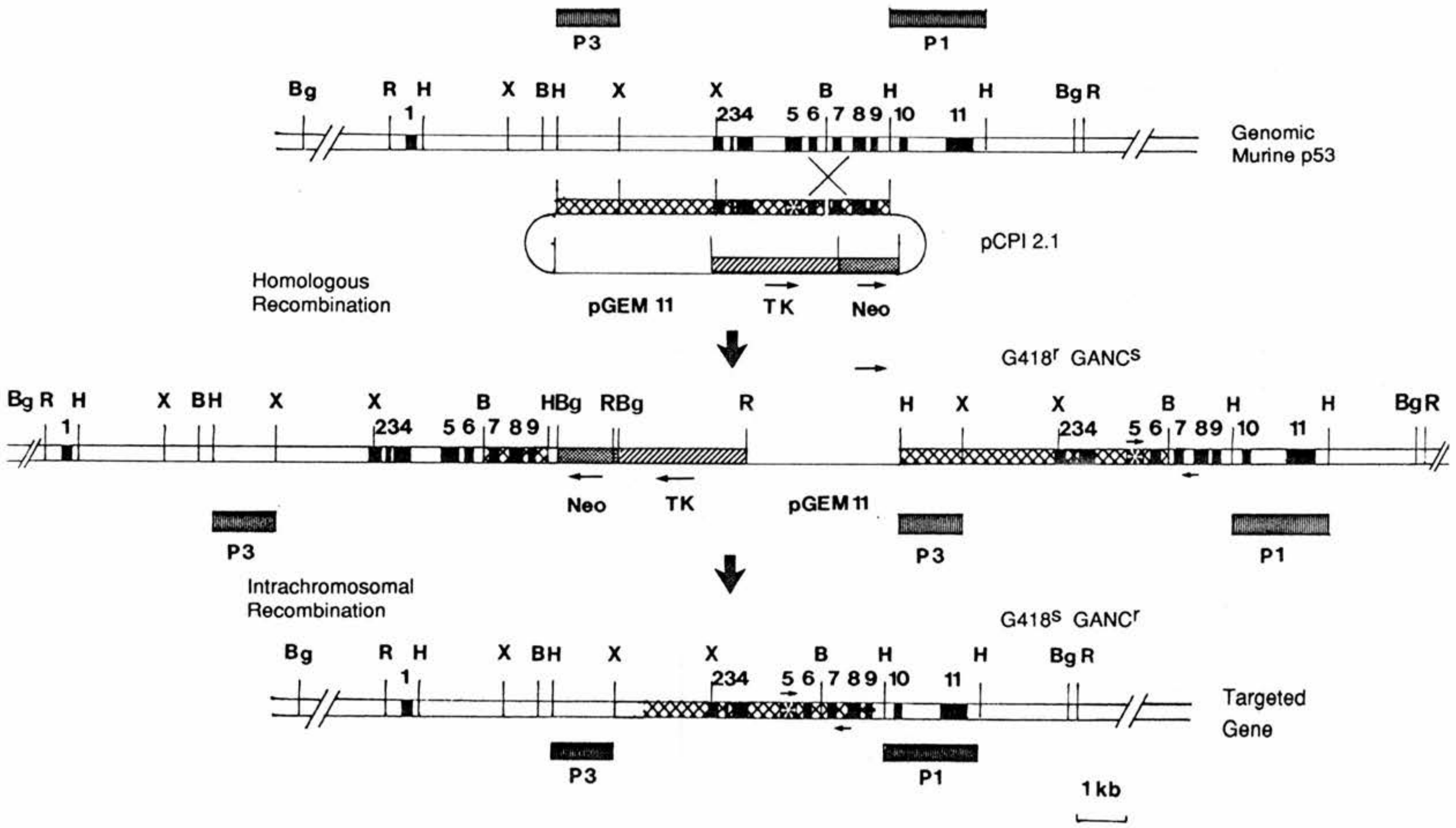
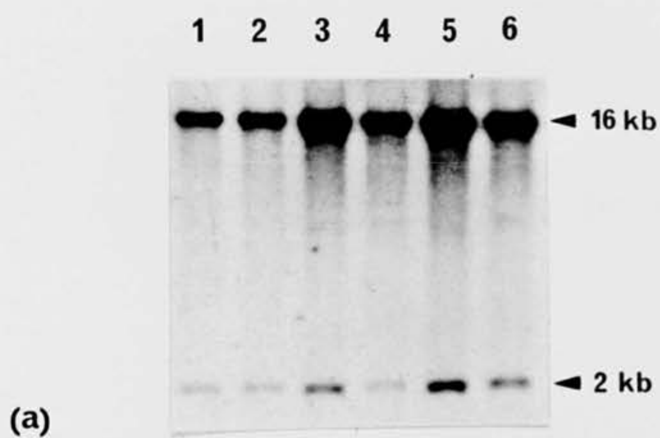
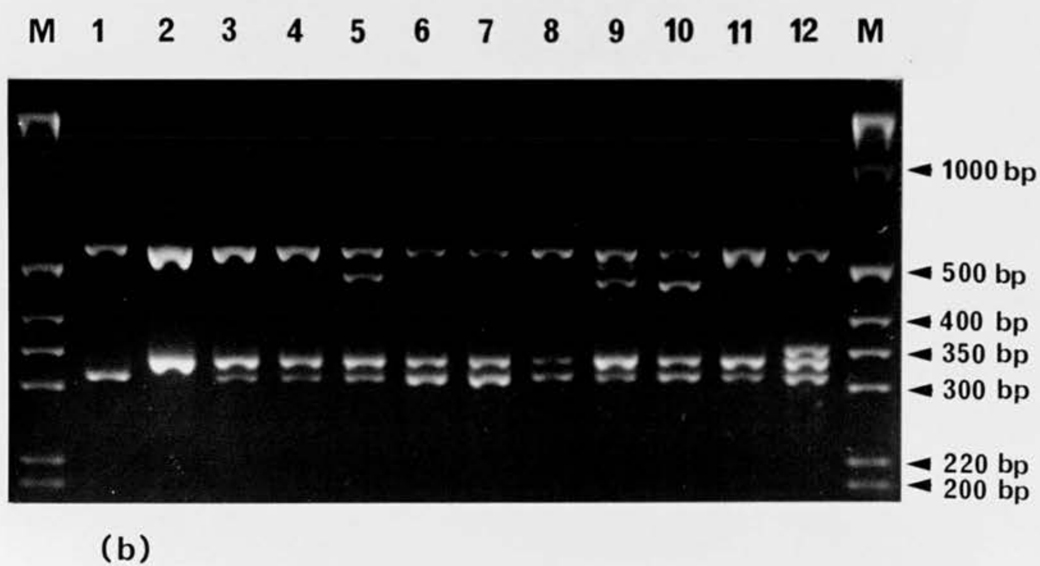

P3
P1
Bg R H X BH X X B H H BgR
1 234 5 6 7 8 9 10 11
Genomic
Murine p53
pCPI 2.1
Homologous
Recombination
pGEM 11
TK
Neo
G418r GANCs
HBg RBg R
Neo
TK
pGEM 11
Intrachromosomal
Recombination
G418s GANCr
Targeted
Gene
1 kb

Figure 6.12 (a) Autoradiograph of Southern blot of DNA extracted from pCPI 2.1 transfected ES cell clones digested with Bgl II and probed with external probe P1. Lanes 1-6, clones 53-58 showing the wild type band at 16 kbp and the pseudo-gene at 2 kbp. **(b)** UV transilluminated, ethidium bromide stained, 4% agarose gel showing Dra III digested PCR amplified fragments from pCPI 2.1 ES cell clones. M, molecular weight marker, sizes in bp. Lane 1, PCR amplified fragment from pCPI 2.1 (positive control); Lane 2, wild-type Ola/129 DNA (negative control); Lanes 3-12, clones 69, 74, 76, 100, 101, 106, 115, 116, 202 and 206 showing the extra (mutant) band.



(a)



(b)

Figure 6.13 Sequence of exon 5 from wild-type genomic p53 and clone Ch53-7. Shown in bold is the sequence of the 5' PCR primer used to detect the alanine to valine (GCG to GTG) mutation at codon 135 (marked by an asterisk). Presence of the mutation results in the creation of a novel Dra III site (CAC NNN GTG) which is not present in the wild-type gene. Following digestion with Dra III the PCR fragment will be shortened by 24 bp only if the mutation is present.

Wild-type gene

Pro	Leu	Asn	Lys	Leu	Phe	Cys	Gln	Leu	Ala*	Lys
CCC	CTC	AAT	AAG	CTA	TTC	TGC	CAG	CTG	GCG	AAG
5'	**CTC**	**AAT**	**AAG**	**CTA**	**TTC**	**TGC**	**CAC**	**CTG**	>	3'

No Dra III site

Mutant gene

Pro	Leu	Asn	Lys	Leu	Phe	Cys	Gln	Leu	Val*	Lys
CCC	CTC	AAT	AAG	CTA	TTC	TGC	CAG	CTG	GTG	AAG
5'	**CTC**	**AAT**	**AAG**	**CTA**	**TTC**	**TGC**	**CAC**	**CTG**	>	3'

Dra III site

Figure 6.14 Sequence of intron 7 from genomic p53 clone Ch 53-7 showing the position and sequence of 3' PCR primer MM 7.7 used to screen pCPI 2.1 transfected ES cell clones.

	Exon 7				**Intron 7**	
	Glu	Asp	Ser	Ser		
5'	GAA	GAC	TCC	AG	GTAGGAAGGC	GCGTGGTAGG
				<--	**CATCCTTCCG**	**CGCACCATCC**

TTAGGTTAGC CTGTTTCTTC CCCAGCTTCT GCCTGTTTCT
AATC (MM 7.7)

GTTCCACGA TCCCGCCCCC TACCACATGC CCAACGCTCT

TTGGTTCCTA CCCTATCTAC CTAAATGAAG TCTCC **3'**

Table 6.2 Summary of results for insertion event obtained for targeting using pCPI 2.1 XD (screened by southern blot) and pCPI 2.1 (screened by PCR for insertion *and* retention of mutation).

Vector	Clones Screened	Clones Targeted	Targeting Frequency
pCPI 2.1 XD	149	20	1 / 7.45
pCPI 2.1	245	10	1 / 24.5

6.4 Discussion

Targeting with pCPI 2.1XD has resulted in disruption of the endogenous p53 gene judged from the result of the Southern blot probed with sequence external to that used in the vector construction (figure 6.9a). The pattern obtained with probe P1 is consistent with both the "hit" event and with "hit and run" (table 6.1). However, confirmatory southern blots using internal probe P3 indicate that the vector has integrated as predicted (table 6.1, figures 6.8 and 6.9b). Confusion over the correct orientation of the gene homology made the interpretation of the blots impossible thus delaying further expansion and ganciclovir selection which is now under way.

One clone gave a different southern blot pattern (figure 6.9a, lane1) with a larger band shift than that predicted for the insertion event. The probe used, P1, is external to the gene homology used in the vector and so this clearly represents endogenous gene disruption. It is possible that the vector has integrated correctly but has lost sequence or that endogenous sequence (including the region homologous to P1) has been picked up by the vector which has then integrated elsewhere in the genome (Szostak et al., 1983).

The insertion targeting event is occurring at a frequency of 1 in 7.45 clones screened; in a system employing a single positve selection marker (Neo). This is therefore comparable to the replacement vector (pCPR 3.1, chapter 7) which targets at a frequency of 1 in 183. The vectors otherwise differ in the lengths of homology, 7.5 kbp in pCPR 3.1 and 5 kbp in pCPI 2.1 XD and the particular sequences involved. Thus, in this series, at one locus, an insertion vector is targeting approximately 25 fold more efficiently than a replacement vector. This is despite having less homology which is one of the

most important determinants of targeting efficiency (Ayares et al., 1986). There are however, other differences between the homologies employed in the vectors which makes direct comparison impossible. The finding that an insertion vector targets more efficiently than a replacement vector is in keeping with previous work suggesting up to 100 fold enhancement (Hasty et al., 1991).

Targeting with pCPI 2.1 generated 10 clones which appeared to have integrated correctly and retained the ala-val mutation at codon 135 (figure 6.12b) from 245 screened. It is possible and likely that a greater number have integrated the vector DNA at the correct site but the mutation has been "repaired" using target sequences as template (Valancius & Smithies, 1991) which would give a negative result on PCR analysis. A further possibility to explain the 10 apparently targeted clones is that recircularisation of the vector occurred prior to random integration (Hasty et al., 1991; Moenis et al., 1992). Both of these problems can be addressed by southern blot screening using external probe P4 (chapter 7) with a Bgl II digest.

The apparently less efficient targeting of pCPI 2.1 compared to pCPI 2.1 XD (table 6.2) despite containing greater homology can be explained by the "repair" of the mutation during or after targeted insertion (Valancius & Smithies, 1991). A further possible, though less likely explanation, is that sequences which inhibit homologous recombination are located in the 2 kbp Xho1 fragment removed to make pCPI 2.1 XD. Southern blot screening of pCPI 2.1 colonies will answer this question.

Of the 10 pCPI 2.1 clones identified, 4 show extra smaller bands on the PCR analysis (figure 6.12b). These are suggestive of loss of genetic material

(between 50 and 200bp) during the targeted insertion. Loss of sequence especially at the free ends of a vector during targeted insertion has been described (Hasty et al., 1992) and could account for these findings. In this case, such sequence loss would result in loss of the BamH1 (vector linearisation) site. Analysis by southern blotting following BamH1 digestion with internal probe P3 would detect whether this is indeed the case.

Although the pCPI 2.1 XD insertion event has clearly occurred as predicted and further selection in ganciclovir should result in cells with one inactive p53 gene copy, inactivation using a replacement vector strategy has been achieved and passed into the mouse germ-line (chapter 7). In the case of the 10 candidate pCPI 2.1 targeted clones, further southern blot analysis will be followed, if the correct pattern is obtained, by ganciclovir selection and it may be possible to screen the colonies generated by PAb240 immunihistochemistry for mutant p53 expression.

Production of chimæras and susequently mice heterozygous for p53 val^{135} will provide unique data on the function of p53. They will give informationon whether this mutant can act dominantly when expressed at physiological levels in the presence of WT p53, will produce cells for culture which contain a temperature sensitive mutant (Mickalovitz et al., 1990) under normal control and produce a model for Li-Fraumeni syndrome (although this mutation is not directly analogous). Furthermore, offspring of a mating between p53 val^{135} heterozygotes and p53 null heterozygotes will address the question of whether p53 mutants act by a dominant negative effect on WT p53 or have an action even in its absence.

Chapter 7

Targeting p53 by Replacement

7.1 Introduction

During mouse embryogenesis, p53 is expressed at high levels at 8.5-10.5 days gestation and also in embryonal cells (Rogel et al., 1985; Schmid et al., 1991) suggesting a role in early development. Wild-type p53 appears to cause G1 cell cycle arrest in the presence of DNA damage via transcriptional control of, as yet, unidentified genes and its inactivation is seen in many common malignancies (section 2.6).

In order to address the role of p53 in normal mammalian development and its function in a complete organism, an attempt was made to inactivate p53 by gene targeting in mouse ES cells. This section discusses the success of this attempt and gives early data on the pathology of the resulting chimæras and both heterozygous and homozygous p53 null animals.

Another study, using a similar system (Donehower et al., 1992), showed that mice with a homozygous p53 inactivation were developmentally normal but developed, at high frequency, malignant tumours usually of mesenchyme and lymphoid tissue. Two heterozygotes out of 96 developed tumours but only after 5 and 9 months. The strategy used in their study however, did result in downstream p53 gene transcription including exons 5-11 (amino acids 158-390), a region involved in p53 oligomerization (Milner et al., 1991) making the phenotype difficult to interpret. In this study care was taken to avoid such potential problems by using a strategy which would not result in any p53 expresion.

7.2 Results

7.2.1 Vector Construction

The genomic clone of murine p53, termed Ch53-7 (Bienz et al, 1984) was used for all vector construction. This clone contains the activating mutation ala-val at codon 135 (Bienz et al, 1984) which confers transforming ability when co-transfected with mutated *ras* (Hinds et al, 1989) and is temperature sensitive (Michalovitz et al., 1990). The remainder of the sequence is wild-type and the published restriction map was confirmed prior to commencement of the vector construction.

In order to ensure that this activating mutation would not interfere with the phenotype by acting as a dominant oncogene, care was taken to remove exon 5, which contains it, in the vector construction (figure 7.1). The vector consisted of 5' homology within the first intron and 3' homology spanning exons 7-11 (figure 7.1). Exons 1, 2-6 and most of intron 1 were removed and PGK Neo inserted between the two regions of homology in the same transcriptional orientation as p53. The neomycin (G418) resistance (neomycin phosphotransferase) vector pSPGKNeo consists of pMC1Neo, a re-engineered coding sequence for the bacterial transposon of Tn5 (Thomas & Capecchi, 1987), driven by the promoter of the mouse *pgk-1* (phosphoglycerate kinase) gene (Adra et al, 1987) to ensure expression of *neo* in ES cells. Maps of the targeting vector, pCPR 3.1 and its intermediates along with confirmatory restriction digests are shown in figures 7.1 and 7.2.

The vector pCPR3.1 only contains a positive selection marker (*neo*) and no negative selection marker outwith the homology to enrich for homologous

recombination (Mansour et al, 1988) (figure 6.2). To incorporate such a counter-selectable marker this vector was re-engineered starting with pCPR 2.1. The thymidine kinase gene from herpes simplex virus (HSV*tk* Colbere - Garapin et al; 1979) under the control of the *pgk* promoter was inserted into the Bgl II/EcoRI sites at the 3' end of pCPR 2.1 (figure 7.3) in the reverse transcriptional orientation to p53 creating pCPR 4.1. Thereafter, PGKNeo was again inserted between the regions of p53 gene homology creating pCPR 5.1 (figure 7.3) confirmed by restriction digestion (figure 7.2).

Figure 7.1 Diagram showing the costruction of replacement targeting vector pCPR 3.1. The 5' Hind III fragment of mouse p53 genomic clone Ch 53-7 was ligated into plasmid pUC 19 to form pCPR 1.1 followed by the ligation of the BamH 1/EcoR 1 fragment creating pCPR 2.1. The Bgl II fragment of the G418 resistance gene, *neo*, under the control of the PGK promoter was then cloned into the BamH 1 site between the regions of homology resulting in targeting vector pCPR 3.1. Open boxes represent introns; filled boxes, exons; thin line, plasmid DNA; hatched boxes, selectable gene (Neo - *neo;* arrow shows the direction of transcription). Abbreviations for restriction endonucleases are as follows: R. EcoR1; H, Hind III; X, Xho1; B, BamH1; Bg, Bgl II; BgB, BamH 1/Bgl II fusion (will not digest with either enzyme).

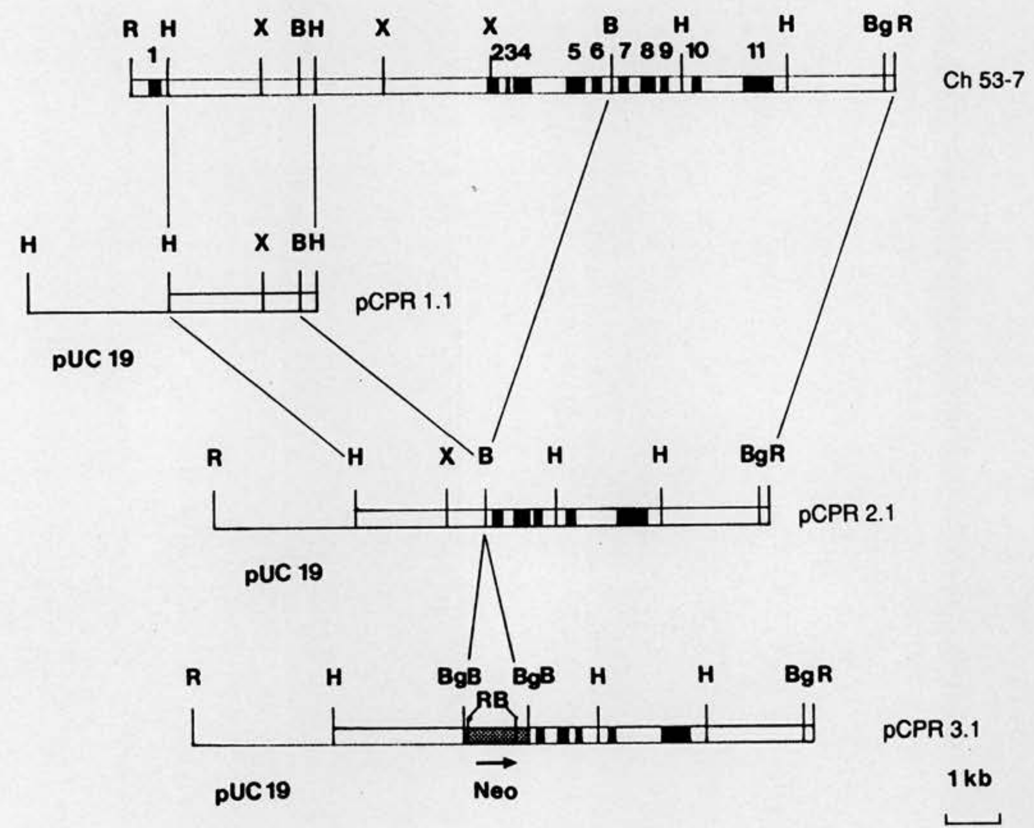
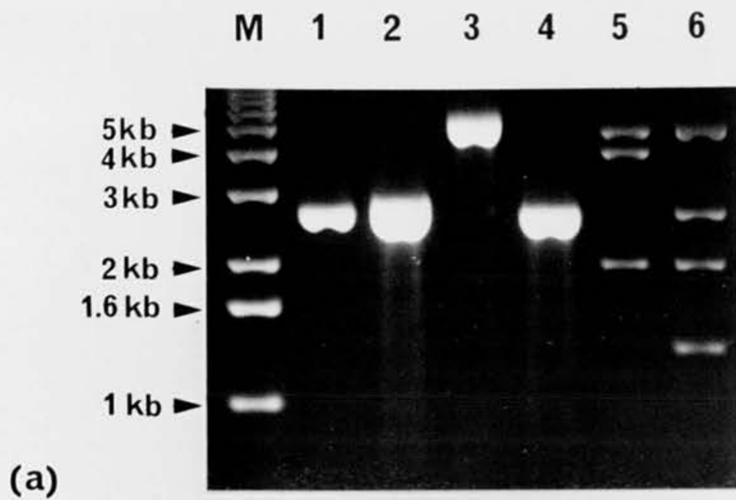

R H X BH X X B H H Bg R
1 234 5 6 7 8 9 10 11
Ch 53-7
H H X BH
pCPR 1.1
pUC 19
R H X B H H BgR
pCPR 2.1
pUC 19
R H BgB BgB H H BgR
RB
pCPR 3.1
Neo
pUC 19
1 kb

Figure 7.2 Diagnostic restriction enzyme digests confirming the maps of the replacement targeting vectors. **(a) M,** molecular weight marker, sizes in kbp; **1**, pUC 19 / Hind III; **2**, pCPR 1.1 / Hind III (two fragments of approximately equal size); **3**, pCPR 1.1 / BamH 1; **4**, pCPR 1.1 / Hind III and BamH 1 (two fragments of approximately equal size); **5**, pCPR 2.1 / Hind III; **6**, pCPR 2.1 / Hind III and BamH 1.**(b) M,** molecular weight marker, sizes in kbp; **1**, pCPR 2.1 / Hind III; **2**, pCPR 2.1 / Hind III and BamH 1; **3**, pCPR 3.1 / Hind III and BamH 1.**(c) M,** molecular weight marker, sizes in kbp; **1**, pCPR 2.1 / EcoR 1 and Bgl II; **2**, pCPR 4.1 / EcoR 1 and Bgl II; **3**, pCPR 4.1 / Hind III and BamH 1; **4**, pCPR 5.1 / Hind III and BamH 1.



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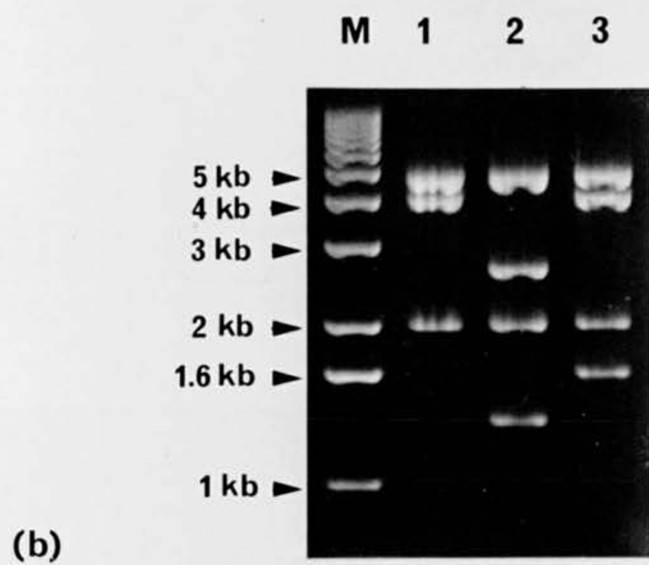
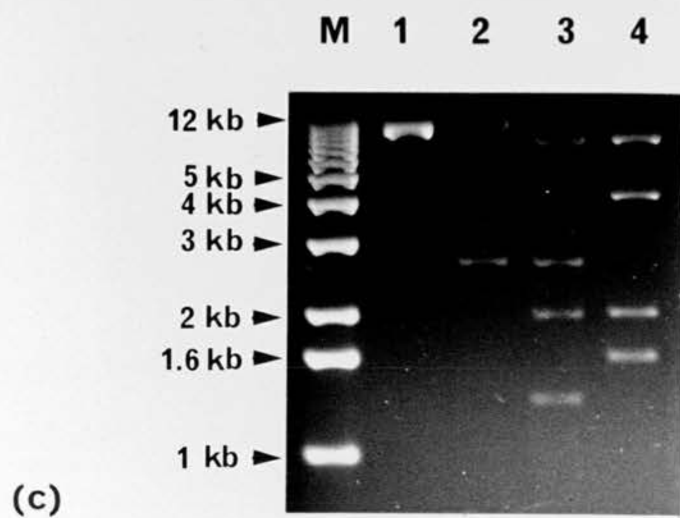
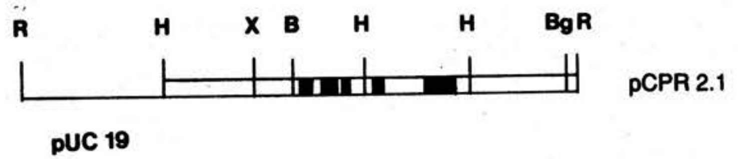


(b)



(c)

Figure 7.3 Diagram showing the construction of replacement targeting vector pCPR 5.1. The Bgl II, EcoR 1 fragment of pSPGKTK containing the HSV*tk* gene under the control of the PGK promoter was ligated into pCPR 2.1 outwith the region of homology to form pCPR 4.1. The *neo* gene was then cloned into the BamH 1 site within the homology as with pCPR 3.1 to create pCPR 5.1. Open boxes represent introns; filled boxes, exons; thin line, plasmid DNA; hatched boxes, selectable genes (TK - HSV*tk*, Neo - *neo;* arrows show the direction of transcription). Abbreviations for restriction endonucleases are as follows: R. EcoR1; H, Hind III; X, Xho1; B, BamH1; Bg, Bgl II; BgB, BamH 1/Bgl II fusion (will not digest with either enzyme).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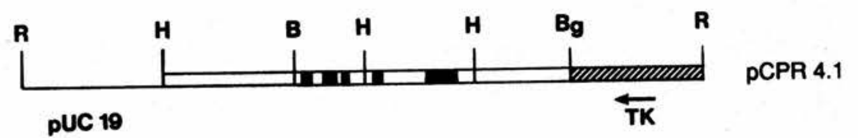
H
X
B
H
H
BgR
pCPR 2.1
pUC 19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B
H
H
Bg
R
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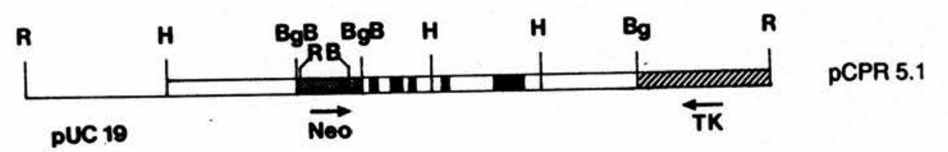
pUC 19
TK


R
H
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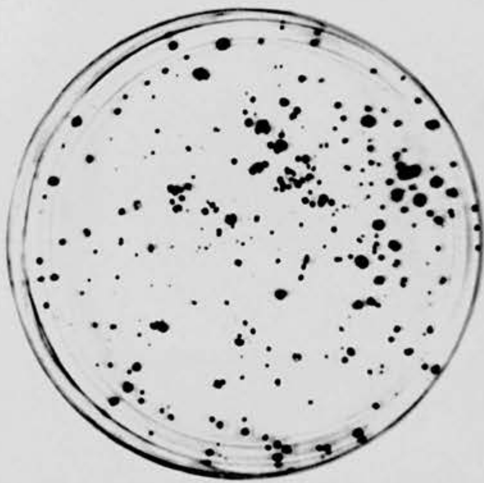
RB
BgB
H
H
Bg
R
pCPR 5.1
pUC 19
Neo
TK
1 kb

7.2.2 Transfection and Culture

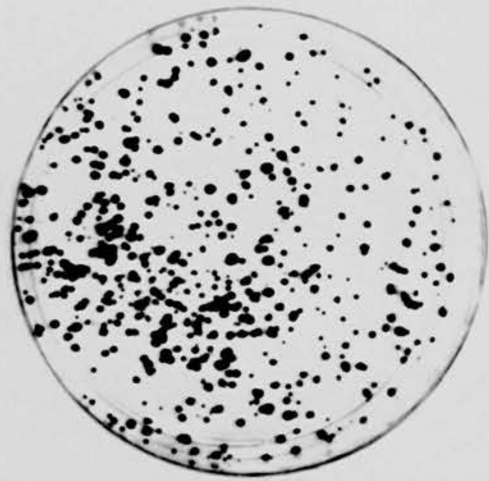
Both vectors were linearised by Pvu I digestion (2 sites within plasmid only) and this was confirmed by agarose gel electrophoresis of an aliquot prior to transfection. Approximately 70 μg of pCPR 3.1 and 50 μg of pCPR 5.1 were transfected independently (conditions optimised for single copy transfection) into 10^8 E14 mouse embryonal stem cells (Hooper et al, 1987). After 24 hours, G418 selection alone (pCPR 3.1) or G418 plus ganciclovir selection (pCPR 5.1) was introduced.

Of the surviving colonies, 183 from the pCPR 3.1 transfection and 204 from the pCPR 5.1 transfection were expanded and DNA harvested for Southern blot analysis. One petri dish from the pCPR 5.1 transfection was maintained in G418 selection only and this demonstrates an approximately 4:1 enrichment when ganciclovir selection is added (figure 7.4).

Figure 7.4 ES cell clones transfected with pCPR 5.1 **(a)** selected in medium cotaining G418 and ganciclvir (142 colonies) and **(b)** G418 only (525 colonies).



(a)



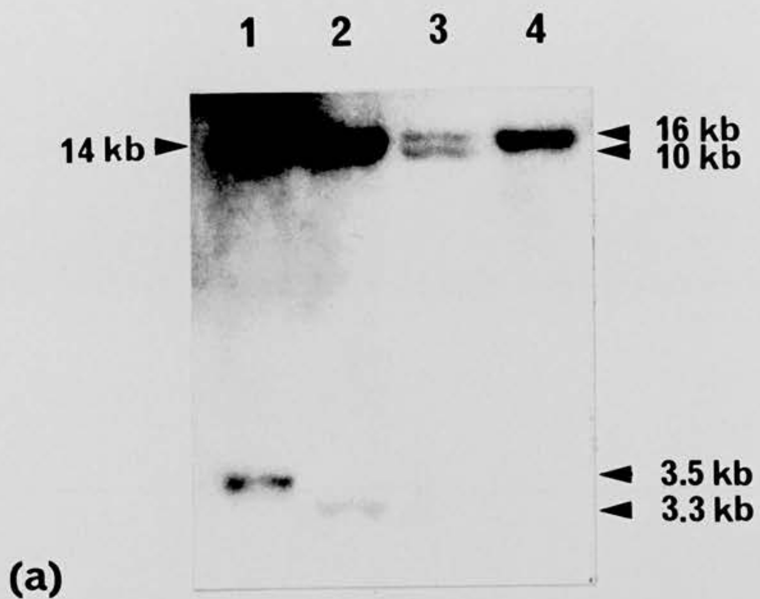
(b)

7.2.3 Colony Screening

Genomic DNA was extracted, digested with EcoR1 and Southern blotted. The filters were hybridised to probe P4 which is external to the region of homology contained within the vector. One clone from the pCPR 3.1 transfection out of 183 gave a pattern suggestive of targeting (figure 7.5a). The mouse pseudogene (Zakut-Houri et al, 1983) also hybridises to P4 making analysis slightly ambiguous (figure 7.5a). The candidate clone, R3.72, was therefore digested with Bgl II and hybridised to P4 confirming that the endogenous gene has indeed been disrupted in the fashion predicted for homologous recombination between it and the targeting vector (figures 7.5a and 7.6).

Genomic DNA for the pCPR 5.1 transfection was screened in an identical fashion. Of the 204 clones, 4 showed the targeted EcoR1 band shift and this was again confirmed by Bgl II digestion (figures 7.5b and 7.7). Unfortunately, 3' p53 sequence outwith the region of homology was not available to investigate the HR event at the 3' end. The results of both experiments are summarised in table 7.1.

Figure 7.5 Southern blots of DNA extracted from ES cells probed with external probe P4. **(a)** **1**, clone R3.72 / EcoR 1 showing targetted band at 3.5 kbp; **2**, negative control (untransfected ES cell DNA) / EcoR 1 showing pseudogene at 3.3 kbp; **3**, R3.72 / Bgl II showing targetted band at 10 kbp; **4**, negative control / EcoR 1. **(b)** pCPR 5.1 targetted clones digested with Bgl II: **1**, negative control; **2-5**, targetted clones showing diagnostic band shift from 16 to 10 kbp and pseudogene at 2 kbp.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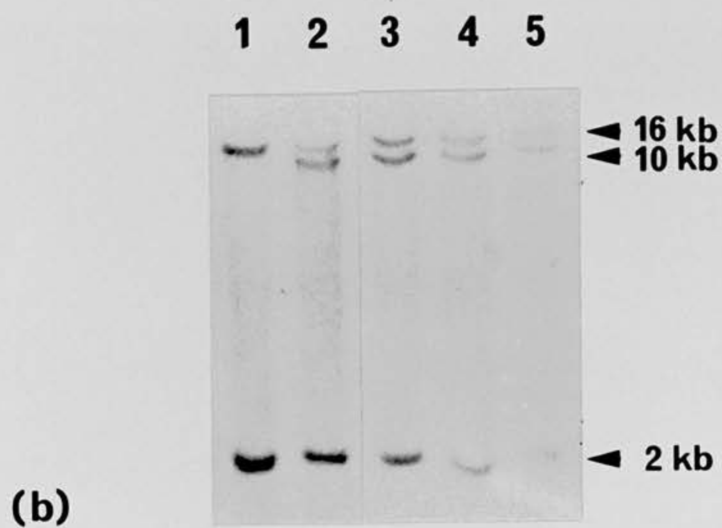
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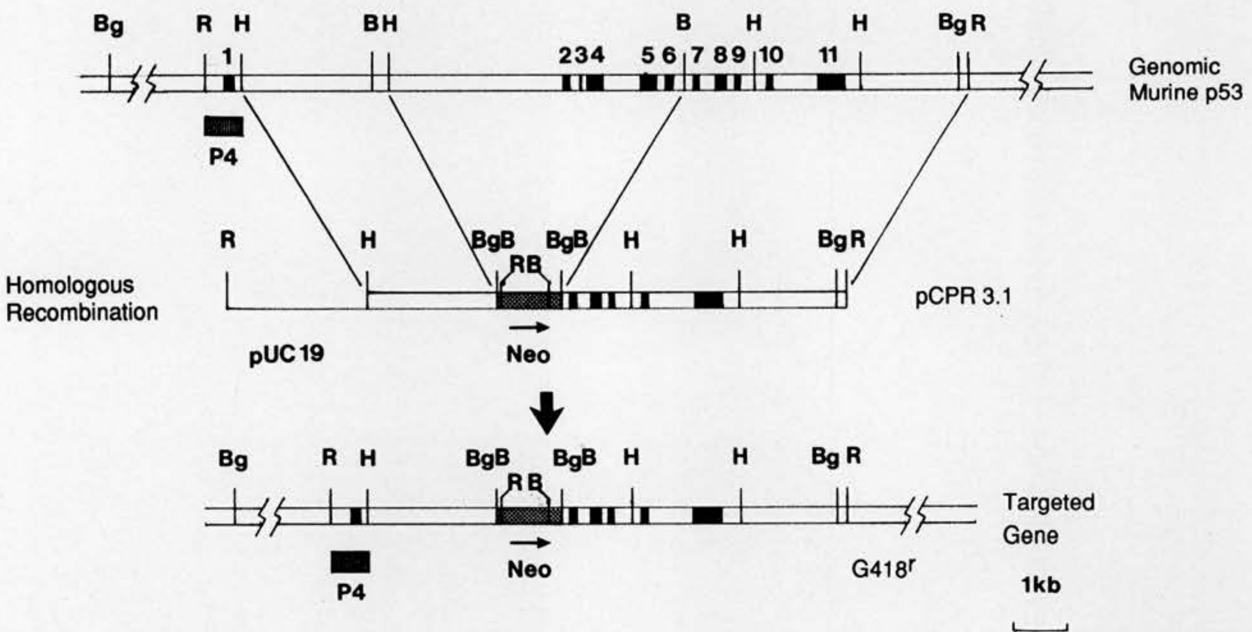
(b)

Figure 7.6 Scale diagram representing the targeting of genomic p53 by homologous recombination with pCPR 3.1. Open boxes represent introns; filled boxes, exons; thin line, plasmid DNA; hatched box, selectable gene (Neo - *neo;* arrow shows the direction of transcription); P4, external probe for Southern blots. Abbreviations for retriction endonucleases are as follows: R. EcoR1; H, Hind III; X, Xho1; B, BamH1; Bg, Bgl II; BgB, BamH 1/Bgl II fusion (will not digest with either enzyme).



Bg
R
H
1
BH
234
B
5 6
7 8 9
H
10
11
H
BgR
Genomic Murine p53
P4
R
H
BgB
RB
BgB
H
H
BgR
Homologous Recombination
pCPR 3.1
pUC19
Neo
Bg
R
H
BgB
RB
BgB
H
H
Bg R
Targeted Gene
P4
Neo
G418^r
1kb

Figure 7.7 Scale diagram representing the targeting of genomic p53 by homologous recombination with pCPR 5.1. Open boxes represent introns; filled boxes, exons; thin line, plasmid DNA; hatched boxes, selectable genes (TK - HSV*tk*, Neo - *neo;* arrows show the direction of transcription); P4, external probe for Southern blots. Abbreviations for retriction endonucleases are as follows: R. EcoR1; H, Hind III; X, Xho1; B, BamH1; Bg, Bgl II; BgB, BamH 1/Bgl II fusion (will not digest with either enzyme).

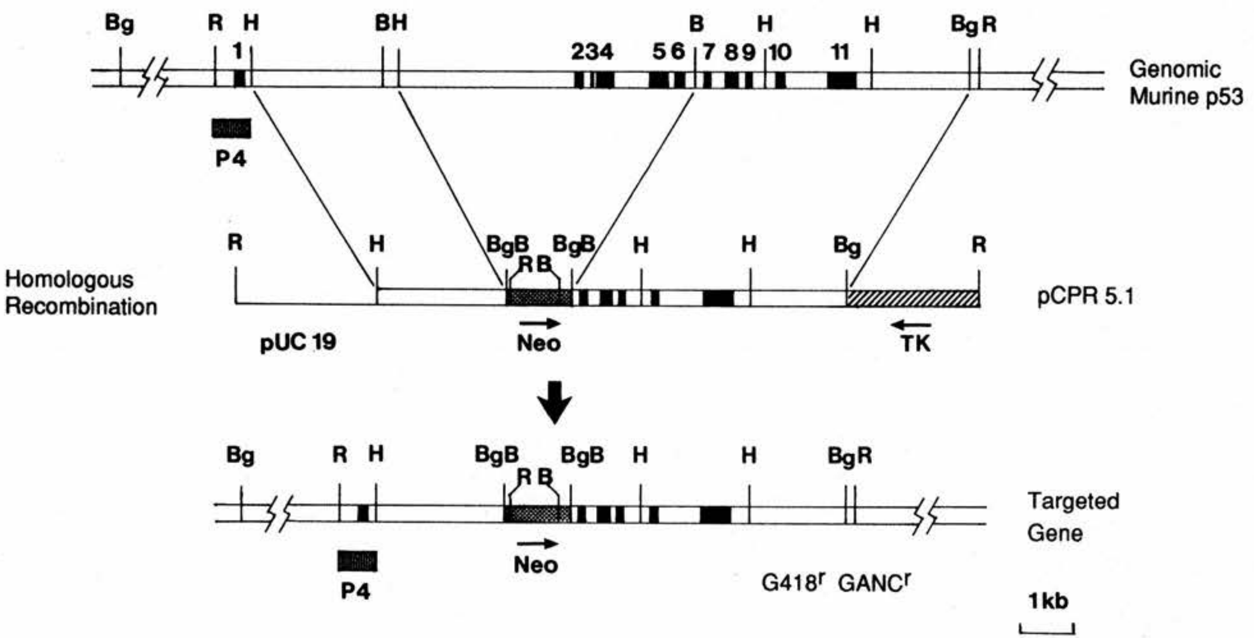

Genomic
Murine p53
Homologous
Recombination
pCPR 5.1
pUC 19
Neo
TK
P4
Targeted
Gene
G418r GANCr
1kb

Table 7.1 Summary of targeting results using replacement vectors

Vector	Selection strategy	Medium	Number of colonies screened	Targeting frequency
pCPR 3.1	Positive	G418	183	1 / 183
pCPR 5.1	Positive Negative	G418 Ganciclovir	204	1 / 51

7.2.4 Germ-line transmission of targeting event

The targeted ES cell clone R3.72 was subsequently expanded and 10-15 cells injected into 3.5 day old blastocysts from F2 (C57/Bl6 x CBA) mice. These were implanted into psuedo-pregnant female mice and chimæric offspring produced identified by their coat colour. Chimæric animals were mated with Ola/129 mice and offspring derived from the targeted cells identified by their chinchilla coat colour. DNA from tail biopsies was screened by southern blotting in the same way as the ES cells for the targeted event. Mating of 2 heterozygotes resulted in mice with homozygous p53 inactivation.

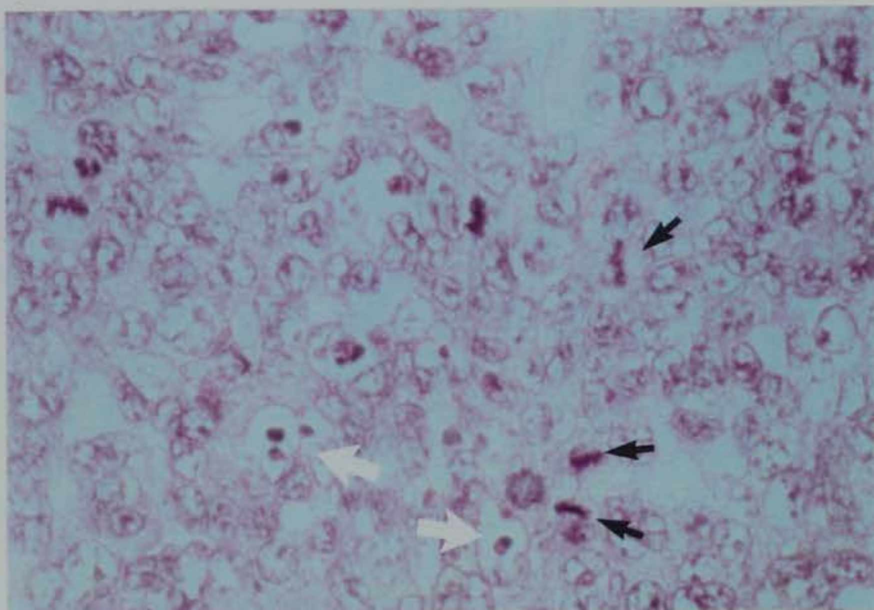
Preliminary analysis suggests that both heterozygous and homozygous mice were developmentally normal. The homozygotes developed tumours at between 1 and 4 months of age (mean 2.7 months) and these were mostly soft tissue sarcomas and lymphomas. The heterozygotes likewise developed a similar spectrum of tumours usually between ages 3 and 12 months (mean 6.8 months) (table 7.2). These tumours tended to be of a high grade; indicated by the presence of numerous mitoses, areas of necrosis and multinucleate cells in the sarcomas (fig. 7.8). Apoptotic figures were also present in the tumours from both heterozygotes (p53+/-) and homozygotes (p53-/-) (fig. 7.8).

Table 7.2 Summary of preliminary analysis of p53 targeted mice. Diagnoses are tentative and await final confirmation.

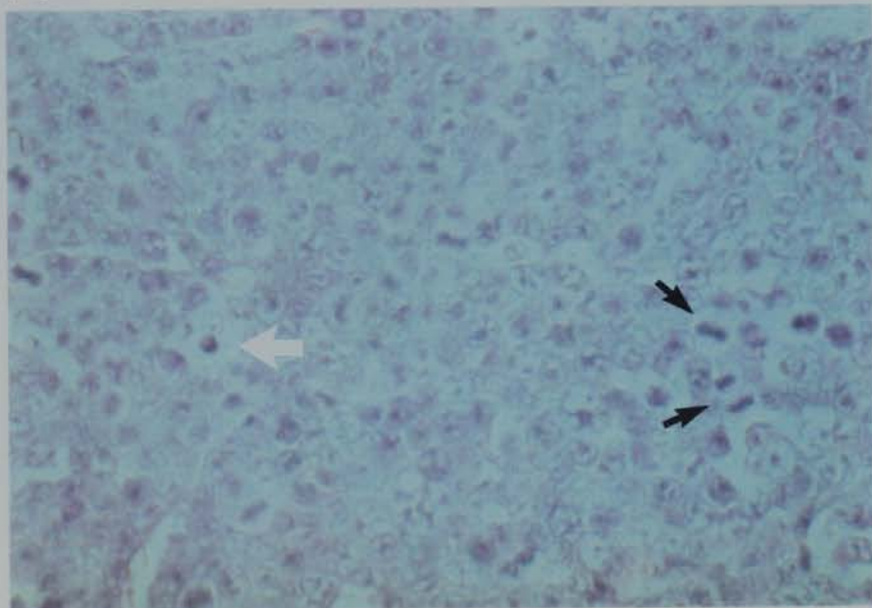
Mouse	Genotype	Age (months)	Tumour
X 220	Homozygote	1	Lymphoma
X 318	Homozygote	2	Sarcoma
X 195	Homozygote	3	Lymphoma
X 228	Homozygote	3	Sarcoma
X 324	Homozygote	3	Lymphoma
X 329	Homozygote	3	Lymphoma
X 196	Homozygote	4	Lymphoma
XS 137	Heterozygote	3	Lymphoma
X 214	Heterozygote	4	Lymphoma
X 166	Heterozygote	4	Lymphoma
X 142	Heterozygote	4	Lymphoma
RX 3	Heterozygote	5	Sarcoma
X 131	Heterozygote	5	Haemangiosarcoma
X 176	Heterozygote	5	Lymphoma
X 181	Heterozygote	5	Lymphoma
X 182	Heterozygote	5	Lymphoma
X 172	Heterozygote	5	Lymphoma
RX 7	Heterozygote	6	Lymphoma
X 59	Heterozygote	8	Sarcoma
X 95	Heterozygote	8	Lymphoma
X 44	Heterozygote	9	Sarcoma
X 317	Heterozygote	9	Lymphoma
X 26	Heterozygote	10	Abdominal tumour
X 55	Heterozygote	10	Lymphoma
X 2	Heterozygote	11	Sarcoma
X 61	Heterozygote	11	Lymphoma
X 5	Heterozygote	12	Liver tumour

Figure 7.8 (a) Photomicrograph of soft tissue sarcoma from mouse X2 (p53 +/-) x200. **(b)** Photomicrograph of a thymic lymphoma from mouse X195 (p53 -/-) x200. Black arrows indicate mitotic figures and white arrows indicate apoptotic figures.

(a)



(b)



7.3 Discussion

The targeted disruption of one copy of the p53 gene has been demonstrated in 5 independent ES cell clones. This was confirmed by showing the predicted band shift in the p53 gene when hybridised to a probe outwith the vector homology thus excluding random integration as a possibility. In the case of the vector pCPR 3.1 this was at a frequency of 1 in 183 using only a positive selectable marker. Previous studies have found targeting a frequency at the *hprt* locus of only 1 in 1000 (Thomas & Capecchi, 1987), 1 in 260 at the *En-2* gene (Joyner et al, 1989) and 1 in 30 at the *ß2 microglobulin* gene (Zijlstra et al, 1989), all employing targeting vectors containing only a positive selection marker. It is clear, therefore, that targeting frequencies vary greatly. This may be due to the recombinogenic potential of different loci, the packaging of the chromatin at these sites and its accessibility for recombination. However, the different amounts of homology in the vectors, the different methods of transfection, the different ES cells and different selection and culture protocols makes more detailed comparison impossible.

By the use of a vector, pCPR 5.1, which differs from pCPR 3.1 by containing a counter selectable marker (HSV-*tk*) the targeting frequency increases from 1 in 183 to 1 in 51 $G418^R$ $Ganc^R$ clones. This approximately 4 fold enrichment is most probably due to the counterselection as the two vectors otherwise differ only in that pCPR 5.1 has 200 bp less homology. In the case of pCPR 5.1 a similar reduction in the number of live clones is seen when ganciclovir is added to the medium (figure 7.4). This level of enrichment is rather lower than other groups. For example 2000:1 enrichment was observed in the *int-2* gene (Mansour et al, 1988) but only 2:1 enrichment

was obtained at the T cell receptor ß sub-unit gene (Mombaerts et al, 1991). Again, differences in methodology preclude further speculation on the underlying causes.

Mice with inactivation of one or both copies of p53 in this study develop normally in keeping with previous work (Donehower et al., 1992). Despite being expressed at high levels during embryogenesis (Schmid et al., 1991) and having a central role in cell cycle control and the onset of apoptosis, p53 inactivation does not influence the development of the mouse. This result has some similarities with finding that inactivation of *pim1* (a gene highly expressed during mouse embryogenesis) does not influence development, suggesting a degree of redundancy in the mammalian genome with the inactivation of seemingly important genes being circumvented without any obvious problem (te Riele et al., 1991). This again demonstrates how gene targeting can lead to unique insights into the control of mammalian development.

The homozygous p53 null mice (p53-/-) develop malignant tumours mainly of mesenchyme and lymphoid tissue with a short latency (mean, 2.7 months) showing a similar pattern to that reported previously (Donehower et al., 1992). This spectrum of neoplasia is strikingly similar to that seen in transgenic mice over-expressing mutant p53 (Lavigueur et al., 1989) (although at a much higher frequency and with a shorter latent period) and in humans with germ-line p53 mutations (Malkin et al., 1991; Srivastava et al., 1991) suggesting a central role for p53 in these tumours.

If p53 inactivation were sufficient alone for tumourigenesis it would be anticipated that all p53 -/- animals would develop the same tumours at the

same site soon after birth which does not happen. The short latency and the fact that not all homozygous p53 -/- have succumbed to malignancy in the time course of follow-up argues strongly that the inactivation of both copies of p53 acting alone is insufficient for carcinogenesis. However, p53 inactivation clearly predisposes to the tumourigenesis possibly by permitting premature DNA replication or replication in the presence of sequence errors with resultant genomic instability and the development of aneuploidy; these processes in turn predisposing to mutation in other genes leading to the acquisition of malignant properties. Such findings suggest that p53 inactivation rather than oncogenic activation by mutation is permissive for carcinogenesis, an observation entirely consistent with the situation in Li-Fraumeni syndrome where the germ-line mutations appear more disabling than activating (Milner & Medcalf, 1991). This evidence also fits with the finding that both copies of p53 are frequently disabled in a common human tumours, such as colorectal cancer (chapters 3-5).

Apoptosis is a fundamental process in normal development and carcinogenesis (Duvall & Wyllie, 1986) and can be induced by wild-type p53 (Yonish-Rouach et al., 1991). However, tumours which lack any functional p53 still undergo apoptosis (fig. 7.8) indicating the presence of other pathways involved in the initiatiation of this process.

Interestingly, Ola/129 mice do tend to develop soft tissue sarcomas and lymphomas with a long latency of more than 22 months (Smith et al., 1973). It remains to be seen if p53 inactivation merely accelarates this inherent tendency or whether there is tissue bias in the emergence of neoplasia. The latter explanation is the more likely in view of the parallels with human Li-Fraumeni syndrome.

In this study, the heterozygous p53 +/- mice succumb to a similar range of tumours as the p53 -/- mice but with a slightly longer latent period (mean 6.8 months). This finding suggests that inactivation of the remaining WT p53 copy is occurring but that this takes a finite time to come about. Analysis of the WT allele in these tumours is under way. The difference between the mean latent periods is relatively short and it might be speculated that inactivation of one p53 allele in the germ-line acting via reduced gene dosage might predispose to loss of the remaining allele. In addition to loss of the WT allele in tumours developed in heterozygous p53+/- mice it would be predicted that the tumours would be DNA aneuploid with multiple divergent stem-lines (chapter 3). This analysis is also underway.

In a previous study (Donehower et al., 1992), the heterozygous (p53 +/-) mice appeared to develop tumours infrequently and with a longer latency (only 2 out of 96 mice after 9 months follow-up). The targeting strategy used in that particular study employed the poly-adenylation signal of the endogenous targetted p53 to act for the *neo* gene within the vector. The result of this was an enrichment for homologous recombination events over random integration. However, it did result in transcription of the 3' end of p53 including exons 5-11 (codons 158-390) which includes the region of p53 responsible for oligomerization. Although p53 could not be detected in immunoblots, expression of low levels of possibly mutated and biologically active p53 cannot be excluded making the phenotype difficult to interpret. This may also explain the slight difference in phenotype of the p53 +/- mice. The vector used here, pCPR 3.1, contains numerous STOP codons following the poly-adenylation signal of *neo* thus preventing any p53 transcription. However, it is also possible that the observed difference in phenotype is due

to the use of mice with a different genetic background or varied carcinogen exposure.

Inactivation of both copies of p53 leads to a developmentally normal animal with a predisposition to a spectrum of malignancies. Such animals and cell lines cultured from them will provide the ideal tool to investigate and dissect the processes by which p53 acts and an *in vivo* system to test carcinogens, cancer prevention measures and therapy.

Chapter 8

Conclusion

It has taken 14 years since its first isolation to identify a role for p53. This appears to be as a "surveyor" of the genome identifying DNA errors or damage and then delaying replication until this has been rectified or if this should prove impossible eliminating the damaged cell by initiating apoptosis. It is not therefore surprising that its inactivation is implicated in so many malignancies.

In this thesis, it has been shown that in human colorectal cancer p53 mutation appears to result in p53 over-expression detected immunohistochemically. The over-expression is closely associated with 17p allele loss in the same tumours and the region deleted on 17p usually involves the p53 gene locus. Thus, inactivation of both p53 alleles is common in colorectal cancer. The timing of this over-expression and mutation is at the adenoma (benign) to carcinoma (malignant) transition suggesting a critical role for p53 in the acquisition of malignant potential in this common human malignancy. Furthermore, both p53 over-expression and 17p allele loss correlate with the presence pf DNA aneuploidy and multiple divergent clones within a tumour. This is consistent with the hypothesis of the effects of loss of p53 genomic surveillance and thus perhaps accelarated acquisition of the genetic alterations conferring tumour aggression. This would also explain the poorer prognosis of patients with tumours showing DNA aneuploidy or 17p allele loss.

Loss of 17q alleles has also been identified as a more frequent event in human colorectal cancer than previously noted and shown to correlate with

the presence of lymph node metastasis. This suggests the inactivation of a gene involved in the suppression of tumour metastasis in human colorectal cancer on chromosome 17q not hitherto detected. Further deletion mapping and detailed analysis of candidate genes will be necessary to identify this important locus.

An attempt to introduce a single activating point mutation into the ES cell p53 gene was partially successful and back-selection of clones containing a correctly inserted vector is underway. This should create an accurate model of the human, family cancer syndrome, Li-Fraumeni syndrome. In addition, cell lines from such animals will contain a single copy of the temperature sensitive mutant p53 val^{135} under the control of its own promoter and in its correct chromosomal site permitting study of cells more similar to the *in vivo* situation.

A p53 null mouse was successfully generated by gene targeting in mouse ES cells. This animal is, perhaps surprisingly, developmentally normal, suggesting a degree of genetic redundancy in mammalian development. However, the p53-/- animals succumb to malignancies at an early age, mostly of lymphoid and mesenchymal origin with similarities to Li-Fraumeni syndrome. Furthermore, heterozygote p53+/- mice also develop the same spectrum of malignancies but with a longer latency suggesting that the difference in timing between p53-/- and p53+/- is the loss of the remaining p53 allele in heterozygous animals.

These p53 -/- and p53+/- animals provide a tool for the investigation of the normal function of p53, the commonest target for genetic events in human malignancy. Comparison of p53-/- mice with p53+/- littermates for rates of

apoptosis, proliferation, spontaneous mutation, response to carcinogens and DNA repair, amongst other parameters, will give insight into p53 function *in vivo* and in tumourigenesis. Such animals could also be used to investigate possible environmental carcinogens, cancer prevention measures and therapeutic strategies in cancer management.

Chapter 9

MATERIALS AND METHODS

9.1 Specimen Collection

All the tumours studied comprised a consecutive series of surgical resections from two local hospitals between June 1988 and January 1990. Surgical colectomy specimens were collected onto ice in the operating theatre immediately upon resection. They were opened and rinsed in ice-cold PBS/EDTA (20mM sodium phosphate buffer pH 7.4, 137mM sodium chloride, 0.5mM EDTA) and dissected within 30 minutes of resection. Samples for DNA extraction, cryostat sectioning and flow cytometry were placed in vials, snap frozen in liquid nitrogen and subsequently stored at -70°C until required. Adjacent tissue was placed into PLPD fixative (37.5mM sodium periodate, 7mM lysine, 1% paraformaldehyde, 2.5% potassium dichromate, 18.75mM sodium phosphate buffer pH 7.4) (Holgate et al., 1986) and fixed overnight at 4°C prior to paraffin embedding. The remainder of the specimen was then fixed in formalin and processed routinely for histopathological assessment.

Tumours were classified as distal if located in the sigmoid colon or rectum and proximal if located in the remainder of the colon.

9.2 Histopathological grading and statistical analysis

Carcinomas were reviewed and graded by a single pathologist (J.Piris) using criteria recommended by the UKCCCR for Dukes' stage, histological type, degree of differentiation and nature of invasive margin. Adenomas were similarly reviewed and graded by a single pathologist (J.T.O'Grady) for morphologic type and degree of dysplasia.

The statistical tests and tables used in this thesis were applied as recommended by Siegel & Castellan, 1988.

9.3 Flow Cytometry

Three or more samples from separate sites in each carcinoma were analysed using the method of Vindelov (1983a & b). Multiple sites were sampled to avoid misclassification as DNA diploid in view of the known heterogeneity of DNA ploidy in colorectal carcinomas (Quirke et al., 1985). Normal colonic mucosa from each patient was analyzed to control for false aneuploid peaks caused by autolysis (Alanen et al., 1989). A piece of tissue approximately 5 mm^3 was disaggregated under a citrate buffer by cutting with a clean scalpel blade and 100 µl transferred to a disposable plastic test-tube. 25 µl of avian erythrocytes were added to act as an internal standard followed by 450 µl of solution A. The sample was mixed by whirlmixer and incubated for 10 minutes at room temperature. Solution B (325 µl) was added, mixed and incubated for 10 mins at room temperature and finally 250 µl solution C added, mixed and incubated on ice for 10 minutes.

Citrate Buffer (pH 7.6)

250mM Sucrose

40mM Trisodium Citrate

5% DMSO

Stock Solution (pH 7.6)

3mM Trisodium Citrate

0.5mM Tris

1% Nonidet P40

Solution A

30 µg/ml Trypsin

Diluted in Stock solution

Solution B

0.5mg/ml Trypsin inhibitor

0.1mg/ml Ribonuclease A

Diluted in Stock solution

Solution C

0.42mg/ml Propidium iodide

1mg/ml Spermine tetrahydrochloride

Diluted in Stock solution.

The sample was then filtered through loose nylon wool and analysed on a Coulter EPICS CS flow cytometer at an excitation wavelength of 488nm. At least 10,000 nuclei were analysed in each sample and cases were scored as aneuploid if any of the samples showed a Go/G_1 peak distinct from the normal diploid peak (as assessed from the internal standard). Coefficients of variation of the diploid peak were typically less than 3.5. DNA index was calculated by dividing the channel number of the aneuploid peak by that of the diploid peak.

In cases where only one frozen sample was available, nuclei were extracted from formalin fixed paraffin embedded tissue taken for routine histology.

Sections 50 µm thick were cut, dewaxed in xylene and rehydrated through grade alcohols prior to digestion in 0.5% pepsin in 0.9% NaCl (pH 1.5) at 37 °C for 60 minutes. The cells were washed three times in fresh PBS, filtered through nylon wool and stained in PBS containing 100 µg/ml propidium iodide overnight at 4 °C. The samples were then analyzed as described above.

9.4 Immunohistochemistry

Frozen Sections

4 μm cyrostat sections of tumour were cut, acetone fixed for 10 minutes then air-dried. Sections were washed in Tris buffered saline (TBS) for 10 minutes before incubation in normal rabbit serum diluted 1:5 in TBS (NRS/TBS) for 10 minutes.

Tris buffered saline

145mM Sodium Chloride

20mM Tris pH 7.6

Appropriately diluted antibody (PAb 1801 1:100 in NRS/TBS, PAb 240 as neat hybridoma culture supernatant) was applied for 30 minutes, washed in TBS and followed by a further 10 minute incubation in NRS/TBS. Bound antibody was detected by application of rabbit antibodies to mouse immunoglobulins conjugated to horse radish peroxidase (1:20 in NRS/TBS) for 30 minutes, washed in TBS then visualised with diaminobenzidene (1mg/ml in 0.2M Tris pH 7.6, 0.03% hydrogen peroxide) for 2-5 minutes. After a brief wash in tap water sections received a light haematoxylin counterstain followed by another tap water wash, dehydration in graded alcohols and xylene before mounting under coverslips (in DPX).

Paraffin Sections

3 μm sections of PLPD fixed, paraffin embedded tissue were cut, floated onto glass slides and allowed to dry. After dewaxing in xylene, rehydration in

graded alcohols and washing for 5 mins in TBS, NRS/TBS was applied for 20 minutes. This was followed by incubation in PAb 1801 (OSI p53Ab-2) diluted 1:100 in NRS/TBS for 60 mins. Bound antibody was detected by incubation in biotinylated rabbit antibodies to mouse immunoglobulins (1:400 in NRS/TBS) for 10 mins followed by avidin-biotin complex linked to horseradish peroxidase (Dako) for 30 mins. After TBS washing, visualisation with diaminobenzidene, haematoxylin counterstaining, dehydration and mounting was identical to that used for frozen sections.

Classification

Cases were scored as antibody positive if any positively staining cells were detected in a section.

9.5 Immunoblot

Sample preparation

SV80 cells were grown to confluence on a 90mm Petri dish. The medium was aspirated and the cells washed twice in phosphate buffered saline (PBS).

Phosphate buffered saline (Delbuccos)

137mM Sodium Chloride

2.7mM Potassium Chloride

1.5mM Potassium dihydrogen orthophosphate

8.1mM Disodium hydrogen orthophosphate

The PBS was aspirated and 1 ml 1 x SDS-PAGE loading buffer was then added.

1 x SDS-PAGE loading buffer

50mM Tris Cl (pH 6.8)

100mM Dithiothreitol

2% Sodium dodecyl sulphate

0.1% Bromophenol blue

10% Glycerol

Cells were scraped up, placed into test tubes and incubated for 10 minutes. After sonication until no longer viscous the sample was spun at 100,000 x g on an ultracentrifuge at 20°C for 30 minutes. The supernatant was removed and a 50 µl aliquot boiled for 3 minutes and then loaded on to a 10% SDS-PAGE gel. The remainder was stored frozen at -70°C. Human colonic tissue was disaggregated in ice-cold PBS using a clean scalpel blade transferred to

a microfuge tube and centrifuged at 13,000 rpm for 5 mins at 4°C. The pellet was resuspended in 500 µl of ice-cold suspension buffer.

Suspension Buffer

100mM NaCl

10mM Tris Cl (pH 7.6)

1mM EDTA (pH 8.0)

1 µg/ml aprotinin

100 µg/ml phenylmethylsulphonyl fluoride (PMSF)

As rapidly as possible thereafter 500 µl of 2 x SDS gel loading buffer was added.

2 x SDS gel loading buffer

100mM Tris Cl (pH 6.8)

200mM Dithiothreitol (DTT)

4% SDS

0.2% bromophenol blue

20% glycerol

The sample was then boiled for 10 minutes, sonicated for 30 seconds to shear high molecular weight chromosomal DNA and spun at 13,000 rpm at room temperature for 10 minutes. The supernatant was transferred to a fresh tube and a 50 µl aliquot boiled for 3 minutes prior to loading on 10% SDS-PAGE for analysis.

SDS PAGE

The gel mould plates were thoroughly cleaned with distilled water and 100% ethanol and assembled with 0.75mm spacers. A 10% resolving gel was poured and allowed to polymerise with an overlay of isobutanol.

10% Resolving Gel

10% Acrylamide : Bisacrylamide (39:1)

375mM Tris Cl pH 8.8

0.1% SDS

10 µl 10% APS/ml of gel

0.4 µl TEMED/ml of gel

After 30 minutes the isobutanol was poured off, the gel washed with distilled water and the stacking gel poured.

5% Stacking gel

5% Acrylamide:Bisacrlamide (39:1)

125mM Tris Cl pH 6.8

0.1% SDS

10 µl 10% APS/ml of gel

1µl TEMED/ml of gel

The comb was inserted, and the gel allowed to polymerise for 30 minutes. On removing the comb the wells were washed with distilled water and the apparatus assembled with electrophoresis buffer in both chambers.

Electrophoresis buffer

25mM Tris pH 8.3

250mM Glycine

0.1% SDS

Samples each containing approximately 50 μg total protein were boiled for 3 minutes in 1 x SDS gel loading buffer and loaded. A molecular weight standard (MW SDS-70L Sigma) was run along with the tissue sample. The gel was run at 100-150V until the bromophenol blue had reached the bottom of the gel. Two identical gels were run at the same time: one was stained with Kenacid Blue to ensure quality and equal loading of total protein; the other was electroblotted for immunostaining.

Kenacid Blue stain

0.002% Kenacid Blue

45% Methanol

10% Acetic acid

2 hrs then destain

Kenacid destain

45% Methanol

7% Acetic Acid

2 changes, 2 hrs.

Western Blotting

The proteins were electrostatically transferred to a nitrocellulose filter (hybond-C) in transfer buffer at 50V overnight at $4^{o}C$.

Transfer Buffer

15mM Tris pH 8.3

120mM Glycine

25% Methanol

After transfer the apparatus was dismantled, the filter washed in distilled water and allowed to air dry. The marker track was cut off and stained briefly in Panceau S.

Panceau S

0.2% Panceau S

3% Trichloroacetic acid

3% Sulfosalicylic acid

Then destained three times in 5% acetic acid. The remainder of the filter was washed in TBS-A .

TBS-A

137mM NaCl

20mM Tris Cl pH 7.6

then blocked overnight in blocking solution.

Blocking Solution

10% milk protein (Amersham)

0.1% Tween in TBS-A.

Copious washing with 0.1% Tween in TBS-A was carried out between each stage. The gel was incubated in PAb 1801 diluted 1:200 in blocking solution for 2 hours, washed, incubated in biotinylated rabbit antimouse antibodies (1:500 in blocking solution) for 20 mins, washed and exposed to streptavidin-alkaline phosphotase conjugate (1:3000 in TBS-A) for 20 mins. After further washing protein binding was detected using NBT and BCIP in diethanolamine buffer.

Enzyme substrate buffer

380mM Bromochloroindolyl phosphate

400mM Nitro blue tetrazolium

10mM Magnesium chloride

137mM Sodium chloride

20mM Tris Cl pH 9.5

for about 5 mins until bands appeared then the reaction stopped by washing in distilled water. The filter was then dried and photographed.

9.6 Southern Blot Deletion Analysis

DNA Extraction

Tissue was homogenised in 10ml lysis buffer using a glass homogeniser.

Lysis Buffer

100mM Tris Cl pH 7.8

20mM NaCl

1mM EDTA

1% SDS

Proteinase K was added to a final concentration of 500 µg/ml and the sample incubated at 48°C for 48 hrs with intermittent mixing. Extraction with equal volumes of phenol, PC-9 and chloroform isoamyl alcohol (24:1) were carried out sequentially.

DNA was then precipitated in 0.5 volumes of 7.5M ammonium acetate and 2 volumes of ethanol at -20°C, spun down, dried and resuspended at a concentration of 1mg/ml.

Blood samples were lysed in an equal volume of lysis buffer for 30 mins, extracted with an equal volume of phenol and precipitated in ½ volume 7.5M ammonium acetate and 2 volumes of ethanol. The pellet was resuspended in lysis buffer containing 0.5mg/ml proteinase K and incubated overnight at 48°C. Thereafter the sample was extracted, precipitated and resuspended in the same way as for colonic tissue.

Southern Blotting

Paired samples of 10µg of normal and tumour DNA were restriction enzyme digested overnight in a total volume of 30 µl in accordance with the manufacturer's recommendations. 5 µl of loading buffer was added to each samples and they were loaded onto a 0.8% agarose gel and electrophoresed overnight at 4°C in 1 x TBE buffer.

Loading Buffer

95% Deionised formamide

20mM EDTA pH 8.0

0.03% Xylene cyanol FF

0.03% Bromophenol blue

1 x TBE

89mM Tris

2mM EDTA pH 8.0

89mM Boric acid

The gel was viewed under UV light to check for complete digestion, denatured and DNA transferred to a charged nylon membrane (Genescreen-Plus, Dupont) by capillary action with denaturing buffer.

Denaturing Buffer

0.5M Sodium hydroxide

1.5M Sodium chloride

After overnight blotting the membrane was air dried and then placed in a sealed plastic bag containing prehybridisation buffer and incubated at 65°C for at least one hour.

Prehybridisation Buffer

10% Dextran Sulphate

6 x SSC

1 % SDS

0.1mg/ml Single stranded human placental DNA

20 x SSC

3M Sodium chloride

0.3M Trisodium citrate pH 7.0

Plasmids containing the DNA probes were cut with the appropriate enzyme to yield the desired fragment, run on a 1% low melting point (LMP) agarose gel containing 0.5 µg/ml ethidium bromide and the fragment cut out. The DNA was extracted from the agarose using the Geneclean II kit (BIO-101) in accordance with the manufacturer's protocol. The yield of DNA was estimated by comparison with standard DNA after electrophoresis of an aliquot in an eithidium bromide agarose gel. Probes were labelled to high specific activity with ^{32}P α dCTP (Amersham) using the random prime method of Fienberg and Vogelstein (1983). A commercial kit (Amersham) was employed to label 25 ng of heat denatured probe DNA in accordance with the protocol. Labelled probe was separated from unincorporated ^{32}P α dCTP by passage over a G50 Sepharose Column, denatured by boiling for 10 minutes and added to 25ml prehybridisation buffer to preincubate for one hour at 65°C prior to addition to the filter. Hybridisation proceeded overnight at 65°C and then filters were washed at different stringencies depending on the probe used:

YNZ 22

2 x SSC, 0.5% SDS at room temperature

0.1 x SSC, 0.5% SDS at 65°C (2 washes)

0.1 x SSC, 0.1% SDS at room temperature

MCT 35

2 x SSC, 0.5% SDS at room temperature

0.1 x SSC,0.1% SDS at 65°C (2 washes)

0.1 x SSC at room temperature

THH 59

2 x SSC, 0.5% SDS at room temperature

1 x SSC, 0.1% SDS at 65°C (2 washes)

0.1 x SSC at room temperature

After the final wash filters were blotted dry, wrapped in transparent plastic wrap and exposed to X-ray film (Fuji) with intensifying screens at -70°C for 2-5 days. X-ray cassettes were warmed to room temperature and the film developed in Kodak D19 for 5 minutes, washed in tap water, fixed in FX 40 for 5 minutes, washed again and dried. Cases were classified as: homozygous (HOM) if only one band was visible in normal DNA; heterozygous (HET) if two bands were resolved in both normal and tumour DNA; and loss of heterozygosity (LOH) if there were two bands in the normal and if one of the bands was either absent or showed marked diminution in the tumour sample.

9.7 PCR Deletion Analysis

DNA Preparation

DNA was extracted from formalin fixed, paraffin embedded tumour tissue which had been taken for routine histopathological assessment. Initially standard haematoxylin and eosin stained sections were examined and the area containing the highest proportion of neoplastic cells was demarcated. 10 µm sections from the corresponding paraffin block were cut and floated onto glass slides from which the chosen area was removed to an 1.5ml microfuge tube. Four 10 µm sections from each case were resuspended in 200 µl paraffin tissue lysis buffer (Jackson et al., 1990).

Paraffin Tissue Lysis Buffer

100mM Sodium chloride

10mM Tris Cl

25mM EDTA

0.5% SDS pH 8.4

Proteinase K was added to a final concentration of 0.1mg/ml and incubated for 5 days at 37°C. DNA was extracted twice with phenol:chloroform:iso-amyl alcohol (25:24:1), once with chloroform:isoamyl alcohol (24:1) and precipitated with 0.1 volume 3M sodium acetate (pH 5.2) and 2 volumes of ethanol. After centrifugation and drying the DNA was resuspended in 20 µl TE for 3 days and 1 or 2 µl used for amplification.

PCR amplification

Two sets of primers were used to amplify p53 sequences around the 2 polymorphisms in exon 4 and intron 6.

Exon 4 primers

954 5' GAG AGA AGC TTG CAG CAG CCA GAC TGC CTT GCG GGT GAC T 3'

959 5' GAA GAG AGA TCT GCA GGG GGA TAC GGC CAG GCA TTG AAG 3'

Intron 6 Primers

883 5' GAG AGA AGC TTC ACT TGT GCC CTG ACT TTC AAC TCT G 3'

870 5' AGA GAG GGA TCC GGG AGG TCA AAT AAG CAG CAG GAG 3'

PCR reactions were set up in a Class 2 hood in a laboratory which never contained amplified products using positive displacement pipettes. Extensive precautions were taken to avoid cross contamination. The 100 µl PCR mix contained: 2 µl of paraffin extracted DNA or 500ng of normal genomic DNA; primers at a final concentration of 1 µM; dNTP's each at 200µM; 0.1 volume of NBL 10 x PCR buffer; and 2.5 units Taq polymerase.

10 x PCR Buffer

100mM Tris Cl pH 8.8

500mM Potassium chloride

15mM Magnesium chloride

1% Triton X-100

Samples were thoroughly mixed, centrifuged and overlain with 100 µl paraffin oil. Amplification was carried out on a programmable heating block starting

with 2 minutes at 94°C followed by 30 cycles of 55°C for 2 minutes, 72°C for 3 minutes, 94°C for 1 minute and a final cycle of 55°C for 2 minutes, and 72°C for 10 minutes to ensure complete extension of all products. 20 µl of amplified PCR mix was digested with BstU 1 or Msp1 in a 30 µl reaction mix in accordance with manufacturer's recommendations. The fragments were analysed by electrophoresis through a 3% agarose gel (3:1, NuSieve GTG: SeaKem GTG) containing 0.5 µg/ml ethidium bromide, viewed under UV light and photographed.

Cases for paraffin DNA analysis were chosen as those which give a heterozygous pattern from amplification and restriction digestion of normal genomic DNA. Paired normal and tumour samples were run together and the cases classified as showing a p53 deletion if one of the two bands were absent or markedly diminished in the tumour sample compared to normal.

9.8 Sequencing Human Genomic p53

Exons 5 - 9 of human p53 were amplified in two sets of reactions with 2 sets of primers situated in adjacent introns.

Exons 5 and 6

883 5' GAG AGA AGC TTC ACT TGT GCC CTG ACT TTC AAC TCT G 3'

870 5' AGA GAG GGA TCC GGG AGG TCA AAT AAG CAG CAG GAG 3'

Exons 7, 8 and 9

890 5' GAG AGA AGC TTG CCA CAG GTC TCC CCA AGG CGA A 3'

891 5' AGA GAG GGA TCC ACT TTC CAC TTG ATA AGA GGT CCC AAG 3'

Again, elaborate precautions were taken to avoid cross-contamination of reactions with reaction products. Negative control (template free) reactions were run with each batch to check for contamination. 100 µl PCR reactions were set up containing: 500ng genomic DNA; primers at 2.5 µM final concentration; 10% DMSO; 1 x Taq polymerase buffer; dNTP's each at a final concentration of 200 µM; and 5 units Taq polymerase.

10 x Taq Polymerase Buffer

100mM Tris Cl pH 8.8

30mM Ammonium sulphate

6mM Magnesium chloride

10mM ß mercaptoethanol

Samples were overlain with paraffin oil and placed in a programmable heating block for 30 cycles of 96°C for 1.3 minutes, 53°C for 1.6 minutes and 71°C for 2.5 minutes. On completion, the entire sample was electrophoresed through a

1% LMP agarose gel (BRL Ultrapure) against a standard marker. The amplified band was cut out and the DNA was extracted by squeeze-freezing.

Squeeze-Freeze DNA Extraction

The agarose plug was equilibrated with 500mM sodium acetate, 1mM EDTA for 30 minutes, frozen in liquid nitrogen then spun out through siliconised glass wool in a microfuge. The DNA was then precipitated twice in the presence of dextran carrier (2 μl of 10 mg/ml dextran) with 0.3 volume 3M sodium acetate and 3 volumes of ethanol at -70°C for 5 minutes. After warming to room temperature for 5 minutes and spinning in a microfuge for 5 minutes, the final pellet was air-dried, suspended in 20 μl TE and restriction digested with BamHI and HindIII as directed by the enzyme manufacturer. After one hour at 37°C the DNA was again separated through a 1% LMP agarose gel and re-extracted by squeeze-freezing. The plasmid vector (pUC19) was prepared by restriction digestion with HindIII and BamHI, electrophoresis through 1% LMP agarose gel and squeeze-freeze extraction.

Ligation

Approximately 100 ng vector and 50 ng insert were ligated in a reaction containing: 1mg/ml bovine serum albumin; 1mM ATP, 1 x ligase buffer; and 1 μl T4 DNA ligase.

10 x Ligase Buffer

500mM TrisCl pH 7.6

100mM Magnesium chloride

100mM Dithiothreitol

The reaction was incubated at 16°C overnight and 5 μl used to transform

competent E.coli. DH5α E.coli were made competent by the calcium chloride method (Sambrock et al., 1989) and transformed by mixing 100 μl thawed cells with the ligation mix and incubating on ice for 30 minutes. The cells were heat shocked to 42°C for 45 seconds then allowed to recover on ice for 2 minutes. 900 μl of SOC was then added, the cells incubated at 37°C for one hour, gently spun down, resuspended in 100 μl of medium then plated out on LB agarose plates containing 50 μg/ml ampicillin.

SOB

2% Bacto-tryptone

0.5% Bacto yeast extract

0.005% Sodium chloride

2.5mM Potassium chloride

Magnesium chloride was added to a final concentration of 10mM prior to use.

SOC

SOB containing 20mM glucose

LB Medium

1% Bacto-tryptone

0.5% Bacto yeast extract

1% Sodium chloride

LB agar

LB medium with 15 g/litre agar

Following overnight incubation at 37°C the number of colonies on each plate

was assessed. Typically the "vector only" ligation produced less than 5 colonies whereas the vector plus insert ligation produced 50-400 colonies. Only those with >100 colonies proceeded to the next stage. Those with <100 colonies were re-transformed with more ligation mix or the ligation repeated until sufficient colonies were obtained.

A mass preparation using all the colonies was prepared by washing the bacteria from each plate into 500ml of LB medium containing 50 µg/ml ampicillin. This was incubated overnight in a shaking incubator at 37°C. The bacteria were pelleted at 7000 rpm for 10 minutes at 4°C, resuspended in 10ml solution I and left at room temperature for 5 minutes.

Solution I

50mM Glucose

25mM Tris Cl pH 8.0

10mM EDTA pH 8.0

Bacteria were then lysed by the addition of 20ml of fresh solution II

Solution II

0.2M Sodium hydroxide

1% SDS

placed on ice for 10 minutes and 15ml of solution III added.

Solution III (100ml)

5M potassium Acetate 60ml

Glacial acetic acid 11.5ml

Distilled water 28.5ml

After thorough mixing, the mixture was placed on ice for 20 minutes then cleared by centrifugation at 7000 rpm for 10 minutes at 4°C. The supernatant was transferred to a clean tube and nucleic acid precipitated by the addition of 27ml (0.6 volumes) isopropanol at room temperature for 15 minutes. After centrifugation at 8000 rpm for 10 minutes at room temperature, the pellet was resuspended in 4ml TE (pH 7.5) then 4.8g CsCl and 200 µl ethidium bromide (10mg/ml) added. Once the CsCl was dissolved the solution was transferred into an ultracentrifuge tube and spun on a Beckman Ultracentifugre for 4 hours at 63,000 rpm (20°C). The plasmid DNA band was visualised under UV light, removed with a syringe and needle and the ethidium bromide removed by 3 extractions with isoamyl alcohol. The plasmid DNA was diluted in 3ml TE and 12ml ethanol added. DNA was precipitated at -20°C for 4 hours. After warming to room temperature the DNA was pelleted by centrifugation at 10,000 rpm, air dried, resuspended in TE and again precipitated with 0.1 volume 3M sodium acetate and 3 volumes ethanol. The resulting pellet was again redissolved in TE and the concentration adjusted to 1mg/ml.

Sequencing

Plasmid DNA was initially denatured and annealed to specific primers for each exon.

5D **1038** 5' TTC ACT TGT GCC CTG AC 3'

5R **1008** 5' GCC CAG GGT CCC CAG GC 3'

7D **1041** 5' GCC ACA GGT CTC CCC AA 3'

8R **931** 5' GAA TCT GAG GCA TAA CTG 3'

9D **1042** 5' GAC CAA GGG TGC AGT TA 3'

Double-stranded DNA was denatured prior to sequencing in one of two ways:

Maxiprep DNA - 10 µg double standard plasmid DNA was denatured in 0.2M NaOH for 10 minutes at room temperature then precipitated with 0.5 volume 7.5M ammonium acetate and 3 volumes of ethanol at -70°C for 5 minutes. The dried pellet was resuspended in 5 µl distilled water and 1 µl taken and annealed to 12.5ng primer in 1 x sequencing buffer plus 10% DMSO in a volume of 10 µl. The mixture was heated to 65°C for 2 minutes and allowed to cool slowly to <35°C.

Miniprep DNA - Hsiao et al., 1991. DNA was extracted from a 1.5 ml overnight culture by the method described in section 9.9 except that at the final stage the pellet was resuspended in 25 µl of TE containing RNAase A. 5 µl was mixed with 2 µl primer (10ng) and denatured with 1 µl 1M hydrochloric acid at 37°C for 10 minutes. The mixture was neutralized with 1 µl 1M sodium hydroxide and 2 µl 5 x sequencing buffer added and incubated at 37°C for 5 minutes. Thereafter the sequencing reaction proceeded as described.

5 x Sequencing Buffer

200mM Tris Cl pH 7.5

100mM Magnesium chloride

250mM Sodium chloride

Sequencing was carried out using the dideoxy chain termination method using the Sequenase 2.0 kit (United States Biochemical). The protocol used was that supplied with the kit with slight modifications: 1 µl DMSO was added to the annealing step and 0.5µl to the extension step; the tubes containing the termination mixes were not pre-warmed as this step was found to be unnecessary; 1µl Mn buffer was added at the extension step when

sequences near to the primer were required. Samples were stored at -20°C until required.

A 0.25mm thick polyacrylamide gel was poured between glass plates and allowed to polymerise.

6% Urea Acrylamide Gel:

6% Acrylamide:Bis acrylamide(39:1)

8M Urea

1 x TBE

1 µl 25% Amonium Persulphate per ml of gel

1 µl TEMED per ml of gel

Prerunning at 40W was carried out until the gel had reached 50°C then the samples were denatured by heating to 75°C for 2 minutes and 2 µl loaded onto the gel. Once the gel had run a sufficient distance it was allowed to cool, the plates separated and the gel fixed in 12% methanol, 10% acetic acid for 20 minutes. Fixative was poured off, and the gel allowed to stick to a piece of Watmann 3MM filter paper. The gel was covered in clear film, vacuum dried at 80°C for 1 hour then exposed to X-ray film overnight at -70°C. without intensifying screens. The sequence was read by eye.

9.9 Vector Construction

The clone used in all the targeting vectors, pMSVp53-G, was initially restriction mapped by single and double restriction digestion, electrophoresis through a 1% agarose gel containing 0.5 µg/ml ethidium bromide and visualisation under UV light. The gel confirmed the published map (Bienz et al., 1984).

Fragments were isolated by separation through a 1% LMP agarose gel following digestion with the appropriate enzyme. The desired band was cut out using a clean scalpel blade and the DNA extracted using the Geneclean II kit (Bio 101). The DNA was resuspended in 10 µl distilled water and 1 µl run into an agarose gel (containing ethidium bromide) along with standard concentrations of plasmid DNA to estimate the concentration. In cases where the vector had compatible, cohesive ends 2 units of calf Intestinal Alkaline Phosphatase was added to the digestion mix (after digestion was complete) and incubated at 37°C for a further 1 hour. The alkaline phosphatase was then inactivated by heating to 85°C for 10 minutes and the DNA electrophoresed and recovered as detailed above.

Ligation were carried out in a total volume of 10 µl containing: 100ng of liniarised vector; an equimolar quantity of insert DNA; 1mM ATP; and T4 DNA ligase buffer.

T4 DNA Ligase Buffer

50mM TrisCl pH 7.6

10mM Magnesium chloride

10mM Dithiothreitol

1 μl of T4 DNA ligase was added and allowed to incubate overnight at 16°C. Vector only controls (containing no insert DNA) were included for each reaction. The total 10 μl ligation was used to transform competent E.coli (HB101 Stratagene) and 200 μl plated out on LB agar containing 50 μg/ml ampicillin. Following overnight incubation at 37°C the colonies were counted to ensure an enrichment in the number from the vector plus insert ligation. Single colonies were picked using sterile tooth picks, placed into 2ml LB ampicillin and allowed to incubate overnight at 37°C in a shaking incubator.

Miniprep

1.5ml of bacteria was transferred to a microfuge tube, pelleted by centrifugation for 30 seconds and the supernatant aspirated. The pellet was resuspended in 100 μl ice cold Solution I then lysed by the addition of 200 μl Solution II and incubated on ice for 5 minutes. Solution III (150 μl) was added, the sample vortexed and placed on ice for a further 5 minutes. Following centrifugation for 5 minutes at 4°C the supernatant was removed, extracted once with phenol:chloroform:iso-amyl alcohol (25:24:1) and precipitated at room temperature by the addition of 2 volumes of ethanol. The DNA was pelleted by centrifugation, dried and resuspended in 50 μl TE containing 2μg/ml DNAase free RNAaseA. Plasmid DNA was analysed for the correct ligation event by restriction digestion and agarose gel electrophoresis.

9.10 Embryonal Stem Cell Culture

The murine embryonal stem cell line E14 was maintained by culture in: CM ß LIF medium:

CM ß LIF Medium

Glasgow Modification of Eagles medium supplemented with:

Glycine 0.1mM

L-alanine 0.1mM

L-aspartic acid 0.1mM

L-asparagine 0.1mM

L-glutamic acid 0.1mM

L-proline 0.2mM

L-serine 0.2mM

Sodium pyruvate 1.0mM

Foetal calf serum 5%

Neonatal calf serum 5%

ß-mercaptoethanol 0.1mM

Leukaemia inhibitory factor (sufficient to prevent differentiation) (Smith et al., 1988; Williams et al., 1988).

Cells were grown at 37°C in 4% carbon dioxide on plastic tissue culture vessels precoated with 0.1% gelatin. The medium was changed as required and cell passaged as they approached 70% confluence.

Electroporation

50-150 µg of plasmid DNA was linearised with the appropriate enzyme and

an aliquot run on a gel to check for complete digestion. The remainder was precipitated, pelleted, dried and resuspended in 200 µl sterile PBS in a class I hood. Approximately 10^8 E14 cells, were disaggregated by incubation in 4ml TVP, spun down and resuspended in 600 µl PBS.

TVP

0.025% Trypsin

1% Chick serum

1mM EDTA

in phosphate buffer saline (Flow labs).

Cells and DNA were mixed, placed in a BioRad electroporation cuvette and electroporated at 0.8mA, 3.0 µFa, time constant 0.1 in a BioRad Gene Pulser. Cells were subsequently placed into 120ml of CM ß-LIF medium and plated out, 8 ml per 100mm pregelled Petri Dish. After 24 hours the medium was aspirated and replaced by CM ß-LIF medium containing 300 µg/ml G418 with or without 2 µM ganciclovir as appropriate.

The medium was replaced as required until obvious clones were visible usually after 10-14 days. When clones were 2-3mm in diameter they were picked by aspiration into a finely drawn glass pipette under a phase contrast dissecting microscope and transferred into a drop of TVP. Following incubation at 37°C for 5 minutes the cells in each droplet were disaggregated by gently pipetting and transferred into a single pre-gelled well of a 24 well plate. Once they had reached 80+% confluence the medium was aspirated and 3 drops of TVP added to each well. After 5 minutes at 37°C the trypsin was inactivated by the addition of 1ml of CM ß-LIF medium and cells disaggregated by gentle pipetting. They were then transferred to $25cm^2$

pregelled plastic culture bottles and refed as required until 80+% confluent. At this stage the medium was aspirated and the cells trypsinised with approximately 2ml TVP. Once disaggregated, 1ml was taken for freezing and 1ml for genomic DNA extraction. Cells for freezing were gently pelleted at 1000rpm for 60 seconds in a microfuge then resuspended in 1ml of freezing medium and transferred to a 1.5ml Nunc cryotube.

Freezing Medium

CM ß-LIF Medium 70%

Foetal calf serum 20%

DMSO 10%

These were placed in a -70°C freezer to slowly cool and freeze overnight and subsequently stored long-term in liquid nitrogen. For DNA extraction, cells were pelleted 13,000rpm for 3 minutes, resuspended in 750 μl of lysis buffer containing 500 μg/ml proteinase K and incubated at 37°C overnight with shaking.

Stem Cell Lysis Buffer

50mM TrisCl pH 8.0

50mM EDTA pH 8.0

100mM NaCl

5mM DTT

0.5mM spermidine

1% SDS

Samples were extracted twice with phenol, once with chloroform: isoamyl alcohol (24:1) and precipitated with an equal volume of isopropanol. The DNA

was spooled on a plastic pipette tip and resuspended in 500 µl TE.

Clones required for further analysis by immunohistochemistry or for blastocyst injection were thawed quickly in a 37°C water bath with agitation, resuspended in 5ml CM ß-LIF medium, pelleted at 1000rpm for 5 minutes then resuspended in 10ml CM ß-LIF and used to inoculate a 25cm^2 flask.

Analysis of Clones

Southern Blot

30 µl of DNA was digested overnight in a total volume of 50 µl using the appropriate restriction enzyme under the recommended conditions. It was separated by 0.8% agarose gel electrophoresis, transferred to a charged nylon membrane, prehybridised and hybridised to a radioactively labelled probe as previously described.

Washing was carried out as follows: 2 x SSC at room temperature; 1% SDS, 0.1 x SSC at 65°C (2 washes, 15 minutes each); then lightly blotted dry and wrapped in transparent plastic film. Filters were exposed to X-ray film for 1-2 days at -70°C.

PCR

Genomic DNA from each clone was amplified using oligonucleotide primers MM5.5 and MM7.7

MM5.5 5' CTC AAT AAG CTA TTC TGC CAC CTG 3'

MM7.7 5' CTA ACC TAC CAC GCG CCT TCC TAC 3'

5 µl genomic DNA was amplified in 100 µl reaction mix containing: primers at

0.5 μM; dNTP's each at 200 μM; and 2.5 units Taq DNA polymerase in PCR buffer.

PCR Buffer

10mM TrisCl pH 8.8

50mM Potassium chloride

1.5mM Magnesium chloride

0.1% Triton X-100

The mix was overlain with 100 μl paraffin oil, placed on a programmable heating block and underwent 30 cycles of: 94°C for 1 minute; 55°C for 1 minute; 72°C for 2 minutes; with a final cycle terminating with 10 minutes at 72°C to ensure complete extension of all products. After amplification, 30 μl of PCR mix was restriction digested in a 40 μl reaction containing 4 μl of 10 x DraIII digestion buffer and 8 units DraIII (NBL) for 1 hour at 37°C.

10 x DraIII Digestion Buffer

500mM TrisCl pH 7.5

100mM Magnesium chloride

1M Sodium chloride

10mM Dithiothreitol

The digested DNA was analysed by electrophoresis through a 4% agarose gel (Nusieve GTG:SeaKem GTG 3:1) containing 0.5 μg/ml ethidium bromide and viewed under UV light. The uncut PCR product was approximately 903bp in length and when digested with DraIII yielded 2 fragments of 555bp and 348bp from the wild type gene or 555bp, 348bp and 324bp in the case of a WT and mutant gene (the 24bp fragment not being visible in the analysis).

9.11 Germ-line transmission of targeted p53

The targeted ES cell clone R3.72 was susequently expanded and 10-15 cells injected into 3.5 day old blastocysts from F2 (C57/Bl6 x CBA) mice. These were implanted into psuedo-pregnant females and chimæric mice produced identified by their coat colour. Chimæric animals were mated with Ola/129 mice and offspring derived from the targeted cells identified by their chinchilla coat colour. DNA from tail biopsies was screened by Southern blotting in the same way as the ES cells for the targeted event. Mating of 2 heterozygotes resulted in mice with homozygous p53 inactivation.

Heterozygote (p53 +/-) and homozygote (p53-/-) mice were genotyped and observed. Animals which had obvious tumours or were unwell were killed by cervical dislocation and underwent post-mortem examination. Diagnoses were made mostly on the basis of the gross pathological appearances and partly on histology and are therefore still provisional.

Chapter 10

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Rapid Communication

p53 Expression in Colorectal Tumors

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The expression of the nuclear phosphoprotein p53 was studied immunohistochemically in a series of 150 benign and malignant colorectal tumors. Using monoclonal antibody PAb1801, tumors divided unequivocally into two groups on the basis of immunohistochemistry. Forty of the carcinomas (46.5%) showed positive staining but only 4 of the adenomas (8.7%) were positive ($P < 0.001$). The few positive adenomas always showed moderate or severe dysplasia. Metaplastic polyps ($n = 9$) and small familial adenomatous polyposis-related adenomas ($n = 9$) were uniformly negative. Carcinomas with p53 expression did not differ from those without in terms of site, differentiation or the prognostic indicators of Dukes' stage, DNA ploidy, or tumor histology. The improved morphologic resolution available in periodate lysine paraformaldehyde dichromate (PLPD)-fixed, paraffin-embedded tissue permitted several conclusions to be made: p53 is confined to neoplastic nuclei; staining in positive tumors is heterogeneous and often more marked at the infiltrative margins; and staining intensity is dramatically reduced in mitotic cells. It is concluded that expression of immunohistochemically detectable p53 (probably representing mutated forms of the protein) occurs in some adenomas around the time of transition to carcinoma. Therefore there is an association with the appearance of infiltrative behavior but not with degree of tumor progression (including metastasis) at the time of resection. (Am J Pathol 1991, 138:807–813)

Overexpression of p53 has been demonstrated immunohistochemically in a number of common human malignancies, including carcinomas of colon, breast, and lung.[1–3] In the case of breast carcinoma, this immunoreactivity has been shown to correlate with a number of clinical and histopathologic indicators of poor prognosis[2] and may become useful in the future in determining therapeutic strategies.

The 53-kd nuclear phosphoprotein p53 is found in all mammalian cells so far investigated as well as in xenopus.[4] It is usually expressed at low levels and has a short half-life (only 6 minutes in the spleen).[5] Its normal function remains obscure but it has been implicated in control of the cell cycle because levels of p53 mRNA and protein increase in late G1 before the onset of DNA synthesis[6] and cells are prevented from entering S phase by microinjection of p53 antibodies or by the presence of anti-sense p53 RNA.[7,8]

p53 forms stable complexes with the products of transforming genes of DNA viruses (SV40 large T antigen[9,10] and adenovirus E1b protein[11]) and can be oncogenically activated by mutations in certain regions of the gene.[12] Under these circumstances, p53 becomes much more stable,[13] loses the capacity to bind viral oncogenes,[14] immortalizes rodent fibroblasts,[15–17] and cooperates with *ras* in their transformation.[18] The nonmutated, wild-type (WT) p53 gene, however, behaves as a tumor suppressor and can reverse the transformed phenotype.[19]

Interest in p53 in colorectal cancer was stimulated by the finding that the region on chromosome 17p, which is consistently deleted in the majority of such tumors (thus indicating the site of a putative tumor suppressor gene), contains the gene for p53.[20] In cases in which one copy of p53 is deleted, the remaining copy is frequently mutated, but mutations also occur in tumors that retain a normal p53 allele.[21]

Previous immunohistochemical studies of p53 have been restricted to frozen sections,[1,2] because the epitope is destroyed by formaldehyde fixation. Here, by the use of the fixative PLPD,[22] we exploited the superior morphologic resolution of immune reaction product pos-

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sible in paraffin sections. We describe p53 expression in a series of 86 colorectal carcinomas and 62 polyps, including 9 from patients with familial adenomatous polyposis, and correlate the staining pattern with well-recognized indicators of clinical outcome.

Materials and Methods

Tissue

Ninety-two consecutive colectomy specimens were received onto ice in the operating theater, opened, and rinsed in ice-cold phosphate-buffered saline/ethylenediamine tetraacetic acid (EDTA) (20 mmol/l [millimolar] sodium phosphate buffer pH 7.4, 145 mmol/l sodium chloride, 0.5 mmol/l EDTA) within 30 minutes. Samples of tumor and adjacent normal mucosa for immunohistochemistry were placed immediately in periodate lysine paraformaldehyde dichromate (PLPD) fixative[22] (1% paraformaldehyde, 75 mmol/l lysine, 37.5 mmol/l sodium periodate, 2.5% potassium dichromate in 18.75 mmol/l sodium phosphate buffer pH 7.4) fixed for 24 hours at 4°C, washed extensively, and processed into paraffin blocks. Further samples of tumor and distant normal mucosa were snap frozen in liquid nitrogen and then stored at −70°C for flow cytometry or cryostat sections. The rest of the specimen was fixed in formalin and processed routinely for hematoxylin and eosin staining and histopathologic assessment. For the purposes of numerical analysis, tumors were defined as right sided if they lay between cecum and splenic flexure or left sided if they were more distal.

Histopathologic Grading

Carcinomas were reviewed and graded by a single pathologist (J Piris) using criteria recommended by the United Kingdom Coordinating Committee on Cancer Research for Dukes' stage, histologic type, degree of differentiation, and nature of invasive margin.[23] Adenomas were similarly reviewed and graded by a single pathologist (J. O'Grady) for morphologic type and degree of dysplasia.[24]

Flow Cytometry

Three or more samples from separate sites in each carcinoma were prepared for assessment of nuclear DNA content by flow cytometry using the method of Vindelov.[25] The tissue was disaggregated under a citrate buffer and treated with detergent (0.1% NP-40) and trypsin (30 μg/ml) followed by RNAase A (0.1 mg/ml). The resulting nuclear suspension was stained with propidium iodide at a final concentration of 2 mg/ml. Avian erythrocytes were added as an internal standard and the samples analysed on a Coulter EPICS CS flow cytometer (Coulter, Hialeah, FL) at an excitation wavelength of 488 nm. At least 10,000 nuclei were analyzed in each sample and cases were scored as aneuploid if any of the three samples showed a G_0/G_1 peak distinct from the normal diploid peak (as assessed from the internal standard). Coefficients of variation of the diploid peak were typically less than 3.5.

Immunohistochemistry

Three-micrometer sections of PLPD-fixed, paraffin-embedded tissue were stained using the human p53-specific mouse monoclonal antibody PAb1801[26] (p53-Ab2 Oncogene Science Inc., Manhasset, NY). Sections were rehydrated in graded alcohols incubated in normal rabbit serum diluted 1:5 in TRIS-buffered saline (TBS) (145 mmol/l NaCl, 20 mmol/l TRIS pH 7.6), and exposed to primary antibody at a dilution of 1:100 for 1 hour. Bound antibody was detected using biotinylated rabbit antibody to mouse immunoglobulin (Dakopatts No. E354, Glostrup, Denmark) and avidin-biotin complex linked to horseradish peroxidase (Dakopatts No. K335). Copious washing in TBS between each step was essential. Visualization was with diaminobenzidine (1 mg/ml) in the presence of 0.03% hydrogen peroxide. Endogenous peroxidase was not inhibited. Cases known to stain positively were included in each run, receiving either primary antibody or simply dilution buffer, to monitor consistency and act as controls. Sections received a light hematoxylin counterstain and were dehydrated in graded alcohols and xylene before mounting. The specificity of PAb1801 for human p53 under the conditions used was confirmed by inclusion in some staining runs of PLPD-fixed SV80 cells (an SV40-transformed, human fibroblast cell line known to express high levels of p53).[27] In addition, 4-μm cryostat sections from a number of carcinomas in the series were fixed in acetone for 15 minutes then exposed to PAb1801 at a dilution of 1:100 (53 cases) or undiluted culture supernatant from the hybridoma secreting antibody PAb240 (39 cases). PAb240 binds to a different p53 epitope from PAb1801, revealed by the conformational changes induced by mutation.[28] Bound antibody was detected using rabbit antibodies to mouse immunoglobulins conjugated with horseradish peroxidase (Dakopatts No. P260) at a dilution of 1:20 and visualized in an identical fashion to that used for PLPD-fixed sections. The distribution of PAb1801 staining within cells and within the tissue was identical in PLPD-fixed and frozen section. No

discrepancy in the assignment of PAb1801 positivity between frozen and paraffin sections was found.

Immunoblots

Total protein was extracted from tumors, normal colonic mucosa, and SV80 cells. Equal amounts of total protein (approximately 50 μg) were separated by electrophoresis through a 10% polyacrylamide gel containing 0.1% sodium dodecyl sulfate along with molecular-weight standards (MW-SDS-70L Sigma Chemical Co., St. Louis, MO) and transferred to nitrocellulose (Hybond-C, Amersham, Arlington Heights, IL) by standard methods.[28] Nonspecific binding was inhibited by overnight incubation in TBS, 10% milk protein, 0.1% Tween-20, then probed with PAb1801 (1:200) and detected using a streptavidin, biotin, alkaline-phosphatase system (Amersham, RPN 22) in accordance with the manufacturers' recommendations.

Results

Reaction product, marking the binding of PAb1801, was confined to the nuclei of neoplastic cells (Figure 1a). Normal colonic mucosa never stained in cytoplasm or nucleus. Rarely pale staining was observed in smooth muscle cells (particularly of blood vessels) at high antibody concentrations but not at the working dilution. Staining

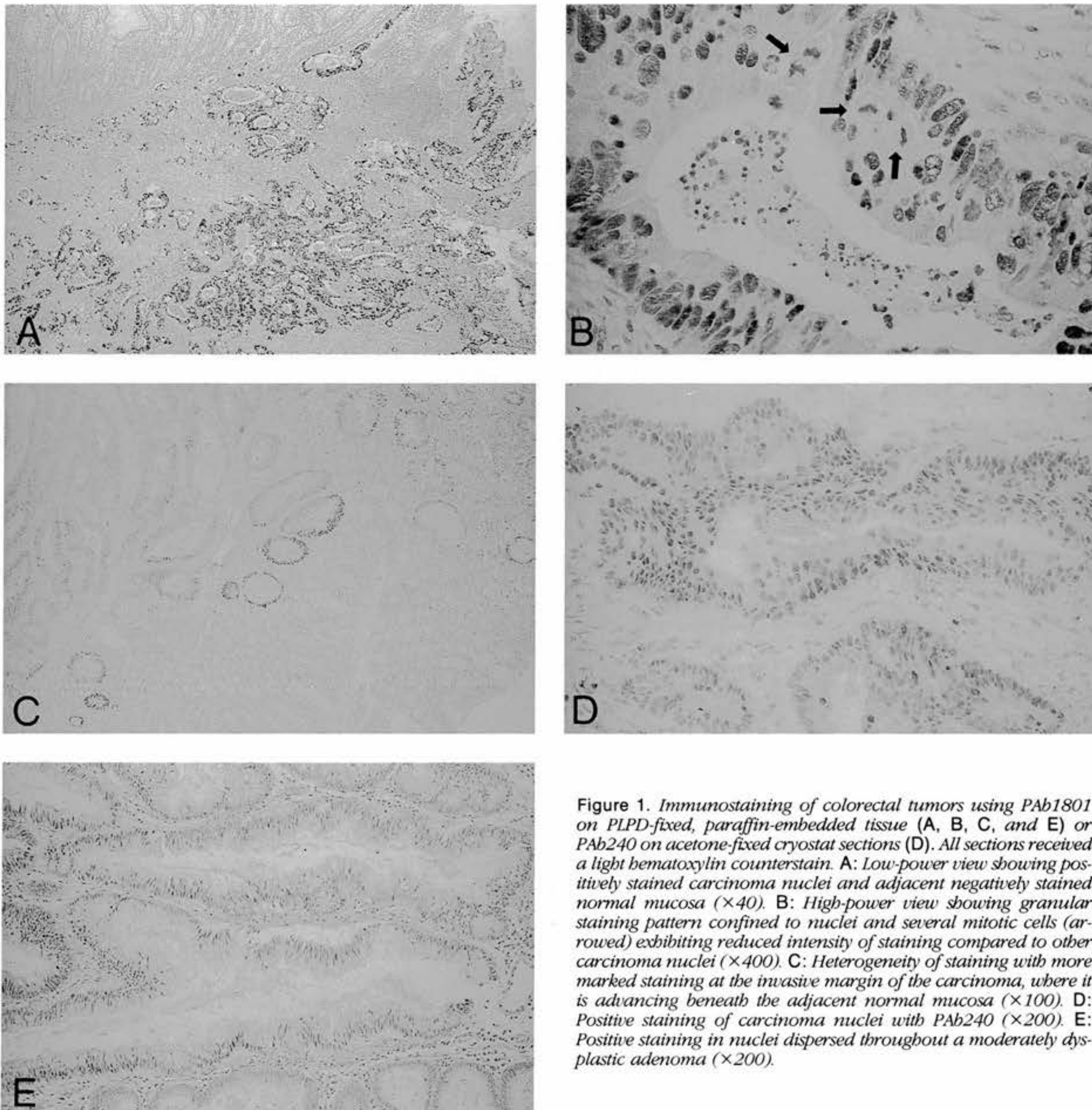

Figure 1. *Immunostaining of colorectal tumors using PAb1801 on PLPD-fixed, paraffin-embedded tissue* (A, B, C, *and* E) *or PAb240 on acetone-fixed cryostat sections* (D). *All sections received a light hematoxylin counterstain.* A: *Low-power view showing positively stained carcinoma nuclei and adjacent negatively stained normal mucosa (×40).* B: *High-power view showing granular staining pattern confined to nuclei and several mitotic cells (arrowed) exhibiting reduced intensity of staining compared to other carcinoma nuclei (×400).* C: *Heterogeneity of staining with more marked staining at the invasive margin of the carcinoma, where it is advancing beneath the adjacent normal mucosa (×100).* D: *Positive staining of carcinoma nuclei with PAb240 (×200).* E: *Positive staining in nuclei dispersed throughout a moderately dysplastic adenoma (×200).*

within the tumor nuclei was granular or reticular in nature (Figure 1b) sometimes with obvious nucleolar sparing. During mitosis, reaction product was reduced dramatically (Figure 1b), whereas interphase tumor cells never showed cytoplasmic staining.

The tumors divided unequivocally into two groups on the basis of PAb1801 immunohistochemistry. Tumors were scored as positive when staining was visible in any nuclei within the tissue section. In such cases, positive nuclear staining of identical incidence and distribution was found even when the concentration of primary antibody was reduced to 1:400 or increased to 1:10. Similarly cases scored negative remained without staining in any cells even at concentrations as high as 1:10.

In positive tumors the pattern of immunostaining was not uniform. In a small number of tumors almost all the nuclei stained positively, but the more common pattern was an admixture of tumor acini that showed no staining with those in which most nuclei were stained. The greatest proportion of positive cells was found in acini at the invasive margins of carcinomas. Sometimes the distinction was obvious within a single gland, with strong staining on the outer side and weak or none on the inner side (Figure 1c). All these patterns were sustained over a 40-fold range of dilution of primary antibody (data not shown). Immunostaining of cryostat sections from a subset of 39 carcinomas using PAb240 demonstrated a very similar pattern and overall incidence of antibody binding (Figure 1d) and all cases that were scored PAb1801 positive were also PAb240 positive by the same criteria.

In total, 86 carcinomas from 86 patients were analyzed with PAb1801 and of these 40 (46.5%) showed positively stained nuclei. Prognostic indicators (Duke's stage, DNA aneuploidy, degree of tumor differentiation, and nature of invasive margin) showed no significant correlation with PAb1801 staining (Table 1). Similarly the proportion of PAb1801-positive tumors was unrelated to their site within the colon or rectum (Table 1).

Table 1. *Correlation of PAb1801 Immunostaining with Histopathological Classification, DNA Ploidy, and Primary Tumor Site for Carcinomas*

Classification	PAb1801 −	PAb1801 +	p Value*
Dukes A	1	4	
Dukes B	23	21	NS
Dukes C	22	15	
DNA diploid	14	6	NS
DNA aneuploid	32	34	
Differentiation: Other	19	23	NS
Poor	27	17	
Margin: Expanding	6	5	NS
Infiltrating	40	35	
Right colon	14	11	NS
Left colon	32	29	

* Chi-square test with Yates' correction.
NS, not significant ($P > 0.05$).

Fifty-five sporadic polyps from 30 patients were studied with PAb1801 comprising 9 metaplastic polyps and 46 adenomas. The metaplastic polyps were all negative. Of the 46 adenomas, 4 (8.7%) were positive (Table 2). The positively staining nuclei were dispersed throughout the adenoma in one case (Figure 1e) and in the remaining three were restricted to only a few glands. All four showed either moderate or severe dysplasia, but there is no statistically significant correlation within our series with other morphologic parameters or tumor size (Table 2). In one large villous adenoma that contained foci of infiltrative carcinoma, however, p53 staining was absent in the adenoma but present in the infiltrative glands only. In addition, PLPD-fixed material was available from nine tubular adenomas, all less than 4 mm in diameter from two patients undergoing colectomy for FAP; all were negative for PAb1801 immunostaining. The difference in incidence of positive staining in adenomas as compared with carcinomas is very significant (Table 2).

Immunoblots of total protein extracted from tissue samples (Figure 2) demonstrated that PAb1801 recognizes a single 53-kd protein that could be extracted from those tumors that were immunohistochemically positive but not from negative tumors or normal colonic mucosa. This detection of p53 in immunoblots correlated exactly with detection in frozen and paraffin sections.

Discussion

The data demonstrate a strong relationship between p53 expression and malignant transformation in colorectal tumors. In contrast to carcinomas, very few adenomas showed p53 expression and those that did all belonged to the more dysplastic categories in which the risk of transition to invasive carcinoma is higher. In one case of a

Table 2. *Correlation of PAb1801 Immunostaining with Histopathology of Adenomas*

Classification	PAb1801 −	PAb1801 +	p Value*
FAP adenomas	9	0	
Metaplastic polyps	9	0	
Tubular adenomas	1	2	NS
Tubulovillous adenomas	11	2	
Villous adenomas	12	0	
Maximum diameter of adenoma†			
≥10mm	29	3	NS
<10mm	13	1	
Dysplasia† Mild	5	0	
Moderate	34	2	NS
Severe	3	2	
Sporadic adenomas	42	4	$P < 0.001$
Carcinomas	46	40	

* Chi-square test with Yates' correction.
NS, not significant ($P > 0.05$).
† Sporadic adenomas only.

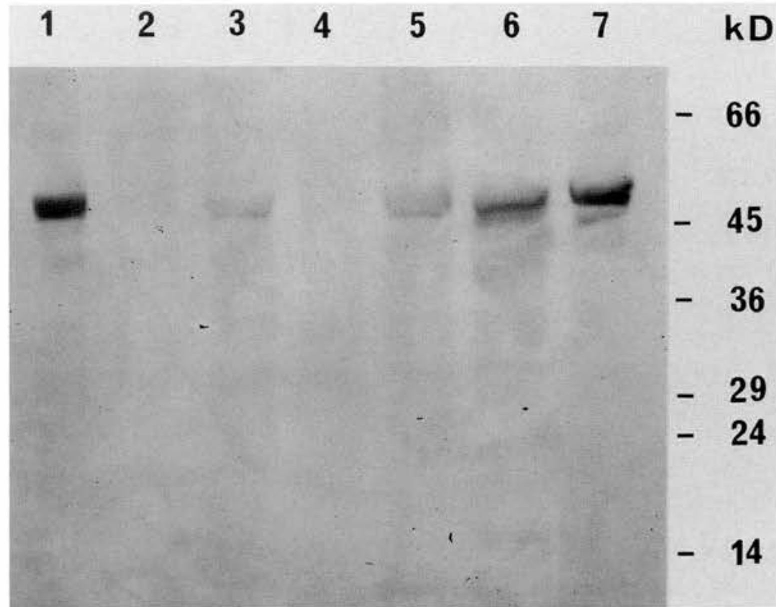

Figure 2. *Immunoblot of total protein stained using PAb1801. Lane 1: SV80 cells. Lane 2: Normal colonic mucosa. Lane 3: Immunohistochemically positive carcinoma. Lane 4: Immunohistochemically negative carcinoma. Lanes 5 and 6: Immunohistochemically positive carcinomas. Lane 7: SV80 cells. Molecular weights are expressed in kilodaltons.*

villous adenoma that had undergone malignant change, p53 expression was restricted to the frankly carcinomatous portion sparing the dysplastic epithelium. In the carcinomas, the advancing edge was the most consistently staining region. Nonetheless, approximately one half of all colorectal carcinomas did not show p53 expression, and comparison of expressing and nonexpressing carcinomas revealed no differences in terms of the tumor site or established prognostic indicators such as histologic grade,[23] Dukes' stage,[30] and DNA ploidy.[31] Because most of the tumors studied in this consecutive series were (as expected) of Dukes' B and C stages, the lack of correlation with staging is essentially an indicator that p53 expression has no association with the tendency to form lymph node metastasis. It appears, therefore, that p53 expression is associated with the transition from adenoma to carcinoma in some colorectal tumors but does not uniquely determine infiltrative activity or relate to other features of carcinoma progression. However, in view of the short time of clinical follow-up of the patients studied here, we have no data yet that directly compare postoperative course and p53 staining.

The monoclonal antibody PAb1801 used here is specific for human p53, binding near the N terminus.[26] It thus detects both WT and mutated forms of the protein, all the mutations described being 3′ to the antibody binding site.[21] Wild-type p53 has a short half-life and was not detected immunohistochemically in any of the normal tissues in this study. By contrast, mutations of p53, which are common in human colorectal cancer,[21] result in a greatly extended protein half-life permitting immunohistochemical detection.[32] PAb240, which binds selectively to mutated p53,[28] stains positively cases that are PAb1801 positive in this series and has a very similar distribution of reaction product. Thus the tumors exhibiting PAb1801 immunostaining almost certainly have p53 mutations; we are now confirming this by sequencing the gene for p53 in selected tumors (manuscript in preparation).

PAb1801 has the advantage that its epitope is stable in PLPD-fixed (but not formaldehyde-fixed) tissue, thus permitting the improved morphologic localization of paraffin sections. The discrete nuclear localization of the PAb1801 epitope observed here contrasts with a previous report on p53 expression in breast carcinomas based on an analysis of frozen sections in which cytoplasmic staining was observed.[2] Complex formation between mutant p53 and the cytoplasmic heat shock protein hsp 72/73 has been demonstrated, which might explain its cytoplasmic location in some tissues.[14] In a large subset of the present series, however, we also applied PAb1801 to frozen sections and found the same pattern of nuclear staining. Breast carcinomas also differ in that p53-expressing tumors tend to be in poorer prognostic groups,[2] an association we do not observe for colorectal carcinomas (Table 1).

Within some of the sections showing positive immunostaining there was marked cell-to-cell heterogeneity. Because the mutation of the p53 gene probably is clonal, this variation in protein level may indicate that mutated p53 is under similar cell-cycle control to that demonstrated in WT p53 with levels increasing before S phase.[6] Further evidence for cell-cycle–related changes in protein levels comes from the mitotic cells that consistently showed much-reduced immunostaining. The distribution of positively stained cells around the advancing margins of carcinomas has not been reported previously and is

more difficult to explain as a cell-cycle–related phenomenon and may represent some form of inductive interaction with stromal tissues.

In transgenic animals, expression of mutated p53, even at high levels, does not produce tumors in some tissues,[33] indicating that changes in p53 alone are not sufficient to cause neoplastic transformation. In human colorectal carcinogenesis, several other genetic changes occur at high frequency, such as Ki-*ras* mutations and deletions in chromosomes 5q, 18q, and 17p (including the locus of the p53 gene).[34] Accumulation of these events seems to be important in determining prognosis, presumably facilitating the rather poorly defined cellular changes that constitute tumor progression. The data presented here show that expression of p53 (probably due to mutation) is an event occurring around the transition from adenoma to carcinoma and therefore at the same stage in tumor progression as 17p deletion.[34] The distribution of the most intensely staining cells also relates to local infiltrative activity. Therefore p53 may be one factor permitting malignant transformation but it is unlikely to account for other features of carcinoma progression such as metastasis and the tendency to develop DNA aneuploidy.

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High frequency of *APC* loss in sporadic colorectal carcinoma due to breaks clustered in 5q21-22

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Familial adenomatous polyposis is transmitted by a gene (*APC*) located within 5q21-22. Hemizygous loss of at least a part of 5q has been reported in 19–36% of sporadic colorectal carcinomas. This suggests that an anti-oncogene is located on that chromosome arm, but the probes used previously gave little information on the status of *APC* in the tumours. Using DNA probes homologous to polymorphic sequences flanking and close to the *APC* locus we show that more than half of a large series of carcinomas had lost at least one flanking allele. Mapping of allele losses provides data that imply clustering of breakpoints in a 10–15 megabase region around *APC*. The commonest chromosome defect responsible for *APC* loss was interstitial deletion. Mitotic recombination or partial arm loss were less frequent mechanisms. Whole chromosome loss was rare. This pattern contrasts with that reported in acquired homozygosity at other anti-oncogene loci in sporadic tumours and implies that *APC* loss is an early event in colorectal carcinogenesis. This view is also supported by the observations that 5q21-22 loss occurs with similar frequency in DNA diploid and DNA aneuploid tumours, and also in tumours at all clinical stages of progression.

Introduction

Two observations suggest that an anti-oncogene concerned with the regulation of colorectal mucosal growth is situated on the long arm of chromosome 5. First, familial adenomatous polyposis—a Mendelian dominant condition characterised by development of large numbers of colorectal adenomas—is transmitted by a gene (*APC*) mapping within 5q21-22 (Bodmer *et al.*, 1987; Leppert *et al.*, 1987). Second, hemizygous loss of at least part of 5q has been reported in 19–36% of sporadic colorectal carcinomas (Solomon *et al.*, 1987; Okamoto *et al.*, 1988; van den Broek *et al.*, 1988; Vogelstein *et al.*, 1988; Law *et al.*, 1988; Rees *et al.*, 1989). There is, however, little information on the status of *APC* itself in these tumours. At other, better-defined anti-oncogene loci, such as the genes conferring susceptibility to Wilms' tumour and retinoblastoma, acquired allele loss within the tumours commonly involves whole chromosomes (Dryja *et al.*, 1984; Fearon *et al.*, 1984). Such losses are probably the result of mitotic non-disjunction. In contrast, recent studies in human colorectal and breast carcinoma have revealed a high frequency of acquired homozygosity of 17p alleles (Monpezat *et al.*, 1988; Vogelstein *et al.*, 1988; MacKay *et al.*, 1988), but here the loss is commonly achieved through partial arm deletion or mitotic recombination, requiring an intrachromosomal breakage event. In this paper we examine the status of *APC* in a large series of sporadic colorectal carcinomas. Using DNA probes homologous to polymorphic sequences flanking and close to *APC* ('closely-flanking markers'), we show that *APC* alleles are lost in a much higher proportion of tumours that hitherto recognised. Whole chromosome loss is rare, the majority of lesions resulting from intrachromosomal breakage (interstitial deletions, mitotic recombinations or partial arm losses). The results have interesting implications on the status of *APC* in colorectal carcinogenesis, and its susceptibility to nearby chromosome breakage events.

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Results

Loss of APC *in sporadic tumours*

There were 119 tumours, confirmed as carcinomas by histology in all but 2 cases, the exceptions both being large villous adenomas. Pairs of closely-flanking markers lying centromeric and telomeric to *APC* within 5q21-22 were used to detect tumours exhibiting hemizygous loss of polymorphic restriction fragment alleles. To establish the physical extent of allele loss, tumour DNA was analysed with probes mapping to regions spanning chromosome 5 at 5p, 5q12-14 (close to the centromere) and 5q35 (telomeric). Details of the probes used are given in Table 1 and examples of allele loss demonstrated with each are shown in Figure 1. The

Table 1 Probes used in analysis of chromosome 5 alleles

Probe	*Specification of target sequence*	*Enzyme*	*Alleles kb*	*Ref*
L1.4	D5 S4	EcoR1	0.7, 0.6	1, 2
MLZ 7.5	—	EcoR1	2.9, 2.2, 3.2*	1
105-153 Ra	D5S39	MspI	8.0, 5.0	1, 2
105–599 Ha	D5S76	TaqI	17.0, 14.0, 10.0	1, 2
105-789 Rb	D5S78	MspI	2.3, 1.8	1, 2
π 227	D5S37	BclI	3.0, 1.8, 1.2	2, 3, 4, 5, 6
C11P11	D5S710	TaqI	4.4, 3.9, 5.0*	3, 7, 8
YN5.48	D5S81	MspI	9.0, 8.0	3, 8
MC5.61	D5S84	MspI	5.5, 5.0	3, 8
λ MS8	D5S43	HinfI	2.4–9.5	2, 9

* Constant allele

References: 1. Leppert *et al.* (1987)
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3. Nakamura *et al.* (1988)
4. Dunlop *et al.* (1989)
5. Meera Khan *et al.* (1987)
6. Stewart *et al.* (1987)
7. Bodmer *et al.* (1987)
8. Wasmuth & Ferrell (1988)
9. Solomon *et al.* (1987)

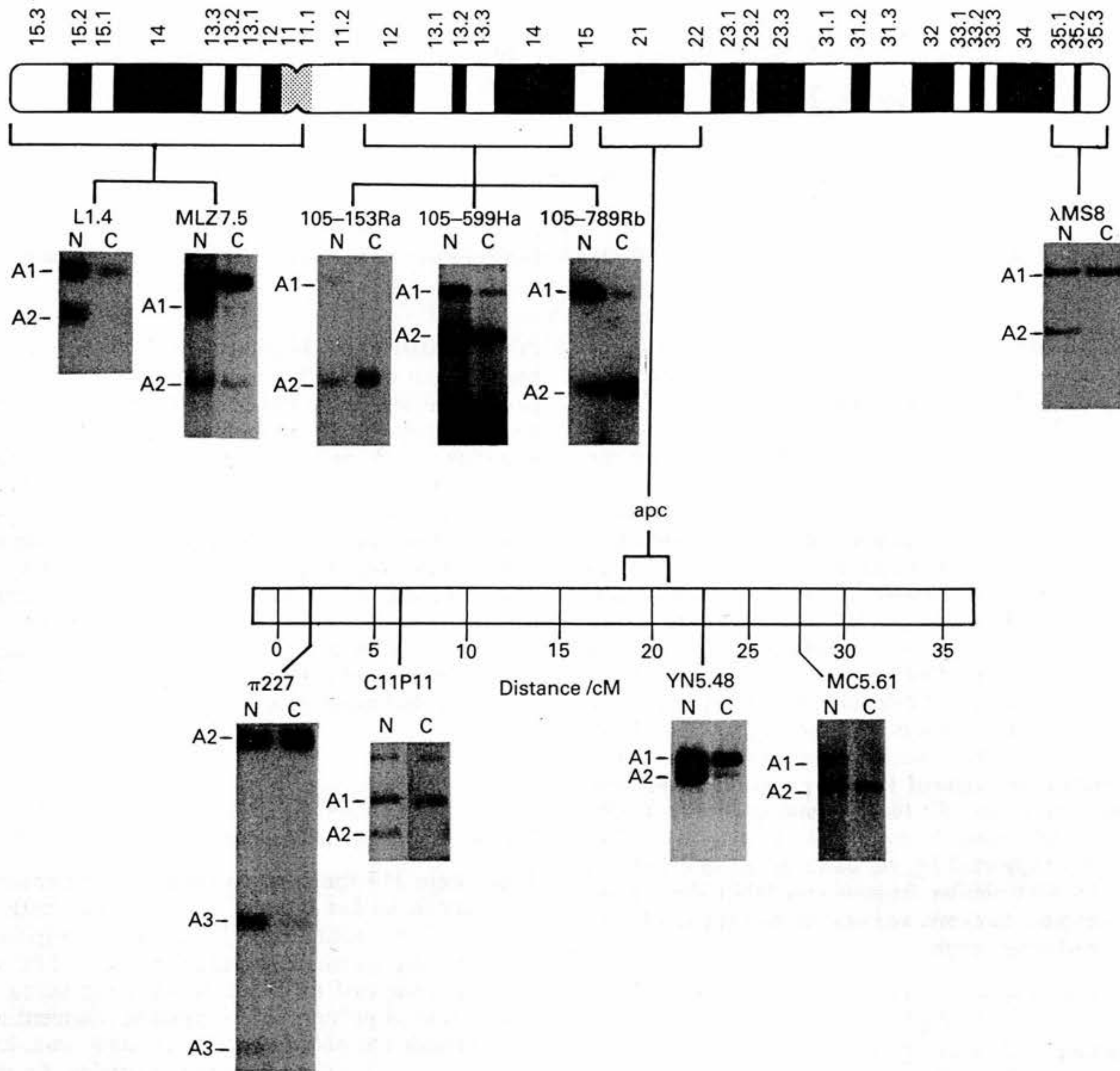

Figure 1 Location of polymorphic sequences in chromosome 5 detected by the ten probes used in this study (Table 1). The genetic distances between the *APC* markers are given in centimorgans (1 cM equivalent to 1 megabase (1 mb) in humans). Distances are reproduced from a single early study (Nakamura *et al.*, 1988) for the sake of consistency. More recent data, however (M.G. Dunlop, Y. Nakamura, unpublished), indicates that the distance between π 227 and C11P11 may be as little as 2 cM and between C11P11 and YN5.48, approximately 10 cM

overall frequency of loss observed for any 5q-linked allele was 48% (Table 2A), substantially higher than that quoted in previous studies. A similar high frequency (45%) was found when analysis was restricted to tumours showing loss of alleles in 5q21-22 (Table 2B). Of the 65 tumours informative with closely-flanking markers on both sides of *APC*, 35 (54%) showed loss of a flanking allele (Table 2C), and in the great majority of these (24/35; 69%) the loss was unilateral. The unilateral loss of closely-flanking markers occurred centromerically and telomerically to *APC* in equal proportions indicating the existence of a cluster of breakpoints within a 10–15 mb region either side of *APC*.

Nature of defects in chromosome 5

Information from tumours mapped with closely-flanking markers, when combined with data from markers mapping to outlying regions of chromosome 5, allowed a physical interpretation of 5q defects (Table 2D). Of the 32 tumours exhibiting loss of one or more of the alleles within 5q21-22, the great majority remained heterozygous for one other chromosome 5 allele at least. This demonstrates that loss of the whole of chromosome 5 plays a minor role in generating *APC* allele loss in sporadic colorectal cancer. The commonest lesion (20/32; 63%) was interpreted as interstitial deletion since the tumours remained heterozygous for markers on both sides of the region showing allele loss. A qualitative depiction of the data (Figure 2) shows the large contribution of breakpoints close to *APC* in these deletions, of which the smallest observed was about 10–15 mb long (the distance between YN5.48 and C11P11; Y. Nakamura unpublished data). The remaining class of chromosome lesions included fewer breaks close to *APC* but showed a striking bias towards the loss of markers telomeric to *APC*. Presumably these represent mitotic recombinations or partial arm losses, which are not distinguished from each other by this type of analysis. The mapping of these allele losses reinforces the view that a specific region, inclusive of the *APC*

Table 2 Analysis of allelic losses in 119 colorectal tumours

	No of cases	Percentage: Of total	Of cases with allele loss
A. Informative within 5q	117	(100%)	
Allele loss within 5q	56	(48%)	
B. Informative at 5q.21-22	107	(100%)	
Allele loss at 5q.21-22	56	(48%)	
C. Informative at closely-flanking loci on both sides of *fap*	65	(100%)	
Loss of at least one flanking allele	35	(54%)	(100%)
Loss of one flanking allele	24		(59%)
Loss of both flanking alleles	11		(31%)
D. Permitting physical interpretation of 5q defect	68	(100%)	
Total with 5q.21-22 allele loss	32	(48%)	(100%)
Interstitial deletion	20		(63%)
Partial arm loss/mitotic recombination	11		(34%)
Whole chromosome loss	1		(3%)

gene, is commonly deleted in sporadic colorectal carcinoma. Of 36 carcinomas showing retention of a pair of closely-flanking markers in 5q21-22 only 1 showed the loss of an outlying 5q allele.

Loss of APC *and tumour progression*

In an attempt to ascertain the phase of tumour evolution at which *APC* alleles are most commonly lost, we studied the frequency of loss in tumours that differed in stage of progression, or in DNA ploidy. There was no correlation ($P > 0.05$) between the degree or occurrence of DNA aneuploidy (as determined by flow cytometry) and *APC* loss (Table 3). Nor did loss of *APC*-flanking markers correlate with Dukes' staging of the extent of tumour spread. It thus appears that the loss of *APC* takes place independently of (and probably before) the emergence of features determining the progression of the carcinoma. This agrees with the observation of 5q21-22 allele loss in both of the villous adenomas in this study. Such tumours do not show infiltration or metastasis but carry a high probability of progressing to carcinoma.

Discussion

The high frequency of 5q21-22 loss recorded in this study is due entirely to the use of closely-flanking markers on both sides of *APC*. Previous studies using unilateral or distant markers, (Solomon *et al.*, 1987; Okamoto *et al.*, 1988; van den Broek *et al.*, 1988; Vogelstein *et al.*, 1988; Law *et al.*, 1988; Rees *et al.*, 1989) suggested lower frequencies of loss, because they failed to detect small deletions including *APC*. This implies that as one approaches the *APC* locus the frequency of observed allele loss may increase still further and indeed that many of the important lesions may be within *APC* itself. A similar situation at 13q14 occurred in sporadic retinoblastoma, where small deletions of the Rb-1 gene, not detected cytogenetically, were revealed by the use of intra-gene molecular probes (T'Ang *et al.*, 1988) and closely-flanking markers (Dryja *et al.*, 1986).

The low frequency of whole chromosome 5 loss argues against mitotic non-disjunction as a major mechanism for the acquired loss of *APC* in tumours. Many reported karyotypes in colorectal cancer cells are hypotriploid or hypotetrapoid, (Reichmann *et al.*, 1981; Petersen & Friedrich, 1986) consistent with origin through non-disjunctive events from a tetraploid parent (Nowell, 1976). The majority of human colorectal cancers do show DNA aneuploidy in this range, as determined by flow cytometry. As the presence of aneuploidy correlates with poor prognosis (Armitage *et al.*, 1985; Quirke *et al.*, 1987), it is tempting to suggest that this mode of chromosome loss contributes to the genesis of homozygosity at anti-oncogene loci during tumour progression (Wyllie, 1989). Our data, however, make it clear that whole chromosome non-disjunction is a rare cause of acquired homozygosity at the *APC* locus. Rather, it appears that the *APC* gene is lost early in the evolution of colorectal tumours. This is further supported by the association of presumptive inherited molecular defects within the *APC* gene in familial adenomatous polyposis where the genetic abnormality is primarily responsible for formation of multiple adenomas. Loss of *APC* early in the adenoma-carcinoma sequence is also suggested by the work of other groups (Remvikos *et al.*, 1988; Vogelstein *et al.*, 1988).

Cytogenetic findings in sporadic colorectal carcinoma (Muleris *et al.*, 1985; Ferti *et al.*, 1988) confirm the present findings of a low frequency of whole chromosome 5 loss. However the fact that such lesions (and the commoner 5q arm losses) are tolerated shows that survival is possible in cells which are hemizygous for genes on 5q outside *APC*. Thus the observed clustering of breakpoints around *APC* is unlikely to be merely the consequence of positive selection for lesions which delete *APC* but conserve important outlying genes.

This clustering of breakpoints is not predicted by a simple interpretation of the anti-oncogene hypothesis of Knudson (1983). Other putative anti-oncogenes involved in colorectal carcinoma, such as those on 17p 12–12.3 (Lothe *et al.*, 1988; Baker *et al.*, 1989), and 18q (Monpezat *et al.*, 1988; Vogelstein *et al.*, 1988), have not yet been analysed in sufficient detail to measure the contribution of different types of chromosomal lesions to the allele loss. Clustered breakpoints are not observed, however, in the somatic loss of anti-oncogenes at 13q14 in retinoblastoma (Dryja *et al.*, 1984) or 11p13 in Wilms' tumour (Fearon *et al.*, 1984), where whole chromosome loss, mitotic recombination and partial chromosome arm loss played a greater role than in the present study. The finding of molecular

Table 3 Prognostic indicators and 5q21-22 status

Tumour status	5q21-22 status*: Retained	Lost
Villous adenoma	0	2 (100%)
Carcinoma — Dukes A	1	8 (89%)
— Dukes B	16	20 (56%)
— Dukes C	14	19 (58%)
DNA diploid	2	6 (75%)
DNA aneuploid	8	11 (58%)

* Tumours were scored as 5q21-22 retained only if an allele within 5q21-22 on *each* side of the *APC* locus retained heterozygosity. Loss of a 5q21-22 allele on either or both sides of *APC* scored as loss

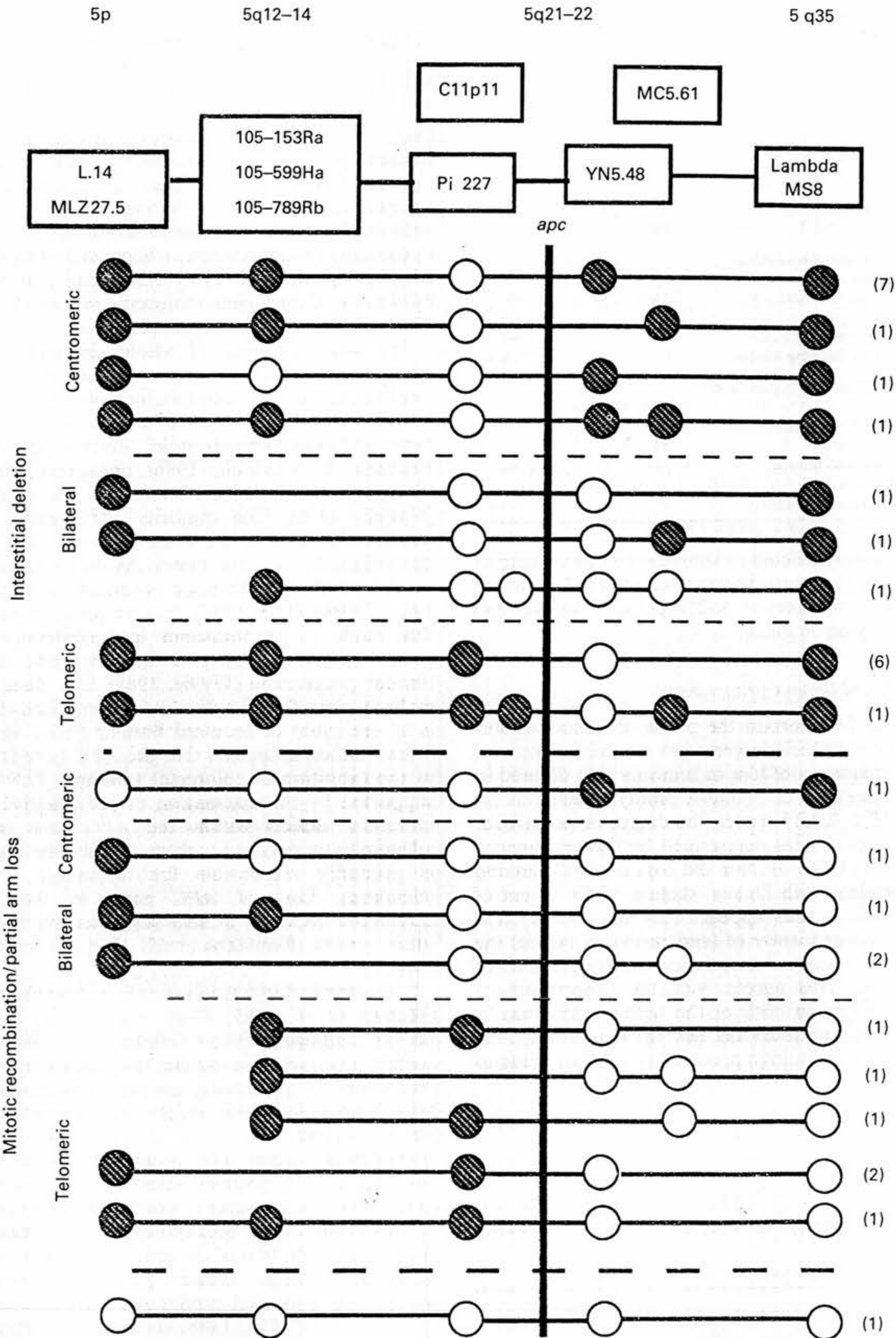

Figure 2 Patterns of allelic loss of chromosome 5 linked markers in 32 tumours in which physical interpretation of the defect was possible. Thirty were carcinomas, two villous adenomas. Uppermost, schematically shown, is the location of polymorphic DNA probes on chromosome 5. Aligned below is the allelic loss (open circle) or retention (shaded circle) revealed by these markers. The number of tumours exhibiting a particular pattern of loss is indicated in brackets on the right-hand side and similar patterns are grouped together as interstitial deletions (centromeric, bilateral, telomeric) or mitotic recombinations/partial arm loss (centromeric or telomeric), involving *APC*

lesions grouped around the *APC* locus in a high proportion of sporadic colorectal carcinomas suggests that this chromosomal region may possess some innate feature predisposing it to breakage and recombination events. This, as well as the putative altered properties of the *APC* gene product(s), may be a factor influencing the contribution of the locus to the progression of colorectal cancer. A constitutive 'fragile' site for chromatin

breakage has been identified in the vicinity of 5q21.2 (Yunis *et al.*, 1987), although its precise relationship to *APC* is unknown. More detailed mapping of the region in and around *APC* may clarify the mechanisms underlying the genetic changes observed in these common human tumours.

Materials and methods

Clinical material

One hundred and nineteen tumours from 117 patients undergoing operation for colorectal carcinoma were sampled. The colons were placed on ice immediately after removal, and within 20 min were opened and rinsed in ice-cold phosphate-buffered saline, supplemented to 0.02% (w/v) with EDTA. Small portions of tumour, and distant apparently normal mucosa were snap frozen in liquid nitrogen. Immediately adjacent tissue was fixed in neutral buffered formalin or PLPD (Holgate *et al.*, 1986) for histology. The remainder of the specimen was examined after fixation for depth of tumour invasion and lymph node infiltration to permit staging in the Dukes' classification. In all there were 117 carcinomas and two large villous adenomas.

DNA analysis

DNA of high molecular weight was prepared from tissue using a method based on SDS-lysis and proteinase K-digestion as described by Goelz *et al.* (1985), digested (10 μg) with the appropriate restriction enzyme and electrophoresed through 0.8% (w/v) agarose gel DNA was transferred on to nylon membranes (Southern, 1975) in 0.5 M NaOH, 1.5 M NaCl. Prehybridization was performed for at least four hours at 65°C in 10% (w/v) dextran sulphate, 1% (w/v) SDS, 0.9 M NaCl–0.09 M sodium citrate, 100 $\mu g\,ml^{-1}$ denatured salmon sperm DNA. Hybridization was at 65°C for 16–24 h in the same solution, together with salmon sperm DNA at 250 $\mu g\,ml^{-1}$ and probe labelled with α-[^{32}P]dCTP (specific activity 10^9 cpm μg^{-1}) by the random priming method (Feinberg & Vogelstein, 1983, 1984). Membranes were washed as described (Nakamura *et al.*, 1988) and exposed at −70°C. Hybridization conditions were altered for probes with high levels of non-specific binding; human placental DNA was used instead of salmon sperm DNA, and the probe preincubated with the hybridization solution at 65°C for one hour before addition to the membrane.

For each probe the target locus specification, preferred restriction enzyme, and allele sizes are given in Table 1. Allele losses were accepted only if the differences in band intensity on the autoradiogram were obvious.

Flow cytometry

Small portions of frozen tissue, taken adjacent to the sites sampled for histology and DNA analysis, were used to prepare suspensions of single nuclei by the detergent-trypsin procedure of Vindeløv *et al.* (1983). Chicken erythrocytes were mixed with the sample at the beginning of the preparation procedure to serve as an internal DNA ploidy standard. The DNA content of these cells is about 35% of that of normal human diploid cells. Nuclei stained with 0.62 M propidium iodide were analysed in a Coulter Epics CS flow cytometer at an excitation wavelength of 488 nm. Coefficients of variation of G_0/G_1 peaks seldom exceeded 3%. Aneuploidy was considered present when two distinct G_0/G_1 peaks were visible.

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