Genetic studies of human colorectal cancer in xenograft and in vitro

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Declaration

This thesis was composed by myself and has not been submitted for any other degree. This thesis records original work carried out by myself unless otherwise stated. Contributions to the work of this thesis made by colleagues are fully acknowledged in the text.

Heather A McQueen

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List of abbreviations

A: Units

A; ampere
b(p); base (pair)
Ci; curie
°C; degrees centigrade
Da; dalton
F; faraday
g; gram
l; litre
M; molar
m; metre
rpm; revolutions per minute
U; units
V; volt

Prefixes:

M= mega (10^6)
K= kilo (10^3)
c= centi (10^{-2})
m= milli (10^{-3})
u= micro (10^{-6})
n= nano (10^{-9})

B: Chemicals

CO2; carbon dioxide
DMSO; dimethyl sulfoxide
DNA; deoxyribonucleic acid
EDTA; ethylene diamine tetra-acetate
EGF; epidermal growth factor
FBS; foetal bovine serum
G418; geneticin sulfate
H & E; haematoxylin and eosin
H2O; water
HCl; hydrochloric acid
HINCS; heat inactivated newborn calf serum
MNNG; N-methyl-N'-nitro-N-nitrosoguanidine
Na; sodium
NaCl; sodium chloride
NaHCO3; sodium bicarconate
NaOH; sodium hydroxide
NRS-TBS; normal rabbit serum in Tris buffered saline
PBS; phosphate buffered saline
PLPD; periodate lysine paraformaldehyde dichromate
RNA; ribonucleic acid
SDS; sodium dodecyl sulfate
TBE; Tris borate EDTA
TE; Tris EDTA
Tris; Tris(hydroxymethyl) aminomethane

List of abbreviations (continued)

C: Techniques, genetic abbreviations and others

APC; adenomatous polyposis coli
cDNA; copy DNA
CIN; cervical intraepithelial neoplasm
DCC; deleted in colorectal carcinoma
DI; DNA index
ds; double stranded
ELISA; enzyme-linked immunosorbent assay
FAP; familial adenomatous polyposis
GAP; GTPase activating protein
GS; Gardner syndrome
GTP; guanosine triphosphate
HPV; human papilloma virus
log; logarithm
LOH; loss of heterozygosity
MCC; mutated in colorectal carcinoma
MMTV; mouse mammary tumour virus
MRC; Medical Research Council
OD; optical density
PCR; polymerase chain reaction
RFLP; restriction fragment length polymorphisms
SCID; severe combined immunodeficiency syndrome
SV40;simian virus 40
UV; ultra violet light
VNTR; variable number tandem repeats
v/v; volume for volume
w/v; weight for volume
YAC; yeast artificial chromosome

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For Bill

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Abstract

Colorectal cancers carry multiple gene abnormalities at both oncosuppressor and oncogene loci. The role of these defects, singly and in combination with each other, in defining colorectal tumour origin and progression is not known. One means to address this important problem experimentally would be to assess the effects of correcting these gene defects in human tumour cell lines against backgrounds of normality, or defined abnormality at other key loci. Such studies demand the existence of colorectal carcinoma lines in which the major colorectal tumour-associated gene defects are defined.

This thesis describes the derivation of 12 such lines and compares them with 6 lines derived elsewhere. Lines derived in this study were propagated firstly as xenografts in immunodeficient mice and briefly as primary cultures. Tumour xenografts were shown to remain stable, faithfully conserving defects or normality, at all sites tested. Stability of the more global index of DNA ploidy was also demonstrated, often over 1-2 years and in one case over 25 monthly passages *in vivo*. Moreover, of the many lines which grew as primary cultures *in vitro* (in which genetic manipulation could take place), 2 were subsequently re-implanted *in vivo* and grew as xenografts with unchanged properties.

In contrast to the stability of these xenografts a cell line, intentionally exposed to potential mutagens *in vitro*, showed a specific rearrangement at the critical locus on 5q21 in association with acquired tumorigenicity. This constitutes the first demonstration of such *in vitro* effects in direct association with specific *APC* and *MCC* alterations.

One of the disadvantages of obtaining "anonymous" long-passaged colorectal lines is demonstrated in that genetic analysis showed 5 lines, considered to arise from different patients, to have become colonized by a single strongly growing line prior to arrival in our laboratory.

Although exploitation of the lines described is largely a future prospect, experiments with a candidate oncosuppressor explored possible effects on cell growth *in vitro* and tumorigenicity *in vivo*, while preliminary investigations were made into optimal gene transfer conditions for the new lines.

The newly characterized colorectal cancer lines described provide uniquely favourable vehicles for future oncosuppressor assays, and renewable purified tumour resources ideal for detailed study of gene defects and many other colon tumour-specific analyses.

Chapter 1: Introduction

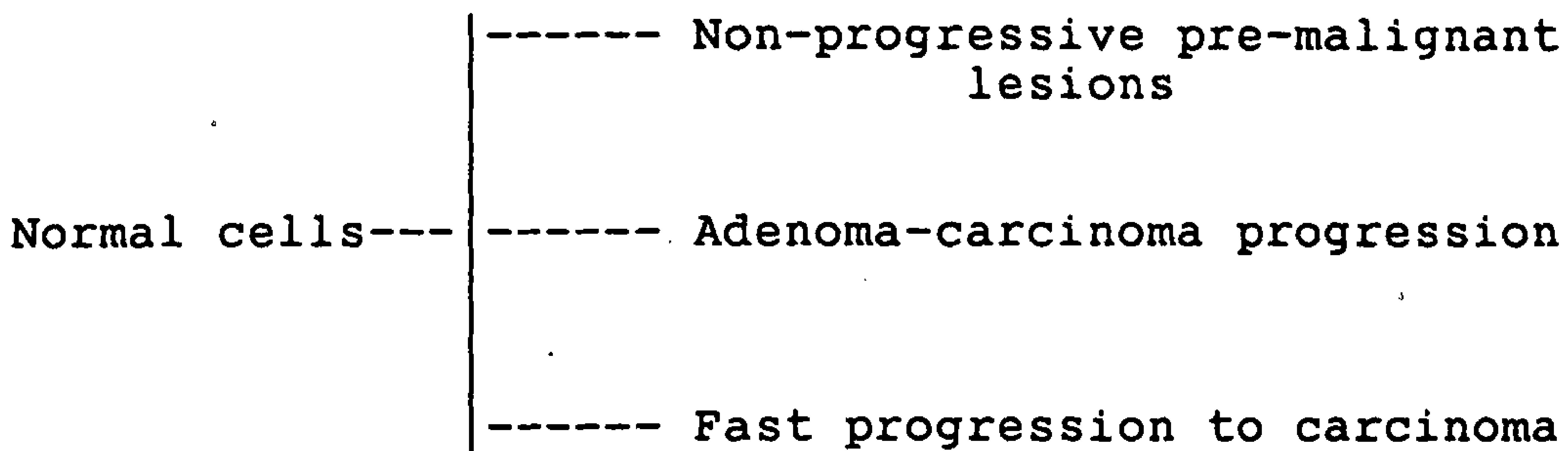
1.1 The study of colorectal cancer

The biology, genetics and common occurrence of colorectal carcinoma make its study important and informative. This carcinoma is the second commonest cause of cancer deaths in the Western world. In Scotland alone (with 5.1 million population) 2,800 new cases are diagnosed every year. Its incidence in the urbanised societies of the Pacific rim- traditionally low- is rising very rapidly. Only in the developing world is this malignant tumour rare. Biopsy specimens are available from putative stages of neoplastic progression from normal colonic epithelium through a prolonged non-infiltrative adenoma stage to malignant adenocarcinoma and finally to metastatic disease. Analysis of phenotypic and genotypic changes at each stage may ultimately elucidate factors involved in tumour progression. There are many known genetic associations with colorectal carcinoma, including the existence of a clearly identifiable familial syndrome with an inherited predisposition to colorectal carcinoma. Investigation into the genetics of both this syndrome and spontaneous cases has helped to provide a genetic framework on which to build an understanding of the loss of control during tumorigenesis. Understanding of this common human cancer may also provide valuable leads for the analysis of other neoplasms for which information and biopsy specimens are less accessable.

1.2 Models of tumour progression

The concept of linear tumour progression in the adenoma to carcinonma sequence (Muto et al 1975) is widely accepted for colorectal tumours. Genetic alterations have been found to occur at various stages of neoplasia (Vogelstein et al 1988) and a genetic model for tumour progression has been proposed (Fearon and Vogelstein 1990). However, none of the tumour-associated genetic lesions has been detected in more than 75% of colorectal tumours and some are found in less than 50% (Vogelstein et al 1988). No single lesion is therefore necessary for the generation of carcinoma and in some cases, none of the known colon-associated genetic changes is detected (Delattre et al 1989, Vogelstein et al 1988). Multiple genetic alterations have been shown to occur significantly more frequently in tumours removed from the distal colon (from the splenic flexure to the end of the sigmoid bowel), while tumours of proximal location were less likely to show such changes (Delattre et al 1989). This observation suggests that different genetic mechanisms may be responsible for the generation of proximal versus distal carcinomas. A linear genetic model for tumour progression such as proposed by Fearon and Vogelstein 1990, would therefore be insufficient to explain the genesis of all colorectal tumours. A more emancipated model of tumorigenesis might allow for both the genesis of carcinoma by different mechanisms and for the possibilty of non-progressive pathways (figure 1).

Figure 1 Alternative pathways of carcinogenesis



Indeed, the vast majority of adenomas never progress to carcinoma even in the inherited syndrome FAP. A precedent for a different molecular basis underlying phenotypically similar neoplasms with variable malignant potential may be seen in CIN (cervical intraepithelial neoplasm) lesions in the pathology of cervical carcinoma. A wide range of HPV (human papilloma virus) types are commonly found in epithelial cells at early CIN stages but only a subset of these types such as HPV18 are found in carcinoma (Coleman et al 1986). Much research on colorectal cancer evolution will be required to fully elucidate the pathways and mechanisms involved. Currently attention is focussed on the loci involved in Vogelstein's genetic model of linear progression, and the evidence for involvement in colon tumorigenesis at many of these loci will now be discussed.

1.3 Inherited colorectal cancer in FAP patients

In familial adenomatous polyposis (FAP), hundreds to thousands of adenomatous polyps develop in the mucosa of the large intestine and rectum. Colorectal adenocarcinoma invariably arises in affected individuals if the adenomatous large bowel is not removed by surgery. The

syndrome is inherited in an autosomal dominant fashion in accordance with a one gene hypothesis. A case report of the constitutional deletion of a region of chromosome 5 in a mentally retarded individual with multiple developmental abnormalities and FAP (Herrera et al 1986) has led to the localisation and recent isolation of this gene.

1.3.1 The APC gene was localised to chromosome 5

The APC gene (adenomatous polyposis coli) was first localised to the long arm of chromosome 5 by two independent efforts (Bodmer et al 1987, Leppert et al 1987). By first constructing a linkage map of chromosome 5 with 16 polymorphic markers and then examining the constitutional DNA of 5 affected kindreds using these markers as probes, Leppert was able to show linkage to the disease gene and localise it within this primary genetic map. Similarly, Bodmer investigated 13 families using 5 unmapped chromosome 5 probes and on finding close linkage to FAP, localized the linked markers to chromosome 5q by in situ hybridisation.

1.3.2 The APC gene in sporadic and inherited cancers

According to Knudson's two mutation model for human cancer (Knudson 1987), both sporadic and inherited forms result from mutations in the same gene. Minisatellite probes to 5q were therefore used to show tumour specific allelic loss in at least 20% of sporadic cases (Solomon et al 1987). Following these discoveries further candidate probes were identified, linkage to APC was

established for each and their order was determined (Nakamura et al 1988, Meera Khan et al 1988, Dunlop et al 1989, Dunlop et al 1990). Using these linked probes it was then possible to detect tumour specific allelic loss at a higher frequency and to characterize the nature of such losses. As predicted by both Knudson's theory of somatic mutation and by the cautious definition of APC as a tumour suppressor gene, inherited forms of the cancer showed one allele (presumably the normal allelic copy of the gene) to be deleted during the development of adenocarcinoma (Miyaki et al 1990). Furthermore, results from our laboratory probing sporadic tumours with markers closely linked to APC established the incidence of loss was high (over 40%), and ascertained that the major cause of loss was interstitial deletion followed by mitotic recombination (Ashton-Rickardt et al 1989), unlike the high incidences of whole chromosome loss being reported for other putative oncosuppressor genes (Muleris et al 1990). Subsequent studies concluded that tumour progression involved losses of a similar molecular nature in inherited cases (Okamoto et al 1990), suggesting that the same genetic alterations occur in both familial and sporadic cancers. The remaining copy of APC in sporadic tumours, as in inherited cases, is assumed to carry a point mutation or other small defect which is sufficient to inactivate the gene. The recombinogenic nature of the favoured methods of allelic loss i.e. mitotic recombinations and interstitial deletions may suggest a

clustering of breakpoints in this region or may indicate a selective advantage for retention of the region telomeric to the APC gene, although existing rare cases of whole chromosome loss argue against the latter.

1.3.3 Tumour-specific APC loss early in tumorigenesis

LOH (loss of heterozygosity) at 5q21 is detected in 29% of sporadic and up to 20% of familial adenomas (Vogelstein et al 1988, Miyaki et al 1990), and in at least 42% of sporadic and 52% of familial carcinomas (Ashton-Rickardt et al 1991, Miyaki et al 1990). Frequent detection of the LOH event in adenomas may suggest its involvement during benign stages of tumorigenesis. Moreover, since most familial polyps do not show LOH and therefore can be considered to have heterozygous defects only, single allele abnormalities appear sufficient to permit polyp formation. Although this implies a role for APC in tumour initiation, the maximal frequency LOH is detected at the carcinoma stage, allowing the possibility that defects around the APC locus exert important effects throughout progression.

1.3.4 Identification of APC and MCC genes

The APC gene was recently isolated by two independent efforts both making use of YACs (yeast artificial chromosomes) to clone large contiguous regions of 5q21 (Joslyn et al 1991, Kinzler et al 1991b). 3 and 6 new genes respectively were isolated by these groups. Using PCR (polymerase chain reaction), searches for mutations in these genes in FAP patients resulted in

selection of the same candidate APC gene by both groups (Groden et al 1991, Nishisho et al 1991). In one case the same mutation was found in FAP and GS (Gardner syndrome) patients suggesting both to be environmental variants of the same disease (Nishisho et al 1991). A cytoplasmic location is suggested for the unusually large predicted protein of the APC gene which bears local sequence similarities to myosins and intermediate filament proteins (Kinzler et al 1991b). The predicted coiled-coil structure of the amino terminal quarter of the APC protein is expected to interact with other proteins (Bourne 1991). Such interaction may be necessary for protein function and, in defective proteins, could lead to dominant negative action analogous to that suggested for p53 protein (section 1.5.1). Mutations were also detected in some sporadic colorectal cancers in another of the 6 genes in which no inherited defects were found (Kinzler et al 1991a). The transcription unit of this gene MCC (mutated in colorectal cancers) is close but points in the opposite direction to that of APC. Both proteins are expressed in normal colonic mucosa and a wide variety of other tissues.

It is interesting to note that in a search of over 200 sporadic tumours for somatic mutations in large portions of either of these genes, only 2 were found (Nishisho et al 1991). Furthermore, of mutations detected in a total of 10 patients, all led ultimately to unscheduled stop codons and unexpectedly, only 2 showed

Figure 2 CHROMOSOMAL ABNORMALITIES IN COLORECTAL CANCER

Vogelstein et al, 1989

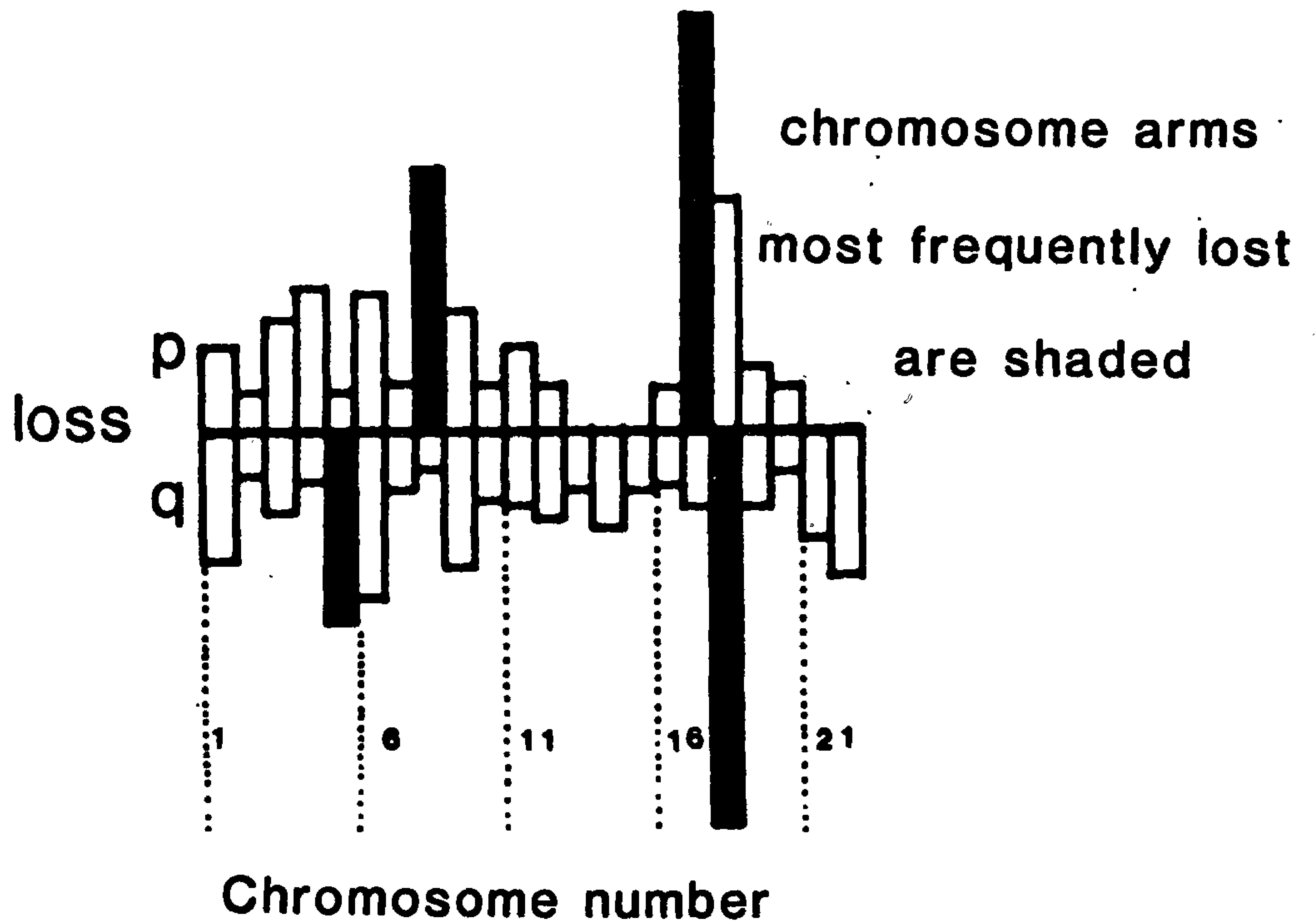
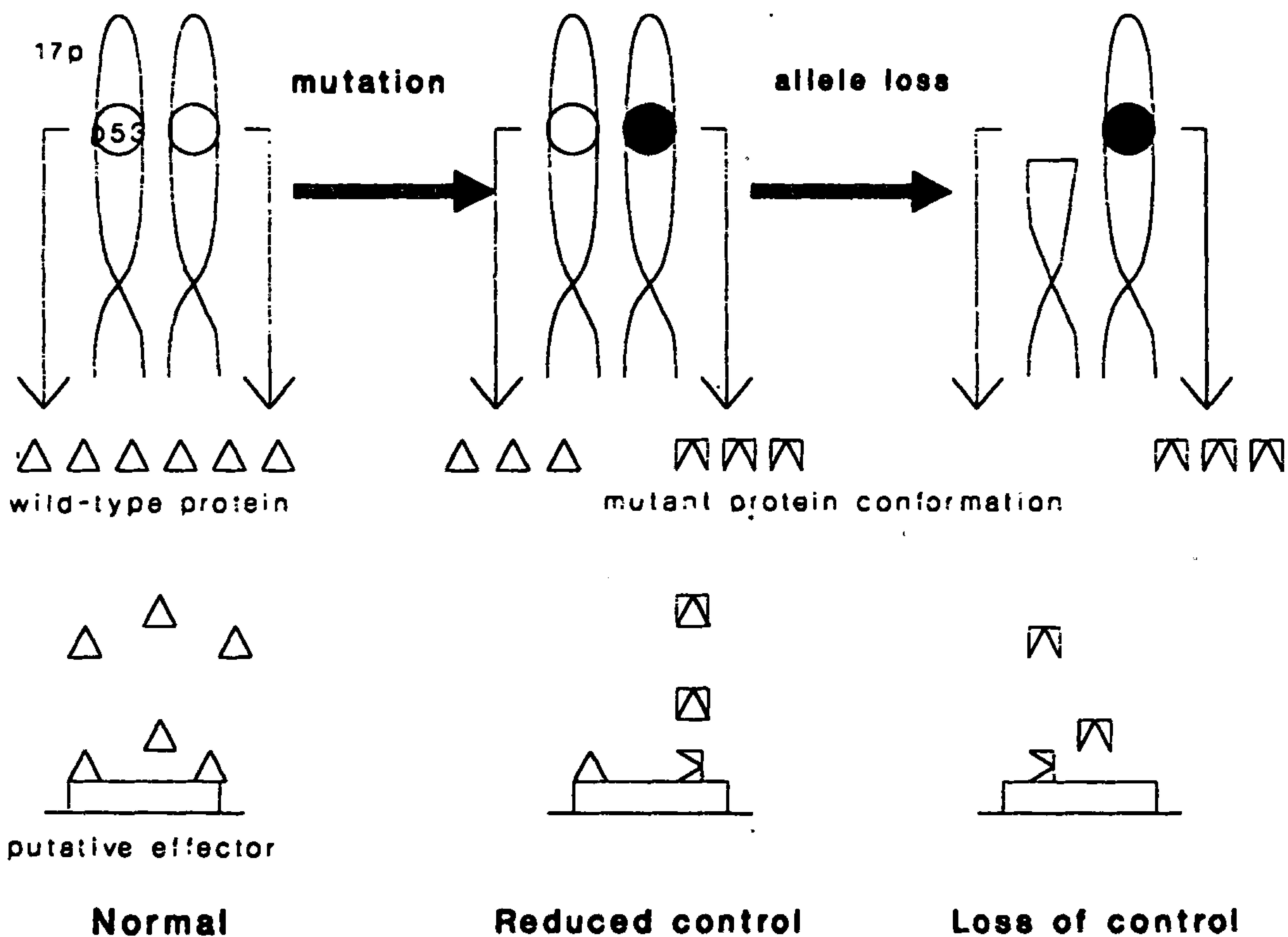


Figure 3 p53 defects on 17p



coincident 5q21 allele loss (Nishisho et al 1991).

1.4 An allelotype for colorectal cancer

Besides the putative tumour suppressor gene on chromosome 5 many other genes are involved in both sporadic and inherited colorectal cancers. In 1989 Vogelstein published an allelotype of colorectal cancer (Vogelstein et al 1989), in which polymorphic markers from every non-acrocentric autosomal arm were used to compile a list of the frequencies of tumour specific loss for each. All markers were lost in at least some tumours but the most frequently detected arm losses, in descending order, were as follows; 17p, 18q, 8p, 18p, 5q, 22q, 6q, 1q, 21q, 9q, 4p. The final members of this list showed about 25% loss (figure 2). This begs the question that if all chromosome arms are sometimes lost are there tumour suppressor activities on each? If, on the other hand, many of the losses can be attributed to random loss due to aneuploidy and the resulting chromosome rearrangements during tumorigenesis, at what frequency of loss can oncosuppressor function be inferred? The answers to these questions are still unclear and may remain so for some time since the study of oncosuppressor genes is still in its infancy, with their discovery hindered by their recessive nature. However, some of Vogelstein's high frequency of loss candidates have been assigned tumour suppressive activity which has been confirmed by functional data.

Oncosuppressor function can only be confidently

assigned to a gene by the demonstration of oncosuppression specifically by that gene in genetic manipulation studies. A colon-associated tumour suppressor gene for which such functional data is available is the p53 gene residing at 17p13.

1.5 The p53 gene is an oncosuppressor

The p53 protein, originally discovered in complex with the large T antigen of SV40 in transformed cells (Lane and Crawford 1979), has undergone intense study over the last decade. Early experiments, in which mixtures of activated ras and the p53 gene were transfected into cell lines, suggested that the p53 gene possessed transforming capabilities. It later transpired that these experiments were misleading since the copy of the p53 gene in use, thought to be wild type, carried a single point mutation. This activating mutation was shown to produce a protein with decreased binding affinity to large T, ability to complex with the cellular heat shock protein hsc70, and a greatly increased half life (Finlay et al 1988). By elegant co-transfection experiments it was shown that contrary to previous ideas, wild type p53 could decrease the transforming function of the original point-mutated p53 and activated ras mixtures. Furthermore, wild type p53 could decrease the transforming function in the same experiment when mutated p53 was substituted with adenovirus transforming protein E1a (Finlay et al 1989). These findings helped to reclassify p53 as a tumour suppressor gene rather than an

oncogene, and suggested a central role in growth control.

1.5.1 p53 protein may have a dominant negative effect

Besides reclass ifying p53 as an oncosuppressor gene, the above observations give rise to an apparent paradox. How can a gene function in wild type as a tumour suppressor by virtue of its central role in growth control yet, after a single point mutation, gain the function of an ac tively transforming oncogene? Since oligomerization of the p53 protein correlates with transformation (Kraiss et al 1988), mutant p53 has been hypothesised to sequester co-existing wild type protein in inactive complex, so forcing the neglect of p53 function in growth control (figure 3). In this way the mutant protein is said to have a dominant negative effect, yet the wild type gene remains an oncosuppressor. This theory is upheld by the observation of a greatly increased half-life of mutant protein, thought to exist in a stabilized protein aggregate.

1.5.2 Dominant activating p53 mutations may also exist

There are, however, some problems with this simplified dominant negative theory of p53 action. The ability to produce progressive tumours only after introduction of mutated p53 has been observed for a cell line which was known not to express p53 (Wolf et al 1984). The mutated p53 protein can not work in this case by sequestering wild type protein, yet transforming ability is demonstrated. Furthermore, transfections with different p53 mutant genes have been shown to display

different transforming capacities in the same _in vitro_ assay, yet all bind wild type p53 with the same efficiency (Halevy et al 1990). Such observations are evidence that the suppressor activity and the transforming potential of _p53_ are 2 distinct functions. A recent hypothesis suggests that wild type control depends on allosteric regulation and mutant p53 adopts an active growth conformation. In this way mutant proteins may have variable activity yet regulate wild type protein in a dominant negative fashion by driving it into a mutant conformation. Such alterations in wild type conformation by mutant protein have been demonstrated (Milner and Medcalf 1991).

1.5.3 p53 mutations are detected in many human tumours

Despite having different transforming potentials, different point mutations in the _p53_ gene are thought to exert common conformational changes in the protein which promote self-oligomerization. These common conformational changes together with the resulting increased half-life allow the detection of mutant protein by immunocytochemistry. A number of antibodies have been raised to p53 and used to detect the expression of accumulated protein in both fixed and frozen pathological specimens. By this method it was found that mutated p53 protein could be detected in 45% to 55% of colorectal cancers (Van Den Berg et al 1989, Purdie et al 1991), and at similar frequencies in a variety of other human tumours including breast (Cattoretti et al 1988, Davidoff

et al 1991), leukemia (Koeffler et al 1986), ovary (Marks et al 1991), lung (Iggo et al 1990), stomach, bladder, testis, soft-tissue sarcomas and melanomas (Bartek et al 1991).

p53 mutations are also often detected by PCR amplification and sequencing. The majority of mutations detected in this way cluster between amino acid residues 130 and 290 (out of 393), and are localized in 4 regions corresponding to high regions of conservation between species (Soussi et al 1990). There exist residue hotspots for p53 mutation which differ between cancer types. Transversions are strikingly frequent at position 3 of residue 249 in the p53 genes of hepatocellular carcinomas of South African (Bressac et al 1991) and Chinese (Hsu et al 1991) patients, who may be exposed to similar carcinogens. Point mutations detected in p53 for the majority of all cancers are usually missense giving rise to altered rather than truncated protein.

The p53 gene has recently been shown to be inherited as a germ-line mutation in a study of five families affected by the dominantly inherited familial Li-Fraumeni cancer syndrome (LFS) (Malkin et al 1990). These mutations, so far, seem also to have restricted distribution in the p53 locus. The restricted distribution of Li-Fraumeni p53 mutations may represent a less severe mutation than many others somatically acquired, and constitutional p53 mutations with more severe effects may not permit survival.

1.5.4 17p allele loss commonly includes loss of p53 gene

Since the p53 gene was known to reside at 17p13, it was provocative that the most frequently detected arm loss in Vogelstein's allelotype was of 17p. Further investigation of this tumour specific loss in colorectal cancer using 20 polymorphic probes established that the area of common loss could be localised to the region containing the p53 gene (Baker et al 1989). Furthermore, sequencing of the remaining copy for two cases revealed point mutations in the highly conserved regions previously implicated in murine p53 mutations. Further studies to evaluate the generality of these observations showed that for a variety of human tumour types (breast, lung, brain, and colon) the majority of 17p losses left hemizygous p53 mutations which were also sometimes present in the heterozygote (Nigro et al 1989).

1.5.5 17p alterations at the adenoma-carcinoma boundary

In order to establish the timing and order of these chromosome 17 alterations in the evolution of colorectal cancers, both 17p loss and p53 mutations were studied for a large series of adenomas and carcinomas (Baker et al 1990a). p53 mutations were found rarely in adenomas and rarely in 17p heterozygotes, but commonly in carcinomas where allelic loss was observed. This suggests a synchrony of the two events occurring at the adenoma-carcinoma boundary. Indeed one carcinoma was discovered to have both lost heterozygosity and acquired a p53 mutation yet contained an area of adenoma in which

neither event had occurred. These observations suggest the occurrence of one of these events to provide strong selection for the other to follow. Such selection demands that the two events must co-operate, an idea which does not fit easily into the domin ant-negative p53, or the oncogenically activated p53 schools of thought. However, there are cases in which mutations within the p53 gene and 17p allele loss do not coexist (Davidoff et al 1991, Shirasawa et al 1991, Coles et al 1990, C Purdie unpublished observations). Furthermore, reports exist of p53 mutations in adenomatous polyps from FPC patients (Shirasawa et al 1991) and in sporadic adenomas (Purdie et al 1991) suggesting occasional earlier involvement.

1.5.6 Functional evidencee for growth suppression by p53

The described LOH and mutations of the p53 gene conform to the predicted pattern for inactivation of a tumour suppressor gene. This descriptive definition must however be supplemented with functional information. To provide such information, constructs expressing wild type p53 have been transfected into a number of cell lines (Finlay et al 1989, Baker et al 1990b, Diller et al 1990, Mercer et al 1990). In all cases selection resulted in a dramatically reduced number of p53 transfectant colonies in comparison to numbers obtained in control transfections. All p53 transfectants displayed either increased doubling times and lower saturation densities, or were growth arrested. Such observations led to proposals that p53 could monitor progression through the

cell cycle and confirmed the suggestion that p53 could suppress growth.

1.5.7 p53 may have a role in cell cycle control

On herpes virus infection the cellular replication machinery becomes localised to sites of viral replication. Both p53 protein and that of another well studied oncosuppressor (Rb) were recently shown to colocalise with the replication machinery under these circumstances (Wilcock and Lane 1991). Furthermore, a temperature sensitive p53 mutant exists which inhibits growth and is localised in the nucleus at 32°C but moves to the cytoplasm of growing cells at 37°C. Movement of this protein from the cytoplasm to the nucleus was shown to coincide with inhibition of DNA synthesis (Gannon and Lane 1991). Expression of wild-type p53 in a human glioblastoma cell line was shown to block cell cycle progression in association with a marked decrease in histone H3 expression, which was subsequently shown to be accompanied by selective down-regulation of PCNA (proliferating-cell nuclear antigen) expression, whose protein is a component of the cellular replication machinery (Mercer et al 1991). Such evidence may suggest a regulatory role for p53 in the assembly or function of the DNA replication-initiation complex. However, there is a different school of thought that p53 may act as a transactivator of gene transcription (for review see Levine et al 1991). p53 has often been compared with, and shares many functions of, the oncosuppressor gene Rb. The

Rb protein has recently been shown to complex with a cellular transcription factor (Bandara and Lathangue 1991) and is now thought to regulate transcription. Wild type p53 has recently been shown to have sequence-specific DNA-binding properties (Kern et al 1991), the effects of which are not yet known.

Regardless of mechanism of action, functional evidence implicates a role for p53 in cell-cycle control. Moreover, induction of apoptotic cell death by transfection and expression of an exogenous wild-type p53 gene has recently been demonstrated (Yonish-Rouach et al 1991).

Despite the obvious importance of the p53 protein, it is worthy of note that many cells both *in vitro* and *in vivo* continue to grow in the complete absence of p53 expression. Furthermore, Li-Fraumeni patients carry constitutional p53 mutations yet develop normally, the only obvious phenotype being a predisposition to carcinogenesis (Malkin et al 1990). Despite the undeniable importance of the role of p53 there may well be other mechanisms at work which may substitute for and co-operate with p53 under certain circumstances.

1.6 The DCC gene: An oncosuppressor on 18q?

After 17p, the chromosome arm loss most frequently detected in colorectal cancer is 18q (figure 2). The gene of interest on 18q has recently been identified (Fearon et al 1990). This involved an exhaustive exon connection strategy by reverse transcriptase PCR using primers to

areas of open reading frame. The gene termed DCC (for deleted in colorectal cancer) consists of at least 8 exons in 370Kb of DNA, with the predicted amino acid sequence of a cell surface glycoprotein similar to the neural cell adhesion molecule. Its loss occurs about as frequently in colorectal carcinomas as that of the p53 gene (about 70%). Unlike 17p losses, 18q losses occur in 47% of late adenomas suggesting loss to be an intermediate event in tumorigenesis (Vogelstein et al 1988). Also unlike the p53 gene, expression of DCC was greatly reduced or absent in the colorectal carcinomas tested. No functional information for the cloned gene has been published to date.

1.7 Colon tumour-associated LOH is confirmed at other loci

Studies have been conducted to check frequencies of allele loss for both FAP tumours and sporadic tumours. These studies confirm the frequent loss of some of the other members of the allelotype series such as 22q (Okamoto et al 1988) and 14 (Sasaki et al 1989). However, the methods used to construct the allelotype were fallible owing to the use of few probes per arm, or sometimes only one. Loss of heterozygosity can be confined to small chromosome regions (Ashton-Rickardt et al 1991), and such losses may not be detected by sparsely located probes. Loss of heterozygosity has subsequently been discovered in 42% of colorectal cancers at 1p35 (Leister et al 1990), a chromosome whose loss was infrequently detected in the allelotype.

1.8 Ki-ras mutations detected early in tumorigenesis

As well as oncosuppressor genes, oncogenes have a role in tumorigenesis. The *ras* oncogene in particular is implicated in colorectal (Bos et al 1987, Forrester et al 1987), amongst many other cancers. *Ras* p21 is a membrane localised protein involved in GTP hydrolysis and with presumpptive signal-transduction capacity (Sigal 1988, Barbacid 1987 & 1990). The gene is activated, acquiring a transforming funtion, by point mutations in codons 12, 13 and 61. Such mutations are found in a variety of human and murine cancers and occur in the *K-ras* gene in about 50% of colorectal carcinomas and late adenomas. In our group mutations have also been detected at a similar incidence in hyperplastic polyps (P Romanowski, unpublished). In contrast, *Ki-ras* mutations have been found in under 10% of adenomas less than 1cm in size (Vogelstein et al 1988). Such a discrepancy may reflect the fact that the small adenomas studied were selected on the basis of being FAP patient adenomas, and this group was then characterized as being less than 1cm in size (Vogelstein et al 1988). Such a set of tumours may not accurately reflect the sporadic incidence of mutations in small adenomas.

1.9 Other oncogenes may be involved in colorectal tumours

Other oncogenes implicated in colorectal tumorigenesis include *c-src*, *c-fos* and *c-myc*. The latter is thought to produce a nuclear transcription factor promoting entry into the G1 phase of the cell-cycle and

retention thereafter in the cycling state. Accordingly, myc transfection of cultured cells leads to immortalisation without transformation. C-myc mRNA is found to be more highly expressed in both colorectal carcinomas and adenomas than in normal mucosa and such overexpressing tumours have been positively correlated with deletions around the APC gene (Erisman et al 1989). However, such altered expression may merely reflect the increased growth rate of the cells. It has been suggested that since an inherited APC defect allows polyposis, 5q defects may be associated with increased proliferation thus leading to the detection of apparent increases in c-myc expression (Maestro et al 1991). An increased activity in colonic tumours is also reported for the membrane localised tyrosine kinase c-src (Bolen et al 1987), and a decrease in expression of c-fos mRNA has been detected (Klimpfinger et al 1990).

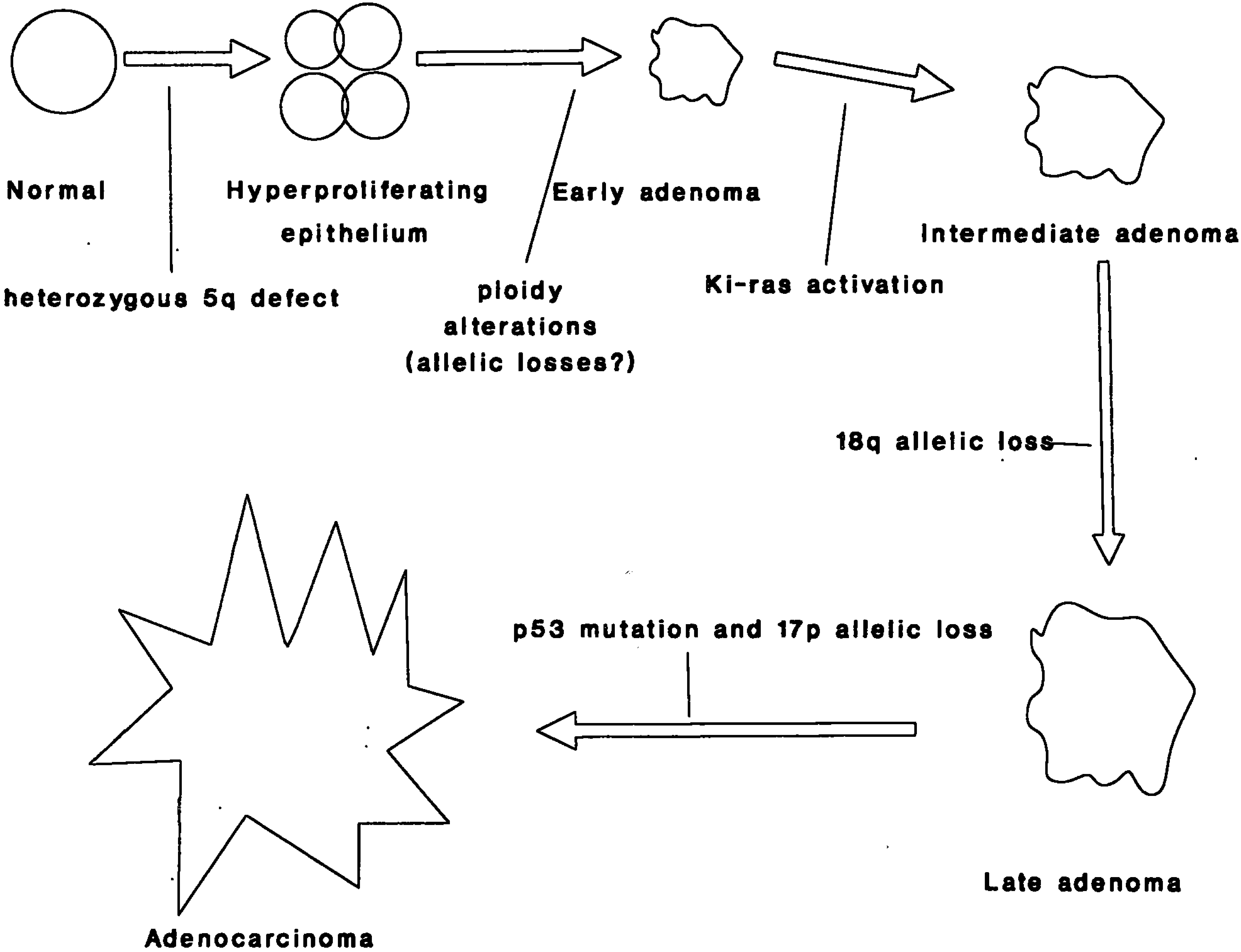
Such altered expressions and activities need not be due to changes in coding sequence and indeed, known cases of specific oncogene amplification or rearrangements in colonic tumours are rare (Fearon and Vogelstein 1990). It is therefore possible that many of these altered activities are a consequence of the neoplastic state, as hypothesised for c-myc over-expression. Alternatively, proto-oncogene expression may be altered in a significant way by mutations in the upstream regulatory sequences rather than the coding region, for which some evidence has been produced for the ras genes (Chakraborty et al

1991). At present there is no clear indication of the extent to which such modification of upstream sequences (by mutation, rearrangement, or binding of transcription factors) is part of tumour progression in colorectal mucosa.

1.10 Aneuploidy is common in colorectal tumours

Another potentially important step is the significant tumour-specific reduction in DNA methylation evident in early benign lesions. This could inhibit chromosome condensation and lead to mitotic non-disjunction. Whether resulting from this or other causes of genomic instability, DNA aneuploidy is detected by flow cytometry in about 60% of colorectal cancers (Giaretti et al 1990) and is a good prognostic indicator (Quirke et al 1987, Armitage et al 1991). Aneuploidy is rarely detected in adenomas and carcinoma ploidy abnormalities increase progressively with size and histological type (Van den Ingh et al 1985). In practice karyotypically aneuploid tumours are known to fall into two broad classes (Reichmann et al 1982, Muleris et al 1990)- near-diploid tumours (where single chromosomes tend to become lost) and tetraploid and severely aneuploid tumours. Only the second class is readily detected by flow cytometry. In either case it is clear that such karyotypic abnormalities may be involved in the generation of allelic losses. While non-specific allelic losses may be generated and evident in Vogelstein's allelotype, specific combinations of allelic loss

Figure 4 Genetic model for colorectal adenocarcinoma progression



critical for tumour progression may be present within this set.

1.11 Accumulation of genetic lesions during progression

A model has been proposed to incorporate the major genes involved in colorectal tumour progression and their observed timing throughout this process (Fearon and Vogelstein 1990, and figure 4). However, the authors found many tumours which did not conform to the prescribed order of genetic changes within this model. None of these changes was entirely restricted to its proposed stage of action and each was sometimes found to occur out of sequence relative to the other events in the model (Fearon and Vogelstein 1990). Given that the timing and order of these events were not obligatory, complex interplay between different combinations of genetic lesions may be suggested. The authors drew the conclusion that the accumulation rather than ordder of these lesions was the critical factor. However, as already discussed, the linear genetic model (figure 4) has limitations and an alternative model (figure 1) may be more generally applicable.

1.12 Co-operation of lesions in oncosuppressor tests

If combinations of genetic lesions are co-operating to make the tumour phenotype, the genetic background of the cell will decide what effects any new lesion may have. This idea may have consequences in functional tests by transfection of these genes. If transfected into uncharacterized recipient cell lines, the unknown genetic

background of recipient cells may confound interpretation of the effects of the transfected gene. Phenotypic effects may vary according to genetic lesions available in the recipient cell for co-operation with the exogenous gene.

1.13 Oncosuppressor genes inferred from cell hybrids

Oncosuppressor genes require functional testing _in vitro_. The existence of such genes was first demonstrated by somatic cell hybrids (reviewed in Stanbridge 1990). Chromosomes 1, 4 and 11 were identified as of major importance in the control of tumorigenesis. In these experiments, however, not only was the genetic background of the recipient cell incompletely characterized but chromosomes were introduced in random combinations. An improvement on this method was the development of microcell transfer and tagging with selectable markers to allow selective introduction of single specific chromosomes into tumour cells. Using this method tumour suppression was demonstrated by chromosomes 1, 3, 6, 9, 11, 13 and 17 on introduction into certain tumour cell lines. Although this method was preferable to the introduction of whole genomes into recipient cells, the genetic material introduced in a single chromosome is still enormous. Effects of the large number of genes introduced interacting with each other and with endogenous genes may be sufficiently complex to preclude meaningful interpretation. Perhaps for this reason, some of the chromosomes implicated in these early studies seem

so far to bear little relevance to current knowledge of locations of oncosuppressor genes.

1.14 Oncosuppression of Wilms' tumour by chromosome 11

Following these experiments, the microcell technique has been used with some success to demonstrate the control of tumorigeniciity by chromosomes predicted from cytogenetic and RFLP analyses. The pediatric nephroblastoma Wilms' tumour is associated with deletions on chromosome 11p13 and 11p15 (Koufos et al 1989). In agreement with the postulate that an oncosuppressor gene resides in this area, control of tumorigenicity was demonstrated in a Wilms' tumour cell line by microcell introduction of a normal chromosome 11 (Weissman et al 1987). Despite the persistence of phenotypic transformed traits including expression of several proto-oncogenes in culture, tumorigenicity in nude mice was completely suppressed. This non-tumorigenic line was also shown to have lost the ability to grow in serum-free medium and to resume growth in the presence of specific growth factors (Dowdy et al 1991). Control microcell fusions of chromosomes 13 and X had no effect. However, despite the clear significance of this work, the lack of specificity of whole chromosome introduction and the lack of recipient cell line characterization attracts criticism. The recipient line was an established Wilms' tumour cell line with two cytogenetically normal copies of chromosome 11. There was therefore no certainty that a chromosome 11 defect was originally present for correction by the

microcell transfer. Indeed, certain familial forms do not appear to map to chromosome 11 at all (Huff et al 1988). Thus, although tumour suppression by chromosome 11 is clearly demonstrated in this experiment, it is not possible to conclude that correction of a specific Wilms' tumour gene defect is demonstrated. Indeed, tumour suppression can be demonstrated by introduction of chromosome 11 into both HeLa and SiHa cell lines (Stanbridge 1990).

1.15 Oncosuppression by the retinoblastoma gene

Specific oncosuppression by the Rb (retinoblastoma) gene has been demonstrated. Mutational inactivation of the Rb gene was proposed as a necessary step in the development of retinoblastoma and a possible step in the genesis of some other tumours. In order to test the inferred oncosuppressor function, the cloned retinoblastoma gene was introduced by retroviral mediated gene transfer into both retinoblastoma and osteosarcoma cell lines containing inactivated Rb (Huang et al 1988). On introduction of normal Rb into the retinoblastoma line the growth rate of the cells was significantly decreased and tumorigenicity in nude mice was suppressed. On introduction into the osteosarcoma line growth in culture was more severely inhibited by Rb. Such results underline the fact that one oncosuppressor gene may have different effects depending on the background on which it acts. Presumably, co-operation with different unknown factors in the two recipient lines led to the different

transfectant phenotypes.

1.16 Oncosuppression by p53

A second oncosuppressor gene which has been functionally tested by specific gene introduction into tumour cell lines is the previously discussed p53 gene (Finlay et al 1989, Baker et al 1990b, Diller et al 1990, Mercer et al 1990). On the introduction of a wild type p53 gene into colonic carcinoma cell lines, 5-10 fold less colonies grew than transfectants receiving the mutant gene which differed in only one nucleotide (Baker et al 1990b). In all growing colonies the exogenous p53 sequences were deleted or rearranged. In the same study, a transient transfection showed expression of the wild type protein to dramatically inhibit progression through the cell cycle as measured by tritiated thymidine incorporation. A cell line derived from a benign tumour, also transfected with the same constructs, showed no measurable growth inhibition (Baker et al 1990b). Furthermore, in a separate experiment wild type human p53 cDNA introduced into SV40- transformed hamster cells proved to inhibit but not arrest growth. A decreased number of colonies were observed with lower saturation densities and increased doubling times (Mercer et al 1990). Presence or absence of effects of exogenous p53 in different recipient lines may tentatively be explained in terms of whether the recipient line has already sustained some event leading to loss of normal p53 control. SV40 transformation is an example of such an event (Lane and

Crawford 1979). The variety of transfectant phenotypes observed could be related to recipient line species differences, but the p53 gene is highly conserved between species such that a more plausible explanation might be the co-operation of other genetic lesions of the recipient cell with the exogenous p53. If so, further p53 transfections of human tumour cell lines of known status at p53 and other colon-associated loci would prove informative. Such experiments would elucidate whether other genetic lesions, singly or in combinations, may modulate or substitute for loss of p53 control in the pathway to the tumour phenotype.

1.17 Oncosuppression by chromosomes 5 and 18

MCC and APC on 5q, and DCC on 18q have only recently been cloned. Consequently no functional analyses are published to date. However, microcell introduction of chromosomes 5, 18 or 11 into a colonic cancer cell line have been described. Introduction of each of chromosomes 5 and 18 were reported to cause morphological changes with cells becoming more closely packed or flatter respectively. Transfectants with either chromosome had also completely lost the abilities to form colonies in soft agar and to produce tumours in nude mice. Chromosome 11 introduction caused no substantial changes (Tanaka et al 1991). As the first evidence of oncosuppression by chromosome 5 or 18 sequences, this result represents an important milestone. Within the recipient cell, however, tumour specific losses of sequences were known to exist

around the 18q locus but for 5q were merely assumed. This is a daring assumption considering the highest reported incidence of 5q loss stands at less than 50% (Ashton-Rickardt et al 1989). Furthermore, a search of over 200 sporadic cases has revealed only one somatic change in the APC gene and one in MCC (Nishisho et al 1991). The large quantity of exogenous DNA introduced by microcell transfer and lack of characterization of colon-associated lesions in the recipient cells in this experiment preclude clear conclusions on the effects of specific genes and their co-operation.

The critical importance of the recipient cell in these experiments may be demonstrated by a further study in which sequences on chromosome 5 were introduced by microcell transfer into v-Ki-ras transformed mouse fibroblasts (Hushino et al 1991). Despite confirmation of the introduction of DNA in which both APC and MCC are known to reside, tumour formation in mice was not suppressed.

1.18 Ideal transfection experiments

As discussed, the value of experiments to test oncosuppressor activity is often limited by lack of specificity of introduced material, undefined genetic background of the recipient cells, or both. In order to rectify this situation it would be desirable to use recipient cell lines which are representative of the relevant neoplasm, characterized at all the major tumour-associated loci and known to be defective for the gene

under test. For example, the APC gene would ideally be tested in a colorectal cancer epithelial cell line in which tumour specific APC gene defects are characterized, and for which gene status is known for the other colon-associated loci.

1.19 Long-established lines are not ideal recipients

Long-established colorectal cell-lines are widely available and commonly employed. These cell lines, however, do not fulfil the requirements of the hypothetical ideal test vehicle for a number of reasons. Firstly, in no case was the tumour from which they derived adequately defined in genetic terms. Tumour specific genetic changes can therefore be assigned in very few cases. Secondly, and contributing to the first problem, is the tendency for chromosomal rearrangement in culture, so that sub-lines with different morphologies and karyotypes can be established from a single primary tumour (Brattain et al 1983). Continuous passage *in vitro* will preferentially select for any rearrangement that leads to increased growth capacity. Due to the artificially contrived environment in culture, these changes need not be representative of those occurring *in situ*. Indeed reports of karyotypes of various colonic lines are very diverse and the overlap with those reported for primary tumours is limited. Some examples of these reports are summarized in table 1. Of particular prominence is the common gain of an extra chromosome 7 in tumour cell lines, yet chromosome 7 has not been

implicated in the genesis of colorectal tumours. A plausible explanation for frequent over-representation in culture may lie in the fact that the gene coding for epidermal growth factor (EGF) maps to chromosome 7. EGF significantly stimulates the growth of human colon carcinoma cells in culture (Murakami and Masui 1980), and the extra chromosome 7 may therefore confer a selective advantage to cultured cells. Presumably in vivo growth of colonic epithelial tumour cells is not constrained by lack of EGF since chromosome 7 gains are not documented for solid tumours or xenografts. This example illustrates the fact that long-established tumour cell lines may be deceptive in demonstrating consistent, apparently specific genetic defects that do not reflect changes in vivo or in situ. Such lines are not therefore good test

Table 1 Karyotypes of colorectal cancer cells

Frequent abnormalities	Cells	Reference*
17p loss and 18 loss	solid carcinoma	Muleris 1990
17p loss and 8 gain (modifications 1 and 5)	solid carcinoma	Reichmann 1981
17p loss and 18 loss	carcinoma xenograft	Lefrancois 1989
7 gain (modifications 1 and 2)	carcinoma cell line	Chen 1982
7 gain	carcinoma cell line	Brattain 1983
7 gain (modifications 1, 3 and 21)	carcinoma cell line	McBain 1984
7p gain and 13 gain (12, 13, 16, 17 gains, 19, 20 loss)	carcinoma cell line	Namba 1983
iso5q	carcinoma cell line	Semple 1978
absent sex chromosome	carcinoma cell line	Tom 1976
iso1q	carcinoma cell line	Jenkyn 1987
iso1q, iso14q, X: 17 translocation	carcinoma cell line	Paraskeva 1984

Notes:
1) All numbers represent chromosomes or chromosome arms
2) Only major modifications are listed
3) *= "et al" omitted from all references

systems for genetic manipulation experiments.

1.20 Ideal recipient cells

For an ideal test system it would be necessary to establish and characterize a set of novel cell lines. These should be characterized with respect to the original genetics of the parent tumour, such characteristics ideally beingg unaltered in culture. Xenografting tumours into immunodeficient animals may help to avoid rearrangements by providing a less artificial environment than that of cell culture. Short-term, secon darily derived cultures from such xenografts would be uniquely favourable vehicles for functional analysis of colon-associated genes in various known genetic backgrounds. Such assays may provide precise information about the functions of oncosuppressor genes and the effects of co-operating loci.

1.21 Aims of this thesis

The aim of the work described in this thesis was to propagate and characterize a number of colorectal adenocarcinomas as ideal experimental systems. In so doing it was hoped to generate information on colonic epithelial cells in xenograft and in culture. Preliminary investigations into transfection of such cells, and the effects of oncosuppression *in vitro* were also planned.

Chapter 2: Establishment of stable tumour xenografts

2.1 Abstract

Human colorectal cancer xenografts were estab lished in immunosuppressed CBA, or SCID (severe combined immunodeficient) mice, resulting in take rates of 38% and 92% respectively. Tumour xenografts were shown, without exception, to retain phenotypic and genotypic features of original primary tumours. The stability of 12 xenografts, maintained in 5 cases for over one year, was defined in terms of allelic status at colon-associated oncosuppressor loci on 5q, 17p and 18q, immunocytochemical detection of p53 mutations, ploidy and histological features. Xenografts could be stored frozen in a viable form yet retained the original defined features after re-implantation. Phenotypic and genotypic features of propagating xenografts reflected those observed for unselected carcinomas with 2 possible exceptions. p53 over-expressing tumours were under-represented and mucinous histology was unexpectedly common for xenografts in immunosuppressed CBA hosts. Regardless of selection of primary tumours, the stable characterized xenografts represent outstanding material for a variety of experiments. Besides their obvious value in proposed experiments to test oncosuppressor gene activity, they provide a renewable resource of purified human tumour ideal for DNA sequence studies. The variety of lesions represented by the xenograft set will provide the opportunity to observe the effects of specific defects and their combinations on tumour biology.

2.2 Introduction

2.2.1 Xenografts in tumour analyses

Tumour xenografts in immunodeficient animals provide valuable models of human colorectal cancer. The practice of propagating tumour tissues as xenografts has many advantages over direct culture of cells. Firstly, preliminary results from other studies have suggested that tumours growing as xenografts may conserve some primary tumour features (Lefrancois et al 1989). Should this represent lack of tumour progression or evolution, xenografts will be faithful representatives of colorectal cancers. Such stable systems would be preferable experimental tumour models to the changing long-established cell lines (discussed in chapter 1 and described in table 1). A second advantage of xenografts over primary culture is the capacity of immunodeficient host animals to limit some bacterial infections by non-specific defence mechanisms such as macrophages and complement. In contrast even low levels of infection by antibiotic-resistant bacteria are generally terminal for cell cultures. This is of particular significance for colonic explants which carry contaminating micro-organisms co-transplanted from the bowel. A third advantage of xenografted tumours is the replacement of their connective tissue stroma by cells from the animal host (Kopf-Maier and Kestenbach 1990). Whereas primary cultures imply growth of tumour cells and normal human stroma cells in competition, the stroma of xenografted

human tumours is excusively from another species. This species difference can be exploited in xenograft tumour samples for differential recognition of human tumour from non-tumour cells, allowing specific elimination of the latter in xenograft derived cultures. Furthermore, loss of human stroma in xenografted tumours will, for many experiments, make them preferable tumour resources to the primary tumours. In DNA studies of primary tumour tissue, such as PCR amplification of sequences or detection of altered RFLPs by Southern blotting, non-tumour DNA from admixed stromal cells often confounds the detection of tumour-specific alterations. Indeed, for allele loss studies in Southern blots, areas of tumour tissue are often selected by cryostat sectioning to minimise contamination of the neoplastic cell population with stromal non-neoplastic cells (Vogelstein et al 1988). The absence of non-neoplastic human cells in xenografted tumours eradicates this problem.

Owing to size, breeding rate and ease of husbandry the animal of choice for graft propagation has been the mouse. Host immunodeficiency is essential to prevent graft rejection and three main kinds of immunodeficient mice are described.

2.2.1.1 The nude mouse as a xenograft vehicle

In 1962 a spontaneous hairless mouse mutant was discovered. The trait is recessive, and homozygous animals were found to lack a thymus gland (Pantelouris 1968). The impaired keratinization that renders these so-

called nude mice hairless may result from the same ectodermal developmental defect that is responsible for the absence of thymus. Lack of thymus and consequent lack of mature T-lymphocytes results in deficient cell mediated immunity and hence the acceptance of foreign tissue grafts. Accordingly it has become common practice to use the nude mouse system for clinical and biological research on human malignant tissues.

Several factors affect the success of human heterotransplants in nude mice including health and receptivity of the animal host, graft preparation, implantation site and type of graft (Reid et al 1978). Breast tumours, for example, were found to require 6 months before developing in nude mice whereas colon tumours showed little to no dormancy before growth. Such differences may be attributed to differing dependence on angiogenesis and other factors such as hormones. Over the last 20 years a number of groups have maintained colorectal cell lines as xenografts in nude mice (Carrel et al 1976, Morikawa et al 1988) and established that these xenografted tumours do not metastasise on subcutaneous implantation (Giavazzi et al 1986).

2.2.1.2 The immunosuppressed mouse as a xenograft vehicle

As an alternative to nude mice as xenograft recipients, techniques were developed in the early 1970s for immunosuppression of immunocompetent CBA mice by thymectomy and whole-body irradiation. Such animals, protected from the lethal effects of bone marrow

radiation by previous injection of cytosine arabinoside, were found to be more receptive to human adenocarcinoma xenografts than were nude mice (Steel et al 1978). Furthermore, CBA mice, even after immunosuppression, are thought to be more robust and less susceptible to minor infections than nude mice. Indeed the high incidence of minor infections such as eye abscesses commonly experienced with nude mice were rarely seen in immuno-suppressed CBA mice. However, some deg ree of immunorecovery was noted about 5 weeks after immunosuppression suggesting these mice to be useful for the propagation of rapidly growing tumours only. Xenografts propagated in this immunosuppressed host were shown, like those in nude mice, to maintain biological characteristics during serial passage (Houghton and Taylor 1978) thus comparing favourably with the nude mouse as a vehicle for propagation of human colorectal cancers.

2.2.1.3 The SCID mouse as a xenograft vehicle

More recently a third type of host for xenografts emerged with the discovery of the severe combined immunodeficiency syndrome (or SCID) mouse. The SCID mutation was found in BalbC derived mice to segregate as a single autosomal recessive gene not linked to the major histocompatability locus. Phenotypically, mice homozygous for the mutation are congenitally deficient in both B and T lymphocytes with thymus and lymphoid organs less than one tenth the normal size (Bosma et al 1983). This lack

of functional lymphocytes is now thought to be the result of an inability to correctly rearrange the immunoglobulin and T-cell receptor genes by site-specific recombination due to defective V(D)J joining (Schuler and Bosma 1989). A pleiotropic effect of this mutation is hypersensitivity to ionizing radiation and a deficiency in DNA double-strand break repair (Biedermann et al 1991). It has been suggested that the SCID gene product performs a similar function in both the double-strand break-repair and V(D)J recombination pathways and may be an ubiquitous protein (Hendrickson et al 1991). The early arrest of B and T cell maturation, although proving leaky in 15-25% of young adults and in all older adults (Bosma and Carroll 1991), qualifies the SCID mouse as a third vehicle to receive xenogeneic grafts. Indeed, the success of human tumour grafts into SCID mice has in some cases improved on and in no case been poorer than their success in nude mice (Phillips et al 1989).

2.2.1.4 Using all three xenograft hosts

Since diverse biological mechanisms underlie the above three types of human xenograft host, variable xenograft success may be observed in each. By comparing growth of groups of tumours in these different vehicles it may be possible to obtain information about tumour properties which may limit xenograft success in any one host.

2.2.2 Novel xenografts of colorectal cancer

This chapter describes the establishment,

propagation and characterization of xenografted colorectal tumours. Take rates and maintenance rates, established for xenografts in both CBA (immunosuppressed by thymectomy and irradiation) and SCID mice, allowed preliminary comparisons between the receptivity of SCID and CBA hosts. Besides maintenance by continuous passage, methods were devised for the cold storage of viable xenograft tissue. Of primary importance to this study was the analysis of characteristics of xenografted tumours to ascertain if they were stably representative of original primary tumours. Phenotypic characteristics were defined by gross morphology, passage interval, histology and electron microscopy. Flow cytometry was used to measure DNA ploidy, while specific colorectal cancer associated genes were studied by Southern analysis and immunocytochemistry. RFLPs (restriction fragment length polymorphisms) in Southern blots were used to detect tumour specific allele loss as an indicator of oncosuppressor gene loss. Over-expression of p53 was detected immunocytochemically by the mouse monoclonal antibody PAb1801 which is specific for human p53 (Banks et al 1986).

2.3 Results

2.3.1.1 Improved xenograft take in SCID mice

Two different xenografting experiments were performed. The first consisted of two runs of the same design in which colorectal carcinomas from consecutive operations were implanted into immunosuppressed CBA, or occasionally nude, mice. In the second experiment individual carcinomas were implanted into both immunosuppressed and SCID mi ce to allow comparisons of host receptivity to individual tumours.

In two consecutive sets of 28 and 26 primary colorectal carcinomas implanted into CBA mice, 11 and 10 xenografts respectively grew to passage. These results give a take rate of 39% in both cases. Of 14 tumours xenografted into both immunosuppressed and SCID mice 11 grew to passage in at least one of its 2 host types giving an overall take rate of 78%. For only 12 of these tumours did the immunosuppressed CBA host mice survive. 4 of these 12 grew to passage giving a 33% take rate in

Table 2 Xenograft take in CBA and SCID host mice

Experiment	Host	1°ry	Xenograft take	% take
Exp.1, run 1	CBA	28	11	39%
Exp.1, run 2	CBA	26	10	39%
Exp.2	CBA	12	4	33%
Total	CBA	66	25	38%*
Exp.2	SCID	13	12	92%
Total	SCID	13	12	92%*
Exp.2	Both	9	9 in SCID	100%
			4 in CBA	44%

Notes:
1) 1°ry= primary tumours
2) * P<0.001

immunosuppressed mice, a figure similar to the 39% recorded in the first set. In contrast, SCID mice survived for 13 of the tumours xenografted, 12 of which grew to passage giving a SCID take rate of 92%. Of 9 tumours xenografted into both immunosuppressed and SCID mice where at least one mouse of both groups survived, 4 grew in both host types and 5 grew only in SCID mice. Take rates are summarized in table 2.

2.3.1.2 Xenografts passage continuously for over 2 years

9 of the first 12 successful implantations survived at least 5 serial passages in mice, and 5 were observed over 10 passages or more. One tumour xenograft has now reached 25 passages (greater than 2 years) in mice. The passage history of all 12 xenografts is summarized in table 3 along with the Dukes stage of the corresponding primary tumours.

Table 3 Passage history of first 12 xenografts

Xen.	Duke's stage	Passages	Reason for loss
GRBO	C	8*	Infection (open wound)
RHSP	C	18*	
JACA	C	2	Slow growth/ necrosis
MUCO	B	25*	
CHKE	B	10	Bacterial infection
DABU	B	5	Tumour necrosis
MASM	B	12*	
JOMCL	C	13*	
JOWR	B	2	Host death
ARNE	C	8	Host immunorecovery
AGDU	C	8*	
CARO	D	2	Slow growth

Notes:
1) Xen.= xenograft line
2) Duke's stage applies to corresponding primary tumour.
3) Passages refers to continuous passages achieved from primary implantation.
4) Reason for loss can only be suggested in some cases
5) *=frozen stored tissue available

Two of the lines surviving only 2 passages were notable for their long passage intervals averaging 60 days. It is known that immunosuppressed CBA mice recover some degree of immunity by about 5 weeks post-thymectomy (Steel et al 1978), and hence such slow growing tumours may be selected against. Bacterial contamination of the xenograft leading to line termination was confirmed histologically in 2 animals, and there were a number of host mouse deaths due to severe radiation sickness amongst both tumour bearing animals and their non-experimental litter mates. In one case (AGDU) the original tumour xenografted was of metastatic origin and doubt exists about the origin of the primary tumour. This could not therefore be included with full certainty as a colonic adenocarcinoma for subsequent analysis and was no longer passaged.

The second run of xenografts implanted in immuno-suppressed mice was established more recently. So far only one tumour has grown for more than 5 passages, while 2 others are growing slowly at third passage. All other tumours were lost after 1 or 2 xenograft passages. The maintenance rate in this experiment is clearly poorer than for the previous set and may reflect the receptivity and health of the host mice: both extensive thymic regeneration and high mortality were noted. The similar take rate to that of the first run may be explained by the fact that 4 hosts rather than 2 (as in the previous run) were implanted with every primary tumour, and those

with subsequent host death were removed from the set.

Low passage maintenance data available for tumour xenografts in SCID hosts was incorporated in a life table (figure 8). A Log-rank test (adjusted for tumour p53 status also included in the table) shows the difference in survival between xenografts in SCID or CBA hosts to be significant ($P<0.001$). According to the preliminary information available, behaviour of xenografts growing in SCIDs is similar to that observed in immunosuppressed CBA hosts. Passage intervals were occasionally 3 days to 1 week shorter in SCID hosts but, in general, tumour sizes were the same.

2.3.2 Xenografts can be stored frozen in viable form

In order to establish a permanent renewable tumour tissue resource, a means of cold storage was devised. As described in the materials and methods section, a procedure was adopted which amalgamates conventional cultured cell freezing and preparation of tumour tissue suspensions for injection into animals or for primary culture. Re-implantation was found to reconstitute tumour growth for 5 lines (table 3) which have been frozen for a number of weeks or longer, after 8, 11, 14, 17 and 18 xenograft passages. In no case has any altered growth characteristic been apparent despite the tissue disruption, freezing and re-injection to which the tumour was exposed. One line is currently passaging without any sign of change 6 months after freezing and re-injection. Histological patterns of re-injected frozen tumours are

shown alongside those of routine xenografts in figure 5.

2.3.3.1 Xenografts faithfully reproduce tumour histology

All xenografts surviving at least one passage were confirmed as adenocarcinomas by histology. While local infiltrative behaviour was evident in most xenografts, no metastases were observed in mice.

The original histological pattern was conserved from the primary tumour throughout serial passage in every case studied (figure 5). The case of MUCO is of particular note since the well differentiated pattern of the primary tumour is conserved at 25th passage corresponding to more than 2 years in mice, and after freezing and re-injection at a number of passages. Although the other extreme of poor differentiation can also be seen in this set, the majority of xenografts fell into the class of adenocarcinomas of average degree of differentiation, with a possible preponderance of mucinous tumours (table 4). Histological examination including those tumours not yielding xenograft growth showed the growing subset to otherwise be a fair representative of the set as a whole.

2.3.3.2 Xenografts display epithelial features

Electron microscopy confirmed the epithelial nature of tumours harvested at 5th passage: they comprised polarised cells arranged around acini or mucin lakes. Apical microvilli with underlying exocytotic vesicles were commonly seen. At the basal end of these cells long fibroblastic cells, copiously secreting collagen, were

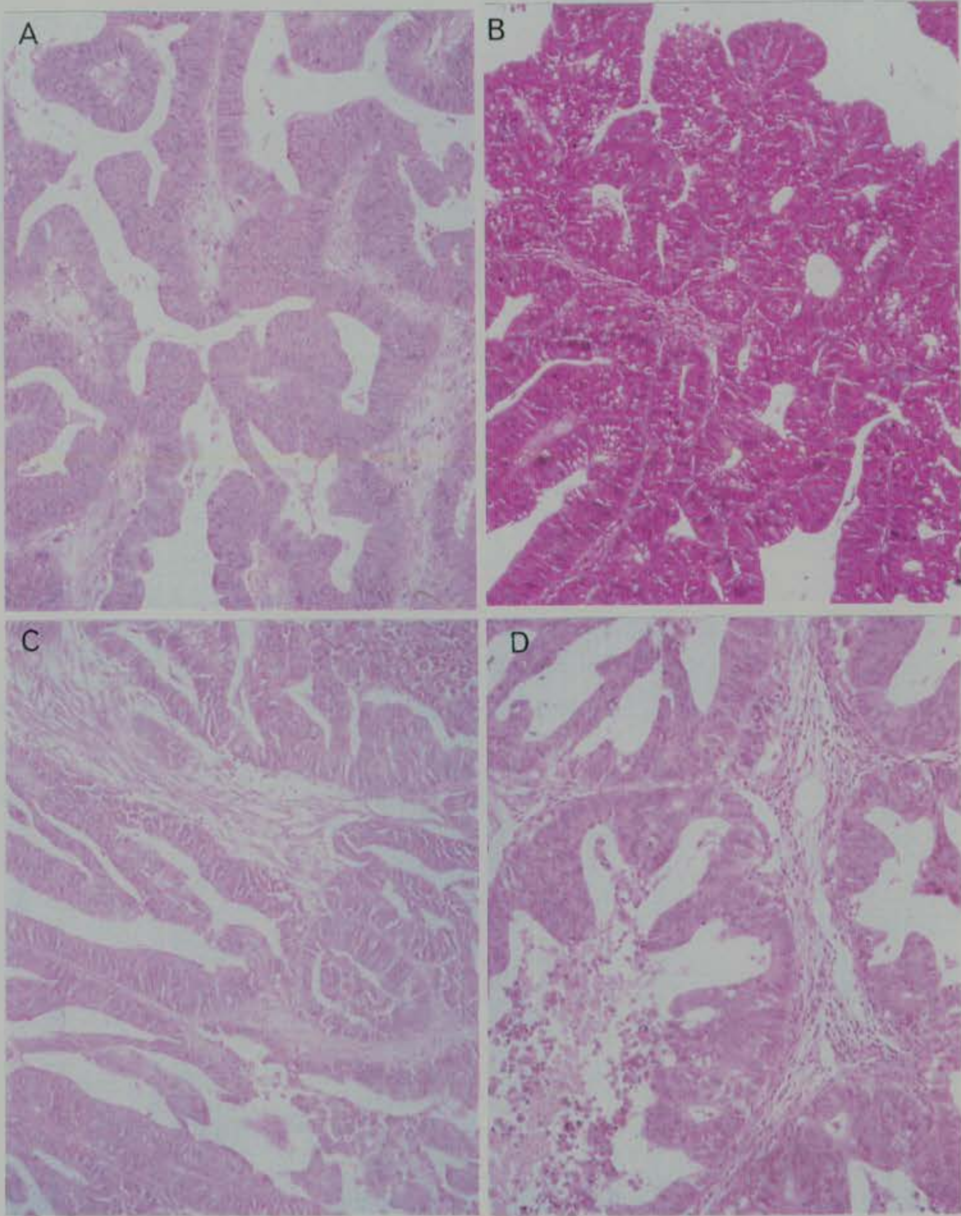
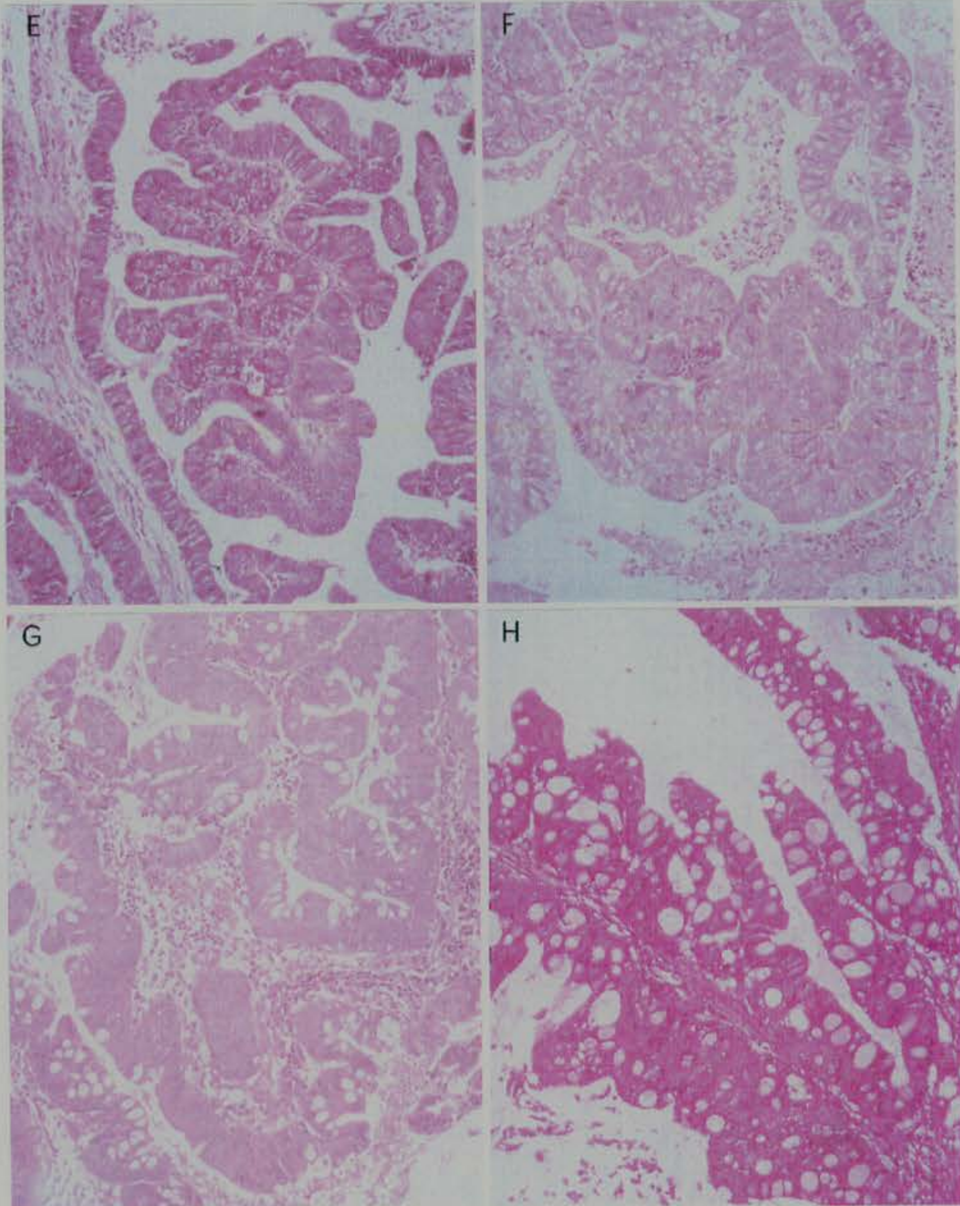
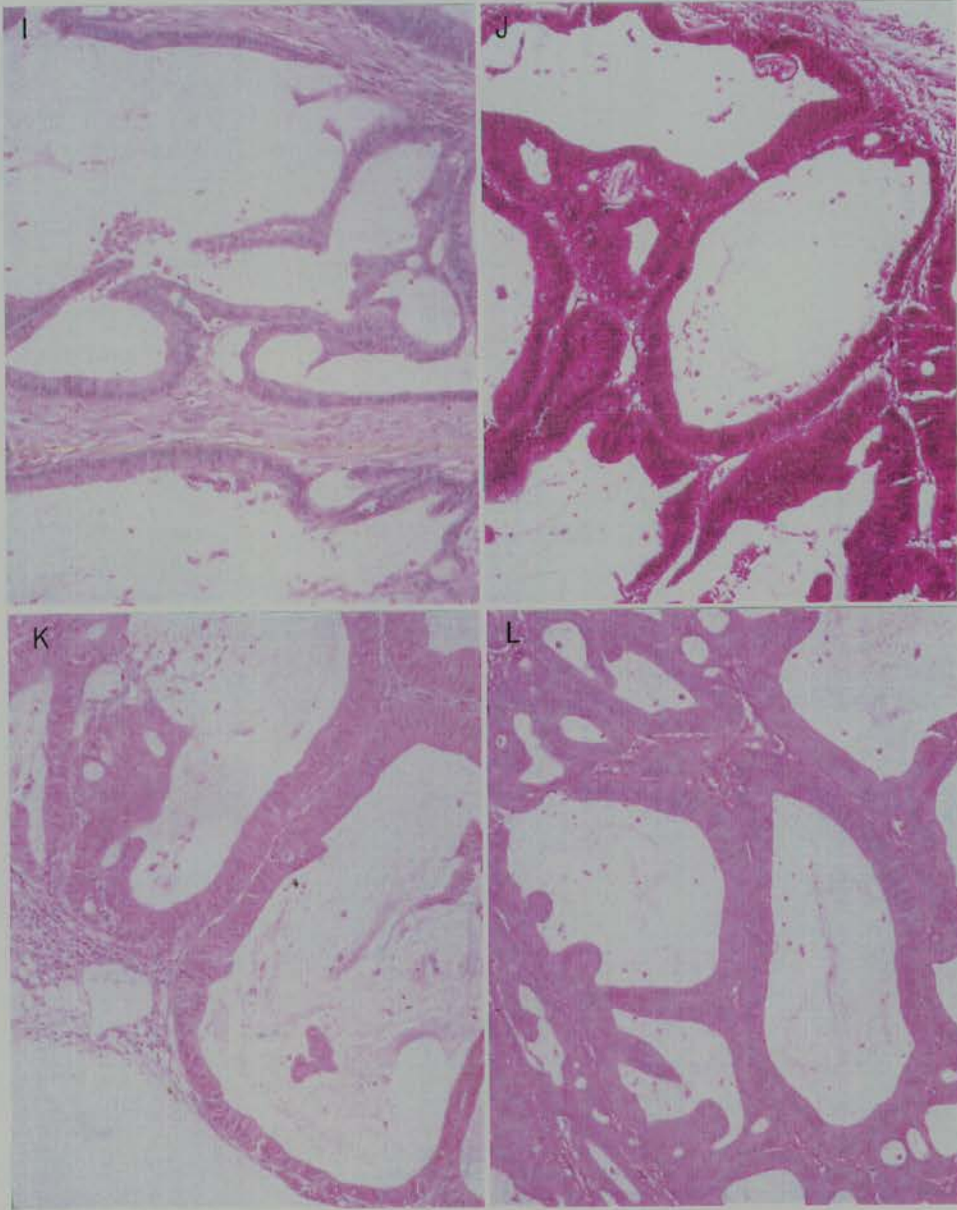


Figure 5 Histological comparisons of original colorectal tumours with the corresponding xenografts (all x100). A; A moderately differentiated primary adenocarcinoma GRBO. B; Corresponding xenograft at 6th passage. C; A well differentiated adenocarcinoma RHSP. D; Corresponding xenograft at 17th passage.

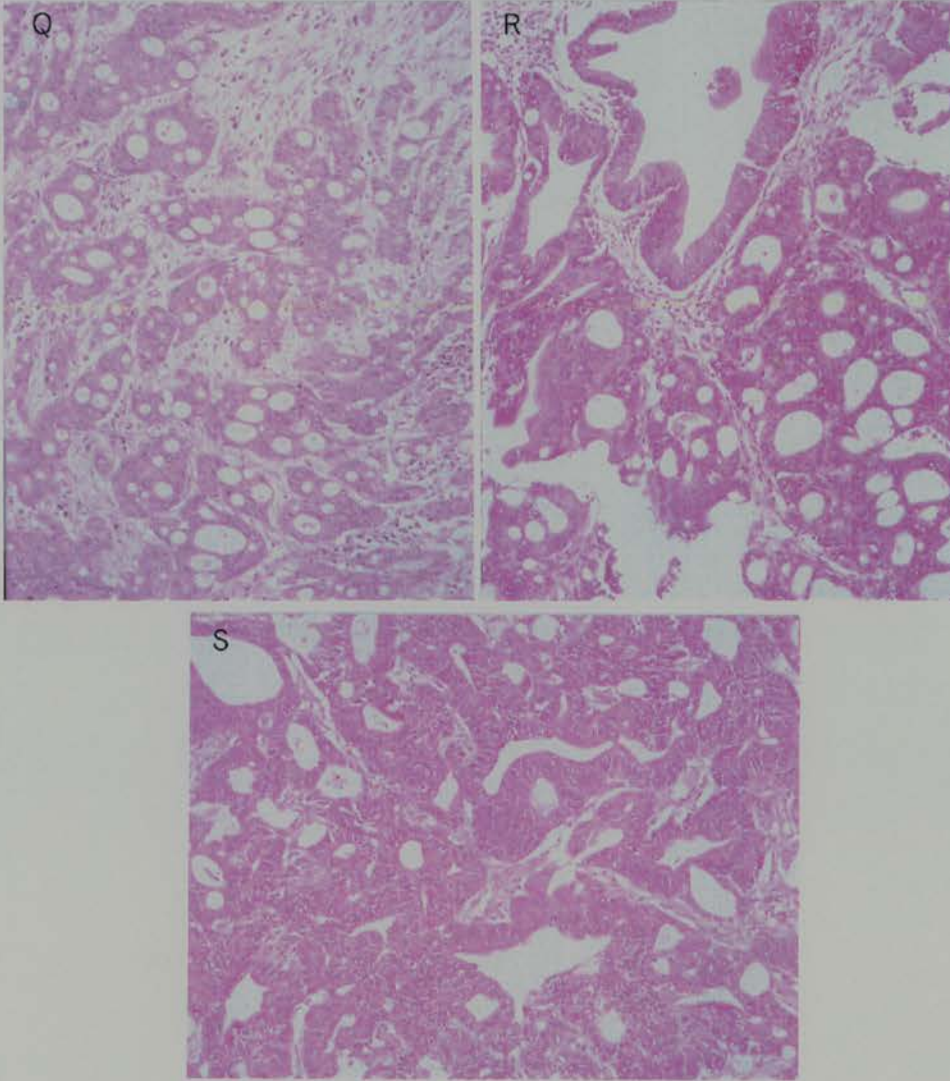


E; A well differentiated primary adenocarcinoma JACA. F; Corresponding xenograft at 2nd passage. G; A well differentiated primary adenocarcinoma CHKE. H; Corresponding xenograft at 9th passage.

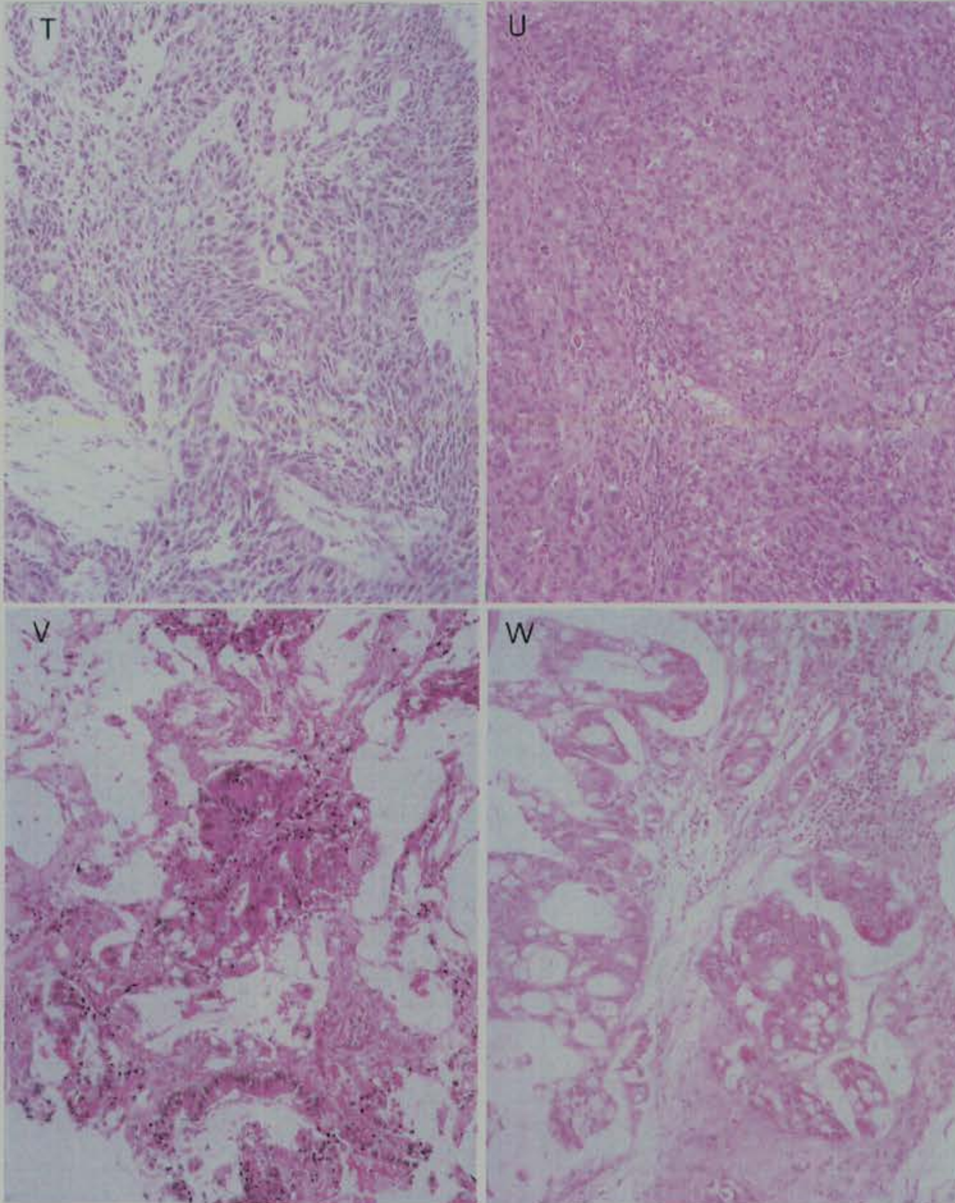


I; A well differentiated primary adenocarcinoma MUCO. J; Corresponding xenograft at 23rd passage. K; Corresponding xenograft after freezing and storage of 9th passage tissue. L; Corresponding xenograft after freezing and storage of 18th passage tissue.

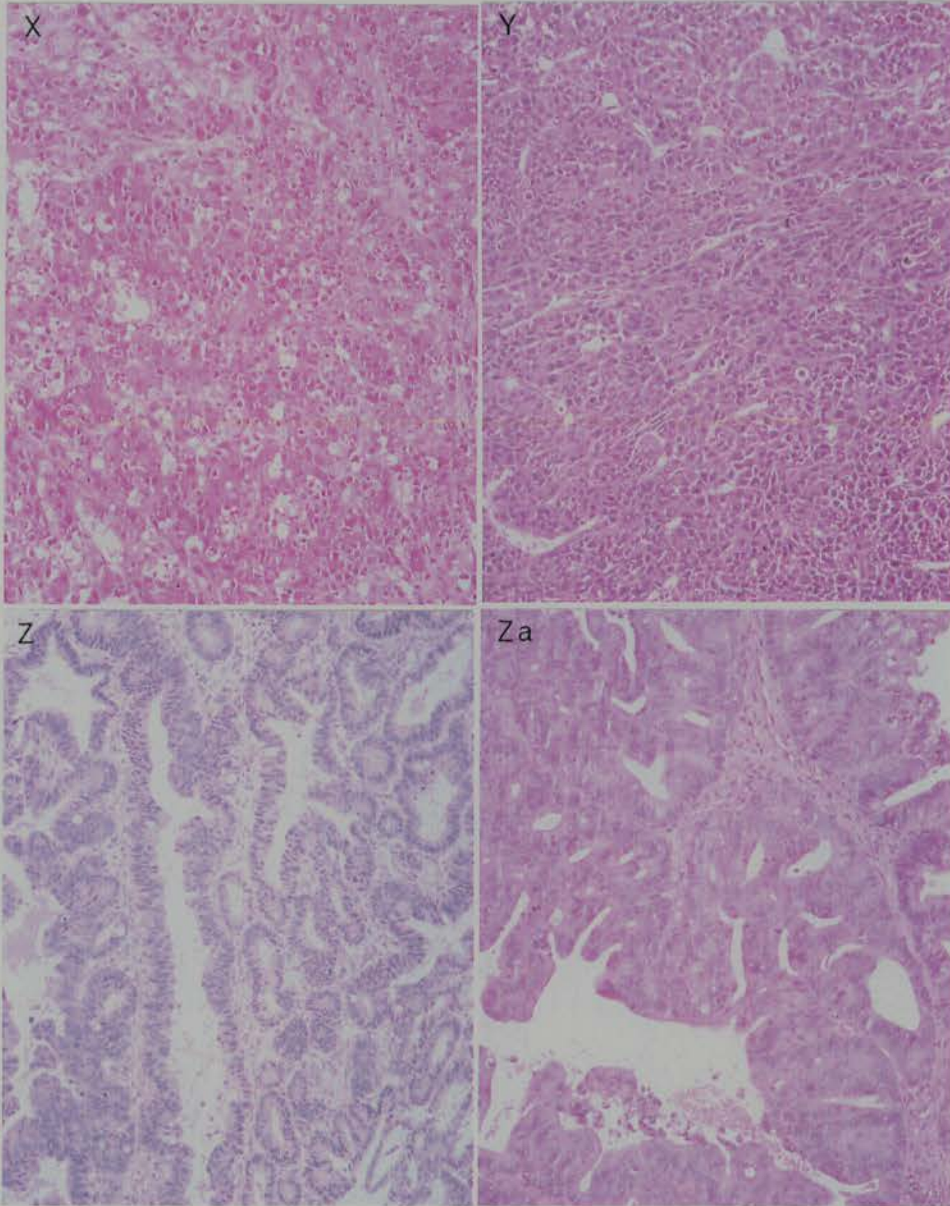
M; A moderately differentiated primary adenocarcinoma DABU. N; Corresponding xenograft at 5th passage. O; A poorly differentiated primary adenocarcinoma MASM. P; Corresponding xenograft at 11th passage.



Q; A moderately differentiated primary adenocarcinoma JOM^{c}L. R; Corresponding xenograft at 11th passage. S; Corresponding xenograft after freezing and storage of 4th passage tissue.



T; A poorly differentiated primary adenocarcinoma JOWR. U; Corresponding xenograft at 2nd passage. V; A moderately differentiated primary adenocarcinoma ARNE. W; Corresponding xenograft at 6th passage.



X; A poorly differentiated metastatic adenocarcinoma AGDU. Y; Corresponding xenograft at 9th passage. Z; A moderately differentiated primary adenocarcinoma CARO. Za; Corresponding xenograft at 2nd passage.

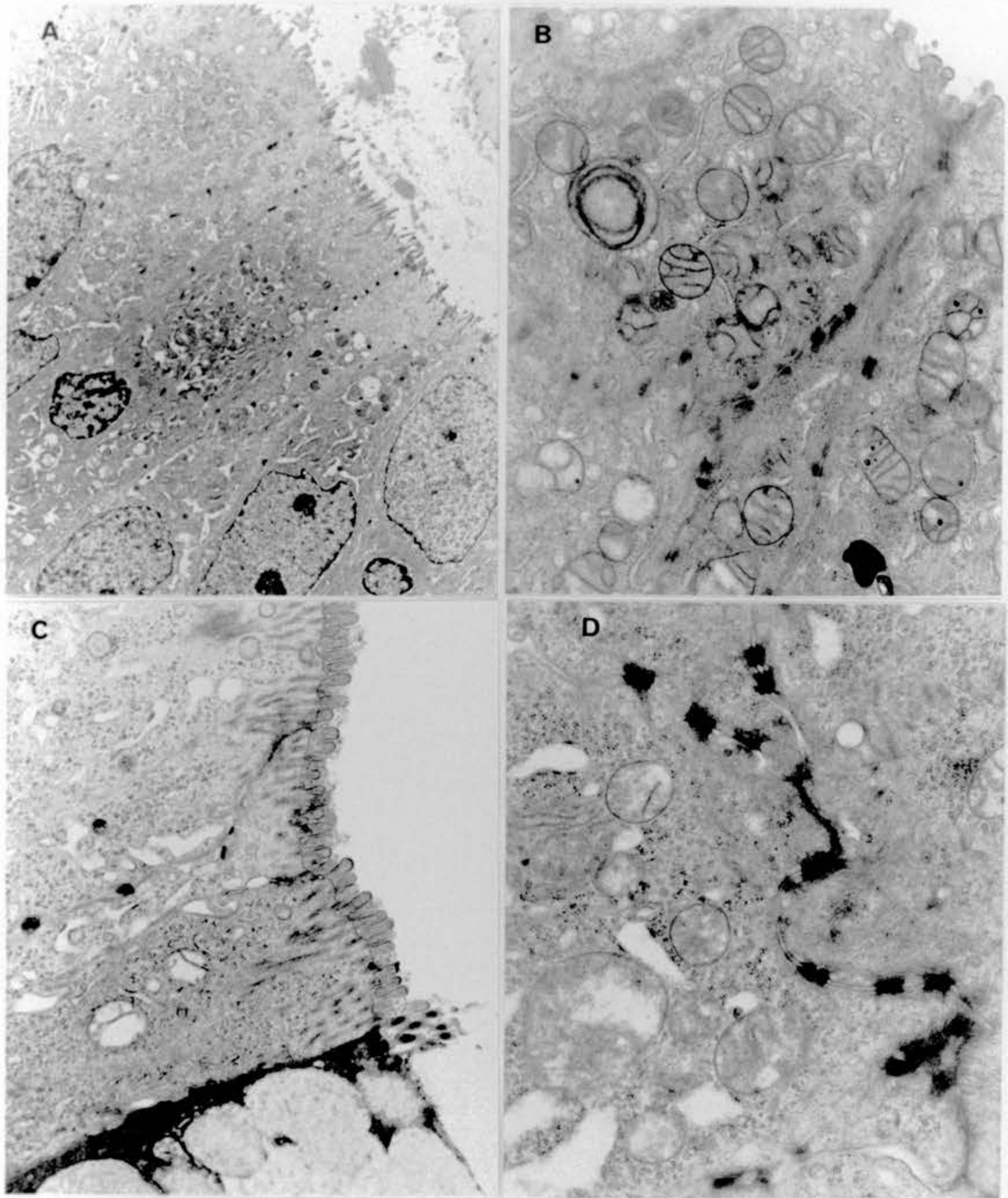


Figure 6 Electron microscopy of 5th passage xenografts showing conservation of epithelial cell markers. A; Polarised cells with apical microvilli in DABU xenograft (x3800), B; Apical tight junctions and desmosomes in RHSP xenograft (x17000), C; Apical microvilli, a variety of junctions and a large pocket of mucin secretion from a goblet cell in CHKE xenograft (x17000), D; Desmosomes in DABU xenograft (x24000).

clearly observed. At apical cell borders tight junctions, a hallmark of secretory epithelial cells, were observed and desmosomes were plentiful (figure 6).

2.3.3.3 Xenografts display characteristic behaviour

The tumours which grew successfully as xenografts tended to show consistent, individually characteristic patterns (table 4a) and rates (table 4b) of growth. Variations in extent of mucin production and tumour morphology were, however, hard to quantify since they depend upon subjective evaluations.

Table 4a Biological characteristics of xenografted tumours

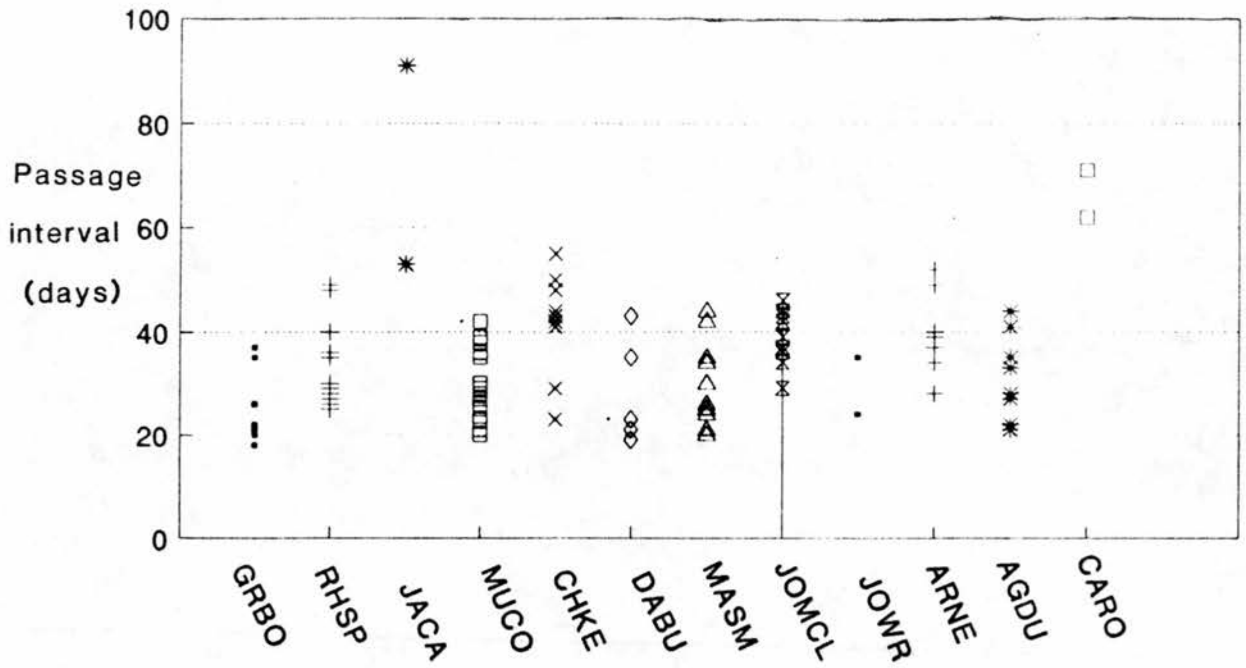
Xen	Histol	Mucin	Passage interval	Additional features
GRBO	M	++	25d (7) **(2.64)** *	Highly angiogenic
RHSP	H	++	34d (18) **(1.66)**	
JACA	M	0	72d (2) **(13.43)**	Grew firm attachments
MUCO	H	++	29d (24) **(1.28)**	
CHKE	H	+	42d (11) **(2.64)** *	
DABU	M	+	28d (5) **(4.14)**	Much necrosis
MASM	P	+	31d (12) **(2.32)**	Well attached, angiogenic
JOMCL	M	+	42d (12) **(1.42)**	Highly angiogenic
JOWR	P	0	29d (2) **(3.90)**	Much necrosis
ARNE	M	+	38d (9) **(2.58)**	Much necrosis
AGDU	P	0	32d (9) **(2.48)**	
CARO	M	+	67d (2) **(3.20)**	

Notes:
1) xen= xenograft lines
2) Histol= degree of histological differentiation;
P= poor, M= moderate, H= high
3) Mucin= degree of mucin production;
0= negligible, += moderate, ++= high
4) Passage= average passage interval (d= in days)
(n)= number xenograft passages contributing to average
(n) = standard error
5) * $P<0.002$, Mann-Whitney U test

2.3.4 Tumour DNA ploidy is conserved in xenografts

DNA index (DI) was measured by flow cytometry and the diploid peak was defined according to an internal chicken standard as described in chapter 7 (materials and

Table 4b Xenograft passage intervals



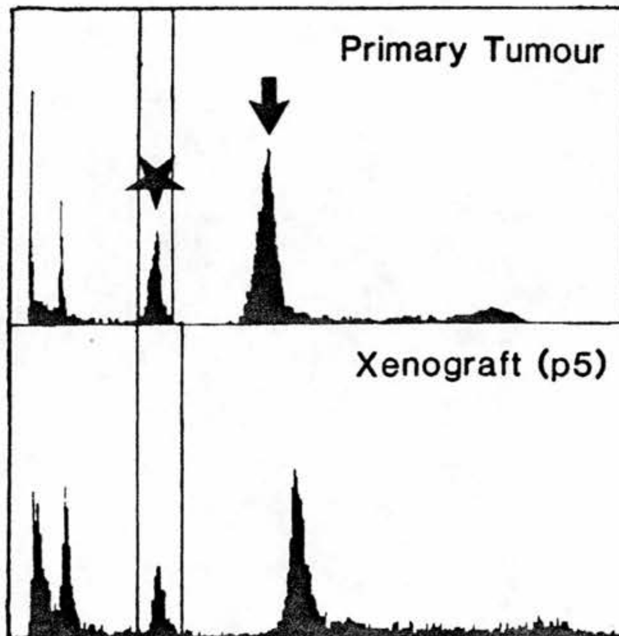


Figure 7 Flow cytometric comparison of an aneuploid primary tumour (DNA index 1.7); arrowed, with the xenograft at passage 5 (DNA index 1.9). Diploid peak position is marked *.

methods). The diploid peak was given a value of 1.0 and DI of any aneuploid peak could be calculated by dividing its channel number by that of the diploid peak. Half peak coefficients of variation were typically less than 3.5.

Of the first 11 primary tumours which gave rise to xenografts, 5 were aneuploid. In all cases studied the DI of the xenograft was similar to that of the relevant primary tumour. This DI conservation includes both diploid and aneuploid examples after more than a year in mice (12 passages), and in one case retention of the diploid status over 24 passages (more than 2 years). DI for tumours and xenografts at various passages are summarized in table 5.

Table 5 DNA index of primary colorectal cancers and corresponding tumour xenografts

Tumour	DNA index Primary	Xenograft (passage number)
GRBO	1.0	1.0 (5)
RHSP	1.7	1.9 (5),(12)
JACA	1.6	-
MUCO	1.0	1.0 (5),(12),(14),(19),(24)
CHKE	1.1	1.2 (5),(9)
DABU	1.0	1.0 (3)
MASM	1.0	1.0 (5),(9)
JOMCL	1.0	1.0 (5),(9)
JOWR	1.0	-
ARNE	1.5	1.6 (5)
AGDU*	1.0	1.0 (5)
CARO	1.4	-

Notes:
*= Metastatic tumour of unknown primary origin
-= Not done

A diploid peak was noted in every sample whether from tumour or xenograft and, by flow cytometry on a sample of mouse liver, the mouse diploid peak position was confirmed to be indistinguishable from that for human

diploid. The ubiquitous diploid peak was therefore assumed to represent the mouse derived stromal component of the tumour.

For the three aneuploid tumours surviving to fifth passage the proportion of cells in the diploid peak remained a constant in two cases, one of which is shown in figure 7. This peak profile was conserved over 11 passages, signifying conservation of histology since it reflects the number of stromal cells in a standard size block of tissue. The alternative interpretation- that balanced proportions of diploid and aneuploid tumour passage together- is unlikely for 2 reasons. Little chance exists; firstly that the correct proportions of both populations would be selected for passage during 11 serial transplants, and secondly that 2 tumour stemlines should grow at exactly equal rates over the entire history of passage, as required to maintain a stable profile. In the third case (ARNE), the fifth passage xenograft has a relatively smaller diploid peak than seen in the tumour yet has conserved histological pattern. This probably reflects ploidy heterogeneity, commonly observed in primary tumours (Quirke et al 1985), with preferential expansion of the aneuploid stemline.

2.3.5 Xenografts maintain genotypes at critical loci

Linked DNA markers were used as probes to detect allelic loss of oncosuppressor loci at 5q21, 17p13 and 18q22. Appropriate genomic digests and conditions for chromosome 5 probes and those on other chromosomes are

summarized in chapter 7 in tables 17a) and 17b) respectively. In these studies, 6 probes spanning the APC locus over no more than 15 Mb, 2 probes to 17p lying close to the p53 gene and a single 18q probe situated within the DCC gene were used. These probes were Pi227, C11p11, L5.62, L5.71, EF5.44, YN5.48; YNZ22, pMCT35.1; and BV15.65 respectively. The patterns detected by some of the probes and examples of allelic losses and retentions are shown in figure 30 in chapter 7.

Samples of constitutional and primary tumour DNA from each patient were analysed together with corresponding xenograft DNA from various passages.

In all cases, at each locus and at each serial passage studied, xenografted tumours maintained the

Table 6 Allelic status of colorectal tumour xenografts at 3 oncosuppressor loci

	chromosome 5					chrom. 17		chrom. 18
Xen.	Pi227	L562	L571	EF544	YN548	YNZ22	MCT35	BV1565
GRBO	R,2	NI,4	R,4	R,2	NI,2	R,4	R,4	R,2
RHSP	NI,10	NI,10	L,10	NI,10	NI,14	NI,10	L,10	L,10
JACA	NI,2	ND	ND	L,2	NI,2	L,2	ND	L,2
MUCO	R,14	NI,10	NI,14	NI,10	R,14	R,14	NI,10	R,14
CHKE	NI,2	R,4	NI,9	NI,4	R,4	R,9	R,4	R,9
DABU	R,r7	NI,r5	NI,r	NI,r5	R,r7	R,r7	ND	NI,7
MASM	NI,9	NI,5	R,9	NI,5	NI,9	R,9	NI,5	R,9
JOMCL	R,7	NI,4	R,7	NI,4	NI,7	R,7	R,4	NI,7
JOWR	R,1	R,1	NI,1	NI,1	R,1	R,1	ND	R,1
ARNE	NI,8	NI,4	R,4	NI,4	NI,8	L,8	L,4	L,8
AGDU*	NI,4	R,4	R,4	NI,4	R,4	R,4	ND	R,4
CARO	NI,1	NI,1	L,1	NI,1	L,4	L,1	ND	L,1

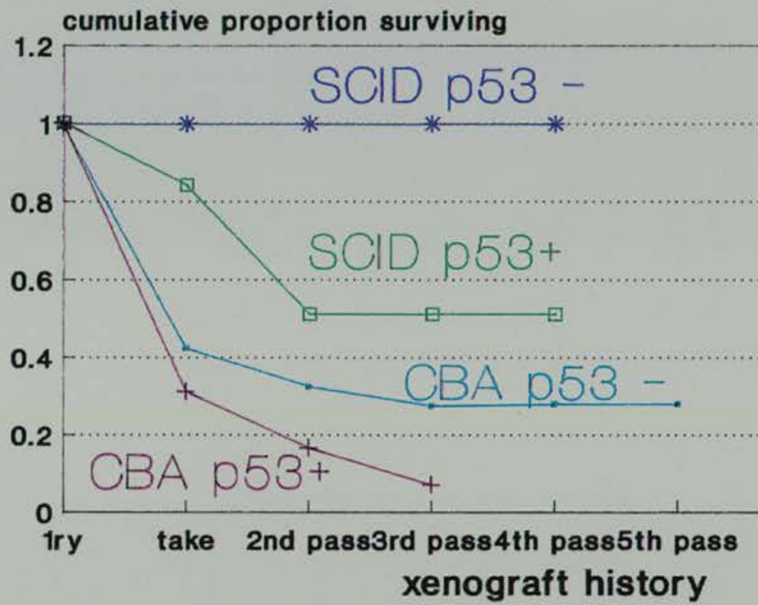
Notes:
1) xen.= xenograft lines
2) R= retention of heterozygosity
3) L= loss of heterozygosity
4) NI= non-informative (constitutional homozygosity)
5) ND= not done
6) numbers= highest xenograft passage number analysed
7) r= grown briefly in culture after 4th passage and re-implanted as a xenograft
8) *= metastatic tumour of unknown primary origin

allelic status of the parental primary ttumour (table 6). Allele losses or rearrangements at the critical loci never appeared during up to 14 passages of tumours that were initially heterozygous. Neither were homozygous losses ever observe d during up to 10 passages of tumours originally showing allele loss.

Loss of heterozygosity was detected for at least one locus in 4 primary tumours within the xenograft set. Cases of retention of both alleles at all 3 loci and of allelic loss at all three loci are represented. In all cases where loss at one oncosuppressor locus was detected, coincident loss of at least one other locus was also always detected.

2.3.6 No p53 mutated tumour grew to 5th xenograft passage

Intra nuclear p53 accumulation was detected immuno-cytochemically in only 2 of the first 11 colorectal adenocarcinomas that grew to xenograft passage. Moreover, neither of these tumours nor any subsequent xenograft carrying a p53 mutation to date, has reached 5th passage in CBA immunosuppressed mice. Of the accumulated series of 66 primary tumours implanted in immunosuppressed mice regardless of xenograft success, 28 were p53 positive giving an incidence of p53 mutation in primary tumours of 42%. This is in keeping with the 46% incidence of mutations observed in a large set of unselected tumours previously gathered by our group (Purdie et al 1991). The lack of p53 mutations in 9 xenografts at passage 5 in immunosuppressed CBA hosts differs significantly from this



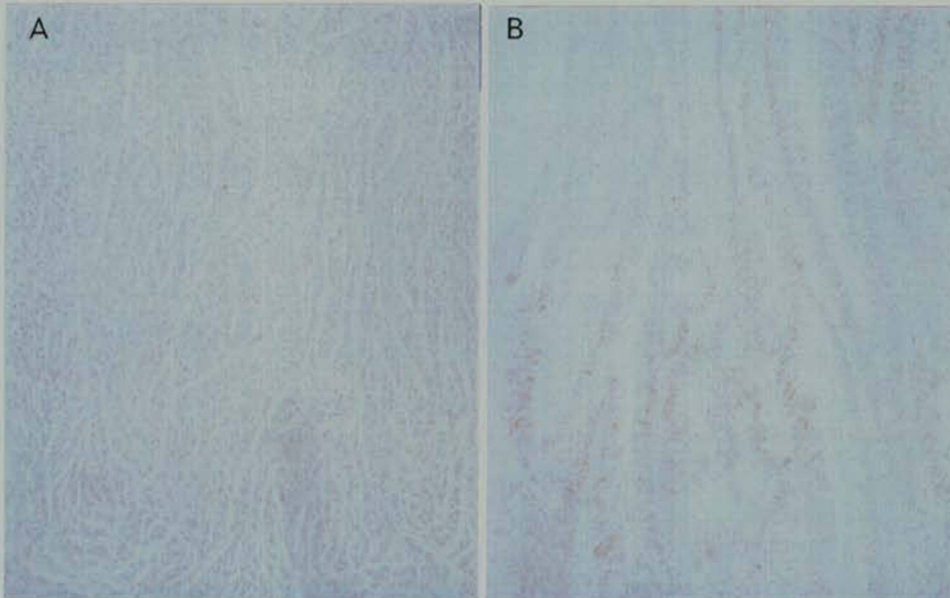


Figure 9 p53 immunocytochemistry with the mouse monoclonal PAb1801. A; Rare positive nuclei in C280 xenograft (x100), B; Positive adenocarcinoma control (x100).

incidence in the primary tumours used for xenografting (P< 0.05, Fishers exact test). In order to incorporate p53 information from the preliminary passage history of more recently established xenografts (for whom the outcome is still unknown), standard life-table analysis was performed. The 4 groups of xenografts (divided according to p53 status and SCID or CBA hosts), were plotted in a single life table (fig ure 8). Log-rank tests showed no significant effect of p53 status on the survival of xenografts in either CBA hosts or SCID hosts alone, but that when numbers (irrespective of host type) were combined in a stratified test, the effect of p53 status on survival was significant (P<0.05).

Direct comparisons of the relative abilities of SCID mice and immunosuppressed mice to support primary tumour growth are available in 9 cases . For these 9 primary tumours, the distribution of p53 mutations is shown in table 7. Tumours carrying p53 mutations are distributed evenly throughout the table. These results, however, apply only to tumours which take, and the p53 bias previously noticed was more pronounced at higher passage numbers (figure 8).

Table 7 p53 expression for primary tumours xenografted into both SCID and immunosuppressed CBA mice

	PAb1801 detection of p53	
	+	-
Growth in CBA mice only	0	0
Growth in SCID mice only	2	3
Growth in both mice groups	2	2
Growth in neither group	0	0

For one p53 over-expressing tumour (JACA) which grew to second xenograft passage in CBAs, sequence information is available (courtesy of C Purdie). This case was of extreme interest since codon 180 in exon 5 was found to carry a mutation leading to an unscheduled stop codon, causing the resulting truncated protein to conserve the epitope detected by PAb1801. Stop mutations are not commonly detected in the p53 gene. Furthermore, 1 of only 3 p53 mutated tumours ever to reach passage 3 is also unusual in that staining is cytoplasmic rather than nuclear.

Rare p53 positive nuclei were detected in many later passage xenografts. The pattern of staining was unusual and ambiguous in that positive nuclei always stained faintly and were widely separated amongst a great majority of negative cells. For comparison, transplantable tumours derived from a cultured colorectal cell line, C280 (see chapter 3), were studied. Although rare positive nuclei were also observed in these tumours (figure 9), the cells cultured in vitro were uniformly negative. This occasional non-specific staining in xenografts was therefore considered artifactual.

Within the xenograft set, 5 tumours showed either loss of a 17p allele or mutation in the p53 gene but not both together (table 8), despite their reported tight linkage in colon cancers (Baker et al 1990a).

2.4 Discussion

2.4.1 Xenografts provide a valuable experimental resource

The value of the xenografts described in this chapter resides in their genotypic and phenotypic stability, homogeneous tumour cell population, differences from each other and capacity for growth after storage at low temperature.

Stability in the xenograft of features present in the primary tumour has been demonstrated at every passage number and for each phenotypic and genotypic parameter studied. This stability extends to tumours passaged for over a year (and in one case for over 2 years), a substantial proportion of the natural history of such human tumours. Moreover, stability was unaffected by storage in frozen form, with subsequent re-implantation to mice. Since aneuploidy is detected by flow cytometry in 60% of colorectal tumours and may be associated with a mixture of specific and random rearrangements, it was extremely gratifying that xenograft DI was never notably different from that of its corresponding primary tumour. In particular, it was demonstrated that xenografts faithfully maintained primary tumour allelic status at three relevant oncosuppressor loci.

The homogeneous tumour cell population of xenografts depends on the entire replacement of the human stromal component of the tumour with mouse stromal cells (Kopf-Maier and Kestenbach 1990). Indeed, after passage of tumour in mice during this study, Southern blots in which

allele losses were detected showed immediate and complete elimination of background alleles due to removal of contaminating normal cells.

The variety of xenografts results from the inclusion within the defined set of members with and without loss of heterozygosity at oncosuppressor loci, diploid and aneuploid members, and members with and without Ki-ras mutations. The variety of lesions represented in the xenograft set are summarized in table 8.

Table 8 Genetic lesions of xenografts and primary locations oof tumours

	1°ry location	5q	17p	18q	DI	p53	Ki-ras
GRBO	distal	R	R	R	1.0	-	+
RHSP	distal	L	L	L	1.9	-	+
JACA	distal	L	L	L	1.6	+	
MUCO	proximal	R	R	R	1.0	-	WT
CHKE	distal	R	R	R	1.2	-	+
DABU	proximal	R	R	NI	1.0	-	WT
MASM	proximal	R	R	R	1.0	-	WT
JOMCL	proximal	R	R	NI	1.0	-	WT
JOWR	proximal	R	R	R	1.0	+	+
ARNE	distal	R	L	L	1.6	-	+
AGDU*	secondary	R	R	R	1.0	+	WT
CARO	distal	L	L	L	1.4	-	WT
Total Incidences		27%	36%	44%	45%	18%	50%

Notes:

1) 1°ry location= location of primary tumour in bowel
2) 5q/17p/18q= colon-associated oncosuppressor loci
3) DI= DNA index
4) p53 mutations were detected immunocytochemically and, for GRBO and JACA xenografts, status was confirmed by sequencing performed by C Purdie of this group
5) Ki-ras analysis was performed on PCR amplified DNA dot blots with radioactive oligo probes for wild type and mutated sequence, by S Lang of this group
6) R= retention of heterozygosity, L= allelic loss, NI= non-informative
7) += mutated, -= no mutation detected, WT= both alleles wild type
8) *= carcinoma of unknown primary origin
9) Total incidences refer to % loss for oncosuppressor loci, % aneuploidy for DIs, and % mutated for p53 and Ki-ras
10) AGDU lesions were not included in calculations of total incidences (see note 8)

The ability to preserve xenografted tumours at low temperature is of particular practical significance. Not only does this mean that tumours can be preserved without continuing provision of host animals, but this process converts each xenograft into a permanent renewable resource for analysis.

<u>2.4.1.1 Experimental uses for xenografts</u>

The variety of renewable, uncontaminated, stable tumours represented in this xenograft set makes them outstanding material for colon tumour analysis.

Firstly, in culture, they will provide uniquely favourable vehicles for proposed tests of oncosuppress or gene function discussed earlier and in chapter 5.

Secondly the xenografts provide ideal material for gene mutation analysis. It is imperative in PCR studies that tumour specific sequences are not contaminated with corresponding sequences from non-tumour cells. In particular, mutations in the recently identified <u>APC</u> gene may be particularly cumbersome to find owing to the enormous size of the gene predicted by its 9.5Kb transcript (Kinzler et al 1991b). The renewable purified tumour xenografts will facilitate simpler identification of sequences without the risks of contamination with non-tumour sequences, or exhaustion of the tumour resource. Xenografts also permit the analysis of tumours with large stromal components which are often uninterpretable in primary tumour studies due to the extent of human stromal contamination. This contamination is removed in the

xenograft so that the human tumour genotype can be ascertained without ambiguity. Moreover, after characterizing specific mutations in tumour xenografts, possibly in combination with those at other loci, the associated tumour biology can be observed _in vivo_ against the background of a whole organism showing far less individual variation than human patients. The variety of genotypes and phenotypes represented in the xenograft set will allow a number of combinations to be addressed. The study of _DCC_ mutations in the xenografts described here is already under way in Dr B Vogelstein's laboratory.

Finally, by virtue of any of the described properties of the xenograft set, they may become useful in diverse and unpredictable studies. The replacement of the stromal component of the tumour with that of the host may allow analysis into stromal-tumour interaction. New genetic tumour associations may come to light or local environmental effects on tumour biology may be discovered. Regardless, the variety of stable, renewable, pure tumour cells growing in the xenograft set will be readily available for analysis.

2.4.2 Xenografts are representative of colorectal tumours

The variety of genetic lesions present within the xenograft set are broadly representative of those observed in unselected primary carcinomas. Incidences of these lesions, derived from large series of primary tumours studied in Edinburgh or elsewhere, are compared in table 9 with those in the xenograft set.

Table 9 Genetic lesions of colorectal cancer

Lesion	xenografts	primary	Reference(et al)
	% Incidence from (n) tumours		
18q allele loss	44% (9)	73% (56)	Vogelstein 1988
17p allele loss	36% (11)	75% (60)	Fearon 1987
5q allele loss	27% (11)	42% (194)	Ashton-Rickardt 1991
p53 mutation *	18% (11)	46% (86)	Purdie 1991
Ki-ras mutation	50% (10)	40% (104)	This group unpub.

Notes:
* = detected immunocytochemically
unpub.= unpublished

The combinations of allelic loss detected in the xenograft set are representative of those combinations detected in large numbers of unselected carcinomas as described by Delattre et al 1989. Delattre found tumours to frequently divide into 2 clear groups: tumours with severe aneuploidy and coincident LOH at more than one locus which were significantly associated with a distal location in the bowel, and a diploid group rarely showing LOH which were most often found to be proximal tumours. In agreement with this, 10 of the 11 primary tumours within the xenograft set were either proximal and diploid (5) or distal and aneuploid (5). All of the proximal tumours showed retention of heterozygosity at all 3 studied loci while 4 of the 5 distal tumours showed LOH at more than 1 site.

While tumours showing co-incident allele loss may have arisen through accumulation of lesions in the adenoma-carcinoma sequence (Fearon and Vogelstein 1990), those without allele loss or aneuploidy must have arisen by alternative mechanisms. The xenograft set therefore supports the evidence for a model of colorectal cancer

which allows for divergent pathways of tumorigenesiss, such as depicted in figure 1. Ki-ras mutations fall equally into both groups suggesting this lesion to be determined by mechanisms independent of those involved in LOH and aneuploidy.

2.4.3 Selection against xenografts carrying p53 mutations

Selection of primary tumours is to some extent inevitable in this study since all tumours included were required to grow as xenografts in immunosuppressed mice. Faster growing tumours will be favoured in these circumstaances since, as discussed in the introduction, some degree of host immunorecovery is observed making slower growing tumours, therefore, vulnerable to rejection. An unexpected finding, however, was the fact that within the xenograft set no continuously passaging tumour carrying a p53 mutation has grown in immunosuppressed mice despite their reported growth in congenitally athymic nudes (Nigro et al 1989). Furthermore, the survival of tumours carrying p53 mutations is significantly poorer than those without in a combined group of CBA and SCID mice, despite many with p53 mutations surviving at the currently available low passage numbers in SCID hosts.

The take rate discovered in this work for colorectal adenocarcinomas in SCID mice is greater than twice that found by ourselves and others in CBA and nude mice (Houghton and Taylor 1978, Lefrancois et al 1989). Such increased receptivity may be attributed to the increased

severity of the immune deficiency in SCID mice (Bosma et al 1983).

Since p53 mutations appear to disable tumour growth in all hosts, but apparently moreso in those with less severe immune defects, it may be suggested that the ability to sustain p53 over-expressing tumours is related to the severity of the immune defect. Implicit in this suggestion is the assumption that such tumours are more immunogenic than tumours expressing wild-type p53. The accepted nuclear location of the p53 protein does not preclude the existence of immunogenic cell surface epitopes specifically associated with aberrant p53. The existence of such epitopes would have important therapeutic implications, particularly when one considers the wide range of tumours known to sustain p53 defects.

However, a metastatic carcinoma of unknown primary origin (AGDU) was observed to over-express the p53 protein over 5 xenograft passages. The most likely primary origin of AGDU is now thought to be breast. It is possible that the nature and effects of p53 mutations associated with breast cancer may vary from those in colon tumours, or that this tumour carried a rare variant of mutation not leading to immunogenicity. Indeed, the sequence of one of the few p53 expressing colonic tumours which grew briefly in xenograft was found to carry an unusual mutation leading to a truncated protein, where the majority of mutations lead to full length proteins with altered conformation. However, the rare growth of

xenografted p53 expressing tumours may indicate that selection is occurring, not against mutated *p53*, but against associated events in colonic tumours.

Within the passaging set in immunosuppressed CBA mice there is a preponderance of mucinous carcinomas (table 4). Mucinous carcinomas are observed to be less p53 immunoreactive to the antibody used in this study than other adenocarcinomas (Campo et al 1991). Lack of *p53* mutated xenografts may therefore be a secondary effect of their lack of association with mucinous tumours whose prominence in the CBA mice xenograft set, may be trivially explained in terms of growth rate. The majority of tumour tissue implanted becomes degenerate on implantation while host fibroblasts invade the area (Kopf-Maier and Kestenbach 1990). Only then do tumour cells close to the stromal cords start to divide and does the tumour regenerate. Logically, a smaller number of cell divisions in conjunction with copious mucin secretion is required to regenerate a tumour of mucinous histology than the number of divisions required to regenerate more densely populated tumours. Mucinous tumour growth can therefore be achieved considerably faster than that of more poorly differentiated types which may become growth limited by host immunorecovery.

Chapter 3: Characterizing established lines

3.1 Abstract

Establ ished colorectal cell lines were characterized as immediate experimental resources. While findings helped to justify our main strategy of deriving new lines, the established lines nonetheless had interesting properties.

5 of the lines, deriving from studies on sporadic colorectal tumours in Nottingham, were shown by genetic analysis to represent a single fast-growing line. After confirming its colorectal epithelial nature, the cells were shown to include a heterozygous Ki-ras mutation, retain heterozygosity at oncosuppressor loci on 17p and 18q, and probably have lost an allele at 5q21. The line was clonable and highly malignant in animals, showing local infiltration and lymph node metastases. This line is therefore suitable for genetic manipulations involving functional tests of exogenous oncosuppressor genes.

Genotypic studies were also conducted in a separate series of human colorectal cell lines from Bristol. These were originally derived from an adenoma of a FAP patient and had been exposed *in vitro* to a variety of agents resulting in increased tumorigenicity. Coincidental with appearance of tumorigenicity, there arose unusual allele losses and imbalances, all remarkably at key loci on 5q, 17p, 18q and Ki-ras. This emphasises the importance of these sites in colorectal cell growth, even *in vitro*, and so highlights the significance of their stability in the newly derived lines described in chapter 2.

3.2 Introduction

In the previous chapter the generation of colorectal adenocarcinoma xenograft lines with defined and stable genotype relative to the patient of origin are described. Although successful, the process of line generation is slow as is growth *in vitro* of these lines. Growth *in vitro* is a pre-requisite for genetic manipulation exper iments. In order to start to define the parameters and methods for gene transfer in colonic epithelial cells, and to explore existing resources, some existing human colorectal lines were analysed. For this purpose cell lines were chosen which had been established within the past 4 years, and for which there was a possibility of defining the genotype of the patient of origin. I am grateful to Dr LG Durrant of Nottingham University and Dr C Paraskeva of Bristol University for kindly supplying these lines.

3.2.1 Nottingham lines

The cell lines from Nottingham, C146, C168, C224, C27.7 and C280 were all established 1-2 years previous to this study (Durrant et al 1986, Durrant et al 1987). They were derived from primary colorectal carcinomas by growth in soft agar in order to isolate stem cells for experimental immunotherapy, and had been characterized with a number of antibodies. They had all been shown to express the tumour associated antigens expressed on colorectal carcinoma cells which are recognised by the monoclonal antibodies C14 and 719T/36 (Durrant et al

1987). CAM5.2, which recognises cytokeratin, was also used to confirm their epithelial origin. Two of these lines, C146 and C168 were further defined in terms of DNA content, proliferative capacity and tumorigenicity (Durrant et al 1986). Although constitutional DNA of the patients from whom the original primary tumours came had not been sampled, some of these patients were known to be alive, and were thus available for sampling if necessary.

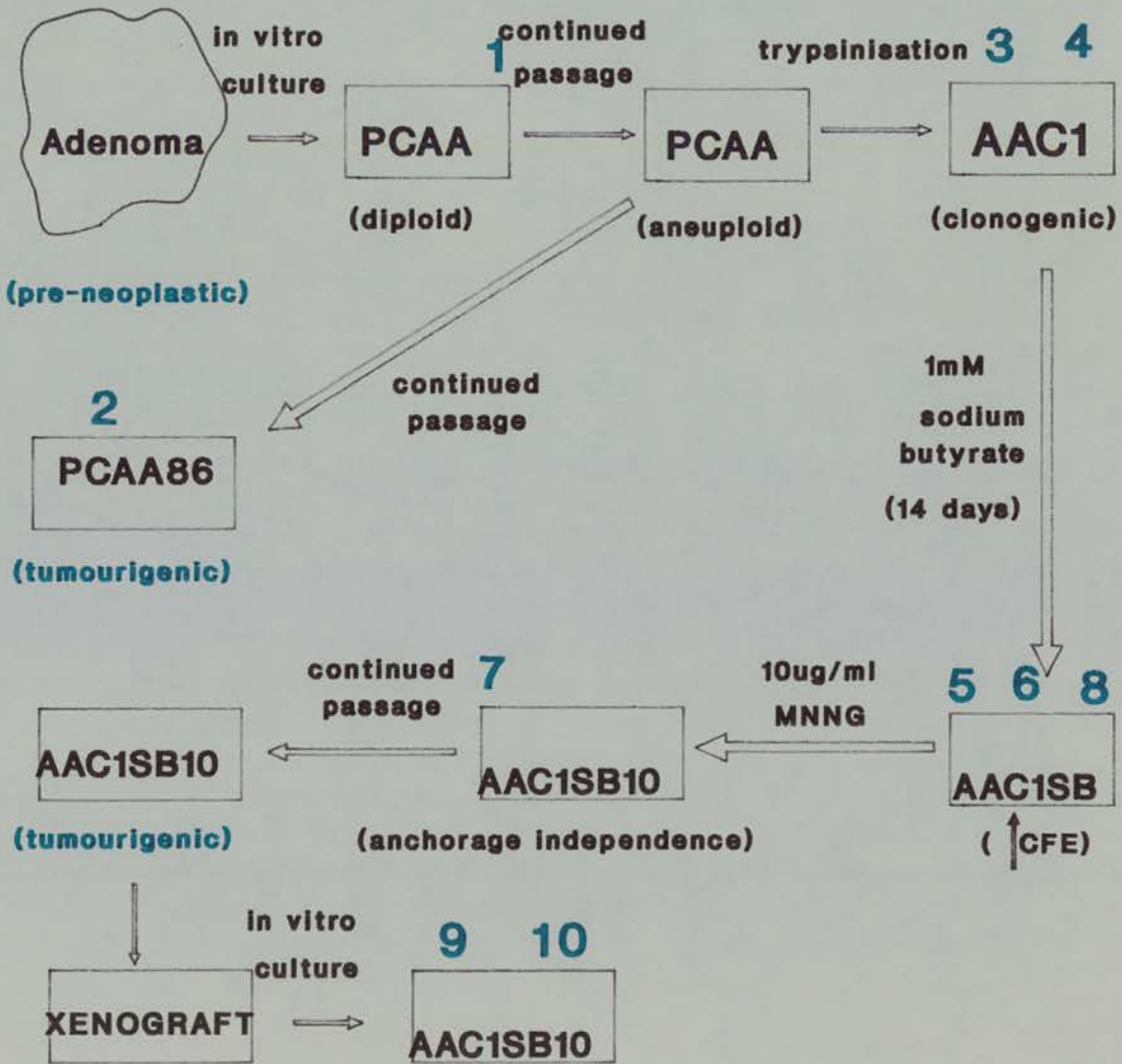
3.2.2 Bristol lines

The cell lines from Bristol were all derived from a single line (PCAA) established, 4 years prior to this study, from an adenoma of a familial adenomatous polyposis patient (Paraskeva et al 1984). Various selection pressures were placed upon this line to generate altered sublines with more aggressive growth. The purpose of deriving these lines was to provide a model for studying the changes associated with progression from adenoma to carcinoma. A summary of the events involved in this progression is illustrated in figure 10.

In brief, the original PCAA cultures were passaged *in vitro* until an immortalized subline emerged (Paraskeva et al 1988). On further continuous passage sublines appeared in which the cells became aneuploid and adopted the morphology of a transformed phenotype. On continuous passage these cells became tumorigenic in nude mice (C Paraskeva, personal communication). Meanwhile, a rare clonogenic variant (AAC1) isolated from the early passage

Figure 10

PROGRESSION OF THE PCAA ADENOMA CELL LINE IN VITRO



diploid line was also found to be aneuploid and, after sodium butyrate treatment, gave rise to cells with an increased colony forming efficiency on plastic. Sodium butyrate was chosen since butyric acid is a naturally occurring fatty acid produced by the intestinal flora, and has been shown to induce differentiation. There may therefore be a link between the production of this substance and dietary factors implicated in colorectal cancer. It is interesting to note that in this experiment the cells pre-treated with sodium butyrate became insensitive to growth inhibitory effects on further treatment with the same concentration and less sensitive to higher concentrations, and that this phenotypic change represented a stable event since the resulting increased colony forming efficiency was maintained throughout the study. These cells however remained anchorage dependent as determined by colony forming efficiency in soft agar, and non-tumorigenic in mice. Subsequent treatment with the potent carcinogen N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) did yield an anchorage independent line which on continuous passage finally became tumorigenic in mice (Williams et al 1990). MNNG treated cells which had not been exposed to sodium butyrate (but otherwise parallel in their passage history) were not tumorigenic. This observation reinforces the idea that the sodium butyrate treatment represents a stable and necessary change in this pathway of progression.

The aims in developing these altered sublines in culture were thus the converse of the aims described in chapter 2 of this thesis. Rather than demonstrating stability of phenotype and genotype, the Bristol lines were treated expressly in order to elicit alterations.

The colonic epithelial origin of these lines had been confirmed by staining with an antibody to epithelial keratin filaments, and also by the ultrastructural presence of desmosomes, microvilli and mucin droplets. Minisatellite DNA probes were used in Bristol to confirm derivation of all lines from a single stock. No karyotypic abnormalities were detected in the original diploid line cytogenetically although it was known to carry a Ki-ras mutation (Farr et al 1988). Because the patient of origin had familial polyposis at least one aberrant copy of the APC gene on chromosome 5 must be present constitutively. Constitutional DNA had also been prepared from a fibroblast cell line derived from the same patient.

3.3 Results

3.3.1.1 Nottingham lines are highly suitable for cloning

These cells were small, refractile and pleomorphic in appearance. They grew under standard conditions in 10% NCS and were passaged by the one step trypsinisation method described in chapter 7. They grew rapidly requiring frequent splitting at high ratios (at least 1:10 once a week). Clonal outgrowth from single cells after trypsinisation was routine. All lines were alike in these repects.

3.3.1.2 Nottingham lines are highly tumorigenic

From each cell line, 10^7 cells were injected subcutaneously into each of 3 immunosuppressed mice. 20 days after injection large infiltrative tumours were removed from every mouse but one. Tumours produced by all 5 lines were histologically indistinguishable. All were poorly differentiated and often infiltrated the underlying muscle and connective tissue. Necrosis was evident as was a high cell turnover indicated by many mitotic and apoptotic bodies. In the case of tumours which had penetrated the full thickness of the peritoneum, secondary masses and enlarged lymph nodes were seen. Although secondary tumour masses were confirmed histologically, examination of heart, lung and liver in each case showed no distant metastases. The histology of these tumours is shown in figure 11.

3.3.1.3 Nottingham lines are epithelial and glandular

In view of the extreme similarity between phenotypes

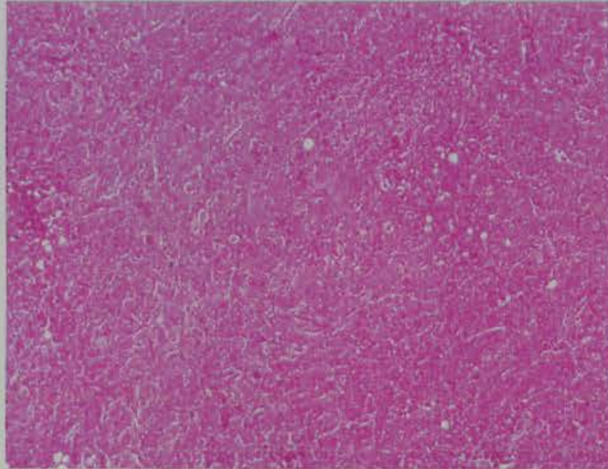


Figure 11 Poorly differentiated histological pattern of C280 cells growing as xenograft (x100).

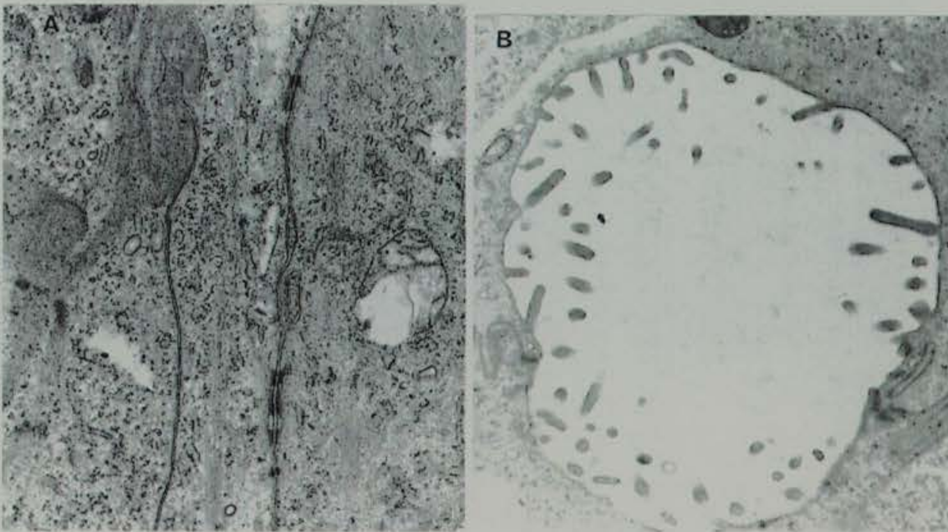


Figure 12 Electron microscopy of C280 xenograft showing conservation of epithelial cell markers. A; A variety of basal cell junctions including desmosomes (x17000), B; Luminal microvilli and a variety of junctions (x13600).

of all 5 lines only one, C280, was prepared for electron microscopy. Although histologically no cell polarization or gland formation could be seen, organised groups of polarised microvillous cell surfaces surrounding small luminal spaces were seen by electron microscopy (figure 12). Exocytosis of microvesicles into the lumen from these microvillous surfaces was also noted. Belt junctions and desmosomes were prominent throughout the specimen, confirming the epithelial origin of this line.

3.3.1.4 Nottingham lines share the same genotype

To ascertain whether these cells had retained heterozygosity at colon-associated loci, RFLP analysis was carried out using the probes Pi227, L5.62, L5.71, EF5.44, YN5.48 for chromosome 5; YNZ22 and MCT35.1 for chromosome 17 and BV15.65 for chromosome 18. The conditions for usage of these probes [table 17a) and 17b)] and some of the patterns detected and examples of allelic losses and retentions [figure 30] are shown in chapter 7.

Allelic status detected for the DNA of these lines was identical for every probe tested on each of the samples. DNA was made and probed for each line on 2 separate occasions, both yielding the same result. These results are shown in table 10.

Allele frequencies for all the chromosome 5 probes used excepting L5.71 are available (Dunlop et al 1990), and L571 allele frequencies were scored from a series of over 100 patients presenting with colonic or lung

Table 10 Alleles detected in Nottingham lines

LINE	chromosome 5					chrom. 17		chrom. 18
	Pi227	L571	L562	EF544	YN548	MCT35	YNZ22	BV1565
C146	A2	A1	A1	A1	A2	A1	A1A2	A1A3
C168	A2	-	-	A1	A2	-	A1A2	A1A3
C224	A2	-	-	A1	A2	-	A1A2	-
C227	A2	-	-	A1	A2	-	A1A2	-
C280	A2	-	-	A1	A2	A1	A1A2	A1A3

Notes:
1)-= not done.
2) Where the DNA was found to be homozygous with a probe, only one allele is shown since the case may in reality be homozygous or hemizygous.

cancers, from whom constitutional DNA was probed by various members of this department. These allele frequencies are included in table 17 in chapter 7.

With respect to the probe EF5.44 the single allele carried by all lines is found in the population at a frequency of only 0.18. The probability that 5 different unrelated patients carry this allele is therefore 0.18^5, which is less than 2 in 10000. The respective probabilities of 5 unrelated patients carrying the alleles shown at Pi227 and YN548, of 3 patients carrying the alleles shown at BV1565, and of 2 patients carrying the MCT35 allele shown are; 0.46^5, 0.55^5, $(2x\ 0.17x\ 0.49)^3$, 0.75^2. The combined probability of observing the identical genotypes shown in table 10 in 5 unrelated patients is $5.09x\ 10^{-10}$, or 5 in ten thousand million. It can therefore be assumed beyond all reasonable doubt that all 5 represent a single line. Reassuringly, the identical bands detected by these 8 probes were of the expected sizes indicating this cell line to be of human origin. The line was henceforth designated C280.

C280 was found by flow cytometry to have a diploid DNA content.

By immunocytochemistry (using the mouse monoclonal human specific antibody PAb1801) faint, scattered p53 positive nuclei were detected in xenografts of this line (figure 9). This pattern was similar to that previously noticed for some primary tumour xenografts (chapter 2). However, fixed preparations of C280 cultured cells in agar were unambiguously negative when stained for p53, despite the positivity of a control line prepared by the same method. Faint, scattered positive nuclei detected in xenografts were accordingly considered to be artifactual.

3.3.2.1 Genetics of Bristol lines: specific 5q21 change

DNA was studied from cell lines at various stages of the progression of the adenoma derived PCAA line to the tumorigenic lines PCAA86 and AAC1SB10. RFLP analysis was carried out using the probes L1.4, MLZ74, Pi227, L5.62, L5.71, EF5.44, YN5.48, MC5.61 and lamdaMS8 for chromosome 5; YNZ22 and MCT35.1 for chromosome 17, YNH24 for chromosome 2, EFD64.2 for chromosome 3 and EFD75 for chromosome 10. The conditions for usage of these probes [table 17a) and 17b)] and examples of allelic losses and retentions detected [figure 30] are shown in chapter 7.

In the results to follow, the DNA samples from different sublines were numbered 1-10 for simplicity, as indicated in figure 10. In summary clone 1 represents the early passage diploid cells, clone 2 the spontaneously transformed line, clones 3 and 4 are aneuploid clonogenic

variants, clones 5-8 represent non-tumorigenic cells after sodium butyrate treatment and 9 and 10 are taken from cells cultured after growth as tumours in animals.

For each probe, the ratio of intensities of hybridising bands was confirmed by quantitative scanning densitometry and these results are shownn in table 11. With some probes, bands were detected which were not predicted by the known allele size. In all cases these unexpected bands were shown, by re-probing filters with labelled pBR322, to be due to contaminating plasmid in the DNA.

Within the DNA set, 2 kinds of changes in allele ratio were observed. The first category of changes involved most probes and constituted transient variations in allele ratio which were not maintained in samples from subsequent stages of the progression. Such random variations were probably due to the arisal of transient subpopulations within the cultures.

In contrast, the second category of changes was noted only with certain probes. In these cases, a consistent bias in allele ratio was seen at later stages of the progression which was not evident in preceding samples. With the probes L5.71 and YN5.48 in particular, clones 5-10 showed increased intensity of allele 2 in comparison with allele 1, whereas clones 1-4 showed roughly equivalent ratios of allele intensity (figure 13). Clones 5 and onwards represented cells treated with sodium butyrate and thereafter throughout the progression

Table 11 Allele ratios of PCAA DNA (chromosome 5)

PCAA DNA	Probe (location on chromosome 5)							
	L1.4	MLZ74	Pi227	L5.62	L5.71	EF5.44	YN5.48	lMS8
	5pt	5qc	5q21	5q21	5q21	5q21	5q21	5qt
1	NI	0.9:1	1:0.8	1.9:1	ND	NI	ND	ND
2	NI	2.5:1	1:0.7	ND	ND	NI	ND	0.9:1
3	NI	1.1:1	1:1.1	2.3:1	1.2:1	NI	1.3:1	1.6:1
4	NI	ND	1:1.4	ND	1.0:1	NI	0.5:1	ND
Mean		1.5:1	1:1.0	2.1:1	1.1:1		0.9:1	1.3:1
s.d		0.7	0.3	0.2	0.1		0.4	0.4
5	NI	1.3:1	1:1.0	3.7:1	1.9:1	NI	ND	0.4:1
6	NI	0.2:1	1:1.2	2.6:1	1.7:1	NI	2.0:1	1.0:1
7	NI	0.8:1	1:1.4	4.3:1	ND	NI	4.9:1	1.2:1
8	NI	0.4:1	1:1.6	ND	1.6:1	NI	2.3:1	0.7:1
9	NI	ND	ND	3.8:1	2.1:1	NI	3.0:1	ND
10	NI	1.0:1	1:1.1	ND	2.7:1	NI	2.0:1	ND
Mean		0.7:1	1:1.3	3.6:1	2.0:1		2.8:1	0.8:1
s.d		0.4	0.2	0.6	0.4		1.1	0.3

(continued) Allele ratios of PCAA DNA (other sites)

PCAA DNA	Probes (chromosomal location)				
	YNH24 (2)	EFD64.2 (3)	EFD75 (10)	YNZ22 (17p)	MCT35.1 (17p)
1	ND	NI	ND	1:1.0	ND
2	ND	NI	2.1:1	ND	ND
3	0.8:1	NI	0.2:1	1:1.5	0.5:1
4	ND	NI	ND	ND	ND
Mean	0.8:1		1.1:1	1:1.2	0.5:1
s.d	0		0.9	0.2	0
5	ND	NI	0.8:1	1:5.2	ND
6	0.8:1	NI	0.8:1	ND	ND
7	0.5:1	NI	1.5:1	1:3.8	1.6:1
8	0.5:1	NI	1.1:1	ND	2.7:1
9	ND	NI	ND	ND	ND
10	ND	NI	1.0:1	1:3.3	3.3:1
Mean	0.6:1		1.0:1	1:4.1	2.5:1
s.d	0.1		0.3	0.8	0.7

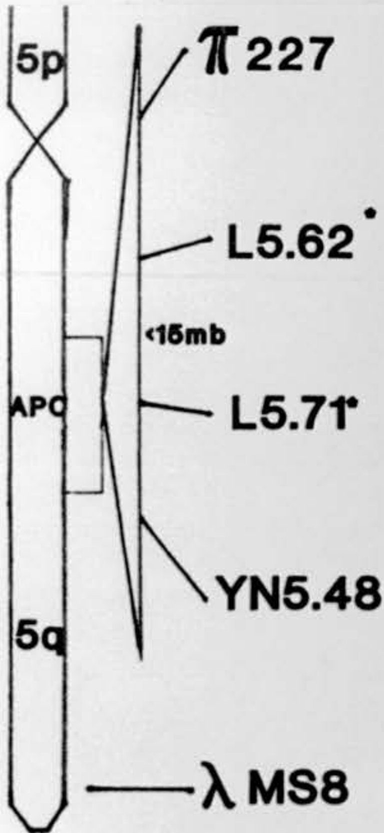
Notes:
1) lms8= lambdaMS8
2) t= telomeric
3) c= centromeric
4) PCAA DNA 1-10= clones 1-10 in progression (figure 10)
5) NI= non-informative (homozygous)
6) ND= not done
7) MLZ74 constant allele excluded
8) L562 relative intensities for paired smaller alleles added and used as one allele
9) s.d= standard deviation

Figure 13

Alleles detected

in PCAA DNA with

chromosome 5 probes



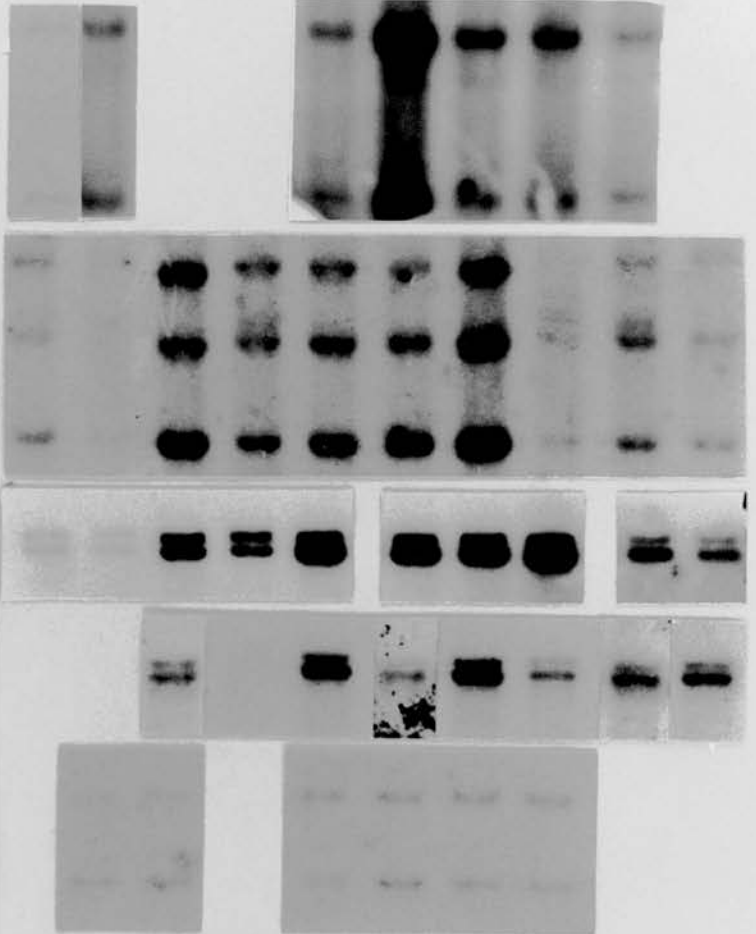


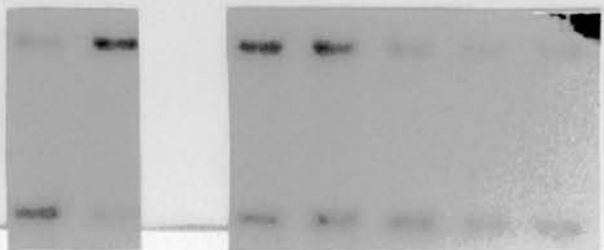
Figure 14

Alleles detected

in PCAA DNA with

chromosome 10 probe

EFD75



but not the spontaneously transformed example (clone 2). Although this bias in allele ratio at clone 5 was consistently seen throughout subsequent samples with these 2 probes, no corresponding chaange was detected by a subtelomeric probe on the same chromosome arm (lambda MS8) or with the probe Pi227 which lies less than 15Mb centromeric (figure 13). In view of the surprising nature of this observation Pi227 results were confirmed by densitometry on 4 different autorads none of which gave notably different results from those shown. For DNA from before the stable phenotypic change associated with sodium butyrate treatment, mean allele ratios (and standard deviations) for the 4 ordered probes Pi227, L571, YN548, lamdaMS8 were; 1: 1.0 (0.3), 1.1 (0.1), 0.9 (0.4), 1.3 (0.4) respectively. For DNA from cells after this change, mean allele ratios (and standard deviations) for the same 4 probes were 1: 1.3 (0.2), 2.0 (0.4), 2.8 (1.1), 0.8 (0.3) respectively. The allele imbalance consistently seen for the 2 middle probes after sodium butyrate treatment, did not therefore extend to flanking regions. The consistency and specificity of the rearrangement imply duplication of a region of 5q21-22 and selective maintenance of this duplication thereafter.

Similarly, chromosome 17p probes detected a 3-5 fold increase in the presence of one allele after sodium butyrate treatment (table 11). Probes to other non tumour-associated loci on chromosomes 2, 10 and 5p did not show consistent rearrangements (table 11). The

pattern detected by a chromosome 10 probe is shown in figure 14.

3.3.2.2 Genotypes show PCAA constitutive DNA to be wrong

In the course of this work DNA was supplied to us from fibroblasts of the patient from whose polyp PCAA was derived. On Southern blot analysis it soon became evident, however, that this DNA could not derive from the same patient as PCAA, since different alleles (incompatable with a shared origin) were detected by many of the probes (particularly VNTR sequences where many alleles are possible). Further studies with minisatellite probes in Dr C Paraskeva's laboratory confirmed this disturbing finding. It would appear that at some point prior to the material being sent to Edinburgh, the donor fibroblasts had been lost and replaced accidentally by cells of another patient.

3.4 Discussion

3.4.1 Results support the strategy of this thesis

The characterization of established lines described in this chapter highlights some of the problems associated with the use of long-established lines as models of colorectal cancer. It is clear from our work that the 5 Nottingham lines had all been colonised by a single cell type. Without this identification the cells would be considered to be 5 different lines with similar phenotype, and characteristics peculiar to this line wwould be erroneously taken to be common characteristics of colonic cell lines. Unintended cross contamination of cultures by fast growing cell lines is well known but the fact that this had happened without being recognised before the lines came to Edinburgh raises doubts as to the true identity of these cells. Similarly, the Bristol lines- although initiated from a known patient- in the end fell short of expectation in that no constitutional DNA resource could be provided and the patient (a private patient from Turkey) was lost to follow-up. Despite these drawbacks, however, both lines possess useful features, and some of the missing information can be deduced from the studies described here.

3.4.2 Further identification of the Nottingham line

DNA finger-printing was considered in order to establish the true origin of this line. Although possible, the tracing of patients, blood collection and analysis involved would have been at some personal

discomfort and expense to the 5 patients and our Nottingham colleagues. Moreover the possibility remained that the dominant line came from yet another source again, as it had grown out at an unspecified time after establishment of the cultures, and during a period when multiple colorectal lines from different patients were being initiated and grown. The line's colorectal carcinoma origin was not in doubt since this was the only tissue type being handled in the Nottingham laboratory. Furthermore, we confirmed its human origin by detecting human alleles in RFLP analysis, and its epithelial origin was confirmed by electron microscopy. Previous cytochemical evidence had also confirmed a colonic origin. As an alternative to a possibly fruitless search for constitutional DNA it was considered worthwhile to compute the probability of allele status by less direct means.

The line clearly conserves both alleles in 17p and 18q and is near diploid. In 5q, however, it is notable that none of the 5 probes detected heterozygosity. There are 2 possible explanations for this; either the line is constitutively homozygous at all 5 loci or allele loss has occurred. As already mentioned, allele frequencies are known (table 17), and can be used to calculate the probabilities of genotypes. The probability of being homozygous for any one allele is the square of its frequency, which for the 5 loci involved calculate as; 0.21, 0.87, 0.16, 0.03, 0.30. The product of these

frequencies gives the probability of co-incident homozygosity at all 5 loci and is $2.63x10^{-4}$, or less than 3 in 10,000. Such a low probability for the detected genotype constitutionally suggests that this cell line has sustained an allelic loss on 5q. This calculation assumes the absence of linkage groups such that homozygosity with any one of these markers does not increase the likelihood of homozygosity with any other. Such an assumption seems reasonable from the experience of our laboratory of over 200 patients studied with probes in 5q21. While the recent isolation of both the MCC and APC genes on 5q will in the near future allow specific definition of any mutations present, the currently known status of this colorectal epithelial line is shown in table 12.

Table 12 Genetic lesions of C280

Lesion	C280
5q	Homozygous (probably hemizygous due to allele loss)
17p	Heterozygosity retained
18q	Heterozygosity retained
DI	1.0
p53	Not detected by immunocytochemistry
Ki-ras	Heterozygous mutation

Notes:
1) 5q,17p and 18q represent oncosuppressor loci
2) DI= DNA index as measured by flow cytometry
3) Ki-ras mutation was detected by PCR DNA amplification and sequencing by the method of Sanger, carried out by P Romanowski of this group.

3.4.3 Nottingham line is useful for genetic manipulation

This colonic epithelial line is rapidly growing and easily clonable and will therefore be useful for gene transfer studies. Analysis of the effects of introduction of exogenous oncosuppressor genes will be particularly

suited to this line owing to its aggressive tumorigenicity in immunodeficient mice. The preceding characterization will make experiments to replace the proposed lost MCC and APC genes, or to specifically target and disable the retained p53 and DCC genes, particularly informative.

3.4.4 Specific changes at critical loci in Bristol lines

Bristol lines which had progressed from benign colorectal cells to a tumorigenic cell line were selected *in vitro*. Multiple genetic changes associated with this altered phenotype were of 2 types; transient changes presumably representing subclones with no growth advantage, and changes that persist in a stable manner.

The striking finding of this investigation is that these stable changes, selected for in the artificial environment of *in vitro* culture, involve the critical loci affected in human colorectal carcinogenesis; 5q, 17p, 18q and Ki-ras.

Since the adenoma derived cell line PCAA originates from a patient with Familial Polyposis Coli where one defective APC gene is assumed to be present by inheritance, it is interesting to note that progression to carcinoma appears, in this case, to have involved a stably maintained acquired allele imbalance in the immediate vicinity of the APC and MCC loci. Indeed, the probe L5.71 which detects the rearrangement has recently been shown to lie within the open reading frame of MCC (Kinzler et al 1991a), while the APC gene is included in

the region of DNA between this probe and YN5.48 (Kinzler et al 1991b) which is also involved in the rearrangement. During tumorigenesis in situ, oncosuppressor genes such as APC are thought to undergo somatic loss or inactivation, and not amplification. Gross changes seem not to be necessary in vitro since the independent spontaneous progression to PCAA86 (clone 2) tumorigenic cells shows no detectable changes in allele copy number (although small rearrangements and point mutations cannot be ruled out). However, the selective maintenance of the 5q21 rearrangement in AAC1SB10 cells (clones 7,9 and 10) does suggest its crucial importance, at least in this pathway, to the tumorigenic phenotype. The apparent duplication demonstrated here during progression in vitro may represent an increase in relative copy number of the pre-requisite aberrant copy of the APC gene, and thereby illustrate a gene dosage effect. Perhaps an excess of mutant protein may act in a dominant-negative fashion sequestering wild type protein and disrupting normal function, ultimately permitting tumorigenic behaviour. Unfortunately, no DNA is available from other family members, precluding rapid discovery of the phase of the inherited defect.

It was previously noted that the phenotypic changes associated with sodium butyrate treatment of this cell line represented a stable event involving acquired resistance to growth inhibition after further treatment, and an increased colony forming efficiency throughout the

duration of study (Williams et al 1990). It is interesting to note that diminished response to this proposed differentiating agent (which is a normal constituent of bowel lumen contents) should be associated with altered gene copy number around the <u>APC</u> and the <u>MCC</u> loci. <u>In situ</u>, a single copy of defective <u>APC</u> is permissive for the generation of large numbers of polyps while reduction to homozygosity at this locus is associated with the occurrence of tumours. Furthermore, <u>MCC</u> is reported to bear similarity to the neural cell adhesion molecule. Should this gene be involved in cell-cell contacts as has been suggested, its disruption or over-expression could easily lead to alterations in cell differentiation.

An allele imbalance in 17p, probably including the <u>p53</u> locus, was also detected after sodium butyrate treatment (clones 5-10). Although p53 over-expression (detected by western blots) increased at stages throughout the progression, no mutation has been found, to date, in any of the 4 hot-spots (C Paraskeva, personal communication). While mutation elsewhere in the gene remains a possibility, the observed 3-5 fold increase in copy number may alone be responsible for this phenomenon.

PCAA and AAC1 cells (clones 1,3 and 4) have been shown karyotypically to have only one chromosome 18, whereas the highly tumorigenic AAC1SB10 cells (clones 9 and 10) have lost both normal chromosomes. However, a

translocation has occurred involving chromosomes 1 and 18 and cells, even at later stages, do express some DCC sequence (C Paraskeva and B Vogelstein, personal communication). This translocation may also involve a rearrangement on the short arm of chromosome 1, detected as a novel sized allele in association with tumour progression (A Williams,, personal communication).

Although PCAA27, PCAA86 and AAC1 are heterozygous for a *Ki-ras* mutation, AAC1SB10 cells are homozygous for the mutated allele (C Paraskeva and C Marshall, personal communication). This outgrowth of a population of cells with allele loss at *Ki-ras* is unexpected since *Ki-ras* mutations are thought to be dominant and effective despite the presence of the remaining wild type copy. The observed allele loss gives rise to the unusual suggestion that a further growth advantage is attained by elimination of normal *Ki-ras* function. This idea gains support from other work. In carcinogen induced mouse skin tumours, the loss of wild type alleles in tumours heterozygous for *H-ras* mutations has been clearly associated with tumour progression (Bremner and Balmain 1990).

It is striking that stable rearrangements were not observed at loci on chromosomes 2, 5p, and 10, chosen for lack of implication in tumour growth (figure 2). Instead stable changes appear to specifically involve colon tumour-associated sites. Epidemiology from such diverse populations as Japan and the U.S.A. implicates the same

small group of key genes as central to colorectal cell growth control. This subset of genes is implicated despite different dietary and other risk factors, and large variations in disease incidences and trends be tween populations. The specific involvement of the same genes even under the artificial conditions of cell culture is re markable and points strongly to the critical nature of these genes in defining colorectal cell behaviour. It is therefore reassuring and significant to note the stability of such critical genes demonstrated in long-passaged tumour xenografts in chapter 2.

3.4.5 Bristol lines demonstrate that 5q21 is responsive

The duplication and specific maintenance of a 5q21 region, containing the APC and MCC genes, occurred in association with acquisition of tumorigenicity. This finding has important implications for the future aim described in this thesis of *in vitro* analysis of oncosuppressor genes. While evidence exists that p53 (chapter 1) and DCC (Fearon, unpublished) have effects on colorectal tumour-derived cells in culture, evidence for APC function comes only from weak effects on transfection into mouse fibroblasts (Hushino et al 1991), and its inferred involvement in polyposis and tumorigenesis. The specific 5q21 change described in association with acquired tumorigenicity of colorectal epithelial cells in culture, although accompanied by changes at other sites, does provide evidence that functionally important genes residing at 5q21 have detectable effects *in vitro*.

3.4.6 Bristol lines are suitable for replacement of AAPC

Tumorigenic cell lines within this set grow clonally and will be useful for manipulation and cloning experiments to test oncosuppressor genes. Since all cell lines retain heterozygosity at 5q21, they must carry the inherited defective copy of APC. According to the gene dosage hypothesis previously mentioned, this defective copy may be duplicated and the excess aberrant APC protein product may influence the phenotype. All mutations reported to date in the APC gene result in unscheduled stop codons (Groden et al 1991), leading to truncated and presumably non-functional protein. The selective increase in mutant gene copy number proposed for the cell line AAC1 suggests that the mutation carried in this APC gene results in a functional protein and is therefore an interesting mutation for study. On the other hand the inherited APC mutation may abrogate function and bear no significance to the selective 5q21 duplication seen, but somatically acquired mutation of the MCC gene may have a dominant effect leading to selection for the observed rearrangement. In either case the situation will be clarified by sequence information and consequent characterization of mutations at either or both loci.

According to the nature of the specific 5q21 mutations involved in this rearrangement a number of possibilities exist for informative genetic manipulation experiments. For example, should the defect reside at the APC locus alone, specific replacement with the wild type

gene by gene target replacement vectors should result in either no change due to replacement of the endogenous wild type copy, or a return to the unrearranged 5q21 status by replacing one of the defective copies. In the latter situation phenotypic effects specific to the 5q21 rearrangement would be apparent since none of the co-inciding rearrangements would be reverted. Should mutations be detected in both genes, their functional significance can be tested by replacement firstly of either alone and then of both together with wild type genes. Numerous other permutations of specific replacement or disablement of colon-associated genes will also be possible and informative.

Chapter 4: In vitro biology of primary cell cultures

4.1 Abstract

Growth *in vitro* was demonstrated for 8 xenografted tumours. Unlike the original human tumour tissue, such xenografts are free from microbial contamination, do not contain human stromal cells and provide a stable renewable resource for repeated culture attempts. The cultures established *in vitro* had an epithelial morphology with one exception, which proved to be a transformed fibroblastic line of non-human (presumably murine) origin. The epithelial cultures displayed individual morphological characteristics. However, most grew very slowly, some were non-progressive and none was cloned. A number of factors were added to or varied in culture in an attempt to assist growth. Although no vital missing factor was discovered, stable xenograft resources will allow an unlimited further search.

2 cultures were expanded sufficiently *in vitro* to allow re-implantation into immunodeficient mice, producing adenocarcinomas with similar histology to the original primary tumours and xenografts. For 1 of these cultures genetic analysis showed the reimplanted tumour to retain its DNA diploid status as well as allelic status on 5 separate chromosomes including the three oncosuppressor loci on 5q, 17p and 18q. Such stability over short periods in culture may be valuable in future experiments to assay *in vitro* the effects of replacement or manipulation of these genes.

4.2 Introduction

A number of factors require attention during derivation of colonic cancer epithelial lines. Problems fall into three broad categories; microbial contamination, stromal overgrowth, and cell requirements, both nutritional and environmental.

4.2.1 Microbial contamination

Due to the abundant microbial flora in the bowel lumen, all tumour tissues taken for culture contain micro-organisms. In order to prevent their co-culture with the epithelial cells, it is necessary to pre-incubate or wash the fresh specimen vigorously in an antibiotic cocktail and to grow primary cultures in an antibiotic rich medium. In addition to this, by first xenografting tumours into immunodeficient animals, low level contamination may be eradicated by the non-specific immunity (macrophages, neutrophils and complement) of the host.

4.2.2 Stromal over-growth

Efforts to retain clumps of dissociated tumour cells, for initiation of growth, often result in the retention of small explants containing both tumour and stromal cell types. The slowly growing epithelial element often becomes dominated and excluded by faster growing fibroblasts.

A technique known as spill-out (Leibovitz et al 1976) which involves sequential settling of dissociated tumour tissue, facilitates the separation of small clumps

of epithelial cells both from single fibroblast cells and larger heterogeneous explants. However, collagenase digestion of the specimen is a popular alternative since collagenase is said to inhibit fibroblastic growth (Namba et al 1983, McBain et al 1984). Fibroblast removal with rubber policemen, subculture using dispase (a neutral protease) which preferentially detaches sheets of epithelial cells, and an antibody-ricin cconjugate (directed to the Thy-1 antigen found on human fibroblasts but not on colonic epithelium), have also been employed (Paraskeva et al 1984). The simplest and most common method of fibroblast elimination however, involves their differential removal during short, repeated treatments of the primary culture with trypsin.

During passage of human tumours as xenografts, prior to culture, the stromal cells of the tumour become replaced with host mouse stromal cells (Kopf-Maier and Kestenbach 1990). Although these stromal cells then feature in cultures established *in vitro*, the species difference between them and the human tumour cells can be exploited. For example, an antiserum raised in rabbit to mouse melanoma cells has been applied to a human cell line grown from a xenografted colorectal adenocarcinoma in order to specifically lyse the mouse fibroblasts (Murakami and Masui 1980).

4.2.3.1 Cell nutrition

Fully defined media have been described for colorectal epithelial cells, incorporating putative

detoxifiers and growth stimulators (Leibovitz et al 1976) and various hormonal supplements (Murakami and Masui 1980). However, the undefined nutrition present in serum, commonly supplemented with hydrocortisone and insulin, is thought to support the growth of most colorectal cancer cells. A strong dependancy is noted of normal epithelial cells on fibroblast conditioned medium (Moyer 1983) which seems also to benefit the growth of tumour cell lines (C Paraskeva, personal communication).

4.2.3.2 Cellular environment

In early attempts at tumour culture *in vitro*, it was observed that some tumour cells flourished only after outgrowth of a fibroblast monolayer (Tompkins et al 1974). Murine fibroblasts with replication incapacitated ("feeders"), were later shown to enhance plating efficiency by three to five-fold (Brattain et al 1982). The beneficial properties of feeder cells are thought to be two fold: the secretion of basement membrane constituents by the fibroblasts may help the epithelial cells to develop polar orientations as in tissue; and feeder cells (which have no growth requirement) produce growth factors and may bind some of the toxic factors present in the uncharacterized serum, or produced by the tumour cells themselves.

Epithelial-mesenchymal interactions have been studied for colorectal carcinoma cells growing in monolayer cultures and in collagen matrices (Richman and Bodmer 1988). The presence of fibroblasts commonly

induced differentiated structures in the adjacent tumour epithelium, as detected by morphology and immunocytochemistry with monoclonal antibodies to components of goblet and columnar cells. The cell lines varied, however, in their capacity to respond, and it is therefore to be expected that feeder cells will be ineffective in supporting cultures of certain unresponsive tumours.

The basal ends of epithelial cells in situ are in contact with the basement membrane, where fibroblasts secrete large amounts of collagen. Collagen coated surfaces (like feeder cells) are often used in vitro to mimic this effect. Although collagen is used routinely in colorectal cultures, it was found to be beneficial in the primary culture of only 50% of a set of fourteen newly established lines (Lewko et al 1989). Alternatively, complex basement membranes are available such as that extracted from the mouse Engleberth-Holm-Swarm tumour. This biomatrix (known as matrigel) has been shown to induce not only differentiation but biochemical function and morophogenesis of mammary epithelial cells and hepatocytes (Stoker et al 1990).

4.2.4 Colonic cultures: common characteristics

Reports of the establishment and characterization of colonic epithelial cell cultures describe many common cell properties. Cells are always described as small, closely packed, polygonal or pleomorphic, with large nuclei and scanty cytoplasm. Initially, primary cultures

always grow as islands of epithelial cells showing a strong preference for high density seeding, and enhanced growth over a fibroblast layer or collagen surface is generally described. Ultrastructural presence of microvilli and secretion of large amounts of carrinoembryonic antigen (CEA) are also commonly reported.

4.2.5 Colonic cultures: diverse properties

Despite these common features in many colonic epithelial cells in culture, there is also a wealth of diversity. Perhaps the most striking is the variability in growth rate of primary cultures including length of lag period before growth and subsequent time before first subculture. Lag periods of between two weeks and six months are reported (Leibovitz et al 1976), while the period of growth before first subculture ranges from less than 4 weeks (MCBain et al 1984) to more than a year (Leibovitz et al 1976). The afore-mentioned differential responses to fibroblast feeders, and to a collagen substrate support this idea of diversity as does the wide variety of colonic culture karyotypes summarized in table 1, and discussed more fully in chapter 1. Other variable characteristics reported include lack of substrate adherence, ability to form tumours in animals (Park et al 1987), and morphological features such as "doming" of cellular aggregates within epithelial islands (Leibovitz et al 1976). This diversity of colonic epithelial cell cultures is not surprising on consideration of the heterogeneity of colonic adenocarcinomas, both

phenotypically and genotypically (Quirke et al 1985, tables 4 and 8 in this thesis).

4.2.6 Xenograft to primary culture in this study

Growth *in vitro* is a pre-requisite for the introduction of exogenous DNA into cells by transfection. We therefore wished to initiate such growth from colorectal tumours. Initial attempts to establish primary cultures directly from fresh tumour specimens were largely unsuccessful in this study, and the growth rate of those which grew was restrictively slow. Accordingly tumours were first xenografted into immunodeficient mice in order to reduce contamination problems in culture and to provide a permanent, well-characterized resource as described in chapter 2. This chapter describes the growth of these tumour xenografts after explanting *in vitro* and also, where possible, their subsequent re-implantation into animals.

4.3 Results

4.3.1 Primary cultures grew from all tumour xenografts

In a series of attempts to culture colorectal tumours directly *in vitro*, without an intermediate xenograft stage, only 5 of 40 primary tumours (12.5%) showed any measure of success. All 5 grew continuously for between 9 and 16 weeks. No culture reached confluence and all were eventually lost in a single bout of bacterial infection of unknown origin. 1 culture derived from an adenoma yet was not notably different in morphology or growth pattern from the others.

Although unproductive, this period allowed observation of the effects of varying some of the factors of the culture process. Variables included percentage serum in the medium, numbers of feeder cells added, growth in a humidified CO2 equilibrated incubator versus closed flasks, collagenase digestion versus mechanical tissue dissociation, seeding of different size fractions of dissociated tissue, and incomplete removal of spent medium during feeding. Assessment of benefit in any particular set of conditions was subjective, but led to the adoption of a general protocol, slightly modified from the original method of Paraskeva et al (1984), for subsequent use (chapter 7).

In contrast to this lack of success with direct culture of primary tumours, cultures were grown on at least 2 occasions from each of the 8 continuously passaging colonic adenocarcinoma xenografts described in

chapter 2. In general, all 8 primary cultures exhibited the expected morphology, growing as epithelial islands of small polygonal cells with large nuclei. However, from observation over 2 to 9 months after a number of repeated seedings, it was apparent that each xenograft line generated cultures with characteristic properties.

4.3.1.1 Characteristics of GRBO in primary culture

GRBO grew well in culture and cells were particularly refractile (figure 15). On two occasions immediate outgrowth followed by 2 passages in culture was achieved over 2 months. Thereafter, however, growth arrested and no further passage was possible. One preparation of frozen dissociated xenograft tissue seeded for culture gave rise to a different pattern. Small pleomorphic cells grew in clusters but without close cell contacts as seen in islands. As the clusters grew into colonies a curious thread-like pattern was evident over the top of the colony (figure 15), which at high power was seen to consist of aggregates of these cells. Feeder cells were required for the maintenance of these pleomorphic cells which, after a few weeks, stopped forming these threads and grew as flatter colonies.

4.3.1.2 Characteristics of RHSP in primary culture

Primary cultures of RHSP were seeded on more than 5 occasions and grew well over 3 or 4 passages in culture. Outgrowth was immediate on seeding but arrested after about three months. RHSP culture is shown in figure 16.

4.3.1.3 Characteristics of MUCO in primary culture

Primary cultures of MUCO were seeded on more than 5 occasions and grew very poorly. Despite the obvious presence of many clusters of epithelial cells in the preparation seeded for culture, only a few islands were ever visible a week later. Most of the cell islands soon become vacuolated and degenerated. Occasional non-vacuolated islands survived but remained static over a number of months. During this period, the islands became surrounded by a cellular debris and fibroblast outgrowth. Eventually the epithelial cells degenerated, developed vacuolation or gradually detached from the substratum. An area of MUCO becoming vacoulated is shown in figure 17.

4.3.1.4 Characteristics of CHKE in primary culture

Primary cultures of CHKE were seeded on 2 occasions. Both attempts produced immediate but slow growth of islands very similar in appearance to RHSP shown in figure 16. Unfortunately both cultures were accidentally lost as was the xenograft line.

4.3.1.5 Characteristics of ARNE in primary culture

Primary cultures of ARNE were seeded on 2 occasions. Both attempts produced immediate but slow outgrowth of islands morphologically similar to RHSP but showing copious mucin production. In one case, 2 passages were achieved over about 100 days before the culture was overcome by yeast infection.

4.3.1.6 Characteristics of DABU in primary culture

Primary cultures of DABU seeded on 2 occasions had a

distinctive cell morphology. After a few weeks of dormancy, fast growing islands were observed. These islands consisted of cells of a more spindly appearance than those in other primary cultures (figure 18) and cells in the centre of the islands often piled up to form rounded domes. DABU cells were sometimes difficult to distinguish from fibroblasts because of their fusiform appearance. A mass culture of mixed epithelial and fibroblastic cells was expanded and is described later in this chapter.

4.3.1.7 Characteristics of MASM in primary culture

Primary cultures of MASM seeded on 3 occasions had a slow growth rate. As in the other 8 xenografts, organoids of epithelial cells were observed during seeding which generally settled to form the flat islands observed. However, in the case of MASM, these organoids continued to grow as three-dimensional clumps from which dense sheets of cells began to emanate. Once the epithelial sheet was established, the central clump budded off leaving an island similar to those observed for other lines. In the islands of MASM, however, the cells were more densely packed and often showed signs of polarity in the form of groups of cells with parallel alignment. Figure 19 shows several of these features. Growth was so slow that passage without splitting, merely to decrease cell density, was only possible after 6 months in one case. This slow growth made these cultures particularly vulnerable to infection, to which the only second passage

representative finally succumbed after 9 months in culture.

4.3.1.8 Characteristics of JOMCL in primary culture

Primary cultures of JOMCL, on more than 5 occasions, grew immediately and strongly upon seeding. The cells within the islands had a variable morphology ranging from rounded or polygonal to spindly and similar to the cells observed in DABU cultures. The two extremes were often present in the same flask and were not related to the length of time in culture. Polygonal cells from a 4th passage culture and spindly cells from 1st passage are shown in figure 20. In the 4th passage culture large numbers of small pleomorphic cells were noticed, growing close to but not always in direct contact with neighbouring cells. These were similar in appearance to the cells described in GRBO cultures but did not form the thread-like structures. Their location was always near to an established epithelial island from which they seemed to emanate, arising most often in coves around the coasts of the islands.

JOMcL cells grew well even in short periods of absence from feeders and collagen. When seeded on culture dishes without collagen, epithelial islands formed as usual but with the addition of three-dimensional buds as described for MASM primary cultures. In JOMCL these buds appeared to grow, often on stalks, from the centre of the islands (figure 20). These buds tended to break off and could be harvested from the medium. Histologically they

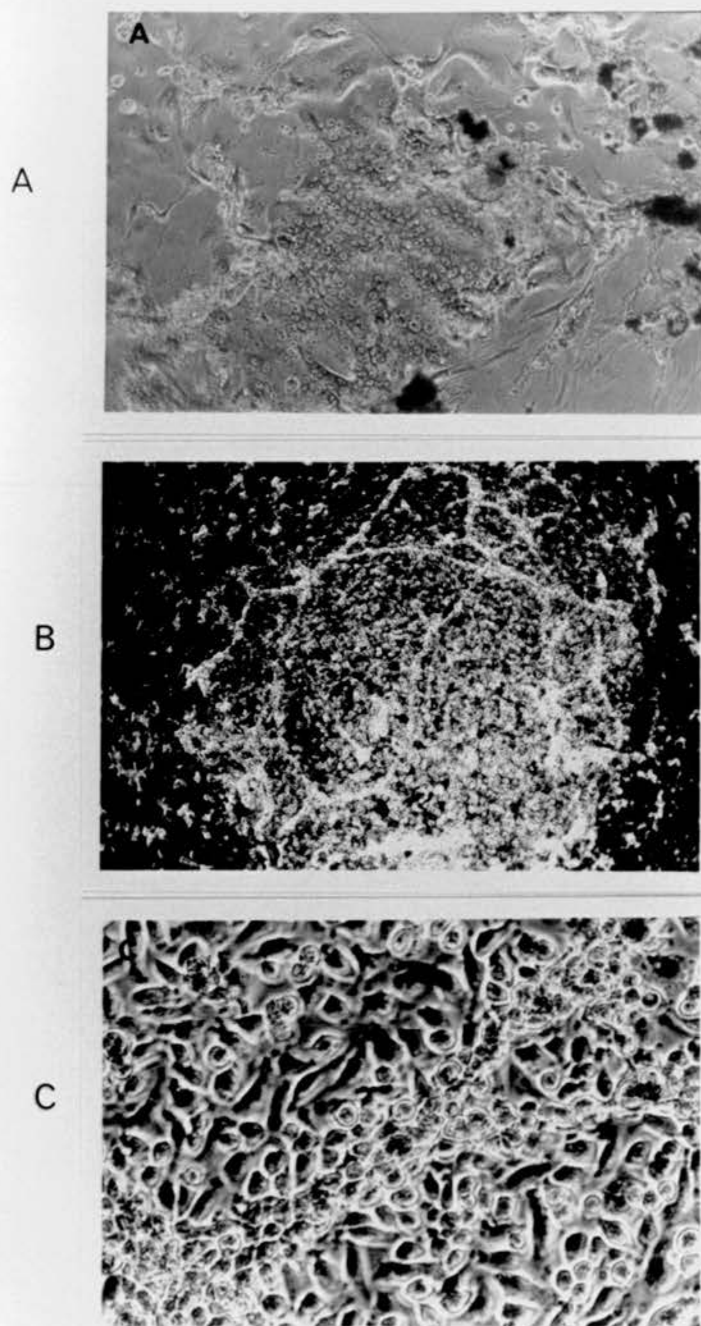


Figure 15 Phase contrast photomicrographs of GRBO cells in primary culture. A; A flat island (x69). B; Altered cells clumping and forming cords (x28). C; Cellular basis of the cord (x221).

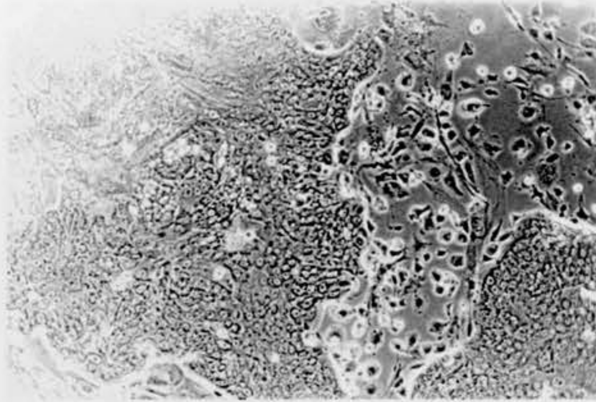


Figure 16 Phase contrast photomicrograph of RHSP cells in primary culture showing flat islands surrounded by feeder cells (x69).

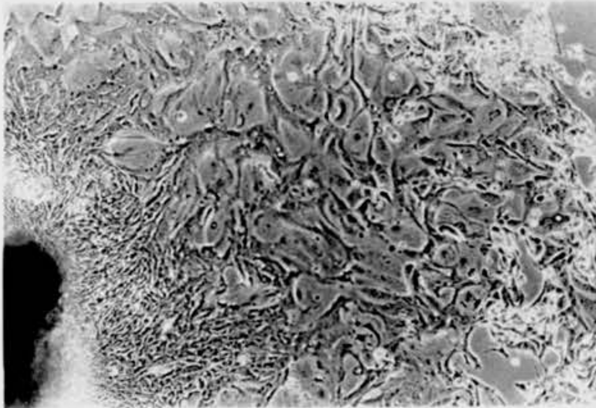


Figure 17 Phase contrast photomicrograph of MUCO cells in primary culture showing vacuolation (x69).

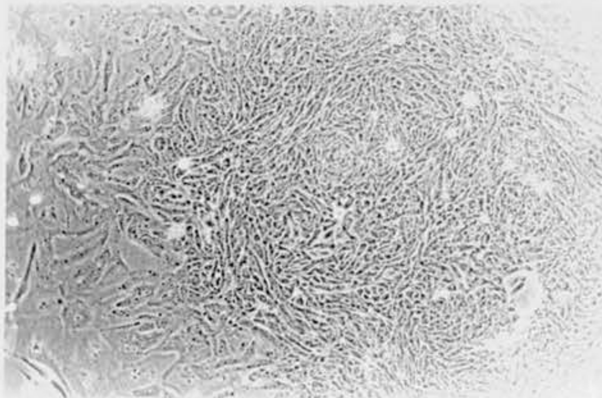


Figure 18 Phase contrast photomicrograph of DABU cells in primary culture showing spindly appearance (x69).

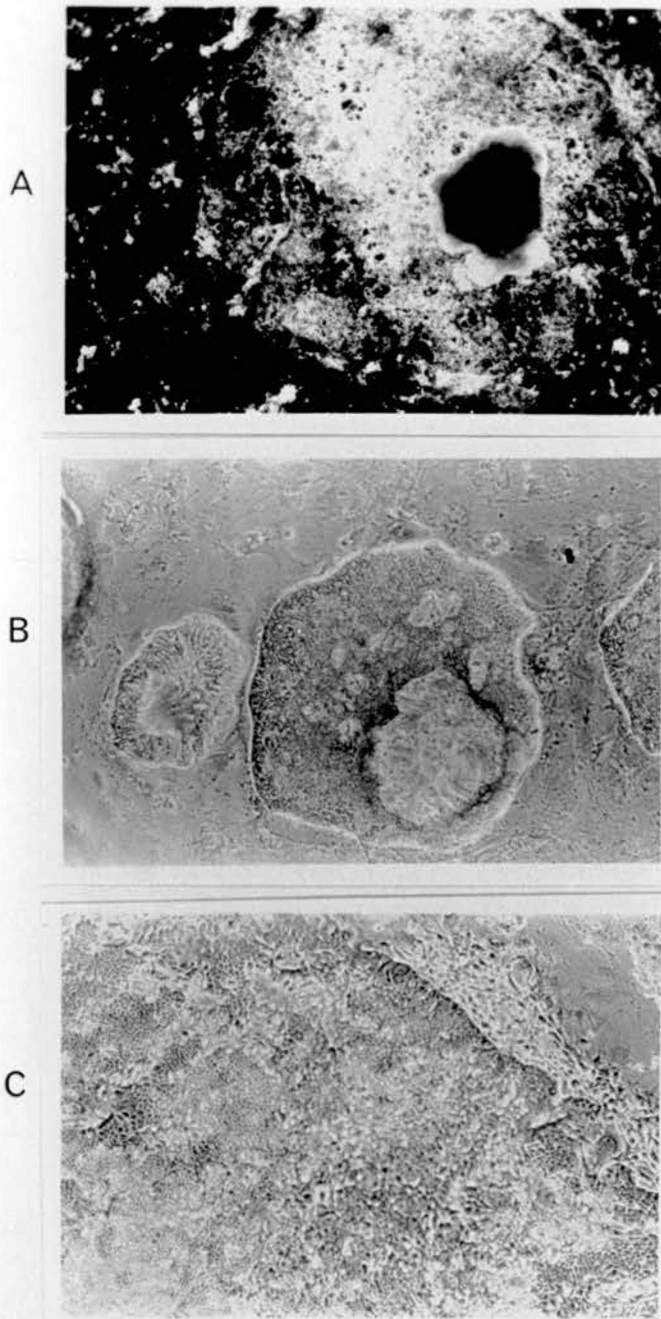
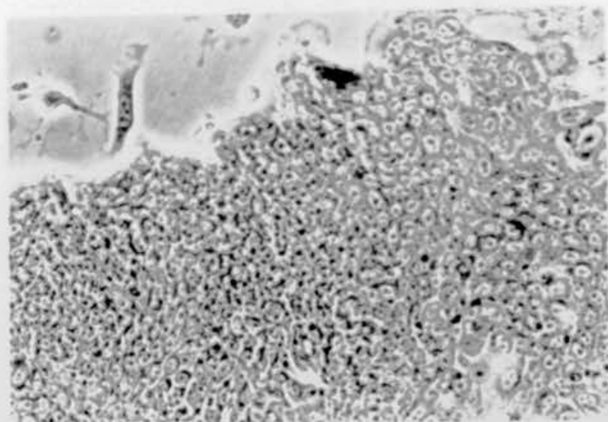
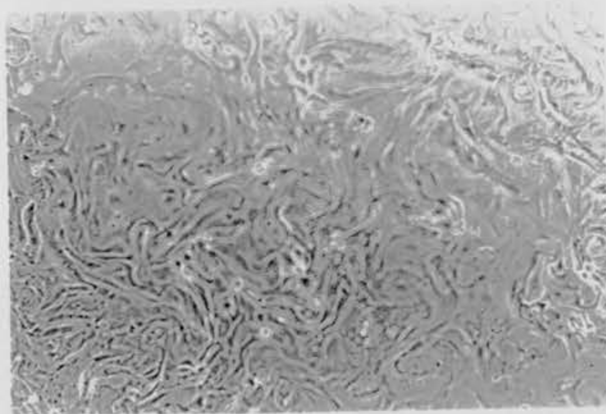


Figure 19 Phase contrast photomicrographs of MASM cells in primary culture. A; Primary culture showing density of cells and presence of 3-dimensional tissue implant (X28). B; Flat islands with indented organisation within (x69). C; Edge of growing island showing polarised cells of columnar appearance (x69).

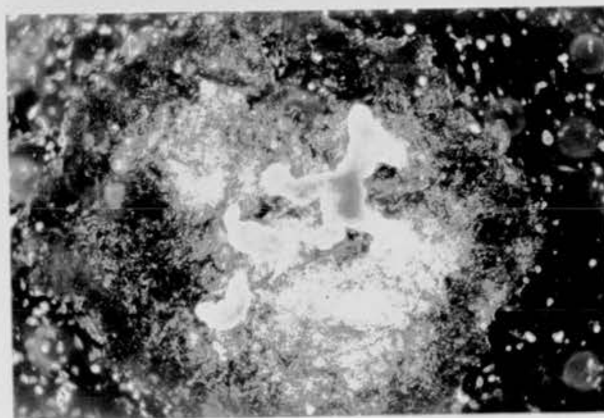
A



B



C



<u>Figure 20</u> Phase contrast photomicrographs of JOM^{c}L cells in primary culture. A; Densely packed polygonal cells of 4th passage culture (x221). B; Spindly cells of 1st passage culture (x69). C; 3-dimensional buds forming within islands when grown without a collagen substrate (x28).

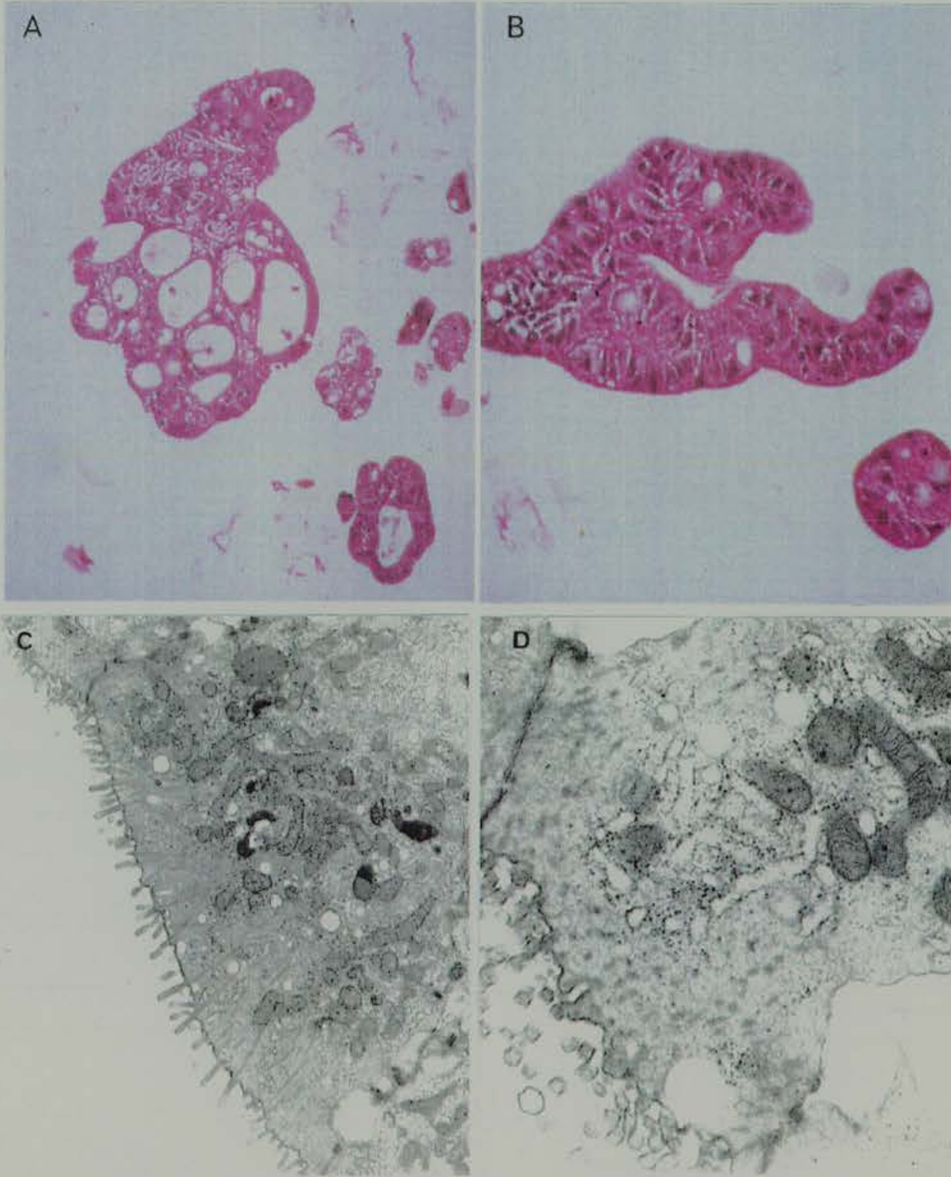


Figure 21 JOM^{c}L organelles produced in primary culture.
A; Histology of organelles showing glandular structure (x100),
B; Histology of organelles showing cell polarity (x252).
C & D; Electron microscopy of organelles showing tight junctions, desmosomes and apical microvilli (x10000 & 25500 respectively).

proved to be small foci of adenocarcinoma, with well-formed central lumens surrounded by polarised tumour cells. In the electron microscope this was confirmed, apical microvilli and lateral tight junctions and desmosomes being clearly seen (figure 21).

JOMCL cells have to date grown continuously in culture over 4 passages with no signs of senescence.

4.3.1.9 Characteristics of AGDU in primary culture

Despite the uncertain origin of the tumour giving rise to the xenograft AGDU, 2 primary cultures were attempted. The appearance and behaviour in culture of this line was distinctly unlike any of the others. Small cauliflower-like florets of cells formed centres from which grew long threads of cells. No flat epithelial islands were seen before cultures were terminated after 10 weeks.

4.3.2 Primary cultures of frozen xenografts

In chapter 2, the growth of xenografts from tissue which had been stored at -70°C is described. In this chapter cell culture growth from identical frozen preparations is described. By the same method as for seeding fresh preparations of tissue, cultures of JOMCL, GRBO and RHSP were succesfully seeded from thawed xenograft specimens. Such dual purpose storage of tissue precludes the necessity to freeze both tissues and cells of any one line.

4.3.3 Mouse feeder cells are adequate and necessary

In an attempt to assist or boost growth of primary

cultures, a number of culture conditions were vvaried.

As it seemed possible that feeder cells might retard growth *in vitro* by inducing terminal differentiation, all cultures were grown for some time, and most were seeded on one occasion, in the absence of feeders. All cultures excepting JOMCL were severely inhibited by the lack of feeder cells.

A second possibility was that human colonic fibroblasts might provide distinctive growth support to colorectal carcinoma cells. Accordingly, human fibroblasts which grew out from an early primary culture established directly from a carcinoma, were treated with mitomycin C and used as substitute feeder cells for a short period of time. No changes were seen in either growth rate or morphology in cultures receiving these human fibroblast feeders in comparison with murine fibroblast feeders, and routine use of the latter was resumed.

4.3.4 Type I collagen is adequate and necessary

Since collagen substratum has been shown to benefit only 50% of primary colonic epithelial cultures (Lewko et al 1989), samples of most of the cultures were seeded on tissue culture plastic alone. Growth in the absence of collagen was noted only for JOMCL; the resulting budded growth of adenocarcinoma was described above. Type IV collagen is secreted in the basement membrane *in situ* and is therefore advocated for coating of flasks for culture purposes. Owing to the expense of this type of collagen,

the ability of cells to grow on the considerably cheaper type I collagen was tested and growth was found in no case to be limited in comparison with growth on type IV. Accordingly type I collagen was routinely used thereafter.

4.3.5 A defined medium does not alter JOMCL growth

Because of the report by Murakami and Masui (1980) that cells grew 3 times faster in a defined hormone containing medium, its effects on primary culture growth were tested. The serum-free medium was supplemented with insulin, glucagon, epidermal growth factor, transferrin, hydrocortisone, triiodothyronine, selenium, and ascorbic acid. Parallel cultures of JOMCL were seeded and grown over 1 passage in either colonic culture medium with 10% serum as described in chapter 7, or in this defined medium. No differences in passage interval or growth pattern were observed between cells in either media.

4.3.6 EGF does not increase success

Epidermal growth factor (EGF) was applied at 10ng/ml to an established growing culture, derived from an adenoma and described in chapter 3 (PCAA). Parallel flasks of PCAA with and without EGF were seeded and grown to passage and for 2 subsequent weeks. Over this time, constituting about 6 weeks, no differences in morphology or passage interval were noted between EGF containing or non-EGF sister cultures.

In the primary culture MUCO no appreciable growth could be attained and degeneration was observed in most

or all seeded cells. To test if EGF could alleviate this situation by providing essential stimulation absent from serum alone, cells were seeded in medium containing 10ng/ml EGF. Vacuolation and degeneration were, however, noted as usual and no useful effects of EGF could be discerned.

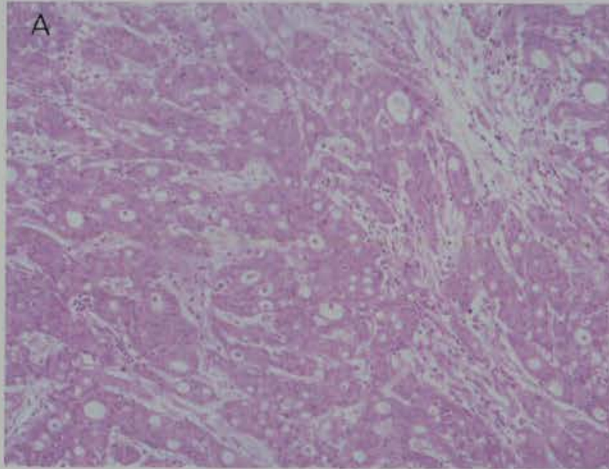
4.3.7 MASM, MUCO and JOMCL are not clonogenic

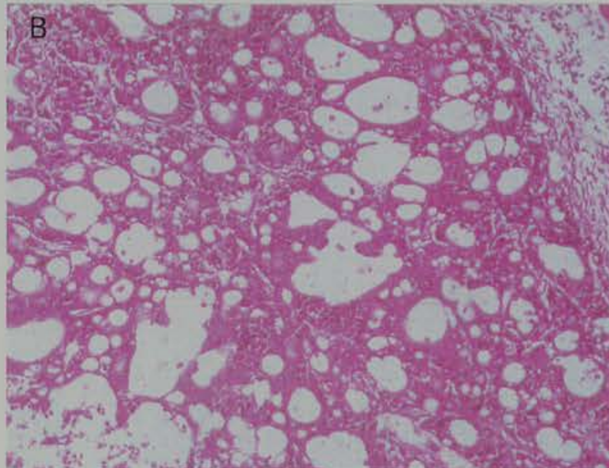
Passage of all cultured cells described in this chapter was effected by dispase digestion, as described in chapter 7, which detaches the cells from the substratum as sheets rather than in single cell suspensions. To test the clonability of some of the cultures, MASM, MUCO and JOMCL were treated with trypEDTA as described in chapter 7. Although single cell suspensions were obtained none of these three cultures grew, with or without addition of feeder cells.

4.3.8 Tumorigenicity of primary cultures

Growth *in vitro* may select for properties not uniformly present in the original xenografted tumours. Under such circumstances, it would not be possible to use these cell lines for the ultimate aim involving comparison of the stable xenografted tumour with derived cells specifically manipulated *in vitro* at oncosuppressor sites. Accordingly it was important at this stage to observe the behaviour on re-implantation *in vivo* of cells cultured *in vitro*. 2 of the primary cultures, JOMCL and DABU grew at a sufficient rate to allow re-implantation, both producing adenocarcinomas.

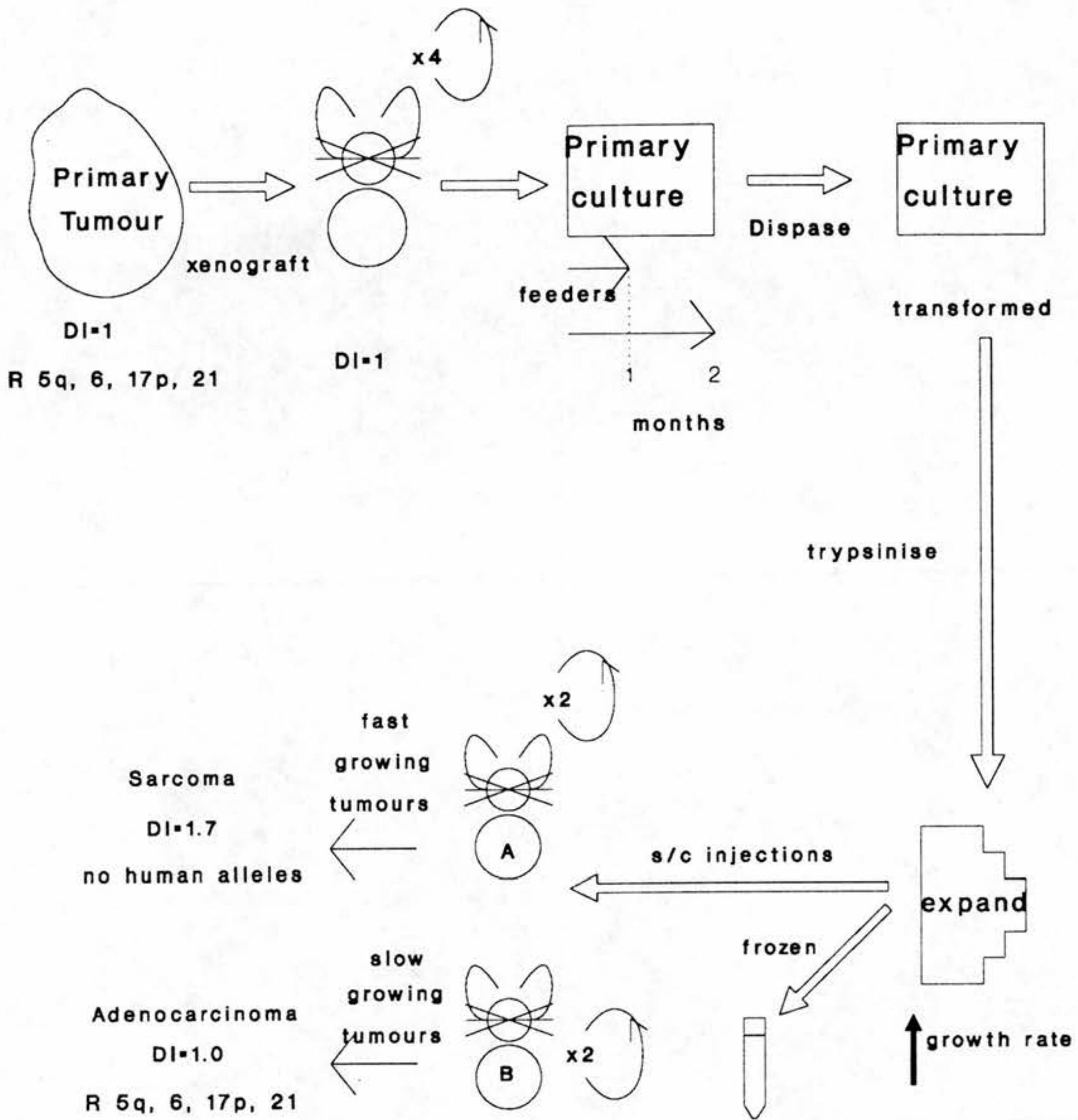
Figure 22 Histological patterns of A; JOM^{c}L primary tumour, B; Xenograft produced by re-implantation of JOM^{c}L primary culture (both x100).





For JOMCL cell re-implantations half the contents of a $150cm^2$ flask of cells were harvested and re-injected into 2 nude mice. Although cell clumping precluded even approximate counting of cell number, by comparison of the cell pellet size with pellets of known cell number this innoculum was low, due to slow growth and resulting lack of availability of cells. After 72 days the resulting tumour was large enough to harvest. Histological examination of fixed sections of this tumour showed morphology indistinguishable from that of both the primary tumour and the passaged xenografts (figure 22). Sufficient material is not yet available from this re-injected tumour to allow genetic analysis.

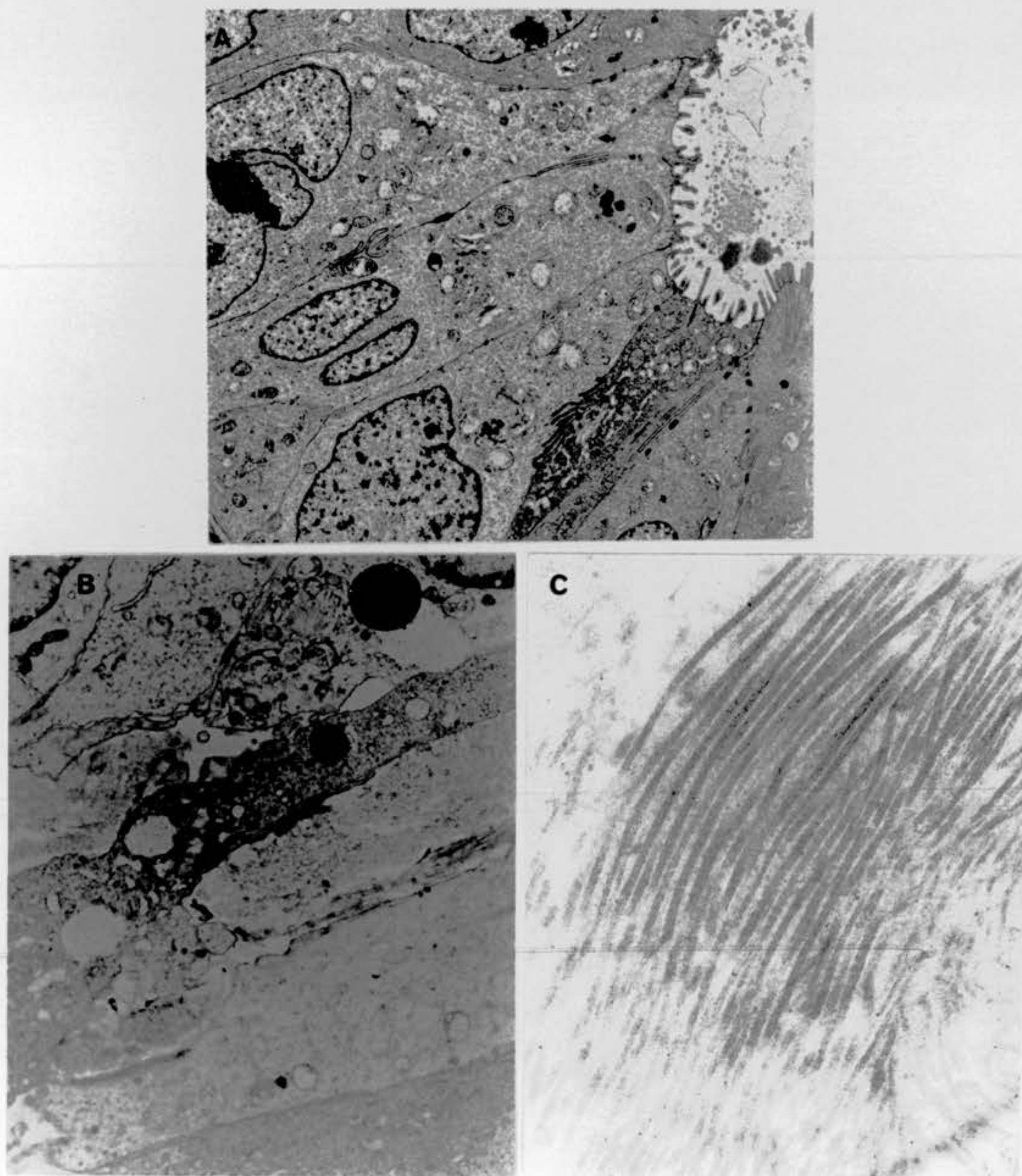
Within DABU cultures, the fusiform appearance of epithelial cells made them impossible to distinguish from neighbouring fibroblasts. This cell mixture grew well after trypsinisation and was rapidly expanded in culture. 10^6 cells were injected subcutaneously into each of 2 immunosuppressed mice. Both mice developed tumours which were passaged twice without any signs of senescence, but the 2 sub-lines grew at widely different rates. A large infiltrative tumour was harvested from the first mouse 32 days after injection, and subsequent passages were conducted after 23 and then only 13 days. In contrast, the second mouse grew a moderately sized tumour which was not large enough to harvest until 54 days after injection and which subsequently took 44 days to grow to passage. The summarized history and important features of this



<u>Figure 23 Derivation of DABU culture and its re-implantation.</u>

Notes:
1) DI= DNA index, R= retention of heterozygosity
2) 5q, 6, 17p, 21= chromosomal locations
3) x2 or x4= 2 or 4 xenograft passages respectively
4) feeders= 3T3 feeder cells
5) s/c= subcutaneous

Figure 24 Electron microscopy of DABU culture re-implanted as xenograft. A; Slow growing adenocarcinoma line showing cell polarity, a variety of junctions and luminal microvilli (x3570), B; Fast growing sarcoma line showing absence of desmosomes or cell polarity (x6800), C; Collagen bundle, found abundantly throughout sarcoma line (x34000).



chain of events are illustrated in figure 23.

Histologically, the fast growing tumour was a poorly differentiated spindle cell tumour that appeared to be a sarcoma. The slow growing tumour was a moderately differentiated adenocarcinoma with morphology closely similar to DABU prior to growth _in vitro_. By electron microscopy the sarcoma was seen to consist of fibroblasts secreting collagen, whereas in the adenocarcinoma the predominant cell type was a polarised columnar cell with apical microvilli and lateral desmosomes (figure 24).

DNA index (DI) for each line was determined by flow cytometry. The sarcoma line DI was 1.6, while the DI for the adenocarcinoma line was 1.0 denoting a diploid tumour similar to the original DABU primary carcinoma. To establish the origin of these lines, DNA was extracted from both the sarcoma and adenocarcinoma and subjected to RFLP analysis with probes to a number of human sequences: YN5.48, L5.71 and Pi227 on chromosome 5; YNZ132 on chromosome 6; YNZ22 on chromosome 17; BV15.65 on chromosome 18 and MCT15 on chromosome 21. Conditions for each probe are summarized (tables 17a and 17b), and examples of patterns detected by some probes are shown (figure 30) in chapter 7.

In no case did any probe hybridise to specific restriction fragments in the digested DNA of the sarcoma. In contrast, under the same conditions all probes detected alleles in the adenocarcinoma DNA identical to those in the blood of the original patient and in the

Table 13 DABU alleles before and after culture

Probe	Primary	Alleles detected in tumour DNA Adenoca xenograft	Sarcoma xenograft
Pi227(Pst1)	A2A2	A2A2	-
Pi227(Bcl1)	A2A3	A2A3	-
L5.62	A1A1	A1A1	-
L5.71	A2A2	A2A2	-
EF5.44	A2A2	A2A2	-
YN5.48	A1A2	A1A2	-
YNZ132*	A1A2	A1A2	-
YNZ22*	A1A2	A1A2	-
BV15.65	A1A1	A1A1	-
MCT15*	A1A2	A1A2	-

Notes:
1) *= VNTR
2) Adenoca= adenocarcinoma
3) -= no alleles detected

corresponding xenograft (table 13). 3 of these probes are VNTRs detecting up to 30 different alleles in the population. This DNA fingerprinting exercise therefore confirms that the adenocarcinoma was identical in origin to the original DABU line while the sarcoma was clearly not of human origin. In view of its fibroblastic appearance and fast growth the fibrosarcoma was presumed to originate from transformed mouse fibroblasts. During the early stages of DABU growth there was an occasion where feeder cells were used that may not have been rendered completely incompetent for replication by mitomycin treatment. The DABU culture may therefore have become contaminated with immortalized 3T3 fibroblasts, which would be liable to selection for spontaneous transformation during long periods of over-crowding in the primary culture. Alternatively, murine stromal fibroblasts from the xenograft explant may have undergone transformation during culture, although the ready

transformation of this 3T3 line experienced under other circumstances supports the former explanation.

Despite this fibroblast contamination, the above results confirm that the slow growing tumour produced by DABU human adenocarcinoma cells had stably conserved DNA ploidy and, more specifically, allelic status at loci on 5 different chromosomes. In particular, variants at the key colon-associated loci on 5q, 17p and 18q had not been selected for during short-term culture.

4.4 Discussion

4.4.1 Valuable, stable xenograft resources

Since tumour characteristics are stably maintained during propagation in immunodeficient animals (chapter 2), the xenografts described provide stable resources for repeated culture attempts. Such xenograft derived cultures avoid previously experienced problems of primary culture contamination with co-cultured bowel microbes. Moreover, the replacement of the stromal element of the tumour with host derived cells prevents culture contamination with human fibroblasts. The species difference can be exploited and in this study has allowed identification of murine fibroblast outgrowth by genetic means which would not have differentially detected human fibroblast contamination. Xenografting prior to culture is therefore a valuable and powerful tool.

4.4.2 Diversity of cultures

Variable phenotypes and growth characteristics were noted in the 8 primary cultures described despite identical environmental conditions. Growth *in vitro*, although observed in all cases, was generally slow and for many cultures was non-progressive despite continuous growth as xenografts. This suggests the general culture protocol lacks vital factors which are available *in vivo*. Human fibroblast feeders, EGF, type IV collagen or a hormone containing defined medium did not provide the missing factors to the cultures tested. Because of the availability of genetically stable tumour xenografts-

some preserved at low temperature- practically unlimited numbers of further candidate growth factors can be screened.

With the high take rate in SCID mice (described in chapter 2), 92-100% of colonic adenocarcinomas have grown as xenografts. This provides an opportunity for unlimited analyses of in vitro requirements for the entirety of unselected adenocarcinomas. Such analyses could provide valuable information about all co-operations of colon tumour cells with other cells, growth factors and various components of the extracellular matrix.

4.4.3 Is clonability necessary?

Of the 8 primary cultures described, growth was observed in vitro for only one after trypsinisation to single cells, with epithelial cultures derived from xenografts commonly exhibiting non-clonal growth from clumps of cells. In contrast to this and the slow growth generally observed in these cultures, long-established colorectal lines are often reported to grow both rapidly and clonally. The ultimate aim in attempting culture in vitro is to provide a suitable host cell in which to test the function of genes associated with colon cancer. Clonability is generally a pre-requisite for experiments involving gene transfer or targetting, since selection of the small number of successfully manipulated cells is necessary. Although it may be possible to achieve clonal growth in vitro for the described cultures by severe selection, such an act would be in opposition to the

original aim of stably maintaining tumour cell properties. It may therefore be more constructive to search for a highly efficient method of transfection, such that clumps of transfectant cells may be selected together, as will be discussed in chapter 5. Genes introduced in retroviral vectors become widely spread throughout the infected population of cells and may therefore be a feasible alternative to transfection. Indeed, viral vectors carring myc and src oncogenes have been shown to induce dysplasia on introduction directly into rat colon allografts (D'Emilia et al 1991).

4.4.4 Stability in vitro

Stability of xenograft characteristics conserved from original primary tumours is shown to extend through short periods in culture for 2 lines. In particular, 1 line retained critical features such as ploidy and allelic status on many chromosomes including key colon-associated oncosuppressor loci on 5q, 17p and 18q despite its rapid growth and clonability in vitro. Particularly if transfection of non-clonal cells proves a possibility, the stable xenograft and short term culture system may be ideal for gene transfer and functional assays of colon tumour-associated genes.

Chapter 5: Oncosuppression by transfection

5.1 Abstract

Preliminary experiments were conducted to explore means of transfection and effects of oncosuppressor genes in colorectal tumour cell lines. Although transfection efficiencies were poor, there were several arresting observations.

The *Krev* gene was found to suppress tumorigenicity, inhibit growth and induce apoptosis in a transformed murine cell line. On transfection into a tumorigenic colonic line the same gene completely arrested growth. No transfectants could be selected from non-clonable colonic cultures. These experiments helped to expose and define problems inherent in selection of cells that might grow less well than their parental lines. The values and difficulties of selecting transfectants from non-clonable cultures were also considered. Methods are discussed which may overcome these problems in the future.

5.2 Introduction

The xenografts described in this thesis display a variety of genetic defects and some have generated *in vitro* passaged lines. Moreover, major changes were not detected in genotype or phenotype on re-implantation *in vivo*. The xen ografts therefore appear suitable for assay of oncosuppressor gene effects against a variety of backgrounds. The question arises as to how such activity would manifest itself and how cells expressing it could be selected.

5.2.1 Effects of oncosuppressor activity

Loss of tumorigenicity is perhaps the most dramatic and definitive effect that an oncosuppressor gene may exhibit. Tumorigenicity is, however, a complex function which may not always be inhibited, despite oncosuppressor activity, due to the effects of other genes within the cell. Such genetic interplay may result in gene transfer of candidate oncosuppressor genes blocking tumorigenicity in one but not another recipient cell background. The possibility of such variable effects justifies the approach described in this thesis of characterizing multiple lines of different genotypes, to allow oncosuppressor assay in a variety of known backgrounds.

An oncosuppressor gene assay may, alternatively, result in growth arrest or retardation *in vitro*. Although a potentially interesting effect, growth inhibition creates problems for further study. In the case of growth arrest, selection of transfectants (which relies on

proliferation) is not possible. In either case, if transfectants are selected the observable spectrum of phenotypic effects is automatically narrowed since growth inhibitory effects are selected against.

Another possible effect of cellular action of an oncosuppressor gene is increased apoptosis, for which assays exist. There is a precedent for modulation of apoptosis by oncosuppressor gene expression in the recently described effects of wild-type p53 on myeloid leukaemic cells (Yonish-Rouach et al 1991). In accord with the previous argument, however, the increased apoptosis would be required to co-exist with continuing replication to avoid loss of transfectants. Furthermore, no means currently exist to select for apoptotic behaviour and with continuing cell culture there is therefore the risk that it will be eliminated from the phenotype.

Finally, oncosuppressor assays may cause decreased cellular response to appropriate growth-supportive signals, or increased response to corresponding inhibitory signals. The oncosuppressor DCC gene codes for a sequence suggesting a cell adhesion molecule (Fearon et al 1990), and thus may exemplify an oncosuppressor gene which modulates responses to cell-cell signals. Such signals may be vital, particularly to cells for which clonal growth is not a feature. At present there is no means of assaying these effects.

Published literature does not indicate whether these

problems will be trivial or extensive. A further long-appreciated problem is the difficulty of expressing exogenous genes in human cells compared to rodent cells. This section of the thesis attempts to make a preliminary assessment of these issues.

5.2.2 Methods for efficient transfection

Experiments intended to define transfection parameters for the proposed biological tests of oncosuppressor function in the new colorectal cell lines are described. Preliminary experiments were conducted to investigate means of transfection and expression of exogenous DNA in these lines.

Transfection methods were chosen which were reported to be applicable to a broad range of cell types, and to repeatedly give high efficiencies of stable transfection. Electroporation was chosen also for its usefulness, due to its physical rather than chemical means of gene transfer, for cells which are refractory to other methods (Potter et al 1984). However, the success of electroporation is associated for most cell types with killing 50-75% of cells by the electrical pulse. Synthetic amphiphiles were chosen for their reported lack of toxicity and ability to transfect primary neuronal cells without altering important cellular physiology (Loeffler et al 1990). The amphiphiles entrap exogenous DNA on the basis of charge and spontaneously associate with natural anionic headgroups in cellular membranes, internalising the DNA by endocytosis (Behr et al 1989,

Felgner et al 1987). Amphiphile-mediated gene transfer is reported to be 5 to >100 times more efficient than the commonly used calcium phosphate method (Felgner et al 1987). Furthermore, this reagent has previously been used for efficient transfection of human epithelial cells such as Hela and of the colon cells CX1, and MIP (J Bubb, personal comunication).

5.2.3 Selectability of a putative oncosupppressor

Experiments were performed in order to compare the selectability and effects of an exogenous oncosuppressor in murine fibroblasts with its selectability and effects in both clonable and non-clonable human epithelial cells. One long term goal of this work was to study the function of oncosuppressor genes known to be involved in colorectal carcinogenesis, in particular the 5q genes MCC and APC. These genes were not cloned until August 1991 and it is clear, on account of its huge size (9.5 Kb of cDNA), that special techniques will need to be employed to transfect APC. Accordingly Krev was chosen for initial study: it is a relatively small, readily available putative oncosuppressor gene, with well described immediate function, but still incompletely defined cellular activities.

The Krev gene was isolated as a human cDNA that suppressed tumorigenicity and caused reversion of phenotype in vitro on transfer into a viral Ki-ras transformed mouse 3T3 cell line (Kitayama et al 1989, Noda et al 1989). The gene- independently isolated by low

stringency hybridization of human DNA with a drosophila ras-related gene (Pizon et al 1988)- is variously termed _rap1_ and, its protein product, _smg_ p21A (Kawata et al 1988). The 21Kd protein product has 50% amino acid homology with _ras_ p21, including the C-terminal cysteine used for membrane anchoring, GTP binding regions and the putative effector domain. Such homology together with its apparent ability to inhibit growth led to the proposal of _Krev_ as a _ras_ antagonist, perhaps by competitive inhibition of GAP (GTPase activating protein) mediated activity. _Ras_ p21 GAP was indeed found to bind _Krev_ p21 tightly without increased hydrolysis (Frech et al 1990), which would allow _Krev_ expression to modulate _ras_ p21 activity by direct competition. It has been hypothesised that deregulation of such _ras_ antagonists may be involved in tumorigenesis and indeed, tumour specific decreases in _Krev_ expression have been demonstrated (Culine et al 1989).

5.3 Results

5.3.1.1 Optimised electroporation of colonic cells

In order to define the optimal parameters for electroporation into colonic epithelial cells, the established line C280 (chapter 3) was electroporated under a variety of conditions with pSV2neo (the Tn5 aph gene driven by the SV40 promoter and enhancer region, Southern and Berg 1982). Expression of the aph gene confers neomycin resistance on transfectant cells and permits their selection with geneticin (G418), a neomycin analogue. The results are shown in table 14.

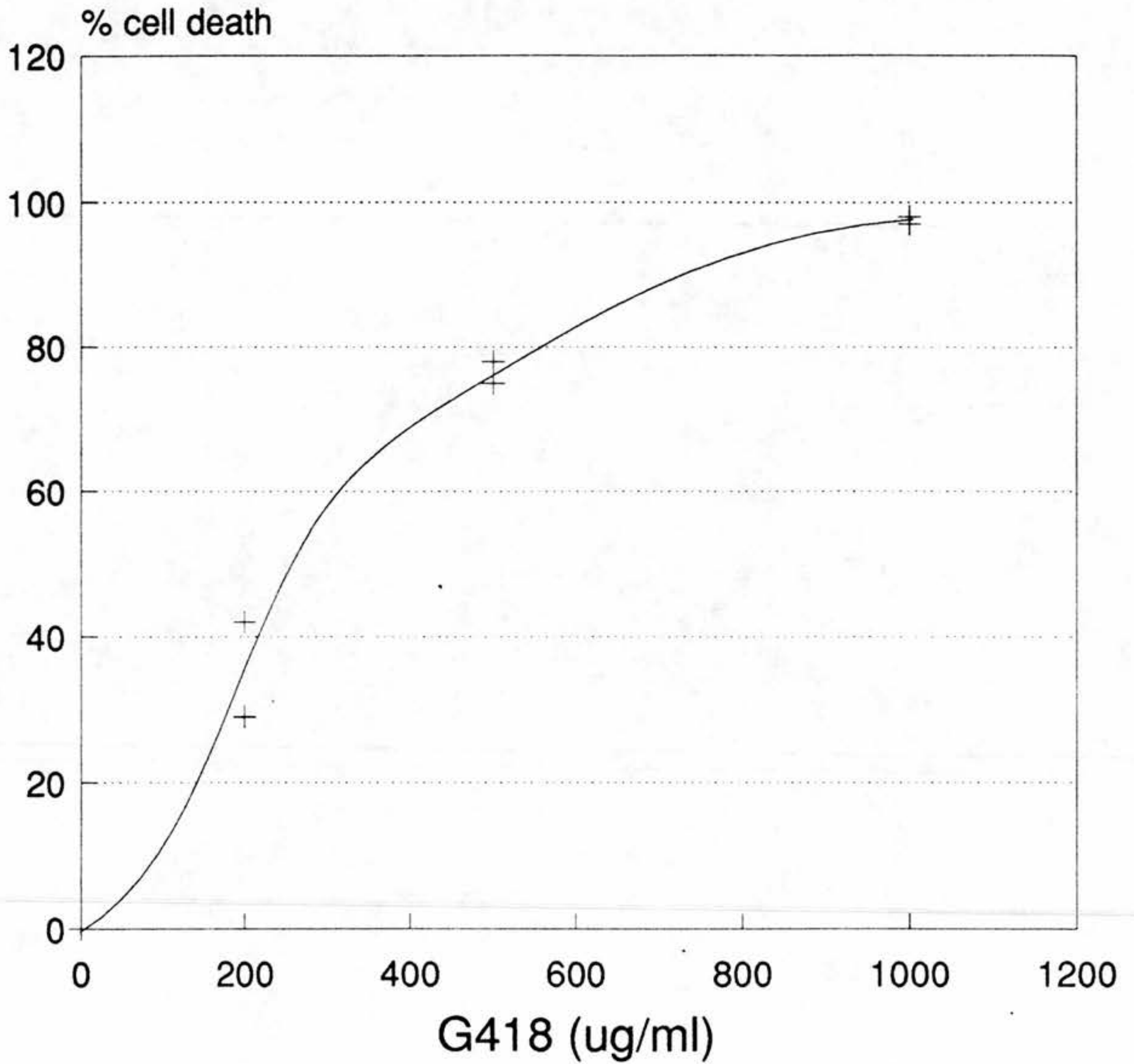
Table 14 C280 electroporation

Number cells treated	plasmid (ug)	pulse (Kv)	Result
107	20	0.4	+
107	20	0.8	+
107	20	1.6	++
5x106	20	0.8	0
5x106	20	1.0	+
5x106	20	1.2	+
5x106	20	1.4	0
5x106	20	1.6	0
5x106	20	1.8	0
106	20	1.4	++
5x105	20	1.4	+
5x106	2	1.4	+
5x106	5	1.4	++
5x106	10	1.4	0
5x106	40	1.4	++

Notes:
1) Colonies were not counted but pooled since separate colonies often arose close together, or cells dispersed causing an apparent increase in colony number. Success was based on the speed of pooled colony growth and therefore indirectly on the number of transfectant cells.
2) 0= No transfectants, += growth, ++= rapid expansion
3) 25uF capacitance used throughout

All rapidly expanding transfections were effected with pulse voltages of 1.4 or 1.6 Kv and, although not discriminated in the table, transfection of only 5ug of

Figure 25



plasmid gave notably the best result.

That the plasmid DNA was successfully introduced into these cells was confirmed by Southern analysis. DNA from transfected cells was blotted from a gel with DNA from non-SV2neo transfectants and control plasmids. The blot was probed with pSV2neo, linearized with HindIII and ^{32}P labelled. Hybridising fragments were detected in the autorad only for G418 resistant cells and control plasmid.

5.3.1.2 G418 selection

Hygromycin resistance was con sidered as a dominant selectable marker but, in preliminary tests, the new cultures were particularly sensitive to low concentrations (50ug/ml), and the preferred marker was neomycin resistance. In order to find the appropriate G418 concentration for selection of a clonable colorectal cell line, 10^6 C280 cells were seeded in each of a series of 32x 50mm diameter Petri dishes, variously treated with 200ug/ml, 500ug/ml, 1000ug/ml G418, or untreated as control. All were grown in 10% HINCS and kept in a CO2 humidified incubator. Dishes were removed at 3 day intervals over a period of 15 days and cells were counted, in duplicate, in a glass slide haemocytometer. For each G418 concentration the number of cells remaining at day 9 was used to calculate percentage cell death relative to the untreated control. The resulting dose response curve is plotted in figure 25. From this curve, the minimum concentration of G418 to kill all cells (and

thus the appropriate concentration for selection), is approximately 1mg/ml, active G418 concentration 500ug/ml according to the batch potency of 500ug/mg (chapter 7).

5.3.2 Transfection efficiencies

A number of test transfections were carried out in order to compare efficiencies by different methods and of various recipient cells. Cells were treated by either electroporation or one of two commercial reagents for amphiphile-mediated transfection. Results are summarized in table 15.

Table 15 Transfection efficiencies

cells	method	vector	effic.	comments
PCAA	EP	pHO5T1	0	high cell kill, non-sel
PCAA	EP	pKrev-1	0	high cell kill, non-sel
PCAA	TRANSF	pHMR	0	non-sel
PCAA	LIPOF	pKrev-1	0	non-sel
PCAA	LIPOF	pSV2neo	0	non-sel
JOMCL	LIPOF	pKrev-1	0	non-sel
JOMCL	LIPOF	pSV2neo	0	non-sel
C280	EP	pSV2neo	+	confirmed (see text)
C280	TRANSF	pKrev-1	10^{-6}	growth arrested (text)
C280	TRANSF	pHO5T1	10^{-5}	(see text)
C280	TRANSF	pSV2neo	$6x10^{-6}$	
3T3	TRANSF	pSV2neo	$1.5x10^{-6}$	

Notes:
1) EP= electroporation
2) TRANSF= amphiphile mediated transfection using TRANSFECTAM reagent (IBF, Columbia, USA)
3) LIPOF= lipofection using LIPOFECTIN amphiphile reagent (GIBCO BRL, Paisley, Scotland)
4) non-sel= non-selectable
8) effic.= transfection efficiency. Number of transfectant colonies divided by number of cells originally transfected
9) += transfectant colonies not counted but pooled
10) pHO5T1; expressing activated _H-ras_ (Spandidos and Wilkie 1984), pKrev-1; (Kitayama et al 1989), pHMR; resistance gene to hygromycin B, (Bernard et al 1985).
11) Negative controls lacking construct were also transfected in every case

Transfection efficiencies for C280 cells were lower than expected comparing poorly with classical methods

such as calcium phosphate procedures where efficiencies of 10^{-4} are reported (Southern and Berg 1982). Efficiencies of 10^{-3} are commonly reported for amphiphile reagents. Efficiencies of C280 transfection compared favourably, however, with control murine 3T3 transfections for which high efficiency transfection is commonly reported. Low efficiencies in our experiment are not therefore due to colonic lines being refractory to transfection but indicate the need for finer tuning of parameters in transfection experiments.

No transfectants were selected in the non-clonable colonic lines PCAA (chapter 3) and JOMCL (chapter 4). Inability to grow from single cells would prevent selection of isolated transfectants and necessitate high efficiencies of transfection such that neighbouring cells may be transfected and selected together. In an attempt to meet this requirement, pHMR transfected PCAA cells were allowed to grow to passage twice over a period of 6 weeks before selection, in the hope that selectable clones of transfectants may grow. Despite a subsequent 3 month period of alternating selection and recovery with fresh feeder cell addition, a final 2 week selection period eliminated all growing colonies.

Despite low transfection efficiencies throughout this work there were a number of striking results of oncosuppressor gene transfer.

<u>5.3.3.1 Krev in murine cells: increased apoptosis</u>

5ug of pKrev-1 (Kitayama et al 1989) were

Figure 26 Morphology of early Krev transfectants,
A; Early Krev transfectants forming clumps of cells (x108),
B; Non-transfectants at a similar density showing no clump formation (x69).

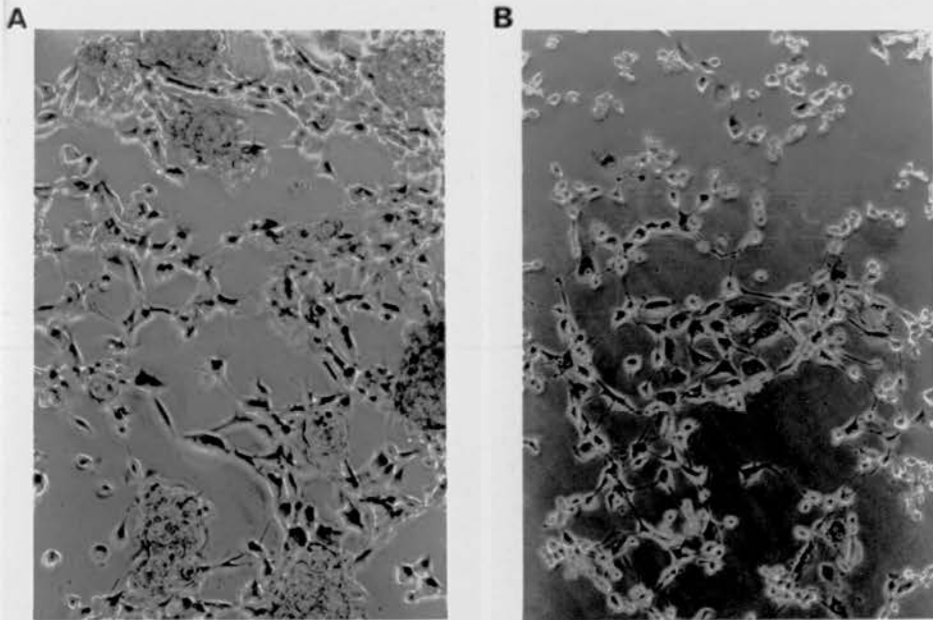


Figure 27 Contact inhibition of Krev transfectants (both x69),
A; Krev transfectant monolayers showing contact inhibition,
B; Monolayer behaviour of non-transfectant parental line showing focus formation.

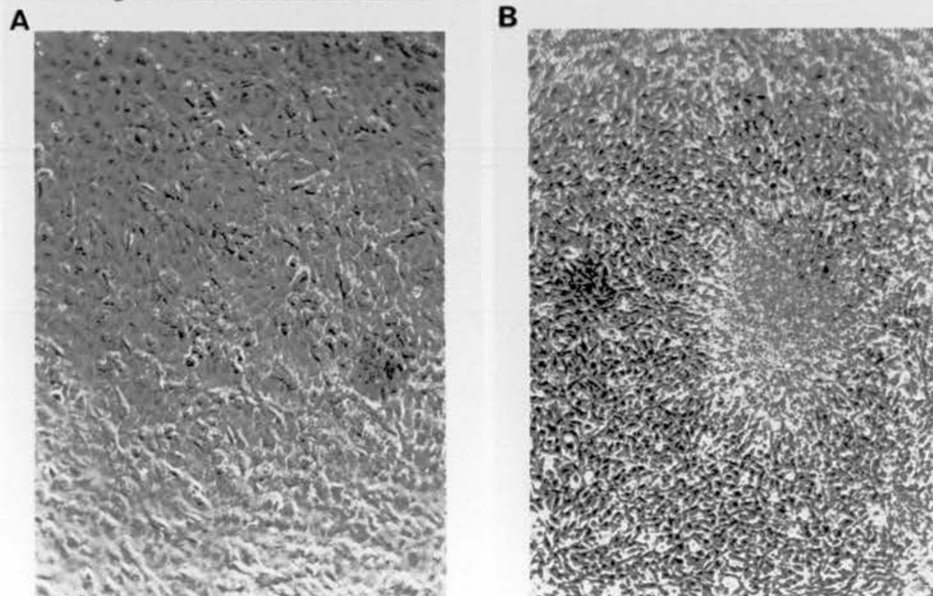
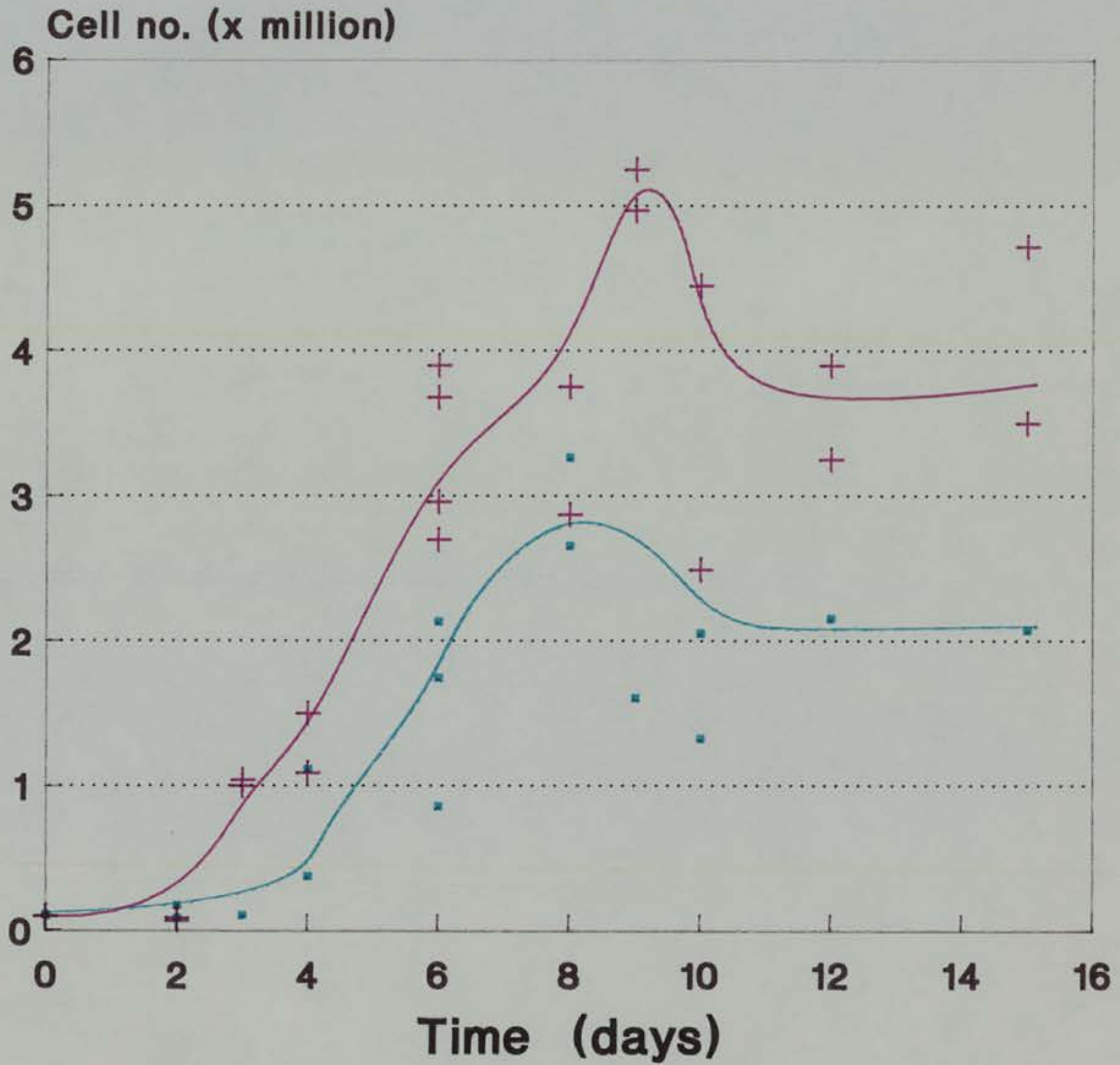


Figure 28



transfected by electroporation into $8x10^5$ DABU transformed murine fibroblasts (chapter 4). An _aph_ gene also expressed from pKrev-1 allowed G418 selection of _Krev_ transfectants. Early transfectants adopted a clustering behaviour with cells growing in clumps, while the parental line at a similar density did not (figure 26). Due to slow growth, transfectant clones were pooled and grown up as a mass culture which required 2 months to grow to passage in contrast with the parental line which was generally passaged twice a week. Monolayer contact-inhibition observed for the transfectant line contrasted sharply with the behaviour of parental cells at confluence, which grew over each other forming large transformed foci (figure 27). Numerous highly refractile rounded cells budding off into the medium were also noted in confluent transfectant cultures, yet were not evident in the parental line. Cells collected from the supernatant of the transfectant line were shown to be apoptotic: of 100 cells counted in wet preparations stained with 10ug/ml acridine orange and viewed in ultraviolet light, 86% demonstrated the characteristic homogeneous nuclear staining and fragmentation.

5.3.3.2 Krev: growth inhibition

The growth rates of the parental and transfectant lines were compared. 50mm Petri dishes were each seeded with 10^5 cells from either line, were fed 10% HINCS at 3 day intervals and grown in a CO2 humidified incubator. Cells were harvested and counted in quadruplicate at

either 2 or 3 day intervals over 15 days. Cell numbers were plotted against time to give comparable growth curves (figure 28).

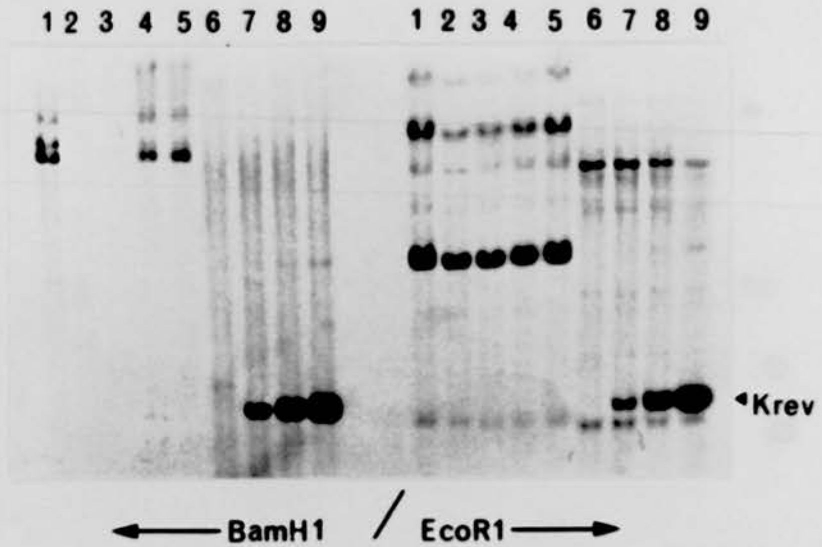
A 2-fold increase in doubling time, and a 2-fold decrease in the plateau phase monolayer density was noted for the Krev transfectants. During growth in log phase (at day 6) the numbers of Krev transfectants were significantly less than those of parentals ($P=0.028$, Mann-Whitney U test). Similarly, the plateau phase concentration (days 10-16) was significantly lower in Krev transfectants ($P=0.005$, U test).

5.3.3.3 Krev: abolished tumorigenicity

In order to test tumorigenicity, $2x10^6$ Krev transfectants were injected either subcutaneously or intra-peritoneally into each of 21 immunosuppressed mice. A further 19 immunosuppressed mice each received $2x10^6$ parental cells. Mice were killed at 21-25 days after injection and all animals were dissected and examined for abnormalities in the peritoneal cavity, heart, liver and lungs. At postmortem, competence of thymectomy was confirmed in each mouse.

The parental cell line consistently induced aggressively growing fibrosarcomas with infiltration at tumour margins and multiple retroperitoneal secondary tumour masses after intraperitoneal injections. Tumours were never detected after injection with transfectant cells and only fat necrosis was found at the site of injection. As a stringent test of differential

Figure 29 Southern blot probed with Krev



Notes:
1= 3T3 mouse fibroblast cell line DNA
2= Spontaneously transformed 3T3 parental cell line
3= 3T3 SV2neo transfectants
4= Transformed line Krev transfectants
5= Transformed line Krev transfectants
6= Human placental DNA
7= Human placental DNA + Krev plasmid equivalent to 1/3 copy/ cell
8= Human placental DNA + Krev plasmid, 1 copy/ cell
9= Human placental DNA + Krev plasmid, 3 copies/cell

tumorigenicity of these 2 lines, subcutaneous injections of both lines were given to opposing flanks of the same mouse in 3 cases. In all 3, tumours were found only in the flank injected with parental cells. Results are summarized in table 16.

Table 16 Tumorigenicity of parental vs. Krev transfectant cells

	i/p	s/c	
Parental	9/9	10/10	
Transfectant	0/10	0/11	($P<0.001$)

Notes:
1) i/p= intraperitoneal
2) s/c= subcutaneous
3) numbers= animals with tumours/ animals injected

5.3.3.4 Krev: instability

Transfectant cells recovered poorly from liquid nitrogen storage and required expansion in the absence of selection, whereupon cell morphology was neither flat nor transformed, and apoptotic bodies were not apparent. In order to ensure presence of only transfectant cells, selection could have been re-applied at this point. However, without further selection DNA was prepared from these cells for Southern analysis. 20ug of DNA from 3T3 cells, the transformed parental line, 3T3 SV2neo transfectants and transformed cell *Krev* transfectants were digested with each of BamH1 or EcoR1. Mixtures of pKrev-1 and digested human placenta DNA equivalent to 0, 1/3, 1 and 3 copies of *Krev* /cell were also included in the blot which was probed with the 2Kb BamH1 fragment of pKrev-1, random prime labelled with radioactive phosphorus.

The pattern of hybridisation with the _Krev_ cDNA probe for both mouse and human derived cells shows the expected small number of endogenous _Krev_ alleles (figure 29) suggesting low gene copy number and strong conservation (Kitayama et al 1989). However, for neither of the transfected DNAs was the pattern different from non-_Krev_ transfected controls. Although this demonstrates that exogenous _Krev_ was not present in these cells recovered from deep frozen storage, it is likely that the _Krev_ DNA was present in the original transfectants, as they grew selectively in G418 and demonstrated a clear phenotype. It appears probable that _Krev_ has been lost due to the non-maintenance of selection. This interpretation suggests instability of exogenous _Krev_ which may be expected since the _Krev_ DNA is associated with an obvious growth disadvantage. Such instability might indicate the need for sustaining selection in oncosuppressor transfections.

5.3.4 Krev in human cells: growth arrest

5ug of either pKrev-1 or the activated _H-ras_ containing pHO5T1 (Spandidos and Wilkie 1984) were transfected into 10^6 C280 cells by amphiphile-mediated transfection. 10 pHO5T1 transfectant colonies were counted after 6 weeks of selection, giving a transfection efficiency of 10^{-5}. At that time, only one living Krev transfectant colony was visible. This single colony was, however, surrounded by many skeleton colonies consisting only of dead cell remains organised in a colonial

fashion. The single *Krev* transfectant colony survived selection over one further month yet remained static. No further growth was noted before the colony eventually detached from the substratum, and was thereafter resistant to repetitive attempts at reseeding.

5ug of pKrev-1 or pSV2neo were introduced into non-clonal human colonic epithelial lines PCAA and JOMCL by amphiphile-mediated transfection. Since, the former line carries a *Ki-ras* mutation yet is non-tumorigenic, while the latter carries only wild-type *Ki-ras* and produces tumours, effects of *Krev* may have been highly informative. However successful control transfectants were not selected as already discussed (5.3.2), and the experiment was deemed inconclusive.

5.4 Discussion

5.4.1 Transfection efficiency

Efficiencies of amphiphile-mediated transfection measured in this study were in all cases 100-1000 times less than expected. Conditions were not optimised prior to these experiments and by tuning parameters such as ratio of DNA: reagent and time in contact with the cells, great improvements may be achievable. Such improvements may lead to transfection of nei ghbouring cells allowing selection of small co-transfected colonies in cell lines which do not grow from single cells.

Despite low transfection efficiencies a number of interesting effects were observed and new questions raised by these experiments.

5.4.2 Krev is an oncosuppressor gene

Krev was isolated from a human cDNA library on the basis of its ability to induce morphological reversion of a transformed murine cell line. Loss of tumorigenicity, observed in original revertants, was not absolute on secondary transfection with the recovered *Krev* cDNA alone and the extent of its suppressive effects were unclear (Kitayama et al 1989). In this chapter, complete loss of tumorigenicity together with many other predicted oncosuppressor gene effects are demonstrated specifically by *Krev* DNA.

A tumour-specific decrease in *K-rev* expression has been detected in 2 tumour types where *K-ras* mutations are not detected (Culine et al 1989). According to the

oncosuppressive capacity of K-rev and its proposed antagonism to Ki-ras, it is possible that the effects of ras activation in human tumours may alternatively be exacted by Krev inactivation. This may explain the fact that Ki-ras activating mutations are detected in only 37% of colorectal tumours (Bos et al 1987). Perhaps defects in the Krev gene are involved in some of the remaining 63%.

5.4.3 K-rev oncosuppressor effects

Many of the possible oncosuppressor effects discussed in the introduction to this chapter were evident following transfection of the Krev gene. Loss of tumorigenicity, decreased growth rate, growth arrest and induction of apoptosis were all observed. Different effects were observed for expression in a human colonic epithelial line than in a transformed murine line. No phenotypic effects were recorded in an earlier study in which Krev was transfected into non-transformed murine cells (Kitayama et al 1989). Such variable effects emphasise the necessity for multiple characterized lines providing a variety of backgrounds in which to assay oncosuppressors.

The balance between cell division and cell death by apoptosis determines whether tumour growth, stasis or regression occurs (Arends and Wyllie 1991). By measuring apoptosis and mitosis in tumours produced by ras and myc transfectant rodent fibroblasts, it has been suggested that ras may inhibit cell death by apoptosis as well as

inducing proliferation (Wyllie et al 1987). In view of the hypothesised antagonism of Krev to ras, it is therefore interesting to note induction of apoptosis by Krev in this study. Induced apoptosis may diminish the ability of Krev transfectants to produce tumours, or lead to the loss of viability observed in C280 Krev transfectants.

Fin ally, no insight into oncosuppressor modulation of responses to cell-cell growth control signals could be obtained owing to the inability to select transfectants from non-clonable cells. Such cells maintaining cell contacts may, in more efficient transfections, prove instrumental in the analysis of oncosuppressor effects on cell signalling.

5.4.4 Prospective oncosuppressor assays

The methods used to observe the effects of oncogenes *in vitro* are clearly insufficient for thorough analysis of the effects of oncosuppressor genes. The main problem in designing experiments that involve transfer of oncosuppressor genes is the need to overcome growth inhibition (in order to expand the transfectants) without selecting against the transferred gene (Sager 1986).

One possible solution may be the alternation of periods of selection for cotransfected selectable markers with periods of negative selection with 5-bromodeoxyuridine and UV to kill growing cells. Negative selection, however, requires oncosuppressor bearing transfectants to grow less well *in vitro* than parental

cells, which is not necessarily the case.

Oncosuppressor inducibility would prove a valuable asset allowing expansion of oncosuppressor bearing transfectants without constitutive inhibitory effects. Such inducibility would be absolutely necessary to allow study of genes whose expression results in complete growth arrest. The fortuitous existence of a temperature sensitive p53 mutation permits tight control of mutant expression (Michalovitz et al 1990), and this gene is therefore particularly suited to regulable gene assays. Although conditional mutations may be discovered for other oncosuppressors, in the meantime, their controlled expression from an inducible promoter may prove valuable. Inducible elements permitting tight regulation of expression in vitro include heat-shock promoters and the MMTV LTR (mouse mammary tumour virus long terminal repeat) 5' control region (Lee et al 1981).

Finally, use of antisense RNA or other specific antagonist could be used to prevent constitutive expression effects of an oncosuppressor. Useful antagonists would include conditional mutant (and therefore regulable) oncogenes acting downstream of the oncosuppressor. On removal or silencing of antagonist, after selection and expansion of oncosuppressor bearing transfectants, the oncosuppressor gene expression would resume and its effects could be observed.

Chapter 6: Concluding discussion

Xenograft resources described in preceding chapters represent a valuable system amenable to widespread exploitation.

6.1 Tumour classification

By virtue of the fact that renewable stable xenograft resources allow unlimited genotypic and phenotypic characterization of individual tumours, di fferent types of tumours can be identified. A diploid DNA status has previously been correlated with a low incidence of p53 mutations (Remvikos et al 1990), which in turn correlates with a right-sided location and mucinous histology (Campos et al 1991); while statistically significant associations are reported of allelic losses with a left-sided location and the absence of extracellular tumour mucin (Kern et al 1989). Tumours within the xenograft set support all these correlations suggesting the existence of 2 genetically distinct classes of tumours.

The classification of many tumour xenografts into a group rarely exhibiting colon-associated genetic changes demonstrates the incomplete nature of the linear genetic model for tumour progression (figure 4), implying the existence of divergent pathways.

6.2 Suggested roles for genes

Under-representation of p53 mutated tumours in CBA mice, and to a lesser extent in SCID mice may reflect immune rejection. The therapeutic implications of immunogenicity specifically associated with the mutated

form of p53 would be immense both for preventative and corrective cancer treatment.

The lack of linkage between 17p allele loss and p53 mutations in tumour xenografts may result from selection pressures within this set but nonetheless, implies independent effects of these 2 events. Furthermore, despite the reported tight linkage in colon cancers (Baker et al 1990a), lack of linkage has been observed in large numbers of unselected breast tumours (Coles et al 1990), and a similar observation has been made in the primary colorectal tumours gathered by our group (C Purdie, unpublished results). It is plausible that the high frequency of 17p allele losses in unselected tumours make their detection common in association with p53 mutations, but that linkage is not necessary.

The class of tumours sustaining colon-associated genetic lesions may be selected against in CBA hosts due to immunogenicity of p53 or slow growth. The more severe immune deficiency of SCID hosts seems, however, to allow indiscriminate xenograft growth. More lesions at key loci may therefore be detected in sets of xenografts in SCID mice. The prominent nature of co-operative defects at these loci in widespread situations was emphasised by their specific involvement in colorectal progression in vitro.

The demonstration, also in vitro, of a specific APC and MCC allele imbalance during colorectal tumour cell progression implies a dominant negative effect for one or

both of these genes. Since functions are known for neither of these genes but the majority of mutations detected to date are expected to annul protein function, this putative functional mutation may provide vital leads to future analysis.

6.3 Sequence analysis

By providing stable renewable resources of tumour without human stroma, the xenografts will be vital for sequence analysis. Together with the available paired normal DNAs, xenografts will facilitate PCR identification of tumour specific sequence differences, in particular of DCC, MCC, and APC genes. Furthermore, the same virtues certify xenograft s as valuable resources (in conjunction with constitutional DNA resources) for unlimited search in candidate tumours, for the genetic defect or defects and further somatic changes responsible for hereditary non-polyposis cancers.

6.4 Oncosuppressor assays

Oncosuppressor gene assays on the variety of known genetic backgrounds provided by xenografts will supply vital information on the effects of co-operating loci.

As well as proposed introduction of wild-type oncosuppressor genes, assays of other tumour-associated genes and their combinations will be informative. Expression of an exogenous mutated copy of a gene known to be unrearranged in the recipient cell would allow investigation of dominance and simulation of in situ events in tumorigenesis. Large numbers of possible

transfection experiments exist according to the variety of backgrounds available, assays of wild-type or mutant genes and double transfections with pairwise combinations of exogenous genes. Besides generating valuable information on gene co-operation, knowledge would be gained of both intra- and inter-gene dominance.

More specific transfections in which selected wild-type oncosuppressor genes are disabled by gene targetting will also be possible. By disabling firstly one allele and observing the phenotype and then inactivating the second allele (Riele et al 1990) precise information on dominance and gene effects would be obtained. Specific correction of defects may also be possible by similar methodology but using replacement vectors.

Less technically demanding experiments which may also generate very useful data would be the construction of somatic cell hybrids. The fusion of cells from the variety of lines available may indicate the existence of complementable defects not easily otherwise predicted. Cell fusions from lines carrying known differences at tumour-associated loci would supply information about both dominance and the effects of various combinations of lesions.

Chapter 7: Materials and methods

7.1 Routine tissue culture

The conditions described below were applied to the routine culture of all established lines other than the adenoma derived colorectal line PCAA. This and primary colorectal cultures required different conditions, described in 7.2.

7.1.1 Medium

340ml Deionised distilled water
40ml x10 concentrated Glasgow minimum essential medium
8ml 0.2M L-glutamine
8ml 1M $NaHCO_3$
0.8ml Penicillin/Streptomycin (60,000 Units/mml, 60,000 ug/ml)

pH to 7.2 with 5M NaOH.

Water was pre-sterilized by autoclaving, medium concentrate was bought sterile and other components were sterilized by 0.2um filtration. A sample of prepared medium without antibiotics was always tested for sterility before use.

7.1.2 Serum

All serum (Gibco or Northumbria biologicals Limited) was batch tested for the ability to sustain growth of routinely grown cell lines, and was heat inactivated and tested for sterility before use. Heat inactivated newborn calf serum (HINCS) or foetal bovine serum (FBS) was added to culture medium to give a final concentration of 10% unless otherwise stated.

7.1.3 Trypsin and EDTA

0.02% EDTA

355ml autoclaved deionised distilled water
40ml x10 PBS (Northumbria Biologicals Ltd.)
4ml 2% EDTA (autoclaved)

Sterility was tested by incubation of a few drops in medium without antibiotics.

0.1% Trypsin

Powdered trypsin (DIFCO) was prepared as a 0.1% solution in PBS, filter sterilized into 25ml aliquots and frozen until use.

TrypEDTA

20ml of 2.5% trypsin in PBS
10ml of 2.0% EDTA in PBS
270ml PBS

Filtered into 20ml aliquots and stored frozen.

7.1.4 Feeding and passaging

Cells were all fed twice weekly by replacement of used medium with the same volume of fresh medium, unless passage was required due to confluence. 25cm^2 flasks were always fed with 4 ml of medium, 75cm^2 with approximately 25ml, and 150cm^2 with approximately 40ml. All plastic-ware was bought sterile from Costar. Cells were routinely passaged by discarding medium and passing an equal volume of 0.02% EDTA over the monolayer. As EDTA chelation of Ca2+ and Mg2+ caused cells to round up, the EDTA was discarded and 4ml of 0.01% trypsin added.
Alternatively, some cells were harvested in one step by addition of 4ml TrypEDTA and incubation at room temperature for 2-5 minutes. With gentle agitation the cells were washed from the surface of the flask and could be pelleted or divided as required. The desired proportion of cells were reseeded and when placed in a new flask, cells were gassed in 5% CO2 in air via a pasteur pipette.

7.1.5 Freezing

Monolayers were routinely harvested and trypsin was inactivated by adding medium containing serum. Cells were counted, using a glass slide haemocytometer, pelleted by centrifugation at 1000rpm for 5 minutes, and resuspended in a 1:1 mixture of 20% DMSO (dimethyl sulfoxide) and HINCS or FBS, at a cell concentration of 5x10^6-10 /ml. 1ml aliquots were placed in polypropylene Bio-freeze vials (Costar) at -70° in a plastic rack overnight, after which vials were transferred to liquid nitrogen. To thaw, vials were removed from liquid nitrogen and thawed rapidly at 37°C. DMSO was washed from the cell suspension by spinning at 1000 rpm for 5 minutes in medium containing 10% serum, and cells were seeded into 25cm2 flasks in 4ml of fresh medium.

7.1.6 Mycoplasma tests

Cells were routinely tested for the presence of mycoplasma by incubation of 0.1x10^6 cells in 4ml medium with serum at 37°C in a 50mm petri dish in a CO2 incubator for 4 days. Medium was then discarded and cells fixed twice in 3 parts methanol: 1 part acetic acid for 5 minutes each time. The dish was inverted and allowed to dry and cells were then stained with 4ml of 10ug/ml Hoechet 33258 dye in PBS for 10 minutes. After washing

the dye off with PBS, a coverslip was placed over cells for viewing under an UV microscope.

7.2. Culture of colonic epithelial cells

The following special methods were applied to primary colorectal tumour cultures and the colorectal line PCAA (Paraskeva et al 1984).

7.2.1 Colonic culture medium

Conditioned medium was obtain ed by feeding confluent Swiss 3T3 feeder cells 24 hours before pass, and retaining this conditioned medium at pass.

200ml conditioned medium (sterilized by 0. 2um filtration)
250ml Glasgow medium, prepared (section 7.1.1)
0.5ml Hydrocortisone stock solution 1mg/ml
0.5ml Insulin stock solution 200 Units/ml
50ml HIFBS

A sample of all media was sterility tested by incubation for a few days in a 37°C incubator prior to use.

7.2.2 Collagen coated flasks

Calf thymus collagen type I (Boehringer) was dissolved in 1:1000 acetic acid: water to give a final concentration of 0.8mg/ml, and was stored at 4°C. Collagen type IV (Sigma) was dissolved in 1:400 acetic acid: water to a final concentration of 0.5mg/ml. Up to 5 hours were often required at 4°C or on ice to allow the collagen to dissolve completely. To coat flasks, 2-4ml of cold collagen was drawn from the solution through a sterile 16 gauge syringe needle and displaced onto the flask surface under sterile conditions. The solution was spread over the entire flask surface by gentle agitation and all excess liquid was drawn off for re-use, via the syringe and needle. Flasks were left in a 37°C room with loosened lids overnight or until dry, sterilized overnight under UV, and stored at 4°C until use. After warming to room temperature, flasks were rinsed in wash solution (section 7.3) to remove all traces of acetic acid before seeding cells.

7.2.3 Feeder cells

Early passage 3T3 cells were split once a week and an extra 75cm^2 flask was set up for mitomycin C mediated mitotic arrest. 24 hours after reaching confluence, medium was replaced with 25ml of fresh medium containing 10ug/ml mitomycin C (stored at 100x concentration in 250ul aliquots in PBS at -70°C). The flask was gently rotated to ensure complete coverage with the poison, and

incubated at 37°C for 2-3 hours. This medium was discarded into a chlorine solution as was all Mitomycin C apparatus, to soak overnight before discarding or washing. The treated monolayer was then washed 3 times in PBS before harvesting in the normal way and resuspending in medium with 10% serum at a concentration of 10^6 cells/ml. These cells were stored in a plastic universal at 4°C for up to 7 days, and added dropwise to cultures as required.

7.2.4 Feeding and passaging

Colonic epithelial cells were fed according to routine methods other than over the first 10 days after seeding or passaging. During this period, it was often necessary to spin used medium for 5 minutes at 1000rpm to retain viable cell aggregates which had not yet become attached. Once pelleted, these aggregates were resuspended in 4ml of fresh medium and were transferred back into the flask. At each feed flasks were checked for an adequate feeder cell content and feeders were added where necessary. Cells were harvested for passage by incubation at 37°C for 20-40 minutes in dispase (sterile grade 1, Boehringer), a neutral protease from Bacillus polymixa. 5mg vials were dissolved in 15ml of colonic culture medium with serum, and 2.5ml of this final solution at 2 Units/ml were used for each passage. Cells became detached as sheets leaving fibroblasts still attached, and could be resuspended into small clumps of cells by gentle repeated flushing over the flask surface with a sterile fine bore tip on a 5ml Finnpipette. The dispase was washed out by centrifugation in medium with serum (1000rpm, 5 minutes), and cells were resuspended and seeded at the required split ratio.

7.2.5 Freezing

Epithelial cells were harvested as for passage (above) except that the cell pellet was resuspended in DMSO: serum (1:1) and frozen as described in section 7.1.5. Since cell number in the harvested cell clumps was not easily counted, the adopted convention was to freeze the cells of 1x 25cm^2 flask in each 1ml of freezing medium.

7.2.6 Mycoplasma tests

For slow growing primary cultures it was not always possible to harvest cells for the described method of test (section 7.1.6). Accordingly medium from these cultures was poured onto preparations of other cells known to be mycoplasma free, and the test could be carried out as described.

7.3 Seeding primary cultures

Maximum amounts of tissue in sterile PBS on ice were taken to a class II safety cabinet. The PBS was poured off into a waste flask and the tissue mass was washed twice in wash solution and deposited in a sterile plastic petri di sh in a small pool of fresh wash solution. The tissue was then broken down as much as possible by repeated slicing with 2 sterile scalpels. This suspension was transferred to a plastic 20ml universal and spun for 5 minutes at 1000rpm in wash solution. The supernatant was discarded and the pellet resuspended in 4ml of primary culture medium and seeded in 25cm^2 collagen coated flasks with 8 drops of feeder cells from a sterile pasteur pipette. Cells were gassed with 5% CO_2 in air supplied from the gas cannister via a fine rubber hose with a pasteur pipette attached, and flasks were kept in a 37°C room.

Wash solution

400ml Glasgow medium, prepared (section 7.1.1)
1ml Stock penicillin /streptomycin
(60,000 U/ml, 60,000 ug/ml)
10% FBS (section 7.1.2)
5ml 2M Hepes (Sigma)

7.4 Fibroblast elimination

7.4.1 Differential settling

Some contaminating indigenous fibroblasts were discarded at initial feeds during the first 10 days of primary culture. Before floating cell aggregates were centrifuged to prevent loss during feeding (section 7.2.4) differential settling of the spent medium was allowed in order to retain heavier particles (cell aggregates) only. Discarded medium was placed in a 20ml plastic universal which was topped up with wash solution (7.7.1). The mixture was shaken and allowed to settle for 10 minutes before using a fine bore tip on a 5 ml Finnpipette to draw off the maximum amount of medium without disturbing the settled pellet. The pellet was then resuspended in wash solution and spun for 5 minutes at 1000 rpm to allow re-seeding.

7.4.2 Differential trypsinisation

Short, repeated treatments with a trypsin solution were used to remove fibroblasts which outgrew from small blocks of tissue after epithelial cell attachment to the substratum. Medium was discarded and replaced with 2.5ml of TrypEDTA solution and flasks were incubated at 37°C for 2-3 minutes. TrypEDTA solution was then discarded and replaced with fresh medium as in feeding.

TrypEDTA

20ml of 2.5% trypsin in PBS
10ml of 2.0% EDTA in PBS
270ml PBS

Filtered into 20ml aliquots and stored frozen.

1 week was allowed for recovery from trypsinisation before repeating the treatment if necessary.

7.5 Carciinoma specimens

Surgical colorectal re section specimens bearing carcinomas were received directly from the operating theatre. These were transported on ice to the trimming room where they were rinsed with cold phosphate-buffered saline or PBS (Northumbria biologicals Ltd.) A section of tissue selected on the basis of viable appearance was removed to wash solution in a 20ml plastic universal.

7.6 Animals

CBA mice were immunosuppressed by thymectomy followed by cytosine arabinoside injection and whole body irradiation (Steel et al 1978). Both immunosuppressed CBA and congenitally immunodeficient nudes (nunu, OLAC or B&K, London) were housed within a laminar flow unit which supplied a constant stream of filtered air. All cages were autoclaved before use and drinking water contained 3ml of 0.01M HCL /50ml bottle. SCID mice were housed within an isolation unit where disinfection barrier regulations were observed and all food and water was autoclaved.

7.7 Xenografts

7.7.1 Wash solution

400ml Glasgow medium, prepared (section 7.1.1)
1ml Stock penicillin /streptomycin
(60,000 U/ml, 60,000 ug/ml)
10% FBS (section 7.1.2)
5ml 2M Hepes (Sigma)

7.7.2 Dorsal xenograft

Portions of carcinoma in wash solution were transported on ice to a class II safety cabinet and washed twice in fresh wash solution before slicing with sterile scalpels into portions no greater than 0.5cm cubed. These portions were kept in ice-cold wash solution until xenografted.

Pairs of CBA or nude recipient mice were anaesthetised with diethyl ether for each xenograft implantation, and the operation was carried out on an

alcohol sterilized area of bench. SCID mice were anaesthetised with a 1:1 mixture of Hypnorm (Janssen) and Hypnovel (Roche) anaesthetics, diluted to a third of the total concentration in deionised distilled water. 0.1ml of anaesthetic/10g mouse was administered intraperitoneally and after 10 minutes the anaesthetic effect was tested by response to tail-pinching. All SCID operations were carried out in the sterile environments either of the isolator or of a laminar flow cabinet. Cages of animals were transferred between the two in autoclave bags containing sterile air and disinfection barrier regulations were observed.

All anaesthetized mice were prepared for operation by alcohol cleansing of the fur. Using sterile scissors and forceps, a small dorsal incision parallel to the backbone was made through the skin of each animal. One tumour portion was implanted into the subcutaneous space on each side of the incision and wounds were closed with sterile 12mm skin clips (Avlox), or sewn in 2 stitches using a sterile silk suture with needle attached (Ethicon). All xenografts were implanted within 60 minutes of original colorectal resection.

7.7.3 Tissue injections

Portions of carcinoma were collected and washed as for xenografted tissues, but slicing with scalpels was continued until a particulate solution was produced which could be drawn into a 16 gauge syringe needle. This was pelleted by centrifugation at 1000rpm in a bench centrifuge for 5 minutes before resuspension in 0.1ml-0.3ml of wash solution for each injection. Injections were given without anaesthetic. Subcutaneous injections were administered to the inner left flank unless otherwise stated and intraperitoneal injections were directed just right of the centre of the abdomen.

7.7.4 Cell injections

Cells were routinely harvested, washed twice in PBS, and counted using a glass slide haemocytometer. Cells were resuspended in cold PBS on ice at a known concentration allowing 10^6-10^7 cells in 0.1-0.3ml volume for each injection. Cultured cell suspensions were injected via a 25 gauge microlance needle.

7.7.5 Serial xenografting

As tumours reached an externally visible diameter of approximately 1cm, or if poor health was observed, animals were killed by cervical dislocation. At dissection tumour masses were freed and removed from surrounding tissues to PBS where approximate sizes were recorded. After trimming away necrotic tissues with a sterile scalpel, remaining tumour was divided for transplantation and analysis. New recipients were

implanted following the method for initial implantation, within 10 minutes of tumour removal.

7.8 Tissue freezing

Maximum amounts of excess viable tissue from xenograft passage were taken in sterile PBS on ice to a class II safety cabinet. The PBS was poured off into a waste flask and the tissue mass was washed twice in wash solution and deposited in a sterile plastic petri dish in a small pool of fresh wash solution. The tissue was then broken down as much as possible by repeated slicings with 2 sterile scalpels. This suspension was transferred to a plastic 20ml universal and spun for 5 minutes at 1000rpm in wash solution. The supernatant was discarded and the pellet resuspended in 10% DMSO: FBS (1:1). Cell concentration was unknown but excess tissues from an average sized tumour xenograft were generally frozen in a volume of 2 ml. 1ml aliquots were placed in polypropylene Bio-freeze vials (Costar) at -70° in a plastic rack overnight or for up to 3 days. At this time vials were transferred to liquid nitrogen for storage. To thaw, vials were removed from liquid nitrogen and thawed rapidly at 37°C. DMSO was washed from the cell suspension by spinning at 1000 rpm for 5 minutes in wash medium, and cells were either seeded as primary cultures (section 7.3), or injected into animals (section 7.7.3).

7.9 Histology and immunocytochemistry

Representative samples of primary tumours and serial tumour xenografts were fixed in 10% buffered formalin for histology. Fixed tissues were processed in paraffin and sections were cut, stained with haematoxylin and eosin and mounted on glass slides.

Representative samples of primary tumours and serial tumour xenografts were fixed at 4°C overnight in periodate lysine paraformaldehyde dichromate (PLPD) for histology and immunocytochemistry.

For immunocytochemistry paraffin processed sections were blocked with normal rabbit serum diluted 1:5 in Tris buffered saline (NRS-TBS) and primary antibody was applied in 1:100 and 1:400 dilutions in NRS-TBS for 30 minutes. After rinsing and reblocking with NRS-TBS, biotinylated secondary antibody was applied in 1:400 dilution in NRS-TBS also for 30 minutes. Antibodies were detected by an avidin-biotinylated horseradish-peroxidase complex (ABComplex/HRP kit, DAKOPATTS). A preparation of the SV80 cell-line, known to over-express p53 protein (Harlow et al 1985), was also fixed in PLPD and included in each run as a positive control. To carry out p53 immunocytochemistry on cultured cells, 10^7 cells were routinely harvested and suspended in 1ml of 1% agar. After chilling at 4°C for 16 hours, the block was removed to PLPD and treated as above.

PLPD (Holgate et al 1986)

0.1M Lysine
0.1M Periodate
2% Paraformaldehyde
Made up to 50ml in Sorensens phosphate buffer (0.05M, pH 7.4)

Combine with 5% potassium dichromate in 50ml distilled water immediately before use.

7.10 Electron microscopy

For electron microscopy, small sections of xenografted tumour freshly excised from the animal were sliced separately with a fresh scalpel to 1mm cubes in gluteraldehyde fixative.

Gluteraldehyde fixative

3% gluteraldehyde in 0.1M Cacodylate buffer
Post-fixed in 1% osmium tetroxide in 0.1M Cacodylate buffer and embedded in araldite.

Representative areas of tumour were selected from 1u semi-thin araldite sections. Ultra-thin sections of chosen areas were secured on 0.35mm copper grids (old 400s, Athene) and viewed on a Joel 100S transmission electron microscope.

7.11 Flow cytometric analysis

7.11.1 Cell dispersal and staining (Vindelov et al 1983)

Portions of tissue of diameter less than 0.25cm were suspended in 200ul of citrate buffer and cut into small pieces using a sterile scalpel. 450ul of trypsin solution and 15ul of chicken red blood cells at 10^6/ml were added in an eppendorf tube and incubated for 10 minutes at room temperature mixing vigorously after 5 and 10 minutes. The same incubation process was carried out after the addition of 325ul of trypsin inhibitor/RNAase solution, and for a third 10 minute period on ice after addition of 250ul dye solution. The final mixture was filtered through nylon wool to remove all cell clumps.

Citrate buffer pH7.6

85.5g sucrose
11.76g trisodium citrate
Dissolve in 800ml distilled water
Add 50ml dimethyl sulfoxide
Make up to 1000ml with distilled water.

Stock solution pH7.6

2000mg trisodium citr ate
121mg Tris
1044mg spermine tetrahydrochloride
2ml nonidet P40
Make up to 2000ml with distilled water.

Trypsin solution

15mg trypsin in 500ml stock solution

Trypsin inhibitor/RNAase solution

250mg trypsin inhibitor
50mg ribonuclease A
500ml stock solution

Dye solution

208mg propidium iodide
500mg spermine tetrahydrochloride
500ml stock solution

7.11.2 Flow cytometry

Final nuclear suspensions were analysed on a Coulter epics CS flow cytometer at an excitation wavelength of 488nm. The chicken red blood cells served as an internal standard allowing the identification of the normal human diploid G0/G1 peak, since the DNA content of that of the chicken is about 35% of the human peak.

7.12 Genomic DNA extraction

At both original resection and most xenograft passages, portions of tumour approximately 0.25cm diameter were placed in sarstedt freezing vials and immersed in liquid nitrogen before subsequent storage at -70°C. Portions of normal colorectal mucosa from the tumour bearing patient were snap frozen in the same way, and peripheral blood was also taken from each patient as a second source of somatic DNA.

7.12.1 DNA from frozen tissue

Pieces of tissue approximately 0.25cm diameter were each sliced in 500ul TE-9, within a class 2 safety cabinet using sterile scalpels. This fine mince was transferred to an eppendorf tube and incubated at 48°C overnight in the presence of 1% sodium dodecyl sulfate (SDS) and 500ug/ml proteinase K (Boehringer) to allow cell lysis and protein digestion.

TE-9

500mM Tris pH 7.6
20mM EDTA

The resulting extract was mixed with an equal volume of TE saturated phenol, spun in a microfuge for 5 minutes and the top phase was removed to a clean eppendorf. This step was repeated twice, firstly with an equal volume of PC-9, and for the resulting top phase with chloroform: iso-amyl alcohol (24:1). DNA was precipitated in 2 volumes of absolute ethanol and 0.25 volumes of 7.5M ammonium acetate at -70°C overnight, and was collected by centrifugation for 15 minutes at 4°C in a microcentrifuge. Pellets were then routinely resuspended in 200ul of TE on ice. Optical density readings at 260nm allowed measurement of DNA concentrations which were then adjusted to 1mg/ml.

TE

10mM Tris pH7.6
1mM EDTA

TE saturated phenol

Equal volumes of phenol and TE were mixed, allowed to separate and top phase was discarded.

PC-9

480ml phenol
320ml TE-9
640ml chloroform

Mixed repeatedly allowed to settle and top phase discarded.

7.12.2 DNA from blood

Blood samples were collected into 0.2% EDTA to prevent coagulation. Whole blood was lysed in an equal volume of lysis buffer for 30 minutes at room temperature. This was mixed with an equal volume of TE saturated phenol and spun at 3000rpm for 20 minutes and the top phase was precipitated at -20°C overnight in 0.25 volumes of 7.5M ammonium chloride and 2 volumes of isopropanol. The pellet was air dried and subsequently treated as for frozen tissues from proteinase K digestion.

Lysis buffer

0.1M Tris pH8
20mM Sodium chloride
1mM EDTA
4% SDS

7.12.3 DNA from cultured cells

1 or 2 x $150cm^2$ flasks of cells were routinely harvested (sections 7.1 and 7.2) and pelleted. 500ul of TE-9 with proteinase K and SDS were added to the cell pellet following the method for tissues, and subsequent procedures for digestion and cleaning were as before.

7.13 Southern analysis (Sambrook et al 1989)

7.13.1 Genomic digests

10ug of each DNA sample were digested overnight under standard conditions, but in a minimum 2-fold excess of restriction enzyme and a total volume of 30ul. The following morning a further 1ul of enzyme was added to each digest and allowed a further 5-9 hours of incubation. Each digest together with 2 ul of loading buffer was then loaded into one well of an 0.8% agarose (electrophoretic grade, Bethesda research laboratory) 500ml slab gel made up in Tris borate EDTA solution (TBE) and immersed in 2.5 volumes of TBE. Large gels were run at 40-90mA at 4°C overnight.

TBE

0.089M Tris-borate
0.089M Boric acid
0.002M EDTA

Loading buffer

30% glycerol
0.25% bromophenol blue
in TBE

After running, genomic gels were stained with ethidium bromide at 0.5ug/ml in TBE for 15 minutes, destained briefly in distilled water and visualized on an UV transilluminator where the image was recorded by a camera linked to a Mitsubishi video copy processor.

7.13.2 Transfer

Gels were trimmed to 20cmx20cm, denatured for 30 minutes in 0.5M NaOH 1.5M Tris on an orbital shaker and transferred onto the blotting apparatus. Whatman 3mm paper was pre-soaked in 0.5M NaOH 1.5M Tris and placed on a plate suspended over a glass dish containing the same alkaline solution, with the ends of the paper serving as a wick to draw up this solution. A 20cmx20cm charged nylon membrane (Hybond N, Amersham) was also pre-soaked and placed gently on top of the gel which was then covered with 4 layers of pre-soaked 3mm paper cut to the size of the gel. Any bubbles between gel and membrane were rolled out using a large glass pipette. Exposed

edges of the gel were sealed with cling film to prevent buffer by-passing the filter, and a 3 inch stack of absorbant paper towels topped with a weight was placed on top to aid capillary movement of the buffer. The apparatus was left for 12-16 hours to allow transfer of the DNA onto the membrane after which the membrane was neutralised in 3M NaCl 0.5M Tris pH 7.4 for about 1 hour and allowed to dry face up on filter paper at room temperature.

7.13.3 Prehybridization

Filters were placed between net meshes in sealed plastic hybridization bags (Hybaid) and prehybridisation solution was added to one port with a plastic syringe ensuring that all air bubbles were withdrawn. Prehybridisation was carried out for 6 hours in a 65°C water bath with 50ml of prehybridization solution per 20cm x20cm membrane, and 30ml for each half-sized membrane.

SSC

0.15M NaCl
15mM Tri sodium citrate

Made up in distilled water as a 20x concentrated solution and diluted in distilled water for use.

Prehybridization solution

10% Dextran sulfate
1% SDS
6x SSC
100ug/ml denatured and snap-cooled salmon sperm DNA

7.13.4 Probe preparation

Probe inserts were digested from plasmids with the appropriate restriction enzymes. In most cases known amounts of digests were run out on 1% low melting point agarose gels containing 0.5ug/ml ethidium bromide. Insert bands were identified under UV light and sliced from the gel in the minimum possible volume of agarose using a scalpel. 3ml of distilled water were added for every 0.1g of agarose and each sample was boiled for 7 minutes in an eppendorf tube and aliquoted into samples containing 25ng of insert in a volume no greater than 25ul. For some probes this insert isolation procedure was not necessary and digests could be diluted directly and aliquoted. In either case, 25ng aliquots were boiled further for 1 minute and transferred immediately to a 37°C water bath for 10-60 minutes prior to labelling. Probes in agarose were always labelled for 12-16 hours at room temperature in a lead casket, while unpurified probes were often labelled for 30 minutes in a 37°C water bath.

Labelling reaction

10ul nucleotide mix
5ul primer
2-25ul DNA
5ul alpha-^{32}P[dCTP] (50uci)
2ul Klenow fragment

The buffers and reagents were supplied in kit form (Amersham).

Labelled DNA was separated from unincorporated radioactivity by passing the sample through a nick-column (Pharmacia) and collecting the DNA containing fraction. This was spun for 2 minutes in a microfuge with an equal volume of phenol, and the top layer was removed and precipitated in 2 volumes of absolute ethanol and 1/10 volume 3M Na acetate at -20°C for no less than 10 minutes. The precipitate was pelleted by spinning in a microfuge for 15 minutes and inverting the tube on a paper towel to dry.

7.13.5 Hybridization

1) Basic method- Probes were denatured in TE containing 100ug/ml salmon sperm DNA at 92-100°C for 10 minutes and added directly to the filter. About 20ml of the prehybridization solution was drawn from the bag via one of the ports using a large plastic syringe and denatured probe was added and mixed well before expelling the liquid back into the bag.

2) VNTR and other probes with high non-specific binding- In these cases it was necessary to pre-incubate the denatured probe in a solution containing denatured human DNA. This was done by denaturing 100ug/ml placenta DNA as above and adding this to 50ml of fresh prehybridization solution at 65 C. Probe pellets were denatured in 1ml of this solution, as above, and added to the human DNA solution and incubated at 65°C for 1 hour. At this point pre-hybridization solution was discarded from the bag and replaced with the pre-incubated probe solution.

In both cases hybridization was carried out for 12-16 hours in a 65°C water bath.

7.13.6 Washing and detection

Hybridization solution containing probe was discarded down a radioactive designated sink and unbound radioactivity was washed off by firstly running 500ml of 2xSSC over the filter using the hybaid vacuum pump system. 250ml of 0.5xSSC, 1%SDS was then added to the bag which was incubated at 65°C for 2x10 minute periods or until no radioactivity above background was detected by a Geiger counter. The SDS was then washed off by 5

Table 17a Probes on chromosome 5

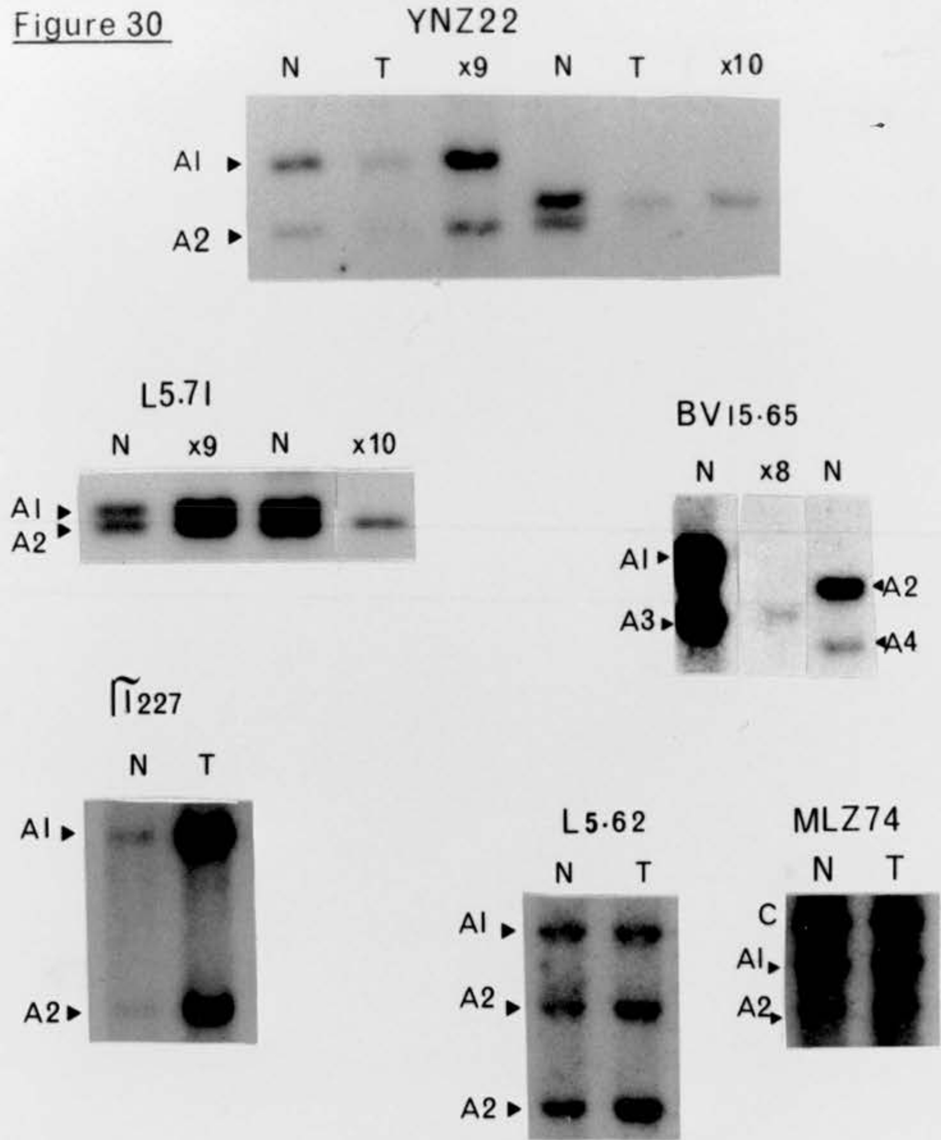
Probe [a)b)]	Insert size (Kb)/enzyme	Genomic digest enzyme	Alleles (size Kb/freq.)	Location & Reference
L1.4 [a) b)]	0.72/EcoR1	EcoR1	0.7/0.72 0.6/0.28	5p Bodmer et al 1987
MLZ7.5 [a) b)]	2.5/EcoR1	EcoR1	2.9/0.49 2.2/0.51 3.15/1.00	5p Leppert et al 1987
Pi227	0.9/HindIII& EcoR1	Pst1/ Bcl1	4.3/0.75 3.0/0.25 (Pst1) 3/0.17 1.8/0.46 1.2/0.37 (Bcl1)	5q21-22 Dunlop et al 1990
C11p11 [a)]	3.6/EcoR1	EcoR1	4.4/0.08 3.9/0.92	5q21-22 Bodmer et al 1987
L5.62 [b)]	Taq1	BglII	9.0/0.93 5+4/0.07	5q21-22 Dunlop et al 1990
L5.71 [b)]	Taq1	Msp1	4.4/0.40 4.3/0.60	5q21-22 Kinzler et al 1991
EF5.44	1.9/EcoR1& HindIII	Msp1	2.9/0.18 2.1/0.82	5q21-22 Dunlop et al 1990
YN5.48	2.4/ Taq1	Msp1	9.0/0.51 8.0/0.49 7.0/0.01	5q21-22 Nakamura et al 1988
MC5.61 [a)]	3.2/ Taq1	Msp1	5.5/0.49 5.0/0.51	5q21-22 Nakamura et al 1988
LambdaMS8 [b)]	purchased [c)]	Hinf1	2.4-9.5 90% polymorphic	5q35 Solomon et al 1987

Table 17b Probes on various chromosomes

Probe a)b)	Insert size (Kb)/enzyme	Genomic digest enzyme	Alleles (size Kb/freq.)	Chromosome & Reference	
YNH24 a) b)	2.0/Msp1	Msp1	>30 allele VNTR	2	Nakamura et al 1987
EFD64.2 a) b)	4.0/Taq1	Msp1	5 allele VNTR	3	Nakamura et al 1987
YNZ132 a) b)	1.6/Msp1	Taq1	6 allele VNTR	6	Nakamura et al 1987
EFD75 a) b)	2.4/Taq1	Taq1	>6 allele VNTR	10	Nakamura et al 1987
YNZ22 b)	1.7/BamH1	Taq1	>10 allele VNTR	17p	Nakamura et al 1987
MCT35.1 a) b)	2.4/Taq1	Msp1	2.4/0.75 1.8/0.25	17p	Carlson et al 1988
BV15.65	2.7/EcoR1& Sal1	Msp1	10.5/0.17 9.7/0.04 7.8/0.49 7.0/0.30	18q	Fearon et al1990
CMM6 a) b)	4.0/Pst1	Taq1	>10 allele VNTR	20	Nakamura et al 1987
MCT15 a) b)	2.7/Taq1	Msp1	4 allele VNTR	21	Nakamura et al 1987

Probe patterns

Figure 30



Notes:

1) N= normal
2) T= tumour
3) x(number)= xenograft passage number
4) A= allele
5) C= constant allele
6) Allelic losses are shown with probes YNZ22, L5.71 & BV15.65
7) Allelic retentions are shown with probes YNZ22, L5.71, Pi22.7, L5.62 & MLZ74

minutes gentle agitation in 0.2xSSC at room temperature. Membranes were drained briefly on filter paper and wrapped in cling film to prevent drying. Probe was detected by x-ray film (Fuji) between intensifying screens at -70°C for 1-10 days, and the autoradiograph was developed using standard photographic solutions.

7.13.7 Re-probing

Since some probes detect polymorphisms with the same enzymes as others, many filters could be stripped of probe and re-used. This was achieved by boiling filters for 20 minutes in approximately 150ml/filter of 1%SDS, 0.1xSSC. Filters were allowed to dry face up on filter paper and could then be used again from the prehybridization step onwards.

7.13.8 Probe information and patterns

Details of the probes used and thei r conditions of usage are tabulated for chromosome 5 probes and for other probes in tables 17a) and 17b) respectively.

Notes for table 17:
a) Probe inserts purified from agarose before labelling.
b) Competition with human DNA required during hybridization
c) Purified probe purchased from Cellmark diagnostics, ICI.

Some of the patterns detected by probes together with some examples of allelic losses and retentions are shown in figure 30.

7.14 Densitometry

Intensity of bands was measured on a LKB Ultroscan XL Laser densitometer. For each sample, the relative intensities of bands were presented as a curve, peaks of this curve corresponding to the bands were defined and the relative area under the curve for each peak was calculated. These relative areas were direct measurements of the ratios of intensities of bands.

7.15 Plasmid DNA (Sambrook et al 1989)

Transformation of competent bacteria, minipreparation and large scale isolation of plasmid DNA were all carried out in adherence with the methods described.

Unless otherwise stated digests of 1ug DNA were routinely cut with 1ul of enzyme in a total volume of 10ul for 1 hour, and run with 1ul of loading buffer in 50ml or 100ml 0.8% agarose gels containing 0.5ug/ml ethidium bromide, for a few hours at room temperature.

7.16 Transfection of cultured cells

7.16.1 Electroporation

Desired capacitance and voltage were set on the pulse controller unit of a gene pulser apparatus (Bio-rad). These were selected such that the peak field strength in volts/cm would fall in the range of 375-625 for human cells, and 625-700 for mouse fibroblasts as recommended by Bio-rad, and the pulse time would approximate 7 milliseconds. Cells were routinely harvested at 80% confluence, washed twice in cold PBS and resuspended in PBS at 10^6/ml. Sterile gene pulsar cuvettes with built in 0.4cm electrodes (Bio-rad) were pre-chilled on ice. 0.8ml of cells were placed in each cuvette and 2-20ug DNA was added. Cells and DNA were held on ice for 10 minutes prior to electroporation. Individual cuvettes containing cells and plasmid were placed in the gene pulsar chamber, pulsed once and returned to ice for an additional 10 minute incubation before plating. Cells were washed out with warmed medium and seeded into either $25cm^2$ flasks which were gassed and placed in a 37°C room, or 100mm petri dishes which were placed in a 37°C CO2 incubator.

7.16.2 Using TRANSFECTAM

Cells at 50-80% confluence were transfected in either $25cm^2$ flasks or in 60mm petri dishes. TRANSFECTAM powder (IBF, Northumbria Biologicals Ltd.) was solubilised in ethanol according to the manufacturers specifications. 2-5ug of plasmid DNA for each transfection were added to 250ul 0.3M NaCl (filter-sterilised). 3ul of the TRANSFECTAM stock per ug DNA were diluted to 250ul and plasmid and TRANSFECTAM solutions were combined. Cells were prepared for transfection by washing the monolayer twice with cold PBS, and the combined solutions were added in 1ml prewarmed culture media without serum. Flasks were placed in a 37°C CO2 incubator for 6 hours at which point a further 3ml of the appropriate growth medium with serum were added. After 48 hours DNA and TRANSFECTAM containing medium was replaced with 4ml of the appropriate growth medium.

7.16.3 Lipofection

Cells at 50-80% confluence were transfected in either $25cm^2$ flasks or in 60mm petri dishes. 2-5ug plasmid DNA and 40ug of Lipofectin reagent (GIBCO BRL) were diluted separately to 50ul in sterile distilled water for each transfection. The 2 solutions were slowly combined in polystyrene tubes ensuring that no precipitation occurred. The combined solutions were left to stand for 15 minutes to allow formation of lipid-DNA complexes. Meanwhile the cell monolayers were washed twice with serum free medium, 3ml of which were added to

each flask of cells. The 100ul of reagent- DNA complex was added dropwise but uniformly, while the flask was gently swirled. Cells were incubated for 24 hours in a 37°C CO2 incubator. At this point 3ml of medium cobntaining 20% FBS were added for a further 48 hours. Medium containing reagent was then replaced with the appropriate growth medium.

7.17 Selection of stable transfectants

Selection for transfectants was initiated 48 hours after transfection. At this time, and twice weekly thereafter, cells were fed with the appropriate growth medium containing a known concentration of either Geneticin G-418 sulphate (Gibco, batch microbiological potency of 500 ug/mg), or hygromycin-B (SIGMA). If cells reached confluence before selection had taken full effect, they were passaged into 2 or 3 flasks all of which were grown in selection medium.

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Appendix

Appendix: Publication arising from work contained in this thesis.

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:ability of critical genetic lesions in human colorectal carcinoma :nografts

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eral genetic lesions are associated with the genesis of nan colorectal carcinomas including mutational activation Ki-*ras* and p53, and loss of heterozygosity involving sumptive oncosuppressor loci on 5q21, 17p13, and 18q22 aron & Vogelstein, 1990). Abnormalities at the 17p13 us are recorded in many types of tumours (Nigro *et al.*, 9) but there is no evidence at present that the 5q and 18q i are involved in sporadic neoplasia other than that of orectal mucosa. Although the 17p13 locus is probably genic with p53, and the product of the 18q22 gene ears to be a cell adhesion molecule, the function for all of se genes in oncosuppression is poorly understood. The al vehicle for assay and analysis of these unknown gene ctions would be a colorectal cancer cell line in which the cosuppressor genes were known to be aberrant or inactive. theory, replacement of even one copy of the appropriate act oncosuppressor gene might then restore phenotype to mality. Recent studies have demonstrated oncosuppres- n of this type on introduction of normal DNA from the noblastoma and Wilms' susceptibility loci, into tumour lines (Huang *et al.*, 1988; Weissman *et al.*, 1987). How- r, for none of the long-established colorectal cancer lines dily available is status at critical oncosuppressor loci own. Moreover long-established lines commonly acquire itional genetic rearrangements during culture (Brattain *et* 1983; Park *et al.*, 1987), which may not be reversible by rection of the original oncosuppressor defect alone. We refore set out to develop a series of lines from primary orectal tumours, defined both in terms of status at onco- pressor loci on chromosomes 5, 17 and 18, and the oility of each locus on serial passage.

Portions of carcinoma obtained from surgical resection specimens were xenografted as described by Lewko *et al.* (1989), into pairs of immunosuppressed (thymectomised, irradiated and arabinoside-C treated) CBA mice (Steel *et al.*, 1978), with the modification that portions no greater than 5 mm^3 were implanted dorsally at initial xenograft and at subsequent transplantations.

From a consecutive series of 28 cancers implanted, 11 grew to passage and eight were xenografted for more than five passages, one of which has now reached passage 16. At each serial passage excess tumour tissue was divided for analysis allowing the characterisation of xenografted tumours at various passage intervals, and the comparison of these with the primary tumour (portions of which were always stored both frozen and fixed at original resection). Haematoxylin and eosin stained paraffin sections of both primary tumour and xenografts fixed in 10% buffered formalin at all passages, showed that the original histological pattern for each primary tumour was conserved throughout serial passage. Although most of the lines fell into the class of adenocarcinomas of average degree of differentiation, extremes of both good and very poor differentiation were represented (Figure 1). No metastases were observed in mice.

To study the status of alleles closely linked to each of three putative colon-associated oncosuppressor loci, genomic DNA was prepared from frozen xenografted tumours at various passages, and compared with DNA from primary tumour tissue and normal colorectal mucosa or peripheral blood leukocytes from the donor patient. Methods for DNA preparation, Southern blotting and probes and conditions for hybridisation have been previously published (Ashton-

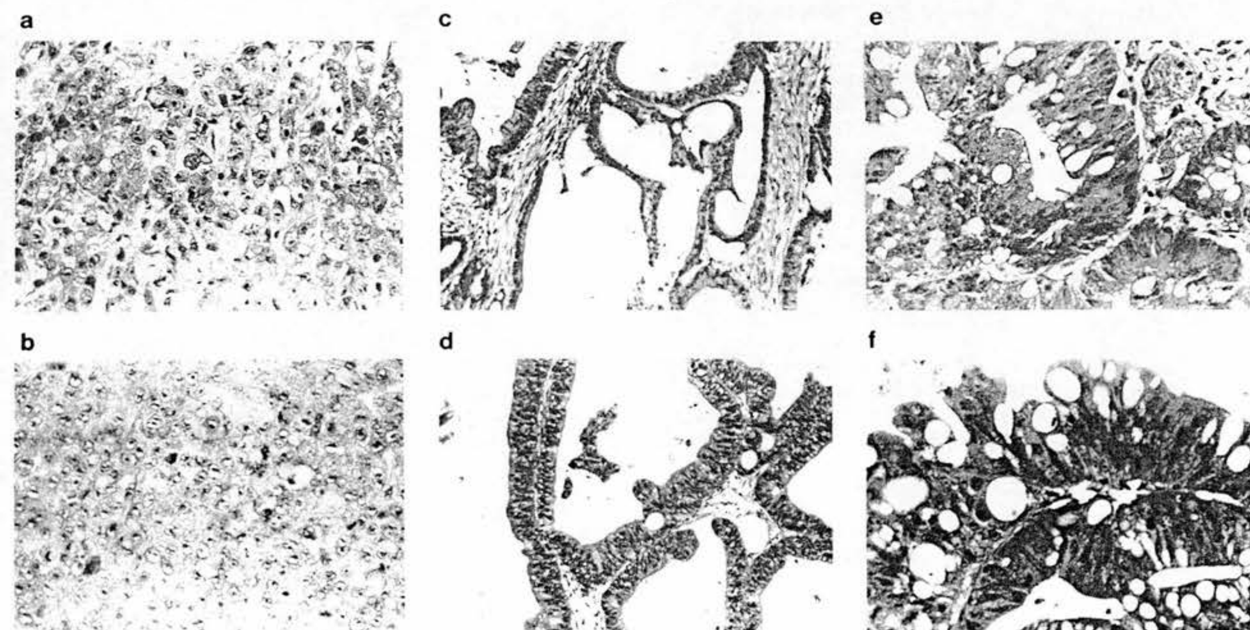

Figure 1 Histological comparisons of original colorectal tumours with the corresponding xenografts. **a**, A poorly differentiated adenocarcinoma AGDU. **b**, Corresponding xenograft at 5th passage XAGDU4. **c**, A well differentiated adenocarcinoma MUCO. **d**, Corresponding xenograft at 11th passage XMUCO10. **e**, A moderately differentiated adenocarcinoma CHKE. **f**, Corresponding xenograft at 6th passage XCHKE5.

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eived 29 May 1990; and in revised form 5 September 1990.

Rickardt *et al.*, 1989). Probes used were: for chromosome 5q: pYN5.48 (Nakamura *et al.*, 1988*a*), pEF5.44 (Dunlop *et al.*, 1990) π227 (Meera Khan *et al.*, 1988; Dunlop *et al.*, 1989), and two new probes pL5.62 and pL5.713 which map between π227 and YN5.48 (Y. Nakamura, personal communication); for 17p: pYNZ22 (Nakamura *et al.*, 1988*b*), pMCT35.1 (Carlson *et al.*, 1988); and for 18q: pBV15.65 (Vogelstein *et al.*, 1988). Almost all of these probes detect highly polymorphic sequences. pBV15.65 is situated within the 18q oncosuppressor gene, pMCT35.1 lies close to the p53 gene and the 5q probes are distributed through the APC locus over no more than 15mb.

Table I summarises allelic losses or retentions found for the parental normal and tumour DNA pairs at each locus. In all cases studied, the allelic status of xenograft DNA was unchanged from that of its primary tumour. For eight tumours this genetic stability was demonstrated over at least four xenograft passages (approximately 4 months) and in two (one of which showed allelic retention at 5q and 17p) over 11 passages (approximately 11 months). A variety of combinations of allelic losses and retentions are represented, broadly similar to those observed by ourselves and others in large unselected series of primary tumours (Ashton-Rickardt *et al.*, 1989; Vogelstein *et al.*, 1988). It appears from these observations that the selection pressures which encourage growth of human colorectal cancer xenografts in immunosuppressed animals do not include allelic losses around the oncosuppressor loci commonly affected in human carcinogenesis *in situ*.

Although our primary aim was to establish the stability of the lines at critical oncosuppressor loci, DNA ploidy was measured by flow cytometry as a global, if crude, index of total nuclear DNA content. Frozen tissues from both passage 5 xenografts and primary tumour were dissociated and stained with 0.62 M propidium iodide as described by Vindelov (1983), and analysed at an excitation wavelength of 488 nm in a Coulter Epics CS flow cytometer. On the basis of an internal chicken red blood cell standard, the presence of a human diploid peak could be confirmed in every case. This was given a ploidy value of 1, and any additional peaks were then assumed to be aneuploid, and their DNA index (DI) derived from their position relative to the diploid peak. In all cases, xenograft DI was similar to that of the relevant primary tumour (example shown in Figure 2), including four tumours remaining diploid after five or nine serial passages in mice, and one after 14 passages (15 months). A similarly high degree of conservation of genetic stability throughout xenograft passage in immunodeficient mice has been confirmed both karyotypically and at the genetic level by Lefrancois *et al.* (1989).

The demonstrated lack of genetic change for defined oncosuppressor loci qualifies these xenografts as favourable tumour models for analysis of oncosuppressor function, by re-introduction of the normal genes. Since growth *in vitro* is a prerequisite for genetic manipulation, tumour tissues removed immediately on to ice from animals at or following passage 5 were processed and seeded as primary cultures on collagen coated flasks with mouse feeder cells, as described by Paraskeva (1984). Epithelial cells from every xenograft line grew *in vitro* as flat islands of cells spreading radially across the collagen surface towards neighbouring islands (Figure 3). Two slightly faster growing lines were observed over three passages *in vitro*, and a third has now grown rapidly over 100 days, requiring a weekly split ratio of up to 1:20. On subcutaneous re-injection into immunosuppressed mice, these cells continued to show the same allelic status and morphology as the primary tumour.

Table I Allelic status at three oncosuppressor loci, and DNA index for a set of 11 xenografts

Line	*5q*	*17p*	*18q*	*DNA Index*
GRBO*	R (5)	R (4)	R (2)	1.0 (5)
MUCO*	R (10)	R (10)	–	1.0 (14)
RHSP*	L (10)	L (10)	–	1.7–1.9 (12)
CHKE*	R (4)	R (4)	R (3)	1.1–1.2 (9)
DABU*	R (5)	R (5)	NI	1.0 (5)
ARNE*	R (4)	L (5)	L (5)	1.5–1.6 (5)
MASM*	R (5)	R (5)	R (2)	1.0 (9)
JOMcL*	R (4)	R (4)	NI	1.0 (5)
JACA	L (2)	L (2)	L (2)	1.6
CARO	L (1)	L (1)	L (1)	1.4
JOWR	R (1)	R (1)	R (1)	1.0

( ) = highest xenograft passage no. analysed; – = not done; NI = non-informative (patient homozygous for alleles studied); R = allele retained; L = allele lost; * = line xenografted over more than five passages. In all cases the allelic status of the xenografts throughout passage was identical with that of the primary tumour.

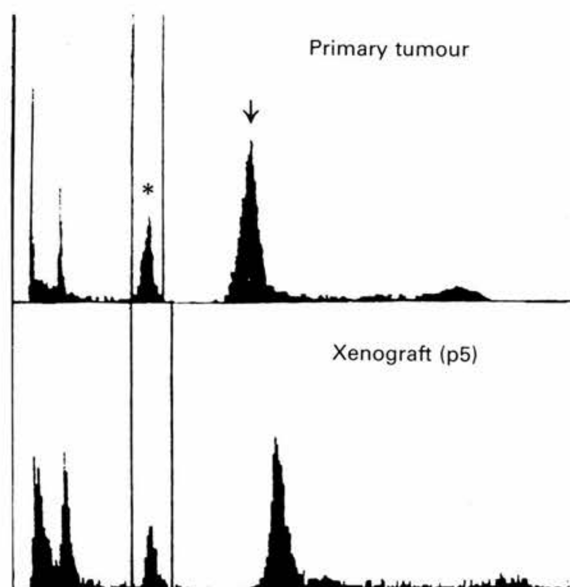

Figure 2 Flow cytometric comparison of an aneuploid primary tumour (DNA index 1.7); arrowed, with the xenograft at passage 5 (DNA index 1.9). Diploid peak position is marked *.

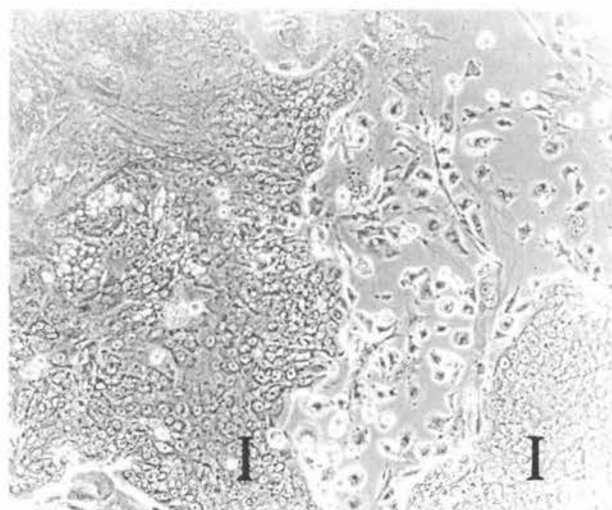

Figure 3 Phase contrast microscopy of XRHSP xenograft tumour epithelial cells growing in culture as islands (I) surrounded by feeder cells.

In conclusion, the xenograft lines described above represent a variety of defined and stable combinations of oncosuppressor gene status and will therefore provide a useful resource for the study of the functions of the genes involved in human colorectal cancer, and their interactions with each other.

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